

Surface imprinted beads for the recognition of human serum albumin

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Abstract

The synthesis of poly-aminophenylboronic acid (ABPA) imprinted beads for the recognition of the protein human serum albumin (HSA) is reported. In order to create homogeneous recognition sites, covalent immobilisation of the template HSA was exploited. The resulting imprinted beads were selective for HSA. The indirect imprinting factor (IF) calculated from supernatant was 1.6 and the direct IF, evaluated from the protein recovered from the beads, was 1.9. The binding capacity was 1.4 mg/g, which is comparable to commercially available affinity materials. The specificity of the HSA recognition was evaluated with competitive experiments, indicating a molar ratio 4.5/1 of competitor was necessary to displace half of the bound HSA. The recognition and binding of the imprinted beads was also tested with a complex sample, human serum and targeted removal of HSA without a loss of the other protein components was demonstrated. The easy preparation protocol of derivatised beads and a good protein recognition properties make the approach an attractive solution to analytical and bio-analytical problems in the field of biotechnology. © 2007 Elsevier B.V. All rights reserved.

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1. Introduction

Molecularly imprinted polymers (MIPs) are polymers prepared in presence of a template that serves as a mould for the formation of template-complementary binding sites (Mosbach, 1994; Sellergren, 2001a,b). Thus, MIPs can be created to recognise a large variety of target molecules often with affinity and selectivity comparable to those exhibited by poly- or monoclonal antibodies. MIPs are less expensive and quicker to prepare than biological receptors. Additionally, they are capable of withstanding much harsher conditions than antibodies, such as high temperature, pressure, extreme pH, and organic solvents. These properties have made them extremely attractive for solving problems in the fields of preparative chemical separations (Sellergren, 2001a,b), solid phase extraction (Lanza and Sellergren, 2001; Andersson and Schweitz, 2003) and sensing (Chianella et al., 2003; Haupt and Mosbach, 2000), or for the removal of specific molecular targets from food (Whitcombe et al., 1997; Ramstrom et al., 2001).

MIPs have been prepared using polypeptides (Kempe et al., 1995; Andersson et al., 1995; Minoura and Rachkov, 2000), bacteria (Dickert and Hayden, 2002), low molecular mass compounds (Katz and Davis, 2000; Chianella et al., 2003) and proteins (Burow and Minoura, 1996; Bossi et al., 2001; Guo et al., 2004) as templates. In the case of proteins, however, only modest success has been obtained due to the specific properties of these templates. First, all proteins are water-soluble compounds that are not always compatible with mainstream MIP technologies, which relies on the use of organic solvents for the polymer preparation. Second, proteins have a large amount of functional groups available for the interaction with functional monomers. Third, proteins have a flexible structure and conformation, which can be easily affected by changes in temperature or the nature of the solvent. Thus, from a thermodynamic and practical standpoint, it is difficult to develop successful imprints for such molecules.

In the last 10 years, some new approaches have been proposed, which use both covalent and non-covalent methods (Wulff, 1993; Mosbach and Ramstrom, 1996). The immobilisation of the protein template on a supporting surface (Shi et al., 1999; Yilmaz et al., 2000; Shiomi et al., 2005) provides a number of advantages, i.e. it allows the imprinting of templates

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independently of their solubility in the polymerisation mix, it minimises protein aggregation and it creates more homogeneous binding sites.

In the present contribution, we report the preparation of imprinted beads for the recognition of human serum albumin (HSA). The protein template is immobilised at the surface of the beads, with an approach modified from Shiomi et al. (2005) in which covalent immobilisation was achieved by derivatising silica beads with aldehyde groups, these were then exploited to form imine bonds with the amino groups of lysine in the HSA. The polymer was made of 3-aminophenyl boronic acid (APBA) (Piletsky et al., 2001; Bossi et al., 2001; Pribyl and Skladal, 2006) using a polymerisation process described earlier by our group (Bossi et al., 2001). The beads thus obtained were characterised in order to evaluate the percentage of polymer derivatised, the binding capacity for HSA, the binding kinetics, the specificity of the HSA binding and the recovery of bound protein.

