Self-assembled membranes with feather-like and lamellar morphologies containing α -helical polypeptides

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Abstract

Biological systems are the ultimate model for an effective selective permeation device. Biomimetic artificial channels based on the assembly of peptides have been previously integrated in vesicles and lipid layers with the expectation of leading in the future to a more efficient water purification and biological separation. We demonstrate here the design of scalable membranes constituted by synthesized copolymers with α -helical polypeptide blocks. They have unique feather-like and lamellar structures and were obtained from poly(styrene-*b*- γ -benzyl-L-glutamate) copolymers via phase inversion or spin-coating. The membranes were then hydrolyzed using acid vapor annealing, which preserved the helical morphology after hydrolysis. Water permeation up to $3.5 \text{ L m}^{-2} \text{ h}^{-1} \text{ bar}^{-1}$ was obtained. Dialysis experiments with membranes prepared via phase inversion had high retention of cytochrome-c. High rejection of cytochrome-c and the negatively charged dye Brilliant Blue was demonstrated for the spin-coated membranes. The bioinspired membranes are developed for effective molecular separation, aiming at applications in the biotech industry.

1. Introduction

Highly ordered self-assembled systems are abundant in nature ¹. They are responsible for important functions, such as DNA replication, embryo morphogenesis and the constitution of biological membranes. Biological architectures and functionalities are inspiring new approaches in synthetic polymeric systems ²⁻⁴. Bioinspired membranes can potentially improve classical separation processes or enable separations not possible with the currently commercial membranes ⁵⁻⁶.

Different approaches of supramolecular self-assembled systems are being explored to mimic biological nanochannels to transport water, ions and/or proton selectively. Artificial channels have been formed by the self-assembly of dendritic dipeptides ⁷⁻⁸, imidazole quartet ⁹, hydrazide-or-peptide-appended pillar[5]arene ¹⁰⁻¹², and amphiphilic multiblock molecules ¹³⁻¹⁴. The self-assembly of dendritic dipeptides by enhanced peripheral π -stacking reported by Percec et al., ⁷⁻⁸ led to the formation of helical pores with 14.5 Å inner pore diameter, that could transport water and protons, but exclude ions across lipid bilayers due to the hydrophobicity of the channel. Barboiu et al., ⁹ reported imidazole (I) quartet channels (2.6 Å diameter) from the self-assembly of alkylureidoimidazoles, stabilized by inner water-wires through H-bond interactions with the imidazole channel. Hydrophobic interactions of the urea tails influenced the channel formation. When incorporated into bilayers, water and proton were reported to transport through the I-quartet channels, while ions were excluded due to steric effects. Another example of artificial water channels is based on hydrazide-appended pillar[5]arene derivatives demonstrated by Hou and coworkers ^{10, 15}. In this case tubular channels with a diameter of ~6.5 Å are formed via H-bond to transport water and/or protons across lipid vesicles. Alternating hydrophobic/hydrophilic domains along the channels could disrupt proton conduction through the channel, making it selectively permeable for water. Peptide-appended pillar[5]arene water channels were reported by Kumar et al. ¹⁶ and demonstrated to have a higher water permeability compared to its predecessor. The channels could self-assemble into 2D arrays in lipid bilayers, implying the possibility of practical separation processes.

Carbon nanotubes ¹⁷⁻²³ and graphene-based ²⁴⁻²⁵ materials were reported as a way to form selective nanochannels having preferential and ultrafast transport of water. Another approach to create self-

assembled artificial channels have been investigated using a cyclopeptide-conjugated block copolymer that led to 2Å peptide channels for proton transport ²⁶.

Although highly innovative, integrating these approaches into real membranes or robust separation devices is still a challenge. Many of the reported artificial channels were investigated as vesicles. The potential scalability is essential when considering the future application of artificial channels in actual separation processes.

The approach we explore in this work builds on our previous experience on the formation of isoporous membranes using the self-assembly of block copolymers combined with non-solvent induced phase separation ²⁷⁻³¹. This method offers a scalable route to form well-defined pore structures, as well as chemically tuned pores by tailoring the block copolymer composition and chemical functionality. The reported block copolymer membranes prepared by this method mostly have pores in the range of ultrafiltration. Much less is explored to fabricate nanofiltration membranes. One of the first successful approaches in this direction was the preparation of membranes based on blends of poly(styrene-*b*-acrylic acid) and poly(styrene-*b*-4-vinyl pyridine) ³². The resulting porous membranes have average pore sizes of 1.5 nm with a sharp size distribution, leading to high water permeability and adequate separation of small proteins and amino acids. Another approach took advantage of the stimuli-responsive behavior of the membranes, closing pores in different pH values to perform separation in a nanofiltration range ³³. Our group also reported membranes with a selective transport for proteins and improved water permeance using a block copolymer containing hydrogen bond forming groups (polystyrene-*b*-polyhydroxystyrene-*b*-polystyrene) ²⁷, and photochemically modifying self-assembled block copolymers containing double bonds (polystyrene-*b*-polybutadiene-*b*-polystyrene) ²⁹.

The ultrahigh water permeation and selectivity of proteins, such as aquaporin, have been intensively investigated³⁴⁻³⁶. Highly innovative approaches demonstrating fully synthetic water channels are being recently reported^{10-11, 37}. However scalability is a big challenge. In most cases, the active permeation unit is integrated into vesicles or lipid layers, with properties that are far from those required for application in real separation processes. The most effective and recent achievements on scalable membrane development with biological³⁸ or artificial water channels³⁹ consist in the integration of these unities in an interfacially polymerized polyamide layer. The permeation characteristics are however still far from those demonstrated for the isolated unities.

