Langmuir
The ACS Journal of Surfaces and Colloids

JULY 18, 2006
VOLUME 22, NUMBER 15

Letters

Highly Efficient Capture and Long-Term Encapsulation of Dye by Catanionic Surfactant Vesicles

Xiang Wang,[†] Emily J. Danoff,[†] Nikolai A. Sinkov,[†] Jae-Ho Lee,[‡,§] Srinivasa R. Raghavan,[‡] and Douglas S. English*,[†]

Departments of Chemistry and Biochemistry, and Chemical & Biomolecular Engineering, University of Maryland, College Park, Maryland 20742-2111

Received February 22, 2006. In Final Form: June 12, 2006

Vesicles formed from the cationic surfactant, cetyltrimethylammonium tosylate (CTAT) and the anionic surfactant, sodium dodecylbenzenesulfonate (SDBS), were used to sequester the anionic dye carboxyfluorescein. Carboxyfluorescein was efficiently sequestered in CTAT-rich vesicles via two mechanisms: encapsulation in the inner water pool and electrostatic adsorption to the charged bilayer. The apparent encapsulation efficiency (22%) includes both encapsulated and adsorbed fractions. Entrapment of carboxyfluorescein by SDBS-rich vesicles was not observed. Results show the permeability of the catanionic membrane is an order of magnitude lower than that of phosphatidylcholine vesicles and the loading capacity is more than 10 times greater.

The development of vesicles and liposomes for controlled release applications (e.g., in drug delivery, agrochemicals, or cosmetics) is a technological objective of great interest. An important challenge in this area is the stability and shelf life of vesicles bearing drugs or other molecules. Conventional phospholipid vesicles formed by sonication or extrusion are essentially kinetically trapped nonequilibrium structures. Over time, these vesicles tend to fuse or rupture to form lamellar phases, and in the process, their contents are likely to be released. Improvements in vesicle stability and encapsulation properties can be achieved by changing bilayer composition[1−3] or by using micron-sized vesicles.[4]

A simple attractive alternative to phospholipid vesicles in some applications may be offered by surfactant vesicles, formed by mixing single-tailed cationic and anionic surfactants. The existence of such "catanionic" vesicles has been known for over fifteen years.[5] These vesicles are spontaneously generated when the individual surfactants are mixed with water in the right proportion. Vesicle formation is thus quicker and easier compared to phospholipid vesicles, since extrusion or sonication steps are not required. Furthermore, the required materials are common surfactants that are cheaper than purified or synthetic phospholipids. Catanionic vesicles also tend to be stable for very long periods of time. Whether these vesicles are truly equilibrium structures is still the subject of some debate.[6,7]

For application of catanionic vesicles as storage and delivery agents of small molecules, the critical issue is their ability to encapsulate molecules. In particular, a key unanswered question is: how do catanionic surfactant vesicles compare to conventional phospholipid vesicles in regards to encapsulation efficiency and

* Corresponding author. E-mail: denglish@umd.edu.
[†] Departments of Chemistry and Biochemistry.
[‡] Chemical & Biomolecular Engineering.
[§] Present address: Laboratory of Diagnostic Radiology Research Center, National Institutes of Health.

(1) Xiang, T. X.; Chen, J.; Anderson, B. D. *J. Membrane Biol.* **2000**, *177*, 137.
(2) Ishida, A.; Otsuka, C.; Tani, H.; Kamidate, T. *Anal. Biochem.* **2005**, *342*, 338.
(3) Apel-Paz, M.; Doncel, G. F.; Vanderlick, T. K. *Langmuir* **2005**, *21*, 9843.
(4) Sato, Y.; Shin-ichiro, M. N.; Yoshikawa, K. *Chem. Phys. Lett.* **2003**, *380*, 279.
(5) Kaler, E. W.; Murthy, A. K.; Rodriguez, B. E.; Zasadzinski, J. A. N. *Science* **1989**, *245*, 1371.
(6) Almgren, M.; Rangelov, S. *Langmuir* **2004**, *20*, 6611.
(7) Laughlin, R. G. *Colloids Surf. A* **1997**, *128*, 27.

