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Polyacrylamide gels containing ionized functional groups for the molecular imprinting of human growth hormone

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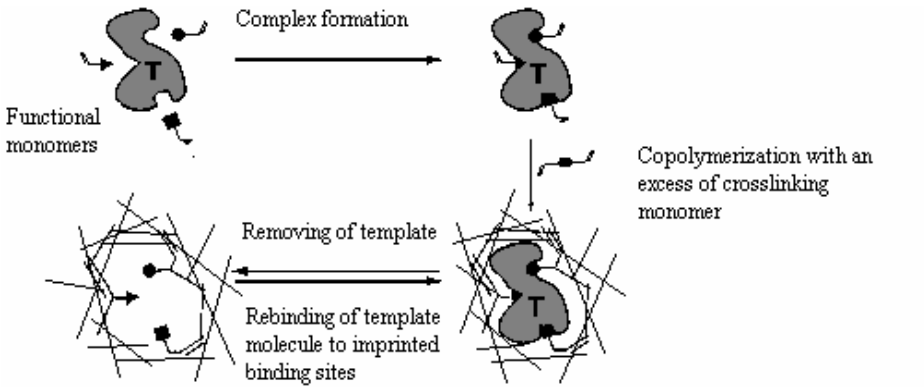
Summary

Networks of polyacrylamide having methacrylic acid and 2-(dimethylamino)ethyl methacrylate incorporated were studied for the possibility of imprinting human growth hormone. Two templates – the monomeric and the dimeric molecular forms of human growth hormone hormone – were for the first time imprinted using a hydrogel containing charged functional groups. The results show that approximately 90 % (w/w) of the templates could be extracted from the molecularly imprinted acrylamide polymers. The molecularly imprinted polymer adsorbed approximately 70 times (for the monomeric form of the hormone) and 60 times (for the dimeric form) more of the template than of the non-imprinted polymer. The selectivity of the molecularly imprinted polymers was also studied.

Introduction

Human growth hormone (hGH) like many bioactive substances, is a heterogeneous polypeptide [1]. The main molecular form is the so-called monomeric hGH with a molecular weight of 22 kDa, consisting of 191 amino acids. However, there are also molecular forms (isoforms) consisting of two or more non-covalently connected monomeric hGH and it is very difficult to separated the usually obtained mixtures.
Molecular imprinting is a recent promising technique for the fabrication of biomimetic polymeric recognition sites or "plastic antibodies/receptors" with a selective affinity for a target molecule (such as a drug, pesticide or biomacromolecule), which is attracting rapidly increasing interest [2-8]. By this technology, recognition matrices can be prepared which possess high substrate selectivity and specificity. Thus, imprinted materials may constitute an alternative to natural recognition elements. In molecular imprinting, recognition sites are tailor-made *in situ* by self-assembly of suitable monomers and the templates followed by copolymerization with cross-linkers to form a polymer network in the presence of the template (T) (s. Scheme 1). The templates are subsequently washed from the molecularly imprinted polymer (MIP),

leaving recognition sites complementary in the positioning of functional groups and in shape. When exposed to the template molecule, the recognition sites of the MIP will rebind the template selectively. Thus, the method creates materials resembling the binding sites of receptors and antibodies.



Scheme 1. General scheme of molecular imprinting technology (T stands for template)

Conventional molecular imprinting technology allows the synthesis in organic solvents of MIPs selective toward relatively low molecular weight compounds. However, synthesis in aqueous media of chemically and mechanically stable MIPs that can recognize biomolecules such as peptides and proteins is still a great challenge [9, 10].

Some human peptide hormones and other larger peptides were successfully molecularly imprinted in aqueous media and show sufficient recognition (and selectivity) with respect to the molecularly imprinted polymer [9-13]. It was shown that human growth hormone and some other proteins can also be adsorbed specifically on crosslinked polyacrylamide gel particles prepared by Hjerten's method using entrapment and molecular imprinting [14]. Here, the monomeric hGH was used for the entrapment and recognition. So far, no investigation of the molecular imprinting of other molecular forms of hGH exists. Typically, polyacrylamide gels are selective because the size and shape of the cavity originally occupied by the substrate is retained by high crosslinking density, but in case of Hjerten's method this does not apply [14]. Therefore, the imprinting mechanism remains uncertain.

