Scientia Pharmaceutica www.scipharm.at

Research article Open Access

Liposomal Polyion-Complexes based on Poly(allylamine) for Oral Peptide Delivery: Basic Investigations

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Sci Pharm. 2008; 76: 751–760 doi:10.3797/scipharm.0806-04

Published: September 18th 2008 Received: June 5th 2008
Accepted: September 15th 2008

This article is available from: http://dx.doi.org/10.3797/scipharm.0806-04

Abstract

The efficacy of polyion-complexes between charged liposomes and oppositely charged polymers for the oral delivery of therapeutic peptides and proteins has been reported previously. It was the aim of the current study, to investigate whether polyion-complexes between negatively charged liposomes and the cationic polymer poly(allylamine) (PALAM) can be formed. Furthermore, the protease inhibitory effect of PALAM, the drug-loading capacity of liposomal PALAM polyion-complexes as well as the toxicity of PALAM and liposomal PALAM polyion-complexes was evaluated. After mixing the negatively charged liposomes with a PALAM solution, the zeta-potential switched from a negative to a positive value, indicating the formation of the polyion-complexes. PALAM was capable of significantly inhibiting Trypsin, although this effect was not very pronounced. Drug-loading capacity using the hydrophilic marker fluorescein isothiocyanate-dextran (FD4) was determined to be 8.0 ± 1.5 %. Cytotoxicity tests using Caco-2 cells and MTS assay revealed that both, PALAM in solution as well as liposomal polyion-complexes displayed cell toxicity. Data gained within this study is believed to contribute to the development of PALAM based colloidal drug delivery systems.

Keywords

Liposomes • Poly(allylamine) • Oral Peptide Delivery

Introduction

The successful oral administration of therapeutic peptides and proteins remains one of the main challenges for pharmaceutical technologists. After oral peptide/protein administration, only a small amount of the drug is absorbed and reaches systemic circulation. The low oral bioavailability of these therapeutics can be mainly attributed to enzymatic digestion during gastro-intestinal (GI) passage [1] as well as to low membrane permeation owing to the hydrophilic character of this substance class [2]. Various approaches to overcome these barriers have been described in the literature, including the co-administration of protease inhibitors [3], the utilization of multifunctional polymers [4] and micro-/nanoparticulate drug delivery systems [5]. Among the colloidal delivery systems for orally administered drugs, the use of polyion complexes between charged liposomes and oppositely charged polymers has been determined to be highly effective. Used polymers include the cationic polymer chitosan and the anionic poly(acrylate) carbopol [6, 7]. Their efficacy for oral peptide delivery has been demonstrated in biofeedback studies in rats, by administering insulin as well as calcitonin. Chitosan is to our knowledge so far the only cationic polymer used in such liposomal polyion complexes. The positive charge of chitosan leads to mucoadhesive properties, which consequently lead to prolonged residence times in the intestinal mucosa and to improved absorption. Another interesting polymer displaying free amino-groups besides chitosan is poly(allylamine) (PALAM). The substructure of PALAM is depicted in Figure 1.

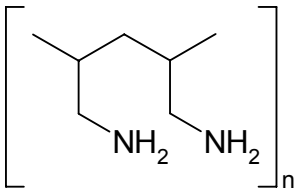

Fig. 1. Substructure of the polymer poly(allylamine)

It was therefore of particular interest, to investigate whether cationic liposomal polyion-complexes based on PALAM can be prepared, and to evaluate the basic features of this proposed novel delivery system. Anionic liposomes were prepared and mixed with a cationic PALAM solution. The size and zeta potential of the prepared liposomes was determined before and after the coating procedure. For drug loading studies, the hydrophilic marker FD4 was used. In addition, the potential protease inhibitory effect of PALAM and the cytotoxicity of PALAM solution and liposomal polyion-complexes based on PALAM were evaluated.

Results and Discussion

Preparation and characterisation of liposomal PALAM polyion-complexes

In order to prepare the liposomal PALAM polyion-complexes, anionic liposomes were prepared in a first step. These anionic liposomes served as “core liposomes” and were mixed with a PALAM solution to form the polyion-complexes. The initially prepared anionic liposomes displayed a size of around 13 µm and a zeta potential of around −70 mV

(Table 1). After mixing with the PALAM solution, the size of the mulitlamellar vesicles (MLV) was determined to be around 18 µm and the zeta potential switched from the negative value to a positive value of around +70 mV. A switch of the zeta potential generally indicates the formation of polyion-complexes. In previous studies with cationic liposomes and carbopol, the formation of a polyio-complex was described as a “coating” of the cationic liposomes with the anionic polymer, leading to a shift of the cationic zeta potential to a negative value [7].

