Spontaneously Formed Vesicles

Vesicles and Polymerized Vesicles from Thiophene-Containing Rod–Coil Block Copolymers**

Dennis M. Vriezema, Johan Hoogboom, Kelly Velonia, Ken Takazawa, Peter C. M. Christianen, Jan. C. Maan, Alan E. Rowan, and Roeland J. M. Nolte**

In recent years the self-assembly of block copolymers has received considerable attention, which has resulted in many exciting discoveries.[1] By careful selection of the type of blocks employed, the properties of these polymers can be readily tuned, making them potentially interesting systems from both an academic and industrial point of view. When a good solvent is chosen for only one of the blocks, aggregation will occur, thus leading to phase separation. A great number of parameters can be varied, which alter the aggregation architecture, namely, temperature, solvent, block length, ratio of one block to another, concentration, and pH.[2] Consequently, a wide variety of morphologies has been reported for this type of self-assembled macromolecules, for example, spheres, rods, lamellae, films, and patterned surfaces,[3] which have a considerable potential in a wide range of applications in the fields of optoelectronics and biomedicine.[4]

Rod–coil diblock copolymers, consisting of a flexible and a rigid block, constitute a special class of block copolymers.[2,3] Recently, we have shown that polyisocyanopeptides can be used as the rigid component in such systems.[5,6] These isocyanide polymers have a very high persistence length, which originates from the well-defined helical β-sheet arrangement of the polymer side chains.[7] Charged diblock copolymers of styrene and isocyano-L-alanyl-L-alanine, and of styrene and isocyano-L-alanyl-L-histidine have also been prepared and found to self-assemble in water to form a variety of structures such as vesicles, multilayers and even helical aggregates.[5]

Herein, we describe the aggregation behavior of a rod–coil diblock copolymer derived from an isocyanoamino acid containing a thiophene group. This diblock copolymer consists of 40 styrene and 50 3-(isocyano-L-alanyl-amino-ethyl)-thiophene (PS-PIAT) units and is an amphiphile in both organic and aqueous solvents because of only a slight difference in polarity between the two blocks (Figure 1a, b). The thiophene rings in PS-PIAT are present as functional groups that can be polymerized after aggregation,[8] to form stable polymerized morphologies with possibly interesting applications, for example, as conducting materials.

PS-PIAT was synthesized and characterized following procedures previously reported by us.[5] As a result of the amide functional groups in the PIAT side chains the thiophene groups are arranged in four stacks, which run parallel to the helical polymer backbone (Figure 1c). When PS-PIAT is dissolved in $CHCl_3$ it forms vesicles as was shown by transmission electron microscopy (TEM).[9] Upon drying, the spherical shape of the vesicles was not retained, as we concluded from the collapsed structures that were visible after platinum shadowing of the TEM grids (Figure 2a, inset I). Aggregation of the diblock copolymer molecules is induced by the poor solubility of the polyisocyanide block in $CHCl_3$. The formed vesicles have a high polydispersity, with diameters varying between 2 and 22 μm, with an average diameter of 7 μm, and an average membrane thickness, as determined from the TEM images, of 27 ± 5 nm, which is approximately twice the length of a single, stretched, PS-PIAT molecule. We propose that the vesicle membrane is composed of a bilayer, in which the polyisocyanide blocks are located in the center of the membrane and the polystyrene blocks are directed towards the solvent (Figure 2b). In separate experiments PS-PIAT was added to a dispersion of ($FeCl_3$) in $CHCl_3$ to polymerize the thiophene groups of the

[*] Dr. A. E. Rowan, Prof. Dr. R. J. M. Nolte, D. M. Vriezema, J. Hoogboom, Dr. K. Velonia
Department of Organic Chemistry
University of Nijmegen
Toernooiveld, 6525ED Nijmegen (The Netherlands)
Fax: (+31) 24-365-2929
E-mail: rowan@sci.kun.nl
nolte@sci.kun.nl
Dr. K. Takazawa, Dr. P. C. M. Christianen, Prof. Dr. J. C. Maan
High Field Magnet Laboratory
University of Nijmegen
Toernooiveld, 6525ED Nijmegen (The Netherlands)

