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Molecularly Imprinted Polymers Based On Amidic Functional Monomers For Selective Recognition Of Cholesterol In Aqueous Media

Corresponding Author

F.Puoci
Dipartimento di Scienze Farmaceutiche, Università della Calabria -Edificio Polifunzionale-Arcavacata di Rende (CS) 87036, (ITALIA)
Fax: 0039 0984493151
E-mail: francesco.puoci@unical.it

Co-Authors

F.Iemma, G.Cirillo, R.Muzzalupo, U.G.Spizzirri, N.Picci
Dipartimento di Scienze Farmaceutiche,
Università della Calabria -Edificio Polifunzionale-
Arcavacata di Rende (CS) 87036, (ITALIA)

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ABSTRACT

The aim of this work was to investigate the possibility of employing amidic functional monomers for the preparation of Molecularly Imprinted Polymers (MIPs) able to bind selectively cholesterol in aqueous media. For this purpose, acrylamide and N,N-dimethylacrylamide were employed in order to maximize the hydrogen bound 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 so obtained matrices showed a good binding capacity towards the template molecule, they bound, indeed, much more cholesterol than the corresponding non-imprinted ones. Finally, the polymers affinity for cholesterol and its selectivity using two steroids quite similar to cholesterol such as progesterone and hydrocortisone. The synthesized materials, showed a good selectivity, because they recognized less effectively the two analogues.

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INTRODUCTION

Molecular imprinting is an efficient technique to create synthetic polymers with high recognition properties[1,2]. The first example of a molecularly imprinted polymer (MIP) was reported half a century ago, however it is only in the last decade that the use of molecular imprinting as a practical tool has become established[3]. In addition to their application as chromatographic stationary phases for enantiomeric separations[4] and for Solid-Phase Extraction (SPE)[5], imprinted polymers have also been used as receptor[6], antibody[7] and enzyme mimics[8]; as affinity and sensing materials and as drug delivery systems (DDS)[9-11]. Polymers could also be imprinted against some special compounds for which there exists no naturally occurring receptor and against which it is difficult or impossible to raise antibodies.

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[12].

The technique to produce MIPs, using the non-covalent approach, involves arranging functional monomers around a templating ligand. This ligand is the selected target substance and it should form a prepolymerization complex with the functional monomer by non-covalent interactions[13]. The formed complex is subsequently radically copolymerized in a solution containing a high ratio of a suitable crosslinker. After copolymerization, the template is removed and the resulting molecularly imprinted polymers (MIPs) are macroporous matrices possessing microcavities with a three-dimensional structure complementary in shape and chemical functionality to that of the template about which they were formed(Figure 1).

Generally, ionic interactions are normally involved if the imprinted polymers were prepared in relatively non-polar solvents (such as chloroform or toluene), and the resulting polymers display good recognition properties. Many of the polymers prepared in relatively polar organic solvents (such as acetonitrile), instead, display poor recognition properties, because the monomer commonly used is methacrylic acid and the hydrogen-bonding ability of its free carboxyl group is not very strong in a polar organic solvent[14].

In some cases, acrylamide and its derivatives was used as an alternative to the "traditional" functional monomer, and the corresponding "amidic MIPs" showed much improved recognition properties, indicating that the amide group of acrylamide is a stronger hydrogen-bonding functional group. It is also important to note that by using acrylamide instead of methacrylic acid, polymers could be made without the existence of charged groups and thus the non-specific, ionic interactions could be reduced[14].

Based on these consideration, the aim of our work is to synthesize a molecularly imprinted polymers able to bind selectively cholesterol using amidic functional monomers.

The preparation of these kind of materials is very

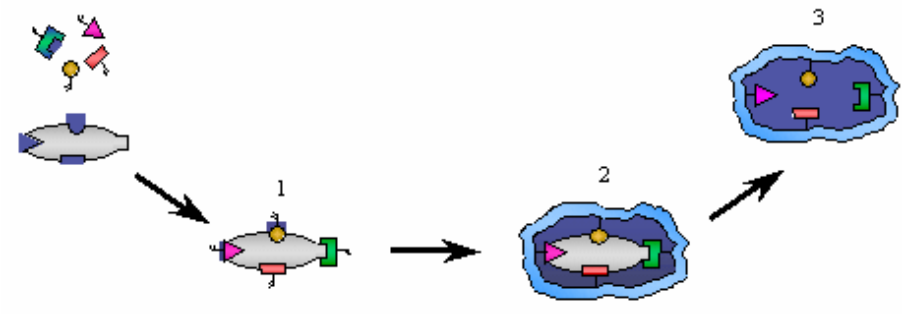

Figure 1: Schematic representation of the molecular imprinting process. (1=Assembly; 2 = Polymerisation; 3 = Template Extraction)

