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# Peptide imprinted polymer synthesized by radiation-induced graft polymerization

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**Abstract**

The design of polymer surfaces with molecular recognition capabilities is an exciting subject with potential application in the analytical and biotechnological fields. Molecular imprinted polymers (MIPs) are still in their infancy and new methodologies are required to broaden their application. In this paper, MIPs were synthesized by simultaneous radiation-induced graft polymerisation in a polar medium. Bacitracin, a cyclic decapeptide, was used as the target molecule to develop MIP grafted onto macroporous membranes. Soluble ternary complexes of vinyl pyridine, bacitracin and copper (II) were obtained and characterized by visible spectroscopy in an aqueous medium. These complexes were grafted onto macroporous polyethylene membranes. MIP materials showed ability to adsorb bacitracin in a higher amount than non-printed polymers. In addition, these materials showed a 5-fold greater selectivity to bacitracin than that of chemically modified bacitracins.


*Keywords:* Imprinted polymers; γ-Radiation; Bacitracin; Graft polymerisation

## 1. Introduction

Materials able to attain selective recognition of peptides, proteins and other biological assemblies are required in biotechnology for product purification, drug delivery, sensors and diagnosis [1].

Molecular imprinted polymers (MIPs) are based on matching geometrical shapes and complementary functional groups in a specific and predictable manner. This is a promising approach for the preparation of highly selective materials, specially for chiral molecules. In this way, the application of molecularly imprinted polymers as separation media in liquid chromatography, capillary electrophoresis and capillary electrochromatography for chiral separation has been extensively reviewed [2–7]. Applications to organic synthesis and catalysis was also recently reported [8].

Although imprinted proteins show a great promise for future work, smaller template molecules and ions continuing to be abundant in the current literature.

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This is mainly due to the fact that large templates are less rigid thus impairing the generation of well-defined binding cavities during the imprinting process. Moreover, the secondary and tertiary structure of large biomolecules such as proteins may be affected when exposed to the thermal or photo-induced treatment involved in the synthesis of imprinted polymers. Rebinding is also difficult since large molecules such as peptides and proteins do not easily penetrate the polymer network for reoccupation of the binding pockets [5]. However, some advance in protein-template removal and reloaded was recently reported [9].

Molecular imprinting of proteins has been achieved by using radio-frequency glow-discharge plasma deposition to build thin polymer films over proteins coated with disaccharide molecules [10]. Unfortunately, this methodology can be applied only to flat surfaces. In a more "classical" approach, Rachkov and Minoura [11] suggested a method to overcome the steric problem of voluminous targets by mimicking the biological systems in a way similar to that in which an antibody does not recognize the entire molecule but a small characteristic substructure (epitope). They used a short tetrapeptide as a template to synthesize a MIP able to recognize oxytocin, a nine amino acid peptide containing the same structural fragment as that of the template. However, the conformational constrains of peptides in a protein structure should be very difficult to emulate for a free peptide.

Non-covalent molecular imprinting has been reported to be an effective and suitable approach because of the greater range of compounds – including chiral molecules – which can be imprinted [4,6]. These polymers display fast rebinding kinetics but the recognition capabilities are limited by the weak nature of the individual interactions. In contrast to the biological environment, stable complexes in solution are mainly limited to non-polar solvents to enhance the hydrogen bond and electrostatic interactions. This difference between polymerisation and analysis media may bear influence on the expected results, specially when peptides or proteins are the target molecules. Also, different effectiveness degrees are achieved when different polymerisation methodologies are applied [12].

