Protease-Sensitive, Polymer-Caged Liposomes: A Method for Making Highly Targeted Liposomes Using Triggered Release

Matthew T. Basel,[†,‡,*] Tej B. Shrestha,[†,‡] Deryl L. Troyer,[‡] and Stefan H. Bossmann[†,*]

[†]Department of Chemistry, 213 CBC Building, and [‡]Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas 66506, United States

ABSTRACT Liposomes have become useful and well-known drug delivery vehicles because of their ability to entrap drugs without chemically modifying them and to deliver them somewhat selectively to tumorous tissue *via* the enhanced permeation and retention (EPR) effect. Although useful, liposome preparations are still less than ideal because of imperfect specificity, slow release kinetics in the tumor, and leakiness prior to reaching the tumor site. Cancer-associated proteases (CAPs), which are differentially expressed in tumors, have also gained traction recently as a method for tumor targeting and drug delivery. By combining the EPR effect with CAPs sensitivity, a much more specific liposome can be produced. The method described here creates an improved liposome system that can target more specifically, with faster release kinetics and lower general leaking, by deliberately producing a very unstable liposome (loaded with hyperosmotic vehicle) that is subsequently stabilized by a cross-linked polymer shell containing consensus sequences for cancer-associated proteases (protease-triggered, caged liposomes). A cholesterol-anchored, graft copolymer, composed of a short peptide sequence for urokinase plasminogen activator (uPA) and poly(acrylic acid) , was synthesized and incorporated into liposomes prepared at high osmolarities. Upon cross-linking of the polymers, the protease-triggered, caged liposomes showed significant resistance to osmotic swelling and leaking of contents. Protease-triggered, caged liposomes also showed significant and substantial differential release of contents in the presence of uPA, while bare liposomes showed no differential effect in the presence of uPA. Thus a protease-sensitive liposome system with fast release kinetics was developed that could be used for more specific targeting to tumors.

KEYWORDS: liposome · cancer-associated proteases · polymer-caged liposome · urokinase plasminogen activator · targeted delivery · triggered delivery

The necessity of targeting chemotherapeutic drugs specifically to a cancer site is widely accepted. Specifically targeting the drugs to the cancer site could lower side effects, increase actual doses in the tumor, and reduce the development of drug resistance.[1,2] One method for delivering drugs specifically is to use a drug delivery device designed to carry the drug to the desired site and then release it into the tumor.[3,4] Liposomes are an example of a well-known drug delivery device. Made up of a simple lipid bilayer that separates an internal aqueous compartment from an external aqueous environment, liposomes can hold a large payload of hydrophilic or hydrophobic drugs. Liposomes also naturally accumulate in tumors due to the enhanced permeation and retention (EPR) effect. Several FDA-approved drug preparations utilize liposomes as the delivery method.[3,4] Although liposomes have been very successful, there are several reasons they are not perfect. For example, the EPR effect is only slightly selective; liposomes often change the pharmacokinetic profile of the drugs, precluding high tumor concentrations; and liposomes are prone to systemic leaking of drugs.[5]

Another method of tumor targeting that has gained traction recently is cancer-associated proteases (CAPs). CAPs are a set of proteases that are usually absent or at very low concentrations in healthy tissues but are often highly upregulated in cancerous tissues. Some well-known CAPs include urokinase plasminogen activator (uPA), many of the matrix metalloproteases, and some of the cathepsins.[6−10] Developing a prodrug or a drug delivery system that could be cleaved and activated by CAPs would be a viable method for targeting drugs to the tumor site, and research has been published on protease-sensitive prodrugs, drug delivery systems, and liposomes.[11−15] Urokinase plasminogen activator is a well-studied CAP that is thought to be involved primarily in angiogenesis and perhaps in tissue remodeling during invasion and metastasis. The consensus sequence for uPA is known to be SGRSA (Ser-Gly-Arg-Ser-Ala).[15,16]

In 2007, Lee *et al.* described a method for creating a liposome coated with a cross-linked polymer coat, termed "polymer-caged liposomes". The polymer-caged liposomes were shown to be extremely

* Address correspondence to mbasel@vet.ksu.edu, sbossman@ksu.edu.

Received for review December 7, 2010 and accepted January 27, 2011.

Published online February 11, 2011
10.1021/nn103362n

© 2011 American Chemical Society

stable, preventing dissociation of the polymer from the membrane, preventing liposome degradation upon freezing or drying, and drastically slowing the natural leak of contents from the liposome. Polymer-caged liposomes may have a future in drug delivery due to their extreme stability. If a suitable drug release trigger can be found, polymer-caged liposomes could be an excellent delivery mechanism.[17]

Here we describe a method for preparing protease-sensitive liposomes based on the polymer-caged liposome concept. Protease sensitivity is conferred by synthesizing a cholesterol-anchored graft copolymer that contains both the protease consensus sequence and poly(acrylic acid), which is easily cross-linked by a diamine. Concurrently, liposomes were synthesized at high osmolarities that would cause the liposomes to osmotically swell and release their contents if diluted into physiological osmolarity. The synthesized polymer was integrated into the outside sheath of these intrinsically unstable liposomes and cross-linked with a diamine. This cross-linked polymer coat should confer additional resistance to osmotic swelling, making the liposomes stable again at physiological osmolarity. Upon liposome contact with the targeted protease, the protease should degrade the protease-sensitive polymer, reversing the stability that the polymer conferred. Thus, in the presence of the targeted protease, the liposomes should rapidly swell and release their contents. If successfully prepared, such a liposome system could (1) increase the specificity of liposomes as drug delivery devices; (2) increase the immediate local concentration of the drug at the tumor site, creating more favorable pharmacokinetics; and (3) prevent unwanted systemic delivery of the drug due to nonspecific leaking of the contents.

RESULTS AND DISCUSSION

Protease-Triggered, Caged Liposome Preparation. Two different methods of making protease-triggered, caged liposomes were tried. First, bare liposomes were synthesized containing 47.5 mol % of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DPPC), 5.0 mol % of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DOPC), and 47.5 mol % of cholesterol hydrated in 10× HEPES buffered saline (HBS) with 100 mM carboxyfluorescein. Three different polymer-incorporated liposomes were made by adding 0.006 equiv (*versus* DPPC+DOPC, measured using the Stewart Assay[19]) of the synthesized polymer (average 9 nm between polymers), 0.013 equiv of the synthesized polymer (average 6 nm between polymers), or 0.053 equiv of the synthesized polymer (average 3 nm between the polymers). These were designated low (L), medium (M), or high (H) samples, respectively.

After incubating overnight to prepare polymer-incorporated liposomes, 3-(ethyliminomethylene amino)-*N*,*N*-dimethylpropan-1-amine methyliodide (EDC*MeI) and ethylenediamine were added. To all three samples (L, M, and H) was added ethylenediamine to give 50, 75, or 100% cross-linking of the poly(acrylic acid) residues (assuming 100% incorporation in the polymer-incorporated liposomes). These samples were labeled 50, 75, and 100, respectively, to give nine different protease-triggered, caged liposome preparations (L50, L75, L100, M50, M75, M100, H50, H75, H100).

To verify that the protease-triggered, caged liposomes were successfully prepared, the hydrodynamic radius (R_H) of each liposome preparation was measured by dynamic light scattering (DLS); these are shown in Figure 1. If the protease-triggered, caged liposomes were successfully prepared, the hydrodynamic radius should increase because the polymer has increased both the actual radius and the ability of the liposome to bind water. As can be seen in Figure 1, each of the protease-triggered, caged liposomes showed an increase in average radius *versus* the control (bare liposomes). The values ranged from a minimum of +6.1 nm for L50 to +15.5 nm for M50. All p values were less than 0.01, indicating that there is very little probability of any of the samples being the same as the control bare liposomes; thus, successful synthesis of the protease-triggered, caged liposomes was accomplished.

As an alternative to cross-linking with ethylenediamine, the peptide GSGRSAGK was also used as a cross-linker. We hypothesized that this peptide sequence would substitute as a cross-linker for the poly(acrylic acid) because it has two amines—the N-terminal amine and the lysine amine. The peptide also contains the uPA consensus sequence, which should render the polymer cage sensitive to uPA. Liposomes were

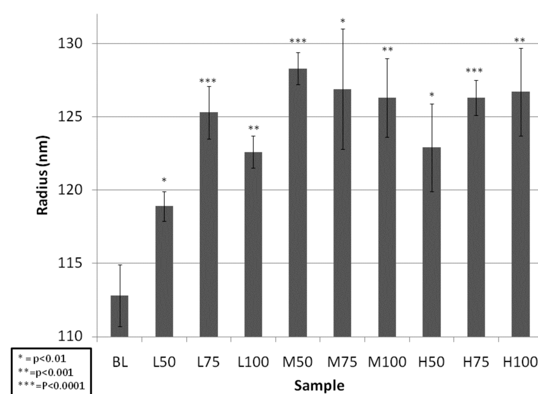


Figure 1. Hydrodynamic radii of various protease-triggered, caged liposome preparations and bare liposomes were measured by dynamic light scattering. Protease-triggered, caged liposomes should have a larger hydrodynamic radius than the corresponding bare liposomes because of the integrated polymer shell. (BL = bare liposomes; L50, L75, L100 = 9 nm between polymers with 50, 75, or 100% cross-linking; M50, M75, M100 = 6 nm between polymers with 50, 75, or 100% cross-linking, H50, H75, H100 = 3 nm between polymers with 50, 75, or 100 cross-linking.)

prepared again containing 47.5 mol % of DPPC, 5.0 mol % of DOPC, and 47.5 mol % of cholesterol hydrated in 10× HBS with 100 mM carboxyfluorescein. Polymer-incorporated liposomes were made by adding 0.032 equiv (*versus* DPPC+DOPC, measured using the Stewart Assay) of the synthesized polymer to the bare liposome preparation and incubating overnight. Polymer-incorporated liposomes were cross-linked with 0.056 equiv (34% cross-linking) of the peptide GSGRSAGK using the EDC coupling reaction.

