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Matrix effects on the selectivity of a cholesterol-imprinted polymer

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Abstract

The effect of cross-linkers ethyleneglycol dimethacrylate (EGDM) and divinylbenzene (DVB) on the recognition properties of cholesterol-imprinted polymers, prepared by the sacrificial spacer method, was investigated. As reported previously EGDM-based polymers selectively bound cholesterol in preference to cholest-5-ene-3-one. The addition of up to 30% DVB led to an increase in the binding capacity with very little trade-off in selectivity. However polymers prepared with pure DVB, or DVB/styrene showed complete reversal of selectivity, in some cases binding cholest-5-ene-3-one to the exclusion of cholesterol from a solution containing both ligands. An explanation based on template conformation has been proposed to account for these observations.

Keywords: Cholesterol; Molecular imprinting; Selectivity

1. Introduction

In the mid 1990s we introduced the concept of molecular imprinting with sacrificial spacers, combining some of the advantages of a covalent template with non-covalent rebinding. The first example of this approach was the preparation of a cholesterol-imprinted polymer using cholesteryl (4-vinyl)-phenyl carbonate (CVPC) as the template monomer [1]. When co-polymerised with ethyleneglycol dimethacrylate (EGDM) and hydrolysed with base, the template is cleaved from the polymer with the loss of the carbonyl spacer as CO_2. The resultant polymer was found to bind cholesterol with a single dissociation constant, which differs fundamentally from the behaviour of most non-covalently imprinted polymers. This was highlighted by a comparison of the affinity spectra of a number of polymers prepared by different imprinting protocols in a study by Umpleby et al. [2]. Another advantage of the method is a greater compatibility with water than the most commonly used imprinting chemistries, allowing the use of aqueous suspension [3] and emulsion [4] polymerisations for the preparation of imprinted beads and core-shell nanoparticles, respectively, as well as with other challenging polymerisation conditions e.g. the preparation of enantioselective menthol-imprinted polymers by ring-opening metathesis polymerisation (ROMP) [5,6] and the preparation of imprinted polymers by nitroxide-mediated living radical polymerisation [7].

The mechanism of non-covalent rebinding to the cholesterol-imprinted polymers was postulated to be the formation of a hydrogen bond between the phenolic residue present in the imprinted sites and the cholesterol −OH group. This hypothesis was supported by several lines of evidence: (i) blocking the phenolic group by esterification with acetyl or benzoyl chloride, or the presence of a competing alcohol in the binding solution both suppressed the uptake of cholesterol by the polymer; (ii) binding was only seen in non-polar solvents; (iii) cholestane and cholesteryl acetate were not bound to the polymer whereas other analogues bearing oxygen at C3 (epicholesterol and cholest-5-ene-3-one) showed some, albeit reduced binding to the polymer.

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One observation that we reported in our earlier papers [1,3,4] was a much reduced binding of cholesterol to divinylbenzene (DVB)-based polymers compared with their EGDM-based equivalents. At the time this was thought to be due to the superiority of methacrylate-based cross-linkers with short flexible linkers over rigid cross-linkers, such as DVB [8]. Recent reports however, in particular those from the group of Spivak [9–11], have highlighted the role of the cross-linker in the imprinting process as being much more subtle than merely providing a means of ''casting'' the shape of the template in the imprinted site.

While the effect of cross-linker on the selectivity of covalently [8,12] and non-covalently [9,13–15] imprinted polymers has been relatively well studied, the same is not true of the semi-covalent methods, where the use of DVB is often preferred as a precaution against degradation of the matrix during template removal [16–20]. There is therefore little known about the effect of the cross-linker on the selectivity of polymers of this type. The aim of this communication is to redress this situation by reporting the results of an investigation into the recognition properties of polymers imprinted with cholesterol by the carbonate ester sacrificial spacer methodology, prepared with EGDM, DVB or a mixture of the two. The data, presented and discussed below, are concerned with the binding of cholesterol and its close structural analogue, cholest-5-ene-3-one to these polymers.

