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Introduction of permanently charged groups into PEGA resins leads to improved biotransformations on solid support

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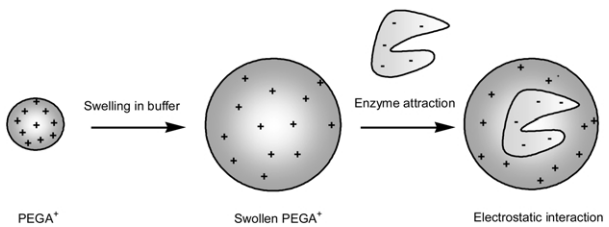
Abstract—The application of biotransformations in solid phase synthesis is an attractive alternative to chemical methodologies. An important issue that needs to be addressed in this context is accessibility of functional groups within a porous polymer to the biocatalyst. This paper shows that such accessibility can be improved for penicillin G amidase by introducing permanent charges into the polymer. The effect appears to be due to better swelling of the polymer in buffer and to electrostatic interactions between polymer and enzyme.

1. Introduction

In the screening approach to biomedical discovery, large libraries of compounds are commonly used to identify novel protein/ligand interactions. These compound libraries can be conveniently synthesized on solid phase polymer beads using combinatorial methods. Ideally, these compound libraries would then be used directly for on-resin biological screening, which requires polymer materials that are accessible to proteins and other biomolecules. Thus, Meldal et al. have successfully developed a range of PEGA polymers (cross-linked acrylamide and polyethylene glycol) for such screening experiments, in particular using peptide libraries for the screening of substrate specificity of small proteases.[1] $PEGA_{1900}$ was found to be completely permeable to proteins with molecular masses up to 35 kDa.[2] However, accessibility appears to be problematic for larger enzymes. Increasing the PEG chain to create $PEGA_{6000}$ or use of soluble PEG polymers has been shown to improve results but has compromised the ease of handling and loading of the polymers.[1d,3] Hence, there is a need for improved polymers that can be used both for combinatorial synthetic chemistry and biological screening.

We have recently introduced a conceptually different approach to improving enzyme access in polymer beads.[4] In this approach, we have exploited the observation that all proteins are charged. The net charge on a protein depends on its pI value. By introducing permanent charges into the PEGA resin, increased swelling is observed due to electrostatic repulsions between identical or similar charges. In addition, oppositely charged enzymes are attracted inside the polymer bead due to favourable electrostatic attraction between polymer and protein (see Scheme 1). For example, we have shown that penicillin G acylase (PGA), a 88 kDa enzyme could be used effectively for the hydrolysis of phenylacetamides within PEGA beads when the resin was derivatised with permanently charged quaternary amines ($PEGA^+$, see Fig. 1).

In this article, two important aspects related to the wider applicability of these novel polymers are investigated. Firstly, the suitability of such charged resins for solid phase chemical synthesis was explored. Secondly, the effects of polymer charges were systematically investigated on two hydrolases of different size.



Scheme 1. $PEGA^+$ resins allow for improved access of proteins due to (i) increased swelling resulting in increased pore size and (ii) improved access of oppositely charged proteins due to electrostatic attraction.

Keywords: Solid phase chemistry; PEGA; Charged PEGA; Penicillin G amidase; Thermolysin.
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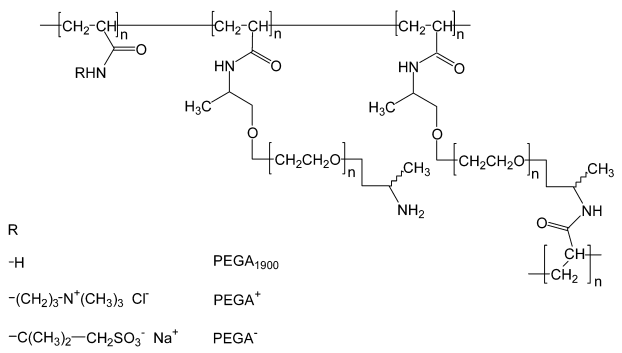


Figure 1. PEGA polymers used in the present study.

