Synthesis of Bilayer-Coated Nanogels by Selective Cross-Linking of Monomers inside Liposomes

Joris P. Schillemans, Frits M. Flesch, Wim E. Hennink, and Cornelus F. van Nostrum*

Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands

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ABSTRACT: In this study, bilayer-coated polyacrylamide hydrogel nanoparticles were prepared by photoinitiated polymerization of acrylamide (AA) and bis(acrylamide) (BA) in the inner compartment of liposomes. The liposomes were formed in AA/BA solutions from lipid/Triton X-100 (TX100) mixed micelles by adsorption of TX100 to Bio-Beads SM2 and were studied by dynamic light scattering and transmission electron microscopy. The hydrodynamic diameters of the liposomes were ∼100 nm with low polydispersity. Addition of ascorbic acid before photopolymerization prevented macroscopic hydrogel formation by inhibition of free-radical polymerization of nonencapsulated monomers. Bare nanogel particles were finally obtained by removal of the lipid bilayer. As opposed to the commonly used dilution method, this convenient and versatile method of nanogel synthesis will allow incorporation of membrane proteins in the bilayer and the use of monomers that readily pass the lipid membrane.

Introduction

In recent years hydrogel micro- and nanoparticles have been identified as valuable materials for a number of applications, including drug delivery and targeting, chromatography, etc.[1−5] Synthesis of these hydrogel particles can be accomplished in several ways, each with their own advantages and drawbacks. Emulsion polymerization is often used for the synthesis of both micro- and nanoparticles. Using this method, the size of the particles can be controlled by the size of the droplets in the w/o emulsions.[6,7] However, such systems are generally incompatible with biological macromolecules like proteins. To ensure compatibility with proteins, water-in-water emulsions can be used for synthesis of microparticles, but control of the particle size down to the nanosize has not been accomplished thus far.[8,9] Another method that is commonly used for both micro- and nanoparticle synthesis is dispersion polymerization.[10,11] Upon initiation by a suitable initiator, dilute systems of monomer and cross-linker form polymer chains in solution, which collapse to form a precursor particle when reaching a critical chain length. Precursor particles continue to increase in size by additional chain growth and aggregation until a colloidally stable particle is formed.[12] The major drawback of this method is that it does not provide straightforward control over particle size, since the colloidal stability is dependent on monomer composition, initiator, and temperature.[13] An alternative, less commonly used approach is available for hydrogel particle synthesis. Monshipouri et al. reported on a method that used the internal compartment of lipid vesicles (liposomes) for the preparation of hydrogel particles, which allows good control over particle size and size distribution, and is compatible with biological macromolecules.[14] Using this method, Kazakov et al.[15,16] described the synthesis of thermo- and pH-sensitive hydrogel nanoparticles, Van Thienen et al.[17] synthesized biodegradable dextran nanogels, and Patton and Palmer synthesized hemoglobin-entrapped nanogels.[18−20]

In addition to providing an alternative method for hydrogel nanoparticle synthesis, bilayer-coated nanogels as such could also have numerous applications. Combining the properties of both hydrogels and liposomes, they could be used as, for example, controlled release devices, artificial cell analogues, and biomimetic sensory systems.[15] Especially for the latter two, the incorporation of functional membrane proteins will be important. The method most commonly used for functional incorporation of membrane proteins in liposomes is detergent dilution.[21,22] The liposomal reactors used for nanoparticle synthesis described thus far, however, were prepared by freeze−thawing, sonication, and extrusion, which are less suitable for functional protein incorporation. Using the detergent dilution method, a dispersion of lipid-containing mixed micelles is diluted below the critical micelle concentration (cmc) of the detergent, which leads to the formation of liposomes. Several detergents can be used for this purpose, e.g., octylglucoside, sodium cholate, C8E12, and Triton X-100 (TX100). Additionally, several methods of detergent dilution are available such as dialysis, rapid addition of solvent, and adsorption by Bio-Beads SM-2.[23−25] Removal of TX100 by Bio-Beads has been described in the literature extensively.[26−29] It has been proven to be an effective, simple, and inexpensive way of incorporating functionally active membrane proteins in liposomes. Nevertheless, up until now this method has not been used to form liposomes in solutions of hydrogel-forming monomers.

