Supramolecular binding of protonated amines to a receptor microgel in aqueous medium†

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Polyanionic microgels containing negatively charged tetrazole binding sites show supramolecular binding of various protonated amines (*e.g.* dibucaine and spermine) in a competitive aqueous medium at millimolar concentration.

Microgels are sponge-like microparticles around 0.1–10 μm in size that swell in a good solvent. Their response often takes less than a second following a change in chemical surroundings (temperature, pH, ionic strength), whereas slab gels tend to respond much more slowly, over hours or days, to reach swelling equilibrium. Although microgels are highly crosslinked polymers, they form colloidal 'solutions' in a good solvent. Intrinsic viscosities of 'water-soluble' microgels are generally low even if the gel is highly charged, and so differ considerably from ordinary polyelectrolytes. Their small size—similar to that of viruses—permits microgels to travel in the bloodstream, which makes them potentially useful in drug delivery and controlled drug release. For example, the release of doxorubicin (an anticancer drug with a single amine group that is protonated at physiological pH) from a lipid-coated polyanionic microgel can be controlled by stimulation with an electrode.[1,2] Microgels have attracted considerable interest in many areas of chemistry over recent years. It is widely recognised that their structural properties and ability to quickly respond to environmental stimuli could be applied in biomedicinal fields.[3,4].

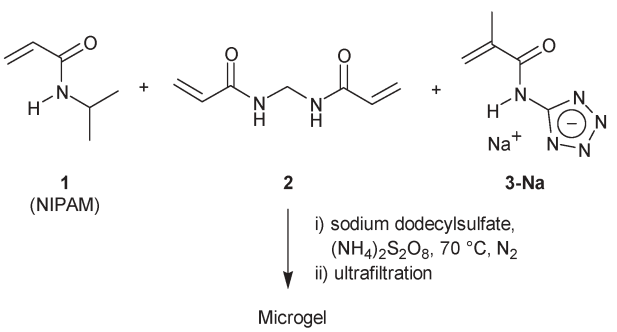
The use of microgels is not purely limited to the temporary physical loading of a drug within a polymer network, a process that relies on the swelling kinetics to trap and release the confined molecules. We anticipated that the incorporation of a monomer with a suitable recognition group could, in fact, afford a polymeric supramolecular receptor with multiple binding sites in the polymer network. In such a host–guest system, the microgel would act as the host and small molecules as the guests, while the choice of recognition group could impart some selectivity for a target guest.

To date, a number of methods have been put forward suggesting polymers as potential supramolecular receptors. Nature already makes extensive use of antibodies and enzymes for the molecular recognition of small molecules, as well as to instigate a plethora of chemical reactions. Molecular imprinting, introduced by Wulff over 30 years ago, is a technique where a monomer is polymerised in the presence of a crosslinker, a template and a porogen.[5] The technique generates crosslinked polymer particles that, after extraction of the template, contain pores resembling an imprint of the template. Molecularly imprinted polymers mimic some of the characteristics of biological supramolecular receptors. Although highly successful for chromatographic separations, the standard molecular imprinting procedure produces insoluble polymer networks. For a polymeric supramolecular receptor, the largest hurdle which has yet to be overcome is the insolubility that imprinted polymer networks display in an aqueous medium.[6] Several reports have suggested an extension of the molecular imprinting concept to microgels.[7–9] However, re-binding of the template generally required organic (co-)solvents and did not work in water, a highly polar and competitive solvent. In almost all cases, a co-solvent such as methanol was essential, and most studies tended to focus on ligands with low intrinsic aqueous solubility.[10]

We present our initial results obtained with a 'water-soluble' polyanionic microgel that possessed the ability to bind various protonated amines (such as the local anaesthetic dibucaine, the β-blocker propranolol, and the oligoamines spermidine and spermine)—*in water at pH 7 and at an ionic strength of 0.15 M*.

N-Isopropylacrylamide, NIPAM (**1**; Scheme 1), was chosen as the major component of a supramolecular receptor microgel because of the wealth of literature available on the properties of NIPAM-based polymers. In addition, the monomer imparts amide groups along the polymer chain—reminiscent of the peptide backbone of proteins. Poly-NIPAM possesses a lower critical solution temperature (LCST) of 32 °C. Below the LCST, the polymer is water-soluble, whereas above the LCST it precipitates from an aqueous solution. Microgels based on NIPAM will precipitate likewise above their LCST.

