A New Method for the Introduction of Recognition Site Functionality into Polymers Prepared by Molecular Imprinting: Synthesis and Characterization of Polymeric Receptors for Cholesterol

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Abstract: A novel approach to the introduction of recognition site functionality into highly cross-linked polymeric matrices *via* molecular imprinting has been developed for compounds with single (or multiple, spatially separated) hydroxyl groups. This new methodology relies on the use of the 4-vinylphenyl carbonate ester (**1**) which functions as a covalently bound template monomer but is easily and efficiently cleaved hydrolytically with the loss of CO_2. This results in the formation of a noncovalent recognition site, bearing a phenolic residue, capable of interacting with the ligand (template) through hydrogen bonding. The polymers obtained by this method were shown to bind cholesterol with a single dissociation constant, thus displaying characteristics similar to a true biological receptor or synthetic host. It has also been shown that the phenolic residues introduced into the recognition site play an important role in the interaction with ligand, as evident from the suppression of cholesterol binding by chemical modification of the polymer with acetyl and benzoyl chlorides and by the addition of solvents which tend to disrupt hydrogen-bonding.

Introduction

Molecular imprinting has received much attention recently as a means of creating spatial memory of template molecules in highly cross-linked polymeric matrices.[1-5] The resulting polymers have been investigated as HPLC stationary phases for chiral resolutions,[6-14] and initial studies have been reported in the areas of synthetic chemistry,[15,16] catalysis,[17-21] sensor design,[22] the use of imprinted polymers as substitutes for antibodies in immunoassays,[23] and for protein separation.[24]

Two distinct imprinting strategies have been employed to introduce specific recognition sites into highly cross-linked porous polymers: The first is the formation of labile covalent bonds between functional groups of the template and those of complementary monomers, which become fixed in their spatial arrangement on polymerization. An important example is the imprinting of sugar derivatives using 4-vinylphenyl boronic acid esters due to Wulff *et al.*[12] The second relies on the self-assembly of monomeric species around the template by a series of noncovalent interactions which, on polymerization, form the basis of the recognition site. Examples of the latter include the imprinting of β-blockers,[10] amino acid derivatives,[8] peptides,[25] nucleotides[26] and derivatives of xanthine[23] and diazepam.[23] The requirement for multiple heteroatom functionality[27]

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Table 1. Preparation Conditions for Imprinted and Nonimprinted Polymers, Yields, and Surface Areas

polymer[a]	template monomer	mol% template	mol% EGDM	solvent	total mass of monomers, g[b]	BET surface area, m^2/g	deg of hydrolysis,[c] %
P1	none		100	*n*-hexane	5	44	
P2	none		100	*n*-hexane:toluene9:1v/v	5	2	
P3	**1**	5	95	*n*-hexane	10	26	104
P4	**1**	5	95	*n*-hexane:toluene9:1v/v	20	52.5	73
P5	**2**	5	95	*n*-hexane	5	34	
P6	**2**	5	95	*n*-hexane:toluene9:1v/v	5	1.8	
P7	none		100	propan-2-ol:toluene3:1v/v	10	408	
P8	**1**	5	95	propan-2-ol:toluene3:1v/v	10	336	60–105[d]

[a] Hydrolyzed polymers are referred to by the reference number, followed by **H**. [b] Yields of polymers were greater than 93% in all cases except **P5** (82%). [c] Determined from the recovery of cholesterol from the hydrolysate. [d] Dependent on the hydrolysis conditions (see text).

limits, to a large extent, the types of template molecules to which existing imprinting protocols can be applied efficiently. In addition, apart from the use of boronate esters, which is specific to 1,2- and 1,3-diols, there is also no general method for the imprinting of alcohols. There is therefore a need both for a more general imprinting strategy for molecules with few functional groups and for one capable of creating recognition sites for templates carrying single or multiple, spatially-separated hydroxyl groups.

We report a new imprinting strategy which addresses the above points and describe the synthesis and characterization of polymers imprinted with cholesterol which we believe to be one of the least functionalized templates used to create recognition sites in a synthetic polymer to date. The key to this method is the use of the 4-vinylphenyl carbonate ester (**1**) which functions as a covalently-bound template monomer but is easily and efficiently cleaved hydrolytically with the loss of CO_2. In this respect the carbonyl group of **1** is acting as a "sacrificial spacer". This results in the formation of a noncovalent recognition site, bearing a phenolic residue capable of binding cholesterol with a single dissociation constant. Furthermore, it has been directly shown that the phenolic residues introduced into the recognition site played a key role in cholesterol rebinding as evident from the "inhibition" studies using acetyl and benzoyl chlorides. Cholesterol was chosen as a suitable template for this investigation due to its rigid structure defined by the fused ring system and as a representative of a class of molecules of great biological and practical significance, namely the sterols.

Results and Discussion

Synthesis and Characterization of the Polymers. The majority of imprinted polymers described in the literature have been prepared with either of the two most readily available difunctional cross-linkers, namely divinylbenzene (DVB) or ethyleneglycol dimethacrylate (EGDM), irrespective of the method of imprinting or the nature of the template. These two cross-linkers were also employed in our initial study. It was found, however, that DVB-based polymers prepared under the same conditions as EGDM-based materials, although they showed qualitatively similar physical and chemical properties, had considerably lower binding capacities when compared to the latter. This was broadly in line with a number of reports which show EGDM-based polymers to be superior to DVB-based materials in terms of the separation factors achieved in imprinted chiral stationary phases.[28] EGDM was therefore selected as the cross-linker for all of the polymers used in this study.