Liposomes formed in monomer solutions have monomers both on the inside and on the outside. Therefore, prevention of polymerization on the outside of the liposomes is required for controlled nanoparticle synthesis. This was accomplished previously by dilution of the exterior compartment to a concentration of monomers too low for hydrogel formation.[15−20] However, dilution causes a steep concentration gradient across the lipid membrane and is only applicable when the monomers cannot diffuse through the liposomal bilayer. When relatively lipophilic monomers are used, diffusion could occur. Additionally, incorporation of membrane proteins can lead to increased permeability of the bilayer.[30] Therefore, in the current study we developed a method for nanoparticles synthesis that will be suitable for the functional incorporation of membrane proteins. First, the liposomes are prepared by detergent removal in order

* Corresponding author: Tel 0031-(0)30 2536970; e-mail c.f.vannostrum@pharm.uu.nl.

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to allow incorporation of functional membrane proteins in the lipid bilayer. Second, for situations where selective removal or dilution of the monomers from the exterior is not feasible, we propose exterior polymerization inhibition using ascorbic acid.

Materials and Methods

Preparation of Macroscopic Hydrogels. Hydrogels were prepared by UV-initiated polymerization of aqueous solutions of acrylamide (AA; MP Biomedicals) and *N*,*N*′-methylenebis(acrylamide) (BA; MP Biomedicals) as cross-linker. AA and BA were dissolved in Hepes Buffered Saline (HBS), pH 7.4. The nomenclature used to describe the composition of AA/BA solutions is as follows: a (5/10) solution contains 5% (w/v) total monomer (AA + BA), of which 10% (w/w) is BA. Irgacure 2959 (Ciba Specialty Chemicals) was added as photoinitiator at a concentration of 0.006% (w/v).

Cross-linking was performed in the absence or presence of varying amounts of the free-radical inhibitor L-ascorbic acid (MP Biomedicals) dissolved in HBS (130 mg/mL, pH adjusted to 6.5 to retard degradation of ascorbic acid). All solutions were filtered before use through a 0.2 μm filter. Samples were purged with N_2 for 5 min and illuminated for 90 s under a N_2 atmosphere using a Bluepoint 4 UVC mercury lamp (150 W, λ range 230−600 nm, Honle UV Technology).

Dynamic Mechanical Analysis (DMA). DMA measurements were performed on a DMA 2980 dynamic mechanical analyzer (TA Instruments, Inc.) in "controlled force" mode as described by Meyvis et al.[31] Macroscopic hydrogels were prepared by free radical polymerization in a cylinder-shaped reaction reservoir as described above. The resulting hydrogels (height 5 mm, diameter 4.5 mm) were placed between a parallel-plate compression clamp, and a force ramp was applied. Young's modulus (E') of the hydrogels was calculated using a plot of the compressional force vs the observed deformation, in which the value of the slope equals E'.

Liposome Preparation. Liposomes were prepared from mixed micelles either by removal of TX100 by Bio-Beads or by extrusion. Appropriate amounts of dioleoylphosphatidylcholine (DOPC) (Lipoid GmbH), egg phosphatidylglycerol (EPG) (Lipoid GmbH), and cholesterol (Sigma), in a molar ratio of 4:1:1, were dissolved in chloroform in a round-bottom flask. A lipid film was prepared under reduced pressure using a rotary evaporator and dried further under a stream of nitrogen. An aqueous solution (20% w/v) of Triton X-100 (TX100) (BDH Laboratory Supplies) was added at a molar ratio of 3:1 (TX100 to total phospholipid (PL)), which is needed to form micelles.[28] Next, either HBS or monomer solution (in HBS) was added to yield a final PL concentration of 2.5 mM. Irgacure was added to a concentration of 0.006% (w/v). Controls consisted of mixed micelles without Irgacure in AA/BA solutions, AA/BA solutions with Irgacure, and AA/BA solutions without Irgacure.

Bio-Beads SM-2 (Bio-Rad) were extensively washed with methanol and subsequently incubated in HBS or AA/BA solution.[26] Washed Bio-Beads (60 mg/mL, moist weight) were added to the micelle solutions, which were subsequently left for 3 h at room temperature (RT) under gentle shaking. Additional (120 mg/mL) Bio-Beads were added, and samples were left for an additional 3 h at RT under gentle shaking. Bio-Beads were allowed to settle, and the supernatant was collected. Microparticles (aggregates) were removed by centrifugation at 18000*g* for 5 min. The PL content of the liposome preparations was determined by phosphate analysis according to Rouser[32] after destruction with perchloric acid.