The metal-ion complex is an interesting alternative to build non-covalent assemblies useful for MIP in polar media [13]. A great degree of versatility can be attained by targeting different functional groups on a protein surface as well as tailoring the strength of the individual interactions. Cu(II), Ni(II) and Zn(II) ions, for example, are targets for histidine residues at a neutral pH and Al(III) and Fe(III) can interact specifically with carboxyl and phosphoryl groups, respectively [14]. Chromatography with immobilized ions through chelating groups (IMAC) allows specific metal coordination binding of biological macromolecules, which has had a great success in the downstream processing [15]. Since long ago, Arnold and co-workers [16] and Mosbach and co-workers [17] utilized IMAC to develop MIP materials based on non-commercial monomers including chelating moieties. Recently, an interesting review gives a survey over recent achievements on the design of templated polymers utilizing coordinative bonds [18].

In order to improve the accessibility of the target molecule to the printed site, the surface grafting technique has an extra advantage over bulk polymerisation [19]. However, a special surface pre-treatment is generally required (vinyl-activated surfaces) and/or specially designed materials, which should contain reactive groups to link the grafted layer [10,16]. In this way, radiation-induced graft polymerisation can overcome this requirement by producing free radicals onto poorly reactive solid polymers.

The use of radiation-induced polymerisation was not as much explored. Rao et al. have used this technique to synthesize successfully metal-ion imprinted polymers recently [20,21].

The aim of this work is to evidence the potentiality of radiation-induced graft polymerisation techniques to prepare MIP materials in a polar medium. Bacitracin A (Bac), a cyclic decapeptide, was used as the target molecule to develop a MIP grafted onto macroporous membranes.

### 1.1. Materials and methods

Bac (M.W. 1423 Da), diethyleneglycol dimethacrylate (DEGDMA) and 4-vinylpyridine (vPy) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Monomers were used as received. All other chemicals were AR grade.

Hollow-fibre polyethylene membranes (140 mm length, 0.33 μm internal pore diameter, 0.6 and 1.2 mm inner and outer diameter, and 70% nominal porosity) were kindly donated by Dr. Maakuchi (JAERI, Takasaki, Japan).

### 1.2. Soluble complexes

Monomer vPy was used for the preparation of soluble complexes for polymerisation. vPy is capa-

ble of forming complexes with copper ions mainly from 1:1 to 1:4 copper(II):vPy molar ratio.

Visible spectra of copper(II)–vPy solutions were obtained as follows: 10 μmol $CuSO_4$ in 1 ml buffer A [30 mM Tris buffer, pH 7.0, 300 mM NaCl/methanol (50/50, v/v)] was mixed with increasing vPy concentrations by adding 10, 20, 30, 40 and 45 μmol vPy to the copper solution. Each solution was scanned between 400 and 800 nm with a Ultrospec 2000 spectrophotometer (Amershan Biosciences, Upsala, Sweden).

Visible spectra of copper(II)–Bac solutions were obtained as follows: 10 μmol Bac in 500 μl buffer A was mixed with increasing copper concentrations by adding 1, 3, 5, 7, 9, 11 and 13 μmol $CuSO_4$ in 500 μl buffer A. Each solution was then scanned as described above.

Visible spectra of vPy–copper(II)–Bac solution were obtained as follows: 10 μmol $CuSO_4$in 500 μl buffer A was mixed with 40 μmol vPy and 10 μmol Bac in 500 μl buffer A.

Copper(II)–vPy complex titration with Bac was performed by preparing different mixtures containing 10 μmol $CuSO_4$ in 100 μl buffer A, 40 μmol vPy and different amounts (from 0 to 14 μmol) of Bac (20 μmol/ml buffer A) in 1.4 ml final volume. The mixtures were analysed by its absorbance at 660 and 708 nm.

### 1.3. Radiation-induced graft preparation of MIPs

A weighed amount of membrane (i.e., 200 mg) was soaked in different monomer solutions (Table 1) previously degassed with a nitrogen stream. Buffer A was used as the solvent. In addition to the complex molecule, a 2% and 1% v/v of DEGMA was added as a "filler" monomer.

Samples were irradiated in a $^{60}Co$ Gamma irradiation source gammacell AECL GC-220. The dose was determined by Friecke dosimetry.

