# Synthesis and ligand recognition of paracetamol selective polymers: semi-covalent *versus* non-covalent molecular imprinting†

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Three molecular imprinting strategies, each based upon a series of ethylene glycol dimethacrylate (EGDMA) cross-linked co-polymers, have been used to produce materials selective for the commonly used analgesic and antipyretic agent paracetamol (*p*-acetaminophen or 4-acetamidophenol) (**1**). The polymers were synthesised using either a semi-covalent imprinting strategy based upon 4-acetamidophenyl-(4-vinylphenyl) carbonate (**4**) or a non-covalent strategy based on methacrylic acid (MAA) as the functional monomer, or by employing a combination of these strategies. Radioligand binding studies demonstrated low template affinity in polymers offering only a single electrostatic interaction point for recognition *via* the phenolic residue in the template, whereas binding was substantially increased upon the introduction of a second binding mode, namely interaction at the acetamide moiety. HPLC analyses revealed no imprinting effect in the purely semi-covalent system, and only a minor effect in the purely non-covalent systems. However, a pronounced imprinting effect was demonstrated for polymers prepared by a combination of semi-covalent and non-covalent imprinting. This study illustrates a limitation of both the non-covalent and the semi-covalent strategies when it comes to achieving imprinted selectivity for small and poorly functionalised templates such as paracetamol. Parallels with conclusions from studies with antibodies are discussed.



## Introduction

Paracetamol (*p*-acetaminophen or 4-acetamidophenol) (**1**) (Fig. 1) has become one of the most frequently used analgesic and antipyretic agents since its introduction in the mid 1950s as a replacement for phenacetin.[1] Although it is considered to be quite safe, it has become one of the most common drugs involved in accidental or intentional cases of overdose which, if not treated early, may result in severe liver damage.[2] Efforts to develop methods for the rapid determination of paracetamol and its metabolites have long been a focus for researchers, with antibody-based strategies initially investigated,[3–5] though more recently a range of highly sensitive instrumental techniques has been used.[6–8] While the selectivities generally offered by antibody-based approaches are an advantage, the issue of stability (shelf-life) cannot be ignored. The antibody-like recognition characteristics[9–11] and general stability[12] of molecularly imprinted polymers (MIPs) make the development of paracetamol-selective MIPs attractive for potential use in sensor devices for measurement of blood concentrations, or as an SPE matrix for the extraction of 4-acetamidophenol from blood.

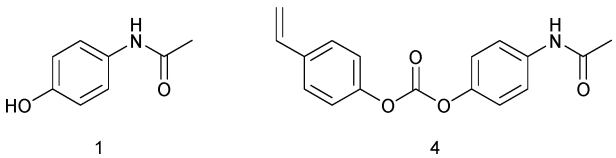


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**Fig. 1** Structures of 4-acetamidophenol (**1**) and the monomer 4-acetamidophenyl-(4-vinylphenyl) carbonate (**4**).

Previous attempts to create polymers capable of recognising 4-acetamidophenol have relied upon the use of non-covalent imprinting strategies and have been only moderately successful.[13–15] In this study, our objective was to examine the efficiency of a series of molecular imprinting systems to determine whether improved MIP materials for paracetamol could be produced, and to simultaneously gain insights concerning the factors steering polymer recognition of small and poorly functionalised systems.

Polymers reported by Yang and Li using 4-vinylpyridine (4-VP) as a functional monomer demonstrated template affinity, though only a minor imprinting effect was noted.[13] Tan *et al.* demonstrated that an increase in both the selectivity and sensitivity of 4-acetamidophenol imprinted bulk acoustic wave sensors could be obtained with polymers based upon two equivalents of MAA in combination with two equivalents of 4-VP (relative to the template). This polymer performed better than those synthesised using four equivalents of either one of the functional monomers.[14] The relative success of the dual functional monomer system was attributed to two favorable processes, the ability of the basic 4-VP to interact more strongly with the weakly acidic phenol residue on the template, and MAA's interaction with the amide functionality. Interestingly, the involvement of both of these functionalities has been implicated in antibody recognition of 4-acetamidophenol.[5] A paracetamol imprinted polymer prepared by the electro-co-polymerisation of aniline and *o*-phenylenediamine has also been examined in conjunction with electrochemical sensor studies.[8]

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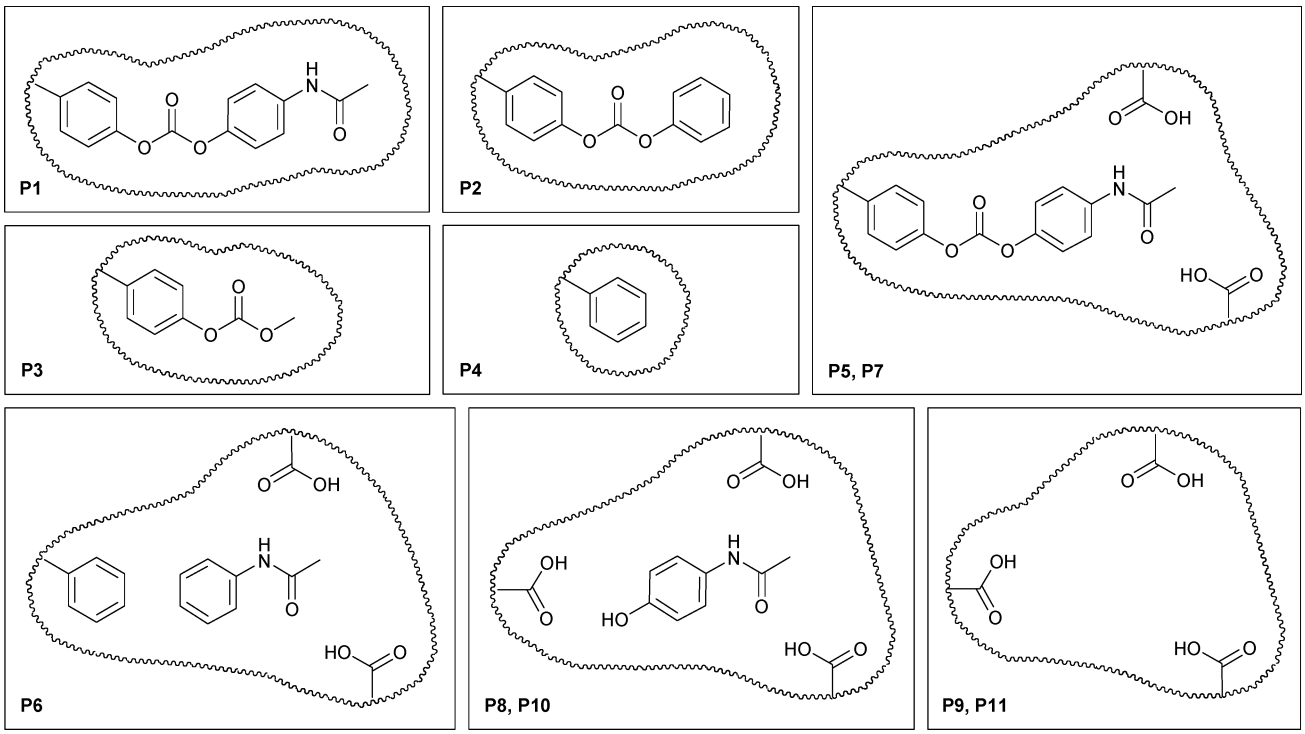


