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Poly(ethylene-co-vinyl alcohol) Membranes with Specific Adsorption Properties for Potential Clinical Application

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Abstract: The preparation of novel polymeric systems through Molecular Imprinting Technology (MIT) for potential application in extracorporeal blood purification is described. Membranes based on poly(ethylene-co-vinyl alcohol) material, produced through a phase inversion method, were modified introducing in their structure specific binding sites for lipid and/or protein molecules. Membranes prepared are intended to selectively remove low density lipoproteins and cholesterol (LDL) from the plasma, by using interactions at a molecular level, between the molecularly imprinted membrane and specific target molecules, created during the preparation procedure. The binding performances of membranes and their potentiality as adsorbents for two different model target compounds, a phospholipid (phosphotidylcholine, PC) and a protein (α-amylase enzyme) were investigated, showing improved adsorption capacity with respect to unmodified control membranes. In addition, molecularly imprinted poly(ethylene-co-vinyl alcohol) materials in the shape of microparticles,

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using the same templates, were prepared and studied for their potential use as adsorbers into a column.

Keywords: Molecular imprinting, molecularly imprinted membranes, plasmapheresis, poly(ethylene-co-vinyl alcohol)

INTRODUCTION

In the biomedical field, novel high selective membranes for chiral drugs, toxins or complex biomolecules, are required. Due to the typical complexity of separations procedures, many technically challenging and commercially attractive separation problems can not be easily solved with existing membranes, and only fractionations into substance groups can be achieved. Active research in the membrane field has to be devoted to the development of innovative membranes for highly specific separations, based on molecular recognition at the nanoscale (1).

In this contest, "molecular imprinting" would open interesting possibilities for the design of selective membranes with enhanced performances.

Molecular Imprinting Technology (MIT) allows the preparation of polymeric materials and devices capable of selective molecular recognition. This means that the final polymeric matrix is able to distinguish between chemical species and to bind those that exhibit specific functional groups. The high selectivity that can be obtained for these selected species is due to recognition sites inserted within the material during preparation procedure, through "template" polymerization of a monomer in the presence of the stamp molecule (template) (2) or through phase inversion of a homogeneous polymer solution containing the molecule to be recognised (3). Under defined conditions the template molecule can be removed, leaving behind selective cavities, complementary in the shape and functionality with the molecules (template) that have to be specifically bound and adsorbed.

The imprinting procedure includes two critical steps: the template extraction from the sites inside the polymer matrix and the rebinding/adsorption of the same molecule. Both imprinting phases must leave unaltered the morphology of the matrix and are necessary to establish the effective recognition and selectivity performances of a molecularly imprinted material or membrane.

The selective memory of molecular imprinted polymers (MIPs) has been proved to be highly resistant to time and use (4), therefore their preparation and production have been studied not only for their academic interest but also for their application to industry. For example, Molecularly Imprinted Membranes (MIMs) were used in the construction of biomimetic systems for the achievement of selective diffusion and bio-separation systems (1, 5, 6), such as chromatographic supports, for the realisation of chemical sensors, and for the preparation of artificial antibodies or cellular receptors.

One of the objects of the present research is the possibility to investigate the innovative biomedical use of imprinted membranes for different potential applications: extracorporeal purification of blood, implantable devices capable of recognition and/or release of active agents, or porous scaffolds exhibiting molecular recognition towards fundamental biological compounds for tissue engineering. Polymer membranes for clinical use must therefore be highly biocompatible and possess a specific permeability and bio-stability (7).

In this paper we deal with the preparation of MIMs (based on poly(ethylene-*co*-vinyl alcohol), EVAL), where the polymer matrix is a membrane obtained by phase inversion. This method needs two principal components: a homogeneous polymer solution and an appropriate non-solvent. The polymer solution is transformed in a two-phase system where the polymer rich phase forms the final solid membrane structure, while the polymer lean phase forms the membrane pores through the exchange of solvent and non-solvent at the interface. However, even if phase inversion is a standard approach for the preparation of separation membranes, it is complicated to adapt such process for the realization of molecularly imprinted membranes. The conditions required for an optimal formation of complementary binding sites may not be compatible or completely relatable with the achievement of an optimal microporous structure of the membrane.

In any case, considering the attractive potentiality of molecular imprinting technique in the introduction of specific cavities inside polymer membranes at nano-scale level, recent efforts of our group were devoted to the preparation and characterisation of molecularly imprinted membranes obtained by phase inversion method (5, 6, 8, 9).

Thus, the membrane preparation procedure (cast solution preparation and following coagulation) was carried out in the presence of different template species (i.e. cholesterol, phosphatidylcholine, α-amylase), involved in various important physiological or pathological processes such as the atherosclerosis plaque formation. In effect, the goal of the present study is the production of membranes with potentiality for the clinical application as selective adsorbents to be used for LDL-apheresis, especially in the case of hypercholesterolemia familial patients.

