Enzymatic Degradation of Liposome-Grafted Poly(hydroxyethyl L-glutamine)

Birgit Romberg,[†] Josbert M. Metselaar,[†] Tom deVringer,[§] Kei Motonaga,[§] J. Jantina Kettenes-van den Bosch,[‡] Christien Oussoren,[†] Gert Storm,[†] and Wim E. Hennink*,[†]

Departments of Pharmaceutics and Biomedical Analysis, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands, and Yamanouchi Europe B.V., Leiderdorp, The Netherlands. Received September 21, 2004; Revised Manuscript Received April 18, 2005

Liposomes coated with poly(hydroxyethyl L-glutamine) (PHEG) show prolonged circulation times and biodistribution patterns comparable to PEG-coated liposomes. While PEG is a nondegradable polymer, PHEG is expected to be hydrolyzed by proteases. In this study the enzymatic degradability of PHEG both in its free form and grafted onto liposomes was investigated, using the proteases papain, pronase E, and cathepsin B. Enzymatic action was monitored with a ninhydrin assay, which quantifies amine groups formed due to hydrolysis of amide bonds, and the degradation products were characterized by MALDI-ToF mass spectrometry. PHEG, both in its free form and when grafted onto liposomes, showed degradation into low molecular weight peptides by the enzymes. Thus, we present a polymer-coated long-circulating liposome with an enzymatically degradable coating polymer, avoiding the risk of cellular accumulation.

INTRODUCTION

Liposomes are nanosized particles consisting of an aqueous volume enclosed by one or more phospholipid bilayers. Because of their biocompatibility and versatility they have been extensively studied as systems for the (targeted) delivery of small conventional drugs but also of macromolecules such as antigens, proteins, and DNA. Both lipophilic and hydrophilic compounds can be incorporated into liposomes, either in or absorbed to the lipid bilayer or in the aqueous compartment(s) (*1*–*4*). Intravenously injected liposomes are generally rapidly removed from the circulation, often hampering their ability to reach the aimed target site. This rapid elimination is due to recognition and capture by macrophages of the mononuclear phagocyte system (MPS), particularly those present in liver and spleen (*2, 3*). Surface modification with the hydrophilic polymer poly(ethylene glycol) (PEG) results in a decrease in uptake by the MPS and consequently in a prolonged circulation time (*2, 5*). Due to this MPS avoidance ('stealth') effect, PEG-liposomes are able to extravasate at pathological sites with areas of leaky endothelium, e.g. in tumors and inflamed tissues (so-called EPR effect (*6, 7*)). Besides PEG, several other polymers have been investigated for prolonging the circulation times of liposomes. Torchilin et al. studied vinyl-based liposome coatings (*8*), Woodle et al. studied poly(oxazoline) conjugates (*9*), and Maruyama et al. studied polyglycerol (*10*). Intravenously injected low molecular weight PEG (MW < 20 kDa) is rapidly cleared by the kidney (*11, 12*). However, its intracellular fate after uptake of PEG-coated liposomes is not known. PEG and the other polymers mentioned are not degradable by mammalian enzymes and therefore there is a risk of accumulation in cells in which the liposomes localize (e.g. macrophages), which in turn can lead to cell function impairment.

In a previous study we reported on two poly(α-amino acid)–lipid conjugates, namely poly(hydroxyethyl L-glutamine)–*N*-succinyldioctadecylamine (PHEG–DODASuc) and poly(hydroxyethyl L-asparagine)–DODASuc (PHEA–DODASuc), as stealth coatings for liposomes (structures shown in Figure 1). Indeed, liposomes coated with these polymers showed circulation kinetics and biodistribution patterns comparable to PEG-liposomes (*13*). The conjugates are based on modified L-α-amino acids and therefore have the potential to be degraded in vivo by proteases and peptidases. Poly(hydroxyethyl L-glutamine) (PHEG) has been investigated as biomaterial for different applications, and its enzymatic degradability has already been demonstrated (*14*–*21*). Relatively high molecular weight PHEG (MW = 25000–75000 Da) is cleaved into low molecular weight peptides by several proteolytic enzymes (e.g. papain, pepsin, pronase E, cathepsin B) of which the cysteine protease papain showed the highest activity (*17, 19*). Besides that enzymatic degradation reduces the risk of polymer accumulation inside cells, an enzymatically degradable coating polymer can also be used to trigger cellular uptake of the liposomes and/or release of the entrapped biologically active agent. To be more precise, in the extracellular space of tumors and inflamed tissues relatively high concentrations of proteases are present (*22, 23*). As just pointed out, long-circulating liposomes preferentially accumulate in such pathological tissues and 'stripping' of the polymer coating by enzymatic action can occur. As a result of the stripping process, surface properties (e.g. charge) and surface-associated targeting moieties that were initially shielded by the polymer coating become exposed (sheddable shielding) which may induce cellular binding and internalization.

In contrast to high molecular weight PHEG, the enzymatic degradation of low molecular PHEG as well

* To whom correspondence should be addressed. Tel: +31-(0)30–2536964. Fax: +31-(0)-2517839. E-mail: W.E.Hennink@pharm.uu.nl.
[†] Department of Pharmaceutics, Utrecht University.
[‡] Department of Biomedical Analysis, Utrecht University.
[§] Yamanouchi Europe B.V.

10.1021/bc0497719 CCC: $30.25
Published on Web 06/11/2005

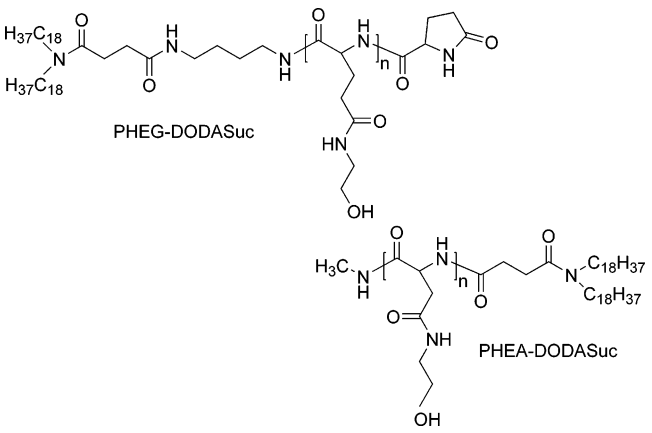

Figure 1. Structures of PHEG−DODASuc and PHEA−DODASuc.

as liposome-grafted PHEG has not been studied so far. The purpose of the present study was to investigate the enzymatic degradability of PHEG−DODASuc used as a coating for long-circulating liposomes and to identify the degradation products. Model enzymes, papain and pronase E, with specificities comparable to those of intra- and extracellular proteases, and the lysosomal cysteine protease cathepsin B, were used. All three enzymes are known to be capable of cleaving high molecular weight PHEG. Papain is a well-studied cysteine endopeptidase from *Carica papaya* latex with a broad catalytic specificity and is frequently used as a substitute for cathepsin B in degradation studies (*20, 21*). Pronase E, isolated from *Streptomyces griseus*, is composed of endo- and exopeptidases and therefore represents a natural mixture of proteases (*24*). Cathepsin B is a cysteine protease, which is present, for example, in mammalian lysosomes and is involved in protein turnover (*25*). It is also extracellularly present in pathological tissues such as tumors and sites of inflammation (*22, 23*).

