OPERATIONAL PARAMETERS FOR THE PREPARATION OF PRECURSOR POLYMER MATRIX USED IN MOLECULAR IMPRINTING

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This paper treats the importance of establishing custom operational parameters for preparing molecularly imprinted pearls with hypericin via wet phase inversion. Hence, characteristic parameters related to the wet phase inversion process were optimised in order to propose an adequate protocol for pearl-shaped imprinted copolymers. To this effect, PAN –co-PMAA precursors prepared by soap-free emulsion polymerization with various compositions were tested as viable matrix for hypericin-molecular imprinting. Precursor copolymer solutions were able to generate robust, spherical and highly porous pearls as desired for these specific applications.

Keywords: molecularly imprinted polymer, phase inversion, precursor copolymer

1. Introduction

During recent years, one of the most promising methodologies for preparing network polymers with specific molecular-recognition properties is considered to be molecular imprinting. Its origin is traced back to the work of

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Polyakov on imprinted silica gel [1] and reported for the first time by Wulff [2] in the 1930s and 1970s, respectively. Molecularly imprinted polymers (MIPs) are synthetic designed sorbents with enhanced selectivity towards template structures [3]. Briefly, this technique consists in the polymerization of functional monomers in the presence of a target-molecule called template. Cross-linking agents and radical initiators are also added. In this particular approach, the self-assembly step of functional monomers around the template molecules, via reversible interactions, generates a stable polymerizable complex; after polymerization/crosslinking and template removal, complementary cavities in shape, size and electronic entourage with the template molecules are imprinted in the polymer matrix [4]. Hence, polymer "memory" for the template molecules is responsible for MIPs high selectivity [5-7]. With regard to the nature of imprinting, the methods have been classified as chemical molecular imprinting and physical-chemical molecular imprinting. The chemical methods imply imprinting during polymerization of monomer-template complexes generated in the self-assembly or the pre-organization step. Physical-chemical imprinting a.k.a. phase inversion refers to creating specific cavities, selective for target molecules, in a post-polymerisation step. In the latter, a precursor solution of a linear polymer is mixed with template molecules to generate specific interactions; subsequently, the solution is precipitated in a proper non-solvent to create robust pearls or membranes [8-10]. Over the last decade, chemical methods for MIP preparation e.g. bulk/monolith polymerization [11, 12], emulsion polymerization [13], suspension/dispersion polymerization [14-16], precipitation polymerisation [17] or sol-gel techniques, have been systematically developed. Over biomolecules, MIPs benefit of (i) less expensive preparing costs, (ii) higher physical robustness, and (iii) thermal, mechanical and chemical stability. These advantages explain the growing interest in the molecular imprinting field as biosensors [18-21], adsorbents in chromatography [22], adsorbents in solid-phase extraction [23,24] or membranes for chiral recognition [25].

The success of a suitable imprinting system is defined by choosing adequate monomer(s) and crosslinker(s) to complement template's functionalities, in order to form stable complexes. Additionally, the imprinting mechanism is usually similar to the following recognition process. Hence, it is important to determine in what extent the functionalities of monomer(s) can interact with the template functional groups in order to evaluate the proper nature of such monomer-template interactions. Hydrogen bonds are the most applied type of molecular interaction in molecular imprinting due to both their strength and their reversibility. Consequently, acrylates and methacrylates with carboxyl functionalities are the most employed monomers, owing to their ability to generate hydrogen bonds with a variety of polar functionalities of the imprint molecules.

The main objective of this paper is to establish the operational procedures for the synthesis of copolymer precursors further used to prepare robust polymer matrix, adequate for molecular imprinting via wet phase inversion. Copolymer robust pearls were imprinted with hypericin during phase inversion, using a purified and concentrated extract from *Hypericum Perforatum*. Hypericin is a powerful naphtodianthrone pigment found in the common perennial plant St. John's Wort along with pseudohypericin, its structural analog (Fig. 1.). The necessity of creating a specific adsorbent for hypericin, to separate it from phyto-extracts having similar structural competitors a.k.a. pseudohypericin is reflected by the acquisition cost of commercially available versions of pure hypericin and its pharmacological effects. Traditionally, hypericin is used as an antidepressant, antitumoral and anti-inflammatory agent [26,27].

