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Molecularly Imprinted Polymers for Selective Adsorption of Cholesterol from Aqueous Environment

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Abstract: In this work the preparation and evaluation of molecularly imprinted polymers (MIPs) based on 2-hydroxyethylmetacrylate (HEMA) for selective recovery of cholesterol from aqueous media are reported. HEMA was used as functional monomer in order to maximize the hydrogen bond forming both in pre-polymerization complex and in rebinding experiments which were performed in polar solvents; in particular, an acetonitrile:water (7:3 v/v) mixture was employed. The templating effect is clearly seen in the capacity of the synthesized polymers to bind cholesterol, and their selectivity was evaluated using two steroids quite similar to cholesterol such as progesterone and hydrocortisone which are less effectively bound by the matrices.

Introduction

Molecularly imprinting polymers are very useful technique to incorporate specific substrate recognition sites into polymers. Molecular recognition characteristics of these polymers are attributed to complementary size, shape, and binding sites imparted to the polymers by the template molecules (Caro et al. [1]).

The concept of molecular imprinting has a long history dating back to the early 1930s. It was not until 1972 when for the first time the preparation of organic polymers with molecular recognition was reported (Takagishi et al. [2]), and the molecular imprinting technology started, as we know it today.

The simplicity of creating tailored recognition sites in synthetic materials by molecular imprinting, as compared with that of complicated multi-step organic synthesis, is very attractive from an application point of view; and when compared with biomolecules, the main advantages of molecularly imprinted polymers (MIPs) are their relatively high stability over a wide range of conditions (temperature, pressure, organic solvents, acidic or basic solutes, etc.) and low cost (Vlatakis et al. [3]).

Thus, molecular imprinting has attracted a broad research interest in recent years, although certain limitations with molecularly imprinted polymers (MIPs) still need to be addressed, such as slow binding kinetics, aqueous compatibility, and heterogeneity of binding site distribution (Karlsson et al. [4]).

Molecular imprinting has now become an established method and has been applied in the area of synthetic chemistry, analytical chemistry; MIP were used as chromatographic stationary phases (Sellergren et al. [5]) for enantiomeric separations (Mosbach et al. [6]) and for Solid-Phase Extraction (Andersson [7]), catalysis

(Bystrom et al. [8]) and sensor design (Hedborg et al. [9]), as well as protein separation (Mallik et al. [10]), receptor (Haupt [11]), antibody (Svitel et al. [12]) and enzyme mimics (Nicholls et al. [13]); as affinity and sensing materials (Puoci et al. [14]) and as drug delivery systems (DDS) (Puoci et al. [15]).

The technique involves preorganization of functional monomers around a template molecule by either covalent, non-covalent or coordination interactions (Cormack et al. [16]). Polymerization of the supramolecular assembly in the presence of an excess of cross-linker and subsequent removal of the template lead to polymers (MIPs) that retain the specific orientation of functional groups within the cavity created and that are capable of selectively rebinding the template (Ye et al. [17]) (Figure 1).

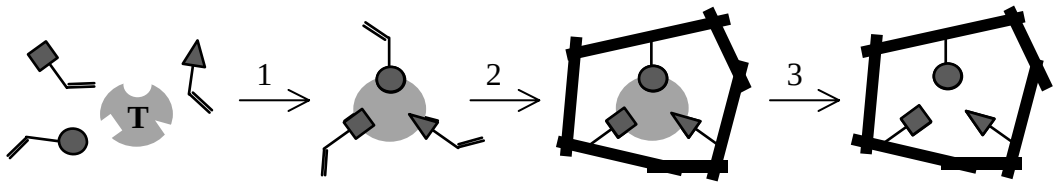


Fig. 1. Schematic representation of the molecular imprinting process. (1 = Assembly; 2 = Polymerization; 3 = Template Extraction).