10.1021/la0605135 CCC: $33.50
Published on Web 06/23/2006

membrane permeability? Despite the extensive literature on catanionic vesicles, there is surprisingly little information on their encapsulating abilities or the permeability of their bilayers. The few studies that have explored encapsulation with well-characterized catanionic vesicles focused principally on the entrapment of glucose.[8−10] Another study by Zhao and co-workers[11] quantified the trapping efficiency of catanionic vesicles for a bromophenol blue dye, but did not study the membrane permeability or long-term stability. In short, the ability of catanionic vesicles to entrap and encapsulate solutes, especially ionic molecules, remains by and large untested.[12]

Here we report preliminary results from our studies on the encapsulation and subsequent release of a model ionic solute from catanionic vesicles. We have chosen carboxyfluorescein (CF) as our model solute. CF is a widely used probe for vesicle encapsulation due to its ability to undergo efficient self-quenching of fluorescence at millimolar concentrations. For example, when 60 mM CF is entrapped in vesicles, its fluorescence intensity is reduced by 60−80%, but as the dye is released from the vesicle, and thus diluted by the surrounding buffer, its fluorescence intensity increases.[13,14] We employ this self-quenching phenomenon to monitor the release of entrapped CF from catanionic vesicles as well as the trapping efficiency of these vesicles. The catanionic vesicles used here are from the well-known CTAT/SDBS system, which is a mixture of the single-tailed cationic surfactant, cetyltrimethylammonium tosylate (CTAT) and the single-tailed anionic surfactant, sodium dodecylbenzenesulfonate (SDBS). For comparison, we conduct similar encapsulation experiments with phospholipid vesicles formed from egg yolk phosphatidylcholine (EYPC). The key result that we report in this paper is that the catanionic vesicles are able to sequester CF more efficiently and for much longer periods of time than the phospholipid vesicles. We use the term sequester to refer to dye that is either captured in the inner water pool of the vesicle or adsorbed to the bilayer since both modes of sequestration are observed here. Our results, though limited in scope, demonstrate the possibility of catanionic vesicles as an extremely efficient alternative for long-term sequestering of small molecules. The broader significance of these results is currently being investigated and will be submitted in a full-paper.

Apparent Encapsulation Efficiency. We studied the apparent encapsulation efficiency of CF in catanionic vesicles at two different CTAT/SDBS compositions, which are pinpointed in the phase diagram (Figure 1).[9,15] The first sample falls in the CTAT-rich vesicle lobe and consists of 1 wt % total surfactant with a 7:3 w/w of CTAT to SDBS. The vesicles in this case are denoted by V^+ since they have a molar excess of the cationic surfactant. The second sample falls in the SDBS-rich vesicle lobe and it is a 3:7 w/w mixture of CTAT to SDBS at 1 wt % total surfactant. These vesicles are denoted by V^-.

Separation of free dye from CF bearing vesicles was achieved by size exclusion chromatography (SEC), as described in the Supporting Information. When encapsulation was achieved, two bands were well-resolved on the column and were visible with the naked eye or by viewing with a UV lamp. The leading band contained vesicles and the second band contained the free dye. DLS experiments were used to confirm these assignments. The DLS results from the leading band always gave values for hydrodynamic radius and total scattering intensity that were consistent with the presence of vesicles. Initial V^+ samples, prior to SEC, were found to have an average radius of 76 ± 5 nm, which was constant throughout the dilute surfactant range of 1.0% to 0.004% total surfactant concentration. This is consistent with the phase diagram in Figure 1. V^+ samples were also studied after elution from the SEC column and the measured average radius was 90 ± 5 nm.

