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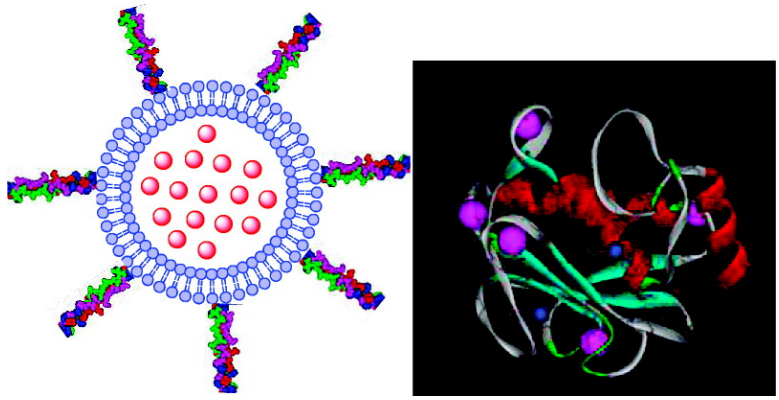

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Mechanistic Studies of the Triggered Release of Liposomal Contents by Matrix Metalloproteinase-9

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Abstract: Matrix metalloproteinases (MMPs) constitute a class of extracellular-matrix-degrading enzymes overexpressed in many cancers and contribute to the metastatic ability of the cancer cells. We have recently demonstrated that liposomal contents can be released when triggered by the enzyme MMP-9. Herein, we report the results of our mechanistic studies of the MMP-9-triggered release of liposomal contents. We synthesized peptides containing the cleavage site for MMP-9 and conjugated them with fatty acids to prepare the corresponding lipopeptides. By employing circular dichroism (CD) spectroscopy, we demonstrated that the lipopeptides, when incorporated into liposomes, are demixed in the lipid bilayers and generate triple-helical structures. MMP-9 cleaves the triple-helical peptides, leading to the release of the liposomal contents. Other MMPs, which cannot hydrolyze triple-helical peptides, fail to release the contents from the liposomes. We also observed that the rate and extent of release of the liposomal contents depend on the mismatch between the acyl chains of the synthesized lipopeptide and phospholipid components of the liposomes. CD spectroscopic studies imply that the observed differences in the release reflect the ability of the liposomal membrane to anneal the defects following the enzymatic cleavage of the liposome-incorporated lipopeptides.

Introduction

Matrix metalloproteinases (MMPs) are a class of Zn^{2+}- and Ca^{2+}-dependent metalloenzymes responsible for the degradation of the extracellular matrix (ECM).[1] These enzymes are normally involved in a variety of physiological processes, e.g., angiogenesis, wound healing, ovulation, etc. The expression levels of these enzymes are tightly regulated at translational and post-translational levels.[2] Overexpression of the MMPs and changing patterns of ECM composition are correlated with many pathological conditions; notable among these are various types of cancers and cardiovascular diseases.[1,3] MMP-2 and -9 are found at elevated levels in many types of metastatic tumors.[4,5]

Among the various drug carriers, liposomes offer several advantages as clinical drug-delivery vehicles, and there are several liposome-mediated drug-delivery products approved for therapeutics and for clinical trials.[6,7] Usually, the encapsulated drugs are passively released to the selected tissue sites upon targeting, and the process is often rather slow. Triggered release of drugs and labeled molecules from liposomes has been recognized to be an attractive therapeutic approach. In this approach, the liposomes do not release their contents until the membranes are destabilized by an external agent (trigger). Consequently, the literature contains several reports on the synthesis of trigger-cleavable lipids, formulation of liposomes, and subsequent release of the encapsulated contents. Changes in pH,[8] mechanical stress,[9] metal ions,[10] temperature,[11] light,[12] enzymes,[13] and conformational changes in peptides[14] have been successfully used as triggers to release liposomal contents.

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For active targeting and subsequent release of liposomal contents, Terada et al.[15] used liposomes functionalized with an MMP-2-cleavable polyethylene glycol (PEG)−peptide−DOPE lipid. Upon interaction with MMP-2, the PEG sheath was removed from the liposomes, and subsequently, the liposomes were internalized by the carcinoma cells. Hatakeyama et al.[16] showed the effectiveness of a similar system and have also used liposomes modified with anti-MMP antibodies to target tumors. Though these systems significantly enhance delivery to tumor sites, they may be susceptible to degradation by the general proteolytic enzymes and other MMPs, which are not localized to tumor tissues. Liposomes with a triple-helical peptide amphiphile have been prepared that bind to CD44 on the cell surface and are internalized by metastatic melanoma cells.[17] Recently, we have demonstrated the release of liposomal contents triggered by the enzyme MMP-9.[18] Our approach is summarized as follows: a triple-helical lipopeptide containing the cleavage site for MMP-9 is synthesized and then incorporated into liposomes (as a minor lipid component). Upon interacting with recombinant human MMP-9, the peptide is cleaved, resulting in destabilization of the liposome bilayer and leakage of the liposomal contents.[18]

Herein, we report the results of our mechanistic studies of the MMP-9-triggered release of liposomal contents. The triple-helical lipopeptides were synthesized employing an automated, microwave-assisted peptide synthesizer, resulting in a drastic reduction in the synthesis time as well as major improvements in the quality of the crude product.[19] Using fluorescence and circular dichroism (CD) spectroscopy, we have demonstrated that the lipopeptides acquire triple-helical conformations in the liposomes and that the release of the liposomal contents is due to enzymatic cleavage of the peptide by MMP-9. We have observed that the rate and extent of leakage of the contents are dependent on MMP-9 concentration. In addition, we have shown that the degree of mismatch between the lipid acyl chains of the lipopeptides and the major phospholipid component of the liposomes determines the extent of leakage of the contents. The lipid-acyl-chain dependence of release from the liposomes offers new insights into the mechanism of destabilization of the liposomes by MMP-9 and provides an additional method for fine-tuning the triggered-release system. Taken together, these observations suggest that the enzyme-triggered release of the liposomal contents can be controlled at multiple levels.

