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# Screening of polymeric supports and enzymes for the development of an *endo* enzyme cleavable linker

Ana Chiva [a], David E. Williams [b,†], Alethea B. Tabor [a], Helen C. Hailes [a,*]

[a] Department of Chemistry, University College London, 20 Gordon Street, London, WC1H OAJ, UK
[b] Unipath Ltd, Bedford MK44 3UP, UK

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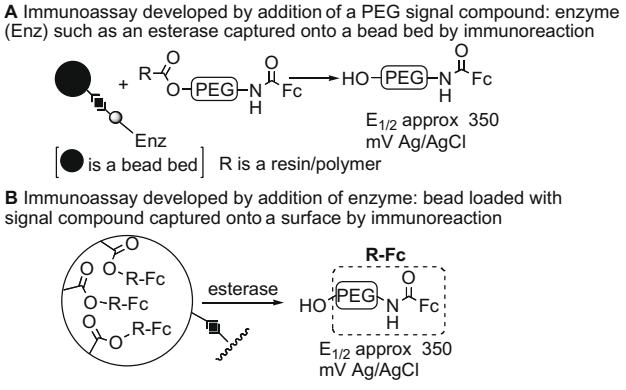
ABSTRACT

Several polymeric supports possessing an ester moiety were prepared and a range of enzymes was investigated to hydrolyse the ester linkage and release a signalling group into solution for applications in immunoassays. *Pseudomonas* lipases were found to most readily cleave the solution-phase analogue and this observation translated well to the corresponding polymeric supports, where the most effective were PEGA resins and the LPOS support PEG-6000.



Immunoassays are used extensively in medical diagnostics to detect or measure biological species. Enzymes are widely used as signal-generating labels in these assays, either to produce a coloured or fluorescent species from a non-coloured or non-fluorescent precursor or to generate a reagent that triggers chemiluminescence or electrochemiluminescence. An alternative approach is to use an enzyme to release a signal compound into solution for detection or to cleave the signal compound from a precursor in which the signal is significantly modified. Despite problems of carrying out analysis of labelled species including fluorescent analysis on resin beads,[1,2] these are potentially extremely powerful, sensitive and general methods that particularly lend themselves to schemes in which amplification is achieved by having multiple labels, bound as a consequence of a single biological recognition event.[3] Ferrocene has been used in a range of carbonylmetallo-immunoassays where the ability to tailor its redox properties has been of advantage.[4] We have been exploring the use of electrochemical detection techniques in which modified ferrocenes, showing significantly altered electrochemistry, are used as the signal compounds.[5,6] These ferrocenes have the potential to be useful in immunoassay schemes in which the ferrocene is cleaved and released into solution.[7] Enzymatic cleavage of side chains containing the label, such as ferrocene, would alter the redox potential of the ferrocene and potentially offer a simple and general method for using ferrocene reagents in an enzyme-coupled immunoassay (Scheme 1).

Due to the extensive range of commercially available hydrolytic enzymes, we have explored enzymatic cleavage of an ester-linked labelled moiety. In the present model study to identify suitable polymeric supports, linkers and enzymes, a fluorescent label was used, but the synthetic methods are easily adaptable to the use of ferrocenes as the label. The strategy used was initially to establish solution-phase chemistries using a solid-phase analogue and then translate this approach onto the solid phase. Enzyme labile linkers that can be cleaved under mild neutral reaction conditions




**Scheme 1.** Possible electrochemical detection schemes for immunoassays.

* Corresponding author. Tel.: +44 (0)20 7679 4654; fax: +44 (0)20 7679 7463.
*E-mail address:* h.c.hailes@ucl.ac.uk (H.C. Hailes).
† Present address: MacDiarmid Institute for Advanced Materials and Nanotechnology, Department of Chemistry, University of Auckland, Private Bag 92019, Auckland, New Zealand.

