Electroformed Giant Vesicles from Thiophene-Containing Rod−Coil Diblock Copolymers

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Introduction

Giant vesicles are very interesting self-assembled structures since their size and membrane curvature make them simple model systems for living cells. Over the past few years several methods have been developed for the construction of large vesicles with diameters in the range of 10−200 μm.[1,2] Electroformation has proven to be one of the most useful in this respect, since it allows the preparation of such vesicles from a large variety of neutral, charged, and zwitterionic lipids.[3] Using this technique, the vesicle size can be readily controlled by varying the voltage and frequency of the electric field.[2] A more recent approach to construct large, micrometer-sized vesicles is to use large amphiphiles, i.e., amphiphiles derived from block copolymers. These macromolecules have been shown to self-assemble into vesicles, referred to as polymersomes, which have a much higher toughness and stability than classical phospholipid-based vesicles, i.e., liposomes.[4] In a fashion similar to liposomes, polymersomes can be loaded with synthetic or biological compounds, like dyes and enzymes.[5,6] The combination of electroformation and block copolymers may offer an ideal route to prepare stable giant polymersomes with potentially unique properties. Discher et al. were the first to demonstrate the feasibility of such an approach and constructed giant polymersomes from poly(ethylene oxide)-*b*-poly(ethylethylene) with diameters between 20 and 50 μm.[5]

Recently, we developed a novel type of amphiphilic diblock copolymer derived from isocyanopeptides and styrene.[7] Charged diblock copolymers of styrene and L-isocyanoalanyl-L-alanine and of styrene and L-isocyanoalanyl-L-histidine were found to self-assemble in water, yielding vesicles, multilayers, and helical aggregates. In this paper we report that this type of diblock copolymer can generate gigantic vesicles up to 100 μm in diameter by the electroformation method. Despite their rigid-rod block copolymer nature, these giant aggregates have a fluidic membrane and are capable of encapsulating solutes, in a manner similar to vesicles prepared by the injection method.[8]

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Experimental Section

Materials. Tetrahydrofuran (THF) was distilled over sodium, CH_2Cl_2 over $CaCl_2$, and methanol over CaH_2. β-3-Thienylethylamine,[9] *N*-formyl-L-alanine,[10] tetrakis(*tert*-butyl isocyanide)nickel(II) perchlorate ($(t\text{-BuNC})_4Ni(ClO_4)_2$),[11] and amine-functionalized polystyrene$_{40}$ (PS_{40}-NH_2)[12] were synthesized using procedures reported in the literature. All other chemicals were commercial products and used as obtained.

Synthesis of Initiator Complex (1). PS_{40}-NH_2 (1.03 g, 0.242 mmol, M_n = 4250, M_w/M_n = 1.04) dissolved in CH_2Cl_2 (10 mL) was added to a stirred solution of $(t\text{-}Bu\text{NC})_4Ni(ClO_4)_2$ (0.143 g, 0.242 mmol) in CH_2Cl_2 (25 mL) under a nitrogen atmosphere. After an additional hour of stirring the solvent was evaporated, yielding a yellow solid (1.17 g, 0.242 mmol, 100%).[7] ^{1}H NMR ($CDCl_3$): δ = 7.82 (s, CH_2NH), 7.3−6.3 (br, $CHPh$), 3.7 (br, $OCH_2CH_2CH_2NH$), 3.3−2.9 (br, CH_2OCH_2 and CH_2OCH_2), 2.3−1.7 (br, $CHPh$), 1.54 (s, $C(CH_3)_3$), 1.7−1.2 (br, CH_2CHPh), 1.26−0.58 ppm ($Bu(CH_2CHPh)_{40}$). ^{13}C NMR ($CDCl_3$): δ = 178 (NCN), 146.5−145 (br, $CHPh_{ipso}$), 130−127 (br, ($CHPh_{ortho+meta}$), 127−124 (br, $CHPh_{para}$), 123 (br, Ni−C═N), 75 (br, $CH_2OCH_2CH_2CH_2NH$), 68 (br, $CH_2OCH_2CH_2CH_2NH$), 54.9 ($CH_2OCH_2CH_2CH_2NH$), 41 ($CHPh$), 46−40 (br, CH_2CHPh), 31.7 ($CH_2CH(CH_3)(CH_2CHPh)_{40}$), 30.3 ($(CH_3)_3CN$), 29.5 ($(CH_3)_3CNH$), 28.2 (br, $CH_2CH(CH_3)(CH_2CHPh)_{40}$), 26.9 ($OCH_2CH_2CH_2NH$), 22.3 ($CH_2CH(CH_3)(CH_2CHPh)_{40}$), 13.9 ppm ($CH_3CH_2CH(CH_3)(CH_2CHPh)_{40}$). FT-IR (cm^{-1}, KBr): 3282 (NH), 3061, 3026, 2923, 2850 (CH), 2253, 2226 (C═N), 1602 (C−C aryl), 1575 (NCN), 1493, 1453 (C−C aryl), 1097 (ClO_4).