**Fig. 2** Schematic illustration of the various binding cavities in the different polymers studied. (**P1**) 4-acetamidophenyl-(4-vinylphenyl) carbonate polymer; (**P2**) phenyl-(4-vinylphenyl) carbonate polymer; (**P3**) methyl-(4-vinylphenyl) carbonate polymer; (**P4**) styrene polymer; (**P5** and **P7**) 4-acetamidophenyl-(4-vinylphenyl) carbonate and MAA polymer; (**P6**) acetanilide imprinted styrene and MAA polymer; (**P8** and **P10**) non-covalently 4-acetamidophenol imprinted MAA polymers (1:3 and 1:4); (**P9** and **P11**) MAA reference polymers.

We postulated that another approach to improving the recognition characteristics of 4-acetamidophenol could be to further strengthen the interaction between the phenolic hydroxyl and the polymer during the polymerisation process. We envisaged that this could be achieved using a so called semi-covalent molecular imprinting strategy, where the template is linked to the functional monomer through a reversible covalent bond. Following polymerisation, the template is cleaved then later rebound through non-covalent interactions. The use of covalent template–monomer interactions during the polymerisation step should result in a more homogeneous recognition site distribution. A further development of this, termed the *sacrificial spacer approach*, was presented by Whitcombe *et al.* for the imprinting of another poorly functionalised template, cholesterol.[16] This approach even takes into account the steric requirements of the non-covalent rebinding interaction. In that study, cholesteryl (4-vinylphenyl) carbonate was used as a covalently bound template monomer during the polymerisation process. Hydrolytic cleavage of the carbonate esters released cholesterol and $CO_2$ to reveal a binding site spacious enough to allow hydrogen bonding to take place between the residual phenolic group in the polymer and the hydroxyl on the template during the rebinding process. The polymers obtained using this method bound cholesterol with a single dissociation constant, thus resembling a true biological receptor in this respect. This imprinting strategy has also been applied to the imprinting of other hydroxyl containing structures such as the anaesthetic propofol (2,6-diisopropyl phenol),[17,18] nandrolone,[19] and bisphenol A.[20]

Based on this strategy, a series of 4-acetamidophenol imprinted EGDMA co-polymers were prepared using the semi-covalent sacrificial spacer approach based on 4-acetamidophenyl-(4-vinylphenyl) carbonate (**P1**), the non-covalently interacting monomer MAA (**P8**, **P10**), or a combination of both strategies (**P5**, **P7**). A series of reference systems was prepared from either styrene (**P4**) or other carbonate ester based monomers (**P2**–**P3**), from non-covalent imprinting of acetanilide (**P6**) or in the total absence of template (**P9**, **P11**), Fig. 2. The affinity for 4-acetamidophenol was evaluated by radioligand equilibrium binding studies whereas HPLC studies were employed in order to determine template selectivity and imprinting effect. The results provide insights into the lower limit of template size and functionality amenable for use in conjunction with semi-covalent imprinting protocols, and through the comparison of covalent and non-covalent polymerisation strategies, furnish information regarding the design of suitable reference systems.

## Experimental

### Chemicals

All solvents used were of analytical or HPLC grade. Tetrahydrofuran (THF) was dried by distillation from Na/benzophenone. MAA was obtained from Merck and was purified by vacuum distillation. 2,2′-azobis(2-isobutyronitrile) (AIBN) was obtained from Janssen Chimica and was recrystallised from methanol before use. EGDMA was obtained from Aldrich and was

purified by extraction with 0.1 M NaOH (3 × 75 ml to 100 ml EGDMA), washed with 75 ml brine, dried over $MgSO_4$, filtered and passed through basic $Al_2O_3$ before use. Acetanilide (99%) was obtained from Merck and was recrystallised from water. 4-aminophenol (99%) was from Riedel-de-Haën. 4-acetoxystyrene (96%), 2-acetamidophenol (97%), 3-acetamidophenol (97%), 4-acetamidophenol (98%), benzanilide (98%), benzoyl chloride (99%), *N*,*N*-diisopropylethylamine (DIEA, 99.5%), triethylamine (99%), triphosgene (98%), 2,6-di(*tert*-butyl)-4-methylphenol (≥99%), $CDCl_3$ (99.8%), methyl chloroformate (99%), phenyl chloroformate (99%) and styrene (99%) were all obtained from Aldrich. [$^3$H]-4-acetamidophenol (50 Ci mmol$^{-1}$) was from American Radiolabeled Chemicals Inc. (USA).

