Molecularly Imprinted Hydrogels Exhibit Chymotrypsin-like Activity

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Hydrogels find diverse applications as biomaterials, sensors, actuators, drug delivery systems, etc.[1−7] Catalytic activity of enzymes immobilized on hydrogels and functional polymers in solution is well documented.[8−10] A polymer mimic which replicates the enzyme-like activity of cytochrome *c* has recently been reported.[11] However, hydrogels exhibiting enzyme-like activity have not yet been reported.

Earlier, we reported hydrolytic activity in hydrogels containing an imidazole group, resulting from the formation of a charge transfer complex with an ester substrate.[12] When substrates which cannot form such a complex need to be hydrolyzed, the two can be brought into proximity by forming a complex in the presence of a metal ion and a print molecule.[13] Since the objective of these efforts was to enhance the rate of hydrolysis from pendent chain linked hydrogels, the substrate to be hydrolyzed was covalently linked to the hydrogel. While the hydrogels exhibited catalytic activity, further rate enhancement was needed for practical applications in drug delivery systems.

In this communication, we show that the hydrolytic activity of imidazole-containing hydrogels can be enhanced by incorporating carboxyl groups. When imidazole, carboxyl, and hydroxyl groups are incorporated in the complex, the mechanism of hydrolysis is akin to that of chymotrypsin.

A complex comprising methacrylic acid (MAA, 0.0022 M), *N*-methacryloylhistidine (MA-His, 0.0022 M), the print molecule 2-(((6-(isobutyrylamino)caproyl)-L-phenylalanyl) 2-amino)pyridine (IBA-6ACA-L-PheAl-2AP, 0.0044 M), and $CoCl_2$ (0.0022 M) was prepared.[10] A deep blue colored complex was isolated. This was further diluted with 2-hydroxyethyl methacrylate (HEMA, 2 g), ethylene glycol dimethacrylate (EGDMA, 0.5 mL), and azobis(isobutyronitrile) (AIBN, 0.125 g). The mixture was taken in a test tube, purged with nitrogen for 10 min, and polymerized at 65 °C. The polymer was isolated as a rod by breaking the test tube and cut into disks of 0.09−0.11 cm thickness on a lathe. The print molecule and cobalt were then leached out in 2,2′-bipyridyl and methanol. The swelling ratios of the hydrogels varied between 32 and 40%. The complexation technique is a modification of the earlier one, in that the carboxyl group also forms a part of the complex.[10] This is expected to enhance the catalytic activity of the imidazole group. The substrate ((6-(methacryloylamino)caproyl)-L-phenylalanyl)-*p*-nitrophenol was then sorbed into the polymer disks and polymerized by γ irradiation from a ^{60}Co source of 0.25 Mrad/h for 6 h. Unpolymerized substrate was extracted in acetone. The loading of the substrate (0.00402 M) was nearly equal to that of the print molecule used (0.0044 M). The hydrolysis of the ester was studied in phosphate buffer of pH = 8.0 and at 37 °C by monitoring the release of *p*-nitrophenol at 400 nm. The fraction of PNP released was plotted against time.

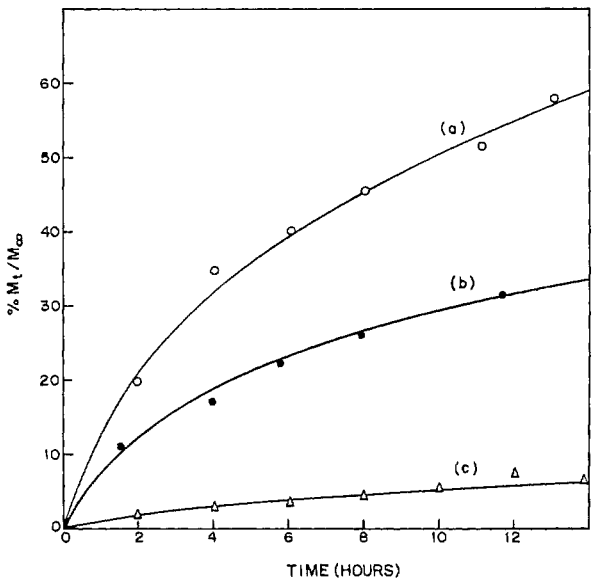

Figure 1. Release of *p*-nitrophenol from the hydrogels: (a) P(HEMA-MA-His-MAA-MA-6ACA-L-PheAl-PNP); (b) P(HEMA-MA-His-MA-6ACA-L-PheAl-PNP) synthesized by template polymerization; P(HEMA-MAA-MA-His-MA6ACA-L-PheAl-PNP) synthesized by conventional polymerization (c).

