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Novel imprinted soluble microgels with hydrolytic catalytic activity†

Stacey C. Maddock, Pamela Pasetto and Marina Resmini*
Dept. of Chemistry, Queen Mary, University of London, Mile End Rd., London, UK E1 4NS.
E-mail: m.resmini@qmul.ac.uk

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The synthesis and kinetic characterisation of soluble imprinted acrylamide based microgels incorporating arginine and tyrosine derivatives as additional functional monomers is reported.

Molecular imprinting represents an attractive approach for the generation of recognition sites in macromolecular systems where a template molecule is used in a casting procedure. Approaches using different types of interactions between the template and the monomer units have been investigated and valuable results have been obtained with particular regard to binding polymers in analytical applications.[1] By contrast, the generation of efficient catalytic imprinted polymers remains a challenge.[2]

Most of the published data in this field refers to the generation of highly cross-linked insoluble polymers; these materials are fairly rigid structures characterised by a low degree of flexibility. Several attempts have been made to build catalytic activity into these polymers, but the rigidity of the structures seems to mitigate against catalytic character comparable with that of enzymes.

We report here the first attempt to improve the potential for higher catalytic activity by using soluble microgels containing two different amino acid sidechains that have been implicated in the catalytic mechanism of catalytic antibodies and enzymes. Results are presented on the synthesis of imprinted soluble acrylamide-based microgels, incorporating arginine and tyrosine side chains as special functional monomers and characterisation of their hydrolytic catalytic activity towards carbonate substrates.

Microgels can be described as cross-linked polymer particles with size comprised between 10 and 300 nm, characterised by very low solution viscosity for their size. One of the main advantages of these macromolecules is the fact that they can lead to homogeneous colloidal solutions when dissolved in the appropriate solvent system.[3] Microgels can be obtained using a variety of techniques including emulsion polymerisation that uses surfactants, precipitation polymerisation leading to microspheres and solution polymerisation. In all cases smaller size particles are obtained compared to bulk polymerisation leading to improved selectivity and catalytic activity.[4]

In polymer imprinting the interactions between the template and the functional monomers play an important role. Moreover the orientation of the functional groups inside the generated cavity has particular influence on the selectivity of the polymer. The choice of template and functional monomers has to reflect these requirements. The association constants need to be high in order to favour a stable interaction possessing a specific geometric directionality during polymerisation. Wulff *et al.*[5] showed that an insoluble polymer containing amidine functionalities, capable of strongly binding a phosphate template, exhibited high hydrolytic activity with the corresponding ester substrates.

Mechanistic studies carried out by our group on the hydrolytic activity of polyclonal antibody preparations, obtained by immunisation with phosphonates and phosphate transition state analogues, have shown that the side chains of a tyrosine and an arginine, are mainly responsible for the observed rate accelerations of hydrolytic reactions. Work by other groups studying the structure and mechanism of monoclonal hydrolytic antibodies has also implicated these two amino acid side chains in catalysis.[6]

We decided to investigate the use of polymerisable derivatives of these two amino acids together with acrylamide, as the backbone monomer, and *N*,*N*′-ethylenebisacrylamide as the cross-linker agent, for the generation of 'soluble' microgels. Based on our previous results with antibodies, phosphate **1** (Fig. 1) was chosen as the transition state analogue for the hydrolysis of the carbonate **2** and used as template for the imprinting. Plausible interactions between the template and the two functional monomers are shown in Fig. 2, some of which might be involved in the catalytic mechanism. Both acrylamide and the cross-linker have been used in the literature to generate soluble polymers for a variety of applications including catalysis.[7] Also the presence of multiple amide groups was thought to provide additional interactions with the template and substrates, complementing those of the functional monomers.

Phosphate **1** was prepared in good yields by reaction of 4-nitrophenylphosphorodichloridate with 4-acetamidophenol in THF[8] followed by hydrolysis of the second chloride. The polymerisable amino acid derivatives **4** and **5** were obtained by acylation of tyrosine and arginine amides with acryloyl chloride. The purity of the arginine derivative, established by ^{1}H NMR, proved to be essential for a good polymer preparation.

