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Iterative reducible ligation to form homogeneous penicillamine cross-linked polypeptides

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ABSTRACT

The syntheses of homogeneous penicillamine disulfide cross-linked polypeptides are reported. Dodecapeptides containing N-terminal, C-terminal, or N- and C-terminal Pen were serially ligated into 36 amino acid polypeptides linked through Cys-Pen, Pen-Cys, or Pen–Pen disulfide bonds. Critical to the syntheses was the incorporation of thiazolidine masked Cys and Pen as the N-terminal residues and selective hydrolysis with silver trifluoromethanesulfonate in acidic aqueous conditions to generate a free thiol for subsequent ligation. This approach allows the synthesis of homogeneous disulfide cross-linked polypeptides that have different reductive stabilities and have application in gene delivery by undergoing a tempered reductive triggered release of DNA.

Fundamental to the success of a gene delivery peptide or polymer is the ability to bind and protect plasmid DNA from serum nucleases.[1–3] The peptide-DNA polyplexes must be stable during systemic circulation to allow the polyplexes to concentrate in the desired cells, but disassemble after cellular uptake to permit transcription of the delivered plasmid. The assembly of multiple gene delivery peptides through disulfide bonds results in transiently stable reducible polypeptides. Disulfide bonds are relatively stable in serum but are readily cleaved upon entry into a cell due to the reducing environment created by high levels of glutathione.[4] Several reports have demonstrated that reducible polypeptides mediate higher levels of gene expression compared to nonreducible systems.[5–10] While higher expression was observed, the random polymerization of Cys-flanked peptides resulted in heterogeneous product mixtures that hindered the further optimization of the polypeptides. Only through the controlled ligation of individual peptides can the amount and location of each component peptide be optimized in the reducible polypeptide.

Recently we introduced a novel iterative reducible ligation methodology of generating homogeneous disulfide cross-linked polypeptides.[11] An N-terminal Cys residue was masked as an Fmoc-thiazolidine, which could be selectively converted to a free Cys in the presence of a disulfide bond without scrambling when silver trifluoromethanesulfonate was used to promote thiazolidine hydrolysis in acidic aqueous conditions. This method avoided the use of acetamidomethyl protected Cys previously reported for the generation of reducible polypeptides, permitting a milder synthesis avoiding the use of concentrated TFA and sulfenyl chlorides that may not permit stoichiometric deprotection of gene delivery peptides containing N-glycans and PEG.[12–17]

In addition to optimizing the amount and location of each component within the polypeptide, the strength of the disulfide bonds is another parameter that can be optimized within a gene delivery polypeptide.[7,18] Increasing the steric hindrance on the carbon bearing the sulfur increases the resistance of the disulfide bond to cleavage.[19,20] Penicillamine (Pen), a Cys like amino acid with β,β-dimethyl substitution, can readily be incorporated into gene delivery peptides through solid phase peptide synthesis and has been shown to form more reductively stable disulfide bonds.[21–24] Gene delivery polypeptides conjugated through disulfide bonds of differing strength may result in the tempered release of the DNA cargo. Endolytic fusogenic peptides that must function immediately upon cellular entry could be attached through readily cleavable Cys–Cys bonds, while other peptides such as nuclear localization signals could be linked through more stable Pen-Cys or Pen–Pen bonds to allow continued association with the plasmid DNA. Due to our previous success at generating homogeneous reducible polypeptides with thiazolidine mediated iterative reducible ligation and the prior use of thiazolidine masked penicillamines in peptide synthesis,[25,26] we explored if a Pen could be incorporated to generate homogeneous polypeptides.

Here we report the synthesis of model polypeptides ligated through disulfide bonds while systemically replacing Cys with Pen (Fig. 1). The synthesis of Cys containing polypeptide **1** has previously been reported;[11] this communication describes the chemistry to synthesize polypeptides containing N-terminal Pen (**2**), C-terminal Pen (**3**), and N- and C-terminal Pen (**4**).