Liposomes were prepared by extrusion as well. Lipid films prepared as described above were hydrated in HBS or AA/BA solution (without TX100), containing 0.006% (w/v) Irgacure. As a control, liposomes with encapsulated ascorbic acid were prepared by hydration of a lipid film in monomer solution containing ascorbic acid at a molar ratio of 600 relative to Irgacure. Subsequently, the formed multilamellar liposomes were extruded using a hand extruder (Avanti Polar Lipids) through polycarbonate filters with a pore size of 0.4 μm.

Nanogel Synthesis. To prevent polymerization outside the liposomes, ascorbic acid dissolved in HBS (130 mg/mL, pH adjusted to 6.5) was added to the liposome dispersion immediately before illumination, in a molar ratio of 200 relative to Irgacure. To compare the method of external inhibition of polymerization to external monomer dilution,[15−19] part of the liposomes prepared in AA/BA (5/10) containing Irgacure were diluted 20 times in HBS. Samples and controls were purged with N_2 for 5 min and illuminated for 90 s under a N_2 atmosphere. To obtain bare nanogels, the PL bilayer was dissolved by addition of TX100 solution (20%, w/v) to a molar ratio of 60 relative to total PL.

Dynamic Light Scattering (DLS). The size and size distribution of particles were measured by dynamic light scattering (DLS) using a Malvern CGS-3 multiangle goniometer (Malvern Ltd.), consisting of a HeNe laser source (λ = 632.8 nm, 22 mW output power), temperature controller (Julabo water bath), and digital correlator ALV-5000/EPP. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. The determination of Z_h by dynamic light scattering is based on the Stokes−Einstein equation

$$Z_h = (k_B T \mathbf{q}^2)/(3\pi\eta\Gamma) \quad (1)$$

where k_B is the Boltzmann constant, η is the solvent viscosity, Γ is the decay rate, and $\mathbf{q}$ is the scattering vector ($\mathbf{q} = (4\pi n \sin(\theta/2))/\lambda$), in which n is the refractive index of the solution, θ is the scattering angle, and λ is the wavelength of the incident laser light. Scattering was measured in an optical quality 4 mL borosilicate cell at a 90° angle. The samples were analyzed at 25 °C. For measurements in AA/BA solutions the hydrodynamic diameter was corrected for differences in solvent viscosity.

Electrophoretic Mobility. The zeta potential of both bilayer-coated and bare nanoparticles in HBS was determined using a Zetasizer 3000 (Malvern Instruments), equipped with a flow-through cell and PCS software (version 1.43). Following solubilization of the bilayer with TX100, bare particles were separated from mixed micelles by 3× ultracentrifugation at 600000*g* for 2 h. The pellet was resuspended in HBS.

Transmission Electron Microscopy (TEM). *Negative Stain.* Lipid-coated and bare nanoparticles were adsorbed on glow discharged Formvar-carbon-coated copper grids, washed twice in HBS, and negatively stained with 2% (w/v) uranyl acetate. The ultrastructure was analyzed with a Tecnai 10 electron microscope (FEI Co.) at a 100 kV acceleration voltage.

Cryogenic-TEM. Samples were prepared in a temperature- and humidity-controlled chamber with an automatic blotting/plunging system using a "Vitrobot". A thin aqueous film of particle dispersion was formed by blotting a glow-discharged 200 mesh copper grid covered with Quantifoil holey carbon foil (Micro Tools GmbH) at 25 °C and 100% relative humidity. The thin film was rapidly vitrified by plunging the grid into liquid ethane. Subsequently, the grids were transferred into the microscope chamber using a GATAN 626 cryoholder system. Samples were analyzed at −180 °C using a Tecnai12 transmission electron microscope (FEI Co.) operating at 120 kV. Low-dose imaging conditions were used to avoid melting of the vitrified film.

