

Polypyrrole-Supported Membrane Proteins for Bio-Inspired Ion Channels

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ABSTRACT

Biomedical platforms constructed by immobilizing membrane proteins in matrices made of synthetic organic polymers is a challenge since the structure and function of these proteins are affected by environmental conditions. In this work an operative composite that regulates the diffusion of alkali ions has been prepared by functionalizing a supporting matrix made of poly(N-methylpyrrole) (PNMPy) with a β -barrel membrane protein (Omp2a) that forms channels and pores. The protein has been unequivocally identified in the composite and its structure has been shown to remain unaltered. The PNMPy-Omp2a platform fulfills properties typically associated to functional biointerfaces with biomedical applications (*e.g.* biocompatibility, biodegradability and hydrophilicity). The functionality of the immobilized protein has been examined by studying the passive ion transport response in presence of electrolytic solutions with Na^+ and K^+ concentrations close to those found in blood. Although the behavior of PNMPy and PNMPy-Omp2a is very similar for solutions with very low concentration, the resistance of the latter decreases drastically when the concentration of ions increases to ~ 100 mM. This reduction reflects an enhanced ion exchange between the biocomposite and the electrolytic medium, which is not observed in PNMPy, evidencing that PNMPy-Omp2a is particularly well suited to prepare bio-inspired channels and smart biosensors.

Keywords: Bioinspired channels; Electroactive polymer; Electrochemical impedance spectroscopy; Ion exchange; Functional biointerfaces

INTRODUCTION

The development of hybrid surfaces made of synthetic polymers and biomolecules for biomedical applications has the potential to revolutionize the treatment of a wide variety of medical conditions. Due to their excellent electrochemical, chemical and optical properties¹⁻³ conjugated conducting polymers (CPs) are promising candidates for biomolecule immobilization and subsequent formation of one- and two-dimensional composites for biomedical devices (*e.g.* biosensors, electrochemical actuators, substrates for tissue engineering and nanowires).⁴⁻¹⁰

On the other hand, biological membranes (lipid bilayers) are unique natural interfaces with exceptional solute transport selectivity and permeability properties, which are mainly due to the embedded membrane proteins (MPs). MPs participate in various cellular processes, such as signaling biochemical cascades, cell-to-cell communication, membrane fusion and ion transport.^{11,12} MPs play a central role in controlling a wide array of gradients such as chemical, electrical, and mechanical gradients and are responsible for cell structure during key events such as cellular division. Furthermore, many signal-transduction processes between cells, such as neurons and muscle tissue, rely on the gating of ion channels (passive transport) and pumps (active transport), which allow the flow of ions across the biomembrane. In these processes, MPs act as channels that move specific molecules and ions into and out of the membrane, and their function is triggered by ligand binding or changes in the transmembrane potential.¹³

Considering that folding processes in MPs of lipid bilayers represent an important challenge in structural biology,^{14,15} it is not surprising that the proper immobilization of purified MPs in platforms for biomedical applications, as for example micro- and nano-arrays, results in a very difficult task. Thus, drastic changes in natural environmental

conditions affect MPs structure, influencing their activity.¹⁶ In addition to that, the complex nature of proteins, as well as their insolubility in aqueous solutions, and instability in the presence of detergents strongly complicate the fabrication of protein-integrated devices based on MPs. Despite these limitations and following biomimicking approaches, some advances have been reported using polymers as soft substrates,¹⁷⁻²⁵ even though only a few of them involved CPs.^{24,25} In a recent study, Arun and Narayan²⁶ anchored bacteriorhodopsin, a MP that functions as a light-driven proton pump, onto conducting surfaces of poly(3,4-ethylenedioxythiophene) (PEDOT) and polyaniline (PAni). More recently, Della Pia *et al.*²⁵ used electropolymerization and amphipatic polymers to functionalize micro- and nano-surfaces with MPs. More specifically, these authors used gold surfaces selectively modified by electrogeneration of polypyrrole (PPy) in the presence of biotin, and the strong affinity of biotin for avidin was exploited to immobilize streptavidin proteins.²⁵ These hybrid platforms were proposed to be particularly suited to fabricate specific antibody bionanosensors. Because of these recent advances, composites combining CPs and MPs are expected to be functional materials with advanced properties and applications.

In this work, a CP-MP nanocomposite with regulated channeling activity has been prepared and characterized. The trimeric outer MP Omp2a from *Brucella melitensis*²⁶ has been selected for this purpose. This β -barrel protein forms pores that allow the diffusion not only of ions but also of molecules as large as 667 Da (*e.g.* nutrients and antibiotics). Indeed, pores are larger in Omp2a than in its homologue Omp2b (85% sequence identity and both encoded in the same genetic locus).²⁷ Although the function of Omp2a is still poorly understood at the structural level, as the 3D structure of *Brucella* β -barrel MPs is not known yet, very recent refolding studies suggested that Omp2a first refolds under a monomeric form and then self-associates into a trimeric

state.^{28,29} On the other hand, poly(N-methylpyrrole) (PNMPy) is the CP chosen to act as the supporting matrix for Omp2a. This PPy derivative displays a compact structure³⁰ and relatively infrequent formation of chemical cross-links when prepared under controlled conditions.³¹ PNMPy has been successfully used in different biomedical and biotechnological applications, as for example the detection of glucose,^[32] neurotransmitters⁶ and opioids.³³

Herein, we describe the procedure to successfully prepare PNMPy-supported MP composites, and the characterization of their surface, electrochemical and biomedical related properties. Subsequently, we demonstrate the potential of this new material to be used as electroactive stable synthetic membranes with enhanced ion permeability for biomedical applications by means of electrochemical impedance spectroscopy (EIS), and using different electrolytic solutions with K^+ and Na^+ concentrations similar to those found in blood. The observed passive ion transport properties indicate that PNMPy-Omp2a system is well suited for the preparation of smart bio-inspired interfaces. Specifically, the ionic transport exerted by PNMPy-Omp2a has wide potential applications in nanofluidics, energy conversion and biosensors.

METHODS

Experimental procedures related with the expression and purification of the trimeric Omp2a outer membrane protein from *Brucella Melitensis*, the refolding of this protein, the synthesis of PNMPy and PNMPy-Omp2a films and some of the measurements, as for example thickness, cyclic voltammetry (CV), circular dichorism (CD), spectroscopic, X-ray photoelectron spectroscopy (XPS), UV-vis spectroscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM), are described in the Electronic Supporting Information.

Wettability. Contact angle measurements were carried out using the water drop method. Images of 0.5 μL distilled water drops were recorded after stabilization with the equipment OCA 15EC (DataPhysics Instruments GmbH, Filderstadt). The software SCA20 was used to analyze the images and determine the contact angle value, which was obtained as the average of at least six independent measures for each sample.

Enzymatic degradation. PNMPy-Omp2a films were immersed in 1 mL of phosphate buffered saline solution (PBS) supplemented with 0.1 mg mL^{-1} of sodium azide (which allowed us to prevent contamination) and 50 $\mu\text{g mL}^{-1}$ of Lipase F-AP15 to examine the enzymatic degradation of the protein. Incubation took place at 37°C in a shaking incubator set at 100 rpm. Samples were analyzed after 1, 4, 8, 24, 48 and 72 hours. After each immersion time, samples were removed from the solution and gently washed with distilled water. Then, samples were dried under vacuum for several days at room temperature before being observed by SEM.

Cellular adhesion. Vero cells (African green monkey kidney epithelial cell line) and Cos-7 cells (African green monkey kidney fibroblast cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air. The cultured medium was changed every two days and, for sub-culture, cell monolayers were rinsed with phosphate buffered saline (PBS) and detached by incubation with 0.25% trypsin/EDTA for 5 min at 37°C. Cell concentration was determined by counting at the Neubauer camera using 4% trypan blue as dye vital. The detached cells with viability $\geq 95\%$ were used for cultures following the conditions for the adhesion assays.