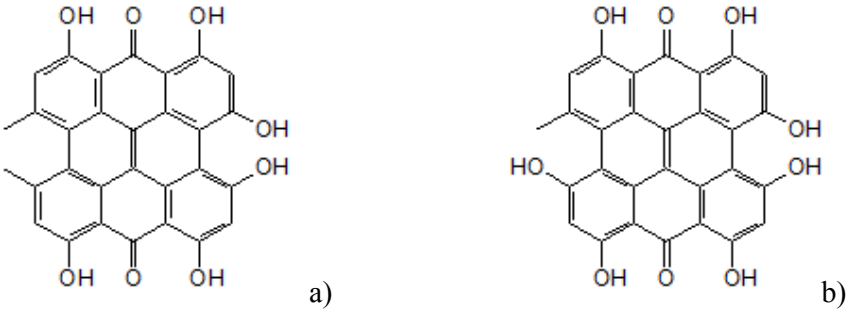

Fig. 1. Chemical structures of hypericin (a) and pseudohypericin (b)

2. Experimental section

2.1. Materials

For copolymer preparation, acrylonitrile (AN, ACROS Organics) and methacrylic acid (MAA, ACROS Organics) were distilated before use. Potassium persulfate (PK, Fluka, 98%) and sodium metabisulfite (MS, Fluka, 97%) were used together as a redox initiation system. Sulphuric acid (H_2SO_4, Fluka, 97%) was used for pH variation of the polymerisation medium. Dimethylsulfoxide (DMSO, reagent grade) and ethanol (EtOH, 99.6%) were purchased from Scharlau or Chimopar and used as such. The hydro-alcoholic (30/70, v/v) extract from the superior part of *Hypericum Perforatum* (St. John's Wort flower) was provided by Plantavorel Romania.

2.2. Preparation of copolymer imprinted pearls (CP x-y, x=1-4, y=4 or 10) [9]

Four PAN-PMAA copolymer precursor powders (CP x), were prepared by soap-free emulsion copolymerization using various weight ratios of AN: MAA monomers (90: 10, 85: 15, 80: 20 and 75: 25) according to a previously described procedure [9]. These copolymer precursors were noted hereafter CP x, where x=1 for 90: 10, x=2 for 85: 15, x=3 for 80: 20 and x=4 for 75: 25, respectively. The monomers were found suitable for imprinting by wet phase inversion due to the structural strength role of AN (used instead of a crosslinker) and to the coordination ability of MAA functionalities (which generated hydrogen bonds with the template molecule). The copolymer precursor powders (8 wt. % relative to DMSO) were dissolved in DMSO (100 mL) at room temperature under continuous stirring in light-protected flasks. Once the powders were totally dissolved, a hydro-alcoholic extract (extract content noted as y, where y= 4 wt. % or 10 wt. % relative to copolymer) was used to prepare the precursor polymer-template feed solutions. In order to obtain the imprinted pearls (CP x-y, x=1-4, and y=4 wt. % or 10 wt. % extract), the feed solutions were introduced into a syringe-like cartridge of the semi-automated dripping system, an installation developed by some of us [9]. The non-imprinted pearls (NP x, x=1-4) were prepared under identical conditions as imprinted ones CP x-y, but without template. Spherical imprinted pearls were obtained when the feed solution droplets, form by dripping, contacted the coagulation bath. In order to choose a proper non-solvent, the pearls were generated using water: isopropyl alcohol phase inversion baths, combined in various ratios: 75: 25, 50: 50, 100: 0. This critical moment is considered to be the imprinting stage which is linked to the phase inversion process. The pearls were maintained in the water bath until complete precipitation of the copolymer. Finally, the pearls were dried at 30 °C until constant weight in order to be physical and chemical characterized.

2.3. Characterization methods

Optical micrographs of imprinted pearls were obtained using an IOR Bucharest microscope, at 10X magnitude order. Rheological studies of copolymer solutions were performed using a RHEOTEST 2.1. device with coaxial cylinders and thermostated bath at 25 and 50 ^{o}C temperature. Surface analysis performed by X-ray photoelectron spectroscopy (XPS) was carried out on Thermo Scientific K-Alpha equipment, fully integrated, with an aluminium anode monochromatic source. Thermo-gravimetric analysis (TGA), were performed using Q500 TA equipment, under nitrogen atmosphere at a constant heating rate of 10°C/min (temperature range 25- 600^{o}C). Differential Scanning Calorimetry (DSC) curves were recorded on Netzsch DSC 204 F1 Phoenix equipment. Heating and cooling

cycles were registered under nitrogen flow in the 25-400°C temperature range, using a 10°C/min heating rate. Fourier Transformed-Infrared (FTIR) spectra, registered on Bruker Vertex 70 equipment in the 400-4000 cm^{-1} range with 4 cm^{-1} resolution and 32 scans (ATR method) were useful to confirm molecular imprinting with hypericin underlining characteristic bands of copolymers and of template molecules.