Again, R_H was measured by DLS, and the protease-triggered, caged liposomes showed a significant increase ($p < 0.0001$) in size: 105.5 ± 1.4 nm for the bare liposomes *versus* 110.8 ± 2.4 nm for the protease-triggered, caged liposomes. Hence, protease-triggered, caged liposomes were synthesized successfully with both a short, inert cross-linker (ethylenediamine) and a large, protease-sensitive cross-linker (GSGRSAGK).

Osmotic Pressure Resistance. Bare liposomes were prepared as described with 51.5 mol % of DPPC, 4.7 mol % of DOPC, and 43.8 mol % of cholesterol hydrated in 10× HBS + 100 mM carboxyfluorescein. Polymer-incorporated liposomes were prepared by adding 0.032 equiv of the synthesized polymer (*versus* DPPC+DOPC, measured using the Stewart Assay[19]) to the bare liposome preparation and incubating overnight. Polymer-incorporated liposomes were cross-linked with 0.056 equiv (34% cross-linking) of the peptide GSGRSAGK using the EDC coupling reaction.

After incubating and purifying, the concentration of carboxyfluorescein in the protease-triggered, caged liposomes and the bare liposomes was measured. Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10, 9.75, 9.5, 9.25, 9, 8.5, 8.25, 8, or 7.75× HBS. The osmotic pressure against the 10× HBS inside the liposomes can be calculated and is 0, 1.69, 3.38, 5.07, 6.76, 10.14, 11.83, 13.52, and 15.21 atm, respectively. The solutions were incubated overnight at 37.5 °C, and fluorescence intensity was measured.

The data were plotted as percent release (fluorescence) *versus* osmotic pressure (Figure 2a). The increase in resistance to osmotic pressure for the protease-triggered, caged liposomes can be calculated from the isorelease pressure difference. The minimum pressure (P_{min}) required to release the liposome contents (that is, the pressure needed to overwhelm the membrane) was estimated from the bare liposomes as the pressure at half maximal release, $P_{Half\ Max}$ (50% fluorescence maximum). Protease-triggered, caged liposomes should show an increase in osmotic pressure resistance due to the pressure exerted by the polymer cage resisting deformation as the liposome swells and pushes against it. This increase in osmotic pressure resistance, the external back pressure exerted by the polymer cage (P_{ext}), can be estimated from the protease-triggered, caged liposomes as the pressure at

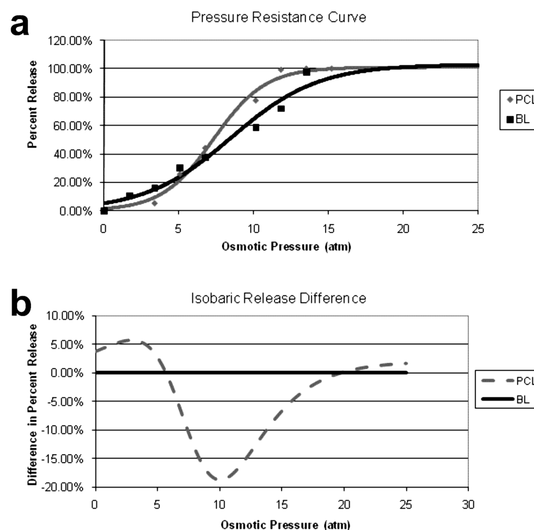


Figure 2. Pressure resistance of protease-triggered, caged liposomes cross-linked with peptide GSGRSAGK. (a) Bare liposomes or protease-triggered, caged liposomes were diluted into decreasing concentrations of HBS (and thus increasing osmotic pressure on the membrane). Fluorescence *versus* osmotic pressure was plotted to determine resistance to osmotic pressure. (b) Isobaric release difference (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) was plotted.

half maximal release. The protease-triggered, caged liposomes' pressure at half maximal release should equal $P_{min} + P_{ext}$ (that is, the pressure needed to overwhelm the liposome membrane plus the pressure needed to overcome the polymer shell), so subtracting the pressure at half maximal release of the bare liposomes from the pressure at half maximal release of the protease-triggered, caged liposomes should give P_{ext} ($P_{min} + P_{ext} - P_{min} = P_{ext}$). The P_{ext} calculations represent a horizontal line across Figure 2a at 50% content release.

Unfortunately, the prepared protease-triggered, caged liposomes showed no increased resistance to osmotic pressure, and the release curves for the protease-triggered, caged liposomes and bare liposomes were identical. Ideally a large change would be seen in the pressure resistance of the protease-triggered, caged liposomes that would show itself as a rightward shift in the fitting curve. Thus at intermediate pressures, a substantial difference in the percent release of the carboxyfluorescein would be seen. Since no right shift in the curve was seen, the protease-triggered, caged liposomes appear to be no more pressure-resistant than the bare liposomes, and the calculated P_{ext} of −1.01 atm confirms this.

More important to the goals of this project, though, are the isobaric release difference, which is defined as the % release of the bare liposomes − % release of the protease-triggered, caged liposomes. Any protease-sensitive liposome system will reach the intended target at a defined osmotic pressure (presumably

physiological pressure). Upon degradation of the polymer cage, the protease-sensitive liposome will substantially resemble a bare liposome at the same defined osmotic pressure. Thus, the difference in release between a bare liposome and a protease-triggered, caged liposome at a defined osmotic pressure is theoretically the maximal protease-specific release available from the protease-triggered, caged liposome system. The isobaric difference in release between the bare liposomes and the protease-triggered, caged liposomes can be calculated for each sample by subtracting the percent release curve for bare liposomes from the percent release curve for the sample. The isobaric difference curves are shown in Figure 2b. As expected from the P_{ext} value, the protease-triggered, caged liposomes also did not show any substantial isobaric release differences.

This result may demonstrate that the polymer cage is too loose to provide any actual pressure resistance. That is, the cage is so loose that it allows the membrane to expand beyond its maximal expansion value, which causes the membrane to form pores and leak the contents of the liposome before the membrane expands enough to stretch the polymer. To create a polymer cage that does add significant pressure resistance, it was hypothesized that the cross-linking needs to be tightened. In order to make tighter cross-linking, a shorter cross-linker, ethylenediamine, was used.

Bare liposomes were prepared with 51.5 mol % of DPPC, 3.7 mol % of DOPC, and 44.8 mol % of cholesterol in 10× HBS with 100 mM carboxyfluorescein. Bare liposomes constituting 3 μmol of DPPC (1 equiv) were added to either 0.17, 0.034, or 0.50 equiv of the protease-sensitive polymer. The resulting polymer-incorporated liposomes, after incubating and purifying, were added to either 46.5 μL (700 nmol; cross-linking percent depends on sample) or 70 μL (1000 nmol; cross-linking percent depends on sample) 1% ethylenediamine, giving six separate protease-triggered, caged liposome samples.

After incubating and purifying, the concentration of carboxyfluorescein in the protease-triggered, caged liposomes and the bare liposomes was measured. Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10, 9.75, 9.5, 9, 8.5, 7.5, or 5× HBS. The osmotic pressure against the 10× HBS inside the liposomes is 0, 1.69, 3.38, 6.76, 10.14, 16.90, and 33.81 atm, respectively. The solutions were incubated overnight at 37.5 °C, and fluorescence intensity was measured. Percent release was plotted against osmotic pressure, and then the isobaric release differences between the various protease-triggered, caged liposomes and the bare liposomes were calculated (Figure 3).

As can be seen in Figure 3a, the protease-triggered, caged curves shifted substantially to the right. This indicates that the polymer cage does add substantial resistance to osmotically induced leakage. Again, the amount of added resistance to pressure (P_{ext}) can be estimated by subtracting the pressure at half-maximum of the bare liposomes (P_{min}) from the $P_{Half\ Max}$ of the protease-triggered, caged liposomes. The P_{ext} and the $P_{Half\ Max}$ for each sample are given in Figure 3b.