PNMPy-Omp2a and PNMPy films deposited onto steel AISI 316 sheets of 1 cm^2 were placed in plates of 24 wells and sterilized using UV irradiation for 15 min in a

laminar flux cabinet. Samples were incubated with 1 mL of culture medium during 30 min under culture conditions to equilibrate the material. Finally, the medium was aspirated and the material was evaluated for cell adhesion by exposing cells to direct contact with the material surface. An aliquot of 50 mL containing 5×10^4 cells was deposited on the substrate of each well. The plate was incubated under culture conditions for 60 min to promote the cell attachment to the film surface. Finally, 1 mL of the culture medium was added to each well. Controls of adhesion were simultaneously performed by culturing cells on the surface of the tissue culture polystyrene (TCPS) plates and uncoated steel. Cell adhesion was evaluated after 24 hours of culture using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which determines the cell viability. The viability results were normalized to TCPS control as relative percentages.

Results were derived from the average of four replicates ($n=4$) for each independent experiment. ANOVA and Turkey tests were performed to determine the statistical significance, which was considered at a confidence level of 95% ($p < 0.05$).

Electrochemical impedance spectroscopy (EIS). Spectra were taken at open circuit potential (OCP) over the frequency range 10 kHz – 10 mHz with a potential amplitude of 0.05 V using an AUTOLAB-302N potentiostat/galvanostat. Typical potassium and sodium blood concentrations vary from 3.5 to 5.2 mM and 135 to 145 mM, respectively. Besides, K^+ is at a higher concentration in intracellular regions than outside the membrane by two orders of magnitude, whereas the opposite is true for Na^+ or Cl^- ions. Therefore, EIS spectra were collected using different aqueous electrolyte mediums at low and high ionic concentrations for KCl (5 and 100 mM) and NaCl (5 and 140 mM).

RESULTS AND DISCUSSION

Synthesis and characterization of PNMPy-Omp2a

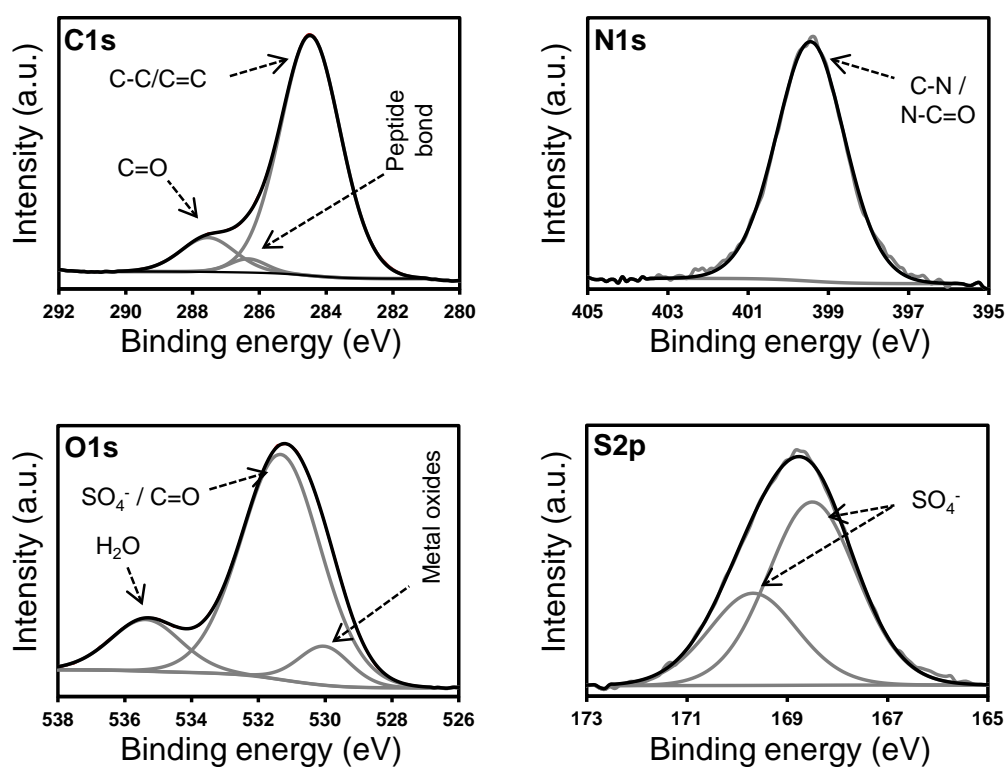
The Omp2a protein was expressed, purified and refolded using the procedure described in the Methods section. The molecular weight and isoelectric point of trimeric Omp2a (367 amino acids) are 113.4 kDa and 4.47, respectively. PNMPy-Omp2a films were prepared by in situ anodic polymerization introducing Omp2a (1 mg mL^{-1}) in the generation medium, which consisted of a 15 mM monomer solution in deionized water with 10 mM of sodium dodecyl sulfate (SDS). Cyclic voltammograms recorded for the anodic oxidation of N-methylpyrrole (NMPy) monomer with and without the MP in the generation medium (ESI) reflected an anodic process with anodic peak potentials of 1.119 and 1.089 V, respectively. According to these results, PNMPy-Omp2a and PNMPy films were deposited onto steel AISI 316 sheets using a potential of 1.10 V and a polymerization time of 20 s. The thickness of PNMPy-Omp2a and PNMPy films, as determined by optical profilometry, is $219 \pm 71 \text{ nm}$ and $298 \pm 72 \text{ nm}$, respectively.

The chemical composition of PNMPy-Omp2a composite has been initially characterized by XPS, as shown in Table 1 and Figure 1. Spectra associated to control PNMPy are provided in the ESI. As expected, the oxygen content in the atomic percent composition is higher for PNMPy-Omp2a than for PNMPy because of the presence of the MP (the protein sequence is displayed in the Supporting Information).

Deconvolution of the high resolution XPS spectrum in the C1s region for the composite led to three Gaussian curves that have been attributed to the saturated and conjugated C–C/C=C bonds^{34,35} (284.5 eV) of NMPy units, the C=O bond (287.5 eV) of oxidized NMPy units^{34,36} (*i.e.* N-substituted PPy derivatives typically undergo an oxidation process), and the C–N, C–NH₃⁺ and C=O from peptide bonds (286.3 eV) in the protein.^{37,38}

Table 1. Atomic and Mass Percent Composition of PNMPy and PNMPy-Omp2a.

		C1s	N1s	O1s	S2p
PNMPy	Atomic composition	70.7	7.9	18.9	2.5
	Mass composition	63.3	8.3	22.5	5.9
PNMPy-Omp2a	Atomic composition	53.9	3.6	39.4	3.1
	Mass composition	45.4	3.5	44.2	6.9

**Figure 1.** High-resolution C1s, N1s, O1s and S2p XPS spectra for PNMPy-Omp2a. Peaks from deconvolution are also displayed (grey lines). Intensity is displayed in arbitrary units.

The high resolution N1s spectrum of control PNMPy (Supporting Information) shows two peaks centered at 399.9 eV (6.9%) and 401.4 eV (1.0%), which have been assigned to the C–N bonds of NMPy units involving neutral and charged nitrogen,

respectively.^{34,39} The ratio $1.0/(6.9+1.0) = 0.13$ represents the fraction of charged nitrogen, which can be associated to the doping level of the electropolymerized material (Supporting Information). In contrast, PNMPy-Omp2a presents a single peak at 399.5 eV due to the overlapping of the binding energies associated to the C–N bond of NMPy units and the N–C=O (amide) of the protein, the latter being also around 400 eV.^{37,40} The annihilation of the peak associated to the charged nitrogen atom indicates that the doping level of the CP in the composite is lower than in the control, which is fully consistent with the low polymerization charge registered for the composite during the electrogeneration process. Furthermore, the absence of a peak at around 401.5 eV reflects that the amount of positively charged residues in Omp2a is very low.^{37,38} Thus, Arg and Lys are essentially present in the composite as uncharged species, photoemitting at the same region that amides in high resolution N1s spectra.