Finally, the imprinted beads were applied to a real biological fluid, human serum, in which HSA is the most abundant protein (ca. 60% of the total proteins) (Andersson and Anderson, 2002; Tirumalai et al., 2003). The high quantity of HSA in serum is considered as a drawback, since it seriously hampers the detection of low abundant proteins, which are often marker of diseases. It has been demonstrated that if serum is selectively depleted of albumin, it would facilitate the analysis of such low-abundant proteins. Albumin removal has been achieved through adsorption to immobilized dyes (Ahmed et al., 2003), immuno-affinity extraction (Wang et al., 2003) or affinity capture by immobilized phage-derived peptides (Sato et al., 2002), but to date there have been no reports concerning the use of molecularly imprinted polymers. In the present case, we attempt the use of a synthetic receptor, the imprinted beads, for the specific removal of HSA from serum.

2. Materials and methods

2.1. Materials and reagents

Acetic acid, aminopropyl silica (particle size 15–40 µm, mean pore size 60 Å), 3-amino propyl trimethoxysilane (APTMS), ammonium persulfate (APS), Bradford assay, [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), ethanol, glycine, glutaraldehyde, human serum albumin (HSA) (Mr 66 kDa), methilic acid, 2N-morpholine ethane sulfonic acid (MES), oxalic acid, propyl triethoxy silane, sodium chloride (NaCl), SDS, Sypro Ruby, thiourea, trizma, urea, were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Acrylamide, *N,N*-methylenebisacrylamide, *N,N,N,N*-tetramethyl-ethylenediamine (TEMED), dithiothreitol (DTT), linear Immobiline dry strips pH gradient 3–10 (7 cm) were obtained from Bio-Rad (Hercules, CA, USA).

2.2. Preparation of human serum albumin (HSA)-imprinted silica using immobilized templates

Silica beads (1 g) were derivatised with 10% (w/v) APTMS in methanol in order to introduce NH₂ functional groups. The

beads were washed three times with ethanol and three times with deionised water and then dried at 50 °C for 24 h. They were then incubated with 10 mM 2N-morpholine ethane sulfonic acid (MES) buffer, pH 5.5 containing 1% glutaraldehyde for 12 h at room temperature, in order to introduce aldehyde groups. The product was washed repeatedly with deionised water. Finally, 1 ml of a 10 mM 3N-morpholino propane sulfonic acid (MOPS) solution (pH 7.0) containing 2.0 mg/ml human serum albumin (HSA) and 0.1 M NaCl was admixed as the template for 3 h at 4 °C, in order to covalently bind the protein on the aldehyde groups. The beads were then incubated with 1 M Tris for 30 min in order to block the un-reacted aldehyde groups. Washes with deionised water followed and finally 1 ml of 50 mM ABPA water solution was added to the beads. After 45 min incubation, 1 ml of 25 mM ammonium persulfate (APS) water solution was added in order to start the polymerisation reaction. The polymerisation was carried out at room temperature for 1 h, after which the beads were washed again with deionised water for five times. Finally, 1 ml of 1 M oxalic acid was added in order to remove the template. This step was carried out at room temperature for 12 h. The derivatisation protocol was checked at each step by FT-IR spectroscopy with a Magna FT-IR 760 Spectrophotometer (Nicolet, Offenbach Germany), samples were prepared mixing 10 mg of beads with 200 mg KBr, sample reading was 32×. The beads were finally conditioned with 10 mM phosphate buffer, pH 8.0, in order to increase the pH and remove the free HSA in solution produced by the treatment with 1 M oxalic acid.

2.3. Evaluation of the percentage of derivatisation

In order to evaluate the percentage of silica beads derivatised, 0.500 g of beads were derivatised (with APTMS, glutaraldehyde, APBA, as previously described). After each step, the beads were dried overnight at 87 °C and they were weighed. The degree of derivatisation was calculated as follows:

$$\text{DG (\%)} = \frac{(\text{mg of "polymer" bound})}{(\text{g beads})} \times 100.$$

2.4. Calculation of the binding capacity

In order to evaluate the binding capacity, 0.500 g of beads were conditioned with 10 mM phosphate buffer, pH 8.0 and incubated with 300 µl of HSA solution at different concentrations (0.036, 0.058, 0.073, 0.18, 0.3, 1.0, 2.6, 2.74 and 4.08 µg/µl) for different times (3, 5, 20, 60 min). The beads were centrifuged at 4000 × *g* for 3 min at room temperature, then the supernatant was transferred in new tubes and it was quantified by Bradford assay at 595 nm. The protein bound was expressed as the difference between the total micrograms of HSA loaded and micrograms of HSA in solution after the binding.