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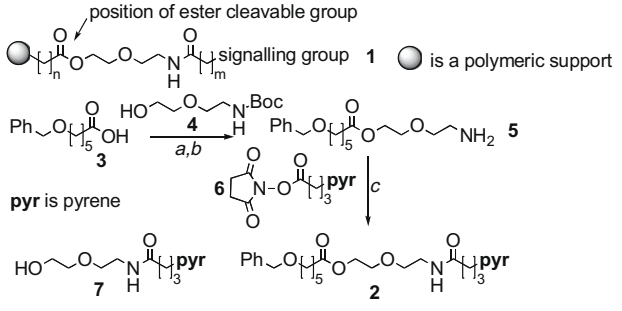
have been used in solid-phase peptide and carbohydrate synthesis.[8] They have been classified as *exo*- or *endo*-linkers which can be cleaved by *exo*- or *endo*-enzymes, respectively.[8] *Exo*-Linkers have been developed by several groups for applications in carbohydrate assembly and combinatorial chemistry.[9,10]

The arrangement of *endo*-linkers is linear and cleavage normally results in partial retention of the enzyme recognition group with the target molecule.[8] Several *endo*-linked systems have also been described,[11,12] including an aminopropyl silica support and ester cleavable linker which was readily hydrolysed using α-chymotrypsin.[13] Our approach was to use an *endo*-linker strategy based upon compound **1** possessing a hydrocarbon chain as a spacer, an ester linker to be enzymatically cleaved, a diethylene glycol unit to enhance the solubility of the signalling molecule when cleaved and in aqueous solution and a signalling moiety. In this study a fluorescent pyrene species was used. To assess the ease of cleavage in aqueous solution, compound **2** was synthesised possessing a benzyloxy group to mimic the solid support (Scheme 2).

Compound **3**[14] was coupled to Boc-protected 2-(2-aminoethoxy)ethanol **4**[15] using DCC and HOBt. Removal of the Boc group gave **5** which was then reacted with commercially available *N*-hydroxysuccinimide pyrene (**6**) to give **2** in 95% yield. Compound **7**, the corresponding hydrolysis product, was readily prepared by reacting pyrene butyric acid with 2-(2-aminoethoxy)ethanol and DCC and HOBt in 65% yield. Several esterases, proteases and lipases were assessed for the cleavage of analogue **2**–**7** to enable selection of a smaller number of hydrolytic enzymes to use with resin analogues. Representative cleavage data after 1 h is summarised in Table 1. Several lipases (entries 4, 7, 9, 10 and 11) demonstrated good levels of hydrolysis and these were explored further in resin-based reactions.

The choice of the resin solid support was crucial: it must be compatible with the biocatalyst, swell in the reaction solvent and enable accessibility of the enzyme to the interior of the resin.[16] Several resins were initially selected for the preparation of enzyme cleavable systems. Polystyrene-based supports were considered, despite the fact that they are known to shrink in aqueous media, because the loading level is higher than that in many available resins and they might be useful in exploring non-aqueous-based chemistries. TentaGel resin, which swells well in aqueous solutions, was investigated, although the polystyrene core can limit the access of macromolecules for cleavage purposes.[16] Polyacrylamide resin PEGA has been recognised as highly suitable for use in enzyme-based chemistries, and was also investigated.[17]

Initially compound **8** was synthesised for attachment to resins (Scheme 3). 6-(Tetrahydropyranyloxy)hexanoic acid[18] (**9**) was coupled to **4** using DCC and HOBt. Deprotection and reaction with **6** gave **8**. The Wang (*p*-benzyloxybenzyl alcohol [HMP] 0.80 mmol/g) and TentaGel HMP (0.35 mmol/g) resins were converted into their




**Scheme 2.** Cleavable signalling molecule **1**, synthesis of solution-phase analogue **2** and structure **7**. Reagents and conditions: (a) $Et_3N$, HOBt, DCC, 52%; (b) TFA, 95%; (c) **6**, DMAP, 95%.

trichloroacetimidate derivatives using trichloroacetonitrile and DBU,[19] then reacted with **8** and boron trifluoride etherate to give the coupled resins **10** and **11**. PEGA-$NH_2$ 1900 g/mol (0.20 mmol/g) and PEGA-$NH_2$ 800 g/mol (0.40 mmol/g) were reacted with the succinimidyl carbonate of **8** to give resins **12** and **13**.