### Apparatus

NMR spectra were acquired on a Bruker AC 250 MHz instrument or on a Varian Unity *Inova*™ 500 MHz instrument. Homonuclear $^1$H connectivities were determined using COSY experiments. Chemical shifts (δ) are presented in ppm and *J* values are reported in Hz. FT-IR spectra were recorded using samples dispersed in KBr on a Nicolet Avatar 320 FT-IR instrument by diffuse reflectance IR spectroscopy. Mass spectra of positive ions obtained by electron impact (EI, 70 eV) were measured using an Agilent 6890 GC-system with an Agilent 5973 MS detector. Liquid scintillation counting was performed on a Tri-Carb 2100TR Liquid Scintillator Analyzer from Packard using Beckman Ready Safe™ scintillation cocktail. Chromatographic evaluations were performed using a Merck-Hitachi LaChrom HPLC system comprised of a series L-7100 pump, L-7200 autosampler, L-7455 diode array detector and a D-7000 interface. BET surface area analysis was performed by $N_2$ adsorption on a Micromeritics ASAP 2400 instrument. High resolution mass spectrometry was performed at the Chemical Center (Lund, Sweden). Elemental analyses were performed by Mikrokemi AB (Uppsala, Sweden). Data analyses were conducted with the software package GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, USA).

**Synthesis of 4-vinylphenol (2).** 4-vinylphenol (**2**) was prepared by hydrolysis of 4-acetoxystyrene with aqueous potassium hydroxide according to the method of Corson *et al.*.[21] Recrystallisation from *n*-pentane afforded shiny colourless plates of **2** (46%); $\lambda_{max}$ (EtOH)/nm 261 (log ε 4.04); $\nu_{max}$/cm$^{-1}$ 3400–3000 br (OH), 2911 (CH), 1661 (C=C) and 1505 (Ar); $\delta_H$ (250 MHz; $CDCl_3$; 25 °C), 7.32–7.29 (2 H, d, *J* 8.5, 2 × ArH), 6.81–6.78 (2 H, d, *J* 8.5, 2 × ArH), 6.71–6.60 (1 H, dd, *J* 10.9 and 17.6, $CH_2$=C*H*), 5.64–5.57 (1 H, d, *J* 17.6, *trans*-C$H_2$=CH), 5.15–5.10 (1 H, d, *J* 10.9, *cis*-C$H_2$=CH) and 4.71 (1 H, s, OH); $\delta_C$ (63 MHz; $CDCl_3$; 25 °C), 155.2, 136.1, 130.7, 127.6, 115.4 and 111.7; *m*/*z* (EI) 120 (M$^+$, 100%), 91 (44), 65 (13) and 39 (6).

**Synthesis of 4-acetamidophenyl chloroformate (3).** A solution of 4-acetamidophenol (**1**) (3.0 g, 19.8 mmol) and DIEA (5.8 ml, 33.7 mmol) in THF (75 ml) was added dropwise to a stirred ice cold solution of triphosgene (7.0 g, 23.8 mmol) in THF (30 ml) under an inert atmosphere ($N_2$). The resultant solution was stirred at RT for 5 h, until the disappearance of **1** was evident on TLC (heptane:ethyl acetate, 2:8). The resultant amine salt was removed by filtration and the filtrate was evaporated to yield a yellow oil which was used without further purification for the preparation of **4**.

**Synthesis of 4-acetamidophenyl-(4-vinylphenyl) carbonate (4).** The yellow oil containing **3** was dissolved in THF (40 ml) and added dropwise to a stirred ice cold solution of 4-vinylphenol (**2**) (2.0 g, 16.6 mmol) and DIEA (5.0 ml, 28.7 mmol) in THF (60 ml) containing a trace amount of 2,6-di(*tert*-butyl)-4-methylphenol under an inert atmosphere ($N_2$). The solution was stirred at RT for 15 h whereafter the resulting amine salt was removed by filtration and the filtrate was evaporated. The resultant yellow crystals were dissolved in $CH_2Cl_2$ (100 ml), extracted with water (2 × 50 ml), $NaHCO_3$ (50 ml, 5% w/v), and brine (50 ml). The organic phase was dried over $MgSO_4$, filtered, evaporated and purified by column chromatography (heptane:ethyl acetate, 2:8). The last traces of **1** were removed by recrystallisation from ethanol (95%) to afford **4** as colourless crystals (57%); mp 161–163 °C; Found: C, 68.25; H, 5.15; N, 4.6. Calc. for $C_{17}H_{15}NO_4$: C, 68.7; H, 5.1; N, 4.7%; $\lambda_{max}$ ($CHCl_3$)/nm 251 (log ε 4.48); $\nu_{max}$/cm$^{-1}$ 3264 (NH), 3067 and 2818 (CH), 1767 and 1658 (C=C), 1610 (NH), 1552 and 1500 (Ar); $\delta_H$ (500 MHz; $CDCl_3$; $Me_4Si$; 25 °C) 7.54–7.52 (2 H, d, *J* 9.0, 2 × ArH), 7.45–7.43 (2 H, d, *J* 8.6, 2 × ArH), 7.23 (2 H, d, *J* 8.7, 2 × ArH), 7.22 (2 H, d, *J* 9.0, 2 × ArH), 6.74–6.68 (1 H, dd, *J* 10.9 and 17.6, $CH_2$=C*H*), 5.74–5.71 (1 H, dd, *J* 0.7 and 17.6, *trans*-C$H_2$=CH), 5.28–5.26 (1 H, dd, *J* 0.6 and 10.9, *cis*-C$H_2$=CH) and 2.16 (3 H, s, $CH_3$); $\delta_C$ (63 MHz; $CDCl_3$; $Me_4Si$; 25 °C) 168.2, 152.1, 150.4, 147.1, 136.0, 135.9, 135.7, 127.3, 121.4, 120.9, 120.8, 114.5 and 24.5; *m*/*z* (FAB) 297.1007, $C_{17}H_{15}NO_4$ requires 297.1001.

