Enzyme responsive polymer hydrogel beads

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We report on a new class of enzyme responsive polymer hydrogels, the molecular accessibility of which can be changed selectively by enzymes present in a sample fluid.

Smart, intelligent or responsive polymer hydrogels change their physical properties in response to applied stimuli such as temperature, ionic strength, solvent polarity, electric/magnetic field or light.[1] Application of the appropriate stimulus gives rise to macroscopic swelling or collapse of the structure resulting in dramatic changes in the molecular accessibility, which is of use in selective removal (or release) of agents from (into) the environment. Future applications are anticipated increasingly in biomedical settings, with potential applications in drug delivery, wound dressings or as implant coatings (smart biomaterials).[2] However, the stimuli that are mentioned above are all rather non-selective and/or disruptive to biological interactions and many existing smart materials are therefore not ideally suited for applications in biomedical settings. In this communication we describe a new class of responsive polymer hydrogels, the molecular accessibility of which can be changed selectively by enzymes. The use of enzymes as biological stimuli to trigger hydrogel swelling or collapse opens up new avenues: (a) enzymes are uniquely chemo-, regio-, and enantioselective; (b) enzymes naturally work under mild conditions (aqueous, pH 5–8); (c) a number of enzymes play key roles as selective catalysts in cell pathways and disease states. Enzyme responsive materials could therefore pave the way to selective removal/delivery of agents in response to disease markers. Previous work in the area of biochemically responsive hydrogels made use of entrapped or covalently immobilised enzymes[3] or engineered enzymes linked to responsive polymers[4] thus describing bioactive hydrogels that respond to small molecules, rather than hydrogels that respond to enzymes. Other work demonstrated hydrolysis[5] or crosslinking[6] of polymer hydrogel networks by enzymes but did not report on changes in molecular accessibility due to changes in hydrogel swelling.

The overall design of the enzyme responsive polymer hydrogels (ERPHs) is given in Fig. 1. Commercially available $PEGA_{800}$ (Polymer Labs, UK; co-polymers of polyethylene glycol and acrylamide, 800 refers to the molecular weight of the PEG chains) beads *i* are used as a starting point. PEGA hydrogels have the ability to capture large amounts of water (many times their own weight) and they provide ideal "wet" environments for biological assays involving proteins. Their high polyethylene glycol content prevents non-specific protein adsorption to the bead surface. Numerous literature reports demonstrate that PEGA beads are accessible to small enzymes and compatible with enzyme activity inside the polymer material.[7]

$PEGA_{800}$ beads were modified with tri-peptides that consist of an enzyme cleavable linker section, comprising combinations of Phe ($R_{2,3}$ = CH_2-C_6H_5) and Gly ($R_{2,3}$ = H), and a charged residue (Arg, R_1 = $(CH_2)_3$-NH-C(=NH)-$NH_2)^+$). These peptides were directly synthesised on the pendant primary amine groups using standard Fmoc/HOBt/DIC chemistry.† It is envisaged that the presence of the cationic Arg groups in high density will cause sufficient electrostatic repulsion between polymer chains to result in bead swelling and enhanced molecular accessibility to the water filled hydrogel pores (*ii*). By choosing a peptide linker that is exclusively recognised by a target enzyme one would expect exposure of the hydrogel material to this enzyme to result in peptide hydrolysis and release of the charged moiety (*ii–iii*, Fig. 1). Loss of these cationic groups is expected to result in pore size

