HYDRA: A novel hydroxy and amine functionalised resin synthesised by reductive amination of PEG aldehyde and a polyamine

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A chemically inert poly(ethylene glycol) (PEG)-based resin was synthesised by reductive amination of a mixture of mono- and dialdehyde PEG_{1500} and the branched cross-linker tris(2-aminoethyl)amine. This unique concept of resin polymerisation yields a polar resin with two functionalities present (OH and NH). The resin loading could be tuned between 0.33 and 0.80 mmol OH per gram and 0.88 and 0.24 mmol NH per gram by varying the monomer composition in the polymerisation mixture. The resin was found to exhibit excellent swelling properties in a broad range of solvents, and was stable towards strongly acidic and basic conditions for weeks. Furthermore, both an octa- and a decapeptide were successfully assembled independently on the different functionalities. In addition, the resin was shown to be fully permeable to a 27 kDa protease.

Introduction

Since Merrifield's achievement to develop a solid support suitable for peptide synthesis,[2] numerous variations over this theme have been published.[3,4] No single resin has yet fulfilled all of the requirements of compatibility with all types of reactions, or possible applications of the solid supports (*e.g.*, synthesis, spectroscopic analysis, solid-phase screening, enzymic assays). Consequently, the search for new and improved resins is still in progress. Some resins are dedicated to special applications such as ion exchange and scavenging; however, most resins find application in various fields of organic synthesis. The most common resin supports for solid-phase organic synthesis (SPOS) are spherical beads of highly cross-linked gel-type polystyrene (1–2% divinylbenzene) and poly(oxyethylene)-grafted polystyrene copolymers. Each of these supports has its respective advantages and disadvantages depending on the particular application.[3,4] Polar, protic solvents, such as alcohols and water, do not swell polystyrene resins and accessibility to all reaction sites may be compromised.[5] Although the PEG-grafted resins such as Tentagel display relatively uniform swelling in a variety of solvents ranging from toluene to water, these resins have shown limitations with regard to their use in enzymic reactions.[6] Nevertheless, a PEG-based support can be more compatible with aqueous solutions and enzymic reactions, as first demonstrated with poly(ethylene glycol)–poly(acrylamide) (PEGA).[7–14] The family of PEG-based resins has been extended to include poly(oxyethylene)–poly(oxypropylene) (POEPOP)[14–16] and poly(oxyethylene)–polyoxetane (SPOCC)[14,16,17] which exhibit similar compatibility with aqueous enzymic chemistry.

PEG-based resins have been used in on-bead assays for the determination of inhibitors of proteolytic enzymes, where two different compounds (the substrate and the inhibitor) on a single bead compete for the enzyme (the one-bead-two-compounds concept).[9,13,18] Previously, bifunctionality has been accomplished by derivatisation of a monofunctional resin with a mixture of two different linkers[13] or by using an orthogonal protecting strategy on N^{α} and N^{ε} of lysine.[9,18,19] Bifunctional Tentagel resins are commercially available;[20] however, here the reactive sites on the outer surface of the bead are orthogonally protected to the reactive sites located in the internal volume of the bead, and bifunctionality is not evenly distributed.

The resin presented here, HYDRA (**hydr**oxy- and **a**mine-functionalised resin), is a new bifunctional resin which possesses an even distribution of functional groups (NH and OH) throughout the polymer matrix. The difference in reactivity between the hydroxy and amino groups can be exploited in, for example, construction of one-bead-two-compound libraries.[13] In addition to its potential use in this type of library construction, the HYDRA resin offers potential use in various other solid-phase techniques, such as tagging, where chemical 'bar-codes' are anchored to one functional group of the solid support along with the synthesis of a combinatorial ligand on the other.[21,22]

This HYDRA support consists of PEG chains linked together in a cross-linked polymeric network by polyamine branching units, the polymer thus being of the *star-block* or *radial* copolymer type.[23] Polymerisation was accomplished *via* reductive amination between PEG aldehyde and a polyamine, and, as a consequence of the branching units being polyamines, the resin embodied two different functionalities, namely amines and alcohols. The success of the polymerisation reaction is facilitated by the quantitative nature of the reductive amination reactions. In the work presented here, the use of the novel resin was demonstrated by peptide synthesis and enzymic cleavage of a resin-bound substrate. Furthermore, an inhibitor for the selected enzyme was assembled on the other functionality, and was shown to inhibit substrate cleavage.

Although linear imine or Schiff base polymers have been prepared from non-PEG-based dicarbonyl compounds and non-PEG-based diamines,[24,25] no account of Schiff base polymers being reduced has to our knowledge been reported. Consequently, this paper reports the first preparation of a copolymer by a two-step process of (1) formation of a polymeric material by formation of the Schiff base, followed by (2) reduction of this to give a more resistant material as well as a point of attachment for chemical entities.

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Results and discussion

Preparation of a bifunctional resin was initially attempted by displacement of dimesylated PEG_{1500} with tris(2-aminoethyl)-amine **2** at elevated temperatures, in the presence of base. Among investigated bases were TEA, DIPEA, DBU and Bu′OK. However, due to the difficulty of obtaining complete conversion in these solid-phase substitution reactions the material produced in this manner was a dark brown, soft, highly swelling resin, with a rather low degree of crosslinking. Furthermore, polymerisations were characterised by long 'sticky periods'[26,27] (typically 2–3 h). Heating dimesylated PEG_{1500} in the presence of the various bases followed by proton NMR analysis revealed that only Bu′OK caused β-elimination of the mesyloxy group to give vinyloxy-terminated PEG, and that β-elimination could therefore not be used as a general explanation for the resin quality. Various conditions and parameters such as solvent, base, stoicheiometry, temperature, *etc*., were altered in attempts to optimise the reaction, yet without success. This strategy was therefore abandoned, and our focus was directed towards a reductive amination approach.