2. Experimental

2.1. Materials and methods

Cholesterol, cholest-5-ene-3-one, cholesteryl chloroformate, 4-acetoxystyrene, ethyleneglycol dimethacrylate (EGDM) and divinylbenzene (80% tech.) (DVB) were obtained from Aldrich Chemical Co. (Gillingham, UK). Azo-bis-isobutyronitrile (AIBN) was obtained from Fluka and recrystallised from methanol. EGDM and DVB were washed with sodium hydroxide solution to remove initiators and dried before use. Hexane and toluene used for polymerisations were distilled over calcium hydride. HPLC analyses were performed using Gilson 303 pumps equipped with an ACS light-scattering mass detector and a Shimadzu SIL-9A autosampler. Samples were analysed on a 250 × 4.6 mm i.d., 5 μm Spherisorb column (Anachem or Hichrom) at room temperature using a flow rate of 1.5 mL min^{-1} and a linear gradient from 20% v/v ethyl acetate: hexane to 100% ethyl acetate over 8 min. Data collection was performed using HP Chemstation software running on the PC platform. Non-linear curve-fitting to extract the Langmuir binding parameters was performed using GraFit software (Erithacus Software Ltd.) by the method described previously [1].

2.2. Cholesteryl (4-vinyl)phenyl carbonate (CVPC)

This compound was synthesised from 4-vinylphenol and cholesteryl chloroformate according to the previously published protocols [1].

2.3. Polymer synthesis, mixed cross-linker series

Polymers were synthesised by bulk polymerisation, using the previously published procedure [1]. Monomer mixtures (5 g in total) and initiator (see Table 1) were placed in quickfit test tubes with *n*-hexane (9 mL) and toluene (1 mL), except for polymer **P8** (prepared on a 10 g scale) where these quantities were doubled. Tubes were fitted with a stopcock and degassed by a sequence of freeze-pump-thaw cycles on a vacuum line. Tubes were transferred to a water-bath at 65 °C, shaken to ensure dissolution of the contents, and allowed to polymerise for 24 h. Polymer was broken-up, washed with methanol, dried and ground in a mechanical mortar. The ground polymer was extracted with methanol in a Soxhlet apparatus for 18 h and dried in vacuo at 70 °C.

2.4. Polymer synthesis, template loading series

Polymers were synthesised and worked up as above, using the monomer and initiator masses given in Table 2. The porogenic solvent mixture used in each case was *n*-hexane (7.2 mL) and toluene (0.8 mL).

2.5. Template cleavage

Polymer (2 g) was weighed into a 50 mL round bottom flask with 1 g of NaOH and a small magnetic follower was added. Methanol (25 mL) was added and the flask clipped to a condenser and lowered into an oil bath at 90 °C. The

Table 1
Polymer composition, masses and molar quantities of monomers and initiator used in the mixed cross-linker study

Polymer	Mass of monomer, g			Molar quantities, mmol (mol%)			Initiator AIBN (1 mol%, g)
	DVB	EGDM	CVPC	DVB	EGDM	CVPC	
P1	0	4.700	0.300	0	23.71 (97.68)	0.56 (2.32)	0.0788
P2	0.514	4.171	0.316	3.95 (15.43)	21.04 (82.25)	0.59 (2.32)	0.0817
P3	1.085	3.581	0.334	8.33 (30.84)	18.07 (66.84)	0.63 (2.32)	0.0850
P4	1.726	2.920	0.354	13.26 (46.27)	14.73 (51.41)	0.66 (2.32)	0.0887
P5	1.957	2.682	0.361	15.03 (51.41)	13.53 (46.27)	0.68 (2.32)	0.0900
P6	2.711	1.905	0.385	20.82 (66.84)	9.61 (30.85)	0.72 (2.32)	0.0943
P7	3.569	1.019	0.412	27.41 (82.26)	5.14 (15.42)	0.77 (2.32)	0.0992
P8	9.115	0	0.885	70.01 (97.68)	0	1.66 (2.32)	0.2097

The total mass of monomer was 5 g in all cases except **P8**, which was prepared on a 10 g scale.