The PHEG-conjugate was evaluated for enzymatic degradability both in its free form and grafted onto the surface of liposomes. Poly-L-glutamic acid was used as a control. Degradation of the free and grafted conjugate was assessed using a ninhydrin assay. Degradation products were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-ToF MS).

MATERIALS AND METHODS

Materials. PHEG−DODASuc (average MW = 3500 Da, determined by NMR and MALDI-ToF MS, corresponding with an average degree of polymerization of 16) was used in this study. Papain from papaya latex (33 units/mg in suspension, 28 mg protein/mL), cathepsin B from bovine spleen (lyophilized powder, 5.6 units/mg solid, 14 units/mg protein), Z-L-Lys-ONp hydrochloride, poly-L-glutamic acid (sodium salt, MW 1500−3000), ninhydrin, hydridantin, 2-methoxyethanol, α-cyano-hydroxycinnaminic acid, *n*-octylglucoside, and cholesterol were all purchased from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands. *N*-α-Benzoyl-D,L-arginine-*p*-nitroanilide (D,L-BAPNA) was obtained from ICN Biomedicals BV, Zoetermeer, The Netherlands. Pronase E (from *Streptomyces griseus*, 6−8 U/mg, lyophilized powder) was a product of Serva Electrophoresis GmbH, Heidelberg, Germany. Dipalmitoylphosphatidylcholine (DPPC) and poly(ethylene glycol)-distearylphosphatidylethanolamine (PEG2000-DSPE) were purchased from Lipoid GmbH, Ludwigshafen, Germany. All other reagents were of analytical grade.

Degradation with Papain. Thirty five microliters of the papain suspension was added to 965 μL of buffer (50 mM KH_2PO_4, 2.5 mM EDTA, 15 mM dithiothreitol, pH 6.5) to give a clear solution (32 U papain/mL). The solution was incubated for 1 h at 37 °C to activate the enzyme (*26*). The enzyme concentration was determined by UV spectroscopy at 278 nm (specific extinction coefficient $\epsilon_{\%} = 25.0$) (*27*). To determine the activity of papain, 50 μL of activated papain solution was added to 950 μL of 2 mM N-α-benzoyl-D,L-arginine-*p*-nitroanilide (D,L-BAPNA) solution in phosphate buffer (50 mM KH_2PO_4, 2.5 mM EDTA, pH 6.5). The formation of the product, 4-nitroaniline, was monitored spectroscopically at 410 nm.

The stability of papain was assessed by incubating the enzyme (400 μL of activated papain solution in 7.6 mL of phosphate buffer) at 37 °C for 120 h. At different time points the activity of papain was determined by mixing 500 μL of this solution with 500 μL of 2 mM D,L-BAPNA followed by the spectrophotometric analysis as described above. PHEG−DODASuc and poly-L-glutamic acid (pGlu) were dissolved in phosphate buffer (50 mM KH_2PO_4, 2.5 mM EDTA, pH 6.5) to a final concentration of 1 mg/mL. Activated papain (150 μL) was added to 2850 μL of the polymer solution. The mixtures were incubated at 37 °C. At different time points samples of 250 μL for ninhydrin analysis and of about 50 μL for mass spectrometric analysis were withdrawn. To inactivate the enzyme, the tubes were closed, heated for 5 min at 100 °C, and stored at −20 °C until analysis.

Degradation with Pronase E. Pronase E was dissolved to a concentration of 1 mg/mL in 10 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid), 5 mM $CaCl_2$, pH 7.5. PHEG−DODASuc and pGlu were dissolved in the same buffer to a final concentration of 1 mg/mL. The pronase E solution (150 μL) was added to 2850 μL of the polymer solutions, which were subsequently incubated at 37 °C for 216 h. At different time points samples of 250 μL for the ninhydrin assay and samples for mass spectrometric analysis were withdrawn and heat inactivated as described for the papain samples. The samples were stored at −20 °C until further analysis.

Degradation with Cathepsin B. To assess the activity of cathepsin B, 1 mg of the enzyme was dissolved in 300 μL buffer (20 mM sodium acetate, 1 mM EDTA, 5 mM cysteine, pH 5). The substrate Z-L-Lys-ONp hydrochloride was dissolved in DMSO to a concentration of 2.3 mg/mL. Substrate solution (50 μL) and enzyme solution (10 μL) were added to 3 mL of acetate buffer. The generation of 4-nitrophenol was monitored spectroscopically over 5 min at 326 nm.

For studying the degradation of PHEG−DODASuc, cathepsin B was dissolved to a concentration of 16.5 mg/mL in buffer (50 mM sodium acetate, 1 mM EDTA, 10 mM dithiothreitol, pH 5.5). PHEG−DODASuc was dissolved in the same buffer without dithiothreitol to a final concentration of 1 mg/mL. Cathepsin B (150 μL) was added to 2850 μL of the polymer solution. The mixture was incubated at 37 °C for 144 h. Samples for ninhydrin and mass spectrometric analysis were withdrawn at different time points, and the enzyme was inactivated as described above. Samples were stored at −20 °C before analysis.

The activity of cathepsin B was also followed in time. Cathepsin B was incubated at 37 °C in buffer (50 mM sodium acetate, 1 mM EDTA, 10 mM dithiothreitol, pH 5.5) at a concentration of 0.825 mg/mL for 144 h. Samples of 10 μL were withdrawn at different time points and added to a mixture of 50 μL of the substrate solution and

3 mL of buffer (50 mM sodium acetate, 1 mM EDTA, pH 5.5). Formation of 4-nitrophenol was monitored spectroscopically at 326 nm.

