

Synthesis of biologically active molecules by imprinting polymerisation

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Highly cross-linked molecularly imprinted polymers (MIPs) are synthetic materials with properties mimicking those of natural receptors. Here we describe an ability of MIP nanoparticles to manifest biological activity. Molecularly imprinted polymers were synthesised by co-polymerisation of urocanic acid, N,N'-bisacryloyl piperazine in the presence of herbicide binding D1 protein, ground and separated from the template by washing and ultrafiltration. It was demonstrated that MIP nanoparticles retained affinity to the template. Moreover, imprinted polymers were able to activate chloroplast photosystem II in in vitro experiments. This provides the first example of the use of imprinted polymers for the attenuation of a biological system and opens new possibilities for their application in pharmacology, biotechnology and medicine.

Key words: molecularly imprinted polymers, D1 protein, Hill reaction.

Introduction. Since being first described in 1972, molecularly imprinted polymers (MIPs) have attracted broad interest from scientists engaged in the development of chromatographic adsorbents, sensors, catalysts, enzymes and receptor mimics [1–4]. The particular features which make them the target of intense investigation are striking resemblance of the MIP binding properties to those of natural receptors [4–6] and their inherent stability, low cost and ease of preparation [7, 8]. However, MIPs suffer in comparison with natural antibodies and receptors in clinical and pharmaceutical applications due to their relatively large particle size and the lack of true water compatibility.

We report here the preparation of water-soluble cross-linked nano-sized MIP particles through the extensive polymer grinding and ultrafiltration. The imprinted polymer was prepared in the presence of thylakoid membrane D1 protein. D1 protein represents a good model system because it is stable *in vitro*, its role in photosynthetic process, where it mediates the electron flow passing through the Photosystem II, is well known and its activity can be easily monitored using Hill reaction [9, 10]. In D1 structure one can envisage two possible interaction sites for MIP: internal (herbicide binding site) and external, exposed to cytoplasm.

Our intention was to investigate the ability of D1-specific MIP upon binding to the D1 to alter the photosynthetic process either through the displa-

cement of the electron acceptor Q_B from herbicide binding site, or through the interaction with D1 external site. Evidence for such a biological activity of synthetic polymers can open a new way for the application of MIP nanoparticles in pharmacology, analytical chemistry and biotechnology as well as in the fundamental research of molecular recognition phenomena. Furthermore, the analysis of the influence of extensive grinding on the recognition characteristics of polymer was of interest in order to establish the minimal particle size necessary for maintaining molecular «memory» and specificity.

Materials and Methods. All chemicals were obtained from commercial sources and used as received.

MIP synthesis. D1 protein was obtained from pea leaves as previously described [10]. Purified (95 %) lyophilised D1 protein (10 mg) was extracted with hexane, mixed with N,N'-bisacryloyl piperazine (BAP) (194 mg), urocanic acid (UA) (6.9 mg), ammonia persulphate $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (10 mg) and H_2O (200 μl). Polymerisation was initiated by adding 2 μl of 30 % N,N,N',N'-tetramethylethylenediamine (TEMED) solution followed by heating at 80 °C (12 h). The control polymers were prepared in the absence of D1 protein (Blank) and in the presence of irrelevant, bovine serum albumin (BSA). Polymers were washed with 0.1 M HCl, ground, filtered through the glass filter (N3) and centrifuged through Ultra-Spin Macrofilters («Roth», Germany) separating molecules with molecular weight < 5 kDa, 5–10 kDa and > 10 kDa (yield is ~ 0.2 mg/fraction). Additionally these fractions were analysed by gel filtration chromatography (GFC) in 100 mM sodium phosphate buffer, pH 7.5, using Superdex Peptide HR 10/30 column, calibrated with peptides and proteins. The absorbance of all solutions was measured at 260 nm.

Chromatography of the polymers on affinity column with immobilised D1 protein (D1-column). To prepare D1-Sepharose, D1 protein (0.1 g) was added to activated CH-Sepharose 4B (5 g) («Pharmacia Fine Chemicals», Sweden) in phosphate buffer (10 mM, pH 7.5) and left overnight at 40 °C. The immobilization rate was 6 mg/g of sorbent. Chromatography column was packed with 3g of D1-Sepharose. The column was equilibrated with eluent (25 mM sodium-phosphate buffer, pH 7.5, flow rate 0.22 ml/min) at room temperature. An aliquot (100 μl) of the polymer solution (fraction 5–10 kDa) was injected into the column and the retention times of the peaks were

measured. All eluted peaks were analyzed additionally by GFC.

