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Chitosan–aprotinin coated liposomes for oral peptide delivery: Development, characterisation and in vivo evaluation

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ABSTRACT

In order to improve the systemic uptake of therapeutic peptides/proteins after oral administration, the polymer-protease inhibitor conjugate chitosan–aprotinin was synthesised and polyelectrolyte complexes between negatively charged multilamellar vesicles (MLV) and positively charged chitosan–aprotinin conjugate were prepared. It could be demonstrated that chitosan–aprotinin was capable of significantly inhibiting Trypsin in vitro in concentrations of 0.05% and 0.1%, whereas no inhibition was observed in the presence of 0.1% chitosan. The size range of the prepared MLV was between 3 and 4.5 μm and the initially negative zeta potential (ca. −90 mV) of the core liposomes switched to a positive value after polymer coating (ca. +40 mV). Confocal laser microscopy studies showed comparable mucoadhesive properties of chitosan–aprotinin coated MLV and chitosan coated MLV. In comparison to calcitonin in solution, the area above the blood calcium concentration–time curve (AAC) after oral administration of calcitonin loaded chitosan coated MLV to rats increased around 11-fold, and around 15-fold in the case of calcitonin loaded chitosan–aprotinin coated MLV. Data gained in the current study are believed to contribute to the development of novel polymer-protease inhibitor based delivery systems.

1. Introduction

Most therapeutic peptides and proteins are nowadays administered via injections, because they are generally only poorly absorbed after oral administration. As the oral administration route is preferred by patients, various approaches have been investigated to make feasible the oral administration of peptides and proteins. One main barrier for most orally administered peptides and proteins is the enzymatic barrier (Woodley, 1994). Digestive enzymes of the stomach and the intestine, including Pepsin, Trypsin, Chymotrypsin, Elastase and membrane bound aminopeptidases as well as carboxypeptidases can lead to rapid protein inactivation. Therefore, researchers investigated already about 15 years ago, if an inhibition of proteolytic enzymes can improve the oral bioavailability of peptide and protein drugs (Yamamoto et al., 1994). In fact, it could be demonstrated that co-administration of various protease inhibitors such as aprotinin or the Bowman Birk Inhibitor (BBI) leads to improved insulin plasma levels after oral administration (Yamamoto et al., 1994). Unfortunately, it was also reported that prolonged administration of protease inhibitors can lead to hypertrophy and hyperplasia of the pancreas (Ge and Morgan, 1993; Melmed et al., 1976). In recent years, the concept of polymer-protease inhibitor conjugates was introduced into the pharmaceutical arena (Bernkop-Schnürch and Scerbe-Saiko, 1998). Potent protease inhibitors are covalently bound to non-absorbable multifunctional polymers such as poly(acrylates) or chitosan (Bernkop-Schnürch and Kast, 2001). This immobilisation is believed to lead to an improved safety profile by simultaneously retaining the inhibitory properties of the inhibitor. Reduced toxicity has been attributed to the fact that the immobilised inhibitors are not absorbed, and that lower concentrations might be used as the inhibitor stays concentrated at the same intestinal segments as the drug is released.

Another effective approach besides protease inhibition is the use of orally administered liposomes, and in particular the use of polymer-coated liposomes (Takeuchi et al., 2005a,b, 2003, 1996). It has been shown that the pharmacological effect of orally administered insulin and calcitonin was greatly increased after administration of polyelectrolyte complexes between mucoadhesive polymers such as chitosan or poly(acrylates) and oppositely charged liposomes compared with liposomal formulations omitting the polymer (Takeuchi et al., 2003, 1996). It has also been shown, that chitosan coated liposomes remain in the intestine for a longer time period than non-coated liposomes, which is mediated by the mucoadhesive effect of chitosan (Takeuchi et al., 2003, 1994).

It was the aim of the current study to investigate the efficacy of liposomes coated with the polymer-protease inhibitor conjugate chitosan–aprotinin for oral peptide delivery. The physico-chemical properties of the novel liposomal delivery system, the Trypsin

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inhibitory capability of the inhibitor conjugate, the calcitonin loading capacity as well as the mucoadhesive properties were evaluated in vitro and compared with the properties of chitosan coated liposomes. In addition, the efficacy of orally administered coated liposomes containing the peptide drug calcitonin was evaluated in vivo in rats.