Liposome Preparation. PHEG−DODASuc- and PEG−DSPE-liposomes were prepared by a film-extrusion method (*28*). In a typical experiment, a lipid mixture composed of DPPC/cholesterol/coating polymer (1,85:1:0.15 molar ratio; total lipid ≈100 mg) was dissolved in about 2 mL of 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 50 °C. After being flushed with nitrogen, the lipid film was hydrated in 4 mL of phosphate buffer (50 mM KH_2PO_4, 2.5 mM EDTA, pH 6.5), yielding a phospholipid concentration of 18.5 μmol/mL for the PHEG-liposomes and 20 μmol/mL for the PEG-liposomes. Liposomes were sized by sequential extrusion through two stacked polycarbonate filters (Poretics, 400, 200, 100, and 50 nm) with a high-pressure extrusion device. The liposomes were subjected to ultracentrifugation at 200000*g* (2 × 30 min, 4 °C) using a Beckman LE-80K Ultracentrifuge and redispersed in 4 mL of buffer (50 mM KH_2PO_4, 2.5 mM EDTA, pH 6.5).

Liposome Characterization. The mean particle size and polydispersity index of the liposome dispersions (10 times diluted with phosphate buffer) were determined by dynamic light scattering using a Malvern 4700 spectrometer. The zeta potential of the liposomes (diluted 1:100 in 5 mM HEPES buffer, pH 7.0) was determined using a Malvern Zetasizer 2000. Phospholipids were quantified spectrophotometrically according to Rouser et al. (*29*) after extraction of the lipids from the phosphate buffer with chloroform (*30*).

Degradation of Liposome-Grafted Poly(amino acid)−Lipid Conjugates with Papain. Activated papain in phosphate buffer (450 μL, 32 U/mL) was added to 8.55 mL of the dispersions of PEG−DSPE- and PHEG−DODASuc-liposomes. The dispersions were incubated at 37 °C and samples (250 μL) were taken periodically. The size and zeta potential of the liposomes were measured as described above. To separate the liposomes from the soluble degradation products, they were spun down by ultracentrifugation. In detail, 5 mL of phosphate buffer was added to the samples. Ultracentrifugation was done for 30 min at 200,000 g at 4 °C. The supernatant was collected, freeze-dried, stored at −20 °C and subsequently analyzed for degradation products by the ninhydrin assay. After 48 h of incubation, *n*-octylglucoside (150 mg) was added to 3 mL of the PHEG−liposome dispersion. Next, fresh papain (150 μL to 2850 μL of solution) was added. Samples of 250 μL were withdrawn at different time points, heat inactivated, stored at −20 °C and subsequently analyzed by the ninhydrin assay.

Quantitative ^{1}H NMR analysis of Papain-Treated PHEG-liposomes. PHEG-liposomes were incubated with papain as described above. After 48 h the papain-treated liposomes were separated from the soluble degradation products by ultracentrifugation (1 mL of liposome dispersion diluted with 5 mL of 5 mM NaCl, 200000*g*, 30 min, 4 °C). Next, the liposome pellet was dispersed in 1 mL of 5 mM NaCl and freeze-dried. The cake was dissolved in a mixture of $CDCl_3/d_6$-DMSO (2:1) and subsequently analyzed by ^{1}H nuclear magnetic resonance spectroscopy (NMR). Spectra were recorded with a Varian G-300 spectrometer (Varian, Palo Alto, CA) at 300 MHz. The number of accumulated transients was 32; the relaxation delay D1 was 10 s. The signals of the liposome mixture were assigned on the basis of chemical shift and intensity data and confirmed by homonuclear double resonance experiments. The relative amount of PHEG−DODASuc was quantified by comparing the integral values of proton signals of cholesterol and PHEG−DODASuc. The cholesterol signal chosen for calculation was at δ 5.2 (position: C6 at the double bond, corresponding to one proton) and the PHEG−DODASuc signal was at δ 3.45 (position: side chain-CH_2 next to OH, corresponding to 16 × 2 protons for $n = 16$). As a control formulation a sample of PHEG-liposomes that were not incubated with papain was prepared and analyzed as described above. A detailed description of this analytical method and its validation will be published elsewhere.

Ninhydrin Assay. When peptide bonds in peptides are cleaved due to action of the enzymes, primary amines are formed. The concentration of amines in the different samples was determined spectrophotometrically by the use of ninhydrin (*31*). The samples were thawed, and 1 M sodium acetate buffer (pH 5.5) was added to give a final volume of 1 mL. Next, 1 mL of freshly prepared ninhydrin solution (2.0 g ninhydrin and 0.3 g hydridantin dissolved in 75 mL of 2-methoxyethanol and 25 mL of 4 M sodium acetate buffer, pH 5.5) was added. The mixtures were vortexed and incubated for 15 min at 100 °C. After being cooled to room temperature, the samples were diluted with 5 mL of 50% ethanol in water, and the absorbance was read at 570 nm with a Perkin-Elmer Lambda 2 UV/vis spectrophotometer. Glycine (in 1 M acetate buffer) was used for calibration. The calibration curve was linear up to 0.5 μmol glycine/mL. Suitable controls containing only substrate or papain were run in parallel, and their readings were subtracted for plotting the degradation of the polymer-conjugates and controls.

MALDI-ToF Mass Spectrometry. α-Cyano-4-hydroxycinnaminic acid was used as a matrix and dissolved to a concentration of 10 mg/mL in 50% acetonitrile in water containing 0.1% trifluoroacetic acid. The samples were mixed with the matrix solution in a volume ratio of 1:3. Three times 1 μL of this mixture was put on a single position of the sample deposition plate and left at room temperature to allow solvent evaporation. Spectra of samples incubated with papain, pronase E, and cathepsin B were acquired with a Voyager-DE STR MALDI-ToF mass spectrometer (Applied Biosystems, Foster City, CA) operated in positive ion mode. Ionization was initiated with a nitrogen laser operating at 337 nm with a repetition rate of 7.6 Hz. Delayed extraction linear mode with an accelerating voltage of 20 kV was used and the acquisition mass range was 700−7000 Da. Calibration was done with a peptide mixture (900−3700 Da). MALDI-ToF spectra were analyzed with the Applied Biosystems Data Explorer Software.