The synthesis of N-terminal Pen polypeptide **2** was analogous to the previous report of the synthesis of **1** (Scheme 1). A donor

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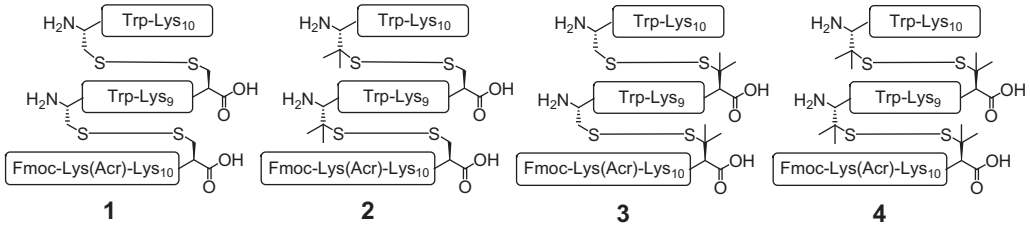
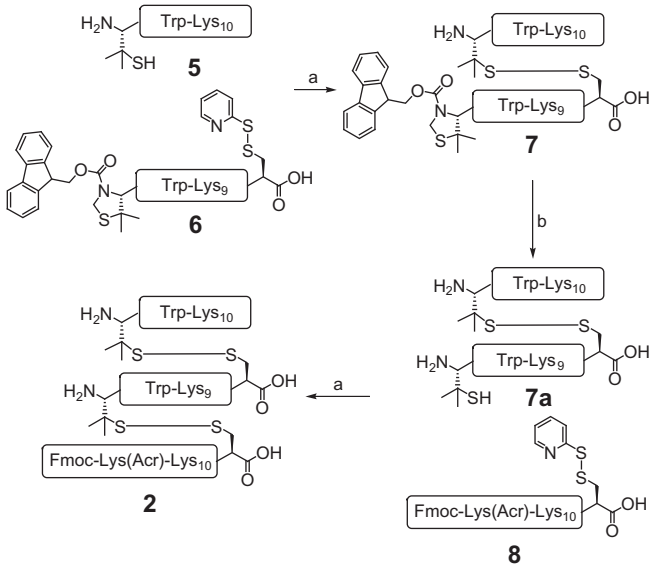
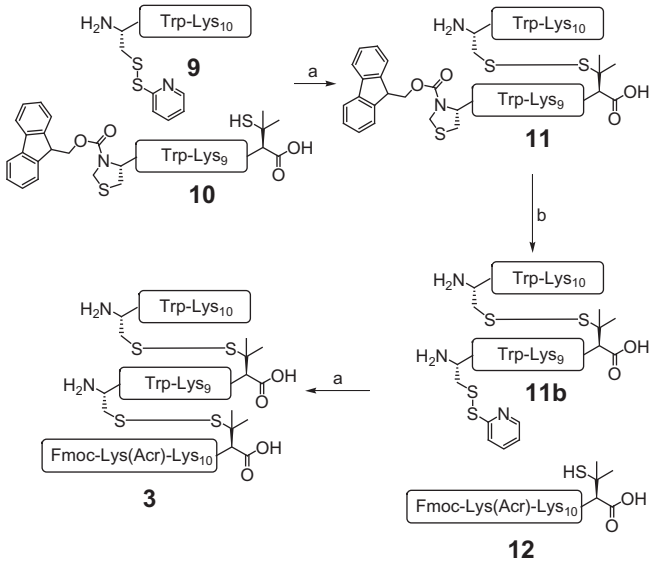


Figure 1. Polypeptides synthesized by thiazolidine mediated iterative reducible ligations. Polypeptide **1** contains N- and C-terminal Cys, **2** contains N-terminal Pen and C-terminal Cys, **3** contains N-terminal Cys and C-terminal Pen, and **4** contains N- and C-terminal Pen.