Enzymatic hydrolysis of the resins was then performed. The cleavage to give **7** was monitored by HPLC analysis. The level of resin loading in each case was determined by resin hydrolysis using 1 M NaOH (24 h) and quantitative determination of **7** by HPLC. Enzymatic cleavage levels were then calculated relative to the total loading of **8**. Resin-conjugate **10** was submitted to enzymatic hydrolysis with a range of enzymes previously screened against **2**. No substantial hydrolysis was observed (Table 2, entry 1) due to poor diffusion of the enzyme into the resin. For enzyme cleavage experiments using **11**, low hydrolysis yields of up to 6% were determined (Table 2, entries 2 and 3). Several studies using TentaGel adducts with enzymes have been reported with variable results, including a 50% cleavage yield with penicillin amidase (MW 60 kDa);[9] lipase RB001-05 to hydrolyse an acetate group in 73% yield, although penicillin acylase (MW 80 kDa) was used to cleave a related *exo*-linker but gave only 1% hydrolysis,[10,20] and using papain (MW 23 kDa) to cleave a resin-bound fluorescently labelled peptide (MW 23 kDa) where very low yields were observed.[21] Our data suggest that the majority of the cleavage with the TentaGel-conjugate **11** is 'surface-based' with poor accessibility to the enzymes used.[1]

PEGA resins can swell up to 60-fold the weight of the polymer in aqueous buffers and allow large proteins to diffuse into the interior of the polymer, although they are not as easy to handle as the TentaGel resins.[1,21] Resin-conjugates **12** and **13** were used with selected enzymes, predominantly from the family of *Pseudomonas*, which proved to work well with the solution-based analogue. Again regioselective cleavage of the ester to give **7** was observed, but the levels of hydrolysis were much higher than those for **10** and **11**. Lipoprotein from *Pseudomonas* sp. and *Pseudomonas cepacia* lipase showed the best catalytic efficiency with both **12** and **13**, and total hydrolysis was achieved using the former with **12** (Table 2, entry 5). Reports in the literature using PEGA in enzyme cleavable reactions have highlighted that the higher the molecular weight of the enzyme, the lower the cleavage. Specifically, near quantitative yields were achieved with enzymes of MW up to 50 kDa, and above this, reaction yields were normally low.[1,20,22] These data agree with our studies where *P. cepacia* lipase (MW 25 kDa)[23] and lipoprotein lipase from *Pseudomonas* sp., (MW 33 kDa)[24] using **12** and **13** gave high hydrolysis yields (Table 2, entries 4, 7 and 5, 8) while the *Pseudomonas fluorescens* and *Aspergillus oryzae* lipase with MWs of around 50 kDa[24,25] gave lower cleavage yields (Table 2, entries 6, 9 and 10).

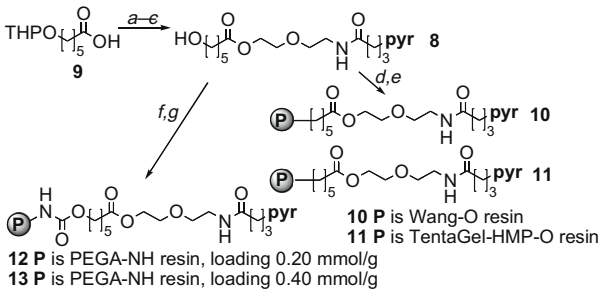
Two further potential supports were then investigated. PAMAMs are commercially available polyamido amine dendrimers with a high loading capacity, and different generations are available, reflecting the degree of loading possible.[26] They have been used as a dendrimeric support to synthesise a polysaccharide with the enzyme cellodextrin phosphorylase.[27] Attachment to latex beads was envisaged to generate multiply-functionalised amine resins for use in immunoassays. To explore the use of PAMAMs alone in preliminary studies, a generation 1 PAMAM was coupled to **8** to yield dendrimer **14** (Scheme 4). Enzymatic cleavage experiments were performed: however, only lipoprotein lipase from *Pseudomonas* sp. hydrolysed the ester moiety in low yield (Table 3) and this approach was not explored further.