**Synthesis of phenyl-(4-vinylphenyl) carbonate (5).** Phenyl-(4-vinylphenyl) carbonate (**5**) was prepared from phenyl chloroformate and 4-vinylphenol (**2**) according to the method of Whitcombe *et al.*[16] The product was purified by column chromatography (heptane:ethyl acetate, 8:2) and was obtained as colourless crystals (47%); mp 50–52 °C; $\lambda_{max}$ ($CHCl_3$)/nm 251 (log ε 4.24); $\nu_{max}$/cm$^{-1}$ 3062 and 2994 (CH), 1769 (C=O), 1627 (C=C) and 1591 (Ar); $\delta_H$ (250 MHz; $CDCl_3$; $Me_4Si$; 25 °C) 7.47–7.39 (4 H, m, 4 × ArH), 7.30–7.22 (5 H, m, 5 × ArH), 6.77–6.66 (1 H, dd, *J* 10.9 and 17.6, $CH_2$=C*H*), 5.76–5.69 (1 H, dd, *J* 0.7 and 17.6, *trans*-C$H_2$=CH), 5.29–5.25 (1 H, dd, *J* 0.6 and 10.9, *cis*-C$H_2$=CH); $\delta_C$ (63 MHz; $CDCl_3$; $Me_4Si$; 25 °C) 152.0, 151.0, 150.4, 135.8, 135.7, 129.6, 127.3, 126.3, 120.94, 120.88 and 114.4; *m*/*z* (FAB) 240.0791, $C_{15}H_{12}O_3$ requires 240.0786.

**Synthesis of methyl-(4-vinylphenyl) carbonate (6).** A solution of methyl chloroformate (2.6 ml, 33.3 mmol) in THF (35 ml) was added dropwise to a stirred ice cold solution of 4-vinylphenol (**2**) (2.0 g, 16.7 mmol) and DIEA (7.3 ml, 41.6 mmol) in THF (55 ml) containing a trace amount of 2,6-di(*tert*-butyl)-4-methylphenol under an inert atmosphere ($N_2$). The solution was stirred at RT for 20 h until the disappearance of **2** was evident on TLC (heptane:ethyl acetate, 8:2). The formed amine salt was removed by filtration and the remaining solution was evaporated. The resultant oil was dissolved in $CH_2Cl_2$ (100 ml), extracted with water (2 × 50 ml), $NaHCO_3$ (50 ml, 5% w/v), and brine (50 ml). The organic phase was dried over $MgSO_4$, filtered, evaporated and purified by column chromatography (heptane:ethyl acetate, 8:2) to afford a colourless oil (77%). Found: C, 67.4; H, 5.8. Calc. for $C_{10}H_{10}O_3$: C, 67.4; H, 5.7%; $\lambda_{max}$ (EtOH)/nm 249 (log ε 4.74); $\nu_{max}$/cm$^{-1}$ 3046 and 2953 (CH), 1757 (C=O), 1627 and 1508 (C=C), 1436 (CH)

 

and 1225 (C–O); $\delta_H$ (250 MHz; $CDCl_3$; $Me_4Si$; 25 °C) 7.42–7.39 (2 H, d, *J* 8.7, 2 × ArH), 7.15–7.11 (2 H, d, *J* 8.8, 2 × ArH), 6.75–6.63 (1 H, dd, *J* 10.9 and 17.6, $CH_2$=C*H*), 5.73–5.66 (1 H, d, *J* 17.6, *trans*-C$H_2$=CH), 5.26–5.22 (1 H, d, *J* 10.9, *cis*-C$H_2$=CH), 3.89 (3 H, s, $CH_3$); $\delta_C$ (63 MHz; $CDCl_3$; 25 °C) 154.1, 150.6, 135.7, 135.5, 127.2, 121.0, 114.2 and 55.3; *m/z* (FAB) 178.0634, $C_{10}H_{10}O_3$ requires 178.0630.

**Synthesis of (4-hydroxyphenyl)benzamide (7).** A mixture of 4-aminophenol (2.7 g, 25.0 mmol) and triethylamine (3.5 ml, 25.1 mmol) in 1,4-dioxane (35 ml) under an inert atmosphere ($N_2$) was treated with dropwise addition of benzoyl chloride (4.5 ml, 38.8 mmol). The mixture was stirred at RT for 3h, then poured into water (100 ml). The product was collected by filtration, the residue dissolved in ethyl acetate and purified by extraction with 10% NaOH (3 × 75 ml). The aqueous phase was treated with 3 M HCl to pH 1 and extracted with ethyl acetate (3 × 75 ml). The organic phase was washed with water (75 ml), saturated $NaHCO_3$ (75 ml), and again with water (75 ml) before it was dried over $MgSO_4$, filtered and evaporated to furnish white crystals of **7** (32%); mp 215–216 °C; $\nu_{max}/cm^{-1}$ 3382 (NH), 3324 (OH), 3052 (CH), 1647 (C=O), 1542 (NH) and 1220 (CO); $\delta_H$ (500 MHz; DMSO-$d_6$; 25 °C) 10.01 (1 H, s, NH), 9.24 (1 H, OH), 7.93–7.92 (2 H, d, 2 × ArH), 7.58–7.49 (5 H, m, 3 × ArH) and 6.75–6.73 (2 H, d, 2 × ArH); $\delta_C$ (125 MHz; DMSO-$d_6$; 25 °C) 164.8, 153.6, 135.1, 131.1, 130.6, 128.2, 127.4, 122.2 and 114.9.

### NMR studies

$^1$H-NMR spectra were recorded at 25 °C using a 29 mM solution of acetanilide in the presence of various concentrations of styrene (0 to 1.75 M) in $CDCl_3$.

### Polymer synthesis

In a typical polymer synthesis, monomer (5 mol%), crosslinker (95 mol%) and template (for **P6**, **P8** and **P10**) were dissolved in chloroform or acetonitrile in a glass test tube (see Table 1 for details). Initiator (AIBN, 1 mol% with respect to polymerisable double bonds) was added and the tube was connected to a vacuum line where the mixture was degassed by three repeated freeze–thaw cycles, and sealed at reduced pressure. Polymerisation was carried out in a water bath at 60 °C for 24 h, and the resultant polymer monolith was manually ground and sieved through a 63 μm sieve. Fine particles were removed by repeated sedimentation from 400 ml acetone (5 × 40 min) and air-dried. The carbonate esters were hydrolysed by stirring the suspended polymers (1.5 g) in 0.1 M NaOH in methanol (100 ml) at RT. Template removal from **P1** was monitored by HPLC, which showed that complete hydrolysis was obtained after 19 h. The polymers were collected by filtration and washed with 0.1 M NaOH in methanol (2 × 20 ml), methanol (2 × 20 ml), water (100 ml), and acetone (100 ml), then air dried.

