

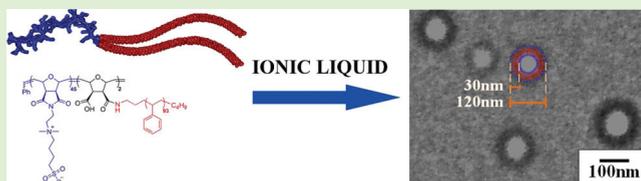
Zwitterionic Polymersomes in an Ionic Liquid: Room Temperature TEM Characterization

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S Supporting Information

ABSTRACT: Conventional transmission electron microscopy (TEM) was utilized to characterize vesicles formed by the spontaneous self-assembly of a novel zwitterionic block copolymer in the ionic liquid (2-hydroxyethyl)-dimethylammonium methanesulfonate as well as in 0.1 M phosphate buffered saline (PBS). This block copolymer was synthesized via ring-opening metathesis polymerization (ROMP) of a norbornene-based sulfobetaine, followed by its end-functionalization with polystyrene to generate the necessary amphiphilic structure. The ionic liquid enabled the visualization of the vesicles in their swollen state by TEM, demonstrating a new method for improved characterization of polymer vesicles.



INTRODUCTION

Here, for the first time, we report the self-assembly of polymer vesicles (PVs) from a zwitterionic amphiphilic block copolymer (ZABC), **1**, in the ionic liquid (2-hydroxyethyl)-dimethylammonium methanesulfonate (see Figure 1). Polymer

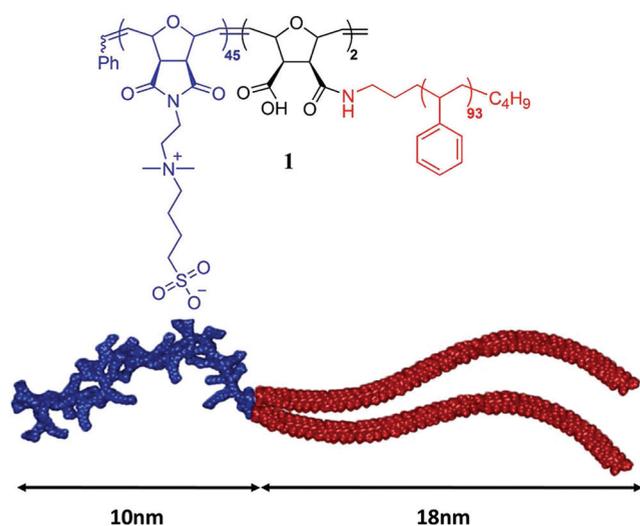


Figure 1. Chemical structure and molecular graphics image of the ZABC, **1**. The dimensions of the hydrophilic zwitterionic block (blue, 10 nm) and the hydrophobic polystyrene block (red, 18 nm) were estimated from conformationally relaxed molecular graphics by Chem3D. The visualization for the molecules was rendered by Discovery Studio Visualizer. The average degree of polymerization of each block was controlled so that the volume fraction of the hydrophilic block is in the range for vesicle formation (25–45%). The M_n is 29.5 kDa. There are 45 and 93 repeat units on average for the sulfobetaine and styrene, respectively.

vesicles, also known as “polymersomes”, have attracted considerable attention in the past decade as they hold potential for a variety of applications, including drug delivery, nano-reactors, biomineralization, imaging, biosensors, and electronics.¹ Ionic liquids, or salts that are liquid at room temperature, are also of great interest due to their many unique properties, among which their negligible vapor pressure makes them promising alternatives to conventional volatile solvents.² The polymer vesicles in ionic liquid will give access to new applications in green chemistry such as biocatalysis and nano/microreactors for organic and inorganic synthesis.³