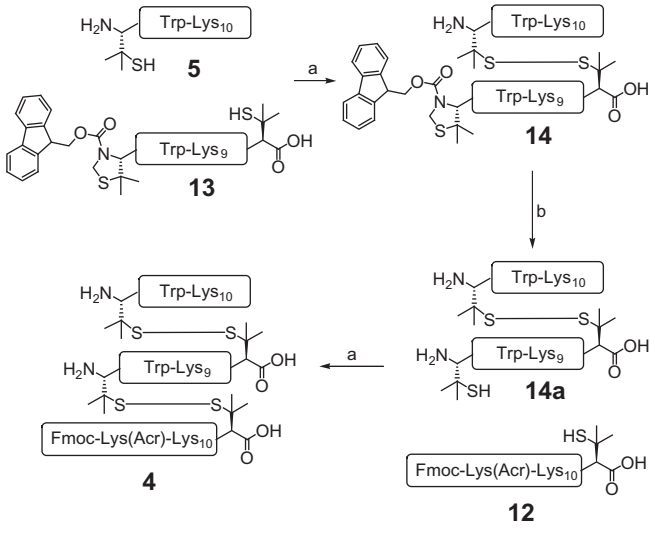
Scheme 1. Synthesis of polypeptide **2**: (a) 100 mM ammonium acetate, pH 5; (b) (i) 5 v/v % piperidine/DMF; (ii) 50 mol equiv AgOTf in 0.1 v/v % TFA.



Scheme 2. Synthesis of polypeptide **3**: (a) 100 mM ammonium acetate, pH 5; (b) (i) 5 v/v % piperidine/DMF; (ii) 50 mol equiv AgOTf in 0.1 v/v % TFA; and (iii) 10 mol equiv DTDP in 10:3 2 M acetic acid/isopropanol.

peptide containing a free sulfhydryl N-terminal Pen (**5**) was ligated with 1.5 mol equiv of an acceptor peptide activated as a *S*-2-sulfanylpyridine (**6**) to generate the polypeptide **7** containing a masked Pen as a Fmoc-5,5-dimethyl thiazolidine. Following preparative purification, **7** was recovered in 57% yield. Removal of the Fmoc and thiazolidine hydrolysis was achieved using 5 v/v % piperidine/DMF for 5 min and 50 mol equiv silver trifluoromethanesulfonate in 0.1 v/v % TFA for 1 h, respectively, to generate the donor polypeptide **7a**. After each deprotection step, the intermediate was purified by gel filtration chromatography using Sephadex G-10 resin eluted with 0.1 v/v % TFA. After purification, donor polypeptide **7a** was immediately reacted with acceptor peptide **8** to generate the desired polypeptide **2** containing N-terminal Pen in 50% preparative yield. These yields are comparable to the yields from the synthesis of **1**, reported as 49% and 54%.

The synthesis of C-terminal Pen polypeptide **3** required a minor modification of the synthetic protocol (Scheme 2). Initial attempts at activating a Pen sulfhydryl as an *S*-2-sulfanylpyridine or more reactive *S*-3-nitro-2-pyridylsulfenyl resulted in acceptor peptides with limited reactivity. Ten mole equivalents of a donor Cys peptide were required to form the mixed disulfide, similar to a previous report documenting the diminished reactivity of the 4-mercaptopyridine disulfide of Pen.[27] Due to the limited reactivity of these Pen products, the acceptor and donor peptides were inverted compared to the synthesis of **2** to maintain Pen on the donor peptide. Acceptor peptide **9** and donor peptide **10** were ligated, resulting in protected polypeptide **11** in 72% yield. The same two-step deprotection unmasked the N-terminal Cys that was subsequently activated with dithiodipyridine to generate the acceptor polypeptide **11b**. Reaction with donor peptide **12** resulted in the desired polypeptide **3** in 45% yield.



Scheme 3. Synthesis of polypeptide **4**: (a) 100 mM ammonium acetate, pH 5; (b) (i) 5 v/v % piperidine/DMF; (ii) 10 mol equiv AgOTf in 0.1 v/v % TFA.