Radical polymerisation in high dilution was used to obtain soluble microgels. This technique[9] does not make use of surfactants. Stabilisation of the growing microgels toward macrogelation is achieved through steric stabilisation when the total monomer concentration is reduced below a critical value, C_m. This value is dependent on factors such as the solvent, the initiator, the amount of cross-linker and the polymerisation temperature, and needs to be determined experimentally for each case.

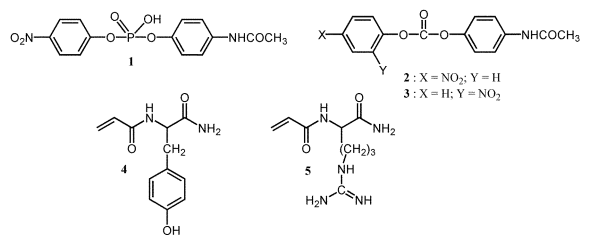

Fig. 1 Structures of the template **1**, substrates **2** and **3**, and polymerisable amino acids **4** and **5** derived from tyrosine and arginine, respectively.

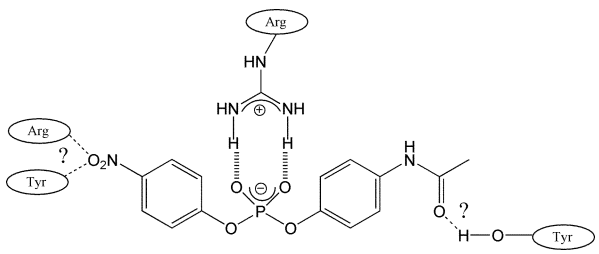

Fig. 2 Plausible interactions of the phosphate template **1** with the arginine and tyrosine monomer derivatives are shown, some of which could be involved in the catalysed reaction.

† Electronic supplementary information (ESI) available: Fig. S1 and S2: raw and corrected kinetic data of Pol396 and Pol397 with substrate. General methodology for polymer preparation and kinetic assays. See http://www.rsc.org/suppdata/cc/b3/b312631e/

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All polymers were prepared in DMSO (ESI†). to facilitate monomers and template solubility. Moreover literature data reported that the affinity constant measured in DMSO between a guanidinium group and a phosphate moiety are in the region of 10^4–10^5 M^{-1}, as determined by ^1H NMR,[10] ensuring a tight interaction during the imprinting stage and therefore good selectivity.

A qualitative preliminary study showed that the imprinted Pol397 containing 70% cross-linker (C_m = 1.5%) exhibited the highest rate accelerations. After isolation the polymer could be easily dissolved in DMSO–water solutions to form a homogenous solution. Kinetic studies (see ESI†) were carried out by UV-Vis spectroscopy, monitoring the rate-determining hydrolytic cleavage of the carbonate substrates **2** and **3**. On the basis of the relative pK_a values of the conjugate acids of the two possible leaving groups, the cleavage would be expected to release 4-nitrophenolate[11] leading to the corresponding monocarbonate ester, which decomposes to CO_2 and the higher-pK_a phenol.

Initial experiments performed using 1.5 mg ml^{-1} of Pol397 showed significant rate enhancement and adherence to the Michaelis–Menten saturation model. Initial rates (v_i) for all the reactions containing microgels were obtained by subtracting the rates of uncatalysed hydrolysis ($v_i^{aq} = k_{uncat}$[**1**]) from the observed rates (v_i^{obs}) at each substrate concentration ($v_i = v_i^{obs} - v_i^{aq}$). Analysis by weighted non-linear regression of the v_i *vs*. $[S]_0$ data provided the values for the kinetic parameters $V_{max} = 5.31 \times 10^{-7}$ (±S.E. 6.5×10^{-8}) M s^{-1} and $K_m = 2.57 \times 10^{-3}$ (±S.E. 4.2×10^{-4}) M. Control experiments were carried out in the presence of: (i) microgels not imprinted with the template (Pol396), (ii) microgels not containing the arginine and tyrosine monomers, (iii) equivalent concentrations of arginine and tyrosine monomer derivatives. Samples (ii) and (iii) exhibited no rate enhancement compared to the non-catalysed rate. With Pol396, as seen in other cases,[2,5] some rate acceleration, albeit small, was observed.