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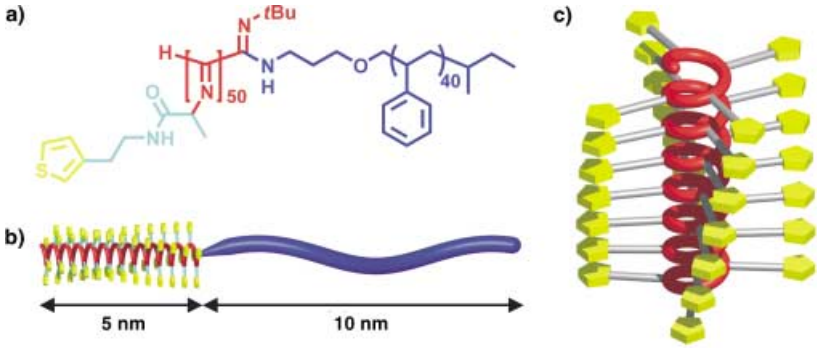

Figure 1. a) Chemical structure of PS-PIAT. b) Schematic representation of PS-PIAT. c) Schematic representation of the PIAT block showing the stacks of thiophene groups.

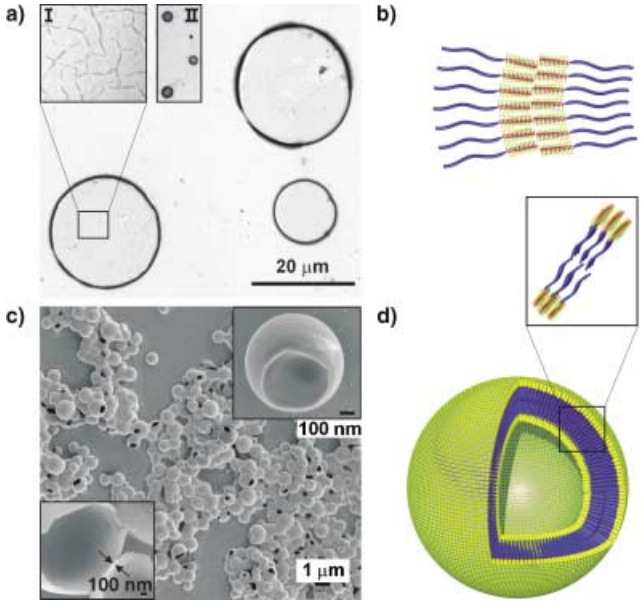

Figure 2. a) Transmission electron micrograph of PS-PIAT vesicles formed in $CHCl_3$ (concentration = 0.1 g L^{-1}) and dried on a carbon-coated copper grid (Pt shadowed). The inset I shows the middle section of the collapsed vesicles and inset II shows vesicles containing $FeCl_3$ (without Pt shadowing). b) Schematic representation of the vesicle wall in $CHCl_3$. c) Scanning electron micrograph of PS-PIAT vesicles formed in THF/water. The inset top right shows a close-up of a vesicle, and the inset bottom left shows the membrane thickness, indicated by arrows. d) Schematic representation of a PS-PIAT vesicle formed in water with a close-up of the vesicle membrane showing the proposed bilayer structure.

aggregates. However, no polymerization occurred and only smaller vesicles were formed, which contained entrapped metal complexes. TEM studies on these samples revealed that there was a high concentration of $FeCl_3$ located near or in the membrane of the vesicles (inset II in Figure 2a).