In the present study, we investigate the formation of artificial channels, by using a copolymer with polypeptide blocks, and industrial methods of membrane manufacture, such as solution casting or spin coating. The chemical functionality and the α -helical conformation of the polypeptide similar to natural protein channels are exciting features to be explored for membrane development that could exhibit selective transport for water and/or specific solutes. Proteins and peptides are highly effective in forming biological nanopores⁴⁰⁻⁴². The helices architecture can form well-defined nanochannels with sizes relevant for the selective transport of water, ions and small biomolecules. Peptides can now be synthesized and engineered with different monomeric unities, forming channels of different sizes and functionalities. They can be easily incorporated in lipid bilayers or in synthetic membranes. Amino acid residues along the channel can create various interactions (e.g. hydrogen bonding or ionic interactions) providing selective transport of water, proton or ions^{41, 43}. π - π interactions and hydrogen bonds are known to stabilize tubular structures of peptide assemblies in β -barrels⁴⁴ and pillar[5]arenes unities¹¹. More than simple incorporation, the application in membrane technology requires robust films and high channel densities. We demonstrate how this can be achieved by using a polypeptide copolymer, poly(styrene-*b*- γ -benzyl-

L-glutamate) (PS-*b*-PBLG), for membrane preparation. A phase segregation in the range of tens of nanometers is driven by the immiscibility of PS and PBLG blocks. In contrast to previously used amorphous block copolymers, an hierarchical structure is observed with another level of self-assembly in the polypeptide phase. Ordered rod-like (helical) structures are formed, resulting in so-called “hexagonal-in-lamellar” morphologies^{41,45-48}. Guiding the self-assembly of the peptide-based block copolymers to form membranes with artificial channels in a scalable manner is a major challenge and has not been previously investigated. We obtained membranes with feather-like and lamellar structures using phase inversion and spin-coating methods. An attractive performance for dialysis and nanofiltration was demonstrated, by rejecting cytochrome-c and negatively charged dye (brilliant blue), while permeating neutral dye (vitamin B₁₂) and smaller molecules (amino acid, vitamin B₂). We promoted the acid vapor hydrolysis of the PBLG, which increased the membrane water permeance from around 0.1 L m⁻² h⁻¹ bar⁻¹ to 3.5 L m⁻² h⁻¹ bar⁻¹ without sacrificing the helical structure.

2. Experimental

2.1. Materials

Pyridine, 1,4-dioxane (anhydrous, 99.8%), 1,1,2-trichloroethane (96%), HBr/HOAc (33wt% hydrogen bromide in acetic acid), ruthenium (IV) oxide hydrate, sodium (meta)periodate ($\geq 99\%$), cytochrome-c (from bovine heart), polyethylene glycol (PEG), cobalamin (vitamin B₁₂), riboflavin (vitamin B₂, from *Eremothecium ashbyii*, $\geq 98\%$), L-phenylalanine (BioUltra, $\geq 98\%$) and Brilliant Blue R-250 were purchased from Sigma-Aldrich. Polyacrylonitrile (PAN) commercial membranes supplied by GMT GmbH Germany were used as the support for composite membrane fabrication. Milli-Q water was used in the experiments involving water.

For the polymer synthesis, N,N-Dimethylformamide (anhydrous, amine free, 99.9%) was purchased from Alfa Aesar and used after distillation. The amino-protected lithium initiator 2,2,5,5-Tetramethyl-1-(3-lithiopropyl)-1-aza-2,5-disilacyclopentane was purchased from FMC Lithium (10wt% in cyclohexane) and used as received. The γ -benzyl-L-glutamate N-carboxy anhydride (BLG-NCA) monomer was synthesized according to a previously reported method ⁴⁹.

2.2. Polymer synthesis

The linear poly(styrene-*b*- γ -benzyl-L-glutamate) (PS-*b*-PBLG) copolymer was synthesized using the combination of anionic polymerization high vacuum techniques and ring opening polymerization ⁵⁰⁻⁵¹. The copolymers used explicitly in this work had a molecular weight of 31 kg mol⁻¹ (PS block) and 20 – 34 kg mol⁻¹ (PBLG block).

2.2.1. Synthesis of amine end-functionalized polystyrene

Amine end-functionalized polystyrene (PS-NH₂) was synthesized by anionic polymerization high vacuum techniques in a similar procedure reported previously ⁵²⁻⁵³.

2.2.2. Synthesis of poly(styrene-*b*- γ -benzyl-L-glutamate) diblock copolymer

The paths for the synthesis of PS-*b*-PBLG are given in Scheme 1. In a 100 ml flame-dried round bottom flask, 1.0 g of PS-NH₂ with $M_n = 31.0 \times 10^3$ g/mol (0.032 mmol of amines) was added, followed by distillation of 30 mL of highly dry benzene into the flask and dissolution of PS-NH₂ (macroinitiator). The solution was stirred for two hours, and benzene was distilled off to dryness. Subsequently, 30 mL of highly pure DMF was distilled into the flask followed by dissolution of the macroinitiator. The flask was placed in a glove box, and 10 mL DMF solution containing 1.0

g of BLG-NCA was added, followed by vigorous stirring. Periodically, the solution was pumped to remove produced CO_2 in order to drive the equilibrium in favor of the final product. The consumption of the NCA monomer was monitored by removal of aliquots from the solution and FTIR analysis. After completion of the polymerization, the final diblock copolymer was precipitated in diethyl ether and dried under vacuum until constant weight.

Scheme 1. General reactions for the synthesis of the PS-*b*-PBLG diblock copolymer.

2.3. Size Exclusion Chromatography (SEC)

The molecular weight average and distribution of the synthesized polymers were determined using an Agilent size exclusion chromatographer (SEC) fitted with refractive index (DRI) and UV-Vis detectors with two identical PLgel columns (5 μm , MIXED-C) connected in series. DMF was used as the mobile phase at a flow rate of 1 mL/min and temperature 35 $^{\circ}\text{C}$. Polystyrene standards were used for calibration of the instrument.

2.4. Nuclear magnetic resonance spectroscopy (NMR)

^1H NMR measurements were performed at room temperature using a Bruker AVANCE III spectrometer operating at 500 MHz in CDCl_3 . ^1H NMR spectra were used to calculate the number-

average molecular weight ($M_{n,NMR}$) of the PBLG block by integration/comparison of the characteristic signals of PBLG block with those of known molecular weight of PS block.