3. Results and discussion

3.1. Rheological studies for CP x-y copolymer solutions

The four precursor copolymer solutions were rheological characterized in order to predict their behaviour when contacting the inversion media. This behaviour is crucial to establish the final shape of the pearls. Furthermore, the most specific parameter in polymer processing is represented by the flow. Rheological behaviour of precursor copolymer solutions was investigated by following the trend of dynamic viscosity as function of applied shear rate. Fig. 2, revealed the pseudo- plastic rheological behaviour of CP 3 and CP 3-4 solutions at 25 ^{o}C and at 50^{o}C. It is well known that the pseudo-plastic behaviour is characterized by a decrease of viscosity with the shear rate. A pseudo-plastic behaviour characterises the flow of solutions with low extents of MAA and extract (CP 1-4 and CP 2-4) whereas the behaviour has a tendency to shift towards Newtonian at maximum contents of MAA and extract (CP 4-10); the viscosity remains constant for a given range of shear rates (data not shown). It was observed in Fig. 2 that CP 3 showed a higher dynamic viscosity than CP 3-4 solution at the same shear rate; these results indicated that the presence of AN, responsible for the intercatenar bonding formation, led to a more pronounced pseudoplastic character of copolymer solutions. Upon extract addition, it was also noticed a sharp decrease of dynamic viscosity followed by a cvasi-Newtonian flow. Consequently, the extract presented a quite important influence upon the general rheological character of copolymer solutions. It is noteworthy the fact that water and ethanol, which are also Newtonian fluids, were the promotor of the cvasi-Newtonian polymer flow at higher shear rates (over 20 s^{-1}). As expected, the increase of temperature led to even sharper deacrease of dynamic viscosity (Fig. 2 b).

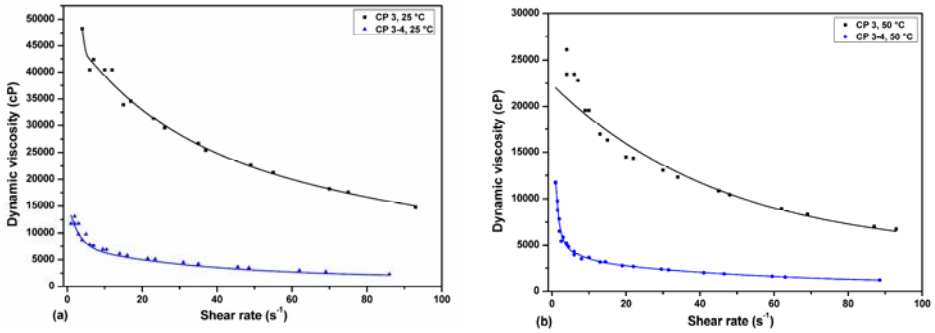

Fig. 2. Rheological behaviour of copolymer DMSO solutions: CP 3 (without template) and of CP 3-4 (with 4% extract) (a) at 25 °C and (b) at 50°C

3.2. Morphology of the obtained pearls

The morphology of CP x-y imprinted pearls was similar in all phase inversion systems (representative example of CP 3-4, fig. 3 a-c). The pearls presented tails due to either the column height of non-solvent in the inversion bath (*h*) or the height between the dripping nozzle and the inversion bath (*H*). Hence, after visual inspection of pearls, *H* was varied until spherical forms were obtained; this operational parameter had an optimum value at *H*=0.5 m. Copolymer inversion at the surface of pearls is almost instant at contact with the non-solvent. Afterwards, the process is governed by diffusion of non-solvent from the surface to the core of copolymer pearls. Complete precipitation can take up to 24 h, meaning the pearl shape is still sensitive to impact. Consequently, if *h* is low the shape of pearls gains an unwanted flat facet. Therefore, *h* was also optimised and established to be at least 20 cm height. Moreover, the pearl shape is highly dependent upon the syringe needle diameter. At low diameters, under 0.05 cm, instead of independent droplets the dripping system generates continuous strings of copolymer, with bulges corresponding to every new feed. This phenomenon was predictable due to copolymer pseudo-plastic behaviour; and hence, the needle properties were optimised as function of applied pressure. Best results were obtained when a 3 Pa constant pressure was applied to extrude the copolymer solutions through a 0.8 cm long needle having the inner diameter approximately 0.05 cm.