In addition, the initially prepared anionic MLV were sonicated for three minutes to gain submicron-sized liposomes (ssLip). Size and zeta potential of the prepared ssLip are provided in Table 1. However, after mixing with the PALAM solution, aggregation was observed. Also the polydispersity index increased to 1, indicating the formation of aggregates with a broad size distribution. Due to this aggregation, no ssLip PALAM polyion-complexes could be prepared. Similar aggregation effects can occur after mixing anionic liposomes with chitosan solutions.

Tab. 1. Size and zeta potential of core liposomes and PALAM-liposome polyion-complexes

Liposomes	Size	PI	Zeta potential [mV]
MLV uncoated	13.06 µm	---	− 71.2 ± 16.6
MLV PALAM	17.60 µm	---	+ 69.4 ± 0.9
sslip uncoated	111 nm	0.159	− 44.5 ± 2.7
ssLip PALAM	5953 nm	1.00	+ 39.1 ± 0.6

FD4 drug-loading studies

It has been previously reported, that polymer coating of liposomes can increase the drug-loading capacity of certain drugs [8]. This effect has been described for cationic liposomes which were coated with poly(acrylic acid) using the model drug FD4. However, no increased drug-loading was observed when using the low molecular mass hydrophilic compound carboxy-fluorescein. Therefore, the increased drug-loading was attributed to interactions between poly(acrylic acid) and FD4. Taking these results into account, also an increased drug-loading of liposomal polyion-complexes based on PALAM might be anticipated. However, in the current study the FD4 drug-loading was only 8.0 ± 1.5 % and did not significantly differ from the drug-loading capacity of anionic core MLV. As the viscosity of PALAM solutions seemed not to increase even when using 2 % PALAM, it might be assumed that a high viscosity of certain polymers such as chitosan or poly(acrylates) contributes to the increased drug-loading of liposome-polymer polyion-complexes.

Trypsin inhibition studies

Certain polymers which are used as pharmaceutical excipients are known to be capable of inhibiting proteolytic enzymes. Such protease inhibitory properties have for example been reported for anionic polymers including poly(acrylates) and derivatives thereof [9, 10]. This effect was explained by interactions between divalent cations which are essential co-factors for proteolytic enzymes and the anionic groups of the polymers. No data regarding

a possible protease inhibitory effect of PALAM has been published so far. Therefore, it was of interest to investigate whether such an inhibitory effect does occur or not. When using a final concentration of 0.5 % PALAM, the absorption caused by nitroaniline, which is cleaved by Trypsin from the Trypsin substrate *N*α-Benzoyl-DL-arginine 4-nitroanilide (NBANA), was significantly decreased during a 5 minute observation period. Results of this study are shown in Figure 2. These results suggest that high concentrations of PALAM are capable of inhibiting Trypsin. However, it remains questionable if such a minor effect caused by relatively high polymer concentrations will lead to improved peptide/protein absorption in vivo.

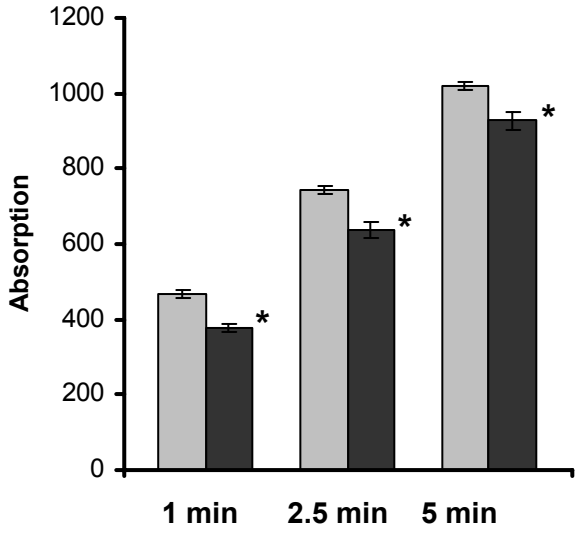

Fig. 2. Time dependent increase of nitroaniline mediated absorption; control (light grey bars), 0.5 % PALAM (dark grey bars, final concentration); * differs $p < 0.05$ from control; each value represents the mean of at least three experiments ± S.D.