Different kinds of template for the synthesis of MIPs were used, in particular, several studies report on the preparation of MIPs for selective recognition of cholesterol (Wang et al. [18]). These materials are very useful because the deleterious effect of cholesterol on human health is well documented (Huval et al. [19]). There are many evidences that hypercholesterolemia is the major risk factor for the early development of atherosclerosis in man and thus the leading cause of coronary heart and peripheral atherosclerotic disease (Holtmeier [20). In several studies it is well-established that drastic lowering of blood cholesterol concentration is followed by a reduction of clinical events and total mortality (Davidson et al. [21]).

Cholesterol homeostasis is regulated by the amount of cholesterol absorbed from the diet, by hepatic cholesterol synthesis and metabolism, and by hepatic sterol excretion (Sellergren et al. [22]). For these reason, in order to obtain a reduction of blood cholesterol concentration, different approaches interfering with both absorption and excretion of cholesterol, may be used. Several attempts are being made to develop cholesterol selective adsorbents that are biocompatible and clinically efficient and many studies have focused on receptors for cholesterol in order to extract the sterol from the food source (Asanuma et al. [23]). Molecularly imprinted polymers have been investigated for this purpose with many advantages (Kugimiya et al. [24]). Being non-absorbed, these polymeric drugs indeed do not exhibit systemic side effects that are associated with other well-known cholesterol lowering agents such as HMG-CoA reductase inhibitors (e.g., statins) (Grundy [25]).

Different approaches to imprint cholesterol have been described. Some researchers (Whitcombe et al. [26]), based on covalent imprinting, conjugated cholesterol with vinyl phenol through a readily hydrolysable carbonate ester linkage. After polymerization and removal of cholesterol by hydrolysis, rebinding was effected by hydrogen bonding between the hydroxyl group of cholesterol and the phenolic group on the polymer. The rebinding of cholesterol was evaluated in hexane and showed a fairly homogeneous population of binding sites. Another work (Sellergren et al. [22]) reports on the synthesis of polymerizable derivatives of cholesterol to be used as

amphiphilic monomers in the imprinting of highly cross-linked methacrylates with cholesterol. The polymers were prepared under conditions favouring apolar intermolecular interactions and cholesterol rebinding from intestinal mimicking fluids was evaluated.

MIPs for the cholesterol recognition based on non-covalent approach were also prepared. In some cases (Asanuma et al. [27]), these materials are cross-linked β-cyclodextrins. These ones were capable of rebinding cholesterol also from aqueous media. One of the limitations of these polymers is their low selectivity resulting from non-specific binding of cholesterol on to the hydrophobic cross-linking monomer used in polymerization. Furthermore, several works (Spizzirri et al. [28]) report on the synthesis of MIPs for cholesterol absorption using methacrylic acid as fuctional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent. Thus made materials were generally used as stationary phases for HPLC analysis.

Finally, other researchers (Sreenivasan [29]) report on the preparation of cholesterol imprinted polymers using HEMA as functional monomer. This one is generally used as co-monomer just to increase the hydrophilic properties of various MIPs (Dirion et al. [30], Yu et al. [31]), but its alcoholic groups could also be able to form hydrogen bond with the hydroxylic group of cholesterol.

In the work of Sreenivasan et.al. the imprinted polymers were tested in organic solvents and showed good recognition properties towards cholesterol. Thus, if HEMA was used to imprint CHO, the recognition and selectivity properties in aqueous media and the optimal ratio crosslinker-functional monomer were not yet studied.

The aim of this work is to evaluate the possibility of employing molecularly imprinted polymers based on HEMA in aqueous environment.

Using HEMA instead of Methacrylic acid it is possible to obtain Polymeric device that is not easily ionisable. MAA, indeed has a pKa = 4.66, that could influence negatively the recognition properties of polymeric matrices at physiological value of pH.