2. Materials and methods

2.1. Materials

Chitosan (Seelab, 150 kDa), aprotinin (Sigma, lyophilised powder, 3–8 TIU/mg solid) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC), L-α-distearolyphosphatidylcholine (DSPC, Nippon Oil and Fats Co.), cholesterol (Chol. Sigma), dicetyl phosphate (DCP, Sigma) coumarin (Sigma), N-benzoyl-arginine-p-nitroanilide (Sigma), were used as received. Calcitonin (elcatonin) was kindly supplied by Asahi Chemical Co., Japan. All used reagents were of analytical grade.

2.2. Synthesis of chitosan–aprotinin conjugate

Synthesis was performed similar as described previously (Werle et al., 2007). The protease inhibitor aprotinin was covalently grafted to chitosan (medium molecular mass, 150 kDa) by using the coupling agent EDAC. In brief, the carboxylic groups of the inhibitor were activated by EDAC and amide bonds between the activated carboxylic groups of aprotinin and the primary amino groups of chitosan were formed. For the synthesis, 60 mg of Chitosan were dissolved in 15 ml 0.1 M HCl under stirring. Then, the pH was carefully adjusted to pH 6.0 with 1 M NaOH. In parallel, EDAC (250 mg in 1 ml H_2O) was added to 1.5 ml of a 2 mg/ml solution of aprotinin in water. The EDAC/aprotinin mixture was incubated for 20 min at room temperature in order to allow activation of the carboxylic groups of aprotinin. Then the mixture was added to the chitosan solution pH 6.0 and incubated at room temperature and stirring for 10 h. Subsequently, the solution was filled into dialysis tubings (MWCO = 12,000–14,000 Da, Spectra/Por, Spectrumlabs) and dialysed 6 times for 12 h against water. Finally the purified chitosan–aprotinin conjugate was lyophilised.

2.3. Trypsin inhibition studies

In order to determine the amount of covalently grafted active aprotinin, the degradation of the Trypsin substrate N-benzoyl-arginine-p-nitroanilide (NBANA) was monitored. In presence of Trypsin, NBANA forms nitroaniline, which leads to an increase in absorbance at 405 nm. Before adding NBANA to the solutions containing Trypsin in buffer only (50 mM phosphate buffer pH 6.5) or in buffer containing different concentrations of chitosan/chitosan–aprotinin, the sample solutions were pre-incubated for 20 min at room temperature. For quantification of the amount of grafted aprotinin, a calibration curve was used. The calibration curve was prepared by adding different amounts of free aprotinin to a Trypsin solution and finally adding NBANA after a pre-incubation period of 20 min. Five minutes after addition of NBANA, the absorbance was measured (Galaxy Fluostar, A = 405 nm).

The following stock solutions were prepared: 0.5 mg of benzoyl-arginine-p-nitroanilide was dissolved in 1 ml of 50 mM phosphate buffer pH 6.5 and filtered through 0.45 μm. 0.2 mg Trypsin was dissolved in 1 ml phosphate buffer pH 6.5. Chitosan and chitosan–aprotinin solutions (0.1% and 0.5%, respectively) in 50 mM phosphate buffer pH 6.5 were prepared. For the determination of the degree of modification, 50 μl of each protease solution were added to 50 μl of a 0.1% chitosan–aprotinin solution and to 50 μl of 8 different dilutions of aprotinin in buffer. These solutions were incubated for 20 min at room temperature. Then, 100 μl of NBANA solution was added, and after 5 min of incubation at room temperature the absorbance was measured immediately with a microplate reader (Galaxy Fluostar, absorbance, A = 405 nm). The amount of covalently grafted active aprotinin in percent (m/m) was calculated by using the calibration curve described above. In addition, the time dependent degradation of NBANA in presence of Trypsin in buffer only as well as in buffer containing chitosan or chitosan–aprotinin (0.1% and 0.05%, respectively) was determined using the same experimental setup as described above, but measuring the increase in absorbance mediated by nitroaniline every 5 min during an observation period of 45 min (Galaxy Fluostar, absorbance, A = 405 nm).

2.4. Preparation and characterisation of liposomes and coated liposomes

Preparation of liposomes coated with chitosan has been described in detail previously (Takeuchi et al., 1996). Anionic multilamellar liposomes (MLV) consisting of DSPC, DCP and Chol. (molar ratio: 8:2:1) were prepared using the thin film hydration method. DSPC, DPC and Chol. were dissolved in chloroform and a thin film lipid layer was obtained by evaporating the organic solvent for 3 h at 40 °C using a rotavapor and water jet vacuum. The obtained thin film layer was dried overnight in a vacuum oven to ensure complete removal of chloroform. Hydration was performed with 66.67 M phosphate buffer pH 6.8 by repeated gentle heating and vortexing. The liposomal suspension was incubated for 30 min at 10 °C.

