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Enzyme-induced shedding of a poly(amino acid)-coating triggers contents release from dioleoyl phosphatidylethanolamine liposomes

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Abstract

The enzymatically degradable poly(amino acid)–lipid conjugate poly(hydroxyethyl L-glutamine)–*N*-succinyl-dioctadecylamine (PHEG–DODASuc) has been shown to effectively prolong liposome circulation times. In this paper, we investigated whether PHEG–DODASuc can stabilize liposomes composed of the fusogenic, non-bilayer-forming lipid dioleoyl phosphatidylethanolamine (DOPE). Moreover, we evaluated the release of an entrapped compound after enzyme-induced shedding of the PHEG-coating, interbilayer contact and membrane destabilizing phase changes. Contents release was monitored using the fluorescent model compound calcein. Liposome destabilization and lipid mixing was studied by dynamic light scattering (DLS), fluorescence resonance energy transfer (FRET) and cryogenic-temperature transmission electron microscopy (cryo-TEM). It was shown that PHEG–DODASuc is able to stabilize DOPE-based liposomes and that contents release can be triggered by shedding of the PHEG-coating.

Keywords: Long-circulating liposomes; Poly(amino acid)s; Shedding; Dioleoyl phosphatidylethanolamine; Liposome destabilization; Triggered contents release

1. Introduction

Hydrophilic polymers such as poly(ethylene glycol) (PEG) greatly improved the applicability of liposomes as drug carriers (Torchilin, 2005). These polymer molecules form a protective layer on the liposome surface and thereby reduce liposome opsonization in the blood and subsequent uptake and clearance of liposomes by macrophages of the mononuclear phagocyte system (MPS). These so-called 'sterically stabilized' or 'stealth' liposomes show prolonged circulation times as compared to non-coated liposomes. Due to the 'enhanced permeability and retention' (EPR) effect the liposomes can extravasate and accumulate at sites where the endothelial cell lining of the vasculature is leaky, such as in tumors and inflamed tissues, which allows selective targeting to these regions (Maeda et al., 2000).

After localizing at the target site, liposomes should release their contents efficiently to achieve a satisfactory therapeutic response. The polymer coating, however, may hamper drug release and interaction of the particles with the target cells. For certain therapeutic applications, extracellular release of the drug from the extravasated liposomes within the target region is sufficient, e.g. in the case of small molecules that easily pass target cell membranes. For such applications steric stabilization of the liposomes may be disadvantageous as it may cause a delay in destabilization of the liposomes and therefore it may limit the therapeutic availability of the drug. For other applications requiring a targeting ligand grafted directly to the bilayer and shielded by the coating to avoid immune responses and rapid clearance, interaction of the ligand with the target receptor can be severely limited by the coating. When the application requires endosomal escape of the incorporated drug to be mediated by fusogenic lipids, the polymer shielding can hinder this process. A potential solution for these situations can be 'shedding', i.e. the loss of the polymer coating after arrival of the liposomes at the target site. Several studies have demonstrated that sheddable coatings can be beneficial for the realization of a therapeutic response (for a review see Romberg et al., 2007). Shedding can be induced by stimuli which are present at the target site. For example, the low

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pH in the endo/lysosomal compartment inside cells or acidic conditions at tumor and inflamed sites can facilitate cleavage of a pH-sensitive bond that links the polymer to its lipid anchor, resulting in a detachment of the polymer coating from the liposome surface (Boomer et al., 2003; Guo and Szoka, 2001; Jeong et al., 2003; Sawant et al., 2006). Also disulfide bonds were used as linkers between the polymer and its anchor. They can be cleaved in reductive environments, for example the glutathione-rich cytosol, after cellular internalization of the carrier (Kirpotin et al., 1996; Zhang et al., 2004; Maeda and Fujimoto, 2006). Furthermore, a peptide-linker, which is sensitive to proteolysis by enzymes present in lysosomes, has been described (Zhang et al., 2004).

