Biotin –specific synthetic receptors prepared using molecular imprinting

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Abstract

The composition of new molecularly imprinted polymers (MIPs) specific for biotin was optimised using molecular modelling software. Three functional monomers: methacrylic acid (MAA), 2-(trifluoromethyl)acrylic acid (TFAA) and 2-acrylamido-2-methyl-propanesulfonic acid (AMPSA), which demonstrated the highest binding scores with biotin, were tested on their ability to generate specific binding sites. The imprinted polymers were photografted to the surface of polystyrene microspheres in water. The affinity of the synthetic "receptor" sites was evaluated in binding experiments using horseradish peroxidase-labelled biotin. A good correlation was found between the modelling results and the performance of the materials in the template rebinding study. The dissociation constants for all MIPs were 1.4-16.8 nM, which is sufficient for most analytical applications where biotin is used as a label.

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1. Introduction

Molecular imprinting provides a means for the preparation of synthetic polymers with predetermined specificity [1]. The polymerisation of monomers around a template forms binding sites-imprints complementary to the template molecules. Generally, molecularly imprinted polymers offer a number of advantages compared to natural receptors and antibodies. These include as a high mechanical, thermal and chemical stability, excellent operational and storage stability, simplicity of manufacturing and comparatively low price for material preparation.

The ability of the molecular imprinted polymers to recognise target analytes make their application in assays and sensors a practical feasibility [2, 3]. An important unsolved aspect, however, which still limits practical application of MIPs is their poor performance in polar media. Although it is desirable to achieve an affinity separation and sensing in water, MIPs usually do not work equally well in aqueous media due to the disruption of hydrogen bonds and competition process between solvent and template molecules for their binding to the polymer functional groups. A significant possible contribution to loss of polymer affinity originates also from the difference in the structure of polymer binding sites in organic solvent (traditionally used for polymer preparation) and in water due to different polymer swelling. To prevent this from happening it is desirable to perform both, synthesis and separation or sensing in the same environment, e.g. in water.

The aim of the present paper is to develop MIPs compatible with water. Several approaches were attempted in the past, which offer possible solutions to this problem. In one of these approaches strong ionic interactions between 2-(diethylamino)ethyl methacrylate or 2-acryloylamido-2-methylpropane sulfonic acid (AMPSA) and corresponding charged functional groups of templates such as ATP and microcystin-LR (cyclopeptide algae toxin) correspondingly, were used and proved to be sufficient for template recognition in water [4,5]. In another approach a polymer was synthesised in water using water-soluble monomers such as *N,N'*-diacryloylpiperazine, AMPSA and bisacryloyl ß-cyclodextrin [6]. The hydrophobic environment of the cyclodextrin cavity improved the specificity of polymer-template interactions. Yet in another approach a thin

MIP film was grafted to the surface of a hydrophobic polypropylene membrane in water [7]. It was shown that the contribution of strong ionic interaction provided by AMPSA and hydrophobic binding provided by polypropylene residues is sufficient for specific recovery of triazine herbicide desmetryn from water. An interesting new possibility is the computational screening of a virtual library of functional monomers and identification of these, which provide strong binding to the template in water [8]. In the present paper we explore this possibility in an attempt to design polymers capable of recognition of a biotin template in water.

2. Materials and methods

2.1. Reagents

Polystyrene non-dyed beads with a carboxylated surface (mean microsphere diameter- 5.3 µm, surface area- $1.08E+12$ μm^2/g, surface COOH per microsphere- $9.85E+07$) were supplied by Luminex Corporation, USA. Benzophenone, AMPSA, MAA, TFAA and N,N´-methylenebisacrylamide (MBAA) were purchased from Aldrich, UK. Water and dimethylformamide (DMF) were bought from BDH, UK. Biotinamidocapryloyl labelled horseradish peroxidase (Biotin-HRP conjugate) and horseradish peroxidase type II (HRP), ammonium persulfate, 3,3´,5´5-tetramethylbenzidine liquid substrate system (TMB), polyoxyethylene-sorbitan monolaurate (Tween 20), sodium phosphate dibasic, sodium phosphate monobasic, albumin bovine fraction V (BSA) were from Sigma, UK. (+)-Biotin was purchased from Fluka, UK.

All reagents were analytical or HPLC grade and used without additional purification.