Fig. 3. Imprinted pearls CP 3-4 obtained in 100% water (a); 75-25% water-isopropyl alcohol (b); and 50-50% water-isopropyl alcohol (c) phase inversion baths

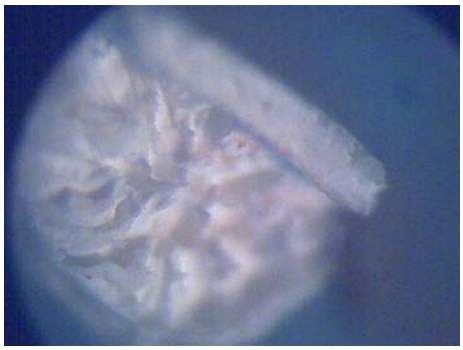

Fig. 4. Morphology for CP 1-4 in section obtained in 75-25% water-isopropyl alcohol phase inversion bath

Optical microscopy studies were also performed in order to visualise pearls morphology (Fig. 4). The inner structure of dry pearls revealed a dense pore structure with star-like channels communicating to the surface. This unique morphology is the result of non-solvent diffusion from the surface to the core of pearls. Also, considering that hypericin molecules are virtually non-soluble in the water media [28], they remained entrapped in the copolymer beads. Additionally, the visual inspection of pearls led to the same conclusion. This particular extract is red-coloured (due to hypericin). During the phase inversion stage the aqueous media remained transparent and colourless and the obtained pearls kept the red-dark colour of the initial feed solution.

3.3. XPS measurements of non-imprinted pearls

The XPS spectra were recorded in order to identify the elemental composition of copolymer pearls surface. The survey was realized for dried non-

imprinted pearls obtained in the 75-25% water-isopropyl alcohol phase inversion bath. In order to appreciate if there is a preferential arrangement on the pearl surface of pendant functional groups belonging to AN and MAA co-monomers, the composition of C, N and O was determined in three points for each sample and the average was taken into account. The relative atomic concentrations (At. %) for carbon, oxygen and nitrogen are listed in Table 1.

Table 1

Composition of NP x pearls determined by XPS

Samples / At. %	NP 1	NP 2	NP 3	NP 4
C1s	90,19	72,42	74,75	80,95
N1s	17,47	24,62	20,63	11,93
O1s	2,33	2,96	4,62	7,12

XPS spectra indicated the presence of the characteristic constituents of the copolymer matrix: C, N, O. High contents of C, registered on the surface of pearls corresponded to carbon present in the copolymer backbone. The O and N contents showed that both MAA and AN functionalities are present on the surface. The values of C for pearls were about 10% higher, indicating a preferential arrangement of functional groups inside the pearls. As expected, the content of O and N from pearls is consistent with the initial copolymer composition.

3.4. Thermal stability

The thermal degradation of imprinted pearls CP 3-4 and CP 3-10 (obtained in 75-25% water-isopropyl alcohol phase inversion bath), compared with the corresponding non-imprinted pearls, NP 3, is presented in Fig. 5 a. All imprinted pearls registered higher mass loss relative to their blanks which is completely understandable since the extract inset contains the template as well. TG plot revealed one decomposition stage of the copolymer matrix. Until 100 °C, all samples lose residual water. For CP 3-4 the water loss is the highest, being the reason for a more pronounced final weight loss. The maximum registered for the imprinted pearls were shifted towards higher values indicating a slight influence of the extract, or naphtodianthrones, upon thermal behaviour of the copolymer matrix. Though the extract (with different content of 4 or 10%) contains a low concentration of hypericins (0,1985 g/L) and the solvent- water/ethanol which is the main constituent of the extract- is removed by drying, all curve profiles were similar during thermal degradation. Hence, the influence of hypericin was studied relative to the non-imprinted copolymer. Naphtodianthrones completely degrade around 320 °C. Hence, in the following temperature range, up to 800 °C, a higher weight loss (completely attributed to polymer degradation) was registered. The

high extent of residues registered for all pearl types, were specific to acrylonitrile based-polymers which undergoes cyclization and graphitization processes.