2. Results and discussion

2.1. Characterization of novel polymers

We recently reported on the successful incorporation of permanently charged monomers into PEGA supports.[4] $PEGA^+$ and $PEGA^-$ have a polyacrylamide backbone in which acrylamide units are partially substituted (10%) with charged units carrying 3-trimethylammonium chloride-propyl and 1,1-dimethyl-2-sulfonate-ethyl side-chains, respectively (Figure 1).

The first step in the further characterization of these novel polymers was to assess their chemical reactivity and loading. The presence of charged groups in $PEGA^+$ and $PEGA^-$ would probably influence the chemical reactivity of the polymer bound free amino groups. The reactivity was therefore monitored after each of four cycles of acylation with Fmoc-L-Phe. After each cycle the Fmoc group was removed with piperidine (20% in DMF) and coupling yield quantified by UV determination (Table 1).

The results obtained (Table 1) suggest that the chemical reactivity of the charged PEGA resins ($PEGA^+$ or $PEGA^-$) is lower than the reactivity of uncharged $PEGA_{1900}$. The presence of quaternary amino groups strongly decreased the reactivity of the polymer linked primary amino groups during the first acylation step. One reaction cycle was therefore not sufficient for the complete acylation of $PEGA^+$ since only 5% of the available amino groups were acylated. The presence of permanent negative charges also decreased the reactivity of the primary amino groups, since after one reaction cycle 50% of the available amino groups were acylated. However, after the second cycle complete acylation was obtained for both polymers. The loadings obtained were comparable to that of $PEGA_{1900}$.

Table 1. Loading determination of PEGA resins

Resin[a]	Loading after each acylation ($mmol/g_{dry}$)				
	1st	2nd	3rd	4th	Loading[b]
$PEGA_{1900}$	0.17	0.14	0.13	0.17	0.15
$PEGA^+$	0.01	0.23	0.24	0.22	0.23
$PEGA^-$	0.05	0.11	0.08	0.10	0.10

[a] Loading provided by manufacturer: 0.10–0.20 $mmol/g_{dry}$.
[b] Average of 2nd, 3rd and 4th acylations.

2.2. Swelling properties of the charged PEGA resins

The swelling properties of the polymers were investigated by measuring the weight of retained buffer after filtration of the polymers. Swelling properties are likely to be pH dependant due to interactions of the permanently charged monomers with functional amino groups.[5] These amino groups are expected to be protonated (and therefore charged) to an extent depending on the pH value of the buffer. In addition, protonation might depend on the presence of permanent charges in the polymer micro-environment: permanent positive charges suppress protonation while permanent negative charges can enhance protonation. The apparent pK value of the surface bound amino groups is around 7.0 and hence a significant proportion is protonated at pH 6.0, while most are deprotonated at pH 8.0. The effect of buffer pH on swelling was investigated by varying the pH value within this range (Fig. 2).

The electrostatic interactions that influence the swelling behaviour of charged polymers largely depend on the ionic strength of the buffer. Increasing concentrations of counter-ions that are present in higher concentration buffers are likely to shield the interactions. The swelling of the