Alternatively, fully degradable coatings can be applied for shedding purposes, for example poly(amino acid)-based coatings which are degradable by proteolytic enzymes. In our group, the poly(amino acid)–lipid conjugate poly(hydroxyethyl L-glutamine)-*N*-succinyl-dioctadecylamine (PHEG–DODASuc) is being investigated as a new coating material for sterically stabilized liposomes. PHEG has been shown to prolong liposome circulation times to a similar extent as PEG (Metselaar et al., 2003). It is composed of L-α-amino acids, which renders it enzymatically degradable by proteases. It was demonstrated that PHEG is degradable by the model enzymes papain and pronase E (Romberg et al., 2005). Incubation with the latter enzyme resulted in small degradation fragments of 1–2 amino acids. In addition, the mammalian enzyme cathepsin B, which is involved in the protein turnover in lysosomes and also present in extracellular fluid of tumors and inflamed tissues, was also capable of degrading the polymer.

One research aim in our group is to design long-circulating liposomes which efficiently release the entrapped drug contents upon arrival at the target site. For this purpose, we selected liposomes based on the non-bilayer-forming, fusogenic lipid DOPE as drug carriers. To achieve efficient drug release from liposomes after extravasation into the pathological site, we hypothesized that liposomes composed of DOPE and PHEG–DODASuc are an attractive carrier system to be exploited for that purpose, with shedding of the PHEG-coating as a drug release tool. In this study, we investigated whether PHEG–DODASuc is able to stabilize DOPE-based liposomes. Moreover, we evaluated whether an entrapped compound is released after enzyme-induced shedding of the PHEG-coating, interbilayer contact and membrane destabilizing phase changes (proposed concept schematically represented in Fig. 1). Contents release was monitored using the fluorescent model compound calcein. Liposome destabilization and lipid mixing were studied by dynamic light scattering (DLS), fluorescence resonance energy transfer (FRET) and cryogenic-temperature transmission electron microscopy (cryo-TEM).

2. Materials and methods

2.1. Materials

PHEG–DODASuc (average MW = 3000 Da, determined by NMR and MALDI-ToF MS, corresponding to an average degree of polymerization of 13) was used in this study. Calcein and Triton X-100 were purchased from Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands. Pronase E (from *Streptomyces griseus*, 6–8 U/mg, lyophilized powder) was a product of Serva Electrophoresis GmbH, Heidelberg, Germany. Dioleoyl phosphatidylethanolamine (DOPE) and poly(ethylene glycol)–distearoyl phosphatidylethanolamine (PEG_{2000}–DSPE) were obtained from Lipoid GmbH, Ludwigshafen, Germany. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) was a product of Invitrogen, Paisley, UK, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), ammonium salt (Rh-PE) was purchased form Avanti Polar Lipids, Alabaster, AL, USA. Sepharose CL-4B was purchased from Amersham Biosciences, Uppsala, Sweden. All other reagents were of analytical grade.

2.2. Liposome preparation

DOPE/PHEG–DODASuc liposomes and DOPE/PEG–DSPE control liposomes, both with a molar ratio of 9:1, were prepared by the film-extrusion method. The molar ratio of 9:1 was chosen as it has been shown that inclusion of 10 mol% PEG in a mixture with DOPE results in the formation of stable liposomes (Johnsson and Edwards, 2001). Lipids were dissolved in a mixture of chloroform/methanol (1:1, v/v) in a 50 mL round-bottom flask. A lipid film was obtained by evaporation of the solvent under reduced pressure at 35 °C. After flushing with nitrogen, the lipid film was hydrated in calcein solution with a quenching concentration (approximately 95 mM, 290 mOsm/kg, pH 7.4), yielding a phospholipid concentration of 10 μmol total lipid/mL. Liposomes were sized by sequential extrusion through two stacked polycarbonate filters (Poretics, 400, 200, 100 and 50 nm) with a high-pressure extrusion device. Non-entrapped calcein was removed from the liposome dispersion by passing it through a Sepharose CL-4B column (1.2 cm × 30 cm) equili-

