Oral drug delivery:
Adhesive versus non-adhesive formulations

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Attempto !

Herrn Univ.-Prof. Dr. Andreas Bernkop-Schnürch danke ich für das Ermöglichen dieser Arbeit, der wissenschaftlichen Unterstützung sowie der darüber hinausgehenden Betreuung und Korrektur dieser Arbeit.

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Besonderer Dank gilt auch meiner Familie und meinen Freunden, die mich immer unterstützen und mir auch aus der Ferne Rückhalt geben.

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Abbreviations and definitions

AC1030	Carbopol Ultrez® 20
Alg	Alginate
Alg-CHO	Aldehyde intermediate of alginate
API	Active pharmaceutical ingredient
COOH-PEG-SH	(O-(3-Carboxylpropyl)-O´-[2-[3-mercaptopropionylamino]ethyl]-polyethylengycol)
Cys	Cysteine
DOPE	Dioleoylphophotidylethanolamine
DOTMA	N-[1-(2,3-Dioleyloxy)propyl]-n,n,n-trimethylammonium chloride
EDAC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
Eu	Eudragit® L 100-55
FDA	Fluorescein diacetate
GIT	Gastrointestinal tract
MDF	Maximum detachment force
MNA	2-Mercaptonicotinic acid
NA-Carbomer	Not hydrophobically modified carbomer
NaFlu	Sodium fluorescein
NHS	N-hydroxysulfosuccinimide
PAA	Poly(acrylic acid)
PAPC	Poly(acrylic acid)-papain complex
PBS	Phosphate buffered saline
PDI	Polydispersity index
pDNA	Plasmid DNA
Pec	Pectin
Pec-Cys-MNAe	Entirely S-protected thiolated pectin
Pec-Cys-MNAg	Preactivated thiolated pectin for gel formulation
PEG	Polyethylene glycol
Polymer-SH	Thiolated polymer
Polymer-S-MNA	Preactivated thiolated polymer
SATA	S-Acetylthioglycolic acid
SNEDDS	Self nanoemulsifying drug delivery system

tan δ	Loss factor
TBA	Thiobutylamidine
TFA	Trifluoroacetic acid
TGA	Thioglycolic acid
TWA	Total work of adhesion

1. Introduction

Oral application of drugs is always desirable as it is patient's route of choice. Oral formulations are convenient and painless. Further, as they do not require sterility they are cost effective and reveal good patient compliance. Parenteral applications – in contrary – are associated with fear, effort and pain. Unfortunately, many modern and high effective drugs are not or very poorly bioavailable when administered per oral [1, 2]. Reasons therefore are diverse, some are sensitive against enzymatic degradation in the gastro intestinal tract (GIT) like protein and DNA drugs or they show low solubility and/or low permeability [3, 4]. Therefore, improving or enabling oral drug delivery for these kinds of drugs is the focus of intensive research. Within the years, different approaches emerged. One of them are mucoadhesive drug delivery systems. Having their entry in pharmaceutical technology more than 20 years ago, meanwhile, they are accepted as a useful strategy to improve bioavailability of drugs. The increased contact time with the absorption site leads to an increased drug concentration gradient and hence to an improved uptake. For these delivery systems, usually multifunctional polymers are used. Besides adhesive properties, these polymers bring some other useful features like sustained drug release, gel formation or permeation enhancing effects [5-7]. However, even if there are promising *in vivo* studies about bioadhesive carriers, the "super glue" amongst mucoadhesive polymers has not been invented yet.

Therefore, another promising attempt has experienced increasing interest within the last years, trying to improve drug uptake via mucus penetrating and permeating drug delivery systems. The aim herein is, to develop a multi-particular delivery system in the nano-range which is able to cross the mucus layer and reach the underlying epithelial cells. Polymeric nanoparticle delivery systems designed for mucosal administration are researched intensively, but in general those carriers are not able to permeate the mucus gel layer as the nano-carrier cut-off size is between 55-100 nm [8]. In most cases, polymeric nanoparticles exhibit a mean particle size higher than 100 nm and therefore, the amount of active pharmaceutical ingredient (API) delivered to the absorption site is not sufficient. Another strategy for mucus penetrating delivery systems are self-emulsifying drug delivery systems. These lipophilic mixtures containing one or more surfactants, lipids and a cosurfactant are able to form stable emulsions with a small droplet size (~ 50 min) [9]. Due to the hydrophobic surface, interactions with the mucus gel layer are low and passage relatively easy [10]. So far, these carrier systems are mainly investigated for delivery of low soluble drugs, only very limited data is published about mucus penetrating properties.

This thesis deals with both, the mucoadhesive and the comparatively newer non-adhesive drug delivery strategy to clarify the status quo of both attempts and investigate how both routes can be improved. Novel mucoadhesive polymeric excipients were generated to overcome the drawbacks of existing ones and to

prove that research in this field is not exhausted yet. Concurrently, different mucus-penetrating systems were developed and characterized in terms of their mucus cleaving potential and facilitation of drug transport. Moreover, both strategies were combined in one formulation to investigate if there is a possibility to combine advantages of both strategies.

Concerning mucoadhesive drug delivery systems, the implementation of a thiol group to mucoadhesive polymers was found to enhance the mucoadhesive effect of these polymers, as these thiolated polymers are able to form covalent bonds with mucus substructures [11]. Unfortunately, these thiol-functionalized excipients suffer from some drawbacks. They are sensitive against oxidation and the reactivity of the thiol groups are pH dependent [12, 13]. Therefore, thiol groups of the polymers have been further modified by the implementation of a pyridylic thiol group via disulfide formation. These pyridylic groups are known to increase the reactivity of the thiol groups [14, 15]. To overcome solubility problems of chitosan, the carbohydrate was grafted with polyethylenglycol (PEG). Resulting novel polymers were investigated especially in terms of mucoadhesion and their suitability for tailor-made drug delivery systems and drug targeting.

Furthermore, mucus penetrating properties of a SNEDDS formulation were improved by incorporation of synthesized novel mucolytic thiol-lipids. Moreover, the SNEDDS formulation was investigated for its ability to shield peptide and DNA drugs against enzymatic degradation. Additionally, novel mucus permeating nanoparticles were synthesized by linking to a mucolytic enzyme and characterized in terms of their ability to facilitate drug transport through intestinal mucus. Finally, these mucus penetrating particles were combined with a mucoadhesive thiomer.

2. Mucosal tissue and mucus

Mucosal tissue covers the walls of the body cavities, whereby the morphology differs from organ to organ. Yet they all have a principal structure in common: They consist of a connective tissue layer, the lamina propria, which is overlaid by an epithelial layer [16]. The gastric and intestinal mucosae develop a single layered epithelium with goblets cells which secret mucus on the surface of the epithelium. Mucosae in the oral cavity or the esophagus exhibit a multilayered/stratified epithelium. Here, mucus is produced by specialized cells which are in or nearby the epithelium like salivary gland cells [17].

The main function of mucus is to prevent draining of the tissue and to hinder xenobiotics to enter unselected in the systemic circulation. The mucus gel layer is one of the first hindrances an API has to overcome. Only substances which are able to cross the mucus layer can be taken up by the underlying epithelium.

Mucus consists to around 95 % of water. The remaining percentage is a mixture of glycoprotein fibers, oligosaccharides, lipids, cells and cell debris, enzymes, antibodies, DNA and electrolytes [18, 19]. The thickness of the mucus gel layer depends highly on its location, in the gastric cavity it is reported to be 50 to 500 µm and in the intestine between 15 and 450 µm [19-21]. On buccal tissue, the thickness of the mucus layer is less than 1 µm [22].

Mucus is characterized by a three-dimensional gel structure. Gel forming mucus fibers can be seen as long flexible strings cluttered with short glycans. These glycans are mostly negatively charged because of carboxyl and sulfate groups. Mucin monomers are cross-linked resulting in an oligomeric gel. Thereby, the monomers are linked via disulfide bonds between disulfide-rich subdomains close to the amino- and carboxyl-termini of the glycoproteins. The net is further stabilized by “naked” globular protein regions which exhibit disulfide bonds as well. For the so formed net a mesh spacing between around 100 and 1000 nm was found, the size highly dependent on the method used. However, not only the size of particles influences their degree and speed of permeation, also the surface characteristic plays an important role in the uptake mechanism as those determine interactions with the gel layer [23]. The zeta potential, density of positively and negatively charges and the hydrophilic properties of the particle surface affect the mucus penetration.

3. Experimental part with results

3.1 Mucoadhesive drug delivery systems

3.1.1 Synthesis

Thiolated polymers

Within this thesis, novel thiolated and S-protected/preactivated thiomers were synthesized and characterized with focus on improving mucoadhesive properties and suitability for mucosal drug delivery. Table 1 provides an overview of all synthesized and investigated thiolated polymers within this thesis. Resulting conjugates can be distinguished by their backbone charge, attached ligands and coupling rate. With chitosan, pectin and alginate naturally occurring carbohydrate derivatives were chosen. Eudragit® L 100-55 and Carbopol Ultrez® 20 are synthetic derivatives based on poly (acrylic acid) (PAA).

Table 1. Summary of all synthesized thiolated polymers within this thesis. All derivatives are conjugated by amide bond formation between the polymeric backbone and the ligand, except Alg-SH. To thiolate alginate, cysteine was attached to the backbone after periodate cleavage and activation of the carbohydrate by reductive amination.

Polymer conjugate	Abbreviation in text	Charge	SH-groups [µmol/g]	Ref.
Alginate-Cysteine	Alg-SH	-	1379 ± 102	[24]
Carbopol Ultrez® 20-Cysteine	AC1030-SH	-	1167	[1]
Chitosan-(PEG-SH)	Chito-PEG-SH	+	244 ± 26	[25]
Chitosan-Thioglycolic acid	Chito-TGA	+	253 ± 19	[25]
Eudragit® L 100-55-Cysteine	Eu-SH-1	-	266 ± 42	[26]
Eudragit® L 100-55-Cysteine	Eu-SH-2	-	140 ± 25	[26]
Eudragit® L 100-55-Cysteine	Eu-SH-3	-	60 ± 13	[26]
Pectin-Cysteine	Pec-SH	-	507 ± 35	[2]
Pectin-Cysteine	Pec-SHg	-	450 ± 30	[27]

Thiomers were synthesized by the covalent attachment of a small thiol-ligand to a polymeric backbone. All chosen polymers are either bearing a carboxyl group (pectin, alginate, PAA-derivatives) or a primary amino group (chitosan) which can be easily attached to a primary amine or a carboxyl group, respectively. These amide bond formations are mediated by the carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysulfosuccinimide (NHS). Thereby, the carboxylic acid moieties of the reactant were activated by formation of an O-acylurea intermediate. This intermediate reacts easily with primary amino groups [28]. For all anionic polymers the amino acid cysteine was chosen as thiol bearing ligand. For the cationic chitosan, a thiol bearing polyethylene glycol derivative (MW 3000 Da) which is functionalized with a carboxyl group (COOH-PEG-SH, (O-(3-carboxylpropyl)-O´-[2-

[3-mercaptopropionylamino]ethyl]-polyethylengycol)) was chosen. This extremely hydrophilic ligand was not only chosen to improve chitosan's mucoadhesive properties but its solubility as well, as the solubility of chitosan is limited at pH values above its pks (5.5-6.5). The schematic pathway of the conjugation is shown in Figure 1. Additionally, chitosan was modified with thioglycolic acid as reference.

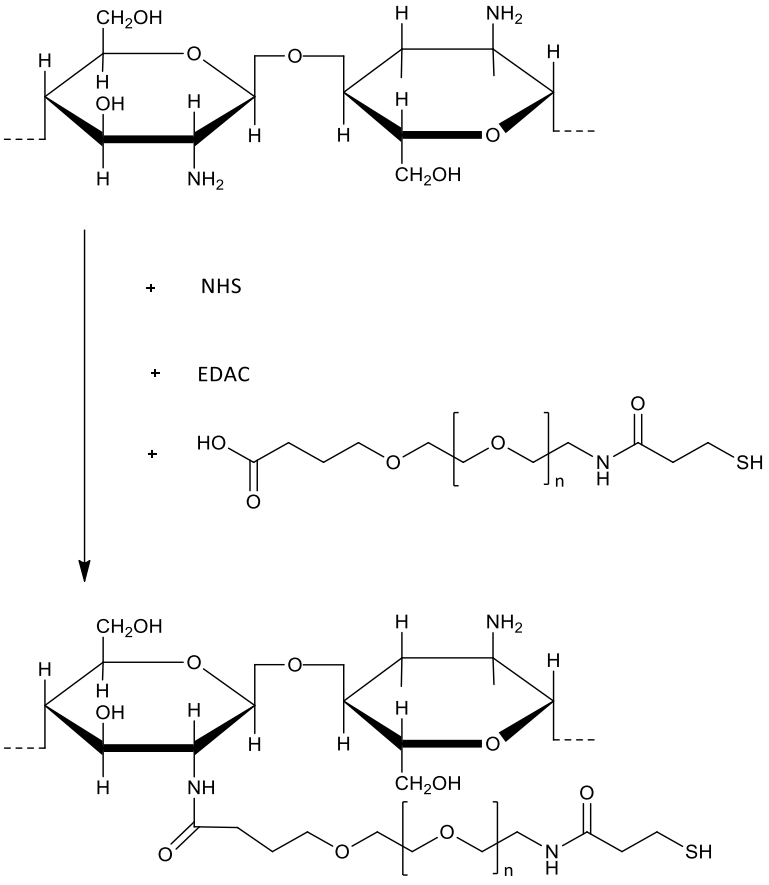


Figure 1
Structure and synthetic pathway of thiolated chitosan (Chito-PEG-SH) as represent of thiolation via amide bond formation mediated by EDAC. Amide bond was formed between the primary amino group of chitosan and the carboxyl group of the COOH-PEG-SH-ligand [25].

For alginate a different pathway was chosen as depicted in Figure 2. In a first step, the carbohydrate was treated with $NaIO_4$. Thereby, some of the vicinal diol groups of the carbohydrate backbone were oxidized, leading to a cleavage of the C-C-bond and subsequently leading to the formation of two neighboring aldehyde groups. Resulting aldehyde groups were further modified via a reductive amination, whereat the primary amine L-cysteine was bound in a Schiff base formation and oxidized with cyanoborohydrate to a secondary amine, resulting in thiolated alginate, Alg-SH [29, 30].

The amount of attached thiol groups was in all cases quantified using a spectrophotometrically method employing Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) [31]. To control purification, a TNBS test was carried out to quantify the primary amino groups of unbound cysteine [32].

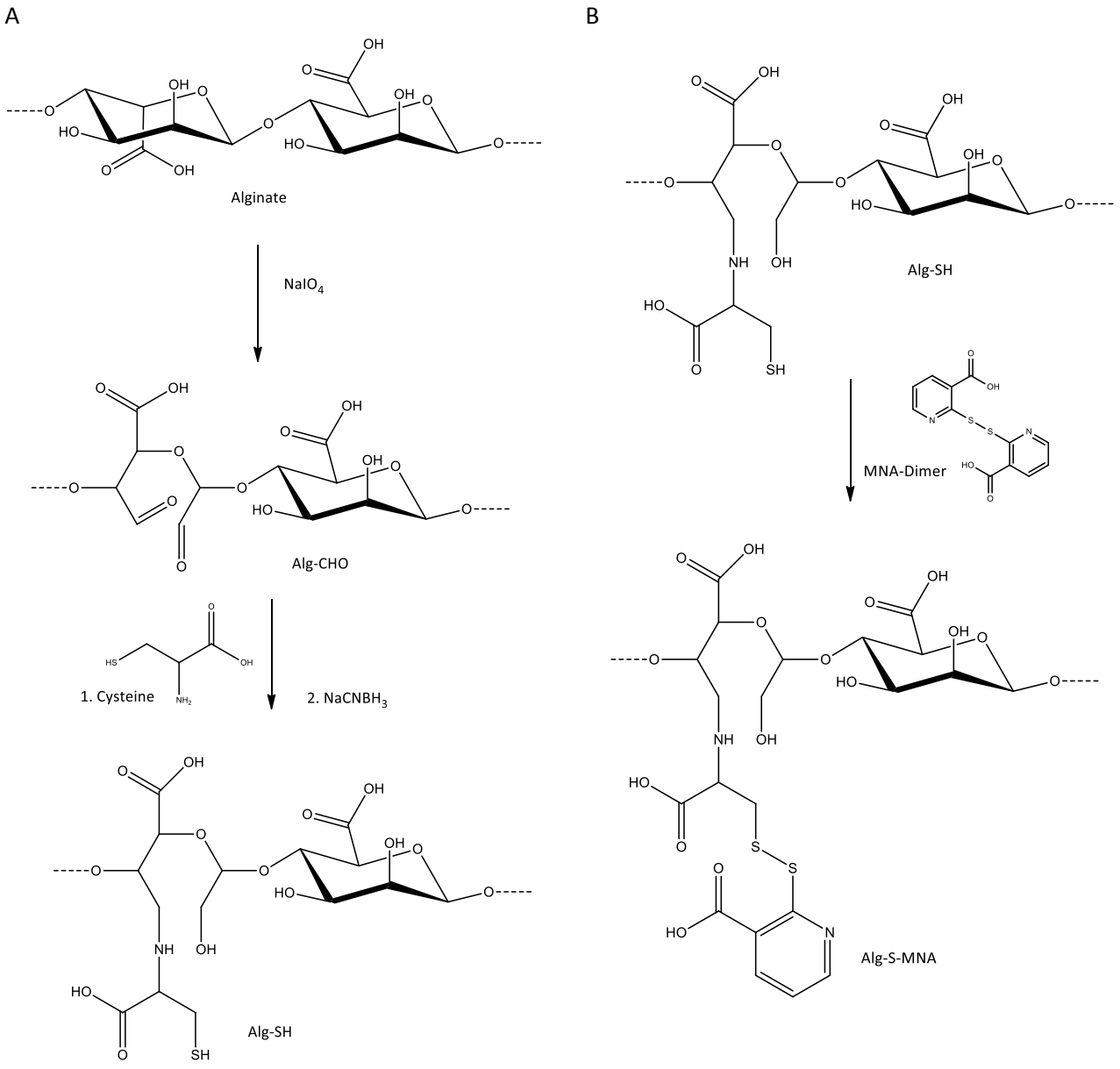


Figure 2.
Pathway of thiolation of alginate. First, the vicinol diols of the carbohydrate backbone were oxidized under opening of the C-C- bond by sodium periodate leading to Alg-CHO. The resulting aldehyde groups were further modified via a reductive amination whereat the primary amine L-cysteine was bound in a Schiff base formation and oxidized with cyanoborohydrate to a secondary amine, resulting in Alg-SH (A). In a next step, the immobilized thiol groups were preactivated by a disulfide exchange reaction using the dimerized ligand 2-mercaptonicotinic acid (MNA) resulting in the alginate-cysteine-mercaptonicotinic acid conjugate Alg-S-MNA (B) [24].

Preactivated/S-protected thiolated polymers

All of the above mentioned thiolated polymers, except Chito-PEG-SH, were further modified by an aromatic thiol bearing ligand, 2-mercaptonicotinic acid (MNA) (Figure 2). The aromatic mercaptane was bond to the thiomers via disulfide bond formation. Therefore, the dimer of the pyridylic ligand was synthesized by oxidation with H_2O_2. In a next step, the oxidized MNA was added to the dissolved thiolated polymer. Via disulfide exchange reaction, a new mixed disulfide, the preactivated thiomer, accurred under release of the MNA monomer. The disulfide exchange reaction is driven by the higher stability of the mixed aliphatic-aromatic disulfide bond compared to the aromatic-aromatic disulfide bond [33]. Resulting rates of bound MNA to the thiomers are given in Table 2. For pectin (Pec-S-MNAe), an additional synthesis pathway was investigated as preactivation after thiolation leads to a relatively high amount of free, unprotected thiol groups. Herein, MNA was conjugated to cysteine via disulfide exchange reaction with the dimerized MNA. The resulting mixed disulfide was subsequently attached to the carboxyl moiety of pectin via amide bond formation mediated by EDAC [27].

Table 2. Summary of all synthesized S-protected thiolated polymers within this thesis. All derivatives are conjugated with 2-mercaptonicotinic acid after thiolation of the backbone except Pec-S-MNAe where cysteine was coupled to 2-mercaptonicotinic acid prior to coupling to the pectin backbone resulting in an entirely S-protected thiomer.

Polymer conjugate	Abbreviation in text	MNA [µmol/g]	S-protection rate [%]	Ref.
Alginate-cysteine-(2-meraptonicotinic acid)	Alg-S-MNA	520 ± 29	38	[24]
Carbopol Ultrez 20-cysteine-(2-mercaptoniotinic acid)	AC1030-S-MNA	865	74	[1]
Eudragit-cysteine-(2-mercaptonicitinic acid)	Eu-S-MNA-1	51 ± 16	20	[26]
Eudragit-Cysteine-(2-mercaptonicitinic acid)	Eu-S-MNA-2	45 ± 18	24	[26]
Eudragit-cysteine-(2-mercaptonicitinic acid)	Eu-S-MNA-3	33 ± 11	55	[26]
Pectin-cysteine-(2-mercapotnicotinic acid)	Pec-S-MNA	263 ± 13	52	[2]
Pectin-cysteine-(2-mercaptonicotinic acid)	Pec-S-MNAg	270 ± 18	60	[27]
Pectin-cysteine-(2-mercaptonicotinic acid)	Pec-S-MNAe	210 ± 11	100	[34]

3.1.2 Swelling, disintegration and solubility

Behavior of a polymeric excipient in water is crucial for its properties in drug delivery. Depending on the pH, all unmodified polymers are soluble in aqueous media. The swelling/erosion behavior of the excipient influences several properties like mucoadhesion and drug release. For instance, the water uptake capacity of a polymer is related to its mucoadhesive properties [35]. The “adhesion by hydration” theory states that adhesion occurs due to water uptake of the polymer from the underlying mucosa by absorption, swelling and capillary effects. Further, swelling leads to chain relaxation and makes polymeric chains available for chain interpenetration with mucus. Thereby, the contact surface is enlarged and hence interactions of the components with each other [35, 36]. Moreover, the rate and extent of water uptake influences rate and extent of the release of an incorporated API. Dissolution of the drug, chain relaxation and disintegration are easing the way out of the formulation.

To investigate the behavior of the synthesized polymer derivatives in aqueous environment, swelling/erosion studies were carried out. Therefore, lyophilized polymers were compressed into test tablets (30 mg, diameter 5.0 mm, thickness 1.5 mm) and incubated in phosphate buffer pH 6.8 0.1 M or HCl 0.1 M, respectively and weighed after predetermined time points. Figure 3 gives weight change of test tablets in percent from the initial weight. For all thiolated polymers a prolonged disintegration time was observed, in general going along with an increase of water uptake capacity. Cohesiveness of thiolated as well as preactivated thiolated derivatives is improved due to the formation of inter- and intra-molecular disulfide bonds. Implementation of the MNA ligand led either to a higher water uptake (Eudragit®, pectin) or to a lower uptake (AC1030) compared to the non-preactivated thiomer but in all cases to a higher water uptake than the unmodified polymer. Further, a prolonged disintegration time compared to both, the thiolated and the unmodified corresponding polymer was observed in all cases of preactivated thiomers. Implemented thiol groups form intra- and intermolecular disulfide bonds leading to a crosslinking of the polymers. The crosslinking leads to a higher cohesiveness of the polymer, a higher molecular mass and hence to a decrease in solubility and increase in disintegration time [37]. Due to prolonged disintegration time, more water can be taken up and entrapped between polymeric chains. Regarding the S-protected polymer, disintegration is reduced by the additional conjugation of a hydrophobic ligand. In case of AC1030, the increased hydrophobicity leads to a reduced water uptake capacity compared to the non-preactivated thiomer.

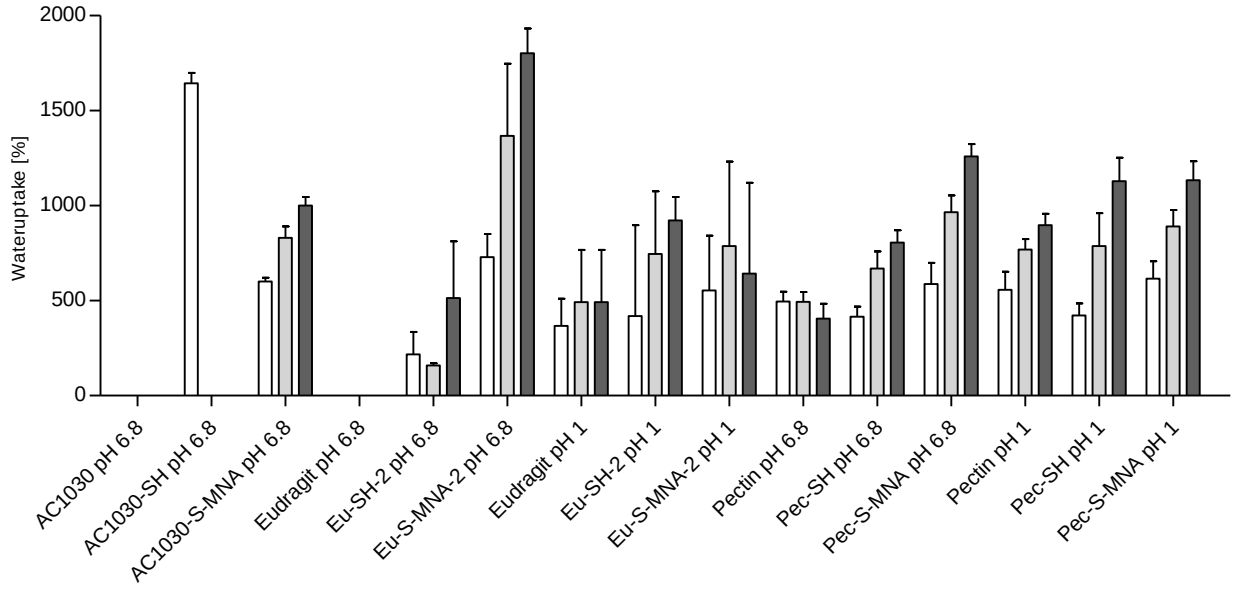


Figure 3.
Graph shows the increase in weight given in percent of initial weight of test tablets. Tablets were incubated either in phosphate buffer pH 6.8 0.1 M or HCl 0.1, respectively. White bars display weight gain after 60 min, light grey bars after 120 min and dark grey bars weight gain after 180 min. Indicated values are means + StDev of at least three experiments. Empty spaces indicate disintegration of the test tablet. Unmodified AC1030 and Eudragit® L 55-100 disintegrated within the first hour [1, 26].

Table 3. Solubility of chitosan and its derivatives. Polymers were incubated overnight in a final concentration of 1 % in different buffers 0.1 M. + = polymer dissolved, - = polymer not dissolved under given conditions [25]. Experiments were carried out in triplicates.

Solvent	Chitosan	Chitosan-TGA	Chitosan PEG-SH
Acetate buffer pH 4	+	+	+
Acetate buffer pH 5	+	+	+
Phosphate buffer pH 6	-	-	+
Phosphate buffer pH 7	-	-	+
Phosphate buffer pH 8	-	-	+
Carbonate buffer pH 9	-	-	-
Carbonate buffer pH 10	-	-	-

Within this thesis, chitosan was grafted to a thiol modified PEG ligand to improve the solubility of chitosan. Chitosan is a multifunctional polymer which shows besides mucoadhesive properties permeation enhancing characteristics. However, most chitosan derivatives are only soluble at pH values lower than its pks ($\leq$ pH 5.5-6.5) when amino groups of the polysaccharide chain are protonated [38]. Hence, the polymer is not soluble in the intestine, the absorption site of most drugs. In logical consequence, it is not possible to take full advantage of – especially the permeation enhancing – properties of the polymer [39]. By grafting chitosan with the extremely hydrophilic ligand COOH-PEG-SH, the solubility of chitosan could be improved (Table 3) and thereby it was possible to combine the advantages of water soluble chitosan with the advantages of thiolated chitosan [25].

3.1.3 Mucoadhesion

The process of mucoadhesion is complex, several steps are involved and it is influenced by many factors like pH of the environment, ionization and physical properties of the polymers [35]. Further, the measured values for mucoadhesion are highly dependent on the chosen method. Therefore, within this thesis, two methods are employed, tensile strength studies and rheological synergism studies.

Tensile studies

Tensile studies carried out within this thesis are based on a method described by Mortazavi and Smart in a slightly modified way [40, 41]. The freshly excised tissue was fixed in a beaker containing phosphate buffer pH 6.8 0.1 M for intestinal mucosa or HCl 0.1 M for gastric mucosa. With a moveable balance and a PC software (Sarto Collect, Satorius AG), the force required to detach the test tablet from mucosal tissue was detected. Thereby, the maximum detachment force (MDF) was determined and the area under the force distance curve representing the total work of adhesion (TWA) was calculated. Results are illustrated in Figure 4 and 5.

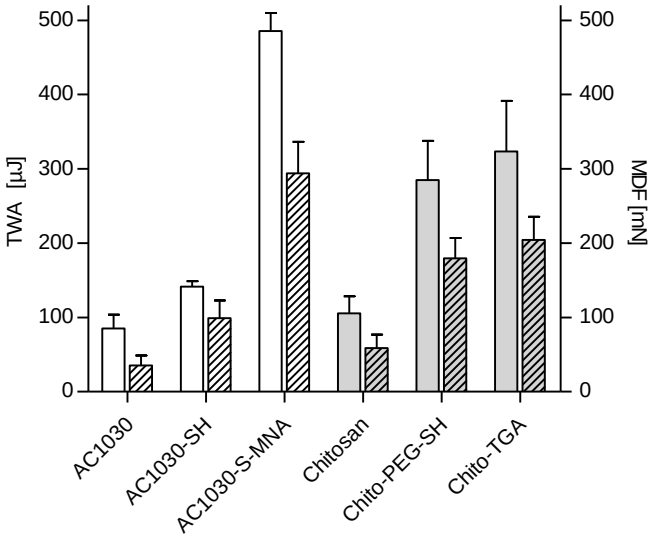


Figure 4.
The bar chart gives the results of tensile studies carried out with polymeric test tablets and intestinal mucosa in phosphate buffer pH 6.8 0.1 M. Bars with no pattern display the total work of adhesion (TWA, area under force distance curve) and striped bars give maximum detachment force (MDF). Indicated values are means ± StDev of at least three experiments [1, 25].

A

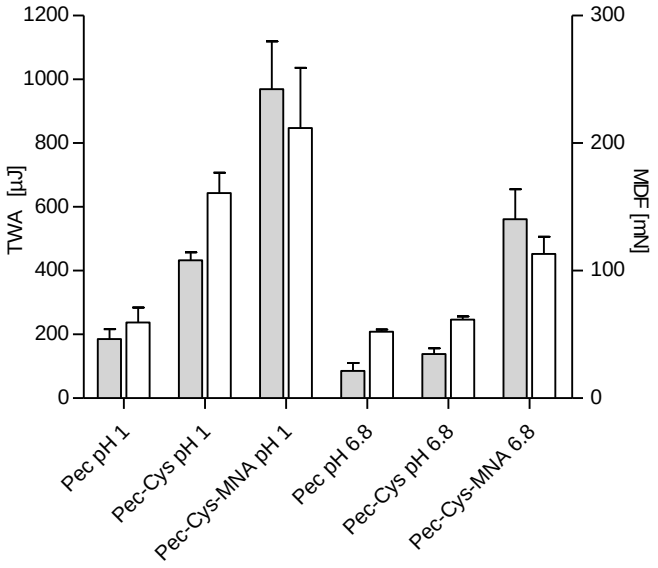
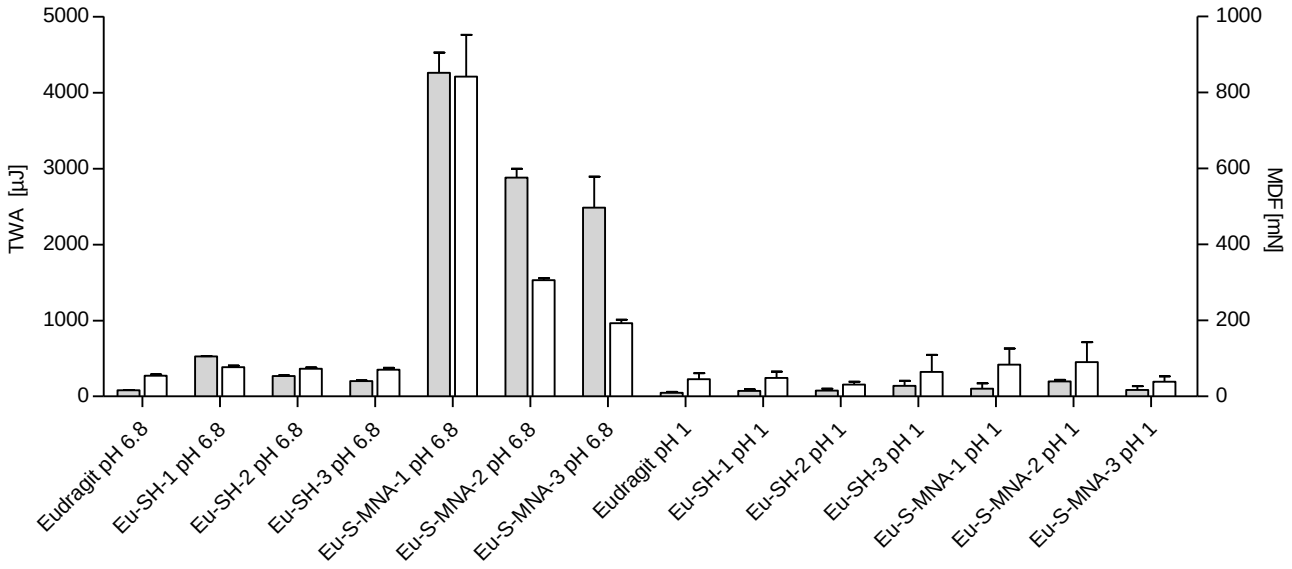


Figure 5A+B.
The bar charts shows the pH dependence of mucoadhesive properties of preactivated thiolated pectin (A) and preactivated thiolated Eudragit® (B). Given is the maximum detachment force (MDF) and the total work of adhesion (TWA) between polymeric test tablets and intestinal mucosa (white bars, phosphate buffer pH 6.8. 0.1 M) and gastric mucosa (grey bars, HCl, 0.1M), detected via tensile studies. Values are means ± StDev of at least three experiments [2, 26].

B



Rheological synergism

To investigate mucoadhesive properties of the synthesized derivatives in fully hydrated form, the rheological synergism method was employed. This method implies that resulting viscosity of a polymer/mucus mixture is higher than the additive viscosity of both single components [42]. Therefore, the dynamic viscosity of polymeric solutions were measured with and without the addition of freshly collected intestinal mucus [25]. A plate-plate viscometer was employed (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) for all rheological measurements. Results and parameters are given in Table 4. Furthermore, the development of interactions between both components was observed over the time and is plotted in Figure 6 [43].

Table 4. Results of rheological investigations of polymer solutions and resulting dynamic viscosity of polymer mucus mixtures. Indicated values are means ± StDev of at least three experiments. *instead of fresh native porcine mucus, commercially available porcine mucin was used (Sigma Aldrich, Austria).

Rheological synergism					
Polymer	**Concentration % [m/v]**	**Dynamic viscosity η [Pa*s]**	**Dynamic viscosity η [Pa*s] + mucus**	**Dynamic viscosity η [Pa*s] of mucus**	**Ref.**
Alg	1.5	1.228 ± 0.501	1.74 ± 0.20	0.060 ± 0.005	[24]
Alg-CHO	1.5	0.01 ±0.010	14.02 ± 2.02	0.060 ± 0.005	[24]
Alg-SH	1.5	0.036 ± 0.011	2.956 ± 0.357	0.060 ± 0.005	[24]
Alg-S-MNA	1.5	182.2 ± 25.2	2.324 ± 0.462	0.060 ± 0.005	[24]
Chito	1	0.027 ±0.059	294.6 ±22.1	13.84 1 ± 0.881	[25]
Chito-PEG-SH	1	0.650 ± 0.119	314.1 ±50.9	13.84 1 ± 0.881	[25]
Chito-TGA	1	0.041 ±0.002	353.2 ± 26.7	13.84 1 ± 0.881	[25]
Eu	2	0.006 ± 0.001	9.028 ± 1.346	9.36 ± 0.360	[26]
Eu-SH-1	2	0.006 ± 0.001	29.187 ±2.794	9.36 ± 0.360	[26]
Eu-SH-2	2	0.005 ± 0.001	24.353 ± 2.968	9.36 ± 0.360	[26]
Eu-SH-3	2	0.005 ± 0.001	20.173 ± 2.735	9.36 ± 0.360	[26]
Eu-S-MNA-1	2	0.005 ± 0.001	34.940 ± 1.631	9.36 ± 0.360	[26]
Eu-S-MNA-2	2	0.005 ± 0.001	30.677 ±0.766	9.36 ± 0.360	[26]
Eu-S-MNA-3	2	0.004 ± 0.001	30.523 ± 4.219	9.36 ± 0.360	[26]
Pec*	1	0.02 ± 0.00	0.22 ± 0.02	0.20 ± 0.08	[2]
Pec-SH*	1	1.38 ± 0.06	207.60 ± 11.60	0.20 ± 0.08	[2]
Pec-S-MNA*	1	317.68 ± 33.90	453.95 ± 64.20	0.20 ± 0.08	[2]
Pec-S-MNAe	1	0.26 ± 0.15	7.44 ± 2.19	0.687 ± 0.08	[34]

All investigated polymers show mucoadhesive properties in their naturally occurring or in the industrially synthesized form. Crucial therefore are their numerous hydrogen bond forming functional groups like hydroxyl, carboxyl and amino groups [35, 44-46]. The adhesion thereby is provided by chemical, but non covalent interactions like ionic bonds, hydrogen bonds and Van-der-Waals bonds [35]. Results of mucoadhesive studies within this thesis confirmed the theory that the implementation of a thiol bearing moiety improves the mucoadhesive properties of the polymers. It was found that thiolated polymers are able to form covalent disulfide bonds with cysteine-rich substructures of mucus glyocoproteins. Thereby, bonds result from simple oxidation or disulfide exchange reactions [47]. These covalent bonds are considerable stronger than non-covalent bonds leading to the marked enhanced adhesive effect. As tensile studies showed, the total work of adhesion and the maximum detachment force were higher than for the corresponding non-modified polymers. For Eu-SH, three different derivatives with increasing amounts of attached thiol groups were investigated. Thereby, a direct correlation between the degree of thiolation and improvement of mucoadhesive properties could be shown. However, it is also reported that capabilities of thiomers are limited, mainly for two reasons: Above all, the reactivity of the thiol group is pH dependent, as the reactive form, the thiolate anion, has to be formed for thiol-disulfide reaction. Formation of the thiolate anion depends on the pH value of the environment and the pka value of the thiol. In general, the formation of the thiolate anion is favored at higher pH values, hence, the reactivity of the thiolate ion and therefore the mucoadhesive properties are low at acidic conditions [48]. Next, the thiol group is very sensitive against oxidation, especially in aqueous environment at pH values above 5, close to their pka values. Disulfide bond formation occurs, intra and inter-molecular crosslinking takes place and limits the interaction with mucus glycoproteins. To overcome these hindrances, a new generation of thiomers, namely “preactivated” or “S-protected” thiomers are introduced. These second generation thiomers are characterized by modification of the thiol group by linking an aryl-thiol via disulfide bond formation. These aromatic ligands should be able to shield against crosslinking in aqueous environment and to improve reactivity of thiomers over a broader pH range [2, 13]. This novel technique combined with a well-chosen polymeric backbone made it possible to design tailor-made polymers for mucoadhesive drug delivery either on the gastric cavity or the intestinal mucosa. As shown in Figure 5, mucoadhesive properties of Pec-S-MNA are improved compared to Pec-SH under gastric conditions as well as under intestinal conditions, indicating a higher overall reactivity of the thiol group with the mucus layer. The mixed disulfide between the thiomer SH-group and the SH-groups of cysteine rich mucus substructures is favored against the thiomer-MNA bond as the more acidic thiol (MNA) is released during the thiol/disulfide exchange reactions [48]. Further, the values for TWA and MDF were found to be higher at acidic conditions than at pH 6.8 despite the fact that the formation of the essential thiolate-anion must

be favored at pH 6.8. This is explained by the pH dependent solubility of pectin. The solubility of the acidic carbohydrate increases with increasing pH. At pH 6.8, the tablets start to dissolve hampering the interactions of the polymer with the mucus layer on the surface of the mucosa.

The pH dependent solubility of polymers is employed in pharmaceutical research for years for controlled release. Eudragit® L 55-100 shows solubility only at pH ≥ 5.5 and has been widely used as acidic-resistant tablet coating. Within this thesis, this property was used to create another preactivated thiolated polymer which exhibits its mucoadhesive potential in the intestine (Figure 4B). At acidic conditions, the polymer is hardly hydrated and no chain interpenetration between the mucus and the polymer is possible. The hydration process is sufficient for the development of fully interaction of both components. Due to the implementation of the hydrophobic MNA ligand, solubility of the derivative is limited at neutral and basic pH values as well but the polymer is still able to take up water. The unmodified derivative is dissolved comparatively fast at pH values above 5.5 [35].

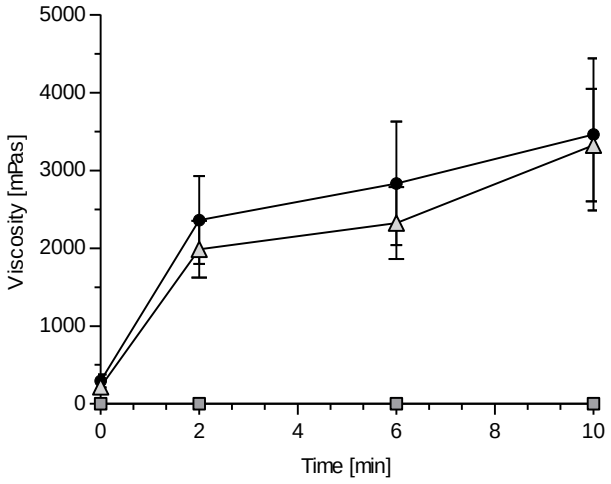


Figure 6.
Graph shows the increase of viscosity of mixtures from freshly collected mucus with polymer-solutions (1.5 % m/v) (2:1) over the first 10 minutes. Viscosity was determined with a plate-plate viscometer RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) at 1 Hz, dynamic oscillatory measurements were carried out. The shear rate was set to 50 s^{-1}, temperature to 37.0 ± 0.1 °C and the gap to 0.5 mm. Graph indicates means ± StDev of at least three experiments.
Thiolated alginate (-●-), S-protected thiolated alginate (-▲ -) and unmodified control (-■ -) after the addition of porcine intestinal mucus [24].

Results of the rheological synergism method confirm the enhancement of interactions between polymer and mucus after implementation of a thiol bearing ligand. For all thiolated polymers an increase of viscosity was quantified after intermingling with mucus. The resulting viscosity increased with increasing amount of thiol groups. Resulting viscosity of preactivated thiomers was higher after the addition of mucus but the increase was not as significant as for the thiolated polymers and therefore the benefit of the preactivated thiomers compared to non preactivated thiomers is not as obvious as regarding results of tensile studies.

The resulting dynamic viscosity of polymer/mucus mixtures is supposed to result from several interaction like chain entanglements, conformational changes and chemical bonds [42, 47]. However, physical properties of the polymers like degree and rate of hydration, cohesiveness and solubility do not come into account within the rheological synergism method and explain the different outcomes for the two set-ups.

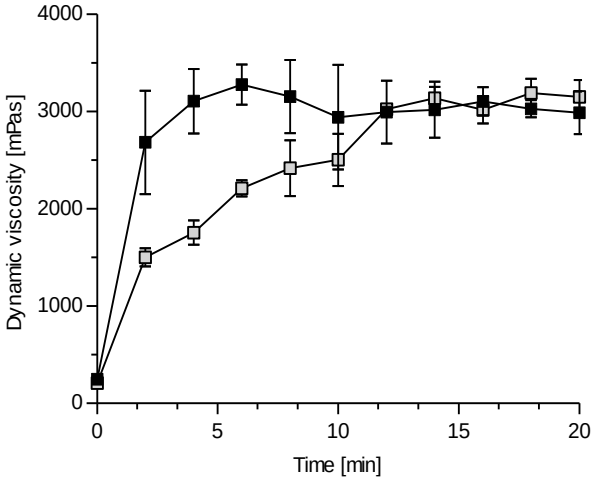


Figure 7.
Graph shows the increase of dynamic viscosity of thiomer solutions (1.5 % m/v) after incubating with two different concentrations of H_2O_2, Alg-SH with 60 nmol H_2O_2 (-■ -) and 30 nmol (-■ -) at predetermined time points. Indicated values are means ± StDev of at least three experiments. Values are determined with a plate-plate viscometer RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) at 1 Hz, dynamic oscillatory measurements were carried out. The shear rate was set to 50 s^{-1}, temperature to 37.0 ± 0.1 °C and the gap to 0.5 mm [24].

3.1.4 Gel formation and sensitivity against oxidation

Thiomers tend to gel formation in aqueous environment especially at pH values above 5, as there the deprotonation of the thiol group is favored. The result is a highly cross-linked polymer with intra- and intermolecular disulfide bonds. The gel sol transformation can be measured rheological, determining the loss tangent. A loss factor (tan δ) < 1 indicates gel formation whereat a loss factor > 1 indicates the sol state [49]. The ability of cross-linking disulfide formation of thiomers can be utilized for gel formulations which are on demand e.g. for buccal or vaginal applications. Further, the *in situ* gelling properties can be used for eye drop formulations [50, 51]. Without the addition of any oxidant like hydrogen peroxide, the formation of a high viscous gel due to disulfide bond formation can be observed after several hours. The addition of H_20_2 leads to an increase of viscosity within a few minutes (Figure 7). Technically, all thiomers show a gel formation after hydration with a loss factor < 1 but resulting viscosity is not always in a satisfying range. A 1 % solution of Pec-Cys revealed a tan δ of 0.29 ± 0.06 with a dynamic viscosity of 1.38 ± 0.06 Pa*s. For the corresponding preactivated thiomer, a significantly higher viscosity was found at the same concentration, 317.68 ± 33.90 Pa*s with a tan δ of 0.35 ± 0.03 [27]. Herein, the increase of viscosity is not only to be explained by disulfide bond formation. Actually, there should be less free thiol groups for inter-

and intramolecular crosslinking available. This increased viscosity is rather to be explained by the heightened hydrophobicity due to the implementation of the aromatic ligand as it was shown that the viscosity of polymers can be increased with hydrophobic modification [29]. Because of the enhanced hydrophobicity, solubility is decreased and therefore the gel state is reached at lower concentrations.

However, the easy oxidation of the thiolated polymers is a huge drawback regarding long time storage of a liquid or semi-solid formulation. Crosslinking of the polymer would not only alter the viscosity of the formulation, furthermore, the decrease in free thiol groups leads to a loss of mucoadhesive properties, as there are less free thiol groups available for the disulfide exchange reaction. To overcome this problem, a designated "entirely S-protected thiomer" (Pec-S-MNAe) was developed within this thesis. As described above, a new synthesis pathway was developed to synthesize preactivated thiomers. In comparison to the original pathway leading to a degree of preactivation between 20 % and 74 %, the entirely protected thiomer exhibits no free thiol groups (Table 2). The resulting free thiol-groups of preactivated thiomers are still available for crosslinking leading to alteration of a liquid or semi-solid formulation. Figure 8 shows that the dynamic viscosity of Pec-S-MNAe was not affected by the addition of H_2O_2. In comparison, a Pec-SH derivative with the same degree of substitution showed a considerably higher viscosity after the addition of hydrogen peroxide. Thereby, a protective effect of the MNA-ligand against oxidation could be proven.

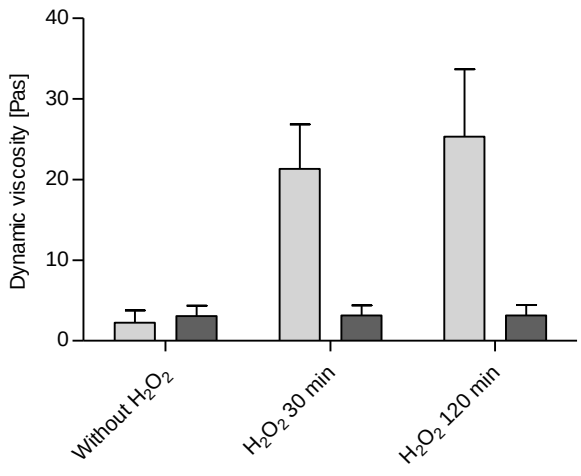


Figure 8.
Comparison of the dynamic viscosity of polymer solutions. Pec-SH (light grey bars) and Pec-S-MNAe (dark grey bars) with and without treatment with H_2O_2. 1 mL of polymer solution (1 % m/v in 0.1 M phosphate buffer pH 6.8) were oxidized with 20 µL H_2O_2 (0.3 % v/v), values were measured 30 and 120 min after the addition of H_2O_2. Indicated values are means ± StDev of at least 3 experiments [34]. Values are determined with a plate-plate viscometer RotoVisco RT20, Haake GmbH, Karlsruhe, Germany, at 1 Hz dynamic oscillatory measurements were carried out [24].

3.1.5 Drug loaded formulations based on mucoadhesive and multifunctional polymers

Based on results of characterization of the novel polymeric excipients synthesized within this thesis, different drug loaded formulations were prepared and investigated for their suitability in targeted drug delivery.

3.1.5.1 Gel formulation for buccal drug delivery

In the oral cavity, mucoadhesive gel formulations are first choice because of their convenient application and their prolonged retention time on the target site. Hydration of Pec-S-MNAg showed a high viscosity compared to the thiolated and unmodified pectin (Table 4) and therefore was promising for a gel formulation. In contrast to native pectin, gelling occurs spontaneously. Pectin and thiolated pectin are able to form gels as well, but pectin requires the presence of divalent cations like Ca^{2+} or other additives like sugar and acid for gel formation [52]. As described above, thiolated pectin can be gelled by oxidation of the thiol groups.

As model drug lidocaine was chosen, which is commonly used in the oral cavity as local anesthetic. Gels were prepared by adding 100 mg of lyophilized polymer to 5 mL of a lidocaine HCl solution 2 % m/v in phosphate buffered saline (PBS) pH 6.8 0.02 M and incubated overnight at 37 °C under shaking to obtain a gel stabilized by disulfide bond formation. To point out benefits of the preactivated thiolated polymer compared to the thiolated and native pectin, lidocaine loaded formulations were prepared the same way based on these polymers.

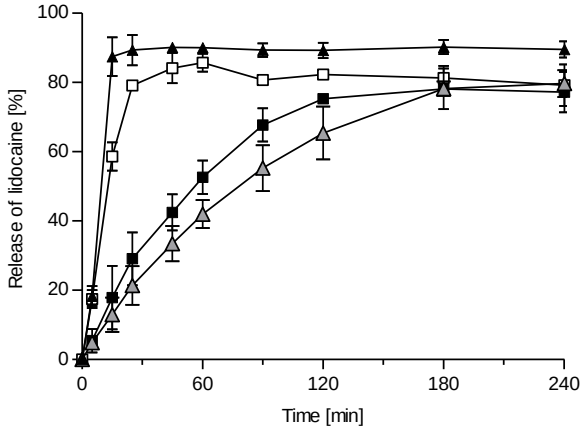


Figure 9.
Release profiles of lidocaine from formulations based on pectin, pectin-cysteine and pectin-cysteine-mercaptonicotinic acid. Tests were carried out using Franz diffusion cells and cellulose acetate membranes (0.2 µm). Pec (-□-), Pec-Cys (-■-), Pec-Cys-MNA (-▲-), lidocaine solution (-▲-). Indicated values are means ± StDev of at least three experiments [27].

The resulting drug loaded formulations of Pec-SH and Pec-S-MNA were transparent gels, the control formulation based on unmodified pectin was more fluid and turbid.

To investigate the release of the API from the formulation, studies with the Franz diffusion cell were carried out. Results are summarized in Figure 9. A sustained release for both thiomer-based formulations could be shown.

Within the rheological synergism method, interactions with mucus and Pec-Cys-MNAg in hydrated form were shown as described above. To confirm these findings for an API loaded formulation, an additional study was carried out. A set-up was chosen where results are influenced by mucoadhesion, cohesiveness of the gel and release profiles. The method was developed in the first place by Rao and Buri for nanoparticles [53]. The gels were loaded with sodium fluorescein (NaFlu) for easy detection. Next, the formulations were applied on freshly excised buccal mucosa which was placed in an incubation chamber (37 °C, 100 % humidity) in an angle of 45°. Then, the tissue with the formulation was rinsed with PBS pH 6.8 0.02 M at a flow rate of 1 mL/min. After predetermined time points, the remaining NaFlu on the buccal tissue was determined. As shown in Figure 10, the marker entrapped in the Pec-S-MNAg-based formulation lasts longest on the tissue, after 1.5 h 55 % of NaFlu still stuck on the tissue compared to 28.5 % for the Pec-SH-based formulation. After 4.5 h, remaining amount of the marker was about twice as high for the preactivated formulation compared to the non preactivated thiolated formulation.

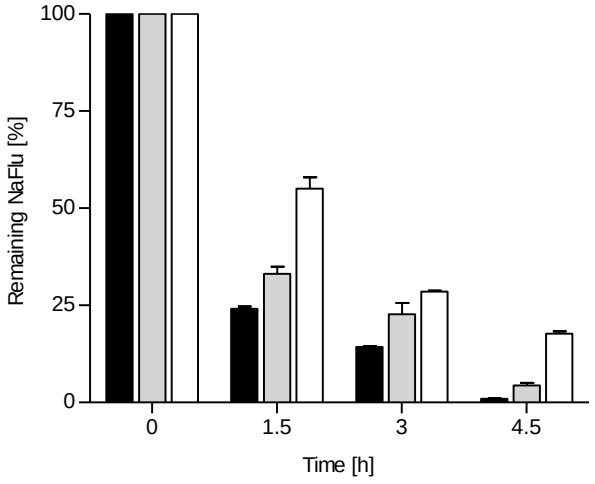


Figure 10.
Amount of NaFlu remaining on porcine buccal mucosa after application of NaFlu containing Pec- (black bars), Pec-SH- (grey bars), and Pec-S-MNA- (white bars) based formulations under continuous flow of PBS pH 6.8 0.02 M. Indicated values are means ± StDev of at least three experiments [27].

3.1.5.2 Gastroretentive drug delivery system

Besides enhanced mucoadhesion in general, one of the main benefits of the preactivated thiomers is their improved mucoadhesion under gastric conditions. Hence, as a logical next step, a formulation particular for drug delivery in the gastric cavity was developed. Gastro retention can not only be achieved by mucoadhesive delivery system, but also due to the size of the formulation [54]. Therefore, with Pec-S-MNA a polymer was chosen which exhibits high water uptake under acidic conditions resulting in an enormous volume gain (Figure 3) combined with a prolonged disintegration time at acidic conditions.

As model drug rosuvastatin calcium was chosen. To prepare drug loaded tablets, excipients were dissolved (30 mg/tablet) and solutions were spiked with rosuvastatin calcium (10 mg/tablet). Solutions were lyophilized and thereafter compressed into tablets of 40 mg. Release studies were carried out with a dissolution tester according the European Pharmacopeia. Release medium was HCl 0.1 M and the concentration of rosuvastatin was detected via RP-HPLC [55]. As shown in Figure 12, a sustained release under acidic conditions could be shown for the Pec-S-MNA formulation as well as for both prepared controls.

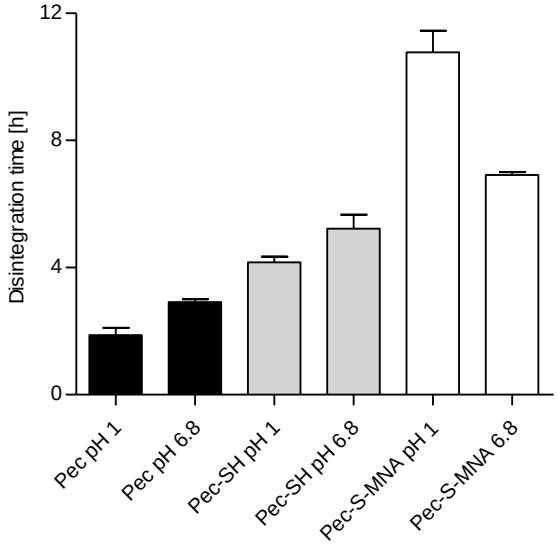


Figure 11.
Histogram shows the time until disintegration of test tablets based on Pec-SH, Pec-S-MNA and unmodified pectin. The time was determined at two different pH values. The endpoint was set when formulations were totally soaked with water and no visible core remained. Indicated values are means ± StDev of at least three experiments [2].

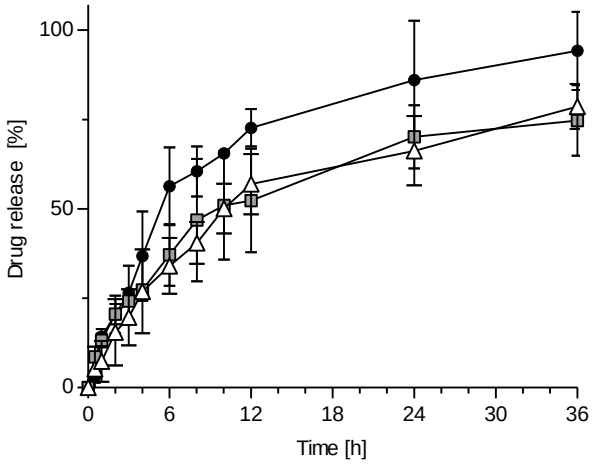


Figure 12.
Release profiles of rosuvastatin calcium from tablets based on unmodified pectin (-●-), Pec-Cys (-■-) and Pec-Cys-MNA (-Δ-) over 36 h. Experiments were carried out in 0.1 M HCl. Indicated values are means ± StDev of at least three experiments. Quantification occurred via RP-HPLC with a CN-RP column (240 x 4.5, 5 µm, Marchery-Nagel). The mobile phase consisted of acetonitrile:water pH 3.5 in a ratio of 40:60. The flow rate was set to 1.0 mL/min and the absorption was measured at 242 nm [2].

3.1.5.3 A lipohilic polymer backbone as matrix for lipohilic drugs

The typical thiomer has a hydrophilic polymer backbone. By implementation of hydrophobic ligands like aromatic thiol structures or by preactivating with these, hydrophilicity is decreased to a certain extent [56]. However, it was stated that lipohilic drugs are more easily entrapped into lipohilic carriers as in hydrophilic polymers. Within this thesis, a hydrophobically modified crosslinked poly(acrylic acid) derivative was thiolated and preactivated. The polymer, Carbopol® Ultrez 20 (AC1030), is characterized by substitution with hydrophobic C10-C30 alkyl chains reducing the hydrophilicity of the crosslinked poly(acrylic acid). These kind of polymers are usually employed as rheology modifier in cosmetics. Within this study, the thiolated and preactivated polymer was investigated as carrier matrix for lipophilic drugs. Therefore, drug load efficiency and the release profile were investigated using the lipohilic model drug valsartan. The unmodified AC1030, AC1030-SH, AC1030-S-MNA as well as a non-alkyl modified Carbomer (NA-Carbomer) were dissolved with 5 % of the lipophilic drug valsartan calculated on the total weight of the resulting tablets and compressed after lyophilzation. *In vitro* release studies were carried out by incubating the tablets in 100 mL phosphate buffer pH 6.8 0.1 M. Samples were taken at predetermined time points and analyzed via HPLC. To determine the total amount of entrapped valsartan employing the described procedure, tablets were dissolved completely and the concentration of released valsartan was analyzed via HPLC. Release profiles are plotted in Figure 13. Release profiles of all AC1030 derivatives showed a sustained release of the API whereat the unmodified carbomer showed a significantly faster release. Further, the entrapment capacity of AC1020 derivatives was close to 100 % whereas only about

65 % of the API was found in the NA-Carbomer formulation. Regarding release profile and drug loading efficiency, the thiolated and the preactivated thiolated polymers are not superior to the commercially available AC1030. Nevertheless, a 4.4-fold prolonged disintegration time for the preactivated and a 2-fold prolonged disintegration time for the thiolated AC1020-derivative was found, respectively. Further, a 3.4-fold and 2.8-fold improved TWA compared to AC1030 was found for the preactivated and the thiolated AC1030, respectively.

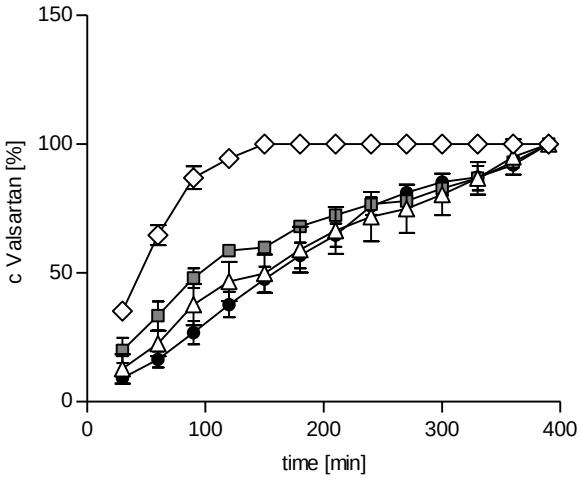


Figure 13.
Time related release of valsartan from 30 mg tablets of AC1030 (-●-), AC1030-cysteine (-■-), preactivated AC1030 (-Δ-) and NA-Carbomer (-◊-) in phosphate buffer pH 6.8. Indicated values are means ± StDev of at least three experiments. Samples were analyzed using a C-18 column (250 x 4.6, 5µm). The mobile phase was 50:50 water-trifluoroacetic acid : acetonitrile-TFA 0.1 %.
The quantification occurred at 260 nm [1].

3.2. Non-adhesive drug delivery systems

3.2.1 Self-nanoemulsifying drug delivery systems

Self-nanoemulsifying drug delivery systems (SNEDDS) are isotropic mixtures of oils, one or more surfactants and a cosurfactant. These mixtures are able to build nanoemulsions spontaneously when diluted in water or aqueous media like intestinal fluids. Resulting nanoemulsions are stable and the droplet size is in the nano-range [9, 57, 58]. These formulations attracted a lot of interest because of their relatively easy preparation technique and several benefits linked with incorporation of an API in the lipid phase. Improved bioavailability is stated especially for poor water-soluble drugs, the main application of these drug delivery systems. The solubility of these kinds of drugs can be enhanced due to an enlarged surface area and hence rate and extent of uptake can be improved [59, 60].

Apart from the use as solubilizer, these formulations show potential for other applications. Due to their hydrophobic surface their interactions with the mucus gel layer are comparatively low. Moreover, due to their small size and their ability of shape deformation SNEDDS are able to cross the mucus barrier [61].

Furthermore, depending on the excipients chosen, SNEDDS can enhance permeation of an API and they are believed to protect protein and DNA drugs against enzymatic degradation. The latter is often stated in the literature but by now, the effect was not proven yet. A reason therefore might be, that the incorporation of such hydrophilic drugs in a hydrophobic carrier is quite challenging.

It was within the aim of this thesis, to improve mucus penetrating properties of a SNEDDS formulation by the incorporation of a novel synthesized mucolytic lipid. Further, the protective effect of peptide and pDNA model drugs against enzymatic degradation was shown after incorporation into the lipophilic carrier system *in vitro* and *in vivo*.

3.2.1.1 The SNEDDS formulation

For all further studies, a SNEDDS formulation developed in a previous study was employed [62]. The mixture contains mid chain mono-, di- and triglycerides, a non-ionic emulsifier and propylene glycol as co-surfactant. The exact composition is depicted in Figure 14. By diluting in phosphate buffer pH 6.8 0.1 M a slightly bluish nanoemulsion was formed spontaneously. The mean droplet size was around 50 nm and showed a Gaussian distribution. Table 5 gives the mean droplet size and the polydispersity index (PDI) of the nanoemulsions with and without the addition of an API in different concentrations and media.

Cytotoxicity of this formulation was investigated via MTT and resazurin assay on a Caco-2 cell monolayer. In the tested concentration range of 0.05 % - 0.5 % SNEDDS over 3 hours diluted in MEM, viability of the cells maintained around 95 %.

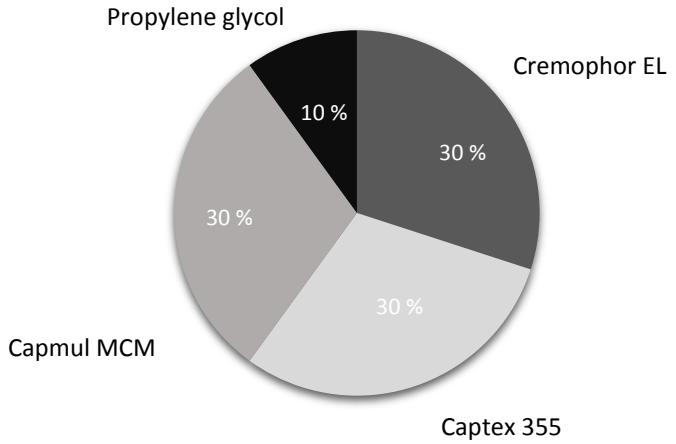


Figure 14.
Ingredients of the self-microemulsifying drug delivery system used within this thesis.
Cremophor EL (macrogolglycerol ricinoleate, non-ionic emulsifier HLB 12-14), Capmul MCM (glycery caprylate), Captex 355 (glyceryl tricaprylate) and propylene glycol as cosurfactant.

3.2.1.2 Enhancement of mucus penetrating properties

The mucolytic properties of thiol components were known for years. 1963 a study was published showing the crucial role of the free thiol group in sulfur-compounds for mucolytic activity. Different compounds were tested regarding their ability to lower the viscosity of gastric hog mucin [63] whereat substances like L-cysteine and N-acetylcysteine showed remarkable effects. Incorporation of such active compounds in a SNEDDS formulation could be interesting for the delivery of other APIs on the one hand but also be used as active agent *per se* on the other hand. For example, diseases like cystic fibrosis are linked with high viscosity sputum and treated with mucolytic agents. However, to incorporate and keep such mercaptanes in the lipid phase of a nanoemulsion, compounds with a high lipohilicity are required. Therefore, a C8 and a C12 alkyl chain were chemically modified. Octylamine was transformed chemically with S-acetylthioglycolic acid-N-hydroxsuccinimdeester (SATA) to a TGA-Octylamine conjugate by amide bond formation. Dodecylamine was modified to a thiobutylamidin-dodecylamine-conjugate (TBA-Dodecylamine) using iminothiolane, resulting in a mercaptane linked by an amidine bond. Resulting conjugates and synthetic pathways are illustrated in Figure 15.

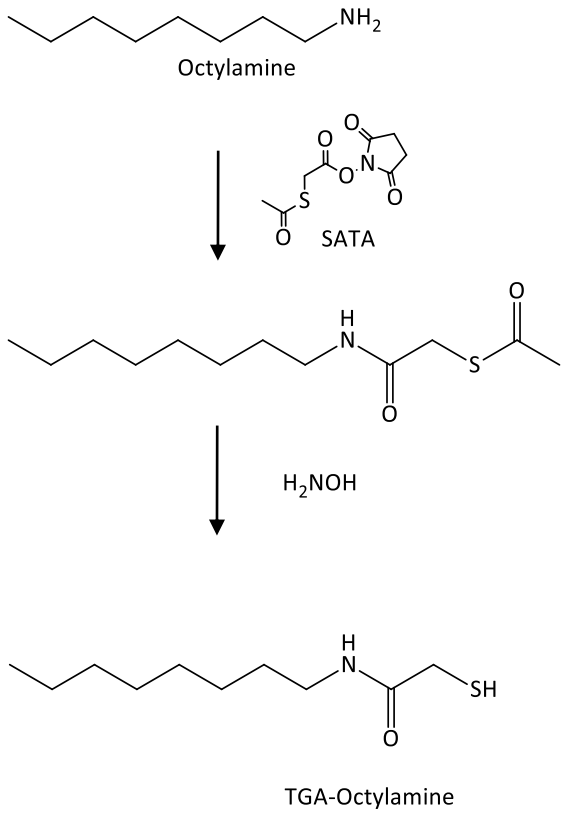


Figure 15 A.
Reaction scheme of thioglycolic acid-octylamine conjugate (TGA-Octylamide). In a first step, the primary amino group of ocytlamine reacted with S-acteylthio-glyocolic acid (SATA) to form an amide bond with the reagent containing a protected sulfhydryl. In a second step, hydroxylamine (H_2NOH) was used to deacetylate the sulfur, leading to the conjugate with a free thiol.