In this work, MIPs in form of membranes based on the polymer EVAL able to provide hydrophilic interactions with hydroxyl groups were developed (following precedent attempts of other authors (10–19). The goal is to observe the effects of different types of templates on MIMs specificity and selectivity properties in order to predict the performances of these membranes in the removal lipoprotein for LDL-apheresis treatment (20–23).

EVAL is used because of its ease processability and good mechanical properties, as well as low immunogenicity and elevated haemocompatibility.

Phosphatidylcholine (PC) and α-amylase are template molecules that were used as model compounds. PC, a molecule present in the external layer of plasma lipoproteins, can aid in the use of the imprinted membranes in the removal lipoprotein from plasma. We refer in a previous work the

study of analogous membranes of EVAL imprinted with free cholesterol (6), a molecule transported by plasma lipoproteins (HDL, LDL) and associated as PC to external layer of these complexes.

Cholesterol is known to be associated with many cardiovascular diseases, such as atherosclerosis, which affects the arterial blood vessel and consists in the formation of multiple plaques due to elevated concentrations of LDL cholesterol and low concentrations of HDL cholesterol. These plaques cause the formation of blood clots that will rapidly slow or stop the blood flow, leading to death of the tissues fed by the artery.

A reduction in blood levels of LDL cholesterol should result in a regression of atherosclerosis (24), to this purpose we studied the possibility of creating a polymer membrane system (or column, packed with molecular imprinting microparticles, in a more recent attempt) capable of recognizing and separating these molecules. In effect, the aim of our present and future research is to develop a device for extracorporeal affinity adsorption that can remove LDL cholesterol from the plasma fraction of the blood and thus lead to significant regression of atherosclerotic coronary lesions, particularly for hypercholesterolic patients, that can not be treated with other conventional methods (oral lipid lowering drug, diet).

With the aim to reduce selectively LDL cholesterol respect to HDL cholesterol, the use as template of the atherogenic apolipoprotein B (or of a peptide sequence contained in this lipoprotein) present mainly in LDL should be evaluated. However, since the availability and precise information about the use of these macromolecules or peptides are very limited, preliminary studies, reported in this work, provide results on the use as template of a model protein, B. subtilis α-amylase (αA). The choice of α-amylase is motivated by the fact that these enzymes have been extensively studied and contribute to the understanding of protein behavior in general rather than to the behavior of this particular group of enzymes. In fact the specificity of action of an enzyme such as amylase could consent the monitoring of the activity and structure of a selected protein model template. In preceding papers of our group, results concerning bioartificial membranes based on poly(ethylene-*co*-vinyl alcohol)/dextran blends imprinted with α-amylase were presented (8, 9): experiments showed the stable immobilization of dextran biopolymer inside the membrane network, but also the pore-former role played by this component. Moreover, functional investigation confirmed the ability of membranes to selectively adsorb the enzyme, and this capability was associated to non-covalent interaction between template molecule and EVAL polymer (thermal and thermogravimetric analysis evidenced these interactions). For this reason, in the present study we concentrated our attention on the preparation of membranes based only on EVAL (setting the coagulation parameters for the control of porosity and then transport properties of membranes) molecularly imprinted with α-amylase, extending the investigation to phosphatidylcholine compound as well.

MATERIALS AND METHODS

Materials

Poly(ethylene-*co*-vinyl alcohol) (EVAL, Sigma Aldrich, intrinsic viscosity 8.44 dL/g at 35°C), with an ethylene molar content of 44%, was used as the biocompatible polymer for the preparation of membrane. Solvents used were dimethyl sulphoxide (DMSO, Riedel-de Haën), tetrahydrofuran (THF, Carlo Erba Reagenti). Phosphatidylcholine (PC, Sigma-Fluka, Biochemika, from egg yolk, lyophilized $\geq$99%, MW = 760), for the first series of membranes, was used as a template, phosphatidylserine (PS, Sigma-Fluka, Biochemika, from bovine brain $\geq$98%, MW = 812), and phosphatidylinositol (PI, Sigma-Fluka, Biochemika, from soybean $\geq$98%, MW = 857) were employed as template analogues for selectivity evaluation (Figure 1). α-Amylase (αA, Sigma-Fluka, Biochemika, from Bacillus Subtilis $\approx$380 units/mg, MW = $48 \cdot 10^3$) was used as model stamp molecule to study the possibility that molecularly imprinted EVAL membranes may be able to specifically recognise also protein molecules (Albumin (ALB, Sigma, from bovine serum $\geq$96%, MW $69 \cdot 10^3$) was the αA analogue).

