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Enhanced capacities and selectivities for cholesterol in aqueous media by molecular imprinting: role of novel cross-linkers

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Abstract

Molecularly imprinted polymers are being increasingly investigated as selective sorbents. For the recovery of cholesterol from aqueous media, the utility of the molecularly imprinted polymers has been limited by modest capacities and selectivities, especially when compared with alternative adsorbents reported for the binding of bile acids [Macromolecules 34 (2001) 1548]. This paper describes the use of cholesterol conjugated monomers and cross-linkers, which bind to the template cholesterol molecule by hydrophobic interactions. This leads to enhanced capacities and selectivities during the recovery of cholesterol from aqueous media. The templating effect is clearly seen in the enhanced capacity and selectivity in the retention of cholesterol vis-a-vis stigmasterol and testosterone.

Keywords: Molecular imprinting; Cross-linkers; Cholesterol

1. Introduction

The deleterious effect of cholesterol on human health is well documented [1–3]. Therefore attempts are being made to develop cholesterol or bile salt selective adsorbents that are biocompatible and clinically efficient [1,2].

Molecularly imprinted polymers are being extensively investigated as selective adsorbents for cholesterol [4–19]. The technique involves preorganization of functional monomers around a template molecule, which resembles shape and size of the guest molecule, by either covalent, non-covalent or coordination interactions. Polymerization of the supramolecular assembly in the presence of an excess of cross-linker and subsequent removal of the template leads to polymers that retain the specific orientation of functional groups within the cavity created by the elution of the template molecule [20–23]. Approaches for the recovery of bile acids using molecularly imprinted polymers have also been reported [1]. However, the molecular interactions involved in the rebinding process are different than those involved in the case of cholesterol in view of the different functional groups involved in rebinding.

Approaches to devise cholesterol selective polymeric adsorbents using molecular imprinting methodology have been summarized in Table 1. Broadly the researchers have exploited hydrogen bonding [6] for rebinding from non-aqueous media, and hydrophobic binding as well as inclusion complexes with cyclodextrin for rebinding from aqueous media [4]. Whitcombe et al. [6] 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. Sellergren et al. [14] synthesized polymerizable derivatives of cholesterol and bile acids to be used as amphiphilic monomers in the imprinting of highly cross-linked methacrylates with cholesterol. The polymers were prepared under conditions favoring apolar intermolecular interactions and cholesterol rebinding from intestinal mimicking fluids was evaluated. The capacity of molecularly imprinted polymer for cholesterol was 17 mg/g as against 13 mg/g exhibited by the non-imprinted polymer. Hwang and Lee [18] adapted a similar approach wherein cholesteryl (4-vinyl) phenyl carbonate was used for covalent imprinting and 4-vinyl pyridine for non-covalent imprinting of cholesterol. As anticipated, covalent imprinting resulted in more selective adsorption of

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Table 1
Cholesterol rebinding by MIPs: prior efforts

Polymer	Functional monomer template	Binding capacity (mg/g)[a]	Rebinding medium	Imprinting efficiency	Rebinding mechanism	Remark (reference)
CD cross-linked with TDI and EPH	CD, Cho	70 (34)	H_20/THF	2.0	Hydrophobic binding to CD cavity	Can be used for aqueous medium [4,16]
4-Vinyl carbonate ester and EGDMA	4-Vinyl carbonate ester, Cho	44	Hexane	–	Hydrogen bonding	Cannot be used for aqueous medium [5]
CD and TDI	CD, Cho	0.15 (<0.01)	H_20/THF	>15	Hydrophobic binding by CD	Can be use for aqueous medium. Selectivity was not studied [23]
Methacryloyl cholesterol and bile acid derivatives and EGDMA	Methacryloyl cholesterol, Cho	17 (13)	Intestinal mimicking solution	1.3	Hydrophobic binding and Hydrogen bonding	Capacity was lower and selectivity was not studied [13]
Allylamine and EPH	Allylamine, Sodium cholate	Not mentioned	BES buffer in sodium cholate	–	Ionic interaction	Sodium cholate was removed not cholesterol [1]
HEMA and EGDMA	HEMA, Cho	3.9 (0.16)	Dichloromethane	24.37	Hydrogen bonding	Very poor capacity but good selectivity [10]
HEMA and NVP	HEMA, Cho	4.86 (0.16)	Methanol	30.37	Cavity interaction/Hydrogen bonding	Very poor capacity but good selectivity [7]
CD-HEMA and HEMA	CD-HEMA, Cho	46.8 (17.6)	Methanol	2.6	Hydrophobic binding	Capacity and selectivity were good [9]
2-(Methacryloxy) ethyl and EGDMA	2-(Methacryloxy) ethyl, Cho	Capacity factor, 15.8 (6.4)	Chloroform and acetic acid for column, Hexane eluent	2.4	Hydrogen bonding	Cannot be used for aqueous medium [16]
Pyridinium (12-4(vinybenzyloxycar nonyl)), dodecanesulfate and pyridinium 12-(cholesteryl-oxycarbonyl) dodecane sulfate and DVB	Pyridinium (12-4(vinybenzyloxycar nonyl)) and dodecanesulfate and pridinium 12-(cholesteryl-oxycarbonyl) dodecane sulfate, Cho	–	2-Propanol: H_20 and isohexane	–	Hydrophobic binding	Can be used for aqueous medium [13]
Cu(II) acrylate monomer and EGDMA	Cu(II) acrylate, Cho	1.22 (1.02)	Dichloromethane	1.1	Metal ion interaction	Very low capacity and selectivity [11]
Cholesteryl acrylate and acryloyl-6-amino-6-deoxy-β or γ-CD and DAPA	Cholesteryl acrylate and acryloyl-6-amino-6-deoxy-β or γ-CD, Cho	19.3 (3.4)	2-Propanol	5.6	Hydrophobic interaction	Low capacity but high selectivity [18]
Acrylic acid and EGDMA	Acrylic acid, Cho	2.9 (2.2)	Dichloromethane	1.3	Hydrogen bonding	Cannot be used for aqueous systems [8]
Cholesteryl 4-(vinyl) phenyl carbonate, Phenyl (4-vinyl) phenyl carbonate and DVB, EGDMA	Cholesteryl 4-(vinyl) phenyl carbonate, Phenyl (4-vinyl) phenyl carbonate, Cholesteryl 4-(vinyl) phenyl carbonate	7.3 (1.0)	Isohexane	7.3	Hydrogen bonding	Cannot be used for aqueous systems [15]
Covalent: Cholesteryl 4-(vinyl) phenyl carbonate and EGDMA	Cholesteryl 4-(vinyl) phenyl carbonate,	Retension Time, 11.3 (3.5), 36.7	Glacial acetic acid	3.2	Hydrogen bonding	Cannot be used for aqueous systems. Capacity and selectivity were good [17]
Non-covalent: Methacrylic acid and 4-vinyl pyridine and EGDMA	Cho	MAA: 10.2 (3.1), 25.1, VP: 13.0 (4.0), 28.2 mg/g		MAA: 3.0, VP: 2.9		
C3, C4, M1, M2 with C1 or C2	C3, C4, M1, M2, Cho	43.7 (11.7)	Intestinal mimicking solution	3.7	Hydrophobic binding	Use for aqueous systems. Very good capacity and selectivity. New functional monomer and cross-linker