Cytotoxicity studies

Novel delivery systems must of course exhibit a favourable safety profile. This is of particular importance for peptide delivery systems, as many therapeutic peptides are used in the treatment of chronic diseases which require frequent drug administration. In the current study, Caco-2 cells were used and the viability of the cells after incubation with PALAM solution and liposomal polyion-complexes based on PALAM was assessed by using the MTS assay. Mitochondrial dehydrogenases of living cells can cleave the yellow formazan from MTS, which can be detected using photometric methods. In the current studies, two different concentrations (0.015 & and 0.03 %) of PALAM in solution and PALAM-liposome polyion-complexes were incubated with Caco-2 cells. A 0.1 % solution of sodium dodecyl sulphate (SDS) served as positive control and HEPES buffer pH 7.4 served as negative control. The results of the cytotoxicity studies are shown in Figure 3. All evaluated PALAM formulations displayed similar toxicity as the positive control. No significant difference between the toxicity of PALAM-liposome polyion-complexes and PALAM in solution could be observed. Toxicity was furthermore not markedly decreased when using lower concentrations (0.015 % instead of 0.03 %).

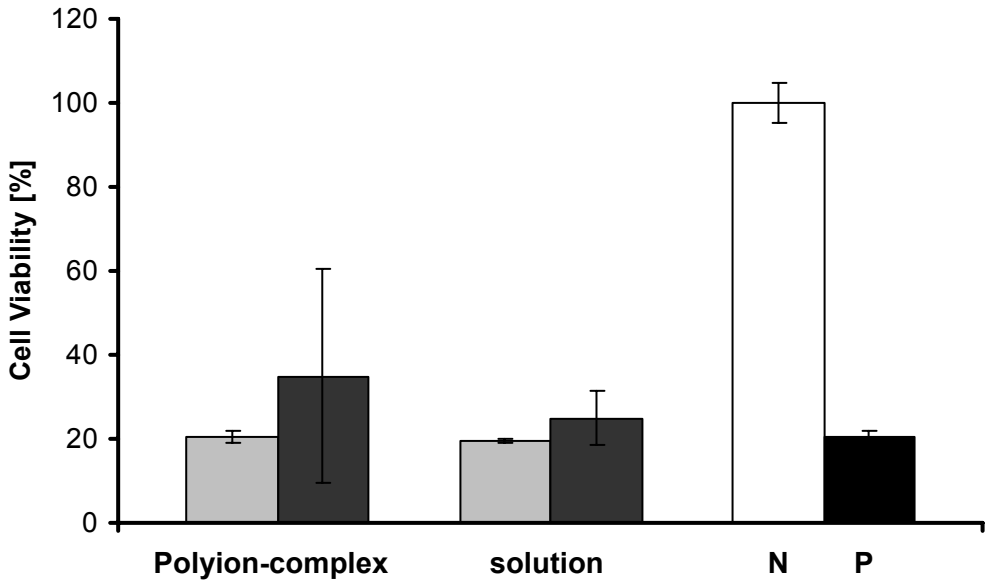

Fig. 3. Cell viability after 2 hours of incubation with various samples; light grey bars: final PALAM concentration = 0.03 %, dark grey bars: final PALAM concentration = 0.015 %; white bar: HEPES pH 7.4; black bar: positive control (0.1% SDS); each value represents the mean of at least eight experiments ± S.D.

Within the current study it was demonstrated that liposomal polyion-complexes based on PALAM can be prepared. The preparation of micron-sized polyion complexes can be performed with state of the art methods. Although aggregation was observed when mixing submicron-sized core liposomes with PALAM solution, this does not necessarily mean that it is not possible to prepare submicron-sized liposomal PALAM polyion-complexes. Similar problems have for example been overcome by sonicating aggregates of chitosan coated liposomes. The observed drug-loading of FD4 into the novel PALAM based polyion-complexes was in the range of similar uncoated liposomes. As it is unclear whether the increased drug-loading capacity of e.g. poly(acrylate) coated liposomes contributes to the increased absorption of peptide drugs in vivo, the observed drug-loading of the novel polyion-complex can be regarded sufficient enough for further studies. Although a significant but minor Trypsin inhibition effect of PALAM was observed, it is rather unlikely that this effect will lead to an increased oral bioavailability in vivo.