2.2 Computer simulation

The workstation used to simulate monomers-template interactions was a Silicon Graphics Octane running IRIX 6.5 operating system. The workstation was configured with two 195 MHz reduced instruction set processors, 712 MB memory and a 12 GB fixed drive. This system was used to execute the software packages SYBYL 6.8 Tripos Inc., St. Louis, MI, USA. The computational design was performed in three steps. Initially, a molecular model of a (+)-biotin (template) was developed and a virtual library of 21 commonly used monomers was designed (Figure 1). All these structures were then charged using the Gasteiger-Huckel approximation method, and refined using the molecular mechanics method applying an energy minimisation with the MAXIMIN2 command using dielectric constant $\varepsilon = 80$. In a second step, the Leapfrog™ algorithm was applied to screen the library of functional monomers for their possible interactions with the template as described earlier [9]. The program was activated for different lengths of runs (10,000, 30,000 and 40,000 steps). The result from each run was examined evaluating the empirical binding scores (Table 1). The monomers giving the highest binding score and capable of forming the strongest complexes with the template were MAA, TFAA and AMPSA. These monomers were used for preparation of the polymers.

2.3 Photografting polymerisation

200 mg of polystyrene beads were incubated for 30 min in 4 ml of 100 mM benzophenone in methanol. The beads were sedimented by centrifugation for 3 min at

12000 rpm. The supernatant was removed and beads were transferred into the reaction mixture (see Table 2). The reaction mixture was heated at 60 °C in order to achieve better solubility of the biotin and benzophenone. The suspension was illuminated upon stirring and heating with UV light (intensity- 0.016W/cm^2) for 2 h using a Cermax Xenon Fiber Optic Light Source (ILC Technology, UK).

In order to remove the template and non-reacted monomers several washes have been performed (all volumes are given per 200 mg of modified beads): 2x 1 ml DMF, 2x 1 ml 100 mM HCl in DMF, 2x 1 ml DMF and 2x 1 ml H$_2$O. All modified beads were stored as 10% suspension in reverse osmosis water at 4 °C.

In order to optimise the time of photografting polymerisation the beads suspension in monomer mixture was illuminated for 10, 30, 60, 120 and 300 min. The subsequent modified beads with polymer coatings were tested in binding assay using HRP-Biotin conjugate.

2.4 Polymer characterisation

The determinations of specific surface area were performed using an ASAP 2000 instrument (Micrometrics Instrument Corp., USA) based on the nitrogen BET.

The quantity of carboxylic groups grafted to the surface was estimated using titration of the MIP 3 and Blank 3 polymer -modified beads (100 mg) suspended in 3 ml of NaOH (10 mM) by 10 µl-portions of 10 mM HCl upon stirring. After every addition of HCl the suspension was stabilised for 5 min and readings of pH were taken.

2.5 Binding assay

2.5.1. Beads conditioning

In order to decrease the non-specific binding of enzyme to modified polystyrene beads the 50-µl aliquots of polymer suspension (solid content- 5 mg) were mixed with 200 µl of 25 mM Na-phosphate buffer, pH 7.5, containing 1 mg/ml BSA and incubated for 1 h at 4 °C in Eppendorf tubes. After incubation the suspension was centrifuged and the supernatant removed.

2.5.2. Biotin-HRP conjugate binding

Each aliquot of BSA-pretreated polymer beads (5 mg) was re-suspended in 200 µl of 25 mM Na-phosphate buffer, pH 7.5, containing different concentrations of Biotin-HRP conjugate (6- 800 µg/ml) and 0.05% Tween 20 (w/v). The incubation was performed for 1 h at 4 °C. After incubation the suspension was centrifuged and supernatant removed. In order to decrease the non-specific binding the beads were washed 3 times with 25 mM Na-phosphate buffer, pH 7.5, containing 0.05% Tween 20 (w/v). The polymer particles were centrifuged for 3 min at 12000 rpm and the supernatant was removed and beads were re-suspended in the fresh buffer. To measure the quantity of bound conjugate the beads were incubated with 200 µl of 3,3´,5´5-tetramethylbenzidine liquid substrate system (TMB) in Eppendorf tubes for 10 min at room temperature. Polymer particles were centrifuged, 100 µl of supernatant was transferred into a microtiter plate and the optical absorbance was measured at 530 nm. The quantity of bound conjugate was calculated using a calibration curve, which was built for several dilutions of the Biotin-HRP conjugate (3- 50 ng/ml).

2.5.3. HRP binding

The analysis of HRP binding to MIP and Blank polymer- modified and non-modified beads were made under the same conditions that were described previously for HPR-Biotin conjugate binding.

3. Results and discussion

The molecular modelling and computational screening were performed as described previously [9]. In order to mimick aqueous conditions, the energy minimisation of monomers and template was performed using dielectric constant of water ($\varepsilon = 80$). The results of modelling clearly indicate, that three monomers: MAA, TFAA and AMPSA have a good chance to form a strong complex with the template molecule in water through ionic and hydrogen bonds. The binding scores calculated for these monomers were reasonably high, in the range -12.2 to -18.6 kcal/mol (Table 1). The possible structures of the complexes formed between monomers and template are presented in Figure 1. These three monomers were used for MIP preparation.

