Stabilization of Polymersome Vesicles by an Interpenetrating Polymer Network

Feng Li,[†,‡] Tijs Ketelaar,[§] Antonius T. M. Marcelis,[*,†] Frans A. M. Leermakers,[‡] Martien A. Cohen Stuart,[‡] and Ernst J. R. Sudhölter[†]

Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands; Laboratory of Physical Chemistry and Colloid Science, Wageningen University, Dreijenplein 6, 6703 HB Wageningen, The Netherlands; and Laboratory of Plant Cell Biology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

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ABSTRACT: Vesicles from Pluronic L121 ($PEO_5-PPO_{68}-PEO_5$) triblock copolymers were stabilized by an interpenetrating polymer network from pentaerythritol tetraacrylate by UV or thermal initiator induced radical polymerization. Fluorescence labeling, atomic force microscopy, and electron microscopy studies were used to study the morphology of the particles and showed that stable vesicles are formed. The block copolymers are noncovalently trapped in the interpenetrating polyacrylate network. The stabilized vesicles retain their size for more than 1 month at room temperature. Upon cooling, the vesicles reversibly lose block copolymer.

Introduction

Polymersomes have been studied extensively in the past 10 years. Many amphiphilic block copolymers, especially those with large hydrophobic and relatively small hydrophilic blocks, can self-assemble into vesicles at low concentrations.[1−18] Recently, pH-sensitive polymersomes,[19,20] biodegradable polymersomes,[21] and near-infrared (NIR) emissive polymersomes were made.[22] Moreover, integral membrane proteins can be inserted in the shells,[23] and viral DNA has been inserted into the polymer vesicles.[24] Vesicles from block copolymers often have a glassy hydrophobic block, which helps to keep them stable for quite a long time. The block copolymers that have been used to prepare polymersomes are usually not commercially available. There are only a few exceptions; one of these is some Pluronics, poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide) triblock copolymers. Pluronics are nontoxic and widely used in drug delivery systems and surface chemistry applications and may provide exciting opportunities for gene therapies. These polymers are readily available and depending on the size and lengths of the blocks form micelles or vesicles in water. Pluronic L121 ($PEO_5-PPO_{68}-PEO_5$), for example, forms small vesicles, ∼100 nm in size,[25,26] which are, however, stable only for a few hours after preparation. They slowly revert to flat lamellar structures. The hydrophobic PPO part does not become glassy at low temperatures, which could contribute to the instability of the vesicles. Furthermore, these triblock copolymers are rather temperature sensitive; at low temperatures they dissolve as unimers in water, while at higher temperatures they form aggregates.[27−29] The poor stability limits their potential use in, e.g., novel polymer-based controlled-release systems.

In this work, we describe a way to stabilize Pluronic L121 vesicles with a permanent interpenetrating polymer network (IPN) of polymerized pentaerythritol tetraacrylate (PETA), which prevents vesicles from reverting into more stable flat bilayers. PETA is a small hydrophobic molecule, which was recently used as a cross-linking agent for stabilizing Pluronic micelles.[30,31] The polymerization of PETA took place mostly in the core of the micelles. A similar approach for stabilizing Pluronic L121 vesicles was used. The formation of the IPN in the vesicles was induced by either UV or thermal initiator induced free radical polymerization of PETA. The morphology of the resulting stabilized vesicles was studied by atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM). Their stability in time and as a function of temperature was studied by dynamic light scattering (DLS).

Experimental Section

Materials. Poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) ($PEO_5-PPO_{68}-PEO_5$) (Pluronic L121) was obtained as a kind gift from BASF Corp. PETA, Nile red, and carboxyfluorescein were purchased from Sigma. The thermal initiator 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was obtained from Wako. The extruder and the extrusion membranes were purchased from Avanti Polar Lipids.