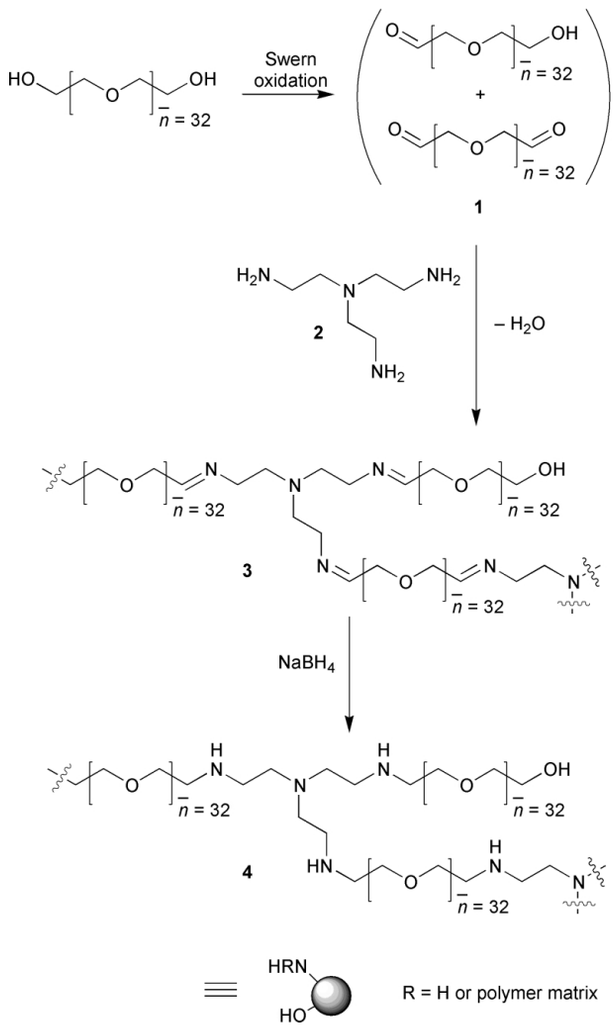
Reductive amination polymerisation between PEG aldehyde and amine **2** produced a resin with desired physical properties. Because the polymeric network consists of PEG chains tethered together by branching units, unlike various other resins *e.g.* polystyrene, Tentagel, PEGA, SPOCC, POEPOP, and poly(oxyethylene)–polystyrene (POEPS-3),[28] the described resin is a *star-block* or *radial* copolymer.[23]

(a) Oxidation of PEG

Oxidation of PEG_{1500} to generate PEG aldehydes was attempted using various protocols. Complete oxidation of all the PEG hydroxy groups was not ideal for the synthesis of a polymer with comparable hydroxy and amine functionalities. Instead, the preferred degree of oxidation was around 50%. Assuming that conversion of either one of the hydroxy groups of a PEG chain is an adiabatic process, at 50% conversion the obtained PEG aldehyde is in theory a 1:2:1 mixture of native PEG, PEG monoaldehyde, and PEG dialdehyde. Low degrees of oxidation were obtained using activated manganese dioxide,[29] 2,2,6,6-tetramethylpiperidin-1-yloxyl (TEMPO)–$CuCl_2$–CaH_2,[30] pyridinium chlorochromate, and the Corey–Kim procedure.[31] Similarly, poor yields of PEG aldehyde were obtained from the ring opening of oxiranemethylated PEG and successive periodate cleavage of the resulting diol, as well as acidolysis of bis(acetaldehyde diethyl acetal)ylated PEG.[32] Oxidation by acetic anhydride–DMSO,[32] and sulfur trioxide–pyridine complex–TEA were found to be impractical due to problems in isolating the oxidation product. Swern oxidation in dichloromethane at −78 to −40 °C resulted in only modest degrees of oxidation of the alcohols, presumably due to PEG precipitation during the course of the reaction. However, changing the solvent to CH_2Cl_2–CCl_4 1:1 solved the problem of precipitation, and batches of PEG aldehyde with degrees of oxidation from 40 to 60% were routinely obtained in 85–95% yield using oxalyl dichloride and DMSO.

(b) Polymerisation

Polymer was initially formed by treating a stirred solution of PEG aldehyde in acetonitrile (1–2 cm^3 solvent per gram of PEG material) with a solution of the branched amine (Scheme 1). The rate of polymerisation in pure acetonitrile was, however, too rapid (1–5 s) to guarantee complete mixing of reagents, even at low temperature. Slower polymerisation rates could be obtained by addition of water. For example, addition of 20% vol/vol water retarded the polymerisation to 3 min at room temperature. The crosslinked imine resin **3** produced in this manner was allowed to cure for 1 h, after which it was placed under high vacuum in order to remove solvent. The dried imine



Scheme 1 Synthesis of HYDRA resin **4** by stepwise reductive amination of PEG aldehyde mixture **1** with polyamine **2**.

resin was chopped into smaller pieces and treated with $NaBH_4$ in MeOH–DMF. After complete reduction, the resin was pushed through a mesh and washed, yielding a mechanically stable, almost colourless resin.

Five different batches of resin (**4a**–**e**) were prepared by varying the ratio of PEG aldehyde to amine crosslinker. The ratios of aldehyde to amine functionalities were 3:7 (**4a**), 4:6 (**4b**), 5:5 (**4c**), 6:4 (**4d**) and 7:3 (**4e**).

In the HYDRA support, the hydroxy functionalities were introduced *via* the non-oxidised PEG terminals, as well as unchanged aldehyde moieties that did not react with amine and were reduced to alcohol. Apart from the secondary amines formed by imine reduction, the resin contains some primary amine due to incomplete imine formation. This is particularly the case for resins **4a** and **4b**, which were polymerised using an excess of polyamine **2**.

