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Synthesis and evaluation of a solid supported molecular tweezer type receptor for cholesterol†

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A new macroporous stationary phase bearing 'tweezer' receptors that exhibit specificity for cholesterol has been constructed from rigid multifunctional vinylic monomers derived from 3,5-dibromobenzoic acid, propargyl alcohol and cholesterol. The synthesis of the novel tweezer monomer that contains two cholesterol receptor arms using palladium mediated Sonogashira methodologies and carbonate couplings is reported. The subsequent co-polymerisation of this tweezer monomer with a range of cross-linking agents *via* a 'pseudo' molecular imprinting approach afforded a diverse set of macroporous materials. The selectivity and efficacy of these materials for cholesterol binding was assessed using a chromatographic screening process. The optimum macroporous stationary phase material composition was subsequently used to construct monolithic solid phase extraction columns for use in the selective extraction of cholesterol from multi-component mixtures of structurally related steroids.

Introduction

In nature, complex molecular assemblies are formed by a multitude of non-covalent interactions.[1] When considered individually, the strength of a single non-covalent interaction is weak in comparison to a covalent bond. However, the simultaneous action of multiple non-covalent bonds facilitates the production of highly stable complexes, as demonstrated elegantly by DNA helices. Attempts by synthetic chemists to imitate nature have led to the generation of numerous synthetic receptor systems including large macrocyclic hosts such as crown ethers,[2] cyclophanes,[3] and calixarenes.[4]

In contrast to the synthetically challenging macrocyclic host systems, Rebek has reported[5] the synthesis of simpler 'molecular cleft' type synthetic receptors. Related studies by Whitlock[6] and Zimmerman[7] and their co-workers enabled the development of a subset of this receptor class that are referred to as *molecular tweezers*. This particular receptor type has been studied extensively and molecular tweezers that exhibit selective recognition for peptides,[8] saccharides,[9] adenine derivatives,[10] cations,[11] anions,[12] electron deficient aromatics,[13] and transition metal ions have been reported.[14]

Cholesterol is one of the most widely occurring steroids and can be isolated by extraction from most animal tissue.[15] There is considerable interest in the reduction of high cholesterol levels in humans which result from the consumption of dairy products.[16] Several studies have focused on receptors for cholesterol in order to extract the sterol from the food source and molecular imprinted polymers (MIPs) have been investigated for this purpose.[17]

To date, molecular tweezers incorporating steroidal units have not been used widely for the recognition of specific sterols, although molecular tweezer type receptors have been developed using the bile acid family of steroids. For example, Maitra and D'Souza reported[18] a family of bile acid-based molecular tweezers capable of binding electron-deficient aromatic substrates such as trinitrofluorenone in organic media (K_a = 220 M^{-1} in $CDCl_3$).

In this paper, we report the synthesis of a cholesterol-based molecular tweezer **1**, (see Fig. 1) and its incorporation into a macroporous stationary phase. A polymerisable unit was incorporated onto the aromatic hub component of the molecular tweezer thereby allowing the tweezer receptor to be co-polymerised with cross-linkers to construct a macroporous polymer network. A 'pseudo' molecular imprinting[19] protocol was used in this co-polymerisation process to produce highly ordered pores within the polymer network capable of rebinding cholesterol in a selective manner. An evaluation of the selective binding characteristics of this new stationary phase is presented.

Experimental

Materials

Commercial dry solvents were used in all preparations except in the case of THF that was distilled from benzophenone and sodium. Triethylamine was pre-dried over 4 Å molecular sieves and then freshly distilled. All other reagents were purchased from either the Aldrich Chemical Company or Acros Chimica and were used as received without purification. Styrene was an exception and was filtered through neutral alumina prior to use.

Cholest-5-en-3-yl (3-{[(cholest-5-en-3-yloxy)carbonyl]oxy}prop-1-ynyl)-5-[(4-vinylanilino)carbonyl]phenyl}prop-2-ynyl carbonate 1. To a cooled (ice bath) solution of 3,5-bis(3-hydroxyprop-1-ynyl)-*N*-(4-vinylphenyl)benzamide **8** (0.12 g, 0.36 mmol) in dry THF (1 cm^3) and triethylamine (200 μL) was added dropwise a solution of cholesterol chloroformate (0.41 g, 0.92 mmol) in dry THF (1 cm^3). The ice bath was removed, and the reaction stirred at room temperature for 4 hours. The solvent was

†Electronic supplementary information (ESI) available: synthetic procedures and analytical data for compounds **3**–**8**. See http://www.rsc.org/suppdata/jm/b2/b210427j/

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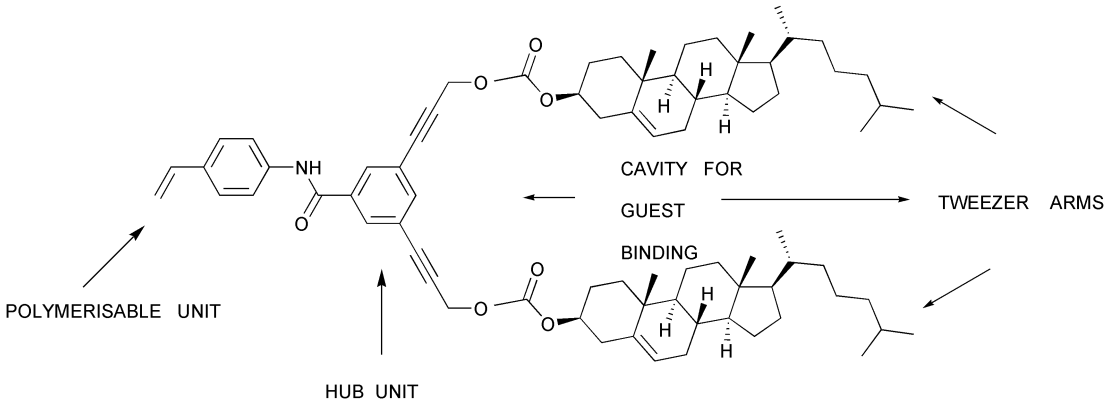

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Fig. 1 The cholesterol-based molecular tweezer system.