Comparing the structure of PALAM with the structure of chitosan, which is widely used in drug delivery formulations, a higher number of amino groups per gram polymer can be found in PALAM. The number of amino groups of the polymer plays a pivotal role for oral drug delivery. One main feature of e.g. chitosan based drug delivery systems is their mucoadhesiveness. It is commonly accepted, that the mucoadhesive properties of chitosan are due to ionic interactions between positively charged amino groups of chitosan an negatively charged groups of the Mucin, in particular of sialic acid. Recently, polymers such as pullulan and gelatine have been chemically modified in order to display free amino groups on their surface and these novel polymers mediated increased pulmonal absorption of insulin [11]. The absorption enhancing properties of these polymers were

found to increase when using polymers with increasing numbers of amino groups. Taking the importance of the number of amino groups as well as the high number of amino groups per gram polymer of PALAM into account, PALAM can be regarded as a potentially promising polymer for mucosal drug delivery. However, as described within the current study, PALAM unfortunately displays cell toxicity. Another research groups reported that the toxicity of PALAM was strongly reduced when using PALAM-DNA complexes [12]. In the case of the investigated liposomal polyion-complexes based on PALAM, however, no reduction of the cytotoxicity in comparison with PALAM solution of the same concentrations could be observed (Fig. 3). Generally spoken, the toxicity of cationic polymers which display free amino groups can be reduced by chemically modifying a certain amount of amino groups, e.g. via glycolylation [13]. Another effective method to reduce the toxicity of PALAM is crosslinking. Sevelamer is a FDA approved, crosslinked PALAM marketed by Genzyme as a phosphate binding drug used to prevent hyperphosphataemia in patients with chronic renal failure. According to the results of the current study, the cytotoxic properties of liposome-PALAM polyion-complexes constitute the main hurdle for the use as oral drug delivery system. Nevertheless, it must be taken into consideration that Caco-2 cells are much more susceptible to cell damage than the GI epithelia, which is protected by a mucus gel layer. Therefore, chemical modifications of PALAM might be performed in order to improve the safety profile of PALAM based delivery systems.

In conclusion, the feasibility of the preparation of a novel peptide/protein delivery system for an intended use in oral drug delivery has been described. Based on the data of the current study, the main limitation seems to be the adverse toxicity profile of the prepared liposome-PALAM polyion-complexes, which is due to the toxicity of PALAM itself. Data gained within this study are believed to contribute to the development of novel PALAM based colloidal drug delivery systems.

Experimental

Materials

L-α-distearolyphosphatidylcholine (DSPC, Nippon Oil and Fats Co.), cholesterol (Chol, Sigma), dicetyl phosphate (DCP, Sigma), poly(allylamine hydrochloride) 70 kDa (PALAM, Aldrich), sodium dodecyl sulphate (SDS, Nacalai tesque), fluorescein isothiocyanate-dextran 4 kDa (FD4, Sigma), Trypsin (Sigma) and *N*α-Benzoyl-DL-arginine 4-nitroanilide (NBANA, Sigma) were used as received. Fetal bovine serum (FBS), DMEM F/12, Hank's balanced salt solution (HBSS) and streptomycin (100 IU/ml) and penicillin (100 IU/ml) were purchased from GIBCO. All used reagents were of analytical grade.

Preparation of liposomes and liposomal PALAM polyion complexes

Preparation of anionic core liposomes coated with chitosan has been described previously [6]. Anionic multilamellar vesicles (MLV) consisting of DSPC, DPC and Chol (molar ratio: 8:2:1) were prepared using the thin film method. DSPC, DPC and Chol were dissolved in chloroform and a thin film lipid layer was obtained by evaporating the organic solvent for 3 hours at 40°C and water jet vacuum. Afterwards, the obtained thin film was dried in a vacuum oven overnight to ensure complete removal of chloroform. Hydration was performed with HEPES buffer pH 7.4 by repeated gentle heating and vortexing. Finally, the liposome suspension was incubated for 30 min at 10°C. Sub-micron sized liposomes

(ssLip) were prepared by sonicating the MLV suspension for three minutes (Sonifier 250, Branson).

Formation of liposomal PALAM polyion complexes was performed by mixing an aliquot of the described liposomal suspensions with a 0.6 % PALAM solution in HEPES pH 7.4 and vortexing. The liposomal PALAM polyion complexes were incubated for 30 min at 10°C. The size of MLV was determined using a LDSA 2400A particle size analyzer (Tohnichi Computer Co. Ltd, Japan) and the size of ssLip was determined by dynamic light scattering (Zetasizer 3000 HSa, Malvern). Zeta potential of all liposomes was measured using a Zetasizer from Malvern.

FD4 drug-loading studies

An anionic thin film lipid layer was prepared as described above. After removal of the organic solvent, the lipid layer was hydrated with a 1 mg/mL solution of FD4 in HEPES buffer pH 7.4. PALAM polyion complexes (MLV) were prepared according to the method described above. Samples were centrifuged for 30 min at 20,000 rpm and 4°C and the fluorescence of the supernatant was measured using a Fluostar, Galaxy (ex. = 485 nm, em. = 538 nm). A calibration curve was prepared with the initial FD4 solution and the results were expressed as percentage of the initial drug-amount.