RESULTS AND DISCUSSION

Enzymatic Degradation of PHEG−DODASuc Followed by the Ninhydrin Assay. *Papain Incubation.* The stability of papain was established using *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide as substrate. It was shown that papain retained 50% of its activity after 72 h of incubation at pH 6.5 and 37 °C. The decrease in activity is likely caused by self-destruction, a phenomenon frequently observed for proteolytic enzymes with a broad specificity (*17*). The degradation PHEG−DODASuc and the control pGlu by papain was monitored by determination of the formed primary amine groups using the ninhydrin assay. The results are presented in Figure 2. During the first 10 h of incubation a strong increase

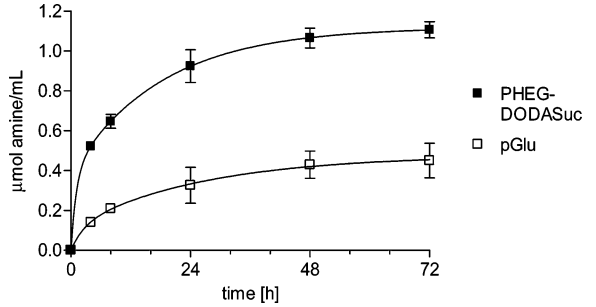

Figure 2. Time course of the concentration of primary amines in papain-incubated samples of PHEG−DODASuc (■) and poly-L-glutamic acid (□) as determined by the ninhydrin assay (average ± SD of three experiments). PHEG−DODASuc incubated in buffer without papain shows a constant concentration of amines in time.

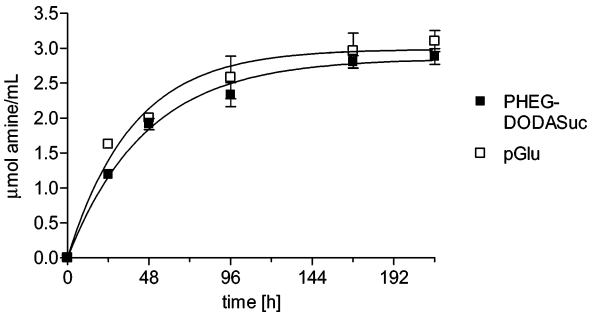

Figure 3. Time course of the concentration of primary amines in pronase E-incubated samples of PHEG−DODASuc (■) and poly-L-glutamic acid (□) as determined by the ninhydrin assay (average ± SD of three experiments).

in free amine concentration was observed for PHEG−DODASuc and pGlu, demonstrating that both relatively low molecular weight polymers are degraded by papain, as previously reported for their high molecular weight counterparts (*14*−*21*, *32*). Figure 2 also shows that a plateau value in amine concentration was obtained after 48−72 h. As mentioned, papain was still active after 72 h indicating that the degradation process did not stop due to inactivity of the enzyme. However, the activity of the enzyme might be inhibited by the formed degradation products. To investigate this, fresh papain was added after 48 h of incubation. No further increase in amine concentration was found, which shows that the obtained fragments cannot be further cleaved by the enzyme. The concentration of the formed amine groups in the PHEG−DODASuc sample after 72 h incubation (1.1 μmol amine/mL) corresponds with about four hydrolysis sites per molecule, yielding fragments with 3−4 amino acids. Fragments of 8−9 amino acids were formed after 72 h incubation of pGlu with papain. The modification of the glutamic acid side chain in PHEG with hydroxyalkylamine can be responsible for its higher enzymatic susceptibility (*16*).

Pronase E Incubation. Figure 3 shows the degradation profiles obtained when PHEG−DODASuc and pGlu were incubated with pronase E. This figure demonstrates that, in agreement with the findings of Pytela et al. for relatively high molecular weight PHEG (*19, 20*), the PHEG−conjugate as well as poly-L-glutamic acid are cleaved by this enzyme. On the basis of the plateau values of the amine concentrations, it is calculated that PHEG and pGlu are finally degraded into fragments of 1−2 and 2−3 amino acids, respectively. The higher susceptibility of PHEG for pronase E (final degradation fragments of 1−2 amino acids) as compared to papain (3−4 amino acids) is likely due to the fact that pronase E contains a cocktail of both endo- and exopeptidases.

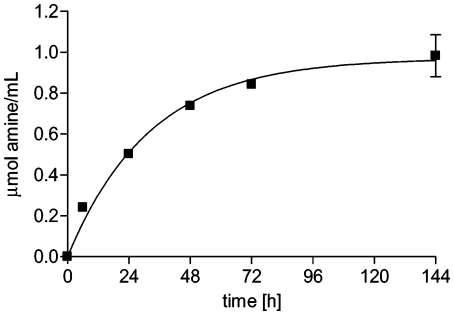

Figure 4. Time course of the concentration of primary amines in cathepsin B-incubated samples of PHEG−DODASuc as determined by the ninhydrin assay (average ± SD of three experiments).

Cathepsin B Incubation. Figure 4 demonstrates the degradability of PHEG−DODASuc by cathepsin B. The number of amine groups increases upon incubation, reaching its maximum value after 144 h. Unfortunately, the final fragment size could not be calculated since the concentration of primary amine groups in a control solution of cathepsin B only increased in time. Likely due to autodigestion, some of the peptide bonds in the enzyme are hydrolyzed, and the formed amine groups give a signal in the ninhydrin assay. In line herewith, an activity assay with a low molecular weight substrate showed that under the selected conditions the activity half-life of cathepsin B was about 30 h. Nevertheless, the concentration of amines in the samples with PHEG−DODASuc was always greater than in the control solution, demonstrating that this conjugate is a substrate for this enzyme. To get insight into the extent of PHEG−DODASuc degradation by cathepsin B, mass spectrometric analysis was done.