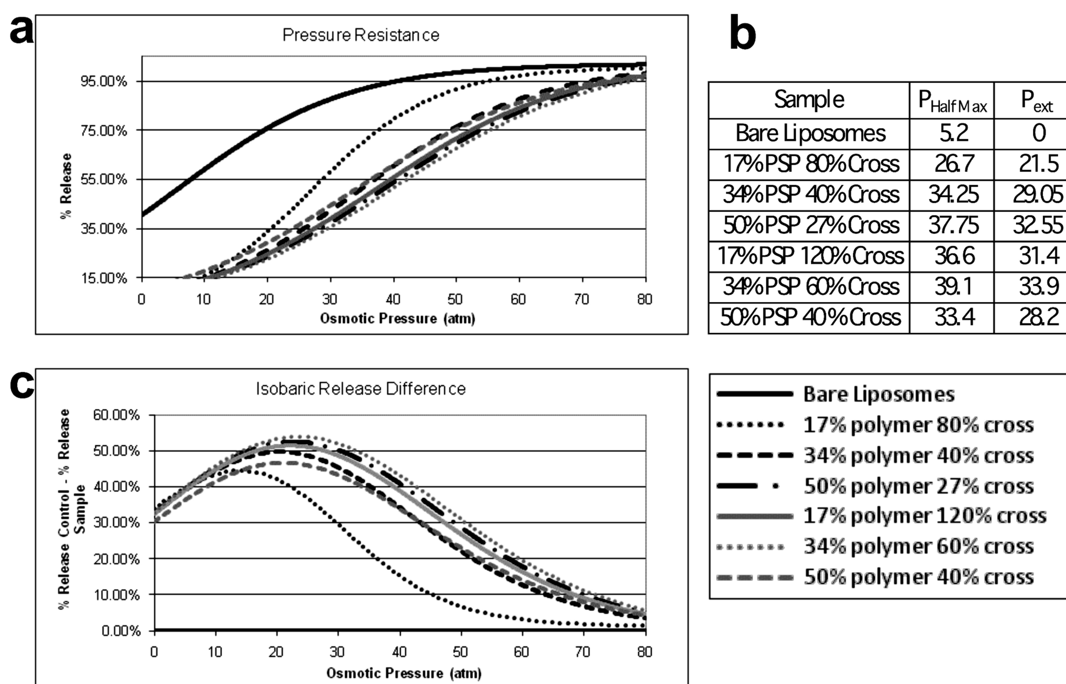
Again, the isobaric release differences are more important to this project, so isobaric release differences were plotted and are shown in Figure 3c. The protease-triggered, caged liposomes prepared with 0.034 equiv of polymer and 60% cross-linking showed the greatest difference in % release *versus* bare liposomes, with a maximum % release difference of 53.84% at 23.5 atm. The smallest difference in % release *versus* bare liposomes was seen with the protease-triggered, caged liposomes prepared with 0.017 equiv of polymer and 80%, which probably simply represents the very low amount of polymer and cross-linking.

Kinetics of Liposome Swelling. Although the swelling of the liposomes and the subsequent release of contents should be very rapid in the presence of large osmotic pressures, this swelling and releasing is transient.[18] This means that the liposome will swell, form pores, release some contents, and then the pores will seal again. If the pressure gradient is not minimized enough to prevent swelling, the process starts again. Thus, even though each swelling and releasing process is very quick, it is conceivable that the total release of contents may happen over a much longer time period.

To determine how quickly the contents of the liposomes are released in response to osmotic pressure, bare liposomes were prepared with 51.5 mol % of DPPC, 3.7 mol % of DOPC, and 44.8 mol % of cholesterol in 10× HBS with 100 mM carboxyfluorescein. Bare liposomes constituting 3 μmol of DPPC (1 equiv) were added to 0.034 equiv of the prepared polymer. The resulting polymer-incorporated liposomes were cross-linked at 60% with ethylenediamine.

Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10, 9.5, 9, 8, 7, 6, or 5× HBS. The solutions were incubated at 37.5 °C, and fluorescence intensity was measured at time 0, 2, 5, 24, 47, and 71 h. Percent release was plotted against osmotic pressure, and then the isobaric release differences between the protease-triggered, caged liposomes and the bare liposomes were calculated (Figure 4).

It takes more than 2 h but less than 5 h for the bare liposomes to reach osmotic equilibrium (no more increase in % release). The protease-triggered, caged liposomes take less than 2 h to reach osmotic equilibrium. The difference in time can be easily understood in light of what is happening to each sample. In the bare liposomes, the osmotic pressure causes the liposome to swell and form pores, releasing some of the contents before the pores close again. This process happens



b

Sample	$P_{Half Max}$	P_{ext}
Bare Liposomes	5.2	0
17%PSP 80%Cross	26.7	21.5
34%PSP 40%Cross	34.25	29.05
50%PSP 27%Cross	37.75	32.55
17%PSP 120%Cross	36.6	31.4
34%PSP 60%Cross	39.1	33.9
50%PSP 40%Cross	33.4	28.2

Figure 3. Pressure resistance of protease-triggered, caged liposomes cross-linked with ethylenediamine. (a) Bare liposomes or protease-triggered, caged liposomes with varying polymer content and cross-linking percentages were diluted into decreasing concentrations of HBS. Fluorescence *versus* osmotic pressure was plotted to determine resistance to osmotic pressure. (b) Pressure at half maximal (50%) release ($P_{Half\ Max}$, in atm) was measured for each liposome preparation. The external pressure (P_{ext}, in atm) added by the polymer coat was calculated as the $P_{Half\ Max}$ of the protease-triggered, caged liposomes minus the $P_{Half\ Max}$ of bare liposomes and represents the increased pressure resistance of the protease-triggered, caged liposomes (a larger value indicates more pressure resistance, the P_{ext} value of bare liposomes is necessarily 0). (c) Isobaric release difference (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) was plotted.

repeatedly, lowering the osmotic pressure stepwise until it reaches an equilibrium state. As the osmotic pressure goes down, the speed of pore formation also slows down. Thus the bare liposomes will take some time to reach equilibrium because their progress toward equilibrium is slowed. At higher pressures, the liposomes will reach equilibrium faster; at lower pressures, the liposomes will reach equilibrium slower because of a lower initial rate, which can be seen in the curve at 2 h (Figure 4a).

On the other hand, protease-triggered, caged liposomes start out the same as bare liposomes. They swell for a moment and release a little bit of their contents. They would continue the bare liposomes trend of "swell, pore, release, close, repeat", but they quickly expand into the polymer cage and are trapped. Thus the point of equilibrium occurs not with equalizing the osmotic pressure inside and out, but rather with running into the polymer cage. Since the time it takes to expand into the polymer cage is much less than the time it takes to release all of the contents, the protease-triggered, caged liposomes reach equilibrium much faster.

Oleic Acid Content. Having demonstrated the ability to prepare protease-triggered, caged liposomes with increased osmotic pressure resistance, optimization was done to maximize P_{ext} and the isobaric release differences. Several different targets for optimization are available: the membrane content of DPPC, DOPC, and cholesterol; the amount of polymer and cross-linker added to the liposomes; and the osmolarity at which the liposomes are prepared. Several different optimizations were done as described below. In each case, optimization is defined as first, increasing the maximum isobaric release difference, and then second, increasing the pressure at which the maximum isobaric release difference occurs (optimal osmotic pressure).

To determine how oleic acid concentration affects P_{ext}, bare liposomes were prepared with either 3.7, 2.5, or 1.2 mol % of DOPC in 10× HBS with 100 mM carboxyfluorescein. Bare liposomes constituting 3 μmol of DPPC (1 equiv) were added to 0.034 equiv of the protease-sensitive polymer. The resulting polymer-incorporated liposomes were cross-linked at 60% with ethylenediamine.

Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10, 9.5, 9, 8, 7, 6, or 5× HBS. The solutions were incubated at 37.5 °C overnight, and fluorescence intensity was measured. Percent release was plotted against osmotic pressure, and then the isobaric release difference between the various protease-triggered, caged liposomes and the bare liposomes was calculated (Figure 5a).

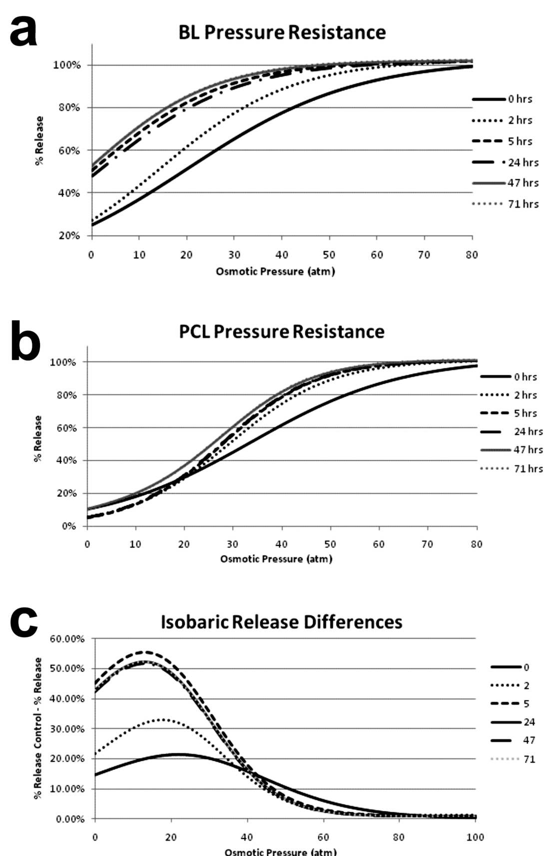


Figure 4. Kinetics of osmotically induced liposomal release. (a) Bare liposomes were diluted into decreasing concentrations of HBS. Fluorescence *versus* osmotic pressure was plotted at 0, 2, 5, 24, 47, and 71 h to determine release kinetics. (b) Protease-triggered, caged liposomes were diluted into decreasing concentrations of HBS. Fluorescence *versus* osmotic pressure was plotted at 0, 2, 5, 24, 47, and 71 h to determine release kinetics. (c) Isobaric release difference curves (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) were plotted for each time point.