Polyethylene glycol has been successfully used as a soluble polymeric support in liquid-phase organic synthesis (LPOS).[28,29] After reactions, the polymer can be precipitated by the addition of diethyl ether, which allows ease of separation and purification and having a solubilised support will enable the enzyme to ap-

**Table 1**
Selected enzymes used to hydrolyse **2**

| Entry | Enzyme[a] | Units used[b] | Hydrolysis[c] |
|---|---|---|---|
| 1 | Esterase from horse liver | 2.6 | + |
| 2 | Esterase from hog liver | 152 | + |
| 3 | Subtilisin A | 26 | 0 |
| 4 | *Pseudomonas cepacia* lipase | 113 | +++ |
| 5 | *Candida antarctica* lipase | 140 | + |
| 6 | *Candida cylindracea* lipase | 14 | + |
| 7 | *Pseudomonas fluorescens* lipase | 180 | ++ |
| 8 | Lipoprotein lipase from *Chromobacterium viscosum* | 14,160 | ++ |
| 9 | Lipoprotein lipase from *Pseudomonas* sp. 17,840 U/mg | 3568 | +++ |
| 10 | Lipoprotein lipase from *Pseudomonas* sp. 282 U/mg | 1410 | +++ |
| 11 | Lipase from *Aspergillus oryzae* | 580 | +++ |

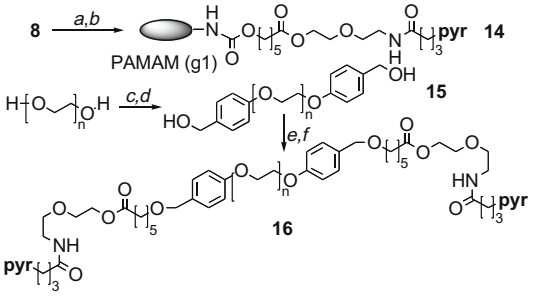
[a] Esterase/protease reactions in 10% $CH_3CN$ in 0.1 M phosphate (pH 8) at 25 °C. Lipases used in 50% hexane and 10% $CH_3CN$ in 0.1 M phosphate (pH 8) at 37 °C. Initial concn **2** $[S]_0$ 1 mM: no background reaction observed. Reaction monitored by TLC analysis.
[b] Where incomplete hydrolysis was observed, additional enzyme had a negligible effect on the level of hydrolysis.
[c] No hydrolysis-0; low levels of hydrolysis (~0 to 30%)-**+**; moderate levels of hydrolysis but **2** remaining (~30 to 90%)-**++**; complete hydrolysis-**+++**.




**Scheme 3.** Synthesis of **8**–**13**. Reagents and conditions: (a) $Et_3N$, HOBt, DCC, **4**, 72%; (b) TFA, 99%; (c) **6**, DMAP, 23%; (d) Wang (HMP) and Tentagel HMP, $CCl_3CN$, DBU, 0 °C; (e) To (d) add **8**, $BF_3{\cdot}OEt_2$; (f) *N,N′*-disuccinimidyl carbonate, $Et_3N$, 74%; (g) PEGA-$NH_2$ resins.

proach the substrate more easily. They have had limited application to date, although penicillin G acylase was used to readily cleave conjugates in high yields and purities.[8,20] It was decided to use an HMP linker as before with a linear PEG-6000, functionalised at both termini. PEG-6000 was converted into the dimesylated PEG,[29] in quantitative yield and then treated with sodium hydride and 4-hydroxybenzaldehyde and directly reduced to the alcohol with sodium borohydride to yield **15** in 74% yield.[30] The terminal hydroxy groups were trichloroacetimidated and reacted with **8** to produce the PEG resin-conjugate **16** (Scheme 4). Good enzymatic hydrolyses were observed again with *Pseudomonas enzymes* (Table 3, entries 2–4). Notably, since the enzyme and support are both in solution, the molecular weight of the enzyme does not appear to be a crucial factor and *P. fluorescens* hydrolysed **16** in a higher yield than *P. cepacia*.

Overall, the approach of initially identifying suitable enzymes from a solution-phase assay then translating this to polymeric supports proved effective. Several polymeric supports were investigated for use in the enzymatic assay. Polystyrene-based resins performed




**Scheme 4.** Synthesis of resin-conjugates **14** and **16**. Reagents and conditions: (a) *N,N′*-disuccinimidyl carbonate, $Et_3N$, 74%; (b) PAMAM (g1); (c) $CH_3SO_2Cl$, $Et_3N$, 100%; (d) 4-(HO)–$C_6H_4CHO$, NaH, 75 °C, then $NaBH_4$, 74%; (e) $CCl_3CN$, DBU, 0 °C, 37%; (f) **8**, $BF_3{\cdot}OEt_2$, 25%.