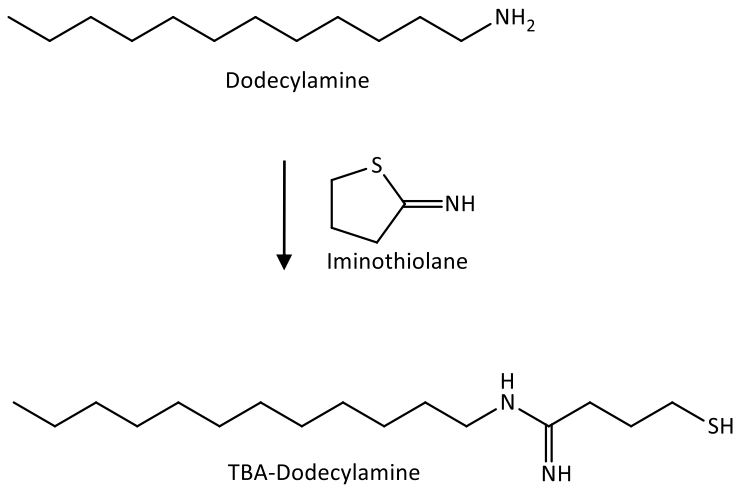


Figure 15 B.
Reaction scheme of thiobutylaminidine-dodecylamine conjugate (TBA-Dodecyl-amine). Traut's Reagent (2-iminothiolane) reacts spontaneously with the primary amino group of dodeclyamine under ring opening resulting in an amidine bond and with introduction of a sulfhydryl group to the alkyl chain.

The resulting conjugates were dissolved in the SNEDDS in a final concentration of 3 %. To investigate the mucolytic activity of the formulations, a transport test in rotating diffusion tubes was carried out [64]. Therefore, SNEDDS were loaded additionally with fluorescein diacetate (FDA) for detection. Silicone tubes were filled with freshly collected intestinal mucus and the ends were sealed with a fitting cap. Next, the 1 % nanoemulsions containing TBA-Dodeyclamine or TGA-Octylamine were added to the one end of the mucus filled tubes. After 5 hours of incubation while rotating at 37 °C, the tubes were frozen and cut into 10 slices. The concentration of the fluorescence marker was detected in each slice after hydrolysis of FDA with sodium hydroxide. As control, an "empty" SNEDDS nanoemulsion and an N-acetylcysteine solution were investigated. The FDA content in each slices showed the movement of the formulation in the mucus. Figure 16 gives the results for all investigated compounds. It could be shown that the addition of mercaptanes led to a faster movement of the FDA through the mucus compared to nanoemulsions without thiol components. The FDA from the "empty" SNEDDS formulation did not migrate into the mucus filled tube at all but stayed at the end where it was applied. The better penetrating of FDA from formulations containing mercatpanes is to be explained by the mucolytic effect of thiol-structures. As the glycoprotein oligomers of the mucus gel layer are mainly cross-linked by disulfide bonds, a disulfide exchange reaction with small molecules like the synthesized lipids leads to cleavage of the glycoprotein net and therefore, to a decreased viscosity. The decreased viscosity facilitates the penetration of the formulation and the API into the mucus layer.

Table 5. Mean droplets size and polydispersity index of nanoemulsions loaded with different APIs in different concentrations (SNEDDS in buffer in %, v/v) measured by dynamic light scattering method using a PSS NicompTM 380 DLS (Santa Barbara, CA, USA). PB = phosphate buffer pH 6.8 0.1M, MEM = minimum essential medium, Bis-Tris = Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan buffer pH 6.0 0.1 M, PDI = polydispersity index.

	Medium	SNEEDS % (v/v)	API	hours after preparation	size [nm]	PDI
1	PB	1 %	-	0	52.83 ±0.57	0.047 ± 0.005
2	PB	1 %	-	8	54.54 ± 1.41	0.048 ± 0.011
3	PB	1 %	-	24	55.75 ± 1.79	0.047 ± 0.016
4	PB	1 %	Lipofectin-pDNA	0	48.85 ± 1.91	0.045 ± 0.017
5	PB	1 %	Lipofectin-pDNA	8	48.33 ± 1.84	0.047 ±0.015
6	PB	1%	Lipofectin-pDNA	24	49.08 ± 2.09	0.064 ±0.004
7	PB	0.025 %	Lipofectin-pDNA	0	29.73 ± 8.26	0.059 ± 0.011
8	MEM	1 %	Lipofectin-pDNA	0	44.63 ± 4.30	0.351 ± 0.03
9	MEM	0.025	Lipofectin-pDNA	0	31.60 ± 8.26	0.350 ± 0.023
10	PB	1 %	leuprolide	0.5	52.56 ±0.61	0.100 ± 0.012
11	Bis-Tris	1 %	TBA-Dodecylamine	0.5	39.7	1.298
12	Bis-Tris	1 %	TGA-Octylamine	0.5	23.5	0.615

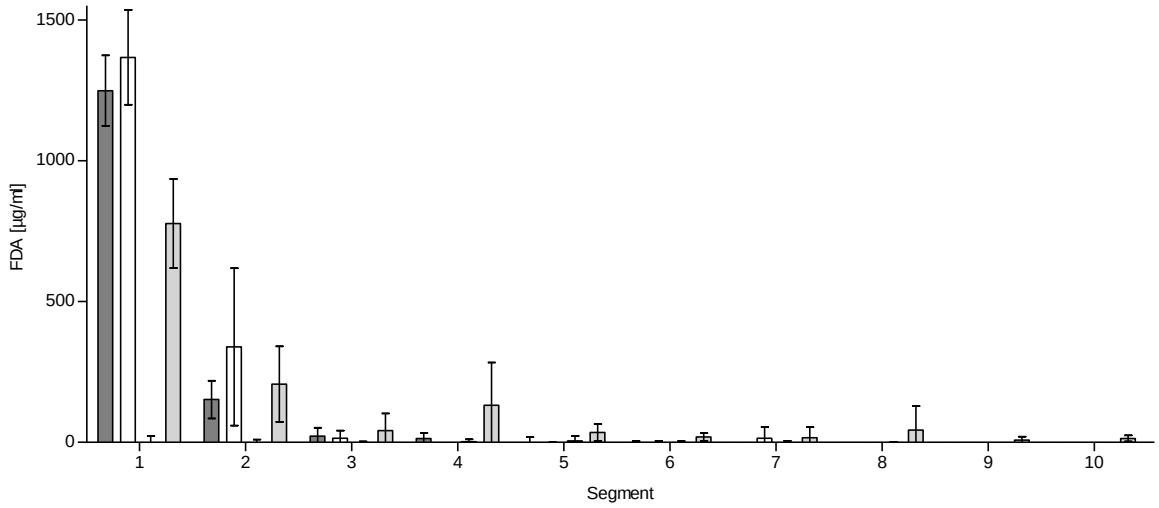


Figure 16.
Results of mucus permeation studies using the rotating diffusion tube assay. FDA loaded samples were loaded on one end of the mucus filled tube and the FDA concentration was measured after cutting the tube in 10 parts of equal size after 4 hours of incubating at 37 °C while tubes are rotated. Dark grey bars = TBA-Dodecylamine loaded SNEDDS, white bars = TGA-Octylamine loaded SNEDDS, black bars empty SNEDDS formulation, light grey bars: N-acetylcysteine solution.

3.2.1.3 Protection of a model plasmid against degradation via RNase I – in vitro evaluation

To prove a protective effect against enzymatic degradation, a model plasmid (pcDNA3-EGFP, Addgene plasmid 13031) encoding for the green fluorescent protein was incorporated in the SNEDDS formulation. The plasmid DNA (pDNA) was propagated in E. coli, harvested and purified using a Midi Prep kit from Qiagen. The pDNA was stored in TE-buffer (10 mM Tris-Cl, 1 mM EDTA) pH 8 and quantified using bisBenzimide H 33258 reagent.

To incorporate the extremely hydrophilic pDNA into the SNEDDS formulation, hydrophobic ion pairing was used to increase the lipohilicity of the API. The precipitation occurred by adding the transfection facilitator Lipofectin®. Lipofectin® is a mixture containing equal parts (m/m) of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and the uncharged dioleoyl-phophotidylethanolamine (DOPE) in a liposomal formulation (1 mg/mL). By mixing equal amounts of Lipofectin with pDNA in water, a cloudy suspension occurred due to complex formation between the cationic DOTMA and the negatively charged pDNA backbone. The precipitate could be obtained after centrifugation and dissolved in the SNEDDS formulation. Resulting API loaded SNEDDS with a concentration of 5 µg pDNA/1 µL SNEDDS were diluted to a 1 % v/v nanoemulsion in phosphate buffer 0.1 M pH 6.8. The Gaussian size distribution of pDNA loaded nanoemulsion is plotted in Figure 17.

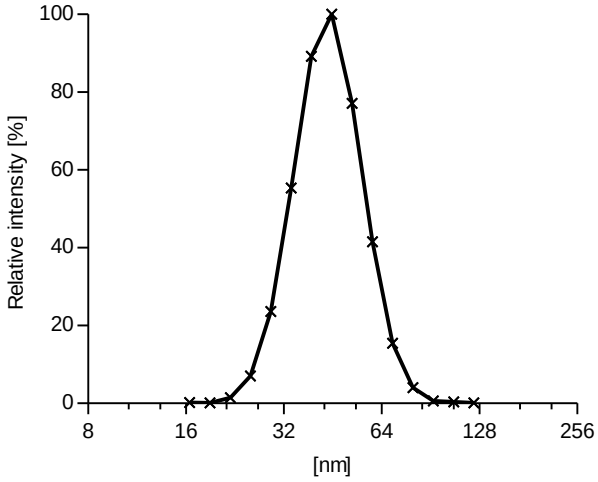
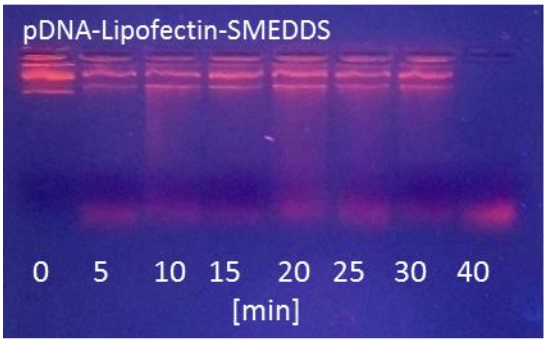
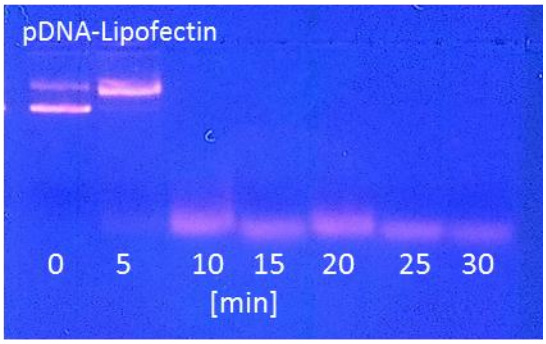


Figure 17.
Droplet size distribution of SNEDDS loaded with Lipofectin®-pDNA complex. Values are means of 3 measurements. The size was measured directly after preparation by dynamic light scattering using a PSS NicompTM 380 DLS (Santa Barbara, CA, USA) [65].

To prove a protective effect against DNase I, degradation studies were carried out employing a PROMEGA® RQ1 RNase-Free DNase kit. Therefore, 40 µl of the 1 % nanoemulsion containing in total 2 µg pDNA were mixed with 4 µL reaction buffer. DNase (1 µL) was added and the mixtures were incubated at 37 °C. The reaction was stopped at predetermined time points. As control, naked pDNA was diluted to the same concentration and treated the same way. Additionally, the pDNA-Lipofectin complex was resuspended to observe the impact of complex formation on degradation. The degree of degradation was determined by running a 1 % agarose gel prestained with GelRED® and detected under UV light. Resulting gels are shown in Figure 18.

The naked pDNA was degraded extremely fast, within the first 5 min, the total amount of pDNA was digested. Complexing of pDNA had only a minor effect on degradation. The complex was stable for the first 5 minutes but was completely degraded within the first ten minutes. In comparison, the pDNA incorporated in the lipid phase showed a clear sustained degradation profile over 30 min, indicating a protective effect of the SNEDDS due to incorporation of the API.





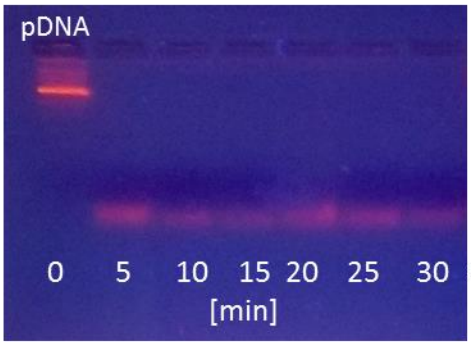


Figure 18.
Results of enzymatic degradation of Lipofectin®-pDNA complex loaded SNEDDS, pDNA-Lipofectin complex and naked pDNA via DNase I. The reaction was stopped at predetermined time points using a stop reagent. Agarose gel electrophoresis was carried out, the gel was prestained with GelRed® und detected under UV light [65].

Before transfection studies were carried out, toxicity studies were performed to observe the impact of tensides and cationic lipids on cell viability. Therefore, HEK-293 cells were seeded in a density of 20×10^4 cells/mL in minimum essential medium with Earls salts, 10 % fetal bovine serum and 1 % antibiotics in a 96 well plate. Cells were kept at 37 °C with exposure to 5% CO_2. After 24 h, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was carried out. Therefore, cells were incubated with empty SNEDDS and Lipofectin®-pDNA complex loaded SNEDDS for 4 hours. Thereafter, MTT was added and after 4 hours viability of cells were determined by the amount of to formazan converted MTT by living cells. As negative control, minimum essential medium was employed and as positive control Triton-X 100 was used. Investigated concentration range and results are given in Figure 19.

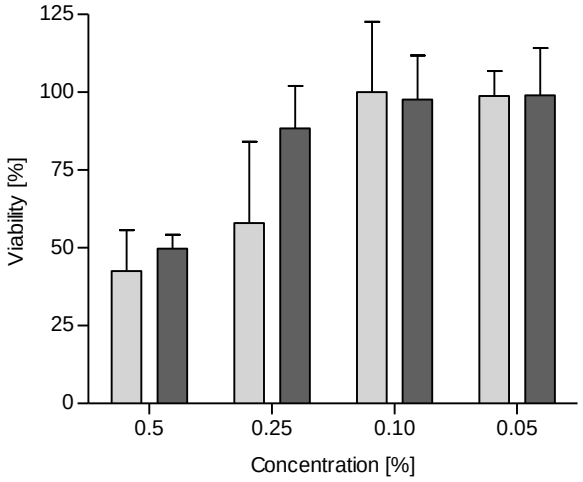


Figure 19.
Viability of HEK-293 cells after 4 h treatment with Lipofectin®-pDNA complex loaded SNEDDS (light grey bars) and "empty" SNEDDS (dark grey bars). The MTT assay was carried out with Triton-X 100 as positive control and minimum essential (MEM) medium as negative control. Viability is calculated in percent of values for MEM [65]. Indicated values are means ± StDev of at least three experiments.

Transfection studies were carried out to investigate the impact of incorporation in SNEDDS. Therefore, HEK-293 cells were kept under conditions as described above. For the transfection studies, cells were seeded in a density of 4 x 10^5 cells/well in a 12 well plate. After 24 hours, cells were incubated with Lipofectin®-pDNA SNEDDS 0.1 % v/v diluted in OPTI MEM® reduced serum medium. As control, a Lipofectin®-pDNA mixture was prepared according to instructions of the provider for ideal transfection using Lipofectin®. Further, cells were treated with naked pDNA in the same concentration. After 4 hours of incubation, samples were discarded, cells were washed and fed with minimum essential medium containing Earls salts, 10 % fetal bovine serum and 1 % antibiotics. After 48 hours, cells were harvested for GFP assay. Therefore, cells were lysed using 100 µL of a Triton-X 100 supplemented phosphate buffer pH 7.4 and a heat-cold-cycle. Afterwards the supernatant was transferred into a 96 well plate and fluorescence of the protein was measured at 488 nm excitation / 510 nm emission. As plotted in Figure 20, no significant difference could be shown for both formulations. This indicates that entrapment of the Lipofectin®-pDNA complex into SNEDDS does not lead to a loss in transfection efficiency. It can be assumed that over the time the pDNA is released from the SNEDDS and accumulated in the aqueous phase. This is confirmed by degradation studies carried out 24 hours after SNEDDS preparation where no difference in degradation profile between naked pDNA and the SNEDDS formulation was to observe, indicating a release of the pDNA.

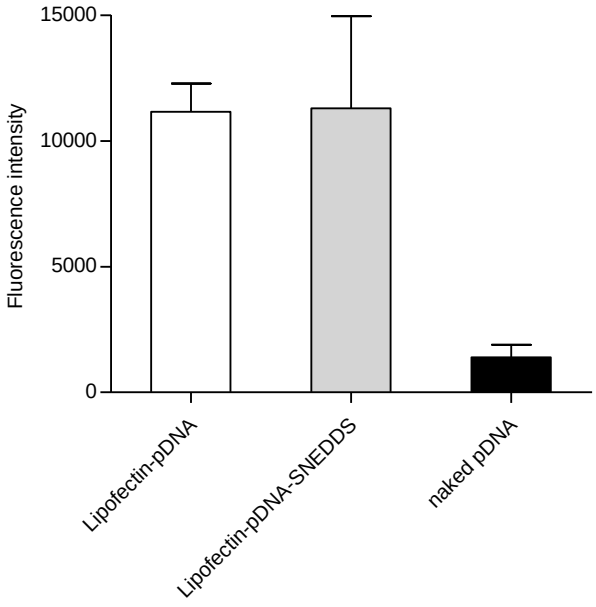


Figure 20.
Results of transfection study. Bar charts compares amount of GFP after transfection HEK-293 cells with the Lipofectin®-pDNA loaded SNEDDS formulation and with Lipofectin®-pDNA mixture solely. Cells were incubated for 4 hours with the pDNA samples and harvested for GFP assay 48 hours after transfection. As control, cells were transfected with naked pDNA [65].

3.2.1.4 Protection of a peptide drug against degradation via luminal enzymes – in vitro and in vivo evaluation

Within this thesis, the nonapeptide leuprolide was incorporated into the SNEDDS after hydrophobic ion pairing of leuprolide acetate with sodium oleate (sodium salt of (9Z)-octadec-9-enoic acid) whereat leuprolide oleate precipitates. The precipitate could be obtained after centrifugation. Analysis via HPLC revealed that the ion pairing with sodium oleate was not complete and therefore, the precipitate was a mixture of leuprolide acetate and oleate. However, the precipitated leuprolide was successfully incorporated into the SNEDDS in a concentration of 4 mg precipitate per gram SNEDDS. For further studies, a nanoemulsion with 1 % of drug loaded SNEDDS was prepared in phosphate buffer pH 6.8 0.1 M. Enzymatic degradation studies were carried out according to a method described previously [66]. Solutions of elastase, trypsin and α-chymotrypsin were prepared leading to an activity in accordance with physiological conditions [67]. The API loaded nanoemulsion as well as a leuprolide acetate control solution were combined with the enzyme solutions. To investigate the impact of the complex formation on the degradation, the resuspended precipitate was treated the same way. After predetermined time points the reaction was stopped with trifluoroacetic acid (TFA). Samples were analyzed via HPLC according to a method described previously [68]. It turned out that elastase had no impact at all on the nonapeptide.

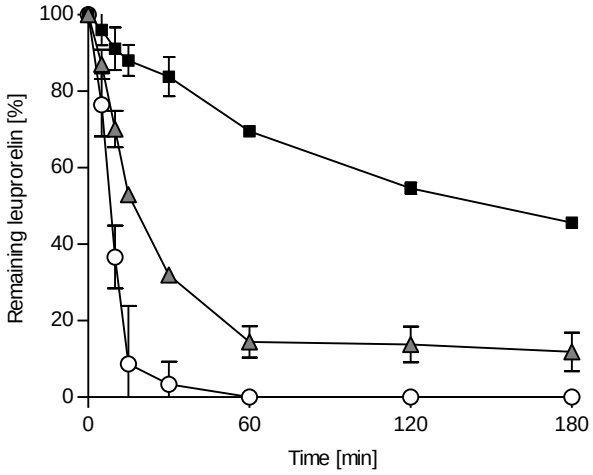
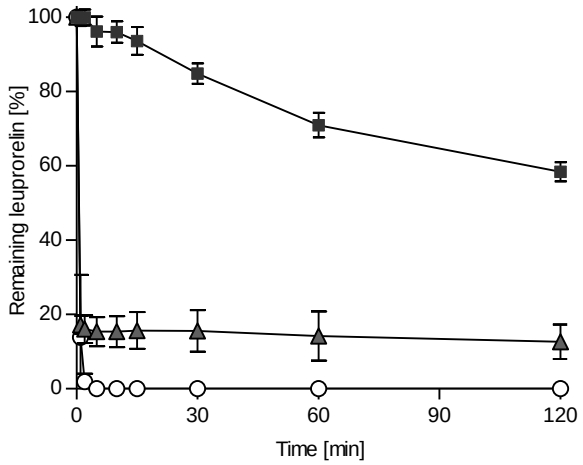


Figure 21.
Degradation profile of leuprolide by trypsin in an aqueous leuprolide acetate solution [-o-] and as Leuprolide-SNEDDS (1:3) emulsified in phosphate buffer pH 6.8 0.1 M. As the leuprolide precipitate incorporated into the SNEDDS contained leuprolide acetate and leuprolide oleate, degradation profiles of both ion pairs are indicated separately: leuprolide acetate [-▲-] and leuprolide oleate [-■-] in the nanoemulsion. Indicated values are means ± StDev of at least three experiments [69].

Figure 21 shows degradation profiles after treatment with trypsin. As the precipitate contained both, leuprolide acetate and leuprolide oleate, degradation curves for both salts are plotted separately. The leuprolide oleate salt showed a significantly sustained degradation, after 3 hours around 50 % were still unaltered whereas the leuprolide acetate control solution was digested totally within 60 min. Leuprolide acetate from the nanoemulsion formulation showed an incomplete degradation indicating a protective effect of the nanoemulsion. Figure 22 displays the degradation profiles after treatment with α-chymotrypsin. Degradation of leuprolide acetate control occurred extremely fast, within the first 5 minutes whereat around 60 % of leuprolide oleate in the nanoemulsion remained unaltered.

To prove this effect *in vivo*, animal studies were carried out with Sprague-Dawley rats to show an enhanced oral uptake. The Animal Ethical Committee of Vienna, Austria approved the protocol for the study which adhered to the Principles of Laboratory Animal Care. Tested SNEDDS formulations and controls are listed in Table 6. Each formulation was administered to 5 rats with a body weight of 200-250 g being starved for 2 hours before the experiment. Blood samples were taken at predetermined time points and treated with ice-cold acetonitrile to precipitate plasma proteins. Samples were concentrated under vacuum and analyzed via LC-MS according to a method published previously [69-71].

A



B

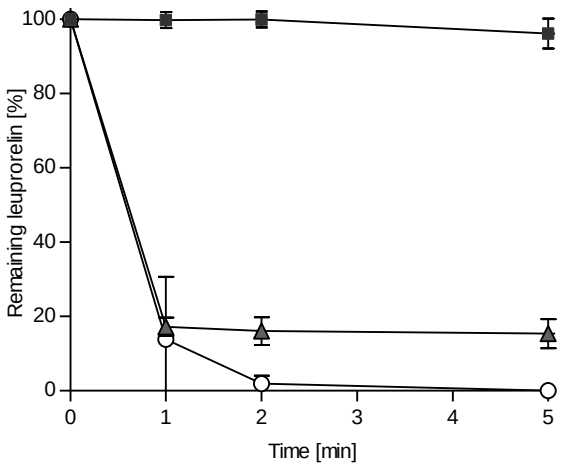


Figure 22A+B.
Degradation of leuprolide by α-chymotrypsin in an aqueous leuprolide acetate solution [-○-] and as Leu-SNEDDS emulsified in phosphate buffer pH 6.8 [-▲-] [-■-]. As the leuprolide precipitate incorporated into the SNEDDS contained leuprolide acetate and leuprolide oleate, degradation profiles of both salts are indicated separately: leuprolide acetate [-▲-] and leuprolide oleate [-■-] in the nanoemulsion. Figure 5B highlights the first 5 minutes of the degradation process due to the rapid metabolism by α-chymotrypsin. Indicated values are means ± StDev of at least three experiments [69].

Table 6. Formulations used for *in vivo* studies in male Sprague-Dawley rats. For every formulation five rats were investigated [69].

Formulation	Route of administration	Dose	Dosage form	Volume
Leuprolide acetate loaded aqueous solution	Iv	0.25 mg	Solution	250 µl
Leuprolide acetate loaded aqueous solution	Oral	1 mg	Solution	250 µl
Leuprolide oleate loaded aqueous suspension	Oral	1 mg	Suspension	250 µl
Leuprolide acetate loaded SNEDDS	Oral	1 mg	SNEDDS	250 µl
Leuprolide oleate loaded SNEDDS	Oral	1 mg	SNEDDS	250 µl

Pharmacokinetic parameters and resulting plasma-concentration/time curves are provided in Table 7 and Figure 23, respectively. A significant improved oral uptake of leuprolide could be shown when administered incorporated in SNEDDS. The leuprolide SNEDDS formulation showed an about 10-fold higher C_{max} compared to the oral leuprolide acetate control solution. Further, the leuprolide oleate SNEDDS and the leuprolide acetate SNEDDS control showed improved uptake compared to formulations without SNEDDS. This is to be explained by the permeation enhancing effect due to the SNEDDS composition which was demonstrated *in vitro* and *ex vivo* in a previous study [62].

The *in vivo* study demonstrated that the hydrophobic ion pairing and subsequently incorporation into SNEDDS led to an improved oral uptake compared to leuprolide acetate SNEDDS and to leuprolide acetate solution. This effect is likely caused by both, the protection against enzymatic degradation and the permeation enhancing properties of the formulation shown *in vitro*.

Table 7. Pharmacokinetic parameters calculated after i.v. and oral administration of investigated formulations in rats. Absolute bioavailability (BA) was calculated with reference to i.v. injection and indicated values are means of five rats. (* $p<0.05$ compared to leuprolide acetate solution; ^{ns} not significant compared to leuprolide acetate solution) [69].

Delivery System	**$AUC_{0\text{-last}}$ [min·ng/mL]**	**C_{max} [ng/mL]**	**T_{max} [min]**	**Absolute BA [%]**
I.v. solution	144653	16226.7	-	-
Leuprolide acetate solution	426	5.98	30	0.074
Leuprolide oleate suspension	0	-	-	-
Leuprolide acetate SNEDDS	2796 ^{ns}	15.66 ^{ns}	30	0.483
Leuprolide oleate SNEDDS	7385 *	51.68 *	30	1.276

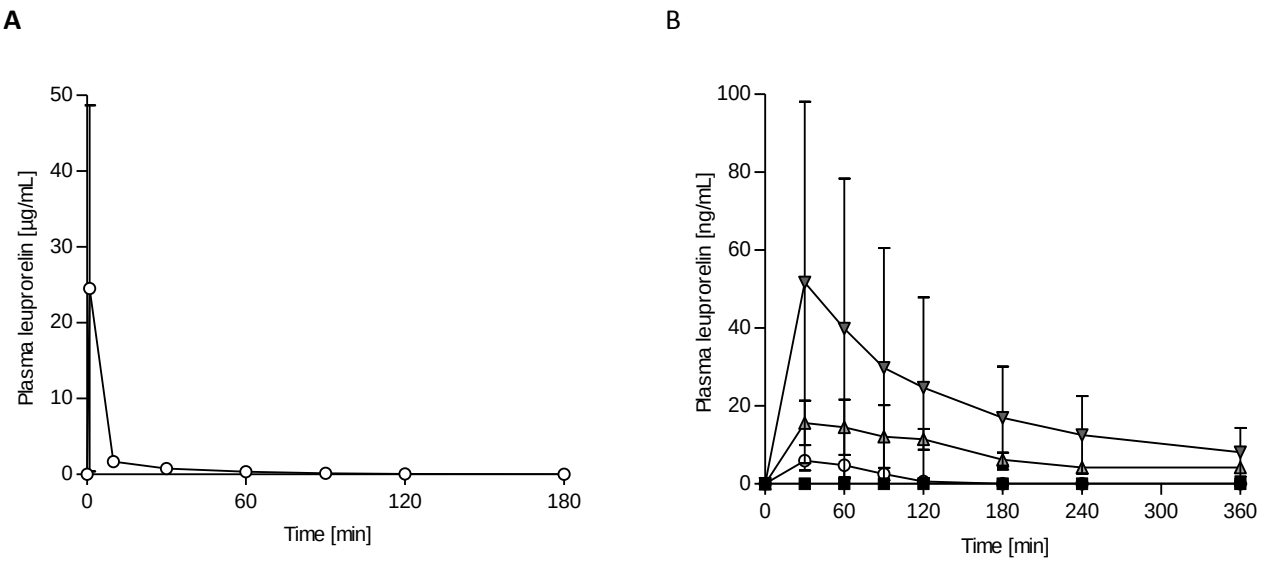


Figure 23A+B.
(A) Plasma concentration curve of leuprolide after intravenous injection of leuprolide acetate to rats (Dose = 1 mg/kg). (B) Plasma concentration curves of leuprolide after oral administration of a leuprolide acetate solution [-o-], a leuprolide oleate suspension (-■-), leuprolide acetate SNEDDS (-▲-) and leuprolide oleate SNEDDS (-▼-) to rats (dose = 4 mg/kg). Indicated values are means ± StDev of five rats. Leuprolide oleate SNEDDS (-▼-) are significant different compared to all other ($p<0.05$); leuprolide acetate SNEDDS (-▲-) are not significant different compared to leuprolide acetate solution [69].

3.2.2. Mucus-penetrating nanoparticles

Nanoparticles are intensively researched as they show promising attributes like easy preparation, easy incorporation of drugs, sustained release, protection against enzymatic degradation and improved bioavailability. However, the efficiency of nanoparticles could be enhanced if their permeation through the mucus gel layer was accelerated and the epithelium could be reached before mucus turnover occurs. Attempts have been made improving the mucus penetrating properties of nanoparticles by modification of the particle surface. But, even if the surface is optimized regarding charge und hydrophilicity, the passage is often hampered by the size of the particles [72]. Therefore, one issue within this thesis was, to combine polymeric nanoparticles with a mucolytic enzyme to help the potential drug carrier to overcome the mucus net by local disrupting of the gel layer by cleavage of protein structures.

After an enzyme activity screening including papain, bromelain, trypsin and α-chymotrypsin in regards to their mucolytic activity on freshly collected porcine intestinal mucus, papain was chosen for further studies [73].

About the mucolytic properties of papain was reported before. Papain is an enzyme showing a broad specificity and preferable catalyzes the cleavage of peptide bonds after basic amino acids [74].

Nanoparticles were prepared by ionic gelation method as illustrated in Figure 24. Thereby, complex formation occurred between poly(acrylic acid) and the enzyme papain. As control, poly(acrylic acid) (PAA) particles were prepared by precipitating the polymer with Ca^{2+} as cross-linker. Resulting nanoparticles were investigated regarding their size and zeta potential, results are given in Table 8.

For diffusion studies, particles were loaded with FDA by incubating with a FDA solution in acetonitrile. Thereafter, particles were separated from unbound FDA via centrifugation and resuspended. After the addition of 1 % m/v trehalose to avoid particle aggregation, the particle suspensions were freeze dried. The total amount of entrapped FDA was determined after sodium hydroxide treatment to convert FDA in fluorescent sodium fluorescein.

To determine the amount of immobilized enzyme in the nanoparticles, a Bradford assay was used to quantify the amount of protein entrapped. The test is based on Coomassei Brilliant Blue dye and carried out as described before [14].
Further, the activity of the bound enzyme was investigated using casein as substrate as described by Itoyama et al [75].

Table 8. Characterization of PAA and PAA-papain nanoparticles regarding mean particle diameter, zeta potential, loading capacity and encapsulation efficiency. Indicated values are means ± StDev of three experiments [73].

	Mean particle diameter [nm]	Zeta potential [mV]	Loading capacity [%]	Encapsulation efficiency [%]
PAA particles				
Blank	162.8 ± 9.7	-16.0 ± 3.1	-	-
FDA labeled	191.4 ± 11.3	-14.2 ± 4.0	13.4 ± 4	3.6 ± 0.7
PAA-papain particles				
Blank	175.6 ± 12.1	-11.7 ± 5.0	-	-
FDA labeled	198.5 ± 7.9	-10.4 ± 1.1	11.8 ± 2	2.4 ± 0.4

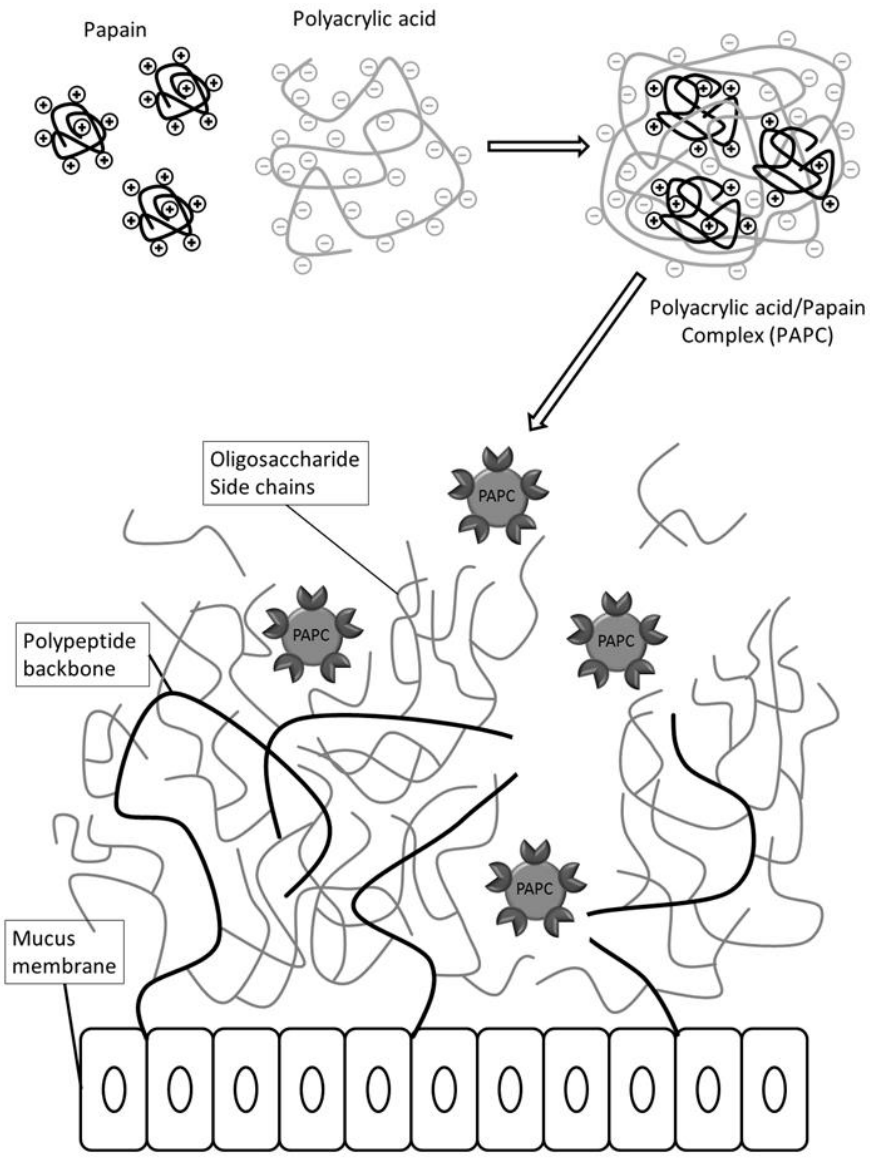


Figure 24.
Schematic representation of the complex formation between the enzyme papain and the polymer poly(acrylic acid) and the following mucus permeation of resulting mucolytic particles (PAPC) [73].

The proteolytic activity of the PAA-papain nanoparticles was investigated using two different methods. First, the loss in mucus viscosity after treatment with the particles was investigated, then, diffusion studies were carried out.

For rheological investigations, porcine intestinal mucus was incubated with PAA-papain particles and with the PAA control particles. After 1 h of incubation at 37 °C, the viscosity was measured using a plate-plate set-up.

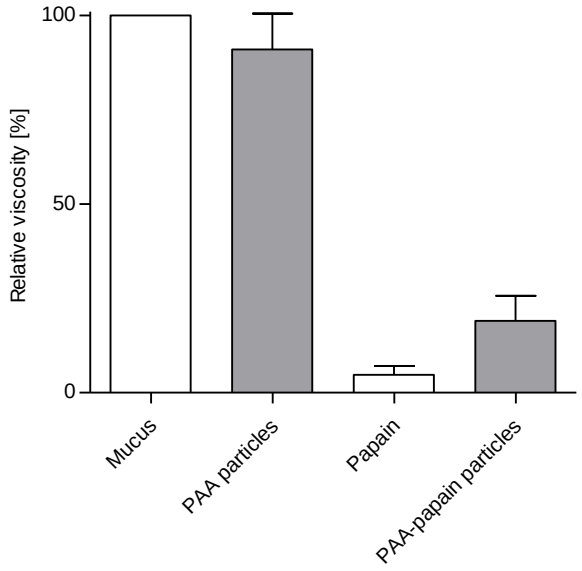


Figure 25.
Influence of papain, PAA and PAA-papain particles (0.2 %; w/v) on the viscoelastic properties of freshly excised intestinal mucus (pH 6.5) over 1 h of incubation and 37 °C. Values are means of at least 3 experiments ± StDev. Results are plotted in percent of initial viscosity of mucus. Values are determined with a plate-plate viscometer, RotoVisco RT20, Haake GmbH, Karlsruhe, Germany. Indicated values are means ± StDev of at least three experiments.
PAA: particles prepared with poly(acrylic acid) and Ca^{2+}, PAA-papain particles: poly(acrylic acid)-papain particles [73].

As plotted in Figure 25, a significant loss in viscosity compared to untreated mucus could be demonstrated after incubation with mucolytic particles and native papain. In comparison, PAA control particles had no significant impact on the viscosity of mucus.

The rotating diffusion tube assay shows the penetrating capacity of the PAA-papain nanoparticles into mucus. Tubes were prepared and treated as stated in section 3.2.1.2. After loading with FDA labeled particles, the tube was incubated for 24 hours. After freezing, the tube was cut into 20 slices and treated with sodium hydroxide to detect the amount of FDA. As plotted in Figure 26, PAA particles without papain remained primarily in the front segments. FDA from papain-complexed particles was found to migrate deeper into the mucus. It could be shown that the papain-particles enable a faster and deeper transport of an API through the mucus gel layer.

The faster transport of FDA and the loss in viscosity of mucus samples is based on the mucolytic properties of the papain. A local splitting of mucus glycoproteins leads to a disruption of the mucus layer and facilitates the particles transport. Hence, administered drugs may reach underlying epithelia before mucus turnover limiting efficiency of mucoadhesive particles.

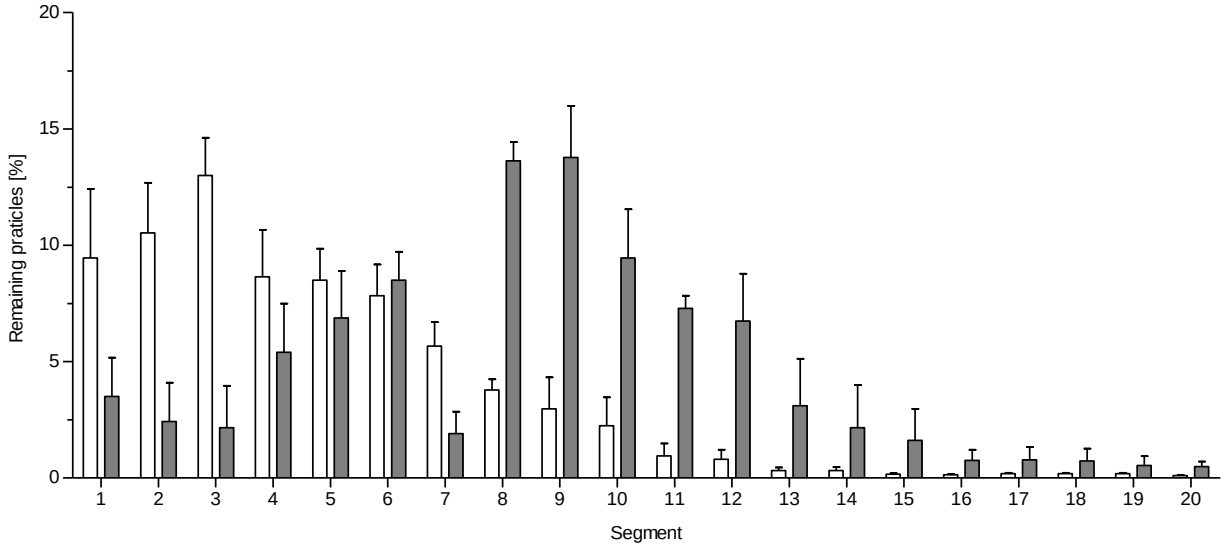


Figure 26.
Particle diffusion experiments of PAA (white bars) and papain modified PAA particles (grey bars) in mucus employing mucus filled silicon tubes over 24 h of incubation at 37 °C and pH 6.5. Indicated values are means ± StDev of at least three experiments [73].

As mentioned above, small mercaptane molecules are able to lower viscosity of the mucus gel due to the breakage of the disulfide bond stabilized network of mucus glycoproteins. In contrast, polymeric thiol structures are adherent to the mucus gel layer, resulting in a higher viscosity due to intensive chemical interaction with (sulfhydryl) mucus substructures. To investigate the impact of a thiol bearing backbone on the diffusion behavior of the papain modified particles, a follow-up study was carried out with thiolated poly(acrylic acid). Thereby, two different modified polymers were synthesized. First, papain was covalently coupled to the poly(acrylic acid) via EDAC mediated amide bond formation as described previously [76]. The amount of immobilized enzyme on the PAA-g-papain conjugate was quantified via Bradford assay and the activity of the enzyme was determined using casein [75].

Next, the PAA-thiomer (PAA-SH) was synthesized by covalent attachment of cysteine. The reaction was mediated by EDAC according to a method described before [77]. Quantification via Ellmans' reagent revealed total amount of around 800 µmol attached thiol groups per gram polymer.

To prepare thiol containing papain particles, both modified polymers were dissolved and mixed in a ratio of 1:2 PAA-g-papain:PAA-SH. For particle formation, a calcium chloride solution was added dropwise under stirring. As control, particles based on the unmodified and the thiolated polymer were prepared. Further, papain containing particles were precipitated using solely PAA-papain crosslinked with Ca^{2+}. The size of all prepared nanoparticles was < 200 nm.

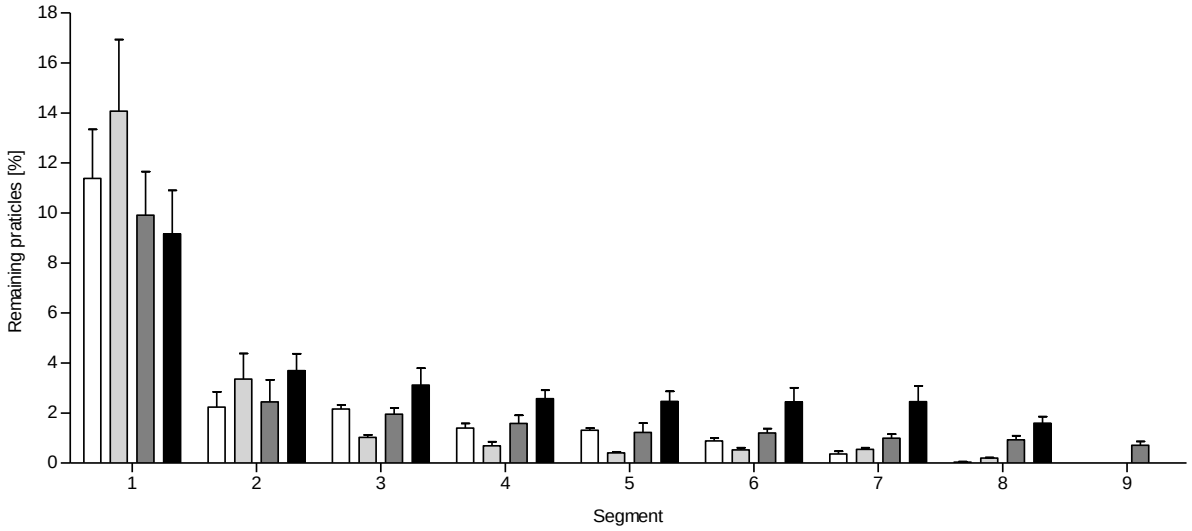


Figure 27.
Particle diffusion experiments of PAA (white bars), PAA-SH particles (light grey bars), PAA-g-papain particles (dark grey bars) and PAA-g-papain/PAA-SH particles in mucus employing mucus filled silicon tubes over 4 h of incubation at 37 °C and pH 6.5. Indicated values are means ± StDev of at least three experiments [78].

To investigate how the mucus penetration of an incorporated API is affected by this thiol- and papain-functionalized carrier system, diffusion studies employing the rotating tube method were accomplished. Therefore particles were loaded with FDA and the test was carried out as described above. After 4 hours of incubation at 37 °C, tubes were frozen and cut into 9 even slices. After alkaline treatment, the amount of FDA in the different segments was determined. Results are summarized in Figure 27. As expected, FDA from PAA-g-papain particles showed a deeper mucus penetration than unmodified PAA particles due to local cleavage of the mucus-network as described above. Furthermore, the amount of FDA from thiomer-based particles in the first section was higher than for other particles. This is explained by the intensive interactions of the thiol functionalized polymer and mucus substructures, as extensively described in section 3.1.3. The combination of thiol-modified and papain-grafted polymer led to a higher amount of FDA in sections 3-8 compared to all control particles indicating a more successful mucus penetration. Furthermore, the FDA distribution within these slices was more even compared to PAA-g-papain particles which show decreasing amount of FDA with increasing compartment number. The more steady distribution of FDA from PAA-g-papain/PAA-SH particles may result from a balanced manifestation of the properties of both involved PAA-derivatives, the mucoadhesive thiomer and the mucolytic PAA-papain. Papain facilitates mucus-penetration due to cleavage of the glycoprotein net. The thiomer adheres to the

gylcoproteins and may keep the incorporated model drug FDA in place. Moreover, the mucoadhesive properties of these particles may help to keep the carrier system *in vivo* on the target site.

The mucoadhesive properties of this carrier system were confirmed by a rheological study (Figure 28). After adding the polymer based particles, a significant increase of the viscosity was found after 2 hours for all formulations except for PAA-g-papain particles. The "rheological synergism" of mucoadhesive polymers with mucus due to various kinds of interactions is intensively described in section 3.1.3 [78].

In comparison to PAA-papain complex particles, the PAA-g-papain particles were not able to decrease the viscosity of the mucus in the tested concentration (Figure 25 and 28). This leads to the assumption that covalent attachment of the enzyme leads to a decrease in activity due to the permanent immobilization. Further, a steric hindrance seems plausible.

Nevertheless, it can be stated that the covalent attachment of the mucolytic enzyme papain reveals a sufficient mucolytic activity to facilitate the transport of an API through a mucus gel layer. Further, compared to PAA-papain complex particles where the enzyme is immobilized due to ionic interactions, a more steady diffusion profile could be observed. For rotating tube diffusion studies with PAA/papain complex particles a concentration of FDA in the middle-section was found. This might be explained by dissociation of the particles after a certain time leading to a parting of polymer and enzyme resulting in liberation of entrapped FDA.

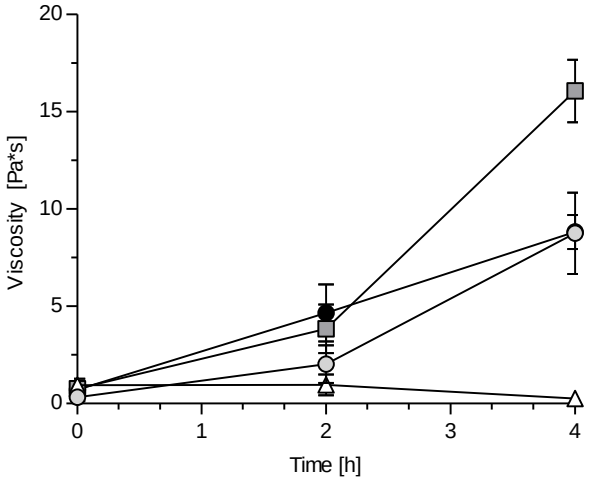


Figure 28.
Influence of papain, PAA (-●-) and PAA-SH (-■-), PAA-g-papain (-Δ-) and PAA-SH/PAA-g-papain particles (-▫-) (0.5 %; w/v) on the viscoelastic properties of freshly excised intestinal mucus (pH 6.5) over 4 h of incubation at 37 °C. Precipitation of all particles occurred via Ca^{2+}. Values are means of at least three experiments ± StDev. Measurements were carried out employing a plate-plate setup of RotoVisco RT20, Haake GmbH, Karlsruhe, Germany [78].

4. Conclusion

Within this thesis, oral drug delivery systems were developed and investigated. Thereby two different attempts were persuaded, mucoadhesive and non-adhesive, more precisely, mucus penetrating drug delivery systems. For the development of novel, tailor-made mucoadhesive drug delivery systems well established mucoadhesive polymers were chemically modified to improve their properties in drug delivery. Different synthetic pathways were investigated to immobilize thiol bearing ligands to the polymers. As these thiol bearing excipients are sensitive to oxidation in aqueous environment and revealing a pH depended reactivity, thiol groups were protected against oxidation and preactivated in regard to their ability of taking part in thiol/disulfide exchange reactions. Therefore, an aromatic mercaptane was covalently attached via disulfide bond formation. It could be shown that this hydrophobic modification led to a more pronounced mucoadhesive effect of the polymers and that sufficient reactivity of the thiol group in acidic environment could be achieved. Further, by attaching thiol groups to the polymer backbones, a higher cohesiveness of the excipient, a higher viscosity, a more pronounced water uptake capacity and a decrease in disintegration was found. All these effects were further enhanced by preactivation of the thiol groups. Furthermore, by synthesis of an entirely S-protected thiomer, alteration due to oxidation could be prevented. Considering the individual properties of synthesized excipients, three promising drug loaded formulations for oral drug delivery were prepared. Thereby, a mucoadhesive gel formulation for buccal drug delivery, a mucoadhesive gastric formulation with comparatively high swelling capacity and a formulation specialized for lipophilic drugs were prepared. Further, by choosing a polymeric backbone with pH dependent solubility, a polymer with pronounced mucoadhesive properties at intestinal conditions but not at gastric conditions was synthesized. All investigated formulations showed sustained release and good mucoadhesive properties on their target mucosa.

The mucosa is a suitable target for drug delivery as it keeps the formulation and the API close to the absorption site. Nevertheless, as the mucus gel layer also displays a hindrance for the APIs on their way to the epithelium, delivery systems facilitating mucus penetration were investigated in this thesis as well. It was possible to improve the mucus penetrating properties of a self-emulsifying drug delivery system by using a novel thiol modified C8 and C12 alkyl chain, as the thiol groups are able to break disulfide-linkage of mucus glycoproteins by disulfide exchange reactions. Further, a protective effect against enzymatic degradation of the SNEDDS formulation for both, peptide and DNA drugs was shown after hydrophobic ion pairing and subsequent incorporation into the lipid-based drug delivery system. Moreover, an *in vivo* study confirmed an improved oral bioavailability of hydrophobically ion paired leuprolide oleate after

incorporating in SNEDDS compared to leuprolide acetate control solution and compared to leuprolide acetate administered with SNEDDS but not incorporated in the lipid phase.

The mucus penetrating properties of polymeric nanoparticles could be significantly improved by complex-formation with a mucolytic enzyme. It was shown that the attached enzyme exhibit enough activity to cleave protein bonds of mucus glycoproteins and thereby lower the viscosity of the mucus gel considerably. By breaking down the mucus net, penetration of the model drug FDA could be improved significantly.

Moreover, the combination of a mucoadhesive thiomer with the papain functionalized polymer leads to a more even distribution of the incorporated model drug compared to both, covalent and non-covalent papain-functionalized particles.

Both, the novel developed and investigated mucoadhesive and mucus penetrating drug delivery systems within this thesis show great potential and significant improvements. It could be shown that after around 30 years of investigating mucoadhesive polymers possibilities are not yet exhausted and mucoadhesion can still be considered as a very promising strategy in improving bioavailability. Additionally, the comparatively new attempt of mucolytic drug delivery systems showed excellent results as well which for sure can compete with mucoadhesive drug delivery systems. The within this thesis developed strategies for the improvement of mucoadhesive and targeted drug delivery systems and the novel approaches to facilitate mucus penetration introduced deliver an interesting platform for further studies on the improvement of oral drug delivery systems.

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6. Curriculum Vitae

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7. List of Scientific Publications

7.1 Original research articles

- **Hauptstein S**, Müller C, Dünnhaupt S, Laffleur F, Bernkop-Schnürch A.
  Preactivated thiomers: Evaluation of gastroretentive minitablets. Int J Pharm. 2013;456:473-9.

- **Hauptstein S**, Hintzen F, Müller C, Ohm M, Bernkop-Schnürch A.
  Development and *in vitro* evaluation of a buccal drug delivery system based on preactivated thiolated pectin. Drug Dev Ind Pharm. 2013. [Epub ahead of print].

- **Hauptstein S**, Bonengel S, Griessinger J, Bernkop-Schnürch A.
  Synthesis and characterization of pH tolerant and mucoadhesive (thiol-polyethylene glycol) chitosan graft polymer for drug delivery. J Pharm Sci. 2014;103:594-601.

- **Hauptstein S**, Griessinger J, Rohrer J, Bernkop-Schnürch, A.
  Preactivated thiolated poly(methacrylic acid-co-ethyl acrylate): synthesis and evaluation of mucoadhesive potential. Eur. J. Pharm. Sci. 2014. [Epub ahead of print].

- **Hauptstein S**, Dezorzi S, Bernkop-Schnürch, A.
  Synthesis and *in vitro* characterization of a novel S-protected thiolated alginate. Under review.

- **Hauptstein S**, Prüfert F, Bernkop-Schnürch, A.
  Self-nanoemulsifying drug delivery system for pDNA – protection against enzymatic degradation. Under review.

- Hintzen, F, **Hauptstein S**, Perera G, Bernkop-Schnürch A.
  Synthesis and *in vitro* characterization of entirely S-protected thiolated pectin for drug delivery. Eur J Pharm Biopharm. 2013;85:1266-73.

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- Hintzen F, Perera G, **Hauptstein S**, Müller C, Laffleur F, Bernkop-Schnürch A.
  In vivo evaluation of an oral self-microemulsifying drug delivery system (SMEDDS) for leuprorelin. Int J Pharm. 2014. [Epub ahead of print].

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- Köllner S, Dünnhaupt S, Waldner C, **Hauptstein S**, Pereira de Sousa I, Bernkop-Schnürch, A. Papain functionalized thiomer nanoparticles. In preparation.

7.2 Oral presentations

- **Hauptstein S**, Hintzen F, Perera G, Bernkop-Schnürch A. *In vivo* evaluation of an oral self-microemulsifying drug delivery system (SMEDDS) for leuprorelin. 6th International Conference on Drug Discovery & Therapy, February 2014, Dubai, U.A.E

7.3 Poster presentations

- **Hauptstein S**, Müller C, Dünnhaupt S, Laffleur F and Bernkop-Schnürch A. Preactivated thiomers: evaluation of gastroretentive mini tablets. 5th ÖGMBT Annual Meeting in cooperation with the 5th Life Science Meeting of the Innsbruck Universities & Biophysics Austria. September 2013, Innsbruck, Austria.

- **Hauptstein S**, Müller C, Dünnhaupt S, Laffleur F and Bernkop-Schnürch A. Präaktivierte Thiomere als mucoadhäsive Hilfsstoffe. 47. Wissenschaftliche Fortbildungswoche der Österreichischen Apothekerkammer February 2014, Schladming, Austria

- Hintzen F, **Hauptstein S**, Perera G, Bernkop-Schnürch A. *In vitro* and *in vivo* evaluation of a self-microemulsifying drug delivery system (SMEDDS) for peptide drugs. 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. April 2014, Lisbon, Portugal

8. Appendix: Original Publications

Preactivated thiomers: evaluation of gastroretentive minitablets

Sabine Hauptstein, Christiane Müller, Sarah Dünnhaupt, Flavia Laffleur and Andreas Bernkop-Schnürch

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Int J Pharm. 2013 Nov 18;456(2):473-9. doi: 10.1016/j.ijpharm.2013.08.032. Epub 2013 Aug 30.

Abstract

The object of this study was to evaluate the potential of a recently developed preactivated thiolated pectin derivative as mucoadhesive excipient in drug delivery to the gastric cavity. Pectin (Pec) was chemically modified with L-cysteine (Cys). The free thiol groups of resulting thiomer were activated with 2-mercaptonicotinic acid (MNA) in order to improve stability and reactivity of attached thiol groups over a broad pH range. Multiunit dosage form properties of the resulting conjugate (Pec-Cys-MNA) were compared to unmodified pectin and the thiolated intermediate using rosuvastatin calcium as a model drug in loaded minitablets. Obtained results were compared with unmodified pectin and the intermediate thiolated pectin. Approximately half of attached thiol groups (507 µmol/g polymer) have been preactivated. Minitablets were evaluated regarding mucoadhesive properties, hardness, disintegration behavior, swelling characteristics and release of rosuvastatin calcium. Mediated by covalent bonds between the polymer and cysteine-rich subdomains in mucus, total work of adhesion increased more than 5-fold. The modification had no impact on hardness of compressed tablets but implementation of the aromatic ligand went along with reduction in hydrophilic properties. Disintegration time was prolonged more than 2-fold while water uptake capacity increased. Weight gain for Pec-Cys-MNA was at least 16-fold. Further, a sustained release of rosuvastatin calcium over 36 hours was determined. Neither biodegradability nor CaCo-2 cell viability was affected. The study shows that Pec-Cys-MNA is a promising excipient for the development of mucoadhesive gastric dosage form.

Key words: mucoadhesive drug delivery system, preactivated thiomers, swellable polymer, rosuvastatin, gastric

1. Introduction

In the 1980s mucoadhesive polymers were pioneered as potential excipients in order to prolong the residence time of drug delivery systems on all kind of mucosal membranes like gastrointestinal, nasal, pulmonary, ocular, buccal, rectal and vaginal mucosa. The increased contact time of the delivery system with mucus results in an increased drug concentration gradient on the absorption membrane and therefore in improved drug uptake [1]. However, in particularly in case of oral delivery mucoadhesive polymers could so far not convince as "super glue". This is likely caused by rapid mucus turnover and strong peristaltic moves pushing even adhering delivery systems forward. Since application in dry form is not possible the effect of adhesion by hydration cannot be utilized. Despite all these challenges there is encouraging data for gastric and intestinal mucoadhesive delivery systems available [2]. For instance, Akiyama et al. showed higher plasma levels of riboflavin and furosemide having their absorption window in the proximal segments of the GI-tract, in rats as well as in volunteers after administration in an adhesive microsphere formulation compared to application of same amount of drug in non-adhesive microspheres. The area under the plasma-concentration curve of furosemide was found to be 1.8-fold higher when administered in a mucoadhesive dosage form [3]. These results are of even higher value since they were achieved with non-covalent binding mucoadhesive polymers exhibiting much lower mucoadhesive properties than covalently binding polymers such as thiolated polymers (thiomers) forming disulfide bonds with cysteine-rich subdomains of the mucus gel layer [4-6].

So far, thiomers could not be applied for gastric mucoadhesive delivery systems as their thiol groups are not sufficiently reactive at low pH. Recently, a novel generation of thiomers – namely preactivated thiomers – was established [7]. These polymers are characterized by bearing a thiol group, which is activated by the attachment of pyridyl substructures via disulfide bond formation, resulting in higher activities of polymeric thiol groups over a broader pH range [7]. According to these developments it can be regarded as logical next step to utilize preactivated thiomers for mucoadhesive gastric drug delivery systems. The aim of this study was to evaluate the potential of a promising candidate out of this new class of modified polymers in regards for usage in mucoadhesive systems for drug delivery in the gastric cavity. As a hydrophilic polymeric backbone the biodegradable fiber pectin was chosen and to activate attached thiol groups 2-mercaptonicotinic acid was utilized. The synthesized excipient was examined in terms of mucoadhesive properties, hardness of compressed minitablets, disintegration time, swelling capacity and release of incorporated rosuvastatin calcium. Further, biodegradability and cytotoxicity of the excipient was examined. Since pH in the stomach fluctuates over a wide range investigations were carried out in acidic and neutral environments. As model drug rosuvastatin calcium, a HMG-CoA reductase inhibitor that

is used in treatment of dyslipidemia, was employed and incorporated into mini tablets, to generate a multi-unit dosage form. Like other statins, the bioavailability of rosuvastatin is low. The absolute oral bioavailability of the sparingly soluble drug is only about only 20 % [8, 9]. By now, several attempts have been made to improve bioavailability of different statins like fluvastatin, simvastatin and atorvastatin calcium in gastro retentive systems [10]. Besides enhanced uptake, modified release formulations of HMG-CoA reductase inhibitors may lead to reduced incidence of serious side effects like rhabdomyolysis. Therefore, rosuvastatin is a promising candidate for improved bioavailability by gastroretentive delivery systems [11].

2. Materials and Methods

2.1. Materials

Lemon pectin (degree of esterification approximately 75 %) was obtained from Herbafood Ingredients GmbH, Weder, Germany and 2-mercaptonicotinic acid 98 % (MNA) from ABCR GmbH & Co KG, Karlsruhe, Germany. L-Cysteine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), hydrogen peroxide, dialysis tubes (MWCO 12 kDa), glutathione (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), pectinase from *Aspergillus aculeatus,* resazurin, phosphoric acid 85 % and acetonitrile HPLC grade were purchased from Sigma Aldrich, Austria. Rosuvastatin calcium was obtained from CHEMOS GmbH, Germany. Water HPLC gradient grade was purchased from Fisher Chemical, United Kingdom. All other reagents used were of analytical grade.

2.2. Synthesis of pectin-cysteine-mercaptonicotinic acid

2.2.1. Synthesis of pectin-cysteine conjugate

Thiolated pectin (Pec-SH) was synthesized by covalent attachment of L-cysteine hydrochloride via amide bond formation according to a method published previously [12]. Amide bond formation was mediated by EDAC [13]. Briefly, 1.5 g of pectin were dissolved in demineralized water and carboxylic acid moieties were activated by addition of 1.5 g of EDAC. After 1 h of incubation 3 g of L-cysteine hydrochloride (Cys) were added. The pH of all components was adjusted to 4.5 using 5 M NaOH. The mixture was stirred for 5 hours. The product was purified via dialysis and lyophilized for 2 days under reduced pressure.

2.2.2. Synthesis of pectin-cysteine-2-mercaptonicotinic acid

The thiomer was preactivated according to a method described previously [7]. First, the dimer of 2-mercaptonicotinic acid was gained via oxidative coupling of the monomer using H_2O_2. The product (2,2'-dithiodinicotinic acid) was freeze-dried for 2 days under reduced pressure. The aromatic ligand was attached covalently by disulfide bond formation. 200 mg of Pec-Cys were dissolved in 50 mL of demineralized water under stirring. Next, 50 mg of MNA dimer were added and pH adjusted to 8 with 1 M NaOH. The mixture was stirred for 6 h at room temperature [7, 14, 15]. To separate the conjugate from unbound MNA dimer, the reaction solution was dialyzed for 7 days using Spectra/Por® 3 membrane (MWCO: 12 kDa) in 5 L of demineralized water under stirring in the dark at 10 °C. The resulting product was freeze-dried for 2 days under reduced pressure.

2.3. Quantification of conjugated L-cysteine hydrochloride

The amount of bound L-cysteine hydrochloride was determined spectrophotometrically using Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) according to a method described previously [16]. To ensure that there is no unbound cysteine in the sample, a TNBS test has been carried out to quantify unbound amino groups allowing direct correlation to unbound cysteine [17].

2.4. Determination of conjugated 2-mercaptonicotinic acid

The amount of immobilized 2-mercaptonicotinic acid was determined spectrophotometrically according a method described previously by our group [7]. To determine the amount of bound MNA, the ligand was released by adding GSH. The absorbance of liberated MNA was measured at 354 nm. To ensure that there is no unbound MNA in the sample, absorption measurements at 354 nm have been carried out prior to reduction with GSH.

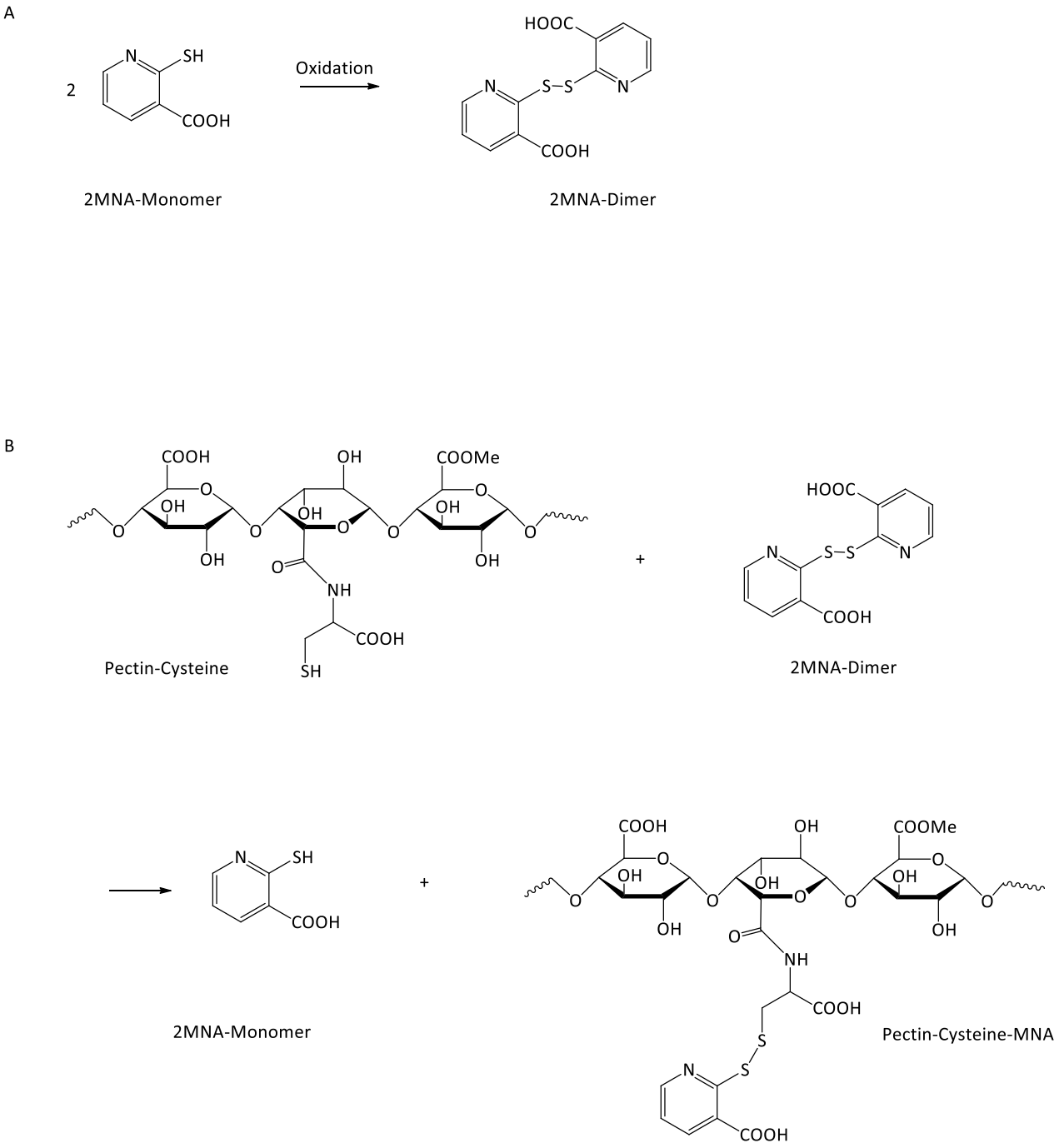

Figure 1.
Synthesis of Pec-Cys-MNA. Dimerization of 2-mercaptonicotinic acid using hydrogen peroxide as oxidizer (A). The resulting dimer was added to Pec-Cys to preactivate the SH-Group (B).

2.5. Manufacturing of minitablets

For the manufacturing of the minitablets 30 mg of lyophilisates were compressed at 10 kN for 25 s yielding 2 mm thick flat-faced discs of 5 mm diameter (Paul Weber, Remshalden-Grünbach, Germany). The hardness of resulting test discs was determined using a Schleuniger 2-E/205 tablet-hardness tester (Dr. K. Schleuniger and Co., Switzerland).