Exhaustive washing with distilled water, methanol and, finally, 0.1 M EDTA was performed to remove the complexes and release the target molecule from the grafted polymer. Finally, one membrane sample was dried to determine the modification degree (MD%), calculated from the difference in weight between the post-irradiated membrane and the original one, the latter being discarded.

### 1.4. Copper saturation capacity

Ten membranes were immersed in 10 ml of 250 mM $CuSO_4$ in the adsorption buffer: (30 mM Tris–HCl, 300 mM NaCl, pH 7.0). After 8 h, they were thoroughly washed with the adsorption buffer and then eluted with 3 ml of 0.1 M EDTA, pH 7.0. Copper was quantified by spectrophotometric determination of the copper(II)–EDTA complex concentration at 715 nm.

### 1.5. Peptide saturation capacity

Grafted membranes were reloaded with copper by soaking them in 250 mM $CuSO_4$ in adsorption buffer. After a careful wash with the adsorption buffer, the samples were incubated overnight with 2 mg/ml peptide solutions in the adsorption buffer. After 10 h, membranes were extensively washed with adsorption buffer and then eluted with a 50 mM sodium acetate buffer, 250 mM NaCl, pH 3.0. Peptide concentration was determined by spectrophotometric measurement at 280 nm.

Trinitrophenylated Bac (TNP-Bac) was obtained as described by Okuyama and Satake [22]. The number of trinitrophenylated residues per molecule of Bac was two, according to the calculation from UV–visible absorption at 345 nm.

Acetylated Bac (Ac-Bac) was obtained as described by Fraenkel-Conrat [23].

Table 1
Monomer mixtures to obtain grafted imprinted polymers based on chelating complexes

| Polymer | Mixture composition[a] | | | DEGMA (%)[b] | Copper adsorption (μmol/ml of membrane) |
|---|---|---|---|---|---|
| | vPy (μmols) | Copper(II) (μmols) | Bac (μmols) | | |
| MIP-1 | 315 | | 105 | 2 | 1.71 |
| MIP-2 | | 105 | 105 | 2 | 1.61 |
| MIP-3 | 315 | 105 | | 2 | 0.34 |
| MIP-4 | 315 | 105 | 105 | 2 | 2.22 |
| MIP-5 | 315 | 105 | 105 | 1 | 2.51 |

[a] Quantity of each component in 20 ml of polymerisation solution.
[b] % v/v of filler (DEGMA) in the polymerisation solution.

## 2. Results and discussion

Bac (Fig. 1) is a 10-amino acid cyclic peptide containing one histidine residue. Its three-dimensional structure in solution shows a significant degree of flexibility. Due to such flexibility, native bacitracins resisted numerous attempts of crystallization [24]. However, in the presence of divalent metal ions, the linear portion folds over to form a characteristic structure with the shape of "eight" [25]. Thus, it is to be expected that Bac can be a good model of macromolecule target for MIP material preparation.

The success of the MIP preparation lies on the assembling of soluble complexes built with three components: monomers, target molecule and the copper ion, which links them to one another. In order to obtain relatively stable monomer-Bac complexes in aqueous solutions, we take advantage of the capability of histidine and vPy to complex the copper(II) ion in polar environments. Amino-copper(II) complexes are easily assembled and detected by spectrometric visible titration experiments [13]. Hence, we demonstrated the existence of these soluble complexes and the optimisation of the ratio between ligands to maximize the amount of ternary complex in solution. Visible spectra of amino-copper(II) complexes with different stoichiometries show wavelength shiftings of their maxima and changes in their molar absortivities. In addition, some of them, especially those of high number of ligands are insoluble, therefore a continuous variation method could not be applied [26].