The results of hydrolysis indicate that the catalytic activity of the imidazole group is enhanced by the presence of the carboxyl groups (Figure 1a,b). A similar cooperative effect was reported for soluble polymers.[14,15] Control gels containing the same amount of methacrylic acid but not incorporated in the complex do not show any rate enhancement (Figure 1c). The rate of hydrolysis is still far too slow in comparison to the time for diffusion of *p*-nitrophenol from a hydrogel disk of thickness 1 mm (≈3.5 h), estimated from the diffusivity of *p*-nitrophenol (≈2.7×10^{-7} cm^2/s). The rate of appearance of *p*-nitrophenol is thus limited by the hydrolysis step.

The functional groups present in the hydrogel, viz., the hydroxyl group in HEMA, imidazole in MA-His, and the carboxylic group in MAA, also constitute the active site of chymotrypsin, viz,. serine-195, histidine-57, and aspartic acid−102, respectively.[16] The activity of chymotrypsin results from the serine hydroxyl group, which is further enhanced by the charge relay system. The catalytic hydrogel thus has the potential to mimic chymotrypsin. In the hydrogel described above, the activity stems from the imidazole group, which is enhanced by the carboxyl. This is because HEMA was not incorporated in the complexation step. Secondly, even if the rate of hydrolysis were to be enhanced, the appearance of *p*-nitrophenol in solution would be diffusion limited. To overcome these limitations, we modified the complexation procedure for the synthesis of the hydrogel and used the hydrogels in the form of microspheres. Thus for a microsphere of 200 μm diameter, the diffusion time of *p*-nitrophenol would be approximately 8 min.

We prepared a complex of HEMA (0.0022 M), MAA (0.0022 M), MA-His (0.0022 M), and IBA-6ACA-L-PheAl-2AP (0.0022 M) and $CoCl_2 \cdot 6H_2O$ (0.0022 M) and diluted it with 2 g of HEMA, 0.5 mL of EGDMA, and 0.125 g of AIBN. Dense microspheres (150−210 μm) were prepared by suspension polymerization.[17] Cobalt and the print molecule were extracted using 2,2′-bipyridyl and methanol. HEMA is now incorporated in the mimic

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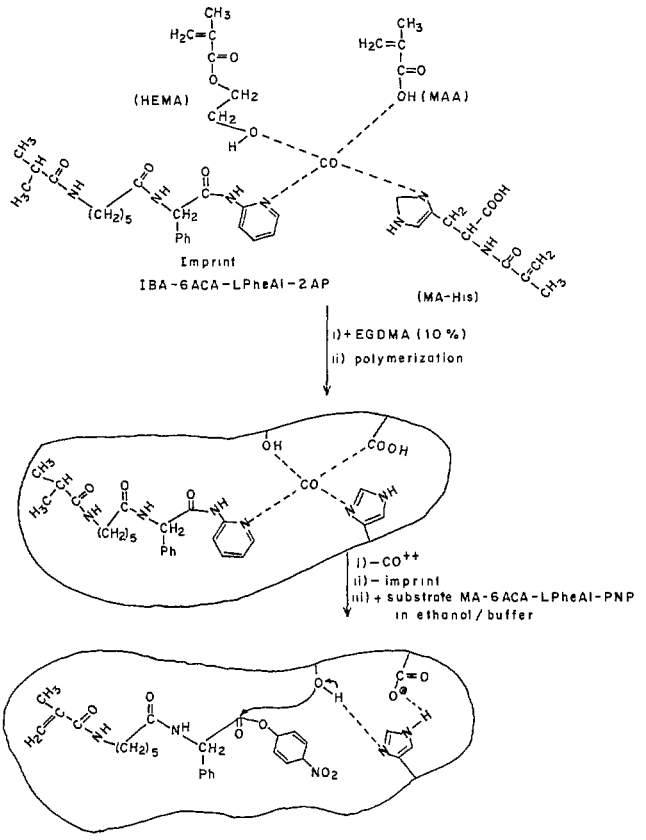