Results and Discussion

General considerations

We choose the non-covalent imprinting method because the limited stock of functional groups for which covalent bond formation and cleavage are readily reversible under mild conditions, has proved to be a serious obstacle to the covalent approach (Sellergren [32]), while, because of the easy access to a broad range of functional monomers from commercial sources, the non-covalent imprinting method has been used by most research groups, which resulted in a large number of non-covalent MIPs displaying favourable molecular recognition properties (Mosbach et al. [33]).
Our bulk MIPs for cholesterol entrapping was prepared using 2-hydroxyethylmetacrylate as functional monomer. In literature, many different ratios of template to functional monomer were used based on the particular structure of the template (Kempe et al. [34]). Thus, in order to find the optimum conditions for our particular template, we synthesized polymers from MIP1 to MIP3 and the correspondent Non Imprinted Polymers (NIPs) at various molar ratios (Table 1). All the polymers were prepared in chloroform, the least polar solvent in which the reagents dissolve, in order to maximize the interactions between the template and the functional monomer (Whitcombe et al. [26]).

Tab. 1. Polymer composition of the imprinted and non imprinted polymers synthesized.

Polymers	CHO (g)	HEMA (g)	EGDMA (g)	CHO:HEMA:EGDMA	$CHCl_3$ (ml)	AIBN (g)
MIP1	0,271	0,730	3,47	1 : 8 : 25	5,25	0,035
NIP1	-					
MIP2	0,271	1,094	3,47	1 : 12 : 25	5,25	0,035
NIP2	-					
MIP3	0,271	1,460	3,47	1 : 16 : 25	5,25	0,035
NIP3	-					

In order to obtain matrices with more accessible cavities, some series of polymers with increasing amount of porogen were prepared, but they showed no good results (data not shown). For these materials, the amount of the bound cholesterol, indeed, was found to be higher, but no imprinting effect was found.

To ensure strong, selective binding of the substrate, it is important that the template molecule preorganizes the functional monomer in a stable configuration prior to polymerization. Since this preorganization takes place in solution, it is necessarily a dynamic process. One way to increase the strength of the template-functional monomer interactions is to decrease the kinetic energy of the system. Some researches (Cheong et al. [35]) examined the effect of the polymerization temperature on the performance of the polymers: higher temperature is expected to drive the equilibrium away from the template-functional monomer complex toward the unassociated species, resulting in a decrease in the number of imprinted cavities. The same researches found a lesser degree of polymerization occurring under UV irradiation than at 40°C. Furthermore, the performance of photopolymerized materials was improved after high-temperature treatment of the initially formed polymer. It may be that there is a relationship between the extent of polymerization and stabilization of the template-functional monomer complex.

Based on these considerations, noncovalent MIPs were synthesized under UV irradiation at 4 °C for 24 h and then with thermal stabilization at 60 °C for others 24 h. Anyway we synthesized MIPs by thermal polymerization and we verified the inefficacy of these materials (data not shown).

Binding experiments

The binding experiments are performed in an acetonitrile/water mixture (7:3 v/v). The data refer to the results obtained after 6 h incubation of the polymers with a cholesterol solution 0.2 mM.
For each polymer the binding percentage and the binding efficiency α_{CHO} were calculated.
α_{CHO} was calculated according the following equation (Equation 1):

$$\alpha_{CHO} = \frac{\%CHO_M}{\%CHO_N} \quad (1)$$

where $\%CHO_M$ e $\%CHO_N$ represent the percentage of bound CHO by MIPs and NIPs respectively.

As can be noted in Table 2 and in Figure 2, the imprinting efficiency is generally equal to two, showing that the synthesized MIPs have a good affinity towards the template, therefore, an increase in the amount of the functional monomers results in a reduction of the binding capacity of the polymers, maybe because a great amount of HEMA is unable to form stable prepolymerization complexes with cholesterol and, subsequently, selective cavities for it.

Tab. 2. Adsorption % of CHO by imprinted and non-imprinted polymers after 6 h.

