Stepwise Solid-Phase Synthesis of Polyamides as Linkers

Keith Rose* and Jean Vizzavona

Contribution from the Department of Medical Biochemistry, University Medical Center, University of Geneva, CH - 1211 Geneva 4, Switzerland

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Abstract: Solid-phase peptide synthesis (SPPS) yields well-defined polyamides of repeat unit NH-Z-CO but requires protected derivatives and deprotection steps. Polyamides of repeat unit NH-Y-NH-CO-X-CO (e.g., Nylon 66) are made by polymerizing diacids with diamines, but the absence of protecting groups leads to heterogeneity of chain length. We have produced a new class of polymers by automated solid-phase synthesis without protecting groups: polyamide chains NH-Y-NH-CO-X-CO containing a precise number of monomer units where X and Y can be varied independently at each step. Dimers and multimeric molecules which display phage-derived binding peptides in a synthetic construction were easily assembled with these precision length polyamide chains, which can usefully replace polypeptides and poly(ethylene glycol) as linkers.

Introduction

In biochemistry and medicine, water-soluble polymer chains are useful as linkers to connect multiple copies of a structural motif[1−4] as well as for shielding proteins from the immune system and increasing biological half-life.[5−7] Ideally, these chains should be of defined structure rather than a mixture of chains of different lengths. Polypeptide chains offer one possibility[4] but are in principle susceptible to proteolytic cleavage and might prove to be immunogenic. Poly(ethylene glycol) (PEG) offers another possibility.[1,3,5,7] Its backbone $(CH_2CH_2O)_n$ is flexible, amphiphilic, unsusceptible to proteases, and nonimmunogenic. Unfortunately, techniques used to prepare PEG or PEG-based chains, even those of fairly low relative molecular mass such as 3400,[1,3] involve a poorly controlled polymerization step which leads to preparations having a spread of chain lengths about a mean value; that is, they involve polymer preparations of $(CH_2CH_2O)_n$ where n does not have a discrete value but rather has a range of values about a mean. This is very evident in mass spectra of PEG chains themselves and of compounds to which PEG chains have been grafted.

We wished to produce a new class of biocompatible polymers which would combine the advantages of both polypeptides (precise length, convenient synthesis) and PEG (flexible, amphiphilic, nonimmunogenic, unsusceptible to proteases) and so replace them as molecular spacers for synthetic and semisynthetic constructions. For convenience, commercially available materials were used and protection and deprotection steps avoided. A three-step solid-phase procedure involving commercially available diacids (or derivatives) and diamines was investigated (Figure 1).

When several copies of the same peptide are present in the same multimeric molecule, avidities (and bioactivities) can be greatly enhanced over the affinity or activity of monomeric peptide. Such was the case (10^5-fold) with the peptabody,[4] a recombinant-DNA produced molecule which possesses five copies of a short cyclic peptide, and with some synthetic erythropoietin mimetic peptides (EMP) dimerized with PEG[3]. We chose to apply the new polyamide materials to the synthesis of dimers and multimeric molecules which display a set binding peptides in a chemically synthesized molecule.

Results and Discussion

General Procedures. The three-step scheme is shown in Figure 1. Resins used were standard polystyrene resins sold for peptide synthesis and had the following declared substitution ratios: 1.02 mmol/g Sasrin, 0.62 mmol/g Fmoc-Abu-Sasrin resin, and 0.6 mmol/g Boc-Leu-PAM resin, all from Bachem (Bubendorf, Switzerland). Abu is a residue of aminobutyric acid. They were thus either hydroxyl resins (Sasrin) or amino resins (resins preloaded with an amino acid). Succinic anhydride was used for step 1, the acylation (Figure 1). In step 2 (activation), activated material is shown as the mixed anhydride. Possible attack by displaced imidazole would give im-$COCH_2CH_2CO$-NH-Resin, but both species would undergo aminolysis to give the same product shown in step 3. A commercially available PEG-based diamine of defined structure NH_2-$CH_2CH_2CH_2$-$(OCH_2CH_2)_3$-CH_2-NH_2 was generally used for step 3 (aminolysis), although 1,6-diaminohexane ("Dah") was also used successfully. The three steps were then repeated to add a second repeat unit, $NHCH_2CH_2CH_2$-$(OCH_2CH_2)_3CH_2NH$-$COCH_2CH_2CO$, and so on. We term the particular residues $NHCH_2CH_2CH_2$-$(OCH_2CH_2)_3$-CH_2NH and $COCH_2CH_2CO$, "PEG" and "succ", respectively, so the repeat unit $NHCH_2CH_2CH_2$-$(OCH_2CH_2)_3$-CH_2NH-$COCH_2CH_2CO$ is referred to as "PEG"-"succ". An automated peptide synthesizer was programmed to perform the steps. Once the desired chain was assembled, a peptide was synthesized on the terminal amino group using standard Boc (*tert*-butyloxycarbonyl) or Fmoc