2.5. Binding kinetics

In order to evaluate the binding kinetics, a 0.500 g of control or MIP beads, conditioned with 10 mM phosphate buffer pH 8.0, were incubated with 300 µl of HSA solution at different

concentrations (0.036, 0.058, 0.073, 0.18, 0.3, 1.0, 2.6, 2.74 and 4.08 $\mu\text{g}/\mu\text{l}$) for different times (3, 5, 20, 60 min). The quantity of bound protein was evaluated as described in the previous paragraph.

2.6. Protocol for HSA elution from the beads

In order to evaluate the quantity of protein bound from beads, a 15 mg of control or MIP beads, conditioned with 10 mM phosphate buffer pH 8.0, were incubated with 300 μl of 0.1 $\mu\text{g}/\mu\text{l}$ HSA solution for 5 min. The beads were washed once with 10 mM phosphate buffer pH 8.0. The supernatant was discarded and the beads were incubated with a solution of 7% acetic acid and 0.1% Tween-20. After 10 min the beads were centrifuged and the supernatant was recovered. The proteins were precipitated with 8 volumes of cold acetone and analysed through SDS-PAGE.

2.7. Binding experiment with human serum

In order to evaluate the binding of HSA from a real complex sample, 0.300 g of MIP beads, conditioned with 10 mM phosphate buffer pH 8.0, were incubated with 300 μl of 0.1 $\mu\text{g}/\mu\text{l}$ human serum solution for 5 min. The beads were washed as previously described. The supernatant was recovered and the proteins were precipitated and analysed through 2D PAGE.

2.8. SDS-PAGE and 2D PAGE

Electrophoresis of proteins was performed using regular SDS PAGE with 12% polyacrylamide gel, with a Tris-glycine cathode buffer (192 mM glycine, 0.1% SDS and 40 mM Tris to pH 8.3). The run was performed by applying 15 mA/gel for 20 min followed by 25 mA/gel until the dye front reached the bottom of the gel.

For 2D-maps, the desired volume of each sample was subjected to protein precipitation in a cold mixture of acetone and methanol (v/v ratio 8:1) for 2 h at -20°C , in order to remove lipids and salts and to regulate the concentration of protein; the solution was then centrifuged at $10,000 \times g$ for 20 min. The pellet was solubilised in the 2D sample buffer (7 M urea, 2 M thiourea, 3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 40 mM TRIS, 5 mM tributylphosphine and 10 mM acrylamide) and allowed to be alkylated at room temperature for 90 min. Dithiothreitol (10 mM) was used to block the alkylation reaction, and then trace amounts of 0.5% ampholine and bromophenol blue were added to the solution. An aliquot (150 ml) of protein solution was subsequently used for rehydrating 7 cm long non-linear immobilized pH gradient (IPG) strips (Bio-Rad), pH 3 to 10, for 4 h. Isoelectric focusing (IEF) was performed with a Protean IEF Cell (Bio-Rad) at low initial voltage, followed by a voltage gradient up to 5000 V; the total product time voltage applied was 25,000 V h for each strip. For the second dimension, the IPGs strips were equilibrated for 26 min in a solution containing 6 mM urea, 2% SDS, 20% glycerol, and 375 mM Tris-HCl, pH 8.8, under gentle shaking. The IPG strips were then laid on an 8–18% T-gradient SDS-gel slab

and cemented *in situ* with 0.5% agarose in the cathode buffer (192 mM glycine, 0.1% SDS, and Tris to pH 8.3). The electrophoretic run was performed by setting a current of 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 20 mA/gel until run completion.

Finally, for SDS-PAGE and 2D-maps, gels were incubated in a fixing solution containing 40% ethanol and 10% acetic acid for 1 h, followed by overnight staining in a ready-to-use Sypro Ruby solution. Destaining was performed in 10% methanol and 7% acetic acid, followed by a rinse of at least 3 h in pure water. The gels were scanned with a Versa Doc Imaging System (Bio-Rad, Hercules, CA) and analysed with the software PDQuest Version 7.1 (Bio-Rad, Hercules, CA). A match set was created from the protein patterns of three replicate gels for each independent sample. A standard gel was generated out of the image with the highest spot number. Spot quantities of all gels were normalized to remove non expression-related variations in spot intensity. The results were evaluated in terms of spot OD (optical density).