evaporated, and the residue dissolved in dichloromethane (2 cm^3). The organic phase was washed with water (3 × 1 cm^3), dried over sodium sulfate, filtered and evaporated to yield a white solid that was purified by column chromatography (dichloromethane : EtOAc 9 : 1) to yield the desired tweezer **1** (0.29 g, 67%) as a sticky cream solid; mp 73–74 °C; ν_{max} (film)/ cm^{-1} 843, 1162, 1255, 1594, 1677, 1748, 2237, 2950, 3360 and 3753; δ_H (250 MHz; $CDCl_3$; Me_4Si) 0.58 (12H, d, 4 × CH_3), 0.60 (6H, s, 2 × CH_3), 1.02 (6H, s, 2 × CH_3), 1.04 (4H, m, 2 × CH_2), 1.12 (4H, m, 2 × CH_2), 1.15 (4H, m, 2 × CH_2), 1.29 (2H, m, 2 × CH), 1.32 (4H, m, 2 × CH_2), 1.44 (4H, m, 2 × CH_2), 1.48 (4H, m, 2 × CH_2), 1.51 (6H, s, 2 × CH_3), 1.57 (4H, m, 2 × CH_2), 1.62 (4H, m, 2 × CH_2), 1.77 (2H, m, 2 × CH), 1.81 (2H, m, 2 × CH), 1.84 (4H, m, 2 × CH_2), 1.96 (4H, m, 2 × CH_2), 2.01 (2H, m, 2 × CH), 2.15 (2H, m, 2 × CH), 2.19 (2H, m, 2 × CH), 3.15 (2H, m, 2 × CH), 4.30 (2H, m, 2 × CH), 4.95 (4H, m, 2 × CH_2), 5.24 (4H, m, 2 × CH_2). 5.35 (1H, dd, J = 18 Hz, 6 Hz, CH=CH_2), 5.86 (1H, dd, J = 18 Hz, 6 Hz, CH=CH_2), 6.55 (1H, t, J = 6 Hz, CH=CH_2), 6.99 (1H, s, NH), 7.14 (2H, AA′XX′, 2 × ArCH), 7.30 (2H, AA′XX′, 2 × ArCH), 7.80 (1H, appt. t, J = 3 Hz, ArCH), 7.99 (2H, appt. d, J = 3 Hz, 2 × ArCH); δ_C (67.5 MHz; $CDCl_3$; Me_4Si) 10.9 (2 × CH_3), 17.7 (2 × CH_3), 18.3 (2 × CH_3), 20.0 (2 × CH_2), 21.6 (2 × CH_2), 21.8 (2 × CH_3), 22.8 (2 × CH_3), 23.3 (2 × CH_2), 26.7 (2 × CH_2), 27.0 (2 × CH_2), 27.2 (2 × CH_2), 28.7 (2 × CH), 29.3 (2 × CH), 30.8 (2 × CH_2), 30.9 (2 × CH), 34.8 (2 × CH_2), 35.2 (2 × CH_2), 35.81 (2 × C–C), 36.9 (2 × C(CH_3)), 38.5 (2 × C(CH_3)), 38.7 (2 × C(CH_3)), 41.30 (2 × C(CH_3)), 48.9 (2 × CH_2), 54.5 (2 × CH_2), 55.7 (2 × C=C), 55.8 (2 × CH_2), 73.2 (2 × C≡C), 77.7 (2 × C≡C), 83.6 (2 × C–O), 112.4 (CH=CH_2), 121.3 (2C, 2 × ArCH), 122.1 (2 × ArCH), 122.3 (ArCH), 124.5 (2 × –C=C), 125.9 (2C, 2 × ArCH), 129.4 (2 × ArC–C≡C), 133.4 (ArC–CH=CH_2), 134.0 (ArC–C=O), 135.0 (ArCN), 136.6 (CH=CH_2), 138.2 (C=O), 152.9 (C=O); *m/z* (MALDI-TOF) calculated for $C_{77}H_{106}O_7N$: 1156.37, found: 1195.48 $[M + K]^+$.

General procedure for the synthesis of 'mini' molecular imprinted polymers

Two solutions were prepared (one with the template and one without) by mixing ethylene glycol dimethylacrylate (EDMA) (142.5 μL, 750 μmol), and AIBN (1.5 mg, 9 μmol) in THF (210 μL). To the template solution was also added tweezer **1** (21.6 mg, 18.75 μmol), and cholesterol (7 mg, 18.75 μmol). A total of 70 μL of each mother solution was dispensed into 1.5 cm^3 HPLC clear glass vials. To each vial was added the required volume of functional co-monomer, and subsequently each vial was sealed under argon. The vials and contents were sonicated at room temperature for 1 hour and purged with argon for a further 5 minutes. The vial contents were allowed to polymerise for 14 hours in a thermostatically maintained water bath at 60 °C, yielding a thin (*ca.* 2 mm) polymeric film on the base of each vial.

Extraction and rebinding experiments

To each of the solid cross-linked polymer networks was added a 1 cm^3 volume of THF and the vials sonicated for one hour without heating. Then, 1 cm^3 of a 4 : 1 mixture of THF : acetic acid (AcOH) was added to each vial, and the vials sonicated for a further hour without heating. The amount of cholesterol released from the polymers was quantified in each extract by HPLC analysis (Table 1). The vials were allowed to stand overnight, and subsequently sonicated for a further hour at 40 °C. No further cholesterol was released from the polymers—as determined by HPLC analysis of the washing solvent. The washing solvent was removed from each polymer by means of a syringe, and a further 1 cm^3 volume of a THF : acetic acid mixture (4 : 1) was added to each polymer. The vials were sonicated for a further 3 hours at 40 °C—no further cholesterol was released from the polymers.

Rebinding experiments were performed by addition of 1 cm^3 of a 5 mg cm^{-3} solution of cholesterol. The vials were sonicated for 1 hour, and then shaken overnight. The amount of cholesterol present in the supernatant liquid was quantified by HPLC and the amount of cholesterol uptake by the polymers was calculated (see Table 1).

General protocol for the selectivity studies

Following the uptake experiments, each polymer was repeatedly washed with 1 cm^3 of a THF : AcOH mixture (4 : 1) to remove the bound cholesterol. Once HPLC analysis showed that the removal of bound cholesterol had been achieved to an appreciable extent, under these detection conditions, the polymers (MIPs and controls) were exposed to 1 cm^3 of a 5 mg cm^{-3} solution of stigmasterol. The polymers were shaken overnight to allow equilibration, before quantification of the residual stigmasterol *via* HPLC analysis (see Fig. 6). This selectivity experiment was repeated using cholesteryl acetate.