* To whom correspondence should be addressed. Keith Rose, Department of Medical Biochemistry, University Medical Center (CMU), 1 rue Michel Servet, CH - 1211 Geneva 4, Switzerland. Tel: (+41) 22 702 5523. Fax: (+41) 22 346 8758.

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NH_2-Resin

– (step 1)→ HO-$COCH_2CH_2CO$-NH-Resin

– (step 2)→ im-CO-$OCOCH_2CH_2CO$-NH-Resin

– (step 3)→ $NH_2CH_2CH_2CH_2$-$(OCH_2CH_2)_3$-CH_2NH-$COCH_2CH_2CO$-NH-Resin

Repetition of this three-step cycle gives a polyamide product:

-NH-diamine-NH-CO-diacid-CO-NH-diamine-NH-CO-diacid-CO-etc

Figure 1. Three-step procedure for synthesis of polyamides without protecting groups, assuming a starting amino resin, NH_2-Resin: step 1, acylation with diacid derivative (here succinic anhydride); step 2, activation with carbonyldiimidazole; and step 3, aminolysis with diamine (here $NH_2CH_2CH_2CH_2$-$(OCH_2CH_2)_3$-CH_2NH_2).

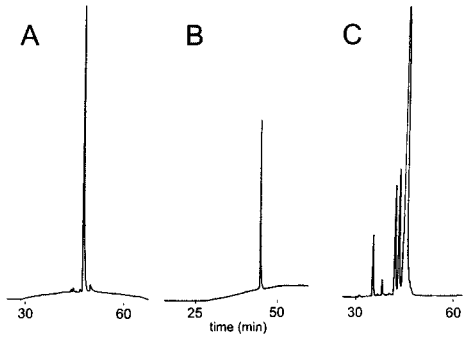

Figure 2. Analytical reversed-phase HPLC traces, A_{214nm} versus time (min). (A) crude Boc-Ser(Bzl) ("PEG"-"succ")$_6$-OH (ether precipitated but not chromatographically purified); the major component was identified as the expected product by mass spectrometry (Table 1). (B) polyamide H-("PEG"-"succ")$_8$-OH after treatment with trypsin for 24 h. (C) polypeptide *H*-PQPQPKPQPQPQPQPKPQPKPEPE-*amide* after treatment with trypsin for 24 h.

(fluorenylmethoxycarbonyl) techniques[8] prior to cleavage from the resin in the standard way. Alternatively, the terminal amino groups were modified with an aminooxyacetyl group or an aldehyde precursor by standard techniques[9] either prior to cleavage from the resin or afterward. The three-step approach of Figure 1 proved greatly superior to the use of PEG-based protected amino acids, such as Boc-NH-$CH_2CH_2CH_2$-$(OCH_2CH_2)_3CH_2NH$-$COCH_2CH_2CO_2H$. These derivatives are not commercially available, the Boc group is slightly labile during isolation, the compounds are inconvenient to handle (sticky), and coupling under standard conditions for peptide synthesis was slow and incomplete (data not shown). The procedure of Figure 1, which does not involve deprotection steps at all, was found to be compatible with both Boc and Fmoc approaches to SPPS with two exceptions, which must be taken into account when planning a synthesis. First, the aminolysis step removed formyl protection from Trp indole (Boc chemistry), so care was needed to avoid indole acylation in any subsequent steps, and second, the aminolysis step was also able to remove the Fmoc group. Other base-labile or nucleophile-labile protecting groups might also be affected by the aminolysis step. We generally synthesized a peptide on a preformed polyamide chain, not vice versa.