3. Results and discussion

3.1. Derivatisation of the imprinted beads

Aminophenylboronate based molecularly imprinted polymers were prepared as described in Section 2. The approach includes first exploiting silica beads as supports for the template (HSA) immobilisation, followed by the polymerisation of the homopolymeric material onto the derivatised beads and finally the cleavage of the protein-silica covalent bonds, resulting in leaving the imprinted cavities freely accessible for binding.

The silica beads were derivatised with 10% APTMS in order to introduce NH_2 groups. Next, a treatment with 1% glutaraldehyde was performed to introduce aldehydic groups. The immobilisation of the protein on the surface of the silica beads happens through a covalent bond between ϵ amino groups of lysines of the template and aldehyde groups on the silica beads.

APBA was used as functional monomer. The polymerisation procedure, is straightforward: a 1:1 (v/v) ratio of the ammonium persulfate (50 mM) and APBA (100 mM) solution in water were allowed to react together in the presence of the protein-derivatised beads for 1 h, gently stirred at room temperature (Bossi et al., 2001, 2004). Studies on the polymerisation of monomers of the same family of ABPA (e.g. aniline) showed that the polymer in solution forms relatively short chains (Mattoso et al., 1995), flocculating in aggregates, while on the supporting surface, it deposits in a reasonably thin and ordered film (MacQuade et al., 2000).

After APBA polymerisation, it was necessary remove the HSA immobilised at the surface of the beads to get free binding cavities. Strong acids have to be used in order to break the silica-protein covalent bonds. Thus, incubation in 1 M oxalic acid for 12 h was adopted as removing procedure (Shiomi et al., 2005).

3.2. Evaluation of the derivatisation

The quality of the derivatisation process was checked by FT-IR measurements, highlighting modifications in the vibra-

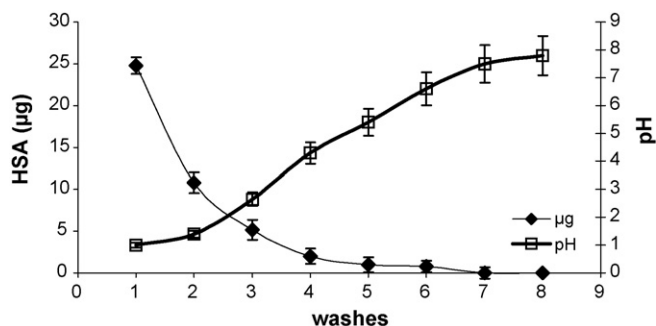


Fig. 1. Influence of the washing protocol on the pH of the beads and on the removal of template.

tional spectrum after each derivatisation step. In particular, the protein covalent immobilisation resulted the detection of signals typical of peptide bonds (1650 cm^{-1}) and carboxy groups ($1300\text{--}1400\text{ cm}^{-1}$). The further polymerisation of APBA was confirmed by the vibration of phenylboronic groups (region $1100\text{--}1200\text{ cm}^{-1}$; a shoulder at 1250 cm^{-1} ; boronate contribution around $3200\text{--}3400\text{ cm}^{-1}$).

3.3. Assessment of a suitable protocol for template removal

Next, washing steps were performed to remove the protein released from the beads and to condition the beads in a buffer suitable for the binding experiments. In order to remove HSA and to increase the pH from 1.2 to 8.0, six washes with 10 mM phosphate buffer were performed. After each washing step, the pH of the solution was measured and the proteins removed were recovered by acetone precipitation and analysed by SDS-PAGE, followed by quantification of the HSA released through densitometry analysis. The data were then plotted as a function of the number of the washing steps, as shown in Fig. 1. No free template was detected in solution after seven washing steps. Moreover, after seven washes, the pH of the solution had risen to 8.0, which was the pH chosen for the binding experiments.

It is probable that a small part of the HSA previously bound to the beads remained embedded in the polymer matrix. The contribution of such remaining template might be negative, occupying the binding sites of the polymer and thus affecting its rebinding properties. Nevertheless there are many documents reporting a positive effect of the non-complete removal of the embedded template. As reported by Hjertén and co-workers, the residual template can improve the mechanical stability of the polymer architecture, conferring rigidity to the binding sites (Hjertén et al., 1997).