Experimental Section

Synthesis of the Lipopeptides. The peptides were synthesized (0.1 mmol scale) using a microwave-assisted peptide synthesizer (Liberty Peptide Synthesizer, CEM Corporation, Matthews, NC) employing standard Fmoc-based solid-phase chemistry. The reactions were conducted inside a 30 mL vessel enclosed within the microwave unit (CEM Discover) of the peptide synthesizer. Commercially available Fmoc−Gly−CLEAR acid resin (Peptides International, Louisville, KY) was used as the solid support and HBTU/HOBT as the coupling reagent. Each coupling step (except for Arg) was performed at 55 °C using 20 W of microwave power for 5 min with a fivefold excess of reagents. The amino acid Arg and the fatty acids at the N-terminal ends of the peptides were subjected to double coupling. The coupling of Arg was performed at 25 °C for 25 min without any microwave power. The activator base used was 5 equiv of diisopropylethylamine in *N*-methylpyrrolidone solvent. The Fmoc groups were deprotected with 5 wt % piperazine in DMF in two treatments, at 30 s and 3 min. A microwave-assisted cleavage from the solid support was performed using a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/water (95:2.5:2.5) for 35 min. The crude peptides were precipitated using ice-cold ether, collected by centrifugation, and washed with cold ether. The white solids obtained by precipitation were purified by reversed-phase HPLC using a Vydac semipreparatory diphenyl column (RP 219TP510) with a linear gradient of 0−70% acetonitrile in water over 60 min at a rate of 8 mL/min (both solvents contained 0.1% TFA). The collected, purified fractions were analyzed using a Vydac analytical diphenyl column (219TP5415) with a linear gradient of 0−70% acetonitrile in water (both solvents containing 0.1% TFA) as the eluant. The flow rate was maintained at 1.5 mL/min for 25 min. The purified peptides were characterized by CD and matrix-assisted laser desorption ionization−time-of-flight (MALDI−TOF) mass spectroscopy.

Liposome Preparation. Stock solutions of the lipopeptide in 9:1 chloroform/methanol were prepared by weighing appropriate amounts of the lipopeptide and dissolving them in glass vials to concentrations of 1 mg/mL. The phospholipids were in 2.5 mg/mL stock solutions in chloroform. Lipid thin films were prepared by transferring the appropriate volumes of the respective lipids into 10 mL round-bottom flasks with Hamilton gastight syringes and adjusting the volume to 5 mL with chloroform. The organic solvents were removed under reduced pressure in a rotary evaporator (Yamato RE 450) at 42 °C with rotation of the flask at 220 rpm. The thin lipid films were kept under vacuum for 24 h to remove residual organic solvents.

The lipid thin film containing the phospholipid and lipopeptide was hydrated in the round-bottom flask by addition of 2 mL of the appropriate buffer solution followed by rotation for 1 h at 220 rpm at 60 °C. The hydrated lipid suspension was then kept at 4 °C for 4 h to ensure proper hydration and folding of the triple-helical peptides, after which it was extruded through a 100 nm pore size polycarbonate membrane at 60 °C to obtain large unilamellar

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vesicles. The size distribution and ζ potential of the liposomes were determined with a Mavern Instruments Nano ZS 90 zetasizer.

The liposomes used for the carboxyfluorescein-release experiments were hydrated as mentioned above with buffer containing 100 mM carboxyfluorescein. The unencapsulated carboxyfluorescein was separated from the liposomes by gel filtration through a Sephadex G100 column. Prior to passing the liposomes through the column, the column was equilibrated with 25 mM HEPES buffer (pH 8.0) whose osmolarity had been adjusted to match that of the carboxyfluorescein-containing buffer with which the liposomes were hydrated. Adjustment of osmolarity was performed by measuring the osmolarity of the solutions using an Advanced Instruments Micro-Osmometer and adjusting the lower-osmolarity solution with addition of solid NaCl. Thus, the buffers that were used to equilibrate the gel filtration columns and to elute the liposomes had osmolarities matching that of the buffer used to hydrate the liposomes. This ensured that background leakage of carboxyfluorescein from the liposomes was minimized.

Buffer Solutions. The lipid thin films were hydrated with different buffer solutions depending on the experiments for which they were used. For the CD experiments, the buffer used was 5 mM phosphate buffer maintained at pH 8.0. For the carboxyfluorescein-release experiments, the buffer solution used to hydrate the liposomes was 25 mM HEPES (pH 8.0) containing 100 mM carboxyfluorescein, 10 mM $CaCl_2$, and 10 μM $ZnCl_2$. For equilibration of the column and elution of liposomes from the column, 25 mM HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$, 10 μM $ZnCl_2$, and NaCl was used. The same buffer was used for the fluorescence measurements to determine carboxyfluorescein release.

CD Spectroscopy Measurements. All of the CD spectra were collected on a Jasco J815 CD spectrometer in a 1 mm path length quartz cuvette at 25 °C. Liposome solutions containing 1 mg/mL total lipid were transferred into the cuvette. The CD spectra were measured in the continuous scanning mode (270−180 nm) with a data pitch of 0.2 nm and a scan speed of 100 nm/min. The response time was set at 0.25 s. For each sample, the average of 50 scans was recorded and corrected for the spectrum of buffer only. The Rpn values were calculated from the CD spectra as the ratio of maximum ellipticity (at 225 nm) to minimum ellipticity (at 200 nm).

Release Studies. The rate and extent of release of carboxyfluorescein from the liposomes were monitored by following the carboxyfluorescein fluorescence emission intensity at 518 nm with an excitation wavelength of 480 nm. In a typical experiment, recombinant human MMP-9 (20 μL from a 25 μM stock solution) was added to a solution of liposomes in buffer [25 mM HEPES (pH 8.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$] in a total volume of 200 μL, and the fluorescence intensity was measured every 20 s over a period of 2 h at 25 °C. The experiments were set up in triplicate in 96-well fluorescent microplates, and the data were collected with a Molecular Devices SpectraMax plate reader. The fractional release was determined by comparing the fluorescence intensity of the liposome solution to the intensity of a solution containing an equal concentration of liposomes in the presence of 1% Triton X. Higher concentrations of Triton X did not cause a further increase in the fluorescence intensity of the carboxyfluorescein. Hence, this concentration was deemed sufficient to cause complete release of the liposome contents. The fractional release was calculated as the ratio of the difference between the fluorescence intensity of carboxyfluorescein at time t (I_t) and its initial intensity at $t = 0$ (I_0) and the difference between the intensity upon treatment with 1% Triton X (I_{triton}) and the intensity at $t = 0$, as shown in the following equation:

$$\text{fractional release} = \frac{I_t - I_0}{I_{\text{triton}} - I_0}$$

Results

Liposome Characteristics. The lipopeptides used in these studies along with their calculated and observed molecular masses are shown in Table 1. Figure 1 shows the structure of **LP1** and the cleavage site for MMP-9 in the peptide. Using dynamic light scattering experiments, we found that the liposomes incorporating 30 mol % lipopeptide have a unimodal size distribution with a mean diameter of 135 ± 30 nm. The ζ potential measurements revealed that the liposomes formulated with 30 mol % lipopeptide **LP1** or **LP2** and 70 mol % POPC, DSPC, DOPC, or DMPC have essentially the same unimodal ζ potential of −10 mV.

