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Switching biomimetic hydrogels

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We propose a new strategy for precise, rapid, quantitatively predictable and reversible on–off regulation of enzymatic reactions. Specifically, we report reversible photo and pH-switchable hydrolytic activity of a chymotrypsin mimic. The switching-off of mimic activity when exposed to UV irradiation is a consequence of deswelling of the hydrogel that results in a 25-fold decrease in the diffusivity of the substrate in the hydrogel. The hydrolytic activity of the enzyme mimic can also be switched on/off in response to pH. Unlike native chymotrypsin, which is irreversibly deactivated, the activity of the mimic is reversible and also more stable in hostile environments. A reaction–diffusion analysis of the problem predicts the course of switching quantitatively.

Keywords: biomimetics; photoswitching; hydrogels; chymotrypsin

1. Introduction

The precise, rapid and reversible on–off regulation of enzymatic reactions has potential applications in bioreactors (Park & Hoffman 1988; Dong & Hoffman 1986), bioseparation techniques (Jacobsen *et al*. 1981; Negishi *et al*. 1981), and biosensors (Kazanskaya *et al*. 1983; Kuan & Lee 1980; Willner & Willner 1992). Such regulation has been used to improve the overall activity of a packed-bed column bioreactor, for improved enzyme purification, and also in the design of novel reusable sensors.

On–off regulation of enzymatic reactions in response to stimuli can be achieved by either regulating the activity of the catalytic site, or by regulating the probability of substrate–active site interaction. In the first strategy, the catalytic site, on the enzyme or enzyme mimic, is switched between active and inactive states by an external stimulus, such as pH or light or temperature, that in turn results in corresponding changes in the rate of product formation. In the second strategy, the rate of product formation is switched on or off by regulating the substrate flux at the active site. Most of the past work in the literature has focused on the first strategy and the use of native enzymes as opposed to enzyme mimics in order to demonstrate on–off regulation.

The commonly used approach of modulation of enzyme activity by changing the pH is an example of the first strategy. Photoregulation of enzyme activity has

been achieved (Porter *et al.* 1992). Willner *et al.* (1991*a*) demonstrated on–off photoregulation of the activity of papain by exploiting the photo-induced *trans–cis* isomerization of the azobenzene group conjugated with the enzyme. But the authors were unable to completely switch off the activity of the enzyme. Kaufman *et al.* (1968), Martinek *et al.* (1971) and Westmark *et al.* (1993) adopted a different approach towards photoregulation of enzymatic reactions. These authors use photo-responsive enzyme inhibitors such as azobenzene groups (e.g. *cis*-p-azobenzyl diphenyl carbamyl chloride), N-cinnamoyl imidazole and transition-state-analogue inhibitors to reversibly photoregulate the activity of enzymes. Since the inhibitor is often consumed during the process, this method of photoregulation is not strictly reversible.

The second strategy towards regulation of enzymatic reactions is to regulate the concentration of the substrate available locally at the active site of the enzyme or enzyme mimic. An interesting approach here is to use a polymeric matrix to control the diffusional flux of the substrate reaching the enzyme active site. This is the approach that our group has been developing over the years, using gels exhibiting volume phase transitions (Kulkarni *et al.* 1992). Such gels are a special category of hydrogels that show sharp volume transitions, abrupt shrinking or swelling, in response to external stimuli (Osada 1993), and, thus, offer a unique method of causing rapid changes in the diffusional flux. Kulkarni *et al.* (1992) achieved a time-invariant diffusional flux through bilayered membranes with reversible barrier layers by exploiting the phenomenon of volume phase transition in response to a pH stimulus.

The deswelling of a hydrogel containing photoisomerizable monomer can also be brought about reversibly by photoirradiation. For example, Aoyma *et al.* (1990) showed that graft copolymers comprising β-benzyl L-aspartate grafted onto poly-(butyl methacrylate) were permeable to a large number of molecules in the dark, while the permeability was suppressed on irradiation with visible light as a result of the change in the conformation of the peptide chain. Willner *et al.* (1991*b*, 1993) exploited such photosensitive gels to demonstrate on–off photoregulation of chymotrypsin encapsulated in acrylamide copolymers containing photoisomerizable azobenzene groups.

Our approach towards achieving precise, rapid and reversible on–off regulation is fundamentally different. We have focused our attention on enzyme mimics, as opposed to native enzymes, because enzyme mimics are, in general, more robust than enzymes and, hence, more suitable for industrial applications. Enzyme mimics are more stable in hostile environments and can be imprinted on a wide variety of surfaces for diverse applications. As opposed to this, the extent of enzyme modification, which can be brought about without denaturing a native enzyme, is limited.

In the past, we reported the synthesis of a biomimetic hydrogel exhibiting chymotrypsin-like activity (Karmalkar *et al.* 1996). This was synthesized by forming a cobalt complex of the three monomers containing hydroxyl, carboxyl and imidazole groups as well as a template molecule and polymerizing the same in the presence of 2-hydroxyethyl methacrylate. Apart from exhibiting hydrolytic activity like chymotrypsin, this biomimetic hydrogel offered additional advantages such as stability. Subsequently, we demonstrated that the catalytic activity of the mimic can be enhanced by using increasingly nucleophilic monomers and by enhancing the cooperative effect of the carboxyl-bearing monomer. The structure of the substrate, and

