Artificial Membranes with Selective Nanochannels for Protein Transport

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Poly(styrene-*b*-tertbutoxy styrene-*b*-styrene) copolymer was synthesized by anionic polymerization and hydrolyzed to poly(styrene-*b*-4-hydroxystyrene-*b*-styrene). Lamellar morphology was confirmed in the bulk after annealing. Membranes were fabricated by self-assembly of the hydrolyzed copolymer in solution, followed by water induced phase separation. A high density of pores of 4 to 5 nm diameter led to water permeance of 40 L m⁻² h⁻¹ bar⁻¹ and molecular weight cut-off around 8 kg mol⁻¹. The morphology was controlled by tuning the polymer concentration, evaporation time, and the addition of imidazole and pyridine to stabilize the terpolymer micelles in the casting solution via hydrogen bond complexes. Transmission electron microscopy of the membrane cross-sections confirmed the formation of channels with hydroxyl groups beneficial for hydrogen-bond forming sites. The morphology evolution was investigated by time-resolved Grazing Incidence Small Angle X-ray Scattering experiments. The membrane channels reject polyethylene glycol with a molecular size of 10 kg mol⁻¹, but are permeable to proteins, such as lysozyme (14.3 kg mol⁻¹) and cytochrome c (12.4 kg mol⁻¹), due to the right balance of hydrogen bond interactions along the channels, electrostatic attraction, as well as the right pore sizes. Our results demonstrate that artificial channels can be designed for protein transport via block copolymer self-assembly using classical methods of membranes preparation. text goes here.

Introduction

The preparation of artificial membranes with chemically tailored pores as bioinspired channels for protein separation and water transport has fascinated research groups worldwide.¹⁻⁹ The expectation is to be able to synthesize analogues to natural protein channels incorporated in polymer films with high stability and scaling-up feasibility. Previous works have been reported on synthesized artificial water channels^{1,2} using dendritic dipeptides,^{3,4} imidazole quartet,⁵ and pillar[5]arene molecules^{6,7}. These smart approaches mimicked water and/or proton transports through biological channels, which are mainly influenced by the combination of hydrogen bonds as well as narrow pore sizes formed by the self-assembly of the molecules. Extending the idea to scalable membranes is not an easy task. A simplified method based on block copolymers is introduced in this work.

Membrane preparation via the phase inversion method introduced by Loeb and Sourirajan¹⁰ has unlocked the fabrication of synthetic membranes with advanced functionality due to its simplicity and flexibility. A particular case was later proposed for the preparation of isoporous membranes with high porosity and sharp selectivity from block copolymers, using self-assembly and non-solvent induced phase separation method.^{11,12} In our group, we have investigated poly(styrene-*b*-4-vinylpyridine),¹²⁻¹⁵ poly(styrene-*b*-ethylene oxide),¹⁶ poly(styrene-*b*-acrylic acid),^{17,18} and polysulfone-based copolymers¹⁹ to fabricate self-assembled nanopores, and we revealed the structure formation mechanism using small angle X-ray scattering and advanced electron microscopy techniques.²⁰⁻²² The method has been explored also by other groups^{23,24} with strong potential for designing membranes with well-defined nanostructures and chemically-tailored pores. Here we explore the possibility of using an analogous strategy to synthesize artificial preferential channels.

Here we use a polystyrene (A) based copolymer with poly(4-hydroxystyrene) block (B) in an ABA architecture, as a model to design preferential nanochannels for protein transport via self-assembly. Poly(4-hydroxystyrene) (PHS) is known to exhibit strong hydrogen bonds with polymers containing hydroxyl, carbonyl, ether, and other compatible groups.²⁵⁻²⁷ Matshushita and co-workers demonstrated the formation of complex micro-phase separated structures of copolymer blends, such as three-phases hierarchical structures, due to the hydrogen bonding formation of PHS blocks with poly(2-vinylpyridine).²⁸⁻³⁰ Preparation of supramolecular ion gel due to hydrogen bonding interactions involving PHS was also investigated³¹. In addition, fabrication of nanoporous films from PHS-based diblock copolymer was demonstrated by the Ober group, by cleaving a sacrificial block, using UV irradiation, while preserving the photo-resistant cross-

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linked PHS.³²⁻³⁴ Nevertheless, the membrane formation from PHS-based copolymers, exploring their hydrogen bonding capability of the PHS for preferential channels, has not been investigated by any group.

Experimental

Materials and methods

Materials. Hydrochloric acid (HCl) (37%), 1,4-dioxane (DOX), pyridine (Pyr), imidazole, as well as polyethylene glycol (PEG), deuterated chloroform (CDCl₃, 99.6 %), bovine serum albumin (BSA), cytochrome *c* and lysozyme were purchased from Sigma-Aldrich. For the copolymer synthesis, tetrahydrofuran (THF) (99.9 %), *sec*-butyllithium (1.4M solution in cyclohexane), styrene (99 %), 4-*tert*-butoxystyrene (99 %), dibutyl-magnesium (1M solution in heptane) were also supplied by Sigma-Aldrich.

