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Magnetic molecularly imprinted polymer beads for drug radioligand binding assay

Richard J. Ansell† and Klaus Mosbach*

Department of Pure and Applied Biochemistry, University of Lund Chemical Centre, P.O. Box 124, S22100 Lund, Sweden

Molecularly imprinted polymer–magnetic iron oxide composite materials which exhibit recognition properties and can be withdrawn from solution by application of a magnetic field were prepared for the first time. Magnetic iron oxide was incorporated using a suspension polymerisation methodology with a perfluorocarbon liquid as the dispersing phase for the preparation of methacrylic acid-1,1,1-trimethylolpropane trimethacrylate copolymer beads molecularly imprinted with the β-blocker (*S*)-propranolol. The resulting superparamagnetic imprinted polymer beads were capable of binding [^{3}H]-(*S*)-propranolol more strongly than a non-imprinted, otherwise identical, polymer. In a competitive radioligand binding assay using a magnet to separate polymer from solution, (*R*)-propranolol and (*R,S*)-metoprolol exhibited cross-reactivities of 19 and 0.7%, respectively, compared with (*S*)-propranolol.

Keywords: *Molecularly imprinted polymer; molecular imprinting; superparamagnetic; magnetic polymer; radioligand binding assay; (*S*)-propranolol*

Molecular imprinting[1,2] has gained acceptance in recent years as a method for the preparation of specific binding sites in polymeric materials. The technique involves polymerisation of functional monomers and a cross-linker around a template or print molecule. Extraction of the template leaves behind recognition sites of functional and shape complementarity to the template. Molecularly imprinted polymers (MIPs) may be produced as bulk polymers and ground into microscopic particles, as micrometre-scale beads, as thin surface layers grafted on to other bead material or as membranes. MIPs have been used for chromatographic separations, solid-phase extraction, in biomimetic sensing devices and as artificial enzymes. The feasibility of using MIPs in competitive radioligand binding assays was first demonstrated for the tranquillizer diazepam and the bronchodilator theophylline,[3] since when similar molecularly imprinted sorbent assays (MIAs) have been developed for morphine and Leu-enkephalin,[4] corticosteroids,[5] the α_2-adrenoceptor agonist yohimbine,[6] the herbicide atrazine,[7] the immunosuppresant cyclosporin[8] and the β-blocker (*S*)-propranolol.[9] Recently, a MIA for (*S*)-propranolol was demonstrated in which the competitive binding takes place directly in blood or urine samples.[10] MIPs have the advantage for such applications of being extremely stable compared with biological antibodies.

Occurring in parallel to the developments in molecular imprinting, magnetic materials have been applied increasingly in medicine and biotechnology. These materials generally contain small particles of ferromagnetic material such as magnetic iron oxide or magnetite, Fe_3O_4. Because of the small size of the magnetite particles, the materials are negligibly magnetic (and so do not aggregate) except in a magnetic field and are termed superparamagnetic. Superparamagnetic polymer composite particles, agarose–polyacrylamide beads, were first described in the 1970s.[11,12] A range of methods have been used since then to prepare magnetic particles: solid magnetite or ferrofluids may be incorporated in suspension polymerisation protocols[11] or polymer beads may be 'post-magnetised' by the precipitation of iron oxide from solution or inclusion of colloidal magnetite or ferrofluids.[12,13] Magnetic polymer particles coated with specific ligands have been used in immunoassay methodologies,[14] the isolation of nucleic acid sequences,[15] cell selection from complex matrices such as whole blood,[16] the isolation of microorganisms from samples in the food industry,[17] batch-mode affinity chromatography of proteins from fermentation broths[18] and magnetically stabilised fluidised-bed separations.[19] Magnetic particles derivatised with proteins have been used in biosensors, either measuring magnetic forces[20] or using magnetism to trap the recognising element.[21] Magnetic polymer particles with entrapped cells[22] or enzymes[23] have been used in biotransformations. Magnetic particles have also been used *in vivo* for the delivery of bioactive agents.[24,25]