Table 2
Quantities of monomers and initiator used in the preparation of the series of DVB-based polymers with variable template loading

Polymer	Mass of monomer, g			Molar quantities, mmol (mol%)			Initiator AIBN (1 mol%, g)
	DVB	Styrene	CVPC	DVB	styrene	CVPC	
PL1	3.223	0.612	0.166	24.76 (80)	5.88 (19)	0.31 (1)	0.0833
PL2	3.119	0.562	0.320	23.96 (80)	5.40 (18)	0.60 (2)	0.0807
PL5	2.845	0.427	0.730	21.85 (80)	4.10 (15)	1.37 (5)	0.0736
PL10	2.482	0.248	1.270	19.06 (80)	2.38 (10)	2.38 (10)	0.0642
PL15	2.201	0.110	1.691	16.91 (80)	1.06 (5)	3.17 (15)	0.0569
PL20	1.977	0	2.024	15.19 (80)	0 (0)	3.80 (20)	0.0511

All polymers were prepared on a 4 g scale.

contents were stirred under reflux for 6 h. The cooled flask contents were added to an excess of dilute HCl. After stirring for 15 min, the polymer was recovered by filtration and washed on the sintered glass funnel with water, methanol and finally diethyl ether. The yield of cholesterol cleaved from the polymer was determined gravimetrically, by extraction of the washings [1]. Hydrolysed polymers were extracted with methanol by Soxhlet extraction and dried in a vacuum oven, as described above.

2.6. Uptake experiments, single ligand study

Polymers both hydrolysed and non-hydrolysed were weighed (20 mg) into 2 mL capacity screw-cap vials. A solution (1 mL) of cholesterol or cholest-5-ene-3-one (2 mM) in *n*-hexane was added to the vials. Two replicates were made for each combination of polymer and ligand. The vials were closed and shaken at 20 °C overnight. The supernatant was separated by filtration through a 2 μm porosity, 13 mm PTFE membrane filter, attached to a disposable syringe. The filtered solutions were transferred to HPLC vials and the concentration of the ligands in the supernatant was determined by HPLC analysis.

2.7. Uptake experiments, competitive binding study

The hydrolysed DVB-based polymer, **P8H**, was weighed into screw-cap vials, as above, using 10, 20, 30, 40, 50, 60, 80 or 100 mg polymer per vial in duplicate. Vials containing the polymers **PL1H** and **PL2H** were similarly prepared using 10, 20, 30, 40, 50 or 60 mg polymer in single replicates only. A solution containing cholesterol (2 mM) and cholest-5-ene-3-one (2 mM) in *n*-hexane (1 mL) was added to each vial. The vials were shaken overnight and the concentration of both ligands in the supernatant was determined by HPLC, as described above.

3. Results and discussion

3.1. Polymer synthesis

Two series of polymers were prepared: the first set of eight polymers was made with a constant (2.32 mol%) amount of the template monomer, but varying the molar ratio of the two cross-linkers EGDM and DVB (Table 1); the second set of six polymers was prepared with a constant (80 mol%) proportion of DVB, varying the template loading, styrene being used to balance the composition (Table 2). In order to more closely match the cross-linking across the range of polymers, the purest form of DVB (80% tech., Aldrich) available at the time was used. For the purpose of calculating the molar compositions, the remainder was assumed to consist of ethylstyrene.

Polymers were prepared by conventional bulk polymerisation using the optimum porogen mixture (toluene:*n*-hexane, 1:9 v/v, 2 mL g^{-1}) found for cholesterol recognition in EGDM-based polymers. Grinding, washing and template removal were all performed in accordance with the previously published protocols [1]. In the following discussion, polymers will be referred to by the codes given in Tables 1 and 2 for the unhydrolysed resins and by the same codes followed by "H" for the resins which have been subjected to basic hydrolysis to remove template.

3.2. Composition and structure

The effect of polymer composition upon polymer structure can be seen in a comparison of the polymer surface areas (Fig. 1a) for the mixed cross-linker set. The starting point for this series, **P1**, was similar to our previously optimised material for cholesterol binding. The porogen mixture used was found to give a moderate (52.5 $m^2 g^{-1}$) surface area (to control the amount of non-specific binding to the resin) and good access to the template sites for hydrolysis in a 5 mol% CVPC, 95 mol% EGDM polymer [1]. This is still more or less true of **P1**, although the slightly higher level of cross-linker has produced a material with a somewhat lower surface area (18 $m^2 g^{-1}$). Whilst there is a smooth variation across the composition range, the dependence of surface area on cross-linker composition is not a simple one, with a partial peak at **P3** and a minimum at **P5**, followed by an increase to the maximum at **P8**.