Figure 2A shows analytical HPLC data for crude (ether precipitated but not chromatographically purified) Boc-Ser(Bzl)-("PEG"-"succ")$_6$-OH, produced using the general procedure on a Sasrin resin and cleaved with 1% trifluoroacetic acid (TFA) so as to preserve the Boc group. After isolation by preparative HPLC, the electrospray mass spectrum showed a single component of expected relative molecular mass (2108.3 found, 2109.6 calculated). We were surprised that crude material which was almost homogeneous could result from a synthesis which did not involve protecting groups: it might have been expected that cross-linking (bridging) of activated carboxyl groups with diamine at each aminolysis cycle would rapidly build up impurities, but this was not the case with the resins and substitutions tested. Starting a synthesis with unloaded (hydroxyl form, HO-CH_2-$(CH_3O)C_6H_3$-O-CH_2-C_6H_4-polymer) Sasrin resin and the activation cycle (instead of the acylation cycle) led to an activated species, presumed to be im-CO-O-CH_2-(CH_3O)-C_6H_3-O-CH_2-C_6H_4-polymer.[10] Aminolysis with diamine NH_2YNH_2 led to the presumed carbamate NH_2YNH-CO-O-CH_2-$(CH_3O)C_6H_3$-O-CH_2-C_6H_4-polymer,[10] which was able to undergo cycles of acylation, activation, and aminolysis (Figure 1). In this way, we were able to synthesize molecules such as H-("Dah"-"succ"-"PEG"-"succ")$_3$-"Dah"-"succ"-"PEG"-H, where "Dah" stands for a residue of 1,6-diaminohexane. Examples of simple compounds made using the general procedure are shown in Table 1. Yields of purified product based on millimoles of starting resin (using for the calculation the susbstitution level declared by the manufacturer) were in the range typical for solid-phase peptide synthesis. For Boc-Ser(Bzl) -("PEG"-"succ")$_6$-OH, H-("PEG"-"succ")$_8$-OH, and H-("Dah"-"succ"-"PEG"-"succ")$_3$-"Dah"-"succ"-"PEG"-H, the yields were 26%, 38%, and 31% of theoretical, respectively, despite clean chromatograms such as Figure 2A.

Table 1. Simple Compounds Synthesized Using the General Procedure

compound	mass found	mass calc
H-Tyr-("PEG"-"succ")$_3$-Leu-OH[a]	1201.8	1201.6
Fmoc-Ser(Bu^t)-("PEG"-"succ")$_8$-Abu-OH[b]	2887.3	2887.5
Boc-Ser(Bzl)-("PEG"-"succ")$_6$-OH[c]	2108.3	2109.6
H -("PEG"-"succ")$_8$-OH[c]	2437.1	2437.0
H-("Dah"-"succ"-"PEG"-"succ")$_3$-"Dah"-"succ"-"PEG"-H[c]	1919.9	1919.5
H-("Dah"-"succ"-"PEG"-"succ")$_4$-OH[c]	2020.4	2020.6

[a] Resins used: Boc-Leu-PAM. [b] Resins used: Fmoc-Abu-Sasrin. [c] Resins used: unloaded Sasrin.

Enzymatic Digestion. As expected, the amide bonds of H−("PEG"-"succ")$_8$-OH and H-("Dah"-"succ"-"PEG"-"succ")$_3$-"Dah"-"succ"-"PEG"-H were completely resistant to the enzymes trypsin, chymotrypsin, and elastase. Figure 2B shows the chromatogram of H-("PEG"-"succ")$_8$-OH after incubation for 24 h at 37 °C with trypsin. The retention time was unchanged, and mass spectrometry showed that the compound was intact. In comparison, Figure 2C shows the result after comparable incubation of the polypeptide *H*-PQPQPKPQPQPQ-PQPKPQPKPEPE-*amide*, which represents the amino acid sequence of the hinge region of camel immunoglobulin, used as a linker for the recombinant peptabody.[4] While the major peak was identified by mass spectrometry as the intact polypeptide, the following digestion products were identified as earlier-eluting components: *H*-PQPQPK-*OH*, *H*-PQPKPEPE-*amide*, and *H*-PQPQPQPQPKPQPKPEPE-*amide*. The camel hinge is an atypical peptide, being devoid of residues of aromatic amino acids (chymotrypsin and elastase susceptible) and Arg (trypsin susceptible), and wherein Lys residues are followed by Pro (which greatly slows tryptic activity); it was nevertheless much less stable (Figure 2C) than the PEG-containing polyamide (Figure 2B).