Results and Discussion

Liposome Preparation. Liposomes composed of DOPC:EPG:Chol 4:1:1 were prepared from mixed micelles using TX100 removal by adsorption to Bio-Beads in HBS and in solutions of monomer (AA) and cross-linker (BA) in HBS. After subsequent removal of the Bio-Beads, the formed vesicles were studied by determination of PL content, DLS measurements, and cryo-TEM (Figures 1 and 2, Table 1).

The Bio-Beads did adsorb not only TX100 but also ∼25% of the PL (Table 1). This corresponds to 2.7 mg lipid/g Bio-Beads, which is in agreement with earlier reports.[28] The DLS

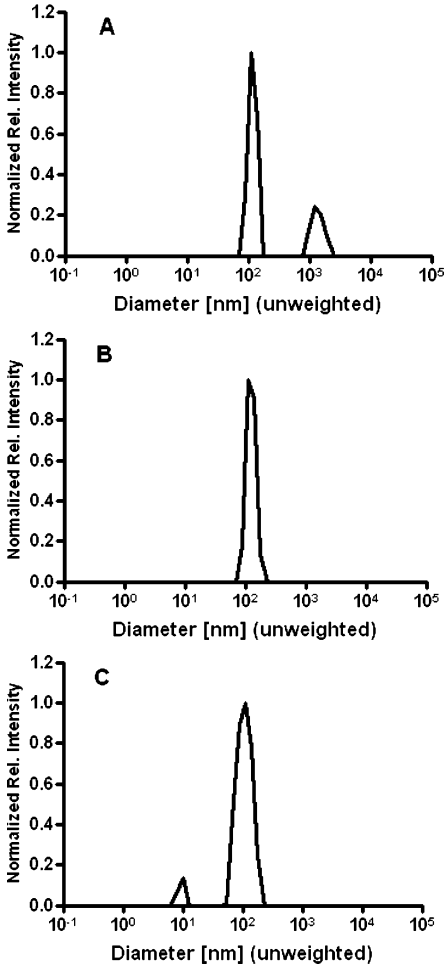

Figure 1. Particle size distribution (hydrodynamic radius (nm), unweighted regularized fit) for the different stages of nanogel preparation by TX100 removal by Bio-Beads. Bimodal distribution after TX100 removal (A), monomodal distribution in the supernatant after subsequent centrifugation (B), and bare nanogels and mixed micelles after resolubilizing the lipid bilayer with TX100 (C).

measurements showed that two populations of particles were present after TX100 removal, i.e., particles that are in the nanometer range (liposomes) and particles in the micrometer range (Figure 1A). The larger particles could be lipid aggregates or (small parts of) Bio-Beads, which were removed by centrifugation. All samples contained a pellet after centrifugation. DLS measurements of the supernatant (Figure 1B) showed only one population of particles in each sample, with hydrodynamic diameters as expected for liposomal vesicles (Table 1). The presence of liposomes in the supernatant was confirmed with cryo-TEM, showing spherical vesicles, homogeneous in size, with no apparent morphological differences between vesicles formed in HBS or AA/BA solutions (Figure 2). Only 33% of the initial amount of PL was found in the supernatant in HBS, while in AA/BA solutions this percentage was significantly higher (65% for (5/10) solution and 60% for (10/20)). Furthermore, the presence of AA/BA in the solution reduced the size of the liposomes: 159 nm in HBS, 115 nm in AA/BA (5/10) solution, and 94 nm in AA/BA (10/20) (Table 1). It has been shown before that the size of liposomes formed by detergent removal by Bio-Beads depends on both the detergent used[29,33] and the rate of removal.[23,28,34−36] The smaller hydrodynamic diameter of the particles formed in AA/BA solutions could be caused by the higher viscosity of these solutions, which influences several processes determining particle size, such as micelle fusion,[35] lipid exchange,[36] and

