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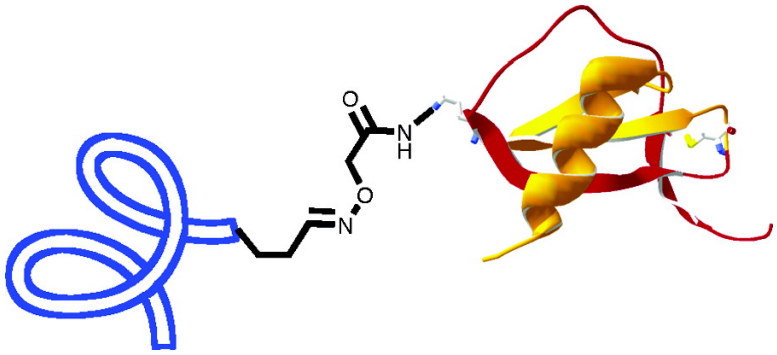

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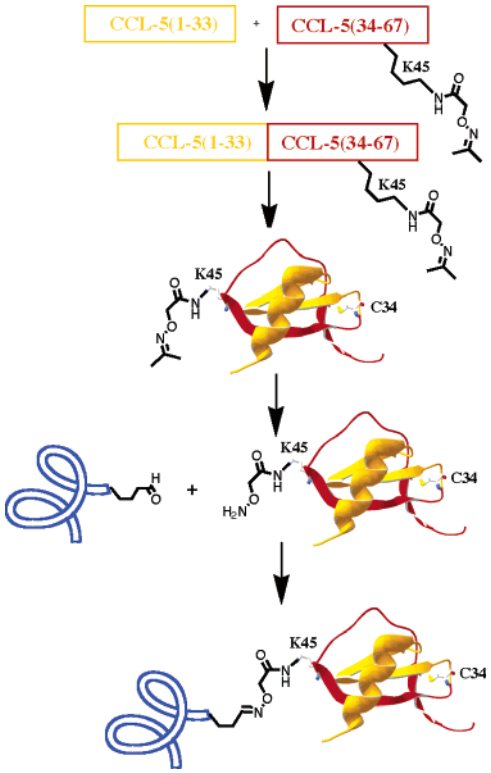
Site-specific attachment of covalent modifiers such as polymers using chemoselective ligation chemistries[1] is preferred over random attachment for the development of protein pharmaceuticals. In particular, oxime-forming ligation[2] has proven useful for the attachment of polymers to proteins or peptides since it is orthogonal to most protein functional groups and protein synthesis schemes.[1−3] Oxime bonds may be formed between an aminooxy group, such as aminooxyacetic acid (Aoaa), and an aldehyde or ketone functionality, such as glyoxylic, levulinic, or pyruvic acid. We have developed an improved synthetic strategy that allows effective protection of the aminooxy group during peptide synthesis, peptide ligation, and protein folding. Mild deprotection of the resulting proteins in solution occurs without interference with the native folded structure for subsequent specific attachment of a modifier such as a polymer. We applied this approach to the site-specific attachment of poly(ethylene glycol) (PEG) to a synthetic CCL-5 (RANTES) analogue. The objective was to attach a PEG polymer in the GAG binding site at position 45 to form CCL-5−PEG, to reduce aggregation[4] and to increase the circulating lifetime.

Incorporation of Aoaa functionalities into peptide building blocks is typically achieved by solid-phase assisted synthesis using a monoprotected carboxylic acid derivative such as Boc-Aoaa-OH, Fmoc-Aoaa-OH, or a derivative carrying a protected Aoaa functionality on the side chain such as Fmoc-Dpr(Boc-Aoaa)-OH.[1,5,6] However, a single protecting group for hydroxylamine functionalities does not provide complete protection due to their inherent nucleophilicity, potentially resulting in multiple acylations during peptide assembly and other potential side reactions, and lability under HF cleavage conditions.[5,6] Recently, a bi-protected aminooxy group has been developed to circumvent this problem, but use of this derivative results in increased hydrophobicity, increased cost, and resin cleavage lability.[6] A simple and cost-effective alternative is the protection of Aoaa with acetone to form isopropylidene Aoaa (Scheme 1). This derivative is prepared by simply dissolving commercially available aminooxyacetic acid hydrochloride in water and subsequent lyophilization with acetone without further purification. Even though this derivative was first described in 1936,[7] its use in peptide synthesis has been limited to date.[8]

We synthesized a PEGylated variant of the chemokine CCL-5 (RANTES) [9,10] using the strategy outlined in Scheme 1. The C-terminal peptide of CCL-5 [CCL-5(34−67)] was assembled by optimized Boc chemistry procedures,[11] and the lysine residue in position 45 was differentially protected with an Fmoc-group (see Figure S1 for details). After Fmoc removal, the isopropylidene Aoaa was coupled to the free amino group after activation with equimolar amounts of *N*-hydroxysuccinimide and diisopropylcarbodiimide in DMF to generate a noncoded isopropylidene Aoaa-lysyl residue.

The isopropylidene protecting group was fully stable to the coupling conditions, subsequent treatment with trifluoroacetic acid

Scheme 1. Synthetic Strategy for the Assembly of PEG-modified CCL-5 Using the Isopropylidene Protection Strategy[a]



[a] The locations of the ligation site (C34) and PEGylation site (K45) are indicated.