2.5. Atomic Force Microscopy (AFM)

The AFM imaging was performed on a Bruker Dimension Icon SPM. The experiment was conducted using the Nanoscope 8.15 software in the tapping mode at an atmospheric environment, a 1 – 10 Ω -cm phosphorus doped silicon cantilever with a resonance frequency of 73-88 kHz and a force constant of 3 N m⁻¹. The acquired images were processed using Gwyddion software.

2.6. Transmission Electron Microscopy (TEM)

TEM images were obtained on a FEI Titan ST microscope, operating at 300 kV or on a FEI Tecnai microscope at 120 kV. For the analysis of the bulk equilibrium morphology of the block copolymers, a 5 wt% polymer solution in 1,1,2-trichloroethane was cast on a Teflon mold and the solution was let slowly drying for 4-5 days at the room temperature in a closed glass container. The dry PS_{31k}-*b*-PBLG_{25k} dense film was kept under 100 °C in vacuum for three days. The RuO₄ staining solution was prepared according to the literature ⁵⁴ by oxidizing hydrated ruthenium dioxide (RuO₂.xH₂O) using sodium periodate (NaIO₄). A typical preparation procedure was performed by mixing a 25 mL chilled NaIO₄ aqueous solution (12.8 mg mL⁻¹) at around 1 °C with 0.15 g RuO₂.xH₂O. The solution was stored in a freezer at -20 °C for regular usage. Ultrathin sections (70-90 nm in thickness) of the films were prepared at a cryogenic condition using a Leica EM UC6 ultramicrotome with a diamond knife at a temperature of -140 to -90 °C. The sections were collected on a 300 mesh copper grid and stained with RuO₄ for about 15 min before imaging.

2.7. Scanning Electron Microscopy (SEM)

SEM images were acquired on a FEI Novanano microscope at an accelerating voltage of 3-5 kV and working distances of 2.5-5 mm. To analyze the membrane cross-section, we fractured the membrane in liquid nitrogen by gently twisting it using tweezers. Before imaging, the samples were mounted on aluminum stubs and sputter coated with a 3 nm platinum layer using a Quorum Technologies Q150T sputter coater in an argon environment.

2.8. Polarized optical microscopy

The polarized optical microscopy was performed using an Olympus BX61 microscope, equipped with a polarizer and an analyzer. The samples were sandwiched between a microscope glass slide and a glass cover. The exposure time was 500 – 700 s.

2.9. Small-Angle X-Ray Scattering (SAXS)

The SAXS experiments were performed at the SAXS1 beamline of the Brazilian synchrotron laboratory (LNLS). The measurements were performed using 8.0 keV beam energy and 1.55 Å X-ray wavelength having a beam area of 1 mm². The sample-to-detector distances were 1.50 and 3.06 m. A PILATUS detector with 172 µm² pixels was used to record the 2D scattering patterns with a sample exposure time of 300 – 500 s. The recorded scattering patterns were then normalized (with respect to the primary beam intensity), and the background subtracted before azimuthal integration to obtain the intensity versus scattering vector (q) profiles.

2.10. Wide-Angle X-Ray Diffraction (WAXD) and X-ray Diffraction (XRD)

X-ray diffraction experiments were performed at the XRD1 beamline of the Brazilian synchrotron laboratory (LNLS)⁵⁵. The wavelength used was 1.033 Å (12 keV) with a beam size of 2.0 mm

(horizontal) and 0.6–0.7 mm (vertical) and an MYTHEN 24K detector from Dectris®. Lab-scale XRD measurements were performed using a Bruker D8 Advance XRD.

2.11. Grazing-Incidence Small-Angle X-ray Scattering (GISAXS)

Time-resolved GISAXS experiments were performed at the D1 beam line of the Cornell High Energy Synchrotron Source. The beam had 10.6 keV energy and 1.17 Å wavelength, and the sample - detector distance was 1.73 m. GISAXS measurements with an incident angle of 0.13 or 0.14 degree and 1 s exposure time were performed every 2 s after casting the polymer solution with 200 µm thickness using a doctor blade system⁵⁶. Igor Pro 6.37 software was used to obtain 1 D profile of the intensity vs. scattering vector for horizontal projections and to determine the peak position and its width by fitting the data with Lorentz functions⁵⁷.

2.12. Rheology

The rheology of 30 wt% PS-*b*-PBLG solutions was studied using a TA Instruments AR 1500ex rheometer at 25 °C. A cone and plate geometry (20 mm diameter and 1° cone angle) was used with a solvent trap to avoid the solution drying out during the measurement. The measurement for each sample was repeated three times.

2.13. Membrane fabrication and hydrolysis

Membranes were fabricated using two different methods: (1) phase inversion and (2) spin coating. Phase inversion membranes were prepared by casting a block copolymer solution (27 wt% in pyridine) on PAN support using a doctor blade with a 50 µm gap. After around 5 s evaporation time, the film was precipitated in a water bath at room temperature. The spin coating was performed using a 5 wt% copolymer solution in dioxane. The solution was dropped and spin coated

on a PAN support with a rotation speed of around 3000 rpm for 60 s. After drying in air, all the membranes were further submitted to high vacuum before hydrolysis.

The membranes were then exposed to HBr/HOAc (33 wt% of HBr in HOAc) vapor at room temperature to promote the hydrolysis of the PBLG block. The membranes were placed in a closed glass chamber together with around 4 mL of HBr/HOAc solution in a separate container. After 3 hr of acid vapor annealing, the chamber lid was opened, and the acid solution was removed. The hydrolyzed membranes were dried for several hours before washing with water. Methanol was then used to further wash the membranes. They were finally kept in methanol overnight before characterizations.

2.14. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was recorded using an i-Nicolet spectrometer with 64 runs and 0.121 cm^{-1} resolution. The measurement for each sample was repeated at least twice. The characterization of the hydrolyzed membranes was done after washing them with methanol and drying under high vacuum.