Preparation of Vesicles from $PEO_5-PPO_{68}-PEO_5$ Copolymer. Pluronic L121 and PETA were intimately mixed by dissolving them in a few milliliters of chloroform, which was evaporated under a nitrogen stream and dried under vacuum overnight. An aqueous solution of the mixture containing 0.2 wt % triblock copolymer and 0.006 wt % PETA was prepared and frozen in liquid nitrogen and subsequently thawed in a 25 °C water bath while stirring. This process was repeated five times. Then the mixture was extruded 25 times through a membrane with 100 nm diameter pores. Larger vesicles for fluorescent studies were made by extrusion through a membrane with 800 nm diameter pores. PETA-loaded vesicle solutions were put into a quartz cuvette, and photopolymerization was performed with UV irradiation (Heraeus TQ150 UV lamp, 150 W) for 40 min. Alternatively, 5 μL of a stock solution of thermal radical initiator (V-70; 0.6 mg/mL) in ethanol was added to 1 mL of a freshly prepared vesicle solution, and the mixture was incubated at 25 °C for 40 min to produce vesicles stabilized by thermally induced polymerization of PETA. The latter method was used in combination with fluorescent probes to avoid photobleaching.

Hydrophobic Nile red-containing vesicle solutions were prepared by adding 5 μL of a stock solution of Nile red (2 mg/mL) to 1 mL

* Corresponding author. E-mail: ton.marcelis@wur.nl.
† Laboratory of Organic Chemistry.
‡ Laboratory of Physical Chemistry and Colloid Science.
§ Laboratory of Plant Cell Biology.

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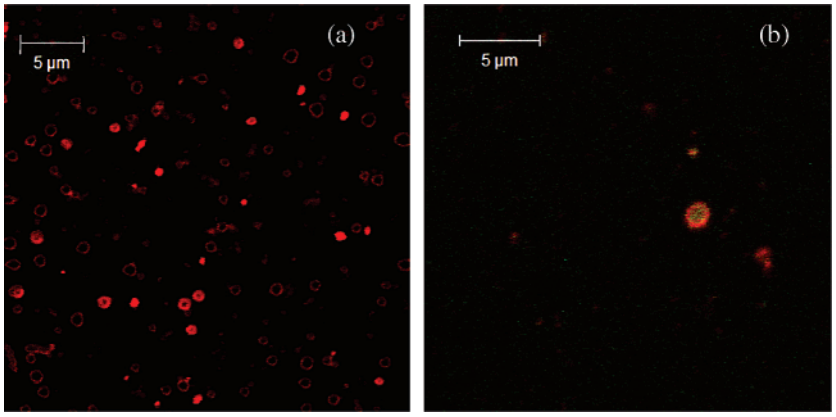

Figure 1. CLSM images of vesicles extruded through a 800 nm membrane: (a) containing membrane encapsulated Nile red; (b) containing Nile red and aqueous entrapped carboxyfluorescein.

of a vesicle solution containing PETA before the extrusion step. After extrusion the polymer network was formed by thermally induced polymerization. Vesicles containing both Nile red and carboxyfluorescein were prepared by performing the same vesicle preparation and thermally induced polymerization reaction in a 20 mM carboxyfluorescein stock solution at pH 8. The carboxyfluorescein that was not trapped in the vesicles was removed by size exclusion chromatography on a Sephadex G10 column with water as eluent.

Confocal Laser Scanning Microscopy. The solutions with stabilized vesicles containing Nile red or Nile red and carboxyfluorescein as fluorescent probe were deposited on a glass surface, covered with a slide, and then visualized directly using a confocal laser scanning microscope (Zeiss LSM 510 Meta). Both samples were imaged 1 day after preparation.

Electron Microscopy. Samples for electron microscopy were dried on a copper grid overnight before doing the measurements. TEM measurements were performed on a TEM microscope (JEOL 1200 EX) operating at 120 kV. For the SEM masurements, the grid holder containing the dried vesicles was placed in a dedicated preparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England), and sputter-coated with 5 nm platinum. The specimens were analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature, with SE detection at 2.5−5 kV. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 s (full frame) at a size of 2528 × 2030, 8 bit.

Atomic Force Microscopy. Tapping mode AFM experiments were carried out on a MFP-3D AFM from Asylum Research (Santa Barbara, CA). Height and phase imaging was done in ac mode in air using OMCL-AC240 silicon cantilevers (Olympus Corp., Japan). One drop of the vesicle solution was deposited on a freshly cleaved mica surface at room temperature and dried overnight before measurements.