Polymers	% CHO bound	α_{CHO}
MIP1	26	1.86
NIP1	14	
MIP2	28	2.00
NIP2	14	
MIP3	21	1.91
NIP3	11	

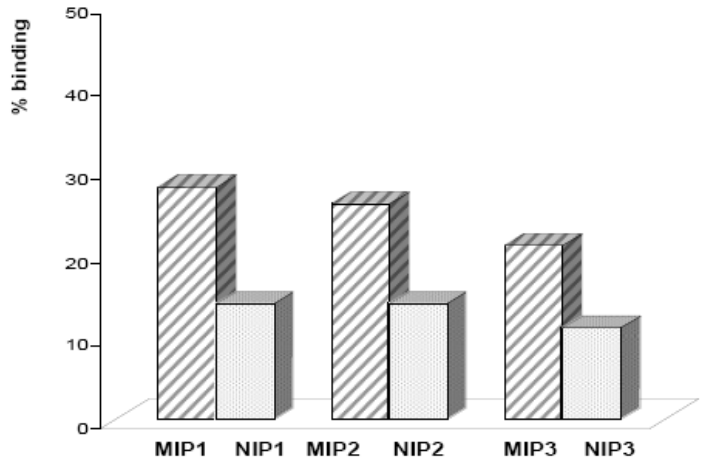
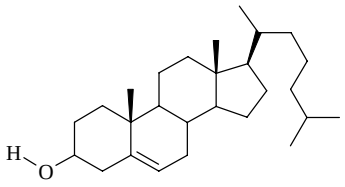


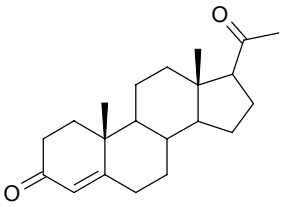
Fig. 2. Bound CHO (%) by imprinted and non imprinted polymers.

Selectivity of the polymers

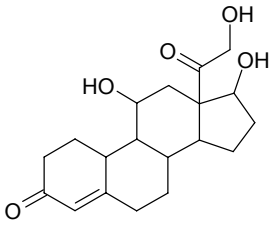
In order to evaluate the imprinting effect, the binding selectivity of polymers was tested by performing the same experiments using two molecules quite similar to cholesterol. For this purpose, we used progesterone (PROG) and hydrocortisone (HY) that differ from cholesterol for some substituents on the steroidal ring. (See Scheme 1). The chemical differences between these steroids may drive their interaction with the polymeric devices. The hydroxylic groups in position 11, 17 of both steroidal ring and alkylic chain of hydrocortisone make this molecule most hydrophilic than progesterone. So it should interact with the most hydrophilic matrix.



CHO



PROG



HY

Scheme 1. Structure of CHO, PROG and HY.

The experiments were performed in the same condition used for cholesterol. For each polymer α_{PROG}, α_{HY}, and ε_{BM} (Equation 2) were calculated. α_{PROG}, α_{HY} were calculated as reported for α_{CHO}; ε_{BM} represents the ratio between the percentage of bound cholesterol by each MIPs and the percentage of bound analogue by the same polymers.

$$\varepsilon_{BM} = \frac{\%\ CHO}{\%\ PROG\ or\ \%HY} \quad (2)$$

To evaluate the specific component of the interaction, in the same manner, (ε_{BN}) was calculated for the non-imprinted polymers. As it is possible to infer from the experimental data (Table 3 and Figure 3), these polymers have a good selectivity for the template molecule.

Tab. 3. Adsorption % of PROG and HY by the imprinted and non-imprinted polymers after 6 h.

Polymers	% PROG bound	% HY bound	α_{PROG}	ε_{BM}	ε_{BN}	α_{HY}	ε_{BM}	ε_{BN}
MIP1	15	2.3	1.60	1.7	1.6	2.85	11.3	17.5
NIP1	9	0.8						
MIP2	4.8	5	0.96	4.4	2.2	2.27	5.2	6.4
NIP2	5.1	2.2						
MIP3	3	11	0.81	8.7	3.8	2.90	1.9	2.9
NIP3	3.7	3.8						

The different affinity of the synthesized polymers for the two analogues is due to the hydrophilic degree of the materials. In particular, MIP1, the most hydrophobic polymer, has more affinity for the most lipophilic analogue (PROG), while MIP3, the most hydrophilic one, has more affinity for the less lipophilic analogue (HY).

