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Molecularly Imprinted Membranes with Affinity Properties for Folic Acid

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The extraction of folic acid from aqueous solutions was proposed through a novel procedure based on the membrane separation process using the approach of molecular imprinting. Molecularly imprinted membranes were prepared via the phase inversion technique using poly(acrylonitrile-co-acrylamide) copolymer as the membrane material and folic acid as the template molecule. Poly(acrylonitrile)-based membranes were also prepared as the reference material. Polymer composition, membrane preparation method, and the pH used in the binding experiments were the parameters which mostly influenced the recognition properties of the imprinted membranes. In particular, the solvent evaporation method allowed to obtain poly(acrylonitrile-co-acrylamide) imprinted membranes which at pH 5.0 showed a specific binding capacity of 5.3 μmol/g_{memb}. Corresponding blank membranes (prepared in absence of the template molecule), only showed a poor non-specific binding of 1.0 μmol/g_{memb}. Polyacrylonitrile-based membranes showed lower folic acid retention (1.5 μmol/$g_{memb.}$ when prepared in the presence of the template and 0.9 μmol/$g_{memb.}$ when prepared in the absence of the template).

Keywords folic acid; molecular imprinting; molecular recognition; molecularly imprinted membranes; poly(acrylonitrile-co-acrylamide) copolymer

INTRODUCTION

The synthesis of materials able to specifically recognize active bio-molecules in aqueous media is one of the most important challenges in chemistry. The specific non-covalent recognition approach at a molecular level is a primary necessity of living systems and therefore many scientists focused their interest on the possibility to mimic this biological mechanism in synthetic materials. The potential applications of these systems include affinity separations, medical diagnostics, drug delivery, catalysts, etc. Among the different approaches used to develop these artificial systems, one of the most promising is molecular imprinting. This technique allows to create template-shaped cavities in polymeric matrices with the memory of the template to be recognized. It is based on the system used by enzymes for substrate recognition, which is called the "lock and key" model. This objective is achieved by means of the addition of the template to a reaction mixture which is constituted of a functional monomer, a cross-linking agent, and a solvent. During the polymerization, the template is integrated in the polymeric matrix and its subsequent extraction gives rise to the formation of template complementary binding sites with high substrate selectivity and specificity (1–3). Molecularly imprinted polymers (MIPs) prepared by this approach are applied in separation processes, catalysis, sensors, and antibodies recognition (4–8).

An alternative way is the application of the imprinting technique for the preparation of membranes with specific selectivity for the targeted compounds in order to obtain molecularly imprinted membranes (MIMs) which combine the advantages of the molecular imprinting and membrane technology (9,10). Based on this approach, Piletsky et al. (11) developed the first imprinted membranes via in situ bulk polymerization of acrylate monomers using adenosine monophosphate as the template. MIMs were also prepared by thermal (12) and interfacial polymerization (13) or by surface functionalization of commercial membranes through the grafting polymerization with MIP in the presence of the template (14,15). A further method used to prepare MIMs is the phase inversion technique. The process foresees the preparation of a membrane in the presence of target molecules with a polymer containing functional groups able to interact with the template. This approach allows to prepare MIMs with selective recognition properties due to the presence of specific recognition sites in the membrane matrix. This method was developed by Kobayashi and co-workers in 1996 (16). Since then, phase inversion molecularly imprinted membranes were prepared for applications in enantiomeric separation (17–19), solid phase extraction (20–25), and detection of various substances in food and aqueous medium (26,27).

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In this work, the molecular imprinting approach was employed as a novel method for the selective extraction of folic acid (FA) from aqueous solutions in order to mimic the extraction from foods and other natural sources such as seaweeds. Traditional methods to extract folate from food and natural sources are based on the chemical extractions and subsequent purification with strong anion exchange resins (28,29). These methods are oftentimes consuming and have complex clean-up procedures which require frequent resins regeneration with the risk of the microbial contamination and thus resulting in being unsuitable for laboratory routine. Alternatively, molecularly imprinted membranes which exhibit specific binding for folic acid are particularly promising as extraction devices thanks to their easy preparation and use.