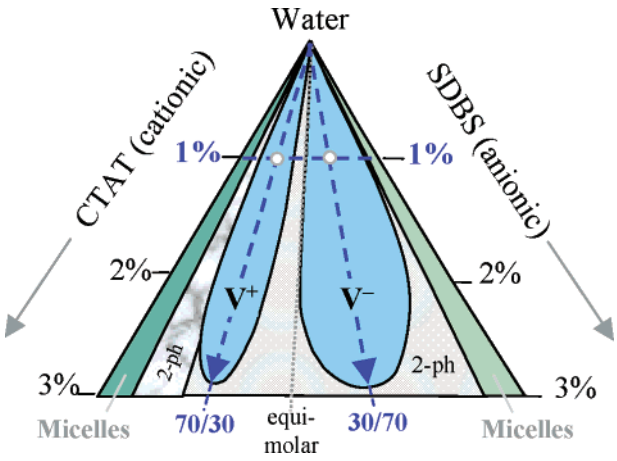

Figure 1. Phase diagram of CTAT/SDBS showing the dilute (water-rich) corner. Vesicles are present in the two lobes, denoted by V^+ and V^-. One composition in each lobe is used in this study and these compositions are indicated by the hollow circles. Adapted from ref 15.

Table 1. Apparent Encapsulation Efficiency and Dye Adsorption for CF on Both EYPC Vesicles and V^+ [a]

	apparent encapsulation efficiency, ϵ	adsorption
EYPC	1.6 ± 0.2%	0.40 ± 0.08%
V^+	21 ± 2%	16 ± 4%

[a] The apparent encapsulation efficiency reflects contributions from dyes that are adsorbed to the bilayer or captured in the inner water pool. Therefore the actual encapsulation efficiency for dye in the V^+ water pool is ca. 5%.

In Table 1, we report the apparent encapsulation efficiency, ϵ, for CF in V^+ and EYPC vesicle preparations. The apparent encapsulation efficiency is calculated using the method described in the Supporting Information. The value of ϵ gives percentage of dye that is captured by the vesicles during their preparation. In the absence of any specific interactions between the solute and the vesicle wall, ϵ is a measure of the aqueous volume enclosed by the vesicles relative to the total solution volume. For EYPC vesicles, ϵ is ca. 1.6%, in agreement with literature values.[16] In comparison, the total enclosed volume of EYPC vesicles calculated from their average DLS radius is about 6%. However, it should be noted that some leakage and rupture of the vesicles is likely to occur during the SEC process, which can explain the difference between these values. Considering next the encapsulation efficiency for the catanionic V^+ vesicles, we note from Table 1 that their ϵ is ca. 21%, which is extremely large compared to the EYPC lipid vesicles. Dye encapsulation was evaluated using 1 mM CF since it was found that CF concentrations above 5 mM inhibited vesicle formation. Experiments to measure ϵ for V^- samples were highly irreproducible, yielding ranges from 0

(8) Caillet, C.; Hebrant, M.; Tondre, C. *Langmuir* **2000**, *16*, 9099.
(9) Kaler, E. W.; Herrington, K. L.; Murthy, A. K.; Zasadzinski, J. A. N. *J. Phys. Chem.* **1992**, *96*, 6698.
(10) Fischer, A.; Hebrant, M.; Tondre, C. *J. Colloid Interface Sci.* **2002**, *248*, 163.
(11) Zhao, G.-X.; Yu, W.-L. *J. Colloid Interface Sci.* **1995**, *173*, 159.
(12) Tondre, C.; Caillet, C. *Adv. Colloid Interface Sci.* **2001**, *93*, 115.
(13) Schwarz, G.; Arbuzova, A. *Biochim. Biophys. Acta* **1995**, *1239*, 51.
(14) Weinstein, J. N.; Yoshikami, S.; Henkart, P.; Blumenthal, R.; Hagins, W. A. *Science* **1977**, *195*, 489.
(15) Koehler, R. D.; Raghavan, S. R.; Kaler, E. W. *J. Phys. Chem. B* **2000**, *104*, 11035.
(16) Goldbach, P.; Brochart, H.; Wehrle, P.; Stamm, A. *Int. J. Pharm.* **1995**, *117*, 225.