Table 1. Hardness of test tablets. Indicate values are mean ± S.D. of 10 tablets.

Polymers	Hardness [N]
Pectin	85 ± 11
Pectin-Cysteine	94 ± 16
Pectin-Cysteine-MNA	86 ± 7

2.6. *In vitro* mucoadhesion studies

To evaluate the mucoadhesive capacity, tensile studies were performed as previously described by our research group [18]. Briefly, freshly excised porcine stomach was cleaned and cut into pieces. The tissue was fixed on a glass base with cyanoacrylate adhesive and placed in beakers with 0.1 M phosphate buffer pH 6.8 and 0.1 M HCl, respectively. The beaker was placed on a balance. The polymer discs were glued to stainless steel basis using the same adhesive. The basis and the test disc were arranged above the mucosa. The disc was attached to the tissue by applying mild force. After an incubation time of 20 min at room temperature the mucosa was pulled down at a rate of 0.1 mm/s by lowering the balance. Data points were collected every second by a computer software (Sarta Collect software; Satorius AG). The total work of adhesion (TWA) represented by the area under the force/distance curve and the maximum detachment force (MDF) were determined [19].

2.7. Disintegration behavior

Disintegration time of test discs was evaluated with a disintegration apparatus in accordance with the European Pharmacopoeia. The oscillating frequency was adjusted to 0.5 s^{-1} [20]. The test was carried out at two different pH values: 0.1 M HCl and 0.1 M phosphate buffer pH 6.8. The temperature was set to 37 ± 1 °C.

2.8. Water uptake capacity

Swelling/erosion characteristics of the different pectin conjugates and control Pec were evaluated by determination of weight change. Test discs were fixed on pin needles and incubated in 0.1 M HCl and phosphate buffer 0.1 M pH 6.8. At predetermined time points the surface water was daped away with paper tissue and the amount of water uptake was determined gravimetrically. Water uptake was calculated according to the following equation with W_0 = initial weight and W_t = weight of the test disk at the time t [21].

Water uptake [%] = $(W_t-W_0)/W_0$ x 100

2.9. Release studies

In vitro release of rosuvastatin calcium was investigated. Mixing with the different excipients was achieved by dissolving 40 mg of rosuvastatin calcium in aqueous solutions containing 120 mg of polymer. After freeze-drying, tablets of 40 mg were compressed as described above. Studies were performed with an ERWEKA DT 700 dissolution apparatus with paddles according to the European Pharmacopoeia. Temperature was set to 37 ± 0.5 °C and speed to 100 rpm. Investigations were made with and without pH change of release medium. Studies without pH change were carried out using 0.1 M HCl (900 mL/vessel). For studies with change in pH the 0.1 M HCl was replaced by phosphate buffer pH 6.8 0.1 M after 2 hours simulating the transition from stomach to intestine corresponding to the European Pharmacopoeia. Samples of 1 mL were withdrawn at predetermined time points and volume was replaced with fresh release medium. The concentration of rosuvastatin calcium was determined via HPLC (Hitachi EliteLaChrom HPLC-System). A CN-RP column (250 × 4.6 mm, 5 µm) (Machery-Nagel, Germany) was used as stationary phase. The mobile phase consisted of acetonitrile : water pH 3.5 (adjusted with phosphoric acid) 40:60 v/v. Flow rate was set to 1.0 mL/min. A diode array detector was used to measure absorption of the eluate at λ = 242 nm [22]. Retention time for rosuvastatin calcium was 7.2 min. Rosuvastatin calcium concentration was calculated from a linear calibration from 3-50 µg/mL. The experiment was carried out in six replicates.

2.10. Viscosity and degradability

Solutions (10 mg/mL) of Pec, Pec-Cys and Pec-Cys-MNA in 0.1 M acetate buffer pH 4.5 were incubated at 37 °C overnight in a thermal mixer. Thereafter, dynamic viscosity was measured at a shear rate of 50 s^{-1} at 37 °C using a plate-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). The enzymatic degradability was determined by the addition of pectinase to the polymer solutions (final concentration of ≥ 3.8 units/mL of pectinase). After an incubation time of 90 min, the dynamic viscosity was measured.

2.11. Cytotoxicity studies – resazurin assay

The resazurin assay is based on the reducing environment of viable cells. The blue, non-fluorescent resazurin is reduced to red and fluorescent resorufin in the environment of metabolic active cells [23]. The colorimetric and fluorescence changes were measured at 540 nm with background subtraction at 590 nm with a Tecan infinite, M200 spectrophotometer, Grödig, Austria [24]. In detail, the assay was performed on Caco-2 cells which were cultured in 24-well plates for 14 days at 37 °C in a 5 % CO_2 environment. The minimum essential medium (MEM) with FCS was replaced every second day. 500 µL of the prepared test solutions (5 mg/mL, Pec, Pec-Cys, Pec-Cys-MNA in MEM without FCS and phenol red) were added. As positive control MEM without FCS and phenol red was added whereas Triton X® 100 in a 4 % (w/w) solution in MEM was used as negative control. Each sample was prepared 4-times, incubation time was 3 h. Subsequently, cells were washed twice with phosphate buffered saline. To each well 250 µL of a 2.2 µM resazurin solution were added. After 3 h the fluorescence was measured as described above.

2.12. Statistical data analysis

Statistical data analysis was performed (GraphPad Prism 5) using one way ANOVA with $P<0.05$ as the minimal level of significance followed by Bonferroni's Multiple Comparison Test with $p<0.05$ as minimal level of significance.

3. Results

3.1. Characterization of pectin-cysteine-mercaptonicotinic acid

3.1.1. Characterization of pectin-cysteine conjugate

A schematic diagram of a thiolated substructure of the formed pectin derivative are given in Figure 1. Ellman's test showed that 507 ± 35 µmol (0.06 g) L-cysteine hydrochloride were bound per gram pectin. The control sample prepared in the same manner omitting EDAC showed a negligible number of remaining traces of L-cysteine. For all experiments the fibrous lyophilized polymer were used.

3.1.2. Characterization of 2,2'-dithiodinicotinic acid

The 2-2-dithionicotinic acid was generated by addition of a H_2O_2 solution to a solution of the MNA monomer (Figure 1A). During the reaction, color changed from yellow to colorless. Further, UV spectra (UVmini1240, Shimadzu Co., Japan) were measured during reaction time. Thereby, the yellow MNA solution showed two absorption maxima at 278 nm and 353 nm, respectively. The oxidized product showed one absorption peak at 257 nm.

3.1.3. Characterization of pectin-cysteine-2-mercaptonicotinic acid

Pec-Cys-MNA formation was achieved by disulfide exchange as illustrated in Figure 1B. As the 2-mercaptonicotinic acid monomer was released during the reaction the solution turned from colorless to light yellow. Approximately 50 % of free thiol groups adhering to pectin were modified: 263 ± 13 µmol (0.041 g) of MNA per gram Pec-Cys-MNA were detected.

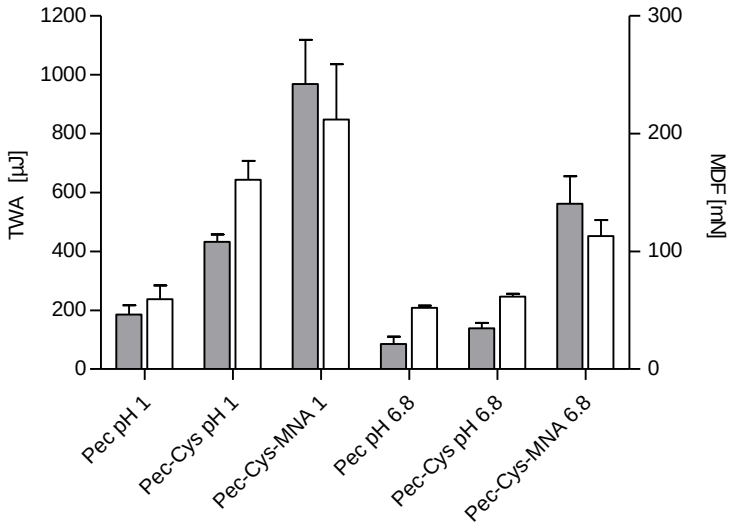

Figure 2.
Diagram shows mucoadhesive properties of Pec, Pec-Cys and Pec-Cys-MNA. White bars display the mean detachment force (MDF), grey bars the total work of adhesion (TWA) at 37 °C. Indicated values are means ± SD, n = 5. TWA-means differ from each other significantly: Pec pH 1 from Pec-Cys-MNA pH 1, Pec-Cys 1 from Pec-Cys-MNA pH, Pec pH 6.8 from Pec-Cys-MNA pH 6.8 and Pec-Cys pH 6.8 from Pec-Cys-MNA pH 6.8. MDF-means differ from each other significantly: Pec pH 1 from Pec-Cys pH 1, Pec pH 1 from Pec-Cys-MNA pH 1 and Pec pH 6.8 from Pec-Cys-MNA (Bonferroni's Multiple Comparison Test, $p < 0.05$).

3.2. *In vitro* mucoadhesion studies

To test mucoadhesive properties tensile studies on porcine mucosa were performed. The results are given in Figure 2 including the maximum detachment force (MDF) and the total work of adhesion (TWA). The improvement ratio for total work of adhesion of Pec-Cys-MNA in comparison to Pec was 5.2-fold at pH 6.8 and 6.5-fold under acidic conditions. After incubation time of 20 minutes, cohesiveness of all tablets was still provided. Swelling process has started but no erosion was observed.

3.3. Hardness of test tablets

The results of hardness test are shown in Table 1. No significant difference between the modified pectin compared to the native pectin could be observed.

3.4. Disintegration studies

The endpoint of the experiment was reached after tablets were fully soaked with no firm core remaining. Results are shown in Figure 3. For pectin-tablets bulking was observed but dissolving of the tablet occurred early. Well-defined shape was lost after 30 min, even though there was still a dry core visible. The disintegration time was prolonged for Pec-Cys 1.9-fold (pH 6.8) and 2.4-fold (pH 1.2), for Pec-Cys-MNA the improvement ratio was 2.3 at pH 6.8 and 2.6 at pH 1.2. Until the endpoint of the test was reached a well-defined shape and a clear borderline between the swelling tablets and the surrounding medium could be observed.

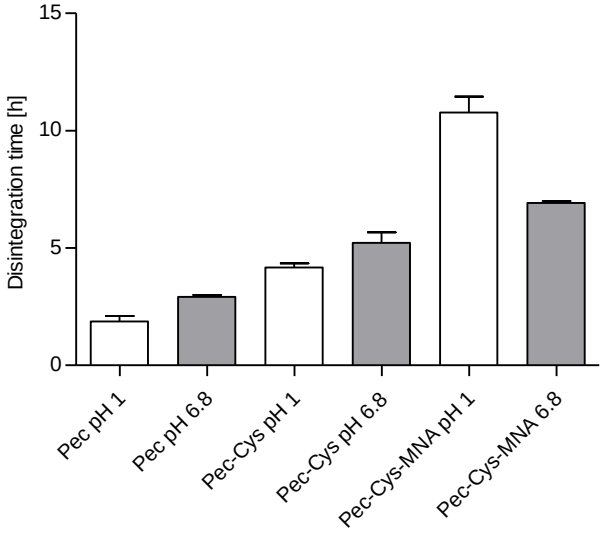

Figure 3.
Histogram shows time until disintegration of all three polymers. The time was determined at two different pH values. Indicated values are means ± SD, n= 6. Means differ from each other significantly (Bonferroni's Multiple Comparison Test, $p < 0.05$).

3.5. Water uptake capacity

Water uptake capacity increased 1.5-fold under acidic conditions for both pectin-derivatives, Pec-Cys and Pec-Cys-MNA, compared to the unmodified pectin. In the phosphate buffer pH 6.8 the water uptake raised 2.4-times for Pec-Cys and 3.5-times for Pec-Cys-MNA. The maximum water uptake for all tested polymers can be seen in Figure 4. After 4.5 h of incubation in aqueous environment pectin was completely moistened and turned into a shapeless, jelly mass. Further weighing was not feasible. In contrast tablets comprising the modified polymer showed a greater cohesiveness and no erosion could be observed for Pec-Cys and Pec-Cys-MNA. After 4.5 hours no further significant weight-change was observed for Pec-Cys and Pec-Cys-MNA. Weight of pectin-tablets increased 11-fold under acidic conditions whereas at pH 6.8 a 7-fold gain was observed. Pec-Cys and Pec-Cys-MNA tablets experienced a 16-fold weight gain under acidic conditions. In the pH 6.8 buffer solution the weight increased for Pec-Cys and for Pec-Cys-MNA 16-fold and 22-fold, respectively.

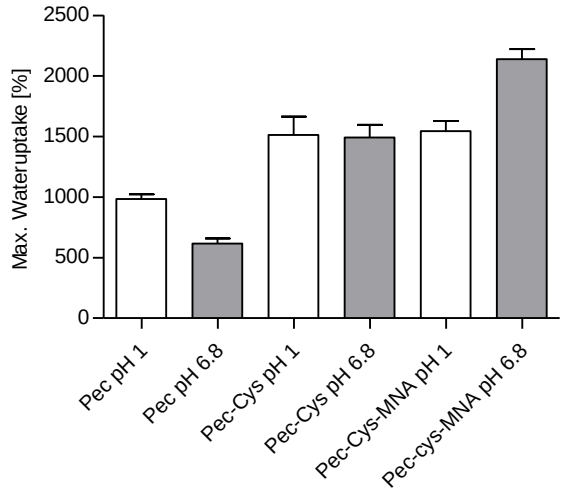

Figure 4.
Maximum water uptake of unmodified pectin, Pec-Cys and Pec-Cys-MNA. Indicated values are means ± SD, n = 4. Means of max. water uptake differ from each other significantly: Pec pH 1 from Pec-Cys pH 1, Pec pH 1 from Pec-Cys-MNA pH 1, Pec pH 6.8 from Pec-Cys pH 6.8 and from Pec-Cys-MNA pH 6.8, Pec-Cys pH 6.8 from Pec-Cys-MNA pH 6.8 (Bonferroni's Multiple Comparison Test, $p < 0.05$).

3.6. Release studies

For all formulations a sustained release could be observed (Figure 5 and 6). Under acidic conditions a less sustained but almost complete (95 %) release was observed for the pectin-based formulation. Formulations based on Pec-Cys and Pec-Cys-MNA had released 50 % after 10 hours. In the pH-change setup of the experiment, release profiles of different formulations did not differ. After pH change to 6.8, release occurred more rapidly, 50 % of rosuvastatin calcium were released after 4 hours.

3.7. Viscosity and degradability

The dynamic viscosity of modified polymers in comparison to the native pectin increased 1250-fold in case of Pec-Cys and 3480-fold in case of the Pec-Cys-MNA (1 % m/v solutions, pH 4.5). Due to the addition of the pectin splitting enzyme pectinase the dynamic viscosity decreased in case of all three polymers.

After the reaction time of 90 minutes, the dynamic viscosity for all 3 tested polymers equaled. Table 2 provides a synopsis of results.

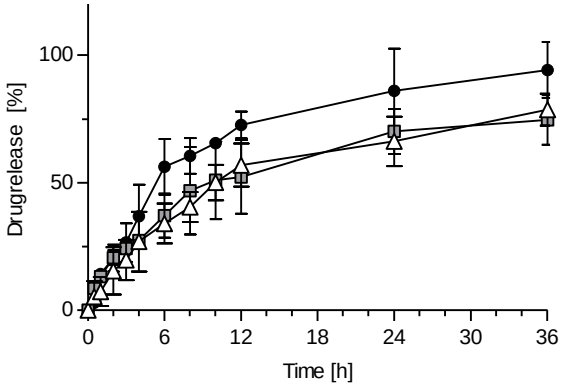

Figure 5.
Release profiles of rosuvastatin calcium from tablets based on unmodified pectin (-●-), Pec-Cys (-■-) and Pec-Cys-MNA (-Δ-) over 36 hours. Experiments were carried out in HCl 0.1 M. Indicated values are means (n=3 ± SD**).**

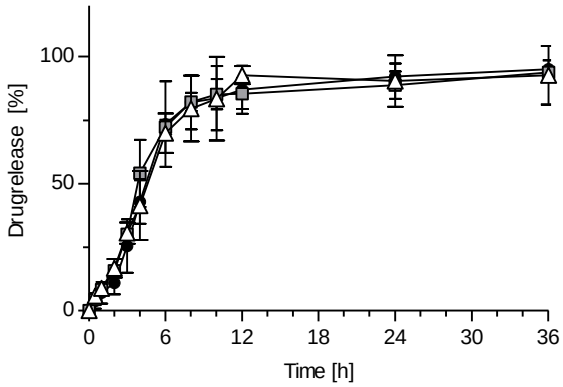

Figure 6.
Release profiles of rosuvastatin calcium from tablets based on unmodified pectin (-●-), Pec-Cys (-■-) and Pec-Cys-MNA (-Δ-) over 36 hours. First two hours were carried out in HCl 0.1 M, than medium was changed to 0.1 M phosphate buffer pH 6.8. Indicated values are means (n=3 ± SD).

Table 2.
Viscosity of pectin and derivatives before and after addition of pectinase. Indicated values are mean ± SD, n = 3.

	Dynamic viscosity [Pas]	
polymer	1 % solution	1 % solution with pectinase
Pec	0.144 ± 0.014	0.005 ± 0.004
Pec-Cys	179.767 ± 17.954	0.007 ± 0.002
Pec-Cys-MNA	499.600 ± 49.201	0.008 ± 0.007

3.8. Cytotoxicity studies – resazurin assay

The resazurin fluorometric cell viability assay is a simple, safe and reliable way to investigate cytotoxicity based on measuring metabolic activity of vibrant cells [25]. There were no significant differences for all tested polymers during the incubation period of 3 hours.

4. Discussion

Within this study, a preactivated thiomer was characterized in order to evaluate its potential as mucoadhesive drug delivery system. Therefore, pectin was thiolated by covalent attachment of cysteine via disulfide bond formation. Thiol groups were further modified by disulfide bond formation with MNA in order to activate sulfhydryl groups immobilized on the polymer and to gain a higher activity over a broader pH range.

The formulation is meant to improve bioavailability of the model drug rosuvastatin calcium, whose oral bioavailability is approximately 20 %, by prolongation of contact time with the gastric mucosa. Gastroretention can be prolonged by mucoadhesive properties of the excipient and can be supported by the size of the tablet after water uptake in the stomach [26]. The chosen polymeric backbone, pectin, shows mucoadhesive properties *per se*. Due to hydroxyl groups pectin is able to form hydrogen bonds with other functional groups such as hydroxyl, carboxyl or amide groups. Thereby interaction between the polymer and gastric mucus is possible. Smart et al. found an adhesiveness of pectin when investigated with mucus gel employing the Wilhemy plate method [27]. In addition, mucoadhesive properties of the polymer on gastrointestinal mucosa could be shown [28, 29]. This study showed that the mucoadhesion is improved by the implementation of free thiol groups and further activation of these thiol groups with an aromatic ligand. Former studies revealed that thiol groups are able to form covalent bonds with cysteine-rich subdomains of the mucus gel layer by disulfide exchange reactions or oxidative coupling resulting in enhanced mucoadhesion [5, 6, 30]. Unfortunately, reactivity of thiol groups strongly depends on pH values of the environment. In aqueous solutions, crosslinking between attached thiol groups takes place easily especially at pH values above 5 being closer to pk_a value of attached thiol groups. Inter- and intramolecular disulfide formation results in a decrease of mucoadhesion. Furthermore, at pH values below 5, reactivity against thiol bearing mucus components is decreased as well as concentration of thiol anion $–S^-$, being required as reactive form, is very low [31]. To overcome this restrained reactivity of thiomers, more recently, so-called preactivated thiolated polymers have been introduced. Formation of inter- and intramolecular disulfide bonds should be limited and further reactivity against other thiols should be increased. Covalent chromatography provides strong evidence that proteins can be linked to thiol group

bearing resins efficiently when activated with pyridyl substructures [7, 32, 33]. Hence, preactivated thiol groups should be able to form disulfide bonds with thiol bearing subdomains of mucin by disulfide exchange reaction whereat the aromatic thiol moiety is released, explaining improved mucoadhesion [32, 33]. Results of tensile studies are in line with the hypothesis about protecting and activating thiol groups by implementing an aromatic leaving group. The improvement of TWA of Pec-Cys-MNA was outstanding for both conditions, pH 1.2 and pH 6.8. Total values for test series in acidic conditions were considerable higher than in phosphate buffer pH 6.8.

Besides enhanced mucoadhesion, coupling MNA to the thiol groups of the synthesized polymer changed other characteristics of the polysaccharide like disintegration time and water uptake.

Water uptake increased considerable from Pec to Pec-Cys to Pec-Cys-MNA. Swelling of polymeric delivery systems is of advantage for several reasons. The higher volume of dosage form may lead to prolongation of retention time in stomach [26]. Further, swelling is important for mucoadhesion, employing simple adhesion by hydration effect and swelling leads to chain relaxation making interpenetration of polymer chains and mucus gel layer easier [34, 35]. Next, swelling of the dosage form is also important for drug release as swelling together with erosion and diffusion are the most important mechanisms for controlled release [36]. Diffusion depends on the water content of the formulation, as the drug has to be dissolved and chain relaxation opens ways out for the drug. After the change of pH in the release medium release profiles of the three investigated formulations are similar.

Solubility of pectin carrying carboxyl groups is decreased in acidic environment. Under less acidic conditions carboxyl groups are deprotonated and chain relaxation may occur more easily. This may facilitate diffusion of the drug resulting in faster and higher drug release [37]. Unmodified, naturally occurring pectin is expected to be degraded under influence of polysaccharidases like pectinases produced by bacterial flora in the colon [38]. To assure biodegradability of the derivative it was tested using pectinase from *Aspergillus aculeatus.* Modification of pectin turned out to be no hindrance for enzymatic degradation.

Pectin and L-cysteine are two naturally occurring substances which are known to be non-toxic and biocompatible. Conjugation of these two substances and further modification with 2-mercaptonicotinic acid showed no significant change in cell viability under tested conditions. Nevertheless, these kind of tests can only give a hint about a substances potential to cause damage or not. It is not possible to predict any long term damages, effects on other kind of tissues and the influence of concentration. Further, under given conditions, it is difficult to investigate cell toxicity over a long time period as pectin is a microbial

susceptible substance. It may be contaminated during reaction time as well as storage, as conditions were non sterile.

5. Conclusion

Within this present study, a recently developed pectin derivative was evaluated *in vitro* for its usage as a novel excipient for mucoadhesive delivery systems on the gastric mucosa using the model drug rosuvastatin calcium. The covalent attachment of 2-mercaptonicotinic acid to thiolated pectin led to improved mucoadhesion especially in an acidic environment where mucoadhesion of non preactivated thiolated thiomers is restricted. Moreover, it could be shown that by preactivation with a pyridylic group the pH dependencies of properties of thiomers are decreased. Furthermore, a considerable gain in water-binding capacity was observed. These properties combined with improved cohesiveness and sustained release for 36 hours under acidic conditions indicate a great potential of the excipient for a mucoadhesive drug delivery system, especially useful for sparingly soluble drug with a low bioavailability like the statin rosuvastatin calcium which can be released very slowly from the formulation to target site which may resulting in enhanced uptake.

6. Acknowledgement

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Development and *in vitro* evaluation of a buccal drug delivery system based on preactivated thiolated pectin

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Abstract

The aim of this study was to evaluate the potential of preactivated thiolated pectin (Pec-Cys-MNA) for buccal drug delivery. Therefore, a gel formulation containing this novel polymer and the model drug lidocaine was prepared and investigated *in vitro* in terms of rheology, mucoadhesion, swelling behavior and drug release in comparison to formulations based on pectin (Pec) and thiolated pectin (Pec-Cys). Both pectin derivatives showed gel formation without addition of any other excipient due to self-crosslinking thiol groups. Under same conditions, pectin did not show gel formation. Viscosity of Pec-Cys-based formulation increased 92-fold and viscosity of Pec-Cys-MNA-based formulation 4958-fold compared to pectin-based formulation. Gels did not dissolve in aqueous environment during several hours and were able to take up water. Mucoadhesion of pectin on buccal tissue could be improved significantly, value of total work of adhesion increased in following rank order: Pec-Cys-MNA > Pec-Cys > Pec. The retention time of a model drug incorporated in gel formulations on buccal mucosa under continuous rinsing with phosphate buffered saline was prolonged, after 1.5 h 3-fold higher amount of a model drug was to be found on tissue after application of Pec-Cys-MNA-based formulation compared to pectin based and 2-fold compared to Pec-Cys-based formulation. The Pec-Cys-MNA-based gel showed a more sustained release of lidocaine than the Pec-Cys-based gel, whereas pectin solution revealed an immediate release. According to these results, the self-crosslinking pectin-derivative is a promising tool for buccal application.

Key words: preactivated thiomer, pectin, buccal application, gel, lidocaine

1. Introduction

Buccal drug delivery is the route of choice for several common diseases in the oral cavity like mucositis, Sjörgen's syndrome, candida infections, gingivitis, periodontitis and toothache. But also for systemic application the oral mucosa has been subject of interest as the tissue provides some advantages like being highly vascularized and relatively permeable. First pass effect can be avoided and pH values between 5.5 to 7 are in a mild range manageable even for sensitive drugs [1-4]. But buccal drug delivery is challenging because of a constant flow of saliva, mobility of the target tissue, limited area and regularly drinking and food intake [5]. One promising approach to overcome these hindrances are mucoadhesive buccal formulations [6]. Ideal dosage forms for buccal application should not cause irritation and should be smooth and flexible so that compliance is not affected. These demands are met by mucoadhesive hydrogels, three-dimensional cross-linked networks of water-soluble polymers [1, 7].

One of these mucoadhesive polymers with the ability of gel formation is pectin [8]. Pectin is naturally occurring, non-toxic and biodegradable. Therefore, it has been extensively investigated as matrix for drug delivery. Unfortunately, pectin has some drawbacks as unmodified pectin is highly water soluble and gelling needs additives. To overcome this hindrance, different pectin derivatives have been synthesized and crosslinking agents have been investigated. Among these, thiolated pectin showed promising results. Former investigations from our research group showed controlled swelling, improved cohesiveness and mucoadhesion as well as gelling by cross-linking of thiol groups [9-12].

Recently, it has been reported that preactivation of the thiol groups could improve some properties of thiolated polymers. Thiol groups of thiol-modified polymers have been preactivated with thiol bearing pyridyl substructures via disulfide bond formation. These disulfide bonds are more reactive against free thiol groups leading to a disulfide/thiol exchange reaction whereas the thiol group bearing pyridyl substructure acts as leaving group. First characterizations of preactivated thiolated poly(acrylic acid) and preactivated thiolated chitosan showed several improvements compared to unmodified and thiolated polymers like improved mucoadhesion, increased viscosity, decreased solubility, and raised water uptake [13-15].

Therefore, the aim of this study was to preactivate thiolated pectin and evaluate its potential for buccal drug delivery. The impact of thiolation and preactivation of thiol groups was investigated by comparison with thiolated and unmodified pectin.

Hydrogels were prepared and loaded with lidocaine as model drug for buccal drug delivery. Resulting formulations were characterized in terms of rheological behavior, stability in aqueous environment and mucoadhesive properties. Further, *in vitro* release studies were carried out.

2. Materials and Methods

2.1. Materials

Lemon pectin (degree of esterification approximately 75 %) was received from Herbafood Ingredients GmbH, Weder, Germany and 2-mercaptonicotinic acid 98 % (MNA) from ABCR GmbH & Co KG, Karlsruhe, Germany. L-Cysteine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), hydrogen peroxide, dialysis tubes (MWCO 12 kDa), glutathione in reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), porcine gastric mucin, sodium borohydride, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, lidocaine hydrochloride, triethylamine, and acetonitril HPLC grade were obtained from Sigma Aldrich, Austria. Water HPLC gradient grade was purchased from Fisher Chemical, United Kingdom. All other reagents used were of analytical grade. Cellulose acetate filters (0.2 µm) were received from Sartorius AG, Germany. Porcine buccal mucosa was donated from a local slaughterhouse.

2.2. Synthesis of pectin-cysteine-mercaptonicotinic acid

2.2.1. Synthesis of pectin-cysteine

According to a method described previously by our group, L-cysteine was attached covalently to the polysaccharide backbone of pectin (Pec) [9]. Thereby, an amide bond was formed between the amino group of L-cysteine and the carboxy group of the D-galacturonic units of the polymer. In brief, 1.5 g of pectin were dissolved in 150 mL of demineralized water. To activate the carboxyl groups 1.5 g of EDAC were added and stirred under room temperature for 1 h. Then, 3 g of L-cysteine hydrochloride (Cys) were dissolved in 20 mL distilled water and the pH adjusted to 4.5. After unifying both solutions, the pH was readjusted to 4.5 using 5 M NaOH. Reaction mixture was stirred for 6 hours. For comparison, a control was prepared treating pectin the same way but omitting EDAC. Thereafter, the mixture was dialyzed for 3 days using Spectra/Por® 3 membranes (MWCO: 12 kDa) for purification of resulting thiomer (Pec-Cys) from unbound L-cysteine hydrochloride and EDAC. 5 L of demineralized water were used as dialysate being replaced every 12 hours. 5 M HCl were added to dialysates in a total concentration of 2 mM. The 3rd and 4th dialysate were additionally loaded with 1 % NaCl. To avoid disulfide bond formation dialysis was performed at 10 °C in the dark. After purification, the product was frozen at -80 °C and lyophilized for 2 days at -77 °C and 0.01 mbar.

2.2.2. Synthesis of pectin-cysteine-mercaptonicotinic acid

The conjugate was synthesized according to a method previously described by Iqbal et al. [13] First, the dimer of 2-mercaptonicotinic acid was prepared using H_2O_2 for disulfide bond formation between two monomer molecules. Therefore, 2 g of 2-mercaptonicotinic acid monomer were dissolved in 50 mL of demineralized water whereat the pH was adjusted to 8 using 5 M NaOH. To the yellow solution 2500 µL of H_2O_2 (30 % v/v) were added and stirred for 10 minutes. To obtain solid MNA dimer (2,2'-dithiodinicotinic acid) the resulting colorless solution was freeze-dried for 2 days.

To attach the aromatic ligand, 500 mg of Pec-Cys were dissolved in 150 mL of demineralized water. Afterwards, 150 mg of MNA dimer was added under stirring and pH was adjusted to 8 using 5 M NaOH. The mixture was stirred overnight.

To separate unbound MNA dimer from the conjugate, the mixture was dialyzed for 6 days using Spectra/Por® 3 membrane (MWCO 12 kDa) in a dialysate of 5 L of demineralized water under stirring at 10 °C in the dark, whereas the dialysate was replaced 3 times a day. Afterwards, the product was freeze-dried for 2 days at 0.01 mbar and -77 °C and stored dry at 10 °C until use.

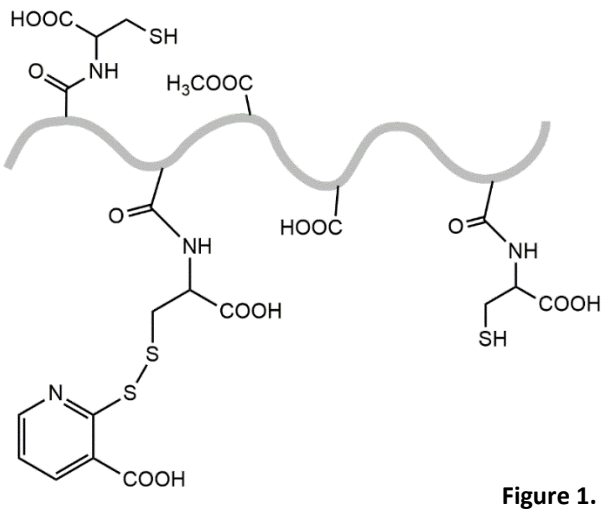

Figure 1.
Structure of Pec-Cys-MNA.

2.3. Quantification of conjugated L-cysteine hydrochloride

To determine the degree of modification, a method described previously was employed. Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) was used to detect the attached thiol groups photometrically [16]. A calibration curve was established using L-cysteine hydrochloride.

2.4. Quantification of conjugated 2-mercaptonicotinic acid

Immobilized 2-mercaptonicotinic acid was detected photometrically. Therefore, samples of 0.5 mg were dissolved in 500 µl of 0.5 M phosphate buffer pH 8. 500 µL of 2 % (m/v) reduced glutathione solution were added in order to release the MNA ligand. The absorbance of quantitatively liberated MNA was measured at 354 nm after incubation of the samples for 60 minutes at 37 °C under stirring. To establish a calibration curve the MNA monomer was used.

2.5. Quantification of lidocaine hydrochloride

The concentration of lidocaine was determined via HPLC. Samples were analyzed using a Hitachi EliteLaChrom HPLC-System. A LichroCART® RP-18 column (125×4 mm, 5 µm) (Merck, Germany) was used as stationary phase. The mobile phase was compounded as follows: acetonitrile: 0.05 M sodium phosphate buffer pH 6 (pH adjusted using triethylamine) 35:65. Flow rate was set to 1.0 mL/min. Detection was carried out with a diode array detector at λ = 320 nm. The observed retention time for lidocaine was 4.5 min. Lidocaine concentrations were calculated from a linear calibration curve with a range of detection between 5 and 400 µg/mL.

2.6. Preparation of lidocaine loaded formulations

To prepare Pec-Cys-MNA and Pec-Cys gels, 100 mg of the lyophilized polymers were added to 5 ml of lidocaine HCl (2 % m/v) containing phosphate buffered saline (0.02 M, pH 6.8 with 8 g NaCl per liter) [17]. Formulations were incubated at 37 °C under shaking for 24 hours (Thermomixer) to induce disulfide formation [18]. Formulations with 100 mg unmodified pectin (Pec) served as reference.

2.7. Rheological characterization of formulations

Resulting formulations were characterized in terms of their viscoelastic behavior using a HAAKE MARS (Haake GmbH, Karlsruhe, Germany) rheometer. Strain sweep measurements were made to determine the maximum strain amplitude for the gel formulations so that investigations of viscoelastic properties could be made within the linear region meaning lower than the determined maximum strain amplitude. Oscillatory investigations were carried out at a frequency of 1 Hz at a temperature of 37 ± 0.1 °C. A plate-plate build-up was used with 35 mm diameter, the gab was set to 0.5 mm and sample size to 750 µL. Thereby the phase angle (δ) and the complex modulus (G*) were obtained. Complex modulus (G''), elastic modulus (G') and dynamic viscosity (η) were received by

$$G' = G^* cos\delta$$

$$G'' = G^* sin\delta$$

$$\eta^* = \frac{G''}{2\pi\nu}$$

with ν = oscillatory frequency. For viscoelastic substances as thiomers it is: 0° < δ < 90° [19]. The loss factor $tan\delta = \frac{G''}{G'}$ gives the ratio between the viscous and elastic modulus. The sol state is characterized by tanδ > 1 and the gel state by tanδ < 1. All measurements were carried out in 3 replicates.

2.8. Swelling Behavior

To investigate formulations in watery environment, samples of 1 g of each formulation were placed in crystallizing dishes (20 mL). The dishes have been filled up with PBS 0.02 M, pH 6.8 and placed in a shaking water bath. At predetermined time points gels were removed and weighed. To minimize erosion caused by handling the formulation, for each data point a separate sample was used [20]. Determinations were done in triplicate.

2.9. Mucoadhesion

2.9.1. Tensile studies - mucoadhesive properties of excipients

To determine mucoadhesive properties of the polymers, tensile studies were carried out according to a method described previously by our research group [21]. Therefore, freshly excised porcine buccal tissue was cut into pieces of approximately 3 cm^2. The tissue was stuck on a glass base (cyanoacrylate adhesive) and placed in PBS 0.02 M pH 6.8. The freeze dried polymers were compressed into test discs (30 mg, diameter 5.0 mm, thickness 1.2 mm) by applying a constant compaction pressure of 10 kN for 25 s (Paul Weber, Remshalden-Grünbach, Germany).

These test discs were stuck with cyanoacrylate adhesive to a stainless steel plate. The steel plate was hung up above the phosphate buffered saline being in a crystallizing dish put on a balance. The setup contained a vertical movement base where the balance was placed. The base was raised until the test disc touched the tissue. To attach the discs to the mucosa mild force was applied. After incubation time of 20 min at room temperature the mucosa was pulled off at a rate of 0.1 mm/s. Computer software (Sarta Collect software; Satorius AG) was employed to collect data points every second. The maximum detachment force (MDF) and the total work of adhesion (TWA) representing the area under the force/distance curve were calculated [22, 23]. The investigation was carried out for each polymer 5-fold.

2.9.2. Mucoadhesive properties of the formulation

To evaluate the mucoadhesive capacity of formulations, a method established by Rao and Buri developed for nanoparticles was employed [24]. Therefore, fluorescence marked formulations were prepared by dissolving sodium fluorescein (NaFlu) in the PBS 0.02 M, pH 6.8 (0.01 % m/v) before adding to the polymer. Porcine buccal mucosa was glued on a half-pipe and placed in an angle of 45° in an incubation chamber with 100 % humidity at a temperature of 37 °C. 300 mg of fluorescence marked formulations were administered to the mucosa and continuously rinsed with the PBS 0.02 M pH 6.8. Temperature of PBS was kept at 37 °C and the flow rate was set to 1 mL/min using a peristaltic pump. After 1.5, 3 and 4.5 h, the mucosa with the remaining formulation on it was incubated in 25 mL of distilled water for dissolving remaining NaFlu for 1 h under stirring in the dark. After centrifugation (13,400 rpm; 5 min) fluorescence of each sample was measured with a microplate reader (Tecan Austria GmbH, Grödig, Austria) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Investigation was carried out for all formulations 4-fold.

2.9.3. Rheology polymer/mucin mixtures

The dynamic viscosity of the formulations was measured with and without the addition of mucin. Therefore, dynamic oscillatory tests were carried out after determination of the linear viscoelastic range for aliquots about 750 µL at a frequency of 1 Hz. Gap was set to 0.5 mm and the temperature to 37.0 ± 0.1 °C. Lidocaine formulations were prepared as described above. To measure the adhesion of the formulations to mucin, 500 µL samples of all formulations were added to 500 µL of a mucin solution (2 g of artificial gastric mucin in 25 mL 0.1 M phosphate buffer pH 6.8). Mixtures were intermingled with a spatula and incubated at 37 °C before measurement for 30 and 120 min, respectively. All measurements were taken in triplicates.

2.10. *In vitro* release studies

To simulate the drug release from the formulations containing licdocaine HCl (2 % m/v), an *in vitro* study was performed employing Franz diffusion cells (diffusion area of 1.57 cm^2, 7.6 mL receptor compartment). As diffusion barrier a cellulose acetate filter (0.2 µm) was used. 150 mg of the formulations and 150 mg of lidocaine solution 2 % m/v were placed on the membrane, receptor compartment containing PBS 0.02 M pH 6.8. Temperature was set to 37 °C with stirring at 300 rpm. Samples of 400 µL were withdrawn at predetermined time intervals, volume was replaced with phosphate buffered saline. The investigation was carried out over 4 hours. The concentration of lidocaine was determined via HPLC analysis. Experiments were carried out in quadruplicates.

2.11. Cell viability

To investigate the influence of the derivatives on cell viability a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed. Mitochandrial activity of living cells is quantified by conversion of soluble MTT into in water insoluble formazan crystals. For this assay, Caco-2 cells were seeded into a 96 well plate in a density of 20 x 10^4 cells/mL. Cells were cultured for 24 hour in minimal essential medium with Earls salts (MEM) with 10 % fetal bovine serum and 1 % penicillin and streptomycin at 37 °C in 5 % CO_2 environment. Then, cells were washed twice with phosphate buffered saline and 100 µL of the prepared polymer solutions 0.5 % m/v of all three polymers were added into the wells. As negative control minimal essential medium was employed whereas Triton X® 100 4 % (v/v) served as positive control. Experiment was carried out in quadruplicates. The cells were incubated with samples for 6 hours. Thereafter, samples were removed and cells were washed two times with prewarmed (37 °C) phosphate buffered saline. Then, 100 µL of a MTT solution in MEM (0.5 mg/mL) was added to each well and incubated for 4 hours. Precipitated formazan crystals were dissolved by addition of 100 µL of DMSO to each well. Absorbance was measured on 570 nm with a background absorbance on 690 nm with Tecan infinite, M200 spectrometer, Grödig Austria. Viability was calculated in percent of negative control [25, 26].

2.12. Statistical data analysis

The GraphPad Prism 5 software was used for all statistical data analysis. One way ANOVA was performed with $P < 0.05$ as the minimal level of significance followed by Newman-Keuls Multiple Comparison Test with $P < 0.05$ as minimal level of significance.

3. Results

3.1. Characterization of the pectin derivatives

L-Cysteine hydrochloride was attached to pectin via amide bond formation whereat the reaction was mediated by EDAC. Resulting amount of attached L-cysteine was about 450 ± 30 µmol per gram polymer.

For further modification of the conjugate, the yellow MNA monomer was oxidized into 2-2-dithionicotinic acid using H_2O_2. During the reaction, color changed from yellow to colorless. Dried product was a white powder. The resulting powder was tested for free thiol groups using Ellman's reagent. As the test was negative, a solution of the product was reduced using sodium borohydride and after incubation time of 30 min, the test for free thiol groups was repeated. Further, the UV-Spectrum of the product changed. UV spectrum of MNA-monomer solution was recorded, whereat two peak maxima could be detected, at wavelength of 278 and 353 nm, respectively. The resulting MNA-dimer showed one peak maximum at 257 nm (UVmini1240, Shimadzu Co., Japan).

To prepare Pec-Cys-MNA, the MNA-dimer was given to a Pec-Cys solution. During the reaction time, the 2-mercaptonicotinc acid monomer was released turning the reaction mixture lightly yellow, the monomer could be found in UV-spectra measured during reaction time. Approximately 60 % of the free thiol groups bound to pectin were preactivated: 270 ± 18 µmol of MNA per gram Pec-Cys-MNA were determined.

All freeze-dried polymers were of fibrous structure and eggshell white. Figure 1 shows the likely structure of Pec-Cys-MNA.

3.2. Characterization of lidocaine loaded formulations

Resulting lidocaine formulation based on thiolated pectin was a clear gel, the gel resulting from Pec-Cys-MNA was transparent as well but lightly beige-colored. The control formulation prepared with unmodified pectin was obviously more fluid and turbid.

3.3. Rheological characterization

Hydrogels turned out to be a convenient dosage form for buccal application. In general, hydrogels are cross-linked systems based on polymers. Rheologically, gels can be characterized by $\tan\delta < 1$ whereas the sol state is characterized by $\tan\delta > 1$. The storage modulus G' represents the solid-like or elastic component of the formulation, G'' represents the liquid-like component of the formulation. It has been previously published that the elasticity is an important factor in determining bioadhesion [27].

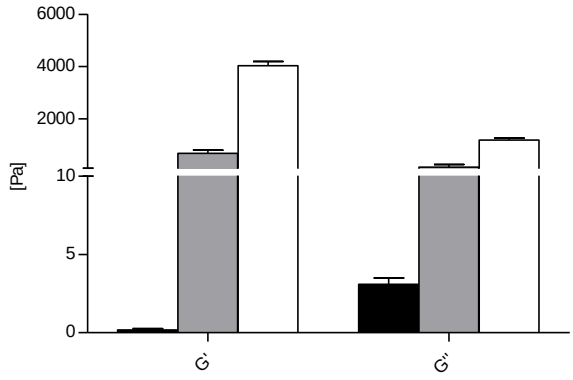

Figure 2.
Comparison of elastic modulus G' and viscous modulus G'' of formulations based on Pec (black bars), Pec-Cys (grey bars), and Pec-Cys-MNA (white bars). Oscillatory measurement were carried out at 1 Hz frequency at 37 °C. All indicated values are means + SD of 3 measurements.

Modified and unmodified polymer formulations exhibit a pseudoplastic flow behavior because of a decrease in viscosity at increasing shear rate (data not shown). As dealing with viscoelastic systems the investigation was carried out in oscillatory mode meaning a sinusoidal movement back and forward of the plate. The elastic modulus (G'), the viscous modulus and the dynamic viscosity (η^*) have been calculated with measured values of phase angle (δ) and the complex modulus (G^*). For the formulation based on unmodified pectin, tanδ was 11.80 ± 1.11 (Table 1), hence a solution. The other two formulations containing modified pectin turned out to be a gel with a value for tanδ < 1. Figure 2 illustrates the ratio between G'' and G' for all formulations. Modification of pectin resulted in a 92-fold increase of dynamic viscosity of the Pec-Cys formulation compared to the Pec-based formulation. For the formulation using Pec-Cys-MNA the enhancement of dynamic viscosity was 4958-fold. Data is provided in Table 2.

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Table 1. Loss tangent of all formulations with and without mucin.

tan δ			
Polymer in formulation	Pec	Pec-Cys	Pec-Cys-MNA
1%	3.81 ± 0.75	0.29 ± 0.06	0.35 ± 0.03
2%	11.80 ± 1.11	0.34 ± 0.05	0.23 ± 0.01
1% with mucin 0.5 h	6.02 ± 2.49	0.11 ±0.01	0.27 ± 0.05
1% with mucin 2 h	5.99 ± 3.03	0.08 ± 0.03	0.24 ± 0.05

Table 2. Dynamic viscosity of different formulations with and without mucin.

Dynamic viscosity η [Pa*s]			
Polymer in formulation	Pec	Pec-Cys	Pec-Cys-MNA
1%	0.02 ± 0.00	1.38 ± 0.06	317.68 ± 33.90
2%	0.13 ± 0.06	12.02 ± 3.70	644.63 ± 23.35
1% with mucin 0.5 h	0.22 ± 0.02	207.60 ± 11.60	394.40 ±54.01
1% with mucin 2 h	0.22 ± 0.02	391.50 ± 29.61	453.95 ± 64.20

3.4. Swelling behavior

For the pectin-based formulation no swelling behavior could be determined. As there was no clear phase boundary between the formulation and PBS, no data could be taken. The other formulations were weighed at predetermined time points and percentage of initial weight calculated. Resulting line chart is given in Figure 3. There was no significant difference in both formulations. After 5.5 hours the investigation was stopped when a plateau was reached. Both formulations absorbed water and gained around 40 % weight compared to the initial weight.

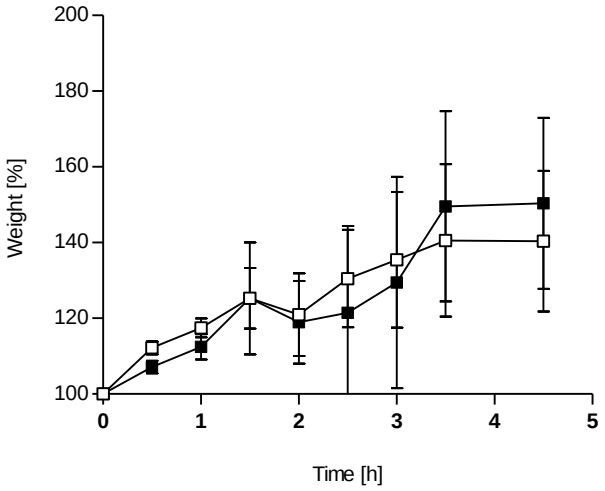

Figure 3.
Time-related water uptake in phosphate buffered saline 0.02 M pH 6.8 of Pec-Cys (-■-), Pec-Cys-MNA (-□-). Indicated values are means ± SD. n = 3.

3.5. Mucoadhesion studies

To show mucoadhesive properties of synthesized excipients tensile studies on porcine buccal mucosa were performed. Figure 4 gives the calculated maximum detachment force (MDF) and the total work of adhesion (TWA). The total work of adhesion increased 4.8-fold for Pec-Cys and 8.9-fold for Pec-Cys-MNA compared to the unmodified pectin. The MDF increased in comparison to unmodified pectin 1.8-fold for Pec-Cys and 1.6-fold for Pec-Cys-MNA, respectively. The cohesiveness of all discs was still provided after incubation time of 20 min. Detachment occurred on the surface between discs and the buccal tissue.

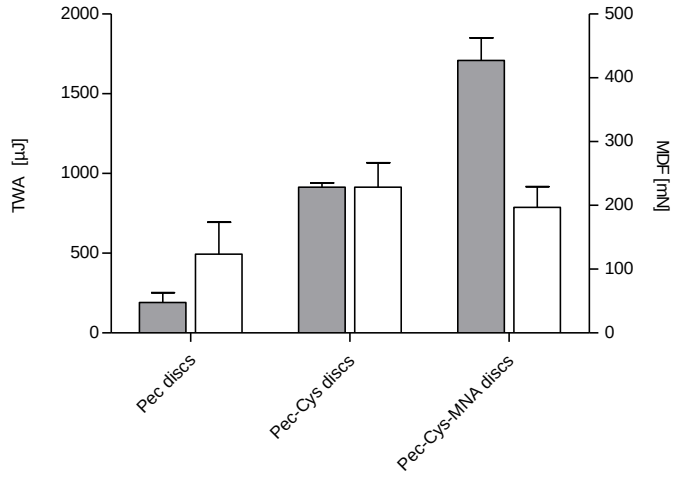

Figure 4.
Bar chart shows results of tensile studies carried out with discs of synthesized excipients and unmodified pectin. Grey bars display the total work of adhesion (TWA), white bars the mean detachment force (MDF). Indicated values are means + SD, n = 5. Means of TWA differ from each other significantly, MDF of Pec differs from MDF of Pec-Cys significantly (Newman-Keuls Multiple Comparison Test $P < 0.05$).

To investigate the adhesive properties of the lidocaine formulations, two methods were employed. The first method is based on the rheological synergism of mucoadhesive polymers. For pectin only a small increase of dynamic viscosity could be measured after incubation time. The mucin gel without polymer showed a dynamic viscosity of 0.20 ± 0.08 Pa*s. After 2 hours of incubation of mucin gel with the pectin formulation, resulting dynamic viscosity was 0.22 ± 0.02 Pa*s. For the Pec-Cys-based formulation, the addition of mucin resulted in an increase of 300 % of dynamic viscosity after 30 min of incubation and 660 % after 2 hours of incubation with mucin compared to dynamic viscosity of solutions/gels containing the same amount of polymer (1 % m/v) without mucin. For Pec-Cys-MNA-based formulations the increase of dynamic viscosity was about 24 % after 30 min and 43 % after 2 hours, whereat the value of dynamic viscosity of Pec-Cys-MNA-formulation/mucin mixture was 15 % higher than the value of dynamic viscosity of Pec-Cys-formulation/mucin mixture after 2 hours. For all investigated polymer/mucin mixtures, tanδ was below 1, showing gel formation.

The second method is based on a set-up developed for investigation of mucoadhesion of nanoformulations. Thereby, the remaining amount of a fluorescence marker (NaFlu) in the formulation was determined. The formulation was applied to buccal tissue and rinsed with PBS 0.02 M pH 6.8 for a certain time period. Results, given in Figure 5, show that there is a significant difference between the formulations. After 1.5 h, only 17.7 % of NaFlu remained on the tissue applied in pectin-solution and 28.5 % applied in Pec-Cys formulation, whereat 55 % of NaFlu in Pec-Cys-MNA formulation were still on the tissue. Even after 4.5 h, about 24-fold more marker remained on the mucosa compared to the amount of marker applied in Pec-based formulation and about 2-fold higher amount of NaFlu compared to the Pec-Cys-based formulation.

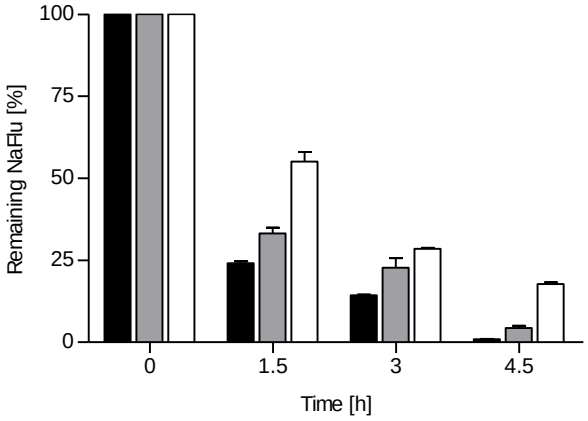

Figure 5.
Amount of NaFlu remaining on buccal porcine mucosa after application of NaFlu containing Pec- (black bars), Pec-Cys- (grey bars), and Pec-Cys-MNA- (white bars) based formulations. Indicated values are means of 4 experiments (+ SD). For time points 1.5 h, 3 h and 4.5 means for Pec, Pec-Cys and Pec-Cys-MNA differ significant from each other. (2 way ANOVA followed by Bonferroni multiple comparison post test, level of significance at least P = 0.05)

3.6. *In vitro* release studies

Release studies using a cellulose acetate membrane in Franz diffusion cells showed a significant sustained release of lidocaine from both gel formulations compared to the pectin-based formulation. To determine effect of the filter membrane on release, studies with pure lidocaine solution were carried out additionally. Percentage of released lidocaine over time is given in Figure 6.

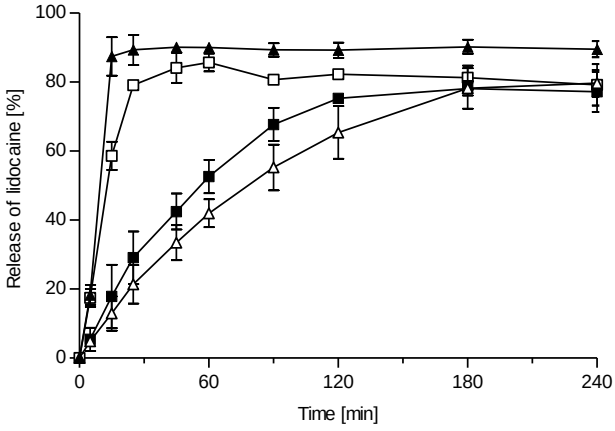

Figure 6.
Release profiles of lidocaine from formulations based on pectin, pectin-cysteine and pectin-cysteine-mercaptonicotinic acid. Tests were carried out using Franz diffusion cell and cellulose acetate membrane (0.2 µm). Pec (-□-), Pec-Cys (-■-), Pec-Cys-MNA (-Δ-), lidocaine solution (-▲-). Indicated values are means ± SD, n = 4.

3.7. Viability test

Results of MTT assay are given in Figure 7. Data analysis revealed, that there was no significant difference between tested substances and negative control.

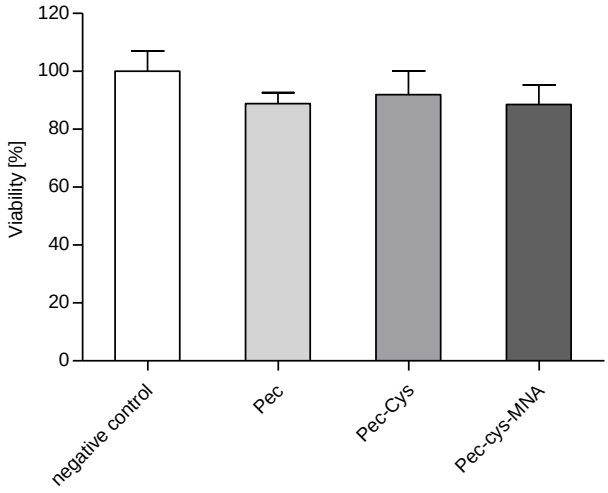

Figure 7.
Bar chart shows cell viability of native pectin and indicated derivatives after 3 h. Cytotoxicity was tested on Caco-2 cells using MTT assay and expressed in percent. Indicated values are means ± SD, n=4. Means of tested polymers do not differ from positive control significantly. (Bonferroni's Multiple Comparison Test, $p<0.05$).

4. Discussion

Requirements for an ideal formulation for local drug delivery are convenient application, retention on the application area and a controllable drug release. For local buccal drug delivery, these characteristics can be met by gel formulations. In this study, a gel formulation based on a recently developed pectin derivative was prepared and investigated for properties in buccal drug delivery. Native pectin needs the presence of cross-linking agents like divalent cations (calcium), sugar or other polymers like alginates or cellulose derivatives for gelling [28]. In the present work it could be shown that it is possible to obtain a pectin based gel without the addition of any other substance. Rheological investigations were performed to characterize the resulting gel structure. As reported previously, crosslinking disulfide bonds are responsible for gel formulation. Marschütz et al. investigated viscosity and the content of free thiol groups of thiolated poly(acrylic acid) in a 3 % (m/v) solution over 24 hours at 37 °C under shaking. A loss of free thiol groups was determined whereat the apparent viscosity increased. The loss tangent values decreased from 2.29 to 0.29 indicating a gel formation [18]. At given conditions, a massive oxidation of thiol groups can be assumed, resulting in gel formation [13, 18]. The dynamic viscosity of Pec-Cys-MNA compared to Pec-Cys increased significantly although there are less free thiol groups for inter- and intermolecular crosslinking available. Therefore, resulting gel structure cannot be caused by intra- and intermolecular

disulfide bonds. Rather this phenomenon can be explained by the heightened hydrophobicity due to the implementation of the aromatic ligand, as in general the viscosity of polymers can be increased with hydrophobic modification to a certain extend [29]. Hydration of the gel was investigated as being important for mucoadhesion and drug release. Results of this study showed that swelling behavior of the formulations is in good accordance with the behavior of typical hydrogels: Due to the crosslinking, the hydrogels do not dissolve in the aqueous incubation medium but absorb water [1]. Incorporated water leads to chain relaxation and the drug can be released through the hydrogel structure. With displaying different hydrophobic characteristics a difference between the two polymers was anticipated but the experiment did not reveal any significant difference at all. Further, the polymer has to expand to induce mobility in the polymer chains to improve interpenetration process between polymer and mucin [30].

In general, drug delivery on the oral mucosa is difficult because of a constant flow of saliva and mobility of the tissue, limiting residence time of drugs administrated to the oral cavity. With buccal mucoadhesive formulations, residence time is expected to be prolonged and drug delivery improved [2]. Pectin has many hydroxyl groups being able to form hydrogen bonds with hydroxyl, carboxyl and amide groups. Due to that, interaction between the polymer and biological mucus is possible. Resulting formation of secondary chemical bonds between hydrogen bonding groups from pectin and functional groups of glycoproteins in mucus are very likely. Mucoadhesive properties of pectin have been reported within different studies: Smart et al. found an adhesiveness of pectin when investigated with mucus gel employing a Wilhemy plate method [31, 32]. Furthermore mucoadhesive properties of pectin on gastrointestinal mucosa have been shown [28, 33]. Thanks to free thiol groups the attached ligands are able to form covalent bonds with cysteine-rich subdomains of the mucus gel layer by disulfide exchange reactions or oxidative coupling [16, 34, 35].

The origin idea of preactivating thiomers was to prevent free thiol groups from inter- and intramolecular disulfide bond formation as already oxidized thiol groups are hardly available for interaction with mucin. Further, reactivity should be ensured over a broad pH range [13]. Covalent chromatography provides the hypothesis that preactivated thiol groups can be bond more efficiently to mucin as it was shown that peptides and proteins are linked to thiol group bearing resins more efficiently when activated with pyridyl substructures [36, 37]. According to this, preactivated thiol groups should be able to form disulfide bonds with thiol bearing subdomains of mucin by disulfide exchange reaction with the aromatic thiol moiety as leaving group. The pectin derivative investigated in this study showed improved mucoadhesion. Improvement could be shown for both experimental setups, tensile studies on porcine buccal mucosa and rheological investigations with porcine mucin. Taking this into account, the preactivated thiolated pectin

seems promising for application not only for buccal but for other kinds of mucosal tissue. These results are in line with the assumption of protecting and activating thiol groups by attaching a protecting group with pyridyl substructure. The TWA was raised significantly for both derivatives compared to unmodified pectin, 4.8-fold increase for Pec-Cys and 8.9-fold for Pec-Cys-MNA, respectively.

The rheological synergism-method is based on the fact that interactions between the mucoadhesive polymer and mucus glycoproteins cause a rheological change as described in 1990 by Hassan and Gallo [38]. It has been reported that an increase in viscosity of polymer/mucus mixtures correlates with mucoadhesive qualities of the polymer [39]. In this investigation, commercial freeze-dried mucins have been used. Unfortunately, when lyophilized mucins are redispersed in water, resulting viscoelastic properties are different from freshly isolated mucus gel. As it is difficult to obtain reproducible amounts of glycoproteins when isolated freshly from the mucosa, commercial mucins have their warrant and therefore have been used in this investigation [40, 41]. The relative increase of viscosity was most pronounced for the Pec-Cys formulation whereat the Pec-Cys-MNA/mucin mixture showed highest value for viscosity. A high viscosity of the formulation without mucin indicates a strongly cross-linked and entangled system. Kelly et al. stated that such high entangled systems make it difficult for mucin chains to penetrate into the polymer, whereas systems with reduced level of crosslinking showed an enhanced mucoadhesion [27]. Moreover, this interlaced system is the base for a sustained drug release. Mucoadhesion studies in the “angular half-pipe” combines under most authenticable conditions mucoadhesion and release studies. This experiment points out that preactivated thiolated pectin can enhance retention time of a model drug at buccal tissue site and therefore is a promising candidate for drug delivery in the oral cavity.

Experiments with Franz diffusion cell using a cellulose acetate filter as barrier were carried out to ensure modified release of the developed formulation. A sustained, close to linear, release for about 2 hours from Pec-Cys-MNA-based formulation was observed. A reason for a lower release of the drug from the pectin, Pec-Cys- and Pec-Cys-MNA-based formulations compared to pure lidocaine solution might be interactions of protonated basic amino group of lidocaine hydrochloride with deprotonated carboxyl groups of pectin [28].

5. Conclusion

In the current study, a recently developed pectin derivative was used for preparation of a drug loaded gel formulation and investigated for its properties in buccal drug delivery. Due to self-crosslinking gel preparation was possible without the addition of any other excipient. Prepared gels showed stability and the ability for water uptake in aqueous environment. It was demonstrated that synthesized derivatives and the formulations possess improved mucoadhesive properties compared to the unmodified polymer. Residence time of model drug loaded formulation on the application area was prolonged significantly. Furthermore, compared to control formulation based on native pectin, both gels showed sustained release. Pec-Cys-MNA showed higher viscosity and a more sustained release than Pec-Cys whereat the total amount of released drug was for both gel formulations equal. Considering results of these investigations, especially the improved mucoadhesion, sustained release and prolonged retention time on tissue, the pectin-based excipient “Pec-Cys-MNA” seems promising and suitable for buccal drug delivery.

6. Acknowledgement

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Synthesis and characterization of pH-tolerant and mucoadhesive (thiol–PEG)-chitosan-graft-polymer for drug delivery

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Abstract

The object of this study was to generate a water-soluble thiolated chitosan to enable the permeation enhancing effect of chitosan at pH ≥ 5.5 without losing the advantages of improved mucoadhesive properties. Therefore, the thiol bearing polyoxyethylene ligand (O-(3-carboxylpropyl)-O'-[2-[3-mercaptopropionylamino)ethyl]-polyethyleneglycol) was conjugated via amide bond formation to the amino group of chitosan. Resulting novel chitosan derivative (Chito-PEG-SH) exhibited 250 µmol free thiol groups per gram polymer. By the attachment of the thiol bearing PEG ligand an improvement of permeation enhancing effect on rat intestine (2.7-fold improvement) as well as on a Caco-2 monolayer model (1.9-fold improvement) could be found. Cytotoxicity studies on Caco-2 cells revealed no change in biocompatibility. Mucoadhesion was improved 3.1-fold by the formation of disulfide bonds with mucus glycoproteins. The mucoadhesive effect of Chito-PEG-SH turned out to be similar to thiolated chitosan and more pronounced than mucoadhesive properties of unmodified chitosan. The graft polymer is soluble in water and aqueous solutions over a broad pH range. In aqueous media the novel polymer does not precipitate at pH ≤ 8.6. According to these results Chito-PEG-SH might show potential as auxiliary agent in oral drug delivery where its solubility even up to pH 8 is likely beneficial.

Key words: pegylation, water soluble chitosan, thiomers, toxicity, mucoadhesion

1. Introduction

Chitosan is a biocompatible, non-toxic biopolymer being of interest in different fields of application. In pharmaceutical research, it found its entries in the early 1990s and it is still subject of great interest because of certain remarkable properties. The polymer exhibits mucoadhesive properties, a permeation enhancing effect for different kinds of drugs, efflux pump inhibition, transfection enhancing properties and sustained release [1-4]. However, besides these promising features chitosan offers, there are some drawbacks hampering its use in drug delivery. The probably most interfering property of chitosan is the limited solubility. In aqueous environment it is only soluble at pH values lower than its pks (≤ pH 5.5-6.5) when amino groups of the polysaccharide chain are protonated [5]. Being not completely soluble under physiological conditions in the intestine, it is not possible to take full advantage of its properties. Kotzé et al. reported that chitosan failed to improve permeation on Caco-2 cell monolayer at pH values ≥ 7.2 whereat water soluble quaternized chitosan was able to decrease transepithelial electrical resistance and increase permeability of mannitol [6]. These findings were confirmed by *in vivo* studies in rats and pigs [7-9]. Besides quaternized chitosan, pegylation of chitosan led to improved water solubility from pH 1.0 to 11.0 depending on the degree of modification [5, 10, 11]. Additionally, reduced cytotoxicity compared to unmodified chitosan could be shown for pegylated chitosan with an even improved ability to enhance permeation of macromolecular substances [12, 13].

Modification of chitosan with thiol-bearing ligands led to improved mucoadhesion, sustained release, permeation enhancing and efflux pump inhibition in several studies compared to unmodified chitosan [14-17]. Considering this, the aim of this study was to combine the advantages of pegylated and thiolated chitosan developing a novel soluble, biocompatible, permeation enhancing chitosan derivative with improved mucoadhesion compared to unmodified chitosan. For this purpose, chitosan was modified by covalent attachment of the thiol bearing PEG-ligand O-(3-carboxylpropyl)-O'-[2-[3-mercaptopropionylamino)ethyl]-polyethylenglycol (COOH-PEG-SH) to the primary amino group of chitosan. This newly synthesized chitosan derivative was investigated in terms of solubility, permeation enhancing properties across Caco-2 cell monolayer and freshly excised rat intestine, mucoadhesion and cytotoxicity.

2. Materials and Methods

2.1. Materials

Low viscous chitosan (average molecular weight 150 kDa, deacetylation degree of 85 %), 5,5'dithiobis-(2-nitrobenzoic acid) (Ellman's Reagent), resazurin, ethanol, acetone, DMSO, Triton-X 100, dialysis cellulose membrane tubes (molecular weight cut-off 12 kDa), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and O-(3-carboxylpropyl)-O'-[2-[3-mercaptopropionylamino)ethyl]-polyethylenglycol (COOH-PEG-SH, 3000 Da) were purchased from Sigma Aldrich (Vienna, Austria). Flouresceinisothiocyanate-dextran (FD4, 4400 Da) was supplied by TdB Consultancy AB (Uppsala, Schweden). Professor Pfaller, Institute of Physiology, Medical University of Innsbruck kindly donated Caco-2 cells. Transwell- and wellplates were purchased from BioGreiner, Austria. All other cell culture care supplies were obtained from PAA, Austria. All chemicals were of analytical grade and used as received.

2.1.1. Preparation of porcine mucus

Small intestine from pigs was obtained from a local slaughter and transported on ice to the laboratory. The mucus was collected from the tissue using a scraper. To homogenize and purify the collected mucus, it was mixed with a sodium chloride solution (0.1 M) and stirred for one hour on ice. Next, the mixture was centrifuged at 4 °C and 9000 rpm for 2 hours. The supernatant and granular material on the bottom were discarded and homogenized, purified mucus was collected for immediate use.

2.2. Synthesis of chitosan-PEG-SH

To prepare the thiol group bearing pegylated chitosan, O-(3-carboxylpropyl)-O'-[2-[3-mercaptopropionylamino)ethyl]-polyethylenglycol (COOH-PEG-SH) was chemically bound to chitosan (chitosan-PEG-SH) via amide bound formation between the primary amino group of chitosan and the carboxyl group of the ligand. The reaction was mediated by a carbodiimide (EDAC) and N-hydroxysulfosuccinimide (NHS). Therefore, 500 mg of chitosan were dissolved at pH 3 (adjusted with HCl) in 25 mL of water. Next, 200 mg of COOH-PEG-SH were dissolved in 25 mL demineralized water, EDAC and NHS were added in a concentration of 100 mM. The pH was adjusted to 5, and mixture was stirred for 30 min. Then, the solutions were combined and stirred overnight. To purify the product of unbound ligand, EDAC and NHS the reaction mixture was diluted and dialyzed against water pH 3 (adjusted using 5 M HCl) for 3 days using Spectra/Por® 3 membranes (MWCO 12 kDa). The dialysate medium of 5 liter was replaced every 12 hours, during the second day of dialysis, 1 % NaCl was added. To avoid disulfide bond formation,

purification occurred at 10 °C in the dark. Finally, the product was frozen at -80 °C and lyophilized for 2 days under reduced pressure.