The preparation of beaded HYDRA was unsuccessfully attempted by adapting previously published procedures for suspension polymerisation.[8,16]

(c) Characterisation

Resins **4a**–**e** were characterised by measuring their swelling properties, stability to acidic and basic conditions, and their loading of OH and NH functionalities.

Swelling. Resin swellings were determined by the syringe method[8] using a broad range of solvents (Fig. 1). A higher aldehyde-to-amine ratio generally increased resin swelling, as expected since a higher ratio implies fewer crosslinking units

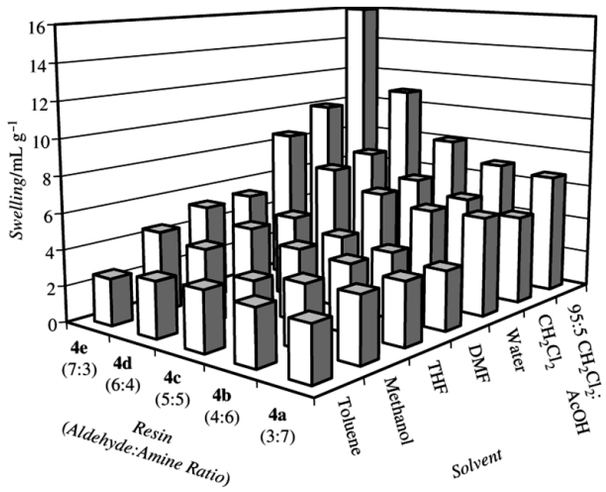


Fig. 1 Swelling properties of resin **4** as a function of aldehyde : amine ratio in the polymerisation synthesis.

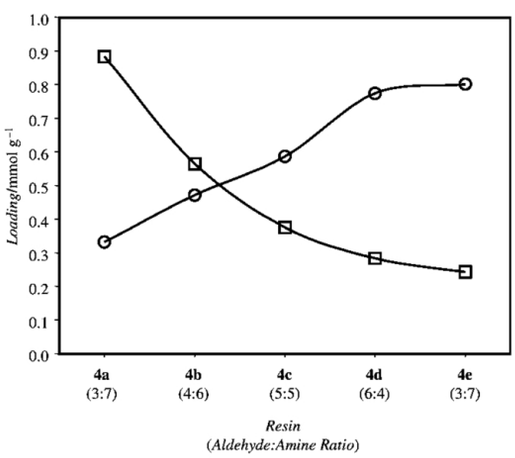
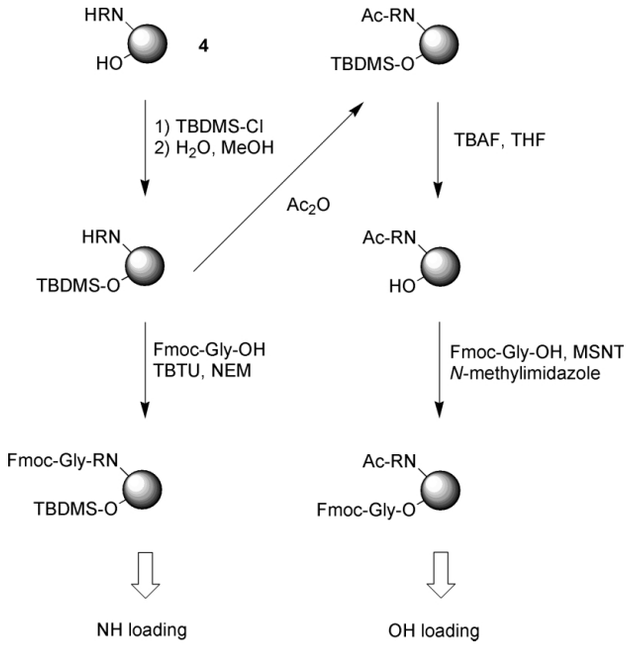


Fig. 2 Loading properties of resins **4a**–**e** as a function of aldehyde : amine ratio in the polymerisation synthesis. ○: OH loading, □: NH loading.

present. The trend was especially pronounced in water (5.5–8.6 $cm^3\ g^{-1}$) and dichloromethane (4.9–10 $cm^3\ g^{-1}$), and particularly for dichloromethane containing 5% acetic acid (6.7–16 $cm^3\ g^{-1}$). PEG-based resins generally exhibit high degrees of swelling in dichloromethane and in acid. In toluene, methanol, THF, and DMF, all resins were characterised by moderate degrees of swelling (typically 3–4 $cm^3\ g^{-1}$). These values are generally lower compared with other PEG_{1500}-based resins,[14] which can be an advantage since too high degrees of swelling cause decreased mechanical stability and necessitate more solvent which results in reagent dilution.

Stability. The synthesis of a compound normally requires several different reaction steps. Some of these steps might involve strong alkaline conditions (*e.g.*, ester hydrolysis), the use of strong acid (*e.g.*, removal of Boc), powerful acylating conditions (*e.g.*, ester- or amide-bond formation), or Lewis acids (*e.g.*, glycosylation reactions). To establish whether or not the resins were stable to these types of reaction conditions, resins **4a**–**4e** were subjected to treatment with aq. 4 M NaOH, neat TFA, aq. 4 M HCl, neat acetic anhydride, and neat boron trifluoride–diethyl ether for 2 weeks. None of the resins dissolved under these conditions, nor were any changes in colour or swelling observed, indicating no bond cleavage.