to 3% with no apparent dependence on any governable variables. Given that the total concentration of surfactant is the same for both V^+ and V^- samples, the differences in the value and reliability of ϵ is unexpected from simple predictions based on enclosed volume. The large and highly reproducible value of ϵ for the V^+ samples is likely due to strong, specific interactions between the V^+ bilayer and the anionic CF dye. If this assertion is correct, one might expect a measurable value for ϵ even when the dye is added after vesicle formation due to strong interactions of CF with the outer leaflet of the V^+ bilayer.

To test whether adsorption of CF to the vesicle bilayer was significant, we conducted measurements in which the dye was added to a vesicle solution after the vesicles were formed. These experiments were done with both EYPC and V^+ vesicles, and in each case the vesicles were then separated on the SEC column and the amount of adsorbed dye was calculated. The results in Table 1 show that only 0.4% of the dye was adsorbed on the EYPC vesicles, indicating that nonspecific interactions of the dye with the lipid bilayer were weak. On the other hand, for the V^+ samples, about 16% of the dye was adsorbed, which is comparable with the encapsulation efficiencies measured earlier. Thus, binding of the anionic CF to the V^+ bilayer contributes significantly to the amount of sequestered dye. Since adsorption accounts for as much as 75% of the captured dye we have referred to ϵ as the apparent encapsulation efficiency. We hypothesize that adsorption occurs via direct, electrostatic interaction between the anionic dye and the excess cationic surfactant in the V^+ bilayer. Electrostatic interactions between charged surfactant vesicles and polyanions in solution have been observed,[17] and Karukstis et al. have reported favorable dye-bilayer interactions in which association was observed to increase with surface charge.[18] Our findings are significant because they illustrate that excess charge in the bilayer effectively increases the loading capacity of the vesicles. To our knowledge, this is a unique property of the catanionic surfactant vesicles that has not previously been reported.

Long-Term Dye Release. We now consider the question of how long the encapsulated fraction remains in the V^+ vesicle interior compared to encapsulation by EYPC vesicles. As time progresses, we expect the encapsulated dye to leak through the vesicle bilayer and into the solution. As discussed earlier, the self-quenching of CF provides a convenient way to monitor its efflux. If the dye is released into the external solution by disrupting the vesicles, there is a large increase in the CF emission intensity. This is illustrated by Figure 2, which shows several time traces obtained over the course of four weeks from V^+ vesicles containing encapsulated CF. For these experiments, a substantial volume of the CF/V^+ sample was prepared on the first day and run on the SEC column to remove free dye; therefore, each trace in Figure 2 is for data acquired from the same preparation at a given number of days after SEC was run. The traces show the emission intensity before and after the addition of Triton X-100, a nonionic detergent that disrupts both lipid and surfactant vesicles.[19] As can be seen, the resulting release of dye into the solution causes a large jump in emission intensity, and the size of this jump is proportional to the amount of dye encapsulated within the vesicles. We note that the intensity jump reports on the encapsulated dye and not on the adsorbed dye, since addition of Triton X-100 to vesicle samples in which the dye was added after vesicle preparation did not produce an intensity jump. As