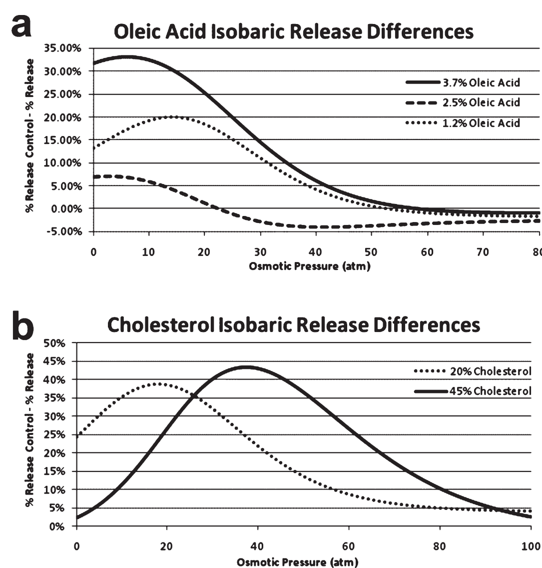


Figure 5. Optimization of membrane content. (a) Bare liposomes and protease-triggered, caged liposomes were prepared with 1.2, 2.5, or 3.7% oleic acid in the membrane and were diluted into decreasing concentrations of HBS. Isobaric release difference curves (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) were plotted for each preparation. (b) Bare liposomes and protease-triggered, caged liposomes were prepared with 20 or 45% cholesterol in the membrane and were diluted into decreasing concentrations of HBS. Isobaric release difference curves (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) were plotted for each preparation.

The concentration of DOPC had a substantial effect on the isobaric release difference of protease-triggered, caged liposomes *versus* bare liposomes by affecting the percent release curves of bare liposomes. Increasing the concentration of DOPC caused the bare liposomes to release their contents at lower osmotic pressures. The bare liposomes at higher DOPC concentrations had higher isobaric release at low osmotic pressures, compared to bare liposomes at lower DOPC concentrations. On the other hand, the concentration of DOPC did not have a strong effect on the percent release curves of protease-triggered, caged liposomes. Thus, the isobaric release difference between protease-triggered, caged liposomes and bare liposomes at the same concentration of DOPC increases with increasing DOPC concentration (Figure 5a). This is consistent with the literature suggesting that DOPC is necessary to allow water permeability and reduce membrane elasticity.[19,20] As the amount of DOPC is lowered, the pressure needed to cause effective pressure in the membrane becomes larger, reducing the percent release at lower pressures. This affects bare liposomes but not protease-triggered, caged liposomes because the protease-triggered, caged liposomes are not as dependent as the bare liposomes on membrane elasticity as the main pressure resistance. Thus, changing the elasticity and water permeability of the membrane does not significantly change the percent release in protease-triggered, caged liposomes.

Because the bare liposomes have lower percent release with lower DOPC concentrations, the isobaric difference curves are also going to be smaller at lower DOPC concentrations, indicating that there is less content available for release upon protease degradation. Therefore, DOPC concentrations at 3.7% or slightly above were determined to be optimal DOPC for this project.

Cholesterol Content. To determine how cholesterol concentration affects P_{ext}, bare liposomes were prepared with either 44.8 or 20.1 mol % of cholesterol in 10× HBS with 100 mM carboxyfluorescein. Bare liposomes constituting 3 μmol of DPPC (1 equiv) were added to 0.034 equiv of the protease-sensitive polymer. The resulting polymer-incorporated liposomes were cross-linked at 60% with ethylenediamine.

Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10,

9.5, 9, 8, 7, 6, or 5× HBS. The solutions were incubated at 37.5 °C overnight, and fluorescence intensity was measured. Percent release was plotted against osmotic pressure, and then the isobaric release differences between the various protease-triggered, caged liposomes and the bare liposomes were calculated (Figure 5b).

Since the cholesterol level was already nearly saturated, a higher concentration of cholesterol was not tested, only a lower concentration of cholesterol. Lowering the cholesterol level shifts $P_{\mathrm{Half\ Max}}$ of both the bare liposomes and the protease-triggered, caged liposomes to the left. The change is approximately the same for both bare liposomes and protease-triggered, caged liposomes, so the P_{ext} value does not change.

Taking these results with the results above for differences in oleic acid concentration, we can determine the activities of cholesterol and oleic acid. Oleic acid concentration affects only the bare liposomes and not the protease-triggered, caged liposomes. Cholesterol concentration affects both bare liposomes and protease-triggered, caged liposomes. This can be explained by oleic acid having more effect on the elasticity of the membrane, while cholesterol has more effect on the water permeability of the membrane. The elasticity of the membrane is going to affect the bare liposomes more than the protease-triggered, caged liposomes because the protease-triggered, caged liposomes do not depend on membrane elasticity to prevent release. Rather, they depend on the "elasticity" of the polymer cage as their main resistance to osmotic leakage. On the other hand, water permeability will affect both bare liposomes and protease-triggered, caged liposomes approximately the same because decreased water permeability reduces the effective osmotic pressure of the system. Reducing the effective osmotic pressure will increase $P_{\mathrm{Half\ Max}}$ for both bare liposomes and protease-triggered, caged liposomes.

The isobaric difference in release can also be calculated for the different cholesterol concentrations. As would be expected from the $P_{\mathrm{Half\ Max}}$ and P_{ext} values, the maximum difference in release is not much different between low and high concentrations of cholesterol in the membrane. The pressure at the maximum difference does shift to the right with increasing cholesterol concentration, though, so higher (saturated) concentrations of cholesterol were deemed to be optimal.

Final Optimization. Above, several different polymer contents and cross-linking percentages were tried. A wide range of polymer equivalent values were tested *versus* the lipid content, but the range of distances between polymer molecules was narrow. In fact, 0.017 equiv of polymer equals an average of 5.3 nm between polymers in the membrane, 0.034 equiv of polymer equals an average of 3.7 nm, and 0.050 equiv of polymer equals an average of 3.1 nm between polymers. Since the fully stretched polymer is nearly 17 nm long, these values allow substantial, if not excessive, overlap. To see if using a less overlapped, more elongated polymer would increase the isobaric difference between bare liposomes and protease-triggered, caged liposomes and/or P_{ext}, polymer equivalents were added to bare liposomes so that there would be, on average, either 3, 6, or 9 nm between the polymers (increasing the range tested by 50%).

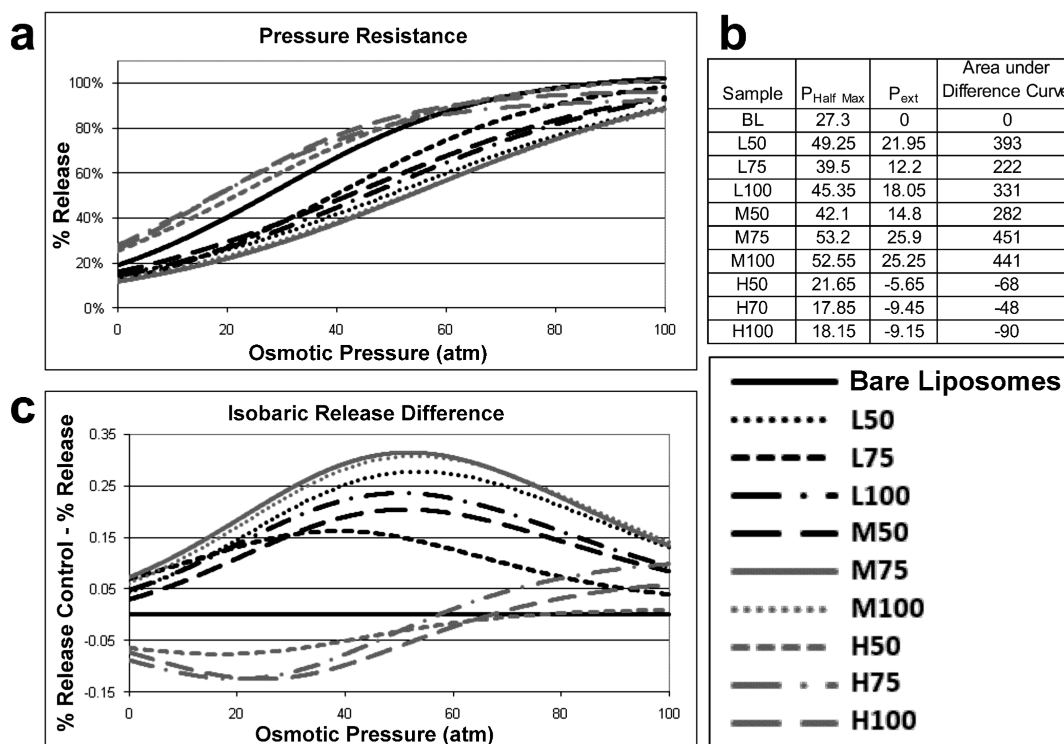
Also, because the cross-linker was chosen as an equivalent to lipid content and not as an equivalent to acrylic acid residue concentration, somewhat random (and sometimes irrational) percent cross-linking was calculated for the liposomes. To more methodically determine ideal percent cross-linking, cross-linker was chosen as an equivalent to acrylic acid residue content at either 25% (50% cross-linking because each cross-linker joins two residues), 37.5% (75% cross-linking), or 50% (100% cross-linking).

Bare liposomes were synthesized containing 47.5 mol % of DPPC, 5.0 mol % of DOPC, and 47.5 mol % of cholesterol hydrated in 10× HBS with 100 mM carboxyfluorescein. Bare liposomes constituting 4 μmol DPPC + DOPC (1 equiv) were added to either 25 nmol (0.006 equiv, average 9 nm between polymers), 53 nmol (0.013 equiv, average 6 nm between polymers), or 212 nmol (0.053 equiv, average 3 nm between polymers) of the synthesized polymer. These were designated low (L), medium (M), or high (H) samples, respectively.