Membrane Preparation

Membranes were prepared via the phase inversion technique, by dipping. Solutions of EVAL (15% w/v) were prepared in DMSO under stirring during 5 h at 50°C. After viscous polymer solution preparation, the template was added in the required amount (αA: 2% in weight related

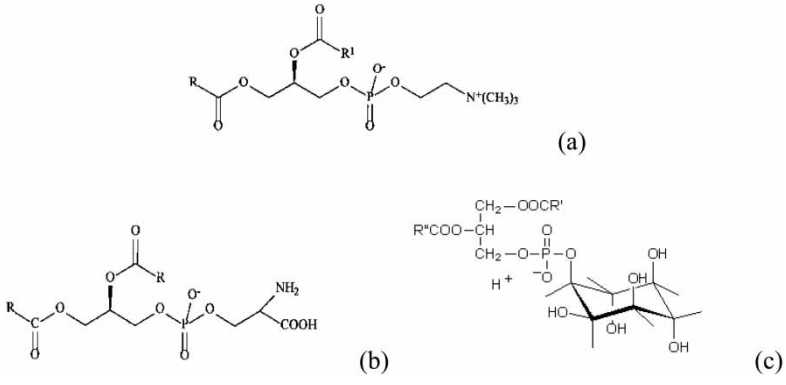

Figure 1. Representation of chemical structures of (a) phosphatidylcholine PC, (b) phosphatidylserine PS, (c) phosphatidylinositol PI.

to the polymeric content; PC: 20% in weight related to the polymeric content) and the solution was stirred at room temperature for 3 h. In particular, due to the poor solubility of both templates in the DMSO solvent, and to avoid a local precipitation in the starting cast solution, αA was pre-dissolved in a water-DMSO 50:50 mixture, while PC was initially dissolved in THF-DMSO 50:50 solution, then added to the corresponding cast solution.

Porous membranes were then prepared by using a Separem Type SP L1 "knife machine": the viscous solutions were uniformly laid on a glass support (the height and velocity of the knife were 400 μm and 1 m/min respectively), and immediately dipped in a DMSO/H_2O 50:50 solution (first phase inversion bath). After one hour, the imprinted polymeric membranes were transferred to a second phase-inversion bath (distilled water) for twenty hours to remove solvent traces. Finally the membranes were removed from the inversion bath and dried in a ventilated oven at 37°C for 5 hrs. The same procedure was adopted to prepare non-imprinted membranes (without the presence of template molecule, but adding the template/pre-dissolution solution to maintain the same conditions).

The real amount of entrapped PC template in the porous network of membranes was determined by measuring the residual quantity in the inversion baths at $\lambda = 250$ nm with a Shimadzu UV-2100 Spectrophotometer. High levels of PC in the inversion bath would imply that the recognition cavities have not formed correctly and that the polymer does not exhibit the ability to entrap the template molecule. In the same way, the leakage of α-amylase into the phase inversion baths was quantified by a spectrophotometric method at 280 nm.

Template Extraction

Templates were extracted from the polymer matrix, placed within a permeability cell, using a permeability apparatus in which a solvent (distilled water for αA or isopropyl alcohol for PC template) passes through the membrane (fixed trans membrane pressure, 0.1 bar). The extraction was monitored by taking samples of the solution at different times and reading with the Spectrophotometer at 280 nm for αA and 250 nm for PC.

Morphological and Physical Characterization

The morphology of the membranes was analyzed using Scanning Electron Microscopy (SEM, JSM-5600 Scanning Microscope). Freeze-dried membranes were torn at room temperature, sputtered with gold, and observed using a voltage of 20 kV by SEM.

Functional Characterization

The functional properties of membranes, in terms of transport properties, recognition and selectivity, were tested by using the permeability apparatus employed for extraction procedure.

The employed permeability cell was a rectangular cell with a useful surface of 11.55 cm^2, and the permeability apparatus connected to the cell was a closed circuit with a peristaltic pump and a regulation valve to control the transmembrane pressure (TMP) in the permeability test. The membranes (about 200 mg) were fixed with rubber seals and kept in a cylindrical pressure-stable cell, where a water flux was induced through the membrane by means of an established trans-membrane pressure (TMP) (from 0,1 to 1,8 bar) controlled by a manometer at the inlet. One side of the membrane was subjected to a constant pressure, while the other side was maintained at atmospheric pressure. The volumetric water flux (Jv) was measured by weighing water permeated with time. The permeation tests were also performed in a cyclic way, passing from the lowest TMP to successive values until highest pressure and starting again from the lowest TMP for eight cycles. These test was carried out by using a feed flows of 400 ml/min.