Synthesis of *N*-Formyl-L-alanine(2-thiophen-3-ylethyl)-amide (FAT). β-3-Thienylethylamine (0.61 g, 4.8 mmol), *N*-formyl-L-alanine (0.56 g, 4.8 mmol), (dimethylamino)pyridine (catalytic amount), and dicyclohexylcarbodiimide (1.19 g, 5.8 mmol) were dissolved in 20 mL of CH_2Cl_2/EtOAc (3/1 v/v). The solution was stirred overnight, followed by removal of the formed dicyclohexylurea by filtration and evaporation of the solvent. After purification by flash column chromatography on silica gel (CH_2Cl_2/MeOH, 95/5 v/v), the product was dissolved in a minimal amount of CH_2Cl_2 and precipitated in petroleum ether (40−65 °C), yielding a white solid (0.65 g, 2.9 mmol, 60%). $[\alpha]_D^{20}$ (CH_2Cl_2 *c* 0.10) = −40°. ^{1}H NMR ($CDCl_3$): δ = 8.13 (s, 1H, C(O)H), 7.29 (m, 1H, thiophene H-5), 7.01 (d, 1H, thiophene H-2, ^3J = 2.3 Hz), 6.95 (m, 1H, thiophene H-4), 6.29 (br, 1H, NHCO), 6.08 (br, 1H, NHCO), 4.48 (m, 1H, C(O)-NHC*H*(CH_3)), 3.52 (m, 2H, CH_2NH), 2.86 (t, 2H, CH_2-thiophene, ^3J = 6.9 Hz), 1.37 ppm (d, 3H, $CHCH_3$, ^3J = 3.4). ^{13}C NMR ($CDCl_3$): δ = 171.7 (COH), 160.9 (C═O), 138.9 (thiophene C-3), 128.1 (thiophene C-4), 126.3 (thiophene C-5), 121.8 (thiophene C-2), 47.8 (C(O)NH*C*H(CH_3)), 40.2 (CH_2NH), 30.2 (CH_2-thiophene), 18.7 ppm (CH_3). FT-IR (cm^{-1}, KBr): 3342 (NH), 3092, 2979, 2936, 2864 (CH), 1685, 1655 (amide I) 1547, 1522 (amide II). EI-MS: *m*/*z* = 226 $[M]^+$ (calcd: 226.30). El anal. calcd for $C_{10}H_{14}N_2O_2S$ (%): C, 53.08; H, 6.24; N, 12.38; S, 14.17. Found: C, 53.06; H, 6.23; N, 12.01; S, 14.37.

Synthesis of L-Isocyanoalanine(2-thiophen-3-ylethyl)-amide (IAT). To a solution of FAT (99.0 mg, 0.438 mmol) in 1.0 mL of CH_2Cl_2, *N*-methylmorpholine (0.97 mL, 0.88 mmol) was added under a nitrogen atmosphere, and the reaction mixture was cooled to −30 °C. Over a period of 10 min diphosgene (43 mg, 0.22 mmol) in 1.0 mL of CH_2Cl_2 was added. After keeping the temperature at −30 °C for an additional 30 min, the temperature was gradually brought to room temperature. A saturated $NaHCO_3$ solution (5 mL) was added, and the reaction mixture was stirred vigorously for 5 min. The organic layer was separated, extracted twice with 10 mL of water, and dried using Na_2SO_4. The solvent was evaporated, and the crude solid was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH, 99.5/0.5 v/v), resulting in a white solid (79