[a] Values in parentheses for non-imprinted polymers.

cholesterol as evaluated chromatographically. Asanuma and coworkers [4] described the cholesterol recognition properties exhibited by polymers prepared by cross-linking of β-cyclodextrin, with diisocyanates in the presence of cholesterol. Zhong et al. [15] prepared polymers comprising acryloyl derivatives of cyclodextrins which were imprinted using cholesteryl acrylate and *N*,*N*′ diacryloyl piperazine as cross-linker. Since the high degree of cross-linker made rebinding from aqueous media difficult, hydrophilic monomers such as 2-hydroxyethyl methacrylate were incorporated. The materials were capable of rebinding cholesterol also from aqueous media. One of the limitations of these polymers is their low capacity and low selectivity resulting from non-specific binding of cholesterol on to the hydrophobic cross-linking monomer used in polymerization [15].

In this communication we report alternative approaches for enhancing capacity and selectivity of molecularly imprinted polymers for cholesterol binding. In the first approach non-specific adsorption was minimized by incorporating hydrophilic cross-linkers. In the second approach cross-linkers containing covalently linked cholesterol rather than cholesterol containing monomers were incorporated so that the degree of cross-linking did not decrease when the loading of the cholesterol bearing moiety was increased. These approaches exploit the same mechanism for rebinding of cholesterol as envisaged by Sellergren et al. [14]. In principle cholesterol conjugate is prepared and brought in contact with cholesterol as a template and the assembly is polymerized in presence of excess cross-linker. The template cholesterol molecule is expected to bind to polymer cholesterol conjugate by hydrophobic binding. During the rebinding experiment cholesterol used as template is washed off and the imprinted polymer is brought in contact with the guest cholesterol molecule in an aqueous medium where upon the latter is expected to bind to polymer cholesterol conjugate through hydrophobic binding. This approach is different than that reported by Whitcombe et al. [6] discussed in the preceding paragraphs. These polymers were evaluated for rebinding of cholesterol from aqueous media. The results demonstrate that both higher cholesterol binding capacities as well as selectivities can be achieved.

2. Experimental

2.1. Materials

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), testosterone (Tes), stigmasterol (Sti), sodium cholate (NaC), *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino) pyridine (DMAP), glycidyl methacrylate (GMA), itaconic acid, were purchased from Aldrich. Sodium deoxycholate (NaDC), α,α′-azobis-(isobutyronitrile) (AIBN), potassium dihydrogen phosphate, potassium carbonate (K_2CO_3), sodium hydroxide, cholesterol, glycerol were supplied by S.D. Fine Chemicals, India, HPLC grade methanol and isopropanol were supplied by Qualigens Chemicals, India. All solvents supplied by local suppliers were purified as per standard procedure [24].

2.2. Instrumentation

^{1}H NMR spectra were recorded on Bruker superconducting FT NMR AC 300 operating at 300 MHz. IR spectra were recorded on Shimadzu 8300 FTIR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer. The absorption wavelength for stigmasterol was 206 nm and for testosterone 241 nm [11]. HPLC analysis was carried out using Waters HPLC system comprising 680 automated gradient controller, 510 solvent delivery pumps, 486 tunable absorbance detector and 746 dual channel integrator. A μ-Bondapak C_{18} column (Phenomenex) in conjunction with methanol-isopropanol (90:10 v/v) as mobile phase was used for estimation of cholesterol at 206 nm. Scanning electron micrographs (SEM) were recorded on Stereoscan 440, Leica. The pore surface area and pore volume of the porous copolymer samples were studied by mercury intrusion porosimetry in the pressure range of 0–4000 kg/cm^2 using an Autoscan 60 mercury porosimeter from Quantachrome, USA. The mercury contact angle was 140°.

2.3. Synthesis of polymerizable derivatives of cholesterol

2.3.1. Synthesis of monocholesteryl itaconate (M1)

In a 250 ml capacity round bottom flask 7.73 g (0.02 moles) cholesterol and 2.60 g (0.02 moles) of itaconic acid were dissolved in 25 ml of THF. The flask was cooled in an ice bath and temperature was maintained between 0 and 5 °C. The 4.12 g (0.02 moles) of DCC was dissolved in 5 ml THF and added to the above solution. One percent DMAP was added as a catalyst. Reaction mixture was stirred for 2–3 h in ice water bath and then at room temperature for 48 h. Dicyclohexyl urea (DCU) formed during the reaction was filtered off and filtrate was concentrated. The solid product was washed with water, 5% acetic acid, 0.5N sodium bicarbonate solutions and brine and water (each 300 ml). White solid was dried under vacuum and characterized.

Yield: 8.26 g (80%).

^{1}H NMR (300 MHz $CDCl_3$): 0.67δ s (3H, 18-H_3), 0.85δ d (3H, 27-H_3), 0.87δ d (3H, 21-H_3), 0.92δ s (3H, 19-H_3), 1δ to 2.28 (steroid), 1.99δ s (3H, **CH_3**–CH_2), 3.18δ s (–CH_2 of itaconate) 5.81δ and 6.42δ s (2H, CH_3=**CH_2**), 5.34δ s (1H, 6-H).

IR (KBr): 3328.9 cm^{-1} –OH of COOH, 1710.7 cm^{-1} ester, 1650.5 cm^{-1} C=C.

Cholesteryl methacrylate (M2) was synthesized as reported by Sellergren et al. [14].

Yield: 5.56 g (94%).

^{1}H NMR (300 MHz $CDCl_3$): 0.67δ s (3H, 18-H_3), 0.84δ d (3H, 27-H_3), 0.89δ d (3H, 21-H_3), 0.91δ s (3H, 19-H_3), 1δ to 2.28 (steroid), 1.98δ s (3H, CH_3–**CH_2**), 5.14δ and 5.20δ s (2H, CH_3–**CH_2**), 5.34δ s (1H, 6-H).