The nonvolatile ionic liquid enables the characterization of these vesicles by conventional TEM at room temperature. To the best of our knowledge, this is the first example demonstrating the use of this technique (room temperature conventional TEM as opposed to cryo-TEM) to visualize polymeric nanostructures in solution. It allows for much simpler sample preparation and can result in fewer artifacts compared to other commonly used electron microscopy methods.⁴ For example, no dedicated and expensive controlled environment vitrification system is required.^{4a} In addition, ionic liquids behave as electronically conducting materials, preventing electron beam damage of sensitive organic nanostructures, even upon extended exposure.^{4c}

Amphiphilic molecules can spontaneously self-assemble into several nanostructures, including PVs.⁵ Although they have been extensively studied, and a number of different chemistries have been used to make vesicles,⁶ the greatest majority of reports have hydrophilic blocks composed of nonionic poly(ethylene oxide) (PEO) or charged poly(acrylic acid),

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while polymers such as polystyrene (PS), poly(lactic acid), polycaprolactone, polybutadiene, and poly(propylene oxide) compose the hydrophobic block.⁷

Recently, zwitterionic polymers, also referred to as polybetaines, have gained considerable attention as a special class of ionic polymers.⁸ They are composed of repeat units carrying both a positive and a negative charge, ensuring an overall neutral charge.^{8b,f} In aqueous solution, they show “antipolyelectrolyte effects” as the salt concentration is increased.^{8f} In addition, they resemble natural functional groups, such as phosphatidylcholines, and hence are thought to be biocompatible.^{1a,c} Zwitterionic PVs are considered analogues to mammalian cell membranes since they both present a bilayered structure and expose the unique zwitterion functional group on the surfaces of the bilayer.⁹ Despite their many interesting properties, there is only one group that has previously reported PVs from the self-assembly of a zwitterionic block copolymer.¹⁰ This polymer was prepared from commercially available monomers, 2-(methacryloyloxy)ethylphosphorylcholine and 2-(diisopropylamino)ethyl methacrylate, and is only an ZABC at elevated pH when the amino group is deprotonated.

EXPERIMENTAL SECTION

Materials. Second generation Grubbs' catalyst and 3-bromopyridine were purchased from Sigma-Aldrich and used as received. Amine-terminated polystyrene (NH₂-PS) (no silane or amide linkage, $M_n = 9500$ g/mol, $M_w = 11\,000$ g/mol, PDI = 1.16, functionality = 0.98) was obtained from Polymer Source, Inc., and used as received. Furan, ethyl vinyl ether (EVE), and 1,4-butane sultone (BS) were purchased from Acros Organics and used without further purification. Maleic anhydride, 2,2,2-trifluoroethanol (TFE), and *N,N*-dimethylethanolamine were obtained from Alfa Aesar and used as received. Acetonitrile, hexane, anhydrous diethyl ether, and methanol were purchased from Fisher Scientific and used as received. 1,3-Dinitrobenzene was obtained from Avocado Research Chemicals and used as received. Tetrahydrofuran (THF) was obtained from Fisher Scientific and was distilled from sodium/benzophenone under nitrogen before use. Dichloromethane (DCM) (Fisher Scientific) was distilled from CaH₂ under nitrogen. Third generation Grubbs' catalyst (G3) (dichlorodi(3-bromopyridino)-*N,N'*-dimethylenoimidazolino-Ru=CHPh) was synthesized according to a previously published procedure.^{7g} The ionic liquid, (2-hydroxyethyl)-dimethylammonium methanesulfonate, was received from the Evonik. Any absorbed water was removed by applying vacuum at 100 °C for 8 h.