Further experiments of other groups where lysozyme was chosen as the protein template, show that the incorporation of functional groups with either negative or positive charge into the polyacrylamide gel leads also to high selectivity for the recognition of the protein [10,15,16]. For that the polyacrylamide hydrogel system was extended by incorporation of two functional monomers, methacrylic acid and 2-(dimethylamino)ethyl methacrylate, at the same time. It is postulated that the recognition of the protein can be realized through the optimal distribution of positive and negative charges and hydrogen bonds.

This work aims at the preparation of molecularly imprinted polyacrylamide hydrogels with ionizable groups for the recognition of human growth hormone, and it is devoted to the development of artificial antibodies against different molecular forms of the hGH. Such molecularly imprinted polymers have possible future application in the

fields of quantitative sorbent assays (analogue - immunoassays) or chromatography (analogue - affinity chromatography).

Experimental

Materials

The monomeric human growth hormone with the molecular weight of 22 kDa and its oligomeric molecular forms with molecular weight of 44 kDa (two globular units) and 66 kDa (three globular units) were prepared and characterized as described earlier [17]. Bovine serum albumine, ≥99% (Sigma), human serum albumin, ~99% (Sigma), lysozyme (from chicken egg white), ~95% (Sigma), acrylamide, ≥99%, (Sigma), 2-(dimethylamino)ethyl methacrylate, ≥98% (Aldrich), methacrylic acid, ≥99% (Aldrich), *N*,*N'*-methylenebisacrylamide, ≥99%, (Merck), *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED) (BioRad), potassium persulfate, 99.99% (Aldrich), sodium chloride, ≥99.5% (Sigma-Aldrich), 1 M NaOH solution (Sigma) were used as obtained.
Deionized water was produced by a Millipore water system. PBS buffer solution (pH 6.2) was prepared from 18.4 ml di-sodium hydrogenphosphate 1/15 M solution (Merck) and 81.6 ml potassium dihydrogenphosphate 1/15 M solution (Merck).

Instruments

Ultraviolet absorbance (by 280 nm) measurements were performed using a UV-VIS spectrophotometer Specord S 100. Every measurement was repeated three times.

Preparation and investigation of molecularly imprinted polymers (MIP)

In a typical polymer synthesis, 2-(dimethylamino)ethyl methacrylate 0.4824 ml (0.00286 mol) and methacrylic acid 0.2428 ml (0.00286 mol) were mixed with 3.55 ml of PBS buffer solution (1/15 M, pH 6.2). The solution was adjusted to pH 6.2 by 1 M NaOH. Then, acrylamide 0.2035 g (0.00286 mol), *N*,*N*'-methylenebisacrylamide 0.1 g (0.000649 mol) and hGH 0.06 g ($2.727 \cdot 10^{-6}$ mol) were added to the the solution in a schlenk tube at 25 °C and purged with nitrogen for 15 min. The concentration of hGH in the solution was 1.29% (w/w). After purging, potassium persulfate [1.5% (w/w) aqueous solution] 0.25 ml ($1.39 \cdot 10^{-5}$ mol) and TEMED [3.75% (w/w) aqueous solution] 0.1 ml ($3.23 \cdot 10^{-5}$ mol) was added to the solution to start the polymerization. After the polymerization proceeded for 30 min, the solution mixture was wet-sieved through a 100-mesh sieve. The resultant gels were washed with 200 ml deionized water three times, 200 ml NaCl solution (0.1 M) three times and 200 ml deionized water three times. UV spectra for the wash liquid were taken to determine the amount of hGH extracted. The gels were lyophilized and sieved to obtain particles of 100-150 μm size which were stored at ambient temperature.
After extraction of the template the hGH-dimer-imprinted gel (**MIP-di**) particles were added to a solution in which a certain amount of hGH dimer (50 % of the amount of template removed from MIP) was dissolved. The amount of the lyophilized gel was 0.05 g. The concentration of hGH in test solution was 0.129% (w/w). The liquid supernatant was separated from the gel after the test tube was centrifugated at 1000 rpm during 5 min. All experiments were reproduced three times.