Table 1. Amino Acid Sequences, Acyl Chains, and Calculated and Observed Molecular Masses of the Lipopeptides Used in This Study

lipopeptide	peptide sequence	acyl chain	calcd M^+	obsd M^+
LP1	GPQGIAGQR$(GPO)_4$GG−COOH	stearic acid	2332.24	2332.27
LP2	GPQGIAGQR$(GPO)_4$GG−COOH	oleic acid	2330.25	2331.20 (MH^+)
LP3	GPKGIAGQK$(GPO)_4$GG−COOH	palmitic acid	2276.21	2277.15 (MH^+)

Mechanism of Liposome Destabilization. First, we sought to optimize the liposome formulation for minimum background leakage and high sensitivity to MMP-9. Carboxyfluorescein-encapsulating liposomes containing 10, 20, and 30 mol % lipopeptide **LP1** and 90, 80, and 70 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), respectively, were prepared. In 25 mM HEPES buffer (pH 8.0), the liposomes formulated with 10 and 20 mol % **LP1** released more than 20% of their contents in 2 h in the absence of any added enzyme. In contrast, the liposomes formulated with 30 mol % **LP1** released less than 3% of the dye in the absence of MMP-9. When we incorporated 40 mol % **LP1** into the liposomes, the release of the encapsulated contents in the absence of MMP-9 was low (<5%). However, in the presence of MMP-9, only 40% of the encapsulated dye was released. Hence, further experiments were conducted with the liposomes containing 30 mol % lipopeptide **LP1**. As will be shown later, the incorporated lipopeptides were demixed and formed domains within the lipid bilayers of the liposomes. When we formulated liposomes containing 20 mol % or less of lipopeptide **LP1**, it is possible that large numbers of small domains were formed and the leakage took place from the domain boundaries.

Next, we proceeded to determine whether the lipopeptides acquired the triple-helical conformation when incorporated into the liposomes. In this endeavor, the CD spectrum of liposomes containing 30 mol % lipopeptide **LP1** and 70 mol % POPC was recorded in 5 mM phosphate buffer (pH 8.0). The spectrum (Figure 2, black trace) shows a pronounced negative peak at 200 nm and a less-intense positive peak at 225 nm, characteristic of triple-helical collagen-related peptides and their fatty acid conjugates.[17,20,21] The presence of these two peaks in the CD spectrum indicates that lipopeptide **LP1** displays a triple-helix conformation even when incorporated in the liposomes.

Quantitative analysis revealed that the Rpn value of the lipopeptide (i.e., the ratio of the intensities of the CD maximum

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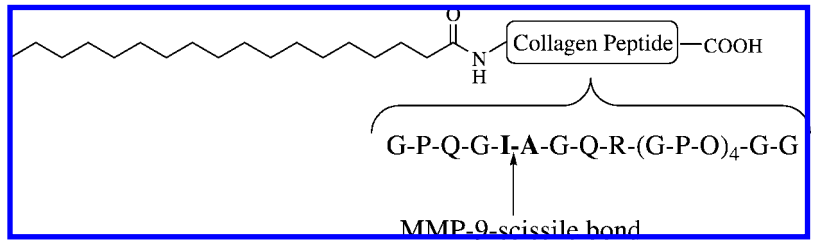

Figure 1. Structure of the lipopeptide **LP1**; the cleavage site for MMP-9 is shown.

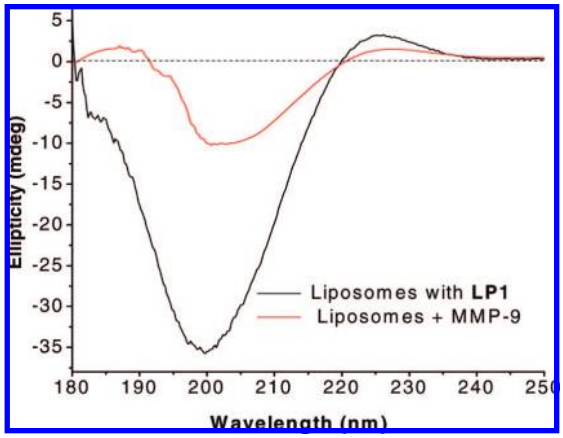

Figure 2. Far-UV CD spectra of liposomes [5 mM phosphate buffer (pH 8.0)] formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1**. The CD spectra of the liposomes were recorded (black) before or (red) 30 min after incubation with MMP-9. Each spectrum represents the average of 50 scans.

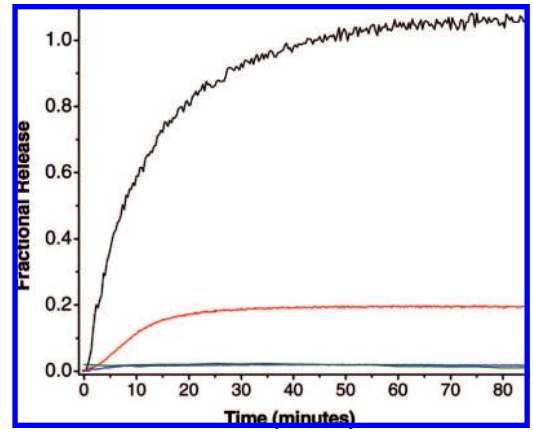

Figure 3. MMP-9-triggered leakage of encapsulated carboxyfluorescein from liposomes formulated with POPC and MMP-9-cleavable lipopeptides. The kinetic trace of carboxyfluorescein fluorescence (λ_{ex} = 480 nm, λ_{em} = 518 nm) was monitored for 85 min for liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1** in the presence of 2.3 μM MMP-9. The reactions were conducted at 25 °C in 25 mM HEPES buffer (pH 8.0) in the (black) presence or (red) absence of 10 mM $CaCl_2$ and 10 μM $ZnCl_2$. The blue trace shows the control experiment using liposomes formulated with 100 mol % POPC. The green trace (which partially overlaps the blue trace) represents the leakage from liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP3**. Each trace shown is the average of three experiments.

at 225 nm and CD minimum at 200 nm)[21] was 0.07. This indicates that a substantial fraction of the lipopeptides within the liposomes acquire the triple-helical conformation.[20,21] When the liposomes were incubated with 3 μM recombinant human MMP-9 for 30 min in 5 mM phosphate buffer (pH 8.0), pronounced reductions in the intensities of both the 200 and 225 nm peaks were observed (Figure 2, red trace). In the latter experiment, the CD spectrum of MMP-9 was subtracted to remove the contribution of the enzyme spectrum. These observations suggest that MMP-9 is able to cleave the liposome-incorporated lipopeptide **LP1** within 30 min. Unfortunately, we could not obtain a kinetic trace of the rate of reduction of either the 200 or 225 nm peak with a good signal-to-noise ratio.