Mass Spectrometric Analysis of Enzymatically Degraded PHEG−DODASuc. Figure 5, 6, and 7 show representative mass spectra of papain-, pronase E-, and cathepsin B-degraded PHEG−DODASuc; Table 1 gives the peak assignments. In the spectrum of the nondegraded PHEG−DODASuc (Figure 5a), the peak distributions of the K^+- and the Na^+-adducts are present. The K^+-adduct is abundant since KH_2PO_4 was used in the degradation buffer. In the spectra of PHEG−DODASuc incubated with papain (Figure 5b and 5c), the high mass peaks originating from the nondegraded conjugate disappeared in time, and new peaks were observed. These new peaks have relatively lower masses and increase in their relative abundance in time (most pronounced at $t = 72$ h). A lactam group forms the terminus of the peptide chain in the native conjugate (Figure 1). The observed peaks in the degraded samples can be assigned to degradation fragments of the PHEG-conjugate without this lactam end group and a lower degree of polymerization. For example in the spectrum of the sample incubated for 8 h with papain (Figure 5b), masses of these fragments were between 1400 and 3000 Da, corresponding to fragment size of 4−13 amino acid residues. The observation that the resulting conjugates do not carry a lactam group indeed demonstrates that the peptide chain was hydrolytically cleaved, yielding PHEG−DODASuc (with an NH_2 terminus) and PHEG with COOH and lactam termini as degradation products. The latter products were not detected due to their low molecular weight. In the spectrum of the papain-degraded conjugate after 72 h incubation (Figure 5c) the peak population shifted to low masses of 1000−2300 Da, corresponding

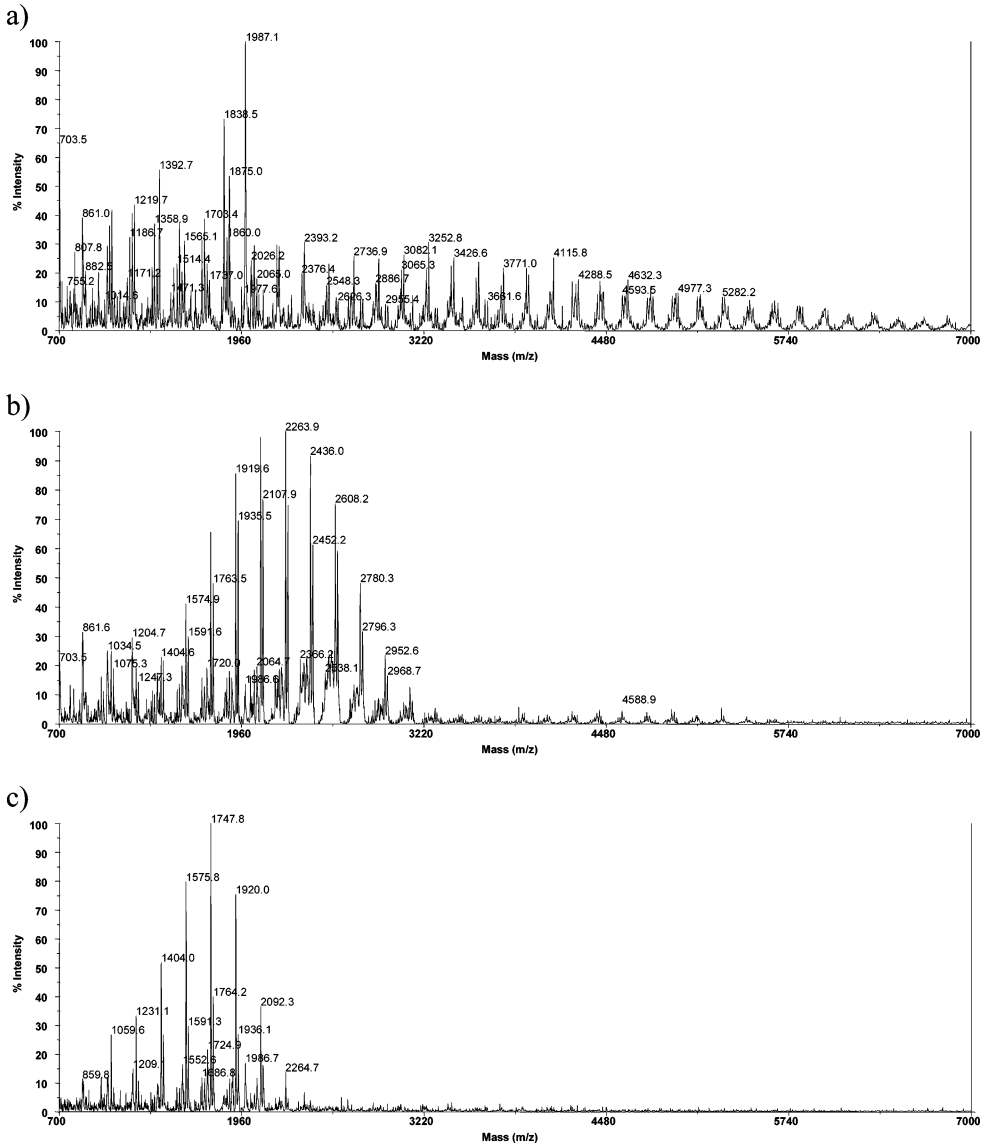

Figure 5. Mass spectra of PHEG−DODASuc (a) and after incubation with papain for 8 h (b) and 72 h (c).

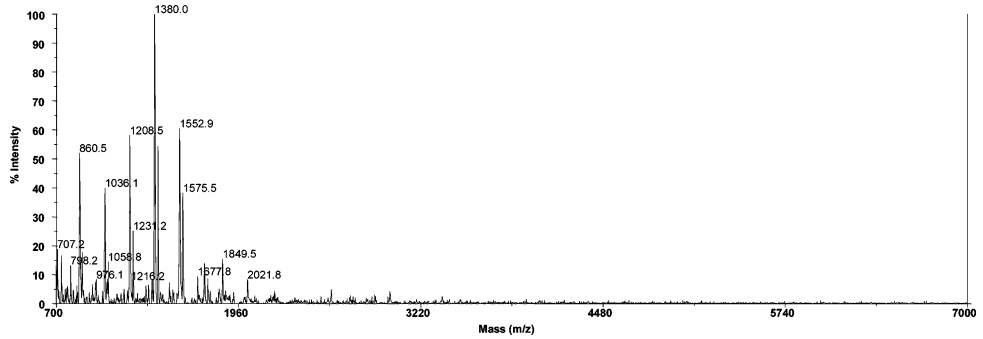

Figure 6. Mass spectrum of PHEG−DODASuc after incubation with pronase E for 216 h.

with fragments of 2−9 amino acids. Figure 6 shows the mass spectrum of PHEG−DODASuc incubated with pronase E. The peak population of the original conjugate disappeared, and peaks of Na^+-adducts of conjugate molecules without the lactam end group appear. Here the Na^+-adducts are the most abundant due to the choice of the buffer. The highest peak represents a fragment size of the DODASuc-anchor with 4 amino acids. Mass spectrometric analysis suggests a lower extent of degradation than the ninhydrin analysis (pronase E-catalyzed degradation of PHEG−DODASuc: compare fragments of 1−2 amino acids based on ninhydrin assay with those found with mass spectrometry analysis (2−5 amino acids)). It should be noted, however, that with the ninhydrin assay both fragments with and without the DODASuc-anchor are detected, whereas in the mass spectrum only the fragments with the anchor were observed. Obviously, the DODASuc-anchor prevents binding of the conjugate to the enzyme when a certain fragment size is achieved. Figure 7 shows the mass spectrum of PHEG−DODASuc degraded by cathepsin B after 144 h of incubation. The highest population of peaks