Folic acid is a water-soluble B vitamin (B12) contained in fresh green leafy vegetables, fruit, yeast, and liver (30). Folate compounds are important for DNA synthesis permitting to preserve the genetic information and for the production of amino acids. Recent studies correlate their deficiency in the etiology of hemolitic anemias and destruction of red blood cells, chronic diseases, and coronary heart disease (31). The supplementation of folic acid to women during preconception time can prevent the risk of some neural tube defects in pregnancy (32). Due to its recognized biological importance, folic acid is used to fortify foods, in particular beverages and cereals, in order to increase its intake in the population. In this study, molecularly imprinted membranes with affinity properties for folic acid were prepared via the phase inversion technique. Authors synthesized an acrylic copolymer with acrylamide as the co-monomer via water-phase precipitation polymerization (20,33) with the aim of promoting specific interactions between the amidic part of the copolymer and the carboxylic groups of folic acid (the template) in the forming membrane. Starting from this polymer, imprinted membranes were prepared by adding folic acid to the casting solution and using two different phase inversion methods: solvent evaporation and immersion precipitation. The subsequent removal of the template from the membranes leaves binding sites complementary to its functional groups in the polymer matrix. Chemical structures of folic acid and of the synthesized polymers are reported in Fig. 1.

The recognition properties of the prepared membranes were evaluated by measuring their adsorption capacity during the filtration of aqueous solutions of FA. For comparison, membranes without template (blank) were prepared and tested.

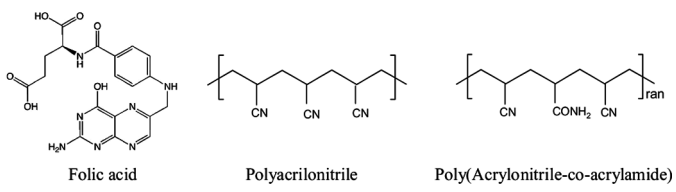

FIG. 1. Chemical structures of folic acid, PAN and P(AN-co-AA).

EXPERIMENTAL

Materials

Acrylonitrile (AN, 99%), acrylamide (AA, 98.5%), acetonitrile (ACN, 99%) potassium persulfate, ferrous sulfate, sodium bisulfite, potassium dihydrogen phosphate, sodium phosphate dibasic, sodium phosphate monobasic (99%), orthophosphoric acid (85%), N-methylpirrolidone (NMP, 99.8%), dimethylacetamide (DMA, 99%), and folic acid were purchased from Sigma (Italy) and used as received.

HPLC Analysis

Folic acid determination was performed on a LaChrom D7000 HPLC system (Hitachi) equipped with a UV-detector. Analysis was carried out using the column Hypersil ODS 5 μm, 250 · 4.6 mm. The mobile phase was KH_2PO_4 50 mM, pH 2.5/ACN (90/10 v/v). The operating conditions were: flow rate: 1 ml/min; T = 25°C, $\lambda = 210$ nm.

Polymer Preparation and Characterization

Polyacrylonitrile (PAN) and poly(acrylonitrile-co-acrylamide) P(AN-co-AA) were synthesized by water-phase precipitation polymerization. The reaction was carried out at 50°C for 3 hours under inert atmosphere and stirring conditions. The couple $K_2S_2O_8$-$NaHSO_3$ was used as catalyst in the presence of Fe^{++}(1 ppm of the total monomer). At a fixed ratio of $K_2S_2O_8$ (0, 47 wt.% of the total monomer) and at water/total monomer ratio of 6:1, different copolymerizations were carried out by varying the $NaHSO_3$/total monomer ratio ($NaHSO_3$/Mo). In the case of the copolymer preparation, the composition of monomer mixture was 90/10 wt.% AN/AA. At the end of each polymerization, the obtained slurry was filtered and the polymer was rinsed several times with de-ionized water in order to remove the un-reacted monomers and the residual catalysts. The polymer was then dried under vacuum at 60°C overnight. The molecular weight of the polymer was determined by intrinsic viscosity measurements on the basis of the following equation (34):

$$[\eta] = 2.75 \times 10^{-2} M_V^{0.767} \quad (1)$$

where: $[\eta]$ is the intrinsic viscosity and M_V is the viscosity average molecular weight.