We decided to investigate whether molecularly imprinted polymers which are superparamagnetic could be prepared. The suspension polymerisation process for the preparation of MIPs, recently developed in our laboratory,[26,27] seemed suited to the inclusion of ferromagnetic material. We chose to study the use of magnetic imprinted materials in MIAs to measure (*S*)-propranolol. In all MIAs developed until now, analyte, radiolabelled analyte and polymer are incubated together, then the polymer is separated by centrifugation. Our goal was to replace the centrifugation step with a magnetic separation. (*S*)-Propranolol was chosen as the analyte because bulk MIPs imprinted with (*S*)-propranolol have been shown to exhibit strong, specific rebinding of the print molecule in aqueous media.[8] We considered it particularly important to demonstrate the use of magnetic MIPs in aqueous media, since other potential applications, including use in batch-mode affinity chromatography, magnetically stabilised fluidised-bed separations and biosensors, are much more likely to be in aqueous media than in organic solvents. In this paper, we describe the successful production of magnetite–molecularly imprinted polymer composite beads and their application in binding studies with (*S*)-propranolol.

Experimental

Preparation of magnetic beads

The apparatus for suspension polymerisation was as described previously.[27] The compositions of the different polymers are given in Table 1. The imprinting phase contained (*R,S*)-propranolol (Fig. 1) [print molecule, obtained by base titration and extraction into methylene chloride from (*R,S*)-propranolol hydrochloride; Fluka, Buchs, Switzerland], methacrylic acid (MAA), (Merck, Darmstadt, Germany), 1,1,1-trimethylolpropane trimethacrylate (TRIM), (Aldrich Chemie, Steinheim, Germany), toluene (2.5 g, HPLC grade, dried over

† Present address: Institut für Analytische Chemie, Chemo- und Biosensorik, Universität Regensburg, 93040 Regensburg, Germany.

sodium) and 2,2′-azobis(2,4-dimethylvaleronitrile) (20 mg), (Wako, Osaka, Japan). For polymers C and D, magnetic iron oxide powder (76 mg, 1 μm particles) [BDH (Merck, Poole, Dorset, UK] dispersed in toluene (1.0 g) was added to the imprinting mixture. The dispersing phase consisted of perfluoro-1,3-dimethylcyclohexane (20 ml) (Fluorochem, Old Glossop, Derbyshire, UK), toluene (1.0 g to saturate the perfluorocarbon liquid) and perfluorinated polymeric surfactant (PFPSW, 50 mg, prepared as described[27]). The phases were mixed by stirring at 2000 rpm for 5 min. Nitrogen was bubbled through the resulting suspension for 5 min.

Polymerisation was performed by stirring at 600 rpm with the polymerisation vessel three-quarters immersed in a water-bath at 50 °C (polymers A and B) or 60 °C (C and D) under a slow stream of nitrogen. After 3 h, the polymer beads were separated by filtration and washed with acetone. Perfluorocarbon liquid was kept for re-distillation and re-use. Polymer aggregates and small fragments were removed by sonication in 50 ml of acetone followed by sedimenting for 1 min and decanting, then for 30 min and decanting; the sedimented fraction after 1 min and the supernatant after 30 min were discarded. For polymers C and D, the most magnetic beads were selected by sedimenting again for 1 min using a magnet. All polymers were washed on sintered glass funnels using the following solutions: ammonium acetate (1 M) in ethanol–acetic acid–water (40 + 25 + 35) (four aliquots of about 200 ml over 24 h); acetic acid–ethanol (30 + 70) (two aliquots of about 200 ml over 12 h; and methanol (two aliquots of about 200 ml over 12 h). Finally, the particles were dried under vacuum and stored at room temperature until use.