3.4. Derivatisation extent and reproducibility of the polymer deposition

The quantity of the polymer deposited on the beads was evaluated too on eight preparation. Starting from a known quantity of beads, the weight of the batch of beads was monitored after the derivatisation step, after drying. The quantity of ABPA deposited around the silica beads was $15.2 \pm 0.5\text{ mg}$ of polymer per gram of beads. It is probable that the treatment with oxalic acid, used

to remove the covalently bound HSA, removes a few superficial layers of APBA MIP-polymer too. A qualitative evaluation of the persistence of the dark brown colour of the APBA-beads suggests that, in any case, part of the ABPA layers are resistant to the strong acidic treatment.

3.5. Binding experiments

Binding experiments were performed in order to evaluate the binding capacity for HSA of both control and imprinted APBA beads. Control and MIP beads (0.500 g), conditioned with 10 mM phosphate buffer pH 8.0, were incubated with increasing quantities of HSA. The quantity of bound protein was evaluated by difference, measuring the residual free protein in the supernatant. The micrograms bound were plotted as a function of the quantity of HSA used, as shown in Fig. 2. No difference in the amount of bound protein was observed between control and MIP beads when the quantity of protein used was below $780\text{ }\mu\text{g}$, while a difference was observed when the quantity of protein used was above $780\text{ }\mu\text{g}$ (Fig. 2). The difference between MIP and control beads correlated with the quantity of available binding sites. When the protein quantity was not enough to saturate the unspecific binding, there was no difference between control and MIP beads, but when HSA was saturating ($>780\text{ }\mu\text{g}$), the presence of specific binding cavities on the MIP became evident. The indirect binding capacities, calculated from the quantity of HSA left unbound in the supernatant, was 1.4 mg/g for MIP beads and 0.9 mg/g for control. These values are in accordance to binding capacities reported for commercial affinity materials.

Kinetic experiments were performed to evaluate the optimum binding time within the range $0.5\text{--}60\text{ min}$. The binding kinetic was evaluated for concentrations of HSA ranging from 0.073 to $4.1\text{ }\mu\text{g}/\mu\text{l}$. As shown in Fig. 3, the best incubation time was 5 min, irrespective of the HSA concentration used. This result is confirmed by the profile of the time course of the imprinting factor (IF, $\mu\text{g bound by MIP}/\mu\text{g bound by ctrl}$) plotted in Fig. 4, showing a maximum IF of 1.6 ± 0.08 for high HSA concentration at 5 min and a maximum IF of 1.2 ± 0.05 for low, non-saturating, concentrations. The binding profile (Figs. 3(b) and 4) for non saturating HSA concentrations indicates that for short incubation times ($<5\text{ min}$) the HSA molecules approach the surface and sticks randomly to it (IF is only slightly in favour of

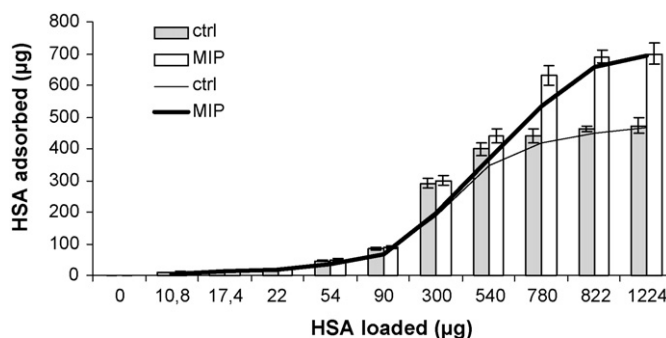


Fig. 2. Evaluation of the binding capacity for control (open) and MIP beads (solid). The amount in micrograms bound from beads is plotted as function of amount in micrograms used for binding.