The high resolution O1s spectra of both control PNMPy (Supporting Information) and PNMPy-Omp2a samples show three peaks. Those of PNMPy have been attributed to the sulphate groups of SDS (531.6 eV), the C=O of oxidized NMPy units (533.7 eV) and the metallic oxides on the steel substrate (529.1 eV). The $C/O_{531.6}$ and $N/O_{531.6}$ composition ratios, where C and N refer to the total compositions displayed in Table 1 and $O_{531.6}$ corresponds to the relative contribution to the total atomic O percent composition of the peak at 531.6 eV (Supporting Information), are 4.3 and 0.5, respectively. For PNMPy-Omp2a, the peaks detected in the high resolution O1s spectra appear at 531.3, 535.4 and 530.0 eV. The first one involves not only the sulfate groups of SDS but also the C=O from the peptide bond.^{37,38} Accordingly, the $C/O_{531.3}$ and $N/O_{531.3}$ ratios are 1.8 and 0.1, respectively, which are lower than in control PNMPy. The peak at 535.4 eV has been attributed to the water oxygen from moisture of the

samples,^{41,42} while the peak at 530.0 eV corresponds to metallic oxides at the substrate surface.

Finally, the S2p high resolution spectrum registered for PNMPy and PNMPy-Omp2a samples are very similar. Both show the spin-split sulfur coupling at 168.3 eV (S2p_{3/2}) and 169.5 eV (S2p_{1/2}), which is associated to the sulfate groups of the surfactant. Moreover, the S2p spectrum for PNMPy (Supporting Information) also present small peaks at 170.4 eV (S2p_{3/2}) and 171.5 eV (S2p_{1/2}), which correspond to metallic sulfates at the steel surface.

UV-vis absorption spectra were registered in the 200-350 nm range to further confirm the presence of Omp2a in the PNMPy-Omp2a composite, which was achieved by identifying the bands associated to the absorption of the peptide group and side groups of aromatic amino acids. PNMPy-Omp2a films were removed from the steel substrate, and suspended in water to conduct the UV-vis measurements. Comparison recorded spectra, which are displayed in Figure 2, indicates typical absorptions of the peptide group and the aromatic side chains of Trp, Tyr and Phe residues were detected as shoulders in the spectrum of PNMPy-Omp2a suspensions when compared to the spectra recorded for the control protein mediums (Figure 2). Thus, peptide groups typically absorb between 180 and 230 nm, while the aromatic side chains of tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) amino acids absorb in the 240-300 nm range.⁴³ Accordingly, spectra of as obtained Omp2a, dialyzed Omp2a and the Omp2a-containing polymerization medium show absorption maxima at such regions. More specifically, the shoulders detected at 289 and 260 nm come from the Trp (10 residues in Table S1) and Phe (18 residues in **Table S1**) residues, respectively.⁴³ The absorbance of the disulfide bond formed by the two Cys residues (Table S1) at 260 nm also

contributes to the latter shoulder. All these peaks appear as shoulders in the spectrum of PNMPy-Omp2a suspension. Protein absorbance was zero above 310 nm.

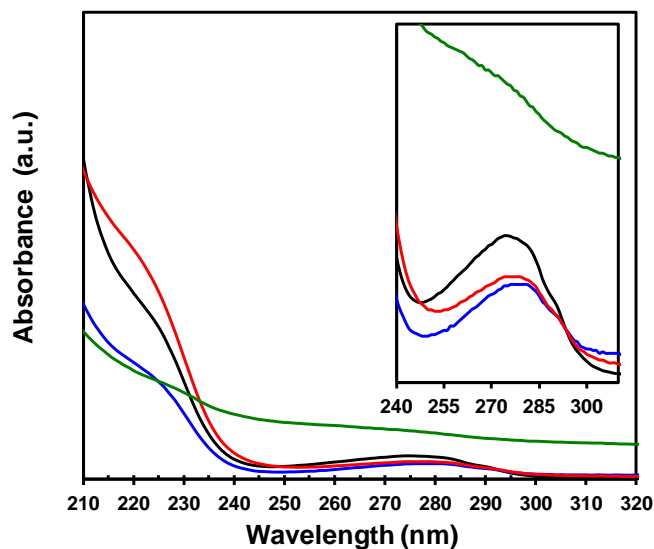


Figure 2. UV-vis spectra for as obtained Omp2a (black), dialyzed Omp2a (blue), the Omp2a-containing polymerization medium (red) and a PNMPy-Omp2a suspension (green).

Although XPS and UV-vis results confirm the correct immobilization of Omp2a into the PNMPy matrix during the electrogeneration process, the native structure of the protein in the PNMPy-Omp2a composite was successfully corroborated by circular dichroism (CD) spectroscopy. Figure 3 compares the CD spectra for the expressed protein as obtained, after dialysis, and once incorporated into the NMPy-containing polymerization medium (Supporting Information). As it can be seen, in all cases the profile exhibits a band at ~ 217 nm, which is typically found in β -stranded proteins.⁴⁴ This characteristic band is preserved in the CD spectra of the composite (Figure 3d), evidencing that the native β -sheet secondary structure is preserved when the pore-forming protein is immobilized into the polymeric matrix. Therefore, the operative and functional state of the PNMPy-Omp2a interface is ensured by having the MP Omp2a in its folded and/or assembled form.

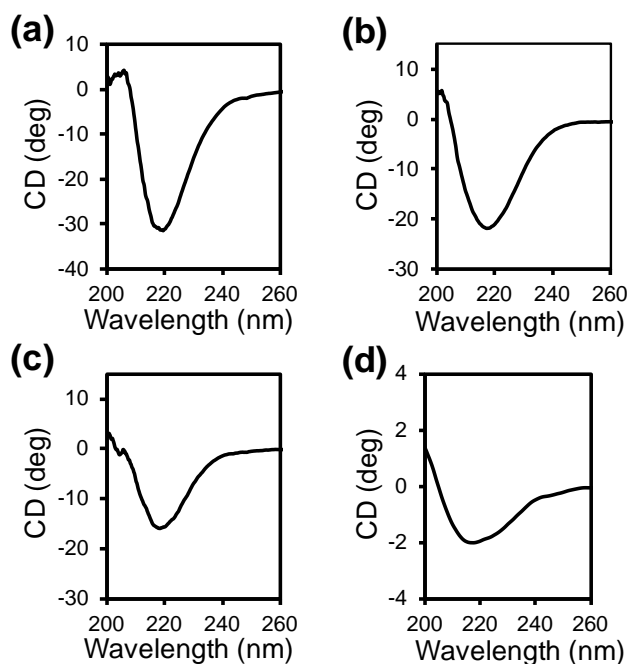


Figure 3. CD spectra for (a) as-obtained Omp2a; (b) dialyzed Omp2a; (c) Omp2a-containing polymerization medium; and (d) PNMPy-Omp2a composite. Protein concentration is 0.05 mg mL^{-1} in all cases with the exception of PNMPy-Omp2a composite.

On the other hand, the UV-vis absorption spectra of steel-supported control PNMPy and PNMPy-Omp2a films (Figure 4) indicate that the protein affects the oxidation process of the CP during the electrochemical polymerization, as it has already been mentioned. The first absorption band corresponds to the $\pi\text{-}\pi^*$ transition of aromatic rings ($\lambda_{\text{max}} = 338$ and 377 nm for PNMPy and PNMPy-Omp2a, respectively). The band gap energy, which was determined from the onset wavelength,⁴⁵ is 2.44 and 2.30 eV for PNMPy and PNMPy-Omp2a, respectively. The second absorption band is related to the first stages of the oxidation process and has been assigned to the formation of polarons. This band is clearly defined for PNMPy at $\lambda_{\text{max}} = 567$ nm, whereas for PNMPy-Omp2a samples this oxidation process is only identified as weak shoulder at ~ 565 nm in the UV-vis spectrum. This feature is fully consistent with XPS results, which suggested that

the doping level of the composite was lower than that of PNMPy. No absorption band in the range of 650-700 nm, which is typically assigned to very high oxidation levels (bipolaric states),³⁶ is displayed by either of these two systems.