Influence of the polymers on the activity of thylakoid membranes. The thylakoid activity was measured as previously described [10]. An aliquot (30 μl) of thylakoid solution (1 μg chlorophyll in probe) containing sucrose (0.35 M) and BSA (1 %) was mixed with polymer solution (20 μl), and DPIP (100 μl , 0.3 mM) in Tris-HCl buffer (0.1 M, pH 7.5) in the microplate wells. The samples were illuminated for 10 min with a 100 W lamp and the absorbance was measured at 530 nm using a Dynatech reader (Germany). The activity of thylakoid membranes was calculated from the amount of reduced DPIP, concentration of chlorophyll and the time of illumination.

Results and Discussion. We have polymerised the functional monomer urocanic acid, containing carboxylic and imidazole residues, essential for the protein recognition and water-soluble cross-linker N,N'-bisacryloyl piperazine [11] in the presence of D1 protein. Judging from our experience, UA is a monomer superior to methacrylic acid, traditionally used in molecular imprinting, being able to form a stronger complex with a template. It can also be co-polymerised with BAP giving polymer with 99 % yield. The synthesised polymer was ground, washed from the template and centrifuged through microfilters to yield three fractions: < 5 kDa, 5–10 kDa and > 10 kDa. These fractions were analysed by GFC giving peaks with average molecular weight 0.8 kDa and 0.2 kDa (fraction < 5 kDa), 6.7 kDa (fraction 5–10 kDa) and 18 kDa (fraction > 10 kDa). Taking into account the fact that the molecular weight of D1 protein is 32 kDa, we did not expect the template to be present in the polymer fractions. Minor quantities of the protein residues (presumably short peptides) however were found in the fraction with molecular weight < 5 kDa, as was determined using BCA-assay [12].

The affinities of the imprinted and blank polymer (prepared in the absence of a template) for templates were analysed by liquid chromatography using a Sepharose column derivatised with D1 protein (D1-column). The elution profiles of the MIP and blank polymers on the D1-column are presented in Fig. 1.

The peaks eluted first correspond to residues of non-polymerised monomers. The capacity factors, which reflect polymer affinity, were much higher for the MIP than for the blank polymer ($K_{\text{MIP}} = 1.11 \pm 0.06$ and $K_{\text{blank}} = 0.56 \pm 0.05$; separation factor $\alpha =$

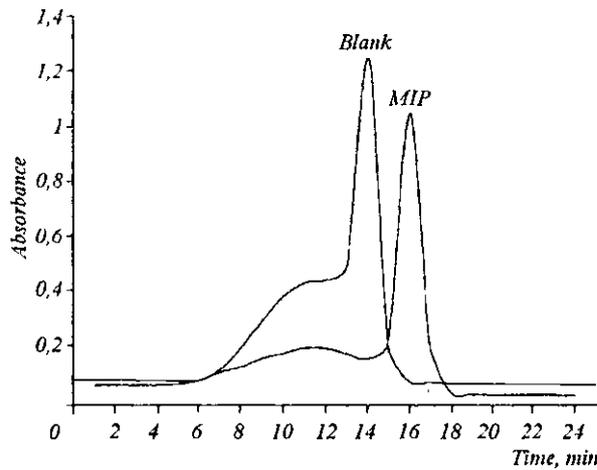


Fig. 1. Chromatography of the polymers on a column with immobilized D1 protein in 25 mM sodium phosphate buffer, pH 7.5. Flow rate 0.22 ml/min. Injections — 100 μ l of the polymer solution (fraction 5—10 kDa) at a concentration of 1 mg/ml. Detection was performed at 260 nm. Capacity factors for blank and imprinted polymers were calculated using equation: $K_i = (T_i - T_0)/T_0$, where K_i — capacity factor of an analyte, T_i — retention time of analyte, and T_0 — retention time of non-interacting species. In our experiments free volume and T_0 were calculated using injection of diluted buffer solution