Synthesis of poly(styrene-*b*-*tert*-butoxy styrene-*b*-styrene) (PS-*b*-PTBOS-*b*-PS) triblock copolymer. The linear triblock copolymer PS-*b*-PTBOS-*b*-PS [PS: polystyrene, PTBOS: poly(4-*tert*-butoxystyrene)], was synthesized by sequential anionic polymerization using high vacuum techniques.³⁵ Tetrahydrofuran, THF, refluxed over sodium, stirred in the presence of CaH₂ and distilled over Na/K alloy. *sec*-Butyllithium (1.4M in cyclohexane) was diluted to appropriate concentration with purified hexane in a home-made glass apparatus under high vacuum. Styrene and 4-*tert*-butoxystyrene were purified by distillation subsequently over CaH₂ and dibutyl-magnesium (1M solution in heptane) and stored in pre-calibrated ampoules.

The synthetic procedure (Fig. 1) involves first the anionic polymerization of styrene (4g, -78 °C) in THF with 0.1 mmol of *sec*-Butyllithium.^{36, 37} After one hour, an aliquot was taken for SEC analysis, followed by the distillation into the glass reactor of 4g *tert*-butoxystyrene under vigorous stirring at -78 °C. One hour later, another aliquot was taken for SEC analysis followed by addition of 4g of styrene at -78 °C. Finally, after one more hour the polymerization quenched with methanol and the polymer precipitated in cool hexane. The final product was dried in a vacuum oven for 24h at 40 °C, to give a colorless powder.

The number average molecular weight (*M_n*) and the polydispersity index (PDI) were determined *via* size exclusion chromatography (SEC) equipped with an isocratic pump, Styragel HR2 and HR4 columns in series (300 x 8 mm), a refractive index detector and THF as the eluent at a flow rate of 1 mL/min at 30 °C. The calibration was performed using polystyrene standards (Mp: 370 to 4.220.000 g/mol). ¹H-NMR spectroscopy characterization was carried out using CDCl₃ on a Brücker AV-500 spectrometer.

The SEC traces along with the molecular characteristics of PS, PS-*b*-PTBOS and PS-*b*-PTBOS-*b*-PS are given in Fig. 2 and in Table 1 respectively, while in Figure 3 is depicted the ¹H-NMR spectrum of the linear triblock copolymer.

From the above results it is clear that the synthesized PS-*b*-PTBOS-*b*-PS triblock copolymer is well-defined with high homogeneity in molecular weight and composition.

Figure 1. Synthetic procedure for the preparation of the linear triblock copolymer PS-*b*-PTBOS-*b*-PS.

Figure 2. Size exclusion chromatographs of the homopolymer (PS), the diblock copolymer (PS-*b*-PTBOS) and the triblock copolymer (PS-*b*-PTBOS-*b*-PS).

Table 1. Molecular characteristics of the polystyrene (PS) block, the PS-*b*-PTBOS diblock copolymer and the PS-*b*-PTBOS-*b*-PS triblock copolymer.

Samples	$\bar{M}_n$ ^a (kg/mol)	PDI ^a	$f_{(PS)}$ ^b %(w/w)	$f_{(PTBOS)}$ ^b %(w/w)
PS	40	1.06	-	-
PS- <i>b</i> -PTBOS	80	1.04	0.52	0.48
PS- <i>b</i> -PTBOS- <i>b</i> -PS	120	1.10	0.69	0.31

^a SEC in THF at 30 °C, ^b ¹H-NMR in CDCl₃

Figure 3. ¹H-NMR spectrum of the linear triblock copolymer PS-*b*-PTBOS-*b*-PS before (bottom) and after (top) hydrolysis.

PS-PtBOS-PS hydrolysis to poly(styrene-*b*-hydroxystyrene-*b*-styrene) (PS-*b*-PHS-*b*-PS). PS_{40k}-*b*-PtBOS_{40k}-*b*-PS_{40k} terpolymer was hydrolysed to PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} in 5 wt% solutions in dioxane, under nitrogen atmosphere at 60 °C using 5-fold excess of hydrochloric acid (HCl) over the *tert*-butoxy groups. After 24 h of hydrolysis, the polymer was precipitated and washed in a large amount of water for at least three times until the pH was neutral. The polymer was then washed with methanol and dried in vacuum at 60 °C overnight. The hydrolyzed terpolymer was analyzed by ¹H NMR (Bruker AV-400) and Fourier Transform Infra Red (FTIR) spectroscopy (Thermo Nicolet iS10).

Membrane fabrication. 11 - 18 wt% PS-*b*-PHS-*b*-PS terpolymer was dissolved in dioxane. Imidazole and pyridine were used as additive in some of the solutions. After stirring overnight, the solution was sonicated for at least 15 minutes and left unstirred for one hour to further remove bubbles. The polymer solution was then cast using a doctor blade with 200 μm gap. After the solvent was evaporated for 5-20 s, the film was plunged into a water bath, in which the phase inversion occurred and the membrane was formed.

Small Angle X-ray Scattering (SAXS) and Grazing-Incident SAXS (GISAXS). SAXS measurements were performed at the SAXS1 beam line of the Brazilian Synchrotron facility (LNLS), which has a fixed energy of 8.0 keV and X-ray wavelength of 1.55 Å. A PILATUS detector with 172 μm² pixel size was used and the sample-to-detector distance was 3.057 m. Each measurement was obtained with 20 frames, 30 s exposure time and a beam area of 1 mm². Profiles of intensity versus scattering vector (*q*) were obtained as the average of the azimuthally integrated 2D scattering patterns. They were corrected by taking in account the primary beam intensity and the background scattering was subtracted.