2.3. Synthesis of chitosan-TGA

For comparison, chitosan-thioglycolic acid (chitosan-TGA), a thiolated but not pegylated chitosan derivative was prepared. Synthesis of the thiomer with a similar degree of substitution was performed according a method described by Kast and Bernkop-Schnürch, the ligand was bound via amide bound formation between the carboxylic acid groups of thioglycolic acid (TGA) and primary amino groups of chitosan [18]. The resulting product was lyophilized after purification via dialysis. Additional, chitosan was treated the same way omitting EDAC and NHS.

2.4. Determination of degree of substitution

The amount of bound COOH-PEG-SH and thioglycolic acid was determined by quantifying the thiol groups spectrophotometrically using Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) according to a method described previously [19]. For the calibration curves, COOH-PEG-SH and thiolglycolic acid were employed.

2.5. Determination of solubility

To investigate the solubility of chitosan-PEG-SH in comparison to non-pegylated thiomer and unmodified chitosan, mixtures of aqueous buffer and polymer (0.5 % (m/v)) were prepared and stirred overnight. To examine solubility in aqueous environment at different pH values acetate buffer 0.1 M (pH 4 and 5), phosphate buffer 0.1 M (pH 6, 7 and 8) and carbonate buffer 0.1 M (pH 9 and 10) were used. Further experiments with acetone, ethanol and DMSO were carried out. To confirm findings of this study, chitosan-PEG-SH, chitosan-TGA and unmodified chitosan were dissolved in a concentration of 0.5 % (m/v) at pH 4. By adding 1 M NaOH stepwise to the solution the pH was raised until the polymer precipitated. The pH-values were measured until precipitation of the polymer occurred.

2.6. Cytotoxicity studies – resazurin assay

To investigate the influence of the derivatives on cell viability, a resazurin assay was performed. The assay is based on the reducing environment of viable cells. Resazurin, a blue non-fluorescent substance is converted into resorufin, a red fluorescent substance in the presence of metabolic active cells [20]. For the assay, Caco-2 cells were cultured in a 24-well plate for 10 days at 37 °C in 5 % CO_2 environment. The minimum essential medium with Earls salts containing FCS was replaced every second day. 500 µL of the prepared polymer solutions 0.05 % (m/v) of different conjugates and unmodified chitosan were added to

each well. As negative control minimal essential medium without phenol red and FCS was employed whereas Triton X® 100 4 % (v/v) served as positive control. The experiment was carried out in quadruplicates. The cells were incubated with samples for 24 hours. Thereafter, samples were removed and cells were washed two times with prewarmed (37 °C) phosphate buffered saline. Then, 250 µL of a 2.2 µM resazurin solution was added to each well and incubated for another 2 hours. The fluorescence of the supernatant was measured at 540 nm with background subtraction at 590 nm with Tecan infinite, M200 spectrometer, Grödig Austria [21, 22].

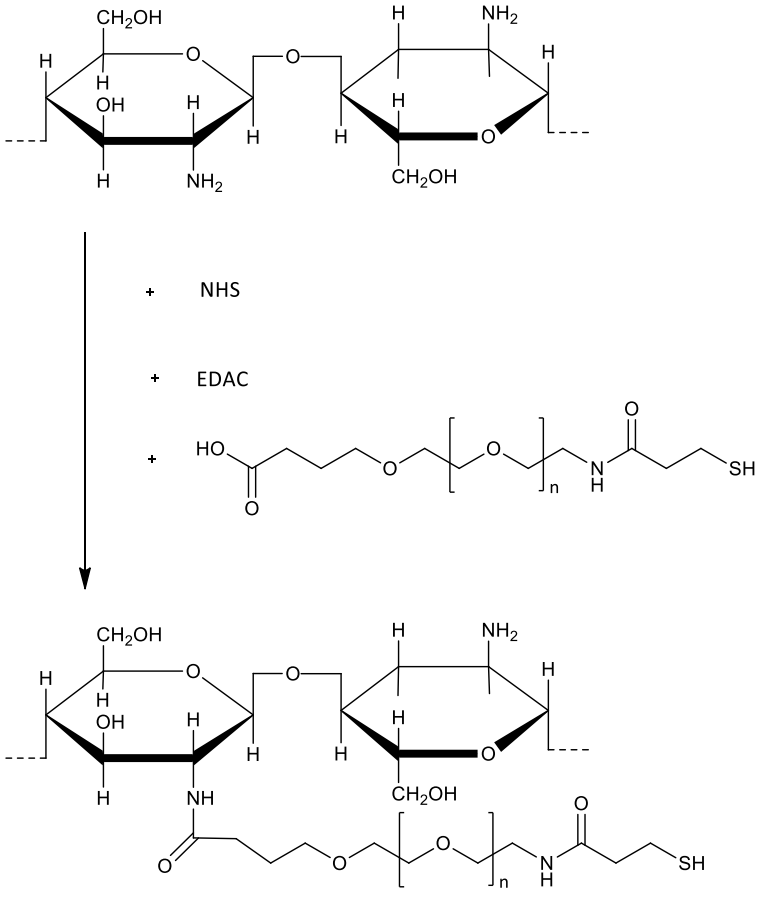

Figure 1.
Synthesis of chitosan-PEG-SH.

2.7. Permeation studies

2.7.1. Permeation across Caco-2 monolayer

The permeation enhancing properties of chitosan-PEG-SH were evaluated in comparison to unmodified chitosan and chitosan-TGA. Therefore a Caco-2 cell monolayer model was employed [23]. Caco-2 cells were kept under conditions as described above. For permeation studies, cells were cultivated on transwell-plates in a density of 1 x 10^5 cells/mL. In order to achieve a complete monolayer and totally differentiated cells, cells have been cultivated for 21 days. During these time the medium was replaced every 48 hours. The transwell system (Greiner bio one®) had a diffusion area of 0.33 cm^2 and the pore size of the PET membrane was 0.4 µm. Transepithelial electrical resistance (TEER) was measured during the 21 days of cultivation and directly before, during and after the experiment using epithelial voltohmmeter (EVOM, World Precision Instruments, Germany) with a pair of side-by-side electrodes. Monolayers employed for transport studies exhibited a TEER value between 500 and 600 Ωs/cm^2. Before the experiment, cells were washed with phosphate buffered saline. Then 600 µL of minimal essential medium without phenol red and FCS (MEM) were added in the outer chamber and 100 µL of MEM were added to the inner chamber. After 30 min of preincubation the medium of the donor chamber was replaced with 100 µL of 0.5 % (m/v) polymer solution containing 0.05 % of the fluorescent and hydrophilic model drug FD4 (m/v) to investigate the transport along the paracellular route. As control, a FD4 solution 0.05 % (m/v) in buffer was used. Over 3 hours every 30 min samples of 100 µL were withdrawn from the acceptor chamber and replaced with MEM. The cumulated amount of permeated FD4 was calculated after measurement of the fluorescence (λ_{ex} = 485 nm, λ_{em} 535 nm) using a microplate reader (Tecan infinite, M200 spectrometer, Grödig, Austria). A calibration curve using FD4 was established. Further, the apparent permeability coefficients (P_{app}, cm/s) for FD4 were calculated as follows: $P_{app} = \frac{Q}{A*c*t}$

With Q = total amount permeated FD4 [µg], A = diffusion area [cm^2], c =initial concentration in the donor chamber [µg/cm^3] and t = time of the experiment [s].

2.7.2. Permeation across freshly excised rat intestinal mucosa

To confirm the results of the permeation studies across the cell monolayer, permeation studies across freshly excised rat intestine were carried out [24]. Therefore, the small intestine (jejunum, ileum) of a non-fasting male Sprague-Dawley rat weighing approximately 250 g was removed directly after sacrificing. The intestine was cut into pieces of 1.5 cm and rinsed free of luminal contents and clamped on Ussing type chambers with a permeation area of 0.64 cm^2. 1 mL of HEPES-Buffer (138 mM NaCl, 1mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 10 mM HEPES) was added to each chamber. Ussing chambers were

placed into a waterbath at 37 °C and allowed to equilibrate for 30 min. Next, the medium of the donor chambers was replaced by solutions of polymers 0.5 % (m/v) containing FD4 at a final concentration of 0.05 % (m/v). The pH of the samples was adjusted to 6.4. For control, the donor chamber was loaded with a FD4 solution in HEPES buffer 0.05 % without the polymer. During 3 h every 30 min samples of 100 µL were withdrawn from the acceptor chamber. The volume was replaced by 100 µL of prewarmed buffer. TEER measurements were made before, after and during the experiment. Analytics and calculations were made as described above. Experiments were carried out in triplicates.

2.8. Mucoadhesion

2.8.1. Viscosity and mucoadhesion

The dynamic viscosity of synthesized chitosan derivatives and unmodified chitosan was examined with and without the addition of porcine intestinal mucus. Therefore, dynamic oscillatory tests were carried out after determining the linear viscoelastic range at a frequency of 1 Hz for samples of 750 µL. A plate-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) was used, shear rate was set to 50 s^{-1}, temperature to 37.0 °C ± 0.1 °C and the gap to 0.5 mm. To determine the mucoadhesive properties of the conjugates and unmodified chitosan, 500 µL samples of 1 % solutions (acetic buffer 0.1 M, pH 4.5) were added to 500 µL of porcine mucus obtained from the intestine of a fresh slaughtered pig. The polymer-mucus mixtures were intermingled and incubated at 37 °C for 30 min before the measurement. All measurements were carried out in triplicates.

2.8.2. Tensile studies

To compare mucoadhesive properties of chitosan-PEG-SH, chitosan-TGA and unmodified chitosan, tensile studies were performed as described previously by our research group [18]. In brief, test discs were prepared by compressing 30 mg of polymer lyophilisates with a pressure of 10 kN for 20 s (Paul Weber, Remshalden-Grünbach, Germany) resulting in flat-faced discs with a diameter of 5.0 mm and 2.0 mm thickness. Fresh excised porcine intestine was cut into pieces and cleaned carefully. The porcine tissue was placed and mounted at the bottom of a beaker containing 0.1 M phosphate buffer at pH 6.8. Tablets were fixated on a string and attached to the mucosa by applying mild force. The system was placed on a balance connected to a computer. After 5 min of incubation at room temperature the tissue was pulled off from the disc at a rate of 0.1 mm/s. Every second data points were taken by a computer software (Sarta Collect; Satorius AG). The area under the force/distance curve was calculated representing the total work of adhesion (TWA). Moreover the maximum detachment force (MDF) was determined [25].

2.9. Statistical data analysis

For all statistical data analysis the GraphPad Prism 5 software was used. ANOVA with $p<0.05$ as the minimal level of significance was performed followed by Bonferroni's Multiple Comparison Test with $p<0.05$ as minimal level of significance or Newman-Keuls Multiple Comparison Test with $p<0.05$ as minimal level of significance where suitable.

3. Results

3.1. Characterization of chitosan-conjugates

PEG-SH and TGA were attached covalently to chitosan. Amide bond formation between the carboxylic acid moieties of the ligands and the amino groups of chitosan was mediated by EDAC/NHS. The synthetic pathway of the reaction leading to chitosan-PEG-SH is illustrated in Figure 1. Ellman's test revealed that 244 ± 26 µmol of PEG-SH and 253 ± 19 µmol of TGA were attached per gram polymer. A control prepared in the same way omitting EDAC/NHS showed a negligible amount of remaining PEG-SH and TGA. Lyophilized polymers were of fibrous structure and eggshell white.

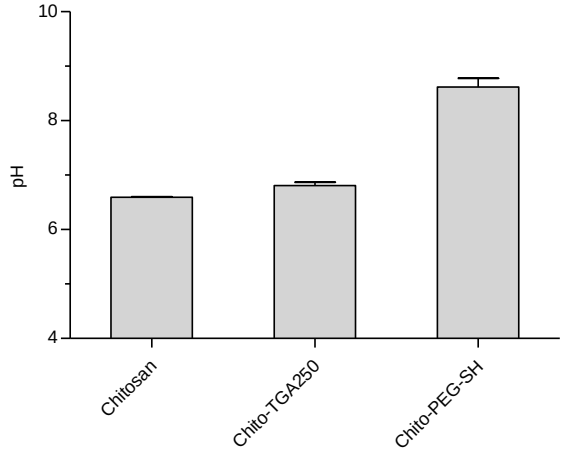

Figure 2.
Graph shows highest pH values measured without precipitation of the polymer. Each point shows mean ± SD of 3 experiments.

3.2. Water- and organosolubility

Table 1 summarizes the results of the solubility test carried out at room temperature in different buffer systems. Implementation of the PEG-SH ligand improved solubility at higher pH values. Additionally, a solubility of chitosan-PEG-SH in DMSO was observed, whereas unmodified chitosan and chitosan-TGA could not be dissolved in tested organic solvents. Modification with TGA did not influence solubility of chitosan. Furthermore, as shown in Figure 2, the pH of chitosan-PEG-SH solution could be raised to 8.6.

Table 1. Solubility of chitosan and its derivatives in different bufferd. + = dissolved, - = not dissolved.

Solvent	Chitosan	Chitosan-TGA	Chitosan-PEG-SH
Acetate buffer pH 4	+	+	+
Acetate buffer pH 5	+	+	+
Phosphate buffer pH 6	-	-	+
Phosphate buffer pH 7	-	-	+
Phosphate buffer pH 8	-	-	+
Carbonate buffer pH 9	-	-	-
Carbonate buffer pH 10	-	-	-

3.3. Cell toxicity test

Metabolic activity of vibrant cells have been investigated with a fluorometric cell viability assay. Over a time period of 24 hours all investigated polymers did not show a cytotoxic effect at the investigated concentrations.

3.4. Permeation studies

3.4.1. Caco-2 monolayer

A Caco-2 cell monolayer was used as an *in vitro* model for intestinal epithelium to investigate the effect of chitosan-PEG-SH on permeation in comparison to chitosan and chitosan-TGA. The transport rate of the model compound FD4 across the monolayer is given in Figure 3A. Both thiolated chitosan derivatives were able to improve transport of FD4 across the monolayer compared to unmodified chitosan and to the FD4 control solution without the addition of a polymeric excipient. After 90 min the amount of permeated FD4 with chitosan-PEG-SH and chitosan-TGA differed significantly from the amount determined using chitosan and control (two-way ANOVA followed by Bonferroni multiple comparison test, $p < 0.05$). The apparent permeability coefficient P_{app} and the enhancement ratio defined as the mean P_{app} of control divided by the mean of P_{app} of the polymer are given in Table 2. The decrease of TEER values is in line with permeated amount of FD4. After 3 hours the TEER value was for the control sample around 94 %, for chitosan, chitosan-TGA and chitosan-PEG the value dropped down to 77 %, 66 % and 61 %, respectively. Within 24 h cells regenerated to TEER values equal to the initial values.

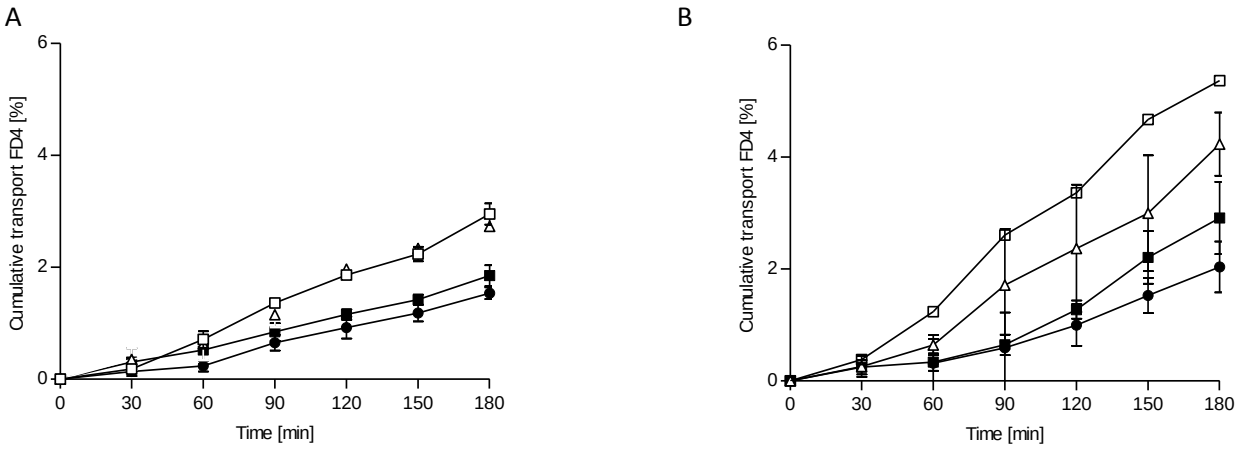

Figure 3.
Effect of chitosan-PEG-SH on transport of FD4 across Caco-2 monolayer (A) and rat intestine (B) in comparison to chitosan and chitosan-TGA. Each point shows mean ± SD of 4 experiments. Control (-●-), chitosan (-■-), chitosan-TGA (- Δ -), chitosan-PEG-SH (-□-).

3.4.2. Rat intestine

Ex vivo permeation studies have been carried out employing freshly excised rat intestine. Figure 4 gives the cumulative transport of the hydrophilic model drug FD4 along the paracellular route. Both thiolated chitosan derivatives improved the transport rate of FD4 across the intestine compared to unmodified chitosan and the control. After 3 hours amounts of permeated FD4 administered with the chitosan derivatives differed significantly from the FD4 control solution and unmodified chitosan (two-way ANOVA followed by Bonferroni multiple comparison test, $p < 0.001$). The apparent permeability coefficient P_{app} and the enhancement ratio are given in Table 2. The TEER value decreased during the measurements to 90 % for the control solution, to approximately 70 % for chitosan and chitosan-TGA and to around 60 % for chitosan-PEG-SH.

Table 2. Apparent permeability coefficient (P_{app}) and enhancement ratio for FD4 across CaCo-2 cell monolayer and rat intestine. Values are means of 4 experiments ± SD. Enhancement are calculated regarding the control solution without polymer. *=P_{app} values differ significantly from others. (One-way ANOVA followed by Bonferroni post test).

Polymer	$P_{app}*10^{-6}$ [cm/]		Enhancement ratio	
	CaCo-2	Rat intestine	CaCo-2	Rat intestine
-	1.1 ± 0.1	5.7 ± 1.3	1	1
Chitosan	1.3 ± 0.1	8.1 ± 1.8	1.2	1.4
Chitosan-TGA	1.9 ± 0.3	11.9 ±1.6	1.8	2.1*
Chitosan-PEG-SH	2.1 ± 0.1	15.1 ± 1.9	1.9	2.7*

3.5. Mucoadhesion studies

As the mucoadhesion of investigated polymers depends highly on the method applied, two different methods, tensile strength studies and rheological synergism, were employed within this study. Results of tensile studies including the maximal detachment force and the total work of adhesion are given in Figure 4. The improvement ratio for TWA and MDF of chitosan-PEG-SH compared to chitosan were 3.1 and 3.5, respectively. In comparison, the improvement ratio of chitosan-TGA were 2.7 and 3.1. Results of rheological investigations are provided in Table 3. Both chitosan derivatives showed an improved mucoadhesive effect compared to unmodified chitosan.

Table 3. Viscosity and mucoadhesion. Values of viscosity of polymers in a total concentration of 1 % with and without porcine mucus. Indicated values are means of at least three experiments ± SD.

	Dynamic viscosity η [Pa*s]	
Polymer	**1 % solution**	**+ mucus after 30 min**
Chitosan	0.027 ± 0.059	294.6 ± 22.1
Chito-TGA_{250}	0.041 ± 0.002	353.2 ± 26.7
Chito-PEG-SH	0.650 ± 0.119	314.1 ± 50.9

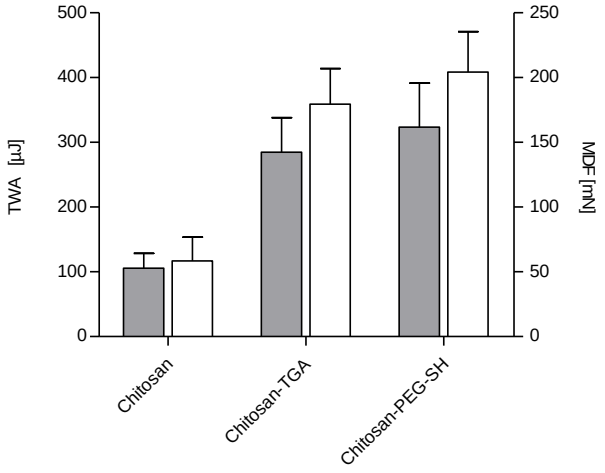

Figure 4.
Bar chart shows total work of adhesion (TWA, grey bars) and maximal detachment force (MDF, white bars) as results of tensile studies carried out with discs of chitosan, chitosan-TGA and Chitosan-PEG-SH. Indicated values are means + SD, n =3.

4. Discussion

Chitosan is listed and investigated as promising pharmaceutical excipient, properties like mucoadhesion, sustained release and a permeation enhancing effect are encouraging. Most of these features are pH depended as chitosan has to be dissolved to develop full potential. The often reported permeation enhancing effect of the polymer is of no use when it cannot be exploit in the intestine, the resorption site

of most drugs. Chitosan is only soluble at acidic conditions making it impossible to employ full benefit of the permeation enhancing effect *in vivo*. Further, mucoadhesive properties of chitosan compared to other polymers lag behind. To overcome these hindrances, many different chitosan derivatives resulting in different qualities were developed. Pegylated chitosan is reported to show improved solubility in organic solvents and a broader range of pH values in aqueous environment dependent on their degree of modification, molecular weight of ligands and the type of chitosan. The derivative synthesized in this study showed solubility up to a pH value of 8 whereas unmodified chitosan and chitosan-TGA could not be dissolved at pH 6. Further, chitosan-PEG-SH showed solubility in dimethyl sulfoxide. The improved solubility is to be explained by the hydrophilicity of the attached ligand and by the size of the implemented side chains. The poor solubility of chitosan results from its partially crystalline structure and tight hydrogen bonds between the amino and hydroxyl groups of chitosan. The attachment of a polyethylen glycol ligand results in reduced order of the structure of the polymer backbone by spreading the polymer and lowering hydrogen bonding capacity leading to enhanced accessibility and affinity for organic and aqueous solvents [26].

Chitosan is considered as a non-toxic biodegradable polymer and suitable for topical or oral application of drug formulations. Several studies reported a decrease in cytotoxicity of chitosan-PEG derivatives, depending on the degree of substitution and molecular weight of the polyethylene glycol (PEG) ligand. In general, with increasing degree of substitution and molecular weight of the ligand, the toxic effect on cells decreased [5]. Biocompatibility is affected by different properties of the polymer such as molecular weight, charge density, structure and conformational flexibility. Mao et al. explained the decrease in cytotoxicity of peglyated trimethyl-chitosan in comparison to trimethyl-chitosan by steric effects, which are able to shield the positive charges [13].

Chitosan is known to ease the transport of hydrophilic substances along the paracellular route due to interactions with the cell membrane resulting in structural changes in tight junction proteins [12]. It has been reported that water soluble chitosan (trimethyl-chitosan) shows a permeation enhancing effect superior to unmodified chitosan in a cell model at physiological pH ≥ 7.2 and *in vivo*. This effect was related to the non-solubility of chitosan under these conditions [27]. Moreover, several studies showed that thiolated chitosan-derivatives exhibit a more pronounced permeation enhancing effect than unmodified chitosan. Here, the effect is explained by interaction of thiol groups with the enzyme protein tyrosine phosphatase which regulates the opening and closing process of tight junctions by a glutathione-dependent process [28]. Casettari et al. published that the effect of peglyated chitosan depends highly on the pH value leading to the conclusion that the effect results from remaining unmodified amino groups on

the chitosan backbone. However, a significant improved permeation enhancing effect for pegylated chitosan compared to unmodified chitosan was shown within the same study carried out at pH 6.0 employing a cell monolayer setup (Calu-3) [12]. To determine permeation enhancing properties of the novel chitosan-PEG-SH derivative, permeation studies have been carried out across Caco-2 cell monolayer and freshly excised rat intestine using the hydrophilic marker FD4 for the paracellular route. Experiments carried out on the monolayer showed a significant higher amount of permeated FDA mediated by both thiol bearing chitosan derivatives compared to chitosan and the control without the addition of any further excipient. There was no significant difference in P_{app} of chitosan-TGA and chitosan-PEG-SH. Transport experiments across the rat intestine revealed a slightly increased effect for chitosan-TGA and a significant higher transport rate for chitosan-PEG-SH with an improvement ratio of 2.7.

Since determined parameters depend on chosen *in vitro* model mucoadhesion was investigated by two different models. Rheological investigation revealed a significant higher viscosity of the developed derivative compared to unmodified chitosan and chitosan-TGA. The higher viscosity can be explained by inter- and intramolecular disulfide bond formation resulting in higher viscosity for both investigated chitosan derivatives. Further, for chitosan-PEG-SH interactions of implemented side chains are assumed via hydrogen bond formation resulting in significant higher viscosity than unmodified chitosan or chitosan modified with short ligands like TGA. Rheological synergism was shown for all investigated polymers whereat the effect was significantly more pronounced for both thiolated chitosans. The ability of improving mucoadhesive properties of a polymer by attaching a sulfhydryl group has been intensively researched by our group and shown in several studies [14]. The mucoadhesion enhancing properties of thiolated polymers can be explained by the formation of covalent bonds between the sulfhydryl groups of the polymer and thiol groups of cysteine rich subdomains of mucus glycoproteins. These covalent bonds are known to be stronger than ionic interactions being responsible for mucoadhesive features of positively charged chitosan with anionic moieties of the mucus layer [14, 29]. Results of rheological synergism investigation were confirmed by tensile studies where for both thiol bearing polymers a significant increased detachment force was detected. Chitosan-PEG-SH showed an effect superior to chitosan-TGA (1.3-fold). Furthermore, attached PEG-SH chains are likely to contribute to the mucoadhesive effect as bioadhesion can be enhanced by chain interpenetration and due to the ability of hydrogen bond formation across the PEG-chain.

In view of drug delivery, the solubility at neutral pH values along with improved mucoadhesion and a permeation enhancing effect recommends chitosan-PEG-SH as a promising agent which might increase bioavailability of drugs. It has been shown in *in vivo* studies that water-soluble chitosan derivatives were

able to improve bioavailability of drugs [27]. The same effect has been reported for thiolated chitosan derivatives [30, 31]. Considering the previous mentioned point and the results of this study, chitosan-PEG-SH combined the advantages of both chitosan modifications. It would be therefore recommended to plan further evaluations of chitosan-PEG-SH as carrier system and additive as *in vitro* well as and *in vivo*.

Many properties of chitosan are dependent on the molecular weight and on the degree of deacteylation of the polymer. In general, solubility increases with higher amount of free amino groups, the permeation enhancing effect is more pronounced when the chain length and degree of N-acetylation increase and the mucoadhesive properties are improved with increasing molecular weight and degree of deacetylation [32]. In the current study, only one chitosan with a molecular weight of 150 kDa and a deacetylation degree of 85 % percent was investigated making it difficult to make general assumptions of the benefit of the modification discussed in this study. Low molecular weight chitosan with a high degree of deacetylation might be better soluble than chitosan with a molecular weight of 400 kDa and therefore substitution with the solubility increasing PEG-ligand might not be sufficient. However, the mucoadhesive and permeation enhancing effect of thiolated chitosan has been investigated in several previous studies. An improvement of mucoadhesive capacity of the unmodified polymer was found for various chitosans of different molar masses [32-35]. Also the permeation enhancing effect due to the thiol group is reported for different molecular weights [17, 32, 34, 36, 37]. Saremi et al. reported a slightly increased P_{app} for thiolated chitosan nanoparticle based on chitosan with 20 kDa compared to chitosan with 50 kDa [38]. Mei et al. investigated in their study the effect of soluble quaternized, thiolated and non-modified chitosans of different molar mass (50, 100, 200, 400 kDa). The degree of deacteylation was 85-88 %. The - due to quaternization - soluble chitosan derivative showed the most pronounced permeation enhancing effect. Permeation enhancing effect of thiolated chitosan of low molecular mass (50 kDa) was not remarkable in first 40 minutes, after 60 min the permeation enhancing effect was apparent, further the mucoadhesive effect of thiolated chitosan (50 kDa) was remarkable [39]. Considering these findings, the effects described in the current study may likely be apparent even for low molecular mass chitosan with a high degree of deacteylation. However, the dimension of permeation enhancing and mucoadhesive effects is influenced by many different factors like molecular weight of both polymers, the ratio of molecular weights of employed polymers, the degree of deacetylation and substitution.

5. Conclusion

Within this study, chitosan-PEG-SH, a new chitosan derivative has been synthesized and characterized. This novel chitosan derivative combines the advantages of water soluble pegylated and mucoadhesive and permeation enhancing thiolated chitosan. The implementation of thiol bearing polyethylenglycol ligand led to significantly improved water solubility and a pronounced mucoadhesive and permeation enhancing effect compared to unmodified chitosan without affection of chitosan's biocompatibility. These features approve chitosan-PEG-SH as a promising excipient and may widen the field of application of chitosan, especially in drug delivery but may also find its entry in different fields of research like food, cosmetics, textile and biotechnology.

6. Conflict of Interest

The authors report no conflict of interests.

7. References

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Preactivated thiolated poly(methacrylic acid-co-ethyl acrylate): synthesis and evaluation of mucoadhesive potential

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Abstract

The study was aimed to develop and investigate a novel polymer for intestinal drug delivery with improved mucoadhesive properties. Therefore Eudragit® L 100-55 (poly(methacrylic acid-co-ethyl acrylate)) was thiolated by covalent attachment of L-cysteine. The immobilized thiol groups were preactivated by disulfide bond formation with 2-mercaptonicotinic acid. Resulting derivative (Eu-S-MNA) was investigated in terms of mucoadhesion via three different methods: tensile studies, rotating cylinder studies and rheological synergism method, as well as water uptake capacity and cytotoxicity. Different derivatives were obtained with increasing amount of bound L-cysteine (60, 140 and 266 µmol/g polymer) and degree of preactivation (33, 45 and 51 µmol/g polymer). Tensile studies revealed a 30.5-, 35.3- and 52.2-fold rise of total work of adhesion for the preactivated polymers compared to the unmodified Eudragit. The adhesion time on the rotating cylinder was prolonged up to 17-fold in case of thiolated polymer and up to 34-fold prolonged in case of the preactivated polymer. Rheological synergism revealed remarkable interaction of all investigated modified derivatives with mucus. Further, water uptake studies showed an over 7 h continuing weight gain for the modified polymers whereat disintegration took place for the unmodified polymer within the first hour. Cell viability studies revealed no impact of modification. Accordingly, the novel preactivated thiolated Eudragit-derivative seems to be a promising excipient for intestinal drug delivery.

Chemical compounds studied in this article:

Eudragit L 100-55, L-Cysteine (PubChem CID 5862), 2-Mercaptonicotinic acid (PubChem CID 673681)

Key words: preactivated thiomer, mucoadhesion, Eudragit® L 100-55

1. Introduction

The concept of mucoadhesion is accepted as a useful strategy to improve bioavailability of drugs by prolonging the residence time on the absorption site. Intensive research was made to improve mucoadhesive properties of natural and synthetic polymers in order to develop tailor-made drug delivery systems. Amongst the different strategies, the introduction of thiol-bearing ligands on the backbone of well-established polymers seems to be a promising one. Besides some other advantages of thiolation like permeation enhancement, efflux pump inhibition and improved cohesion of polymeric formulations, mucoadhesion is tremendously augmented [1]. The adhesive effect of most mucoadhesive materials is based on chemical interactions like ionic bonds, hydrogen bonds, hydrophobic bonds and van-der-Waals bonds [2]. These interactions are relatively weak compared to covalent interactions formed by thiolated polymers with mucus substructures via thiol/disulfide exchange reactions or a simple oxidation process [3, 4]. However, as the intestine is the absorption site of most drugs, a selective mucoadhesion of these polymers to the intestinal mucosa is on demand. Formulations should pass the stomach unhampered and develop their full mucoadhesive potential in the small intestine. A prerequisite for mucoadhesion, however, is the hydration of the polymer directly on the mucosa, known as “adhesion-by-hydration”. In comparison to other mucosal tissues such as the nasal, intraoral or vaginal mucosa, however, it is very challenging to bring mucoadhesive polymers in unhydrated form into contact with the intestinal mucosa. They simply hydrate already in the stomach and an enteric coating does not improve the situation as such coatings are isolating the mucus gel layer from the mucoadhesive polymer when arrived in the intestine. A solution of this problem might be the design of mucoadhesive polymers that do not swell at a pH < 5.5. It was therefore the aim of this study to design mucoadhesive thiolated polymers exhibiting demanded swelling properties. As the reactivity of thiol groups can be strongly further improved by the covalent attachment of mercaptonicotinic acid to them, in particular this type of thiomer was tested. Therefore, poly(methacrylic acid-co-ethyl acrylate) (Eudragit® L 100-55), which is only soluble at pH values higher than 5.5 is thiolated and in a next step, to these thiol groups mercaptonicotinic acid was attached. The mucoadhesive properties of the modified polymer was determined at gastric and intestinal pH employing three different methods, tensile studies, the rotating cylinder method and the rheological synergism method. Additionally, the swelling behavior and the cytotoxic potential of this novel polymer were investigated.

2. Materials and Methods

2.1. Materials

Eudragit® L 100-55 (poly(methacrylic acid-co-ethyl acrylate)) was received from Evonik, Darmstadt, Germany. 2-Mercaptonicotinic acid 98 % (2MNA) from ABCR GmbH & Co KG, Karlsruhe, Germany. L-Cysteine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), hydrogen peroxide, dialysis tubes (MWCO 12 kDa), glutathione in reduced form (GSH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), Triton-X 100, sodium borohydride, sodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride were obtained from Sigma Aldrich, Austria. Professor Pfaller, Institute of Physiology, Medical University of Innsbruck kindly donated Caco-2 cells. Wellplates were purchased from BioGreiner, Austria. All other cell culture care supplies were obtained from Biochrom, Berlin, Germany. All chemicals were of analytical grade and used as received. Porcine intestine was kindly donated from a local slaughterhouse.

2.2. Synthesis of preactivated thiolated Eudragit®

2.2.1. Synthesis of thiolated Eudragit ®

To prepare thiolated Eudragit® (Eu-SH) L-cysteine was attached covalently via amide bond formation between the primary amino group of L-cysteine and the carboxyl group of the polymer. The procedure was performed according to a method described previously by our group with minor modifications [5]. In brief, 1 g of Eudragit® L 100-55 was dissolved in 75 mL of demineralized water, the pH was adjusted to 6.5 with sodium hydroxide (5 M). To activate the carboxyl groups 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) were added in a final concentration of 50 mM. In order to achieve different degrees of modification, different amounts of L-cysteine hydrochloride were added. 1.0, 0.5 and 0.25 g of the ligand were dissolved in 25 mL of demineralized water and the pH adjusted to 6.5 before unifying with the polymer solution. The reaction mixture was stirred at room temperature for 3 hours. A control was prepared the same way omitting EDAC/NHS. To purify the conjugate of unbound ligand, EDAC and NHS the reaction mixture was dialyzed against 5 L of demineralized water pH 6 (adjusted using 1 M NaOH) for 3 days using Spectra/Por® 3 membranes with a molecular weight cut-off of 12 kDa. The dialysate medium was replaced every 12 hours. The third and fourth dialysate were additionally loaded with NaCl in a final concentration of 1 % (m/v). To minimalize disulfide bond formation, purification occurred at 10 °C in the dark. Finally, the product was frozen at -80 °C and lyophilized for 2 days under reduced pressure.

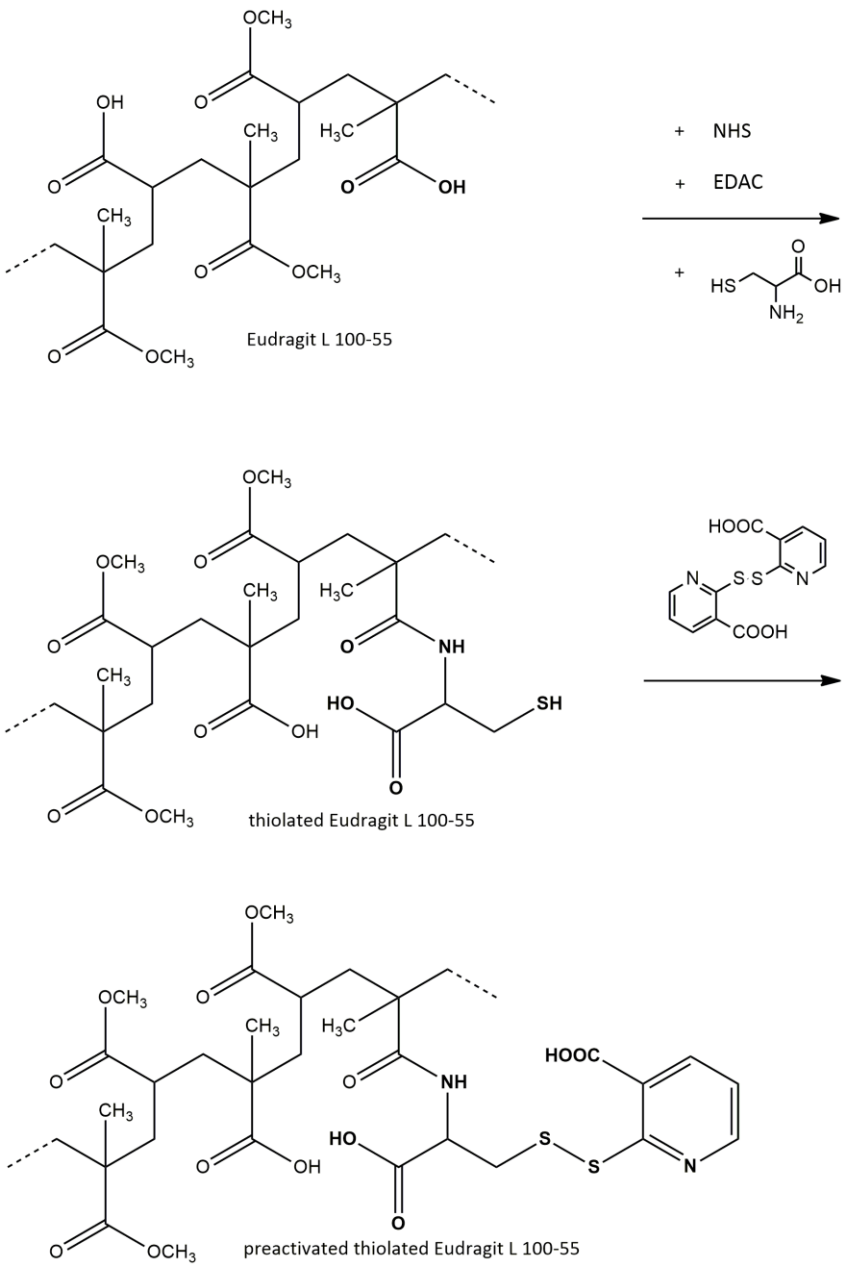

Figure 1.
Synthesis of thiolated and preactivated Eudragit® L 100-55.

2.2.2. Preactivation of thiolated Eudragit ®

To obtain preactivated thiolated Eudragit®, the thiolated conjugates were further modified according to a method firstly described by Iqbal et al [6]. The pyridylic ligand 2-mercatptonicotinic acid (2MNA) was bound covalently to the implemented thiol group via disulfide bond formation. Therefore, the ligand was dimerized by the addition of hydrogen peroxide to a solution of 2MNA (1 % m/v) at pH 8 (adjusted with NaOH) to form disulfide bonds between 2MNA molecules. By the addition of hydrogen peroxide in a final

concentration of 1.4 % (v/v) the color of the reaction mixture turned from yellow to colorless. The product 2,2'-dithiodinicotinic acid freeze-dried for 2 days under reduced pressure.

Next, the aromatic ligand was attached to the thiolated polymer via disulfide exchange reaction. In brief, 400 mg of each Eu-SH conjugate were dissolved in 100 mL demineralized water. Then, 100 mg of 2MNA dimer were added under stirring and the pH was adjusted to 8 using 5 M NaOH. The reaction mixture was stirred for 6 hours. To separate the conjugate from unbound 2MNA, the solution was dialyzed for 6 days using Spectra/Por® 3 membrane with a molecular weight cut off of 12 kDa against 5 L of demineralized water pH adjusted to 6.0 with NaOH. The dialysate was replaced 3 times a day. The product was freeze-dried for 2 days under reduced pressure and stored dry at 10 °C until use.

2.3. Quantification of conjugated L-cysteine hydrochloride

To determine the total amount of attached thiol groups a method described previously was employed [7]. To reduce possible intra- and inter molecular disulfide bonds the conjugate solutions were treated with $NaBH_4$, then the amount a thiol groups were determined using Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)). A calibration curve was established using L-cysteine hydrochloride. To guarantee complete purification via dialysis a TNBS test was carried out to determine remaining traces of unbound L-cysteine according to a method described previously [8].

2.4. Quantification of conjugated 2-mercaptonicotinic acid

The amount of conjugated 2MNA was determined spectrophotometrically as described previously [9]. Samples of 0.5 mg were dissolved in 500 µl of phosphate buffer 0.5 M pH 8. Then 500 µL of a 2 % (m/v) reduced glutathione solution were added to liberate 2MNA and the mixture was incubated for 1 h under stirring. The absorbance of free 2MNA was measured at 354 nm. To guarantee complete purification via dialysis a control without the addition of GSH was measured at 354 nm.

Table 1. Reaction conditions and resulting coupling rates for thiolated and preactivated Eudragit® L 100-55. Given values are means ± SD.

Degree of modification					
thiolated polymer			preactivated polymer		
polymer	polymer ligand weight ratio	L-cysteine [µmol/g]	polymer	polymer ligand weight ratio	2MNA [µmol/g]
Eu-SH-1	1:1	266 ± 42	Eu-S-MNA-1	4:1	51 ± 16
Eu-SH-2	2:1	140 ± 25	Eu-S-MNA-2	4:1	45 ± 18
Eu-SH-3	4:1	60 ± 13	Eu-S-MNA-3	4:1	33 ± 11

2.5. Mucoadhesion

2.5.1. Viscosity and mucoadhesion

The dynamic viscosity of synthesized Eu-SH and Eu-S-MNA conjugates as well as unmodified Eudragit® was investigated with and without the addition of porcine intestinal mucus. After the determination of the linear viscoelastic range at 1 Hz dynamic oscillatory measurements were carried out employing a plate-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). Shear rate was set to 50 s^{-1}, temperature to 37.0 °C ± 0.1 °C and the gap to 0.5 mm. To determine the mucoadhesive properties by the rheological synergism method of the conjugates in comparison to the unmodified polymer 500 µL samples of 2 % (m/v) solutions (phosphate buffer 0.1 M, pH 6.8) were added to the same amount of porcine mucus obtained from the small intestine of a fresh slaughtered pig. Small intestines of three pigs were donated from a local slaughter. To obtain the mucus, the emptied intestines were cut into pieces and opened lengthwise. Using a scraper, the mucus was separated from the tissue. To homogenize and purify the collected mucus, it was mixed with a sodium chloride solution (0.1 M) on ice. The mixture was centrifuged at 4 °C and 13 000 g for 2 hours. Granular material at the bottom and the supernatant were discarded. The homogenized and purified mucus was used immediately. The polymer-mucus mixtures were intermixed and incubated at 37 °C for 30 min before measurement. All measurements were carried out in triplicates [10].

2.5.2. Tensile studies

Tensile studies were carried out according to a method described by Mortazavi and Smart in a slightly modified way [11, 12]. Therefore, freeze dried conjugates and the unmodified control were compressed into test discs (30 mg, diameter 5.0 mm, thickness 1.5 mm) by applying a constant compaction pressure of 10 kN for 20 s (Paul Weber, Remshalden-Grünbach, Germany). Freshly excised porcine intestinal mucosa was cut into pieces of approximately 3 cm^2. The tissue was fixed in a beaker with a cyanoacrylate adhesive and covered with HCl 0.1 M and phosphate buffer 0.1 M pH 6.8, respectively. The beaker was placed on a balance being connected to a computer. The test discs were fixed on a string and attached to the mucosal tissue by applying mild force. After 20 min of incubation at room temperature, the mucosa was pulled off from the test disc at a rate of 0.1 mm/s. A computer software (Sarta Collect software; Satorius AG) was employed to collect data points every second. The maximum detachment force (MDF) and the total work of adhesion (TWA) representing the area under the force/distance curve were calculated. The investigation was carried out for each polymer 5-fold. To investigate the pH dependent mucoadhesion of the polymers, tensile studies were carried out with 0.1 M HCl instead of phosphate buffer, additionally.

2.5.3. Rotating cylinder

Via the rotating cylinder method, the time of adhesion of test discs on intestinal mucosa was evaluated. Therefore, freshly excised porcine intestinal mucosa was fixed on a steel cylinder fitting as stirrer in a dissolution tester according to the European Pharmacopeia (Erweka DT 700, Erweka GmbH, Heusenstamm, Germany). The vessels were filled with 900 mL of phosphate buffer 0.1 M pH 6.8. Test discs (prepared as described above) of each conjugate and the unmodified control were attached to the tissue by applying mild force. The "stirrer" with the mucosa was fixed in the apparatus, temperature was set to 37 °C and rotating speed to 50 rpm. The time of detachment was determined by the help of a web cam [13, 14].

2.6. Swelling Behavior

Swelling/erosion characteristics of the synthesized conjugates and the unmodified polymer were evaluated by determination of weight change in aqueous environment. The test discs were fixed on needles and incubated in HCl 0.1 M and phosphate buffer 0.1 M pH 6.8, respectively. Discs were weight at predetermined time points [9]. Weight was calculated in percent from initial weight.

2.7. Cytotoxicity

To investigate the influence of the modifications on cell viability a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed. Within this assay, the mitochondrial activity of living cells is quantified by their ability to convert water soluble MTT into in water insoluble formazan crystals. Caco-2 cells were seeded into a 96 well plate in a density of 20 x 10^4 cells/mL of minimal essential medium with Earls salts (MEM) containing 10 % fetal bovine serum (FCS) and 1 % penicillin and streptomycin. Seeded cells were incubated for 24 hours in 5 % CO_2 environment at 37 °C. After washing the cells twice with prewarmed (37 °C) phosphate buffered saline (PBS), 100 µL of the prepared polymer solutions in four different concentrations ranging from 0.05 % to 0.20 % (m/v) in MEM were added to the wells. As negative control MEM was employed whereas Triton X® 100 4 % (v/v) served as positive control. The experiment was carried out in triplicates. The cells were incubated with the polymer samples for 24 h. Thereafter, polymer solutions were removed and cells were washed twice with PBS. Next, each well was loaded with 100 µL of a MTT solution (0.5 mg/mL) in MEM. After incubation time of 4 h, the supernatant was removed and precipitated formazan crystals were dissolved in 100 µL DMSO. The absorbance of formazan solutions were measured at 570 nm with a background subtraction at 690 nm with a microplate reader (Tecan infinite, M200 spectrophotometer, Grödig Austria). The viability for each sample was calculated in percent of the MEM control [15].

2.8. Statistical data analysis

The GraphPad Prism 5 software was used for all statistical data analysis. One way ANOVA was performed with $P < 0.05$ as the minimal level of significance followed by Bonferroni post-test with $P < 0.05$ as minimal level of significance.

3. Results

3.1. Characterization of thiolated Eudragit® L 100-55

The poly(methacrylic acid-co-ethyl acrylate) was modified by covalent attachment of L-cysteine via amide bond formation between the carboxylic group of the polymer and the primary amino group of the ligand mediated by EDAC/NHS. Three thiolated derivatives, Eu-SH-1, Eu-SH-2 and Eu-SH-3, with increasing degrees of modification could be obtained by the addition of different amounts of the thiol-group bearing ligand. Higher amounts of ligand lead to a higher degree of modification. The total amount of attached L-cysteine in µmol per gram polymer is given in Table 1. A control polymer treated the same way but omitting EDAC/NHS in the reaction mixture displayed only a negligible amount of remaining traces of the ligand. All thiomers were obtained as eggshell white, lyophilized polymers of fibrous structure. TNBS test revealed only negligible amounts of remaining unbound L-cysteine, values were subtracted from determined L-cysteine.

3.2. Characterization of preactivated thiolated Eudragit® L 100-55

Obtained thiolated polymers were further modified by the attachment of 2MNA as illustrated in Figure 1. The aromatic dimer was added without any further addition of an oxidizing agent. As the aromatic disulfide is less stable then the mixed aliphatic-aromatic disulfide, a disulfide exchange reaction took place leading to preactivated thiolated Eudragit [16]. The release of the aromatic monomer could be followed by UV-spectroscopy as the MNA-dimer shows an absorption maximum at 257 nm whereas the monomer displays two absorption maxima, at 278 nm and 353 nm, respectively. The coupling rates are given in Table 1 in µmol per gram polymer. A higher amount of total thiol groups led to a higher amount of conjugated 2MNA. To detect remaining traces of unbound 2MNA a control was investigated by omitting the reductant. The experiment revealed only a negligible amount of 2MNA, values were subtracted from determined coupling rate.

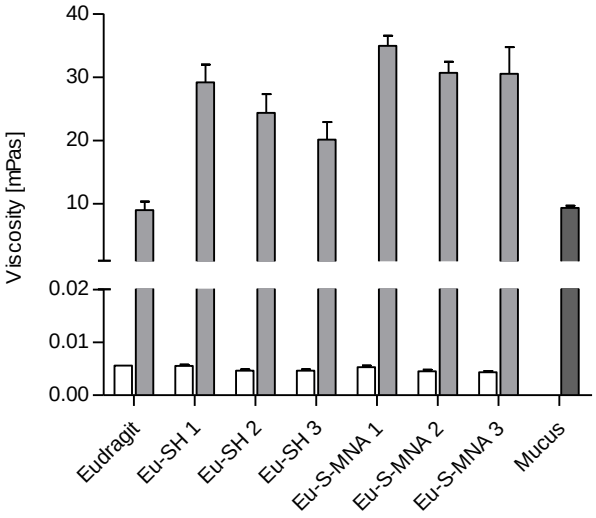

Figure 2.
Viscosity of mucus, tested polymers with (grey bars) and without (white bars) the addition of porcine intestinal mucus. Indicated values are means of at least 3 experiments ± SD of dynamic viscosity determined in rheological studies after an incubation time of 30 min for each mixture.

3.3. Mucoadhesion

Results of rheological studies are provided in Figure 2. A rheological synergism of polymer-mucus mixtures could be measured for all Eudragit® derivatives whereas the viscosity of unmodified Eudragit ® L 100-55 mucus mixture did not differ significantly from the viscosity of pure mucus. Further, tan δ of all investigated polymer solutions was > 1 (data not shown) being characteristic for the sol state. Tan δ of the porcine mucus and of all polymer-mucus mixtures was < 1 indicating a gel structure.

To investigate mucoadhesion of polymer tablets, tensile studies were carried out. The results including the maximal detachment force (MDF) and the total work of adhesion (TWA) are illustrated in Figure 3A. It turned out that TWA and MDF are in good correlation with each other. The TWA of all preactivated Eudragit-derivatives and of Eu-SH-1 differ significantly from the unmodified Eudragit®. The improvement ratios of TWA of the thiolated polymers were 6.5 for Eu-SH-1, 3.2 for Eu-SH-2 and 2.5 for Eu-SH-3. Improvement ratios for the preactivated thiolated polymers Eu-S-MNA-1, Eu-S-MNA-2 and Eu-S-MNA-3 were 52.2, 35.3 and 30.5, respectively. The cohesiveness of all polymeric discs was still provided after the incubation time of 20 min, the detachment occurred on the surface between the discs and the intestinal tissue. To show pH dependent mucoadhesive properties, tensile studies were carried out additionally under acidic conditions. Results are shown in Figure 3B. The total work of adhesion was more than 10 times lower under acidic conditions compared to values obtained at pH 6.8. To confirm results of rheological and tensile studies an additional mucoadhesion test was carried out. Results of the rotating cylinder experiment are shown in Figure 4. A more than 17-fold prolonged adhesion time on the mucosa

could be achieved for the thiolated polymer (Eu-SH-1) and a more than 34-fold prolonged adhesion time for the preactivated polymer (Eu-S-MNA-1) could be observed. Time of adhesion of preactivated polymers differed significantly from adhesion time of the corresponding thiolated derivative.

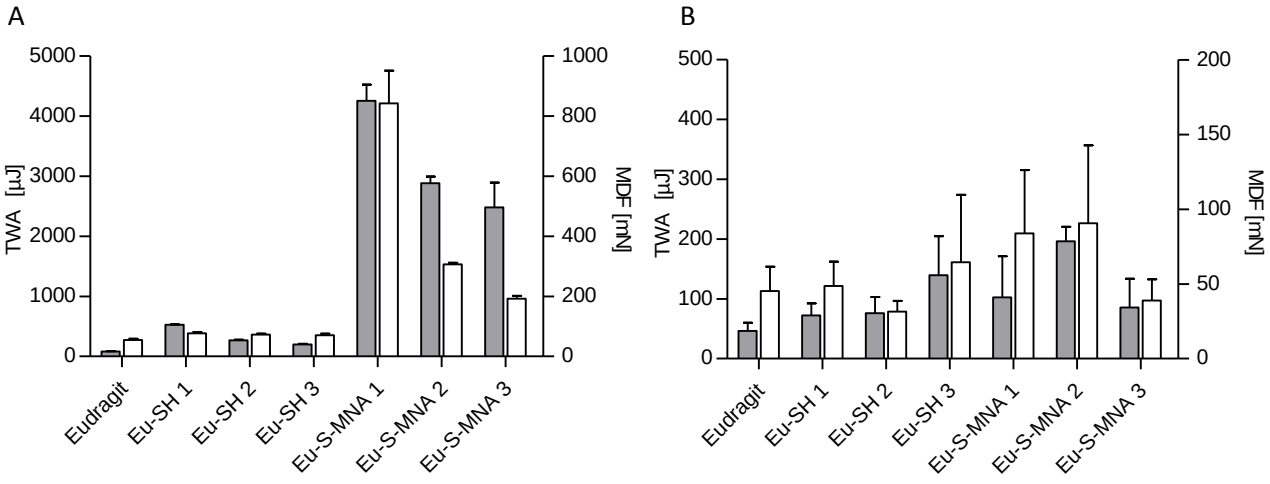

Figure 3A+B.
Bar chart shows results of tensile studies carried out with discs of thiolated, preactivated thiolated and unmodified Eudragit® in phosphate buffer (A) and 0.1 M HCl (B). Grey bars display the total work of adhesion (TWA), white bars the mean detachment force (MDF). Indicated values are means + SD, n = 5. Means of TWA of unmodified Eudragit® differ significantly from all modified polymers except Eu-SH-3 (Bonferroni Multiple Comparison Test, $p<0.0001$). Means of TWA of preactivated polymers differ significantly from thiolated, non-preactivated polymers (Bonferroni Multiple Comparison Test, $p<0.0001$).

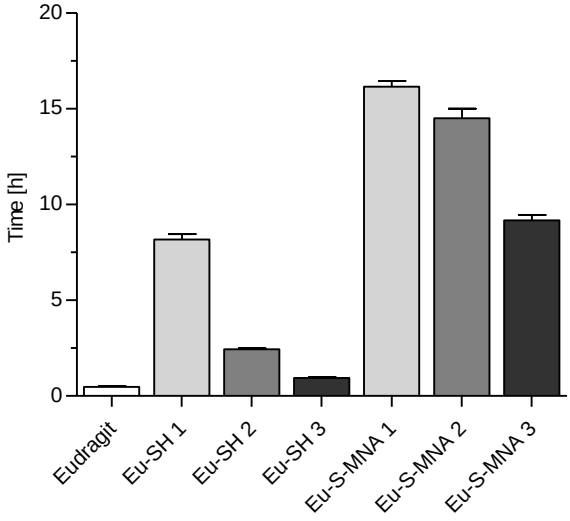

Figure 4.
Comparison of the mucoadhesive properties of unmodified, thiolated and preactivated Eudragit® L 100-55 employing the rotating cylinder method. Polymeric test discs were attached to excised porcine intestinal mucosa, which was fixed on a vertical cylinder rotated with 100 rpm in 100 mM phosphate buffer pH 6.8 at 37 °C. The indicated time of adhesion represents the mean + SD of at least three experiments. All means differ from each other except unmodified Eudragit® L 100-55 from Eu-SH-3 (Bonferroni Multiple Comparison Test, $p<0.05$).

3.4. Swelling behavior

The test discs were weighed at predetermined time points and the percentage of initial weight was calculated. Resulting line charts of the unmodified polymer, Eu-SH-2 and Eu-S-MNA-2 at both conditions, phosphate buffer pH 6.8 and 0.1 M HCl are depict in Figure 5A, 5B and 5C, respectively. All investigated polymers showed a weight gain before erosion occurred. Polymers with a small degree of thiolation and the unmodified control showed a faster disintegration at pH 6.8, after three hours Eu-SH-2 and Eu-S-MNA-3 were totally dissolved and Eu-SH-3 was disintegrated after less than one hour. The thiomer with the highest degree of modification, Eu-SH-1, started the dissolving process after 3 hours. The preactivated polymers with higher degree of modification experienced a continuous weight gain during the 5 hours of observation. After 5 hours, the weight of the test discs were 8.8-fold (Eu-S-MNA-1) and 7.3-fold (Eu-MNA-2) higher than the initial weight. Under acidic conditions, none of the tested polymers dissolved within observed time range. After a phase of continuous weight gain, a steady weight was achieved for all investigated polymers except Eu-SH-1, which was dissolved after less than 2 hours. Weight gain of Eu-SH-2 and Eu-SH-3 was significantly higher (9.7-fold and 11.7-fold of initial weight) as corresponding preactivated thiomers (7.3-fold and 3.0-fold of initial weight) after 5 hours. Water uptake occurred significantly slower for Eu-S-MNA 1 in acidic conditions than at pH 6.8, after 1 hour a constant value was reached.

3.5. Assessment of cytotoxic effects

The cell viability was investigated at 4 different concentrations over 24 hours. A MTT assay with Caco-2 cells was performed. Viability was calculated in percent of control (MEM with FCS). Results of the investigation are summarized in Figure 6 and Table 2. There was no significant difference between the different polymers but toxicity seems to be concentration depended. Viability dropped down to approximately 80 % at the highest concentration of 0.20 % after 24 hours whereat viability remained over 85 % at a concentration of 0.05 %.

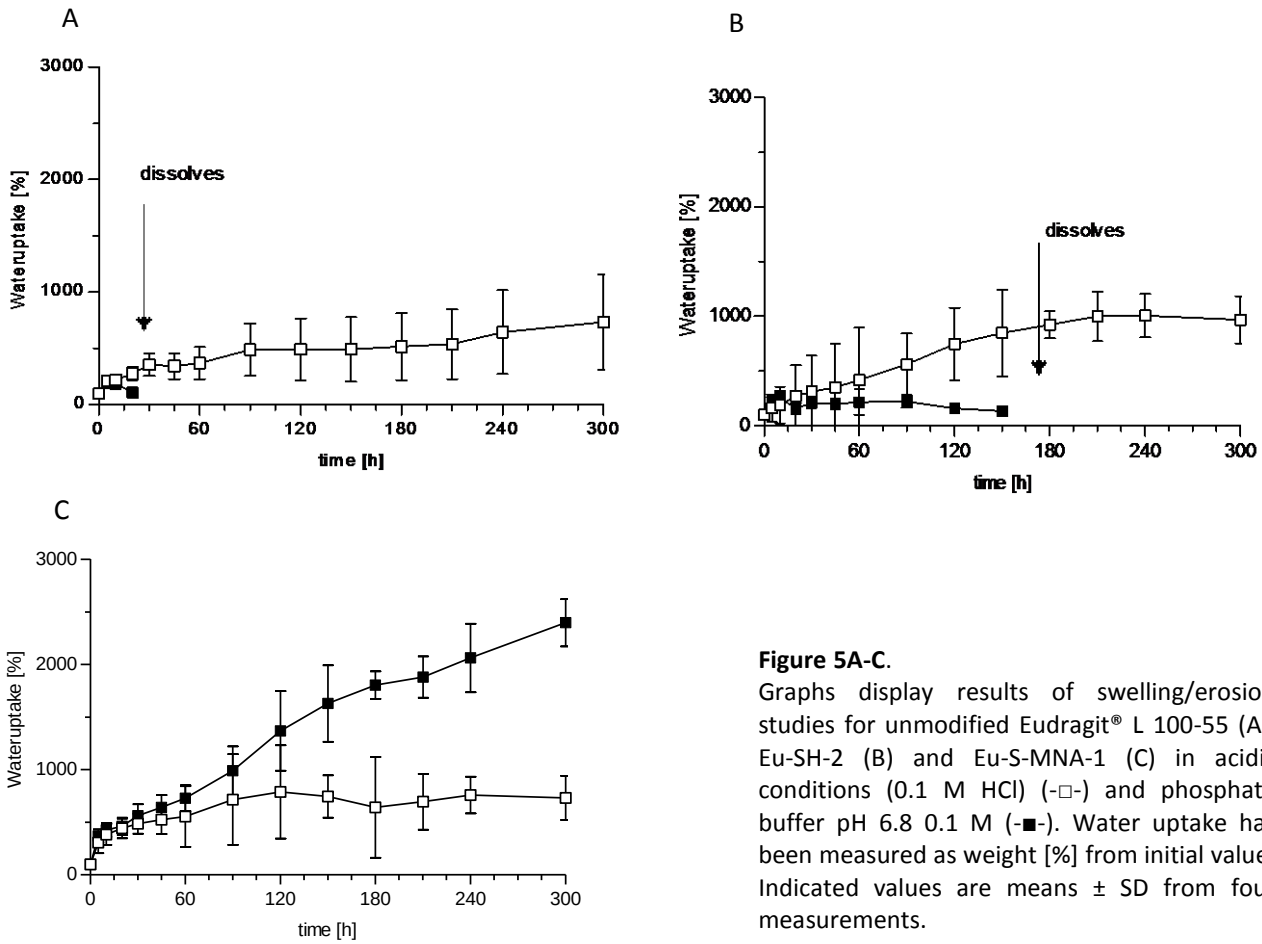

Figure 5A-C.
Graphs display results of swelling/erosion studies for unmodified Eudragit® L 100-55 (A), Eu-SH-2 (B) and Eu-S-MNA-1 (C) in acidic conditions (0.1 M HCl) (-□-) and phosphate buffer pH 6.8 0.1 M (-■-). Water uptake has been measured as weight [%] from initial value. Indicated values are means ± SD from four measurements.

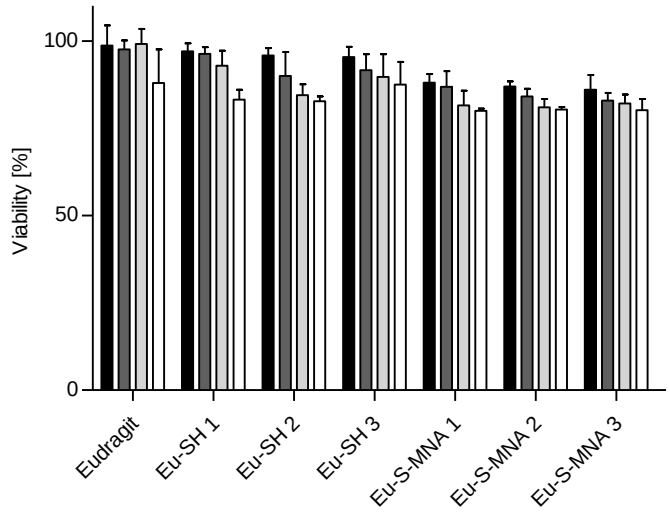

Figure 6.
The histogram displays the influence of all polymers on cell viability after 24 hours. Different concentrations were investigated (0.05 % black bars, 0.10 % dark grey bars, 0.15 % light grey bars, 0.20 % with bars). Indicated values are means + SD of at least three experiments.

Table 2. Significant differences in viability of modified polymers in comparison to unmodified Eudragit L 100-55.

Significance of loss in viability				
polymer	**concentration**			
	0.05 %	0.10 %	0.15 %	0.20 %
Eu-SH 1	ns	ns	ns	ns
Eu-SH 2	ns	ns	P < 0.01	ns
Eu-SH 3	ns	ns	ns	ns
Eu-S-MNA 1	ns	P < 0.05	P < 0.0001	ns
Eu-S-MNA 2	P < 0.05	P < 0.01	P < 0.0001	ns
Eu-S-MNA 3	P < 0.01	P < 0.01	P < 0.0001	ns

4. Discussion

The concept of improved mucoadhesion due to covalent bond formation between the excipient and mucus compounds has shown excellent results and the concept has been proven *in vivo* several times [17-19]. However, it is known that the reactivity of the free thiol groups is limited due to too early oxidation [9]. Thereby a driving force was given to develop improved drug delivery systems based on the concept of covalent bond formation and help to develop thiomers full potential. The “preactivated” or “S-protected” thiomers were introduced. First investigations were made to characterize the potential of this new class of polymers employing different polymers and ligands [20, 21]. Within this study, it was possible to synthesize thiolated poly(methacrylic acid-co-ethyl acrylate) with different degrees of modification and to preactivate the resulting thiomers in dependence of their thiol content.

To investigate mucoadhesive qualities of the polymer in fully hydrated form, the “rheological synergism” method was employed. Therefore, viscosity of the synthesized polymers was determined. It turned out that the modification had no significant impact on the rheological behavior of the Eudragit-derivatives. The dynamic viscosity of polymer-mucus mixtures was measured since it is reported that an increase in viscosity of polymer-mucus mixtures correlates with mucoadhesive qualities of the polymer [22]. The resulting dynamic viscosity of polymer/mucin mixtures is supposed to result from certain interactions between the two components like chain entanglements, conformational changes, non-covalent interactions and in case of thiomers from covalent interactions as well [3, 23]. All modified polymers exhibit a significantly higher dynamic viscosity than the unmodified control. The increase in viscosity was more pronounced with a higher degree of thiolation, whereas the viscosity further increased for preactivated thiomers compared to their corresponding non-preactivated thiomers. Mucoadhesion is a complex process and several theories trying to describe it are established [2]. However, due to the

complexity of the process, it seems neither possible to describe the process by a single theory nor to characterize the properties of a mucoadhesive excipient by a single experiment. It makes a significant difference if the dosage form is dry, partially hydrated or fully hydrated. Therefore, two additional tests were carried out to determine mucoadhesive properties of modified polymers compressed to test discs. Within tensile studies the force being required to detach the tablet from the mucosal surface is determined. To confirm findings of these studies, the "rotating cylinder" method was employed. This method is supposed to meet *in vivo* conditions more satisfyingly as the adhesion time under shear forces is investigated [24]. Both tests showed the same results, a higher degree of thiolation led to a more pronounced mucoadhesive effect which could be further improved by preactivation. The effect of preactivation was more pronounced at tensile studies. An explanation therefore might be that disintegration and swelling do not come into effect at tensile studies in contrast to the rotating cylinder method, where adhesion time is crucially influenced by these factors. The endpoint especially for the derivatives with lower degree of modification was not reached because tablets detached from mucosa when disintegration took place. Results of tensile studies are even more remarkable when compared to results of other representatives of preactivated thiomers developed and investigated so far. Dünnhaupt et al. reported a TWA value of around 400 µJ for preactivated thiolated chitosan and Iqbal et al. determined 325 µJ for the TWA compared to values over 4000 µJ found in this study [6, 25].

As shown in results of all mucoadhesion experiments, the adhesive properties of preactivated derivatives is more pronounced than for the thiolated, non-preactivated polymers. These findings are in line with the theoretical background of the development of preactivated thiomers. The aromatic-aliphatic mixed disulfide bond is more reactive as the aliphatic-aliphatic disulfide bond facilitating disulfide-exchange reaction of the preactivated thiomer with thiol groups of glycoproteins [26, 27]. Further, results of tensile studies carried out under acidic conditions were in line with the theory that a reduced hydration leads to a reduced mucoadhesive effect as investigated during swelling behavior studies. A significant lower adhesive effect under acidic conditions compared to tensile studies at pH 6.8 was shown.