Results and discussion

Synthesis of the imprinted polymers

The imprinted functionalized polyacrylamide hydrogels were prepared by free radical polymerization of acrylamide, 2-(dimethylamino)ethyl methacrylate and methacrylic acid (1:1:1 ratio) together with the crosslinker *N*,*N*'-methylenebisacrylamide in buffer solution in the presence of the selected molecular form of the hGH. An highly effective redox initiation system was used to allow polymerization at room temperature for only 30 min. The imprinted materials were obtained as gel particles of 100 to 150 μm in diameter.

The removal of the template

After the polymerization was completed, the template had to be removed by intensive washing. The volume change in the MIP hydrogel is very dependent on the ion strength of the solution [15]. To remove the template molecules from the MIP as effectively as possible, the MIP particles were rinsed therefore by changing the ion strength of the solution by chainging from deionized water to 1 M NaCl solution.
The removal of the template (dimeric or monomeric hGH) from the imprinted particles was confirmed by quantitative measurements of the UV absorbance at 280 nm of the NaCl solution used for washing the hGH from the particles. A part of template (approximately 10 % for the monomeric hGH and 9 % for dimeric hGH) could not be extracted from the gel and thus, was fixed to MIP (Table 1). As possible reasons for that one can assume that some free radicals attacked these hGH molecules leading to covalent binding to the gel, or some hGH molecules are entrapped so deeply in the cross-linked hydrogel that it could not be removed [10]. However, the amount of template which could not be extracted from the gel was significantly smaller than in the case of an imprinting experiment of lysozyme in the same polymer system [10]. This can be explained by the more favourable structure of the hGH globular units – the protein has a single tryptophan located in the hydrophobic interior of the molecule [1]. If the protein is not denaturated free radicals can not attack the tryptophan due to a shielding effect [10]. Lysozyme, however, has several non-shielded trytophan units specially prone to be attacted by radicals.

Table 1. Efficiency of the template removal from the prepared MIPs

Template	Template in MIP sample, mg	Template in washing solution, mg/ml	From MIP extracted template in mg	From MIP extracted template in %
hGH monomer	30 ± 0.01	0.0899 ± 0.002	26.97 ± 0.6	89.9 ± 2
hGH dimer	30 ± 0.01	0.0911 ± 0.002	27.33 ± 0.6	91.1 ± 2

Recognition of hGH templates

The amount of hGH adsorbed onto the **MIP-di** particles (A) was calculated by subtracting the quantity of dimeric hGH in the supernatant from that in the original solution (s. Table 2).The same experiment was carried out with hGH-monomer-imprinted gel (**MIP-mon**) particles using the monomeric hGH as analyte.

Table 2. hGH adsorbed onto the MIPs and the non-imprinted polymers

		Adsorbed amount of hGH (A) in % from original solution	
MIP	Protein	after 2 h	after 18 h
MIP-mon	monomeric hGH	51.9 ± 0.4	69.9 ± 0.4
MIP-di	dimeric hGH	50.4 ± 0.4	61.4 ± 0.4
NIP	monomeric hGH	0.9±0.1	0.9±0.1
NIP	dimeric hGH	0.9±0.1	0.9±0.1

A non-imprinted polymer was synthesized the same way as the MIP and after polymerisation was treated exactly the same way as the MIP. The non-specific binding of hGH to the non-imprinted polymer (**NIP**) particles was also measured and found to be only 0.9%. This low value can be explained with the hydrophilic character of the NIP. By subtracting the amount of hGH adsorbed onto the non-imprinted reference gel particles from that adsorbed onto the hGH-imprinted polymer particles, the amount of specifically bound hGH (B) was calculated and transferred to % from original concentration values (s. Table 3). It was found that after 2 h about 50 % of the hGH were absorbed specifically on the MIP, and this increased to even 60% (MIP-di) and 69% (MIP-mon), respectively, after 18 h.