Coating with chitosan and chitosan–aprotinin was performed by mixing an aliquot of the described liposomal suspensions with a 0.6% polymer solution in 100 mM ABS buffer pH 4.5 and vortexing. The coated liposomes were incubated for 30 min in a cooled water-bath (10 °C). The size of non-coated and coated MLV was determined using a LDSA 2400A particle size analyser (Tohnichi Computer Co. Ltd., Japan). Zeta potential was measured with suspensions that were prepared by adding 20 μl of the liposomal suspension to 8 ml of purified water via dynamic light scattering analysis (Zetasizer 3000 Has, Malvern). For control experiments with non-coated liposomes, 1:1 dilution of the liposomal suspension with buffer was performed in order to achieve same concentrations as with coated liposomes.

Calcitonin containing MLV for in vivo studies were prepared similar as described above. Instead of using buffer only, hydration was performed with 66.67 M phosphate buffer pH 6.8 containing 160 μg/ml calcitonin. MLV were diluted 1:1 with 66.67 M phosphate buffer pH 6.8 and mixed with an aliquot of 0.6% chitosan or chitosan–aprotinin solution in 100 mM ABS buffer pH 4.5 (final calcitonin concentration of coated MLV was 40 μg/ml). As a control, a calcitonin solution (40 μg/ml) in 66.67 M phosphate buffer pH 6.8 was prepared. Calcitonin loaded MLV were ultracentrifuged for 45 min at 231,000 × g and 4 °C and the calcitonin concentration of the supernatant was analysed by HPLC (Jasco, Japan) under the following conditions: ODS-2 column (GL Science), mobile phase = acetonitrile:0.1% trifluoracetic acid solution (35:65, v/v), flow rate = 1 ml/min, injection volume = 20 μl, wavelength = 220 nm.

2.5. Mucoadhesion studies

MLV were prepared in a similar manner as described above, but the hydrophobic fluorescence marker coumarin was additionally added when preparing the thin film lipid layer. After hydration with 100 mM ABS buffer pH 4.5, the coumarin concentration of the liposomal suspension was 0.1 mg/ml. MLV were administered

Table 1
Concentrations of reagents used for the synthesis of chitosan–aprotinin and control and percent of aprotinin in chitosan–aprotinin [m/m]; values represent the mean ± S.D. of at least three experiments.

Polymer	Chitosan (mg/ml)	EDAC (mg/ml)	Aprotinin (mg/ml)	Percent of aprotinin (m/m) in chitosan–aprotinin
Chitosan–aprotinin	4.8	19.5	0.24	4.68 ± 0.29
Control (Chitosan only)	4.8	–	–	No inhibitory effect

intragastrically to 10-week-old rats which had been fasted for 48 h prior to MLV administration. The rats received either 500 μl of chitosan coated MLV suspension or 500 μl of chitosan–aprotinin coated MLV suspension (final concentration of each polymer was 0.3% and final coumarin concentration was 0.05%, respectively). The control rats did not receive any treatment. One hour after administration, the rats were sacrificed and the intestine was excised and divided into three 15 cm segments referred to as "duodenum", "jejunum" and "ileum". Each segment was washed with 10 ml of 0.9% saline solution. Samples were frozen and sliced into sections of 10 μm thickness by using a Cryostat (LEICA). Samples were analysed using a confocal laser scanning microscope (LSM510, Carl Zeiss Jena) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.6. In vivo studies

All animal experiments performed within the current study were approved by the animal welfare commission of Gifu Pharmaceutical University. Polymer coated calcitonin containing MLV (final polymer concentration was 0.3%) as well as a control solution were intragastrically administered to 10-week-old male Wistar rats which were fasted 24 h before the experiment. During the experiment, rats had free access to water. Rats were divided into three cohorts of $n = 3$, respectively. The cohorts received either 1 ml of a calcitonin solution, 1 ml of chitosan coated MLV or 1 ml of chitosan–aprotinin coated MLV (administered amount of calcitonin was 40 μg per each rat). Blood samples (200 μl) were withdrawn from the jugular vein after predetermined time-points (0.5, 1, 2, 4, 8, 12 and 24 h). Blood calcium levels were determined by using a commercially available calcium kit (Calcium C-Test, WAKO, Wako Pure Chemicals, Japan).

2.7. Statistical data analyses

Statistical data analyses were performed using the Student *t*-test with $p < 0.05$ as the minimal level of significance. Calculations were done using the online calculation programme at http://www.physics.csbsju.edu/stats/t-test_bulk_form.html.