General procedure for the synthesis of monolithic molecular imprinted polymers

A mixture of the tweezer receptor **1** (0.116 g, 0.1 mmol), cholesterol (0.039 g, 0.1 mmol), EDMA (750 μL, 4 mmol), methacrylic acid (68 μL, 0.8 mmol) and AIBN (0.008 g, 0.048 mmol) in dry THF (1.12 cm^3) was sonicated for 30 minutes at room temperature. A stainless steel column (50 mm × 4.6 mm id) was filled with the mixture and placed in a water bath at 60 °C. After 14 hours, the column was removed, and connected to a HPLC pump and washed with THF, followed

Table 1 Composition of MIPs prepared for the screening process

Polymer[a]	Functional co-monomer[b]	Ratio template : functional co-monomer	Cross-linker[b]	% Cholesterol recovery	% Cholesterol binding
1	MAA	1 : 2	73% EDMA	15	60
3	MAA	1 : 3	73% EDMA	29	48
5	MAA	1 : 3.5	73% EDMA	20	30
7	MAA	1 : 4	73% EDMA	31	74
9	MAA	1 : 4.5	73% EDMA	30	61
11	MAA	1 : 5	73% EDMA	12	41
13	MAA	1 : 6	73% EDMA	10	55
15	MAA	1 : 8	73% EDMA	24	39
17	MAA	1 : 2	50% EDMA	17	46
19	MAA	1 : 3	50% EDMA	27	19
21	MAA	1 : 4	50% EDMA	25	54
23	MAA	1 : 5	50% EDMA	25	27
25	MAA	1 : 6	50% EDMA	17	45
27	MAA	1 : 8	50% EDMA	19	51
29	MAA	1 : 4	73% DVB	10	42
31	MAA	1 : 6	73% DVB	9	52
33	MAA	1 : 8	73% DVB	16	47
35	MAA	1 : 4	TRIM	36	0
37	MAA	1 : 6	TRIM	28	27
39	MAA	1 : 8	TRIM	20	10
41	MMA	1 : 4	73% EDMA	0	0
43	MMA	1 : 6	73% EDMA	28	22
45	MMA	1 : 8	73% EDMA	15	0
47	*t*BuMA	1 : 4	73% EDMA	20	30
49	*t*BuMA	1 : 6	73% EDMA	16	0
51	*t*BuMA	1 : 8	73% EDMA	13	0
53	*t*BuMA	1 : 4	50% EDMA	38	41
55	*t*BuMA	1 : 6	50% EDMA	54	0
57	*t*BuMA	1 : 8	50% EDMA	53	26
59	Styrene	1 : 4	73% DVB	28	13
61	Styrene	1 : 6	73% DVB	24	49
63	Styrene	1 : 8	73% DVB	7	51

[a]Note: for each MIP a subsequent control polymer was prepared in the absence of the tweezer receptor and cholesterol guest (for example, in the case of polymer 1, polymer 2 represents the corresponding control). The concentration of the cholesterol guest in each of the above polymers was 0.012 mmol dm^{-3}. The porogenic solvent used in the preparation of the polymers was THF. [b]Abbreviations: MAA = methacrylic acid; *t*BuMA = *tert*-butyl methacrylate; EDMA = ethylene glycol dimethylacrylate; DVB = divinylbenzene; TRIM = trimethylolpropane trimethacrylate.

by a THF : AcOH mixture (4 : 1) to elute the cholesterol guest. Non-imprinted control 'blank' monoliths were prepared in the same way, but with omission of the tweezer host and cholesterol guest.

The procedures outlined above were performed in triplicate for each imprinted and blank polymeric film or monolithic column. The chromatographic results were reproducible between replicate samples with errors $\leqslant 5\%$.

Characterisation

General. Thin-layer chromatography (TLC) was performed on aluminium sheets coated with Merck 5735 Kieselgel 60F. Developed TLC plates were air-dried and scrutinized under a UV lamp. Sorbsil 60 (0.040–0.063 mm mesh, Merck 9385) was used to perform column chromatography. Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected. Mass spectra (MS) of lower molecular weight materials were obtained using a VG Autospec mass spectrometer operating in the chemical ionisation mode employing ammonia as the impact gas. Accurate mass spectra (MS) were obtained using a Micromass Q-Tof1 LC-MS-MS spectrometer operating in the electrospray mode. MALDI-TOF mass spectra of higher molecular weight materials were obtained on a SAI LT3 LaserTof using *trans*-3-indoleacrylic acid as the matrix. A typical sample preparation for MALDI-TOF mass spectrometric analysis is described as follows: 3 μL of a solution of the analyte in THF (10 mg cm^{-3}) was combined with 20 μL of the freshly prepared matrix (0.2 M in THF) in a mini-vial, and from the mixture was taken a 2 μL aliquot which was carefully transferred onto a sample plate and left to air-dry prior to analysis. ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC250 (250 MHz) spectrometer (using the solvent proton signal or tetramethylsilane as internal reference). ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC250 (62.5 MHz) spectrometer. Infrared (IR) spectroscopic analyses were performed on a Perkin Elmer 1720-X Infrared Fourier Transform spectrometer.

X-Ray diffraction analysis. X-Ray diffraction patterns were obtained using the MARresearch Image Plate System equipped with a Mo-Kα radiation source. The crystals were positioned at 70 mm from the Image Plate and 3948 reflections were measured. 100 frames were measured at 2° intervals with a counting time of 2 minutes. Data analysis was carried out using the XDS program[20] to provide 1613 independent reflections ($R_{int} = 0.0835$). The structure was solved using direct methods with the SHELX86 program.[21] The non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms bonded to carbon were included in geometric positions and given thermal parameters equivalent to 1.2 times those of the atom to which they were attached. The structure was refined on F^2 using SHELXL[22] to R_1 0.0764 and wR_2 0.2277 for 941 reflections with $I > 2\sigma(I)$.

Data for **5**: $C_{13}H_{10}O_4$, $M = 230.21$, orthorhombic, $a = 15.47(2)$, $b = 7.797(14)$, $c = 18.86(3)$ Å, $U = 2275$ Å^3, space group *Pbca*, $d_{calc} = 1.344$ g cm^{-3}, $Z = 8$.

CCDC reference number 203506.

See http://www.rsc.org/suppdata/jm/b2/b210427j/ for crystallographic data in CIF or other electronic format.

Liquid chromatography. Chromatographic analyses were performed on a Beckman Gold HPLC System equipped with programmable solvent module 126, a Rheodyne injector (injection loop = 20 μL) and a UV/vis programmable detector module 166 operating at 210 nm. During the 'pseudo-combinatorial' molecular imprinted study, the cholesterol uptake and recoveries were quantified using a Luna 3u C18(2), with a 150 × 4.60 mm HPLC column, mobile phase of 50 : 50 acetonitrile : isopropyl alcohol, flow rate 1 cm^3 min^{-1}, and column temperature 20 °C.

Capacity factors for the molecular imprinted monoliths were calculated from the equation $k_a = (t_R - t_m)/t_m$, where t_R is the retention time of the analyte and t_m the dead volume determined from the void marker (toluene). The separation factor α was calculated from the equation $\alpha = K_{a(MIP)}/K_{a(CONTROL)}$.