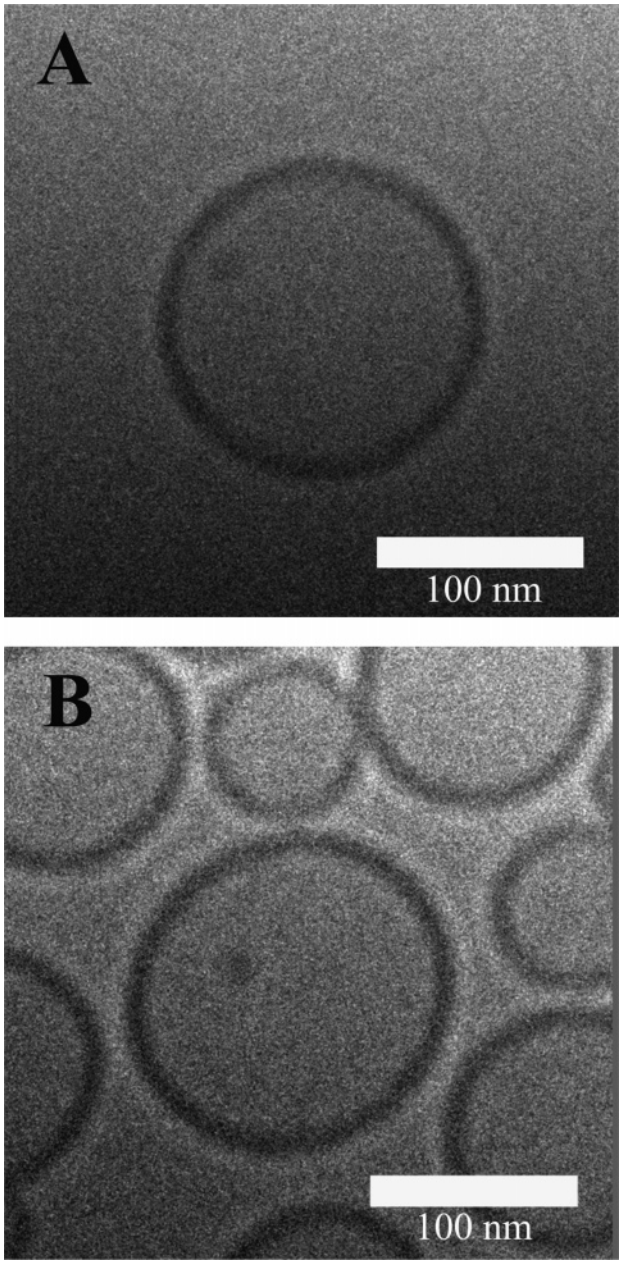

Figure 2. Cryogenic-temperature transmission electron microscope images of particles formed after TX100 removal by Bio-Beads in HBS (A) and AA/BA solution (B). Representative images are shown.

postvesiculation.[34] These processes are also the likely cause of the higher PL content of the supernatant after centrifugation.

Inhibition of Free-Radical Polymerization. Liposomes that are formed in AA/BA solution contain monomer both in the inner compartment and in the outer liquid. To produce nanoparticles within the liposomal reactor, it is important to restrict the hydrogel formation of the monomers to the inner compartment of the liposomes. Therefore, polymerization outside the liposomes should be prevented, which can be accomplished in two ways: (1) dilution of the monomers to a concentration too low for hydrogel formation and (2) inhibition of free radical polymerization. It is very likely that dilution leads to leaking of the monomer and particularly of the relatively hydrophobic cross-linker out of the liposomes, thereby also impairing hydrogel formation inside the liposome (as will be discussed below in the Nanogel Synthesis section). Consequently, inhibition of polymerization is the preferred method to prevent hydrogel formation outside the liposome.

Table 1. Effect of Hydration Medium on Phospholipid (PL) Recovery, Hydrodynamic Diameter (Z_h), and Polydispersity Index (PDI) of PL Vesicles Formed by TX100 Removal by Bio-Beads[a]

medium	PL after Bio-Beads (%)[b]	PL after centrifugation (%)[b]	Z_h (nm)[c]	PDI[c]
HBS	73 ± 2	33 ± 7	159 ± 3	0.04 ± 0,02
AA/BA (5/10)	78 ± 2	65 ± 3	115 ± 7	0.10 ± 0.04
AA/BA (10/20)	72 ± 4	60 ± 2	94 ± 6	0.07 ± 0.06

[a] $n = 4$, values represent average ± sd. [b] Percentage of the initial amount of PL. [c] Measured in the supernatant after centrifugation.