The zwitterionic monomers oxanorbornene sulfobetaine (NSB) and oxanorbornene anhydride (NA) were synthesized according to previously published procedures.¹¹ NSB (0.400 g, 1.074 mmol), NA (0.009 g, 0.05 mmol), and the catalyst G3 (0.024 g, 0.027 mmol) were placed in different reaction flasks and purged with N₂ gas. NSB was dissolved in 6 mL of TFE, while NA and the catalyst were each dissolved in 1 mL of DCM. Upon complete dissolution of the solids, the solutions were further degassed by three freeze–pump–thaw cycles. After letting the solutions warm up to room temperature (RT), the solution of NSB was added to the catalyst solution and the reaction mixture was stirred for 15 min at room temperature to yield zwitterionic block (Poly(NSB)₄₅). An aliquot was taken for ¹H NMR analysis. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker DPX300 spectrometer (Bruker, Madison, WI). ¹H NMR (300 MHz, D₂O) δ (ppm): 6.17 (br s, 2H,

–CH=CH–, trans), 5.98 (br s, 2H, –CH=CH–, cis), 5.13 (br s, 2H, C–CH–O–), 3.72 (br s, 2H, N–CH₂–CH₂–), 3.58 (br s, 2H, –CH–C=O), 3.47 (br s, 2H, –CH₂–N(CH₃)₂), 3.21 (br s, 6H, –N(CH₃)₂–), 3.17 (br s, 2H, –CH₂–CH₂–SO₃[–]), 2.98 (br s, 2H, –N(CH₃)₂–CH₂–), 1.97 (br s, 2H, –CH₂–CH₂–CH₂–SO₃[–]), 1.83 (br s, 2H, –CH₂–CH₂–CH₂–SO₃[–]).

At the end of the 15 min, the solution of the second monomer, NA, was added to the reaction mixture. The polymerization was allowed to continue for another 5 min and then terminated by the addition of excess EVE (0.75 g, 1 mL, 10.46 mmol). The reaction mixture was allowed to stir for 30 min. An aliquot was taken for ¹H NMR analysis. ¹H NMR (300 MHz, D₂O) δ (ppm): 6.20 (br s, 2H, –CH=CH–, trans), 6.01 (br s, 2H, –CH=CH–, cis), 5.15 (br s, 2H, C–CH–O–), 3.74 (br s, 2H, N–CH₂–CH₂–), 3.61 (br s, 2H, –CH–C=O), 3.50 (br s, 2H, –CH₂–N(CH₃)₂), 3.23 (br s, 6H, –N(CH₃)₂–), 3.17 (br s, 2H, –CH₂–CH₂–SO₃[–]), 3.00 (br s, 2H, –N(CH₃)₂–CH₂–), 1.99 (br s, 2H, –CH₂–CH₂–CH₂–SO₃[–]), 1.85 (br s, 2H, –CH₂–CH₂–CH₂–SO₃[–]).

After 30 min, an excess amount of NH₂-PS (0.50 g, 0.053 mmol) dissolved in 10 mL of DCM was added to the mixture. The reaction was allowed to proceed for an additional 24 h at RT to yield the diblock copolymer **1** (Poly(NSB₄₅-*b*-S₉₃)). The polymer was precipitated from excess diethyl ether to yield a light yellow powder. Yield = 86%. ¹H NMR (300 MHz, *d*₇-DMF with LiBr) δ (ppm): 7.14 (br s, 3H), 6.67 (br s, 2H), 6.19 (br s, 2H), 1.94 (br s, 1H), 1.58 (br s, 2H). The observed peaks in the ¹H NMR corresponded to the PS block because of the decreased solubility of the sulfobetaine block in the solvent and subsequent micellization of the polymer.

Vesicle Preparation and Characterization. *Ionic Liquid.* The formation of zwitterionic polymer vesicles in ionic liquid was done by using a method similar to the solvent-switch technique published in the literature.¹² The zwitterionic amphiphilic block copolymer **1** was not directly soluble in ionic liquid. Hence, **1** (3 mg) was initially dissolved in a mixture of solvents, DCM (2 mL) and TFE (1 mL), to prepare a polymer solution of concentration 1 mg/mL. TFE was used as a good solvent for the hydrophilic zwitterionic block and DCM for the hydrophobic PS block. Upon complete dissolution of the block copolymer in the organic solvents, 1 mL of polymer solution was mixed with 1 mL of (2-hydroxyethyl)dimethylammonium methanesulfonate ionic liquid (Figure S2).¹³ After assuring a homogeneous mixture, DCM and TFE were removed by applying vacuum at 80 °C for 12 h.

