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"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 here reported. In order to create homogeneous recognition sites the covalent immobilisation of the template HSA was exploited. Imprinted beads resulted selective for HAS. The indirect imprinting factor (IF) calculated from supernatant was of 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, 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 tested also with a complex sample, the human serum sample, showing a targeted removal of HSA, without a loss of the other protein components. The easy preparation protocol of derivatised beads and a good protein recognition make the approach attractive to resolve analytical and bio-analytical problems in the field of biotechnology.

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, 2001]. 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 mono-clonal antibodies. MIPs are less expensive and quicker to prepare than biological receptors. Additionally, they are capable of withstanding much harsher conditions, such as high temperature, pressure, extreme pH, and organic solvents as compared to antibodies. These properties have made them extremely attractive for solving problems in the fields of preparative chemical separations (Sellergren, 2001 a-b), solid phase extraction (Lanza and Sellengren, 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 as template molecule 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).

In the case of proteins, however, only some modest results have been obtained. The main problem is strictly dependent on 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. Secondly, proteins have a large amount of functional groups available for the interaction with functional monomers. Thirdly, proteins have a flexible

structure and conformation, which can be easily affected by changes in environmental temperature, or in dependence to the solvent. Thus, from a thermodynamic and practical standpoint, it is difficult to develop successful imprints for such molecules.

In the last ten years, some new approaches have been proposed. These approaches follows both the covalent and non covalent approaches [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], gives a number of advantages, i.e. allows the imprinting of templates independently to their solubility into 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 and co-workers in 2005 [Shiomi et al., 2005], in which covalent immobilisation was achieved by derivatising silica beads with aldehyde groups, exploited next for forming imine bonds with the amino groups of lysine of HSA. The polymer was made of 3-aminophenyl boronic acid (APBA) [Piletsky et al., 2001; Bossi et al., 2001; Pribyl and Skladal, 2005] with a polymerisation process was described earlier by our group [Bossi et al., 2001].

The beads thus obtained, were characterised in order to evaluate the polymer derivatisation percentage, 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) [Anderson 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 N., *et al*., 2003], or immuno-affinity extraction [Wang et al*.,* 2003], or affinity capture by immobilized phage-derived peptides [Sato et al., 2002], but to date no reports concern 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.

Materials and Methods.

Materials and reagents

Acetic Acid, Aminopropyl silica (particle size 15-40 um, mean pore size 60 A), 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, 2 N-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).

Preparation of Human Serum Albumin (HSA)-imprinted silica using immobilized templates.

A 1 g of silica beads were derivatised with 10% (w/v) APTMS in methanol, in order to introduce NH_2 functional groups. Afterwards, the beads were washed three times with ethanol and three times with deionised water, after that they were dried at 50°C for 24 hours. They were then incubated with 10 mM 2-N-Morpholino Ethane Sulfonic Acid (MES) buffer pH 5.5 containing 1% glutaraldehyde, and the mixture was incubated for 12 hours at room temperature, in order to introduce aldehyde groups. The product was washed repeatedly with deionised water, and finally 1 ml of a 10 mM 3-N-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 bind covalently the protein on the aldehyde groups. After, the beads were incubated with 1 M Tris, in order to block the un-reacted aldehyde groups. Washes with deionised water followed, and finally 50 mM ABPA water solution was added to the beads. After 45 min incubation, 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 that the beads were washed again with deionised water. 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 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.

Evaluation percentage of derivatisation

In order to evaluate the percentage of silica beads derivatisation, 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 weighted. The percentage of derivatisation was calculated as *(mg of "polymer" bound/g beads)*100*.

Calculation of the binding capacity.

In order to evaluate the binding capacity, a 0.500 g of beads were conditioned with 10 mM phosphate buffer pH 8.0 and incubated with 300 ul 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 ug/ul) for different times (3, 5, 20, 60 minutes). The beads were centrifuged at 6000 rpm for 3 minutes 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.

The binding kinetic.

In order to evaluate the binding kinetic, a 0.500 g of control or MIP beads, conditioned with 10 mM phosphate buffer pH 8.0, were incubated with 300 ul 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 ug/ul) for different times (3, 5, 20, 60 minutes). The quantity of bound protein was evaluated as described in the previous paragraph.