Swelling behavior influences several qualities of a polymer in drug delivery. The swelling capacity of a polymer is related to its mucoadhesive properties. The "adhesion by hydration" theory is based on water uptake of the excipient from underlying mucosal tissue by absorption, swelling and capillary effects resulting in a marked adhesion [22]. Water uptake leads to relaxation of polymer chains that enables chain interpenetration with mucus [22, 28]. Furthermore, drug release is influenced by the extent and rate of water uptake. The release of incorporated drugs is mainly affected by the swelling/erosion behavior and the diffusion process [29]. The diffusion process in turn is affected by the water content of the dosage

form since the drug should be dissolved, additionally chain relaxation eases the way out for the matrix. As the polymeric backbone exhibits pH-dependent hydration properties (not soluble at pH values < 5.5), water uptake studies were carried out in 0.1 M HCl to simulate gastric conditions and in phosphate buffer 0.1 M pH 6.8 to simulate intestinal conditions. A poorer water uptake in acidic conditions is favored as the dosage form should stick to the tissue in the intestine where pH-values are close to neutral and not to the mucosa in the stomach. Therefore, the poly(methacrylic acid-co-ethyl acrylate) was chosen, which is employed for many years as gastric juice-resistant coating of tablets which are supposed to pass the stomach unaltered and start drug release in the intestine. As shown in Figure 5A, the test tablet based on unmodified Eudragit® L 100-55 showed a moderate water uptake at acidic conditions whereat at pH 6.8, where the polymer showed good solubility, disintegration took place immediately. Due to the thiolation, the time until disintegration started was delayed. This can be explained by crosslinking disulfide bonds within the polymer that are known to lead to a higher cohesion. As shown in Figure 5, in acidic - simulated gastric conditions - the behavior of the modified polymers equals the behavior of the unmodified polymer which indicates that there is no loss of the pH dependent properties of Eudragit® L 100-55. The “adhesion by hydration” force should be significantly lower in gastric environment and the formulation is likely to pass the stomach unaltered. Furthermore, the water uptake of the preactivated polymer could be improved and disintegration delayed. A slow dissolution of tablets can be employed to achieve a sustained release of an incorporated drug. In general, an increasing water uptake could be observed with an increasing degree of modification. This is explained by the enhanced cohesive properties due to inter- and intramolecular disulfide bonds [13]. Regarding the preactivated polymers the additional implementation of a hydrophobic component reduces the solubility of the polymer and prevents from disintegration, this in turn has positive effects on mucoadhesion.

Eudragit ® L 100-55 is a well-established excipient in tablet manufacture and considered as not harmful. To exclude any changes in toxicity because of the modification, a viability assay was performed. No significant decrease in viability due to the modification could be observed.

In view of drug delivery the shown remarkable improved mucoadhesive properties along with a pH dependent water uptake capacity, the prolonged disintegration time and the unaltered biocompatibility suggest this novel excipient, preactivated thiolated poly(methacrylic acid-co-ethyl acrylate), for further investigations in drug delivery. It has been shown that prolonged retention time could increase bioavailability of drugs [12]. Several *in vivo* studies showed an enhanced oral bioavailability for peptide drugs when administered incorporated in thiomer or preactivated thiomer tablets. According to results

from former studies and the encouraging results of the present study, the developed derivative Eu-S-MNA is likely to be of interest for drug delivery.

5. Conclusion

The synthesized thiolated and preactivated derivative of Eudragit® L 100-55 showed an enhanced water uptake and prolonged disintegration time at pH 6.8 compared to the unmodified and thiolated polymer. The water uptake at acidic conditions was hardly altered. Further, mucoadhesion was improved due to the implementation of the aromatic ligand, which was more than 10-times higher under intestinal environment compared to the adhesion at gastric conditions. These pH dependent properties enables targeted delivery to the intestinal mucosa. Furthermore, no change in biocompatibility could be detected due to modification with L-cysteine and 2-mercaptonicotinic acid. Hence, the novel derivative may improve drug targeting to the absorption site what may reduce the dose and improve the uptake of drugs.

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Synthesis and *in vitro* characterization of a novel S-protected thiolated alginate

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Abstract

The object of this study was to synthesize and characterize a novel S-protected thiolated polymer with a high degree of modification. In this regard, an alginate-cysteine and an alginate-cysteine-2-mercaptonicotinic acid conjugate were synthesized. To achieve a high coupling rate of the thiol group bearing ligand cysteine to the polymer, the carbohydrate was activated by an oxidative ring opening with sodium periodate followed by a reductive amination to bind the primary amino group of cysteine to resulting reactive aldehyde groups. The obtained thiolated polymer displayed 1561 ± 130 µmol thiol groups per gram polymer. About one third of these thiol groups were S-protected by the implementation of a thiol bearing aromatic protection group via disulfide bond formation. Test tablets of both modified polymers showed improved stability against oxidation in aqueous environment compared to the unmodified alginate and exhibit higher water uptake capacity. Rheological investigations revealed an increased viscosity of the S-protected thiolated polymer whereat the thiolated non S-protected polymer showed gelling properties after the addition of hydrogen peroxide. The mucoadhesive properties could be improved significantly for both derivatives and no alteration in biocompatibility tested on Caco-2 cell monolayer employing an MTT assay could be detected after modification. According to these results, both new derivatives seem promising for various applications.

Key words: Alginate, S-protected thiomer, preactivated thiomer, mucoadhesion

1. Introduction

By choosing the right drug delivery system, pharmacological and pharmacokinetic properties of drugs can be improved compared to the "free" drug [1]. Within the last decades, various strategies have been pursued to find more effective formulations for different kinds of drugs. Amongst others, drug delivery systems based on polymeric excipients are intensively and ongoing investigated. A reason therefore might be the diversity of polymers and the ability, to modify synthetic as well as naturally occurring polymers to generate a tailor-made drug delivery system. In general, mucoadhesive polymers are high on demand as a site-specific application of the active pharmaceutical ingredient is desirable because of an increased drug concentration gradient on the absorption site and hence an improved systemic uptake [2]. However, the process of mucoadhesion is a complex one and driven by several mechanisms and forces [3]. Most mucoadhesive materials are adherent to the mucosal surface because of relatively weak non-covalent interactions like hydrogen bonds, van der Waal's forces and ionic interactions [4]. It was shown in many different studies that thiolation improves the mucoadhesive properties of well-established mucoadhesive polymers because of a formation of covalent disulfide bonds with thiol bearing mucus substructures [2]. Meanwhile, with the preactivated or S-protected thiolated polymers, a next generation of mucoadhesive polymers has been introduced to overcome drawbacks of the thiomers like too early oxidation, pH dependent reactivity and subsequently to improve mucoadhesion [5-7].

It is reported that the degree of modification correlates with the improvement of mucoadhesion. For some polymeric backbones a high degree of thiolation could be achieved, like 980 µmol/g polymer for chitosan-TGA [8] or 767 µmol/g polymer for poly (acrylic acid) [9]. For others, however, achieved coupling rate is comparatively low. One of them is alginate. Thiolation of alginate via carbodiimide mediated coupling led to a range of immobilized thiol groups between around 230 and 340 µmol/g polymer [10-13]. Hence, the object of this study was to use a different synthetic pathway to generate thiolated alginate with a substantially improved degree of modification. Besides covalent coupling of the thiol bearing ligand to the polymeric backbone by amide bond formation, reductive amination with a sulfhydryl ligand exhibiting a primary amino group after activation of carbohydrates using sodium periodate is described. This method leads to comparatively higher rates of substitution [14, 15].

Alginate itself offers great properties for drug delivery. It is a biodegradable, biocompatible, mucoadhesive and widely used natural occurring polymer [16]. In pharmaceutics, the carbohydrate is employed for different applications, as emulsion thickener, carrier matrix, wound dressing and as agent against heartburn and gastric reflux [17].

Therefore, the aim of the study was to convert this potential excipient into a novel S-protected thiolated polymer with a high degree of modification. A ring opening reaction was carried out to oxidize the proximal hydroxyl groups of the polymeric backbone in order to form aldehyde groups being subject to a reductive amination with the primary amine L-cysteine. By a disulfide exchange reaction, the implemented thiol group is protected by an aromatic thiol substructure. Both, the novel thiolated and the S-protected thiolated polymer are characterized in terms of solubility, swelling behavior, mucoadhesive properties, rheological properties and cytotoxicity.

2. Materials and Methods

2.1. Materials

Sodium alginate, L-cysteine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), hydrogen peroxide, dialysis tubes (MWCO 3500 Da), glutathione in reduced form (GSH), MES, sodium cyanoborohydride, sodium periodate, sodium borohydride, sodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride were obtained from Sigma Aldrich, Austria. 2-mercaptonicotinic acid 98 % (MNA) was purchased from ABCR GmbH & Co KG, Karlsruhe, Germany. Professor Pfaller, Institute of Physiology, Medical University of Innsbruck kindly donated Caco-2 cells. Wellplates were purchased from BioGreiner, Austria. All other cell culture care supplies were obtained from Biochrom, Berlin, Germany. All chemicals were of analytical grade and used as received. Porcine intestine was kindly donated from a local slaughterhouse.

2.2. Synthesis of preactivated and thiolated alginate

2.2.1. Synthesis of aldehyde polymer – periodate oxidation

The native sodium alginate was modified to an aldehyde form via oxidation with sodium periodate according a method described by Ito et al. and modified by our group [14, 18]. In brief, 1.5 g of sodium alginate were dissolved in 140 mL water, then 800 mg of sodium periodate dissolved in 10 mL were added under light protection and stirred for 2 h. Thereafter, to inactivate excess of unreacted periodate, 200 µL of ethylene glycol were added. The reaction mixture was stirred for another hour and finally the oxidized polymer was purified via dialysis employing a cellulose membrane tube (molecular weight cut off: 3500 Da) against water for 3 days. The dialysis medium (5 L of demineralized water) was changed 3-times a day. Finally, the product was freeze dried for 2 days under reduced pressure.

2.2.2. Thiolation of the oxidized polymer via reductive amination

The aldehyde polymer was thiolated according to a method described previously. First, 1 g of alginate-aldehyde (Alg-CHO) was dissolved in 40 mL MES buffer 0.1 M. Next, 2 g of L-cysteine were added and the volume of the reaction mixture was adjusted to 50 mL. The pH was adjusted to 5 and the mixture was stirred for 3 hours at room temperature. Thereafter, 4.0 g of sodium cyanoborohydride were added and the mixture was stirred for 72 h. The thiolated polymer (Alg-SH) was purified from unbound L-cysteine and periodate by exhaustive dialysis for 3 days with a change of dialysis media every 12 hours. The dialysis medium consisted of 5 L 2 mM HCl. The 4th and 5th medium contained 1 % of NaCl. Purification took place at 10 °C in the dark. The thiolated polymer was finally freeze dried under reduced pressure. To verify the purification steps and to produce a control polymer, the same protocol was followed but omitting sodium periodate [14].

2.2.3. Preactivation of the thiolated product via disulfide exchange

To synthesize S-protected thiolated alginate, the thiolated conjugates were further modified according to a method firstly described by Iqbal et al. [19] Therefore, the aromatic ligand 2-mercatptonicotinic acid (MNA) was bond covalently to the conjugated thiol group of the polymer via disulfide bond formation. MNA was dimerized with hydrogen peroxide as described previously [7]. 400 mg of the Alg-SH conjugate were dissolved in 100 mL demineralized water and 100 mg of MNA dimer were added under stirring, the pH was adjusted to 8 using 5 M NaOH. After 6 hours, the conjugate was separated from unbound MNA via dialysis for 6 days using cellulose dialysis tubes (molecular weight cut off: 3500 Da) against 5 L of demineralized water. The dialysate was replaced 3 times a day. The product was freeze-dried for 2 days under reduced pressure and stored dry at 10 °C until use.

2.3. Evaluation of thiol group content

The amount of free thiol groups resulting from conjugation of L-cysteine was determined spectrophotometrically using Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) according to a method described previously [20]. To determine the total amount of immobilized L-cysteine, the amount of thiol groups was determined again after reduction with $NaBH_4$. To ensure that there are no unbound L-cysteine molecules in the sample, free amino groups were quantified via TNBS test allowing direct correlation to unbound cysteine [21].

2.4. Evaluation of MNA content

The amount of immobilized 2-mercaptonicotinic acid was determined spectrophotometrically according a method described previously by our group [19]. The amount of bound MNA was determined after reduction with glutathione. The absorbance of liberated MNA was measured at 354 nm. To verify that there is no unbound MNA in the sample, absorption measurements at 354 nm were carried out prior to reduction with GSH.

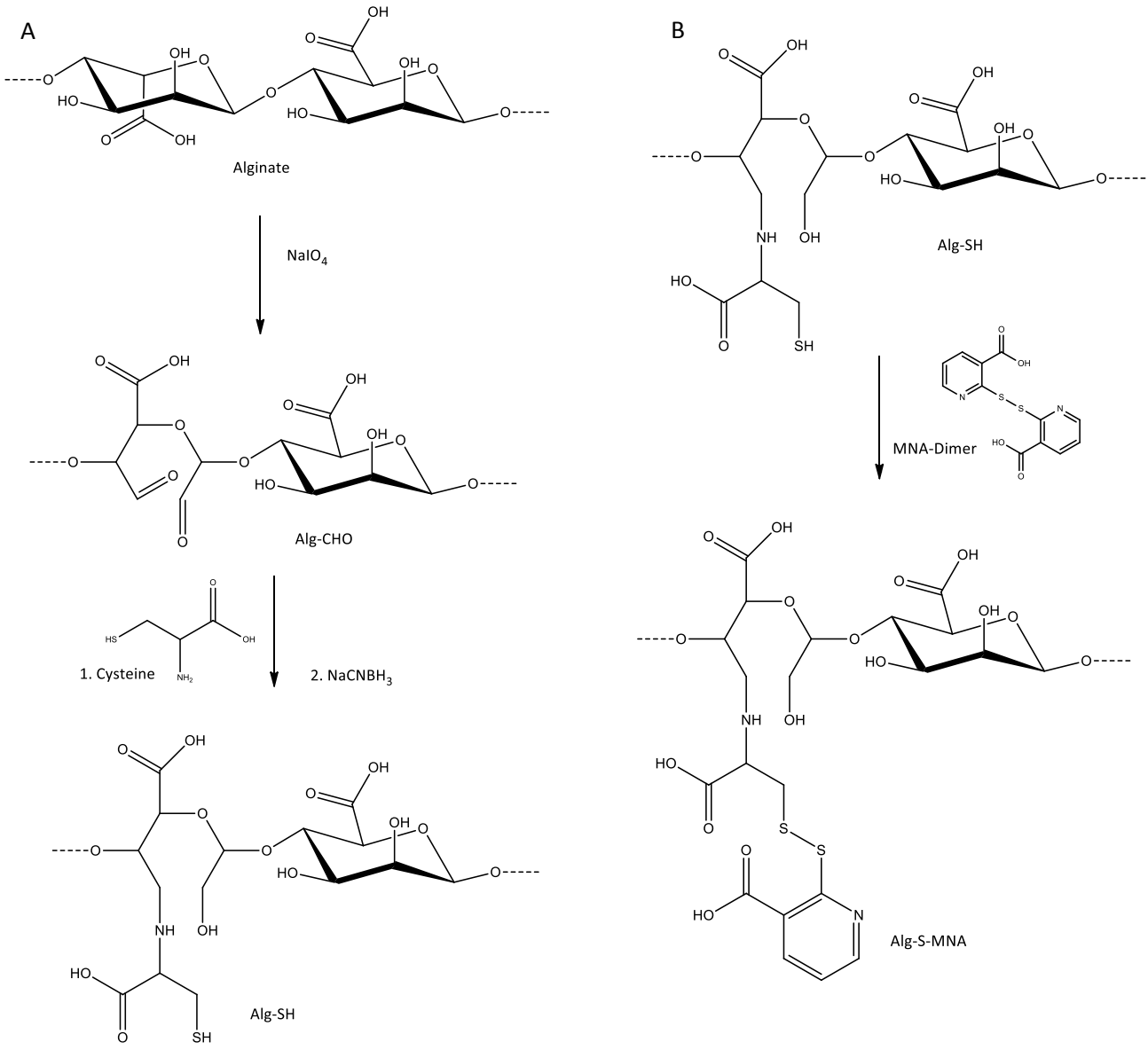

Figure 1.
Schema of modification of sodium alginate. First, the carbohydrate chains were opened by sodium periodate oxidation. Resulting aldehyde groups were further modified via a reductive amination, the primary amine L-cysteine was bound covalently (Alg-SH) (A). The immobilized thiol groups were S-protected in a next step by a disulfide exchange reaction using the dimer of 2-mercaptonicotinic acid (Alg-S-MNA) (B).

2.5. Solubility and swelling/erosion behavior

To investigate changes in the properties of the modified polymer, alginate, thiolated alginate (Alg-SH), preactivated thiolated alginate (Alg-S-MNA) and the intermediate Alg-CHO were hydrated in buffers with different pH values. The mixtures of aqueous buffer (acetate buffer 0.1 M pH 4 and 5, phosphate buffer 0.1 pH 6, 7 and 8) and polymer (0.5 % m/v) were incubated overnight.

To investigate the swelling/erosion behavior of the excipients in aqueous environment, water uptake studies were carried out. Therefore, test discs were compressed with 30 mg of the lyophilized polymers. The compaction pressure of 10 kN was applied for 20 s (Paul Weber press, Remshalden-Grünbach, Germany). Resulting tablets were flat-faced discs with a diameter of 5.0 mm and 2 mm thickness. The water uptake/erosion was determined by the weight change. After fixing on needles, the test discs were incubated in phosphate buffer 0.1 M pH 6.8. The discs were weight at predetermined time points and the swelling ratio was calculated in relation to the initial weight [7].

2.6. Collection and purification of intestinal porcine mucus

Porcine mucus was obtained as described previously [22]. In Brief, the mucus from fresh excised small intestine was collected from the tissue using a scraper. To purify and homogenized the obtained mucus, the mucus was dispersed in a sodium chloride solution (0.1 M) and stirred for one hour on ice. Afterwards, the suspension was centrifuged at 4 °C and 9000 rpm for 2 hours. The supernatant was discarded and the granular material at the bottom was removed.

2.7. Rheological investigations

A plate-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) was employed to characterize the polymers regarding their rheological properties. After the determination of the linear viscoelastic range at 1 Hz dynamic, oscillatory measurements were carried out. The shear rate was set to 50 s^{-1}, temperature to 37.0 ± 0.1 °C and the gap to 0.5 mm. The polymers were dissolved in a concentration of 1.5 % (m/v) in phosphate buffer 0.1 M pH 8 and samples of 800 µL were taken for each measurement. Each investigation was carried out in triplicates.

To investigate gelling properties of the polymers, hydrogen peroxide was added in different concentrations (30 nMol and 60 nMol) to the polymer solutions. The increase in viscosity was measured after predetermined time points.

The rheological synergism method was employed to investigate mucoadhesive properties of the modified polymer. Therefore, 500 µL of dissolved polymer were intermixed with 1000 µL of fresh porcine intestinal mucus. Parameters for the rheological measurement were set as described above. Dynamic viscosity was measured at predetermined time points.

2.8. Cytotoxicity

To exclude a negative impact of the modification on biocompatibility, a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed. The assay is based on the ability of vibrant cells to convert water soluble MTT into water insoluble formazan cristals due to the reductive activity of mitochondria. Therefore, Caco-2 cells were seeded in a density of 2 x 10^5 cells per mL minimal essential medium (MEM, containing phenol red, Earls salts, 10 % fetal bovine serum and 1 % penicillin and streptomycin) into a 96 well plate. Cells were allowed to attach for 24 hours in 5 % CO_2 environment at 37 °C. Thereafter, cells were washed twice with prewarmed (37 °C) phosphate buffered saline (PBS). Next, 100 µL of the prepared polymer solutions in 5 different concentrations (1 %, 0.75 %, 0.5 %, 0.25 % and 0.125 %) dissolved in MEM were added to the wells. As negative control MEM was employed whereas Triton X® 100 4 % (v/v) served as positive control. The Caco-2 cells were incubated with the polymer samples for 3 hours. Afterwards, the supernatant was removed and cells were washed twice with phosphate buffered saline. Then, wells were incubated with 100 µL of a MTT solution (0.5 % m/v) in MEM. After 4 h, the supernatant was removed and precipitated formazan crystals were dissolved in 100 µL DMSO. The absorbance of resulting formazan solutions were measured at 570 nm with a background subtraction at 690 nm employing a microplate reader (Tecan infinite, M200 spectrometer, Grödig Austria). The viability of the cells was calculated in percent of negative control (MEM) [23].

2.9. Statistical data analysis

Statistical data analysis was carried out with “GraphPad Prism 5” software. One way ANOVA was performed followed by a Bonferroni post-test with $p < 0.05$ as minimal level of significance.

3. Results

3.1 Characterization of thiolated and S-protected alginic acid

Cysteine was covalently bound to the polymer with a coupling rate of 1561 ± 130 µmol attached thiol groups per gram polymer. Thereof 1379 ± 102 µmol/g are detected as free, non oxidized SH-groups. The lyophilized thiolated polymer and the control (Alg-CHO) prepared the same way omitting cysteine were eggshell white, odorless and of fibrous structure. To ensure that there are no unbound L-cysteine molecules trapped in the polymer, the TBNS test for free amino groups was carried out and revealed only a negligible amount of remaining traces of unbound L-cysteine, values were subtracted from determined L-cysteine.

The obtained thiolated polymer was further modified by the covalent attachment of MNA via disulfide bond formation as illustrated in Figure 1. Thereby, the dimerized pyridylic structure was added without any oxidizing agent to the reaction mixture to achieve a disulfide exchange reaction. As the aromatic disulfide is less stable then the mixed aliphatic-aromatic disulfide preactivated thiolated alginate could be obtained [24]. The release of the MNA monomer was followed by UV-spectroscopy as the dimer and the monomer of MNA show different absorption spectra. The dimer shows an absorption maximum at 257 nm and the monomer exhibits two absorption maxima, at 278 nm and 353 nm, respectively. The amount of immobilized MNA was determined to be 520 ± 29 µmol per gram polymer. To exclude remaining unbound MNA a control was investigated by omitting the reductant.

Table 1. pH-dependent solubility of modified polymers and unmodified control in different buffer systems (1 % m/v). Acetate buffer pH 4 and 5, 0.1 M and phosphate buffer pH 6, 7 and 8, 0.1 M. + = dissolved, - = not dissolved but hydrated.

	pH 4	pH 5	pH 6	pH 7	pH 8
Alg-SH	-	-	-	+	+
Alg-S-MNA	-	-	-	-	-
Alg-CHO	+	+	+	+	+
Alginat	+	+	+	+	+

3.2. Solubility and swelling/erosion behavior

The changes in hydrophilicity due to the modification was investigated for the S-protected thiolated and thiolated polymer, the native alginate and the alginate-aldehyde intermediate. Results are given in Table 1. Due to thiolation, the pH range in which the polymer shows good solubility was limited on the acidic side. For the preactivated thiolated polymer, no clear solution could be observed at the investigated pH range.

To determine the behavior of test tablets in aqueous environment, the discs were incubated in phosphate buffer pH 6.8 0.1 M and weight at predetermined time points. The swelling ratio was calculated and plotted in Figure 2. Alg-CHO was dissolved within the first hour without revealing any swelling capacity. The unmodified alginate showed only a slight increase in weight indicating water uptake and dissolved after 4.5 hours. The thiolated polymer showed the highest weight gain which occurred within the first minutes and remained stable during the time of investigation. The preactivated thiolated polymer showed a similar behavior but with a lower water uptake.

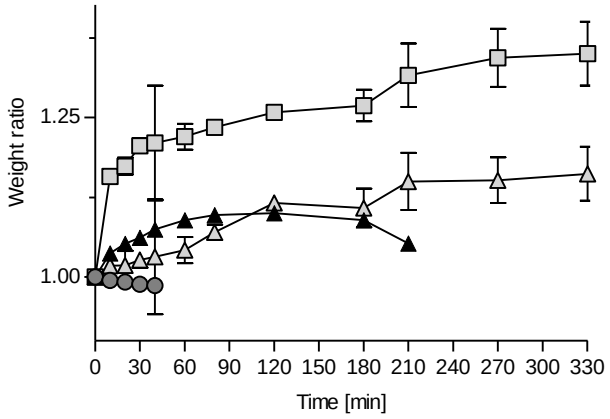

Figure 2.
The line chart displays the results of swelling/erosion studies for the unmodified alginate (-▲-), Alg-CHO (-△-), Alg-SH (-■-), and Alg-S-MNA in pH 6.8 0.1 M (-●-). Water uptake was measured by weight change and weight ratio from initial weight was calculated. Indicated values are means ± SD from 3 measurements.

3.3. Rheological investigations

The results of rheological studies are provided in Figures 3-5. The dynamic viscosity of polymer solutions were measured. It could be observed that viscosity of the polymer decreased to 0.01 mPas after cleavage with sodium periodate but increased after thiolation and even more after preactivation to 0.03 and 183 mPas, respectively.

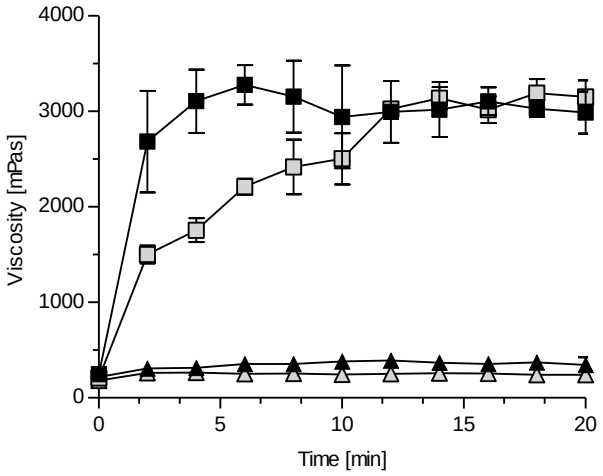

Figure 3.
Graph shows the dynamic viscosity of polymer solutions (1.5 %) after incubating with two different concentrations of hydrogen peroxide, Alg-SH with 60 nmol H_2O_2 (-■-) and 30 nmol (-■-), Alg-S-MNA with 60 nmol H_2O_2 (-▲-) and 30 nmol (-▲-).Indicated values are means ± SD from 3 measurements.

Thiolated and preactivated thiolated alginate were mixed with two different concentrations of hydrogen peroxide in order to investigate the cross-linking properties of the polymers. The dynamic viscosity of the polymers was measure over 20 min at predetermined time points. For the thiolated derivative, a 12-fold increase in the viscosity could be observed compared to unmodified alginate whereat the increase was steeper at the higher concentration of H_2O_2 as shown in Figure 3. The preactivated thiolated polymer exhibited only a 1.5-fold increase in viscosity after the addition of the oxidant and remained stable during the observed time range.

To investigate mucoadhesive properties of the modified polymers, the rheological synergism method with porcine mucus was employed. Both, the thiolated and preactivated thiolated alginate showed a significant increase of viscosity and showed a pronounced rheological synergism. For the unmodified and the Alg-CHO intermediate, no synergism could be shown (Figure 4). Furthermore, tan δ of Alg-SH-mucus mixture and Alg-S-MNA-mucus mixture was < 1 indicating a gel structure. Furthermore, the increase of viscosity of the polymer-mucus mixtures was observed over time. Within the first 10 min an increase in viscosity could be shown (Figure 5).

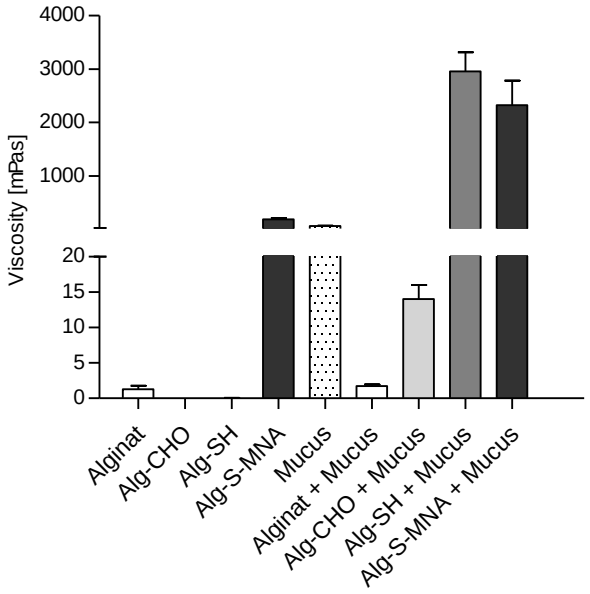

Figure 4.
Viscosity of mucus and tested polymers with and without intermingling with porcine intestinal mucus. Rheological studies were carried out after an incubation time of 15 min for each mixture. Indicated values are means ± SD from 3 measurements.

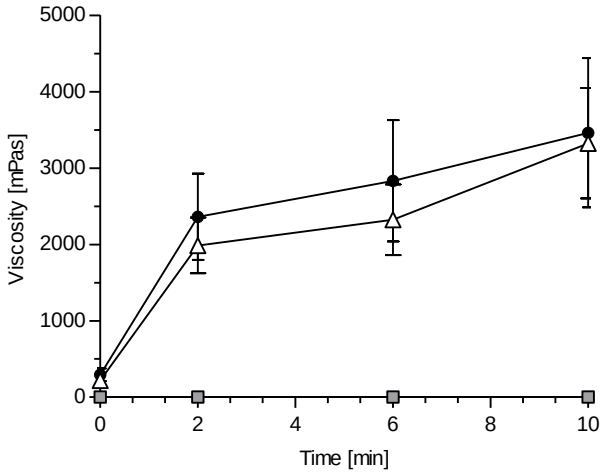

Figure 5.
The graph shows increase of viscosity of thiolated alginate (-●-), S-protected thiolated alginate (-△ -) and the unmodified control (-■-) after the addition of porcine intestinal mucus. Indicated values are means ± SD from 3 measurements.

3.4. Assessment of cytotoxic effects

The effect of the polymers on cell viability was investigated at 5 different concentrations over 3 hours employing a MTT assay. Over the investigated time and concentration range no significant impact on cell viability due to the modification could be observed.

4. Discussion

Meanwhile, thiomers are well established multifunctional polymers having reached the pharmaceutical market. The implantation of a thiol group to well know polymers leads to the improvement of several properties like permeation enhancement, gelling properties, cohesiveness and mucoadhesion. However, the extent of these improvements is directly linked to the amount of immobilized thiol groups [25,26]. To overcome the non-satisfying degree of thiolation of alginate, a novel strategy was pursued to enhance the coupling rate using a partially oxidized alginate. Alginate was activated by $NaIO_4$, which oxidizes vicinal diol groups by breaking up the C-C-bond and subsequent leads to the formation of two neighboring aldehyde groups. These aldehyde groups are able to react with primary amino groups forming a Schiff base [27]. In a reductive amination, the imine is reduced by the addition of cyanoborohydrate [28]. The resulting thiolated polymer with a remarkable degree of modification was further modified by implantation of an aromatic protecting group as it could be shown for other thiolated polymers that the aromatic-aliphatic disulfide bond leads to improved stability against too early oxidation and enhances reactivity of the thiomer [5, 7]. The novel polymers were investigated regarding to their solubility, stability and swelling behavior in aqueous environment. Solubility and swelling capacity is crucial for several qualities of a polymer in drug delivery. The swelling capacity of a polymer is, amongst others, related to its bioadhesive properties [29]. Due to water uptake of the formulation, chain relaxation of polymeric fibers takes place and enables chain interpenetration and hence, interaction with the mucus [29, 30].

Moreover, the speed and degree of water uptake influences rate and extent of the release of an incorporated active pharmaceutical ingredient. The release is mainly affected by the swelling/erosion process, diffusion of a drug can be eased by hydration of the matrix due to dissolution and chain relaxation [31]. After oxidation of the carbohydrate, the Alg-CHO intermediate displayed a fast disintegration without any swelling capability. As published previously, this effect can be explained by the oxidative breaking of the C-C-bonds and probably hydrolysis of the carbohydrate [27]. A decrease in molar mass after oxidation of polysaccharides was reported [32]. However, this effect was overcompensated by the implementation of the thiol bearing ligand. For both, the thiolated and the thiolated preactivated polymer a decreased solubility at lower pH values was observed. At higher pH values, remaining free carboxyl groups of the

alginate were deprotonated and the charged polymer reveals improved water solubility. Due to the thiolation, intra- and intermolecular disulfide bonds were formed. This crosslinking led to an improved cohesiveness of the polymer, a higher molecular mass and hence to a decrease in solubility and increase in disintegration time [33]. Regarding the S-protected-polymer, required pH values for complete dissolution were even higher. This effect can be explained by the additional conjugation of a hydrophobic ligand. Furthermore, water uptake could be improved for both alginate derivatives whereat the swelling capacity of the preactivated polymer was not as pronounced due to the hydrophobic component. A prolonged disintegration time of polymeric tablets is favored when a sustained release of an incorporated active pharmaceutical ingredient is on demand.

Results of rheological studies can be explained by the same phenomenon. Dynamic viscosity of the Alg-CHO decreased because of the degrading oxidation but increased after thiolation due to inter- and intramolecular disulfide bond formation. Viscosity increased even more for Alg-S-MNA. This is not solely to be explained by crosslinking due to disulfide bond formation. At pH 8, the Alg-S-MNA polymer was not completely dissolved, gel formation was observed due to the higher hydrophobicity and the crosslinking disulfide bonds. After treatment with hydrogen peroxide, the thiomer showed an over the time increasing viscosity due to oxidation of free thiol groups leading to an extensive crosslinking [34]. A higher amount of hydrogen peroxide led to a more rapid but not more pronounced oxidation. The preactivated polymer showed a relatively small change in viscosity. This is in accordance with the theory that the aromatic group prevents the polymer from formation of additional disulfide bonds in comparison to non S-protected thiomers which are unstable especially in solutions with pH values above 5 as it is the case under physiological conditions [35].

Improvement of mucoadhesion by immobilizing thiol groups to a polymeric backbone has been reported in several studies and the concept was proven *in vivo*. Furthermore, a correlation between degree of thiolation and resulting mucoadhesion was found [25, 26, 36]. To proof a mucoadhesive effect of the highly thiolated and S-protected thiolated polymer, the “rheological synergism” method was employed. It is reported that the increase of viscosity of polymer-mucus mixtures compared to the viscosity of both single components correlates with the mucoadhesive properties of the polymer [29]. The dynamic viscosity of the polymer/mucus mixture results from interactions between the two components. Chain entanglements, conformational changes, non-covalent interactions like van der Waal forces, hydrogen bonds and ionic interactions and for thiomers covalent interactions due to disulfide bond formation with mucin substructures [37, 38]. Both, the thiolated and the preactivated thiolated derivative showed a pronounced rheological synergism. The increase in interactions between both components over time

could be explained by the formation of disulfide bonds and chain entanglement. Alginate is a well-established excipient and listed in the Pharmacopoeia Europea. To exclude any changes in biocompatibility of the polymer due to the modification, impact of the substances on Caco-2 cells were investigated and demonstrated no significant changes in toxicological profile.

In view of drug delivery, both, the thiolated and the S-protected thiolated polymer show partly different, but promising properties for application in drug delivery. Properties like reduced solubility and increased water uptake, improved mucoadhesion, gelling properties or stability against oxidation may open applications in tailor-made drug delivery systems.

5. Conclusion

Within this study, a novel attempt has been persuaded to improve the coupling rate of cysteine on sodium alginate as properties of thiomers are highly dependent on the degree of thiolation. The polymer could be thiolated with more than 1300 µmol attached thiol groups per gram polymer. Thereby, the carbohydrate rings were opened via sodium periodate followed by reductive amination of the oxidized alginate. The thiomer was successfully S-protected showing improved cohesiveness, prolonged integration, increased viscosity and improved mucoadhesive properties compared to the unmodified alginate. According to this and taking stability against oxidation due to S-protection as well as the unaltered cytotoxicity of the excipient into account, the novel polymer has potential to become a valuable excipient.

6. Acknowledgement

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Synthesis and *in vitro* characterization of entirely S-protected thiolated pectin for drug delivery

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Abstract

The study was aimed to synthesize a thiolated polymer (thiomer) that is resistant to oxidation in solutions above pH 5. In order to protect a pectin-cysteine conjugate against premature oxidation, the thiomer was S-protected by a disulfide connected leaving group. Therefore, 2-mercaptonicotinic acid was first coupled to L-cysteine by a disulfide exchange reaction and the purified product was subsequently attached to pectin by a carbodiimide mediated amide bond formation. The obtained fully S-protected thiolated pectin was *in vitro* characterized with respect to co- and mucoadhesive properties and stability towards oxidation. The results indicated a 1.8-fold and 2.3-fold enhanced disintegration time at pH 6.8 of the S-protected thiolated pectin (Pec-Cys-MNA) compared to thiolated pectin (Pec-Cys) and unmodified pectin (Pec). Moreover, rheological measurements of polymer/mucus mixtures showed a 1.6-fold (compared to Pec-Cys) and 6.7-fold (compared to Pec) increased dynamic viscosity of Pec-Cys-MNA. On the other hand, in the presence of a strong oxidizing agent such as H_2O_2 (0.3 % v/v), no increase in viscosity of Pec-Cys-MNA could be observed. Further investigations proved that the first time all thiol groups on a thiolated polymer could be protected by a leaving group owing to the novel synthesis. Accordingly, these features may help to develop thiomer based liquid or gel formulations targeting mucosal surfaces such as nasal, ocular or vaginal drug delivery systems.

Keywords: thiomers; S-protected thiomers; mucoadhesion; oxidation; disulfide exchange

1. Introduction

The concept of mucoadhesive excipients for drug delivery systems was invented to prolong the residence time of dosage forms at the site of action [1, 2]. The intimate contact combined with controlled drug release at delivery site may also lower drug concentrations and administration frequency for disease treatment. A promising approach to enhance the interactions between dosage form and mucosal membrane or mucus was the introduction of thiol bearing ligands to pharmaceutically used polymers, so-called thiomers [3]. Thiomers can form covalent disulfide bonds between cysteine-rich subdomains of mucus and the auxiliary agent, which are much stronger than van der Waal's forces, hydrogen bonds or dipole forces. However, in an aqueous environment above pH 5 thiomers are sensitive to oxidation unless they are sealed under inert conditions [4]. This deteriorating factor with respect to their pharmaceutical use limits the fields of application since they cannot be stored as aqueous solution (e.g. eye drops) and probably reduces the efficiency of the system, especially in body regions where pH is raised.

It was therefore the aim of the study to synthesize a thiomer that is stable towards oxidation and to investigate mucoadhesive properties. In order to reduce uncontrolled oxidation of thiomers, a protecting ligand was introduced. The idea is adapted from covalent chromatography, where thiol bearing resins can be preactivated by disulfide connected pyridyl substructures [5, 6]. These resins bind very efficiently peptides or proteins via disulfide exchange reactions. In analogy, preactivated thiomers may bind to high extent to cysteine-rich subdomains of mucus by disulfide exchange reactions and are protected against premature oxidation. However, a pyridyl substructure as leaving group is problematic from toxicological point of view and was therefore replaced by 2-mercaptonicotinic acid [7]. Previous studies attempted to protect thiomers by binding the leaving group to an already thiolated polymer backbone. However, the S-protection rate was only around 50 % by using this method [8, 9]. In order to improve the S-protection rate, the thiol bearing ligand was bound to the leaving group before coupling to the polymer backbone and purified. Accordingly, the ligand L-cysteine was connected to 2-mercaptonicotinic acid by a disulfide exchange reaction and excess of L-cysteine was removed by ion exchange chromatography. The S-protected ligand was subsequently coupled to pectin as model polymer backbone. The final product was *in vitro* analyzed and compared with the conventional thiomer and unmodified pectin.

2. Materials and methods

2.1. Materials

2-Mercaptonicotinic acid (MNA) was purchased from abcr GmbH & Co. KG, Germany. Pectin (classic, from citrus fruits, degree of esterification: 35-40%) was supplied by Herbstreith & Fox, Germany. Resazurin sodium salt powder was purchased from Acros Organic, Austria. Fetal bovine serum and MEM with phenol red was purchased from PAA laboratories, Austria. All other chemicals were purchased from Sigma-Aldrich, Austria.

2.2. Synthesis of Cys-MNA

The ligand 2-((2-amino-2-carboxyethyl)disulfanyl)nicotinic acid (Cys-MNA) for conjugation with pectin was synthesized in a two-step reaction. 2-Mercaptonicotinic acid (MNA) was first oxidized to the corresponding MNA-dimer (2,2'-disulfanediyldinicotinic acid) with hydrogen peroxide according to a method described previously [8, 9]. The obtained MNA-dimer was subsequently modified by a disulfide exchange reaction with L-cysteine to Cys-MNA as shown in Figure 1.

Briefly, 2 g of MNA was dispersed in 80 mL of demineralized water by ultrasonication for 10 min. Afterwards the pH was adjusted to pH 8 with NaOH (5 M) obtaining a yellow clear solution. In order to oxidize MNA, 2.5 mL of hydrogen peroxide (30 % v/v) was added and stirred for 10 min until the solution became colorless. The pH was maintained during the reaction at pH 8-9 with NaOH (5 M). Following that, a solution containing 1 g of L-cysteine in 40 mL of demineralized water was adjusted to pH 8 and added drop-wise to the reaction mixture within 60 min. The pH was maintained during the reaction at pH 8.5-9.5 and a slightly yellow clear solution was obtained.

Thin layer chromatography (TLC) was used to verify that Cys-MNA was successfully generated. The stationary phase was silica gel with a fluorescent indicator coated on aluminum foils and a mixture of butanol:acetic acid:water (4:1:1) was used as mobile phase. The plates were analyzed under blacklight (UV254) and subsequently sprayed with a ninhydrin solution (0.3 g in 100 mL ethanol) in order to visualize primary amino groups after 10 min drying at 110 °C.

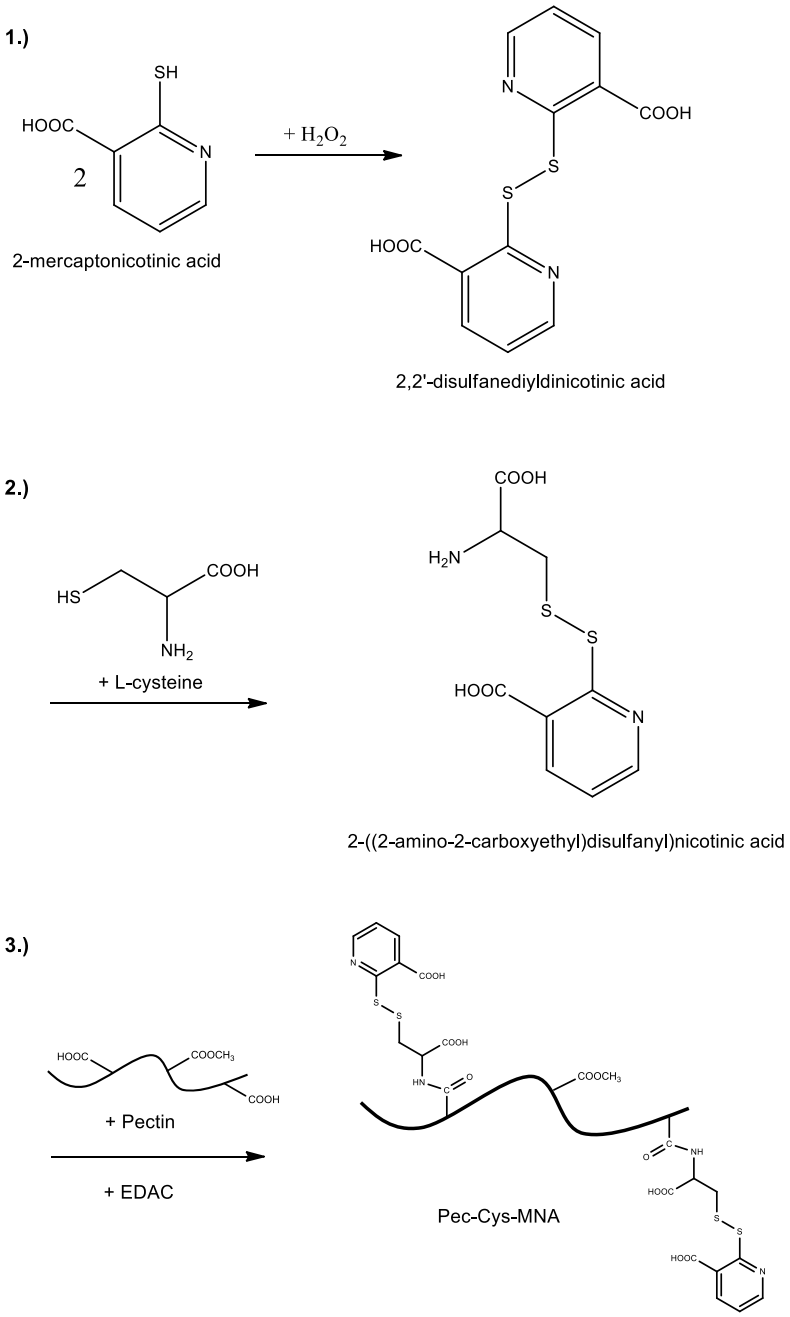

Figure 1.
Schematic synthesis of S-protected thiolated pectin (Pec-Cys-MNA). First, 2-mercaptonicotinic acid was oxidized with hydrogen peroxide to the corresponding MNA-dimer (1). Subsequently, L-cysteine was added to form Cys-MNA ligand via disulfide exchange reaction (2). The purified product was coupled to pectin by a carbodiimide mediated amid bond formation (3).

2.3. Purification of Cys-MNA

In order to eliminate unbound L-cysteine and L-cystine from the reaction mixture, two purification steps were performed. The pH of the slightly yellow solution was decreased to pH 7.5 to precipitate L-cystine, since it is poorly soluble in an aqueous solution in a pH range of 2-8 [10]. The precipitation was removed by filtration and the filtrate was further purified with an ion exchange column. At pH 7.5 L-cysteine is uncharged, whereas MNA, MNA-dimer and Cys-MNA are mainly negatively charged (calculated by ChemAxon, www.chemicalize.org). Therefore, the column was packed with 30 g anionic exchange resin (Dowex® 1X4 chloride form, 100-200 mesh), loaded with 250 mL of a NaCl-solution (2 M) and washed with 250 mL of demineralized water. Afterwards, the filtrate was filled onto the column and washed two times with 250 mL of demineralized water. The bound compounds were released by adding three times 250 mL of a NaCl-solution (2 M) and the collected eluent was reduced to 200 mL with a rotary evaporator. The solution was taken for synthesis of S-protected thiolated pectin conjugate, since only compounds displaying primary amino groups can couple to the preactivated pectin.

2.4. Synthesis and purification of S-protected thiolated pectin

The S-protected thiolated pectin conjugate (Pec-Cys-MNA) was synthesized by the covalent attachment of primary amino groups of Cys-MNA to the carboxylic acid group of pectin according to a modified method, as described previously by our research group [11]. Briefly, 2 g of pectin (Pec) were dissolved in 200 mL of demineralized water and pH was adjusted to 5.5. The carboxylic acid moieties were activated for conjugation by the addition of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) in a final concentration of 150 mM and N-hydroxysuccinimide (NHS) in a final concentration of 40 mM. After stirring for 15 min at pH 5.5 the pH was adjusted to pH 7 and the solution obtained in above described purification process was slowly added within 30 min. The reaction mixture was stirred for 3 hours at room temperature. In order to purify the Pec-Cys-MNA conjugate, the solution was filled in a dialysis tube (MWCO 10,000-12,000 Da) and dialyzed ten times against 5 L of demineralized water for 12 hours. Thereafter, the dialyzed product was freeze dried and stored under dry conditions until use.

2.5. Synthesis and purification of thiolated pectin

The pectin-cysteine conjugate (Pec-Cys) was synthesized according to a method described previously [11, 12]. In brief, 2 g of pectin were dissolved in 200 mL of demineralized water, the pH was adjusted to pH 4.5 and EDAC was added in a final concentration of 150 mM. After 15 min of activation, 1 g of L-cysteine dissolved in 10 mL of demineralized water was added and the solution was stirred for 3 hours at room temperature at pH 5. For purification, the reaction mixture was filled in a dialysis tube (MWCO 10,000-

12,000 Da) and dialyzed ten times against 5 L of HCl (200 µM) in the dark at 10 °C to prevent oxidation of free thiol groups. The product was lyophilized by drying frozen solutions at -30 °C and 0.01 mbar. The polymer was stored in the dark at 4 °C under dry conditions until further use.

2.6. Characterization of thiolated and S-protected thiolated pectin

The thiolated (Pec-Cys) and S-protected thiolated pectin (Pec-Cys-MNA) were analyzed for functional groups to characterize the polymers. Moreover, the S-protected thiolated pectin was analyzed with respect to the extent of preactivation with MNA. The free thiol group content was determined spectrophotometrically using Ellman's reagent according to a method described previously [3]. The disulfide bond test was performed to quantify disulfide bonds formed due to oxidation during the thiolation process of Pec-Cys and secondly to quantify disulfide bonds of Pec-Cys-MNA. The test was performed in a similar way as described for thiol groups after reduction with $NaBH_4$ [3]. To verify that the isolation by dialysis was successful, the amount of primary amino groups was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) detecting unbound L-cysteine and L-cystine [13, 14]. In order to underline the results of disulfide bond tests, the amount of conjugated MNA at Pec-Cys-MNA was analyzed with reduced glutathione [8, 9]. Therefore, 0.5 mg of the polymer were dissolved in 500 µL of phosphate buffer pH 8 (0.5 M) and mixed with 500 µL of reduced glutathione (2 % m/v). After 90 min of incubation absorbance was measured at 354 nm. MNA was employed to establish the calibration curve. All experiments were performed in triplicate.

2.7. Cytotoxicity screening – resazurin assay

Evaluating the cytotoxic potential of Pec-Cys-MNA, Pec-Cys and unmodified pectin (Pec) the resazurin assay on Caco-2 cells was performed. In the environment of metabolic active cells the blue, non-fluorescent resazurin is reduced to red and fluorescent resorufin [15, 16].

Briefly, Caco-2 cells (d = 1 x 10^5 cells/well) were cultured on 24-well plates in a final volume of 500 µL MEM supplemented with 20 % fetal bovine serum (FBS) at 37 °C in 5 % CO_2 environment. The medium was exchanged every other day for 2 weeks. Before incubation with test compounds, the cells were washed twice with 500 µL of prewarmed phosphate buffer saline (PBS). Following that, 500 µL of 0.5 % (m/v) test solutions (Pec, Pec-Cys, Pec-Cys-MNA), negative control (MEM without phenol red) and positive control (4 % m/v Triton X-100) were filled into wells in triplicate. The cells were incubated for 3 h and 24 h, respectively, and washed again two times with PBS. The resazurin solution (2.2 µM in MEM without phenol

red) was added in 250 µL quantity and incubated for another 3 hours. The supernatant was removed and analyzed at 540 nm with background subtraction at 590 nm using a fluorimeter (Tecan infinite M200).

2.8. Preparation of tablets

The lyophilized polymers (Pec, Pec-Cys, Pec-Cys-MNA) were compressed into 30 mg flat-faced tablets (5.0 mm diameter) using a single punch excentric press (Paul Weber, Remshalden-Grünbach, Germany). The compaction pressure of 12 kN was kept constant during the manufacturing process and all tablets were stored under dry conditions until further use.

2.9. Evaluation of the disintegration behavior

The disintegration behavior of polymer tablets was analyzed according to the European Pharmacopoeia (Ph. Eur.). The stability of each polymer tablet was tested in 0.1 M phosphate buffer pH 6.8 and 0.1 M HCl pH 1 at 37 °C using a disintegration test apparatus with discs (Ph. Eur. Apparatus A). The oscillating frequency was 0.5 s^{-1} and the time was taken when tablets were completely disintegrated. The experiments were performed with 3 tablets of each polymer.

2.10. Evaluation of the water uptake capacity

The water uptake capacity of polymer tablets in 0.1 M phosphate buffer pH 6.8 was measured gravimetrically as described previously [17]. In brief, the tablets were fixed on a needle, accurately weighed and immersed in phosphate buffer at 37 °C. At several time points tablets were taken out, excess water was removed by gentle soaking with a tissue paper and weighed again. The amount of water uptake was calculated by following equation: Water uptake [%] = $(W_t-W_0)/W_0 \times 100$

where W_t is the weight of swollen tablet at given time point and W_0 is the initial weight of the tablet. The results were expressed as a function of time and all tests were performed in triplicate.

2.11. *In vitro* evaluation of mucoadhesive properties

2.11.1. Rotating cylinder method

In order to investigate mucoadhesive properties of produced polymers, samples were analyzed by the rotating cylinder method as reported [11]. Native small intestinal porcine mucosa was fixed on stainless steel cylinders and tablets were attached to the mucosa by little force. The cylinder was used as stirrer in a dissolution tester (Erweka GmbH, Heusenstamm, Germany) according to the European Pharmacopeia (Paddle Apparatus). The dissolution tester was preheated at 37 °C and the vessels were filled with 900 mL

of 0.1 M phosphate buffer pH 6.8. Afterwards, the cylinders were immersed with a rotational speed of 100 rpm and the detachment of tablets was observed visually.

2.11.2. Rheological investigations of polymer/H_2O_2 and polymer/mucus mixtures

Confirming that all thiol groups immobilized on Pec-Cys-MNA were S-protected by MNA, polymer solutions were oxidized with hydrogen peroxide and rheologically investigated. Therefore, 1000 µL of polymer solutions (1 % m/v Pec, Pec-Cys and Pec-Cys-MNA in 0.1 M phosphate buffer pH 6.8) were oxidized with 20 µL of hydrogen peroxide (0.3 % v/v) and incubated at 37 °C until the dynamic viscosity was measured after 30 min and 120 min. Controls without oxidant were directly placed on the rheometer.

In analogy, to evaluate the adhesion properties of Pec, Pec-Cys and Pec-Cys-MNA 500 µL of polymer solutions (1 % m/v Pec, Pec-Cys and Pec-Cys-MNA in 0.1 M phosphate buffer pH 6.8) were mixed with 500 mg of native small intestinal porcine mucus and incubated for 30 min and 120 min at 37 °C. Controls without mucus were mixed with 500 µL of 0.1 M phosphate buffer pH 6.8.

Afterwards, 750 µL of the polymer/H_2O_2 mixtures, polymer/mucus mixtures and control solutions were transferred to a cone-plate rheometer (Haake Mars Rheometer, 379-0200, Thermo Electron GmbH, Karlsruhe, Germany) to measure the dynamic viscosity (η^*). The oscillating measurements were performed with a shear stress in the range of 0.5-10 Pa and the temperature was maintained at 37 ± 0.1 °C.

2.12. Statistical analysis

Statistical data analysis were performed using one-way ANOVA with 95 % confident interval (p value < 0.05) as the minimal level of significance followed by Dunns multiple-comparison post test ($p < 0.05$). Calculations were done using the software GraphPad Prism 5 version 5.01. The results are expressed as the mean of at least 3 experiments ± SD.

3. Results and discussion

3.1. Synthesis and purification of Cys-MNA

The first step to synthesize entirely S-protected thiolated pectin was the generation and purification of the ligand Cys-MNA as shown schematically in Figure 1. The successful formation of Cys-MNA by a disulfide exchange reaction between MNA dimer and L-cysteine was confirmed by thin layer chromatography (Figure 2). The left hand plate compared the reaction mixture (I), MNA II), MNA dimer (III) and L-cysteine (IV) under UV-light and the right hand plate was subsequently sprayed with ninhydrin. The reaction mixture showed three spots on the left hand plate indicating that the mixture contained MNA, MNA dimer

and probably Cys-MNA. The ligand Cys-MNA is the only compound in the reaction mixture that can be detected under UV-light and visualized by ninhydrin since it displays a primary amino group. MNA and MNA-dimer, however, could not be detected with ninhydrin as they have no amino groups. On the other hand, L-cysteine has an amino group, but it cannot be seen under UV-light. The ninhydrin sprayed TLC plate confirmed these considerations. Accordingly, the only compound that could be seen under UV-light and after ninhydrin detection was identified as Cys-MNA.

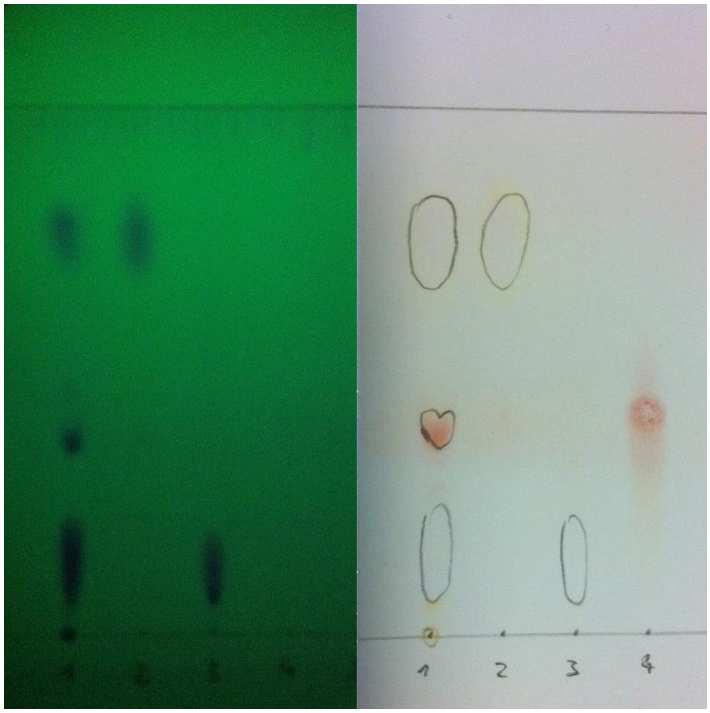

Figure 2.
Thin layer chromatography (TLC) on silica gel to separate the reaction mixture (1) after addition of L-cysteine and to compare it with MNA (2), MNA-dimer (3) and L-cysteine (4). The mobile phase was butanol:water:acetic acid (4:1:1). The left hand plate was analyzed under UV254-light, whereas the right hand plate was subsequently sprayed with ninhydrin

In order to prepare the reaction mixture for the covalent attachment to pectin mediated by EDAC (Figure 1), all compounds that display primary amino groups, except Cys-MNA, should be removed as they might be also coupled to pectin. Since L-cysteine can be oxidized to L-cystine by oxygen in air or by residual hydrogen peroxide, these two molecules are of interest. Due to the very low solubility of L-cystine at a pH between pH 2 and 8, this product was eliminated by filtration at pH 7.5. Moreover, at this pH L-cysteine is uncharged, whereas MNA, MNA dimer and Cys-MNA are mainly negatively charged (calculated by ChemAxon, www.chemicalize.org). Therefore, unbound L-cysteine was efficiently removed by anionic

exchange chromatography. MNA and MNA dimer, however, did not interfere with the carbodiimide mediated coupling reaction to pectin and were therefore later eliminated by dialysis.

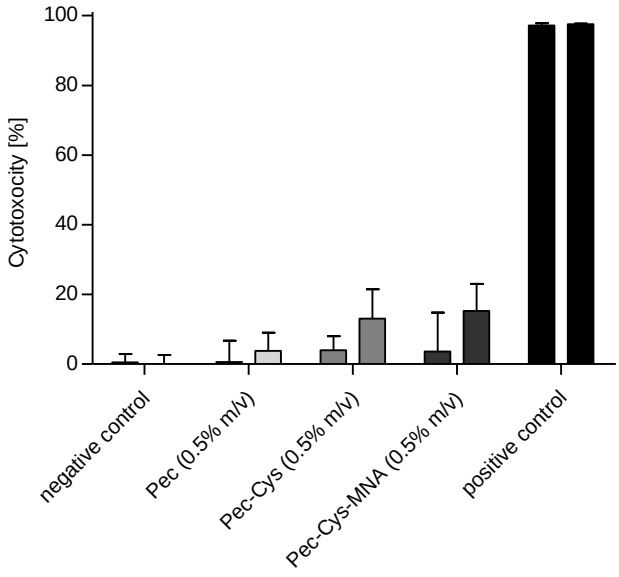

Figure 3.
Histogram shows the cytotoxic potential of S-protected thiolated pectin (Pec-Cys-MNA) compared to thiolated pectin (Pec-Cys) and unmodified pectin (Pec) after 3 hours (first bar) and 24 hours (second bar). Cytotoxicity assays were performed on Caco-2 cells using resazurin. As negative control MEM without phenol red was used and the positive control was Triton X-100 (4 % m/v). Indicated values represent an average of at least three experiments (± SD).

3.2. Synthesis and characterization of thiolated and S-protected thiolated pectin

The lyophilized pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) were white and of fibrous structure. The thiolated pectin (Pec-Cys) showed 223.5 ± 13.4 µmol/g free thiol groups determined by Ellman's reagent, whereas no free thiol groups could be detected at the S-protected thiolated pectin (Pec-Cys-MNA). After reduction with sodium borohydride, however, Pec-Cys-MNA showed 407.8 ± 22.8 µmol/g thiol groups; accordingly 50 % of that was immobilized as L-cysteine on the polymer backbone and the other half belonged to the leaving group MNA. This finding was confirmed using an assay with reduced glutathione with which 210.6 ± 11.1 µmol/g MNA was quantified. In both products no unbound L-cysteine or L-cystine could be detected with TNBS confirming quantitative isolation. The characterization tests proved that the thiolation rate of designed polymers was comparable and that the S-protection of Pec-Cys-MNA was complete.

The cytotoxicity screening results of pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) are shown in Figure 3. The findings indicate that none of the polymers was significantly ($p < 0.05$) cytotoxic on Caco-2 cells within 24 hours.

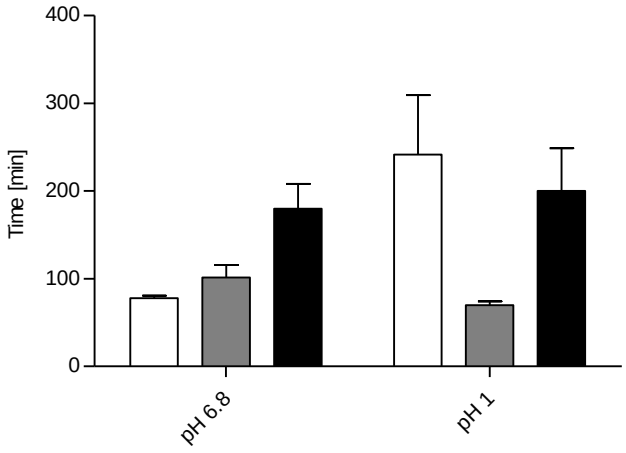

Figure 4.
Disintegration time of 30 mg tablets of Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) in 0.1 M phosphate buffer pH 6.8 and 0.1 M HCl pH 1. Indicated values are means ± SD of at least three experiments.

3.3. Evaluation of the disintegration behavior

The disintegration time of pectin (I), thiolated pectin (II) and S-protected thiolated pectin (III) discs in HCl 0.1 M (pH 1) and phosphate buffer 0.1 M (pH 6.8) are shown in Figure 4. Unmodified pectin disintegrated very quickly at pH 6.8, whereas at pH 1 the disintegration was prolonged due to the mainly protonated and therefore uncharged carboxylic acid groups of the polymer. Hence, hydrophilicity was reduced and the polymer chains do not repulse each other, resulting in a tightly packed and relatively insoluble gel [12, 18]. These variations with respect to the solubility in different body regions can be reduced by the modification of carboxylic groups as described for thiomers since they are less susceptible to changes in ionic strength and/or pH [19]. The increased stability of Pec-Cys at pH 6.8 in comparison to unmodified pectin can be explained by improved crosslinking properties of the polymer after the thiolation process. The formation of covalent bonds (disulfide bonds) within the polymer leads to an enhanced stability and cohesion that is responsible for the disintegration time. In accordance, the introduction of MNA as S-protective leaving group further prolonged the disintegration of discs in both media. This might be caused by the lipophilic nature of MNA that increases hydrophobicity of the conjugate, in combination with an increased formation of inter- and/or intramolecular disulfide bonds since crosslinking influences chain mobility and resistance to dissolution [20].

3.4. Evaluation of the water uptake capacity

The swelling behavior and water uptake capacity, respectively, is a good indicator to predict mucoadhesive and cohesive properties of a polymer. Furthermore, the stability of the polymer and release of an embedded drug are related to this capacity. The amount of uptaken water in relation to the initial weight of compressed polymer tablets in phosphate buffer pH 6.8 is shown in Figure 5. The results indicate that

the swelling behavior of thiolated and S-protected thiolated pectin was greatly improved compared to unmodified pectin. As unmodified pectin has many deprotonated COO- groups at pH 6.8 that can repel each other, the polymer is hydrophilic and the tablet can swell very rapidly. On the other hand, stability is decreased and erosion of the tablet starts rapidly, which leads to a reduced amount of absorbed water. The thiolated pectin, however, can form a stable disulfide crosslinked network allowing it to retain its structure in the presence of water. Hence, more water can be incorporated owing to the increased cohesiveness. The introduction of the lipophilic MNA as S-protecting ligand further increases the ability to bind water, resulting in a 2256.3 % water uptake capacity. The considerably higher ability to swell might be caused by the decreased solubility in an aqueous environment and the increased behavior to form stabilizing disulfide bonds as reported for preactivated covalent chromatography resins or preactivated thiomers [5, 6, 9]. Moreover, the greatly improved swelling behavior may help to enhance the gastrointestinal transit time due to the increase in volume and viscosity. This may prolong the residence time at the site of action and the carrier can act as controlled release matrix [21, 22].

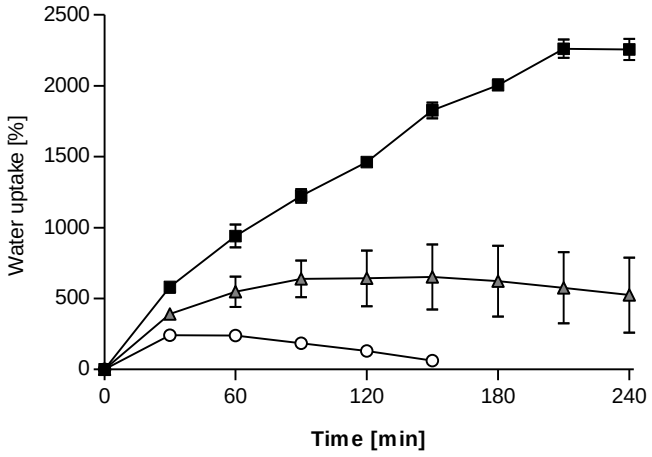

Figure 5.
Water uptake capacity of Pec (○), Pec-Cys (▲) and Pec-Cys-MNA (■) in 0.1 M phosphate buffer pH 6.8 at 37 °C. Indicated values are means ± SD of at least three experiments.

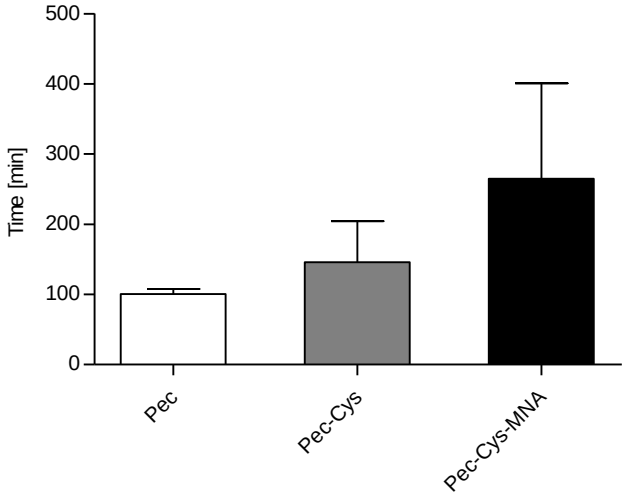

Figure 6.
Adhesion time of 30 mg tablets of Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) on intestinal porcine mucosa using rotating cylinder method. The cylinders were immersed in 0.1 M phosphate buffer pH 6.8 and rotated with 100 rpm. Indicated values are means ± SD of at least three experiments.

3.5. *In vitro* evaluation of mucoadhesive properties

3.5.1. Rotating cylinder method

In order to evaluate the mucoadhesion properties of novel synthesized polymers, freshly excised porcine intestinal mucosa was fixed on a rotating cylinder, loaded with polymers tablets and immersed in physiological buffer simulating *in vivo* conditions. The time until tablets were detached from the mucosa is illustrated in Figure 6. Although pectin is already mucoadhesive due to physical entanglement and secondary interactions (hydrogen bonds) that contribute to the formation of strengthened network [20], Pec-Cys and Pec-Cys-MNA showed more favorable adhesive properties. Thiolation and S-protection enhance the adhesion time by the formation of covalent disulfide bonds between the polymer and mucus. However, it is noteworthy that cohesiveness of polymer discs also influences the residence time at the mucosa since the discs were mainly not detached, but gradually hydrated and eroded. Therefore, improved adhesive properties of Pec-Cys and Pec-Cys-MNA were caused by the decreased solubility and increased stability.