We proceeded to determine the effect of lipopeptide cleavage on the stability of the liposomes. Liposomes containing 30 mol % **LP1** and 70 mol % POPC and encapsulating the self-quenching dye carboxyfluorescein were prepared.[22] The kinetics of carboxyfluorescein release from these liposomes was measured in the presence of 2.3 μM MMP-9 in 25 mM HEPES buffer (pH 8.0). We observed complete release of the encapsulated dye within 80 min in the presence of MMP-9 (Figure 3, black trace). In order to demonstrate that the release of the liposomal contents was due to cleavage of **LP1** by MMP-9 and subsequent destabilization of the membrane, we conducted the following control experiments. Liposomes encapsulating carboxyfluorescein were formulated with POPC only, and the dye release from these liposomes was measured in the presence of 2.3 μM MMP-9. We observed that only 2% of the encapsulated dye was released after 2 h (Figure 3, blue trace). Next, we formulated the liposomes encapsulating carboxyfluorescein with 30 mol % **LP1** and 70 mol % POPC, but $CaCl_2$ and $ZnCl_2$ were omitted from the buffer. The release of carboxyfluorescein from these liposomes in the presence of 2.3 μM MMP-9 measured in Ca^{2+}- and Zn^{2+}-free buffer is also shown in Figure 2 (red trace). Less than 20% of the liposomal contents was released when the buffer did not contain any $ZnCl_2$ and $CaCl_2$. Since MMP-9 is known to require Ca^{2+} ions to maintain its full catalytic activity,[23] the reduced leakage was likely due to low catalytic activity of the enzyme, resulting in its inability to cleave the lipopeptide and subsequently destabilize the liposomes.

As a further test of our hypothesis, we formulated liposomes with 70 mol % POPC and 30 mol % lipopeptide **LP3**. This peptide has two lysine residues in its sequence, replacing a glutamine residue and an arginine residue of lipopeptide **LP1** (Table 1). MMP-9 cleaves triple-helical polypeptide substrates between the glycine and isoleucine residues.[24] It has been demonstrated that nonpolar residues are preferred in the P2 position (where a lysine was substituted for a glutamine) and that introduction of basic or acidic residues in this position leads

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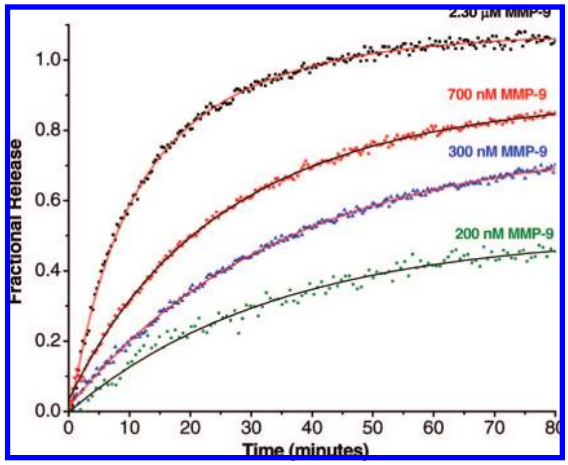

Figure 4. Dependence of the release of liposomal contents on the concentration of MMP-9. The kinetic traces of carboxyfluorescein fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 518$ nm) were recorded for 85 min for liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1** in the presence of 2.3 μM (black squares), 700 nM (red diamonds), 300 nM (blue triangles), and 200 nM (green squares) MMP-9. The experiments were conducted at 25 °C in 25 mM HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$. The solid lines are single-exponential fits of the data by nonlinear regression.

Table 2. Dependence of Carboxyfluorescein Release from Liposomes on MMP-9 Concentration

MMP-9 concentration (μM)	rate constant (min^{-1})	fractional release
0.20	0.029 ± 0.008	0.51 ± 0.01
0.30	0.028 ± 0.001	0.76 ± 0.03
0.70	0.039 ± 0.002	0.84 ± 0.02
2.30	0.075 ± 0.001	1.03 ± 0.09

to reduction in cleavage activity of the MMP by up to 14-fold.[25] Hence, we expected MMP-9 to demonstrate reduced catalytic activity for the lipopeptide **LP3** and consequently cause less dye release from liposomes formulated with this lipopeptide than from liposomes prepared with **LP1**. The release studies with liposomes incorporating **LP3** in the presence of 2.3 μM MMP-9 revealed that less than 5% of the contents were released after 2 h (Figure 3, green trace). These results demonstrate that the carboxyfluorescein release in the presence of MMP-9 was indeed due to destabilization of the lipopeptide-containing liposomes triggered by the cleavage of the lipopeptides by the enzyme.

Dependence of Release of Liposomal Contents on MMP-9 Concentration. A precondition for successful targeted delivery of drugs or diagnostic agents to sites overexpressing MMP-9 is that the release of the liposomal contents be dependent on the concentration of MMP-9. To test whether our system demonstrates this desirable property, we measured the release of encapsulated carboxyfluorescein from liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1** as a function of the concentration of MMP-9. Figure 4 shows kinetic traces for the release of carboxyfluorescein from the liposomes in the presence of 0.20, 0.30, 0.70, and 2.30 μM of enzyme.

We observed that both the extent and rate of contents release from the liposomes increased with increasing MMP-9 concentration. The traces in Figure 4 could be fit by the single-exponential rate equation $F = F_0 - Ae^{-kt}$, where F represents fractional release, to obtain the rate constants (k) for the dye release and the amplitudes (A), which represent the extent of release from the liposomes. The values of the rate constants and extents of release obtained from these traces are summarized in Table 2. Clearly, the rate constants and extents of contents release from the liposomes depend on the concentration of the active MMP-9. In the presence of 200 nM MMP-9, we observed that 50% of the encapsulated dye was released. Given that MMP-9 expression is several-fold higher in tumors and arthritic joints than in normal tissues (for some lung cancer patients, the concentration of MMP-9 in bronchial lavage fluid can be as high as 100 nM; in arthritic joints, MMP-9 concentrations in the range 100−200 nM have been reported),[26] we expect that the release of contents from our liposomes will be more prevalent in the abnormal tissues.

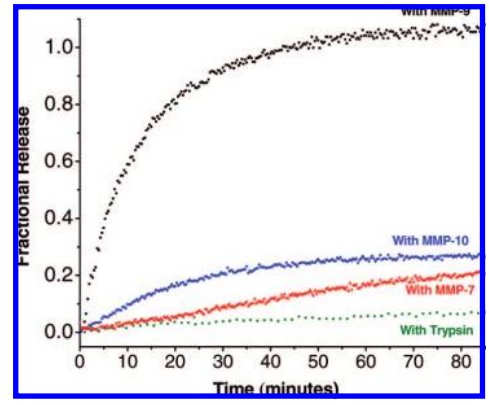

Figure 5. Comparison of the release of liposomal contents triggered by MMP-7, -9, or -10 or by trypsin. The kinetic traces for the carboxyfluorescein fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 518$ nm) were recorded for 85 min for liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1**. The reactions were conducted at 25 °C in 25 mM HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$, 10 μM $ZnCl_2$, and 2.3 μM MMP-9 (black squares), MMP-10 (blue diamonds), MMP-7 (red triangles), or trypsin (green squares).