GISAXS experiments were performed at the beam line D1 of the Cornell High Energy Synchrotron Source (CHESS) with a beam energy of 10.6 keV and X-ray wavelength of 1.162 Å. In this case two kinds of samples were observed: (1) dry spin-coated thin films and (2) in situ cast solutions of the terpolymer in a mixture of solvents. For the time-resolved GISAXS, an in situ doctor blading system³⁸ was used to cast films with a thickness of 200 μm using a casting speed of 1500 μm/s as the beam was immediately exposed to the film surface with an incident angle of 0.13°. A proper amount of the copolymer solution was first dropped using a pipette onto a glass substrate, fixed on the sample goniometer with a sample-to-detector distance of 1.82 m. The GISAXS measurements were triggered immediately after the film was cast, using an automatic system. The 2D scattering patterns were acquired every 10 s, with a 1.5 s exposure time. A MedOptics CCD camera of (1024)² pixels with pixel size of (46.9 μm)² was used as X-ray detector. The scattered intensity for horizontal the projections of the 2D patterns were obtained using Igor Pro 6.37 software³⁹. The positions of the peaks were obtained with the same software by fitting the data with a sum of Lorentz functions. The characteristic domain spacing (*d*-spacing) was calculated as $2\pi/q^*$, where *q*^{*} corresponds to the maximum of first Bragg reflection.

Scanning Electron Microscopy (SEM). SEM images of the membranes were obtained using FEI Novanano microscope with 5 kV accelerating voltage and 3-mm working distances. The wet membranes were frozen at -20 °C and freeze-dried. The membranes were mounted on an aluminum stub using aluminum tape and

sputter coated with iridium (3-nm thickness) before imaging. The imaged membrane pore sizes were analyzed using ImageJ software.

Transmission Electron Microscopy (TEM). TEM images of PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} dense films, membranes, and micelles from diluted solutions were obtained using a Tecnai 12 (FEI company) microscope, operating at 120 kV. The dense film was cast on a Teflon plate from a 5 wt% polymer solution. The solvent was slowly evaporated for 4 days at room temperature, followed by annealing at 120 °C for 5 days. Once annealed, the film was quenched in liquid nitrogen and kept at room temperature.

The PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} membranes and dense films were first stained with ruthenium tetroxide (RuO₄) for 2 hours before embedding in epoxy resin and cured at 60 °C for 24 h. Ultrathin sections (80 nm thick) of the embedded films/membranes were cut with diamond knife in an ultra microtome (Leica EM UC6). The thin slices were placed on a copper grid and further stained with RuO₄ for another 2 hours prior to imaging by TEM.

For micelles characterization, 0.5 to 5 mg/mL PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} were dissolved in dioxane and stirred overnight. An appropriate amount of imidazole and pyridine was added into the solution. A drop of the solution was placed onto a carbon-coated copper grid, and the solvent was then blotted by a filter paper, placed underneath the copper grid. The solvent was completely evaporated at room temperature before TEM imaging.

Atomic Force Microscopy (AFM). AFM images of the PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} thin films were obtained using Agilent 5500 AFM in tapping mode, controlled by PicoView 1.8 software. The thin films were prepared using 5 wt% PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} solution in dioxane, spin coated on a silicon wafer with a rotation speed of 1600 rpm for 60 s. For the AFM measurement, silicon cantilevers with a resonance frequency of 76–263 kHz and force constant of 1.2–29 Nm⁻¹ were used, and the acquired images were post-processed using Gwyddion software.

Dynamic Light Scattering (DLS). DLS measurements were carried out using Malvern Zetasizer for the analysis of the terpolymer micelles hydrodynamic radii in dilute solution, as well as the membrane surface zeta potential. For the size measurements, the sample was loaded using a glass cuvette. A backscattering measurement angle of 173° was used, repeating the measurement three times for each sample, with 12–15 runs per measurement. The dilute PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} solution was prepared by dissolving 0.5 mg/mL copolymer in dioxane, stirred overnight. The solutions were passed through a 0.2 µm syringe filter, before loading.

The apparent PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} membrane zeta potential was measured using electrophoresis and electro-osmosis techniques, as described elsewhere.⁴⁰ Standard polystyrene latex, as well as other commercial materials were used as tracer particles.⁴⁰ The solution pH was adjusted using 1 M HCl and 1 M NaOH. The membrane zeta potential was evaluated by measuring the tracer particles zeta potentials at different distances from the membrane surface. A linear change with distance is observed and the value at distance equal to zero is extrapolated.

Rheology. The solution rheology was investigated at 25 °C on a AR 1500ex rheometer, using a cone and plate measurement system with 20 mm diameter and 1° cone angle. The range of shear rates

for the viscosity measurements was 0.1–1000 s⁻¹ and the oscillation strain for frequency sweep measurements was fixed at 10%. The samples compositions were similar to the cast solutions used for membrane preparation.