Bac can form a 1:1 complex with several divalent metal ions, including cobalt(II), nickel(II), and copper(II) [27,28]. Different spectroscopic studies of metal(II)–Bac have suggested that His-10 and the thiazoline ring sulphur are coordinated to the metal [28,29]. Thus, we studied the copper(II)–Bac complexes formation by following the absorption spectra in the visible region. A buffer/methanol solution (buffer A) was used as the solvent and pH 7.0 was selected to preserve all reagents as soluble free bases. Bac does not absorb light in the 400–800 nm wavelength range, however, when the copper(II) solution was slowly added to a Bac solution, a broad peak with a maximum at 648 nm was detected when the Bac:copper(II) ratio was 10:1, and progressively changed to 665 nm when the Bac:copper(II) molar ratio reached 1:1 (Fig. 2). According to the empirical equation reported to analyze equatorially-coordinated copper(II) complexes [30], the main component in the mixture should be assigned to complexes $Bac_2$–copper(II) and Bac–copper(II), respectively. Similar titration experiments using imidazole instead of Bac showed the same behaviour (data not shown).

On the other hand, the formation of copper(II)–vPy complexes was also followed by the absorption spectra in the visible region. VPy is a monodentate ligand, in consequence copper complexes yield a mixture of copper(II)–$vPy_n$ complexes with different stoichiometry where $n$ ranged from 1 to 4 [31]. As the pyridine (Py) moiety of vPy has a $pK_a$ value of 5.51, at a neutral pH and low concentration the amount of pyridinium ion is negligible and therefore complex formation is not impaired.

Fig. 3 shows the absorption spectra of copper(II) solutions with a step-by-step addition of vPy from 1:1 up to 4:1 vPy:copper(II) molar ratio. Higher molar ratios produced insoluble complexes under our working conditions, precipitating green needles. Absorption spectra in the visible region showed a broad peak where the maximum absorption shifted to the blue region after each vPy addition.

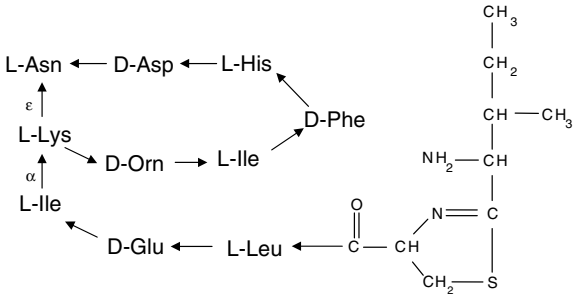


Fig. 1. Bacitracin A peptide.

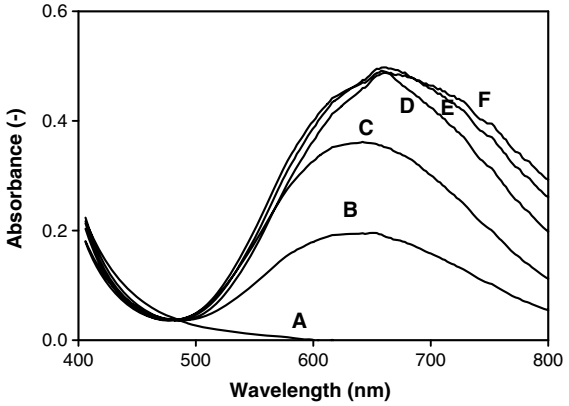


Fig. 2. Visible spectra of Bac 10 mM (A) and Bac–copper(II)mixtures corresponding to 10 mM Bac and copper(II) 3 mM (B), 5 mM (C), 7 mM (D), 9 mM (E) and 11 mM (F), respectively.