3.5.2. Rheological investigations of polymer/H_2O_2 and polymer/mucus mixtures

Measurements of the apparent polymer viscosity and of mixtures with hydrogen peroxide demonstrated, that no free thiol groups were immobilized on the S-protected thiolated pectin since the dynamic viscosity was constant after the addition of oxidant (Figure 7A). The thiolated pectin, however, showed an increase in viscosity after oxidation with hydrogen peroxide. The increase in viscosity can be explained by the formation of inter- and/or intramolecular disulfide bonds that stabilize the polymeric network [23, 24]. On

the other hand, oxidation of thiomers can also occur *in vivo* at pH > 5 that could reduce the ability to bind to the mucus and limits their use in pharmaceutical formulations. The viscosity of a 1 % (m/v) pectin solution was so low, that it could not be seen at Figure 7A, but the addition of hydrogen peroxide did not affect the viscosity of this solution. Hence, it could be proven that Pec-Cys-MNA had constant characteristics without uncontrolled oxidation in solution due to the S-protective leaving group.

Figure 7B shows the results of rheological investigations of polymer solutions with native porcine mucus. The increase in viscosity of pectin is likely caused by the formation of hydrogen bonds with the mucus and the high initial viscosity of the native mucus. The comparatively higher increase in viscosity of thiolated pectin revealed once again of disulfide bonds between mucus and polymer. Furthermore, the viscosity of S-protected thiolated pectin overtopped the thiolated ones, indicating that S-protection by MNA improved the mucoadhesion characteristics of the polymer.

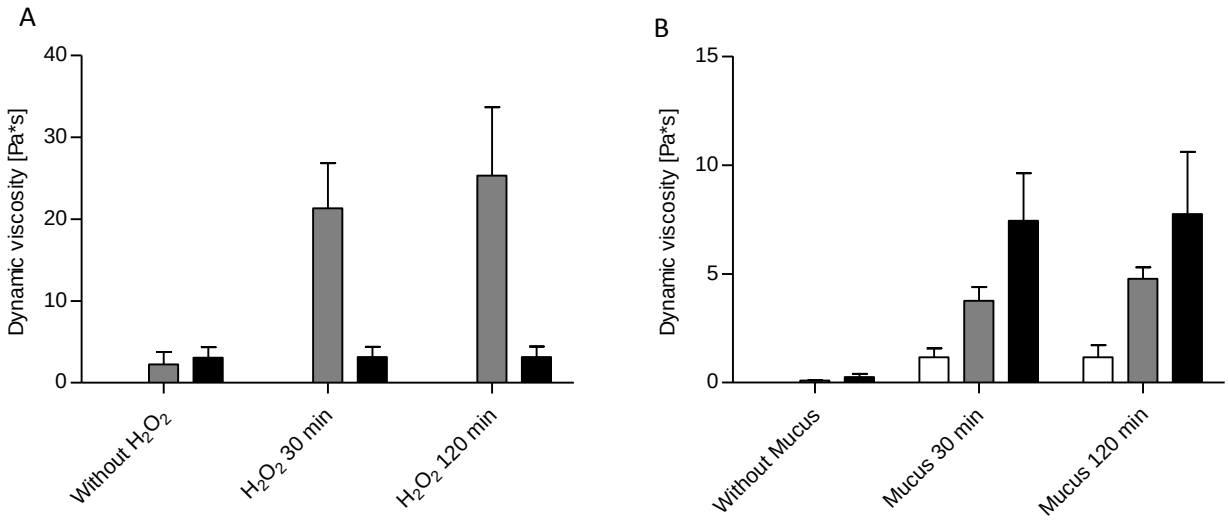

Fig. 7A+B
Comparison of the dynamic viscosity of polymer solutions (Pec (□), Pec-Cys (■) and Pec-Cys-MNA (■) which were oxidized with H_2O_2. 1 mL of polymer solutions (1 % m/v in 0.1 M phosphate buffer pH 6.8) were oxidized with 20 µL H_2O_2 (0.3 % v/v). The polymer solutions were also mixed with native porcine mucus (A) in a weight ratio of 1:1 and rheologically investigated (B). Indicated values are means ± SD of at least three experiments.

The degree of hydration is an important parameter influencing the mucoadhesive strength of auxiliary agents. The rotating cylinder method represents a method of relatively low hydrated conditions since the dry polymer come in contact with the wet mucosal surface. In that case, the adhesion might be a result of a combination of capillary attraction and osmotic forces that dehydrate and strengthen the mucus

layer [20]. This process must be distinguished from swollen polymers that are attached to mucus such as in rheological investigations of polymer/mucus mixtures. Although hydration is important for mucoadhesion, excess of hydration can lead to the formation of a slippery mucilage. The highly crosslinked Pec-Cys-MNA may be also advantageous for a prolonged mucoadhesive effect since it only permit a certain degree of hydration. Moreover, the relatively hydrophobic nature of the conjugate may support this effect. The *in vitro* characterization of Pec-Cys-MNA showed that the introduction of a S-protected thiol bearing ligand to pectin further improved the mucoadhesion properties in “dry-on-wet” and “wet-on-wet” tests compared to the thiolated and unmodified pectin. Furthermore, the stability of the thiomer towards oxidation in an aqueous environment above pH 5 likely enable the application as eye drops or vaginal gel that are not designed as instant formulation. This allows the usage in numerous pharmaceutical formulations, such as nasal, vaginal or ocular drug delivery systems [20].

4. Conclusion

The present work describes a novel synthesis to produce for the first time a fully S-protected thiomer. Interestingly, the S-protection not only improved the stability of the thiomer in solution at high pH, but also enhanced adhesive and cohesive properties of the thiomer. Moreover, the S-protecting ligand did not affect the cytotoxicity on Caco-2 cells. According to these results, the S-protection seems to be a promising modification tool to further improve the favorable characteristics of thiomers with respect to their use as excipient in mucoadhesive drug delivery systems and enables the development of liquid thiomer based formulations.

5. Acknowledgement

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Thiolated and S-protected hydrophobically modified cross-linked poly(acrylic acid) - a new generation of multifunctional polymers

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Abstract

The aim of this study was to create a novel multifunctional polymer by covalent attachement of L-cysteine to the polymeric backbone of hydrophobically modified cross-linked poly(acrylic acid) (AC1030). Secondly, the free thiol groups of the resulting thiomer were activated using 2-mercaptonicotinic acid (2-MNA) to provide full reactivity and stability. Within this study, 1167.36 µmol cysteine and 865.72 µmol 2-MNA could be coupled per gram polymer. Studies evaluating mucoadhesive properties revealed a 4-fold extended adherence time to native small intestinal mucosa for the thiomer (AC1030-cysteine) as well as an 18 - fold prolonged adhesion for the preactivated thiomer (AC1030-Cyst-2-MNA) compared to the unmodified polymer. Modification of the polymer led to a higher tablet stability concerning the thiomer and the S-protected thiomer, but a decelerated water uptake could be observed only for the preactivated thiomer. Neither the novel conjugates nor the unmodified polymer showed severe toxicity on Caco-2 cells. Evaluation of emulsification capacity proofed the ability to incorporate lipophilic compounds like medium chain triglycerides and the preservation of the emulsifying properties after the modifications. According to these results thiolated AC1030 as well as the S-protected thiolated polymer might provide a promising tool for solid and semisolid formulations in pharmaceutical development.

Keywords: Thiomers, C10–C30 alkyl acrylate cross polymers, Mucoadhesion, Preactivated thiomers, Multifunctional polymers, Drug delivery

1. Introduction

Polymeric systems with the ability to change their properties depending on alterations in the environment are defined as smart polymers. Due to different functional groups these polymers respond to changes in pH, temperature or solvents, which makes them interesting for tissue engineering or drug delivery [1].

On the one hand, polymers with amphiphilic balance, like block copolymers with hydrophilic and hydrophobic moieties are one representative for this type of polymers [1]. Recently, a new class of carbomers, exhibiting this feature was introduced into the market. In general, carbomers consist of poly(acrylic acid) cross-linked with divinyl glycol or polyalkenyl ethers. They are available at different molecular weights and with different properties, which makes them interesting excipients for personal care formulations and of course for pharmaceutical development. Concerning the pharmaceutical field, carbomer polymers are currently used as rheology modifiers for hydrogel formulations or in solid dosage forms in order to control drug release rates.

The novel types of carbomers are characterized by C10-C30 alkyl chains, which provide additional lipophilic features. One representative within this category is AC1030, which was designed as a rheology modifier for the use in cosmetic gels and creams. The most outstanding feature of this hydrophobically modified polymer is the ability to form stable emulsions with different oils due to its lipophilic moieties even in the presence of surfactant levels up to 20 %, as stated by the manufacturer.

On the other hand multifunctional polymers are represented by thiolated polymers, so called thiomers, which have shown great potential concerning drug delivery and mucoadhesion. These thiomers, which are obtained by the immobilization of thiol bearing ligands to a polymeric backbone, are able to form disulfide bonds with cysteine rich subdomains of the mucus gel layer [2]. With these covalent bonds they provide higher mucoadhesive properties compared to non - covalent bonds via ionic interactions or hydrogen bonds [3]. As thiol groups are highly susceptible towards oxidation, the concept of S – protected thiolated polymers was established by our research group [4]. To protect the thiol groups from uncontrolled oxidation reactions, disulfide bonds between the immobilized thiol group and an aromatic thiol bearing ligand namely 2-mercaptonicotinic acid (2-MNA) were formed providing a higher reactivity and stability towards oxidation [4].

In order to combine both promising strategies, thiolated AC1030 as well as the preactivated thiomer were synthesized within this study and evaluated as a multifunctional excipient in the field of pharmaceutical technology. In consequence, L-cysteine was covalently attached to the polymeric backbone of AC1030 and the thiol groups were activated using 2- mercaptonicotinic acid. These novel AC1030 conjugates and

AC1030 itself were evaluated in terms of mucoadhesive properties and swelling behavior, as well as cytotoxicity. Furthermore interactions with lipophilic excipients were examined throughout this study. On the part of interactions with lipophilic drugs, valsartan as a model drug was evaluated in tablets of AC1030 conjugates. Considering semisolids, AC1030 polymers were investigated for their emulsifying properties and emulsions formed with medium chain triglycerides were tested for their stability.

2. Materials and Methods

2.1. Materials

Hydrophobically modified cross-linked poly(acrylic acid) (Carbopol® Ultrez 20) and carbomer without hydrophobic modifications (Carbopol® 981) were kindly supplied by Lubrizol Europe, Brussels, Belgium. 2-Mercaptonicotinic acid (2-MNA) was purchased from ABCR GmbH & Co KG, Karlsruhe, Germany. Water HPLC gradient grade was purchased from Fisher Chemical, United Kingdom. Valsartan was obtained from TCI Chemicals, Eschborn, Germany. All other chemicals were purchased from Sigma-Aldrich, Austria.

2.2. Synthesis of thiolated AC1030

AC1030-cysteine was synthesized by covalent attachment of L- cysteine via amide bond formation mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC). First, AC1030 was neutralized using 5 M NaOH. Then, the carboxylic moieties were activated by addition of 200 mM of EDAC and 50 mM of N-hydroxysuccinimide (NHS). Afterwards, 1 g of L-cysteine was added to the mixture and the pH was adjusted to 5.5. The reaction mixture was stirred for 6 hours at room temperature. For purification, the product was dialyzed at 10 °C in the dark. Dialysis was performed two times against 0.2 mM HCl, two times against the same medium but containing 1 % NaCl to quench ionic interactions and then again two times against 0.2 mM HCl. In the end purified thiolated AC1030 was obtained by lyophilization under reduced pressure (Christ Gamma 1-16 LSC Freeze dryer). A control polymer was prepared in exactly the same way but EDAC and NHS were omitted in the reaction. Table 1 displays the composition of the reaction mixtures.

Table 1. Composition of reaction mixtures for the synthesis of thiolated AC1030 and preactivated AC1030.

	Polymer (g)	EDAC (mM)	NHS (mM)	Cysteine (g)	2 MNA (g)	free thiol groups (µmol/g)	L-cysteine (µmol/g)	Unbound cysteine (µmol/g)	2-MNA (µmol/g)
AC1030	1.0	-	-	1.0	-	12.15	20.45	10.12	
AC1030-cysteine	1.0	200	50	0.5	-	814.82	1167.36	65.87	
AC1030-Cyst-2-MNA	1.0 AC1030-cysteine	-	-	-	0.5	45.13	1137.43	36.65	865.72

2.3. Synthesis of S-protected thiolated AC1030

Thiolated AC1030 was preactivated via disulfide bond formation between the polymeric thiol groups and the aromatic thiol bearing ligand 2-mercaptonicotinic acid, according to a previously described method [4]. First, the dimeric form of 2-mercaptonicotinic acid was synthesized via oxidation using hydrogen peroxide. Then, the aromatic ligand was attached covalently by disulfide bond formation. Therefore, 500 mg of AC1030-cysteine were hydrated in distilled water by stirring with a magnetic stirrer at room temperature. In the next step, 250 mg of 2,2'-dithiodinicotinic acid were added and pH was adjusted to 8 using 5 M NaOH. After stirring for 6 hours, the reaction mixture was dialyzed against water at 10 °C in the dark to remove unbound 2-MNA. The preactivated thiomer was obtained by freeze drying of the aqueous polymer solution.

2.4. Characterization of AC1030 conjugates

The amount of covalently attached L-cysteine was determined using Ellman´s reagent (5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) as described previously [5]. To determine the total amount of attached L-cysteine, the reaction was performed after reduction with $NaBH_4$ followed by addition of DTNB. To ensure the removal of unbound cysteine a TNBS test was performed [6].

The degree of preactivated thiol groups was quantified spectrophotometrically using a method previously described by our research group [4]. By the addition of glutathione 2-MNA was released from the polymer and the absorbance of the aromatic ligand was measured at 354 nm. For control, samples without GSH were used.

2.5. Manufacturing of tablets

For further studies, polymer conjugates were compressed at 10 kN by a single punch excentric press (Paul Weber, Remshalden-Grünbach, Germany) into 30 mg tablets.

2.6. Disintegration of tablets

The disintegration behavior of tablets was evaluated according to the European Pharmacopeia (Ph. Eur.). Each polymer tablet was tested in 100 mM phosphate buffer, pH 6.8 at 37 °C using a disintegration apparatus (Ph. Eur. Apparatus A). The oscillating frequency was 0.5 s^{-1} [7] and the test was performed until complete disintegration. Three tablets were tested for each polymer.

2.7. Swelling behavior

The water absorption capacities of the synthesized polymers as well as control were determined gravimetrically. Therefore, the prepared tablets were fixed on a paper clip and incubated in 100 mM phosphate buffer pH 6.8 at 37 °C [8]. At predetermined time points, the surface water was removed and the tablets were weighted. Water uptake was calculated using the following equation:

$$Water\ uptake\ [\%] = (Wt - W0)/W0) \times 100$$

where w_t is the weight at the given time point and w_0 is the initial weight of a tablet.

2.8. *In vitro* evaluation of mucoadhesive properties

2.8.1. Tensile studies

In order to evaluate the mucoadhesive properties of AC1030 and the newly synthesized AC1030 conjugates, tensile studies were performed [9]. In detail, porcine native intestinal mucosa was mounted on the bottom of a beaker using a cyanoacrylate adhesive. Test discs of different polymers were attached to a stainless steel flat cylinder (8 mm in diameter, 0.3 g of weight in the system), which was hung by a nylon thread over a laboratory stand. The beaker was slowly filled with 100 mM phosphate buffer pH 6.8 at 37 °C. The beaker was placed on a balance and carefully lifted by a mobile platform to provide contact between the tablet and the intestinal mucosa. After a contact time of 20 minutes, the platform was lowered at a rate of 1.0 mm/s. Data points were collected every second by a personal computer (Sarta Collect software; Sartorius AG, Austria) linked to the balance. Afterwards, the total work of adhesion (TWA) as well as the maximum detachment force (MDF) were calculated by using trapezoidal rule.

2.8.2. Rotating cylinder

In addition to the tensiometer studies, mucoadhesive properties were evaluated using the rotating cylinder method as described previously by our research group [10]. In brief, native porcine small intestinal mucosa was fixed on a stainless steel cylinder and test disks of each polymer were attached to the tissue. The cylinder was placed in a dissolution tester (Erweka GmbH, Heusenstamm, Germany) according to the

European Pharmacopeia (Paddle Apparatus). The vessels were filled with 900 ml of a 100 mM phosphate buffer, pH 6.8, at 37 °C. Then, the cylinders were rotated with a speed of 100 rpm and the detachment of the test disks was observed visually.

2.9. *In vitro* release studies

Drug release studies were performed for unmodified AC1030 as well as for the newly synthesized AC1030 polymers and carbomer without hydrophobic alkyl chains (NA - carbomer) as control using valsartan as model lipophilic compound. First of all, 28.5 mg of polymer were dissolved in distilled water, 1.5 mg of valsartan were added subsequently and stirred till homogenous. The polymer/drug mixtures were lyophilized and compressed into 30 mg tablets as described above. The tablets were placed in Erlenmeyer flasks containing 10 ml of 100 mM phosphate buffer, pH 6.8. The flasks were placed on a rocker at 37 °C and 100 % relative humidity. Sink conditions were maintained throughout the study. Samples of 200 µl were taken at predetermined time points and replaced by an equal volume of fresh release medium. The analysis of all samples was performed via HPLC analysis (LaCHROM Elite) using a ProntoSIL C18-column (250 × 4.6 mm, 5 µm; Bischoff Chromatography, Leonberg, Germany). A mixture of water containing 0.1 % of TFA (triflouroacetic acid) and acetonitrile with 0.1 % TFA (50:50) served as mobile phase and the eluate was detected at 260 nm using a DAD. Quantification was performed by integrating peak areas and the amounts of released drug was calculated by interpolation from a standard curve containing increasing concentrations of valsartan. Cumulative corrections were made for previously removed samples.

2.10. Drug load capacity

To determine the drug load capacity of the different polymers, unmodified polymer, AC1030 conjugates and NA - carbomer as control were spiked with different amounts of valsartan. Therefore, polymers were dissolved in distilled water and spiked with 5 or 10 % of drug, calculated on the total weight of the tablet. These aqueous solutions were lyophilized and compressed into tablets. For complete drug release, the tablets were placed in a beaker containing 10 ml of 200 mM Tris buffer, pH 9 for the tablets with 5 % valsartan and 15 ml of the same buffer for tablets with 10 % drug. After 24 hours, samples of 200 µl were withdrawn and analyzed via HPLC as mentioned above.

2.11. Emulsification capacity

To ensure the preservation of the emulsifying properties, all polymer conjugates were tested for their emulsification capacity. Therefore, medium chain triglycerides were incorporated into aqueous polymer solutions. First, each polymer was dissolved at a concentration of 0.5 % in distilled water by stirring with a

magnetic stirrer. The oil was added drop wise under constant stirring. The resulting formulation consisted of 70 % aqueous polymer solution and 30 % medium chain triglycerides. Stability of all formulations was determined by centrifugation at different G forces as well as different time intervals. First each emulsion was centrifuged for 1 minute, then for 5 minutes followed by 10 minutes. If stability was still provided after centrifugation for 30 minutes, G force was increased to the next step.

2.12. Assessment of cell viability

Potential cytotoxic effects of unmodified AC1030 as well as the synthesized AC1030 conjugates on Caco-2 cells were determined using MTT assay. Caco-2 cells were cultured on a 96 well plate in a density of 20 x 10^4/ml cells in 100 µl of minimum essential medium (MEM) for 24 hours at 37 °C and 5 % CO_2. Thereafter, cells were incubated with 0.05 % (m/v) solutions of S-protected thiomer, thiomer and unmodified polymer. Untreated cells served as positive control and a 1 % solution of Triton X-100 was used as negative control. After 24 hours of incubation, cells were washed twice with PBS and medium was replaced by 100 µl 0.5 % (m/v) MTT in MEM. After an incubation time of 4 hours, the medium was removed and formazan crystals were dissolved in 100 µl of DMSO. Absorbance was measured at a wavelength of 570 nm (Tecan infinite, M200 spectrometer, Grödig, Austria). Cell viability was calculated using the following equation:

$$\% \text{ viability} = \frac{\text{Average absorbance of each triplicate}}{\text{Absorbance of positive control}} \text{ X } 100$$

2.13. Statistical analysis

Statistical data analysis was performed using Students *t*-test with a confidence interval ($p < 0.05$) for the analysis of two groups. One way ANOVA was performed to analyze the data of more than two mean values.

3. Results

3.1. Synthesis and characterization of AC1030 conjugates

AC1030-cysteine was synthesized by the covalent attachment of L-cysteine via amide bond formation. In a second step the aromatic ligand 2-MNA was attached to the thiolated polymer by disulfide bond formation, which is shown in Figure 1. The total amount of cysteine immobilized on the polymeric backbone, the amount of free thiol groups as well as the amount of the coupled aromatic ligand 2-MNA is shown in Table 1. A control polymer, which was synthesized in the same way but omitting EDAC and NHS showed only a negligible amount of attached L-cysteine. The result of the TNBS test confirmed the removal of unbound L-cysteine via dialysis (Table 1).

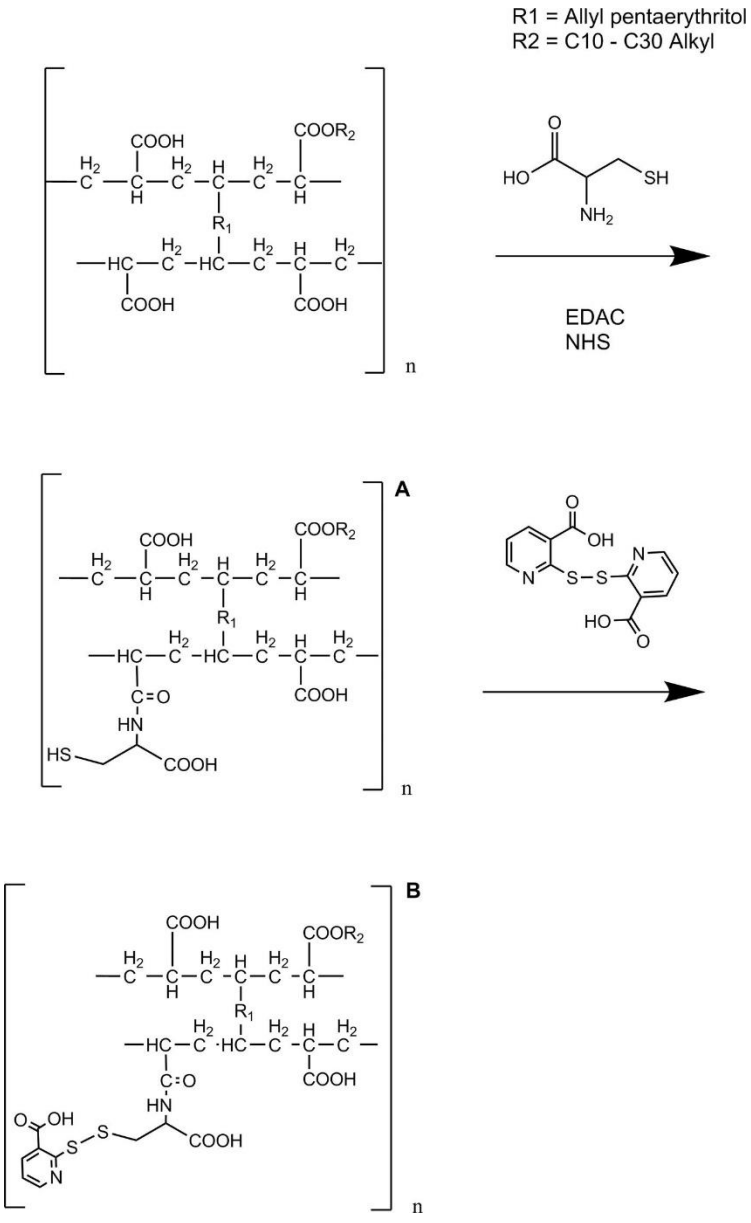

Figure 1.
Synthesis of AC1030-cysteine (A) and AC1030-Cyst-2MNA (B).

3.2. Evaluation of the swelling behavior

The novel AC1030 conjugates as well as the unmodified polymer were investigated in terms of their swelling behavior. Time related water uptake for all tested polymers is depicted in Figure 2. Within this experiment, differences regarding swelling speed and swelling ratio could be observed for tablets of AC1030, AC1030-cysteine and AC1030-Cyst-2-MNA. Being incubated in aqueous environment, all test discs expanded their weight before disruption, however tablets of modified polymers exhibited a greater cohesiveness, which was expressed in a prolonged swelling time. Furthermore, a decelerated water uptake could be observed for test discs of S-protected thiomer.

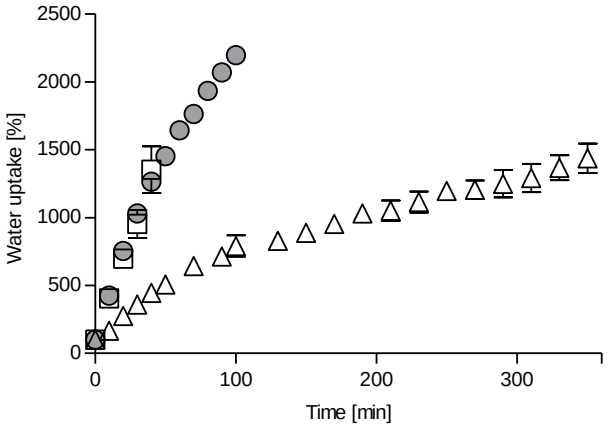

Figure 2.
Time dependent water uptake of AC1030 (□), thiolated AC1030 (●) and preactivated AC1030 (Δ) test discs in phosphate buffer pH 6.8. Illustrated values are the means of at least three experiments ± standard deviation.

3.3. Mucoadhesive properties

3.3.1. Tensile studies

The mucoadhesive properties of the newly synthesized polymers and unmodified AC1030 were evaluated by tensile studies on porcine intestinal mucosa. The total work of adhesion (TWA) and the maximum detachment force (MDF) as a measure of mucoadhesion are displayed in Figure 3.

It was shown that the preactivated thiomer exhibited the highest improvement in mucoadhesive properties. It displays a 5.7-fold increase in the total work of adhesion as well as a 2.9-fold increase in the maximum detachment force compared to the unmodified polymer. In contrast, the thiomer showed a 3.4-fold improved TWA and 2.8-fold higher MDF. Cohesiveness of test discs was not affected during the incubation time. Although swelling had started, no signs of erosion were visible.

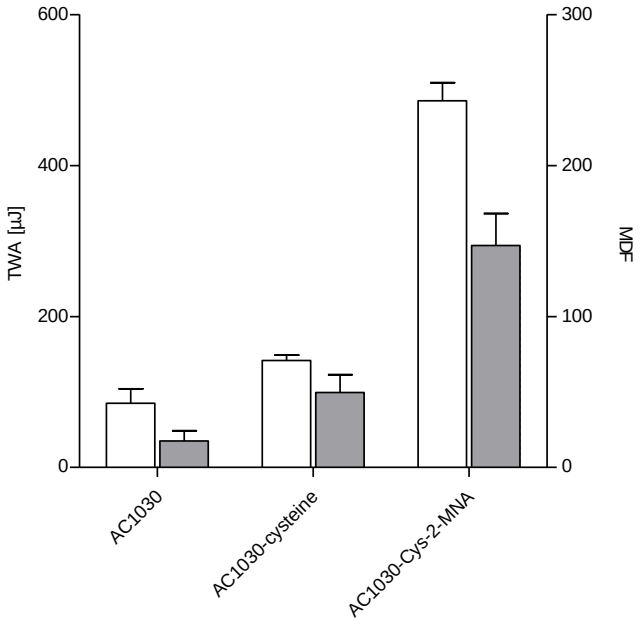

Figure 3.
The graph displays the mucoadhesive properties of AC1030, AC1030-cysteine and preactivated AC1030 determined via tensile studies. White bars show the total work of adhesion (TWA) and grey bars represent the maximum detachment force (MDF). Values are means ± standard deviation of at least five experiments.

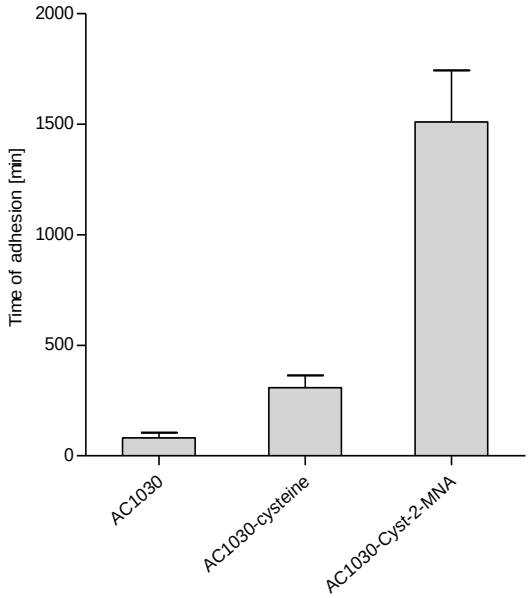

Figure 4.
Mucoadhesive properties of AC1030, thiolated AC1030 and preactived AC1030. The time of adhesion on freshly excised porcine intestinal mucosa was determined via rotating cylinder in 0.1 M phosphate buffer pH 6.8. The graph shows the means of at least six experiments ± standard deviation.

3.3.2. Rotating cylinder

To simulate an *in vivo* situation for mucoadhesion, time of adhesion to native small intestinal porcine mucosa was evaluated for test discs of the new conjugates as well as the unmodified polymer. Cohesive as well as adhesive properties of the polymers affect adhesion time in this experimental setup. Tablets of thiolated polymer showed an almost 4-fold extended time of adherence compared to the unmodified polymer. A further improvement considering mucoadhesive properties could be determined for the S-protected thiolated polymer (Figure 4). Within the experiment test discs of all polymers were converted into a shapeless jelly mass, which was partly eroded before detachment. With regard to AC1030-Cyst-2-MNA, complete detachment could not be observed, but a negligible jelly residue still adhered to the mucosal tissue by the end of the experiment.

3.4. Disintegration of tablets

Assessing disintegration behavior of tablets compressed of modified as well as unmodified AC1030 polymers revealed significant differences. Unmodified AC1030 tablets disintegrated within 70 minutes. AC1030-cysteine tablets and tablets of the preactivated thiomer showed a 2- and 4.4- fold prolonged disintegration time. Disintegration was observed after 112 minutes for tablets of AC1030-cysteine and 308 minutes for AC1030-Cyst-2-MNA, respectively. Disintegration was stated as fully swollen tablets without firm core remaining.

3.5. *In vitro* release studies

In vitro release studies for the model drug valsartan were carried out for the unmodified polymer, the thiomer, the preactivated thiomer and carbomer without hydrophobic modifications as a control polymer. Drug release from tablets of NA - carbomer occurred within 3 hours. In contrast, all AC1030 polymers showed a sustained release profile and tablets of these polymers released the drug completely within 6 hours (Figure 5). Considering drug release from AC1030 conjugates as well as unmodified AC1030, no significant differences could be observed.

3.6. Loading efficiency

Drug load efficiency of AC1030, AC1030-cysteine, AC1030-Cysteine-2-MNA and NA - carbomer were evaluated by spiking polymers with 5 and 10 % of valsartan.

Regarding 5 % drug content, all AC1030 conjugates showed almost 100 % of utilized drug amount in the tablets, whereas only 65 % of utilized drug amount could be loaded into NA - carbomer tablets. When 10 % valsartan should be loaded into the tablets the polymers, the following order could be determined regarding drug load: AC1030 (67.8 %) > AC1030-cysteine (44.8%) > AC1030-Cyst-2-MNA (31.9%) > NA-carbomer (12.5 %).

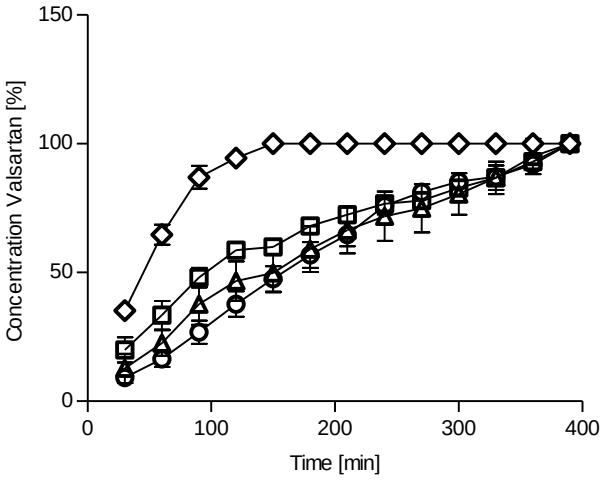

Figure 5.
Time related release of valsartan from 30 mg tablets of AC1030 (○), AC1030-cysteine (□), preactivated AC1030 (Δ) and NA-carbomer (◊) in phosphate buffer pH 6.8. Indicated values are the means of at least three experiments ± standard deviation.

3.7. Emulsification capacity

Incorporation of 30 % (v/v) medium chain triglycerides into aqueous solutions of AC1030, AC1030-cysteine, AC1030-Cyst-2-MNA and NA - carbomer revealed that all AC1030 polymers were able to form white creamy emulsions. However, emulsions formed with NA - carbomer solutions showed signs of creaming right after the preparation, which referred to a deficiency concerning emulsification properties. Stability of the prepared formulations was evaluated by centrifugation cycles. Time and centrifugational force leading to phase separation in the tested emulsions are illustrated in Table 2.

Table 2. Emulsion stability of AC1030, AC1030-cysteine, AC1030-Cyst-2MNA and NA - carbomer

	G force (g)	Time (min)
NA - carbomer	61	5
AC1030	835	30
AC1030-cysteine	614	10
AC1030-Cyst-2-MNA	273	10

3.8. Assessment of cell viability

Cell viability studies for the unmodified polymer, the thiomer as well as the preactivated thiomer revealed no severe toxicity after 24 hours. Mitochondrial activity was slightly affected by the novel conjugates as well as the unmodified polymer, but no significant differences could be observed between the tested substances (Figure 6).

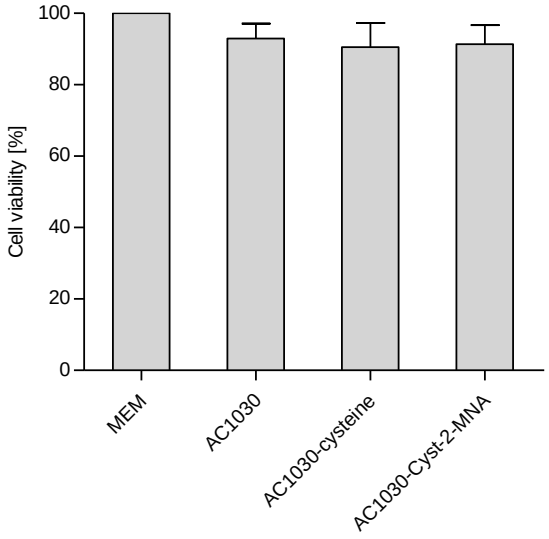

Figure 6.
Histogram shows influence on cell viability of thiomer, preactivated thiomer and unmodified polymer.

4. Discussion

Carbomers are widely used for pharmaceutical as well as personal care formulations. They are available at different molecular weights and therefore with different properties concerning water uptake capacity and viscosity. With AC1030 another important feature is added. AC1030 is a hydrophobically modified carbomer and has - due to its C10-C30 alkyl esters - a higher ability to stabilize oil - containing formulations, as stated by the manufacturer. Within this study AC1030 was tested for its potential suitability as a multifunctional polymer in the field of pharmaceutical technology. By the immobilization of a thiol bearing ligand namely L-cysteine and the S – protection via 2-MNA it was possible to improve the properties of the polymer.

Modification of AC1030 led to a significant improvement in tablet stability. Tablets compressed of thiolated AC1030 showed a 2-fold prolonged disintegration time. The increase in disintegration time of thiolated AC1030 compared to the unmodified polymer can be explained with the higher polymeric crosslinking achieved via disulfide bond formation between the cysteine moieties attached to the polymeric backbone. The higher crosslinking leads to improved stability and cohesiveness, which is expressed in a prolonged disintegration time. Regarding preactivated AC1030 a further enhancement concerning disintegration time could be observed. Stability of tablets of S-protected thiolated AC1030 was 4.4-fold increased compared to unmodified polymer, which correlates well with former studies for a different polymer, namely pectin [11]. By introducing the aromatic ligand 2-MNA the lipophilicity of the polymer is increased and along with higher crosslinking via disulfide bonds the flexibility of the polymer chains is reduced, which in the end results in a higher tablet stability [12].

Another possible reason for improved disintegration properties could be seen in the different swelling behavior. The S-protected thiolated AC1030 shows a decelerated water uptake (Figure 2) compared to AC1030 and AC1030-cysteine. Similar results could be observed for pectin, where a less rapid swelling was achieved by hydrophobic modification leading to higher stability of tablets [8]. The aromatic ligand seems to reduce water uptake into the polymer matrix, which might be due to the increased lipophilicity of the conjugate. Concerning the thiolated polymer, water uptake capacity could be increased 1.6-fold compared to the unmodified polymer. Similar results were obtained for thiolated poly(acrylic acid) [7]. The decelerated swelling of the preactivated thiomer might be caused by the lipophilic structure of the aromatic ligand 2-MNA. In addition to the C10-C30 alkyl chains, the S-protection led to an additional increase in lipophilicity, which might reduce water uptake capacity.

Mucoadhesion is a complex process, which is influenced by a broad range of parameters, like swelling behavior, ionization or lipophilicity [13]. Besides hydrogen bonds between carboxylic acid groups of AC1030 and hydroxyl, carboxyl or amide groups of mucus components, mucoadhesive properties can also

be attributed to the lipophilic moieties in this polymer. Due to their alkyl chains, AC1030 polymers provide additional interactions with mucus components via Van der Waals bonds. Thiolation as well as S-protection of AC1030 resulted in improved mucoadhesive properties. Regarding mucoadhesion different kinds of thiolated and S-protected thiolated polymers were evaluated. Hence, enhanced mucoadhesion is the result of disulfide bond formation between thiol groups and cysteine rich domains of the mucus gel layer. In order to prevent intra- and intermolecular disulfide bond formation in thiolated polymers, thiol groups were protected using an aromatic ligand, namely 2-MNA. With respect to mucoadhesion, the S - protection should provide full reactivity towards thiol rich subdomains of mucin by disulfide exchange reaction. In return the aromatic thiol moiety acts as a leaving group, which results in improved mucoadhesive properties [14, 15]. With a 5.7-fold higher TWA and a 2.9-fold higher MDF the AC1030-Cyst-2-MNA conjugate performed superiorly over AC1030-cysteine, which showed a 3.4-fold higher TWA and a 2.8-fold higher MDF compared to the unmodified polymer. These results are in good correlation with the results of the rotating cylinder. Again, the preactivated thiomer displayed the longest adhesion time to the intestinal mucosa, followed by the thiomer and the unmodified polymer (Figure 4). With regard to the S-protected thiomer, ameliorated mucoadhesion could also be due to the decelerated water uptake. A controlled swelling process can provide an extended adhesion as overhydrating in adhesive polymers was found to be a crucial factor in the removal process [13].

The most outstanding feature of AC1030, AC1030-cysteine and AC1030-Cyst-2-MNA is the ability to incorporate lipophilic excipients more efficiently compared to earlier generation of carbomers without hydrophobic modification. Due to their lipophilic residues, AC1030 conjugates as well as the unmodified polymer can develop more interactions via Van-der-Waals bonds. On the one hand larger amounts of lipophilic drugs like valsartan could be incorporated into tablets of AC1030 polymers and sustained release properties for the model drug were identified (Figure 5).

Valsartan was not only released over a longer period it was also released more slowly compared to NA-carbomer. In the first 60 minutes more than 50 % of valsartan were released from NA-carbomer tablets compared to about 20 % from AC1030 polymers. NA - carbomer does not have lipophilic moieties and therefore it seems to have lower interaction potential with valsartan compared to AC1030 polymers, which leads to a more rapid release of the lipophilic compound. The interactions between polymers and drugs were researched by Paulsson [16]. In his work he obtained similar results regarding the interaction between lipophilic polymers and lipophilic drugs. Drug release profiles were determined for different paraben esters with the following outcome. The rate of release decreased with increasing lipophilicity. Comparing the liberation of alprenolol and butyl paraben from different polymer matrices, he discovered

a slower release from a lipophilic polymer. Similarly, in case of AC1030 polymers the interaction between lipophilic residues of the polymer and valsartan seem to be the regulatory factor for drug release. As a BCS class III drug, valsartan is characterized by low solubility and high permeability. Valsartan displays an absolute bioavailability of 25 % and an average elimination half-life of about 6 hours [17]. The oral application via a drug delivery system with mucoadhesive and sustained release properties could therefore increase the bioavailability and reduce the dosing frequency.

On the other hand, the higher potential of interactions between AC1030 polymers and hydrophobic molecules is also evident in the capability to form stable emulsions with oils. Efficient incorporation of medium chain triglycerides into aqueous solutions of the novel AC1030 conjugates as well as the unmodified polymer was feasible within this study. Unlike the hydrophobically modified carbomers, NA-carbomer did not provide an equivalent result, most likely due to the lack of hydrophobic regions within the polymer. Both AC1030 and AC1030 cysteine emulsions displayed a high stability, whereas emulsions with S-protected thiomer tended to creaming in early stages of centrifugation cycles (Table 3). The orientation of the amphiphilic polymer on the oil water interphase seems to be disturbed by the rather large substituent 2-MNA. Due to steric hindrance of the substituent, less molecules might assemble on the interphase, which could result in a declined emulsion stability [18].

5. Conclusion

Within this study the covalent attachment of L-cysteine to AC1030 as well as the S-protection could be achieved by our research group for the first time. With these modifications, promising tools for mucosal drug delivery especially in combination with lipophilic compounds can be provided. Furthermore, the lipophilic C10-C30 chains provide the potential for using these AC1030 polymers in semisolid drug delivery systems with additional mucoadhesive properties, which makes them highly interesting polymeric excipients for semisolid formulations which are intended for topical applications. With improved mucoadhesion, tablet disintegration, sustained release of lipopohilic drugs together with the emulsification capacity, these thiolated and S-protected AC1030 polymers might be a useful tool to improve solid formulations for systemic applications as well. Therefore, this polymer modification has the potential to become a very versatile excipient for future pharmaceutical technologies.

6. Acknowledgement

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In vivo evaluation of an oral self-microemulsifying drug delivery system (SMEDDS) for leuprorelin

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Abstract

The objective of this study was to develop a self-microemulsifying drug delivery system (SMEDDS) for the model peptide drug leuprorelin to prove a protective effect against luminal enzymatic metabolism. In order to incorporate leuprorelin into nanoemulsion droplets (o/w), the commercially available hydrophilic leuprolide acetate was modified by hydrophobic ion paring with sodium oleate. The obtained hydrophobic leuprolide oleate was dissolved in the SMEDDS formulation (30 % (m/m) Cremophor EL, 30 % (m/m) Capmul MCM, 10 % (m/m) propylene glycol and 30 % (m/m) Captex 355) in a concentration of 4 mg/g showing a mean droplet size of 50.1 nm when dispersed in a concentration of 1 % (m/v) in phosphate buffer pH 6.8. The nanoemulsion was able to shield leuprolide oleate from enzymatic degradation by trypsin and α-chymotrypsin, so that after 120 min 52.9 % and 58.4 %, respectively, of leuprolide oleate were still intact. Leuprolide acetate dissolved in an aqueous control solution was completely metabolized by trypsin within 60 min and by α-chymotrypsin within 5 min. Moreover, an *in vivo* study in rats showed a 17.2-fold improved oral bioavailability of leuprolide oleate SMEDDS compared to a leuprolide acetate control solution. This is the first time, to our knowledge, that hydrophobic ion pairing is utilized in order to incorporate a peptide drug in SMEDDS and evidence of a protective effect of nanoemulsion droplets (o/w) against enzymatic degradation of a peptide drug was provided. According to these results, the system could be likely a novel platform technology to improve the oral bioavailability of peptide drugs.

Keywords: self-microemulsifying drug delivery system, protection against enzymatic degradation, leuprorelin, hydrophobic ion pairing

1. Introduction

The oral administration of peptide and protein drugs remains a significant challenge for pharmaceutical researcher due to several physiological barriers limiting gastrointestinal absorption. The rapid degradation by luminal enzymes is one of the main problems that need to be overcome to enhance systemic uptake [1]. An increasingly popular approach to improve the bioavailability of peptide drugs via the oral route are self-microemulsifying drug delivery systems (SMEDDS).

SMEDDS are isotropic mixtures of oil(s), one or more surfactants and a co-surfactant (or co-solubilizer) [2]. Dispersion of these mixtures in an aqueous environment leads to transparent or slightly bluish, thermodynamically stable oil-in-water (o/w) microemulsions with a droplet size range from 10 to 300 nm [2-4]. Since just gentle agitation is required to emulsify these formulations, the digestive motility of stomach and intestine is sufficient after oral application [5-7]. Among many factors contributing to improved oral bioavailability are the large surface area, permeation enhancement and protection against luminal enzymatic degradation. Although the latter point is often mentioned in the literature, to our knowledge it is not yet demonstrated. In addition, it was rarely successful to incorporate peptides into o/w microemulsion droplets due to their generally hydrophilic nature.

Leuprorelin (leuprolide acetate) is a synthetic gonadotropin-releasing hormone (GnRH) analogue used in the treatment of sex hormone-related disorders such as advanced prostatic cancer, endometriosis and precocious puberty [8, 9]. The highly water-soluble nonapeptide has two ionizable basic side chains, imidazole group of histidine (pKa ~ 6.0) and guanidine group of arginine (pKa ~ 13.0) [10]. As most peptide drugs, its bioavailability is low and it is usually given intramuscularly as depot injection (e.g. Lupron®). Transport studies already showed that inhibition of proteolytic enzymes could improve the intestinal absorption of leuprorelin [1].

Therefore, it was the aim of the study to prove a protective effect of a self-microemulsifying drug delivery system for the model peptide drug leuprorelin against metabolism by intestinal enzymes. In order to incorporate leuprorelin into the lipophilic core of the SMEDDS droplets, the commercially available hydrophilic leuprolide acetate was modified by hydrophobic ion paring with sodium oleate to obtain the hydrophobic leuprolide oleate. Since peptide drugs in general show poor permeability across intestinal membranes a formulation with permeation enhancing properties seems appropriate for the preparation of leuprorelin SMEDDS. Therefore, leuprolide oleate SMEDDS were prepared employing a formulation with a permeation enhancing effect. Increased permeation for the hydrophilic macromolecular compound fluorescein isothiocyanate-dextran 4 (FD4) via tight junction opening was shown in a previous study [11],

since leuprolide acetate is also mainly absorbed by the paracellular route [1] this formulation seems suitable.

2. Materials and Methods

2.1. Materials

Capmul MCM (Mono/diglycerides of caprylic acid, HLB = 5-6) and Captex 355 (Caprylic/Capric Triglyceride) was supplied by Abitec Corporation, USA. Cremophor EL (non-ionic emulsifier obtained by causing ethylene oxide to react with castor oil in a molar ratio of 35 to 1, HLB = 12-14) was purchased from BASF, Germany. Propylene glycol was obtained from Gatt-Koller, Absam, Austria. Acetonitrile and water for HPLC analysis were purchased from Avantor Performance Materials, Netherlands. Trifluoroacetic acid was obtained from Carl Roth, Germany. Leuprolide acetate (M_r 1209.4 Da (free peptide)) was supplied by Chemos, Germany. All other chemicals were purchased from Sigma-Aldrich, Austria. All chemicals were of analytical grade.

2.2. Hydrophobic ion pairing of leuprorelin

In order to increase hydrophobicity of leuprolide acetate to improve the lipid-solubility, hydrophobic ion pairing with sodium oleate was performed as described previously [10]. Therefore, sodium oleate dissolved in demineralized water was added to leuprolide acetated solutions 1 mg/mL in a molar ratio of 1:1, 2:1, 3:1 and 4:1 under continuous stirring. The obtained solutions with precipitated ion pairs were centrifuged at 5,000 rpm. The aqueous supernatant was analyzed for remaining dissolved leuprorelin via HPLC as described below. The precipitated pellet was freeze-dried at -30 °C and 0.01 mbar (Christ Gamma 1-16 LSC Freeze dryer) and stored at -24 °C.

2.3. Preparation and characterization of SMEDDS

A previously developed SMEDDS formulation showing a permeation enhancing effect via tight junction opening was employed. The self-emulsifying mixtures was composed of 30 % (m/m) Cremophor EL, 30% (m/m) Capmul MCM, 10 % (m/m) propylene glycol and 30 % (m/m) Captex 355 [11]. The lyophilized leuprolide oleate was dissolved in a concentration of 4 mg/g in the SMEDDS by using a thermomixer at 37 °C for 12 hours. Subsequently, the SMEDDS were emulsified in phosphate buffer pH 6.8 in a concentration of 1 % (m/v) under gentle stirring (200 rpm) resulting in a leuprolide loaded microemulsion (0.04 mg/mL). The mean droplet size was measured by dynamic light scattering using a PSS NICOMP TM 380 DLS (Santa Barbara, CA, USA). A microemulsion without leuprolide served as control.

Additionally, the lyophilized leuprolide oleate (molar ratio 3:1) was dispersed in phosphate buffer pH 6.8 in a concentration of 0.04 mg/mL. The leuprolide oleate suspension and leuprolide oleate microemulsion were analyzed by HPLC with respect to the solubility of leuprolide oleate in both media and the purity of the lyophilized product.

2.4. Quantification of leuprorelin via RP-HPLC

HPLC analysis was performed according to a method previously described by our research group [12]. In brief, samples containing leuprorelin were analyzed on a Nucleosil 100-5 C18 column (250x4 mm) at 40 °C with gradient elution (1 mL/min): 0-10 min; linear gradient; from 25 % A / 75 % B to 40 % A / 60 % B (eluent A: acetonitrile; eluent B: 0.1 % trifluoroacetic acid) at 278 nm. The calibration curve was established with leuprolide acetate in a range from 1.5 to 200 µg/mL. The detection and quantification limit was investigated in the validation study, and can be specified with 0.48 µg/mL and 1.44 µg/mL [12].

2.5. Drug release and payload

Determining the content of incorporated leuprorelin in droplets, microemulsions were filled into dialysis tubes (MWCO 10,000 Da) and placed in a beaker with phosphate buffer pH 6.8. This system was incubated under stirring and the amount of leuprorelin in the outer water phase was quantified. The mixture resulting from sodium oleate: leuprolide acetate in a ratio of 3:1 showed the lowest amount of residual leuprolide acetate in the water phase during hydrophobic ion pairing. Therefore, this precipitate was used for further release studies. Briefly, a thoroughly mixed formulation containing leuprolide oleate in SMEDDS (4 mg/g) was emulsified in phosphate buffer pH 6.8 (1 % m/v). Afterwards, 50 mL of the microemulsion was filled into the dialysis tube and placed in a beaker with 50 mL phosphate buffer pH 6.8. The release of leuprorelin to the outer phase was analyzed via HPLC over 30 hours. An aqueous leuprolide acetate solution (0.04 mg/mL) served as control.

2.6. Enzymatic degradation by intestinal enzymes

Enzymatic degradation studies were performed according to a modified method, as described previously by our research group [13, 14].

Leuprolide acetate and leuprolide oleate SMEDDS were dissolved in a buffer containing 1.36 g/L of Na_2HPO_4, 0.22 g/L of KH_2PO_4 and 8.5 g/L of NaCl to obtain a final concentration of 0.04 mg/mL. Enzyme solutions were prepared in a final activity of 9.35 IU/mL for trypsin, 7.16 BTEE U/mL for α-chymotrypsin and 0.29 IU/mL for elastase. The enzyme activities were in accordance with physiological conditions [15].

Degradation studies were performed by adding 100 µL of each enzyme solution to 100 µL of leuprolide acetate solution (0.04 mg/mL) and leuprolide oleate microemulsion (0.04 mg/mL leuprolide oleate // 1 % m/v microemulsion), respectively, and incubated in a thermomixer at 37 °C. The enzymatic reaction was stopped at predetermined time points by adding 100 µL of 2 % trifluoroacetic acid (TFA) to the reaction mixture. The samples were analyzed by HPLC. The observed retention time for leuprolide acetate was 5.7 min and for leuprolide oleate 9.7 min.

2.7. *In vivo* evaluation of leuprorelin SMEDDS in rats

The protocol for the *in vivo* study on animals was approved by the Animal Ethical Committee of Vienna, Austria and adheres to the Principles of Laboratory Animal Care. The *in vivo* study was performed on 25 Sprague Dawley rats weighing 200-250 g, which were divided into 5 cohorts of 5 animals each (Table 1). Rats were housed in polycarbonate cages (46 x 25 x 21 cm) at room temperature and fasted for 2 hours before administration of all dosage forms, but with free access to water. Aliquots (250 µL) of each oral formulation were administered through a flexible plastic stomach tube with a round tip in order to minimize trauma, followed by administration of 250 µL of water. Intravenous injections were applied into tail veins. Blood samples were taken at predetermined time points from the tail vein. Blood samples (approx. 120 µL) were spiked with 20 µL of a 3.8 % (m/v) sodium citrate solution in order to prevent blood clotting. The samples were immediately centrifuged at 10,000 rpm to obtain the plasma.

Table 1: Formulations used for *in vivo* studies in male Sprague-Dawley rats.

Formulation	Route of administration	Dose	Dosage form	Volume
Leuprolide acetate loaded aqueous solution	Iv	0.25 mg	Solution	250 µl
Leuprolide acetate loaded aqueous solution	Oral	1 mg	Solution	250 µl
Leuprolide oleate loaded aqueous suspension	Oral	1 mg	Suspension	250 µl
Leuprolide acetate loaded SMEDDS	Oral	1 mg	SMEDDS	250 µl
Leuprolide oleate loaded SMEDDS	Oral	1 mg	SMEDDS	250 µl

2.8. Standard and sample preparation

The stock solution of leuprolide acetate (1 mg/mL) was prepared in 50 % acetonitrile and 50 % water with 0.1 % formic acid. Calibration curves (1-1000 ng/mL) were established by adding 20 µL of leuprolide standards to 100 µL of rat plasma. Afterwards, standards and samples were treated 2 times with 200 µL of ice-cold acetonitrile in order to precipitate plasma proteins. Subsequently, samples were centrifuged at 13,400 rpm for 10 min and the supernatant was transferred into glass vials, followed by evaporating to dryness with a Univapo 100 ECH (Uniequip, Germany) for 60 min at 40 °C. The residue was dissolved with 125 µL of mobile phase (80 % A and 20 % B) before analyzing via LC-MS [16, 17].

2.9. Quantification of leuprorelin via LC-MS

Leuprorelin was quantified in blood samples of the *in vivo* study via LC-MS by a slightly modified method described previously by our research group [16, 17].

In brief, the analysis was carried out by LC on an Agilent 1200 Series system (Agilent Technologies, Waldbronn, Germany) equipped with a G1312B SL binary pump, G1329B autosampler, vacuum degasser and G1316B column oven. The mobile phase consisted of solvents A: 10 % acetonitrile, 90 % water, 2 mM ammonium acetate, 0.1 % formic acid and B: 90 % acetonitrile, 10 % water, 2 mM ammonium acetate, 0.1 % formic acid. Plasma samples and standards were separated on a YMC-Pack C4 column (250 x 4.6 mm, 5 µm, 30 nm pore size) using isocratic elution (80 % A : 20 % B) for 15 min at a flow rate of 1 mL/min, an oven temperature of 60 °C and injection volume of 40 µL. Mass spectrometry was performed on a Bruker MicoOTOF-Q II system operated in positive ion mode under the following conditions: end plate offset, -500 V; capillary voltage, -4500 V; nebulizer pressure, 29 psi; dry gas (nitrogen) flow rate, 6.0 L/min; dry temperature, 200 °C; funnel 1 RF, 200 Vpp; funnel 2 RF, 300 Vpp; ISCID energy, 0 eV; hexapole RF, 500 Vpp; ion energy, 6.0 eV; collision energy, 10.0 eV; collision RF, 600 Vpp; transfer time, 60 µs; pre pulse storage, 12.0 µs; mass range, 200-2000 m/z.

2.10. Pharmacokinetic and statistical data analysis

Pharmacokinetic parameters of leuprorelin were calculated by applying a non-compartmental pharmacokinetic analysis to the plasma concentration-time data using the software GraphPad Prism 5 version 5.01. The area under the concentration versus time curve ($AUC_{0\text{-}last}$) was calculated in accordance to the linear trapezoidal rule, using kinetic data collected from individual values. The absolute bioavailability was calculated from the dose corrected AUCs for oral versus intravenous administration.

Statistical data analysis was performed using one-way ANOVA and the Kruskal-Wallis test with 95 % confident interval (p value < 0.05) as the minimal level of significance followed by a multiple-comparison Bonferroni and Dunns post hoc test ($p < 0.05$). The results were expressed as the mean of at least 3 experiments ± SD.

3. Results and discussion

3.1. Hydrophobic ion pairing of leuprorelin

The hydrophobic ion pair exchange of leuprolide acetate with sodium oleate led to precipitation in aqueous media. In the following, this precipitate could be separated from the water soluble fraction by centrifugation. The extent of ion pairing was determined by measuring residual leuprolide in the water phase. As shown in Figure 1, the leuprolide water solubility decreased up to a molar ratio of 3:1 and increased with further increasing concentration of oleate. Although leuprolide has two basic amino acid groups that should be able to bind two molecules of oleate, the aqueous solubility decreased by adding more oleate. Choi et al. explained this effect with the formation of a hydrophobic complex when reaching a molar ratio of 3:1. However, further addition of sodium oleate (molar ratio 4:1) led to an efficient micellar solubilization of leuprorelin by sodium oleate [10].

Further investigation of the lyophilized leuprolide oleate pellet (molar ratio 3:1) as aqueous dispersion and microemulsion by HPLC indicated that the hydrophobic ion pairing was not complete. The leuprolide oleate pellet was dispersed in water and centrifuged at 10,000 rpm for 5 min. The chromatogram of the aqueous supernatant after centrifugation showed a single peak at the retention time of leuprolide acetate, an undissolved pellet remained. However, when the leuprolide oleate pellet was dissolved in SMEDDS, no precipitation in the generated microemulsion after centrifugation could be observed and the chromatogram showed two peaks: one for leuprolide acetate and one for leuprolide oleate (data not shown). Accordingly, the microemulsion as well as the lyophilized pellets contained both ion pairs.
Results demonstrated that leuprolide oleate is only soluble in the microemulsion, whereas leuprolide acetate could be dissolved in the aqueous control solution and in the aqueous phase of the o/w microemulsion. The ratio of leuprolide acetate to oleate in the microemulsion was 59:41.

3.2. Preparation and characterization of SMEDDS

The lyophilized leuprolide oleate could be dissolved in the SMEDDS formulation in a concentration of 4 mg/g. All four products of hydrophobic ion pairing (molar ratio 1:1, 2:1, 3:1 and 4:1) led to clear, homogenous mixtures that were able to self-emulsify in phosphate buffer pH 6.8 within a minute. The

obtained microemulsions were slightly bluish. Moreover, as shown in Figure 2, the droplet size of control microemulsion without leuprorelin and all other leuprorelin microemulsions did not differ significantly. The fact that the droplet size does not increase when the leuprolide oleate is incorporated seems beneficial from the drug delivery point of view providing a large surface area dispersion to improve drug absorption. PDI values are 0.060 ± 0.002 for unloaded SMEDDS and 0.103 ± 0.005 for loaded SMEDDS.

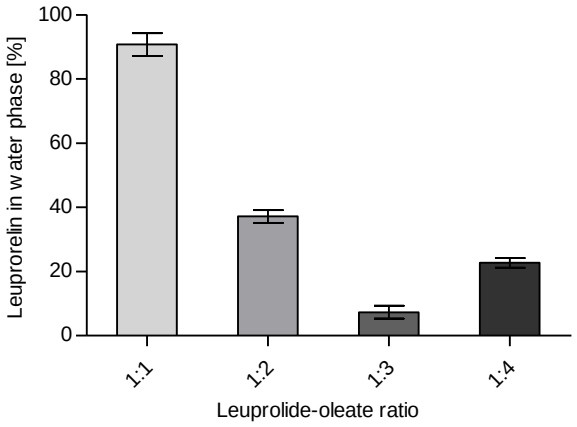

Figure 1.
Comparison of residual leuprolide acetate in the water phase depending on the ratio between leuprolide acetate and sodium oleate during hydrophobic ion pairing process. Indicated values are means (± SD, n = 3).

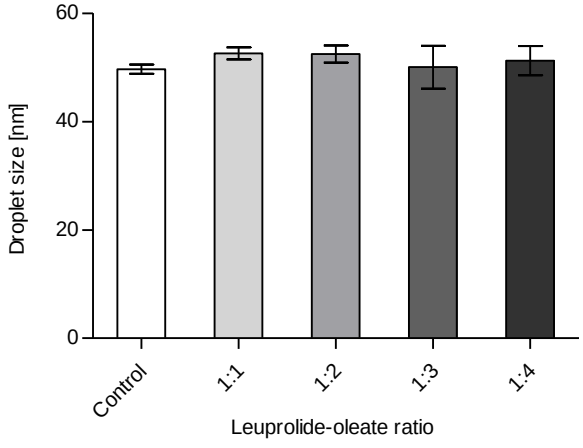

Figure 2.
Comparison of the droplet sizes of Leu-SMEDDS emulsified in phosphate buffer pH 6.8 with respect to the ratio between leuprolide acetate and sodium oleate during hydrophobic ion pairing process. Indicated values are means (±SD, n = 3).

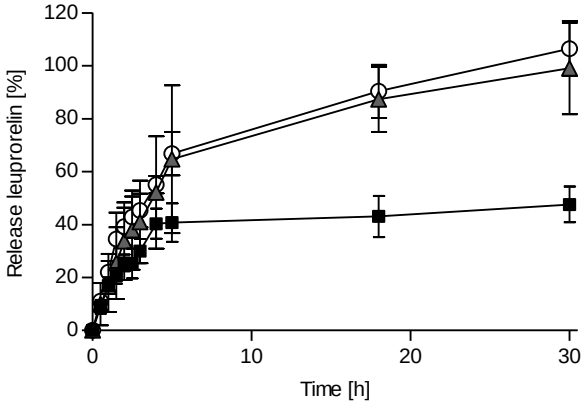

Figure 3.
Leuprorelin release profiles from an aqueous leuprolide acetate solution [○] and Leu-SMEDDS (1:3) emulsified in phosphate buffer pH 6.8 [▲] [■]. As the leuprorelin precipitated incorporated into the SMEDDS contained leuprolide acetate and leuprolide oleate, release profile for both ion pairs are indicated serparately: leuprolide acetate [▲] and leuprolide oleate [■] from Leu-SMEDDS microemulsion. Indicated values are means (± SD, n = 3).

3.3. Drug release and payload

The drug release of leuprorelin across a dialysis membrane (MWCO 10,000 Da) in an aqueous environment over time is shown in Figure 3. As already mentioned, the microemulsion contained leuprolide acetate and oleate; therefore two release profiles are shown. The release of leuprolide acetate is slower, but similar to the control solution indicating that leuprolide acetate is incorporated into the microemulsion droplet to a very low extent. However, the profile of leuprolide oleate is significantly sustained and after 30 hours approximately 50 % were released. Since the plateau phase was reached, the remaining leuprolide oleate was likely still incorporated in SMEDDS droplets. Consequently, drug release studies confirm the assumption that leuprolide oleate was at least partially incorporated into the microemulsion droplets.

The sustained release of leuprorelin by embedding it into the droplets is advantageous since for oral delivery of peptide drugs a drug release as close as possible to the site of absorption is favored. The microemulsion avoids an immediate release and could shield the peptide drug from rapid degradation until the absorption site is reached. Although the peptide might be degraded to a certain extent, the probability of the peptide drug reaching the absorption site unaltered is increased. Furthermore, the permeation enhancing effect of the microemulsion may support the absorption to increase bioavailability [11]. A drawback of the developed microemulsion might be the incomplete release of leuprolide oleate under the chosen conditions. However, *in vivo* the excipients are likely digested by lipases or mixed micelles with bile acids and phospholipids could be formed, which leads to an enhanced release of the embedded leuprorelin.

3.4. Enzymatic degradation by intestinal enzymes

One of the main obstacles to improve the oral bioavailability of leuprorelin is the enzymatic barrier of the gastrointestinal tract due to an intensively degradation by proteolytic enzymes [1, 18, 19]. Therefore, a self-emulsifying drug delivery system was developed to protect leuprorelin from enzymatic degradation in the intestine. Trypsin, α-chymotrypsin and elastase were separately investigated to measure a specific protective effect and to evaluate the extent of degradation by each enzyme.

Figure 4 shows the degradation profiles of leuprorelin in a microemulsion prepared with the lyophilized product of hydrophobic ion paring (molar ratio 3:1) and an aqueous control solution containing leuprolide acetate by trypsin as a function of time. The microemulsion contained leuprolide acetate as well as leuprolide oleate as described in section 3.1. The leuprolide acetate control was completely degraded within 60 minutes, whereas leuprolide acetate in the microemulsions was metabolized rapidly within the first 60 minutes it remained stable thereafter. Leuprolide oleate seemed to be protected by the microemulsion droplets right from the beginning, resulting in a comparatively lower extent of degradation. Accordingly, the leuprolide oleate is likely embedded into the lipid phase due to the higher hydrophobicity and therefore protected against degradation by trypsin.

The degradation of leuprolide acetate control and of leuprorelin SMEDDS (3:1) by α-chymotrypsin was similar but more rapidly (Figure 5). Therefore, the reaction was stopped at different time points and the first 5 minutes are separately presented in Figure 5B. After 5 minutes no remaining leuprolide acetate was to be detected in the solution, whereas in the microemulsion a small amount remained stable. In contrast, leuprolide oleate was metabolized very slowly more than 50 % remained unaltered within 120 minutes due to the protective effect of SMEDDS droplets.

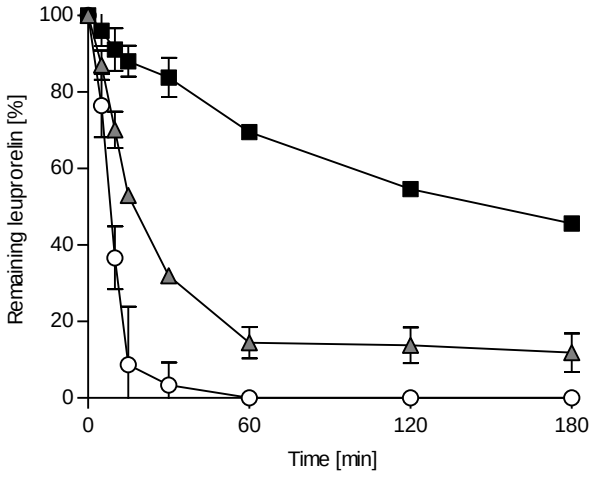

Figure 4.
Degradation profile of leuprolide acetate by trypsin in an aqueous leuprolide acetate solution [○] and as Leu-SMEDDS (1:3) emulsified in phosphate buffer pH 6.8 [▲] [■]. As the leuprorelin precipitated incorporated into the SMEDDS contained leuprolide acetate and leuprolide oleate, degradation profiles of both ion pairs are indicated serparately: leuprolide acetate [▲] and leuprolide oleate [■] in the microemulsion. Indicated values are means (±SD, n =3).

However, leuprorelin was not metabolized by elastase at all as the whole amount of leuprolide acetate and oleate in solution and microemulsion could be found after 180 minutes of incubation (data not shown). Consequently, a protective effect was only shown for trypsin and α-chymotrypsin.

Degradation studies with trypsin and α-chymotrypsin showed that leuprorelin SMEDDS had a limited protective effect for the hydrophilic leuprolide acetate. This might be caused by an insufficient – if at all – incorporation of leuprolide acetate into SMEDDS droplets. The nevertheless lower degradation might be a result of enzyme inhibitory properties of microemulsion components. However, ion pairing of leuprolide acetate with sodium oleate led to a hydrophobic product that was likely incorporated into the microemulsion droplets. Since leuprolide oleate was shielded from degradation right from the start of the experiment, the peptide seems to be embedded into the microemulsion droplets. As mentioned in the hydrophobic ion pairing section, both ion pairs (leuprolide acetate and oleate) were present in the microemulsion that allows good comparability in the same formulation in respect to the investigated protective effect.

Self-microemulsifying drug delivery systems were extensively investigated in the last decade to improve the oral bioavailability of poorly water soluble drugs. Despite of the promising concept of improving oral bioavailability due to increased solubility [20-22], with a few exceptions (e.g. Neoral®) SMEDDS play a minor role on the pharmaceutical market. One reason therefore could be that most studies focus on a higher bioavailability by increasing water-solubility. Other promising properties of SMEDDS, e.g. protection of peptide drugs against enzymatic degradation are rarely examined. This lack of information is a obstacle regarding the development of self-emulsifying drug delivery systems. Furthermore, the incorporation of peptides into the lipophilic droplets is a great challenge due to their hydrophilic nature and was rarely successful so far [23-25]. All other studies in which emulsions were investigated as peptide carriers the drug is assumed to be in the aqueous phase [24, 26, 27]. Within the present study it could be demonstrated that the protective effect against enzymatic metabolism is improved, when the peptide is embedded in the lipophilic phase.