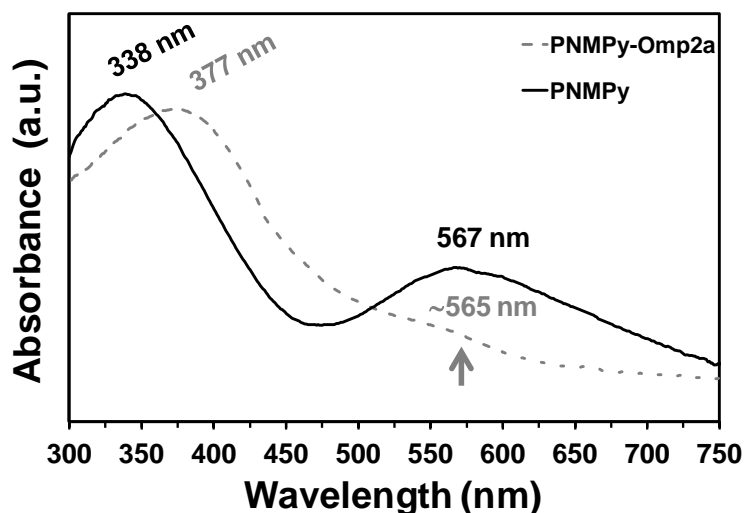


Figure 4. UV-vis spectra for control PNMPy and PNMPy-Omp2a films deposited onto steel sheets. The values of λ_{\max} are indicated.

Surface properties

To better understand the passive ion transport response of PNMPy-Omp2a composite, its surface properties were evaluated.

Surface morphology and topography of PNMPy-Omp2a were deeply examined by SEM and AFM, respectively. Control PNMPy films (Figure 5) show a very compact and dense globular structure that is consistent with a homogeneous distribution of the polymer chains. RMS roughness of PNMPy (23 ± 9 nm) is similar to that of steel substrate (18 ± 5 nm). These features indicate a multi-directional growing of polymers that is only possible through the formation of some chemical couplings involving the β - and β' -positions of the repeat units. Accordingly, the transport of ions through this

partially cross-linked polymeric matrix is expected to be hindered by the densely packed structure.

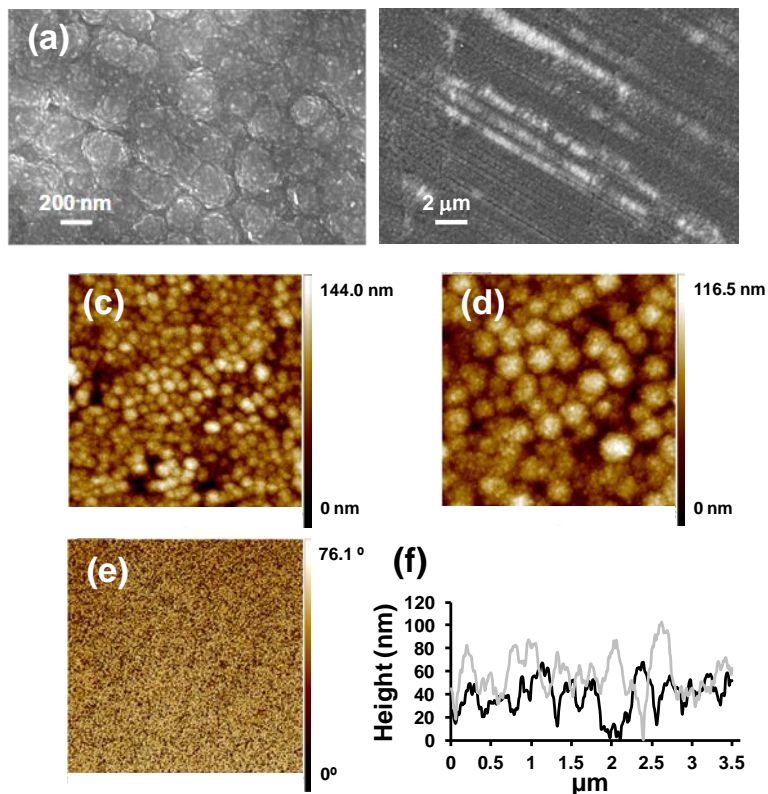


Figure 5. PNMPy surface characterization: SEM micrograph at (a) 100 kX and (b) 10 kX; (c) 5.0×5.0 and (d) 2.5×2.5 μm^2 AFM height images; (e) 2.5×2.5 μm^2 AFM phase image corresponding to (d); and (f) cross-section profiles obtained from (d). In the cross sectional profiles the black line corresponds to the diagonal drawn from the down left corner to the up right corner, while the grey line corresponds to the diagonal drawn from the up left corner to the down right corner

Incorporation of the protein provokes significant changes in the surface morphology. SEM micrographs and AFM height images of PNMPy-Omp2 (Figure 6) show elements of both submicrometric and micrometric dimensions with very homogeneous and smooth texture, and without the nodular outcrops typically found in PNMPy aggregates. These particles correspond to folded protein molecules, as evidenced by comparison with SEM micrographs derived from dialyzed Omp2a solutions (Supporting Information).

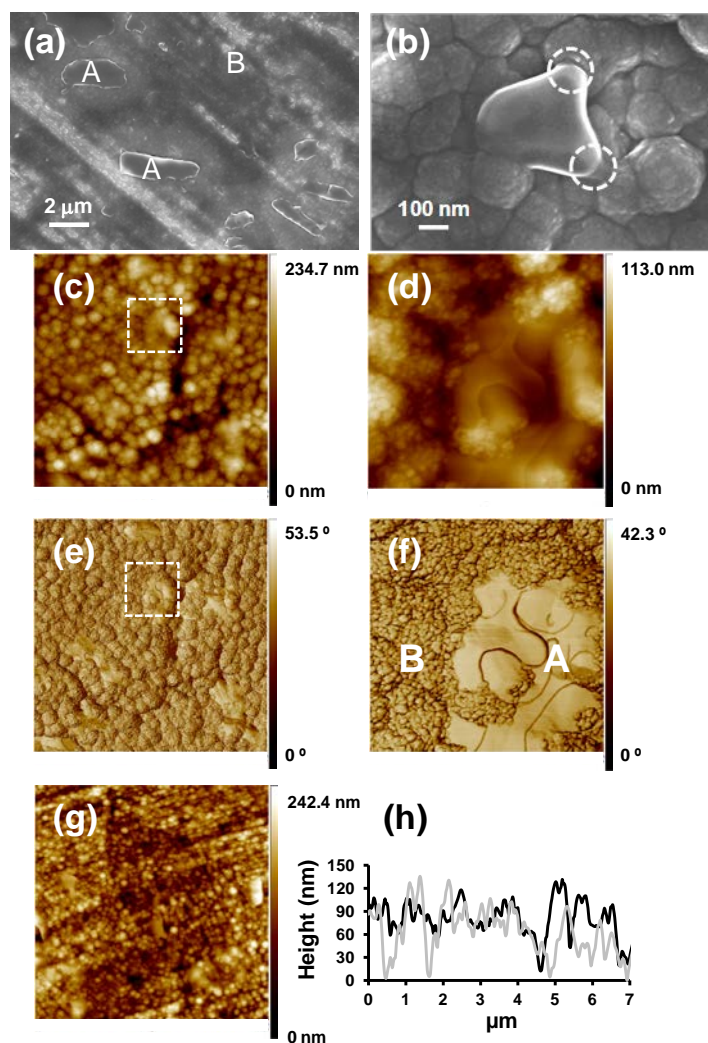


Figure 6. PNMPy-Omp2a surface characterization: (a) Low and (b) high magnification SEM micrographs; (c) $5.0 \times 5.0 \mu\text{m}^2$ and (d) $1.0 \times 1.0 \mu\text{m}^2$ AFM height images; (e) $5 \times 5 \mu\text{m}^2$ and (f) $1.0 \times 1.0 \mu\text{m}^2$ AFM phase images corresponding to (c) and (d), respectively; (g) $10.0 \times 10.0 \mu\text{m}^2$ AFM height image; and (h) cross-section profiles obtained from (c). Dashed circles in (b) illustrate the connection between PNMPy and Omp2a particles. Squares in (c) and (e) indicate the regions displayed in (d) and (f), respectively. Labels A and B in (f) indicate the PNMPy and Omp2a phase, respectively. In the cross sectional profiles the black line corresponds to the diagonal drawn from the down left corner to the up right corner, while the grey line corresponds to the diagonal drawn from the up left corner to the down right corner.