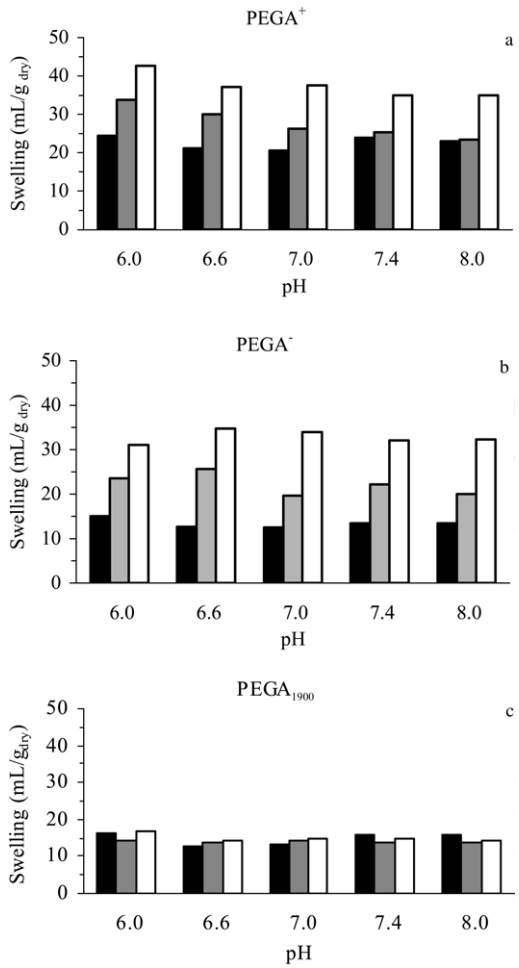


Figure 2. Swelling behaviour of PEGA polymers as a function of buffer concentration (0.1 M: black; 0.01 M: grey; 0.001 M: white) and the pH of the buffer (6.0–8.0) of the buffer solutions (a: $PEGA^+$; b: $PEGA^-$; c: $PEGA_{1900}$).

polymers was therefore studied in buffer concentrations varying from 0.1 to 0.001 M (Fig. 2).

The expected effect of buffer concentration on the swelling of charged PEGA polymers is evident for both charged polymers as more dramatic effects are observed at lower ionic strength (Fig. 2a and b). A pH change from 6.0 to 8.0 has a significant effect on $PEGA^+$ resulting in a better swelling at lower pH values. In this case, the presence of permanent quaternary amino groups and protonation of primary amino groups at lower pH values increases the electrostatic repulsion within the polymer, resulting in improved swelling. In $PEGA^-$ protonation of amino groups creates opposite charges counteracting repulsion resulting in decreased swelling at lower pH values.

In the absence of permanent charges ($PEGA_{1900}$) no measurable effect of either ionic strength or pH of the buffer on swelling is observed (Fig. 2c). The positively charged amines at low pH appear to be too distant from each other within the polymer beads to have a measurable effect on the swelling behaviour.

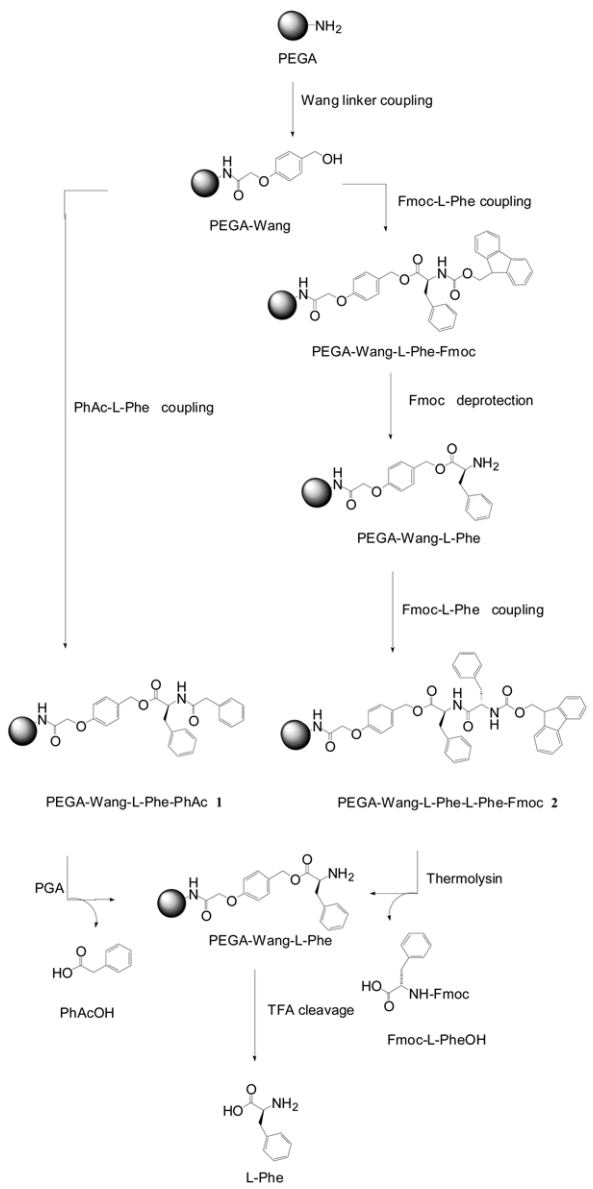
2.3. Biotransformations on novel polymers

Having established improved swelling, the charged polymers were subsequently studied for protein access to the core of the polymer beads. Two hydrolytic enzymes that had been previously investigated in our group and had yielded different accessibility were used as model systems. Thermolysin from *B. thermoproteolyticus* had been used in peptide synthesis/hydrolysis experiments and was found to be able to penetrate to the core of $PEGA_{1900}$ beads.[2b,c] In contrast, Penicillin G amidase (PGA) from *E. coli*,[6,7] had always given low yields. The two enzymes were particularly useful as model systems because of their difference in size (35 kDa for thermolysin and 88 kDa for PGA).