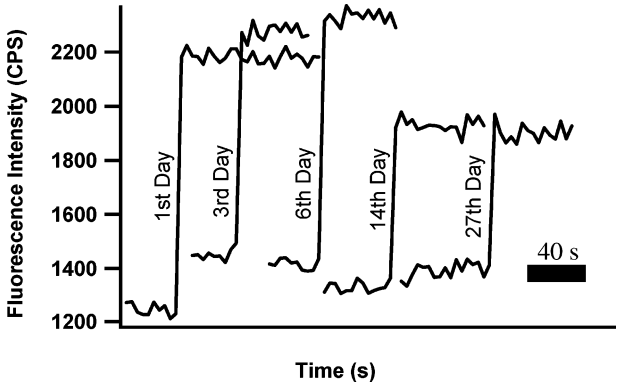

Figure 2. Denaturation of catanionic vesicles and release of carboxyfluorescein. Each time trace depicts detergent-induced denaturation of a 1.5 mL aliquot of carboxyfluorescein-loaded, CTAT-rich, catanionic vesicles taken from a single batch. The spike in fluorescence is due to the increase in carboxyfluorescein fluorescence efficiency as it is released upon disruption of the catanionic vesicles. The scale bar shows the time scale for the individual traces and the day denotes the age of the sample.

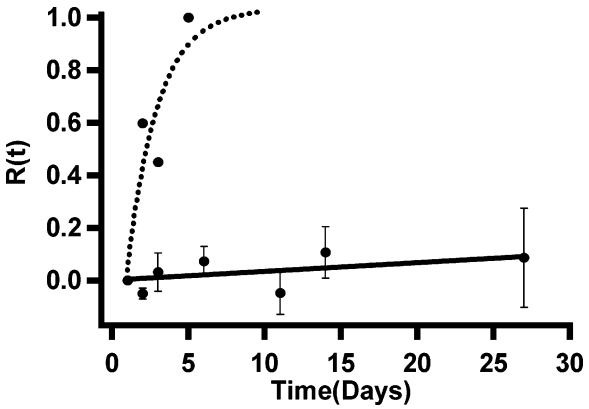

Figure 3. Comparison of dye released as a function of time, $R(t)$, between equilibrium vesicles (solid line) and phospholipid vesicles (dotted line). Release of carboxyfluorescein (CF) is shown as a function of time over a period of 27 days. The half-life for release in catanionic vesicles is 84 days compared to 2 days in phospholipid vesicles, illustrating the enhanced stability of V^+ samples.

expected, the largest jump occurs for the freshly prepared vesicle solution where all the dye is encapsulated in the vesicles. As described in the Supporting Information, we compare the magnitude of the jump on Day x with the highest jump (Day 0) and thereby obtain the fraction of the dye released on day x, $R(t = x)$. It should be noted that $R(t)$ may actually underestimate the degree of dye retention since it does not account for dequenching occurring within the vesicles as the dye leaks out. This effect will be negligible in the catanionic samples since the dye concentration remains nearly unchanged over the time course of Figure 3.

Plots of $R(t)$ are shown in Figure 3 for CF in V^+ (solid line) and EYPC (dotted line) vesicles. First, consider the results for vesicles formed from EYPC. Here, the dye is released rapidly over a period of about 5 days, yielding an estimated half-life of ca. 2 days for the entrapped dye. When $R(t)$ reaches 1 there is no longer an increase in fluorescence emission upon addition of detergent, i.e., the dye concentration inside the vesicles has equilibrated with that of the bulk solution. Note that the equilibration takes place by transport across the membrane not by vesicle degradation, since the vesicles themselves are stable for up to several weeks. In contrast to EYPC vesicle samples, V^+ samples are able to encapsulate CF over an extremely long period of time. The release of CF is approximately 20% after 27

(17) Antunes, F. E.; Marques, E. F.; Gomes, R.; Thuresson, K.; Lindman, B.; Miguel, M. G. *Langmuir* **2003**, *20*, 4647.
(18) Karukstis, K.; Zieleniuk, C. A.; Fox, M. J. *Langmuir* **2003**, *19*, 10054.
(19) Jang, H.; Pell, L. E.; Korgel, B. A.; English, D. S. *J. Photochem. Photobiol., A* **2003**, *158*, 111.

days giving an estimated half-life of 84 days for the entrapped dye. DLS data taken over the 27-day course of the experiments show that the catanionic vesicle average radii remain unchanged and indicate that vesicle fusion or rupture is not occurring to any significant degree. This indicates a fundamental difference in the permeability of V^+ membranes to anionic solutes and in the overall vesicle stability compared with lipid vesicles. We acknowledge that it is possible to obtain slower efflux rates in EYPC vesicles. For instance, the addition of cholesterol or changes in lipid composition will improve the long-term encapsulation by EYPC vesicles.[1−3] Xiang and Anderson[1] show that the addition of 0.25 volume fraction of cholesterol to EYPC decreases membrane permeability by nearly 10-fold. Here we show that catanionic vesicles achieve much better encapsulation stability without additional components.