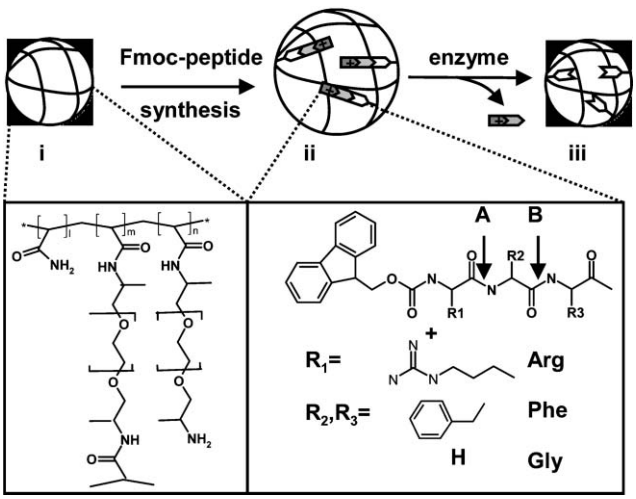

Fig. 1 Schematic description of enzyme responsive polymer hydrogels. The polymer beads *i* consist of acrylamide backbones crosslinked with polyethylene glycol ($PEGA_{800}$) and carrying PEG linkers with pendant primary amine groups for chemical functionalisation. Polymer beads are functionalised with enzyme cleavable peptide linkers using standard Fmoc-peptide synthesis methods. Peptides are composed of an enzyme cleavable section consisting of Gly (R = H) or Phe (R = CH_2-C_6H_5) and carry positive charge through an Arg residue (R_1 = $(CH_2)_3$-NH-C(=NH)-$NH_2)^+$) that give rise to electrostatic repulsion of polymer chains resulting in swelling of the beads (*ii*). Upon exposure to proteases that are able to cleave the peptide sequence (at cleavage sites A or B) the charged Arg group is removed, resulting in collapse of the bead structure (*iii*) and reduced molecular accessibility of the hydrogel pores.

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collapse and diminished molecular accessibility of the hydrogel pores.

It is well known that PEGA beads have a strict molecular weight cut-off and it has been consistently estimated that the hydrogel pores are exclusively accessible to globular proteins of 35 kDa molecular weight, or less.[8] To study changes in the molecular accessibility in response to enzyme action we made use of fluorescein labelled dextrans. These linear sugars are significantly different from globular proteins and their molecular accessibility to $PEGA_{800}$ has not yet been reported. Thus, we started by monitoring penetration of a series of labelled dextrans with molecular weights of 4, 10, 20, 40 and 77 kDa into individual $PEGA_{800}$ beads. Beads were exposed to 1 mM solutions of these fluorescent markers and the diffusion of the fluorophores to the centre of individual beads was monitored using two-photon fluorescence microscopy, a method that was previously described as a useful tool to analyse fluorescence events inside polymer beads.[9] It was found that *i* allowed exclusive access of the 4, 10, 20 kDa markers but not of the 40 and 77 kDa makers (See Fig. 2) , the molecular weight cut-off for labelled dextrans is therefore <40 kDa.

Next, the effect of incorporated cationic arginine residues on the swelling and molecular accessibility of $PEGA_{800}$ was investigated. We demonstrated previously that introduction of permanently charged residues such as quaternary amines and sulfonates in the polymer backbone increases the molecular accessibility due to electrostatic repulsion between charged polymer chains resulting in increased pore sizes. For $PEGA_{1900}$ a significant increase of molecular accessibility was reported when 10% of the acrylamide monomers were replaced by quaternary amine analogues.[10] For modified $PEGA_{800}$ where the pendant amine groups were derivatised with cationic peptide, it was observed by two photon microscopy that all five labelled dextrans could access the bead pores $PEGA_{800}$ (*ii* in Fig. 1).‡ The control in Fig. 3 (top row) shows fluorescence cross-sectional contrast images through the equator of individual beads *ii* upon exposure to the 77 kDa fluorescent marker in the course of 10 min, leaving no doubt that the marker can fully penetrate the pores of cationic $PEGA_{800}$. The molecular weight cut-off for labelled dextrans has therefore increased from <40 kDa to >77 kDa when comparing *i* and *ii* (compare pixel intensity plots of controls *i* and *ii*, Fig. 3). The change in swelling of the beads upon incorporation of the cationic peptides was determined by light microscopy. Comparison of the

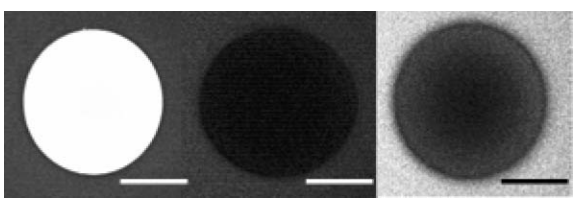


Fig. 2 Two-photon microscopy images of single representative beads show fluorescence cross-sectional contrast through the equator of individual $PEGA_{800}$ beads. The left hand image shows full accessibility of a 10 kDa dextran marker after 10 min. The middle image shows that a 40 kDa dextran does not enter the interior of the beads. The right hand image shows the same image at 4× higher laser intensity. These images clearly show that $PEGA_{800}$ beads have a distinct cut-off value in molecular accessibility for dextrans. The size bar represents 100 μm.

