Liposome-Templated Supramolecular Assembly of Responsive Alginate Nanogels

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Nanosized gel particles (nanogels) are of interest for a variety of applications, including drug delivery and single-molecule encapsulation. Here, we employ the cores of nanoscale liposomes as reaction vessels to template the assembly of calcium alginate nanogels. For our experiments, a liposome formulation with a high bilayer melting temperature (T_m) is selected, and sodium alginate is encapsulated in the liposomal core. The liposomes are then placed in an aqueous buffer containing calcium chloride, and the temperature is raised up to T_m. This allows permeation of Ca^{2+} ions through the bilayer and into the core, whereupon these ions gel the encapsulated alginate. Subsequently, the lipid bilayer covering the gelled core is removed by the addition of a detergent. The resulting alginate nanogels have a size distribution consistent with that of the template liposomes (ca. 120−200 nm), as confirmed by transmission electron microscopy and light scattering. Nanogels of different average sizes can be synthesized by varying the template dimensions, and the gel size can be further tuned after synthesis by the addition of monovalent salt to the solution.

1. Introduction

Polymer hydrogels (i.e., three-dimensional networks of polymer chains swollen in water) are ubiquitous in biology as well as in technology. Such hydrogels have long been envisioned as a means of storing an active ingredient, such as a therapeutic drug, flavor molecule, cosmetic ingredient, or agrochemical, and slowly releasing these molecules into the surrounding environment.[1−3] For example, hydrogels made of synthetic degradable polymers have been used in medicine as implantable drug delivery vehicles.[1,4] In many emerging biomedical applications, the size of the hydrogel is turning out to be an important control variable.[5−8] For instance, gel particles smaller than about 200 nm can evade capture by macrophages in the bloodstream and are thus more likely to remain in circulation for longer times compared to larger particles.[7] For cancer therapy also, gels ranging in size from about 100 to 200 nm could be particularly useful, since these tend to preferentially accumulate in many tumors.[5,6] Thus, a need exists for small hydrogel particles in the nanoscale size regime.

In this paper, we describe the formation of nanosized gels using lipid vesicles (liposomes) as a template. As is well-known, liposomes are closed structures formed by the self-assembly of amphiphilic lipid molecules in water, with the lipids organized at the liposomal shell in the form of a bilayer membrane.[9] We employ the cores of nanosized liposomes as reaction vessels within which we induce the gelation of the biopolymer sodium alginate under the action of divalent calcium ions. The gelation transforms the liposomal interior from a fluid state to a soft, elastic solid. Upon removal of the lipid bilayer covering the gelled core, we are left with alginate nanogel particles that closely match the size of the liposomal template. We characterize our nanogels by light scattering as well as microscopic techniques. A light-scattering technique coupled with field-flow fractionation[10] allows us to precisely compare the size distributions of the liposomes and the templated nanogels. The data show that our synthesis scheme corresponds to a true templating reaction and that our procedure can be extended to preparing nanogels of different sizes and chemistries. Moreover, these alginate nanogels can be subsequently reconstituted as stable dispersions in water or buffer solutions.

A few reports have recently appeared on gel synthesis using liposomes as templates,[11−17] although in most of these cases the focus was on relatively large gel particles. The term "lipobead" has been used by some authors to refer to large gel particles that retain a lipid bilayer coating on their surface.[13] Only one group has systematically studied the synthesis of *nanosized* gels via liposomal templates, and the gels in this case were based on the synthetic polymer poly(*N*-isopropylacrylamide) (NIPAAm) and