Three monomer compositions were polymerized under two sets of solvent conditions (Table 1): EGDM homopolymers were prepared as nonimprinted polymers, **P1** and **P2**. Cholesterol-imprinted polymers **P3** and **P4** were the products of the copolymerization of cholesteryl (4-vinyl)phenyl carbonate (**1**) and EGDM. Similarly the phenol-imprinted polymers **P5** and **P6** were synthesized from phenyl (4-vinyl)phenyl carbonate (**2**) and EGDM. The polymers were all prepared "in bulk" by thermally-initiated free radical polymerization, using an azo-initiator. Solvents used in the above polymer preparations gave macroporous polymers of low to moderate surface area (Table 1).

Polymers, as prepared, contain the template molecule covalently attached to the polymer matrix, requiring hydrolysis of the carbonate ester group to remove the template and generate the recognition sites. The loss of the carbonyl group (as CO_2) during hydrolysis of the carbonate ester provides sufficient space to allow the template- and vinylphenol-derived hydroxyl groups to form a hydrogen bond in the rebinding/recognition event (Scheme 1a). The polymers **P1–P6** were hydrolyzed to form **P1H–P6H**, respectively.

Polymers **P1–P6** were hydrolyzed using the standard conditions of 6 h reflux with 1 M NaOH in methanol, this was found to be sufficient to quantitatively remove the template from the lowest surface area material.[29] The mass of cholesterol recovered from the hydrolysate by ether extraction (as described in detail in the Experimental Section) was used to determine the percentage of accessible template sites (see Table 1). This method was not applicable to the phenol-imprinted polymers for which the template removal was confirmed by IR spectroscopy. The aromatic carbonate carbonyl band of polymer **P6** was clearly resolved as a shoulder in the IR spectrum at 1779 cm^{-1}, which disappeared on hydrolysis (Figure 1b,c). In the cholesterol-imprinted polymers, however, the broad envelope due to the carbonyl groups of the poly(methacrylate) matrix completely obscured the carbonate carbonyl band (Figure 1a).

Binding Properties of the Polymers. The polymers **P1H–P6H**, prepared as described above were first exhaustively tested in adsorption experiments, performed in batch using 2 mM cholesterol solution in hexane (Figure 2a,b). In both series the cholesterol-imprinted polymers exhibited a significant uptake of cholesterol, evident from the decrease in concentration of the ligand in solution, with increasing amounts of polymer. As little as 20 mg/mL of the imprinted polymers **P3H** and **P4H** was sufficient to bind more than half of the cholesterol initially present. Practically no binding of cholesterol to the unhydrolyzed cholesterol-imprinted polymers **P3** and **P4** was observed

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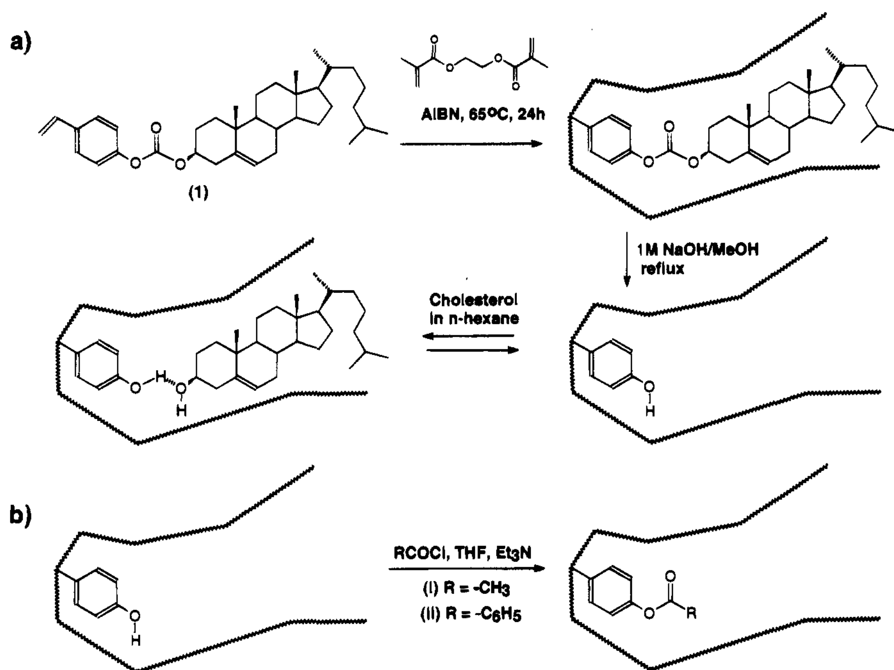
(29) A partial optimization was attempted for the high surface area polymers **P7** and **P8** and the implications for the binding of cholesterol to these polymers is discussed in the following section. It should be noted that for the high surface area polymers, very much milder conditions could be used, and that 10 min reflux with 0.1 M NaOH in methanol was sufficient to remove 60% of available template from polymer **P8**, based on cholesterol recovery.

Scheme 1. (a) Preparation of Cholesterol-Imprinted Polymers Using Cholesteryl (4-Vinyl)phenyl Carbonate (**1**), and (b) Chemical Modification of the Recognition Site by Acylation.



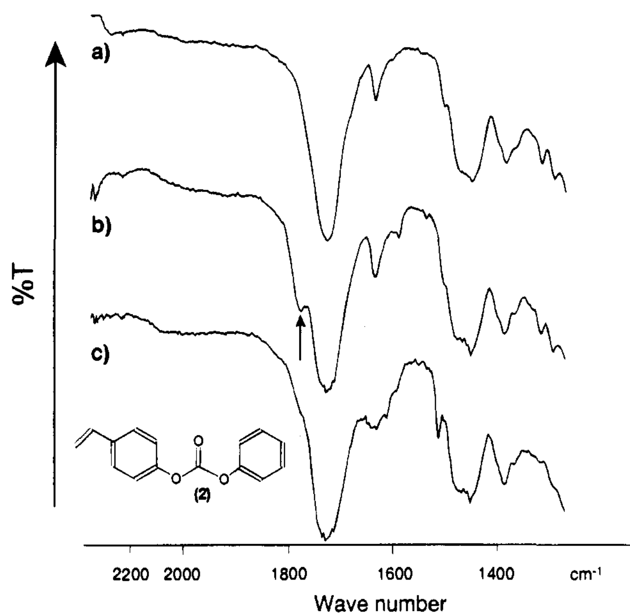


Figure 1. FT-IR spectra of cholesterol-imprinted polymer: (a) **P3** and phenol-imprinted polymers (prepared using the template monomer **2**), (b) **P6**, and (c) **P6H**.

under these conditions. An apparent increase in the concentration of cholesterol in solution with increasing amounts of these polymers may be due to some swelling with solvent, to the exclusion of cholesterol.