3. Results

3.1. Synthesis of chitosan–aprotinin conjugate

The synthesis was performed as describe previously (Werle et al., 2007), but using a chitosan with a lower molecular mass (150 kDa instead of 450 kDa). Basic parameters of the synthesis are summarised in Table 1. The synthesised conjugate contained 4.68% (m/m) aprotinin. It has been demonstrated in a previous study (Werle et al., 2007), that the Trypsin inhibition test is an appropriate method for the determination of immobilised aprotinin. Moreover, it has been demonstrated that the performed purification step via dialysis is efficient in order to remove low molecular mass educts and unbound aprotinin (Werle et al., 2007).

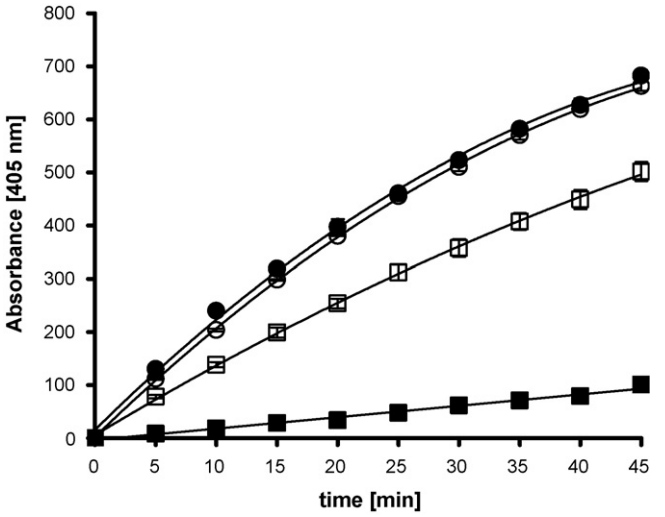

Fig. 1. Increase of nitroaniline mediated absorption in the presence of NBANA, Trypsin and: (●) buffer, (○) 0.1% Chitosan, (■-) 0.1% Chito–Aprotinin, (□) 0.05%; each point represents the mean of at least three experiments ±S.D.

3.2. Trypsin inhibition studies

Results of the Trypsin inhibition studies are shown in Fig. 1. A solution of 0.1% chitosan did not show any Trypsin inhibitory properties in comparison to buffer solution, whereas both concentrations of chitosan–aprotinin, 0.1% and 0.05%, significantly inhibited Trypsin during the 45 min observation period. These results are in good correlation with data gained in studies using a higher molecular mass chitosan–aprotinin conjugate (Werle et al., 2007). It has already been demonstrated that the immobilisation of aprotinin on chitosan does not decrease the ability of the conjugate to inhibit both, Trypsin and Chymotrypsin (Werle et al., 2007).

3.3. Characterisation of liposomes

Anionic MLV serving as core liposomes have been prepared as described previously (Takeuchi et al., 1996). The zeta potential of the core MLV switched after mixing with chitosan as well as chitosan–aprotinin from a negative value to a positive value (see Table 2). The size was in the range of 3–4.5 μm, which has also been reported previously for anionic MLV and chitosan coated MLV. Drug loading experiments revealed that at least 75% of the drug is associated with non-coated as well as coated MLV.

3.4. Mucoadhesion studies

Comparing the images of confocal laser scanning microscopy studies, similar distribution patterns of coumarin mediated fluorescence were found after intragastric administration of coumarin labelled chitosan coated MLV and chitosan–aprotinin coated MLV (see Fig. 2). Fluorescence was in both cases observed on the mucosal side of all three intestinal segments, duodenum, jejunum and ileum, whereas no fluorescence was observed as expected in control rats which did not receive any formulation at all. It has already been

Table 2
Size, zeta potential and calcitonin drug-loading capacity of non-coated and coated MLV; values represent the mean ± S.D. of at least three experiments.