Binding assays using [^{3}H]-*(S)-propranolol*

Polymers (different amounts) were incubated with [^{3}H]-(*S*)-propranolol (1.68 pmol, 930 Bq), (DuPont NEN, Stockholm, Sweden) in different buffer systems (total volume 1 ml). After incubation on a rocking table overnight, the polymer was separated from the supernatant by centrifugation (5 min at 13 000 rpm) or by placing the Eppendorf tubes in a Dynal magnetic particle concentrator (MPC-E). Supernatant (400 or 500 μl) was withdrawn, added to scintillation fluid (10 ml) (National Diagnostics, Atlanta, GA, USA) and counted by liquid scintillation counting.

Competitive binding assays

Polymer C (20 μg) was incubated with [^{3}H]-(*S*)-propranolol (1.68 pmol, 930 Bq) and different concentrations of non-radiolabelled (*R*,*S*)-metoprolol (Fig. 1) (Leiras, Turku, Finland), (*S*)-propranolol or (*R*)-propranolol (Fluka) in sodium citrate (25 mM) of pH 5.0 containing 2% v/v ethanol (total volume 1 ml). After incubation on a rocking table overnight, the polymer was separated from the supernatant by placing the Eppendorf tubes in a Dynal MPC-E cocentrator for 1 min. Supernatant (500 μl) was withdrawn, added to scintillation fluid (10 ml) and counted by liquid scintillation counting.

Results and discussion

Production of magnetic beads

(*R*,*S*)-Propranolol was used as a print molecule, rather than just (*S*)-propranolol, since the racemate is less expensive and yields polymers which can be used equally well in an MIA for (*S*)-propranolol.[9] This is considered to be because the small amount of [^{3}H]-(*S*)-propranolol employed in the assays binds only to the (*S*)-propranolol-specific sites. Although imprinted polymer beads may be produced using either thermal or UV-initiated polymerisation,[27] when magnetic iron oxide was included only the former initiation technique could be used since the suspension adsorbed incident UV radiation. This limited the porogenic solvents which could be used to those with boiling-points above 60 °C. We chose to use toluene since this solvent has been shown to work well in the imprinting of (*S*)-propranolol.[9] The washing of the polymers to extract the template is of particular importance in order to leave behind as many active recognition sites as possible. Our washing protocol was essentially identical with that used by Andersson[9] and shown to remove ⩾99% of propranolol from his imprinted polymers.

The compositions of the polymers made in this study are given in Table 1. Initially, non-magnetic beads (polymers A and B) were made by a method similar to that used previously to prepare beads imprinted with Boc-*l*-Phe.[24] The product was mostly single beads of diameter 5–50 μm [Fig. 2(*a*)]. Although the size distribution of the beads is fairly broad, we believe this could be narrowed by optimising the reactor design. The few aggregates and fragments were removed by decanting and sedimentation. When magnetite was added to the imprinting mixture, polymerisation did not occur at 50 °C, and the temperature had to be raised to 60 °C. More aggregates and fragments were produced during polymerisation. By increasing the cross-linking (achieved in practice by decreasing the amounts of template and functional monomer), good beads were again produced [polymers C and D, Fig. 2(*b*)]. However, the most magnetite that could be incorporated in the protocol without further optimisation was 5% by weight.

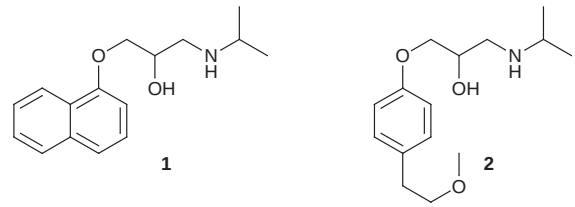


Fig. 1 Structures of propranolol (**1**) and metoprolol (**2**).