The nonimprinted hydrolyzed polymers also showed either negligible (**P2H**) or much reduced binding (**P1H**). Presumably the slight difference in the behavior of these two EGDM homopolymers was due to the increased surface area of **P1H** (44 m^2/g) as compared to **P2H** (2 m^2/g).[30] However quantitative comparisons under standard conditions (cholesterol, 2 mM and polymer 10 mg/mL) showed practically no dependence on surface area. Thus, the high surface area nonimprinted **P7** and imprinted polymer **P8**,[31] although displayed somewhat enhanced cholesterol binding (25 and 26 μmol/g, respectively) before hydrolysis, after hydrolysis[32] gave a specific uptake of 59 μmol/g (defined as the difference between binding to imprinted and nonimprinted polymers, subjected to identical hydrolysis). This appeared to be very similar to **P3H** and **P4H** described above, despite significant differences in the surface areas of these materials.

To further demonstrate the specificity of the recognition sites and to elucidate the importance of the nonfunctional part of the template molecules, we investigated the corresponding set of phenol-imprinted polymers (Figure 2a,b). Significantly the phenol-imprinted polymers **P5H** and **P6H** showed binding characteristics almost identical to those exhibited by their nonimprinted counterparts. Once again the slightly higher uptake of cholesterol by **P5H** as compared to **P6H** was attributed to nonspecific adsorption of the ligand by the higher surface area polymer. It should be noted that the effect of the size and bulkiness of nonfunctionalized substituents in template molecules on recognition properties of imprinted polymers still remains a largely unexplored area. More experiments are currently in progress to elucidate this relationship using a series of analogous aromatic hydroxy compounds.

We then proceeded to demonstrate that the recognition (and binding) properties of the cholesterol-imprinted polymers were also due to the phenolic hydroxyl group situated at the template-derived cavity (Scheme 1a). To this end, a selective chemical modification of this functionality was undertaken (Scheme 1b). One would expect to inhibit binding by both blocking the formation of the hydrogen bond and disrupting the "fit" of ligand in the recognition site by the introduction of an additional group partially filling the cavity.

The chemical modifications of the recognition sites were performed on the cholesterol-imprinted polymer **P4H**, using

(30) It is interesting to note in this context that the addition of 10% of toluene to the solvent mixture had opposite effects on the surface area in the cholesterol-imprinted polymers as compared to the corresponding nonimprinted and phenol-imprinted materials (Table 1).

(31) Prepared in propan-2-ol:toluene, 3:1 v/v.

(32) Hydrolysis conditions: 1.0 M NaOH/MeOH, 10 min reflux, 92% template recovery from **P8**.