Effect of Noncollagenase MMPs. The premise for selecting a collagen-related triple-helical peptide for our strategy was the susceptibility of such peptides to MMP-9 cleavage combined with their resistance to cleavage by noncollagenase MMPs or other proteolytic enzymes. Hence, the release of the liposomal contents should not be triggered by MMP-7 or -10 (both of which lack the ability to hydrolyze triple-helical peptide substrates[27]) or by trypsin. In order to test this hypothesis, release of carboxyfluorescein from liposomes formulated with 70 mol % POPC and 30 mol % lipopeptide **LP1** was measured in the presence of each of these enzymes (Figure 5). We observed that 2.3 μM MMP-7 released only 15% of the encapsulated dye (Figure 5, red triangles) and that 2.3 μM MMP-10 was able to release ~20% of the dye (Figure 5, blue diamonds) in 85 min. Trypsin also released less than 10% of the encapsulated dye from the liposomes (Figure 5, green squares).

Effect of Lipid Acyl Chain Mismatch on Carboxyfluorescein Release. In biomembranes, the distribution of lipid and protein components is not random, and components may form domains whose lifetimes may be very short (on the order of 10 μs) or

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much longer.[28] In model membranes containing two lipids, the formation and stability of the domains depend on differential interactions between the two lipid components.[29] The difference in the interaction energies of two similar lipids and two different lipids provides the thermodynamic driving force for the formation of the domains.[29,30] In our contents-release studies, the liposomal membrane was composed of 70 mol % POPC and 30 mol % lipopeptide **LP1**. Considerable hydrophobic mismatch exists between the stearoyl chain (18:0) of **LP1** and the acyl chains of the major lipid component of these liposomes (POPC). POPC has a palmitoyl chain (16:0) and an oleoyl chain (18:1) in the structure. Hence, we expected some degree of demixing of POPC and the lipopeptides within the lipid bilayers of the liposomes.[31]

When the lipopeptide is incorporated into the liposomes, the formation of collagen-like triple helices by lipopeptide molecules is subject to the availability of lipopeptide neighbors in the immediate vicinity. The increase in the number of lipopeptide nearest neighbors shifts the monomer/trimer equilibrium toward the latter.[20] In addition, upon enzymatic cleavage of the lipopeptides, the membrane destabilization should be affected by the amount of demixing of the phospholipids and the lipopeptides. The cleavage of isolated lipopetide molecules in the liposomal membrane should produce "transient" destabilization of the membrane that can be "healed" quickly by the surrounding phospholipid molecules. However, the cleavage of "patches" of lipopeptides in areas with high local concentrations of lipopeptide molecules will produce larger defect areas on the membrane; the surrounding phospholipid molecules will take a longer time to "anneal" these larger defects. Thus, we hypothesized that the major phospholipid used in the liposomal formulation will significantly affect the MMP-9-triggered leakage of the contents.

To study the effect of the acyl chains of the major lipids on MMP-9-triggered release, liposomes encapsulating carboxyfluorescein were formulated with 30 mol % lipopeptide **LP1** and the major lipid component (70 mol %) of the liposomes was varied [POPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was used]. This ensured that the concentration of the major lipid (i.e., POPC, DOPC, or DSPC) in the liposomes was equivalent in all of the formulations. Figure 6 shows the kinetic traces for the release of the encapsulated dye from these liposomes in the presence of 700 nM MMP-9.

For the liposomes formulated with the lipopeptide **LP1**, the greatest extent of contents release was observed when POPC was used as the major lipid (Figure 6, black squares). Using DOPC as the major lipid reduced the rate as well as the amount of dye release (Figure 6, red diamonds). When we used DSPC (which contains stearoyl chains, the same acyl chain as in lipopeptide **LP1**) as the major lipid component of the liposomes, the extent of release was very small (Figure 6, blue triangles). The kinetic traces for DOPC and DSPC liposomes could not be fit by the single-exponential rate equation. A double-exponential fit of the release data for the DSPC liposomes showed a total amplitude of 0.1, indicating that only 10% of

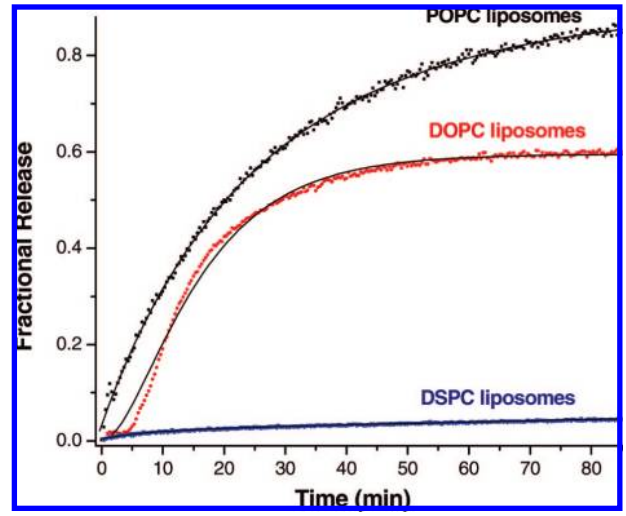

Figure 6. Dependence of MMP-9-triggered release from liposomes on the major phospholipid component. The kinetic traces for carboxyfluorescein fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 518$ nm) were recorded for 85 min for liposomes formulated with 30 mol % lipopeptide **LP1** and 70 mol % POPC (black squares), DOPC (red diamonds), or DSPC (blue triangles). The reactions were conducted at 25 °C in 25 mM HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$ in the presence of 700 nM MMP-9. The solid black lines are single-exponential fits of the data by nonlinear regression.

the encapsulated dye was released from the liposomes over the 2 h interval, compared to 87% and 72% for the POPC and DOPC liposomes, respectively (Table 3). Release from the DOPC liposomes in the presence of MMP-9 proceeded with a lag phase; we do not yet understand the molecular mechanism for the origin of this lag phase. The discrepancy between the observed data points and the fitted curve for the DOPC liposomes indicates that the release from these liposomes is kinetically more complex than a double-exponential rate equation.