All three polymers were derivatised to give substrate **1** (specific substrate for PGA) and substrate **2** (specific substrate for thermolysin) using previously developed experimental procedures (Scheme 2). To enable full analysis the substrates were attached to the solid support by a cleavable Wang linker.

Using PEGA derivatives **1** and **2** as substrates for hydrolases the possible correlation of swelling behaviour of the charged polymers with the yield of enzymatic hydrolysis was further investigated. At first, hydrolysis of **1** by PGA was studied at pH 8.0, the value where the enzyme shows the highest hydrolytic activity. The reaction was studied at buffer concentrations from 0.5 to 0.001 M (Fig. 3).

While for $PEGA^-$ and $PEGA_{1900}$ no apparent effect was observed (conversions of $<15\%$ at all buffer concentrations studied), for $PEGA^+$ an increase in hydrolysis yields was obtained at decreasing ionic strength. When comparing Figures 2a and 3 correlation between the increased swelling of the resin in diluted buffers and the final conversion of the hydrolytic reaction is evident. Despite the increase of swelling of $PEGA^-$ (Fig. 2b) in diluted buffers, enzyme hydrolysis did not increase by lowering the buffer concentration from 0.5 to 0.001 M. These observations



Scheme 2. Chemical synthesis of PEGA-Wang-L-Phe-PhAc, **1**, of PEGA-Wang-L-Phe-L-Phe-Fmoc, **2** and enzymatic hydrolysis by PGA and thermolysin.

suggest that PGA accessibility is determined by pore size (swelling) but also by electrostatic interactions between polymer and protein. Indeed, PGA has an overall negative charge at pH 8.0 (pI=5.2–5.4), and would be attracted by $PEGA^+$ side chains, while it would repelled in $PEGA^-$.

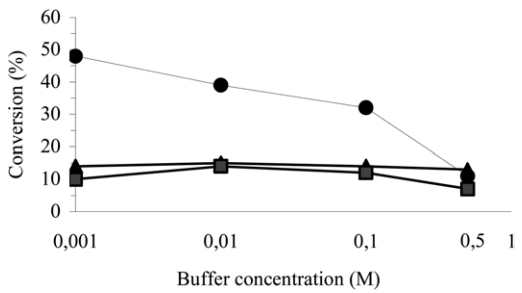


Figure 3. PGA catalysed release of PhAcOH from substrate **1** in Kpi buffer, pH 8.0, at different buffer concentration. $PEGA^+$: circles; $PEGA^-$: triangles; $PEGA_{1900}$: squares.

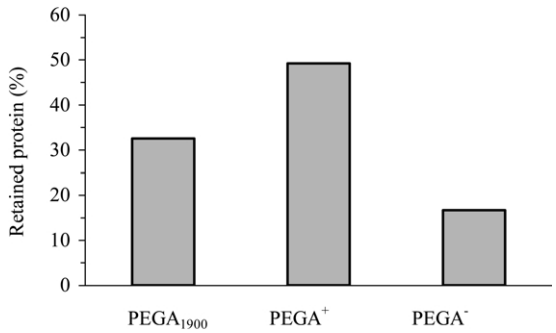


Figure 4. Retained PGA (in Kpi buffer, 0.001 M, pH 8.0) by PEGA polymers after filtration.