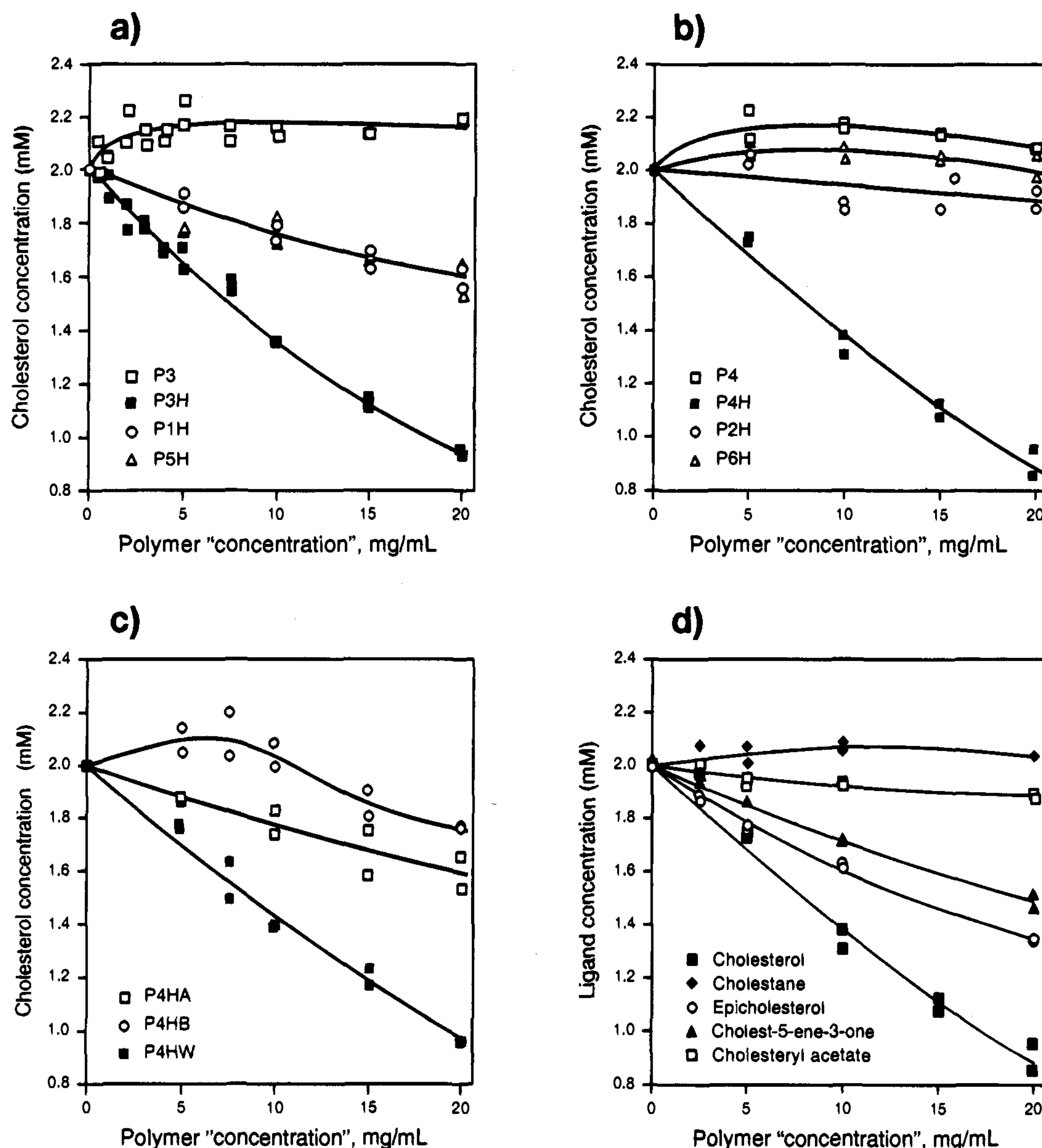


Figure 2. Results of batch binding experiments: The concentration of cholesterol measured in the supernatant at equilibrium as a function of the mass of added polymer: (a) Polymers prepared in *n*-hexane: **P3** (□), cholesterol-imprinted polymer before hydrolysis; **P3H** (■), cholesterol-imprinted polymer after hydrolysis; **P1H**, (○), hydrolyzed nonimprinted polymer; **P5H** (△), hydrolyzed phenol-imprinted polymer. (b) Polymers prepared in 9:1 *n*-hexane:toluene: **P4**, (□), cholesterol-imprinted polymer before hydrolysis; **P4H** (■), cholesterol-imprinted polymer after hydrolysis; **P2H**, (○), hydrolyzed nonimprinted polymer; **P6H** (△), hydrolyzed phenol-imprinted polymer. (c) Polymers chemically modified at the recognition site: **P4HA**, (□), cholesterol-imprinted polymer modified by acetylation; **P4HB** (○), cholesterol-imprinted polymer modified by benzoylation; **P4HW**, (■), cholesterol-imprinted polymer control, subjected to identical workup conditions. (d) Equilibrium binding of cholesterol, and its structural analogues, to the cholesterol-imprinted polymer **P4H** determined in batch experiments over a range of polymer "concentration".

acetyl chloride and benzoyl chloride to produce **P4HA** and **P4HB**, respectively, as described in the Experimental Section. Aqueous workups were used in preference to quenching the reactions with methanol. This ensured that methacrylic acid residues, formed by partial hydrolysis of the polymer matrix,[33] were not converted to the methyl ester *via* exchange with the acyl chloride. An unmodified polymer, **P4HW** subjected to the same workup procedure, was also included as a control. The effect of the chemical modification on cholesterol uptake by the three polymers is presented in Figure 2c. The control polymer **P4HW** displayed binding properties identical to **P4H** (Figure 2b), proving that the workup has no effect on the binding properties of the polymer. Acylation, either with acetyl chloride (**P4HA**) or benzoyl chloride (**P4HB**), however, did result in a significant suppression of binding. The benzoylated polymer **P4HB** had the lowest affinity for cholesterol over the concentration range employed, although the complex shape of the graph suggests that interactions with both ligand and solvent are important. The acetylated polymer, **P4HA**, showed a more straightforward concentration dependence with marginally higher uptake than **P4HB**. Significantly, the binding curve for **P4HA** was almost coincident with that for the nonimprinted

(33) The extent of hydrolysis of the poly(methacrylate) matrix was determined by conductimetric titration of the polymers (0.1 g), suspended in 5:1 v/v aqueous methanol (degassed to remove dissolved CO_2) with KOH solution, while being purged with nitrogen. Conductivity was measured using a WPA. CM35 conductivity meter and CM25B cell (cell constant $K = 1.0$), both supplied by WPA., Linton, Cambridge, UK. End-points were determined from the minima in the graphs of conductivity against titre. For a description of the method see: Vanderhoff, J. W.; Van den Hul, H. J.; Tausk, R. J. M.; Overbeek, J. T. G. *Clean Surfaces: Their Preparation and Characterization for Interfacial Studies*; Goldfinger, G., Ed.; Marcel Dekker: New York, 1970; pp 15–44. The unhydrolyzed polymer **P4** gave a free carboxyl number of 1.6 ± 0.5 μmol/g, whereas after hydrolysis the corresponding measurement on the hydrolyzed polymer **P4H**, was 1.3 ± 0.5 μmol/g. The phenolic groups of **P4H**, liberated by hydrolysis of the carbonate ester, were not detected by this method.

P1H and phenol-imprinted **P5H** polymers suggesting that the residual binding in **P4HA** was predominantly due to nonspecific interactions. Similar results were obtained on acylation of higher surface area polymers. Independent confirmation of the role of hydrogen-bonding in the recognition event was obtained by the fact that the addition of as little as 3% of a hydrogen-bonding solvent (pyridine, 1-hexanol or DMF) completely suppressed the binding of cholesterol to **P4H**. In addition noncovalent imprinting, even with 10% of the hydrogen-bonding monomer, methacrylic acid, in the polymerization mixture, failed to produce a polymer[34] capable of binding cholesterol under the same conditions as **P4H**.

