A fluorescence polarisation molecular imprint sorbent assay for 2,4-D: a non-separation pseudo-immunoassay†

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The first pseudo-immunoassay which employs a molecularly imprinted receptor and a fluorescent probe, and quantifies the bound analyte directly using the fluorescence anisotropy of the polymer–probe–analyte suspension, is described.

Molecularly imprinted polymers (MIPs) have been widely exploited as antibody mimics in pseudo-immunoassays, since the seminal paper by Mosbach *et al.* in 1993.[1–3] MIP-based competitive binding assays (molecularly imprinted sorbent assays or MIAs) have been developed for a range of analytes including theophylline,[1] morphine,[4] propranolol,[5] atrazine[6] and 2,4-dichlorophenoxyacetic acid (2,4-D)[7] which exhibit selectivity and sensitivities comparable with assays based on biological antibodies.

In any competitive binding assay, the analyte and a probe compete for available binding sites, and the greater the concentration of analyte the less of the probe binds. Some perceived disadvantages of MIPs do not hinder their application in such assays: Many MIAs function in aqueous solvents and binding site heterogeneity is not a problem as long as the sites binding the probe most strongly are selective.[8] Compared to some established assays based on biological antibodies such as the enzyme monitored immunotest (EMIT), however, two main disadvantages have held back the commercial acceptance of MIAs. First, the dominance of radiolabelled forms of the analyte as probes, and second the need for separation of the MIP, with bound probe, from unbound probe in solution, before the amount bound (and hence concentration of the analyte) can be calculated. Thus, the adaption of MIAs to modern assay formats should be of great interest. The only reported MIA not requiring the separation of MIP from solution is the scintillation proximity assay for propranolol developed by Ye *et al.*[9,10] Here, the measured signal is generated by scintillation fluors covalently incorporated into the MIP and a radiolabelled probe is still needed. Other MIAs have been developed based on fluorescent probes but still requiring separation *e.g.* a MIA for 2,4-D which employs the structurally related 7-carboxy-4-methylcoumarin (CMMC) as a probe (Fig. 1).[11] In the current work we have combined the principles of a non-separation MIA and use of a fluorescent probe for the first time by developing a fluorescence polarisation molecular imprinted sorbent assay (FPMIA) for 2,4-D.

Fluorescence polarisation immunoassays using biological antibodies were introduced in 1973 by Dandliker *et al.*[12] and have been reported for analytes including proteins, hormones and pollutants such as 2,4-D.[12–19] When the fluorescent probe binds to an antibody (or a MIP) in solution its tumbling rate falls, and consequently the measured fluorescence will be more isotropic than that of the free probe, which tumbles faster. The fluorescence anisotropy (or polarisation) hence increases with the percentage of probe bound, or decreases with the amount of competing analyte.

In order to perform fluorescence measurements on a mixture of a fluorophore and polymer particles in solution, it is important that fluorescence can be distinguished from the scattering of the excitation light by the polymer particles.[20] Hence the excitation and emission wavelengths should be well separated (*e.g* for CMMC, λ_{ex} 323 nm and λ_{em} = 385 nm) and the particles very small. In earlier publications on different MIAs for 2,4-D, we used micrometer-sized particles obtained by grinding of bulk MIPs,[11] or by precipitation polymerisation.[21] However, these are too large to be used in a fluorescence polarisation assay since they sediment quite quickly and scatter the excitation light. Therefore, we modified the MIP synthesis method, in comparison to our earlier work, such that the MIP was obtained in microgel form.

Precipitation polymerisation can be optimised to produce particles ranging in size from true microgels with diameter d = 10–100 nm[22–24] to nano/microspheres of d = 500–1000 nm which have been utilised in CEC and binding assays[9,10,25] and even larger spheres of d = 5 μm suitable for HPLC.[26] We desired particles below the excitation wavelength of CMMC (323 nm). However one concern was that as MIP particles become smaller and consequently less rigid, it is by no means obvious they will retain binding sites with high affinity and selectivity. The only MIP particles of $d < 300$ nm that have been studied have been used for

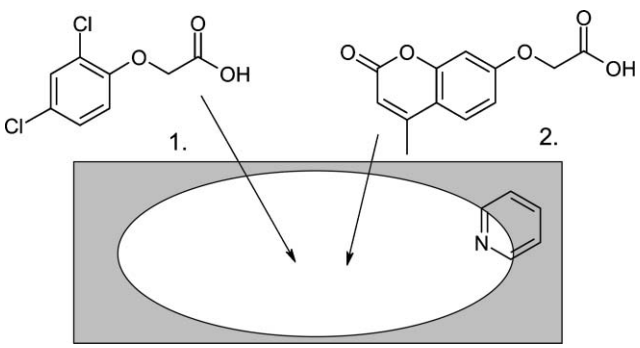

Fig. 1 Principle of the assay for 2,4-D described by Haupt *et al.*[11] and of the current assay. 2,4-D (**1**) and CMMC (**2**) compete for the binding sites produced by imprinting poly(TRIM-co-vinylpyridine) with 2,4-D.