Figure 2. Schematic diagram showing the complex formation, polymerization, and hydrolysis.

during the complexation step. The structure of the complex was characterized by UV spectroscopy and ESR.[18] The studies showed that cobalt formed a complex with HEMA, MAA, MA-His, and the template in the molar ratio 1:1:1:1:1. A schematic of the complex formation and the mechanism of hydrolysis are shown in Figure 2. The concentration of the active site per gram was obtained by hydrolysis of MA-His in hydrochloric acid. The histidine liberated was estimated by the ninhydrin test.

The results of hydrolysis of MA-6ACA-L-PheAl-PNP are summarized in Figure 3b,d and compared with control gels of identical composition but formed in the absence of the complexation step (Figure 3a,c). The catalytic effect of hydrogels synthesized by template polymerization is evident. It should be noted here that the ester substrate is now used in the monomeric form. Although MA-6ACA-L-PheAl-PNP is not a typical substrate for chymotrypsin, it was used since this allowed us to work with the same print molecule as before.

The modified complexation technique leads to the catalytic triad in which the hydroxyl group is the effective nucleophile as in the case of chymotrypsin. Furthermore, the use of microspheres significantly enhanced the observed rate of ester hydrolysis (Figure 3b,d), as compared to the control gels (Figure 3a,c). However, at identical active-site concentration, the catalytic activity is much lower in comparison to that for chymotrypsin (Figure 3e). While the use of smaller microspheres (37−45 μm) further enhanced the observed hydrolysis rate, this was still lower than that of chymotrypsin.

While we have succeeded in synthesizing a hydrogel which can exhibit catalytic activity comparable to that of chymotrypsin, we have not been able to overcome the

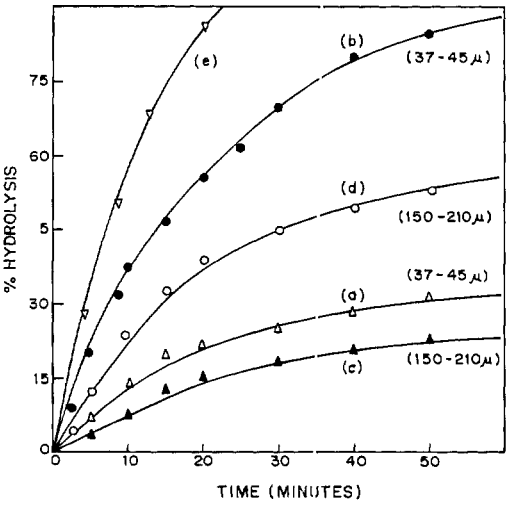

Figure 3. Hydrolysis of MA-6ACA-L-PheAl-PNP using microspheres synthesized by template polymerization, (150−210 μm (d), 37−45 μm (b)), conventional polymerization (150−210 μm (c), 37−45 μm (a)), and chymotrypsin (e). The hydrolysis studies were carried out in a mixed solvent system comprising 40:60 vol/vol ethanol/buffer at 37 °C with constant stirring. The ratio of the substrate to the catalyst (moles of imidazole present per gram of catalyst) was 1:1 mol/mol for all the experiments. *p*-Nitrophenol that was released was detected at λ_{max} = 400 nm on a UV−vis spectrophotometer.

diffusional limitations. Since the diffusion time of *p*-nitrophenol for these microspheres (37−45 μm) is negligibly small (20 s), the observed rate of hydrolysis seems to be limited by the diffusion of the substrate through the microsphere.