In the rebinding/adsorption test, the peristaltic pump maintained a difference of pressure equal to 0.8 bar, and a constant flow of an aqueous solution containing 0.15 mg/ml of PC. The amount of the rebound (recognized) template was measured taking samples of circulating solution at different times and reading them with the Spectrophotometer. The rebinding test was performed with αA imprinted membranes as well, following the same procedure and using a 0.15 mg/ml solution (the test was performed in triplicate).

Finally a selectivity test was performed to see whether the imprinted membrane is capable of recognising only PC or other similar molecules, and αA compared with an other protein (ALB). The procedure was the same as in the binding experiment, but in the latter case, solutions were prepared with an equal amount of template analogues (also in this case the experiments were carried out in triplicate). The selectivity test was carried out in competitive conditions as well, putting in the solution both PC and PS (in the same concentration, 0.15 mg/ml) for PC-imprinted membranes and αA and ALB (in the same concentration, 0.15 mg/ml) for αA-imprinted samples.

RESULTS

PC-Imprinted Membranes

Scanning Electron Microscopy

Figures 2 and 3 show the morphology of the cross section of the PC-imprinted membrane and non-imprinted membrane respectively. No substantial

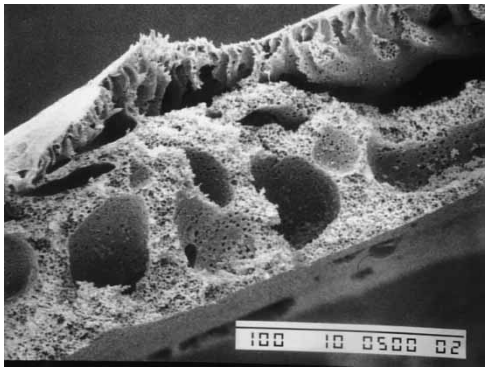

Figure 2. SEM image of PC imprinted membrane after preparation procedure via phase inversion.

differences in the morphology of imprinted and control membranes were evident from microscopy investigation. In particular, the images show an asymmetrical structure of the membranes consisting of the typical finger-like macrovoids and an underlying homogeneous porous polymer matrix due to the liquid-liquid demixing during the phase inversion process. The observed final porous structure of the membranes can be ascribed to the use of water/DMSO 50:50 coagulation bath; in fact the use of this mixture for the inversion phase was aimed at reducing the phase separation process kinetic (formation of pores) and making the membrane more porous. The porous structure is a very important characteristic for biomedical applications of the membranes, and in particular for molecularly imprinted membranes, leaving the recognition cavities exposed and accessible for the template to be adsorbed. The morphology of the PC-imprinted membrane was observed

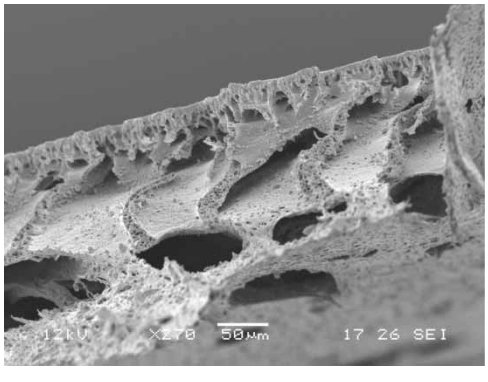

Figure 3. SEM image of non-imprinted membrane after preparation procedure via phase inversion.

by SEM after the template extraction procedure as well and this investigation showed no difference in morphology after the removal of the phospholipid, a promising aspect for the following rebinding of new template molecules (stable porous structure of polymer membrane).

Functional Characterization

The removal of phosphatidylcholine from the polymeric matrix mainly depends on the strength of the interactions between polymer and template. As a consequence of the higher solubility of PC in isopropyl alcohol compared to water, better results were obtained using the first solvent. During a 2 h experiment, the amount of the template extracted from a PC-imprinted membrane with water was 7.5% of the initial amount. In the case of isopropyl alcohol during a 1 hr experiment 35% was extracted from the matrix.

The extraction of the PC template by isopropyl alcohol flux through the membranes during the course of 5 hrs was practically complete, related to the initial amount of PC in the polymeric matrix, in the case of the closed circuit (the advantage of this system lies in the smaller amount of solvent necessary and the better quantitative results of extraction, compared with other contact schemes investigated). After the removal of template molecules from the membrane network, recognizable cavities were present in the porous structure. The advantage of this presence lies in the useful morphology, but also in the spatial arrangement at a nanoscale level of functional groups of the polymer chains, making these cavities potentially capable to present a memory towards extracted "stamp" molecule.