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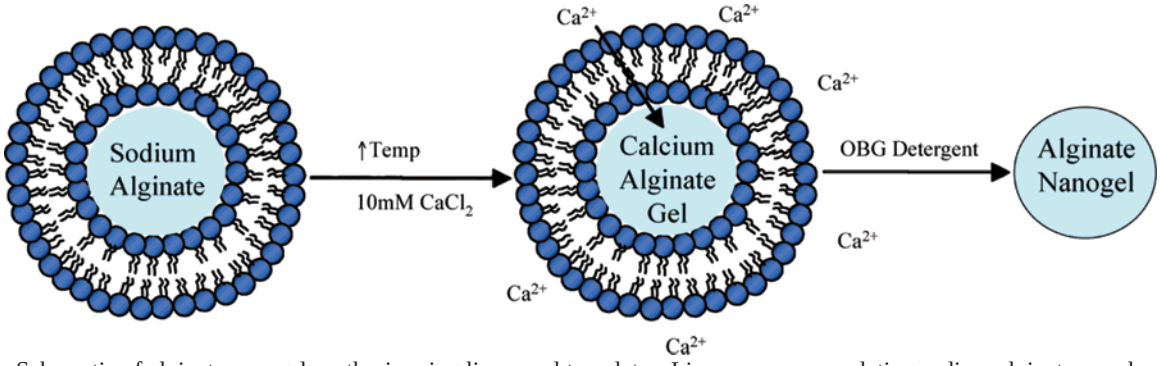

Figure 1. Schematic of alginate nanogel synthesis using liposomal templates. Liposomes encapsulating sodium alginate are placed in a 10 mmol/L $CaCl_2$ solution and exposed to temperatures near the T_m of the lipid. The increased transmembrane permeability allows Ca^{2+} to diffuse into the liposomes and ionically cross-link the alginate to form a nanogel. Subsequent removal of the lipid shell yields alginate nanogels.

were formed by free-radical polymerization.[12,18] Our interest in this study is on ionically cross-linked biopolymer nanogels, and the emphasis of the present work is on improvement and detailed characterization of the templating process of such gels within liposomes.

To improve the templating process, our approach is to use a trigger mechanism to initiate cross-linking within the liposomal cores. We exploit a well-known property of lipid bilayers, which is that the bilayers become more permeable close to their bilayer melting temperature (T_m).[19−22] The increased permeability arises because, near T_m, the bilayer exhibits a coexistence of "gel" domains (in which the lipid tails are elongated and rigid) and liquid crystalline domains (where the lipid tails are fluid).[19,20] Between these domains, there are grain boundaries, which causes the formation of pores in the membrane,[20] in turn leading to an increase in transmembrane permeability. Here, we employ lipids that have a T_m above room temperature to form our liposomes, and we encapsulate sodium alginate in these liposomes (Figure 1). Next we introduce Ca^{2+} ions into the solution, and we raise the temperature to the T_m. Thereby, we facilitate the diffusion of Ca^{2+} ions into the liposomal core (Figure 1), where the ions cross-link alginate chains at junction zones and create a gel (this is the well-known "egg-box" mechanism[23]). We have used alginate in our studies because it is appealing as a biomaterial due to its ability to undergo cross-linking under mild conditions.[23−25]

The ability to gel the cores of liposomes has applications beyond drug delivery. Specifically, it is worth noting that eukaryotic cells can generally be considered as gels enclosed by a bilayer membrane, where the gel is formed by the polymerization of cytoskeletal proteins such as actin, filamin, and tubulin. Thus, a liposome with a gelled core might be a better model for a biological cell compared to a buffer-filled liposome.[14] Additionally, a gelled liposome could also be used as a container for single molecule fluorescence studies, e.g., for localizing a single DNA or protein molecule within the interior. These types of fundamental studies will be the focus of future efforts in our lab.

2. Experimental Section

Materials. The lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, > 99% purity) and cholesterol (>98%) were purchased from Avanti Polar Lipids.[26] Other amphiphiles and chemicals were purchased from Sigma-Aldrich, including the lipid dicetyl phosphate (DCP), the detergent octyl β-glucopyranoside (OBG), sodium azide (NaN_3), and ethylenediaminetetraacetic acid (EDTA). The alginate biopolymer was also obtained from Sigma-Aldrich, and it was a low-viscosity sodium alginic acid, composed primarily of 1−4-β-D-mannuronic acid residues. The molecular weight of the polymer was determined to be around 145 000 Da by light scattering (Zimm plot). Salts NaCl and $CaCl_2$ were purchased from Fisher Scientific. The buffer ingredients tris(hydroxymethyl)aminomethane (Tris) from Amresco and *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) (TAPS) from Midwest Scientific (Valley Park, MO) were used to prepare Tris−TAPS−NaCl buffer (at pH 8.0) by combining 50 mmol/L of Tris and TAPS with 15 mmol/L of NaCl. A Tris−TAPS−$CaCl_2$ buffer was also prepared, with the same Tris and TAPS concentrations combined with 10 mmol/L of $CaCl_2$. NaN_3 (3 mmol/L) was added to all buffer solutions to prevent bacterial contamination.