Table 1 Preparation and physical properties of polymers A–D

Polymer	(*R*,*S*)-propranolol/mmol	MAA/mmol	TRIM/mmol	Magnetic iron oxide/mg	Average bead diameter*/μm	Specific surface area†/m^2 g^{-1}	Pore volume†/ml g^{-1}	Pore diameter†/Å
A	1.0	4.0	4.0	-	13.0	108	0.56	261
B	–	4.0	4.0	-	12.6	159	0.82	268
C	0.5	2.0	4.0	76	9.4	301	1.02	193
D	–	2.0	4.0	76	9.5	333	0.95	173

* The average bead size of each polymer produced was determined by estimating the sizes of 50–100 beads under a microscope. † Surface areas and pore volumes were determined by nitrogen adsorption using a Micromeritics (Norcross, GA, USA) ASAP 2400 instrument covering pores between 17 and 3000 Å. Samples were de-gassed at 120 °C and an 80-point pressure table was used with a 45 s equilibration time. Surface area was determined from a BET plot. Pore volume was the average of Barrett, Joyner and Halenda (BJH) cumulative adsorption and desorption pore volumes. Average pore diameter was calculated as 4 × BJH adsorption pore volume/surface area.

Physical properties of magnetic and non-magnetic beads

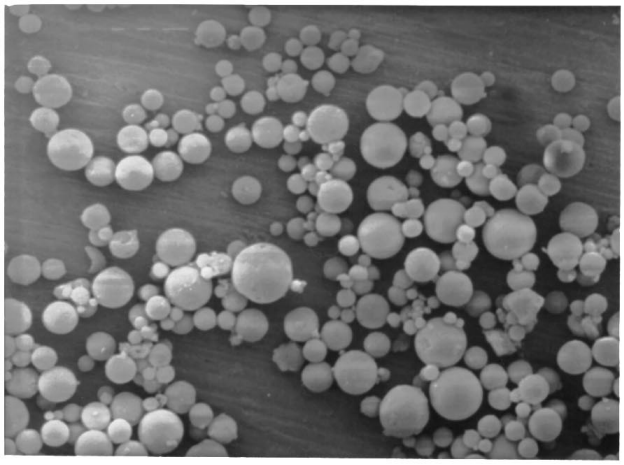
Fig. 2 shows that the magnetic and non-magnetic beads appeared very similar under an electron microscope, although under a light microscope the magnetic beads were much more opaque. Table 1 shows that the magnetic polymers C and D were comprised of beads of smaller size than the non-magnetic polymers A and B. This is probably due to the higher polymerisation temperature used in the preparation of these polymers, rather than the inclusion of magnetite or the different ratios of cross-linker. The surface areas and pore volumes of C and D were greater than those of A and B, and the average pore diameter was smaller. These effects could be due to the higher polymerisation temperature or the higher cross-linking for C and D.

[^{3}H]-*(S)-propranolol binding to non-magnetic beads*

The effects of ethanol concentration (Fig. 3) and buffer composition (Fig. 4) on the binding of [^{3}H]-(*S*)-propranolol to polymers A and B were investigated, in order to compare our non-magnetic beads with the published results for bulk (*S*)-propranolol-imprinted polymers.[9] Although it is most interesting to perform the binding assays in aqueous media, a small amount of ethanol must be added to wet the polymers, which are hydrophobic and otherwise form aggregates in solution.

Binding was measured in sodium citrate (25 mM) of pH 6.0 containing different concentrations of ethanol. This buffer was chosen as it had been shown to give the highest specific binding of (*S*)-propranolol to imprinted bulk polymers.[9] As can be seen from Fig. 3, the binding of [^{3}H]-(*S*)-propranolol to the non-imprinted polymer increases with decreasing concentration of ethanol, whereas the specific binding (*i.e.*, the difference between binding to imprinted and non-imprinted polymers) decreases. The non-specific binding at low ethanol concentrations is much greater than that for bulk imprinted polymers,[9] suggesting that the beads are more hydrophobic. However, A and B contain 20.3% m/m MAA and the bulk polymers 8.0% m/m MAA, which suggests that the beads should be less hydrophobic. Apart from the use of ethylene glycol dimethacrylate as cross-linker in the bulk polymers and TRIM in the bead polymers, the main difference is in the preparation protocol. Specifically, it may be that some of the perfluorinated

(*a*)



(*b*)