2.15. Dialysis and filtration tests

The dialysis experiments were performed using a PermeGear Side-Bi-Side cells with an orifice diameter of 15 mm and 5 ml volume at room temperature. The feed side was filled with a solution of cytochrome-c or phenylalanine with a concentration of 1 g L^{-1} in phosphate buffered saline (PBS) solution at a physiological pH of 7.4, while a pure PBS solution was used in the permeate side. For the diffusion study of vitamin B₁₂ and vitamin B₂, a 100 ppm vitamin B solution in water was used as the feed, while Milli-Q water was used in the permeate side. The solutions at the feed

and permeate side were stirred at 360 rpm, and the concentrations were measured periodically using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). The recovery percentage of the solute at time t was calculated as the ratio of the solute concentration in the permeate and the initial feed concentration.

The filtration tests were performed using an in-house stainless steel filtration cell with an effective membrane area (A) of 1 cm^2 and a transmembrane pressure (ΔP) of 8 bar. The permeance was calculated from the measured permeance volume (V) during the filtration period (t) as in equation (6.1).

$$J_w = \frac{V}{A \cdot t \cdot \Delta P} \quad (6.1)$$

The rejections of dyes (brilliant blue and vitamin B₁₂), PEG (200, 3000, 10 000, and 35 000 g mol⁻¹), and cytochrome-c were examined using feed solutions of 100 ppm dyes in water, 1 g L⁻¹ PEG in water, or 1 g L⁻¹ cytochrome-c in PBS solution. The dyes and cytochrome-c concentrations in the feed and permeate were measured using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). The concentrations of PEG were measured using an Agilent 1260 Infinity gel permeation chromatography with two columns in series (PL aquagel-OH 40 and 60) operated at 30 °C. The rejections (R) were calculated using the equation (6.2).

$$R = \left(1 - \frac{c_p}{c_f}\right) \times 100\% \quad (6.2)$$

3. Results and Discussion

3.1. PS-*b*-PBLG synthesis

The copolymers used in this work are PS_{31k}-*b*-PBLG_{20k}, PS_{31k}-*b*-PBLG_{21k}, PS_{31k}-*b*-PBLG_{25k}, and PS_{31k}-*b*-PBLG_{34k}. The successful synthesis of the PS-*b*-PBLG diblock copolymers was confirmed by SEC (Figure 1a) and ¹H NMR (Figure 1b) measurements.

Figure 1. (a) SEC traces of the macroinitiator PS-NH₂ and the corresponding PS_{31k}-*b*-PBLG_{20k} diblock copolymer; (b) ¹H NMR spectrum (CDCl₃) of the PS_{31k}-*b*-PBLG_{25k} diblock copolymer.

3.2. Morphology of dense PS-*b*-PBLG films

The morphology of a dense PS_{31k}-*b*-PBLG_{25k} copolymer investigated after slow solvent evaporation and days in oven at 100 °C. The Hansen solubility parameters of 1,1,2-trichloroethane are seen in Table 1, as well as for PS and PBLG⁵⁸. The values indicate that 1,1,2-trichloroethane is a common solvent for both blocks. A slow solvent evaporation, followed by long drying and heating close to the PS glass transition temperature led to a morphology that is mainly influenced by the interaction between blocks. A highly ordered and lamellar morphology was confirmed by the TEM images in Figure 2a. A lamellar morphology was also obtained from PS_{31k}-*b*-PBLG_{20k} and PS_{31k}-*b*-PBLG_{34k} (Figure S1 Supporting Information). It also showed that a small variation of the PBLG molecular weight (20 – 34 kg mol⁻¹) did not change the lamellar morphology. A similar structure has been previously observed for other rod-coil block copolymers^{45, 59-61}. AFM images (Figure S2 Supporting Information) reinforce the formation of lamellar morphology by the PS-*b*-PBLG copolymers. Figure 2b shows that the dense PS_{31k}-*b*-PBLG_{25k} film was birefringent, which indicates that the copolymer is semi-crystalline. SAXS experiments were performed to confirm the overall arrangement of the blocks and more precisely quantify the domain sizes. The SAXS profile in Figure 2c confirms the lamellar structure ($q/q^* = 1, 2, 3, 4$) with a domain spacing of 17.45 nm and 16.64 nm for PS_{31k}-*b*-PBLG_{25k} and PS_{31k}-*b*-PBLG_{20k}, respectively, close to the estimated value from the TEM images. At a higher q between 4–5 nm⁻¹, a peak was observed, which might correspond to the presence of the rod PBLG with a domain size of around 1.3 nm. This result alone is not a conclusive indication of the α -helical PBLG, but it could indicate the existence of uniform domains in approximately 1 nm scale, close to what is expected from the helical PBLG. FTIR measurement of the solid bulk copolymer confirmed the α -helical structure of PBLG by the presence of amide I and II peaks around 1650 and 1547 cm⁻¹⁶², respectively

(Figure 2d). In addition, high-resolution X-ray diffraction was performed to evidence the formation of helices as discussed in the following paragraph.

Figure 2. Characterization of PS-*b*-PBLG dense films: (a) TEM image and (b) polarized optical microscope of dense PS_{31k}-*b*-PBLG_{25k} films prepared from solution in 1,1,2-trichloroethane dried at 100 °C vacuum for 3 days; (c) SAXS profile of PS_{31k}-*b*-PBLG_{25k} and PS_{31k}-*b*-PBLG_{20k}, (d) FTIR of PS_{31k}-*b*-PBLG_{25k} and (e) WAXD of PS_{31k}-*b*-PBLG_{34k}. The schematic diagram shows coil-helix configuration of PS-*b*-PBLG in a lamellar structure where PBLG helices self-assemble into hexagonally ordered structure inside the lamellar domain.