Liposome Preparation. A lipid formulation consisting of DPPC:cholesterol:DCP (7:2:1 molar ratio) was used to prepare liposomes by the solvent injection method.[27] This method involves dissolving a 50 mmol/L aliquot of the lipid mixture in chloroform within a glass tube and evaporating the solvent under a dry nitrogen stream. The resulting thin lipid film was placed in a vacuum desiccator at room temperature overnight to completely remove any residual solvent. The dried lipid film was resolubilized in 50 μL of dry isopropyl alcohol (IPA) and injected into 1 mL of Tris−TAPS−NaCl buffer while vortexing at the highest setting. This yielded control liposomes at a concentration of 5 mmol/L. To prepare liposomes containing the alginate in their cores, the alginate was added to the buffer solution prior to lipid injection. Details on alginate nanogel preparation are given in the Results section.

Light Scattering and Asymmetric Flow Field-Flow Fractionation (AF4). An Eclipse AF4 setup integrated with a light-scattering instrument (DAWN EOS, Wyatt Technology) was used for size separation and characterization of the liposomes and nanogels.[10] The utility of AF4 is to provide a more accurate size distribution of particles in a sample. AF4 fractionates particles on the basis of their diffusion coefficients in a flow channel. A sample of polydisperse particles is injected into the channel, and a perpendicular crossflow focuses the sample against an accumulation membrane at the bottom of the channel. The crossflow is then gradually reduced, whereupon smaller particles reach equilibrium between diffusion and drift velocity farther above this membrane, where the channel flow is faster. Thus, fractionation occurs as smaller particles elute first, followed by larger particles. The eluting stream is directly coupled to the light-scattering instrument to obtain the size distribution.

The AF4 channel used in this study had a 250 μm thick separation spacer, and a cellulose membrane with a 10 kDa cutoff was used

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at the bottom of the channel. The Tris−TAPS−NaCl buffer was used as the carrier solution in all AF4 runs. For the control liposomes, 10 μL of the solution was loaded into the AF4 injection loop, and the separation was conducted with a 1 mL/min channel flow. The crossflow was continuously varied from 0.8 mL/min to zero over 70 min. For the templated nanogels, the same conditions were used, except with a 30 μL sample volume and 60 min elution time. Light-scattering data were collected simultaneously at 10 scattering angles θ on each eluting sample and were analyzed as follows, using the instrument software. In the limit of dilute, noninteracting particles, the intensity will depend only on the particle size and shape (form factor). Thus, a Guinier plot of the data (i.e., a semilog plot of the intensity vs $\sin^2 \theta/2$) will describe a straight line, the slope of which will be equal to $R_g^2/3$, where R_g is the radius of gyration of the particles. The R_g for each eluting slice can thus be obtained, and these values can be combined to construct a particle size distribution.

Microscopy. Transmission electron microscopy (TEM) of alginate nanogels was performed on a Philips EM 400T microscope operating at 120 kV equipped with a Soft Imaging System CCD camera (Cantega 2K). TEM samples were prepared by dropping diluted dispersions of the nanogels onto 600-mesh carbon-coated copper grids, following which the grids were immediately freeze-dried (lyophilized). Optical micrographs of larger liposomes and alginate gel particles were obtained using a Carl Zeiss Axiovert 200 inverted microscope with a 40× differential interference contrast (DIC) objective.

3. Results and Discussion

Nanogel Synthesis. The template liposomes used here are formed from a mixture of DPPC:cholesterol:DCP in a molar ratio of 7:2:1. The major component, DPPC, is a zwitterionic lipid having a $T_m \approx 42$ °C, which means that the liposome bilayers are in their gel state at room temperature.[19] We incorporated cholesterol in our lipid formulation because low amounts of cholesterol further enhance membrane permeability near T_m (see below).[19,21] DCP is an anionic lipid that gives a net negative charge to the bilayers, which in turn prevents aggregation of liposomes through electrostatic (double-layer) repulsions.[19,21]