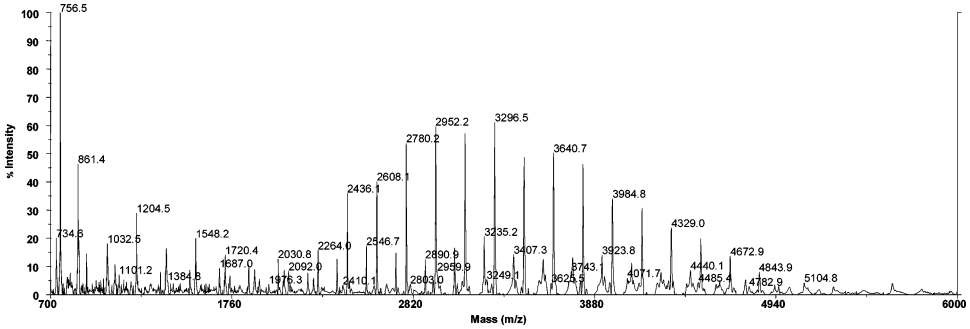

Figure 7. Mass spectrum of PHEG−DODASuc after incubation with cathepsin B for 144 h.

Table 1. Peak Assignment in Mass Spectra of PHEG−DODASuc Incubated with Papain, Pronase E, and Cathepsin B, as Shown in Figures 5, 6, and 7, Respectively

incubation	*m/z*	assignment
papain (t = 0 h)	1186.7	[DODASuc−$(PHEG)_2$-lactam+K]$^+$
	1875.0	[DODASuc−$(PHEG)_6$-lactam+K]$^+$
	2548.3	[DODASuc−$(PHEG)_{10}$-lactam+Na]$^+$
	3252.8	[DODASuc−$(PHEG)_{14}$-lactam+K]$^+$
	4632.3	[DODASuc−$(PHEG)_{22}$-lactam+K]$^+$
	5282.2	[DODASuc−$(PHEG)_{26}$-lactam+Na]$^+$
papain (t = 8 h)	4588.9	[DODASuc−$(PHEG)_{22}$-lactam+H]$^+$
	1075.3	[DODASuc−$(PHEG)_2$+K]$^+$
	1574.9	[DODASuc−$(PHEG)_5$+Na]$^+$
	2107.9	[DODASuc−$(PHEG)_8$+K]$^+$
	2263.9	[DODASuc−$(PHEG)_9$+Na]$^+$
	2796.3	[DODASuc−$(PHEG)_{12}$+K]$^+$
	2952.6	[DODASuc−$(PHEG)_{13}$+Na]$^+$
papain (t = 72 h)	1059.6	[DODASuc−$(PHEG)_2$+Na]$^+$
	1591.3	[DODASuc−$(PHEG)_5$+K]$^+$
	1747.8	[DODASuc−$(PHEG)_6$+Na]$^+$
	1936.1	[DODASuc−$(PHEG)_7$+K]$^+$
	2264.7	[DODASuc−$(PHEG)_9$+Na]$^+$
pronase E (t = 216 h)	1036.1	[DODASuc−$(PHEG)_2$+H]$^+$
	1058.8	[DODASuc−$(PHEG)_2$+Na]$^+$
	1380.0	[DODASuc−$(PHEG)_4$+H]$^+$
	1552.9	[DODASuc−$(PHEG)_5$+H]$^+$
	1575.5	[DODASuc−$(PHEG)_5$+Na]$^+$
cathepsin B (t = 144 h)	2546.7	[DODASuc−$(PHEG)_{10}$-lactam+Na]$^+$
	3235.2	[DODASuc−$(PHEG)_{14}$-lactam+Na]$^+$
	4782.9	[DODASuc−$(PHEG)_{23}$-lactam+Na]$^+$
	2264.0	[DODASuc−$(PHEG)_9$+Na]$^+$
	3296.5	[DODASuc−$(PHEG)_{15}$+Na]$^+$
	4843.9	[DODASuc−$(PHEG)_{24}$+Na]$^+$

can be assigned to Na^+-adducts of degradation fragments without lactam endgroup. The peak population of the Na^+-adduct of the nondegraded conjugate is also present; however, their peak intensities are much lower than those of the degraded conjugate. Masses of the degraded fragments are between 1500 and 5000 Da, corresponding with conjugates of DODASuc with 5 to 25 hydroxylethyl L-glutamine residues. These results demonstrate that under the selected conditions PHEG−DODASuc is degraded by cathepsin B, however, not as considerable as observed with papain or pronase E. Because cathepsin B is rather unstable under the experimental condtions ($t_{1/2}$ = approximately 30 h), maximal fragmentation of PHEG−DODASuc is not achieved. It is therefore expected that under physiological conditions where the enzyme concentration is substantially higher (10−40 mg/mL in lysosomes (*25*), as compared to 0.8 mg/mL in this study) and its turnover is high, PHEG−DODASuc can be degraded further.

Degradation of PHEG Conjugate Grafted onto a Liposome Surface. Polymer-coated (PHEG/PEG) liposomes were prepared by the film-extrusion method. The loss of phospholipids during liposome preparation was approximately 30%; Table 2 shows the physical characteristics (size and charge) of the different liposome preparations. The PHEG-coated liposomes were slightly bigger than the PEG-liposomes. This is probably due to aggregate formation during the ultracentrifugation step (UC induced size increase of ≈30 nm, increase in polydispersity of ≈0.2). The negative values of the zeta potential of the different liposomes are probably due to the low ionic strength (5 mM HEPES, pH 7.0) at which the measurements were done (*33*).