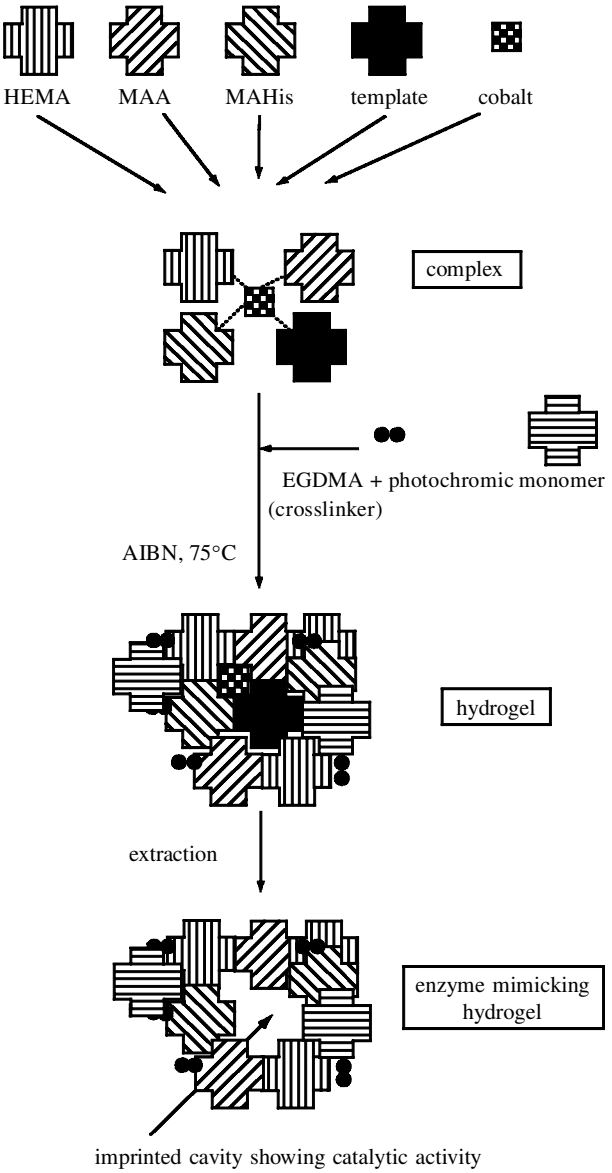

Figure 1. Schematic of the synthesis of the UV-responsive biomimetic hydrogel.

also the composition of the support polymer, played a significant role in determining the catalytic activity of the mimic (Lele *et al.* 1999*a*,*b*).

In this paper, we demonstrate a chymotrypsin mimic whose hydrolytic activity can be suitably tailored so as to be triggered on/off by UV light and pH. The paper also describes a mathematical model that captures the physics of the problem and allows quantitative predictions without the use of any adjustable parameters. We, thus, illustrate a model system for precise, rapid and quantitatively predictable reversible on–off regulation of enzymatic reactions by two independent stimuli.

2. Experimental details

(*a*) *Synthesis of the stimuli-responsive hydrogels*

The UV-responsive biomimetic hydrogel was prepared as follows. A metal coordination complex between cobalt, vinyl monomers mimicking the active site of chymotrypsin, and template molecules was prepared. The complex was then copolymerized with a hydrogel-forming monomer and a photosensitive monomer to form gel microspheres by suspension polymerization. Figure 1 illustrates schematically the synthesis of the stimuli-responsive enzyme-mimicking hydrogel. We have discussed our choice of monomers in an earlier communication (Karmalkar *et al.* 1996). The gels were prepared as microspheres, since we wanted to ensure rapid response to the external stimuli.

The detailed procedure was as follows. A 5 ml methanol solution of 2-hydroxyethyl methacrylate (0.286 g, 0.0022 M), methacrylic acid (0.189 g, 0.0022 M) N-methacryloyl L-histidine (0.5 g, 0.0022 M) and isobutyryl 6-amino caproyl L-phenylalanyl 2-amino pyridine (0.932 g, 0.0022 M) was prepared. Addition of 0.524 g (0.0022 M) of cobalt chloride hexahydrate to the above solution while stirring yielded the complex. Methanol was then evaporated from the solution. The complex was dissolved in a solution of 0.3 g 2-methacryloyl hydroxyethyl p-phenyl azobenzoate, 1.7 g 2-hydroxyethyl methacrylate, 0.3 g ethyleneglycol dimethacrylate and 0.03 g azobisisobutyronitrile.

The procedure for the suspension polymerization was as follows. 47 ml of 37% sodium chloride was added to a three-neck round-bottom flask equipped with a glass stirrer. To this, 3 ml of 1 N NaOH and 2 g magnesium chloride hexahydrate were added with stirring to give a uniform suspension of magnesium hydroxide. The solution was purged with nitrogen for 15 min. The monomer mixture prepared as described earlier was then added dropwise. The polymerization was carried out at 75 °C for 3 h with constant stirring at 1000 RPM. The microspheres were isolated by filtration. The template molecules and cobalt were extracted from the microspheres using a 1% 2,2′-bipyridyl solution in methanol followed by dilute hydrochloric acid for 12 h. The microspheres were vacuum dried and sieved to collect microspheres with mesh sizes between 35 and 40. These microspheres were then used for all subsequent experiments. The average radius of the dry microspheres was found to be *ca.* 40 μm.

We confirmed the swelling–deswelling behaviour of microspheres thus produced by ^{1}H NMR. The NMR experiments were carried out as follows: 100 mg of microspheres containing the photosensitive group were swollen to equilibrium for 24 h in ethanol:buffer (40:60). The swollen microspheres were isolated and the NMR spectrum was recorded. The microspheres were then exposed to UV irradiation for 10 min and the spectrum was recorded again. The spectrum showed that in the swollen state the peak for water was broader, and in the deswollen state it was sharper. When the azobenzene moiety in the polymer is converted from the *trans* form to the *cis* form on photoirradiation, a large dipole moment is induced (Hartley 1938). The hydroxy groups of 2-hydroxyethyl methacrylate interact with the dipole of *cis*-azobenzene, leading to loss of hydration of the hydroxyl groups (Ishihara *et al.* 1984). The hydrogel used for demonstrating pH-sensitive catalytic activity was prepared in a similar manner to the UV-responsive gel discussed above, except that the photosensitive monomer was excluded from the system.