Dynamic Light Scattering. DLS was carried out on an ALV/DLS-5000 light-scattering apparatus (ALV, Langen, Germany), equipped with an argon ion laser (LEXEL, Palo Alto, CA) operating at a wavelength of 514.5 nm. All experiments were performed at a scattering angle of 90°. Temperature was controlled by using a Haake C35 thermostat. The hydrodynamic radius was calculated from cumulant fits or a CONTIN multiexponential fit. The relative intensity was obtained according to the intensity ratio of vesicles and the toluene.

Results and Discussion

Morphology Study. L121 vesicles were prepared at 25 °C in the L_1/L_α two-phase regime.[25] To avoid undesired aggregation, the freeze−thaw step and extrusion processes were strictly performed below 25 °C. Pluronic L121 was well mixed with PETA during freeze−thaw cycling; no phase separation was observed within 1 h after this process. The mixture was extruded and stabilized by either UV-induced or thermally induced free radical polymerization.

To prove that the particles are indeed hollow, fluorescent labeling experiments were done. For this purpose, larger vesicles were prepared because they can be directly visualized by fluorescent microscopy. Both hydrophobic probes Nile red and water-soluble carboxyfluorescein were used.

Figure 1 shows the CLSM images of vesicles prepared by extrusion through an 800 nm membrane. The images show vesicles with a size of around 1 μm, which is comparable to the pore size of the membranes used for the preparation. The hydrophobic dye, Nile red, has a very low fluorescence in aqueous solution, but an enhanced fluorescence in a hydrophobic environment,[32,33] so only the hydrophobic shells of the vesicles are visualized in Figure 1a. Carboxyfluorescein is a water-soluble probe with green fluorescence. Vesicles containing both Nile red and carboxyfluorescein are shown in Figure 1b. The presence of the red shell and green fluorescence of carboxyfluorescein entrapped in the aqueous interior of the particles is direct evidence that hollow nanoparticles are present in aqueous solution.

Detailed structural information was obtained by electron microscopy. Figure 2 shows TEM images of smaller vesicles. The vesicles were prepared with a 100 nm extrusion membrane. The two TEM images show circular particles having a diameter of ∼50 nm up to 250 nm. The electron contrast of the deformed vesicles is so weak that the thickness of the vesicle walls can hardly be seen. This is typical for soft vesicles.[35] Some irregular features can be discerned on the surface of the polymerized vesicles, shown more clearly in Figure 2b. These may originate from drying artifacts or beam damage or could come from irregularities formed during the polymerization of PETA. It is possible that during the polymerization reaction some phase separation of PETA occurs, which could lead to some of the inhomogeneities observed in the TEM pictures. Complete phase separation does not occur; otherwise, the vesicles would not be stabilized or be observable by AFM (vide infra). The IPN of polymerized PETA allows the Pluronic copolymers to remain associated by hydrophobic interactions of the PPO block with the polymerized PETA network, and this association stabilizes the vesicle capsules.

Figure 3 shows a SEM image of vesicles obtained by extrusion through an 100 nm membrane. For SEM visualization, the sample was covered with 5 nm platinum. From this picture it is clearly seen that the particles are deformed by the drying process. The indentations in the center indicate that they are deflated hollow spherical particles. The particles are somewhat

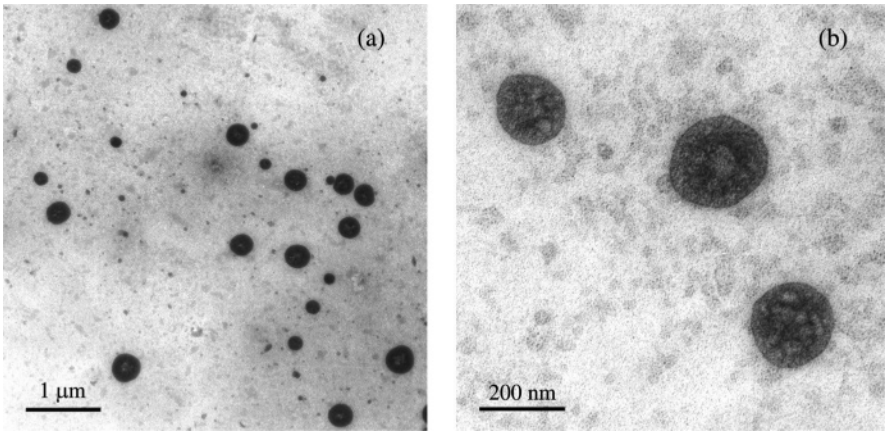

Figure 2. (a) TEM image of vesicles prepared by extrusion through an 100 nm membrane. (b) Higher magnification.