Although PNMPy and Omp2a proteins are inter-connected in the composite (Figure 6b), well-defined morphological and texture differences reveal a complete phase separation. The latter fact is corroborated by the high contrasts observed in AFM phase images, which allow us to distinguish between the two phases in the composite. The smooth areas in Figure 6f (labeled as A) correspond to the Omp2a phase, whereas the

rough and irregular areas (labeled as B) represent the PNMPy phase. The mean RMS roughness, which was measured considering several $5 \times 5 \mu\text{m}^2$ windows, increases to $31 \pm 5 \text{ nm}$ upon the incorporation of the Omp2a protein.

Overall, morphological and topographical observations reveal that Omp2a proteins are well-distributed throughout the PNMPy-OMP2a composite surface, thus establishing pore structures. Although the PNMPy phase is characterized by a dense and compact matrix, MP functionality is expected to enhance the passive ion transport across the artificial membrane creating permanent ion channels.

Furthermore, contact angle measurements indicate that the surface of both steel ($\theta = 74^\circ \pm 7^\circ$) and PNMPy ($\theta = 67^\circ \pm 4^\circ$), while PNMPy-Omp2a water-wettability increases significantly upon the incorporation of the MP ($\theta = 32^\circ \pm 8^\circ$). This effect is noticeably more pronounced for PNMPy-Omp2a than for composites prepared using PNMPy and other proteins (Supporting Information), as for example bovine serum albumin (BSA). This indicates that not only Omp2a is immobilized on the CP matrix, but it retains its natural disposition where the protein hydrophilic regions are positioned towards the outer region of the membrane, just as in biological environments. Although the isoelectric point of BSA (4.7) is very similar to that of Omp2a (4.5),⁴⁶ the contact angle value for PNMPy-BSA films prepared under similar experimental conditions ($\theta = 54^\circ \pm 7^\circ$) represents a reduction of only 20 % with respect to PNMPy.

Electrochemical properties

Cyclic voltammetry studies were run to determine the influence of these new formed ion channels in the electrochemical properties of the PNMPy-Omp2a composite. Firstly, as occurred for the monomer (Supporting Information), the anodic process at around $\sim 1.1 \text{ V}$ is less marked for PNMPy-Omp2a than for PNMPy (Figure 7). The ability to

exchange charge reversibly, hereafter denoted electroactivity, increases with the similarity between the anodic and cathodic areas of the first control voltammogram. Thus, the electroactivity of PNMPy-Omp2a is 10% higher than that of PNMPy. This result, which is practically independent of the scan rate (Supporting Information), evidences the role played by the pore-forming protein in maintaining a flow of ions in and out of the CP matrix.

Besides, the electrochemically-induced reduction of the thickness has been used to evaluate the relative porosity (*Por*) of PNMPy-Omp2a and PNMPy systems. Specifically, *Por* (in %) has been estimated as the difference between the thicknesses of the films as prepared and after 15 consecutive redox cycles (193 nm \pm 51 nm and 253 nm \pm 93 nm for PNMPy-Omp2a and PNMPy, respectively) relative to the thickness of the as prepared film. The values obtained for *Por* are very similar for the two systems (*i.e.* 12% and 15%, respectively). Therefore, the difference observed in electroactivity and LEA values between PNMPy and PNMPy-Omp2a systems are mainly due to the operative state of Omp2a.

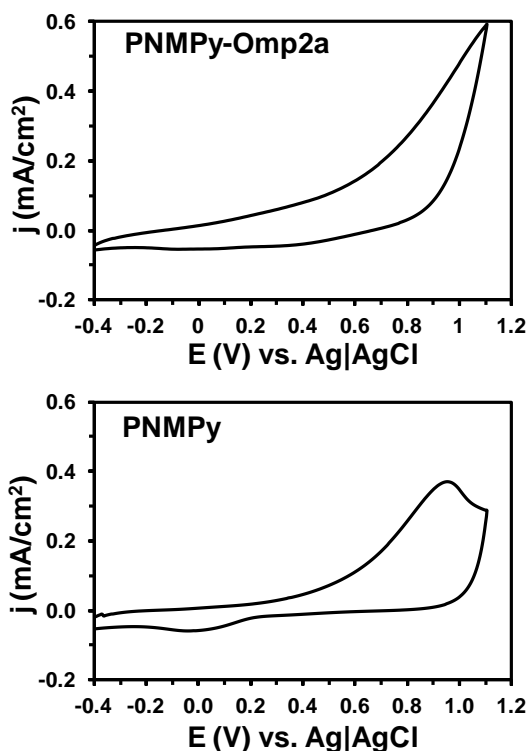


Figure 7. Cyclic voltammograms of PNMPy-Omp2a and PNMPy in aqueous solution with 0.1M LiClO₄. Initial and final potentials: -0.40 V; reversal potential: 1.10 V. Scan rate: 25 mV s⁻¹.

SEM micrographs recorded for PNMPy-Omp2a samples submitted to 15 redox cycles (Figure 8) reveal significant structural changes, which mainly affect the elements associated to the protein phase. Specifically, after such amount of cycles, the smooth homogeneous particles (Figures 8a and 8b) disaggregate into a heterogeneous distribution of small spherical aggregates that have been associated to unfolded protein molecules. Accordingly, AFM images suggest that the 3D structure of the protein collapses, and only the space once occupied by the Omp2a remains in the PNMPy surface. However, this aggressive electrochemically-induced degradation process is not expected to occur when assessing the ion transport response of PNMPy-Omp2a by EIS since it is a non-destructive technique. Consequently, the functionality of the biomolecule in the composite is assured for passive ion transport applications (see below).

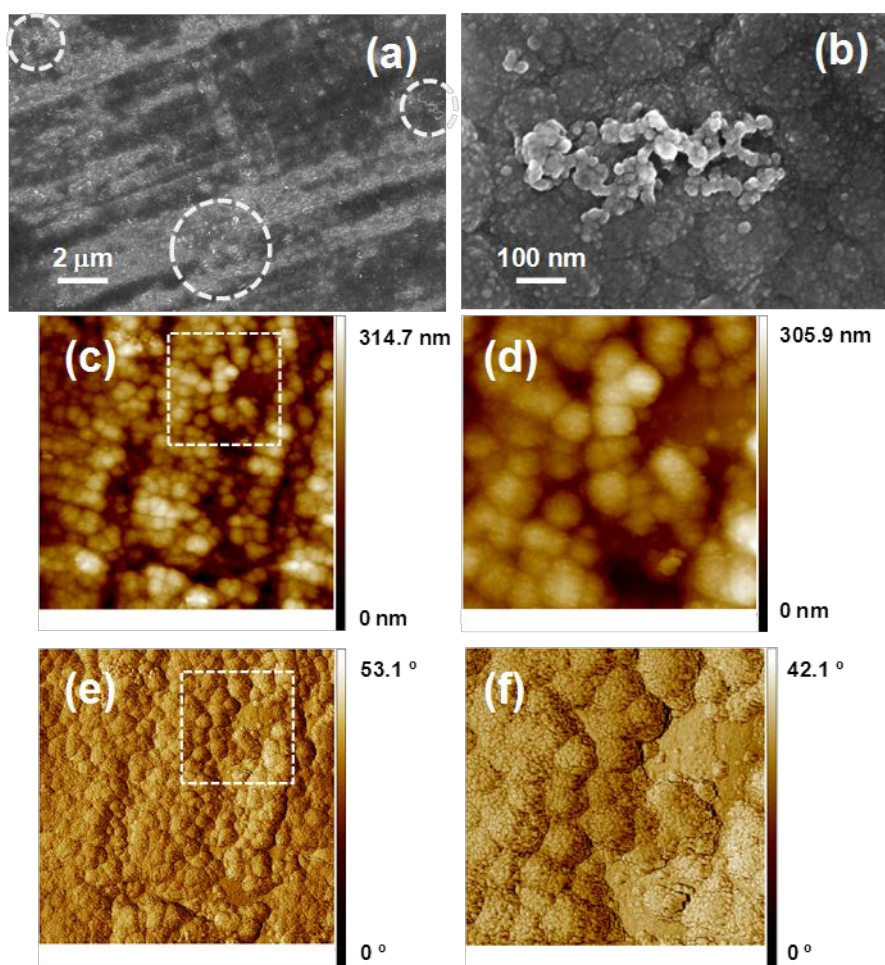


Figure 8. Surface characterization of PNMPy-Omp2a after 15 consecutive oxidation-reduction cycles: (a) Low and (b) high magnification SEM micrographs; (c) $5 \times 5 \mu\text{m}^2$ and (d) $2.0 \times 2.0 \mu\text{m}^2$ AFM height images; (e) $5 \times 5 \mu\text{m}^2$ and (f) $2.0 \times 2.0 \mu\text{m}^2$ AFM phase images corresponding to (c) and (e), respectively. Dashed circles in (a) illustrate the regions with unfolded protein. Squares in (c) and (e) indicate the regions displayed in (d) and (f), respectively.