(*b*) *Synthesis of the substrate*

The synthesis of the substrate N-methacryloyl 6-aminocaproyl L-phenylalanyl p-nitrophenolate has been reported earlier (Karmalkar *et al*. 1996).

(*c*) *Estimation of the mimic concentration in the hydrogel*

The functional-group concentration present per unit weight of the microspheres was estimated from the amount of L-histidine content in the polymer. In a typical procedure, 0.1 g of microspheres were suspended in 10 ml 0.2 N HCl, and refluxed under stirring for 24 h to hydrolyse histidine from the polymer backbone. The suspension was allowed to cool to room temperature and the pH was adjusted to 5.5 by adding 1 N NaOH. The microspheres were separated by filtration and the filtrate was used to determine histidine content by the ninhydrin test (Hamilton 1963). Thus, the concentration of the enzyme mimic in the microspheres was determined to be 1.45×10^{-4} mol cm^{-3}.

(*d*) *Swelling studies*

The dynamic swelling behaviour of the photosensitive polymer in aqueous media (ethanol:buffer (40:60 v/v), pH = 6.8) was studied at 37 °C. For this purpose, the polymers were prepared in the form of discs, as described in an earlier communication (Karmalkar *et al*. 1997). A dry polymer disc was weighed and immersed in ethanol:buffer (40:60 v/v) maintained at 37 °C. The disc was removed at various time intervals, blotted with a tissue paper and weighed. This procedure was repeated until there was no further weight gain. The equilibrium swelling was calculated as the ratio of the difference between the weight of the swollen polymer (W_s) and that of the dry polymer (W_d) to that of the swollen polymer (W_s). The dynamic swelling behaviour was also studied for the deswollen gels under the influence of UV light:

$$\text{water content (\%)} = \frac{(\text{weight of swollen gel} - \text{weight of dry gel})}{(\text{weight of swollen gel})} \tag{2.1}$$

The water content of the swollen gel was determined to be 32%, whereas that of the deswollen gel following photoirradiation was found to be 13%.

(*e*) *Measurement of diffusion coefficients*

Diffusion coefficients of the substrate N-methacryloyl 6-aminocaproyl L-phenylalanyl p-nitrophenolate in the aqueous media were experimentally determined by the desorption technique (Yasuda *et al*. 1968). Polymer discs were soaked in an ethanol:buffer (40:60) solution containing MA-6ACA-L-PheAl-PNP until equilibrium was reached. The desorption runs were carried out at 37 °C in 100 ml ethanol:buffer (40:60 v/v). The concentration of the diffusant released at intervals of 5 min was determined by monitoring the absorbance of the medium using an UV–VIS spectrophotometer. A graph of the fraction of diffusant released (M_t/M_f) at time t versus the square root of time was plotted. The diffusion coefficient, D, was calculated from the equation (Yasuda *et al*. 1968)

$$D = \tfrac{1}{16}\pi L^2, \tag{2.2}$$

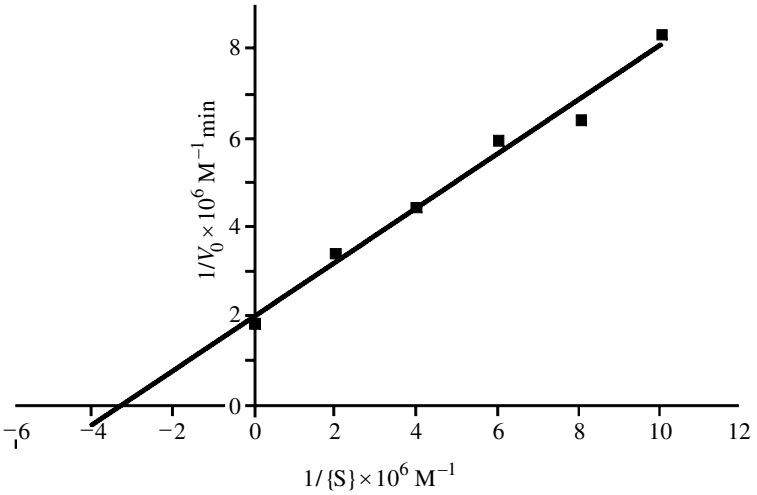

Figure 2. Lineweaver–Burks plot for the hydrolysis of MA-6ACA-L-PheAl-PNP by the mimic. The straight line through the experimental data confirms the Michaelis–Menten kinetics.

where

$$L = \frac{\mathrm{d}(M_t/M_\mathrm{f})}{\mathrm{d}(t^{0.5}/l)}, \tag{2.3}$$

and where M_t denotes the diffusant released at time t, M_f denotes the diffusant released at infinite time, l denotes the half thickness of the disc, and t denotes the time.

Using the above procedure, the diffusion coefficient of the substrate in the swollen hydrogel was found to be $4.8 \times 10^{-8}\ \mathrm{cm^2\ s^{-1}}$, whereas the diffusion coefficient of the substrate in the deswollen hydrogel was an order of magnitude lower, $1.8 \times 10^{-9}\ \mathrm{cm^2\ s^{-1}}$.

(*f*) *Verification of Michaelis–Menten kinetics and estimation of the constants*

Enzymatic hydrolyses follow Michaelis–Menten kinetics (Lehninger *et al.* 1993). The Michaelis–Menten equation for an enzymatic hydrolysis is given as

$$V_0 = \frac{k_2[E_\mathrm{total}][\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]}, \tag{2.4}$$

where V_0 ($\mathrm{mol\ cm^{-3}\ s^{-1}}$) is the initial velocity of the reaction, k_2 ($1/s$) is the rate constant of the reaction, $[E_\mathrm{total}]$ is the active site concentration, [S] is the substrate concentration, and K_m ($\mathrm{mol\ cm^{-3}}$) is the Michaelis–Menten, or binding, constant. V_0 can also be defined as the number of moles of product formed per unit volume per second. At high substrate concentrations, V_0 is independent of the substrate concentration. The constants are calculated by plotting the Lineweaver–Burks plot (Lehninger *et al.* 1993).