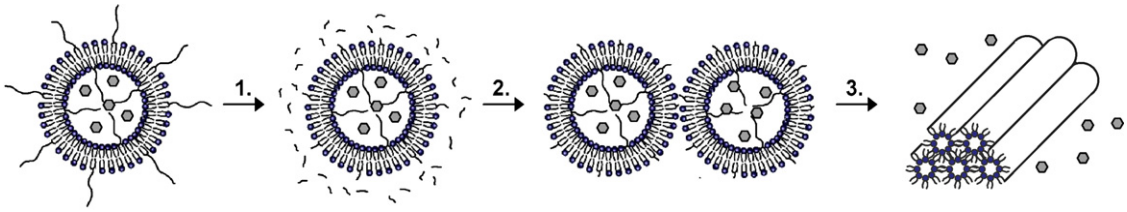

Fig. 1. Schematic representation of the proposed concept of shedding of the PHEG-coating and subsequent drug release. (1) Degradation of the PHEG-coating by proteases; (2) bilayer contact; (3) phase change from the lamellar to the inverted hexagonal phase, destabilization and drug release.

brated with a buffer composed of 10 mM HEPES, 5 mM $CaCl_2$, 142.5 mM NaCl and pH 7.4.

Fluorescently labeled and unlabeled liposomes for the fluorescence resonance energy transfer (FRET) experiment and the cryo-TEM experiments were prepared in a similar way. For the preparation of the labeled formulation, NBD-PE was dissolved in chloroform to a concentration of 1 mg/mL and Rh-PE in was dissolved in ethanol to a concentration of 0.2 mg/mL. One mol% of each label was added to the lipid mixture in chloroform. The lipid films were hydrated with buffer.

2.3. Liposome characterization

The mean particle size and the polydispersity index of the liposome dispersions were determined by dynamic light scattering using a Malvern ALV/CGS-3 Goniometer (diluted 1:100 in buffer). Phospholipids were quantified spectrophotometrically as described by Rouser et al. (1970).

2.4. Release assay

Pronase E was dissolved to a concentration of 4 mg/mL in the above-mentioned buffer. Liposomes were incubated with 11, 18 or 36 μg pronase E/μmol total lipid at 37 °C. As a control, liposomes were incubated in the absence of pronase E. At different time points, 5 μL of the incubation mixtures were pipetted into a 96-well plate in triplicate and diluted with 150 μL buffer. As a 100% destabilization control, 5 μL of the incubation mixtures were pipetted into wells in triplicate, 140 μL buffer and 10 μL 10% Triton X-100 (w/w in reversed osmosis water) were added and well contents were mixed thoroughly. Fluorescence was assessed using a Fluostar Optima fluorimeter (BMG Labtech GmbH, Offenburg, Germany). Calcein fluorescence was measured at 520 nm after excitation at 485 nm. The %leakage was calculated according to the following formula:

$$\%\text{leakage} = \frac{F/F_{\max} - F_0/F_{0,\max}}{1 - F_0/F_{0,\max}} \times 100$$

where F is the fluorescence measured at a certain time point, $F_{\max}$ the fluorescence after Triton treatment and F_0 fluorescence immediately after addition of pronase E and $F_{0,\max}$ the fluorescence of the latter sample after Triton addition.

To confirm PHEG degradation, samples were withdrawn from the PHEG-liposomes incubated with 18 μg pronase/μmol total lipid at the start of incubation and after 45 h, and a ninhydrin assay was performed as described earlier (Romberg et al., 2005).

2.5. Dynamic light scattering study

Liposomes were incubated as described above with or without 11 μg pronase E/μmol total lipid. At different time points, samples of 10 μL were withdrawn, diluted in 800 μL buffer and subsequently analyzed by dynamic light scattering for hydrodynamic liposome diameter, polydispersity index and scattering intensity.