Loading. Owing to the bifunctional nature of the resin, a few synthetic steps were involved in determining the loading of both functionalities (Scheme 2). The hydroxy functionality was



Scheme 2 Synthetic steps involved in independently determining the loading of OH and NH on HYDRA resin (R = H or polymer matrix).

first capped by silylation with *tert*-butyldimethylsilyl chloride (TBDMSCl). Silylated amine was subsequently hydrolysed with water. *O*-Silylation was performed as a precaution to avoid even minor amounts of acylation of the hydroxy functionality which can occur with *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide (TBTU)[33] as the coupling reagent. The amine was coupled with TBTU-activated Fmoc-glycine in DMF. Amine loading (Fig. 2) was hereafter calculated by Fmoc cleavage and optical-density measurements at 290 nm.[34] The measured loading was corrected to compensate for the mass increase imposed on the resin by the silyl group and Fmoc-glycine (see Experimental section). In order to determine the hydroxy loading (Fig. 2), the free amines of the *O*-silylated resin were capped with acetic anhydride, after which the silyl group was removed with TBAF. The resin was esterified with 1-(2,4,6-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT)[35]-activated Fmoc-glycine in CH_2Cl_2, and, after Fmoc-loading determination, correction of the measured loadings to compensate for the mass of Fmoc-glycine and acetyl groups was performed. As expected, increasing the content of polyamine **2** resulted in an increase in amine loading and consequently a decrease in hydroxy loading.

The resins presented here exhibited a higher degree of total loading (on average 1.1 mmol g^{-1}) compared with other PEG_{1500} based resins, which typically have loadings of 0.2–0.6 mmol g^{-1}.[14] The theoretical total loadings of **4a**–**4e** were calculated on the basis of the amount of amine and aldehyde used in the polymerisations and were expected to be in the vicinity of 1.3 mmol g^{-1}, a number which corresponds well with the observed total loadings.

Spectroscopy. Magic Angle Spinning (MAS) NMR is becoming an increasingly important analysis tool in solid-phase chemistry, and the possibility to work with a resin which allows analysis of a compound without cleavage from the resin is valuable.[14,36–38] It was speculated that the *star-block* structure of the polymer would result in fewer microenvironments than in polymers containing backbone segments, yielding a more chemically homogenous polymer network, which should have a positive outcome on MAS NMR. Resin **4c** was *N*-acylated with 2-(*N*-*tert*-butoxycarbonylamino)benzoic acid (Boc-Abz) and studied by this technique (Fig. 3) for comparison with a

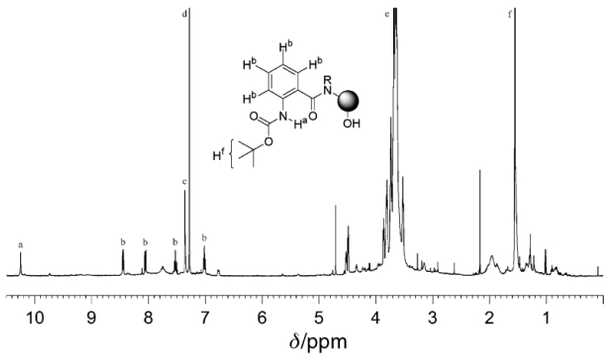


Fig. 3 ^{1}H MAS nanoprobe NMR (500 MHz) spectrum of resin Boc-Abz-**4c** (R = H or polymer matrix). (a) ArN*H*; (b) Abz; (c) chloroform, resin interior; (d) chloroform, outside resin; (e) $[CH_2CH_2O]_n$, NCH_2CH_2N; (f) Boc, water.

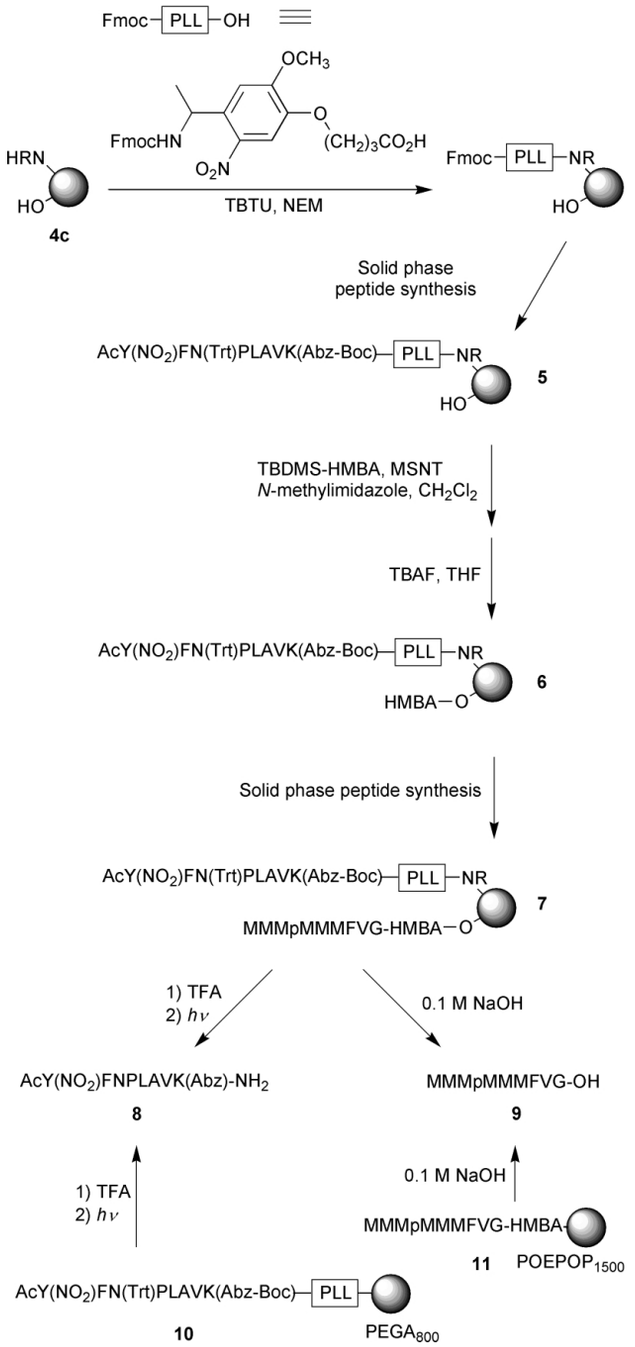
previous study.[14] The quality of the resins' spectral properties were quantified by three parameters, (i) general spectral quality as estimated visually; (ii) linewidth as measured using the Boc singlet at δ 1.55 and (iii) the multiplet resolution as measured using the Abz aromatic proton doublet at δ 8.45 and the degree of separation between the doublet peaks. In $CDCl_3$, the spectral quality was of high resolution, and the linewidth was measured to 1.3 Hz; however, because the Boc signal was overlapped with the water peak, and the observed value may be higher than the actual value. Nevertheless, the value is as good or better than the respective linewidth measured on similarly derivatised PEG_{1500}-based resins (POEPOP: 1.3, SPOCC: 1.8, POEPS-3: 2.2).[14] The multiplet resolution for Boc-Abz–**4c** was measured to 0.16 ppm, which is comparable to the values obtained for POEPOP (0.08 ppm), SPOCC (0.15 ppm) and POEPS-3 (0.18). It should be noted that 256 scans were recorded for these resins and only 64 scans were recorded for **4c**. The linewidth and multiplet resolution of resin **4c** was better than that reported for $PEGA_{1900}$, TentaGel, ArgoGel and PLASM.[14]