useful because, although cholesterol is an important component of every biological membrane and contributes to their physical stability, it shows severe toxic effects[15]. An excess of cholesterol, indeed, is the major risk factor for health in humans because it is involved in the atherosclerosis development and in heart degenerative processes[16]. For these reasons, many studies report on the synthesis of MIPs for cholesterol entrapping. These polymeric devices were generally prepared using methacrylic acid as functional monomer, ethylene glycol dimethacrylate (EGDMA) as crosslinker and were used as stationary phases for chromatographic application[17].

In our work, amidic functional monomers (acrylamide and N,N-Dimethylacrylamide) were used. As before explained, these monomers were employed to maximize the hydrogen bound forming in rebinding experiments which were performed in polar solvents; in particular, an acetonitrile:water (7:3 v/v) mixture was employed.

Finally, the polymers affinity for cholesterol and its selectivity using progesterone and hydrocortisone as analogues were tested.

EXPERIMENTAL

Materials

Ethylene glycol dimethacrylate (EGDMA), acrylamide (AAm), N,N-dimethylacrylamide (DMAA), 2,2'-azoisobutyronitrile (AIBN), chole sterol, 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 polymer

The MIP stationary phase was prepared by bulk polymerization. Acryilamide (AAm) or N,N-dimethylacrylamide (DMAA) were used as functional monomers to prepare the MIP by the non-covalent imprinting method. Briefly, template cholesterol, functional monomers, EGDMA and AIBN were dissolved in 5, 25 ml of the selected solvent in a thick-walled glass tube. The tube was sparged 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 24h[18]. 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:tetrahy drofuran (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 prepared polymers are showed in TABLES 1 and 2.

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.

TABLE 1: DMAA polymers composition

Polymers	CHO (g)	DMAA (g)	EGDMA (g)	CHO: DMAA: EGDMA	$CHCl_3$ (ml)	AIBN (g)
MIP1	0,271	0,557	3,47	1 : 8 : 25	5,25	0,035
NIP1	-					
MIP2	0,271	0,836	3,47	1 : 12 : 25	5,25	0,035
NIP2	-					
MIP3	0,271	1,114	3,47	1 : 16 : 25	5,25	0,035
NIP3	-					

TABLE 2: Aam polymers composition

Polymers	CHO (g)	AAm (g)	EGDMA (g)	CHO: AAm: EGDMA	DMF (ml)	AIBN (g)
MIP4	0,271	0,400	3,47	1 : 8 : 25	5,25	0,035
NIP4	-					
MIP5	0,271	0,600	3,47	1 : 12 : 25	5,25	0,035
NIP5	-					
MIP6	0,271	0,800	3,47	1 : 16 : 25	5,25	0,035
NIP6	-					

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[19]. A 25 x 0.4 mm C4 Kromasil column, particle size 5 μm (Teknocroma, Barcellona, Spain) was employed. The mobile phase was acetonitrile and the flow rate was 1.0 ml/min.

RESULTS AND DISCUSSION

General consideration

Bulk molecularly imprinted polymers for cholesterol entrapping were prepared using acrylamide and N,N-dimethylacrylamide as functional monomers. We choose the non-covalent imprinting method, pioneered and extensively developed by Mosbach and co-workers[19], for the bulk of this work, as is it the more convenient and bases the binding of our templates on H-bonding, which is a dominant interaction in biological systems.

The first parameter we investigated was that of the ratio of template to functional monomer. This parameter was widely studied, in particular for MIPs based on methacrylic acid. According to the work of Mosbach and Sellergren[20], each template requires 3 or 4 equiv. of functional monomer in order to produce a sufficiently selective polymer. Anyway, many different molecules having different functional groups[21] have been used as templates, with different ratios of functional monomer to template. Thus, in order to find the optimum conditions for our particular template, we synthesized polymers from MIP1 to MIP6 and the correspondent NIP (TABLE 1) containing 8, 12, and 16 mmol of functional monomers and 25 mmol of crosslinker.