To demonstrate the specificity of the cholesterol imprints, the binding of a series of structural analogues of cholesterol (from 2 mM solutions, in hexane) to the polymer **P4H** were measured. The equilibrium binding of each ligand over a range of polymer concentrations were determined using HPLC, and the results are presented in Figure 2d. Cholestane and cholesteryl acetate showed practically no binding to the cholesterol-imprinted polymer **P4H**, consistent with the hydrogen-bonding mechanism. Where an oxygen atom was present at C3, as in epicholesterol and cholest-5-en-3-one, some binding was measured, but much less than was shown by cholesterol. Ligand discrimination is therefore possible on the basis of the inversion of configuration at a single carbon atom, namely that bearing the hydroxyl group, or on the basis of ketone substituting for hydroxyl (the latter also introduces a slight conformational change in the steroid A-ring). These results suggest that the new imprinting methodology described here is highly selective in terms of the template/ligand structure and may therefore be of general applicability for hydroxy compounds.

Encouraged by the fact that low levels of cholesterol uptake were observed with nonimprinted, phenol-imprinted, and acetylated polymers and the fact that the three classes of polymer showed near identical behavior, we investigated the diversity of the recognition sites using Scatchard analysis,[35] often employed in biochemical studies to determine the equilibrium binding constants and the number of binding sites in antibodies and other biological receptors. However, to apply Scatchard analysis to our system, it was necessary first to establish unambiguously the completely reversible nature of the interaction of ligand with the recognition sites of the imprinted polymers. To this end a series of experiments in which the concentrations of cholesterol and the polymer **P4H** were perturbed by a dilution or by the addition of a concentrated solution of the ligand were performed. The results (Figure 3) confirmed that at equilibrium (overnight) the concentration of cholesterol in the solution and, therefore, the amount bound to the polymer was the same irrespective of the history of the sample. Similar results were obtained with a cholesterol-imprinted polymer with a higher surface area.[36] The advantage of using the lower surface area polymers is that the response to perturbations can be more readily seen. In particular the addition of a small volume of a 12 mM solution (Figure 3, dashed line, solid triangles) resulted in a momentary excess of free cholesterol relative to the equilibrium concentration, which accounts for the "spike" rising above the unperturbed line determined using the final concentrations (solid line, open squares).

The binding properties of **P4H** were determined by measuring the ligand uptake over a range of cholesterol concentrations from

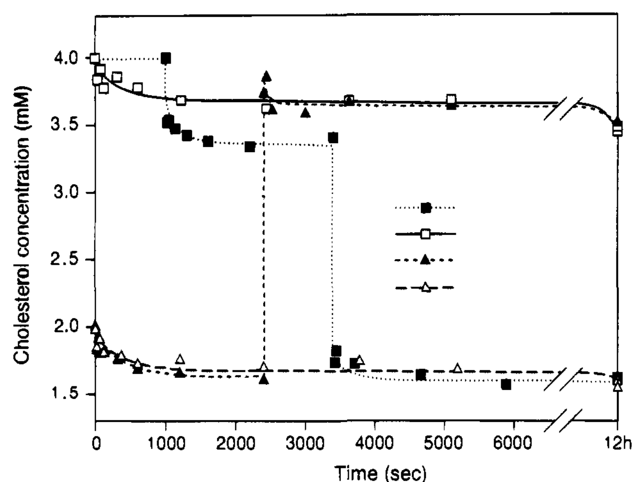


Figure 3. Graph showing the kinetics and reversible nature of cholesterol binding to **P4H**: (i) polymer (4 mg/mL) with 4 mM cholesterol in hexane (□); (ii) polymer (5 mg/mL) with 2 mM cholesterol in hexane (△); (iii) polymer (10 mg/mL) with 4 mM cholesterol in hexane, diluted by the addition of an equal volume of hexane after 2400 s, line offset by 1000 s, (■); (iv) polymer (5 mg/mL) with 2 mM cholesterol in hexane, with the addition of 12 mM cholesterol (25% by volume) after 2400 s (▲).

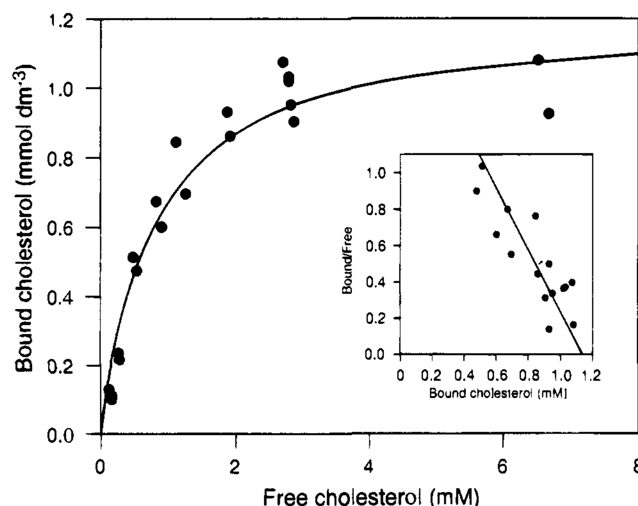


Figure 4. Binding isotherm for cholesterol-imprinted polymer **P4H**, 20 mg polymer + 2 mL cholesterol in hexane. (inset) Scatchard plot over 1−8 mM concentration range. Curve-fitting gives a dissociation constant, K_d, of 0.59 ± 0.12 mM and a capacity of 114 ± 6 μmol/g.

0.5 to 8 mM (Figure 4). A Scatchard plot[35] was constructed (Figure 4, inset) by plotting the ratios of bound to free ligand concentrations against the bound ligand concentration for a constant mass of polymer. Curve-fitting[37] gave a dissociation constant of 0.59 ± 0.12 mM with a site population of 114 ± 6 μmol/g. This appears to be a remarkably uniform binding constant,[38] over a large proportion of the imprint sites, 61% based on the hydrolysis data, or 45% overall. The binding characteristics of polymer **P3H**, shown in Figure 2a, can be similarly curve-fitted using an appropriate equation[39] to give $K_d = 0.84 \pm 0.24$ mM at a site population of 100 ± 13 μmol/g, which is strikingly similar to the parameters determined for

(34) Prepared in the same solvent (and at the same concentration), from methacrylic acid (10 mol%) and EGDM (90 mol%), in the presence or absence of cholesterol (5 mol% with respect to monomers).