IR (KBr): 1720.3 cm^{-1} ester, 1649 cm^{-1} C=C.

2.3.2. Synthesis of glyceroldimethacrylate (C2)

In a 500 ml round bottom flask 110 g (0.8 moles) anhydrous K_2CO_3 was placed in 500 ml of dry acetone. To this solution 50.90 ml (0.6 moles) of MAA was added in a drop wise manner over 30 min under vigorous stirring at room temperature. The reaction was continued for 2 h to complete the formation of potassium methacrylate. Further 26.4 ml (0.2 moles) GMA was added over 2 h in a dropwise manner. The reaction was continued at room temperature for 12 h and then at reflux for another 4 h. The reaction mixture was cooled to room temperature and filtered to remove unreacted potassium methacrylate and potassium carbonate. Acetone was evaporated under vacuum at 35 °C. The crude product was dissolved in diethyl ether, washed repeatedly with water to remove traces of K_2CO_3 and potassium methacrylate. Ether layer was dried over Na_2SO_4. Ether was removed under vacuum to yield an oily liquid.

Yield: 77.3 g (60%).

^{1}H NMR (300 MHz $CDCl_3$): 2.00δ s (6H, –CH_2), 3.36δ m (1H, **CH**–OH), 3.99δ to 4.6δ 2dd (4H –CH_2–O– and O–**CH_2**–), 5.8 δ, 6.2δ 2s (4H, **CH_2**=C–).

IR (Neat): 3425.3 cm^{-1} –OH, 1720.4 cm^{-1} ester, 1639.4 cm^{-1} of C=C.

2.3.3. Synthesis of glyceryldicholesteryl itaconate (C3)

In a 250 ml capacity round bottom flask 1.5 g (0.003 moles) of monocholesteryl itaconate and 0.21 ml (0.003 moles) of glycerol were dissolved in 25 ml of THF. The flask was cooled in an ice bath and temperature was maintained between 0 and 5 °C. The 0.62 g (0.003 moles) of DCC was dissolved in 5 ml THF and added to the above solution. One percent DMAP was added as a catalyst. It was stirred for 2–3 h in ice water bath and then at room temperature for 48 h. DCU formed during reaction was filtered off and workup was followed by same method reported for M1.

In the next step, 2.0 g (0.003 moles) of glycerylmonocholesteryl itaconate was coupled with 1.5 g (0.003 moles) of monocholesteryl itaconate by DCC coupling by the same procedure as reported earlier.

Yield: 2.2 g (65%).

^{1}H NMR (300 MHz $CDCl_3$): 0.67δ s (6H, 18-H_3), 0.84δ d (6H, 27-H_3), 0.87δ d (6H, 21-H_3), 0.92δ s (6H, 19-H_3), 1δ to 2.28 (steroid), 1.82δ s (6H, **CH_3**–CH_2), 3.7δ m (1H **CH**–OH), 3.50δ to 4.16δ 2dd (4H –CH_2–O– and O–CH_2–), 3.02δ s (2H–CH_2 of itaconate) 6.53δ and 6.98δ s (2H, CH_3–**CH_2**), 5.34δ s (1H, 6-H).

IR (KBr): 1697.2 cm^{-1} Ester, 3327 cm^{-1} OH, 1625 cm^{-1} C=C.

2.3.4. Synthesis of monocholesteryl itaconate glycerol methacrylate (C4)

2.3.4.1. Synthesis of glyceryl itaconate ester. In a 1000 ml round bottom flask 10 ml (0.076 moles) of GMA and 10 g (0.076 moles) of itaconic acid were added along with 2 g of hydroquinone and 500 ml of benzene. To this solution, 1.5 ml of pyridine was added as catalyst and the solution was refluxed for 5 h. Benzene was recovered and the residue washed first with 1% sodium bicarbonate solution and then with water to remove itaconic acid. The filtrate was dried on anhydrous sodium sulfate and concentrated under vacuum. The product obtained was characterized by IR and NMR.

Yield: 15 g (75%).

^{1}H NMR (300 MHz $CDCl_3$): 2.00δ s (6H, 2CH_3), 3.3δ m (1H–**CH**–OH), 4.01δ to 4.07δ dd (2H, –**CH_2**–O–), 4.5δ to 4.56δ dd (2H, –CH_2–O–), 5.66δ and 6.2δ s (4H, =**CH_2**).

IR (Nujol): 1720.3 cm^{-1} Ester, 1695 cm^{-1} –COOH, 1645 cm^{-1} C=C.

2.3.4.2. Condensation of glyceryl itaconate and cholesterol. In a 250 ml capacity round bottom flask 14.98 g (0.039 moles) of cholesterol and 10 ml (0.039 moles) of glycerylitaconate ester were dissolved in 25 ml of THF. The flask was cooled in an ice bath and temperature was maintained between 0 and 5 °C. The 7.99 g (0.039 moles) of DCC was dissolved in 5 ml THF and added to the above solution. One percent DMAP was added as a catalyst. Solution was stirred for 2–3 h in ice water bath and then at room temperature for 48 h. Dicyclohexyl urea (DCU) formed during reaction was removed by filtration and workup was performed by the same method reported for M1.

Yield: 16 g (65%).

^{1}H NMR (300 MHz $CDCl_3$): 0.67δ s (3H, 18-H3), 0.82δ d (3H, 27-H3), 0.86δ d (3H, 21-H3), 0.93δ s (3H, 19-H3), 1δ to 2.28δ (steroid), 1.89δ s (3H, **CH_3**–CH_2),

IR (Nujol): 1720.3 cm^{-1} Ester, 1645 cm^{-1} C=C.

2.4. Synthesis of imprinted polymers

In 20-ml test tube, predetermined quantities of monomer, cross-linker and cholesterol were dissolved in ethanol. (For details of precise quantities used in each experiment, please refer to Table 2.) For the synthesis of non-imprinted polymers no cholesterol was used to serve as a template during polymerization. The test tubes were purged with nitrogen for 20 min and 1% by weight of AIBN was added. Tubes were maintained in a hot water bath at 60 °C for 16 h. The template cholesterol was extracted from the imprinted polymers by Soxhlet extraction for 48 h in methanol. Complete extraction was confirmed by verifying that further extraction did not yield any cholesterol. The polymer was crushed and sieved through a mesh to 37-μm size.