N,*N*′-Methylenebis(acrylamide), **2**, served as crosslinker in the preparation of the microgel. It is also an acrylamide and its incorporation into a poly-NIPAM network preserves the polymer's environmentally sensitive nature.



Scheme 1 Synthesis of a poly-NIPAM based microgel.

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† Electronic supplementary information (ESI) available: Additional ^{1}H NMR, light-scattering, SEM, copolymerisation, microcalorimetry and crystal structure data. See DOI: 10.1039/b604393c

Tetrazole-containing monomer **3-Na**[11] was the third component and introduced negatively charged binding sites for cationic ligands. Tetrazoles have pK_a values similar to carboxylic acids but are generally more lipophilic. The bioisosteric replacement of a carboxylic acid functional group by a tetrazole is a well-known concept in modern drug design.[12] We chose tetrazole-containing monomer **3** (pK_a 4.5) because of its easy synthetic access and binding properties. The charge distribution of a tetrazole over a larger molecular surface area is considered to be an advantage for an H-bond donor in drug binding and supramolecular chemistry.[13,14] The crystal structure of a 4 : 1 model complex between monomer **3** and spermine (**4**) illustrates this clearly (Fig. 1) and shows each spermine NH being hydrogen-bonded to a tetrazole nitrogen.‡ In addition, the terminal spermine NH groups are involved in bifurcated H-bonds to nearby carbonyl oxygens of the methacrylamide monomer. Incorporation of a tetrazole functionality into a NIPAM-based microgel was therefore expected to create binding sites superior to those found in previously reported methacrylic acid-based microgels.[2] Moreover, whilst methacrylic acid has been the preferred comonomer for most NIPAM-based microgels reported in the literature, its copolymerisation with NIPAM is characterised by unfavourable reactivity ratios differing by three orders of magnitude. As a result, the integration of the crucial binding sites is delayed towards the late stages of the polymerisation and is difficult to control.[15] In contrast, the reactivity ratios of **3-Na** and NIPAM are close to an ideal copolymerisation (see ESI) and therefore allow a statistical distribution of key binding sites into the polymer network.

Microgels were prepared by radical polymerisation of **1** (*ca.* 80–84 mol%), **2** (10 mol%) and **3-Na** (*ca.* 6–10 mol%) at 70 °C in dilute aqueous solution, using ammonium persulfate as initiator and sodium dodecylsulfate as dispersant (Scheme 1).[16] The LCST of the microgel causes the microgel to precipitate during the polymerisation and thus helps to instill a measure of control over the final size of the polymer particles, narrowing their size distribution at the same time. The dilute microgel solution was purified and concentrated by ultrafiltration against deionised water, using cross-flow membranes with a molecular weight cut-off of 100,000 g/mol. The microgels were finally isolated as solids after freeze-drying.

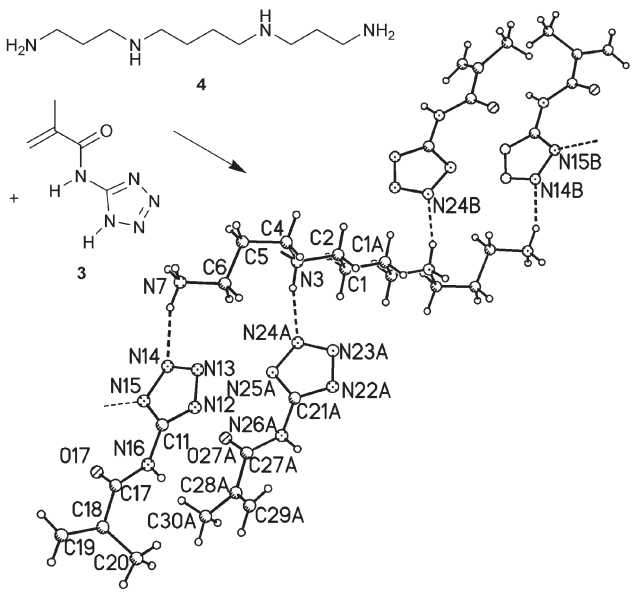

Fig. 1 Perspective view of the model compound (from spermine **4** and monomer **3**) which lies on a centre of inversion. Dotted lines indicate hydrogen bonds. Atoms labelled A are generated by the symmetry code: +x, 0.5 − y, 0.5 + z; and those labelled B: 2 − x, 1 − y, 1 − z.