After incubating overnight to prepare polymer-incorporated liposomes, EDC*MeI and ethylenediamine were added. To all three samples (L, M, and H) was added ethylenediamine to give 50, 75, or 100% cross-linking of the poly(acrylic acid) residues (assuming 100% incorporation in the polymer-incorporated liposomes). These samples were labeled 50, 75, and 100, respectively, to give nine different protease-triggered, caged liposome preparations.

Both the samples and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of either 10, 9.5, 9, 8, 7, 6, 5, 3, 1, or 0.5× HBS. The solutions were incubated at 37.5 °C overnight, and fluorescence intensity was measured. Percent release was plotted against osmotic pressure, and then the isobaric release differences between the various protease-triggered, caged liposomes and the bare liposomes were calculated (Figure 6).

The top six values for maximum difference in percent release *versus* control were all from samples with spacing farther apart (lower percent polymer) than the original protease-triggered, caged liposomes. This would indicate that having less overlap between the polymers results in less leaky liposomes. This is a reasonable result because polymers that start out more stretched out are going to have less expansivity than



Sample	$P_{Half\ Max}$	P_{ext}	Area under Difference Curve
BL	27.3	0	0
L50	49.25	21.95	393
L75	39.5	12.2	222
L100	45.35	18.05	331
M50	42.1	14.8	282
M75	53.2	25.9	451
M100	52.55	25.25	441
H50	21.65	-5.65	-68
H70	17.85	-9.45	-48
H100	18.15	-9.15	-90

Figure 6. Optimization of polymer content and cross-linking. (a) Bare liposomes or protease-triggered, caged liposomes with varying polymer content and cross-linking percentages were diluted into decreasing concentrations of HBS. Fluorescence *versus* osmotic pressure was plotted to determine resistance to osmotic pressure. (b) P_{ext} was calculated as the pressure at half maximal release ($P_{Half\ Max}$) of the protease-triggered, caged liposomes minus the $P_{Half\ Max}$ of bare liposomes and represents the increased pressure resistance of the protease-triggered, caged liposomes. (c) Isobaric release difference (calculated as the % release of bare liposomes − % release of protease-triggered, caged liposomes) was plotted. (BL = bare liposomes; L50, L75, L100 = 9 nm between polymers with 50, 75, or 100% cross-linking; M50, M75, M100 = 6 nm between polymers with 50, 75, or 100% cross-linking, H50, H75, H100 = 3 nm between polymers with 50, 75, or 100% cross-linking.)

loose polymers (and polymers that have to stretch farther to cross-link will theoretically be more stretched out). Thus, the membrane will expand less due to osmotic pressure before interacting with the polymer cage and will thus release less of the liposomal contents in the expansion process. The drawback could be a weaker polymer cage, but the data do not support this. Liposomes with lower polymer levels are less leaky at low osmotic pressures and continue to be less leaky when the osmotic pressure is raised. There does seem to be a drop off in polymer cage strength in the very low integration levels (spacing average 9 nm apart).

Liposomes with very high concentration of polymer (average spacing 3 nm apart) actually show very little difference from bare liposomes. This is probably due to a very large expansivity of the polymer cage before it will resist osmotic pressure. Although the polymer cage is well cross-linked, since the polymer is very close together, it can be stretched apart a little bit before the cross-linking is truly tight. In very high concentrations of polymer, the expansivity appears to be greater than the maximum membrane expansivity, so the liposomes will leak out all of their contents before the polymer shell will provide resistance to osmotic pressure.

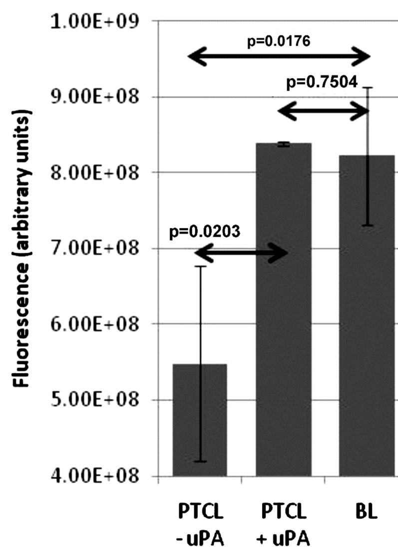
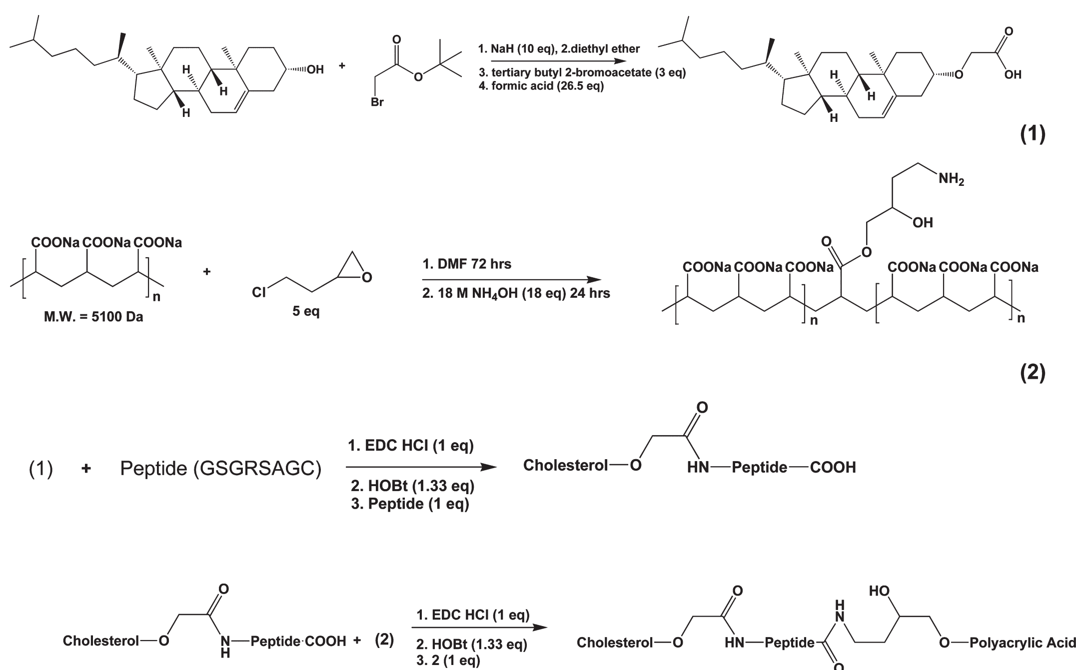


Figure 7. Protease-triggered, caged liposomes are sensitive to urokinase plasminogen activator (uPA). Bare liposomes and protease-triggered, caged liposomes were prepared as optimized in 8× HBS and were diluted into 1× HBS to represent physiological osmolarity. The uPA was added to the protease-triggered, caged liposomes and allowed to degrade the polymer shell. Fluorescence was measured to determine percent release of the bare liposomes (BL), protease-triggered, caged liposomes without uPA (PTCL no uPA), and the protease-triggered, caged liposomes with uPA (PTCL + uPA).



Scheme 1. Synthesis of protease-sensitive graft copolymer. Cholesterol was acid-functionalized by treating with 2-bromoacetate. Polyacrylic acid was amino-functionalized by treating with 2(2-chloroethyl)oxirane followed by concentrated ammonium hydroxide. The peptide was then attached by EDC coupling to (1) the acid-functionalized cholesterol and (2) the amine-functionalized polyacrylic acid.

Thus, the optimal spacing between polymers appears to be between 5 and 6 nm on average (1.3−1.9% polymer). These values of polymer incorporation have consistently had the highest isobaric pressure differences and the highest P_{ext} values. The optimal value of cross-linking seems to depend strongly on polymer incorporation level. At higher incorporation, less cross-linking seems to be preferred, while at lower incorporation, more cross-linking seems to be preferred.

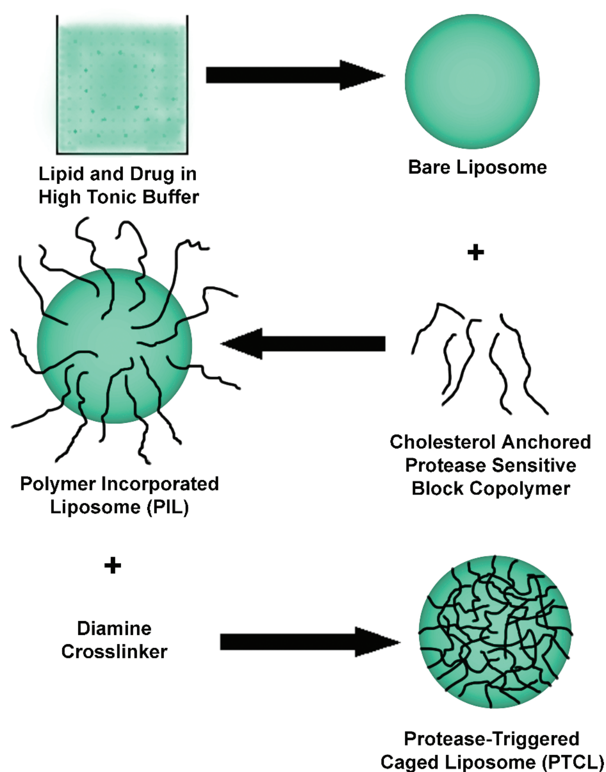
Urokinase Sensitivity. The most important aspect of the protease-sensitive, polymer-caged liposomes is that they be sensitive to the desired protease, in this case uPA. This sensitivity would be measured as an increase in delivery upon interacting with uPA. To test the urokinase sensitivity of the prepared protease-triggered, caged liposomes, the liposomes were prepared as optimized above. Bare liposomes were synthesized containing 47.5 mol % of DPPC, 5.0 mol % of DOPC, and 47.5 mol % of cholesterol hydrated in 8× HBS with 100 mM carboxyfluorescein. Polymer-incorporated liposomes were prepared by adding 0.013 equiv (*versus* DPPC+DOPC, measured using the Stewart Assay[19]) of the synthesized polymer to the bare liposome preparation and incubating overnight. Polymer-incorporated liposomes were cross-linked with ethylenediamine to give 75% cross-linking using the EDC coupling reaction.