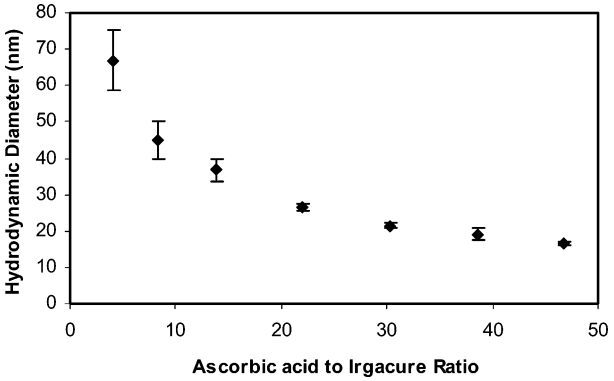

Figure 3. Particle size as a function of the ratio of ascorbic acid to Irgacure in the absence of liposomes. Samples containing AA/BA (5/10) solution, 0.006% (w/v) Irgacure, and varying amounts of ascorbic acid were illuminated for 90 s. Particle size was measured by DLS. Error bars represent standard deviation ($n = 3$).

Ascorbic acid is a well-known, water-soluble radical scavenger. It is a bivalent acid, with respective pK_a's of 4.17 and 11.57,[37] by which ascorbic acid carries one negative charge at pH 7.4. The lipid bilayer contains EPG, a lipid with a negatively charged head group at physiological pH, which prevents ascorbic acid from readily diffusing over the bilayer. These characteristics make ascorbic acid a suitable candidate for the inhibition of free radical polymerization outside the liposomes. To assess to what extent ascorbic acid inhibits UV-induced free radical polymerization, we tested its effect on macroscopic hydrogel formation. AA/BA (5/10) solutions containing ascorbic acid at a molar ratio of 4 and higher relative to Irgacure remained liquid upon UV illumination. However, DLS measurements showed the presence of particles, whose size decreased from 66 to 15 nm with increasing ascorbic acid concentration (Figure 3). Using a (5/10) AA/BA solution containing 0.006% (w/v) Irgacure, and 90 s of illumination, the particle size leveled off at a ratio of ~40 while in a (10/20) AA/BA solution the same plateau value was reached at a ratio of ~150 (data not shown). Apparently, it is not possible to inhibit the polymerization completely. Upon UV illumination some monomers will polymerize before ascorbic acid terminates the reaction by radical scavenging. At higher concentrations the availability of monomers is higher, which explains the higher amount of ascorbic acid needed to reach the plateau using a (10/20) solution. It must be noted, however, that the scattering intensity at this plateau is just at the detection limit of the DLS equipment.

Besides radical scavenging, ascorbic acid also absorbs UV light (λ_{max} at neutral pH is 265 nm).[37] To accomplish inhibition of polymerization outside liposomes while allowing polymerization inside, it is important to make sure that ascorbic acid does not inhibit polymerization inside by UV absorption. Therefore, we determined the storage modulus of a macroscopic hydrogel when the initiating light first passed through a solution of ascorbic acid having a concentration equal to the highest concentration of the inhibition experiments (200 equiv relative to Irgacure). The modulus of a 5/10 hydrogel as determined by DMA was not affected when the beam of UV light passed through a quartz cuvette before reaching the sample (1.9 ± 0.2 kPa), nor when this cuvette was filled with buffer, AA/BA (5/10) solution, or ascorbic acid-containing AA/BA (5/10) solution.

Nanoparticle Synthesis. The monomer dilution method to prevent external polymerization failed in our hands. When liposomes in AA/BA solution were diluted 20 times in HBS and illuminated, and subsequently the lipid bilayer was solubilized with TX100, DLS measurements did not detect the presence of nanoparticles; only mixed micelles were observed (hydrodynamic diameter = 9 nm). It is most likely that dilution of the external compartment caused AA and BA to diffuse over the bilayer, leading to dilution of the monomers in the liposomal compartment to a concentration too low for hydrogel formation. This is in contradiction to earlier work by Kazakov et al., who did observe nanoparticles by DLS using the same method.[15] This may be explained as follows. Kazakov et al. used dialysis in 25 kDa cutoff bags in an attempt to remove the lipids that were solubilized by TX100. However, dialysis of mixed micelles is also one of the methods to produce liposomes by detergent removal,[24] which could mean that the remaining particles observed by Kazakov et al. were re-formed (empty) liposomes instead of nanogels.