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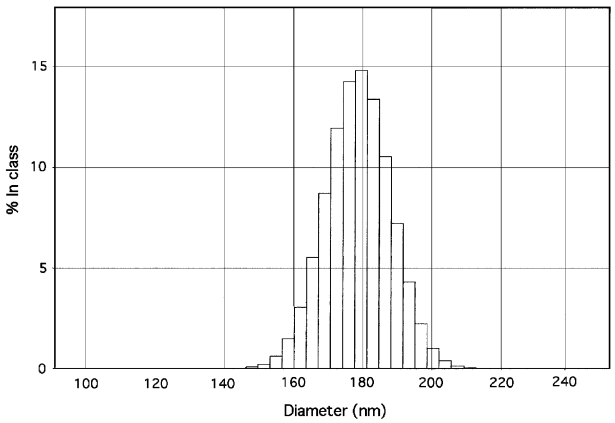

Fig. 2 Dynamic light scattering at 90° of the MIP microgel suspension in methanol (Zetasizer, Malvern Instruments). Peak analyses by intensity, volume and number all showed single, symmetrical peaks with a narrow size distribution.

catalytic applications where the requirements are different than for binding assays.[22–24]

We used a similar polymer recipe as in our earlier work, with trimethylolpropane trimethacrylate as cross-linking monomer and 4-vinylpyridine as functional monomer, but with acetonitrile as solvent. The dilution of the monomers (5% v/v of monomers in the total solution) and polymerisation conditions (see ESI†) were such that the MIP was obtained in the form of microgel particles. Dynamic light-scattering data (Fig. 2) show that the mean size of the fully solvated particles was 180.8 nm, thus suitable for our needs (although when allowed to dry completely, the particles shrink and aggregate, see ESI†). The particles have a very narrow size distribution (low polydispersity) and the data were fitted by a simple monomodal model.

To confirm the presence of selective binding sites, a conventional radioligand MIA was performed using ^{14}C-2,4-D. We found that the imprinted microgel adsorbed considerably more of the radioligand than a non-imprinted control microgel prepared under identical conditions but in the absence of the 2,4-D template (Fig. 3). This is congruent with our earlier results[7] and confirms the imprinting effect generated by the template. In addition, a competitive assay showed that the MIP microgel was also able to bind the fluorescent probe CMMC, although to a lesser extent

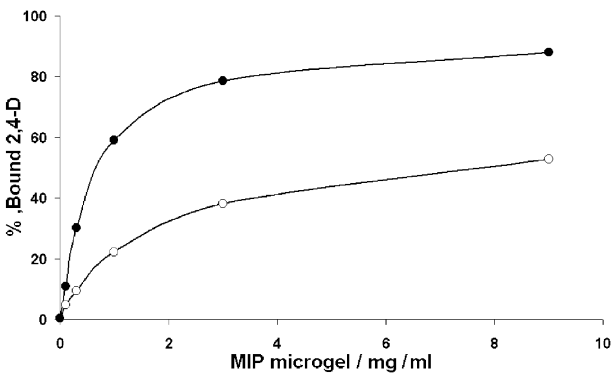

Fig. 3 Binding of ^{14}C-2,4-D to the MIP (filled circles) and the control (open circles) microgels in 10 mM sodium phosphate buffer at pH 7, as a function of the polymer concentration.

than 2,4-D (about 10%, figure not shown). This is also in concordance with our earlier results.[11] These results show that a molecularly imprinted polymer microgel has been obtained with affinity for 2,4-D, and that CMMC can be used as a fluorescent probe.

To develop the FPMIA, we first investigated the appropriate concentrations of polymer and CMMC to use. Various combinations, in different buffer systems, were investigated and the fluorescence intensities were recorded of the mixtures, and of the supernatants after polymer was removed by centrifugation (data not shown). We sought to find conditions under which a) the fluorescence signal overlaps minimally with the scattering of excitation light by the polymer (higher CMMC, lower polymer) b) the fluorescence intensity is not affected by the presence of the polymer (polymer does not quench the fluorescence, nor does the CMMC fluorescence increase on binding to the polymer. This was found to depend on the buffer solution) c) approximately 50% of the probe binds to the polymer, as calculated from the fluorescence yield after centrifugation. The optimum conditions found were using 2 mg mL^{-1} polymer, 160 ng mL^{-1} CMMC, and in 10 mM sodium phosphate buffer at pH 7. Next we incubated polymer and CMMC under these conditions together with varying concentrations of 2,4-D and of the closely related competitor