EMP Dimer with Conventional PEG Linker. Figure 3 shows the chemistry used to construct the dimers. The amino

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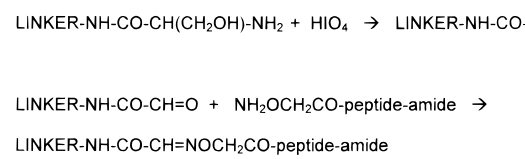

Figure 3. Chemistry used to construct the EMP dimers and the tetrameric constructs. Terminal Ser residues (one shown) on a linker are oxidized with periodate to a glyoxylyl function, which is then oximated with aminooxyacetyl peptide.[9]

alcohol (Ser) functions on the termini of the symmetrical linker were oxidized with periodate to glyoxylyl groups. These aldehyde groups were then condensed with aminooxyacetyl peptide through formation of an oxime bond.[9] Figure 4A shows the reversed-phase HPLC chromatogram and the mass spectrum of an erythropoietin mimetic peptide dimer (EMP dimer[3]) made using the commercial PEG linker of mean relative molecular mass 3400. The matrix-assisted laser desorption ionization time-of-flight technique (MALDI-TOF, which leads to singly protonated species) was employed because the spectrum was too complex for analysis on our electrospray quadrupole machine (which multiply protonates all species present). Previous work with such dimers[3], made with commercial PEG from the same supplier, demonstrated an ED50 (concentration required to obtain 50% of maximum response) of 0.1 nM (EPO-dependent cell proliferation assay), 1000-fold lower than that of the peptide monomer. As expected, the mass spectrum shows more than 40 components, each differing by the PEG repeat unit, CH_2CH_2O, a spacing of 44 atomic mass units. It was not possible to separate the individual components by HPLC. The mass peak at the center of the distribution (8024 amu) corresponds to a molecule with 78 CH_2CH_2O groups, consistent with the declared relative molecular mass of the PEG (3400, i.e., 77 repeat units, 231 bonds, found here to be ±20 repeat units or 60 bonds).

EMP Dimer with PEG-Based Polyamide Linker. Figure 4B shows the analytical HPLC chromatogram and mass spectrum of the EMP dimer made using one of our PEG-based polyamide chains even longer than that used in Figure 4A. The polyamide linker was symmetrical ("PEG"-"succ")$_6$-"PEG"-("succ"-"PEG")$_6$. Although it has more bonds than the commercial PEG (243 versus 231) and 42 CH_2CH_2O units, it is much more homogeneous. In the mass spectrum there is no sign of material with one too few CH_2CH_2O repeats (44 mass units). The signals slightly more massive than the principal signal are due to cationization with sodium and potassium, a common feature of electrospray ionization mass spectra. The relative molecular mass found (8417) is close to the theoretical value of 8420. It proved possible to make dimers with longer and shorter chains (16 and 4 "PEG"-"succ" repeat units). Excellent chromatograms and mass spectra were obtained using spacers up to ("PEG"-"succ")$_8$-"PEG"-("succ"-"PEG")$_8$, the longest tried.

Tetrameric Constructs. Figure 4C shows the HPLC chromatogram and mass spectrum of a tetrameric construct displaying four copies of the phage-derived peptide ADGACRNPWC (with a disulfide bond between the cysteines) which binds to the BCL_1 tumor cell line.[4] This tetrameric molecule is represented schematically in Figure 5. In comparison with the peptabody, which has five peptides, a linker length of 72 bonds, and no reporter group for a relative molecular mass of 85 000, the synthetic molecule has 4 binding peptides, a PEG-polyamide spacer length of 167 bonds, and a fluorescein reporter group for a relative molecular mass of 15 828 (found; close to the theoretical 15 839). The small signal at mass 15 521 corresponds to a very minor component with a single "PEG"-"succ" repeat unit missing out of the 32 present. Such deletions are common in solid-phase peptide synthesis. As with standard peptide chemistry, optimization of the coupling steps (higher concentrations, longer times, higher temperatures, better solvents, additives such as 4-(dimethylamino)pyridine) would be expected to yield even longer chains. With these unhindered and very soluble compounds, there should be none of the "difficult sequences" found with peptides and generally associated with beta-structure formation.