In order to investigate the observed catalytic activity the same experiments were performed with increased polymer concentration. Kinetic data (Fig. 3(a)) obtained at a concentration of 4 mg ml^{-1}, a value at which the polymers are still soluble, gave for Pol397 $V_{max} = 1.34 \times 10^{-6}$ (±S.E. 1.28×10^{-7}) M s^{-1} and $K_M = 2.38 \times 10^{-3}$ (±S.E. 3.1×10^{-4}) M and for Pol396 $V_{max} = 2.53 \times 10^{-7}$ (±S.E. 1.41×10^{-8}) M s^{-1} and $K_M = 7.89 \times 10^{-4}$ (±S.E. 8.9×10^{-5}) M. As expected for Pol397 the K_M value has not changed while the V_{max} has increased by a factor proportional to the concentration of the polymer. An accurate calculation of the catalytic activity would require knowledge of the concentration of active sites and not rely on an approximation derived from the composition of the polymerisation mixture. Nevertheless an estimate of the ratio k_{cat}/k_{uncat} giving a value of 530 was obtained for Pol397 by considering 1% of the estimated arginine residues present to be catalytically active. More significantly the value V_{max}/K_mK_{uncat} = 8.9 obtained for Pol397 at 4 mg ml^{-1} shows a significant enhancement, about an order of magnitude, over the uncatalysed reaction (without microgels). The kinetic homogeneity of Pol397 in the substrate range investigated is demonstrated by the linearity of the Hanes plot (Fig. 3(b)). Curvature in this plot would have indicated non-identical binding events suggesting the possibility of functional heterogeneity. This result is quite interesting given the expected "polyclonal" nature of these preparations.

In order to prove that the catalytic activity of Pol397 is specifically due to the imprinting effect, the specificity of the polymer was tested on the potential substrate **3** that differs from substrate **2** and the template by having the nitro group in the *ortho* position instead of the *para*. Pairs of isomeric substrates such as **2** and **3** have been successfully used before with polyclonal catalytic antibodies to prove specificity and exclude interference from contaminating enzymes.

Results showed that for both polymers there is a small rate enhancement compared to the uncatalysed reaction but there is no significant difference between the imprinted and non-imprinted polymer. The v_i *vs*. [S] data corrected for the uncatalysed reaction did not show saturation in the substrate range studied and were fitted by linear regression to a linear curve leading to a $k_{ortho} = 6.4 \times 10^{-5}$ s^{-1}. The substrate selectivity for Pol397 expressed as $(V_{max}^i/K_M^i)_p/k_{ortho}$ resulted to be 8.75. This clearly demonstrates, that despite the moderate rate enhancement, a specific three-dimensional cavity providing substrate selectivity has been generated by the imprinting process in soluble microgels. Further work is currently in progress on the optimisation of these systems to obtain catalytic polymers with higher efficiency.

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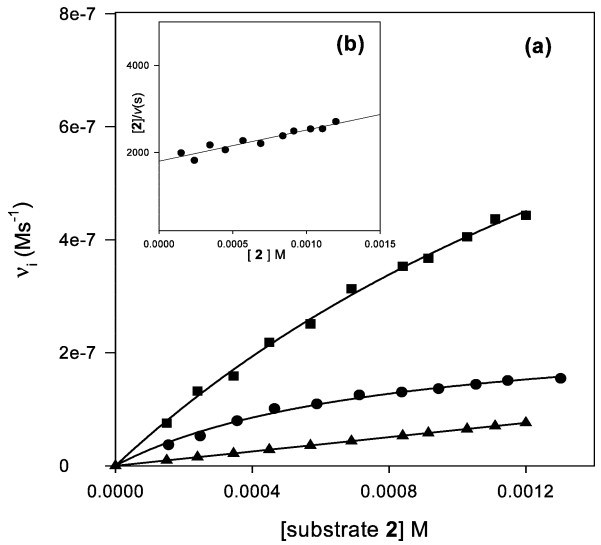

Fig. 3 (a) Adherence to the Michaelis–Menten equation of the initial rate v_i for Pol397 (■, imprinted) and Pol396 (●, non-imprinted). (b) Hanes plot for Pol397. All experimental data obtained with microgels are corrected by subtracting the corresponding rate obtained without polymer (▲) (k_{uncat} = 6.34×10^{-5} s^{-1}). Reactions were performed in a 9:1 solution of DMSO and Tris-HCl buffer.

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