The 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 ul of 0.1 ug/ul HSA solution for 5 minutes. 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 minutes the beads were centrifuged and the supernatant was recovered, and the proteins were precipitated with 8 volumes of cold acetone and analysed through SDS-PAGE.

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 ul of 0.1 ug/ul human serum solution for 5 minutes. The beads were washed as previously described. The supernatant was recovered and the proteins were precipitated and analysed through 2D-PAGE.

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, instead, 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 removing lipids and salts and for regulating the concentration of protein samples, for 2 hours at -20°C; the solution was then centrifuged at 10 000 *g* for 20 minutes. The pellet was solubilised in the 2-D sample buffer (7M urea, 2M thiourea, 3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 40mM TRIS, 5mM tributylphosphine, and 10mM acrylamide) and allowed to be alkylated at room temperature for 90 minutes. For blocking the alkylation reaction, 10 mM dithiothreitol was used, and then 0.5% Ampholine and bromophenol blue in traces were added to the solution. An aliquot (150 ml) of protein solution was subsequently used for rehydrating 7 cm long immobilized pH gradient (IPG) strips (Bio-Rad), pH 3 to 10 not linear, for 4 hours. 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 25000

voltage hour for each strip. For the second dimension, the IPGs strips were equilibrated for 26 minutes 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% to 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 hour, followed by 10 mA/gel for 1 hour and 20 mA/gel until run completation.

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 samples. 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).

Results and discussion

Aminophenylboronate based molecularly imprinted polymers were prepared according to the scheme depicted in Figure 1. The approach includes at 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 covalent bond between ε amino groups of lysine of 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 let react together in presence of the protein-derivatised beads for 1 h, gently stirred at room temperature (Bossi et al., 2001; Bossi et al., 2004). Study 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 (Mc Quade et al., 2000).

After APBA polymerisation, it is necessary remove the HSA immobilised at the surface of the beads for getting free binding cavities. Strong acids have to be used in order to break the silica-protein covalent bonds. Thus, incubation in 1M oxalic acid for 12 hours was adopted as removing procedure (Shiomi et al., 2005). Subsequently, 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 Figure 2 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, that is the pH chosen for the binding experiments.

It is probable that a small part of the HSA previously bound to the beads remains 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 Hjerten and co-workers, the residual template can improve the mechanical stability of the polymer architecture, conferring rigidity to the binding sites (Hjerten et al., 1997).

The quantity of the polymer deposited on the beads was evaluated too. Starting from a known quantity of beads, the weight of the beads batch was monitored after each derivatisation step, after drying. The quantity of APTMS bound was 55.3 ± 0.5 mg/g beads; the quantity of glutaraldehyde was 54.0 ± 0.5 mg/g beads. The quantity of ABPA deposed around the silica beads was 15.2 ± 0.5 mg of polymer per gram of beads. It is probable that the treatment with oxalic acid, used to remove the covalently bound HSA, removes 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 ABPA layers resist to the strong acidic treatment.

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 Figure 3 No difference in the amount of bound protein was observed between control and MIP beads when the quantity of protein used was below 780 ug, while a difference was observed, when the quantity of protein used

was above 780 ug. The difference between MIP and ctrl beads correlates with the quantity of available binding sites. When the protein quantity is not enough to saturate the unspecific binding, there is no difference between ctrl and MIP beads, but when HSA is saturating (> 780 ug), the presence of specific binding cavities on MIP becomes 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 - 60 min. The binding kinetic was evaluated for concentrations of HSA ranging from 0.073 ug/ul to 4.1 ug/ul. As shown in Figure 4, the best incubation time was 5 minutes, irrespectively to the HSA concentration used. The result is confirmed by the profile of the time course of the imprinting factor (IF, ug bound by MIP / ug bound by ctrl) plotted in Figure 5, 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 (Figure 4b and 5) for non saturating HSA concentrations indicates that for short incubation times (<5 min) the HSA molecules approach the surface and sticks randomly to it (IF is only slightly in favour of MIPs). When the incubation time rises up to 5 min, the binding to specific cavities is appreciable and maximised (IF reach its maximum), as if the total quantity of protein bound does not change significantly respect 3 min, but as if a thermodynamic driven rearrangement of the bound proteins took place and proteins on MIP move to the specific cavities. Finally, for longer incubation time (20-60 min), the specific binding sites are saturated and proteins get bound un-specifically. The same does not apply to the high concentration of HSA (4.1 ug/ul), accounting for enough protein to get a fast saturation of unspecific binding sites. In the case, once the maximum IF reached at 5 min it maintains almost constant over the time course.

