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Poly(amino acid)s: Promising enzymatically degradable stealth coatings for liposomes

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Abstract

Poly(amino acid)s (PAAs) were evaluated as coating polymers for long-circulating liposomes. The pharmacokinetics of PAA-coated liposomes were assessed in rats. Prolonged circulation times were obtained, comparable to those reported for poly(ethylene glycol) (PEG)-liposomes. Besides, the enzymatic degradability of PAAs was studied. PAAs – in free as well as liposome-associated form – are degradable by proteases, which is beneficial for reducing the risks of accumulation in vivo. Furthermore, complement activation by PAA-liposomes was evaluated in vitro and in vivo. Like other liposome types, they appear to activate the complement system. However, a role of endotoxin contamination of the PAA-liposome formulations used cannot be excluded in our complement activation studies.

Keywords: Long-circulating liposomes; Pharmacokinetics; Poly(amino acid)-coatings; Enzymatic degradability; Complement activation

Long-circulating, sterically stabilized liposomes (often also referred to as 'stealth' liposomes) are frequently used as pharmaceutical carriers (Torchilin, 2005). Their delivery concept makes use of their passive targeting properties to tumors and sites of inflammation, where they can extravasate due to the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). A steric stabilization layer of a hydrophilic polymer on the liposome surface such as the frequently used poly(ethylene glycol) (PEG), is responsible for a reduced uptake by cells of the reticulo-endothelial system which in turn results in prolonged circulation times as compared to non-coated liposomes. There are, however, some limitations associated with the use of polymers as liposome coatings. These polymers are generally not degradable by mammalian enzymes and upon uptake of the coated liposomes by cells, the polymer may accumulate and cause cell function impairment on the long term (Moghimi and Szebeni, 2003). Polymer coatings may also hinder drug release and target cell interaction after liposome localization in the target region therefore leading to a reduced therapeutic efficacy (Boomer et al., 2003). Another point of concern, which does not only apply to coated liposomes but also uncoated, is that liposomes have been reported to activate the complement system in preclinical studies (Szebeni, 1998). In the clinical setting, adverse respiratory, hemodynamic and hematological changes may occur in patients upon complement activation and potentially lead to hypersensitivity reactions (HSR).

We recently designed alternative sterically stabilizing polymers based on poly(amino acid)s (PAAs), in particular poly(hydroxyethyl L-glutamine) (PHEG) and poly(hydroxyethyl L-asparagine) (PHEA), both coupled to a hydrophobic anchor to graft them onto the liposome bilayer (Fig. 1) (Metselaar et al., 2003). This paper summarizes the current status of PAA-coated liposomes regarding circulation kinetics, enzymatic degradability of the PAA-coating and activation of the complement system.

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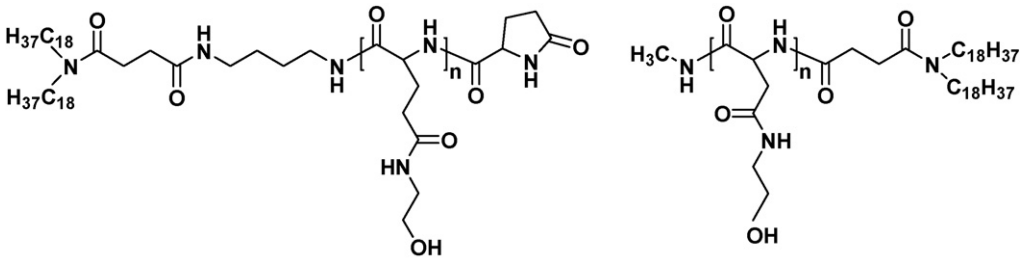

Fig. 1. Structures of the PHEG- and PHEA-lipid conjugates ($n \approx 20$).

1. Circulation kinetics of PAA-liposomes

Prolongation of liposome circulation times is the most important feature for a stealth liposome coating. To assess the stealth properties of PAA-liposomes, a pharmacokinetic study in rats was carried out. DPPC/cholesterol liposomes (2:1) with or without 5 mol% of either a PHEA- (molecular weight 3000 Da) or PHEG- (molecular weight 4000 Da) lipid conjugate and trace amounts of [^{3}H]-cholesteryloleyl ether as a radioactive label were prepared by lipid film-hydration and sized by extrusion to approximately 150 nm. Male Wistar rats (body weight approximately 200 g) received a single dose intravenous (i.v.) injection of liposomes containing 25 μmol/kg total lipid. Blood samples were collected from the tail vein at different time points after injection and analyzed by liquid scintillation counting. The area under the curve ($AUC_{0–48h}$) values and the terminal half-life were calculated from the liposome blood concentrations assuming a rat blood volume of 60 mL/kg (Table 1). Both Fig. 2 and Table 1 show that coating liposomes with 5 mol% PHEG or PHEA results in significantly prolonged circulation times as compared to non-coated liposomes and that circulation time of PHEA-liposomes is somewhat superior over that of PHEG-liposomes. Circulation times of PAA-liposomes were comparable to those observed for PEG-liposomes (Metselaar et al., 2003).