To prepare liposomes containing alginate, we first added 1% w/v sodium alginate to Tris−TAPS−NaCl buffer and then injected the lipid formulation in IPA into this buffer solution. This procedure results in liposomes containing some sodium alginate in their cores (see below for further size characterization). We then subjected the liposomes to five freeze−thaw cycles with liquid nitrogen and hot tap water. Freeze−thaw cycles are useful for enhancing encapsulation of solutes like alginate, since freezing tends to disrupt membrane bilayers, which may cause the solute to enter the liposome upon thawing and membrane re-formation.[28] Unencapsulated alginate was thereafter removed via three centrifugation/buffer rinses, each at 13 200 rpm for 10 min, with resuspension in 1 mL Tris−TAPS−NaCl buffer. The last resuspension was done with 1 mL of Tris−TAPS−$CaCl_2$ buffer to initiate gelling of the sodium alginate chains in the liposomes via the divalent Ca^{2+} cations.

As discussed in the Introduction, we exploited the increased permeability of bilayers near their T_m to facilitate entry of Ca^{2+} into the liposome core.[19−22] To expose the sample to a temperature cycle across T_m, we placed alginate-containing liposomes in a heated water bath (60 °C) followed by an ice bath (0 °C), both under continuous stirring. The rate of temperature change was measured by a digital thermometer and was approximately 1 °C/s in both cases. Samples were exposed to 10 such temperature cycles across the T_m of DPPC. The net effect is that Ca^{2+} ions

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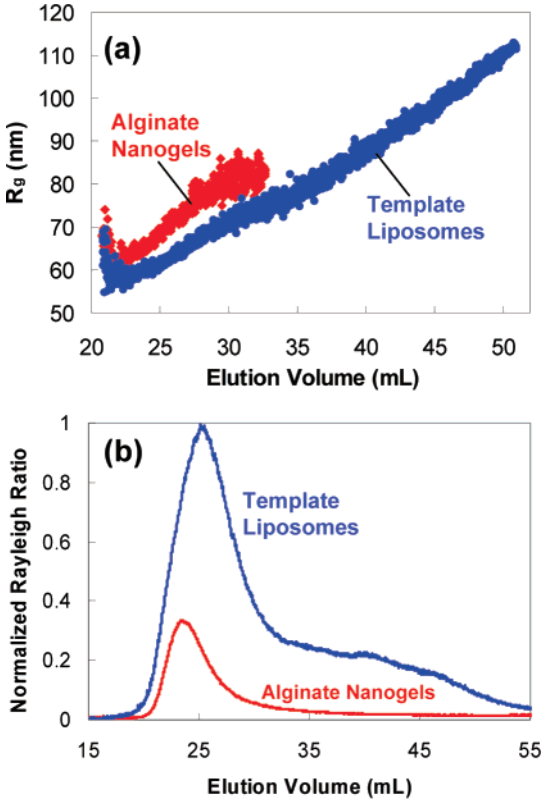

Figure 2. Data from light scattering for the template liposomes and alginate nanogels, following AF4 fractionation. The radius of gyration, R_g, is shown in part a, while the scattering intensity (normalized Rayleigh ratio) at 90° is shown in part b.

diffuse increasingly through the bilayer and cross-link the alginate chains, as shown in Figure 1. We are thus able to accomplish alginate gelation in the liposome cores using a low Ca^{2+} concentration gradient. The lipid bilayer still covers the gel particles, and so what we have at this stage are nanosized lipobeads. The lipobeads were rinsed three times by centrifugation (13 200 rpm for 10 min) using 1 mL of Tris−TAPS−NaCl that also contained 2 mmol/L of the Ca^{2+} chelator EDTA.

Next, we converted the lipobeads to nanogels. The lipid bilayers around the lipobeads were removed by adding 30 mmol/L of OBG. OBG is a single-tailed detergent that is known to disrupt lipid bilayers because the detergent has a very different (cone-shaped) molecular geometry compared to conventional lipids (which are cylinder-shaped). The OBG treatment results in a stable dispersion of calcium alginate nanogels. The nanogels were rinsed by centrifugation (13 200 rpm for 10 min) and resuspended in 0.3 mL of Tris−TAPS−NaCl buffer. Nanogel dispersions in buffer remained stable over the period of observation (several weeks). For comparison with the nanogels, we also ran two controls through the same above procedure, *viz.* empty DPPC:cholesterol:DCP liposomes and the same liposomes encapsulating ungelled alginate (in the latter case, the Ca^{2+} gelation step alone was omitted).