We wanted to see if the enzyme mimic prepared by us followed the Michaelis–Menten kinetics, and, if so, we also wanted to determine the value of the rate constant and the binding constant for our system. For this purpose, we prepared porous

P(GMA-EGDMA) microspheres with the pore surface coated with the enzyme-mimicking polymer. This was achieved by absorbing the complex comprising the three monomers, cobalt chloride and the template molecule along with the crosslinker EGDMA onto porous P(GMA-EGDMA) microspheres followed by polymerization and extraction. For the synthesis of the microspheres, 3.6 g of glycidyl methacrylate (GMA), 8.4 g ethylene glycol dimethacrylate (EGDMA), and 0.12 g of azobisisobutyronitrile were mixed with 16 g cyclohexanol and suspended in a solution of 88 ml of 1% polyvinylpyrrolidone (MW 3.6×10^5) in a 100 ml round-bottom flask. Nitrogen was purged through the mixture for 15 min. The polymerization was carried out at 70 °C for 2 h and at 80 °C for 6 h. The mixture was cooled to room temperature, the microspheres were isolated by filtration and washed with water and alcohol repeatedly. 1 g of microspheres were added to 5 ml of methanol containing the monomers, ethylene glycol dimethacrylate and the initiator. Methanol was evaporated under vacuum at room temperature and the microspheres were heated in an oven at 75 °C for 24 h to initiate polymerization. The surface-area measurements were carried out using the nitrogen adsorption method on the microspheres with and without the complex adsorbed. The surface area before adsorption of the complex was 324 $m^2\ g^{-1}$ and reduced to 265 $m^2\ g^{-1}$ after the adsorption of the complex. A porous support was used in order to avoid the influence of diffusional limitations on the kinetics so that the intrinsic active site reaction kinetics was studied. The concentration of the active site as estimated by the ninhydrin test, was 0.025 mmol g^{-1}.

The porous microspheres (50 mg) prepared as described above were then suspended in a 25 ml solution of ethanol:buffer (40:60 v/v) of known substrate concentrations (over the range 0.08–1.008 mM). The rate of p-nitrophenol formation was monitored using a UV–VIS spectrophotometer. Linearity of the Lineweaver–Burks plot confirmed that the enzyme mimic followed Michaelis–Menten kinetics (figure 2).

The rate constant was found to be $1.506 \times 10^{-4}\ s^{-1}$, and the binding constant was found to be 2.88×10^{-8} mol cm^{-3}. We also conducted control experiments to account for the facile hydrolysis of the substrate in the reaction medium. The blank experiments were run in ethanol:buffer (40:60) medium and the absorbance values due to appearance of p-nitro phenol in this experiment were subtracted from the values obtained in the hydrolysis experiments using the microspheres.

(*g*) *Evaluation of the UV-responsive activity*

The chymotrypsin mimicking hydrogel (50 mg) was added to a 50 ml flask containing 25 ml ethanol:phosphate buffer (40:60 v/v, 0.01 M, pH = 6.8), maintained at 37 °C in a thermostated flask. The concentration of the catalyst as estimated by the ninhydrin method was 2.1×10^{-4} mol g^{-1}. A 2.1×10^{-3} M stock solution of the substrate MA-6ACA-L-PheAL-PNP was prepared in dry ethanol. 100 µl of the stock solution was added to the thermostated flask maintained in the dark and the hydrolysis was allowed to continue for 20 min. The effective concentration of the substrate in 25 ml solution was 8.36×10^{-6} mol l^{-1}. The flask was then exposed to UV irradiation (λ = 280–350 nm) for the next 20 min followed by 20 min in the dark. A medium-pressure, 450 W Hanovia UV lamp was used as the source. The sequence was repeated five times to confirm the switchability of the hydrolytic activity of the gel. The p-nitrophenol released was monitored using UV–VIS spectrophotometer at λ = 400 nm. Subjecting the same hydrogel sample to the above experiment

eight times repeatedly, tested the reversibility of the on–off regulation. Indeed, the results of the experiment were identical, within the bounds of experimental error, thus confirming complete reversibility.

The stability of the polymer mimic was confirmed by repeating the hydrolysis of MA-6ACA-L-PheAL-PNP 45 times. The activity was retained within 98% of the original after 20 cycles and within 95% of the original after 45 cycles (Karmalkar 1995).

(*h*) *Evaluation of the pH-responsive activity*

To evaluate the pH-sensitive hydrolytic activity, 25 ml buffer solutions of, firstly, ethanol:phosphate buffer (0.01 M, pH = 6.8), and, secondly, of ethanol:citrate buffer (0.01 M, pH = 3) were thermostated at 37 °C and stirred magnetically. 100 μl of the substrate stock was added to the flask maintained at pH = 6.8. 50 mg of microspheres swollen to equilibrium at pH = 6.8 were suspended in the flask and vigorously stirred. The microspheres were then quickly transferred to the buffer solution of pH = 3. 100 μl of fresh substrate solution was added and the reaction allowed to proceed for the next 20 min. The sequence was repeated five times. The p-nitrophenol released was monitored using UV–VIS spectrophotometer at $\lambda = 400$ nm, pH = 6.8 and $\lambda = 320$ nm, pH = 3.

3. Results and discussion

Before demonstrating the switchability of the mimic, we would like to briefly summarize our approach for the synthesis of chymotrypsin mimics. The active site of chymotrypsin comprises hydroxyl group in serine (195), carboxyl group in aspartic acid (102), and imidazole group in histidine (57). The three are brought into the proximity of one another as a result of the conformation acquired by chymotrypsin molecule due to chain folding. The nucleophilicity of the serine hydroxyl group is enhanced by the cooperative effect amongst the three; referred to as the triad. Chymotrypsin hydrolyses esters and amides comprising phenyl alanine and tyrosine residues next to the carbonyl group in the ester or the amide (Fersht 1985).