Liposomes	Size [μm]	Zeta potential [mV]	Drug-loading [%]
Non-coated	3.12	−93.0 ± 4.7	>75%
Chitosan	3.16	+40.8 ± 1.8	>75%
Chito–Aprotinin	4.46	+39.9 ± 1.6	>75%

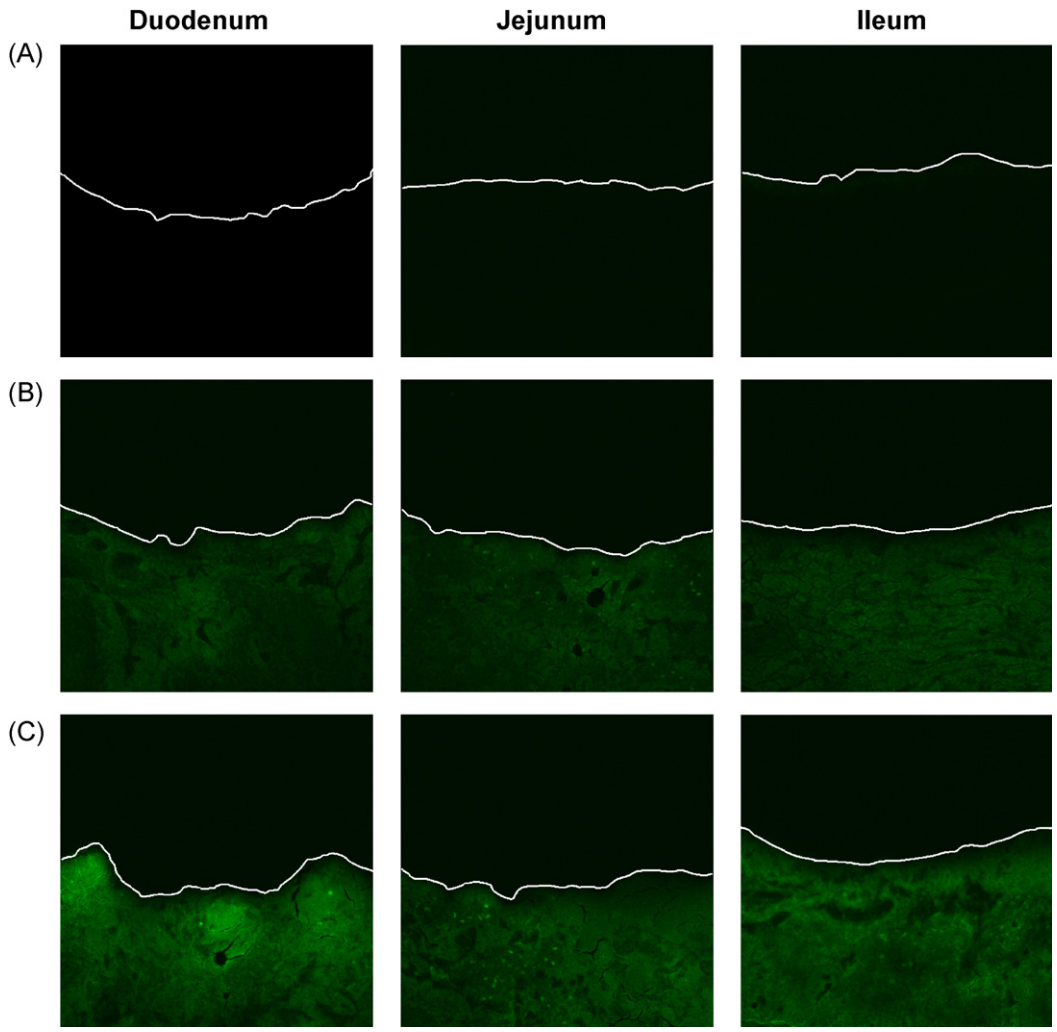

Fig. 2. Confocal laser scanning micrographs of different segments of rat intestinal mucosa 1 h after peroral administration of coumarin loaded liposomes; (A) control without formulation, (B) chitosan coated MLV, (C) chitosan–aprotinin coated MLV; the white line divides the luminal side (upper part) from the mucosal side (lower part).

demonstrated in previous studies that significantly higher amounts of fluorescence labelled chitosan coated liposomes in comparison to fluorescence labelled uncoated liposomes can be found in the intestine 1 h after intragastric administration in rats (Takeuchi et al., 2005a,b).

3.5. *In vivo studies*

A direct comparison of the time dependent blood calcium level over a 24 h period after intragastric administration of calcitonin loaded chitosan coated MLV and chitosan–aprotinin coated MLV showed that the blood calcium level significantly differed at three time points. A significant decrease of the blood calcium level after oral administration of chitosan–aprotinin coated MLV was observed after 30 min, as well as after 12 and 24 h compared with calcium levels gained after oral administration of chitosan coated MLV. The results of this study are provided in Fig. 3. The pharmacokinetic parameters after oral administration of a calcitonin solution and the chitosan and chitosan–aprotinin coated MLV are summarised in Table 3. The most pronounced pharmacological effect in the case of the calcitonin solution and chitosan–aprotinin coated MLV was observed after 0.5 h, whereas it was observed after 4 h in the case of chitosan coated MLV. Notably, no significant differences between chitosan coated MLV and chitosan–aprotinin coated MLV was observed between 1 and 8 h after calcitonin administration.