Conclusions. Results of biological assays of the several PEG-polyamide linked EPO-mimetic dimers and the many multimeric constructs we have made will be reported elsewhere. What we describe here is a facile stepwise method for making a new class of polymers: biocompatible polyamides with a defined number of repeat units, which uses commercially available diacids and diamines (for an extensive list see ref 6) and involves solid-phase chemistry which can be performed by machine. Previous syntheses of polyamides of repeat unit NHYNH−COXCO, for example, Nylon 66 and more recent solution polymerization of diacids with diamines, yield products with a wide range of chain lengths.[6] Acute toxicity screening in rodents suggests that such polyamides are neither toxic nor immunogenic.[6] We have shown that it is possible, by selecting an appropriate diacid and diamine for each step, to modulate factors such as hydrophobicity along the length of the chain (e.g., diamines such as 1,6-diaminohexane), to add reactive groups to the chain termini (e.g., oxime-forming groups), and to extend the chain by standard techniques of peptide synthesis. One is no longer limited to the few standard lengths of commercial PEG linkers, and so length can be fine-tuned quite closely. As expected for PEG-based molecules, these polyamides were perfectly soluble in water and in organic solvents such as dimethylformamide, but not in diethyl ether. The oxime chemistry used in making the di- and tetra-meric constructs has been shown to be suitable for attaching up to eight polypeptides.[9] Phage-derived peptides may thus be displayed on a totally synthetic molecule on the tips of biocompatible chains without the problems associated with recombinant expression and refolding: we were able to make tetrameric constructs just as easily with the phage peptide SVWRWLPYDKYE as with ADGACRNPWC, whereas the corresponding peptabody could not be produced in soluble form.[4] A very wide range of precisely made dimers and multimeric structures is now synthetically readily accessible.

Experimental Section

HPLC. Preparative reversed-phase HPLC was performed at 20 mL/min on Waters equipment using a radial compression module fitted with a C_{18} column (25 × 2.5 cm Novapak 7 μm particle size). Solvent A was 1 g of TFA in 1 L of HPLC grade water, and solvent B was 1 g of TFA mixed with 100 mL of water then brought to 1 L with acetonitrile. A 50 mL sample loop was used, and UV monitoring was at 229 nm. Appropriate linear gradients (usually 0.5%/min) were applied to permit resolution and isolation of components. Peaks were collected manually at the detector exit, organic solvent was removed at room temperature on a rotary evaporator, and product was recovered by lyophilization. Analytical HPLC was performed with the same solvents, but a C_8 column was used (250 × 4 mm Nucleosil 300A 5μm particle size) at a flow rate of 0.6 mL/min, monitoring at 214 nm. Generally, after injection, 100% A was maintained for 5 min, whereupon a linear gradient of 2%/min was applied to 100% B.

Mass Spectrometry. Electrospray ionization mass spectrometry was performed on a Trio 2000 instrument or a Platform II instrument (both from Micromass, Manchester, England) using a solvent consisting of acetonitrile/water 1:1 acidified with either acetic acid (1%, Trio) or formic acid (0.2%, Platform). The infusion rate was either 2 (Trio) or