Past efforts to synthesize chymotrypsin mimics based on synthetic linear polymers have been recently reviewed by Fife (1995). We sought to bring the functional groups responsible for the chymotrypsin activity into close proximity by complexing the monomers bearing the respective functional groups, the template and the metal ion and polymerizing the assembly in the presence of crosslinker on the surface of a porous crosslinked polymer support.

While in chymotrypsin, the functional groups responsible for the catalytic activity are brought into proximity as a result of chain folding governed by sequence distribution of amino acids in the chain; in the mimic synthesized by us this is achieved by polymerization of a supramolecular structure comprising the complex formed by the monomers bearing respective functional group and the template in the presence of a crosslinker.

In a specific case the mimic (Lele *et al.* 1999*a*) comprised monomer template assembly comprising methacryloyl serine, methacryloyl histidine and methacryloyl aspartic acid, and the template N-nicotinoyl tyrosyl benzyl ester complexed in the presence of Co(II). The hydrolysis of N-benzyloxy carbonyl L-tyrosyl-p-nitrophenyl

ester (N-Cbz-Tyr-PNP) by the mimic followed Michaelis–Menten kinetics. The mimic imprinted with the template N-nicotinoyl tyrosyl benzyl ester showed two-fold higher substrate affinity than the mimic not imprinted with the template, thus highlighting the role of the imprinting process.

In another case (Lele *et al.* 1999*b*), the mimic comprised 2-hydroxy ethyl methacrylate, methacryloyl histidine and methacrylic acid. A four-fold enhancement of the catalytic activity of this mimic in the hydrolysis of N-Cbz-Tyr-PNP was observed when methacrylic acid was replaced by methacryloyl β-alanine. Thus, the nucleophilicity of the hydroxyl group was enhanced when methacrylic acid in the triad was replaced by methacryloyl β-alanine, indicating that a cooperative effect operated amongst the three, although its magnitude was much lower than in the case of chymotrypsin.

To illustrate that the hydrolytic activity results from the cooperative activity amongst functional groups located on the same polymer chain, we synthesized polymers comprising methacryloyl histidine, methacrylic acid and 2-hydroxy ethyl methacrylate firstly in the presence of the cobalt and the template molecule and, secondly, in the absence of cobalt and the template molecule (Karmalkar *et al.* 1996). No crosslinker was used in either case. The intrinsic viscosity of the polymer synthesized in the presence of cobalt and the template molecule was 0.15 dl g^{-1} in methanol at 25 °C and dropped to 0.04 dl g^{-1} in the presence of cobalt. This is due to the fact that the hydroxyl, carboxyl and imidazole groups form a complex during template polymerization. The vinyl monomers bearing these groups are so distributed along the polymer chain that they are brought into close proximity in the presence of cobalt. This leads to a decrease in the chain dimension, as reflected in the drop in intrinsic viscosity. Since this does not happen in the case of polymers synthesized in the absence of cobalt, the intrinsic viscosity drops marginally from 0.13 dl g^{-1} to 0.11 dl g^{-1}.

The hydrolytic activity of the mimic was lost in the presence of Tosyl L-phenyl alanine chloromethyl ketone (TPCK) and also on the acetylation of the hydroxyl group in HEMA, further elaborating the similarities in the catalytic activity of the mimic *vis a vis* chymotrypsin (Karmalkar *et al.* 1996).

(*a*) *Photoreversible on–off regulation of the enzyme mimic*

The effect of irradiation on the catalytic activity of the chymotrypsin-mimicking hydrogel in the hydrolysis of N-methacryloyl 6-aminocaproyl L-phenylalanyl p-nitrophenol is shown in figure 3. While p-nitrophenol is released on hydrolysis in visible light, the same is suppressed on UV irradiation.

UV irradiation leads to a wide range of photochemically induced changes in the polymer structure depending upon the nature of the photoisomerizable monomer present. The effect of UV irradiation on the activity of chymotrypsin immobilized in the polyacrylamide containing three photosensitive monomers—(i) 4-(methacryloyl amino) azobenzene, (ii) 1-[β-(methacryloxy) ethyl] 3-3 dimethyl-6′-nitrospiro [indoline-2-2′ [2H-1] benzopyran], and (iii) bis-[4-(dimethyl amino) phenyl] (4 vinyl phenyl) methyl leuco hydroxide—was investigated by Willner *et al.* (1993).

UV irradiation resulted firstly in *trans–cis* isomerization of copolymer containing 4-(methacryloyl amino) azobenzene, and, secondly, in formation of merocyanine copolymer in the case of copolymer containing 1-[β-(methacryloxy) ethyl] 3-3

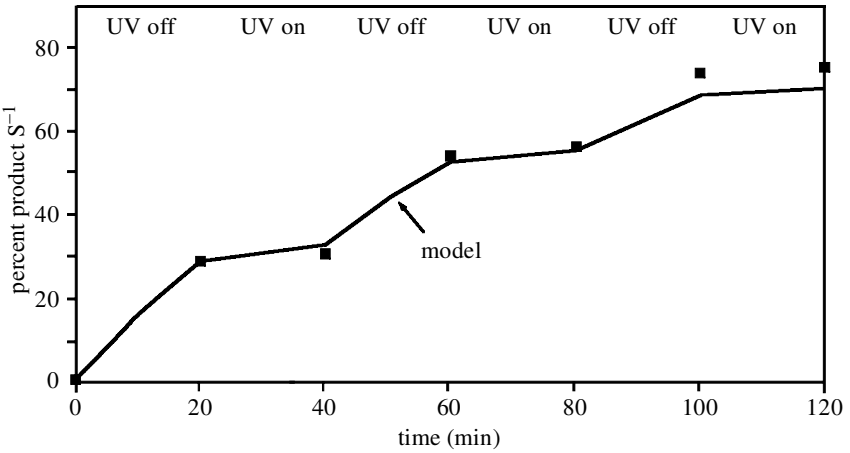

Figure 3. Photoreversible on–off catalytic activity of the mimicking hydrogel. The symbols are the experimental results. The line is the model prediction.