The recovery of the bound protein onto the beads was next evaluated. A protocol for protein elution was optimised. Particularly, after binding, five different conditions for elution HSA from beads were tried, in order to select the most effective washing procedure. The first protocol is made of 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 is 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 and co-workers (Fortis et al., 2005 a-b). The third one is 7% Acetic Acid, 0.1% Tween 20. This solution was reported for washing APBA-MIPs polymers from Bossi and co-workers (Bossi et al., 2001). The forth one is 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 one is a high ionic strength solution 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 to recover the HSA. However, among all the protocols applied only the protocol number 3 has supplied good results, similar to those attended from Bradford Assay. The other protocols allowed to recover a little 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 a direct binding capacity for MIP and ctrl (calculated from the quantity of HSA recovered from the beads), which was 0.696 mg/g on MIP beads and 0.363 mg/g on ctrl. The advantage of a direct calculation of binding capacity is the elimination of part of the unspecific contributions. The consequence is fairly lower values of binding capacities, respect 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.

In order to evaluate the specificity of binding, a competitive experiment was performed (see Figure 6). 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 from beads and SDS-PAGE. The competitor chosen was Carbonic Anhydrase (CA), which has approximately the same isoelectric point of 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 (approximately 3.5 $\pm$ 0.5 ug) is not perturbed by the addition of CA, accounting for the un-specificity of the binding. In the MIP, no displacement was observed for CA/HSA molar ratio lower than 3.5/1, while the addition of the CA competitor at ratio 4.5/1 respect HSA displaces half of the HSA bound, and CA/HSA ratio above 8/1 give rise to a complete displacement of the HSA binding, which decreases from 10.3 ug to the unspecific level of 3.5 ug. These preliminary results indicate that only for relative high concentrations of competitor the HSA binding is displaced. It follows that samples like human serum, which contains approximately the 60-80% of albumin, the problem 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 the 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 vessel communication system, accounting for a high complexity of such fluid. Despite albumin and immunoglobulins constitute the vast majority of the proteic fraction of serum (ca. 90%), the main interest is focused on the low represented proteins, as their presence or their quantitative variations are often correlated to pathological states. Therefore, methods for an efficient removal of HSA are highly welcomed, as they permit tracing and analysing low abundant 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 Figure 7. The total number of protein spots counted for serum was 124 and 116 for MIP-treated serum, indicating again the imprinted beads are targeted for the binding of HSA and does not remove randomly 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 decreases of a factor 1.7 $\pm$ 0.3 upon treatment (i.e. the 58% HSA was removed). The quantity of albumin removed with the beads was of 183 ug, starting from 311 ug of HSA. It can be concluded that the imprinted beads remove the protein target also in a complex fluid, leaving nearly unchanged the other proteins. The removal process is easy and straightforward, being the optimal incubation time of 5 min. Of course, these preliminary experiments with a complex fluid needs further investigation, in which the quantity of imprinted beads would be optimised to obtain an almost complete depletion of HSA from serum.

In conclusion, the results presented here, demonstrate the efficiency of the immobilisation approach for generating a surface able to recognize a specific protein target in simple and complex samples. This approach, in the future, may have great potential applications, in biomaterials for recognition of proteins, in sensing and in proteome applications.

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FIGURE CAPTIONS

Figure 1. Steps of the protein imprinting process with immobilisation of the albumin template (modified from Shiomi *et al*., 2005).

Figure 2. Influence of the washing protocol on the pH of the beads and on the removal of template.

