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Improved biotransformations on charged PEGA supports

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PEGA supports functionalised with permanent charges show superior swelling properties in aqueous media when compared to neutral PEGA; a novel positively charged PEGA resin significantly improves penicillin G amidase (PGA) catalysed biotransformation on solid support, by favouring accessibility of the negatively charged enzyme.

Applications of enzyme catalysis on substrates that are immobilised onto solid supports are on the increase. Examples are chemo-enzymatic synthesis of compound libraries,[1] 'on-bead' screening for enzyme substrates or inhibitors in combinatorial libraries,[2a,b] enzyme cleavable linkers and protecting groups for release of polymer bound libraries,[3] applications in micro-array technologies[4] and enzymatic optical resolution.[5] The ability of the enzyme to catalyse reactions within the solid phase resin beads strongly depends on the dimension of the protein compared to the pore size of the resin. This in turn is determined by the swelling properties of the resin.[2,6]

PEGA resins are hydrophilic solid supports that have been designed for batch and continuous flow peptide synthesis.[7] It has been reported that $PEGA_{1900}$ (the subscript refers to the PEG molecular weight), is freely permeable to enzymes with molecular weight up to 35 KDa and compatible with their activity. Thus, $PEGA_{1900}$ was found to be ideally suitable for the screening of peptide libraries, affinity purification and on-resin enzyme assays.[2,5] For the use of large enzymes such as penicillin G amidase (PGA, MW = 88 KDa) in solid phase biocatalysis, the access of the protein to the inside of solid polymers is the limiting step. This translates into the low yields reported in the literature on $PEGA_{1500}$.[3] Relatively better results have been obtained either by using PEGA resins with longer PEG chains ($PEGA_{1500}$) that allow access of large proteases[2c] or by using soluble resins for PGA catalysed hydrolysis of enzyme scissible linkers.[3b] However, both the loading and the ease of handling of these resins were compromised.

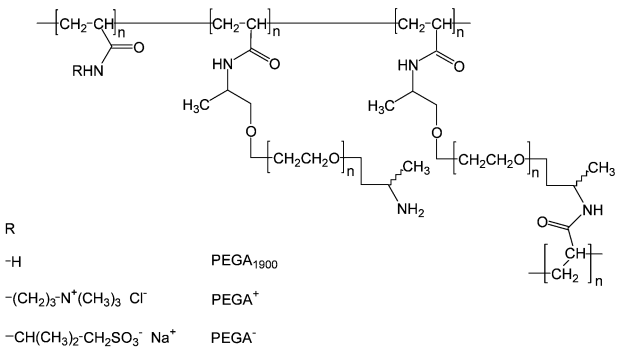
In this communication we suggest a different approach to achieve better accessibility of large proteins into PEGA supports. Upon introducing permanent positive or negative charges into the acrylamide backbone, electrostatic repulsion between adjacent chains is expected to increase the swelling of the polymer, thereby increasing the pore size. In addition, electrostatic attraction between oppositely charged polymer and protein molecules is expected to aid enzymatic diffusion into the beads.

To study both these effects, neutral $PEGA_{1900}$ and novel positively charged $PEGA^+$ and negatively charged $PEGA^-$ were prepared by Polymer Laboratories (Scheme 1). The charged polymers have the backbone of $PEGA_{1900}$ and a partial substitution with charge side chain monomers (10% substitution of the acryl amide monomer).

The swelling behaviour of $PEGA_{1900}$, $PEGA^+$ and $PEGA^-$ was studied at different concentrations of phosphate buffer at pH 8.0 (Fig. 1).† This pH value was chosen because it represents the optimum value for hydrolysis reactions catalysed by PGA.

A significant improvement in swelling was observed for $PEGA^+$ (from 18 to 35 mL $g^{-1}{}_{dry}$) and $PEGA^-$ (from 13 to 32 mL $g^{-1}{}_{dry}$) when the buffer concentration was lowered from 0.1 to 0.001 M (Fig. 1). This observation reflects the increased ionic interactions between chains at low ionic strength. Both charged polymers have the same amount of acrylamide monomers substituted with charged residues, thus explaining the very similar swelling behaviour. As expected, no ionic strength dependence on polymer swelling was observed for neutral $PEGA_{1900}$.