MIP grafting was performed as described previously [7]. The optimal polymerisation time was found to be 1-2 hours because these polymers demonstrated the best binding towards biotin. The shorter time (10 or 30 min) was not sufficient to obtain polymer coating on the beads surface, longer UV irradiation (5 h) has led to the polymer formation not only on polystyrene surface but also in solution (Figure 2). In first case the binding was lower due to insufficient amount of functional sites or in second case due to the presence of non-specific, non-grafted polymer formed in solution.

Three MIPs and 3 corresponding Blank polymers were photografted to the polystyrene beads surface, washed and studied for Biotin-HRP conjugate binding. The results of analysis indicates a good correlation between the empirical binding score calculated for monomer-template interaction and the value of dissociation constants calculated for corresponding polymers (Table 1). Thus the best results were obtained with MAA as a monomer which has shown the highest binding energy to the template in modelling experiment (MIP 3). Obviously the strength of monomer-template interactions is one of the most important factors, which determine the success of imprinting.

The dissociation constants for Biotin-HRP conjugate binding to MIP and Blank polymers were calculated from Scatchard plot. The Scatchard plot of the MIP 3 shows two lines with different slopes corresponding to high and low affinity populations of binding sites (Figure 3). The Scatchard plots of the blank polymer, prepared in the absence of template is linear reflecting the presence of only one population of binding sites (Figure 3). The dissociation constants for all MIPs were reasonably low: 1.4-16.8 nM. These values are sufficient for most analytical applications where biotin is used as a label. It is interesting that the K_D value obtained in our experiment made in water is 4 orders of magnitude lower than previously reported for MIP and biotin derivative in experiment made in organic solvent [10].

We believe three reasons are responsible for excellent performance of the new MIP system. Firstly, polymers were prepared and tested in aqueous environment and due to this the structure of binding sites should be the same in both, synthesis and assay conditions. Secondly, the monomers were selected on the basis of their binding energy to template, calculated for aqueous environment. Thirdly, the recognition was improved

also due to participation of support polystyrene layer in template binding through the hydrophobic interactions, as it was shown previously [9]. To prove the last point an emulsion of monomer mixture in water was polymerised as described previously in the absence of polystyrene beads. The resulting polymer was washed and tested in the binding assay. No preferential binding of Biotin-HRP conjugate as compared with HRP alone was observed in this assay. Clearly the support plays a critical role in the template recognition by MIP in water.

The polymer specificity has been compared with results for non-biotinilated HRP. The results clearly indicate the superior affinity of imprinted polymer for biotinilated enzyme as compared with free HRP (Table 3).

It was found that beads, modified with MAA and TFAA were stable. Practically no changes were observed in polymer performance over a three weeks period of time. The beads modified with AMPSA gradually lost their affinity, probably as result of polymer hydrolysis catalysed by the strong sulfonic group.

The disadvantage of the proposed method is the limited number of binding sites introduced into the polymers (1-10 pmol/g). Assuming that the surface area of beads is 1.2 m^2/g (result from BET measurements), the total coating is very low - $6.32 \ 10^{11}$ sites/m^2 or 0.63 sites/μm^2). This value is in agreement with the titration data for the quantity of carboxylic groups grafted to the bead surface. Thus non-modified polystyrene beads contain 46 μmol/g carboxylic acid and the quantity is only slightly higher for grafted beads: 58.5 μmol/g for MIP, and 63.2 μmol/g for Blank polymers. Obviously the amount of material grafted to the surface of polystyrene beads is very low. Further work should be done aimed at increasing the quantity of the grafted material. Perhaps better

results could be obtained by using porous beads with a larger surface area for modification.

4. Conclusions

The results of this study indicate a possibility of using molecular modelling software for rational selection of monomers capable of template recognition in water. The MIP was successfully grafted to the polystyrene surface in aqueous environment. The modified polymers demonstrated high affinity to the biotinilated protein, which is sufficient for an analytical application of these materials in assays and separation. The future work will be aimed at increasing the quantity of binding sites introduced by imprinting.

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Figures legends

Figure 1. The computationally derived structures of biotin - monomer complexes: a) biotin - methacrylic acid; b) biotin - 2-(trifluoromethyl)acrylic acid; c) biotin - 2-acrylamido-2-methyl-1-propanesulfonic acid.