Table 2
Preparation of adsorbents for the cholesterol binding experiments[a]

Polymer	Mole ratio	Weights (g)	Monomer	Cross-linker
P_1	20:4:1	2.5:1.146:0.243	M2	C1
P_2	5:1:1	2.5:0.286:0.243	M2	C1
P_3	20:4:1	0.4:0.162:0.033	M2	C2
P_4	20:4:1	2.5:1.257:0.243	M1	C1
P_5	9:1:1	0.4:0.125:0.075	–	C2,C4
P_6	7:3:3	0.4:0.483:0.290	–	C2, C4
P_7	5:5:5	0.4:1.127:0.678	–	C2, C4
P_8	2:8:8	0.9:4.50:2.713	–	C2, C4
P_9	9:1:1	2.5:0.900:0.541	–	C1, C4
P_{10}	7:3:3	1.25:1.736:1.044	–	C1, C4
P_{11}	5:5:5	1.25:4.052:2.438	–	C1, C4
P_{12}	2:8:8	0.416:5.40:3.251	–	C1, C4
P_{13}	2:1:1	1.25:3.321:1.210	–	C1, C3

[a] The polymers were prepared as described in the experimental section. Where, C_1: EGDMA; C_2: glyceroldimethacrylate; C_3: glyceryldicholesteryl itaconate; C_4: monocholesteryl itaconate glycerol methacrylate; M_1: monocholesteryl itaconate; M_2: cholesteryl methacrylate.

2.5. Cholesterol binding studies

2.5.1. Preparation of intestinal mimicking solution (A)

A total of 200 ml water was added to 125 ml of a 0.2 mol/l potassium dihydrogen phosphate solution and 95 ml of 0.2 mol/l sodium hydroxide solution. Then 24.5 g of sodium deoxycholate (NaDC) and 16.5 g sodium cholate (NaC) were added. The pH was adjusted to 7.5 ± 0.1 with 0.2 mol/l sodium hydroxide solution and final volume was made up to 500 ml using water. After sparging with nitrogen for 30 min, the solution was stored in dark at room temperature [14].

2.5.2. Preparation of cholesterol standard solution (B)

To 500 ml of (A) above, 900 mg of cholesterol was added and the solution was sonicated for 3 h at 50 °C. The solution was then sparged with nitrogen for 30 min and stored in dark at room temperature.

2.5.3. Adsorption of cholesterol from intestinal mimicking solution

A total of 10 mg of dry polymer was suspended into 5 ml of intestinal mimicking solution (IMS). The samples were then stirred in a circulatory shaking bath at room temperature for 24 h. The solution was centrifuged to separate the polymer and the supernatant solution was estimated for cholesterol by HPLC.

2.6. Selectivity studies

The selectivity studies were performed in water/THF mixture (5:6; v/v) since the steroids testosterone and stigmasterol were insoluble in IMS. In a 50-ml conical flask, 10 mg of polymer was weighed and 4 ml of above steroid solution was added. Flask was stirred in a circulatory shaking bath at room temperature for 24 h. The polymer suspension was centrifuged (1000 rpm for 30 min) and concentration of ligand in the supernatant was determined by UV spectroscopy monitoring for stigmasterol at 206 nm and testosterone at 214 nm. The amount of steroid bound to the polymer was calculated by difference.

Table 3
Adsorbents for cholesterol binding: swelling ratio and surface area

Polymer	Swelling ratio[a]	Pore volume of MIP (cm^3/g)	Pore surface area of MIP (m^2/g)
P_1	1.00	2.1892	32.47
P_2	3.00	2.3743	38.24
P_3	3.00	1.3101	20.81
P_4	3.00	2.0704	46.59
P_5	7.00	2.4929	53.25
P_6	4.00	1.2165	52.40
P_7	3.00	1.2527	19.58
P_8	–	1.5378	18.00
P_9	4.00	2.4457	48.62
P_{10}	3.00	1.9149	50.84
P_{11}	1.00	1.3729	37.35
P_{12}	2.00	–	–

[a] Weight of swollen polymer/weight of dry polymer.

2.7. Swelling studies

Equilibrium swelling studies were carried out for all polymers in water at 25 °C as per standard procedure [14]. The results are summarized in Table 3.

3. Results and discussion

The importance of lowering cholesterol is well established [1–3]. In view of the problems associated with the administration of cholesterol lowering agents such as statins, there is an increasing emphasis on use of polymeric adsorbents as sequesterants for bile acids as well as cholesterol. Since the functionalities available on the two are not the same, the choice of functional groups for sequestering cholesterol and bile acids differs. Polymers, which bind to bile acids through ionic interactions, have been synthesized and binding capacities of polymeric adsorbents have been reported [1].

Since cholesterol contains no ionizable groups, the only interactions for binding to cholesterol are either hydrogen bonding or hydrophobic interactions. Further, for binding in aqueous media, only the later can be exploited. Imprinting using a covalent approach is reported to be more efficient than that using a non-covalent approach [3,6]. non-covalent imprinting methodology has also been extensively investigated for the imprinting of cholesterol and subsequent rebinding from non-aqueous media (Table 1).

In particular, Sellergren et al. [14] synthesized a large number of imprinted polymers containing cholesterol, bile acid derivatives and EGDMA as the cross-linker. These were then evaluated for the selective adsorption of cholesterol from simulated intestinal fluids. Typically the ratio of the

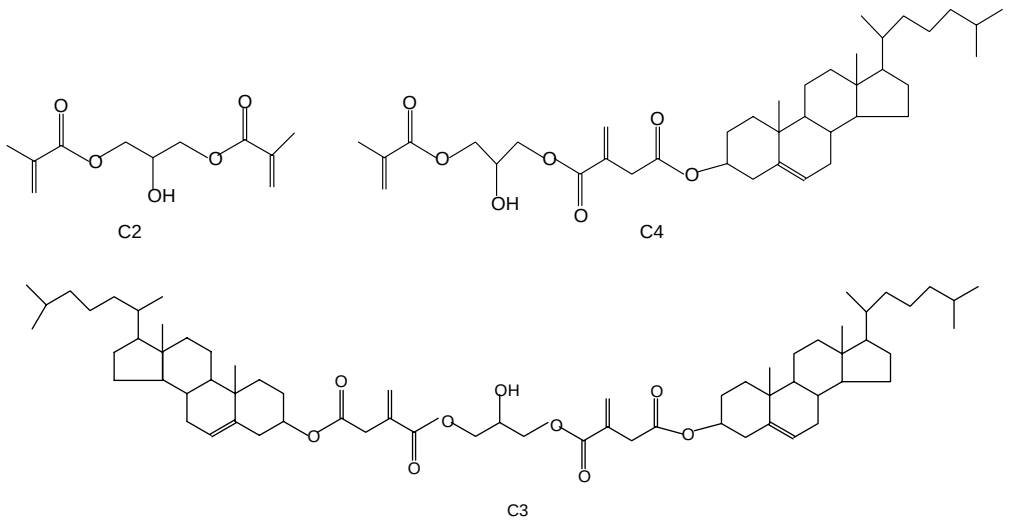


Fig. 1. Hydrophobic, hydrophilic and cholesterol bearing cross-linkers used for imprinting.

cross-linker, methacrylic acid and the steroid monomer was (10:2:1) and the ratio of the steroid monomer to cholesterol used as template was (2:1). Methacrylic acid was incorporated as to bind to cholesterol through hydrogen bonding and also to repel bile acids in the intestinal mimicking solution.