We have illustrated the ability for V^+ to achieve dramatically different encapsulation characteristics for CF relative to those of EYPC vesicles. At this point we do not know how general this observation is or whether these improved characteristics will emerge for other dyes or vesicle compositions. The original motivation for investigating CF encapsulation was to provide a direct comparison with the well characterized CF/EYPC system. Recently, Fischer et al. have reported that CTAT-rich vesicles are less permeable to glucose than SDBS-rich vesicles or vesicles prepared from the pure ion-pair amphiphiles.[10] Future work is under way to determine if the extraordinary ability of V^+ to sequester CF is more than a mere curiosity and actually the first evidence of a general trend leading to important applications.

Conclusions

In summary, spectroscopic evidence has been presented supporting the unprecedented capacity and long-term encapsulation of positively charged catanionic vesicles for anionic CF. The remarkable apparent encapsulation efficiency of 21% is assigned to electrostatic interaction between the anionic solute and the excess positive charge of the V^+ bilayer. The long-term stability of the encapsulation is due to low membrane permeability. Previous studies have shown that fusion of catanionic vesicles occurs on a relatively long time scale of months.[6] Here we have shown that the encapsulation of anionic solutes does not appear to radically alter this process. Thus catanionic vesicles are promising candidates for high efficiency capture and long-term encapsulation of ionic solutes.

Acknowledgment. This work was supported in part by the National Science Foundation MRSEC-DMR #0080008 and by a grant from the Center for Nano-Manufacturing & Metrology to D.S.E. The authors also wish to thank the reviewers for their helpful comments and Mr. Charles Luckett for his critical reading of the manuscript.

Supporting Information Available: Materials used and methods of investigation. This material is available free of charge via the Internet at http://pubs.acs.org.

LA0605135

Supporting Information for
Highly Efficient Capture and Long-Term Encapsulation of Dye by Catanionic Surfactant Vesicles

*Xiang Wang, Emily J. Danoff, Nikolai A. Sinkov, Jae-Ho Lee, Srinivasa R. Raghavan, & Douglas S. English**

MATERIALS AND METHODS

Materials. The surfactants CTAT, SDBS, and Triton X-100 were obtained from Aldrich Chemicals, and the phospholipid EYPC was acquired from Avanti Polar Lipids. The fluorescent dye 5(and-6)carboxyfluorescein (CF) was purchased from Molecular Probes. All chemicals were used without further purification.

Vesicle Preparation. Figure 1 shows the water-rich corner of the ternary phase diagram for mixtures of CTAT, SDBS, and water. Catanionic vesicles are present at compositions within the two lobes on either side of the equimolar line. Vesicle samples were prepared by weighing and mixing the two surfactants in water, followed by gentle stirring. For large unilamellar vesicles (LUV) of EYPC an extrusion method described previously was used.[1]

Dye Encapsulation. For dye encapsulation, vesicles were prepared using aqueous solutions containing 1.0 mM CF since it was found that the formation of CTAT-rich vesicles (7:3 CTAT to SDBS w/w) was inhibited at CF concentrations of 5 mM or greater. The CF/CTAT/SDBS solutions were stirred for 15-30 min and the resulting

vesicle solutions were allowed to equilibrate in the dark at room temperature for at least 48 h. Dynamic light scattering (described below) was then used to confirm vesicle formation. SDBS-rich vesicles (e.g., 3:7 CTAT to SDBS w/w) could be prepared with higher CF concentrations (up to ca. 100 mM); however CF encapsulation in these vesicles was greatly reduced relative to that of CTAT-rich vesicles and in many cases CF encapsulation by SDBS-rich vesicles (i.e., the formation of distinct bands in the chromatography column, see below) was not achieved.