In the 1950s, Leussing and Hansen reported the characteristic absorption spectra of each copper(II)–Py$_n$complex from the analysis of cooper–pyridine mixtures [31]. Each complex shows a steady shift to lower wavelengths of the maximum absorption peak in the visible region. In addition, an increase in the extinction coefficient of the complexes concomitant with higher $n$ values was reported. They also showed an interesting isosbestic point around 700 nm for the transition involving complexes containing 2, 3 and 4 pyridine moieties. Therefore, spectra recorded with increasing vPy amounts correspond to the increase in the concentrations of copper(II)–vPy$_n$ complexes with higher $n$ values. Taking the association constants reported under similar solvent conditions [31] into account, approximately 80% and 90% of the initial copper concentration is complexed as copper(II)–vPy$_n^{2+}$, where $2 \leqslant n \leqslant 5$, in the solutions of 1:3 and 1:4 copper(II):vPy molar ratio, respectively. Under these conditions, the more abundant complex (over 30%) corresponds to the form copper(II)–vPy$_2^{2+}$. Visible spectra of these solutions show maxima at 726 and 708 nm (spectra D and E in Fig. 3), in accordance with the maximum absorption at 700 nm reported for the isolated copper(II)–Py$_2^{2+}$ complex [31]. In addition, D and E spectra cross each other at 698 nm, pointed out with an arrow in Fig. 3 (isosbestic point for the third and fourth equilibriums of copper(II)–vPy$_n$ complexes).

Fig. 4a shows the spectra corresponding to copper(II) (Fig. 4a, A), 1:3 copper(II):vPy (Fig. 4a, B), 1:1 copper(II):Bac (Fig. 4a, C) and vPy:copper(II):Bac 3:1:1 (Fig. 4a, D) molar ratio solutions. Considering the additive contribution of each ligand in an equatorially-coordinated copper(II) complex to the blue-shift effect, spectrum D in Fig. 4a is shifted with regard to the summatorial spectrum of B and C. Thus, a spatial arrangement in the equatorial plane of the octahedral distorted copper(II) ion is the most likely conformation.

Finally, Fig. 4b shows the titration of a 1:4 copper(II):vPy solution with Bac monitoring the absorbance ratio 660:708 nm (maxima of Fig. 4a, D and Fig. 3, E respectively). This ratio increases linearly with Bac concentration and reaches a plateau when copper(II) and the peptide are at equimolar concentration. Thus, Bac displaces one vPy from the original complex.

Taking into account all the previous visible spectra analysis, we choose 3:1:1 vPy:copper(II):Bac ratio as the best condition to obtain the ternary complex in solution.

### 2.1. Grafting procedure

Imprinted graft polymerisation was performed by γ-irradiation of membranes soaked in copper(II), Bac, vPy mixtures with DEGMA solutions prepared according to Table 1 for each imprinted polymer. DEGMA was added to the aqueous methanolic solutions as a *filler* and cross-linking reagent to build the skeleton and reduce the flexibility of the imprinted polymer.

An irradiation dose of 6 kGy was enough to develop the grafting polymerisation and deplete the solution of reactive monomers [32]. After irradiation, samples were thoroughly washed as described

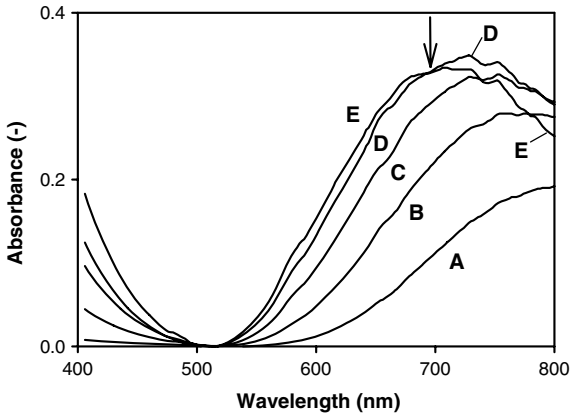


Fig. 3. Visible spectra of copper(II) 10 mM (A) and vPy–copper(II) mixtures corresponding to 10 mM copper(II) and vPy 10 mM (B), 20 mM (C), 30 mM (D) and 40 mM (E), respectively.