0.1 M PBS Buffer. The zwitterionic polymer vesicles in 0.1 M PBS buffer (at pH = 7.4) were formed by using the film rehydration method as described by Discher et al.^{1a} **1** (3 mg) was dissolved in a mixture of DCM (2 mL) and TFE (1 mL) to prepare a polymer solution of concentration 1 mg/mL. This polymer solution was first thoroughly dried onto the wall of a 25 mL glass vial under vacuum for 4 h in a rotary evaporator. These films were then rehydrated with 0.1 M PBS buffer (3 mL) and sonicated at 80 °C for 10 min, repeating for three times with a gap of 5 min between each trial.

Dynamic Light Scattering. To investigate the vesicle formation and the average size, dynamic light scattering (DLS) was used. Measurements were performed at room temperature using an ALV unit equipped with an ALV/SP-125 precision goniometer (ALV-LASER Vertreibsgesellschaft m.b.h., Langen, Germany), an Innova 70 argon laser ($\lambda = 514.5$ nm; maximum power 3 W, Coherent Inc.) operated at 300 mW, and

a photomultiplier detector (Thorn EMI electron tubes). Signals from the detector were processed by an ALV 5000 multiple tau digital correlator board and its associated software.

For the DLS measurements of polymer vesicles prepared in the 0.1 M PBS buffer and ionic liquid, the solutions of 1 mg/mL concentration were filtered through 0.45 μm PTFE syringe filters into a cylindrical scattering cell with an outer diameter of ~ 0.25 in. and were sealed. Because of high viscosity of the ionic liquid, it was filtered prior to the preparation of vesicles. Since the ionic liquid is highly hygroscopic, the vesicle dispersion was heated at 80 $^{\circ}\text{C}$ under vacuum for 8 h to remove any absorbed water and then sealed. The scattering cell containing vesicle dispersion was then immersed in a large diameter thermostated bath at 25 $^{\circ}\text{C}$ containing the index matching fluid, decalin. The intensity correlation functions $g_2(t)$ were taken in 30 $^{\circ}$ angular increments from 30 $^{\circ}$ to 120 $^{\circ}$, for a duration of 5 min. From these autocorrelation functions, the diffusion coefficient D is derived and from which the hydrodynamic radius, R_h , is calculated.¹³

Transmission Electron Microscopy. The structure and size of zwitterionic polymer vesicles were characterized by conventional transmission electron microscopy (JEOL 100CX) operating at 100.0 kV.

Vesicles in Ionic Liquid. To observe the vesicles dispersed in the ionic liquid, a drop of the sample was placed on parafilm and then applied to the carbon-coated TEM grid by touching the drop with one face of the TEM grid. These grids were stored under vacuum overnight prior to characterization by TEM to remove any absorbed water. Lower emission current was used by using smaller spot size (spot size 3) to reduce the movement of ionic liquid due to interaction with electron beam.

Vesicles in 0.1 M PBS Buffer. To observe the vesicles dispersed in 0.1 M PBS buffer, 500 μL of solution containing vesicles was spread on parafilm and mixed with 500 μL of 1 wt % sodium tungstate (maintained at pH 6.8 with 1.0 N KOH) (vesicle dispersion/sodium tungstate in 1:1 v/v ratio), a TEM negative stain. After sitting for 2 min, a drop of this solution was placed on a 400 mesh copper grid coated with Formvar and stabilized with carbon film coating. The excess fluid was drained off with filter paper. These TEM grids were then floated on top of deionized water to remove any excess stain. The grids were then wicked and allowed to dry at room temperature and stored under vacuum.