A high-resolution WAXD experiment conducted at the synchrotron facility revealed information about the crystalline structure of the rod PBLG block, as shown in Figure 2e. A copolymer with slightly higher PBLG content ($\text{PS}_{31\text{k}}\text{-}b\text{-PBLG}_{34\text{k}}$) was used for the experiment with a good signal to noise ratio. The presence of the primary peak q_1 corresponded to a 1.3 nm periodic distance and was close to the domain spacing of PBLG rods observed by SAXS. The WAXD profile indicated that the PBLG rods self-assembled into a hexagonally ordered structure, which correlates with the presence of the peaks at a characteristic ratio of 1, $\sqrt{3}$ and 2 with respect to the primary peak (q_1), similar to what was observed from the XRD profile of $\text{PS}_{31\text{k}}\text{-}b\text{-PBLG}_{25\text{k}}$ in Figure S3 (Supporting Information). A small shoulder at q_2 corresponds to a helix pitch of 5.3 Å, similar to what has been observed in the literature for an ABA triblock terpolymer, where the A block is PBLG and the B block is polyethylene oxide ⁶³. This confirmed the formation of the secondary structure of PBLG organized as α -helices. Another peak (q_3), which corresponds to 1.35 Å, could also be associated with the α -helix ⁶³. The previous reported value for $\text{PBLG}_{54}\text{-}b\text{-Poly(ethylene glycol)}_{21}\text{-}b\text{-PBLG}_{54}$ was 1.54 Å. A slight deviation from this value could be due to the difference in the copolymer composition and the architecture.

3.3. PS-*b*-PBLG self-assembly in solution

For the membrane preparation, the copolymer solutions are cast and immersed/quenched in water or spin coated with fast solvent evaporation. The final morphology is a non-equilibrium one and is highly dependent on the used solvent. The copolymer self-assembly in solution is expected to have a strong influence on the final structure. We investigated the self-assembly in solution by SAXS and by rheological experiments. In the present study, membranes were prepared using

pyridine or dioxane as a solvent. PBLG-based polymers have been reported to form an α -helical secondary structure both in dioxane and pyridine, being aggregated in dioxane but not in pyridine⁶⁴. The degree of PBLG association depends on the polarity contribution of the solvents. It could influence the copolymer self-assembly. This is therefore of great importance when the formation of nanochannels from PS-*b*-PBLG copolymers is aimed. The aggregation of PBLG is influenced by the hydrogen/polar interactions, particularly at the end of the polymer chains.⁶⁴⁻⁶⁶ According to the solubility parameters listed in Table 1, pyridine has a higher polar contribution (δ_p) and a higher combined hydrogen bond/polar contribution (δ_a) compared to dioxane. It could then compete with the interchain hydrogen bond/polar interactions and break the aggregation, making the helices more flexible.

Table 1. Hansen solubility parameters of the solvent and polystyrene^{58, 67}

Solvent	δ_D (MPa ^{0.5})	δ_P (MPa ^{0.5})	δ_H (MPa ^{0.5})	δ_a $= \sqrt{\delta_P^2 + \delta_H^2}$	δ $= \sqrt{\delta_D^2 + \delta_P^2 + \delta_H^2}$
1,1,2-trichloroethane	18.2	5.3	6.8	8.6	20
1,4-dioxane	17.5	1.8	9	9.2	20
Pyridine	19	8.8	5.9	10.6	22
Polystyrene	21.3	5.8	4.3	7.2	24
β-PBLG⁵⁸	21.6	6.4	9.1	11.1	24

SAXS measurements for concentrated copolymer solutions in dioxane and pyridine (Figure 3a) indicate the formation of a lamellar structure with a repeating distance of around 20 nm, similar to

the domain sizes of the lamellar structure in the dense film prepared from 1,1,2-trichloroethane. The polarized optical microscopy of the concentrated copolymer solution (Figure 3b and c) suggests that the copolymer forms liquid crystals both in dioxane and pyridine, due to the helical structure of PBLG.

Figure 3. Characterizations of PS-*b*-PBLG in solutions. (a) SAXS profiles of the PS_{31k}-*b*-PBLG_{21k} solution in dioxane (DOX) and pyridine (PYR); (b-c) polarized optical microscope images of 45 wt% PS_{31k}-*b*-PBLG_{20k} solutions in (b) DOX and (c) PYR.

Rheological investigation of the copolymer solutions shows that when pyridine is used as solvent, the storage and loss moduli, as well as the viscosity are lower than those in dioxane (Figure S4a-c, Supporting Information). This is consistent with the hypothesis that the pyridine polarity might disrupt the PBLG segment-segment interaction, as well as plasticize the polystyrene blocks. The amplitude sweep measurement (Figure S4b) showed that the copolymer in dioxane or pyridine has a liquid-like rheological behavior and is profoundly non-associated, indicated by $\tan \delta (G''/G') > 1$. The frequency sweep (Figure S4b) performed at 1% strain indicated that the storage modulus

(G') is frequency dependent, suggesting that the solutions are fluid-like. Moreover, the flow sweep measurement (Figure 3f) revealed that the copolymer in pyridine behaved as a Newtonian-fluid, as the viscosity was independent on shear rate and a linear correlation between shear rate and shear stress was observed. On the other hand, shear thinning phenomena were found for the copolymer in dioxane.

3.4. Kinetically trapped self-assembled morphology

Figure 4a – c shows the AFM images of the membranes prepared by casting 27 wt% PS_{31k}-*b*-PBLG_{20k} solutions in pyridine on a PAN porous asymmetric support and immersing in water (phase inversion). A unique hierarchical and highly ordered feather-like morphology was observed, formed by a self-assembly in two scales: (i) the phase segregation of PS and PBLG blocks and the PBLG helix formation in the resulting confined phases. The self-assembly takes place in solution and the immersion in water, a strong non-solvent for both blocks, leads to fast solidification, kinetically trapping the hierarchical morphology. The zigzag morphology on the film surface resembles that previously reported for the bulk of poly(styrene-*b*-hexyl isocyanate) copolymer films⁶⁸, with blocks prone to form coils (PS) and rods (hexyl isocyanate) in solution. The rod tilting phenomena can be explained in light of the Halperin's theory.⁶⁹⁻⁷⁰ When a rod-coil block copolymer is dissolved in a preferential solvent for the coil-forming block, as the coil is swollen, its entropic contribution to the total free energy per chain increases. When the rods are tilted, and the contribution of the rod-coil interfacial energy is not dominant, the free energy is minimized, because the area per chain increases and the degree of coil stretching decreases. In our

case, pyridine is a preferential solvent for the polystyrene and could promote tilting of the PBLG rods, as proposed by Halperin, resulting in a feather-like (zigzag) morphology.