The data suggests that acyl-chain matching between the lipopeptide and major phospholipid components plays a significant role in determining the rate and the extent of MMP-9-triggered release of the liposomal contents. Another factor that may also contribute to the noted differences in release is the difference in the phase composition of the liposome membranes. DSPC bilayers ($T_m = 55$ °C) are expected to be in the gel phase at room temperature, while DOPC ($T_m = -20$ °C) and POPC ($T_m = -3$ °C) bilayers should be in their liquid-crystalline states at room temperature.[32,33]

In order to determine if the lipid phase affects MMP-9-triggered liposomal contents release, we synthesized the lipopeptide **LP2**. **LP2** has the same peptide sequence as **LP1**, but the acyl chain contains the oleoyl moiety (Table 1). Liposomes encapsulating carboxyfluorescein were formulated with 30 mol % **LP2** and 70 mol % POPC, DOPC, or DSPC, and the contents-release studies were conducted in the presence of 700 nM MMP-9 (Figure 7). When POPC was used as the major lipid, 22% of the encapsulated dye was released from the liposomes in 1 h (Figure 7, blue squares). Similarly, for the liposomes with DOPC as the major lipid component, we

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Table 3. Summary of the Kinetic Parameters for Liposomes Formulated with Stearic and Oleic Acid Lipopeptide Conjugates **LP1** and **LP2** and Various Major Phospholipid Components in the Presence of 700 nM MMP-9

	POPC liposomes		DOPC liposomes		DSPC liposomes[a]	
lipopeptide	rate (min^{-1})	amplitude	rate (min^{-1})	amplitude	rate (min^{-1})	amplitude
LP1 (stearic acid conjugate)	0.036 ± 0.02	0.87 ± 0.02	0.063 ± 0.006	0.72 ± 0.05	0.13 ± 0.01 0.004 ± 0.001	0.016 ± 0.003 0.086 ± 0.001
LP2 (oleic acid conjugate)	0.16 ± 0.01	0.22 ± 0.02	0.09 ± 0.01	0.27 ± 0.02	0.09 ± 0.001	0.70 ± 0.01

[a] The two sets of kinetic parameters for DSPC with **LP1** were obtained using a double-exponential fit of the data.

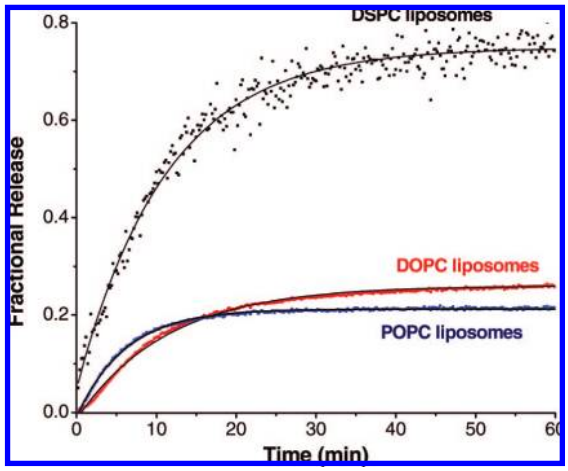

Figure 7. Dependence of MMP-9-triggered release from liposomes formulated with lipopeptide **LP2** on the acyl chains of the major phospholipid. The kinetic traces of carboxyfluorescein fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 518$ nm) were recorded for 60 min for liposomes formulated with 30 mol % lipopeptide **LP2** and 70 mol % DSPC (black squares), DOPC (red circles), or POPC (blue squares). The reactions were conducted at 25 °C in 25 mM HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$ and 10 μM $ZnCl_2$ in the presence of 700 nM MMP-9. The solid black lines are single-exponential fits of the data by nonlinear regression.

observed 27% release of the contents in 1 h (Figure 7, red circles). However, when the major lipid had the acyl chains mismatched compared to the lipopeptide **LP2** (i.e., when DSPC was used as the major lipid), 70% of the dye was released in 1 h (Figure 7, black squares). The amplitudes and single-exponential rate constants obtained from quantitative analyses of the data are given in Table 3. The data presented in Figures 6 and 7 provide confirmation that the triggered release of the liposomal contents is favored by a greater degree of acyl chain mismatch between the lipopeptide and major phospholipid component of the liposomes.

Circular Dichroism Studies. As discussed above, MMP-9-triggered carboxyfluorescein release from the liposomes is favored when the acyl chain of the lipopeptide does not match with that of the major phospholipid in the liposomes. We considered that this could be due to one or a combination of two related mechanisms. The incorporation into the lipid bilayer of phospholipids and lipopeptides having mismatched chains could favor the formation of domains and tight collagen-like triple helices in the lipopeptide domains (due to high local concentrations of the lipopeptides). The concentration dependence of triple-helix formation by collagen-related peptides has been previously demonstrated.[20,21] Such a higher degree of triple helicity would in turn create an additional kinetic barrier for unwinding and subsequent cleavage of the lipopeptides by MMP-9. It is also possible that the increased carboxyfluorescein release from the acyl-chain-mismatched liposomes is due to the decreased annealing ability (after the cleavage of the lipopeptides by MMP-9) of these membranes relative to those with matched acyl chains. To determine which of these two possible mechanisms is responsible for the observed acyl-chain dependence of liposomal release, we determined the Rpn values[20] of the lipopeptides in different liposome formulations using CD spectroscopy (Table 4). The Rpn value indicates the presence of triple-helical conformations of peptides, with larger values implying higher degrees of triple helicity.[20,21]

Table 4. Rpn Values for Lipopeptides Incorporated into the Liposomes

	POPC liposomes	DSPC liposomes	DOPC liposomes
LP1	0.067	0.085	0.120
LP2	0.065	0.097	0.083
LP3	0.039	0.085	0.041

For the liposome-incorporated lipopeptide **LP1**, the Rpn values were 0.067 and 0.085 for liposomes with POPC and DSPC, respectively, as the major lipids. For liposomal **LP2**, the Rpn value was 0.097 when the major phopholipid was DSPC and 0.065 when the major phospholipid was POPC. Clearly, there was no correlation between the acyl-chain mismatch and the calculated Rpn values for the lipopeptides in the liposomes. Thus, it appears that the higher degree of triple helicity of the incorporated lipopeptides did not contribute to the reduced extent of contents release for the acyl-chain-mismatched liposomes. It is likely that when the acyl chains of the lipopeptides and the major lipid component of the liposomes are matched, the defects created by the enzymatic cleavage of the lipopeptides are annealed quickly, leading to the reduction of the extent of release of the encapsulated dye from these liposomes.