### Polymer titrations

Polymer particles were suspended in toluene (25 mg/ml), and from this stock solution appropriate volumes were distributed to Eppendorf tubes. Each incubation mixture contained [$^3$H]-4-acetamidophenol (0.27 pmol) and the desired amounts of polymer (0.5 to 20 mg) in a total volume of 1 ml toluene. The samples were incubated on a rocking table at 23 °C until equilibrium was reached (2 h). After centrifugation at 20,800 × *g* for 5 min, 500 μl of the supernatant was removed from each incubation tube and mixed with 2 ml scintillation cocktail then measured by liquid scintillation counting. All experiments were performed in triplicate.

### Equilibrium binding studies

Binding studies in different solvents were performed essentially as described above. Polymers (2.5 or 5 mg) were incubated with [$^3$H]-4-acetamidophenol (0.27 pmol) in a total volume of 1 ml solvent in Eppendorf tubes.

### Competitive experiments

Competitive binding studies were performed essentially as described above in toluene containing 0.1% dimethyl formamide. Polymer samples (2.5 mg) were incubated together with [$^3$H]-4-acetamidophenol (0.27 pmol) and competing, unlabelled 4-acetamidophenol (10 or 100 μM).

### Chromatographic evaluation

Polymer particles were suspended in chloroform/acetonitrile (85:15, v/v) in a slurry reservoir and packed into stainless steel

**Table 1** Polymer compositions and physical data

| | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10[c] | P11[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Acetanilide (mmol) | – | – | – | – | – | 0.43 | – | – | – | – | – |
| 4-acetamidophenol (mmol) | – | – | – | – | – | – | – | 0.43 | – | 1.00 | – |
| **4** (mmol) | 1.23 | – | – | – | 0.43 | – | 0.43 | – | – | – | – |
| **5** (mmol) | – | 1.25 | – | – | – | – | – | – | – | – | – |
| **6** (mmol) | – | – | 1.27 | – | – | – | – | – | – | – | – |
| Styrene (mmol) | – | – | – | 1.29 | – | 0.43 | – | – | – | – | – |
| MAA (mmol) | – | – | – | – | 0.85 | 0.85 | 0.85 | 1.30 | 1.30 | 4.00 | 4.00 |
| EGDMA (mmol) | 23.36 | 23.71 | 24.07 | 24.54 | 24.21 | 24.21 | 24.21 | 24.66 | 24.66 | 20.00 | 20.00 |
| Chloroform (ml) | 10 | 10 | 10 | 10 | 10 | 10 | – | – | – | – | – |
| Acetonitrile (ml) | – | – | – | – | – | – | 10 | 10 | 10 | 10 | 10 |
| Ratio M:C[a] | 1:19 | 1:19 | 1:19 | 1:19 | 1:2:57 | 1:2:57 | 1:2:57 | 1:19 | 1:19 | 1:5 | 1:5 |
| BET surface area[b] ($m^2/g$) | 423 ± 4 | 411 ± 6 | 436 ± 5 | 466 ± 5 | 506 ± 7 | 531 ± 7 | 510 ± 5 | 493 ± 5 | 477 ± 5 | 350 ± 4 | 364 ± 4 |
| Pore diameter[b] (Å) | 40.5 | 40.9 | 48.9 | 50.6 | 63.3 | 64.7 | 93.6 | 84.5 | 99.3 | 89.1 | 76.2 |

[a] Denotes the ratio between monomer (M) and cross-linker (C). [b] After hydrolysis. [c] Polymer synthesis according to Tan *et al.*[14]

HPLC columns (100 × 4.6 mm) at 300 bar using a single action reciprocating plunger pump (Haskel Engineering Supply Co., USA) with 400 ml acetone as the packing solvent.

Chromatographic analyses were performed in triplicate using 1 mM of the analytes: acetanilide, benzanilide, (4-hydroxyphenyl) benzamide, 2-acetamidophenol, 3-acetamidophenol or 4-acetamidophenol dissolved in chloroform/methanol (94:6, v/v). Chloroform/methanol (99:1, v/v) was used as the mobile phase (1 ml/min), and elution was monitored at 246 nm at 24 °C. The void volume ($V_0$) was determined by injections of acetone, and the retention volumes ($V_R$) for analytes were assigned to the point of the peak corresponding to 50% of the peak area by manual integration using gravimetric analysis.



## Results and discussion

Ideally, a method for the rapid and accurate determination of paracetamol (and its metabolites) in acute situations should be robust, sensitive and selective. Although MIPs offer the potential to help fulfil these requirements, significant success with this target using various molecular imprinting systems has thus far been elusive. We suggest that the limited size and functionality of the target are issues that challenge the molecular imprinting technique. The reported[5] significance of both the phenolic and amide moieties of paracetamol for recognition by antibodies provides some support for this argument.

The paracetamol molecular imprinting studies thus far reported[13–15] have employed non-covalent strategies, an advantage of which is the relative ease of polymer preparation and the vast array of functional monomers available. Nonetheless, this approach is inherently complicated by the presence of competing interactions (*e.g.* template–template, solvent–template, functional monomer–functional monomer), which contribute to the site heterogeneity.[22–24] Tan *et al.*'s use of 4-VP to provide stronger interactions with the mildly acidic phenolic hydroxyl, in conjunction with MAA for interaction with the amide, provided an improvement in the recognition performance of the polymer.[14] Another general strategy for improving the quality of the template–functional monomer interactions is the use of multidentate functional monomers capable of stoichiometric non-covalent interaction, in particular as developed and exploited by Wulff *et al.*[25,26] and Sellergren *et al.*,[27,28] and more recently by Takeuchi *et al.*[29] and ourselves.[30]

Strong template–monomer interactions and more homogenous recognition site distributions are best exemplified through the use of reversible covalent bonds for molecular imprinting.[31] Semi-covalent imprinting strategies, in particular when using the sacrificial spacer approach, have proven useful for poorly functionalised structures, *e.g.* cholesterol.[16] The possibility of applying a sacrificial spacer-based semi-covalent approach to the imprinting of paracetamol seemed achievable through the use of the phenolic hydroxyl as a basis for attachment of the polymerisable spacer, a carbonate ester-containing template monomer that upon hydrolysis releases 4-acetamidophenol and $CO_2$ while exposing the binding site. Moreover, we envisaged that the sacrificial spacer approach could be further augmented by additional non-covalent interactions (hydrogen bonding between methacrylic acid and the amide functionality of paracetamol).