After the extraction procedure, to investigate the hydrodynamic properties of molecularly imprinted membranes and controls, an under pressure permeability test was performed. The hydraulic permeability across the membrane was determined by measuring the volumetric water flux per unit of area (Jv) through the porous membrane. In the hydraulic permeation tests the driving force of the process was the imposed pressure gradient TMP. From Jv values, the hydraulic permeability K_D was determined according to Darcy's law:

$$K_D = \frac{\mu \cdot S \cdot J_V}{TMP}[\text{darcy}] \quad (1)$$

Here μ is the water viscosity and S is the thickness of membrane employed for the experiment.

The variation of Jv and K_D as a function of TMP provides information on the type of convective transport through the membrane.

Measured Jv values depend on TMP (Table 1), showing a linear variation with the increase of applied pressure gradient.

Table 1. volumetric flux (Jv) and Darcy permeability (K_D) for PC- and αA-imprinted membranes and respective controls as a function of trans membrane pressure (TMP)

PC-imprinted			Control		
TMP (bar)	Jv $\times 10^4$ (cm^3/cm^2sec)	K_D (darcy)	TMP (bar)	Jv $\times 10^4$ (cm^3/cm^2sec)	K_D (darcy)
PC-imprinted systems					
from 0.1 to 1.8	from 3.98 to 47.26	from 0.199 to 0.131	from 0.1 to 1.8	from 4.42 to 48.08	from 0.186 to 0.112
	αA-imprinted			Control	
αA-imprinted systems					
from 0.1 to 1.8	from 8.35 to 75.22	from 0.256 to 0.128	from 0.1 to 1.8	from 9.11 to 73.02	from 0.244 to 0.109

Permeation tests performed in a cyclic way, as described above, showed no significant variations in the water volumetric flux through the membranes (no structure modification was observed by SEM analysis), without deviation from the linear trend of volumetric flux Jv through both series of the membranes. Table 1 showed Jv and K_D values. These values are high, as could be expected from the pore large dimensions, clearly indicating a direct relationship between the morphology and the transport properties of the membranes. No evident differences between molecularly imprinted and control membranes in the volumetric flux through the polymer structure were detected, showing very similar behavior in terms of hydrodynamic permeability.

The rebinding procedure was performed on the "extracted" molecularly imprinted membrane and the blank membrane (control) for 15 hrs. The comparison between the two was necessary to estimate the effective recognition capabilities due to imprinting and not to the morphological characteristics of the polymeric material. Figure 4 shows the amount of rebound template in both instances.

In effect, concerning the molecularly imprinted membrane, the amount of bound PC increased rapidly with the increasing filtration time to become constant after 10 h. Over 50% of the initial amount of template inside the rebinding solution was found to be up-taken after the first 2 hours, making the potential medical applications for this membrane more probable, due to the rapid kinetic of rebinding. Practically, about the 78% of the initial PC present in the rebinding solution was adsorbed by imprinted membrane at the end of the test.

Results obtained indicate that the presence of imprinted sites in the membrane plays a key role in the rebinding process of phosphatidylcholine and that the porosity does not seem to have any major influence (as showed by SEM analysis, the morphology of imprinted and non-imprinted

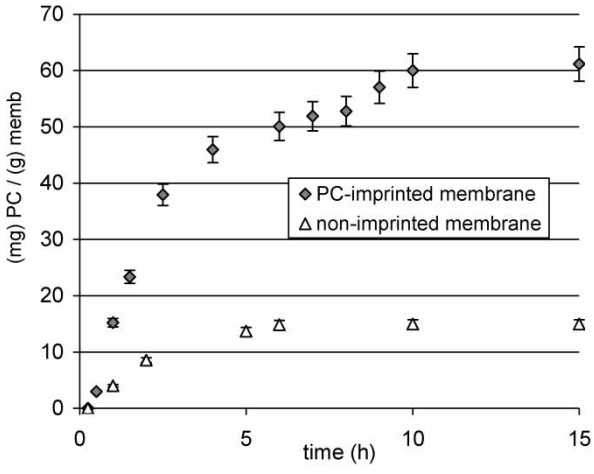

Figure 4. Cumulative adsorption of PC template for imprinted and control EVAL membranes.

membranes was very similar, and a hydrodynamic permeability test confirmed similar convective transport properties).

The distribution coefficient was estimated as a measurement of the material's capacity of template recognition and rebinding:

$$K_p = \frac{C_1 - C_2}{C_1} \tag{2}$$

where C_1 and C_2 were the concentration of the template in the starting solution and after the adsorption/rebinding test passing through the membrane, respectively. At equilibrium conditions, for the blank and the imprinted membranes, K_p values were found to be 0.06 and 0.78 respectively, indicating a high imprinting effect in the membranes. The difference in the up-taking performances of the imprinted and the non-imprinted membranes may be associated only to the chemical functionality organization and positioning in the imprinted polymer chains (during molecular imprinting via phase inversion), considering that no evident differences in morphological and convective transport properties were observed.