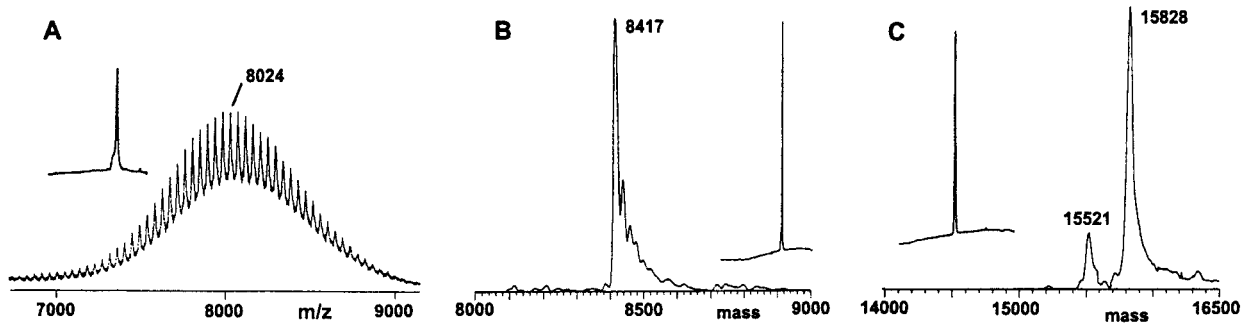

Figure 4. Analytical HPLC traces (upper traces, A_{214nm} versus time) and mass spectra (lower traces, vertical axis is relative intensity) of EMP dimers. (A) Dimer made with commercial PEG linker, MALDI-TOF mass spectrum; horizontal axis is *m/z*. (B) Dimer made with PEG-based polyamide linker, electrospray ionization; mass spectrum transformed to a true mass scale. (C) Tetrameric construct, electrospray ionization mass spectrum transformed to a true mass scale.

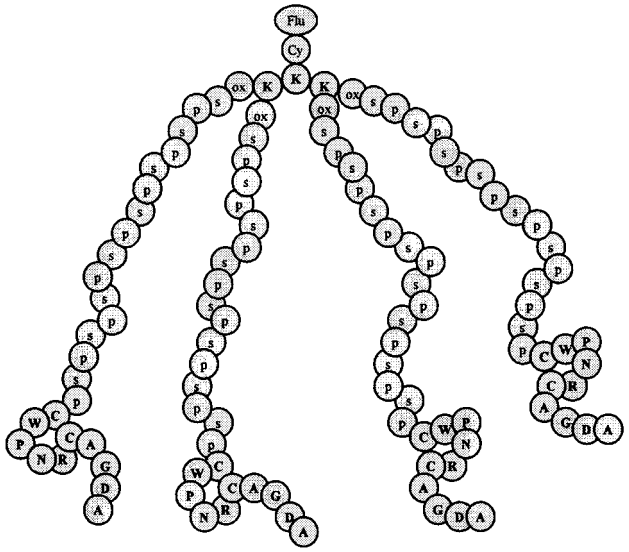

Figure 5. Schematic structure of a tetrameric construct: Flu, fluorescein; Cy, cysteamine linker; K, diacylated lysyl residue; ox, oxime linker; s, $COCH_2CH_2CO$; p, $NH\text{-}CH_2CH_2CH_2\text{-}(OCH_2CH_2)_3\text{-}CH_2\text{-}NH$; ADGACRNPWC, one-letter code for amino acid residues of the phage-derived peptide (a disulfide bond exists between Cys residues).

10 (Platform) μL/min. External calibration was with a solution of horse heart apomyoglobin. MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager Elite machine (Perkin-Elmer) equipped with delayed extraction. External calibration was performed using the mixture of peptides supplied by the manufacturer.

General Procedures. Standard techniques of solid-phase synthesis[8] were used both with a suitably programmed ABI 430A instrument and a home-built machine. For polyamides the following methods were employed. Acylation: amino resin (~0.3 mmol) was acylated with succinic anhydride (4 mmol in 8 mL of dimethylformamide, DMF, Burdick and Jackson High Purity grade containing 0.5 M hydroxybenzotriazole, HOBT, to which 0.4 mL of diisopropylethylamine was added) by vortex mixing at room temperature for 30 min. If an unloaded hydroxyl resin was used (e.g., Sasrin from Bachem, Bubendorf, Switzerland), 4-(dimethylamino)pyridine (0.5 M) replaced the HOBT and the coupling was repeated once. After the resin was drained and washed with DMF, the Kaiser ninhydrin test showed (in the case of an amino resin) that acylation was complete (otherwise the acylation step was repeated). Activation: the free carboxyl groups were activated with carbonyldiimidazole (from Fluka, Buchs, Switzerland, 8 mmol in 8 mL of DMF) for 30 min with vortex mixing. It is preferable to employ a large excess of activating agent to activate essentially all of the resin-bound carboxyl groups and so avoid accumulation of chains lacking a repeat unit. Aminolysis: after draining the activating agent and washing the resin with DMF, the resin-bound mixed anhydride or imidazolide (Figure 1) was aminolysed with the PEG-based diamine (e.g., 4,7,10-trioxa-1,13-tridecanediamine, Fluka, 4 mL of diamine premixed with 4 mL of DMF and made 0.5 M in HOBT) for 60 min with vortex mixing. After thorough washing with DMF, the Kaiser test showed the characteristic blue color and the amino resin was ready for the next acylation/activation/aminolysis cycle. When a more hydrophobic diamine such as 1,6-diaminohexane was being used, *N*-methylpyrrolidone replaced DMF for all three steps (acylation, activation, and aminolysis). Products were cleaved from the resin using the appropriate standard techniques for the resin in question and precipitated with cold diethyl ether (at its melting point, a small amount of solid ether was present). After being washed with cold ether, the product was dissolved generally in 50% aqueous acetonitrile, filtered to remove resin, rotary evaporated at room temperature, and recovered by lyophilization. The crude H-("Dah"-"succ"-"PEG"-"succ")$_4$-OH was dissolved in 40% acetic acid rather than 50% acetonitrile.