The extremes of this plot can be explained by a switch in the phase behaviour of the polymerisation from the macroporous domain at **P1** to the microporous (gel-like) domain at **P8** in their respective phase diagrams. This reflects the different interactions of the two cross-linkers and their polymers with the porogen mixture. The situation between these two extremes is not clear cut, as can be seen from **P5**, which has

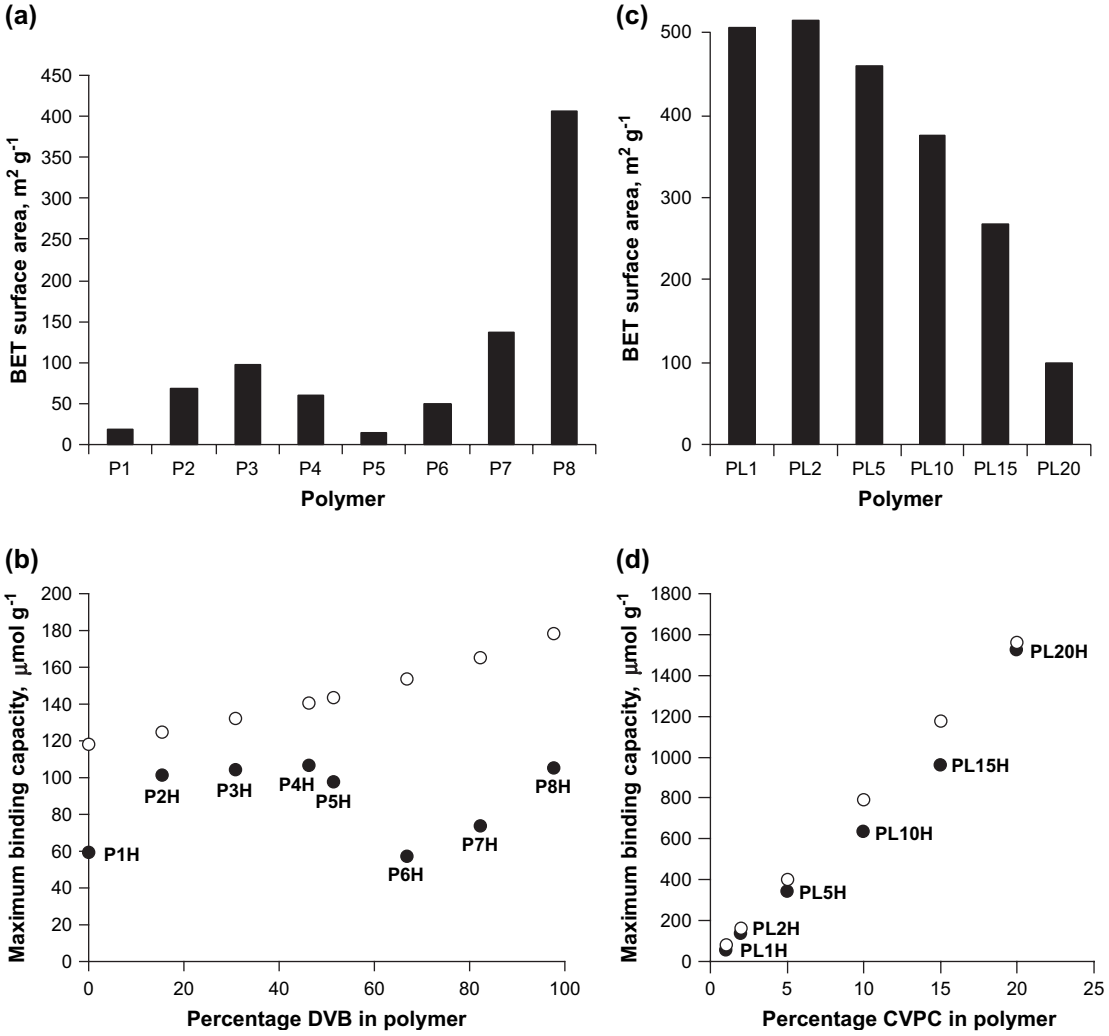

Fig. 1. BET surface areas measured for: (a) polymers prepared with mixtures of cross-linker and (c) the template loading series and (b) and (d): the theoretically (○) and experimentally determined (●) maximum yield of imprint sites as a function of polymer composition for the same two series, respectively. The theoretical capacities have been calculated from the yield of recovered cholesterol in all cases except **PL20H**, where it has been calculated from the mass loss following hydrolysis.