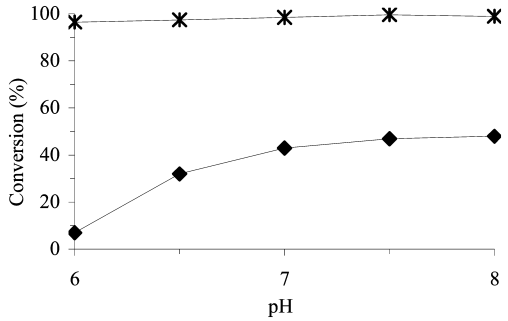


Figure 5. PGA (diamonds) and thermolysin (stars) catalysed hydrolysis of peptides **1** and **2** linked to PEGA$^+$in 0.001 M Kpi buffers of varying pH values.

Further evidence of increased access of PGA protein to the polymer beads was provided by the determination of protein retained in the polymer bead.[8] Figure 4 shows that 49% of the total amount of PGA present in a PEGA$^+$ suspension at pH 8.0 was absorbed by the polymer. In contrast, the unfavourable interactions between PGA and PEGA$^-$ is directly measured by only 20% of the enzyme being retained within the polymer. For PEGA$_{1900}$ an intermediate protein uptake of 32% was observed.

Clearly, the combined effects of polymer swelling and favoured electrostatic interactions between protein and permanent polymer charges result in protein retention by the charged polymer. It is not straightforward to establish what the relative importance of each of these two effects because both effects depend on the buffer concentration and cannot be studied independently. However, an indication of the relative importance of each of these effects could be obtained by lowering the pH of the buffer. For example, at pH 6.0 polymer swelling is significantly increased, while PGA is still overall negatively charged.

When the enzymatic hydrolysis was performed at optimal swelling conditions for PEGA$^+$ (corresponding to 0.001 M ionic strength and pH 6.0), the yield was less than 10%. Hence, despite increased swelling and electrostatic attractions between polymer and protein poor conversions are obtained. This unforeseen observation can be explained in terms of two possible effects. Firstly, the enzyme activity could be reduced at lower pH values. It is indeed known that amide hydrolysis is more favoured at pH 8.0 than at pH 6.0 (PGA has an optimal pH for the hydrolysis in the range 7.5–8.5). Secondly, the decrease of pH from 8.0 to 6.0 reduces the net negative charge on the enzyme. As a result the electrostatic attraction with the permanent charges within PEGA$^+$ decreases resulting in a decreased access of the enzyme.

In order to find out which of these two effects was more important, the results were compared with the thermolysin catalysed hydrolysis reaction of **2** on PEGA$^+$ (Fig. 5). Thermolysin has a pI of 5.1, similar to that of PGA, while its size is significantly smaller than that of PGA (only 35 kDa).

As stated before, thermolysin had been shown to access all chemical groups within PEGA$_{1900}$.[2a–c] One would anticipate this protein to access PEGA$^+$ equally well at pH values significantly over its pI, where the enzyme is negatively charged. This was indeed observed, and complete hydrolysis of peptide **2** was observed. Upon lowering the pH, and hence lowering the net negative charge of the protein, no significant decrease in conversion yields was observed. The dramatic decrease in enzymatic conversions as observed for PGA was clearly absent in thermolysin. These observations suggest that the decrease in hydrolysis observed for PGA is not related to limited enzyme accessibility. Instead, the decreased hydrolysis yields observed are probably related to reduced catalytic activity of the enzyme inside the polymer bead rather than net protein charge.

In summary, we have investigated novel PEGA polymers with superior swelling properties that vary with ionic strength and pH of the buffer used. We have also demonstrated that enzyme absorption by polymer beads can be improved by exploiting enzymes net charge.

3. Experimental

3.1. General

PEGA$_{1900}$, PEGA$^+$ and PEGA$^-$ were prepared by Polymer Laboratories (UK). 4-Hydroxymethyl phenoxyacetic acid (HMPA), *N*,*N*′-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBT), *N*-Fmoc-L-phenylalanine (Fmoc-L-Phe), 4-dimethylaminopyridine (DMAP), *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), *N*-ethyldiisopropylamine (DIPEA) phenylacetyl-L-phenylalanine (PhAc-L-Phe) and phenylacetic acid (PhA-cOH) were purchased from Sigma-Aldrich. Penicillin G amidase (PGA) (solution: 16–32 U/mg) was obtained from Fluka and Thermolysin was obtained from Sigma (100 units/mg protein). All the solvents were of HPLC grade and obtained from Labscan. *N*,*N*′-dimethylformamide (DMF) was Biotech grade, >99% (Aldrich).