(d) Derivatisation

The usefulness of the resins for peptide synthesis was demonstrated by the assembly of an octameric peptide through the amine functionality (Scheme 3). A photolabile linker[39] was first attached using TBTU in DMF. Peptide synthesis was then accomplished using previously described procedures.[34] In parallel, the same peptide sequence was assembled on $PEGA_{800}$, and comparison of the RP-HPLC traces of peptide **8** photolysed from the two resins showed no significant difference in purity (95% in both cases). The hydroxy functionality of **5** was hereafter esterified with the base-labile linker 4-(hydroxymethyl)-benzoic acid (HMBA) to give **6**, and a decameric peptide was assembled through this linker. Again, the synthesis was performed in parallel, this time on HMBA-$POEPOP_{1500}$. Cleavage of peptide **9** from resins **7** and **11** with 0.1 M NaOH and analysis by RP-HPLC showed that the purities of crude peptides **9** from these two resins were comparable (61% and 74%, respectively). The crude material cleaved from resin **7** was contaminated with a small amount of peptide **8**. The presence of peptide **8** may be due to imperfect exclusion of light during cleavage of peptide **9**. The reason for the limited purity in both cases could result from oxidation of one or more of the multiple methionine residues in peptide **9**.

(e) Enzymic studies

On the basis of a previous study of the substrate specificity of the 27 kDa endoprotease Subtilisin Carlsberg,[13] resin-bound peptide **8** was examined as a substrate for this protease. To investigate HYDRA's enzyme permeability, resin **5** was treated with aq. TFA to remove protecting groups and the resin was then incubated with the protease for 6 h. By RP-HPLC analysis of cleaved materials it was determined that nearly all peptide



Scheme 3 Independent synthesis of two different peptide sequences on HYDRA resin (R = H or polymer matrix) and comparative syntheses on other PEG-based resins (p = D-Pro).

(t_R = 14.2 min) had been digested [compare 280 nm plots of Fig. 4a (before enzyme cleavage) and Fig. 4c (after enzyme cleavage)], indicating that the enzyme could react with peptide attached to the exterior and the interior of the resin granulate. If the enzyme were not capable of penetrating the resin, only the small amount of peptide on the exterior of the granulate would be digested.[40] The same experiment conducted on PEGA resin **10** gave a similar result (Fig. 4b). The new peak at t_R = 14.0 min was determined by ESI-MS to contain the expected peptide fragment LAVK(Abz)-NH_2.

The sequence MMMpMMMF has an IC_{50}-value of 0.055 ± 0.003 mmol dm^{-3} against Subtilisin Carlsberg,[13] and was therefore assembled through the hydroxy functionality, using a short C-terminal extension. After removal of the protecting groups, resin **7** was incubated with the protease. In this case it was evident that digestion of substrate was inhibited, as seen by the large remaining amount of substrate (Fig. 4d).