The aggregation behavior of PS-PIAT was subsequently studied in water by injecting a PS-PIAT solution in THF (0.5 g L^{-1}) into ultra-pure water, which gave a final water/THF ratio of 5:1 (v/v).[9] After the solution was allowed to equilibrate over two days, the morphology of the aggregates was examined by cryogenic scanning-electron microscopy (cryo-SEM) and SEM (Figure 2c). In both the SEM and cryo-SEM images spherical particles were visible. A number of these particles contained holes, which shows that they had a hollow interior. From this evidence, it was concluded that the aggregates were vesicular in nature. Inclusion experiments with the water-soluble fluorescence probe ethidium bromide, followed by size-exclusion chromatography, also indicated that the spherical particles were vesicles.[10] Fluorescence microscopy studies of these filled vesicles and of vesicles filled with methylene blue support the vesicular nature of the spheres. Their membranes had a thickness of 30 ± 10 nm (see bottom-left inset in Figure 2c), which corresponds to twice the length of a single PS-PIAT molecule. The most likely membrane structure of the vesicles in water is therefore a bilayer of PS-PIAT molecules in which the polystyrene blocks are pointed towards the center of the membrane and the polyisocyanide blocks towards the solvent (Figure 2d).[2]

The SEM images revealed that upon drying the formed PS-PIAT vesicles retained their shape. These vesicles have therefore a much higher stability than the vesicles formed in $CHCl_3$, which arises from the different constitutions of their vesicle membranes. In pure water, or when only a small amount of organic solvent is present, the polystyrene blocks are in their glassy state, and consequently, there is no reorganization after evaporation of the solvent water, so the vesicles preserve their shape. In $CHCl_3$ the polystyrene blocks are in direct contact with the solvent, which gives them a high degree of flexibility, allowing the vesicles to collapse when the $CHCl_3$ evaporates.

The vesicles in THF/water (1:5 v/v) were found to fuse when left to stand to yield particles that had increased in size by a factor of 20. Fusion of the vesicles of diblock copolymers has been reported,[11] but the increase in size of these aggregates was not as dramatic as shown here. Directly after preparation the average vesicle diameter was 80 nm (Figure 3a), but a few hours after preparation larger vesicles were seen amongst the vesicles that still had a diameter of 80 nm (Figure 3b). After 50 h only large vesicles, with an average diameter of 1.5 μm, were present (Figure 3c). The growth curve of the vesicles was determined by measuring the average vesicle diameter at several intervals of time after the initial injection into water, as observed by SEM images (Figure 3d). The driving force behind the fusion process is the release of strain in the initially formed vesicles, which have a high curvature and a large number of membrane defects. By fusing into larger vesicles, the curvature energy decreases, thus leading to a thermodynamically more stable state.[12] A factor that facilitates the fusion process is the presence of THF, which gives the PS-PIAT molecules the mobility to reorganize by solvation of the polystyrene blocks. Indeed, dialysis against ultra-pure water of the PS-PIAT vesicles prepared in THF/water directly after preparation, showed that the vesicles remained small, but many vesicles were seen that were in an intermediate stage of fusion (Figure 3e, f). Even after the vesicles were allowed to stand in pure water for