3.2. Chemical synthesis

Before and after any chemical step resins were washed three times with MeOH/DMF (1:1), MeOH, DCM and DMF and filtered. All the reactions (loading determination, chemical syntheses and enzymatic hydrolyses) were performed at room temperature, under constant stirring in a blood rotator (40 rpm) and repeated at least three times.

3.2.1. Ninhydrin test. Three solutions were prepared:

solution A: 5 g ninhydrin in 100 mL of ethanol; solution B: 80 g phenol in 20 mL of ethanol; solution C: 2 mL of a KCN solution (0.001 mequiv. in pyridine) diluted to 100 mL. One drop of each solution was added on a small sample of resin. The mixture was then heated to 200 °C. A blue colour was evidence of the presence of unreacted amines. Yellow indicated all amines had reacted.

3.2.2. Determination of loading. The availability of amino groups of the resin was measured by reacting them with Fmoc-L-Phe (3 equiv.) in the presence of DIC (4 equiv.) and DMAP (0.1 equiv.) in dry DMF. The process was repeated until the ninhydrin test gave a negative result. The Fmoc group was removed and quantified by suspending the resins in a 20% solution of piperidine in DMF and filtering the mixture after 2 h. The loading of the resin was calculated on the filtrate using Eq. 1.

$$\text{Loading (mmol/g}_{\text{dry}}) = \frac{\text{Ads (290 nm)} \times V\ \text{(mL)}}{4950 \times g\ \text{(dry resin)}} \qquad (1)$$

where *Ads* (290 nm) represents the absorbance at 290 nm, *V* (mL) the volume of piperidine (20% in DMF) used, 4950 the ε and *g* (dry resin) the amount of dry resin.

3.2.3. Synthesis of peptides 1 and 2. Before chemical synthesis the resins were washed three times with MeOH/DMF, MeOH, DCM and DMF. The syntheses were performed on a gram scale (wet weight polymer) (Scheme 2). The solvent content after each chemical synthesis was calculated by drying samples at 110 °C (mmol/g_{dry}).

Wang linker coupling. The resins were weighed in reactor syringes and suspended in DMF. The Wang linker was attached using HMPA (3 equiv.) in the presence of DIC (4 equiv.) and HOBT (6 equiv.). The mixtures were allowed to mix on a blood rotator overnight. The resins were then filtered (VacuumSystem, Stepbio) and washed with MeOH/DMF, MeOH, DCM and DMF. The synthesis step was repeated until the ninhydrin test gave a negative result indicating all amines had reacted.

*Synthesis of **1***. Next, PhAc-L-Phe (3 equiv.) was added in the presence of DIC (4 equiv.) and DMAP (0.1 equiv.) in DMF. The reaction was performed in two cycles, the first of 2 h and the second overnight. After each cycle the resin was filtered and washed with MeOH/DMF, MeOH, DCM and DMF. The non-reacted OH groups were capped with acetic anhydride (10 equiv.) in DMF overnight.

*Synthesis of **2***. Fmoc-L-Phe was coupled in the presence of HOBT (12 equiv.), HBTU (12 equiv.) and DIPEA (7.5 equiv.) in dry DMF. Subsequently unreacted OH groups were capped with acetic anhydride as described above.

3.2.4. Enzymatic reactions. Prior to the enzymatic reactions, the peptide carrying polymers were washed three times with the buffer used for the enzymatic hydrolysis.

*PGA catalysed hydrolysis of **1***. The PGA solution was lyophilised and the specific activity of the lyophilised powder was 17 U/mg (benzylpenicillin units). The resin was suspended in 6 mL of the appropriate buffer in the presence of 5 mg of lyophilised PGA (85U). The reactions were incubated at room temperature for 24 h on a blood rotator. Afterwards, the reaction mixtures were filtered and washed using 36 mL (12×3 mL) of MeCN/H_2O (1:1). The filtrate was recovered in a flask, dried under vacuum, re-dissolved in 1 mL of MeCN/H_2O (1:1), centrifuged, and filtered through 0.45 μm membrane filters. The samples were then analysed with a RP-HPLC system as detailed below.