Enzymatic Digestion. Portions of peptide or polyamide (1 mg/mL in 1% w/v ammonium bicarbonate solution) were treated separately with trypsin, chymotrypsin, and elastase (enzyme/substrate 1:100 w/w, enzyme stock solutions freshly prepared at 1 mg/mL in water with enzymes from Sigma, St Louis) at 37 °C. Aliquots were withdrawn at intervals (1, 4, and 24 h) and analyzed by reversed-phase HPLC, monitoring at 214 nm.

Synthesis of EMP Peptide. The monomeric peptide[3] carrying an N-terminal aminooxyacetyl (AoA) group[9] *NH_2OCH_2CO*-GGLY-ACHMGPMTWVCQPLRG-*amide* was synthesized by Boc chemistry on methylbenzhydrylamine resin (MBHA resin, Perkin-Elmer ABI division) using an ABI 430A instrument. The product was cleaved from the resin, deprotected with HF (containing 5% *p*-cresol, 0 °C for 60 min), precipitated with diethyl ether, and purified by preparative HPLC. The mass found by electrospray ionization mass spectrometry was 2249.1, close to the calculated value of 2249.6. Disulfide bond formation was achieved as follows: 45 mg of peptide (20 μmol) was dissolved in 45 mL of water and the pH brought to 7 (glass electrode) with 1% ammonia solution. A solution of hydrogen peroxide (25 equiv, 544 μL of a 3% solution) was mixed in. After 30 min at room temperature the reaction was stopped with 450 μL of acetic acid and the solution injected immediately onto preparative HPLC: yield 23 mg of oxidized peptide, mass found 2247.4, calcd 2247.7.

EMP Dimer with Commercial PEG Linker. Oximation with commercial PEG dialdehyde was as follows: 2.4 mg of EMP peptide (1.06 μmol; 1.2-fold excess over aldehyde groups) was dissolved in 0.2 mL of water and added to 1.5 mg (0.44 μmol) of PEG-dialdehyde (ALD-3400, average relative molecular mass 3400, Shearwater Polymers, Huntsville, AL) dissolved in 0.45 mL of acetate buffer (0.15 M, counterion sodium, 6 M in guanidine hydrochloride). After 16 h in the dark at room temperature, the product was isolated by preparative HPLC and characterized by analytical HPLC and MALDI-TOF mass spectrometry (results shown in Figure 4A).

EMP Dimer with PEG-Based Polyamide Linker. To make the PEG-polyamide linker, Boc-Ser(Bzl)-("PEG"-"succ")$_6$-OH was prepared on Sasrin resin using the techniques described above (and see mass data Table 1). The material was cleaved from the resin with 1% trifluoroacetic acid (TFA) in dichloromethane as recommended by the