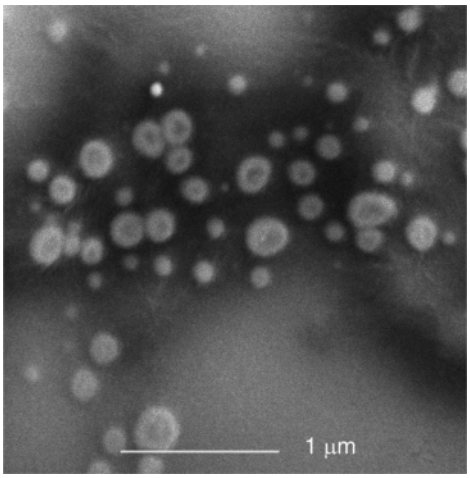

Figure 3. SEM image of vesicles obtained by extrusion through an 100 nm membrane.

polydisperse. Some big aggregates attributed to the drying step were also observed (data not shown).

AFM images of the vesicles obtained by extrusion through an 100 nm membrane and deposited on mica are shown in Figure 4. From the height image we can extract information about the deformed vesicles. The height of the vesicles was in the range 2−5 nm, much smaller than the average diameter of the vesicles (∼160 nm). This may have two reasons: (1) the height measured with tapping mode AFM may be lower than the real height of the vesicles due to the deformation of the vesicle surface by the AFM tip,[34] and (2) the vesicles were deformed as they were dried on the mica surface. The phase image (Figure 4b) shows something similar as the height image. The edge and the center of deformed vesicles have different responses to the force of the AFM probe. These features have been observed before and discussed in the literature.[35,36] The height of the vesicles observed by AFM is slightly smaller than twice the wall thickness of L121 vesicles estimated from cryo-TEM studies.[25] This difference may be attributed to the different conditions and different techniques by which they were measured. From the amount of PETA used as compared to the amount of L121 (about 3 wt %) only an insignificant change in wall thickness is expected. In combination with SEM, the data confirm that the vesicles are stable and detectable in the dry state. Both SEM and AFM yield consistent vesicle sizes within the range of 50−250 nm, which is in agreement with DLS measurements, that also show that the particles are relatively polydisperse. The cross sections of the dried vesicles are slightly larger than their diameters in solution.[35]

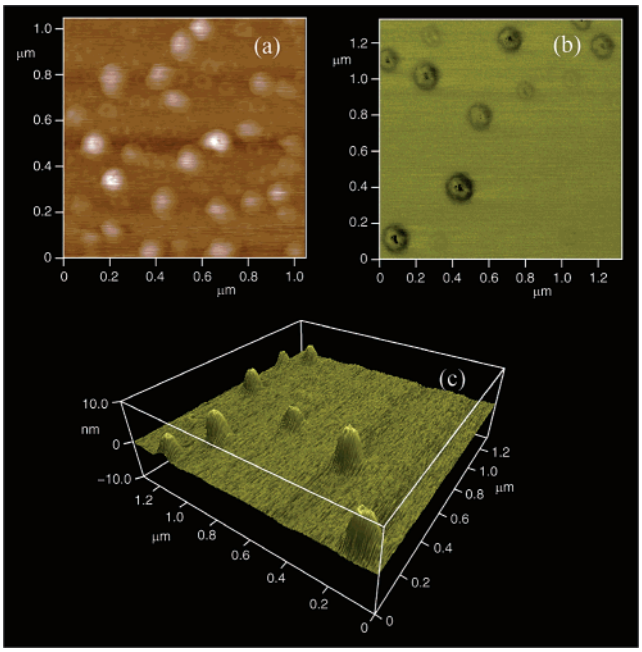

Figure 4. AFM image of stabilized vesicles obtained by extrusion through an 100 nm membrane: (a) height image, (b) phase image, and (c) 3D height image area in (b).

Thermal Stability Study. All the samples used for the morphology study were usually between 1 day and 2 weeks old before the measurements. As shown in the images for AFM, TEM, and SEM studies, they were deformed but still retained their spherical shape after drying and under the measuring conditions. This means that at least a certain number of vesicles do not fall apart in time and under these conditions. Therefore, a more detailed study of their stability was performed as a function of time using dynamic light scattering (DLS).