The hydrodynamic radius of the microgel was determined by light scattering in solution, with the size distribution exhibiting a peak at 0.28 μm. Although additional peaks were observed at higher radii, these were thought to originate mainly from aggregation of microgel particles in solution; such large aggregates and macroscopic gel particles were readily removed by filtration of the aqueous microgel mixture through a 0.45 μm membrane filter. While this procedure was likely to eliminate the large-particle part of the size distribution, it greatly improved the solubility of the residual microgel fraction. Scanning electron microscopy (SEM) of the microgel before filtration revealed microgel particles with sizes in the 0.2–0.3 μm (Fig. 2) range in agreement with the light-scattering results. ^{1}H NMR spectra of the microgels confirmed the absence of low-molar-mass impurities, suggesting that ultrafiltration was an efficient way of purifying microgels. The microgels gave clear solutions in water.

^{1}H NMR spectroscopy was used to investigate the supramolecular binding of amines to the microgel. Evidence of binding came from complexation-induced shifts and line broadening of the ligand's NMR signals. Complexation-induced shifts refer to a change in the chemical shift of the host/guest system when binding occurs under fast exchange on the NMR time scale. The observed chemical shift is then a weighted average of the free and bound species in the system. To ensure that signal shifts during the NMR titration were not the result of changes in pD, binding studies were carried out in a pD 7.4 buffer (at 0.15 M ionic strength). Fig. 3 depicts how the signals for spermine (**4**), a tetraamine that is almost completely protonated at neutral pH (90.6% at pH 7.0), shift by over 0.3 ppm with increasing microgel-to-spermine ratio.

Line broadening of the NMR signals of the ligand is a further indication of binding—provided that contributions from other factors such as shimming errors and high viscosity can be ruled out.[17] When a low-molar-mass ligand interacts with a protein or a polymer, it takes on the macromolecule's lower tumbling rate, coinciding with a broadening of the NMR signal. While line broadening was observed for a range of compounds (*e.g.* propranolol, spermidine, see ESI), it was most noticeable for dibucaine (**5**). Fig. 4 shows the line broadening of dibucaine's aromatic 3-H signal in D_2O at pD 7.4. Like complexation-induced shifts, the extent of line broadening depends on concentration,

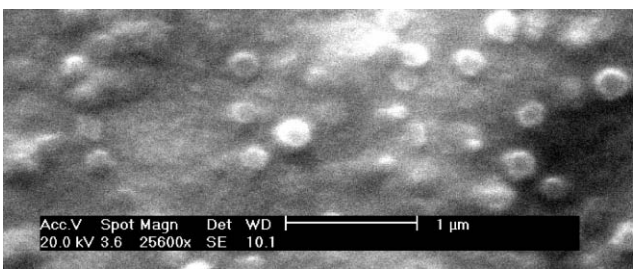

Fig. 2 SEM image showing the spherical shape of microgel particles and their narrow size distribution (typical diameter *ca.* 0.2–0.3 μm).

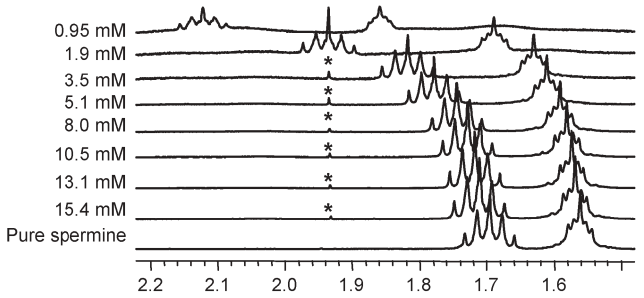

Fig. 3 ^{1}H NMR (400 MHz, D_2O, pD 7.4) spectra, obtained after adding increasing amounts of spermine (**4**) tetrahydrochloride to a microgel solution (3 mg/0.7 mL), show complexation-induced shifts of spermine signals characteristic of binding. With a content of approx. 8 mol% **3-Na** in the microgel, the concentrations correspond to spermine-to-tetrazole molar ratios of 0.4 : 1 to 7 : 1. The ^{1}H NMR spectrum of pure spermine in pD 7.4 buffer recorded in the absence of microgel is displayed for comparison. The signals of acetone impurities are marked by asterisks.