The inert solvent used in the polymerization mixture may play a major role in determining the properties (surface area, internal pore volume, etc.) of the resulting polymer. Moreover, since polar solvents are more able to solvate polar molecules, this leads to the disruption of H-bonds between, in this case, the template and the functional monomer[22].

The general procedure is to choose the least polar solvent in which the reagents dissolve, in order to maximize the interactions between the template and the functional monomer(s). N,N-Dimethylacrylamide containing polymers were synthesized in $CHCl_3$, while acrylamide ones in dimethylformamide because of the poor solubility of acrylamide - cholesterol complex in $CHCl_3$.

In order to obtain matrices with more accessible cavities, some series of polymers using two or three 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 this effect is due to the aspecific interaction, not to the specific ones: no imprinting effect was relieved.

To ensure strong, selective binding of the substrate, it is important that the template molecule preorganize the functional monomer(s) 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[23] examined the effect of the polymerization temperature on the performance of the polymers. The 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[23] 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 tradeoff between the extent of polymerization and stabilization of the template-functional monomer complex.

Based on these consideration, MIPs were synthesized under UV irradiation at 4°C for 24 h and then with thermal stabilization at 60°C for others 24 h.

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 polymers the binding percentage and the binding efficiency α_{CHO} were calculated.

α_{CHO} was calculated according the following equation:

$$\alpha_{CHO} = \frac{\%CHO_M}{\%CHO_N}$$

where:

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

N,N-Dimethylacrylamide imprinted polymers

As it is possible to note in TABLE 3 and in figure 2, these polymers have a good binding capacity for cholesterol, and the increase of the amount of the functional monomer corresponds to a reduction of the capacity of the polymers to bind the cholesterol. MIPs3, indeed, shows a very low binding capacity, because a great amount of N,N-dimethyacrilamide is unable to form stable pre-polymerization complex with the template and, consequently, the final material does not have stable and selective cavities for cholesterol.

TABLE 3: Adsorption % of CHO by the DMAA imprinted and non-imprinted polymers after 6 hours

Polymers	% CHO Bound	α_{CHO}
MIP1	26	1.63
NIP1	16	
MIP2	24	1.85
NIP2	13	
MIP3	16	1.46
NIP3	11	

TABLE 4: Adsorption % of CHO by the AAm imprinted and non-imprinted polymers after 6 hours

Polymers	% CHO Bound	α_{CHO}
MIP4	15	3.00
NIP4	5	
MIP5	14	3.50
NIP5	4	
MIP6	11	5.50
NIP6	2	