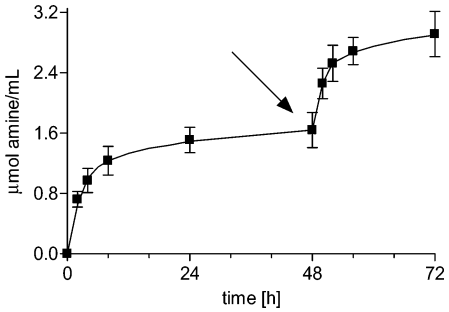

Figure 8. Time course of the concentration of amines in the papain-incubated samples of PHEG−DODASuc-coated liposomes as determined by the ninhydrin assay (average ± SD of three experiments). The arrow indicates the time point when liposomes were solubilized with *n*-octylglucoside and fresh papain was added.

Table 2. Liposome Characteristics

liposome type	mean diameter [nm]	polydispersity index	zeta potential [mV]
PHEG−DODASuc	158 ± 1	0.31 ± 0.03	−29 ± 1
PEG−DSPE	127 ± 1	0.07 ± 0.02	−35 ± 5

Figure 8 shows that the concentration of amines increased in time when liposomes grafted with PHEG−DODASuc were incubated with papain. This means that PHEG−DODASuc grafted onto a liposomal surface is still susceptible for degradation by papain and probably by other proteolytic enzymes. In agreement with expectations, PEG−DSPE coated on the liposomes was not degraded by this enzyme. For PHEG−DODASuc, a plateau in amine concentration was reached after approximately 24−40 h of incubation. In Figure 9 the size and zeta potential of the liposomes as a function of incubation time with papain are shown. For the PEG−DSPE coated liposomes, no significant changes in size and zeta potential upon incubation were observed. However, the PHEG−DODASuc-coated liposomes decreased around 20 nm in size during the first 5−10 h of degradation. The degradation of the conjugate on the liposome surface results in a smaller hydrodynamic diameter and is therefore a possible explanation, but this alone cannot be the reason for the observed drop in size. The end-to-end distance of the peptide part of the conjugate is approximately 6.5 nm; thus, a complete shedding of the peptide would result in a maximum size decrease of ≈13

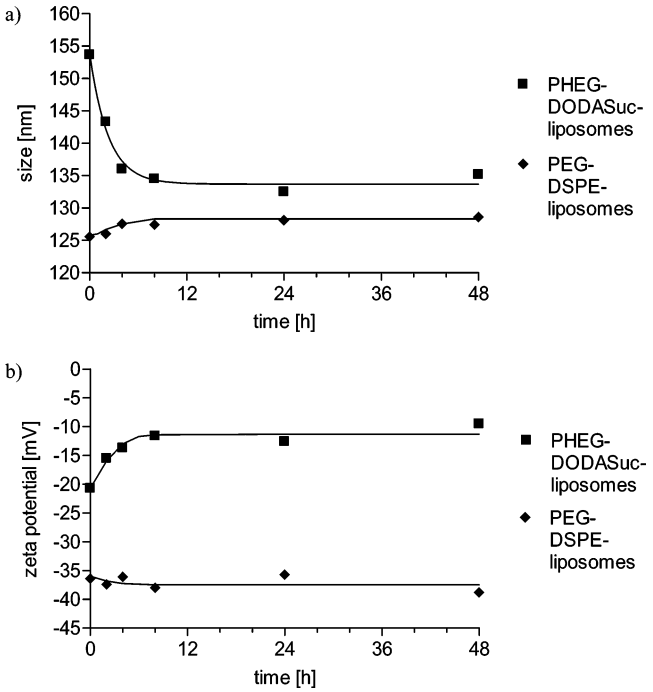

Figure 9. Size (a) and zeta potential (b) of PHEG−DODASuc liposomes (■) and PEG−DSPE liposomes (◆) as a function of incubation time with papain.

nm. As the polydispersity index of the PHEG-coated liposomes decreased from 0.3 to around 0.2 upon incubation with the enzyme, a dissociation of the aggregates, which were formed during ultracentrifugation, is likely to occur as well. The zeta potential of the PHEG-liposomes goes from −20 mV to about −10 mV. Mass spectrometry (Figure 5b/5c) demonstrates that after enzymatic digestion the formed DODASuc-peptide fragment has a terminal amine group (instead of the neutral lactam group of the nondegraded conjugate). This amine group is protonated at pH of 7.0 and likely responsible for the less negative zeta-potential. The progression of changes in size and zeta potential in time are in very good agreement with the progression of the amine content of the samples as seen in the ninhydrin degradation assay (compare Figures 8 and 9). The preparation method applied will result in the formation of unilamellar liposomes (*34*), with polypeptide conjugate present at both the inner and outer layer. It is likely that only the polypeptide present at the outside of the liposomal bilayer is accessible for enzymatic attack. When the liposomes were destabilized by *n*-octylglucoside after 48 h incubation with papain, formation of amines continued (Figure 8). This indicates that indeed only the polypeptide present at the outside of intact liposomes is degraded by papain.

According to the phosphate determination 70% of the initial phospholipid amount is recovered in the final dispersion. Assuming that therefore around 70% of the initial PHEG-conjugate amount is remaining in the liposome dispersions after preparation, the extent of degradation of surface grafted PHEG is the same as that of the conjugate in solution (final degradation products contain 3−4 amino acids). ^{1}H NMR-analysis of the liposome dispersion incubated with papain for 48 h showed that 63 ± 10% of the PHEG was still associated with the liposomal bilayer. As concluded from the data presented in Figure 8, PHEG present at the inside the liposome (≈50% of the totally present polymer) is not accessible for enzymatic attack. From the ^{1}H NMR analysis it can be derived that the PHEG fragment present at the outer layer of the liposomal membrane after enzymatic degradation contains 4−5 amino acid residues. This is in excellent agreement with the data of the enzymatic degradation of the PHEG-conjugate after mass spectrometric analysis.

In conclusion, this paper demonstrates that PHEG−DODASuc is enzymatically degradable even when it is attached to a liposomal surface, avoiding the risk of intracellular accumulation and making liposomes with a sheddable coating feasible.

ACKNOWLEDGMENT

The authors thank Mirjam Damen of the Department of Biomedical Analysis for her valuable assistance with mass spectrometry analysis.

LITERATURE CITED

(1) Sharma, A., and Sharma, U. S. (1997) Liposomes in drug delivery: progress and limitations. *Int. J. Pharm. 154*, 123−140.

(2) Torchilin, V. P., and Trubetskoy, V. S. (1995) Which polymers can make nanoparticulate drug carriers long-circulating? *Adv. Drug Delivery Rev. 16*, 141−155.

(3) Marjan, J. M., and Allen, T. M. (1996) Long circulating liposomes: past, present and future. *Biotechnol. Adv. 14*, 151−175.