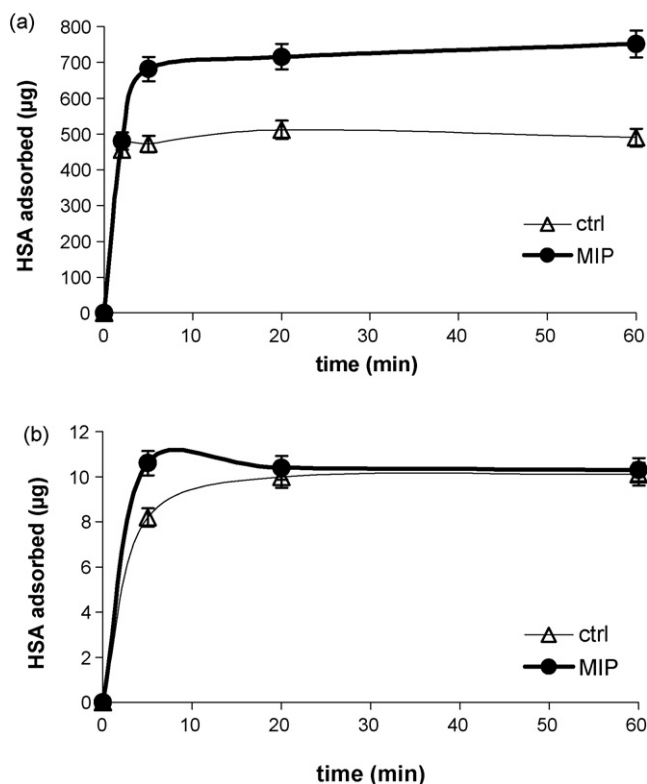


Fig. 3. Binding kinetics. The amount in micrograms of HSA bound are plotted as function of the time, under saturated conditions (a) and non-saturated conditions (b).

MIPs). When the incubation time rises to 5 min, the binding to specific cavities is appreciable and maximised (IF reaches its maximum). Since the total quantity of protein bound did not change significantly with respect to 3 min incubation, this suggests a thermodynamically driven rearrangement of the bound proteins took place and proteins on the MIP moved to the specific cavities. The course of the binding kinetic might be explained by several different interaction being established during the binding of the protein to the surface, thus weak forces, hydrogen bonding and hydrophobic interactions have to take place (Thulasiraman

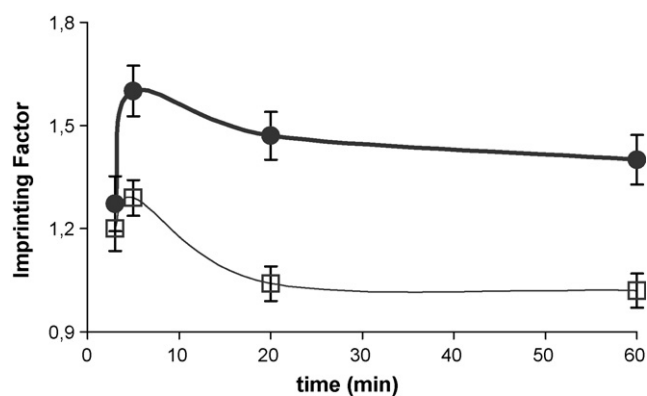


Fig. 4. The IF calculated as micrograms of HSA bound by MIP/micrograms of HSA bound by the control is plotted as function of the time, both for saturated conditions and non-saturated conditions.

et al., 2005). The nature of APBA polymer is both hydrophobic and hydrophilic, allowing to establish multiple interactions with the protein. The protein binding mechanism to MIP p-APBA beads is supposed to include spatial recognition between the protein and the imprinted cavities and several binding contributions that derives from the interaction of several aminoacid lateral chains (i.e. hydroxyl of serine, threonine, tyrosine) with boronate groups (Bossi et al., 2001). Plus, hydrophobic interactions occur between the aromatic structure of the polymer and both the aromatic side chains and the backbone of the protein (Bossi et al., 2004).

Longer incubation times (20–60 min) did result in a loss of specific binding, most likely due to a co-aggregation of proteins from the solution to the bound proteins, as reported for other protein-MIPs (Turner et al., 2006).

3.6. Recovery of bound protein

The recovery of the bound protein onto the beads was evaluated. A protocol for protein elution was optimised. In particular, after binding, five different conditions for elution of HSA from beads were tried in order to select the most effective washing procedure. The first protocol was 7 M urea, 2.2 M thiourea, 3% CHAPS, 40 mM Tris, pH 8.0 (TUC buffer), that is, a strongly denaturant buffer. It is usually applied to treat sample before 2D-gel electrophoresis (Righetti et al., 2004). The second one was TUC buffer, followed by 9 M urea and 5% acetic acid, pH 3.0, which is usually applied for protein elution from affinity capture beads, as described by Fortis et al. (2005a,b). The third was 7% Acetic Acid, 0.1% Tween-20. This solution was reported for washing APBA-MIPs polymers by Bossi et al. (2001). The forth protocol was an acidic buffer supplemented with salt to increase the ionic strength: 0.1 M glycine, pH 3.0, 0.5 M NaCl and finally the last washing procedure was a high ionic strength solution of 1 M NaCl. The quantity of HSA eluted was evaluated by SDS-PAGE. As reported in Table 1, no protein release was observed for alkaline washing, while washing with acid or high ionic strength allowed the HSA to be recovered. However, among all the protocols applied only the third gave good results, similar to those achieved with the Bradford assay. The other protocols recovered a small quantity of protein, thus in all the following experiments, washing with 7% acetic acid 0.1% Tween-20 was adopted for elution of the protein from beads.