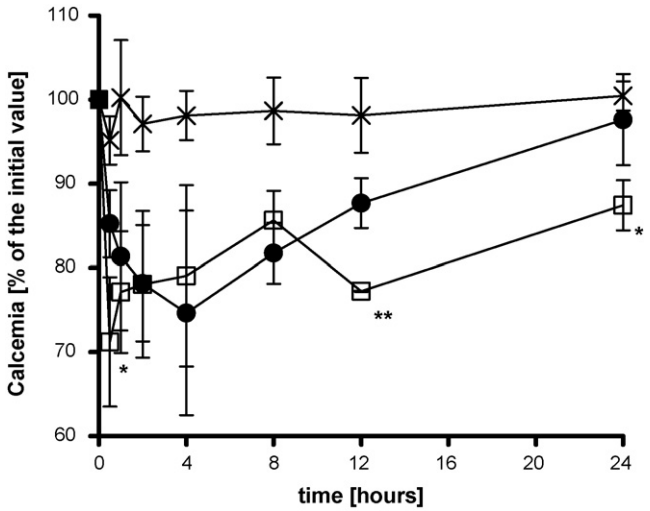

Fig. 3. Blood calcium profiles after intragastric administration of calcitonin solution (-x-), calcitonin loaded chitosan coated MLV (-●-) and chitosan–aprotinin coated MLV (-□-); administered dose: 40 μg calcitonin per rat; chitosan–aprotinin coated MLV differ $^{*}p < 0.05$, $^{**}p < 0.005$ from chitosan coated MLV; each point represents the mean ± S.D. of at least three experiments.

Table 3
Pharmacokinetic parameters after oral administration of various multilamellar vesicles containing calcitonin.

Formulation	T_{min} [h]	C_{min} [%]	AAC_{0-24} [%]	AAC improvement factor
Solution	0.5	95 ± 3	30 ± 32	1
Chitosan coated MLV	4	75 ± 12*	316 ± 44*	11
Chitosan–aprotinin coated MLV	0.5	71 ± 8*	442 ± 86*	15

* Differs $p < 0.05$ from solution; values represent the mean ± S.D. of at least three experiments.

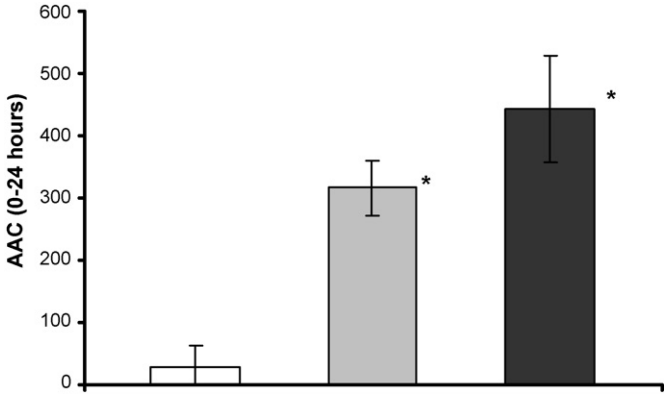

Fig. 4. Area above the blood calcium concentrations–time curves (AAC) after intragastric administration of calcitonin solution (white bar), chitosan coated MLV (light grey bar) and chitosan–aprotinin coated MLV; (* differs $p < 0.005$ from solution); each point represents the mean ± S.D. of at least three experiments.

Moreover, as shown in Fig. 3, the calcium level returned to the initial value after 24 h in the case of chitosan coated MLV, whereas in the case of chitosan–aprotinin coated MLV the calcium level was still decreased after 24 h. Comparing the area above the blood calcium concentration–time curve (AAC) of the calcitonin solution with the AAC of the coated liposomes, it can be seen that that both formulations were capable of significantly increasing the AAC (Fig. 4). Chitosan coated MLV increased the AAC about 11-fold in comparison to the calcitonin solution, and administration of chitosan–aprotinin coated MLV led to an even 15-fold increase. However, the AAC of chitosan coated MLV was not significantly different from that of chitosan–aprotinin coated MLV.