RESULTS AND DISCUSSION

Previously, we reported that ring-opening metathesis polymerization (ROMP) could be successfully used to synthesize well-defined, norbornene-based zwitterionic polymers.^{11a,14} Here, this chemistry was extended to generate a new ZABC, **1**, where the zwitterionic block was combined with a PS block (Scheme S1). The zwitterionic block was synthesized by ROMP of a norbornene-based sulfobetaine ($M_n = 16.8$ kDa), which was subsequently end-capped with maleic anhydride functionalities ($M_n = 17.1$ kDa). The anhydride containing monomer was added in a stoichiometric ratio to yield, on average, two repeat units per chain end. Although other more precise methods exist to “end-cap” polymer chains produced by ROMP, they also require more effort (reagent synthesis, postpolymerization reactions, etc.).¹⁵ Finally, an amine-terminated PS chain was attached to the zwitterionic block via ring-opening of the anhydrides to yield the ZABC, **1** ($M_n = 29.5$ kDa), containing on average two PS tails as shown in Figure 1.^{10a,16} The degrees

of polymerization of the zwitterionic block and polystyrene blocks were 45 and 93, respectively—chosen so that the volume fraction of the hydrophilic block was around 35%, favoring vesicle formation (25–45%).¹⁷ Detailed synthetic procedures are also found in the Supporting Information.

Although PV formation in aqueous media has been extensively investigated, only recently have the studies been extended to nonaqueous media such as organic solvents, supercritical CO₂, and ionic liquids.^{18,19} Not surprisingly, the nature and composition of the solvent plays an important role in determining the size and structure of PVs.²⁰ To date, however, the study of PVs in ionic liquids has been confined to neutral amphiphilic block copolymers based on PEO.¹⁹ How the ionic liquid impacts vesicle assembly when the block copolymer contains a charged hydrophilic block remains an open question. Ionic liquids possess at least two contrasting properties expected to influence PV self-assembly of **1**. While their ionic strengths have been calculated between 1 and 10 M, their E_TN parameters (normalized empirical parameter of solvent polarity) are more typical of polar organic solvents like DMSO. For example, quaternary ammonium sulfonates have E_TN values of 0.45–0.65 (DMSO, $E_TN = 0.44$).^{2a} Therefore, while they are much less polar than aqueous solutions, their high ionic strength is expected to reduce PV size due to electrostatic screening.^{3b,21} Even though ammonium-based ionic liquids are considered to be close ion pairs, their ionic strength is still larger than standard PBS solutions.

Here, ZABC (**1**) was initially dissolved in a mixture of solvents, 2,2,2-trifluoroethanol, and dichloromethane (1:2 volume ratio). Upon addition of this solution into the ionic liquid, PVs were spontaneously formed. This is similar to the solvent-switch technique for vesicle preparation, in which an organic solvent is used to dissolve the copolymer and then water is added gradually to the copolymer solution followed by the removal of organic solvents.^{12,22} Representative TEM images of the vesicles formed in the ionic liquid are shown in Figure 2a,b. The hydrophilic zwitterionic block has a preference for the ionic liquid allowing the hydrophobic polystyrene block to form the membrane core. The dark contrast observed in the TEM micrograph is due to the PS block (see Figure 2a) because the outer hydrophilic block was dissolved in the ionic liquid and thus invisible in TEM images. The average PV diameter in the ionic liquid was determined by both TEM and dynamic light scattering (DLS). As shown in the TEM micrograph of Figure 2a, the average PV diameter is 120–150 nm. This was consistent with the average diameter determined by DLS shown in Figure 3a. The hydrodynamic radius, R_h , of 70 nm (140 nm diameter) was calculated using the slope of the plot of Γ vs q^2 for four angles (30 $^{\circ}$, 60 $^{\circ}$, 90 $^{\circ}$, and 120 $^{\circ}$). The inset in Figure 3a shows the size distribution collected at 90 $^{\circ}$ for PVs with an R_h of 60 nm (120 nm diameter).