Figure 3. Evaluation of the binding capacity for control (open) and mip beads (solid). The micrograms bound from beads are plotted as function of micrograms used for binding.

Figure 4. Binding kinetic. The micrograms of HSA bound are plotted as function of the time, in saturated condition (a) and no-saturated condition (b).

Figure 5. The IF calculated as micrograms of HSA bound by MIP/ micrograms of HSA bound by ctrl is plotted as function of the time, both for saturated condition and no-saturated condition.

Figure 6. Competitive adsorption of HSA in presence of a non template protein, Carbonic Anhydrase (CA). Rebinding was quantified in ug of HSA adsorbed on MIP and ctrl beads, after elution of bound proteins and analysis in SDS-PAGE

Figure 7 2D-maps of human serum a) untreated serum sample, b) serum treated with imprinted beads, for the depletion of HSA.

Table 1 (Bonini F., *et al.*)

Table 1. Recovery of HSA from MIP and control beads.

Solution used for elution	**ctrl beads ug**	**MIP beads ug**
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

Figure 1 (Bonini F., *et al*.)

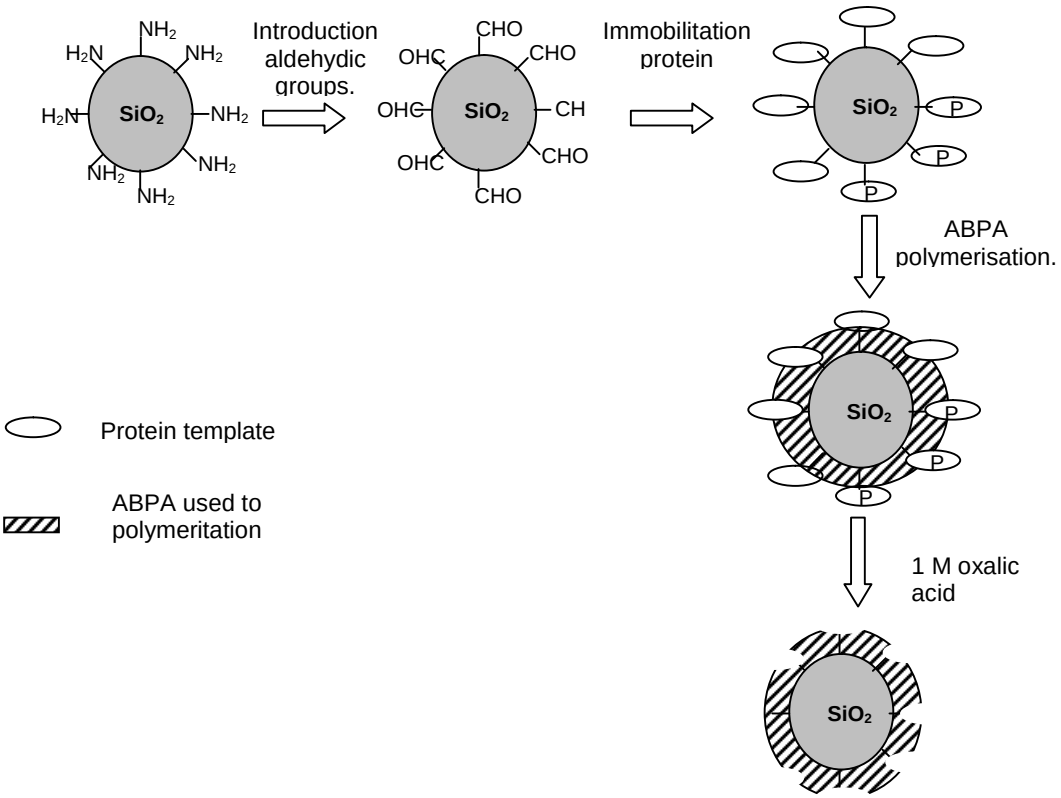


Figure 2 (Bonini F., *et al*.)