(35) Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660−672.

(36) Whitcombe, M. J.; Rodriguez, M. E.; Vulfson, E. N. *Proceedings of Separations for Biotechnology III*; Pyle, D. L. Ed.; SCI: 1994; p 565.

(37) Curve-fitting by nonlinear regression using the Grafit software package, from Erithacus Software on an IBM-PC clone microcomputer.

(38) Noncovalent imprinting tends to give a range of binding affinities with widely differing binding constants, see for example ref 23.

(39) Equation for the curve fitting is

$$[\text{bound ligand}] = \frac{([\text{polymer}]*\text{capacity}*[\text{free ligand}])}{(K_d + [\text{free ligand}])}$$

P4H by Scatchard analysis. A consideration of these results alongside the data on the chemical modifications strongly suggests that it is feasible to achieve a high degree of uniformity between recognition sites of imprinted polymers, which resemble complex biological receptors and synthetic organic hosts.

Conclusions

A new method of molecular imprinting for compounds with single (or spatially separated) hydroxyl groups is described, which involves covalent attachment during the imprinting (polymerization) step, but which gives rise to a noncovalent recognition (binding) site. This method is based upon the (4-vinyl)phenyl carbonate ester functionality which can be conveniently prepared from 4-vinylphenol and the corresponding chloroformate. The template is removed by base hydrolysis, the loss of CO_2 ensuring that hydrogen-bond formation can occur between the polymer recognition site functionality and the template hydroxyl, without gross distortion of the binding geometry. The applicability of this imprinting method has been investigated using cholesterol as the template, which bears a single hydroxyl group on a hydrocarbon skeleton of 27 carbon atoms, and is therefore one of the least functionalized templates imprinted to date.

The recognition of cholesterol by the imprinted polymers depends on at least two separate parameters: The first is the size of the template. Imprinting with the smaller template, phenol, gives polymers with identical surface functionalities but no further affinity for cholesterol in binding experiments than nonimprinted polymers. Although surface areas, which influence nonspecific binding, vary across the range of polymer compositions, and also with polymerization solvent, the range of surface area shown by nonimprinted and phenol-imprinted polymers overlaps with that of the cholesterol-imprinted polymers, allowing a valid comparison to be made. In any case, despite having different surface areas the cholesterol-imprinted polymers studied showed very similar binding characteristics, both in terms of the binding curves and in the capacities and dissociation constants determined by the two methods employed. The second effect is the contribution to binding due to the hydrogen bond, which is at the heart of the imprinting strategy and contributes the majority of the binding energy. Chemical modification of the phenolic hydroxyl group of the recognition site almost completely suppresses binding, as does the addition of small amounts of 1-hexanol, DMF, or pyridine which compete with ligand and receptor for hydrogen-bonding. The ability of the imprinted polymers to recognize the template, cholesterol, was confirmed by the differential binding displayed by a range of close structural analogues including epicholesterol and cholest-5-en-3-one.

Binding to the imprinted polymers is clearly equilibrium driven, as demonstrated by the effects of perturbations on the conditions of uptake experiments, which show the final extent of binding to be dependant solely on the overall concentrations of polymer and ligand, independent of the history of the sample. For such an equilibrium it should be possible to determine the binding constant and the population density of active sites within the resin. This was indeed achieved for the imprinted polymer **P4H** by the construction of a Scatchard plot (Figure 4, inset) which gave figures of $K_d = 0.59 \pm 0.12$ mM at a capacity of 114 ± 6 μmol/g. It was initially expected that a range of binding affinities would have been seen, but this does not appear to be the case, with a relatively uniform binding affinity over a large proportion of the theoretically accessible sites. The corresponding parameters were also determined from the binding data for polymer **P3H** and are in very good agreement with a measured K_d of 0.84 ± 0.24 mM at a capacity of 100 ± 13 μmol/g. The polymers obtained therefore displayed binding characteristics similar to true biological receptors or synthetic hosts.

Experimental Section

Materials and Methods. *p*-Acetoxystyrene, cholesteryl chloroformate, phenyl chloroformate, and ethyleneglycol dimethacrylate were obtained from Aldrich Chemical Co. Ltd. AIBN was obtained from Fluka and was recrystallized from methanol before use. Solvents used in the polymer preparations (*n*-hexane and toluene) were distilled before use. All other solvents and reagents were either laboratory reagent or analytical grade and used as received. FT-NMR spectra were obtained on a Jeol EX-270, FT-IR spectra of samples dispersed in KBr were recorded on a Perkin-Elmer Series 1600 FTIR spectrophotometer by diffuse reflectance IR spectroscopy (Spectra-Tech Inc.). Polymer was filtered from solutions prior to HPLC analysis using Minisart RC15 2 μm membrane filter cartridges (Sartorius). HPLC analyses (for cholesterol) were performed using Gilson 303 pumps equipped with an ACS light-scattering mass detector and a Shimadzu SIL-9A autosampler. Samples were analyzed on a 25 cm, 5 μ Spherisorb column (Anachem or Hichrom), at room temperature, using a flow rate of 1.5mL/min. Elution was with a linear gradient from 20% ethyl acetate:hexane to 100% ethyl acetate over 8 min.

4-Vinylphenol. This compound was prepared by the hydrolysis of *p*-acetoxystrene with aqueous potassium hydroxide according to the method of Corson *et al.*[40] and obtained as shiny colorless plates. The product was washed thoroughly with water, dried under vacuum and stored at −20 °C until required.