The hydrophobic core thickness of the PVs was estimated to be around 30 nm from TEM image analysis (Figure 2a) and is in the range of PV wall thicknesses generally observed.^{22e} This is consistent with a bilayer membrane structure based on the length of the hydrophobic PS, which was calculated to be 18 nm (see Figure 1) with MM2 energy minimization using Chem3D for a Gaussian conformation. These dimensions show that the vesicle is composed of a double layer with limited interpenetration of the PS chains.^{18e} This results from a combination of factors including the volume occupied by each block of the ZABC in the vesicle.

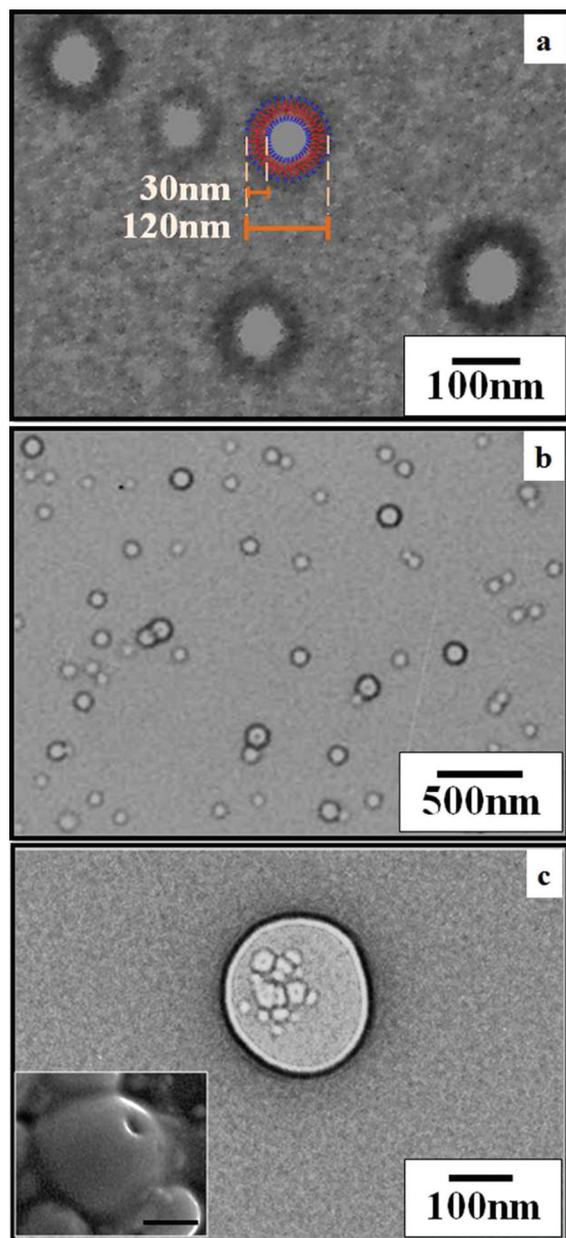


Figure 2. TEM images of zwitterionic PVs from 1: (a) formed in ionic liquid at higher magnification, (b) at lower magnification, and (c) formed in PBS buffer. The inset in (c) shows the same vesicles imaged by SEM, where the scale bar corresponds to 100 nm.

Polymer vesicles were also prepared in 0.1 M PBS buffer, which allowed a direct comparison between the two solvents (ionic strength) and also enabled us to confirm the ability of this novel ZABC to form PVs under more traditional conditions. The nanostructures in PBS buffer were formed by the film rehydration method.^{1a} DLS indicated that the average diameter of PVs in these solutions was 250 nm as shown by the peak maximum of 125 nm in the inset of Figure 3b for R_h . A hydrodynamic radius of 150 nm (300 nm diameter) was determined using the plot of Γ vs q^2 for four angles (30° , 60° , 90° , and 120°). TEM analysis upon drying and negative staining yielded PV diameters of ~ 200 nm as shown in Figure 2c, in agreement with DLS. In addition, PVs formed in PBS buffer were larger than those in the ionic liquid, which is consistent with the expectation that the higher ionic strength of