Table 3. Specifically bound amount of hGH on the MIPs

		Specifically bound amount of hGH (B) in % from original solution	
MIP	Protein	after 2 h	after 18 h
MIP-mon	monomeric hGH	51.0 ± 0.4	69.0 ± 0.4
MIP-di	dimeric hGH	49.5 ± 0.4	60.5 ± 0.4

Selectivity of the imprinted polymers

To confirm the selectivity of the imprinted polymer, other molecular forms of hGH and other proteins such as bovine serum albumin (**BSA**), human serum albumin (**HSA**), or lysozyme (**LSZ**) was dissolved in the solutions, and the same experiments as those for the template hGH forms were carried out using the same concentration of the protein.

As we can see from Table 4, the molecular forms of hGH show some crossreactivity with relative MIPs:

MIP-di binds monomeric hGH approximately 2 (61,4\34,2) times less as dimeric hGH;

MIP-di binds trimeric hGH approximately 3 times less as dimeric hGH;

MIP-mon binds dimeric hGH c.a. 4 times less as monomeric hGH;

MIP-mon binds trimeric hGH c.a. 20 times less as monomeric hGH;

The other proteins, however, show a very low tendency to bind to the MIPs with values in the range of 0.4-1.5 %.

Two of the weakly adsorbing proteins - BSA and HSA - have approximately the same isoelectric point (pH 5.1 or 5.2) and molecular weight (ca. 66 kDa) as the hGH trimer, which adsorbed relatively well onto the MIP-di (20.1 %). This can be considered as a good proof for an effective imprinting mechanism and not just plain adsorption capacity of the hydrogels. For the crossreactivity of the dimeric hGH to MIP-mon

(15.5 ± 0.1 %) and the hGH trimer to MIP-di (20.1 ± 0.4 %) one can discuss the following reasons:
- there is in principle enough space for the larger molecular form of hGH in the hydrogel at the recognition place;
or
- due to some aggregation of the used hGH template molecules at the higher concentrations during the imprinting process some larger cavities are imprinted in the gels [1].

Table 4. Selectivity of the imprinted polymers MIP-mon and MIP-di in adsorption experiments using different analytes

Test protein	Adsorption onto MIP after 18 h in % from the original solution	
	MIP-mon	MIP-di
BSA	0.9 ± 0.1	1.0 ± 0.1
HSA	1.5 ± 0.1	1.3 ± 0.1
LSZ	0.5 ± 0.1	0.4 ± 0.1
monomeric hGH	69.9 ± 0.4	34.2 ± 0.3
dimeric hGH	15.5 ± 0.1	61.4 ± 0.4
trimeric hGH	3.1 ± 0.1	20.1 ± 0.4

Such molecularly imprinted polymers have possible future application in the fields of quantitative sorbent assays (analogue - immunoassays) or chromatography (analogue - affinity chromatography). The polymeric nature of molecularly imprinted polymers results in several advantages over natural antibodies. In particular, molecularly imprinted hydrogels can provide selective recognition of the different molecular forms of human growth hormone and, thus, solve problems related to the absence of the natural antibodies that can specifically recognize different molecular forms of this hormone [1]. Furthermore, the proposed imprinted polymers have general advantages as compared to immobilized antibodies like robustness, longer lifetime and like multiple use.

Conclusions

Two molecular forms of the of human growth hormone - the 22 kDa monomer and and the 44 kDa dimer - were molecularly imprinted into polyacrylamide hydrogels (MIP) with ionic functional groups. By this, hGH dimers were molecularly imprinted for the first time. More than 90% of the template could be removed by extraction from the MIPs allowing for a large number of binding sites.
Significant imprinting efficiency was detected using ultraviolet absorbance. The selectivity study shows excellent results for selective binding when the analyte and the template had been identical and a very low binding of other proteins. However, the different molecular forms of hGH show some crossreactivity within the imprinted hydrogels.

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