Cholesteryl (4-vinyl)phenyl Carbonate, (1). To a cooled solution (ice-bath) of 4-vinylphenol (2.0 g, 16.6 mmol) in dry THF (60 mL) and triethylamine (4 mL) containing a trace of 2,6-di-*tert*-butyl-4-methylphenol was added dropwise a solution of cholesteryl chloroformate (7.5g, 16.6 mmol) in THF (40 mL), and the mixture was stirred overnight at room temperature. The solvent was removed, and the residue dissolved in dichloromethane. The organics were washed with water, dried, and evaporated to yield the crude product. Recrystallization from aqueous acetone followed by propan-2-ol gave the product as colorless plates (5.0 g, 57%), mp 146−147 °C;[41] IR (KBr) 2942 (CH), 1751 (C=O), 1601 (Ar), 1630 (C=C), 1506 (Ar) cm^{-1}; 270 MHz ^{1}H-NMR (CDCl$_3$) δ (ppm) 7.41, 7.21, (aromatic AB system, 4H, $J_{A,B}$ = 8.6 Hz), 6.70 (dd, 1H, $J \approx 10.9$, 17.6 Hz, CH$_2$=C*H*), 5.71 (d, 1H $J_{7,8'trans} \approx 17.6$ Hz, *trans*-C*H*$_2$=CH), 5.43 (m, 1H, H-6, C=C*H*), 5.25 (d, 1H $J_{7,8'cis} \approx 10.9$ Hz, *cis*-C*H*$_2$=CH), 4.59 (m, 1H, H-3α), 2.51 (m, 2H, H-4, CH$_2$), 2.05−1.00 (m's, 27H), 1.05 (s, 3H, H-19, -CH$_3$), 0.93 (d, 3H, J=6.3Hz, H-21, −CH$_3$), 0.88 (d, 6H, $J \approx 6.6$ Hz, H-26,27, -CH$_3$), 0.69 (s, 3H, H18, -CH$_3$); 67.9 MHz ^{13}C-NMR[42] (CDCl$_3$) δ (ppm) 11.83, 18.67, 19.25, (3× -CH$_3$, C18, C21, C19), 21.01 (-CH$_2$, C11), 22.51, 22.77 (2× -CH$_3$, C26, C27), 23.79, 24.24, 27.59 (3× -CH$_2$, C23, C15, C16), 27.98 (-CH, C25), 28.20 (-CH$_2$, C2), 31.77 (-CH, C8), 31.86 (-CH$_2$, C7), 35.76 (-CH, C20), 36.14 (-CH$_2$, C22), 36.48 (Q, C10), 36.77, 37.88, 39.48, 39.66 (4× -CH$_2$, C1, C4, C24, C12), 42.25 (Q, C13), 49.90, 56.07, 56.62, 78.82 (4× -CH, C9, C17, C14, C3), 114.02 (=CH$_2$, C8′), 121.08 (aromatic -CH, C2′,C6′), 123.15 (-CH, C6), 127.12 (aromatic -CH, C3′,C5′), 135.33 (Q, C4′), 135.76 (=CH, C7′), 139.07, 150.57 (2× Q, C5, C1′), 152.85 (carbonyl). Anal. Calcd for $C_{36}H_{52}O_3$: C, 81.16; H, 9.83. Found: C, 81.34; H, 9.98.

Phenyl (4-Vinyl)phenyl Carbonate (2). The phenyl carbonate monomer was prepared by the same method as the cholesteryl compound (above), from 4-vinylphenol (2.0 g, 16.6 mmol) and phenyl chloroformate (2.86 g, 2.29 mL, 18.27 mmol). The compound was obtained as colorless crystals, following recrystallization from aqueous methanol: yield 2.66 g, 67%, mp 50−52 °C; IR (KBr) 3059 (CH),

(40) Corson, B. B.; Heintzelman, W. J.; Schwartzman, L. H.; Tiefenthal, H. E.; Lokken, R. J.; Nichels, J. E.; Atwood, G. R.; Pavlik, F. J. *J. Org. Chem.* **1958**, *23*, 544−549.

(41) The product melted to an unidentified liquid crystalline phase.

(42) ^{13}C-NMR assignments were made on the basis of published data for related compounds in: Blunt, J. W.; Strothers, J. B. *Org. Magn. Reson.* **1977**, *9*, 439−464.

(43) A sample, recrystallized to remove a colored impurity, was identical (mp, mixed mp, IR, ^{1}H-NMR, and ^{13}C-NMR) with an authentic sample of cholesterol.

1774 (C=O), 1631 (C=C), 1590 (Ar) cm^{-1}; 270 MHz ^1H-NMR ($CDCl_3$) δ (ppm) 7.44−7.37 (m, 4H, aryl), 7.28−7.21 (m, 5H, aryl), 6.69 (dd, 1H, $J \approx 10.9$, 17.5Hz, $CH_2=CH$), 5.71 (d, 1H $J_{7',8'\text{-trans}} \approx 17.5$ Hz, *trans*-$CH_2=CH$), 5.25 (d, 1H $J_{7',8'\text{-cis}} \approx 10.9$Hz, *cis*-$CH_2=CH$); 67.9 MHz ^{13}C-NMR ($CDCl_3$) δ (ppm) 114.41 (-CH_2, C8′, $CH_2=CH$), 120.88, 120.94 (2× -CH), 126.31 (-CH, C4), 127.28, 129.56, (2× -CH), 135.71 (-CH, C7′, $CH_2=CH$), 135.83, 150.46, 151.00 (3×Q), 151.97, (carbonyl). Anal. Calcd for $C_{15}H_{12}O_3$: C, 74.99; H, 5.03. Found: C, 75.21; H, 5.01.