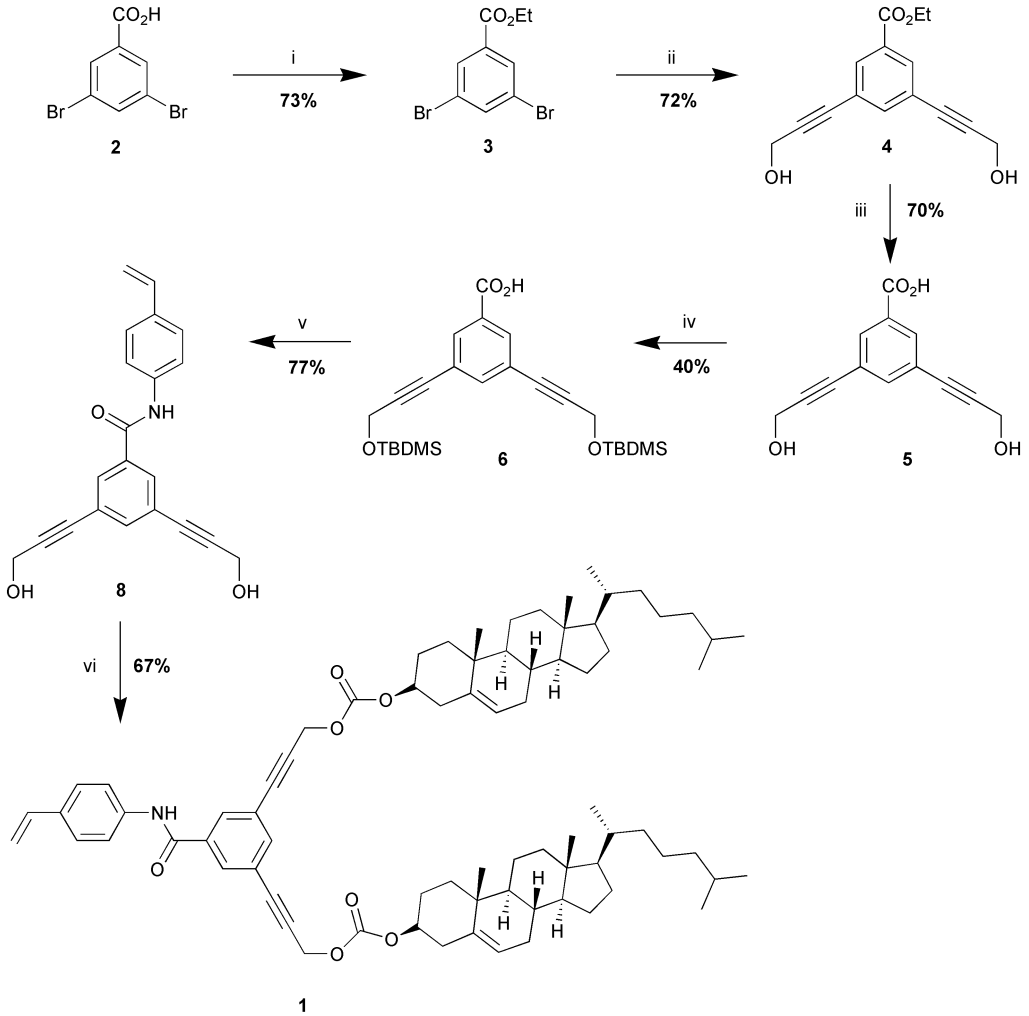
Results and discussion

Synthesis of a novel molecular tweezer for cholesterol

The synthetic approach employed to afford the cholesterol-based molecular tweezer **1** is illustrated in Scheme 1.

We have recently reported the synthesis of 3,5-bis(3-hydroxyprop-1-ynyl)-*N*-(4-vinylphenyl)benzamide **8**, which is referred to in this paper as the 'tweezer hub' unit, and the precursor to **1** (Scheme 1).[23] Synthesis of this compound was realised *via* a six-step synthetic strategy,† which included a synthetically challenging Sonogashira coupling between propargyl alcohol and 3,5-dibromobenzoic acid ethyl ester **3**. Following the publication of this procedure, an increase in the yield of diol **4** was achieved *via* optimisation of conditions reported by Moore and co-workers.[24] Use of $Pd(dba)_2$ and triphenylphosphine in conjunction with copper(I) iodide and triethylamine in refluxing THF afforded excellent yields (70–76%) of product **4** in comparison to our published procedure. This enhancement in yield could be attributed to the longer lifetime of this active catalytic system. Low donating ligands such as tri-2-furylphosphine (TFP) are known to have only short-lived catalytic activities[25] (albeit longer than the triphenylarsine ligand), and hence it is likely that the TFP ligand did not remain sufficiently active to afford good yield of the bis-acetylenated product. The electron donating triphenylphosphine ligand, in comparison, is likely to undertake more frequent catalytic cycles, and hence an enhancement in yield was observed. The main drawback in this case was, however, the long reaction times and harsh conditions that were necessary to force this palladium catalytic system to approach reaction completion. At least 24 hours under continuous reflux were essential for attainment of a 76% yield.

The ethyl ester of free diol **4** was hydrolysed smoothly to the free carboxylic acid **5** in basic media. Subsequent TBDMS protection of each hydroxy moiety under standard conditions[26] afforded the required acid **6** in acceptable yield. Use of the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) in combination with HOBt[27] proved highly effective for the amide bond formation in conjunction with 4-vinylaniline, and a good yield of the TBDMS protected amide **7** was realised if DMF was employed as the solvent. Subsequent cleavage of the TBDMS protecting



Scheme 1 *Reagents*: i. EtOH, DCC, DMAP, Et_2O; ii. propargyl alcohol, $Pd(dba)_2$, PPh_3, CuI, Et_3N, THF; iii. LiOH, H_2O, $(CH_3)_2CO$; iv. TBDMSCl, imidazole, DMF; v. 4-vinylaniline, EDCI, $HOBt{\cdot}H_2O$, DMF → **7** then TBAF, THF, AcOH; vi. cholesterol chloroformate, Et_3N, THF.

groups[28–30] afforded the free diol **8** in good yield. The required molecular tweezer **1** was obtained in acceptable yield *via* reaction of the tweezer hub unit **8** with cholesteryl chloroformate under basic conditions.[17]

X-Ray crystallographic analysis of 3,5-bis(3-hydroxyprop-1-ynyl)benzoic acid 5

The structure of **5** consists of discrete molecules as shown in Fig. 2a together with the atomic numbering scheme. The acid diol molecule **5** is essentially planar with slight deviations observable in the acetylenic substituents. Thus the acid group is rotated by 4.6° from the plane of the benzene ring and atoms C(41), C(42), C(43), O(44) are −0.06, −0.14, −0.24, −0.91 Å and C(61), C(62), C(63), O(64) −0.03, −0.08, −0.15, −0.68 Å from this ring plane, respectively. The molecules are held together in the crystal *via* three strong intermolecular hydrogen bonds (Fig. 2b) with dimensions (O···O, H···O distances/Å, O–H···O angle/°) as follows: O(23)–H(23)···O(64) ($1-x$, $y-0.5$, $0.5-z$) 2.68, 1.86, 171; O(44)–H(44)···O(22) ($x+0.5$, $1.5-y$, $-z$) 2.76, 1.98, 159 and O(64)–H(64)···O(44) ($x-0.5$, y, $0.5-z$) 2.72, 1.91, 166, respectively.