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mg, 0.38 mmol, 87%). Single crystals were obtained by slow evaporation of a diethyl ether solution of the compound. $[\alpha]_D^{20}$ (CH_2Cl_2 *c* 0.5) = +11°. ^{1}H NMR ($CDCl_3$): δ = 7.31 (m, 1H, thiophene H-5), 7.04 (d, 1H, thiophene H-2, ^3J = 2.0 Hz), 6.97 (m, 1H, thiophene H-4), 6.43 (br, 1H, NHCO), 4.19 (q, 1H, C(O)NHC*H*(CH_3), ^3J = 7.1 Hz), 3.58 (m, 2H, C*H*$_2$NH), 2.91 (t, 2H, C*H*$_2$-thiophene, ^3J = 6.9 Hz), 1.61 ppm (d, 3H, CHC*H*$_3$, ^3J = 7.1). ^{13}C NMR ($CDCl_3$): δ = 166.1 (C=O), 161.5 (NC), 138.5 (thiophene C-3), 128.1 (thiophene C-5), 126.7 (thiophene C-2), 122.0 (thiophene C-4), 53.7 (C(O)NH*C*H(CH_3)), 40.5 (CH_2-NH), 30.1 (*C*H$_2$-thiophene), 20.0 ppm (CH_3). FT-IR (cm^{-1}, KBr): 3282 (NH), 3093 (thiophene), 2145 (CN), 1661 (amide I), 1567 (amide II). EI-MS: *m*/*z* = 208 $[M]^+$ (calcd: 208.28). El anal. calcd for $C_{10}H_{12}N_2OS$ (%): C, 57.67; H, 5.81; N, 13.45; S, 15.40. Found: C, 57.91; H, 5.89; N, 13.11; S, 15.58.

Synthesis of Polystyrene$_{40}$-*b*-poly(L-isocyanoalanine-(2-thiophen-3-ylethyl)amide) (PS−PIAT). To a stirred solution of IAT (50.0 mg, 0.240 mmol) in CH_2Cl_2 (25 mL) a solution of initiator complex **1** (22.9 mg, 4.79 μmol) in CH_2Cl_2 (5 mL) was added. Complete consumption of IAT, as observed by IR spectroscopy, was achieved after 2 days stirring. The reaction mixture was evaporated to dryness, and the resulting polymer was dissolved in a minimal amount of CH_2Cl_2. The polymer was then precipitated by dropping this solution into a well-stirred mixture of methanol/water (1/1 v/v, 10 mL). The product was filtered off and washed with methanol/water (1/1 v/v, 20 mL). After drying in vacuo the polymer was obtained as an orange/brown solid (40 mg, 60%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.3−6.3 (br, CH*Ph*), 6.94 (br, thiophene H-5), 6.83 (br, thiophene H-2), 6.79 (br, thiophene H-4), 4.3−4.1 (br, C=NC*H*(CH_3)), 3.9−3.3 (br, C*H*$_2$CH$_2$-thiophene), 3.7 (br, OCH$_2$-CH$_2$C*H*$_2$NH), 3.3−2.9 (br, C*H*$_2$OCH$_2$ and CH$_2$OC*H*$_2$), 3.1−2.6 (br, C*H*$_2$-thiophene), 2.1−1.7 (br, CH$_2$C*H*Ph), 1.7 (br, C(CH_3)$_3$), 1.7−1.2 (br, C*H*$_2$CHPh), 1.62 (br, NHCH(C*H*$_3$)CO), 1.0−0.6 ppm (*Bu*(CH_2CHPh)$_{40}$). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 155.2 (C=N), 147−143 (br, CH*Ph*$_{ipso}$), 140 (thiophene C-3), 130−127 (br, (CH*Ph*$_{ortho+meta}$), 127−124 (br, thiophene C-4, C-5 and CH*Ph*$_{para}$), 113 (br, thiophene C-2), 72 (br, *C*H$_2$OCH$_2$CH$_2$CH$_2$-NH), 68 (br, CH$_2$O*C*H$_2$CH$_2$CH$_2$NH), 63 (br, C=N*C*H(CH_3)), 42−38 (br, *C*HPh and *C*H$_2$CHPh), 39.8 (br, CH$_2$*C*H$_2$NHCO), 37.5−34 (br, CH$_2$OCH$_2$*C*H$_2$CH$_2$NH), 32−30 (C=NC(*C*H$_3$)$_3$) and CH$_2$*C*H(CH_3)(CH_2CHPh)$_{40}$), 28.7 (*C*H$_2$CH$_2$NHCO), 25.9 (br, *C*H$_2$CH(CH_3)(CH_2CHPh)$_{40}$), 22.6 (CH_2CH(*C*H$_3$)(CH_2CHPh)$_{40}$), 21.4 (CH(*C*H$_3$)), 13.4 ppm (CH_3CH_2CH(*C*H$_3$)(CH_2CHPh)$_{40}$). FT-IR (cm^{-1}, KBr): 3288 (NH), 3060, 3027, 2950, 2924, 2850 (CH), 1660 (amide I), 1604 (C−C aryl), 1532 (amide II).