2. Enzymatic degradability of PAAs

PAAs are composed of modified L-α-amino acids and are consequently expected to be degradable by lysosomal proteases, reducing the risk of side effects associated with polymer accumulation in vivo. Besides that, an enzymatically degradable coating polymer can be used to enhance target cell interaction of the liposomes and/or release of the entrapped drug after degradation by extracellular proteases present in pathological tissues such as in tumors and at sites of inflammation (i.e. shedding). Surface properties (e.g. a positive charge) and surface-associated targeting moieties, which are shielded by the polymer coating in the circulation, become exposed upon enzyme-triggered shedding of the coating. This can induce cell binding and internalization and/or promote drug release. The degradability of the PAAs was studied by incubation with the proteases cathepsin B, papain and pronase E (Romberg et al., 2005). Cathepsin B is involved in the lysosomal protein turnover and is extracellularly present in tumors and sites of inflammation. Papain is frequently used as a model enzyme for cathepsin B in degradation studies (Pytela et al., 1998). Pronase E is a natural mixture of exo- and endopeptidases. The PHEG-conjugate was incubated with the enzymes and samples were withdrawn at different time points. When peptide bonds are cleaved due to the action of proteases, primary amine groups are formed. The concentration of amines in the samples was determined spectrophotometrically after incubation with ninhydrin (Prochazkova et al., 1999). From the number of generated amine groups, which corresponds to the number of cleavage sites, the average fragment size was calculated. Degradation of the PHEG-conjugate by papain resulted in small peptide fragments of three to four amino acids. Pronase E and cathepsin B were also capable of degrading PHEG. Besides degradation of the free conjugate, the degradability of PHEG was also determined when it was grafted onto a liposome surface.

Table 1
Pharmacokinetic parameters

	AUC (μmol/mL h)[a]	Terminal half-life (h)[b]
PHEA-liposomes	7.5 ± 0.2	14.4
PHEG-liposomes	6.0 ± 1.1	11.9
Non-coated liposomes	3.1 ± 0.7	9.7

[a] PHEA- versus non-coated liposomes and PHEG- versus non-coated liposomes: $p < 0.01$, PHEA- versus PHEG-liposomes: $p < 0.05$, as determined by a two-tailed Student's t-test assuming equal variances.

[b] Terminal half-life based on data from Fig. 2. Blood concentrations were fitted to a two-phase exponential decay curve by nonlinear regression analysis.

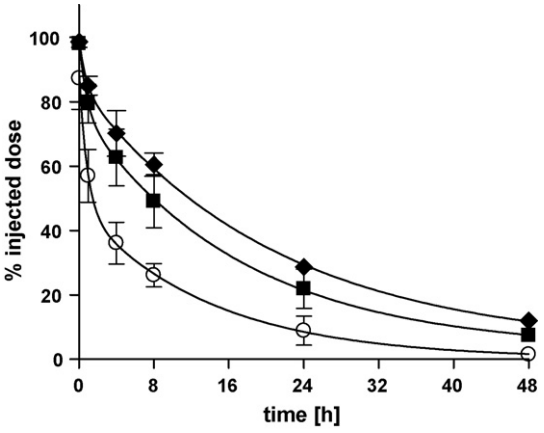

Fig. 2. Circulation kinetics of DPPC/cholesterol liposomes coated with PHEA- (♦), PHEG- (■) and non-coated liposomes (◯). Results are expressed as the mean percentage of the injected dose of four rats ± S.D. Blood concentrations were fitted to a two-phase exponential decay curve by nonlinear regression analysis ($R > 0.999$).