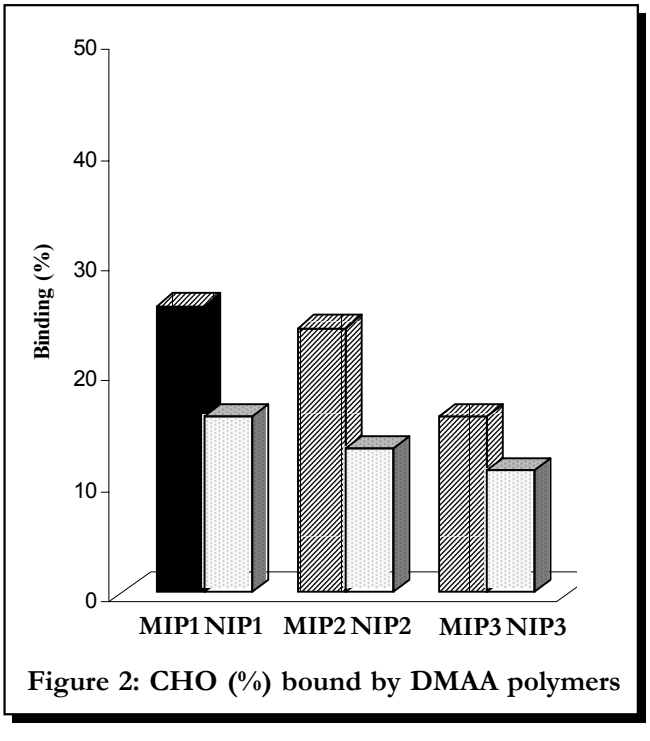

Figure 2: CHO (%) bound by DMAA polymers

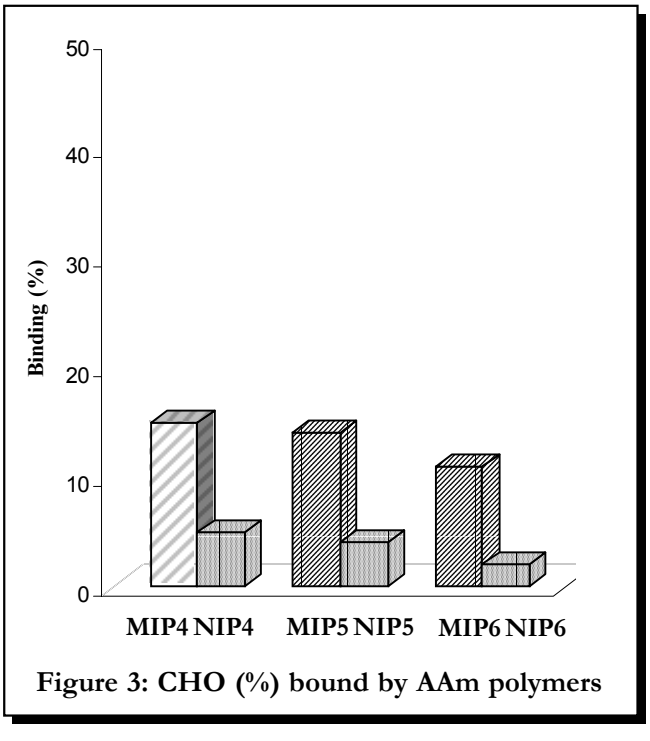

Figure 3: CHO (%) bound by AAm polymers

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Acrylamide imprinted polymers

These materials show a very high value of α_{CHO} (TABLE 4), according to the theory that amidic functional monomers are useful in the preparation of molecularly imprinted polymers. Nevertheless, the needed to use a polar solvent as DMF carries out to an interference in hydrogen bond forming and, consequently, the binding percentages are low. In this case, it possible to note a reduction of these percentage with the increase of the functional monomer for the same reasons explained in DMAA polymers. (Figure 3)

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 substituent on the steroidal ring. (Figure 4).

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.

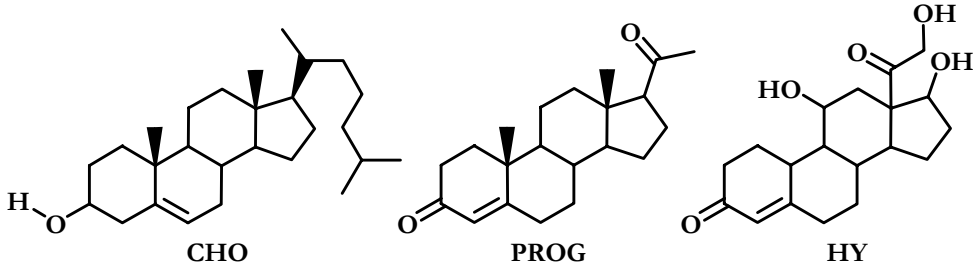
The experiments were performed in the same condition used for cholesterol. For each polymer α_{PROG}, α_{HY}, and ε_{BM} were calculated. α_{PROG}, α_{HY} were calculated as reported for α_{CHO}; α_{BM} represents the ratio between the percentage of bound cholesterol by each MIPs and the percentage of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

$$\varepsilon_{BM} = \frac{\%CHO}{\%PROG/HY}$$

In order to evaluate the aspecific component of the interaction, in the same manner, (ε_{BN}) was calculated for the non-imprinted polymers.

Selectivity of N,N-dimethylacrylamide imprinted polymers

These polymers show a good selectivity for the template molecule (TABLE 5). In this case, an increase of the functional monomers carries out to an increase of the binding percentage of the most hydrophobic analogiue (progesterone), may be



CHO PROG HY

Figure 4: CHO, PROG, HY

TABLE 5: Adsorption % of PROG and HY by the DMAA imprinted and non-imprinted polymers after 6 hours.