4. Discussion

Aprotinin is a polypeptide inhibitor of Trypsin and Chymotrypsin. These two proteolytic enzymes can be regarded as the two intestinal enzymes which are mainly responsible for the enzymatic degradation of the two therapeutically relevant protein drugs calcitonin and insulin (Bai et al., 1996; Werle et al., 2006). Recently, the synthesis and characterisation of a chitosan–aprotinin conjugate has been described (Werle et al., 2007). In that case, a chitosan with an average molecular mass of 450 kDa was used, whereas in the current study a chitosan with an average molecular mass of 150 kDa was used. It was assumed that using a low molecular mass chitosan would lead to the formation of more uniform polymer–liposome complexes. However, it was not the aim of the study to investigate such phenomena in detail. The amount of immobilised aprotinin on the polymeric backbone in the current study was higher than that of the previously synthesised conjugate (5% versus 1–2% (m/m)), using the same synthetic pathway and exactly the same conditions. Besides using a different molecular mass, also the supplier of chitosan was different in both cases, so that also differences in the purity of the used chitosans might be responsible for the different yields. As demonstrated in the Trypsin-inhibitory studies, the novel chitosan–aprotinin was capable of inhibiting Trypsin even in low concentrations, whereas unmodified chitosan did not exhibit any inhibitory activity at all (Fig. 1). In the previous study it was demonstrated, that Chymotrypsin can be inhibited too by chitosan–aprotinin. Moreover, it was demonstrated that the purification method was suitable to remove unbound chitosan and other low molecular mass educts (Werle et al., 2007).

After demonstrating the inhibitory effect of the novel polymer in vitro, liposomal polyelectrolyte complexes between negatively charged liposomes and the cationic polymers were formed. Size and changes in zeta potential of the liposomes coated with chitosan–aprotinin were comparable with that of chitosan coated MLV. Generally, all results gained with chitosan coated MLV in the current study are consistent with previously reported data (Takeuchi et al., 1996). The mucoadhesive effect of chitosan has been found to be at least partly responsible for the superior efficacy of chitosan coated MLV in comparison to non-coated MLV. Therefore, it was of interest to investigate if the immobilisation of aprotinin leads to a loss of the mucoadhesive properties. Recently, the methods for evaluating the mucoadhesive properties of micro- and nanoscaled colloidal delivery systems have been summarised (Takeuchi et al., 2005a,b). However, there is still controversy about the most appropriate method. In the current study, confocal laser scanning microscopy was used. Although this is not a quantitative method, first insightful data can be gained, especially because the described experimental set-up allows the determination after oral administration in vivo. It has been reported previously by using a different method that the immobilisation of BBI on chitosan led to a decrease of the mucoadhesive properties of the corresponding conjugate (Bernkop-Schnürch and Pasta, 1998). BBI has a similar molecular mass (8 kDa) like aprotinin (6.5 kDa) and also the amount of immobilised BBI was comparable to that of aprotinin in the current study. However, it must be taken into consideration that in the current study, the mucoadhesiveness of a colloidal system was evaluated, whereas in the previous study with BBI the mucoadhesiveness of tablets based in this conjugate was determined. The results gained in the current mucoadhesive studies, which suggest that the mucoadhesive properties of chitosan coated MLV and chitosan–aprotinin coated MLV are in the same range, are further backed up by two observations. First, the pharmacological effect of chitosan–aprotinin coated MLV was even prolonged in comparison to chitosan coated MLV and of course to the calcitonin solution. Second, no difference in the zeta potential of chitosan–aprotinin and chitosan coated MLV was observed, suggesting that after immobilisation of aprotinin, still a comparable amount of amino groups, which are at least partly responsible for the mucoadhesive properties of chitosan, occurs in the two polymers. In conclusion, data gained within the current study support the hypothesis that the mucoadhesive properties of chitosan–aprotinin coated MLV is not markedly different from that of chitosan coated MLV.

Finally, the novel delivery system was evaluated in vivo. Calcitonin was chosen as model peptide drug and was intragastrically administered to rats. It has been previously demonstrated in biofeedback studies using calcitonin as well as insulin, that chitosan coated MLV are more effective in vivo than non-coated MLV (Takeuchi et al., 2003, 1996). This superior effect has been mainly attributed to the prolonged residence time of the mucoadhesive liposomes in the intestine, but also effects such as protection of the peptide drug from enzymatic attack by incorporation into the liposomes have been discussed. Within the current study, the blood calcium level in the chitosan–aprotinin MLV group 30 min after drug administration was significantly lower than that of the chitosan MLV group. In addition, such a significant effect was observed