In view of the large excess of hydrophobic cross-linker, viz. EGDMA used in the synthesis of the cross-linked polymer, we believed that at least part of non-specific binding of cholesterol would be due to hydrophobic binding with the cross-linker. In order to explore if this were indeed so, we synthesized the cross-linker glyceroldimethacrylate (C_2) which would have hydrophilic group on the cross-linker backbone (Fig. 1). Apart from the selectivity in recognition, the capacity of the adsorbent is equally critical. An increase in the content of the steroid monomer which would present the rebinding sites for higher capacity, leads to a lower degree of cross-linking and consequently a loss in selectivity. In order to circumvent this problem, we synthesized cross-linkers containing cholesterol. Copolymerization of these along with conventional cross-linkers was expected to lead to enhanced cholesterol binding capacity without loss in selectivity, since the cross-link density remained the same. Apart from these two variables which govern the chemical composition of the polymer, the polymerization conditions as well as the monomer composition influence the morphological structure of the cross-linked polymer as reflected in pore volume, pore size, surface area, etc. which also influence the adsorption capacity. In the following sections we explain the effect of these variables on the binding capacity for cholesterol as well as selectivity vis-a-vis other steroids.

3.1. Polymer synthesis and characterization

Sellergren et al. [14] incorporated methacrylic acid in the cross-linked polymer structure to provide a hydrogen bonding site for cholesterol and also repel any bile acids. The cross-linker used in this work viz. EGDMA was highly hydrophobic. It may be noted here that methacrylic acid is ionized at pH beyond 4 and renders polymers containing methacrylic acid hydrophilic. Since we wished to investigate the effect of hydrophilicity of cross-linker on suppressing non-specific binding, we did not want this to be overshadowed/complicated by the swelling caused by ionization of methacrylic acid. In the absences of methacrylic acid, swelling of network would be a good measure of the hydrophilicity of the matrix. Hydrophilicity was enhanced by incorporating either ionizable groups in the monomer itself, as in the case of monocholesteryl itaconate or by incorporating hydroxyl groups in the cross-linker, as in the case of glycerol dimethacrylate. In order to increase the concentration of cholesterol bearing conjugates (Fig. 2) without lowering the degree of cross-linking, we synthesized hydroxyl bearing cross-linkers monocholesteryl itaconate glycerol methacrylate and glyceryl dicholesteryl itaconate. The latter allowed us to double the loading of the binding

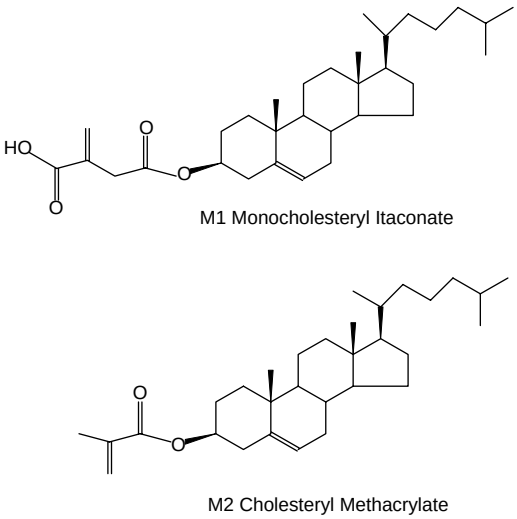
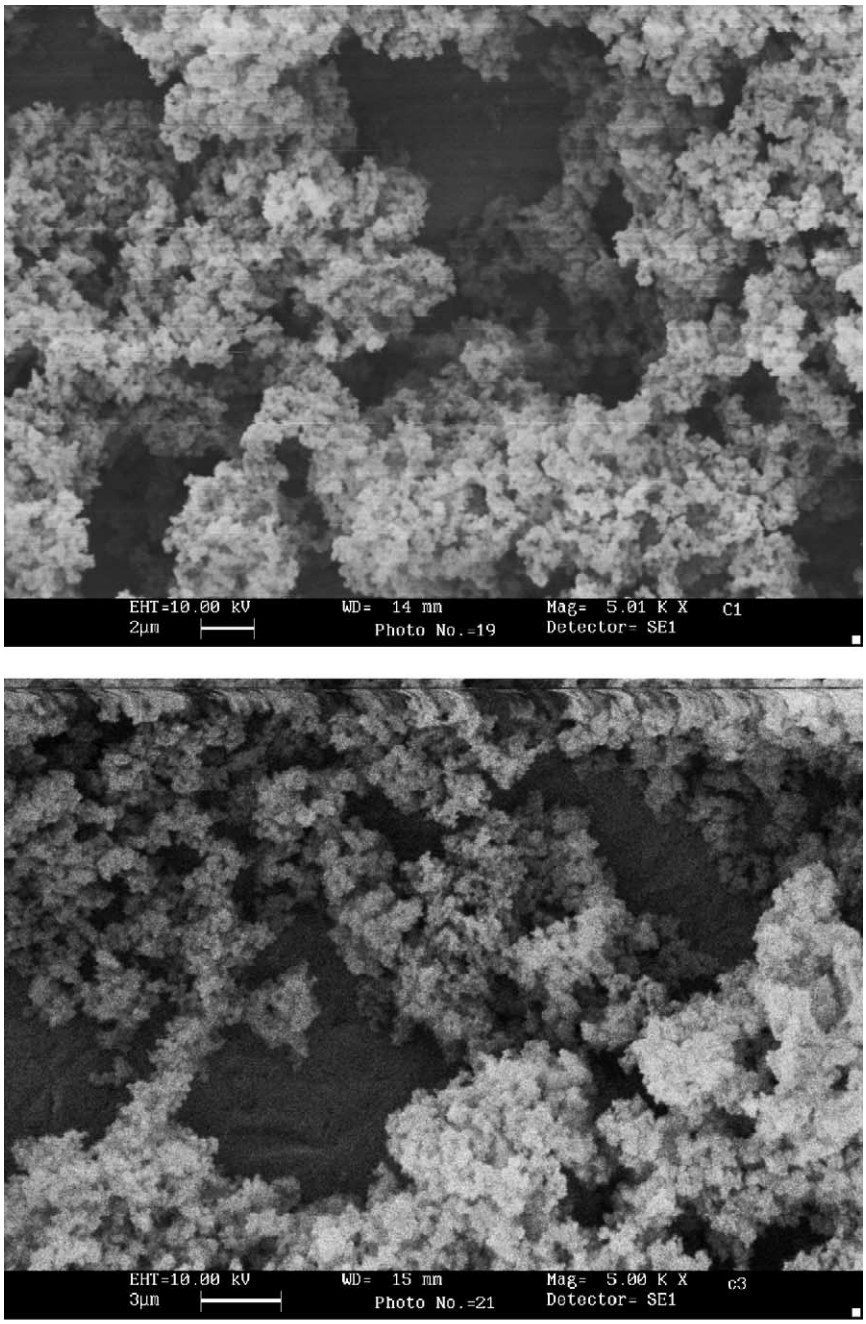