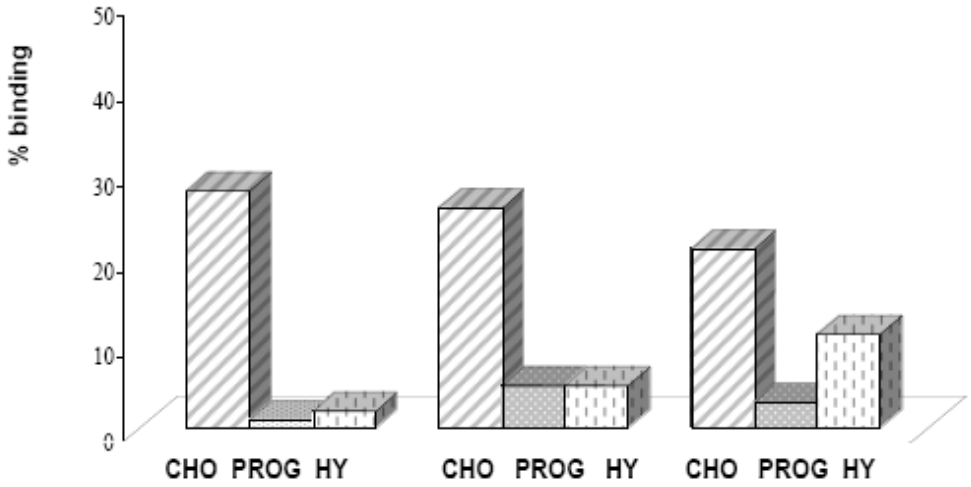


Fig. 3. CHO, PROG, HY (%) bound by imprinted polymers.

Experimental Part

Materials

Ethylene glycol dimethacrylate (EGDMA), 2-hydroxyethylmetacrylate (HEMA), 2,2'-azoisobutyronitrile (AIBN), cholesterol, progesterone and hydrocortisone were obtained from Aldrich. All solvents were reagent grade or HPLC-grade and used without further purification and they were provided by Fluka Chemie.

Synthesis of cholesterol imprinted polymers

The MIP stationary phase was prepared by bulk polymerization. HEMA was used as functional monomer to prepare the MIP by the non-covalent imprinting method. Briefly, template cholesterol, functional monomer, EGDMA and AIBN were dissolved in 5,25 ml of the choloform in a thick-walled glass tube. The tube was spurged with nitrogen, sonicated for 10 min, and then photolyzed for 24h with 360 nm light at 4 °C. After the photolysis, the tubes were incubated at 60 °C for 24 h (Schmidt et al.[32]). The resultant bulk rigid polymers were crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIPs materials were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant MIPs materials were soxhlet extracted with 200 ml of an acetic acid:tetrahydrofuran (1:1) mixture for at least 48 h, followed by 200 ml of tetrahydrofuran for another 48 h. The extracted MIPs materials were dried in an oven at 60 °C overnight. The washed MIPs materials were checked to be free of cholesterol and any other compound by HPLC analysis. Blank polymers (to act as a control) were prepared under the same conditions without using the template. The molecular ratios of the different polymers that were prepared are showed in Table 1.

Binding experiments

The binding experiments were performed in an acetonitrile:water mixture (7:3 v/v). The polymer particles (20 mg) were mixed with 1 ml cholesterol solution (0.2 mM) in a 1 ml eppendorf® and sealed. The eppendorf were oscillated by a wrist action shaker (Burrell Scientific) in a water bath for 24 h. Then the mixture was centrifuged for 10 min (10000 rpm) in an ALC® microcentrifugette® 4214 and the cholesterol concentration in the liquid phase was measured by HPLC. The amount of cholesterol bound to the polymer was obtained by comparing its concentration in the polymer samples to the reference samples. The same experiments were performed using progesterone and hydrocortisone solutions.

HPLC Analysis

The liquid chromatography consisted of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 208 nm for CHO; at 268 nm for PROG and HY. A 25 x 0.4 mm C4 Kromasil column, particle size 5 µm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was acetonitrile for CHO; acetonitrile/water mixture (7/3 v/v) for PROG and HY. The flow rate was 1.0 ml/min.

Acknowledgments

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