Biodegradability and biocompatibility

CP-based platforms for biotechnological and biomedical applications typically require biodegradability and/or biocompatibility to minimize their impact on the environment and/or the adverse reaction of the body.⁴⁷ Accordingly, bearing those requirements in mind, and before performing EIS measurements, both enzymatic degradation and cell viability tests have been conducted to validate the integrated-protein composite for biointerface applications.

In the particular case of PNMPy-Omp2a, excessively rapid enzymatic degradation of the MP could greatly influence the applicability of the composite when used in vivo physiological mediums. In consequence, the enzymatic biodegradability of Omp2a in the composite was investigated by monitoring the protein morphology change induced during 72 h of continuous immersion in a Lipase-containing solution. Samples extracted after 1, 4, 8, 24 and 72 h were observed by SEM, and results indicated that severe degradation of the protein started after 24 h of immersion in the enzymatic solution (Supporting Information). Thus, the protein retained the initial aspect and texture in samples extracted after 1, 4 and 8 h. This feature suggests that the CP matrix protects Omp2a aggregates from the action of the enzyme at the earlier stages of degradation. Micrographs of samples extracted after 72 h revealed that the degraded protein is mostly detached from the surface of the films (Figure 9), indicating that the benefits provided by the protein to the composite are expected to be null after this time of exposure to aggressive bioconditions.

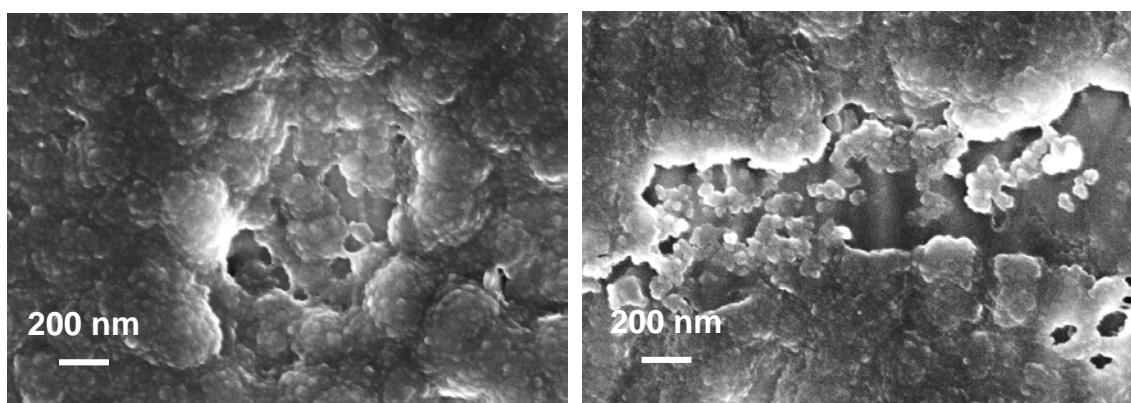


Figure 9. SEM micrographs for PNMPy-Omp2a composite after 72 h of immersion in a Lipase F-AP15 solution.

Quantitative results for cell adhesion assays are displayed in Figure 10a. The number of Vero and Cos-7 cells adhered to the surface of the PNMPy-Omp2a is

significantly higher than that of PNMPy. This tendency is more evident in the case of Vero cells. On the other hand, the adhesion of both Vero and Cos-7 cells on the surface of PNMPy and PNMPy-Omp2a is favored with respect to the TCPS and Steel control substrates, which show similar relative viabilities.

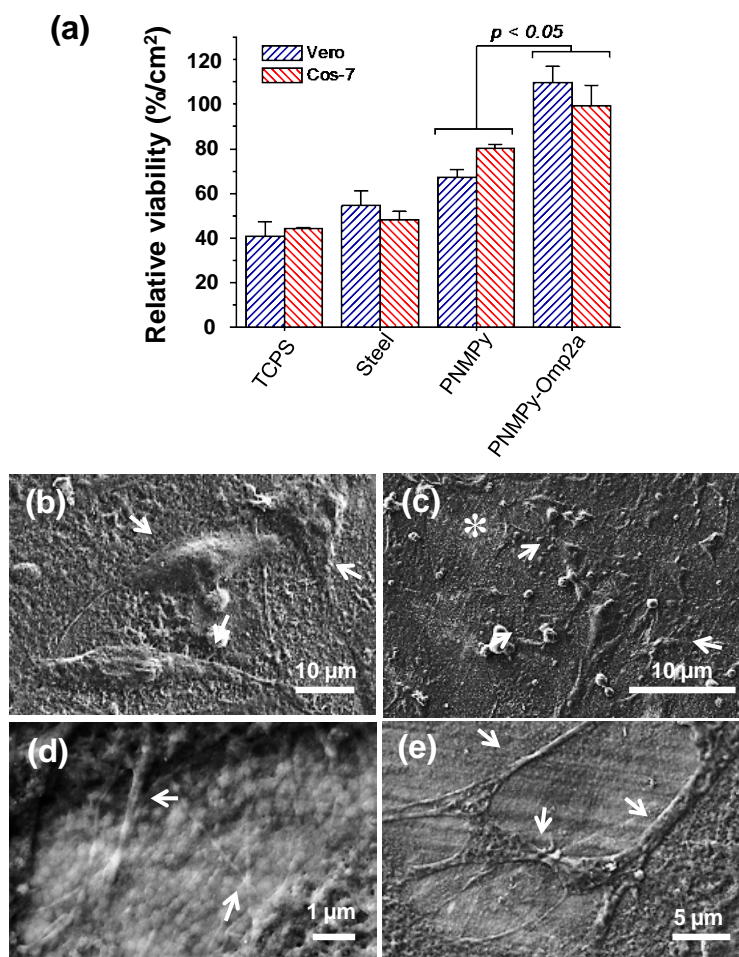


Figure 10. (a) Cellular adhesion onto PNMPy-Omp2a, PNMPy and steel surfaces using Vero (epithelial-like) and Cos-7 (fibroblast-like) cells. The relative viability was established in relation to the TCPS control (tissue culture polystyrene). ANOVA-Tukey test, $p < 0.05$. SEM micrographs of Cos-7 cells seeded for 24 h onto (b) PNMPy-Omp2a and (c) PNMPy surfaces. The asterisk in (c) indicates substrate surface, while arrows in (b) and (c) indicate the cells adhered onto the surface. Micrographs showing cellular interactions (arrows) at cultured PNMPy-Omp2a films: (d) connection sites (filopodia) between Cos-7 cells and the substrate; and (e) intercellular junctions used for cell-cell communication processes.

On the other hand, SEM micrographs show the significant spreading of Cos-7 cells, which form cellular monolayers on the surface of PNMPy-Omp2a (Figure 10b),

whereas large domains without cells appear on the surface of PNMPy (Figure 10c). The actin filaments emerging from the cells and with the barbed ends oriented towards the surface of PNMPy-Omp2a (Figure 10d), which are known as filopodia, are responsible of local adhesion. The grouping of these filaments results in stress fibers, denoted lamellipodia, that are used by cells to move along the substrate. Figure 10e shows typical connections between two Cos-7 cells to establish communication, which is a crucial step in the coordination of differentiation processes in eukaryotic cells. Similar features are displayed in the Electronic Supporting Information for cultured Vero cells. The overall of the results presented in this section indicates that PNMPy-Omp2a is a potential candidate for biotechnological and biomedical applications in which biocompatibility is a requisite.