dimethyl-6′-nitrospiro [indoline-2-2′ [2H-1] benzopyran] and heterolytic bond cleavage in the case of copolymer containing bis-[4-(dimethyl amino) phenyl] (4 vinyl phenyl) methyl leuco hydroxide. Each of these structural changes led to an enhancement in the permeability of the substrate N-(3-carboxy propionyl)-2 phenyl alanine p-nitroanilide through the polymer, as demonstrated by the flow dialysis experiments. Consequently, the rate of chymotrypsin catalysed hydrolysis of N-(3-carboxy propionyl)-L-phenyl alanine p-nitro anilide was enhanced when chymotrypsin immobilized in these copolymers was exposed to UV irradiation and suppressed when exposed to visible light.

In the present case, the activity of the chymotrypsin mimic is switched off when exposed to visible light. This observation is opposite to that reported by Willner *et al*. (1993) quoted earlier. This can be explained on the basis of the differences in the swelling characteristics of the polymer used by us *vis a vis* that used by Willner *et al*. (1993). The polymer used by us has a total water content of 32%. The hydroxy group in poly-(2-hydroxy ethyl methacrylate) is solvated by water molecules. When exposed to UV irradiation, the azobenzene in the *trans* form is isomerized to the *cis* form. As a result, the distance between the 4 and 4′ positions in azobenzene is decreased from 9 Å to 5.5 Å. A large dipole moment across the azo bond is induced, leading to an increase in the dipole moment from 0.5 D to 3.1 D. This interaction strips the solvating water molecules from the hydroxy group in HEMA, causing deswelling of the polymer. The total water content in the deswollen polymer is 13%. It is pertinent at this stage to comment on the state of water in the swollen and deswollen polymer. The water content in hydrogels is classified into three categories. The bound water comprises water polarized around charged ionic groups and water oriented around hydrogen bonding groups or other dipoles. The interfacial water comprises water structured in ice-like configurations around hydrophobic groups. Bulk water comprises water imbibed in larger pores. For poly HEMA gels the fractions of bound, interfacial and bulk water as a function of total water content have been reported (Ratner & Hoffman 1976; Lee *et al*. 1975). Based on these results we conclude that when the total water content is 32%, *ca*. 5% of the total water is present as bulk water,

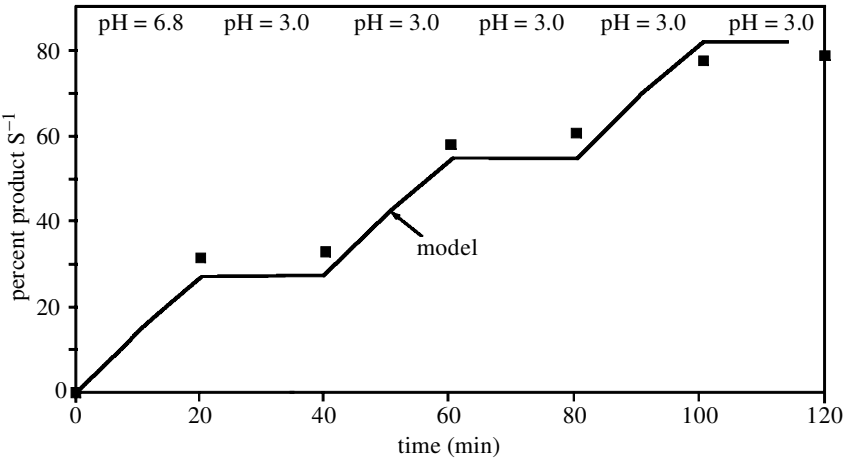

Figure 4. pH sensitive on–off catalytic activity of the mimicking hydrogel. The symbols are the experimental results. The line is the model prediction.

33% as interfacial water and 60% as bound water. UV irradiation leads to deswelling. The total water content at this stage is 13%. At this stage, all water present is in the form of bound water. Bulk and interfacial water as well as a part of the bound water is excluded from the system. The decrease in the total water content from 32% to 13% leads to a decrease in the diffusivity of the substrate N-methacryloyl 6-amino caproyl L-phenyl alanyl p-nitrophenolate from $4.8 \times 10^{-8}\,\mathrm{cm^2\,s^{-1}}$ to $1.8 \times 10^{-9}\,\mathrm{cm^2\,s^{-1}}$, leading to observed suppression of the hydrolysis.

(*b*) *pH-responsive catalytic activity*

Chymotrypsin-catalysed hydrolysis of specific ester or amide substrates exhibits bell-shaped pH-rate profiles with an optimum of pH 7.9–8. In the pH range 2–4, very little catalytic activity is observed. An increase in the pH from 8 to values greater than 9.5 causes irreversible denaturation of the enzyme (Bender *et al*. 1964). In the case of the biomimetic hydrogel, very little catalytic activity is observed in the pH range 1–3, however, the enzyme mimic remains stable even at pH values of 11 (Karmalkar 1995).

Figure 4 illustrates the fraction of product released by the biomimetic hydrogel during the pH-switching experiment. Changing the pH from 3 to 6.8 could reversibly control the catalytic activity. At pH 3, the imidazole group in histidine is protonated, which disrupts the charge relay amongst the functional groups constituting the triad and the activity is lost. At pH 6.8, the activity is again restored.