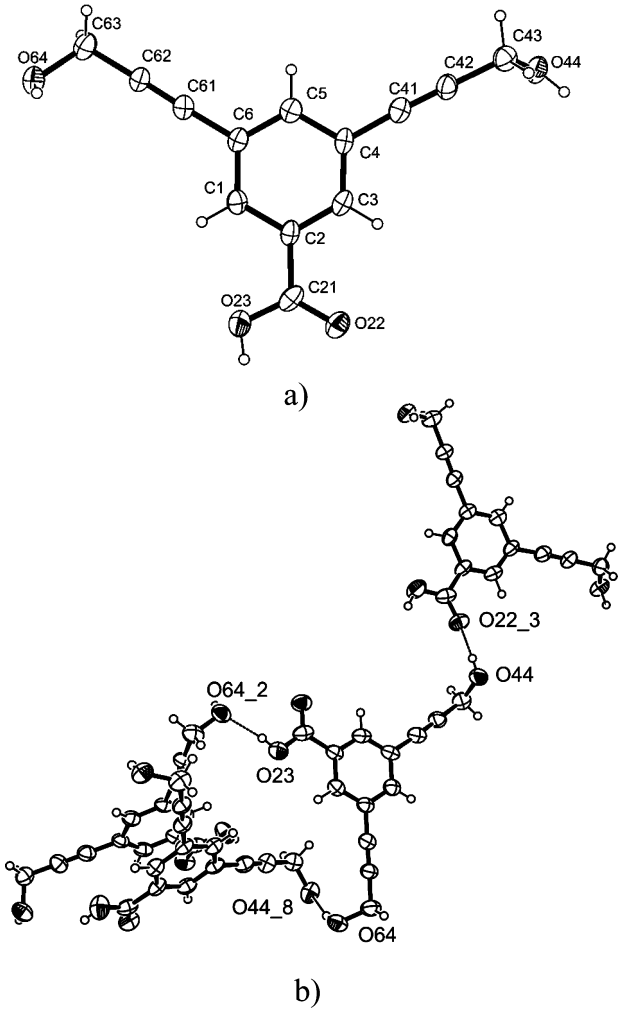


Fig. 2 a) The structure of **5** with ellipsoids at 15% probability and b) the hydrogen bonded array of **5** in the solid state.

Evaluation of cholesterol binding properties of molecular tweezer receptor 1

The molecular tweezer receptor **1** was designed so that the rigid aromatic bis-acetylene arms created a cavity of suitable dimensions for cholesterol uptake between the two hydrophobic cholesterol 'tweezer arms'. Evaluation of the receptor–cholesterol guest binding by the steroidal molecular tweezer receptor **1** in the solution state indicated negligible binding between the molecular tweezer host and cholesterol guest. The solution state binding assay was hindered severely by the solubility of both the tweezer host and the cholesterol guest in the solvents employed. An NMR spectroscopic binding assay was performed in neat $CDCl_3$ as this solvent proved to be the only suitable medium to obtain solubility of both host and guest. Negligible binding was detected between the tweezer host and cholesterol guest. It was decided to pursue the evaluation of the binding properties of this receptor following polymerisation since it has been noted[31] by other research groups that other tweezer-type receptors exhibit enhanced binding properties in the solid phase in comparison to solution phase analysis. Subsequently, despite the poor binding efficiencies demonstrated in the solution state in an apolar solvent, it was predicted that by synthesising *molecular imprinted polymers* using a 1 : 1 mixture of molecular tweezer host : cholesterol guest, effective binding would occur in the solid-state environment. Hence this detrimental solubility factor would not be so significant, and use of polar solvents known to promote hydrophobic binding (such as acetonitrile and isopropyl alcohol) would be feasible.

The 'pseudo-combinatorial' technique pioneered by Lanza and Sellergren[32] was used to generate a large array of molecular imprinted polymers on a small scale in order to assay the cholesterol binding potential of these polymeric receptors. The overall aim of this 'pseudo'-MIP study was the polymerisation of a *1 : 1 tweezer host–cholesterol complex* (the 'template'),‡ in the presence of various functional co-monomers and an excess of the cross-linking agent. This polymerisation would be performed within 1.5 cm^3 HPLC vials, and it was predicted that a 1–2 mm thick polymeric film would be formed upon the base of each vial. Following the polymerisation, it was envisaged that the predominantly hydrophobic interactions could be disrupted by washing the polymers with a good solvent for cholesterol such as THF with addition of a small quantity of acetic acid. After HPLC analysis of the washing solvent revealed that extraction of cholesterol from the polymers was sufficient, it was perceived that 'imprinted' polymeric receptors would result featuring the tweezer receptor **1** within their structure. Following the addition of a solution of known concentration (12.9 mM) of cholesterol to each polymer, the uptake of cholesterol was quantified by HPLC analysis of the supernatant following a suitable period of equilibration (24 hours) of the MIP host and cholesterol guest.

The initial variables that were considered for the composition of the MIPs were as follows: the functional co-monomer, the ratio of template : functional co-monomer, the choice of cross-linker and cross-linking density and porogen selection. Each of these variables was considered individually during the screening process. The composition of a selection of the polymers synthesised is illustrated in Table 1. In all cases the polymerisations were initiated by AIBN at 60 °C.

Choice of functional co-monomer. In this screening assay, various functional co-monomers were tested: methacrylic acid (MAA), styrene, 4-vinylbenzoic acid (VBA), 4-vinylphenol (VP), ethylene glycol dimethylacrylate phosphate (EGDMP),[33] *tert*-butyl methacrylate (*t*BuMA),[34] methyl methacrylate (MMA), and a 1 : 1 mixture of methacrylic acid and styrene.

The key results from the study of the influence of the functional co-monomer on the imprinting efficiency are illustrated in Fig. 3.

The optimum results for cholesterol rebinding were achieved

‡A binding stoichiometry of 1 : 1 was assumed to occur under these conditions between the molecular tweezer and the cholesterol guest system.