Nanogel Characterization by Light Scattering. Figure 2 shows real-time light-scattering data for the template liposomes and for the alginate nanogels, following size-fractionation by AF4. As described in the Experimental Section, the light-scattering data for each AF4 elution slice are analyzed to obtain the corresponding radius of gyration R_g. Figure 2a plots the R_g as a function of elution volume while Figure 2b shows corresponding data for the scattered intensity (normalized excess Rayleigh ratio at 90°). The template liposomes scatter strongly, and their R_g ranges from 55 to 112 nm. The nanogels prepared from these

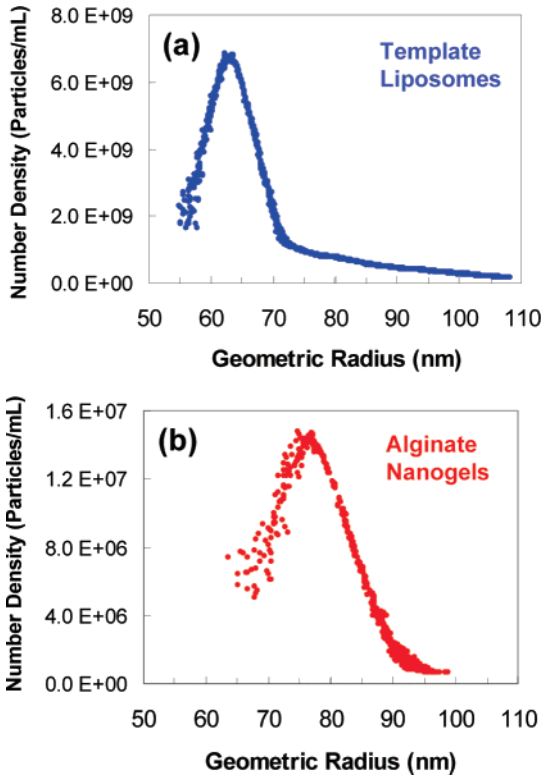

Figure 3. Size distributions of the template liposomes (a) and the alginate nanogels (b) obtained from the light-scattering data in Figure 2.

liposomes have a more narrow range from 65 to 85 nm for their R_g. Thus, the nanogel sizes fall within those of the template liposome. Note that the peak scattered intensity in Figure 2b from the nanogels is about 4-fold weaker than that from the template liposomes, which means that the number density of nanogels is low compared to the liposomes.

The data in Figure 2 are converted into size distributions in Figure 3 for both the template liposomes and the alginate nanogels. The template liposomes (Figure 3a) have a size distribution that peaks around a radius of about 63 nm followed by a long tail. The nanogels (Figure 3b) show a slightly wider peak centered around a radius of ca. 75 nm, but the long tail is absent. Thus, on average, the nanogels are slightly larger than the template liposomes, due in part to their tendency to swell in solution (see below). On the whole, however, the sizes of the two structures are quite comparable. In other words, the nanogels do correspond in size to their liposome molds; i.e., a true templating has been achieved. Note that the peak number density of the template liposomes is at least 2 orders of magnitude greater than that of the alginate nanogels. This is consistent with Figure 2b and implies that the yield of nanogels is quite low. We attribute the low yield to the low encapsulation efficiency of alginate in the liposomes. The encapsulation efficiency of polymers in liposomes is generally quite low (<10%), especially for large polymer coil sizes.[9]

We further confirmed nanogel formation by comparing the nanogel sample to the two controls (empty liposomes and liposomes with ungelled alginate), following OBG detergent treatment. Figure 4 plots the scattered intensity (normalized excess Rayleigh ratio at 90°) for each of these samples as they elute from the AF4 device. The OBG should disrupt the liposomes in both controls and convert them into smaller micelles, which in turn should get removed by the centrifugation/buffer rinses. Thus, we expect to see minimal scattering from the control samples. On the other hand, the nanogels should remain intact upon OBG