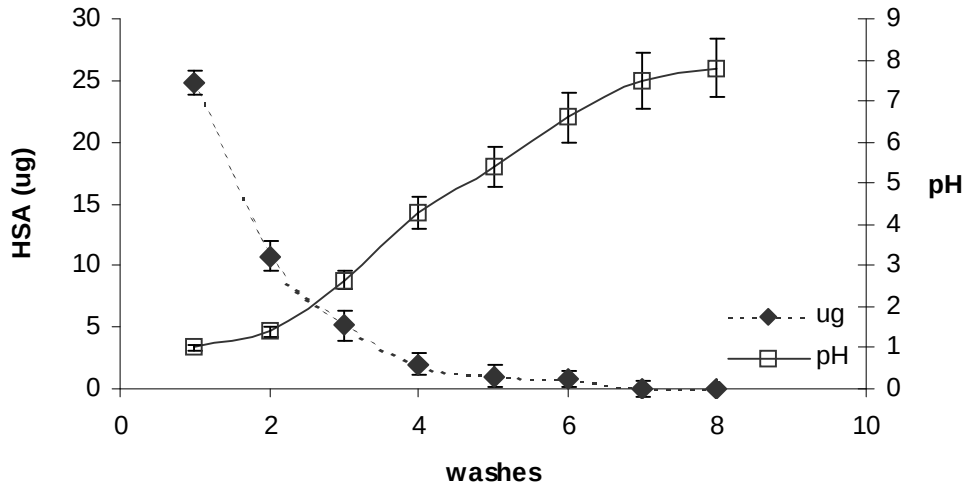


Figure 3 (Bonini F., *et al*.)

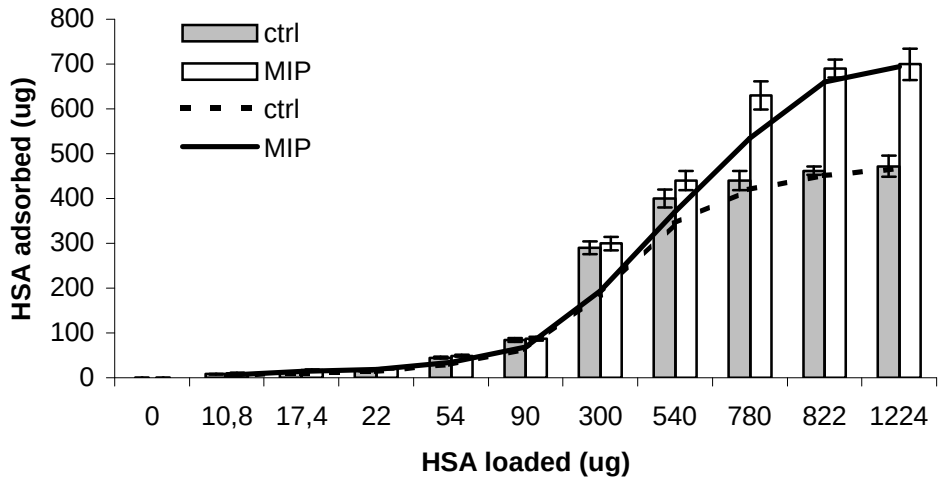
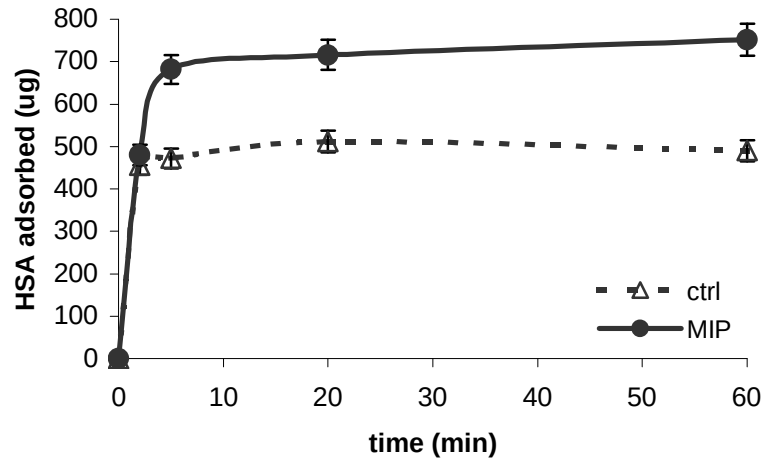