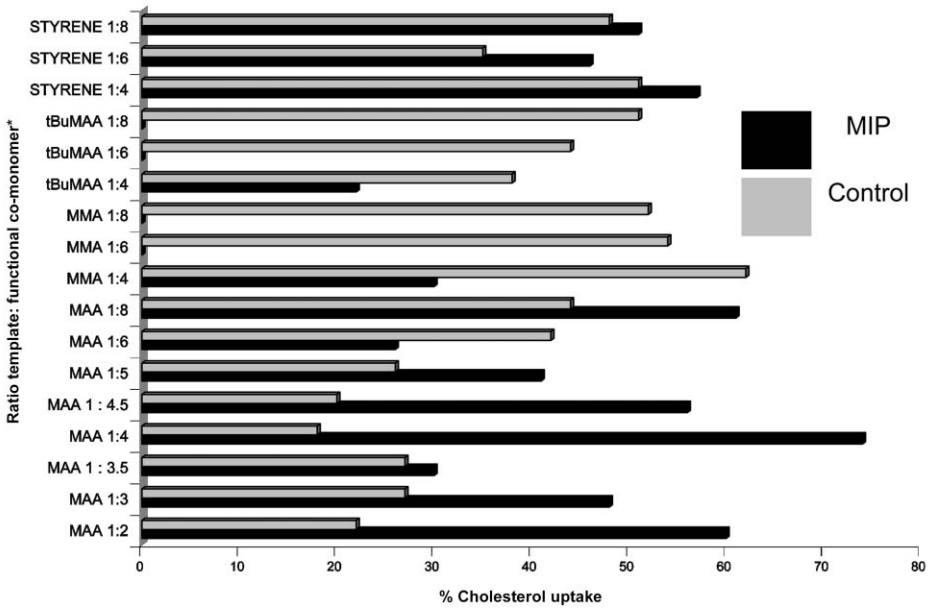


Fig. 3 Effect of choice of functional co-monomer on cholesterol uptake.

using *methacrylic acid* as the functional co-monomer in a 1 : 4 template : monomer ratio, in conjunction with a 73% EDMA density and using THF as the porogen (solvent). The large difference in the percentage binding of cholesterol by the MIP and control polymers indicates that an 'imprinting' effect has occurred. A degree of non-specific binding occurred within these resins, as indicated by the binding of cholesterol by the control polymers albeit to a lesser degree. Furthermore, addition of an extra quantity of cholesterol to these resins did not result in any extended uptake of cholesterol, suggesting that the maximum capacity of these resins had been realised. Methacrylic acid derivatives proved to be not so efficient as functional co-monomers. For example, *tert*-butyl methacrylate yielded inefficient results overall both in terms of cholesterol uptake and recovery. In the case of this functional co-monomer, poor cholesterol recoveries were achieved when the EDMA density was 73%. Furthermore, the uptake of cholesterol by these MIPs proved very inefficient in the case of the higher template : co-monomer ratios. Only in the case of the 1 : 4 template : co-monomer ratio was any significant uptake of cholesterol observed, however, greater uptake was noted for the corresponding control polymer, thereby indicating the presence of only non-specific binding. Decreasing the size of the ester group from the *tert*-butyl to the methyl ester in a 1 : 4 template : monomer ratio yielded slightly higher cholesterol uptake from the MIPs, although higher non-specific binding from the corresponding control was also prevalent.

In the absence of definitive NMR spectroscopic data, we can only speculate upon the mode of binding of the polymers imprinted with the tweezer receptor with the cholesterol substrate. In the case of the methacrylic acid imprinted polymers, it is probable that polar residues (arising from the methacrylic acid) surround the binding cavities created by the tweezer host, from the methacrylic acid. Consequently, selective inclusion of cholesterol into the apolar binding cavities is more probable than non-selective binding to the surrounding polar residues. In the case of hydrophobic co-monomers such as styrene, there are likely to be apolar hydrophobic residues arising from the styrene surrounding the apolar-binding site. Consequently in this situation, there is now competition for the inclusion of the apolar cholesterol guest into the imprinted binding cavities, and a certain degree of non-specific interaction of the cholesterol with these additional hydrophobic residues will occur. In fact, equal binding of cholesterol by the imprinted and blank polymers was observed, thereby supporting this hypothesis. In the case of the methacrylate esters, the hydroxy moiety of the carboxylic acid residues on the polymer is blocked, increasing the apolar nature of these sites, subsequently multiplying the possibility for non-specific binding. Furthermore, there is the possibility of a detrimental steric factor placed upon the cholesterol binding from the bulky *tert*-butyl ester group. It is thought that the overall binding mechanism of cholesterol to the imprinted polymers is a delicate balance between simple hydrogen bonding of the cholesterol hydroxy at C-3 of the steroid backbone with the methacrylic carboxylic acid functionality and the tweezer binding mechanism proposed at the onset of this work.

Inefficient binding of cholesterol was noted for both MIPs and controls synthesised from 4-vinylbenzoic acid, suggesting that this monomer is unsuited to this imprinting protocol. Similarly inferior results were also obtained from a 1 : 1 mixture of methacrylic acid : styrene. These results indicate that a combination of hydrophobic and hydrogen-bonding moieties in the functional co-monomer yield an insufficient imprinting performance. This result was further confirmed by the low cholesterol uptake observed using 4-vinylphenol as the functional co-monomer. EGDMP showed poor binding affinities with cholesterol, in contrast to the literature reports.[33] Ineffective cholesterol recoveries were obtained initially, suggesting a lower number of possible cholesterol binding sites and in general poor cholesterol binding was observed by the MIPs (possibly linked to the lower cholesterol recoveries). Although more moderate cholesterol binding was achieved at higher template : monomer ratios, the binding mode was most likely non-specific, since identical cholesterol binding was observed with the MIPs and the corresponding control polymers.

Choice of cross-linker and cross-linking density. Initially ethylene glycol dimethylacrylate (EDMA) was chosen as the cross-linker. EDMA is the most common cross-linker employed in imprinting protocols,[19] and was found to be more effective than divinylbenzene (DVB) used in the imprinting of cholesterol by Whitcombe and co-workers.[17] DVB and trimethylolpropane trimethacrylate (TRIM) were also tested in this study. The key results from the study of the influence of the cross-linking agent on the imprinting efficiency are illustrated in Fig. 4.