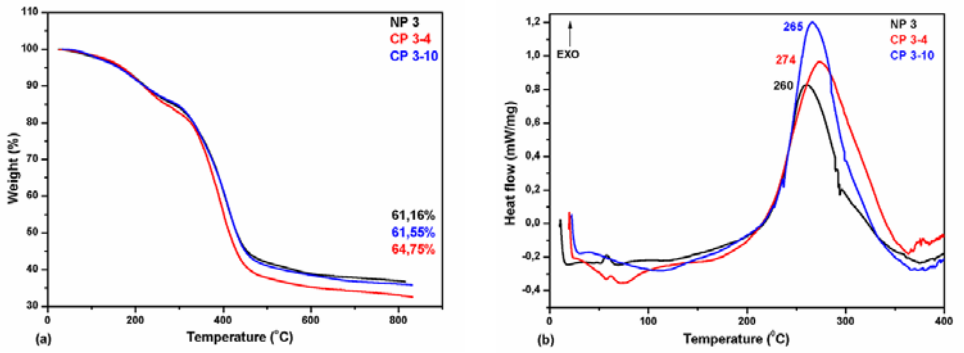

Fig. 5. TGA profile (a) and DSC curve (b) of the non-imprinted pearls NP 3 compared to imprinted ones CP 3-4 and C 3-10, respectively

Thermal behaviour of the copolymer pearls can be evaluated by differential scanning calorimetry measurements in the 20-400°C temperature range. Fig. 5 b shows the corresponding DSC curve of NP 3 in comparison to CP 3-4 and CP 3-10. The small endothermic peak from 68 °C and 73 °C, registered for NP 3 and CP 3-4, respectively was assigned to the free water loss. This process was also present in the CP 3-10 pearls, but accompanied by a large endothermic peak at 113 °C representative for bonded water loss. Thus, higher extract extents led to enhanced intramolecular water adsorption. The exothermic peak recorded around 260°C (see NP 3 curve, Fig. 5 b) on the DSC pearls thermogram corresponded to polyacrylonitrile cyclization process. Maximum temperatures of this particular process were shifted towards higher values i.e. 265°C for CP 3-10.

3.5. FTIR Analysis

FT-IR investigations highlighted the presence of characteristic copolymer bands and of those affected by the hypericin-imprinting process. All samples present similar spectra with characteristic bands specific for copolymers and for extract components. Characteristic bands of copolymer i.e. ν_{-CH} stretching vibrations, $\nu_{-C\equiv N}$ band (corresponding to AN monomer) and characteristic vibrations of ν_{-OH} and $\nu_{-C=O}$ band (both associated with the carboxyl functionality of MAA monomer) appear in the 2920-2935 cm^{-1} range, in the 2240-2244 cm^{-1} region, and in the 3736-3610 cm^{-1} and 1710-1720 cm^{-1} range, respectively. Comparing the blank pearls, NP 3, with the imprinted ones, CP 3-4, (Fig. 6), the appearance of a shoulder between 1579-1672 cm^{-1}, corresponding to $\nu_{-C=O}$ specific bands in polyphenols, confirmed the presence of hypericin in the imprinted pearls.

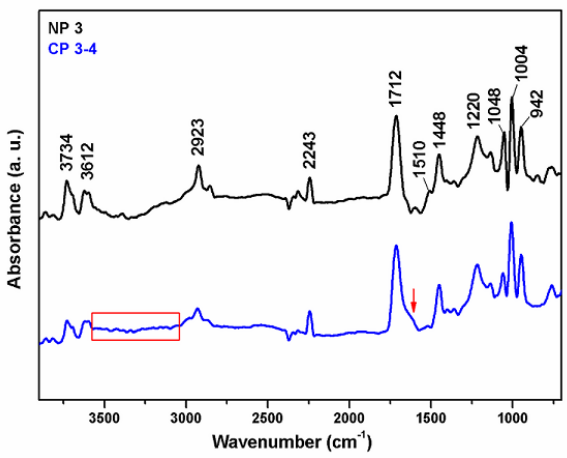

Fig. 6. FTIR spectra of CP 3-4 compared to NP 3

Furthermore, the intensity of –OH characteristic bands from MAA (3035-3581 cm^{-1} range) decreased in CP 3-4 spectrum as a results of monomer-template interactions. The imprinted pearls also exhibited changes relative to other characteristic copolymer bands i.e. disappearance of specific bands around 1510 cm^{-1} and 1048 cm^{-1}, corresponding to ν_{C-C} and ν_{C-O} vibrations; these bonds are also presumed to be affected by copolymer-template interactions.

4. Conclusions

Rheology study of pearls precursor solutions was paramount for understanding the behaviour of feed solutions in the dripping process. Together with morphology investigations, operational procedures and specific phase inversion parameters were optimised. Best results, obtained for solutions with lower AN ratio, CP 3 were completely justified by the cvasi-Newtonian flow of initial solutions which generated spherical and almost tailless pearls. Both the structural and the thermal properties of imprinted pearls underlined the presence of template in the copolymer matrix, which was retained and physically imprinted during phase inversion.

Acknowledgments

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