*Thermolysin catalysed hydrolysis of **2***. As above, but using 2 mg of thermolysin lyophilised powder directly as supplied.

To ensure that enzymatic reactions had been completed some reactions were left for longer reaction times after further addition of fresh enzyme.

3.2.5. Cleavage of Wang-linker. After the enzymatic reactions, the remaining peptide on the solid support was cleaved with a solution of TFA/H_2O (95:5) for 2 h. The filtrate was collected, the resins were then washed with a 1:1 mixture of MeCN/H_2O, filtered, collected, and dried under vacuum. The residues was re-dissolved in 1 mL of MeCN/H_2O (1:1) and analysed by RP-HPLC as detailed below.

3.2.6. HPLC analysis. Reverse phase HPLC analysis was carried out on a Waters 2690 LC system equipped with a Waters 468 UV detector using a C_{18} column. A gradient from 20 to 50% MeCN/H_2O (with 0.1% TFA in both phases) was used and enzymatic conversions were then calculated by comparison with standard solutions. Analysis was at 256 nm.

3.2.7. Determination of protein inside polymer beads. The amount of protein that had accessed the PEGA polymers (Fig. 4) was determined by the Pierce method, using bicinchoninic acid kit (Sigma). Standard PGA solutions with known enzyme concentrations were used as standards. Analyses were performed as reported in literature.[8]

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References and notes

1. (a) Meldal, M. *Tetrahedron Lett.* **1992**, *33*, 3077–3080. (b) St. Hilaire, P. M.; Willert, M.; Juliano, M. A.; Juliano, L.; Meldal, M. *J. Comb. Chem.* **1999**, *1*, 509–523. (c) Meldal, M. *Biopolymers* **2002**, *66*, 93–100. (d) Meldal, M.; Svendsen, I.; Juliano, L.; Juliano, M. A.; Nery, E. D.; Scharfstein, J. *J. Pept. Sci.* **1998**, *4*, 83–91.
2. (a) Kress, J.; Zanaletti, R.; Amour, A.; Ladlow, M.; Frey, J. G.;

Bradley, M. *Chem. Eur. J.* **2002**, *8*, 3769–3772. (b) Ulijn, R. V.; Baragaña, B.; Halling, P. J.; Flitsch, S. L. *J. Am. Chem. Soc.* **2002**, *124*, 10988–10989. (c) Ulijn, R. V.; Bisek, N.; Halling, P.; Flitsch, S. L. *Org. Biomol. Chem.* **2003**, *1*, 1277–1281. (d) Ulijn, R. V.; Brazendale, I.; Margetts, G.; Flitsch, S. L.; McConnel, G.; Girkin, J.; Halling, P. J. *J. Comb. Chem.* **2003**, *5*, 215–217.

3. Grether, U.; Waldmann, H. *Chem. Eur. J.* **2001**, *7*, 959–971.
4. Basso, A.; De Martin, L.; Gardossi, L.; Margetts, G.; Brazendale, I.; Bosma, A. Y.; Ulijn, R. V.; Flitsch, S. L. *Chem. Commun.* **2003**, 1296–1297.
5. Eichenbaum, G. M.; Kiser, P. F.; Shah, D.; Simon, S. A.; Needham, D. *Macromolecules* **1999**, *32*, 8996–9006.
6. Kadereit, D. W.; Waldmann, H. *Chem. Rev*. **2001**, *101*, 3367–3396.
7. (a) Reents, R.; Jeyaraj, D. A.; Waldmann, H. *Adv. Synth. Catal.* **2001**, *343*, 501–513. (b) Waldmann, H.; Reidel, A. *Angew. Chem. Int. Ed.* **1997**, *36*, 647–649. (c) Basso, A.; De Martin, L.; Ebert, C.; Linda, P.; Gardossi, L.; Ulijn, R. V.; Flitsch, S. L. *Tetrahedron Lett.* **2003**, *44*, 6083–6085.
8. Giacomini, C.; Villarino, A.; Franco-Fraguas, L.; Batista-Vieira, F. *J. Mol. Catal. B: Enzymatic* **1998**, *4*, 313–327.