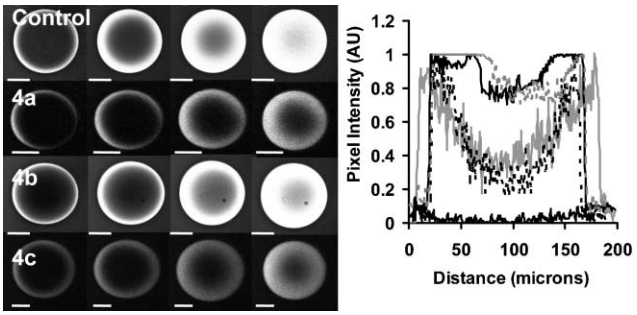
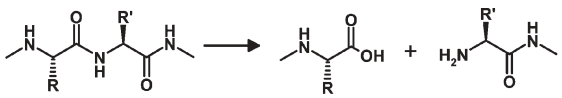


Fig. 3 Left: Two-photon microscopy images of single representative beads show diffusion of fluorescein labelled 77 kDa dextran into the $PEGA_{800}$ bead after 1, 2, 5, 10 min. The right hand image shows the pixel intensity analysis. The data represent from top to bottom: grey, dotted line: control *ii* (no enzyme), black solid line: **4b**, grey solid line: **4c**, black dotted line **4a**, black, solid line: control *i* (no Arg). These images clearly show that PEGA beads that are modified with peptide **4** show decreased molecular accessibility after treatment with trypsin and thermolysin, not chymotrypsin. The size bars represent 50 μm.

Table 1 Primary substrate specificities and molecular weights of three different proteases. P1 and P′1 refer to the amino acids directly adjacent to the cleaved amide bond



Enzyme	M_w/kDa	P1	P′1
Thermolysin	35	Any	Hydrophobic
Chymotrypsin	25	Hydrophobic	Any
Trypsin	22	Charged (+)	Any

mean average diameter of a random selection of 200 beads gave 0.28 mm (s.d. 0.07) for beads *i* and 0.32 mm (s.d. 0.07) for beads *ii*, corresponding to a 13% change in diameter and 33% change in average bead volume. Standard deviations reflect the polydispersity of PEGA beads.

Next we moved on to assess the feasibilty of exploiting selective enzymatic hydrolysis inside modified beads to control molecular accessiblity. Peptide linkers were designed to respond in different ways to three enzymes with different and complementary selectivities for the amino acids flanking the cleaved amide bond (these amino acids are referred to as P1 and P′1, see Table 1). Chymotrypsin from bovine pancreas is well known to cleave peptides preferentially at the carboxylic side (P1) of hydrophobic residues (in this case Phe), whereas it is rather non-specific for the P′1 amino acid (we chose Gly). By contrast, thermolysin from *Thermoproteolyticus rokko* prefers hydrophobic residues (Phe) at the P′1 end of the cleaved peptide bond and is unselective for the P1 position (Gly).[11] As a third enzyme, hog pancreatic trypsin cleaves selectively at the carboxylic acid side of positively charged residues and is therefore expected to cleave peptides at cleavage site A (Fig. 1). Enzymatic hydrolysis of PEGA bound di-peptides **1** and **2** (Table 2) demonstrated the expected selectivity for the cleaved bond B (see Fig. 1) with thermolysin being less selective (yield of **1a** < **2a**) compared to chymotrypsin (**1b** > **2b**) for this combination of amino acids.§ Peptides **3** and **4** carry

Table 2 Enzymatic cleavage of peptide linkers on $PEGA_{800}$ beads by three different enzymes. A and B refer to the possible enzyme cleavage sites (see Fig. 1) and give % conversion based on HPLC analysis¶