Figure 4. AFM (a,d) topological, (b, e) phase contrast, and (c, f) 3D images of the PS_{31k}-*b*-PBLG_{20k} membranes prepared by (a-c) casting of 27 wt% copolymer solutions in pyridine and immersing in water, and (d-f) spin coating of a 5 wt% copolymer solution in dioxane. The dark/bright area in the topographic profile corresponds to the depth, while in the phase contrast image is mainly associated with differences in rigidity.

The phase contrast in AFM phase image (Figure 4b) is not pronounced, indicating that both PS and PBLG phases have a similar rigidity. Polystyrene is a glassy polymer, while PBLG is

crystalline with helices conformation, both cases with comparable rigidity. The 3D image of the membrane surface (Figure 4c) supports the formation of a highly-ordered zigzag morphology.

The morphology evolution during the solvent evaporation was examined by time-resolved GISAXS experiments after casting the polymer solution. The in-situ GISAXS experiments were performed with 20 wt% PS_{31k}-*b*-PBLG_{20k} casting solutions in pyridine and 20 wt% PS_{31k}-*b*-PBLG_{20k} in dioxane. At the initial stage of evaporation, both solutions present a broad peak in the scattering profile, which might indicate the presence of phase separated morphology (Figure 5b and e). This phenomenon was expected because of the used solvents are more selective for the PBLG blocks according to the Hansen solubility parameters (Table 1). By increasing the evaporation time, for the solution in dioxane, the peak narrows as the regularity of the surface structuration is improving, and at 20 s a secondary peak starts to be observed. This process is also described by the corresponding scattering images in Figure 5a and the video in Supporting Information. Initially, a diffuse ring is visible at low q (close to the beam stop), and then, this primary ring sharpens, followed by the appearance of a secondary ring. This feature sharpens and become more intense as the long-range order is promoted through the surface.

The change of domain spacing and the primary peak width (FWHM) with time, represented in Figure 5c, indicate the existence of two regimes: 1) a fast structure formation at low evaporation time, probably due to solvent evaporation, and 2) a much slower process starting around 40 s. At this time, when most of the solvent is already evaporated, the polymer chains still have enough mobility to relax into the favored lamellar morphology. The d -spacing decreases with time (the primary peak position shifts to higher q values) as a result of the structure compaction, while the order is improved, as indicated by the decrease of FWHM. For the completely dried film, the d -

spacing value (18.6 nm) is close to that of the dense films (17.4 nm) measured by SAXS as discussed in Section 3.2.

A different evolution of the membrane structure during the evaporation was observed when pyridine was used as a solvent (Figure 5d and e). Even if the polymer chains have higher mobility in solution, as indicated by the lower viscosity and modulus, the fast solvent evaporation did not allow the polymer chains to relax in a more ordered arrangement before the complete drying of the film. At the same time, the presumption that pyridine polarity disrupts the PBLG inter segments interaction and plasticizes the polystyrene blocks might also support this behavior.

Figure 5. (a) GISAXS 2D images of 20 wt% PS_{31k}-b-PBLG_{20k} in dioxane at different evaporation times; (b) in-plane curves and (c) the time evolution of d -spacing and full width at half maximum (FWHM) (determined for the first peak) during the solvent evaporation; (d) GISAXS 2D images for 20 wt% PS_{31k}-b-PBLG_{20k} in pyridine and (e) in-plane curves at 4 s interval during solvent evaporation; (f) Horizontal (in-plane) scattering profiles intensity vs. q for thin films spin-coated from 5 wt% PS_{31k}-b-PBLG_{20k} and PS_{31k}-b-PBLG_{34k} in dioxane.

The second type of membranes explored in this work was prepared by spin coating of 5 wt% PS_{31k}-*b*-PBLG_{20k} copolymer solutions in dioxane also on a porous PAN ultrafiltration asymmetric support. In this case the more dilute PS_{31k}-*b*-PBLG_{20k} solution forms a thinner layer than by casting. The morphology is also lamellar and perpendicular to the surface as shown in Figure 4d-f and 6b, but it is smoother than that obtained by the casting procedure. The lamellar order follows the self-assembly detected in solution, as confirmed by SAXS (Figure 2a). The formation of the lamellar morphology was confirmed by the scattering profiles of the integrated in-plane GISAXS cut for thin spin-coated films on silicon wafers from 5 wt% PS_{31k}-*b*-PBLG_{20k} and PS_{31k}-*b*-PBLG_{34k} in dioxane (Figure 5f). The fitting procedure for the profile deconvolution is exemplified in Figure S5 in the Supporting Information. Both of the PS_{31k}-*b*-PBLG_{20k} and PS_{31k}-*b*-PBLG_{34k} copolymers are characterized by a lamellar morphology, as indicated by the relative position of the two peaks q/q^* which is very close to 1, 2. The inter-lamellar domain spacing estimated as $d=2\pi/q^*$, where q^* is the value of q -vector for the primary peak, are 17.1 and 20.7 nm for PS_{31k}-*b*-PBLG_{20k} and PS_{31k}-*b*-PBLG_{34k}, respectively, close to the value obtained by SAXS and TEM for the dense films.