The next step was then to assess whether improved swelling had any effect on the efficiency of the biotransformation. PGA was chosen as a model enzyme because of its applicability on enzyme cleavable linkers and in enzymatic deprotection strategies.[3] As the solid phase substrate for PGA, a N-phenylacetylated L-Phe (**1**) was linked to the resin *via* a Wang-type linker. This linker allows for the separate analysis of both



Scheme 1 a) $PEGA_{1900}$ is a co-polymer of ethylene glycol and acrylamide. The molecular weight of the building blocks determines the chain extension and the pore size (chain length between cross-links) respectively. b) $PEGA^+$ and $PEGA^-$ are prepared by the substitution of 10% of the acrylamide with monomers containing (3-trimethylammonium chloride) propyl and 1,1-dimethyl-2-(sulphonate) ethyl residues, respectively. Loadings: 0.15 mmol $g^{-1}{}_{dry}$ ($PEGA_{1900}$) 0.23 mmol $g^{-1}{}_{dry}$ ($PEGA^+$) and 0.10 mmol $g^{-1}{}_{dry}$ ($PEGA^-$).

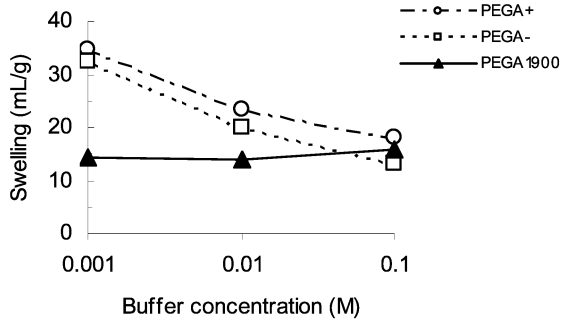


Fig. 1 Swelling of $PEGA^+$, $PEGA^-$ and $PEGA_{1900}$ in Kpi buffer at pH 8.0 as a function of buffer concentration.

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the cleaved phenylacetic acid (**2**) and the remaining amino acid (**3**) (Scheme 2).

Enzymatic hydrolysis of (**1**) was observed with all three resins (Fig. 2) indicating that all three supports are compatible with PGA activity.

For $PEGA^+$ a correlation between the increase in the swelling of the resins in the presence of diluted buffers and the yield of the hydrolytic reaction was found. A conversion of 48% in 0.001 M phosphate buffer was observed, which represents a dramatic improvement if compared to the low yields previously reported on solid phase supported chemistry by using PGA (1–15%).[3] For $PEGA^-$ the reaction yield did not increase by lowering the buffer concentration despite the increase of swelling.

The difference between the two resins can be explained in terms of electrostatic interactions between the polymer and the protein molecules. PGA has an overall negative charge at pH 8.0 (pI = 5.2–5.4),[8] and it is attracted by $PEGA^+$, while it is repelled in $PEGA^-$. As expected, the yield obtained with neutral $PEGA_{1900}$ was independent of the buffer concentration.

The amount of protein that entered the different resin beads was determined through the Pierce test.[9] It was found that, after 24 h of stirring, almost 50% of the protein was inside the beads in the case of $PEGA^+$, while this percentage was lower than 30% in the case of neutral $PEGA_{1900}$ and of $PEGA^-$. These results are in line with the hydrolysis conversions observed and provide evidence that electrostatic attraction occurs between the positively charged $PEGA^+$ and PGA.

When conversion data are expressed in terms of micromoles of product (**2**) released per gram of dry resin an 8 fold increase in the conversion is observed when comparing neutral (14.6 μmol g^{-1} $_{\text{dry resin}}$) or negatively charged resin (14.4) to the $PEGA^+$ (109.5). The maximum concentration of cleaved PhAcOH (**2**) observed was 0.033 mM (for $PEGA^+$ at pH 8.0 and 0.001 M) which is below the inactivation constant of PhAcOH for PGA (0.098 mM),[10] suggesting that product inhibition does not play a role here.