Preparation of Aggregates. In a typical aggregation experiment 1.0 mL of a 1.0 mg/mL PS−PIAT solution in THF was injected into 5 mL of ultrapure water at room temperature, and the dispersion was left to equilibrate. No additional energy was applied to induce aggregate formation. SEM samples were prepared by drying a drop of the dispersion on a microscopy glass, which was cleaned by sonication for 15 min in CH_2Cl_2. The samples were left to dry for a day in a desiccator before studying them.

Electroformation. The used electroformation setup consisted of a bath with two platinum electrode wires (5 × 1 mm), which were separated 5 mm from each other and were connected to an external alternating or direct current supply. A thin film of PS−PIAT in $CHCl_3$ was dried on the electrodes, the bath was filled with a 0.1 M sucrose solution, and an alternating current potential of 10 V with a frequency of 10 Hz was applied for 6 h. Thereafter, the frequency was lowered to 2 Hz for 12 h to release the vesicles from the electrodes, followed by dilution into an isoosmotic sucrose solution. The setup was mounted on the stage of a light microscope for optical observation.

Measurements. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-300 instrument (^{1}H/300 MHz, ^{13}C/75 MHz) at room temperature. Infrared spectra were measured on a BioRad FTS 25 spectrometer. For SEM studies a JEOL JSM-6330F was used. A 1.5 nm layer of Pd/Au was sputtered on the SEM samples before studying by using a Cressington 208 HR sputter coater. Light and fluorescence micrographs were made with a Zeiss Axiovert 100.

X-ray Analysis of IAT. Crystals of IAT suitable for X-ray diffraction studies were obtained from diethyl ether by slow evaporation of the solvent. A single crystal was mounted in air on a glass fiber. Intensity data were collected at room temperature and corrected for Lorentz and polarization effects. An Enraf-Nonius CAD4 single-crystal diffractometer was used, Mo Kα radiation, omega scan mode. Unit cell dimensions were determined from the angular setting of 25 reflections. Semi-empirical absorption correction (ψ-scans) was applied.[13] The structure was solved by the program CRUNCH[14] and was refined with standard methods (refinement against F^2 of all reflections with SHELXL97[15]) with anisotropic parameters for the non-hydrogen atoms. The hydrogens of the methyl group were refined as a rigid rotor with idealized sp^3 hybridization and a C−H bond length of 0.97 Å to match maximum electron density in a difference Fourier map. All other hydrogens were placed at calculated positions and were refined riding on the parent atoms. The thiophene ring is disordered over two positions. This disorder can be interpreted as a swap of the ring and can be described by a suitable disorder model. Crystallographic data for IAT has been deposited with the Cambridge Crystallographic Data Centre and can be retrieved under number CCDC 225216.