In most cases, the cross-linking density was maintained[19] at 73% in order to assess the effect of variation of functional

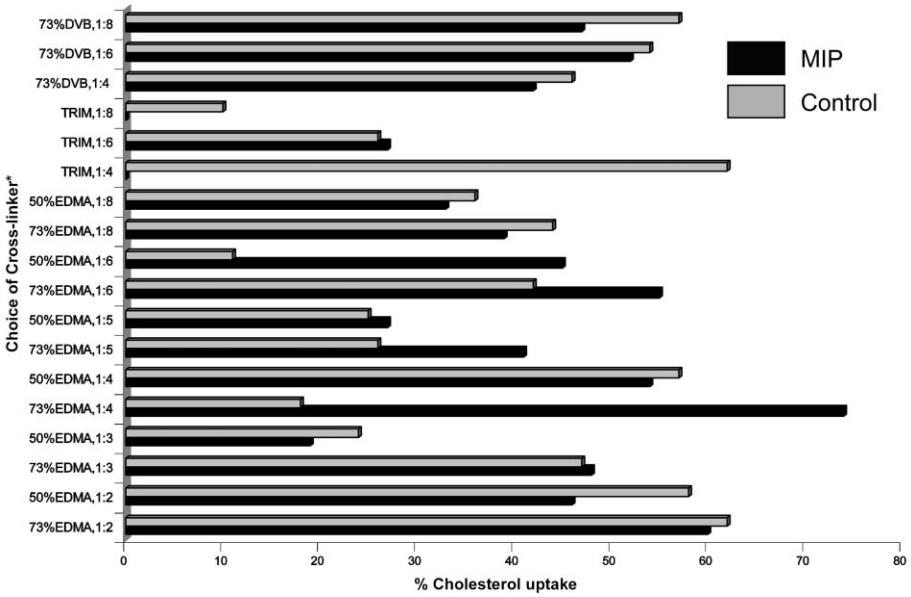


Fig. 4 Effect of choice of cross-linking agent and density on cholesterol uptake.

co-monomer on cholesterol rebinding. It was observed, however, that recoveries of cholesterol from the polymers after completion of the polymerisation were not optimal. These low recoveries are attributed to the high cross-linking density of the polymer hindering the release of the template, despite the use of relatively harsh washing conditions (4 : 1 THF : AcOH). Hence, it was decided to decrease the cross-linking density from 73% to 50% for selected polymers and assess the effect upon the cholesterol recovery. In most cases, this reduction in cross-linking density served only to reduce the overall uptake of cholesterol by the MIPs, thereby suggesting that a more rigid polymer network is a pre-requisite for efficient molecular imprinting techniques.

From this study, it was apparent that EDMA used at 73% cross-linking density in conjunction with a 1 : 4 ratio of template : MAA represented the optimum imprinting reagent combination. The two other cross-linkers tested—DVB and TRIM—both proved less efficient in the production of efficient imprinted materials. In particular, extremely poor results were obtained using the trifunctional cross-linker TRIM. For DVB, in the majority of cases very similar uptakes of cholesterol were noted for the MIPs and their corresponding controls. This further confirms the likely presence of non-specific binding sites present within these MIPs. This situation is particularly significant for the DVB cross-linked polymers based on styrene, thereby emphasising that aromatic functional co-monomers and cross-linkers participate predominantly in *non-specific* hydrophobic interactions with the cholesterol guest and are hence unsuited to this imprinting protocol.

Choice of porogen. The porogen selection was hindered severely by the limited solubility range of both the tweezer host and cholesterol guest. Both substrates presented sufficient solubility in THF and hence this solvent was the porogen of choice at the onset of this study. However, efficient promotion of hydrophobic binding required use of a porogen such as acetonitrile. The solubility of the tweezer and cholesterol was not sufficient in acetonitrile to permit its use alone. A mixture, however, of 50 : 50 THF : acetonitrile could be employed. Likewise isopropyl alcohol is another suitable porogen as this has been shown to be a highly effective solvent for hydrophobic binding involving steroids.[33] Further solubility studies revealed that a combination of 50 : 25 : 25 of THF : acetonitrile : isopropyl alcohol was suitable for use in the imprinting assays.

Despite the use of mixed solvent systems as the polymerisation porogen, the results obtained from the screening assays indicate that the optimum porogen for this pseudo-imprinting protocol was THF. Combinations of THF and acetonitrile afforded mixed results, and higher uptakes of cholesterol were observed from polymers derived from the use of a neat THF porogen. In general, addition of isopropyl alcohol to the porogen proved to have a particularly detrimental effect on cholesterol uptake. This effect was pronounced particularly in the case of the methacrylic acid-based MIPs. It is proposed that the isopropyl alcohol was acting as a hydrogen-bonding competitor with the cholesterol guest. This result further emphasises the importance of secondary hydrogen bonding interactions between the template and the functional co-monomer in this imprinting protocol.

Selectivity studies. Although certain polymers revealed high uptake of cholesterol in comparison to their corresponding blank control polymers, the nature of this cholesterol binding is not certain. It was decided, therefore, to study the uptake of certain cholesterol steroidal analogues for comparison with cholesterol itself. The steroids chosen in this case were cholesteryl acetate and stigmasterol (Fig. 5).

Ideally, the MIPs should exhibit a higher selectivity for cholesterol in comparison to its structural analogues. The MIPs

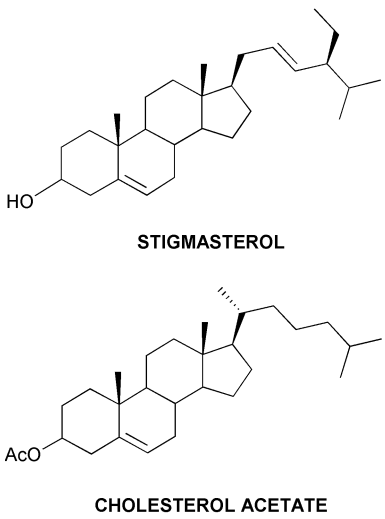


Fig. 5 Structures of the steroids employed in the selectivity study.

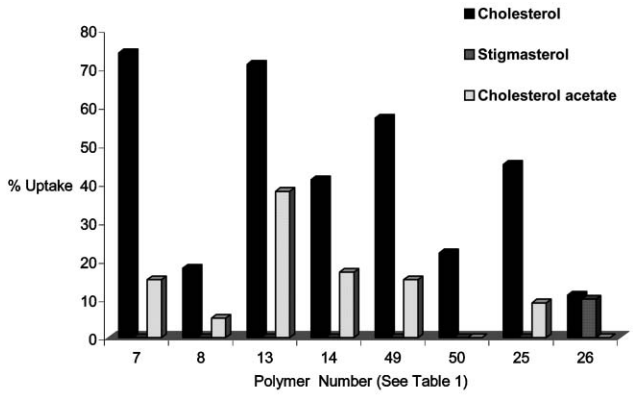


Fig. 6 Substrate selectivity assays carried out using the cholesterol-based molecular tweezer imprinted polymers.

that exhibited the most significant selective binding of cholesterol in comparison to the relevant control *and* the highest levels of cholesterol recovery following the imprinting protocol were chosen for this selectivity study (see Table 1). The results from the selectivity studies are illustrated in Fig. 6.