Discussion

For most of the literature reports on MMP-directed tumor targeting using liposomes, the goal was to enhance the uptake of the intact liposomes into the cells in order to deliver the encapsulated cytotoxic agents.[15,16] Uptake of liposomes by endocytosis usually exposes the liposomes to the acidic lysosomal environment, which often degrades the encapsulated drug.[34] Our goal is to deliver the drugs (especially inhibitors for MMPs and other extracellular cancer-associated enzymes) in the ECM, employing the extracellular MMPs as the triggers. In addition, the compounds released in the tumor ECM by means of our methodology should be able to diffuse through the membranes of nearby cells if the molecules have favorable partition coefficients.[35]

The triple-helical conformation for collagen-mimetic lipopeptides has previously been observed in solution.[20,21] However, incorporation of the lipopeptides in liposomes may not render them triple-helical. For instance, because of restrictions imposed

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by the neighboring phospholipid or lipopeptide molecules in the membrane, the conformational entropy of the lipopeptides may be reduced upon incorporation into the lipid bilayers of the liposomes.[36] The CD spectra of the lipopeptide-containing liposomes (Figure 1) show that this is not the case. For both lipopeptides **LP1** and **LP2**, the CD spectra show pronounced minima at 200 nm and positive peaks at 225 nm, characteristic of the triple-helical conformation.[20,21] Upon incubation of the liposomes with MMP-9, we observed reductions of the intensity for both the negative and positive peaks (Figure 1). Clearly, upon cleavage of the liposome-incorporated lipopeptides, the triple helicity is reduced. Because of high background noise and light scattering from the liposomes, the CD spectra shown in Figure 1 were recorded using a 1 mm path length cuvette and averaged over 50 scans. As a result of the unfavorable signal-to-noise ratio, our attempts to follow the kinetics of enzymatic cleavage of the liposome-incorporated lipopeptides by CD spectroscopy were unsuccessful.

To determine the effects of peptide cleavage and the subsequent release of the liposomal contents, we employed the fluorescence emission signal from the self-quenching dye carboxyfluorescein.[22] Carboxyfluorescein has been used extensively to monitor the kinetics of release of encapsulated aqueous contents from liposomes.[37,38] At high concentrations (such as those within the liposomes), the fluorescence emission of the dye is self-quenched. Release of the dye from the liposomes to the surrounding medium results in dequenching of its fluorescence emission.[22] Consequently, a large enhancement in the fluorescence emission intensity of carboxyfluorescein is observed. We determined that the increase in emission intensity of carboxyfluorescein is linear in the concentration range 3−30 μM (data not shown) and that the dye is not photobleached during the course of our release studies (90 min). The complete release of the liposome-encapsulated carboxyfluorescein was achieved by the addition of 1% of a detergent (Triton X), which destabilizes the membrane.[39]

Our studies with liposomes formulated with POPC and the lipopeptide **LP1** showed that complete release of the contents occurred in 40 min with 2.3 μM MMP-9. During these experiments, we included 10 mM $CaCl_2$ and 10 μM $ZnCl_2$ in the assay buffer to maintain the optimal enzyme activity.[23] However, some peptides and proteins are known to partition into or cross the membranes of liposomes. Such transient membrane perturbations can cause the release of contents from the liposomes. For example, δ-lysin (a hemolytic staphylococcal peptide) causes leakage of carboxyfluorescein from liposomes by partitioning into the liquid-disordered "l_d" regions in the membrane.[40] To determine whether such a mechanism was operating in our MMP-9-triggered release of liposomal contents (i.e., whether the enzyme was becoming embedded in the lipid bilayers of the liposomes and causing contents release), we conducted the appropriate control experiments. Liposomes were prepared with POPC only and incubated with 2.3 μM MMP-9 in a buffer containing $CaCl_2$ and $ZnCl_2$. These liposomes showed no significant leakage of the contents in 85 min (Figure 3, blue trace). In interpreting these results, we noted that the liposome phase composition is expected to be different when the lipopeptide is excluded from the lipid bilayers. This difference may be sufficient to alter the membrane properties such that the added MMP-9 cannot become embedded in the bilayers of the liposomes formulated with POPC only.[40] However, our results from the other control experiment, which used liposomes formulated with POPC and lipopeptide **LP1**, ruled out this mechanistic possibility. When we incubated these liposomes with 2.3 μM MMP-9 in a buffer that *did not* contain any Ca^{2+} or Zn^{2+} ions, only 15% of the encapsulated dye was released (Figure 3, red trace). The minimal leakage observed in this case is likely due to residual enzyme activity caused by contaminant Ca^{2+}, Zn^{2+}, and/or other metal ions in the buffer solution or the presence of these two ions in the stock solutions of MMP-9.

Lipopeptide **LP3** (Table 1) was synthesized and incorporated into the liposomes in order to determine the influence of the peptide sequence on the enzymatic cleavage and subsequent destabilization of the liposomal membranes. **LP3** [sequence: $GPKGIAGQK(GPO)_4GG$] contains the MMP-9 cleavage site (glycine−isoleucine) in the structure.[24] The glutamine and arginine residues of **LP1** are replaced with lysine in **LP3**. The rationale for this replacement was that MMP-9 prefers nonpolar residues flanking the cleavage site,[25] so substitution with a polar residue was expected to reduce the activity of the enzyme for this substrate. The ε-amino groups of the lysine residues of **LP3** (pK_R 10.5) would have had positive charges under our experimental conditions (pH 8.0). When incubated with MMP-9, the liposomes formulated with 30 mol % lipopeptide **LP3** and 70 mol % POPC released less than 5% of their contents (Figure 3, green trace), confirming that the enzymatic cleavage of the peptide by MMP-9 is the trigger for membrane perturbation and release of liposomal contents. By CD spectroscopy, we observed that in solution, lipopeptide **LP3** is not as triple-helical as **LP1** (data not shown), possibly because of the repulsion caused by the positive charges of the lysine residues. In addition, after cleavage of the liposome-incorporated **LP3** by MMP-9, the cleaved product would have had a positively charged headgroup and may not have caused enough perturbation of the liposomal membranes for efficient release of the encapsulated dye.

A triggered-release system should exhibit release characteristics that are dependent on the concentration of the triggering enzyme. Our results indicated that both the rate and extent of release of the encapsulated dye consistently increased with increasing concentration of MMP-9 (Figure 4 and Table 2). Between 200 nM and 2.3 μM concentrations of MMP-9, the kinetic traces could be fit by a single-exponential rate equation. The rates obtained from these fits (Table 1) represent the average of a series of microscopic rate constants encompassing several processes, e.g., unwinding of the triple helix, cleavage of the peptide, perturbation of the membrane, and annealing of the defect in the membrane.[40,41] Clearly, the extent of contents release from our liposomes can be controlled by the amount (activity) of the MMP-9 present. In addition, if the liposome contents include inhibitors of MMP-9, our controlled-release methodology will function like a "negative-feedback" system.

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The released inhibitors will inhibit the cleavage of the liposome-incorporated lipopeptides by MMP-9; this in turn will slow the rate as well as the extent of the release of the encapsulated contents. Thus, the liposomes can act as a self-adjusting triggered-release system for delivering drugs and other molecules.