resin manufacturer and purified by preparative HPLC. To a solution of Boc-Ser(Bzl)-(PEG-succ)$_6$-OH (9.7 mg, 4.4 μmol) in *N*-methylpyrrolidone (NMP) was added HATU reagent (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 1.6 mg, 4.4 μmol) and a solution of DIEA diluted 10-fold in NMP (15 μL, 8.8 μmol) with stirring. After 5 min corresponding to the preactivation of the carboxylic acid group, the active ester was aminolysed with the PEG-based diamine (4,7,10-trioxa-1,13-tridecanediamine, "PEG", diluted 100 fold with NMP, 24 μL, 1.15 μmol) overnight. The symmetrical Boc-Ser(Bzl)-("PEG"-"succ")$_6$-"PEG"-("succ"-"PEG")$_6$-Ser-(Bzl)-Boc was directly purified by preparative HPLC (yield 7 mg, 1.6 μmol, 73%). The coupling agent HATU gave a better yield than the cheaper HBTU. The two Boc groups and benzyl groups were removed with the standard trifluoromethanesulfonic acid (TFMSA) cleavage procedure (300 μL of TFA for 4 min followed by addition of 30 μL of TFMSA for 25 min). The product was precipitated with cold diethyl ether (at its melting point, a little solid ether was present), washed three times with cold ether, and dried in a desiccator. The Ser residues of the deprotected linker Ser-("PEG"-"succ")$_6$-"PEG"-("succ"-"PEG")$_6$-Ser were oxidized to glyoxylyl functions (O═CH−CO) using standard techniques,[9] and the resulting dialdehyde linker was repurified by HPLC. A solution of the aminooxyacetyl-EMP peptide derivative with its disulfide bond formed (96 μL, 21.3 mM in 0.1 M acetate buffer, pH 4.0, counterion sodium; a 1.5-fold excess over aldehyde groups) was mixed with the dialdehyde (200 μL, 3.5 mM in water) and left to react at room temperature for 48 h. The dimeric product was isolated by reversed-phase HPLC with a yield of 1.2 mg (20%) and characterized by analytical HPLC and electrospray ionization mass spectrmetry (results shown in Figure 4B).

Tetrameric Construct. The monomeric peptide carrying an aminooxyacetyl (AoA) group *H*-ADGACRNPWC-("PEG"-"succ")$_8$-Lys-(AoA)-*amide* was synthesized on Fmoc-Lys(Mtt)-MBHA resin (0.5 mmol) by standard techniques.[8,9] Fmoc protection was removed, 8 cycles of "PEG"-"succ" were performed, and then Boc-Cys(4-MeBzl) was coupled to the terminal amino group. Mtt protection was removed (multiple rounds of 1% TFA in dichloromethane until the solution was no longer yellow) and the amino group acylated with Fmoc-Osu (2 mmol in 5 mL of DMF with *N*-methylmorpholine as base). The peptide chain was extended out to the N-terminus by Boc chemistry. Fmoc protection was removed with 10% piperidine in DMF for 7 min since stronger conditions led to succinimide formation at Asp-Gly. This piperidine treatment removes formyl protection from the Trp indole. The amino group was acylated with Boc-AoA-Osu (0.6 mmol in 5 mL of dry DMSO with *N*-methylmorpholine as base, not more forcing conditions or acylation of Trp occurs). After cleavage from the resin and deprotection with HF, purification by HPLC, and disulfide bond formation with hydrogen peroxide as described for the EMP peptide, the product was isolated by HPLC and characterized by analytical HPLC and electrospray ionization mass spectrometry: mass found 3709.1, theoretical 3709.4. A tetravalent template Ser-Lys(Ser)-Lys-(Ser-Lys(Ser))-NHCH$_2$CH$_2$SH was prepared by standard techniques starting with Fmoc-cysteamine-Sasrin resin (Bachem) and coupling two rounds of Fmoc-Lys(Fmoc) then Boc-Ser(Bu^t). After deprotection and cleavage (270 mg of resin, 2.7 mL of TFA, 30 min, filtered, evaporated under a nitrogen stream to small volume, precipitated with cold diethyl ether), product was purified by HPLC. The thiol group was alkylated as follows: to a solution of phosphate buffer (2.5 mL, 0.25 M phosphate, pH 7.0, 1 mM in EDTA) was mixed in first 1 mL of purified template (10 mM in water), and immediately afterward, 5-iodoacetamidofluorescein (Fluka, 1 mL, 10 mM in DMF) was mixed in. After 90 min in the dark, the fluorescein-labeled template was purified by HPLC: yield 7.8 mg, 66%. After oxidation of the Ser residues[9] and isolation by HPLC, the tetra-glyoxylyl fluorescein-labeled template (28 μL, 3.4 mM in water) was oximated with *H*-ADGACRNPWC-("PEG"-"succ")$_8$-Lys(AoA)-*amide* (disulfide form, 66 μL, 6 mM in 0.1 M acetate buffer, pH 4.0, counterion sodium, 1.04-fold excess over each aldehyde group present) at room temperature for 48 h. Product was purified by HPLC and characterized by analytical HPLC and electrospray ionization mass spectrometry (results shown in Figure 4C).

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