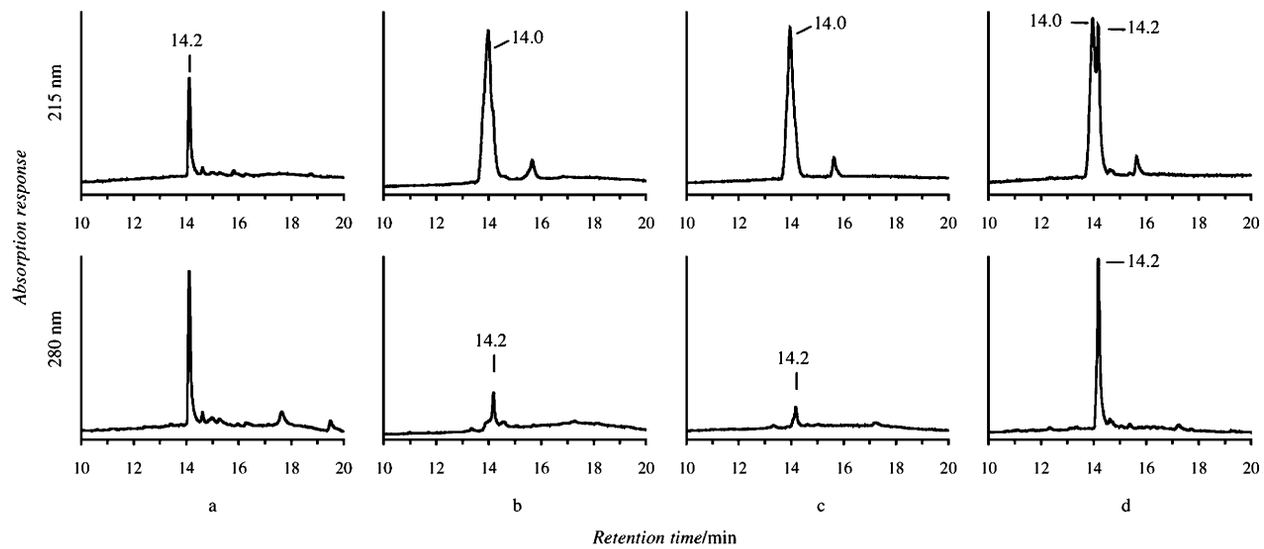


Fig. 4 HPLC traces of substrate for Subtilisin Carlsberg at 215 (top) and 280 nm (bottom). (a) On resin **4c** before enzymic incubation; (b) on PEGA resin after enzymic incubation; (c) on resin **4c** after enzymic incubation; (d) on resin **4c** with attached inhibitor after enzymic incubation.

Conclusions

In the work presented here, the first resin that embodies two different functionalities as produced directly by reductive amination with PEG aldehyde and a polyamine is reported. Preparation of PEG aldehyde by Swern oxidation was found to be most efficient and convenient. Resin batches with an OH loading between 0.33 and 0.80 mmol g^{-1} and an NH loading between 0.88 and 0.24 mmol g^{-1} were obtained by varying the stoicheiometry of the polymerisation mixture. The bifunctional HYDRA support showed excellent swelling properties in a broad range of solvents ranging from water to dichloromethane. Peptides were successfully synthesised independently on the different functionalities of the HYDRA resin. A substrate for Subtilisin Carlsberg was attached to the amino functionality and enzymically cleaved by this protease. Attachment of an inhibitor on the hydroxy functionality impeded the enzyme activity.

Thus, HYDRA is a novel type of resin that has the advantage of possessing two different functionalities uniformly distributed through the polymer matrix. Furthermore, owing to its relatively low swelling in common solvents compared with other PEG_{1500}-based resins, HYDRA can be treated in solution at higher concentrations without the use of more equivalents of reagent. Owing to its good solvation in water, enzymes can completely penetrate the matrix.

The novel characteristics of the HYDRA resin will be investigated for purposes other than the ones presented here. The resin offers potential for encoded libraries taking advantage of the bifunctionality as well as for one-bead-two-compounds libraries.

Experimental

General

All solvents were anhydroscan-grade from Labscan and were used as purchased. Unless indicated, chemicals were obtained from Fluka or Aldrich. Tris(2-aminoethyl)amine (95% purity) was from Fluka and used without any further purification. TBTU, MSNT, HMBA and Fmoc-amino acids and corresponding pentafluorophenyl (Pfp) esters were from Bachem (Switzerland) or NovaBiochem (Switzerland), and Subtilisin Carlsberg was from NOVO Industries (Denmark). $PEGA_{800}$ was from Polymer Laboratories (UK). PEG_{1500} was purchased from Fluka and was dried (determined by Karl-Fischer titration) by stirring 20–40 g portions of this material at 100 °C *in vacuo* for *ca.* 2 h. Literature procedures were used to prepare the photolabile linker,[39] TBDMS-HMBA,[41] and Fmoc-Lys(Boc-Abz)-OH.[42]

^{1}H NMR spectroscopy of PEG aldehyde was taken at 250 MHz in $CDCl_3$, and was performed on a Bruker DPX 250 MHz instrument. Proton shifts are downfield from TMS (0.00 ppm) as internal standard. *J*-Values are in Hz. A 32 s presaturation delay was employed. ^{1}H MAS NMR was recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a 4 mm ^{1}H-observe nano NMR probe, at 25 °C, using a spin rate of approximately 2 kHz. Resin particles were transferred into a nanotube, dried overnight *in vacuo*, and treated with $CDCl_3$ (40 mm^3). The spectrum was recorded as a one-pulse experiment with presaturation of the main PEG resonance. Acquisition data for the spectrum was as follows: 2.0 s acquisition time, 2.0 s presaturation delay, sweep width of 8000 Hz.

PEG_{1500} aldehyde 1

Oxalyl dichloride (1.65 cm^3, 19.2 mmol) was dissolved in CH_2Cl_2–CCl_4 (1 : 1; 90 cm^3) under argon, the solution was cooled to −47 °C, DMSO (2.90 cm^3, 40.8 mmol) in CH_2Cl_2–CCl_4 (1 : 1; 9 cm^3) was slowly added over a period of 3 min, and the mixture was stirred for an additional 6 min. Dried PEG_{1500} (7.20 g, 4.8 mmol) in CH_2Cl_2–CCl_4 (1 : 1; 30 cm^3) was added during 1 h using a syringe pump. The mixture was stirred at −47 °C overnight, treated with TEA (10 cm^3, 71.7 mmol), and allowed to warm to ambient temperature. Water (10 cm^3) was added and the mixture was concentrated *in vacuo*. The resulting residue was diluted with water (20 cm^3) and extracted with CH_2Cl_2 (3 × 50 cm^3). The extracts were dried ($MgSO_4$) and concentrated to leave an oily residue to which was added Et_2O (100 cm^3) with stirring for 2 h. The white precipitate was filtered off and dried *in vacuo*. Yield: 6.83 g (95%). ^{1}H NMR revealed a 46% degree of oxidation. ^{1}H NMR ($CDCl_3$; 250 MHz) δ 9.74 (0.92 H, t, *J* 0.8, aldehyde), 4.16 (1.84 H, d, *J* 0.8, CH_2CHO), 3.64 (130 H, s, $[CH_2O]_{65}$).