Both the sample and the control were diluted to 4 μM carboxyfluorescein and a final tonicity of 1× HBS, to represent physiological osmolarity. Three samples were prepared: bare liposomes in 1× HBS, protease-triggered, caged liposomes in 1× HBS, and protease-triggered, caged liposomes in 1× HBS + 25 μg/mL uPA. The samples were incubated overnight, and fluorescence intensity was measured (Figure 7).

As was expected from the above results, the bare liposome sample showed 50% more fluorescence than the protease-triggered, caged liposomes sample without uPA, which again represents the maximal protease-specific delivery of the protease-triggered, caged liposomes. The percent delivery upon interacting with the protease (uPA) can be calculated by taking the increase in fluorescence of the protease-triggered, caged liposomes with uPA *versus* fluorescence of the protease-triggered, caged liposomes without uPA and comparing this increase to the 50% calculated increase for protease-triggered, caged liposomes without uPA *versus* bare liposomes. Upon adding uPA to the protease-triggered, caged liposomes, the fluorescence increased by 53%. Comparing to the 50% more fluorescence of bare liposomes, 53%/50% = 106% of the possible protease-sensitive delivery was in fact delivered, indicating that the liposome is highly sensitive to uPA. That is, the entire payload is delivered when the liposome encounters high uPA concentrations.

CONCLUSIONS

The purpose of this study was to develop a drug delivery device that would quickly release its contents upon interaction with a specific protease but would be stable until then. The proposed method was to prepare liposomes in a high tonic buffer so that they are



Scheme 2. Synthesis of protease-sensitive, polymer-caged liposomes. Liposomes were prepared in high tonicity buffers. After purifying, a graft copolymer synthesized to contain the uPA consensus sequence and polyacrylic acid was dropped into the outer sheath of the liposome membrane. The polymer was then cross-linked using a suitable diamine (ethylenediamine or the peptide GSGRSAGK).

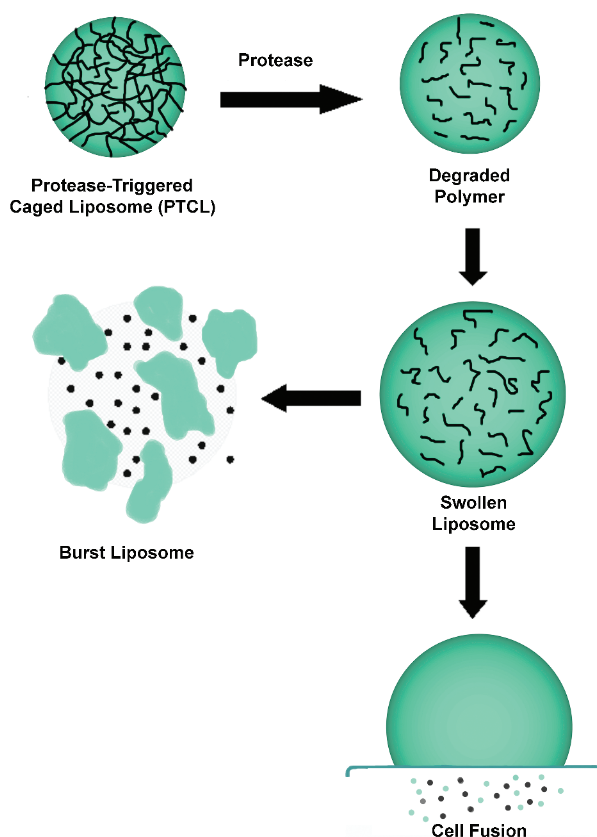
intrinsically unstable in low tonic buffers. The intrinsically unstable liposomes would then be stabilized by forming a covalently bound polymer cage around the liposome. This would be done by synthesizing a cholesterol-anchored, protease-sensitive graft copolymer, then allowing the cholesterol anchor to diffuse into the membrane of the bare liposomes (Scheme 1). The integrated polymer would then be cross-linked with a diamine (since the core of the polymer is acrylic acid, a diamine will cross-link the polymer) to give protease-triggered, caged liposomes (Scheme 2).

Each of these steps has been successfully accomplished. A cholesterol-anchored, protease-sensitive, graft copolymer containing poly(acrylic acid) was synthesized. Bare liposomes were successfully prepared in high tonic buffer, and the synthesized polymer was successfully integrated into them. The poly(acrylic acid) in the polymer was successfully cross-linked with both a short peptide sequence containing a lysine to make a diamine and with ethylenediamine.

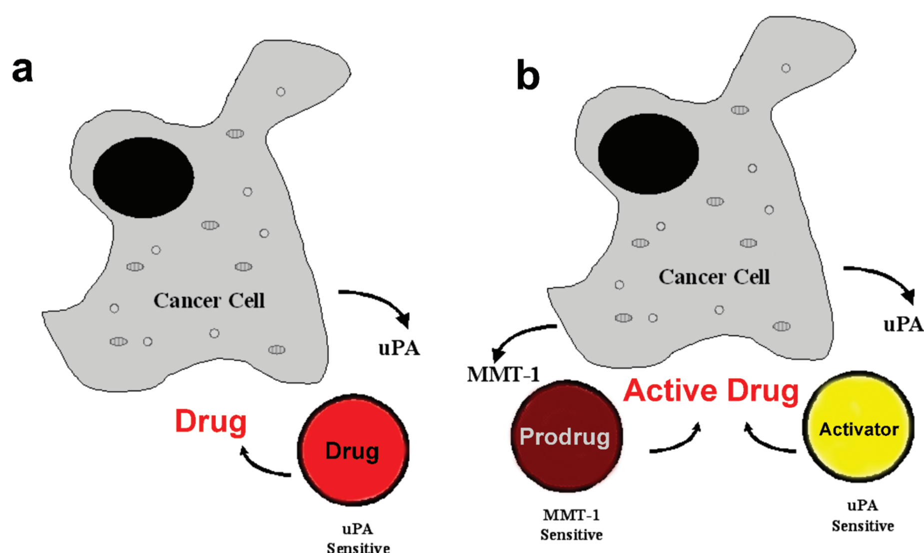
The proposed method of release was by protease degradation of the polymer (Scheme 3). This was accomplished by incorporating a peptide sequence containing the consensus sequence for uPA as the graft of the synthesized copolymer. The polymer cage was shown to confer substantial osmotic pressure resistance, and the factors affecting how much pressure resistance the polymer cage confers were studied. It was then shown that uPA can degrade the polymer cage and release the contents of the protease-triggered, caged liposomes.

This liposome system holds enormous potential as a drug delivery system for cancer therapy and perhaps many other diseases as well (as long as there are specific proteases associated with the disease, such as Alzheimer's disease). Liposomes already have some selectivity toward cancer because of the EPR effect.[3,4] Adding sensitivity to CAPs can increase the specificity of the liposome as a drug delivery device, minimizing unwanted side effects from general delivery and increasing the drug delivery to the site. This system could also be stacked to provide multiple layers of specificity by making liposomes sensitive to different proteases. By filling one liposome with a prodrug and another liposome with an activator, three levels of specificity can be obtained (Scheme 4).

A major reason cancer therapy sometimes fails is that the undesired side effects of the drug limit the amount of the drug that can be delivered systemically, thus limiting the amount of drug that can be delivered to the tumor.[21] A more specific delivery method could decrease systemic delivery (and thus unwanted side effects) and increase tumor delivery (increasing effectiveness of the drug). Thus the cancer therapy would be much less likely to fail because of low dosages or stopped because of overwhelming side effects.



Scheme 3. Proposed method of protease-sensitive liposome action. Upon dilution into physiological osmolarity, the protease-triggered, caged liposomes do not release their contents. When treated with the correct protease (uPA), the polymer is degraded, allowing the liposome to osmotically swell and release its contents, or possibly to fuse (*in vivo*) with nearby planar membranes.[25−28]



Scheme 4. Proposed possible method for *in vivo* use of protease-sensitive liposomes. (a) The current liposome can be loaded with a water-soluble chemotherapeutic drug. The liposome prevents systemic release of the drug. The liposome naturally builds up concentration at the tumor site due to the EPR effect, while the high uPA concentration at the active site releases the drug. (b) Since a simple drop-in method was used to add the protease consensus sequence to the polymer, polymers sensitive to other proteases could be easily made as well. By loading liposomes sensitive to different proteases with prodrugs and activators, the level of specificity could be exponentially increased.

METHODS

Materials. All lipids obtained for liposome synthesis were of greater than 99% purity. DOPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DPPC was purchased from Sigma Life Science (St. Louis, MO). Cholesterol was purchased from Pfaltz and Bauer (Waterbury, CT). Lipids were dissolved in chloroform upon receipt and stored at −20 °C to prevent degradation or absorption of water.