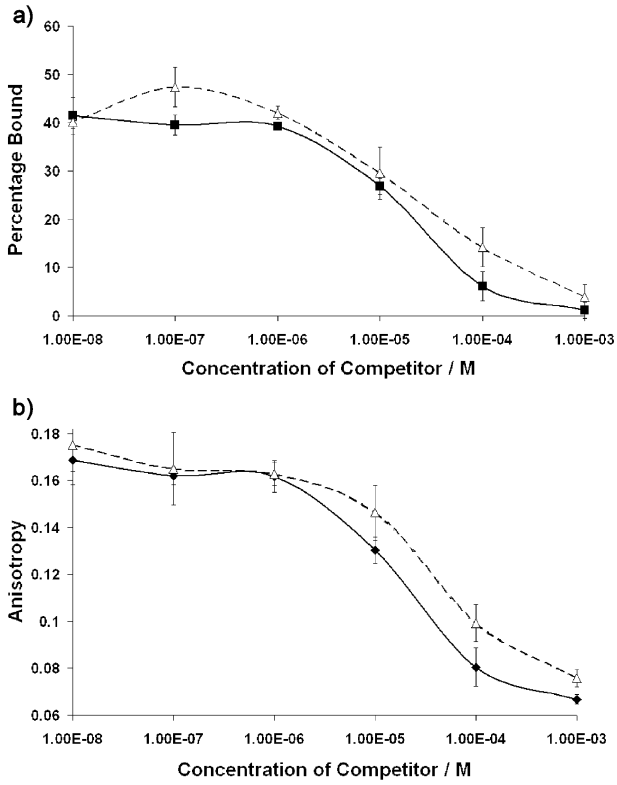

Fig. 4 a) % CMMC bound (calculated by centrifugation and measurement of the supernatant fluorescence) and b) fluorescence anisotropy for MIP (2 mg mL^{-1}), CMMC (160 ng mL^{-1}) and varying concentrations of 2,4-D (solid curve, filled circles) or 4-CPOAc (dashed curve, open triangles) in 10 mM sodium phosphate buffer at pH 7. Each point is the average of three separate assays. Error bars represent ± standard deviation in the calculated values. Fluorescence data were recorded using a Jobin-Yvon Horiba Fluorolog-2 fluorescence spectrophotometer with polarization accessories.

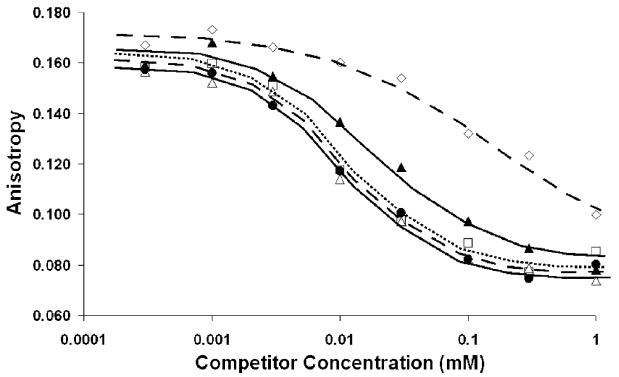

Fig. 5 Fluorescence anisotropy for MIP, CMMC and varying concentrations of 2,4-D or other competitors. Curves fitted by eye. 2,4-D; filled circles, solid curve. 2,4-DB; open triangles, dashed curve. 3,4-D; open squares, dotted curve. 4-CPOAc; filled triangles, solid curve. POAc; open diamonds, dashed curve. Experimental conditions as for Fig. 4.

4-chlorophenoxyacetic acid (4-CPOAc), and measured the fluorescence intensity and fluorescence polarisation of the mixtures, and the fluorescence after centrifugation. From the latter data we could calculate the % bound (Fig. 4a), and demonstrate that 2,4-D is better at displacing CMMC from the binding sites than its structural analogue 4-CPOAc. Measurements of the fluorescence intensity of the mixture before centrifugation showed the intensity was always the same (data not shown), confirming that the quantum yield of CMMC is unaffected either by the presence of the polymer or that of 2,4-D or 4-CPOAc. Measurements of the fluorescence anisotropy (Fig. 4b) showed that the anisotropy correlates closely with the % CMMC bound.

Finally we set up the assay using a wider range of competitors, all closely related structurally to 2,4-D (Fig. 5). The results show that the assay is reasonably selective for 2,4-D, with an IC_{50} value about 10 μM, approximately 30 times lower than that for phenoxyacetic acid (POAc), although 3,4-dichlorophenoxyacetic acid (3,4-D) and 2,4-dichlorobutyric acid (2,4-DB) compete relatively strongly. These results are again in good agreement with the ones from our earlier work when the competitive binding assay was done in buffer.[11] Thus we have shown for the first time that MIP microgels of of $d < 300$ nm can indeed have affinities and selectivities similar to those of bulk polymers.

It is interesting that the ability to displace CMMC appears to correlate with the number of chloro-groups on the molecule. The difference observed is not due to chlorine quenching the CMMC fluorescence, since no difference in the fluorescence intensity was observed, and in any case quenching of the CMMC in solution would tend to give higher anisotropy measurements at any given concentration, rather than lower.

In conclusion we have demonstrated that an imprinted polymer can be used as the recognition element in a fluorescence polarization molecularly imprinted sorbent assay, analogously to how antibodies are used in conventional FPIAs. The assay exhibits reasonable selectivity for 2,4-D and could certainly be used as a 'group-specific' screen for 2,4-D and related pesticides. Work is currently under way aiming at improving the sensitivity of the assay.

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