Membrane filtration test. Membrane performance tests were performed at 2 bar using a dead-end Amicon cell for ultrafiltration with an effective membrane area of 4.1 cm². Non-woven polyester supports were placed underneath the PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} membranes to ensure their mechanical stability during filtration. Milli-Q water was used as feed for water permeance measurement. The water flux was measured at different pressures, varying from 0.5 to 3 bar. Water flux (Q) and permeance (J_w) were calculated using Equation 1 and 2 as follows.

$$Q = \frac{V}{A \cdot t} \quad (1) \quad J_w = \frac{Q}{\Delta P} \quad (2)$$

where V is the amount of water collected during the filtration time (t), A is the effective membrane area, and ΔP is the transmembrane pressure. The molecular weight cut-off (MWCO) was measured by filtering an aqueous solution of 1 g/L polyethylene glycol (PEG) with different molecular weights (35 kg mol⁻¹, 10 kg mol⁻¹, 6 kg mol⁻¹, 3 kg mol⁻¹, and 0.6 kg mol⁻¹). The PEG compositions in the feed (c_f) and permeate (c_p) were measured using gel permeation chromatography Agilent 1200 with two columns in series and refractive index detector. Rejections (R) were calculated with the Equation 3 and the MWCO was determined as the minimum molecular weight with 90% rejection.

$$R = \left(1 - \frac{c_p}{c_f}\right) \times 100\% \quad (3)$$

Bovine serum albumin (BSA) and lysozyme (Lys) rejections were tested using 1 mg/mL solution of BSA and Lys separately in phosphate buffered saline (PBS) solution at physiological pH of 7.4. Protein concentrations at the feed and permeate were measured using NanoDrop^{TM} 2000/2000c spectrophotometer (Thermo Fisher Scientific).

Results and Discussion

Polymer Synthesis and Hydrolysis. The completion of the PS_{40k}-*b*-PtBOS_{40k}-*b*-PS_{40k} hydrolysis to PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} was confirmed by the disappearance of the *tert*-butoxy groups and appearance of phenol groups in the ¹H NMR spectrum (Fig. 3). The hydrolysis yield was nearly 100%. A broad O-H band appears in FTIR (not shown here) between 3500–3200 cm⁻¹, corresponding to the phenolic groups in the hydrolyzed polymer.

Morphology of dense films. The morphology of the self-assembled terpolymers was analyzed in the bulk of annealed dense films and in as-cast thin films. SAXS analysis (Fig. 4a) of the PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} bulk film cast from dioxane and thermal annealed at 120 °C film showed peaks at $q = 0.13, 0.25, 0.38, 0.49, 0.63, 0.76, 0.88 \text{ nm}^{-1}$. The ratio of the peak position is 1:2:3:4:5:6:7, which corresponds to lamellar morphology⁴¹. The d-spacing calculated from the first peak was 49.9 nm, which is close to the distances between the centers (41 ± 1 nm) of the repeating micro-domains measured in the TEM image (Fig. 4b).

The TEM image of the annealed film showed a three-phases lamellar structure formed by the polystyrene (PS) and poly(4-hydroxystyrene) (PHS) blocks. Both PS and PHS were stained by ruthenium, but since the hydroxyl groups are more reactive⁴², the PHS domains are darker than the PS. Three different alternating domain lengths are seen, i.e. dark thin lines formed by PHS, separating narrow from wider gray lamellae of PS. Under the equilibrium state, both enthalpy and entropic parameters influence the terpolymer self-assembly. The hydrolyzed terpolymer (PS_{40k}-*b*-PHS_{23.7k}-*b*-PS_{40k}) has a middle block shorter than that of PS blocks due to the disappearance of *tert*-butyl groups from its precursor (PS_{40k}-*b*-PtBOS_{40k}-*b*-PS_{40k}). Polymer-polymer interaction and block lengths ratio are the parameters guiding for the formation of the lamellar structure in the bulk of annealed dense films, which practically corresponds to the equilibrium morphology. The main differences in solubility parameter between the PS and PHS blocks result from polarity and capacity of hydrogen bond formation. The Flory-Huggins interaction parameter is $\chi_{PS/PHS} = 0.124$ ⁴³. The morphology of symmetric ABA triblock copolymers has been examined by self-consistent field theory.^{44, 45} The factors affecting the morphology are similar for ABA triblock and AB diblock copolymers in many aspects, but there are additional considerations. The middle block can assume conformations as bridge or loops. Looped middle blocks themselves can adopt a preferential hairpin or train conformation, with the later meaning segments in very close proximity to the interphase. Hydroxyl-hydroxyl hydrogen bonding between the PHS blocks might also affect the formation of lamellar channel-like domains in a train conformation, explaining the dark lines in the TEM image in Fig. 4b. The wide lamella is constituted by the polystyrene blocks, while the narrow lamella is probably mostly contains looped hydroxystyrene, organized in a mixture of hairpin and bridged conformations.