The template containing monomer 4-acetamidophenyl-(4-vinylphenyl) carbonate (**4**) was synthesised in two steps from 4-acetamidophenol (**1**), triphosgene and 4-vinylphenol (**2**). For the synthesis of reference polymers, two similar carbonate ester analogs were prepared: from 4-vinylphenol and phenyl chloroformate or methyl chloroformate to afford phenyl-(4-vinylphenyl) carbonate (**5**) and methyl-(4-vinylphenyl) carbonate (**6**), respectively.

The semi-covalent 4-acetamidophenol imprinted polymer **P1** was synthesised by co-polymerisation of 4-acetamidophenyl-(4-vinylphenyl) carbonate (**4**, 5 mol%) and EGDMA (95 mol%) in chloroform by thermally-initiated free radical polymerisation using AIBN (1 mol%). Suitable reference polymers (**P2**–**P3**) were prepared similarly from phenyl-(4-vinylphenyl) carbonate (**5**) or methyl-(4-vinylphenyl) carbonate (**6**) and EGDMA. These reference polymers were expected to contain smaller binding sites than **P1** since the monomers were based on either phenyl- (**P2**) or methyl carbonate (**P3**). To evaluate the influence of the phenolic moieties incorporated in the polymer cavities, a styrene based polymer (**P4**), lacking the ability to engage in hydrogen bonding interactions with paracetamol, was also prepared in chloroform. 4-acetamidophenol imprinted polymers were also synthesised using a combination of one equivalent 4-acetamidophenyl-(4-vinylphenyl) carbonate and two equivalents of the non-covalently interacting functional monomer MAA in either chloroform (**P5**) or acetonitrile (**P7**). As a suitable reference for these systems, acetanilide was imprinted in a styrene MAA co-polymer using chloroform as a porogen (**P6**). Non-covalently imprinted 4-acetamidophenol polymers were also prepared in acetonitrile, using either three or four equivalents of MAA (**P8** and **P10**), with corresponding reference polymers prepared in the absence of template (**P9** and **P11**).

Post-polymerisation treatment of **P1** with methanolic sodium hydroxide was performed to remove the template molecule by hydrolysis of the carbonate ester. The release of 4-acetamidophenol from **P1** was monitored by HPLC analysis, which after 19 h no longer showed any product, indicating that complete hydrolysis had occurred. All polymers (**P2**–**P11**) were subjected to the same treatment as described above to assure template removal. Hydrolysis of **P1**, **P2**, **P5** and **P7** was also confirmed by IR measurements where the aromatic carbonate shoulder at 1779 $cm^{-1}$ disappeared after treatment. In the case of **P3** the carbonate shoulder was not observed, either before or after hydrolysis.

Polymers were then subjected to a series of radio-ligand binding studies in a range of different solvents and solvent mixtures using [$^3$H]-4-acetamidophenol. All polymers demonstrated high binding affinity for 4-acetamidophenol in toluene (data not shown), which was expected as recognition of this template relies mostly upon electrostatic interactions. Accordingly, lower binding was observed in more hydrophilic media such as acetonitrile and buffer systems, due to the ability of these solvents to compete for electrostatic interactions.[32] In an attempt to minimise non-specific ligand binding in toluene, additional equilibrium binding studies were performed in toluene containing 0.1% dimethyl formamide (Fig. 3, black bars). In these studies, the highest affinity for the template was obtained for the non-covalently imprinted polymer **P10**, where a binding of 61 ± 1% was determined. Only a minor difference in binding (4%) between the imprinted (**P10**) and non-imprinted (**P11**) polymers was observed. We attribute this result

 

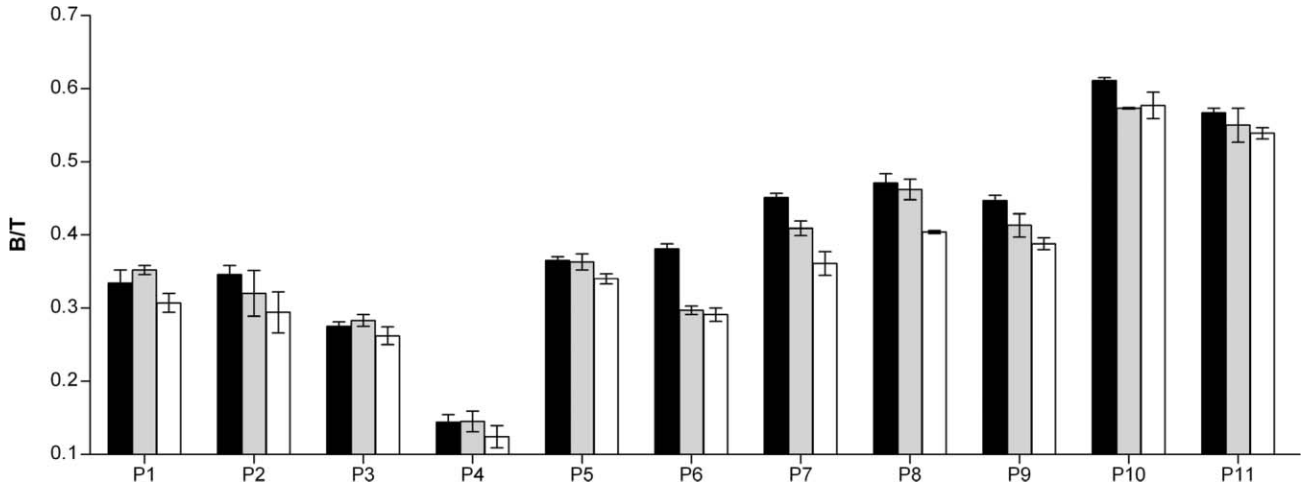


**Fig. 3** Binding of [$^3$H]-4-acetamidophenol to **P1**–**P11** in toluene containing 0.1% dimethyl formamide in the presence of various concentrations of cold 4-acetamidophenol. No cold ligand present (black bar), 10 μM cold ligand present (grey bar), 100 μM cold ligand present (white bar). B/T denotes the amount of bound radioligand (B) relative to the total amount added (T). Error bars reflect SEM.



to the high abundance of carboxylic acid residues present in these polymers relative to the other polymer systems studied, and the resultant high incidence of non-specific electrostatic interactions.