From these results, it was possible to calculate the direct binding capacity for MIP and control (calculated from the quantity of HSA recovered from the beads), which was 0.696 mg/g on

Table 1
Recovery of HSA from MIP and control beads

Solution used for elution	Control beads (µg)	MIP beads (µg)
TUC buffer pH 8.8	0.3 ± 0.25	0.23 ± 0.20
TUC buffer + 9 M urea, pH 3.0	5.5 ± 0.52	6.0 ± 0.40
7% acetic acid + 0.1% Tween-20	5.8 ± 1.20	9.2 ± 1.50
0.1 M glycine + 0.5 M NaCl, pH 3.0	0.4 ± 0.12	0.24 ± 0.15
1 M NaCl	0.57 ± 0.15	0.85 ± 0.16

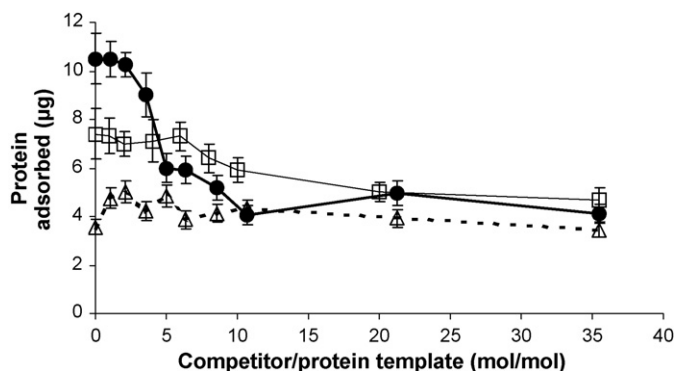


Fig. 5. Competitive experiments. Open triangles indicate the HSA adsorbed onto control beads upon addition of increasing amount of the competitor protein carbonic anhydrase (CA). Filled circles indicate the amount of HSA adsorbed onto MIP-HSA beads when challenged with CA competitor. Open squares indicate the amount of CA adsorbed onto MIP-CA beads when challenged with HSA competitor.

MIP beads and 0.363 mg/g on the control. The advantage of a direct calculation of binding capacity is the elimination of part of the unspecific contributions. The consequence is fairly low values of binding capacities with respect to those calculated indirectly, whereas a significant increase in the IF 1.91 was observed, thus indicating the binding cavities on the imprinted beads are specific for HSA recognition.

3.7. Competitive experiments to evaluate specific binding

In order to evaluate the specificity of binding, a competitive experiment was performed (Fig. 5). The beads were assayed for the rebinding with a mixture made of a fixed concentration of the template and an increasing concentration of a competing protein. The quantity of the protein bound to the beads was evaluated after elution by SDS-PAGE. The competitor first chosen was carbonic anhydrase (CA), which has approximately the same isoelectric point as HSA but a smaller molecular mass (31 kDa) (Righetti and Caravaggio, 1976). The shape effect on the binding was evaluated. In control beads, the HSA binding (Fig. 5, open squares) did not change significantly by the presence of CA, the HSA bound ($3.5 \pm 0.5 \mu\text{g}$) was considered as non-specific adsorption. In the MIP, the HSA bound was $10.3 \mu\text{g}$ (Fig. 5, solid circles); the displacement of HSA from the CA competitor was appreciable only upon an addition of CA of 4.5/1 mol/mol respect to HSA. In the case half of the HSA was displaced. When CA/HSA ratio was above 8/1 mol/mol a complete displacement of the bound HSA was observed. These results indicate that the HSA binding is displaced only for relative high concentrations of competitor.