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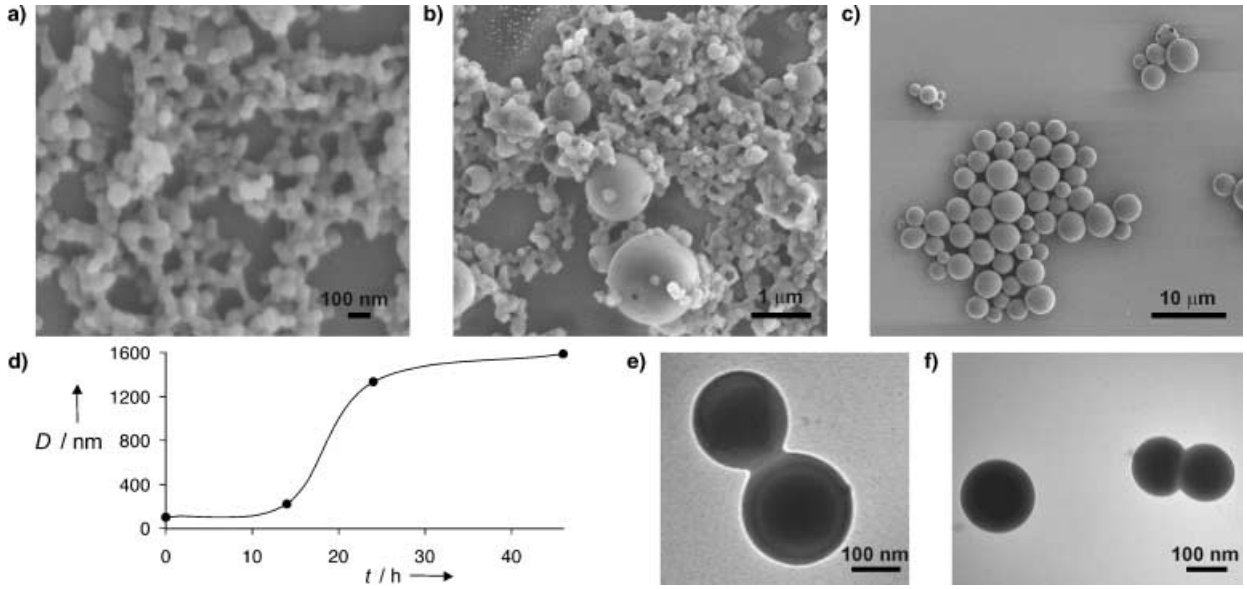

Figure 3. Fusion of PS-PIAT vesicles. a) Freshly prepared vesicles. b) Vesicles 19 h after preparation. c) Vesicles 50 h after preparation. d) Average vesicle diameter (D) as a function of time. e), and f) Intermediate stages of vesicle fusion as recorded in a dialyzed solution.

two weeks, their size remained unchanged. Therefore, THF is a necessary component for the vesicles to fuse.

Polymerization of the thiophene functional groups in the skin of PS-PIAT vesicles formed in water was investigated by electrochemical oxidation. Blank experiments with precursors of PIAT were carried out to estimate the oxidation potential necessary for thiophene polymerization inside vesicles; this potential amounted to 1.55 V (versus ferrocene/ferrocenium; Fc/Fc^+). Electrochemical polymerization was subsequently carried out by drying a drop of PS-PIAT vesicles in THF/water (1:5 v/v) on an indium-tin oxide (ITO) plate.[13] The results of SEM studies on the ITO plate after polymerization showed that the vesicles were very deformed because of intervesicular cross linking, and therefore the polymerization of the thiophene groups occurred readily, but not very selectively (Figure 4a).[14] To have more control during the polymerization, we tried chemical oxidation. Polymerization by use of $FeCl_3$ (see above), was not successful, but we found that the complex [bis(2,2′-bipyridine)ruthenium(II)-bis(pyrazolyl)] (BRP)[15] can act as a chemical oxidant.[16] In water, one of the pyrazolyl groups of BRP is protonated and the resulting species $[(bpy)_2Ru(pz)(pzH)]^+$ (BRP-H) has an oxidation potential of 1.60 V (versus Fc/Fc^+), thus making it suitable to polymerize the thiophene groups in PS-PIAT. Vesicles of PS-PIAT were prepared by injecting a 0.5 g L^{-1} solution of the diblock copolymer in THF into a solution of 2×10^{-7} M BRP in ultra-pure water at 70 °C. After the sample was allowed to stand for three days, SEM studies were carried out, the results of which showed that the vesicles were not deformed and had retained their original spherical shape (Figure 4b). The presence of polythiophene was investigated