HBS (10×) was prepared as 0.012 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.36 M sodium chloride, and 0.045 M potassium chloride. HEPES (>99.5% purity) was purchased from Sigma Life Science. Sodium chloride and potassium chloride (>99% purity) were purchased from Fisher Scientific (Pittsburgh, PA). 5(6)-Carboxyfluorescein (>99% pure) was purchased from Sigma Life Science and was stored at −20 °C upon receipt. Brij-58 was purchased from Acros Organics (Geel, Belgium).

Peptides GSGRSAGC (gly ser-gly arg-ser-ala-gly cys) and GSGRSAGK (gly ser-gly arg-ala-gly lys) (synthetic, >90% pure) were purchased from GenScript (Piscataway, NJ). 3--(Ethyliminomethyleneamino)-*N*,*N*-dimethylpropan-1-amine hydrochloride (EDC*HCl) was purchased from Sigma Aldrich (St. Louis, MO). EDC*MeI was purchased from MP Biomedicals (Solon, OH). Hydroxybenzotriazole (HOBt) was purchased from Fisher Scientific. Both peptides and all coupling reagents were stored at 4 °C upon receipt. Sodium polyacrylate (M_w = 5100 Da, M_n = 1400 Da, PDI = 3.64) was purchased from Sigma-Aldrich.

Acid-Functionalized Cholesterol. A method for adding an acid function to cholesterol was adapted from Hussey *et al*:[22] 1.15 g of cholesterol (3 mmol, 1 equiv) was dissolved in 30 mL of tetrahydrofuran (THF) along with 0.72 g of NaH (30 mmol, 10 equiv), and the reaction mixture was stirred for 1 h. After 1 h, 10 mL of diethyl ether was added and the reaction mixture was stirred for 1.5 h. Then, 1.76 g of tertiary butyl 2-bromoacetate (9 mmol, 3 equiv) was added to the mixture, and the mixture was refluxed for 15 h. After refluxing, 20 mL of water was added and the crude product was extracted with 3 × 25 mL diethyl ether. The ether was removed by rotary evaporator, and the compound was purified by descending silica gel column chromatography using 10:1 hexane/ethylacetate (vol/vol) as a mobile phase, yielding a white solid compound. Product was confirmed by NMR (Supporting Information Figures 1 and 2).

To deprotect the tertiary butyl to obtain the acid-functionalized cholesterol, 3 mL of formic acid (79.5 mmol, 26.5 equiv) was added to the product isolated above with 7 mL of diethyl ether. The mixture was refluxed for 2 h, and then the diethyl ether and formic acid were removed by rotary evaporator. Product was confirmed by NMR (Supporting Information Figures 3 and 4).

Amine-Functionalized Polyacrylic Acid. Five grams of sodium polyacrylate (1 equiv) and 0.38 mL of 2-(2-chloroethyl)oxirane (5 equiv) were dissolved in 20 mL of dry dimethylformamide (DMF). The mixture was stirred for 72 h at room temperature, and then 1 mL of 18 M NH_4OH (18 equiv) was added dropwise to the mixture. The solution was stirred another 24 h at room temperature and then rotary evaporated to dryness. Once dry, the powder was put under high vacuum for 72 h to remove any remaining solvent. The resulting white powder was stored under nitrogen. Product was confirmed by NMR (Supporting Information Figure 5).

To determine the number of 1-amino-3-hydroxybutyl groups added to the polymer, a ratio between the area of the peak at 1.5 ppm (identified as the methylene peak of the poly(acrylic acid)), which contains two protons, and the area of the peak at 3.6 ppm (identified as the methylene peak of C1 of the butyl group, beside the amine), which also contains two protons, was calculated. This ratio gives the number of acrylic acid residues per 1-amino-3-hydroxybutyl group and was calculated to be 31.22. The poly(acrylic acid) used was 5100 Da, which has 70 poly(acrylic acid) residues per chain. Thus, taking 70 divided by 31.22 gives 2.25 (or approximately 2) 1-amino-3-hydroxybutyl groups per polymer chain. Although the target was one 1-amino-3-hydroxybutyl group per chain, two was deemed sufficient and the polymer was used. Molecular weight of the polymer was calculated as M_w = 5300 Da, M_n = 2700 Da, and PDI = 1.83 (the decrease in PDI is due to some purification of the polymer during synthesis).

Condensation. Acid-functionalized cholesterol (2.5 mg, 1 equiv) of the acid was dissolved in 500 μL of dry DMF and cooled to 0 °C. Then, 1.7 mg (1 equiv) of EDC*HCl was added and the solution was stirred at 0 °C for 30 min. One milligram (1.33 equiv) of HOBt was added, and the solution was stirred at room temperature for 3 h. Four milligrams (1 equiv) of the peptide GSGRSAGC was added to the mixture, and the solution was stirred overnight to give the cholesterol−peptide complex.

After 24 h, the mixture was again cooled to 0 °C. Then, 1.7 mg (1 equiv) of EDC*HCl was added, and the solution was stirred at 0 °C for 30 min. One milligram (1.33 equiv) of HOBt was then added, and the solution was stirred at room temperature for 3 h. Twenty-nine milligrams (1 equiv) of amine-functionalized poly(acrylic acid) was added to the mixture, and the mixture was stirred overnight. The mixture was then rotary evaporated to dryness, and the resulting powder was put under high vacuum for 72 h to remove any solvent remaining.

Bare Liposome Preparation. Bare liposomes were prepared according to standard methods.[23] To prepare bare liposomes, DPPC or DPPC and DOPC (various ratios) were dissolved in 600 μL of chloroform along with cholesterol (various ratios). The solution was vortexed for 30 s to ensure even distribution of the lipids. The chloroform was then evaporated off at 50 °C. Once the lipid film was dry, it was placed under high vacuum for 1 h to remove any remaining chloroform. The lipid film was then hydrated with 600 μL of *n*× HBS (prepared by diluting 10× HBS) with or without 100 mM carboxyfluorescein. The hydrated film was vortexed for 3 min, sonicated for 1 min, and then vortexed again for 2 min to suspend the lipid film in the HBS. The suspension was then put through 10 freeze−thaw cycles, 8 min/cycle (4 cold/4 hot) with the high temperature being 50 °C and the low temperature being −80 °C. After the last freeze−thaw cycle, the suspension was warmed to 50 °C. The suspension was then forced through two polycarbonate membranes with 200 nm pores using an Eastern Scientific, Inc. (Rockville, MD) mechanical extruder. The liposome preparation was then purified from the unentrapped analytes by passage through a 15 cm × 1 cm Sephadex G-50 gel filtration column using *n*× HBS as the mobile phase (the same osmolarity that the liposomes were prepared). Liposome preparation was verified by dynamic light scattering of the resulting suspension as described below. The suspension was stored at 4 °C until used.

Polymer-Incorporated Liposomes. Polymer-incorporated liposomes were prepared similarly to the procedure in Lee *et al.*[17] To prepare polymer-incorporated liposomes, the protease-sensitive polymer synthesized above (various amounts) was added to bare liposomes. The mixture was heated to 37.5 °C and rocked overnight. The polymer-incorporated liposomes were then separated from un-incorporated polymer by passing over a 15 cm × 1 cm Sephadex G-50 gel filtration column. Polymer-incorporated liposome preparation was verified by dynamic light scattering. The suspension was stored at 4 °C until needed.

Protease-Triggered, Caged Liposomes. Protease-triggered, caged liposomes were prepared similarly to the procedure in Lee *et al.*[17] To prepare protease-triggered, caged liposomes, polymer-incorporated liposomes were heated to 37.5 °C. One equivalent of EDC*MeI (in relation to the poly(acrylic acid) residues, assuming 100% incorporation) was added to the suspension, and the suspension was rocked for 2 h at 37.5 °C. Either the peptide GSGRSAGK or ethylenediamine was then added to the suspension, and the suspension was rocked at 37.5 °C overnight. The protease-triggered, caged liposomes were separated from reagents by passing over a 15 cm × 1 cm Sephadex G-50 gel filtration column. Protease-triggered, caged liposome preparation was verified by dynamic light scattering. The suspension was stored at 4 °C until needed.

Lipid Concentration. Lipid concentration was assayed by the Stewart Assay adapted from Lasch *et al.*[24] Iron(III) thiocyanate ion was prepared from 27.03 g (0.1 mol) of $FeCl_3*(H_2O)_6$ and 30.4 g (0.4 mol) of NH_4SCN and diluted to 1 L in water to give 0.1 M $Fe(SCN)(H_2O)_5^{2+}$. One hundred microliters of either bare liposomes, polymer-incorporated liposomes, or protease-triggered, caged liposomes was added to 2 mL of the

$Fe(SCN)(H_2O)_5^{2+}$ solution and 2 mL of chloroform. Standards were made with DPPC to cover the range of possible concentrations. The mixtures were then vortexed vigorously for 1 min and centrifuged at 2000 rpm for 10 min to separate the organic and aqueous layers. The lower organic layer was removed, and 725 μL of the organic layer was diluted to 1.45 mL with chloroform. The absorbances (ABS) at 485 and 690 nm were taken. The value ABS(485)−ABS(690) was plotted against the standard curve to determine phospholipid concentration.

Dynamic Light Scattering. Once the liposome concentration was found, liposomes were diluted to 1 mg phosphorus/L in isotonic buffer. The liposome suspension was allowed to stabilize to room temperature, and dynamic light scattering measurements were taken with a Brookhaven (Holtsville, NY) ZetaPlus particle size analyzer.