the lowest measured surface area of any member of this series (14 $m^2 g^{-1}$), yet the yield of imprint sites for **P5H**, (98 μmol g^{-1}), as determined by the release of template on hydrolysis (Fig. 1b) is only slightly lower than the maximum measured capacity (106 μmol g^{-1}, **PL4H**) whereas its other neighbour in this series, **P6**, has a higher surface area (50 $m^2 g^{-1}$) but the lowest degree of hydrolysis of any of the materials (57 μmol g^{-1}). The lack of correlation between the surface areas of the polymers and the yield of hydrolysis may be a reflection of the interaction of the solvent used in the hydrolysis step (methanol) with the microstructure of the polymers. Shea et al. used the quenching of fluorescent probes in macroporous DVB-based polymers to determine the diffusivity of ionic reagents [21] and to investigate chain solvation in a similar system [22]. The latter study concluded that the degree of hydrolysis of a ketal-based template in methanol did not correlate with the macroscopically observed properties of the polymers, such as their swelling behaviour in the same solvent. It appears that similar chain solvation effects may be operating in the mixed cross-linker series of polymers prepared in the present study.

The surface areas (Fig. 1c) and capacities (Fig. 1d) of the loading series both show much simpler behaviour, with a high surface area in the initial members of the series, dropping off as the percentage of template (and the corresponding capacity) increases. A comparison of the most similar pair of materials from the two series shows an increase in the surface area of around 27% (from 406 to 516 $m^2 g^{-1}$) when the level of cross-linker was reduced from 97.7 mol% (**P8**) to 80 mol% (**PL2**). The later members of this series are vastly overloaded from an imprinting point of view, which is compounded by the high molecular weight of the template. While DVB appears to dominate the structure and phase behaviour of the polymer at 1 or 2 mol% loading (**PL1** and **PL2**), as may be inferred by their almost identical surface areas (507 and 516 $m^2 g^{-1}$, respectively), higher template loadings lead to a reduction in surface area, already noticeable at 5 mol% template (461 $m^2 g^{-1}$ for **PL5**) and steadily decreasing (to 98 $m^2 g^{-1}$