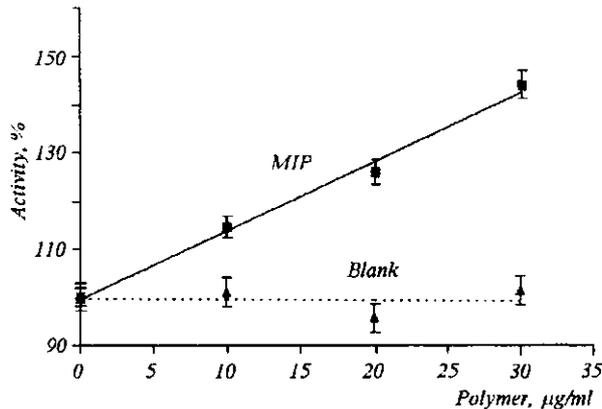


Fig. 2. Influence of the polymers on thylakoid activity *in vitro*. Reaction mixture (150 μ l) contained: thylakoid suspension (1 μ g chlorophyll) and DPIP (9 μ g) in Tris-HCl buffer (0.1 M, pH 7.5). All measurements were done in triplicate

= 1.98). For a non-derivatised column the capacity factor for MIP (fraction 5—10 kDa) was the same as for a blank polymer ($K_{MIP} = 0.60 \pm 0.06$). This provided a clear indication of the role of the imprinting process and proved that the affinity of the MIP to D1-column results from the interaction of the polymer with the immobilised template. From these results we can assume that at least a part of the polymer specific structure is preserved upon grinding.

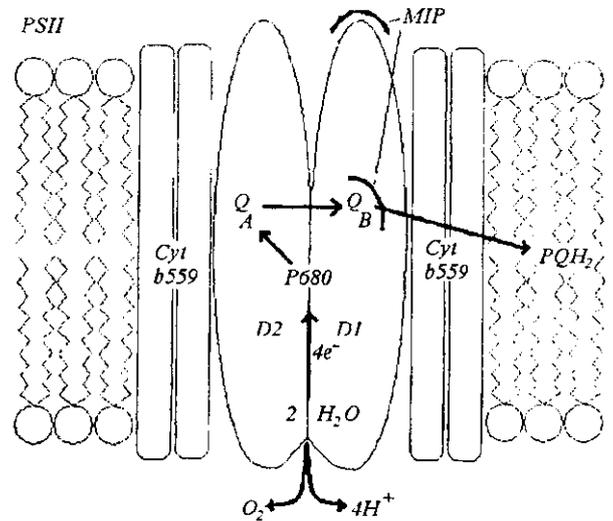


Fig. 3. Schematic structure of the Photosystem II core with D1 protein. Q_A and Q_B — bound plastoquinines; PQH_2 — reduced plastoquinone, *Cyt b559* — b-type cytochrome; *D1* and *D2* — core proteins of the PS II, involved in the electron transport; *MIP* — polymer formed in the presence of D1 protein with possible sites for its interaction

The biological activity of the polymers was analysed *in vitro* using the Hill reaction [9]. Upon illumination thylakoids reduced the dichlorophenolindophenol (DPIP)-electron acceptor, changing its absorbance spectra. The activity of thylakoids can be calculated from the amount of DPIP reduced. We had anticipated that the imprinted polymer upon binding to D1 protein would influence this reaction. In our experiments the clear increase (up to 45 % for 30 μ g/ml of polymer concentration) in activity of thylakoid membranes was observed (Fig. 2) which suggests that the target of MIP action is not the herbicide-binding site but rather the external site of the D1 protein (Fig. 3).