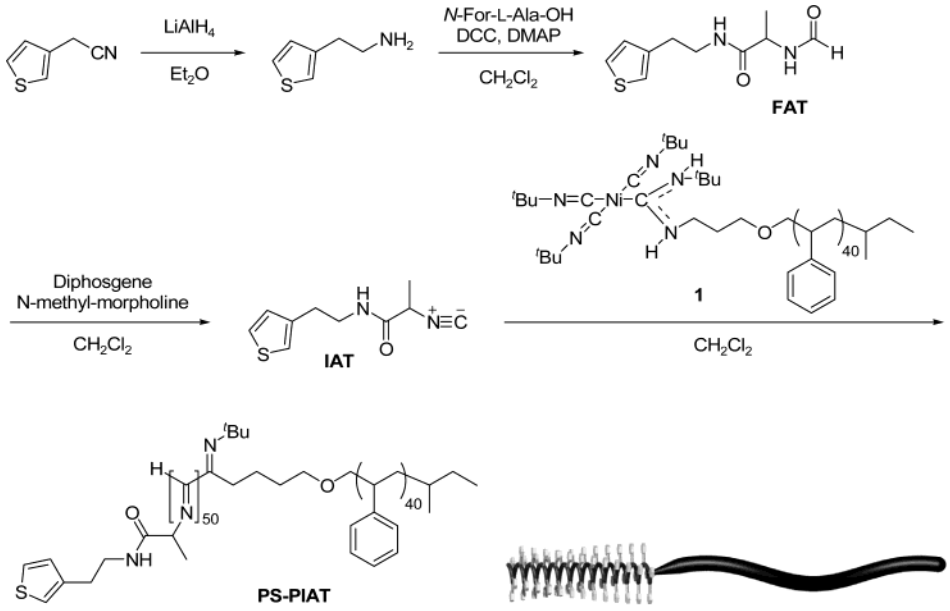
Results and Discussion

In recent studies it has been found that peptide-based polyisocyanides have an enhanced stability and rigidity of the helical polymer backbone, when compared to other polyisocyanides. This rigidity is the result of a hydrogen-bonding network between the stacking amide groups of the peptide side chains. This additional stabilization increases the persistence length of these types of macromolecules, which were found to be even more rigid than DNA.[16,17] In this study we used the rod−coil diblock copolymer polystyrene$_{40}$-*b*-poly(L-isocyanoalanine(2-thiophen-3-ylethyl)amide) (PS−PIAT). Its synthesis was carried out in four steps as outlined in Scheme 1.

Isocyanides are polymerized using Ni(II)-based catalysts, in which nucleophiles, i.e., alcohols or amines, act as initiators.[18,19] These nucleophiles are also incorporated as the first unit in the resulting polyisocyanide. To construct PS−PIAT, an amine-functionalized polystyrene$_{40}$ (PS$_{40}$-NH$_2$) was used to prepare the macromolecular initiator complex **1** (see Experimental Section), which was subsequently treated with 50 equiv of IAT to give PS−PIAT in a yield of 60%. Attempts to determine the molecular weight by GPC and MALDI−TOF spectrometry were unsuccessful because these types of polyisocyanides do not run on a GPC column and they do not fly in a MALDI spectrometer. However, using ^{1}H NMR, an estimation of the molecular weight could be made by comparing integrals of the polystyrene block, of which the molecular weight and polydispersity were known, with integrals of the polyisocyanide block. In this way the PIAT block was estimated to consist of 62 ± 15 repeating units, corresponding to a total molecular weight for PS−PIAT of 18 ± 4 kg/mol. The crystal structure of IAT already revealed the preference of this molecule to form hydrogen-bonding networks between the amide groups of stacked molecules (Figure 1).