Ion channels

EIS measurements have been carried out to evaluate the proper function of ion channels and pores formed by the MP in the PNMPy-Omp2a composite.⁴⁸⁻⁵² This methodology provides information not only of the ion transport a complete description of the investigated system, as was recently discussed in a comparative study.⁵³ Impedance was measured as a function of frequency for steel-supported PNMPy and PNMPy-Omp2a films. EIS spectra were registered using different aqueous electrolyte mediums: 5 and 100 mM KCl solutions and 5 and 140 mM NaCl solutions. It should be noted that Na⁺ and K⁺ are two main blood electrolytes, their normal blood levels being 135-145 and 3.5-5.2 mM, respectively. Moreover, the concentration of K⁺ is two orders of magnitude higher in intracellular regions than outside the membrane while the opposite occurs for Na⁺.

Figures 11a-11e compare representative Nyquist plots obtained for PNMPy and PNMPy-Omp2a in different Na⁺- and/or K⁺-containing media. In all cases Nyquist spectra show a semicircle in the high frequency range and a straight ascending line in the low frequency range. The electrical equivalent circuit (EEC) used to fit the experimental data is shown in Figure 11f, where R_S corresponds to the electrolyte solution resistance and R_M represents the ability of the PNMPy-Omp2a system to impede ion transport at the interface between the electrolyte and the membrane. The ECC also includes double layer capacitances from both the membrane and steel (Q_M and Q_{ST}, respectively) and a Warburg impedance element (W), corresponding to the diffusion of water molecules. Therefore, both R_M and Q_M are associated to the overall contribution of the protein-integrated interface. It should be noted that the incorporation of ion channels through the immobilization of the MP is expected to alter the membrane resistance value, R_M. Accordingly, channel activity can be monitored by comparing the membrane resistance of PNMPy and PNMPy-Omp2a in the different media.

Table 2 lists the contribution of each element for PNMPy and PNMPy-Omp2a. Both capacitances were replaced by a constant phase element (CPE) that describes a non-ideal capacitor when the phase angle is different from -90°. The CPE impedance is attributed to the surface reactivity, surface heterogeneity and roughness, which in turn are related to the electrode geometry and porosity. Also, the CPE impedance accounts for the non-uniform diffusion among the films adhered to the electrode surface. The CPE impedance, which has been expressed as $Z_{CPE} = [Q(j\omega)^n]^{-1}$, represents an ideal capacitor and a pure resistor for n= 1 and n= 0, respectively, while it is associated with a diffusion process when n= 0.5. The fitting quality was judged based on the error percentage associated to each circuit component, showing errors lower than 5% in most of the elements.

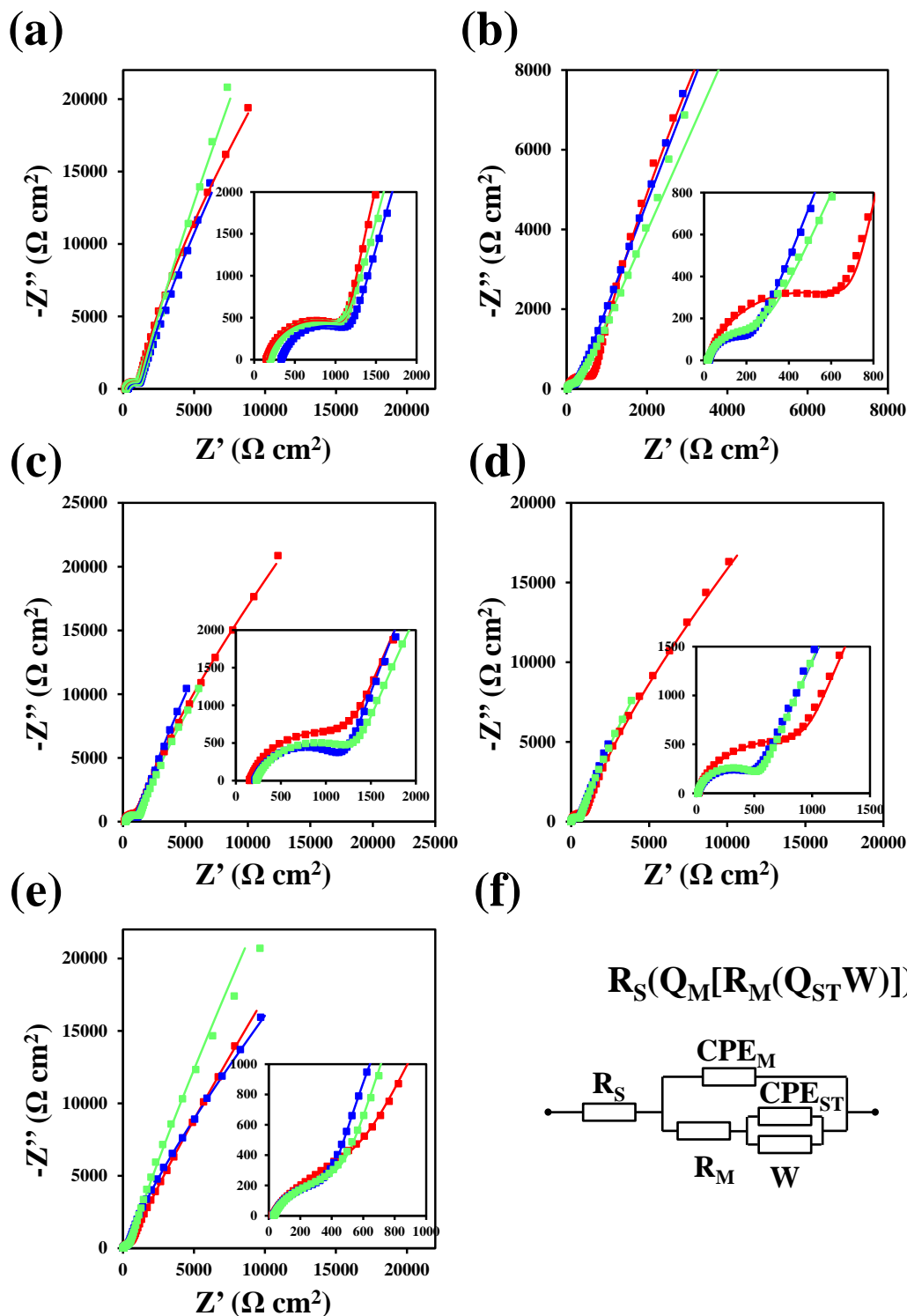


Figure 11. Nyquist plots for PNMPy (red) and PNMPy-Omp2a (blue and green) in an aqueous solution containing: (a) 5 mM K^+ ; (b) 100 mM K^+ ; (c) 5 mM Na^+ ; (d) 140 mM Na^+ ; and (e) 5 mM K^+ and 140 mM Na^+ . (f) Electrical equivalent circuit (EEC) used for fitting experimental data for PNMPy and PNMPy-Omp2a. R_S is the electrolyte resistance, CPE_M and R_M are the membrane constant phase element and resistance, respectively, CPE_{ST} is the steel constant phase element, and finally, W is the Warburg impedance.

EIS results evidence that the pore-forming MP is properly immobilized in the PNMPy matrix, retaining the functionality associated to the β -barrel structure. More specifically, the response of PNMPy and PNMPy-Omp2 films is very similar for both 5 mM K^+ and the 5 mM Na^+ electrolytic media (Figures 11a and 11c, respectively). Accordingly, the R_M values (Table 2), which indicate the facility to exchange ions between the system and the electrolyte solution, are very similar for the control PNMPy and the MP-containing composite. Similarly, the ability to store electrical charge and the diffusion impedance of PNMPy, which are defined by Q_M and W , respectively, are not significantly affected by the incorporation of the protein when the concentration of ions is low. In spite of this, the diffusion of hydrated Na^+ is slightly higher for the MP-composite than for the CP, suggesting that Omp2a is more efficient for the transport of Na^+ than for K^+ .