Fig. 4d shows an AFM image of the PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} thin film spin-coated on a silicon wafer from a solution in dioxane. The film topography is characterized by a highly ordered lamellar structure. The GISAXS analysis of the film (Fig. 4c) confirmed the lamellar structure by the appearance of three peaks at q/q^* of 1, 2, and 3 with d -spacing of 41 nm. This value is very close to the distances between the centers of domains (44 ± 4 nm) imaged by AFM (Fig. 1d) and to distances imaged by TEM for the bulk of annealed samples. However the morphology investigated by GISAXS and AFM is expected to be influenced by the solvent dioxane. From values of solubility parameters in Table 2, using Equation 4, it is possible to estimate the Flory-Huggins interaction parameters between each polymer block and the solvent (χ_{si}). When multiplied by the polymerization degree of the blocks (N_i), the polymer-solvent repulsion can be estimated, being lower for PHS ($\chi_{sPHS}N_{PHS} = 177$) than for PS ($\chi_{sPS}N_{PS} = 323$). This is an indication that in solution the middle PHS blocks looped to form the corona, while the PS blocks aggregated into the micelle core, forming a flower-like arrangement.

$$\chi_{si} = \frac{VA_{si}}{RT} \quad (4)$$

where R and T are gas constant (8.314 J/mol.K) and temperature (K), respectively; V is the solvent molar volume; and A_{si} is the Hansen solubility parameter difference between for the solvent (s) and the polymer (i) calculated from Equation 5 involving the dispersion (δ_D), polar (δ_P), and hydrogen bond (δ_H) terms.

$$A_{si} = (\delta_{Ds} - \delta_{Di})^2 + 0.25(\delta_{Ps} - \delta_{Pi})^2 + 0.25(\delta_{Hs} - \delta_{Hi})^2 \quad (5)$$

Figure 4. (a) SAXS curve and (b) TEM image of PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} annealed film, stained with RuO₄; (c) GISAXS curves of the horizontal projection planes and the 2D-pattern (inset) and (d) AFM image of thin films spin coated on Si wafers from dioxane

Table 2. Hansen solubility parameters of the polymers and the solvents⁴⁶

Component	$\delta_D^{0.5}$ (MPa ^{0.5})	$\delta_P^{0.5}$ (MPa ^{0.5})	$\delta_H^{0.5}$ (MPa ^{0.5})	δ $= \sqrt{\delta_D^2 + \delta_P^2 + \delta_H^2}$
PS	18.5	4.5	2.9	19.3
PHS	17.6	10.0	13.7	24.5
Dioxane	17.5	1.8	9.0	19.8
Pyridine	19.0	8.8	5.9	21.8

We performed time-resolved GISAXS experiments of the PS_{40k}-b-PHS_{27.3k}-b-PS_{40k} film casting. The measurement followed the morphological changes on the film surface cast from the terpolymer solution as the solvent evaporated. Fig. 5a and 5b show the evolution of GISAXS 2D-patterns and the scattering curves, respectively, of the PS_{40k}-b-PHS_{27.3k}-b-PS_{40k} film cast from 18 wt% terpolymer in dioxane. As the evaporation evolved, two peaks corresponding to lamellar order ($q/q^* = 1$ and 2) were observed with d-spacing of around 44 nm, similar to the distances between domains measured from the AFM topography image of the spin-coated film. The d-spacing plot versus time (Fig. 5c) shows that the development of the structure occurred only at the beginning of the

casting, while the structure remained the same at higher evaporation times, with constant d-spacing. This result suggested that the lamellar structure could be hindered if the morphology evolution could be interrupted after short evaporation time.

Since the PS_{40k}-b-PHS_{27.3k}-b-PS_{40k} terpolymer form lamellar structures, they most frequently tend to align parallel to the surface. Membrane fabrication with vertically aligned channels from this terpolymer is challenging. Previous works of our group^{12, 47, 48} and others demonstrated the pore formation in membranes with additives in solution, which promote lamellar or hexagonal spherical morphologies by controlled hydrogen bond. The equilibrium morphology of the PS_{40k}-b-PHS_{27.3k}-b-PS_{40k} terpolymer has interesting domains containing O-H functional groups. We expected to use the OH groups in this copolymer to induce the formation of preferential channels. Their self-assemblies in equilibrium lead to a well-defined phase, seen in Fig. 4b. The challenge is to assemble channels aligned in a suitable path for selective transport.

Figure 5. Time-resolved GISAXS (a) 2D-patterns, (b) in-plane curves, and (c) d-spacing versus evaporation time for thin films cast from 18 wt% PS_{40k}-b-PHS_{27.3k}-b-PS_{40k} in dioxane.

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Fig. 6a and 6b show SEM images of the membranes prepared by casting 11 wt% and 18 wt% $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ copolymer solutions in dioxane, respectively, with 5 s evaporation time before immersion in water. Membranes cast from 18 wt% solutions exhibited some opened pores with a lamellar structure dominating the surface, whilst 11 wt% solutions led to membranes with more open pores and better ordered structure. The pores were practically monodisperse with average diameter 9 ± 2 nm, estimated from the SEM image. At lower concentration the inter-micelle interaction should be less pronounced, and the tendency of the micelles to form lamellar structure decreases.

Our next aim was to further reduce the membrane pore sizes, leading to more selective channels, with high density of open pores on the surface. One of the strategies was to make the membrane surface more densely packed with micelles. In this case, the micelle deformation needs to be hindered to avoid merging into lamellar morphology or worm-like structures. The stabilization of analogous membranes based on $\text{PS-}b\text{-P4VP}$ was previously promoted by our group, by complexing pyridine groups with copper salts¹² or by adding hydrogen bonding additives to the casting solution.⁴⁷ Hydrogen bonding molecules were previously proposed by Ikkala, ten Brinke, and co-workers, to induce self-assembly.⁴⁹⁻⁵² In this study, we use imidazole and pyridine to stabilize micelles, by

forming hydrogen bond with the hydroxyl groups of PHS. Phenol is known to form strong hydrogen bond with Lewis bases.⁵³⁻⁵⁵ Hydrogen bonds between polymers containing hydroxystyrene and pyridine or imidazole have been previously confirmed by spectroscopic and calorimetric methods, as well as by observing various morphologies affected by such interactions^{36, 56-59}.