The existence of a hydrogen-bonding network in PS−PIAT was confirmed by IR studies in an analogous way as described previously.[16] The thiophene groups were included in the block copolymer so that once a 3D architecture had been self-assembled, it could be cross-linked by polymerization of the fore-mentioned groups, resulting in stable and potentially conductive nanometer-sized vesicles and fibers.[8]

Scheme 1. Synthetic Route to PS−PIAT; the Image on the Right Bottom Is a Schematic Illustration of PS−PIAT



Assemblies of this amphiphilic diblock copolymer were initially prepared via the injection method.[20] PS−PIAT was dissolved in a good solvent (THF) and then injected into a poor solvent (water). Immediately after addition of the PS−PIAT solution very small vesicles (<100 nm) were observed by electron microscopy. Upon standing, these vesicles fused over a period of 50 h to give vesicles with a final average diameter of 1.5 μm (Figure 2a).[8] Removing the THF immediately after injection into water resulted in vesicles with a small average diameter of 80 nm, which was retained over a period of weeks.[8]

Giant vesicles of PS−PIAT were prepared by electroformation as described in the Experimental Section. Using this approach, large quantities of well-defined aggregates were obtained. Their diameters ranged from 1 to 100 μm (Figure 2b,c), which is significantly larger than the vesicles prepared via the injection method and about 2 times larger than the polymersomes reported by Discher and Dimova.[5,21] The electroformation procedure used to form the giant vesicles is that commonly used in the electroformation of phospholipid vesicles. In contrast to the approach of Dimova et al., who prepared vesicles in water while applying an increasing potential, the giant vesicles described here were assembled in a 0.1 M sucrose solution while applying a constant potential. This latter approach is similar to that developed by Discher et al. and only differs in the method of dissociation of the vesicles from the electrode.[2] A standard method for the preparation of vesicles from low molecular weight amphiphiles, e.g., dioleoylphosphatidylcholine (DOPC), is the hydration method,[22] which was used to check whether vesicles of PS−PIAT could be formed under the same conditions. A drop of a PS−PIAT solution in $CHCl_3$ was dried on a roughened Teflon surface and subsequently immersed into a 0.1 M sucrose solution of 40 °C. This experiment can be considered as a blank experiment since the electroformed vesicles were also prepared in a sucrose solution. This approach did not result in the formation of vesicles. The lack of vesicle formation by the hydration method is likely the result of the low mobility of the polystyrene$_{40}$ chains below their glass transition temperature ($T_g \sim 70$ °C). The mechanism of the electroformation of giant vesicles is not yet fully understood; however, it is believed that the electroosmotic motion of water induces bilayer separation and vesicle growth.[3] Since the electroformation experiments were done at a temperature below the T_g of polystyrene$_{40}$, it is assumed that the applied alternating current supplies the energy that is needed to plasticize the polystyrene chains, resulting in the growth of the vesicles.

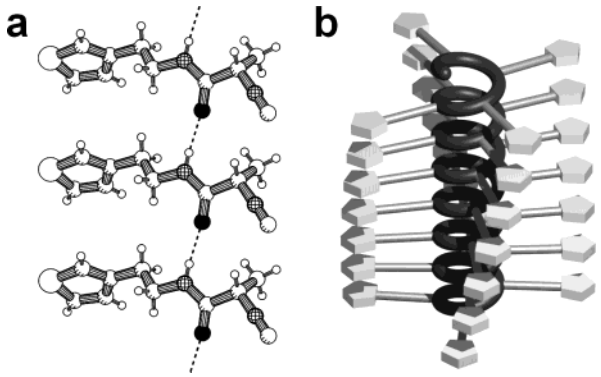


Figure 1. (a) PLUTON representation of the single-crystal X-ray structure of IAT, showing the most abundant conformation of the thiophene ring.[25] (b) Computer modeled image of the PIAT block.

Although constructed from rigid diblock copolymers, fusion of two giant vesicles was possible when they were brought in close proximity using a micropipet. This suggests that despite the high T_g value of the polystyrene block, the vesicle membranes are still highly fluidic in nature and behave like the membranes of simple lipid molecules (Figure 2d). One of the requirements for fusion is thought to be a good contact between the two membranes and a pressure above a certain threshold. The large size of the giant vesicles means that the curvature of the membranes is small, which is known to more readily enable the diblock copolymers to easily insert into the membrane of the adjacent vesicle without disruptive reorganization. It is most remarkable that this process so readily occurs, given that the polystyrene is probably in a glassy state. The precise mechanism of this fusion is currently being further investigated. This fusion behavior is in contrast to vesicles prepared via the injection method, where THF is an essential com-