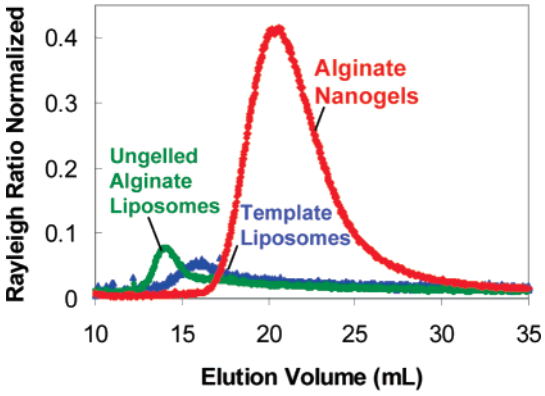

Figure 4. Light-scattering intensities (normalized Rayleigh ratios) at 90° for samples passed through the AF4 setup following OBG detergent treatement. Data are shown for the template liposomes (blue), liposomes containing ungelled alginate (green), and alginate nanogels (red). Only the nanogels show a significant scattering intensity.

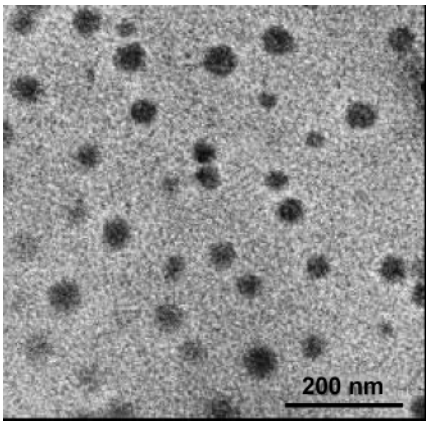

Figure 5. TEM images of alginate nanogels. The sample was freeze-dried on the TEM grid before imaging.

treatment, and the rinsed nanogel dispersion should still scatter strongly. This is indeed what we find in Figure 4: the scattering from the nanogels is quite high, whereas the scattering from each of the two controls is negligible. Figure 4 thus confirms that we have indeed formed nanogel particles by our procedure.

Nanogel Characterization by TEM. In addition to the indirect characterization by light scattering described above, we have also obtained direct images of nanogels using TEM. To obtain these images, drops of the rinsed nanogel dispersion were placed on TEM grids, which were then freeze-dried. No further contrast enhancement or staining was done. TEM images of freeze-dried alginate nanogels are presented in Figure 5, where we observe distinct spherical structures, well-separated from one another. The spheres have radii around 25−50 nm, which are smaller than the values measured by light scattering. However, the TEM sizes correspond to dried nanogels, whereas the light scattering was done on nanogels in solution. Indeed, alginate nanogels in aqueous solution are known to swell up to several times their dehydrated size.[29,30] We also performed TEM studies on a control sample of freeze-dried template liposomes, but no structures could be observed (results not shown). This is consistent with the notion that liposomes are fragile, self-assembled structures that get disrupted during the freeze-drying process.

Nanogel Response to Salt (NaCl). The above data confirm the successful synthesis of alginate nanogels using liposomes as

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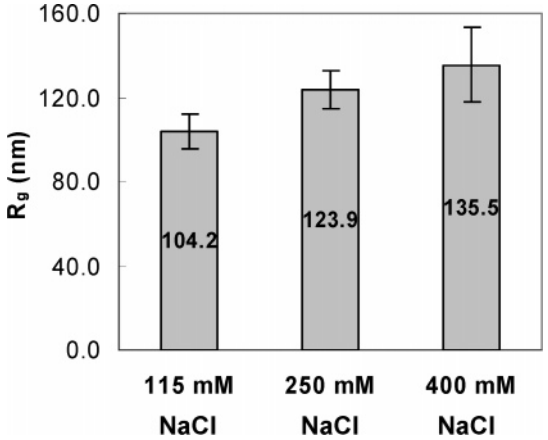

Figure 6. Sizes of alginate nanogels at different concentrations of NaCl. The data show an increase in size with increasing salt concentration.