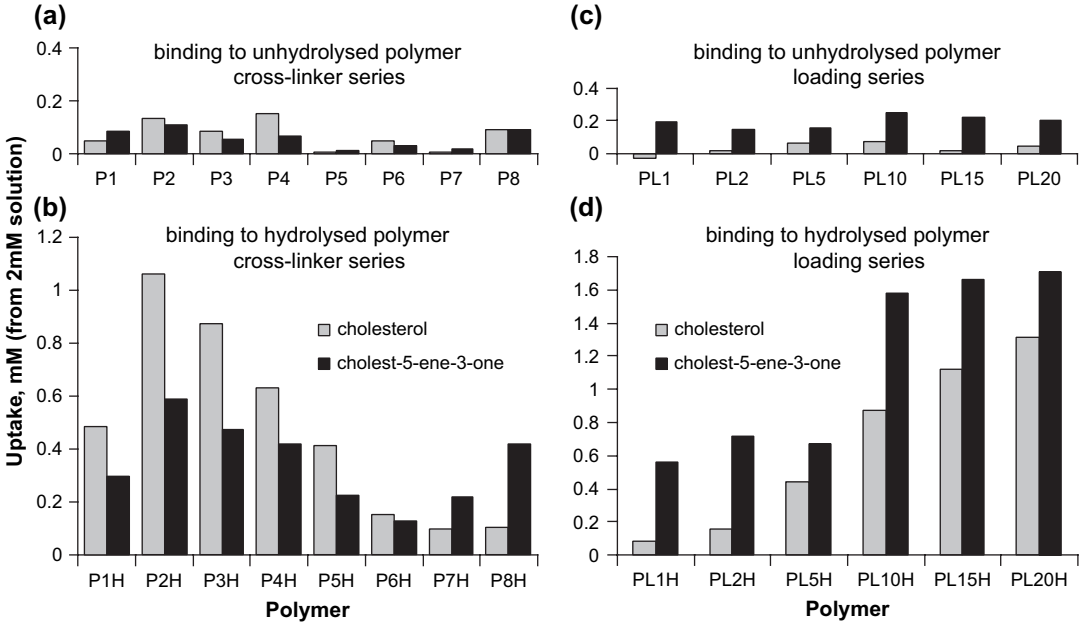

Fig. 2. Results of single ligand binding experiments with cholesterol and cholest-5-ene-3-one (2 mM) and polymer (20 mg mL^{-1}) for: (a) unhydrolysed polymer (mixed cross-linker series); (b) hydrolysed polymer (mixed cross-linker series); (c) unhydrolysed polymer (DVB-based template loading series); (d) hydrolysed polymer (DVB-based template loading series).

for **PL20**) as the mass of template monomer in the polymerisation mixture increases.

3.3. Binding experiments, single ligands

The single ligand binding data for both series of imprinted polymers are presented in Fig. 2, along with the uptake measured for the corresponding unhydrolysed polymers as control. The EGDM-rich polymers, **P1H**–**P3H** (Fig. 2b) behaved as expected, showing a higher binding of cholesterol than the analogous ketone. It appears that a small percentage of DVB in the cross-linker is actually beneficial in this series as **P2H** appears to show both the highest capacity for cholesterol and the most selective binding, presumably due to the higher surface area and greater degree of template hydrolysis. If the binding to the unhydrolysed polymer (Fig. 2a) is subtracted however, to compare "specific" uptake, **P1H** becomes the most selective polymer with a cholesterol:cholest-5-ene-3-one binding ratio of 2.1, however **P2H** and **P3H** are only slightly worse with ratios of 2.0 and 1.9, respectively. Both the binding capacity and selectivity drop off across the series until the DVB-rich region, represented by **P7H** and **P8H**, is reached. In this region of the phase diagram the binding selectivity switches from cholesterol to favouring binding of the ketone. When DVB alone is used as the cross-linker (**P8H**) the ratio of binding is 4.2 in favour of the ketone. If the correction for binding to the unhydrolysed polymer is applied, this number increases to 39.3. These results confirm our previous observation that DVB-based polymers did not show significant uptake of cholesterol, but the apparent highly selective uptake of the ketone analogue was a surprise.

The behaviour of **P8H** is mirrored in the loading series (Fig. 2c and d), with higher binding for cholest-5-ene-3-one across all samples. The first two members of this series, **PL1H** and **PL2H** appear to be highly selective for the ketone, but **PL5H** shows poor selectivity, perhaps due to overloading, resulting in some loss of site isolation. The theoretical capacity of **PL10H** at 20 mg mL^{-1} is approximately equal to (and the capacities of **PL15H** and **PL20H** are in excess of) the amount of ligand in a 2 mM solution, and therefore no definite conclusion about the selectivities of these materials can be drawn from the current experiment, except that they are clearly also binding ketone in preference to cholesterol. While hydrolysis of template is clearly responsible for the binding observed, i.e. there is an imprinting effect, the matrix determines the ligand selectivity.

3.4. Competitive binding experiments

To investigate how selective the binding of cholest-5-ene-3-one to DVB-based polymers was, a series of mixed ligand competitive binding experiments were carried out with selected polymers. The results of these experiments using **P8H**, **PL1H** and **PL2H** are shown in Fig. 3, where solutions containing both cholesterol and cholest-5-ene-3-one (2 mM + 2 mM) were incubated with polymer concentrations between 10 and 100 mg mL^{-1}. Under competitive conditions binding of cholesterol was almost completely suppressed with **P8H** and **PL1H** and a minor uptake was seen for **PL2H** at the higher concentrations of polymer, whereas binding of the ketone increased with increasing polymer concentrations in all cases. The binding curves could be fitted to a one-site binding model [1] which gave the following parameters: $K_d = 0.7 \pm 0.1$ mM,