Figure 2. Optimisation of illumination time for polymer grafting. All percentages were calculated accordingly to the highest value of Biotin-HRP binding demonstrated by polymer prepared by 120-min illumination.

Figure 3. Representative Scatchard plot of MIP 3 and Blank 3 polymer-template interactions. Every point is made in triplicate. The standard deviation between replicates is 5 %.
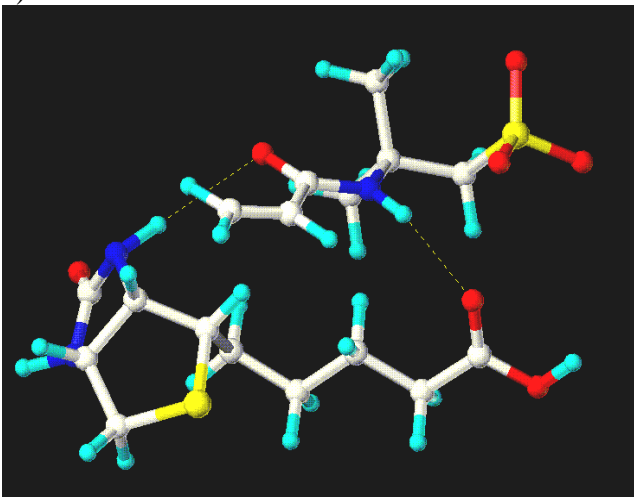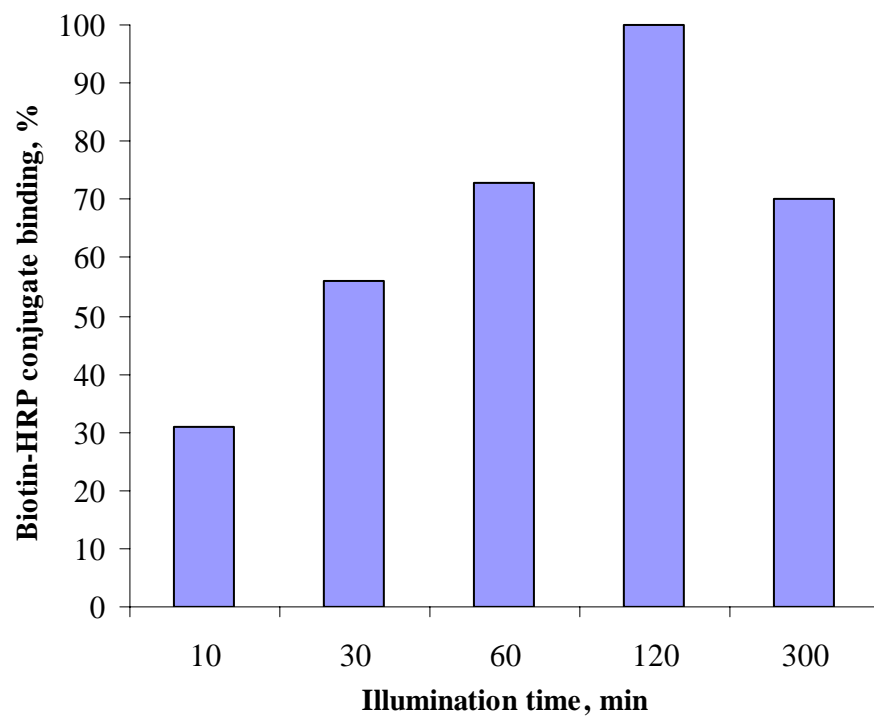
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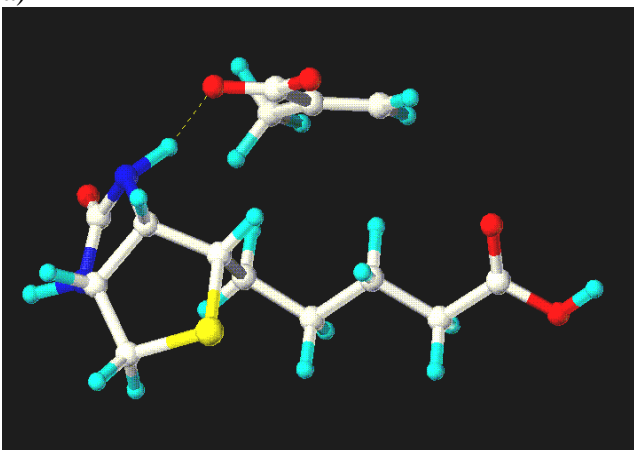
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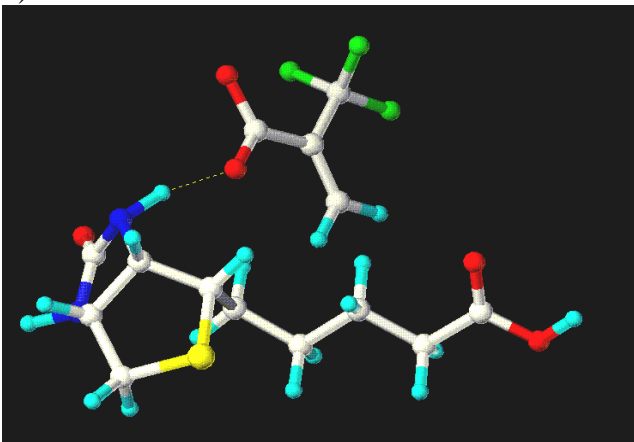
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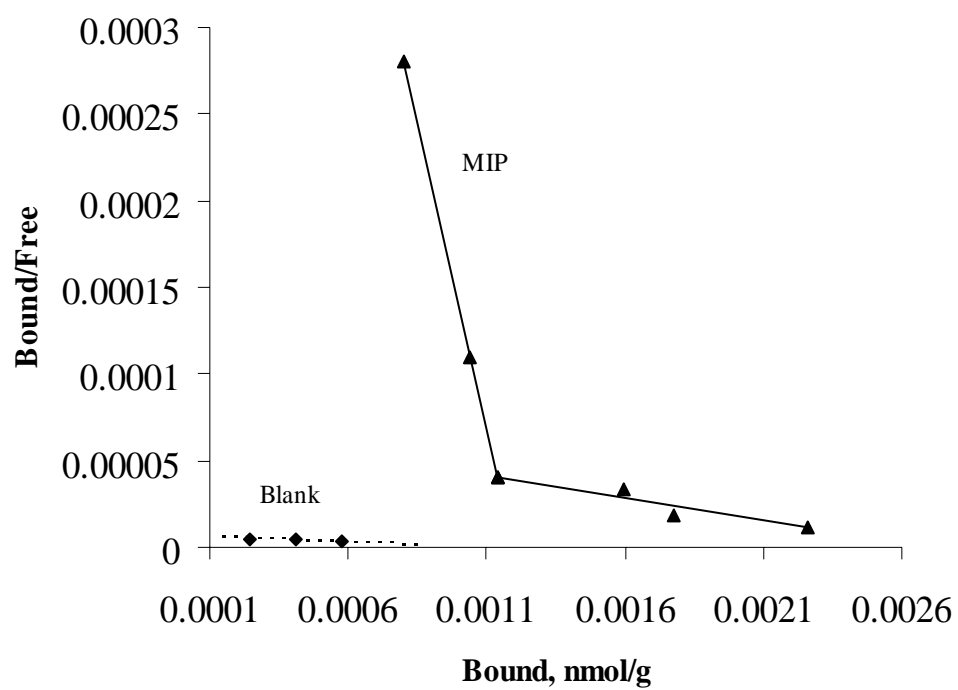Fig.1 Piletska *et al.*