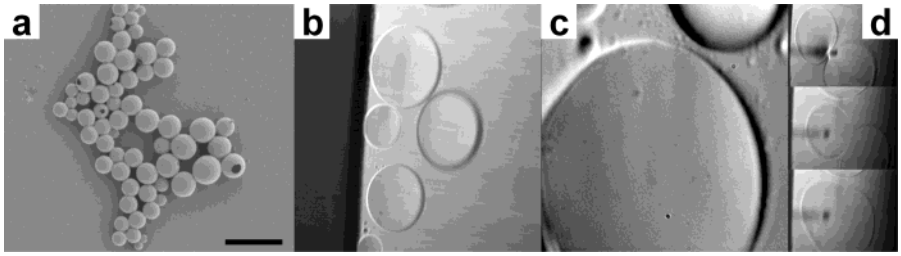


Figure 2. (a) Scanning electron micrograph of PS−PIAT vesicles, formed by the injection method, after 2 days in dispersion; the scale bar represents 5 μm. (b, c) Optical micrographs of electroformed giant vesicles of PS−PIAT; the image width corresponds to 60 μm. (d) Two giant vesicles fuse into one vesicle with the help of a micropipet. The width of the optical micrographs amounts to 80 μm.

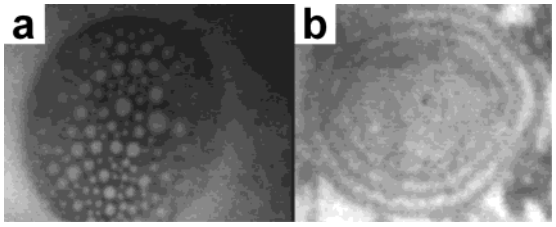


Figure 3. Fluorescence micrographs of fluorescein-labeled dextran inside electroformed giant vesicles of PS−PIAT. (a) Multicompartmental giant vesicle; the width of the image corresponds to 80 μm. (b) Multilamellar giant vesicle; the width of the image corresponds to 45 μm.

ponent for fusion by dissolving the polystyrene block, allowing it to reorganize.[8]

These giant vesicles have the potential to be used as microcapsules, as was demonstrated in a separate experiment. Giant vesicles of PS−PIAT were prepared by electroformation in a 0.1 M sucrose solution containing fluorescein-labeled dextran ($M_w = 10^4$ g/mol). The vesicles that grew from the electrode surface were found to encapsulate the dye, as could be observed by fluorescence microscopy. The encapsulation of the labeled biopolymer allowed the observation of two distinct types of giant vesicles, viz. multicompartment ones (Figure 3a) and multilamellar ones (Figure 3b).

These results are in contrast to experiments carried out with classical phospholipids and with block copolymers studied by Discher et al. In both cases the giant vesicles were unilamellar in nature.[5,23] The mechanism of formation of the compartmental vesicles shown in Figure 3 requires further study, but it is likely that the structure of PS−PIAT plays an important role. Both blocks of PS−PIAT have a very low solubility in water, in contrast to the classical phospholipids and the poly(ethylene oxide)-*b*-poly(ethylethylene) block copolymers studied by Discher and co-workers, which are more water compatible. The multilamellar vesicles are probably formed from a multilayer sheet of PS−PIAT, which shrinks at a rate that is similar for the whole sheet, yielding the observed "onion type" vesicles.[24] Encapsulation of smaller, clustered vesicles on the electrode surface by a giant vesicle could explain the formation of the multicompartmental vesicles.

Conclusions

Our experiments have shown that electroformation can be used to prepare large quantities of stable, giant vesicles from PS−PIAT with sizes up to 100 μm. With other methods of aggregate formation such diameters are inaccessible. Despite their polymeric nature, the membranes of these giant vesicles are still fluidic, allowing microinjection techniques to be used to modify them. The thiophene groups inside the vesicles prepared with the injection method are polymerizable as we showed previously, opening the possibility to regulate the rigidity of the vesicle membrane, in that way freezing them in a desired morphology.[8]

Supporting Information Available: X-ray crystallographic files for IAT in CIF format. This information is available free of charge via the Internet at http://pubs.acs.org.

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