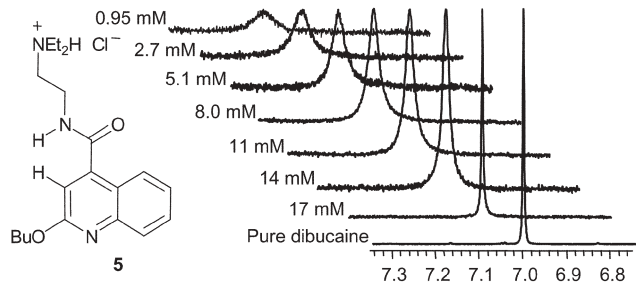

Fig. 4 Line broadening of the NMR signal of dibucaine's aromatic 3-H proton is evident in the presence of the microgel (2.2 mg/0.7 mL) at millimolar dibucaine concentrations.

with the maximum line broadening being again observed in the millimolar range.

When ^{1}H NMR measurements were repeated at pD 9.4, line-broadening of dibucaine and spermine was no longer observed. Protonation of the amine groups was thus deemed to be essential for binding. Therefore the polymer–ligand interactions were not purely hydrophobic interactions but involved a largely electrostatic component. In order to rule out a simple ion-exchange process as the main driving force responsible for binding, we repeated the NMR measurements with a tetramethylammonium salt (which is unable to hydrogen bond but capable of ion exchange) and a benzamidinium salt[14] capable of H-bonding but known to bind poorly to tetrazoles. In both cases, neither line broadening nor complexation-induced shifts were observed (see ESI).

The binding constant of the spermine–microgel interaction was determined by isothermal titration calorimetry in H_2O containing 0.1 M morpholine *N*-propanesulfonate buffer (pH 7.15). Positive values for both ΔH (3.2 kJ mol^{-1}) and $T\Delta S$ (19.2 kJ mol^{-1}) account for an endothermic, entropy-driven process. This is indicative of water being released in the binding step.[18] Similar, large endothermic binding processes are well known for protein–ligand systems when a rearrangement or closure of the binding site is necessary.[19] The flexible structure of the microgels demands some degree of rearrangement upon binding to a ligand, which helps to explain why the binding of spermine is dominated by an entropic contribution. The data were corrected for dilution and, when fitted to an n : 1 binding model, gave an estimate for the binding constant of about 700 M^{-1}, with n being close to the value expected for four tetrazoles per binding site.

In conclusion, our results demonstrated binding of various protonated amines to a tetrazole-containing anionic receptor microgel in a competitive aqueous environment. ^{1}H NMR spectra gave evidence of line broadening and complexation-induced shifts at millimolar concentrations in aqueous solution. This was backed up by microcalorimetric data. Work is currently underway to enhance the microgel's binding affinity and to introduce selectivity by using a suitable templating (molecular imprinting) technique.

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Notes and references

‡ *Crystal data* for model complex: Single crystal X-ray diffraction data were collected on $C_{10}H_{30}N_4{\cdot}4(C_5H_6N_5O)$ with an Oxford Diffraction Xcalibur2 diffractometer with Mo Kα radiation at 120 K. Crystal dimensions: 0.08 × 0.03 × 0.03 mm^3, M = 814.91, monoclinic, $P2_1/c$, a = 11.1364(14) Å, b = 13.482(2) Å, c = 14.299(2) Å, β = 110.275(12)°, V = 2013.8(5) Å^3, Z = 4, 10445 reflections collected; 1959 independent [R_{int} = 0.0707]. Final R indices [$I > 2\sigma(I)$, 1513 data]: $R1$ = 0.1029, w$R2$ = 0.1967; R indices (all data), $R1$ = 0.1461, w$R2$ = 0.2167. Refinement with SHELXTL. No observed intensity beyond θ = 20° because of the small weakly diffracting nature of the crystals so completeness to θ = 22.32° was only 76.1%. CCDC 603033. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b604393c

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