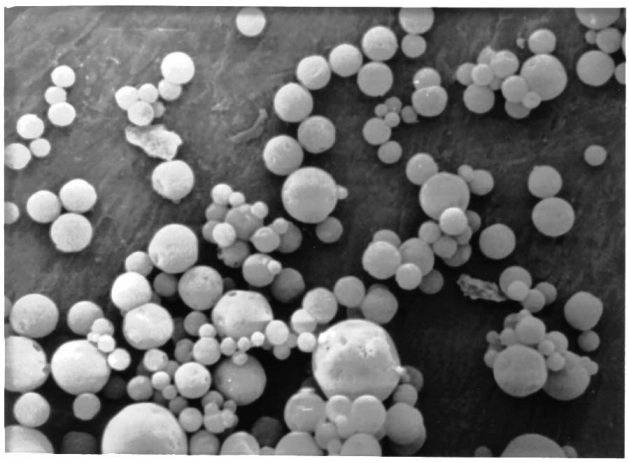


Fig. 2 Scanning electron micrographs of molecularly imprinted polymer beads prepared by suspension polymerisation: (*a*) polymer A, (*R*,*S*)-propranolol imprinted, non-magnetic; (*b*) polymer C, (*R*,*S*)-propranolol imprinted, magnetic. Beads were placed on aluminium pegs and sputter coated with 15 nm of gold using a Polaron E5150 coater. The images were obtained using an ISI 100A SEM at 25 kV. Magnifications ×320.

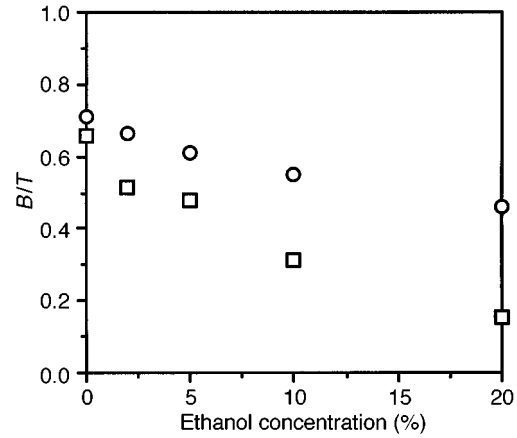


Fig. 3 Binding of (*S*)-propranolol to polymers A (○) and B (□) as a function of ethanol concentration. [^{3}H]-(*S*)-propranolol (1.6 pmol) and polymer (20 μg) were incubated in 1 ml of sodium citrate (25 mM) of pH 6.0 containing various concentrations of ethanol. *B/T* is the ratio of the amount of radioligand bound (*B*) to the total amount added (*T*) to the test-tubes. The precise total activity, *T*, was determined for each ethanol concentration in tubes without polymer but otherwise treated identically. Each point represents the average of two assays.

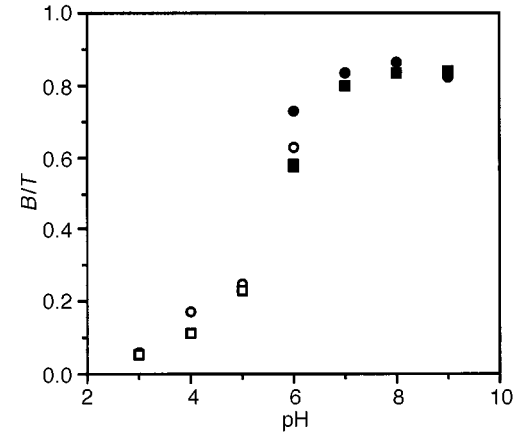


Fig. 4 Binding of (*S*)-propranolol to polymers A (circles) and B (squares) as a function of buffer composition. [^{3}H]-(*S*)-Propranolol (1.6 pmol) and 20 μg of polymer particles were incubated in 1 ml of 25 mM buffer containing 2% v/v ethanol. The buffers were sodium citrate (pH 3.0–6.0) (open symbols) and sodium phosphate (pH 6.0–9.0) (filled symbols). The precise total activity, *T*, was determined for each buffer in tubes without polymer but otherwise treated identically. Each point represents the average of two assays.

polymeric surfactant PFPSW remains associated with the surface of the beads. PFPSW incorporates chains of Brij 35, which would certainly make the beads more hydrophobic.