The surface of $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ membranes, cast from 11 wt% copolymer solutions in 1,4-dioxane, using imidazole as additive, are shown in Fig. 7a. The addition of imidazole led to the formation of membranes with ordered pores with diameters of 4 ± 2 nm, with evaporation time of 5 s. When the evaporation time was 20 s, the membrane surface were more densely packed with micelles, more ordered, and even smaller pore could be formed. The pore sizes were significantly reduced by nearly 50%, compared to the pristine membrane.

When imidazole was used in copolymer solutions of increasing concentration, a clear trend was observed in Fig. 7. At a higher polymer concentration, instead of stabilizing single micelles, strong inter-micelle interactions predominated. This led to the formation of bigger aggregates. Membranes with larger pores were obtained from 13 wt% copolymer solutions. Lamellar structures were formed from 15 wt% copolymer solutions, similar to the equilibrium morphology in the bulk, as in Fig. 4a.

Figure 6. SEM images of membranes cast from (a) 11 and (b) 18 wt % $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ solutions in dioxane, with 5 s evaporation time.

Figure 7. SEM images of membranes cast from (a) 11, (b) 13 and (c) 15 wt% $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ solutions in dioxane with imidazole (1:1 imidazole to hydroxystyrene molar ratio), with 5 s evaporation time.

Figure 8. TEM (a-e) and SEM (f) images of $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ membranes cross-sections from different positions: (a, d, e) pristine, (b) 1:1 imidazole, (c) 1:1 pyridine.

Figure 9. TEM images of $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ micelles prepared from (a) dilute solutions in dioxane and (b) the corresponding size distribution obtained by DLS. Micelles at higher concentration (5 mg/mL): (c) pristine copolymer (d) 1:1 imidazole and (e) 1:1 pyridine.

Analogous to imidazole, pyridine was used to induce hydrogen bonds with the hydroxystyrene groups for micelle stabilization in 11 wt% $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ solutions in dioxane. First 1:1 molar ratio with respect to hydroxystyrene was used, the same as for imidazole. A highly ordered membrane structure resulted with nanopores of 3 ± 1 nm. Pyridine ($\text{pK}_a = 5.2$) is less basic than imidazole ($\text{pK}_a = 7.1$). Imidazole has 2 basic nitrogen atoms, while pyridine has just one. Imidazole is more prone to promote stronger interactions with hydroxyl groups, as well as to promote intermicelle interactions, while the interaction with pyridine is softer and more active in stabilizing single micelles instead of linking them into arrays and lamellae. With higher concentration of pyridine, close to a 5:1 molar ratio, with respect to hydroxystyrene, a sharp pore size distribution was obtained with an average pore size of 5 ± 1 nm. At this ratio, pyridine can be considered more as a co-solvent. We recently reported that isoporous copolymer membranes can be prepared by choosing a mixture of solvents, which fits a trend line estimated by taking into consideration Flory-Huggins parameters and block volume ratios.⁶⁰ For the $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ terpolymer investigated in this study, a mixture of 9:1 dioxane to pyridine (5:1 pyridine to hydroxystyrene, 11 wt % copolymer) is suitable to provide the required segregation strengths to fit the trend line.

TEM images of $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ membrane cross sections showed vertically aligned channels (Fig. 8). The darker domains corresponded to the phenolic groups, since hydroxyl groups react more with ruthenium tetroxide⁴² than styrene alone. The middle structure of the membranes (Fig. 8d) has continuous channels, whereas the bottom surface of the membrane seems to be more opened with string-like micelles (Fig. 8e). The TEM results confirm the formation of vertically-aligned nanochannels having OH-functional groups, which can be useful for the transport of water and/or other hydrogen bond interacting molecules.

The self-assembly of block copolymers in dilute solution was studied using TEM and DLS as shown in Fig. 9. The $\text{PS}_{40\text{k}}\text{-}b\text{-PHS}_{27.3\text{k}}\text{-}b\text{-PS}_{40\text{k}}$ copolymers formed monodispersed spherical micelles ($\text{PDI} < 0.2$) in dioxane at lower polymer concentration of 0.5 mg/mL. The hydrodynamic radii were 21.7 nm, close to the micelle sizes measured from the TEM image (28 ± 1 nm). As the copolymer content was increased ten times, the micelles from solution in dioxane without additives merged into worm-like aggregates as shown in Fig. 9c, whereas the micelles complexed with imidazole and pyridine forming aggregates of more stable spherical micelles. Imidazole and pyridine' interaction with the phenol group can be seen as acid-base as in the case of $\text{PS-}b\text{-P4VP/PS-}b\text{-PAA}$ blends.¹⁸

Rheology investigation of the $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ casting solution showed that the addition of imidazole and pyridine (1:1 mol ratio with respect to hydroxystyrene) had higher viscosity, compared to the pristine solution (Fig. 10a). This is a result of the hydrogen bonds promoted by imidazole and pyridine solutions. The storage and loss modulus curves for solutions with and without additives further demonstrate the effect of the hydrogen bonds (Fig. 10b). Solutions with imidazole or pyridine have higher modulus than the pristine solution with the same polymer concentration (11 wt%). The viscosity of the 18 wt% solutions (Fig. 10a) was higher than that with imidazole at low shear rates, due to more entanglement, but it became almost equal to those prepared with 11 wt% containing imidazole at high shear rates. At high shear rates the interconnectivity between micelles is less pronounced.