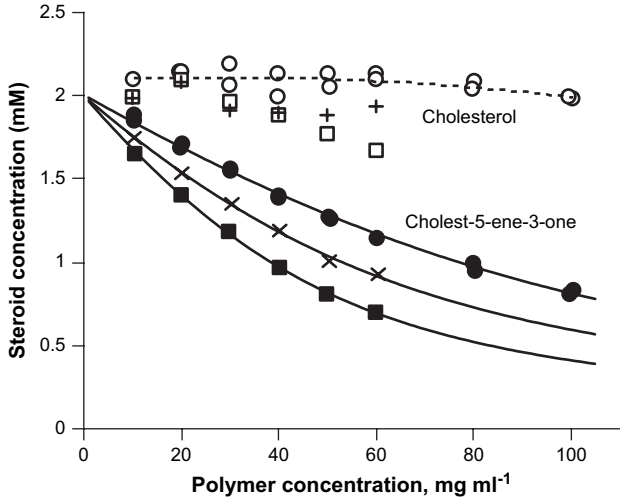

Fig. 3. Concentrations of cholesterol and cholest-5-ene-3-one measured in the supernatant of solutions (2 mM in both ligands) treated with different concentrations of the polymers **P8H**: cholesterol (○), cholest-5-ene-3-one (●); **PL1H**: cholesterol (+), cholest-5-ene-3-one (×); **PL2H**: cholesterol (□), cholest-5-ene-3-one (■), showing selective uptake of the ketone in each case. The solid lines are the theoretical binding curves calculated for ligand–polymer dissociation constant (K_d) of 0.7 mM and a maximum binding capacity (B_{max}) of 22 μmol g^{-1} for **P8H**; $K_d = 1.0$ mM, $B_{max} = 37$ μmol g^{-1} for **PL1H** and $K_d = 0.8$ mM, $B_{max} = 47$ μmol g^{-1} for **PL2H**, assuming a one-site binding model in each case (parameters were obtained by fitting the experimental points for the ketone data for each polymer). The dashed line shows the trend for the cholesterol concentration with **P8H** and is for guidance only.

$B_{max} = 22 \pm 2$ μmol g^{-1} for **P8H**; $K_d = 1.0 \pm 0.3$ mM, $B_{max} = 37 \pm 6$ μmol g^{-1} for **PL1H** and $K_d = 0.8 \pm 0.1$ mM, $B_{max} = 47 \pm 2$ μmol g^{-1} for **PL2H**, the last two materials having a reduced data set with respect to **P8H**. These parameters were used to calculate the unbroken lines in Fig. 3 which show excellent agreement with the experimental data in all cases. The fact that all three of these materials show selective binding for cholest-5-ene-3-one in competitive binding experiments suggests that the effect is general for CVPC–DVB copolymers at low template loadings.

The apparent dissociation constants for cholest-5-ene-3-one binding to the three materials above are very similar and quite close to that determined for cholesterol binding to EGDM-based polymers under similar solvent conditions (0.6–0.8 mM) [1]; however the measured capacities (B_{max}) are significantly different and account for the shape of the binding curves for the three materials. The capacities for all these materials are a fraction of the theoretical or experimentally determined maxima (21% for **P8H**, 70% for **PL1H** and 35% for **PL2H**, based on template recovery) which suggests that only a subset of the potential sites are active in binding. The same observation was also made for cholesterol binding to the EGDM-based polymers [1] where a site population of 114 μmol g^{-1}, determined from fitting the binding isotherm, accounted for only 61% of the experimentally determined maximum binding capacity, based on cholesterol recovery, for a 5 mol% template-loaded polymer. This suggests that there is a conformational aspect to the templating in these polymers which is influenced by the polarity or structure of