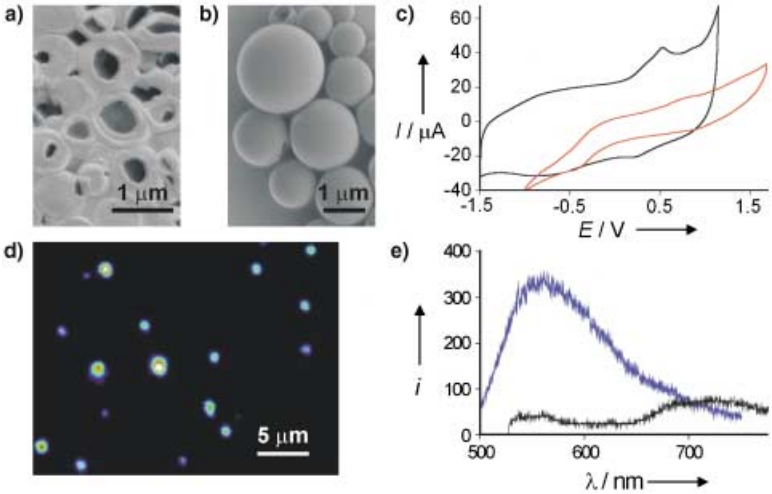

Figure 4. Polymerization of the thiophene groups of PS-PIAT vesicles. a) ITO plate containing PS-PIAT vesicles after electrochemical treatment. b) Vesicles polymerized by BRP-H. c) Cyclic voltammograms of unpolymerized vesicles (red), and of vesicles polymerized by BRP-H (black), scan-rate 250 mV s^{-1} (potential versus Fc/Fc^+). d) Fluorescence micrograph of polymerized vesicles excited with laser light at 460 nm. e) Fluorescence spectra of (excitation at 460 nm) a single unpolymerized vesicle (blue) and a single vesicle polymerized with BRP-H (black).

by electrochemical measurements on a sample of dried-in vesicles. This sample showed a new oxidation wave at 0.523 V (versus Fc/Fc^+), and a broad reduction wave at 0.23 V ($E^{1/2}=$ 0.38 V), indicative of polymerized thiophene groups (Figure 4c).[17] Infrared studies on the same sample revealed a vibration at 802 cm^{-1}, which also confirms the presence of polymerized thiophene groups.[18] To definitively prove the formation of polymerized vesicles, fluorescence experiments were carried out on single vesicles formed with and without BRP-H.[19] The polymerized vesicles are observed as fluorescent spheres upon excitation at 460 nm (Figure 4d). The emission spectrum of a single vesicle polymerized by use of

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the ruthenium complex displays a maximum at 725 nm (Figure 4e, black curve), which does not arise from the ruthenium complex, and is in agreement with the presence of polymerized thiophene groups.[17,20] Interestingly, a single vesicle without BRP-H has an emission spectrum with a maximum at 550 nm (Figure 4e, blue curve). We attribute the signal at 550 nm to the highly organized stacks of thiophene groups in PIAT (Figure 1c).

A potentially interesting application of the vesicles of PS-PIAT would be their use as microreactors. To this end, vesicles of PS-PIAT were loaded with *Candida antarctica* Lipase B (CAL B) enzymes.[21] When this sample was examined by TEM, dark and light vesicles were seen (Figure 5a). The same experiment was carried out with CAL B enzymes labeled with the fluorescent dye Alexa Fluor 488. Fluorescence microscopy experiments again showed that two types of vesicles were present: vesicles with, and without fluorescence (Figure 5b). From the results of these combined experiments, it was concluded that the dark vesicles in the TEM images contain entrapped enzymes and the light vesicles are empty. The activity of the entrapped enzymes was tested by adding the substrate 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU octanoate) to the mixture. This compound fluoresces when the DiFMU group is cleaved upon hydrolysis, which is catalyzed by the enzyme CAL B. After 30 min a significant increase in fluorescence intensity from the single vesicles could be detected, compared to the normal intensity arising from the auto-hydrolysis of the DiFMU octanoate (Figure 5c). This proves that the entrapped enzymes were still active and that the substrate can permeate through the vesicle membrane. Interestingly, the resulting more polar product does not permeate the vesicle.