Carboxyfluorescein Concentration. A percent encapsulation procedure was adapted from Lasch *et al.*[24] In order to determine percent encapsulation, 100 μL of the liposome suspension was added to 100 μL of 5 M Brij-58. The solutions were mixed well and then diluted to 2 mL in water. Serial dilutions were made until the absorbance at 480 nm was less than 1. The absorbances at 480 and 690 nm were then measured. The concentration of carboxyfluorescein was determined by the ABS(480)−ABS(690) as compared to a standard curve.

Pressure-Sensitive Carboxyfluorescein Release. Bare liposomes, polymer-incorporated liposomes, and protease-triggered, caged liposomes were prepared as above in 10× HBS. Various dilutions of HBS were then made by diluting 10× HBS with 0.012 M HEPES buffer to make the solutions have the desired pressures against 10× HBS. The desired samples were then diluted so that each sample contained 2 μmol carboxyfluorescein in 2 mL in each sample. The diluted samples were incubated at 37.5 °C for the desired time, and then fluorescence measurements were taken (ISA SPEX Fluoromax-2) to determine percent release. Carboxyfluorescein was excited at 450 nm; the fluorescence was recorded from 470 to 620 nm and was integrated to give total fluorescence. Curves of total fluorescence *versus* pressure were fitted using a logistic function:

$$F(\Pi) = \frac{A}{B + Ce^{-D\Pi + E}}$$

where Π is the difference in osmotic pressure and A, B, C, D, and E are fitting constants. The fluorescence *versus* pressure curves were then compared to determine the difference in pressure sensitivity of various liposome preparations.

Urokinase-Sensitive Carboxyfluorescein Release. Protease-triggered, caged liposomes were prepared as above. These liposome preparations were then diluted into HBS with various amounts of uPA so that the final concentration of HBS was 1× and the final content of carboxyfluorescein was 2 μmol in 2 mL. The diluted samples were incubated at 37.5 °C for the desired time, and then fluorescence measurements were taken to determine percent release. Carboxyfluorescein was excited at 450 nm; fluorescence was recorded from 470 to 620 nm and was integrated to give total fluorescence.

Acknowledgment. The authors thank C. Sorenson for valuable input on dynamic light scattering methods. M.B. thanks the National Science Foundation Graduate Research Fellowship Program for support during this research.

Supporting Information Available: NMR data supporting the synthesis of the polymer described is available. Supplementary Figure 1: ^{1}H NMR of tertiary butyl protected, acid-functionalized cholesterol. Supplementary Figure 2: ^{13}C NMR of tertiary butyl protected, acid-functionalized cholesterol. Supplementary Figure 3: ^{1}H NMR of acid-functionalized cholesterol. Supplementary Figure 4: ^{13}C NMR of acid-functionalized cholesterol. Supplementary Figure 5: ^{1}H NMR of amine-functionalized poly(acrylic acid). This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES AND NOTES

1. Hamad, I.; Moghimi, S. M. Critical Issues in Site Specific Targeting of Solid Tumors: The Carrier, the Tumour Barriers and the Bioavailable Drug. *Expert Opin. Drug Delivery* **2008,** *5*, 205–219.
2. Cassidy, J.; Schatzlein, A. G. Tumor Targeted Drug and Gene Delivery: Principles and Concepts. *Expert Rev. Mol. Med.* **2004,** *6*, 1–17.
3. Moses, M. A.; Brem, H.; Langer, R. Advancing the Field of Drug Delivery: Taking Aim at Cancer. *Cancer Cell* **2003,** *4*, 337–341.
4. Allen, T. M.; Cullis, P. R. Drug Delivery Systems, Entering the Mainstream. *Science* **2004,** *303*, 1818–1822.
5. Gabizon, A. A.; Shmeeda, H.; Zalipsky, S. Pros and Cons of the Liposome Platform in Cancer Drug Targeting. *J. Liposome Res.* **2006,** *16*, 175–183.
6. Bogenrieder, T.; Herlyn, M. Axis of Evil: Molecular Mechanisms of Cancer Metastasis. *Oncogene* **2003,** *22*, 6524–6536.
7. Duffy, M. J. Proteases as Prognostic Markers in Cancer. *Clin. Cancer Res.* **1996,** *2*, 613–618.
8. Johansson, N.; Ahonen, M.; Kahari, V. M. Matrix Metalloproteinases in Tumor Invasion. *Cell. Mol. Life Sci.* **2000,** *57*, 5–15.
9. Turk, B. E.; Huang, L. L.; Piro, E. T.; Cantley, L. C. Determination of Protease Cleavage Site Motifs Using Mixture Based Oriented Peptide Libraries. *Nat. Biotechnol.* **2001,** *19*, 661–667.
10. Khasigov, P. Z.; Podobed, O. V.; Gracheva, T. S.; Salbiev, K. D.; Grachev, S. V.; Berezov, T. T. Role of Matrix Metalloproteinases and Their Inhibitors in Tumor Invasion and Metastasis. *Biochemistry (Moscow)* **2003,** *68*, 711–717.
11. Thor, B. Ricin-like Toxin Variants for Treatment of Cancer, Viral or Parasitic Infections. U.S. Patent 6593132, 2003.
12. Turk, V.; Kos, J.; Turk, B. Cystein Cathepsins (Proteases)—On the Main Stage of Cancer?. *Cancer. Cell.* **2004,** *5*, 409–410.
13. Sarkar, N.; Banerjee, J.; Hanson, A. J.; Elegbede, A. I.; Rosendahl, T.; Krueger, A. B.; Banerjee, A. L.; Tobwala, S.; Wang, R.; Lu, X.;*et al.*Matrix Metalloproteinase-Assisted Triggered Release of Liposomal Contents. *Bioconjugate Chem.* **2008,** *19*, 57–64.
14. Elegbede, A. I.; Banerjee, J.; Hanson, A. J.; Tobwala, S.; Ganguli, B.; Wang, R.; Lu, X.; Srivastava, D. K.; Mallik, S. Mechanistic Studies of the Triggered Release of Liposomal Contents by Matrix Metalloproteinase-9. *J. Am. Chem. Soc.* **2008,** *130*, 10633–10642.
15. Duffy, M. J. Urokinase-Type Plasminogen Activator: A Potent Marker of Metastatic Potential in Human Cancers. *Biochem. Soc. Trans.* **2002,** *30*, 207–210.
16. Liu, S.; Bugge, T. H.; Leppla, S. H. Targeting of Tumor Cells by Cell Surface Urokinase Plasminogen Activator-Dependant Anthrax Toxin. *J. Biol. Chem.* **2001,** *276*, 17976–17984.
17. Lee, S. M.; Chen, H.; Dettmer, C. M.; O'Halloran, T. V.; Nguyen, S. T. Polymer Caged Liposomes: A pH-Responsive Delivery System with High Stability. *J. Am. Chem. Soc.* **2007,** *129*, 15096–15097.
18. Koslov, M. M.; Markin, V. S. A Theory of Osmotic Lysis of Lipid Vesicles. *J. Theor. Biol.* **1984,** *109*, 17–39.
19. Rawicz, W.; Olbrich, K. C.; McIntosh, T.; Needham, D.; Evans, E. Effect of Chain Length and Unsaturation on Elasticity of Membrane Bilayers. *Biophys. J.* **2000,** *79*, 328–339.
20. Rawicz, W.; Smith, B. A.; McIntosh, T. J.; Simon, S. A.; Evans, E. Elasticity, Strength, and Water Permeability of Bilayers that Contain Raft-Microdomain Forming Lipids. *Biophys. J.* **2008,** *94*, 4725–4736.
21. Frame, D. New Strategies in Controlling Drug Resistance. *J. Manage. Care Pharm.* **2007,** *13*, 13–17.
22. Hussey, S. L.; He, E.; Peterson, B. R. Synthesis of Chimeric 7-α-Substituted Estradiol Linked to Cholesterol and Cholesterylamine. *Org. Lett.* **2002,** *4*, 415–418.
23. New, R. R. C. Preparation of Liposomes. In *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1990; pp 33−104.
24. Lasch, J.; Weissig, V.; Brandl, M. Preparation of Liposomes. In *Liposomes: A Practical Approach*, 2nd ed.; Torchilin, V., Weissig, V., Eds.; Oxford University Press: New York, 2003; pp 3−30.

25. Ertel, A.; Marangoni, A. G.; Marsh, J.; Hallett, F. R.; Wood, J. M. Mechanical Properties of Vesicles I. Coordinated Analysis of Osmotic Swelling and Lysis. *Biophys. J.* **1993,** *64*, 426–434.
26. Hallett, F. R.; Marsh, J.; Nickel, B. G.; Wood, J. M. Mechanical Properties of Vesicles II. A Model for Osmotic Swelling and Lysis. *Biophys. J.* **1993,** *64*, 435–442.
27. Cohen, F. S.; Akabas, M. H.; Finkelstein, A. Osmotic Swelling of Phospholipid Vesicles Causes Them To Fuse with a Planar Phospholipid Bilayer Membrane. *Science* **1982,** *217*, 458–460.
28. Akabas, M. H.; Cohen, F. S.; Finkelstein, A. Separation of the Osmotically Driven Fusion Event from Vesicle-Planar Membrane Attachment in a Model System for Exocytosis. *J. Cell Biol.* **1984,** *98*, 1063–1071.