Fig. 2 Piletska *et al.*

Fig. 3 Piletska *et al.*

Table 1. Binding scores calculated for monomer-template interactions and dissociation constants (K_D) measured for MIPs and biotin-HRP.

Monomer	Binding score, kcal/mol	K_D (MIP-HRP-Biotin), nM
MAA	-18.6	1.39 ± 0.1
TFAA	-15.89	5.01 ± 0.36
AMPSA	-12.22	16.78 ± 2.18

Table 2. Polymer composition.

Polymer	MIP1	Blank1	MIP2	Blank2	MIP3	Blank3
(+)-Biotin (mg)	9.76	-	9.76	-	9.76	-
AMPSA (mg)	41.2	41.2	-	-	-	-
TFAA (mg)	-	-	28	28	-	-
MAA (mg)	-	-	-	-	34.4	34.4
MBAA (mg)	61.6	61.6	61.6	61.6	61.6	61.6
BP (mg)	4	4	4	4	4	4
Water (ml)	4	4	4	4	4	4

Table 3. The binding characteristics of the MIP -coated PS beads. The calculation represents the affinity (K_D) and maximal concentration (Bmax) of high-affinity population of binding sites only.

Monomer	HRP-Biotin		HRP	
	K_D, nM	Bmax, pmol/g	K_D, nM	Bmax, pmol/g
MAA (MIP 3)	1.39 ± 0.1	1.26 ± 0.1	1250 ± 175	10 ± 1.4
MAA (Blank 3)	163 ± 3.3	1.15 ± 0.1	5000 ± 250	4.9 ± 1.9
PS	714 ± 85	5.1 ± 0.7	244 ± 7	7.3 ± 0.2