To test the selectivity of the membranes, solutions (0.15 mg/ml) of phosphatidylserine (PS) and phosphatidylinositol (PI) were passed through the membrane and the amount of the solute molecules adsorbed by the polymeric matrix was evaluated. The results (Table 2) show a very high selectivity of PC-imprinted membrane towards the template, exhibiting a very higher adsorption of PC with respect to PS and PI analogues.

To better investigate the recognition and selectivity properties of molecularly imprinted membranes, a competitive selectivity test was performed using, together with the PC template, the PS analogue in the same

Table 2. Percentage (%) adsorption of template PC, or analogues, during rebinding, selectivity, and competitive selectivity tests in respect to initial solute in the test solution

	Percentage adsorption (%) *(rebinding test)*		Percentage adsorption (%) *(competitive rebinding test)*	
Molecule	PC-imprinted membrane	Control membrane	PC-imprinted membrane	Control membrane
Phosphatidylcholine (PC) *template*	78.05	6.09	68.22	4.11
Phosphatidylserine (PS)	0.15	0.29	5.91	0.96
Phosphatidylinositol (PI)	0.10	0.16	—	—

concentration (both 0.15 mg/ml). Results of the competitive test were introduced in Table 2. A light variation in the adsorption capacity of membranes was observed, but molecularly imprinted networks confirmed the capacity to discern the template molecule against the analogue in an aqueous medium (and this is a very interesting result considering that the recognition in water is one of the biggest problems to be completely solved in the molecular imprinting field).

αA-Imprinted Membranes

Scanning Electron Microscopy

The αA template was physically entrapped (and extracted) in the polymer membrane without structural changes, as shown in Figs. 5 and 6 (sections of αA-imprinted membrane and control membrane). In effect, a comparison between the αA-imprinted membrane and a non-imprinted membrane indicates that the addition of the enzyme did not substantially modify the morphological structure of the final polymer matrix. An asymmetrical morphology of the membranes, consisting of the typical finger-like macrovoids and a homogeneous porous structure, was evident like in the case of the PC-imprinted membrane. The observed morphology has to be ascribed to the liquid-liquid demixing during the phase inversion process. Near the top surface contacted with the non-solvent, conic macrovoids were formed and the support layer with finger-like cavities appears. The asymmetric porous structure of the membranes stems of the utilization of a solvent/non-solvent 50:50 coagulation bath, which is responsible for the presence of spherical macrovoids and tubular macrovoids of small dimensions. The utilisation of the DMSO/H_2O inversion bath, resulting in a porous structure of the

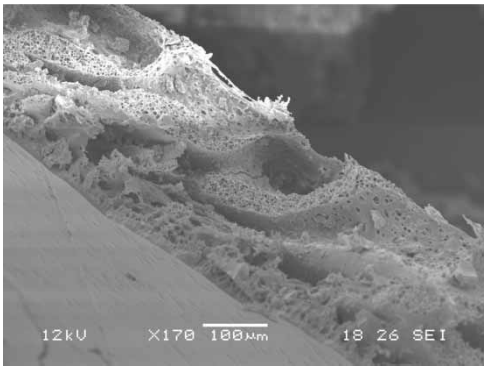

Figure 5. SEM image of αA imprinted membrane after preparation procedure via phase inversion.

membrane (the phase inversion by dipping of EVAL solution in pure water give membrane whit very poor microporosity and a dense structure, not useful for imprinting application), can delay the phase separation, decreasing the polymer concentration of the concentrated phase. The presence of DMSO in the aqueous inversion bath allows for reducing the presence of fingers and for the formation of top surface defects due to the whirling interdiffusion between solvent and non-solvent. The driving force responsible for the diffusion of the solvent out of the membrane, given by the different concentration of DMSO out and inside the membrane, can be reduced in this way. The second inversion bath (100% water) ends the diffusive processes of DMSO out of the film and of water into the film. In this way, a total replacement of the solvent with the non-solvent is guaranteed. The porous structure is a key characteristic for molecular imprinted membranes. The morphological characterization of the membrane was also performed after the extraction of

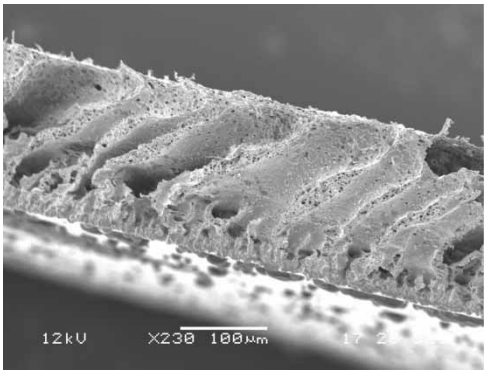

Figure 6. SEM image of non-imprinted membrane after preparation procedure via phase inversion.