Since the intention was to demonstrate the application of magnetic imprinted polymers in essentially aqueous mixtures, we chose in any case to work with 2% v/v ethanol in our binding assays, and investigated the effect of the buffer salt and pH (Fig. 4). Unlike the bulk propranolol imprinted polymers,[9] the beads seem to exhibit hardly any specific binding in sodium citrate (25 mM) at pH 3.0, 4.0, 5.0 or 6.0. However, there is more specific binding in sodium phosphate (25 mM), particularly at pH 6.0 (although phosphate buffers only very weakly at this pH).

Comparison of binding to magnetic and non-magnetic beads

Different amounts of polymers A, B, C and D were incubated with [^{3}H]-(*S*)-propranolol in sodium phosphate (25 mM) of pH 6.0 containing 2% v/v ethanol (Fig. 5). Initially, both magnetic and non-magnetic polymers were removed from solution by centrifugation. It can be seen that the binding to the imprinted polymer is higher, and to the non-imprinted polymer lower, for the magnetic polymers. Hence the specific binding is approximately three times higher for the magnetic polymers. This might be due to the greater cross-linking compared with A and B. Alternatively, the inclusion of magnetite itself could have an effect; this would certainly make the polymers less hydrophobic, and so could reduce non-specific hydrophobic interactions.

The experiment was repeated using the Dynal MPC-E concentrator to separate the polymers from solution (Fig. 6). Comparison of Figs. 5 and 6 suggests that higher activity was found in the supernatant for all the polymers than when polymer was removed by centrifugation. This is because magnetic separation did not remove all of the polymer. For complete separation of bound and non-bound radioligand, complete removal of the polymer from solution is required. Clearly, more of the polymer is removed in the case of the magnetic polymers, such that the calculated values of *B/T* are 70–90% of those when the centrifuge was used, *i.e.*, 70–90% of the polymer has been separated. For the non-magnetic polymers, 40–60% of the polymer was separated using the magnetic separator. This is simply the amount of polymer which sediments in 1 min, so that it is no longer in suspension and not drawn up by the pipette. The data are more erratic for the non-magnetic beads because the sedimented polymer is easily disturbed and taken up by the pipette, while the magnetic beads are held more strongly in place.

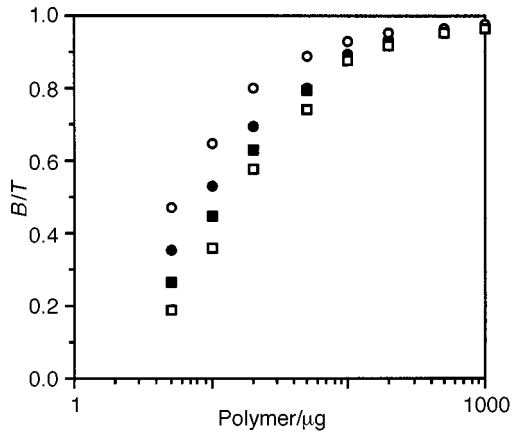


Fig. 5 Binding of (*S*)-propranolol to polymers A (●), B (■), C (○) and D (□) as a function of the amount of polymer added. [^{3}H]-(*S*)-propranolol (1.6 pmol) and polymer particles were incubated in 1 ml of sodium phosphate (25 mM) of pH 6.0 containing 2% v/v ethanol. The polymer was removed from solution by centrifugation and 400 μl of supernatant were removed for scintillation counting. Each point represents the average of two assays.