We previously described the importance of using a low resin to volume ratio when studying amide hydrolysis to overcome the preference for amide synthesis on solid support.[5b] Upon increasing the reaction volume from 6 to 18 mL, no further increase in hydrolysis yield was observed (43%) thus making the conversion yields obtained with PGA not fully limited by equilibrium but still diffusion dependent. Leaving the enzymatic reaction for longer periods did not result in increased hydrolysis either.

In summary, we show that by taking advantage of the improved swelling properties of positively charged resins ($PEGA^+$) when using enzymes as bulky as PGA, a significant enhancement in hydrolysis yields can be obtained. This effect is a consequence of increased protein accessibility due to a better swelling and electrostatic attraction between the negatively charged enzyme and the positive resin. Overall we provided a further step in the understanding of enzyme catalysis on solid supports.

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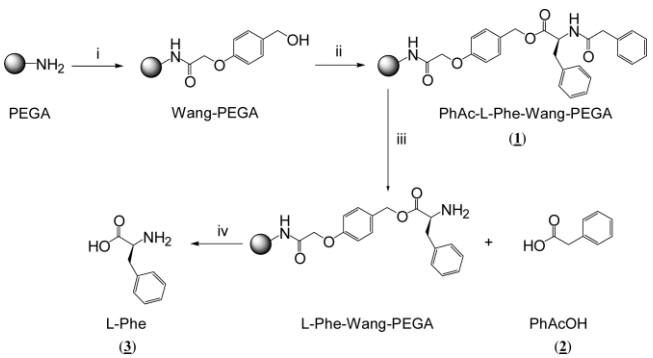

Scheme 2 Synthesis of PhAc-L-Phe-Wang linker on PEGA polymers. (i) washing and HMPA coupling in DMF; (ii) PhAc-L-Phe coupling and capping with acetic anhydride; (iii) PGA hydrolysis in 6 ml Kpi buffer pH 8.0, rt, 40 RPM, 24 h; (iv) cleavage of the Wang linker with TFA–H_2O (95 : 5).

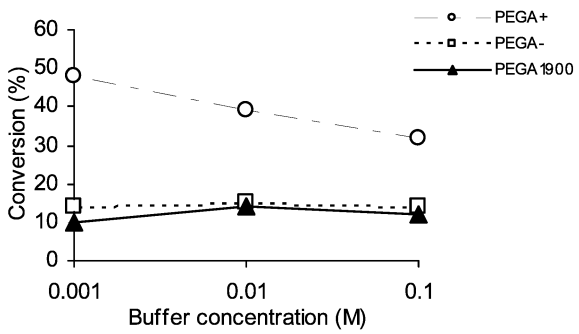

Fig. 2 Yields of PGA catalysed hydrolysis of (**1**) on solid supports at pH 8.0 in Kpi buffers with different buffer concentration.‡

Notes and references

† Polymers were washed in the appropriate buffer and then allowed to swell. The swelling was quantified by measuring weight differences of dried and swollen resins.

‡ Enzymatic hydrolysis was performed by washing about 100 mg of the wet functionalised resin with Kpi buffer. The resin was then suspended in 6 mL of the same buffer and in the presence of 5 mg of lyophilised enzyme. The reactions were allowed to mix in a blood rotator for 24 h at rt. The samples were analysed with a RP-HPLC system. Products amounts were calculated using calibration curves. At the end of the reaction the mixtures were filtered and the resins washed with 36 mL (12 × 3 mL) of ACN–H_2O (1 : 1). The liquid phase was recovered in a flask, dried under vacuum, redissolved in 1 ml of ACN–H_2O (1 : 1), eventually centrifuged, and filtered through 0.45 μm membrane filters. The peptide structures were then cleaved through the Wang linker with a solution of TFA (95%) to confirm conversion results.

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