To illustrate that the hydrolytic activity of the hydrogel results from the cooperative effect among functional groups located on the same polymer chain, we synthesized polymers of identical composition as in the synthesis of hydrogels excluding the cross-linker. The intrinsic viscosity of the polymer synthesized in the presence of cobalt and the print molecule was 0.15 dL/g in methanol at 25 °C and dropped to 0.04 dL/g in the presence of cobalt. This is because during template polymerization the hydroxyl, carboxyl, and imidazole groups form a complex. The vinyl monomers bearing these groups are so distributed along the polymer chain that they are brought into close proximity again in the presence of cobalt. This leads to a collapse of the polymer chain and a decrease in the chain dimension. When polymerization is carried out in the absence of cobalt, the monomers are so distributed that they cannot be brought together in the presence of cobalt. Correspondingly, the intrinsic viscosity decreases only marginally from 0.13 to 0.11 dL/g.

The monomer reactivity ratios (r_1:r_2) for the binary systems HEMA:MAA (1.5:0.6), HEMA:MA-His (0.95:0.68), and MA-His-MAA (0.75:0.51) indicate a preference for homopolymerization for HEMA, but MAA and MA-His indicate random copolymerization.

The hydrolytic activity of templated polymer as well as the control in solution was much lower than that of the corresponding hydrogels (Figures 4a,b and 3b). Although the necessary functional groups are present, in solution, there is no mechanism whereby the polymer chain can be made to assume the conformation needed to bring them into proximity.[19] The effect of the template is not observed for the soluble polymers. During template polymerization, complexation brings the functional groups into proximity, cross-linking

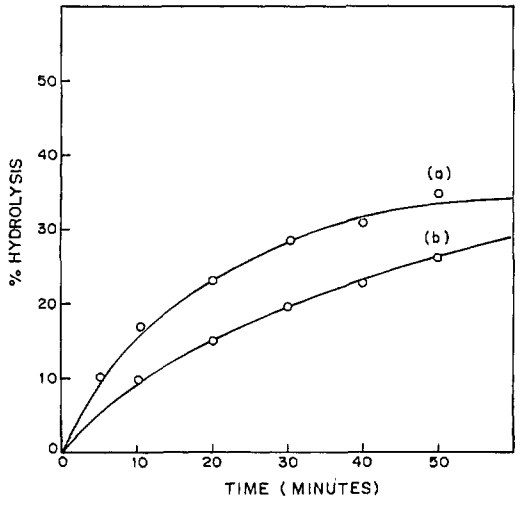

Figure 4. Hydrolysis of MA-6ACA-L-PheAl-PNP in solution by polymers P(HEMA-MAA-MA-His) synthesized by template polymerization (a) and P(HEMA-MAA-MA-His) synthesized by conventional polymerization (b). The hydrolysis was carried out in 40:60 vol/vol ethanol/buffer at 37 °C.

freezes the conformation, and the print molecule creates the cavity which confers substrate specificity. The catalytic hydrogels reported herein mimic chymotrypsin more closely than those reported earlier.[12,13]

The substrate diffusion limitations cited earlier in the case of dense hydrogel microspheres were overcome by synthesizing porous microspheres. These hydrogels exhibited Michaelis–Menten constants comparable to that of chymotrypsin. The activity of the catalytic hydrogel was higher for substrate containing L-phenylalanine in the neighborhood of the ester group being hydrolyzed and was completely lost in the presence of tosyl-L-phenylalanyl chloromethyl ketone (TPCK), a known inhibitor for chymotrypsin, and also when the hydroxyl group in HEMA was acetylated.[20]

In summary, the hydrogels reported in this work exhibit catalytic activity similar to that of chymotrypsin. The activity of the hydrogels via-à-vis chymotrypsin against active and inactive esters and amides typical for chymotrypsin is being compared quantitatively. In comparison to the native enzyme, the GELZYMES described by us retain their activity even when subjected to variations in pH and temperature and on repeated recycle. For example, the activity was retained within 95% even after 45 recycles. The active site of the hydrogel can be optimized for the desired substrate by the choice of the appropriate imprint, a much simpler alternative to site-directed mutagenesis in enzymes. Efforts to demonstrate these effects are in progress.

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