Optimisation of [^{3}H]-*(S)-propranolol binding to magnetic beads*

In view of the apparent difference in specific binding between the non-magnetic and magnetic polymers, we investigated the effect of the buffer salt and pH on the binding of [^{3}H]-(*S*)-propranolol to polymers C and D (Fig. 7). Unlike the non-magnetic polymers, there was a very significant difference in binding to the imprinted and non-imprinted polymers in sodium citrate (25 mM) at pH 3, 4, 5 and 6. In fact, the highest specific binding to the imprinted polymer occurred in sodium citrate (25 mM) at pH 5.0. The different performance of magnetic and non-magnetic polymers in different buffers may be a consequence of the increased proportion of cross-linker, the

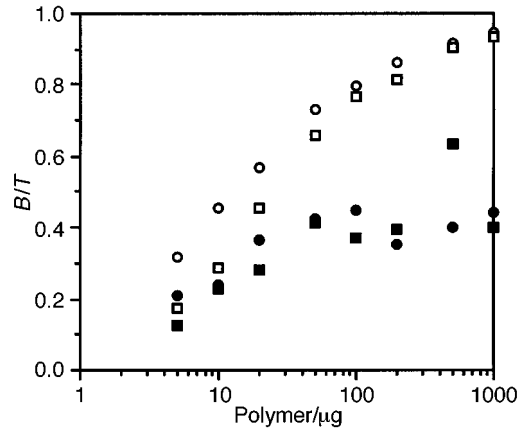


Fig. 6 Binding of (*S*)-propranolol to polymers A (●), B (■), C (○) and D (□) as a function of the amount of polymer added. [^{3}H]-(*S*)-Propranolol (1.6 pmol) and polymer particles were incubated in 1 ml of sodium phosphate (25 mM) of pH 6.0 containing 2% v/v ethanol. The polymer was removed from solution by placing the tubes in a Dynal MPC-E concentrator for 1 min and 400 μl of supernatant were removed for scintillation counting. Each point represents the average of two assays.

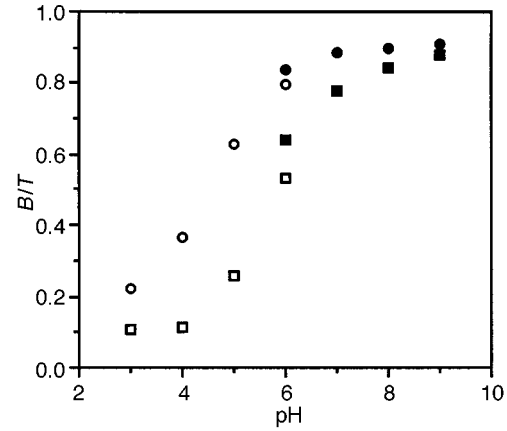


Fig. 7 Binding of (*S*)-propranolol to polymers C (circles) and D (squares) as a function of buffer composition. [^{3}H]-(*S*)-Propranolol (1.6 pmol) and polymer (20 μg) were incubated in 1 ml of 25 mM buffer containing 2% v/v ethanol. The buffers were sodium citrate (pH 3.0–6.0) (open symbols) and sodium phosphate (pH 6.0–9.0) (filled symbols). The precise total activity, *T*, was determined for each buffer in tubes without polymer but otherwise treated identically. Each point represents the average of two assays.

increased surface area or the inclusion of magnetite for the magnetic polymers.

Competitive binding assays

The binding of [^{3}H]-(*S*)-propranolol to the (*S*)-propranolol imprinted sites in polymer C in sodium citrate (25 mM) pH 5.0 containing 2% v/v ethanol was studied in the presence and absence of different competing ligands (Fig. 8). The IC_{50} values (concentration of competing ligand required to displace 50% of the radioligand) were calculated from the *x*-intercepts after log–logit transformation. The values for (*R*,*S*)-metoprolol and (*R*)- and (*S*)-propranolol were 26.5, 1.00 and 0.19 μM, respectively. These values are remarkably similar to those obtained previously for a bulk (*R*,*S*)-propranolol imprinted polymer (64, 1.22 and 0.43 μM, respectively).[9] The cross-reactivity of (*R*)-propranolol (19%) is greater than that of (*R*,*S*)-metoprolol (0.7%), hence the polymer exhibits higher substrate selectivity than stereoselectivity. Andersson[9] has suggested that this is due to the strength of hydrophobic interactions between the polymer and the naphthyl ring structure of propranolol, making recognition at this end of the molecule of paramount importance for rebinding in aqueous media.