In addition, the copolymer was characterized by FT-IR analysis using a Perkin-Elmer Spectrum One FT-IR spectrometer.

Membrane Preparation and Characterization

The synthesized polymers were dissolved at 60°C in NMP and membranes were prepared via phase inversion process using two different methods: solvent evaporation (SE) and immersion precipitation (IP). When using the first method, the polymer solution was cast onto a glass plate with a casting knife having a gap of 400 μm and membranes were formed by evaporation of the solvent at 60°C for 3 hrs. In the case of the immersion precipitation method the glass plate was immediately immersed into a coagulation bath containing water as the non-solvent. The polymer concentration in the casting solutions was 15 wt.%. Imprinted membranes were prepared by adding 1.6 wt.% of FA during the preparation of the casting solution while blank membranes were prepared in absence of the template.

The membrane morphology was investigated by scanning electron microscopy (SEM) using a FEI QUANTA 200 F microscope at 20 kV. Membrane permeability (L_P) was determined by water flux measurements (J) at different transmembrane pressures (TMP) in a cross-flow filtration cell. The plot of J vs TMP directly gives the permeability according to the following equation:

$$L_P = J/(TMP) \tag{2}$$

The membrane thickness was determined by a digital micrometer (MAHR 40E, Germany) as the average of at least ten measurements.

Folic Acid Binding

The prepared membranes were previously extracted in a cross-flow filtration cell by prolonged washing with sodium phosphate buffer 50 mM at pH 8.0 till no folic acid was detected by HPLC measurements in the permeate and retentate stream. Afterwards, membrane permeability was measured as described above and finally binding tests were performed. The recognition properties of the membranes were evaluated by measuring their binding capacity during the filtration of 100 mL of a $4.5 \cdot 10^{-6}$ mol/L aqueous solutions of folic acid at different pH. Experiments were carried out in a cross-flow filtration cell at 0.5 bar according to the total recycle mode. The amount of FA retained by the membrane was calculated from the mass balance of the Eq. (3):

$$Q_{FA} = \frac{V(C_0 - C_e)}{m} \tag{3}$$

where: Q_{FA} is the mass of the retained folic acid per gram of membrane; C_0 is the folic acid concentration at the beginning of the filtration test; C_e is the concentration at the end of the test; V is the volume of folic acid solution. After binding experiments membranes were first washed with de-ionized water in order to remove the weakly non-specific bound FA and then with sodium phosphate buffer 50 mM at pH 8.0 to extract the template specifically bound to the membrane. Afterwards, the binding experiments were repeated at least three times.

RESULTS AND DISCUSSION

Polymer Preparation and Characterization

PAN and copolymers AN/AA at 90:10 weight ratio were prepared by water-phase precipitation polymerization. The reaction is a free-radical polymerization which takes place in the water phase, all the components being water soluble except for AN which is partially soluble (7 wt.% in water) giving rise to the formation of random copolymers. Both the initiation and termination reactions are sensitive to the concentration of free-radicals which are generated by the redox couple $NaHSO_3/K_2S_2O_8$ in the presence of Fe^{++} ions. In the present work, several polymerization tests were carried out at different $NaHSO_3$/Mo ratio and the results are reported in Table 1.

As can be seen, the different $NaHSO_3$/Mo ratio in the reaction environment influenced both the yield and properties of the synthesized polymers. In particular, the polymerization yield increases as the above ratio increases while the intrinsic viscosity and the molecular weight of the homopolymer and of the copolymer decreases. These results could be explained taking into account that the increase of the $NaHSO_3$ concentration leads to the formation of a higher concentration of free radicals. As a consequence, a higher number of polymer chains are formed and a higher polymerization yield is obtained. At the same time, the concentration of the chain terminators also increases, giving rise to the formation of polymer chains with lower molecular weight.