3.5. Membrane hydrolysis

Figure 6. Scheme of the PS_{31k}-*b*-PBLG_{20k} hydrolysis by exposition to acid vapor and SEM images of the PS_{31k}-*b*-PBLG_{20k} membrane cross-sections, prepared by (a, c) casting of 27 wt% copolymer solutions in pyridine followed by immersion in water, and (b, d) spin coating of a 5 wt% copolymer solution in dioxane; images before (a, b) and after (c, d) the hydrolysis; arrows indicate the thickness of the PS_{31k}-*b*-PBLG_{20k} layer on the PAN porous support.

The preformed membranes were exposed to acid (33 wt% HBr in HOAc) vapor for three hours. A deprotection of PBLG into poly-L-glutamic acid (PLGA) by addition of HBr/HOAc to the solution has been reported by other groups⁷¹⁻⁷². In our work, we demonstrated the deprotection in a swollen solid film, similar to the vapor phase hydrolysis applied for cellulose regeneration⁷³. The

membrane morphology was preserved after the hydrolysis with a slightly more opened structure as confirmed by the SEM images of the membrane cross-sections (Figure 6a and c). SEM images of the membrane surface are presented in Figure S4 (Supporting Information). Since the hydrolysis was performed in the solid preformed membrane, the self-assembled morphology was preserved.

The completion of the hydrolysis reaction was confirmed by FTIR measurements presented in Figure 7a (green spectrum). A broad O–H band appeared around $2700 - 3680\text{ cm}^{-1}$. This wavenumber range is characteristic of carboxylic acid groups. There is an overlap with the strong N–H peak of the polypeptide at around 3290 cm^{-1} . Another clear evidence of the successful deprotection was observed by the shifting and broadening of the C=O peak between 1732 and 1710 cm^{-1} , as well as the disappearance of the peak at 1166 cm^{-1} (stretching of C–O ester group). Furthermore, Figure 7b shows that the water contact angle decreased after hydrolysis due to the presence of carboxylic acid.

The hydrolyzed membranes still preserve the polypeptide (PLGA) α -helical structure, since the Amide I and II bands at 1653 and 1548 cm^{-1} , respectively, were still observed. It is known that the secondary structure of PLGA is pH-dependent. A random PLGA coil in solution can transform into α -helices as the pH decreases from neutral to less than 5⁷⁴⁻⁷⁸. During the acid vapor annealing, the membrane swells with acid, and the PBLG is converted to PLGA. If the flexibility would be high enough, the freshly formed PLGA could adopt the α -helical conformation. The α -helices were then entrapped as the membrane dried in air. Further washing with water and methanol did not disrupt the α -helical structure of the PLGA in the membrane as the structure was already trapped in the solid state.

Figure 7. (a) FTIR spectra and (b) water contact angle of the PS_{31k}-*b*-PBLG_{20k} membranes before and after the hydrolysis; pristine-1 and hydrolyzed-1 are membranes cast from 27 wt% copolymer solutions in pyridine; pristine-2 and hydrolyzed-2 are spin-coated films from 5 wt% copolymer solutions in dioxane.

Similar to the membrane prepared via phase inversion, the lamellae of the membrane prepared by spin coating from dioxane solution are better revealed after hydrolysis (Figure S4b and d). The completion of the PBLG deprotection was confirmed by the FTIR spectra shown in Figure 7a (red spectrum). The spectrum of the hydrolyzed film was similar to that for the phase inversion membrane (hydrolyzed-1, green spectrum), except that the ester C=O peak at 1732 cm⁻¹ was still visible and only broadened or overlapped with the carboxyl C=O peak at 1710 cm⁻¹. This could indicate a partial deprotection of PBLG for the hydrolyzed-2 membrane. The spin-coated film is probably denser than that prepared by casting and immersion in water. The acid access to chains far from the surface is more restrictive than that in phase inversion membranes. Furthermore, as

previously discussed, dioxane could promote a tighter distribution of PBLG helices, making it again less accessible to the acid vapor. A decrease in water contact angle was observed after the hydrolysis (Figure 7b). The hydrolyzed membrane still contained α -helices, as confirmed by the FTIR spectroscopy (Figure 7a).

3.6. Membrane separation performance

The membranes were tested in dialysis and nanofiltration experiments. Figure 8a shows the hydrolyzed-1 membrane performance (cast from 27 wt% PS_{31k}-*b*-PBLG_{20k} in pyridine and hydrolyzed) in dialysis tests. The y-axis is the ratio of permeation concentration normalized by the initial feed concentration. Small molecules, such as phenylalanine, vitamin B₁₂, and vitamin B₂, could diffuse through the membrane as the concentration of the solutes in the permeate side increases over time, reaching the osmotic equilibrium, after 60 hours or more. The diffusion of vitamin B₂ and phenylalanine, which are smaller than 380 g/mol, was faster than that of vitamin B₁₂ (1355 g/mol). Both vitamin B₂ and B₁₂ have zero net charge at the testing pH=7. The isoelectric points of phenylalanine and cytochrome-c are respectively 5.5 and 9.6. They are therefore respectively negative and positive under the test conditions. The membrane could completely retain cytochrome-c, which was not detectable in the permeate over a long dialysis period. This indicates that the channel diameter of the hydrolyzed self-assembled membranes was smaller than 3.2 nm, the molecular diameter of cytochrome-c⁷⁹. The nanopores of the non-hydrolyzed membrane were clearly narrower and no transport of vitamin B₁₂ and phenylalanine from the feed to the permeate side was detected over dialysis periods longer than two days.

Figure 8. (a) Permeate/Feed concentration ratios as a function of time for several solutes in dialysis tests of the hydrolyzed-1 membrane (27 wt% PS_{31k}-*b*-PBLG_{20k} in pyridine solution cast, immersed in water and hydrolyzed); the top left image shows the dialysis set up; (b) rejection profile of neutral and charged molecules using hydrolyzed-2 membranes (hydrolyzed spin coated 5 wt% PS_{31k}-*b*-PBLG_{20k} solution in dioxane) in nanofiltration tests; the top right image shows the typical retentate and permeate.