2.6. Lipid mixing assay

An assay based on fluorescence resonance energy transfer was used to investigate if lipid mixing of DOPE-based liposomes was involved in the process of contents release. Liposomes labeled with Rh-PE and NBD-PE were mixed with unlabeled liposomes in a ratio of 1:3 and incubated with or without 80 μg pronase E/μmol total lipid at 37 °C. Samples of 5 μL were withdrawn at different time points and diluted in buffer with or without Triton X-100 in a 96-well plate as described above. The fluorescence was measured at 520 nm (NBD emission) and 600 nm (Rh emission) after excitation at 485 nm (NBD excitation). The percentage of lipid mixing was calculated by the following equation:

$$\%\text{lipid mixing} = \frac{F520/F600 - F_0520/F_0600}{F_{\max}520/F_{\max}600 - F_0520/F_0600} \times 100$$

where $F520/F600$ is the ratio of fluorescence intensities of the sample at the two wavelengths, F_0520/F_0600 is the ratio at time 0 and $F_{\max}520/F_{\max}600$ is the ratio after Triton X-100 treatment indicating 100% lipid mixing. All manipulations with the NBD/Rh-labeled liposomes were done under minimal light conditions to avoid photobleaching.

2.7. Cryogenic-temperature transmission electron microscopy (cryo-TEM)

PHEG-liposomes were incubated with 120 μg pronase E/μmol total lipid for 48 h. Non-incubated PHEG-liposomes (in a similar liposome concentration as in the incubated liposome sample) were used as a control. Samples for cryo-TEM were prepared in a temperature- and humidity-controlled chamber with an automatic blotting/plunging system using a "Vitrobot". A thin aqueous film of particle dispersion was formed by blotting a glow-discharged 200 mesh copper grid covered with Quantifoil holey carbon foil (Micro Tools GmbH, Jena, Germany) at 25 °C and 100% relative humidity. The thin film was rapidly vitrified by quick freezing in liquid ethane and transferred into the microscope chamber using a GATAN 626 cryoholder system. Samples were analyzed using a Tecnai12 transmission electron microscope (FEI Co., Eindhoven, The Netherlands) operating at 120 kV at −180 °C.

3. Results and discussion

3.1. Liposome characterization

The size of the liposomes was between 120 and 140 nm with a polydispersity index below 0.14, indicating a relatively homogenous size distribution. Liposomes were stable for at least 2 weeks when stored at 4 °C as determined by DLS analysis.

3.2. Release assay

The ninhydrin assay confirmed degradation of PHEG into fragments of 1–2 amino acids after 45 h of incubation with 18 μg pronase/μmol total lipid. Contents release was determined

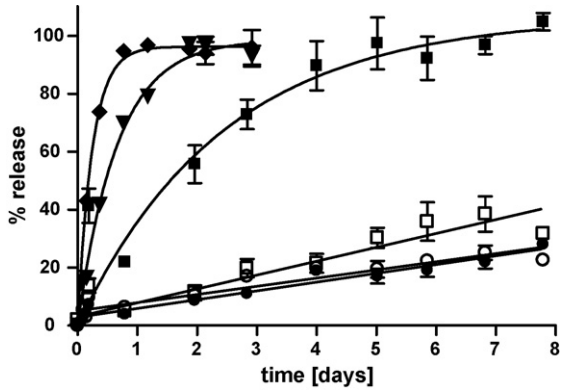

Fig. 2. Calcein release from liposomes: PEG-liposomes incubated without pronase (◯), PEG-liposomes incubated with 11 μg pronase E/μmol total lipid (●), PHEG-liposomes incubated without pronase E (□), PHEG-liposomes incubated with 11 μg pronase E/μmol total lipid (■), PHEG-liposomes incubated with 18 μg pronase E/μmol total lipid (▼) and PHEG-liposomes incubated with 36 μg pronase E/μmol total lipid (♦). Data points are expressed as mean %leakage ± S.D. ($n = 3$).

via fluorescence dequenching experiments using calcein as a fluorescent probe. Fig. 2 shows the results of calcein release upon incubation with or without pronase E. Incubation of PEG-liposomes resulted in less than 30% calcein release after 7 days of incubation, both in the presence and absence of the enzyme. PHEG-liposomes incubated without pronase E show a similar extent of release during 8 days (less than 40%). When PHEG-liposomes were incubated with pronase E, an enzyme concentration-dependent calcein release was observed. Fig. 2 shows that release was complete after 8 days of incubation with 11 μg pronase E/μmol total lipid. After approximately 2 days of incubation with 18 μg pronase E/μmol total lipid and after 24 h of incubation with 36 μg pronase E/μmol total lipid, also complete release was observed.