With reference to the polymer composition, FT-IR spectra reported in Fig. 2, show that in the case of P(AN-co-AA) (b), further to the presence of the typical bands of PAN (a) such as the nitrile stretching at 2244 cm^{-1}, there

TABLE 1
Intrinsic viscosity, molecular weight and polymer yield of PAN and P(AN-co-AA) synthesized at different $NaHSO_3$/Mo

Polymer	$NaHSO_3$/Mo (wt.%)	[η] (ml/g)	$M_V \times 10^{-3}$ (g/mol)	Yield (%)
PAN	0.2×10^{-3}	940	815	45
	0.3×10^{-3}	520	377	70
	0.4×10^{-3}	210	115	84
P(AN-co-AA)	0.2×10^{-3}	850	714	52
	0.3×10^{-3}	600	454	65
	0.4×10^{-3}	320	200	76

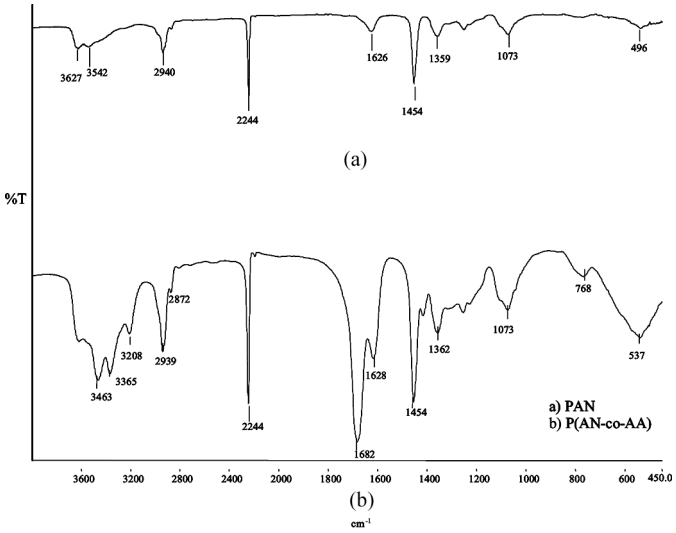

FIG. 2. FT-IR spectra of the synthesized PAN and P(AN-co-AA).

is also a large band at 1682 cm^{-1} (stretching of the carbonyl group in the amide compounds) and two bands at 3365 cm^{-1} and 3463 cm^{-1} (N-H stretching) which indicate the presence of the acrylamide in the polymer chain.

All the prepared polymers have been dissolved in NMP at a weight ratio of 10% in order to evaluate if the obtained solutions are suitable for the preparation of flat membranes. Polymers with $M_V > 400{,}000$ g/mol gave highly viscous solutions with a strong tendency to form gels, unsuitable for the preparation of uniform flat membranes. On the other hand, polymers with lower M_V may also be dissolved up to 15 wt% giving rise to the formation of uniform flat membranes.

Membrane Preparation and Characterization

Membranes prepared with polymers having a molecular weight $> 400 \cdot 10^3$ g/mol exhibited many structural defects due to the high viscosity of the casting solutions. So far, only the polymers with $M_V < 200 \cdot 10^3$ g/mol were used for the subsequent studies.

The phase inversion process adopted for the preparation of membranes was carried out according to two different methods: SE and IP. These methods were selected to prepare membranes with different morphologies and transport properties. The aim was to identify the best conditions to obtain imprinted membranes with appropriate recognition properties toward template molecules.

In Fig. 3 are reported SEM micrographs of the cross section of the membranes prepared according to the two methods.

Membranes prepared via solvent evaporation (a) exhibit a dense homogeneous structure due to the progressive increase of the polymer concentration in the forming membrane as a consequence of the evaporation of the solvent.

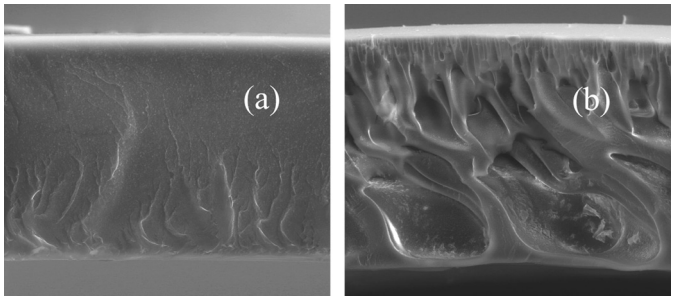

FIG. 3. SEM images of the cross section of membranes prepared via solvent evaporation (a) and immersion precipitation using water as the coagulation bath (b).