Because the dilution method results in the diffusion of monomers out of the liposomes, we used polymerization inhibition by radical scavenging. Ascorbic acid was added to the undiluted liposome preparations at an ascorbic acid/Irgacure ratio of 200, and the dispersions were UV-irradiated. The hydrodynamic diameter measured by DLS after illumination was unchanged, indicating that the polymerization of AA/BA did not affect the size of the liposomal reactor. The small particles that may have been formed outside the liposomes in the presence of ascorbic acid (see above) could not be detected by DLS because the corresponding signal was too low in the presence of the liposomes. After solubilizing the lipid bilayer with TX100, particles of about 100 nm remained (Figure 1C). The particle size decreased by 13 ± 3 nm upon removal of the bilayer, whereas samples without monomers (HBS) or without photoinitiator decreased dramatically, showing only micelles after solubilization of the liposomes (Figure 4A). The decrease of 13 nm upon removal of the bilayer is slightly more than 2 times a DOPC bilayer, which is known to be ~4 nm thick.[38,39] Macroscopic polyacrylamide gels of the same compositions were dimensionally stable in contact with buffer (results not shown). Therefore, the observed decrease in particle size may be due to the fact that liposomes are not always strictly unilamellar but may have an average of more than one bilayer depending on their size.[40]

To confirm that the polymerization indeed took place in the inner compartment of liposomes, larger liposomes were prepared by extrusion, which should result in larger nanoparticles accordingly. Besides, in one control sample ascorbic acid was also encapsulated in the inner compartment to prevent hydrogel formation inside the liposomes. As expected, extruded liposomes are larger than the liposomes prepared by TX-100 removal (115 and 250 nm), and consequently the formed bare nanoparticles are larger as well (compare parts A and B of Figure 4). The difference between bilayer-coated and bare nanogels is 38 ± 13 nm, which is most likely due to increased oligolamellarity

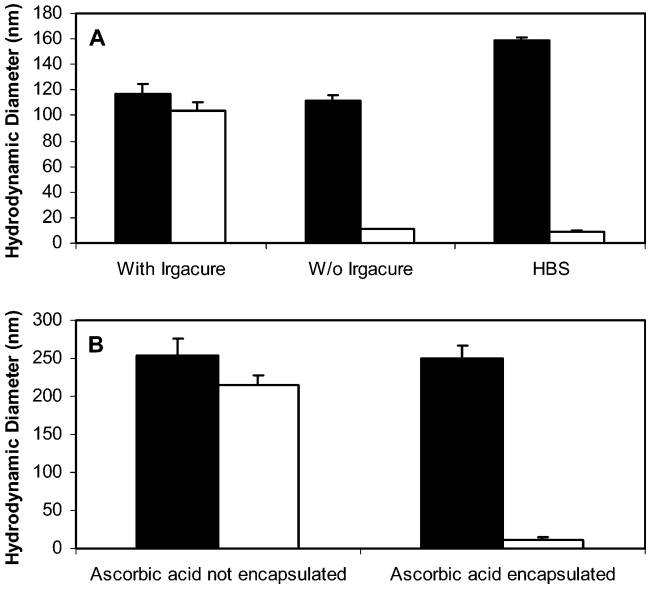

Figure 4. Hydrodynamic diameter as determined by DLS before (■) and after (□) lipid solubilization with TX100. Error bars represent the standard deviation ($n = 4$). (A) Liposomes prepared by TX100 removal by Bio-Beads either in AA/BA solution with or without Irgacure or in HBS only (with Irgacure). (B) Liposomes prepared by extrusion in Irgacure-containing AA/BA solution in the presence of ascorbic acid either only outside or both outside and inside the liposomes.

of the extruded liposomes. As expected, UV polymerization of AA and BA inside liposomes could be completely inhibited by the enclosure of ascorbic acid. After illumination and solubilization of the lipid bilayer of liposomes that contained both monomers and ascorbic acid, only micelles with a hydrodynamic diameter of 12 nm were detected. These results demonstrate that nanoparticles were indeed formed inside the liposomes.

TEM images acquired using negative staining with uranyl acetate confirmed the results presented above. Samples of liposomes in Irgacure-containing AA/BA solutions showed lipid-coated nanoparticles after addition of ascorbic acid and illumination and bare nanoparticles when the lipid bilayer is subsequently solubilized with TX100 (Figure 5A,B).