(*c*) *Stimuli-responsive hydrolytic activity of the biomimetic gels: a quantitative interpretation*

To interpret our experimental observations quantitatively we now develop a mathematical model for the demonstration of pH and photoreversible chymotrypsin-like activity.

Consider a system consisting of N polymeric particles of swollen radius R (R_{wet}) suspended in a liquid of volume V_{bulk}. Each of the particles has an enzyme-mimic

concentration of $[E_{\mathrm{total}}]$. The bulk liquid has a substrate concentration of $(\mathrm{S}_{\mathrm{bulk}})_0$ initially, where $(\mathrm{S}_{\mathrm{bulk}})_0$ is very small compared with the solvent concentration.

As time progresses, the substrate molecules penetrate the polymer particle by diffusion. Within the particle, the substrate S is catalytically converted by the enzyme mimic, E, to product P. The conversion follows Michaelis–Menten kinetics, namely

$$\mathrm{E} + \mathrm{S} \underset{k_{1\mathrm{b}}}{\overset{k_{1\mathrm{f}}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}, \tag{3.1}$$

$$V_0 = \frac{k_2 \lfloor \mathrm{E}_{\mathrm{total}} \rfloor [\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} = k_2[\mathrm{ES}] = \left.\frac{\partial [\mathrm{P}]}{\partial t}\right|_{\mathrm{local}}, \tag{3.2}$$

where V_0 is the rate at which product is formed per unit volume, $k_{1\mathrm{f}}$ and $k_{1\mathrm{b}}$ are the forward and backward reaction rate constants of the first step of the reaction, respectively, and k_2 is the reaction rate constant of the second step of the reaction. [A] is the concentration in moles per unit volume of species A (where A can be S, P, ES or $\mathrm{E}_{\mathrm{total}}$). $K_{\mathrm{m}} = (k_2 + k_{1\mathrm{b}})/k_{1\mathrm{f}}$ is the Michaelis–Menten or binding constant.

Following the reaction, the product P then diffuses outwards into the bulk and, consequently, the product concentration in the bulk increases with time. This product concentration, $[\mathrm{P}_{\mathrm{bulk}}]$, has been monitored in our experiments and reported as a ratio with respect to the initial substrate concentration, $[\mathrm{S}_{\mathrm{bulk}}]_0$.

We need to consider three rate processes in our model, namely the diffusion of the substrate into the polymeric matrix, the catalytic conversion of the substrate into the product and the diffusion of the product out of the polymeric matrix. Of these three processes, we can neglect the diffusion of the product out of the polymeric matrix since the diffusivity of the product (which is of the order of 10^{-7} cm^2 s^{-1}, Vadalkar *et al.* (1996)) is at least an order of magnitude higher than the diffusivity of the substrate as measured in this work (1.8–48.0×10^{-9} cm^2 s^{-1}).

Now, the diffusion–reaction problem can be formulated as

$$\frac{\partial [\mathrm{S}]}{\partial t} = \frac{D_{\mathrm{S}}}{r^2}\frac{\partial}{\partial r}\left(r^2 \frac{\partial [\mathrm{S}]}{\partial r}\right) - \frac{k_2 [E_{\mathrm{total}}][\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]}, \tag{3.3}$$

along with the initial conditions

$$[\mathrm{S}] = [\mathrm{S}_{\mathrm{bulk}}]_0, \qquad r = R, \quad t = 0,$$
$$[\mathrm{S}] = 0, \qquad 0 < r < R, \quad t = 0,$$

and boundary conditions

$$[\mathrm{S}] = [\mathrm{S}_{\mathrm{bulk}}], \quad r = R,$$
$$\frac{\partial [\mathrm{S}]}{\partial r} = 0, \quad \text{at } r = 0.$$

In equation (3.3) above, the term on the left-hand side represents the accumulation of the substrate per unit time in a unit volume in the microsphere. The first term on the right-hand side represents the net diffusive flux of the substrate through a unit radial thickness in the microsphere. The second term on the right-hand side is the rate of consumption of the substrate due to the enzymatic reaction in that unit volume.

The substrate and product concentrations in the bulk can then be determined using the following equations:

$$V_{\text{bulk}}[\text{P}_{\text{bulk}}] = N\int_0^t\left(\int_0^R\left(\frac{k_2[E_{\text{total}}][\text{S}]}{K_{\text{m}}+[\text{S}]}\right)4\pi r^2\,\text{d}r\right)\text{d}t, \tag{3.4}$$

$$V_{\text{bulk}}([\text{S}_{\text{bulk}}]_0 - [\text{S}_{\text{bulk}}]) = N\left(\int_0^t\left(D_{\text{S}}\frac{\partial[\text{S}]}{\partial r}\right)_{r=R}4\pi R^2\,\text{d}t\right). \tag{3.5}$$

Equation (3.5) gives the product concentration in the bulk solution that is actually monitored in our experiments. It is immediately clear that the rate of product released into the bulk solution is zero when the enzyme concentration is zero. This is what would be expected when low pH reduces the activity of the enzyme mimic. It is also apparent that the rate of release of the product depends upon the concentration of S at different locations along r. If the concentration of S is low within the microsphere, then the rate of product release is also low. Therefore, if the substrate concentration were to be reduced by increasing the diffusional resistance of the polymer matrix, then the rate of product release would go down. This is what would be expected when exposure to UV light causes a sudden decrease in the diffusivity of the hydrogel on deswelling.