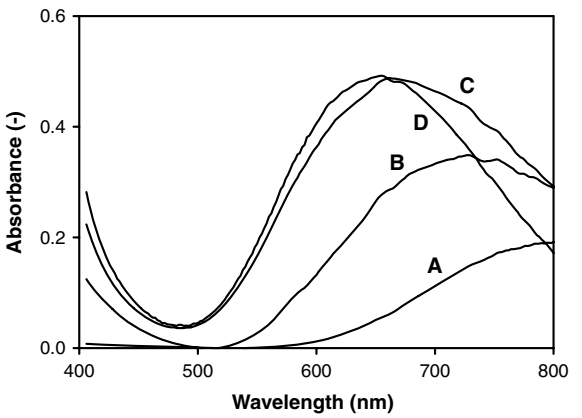


Fig. 4a. Visible spectra of copper(II) 10 mM (A), vPy–copper(II) mixture 10 mM copper(II) and vPy 30 mM (B), Bac–copper(II) 10 and 10 mM, respectively (C) and vPy–copper(II)–Bac mixture 10 , 30 and 10 mM, respectively (D).

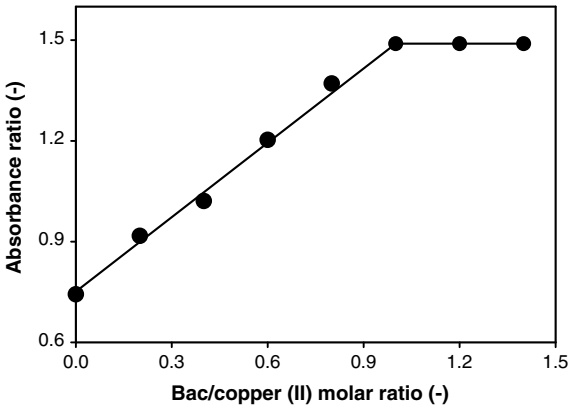


Fig. 4b. 660:708 nm absorbance ratio in terms of Bac–copper(II) molar ratio during the titration vPy–copper(II) solution (molar ratio 4:1) with Bac.

in Section 1.1 to remove all possible homopolymer content. Samples were conserved submerged in an aqueous solution to preserve the original polymer conformation. Additionally, two controls of the grafting polymerisation procedure were done. In one of them, membrane sample was irradiated only with the filler monomer (2% v/v DEGMA) yielding a MD% 18% (increase in dry weight). The other sample was irradiated only with vPy (in the same concentration used for preparation of imprinted samples), yielded no weight difference thus indicating a negligible grafting degree.

Complexes were removed from the polymer network by sequestering the copper with EDTA. Specific adsorption capacity to copper(II) was determined by reloading, washing and eluting the ion. Table 1 shows that results. MIP-4 and MIP-5 display the highest adsorption capacities thus suggesting that a spatial arrangement should be present. Additionally, MIP-5, with a lower cross-linker concentration, shows the highest copper adsorption. It is also interesting to note that MIP-3, having the lowest copper rebinding value, shows a pale green colour after the polymerisation step that could not be removed neither by washing nor with EDTA solution, thus evidencing that immobilized pyridine moieties can complex copper ions with an affinity constant higher than that of EDTA.

Copper-loaded MIPs were soaked in Bac solutions and, after elution by lowering the pH to 3, the adsorption capacity was calculated. Fig. 5 shows the results of Bac adsorption to different MIPs: as in the case of copper adsorption, MIP-4 and MIP-5 showed the highest adsorption capacity. In addition, in both cases, MIP-5 shows higher capacity