Fig. 10b shows that the 18 wt% solution in dioxane has a soft gel behavior, with almost equal values of storage (G') and loss modulus (G''), while for the 11 wt% solutions imidazole or pyridine, the loss modulus exceeds the storage modulus (G'), indicating a liquid-like profile, with the dominance of the viscous nature over the elastic component. For ordered block copolymer systems, morphology can be correlated to the modulus dependence on frequency dependence. For lamellar microdomain structures, it was found experimentally that at low frequencies $G' \sim \omega^n$, with ω being the angular frequency and $n = \frac{1}{2}$ in this case.⁶¹⁻⁶⁴ For cylindrical domains, the exponent values were reported as $n = 1/3 \sim 1/6$.⁶⁴⁻⁶⁶ For the systems investigated here, the 18 wt% solution in dioxane has the behavior of an elastic gel with G' almost independent of frequency, while the 11 wt% pristine solution is similar to viscoelastic liquids. At low frequency⁶⁷, $G' \sim \omega^2$ and $G'' \sim \omega^1$. For the 11 wt% solutions with added imidazole and pyridine, the response is intermediate between that of a gel and that of a liquid, but the exponent n is still higher than that found for ordered structures ($n =$

0.77 with imidazole and $n=1$ with pyridine). These observations suggest that the inter-micellar interactions are reduced for the solutions and the assembly and merging into lamellar are disfavored. When comparing pyridine and imidazole, the latter leads to higher storage and loss values and to higher viscosity, due to stronger basic character.

Membrane permeance and rejection

Fig. 11a shows the pure water permeance and molecular weight cut-off (MWCO) of the $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ membranes. The use of additives enhanced the separation performance of the membranes without sacrificing the permeance. The membranes were stable at the testing pressures between 0.5-3 bar, confirmed by a linear increase of water flux as pressure increases (Fig. 11c). For the membranes prepared with imidazole and 5 s evaporation, the pure water permeance increased more than twice, compared to that of pristine membrane. The selectivity was improved, showed by the decrease in the MWCO. The MWCO was even lower for membranes with imidazole prepared with 20 s evaporation as well as for the one with 1:1 mol pyridine to hydroxystyrene, due to the pore size reduction, keeping the permeance still similar to that of the pristine membrane. Membranes prepared with 5:1 pyridine to hydroxystyrenes had MWCO and water permeance similar to those prepare from solutions with 1:1 imidazole to hydroxystyrene and 5s evaporation. The high water flux of these two membranes can also be first associated with the hydrophilicity of the membranes with contact angles lower than that of other membranes (Table 3) measured by Krüss EasyDrop equipment. All the SHS-membranes in general exhibited hydrophilic surfaces with contact angles of approximately 60 to 80 ° due to the presence of OH groups, whilst the original $\text{PS-}b\text{-PtBOS-}b\text{-PS}$ membranes, before the hydrolysis, had a more hydrophobic surface with a contact angle larger than 90 °.

Figure 10. Rheology of $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ solutions in dioxane: (a) viscosity as a function of shear rate and (b) storage (G' , open symbols) and loss moduli (G'' , filled symbols) as a function of angular frequency. Comparison between pristine solutions (11 and 18 wt%) and those with 1:1 imidazole to hydroxystyrene and 1:1 pyridine to hydroxystyrene additives.

Table 3. Membranes contact angles

Membranes	Contact angle (°)
PS _{40k} - <i>b</i> -PtBOS _{40k} - <i>b</i> -PS _{40k} evaporation	93.5 ± 0.7
PS _{40k} - <i>b</i> -PHS _{27.3k} - <i>b</i> -PS _{40k} evaporation	78.2 ± 0.5
PS _{40k} - <i>b</i> -PHS _{27.3k} - <i>b</i> -PS _{40k} evaporation 1:1 Imidazole to hydroxystyrene	65.5 ± 1.3
PS _{40k} - <i>b</i> -PHS _{27.3k} - <i>b</i> -PS _{40k} evaporation 1:1 Imidazole to hydroxystyrene	78 ± 0.6
PS _{40k} - <i>b</i> -PHS _{27.3k} - <i>b</i> -PS _{40k} evaporation 1:1 Pyridine to hydroxystyrene	74.4 ± 1.3
PS _{40k} - <i>b</i> -PHS _{27.3k} - <i>b</i> -PS _{40k} evaporation 5:1 Pyridine to hydroxystyrene	59.4 ± 0.6

The membranes with higher water permeance (1:1 imidazole and 5:1 pyridine) had also the lowest contact angle, close to 60°. They were then tested for protein rejections using bovine serum albumin (BSA, 66.5 kg mol⁻¹) and lysozyme (14.3 kg mol⁻¹). The membranes obviously rejected BSA due to the high molecular weight, but lysozyme permeated through the 1:1 imidazole and 5:1 pyridine membranes with a rejection of about 25% and 11%, respectively (Fig. 11c). When tested with neutral 10 kDa polyethylene glycol (PEG) molecules the rejection was 100 %, consistent with the MWCO value of 7.8 kg mol⁻¹ reported in Fig. 11a. This result shows that the membrane nanochannels were preferential for lysozyme, possibly due to the following reasons.