In contrast, R_M values are lower for PNMPy-Omp2a than for PNMPy in the 100 mM K^+ and 140 mM Na^+ solutions (Figures 11b and 11d). Accordingly, PNMPy-Omp2a promotes the passive ion transport in solutions with high ionic concentrations, thus the MP functionality is enhanced when the concentration of ions in the medium is high. EIS spectra recorded in a solution containing 5 mM K^+ and 140 mM Na^+ , which correspond to the concentrations of such ions in blood, evidence a similar behavior (Figure 11e). Thus, the ion transport resistance of PNMPy ($1037 \Omega \text{ cm}^2$) decreases one order of magnitude when the MP is immobilized in the PNMPy-Omp2a composite ($\sim 600 \Omega \text{ cm}^2$), which activates the transport of ions.

Inspection of the Q_M values obtained for the 100 mM K^+ and 140 mM Na^+ solutions indicates that, as occurred for the solutions with lower concentrations of ions, the MP does not alter significantly the ability to store charge of the CP. Indeed, inspection of

results displayed in Table 2 evidences that such ability is intrinsic to PNMPy since it is practically independent of the concentration and nature of the ions as well as of the Omp2a presence. Comparison of the diffusional impedance values obtained for more concentrated solutions also suggest that Omp2a favors the diffusion of hydrated Na^+ with respect to that of hydrated K^+ . Thus, results for PNMPy indicate that the bulk diffusion of ions through the CP pores is practically independent of both the concentration and nature of ions. Accordingly, although ions affect the R_M of PNMPy, the diffusional activity of this material is not selective. In contrast, the impedance behavior observed for PNMPy-Omp2a evidences that R_M is very sensitive to the ions concentration while W is affected by the nature of the ions. These features indicate that the MP brings not only ion transport capacity but also sensitivity and selectivity to the CP.

With this work, we have successfully produced a protein-integrated system which preserves the native structure and functionality of the MP. The combination of biological elements with synthetic materials is a challenging research field, which requires further understanding of interactions and compatibility between materials. However, as a first step, PNMPy-Omp2a approach has resulted in a biointerface with proven passive ion transport. Interestingly, as Omp2a allows the passive transport of other molecules such as nutrients and antibiotics, PNMPy-Omp2a composite can also be exploited to design biohybrid materials with multifunctional artificial channels for bioapplications.

Table 2. Fitting parameters used to simulate the EIS spectra displayed in Figures **10a-10d** using the electrical equivalent circuit represented in **Figure 10f**: R_S ($\Omega \text{ cm}^2$), R_M ($\Omega \text{ cm}^2$), Q_M ($\text{F cm}^{-2} \text{ s}^{n-1}$), n , Q_S ($\text{F cm}^{-2} \text{ s}^{n-1}$) and W ($\Omega \text{ cm}^2$). For PNMPy-Omp2a, the first and second values provided for each element correspond to the blue and green spectra, respectively. The error percentage associated to each circuit element is included in parenthesis. Fitting parameters used to simulate the EIS spectra displayed in Figure 11e are listed in the ESI.

	PNMPy				PNMPy-Omp2a			
	5 mM K^+	100 mM K^+	5 mM Na^+	140 mM Na^+	5 mM K^+	100 mM K^+	5 mM Na^+	140 mM Na^+
R_S	154 (0.2)	21.2 (1.0)	156 (0.4)	19.1 (1.0)	336 (0.4)	22.1 (0.6)	217 (0.3)	14.7 (0.7)
					201 (0.4)	18.2 (1.3)	229 (0.2)	14.5 (0.6)
R_M	1158 (0.7)	821 (2.5)	1553 (1.7)	1249 (3.6)	1005 (0.6)	243 (2.1)	1096 (0.8)	589 (1.7)
					1074 (1.4)	328 (4.3)	1282 (0.6)	589 (1.3)
$Q_M \cdot 10^5$	3.749 (1.6)	5.158 (4.5)	4.732 (2.5)	5.137 (4.4)	4.941 (3.7)	3.683 (5.5)	3.964 (2.0)	4.853 (3.1)
					3.788 (3.3)	5.906 (7.9)	5.006 (1.3)	2.886 (2.8)
n	0.8500 (0.3)	0.8254 (0.8)	0.8497 (0.5)	0.8597 (0.8)	0.8291 (0.9)	0.8884 (0.9)	0.8460 (0.5)	0.8494 (0.5)
					0.8363 (0.7)	0.8363 (0.7)	0.8161 (0.3)	0.8931 (0.4)
$Q_S \cdot 10^4$	3.787 (0.6)	4.229 (2.7)	2.227 (1.3)	2.441 (4.0)	5.406 (1.2)	4.844 (0.7)	7.385 (0.9)	7.790 (3.4)
					4.013 (1.4)	4.533 (1.4)	5.852 (0.6)	5.580 (2.5)
n	0.9787 (0.5)	0.9579 (2.2)	0.9740 (1.2)	0.9869 (3.3)	0.9017 (1.3)	0.7626 (0.4)	0.8921 (1.0)	0.8169 (2.3)
					0.8720 (1.1)	0.7286 (0.8)	0.9292 (0.7)	0.8628 (1.7)
$W \cdot 10^5$	9.356 (2.6)	9.579 (12.5)	10.76 (2.9)	13.89 (7.2)	9.040 (8.9)	2.369 (.89)	12.16 (7.8)	10.37 (19.5)
					3.626 (17.2)	1.630 (5.7)	17.39 (2.7)	13.77 (11.1)

CONCLUSIONS

In this work, novel CP-MP biocomposites have been prepared using PNMPy, which shows a very compact structure, and Omp2a from *Brucella melitensis*, a β -barrel protein that forms trimeric pores. The MP has been successfully immobilized onto the surface of PNMPy during the electrochemical polymerization, as has been proven by XPS and UV-vis spectroscopy results. Furthermore, Omp2a retained its native state β -sheet structure, thus preserving its folded operative form. SEM and AFM characterization techniques have allowed us to identify the PNMPy-Omp2a composite surface properties, which are related with the passive ion transport. As a result, Omp2a pore-forming structures are distributed throughout the compact PNMPy matrix. In addition to that, PNMPy-Omp2a biocomposites are hydrophilic, electroactive and biocompatible biointerfaces.

Although the porosity of PNMPy and PNMPy-Omp2a are very similar, their responses towards electrolyte solutions are completely different. Thus, the behavior of PNMPy is independent of the electrolyte concentration, while PNMPy-Omp2a promotes the exchange of ions between the systems and the electrolyte solution. This passive transport ability, which is associated to the β -barrel structure of the immobilized protein, is regulated by the concentration of ions in the electrolyte solution.

PNMPy-Omp2a behaves as a smart bio-inspired ion-channel, which has potential applications in different areas of the biomedical field, such as nanofluidics, energy conversion and biosensing. Furthermore, changing the concentration of ions in the electrolyte solution regulates the activity of Omp2a nanochannels integrated in the PNMPy-Omp2a composite. This has been proved by examining the ion transport process using K^+ and Na^+ concentrations around those typically found in blood. Furthermore, results suggest that that Omp2a is more efficient for the diffusion of Na^+

than for K^+ . In addition to the above mentioned applications, Omp2a immobilized onto PNMPy can be used to simulate the process of ions and small molecules transport in living organisms, enabling to investigate details of the chemistry, size and conformational states of β -barrel nanochannels.

SUPPORTING INFORMATION

Sequence of the Omp2a protein, high resolution XPS spectra and assignment of the peaks, control voltammograms of monomer solutions, SEM micrographs of SDS and Omp2a, contact angles, cyclic voltammograms of PNMPy-Omp2a and PNMPy in aqueous solution with $LiClO_4$, SEM micrographs for PNMPy-Omp2a after 8 h and 24 h of immersion in a Lipase F-AP15 solution, SEM micrographs of Vero and Cos-7 cells seeded for 24 h onto steel, and SEM micrographs of Vero cells seeded for 24 h onto PNMPy and PNMPy-Omp2a surfaces. This material is available free of charge via the Internet at <http://pubs.acs.org>

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Graphical Abstract