3.3. DLS study

PEG-liposomes incubated with or without pronase showed no changes in size, polydispersity and scattering intensity as determined by DLS measurements (Fig. 3). The same holds true for PHEG-liposomes incubated in the absence of the protease. When PHEG-liposomes were incubated with pronase E, however, the hydrodynamic diameter of the liposomes increased in time, indicating liposome aggregation and possibly fusion (Fig. 3a). Also the polydispersity index increased, confirming aggregation and/or fusion (Fig. 3b). Upon incubation of PHEG-liposomes with the enzyme, the scattering intensity increases initially, again suggesting aggregation of liposomes (Fig. 3c). After approximately 2.5 days of incubation, the scattering intensity decreases. This is likely related to a decreasing concentration of liposomes in the dispersion as a result of liposome destabilization.

3.4. Lipid mixing assay

Contents release from PHEG-liposomes can be a result of lipid mixing followed by destabilization of the liposomes. To investigate this, a lipid mixing assay was carried out. This assay is based on the decrease of fluorescence resonance energy transfer (FRET) between the two fluorophores NBD and Rh after label dilution (Düzgünes et al., 2003). When the two labels are in close proximity to each other (less than 10 nm) the emission fluorescence of the NBD partly excites the Rh molecules and Rh fluorescence can be observed. When the distance between the two labels increases after lipid mixing and possible fusion with an excess of 'sink' (non-labeled) liposomes, FRET does not occur and Rh fluorescence cannot be observed. A label concentration of 1 mol% was used as significant quenching of NBD by Rh occurs. As can be seen from Fig. 4, in the case of PEG-

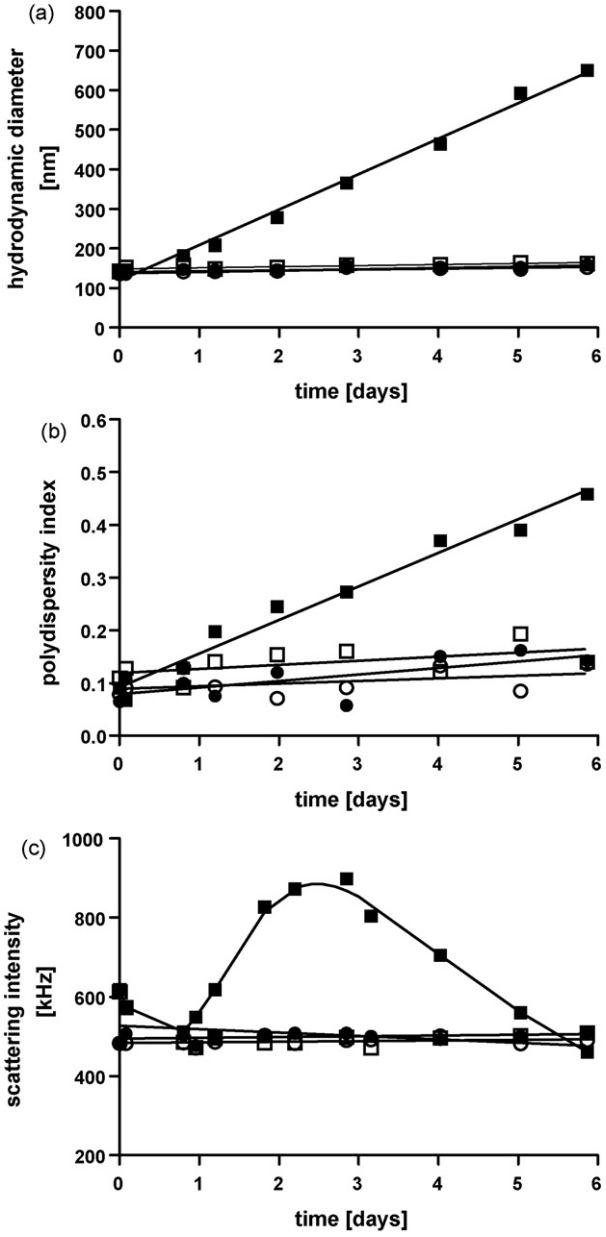

Fig. 3. Hydrodynamic diameter (a), polydispersity index (b) and scattering intensity (c) of liposome samples: PEG-liposomes incubated without pronase (◯), PEG-liposomes incubated with 11 μg pronase E/μmol total lipid (●), PHEG-liposomes incubated without pronase E (□) and PHEG-liposomes incubated with 11 μg pronase E/μmol total lipid (■).