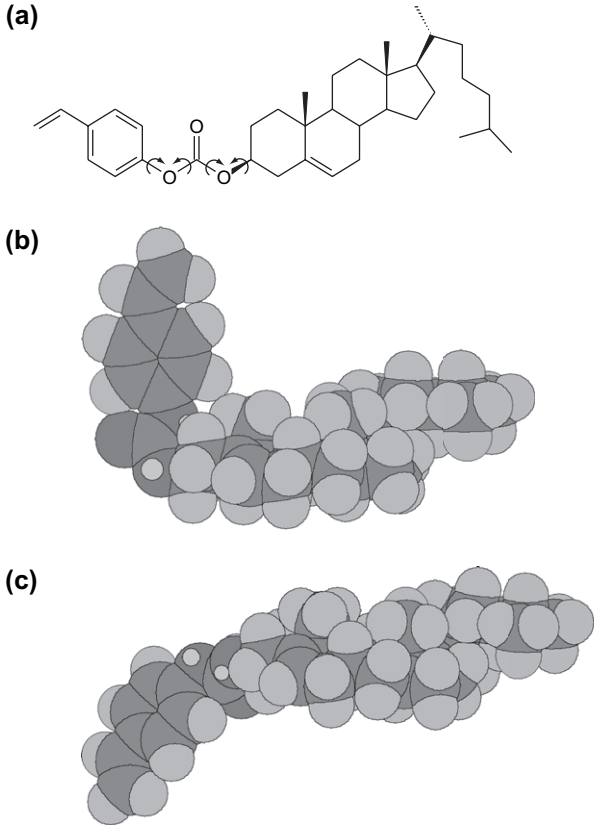

Fig. 4. Structure of cholesteryl (4-vinyl)phenyl carbonate (CVPC) showing: (a) single bond rotors in the carbonate unit and (b) and (c) two possible conformations of the template molecule as space-filling models (local minima determined using CS Chem3d Pro, Vn 3.2, Cambridgesoft Corp.) showing the relative disposition of the cholesteryl and vinylphenyl groups.

the cross-linker. Indeed rotation can occur about the four single bonds of the carbonate group (Fig. 4a) and a number of possible conformational forms of the molecule are possible (Fig. 4b and c). While these should be rapidly interconverted in solution, as the polymerisation proceeds and the solid matrix is formed, rotation will cease and the local conformational minima will become fixed (the energy barrier for rotation will become very great) leading to discrete subsets of the imprinted sites. The relative population size of each of these imprinted site structures will be determined according to whether CVPC is co-polymerised into a predominantly styrenic or methacrylic polymer backbone. Selectivity in rebinding can then either arise from the relative populations of the different conformations or their ease of hydrolysis, or a combination of both factors.

While the strengths of hydrogen-bond interactions between a phenol and cholesterol or cholest-5-ene-3-one will be significantly different in solution, in the polymer a non-ideal configuration of the hydrogen bond (rather than the linear O–H–O configuration preferred in solution) will be imposed by the constraints of the recognition site. This shows how rather subtle changes in geometry might give rise to selectivity effects. In contrast, in non-covalently imprinted polymers one would expect a near ideal geometry to be achieved, since the pre-polymerisation complex more closely resembles the rebound

state of the template to the polymer and accounts for the lower K_ds seen more generally in non-covalently imprinted polymers. Subtle structural effects have been observed in these polymers too however. A study by Spivak et al. examined the selectivity of polymers, imprinted by interaction with a single functional monomer, using a range of amine templates imprinted, by conducting a comprehensive structure–binding relationship study [23]. The authors point out that Van der Waals forces are much more significant at the rebinding stage than in solution (pre-polymerisation complex) and highlight the importance of the supporting matrix in determining polymer selectivity.

4. Conclusions

The nature of the cross-linker has been found to exert a profound influence over the binding properties of imprinted polymers prepared by the sacrificial spacer method using CVPC as the template. Substituting a small amount of DVB for EGDM resulted in a greater than two-fold increase in binding capacity for cholesterol, with little trade-off in selectivity. More significantly however, in DVB–styrene mixtures or DVB alone, a complete reversal in ligand preference was seen. This suggests that the binding selectivity, at least in the case of polymers imprinted using semi-covalent methodologies, can be manipulated by the choice of cross-linker. Furthermore the use of mixtures of cross-linkers is an area largely unexplored in imprinted polymer synthesis and can yield some unexpected results, and as recent reports have shown [11], cross-linkers are functional monomers too!

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