A sample of stabilized vesicles prepared from extrusion through a 100 nm membrane was repeatedly analyzed by DLS over a period of 25 days after preparation. The relative scattering intensity and radius as a function of time obtained from the cumulant fits are shown in Figure 5a,b. This clearly indicates that the PETA polymerized L121 vesicles are stable in time at 25 °C. A CONTIN analysis of the measurement after 25 days, shown in Figure 5c, exhibits a single peak. Similar CONTIN analyses were obtained for the measurements at other times. Therefore, the average size of the vesicles and their size distribution do not change during storage. The stability of the PETA polymerized L121 vesicles is definitely increased as

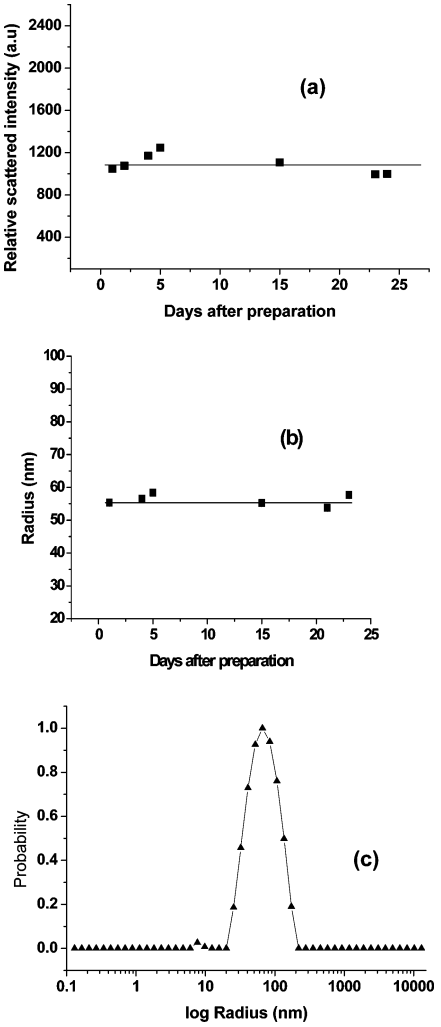

Figure 5. DLS measurements of stabilized vesicles obtained by extrusion through an 100 nm membrane: (a) relative scattering intensity at 25 °C as a function of time; (b) hydrodynamic radius at 25 °C as a function of time; (c) CONTIN analysis of the sample measured 25 days after preparation at 25 °C.

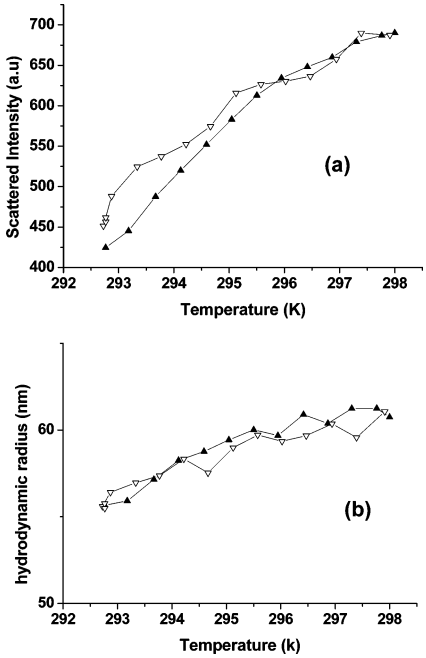

Figure 6. DLS measurements of stabilized vesicles obtained by extrusion through an 100 nm membrane as a function of temperature: (a) scattered light intensity (arbitrary units); (b) hydrodynamic radius. The temperature was gradually increased from 20 to 25 °C in 2 h and subsequently reduced to 20 °C. Filled triangles represent data upon temperature increase; empty triangles represent data upon temperature decrease.