(4) Lasic, D. D. (1994) Sterically stabilized vesicles. *Angew. Chem., Int. Ed. Engl. 33*, 1685−1698.

(5) Woodle, M. C., and Lasic, D. D. (1992) Sterically stabilized liposomes. *Biochim. Biophys. Acta 1113*, 171−199.

(6) Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K. (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Controlled Release 65*, 271−284.

(7) Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A., and Barenholz, Y. (1994) Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res. 54*, 987−992.

(8) Torchilin, V. P., Shtilman, M. I., Trubetskoy, V. S., Whiteman, K., and Milstein, A. M. (1994) Amphiphilic vinyl polymers effectively prolong liposome circulation time in vivo. *Biochim. Biophys. Acta 1195*, 181−184.

(9) Woodle, M. C., Engbers, C. M., and Zalipsky, S. (1994) New amphipatic polymer−lipid conjugates forming long-circulating reticuloendothelial system-evading liposomes. *Bioconjugate Chem. 5*, 493−496.

(10) Maruyama, K., Okuizumi, S., Ishida, O., Yamauchi, H., Kikuchi, H., and Iwatsuru, M. (1994) Phosphatidylpolyglycerols prolong liposome circulation in vivo. *Int. J. Pharm. 111*, 103−107.

(11) Mehvar, R. (2000) Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation. *J Pharm. Pharm. Sci. 3*, 125−136.

(12) Caliceti, P., and Veronese, F. M. (2003) Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv. Drug Delivery Rev. 55*, 1261−1277.

(13) Metselaar, J. M., Bruin, P., de Boer, L. W., de Vringer, T., Snel, C., Oussoren, C., Wauben, M. H., Crommelin, D. J., Storm, G., and Hennink, W. E. (2003) A novel family of L-amino acid-based biodegradable polymer−lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. *Bioconjugate Chem. 14*, 1156−1164.

(14) Rypacek, F. (1998) Structure-to-function relationships in the biodegradation of poly(amino acids)s. *Polym. Deg. Stab. 59*, 345−351.

(15) Dickinson, H. R., and Hiltner, A. (1981) Biodegradation of a poly-(alpha-amino acid) hydrogel. II. In vitro. *J. Biomed. Mater. Res. 15*, 591−603.

(16) Chiu, H. C., Kopeckova, P., Deshmane, S. S., and Kopecek, J. (1997) Lysosomal degradability of poly(a-amino acids). *J. Biomed. Mater. Res. 34*, 381−392.
(17) Rypacek, F., Pytela, J., Kotva, R., and Skarda, V. (1997) Biodegradation of poly(amino acid)s: evaluation methods and structure-to-function relationships. *Macromol. Symp. 123*, 9−24.
(18) Yaacobi, Y., Sideman, S., and Lotan, N. (1985) A mechanistic model for the enzymic degradation of synthetic biopolymers. *Life Support Syst. 3*, 313−326.
(19) Pytela, J., Saudek, V., Drobnik, J., and Rypacek, F. (1989) Poly(N5-hydroxyalkylglutamines). IV. Enzymatic degradation of N5-(2-hydroxyethyl)-L-glutamine homopolymers and copolymers. *J. Controlled Release 10*, 17−25.
(20) Pytela, J., Kotva, R., and Rypacek, F. (1998) Enzymatic degradation of poly[N5-(2-hydroxyethyl)-L-glutamine] and poly[N5-(2-hydroxyethyl)-L-glutamine-stat-L-glutamic acid]: analysis of final degradation products. *J. Bioact. Compat. Pol. 13*, 198−209.
(21) Hayashi, T., Tabata, Y., and Nakajima, A. (1985) Biodegradation of poly(a-amino acid) in vitro. *Polymer J. 17*, 463−471.
(22) Koblinski, J. E., Ahram, M., and Sloane, B. F. (2000) Unraveling the role of proteases in cancer. *Clin. Chim. Acta 291*, 113−135.
(23) Trabandt, A., Gay, R. E., Fassbender, H. G., and Gay, S. (1991) Cathepsin B in synovial cells at the site of joint destruction in rheumatoid arthritis. *Arthritis Rheum. 34*, 1444−1451.
(24) Awad, W. M., Jr., Soto, A. R., Siegel, S., Skiba, W. E., Bernstrom, G. G., and Ochoa, M. S. (1972) The proteolytic enzymes of the K-1 strain of Streptomyces griseus obtained from a commercial preparation (pronase). I. Purification of four serine endopeptidases. *J. Biol. Chem. 247*, 4144−4154.
(25) Otto, H. H., and Schirmeister, T. (1997) Cysteine Proteases and Their Inhibitors. *Chem. Rev. 97*, 133−172.
(26) Zhao, G., and Zhou, Z. S. (2001) Vinyl sulfonium as novel proteolytic enzyme inhibitor. *Bioorg. Med. Chem. Lett. 11*, 2331−2335.
(27) Mitchel, R. E., Chaiken, I. M., and Smith, E. L. (1970) The complete amino acid sequence of papain. Additions and corrections. *J. Biol. Chem. 245*, 3485−3492.
(28) Amselem, S., Gabizon, A., and Barenholz, Y. (1993) *Liposome Technology* (Gregoriadis, G., Ed.) pp 501−525, CRC press, Boca Raton, FL.
(29) Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids 5*, 494−496.
(30) Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Med. Sci. 37*, 911−917.
(31) Prochazkova, S., Varum, K. M., and Ostgaard, K. (1999) Quantitative determination of chitosans by ninhydrin. *Carbohydr. Polym. 38*, 115−122.
(32) Miller, W. G. (1961) Degradation of poly-a, L-glutamic acid. I. Degradation of high molecular weight PGA by papain. *J. Am. Chem. Soc. 83*, 259−265.
(33) Moribe, K., Maruyama, K., and Iwatsuru, M. (1997) Estimation of surface state of poly(ethylene glycol)-coated liposomes using an aqueous two-phase partitioning technique. *Chem. Pharm. Bull. (Tokyo) 45*, 1683−1687.
(34) Jousma, H., Talsma, T., Spies, F., Joosten, J. G. H., Junginger, H. E., and Crommelin, D. J. A. (1987) Characterization of liposomes. The influence of extrusion of multilamellar vesicles through polycarbonate membranes on particle size, particle size distribution and number of bilayers. *Int. J. Pharm. 35*, 263−274.

BC0497719