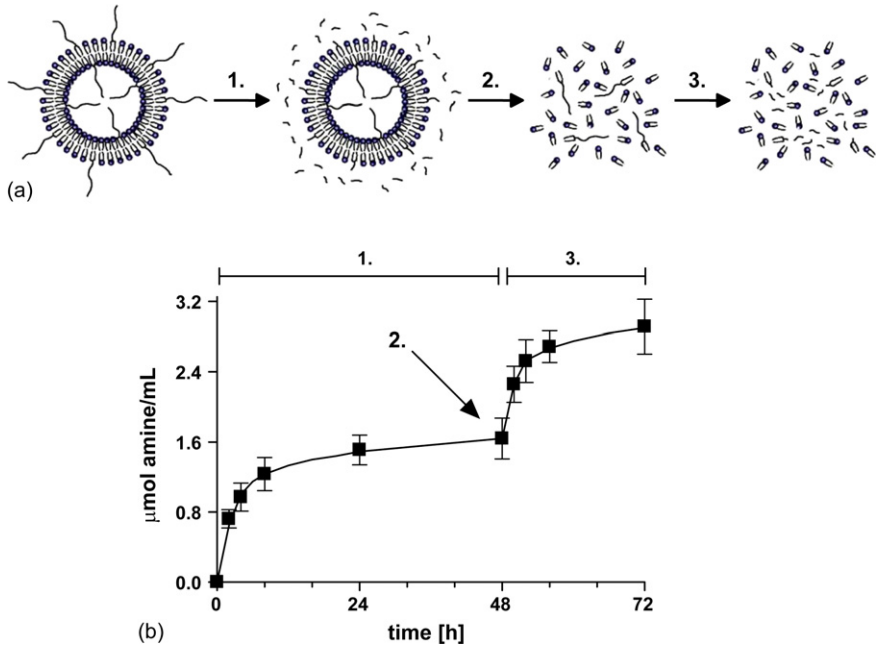

Fig. 3. Degradation of PHEG on a liposome surface. (a) Schematic representation of the degradation and (b) time course of the concentration of amines in the papain-incubated samples of PHEG-coated liposomes as determined by the ninhydrin assay (average ± S.D. of three experiments). (1) Degradation of PHEG on the surface of the liposome, (2) solubilization of liposomes with *n*-octyl glucoside and addition of fresh enzyme and (3) degradation of internal PHEG. Part (b) adapted and reprinted with permission from Romberg et al. Copyright (2005) American Chemical Society.

DPPC/cholesterol liposomes coated with 5 mol% PHEG were prepared by film-hydration and extrusion and subsequently incubated with papain. Samples were withdrawn at different time points and analyzed with the ninhydrin assay. Fig. 3a shows the schematic representation of the enzymatic degradation of liposome-grafted PHEG. Fig. 3b shows the increase in amine concentration in time upon incubation of PHEG-liposomes with papain. After 48 h of incubation a plateau in the number of generated amine groups was reached. When the liposomes were subsequently solubilized with *n*-octyl glucoside and fresh papain was added, the formation of amine groups continued, indicating that now the conjugate grafted onto the inside of the liposomes could also be degraded. Fragment sizes obtained were similar to those observed after PHEG degradation in solution. Initial shedding experiments with PHEG-liposomes demonstrate that the exposure of surface characteristics after PHEG degradation, which can promote interaction with cells and/or enhance the release of an entrapped drug, is feasible (Romberg et al., in preparation). Surprisingly, the PHEA-conjugate could not be degraded by any of the chosen enzymes. Nevertheless, degradation in vivo is still expected in view of a very diverse pool of proteases and their high concentrations.

3. Complement activation by PAA-liposomes

Liposomes have been reported to cause HSR, which occur immediately upon intravenous administration and are reflected in changes in arterial pulmonary and systemic blood pressure and respiration rate, facial edema and chest and back pain (Szebeni et al., 1999). In a porcine model of liposome-induced cardiopulmonary distress, it was demonstrated that the liposome-induced hemodynamic reactions are very similar to the observed human hypersensitivity responses, and likely a consequence of complement activation (Szebeni et al., 1999). To evaluate whether the PAA-coated liposomes activate the complement system, preliminary experiments with PHEA- and PHEG-liposomes in vitro and in the porcine model were carried out. Activation of all three complement pathways (classical, alternative and lectin pathway) in human serum samples can be assessed in vitro with an ELISA assay for the quantification of SC5b-9 (Szebeni et al., 1997). SC5b-9 is the soluble, non-lytic form of the terminal complement complex (TCC) and is generated by the assembly of the complement factors C5–C9 and subsequent binding to the regulatory S protein (Kolb and Müller-Eberhard, 1975). The amount of SC5b-9 is proportional to the total generated TCC and therefore total complement activation. Serum from healthy volunteers was incubated with the PAA-liposome formulations (4:1, v/v) for 30 min at 37 °C and subsequently tested using an SC5b-9 ELISA kit (Quidel Co., San Diego, CA, USA) (for a detailed description of the method see Szebeni et al., 1997). Fig. 4 shows the results of a typical experiment. PHEA- and PHEG-liposomes induce formation of SC5b-9 in a concentration-dependent manner, indicating complement activation. In this assay, PHEG-liposomes appear to activate the complement system to a higher extent than PHEA-liposomes.