Interestingly, polymer **P7**, which was prepared in acetonitrile using a combination of semi- and non-covalent imprinting, also showed high binding (45 ± 1%). When chloroform was employed as the porogen (**P5**) the binding and surface areas were lower. Binding to **P6** was similar to **P5**, despite the lack of the hydroxyl functionality provided by the residual phenol. The contribution of the MAA-derived non-covalent interactions to the total binding is apparent, in particular upon comparison of **P7** and **P5** with the purely semi-covalently based systems that showed lower binding (35% for **P1** and **P2**, 28% for **P3**), and even after consideration is taken of the gas-accessible surface areas (BET-data). In the case of **P3**, the lower binding may be explained by the formation of a somewhat smaller cavity in this polymer, leading to exclusion of the ligand from the binding site. The styrene-containing reference polymer **P4** bound only 14 ± 1% paracetamol which indicates that incorporation of a phenolic residue in the polymer cavity can contribute to ligand recognition. This is further supported by the fact that **P4** has a larger BET surface area (466 $m^2/g$) than **P1** (423 $m^2/g$) and still displays lower binding of 4-acetamidophenol. The results obtained for **P4** also indicate that non-specific binding to the EGDMA backbone is substantial. This kind of weak interaction between template and crosslinker has previously been shown by molecular dynamics studies to be common in EGDMA systems, due to the vast number of cross-linker molecules present in the polymerisation mixture.[33] The omni-MIPs developed by Spivak and co-workers provide a good example of the role cross-linking functional monomers can play on the induction of ligand selective binding sites.[34,35]

The difference in BET surface areas between **P1** (423 $m^2/g$) and **P7** (510 $m^2/g$) was substantial and may provide a likely explanation for the greater binding capacity of **P7**. However, the binding observed using **P10**, with a considerably smaller surface area (350 $m^2/g$), indicates that specific binding may be involved in this case.

Competitive binding studies were performed with [$^3$H]-4-acetamidophenol in toluene containing 0.1% DMF using unlabelled 4-acetamidophenol (10 or 100 μM) as the competing ligand (Fig. 3, grey and white bars). In general, the polymers demonstrated only a minor reduction in binding (≤10%) with increasing concentrations of unlabelled ligand. This result indicates that the majority of the observed binding under these conditions is due to non-specific interactions with the EGDMA backbone and randomly incorporated hydroxyl groups. However, both **P6** and **P7** display sites selective for 4-acetamidophenol. The fact that **P6** (an acetanilide imprinted reference polymer containing styrene and MAA) also rebinds 4-acetamidophenol selectively suggests that π–π interactions between the aromatic residues present in 4-acetamidophenol and the polymer also contribute to the recognition. Attempts to study π–π interactions between paracetamol and styrene using NMR titrations failed due to poor solubility. However, the presence of such weak aromatic interactions in chloroform was confirmed by NMR titration of acetanilide and styrene (see ESI†). Unfortunately, again due to solubility issues, it was not possible to further increase the concentration of unlabelled ligand in the competitive experiments to quantify the extent of interaction. Instead we turned to chromatographic studies in order to gain more knowledge regarding the ligand specificities of the polymers.

Chromatographic evaluation of the recognition characteristics of the polymers was performed in chloroform/methanol (99:1, v/v) where all polymers demonstrated selectivity for 4-acetamidophenol over the structurally similar ligands acetanilide, benzanilide, (4-hydroxyphenyl)benzamide, 2-acetamidophenol and 3-acetamidophenol, Fig. 4. A substantial increase in capacity factor was observed in all the polymers for all ligands containing a phenolic residue, and selectivity was even seen for 4-acetamidophenol over both 3-acetamidophenol and 2-acetamidophenol, Table 2. Moving the hydroxyl group from the *para* to *meta* position in the ligand results in a weakening of the interaction point to the polymer cavity followed by a reduction in retention. 2-acetamidophenol shows a very low capacity factor in all the polymers which is explained by the inability of this ligand to

**Table 2** Capacity factors (k′)[a] obtained from HPLC analysis in chloroform/methanol (99:1) with ligand concentration 1 mM

| Analyte | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Acetanilide | 0.39 | 0.45 | 0.45 | 0.35 | 0.42 | 0.39 | 0.30 | 0.25 | 0.26 | 0.40 | 0.39 |
| Benzanilide | 0.19 | 0.23 | 0.24 | 0.16 | 0.21 | 0.20 | 0.13 | 0.10 | 0.10 | 0.10 | 0.11 |
| (4-hydroxyphenyl)benzamide | 4.59 | 5.47 | 5.41 | 3.65 | 5.24 | 4.06 | 3.86 | 3.32 | 3.26 | 3.73 | 3.84 |
| 2-acetamidophenol | 1.09 | 1.25 | 1.27 | 0.97 | 1.26 | 1.07 | 0.99 | 0.88 | 0.90 | 1.04 | 1.09 |
| 3-acetamidophenol | 6.20 | 7.05 | 7.06 | 4.97 | 7.66 | 5.96 | 6.90 | 6.73 | 6.30 | 9.11 | 8.88 |
| 4-acetamidophenol | 7.74 | 8.87 | 9.01 | 5.70 | 9.70 | 7.07 | 8.58 | 8.47 | 7.76 | 13.53 | 12.52 |

[a] $k' = (V_R - V_0)/V_0$. The SEM of the capacity factors presented are all ≤0.02.