(TFA) to deprotect the N-terminal amine of the peptide, treatment with anhydrous hydrogen fluoride during peptide deprotection and cleavage, and purification by RP-HPLC in the presence of 0.1% TFA (see Supporting Information for sequence information and more detailed procedures). The purified C-terminal peptide was then linked to the N-terminal α-thioester peptide [CCL-5(1−33)] by native chemical ligation at 1 mM peptide concentration in 6 M guanidinium, 100 mM phosphate pH 7.5 in the presence of 0.5% thiophenol as a catalyst.[12] After ligation and purification of the linear peptide by RP-HPLC, the protein was folded into the correct tertiary structure in the presence of denaturant under reducing conditions employing a redox couple to adjust the redox potential.[13]

After folding and subsequent purification, the isopropylidene group was removed by treatment with 1 M methoxylamine for 1 h. Complete removal of the isopropylidene group was confirmed by ESI-MS and RP-HPLC (Figure 1A). After desalting by RP-HPLC, the protein was reacted with a commercial 20 kDa propionaldehyde-derivatized methoxy-poly(ethylene glycol) (PEG). The reaction was complete in 1 h.

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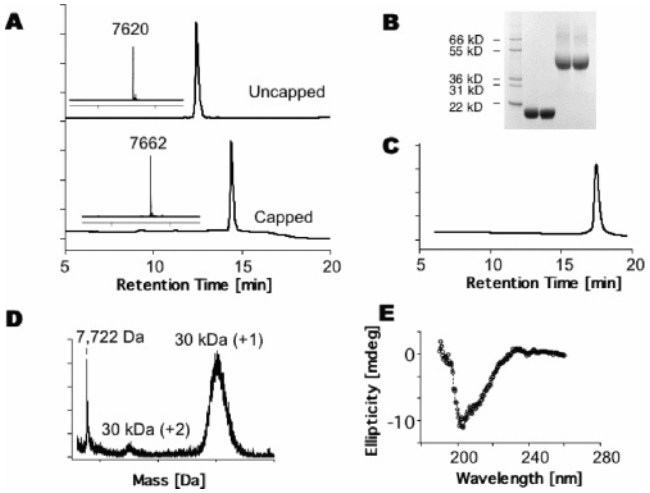

Figure 1. (A) Folded CCL-5 prior (bottom) and after (top) treatment with methoxylamine to remove the isopropylidene protecting group (42 amu) (B) Reduced and non-reduced SDS-PAGE of uncapped CCL-5 and CCL-5 with 20-kDa mPEG propionaldehyde. Lane 1: MW markers. Lane 2+3: uncapped, folded CCL-5. Lane 4+5: crude reaction mix of PEG-modified CCL-5. (C) RP-HPLC of PEG-modified, purified CCL-5. (D) MALDI mass spectrum of PEG-modified CCL-5. (E) Far-UV CD spectrum of PEG-modified CCL-5.

Table 1. Activity of Wild-Type and PEG-modified CCL-5 Variant in an HIV in Vitro PBMC Assay

compound	IC_{50} (nM)	IC_{90} (nM)
CCL-5	0.62	4.87
PEG−CCL-5	0.52	2.36

An SDS-PAGE gel and MALDI mass spectrum (Figure 1,B and D) identified the target protein−polymer construct. The apparent presence of free protein in the MALDI mass spectrum is due to the documented cleavage of oxime bonds by UV−laser excitation.[14] Purification by ion-exchange chromatography yielded a purified product of high overall purity (>95%) as judged by RP-HPLC (Figure 1C), and correct secondary structure was determined by CD (Figure 1E). A tryptic digest of the resulting protein was consistent with dominant PEG attachment at the desired site in position 45 (Figure S3). The overall assembly yield for the synthesis was 20% based on peptide starting materials.

The anti-HIV activity of CCL-5-PEG in PBMCs was compared to that of the unmodified parent molecule, PSC RANTES, prepared analogously, but without the chemoselective linker and PEG attachment, using the clinical HIV-1 primary isolate 92/US/712.[15] In this assay, the PEGylated variant was found to have comparable antiviral activity relative to that of the parent compound (Table 1). This result confirmed that Aoaa modification, deprotection, and PEG attachment in position 45 did not interfere with correct folding, tertiary structure, and antiviral activity. This is in marked contrast to typical results for the random modification of recombinant proteins with polymers such as PEG, where the in vitro activity of the modified protein can drop dramatically upon attachment of large polymers.[16]

The approach presented for the reversible protection of Aoaa should be broadly applicable to the chemical synthesis of modified peptides and proteins, in particular for the site-specific attachment of carbonyl-carrying polymers and sugars. In contrast to previous assembly strategies for attachment of polymers to chemically synthetic proteins that typically used polymer attachment at the peptide level,[1a] this new approach permits the site-specific attachment at any stage of the synthesis. This is particularly advantageous for the attachment of heterogeneous polymers such as PEGs or oligosaccharides, since their attachment during synthesis or folding presents significant purification and characterization problems due to their heterogeneity (of molecular mass and structure). Further studies assessing the oligomerization behavior, pharmacokinetic properties and anti-HIV efficacy of PEGylated CCL-5 are ongoing.

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Supporting Information Available: Experimental details and further characterization for CCL-5-PEG. This material is available free of charge via the Internet at http://pubs.ACS.org.

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