Conclusions

We have successfully demonstrated that superparamagnetic molecularly imprinted MAA–TRIM copolymer beads can be prepared by inclusion of magnetic iron oxide in a suspension polymerisation protocol utilising a perfluorocarbon liquid as the dispersing phase. We have also shown that the recognition properties of the imprinted polymer particles are not affected by the inclusion of iron oxide. In the case studied, non-magnetic beads appeared to exhibit more non-specific hydrophobic binding of [^{3}H]-(*S*)-propranolol than previously reported bulk imprinted polymers. However, the non-specific binding was lower, and the specific binding higher, for the magnetic polymer beads. It is unclear whether this is due to the incorporation of magnetic iron oxide or the higher degree of cross-linking in the magnetic polymers.

Most of the magnetic polymer could be separated from solution when an Eppendorf tube was placed in a Dynal MPC-E magnetic particle concentrator for 1 min. This was considered to be the longest practically convenient interval for which the tube could be placed in the MPC-E concentrator; undoubtedly, more polymer could be removed by using longer times or by increasing the magnetic iron oxide content of the polymer. We were unable to prepare beads containing more than 5% w/w of magnetic iron oxide. However, we believe that further optimisation of the method, perhaps using a polymeric stabiliser for the iron oxide, could lead to a higher iron oxide content. Alternatively, materials with higher magnetic susceptibilities could be employed.

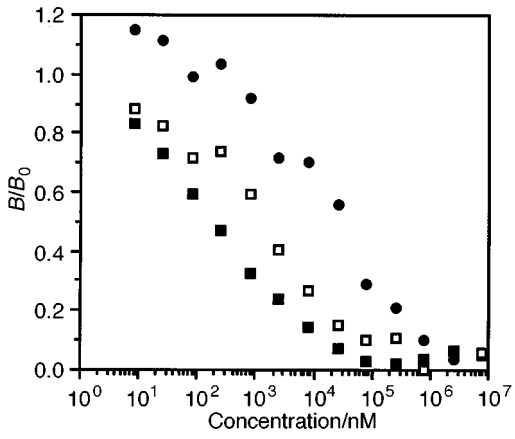


Fig. 8 Displacement of [^{3}H]-(*S*)-propranolol binding to polymer C in sodium citrate (25 mM) of pH 5.0 containing 2% v/v ethanol by increasing concentrations of various competing ligands. B/B_0 is the ratio of the amount of [^{3}H]-(*S*)-propranolol bound in the presence of displacing ligand, *B*, to the amount bound in the absence of displacing ligand, B_0. Displacing ligands: (*S*)-propranolol (■), (*R*)-propranolol (□) and (*R*,*S*)-metoprolol (●). The polymer was removed from solution by placing the tubes in a Dynal MPC-E concentrator for 1 min and 500 μl supernatant were removed for scintillation counting. Each point represents the average of three assays.

In a competitive radioligand binding assay for (*S*)-propranolol, the magnetic (*R*,*S*)-imprinted polymer was found to exhibit very similar cross-reactivity for (*R*)-propranolol and (*R*,*S*)-metoprolol to previously reported non-magnetic bulk polymers.[9]

This work represents a significant advance in the use of MIA in diagnostic assays. Bulk (*S*)-propranolol imprinted polymers have recently been used for the direct assay of (*S*)-propranolol in blood and urine samples.[10] The use of magnetic polymers in such samples would be of further use in simplifying the method. We would also suggest that magnetic molecularly imprinted polymers may have future applications in other fields such as batchwise affinity chromatography, for cell sorting and as recognition elements in biosensors.

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