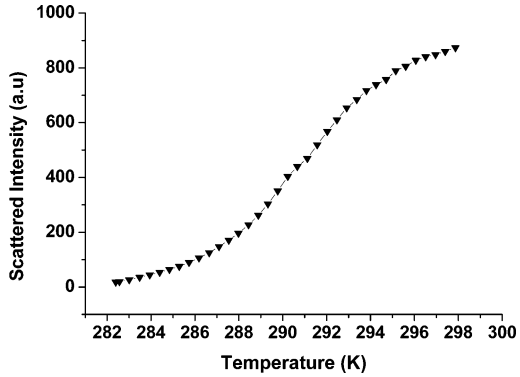

Figure 7. DLS measurements of 100 nm vesicles scattered light intensity value (with arbitrary units). The temperature was gradually decreased from 25 to 10 °C in 6 h.

compared to the pure L121 vesicles: the latter were only stable for few hours, whereas the polymerized L121 vesicles remain stable for at least 1 month.

It is found that the size of the polymerized vesicles can be changed reversibly in a temperature range of 20−25 °C, as can be seen from Figure 6a,b. When the vesicle solution is slowly cooled down from 25 to 20 °C, the scattering intensity and the hydrodynamic radius of the particles decrease. Upon slow heating the radius and scattering intensity return again to their original value. The solubility of Pluronics (and also Pluronic L121) in water increases at low temperatures. Since the Pluronics are not covalently attached to the IPN and they are prepared at 25 °C, some molecules can slowly dissociate from the network upon cooling to 20 °C. Upon increasing the temperature they associate again with the vesicle, and this process is reversible.

In a similar experiment, the temperature of the vesicle solution was slowly decreased to 10 °C in 6 h, and the light scattering was measured (Figure 7). It is seen that the scattering intensity decreases strongly. It is found that at about 14 °C the vesicles start to disintegrate. When the temperature decreases, the Pluronic becomes more soluble and can dissociate from the permanent polymer PETA network. The phase diagram of Pluronic L121 itself indicates that at a temperature below 14 °C the surfactants prefer to form micelles instead of lamellar aggregates.[25,26]

Loss of Pluronic molecules from the polymer network results in a decreased scattering intensity, while the free Pluronic molecules will partly self-assemble into small micelles, depending on the concentration and the exact temperature. When too many Pluronic molecules leave the network, the insoluble polymeric PETA networks will precipitate out of the solution. When the temperature is brought back to room temperature, a small fraction of the free Pluronic molecules will reassociate with the polymeric PETA network, and some of the capsules appear in solution again while stirring. The remaining Pluronic molecules will self-assemble into lamellar aggregates or vesicles as the pure Pluronic L121 molecules do.[26] These aggregates are not stable, and their size grows very quickly in time (data not shown).

These observations indicate that the triblock copolymers can be removed from and associate again with the polymeric PETA capsules as a function of temperature. In view of the low amount

of PETA that is used in relation to the amount of Pluronic, a picture emerges of stabilized vesicles that are held together by a loose polymeric network, although theoretically the amount of PETA is sufficient to form a rather tight 2-D network. Because of the self-aggregating properties of the Pluronics, this is enough to stabilize the vesicles for a long time. However, the Pluronic molecules are not covalently bound to the IPN, so when their solubility in water increases as happens when the temperature decreases, they can reversibly dissociate from the polymer network. However, when too many Pluronics leave the network, precipitation of the network occurs. This temperature-dependent behavior would also allow one to influence the permeability and thus release of entrapped species through the vesicle walls.

The easy and versatile preparation from inexpensive materials make these stabilized Pluronic L121 vesicles attractive for a broad range of possible applications. Clear advantages are the possibility to vary the strength of the network by using other acrylate network-forming compounds and the possibility to modify the properties of the vesicles by using functionalized Pluronics. The easy control of vesicle size, the nontoxic nature of Pluronics, and their long-term stability make these vesicles potentially interesting for drug delivery systems. We have shown that it is possible to load the vesicles with hydrophobic or hydrophilic dyes, which makes them potentially useful for molecular imaging, biological assays, as biomarker, for cell-labeling,[37] drug delivery, and food applications.

Conclusions

Pluronic L121 vesicles were stabilized by means of a network of polymerized PETA. Polymerization could be achieved not only by UV irradiation but also by a thermal radical initiator. The permanent interpenetrating network allows the Pluronic L121 copolymers to reversibly associate with the vesicles. The size of the vesicles can easily be varied from 100 nm to 1 μm, depending on the pore size of the extrusion membrane. CLSM, SEM, DLS, and AFM studies prove that unilamellar vesicles are formed and that the stabilized vesicles can retain their sizes for more than 1 month at room temperature.

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