**Table 3** Normalised selectivity factors (R)[a] between imprinted polymers and their respective reference systems as a measure of imprinting effect

| | P1 vs | | | P5 vs | P7 vs | P8 vs | P10 vs |
|---|---|---|---|---|---|---|---|
| Analyte | P2 | P3 | P4 | P6 | P6 | P9 | P11 |
| Acetanilide | 1.00 | 1.00 | 0.83 | 0.79 | 0.65 | 0.88 | 0.94 |
| Benzanilide | 0.97 | 0.93 | 0.85 | 0.75 | 0.53 | 0.86 | 0.90 |
| (4-hydroxyphenyl)benzamide | 0.96 | 0.99 | 0.93 | 0.94 | 0.78 | 0.93 | 0.90 |
| 2-acetamidophenol | 1.00 | 1.00 | 0.83 | 0.86 | 0.76 | 0.90 | 0.89 |
| 3-acetamidophenol | 1.01 | 1.02 | 0.92 | 0.94 | 0.95 | 0.98 | 0.95 |
| 4-acetamidophenol | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

[a] $R = (k'_{\text{analyte MIP}}/k'_{\text{analyte REF}}) \times (k'_{\text{template REF}}/k'_{\text{template MIP}})$.

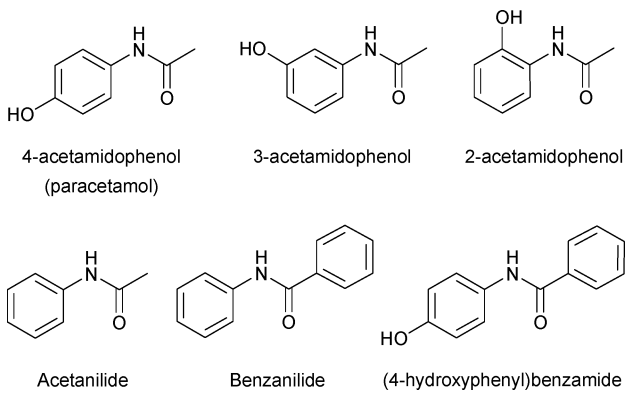


**Fig. 4** Structures of 4-acetamidophenol and related analogues.

create favourable recognition interactions in the polymer functionalities. This is most likely due to the relative inaccessibility of the phenolic hydroxyl due to the proximity of the *ortho*-acetamido substituent. This problem is exacerbated by the presence of a stabilising and competing internal hydrogen bond within the ligand. The lower capacity factor obtained for (4-hydroxyphenyl)benzamide over that for 4-acetamidophenol is noteworthy as they both display a phenolic residue in the *para* position. This difference can to some extent be explained by the fact that (4-hydroxyphenol)benzamide possesses a two and a half times larger rotational volume[36] than 4-acetamidophenol, leading to exclusion of the former ligand from some of the binding sites. When comparing the series of polymers studied, a general trend observed is that the capacity factors were greatest for the mixed and purely non-covalent systems and more modest for the semi-covalent polymers. The highest capacity factors were obtained for the non-covalent polymer **P10**, although only a moderate imprinting effect was seen for this system, Table 3. Interestingly, the effect of imprinting was not evident at all for the purely semi-covalent system (**P1**) which indicates that more than one additional electrostatic interaction point is necessary for obtaining recognition, even for a template as small as paracetamol. Incorporation of a second complexation point resulted in a more pronounced imprinting effect. This is illustrated by results obtained with the mixed system **P7**, where MAA had the ability to interact with the amide functionality in 4-acetamidophenol. When comparing **P5** and **P7** it was seen that a more pronounced imprinting effect is obtained in the acetonitrile based polymer system than in that synthesised in chloroform. Interestingly, the choice of acetonitrile over chloroform as a porogen has previously been reported to increase the selectivity of MAA-EGDMA imprinted co-polymers.[37–39]

Collectively, the results demonstrate that polymers offering only a single interaction point, as in the case for the semi-covalent systems (**P1**–**P3**), while capable of engendering the polymers with selectivity for 4-acetamidophenol, did not elicit a clear imprinting effect. We conclude that this is due to the small size and low degree of functionalisation of the polymerisable paracetamol template (**4**). We propose that these factors severely limit the quantity of favourable contributions from van der Waals interactions between polymer backbone and template structure, which seem to be necessary in purely semi-covalent systems involving poorly functionalised templates in order to obtain template selectivity.[17,38] In contrast, the non-covalent systems (**P8**, **P10**) showed high template rebinding, but only a weak imprinting effect, as randomly distributed carboxyl moieties exerted a dominating influence. From the results obtained with the polymers investigated in this study, the best strategy for creating MIPs selective for the small and poorly functionalized 4-acetamidophenol, was to rely upon the simultaneous use of semi-covalent and non-covalent imprinting. Here, the combination of the control of template–polymer interaction at the phenolic hydroxyl of paracetamol afforded by the sacrificial spacer approach in conjunction with the amide–carboxyl interactions furnished by MAA, and even cross-linker, yielded the most promising result, as based upon the good imprinting effects obtained for 4-acetamidophenol using **P5**



 

and **P7**. This reflects the results obtained with antibodies[5] where the significance of both functionalities for binding was established.

## Conclusions

Polymers with selectivity for 4-acetamidophenol (paracetamol) have been prepared using three different imprinting approaches. A series of batch binding and chromatographic studies demonstrated that a pure non-covalent imprinting system with MAA bound paracetamol most efficiently although displayed only a weak imprinting effect, suggesting that binding was due mainly to non-specific interactions. Polymers prepared using solely the semi-covalent approach displayed low binding and no imprinting effect under the conditions studied, highlighting the necessity for more than one electrostatic interaction point for creating polymers capable of the selective molecular recognition of 4-acetamidophenol. The use of MAA in combination with a semi-covalent strategy based upon a sacrificial carbonate ester (**4**), resulted in an improved imprinting effect. It is therefore evident that in order to obtain successful imprinting of small and poorly functionalised template structures, such as 4-acetamidophenol, neither the non-covalent nor the semi-covalent imprinting method alone is powerful enough to create selective recognition. At least in this case, the best solution to this problem requires the use of a combination of these methods, thereby taking advantage of the template fixation obtained from the semi-covalent approach and the complexing power obtained from the non-covalent approach to introduce additional interaction points to the template. This study not only helps to define the lower limit of template size and functionality amenable for use with semi-covalent imprinting protocols, but also highlights the potential benefits that can be obtained by combining the strengths of different imprinting strategies. We believe that the improvements, albeit modest, obtained with this strategy warrant the use of this approach in other systems with similar challenges. The basis for this argument is strengthened through comparison with conclusions drawn from studies with antibodies selective for paracetamol (4-acetamidophenol) and its metabolites.

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