Cardiovascular changes after liposome administration were assessed in male Yorkshire swine (weight between 10 and 25 kg). Pigs were sedated with an intramuscular injection of ketamine, intubated, anesthetized with isoflurane and subsequently instrumented as described previously (Szebeni et al., 1999). In brief, a catheter was inserted via the right internal jugular vein into the pulmonary artery; another catheter was introduced through the

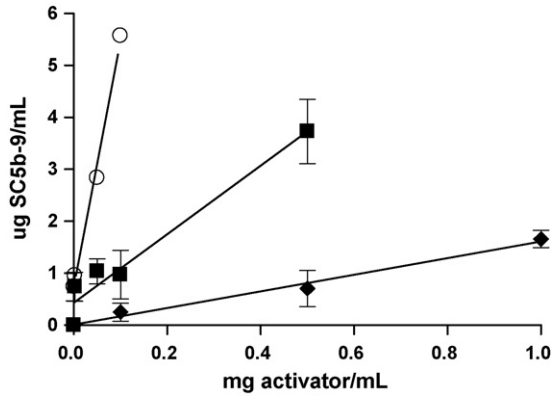

Fig. 4. In vitro complement activation assessed by the SC5b-9 ELISA assay. SC5b-9 level of PHEA- (♦) and PHEG- (■) liposomes as a function of liposome concentration in a representative serum sample. Zymosan, a potent complement activator, was used as a positive control (◯). Bars represent means ± S.D. for three wells.

Table 2
Hypersensitivity reactions in the porcine model

Liposome type	Lipid dose (μmol/kg)	Frequency of reactions	Severity of reactions
PHEA	0.29–0.85	2/3	None (1), mild (2)
PHEG	0.26–1.38	3/3	Mild (2), severe (1)

right femoral artery into the proximal aortic arch to measure the central systemic arterial pressure. Furthermore, heart rate, blood oxygen and CO_2 levels were monitored. PHEA- and PHEG-liposomes were diluted in PBS to the desired dose (see Table 1), injected into the jugular vein in 1 mL boluses and flushed into the circulation with 10 mL PBS at an injection rate of approximately 2.5 mL/s. The observed physiological reactions were similar to those observed in earlier studies: temporary decreases in systemic arterial pressure, heart rate, respiration rate and changes in pCO_2 (Szebeni et al., 1999). Based on these changes, reactions were classified as follows: none (no significant changes in any of the measured parameters), mild (transient <50% changes in at least one of the measured parameters), severe (up to 10 min and >50% changes in at least one of the measured parameters) and lethal (circulatory collapse within 2 min requiring resuscitation with i.v. epinephrine and cardiac massage). In Table 2 the results are shown. Both formulations induced acute HSR in the pigs, with again PHEG-liposomes exerting a stronger effect than PHEA-liposomes. Thus, the in vitro and in vivo experiments reveal that PHEA- and PHEG-liposomes can activate the complement system and induce HSR in the porcine model. This is in line with earlier findings on the complement activation by other types of liposomes, e.g. PEG-liposomes (Moghimi and Szebeni, 2003). As yet, it is not clear how these reactions depend on factors like lipid dose/composition and animal species. As pointed out previously, the pig model is an extremely sensitive and reactive model compared to humans (Szebeni et al., 1999). Another element to be considered is the fact that the synthesis and work-up of the PAA conjugates do not exclude some residual contamination with endotoxins. As endotoxins are known to be potent complement activators, this is an issue, which awaits final resolution.

4. Conclusion

PAA-conjugates represent promising coatings for long-circulating liposomes. Prolonged circulation times were obtained, comparable to those reported for PEG-liposomes. A key advantage of PAAs is that they are enzymatically degradable, thereby reducing the risks of accumulation in vivo. Like other liposome systems, they appear to activate the complement system, however, a role of endotoxin contamination cannot be ruled out in our studies.

Acknowledgement

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