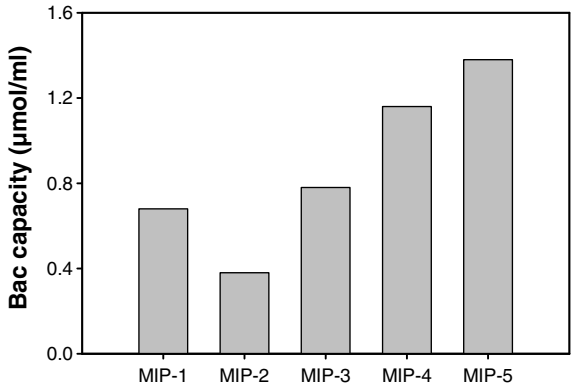


Fig. 5. Adsorption capacity to Bac of the different MIPs described in Table 1.

than MIP-4 thus suggesting that 1% DEGMA is the concentration of cross-linker showing the best balance between grafting degree and adsorptive properties. Adsorption capacity to Bac of a commercial copper(II)–NTA agarose under the same buffer condition was 12.6 µmol/ml, tenfold higher than that of MIP-5. However, in this comparison we only consider the histidine-cation interaction. In order to check the selectivity of MIP-4 and MIP-5 in comparison with commercial copper(II)-NTA agarose, two Bac analogs – Ac-Bac and TNP-Bac – were synthesized by acetylation and trinitrophenylation, respectively. Fig. 6 depicts their adsorption onto MIP-4, MIP-5 and copper(II)-NTA-agarose. The commercial matrix did not show any selectivity in its adsorptive behaviour while both MIPs revealed a significantly lower adsorption of both analogs than that of Bac (fivefold lower adsorption) thus, suggesting that additional steric

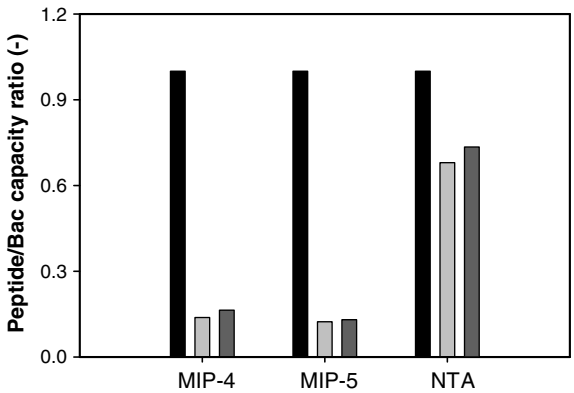


Fig. 6. Relative adsorption capacity: Bac/Bac (black bars), Bac-TNP/Bac (grey bars) and Bac-Ac/Bac (dark grey bars) of MIP-4, MIP-5 and commercial NTA-agarose.

interactions must be involved in the adsorption of Bac to MIP materials.

## 3. Conclusions

The design of artificial receptors for peptides and proteins is a very difficult but interesting issue because of the relative complexity of the protein surface and the large number of potential competing sites on other non-target proteins.

In this work, MIPs with recognition capabilities towards a 1.4 kDa peptide were synthesized on hollow-fibre membranes by radiation-induced graft polymerisation.

MIP materials showed differential adsorption towards structurally related molecules, a behaviour not evidenced in a non-imprinted commercial matrix.

This very simple method is focused on polymerisation in a polar environment in order to preserve the native conformational shapes and interactions. The advantages of this new approach to obtain MIP materials can be summarized as follows: printed complexes are prepared by simple mixing of commercial monomers vPy and DEGMA, a metal ion and the target molecule. Visible spectroscopy becomes a sensitive tool to analyse the copper complexes in solution and optimise the vPy–copper(II) – target molecule molar ratio. Simultaneous irradiation of a monomer solution with a trunk polymer allows the grafting of a nascent MIP polymer onto a poorly reactive polymeric surface. However, the possible complication of grafting to/from the template via chain transfer or H-abstraction should also be considered, especially under these rather polar conditions and as the size of the template increases.

Radiation-induced graft polymerisation is highly independent of trunk polymer shapes, thus the grafting procedure, which generates the recognition properties, can be done onto different solid shapes.

In conclusion, radiation-induced graft polymerisation should be considered a powerful tool to prepare MIP materials.

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