In a second set of experiments CA was used as template and HSA as competitor, as shown in Fig. 5 (open squares). The amount of CA bound to the MIP is $8 \mu\text{g}$. The addition of HSA does not displace CA significantly. The results indicate that the binding to the polymer is very much dimension-dependent, thus HSA being far bigger than CA, a scarce competition effect was evident. The shape of the protein is therefore crucial for the binding. This might limit the selectivity properties of the MIP-beads, thus efforts should be addressed to further tailor the selection.

3.8. The specific removal of a protein exploited for proteome analysis: depletion of human serum albumin

Despite the selectivity of the MIP beads was not extremely tuned, an interesting application was still envisaged, as we focus the interest on samples like human serum, which contains a large predominance of albumin (the 60–80%, w/w). In the case, the problems of competition for the imprinted binding sites might be negligible. For these reasons, the last part of our work was devoted to the evaluation of the binding performance of imprinted beads on real biological fluid, human serum. Serum samples are composed of thousands of proteins and peptides, released from various organs and travelling through the body through the vasculature, accounting for the high complexity of such fluid. Despite the fact that albumin and immunoglobulins constitute the vast majority of the proteic fraction of serum (ca. 90%), interest is focused on proteins that form the minor fraction, as their presence or their quantitative variations are often correlated to pathological states. Therefore, methods for efficient removal of HSA are highly welcomed, as they permit tracing and analysis of low abundance proteins, usually masked by serum HSA. In the present research we attempted the use of the imprinted beads for albumin removal. Serum samples were incubated with imprinted beads, the supernatants recovered and their proteins precipitated with TCA. Recovered proteins were subjected to bi-dimensional (2D) electrophoresis analysis. The 2D maps of untreated serum and serum depleted of albumin with imprinted beads were analysed with the software PDquest (Biorad, Hercules, CA) and are shown in Fig. 6. The total number of protein spots counted for serum was 124 and 116 for MIP-treated serum, indicating again that the imprinted beads targeted HSA and did not randomly remove other serum components. By analysing the optical density of the albumin spot, a decrease was reported for serum depleted with imprinted beads, in fact the spot intensity decreased a factor 1.7 ± 0.3 upon treatment (i.e. the 58% HSA was removed). The quantity of albumin removed with the beads was of $183 \mu\text{g}$, starting from $311 \mu\text{g}$ of HSA. It can be concluded that the imprinted beads also removed the protein target in a complex fluid, leaving the other proteins nearly unchanged. The removal process was easy and straight-

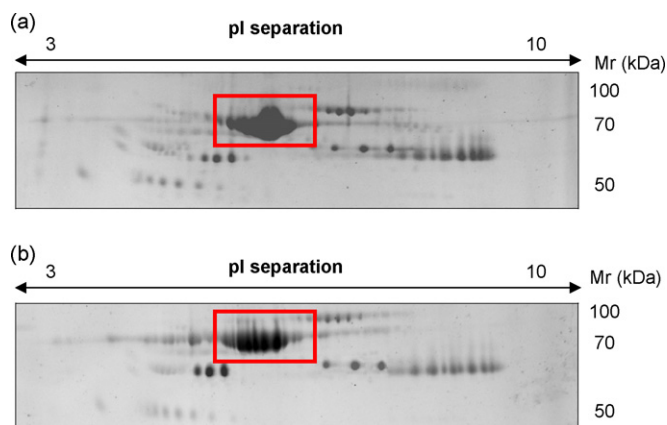


Fig. 6. 2D-maps of human serum: (a) untreated serum sample, (b) serum treated with imprinted beads, for the depletion of HSA.

forward with an optimal incubation time of 5 min. Naturally, the preliminary experiments with a complex fluid need further investigation where the quantity of imprinted beads would be optimised to obtain an almost complete depletion of HSA from serum.

4. Conclusions

The results presented here are a further achievement in the area of protein imprinting. The p-APBA derivatised beads shows the possibility to create specific recognition sites directed towards the binding of a protein. To obtain more homogeneous population of binding sites, the protein template was immobilised to the silica scaffold, followed by the polymerisation of APBA. The MIP beads show a recognition mainly due to shape interactions. Results demonstrate the binding ability of the imprinted beads both in simple and complex samples, i.e. biological fluids. The MIP beads were used for the first time as a tool for the pre-fractionation of the sample prior to proteome analysis (removal of the 58% of albumin in serum), thus opening the route for a new area of application of MIP recognition materials.

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