Typical polymerisation procedure (4c)

PEG aldehyde **1** (200 mg, 133 μmol aldehyde) was dissolved in a mixture of MeCN (120 mm^3) and water (30 mm^3) under argon and the solution was cooled to 0 °C with an ice-bath before the addition of a solution of tris(2-aminoethyl)amine **2** (6.8 mg, 133 μmol NH_2) in MeCN (50 mm^3) with stirring. After 5 min, the ice-bath was removed and resin **3** was allowed to cure for 1 h at ambient temperature. The resin was lyophilised overnight,

cut into smaller lumps (≈3 × 3 mm) and treated with 0.5 M $NaBH_4$ (5 cm^3; DMF–MeOH 1:1) overnight. Water (5 cm^3) was added and the resin was pushed through a 1 × 1 mm mesh sieve. The resulting granulated resin **4c** was washed with MeCN (3×) and CH_2Cl_2 (3×) and dried *in vacuo*. Yield: 158 mg (79%) of a colourless resin. Resins **4a**, **4b**, **4d** and **4e** were synthesised from the following aldehyde:amine molar ratios and gave the following yields: **4a** (3:7 aldehyde:amine): 94%, **4b** (4:6 aldehyde:amine): 92%, **4d** (6:4 aldehyde:amine): 83%, **4e** (7:3 aldehyde:amine): 46% as determined by mass balance. The procedure was easily scaled up to gram scale. Resin-swelling measurements were performed by the syringe method.[8]

Loading determinations

Resins **4a**–**4e** were each treated as follows: Approximately 30 mg of resin was treated overnight with a CH_2Cl_2 solution (600 mm^3) containing TBDMSCl (0.5 M), DMAP (0.05 M) and TEA (0.5 M). The resin was washed successively with CH_2Cl_2 (3×), MeOH (3×), water (10 min), MeCN (3×) and DMF (3×). The resin was divided into two portions (*a* and *b*). Portion *a* was treated with 2:4:4 Ac_2O–pyridine–DMF overnight, washed successively with DMF (3×), MeCN (3×) and THF (3×), treated with a mixture of 1 M TBAF in THF (500 mm^3, 0.5 mmol) and AcOH (28 mm^3, 0.5 mmol) for 4 h, and washed successively with THF (3×) and CH_2Cl_2 (3×). The resin was treated with 500 mm^3 of CH_2Cl_2 containing Fmoc-Gly-OH (0.5 M), MSNT (0.5 M) and *N*-methylimidazole (0.5 M) for 30 min, washed with CH_2Cl_2 (3×) and coupled again with a fresh batch of reagents for 1 h. The resin was washed successively with CH_2Cl_2 (3×), 95:5 CH_2Cl_2–DIPEA, CH_2Cl_2 (3×) and MeCN (3×), and dried *in vacuo*. Portion *b* was treated overnight with 500 mm^3 of DMF containing Fmoc-Gly-OH (0.5 M), TBTU (0.47 M) and *N*-ethylmorpholine (0.5 M), and washed with DMF (5×), MeCN (3×) and CH_2Cl_2 (3×), and dried *in vacuo*. The Fmoc loading of portions *a* and *b* was determined by Fmoc cleavage and optical-density measurements at 290 nm and were calculated by employing a calibration curve.[34] OH and NH loadings were calculated from the Fmoc loadings by the following equation: $Loading_{Corrected} = (Loading_{Measured}{}^{-1} - \Delta M)^{-1}$, where ΔM is the combined mass increase to the resin imposed by Fmoc-Gly, Ac and TBDMS. For X = OH, $\Delta M = 0.321$ g $mmol^{-1}$; for X = NH, $\Delta M = 0.394$ g $mmol^{-1}$. Corrected loadings of **4a**–**4e** are depicted in Fig. 2.

Boc-Abz-functionalised resin

Boc-Abz-OH (6.2 mg, 26 μmol), TBTU (9.0 mg, 24 μmol) and *N*-ethylmorpholine (3.8 mm^3, 26 μmol) were mixed in DMF (200 mm^3), incubated for 10 min, and added to resin **4c** (23 mg, 8.7 μmol NH). After 17 h, the resin was washed successively with DMF (5×) and CH_2Cl_2 (5×) and lyophilised. ^{1}H MAS NMR ($CDCl_3$; 500 MHz) δ 10.22 (s, 1 H, ArN*H*), 8.45 (d, 1H, *J* 8, Abz), 8.05 (dd, 1H, *J* 8, 1.5, Abz), 7.52 (dt, 1H, *J* 8, 1.5, Abz), 7.02 (t, 1H, *J* 8, Abz), 3.64 (br s, $[CH_2CH_2O]_n$, NCH_2CH_2N), 1.55 (s, Boc, water).

Solid-phase peptide synthesis

Peptides were synthesised by use of either the Fmoc-amino acid OPfp[43] ester protocol[34] or Fmoc-amino acid–TBTU–*N*-ethylmorpholine protocol.[34] Fmoc deprotection was accomplished by washing with 20% piperidine in DMF (3 and 17 min) followed by washing with DMF (8×). Acetylation was performed with Ac-ODhbt (3 equiv.) in DMF.