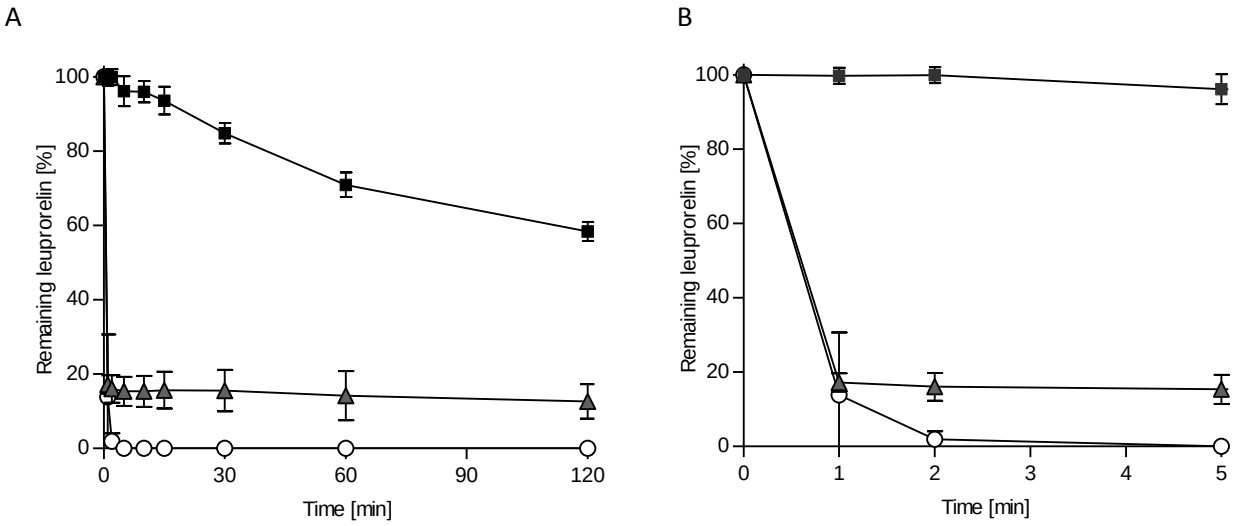

Figure 5A+B
Degradation of leuprorelin by α-chymotrypsin in an aqueous leuprolide acetate solution [○] and as Leu-SMEDDS (1:3) emulsified in phosphate buffer pH 6.8 [▲] [■]. As the leuprorelin precipitate incorporated into the SMEDDS contained leuprolide acetate and leuprolide oleate, degradation profiles of both salts are indicated serparately: leuprolide acetate [▲] and leuprolide oleate [■] in the microemulsion. Figure 5B highlights the first 5 minutes of the degradation process due to the rapid metabolism by α-chymotrypsin. Indicated values are means (± SD, n = 3).

3.5. *In vivo* evaluation of leuprorelin SMEDDS

The detail information about administered formulations and the calculated pharmacokinetic parameters of the *in vivo* study are shown in Table 1 and Table 2, respectively. The plasma-concentration profile of intravenous injections of leuprolide acetate to male Sprague Dawley rats indicate a rapid metabolism since more than 99 % were metabolized within 90 min (Fig. 6A). Moreover, an orally administered leuprolide acetate solution proved that the peptide drug cannot be easily absorbed from the gastrointestinal tract (C_{max} 5.98 ng/mL; absolute bioavailability 0.074 %), which might be caused by severe degradation by luminal enzymes and a low permeability across intestinal membranes (Fig. 6B). However, leuprolide acetate given orally as SMEDDS showed a 6.5-fold improved absolute bioavailability and reached a C_{max} of 15.66 ng/mL after 30 min (Table 2). Moreover, the absorption was sustained compared to the solution (Fig. 6B). This is likely caused by an enhanced permeability across intestinal membranes since the system was able to open tight junctions *in vitro* [11] representing the predominant way of uptake for hydrophilic macromolecular drugs like leuprorelin [1]. Furthermore, the SMEDDS showed a slight protection of leuprolide acetate against degradation by intestinal serine proteases *in vitro* that might support the gastrointestinal uptake. If the freeze-dried pellet, obtained in the hydrophobic ion pairing process, was dissolved in the self-emulsifying system and orally administered to rats, the uptake was even further

enhanced. The results showed a significant improvement of the area under the plasma concentration-time curve of leuprorelin (Fig. 6B), resulting in a 17.2-fold increased absolute bioavailability and a 8.6-fold increase of C_{max} compared to the control solution (Table 2). Moreover, the control leuprolide acetate was no more detectable after 2 h when given as solution, whereas in case of leuprolide oleate SMEDDS plasma levels of around 8 ng/mL were found after 6 h. The enhanced and sustained uptake is likely caused by the combination of an improved transport across the intestinal gut wall, the protective effect against proteases and a sustained release of the peptide drug from the SMEDDS droplets.

Up to now, most peptide and protein drugs are given intravenously or subcutaneously by injections due to several physiological barriers limiting the oral bioavailability of hydrophilic macromolecular drugs. In order to overcome the user-unfriendly route of administration, especially for long-term disease treatment, it was the aim to develop a system that enables oral administration of peptides. The main obstacles are a low intestinal permeability and a rapid degradation by luminal enzymes. The developed self-emulsifying drug delivery system addresses these problems since the system can increase the permeability, particularly by tight junction opening, and shield leuprorelin against proteases.

Table 2: Pharmacokinetic parameters calculated after iv and oral administration of investigated formulations in rats. Absolute bioavailability (BA) was calculated with reference to iv injection and indicated values are means of five rats. (* $p<0.05$ compared to leuprolide acetate solution; ^{ns} not significant compared to leuprolide acetate solution).

Delivery System	**$AUC_{0\text{-last}}$ [min·ng/mL]**	**C_{max} [ng/mL]**	**T_{max} [min]**	**Absolute BA [%]**
Iv solution	144653	16226.7	-	-
Leuprolide acetate solution	426	5.98	30	0.074
Leuprolide oleate suspension	0	-	-	-
Leuprolide acetate SMEDDS	2796 ^{ns}	15.66 ^{ns}	30	0.483
Leuprolide oleate SMEDDS	7385 *	51.68 *	30	1.276

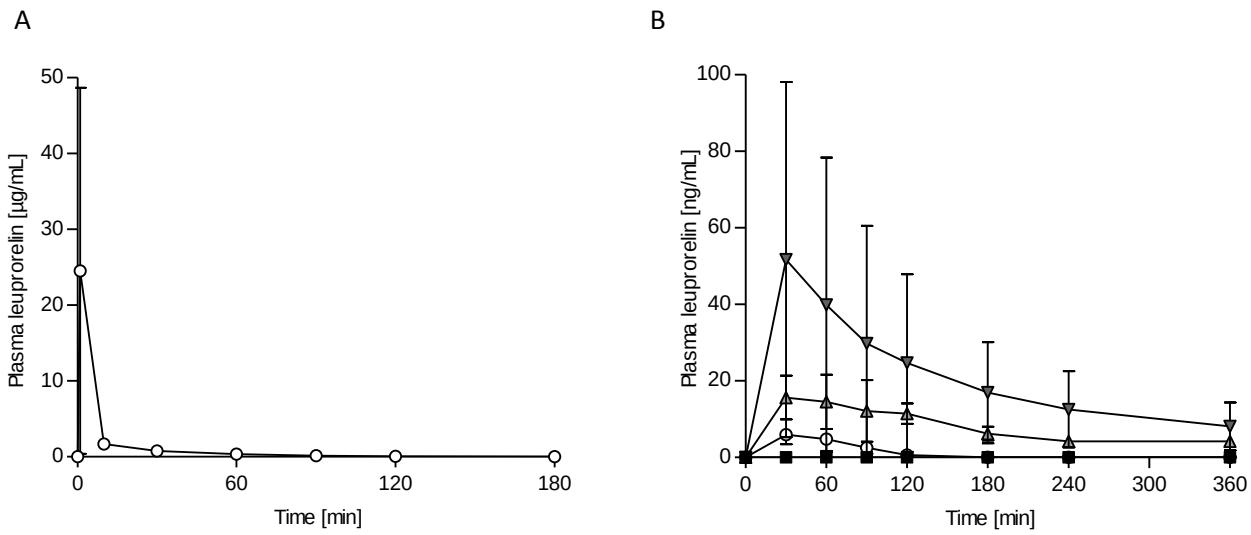

Fig.

Figure 6A+B

(A) Plasma concentration curve of leuprorelin after intravenous injection of leuprolide acetate to rats (Dose = 1 mg/kg). (B) Plasma concentration curves of leuprorelin after oral administration of a leuprolide acetate solution [○], a leuprolide oleate suspension [■], a leuprolide acetate SMEDDS [▲] and a leuprolide oleate SMEDDS [▼] to rats (Dose = 4 mg/kg). Indicated values are means (± SD) of five rats. Leuprolide oleate SMEDDS [▼] are significant different compared to all other ($p<0.05$); leuprolide acetate SMEDDS [▲] are not significant different compared to leuprolide acetate solution.

4. Conclusion

This is the first time, to our knowledge, that hydrophobic ion pairing of a peptide drug was used in order to embed a peptide drug successful into microemulsion droplets. Moreover, the self-microemulsifying drug delivery system for leuprorelin proved that SMEDDS can shield peptides from degradation by intestinal proteases. Furthermore, a sustained release of leuprorelin could be demonstrated that avoids an initial rapid degradation of the drug. These considerations could be confirmed by an *in vivo* study in rats and led to significant enhanced plasma profiles of leuprorelin via oral delivery. According to these results, the novel system could be likely a novel platform technology to improve the oral bioavailability of peptide drugs.

5. Acknowledgement

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Preparation and characterization of mucus-penetrating papain/poly(acrylic acid) nanoparticles for oral drug delivery applications

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Abstract

Particle diffusion through the intestinal mucosal barrier is restricted by the viscoelastic and adhesive properties of the mucus gel layer, preventing their penetration to the underlying absorptive endothelial cells. To overcome this natural barrier, we developed nanoparticles which have a remarkable ability to cleave mucoglycoprotein substructures responsible for the structural and rheological properties of mucus. After rheological screening of various mucolytic proteases, nanoparticles composed of poly(acrylic acid) and papain were prepared and characterized regarding particle size and zeta potential. Analysis of nanoparticles showed mean diameters sub-200 nm (162.8 - 198.5 nm) and negative zeta potentials advancing the mobility in mucus gel. Using diffusion chamber studies and the rotating diffusion tubes method, we compared the transport rates of papain modified (PAPC) and unaltered poly(acrylic acid) (PAA) particles through freshly excised intestinal porcine mucus. Results of the diffusion assays demonstrated strongly enhanced permeation behavior of PAPC particles owing to local mucus disruption by papain. Improved transport rates, reduction in mucus viscosity and the retarded release of hydrophilic macromolecular compounds make proteolytic enzyme functionalized nanoparticles of substantial interest for improved targeted drug delivery at mucosal surfaces. Although cytotoxicity tests of the nanoparticles could not be performed, safety of papain and poly(acrylic acid) was already verified making PAPC particles a promising candidate in the pharmaceutical field of research. The focus of the present study was the development of particles which penetrate the mucus barrier to approach the underlying epithelium. Improvements of particles that penetrate the mucus followed by cell uptake in this direction are ongoing.

Keywords: Oral drug delivery, Mucus barrier, Mucus penetrating particles, Poly(acrylic acid), Papain

1. Introduction

Within recent years, polymeric nanoparticles are extensively used as drug delivery devices due to their ability to protect and deliver drugs, proteins and genes through the peroral route [1]. Entrapment of therapeutic agents in nanoformulations leads to controlled release profiles, improved bioavailability and minimizes side effects. Nanocarriers have been exposed as potential vehicle for crossing membrane barriers of the small intestine and for entering the systemic circulation [2]. Nevertheless, rate and extend of mucosal drug delivery is restricted by a layer of highly viscoelastic and adhesive mucus, that hinders the transport of macromolecules through the gel [3]. The mucus layer consists of an aqueous solution of glycoproteins, inorganic salts, proteins, lipids and mucopolysaccharides which protect the region of underlying cells. Particularly, the mucoglycoprotein chains are thought to be responsible for the structural properties of mucus and determine its function as a molecular sieve [4].

The mucin network acts as protective barrier through rapid elimination of foreign particles and reduces its residence time at the target site. To overcome these clearance mechanism and achieve sufficient drug concentrations in the mucosa, nanocarriers that are capable of permeating the viscoelastic intestinal gel layer are required [5]. An increasing number of nanoparticle-based delivery systems have been developed to traverse the mucus gel barrier before mucus turnover occurs. Preparation technologies focused on the optimization of the surface chemistry in order to create mucu-inert particles. A high density of cationic and anionic surface charge groups generated particles with strong hydrophilicity and neutral charge avoiding adhesion within the mucosal network. Muco-inert particles do not interact significantly with the mucus and diffuse in low viscosity channels or pores in the mucus gel [6]. Nevertheless, highly branched networks or viscous areas hinder the particle transport by size filtering. A promising strategy for efficient drug delivery across the mucus is the development of nanoparticles that are capable of local disruption of the mucosal matrix. Nanocarriers based on biocompatible polymers in combination with proteolytic enzymes should facilitate deep mucus penetration by cleavage of protein structures within the mucus. The novel nanoparticulate drug carrier system is supposed to quickly traverse the mucosal barrier leading to improved particle transport rates. As a result of local mucus disruption, cleavage of mucoglycoproteins occurs only on the delivery site in the intestinal mucus layer whereby only a minimal part of the mucus is undermined by the particles.

This study presents a novel generation of mucus-penetrating particles composed of poly(acrylic acid) and mucolytic enzyme (papain) with average sizes <200 nm in diameter. Particles were prepared by ionic gelation method and characterized regarding size, zeta potential and protein quantity. We focused our

investigations on the diffusion characteristics of papain modified nanoparticles through freshly excised porcine intestinal mucus by utilization of modified Ussing-type chambers and the rotating tubes method.

2. Materials and methods

2.1. Materials

Poly(acrylic acid) (molecular mass ~ 100 kDa), papain (from *Carica papaya*, 3.6 units/mg solid), bromelain (from pineapple stem, 3.4 units/mg solid), trypsin (from porcine pancreas, 1,240 BAEE units/mg solid), αchymotrypsin (from bovine pancreas, 57.24 units/mg solid), pepsin (from porcine gastric mucosa, 920 units/mg solid), proteinase (from *Aspergillus Melleus*, type XXIII, 3.3 units/mg solid), trehalose, 2-(N-morpholino)ethanesulfonic acid hydrate (MES hydrate), Coomassie Brilliant Blue G, casein (from bovine milk), Ellman's reagent (DTNB, 5,5'-dithiobis(2-nitrobenzoic acid)) and fluorescein diacetate (FDA) were obtained from Sigma – Aldrich (Vienna, Austria). Fluorescein isothiocyanate dextran (FD4, 4400 Da) was purchased from TdB Consultancy AB (Uppsala, Sweden).

2.2. Proteolytic enzyme screening

Intestines from freshly slaughtered pigs were collected on ice, cut into smaller segments and rinsed carefully with physiological saline (0.9 % NaCl). After the washing process, intestinal mucus was isolated by gently scraping from underlying tissue. Subsequently the pH of the resulting scrapings was adjusted to 5.0, 6.5 and 8.0 by addition of 0.1 M NaOH.

To analyze the mucolytic activity of papain, bromelain, trypsin, α-chymotrypsin, proteinase and pepsin, the decrease in viscosity was evaluated by mixing the enzymes with freshly scrapped mucus. Therefore, 20 mg of mucolytic enzymes were hydrated in 500 µL demineralized water and incubated with 9.5 mL of mucus at 37 °C for 12 hours. At regular time intervals, the viscoelastic properties of samples (0.2 %, w/v) were measured by adding 700 µL of the enzyme/mucus mixture to a cone-plate viscometer (RotoVisco RT20, Haake GmbH, Karlsruhe, Germany). Then, dynamic oscillatory tests within the linear viscoelasticity region were performed as described by Marschütz [7]. Mucus samples without addition of proteolytic enzymes were treated in the same way and served as references.

2.3. Preparation of nanoparticles and FDA loading

Nanoparticles were prepared via formation of complexes [8, 9] between poly(acrylic acid) and papain in aqueous system. Briefly, poly(acrylic acid) was dissolved in demineralized water in various concentrations (0.1, 0.25, 0.5, 1.0 and 1.5 % (w/v)). Each polymer concentration was also tested at different pH values

(3.5, 4.5, 5.5, 6.5 and 7.5). Papain solution was prepared by hydrating 500 mg of protein in 250 mL demineralized water and the pH was adjusted to 4.5. Dissolved papain was added dropwise to the polymer solutions under intense stirring conditions until turbidity occurred. The poly(acrylic acid)/papain mixture was stirred for 1 h to allow complexation of positively charged protein and polyanion.

Unmodified poly(acrylic acid) (PAA) nanoparticles were produced by using Ca^{2+} as crosslinker. In brief, PAA was dissolved in demineralized water to obtain a 0.5 % (w/v) solution. The pH was raised to 8.0 with 2.5 M NaOH. Then, 0.5 % (w/v) calcium solution was slowly added to 100 mL of the polymer solution until turbidity occurred. In the following step, nanoparticles were purified by centrifugation at 5500 rpm for 20 min with 1.0 % trehalose to avoid particle aggregation. The supernatant was discarded and the remaining pellet was resuspended in demineralized water. Then, nanoparticle suspensions were lyophilized at -77°C, 0.01 mbar (Virtis Bench top freeze-drier, Bartelt, Graz, Austria) and kept at 4 °C until further use.

For diffusion studies, the marker fluorescein diacetate (FDA) was incorporated in the obtained PAA and PAPC particles [10]. First, 10 mL particle suspension as described above was transferred to 5 mL of a 0.5 % (w/v) FDA solution in acetonitrile. The suspension was incubated on a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany) at 18 °C for 1.5 h. Afterwards, labeled nanoparticles were centrifuged, resuspended and freeze dried. In the next step, the amount of embedded FDA was determined by fluorescence measurements. Therefore, lyophilized nanoparticles with incorporated FDA were resuspended in 5 M NaOH at 1 mg/mL under stirring conditions at 37 °C. After 1 h of incubation, the reaction mixture was centrifuged at 13,500 rpm for 15 min and the supernatant was collected for fluorescence measurement with a microplate reader (Infinite™ M200, Tecan, Grödig, Austria). Alkaline treatment quantitatively hydrolyses fluorescein diacetate to the fluorescent sodium fluorescein which enables detection at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The amount of incorporated model compound was calculated using a calibration curve containing decreasing concentrations of FDA.

2.4. Quantification of immobilized enzyme

The amount of adsorbed papain was quantified by determination of reduced and oxidized thiol groups using Ellman's reagent. Briefly, 2.5 mg of poly(acrylic acid)/papain complex (PAPC) particles were hydrated in 500 µL Tris buffer. Samples were treated with 1.0 mL 4 % sodium borohydride solution. After an incubation period of 1 h at 37 °C, the reaction was stopped with 200 µL 5 N HCl and neutralized by adding

1 mL phosphate buffer (pH 8; 1 M). In order to visualize sulfhydryl groups, 100 µL of freshly prepared Ellman's reagent were added. Absorbance of test solutions was measured at 450 nm using a microplate reader (Infinite™ M200, Tecan, Grödig, Austria).

In addition, the Bradford assay was used to determine the amount of protein adsorbed to poly(acrylic acid), which is based on the Coomassie Brilliant Blue dye [11]. First, 100 mg of dye were dissolved in 50 mL of 95 % ethanol, followed by 100 mL of phosphoric acid (85 %, w/v). The solution was diluted to 1 L with demineralized water. The reagent was filtered and stored at 4 °C under exclusion of light. A volume of 1 mL Bradford solution was added to 0.2 mL particle suspension. After an incubation period of 45 min, absorbance of the resulting papain-dye complex was measured at 595 nm. Spectroscopic experiments were corrected by measuring the sample absorbance also at 850 nm to eliminate the influence of poly(acrylic) acid which aggregates with the dye and influence the results [12].

2.5. Measurement of the enzymatic activity of immobilized enzyme

The enzymatic activity of free and immobilized papain was determined according to the method described by Itoyama et al., using casein as substrate [13]. Briefly, 2 mL of 10 mM PBS (pH 8.0), 1 mL of papain or nanoparticle suspension in 50 mM PBS containing 2 mM EDTA and 5 mM L-cysteine and 1 mL of 2.0 % (m/v) casein solution were combined. The reaction mixtures were allowed to proceed for 30 min at 37 °C under permanent stirring. Afterwards, the reaction was stopped by adding trichloroacetic acid in a final concentration of 10.0 %. The samples were centrifuged at 13,400 rpm for 10 min and the absorbance of the supernatant measured at 280 nm. The enzyme activity of immobilized papain was defined as relative activity corresponding to native papain displaying 3.6 Units/mg.

2.6. Characterization of nanoparticles

Size and zeta potential analysis of PAA and PAPC nanoparticle suspensions were performed by photon correlation spectroscopy using PSS Nicomp 380 ZLS particle sizer (Santa Barbara, CA, USA) with laser wavelength of 650 nm and an E-fields strength of 10 V/cm. Particles were measured in demineralized water after lyophilization at a final concentration of 0.5 % (w/v). To analyze the morphological characteristics of the obtained particles transmission electron microscopy (TEM) was carried out. Therefore, nanoparticles were mounted on carbon/formvar filmed copper grids, dried and examined with a Zeiss Libra 120 energy filter transmission microscope at 80 kV using zero loss electron to enhance contrast. Images were obtained with a Troendle TRS 2K camera and iTEM software (Olympus).

2.7. Mucolytic activity of nanoparticles

The proteolytic activity of the nanoparticle formulations on porcine intestinal mucus was evaluated by determination of loss in mucus viscosity. For this purpose, 2.0 mg of PAA and of PAPC particles were dispersed in 50 µL demineralized water (pH 6.5), mixed with 950 µL of fresh mucus solution and incubated for 1 h at 37 °C. Viscosity analysis was conducted as described above.

2.8. Analysis of particle penetration in mucus

Diffusion chamber studies

Permeation of polymeric materials in natural mucus was investigated by utilization of modified Transwell-Snapwell diffusion chambers as previously described by Norris and Sinko [14]. For penetration studies, 100 µL of freshly prepared mucus were placed between polycarbonate filters (Polycarbonate Track-Etch membranes, pore size: 450 nm, Sartorius, Germany) attached to the Snapwell ring located between donor and acceptor chamber. The donor compartment was loaded with 2 mL of 100 mM MES buffer pH 5.5 containing 20 mg FDA labeled nanoparticles and the acceptor site with experimental buffer only. All chambers were kept in an incubator for 3 h at 37 °C to allow nanoparticle diffusion through the mucus layer. Samples of 100 µL were withdrawn from the receiver site at 30, 60, 90, 120, 150 and 180 minutes and replaced by an aliquot of 100 µL of transport buffer. Obtained diffusants were treated with 1 mL 5 M NaOH and reacted for 1 h, in order to transform undetectable FDA to fluorescent sodium fluorescein. The concentration of the model compound was measured by fluorometric detection (Infinite™ M200, Tecan, Grödig, Austria) at excitation and emission wavelengths of 485 and 520 nm, respectively. The amount of permeated particles was calculated by corresponding calibration curves elaborated in the same conditions. Cumulative corrections were made for previously removed samples.

Particle transport in rotating diffusion tubes

To further analyze the quantitative particle transport in freshly excised intestinal porcine mucus, the rotating tube method was used [15]. For this purpose, silicon tubes (length: 40 mm; diameter: 6 mm) were filled with 300 µL of natural mucus and the ends sealed with silicon caps. Fluorescein diacetate labeled nanoparticle suspensions in a concentration of 0.25 % (w/v) were added to one end of the mucus containing tubes. Nanoparticle permeation was proceeded under continuous rotation of all test tubes at 37 °C. After 24 hours of incubation, tubes were frozen at -80 °C and cut into 20 slices of 2 mm. Each piece was treated with 500 µL 5 M NaOH and incubated for 1 hour. The resulting sodium fluorescein was detected and quantified as described above.

2.9. Release studies

The release profile of PAPC and PAA particles was evaluated by using fluorescein isothiocyanate dextran (FD4) as hydrophilic model compound. Briefly, 5 mg FD4 were added to 10 mL of 0.5 % (w/v) aqueous poly(acrylic acid) solution. The polymeric solution was stirred for 30 min and the particles were prepared as described above. The drug load was determined as described previously by our research group [16].

Release rates from the drug delivery systems were then analyzed by suspending 5 mg of FD4 loaded nanoparticles in 1 mL of 100 mM phosphate buffer pH 6.5 serving as release medium. The samples were incubated in an oscillating water bath at 37 °C for 6 hours under protection of light. At predetermined time points aliquots of 100 µL were withdrawn and replaced with an equal volume of experimental buffer. Sink conditions were maintained throughout the experiment. The suspensions were centrifuged (13,500 rpm; 15 min) and the quantity of FD4 in the supernatant was assayed by measuring the fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 525 nm by a microplate reader (Infinite™ M200, Tecan, Grödig, Austria). The amount of incorporated model compound was calculated using a standard curve containing decreasing concentrations of FD4.

2.10. Statistical data analysis

All data are expressed as mean ± standard deviation. Statistical analysis was conducted using software GraphPad Prism version 5 (GraphPad Software, Inc., CA, USA). A probability of less than 0.05 (p<0.05) was considered statistically significant.

3. Results and Discussion

3.1. Proteolytic enzyme screening

Orally administered proteolytic enzyme formulations are known to decrease the elastic and viscous properties of mucus through the enzymatic breakdown of complex protein substances [17]. In order to identify the most promising candidates, the enzymes were analyzed regarding their mucolytic efficiency. Freshly prepared mucus was used for all experiments to ensure preferably natural conditions. Figure 1 shows the rheological comparison of papain, bromelain, trypsin, chymotrypsin, pepsin and proteinase mixed with freshly isolated porcine intestinal mucus at pH 5.0, 6.5 and 8.0. The relative decrease of mucus viscosity provides an overview of the proteolytic activity at different intestinal pH values. Among all enzymes tested, papain and proteinase revealed a significant reduction of mucus viscosity after 1 h of incubation regardless of pH. Bromelain showed sufficient mucolysis, but only at pH 5.0 and 6.5. At higher pH values it became less effective corresponding to its pH optimum 4.5 to 5.5 [18].

In contrast, trypsin and chymotrypsin showed enhanced mucus liquefaction in more basic environment. The optimum pH values for peptide hydrolysis using trypsin and chymotrypsin are 7.0 - 9.0 and 7.8, respectively [19, 20]. The slightest influence on mucus viscosity revealed pepsin since it hydrolyzes substrates preferably at pH values below 3.5 [21]. Based on the rheological screening, the highly mucolytic active papain was chosen for the development of nanoparticles. Papain exhibits the strongest protein digestion at pH 6.0 – 7.0 [22] which is advantageous for its application in the small intestine where pH 6.4 – 7.5 is prevalent. Besides its considerable ability to degrade the intestinal mucus, papain represents a biocompatible, safe and already well-established auxiliary agent [23].

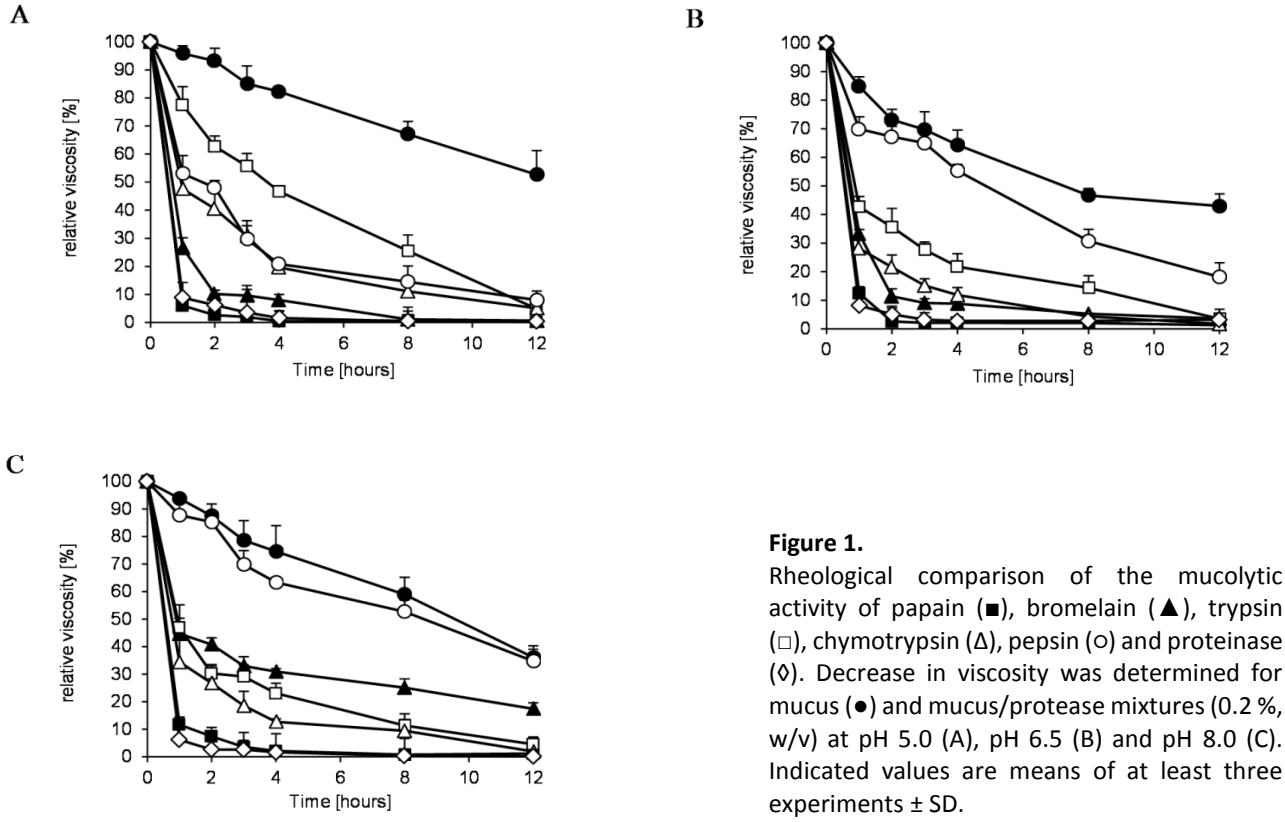

Figure 1.
Rheological comparison of the mucolytic activity of papain (■), bromelain (▲), trypsin (□), chymotrypsin (Δ), pepsin (○) and proteinase (◊). Decrease in viscosity was determined for mucus (●) and mucus/protease mixtures (0.2 %, w/v) at pH 5.0 (A), pH 6.5 (B) and pH 8.0 (C). Indicated values are means of at least three experiments ± SD.

3.2 Characterization of nanoparticles

The development of PAPC particles is based on the complexation of proteins with synthetic polyelectrolytes yielding aggregates or coacervates. Among all concentrations and pH values tested, particles showed sufficient mean diameters and zeta potentials by applying PAA in a final concentration of 0.5 % at pH 5.5. As shown in Figure 2, particle formation occurs through the stoichiometric formation of ion pairs or salt linkages between oppositely charged groups under retention of the biochemical function of papain. Papain displays several basic groups, in particular 11 amino groups, 1 imidazolyl and

12 guanidyl groups, which can be protonated depending on the pH of the surrounding environment [24]. In consequence of its isoelectric point (IEP) of 9.5, papain carries more positive charge due to the gain of protons at pH 4.5. Macroscopic appearance of PAA and PAPC nanoparticle suspensions is displayed in Figure 3.

The amount of attached protein to poly(acrylic acid) was determined with Ellman's reagent which quantifies the free and oxidized thiol groups on the cysteine residues of papain. Papain exhibits three intrachain disulfide bonds and a free sulfhydryl group which are available for photometrical detection [25]. Results displayed a protein percentage of 28 % in the particles. The Bradford assay revealed papain amounts in the same range, leading to a polymer/protein ratio of approximately 70/30.

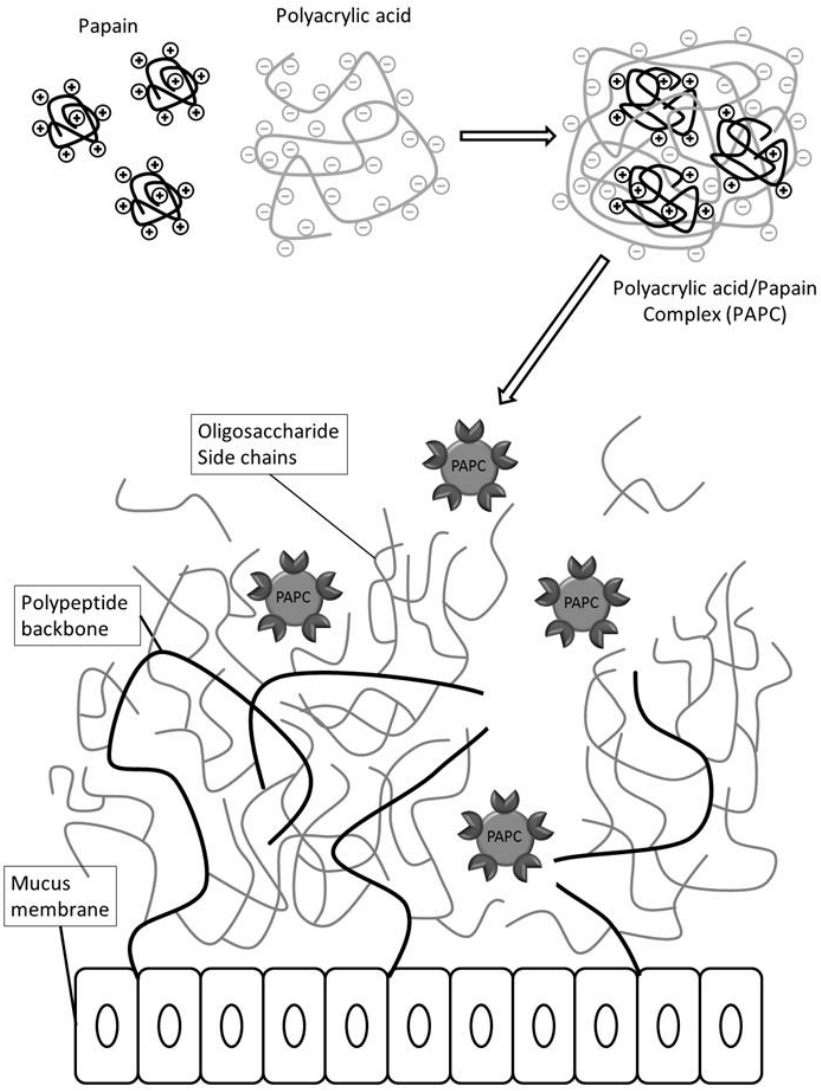

Figure 2.
Schematic representation of papain / poly(acrylic acid) complexation in aqueous media and following mucus permeation of PAPC nano-particles.

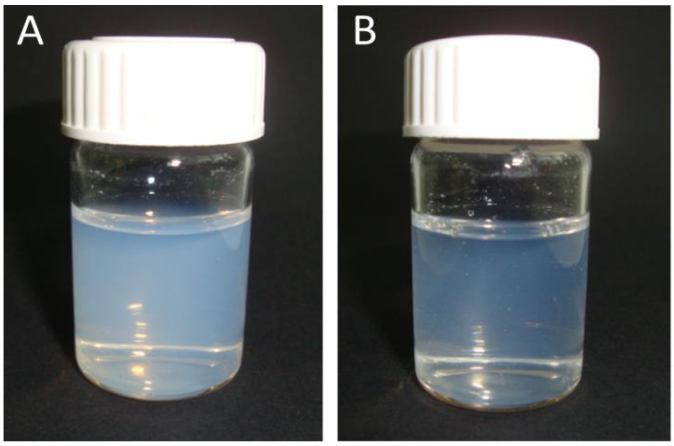

Figure 3.
Macroscopic (digital camera) images of poly(acrylic acid) (A) and poly(acrylic acid)/papain complex (B) particle suspensions.

The amount of attached protein to poly(acrylic acid) was determined with Ellman's reagent which quantifies the free and oxidized thiol groups on the cysteine residues of papain. Papain exhibits three intrachain disulfide bonds and a free sulfhydryl group which are available for photometrical detection [25]. Results displayed a protein percentage of 28 % in the particles. The Bradford assay revealed papain amounts in the same range, leading to a polymer/protein ratio of approximately 70/30.

The detailed values are summarized in Table 1. A major advantage of the PAA/papain complexes (PAPC) is that the native structure and function of the protein is not markedly lowered by association with the polyelectrolyte. Covalent attachment of papain to polymers would modify the three-dimensional structure of the protein leading predominantly to loss of the enzymatic potential. [26]. Enzymatic tests with casein as substrate verified the maintained activity of papain within the complex (Table 1). Besides the favorable mucolytic activity of PAPC, this delivery system is composed of two components which have to be verified as nontoxic. Poly(acrylic acid) is often used as pharmaceutical excipient because of its very safe toxicity profile [27]. Genotoxic and cytotoxic evaluation of papain confirmed safety of the proteolytic enzyme [23].

Table 1. Determination of attached protein and enzymatic activity of PAPC particles. Indicated values are means of at least three experiments ± SD.

	Papain	PAPC particles
Proteinquantification		
Ellman ([µg/mg]	1006.5 ± 97.2	281.0 ± 47.4
Bradford [µg/mg]	989.6 ± 36.3	332.5 ± 23.9
Enzyme activity		
Casein [units/mg solids]	3.6 ± 0.8	1.4 ± 0.3

Particle size and zeta potential

Interaction of particles with mucin molecules can be controlled by regulation of particle size and surface charge. An overview of the physicochemical qualities of the nanoparticles is provided in Table 2. As expected, all particles show negative charge related to the carboxylic acid moieties of poly(acrylic acid) which is advantageous for particle mobility in mucus. Crater and Carrier reported about faster diffusing times of anionic particles compared to cationic particles [28]. Preparing particles of positively charged polymers such as chitosan leads to enhanced mucoadhesiveness and restrict unobstructed diffusion. Chitosan exhibits positively charged amino groups that stick on mucosal surfaces which contain a high density of negatively charged groups as sulfonic acid and sialic acid moieties [29]. For this reason, poly(acrylic acid) was chosen as polymeric excipient for the development of muco-inert particles since it exhibits only weak mucoadhesive strength. Within a comparative study of anionic, cationic and non-ionic polymers, poly(acrylic acid) showed lower adhesion times on porcine intestinal mucosa than other biomaterials such as chitosan, sodium alginate, hydroxyethylcellulose and hydroxypropylcellulose [30].

Table 2. Characterization of PAA and PAPC nanoparticles regarding mean particle diameter, zeta potential, loading capacity and encapsulation efficiency. Indicated values are means ± SD (n ≥ 3).

	Mean particle diameter [nm]	Zeta potential [mV]	Loading capacity [%]	Encapsulation efficiency [%]
PAA particles				
Blank	162.8 ± 9.7	-16.0 ± 3.1	Not determined	Not determined
FDA labeled]	191.4 ± 11.3	-14.2 ± 4.0	13.4 ± 4	3.6 ± 0.7
PAPC particles				
Blank	175.6 ± 12.1	-11.7 ± 5.0	Not determined	Not determined
	198.5 ± 7.9	-10.4 ± 1.1	11.8 ± 2	2.4 ± 0.4

The zeta potential of PAPC dosage forms is slightly decreased as result of papain incorporation. The reduction of electrical charge is advantageous for mucus transition since neutral particles show the most efficient permeation rates. Experimental studies have shown that capsid virus-like particles are capable of permeating through mucus as fast as in saline based on their “slippery” properties. The human papilloma virus and the Norwalk virus are densely covered with positively and negatively charged moieties leading to a neutral surface which minimize the electrostatic interactions of virus with the mucus gel [31].

Besides the essential role of surface chemistry, the particle size presents also a determining parameter on the mucus permeation properties of nanocarriers. According to the microarchitecture and mesh spacing of the mucin network, non-mucoadhesive particles < 500 nm can diffuse between mucin fibers [6, 14, 32]. As shown in Table 2, PAA and PAPC particles displayed mean sizes in the range of 160-200 nm that is

favorable for unhindered transport across mucus gel and excludes size filtering. Additionally, FDA labeled nanoparticles displayed mean diameters in a close range in order to create comparable results respecting diffusion analysis. Figure 4 illustrates the morphological properties and surface appearance of PAA and PAPC particles which were investigated by transmission electron microscopy. As can be seen from the TEM images, particles display spherical shape and mean diameters < 200 nm.

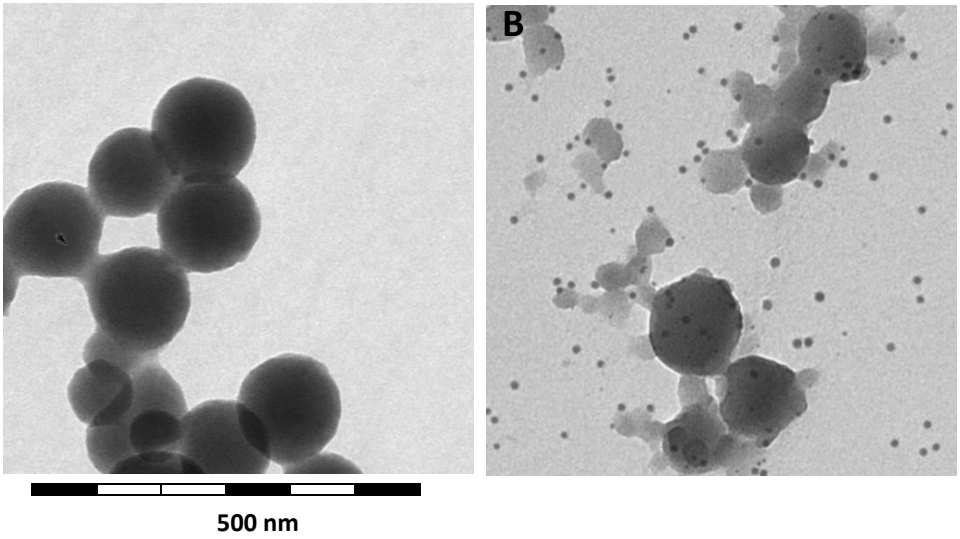

Figure 4.
Transmission electron microscopy images of the spherical shape of nanoparticles based on PAA (A) and PAPC (B). Displayed bar represents 500 nm.

3.4. Mucolytic effect of nanocarriers

Gastrointestinal mucus forms a dynamic semipermeable barrier which is secreted and shed continuously. The viscoelastic properties of mucus depend on the content of mucin or mucus glycoproteins [32]. The nature of these macromolecules is crucial for the integrity of mucus since reduction of disulfide bridges or proteolytic cleavage hinders gel formation and degrades the tightly bound gel [33]. The mucolytic effect of papain modified particles could be demonstrated by adding the enzyme formulation to freshly prepared mucus. Findings revealed a significant loss in viscosity in case of native papain and papain incorporated into the particles. Figure 5 shows the rheological properties of intestinal mucus and its corresponding mixtures with nanoparticles. As expected, papain showed the strongest impact on mucus viscosity, indicated by a relative viscosity percentage of 5 %. The lower degree of mucus liquefaction of PAPC compared to papain only can be explained by the lower amount of proteolytic enzyme within the formulation. Despite a protein content of 30 %, PAPC particles cause a relative reduction in viscosity of 81 % after 1 hour of incubation. In contrast, PAA nanoparticles showed no marked effect of mucus consistency. The liquefaction of mucus after PAPC particle addition also confirmed the maintained enzymatic activity and stability of integrated papain.

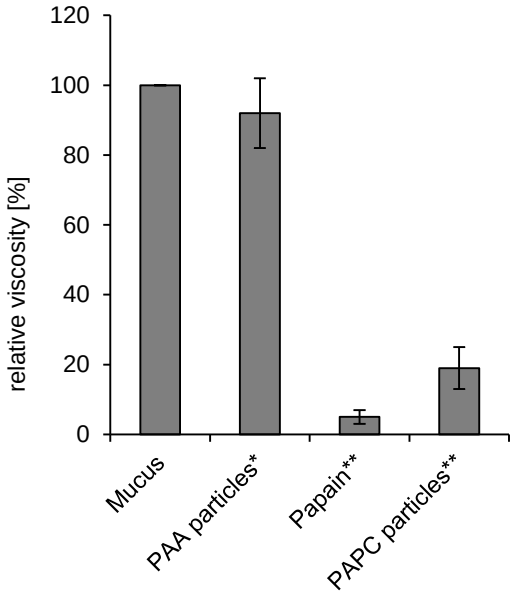

Figure 5.
Influence of papain, PAA and PAPC particles (0.2 %; w/v) on the viscoelastic properties of freshly excised intestinal mucus (pH 6.5) over 1 h of incubation and 37 °C. Values are means of at least three experiments ± standard deviation. (Differ from native mucus with *p < 0.05 and **p < 0.001) Moreover, samples were incubated for 12 hours to visualize and prove the mucolytic activity of the nanoparticles and papain. As shown in Figure 6, papain and papain containing nanoparticles showed a visible liquefaction of the mucus gel. The distinct liquefaction would not occur in humans or animals because of the lower particle concentration and mucus turnover process.

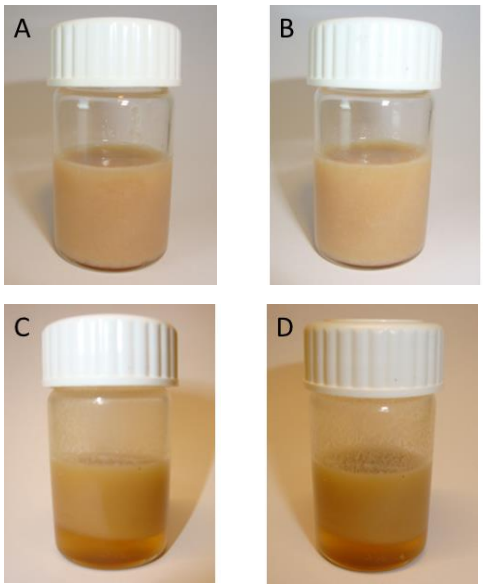

Figure 6.
Photographs of mucus samples treated with the particle formulations and papain (0.2 %; w/v) after 12 hours of incubation. Native mucus (A), mucus with PAA particles (B), mucus with papain (C) and mucus with PAPC particles (D).

3.5. Nanoparticle diffusion through intestinal mucus

Two different test techniques were conducted to evaluate the particle diffusion behavior in natural porcine mucus. The first method showed the particle transport from one compartment to a second by crossing a thin mucus layer. The particles were suspended in buffer and permeated the mucus barrier within 3 hours. The polycarbonate filter excluded diffusion of particle aggregates in the transport medium and ensured permeation of nanocarriers ≤ 450 µm. The rotating tubes assay revealed the penetration capacity of nanoparticles over a prolonged time period (24 h). The particles stay in direct contact with the mucus and traverse the tubes under exposure of rotation. All diffusion experiments require particle fluorescence labeling in order to visualize them *in vitro*. As shown in Table 2, FDA incorporation of 13.4 % and 11.8 % was achieved for PAA and PAPC particles, respectively. Both applied test techniques revealed significant improved transport ratios of papain modified particles. After an incubation period of 3 h in the diffusion chambers, particle transport rates of enzyme modified carriers were approximately 2.5-fold improved as a result of papain embedding (Figure 7).

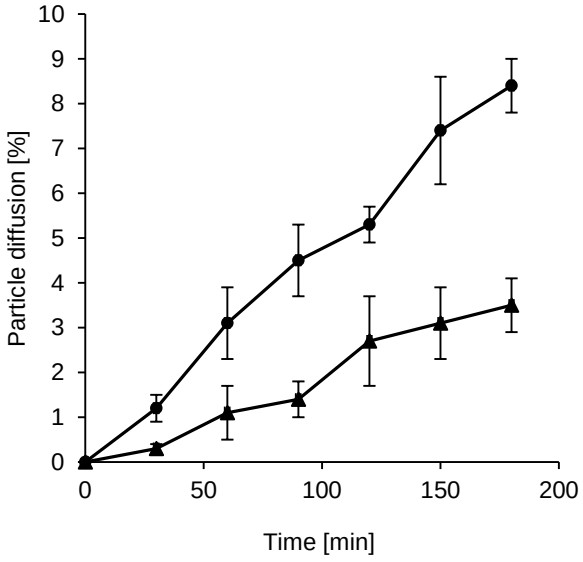

Figure 7.
Transport of FDA labeled PAA (▲) and PAPC* (●) nanoparticles (1%; w/v) through natural porcine intestinal mucus at pH 5.5 (100 mM MES buffer) at 37 °C. Indicated values are means ± SD of at least three experiments. (Differ from PAA particles with *p < 0.001)

The particle distribution in the rotating tubes revealed deeper mucus penetration of the PAPC particles. As depicted in Figure 8, unmodified PAA particles remained primarily close to the starting point of the tubes (segments 1 to 7) whereas PAPC nanocarriers were located in the middle section (segments 5 to 12). The profound mobility of PAPC particles within the mucus hydrogel is markedly based on the presence of papain. Rapid mucolyis by papain application was already proven by Rosenthal and Traut who benefited

papain's mucolytic properties for diagnosis of gastric cancer [34]. This enzyme exhibits a broad specificity and preferably cleaves peptide bonds containing basic amino acids and it shows esterase activity [35]. A local disruption of the mucus glycoproteins as a result of protein splitting into subunits facilitates the particle transport and provides access for mucosally administered drugs to the underlying enterocytes (Figure 2). Many of the mucus-penetration studies to date have focused on the development of muco-inert particles. These particles showed increased immobility in mucus gels through the modification of their surface properties. Engineering of densely charged neutral coatings or a high density of PEG molecules onto the surfaces of synthetic particles may reduce particle mucus adhesive interactions and improve the particle transport rates. Although the diffusion potential of muco-inert particles can be markedly improved due to a high density of both cationic and anionic surface, densely charged synthetic particles may exhibit immunogenic properties similar to viruses [6].

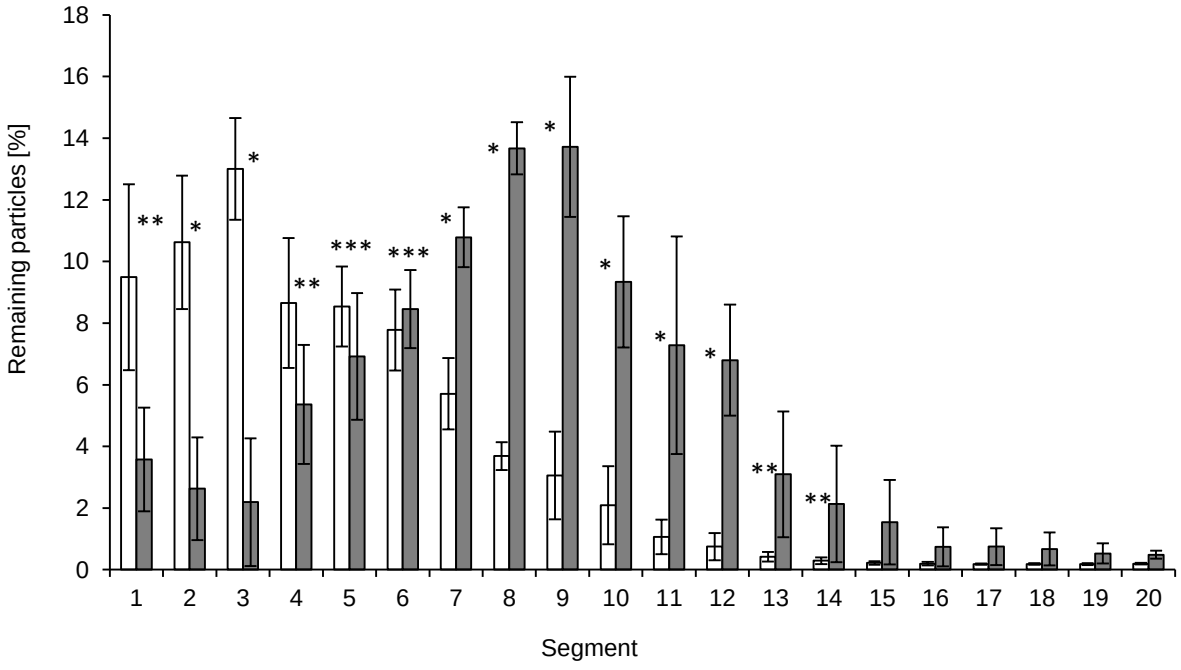

Figure 8.
Particle diffusion experiments of PAA (white bars) and papain modified PAA (grey bars) particles in mucus containing silicon tubes over 24 h of incubation at 37 °C and pH 6.5. Indicated values are means ± SD of at least three tests. (Differ with *p < 0.001, **p <0.01, ***p<0.05)

Furthermore, comparative experiments with different surface coated polystyrene particles indicated that many of the diffusing nanocarriers adhere to mucin fibers or get trapped in cages formed by mucoglycoproteins [36]. Utilization of proteolytic enzyme functionalized particles overcomes these obstacles through reducing of mucin crosslinks accompanied with decrease in the average mesh pore spacing.

3.6. Assessment of the release behavior

The release kinetics of FD4 from the PAPC and PAA nanoparticles is shown in Figure 9. The release profiles of both particle types show a biphasic pattern with an initial burst followed by a slow release rate. The rapid release phase could be attributed to the fast liberation of FD4 located on or near the particle surface. The following slow release rate might be due to diffusion of the trapped FD4 molecules from the inner part of the polymeric matrix. It has been demonstrated that PAPC particles revealed a slower release of the hydrophilic model compound in comparison to the particles based on PAA only. After 1 hour of incubation, papain modified particles liberated 40 % of the marker whereas PAA containing particles released 70 %. Such a retarded release rate could be ascribed to the higher hydrophilicity of the PAPC particles.

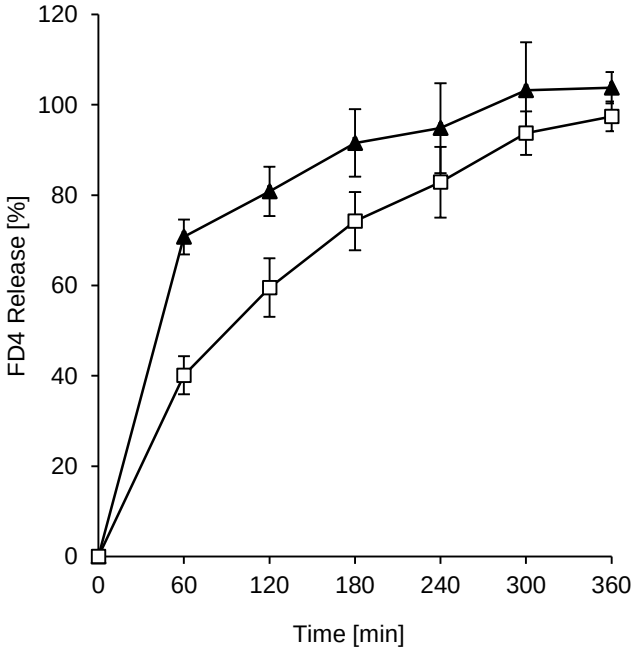

Figure 9.
Release behavior of FITC-dextran loaded nanoparticles based on PAA (▲) and PAPC* (□) in 100 mM phosphate buffer pH 6.5. Indicated values are means ± SD of at least three experiments. (Differ from PAA particles with *$p < 0.05$)

The nanocarriers are composed of a hydrophilic polymeric excipient and a proteolytic enzyme leading to a hydrophilic character of the particles. The carboxylic groups of PAA and the functional groups of the amino acids within the papain polypeptide chain are available for ionic interactions, van der Waal`s forces and/or hydrogen bonds. In case of incorporated hydrophilic agents the interactions are more distinctive which cause likely a retarded release compared to PAA nanoparticles. According to this, PAPC particles are suitable carriers for hydrophilic and macromolecular drugs and might increase their absorption on the site of absorption.

4. Conclusion

Within this study the potential of a proteolytic enzyme modified nanoparticles as mucus-penetrating delivery system was evaluated. Papain/poly(acrylic acid) particles were obtained via ionic gelation method resulting in mean diameters below 200 nm and negative zeta potentials. The mucolytic activity of the established nanocarriers significantly improved the particle transport rates and decreased the viscoelastic properties of porcine intestinal mucus compared to unmodified particles. The advantages of the papain functionalized nanoparticulate delivery system provide a promising tool to overcome the mucosal barrier and avoid rapid mucus clearance mechanism.

5. Acknowledgments

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Thiomers and Thiomer-based Nanoparticles in Protein- and DNA- Drug Delivery

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Abstract

Introduction

In recent years, thanks to advances in biotechnology, more and more highly efficient protein- and DNA-based drugs have been developed. Unfortunately, these kinds of drugs underlie poor non-parental bioavailability. To overcome hindrances like low mucosal permeability and enzymatic degradation polymeric excipients are utilized as drug carrier. Among investigated polymers, thiolated excipients showed several promising qualities in comparison to the analogical unmodified polymer.

Areas covered

The article deals with the comparatively easy modification of polymers to synthesize designated thiomers. Preparation is based upon well-established polymeric excipients like chitosan or poly(acrylates). Further, the recently developed "next generation" thiomers e.g. preactivated or S-protected thiomers are introduced. Designative properties like mucoadhesion, uptake and permeation enhancement, efflux pump inhibition and protection against enzymatic degradation of thiomers will be discussed and differences between first and next generation thiomers will be pointed out. Additionally, nanoparticles prepared with thiomers will be dealt with regarding to protein and DNA-drug delivery as thiomers seems to be a promising approach to avoid parenteral application.

Expert opinion

Properties of thiomers per se and results of *in vivo* studies carried out so far for peptide and DNA drugs demonstrate their potential as multifunctional excipients. However, further investigations and optimizations have to be done before establishing a carrier system ready for clinical approval.

Key words: DNA drugs, nanoparticles, oral delivery, protein drugs, thiomers

1. Introduction

By now, there are many highly efficient drugs being only bioavailable when administrated parenterally. As parenteral application is associated with pain, fear and efforts, alternative routes are desirable. For example, oral, pulmonary or nasal application can help improving compliance and cut down side effects linked with parenteral application. Two important classes with growing demand in health care are protein- and DNA-based drugs. Non-parenteral application of these pharmaceutics leads to poor bioavailability [1, 2]. Main problems are low mucosal permeability and instability due to enzymes and varying pH values in the gastrointestinal tract (GIT) [3]. To achieve therapeutic efficient plasma levels different approaches have been investigated. Besides derivatization of drugs, enzyme inhibitors, mucolytic compounds as well as stabilizing agents co-administrated to the drug and agents to facilitate permeation, different formulation techniques like mirco- and nanoparticles are studied [3, 4]. Nanoparticles have several advances due to their size and their properties in surface modification. Nanoparticulate formulations can enhance bioavailability, can be used in controlled/delayed release formulations and allow drug accumulation at specific locations. Drug efficiency increases and both frequency and extent of side effects decrease [5-7]. Furthermore, nanoparticles are relatively stable in gastrointestinal tract compared to other colloidal carriers like liposomes [8, 9]. Nanoparticulate carrier systems consistent of multifunctional polymers showed considerable potential. Suitable excipients are demanded for protecting and targeting drugs. Multifunctional polymers show several useful properties: mucoadhesion, protective activity, controlled release as well as permeation and uptake enhancement.

One of the most promising groups of multifunctional polymers are thiolated polymers or so called thiomers. Established functional polymers like chitosan and poly(acrylic acid) (PAA) are derivatized in order to improve properties for drug delivery. Immobilization of thiol bearing ligands to the backbone of these polymers leads to strongly improved mucoadhesion, protective and permeation enhancing effects [10-14]. Additionally, efflux pump inhibition could be shown *in vitro* and *in vivo* [15, 16]. Further, nanoparticle preparation, stabilization and drug incorporation is possible employing simple methods [17]. In here, we will give an overview of different kinds, synthesis and properties of thiomers. Further, recent results of research in thiomer nanoparticles for drug delivery will be provided and discussed.

2. Thiomers

First generation thiomers

In general, thiomers are synthesized by covalent attachment of thiol group bearing ligands to the backbone of a polymeric substance under relatively simple and mild conditions. Due to the chemical structure of modified polymers, they can be subdivided into cationic and anionic thiomers. In case of the first generation thiomers, all employed ligands used are of aliphatic structure. By now, cationic thiomers have mainly been based on chitosan. Thiolated chitosan derivatives are achieved by attaching a sulfhydryl-bearing ligand to chitosan's C2 amino groups. Chitosan-4-thiobutylamidine (Chitosan-TBA) can be synthesized by chemical reaction of 2-iminothiolane with chitosan leading to a cationic amidine conjugate in a one-step reaction [18]. Another thiolated chitosan derivative generated by amidine bond formation with isopropyl-S-acetylthioacetimidate is chitosan-thioethylamidine [19]. N-acteylcysteine, glutathione, thioglycolic acid, and 6-mercaptonicotinic acid are immobilized by amide bond formation between the ligands carboxyl group and chitosan's amino group mediated by carbodiimides [1, 20, 21].

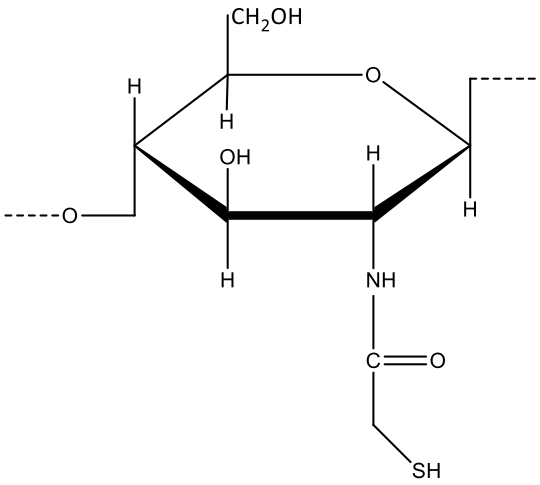
Recently, a new cationic thiomer was developed based on hydroxyethylcelullose (HEC). Therefore, the polymer was oxidized to the aldehyde form. To thiolate the polymer, cysteamine was used. In a reductive amination reaction with sodium cyanoborohydride the cationic HEC-cysteamine was generated [22].

So far, all anionic thiomers are based on carboxyl group bearing polymers. Sulfhydryl bearing ligands can be easily attached using the carboxyl group for amid bond formation. Therefore ligands bearing both, thiol and amino groups like cysteine, cysteamine and homocysteine are employed [23-26]. The amide bond formation is mediated by carbodiimides. Oxidation of thiol groups to disulfide bonds can be avoided as described below. Employing this synthesis pathway, poly(acrylic acid), polycarbophil, alginate, carboxymethylcellulose and pectin are derivatized into thiomers [27-31]. Instead of forming an amino bond for thiolization, Sharma et al. induced an ester bond between the carboxyl group of pectin and thioglycolic acid in hydrochloric environment [32].

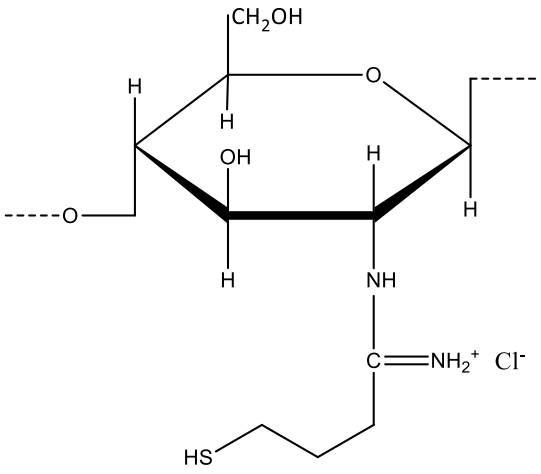
Next generation thiomers

As a matter of fact, free thiol groups are comparatively unstable in solutions as they are oxidized at pH ≥ 5 leading to self-crosslinking of the polymer [33]. Different approaches have been made for both, using the affinity of disulfide bond formation and trying to delay the oxidation. Self-crosslinking properties of thiolated poly(acrylic acid) could be shown, resulting in a 10- to 12-fold increase in viscosity after incubation at 37 °C for 24 hours (3 % m/v solutions in phosphate buffer pH 6.8), a quality that might be used in *in situ* gelling formulation [34]. Additionally, aromatic thiol bearing ligands turned out to be extraordinary good electron donors, leading to a stronger notion to oxidation [35]. To benefit from this

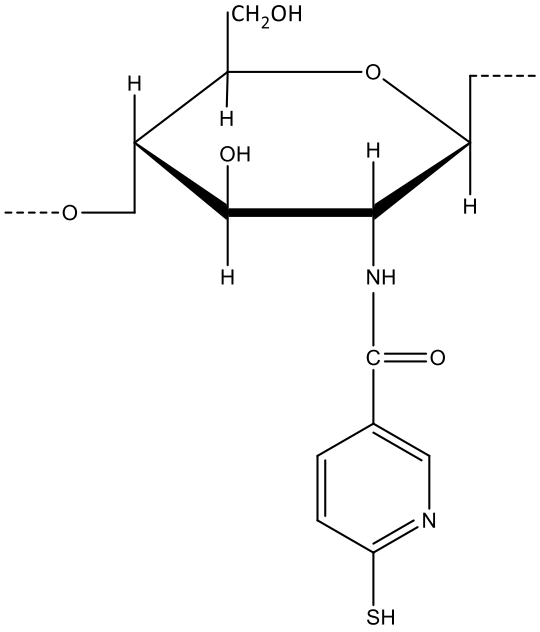
affinity, aromatic ligands have been attached to the polymer backbone instead of aliphatic ligands [36, 37]. For example, Perera et al. linked the aromatic thiol group bearing 4-aminothiophenol chemically to pectin in order to improve the *in situ* gelling properties and to be able to form pectin gel formulations with stronger cohesiveness. *In vitro* characterization of the new conjugate showed after oxidation *in situ* with hydrogen peroxide a 500-fold increased dynamic viscosity and disintegration time in comparison to unmodified pectin, further water uptake and disintegration time was increased significantly whereas the modified pectin without induced oxidation showed a decreased water uptake [38]. A similar approach has been taken by Millotti et al. from same group by attaching the aromatic heterocyclic compound 6-mercaptonicotinic acid. The molecule does have two tautomeric structures: thiol (S-H) and thione (C=S), whereat the thione form is the predominant structure in solution with polar solvents like water. As this structure can react as a nucleophile and a proton donor, disulfides can be formed without the presence of sulfhydryl groups meaning pH independent reactivity of thiomers [20]. An improved mucoadhesion towards chitosan with aliphatic substituents was shown [36]. Following the idea of pH independent and in general potentiated reactivity, preactivated thiomers have been developed recently by our group. Preactivated or designated S-protected thiomers are based on common thiomers at which free thiol groups are activated via pyridyl substructures. Resulting pyridyl disulfides are able to react rapidly with sulfhydryl groups by formation of disulfide bonds via disulfide exchange. These properties have already been employed by covalent chromatography [39, 40]. The first preactivated anionic thiomer was poly(acrylic acid)-cysteine (PAA-Cys), as preactivating agent 2-mercaptonicotinic acid (2MNA) was employed. Therefore, 2MNA was oxidized to the dimer and coupled via disulfide exchange reaction to the cysteine-moiety [41]. 6-mercaptonicotinamide (6MNA) was employed to preactivate chitosan-thioglycolic acid (Chitosan-TGA). 6MNA was synthesized out of 6-chloronicotinamide and thiourea according a method developed by Forrest et al. [42]. After oxidation of the 6-MNA monomer into the 6,6-dithionicotiniamide (6-DTNA) dimer it was coupled via disulfide exchange reaction to chitosan-TGA as well. The degree of preactivated thiol groups can be determined via spectrophotometrically detection of the aromatic substructure (Figure 1) [43].



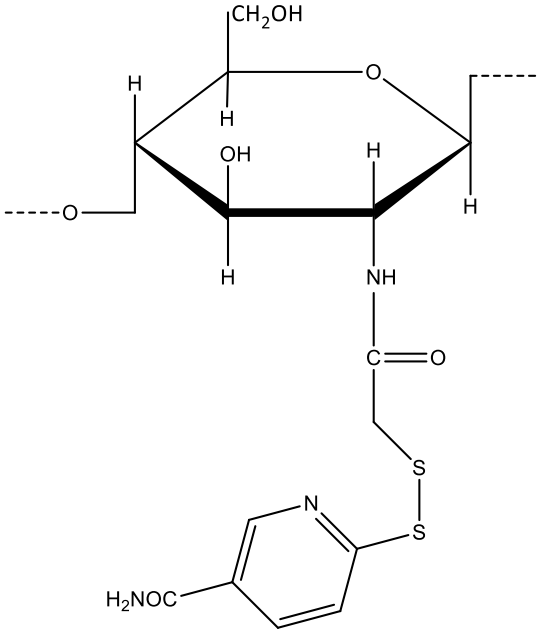
Chitosan-thioglycolic acid



Chitosan-thiobutylamidine



Chitosan-6-mercaptonicotinic acid



Chitosan-thioglycolic acid
thioglycolic-6-mercaptonicotinamide

Figure 1.
Substructure of selected thiolated chitosan-derivatives.