The above equations are valid in general for both the pH and photoreversible cases. Only the parameters have to be changed appropriately as the external environment (pH or UV) changes, as given in table 1. The mutual diffusion coefficient of the gel in a solvent determines the swelling/deswelling kinetics of the gel (Singh & Weber 1996; Tanaka & Fillmore 1979). The mutual cooperative diffusion coefficient for our gels is always expected to be greater than 10^{-7} cm^2 s^{-1} (Tanaka & Fillmore 1979). Therefore, the time-scale for swelling and deswelling will be *ca.* 1–3 min, which is much smaller than our experimental time-scales of 20 min. Since the swelling and deswelling of the polymer matrix takes place very rapidly compared with the time-scales of our experiment, the consequences of the swelling and deswelling process only influence the product-release kinetics through the volume and diffusivity changes.

The above equations were then solved numerically. All parameter values were known from independent experiments (as described in § 2) and are tabulated in table 1. Figures 3 and 4 compare the model predictions with the experimental data for the case of photo and pH reversible behaviour, respectively. As can be seen, the model predicts the experimentally observed results well. What is particularly satisfying is that the model fits the data without having to take recourse to any adjustable parameters, as is often the case, in such complex systems.

The case of photoswitching (figure 3) is more interesting. When the UV light is on, the rate of product formation decreases considerably although the catalytic activity remains unchanged. As shown in table 1, the time-scale of substrate diffusion increases to 171 min, as compared with 7.6 min when UV light is switched off.

In the case of pH switching (figure 4), at pH 3 the mimic activity is completely switched off by reducing the concentration of the mimic to zero. The experimental results show a very small amount of product formation at pH 3, which could be due to a small residual concentration of active sites in the mimic. Changing the pH to 6.8 restores the original activity and, thus, the rate of product formed in the model predictions. There is a small drop in the rate of product formed during the 40–60 min time period compared with the 0–20 min time period. This could be due to the inability of the mimic to recover its activity completely in the time-frame of the experiment.

Table 1. *Values of the various model parameters corresponding to the experiments illustrating pH and photoreversible behaviour of the enzyme-mimicking polymer*

(Additional notation relevant to this table: V_p = dry volume of a polymer particle; V_w = volume of solvent in a swollen particle; ρ_w = density of solvent; equilibrium swelling = $V_w\rho_w/(V_w\rho_w + V_p\rho_{dry})$; V_{bulk} = total volume of solvent in which particles are immersed; W_{dry} = total dry weight of particles; R^2_{wet}/D_S = diffusion time-scale.)

time period (min)	experiment: pH reversible behaviour (UV off)	experiment: photoreversible behaviour (pH=6.8)
time invariant quantities	$D_S = 4.8 \times 10^{-8}$ cm^2 s^{-1} equilibrium swelling = 0.32 $R_{dry} = 4.1 \times 10^{-3}$ cm $\rho_{dry} = 1.023$ g cm^{-3} $V_{bulk} = 25.1$ cm^3 $W_{dry} = 50 \times 10^{-3}$ g $[S_{bulk}]_0 = 8.3664 \times 10^{-9}$ mol cm^{-3} $k_2 = 1.506 \times 10^{-4}$ s^{-1} $K_m = 2.88 \times 10^{-8}$ mol cm^{-3} $R^2_{wet}/D_S = 7.5725$ min	$R_{dry} = 4.1 \times 10^{-3}$ cm $\rho_{dry} = 1.023$ g cm^{-3} $V_{bulk} = 25.1$ cm^3 $W_{dry} = 50 \times 10^{-3}$ g $[S_{bulk}]_0 = 8.3664 \times 10^{-9}$ mol cm^{-3} $k_2 = 1.506 \times 10^{-4}$ s^{-1} $K_m = 2.88 \times 10^{-8}$ mol cm^{-3}
$t = 0$ min	set pH = 6.8 set $[S_{bulk}] = [S_{bulk}]_0$	set UV off swollen matrix
$0 < t < 20$ $40 < t < 60$ $80 < t < 100$	pH = 6.8 $[E_{total}] = 1.45 \times 10^{-4}$ mol cm^{-3}	UV = off $D_S = 4.8 \times 10^{-8}$ cm^2 s^{-1} equilibrium swelling = 0.32 $[E_{total}] = 1.45 \times 10^{-4}$ mol cm^{-3} $R^2_{wet}/D_S = 7.5725$ min
$t = 20$ $t = 60$ $t = 100$	set pH = 3.0 set $[S_{bulk}] = [S_{bulk}]_0$	set UV on deswelling of matrix concentrations (including enzyme concentration) scaled up
$20 < t < 40$ $60 < t < 80$ $100 < t < 120$	pH = 3.0 $[E_{total}] = 0.0$ mol cm^{-3}	UV = on $D_S = 1.8 \times 10^{-9}$ cm2 s^{-1} equilibrium swelling = 0.13 $[E_{total}] = 1.863 \times 10^{-4}$ mol cm^{-3} $R^2_{wet}/D_S = 171.124$ min
$t = 40$ $t = 80$	set pH = 6.8 set $[S_{bulk}] = [S_{bulk}]_0$	set UV off swelling of matrix concentrations scaled down
$t = 120$	experiment stopped	experiment stopped

4. Conclusion

In this work, we have demonstrated a novel polymeric chymotrypsin mimic whose hydrolytic activity can be rapidly, precisely and reversibly triggered on/off by UV light and pH. Unlike the enzyme-based systems, the enzyme-mimicking hydrogel devised by us offers additional features: greater tailorability; complete reversibility; and stability in hostile environments. The photoswitching is achieved by controlling the diffusion of the substrate into the polymer matrix, and the pH switching by controlling the activity of the enzyme mimic. Both these effects are reversible.

We have also described a mathematical model that captures the physics of the process and predicts the switching behaviour of the biomimetic hydrogel quantitatively without using any fitting parameters. Similar systems having a two-way control of the reaction can be used for sensor applications, separation processes and as designer catalysts. Multi-stimuli responsive enzyme mimics are also likely to find applications in systems involving multiple enzymatic reactions in which selective switching could be used to alter reaction pathways. New possibilities of selectivity control in catalysed reactions may then open up.

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