In summary, we have synthesized new diblock copolymers that are uniquely able to form aggregates in both water and organic solvents. In THF/water (1:5 v/v) the initially formed vesicles fuse to form aggregates with a diameter 20× larger than that of the original vesicles. The thiophene groups located in the skin of the aggregates can be coupled to give polymerized vesicles. The vesicles are capable of including enzymes, thus resulting in catalytically active microreactors, which are permeable to substrate molecules. The combination of polymerizable vesicle-forming diblock copolymers with the inclusion of enzymes opens the possibility to create stable micrometer-sized reaction vessels. The presence of the thiophene functional groups in the diblock copolymers additionally enables the construction of conducting vesicles with potentially tunable properties.

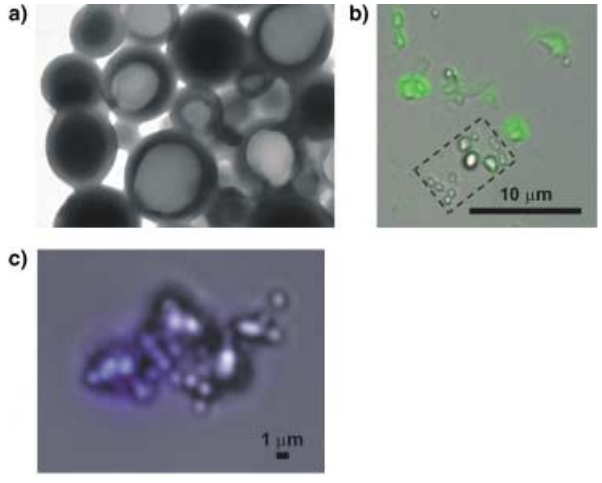

Figure 5. Inclusion of CAL B enzymes inside vesicles of PS-PIAT in THF/water. a) TEM micrograph of a dried drop of this mixture on a carbon-coated copper grid. b) Superposition of an optical micrograph and a fluorescence micrograph excited at 488 nm of the same region of the sample showing a distribution of both empty PS-PIAT vesicles and vesicles containing Alexa Fluor 488 labeled enzymes. Vesicles without enzymes are indicated by a rectangle. c) Fluorescent emission of product formed from DiFMU octanoate hydrolysis by CAL B entrapped in PS-PIAT vesicles.

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[8] Polymerized vesicles were prepared from low-molecular-weight surfactants, for a review see: H. Ringsdorf, B. Schlarb, J. Venzmer, *Angew. Chem.* **1988**, *100*, 117; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 113.
[9] During the preparation no extra energy, for example, from stirring or sonication, was put into the suspension.
[10] A PS-PIAT solution of 0.5 g L^{-1} in THF was injected into a solution of 10^{-4} M ethidium bromide in water, with a final THF/water ratio of 1:5 (v/v). This mixture was allowed to equilibrate over 50 h before purification by Sephadex gel chromatography. A fluorescence detector at 280 nm was used to monitor the fractions. The vesicles with entrapped ethidium bromide had a retention time of 3 h; the nonincluded dye had a retention time of 56 h.
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[13] An electrochemical cell, fitted with a $Ag/AgNO_3$ reference electrode, a platinum counter electrode, and an ITO plate as the working electrode, was used for the electrochemical polymerizations. Acetonitrile served as the solvent and tetrabutylammoniumhexafluorophosphate was added as the electrolyte. The polymerization was carried out under an inert atmosphere of nitrogen.
[14] In acetonitrile, in which the electrochemical experiments were conducted, the vesicles shrink, but do not collapse or fall apart.
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[16] We found that BRP-H is also a polymerization catalyst for other thiophenes, for example, 3-dodecyl-thiophene.
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[19] In the measurement setup an argon ion laser beam was focused by a 25× microscope objective to a 50 μm diameter spot on the sample. The fluorescence emission was imaged by a 50× objective on a CCD camera.
[20] No UV/Vis spectrum could be recorded from a single vesicle, nor from a suspension of vesicles, because of scattering problems.
[21] A 0.5 $g L^{-1}$ solution of PS-PIAT dissolved in THF was injected into a 30 $mg L^{-1}$ solution of CAL B enzymes; the final water/THF ratio was 12:1 (v/v). After the mixture was left for two days to equilibrate, it was dialyzed to dispose of all nonincluded enzymes.

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