Fig. 2. Polymerizable derivatives of cholesterol.

sites compared to the former. The ratio of cholesterol conjugate to template cholesterol as well as the cholesterol bearing cross-linker to conventional cross-linker was varied (Table 2). All polymerizations were carried out in presence of ethanol as the porogen as this is supposed to lead to macroporous resins with low swelling [14]. Those prepared in the presence of dichloromethane are gel type and exhibit high degree of swelling [14]. Since the cross-linked polymers are to be used as cholesterol sequesterants in 'intestinal mimicking' media, the swelling measurements were made in water rather than other solvents. Polymers were characterized for their surface area and pore volume (Table 3). The scanning electron microscope imaging indicated that the cholesterol imprinted polymers were more porous than the corresponding non-imprinted polymers (Fig. 3).

3.2. Evaluation of polymers prepared using cholesterol bearing monomers

In this series of experiments, ethylene glycol dimethacrylate was copolymerized with cholesteryl methacrylate in the mole ratio 5:1. Polymerization was carried out using



P_9' = Non-imprinted polymer

Fig. 3. Scanning electron microscope (SEM) of imprinted vs. non-imprinted polymers (P_9).

Table 4
Rebinding of cholesterol

Polymer	Rebinding by MIP (mg/g)	Rebinding by non-MIP (mg/g)	Imprinting efficiency, α[a]
P_1	16.7	11.5	1.4
P_2	23.0	18.2	1.3
P_3	8	3	2.6
P_4	32.5	23.8	1.4
P_5	28.6	21.5	1.3
P_6	23.6	15.7	1.5
P_7	18.2	15.2	1.2
P_8	33.4	19.0	1.7
P_9	43.7	11.7	3.7
P_{10}	20.0	10.3	1.9
P_{11}	31.2	13.6	2.2
P_{12}	32.0	22.9	1.4
P_{13}	19.9	10.7	1.9

[a] α = MIP/non-MIP.

the functional monomer: template mole ratio of (4:1) (P_1) and (1:1) (P_2) respectively. It was observed that imprinting of cholesterol in stoichiometric proportion (1:1) led to a higher binding capacity for cholesterol for the polymer P_2 (23 mg/g) as compared to the case wherein excess of functional monomer (4:1) was used for the polymer P_1 (16.7 mg/g) (Table 4). This can be attributed to the preferential self association involving the cholesterol bearing monomers rather than the intermolecular association between the cholesterol bearing monomer and cholesterol. In contrast, when the functional monomer and the template are incorporated in the stoichiometric proportions, the intermolecular association between the cholesterol bearing monomer and the template molecule viz. cholesterol will predominate, leading to higher imprinting efficiency. The formation of highly porous structure with higher surface area leads to higher swelling ratio (viz. P_3) as compared to the polymer synthesized using a high monomer template ratio, which exhibits a low swelling ratio (viz. P_1). The exposure of the binding sites during rebinding leads to higher cholesterol binding capacity.

In contrast, when ethylene glycol dimethacrylate was replaced by glycerol dimethacrylate, the porosity as well as surface area both decreased and yet swelling ratio was high (viz. 3), since the cross-linker is now more hydrophilic as compared to EGDMA. As a result, the binding capacity of the imprinted polymer P_3 decreased (8 mg/g). Yet the non-specific hydrophobic binding was suppressed as indicated by very low cholesterol binding capacity of the corresponding non-imprinted polymer (3 mg/g). Substitution by glycerol dimethacrylate leads to higher selectivity for cholesterol over the non-imprinted polymer (2.6) as compared to the hydrophobic cross-linker EGDMA (1.45). Thus while we have succeeded in improving the selectivity by using a more hydrophilic cross-linker, we still need to enhance the binding capacity of the imprinted polymer for cholesterol. In order to further explore the effect of hydrophilicity of the matrix on cholesterol binding capacity, cholesteryl methacrylate was replaced by cholesteryl mono itaconate. While the swelling ratio of this polymer (P_4) was the same (viz. 3) as that in case of P_2 and P_3, the porosity was comparable and the surface area was much higher than in case of both P_2 and P_3 which leads to increased exposure of the cholesterol binding sites within the porous matrix. Accordingly the cholesterol imprinted copolymer comprising mono cholesteryl itaconate and ethylene glycol dimethacrylate (P_4) exhibits the highest binding capacity (32.5 mg/g) in this series of adsorbents without loss of imprinting efficiency (1.4). It may be noted that the ratio of the cholesterol bearing monomer and template in this case is 4:1, and bringing this down to 1:1 may lead to further enhancement in capacity.

3.3. Evaluation of polymers prepared using cholesterol conjugated cross-linkers

In the previous section it was shown that the substitution of EGDMA by glycerol dimethacrylate resulted in enhanced selectivity of the imprinted polymer to cholesterol vis-a-vis non-imprinted one, but the cholesterol binding capacity was low. This could be increased by increasing the concentration of cholesteryl methacrylate. However, this would lead to a decrease in the cross-linker concentration and loss in selectivity. To over come this problem, we synthesized cholesterol bearing cross-linker monocholesteryl itaconate glycerol methacrylate (C_4). This cross-linker can be looked upon as glycerol methacrylate containing an additional methylene group conjugated with cholesterol.

The incorporation of this cross-linker would help in increasing cholesterol binding without sacrificing the cross-link density. Although increasing cholesterol loading is expected to enhance capacity without sacrificing the cross-link density and hence the selectivity, it must be borne in mind that this will also depend on the porosity and surface area of the resulting polymer and that these morphological features cannot be independently controlled.