Trypsin inhibition studies

A Trypsin solution (0.2 mg/mL Trypsin from Sigma in HEPES pH 7.4) and a solution of NBANA (0.5 mg/mL in H_2O; filtered through 0.45 µm filter) was prepared. To 50 µl of a 2% PALAM solution (in HEPES buffer pH 7.4; pH was adjusted to 7.4), 50 µl of the Trypsin solution were added. Finally, 100 µl of the NBANA solution were added and the mixture was incubated at RT. The absorption of the samples was analysed using a spectrophotometer.

Cytotoxicity studies

A human colon-carcinoma cell line (Caco-2) was cultured under standard conditions (5% CO_2, 95% humidified air, 37 °C) in DMEM supplemented with FBS (10%), NEAA (1 %), L-glutamine (1 %) and streptomycin (100 IU/ml) and penicillin (100 IU/ml).

Cytotoxicity of PALAM solutions and PALAM liposomal polyion-complexes was determined by using MTS assay. The cells were seeded at 3.15×10^4 cells/cm^2 cells onto 96-well plates (Becton Dickinson). Cells were cultured for 4 days and the culture medium was changed on alternative days (total 7 days). The culture medium was removed and washed twice with 200 µl of HBSS. The cells were then exposed to 100 µl of samples. After 120 min of incubation, the cells were washed three times with 150 µl of HBSS. The cells were incubated with 20 µl of CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Madison, MI, USA) composed of 317 µg/ml MTS in 100 µl of culture medium. After incubation in a CO_2 incubator for 2 hours, absorbance values were measured with a microplate reader (Corona Electric, MTP 120, Tokyo, Japan) at a wavelength of 492 nm. Background absorbance in cell-free wells was measured and subtracted from the measurement absorbance. Reference wavelength was 660 nm. A solution of 0.1 % of SDS in Hepes in HBSS served as a positive control. The percentage of cell viability was expressed as the percentage calculated by the following equation:

$$\% \text{ Cell viability} = (ABS_{samples} / ABS_{control}) \times 100 \quad (2)$$

where $ABS_{samples}$ was the absorbance values of those wells exposed to the liposomal suspensions, and $ABS_{control}$ was the absorbance values of those wells treated with 10 mM Hepes in HBSS.

Acknowledgement

This study was supported by grant Nr. J2652 (Erwin-Schrödinger) from the Fonds zur Förderung der wissenschaftlichen Forschung (FWF) to Martin Werle.

Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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Liposomale Polyionen-Komplexe basierend auf Poly(allylamin) für die orale Verabreichung von Peptiden: Grundlegende Studien

Zusammenfassung

Das Potential von Polyionen-Komplexen zwischen geladenen Liposomen und entgegengesetzt geladenen Polymeren für die orale Verabreichung von therapeutischen Peptiden und Proteinen wurde in vorangegangen Studien bewiesen. Das Ziel der vorliegenden Arbeit war es zu untersuchen, ob Polyionen-Komplexe zwischen negativ geladenen Liposomen und dem kationischen Polymer Poly(allylamin) (PALAM) hergestellt werden können. Darüber hinaus wurde der Protease-Hemmeffekt von PALAM, die Wirkstoffbeladungs-Kapazität von liposomalen PALAM Polyionen-Komplexen so wie die Toxizität von PALAM und von liposomalen PALAM Polyionen-Komplexen untersucht. Nach dem Mischen der negativen Liposomen mit einer PALAM-Lösung, wechselte das negative Zeta-Potential zu einem positiven Wert, was auf eine Formation von Polyionen-Komplexen schließen lässt. PALAM zeigte einen geringen aber signifikanten Hemmeffekt gegenüber Trypsin. Die Wirkstoffbeladungs-Kapazität mit der hydrophilen Substanz Fluoreszein-Isothiocyanat-Dextran (FD4) betrug 8.0 ± 1.5 %. Zytotoxizitäts-Studien mit Caco-2 Zelllinien wurden mittels MTS-Assay ausgewertet und zeigten dass sowohl PALAM in Lösung, als auch die liposomalen Polyionen-Komplexe toxische Eigenschaften aufwiesen. Die Daten der vorliegenden Studie sollen zur Entwicklung von auf PALAM basierenden, kolloidalen Wirkstoff-Abgabesystemen beitragen.