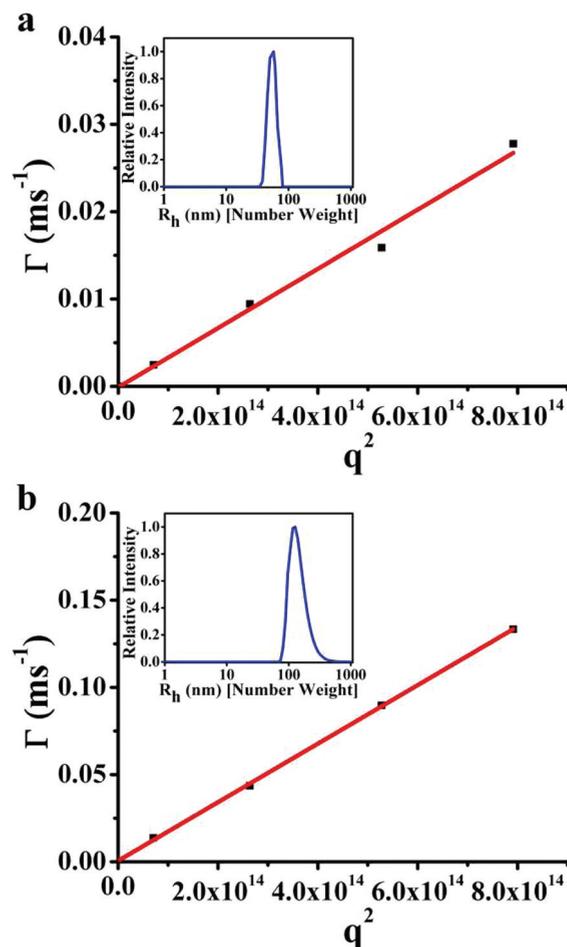


Figure 3. DLS data from the ZABC, 1, in (a) the ionic liquid and (b) PBS buffer. The plots show Γ vs q^2 for four angles (30° , 60° , 90° , and 120°) along with the fitting line. The slope of this line was used to determine the diffusion coefficient, D , which provided the hydrodynamic radius, R_h , using the Stokes–Einstein equation. The insets in (a) and (b) show relative scattering intensity vs R_h obtained at 90° .

the ionic liquid allowed more effective screening of the charges (strong dipoles) on the zwitterionic block.

The effects of drying during sample preparation are evident in the TEM micrograph of Figure 2c as patchy, lighter spots on the PV. These surface defects, or holes in the membrane, were further confirmed by scanning electron microscopy (SEM). The inset of Figure 2c shows the expected spherical morphology but with a dimple or pore. The patchy spots in TEM and the pores in SEM were seen routinely.¹³ As expected, such defects were not observed in the TEM of PVs dispersed in the ionic liquid, demonstrating another benefit of using the ionic liquid for characterization of PVs. The ability to visualize these defects in the PVs prepared in PBS buffer may be related to the use of PS as the hydrophobic core, which has a high glass transition temperature (T_g). Under the high vacuum of TEM or SEM, the remaining interior buffer solution of the PVs must escape rapidly and likely leads to the observed membrane damage. However, due to the high T_g of the PS block, it is unable to reorganize, thereby allowing the “pore” to be visualized.

CONCLUSIONS

In summary, a novel ZABC having a hydrophilic zwitterionic block and hydrophobic PS block was synthesized. The formation of zwitterionic vesicles in an ionic liquid, as well as PBS buffer, was confirmed by TEM and DLS. The ionic liquid allowed vesicle formation and simultaneously the use of conventional, room temperature TEM to visualize them. Extension of this approach is easily envisaged for the characterization of other hydrophilic nanostructured assemblies such as gels and biological materials.

ASSOCIATED CONTENT

Supporting Information

All the experimental procedures, including monomer and polymer synthesis, structure of the ionic liquid, vesicle preparation, and their characterization by DLS and SEM. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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