**Table 2**
Enzymatic hydrolysis of resin-conjugates **10**–**13**

| Entry | Resin conjugate | Enzyme[a] | Units used[b] | Hydrolysis (time) |
|---|---|---|---|---|
| 1 | **10** | *Pseudomonas cepacia* lipase (MW 25 kDa) | 600 | 1% (4 h) |
| 2 | **11** | Lipoprotein lipase from *Pseudomonas* sp. (MW 33 kDa) 17,840 U/mg | 3920 | 4% (4 h) |
| 3 | **11** | *Pseudomonas cepacia* lipase (MW 25 kDa) | 600 | 6% (4 h) |
| 4 | **12** | *Pseudomonas cepacia* lipase (MW 25 kDa) | 600 | 29% (4 h)<br>41% (16 h) |
| 5 | **12** | Lipoprotein lipase from *Pseudomonas* sp. (MW 33 kDa) 17,840 U/mg | 11,600 | 59% (4 h)<br>100% (16 h) |
| 6 | **12** | *Pseudomonas fluorescens* lipase (MW 50 kDa) | 290 | 1% (4 h) |
| 7 | **13** | *Pseudomonas cepacia* lipase (MW 25 kDa) | 600 | 21% (4 h)<br>43% (16 h)<br>78% (72 h) |
| 8 | **13** | Lipoprotein lipase from *Pseudomonas* sp. (MW 33 kDa) 17,840 U/mg | 11,600 | 71% (4 h)<br>78% (16 h) |
| 9 | **13** | *Pseudomonas fluorescens* lipase (MW 50 kDa) | 290 | 4% (4 h) |
| 10 | **13** | Lipase from *Aspergillus oryzae* (MW 51 kDa) | 180 | 7% (4 h) |

1–4 μmol-loaded resins **10**–**13** used.
[a] Wang resin reaction: 1% $CH_3CN$, in 0.1 M phosphate (pH 8) at rt. Tentagel resin reactions: 50% hexane in 0.1 M phosphate (pH 8) at 37 °C. PEGA reactions in 0.1 M phosphate (pH 8) at 37 °C. Reactions monitored by HPLC.
[b] Addition of further enzyme gave negligible increase in the level of hydrolysis.

**Table 3**
Enzymatic hydrolysis of resin conjugates **14** and **16**

| Entry | Resin conjugate | Enzyme[a] | Units used[b] | Hydrolysis (time) |
|---|---|---|---|---|
| 1 | **14** | Lipoprotein lipase from *Pseudomonas* sp. (MW 33 kDa) | 11,620 | 15% (4 h) |
| 2 | **16** | *Pseudomonas cepacia* lipase (MW 25 kDa) | 200 | 56% (4 h) |
| 3 | **16** | Lipoprotein lipase from *Pseudomonas* sp. (MW 33 kDa) | 11,600 | 28% (4 h) |
| 4 | **16** | *Pseudomonas fluorescens* lipase (MW 50 kDa) | 180 | 79% (4 h) |

1–3 μmol-loaded resins **14** and **16** used.
[a] 0.1 M phosphate (pH 8) at 37 °C. Reaction monitored by HPLC.
[b] Addition of further enzyme gave negligible increase in the level of hydrolysis.

poorly which concurred with several other reports using a range of enzymes: however, these resins may be useful in non- or low-aqueous media biocatalytic reactions.[31] The PEGA resin-conjugates were readily cleaved using several enzymes. Almost quantitative yields were obtained with enzymes having a molecular weight up to 50 kDa. The PAMAM conjugate allowed partial enzymatic hydrolysis only, while the use of PEG-6000 as a support enabled the enzyme to cleave most of its loaded sites. Factors including the molecular weight of the enzyme appeared to play a key role in the yield of the cleavage reaction, particularly in the case of the heterogeneous supports such as PEGA. This model system can now be developed further into biosensors using the linker and resin methodology.

## Acknowledgement

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## Supplementary data

Supplementary data (synthesis of **2**, **5**, **7**, **8** and **10**–**16**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.03.040.

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