Our recombinant MMP-9 has the catalytic and fibronectin domains of the full-length enzyme. It has been hypothesized in the literature that the fibronectin domain of MMP-9 is important for the unwinding of triple-helical peptide substrates prior to the cleavage by the catalytic domain of the enzyme.[41] MMP-7 and -10 lack the fibronectin domain in their structures.[41] We observed that less than 20% of the liposome-encapsulated dye was released in the presence of recombinant MMP-7 or -10 (Figure 5). This small observed release may be attributed (in part) to cleavage of the monomeric lipopeptide, which is in equilibrium with the trimer.[17,20] Similarly, even though our triple-helical peptides contained the cleavage site for trypsin, the enzyme failed to release significant amounts of the encapsulated carboxyfluorescein from the liposomes (Figure 5, green squares) because of the inability of the enzyme to unwind the triple helix.

The following question arises: why does the enzyme-triggered release from the liposomes depend on the major phospholipid in the formulations? Our results (Figures 6 and 7 and Table 3) showed that when the acyl chains of the lipopeptides and phospholipids were similar, small amounts of the liposomal contents were released. When the corresponding acyl chains were different, the amounts of contents release increased. However, the Rpn values (i.e., triple helicity) measured for the liposome-incorporated lipopeptides (Table 4) did not show any obvious correlation between the extent of triple helicity and the corresponding extent of contents release.

In order to explain these contradicting observations, we noted that after enzymatic cleavage of the liposome-incorporated triple-helical lipopeptides, one of the products contains a short peptide linked to the acyl chain. For example, enzymatic cleavage of **LP1** results in the liberation of the polypeptide sequence IAGQR$(GPO)_4$GG−OH while the stearoyl moiety remains conjugated to the peptide GPQG. The estimated isoelectric point (p*I*) for this remnant four-residue polypeptide−strearic acid conjugate is ∼5.5, and that for the uncleaved lipopeptide is ∼9.5.[42] Hence, under our experimental conditions (pH 8.0), the cleavage of lipopeptide results in a reversal of the net charge on the lipopeptide from positive to negative. It is also possible that the reduced headgroup size of the cleaved lipopeptide product induces curvature stress in the bilayer, since the enzyme does not have access to the inner lipid layer of the liposomes.[43] In order to minimize the total free energy due to these changes in the lipid membranes, domain reorganization is likely to occur. For example, domains in raft-containing membranes exhibit a circular shape in order to minimize line tension. If these domains are perturbed by mechanical or chemical means, they quickly reassume the circular shape to minimize the boundary length.[44]

The microscopic details of the domain reorganization following the MMP-9-mediated cleavage of our liposome-incorporated lipopeptides are complex, and we do not yet understand the molecular mechanism. However, it is likely that this step will lead to defects in the membrane that are sufficient for contents of the liposome to leak. The membrane changes that result from the hydrolysis of the lipopeptides are expected to be similar to the membrane perturbation that occurs by means of phospholipase A_2-mediated hydrolysis of the headgroups of phosphocholines, which also leads to the leakage of encapsulated contents from liposomes.[45]

In the liposomes formulated with lipopeptide and phosphocholine having mismatched acyl chains, the extent of a priori demixing of the lipopeptides into lipopeptide-rich domains is expected to be greater.[46] Consequently, in these liposomes, upon enzymatic cleavage of the lipopeptides, the perturbation in the membrane should be larger (the effect should be localized in domains rather than distributed over the entire membrane surface). In this scenario, the probability of reorganization of the surrounding lipids to anneal the defects is less. When the liposomes are formulated with lipopeptides bearing acyl chains similar to those of the major phospholipid, the lipopeptides are more mixed with the phosphocholine.[46] In this situation, the membrane defects generated by the cleavage of the lipopeptides can be annealed rapidly.[47] While the amplitudes obtained from exponential fits of the acyl-chain-dependent release data (Table 3) are consistent with this hypothesis, the rate constants are not. For example, for the oleic acid-conjugated peptide (**LP2**), the rate constants for dye release from the liposomes were 0.09, 0.09, and 0.16 min^{-1} with DSPC, DOPC, and POPC, respectively, as the major lipid. It should be noted that the amplitudes for these dye release traces were very different: 0.70 for the DSPC liposomes and 0.27 and 0.22 for DOPC and POPC liposomes, respectively. With such different amplitudes, it is unrealistic to compare the rate constants obtained from the fits of the release data.[48] The first-exponential-phase rate constant (i.e., 0.13 min^{-1}) calculated for the release from the liposomes incorporating DSPC and lipopeptide **LP1** may also be attributed to a similar artifact, as the total amplitude in this case is only 0.1 (Table 3). For the liposomes formulated with DOPC as the major lipid, a lag phase was observed in the kinetic trace, creating a noticeable deviation from a single-exponential process (Figure 6). As discussed previously, the enzyme-triggered release of the liposomal contents is a complex, multistep process, and analyzing the rates as single-exponential processes is an approximation, except when one of the steps is clearly rate-limiting.

Conclusions

In conclusion, we have designed liposomes whose contents are released upon triggering with human MMP-9. The liposomes are formulated with phospholipids and a lipopeptide that contains a collagen-related peptide sequence containing the MMP-9 cleavage site. Because of the amino acid sequence, the

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lipopeptide is able to acquire a triple-helical conformation (similar to collagen) when incorporated in the liposomes. The encapsulated dye is released from these liposomes in the presence of MMP-9 in a concentration-dependent manner. We observed that the dye can be completely released in 40 min in the presence of 2.3 μM MMP-9. MMP-7 and -10 and trypsin failed to release the liposome-encapsulated dye because of their inability to unwind the triple-helical lipopeptides. Interestingly, the extent of MMP-triggered release is strongly dependent on the acyl-chain mismatch between the lipopeptide and the major phospholipid component of the liposomes. Our results led to the conclusion that formation of lipopeptide-rich domains in the liposomal membrane facilitates MMP-9-triggered release of the liposome contents. It is likely that as the local "effective" concentration of lipopeptides exceeds a certain critical mole fraction in the membranes, repair of defects caused by the enzymatic cleavage of the peptide becomes inefficient, resulting in complete release of the encapsulated contents. The results demonstrate that the sensitivity of the liposomal release to MMP-9 can be altered simply by changing the major phospholipid used in the formulation. On the basis of the ease of purification of lipopeptide **LP1** and the observed release rates, it appears that the **LP1**−POPC liposomes are better than the **LP2**−DSPC liposomes in releasing the encapsulated contents. Overall, our methodology will find applications in drug delivery and in diagnostics systems that are based on the release of liposomal contents using enzymatic as well as other triggers.

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