The ability of polymers to enhance the stability and activity of thylakoids and enzymes has previously been demonstrated [13—15], though this effect has been ascribed to the inhibition of proteolysis or non-specific stabilisation of the protein structure by restricting unfolding [16]. In our experiments, however, we were able to select the molecular target in the photosystem complex for the interaction with MIPs and enhance the biological effect using imprinting with D1 protein. A good indication of the selectivity of the interaction is the negligible influence of the blank polymer and monomers (UA and BAP) on the Hill reaction (Fig. 3 and 4). Additionally the blank polymer and D1 protein were not able to

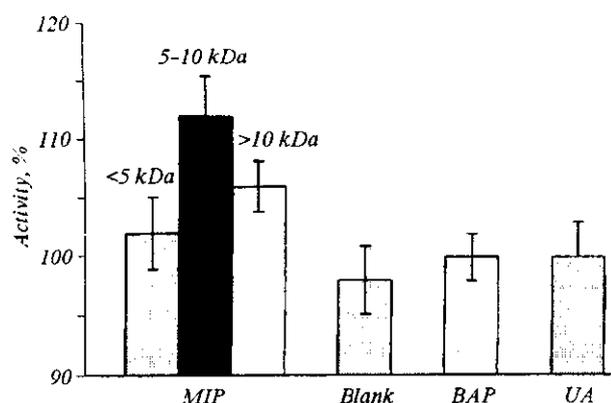


Fig. 4. Influence of the size of the polymer fractions and monomers (concentration 10 $\mu\text{g}/\text{ml}$ each) on thylakoid activity. Blank — fraction of the blank polymer with size 5–10 kDa (for reaction conditions see legend to Fig. 3). All measurements were done in triplicate

change the activity of thylakoids. We also found that polymer prepared in the presence of BSA had no biological activity. We thus conclude that the activation of the thylakoids by D1-specific MIP is induced by the selective interaction with D1 protein so as to provide partial stabilisation of the photosystem complex structure, and preventing its interaction with proteases or free radicals. The influence of MIP particle size on thylakoid activity was also investigated (Fig. 4).

The lower activation achieved for the polymer fraction with a particle size smaller than 5 kDa can be explained by the partial destruction of the binding sites. The reason for slightly lower activity of > 10 kDa fraction in comparison with 5–10 kDa fraction could be relative difficulty in permeation of this fraction through the thylakoid membranes. It can be concluded that in order to possess affinity for D1 protein MIPs should contain at least 25–30 monomer units with pre-determined primary monomer sequence.

Conclusions. To our knowledge, this is the first report of a MIP capable of mediating biological activity. These water-soluble MIPs have an affinity to the original template and superior activity to randomly synthesised polymers. They can be prepared easier than specifically designed discrete organic structures. Although the yield of the polymer is low, MIP nanoparticles can be used in the area of fundamental research of molecular recognition phenomena. It is anticipated that imprinted polymers can be

practically useful in analytical chemistry, biotechnology, in the pharmaceutical and food industries.

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Синтез біологічно активних молекул методом молекулярного імпринтингу

Резюме

Молекулярно-імпринтовані полімери (МІП) є сітчастими полімерами з високим ступенем зшивання, які імітують біологічні рецептори. В даній роботі вперше продемонстровано біологічну активність матричних полімерних наночастинок. МІП отримано внаслідок спієлімеризації уроканової кислоти та N,N'-бісакрилоїлпіперазину за присутності гербіцид-зв'язувального білка D1 як матричної молекули. Далі його подібноували і за допомогою ультрафільтрації виділяли фракції матричних полімерних наночастинок. Показано, що такі частинки мали афінність до матричних молекул, а також здатність активувати фотосистему II хлоропластів в експериментах *in vitro*. Подібні властивості матричних полімерних наночастинок відкривають широкі перспективи їхнього використання в фармакології, біотехнології та медицині.

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Синтез биологически активных молекул методом молекулярного импринтинга

Резюме

Молекулярно-импринтированные полимеры (МИП) представляют собой сетчатые полимеры с высокой степенью сшивания, имитирующие биологические рецепторы. В данной работе впервые продемонстрирована биологическая активность матричных полимерных наночастиц. МИП получены вследствие сополимеризации урокановой кислоты и N,N'-бисакрилол пиперазина в присутствии гербицид-связывающего белка D1 как матричной молекулы. Далее их измельчали и с помощью ультрафильтрации выделяли фракцию матричных полимерных наночастиц. Показано, что такие частицы обладали аффинностью к матричным молекулам, а также способностью активировать фотосистему II хлоропластов в экспериментах *in vitro*. Подобные свойства матричных полимерных наночастиц открывают широкие перспективы их использования в фармакологии, биотехнологии и медицине.

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