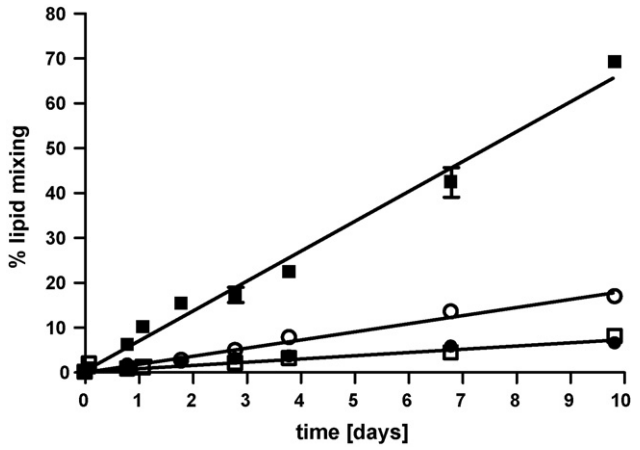

Fig. 4. Lipid mixing of liposomes as measured with FRET. PEG-liposomes incubated without pronase (◯), PEG-liposomes incubated with 80 μg pronase E/μmol total lipid (●), PHEG-liposomes incubated without pronase E (□) and PHEG-liposomes incubated with 80 μg pronase E/μmol total lipid (■). Data points are expressed as mean %lipid mixing ± S.D. ($n = 3$).

and PHEG-liposomes incubated without pronase E and PEG-liposomes incubated with pronase E, less than 20% lipid mixing occurred within 10 days of incubation. On the other hand, for PHEG-liposomes incubated with pronase E a clear increase in lipid mixing was observed. This increase in lipid mixing was accompanied by a slow formation of a visible precipitate. After 10 days of incubation approximately 70% lipid mixing was observed accompanied by extensive precipitation leading to termination of the experiment. The observed lipid mixing is likely a consequence of the interbilayer contact of liposomes, which is enabled after removal of the PHEG-coating by the action of pronase E.

3.5. Cryogenic-temperature transmission electron microscopy (cryo-TEM)

Fig. 5 shows cryo-TEM images of native PHEG-liposomes and PHEG-liposomes after incubation with pronase E. In the sample of native liposomes, mainly nicely shaped unilamellar liposomes were observed (see insert in top image of Fig. 5). Images of liposome samples after incubation with pronase E show different liposome morphologies. In the top image, bilayer contact between liposomes and possibly fusion events can be seen (indicated by arrows). The bottom image shows a dense, large structure indicating a cluster of possibly collapsed liposomes, presumably after change from the L_α-phase to an H_{II}-like phase (Zhang et al., 2004; Johnsson and Edwards, 2001).

DOPE is a non-bilayer forming, cone-shaped lipid. Above pH 9, it adopts the lamellar L_α-phase, whereas below pH 8, DOPE forms inverted micelles, also known as the inverted hexagonal (H_{II}) phase (Zhang et al., 2004; Johnsson and Edwards, 2001). However, it has been shown that DOPE can be stabilized in the lamellar bilayer conformation by inclusion of molecules that have a complementary inverted cone-shape, such as PEG–lipid conjugates (Ellens et al., 1984; Shin et al., 2003). A conversion from the lamellar to the hexagonal phase after shedding of the PEG-coating and subsequent interbilayer contact can cause DOPE-based liposomes to destabilize and release their contents (Guo and Szoka, 2001; Kirpotin et al., 1996; Shin et al., 2003).