First we estimate the Stokes radius for PEG,⁶⁸ using Equation 6, which combines the Stokes-Einstein equation⁶⁹ and empirical equations relating intrinsic viscosities and molecular weight:

$$a = 16.73 \cdot 10^{-10} \text{ M}^{0.557} \quad (6)$$

where a is the Stokes radius (cm) and M is the molecular weight (g mole⁻¹).

The Stokes radii for PEG are listed in Table 4. The values for lysozyme and cytochrome *c* were taken from previous reports^{70, 71}.

Table 4. Stokes radii and rejection of PEG and proteins

Solutes	Radius (nm)	Rejection	
		1:1 Imidazole	5:1 Pyridine
PEG 10,000	2.8	97 ± 0.3%	98 ± 2%
PEG 6,000	2.1	76 ± 4%	79 ± 0.3%
PEG 3,000	1.5	42 ± 9%	47 ± 0.4%
Lysozyme	1.9	25 ± 17%	11 ± 3%
Cytochrome <i>c</i>	1.6	32 ± 14%	33 ± 10%

According to Table 4, PEG 10,000 has a Stokes radius 1.5-fold larger than lysozyme. Lysozyme can permeate much better than PEG 10,000 with a 4-fold lower rejection. Other PEG molecules with radii closer to or even lower than lysozyme (PEG 6,000 and 3,000) also have high rejection. This indicates that the transport of lysozyme is helped by other factors besides size exclusion, such as the hydrogen bond interactions along the channels. The same phenomena were also observed for cytochrome *c*. Lysozyme is expected to have hydrogen bond interaction with the OH groups of PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k}, aligned along the channels wall, as well as with imidazole and pyridine molecules that can still remain in the membranes due to the strong H-bond complexes with the OH groups. The rejection of lysozyme is preferentially low in the presence of pyridine. Interaction in the form of hydrogen bond could facilitate the transport. Too strong interaction could however favor a strong binding and disfavor mobility. A final factor influencing the transport is the electrostatic character of membrane and solutes. The membranes are negatively charged at the measured pH, confirmed by zeta potential analysis (Fig. 11d). The isoelectric points of lysozyme^{71, 72} and cytochrome *c*⁷³ are 11.35 and 9.59. Therefore both proteins are positive at the experiments conditions. PEG is considered rather neutral molecule. In summary, the right pore sizes of the membranes of 4-5 nm enabled the lysozyme to pass through, while rejecting other molecule with comparable sizes but having less hydrogen bond and electrostatic interaction with the channels. The same effect was also observed for cytochrome *c* which permeated through the membranes with a low rejection of around 30%. Although PEG was reported to have hydrogen bonding interaction with PHS,^{74, 75} the 10kg mol⁻¹ PEG is large enough to be rejected by the channels. This is possibly because PEG has only ether as hydrogen bond forming site, which has to compete with the hydroxyl-hydroxyl interaction of the copolymer blocks, whilst lysozyme and cytochrome *c* have more hydrogen bond forming sites, which allow them to pass through the channels.

Figure 11. (a) Water permeance and molecular weight cut-off (MWCO) of $\text{PS}_{40k}\text{-}b\text{-PHS}_{27.3k}\text{-}b\text{-PS}_{40k}$ membranes prepared with different additives and evaporation times; (b) Rejection of BSA, lysozyme, cytochrome c and 10 kg mol^{-1} PEG; (c) Water flux at varying pressures of membranes with 1:1 imidazole to hydroxystyrene and 5:1 pyridine to hydroxystyrene; (d) zeta potential of pristine membranes at different pH.

Conclusions

Flat-sheet membranes with preferential nanochannels of 4-5 nm diameter were fabricated, using a combination of self-assembly and non-solvent induced phase separation, in the presence of imidazole and pyridine. PS_{40k}-*b*-PHS_{27.3k}-*b*-PS_{40k} terpolymer synthesized via sequential anionic polymerization was used as the membrane materials. It exhibited a three-phase lamellar morphology in the solid bulk equilibrium state. Complexation with imidazole and pyridine in the membrane casting solution, along with the optimum solvent, polymer concentration and evaporation time, gave rise to highly ordered isoporous membranes, containing nanochannels with pure water permeance up to 40 L m⁻² h⁻¹ bar⁻¹ and a molecular weight cut off of about 8 kg mol⁻¹. The membranes exhibited an interesting preferential transport for proteins, such as lysozyme and cytochrome *c*, while rejecting polyethylene glycol with a comparable molecular size. The high selectivity for lysozyme was possibly affected by several factors, including hydrogen bonding within the channels, electrostatic attraction, and a right pore dimension.

Acknowledgements

This work was funded by King Abdullah University of Science and Technology (KAUST) Grant 1671 - CRG2. The authors thank Dr. Yihui Xie and Dr. Stefan Chisca for the discussion on polymer modification and NMR analysis, as well as CHESS at Cornell, USA and LNLs in Brazil for access to the GISAXS and SAXS synchrotron facilities. The Cornell High Energy Synchrotron Source (CHESS) is supported by the National Science Foundation and the National Institutes of Health/National Institute of General Medical Sciences under NSF award DMR-1332208.

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