The results for the polymer samples P_5 to P_8 (Table 4) demonstrate, that at constant ratio of cholesterol bearing cross-linker to cholesterol (1:1), the selectivity of the imprinted polymers to cholesterol vis-a-vis non-imprinted polymers does not show a systematic variation, as one would anticipate if the cross-linker concentration were to decrease. This is because, when the ratio of the cross-linker to cholesterol bearing cross-linker is varied, there is no change in cross-link density. In contrast, the cholesterol binding capacity for both imprinted as well as non-imprinted polymer decreases, when the concentration of the cholesterol bearing cross-linker is increased. This could be attributed to decrease in the pore volume as well as surface area, which implies that although as indicated by the chemical composition the amount of cholesterol available for the hydrophobic binding has increased in principle, the fraction available on the pore surface has decreased as a result of decrease in porosity and surface area. In the case of polymer P_8,

the ratio of cross-linker to cholesterol bearing cross-linker has increased four folds (1:4) as compared to that in case of polymer P_7. Thus while the porosity has marginally increased and surface area decreased, a four-fold increase in cholesterol concentration results in a larger available population of cholesterol binding sites on the surface which leads to higher cholesterol binding capacity in case of both imprinted and non-imprinted polymers. Yet another feature to be noted is that these polymers are highly hydrophilic as indicated by their swelling ratios (viz. 7–3). It is possible that very high hydrophilicity suppresses the binding of cholesterol.

To test this hypothesis, we replaced glycerol dimethacrylate by EGDMA. The results for the polymer P_9 to P_{12} indicate that for identical composition, the swelling ratio decreases when glycerol dimethacrylate is replaced by EGDMA. Thus polymers P_5 and P_9 are identical in monocholesteryl itaconate glycerol methacrylate, content as well as porosity and to a certain extent the surface area, which implies that parts of the sites are not available on the surface for binding with cholesterol. Yet the cholesterol binding capacity of the polymer P_9 is 43.7 mg/g, which is, 50% higher than polymer P_5 (28.6 mg/g). It is also note worthy that the cholesterol imprinted polymer P_9 exhibits higher selectivity (3.7) with respect to cholesterol over the corresponding non-imprinted polymer. In the case of polymer P_5 this value is (1.3). Although the proportion of cholesterol bearing cross-linker is increased in case of polymer P_{10}, decreased porosity accompanied by a modest increase in surface area leads to lower cholesterol binding capacity as well as selectivity vis-a-vis non-imprinted polymer. A further increase in cholesterol content as in case of polymer P_{11} more than compensates for this lowering and leads to a higher cholesterol binding capacity. Further increase in the cholesterol bearing cross-linker has no effect. Thus although the effect of polymer composition and morphology cannot be independently controlled, we have achieved higher cholesterol binding capacity as well as selectivity compared to the non-imprinted polymer, by judicious choice of a cholesterol bearing hydrophilic cross-linker and EGDMA.

The results of cholesterol binding capacity of polymers P_9–P_{12} also highlight the role of molecular imprinting vis-a-vis self stacking of cholesterol in the rebinding experiment. In this series of polymers, the degree of cross-linking remains constant irrespective of the amount of cholesteryl ligand incorporated, since it has been conjugated with a cross-linker. In a typical rebinding experiment 10 mg of the polymer is brought in contact with 5 ml of intestinal mimicking fluid, which contains 4.6 mmol/l cholesterol. In the case of polymer P_9, 10 mg polymer, which exhibits a swelling ratio of 4, the concentration of cholesteryl ligand is 0.1 mol/l. Since the ligand is a part of cross-linked structure, self association between cholesteryl ligand will not be favored. Similarly since free cholesterol concentration is much lower than the cholesteryl ligand concentration, the self association between free cholesterol will not be favored over intermolecular association between cholesterol ligand and free cholesterol. The latter is favored due to large apolar contact. It may be further noted that as the cholesteryl ligand concentration increases from P_9 to P_{11} the swelling ratio decreases. As a result of these two effects, the cholesteryl ligand concentration within the swollen particle increases 20 folds, yet the cholesterol rebinding capacity in the case of non-imprinted polymers remains practically unaltered. This indicates that in the case of non-imprinted polymers the guest cholesterol molecule does not stack over the cholesteryl conjugate. In contrast, the cholesterol binding capacity of cholesterol imprinted polymers is always higher than the corresponding non-imprinted polymers which indicates that apolar association between the guest cholesterol molecule and the cholesteryl ligand is effective only in the case of cholesterol imprinted polymers. The increase in the binding capacity of non-imprinted polymer P_{12} can be attributed to very large excess in cholesterol ligand concentration.

Under the conditions of synthesis of the cholesterol imprinted polymer, the concentration of cholesterol in alcohol was 0.073 mol/l. The solubility of cholesterol is eight times higher. Also the mole ratio of cholesterol to cholesterol bearing ligand increases from 1:1 to 1:4. We therefore expect no preferential self association of cholesterol over intermolecular association with cholesteryl ligand.

In order to further explore if the binding capacity of the polymer could be increased by increasing the amount of cholesterol in the cholesterol bearing cross-linker, we replaced the cross-linker monocholesteryl itaconate glycerol methacrylate (C_4) by glycerol dicholesteryl itaconate (C_3). A comparison of the molecular structure of the two reveals that for the same molar composition, the cross-linker C_3 offers twice the number of cholesterol binding sites. The structure of this cross-linker is analogous to the multifunctional vinyl monomer derived from 3,5 dibromobenzoic acid, propargyl alcohol and cholesterol, which contained two cholesterol receptor sites [19]. The molecularly imprinted polymer based on this tweezer monomer exhibited very high selectivity (5.4). We would thus expect the polymer (P_{13}) to offer higher cholesterol binding capacity. However the results do not validate the same. This can be attributed to the following factors. For the same molar composition, the polymer P_{13} is more hydrophobic than the polymer comprising cross-linker C_4. Also the presence of two cholesterol molecules in the vicinity of one another is likely to result in hydrophobic association between the two. This would result in lower availability of cholesterol as template binding site. The cross-linker C_3 can also be looked upon as two moles of cholesteryl methacrylate (M_2) conjugated through a hydrophilic spacer. A comparison of the results for polymers P_2 and P_{13} reveals that even when amount of cholesterol binding sites available for imprinting is increased two folds, the cholesterol binding capacity has actually marginally decreased. Yet the selectivity has increased which could be