Our results show that shedding-induced lipid mixing, liposome destabilization and membrane fusion is much slower than release of an encapsulated dye. It is likely that release of an encapsulated compound already occurs when the PHEG-coating is being cleaved and liposomes start to aggregate, whereas the process of lipid mixing and fusion occurs at a slower rate. Such observations have also been reported for PEG/DOPE liposomes (Boomer et al., 2003).

It is known that DOPE can promote membrane fusion and renders liposomes fusogenic (Hafez and Cullis, 2001). After endocytic internalization of the liposomes, fusion with the endosomal membrane can lead to release of the incorporated

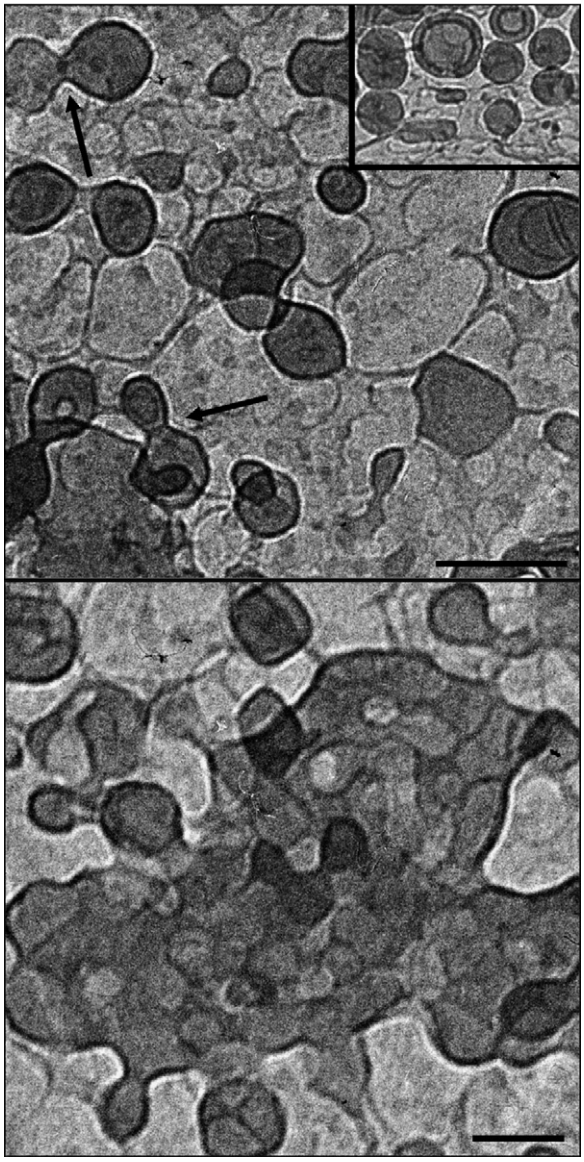

Fig. 5. Cryogenic-temperature transmission electron microscope images of PHEG-liposomes incubated for 48 h with 120 μg pronase E/μmol total lipid (top and bottom). Arrows in the top image indicate bilayer contact between liposomes and possibly fusion events. The insert in the top image shows native PHEG-liposomes. Representative images are shown. Bars represent 200 nm.

compound into the cytosol in its intact form. As lipid mixing and possibly fusion processes were observed in our experiments, it appears that the fusogenic activity of DOPE-based liposomes is enabled by shedding of the PHEG-coating.

Our results indicate that like PEG-conjugates, PHEG–DODASuc can be used to transiently stabilize DOPE-based liposomes and provide them with the necessary long circulation times for efficient accumulation at the target site. Furthermore, we have demonstrated that degradation of the PHEG-coating by proteases leads to liposome destabilization and contents release. One can envisage accelerated drug release due to shedding of PHEG induced by extracellular proteases after arrival at the target site, when compared to a formulation with a non-sheddable coating. Furthermore, following internalization by target cells, escape of the incorporated drug from the endo/lysosomal compartment into the cytosol, mediated by the fusogenic activity of DOPE, may occur upon proteolytic shedding of the PHEG-coating.

In conclusion, DOPE/PHEG-liposomes are a promising candidate for the development of a long-circulating liposome system which efficiently releases the entrapped drug contents upon arrival at the target site.

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