αA template, confirming that, the αA-imprinted membrane did not show modification in the morphology due to the extraction procedure with water, in the permeability apparatus, leaving unaltered the recognition cavities for the rebinding of the new template molecules.

Functional Characterization

Following the phase inversion procedure, αA was extracted by a water flux through the membranes in the permeability apparatus introduced before, and the percentage extraction of the template was very high (>98%), related to the initial amount in polymeric matrix.

A hydrodynamic permeation test, like in the case of PC-imprinted membranes series, evidenced a high volumetric flux across the membranes (Table 1). Convective water flux through the membranes (imprinted and non-imprinted) resulted to be lightly superior to the flux measured for the PC-imprinted series. This difference can be ascribed to the addition of water-DMSO (in the case of αA-series) or THF-DMSO (PC-series) pre-dissolution solutions, that created a variation in the porous structure formation at the micro-scale level. No differences between imprinted and non-imprinted membranes was observed in terms of convective flux, showing no effect associated to the presence of αA template during phase inversion mechanism.

The results of the rebinding/adsorption experiments for αA-imprinted membranes were reported in Fig. 7. Also in this case, the amount of the adsorbed template increased with filtration time to become constant after a few hours. The equilibrium partition coefficient K_P was estimated, showing the possibility to insert, inside an EVAL porous membrane, imprinted cavities capable of adsorbing a high amount of a specific molecule. The higher values of K_P (0.43) for αA-imprinted membranes with respect the control one (0.12) confirmed the imprinting effect and the potentiality of Molecular Imprinting to produce porous membranes showing an increased adsorption capacity towards protein molecules. The greater amount of α-amylase adsorption in the imprinted membrane with respect to the blank one indicates that the specific sites for template exist (and were introduced in the structure during phase inversion process) and accomplish their recognition function.

In addition to the imprinting effect, selectivity of membranes (indicating the "recognition" capacity of these to adsorb a specific molecule of large dimension, the template, instead of molecules of similar structure) was confirmed. The solution of the template analogue (albumine, ALB) was passed through the membrane and the amount of molecules solute entrapped into the polymer matrix was evaluated. Table 3 shows the percentage adsorption of ALB taken up in the imprinted membrane with time in perm-selectivity experiments, compared with αA template.

Also in this case, the imprinted membrane retained more template than the analogue molecule, confirming that the presence of selective cavities

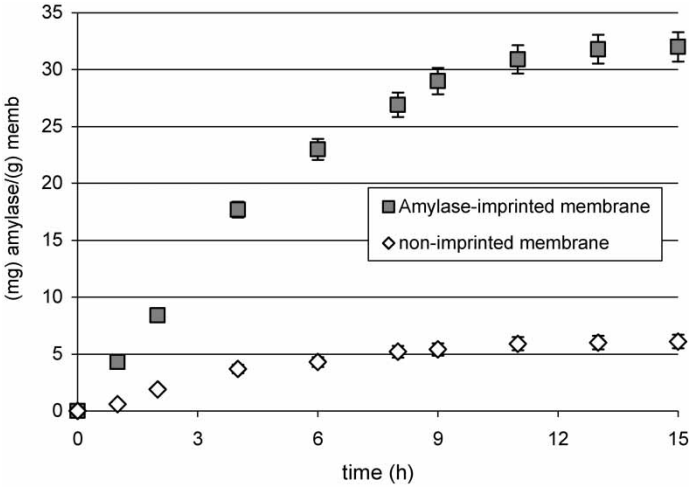

Figure 7. Cumulative adsorption of αA template for imprinted and control EVAL membranes.

play the role to make the membrane structure able to adsorb in a specific way the protein selected as the template. The test was performed by using a non-imprinted membrane as well. The imprinted membrane adsorbed more of the ALB molecule (8.3%) than the non-imprinted one (6.1%), suggesting that the first ones exhibited a conformational organization of the macromolecular chains and a spatial orientation of hydroxyl groups that are able to establish non-covalent interactions with a functional group of protein molecules, and also in the absence of specific recognition mechanism the αA-imprinted membrane should show a poorly higher solute retention than the non-imprinted membrane. This result was not confirmed in the competitive selectivity procedure, that evidenced a comparable rebinding/adsorption for

Table 3. Percentage (%) adsorption of template αA, or analogue, during rebinding, selectivity, and competitive selectivity tests in respect to initial solute in the test solution

	Percentage adsorption (%) *(rebinding test)*		Percentage adsorption (%) *(competitive rebinding test)*	
Molecule	αA - imprinted membrane	Control membrane	αA - imprinted membrane	Control membrane
α-Amylase (αA)*template*	43.46	12.21	39.55	9.33
Albumin (ALB)	8.30	6.12	6.71	6.89

ALB molecule using imprinted or non-imprinted membrane, while the selectivity and imprinting effect (with clearness of high specific adsorption and low not-specific rebinding) were confirmed in this test by the largely higher up-taking of αA in respect to ALB (for imprinted membrane) and the similar behaviour of non-imprinted membrane for the two proteins.