Assembly of substrate [AcY(NO_2)FN(Trt)PLAVK(Boc-Abz)] on HYDRA (5)

A batch of resin **4c** (290 mg, 0.17 mmol OH, 0.11 mmol NH) was treated with a mixture of photolabile linker (344 mg, 6 equiv.), TBTU (198 mg, 5.6 equiv.) and *N*-ethylmorpholine (84 mm^3, 6 equiv.) in DMF (3 cm^3, incubated 10 min) overnight. The Fmoc group was removed and the peptide was acylated with TBTU–*N*-ethylmorpholine-activated Fmoc-Lys(Boc-Abz)-OH (259 mg, 4 equiv.). After removal of the Fmoc group the remaining peptide was assembled by using Fmoc-amino acid OPfp esters (3 equiv.) and 1-hydroxy-7-azabenzotriazole (HOAt) (0.1 equiv.), and was terminally acetylated with 3-acetoxy-3*H*-benzo[*d*][1,2,3]triazin-4-one (Ac-ODhbt). The resin was treated with 0.1 M NaOH (2 cm^3) for 30 min, and washed successively with water (5×) and MeCN (5×). A small sample of resin (≈1 mg) was treated with 95:5 TFA–water for 5 min, washed successively with DMF (3×) and water (3×), and subjected to photolysis with a 225 W Hg UV lamp for 30 min to yield a small sample of peptide **8** which by RP-HPLC was found to be >95% pure. $t_R = 14.2$ min; *m*/*z* (ESI) 1156.5 [$(M + H)^+$, 100%. $C_{56}H_{77}N_{13}O_{14}$ + H requires *m*/*z*, 1156.3], 1178.6 [$(M + Na)^+$, 10].

Assembly of Subtilisin Carlsberg inhibitor[13] (MMMpMMMFVG) on HYDRA (7)

Resin **5** (0.14 mmol OH) was treated with TBDMS-HMBA (139 mg, 3 equiv.), MSNT (151 mg, 3 equiv.) and *N*-methylimidazole (81 mm^3, 6 equiv.) in CH_2Cl_2 (2 cm^3) for 90 min and filtered off. A fresh portion of reagents was added and the resin was left overnight. The resin was washed successively with CH_2Cl_2 (3×) and THF (3×) and treated with THF containing TBAF (1 M) and AcOH (1 M) for 3 h. After being washed successively with THF (3×), MeCN (3×) and CH_2Cl_2 (3×), the resin was treated twice with Fmoc-Gly-OH (152 mg, 3 equiv.), MSNT (151 mg, 3 equiv.) and *N*-methylimidazole (81 mm^3, 6 equiv.) in CH_2Cl_2 (2 cm^3; 2 × 1 h). The resin was washed successively with CH_2Cl_2 (3×), MeCN (3×) and DMF (3×), and the Fmoc group was removed. Assembly of the remaining part of the peptide was accomplished by use of Fmoc amino acid OPfp esters (3 equiv.) and HOAt (0.1 equiv.), except for Fmoc-Pro-OH (3 equiv.) which was activated with TBTU (2.8 equiv.) and *N*-ethylmorpholine (3 equiv.). Upon completion of the peptide sequence and removal of the Fmoc group, the resin was washed successively with DMF (3×) and MeCN (3×). Two small samples of resin (≈1 mg each) were taken out. One was subjected to photolysis as described for resin **5**, resulting in identical chromatographic purity and mass spectrometric data as material photolysed off resin **5**. The other sample was treated with 0.1 M NaOH (50 mm^3) in the dark for 1 h, and added to saturated aq. NH_4Cl (10 mm^3) and MeCN (50 mm^3). RP-HPLC of the supernatant showed the purity of peptide **9** to be 61% and peptide **8** was also present. Peptide **9**: $t_R = 15.7$ min; *m*/*z* (ESI) 1205.6 [$(M + H)^+$, 100%. $C_{51}H_{84}N_{10}O_{11}S_6$ + H requires *m*/*z*, 1205.7].

Assembly of substrate [AcY(NO_2)FN(Trt)PLAVK(Abz-Boc)] on $PEGA_{800}$ (10)

$PEGA_{800}$ (55 mg, 0.022 mmol) was treated according to the procedure described for assembly of substrate on HYDRA (**5**). RP-HPLC data (purity, retention time) and ESI data of photolysed peptide **8** were identical with those of material photolysed from **5**.

Assembly of inhibitor (MMMpMMMFVG) on $POEPOP_{1500}$ (11)

$POEPOP_{1500}$ (80 mg, 0.036 mmol) was treated according to the procedure described for assembly of substrate on resin **6**. RP-HPLC retention time and ESI data of NaOH-cleaved peptide **9** were identical with those of material cleaved from resin **7**. RP-HPLC purity was 74%.

Enzyme incubations

Approximately 5 mg of each resin (**5**, **7**, and **10**) were treated with 95:5 TFA–water for 10 min, washed successively with

DMF (3×), 20% piperidine in DMF, DMF (3×), MeCN (10×), water (3×) and enzyme buffer (50 mM Bicine, 2 mM $CaCl_2$) (3×). The resins were each added to enzyme buffer containing Subtilisin Carlsberg (5×10^{-8} M; 1 cm^3) and incubated at ambient temperature for 6 h. The resins were washed successively with water (3×), 2% TFA in water (3×), water (3×), 2% aq. $NaHCO_3$ (3×) and water (3×), then photolysed in 100 mm^3 of water with a 225 W Hg UV lamp for 30 min, after which the supernatant was analysed by RP-HPLC (see Fig. 4b–d). t_R = 14.0 min; *m/z* (ESI) 548.4 {$(M + H)^+$, 96%. $C_{27}H_{45}N_7O_5$ [LAVK-(Abz)-NH_2] + H *m/z* requires 548.4}, 588.5 [$(M + K)^+$, 31].

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Notes and references

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