The polymers showed a negligible uptake of stigmasterol, indicating minimal affinity for this steroid by these MIPs. Cholesteryl acetate was bound to a small extent by all polymers screened in this separate study. This result was predicted, given the extremely close structural similarities of this steroid to the cholesterol template. Cholesteryl acetate differs from cholesterol only in the modification of the C-3 hydroxy of the steroid backbone; hence a potential hydrogen-bonding site is blocked, thereby further supporting evidence of selective hydrogen bonding in the polymers.

Given the overall results obtained from this chromatographic screening process, it is apparent that the optimum conditions for the synthesis of molecular imprinted polymers featuring the steroid molecular tweezer are the use of methacrylic acid as the functional co-monomer in a ratio of four equivalents to the template with a cross-linking density of 73% EDMA in a THF porogen, initiated by AIBN at 60 °C.

Significance of the tweezer cavity. To assess the influence of the tweezer-binding cavity on cholesterol uptake, the monomer cholesteryl (4-vinyl)phenyl carbonate used by Whitcombe and co-workers for the imprinting of cholesterol was synthesised according to the published procedure.[17] Cholesteryl (4-vinyl)phenyl carbonate can be considered a 'single-armed' analogue of tweezer receptor **1**, and consequently differs from receptor **1** as a result of the lack of a formal 'binding cavity'. Molecular imprinted polymers were synthesised for cholesteryl (4-vinyl)phenyl carbonate using the conditions deemed to be optimum for receptor **1** (4 equivalents methacrylic acid, 73% EDMA, THF), and the binding capacities of cholesterol compared for each receptor.

Promisingly, imprinted polymers based on cholesteryl (4-vinyl)phenyl carbonate revealed minimal uptake of cholesterol (average 8% from solution). This result compares favourably with the average cholesterol uptake of 74% exhibited by the two-armed tweezer receptor **1**, thereby highlighting the need for a well-defined cavity such as that created by the two arms of receptor **1**.

Application of the imprinting protocol to molecular imprinted monoliths

Having developed the optimum molecular imprinting protocol for efficient cholesterol uptake, it was decided to apply these conditions to the synthesis of novel monoliths[35] featuring our steroidal tweezer receptor. The optimum macroporous stationary phase material composition thus determined was used subsequently to construct monolithic solid phase extraction (SPE) columns for use in the selective extraction of cholesterol in the presence of the previously studied structural analogues. In recent years, monolithic stationary phases[35] have been used to overcome the problems associated with conventional particle-packed HPLC columns, for example, issues relating to permeability. Furthermore, monolithic stationary phases are used to accelerate mass transfer processes yielding increased performance at elevated flow rates.[34] Monolithic materials of this type also have the advantage over their more conventional counterparts in the speed of their preparation. A typical protocol for the synthesis of a macroporous monolithic stationary phase involves simply mixing the polymerisation components in an empty column, and then heating this column until polymerisation is complete. This approach compares favourably with the more conventional stationary phase synthesis, in which the material is first polymerised, then crushed and ground before packing into the analysis column under high pressure. Although this process often affords highly efficient stationary phases, it is time-consuming.

For synthesis of monoliths featuring the steroid tweezer receptor, it was decided to employ clean, short (50 mm × 4.6 mm id) stainless-steel columns both to minimise analysis times and reduce the back pressures during the chromatographic analysis. The optimum molecular imprinting conditions described above were applied to the synthesis of two sets of monolithic columns (one MIP and one corresponding control/blank column per set). The results from this initial study are illustrated in Table 2.

Encouragingly, the imprinted monoliths exhibited similar properties to their corresponding polymers in the pseudo-imprinting screening assay, both in terms of cholesterol affinity and selectivity. In both cases, the templated monoliths revealed acceptable capacity factors for cholesterol in comparison to the relevant blank columns, prepared in the absence of the tweezer receptor and cholesterol guest (average $K_a = 1.36$). This is reflected in the observed separation factor α of 5.44 for the imprinted monoliths in comparison to the blank untemplated polymers. 100% THF was employed as the mobile phase to prevent undesirable swelling of the polymeric resins. Use of low chromatographic flow rates (0.05 cm^3 min^{-1}) was desirable as a result of the short column length—when higher flow rates were employed there was insufficient time to enable the establishment of an efficient binding equilibration between cholesterol and the imprinted stationary phase. Consequently the good uptake of cholesterol observed at such low flow rates may be attributed to the realisation of effective thermodynamically controlled binding equilibration thereby indicating that uptake maybe predominantly occurring under kinetic control. It is, however, also possible that kinetics were inhibiting mass transfer at such higher flow rates.

Furthermore the imprinted monoliths revealed excellent selectivity for cholesterol, in that the structurally related sterols stigmasterol and cholesteryl acetate were retained on the column to a far lesser extent. Indeed, negligible retention times were noted for stigmasterol on the column, and this is consistent with the selectivity study performed during the screening assays.

Table 2 Performance of molecular imprinted monoliths for application in SPE[a]

	$K_{a(MIP)}$	$K_{a(CONTROL)}$
Cholesterol	1.36	0.25
Cholesterol acetate	0.04	0.10
Stigmasterol	0	0.08
Selectivity factor α	5.44	—

[a]Average values are quoted for the capacity factor k_a and the selectivity factor α.

Conclusions

A novel macroporous stationary phase material bearing tweezer receptors that are specific for cholesterol has been constructed from rigid multifunctional vinylic monomers derived from 3,5-dibromobenzoic acid, propargyl alcohol and cholesterol. Co-polymerisation of this tweezer monomer in conjunction with methacrylic acid and a large excess of cross-linking agent *via* a 'pseudo' molecular imprinting approach afforded a diverse set of macroporous materials whose selectivity and efficacy for cholesterol binding was assessed using a chromatographic screening process. The optimum macroporous stationary phase material composition was found to be a methacrylic acid-based imprinted polymer, prepared using a cross-linking density of 73% EDMA, in conjunction with THF as the porogen under AIBN-initiated thermal conditions. Furthermore, the efficiency of the formal binding cavity created by the tweezer receptor was assessed by comparison to an analogous 'one-armed' tweezer receptor. The MIP composition thus determined was subsequently used to construct monolithic solid phase extraction cartridges for use in the selective extraction of cholesterol in comparison to certain structurally related steroids.

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