templates. But do these nanogels show the same responsive properties as much larger alginate gels? To test nanogel responsiveness, we have examined the effect of adding NaCl to the nanogel dispersions. If Na^+ ions from the bulk solution were to exchange with the Ca^{2+} cross-links in the alginate gel, the net degree of cross-linking of the gel would be lowered, and consequently, the gel would swell more.[29,30] We have therefore monitored changes in the nanogel radius at increasing NaCl concentrations. The control sample of nanogels in Tris−TAPS−NaCl buffer corresponds to an overall NaCl concentration of 115 mmol/L. Additional NaCl was added to this sample to bring the Na^+ concentration to 250 mmol/L, and the sample was incubated overnight and then analyzed by light scattering. The same procedure was then repeated for an Na^+ concentration of 400 mmol/L. Figure 6 shows results for the radius of gyration (R_g) of the nanogels estimated from the light-scattering data. As expected, we find a significant increase in nanogel radius with increasing NaCl concentration. These results demonstrate that nanogel properties can indeed be manipulated by tuning external variables such as the salt concentration.

Larger Gel Particle Synthesis. All the results thus far have been for nanogels templated from relatively small liposomes (∼100 nm in radius). A final question we address is whether we can control the size of the gels by varying the size of the template liposomes. By using a lipid formulation of DPPC:DCP (9:1 molar ratio), we can obtain liposomes of ca. 400−500 nm in radius using the solvent injection method. Note that, at these larger sizes, the liposomes could well be a combination of unilamellar and multilamellar structures. Nevertheless, we have been able to encapsulate alginate in these liposome cores, and we have subsequently cross-linked the alginate chains by exposure to a Ca^{2+} gradient to yield micron-sized gel particles. Evidence for gel formation in this case can be obtained directly from optical (DIC) microscopy. Figure 7a shows a micrograph of the gel particles with intact lipid bilayers (before OBG treatment). We can resolve a large number of distinct spherical structures in this

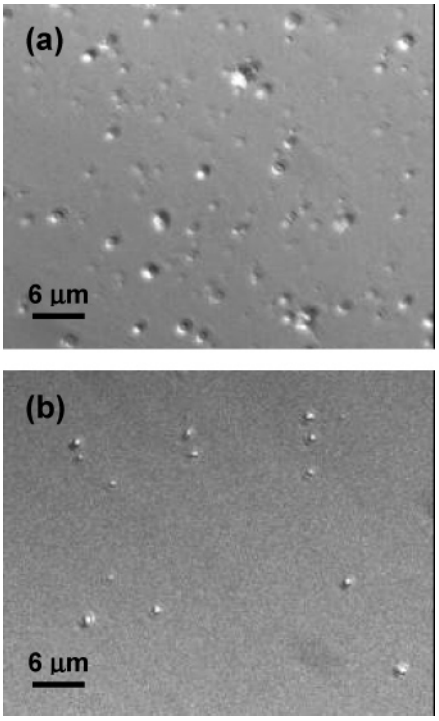

Figure 7. Optical (DIC) micrographs of alginate gels templated by larger (micron-sized) vesicles: (a) before OBG treatment (i.e., with lipid shell intact) and (b) after OBG treatment (i.e., bare gel particles).

image. Figure 7b shows the same sample after treatment with 30 mmol/L OBG. Here again, we find distinct gel particles with approximately the same size as in Figure 7a, although the number density of such particles is significantly lower. For comparison, we also obtained DIC micrographs of a control sample of liposomes alone; in this case, upon exposure to OBG, the liposomes were destroyed, and no structures could be resolved by DIC. Light scattering also confirmed that the average radius of the gel particles was around 500 nm, i.e., comparable to that of the template liposomes. The data suggest that our templating strategy can be generalized to gels over a range of sizes.

4. Conclusions

We have used liposomes to template relatively monodisperse populations of alginate nanogels. The solvent-injection method was used to form the liposomes and simultaneously encapsulate sodium alginate in the liposome cores. Alginate gelation in the liposome cores was accomplished using a low Ca^{2+} concentration gradient by exploiting the increased transbilayer permeability near T_m. The lipid coating around the nanogels was then removed by the addition of OBG detergent. Light scattering and TEM confirmed the formation of nanogels. Experiments with two different liposome sizes showed that the gel particles conform to the sizes of the templates. The nanogels described here could be useful for biomolecule encapsulation either in controlled release experiments or for single molecule fluorescence studies.

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