The zeta potential of the particles in HBS increased after solubilization of the bilayer (−8.7 and −4.2 mV for bilayer-coated and bare particles, respectively), which is in agreement with earlier reports for acrylamide particles in PBS.[20] The particle size of both bilayer-coated and bare particles remained constant after storage for 2 months at 4 °C, which suggests that particles are stable.

Conclusion

Our experiments show that TX100 removal from mixed micelles by Bio-Beads can be used as a method of liposome preparation in monomer solutions. Phospholipid vesicles with a narrow particles size distribution were obtained. The size of these vesicles depends on the monomer concentration.

Formation of hydrogel nanoparticles can be accomplished when polymerization takes place in the inner compartment of the liposomes. In this paper we have shown that ascorbic acid is a good candidate to inhibit polymerization specifically outside the liposomes. The degree to which ascorbic acid inhibits free radical polymerization depends on both the ascorbic acid and the monomer concentration.

In this paper we have broadened the possibilities for hydrogel nanoparticles synthesis in a liposome reactor using detergent removal and polymerization inhibition by ascorbic acid, which allows incorporation of membrane proteins and the use of monomers that easily diffuse through the lipid bilayer.

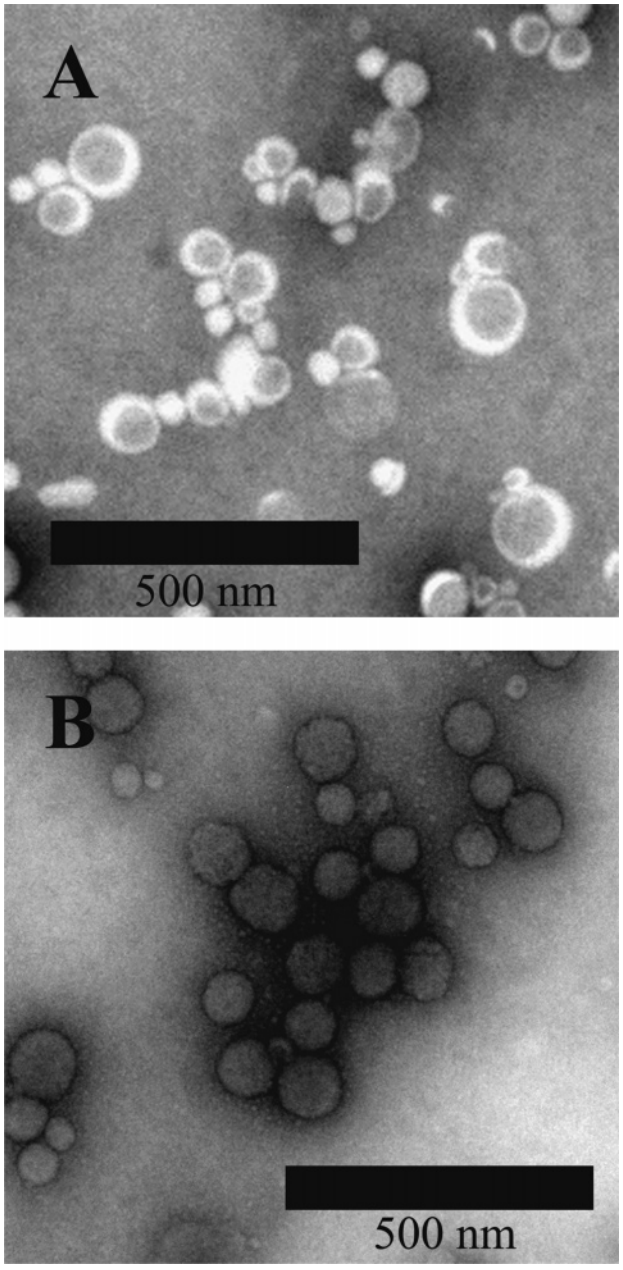

Figure 5. Transmission electron microscope images of lipid-coated (A) and bare (B) polyacrylamide (10/10) nanoparticles. Bare particles were obtained by treating lipid-coated particles with TX100. Samples were stained using 2% uranyl acetate. Lipid membranes appear light since uranyl acetate does not stain the hydrophobic core of membranes, whereas nanoparticles appear dark because uranyl acetate easily penetrates their hydrophilic environment.

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