Size exclusion chromatography (SEC) was used to separate the free dye from the encapsulated dye. A 2 x 25 cm column packed with Sephadex G50 resin (medium mesh, Amersham Biosciences) was used. During elution, vesicle solutions divided into two clear bands, one containing the dye-bearing vesicles and the other consisting of free dye. The band containing the vesicles was collected for further studies of vesicle leakage, as described below. The apparent encapsulation efficiency (ε) was also determined by measuring the amount of encapsulated dye relative to the total initial amount, using UV-Vis absorption (Hitachi U-3010 Spectrometer). It is thus defined as:

$$\varepsilon = \frac{V_f C_f}{V_i C_i} \tag{1}$$

where V and C are volume and concentration and i denotes initial values taken from the original preparation and f denotes values taken from the leading band in the SEC column. To avoid artifacts in UV-Vis spectroscopy from light scattering or from dye aggregation inside the vesicles, the absorbance of the encapsulated dye was determined after first disrupting the vesicle membranes by the addition of Triton X-100 surfactant. As

mentioned in the main text ε reflects contributions from CF that is both encapsulated in the water pool of the vesicle and electrostatically adsorbed to the bilayers of V^+ samples.

Long-Term Dye Encapsulation and Release. We exploit the self-quenching behavior of CF to monitor dye efflux from vesicles.[2,3] The following experimental protocol was adopted, which is similar to that in an earlier study[1], except for changes made to allow calculation of the fraction of dye released over a long period (weeks). Samples were checked on a specific day by placing a fixed aliquot (1.5 mL) into a 1 cm cuvette and monitoring its emission at 520 nm while exciting at 490 nm using a Spex Fluorolog-3 spectrometer. The intensity was monitored for several minutes to establish the baseline fluorescence intensity, which contains a contribution from both free and encapsulated dyes. After the baseline was established, 100 μL of 10% (w/w) aqueous Triton X-100 was added to disrupt the vesicles. Vesicle disruption results in the release of all dye molecules into solution and a concomitant increase in fluorescence (see Figure 2). To monitor long-term leakage rates, the fraction of dye released as a function of time, *R(t)*, was calculated for a given day. This quantity measures the fraction of encapsulation on *Day x* relative to the initial value on *Day 0*:

$$R(x) = 1 - \left\{ \frac{F_x(final) - F_x(initial)}{F_0(final) - F_0(initial)} \bullet \frac{F_o(final)}{F_x(final)} \right\} \qquad (2)$$

where *F*(*initial*) and *F*(*final*) are the fluorescence intensities before and after adding the Triton X-100. This approach allows the direct determination of the proportion of the dye released on a daily basis and accounts for deviations due to long-term drift in the spectrophotometer.

Dynamic Light Scattering (DLS). The sizes of vesicles in solution were monitored using DLS on a Photocor-FC instrument. The light source was a 5 mW laser at 633 nm and the scattering angle was 90°. A logarithmic correlator was used to obtain the autocorrelation function, which was analyzed by the method of cumulants to yield a diffusion coefficient.[4] The apparent hydrodynamic size of the vesicles was obtained from the diffusion coefficient through the Stokes-Einstein relationship. The intensity (total counts) of the signal was also recorded for each sample.

References

(1) Jang, H.; Pell, L. E.; Korgel, B. A.; English, D. S. *J. Photochem. Photobiol., A* **2003**, *158*, 111.
(2) Schwarz, G.; Robert, C. H. *Biophys. J.* **1990**, *58*, 577.
(3) Schwarz, G.; Robert, C. H. *Biophysical Chemistry* **1992**, *42*, 291.
(4) Koppel, D. E. *J. Chem. Phys.* **1972**, *57*, 4814.