Polymers	% PROG bound	% HY bound	α_{PROG}	ε_{BM}	ε_{BN}	α_{HY}	ε_{BM}	ε_{BN}
MIP1	3	8	0.38	8.7	2	1.33	3.3	2.7
NIP1	8	6						
MIP2	6	7	2.00	4.0	4.33	1.40	3.4	2.6
NIP2	3	5						
MIP3	10	5	1.43	1.6	1.6	1.00	3.2	2.2
NIP3	7	5						

TABLE 6: Adsorption % of PROG and HY by the AAm imprinted and non-imprinted polymers after 6 hours.

Polymers	% PROG bound	% HY bound	α_{PROG}	ε_{BM}	ε_{BN}	α_{HY}	ε_{BM}	ε_{BN}
MIP4	8	1	2.67	1.9	1.7	0.33	15.0	1.6
NIP4	3	3						
MIP5	8	3	4.00	1.8	2.0	0.6	4.7	0.8
NIP5	2	5						
MIP6	9	10	9	1.2	2.0	1.7	1.1	0.3
NIP6	1	6						

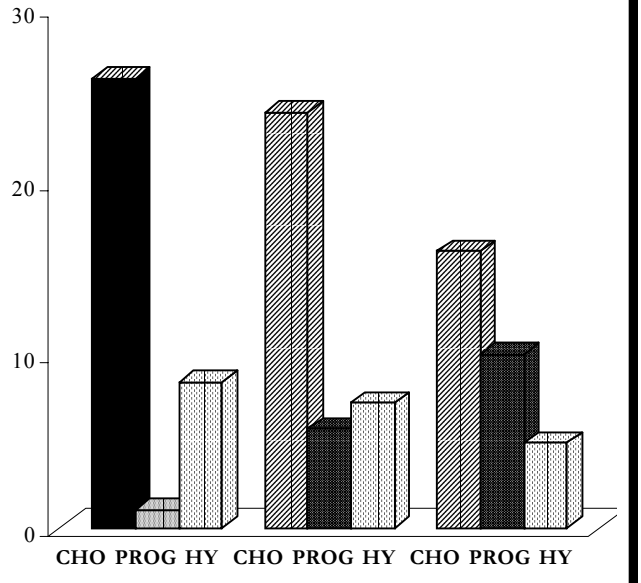


Figure 5:CHO, PROG, HY (%) bound by DMAA MIPs

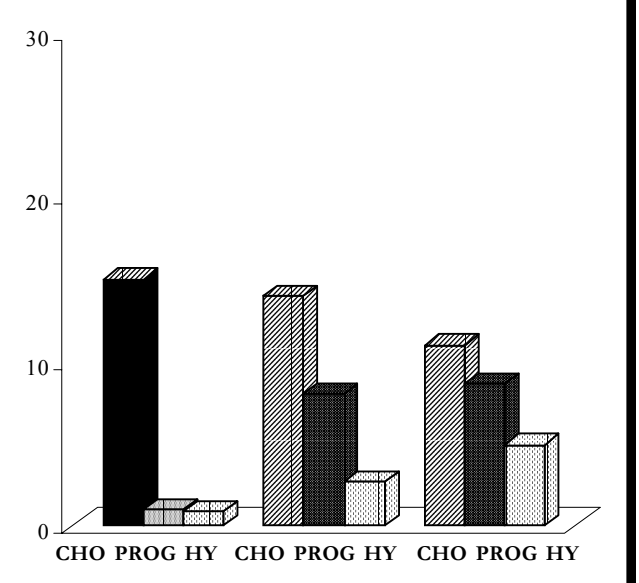


Figure 6: CHO, PROG, HY (%) bound by AAm MIPs

because the methylic groups of N,N-dimethya crylamide is responsible of the formation of hydro phobic matrices. Thus, progesterone interacts more strongly with MIP3, the polymer with the higher amount of functional monomer(Figure 5).

Selectivity of acrylamide imprinted polymers

The polymers based on acrylamide show a lower selectivity for the template compared to DMAA polymers (TABLE 6). All the MIPs of these series have a very low affinity for progesterone, while the affinity on the NIPs gradually decrease with the increase of the amount of the functional monomer. This behaviour should be due to the hydrophilic properties of the polymeric matrices which depend on the amount of acrylamide.

Hydrocortisone is the most hydrophilic analogue, so it shows higher affinity for the most hydrophilic polymers (MIP6) (Figure 6).

Finally, it is possible to note that NIP5 and NIP6 bind hydrocortisone more strongly than cholesterol, so ε_{BN} is < 1.

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