Figure 4 (Bonini F., *et al*.)

a)



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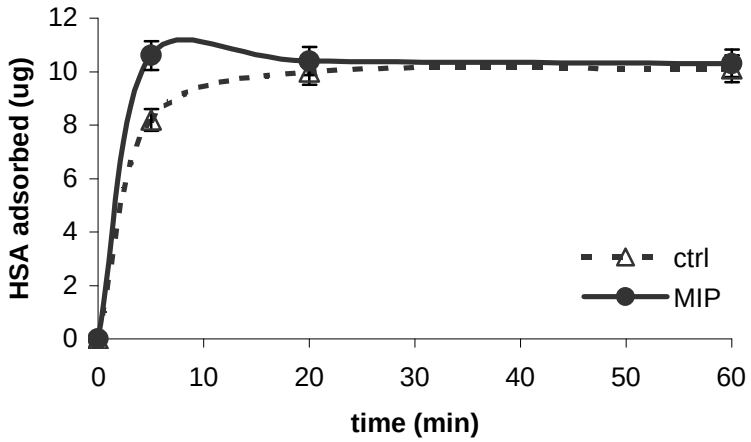

Figure 5 (Bonini F., *et al*.)

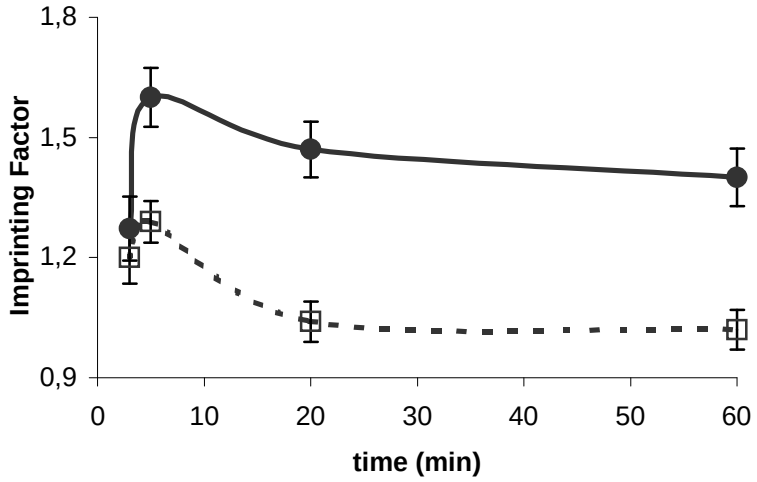

Figure 6 (Bonini F., *et al*.)

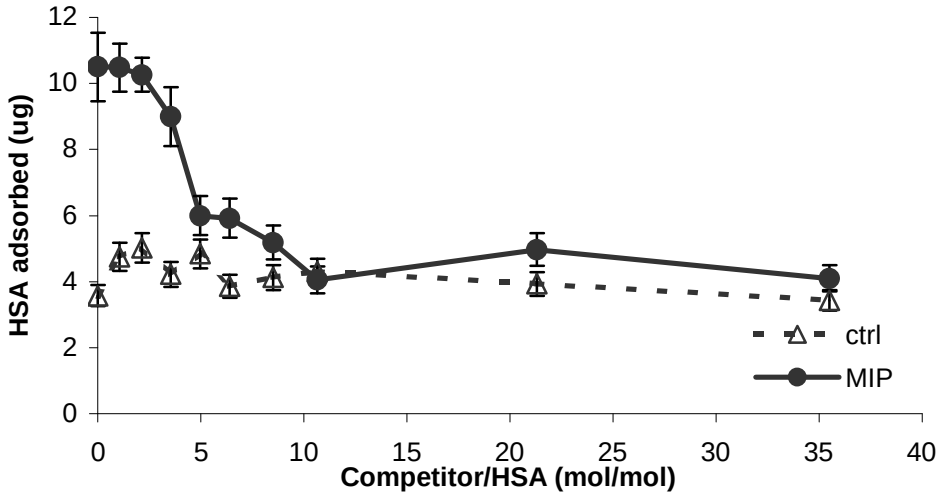

Figure 7 (Bonini F., *et al*.)