On the other hand, membranes prepared via immersion precipitation (b) show a porous structure with finger-like macrovoids due to the rapid liquid-liquid demixing typical of the non-solvent induced phase separation (35).

Table 2 shows thickness and water permeability of the prepared membranes. As can be seen, both are strongly influenced by the preparation method and there are only small differences between the homopolymer and the copolymer-based membranes. In fact, membranes obtained via IP show a much higher thickness (ranging from 270 to 300 μm) than those prepared via SE (ranging from 80 to 90 μm). This large difference is determined by the formation of the macrovoids in the porous membranes. Thickness measurements obtained by SEM micrographs are in agreement with the results obtained by manual measurements performed with a digital micrometer. Water permeability ranges between 200 and 2501 $\cdot h^{-1} \cdot m^{-2} \cdot bar^{-1}$ in the case of the two membranes prepared via IP, which are typical of porous membranes. On the other hand, those prepared via solvent evaporation exhibit water permeability ranging from 10 to 201 $\cdot h^{-1} \cdot m^{-2} \cdot bar^{-1}$ which reflects the more dense structure of these membranes.

Binding Tests

After removal of folic acid, the recognition capacities of the imprinted and blank membranes were evaluated by binding experiments carried out in a cross-flow filtration cell as already described. Binding tests were first carried out at different pH (from 4 to 8) using P(AN-co-AA) folic acid-imprinted membranes prepared via immersion precipitation. The highest binding capacity (4.5 μmol/$g_{memb.}$) of imprinted membranes was observed at pH 5. At more acidic or basic conditions membranes exhibited much lower affinity for the template molecules. This behavior can be explained on the basis of the ionic state of FA at the different pH. In particular, at pH < 4 it is present in the protonated form ($pKa_1 = 2.35$; $pKa_2 = 8.3$) which undergoes photolytic degradation (36), while at pH > 6 it is partially present in its anionic form which might be less

TABLE 2
Water permeability and thickness of the membranes prepared with the solvent evaporation and the immersion precipitation methods

	Solvent evaporation		Immersion precipitation	
Membrane	L_p ($l \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$)	Thickness (μm)	L_p ($l \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$)	Thickness (μm)
PAN	10	80	250	270
P(AN-co-AA)	20	90	200	300

active for the above-mentioned interactions. For comparison, experiments using blank membranes prepared in the same conditions were carried out. These membranes only showed a poor non-specific binding (about 0.9 μmol/$g_{memb.}$ at pH 5). The retention of FA by blank and imprinted membranes at the different pH is shown in Fig. 4.

The difference in FA retention between an imprinted membrane and the corresponding blank gives the specific binding capacity. This difference is the strongest evidence that membranes were actually imprinted. In fact, imprinted membranes show high affinity toward template molecules due to 3-D structure and to the arrangement of the functional groups around the template. The lack of these recognition sites into blank membranes is the reason for the poor interaction between the template and the polymer chains.

Binding tests at pH 5 were also performed using membranes prepared via SE. The comparison with the results obtained with membranes prepared via *IP* evidenced that the recognition properties of the membranes are also influenced by the method used for their preparation. Membranes prepared via SE exhibit a higher specific binding capacity (5.3 μmol/$g_{memb.}$) than those prepared via immersion precipitation (3.6 μmol/$g_{memb.}$). In the first case, after the evaporation of the solvent, the template remains homogeneously distributed in the polymeric matrix thus allowing a better assembling of the polymer chains around the template molecules. When using the IP method, part of the template is released in the coagulation bath reducing the possibility of creating an adequate number of recognition sites in the polymeric matrix. In addition, the solvent-nonsolvent exchange during the phase separation process may interfere with the spatial arrangement of polymer chains around the template thus reducing the imprinting effect.

After each binding experiment the membrane was washed with sodium phosphate buffer 50 mM at pH 8.0 in order to recover the retained FA and to restore its initial conditions. The subsequent repetition of the binding experiments for three times revealed a negligible reduction of the binding capacity thus indicating that the membrane could be reused without significant loss of the performance.