3. Properties of thiomers

3.1. Mucoadhesive properties

For mucoadhesion, the possibility of interaction between participants must be provided. For most known mucoadhesive polymers like poly(acrylates), sodium cellulose derivatives, alginate and chitosan the attachment is achieved by chemical, non-covalent bonds such as ionic bonds, hydrogen bonds, and van-der-Waals bonds [23, 44]. Further, physical interpenetration effects can be involved [17]. Covalent bonds with mucus components are possible with thiomers [24]. The reactive thiol groups are capable of forming disulfide bonds with proteins in the intestinal mucus layer leading to a strong adhesion effect [45]. Mucoadhesive properties of these thiolated polymers are improved in comparison to unmodified polymers. A positive correlation between thiol group content and mucoadhesive properties could be shown [23, 45-47]. Dünnhaupt et al. investigated mucoadhesion of preactivated thiolated chitosan in comparison to thiolated and unmodified chitosan. Figure 2B illustrates the results of tensile studies [48]. Figure 2A shows the results of the same investigation for the anionic preactivated thiolated poly(acrylic acid) performed by Iqbal et al. [41]. Even if only about 50-70 % of attached thiol groups have been activated, significant improvement of mucoadhesion could be demonstrated. So far, there have been no mucoadhesion studies carried out for nanoparticles based on preactivated thiomers, but as for other types of thiomers mucoadhesion could be shown in nanoparticulate formulations, mucoadhesive properties can be assumed [49]. Dünnhaupt et al. described in a previous work the distribution of thiolated mucoadhesive nanoparticles on intestinal mucosa. Within this study, adhesion of thiol group bearing nanoparticle formulations to the mucus layer could be shown via fluorescent labeling of the particles. It turned out that nanoparticles prepared using modified polymers showed a 6-fold increase of mucoadhesion compared to nanoparticles consisting of unmodified polymers. Tests were performed with chitosan and poly(acrylic acid) [49]. The results are consistent with data published by Barthelmes et al. investigating mucoadhesion of thiomer nanoparticles on porcine urinary bladder. Therefore, chitosan and thiolated chitosan (TGA) nanoparticles have been labeled using the fluorescent marker FDA. For formulations based on thiolated chitosan, after 1 hour around 80 % of FDA was still adhered to the mucosa whereas for formulations based on unmodified chitosan only around 25 % remained during the first hour [50].

Lately, Barthelmes et al. were able to confirm the *in vitro* mucoadhesive studies with an *in vivo* study carried out with female rats. Unmodified and thiolated chitosan nanoparticle suspension and pure fluorescent marker FDA suspension as control was delivered intravesical using a catheter. Remaining fluorescent marker was determined after sacrificing rats at predetermined time-points. Chitosan-TGA nanoparticles showed a significant 4-fold increased mucoadhesive effect than unmodified chitosan nanoparticles and a 170-fold increased retention time compared to pure FDA suspension [51].

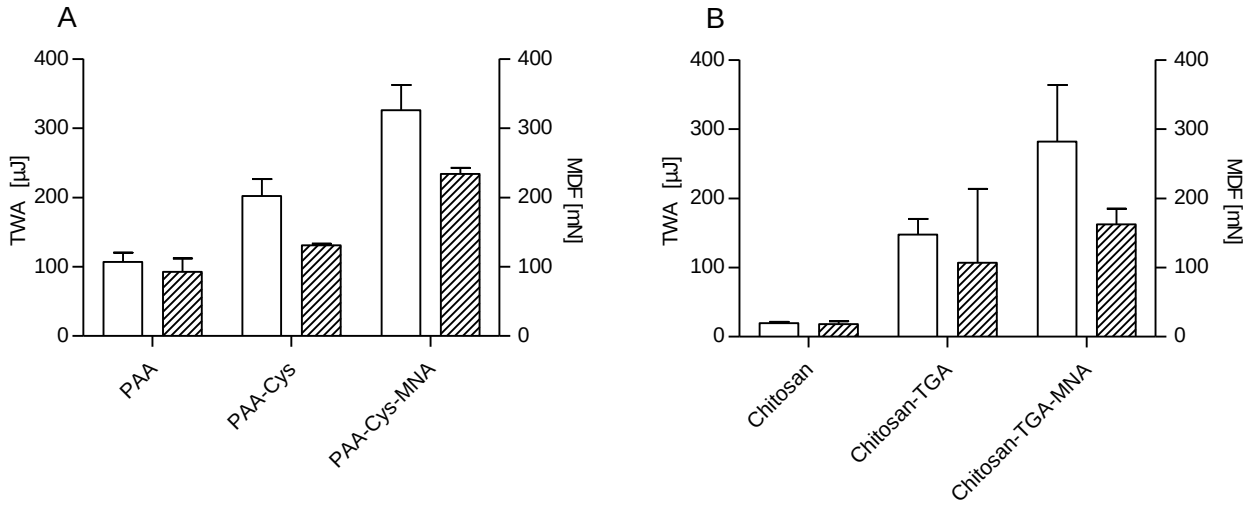


Figure 2 A+B.
Bar charts show results of tensile studies, carried out on porcine intestinal mucosa. TGA: thioglycolic acid, TGA-MNA: thioglycolic acid-6-mercaptonicotinamide, TWA: total work of adhesion, representing the area under the force/distance curve, white bars. MDF: maximum detachment force, striped bars. Data adapted from [41, 48].

3.2. Permeation enhancement and efflux pump inhibition

The oral bioavailability of several drugs is not only reduced by enzymatic degradation but the absorption barrier consistent of epithelial cell membranes. The interconnecting tight junctions hinder paracellular uptake of hydrophilic drugs. Chitosan is one of the well-investigated permeation enhancers [52, 53]. Several *in vitro* tests showed a further improvement of permeation enhancement by immobilizing thiol groups [19, 54, 55]. The underlying mechanism in uptake enhancement due to unmodified and thiolated chitosan is assumed to be the same: interactions with tight junctions [56-58]. Wang et al. investigated the permeation enhancer qualities of preactivated thiolated poly(acrylic acid) using sodium fluorescein as model drug in Ussing type chambers with freshly excised rat intestinal mucosa and Caco-2 cell monolayer as model barrier. It could be shown by both methods that the addition of PAA-Cys-MNA leads to a significant higher uptake of the model drug. The enhancement ratio was dependent on coupling rates of 2-MNA to the polymer [59].

Thiomers have also been tested for their efficacy in improving uptake of efflux pump substrates. The bioavailability of some drugs is restricted because of being substrate of the multidrug efflux pump P-glycoprotein (P-gp). P-gp is located in the apical membrane of enterocytes and transports its substrates from the inner side of the membrane to the outer side [60]. To overcome this hindrance, excipients, which are able to inhibit the transporter, are on demand. It could be shown, that using inhibitors improves intestinal absorption and tissue distribution and reduces elimination of drugs [15, 61]. *In vivo* studies have

been carried out by Föger et al. by applying a solid, enteric-coated chitosan-TBA/GSH oral formulation to rats and the P-gp substrate rhodamine 123 (rho-123) intravenous. The area under the plasma concentration-time curve of rho-123 increased by 58 % in comparison to unmodified chitosan and by 217 % in comparison to control buffer [16].

Dünnhaupt et al. studied permeation enhancing effect and efflux pump inhibition properties of thiolated and preactivated thiolated chitosan to achieve improved oral delivery of hydrophilic macromolecules. FD_4 was used as hydrophilic model drug, P-gp inhibition was investigated using Rhodamine-123 as P-gp substrate. Permeation studies using excised rat intestine showed within 3 hours a P_{app} being 1.3-fold higher for preactivated thiolated chitosan in comparison to thiolated chitosan and more than 2-fold higher than the P_{app} of unmodified chitosan. For efflux pump inhibition, the increase was 1.4-fold for preactivated thiolated chitosan compared to thiolated chitosan [43].

4. Preparation of thiomer nanoparticles

The most common used method to prepare nanoparticles is “ionic gelation” by inducing ionic complex formation, a simple and mild process. Electrostatic interactions between the positive charged amino group of chitosan and negative charged molecules like cyclodextrin, sodium sulfate and tripolyphophate (TPP) lead to complex formation [62]. For nanoparticle formation, a TPP-solution is dropped into an acidic solution of the thiomer under stirring. To stabilize the particles, the suspension can be stirred for several hours to induce disulfide bond formation within the particles. Size range of chitosan-6-mercaptonicotinic acid prepared according to this method was between 224 and 271 nm, for chitosan-TBA a mean diameter of 164 ± 6.9 nm was achieved. Particles can be collected by centrifugation and resuspended in water. After adding trehalose, particles can be lyophilized [63, 64]. Bernkop-Schnürch et al. developed a method that allows formation of nanoparticles without ionically crosslinking. After inducing particle formation via TPP, thiol groups of resulting particles are partially oxidized. Using hydrogen peroxide or iodine solution leads to disulfide bond formation. Afterwards, the polyanion was removed via dialysis. Resulting chitosan-TBA particle mean size was 366 ± 30 nm and a zeta potential around + 11.3 ± 1.3 mV [65]. Barthelmes et al. employed this method for chitosan-TGA particles in order to develop stable thiomer nanoparticles. Resulting particles had an average size of 158 ± 8 nm showing a zeta potential of ~ + 16 mV. Investigating the stability of these covalently cross-linked nanoparticles showed that more than 99 % of these particles were stable over a 60-min period in simulated gastric fluid whereat only 10 % of the ionically cross-linked chitosan-TGA nanoparticles were stable over this time period [66].

The ionic gelation method can be used for anionic thiomers as well; the procedure is basically the same but using cationic cross-linking agents. Sharma et al. used magnesium chloride to form pectin-TGA nanoparticles whereat the polymer solution was added drop-wise to a magnesium chloride solution [67]. Thaurer et al. prepared PAA-Cys nanoparticles using calcium chloride as ionic cross linker with an average size of 139 ± 34 nm [68]. Stabilizing via disulfide bond formation is also possible using hydrogen peroxide for partial oxidation. Dünnhaupt et al. prepared PAA-Cys nanoparticles using H_2O_2 for stabilization; resulting particle size was about 300 nm [49]. The ionic linker Ca^{2+} can be removed using ethylenediaminetetra-acetic acid and dialysis [17].

Further, thiolated chitosan nanoparticles were prepared using a polymerization reaction. Radical polymerization used to prepare nanoparticles was first described by Chauvierre et al. in 2003. The polymerization process of isobutyl-cyanoacrylate was provoked by polysaccharides and cerium ammonium nitrate. Following this method, Bravo-Osuna et al. prepared nanoparticles based on thiolated chitosan/isobutyl-cyanoacrylate copolymer. Therefore, thiolated chitosan was dissolved in nitric acid, cerium ammonium nitrate and isobutylcyanate were added under stirring and argon gassing at 40 °C for 40 minutes. After reaction time, the system was cooled down to room temperature and pH adjusted to 4.5 with NaOH solution. Nanoparticle size was about 229 nm and the resulting zeta-potential was determined to be 32.0 ± 0.2 mV [69]. Mazzaferro et al. also used isobutyl-cyanoacrylate to form nanocarrier with thiolated chitosan. Instead of a radical polymerization, an anionic polymerisation was induced. To achieve the anionic polymerization in presence of chitosan-TBA, the polymer was solved in nitric acid, and then isobutyl-cyanoacrylate was added under stirring and argon. Reaction took place at 40 °C, after reaction time and cooling down to room temperature; pH was adjusted to 6.5 using NaOH. Resulting particles had a size between 135-195 nm with a positive zeta-potential of about 40 mV [70].

Another preparation method for nanoparticles is the complex coacervation method. Like the ionic gelation method described above, particles are stabilized by Coulombic interactions. Intermolecular complexes are formed due to electrostatic interactions for example of cationic amino groups of chitosan and negatively charged groups like carboxyl groups of anionic polymers such as alginate or sulfate groups of dextran sulfate. [62, 71]. Saboktakin et al. formed insulin loaded dextran sulfate-chitosan-TGA particles by complex coacervation. Complexation occurred when chitosan solution was dropped in dextran sulfate-insulin solution under stirring. Resulting particles had a size range from 64 to 169 nm [72].

In general, loading of the particles with an active pharmaceutical ingredient (API) can be done during particle formation process or afterwards, the latter happens while incubating the particles suspension with

the drug. The achieved drug load rate is higher when the API is added to the solution before inducing particle formation but a drawback for this method is, that the API has to undergo the complete process and may be affected [73]. In the case of DNA-drugs, the drug itself can be used to precipitate nanocomplexes: Chitosan-TGA/pDNA complexes prepared by Lee et al. had an average size about 100 nm and a positive Zeta-potential of about 15 mV [74]. This polyelectrolyte complexation method was also employed by Yin et al. who prepared different trimethly-chitosan-cysteine-nanoparticles in a size range between approximately 100 and 200 nm with a positive Zeta-potential between 10 and 20 mV through self-assembly of positively charged polymer and negatively charged insulin [75].

5. Thiomer Nanoparticles - *in vitro* and *in vivo* studies

5.1. Protein delivery

Progress in biotechnology made production of peptide drugs in commercial scale possible, making their use as highly efficient drugs more interesting and draw researches attention on finding ways to improve their oral bioavailability [4].

Perera et al. could demonstrate that insulin encapsulated in thiomer nanoparticles could be protected from enzymatic degradation. Insulin loaded PAA-Cys/insulin particles (122-136 nm) were prepared using poly(vinyl pyrrolidon) as linking agent. Particles were stabilized via disulfide formation followed by incorporation into a triglyceride. Around 45 % of encapsulated insulin could be protected from trypsin and elastase and approximately 21 % from chymotrypsin degradation in *in vitro* studies [76]. The influence of the degree of thiol group content on release from core-shell nanoparticles has been investigated. Therefore, chitosan-TBA has been employed for encapsulation of drug Leu-Enkephalin in core-shell isobutylcyanoacrylate/chitosan-TBA nanoparticles. Particles were obtained by anionic emulsion polymerization [70, 77, 78]. To associate the protein with the resulting particles, two different methods have been employed, either during the nanoparticle formation process (inclusion method, “loading”) or via adsorption on preformed nanoparticles. The higher content of drug was obtained by the inclusion method. It turned out that with increasing degree of thiolation a decrease in both, loading and adsorption efficiency occurred and was linked with an increased initial release. It is assumed that the protein drug interacts with the positively charged, free amino groups of chitosan shell of the formulation [70]. As increasing TBA-attachment leads to reduction in free amino groups, the strength of linkage between excipient and active pharmaceutical ingredient declined. However, Millotti et al. found a reverse effect using the protein drug insulin. Chitosan-6-mercaptonicotinic acid (6MNAcid) particles were prepared using insulin-containing TPP-solution as linking agent, average size of resulting particles was 271 nm. *In vitro*

release studies showed that within the first 30 minutes, 50 % percent of the incorporated insulin was released, whereas 70 % of incorporated insulin was released form unmodified chitosan nanoparticles prepared for comparison. Both formulations released 100 % of insulin whereat a more sustained release was observed from 6MNA modified chitosan within the first 2 hours. *In vivo* evaluation of this carrier system was carried out using non-diabetic rats and determining the human insulin content in the plasma via ELISA after oral administration. Maximal insulin level was achieved at 120 min for unmodified chitosan particles and after 180 min for particles with thiolated chitosan. There was a significant 7.8-fold higher maximal plasma level of insulin detected after administration of thiolated particles compared to non-thiolated particles [63]. The highest concentration of human insulin determined in plasma after oral application (C_{max}) was for thiolated chitosan-based (chitosan-6MNA) nanoparticles 76.6 mU/l whereas chitosan nanocarrier led to C_{max} = 9.75 mU/l. The AUC calculated for insulin after administration with chitosan-6MNA particles was with 1810 mU 4-times higher than the AUC after administration of insulin loaded chitosan-nanoparticles.

To combine the positive qualities of trimethyl chitosan and thiolated chitosan, Yin et al. synthesized thiolated trimethyl chitosan (TMC-Cys). Therefore, TMC synthesized by using CH_3I in NaOH solution. After reaction time I^- was exchanged to Cl^- with anion-exchange resin and purified via dialysis. TMC was thiolated using L-cysteine HCl. Insulin loaded nanoparticles were prepared via ionic interactions due to positively charged polymers and negatively charged insulin. Resulting particles size range was between 100-200 nm with insulin encapsulation efficiency about 90 %. *In vitro* insulin release was investigated in phosphate buffer 0.2 M, pH 7.4 at 37 °C. TMC-Cys-insulin particles (chitosan 500 and 200 kDa) showed peptide release of 80-90 % after 4 hours, TMC-Cys-insulin particles (chitosan 30 kDa) showed faster release with almost 100 % peptide release after 1 h. Furthermore, an increased permeation enhancing effect for insulin from TMC-Cys-nanocarrier compared to TMC could be found (rat intestine). Pharmacological *in vivo* effect of insulin administrated orally with TMC-Cys-nanoparticles was determined in rats. For comparison, oral insulin solution and TMC-insulin-nanoparticles were employed. In addition, insulin suspension, TMC-Cys- and TMC-insulin nanoparticles were administrated into ileum. Both, oral and ileal application led to considerable decrease of blood glucose level compared to insulin solution. The effect lasted for 7 to 8 hours after administration and a maximal blood glucose depression of 35 % for oral administration and 70 % for ileal application was measured. Compared to TMC-insulin-nanoparticles TMC-Cys-insulin-nanoparticles showed a prolonged and higher hypoglycemic effect (Figure 3) [75].

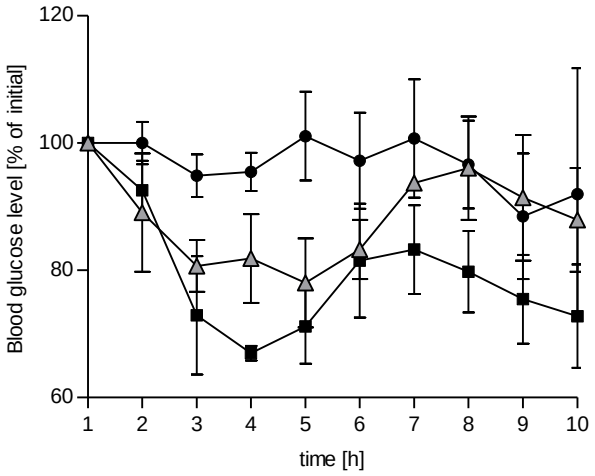


Figure 3.

Serum glucose levels in normal rats after oral administration of trimethyl-chitosan nanoparticles (-▲-), trimethyl-chitosan-cysteine nanoparticles (-■-) with insulin dose of 50 IU/kg, insulin solution.

Recently, Shahnaz et al. published their work about nasal administration of leuprolide in rats. Size of chitosan-TGA nanoparticles was about 252 nm, zeta-potential 10.9 ± 4 mV and sustained leuprolide release was determined over 6 h. Particles were prepared by ionic gelation and leuprolid was incorporate during particle formation process. It turned out that unmodified chitosan nanoparticle and chitosan-TGA nanoparticles enhanced leuprolide transport through nasal mucosa compared to leuprolide solution 2.0- and 5.2-folds, respectively. The relative bioavailability of leuprolide administered nasal via thiomer nanoparticle (versus subcutan injection) was about 19.6 % compared to leuprolide solution with 2.8 %. The maximum plasma concentration of leuprolide administered nasal via chitosan-TGA nanoparticles was about 3.8-fold improved compared to leuprolide solution administered nasal [79].

Makhlof and coworkers achieved an improved pulmonary delivery of calcitonin after encapsulation in glycol-chitosan-thioglycolic acid nanoparticles compared to non-thiolated glycol-chitosan nanoparticles (size range of 230-330 nm). Mucoadhesion of nanoparticle bearing free thiol groups was increased 2-fold compared to non thiolated ones in rats (intra tracheal application). For *in vivo* study, calcitonin solution, calcitonin loaded glycol-chitosan (GCS) or thiolated glycol-chitosan nanoparticles (GCS-TGA) were administrated to rats via a liquid Micros-Sprayer technique. Blood calcium level was investigated over a 24-hour time period. All formulations were able to reduce blood calcemia, whereas free calcitonin showed a short duration time that may be caused by the rapid elimination due to mucociliary clearance and/or drug degradation by peptidases. GCS and GCS-TGA formulations were able to prolong the hypocalcemic effect of the peptide with a pharmacological availability of 27 and 40 %, respectively. Makhlof et al. assume, that this improvement is due to mucoadhesive properties and permeation enhancing effects of

glycol-chitosan, further, the higher efficacy and longer lasting effect of thiolated glycol-chitosan could be the result of its higher mucoadhesive qualities. Table 1 presents the area above blood calcium curve after pulmonary application [80].

Table 1. Area above blood calcium curve over 24 hours after administration. Pharmacological bioavailability in % relative to subcutaneous administration [80].

	Calcitonin (µg/kg)	Area above blood calcium curve	Pharmacological bioavailability (%)
Solution (subcutaneous)	5	447.4 ± 110.3	-
Solution (pulmonary)	25	215.5 ± 145.2	9.63 ± 6.49
Glycol chitosan nanoparticles (pulmonary)	25	609.9 ± 144.1	27.27 ± 6.44
Glycol chitosan-TGA nanoparticles (pulmonary)	25	909.6 ± 26.2	40.67 ± 1.17

5.2. Gene delivery

More and more attention is drawn on therapeutic nucleic acids for vaccination and therapeutic gene expression. Carlisle et al. demonstrated, that plasmid DNA conjugated with thiolated polyethylenamine to nanoparticles coated with thiol-reactive poly[*N*-(2-hydroxypropyl)methacrylamide] bearing 2-pyridyldisulfanyl groups, leading to reducible disulfdide-linked coating, could improve transfection activity 40-100 fold compared to thioether-linked particles [81]. Further, it could be shown that thiolation of gelatin used as DNA-drug carrier brought benefits compared to investigated non-thiolated gelatin based formulations. It turned out that transfection was more effective in NIH-3T3 murine fibroblast cells using the nanoparticles of thiolated gelatin [82]. Based on these results, thiomers investigation as non-viral carriers for DNA seems promissing. Nanoparticles can be prepared readily using thiolated chitosan: Coacervation between positively charged amino groups of chitosan and negatively charged phosphate groups of nucleic acids leads to chitosan-DNA complexes in the nano-range [83]. For their evaluation of thiolated chitosan nanocarrier in antisense therapy Talaei et al. employed N-acetylcysteine-chitosan (chitosan-NAC) and N-acetyl penicillamine (chitosan-NAP). Nanoparticles were prepared by ionic gelation containing antisense oligonucleotide (ASOND) complementary to positions 3811-3825 of the human EGFR (epidermal growth factor receptor) cDNA, resulting particle size range was from 200 to 300 nm. *In vitro* release studies were carried out in phosphate buffered saline pH 7.4 (PBS) as well as under stimulated reducing cytosol conditions (dithiothreitol containing environment). During 15 h in PBS there was a lower (22 % instead of complete) but more sustained release to detect compared to unmodified chitosan-ASOND particles. In reducing cytosol stimulating medium particles dissociated leading to release of about 50 %. The impact on EGFR expression was investigated *in vitro* using T47D breast cancer cells. The expression of

EGFR protein was down-regulated by ASOND containing particles (NAC-chitosan, NAP-chitosan, unmodified chitosan) about 2-fold compared to free ASOND [84].

Martien et al. achieved protection of DNA against nucleases via encapsulation into chitosan-TGA carrier, particles were stabilized via oxidative cross-linking. Chitosan-TGA-particles resisted DNase I for 30 min whereat unprotected DNA and chitosan/DNA particle showed substantial degradation [85]. It is assumed, that the enzyme is inhibited via complexation of divalent cations, essential for enzyme activity, by the modified polymer [85, 86]. Furthermore, a 5-fold increase of transfection rate (expression of green fluorescence protein) for of chitosan-TGA/pDNA nanoparticles (average size of 100-200 nm) compared to unmodified chitosan/pDNA nanoparticles in Caco-2 cells was detected [85]. Lee et al. found enhanced and sustained gene delivery while investigating chitosan-TGA/DNA nanoparticles (113-220 nm): For the investigation, pDNA encoding the reporter gene for the green fluorescent protein (GFP) was employed. A study to determine the effect of thiolation of chitosan and thiolation followed by cross-linking on protection of pDNA from DNAse showed that unmodified chitosan and cross-linked thiolated chitosan protected pDNA form digestion whereas particles with thiolated non-cross linked chitosan turned out to provide less protection. Additionally, Lee et al. compared transfection efficiency of thiolated chitosan and thiolated chitosan with Lipofectin (a liposomal transfection reagent) in HEK 293 cells. Results showed higher transfection efficiency for thiolated chitosan nanocomplexes compared to Lipofectin, (approximately 30 % after 60 hours compared to about 10 % for Lipofectin system) and unmodified chitosan. Results from Lee et al. were in accordance with data published by Martien et al. *In vitro* transfection study using HEK 293 cells showed that a higher degree of free thiol groups led to a higher efficiency in transfection whereas oxidation of free thiol groups entailed a reduction in transfection efficiency. The *in vivo* gene transfer potential was determined using intranasal application to mice. Particles prepared with thiolated chitosan led to increased gene expression of green fluorescent protein after 3 days, particles containing cross-linked thiolated chitosan after 7 days compared to particles prepared with unmodified chitosan (Figure 4). Additionally, mucoadhesion study showed that mucoadhesion enhancing effect of thiolation is not lost in particle formulation [74].

These results confirm studies from Schmitz et al. and Loretz et al. who investigated chitosan-TBA and chitosan-NAC, respectively. Improved stability, enhanced pDNA release under reducing conditions and higher transfection rates in Caco-2 cell could be shown for these chitosan derivatives [87, 88]. Loretz et al. showed, that chitosan/DNA particles prepared with chitosan-NAC followed by crosslinking via oxidation were more stable in environments with an excess of polyanion heparin or with pH 10 then non-oxidized chitosan-NAC particles but not more stable than non-thiolated chitosan/DNA particles. It turned out in the

in vitro transfection study that oxidized chitosan-NAC/DNA particles could not improve transfection efficacy, apparently the vehicle is to stable to release DNA, whereat chitosan-TBA/DNA particles raised transfection 2.5-fold in comparison to chitosan/DNA particles [88].

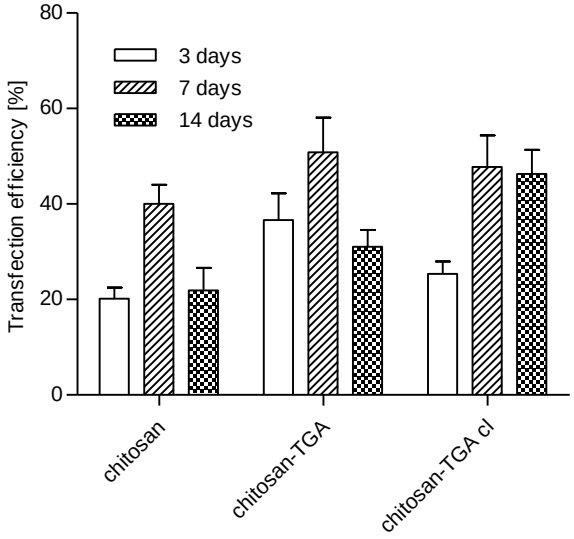


Figure 4.

Application of green fluorescence protein pDNA/chitosan nanoparticles intranasal in mice. Chart shows level of gene expression in BAL cells for 14 days after administration. N = 4. cl: cross-linked nanoparticles, TGA: 4-thiobutyl-amidine. Figure adapted from [74].

6. Expert opinion

This paper reviews thiomers and thiomer-based nanoparticulate delivery systems. Thiomers are a class of different polymeric excipients characterized by bearing free thiol groups. In many studies common properties have been demonstrated. First of all, remarkable improvements in mucoadhesion came along with thiolation independent of the underlain polymer. With introduction of preactivated thiomers, mucoadhesion could be further improved, although the currently used synthesis pathway does not allow to preactivate the total amount of attached thiol groups. To investigate the influence of preactivation in more detail, it would be desirable to modify up to 100 %. Additionally, permeation enhancing and efflux pump inhibition could be shown in several studies for different kind of thiomers. However, by now, it was only possible to demonstrate these properties *in vitro*. It is not for sure that these effects of widely biodegradable polymers under physiological conditions including enzymes and chyme as well as gastric and intestinal peristalsis still be notable. As a matter of fact, qualities of thiomers are depending on the polymeric backbone, the chosen thiol group bearing ligand and the degree of modification, making general conclusions about thiomers difficult but expand application area. For instance, modification with hydrophilic aromatic ligands led to increased viscosity being an advantage in gel or *in situ* gelling

formulations. Further, viscosity can have an impact on drug release. As the viscosity and solubility can be adjusted by varying ligands and degree of modification, tailor-made thiomer-based release medium may be possible someday. Sustained release could already be detected for thiomer nanoparticlulate formulations being a first step in controlled-release area. It turned out that thiol groups attached to the excipients could be employed for preparation of relatively stable drug loaded nanoparticles. Therefore, particles can be cross-linked via oxidation of thiol groups into disulfides. Cross-linking of particles does, besides having an impact on stability, have an influence on drug release. It seems to be a balancing act in introducing enough disulfide bonds for stabilizing particles and granting release. Further, drug release does not only depend on carrier system, individual interactions between the active pharmaceutical ingredient and excipients are important. Several *in vivo* studies with thiomer-drug formulations have been carried out showing that these formulations can succeed in protecting the DNA- or peptide-based drug and showed increased bioavailability in comparison to the unmodified polymer. These results seem promising for future investigations. It has to be considered that there are, by now, no systematic *in vivo* studies available that would make a general prediction possible. It is hardly possible to compare given results from different designed studies using different polymers with different kind and degree of modification and to draw general conclusion. As far as there are no clinical studies available evaluation and forward-looking statements are difficult. Nevertheless, encouraging results in drug formulation of protein- and DNA-based drugs have been achieved, even though for DNA-drugs mainly model drugs are used and by now there is no possibility to predict a therapeutic relevance of obtained bioavailability. Another advantage of thiomers-based nanoparticles, besides permeation enhancing, mucoadhesion, biocompatibility, uptake and transfection enhancement is the cheap and simple synthesis of thiomers and the mild conditions during nanoparticle formulation being essential for sensitive drugs like proteins.

A further aspect to keep in mind is that most formulations are only produced in laboratory scale. Therefrom, before any clinical trials are to debate, future research should concentrate on optimizing production and up scaling of the carrier system as well as providing more and better comparable i*n vivo* studies. *In vitro* toxicological investigations have been carried out using different kind of cell lines showing all no noteworthy toxic effect. It has to be mentioned that these kinds of investigation only have been performed with an incubation time up to 24 h [63, 74, 85]. Therefrom, no long-term data is provided making it even more difficult to transpose conclusion to human application.

In conclusion, various thiomers have been developed and investigated in terms of nanoparticulate carrier systems. Considering the low bioavailability of peptide- and DNA- based drugs when administrated non-parenteral, very encouraging results have been reached. As the individual thiomers differ in their

properties, the excipient fitting best for a certain formulation can be evaluated, leading to tailored carrier system for each drug and each application route. With promising qualities of thiomers per se and results of *in vivo* studies carried out so far, we are dealing with a substance class which will not be ruled out easily in the field of drug delivery development. It is also obvious, that further optimizations are necessary for establishing a carrier system ready for clinical approval.

Table 2. Main features of thiomer nanoparticles in drug delivery. PAA-Cys: poly(acrylate)-cysteine, 6MNAcid: 6-mercaptonicotinic acid, TMC-Cys: trimethylchitosan-cysteine, TGA: thioglycolic acid, NAC: chitosan-N-acteylcysteine, NAP: N-acetyl penicillamine, TBA: 4-thiobutylamidine, ASOND: antisense oligonucleotide.

Thiomer	Drug/model drug	outcome	Ref.
PAA-Cys	Insulin	*In vitro* degradation studies, nanoparticles protected 44.47 % of the initial insulin amount from trypsin, 21.33 % from chymotrypsin, 45.01 % from elastase compared to insulin solutions	[76]
Chitosan-6MNAcid	Insulin	AUC *in vivo* after oral administration to rats 4-fold improved compared to unmodified chitosan nanoparticles	[63]
TMC-Cys	Insulin	Oral and ileal application in rats blood glucose depression of 35 % for oral administration and 70 % for ileal application in *vivo*, hypoglycemic effect higher and longer lasting compared to TMC-insulin-nanoparticles	[75]
Glycol chitosan-TGA	Calcitonin	Pulmonary application to rats, pharmacological availability of 40 % compared to 27 % after administrating non-thiolated glycol chitosan/calcitonin particles	[80]
Chitosan-NAC/ Chitosan-NAP	ASOND	*In vitro* release after 15 hours showed a more sustained release of 22 % whereas ASOND was completely released from non-thiolated chitosan particles	[84]
Chitosan-TGA	pDNA	5-fold higher transfection rate (Caco-2) due to protection from nucleases	[85]
Chitosan-TGA	pDNA	Intranasal application in mice lead to gene expression over 14 days	[74]
Chitosan-NAC	pDNA	2.5-fold increase of transgene expression (Caco-2) in comparison to non-thiolated chitosan carrier	[88]
Chitosan-TBA	pDNA	Increased transfection efficiency in Caco-2 cells compared to naked plasmid	[87]

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Self-nanoemulsifying drug delivery system for pDNA – protection against enzymatic degradation.

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Abstract

As especially oral gene delivery is still challenging because of poor uptake and rapid enzymatic degradation, alternative gene delivery vehicles providing sufficient efficacy and safety are on demand. It was therefore the aim of this study to investigate a novel strategy for oral gene delivery utilizing a self-nanoemulsifying drug delivery system (SNEDDS) consisting of Cremophor EL (30 %), Captex 355 (30 %), Capmul MCM (30 %) and propylene glycol (10 %). After hydrophobic ion pairing of a model plasmid encoding for green fluorescence protein with six different cationic lipids resulting pDNA-lipid complexes were incorporated into the SNEDDS. The formulations were evaluated in terms of resulting droplet size, cytotoxicity, protection against enzymatic degradation via DNase I and transfection efficiency. The mean droplet size of resulting nanoemulsions was determined to be between 45.8 and 47.5 nm. A concentration dependent cytotoxicity of the formulations was found on HEK-293 cells via MTT assay. Degradation studies via DNase I showed that incorporation into the SNEDDS led to significantly, up to 8-fold prolonged resistant time against enzymatic digestion compared to naked pDNA and pDNA-lipid complexes. Transfection studies carried out with the three most promising formulations revealed a significantly improved transfection compared to naked pDNA. Further, no decrease in transfection efficiency was shown due to incorporation of pDNA-lipid complexes into SNEDDS compared to transfection using the liposomal Lipofectin® transfection reagent. According to these results, self-emulsifying drug delivery systems represent a promising novel strategy for DNA drug delivery.

Key words: SNEDDS, nonviral vector, hydrophobic ion pairing, gene delivery

1. Introduction

Gene therapy is about controlling expression of specific proteins by introducing genetic material into certain cells. This makes gene therapy to a promising tool as the progression of a wide range of diseases like cancer, AIDS and neurological disorders like Parkinson's or Alzheimer's disease can be controlled by the induction or inhibition of specific genes [1, 2]. Because of their high selectivity and specifity for their target, gene drugs are believed to show reduced side effects and higher efficiency in comparison to most small molecule drugs [2]. However, safe and efficient delivery of DNA drugs into targeted cells is still a major task in pharmaceutical research. Poor cellular uptake and rapid enzymatic degradation of DNA drugs in general demands for drug delivery system which facilitates cellular uptake and shields against enzymatic degradation [2]. Especially when it comes to oral drug delivery, whereat the drug is exposed to different enzymes and extreme pH changes, innovative drug delivery systems are required.

Because of the high expectations which are linked with successful gene therapy, there is an ongoing interest and research for different kinds of vectors for gene delivery. As they are considered to be less immunogenic, focus is laid on non-viral vectors. Most of them include the use of cationic molecules which are able to form inter-polyelectrolyte complexes with the negatively charged DNA backbone. Thereby, different formulations have been investigated like liposomes and polymer-based nanoparticles. Considering the low number of available finished dosage forms based on gene therapy whereat none of the authority approved therapeutics is an oral dosage form, novel strategies are highly welcome. One innovative attempt in gene delivery might be the incorporation of DNA into a self-nanoemulsifying drug delivery system (SNEDDS). These delivery systems gained much attention during the last years as they are able to improve bioavailability of drugs due to several factors. The usage of permeation enhancing fatty acids and other surfactants, a large surface area due to ultrafine dispersion and a protective effect against enzymatic degradation are the most important ones [3,4].

So far, these systems are mainly investigated for oral delivery of lipophilic drugs which are incorporated easily into the lipid based carrier [5-7]. Hydrophilic drugs, however, are not able to be dissolved in SNEDDS or migrate into the aqueous phase immediately after contact with aqueous fluids. Therefore, modification of the hydrophilic DNA is required to enable dissolution in the SNEDDS and thus entrapment in the lipid phase of the nanoemulsion, which could provide protection from enzymes in the aqueous fluids of the gastrointestinal tract. The replacement of counter ions of peptides, proteins and polynucleotides is a known tool to alter solubility of these biomolecules without changing their structure and hence their activity. Using ionic detergents drops aqueous solubility distinctly and increases solubility in organic phases at the same time [8].

Accordingly, the aim of this study was to employ this strategy to incorporate DNA into a SNEDDS formulation to prove a protective effect against enzymatic degradation. Therefore, a model plasmid encoding for green fluorescent protein (GFP) was complexed with different cationic lipids to reduce hydrophilicity and enable incorporation into the lipophilic carrier. The resulting pDNA-lipid complexes were investigated regarding precipitation efficiency and solubility in SNEDDS. The nanoemulsions were characterized in terms of resulting droplets size and stability. The cytotoxic potential of the delivery system was evaluated via a MTT assay using HEK-293 cells. Moreover, the protective effect of pDNA incorporation against degradation via DNase I was examined and the impact on transfection efficiency using HEK-293 cells was investigated.

2. Materials and Methods

2.1. Materials

Capmul MCM and Captex 355 were supplied by Abitec Corporation, Janesville, USA. Cremophor EL was purchased from BASF, Ludwigshafen, Germany. Propylene glycol was obtained from Gatt-Koller, Absam, Austria. The green fluorescent plasmid was amplified in *Escherichia coli*, which were received from Addgene (pcDNA3-EGFP, Addgene plasmid 13031). Agarose, Agar-Agar, and LB-Medium were ordered from Carl Roth, Karlsruhe, Germany. GelRed® was supplied by GenON, Ludwigshafen am Rhein, Germany, the plasmid-extraction kit by Qiagen, Hilden, Germany, DNase I by Promega, Mannheim, Germany and Lipofectin® by Life Technologies, Carlsbad, CA, USA. Dodecyltrimethylamonium bromide, cetylpyridinium chloride monohydrate and cetrimide were supplied by ABCR, Karlsruhe, Germany. All other reagents were purchased from Sigma-Aldrich, Vienna, Austria and were of analytical grade.

2.2. Preparation of the plasmid

The enhanced green fluorescence protein plasmid (6.1 kb) was used to investigate the protection of the vehicle against degradation by DNase I. The plasmid was propagated in E. coli and isolated using a Midi Prep kit from Qiagen. The purified pDNA was resuspended in TE-buffer (10 mM Tris-Cl, 1 mM EDTA) pH 8 and quantified using bisBenzimide H 33258 dye and a mircoplate reader (Tecan, Grödig, Austria). DNA was diluted with demineralized water to the requested concentration and stored at -20 °C until use.

2.3. Hydrophobic ion pairing

Before incorporation of the plasmid in the lipophilic vector, lipophilicity of the active pharmaceutical ingredient (API) was increased by hydrophobic ion paring. Therefore, 6 different lipids were investigated regarding their suitability as ion pairing partner. The primary amine hexylamine and the quaternary amines dodecyltrimethylamonium, cetylprydinium chloride monohydrate, stearalkonium chloride, cetrimide and Lipofectin® were used. Lipofectin® is a liposomal formulation which contains the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phophotidyl-ethanolamine (DOPE) 1:1. Complex formation should occur due to the cationic amino group of the lipid and the negatively charged backbone of the pDNA. Different molar ratios of pDNA:lipid were investigated, 1:1, 2:1 and 3:1, in order to identify the most suitable ratios for ion pairing. The pDNA was diluted to a concentration of 0.7 mg/mL and precipitated with equal volume of lipid solution. After an incubation period of 10 min, the resulting precipitate was removed by centrifugation at 3000 g for 30 min. Total amount of precipitated DNA was calculated by determining remaining pDNA in the supernatant after centrifugation using a detection kit with bisBenzimide H 33258.

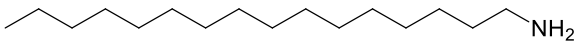
2.2. Preparation and loading of SNEDDS

The formulation employed in this study consists of 30 % (m/m) Cremophor El, 30 % (m/m) Captex 355, 30 % (m/m) Capmul MCM, and 10 % (m/m) propylene glycol [3]. The obtained precipitated pDNA-lipid complexes were dissolved in the SNEDDS in a concentration of 5 µg/µL by incubation of the precipitate with the SNEDDS overnight at 37 °C.

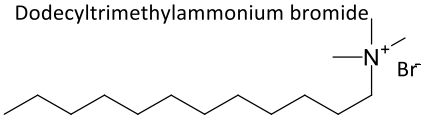
2.3. Preparation and characterization of nanoemulsion

For further investigations, nanoemulsions were prepared by diluting the pDNA-lipid complex loaded SNEDDS in phosphate buffer pH 6.8 0.1 M in a concentration of 1 % at room temperature. Resulting nanoemulsions contained pDNA in a final concentration of 50 µg/mL. The droplet size of all prepared emulsions was determined by dynamic light scattering using a PSS NicomPTM 380 DLS (Santa Barbara, CA, USA) directly after preparation and 4, 8 and 24 hours after preparation. As control, a nanoemulsion without pDNA-lipid complex was prepared and investigated. Further, the droplet size of nanoemulsions prepared in OPTI MEM® was investigated at various concentrations.

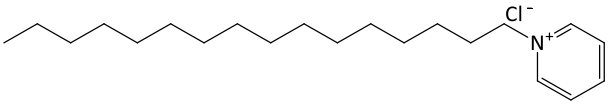
A Hexadecylamine



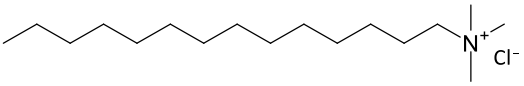
B Dodecyltrimethylammonium bromide



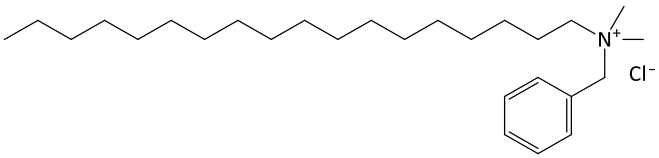
C Cetylpyridinium chloride



D Cetrimide



E Stearalkonium chloride



L Lipofectin

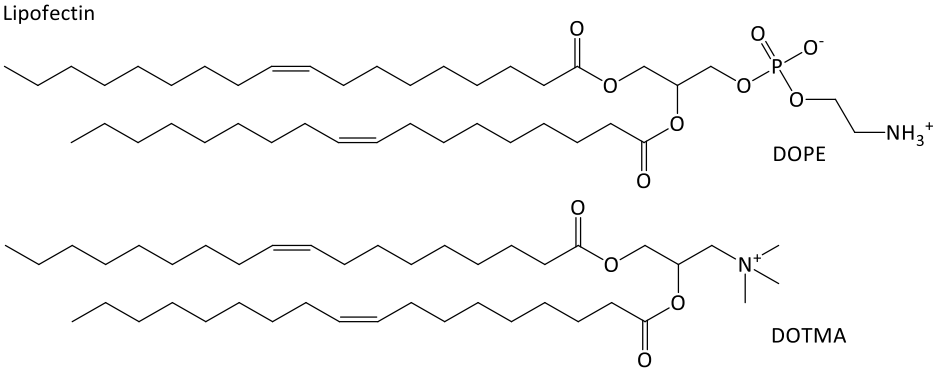


Figure 1.
Structures of lipids used in this study for hydrophobic ion pairing. Lipofectin® is a commercially available transfection facilitator containing equal parts of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE).

2.4. Cytotoxicity

Cytotoxic potential of all formulations was investigated via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Within the assay the reductive activity of vibrant cells is determined. Due to the reductive activity of mitochondria, water soluble MTT is converted into water insoluble formazan crystals. HEK-293 cells were maintained at 37 °C under 5 % CO_2 and 90 % relative humidity in minimum essential medium (MEM, containing phenol red, Earls salts, 10 % fetal bovine serum and 1 % penicillin and streptomycin). Cells were seeded in a density of 2 x 10^5 cells/mL MEM into a 96 well plate. After 24 hours at 37 °C in an incubator with 5 % CO_2, cells were attached and washed twice with prewarmed (37 °C) phosphate buffered saline (PBS). Subsequently, 100 µL of the prepared nanoemulsions in four different concentrations 0.05, 0.10, 0.25, and 0.50 % v/v in MEM were added to the wells. As negative control MEM and as positive control Triton X® 100 4% (v/v) were employed. The cells were incubated with the samples for 4 hours. Thereafter, the supernatant was discarded and the cells were washed twice with PBS. For detection, cells were loaded with 100 µL of a MTT solution (0.5 % m/v) in MEM. After 4 h of incubation, the supernatant was removed and the precipitated formazan crystals were dissolved in 100 µL DMSO. The absorbance of resulting colored formazan solutions in DMSO was measured at 570 nm with a background subtraction at 690 nm by a microplate reader (Tecan infinite, M200 spectrometer, Grödig Austria). The viability of the cells was calculated in percent of negative control (MEM) [9].

2.5. Stability assay against DNase I degradation

Considering precipitation efficiency, solubility in SNEDDS and toxicological profile, the most promising formulations were chosen for further investigations. Degradation studies of pDNA were carried out employing the PROMEGA® RQ1 RNase-free DNase kit. Therefore, the formulations loaded with the pDNA-lipid complex were prepared as described above. The pDNA-lipid complexes without SNEDDS, naked pDNA and a naked pDNA SNEDDS emulsion with the same amount of pDNA served as control. Equivalents containing 2 µg pDNA were incubated with 1 µL of DNase I (1 U) and 4 µL DNase reaction buffer (400 mM Tris-HCl pH 8, 100 mM $MgSO_4$ and 10 mN $CaCl_2$) at 37 °C. The reaction was stopped at predetermined time points with 1 µL of stop solution (20 mM EGTA, pH 8). The degree of degradation was investigated by 1 % agarose gel electrophoreses. The gel was prestained with GELRed® and visualized under UV light.

2.6. Transfection studies

HEK-293 cells were maintained as described above and seeded in a 12 well plate in an initial concentration of 4 x 10^5 cells per mL and well and incubated for 24 h before loading with samples. The pDNA-lipid complex loaded SNEDDS were diluted with OPTI MEM® reduced serum medium without antibiotics to 0.1 % SNEDDS resulting in a final concentration of 0.5 µg pDNA/mL. Each well was loaded with 1000 µL of the nanoemulsion. After incubation of 4 hours, the supernatant was discarded and MEM with FCS and antibiotics was added instead. After 48 hours, cells were harvested for GFP assay. As control, cells were treated with the naked pDNA and the pDNA-Lipofectin® complex prepared according to suggestions of Lipofectin® provider.

2.7. Green fluorescence protein assay

To detect transfection efficiency, a GFP assay as described previously was carried out [10]. In brief, the medium was discarded and the cells were washed twice with prewarmed PBS and thereafter lysed with 100 µL phosphate buffer 0.1 M, pH 7.5 containing 0.2 % Triton X-100 and 1 mM DTT (dithiothreitol) by a freeze-thawing cycle. 50 µL of each lysate were transferred to a 96-well plate and the amount of GFP was determined by measuring fluorescence at 488/510 nm (excitation/emission) with a mircoplate reader (Tecan infinite, M200 spectrometer, Grödig Austria).

2.8. Statistical data analysis

All experiments were performed in triplicates and the related values are reported as the means ± SD. The GraphPad Prism 5 software was used for all statistical data analysis. One way ANOVA was performed with $P < 0.05$ as the minimal level of significance followed by Bonferroni post-test with $P < 0.05$ as minimal level of significance.

3. Results

3.1. Hydrophobic ion pairing

Hydrophobic ion pairing was carried out successfully by the addition of five different lipids and Lipofectin® to a pDNA-solution. Structures of all investigated lipids are depict in Figure 1. Interaction of positively charged lipid with the negatively charged backbone of pDNA leads to complex formation. Thereby, the complex partially precipitates in form of a water-insoluble pDNA-lipid complex. By the addition of all lipids to the pDNA solution, a suspension was formed. Within the investigated range, no significant difference in the amount of precipitated complex was observed by the addition of different molar ratios of lipids to the pDNA solution. Therefore, for all further investigations, a molar ratio of 1:1 pDNA:lipid was used. In

case of all lipids, a precipitate was obtained after centrifugation. The extent of complex formation, however, was strongly dependent on the type of lipid used. The primary amine hexadecylamine, for instance was able to precipitate just 18 % of pDNA whereas all quaternary amines were able to precipitate a significantly higher amount as listed in Table 1.

Table 1. Characterization of different pDNA-lipid complexes and resulting nanoemulsions. Table gives the amount of precipitated pDNA, characterizes the solubility of formed complex in the SNEDDS and gives the droplet size of a prepared 1 % v/v nanoemulsions in phosphate buffer pH 6.8 0.1 M as well as the polydispersity index (PDI). DOTMA/DOPE = N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride /dioleoyl phophotidyl-ethanolamine. Indicated values are means of three experiments.

Formu-lation	Lipide	Precipitation efficiency [%]	Dissolution time of precipitate in SNEDDS	Droplet size [nm]	PDI
A	Hexadecylamine	17.7 ± 5.2	16 h	47.3 ± 0.24	0.059
B	Dodecyltrimethyl-ammonium bromide	70.3 ± 10.1	16 h	47.4 ± 0.31	0.094
C	Cetylpyridinium chloride monohydrate	45.1 ± 6.8	16 h	47.3 ± 0.29	0.094
D	Cetrimide	89.1 ± 9.8	16 h	47.3 ± 0.41	0.135
E	Stearalkonium chloride	91.1 ± 8.9	1 h	47.5 ± 0.38	0.126
L	DOTMA/DOPE 1:1	58.2 ± 5.6	immediately	45.8 ± 0.29	0.059

3.2. Preparation of drug loaded SNEDDS and characterization of nanoemulsion

All obtained pDNA-lipid complexes were dissolved in the SNEDDS formulation in a final concentration of 10 µg/1 µL SNEDDS. The Lipofectin®-pDNA complex dissolved immediately, whereas all other formulations were entirely dissolved after 16 h of incubation at 37 °C resulting in a clear oily formulation. The drug loaded and the “empty” SNEDDS formulation were diluted in phosphate buffer pH 6.8 0.1 M resulting in a slightly bluish nanoemulsion. The mean diameter (intensity weight) of droplets in the emulsion was determined to be around 50 nm for all formulations. Exact values for the mean droplet size and the polydispersity index are given in Table 1. Mean droplets size of SNEDDS without any pDNA-lipid complex was 52.83 ± 0.57 nm. Figure 2 shows size distributions of nanoemulsion in phosphate buffer pH 6.8 0.1 M. By incorporation of the different complexes in the lipid phase, size distributions was just slightly changed. The Lipofectin®-pDNA complex loaded SNEDDS showed a narrower size distribution and a relatively lower polydisperity index compared to all other formulations. To investigate the stability of the nanoemulsion, the size was measured again after 4, 8 and 24 hours whereat no significant change in size distribution could be observed. Moreover, diluting to 0.5 and 0.25 % also did not lead to a significant change in size

distribution. Furthermore, the droplet size of nanoemulsions diluted in OPTI MEM® reduced serum medium showed no alteration in mean droplet size. Diluting in both, phosphate buffer and OPTI MEM® to a concentration below 0.1 % resulted in a significant decrease in the droplet size to around 30 nm.

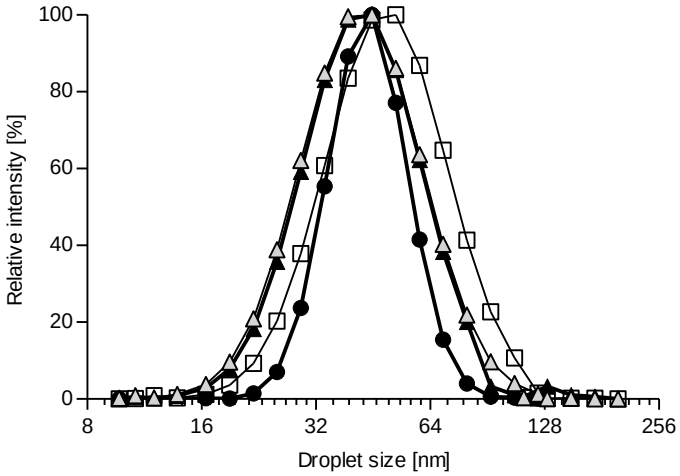


Figure 2

Size distribution of "empty" SNEDDS (-□-), SNEDDS loaded with pDNA- Lipofectin® complex (-●-), pDNA-stearalkonium complex (-▲-) and pDNA-cetrimide complex (-△-) in a 1 % emulsion with phosphate buffer 0.1 M pH 6.8. Indicated values are means of three measurements.

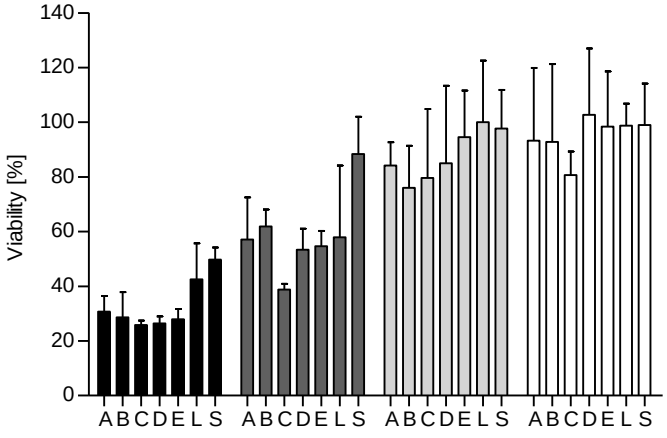


Figure 3.

Results of cytotoxicity studies. HEK-293 cell monolayer was incubated with different concentrations of the different pDNA-lipid complex loaded SNEDDS and the basic SNEDDS formulation. Viability was detected via MTT assay. White bars 0.05 %, light grey bars 0.10 %, dark grey bars 0.25 % and black bars 0.5 % v/v. SNEDDS were loaded with

A = pDNA-hexadecylammonium,
B = pDNA-dodecyltrimethlyammonium
C = pDNA-cetylpyridinium
D = pDNA-cetrimide
E = pDNA-stearalkonium and
L = pDNA-Lipofectin® complexes and diluted in OPTI MEM®.
S = "empty" SNEDDS in OPTI MEM®.

3.3. Cytotoxicity

Results of MTT assay are plotted in Figure 3. A concentration dependent toxicity was observed for all investigated formulations. Thereby, at higher concentrations, formulations containing cationic lipids resulted in a significant lower viability as the empty SNEDDS formulation, as shown in Figure 3. Further, the Lipofectin® loaded formulation showed at a concentration of 0.25 % a significantly higher cell-viability compared to the other loaded formulations.

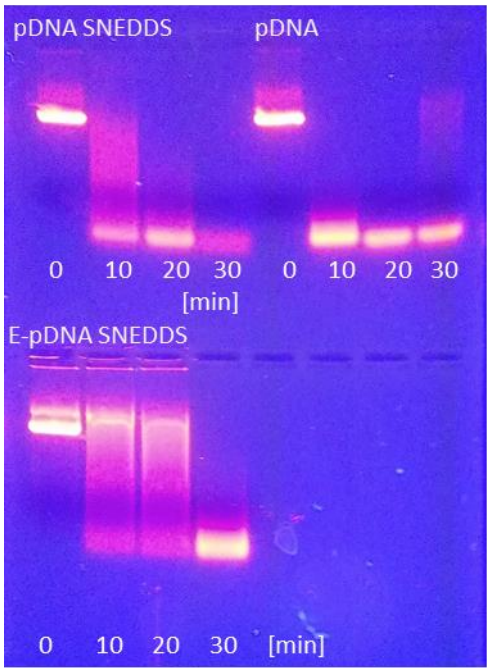


Figure 4.
Results of enzymatic degradation studies with DNase I. Reaction was stopped after predetermined time points and extent of degradation was visualized by agarose gel electrophorese. Gel was prestained with GelRED® and detected under UV light. Gel shows results for a 1 % nanoemulsion mixed with pDNA (pDNA-SNEDDS), pDNA solution and nanoemulsion loaded with pDNA-stearalkonium (E-pDNA SNEDDS).

3.4. Stability against DNase I degradation

Via enzymatic degradation studies using DNase I, the success of incorporation of pDNA into the lipophilic carrier was confirmed and a protective effect against enzymatic degradation was observed. Under test conditions, degradation of the pDNA-cetrimide and the pDNA-stearalkonium complex incorporated in SNEDDS showed a sustained degradation over 30 min (Figure 4) whereas the naked pDNA was completely digested by the enzyme within the first 5 minutes. For the Lipofectin®-pDNA complex loaded SNEDDS no degradation was observed during the first 30 min (Figure 5) of investigation but was completely degraded after 40 min. To ensure that this effect results from incorporation into the delivery system, degradation studies were carried out with a mixture of non hydrophobically modified pDNA with SNEDDS and with the

resuspended pDNA-lipid complexes without the lipophilic carrier. It turned out that the SNEDDS had no protective effect at all to the unmodified pDNA. Furthermore, it was shown that the complex formation of the pDNA with cationic lipids only led to a minor protective effect against DNase I. For all three investigated lipoplexes, a delay of 5 minutes in degradation was to observe. Additionally, degradation studies were repeated 24 hours after preparation of the pDNA-lipid complex loaded nanoemulsion. Degradation profiles of these formulations were similar to the naked pDNA, indicating a release of the pDNA from the lipophilic carrier system.

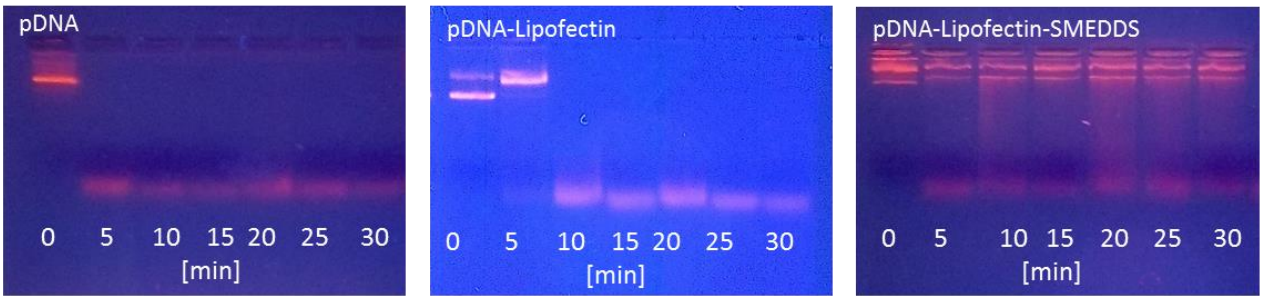


Figure 5.
Results of enzymatic degradation studies with DNase I. Reaction was stopped after predetermined time points and extent of degradation was visualized by agarose gel electrophorese. Gel was prestained with GelRED® and detected under UV light. Gel shows results for a 1 % naked pDNA solution, 1 % resuspended pDNA- Lipofectin® complex and 1 % nanoemulsion of pDNA- Lipopfectin® complex loaded SNEDDS.

3.5. Transfection studies

Cells were harvested for GFP assay 48 hours after transfection. As illustrated in Figure 6, all formulations showed a significantly improved transfection compared to the naked pDNA. The formulation containing pDNA-stearalkonium complex led to significant lower expression of the GFP compared to the other pDNA-lipid complex loaded formulations. Between the SNEDDS formulation containing the pDNA- Lipofectin® complex and the pDNA-cetrimide complex, no significant difference to the unformulated Lipofectin® facilitated transfection was observed.

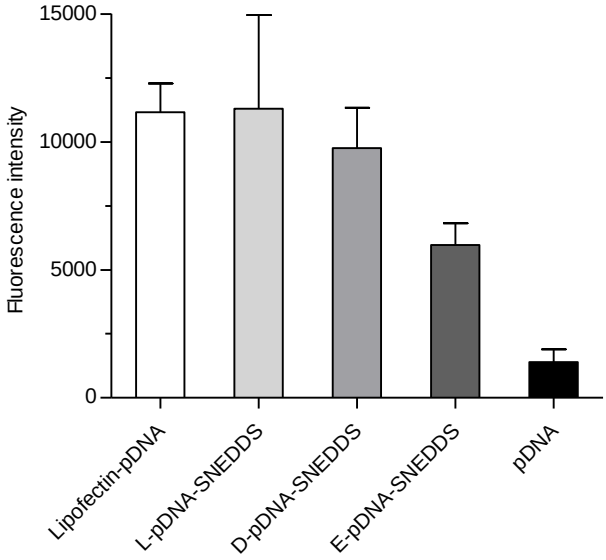


Figure 6.
Results of 4 hours transfection studies carried out on HEK-293 cells. Cells were harvested 48 hours after transfection and the fluorescence intensity of the green fluorescence protein was measured after cell lysis using a microplate reader (488 excitation/510 emission wavelength). Indicated values are means of three replicates ± SD. SNEDDS loaded with pDNA-Lipofetcin® complex (L-pDNA-SNEDDS), pDNA-cetrimide complex (D-pDNA SNEDDS) and pDNA-stearalkonium complex (E-pDNA-SNEDDS).

4. Discussion

Self-emulsifying drug delivery systems are of increasing interested in pharmaceutical research. Preparation does not require cost intensive equipment and lipophilic drugs are incorporated easily. Moreover, an improvement in bioavailability was shown for incorporated drugs in different studies [5-7]. Recently, a protective effect of a peptide drug against enzymatic degradation was shown *in vitro* and *in vivo* studies carried out with this formulation showed improved bioavailability of the peptide drug in rats. Thereby, it was possible to incorporate the nonapeptide leuprorelin into SNEDDS after hydrophobic ion paring to increase its lipohilicity [3].

DNA drugs are similar to peptide drugs regarding their sensibility against enzymatic degradation and their hydrophilicity. Hence, DNA drugs should benefit from incorporation into SNEDDS as well but are likewise difficult to incorporate in the lipid based carrier. Therefore, within this study, hydrophobic ion pairing was carried out employing different amino group functionalized lipids. The negatively charged phosphate group of the DNA backbone has been employed for complex formation for several years. Thereby, basic amino groups are commonly used as ion pairing partner. For example, the polymer chitosan was intensively investigated for pDNA nanoparticle formulations [11, 12]. Unfortunately, the charge of primary amino groups is pH dependent. Therefore, besides a primary amine, hexadecylamine, lipids with a quaternary amino group were investigate for their ability to precipitate the pDNA. Even if the pka value of hexadecylamine is above 10, providing a degree of protonation at pH values between 6-8 up to 99 % (calculated via chemicalize.org), precipitation efficiency was significant lower for this primary amine

compared to all quaternary amines. Lipofectin® is a mixture of a quaternary amine, DOTMA and an amphiphilic lipid, DOPE. The mixture precipitated pDNA to higher degree compared to the primary amine hexadeclyamine and the quaternary amine cetylpyridinium chloride. All other investigated quaternary amines precipitated pDNA to a higher degree. Hence, quaternary amines, in general, can be considered as superior in precipitating pDNA. Besides the interaction of the cationic with the anionic group, solubility of the lipid itself in water determines precipitation efficiency. Lipofectin® is a liposomal mixture and easily dispersed into water. Most of the other amines used in this study had to be sonicated or heated to 37 °C in order to dissolve them in a concentration of 1 mg/mL. This liposomal formulation may be able to bind pDNA without leading to precipitation. Within this study, precipitation was crucial as the pDNA-lipid complex had to be separated from the water phase in order to incorporate the pDNA into the lipophilic carrier. After overnight incubation, all precipitates could be dissolved in the SNEDDS lipid mixture. This indicates that hydrophobic ion pairing with all lipids investigated within this study altered the solubility of the pDNA in an extent making dissolution in a liquid lipid mixture possible [8]. Furthermore, the droplet sizes of resulting nanoemulsions were not changed significantly for all prepared formulations confirming uniform incorporation of the precipitate in the carrier system.

As cytotoxic effects of surfactants and cationic substances are well known, a MTT assay was carried out to determine a reasonable concentration range for transfection. Non-ionic surfactants as well as cationic substances show a concentration dependent effect on cell viability as these substances are able to interfere with the cell membrane, breaking their integrity and leading to detachment from the culture flask [13, 14]. Within this study, the influence on cell viability increased after incorporation of the cationic lipids into the SNEDDS indicating a clear impact of the cationic substance. At higher concentrations, viability after incubation with pDNA-Lipofectin® complex loaded SNEDDS was superior to the other loaded formulations. Besides the cationic head group of these lipids, the structure of the whole component influences toxicity. It has been reported that the double tailed cationic lipids are less toxic than their single-tailed corresponding lipid [15]. Furthermore co-lipids like DOPE are reported to exhibit low toxicity.

Regarding the toxicological profile as well as precipitation efficiency and solubility in the SNEDDS, three formulations were chosen for further investigations. As enzymatic degradation of DNA is a crucial factor in limiting oral gene delivery and naked DNA is degraded rapidly, the investigated SNEDDS formulations were mainly designed to protect from enzymatic degradation. DNA-lipid complexes in general are reported to be able to shield enzymatic attacks [16]. However, within this study only a minor effect of protection against enzymatic degradation via DNase I was found for all investigated pDNA-lipid complexes, including the commercial available transfection facilitator Lipofectin®. After incorporation of the pDNA-lipid complexes into the SNEDDS, a remarkable delay in enzymatic degradation was found. For the pDNA-

cetrimide complex and the pDNA- stearalkonium- complex, the effect was not as pronounced as for the pDNA- Lipofectin® complex. For the former two formulations, degradation was sustained but completed within 30 min whereas the pDNA- Lipofectin® complex loaded SNEDDS formulation showed no sign of degradation for 30 min. The more pronounced protection of the pDNA in the pDNA-Lipofectin® complex formulation could be explained due to a higher solubility of the pDNA- Lipofectin® complex in the SNEDDS. The precipitate was dissolved immediately after adding the SNEDDS formulation whereas pDNA-stearalkonium complex was dissolved after 1 h of incubation at 37 ° and the pDNA-cetrimide complex needed 16 h incubation before complete dissolution. Higher solubility may result in a higher amount of precipitate in the lipid phase of the nanoemulsion and a more sustained release of the pDNA from the lipid phase into the aqueous phase where the pDNA can be digested by the enzyme.

The usage of cationic auxiliary agents have advanced DNA and mRNA transfection research *in vitro* and *in vivo*. Usually, liposomal formulations containing two different lipids, a cationic amphiphile and a neutral phospholipid like in Lipofectin® are used. These kinds of transfection agents are successful due to the ability of capturing the negatively charged polynucleotides, increase of cellular uptake due to interaction of the resulting positively charged complexes with the negatively charged membrane components. Furthermore, they are able to perform membrane fusion with endosomes or plasmalemma [17]. Due to incorporation into SNEDDS, liposomal structure of Lipofectin® is likely lost. Transfection studies, however, revealed that these structural changes do not affect the transfection efficiency of this auxiliary agent.

5. Conclusion

This is the first time, to our knowledge, that pDNA was incorporated into the lipid phase of a nanoemulsion. Hydrophobic ion pairing was carried out successfully to increase the lipophilicity of the model pDNA with different lipids. Furthermore, a protective effect against degradation via DNase I could be shown due to incorporation in the lipid phase. Moreover, it was found that incorporation into the lipid phase and hydrophobic ion pairing did not lead to a decrease in uptake and therefore in no decrease in transfection efficiency compared to the commercially available liposomal transfection reagent Lipofectin®. According to these outcomes, self-emulsifying drug delivery might be a novel promising strategy for DNA drug delivery, in particular when protection towards DNases is essential.

6. Acknowledgement

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