The second type of the membrane (hydrolyzed-2), prepared by spin coating of 5 wt% copolymer solutions in dioxane, followed by exposure to acid vapor, was tested in nanofiltration dead-end cell at 8 bar transmembrane pressure. The membrane exhibited a pure water permeance of $3.5 \pm 0.7 \text{ L m}^{-2} \text{ h}^{-1} \text{ bar}^{-1}$. The water permeance of the non-hydrolyzed membrane was around 0.1 L m^{-2}

$\text{h}^{-1} \text{bar}^{-1}$. The conversion of PBLG to the more hydrophilic polymer, PLGA, increased the water permeance 35 times. The selectivity of the the hydrolyzed membranes was further investigated under these testing conditions. The retention of cytochrome-c was almost 100%. The rejection of PEG with comparable or higher molecular weight was lower than 80 %. The reason for that is that PEG is a linear and neutral molecule and could enter the lamellar channels easier than the globular cytochrome-c molecules. Although the solute molecular weight and corresponding retentions are the most applied parameters to characterize the nanofiltration membrane selectivity and the most frequently used in the literature, the estimation of other size parameters, such as Stokes diameter or the equivalent molar diameter⁸⁰, is reported to supply a more precise information, particularly when the used solutes are not fully simetric and globular. In addition to the size, the selectivity of nanofiltration membranes can be highly influenced by charge. The hydrolyzed membrane was permeable for the neutral vitamin B₁₂ (1355 g/mol) with less than 20 % retention, but rejected almost 100 % of the smaller, negatively charged dye, Brilliant Blue (826 g/mol). The high rejection was due to the additional charge repulsion effects, provided by the carboxylic acid groups of the hydrolyzed membrane (Figure 8b).

Table 2 summarizes the performance of a series of commercial nanofiltration membranes, which have been carefully optimized. The Nitto NTR-7450 membrane has a performance close to that reported here. It has a molecular weight cut-off (MWCO) around 1000 g/mol. It rejects 88.2 % of Eosin with molecular weight 646 g/mol⁸⁰ with a water permeance of $4.3 \text{ L m}^{-2} \text{ h}^{-1} \text{ bar}^{-1}$. The water permeation is highly dependent on the thickness of the membrane selective layer, which imposes the highest flow resistance. The thickness of the membranes prepared in this work by solution casting and spin coating was respectively 680 nm and 340 nm. Table 2 lists estimated values of effective thickness for the commercial membranes⁸¹. There is a large variation in the thickness of

artificial channels layers reported in the literature. For a fair comparison, the thickness needs to be clearly taken into consideration. The performance characterization in this work was evaluated in dead-end filtration cells, under pressure, in a procedure analogous to that frequently applied to commercial membranes in the industry. The comparison with non-commercial systems of artificial water and ion channels is not always straightforward, also because in many cases the methods adopted for the characterization are far from the traditional one applied to membranes. When considering future applications, the membrane needs to be robust. It is important to mention that the membrane reported here had good mechanical stability, with no compaction under the operating pressure (8 bar), maintaining a stable flux during a twelve-hour filtration test.

Table 2. Performance of commercial nanofiltration membranes⁸⁰⁻⁸⁵.

Membrane	Effective thickness (μm)	Permeance ($\text{L m}^{-2} \text{ h}^{-1} \text{ bar}^{-1}$)	MWCO (g/mol)
Desal 5DK	1.7-2.6	4.2-5.4	200
NTR-7450	3.9-5.5	3.6	600-1000
NF-270	0.74	10.6-11.5	200-400
Desal 5DL	2.5	5.8	400
Nadir NP030		1.7	520
Desal G-5	40.5	1.23	1000

Our research demonstrates the potential of creating artificial channels with a hierarchically self-assembled structure based on hybrid biomaterials containing α -helical polypeptide. We have shown the influence of two different types of solvents that interact differently with the helices and

led to two morphologies exhibiting tunable molecular separation. The hydrolysis method proposed in this work could tune the membrane performance and make it more permeable for water, while keeping the helical morphology. Further future optimization may include the development of even thinner membranes, using methods such as interfacial polymerization or dip-coating.

4. Conclusions

A rod-coil block copolymer, PS-*b*-PBLG, has been investigated in this work for the preparation of phase inversion and spin coat membranes with a self-assembled structure forming nanochannels. Dense and thick copolymer films prepared from solutions in 1,1,2-trichloroethane, dried in oven at 100 °C for 3 days, have hierarchical lamellar bulk morphology, with a confined hexagonally arranged helical structure relative to the PBLG block inside the PBLG lamellae phase, confirmed by high-resolution WAXD and FTIR measurements. The membranes prepared by casting a solution in pyridine and immersing in water have a feather-like zigzag structure. Membranes made by spin coating a solution in dioxane have a vertically-aligned lamellar structure forming continuous lamellar channels. Dialysis experiments were characterized by high retention of cytochrome-c and effective diffusion of smaller molecules, such as amino acid, vitamin B₂, and B₁₂. The spin-coated membranes had a high rejection of cytochrome-c and the negatively charged dye Brilliant Blue, while allowing the neutral vitamin B₁₂ to pass through. The lamellar channels exhibited a lower rejection of PEG compared to cytochrome-c of similar molecular weight, indicating that linear molecules could pass through the membrane better than globular ones. Our efforts support the further development of bioinspired membranes based on polypeptide copolymers as an excellent candidate to form scalable artificial channels with a hierarchical

structure, aiming at applications where the high purity of products is essential, such as in the biotech, and pharmaceutical industry and water purification.

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Supporting Information

TEM images of dense films (Figure S1)

AFM images of spin coated PS-*b*-PBLG films (Figure S2)

XRD profile of PS-*b*-PBLG films (Figure S3)

Rheology profiles of PS-*b*-PBLG solutions (Figure S4)

Fitting procedure of the experimental scattering data for spin-coated films (Figure S5)

SEM images of membranes before and after hydrolysis (Figure S6)

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