In order to prove the role of the acrylamide functional monomer in the recognition capacity of the above-described imprinted P(AN-co-AA)-based membranes, a series of binding tests was performed at pH 5, using PAN-based membranes prepared via SE. Results, reported in Fig. 5, were compared with those obtained with P(AN-co-AA)-based membranes.

PAN-based membrane prepared in the presence of the template shows a poor FA retention (1.5 μmol/$g_{memb.}$), only a little bit higher than that of the corresponding blank membrane (0.9 μmol/$g_{memb.}$). P(AN-co-AA) imprinted membrane retained 6.3 μmol/$g_{memb.}$ and its corresponding blank retained only 1.0 μmol/$g_{memb.}$. These results evidence three important aspects:

1. blank membranes exhibit similar low retention, independent on the polymer composition;

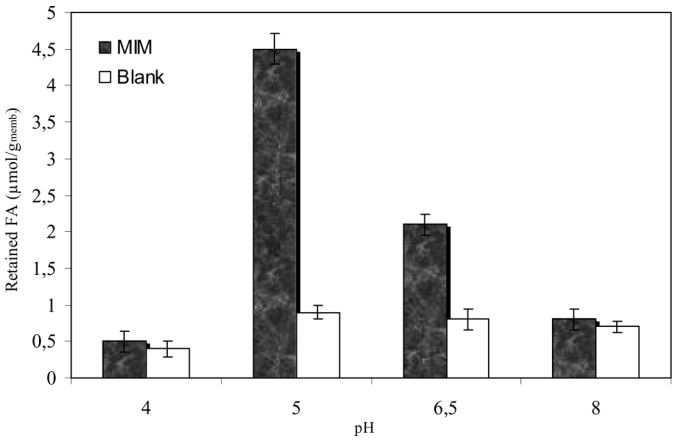

FIG. 4. Effect of the pH on the binding capacity of and poly(P(AN-co-AA) blank and imprinted (MIM) membranes.

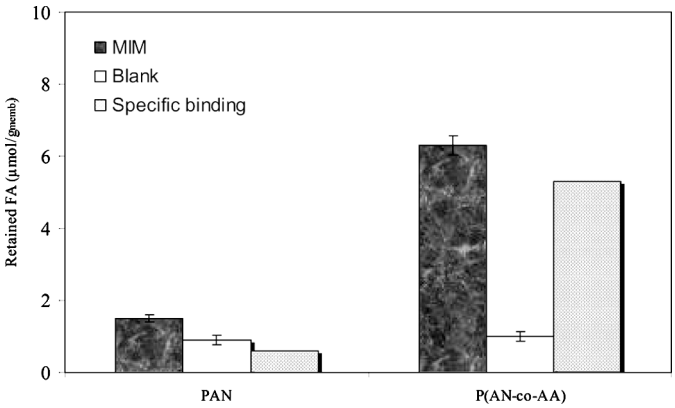

FIG. 5. Folic acid retained by PAN and P(AN-co-AA)-based membranes prepared via solvent evaporation.

2. only P(AN-co-AA) imprinted membrane exhibits high binding capacity;
3. only the simultaneous presence of acrylamide in the polymer chain and of the template in the casting solution may confer molecular recognition properties to the forming membrane.

Moreover, the interactions between the acidic groups of the target molecule and the acrylamide comonomer are a key factor in the process of recognition.

CONCLUSIONS

The combination of molecular imprinting and membrane technology allowed to prepare molecularly imprinted membranes with affinity properties for FA. This has been achieved through the synthesis of a P(AN-co-AA) copolymer containing acrylamide as functional monomer which specifically interacts with the target molecule thanks to its functional groups.

The polymer composition, membrane preparation method, and pH used in the binding experiments play an important role in the molecular recognition. In particular, P(AN-co-AA) imprinted membranes prepared via solvent evaporation exhibited the highest specific binding capacity ($5.3\,\mu mol/g_{memb.}$) during recognition tests performed at pH 5. Blank membranes showed poor non-specific binding $\leq 1.0\,\mu mol/g_{memb.}$. PAN-based membranes prepared in the presence of the template only showed low FA retention ($1.5\,\mu mol/g_{memb.}$).

Thanks to their good performance and to their ease and low energy operation, the imprinted membranes prepared in this work may find application in the extraction of folic acid from foods and beverages.

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