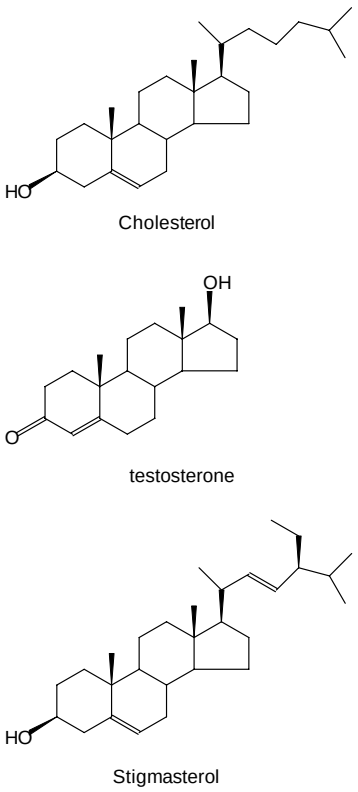


Fig. 4. Steroids used for selectivity studies.

attributed to the presence of hydrophilic hydroxyl group in the spacer. The cross-linker C_3 does not behave as a tweezer.

3.4. Selectivity measurements: role of sorbate structure

The selectivity of molecularly imprinted polymer was evaluated in two ways. (1) We evaluated the binding capacity of cholesterol imprinted polymers for binding of cholesterol as well as two related molecules stigmasterol and testosterone. Stigmasterol has the same structure as cholesterol, but for the unsaturation in the side link and incorporation of ethyl linkage at C_{24} position (Fig. 4). Testosterone on the other hand has no side chain, and is over all more hydrophilic than cholesterol or stigmasterol. If the rebinding involved hydrophobic binding, we would expect testosterone binding capacity to be lower than stigmasterol binding capacity. (2) We also evaluated the capacities of corresponding non-imprinted polymers towards all three sorbents. The results summarized in Table 5 indicate that in all cases cholesterol imprinted polymers have higher binding capacities for cholesterol than for either stigmasterol or testosterone. This is only to be expected since imprinting is expected to create shape selective cavities within the polymer structure. As a result $\alpha_{cho/sti}$ and $\alpha_{cho/test}$ are always higher than unity. Further it is also note worthy that binding capacities for testosterone are always lower than those for stigmasterol. An analysis of three dimensional structures of the three steroids reveals that the space fitting requirements of cholesterol versus stigmasterol are only marginally different. Testosterone in contrast can fit into the same cavity as cholesterol and yet the binding capacity for testosterone is lower. This is because testosterone is more hydrophilic than either cholesterol or stigmasterol. Hence $\alpha_{cho/test}$ values for all imprinted polymers are greater than $\alpha_{cho/sti}$. Table 5 also summarizes the binding capacities of non-imprinted polymers for cholesterol, stigmasterol and testosterone. In the absence of imprinting, rebinding is driven by hydrophobic binding alone. In the case of non-imprinted polymers, therefore the value $\alpha_{sti/test}$ could be either greater or lower than unity.

In summary the use of stoichiometric proportion of functional monomer especially cholesterol monoitaconate (M1) and template has led to polymers having higher binding capacity for cholesterol. The choice of the functional cross-linker has led to polymers with enhanced capacities. A more effective method of increasing the binding capacity without affecting the selectivity was to use cross-linkers containing cholesterol. The selectivities of these MIPs for cholesterol vis-a-vis stigmasterol and testosterone further demonstrate the merit of these cross-linkers. Though the polymer composition and morphology could not be independently controlled, this approach has led to MIPs having higher recognition ability for cholesterol in the aqueous

Table 5
Selectivity studies

Polymer	Cholesterol (mg/g)	Stigmasterol (mg/g)	Testosterone (mg/g)	α_{sti} (mg/g)	α_{test} (mg/g)	$\alpha_{sti/test}$ (mg/g)
P_1	13.5 (9.1)	7.8 (4.2)	6.5 (3.2)	1.73 (2.16)	2.0 (2.84)	1.2 (1.3)
P_3	13.2 (5.9)	9.7 (3.9)	8.1 (4.3)	1.36 (1.51)	1.6 (1.37)	1.1 (0.9)
P_4	22.7 (17.2)	8.7 (4.5)	7.1 (5.1)	2.60 (3.82)	3.1 (3.37)	1.2 (0.8)
P_5	25.0 (17.2)	12.2 (7.8)	7.8 (5.4)	2.04 (2.20)	3.2 (3.18)	1.5 (1.4)
P_6	12.7 (5.2)	8.9 (4.2)	6.2 (3.3)	1.42 (1.24)	2.0 (1.58)	1.4 (1.2)
P_7	14.2 (10.1)	7.5 (3.8)	6.2 (3.5)	1.89 (2.66)	2.3 (2.88)	1.2 (1.0)
P_9	38.5 (14.0)	17.0 (15.0)	8.1 (6.4)	2.30 (0.93)	4.7 (2.19)	2.0 (2.3)
P_{10}	13.3 (5.3)	7.8 (3.8)	6.7 (4.1)	1.70 (1.40)	1.9 (1.3)	1.1 (0.9)
P_{11}	26.5 (14.5)	8.6 (4.8)	7.2 (3.4)	3.08 (3.02)	3.7 (4.26)	1.2 (1.4)
P_{13}	21.2 (11.8)	11.2 (5.2)	7.5 (3.2)	1.90 (2.26)	2.8 (3.6)	1.5 (1.6)

α_{sti} = MIP cholesterol/MIP stigmasterol; α_{test} = MIP cholesterol/MIP testosterone; $\alpha_{sti/test}$ = MIP stigmasterol/MIP testosterone. The numbers in the parentheses show the selectivity of the non-imprinted polymer.

medium. The rebinding studies using stigmasterol and testosterone have further highlighted the role of hydrophobic interactions during rebinding.

4. Concluding remarks

Polymers containing cholesterol bearing monomers and cross-linker imprinted with cholesterol exhibit enhanced affinity and capacity for cholesterol from intestinal mimicking media. It was shown that the non-specific binding on to imprinted and non-imprinted polymers could be reduced by hydrophilic modification. Cholesterol conjugated cross-linkers were synthesized to improve the recognition ability of MIPs. This approach led to polymers having stronger affinity for cholesterol than reported earlier. The polymers also had very good selectivity for cholesterol as compared to other steroids. The adsorptive capacity seems to arise from binding sites induced by the hydrophobic association between cholesterol units in the polymer backbone and the presence of cholesterol as a template during polymerization. The adsorbents exploiting this approach may find applications for selective binding of steroids from aqueous medium.

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