In any case, the imprinting effect (higher template adsorption for imprinted membrane in respect to control membrane) and the good selectivity observed (higher template adsorption in respect to ALB molecule) were very interesting results for the Molecular Imprinting field and for the area of molecularly imprinted membrane, because while the polymeric systems imprinted with small organic molecules are usually very sensitive towards the molecular structure (3), the recognition and selectivity for larger molecules is usually poor, highly dependent on operating conditions, and difficult to determine.

During this work, we prepared molecularly imprinted membranes for two different model target compounds, a phospholipid (phosphotidylcholine, PC) and a protein (α-amylase enzyme, αA). After the phase inversion procedure, an easy and very high extraction of PC (total, 100% in respect to initially entrapped lipid) and αA (superior to 98%) was observed.

The imprinted systems showed high adsorption capacity in terms of quantitative rebinding (about 60 mg of PC per g of membrane and 32 mg of αA per g of membrane) and in terms of solution purification (78% of PC initially present in the rebinding/adsorption test was up-taken by imprinted membrane, while the 43% of αA was bound). In addition, both membrane typologies showed an evident "imprinting effect" associated with a higher adsorption capacity of the imprinted system in respect to control one. The imprinted sites introduced during the preparation procedure exhibited high specificity as well, being the membranes able to recognize PC in respect to PS and PI, and αA in respect to ALB, respectively. The results of the present investigation resulted to be interesting thinking that the specific adsorption and the recognition of templates of small dimension in water is very difficult to obtain and, clearly, the imprinting effect in aqueous media is the most important feature to be obtained in the case of high molecular weight compounds (such as proteins).

CONCLUSIONS

In this work we prepared and characterized polymeric membranes able to recognise phospholipids or proteins for potential use in extra corporeal purification systems. The method employed for the preparation of porous membranes was phase inversion (by dipping), the most utilized procedure for the preparation of separation membranes. Some times (1, 7), the adaptation of the inversion process to the preparation of molecularly imprinted membranes is not easy because the formation of optimal MIP sites may not be compatible with the ones for obtaining an optimal pore structure.

In this work, the realization of membranes through the phase inversion process resulted to be a useful method to introduce in the polymer network specific cavities able to rebind and recognize two different templates (phosphotidylcholine PC and the protein α-amylase αA) showing in addition a good selectivity in water medium as well.

The systems realized could be attractive solutions for ex vivo (dialysis, blood filtration, and fractioning) or in vivo application in the biomedical field, but the imprinting efficiency, the membrane morphology (microporosity) and separation conditions (using plasma solution or blood as well) can be further investigated and optimized in order to improve the adsorption and selective performances. In particular, binding selectivities are promising but the separation factors of membranes must still be improved to compare these systems with commercially available membranes.

The very important feature of molecularly imprinted membranes (MIM) is the interplay of selective adsorption and transport properties, making them potentially superior to state-of-the-art synthetic membranes already applied in various biological and biomedical fields (1).

The adsorption capability and transport properties of microporous imprinted membranes can be based on template-specific binding sites inside the porous structure, then the main problem will be the optimization of MIP recognition cavities and membrane transport properties at the same time, which is a challenging but not impossible obstacle to overcome.

In conclusion, the aim of the present research was the utilization of an alternative method (molecular imprinting) for the preparation of selective polymer materials for blood treatment. The reported results concerned the first step on the road of the production of high performance adsorbents for LDL-cholesterol. The next step will be the preparation of polymers (the preparation of specific adsorbents in the shape of microparticles via phase inversion, to prepare packed columns to be compared with membrane performances, is a work in progress of our group) or membranes with specific recognition properties towards more specific lipoproteins (atherogenic apolipoprotein B) or sequences (epitope approach), and also the test of membranes in blood or serum, instead of a simple physiological solution. In any case, the high cumulative adsorption of proteins and phospholipids compounds in water, starting from a commercial origin polymer (EVAL), seemed to be encouraging for the realization of specific adsorbents for hydrophobic (but also hydrophilic) templates.

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