Entry	Peptide linker	Enzyme	A[a]	B[a]
1a	Phe-Gly	Thermolysin	n.a.	66
1b	Phe-Gly	Chymotrypsin	n.a.	15
2a	Gly-Phe	Thermolysin	n.a.	98
2b	Gly-Phe	Chymotrypsin	n.a.	6
3a	Arg(+)-Phe-Gly	Thermolysin	68	32
3b	Arg(+)-Phe-Gly	Chymotrypsin	7	14
3c	Arg(+)-Phe-Gly	Trypsin	99	<0.1
4a	Arg(+)-Gly-Phe	Thermolysin	28	43
4b	Arg(+)-Gly-Phe	Chymotrypsin	3	2
4c	Arg(+)-Gly-Phe	Trypsin	53	<0.1

[a] Yields determined by HPLC.

the Fmoc-Arg group and were hydrolysed selectively according to the enzymes' specificities, with thermolysin now also cleaving the Arg-Phe (A) bond. As expected, trypsin cleaved the Fmoc-Arg from both peptides **3** and **4**. Next we moved on to assess whether enzymatic cleavage had the expected effect on molecular accessibility of the ERPHs. To this end, $PEGA_{800}$ beads carrying peptide **4** were treated with the three enzymes and subsequently exposed to the 77 kDa fluorescein labelled dextran.

Changes in the molecular accessibility were then assessed using two-photon microscopy. Fig. 3 shows fluorescence cross-sectional contrast images through the equator of individual beads and leaves no doubt about the reduced accessibility observed in the presence of thermolysin (**4a**) and trypsin (**4c**), of which both enzymes were shown to cleave peptide linker **4** (Table 2). By contrast, chymotrypsin (**4b**) was not able to cleave this linker and the molecular accessibility of the fluorescent dextran was found to be similar to the control (no enzyme). These results confirm that reduced accessibility in **4a** and **4c** can be explained in terms of selective enzymatic cleavage of the peptide linker. Mean average bead diameters determined by light microscopy confirmed these results. The thermolysin and trypsin treated beads demonstrated a significant decrease from 0.32 to 0.26 mm (s.d. 0.06) (**4a**) and 0.29 mm (s.d. 0.06) (**4c**), while no significant change was observed for chymotrypsin (0.31 mm, s.d. 0.07, **4b**).

In summary, we have demonstrated that the molecular accessibility of $PEGA_{800}$ polymer hydrogel beads can be controlled selectively using enzymes. These enzyme responsive polymer hydrogel beads are programmable to respond uniquely to target enzymes by selection of appropriate enzyme cleavable linkers. They therefore have the potential to respond uniquely to a target protease in a complex mixture that may contain many other enzymes (*e.g.* a tissue fluid). We envisage that this approach will have applications in the selective removal of (harmful) macromolecules in biomedical contexts.

Notes and references

† Fmoc Amino acids (3 equiv.) were linked to $PEGA_{800}$ beads using DIC (di-isopropyl carbodiimide) (6 equiv.) and HOBt (hydroxybenzotriazole) (6 equiv.) in DMF. Each coupling was performed overnight on a blood rotator at room temperature and repeated. Between steps resin was washed extensively using 5 ml volumes of 5 × EtOH, 5 × MeOH, 5 × 50 : 50 (v/v) DMF : MeOH, 5 × DMF. A 2 ml mixture of TFA : water 95 : 5 (v/v) was used for deprotection of side chains and a mixture of 10% piperidine in DMF for Fmoc deprotection, both during 3 h. The efficiency of each coupling step was assessed by UV/HPLC analysis of cleaved Fmoc. The peptide loading obtained was 200–240 mmol g^{-1} PEGA, corresponding to >90% of the total primary amine loading. Enzyme reactions: 0.1 mM of enzyme was dissolved in 1 ml of 0.2 M phosphate buffer solution of pH 7.5 and added to approx. 10 mg of $PEGA_{800}$. Reactions were incubated overnight at room temperature on a blood rotator, washed with 8 ml acetonitrile/water 50 : 50 (v/v). The samples were analyzed by HPLC, the total peptide loading was set at 100%.

‡ The 10% derivatised acrylamide in a previous report[10] and the >90% derivatisation of pendant amines described here both amount to approximately 8–9% (w/w) cationic groups per weight of polymer.

§ Note that the yields for the chymotrypsin reaction are lower compared to those of thermolysin in $PEGA_{800}$. In $PEGA_{1900}$ complete conversions have been described for both these enzymes showing the expected selectivity. See *e.g.* reference 11.

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