General Polymer Synthesis. The monomer mixture and solvent, 2 mL/g of monomers (see Table 1), were transferred to a test tube, and azo-*bis*-isobutyronitrile (AIBN) (1 mol% with respect to polymerizable double bonds) was added. The tube was closed with a tap fitted with a ground glass joint for connection to a vacuum line. The polymerization mixture was degassed by a series of freeze−thaw cycles and sealed at reduced pressure. The polymerization was carried out in a water-bath at 65 °C for up to 24 h. The polymer was obtained as a brittle solid which was broken up with a spatula and washed with methanol on a sintered glass filter. The air-dried polymer was ground to an average particle size of 30 μm in an agate mortar on a Fritsch Pulverisette "O" grinding mill. The ground polymer was extracted with methanol in a soxhlet apparatus for 12−18 h and finally dried *in vacuo* at 70 °C.

Polymer Hydrolysis, Standard Conditions. Polymers were suspended in 1 M sodium hydroxide in methanol and heated to reflux during 6 h. The cooled suspensions were added to an excess of dilute hydrochloric acid, and the products were filtered and washed with water, methanol, and a small volume of ether. Polymers were extracted in a Soxhlet apparatus with methanol followed by hexane. The polymers were dried as above. In the case of cholesterol-imprinted polymers, the hydrolysate and washings (the filtrate) from each preparation were transferred to a separating funnel. The aqueous mixtures were extracted with three portions of diethyl ether, after the addition of brine. The combined ether extracts were dried ($MgSO_4$) and evaporated, in a preweighed flask, to yield cholesterol.[43] The solid residues were dried to constant weight under high vacuum. The purity of the recovered cholesterol was confirmed by ^1H and ^{13}C NMR, and its mass was used to calculate the degree of hydrolysis of the resins. Hydrolyzed polymers are designated by **H** following the name of the parent polymer. High surface area polymers, **P7** and **P8**, were also hydrolyzed with both 1 and 0.1 M sodium hydroxide in methanol, for reaction times of 1 h or 10 min reflux as well as under the standard conditions.

Chemical Modification of Polymer P4H. Polymer **P4H** (0.5 g) was suspended in THF (25 mL) and triethylamine (2.5 mL) with vigorous stirring during the addition of 5 mL of acetyl chloride (**P4HA**) or benzoyl chloride (**P4HB**). The polymer mixtures were stirred at room temperature for 1 h. Water was cautiously added to the reaction mixtures. The slurries were poured into a mixture of water and acetone. The products were obtained by filtration and washed with water, acetone, methanol, water, methanol, and finally diethyl ether. The polymers were dried *in vacuo*. The yields were nearly quantitative.

Batch Binding Experiments: General Method. The required mass of polymer (1−40 mg) was weighed into a 2 mL screw-cap vial. A solution of cholesterol in hexane (usually 2 mL) was added, and the vial was sealed and placed on its side in a sample shaker. Except in the kinetics experiments, samples were shaken overnight at room temperature to allow the equilibrium binding to be established. Polymer suspensions were rapidly transferred to a 5 mL syringe barrel fitted with a disposable 2 μm filter cartridge and filtered directly into HPLC vials to remove all polymer particles. Solutions were then analyzed by HPLC to determine the concentration of cholesterol remaining in the supernatant.

Similar equilibrium binding experiments were performed with a range of structural analogues of cholesterol (all 2 mL, 2 mM in hexane) to determine their binding to the polymer **P4H** (5−40 mg). Epicholesterol (cholest-5-en-3α-ol) and cholest-5-en-3-one solutions were analyzed using the same HPLC conditions as cholesterol. Cholestane and cholesteryl acetate were detected using a linear gradient from 10% ethyl acetate:hexane to 90% ethyl acetate over 8 min, column and detector as for cholesterol. All compounds gave a single peak, and sample concentrations were determined from individual calibrations.

Kinetic Measurements. In order to demonstrate the reversible nature of binding to the cholesterol-imprinted polymer **P4H**, a series of kinetic measurements were undertaken.

(i) In the first experiment, polymer **P4H** (8 mg, 4 mg/mL) was shaken with 2 mL of a 4 mM solution of cholesterol in hexane. After a predetermined period the solution was rapidly filtered from the polymer and analyzed, as above. Each individual point was established with a fresh sample. Points on the kinetic curve were determined for 5000 s, and two further points were plotted for samples left overnight.

(ii) In a similar fashion the kinetics of binding of polymer **P4H** (10 mg, 5 mg/mL) with 2 mL of 2 mM cholesterol were determined.

(iii) In a third experiment, **P4H** (20 mg, 10 mg/mL) was allowed to bind cholesterol from a 4 mM solution, with sampling up to 2400 s. Samples were prepared under the same initial concentrations, but at reduced volumes (**P4H** (10 mg, 10 mg/mL) and 1 mL, 4 mM cholesterol) were shaken for 2400 s before the addition of 1 mL of pure hexane and a further predetermined delay before analysis. In this experiment the final overall concentration of both cholesterol (2 mM) and polymer (5 mg/mL) were identical to that described in (ii) above.

(iv) In the final experiment the initial samples were identical to those described in (ii), above, **P4H** (10 mg, 5 mg/mL) with 2 mL of 2 mM cholesterol, with samples analyzed for up to 2400 s. Later samples, **P4H** (8 mg, 5 mg/mL) with 1.6 mL of 2 mM cholesterol were shaken for 2400 s before the addition of 0.4 mL of a 12 mM solution of cholesterol. This gave overall final concentrations of 4 mg/mL of polymer and 4 mM cholesterol, identical to (i) above.

Measurement of Binding Constant. A Scatchard plot for polymer **P4H** was constructed by determining the batch binding at equilibrium for 20 mg samples of polymer shaken with 1, 1.5, 2, 3, 4, and 8 mM solutions of cholesterol in hexane. From the analysis results the bound and free cholesterol can be determined. A correction for nonspecific binding was applied to the bound cholesterol figure by subtracting the amount of cholesterol bound to the nonimprinted polymer **P2H**, under the same conditions. The results, shown in Figure 4, were analyzed by curve-fitting software,[36] employing nonlinear regression techniques, using a one site binding model, which gave estimates of the dissociation constant and binding capacity of the polymer.

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