after 12 and 24 h (Fig. 3). This effect was most likely mediated by the protease inhibitory activity of chitosan–aprotinin, as drug-loading and mucoadhesive properties of the two formulations did not markedly differ. The reason, why the effect of chitosan–aprotinin MLV was most pronounced in the beginning as well as at the end of the absorption experiment might be that at these time points low concentrations of the formulation are located in the intestine. Therefore, the molar ratio of calcitonin/proteases is lower than at other time points, and enzymatic degradation is believed to be more pronounced. Consequently, protease inhibition will lead to significantly higher calcitonin plasma levels at these time points. Between around 1 and 8 h, other strategies than protease inhibition such as permeation enhancement might be more effective in order to increase the plasma level of orally administered peptide drugs. Comparing the AAC of the coated MLV with that of a calcitonin solution, the AAC of chitosan MLV was significantly increased 11-fold and 15-fold in the case of chitosan–aprotinin MLV during a 24-h period (Table 3, Fig. 4). The AAC of chitosan–aprotinin coated MLV was increased around 1.4-fold in comparison to the AAC of chitosan coated MLV, however, this effect was not significant ($p > 0.05$). However, the AAC was calculated for the duration of the observation period (24 h). After 24 h, the blood calcium level in the chitosan MLV groups was found to be in the same range as the initial measured blood calcium level, whereas in the case of chitosan–aprotinin coated MLV the blood calcium level was still decreased. In summary, results gained in vivo studies suggest that the efficacy of chitosan coated MLV can be further improved by a covalent attachment of specific protease inhibitors such as aprotinin to the polymer.

In order to develop liposomal polymer-protease inhibitor conjugate complexes with further enhanced efficacy, some critical points should be taken into consideration. The possibility that encapsulation of calcitonin into the liposomes leads to a protection of a certain amount of the peptide from enzymatic degradation has already been discussed and seems likely (Thongborisute et al., 2006). Therefore, it would be generally of interest to investigate if an increased share of the inhibitor on chitosan would lead to an even higher increased effect in vivo. An optimised liposomal chitosan–aprotinin delivery system which displays a maximum amount of covalently bound aprotinin without exhibiting decreased mucoadhesive properties might lead to a more pronounced effect in vivo. Another critical parameter in the described delivery system is the polymer chitosan itself. There are numerous studies that clearly demonstrate the potential of chitosan for oral drug delivery (Aungst, 2000; Bernkop-Schnürch, 2000; Luessen et al., 1996). It has been used for the successful delivery of not only peptides and proteins, but also for the delivery of other hydrophilic macromolecules such as siRNA or pDNA (Tahara et al., 2007; Zheng et al., 2007). Nevertheless, a constant point of criticism is the pH dependent solubility of chitosan. The solubility depends on various factors, such as degree of deacetylation, molecular mass and source of chitosan, but as a general rule it can be stated that commercially available chitosan is not soluble above a pH of 6.5. As this can be regarded as a limiting factor for its use in oral drug delivery in general, this solubility behaviour might limit the efficacy of chitosan-protease inhibitor conjugates in particular. The optimum pH range of Trypsin and Chymotrypsin depends on the enzyme source, but in most mammals it is between 7 and 9. Therefore, it is rather unlikely that a chitosan-protease inhibitor conjugate is capable of inhibiting proteolytic enzymes in intestinal segments with higher pH, and consequently, at intestinal segments with the highest enzyme activities. However, especially the observed significant decrease of the blood calcium level in the chitosan–aprotinin group after 30 min seems to indicate that inhibition of proteolytic in the upper part of the small intestine leads to improved drug uptake. Taking this into consideration, the pharmacological effect might be much more pronounced, if the proteolytic enzymes can be inhibited at higher pH. To achieve this, polymer-protease inhibitor conjugates based on poly(acrylates) instead of chitosan might be a promising alternative.

5. Conclusion

Within the current study, two promising approaches for the oral delivery of peptide drugs were combined. The successful formation of polyelectrolyte complexes between anionic liposomes and the cationic polymer-protease inhibitor conjugate chitosan–aprotinin was demonstrated, and the properties of the novel delivery system were described. Chitosan–aprotinin was capable of inhibiting Trypsin in vitro, and the mucoadhesive properties of chitosan–aprotinin coated MLV did not differ from that of chitosan coated MLV. A different and more pronounced effect of calcitonin loaded chitosan–aprotinin MLV in comparison to chitosan coated MLV was observed. Data gained in the current study indicate the potential of liposomal chitosan–aprotinin as an oral delivery system for peptide drugs and are believed to contribute to the development of novel polymer-protease inhibitor based delivery systems.

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