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Synthesis of Peptide Bioconjugates

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Summary

Bioconjugates play an important role in several fields of biomolecular and biomedicinal sciences. Protein/polypeptide-based conjugates with covalently attached epitope peptides are considered as potential synthetic vaccine candidates and/or target antigens in affinity-based bioassays. This chapter describes the synthesis of two- and three-component bioconjugates using water-soluble branched chain polymeric polypeptides with multiple amino and/or carboxyl groups as macromolecular partners and oligopeptides as epitopes with small molecular mass. The synthetic procedures outline three major strategies for the incorporation of multiple copies of uniformly oriented peptide epitopes. In the first example, chloroacetylated polypeptide is conjugated with SH-peptide to form a thioether linkage. Second, two independent oligopeptides are introduced into a macromolecule by amide and disulfide bonds, respectively. In the third example, a new procedure is reported for the formation of disulfide bridges by the use of Npys-modified polypeptide and SH-peptide.

Key Words: Peptide–macromolecule conjugate; amide bond; thioether bond; disulfide bridge; antibody epitope; T-cell epitope peptide.

1. Introduction

Bioconjugates play an important role in several fields of biomolecular and biomedicinal sciences. Protein/polypeptide-based conjugates with covalently attached epitope peptides are considered as potential synthetic vaccine candidates and/or target antigens in affinity-based bioassays (e.g., ELISA, BIACORE) ***(1,2)***. Drugs such as daunomycin, methotrexate, and a GnRH analog coupled either to monoclonal antibodies or to synthetic linear or branched polypeptides

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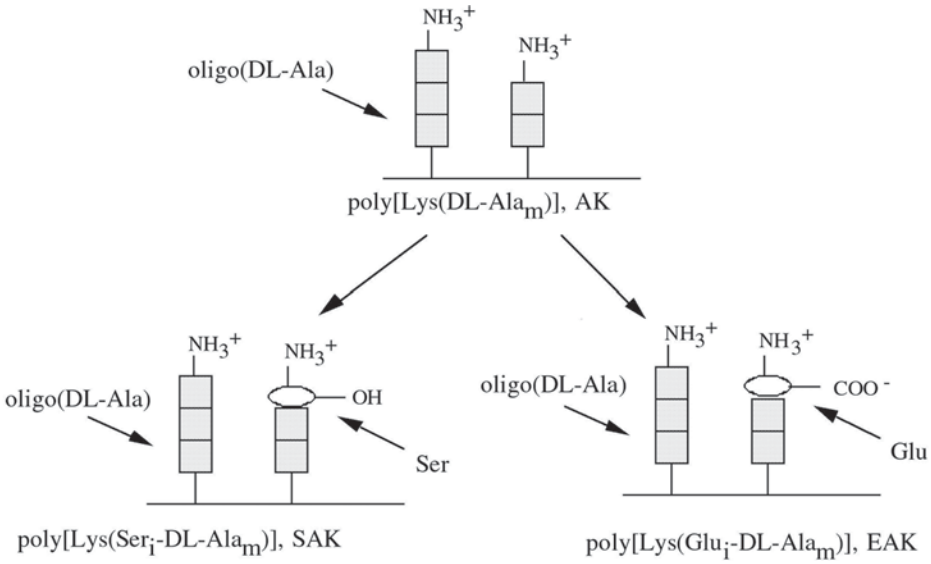

Fig. 1. Schematic representation of branched-chain polymeric polypeptides.

exhibit altered biodistribution and decreased nonspecific toxicity (e.g., cardiotoxicity). Consequently, the therapeutic effects of such drugs can be markedly improved by conjugation ***(3–5)***. Bioconjugates could be classified either by the number and size of components involved or by the type of covalent linkage applied between the partner entities. This chapter describes the synthesis of two- and three-component bioconjugates combining water-soluble branched-chain polymeric polypeptides as the macromolecular partners of small molecular mass bioactive oligopeptides. Collectively, the bioconjugates presented possess not only the most frequently utilized amide and/or disulfide linkage, but also the thioether bond. The synthetic procedures outlined could also be adapted to the preparation of conjugates containing macromolecular partners with similar functional groups (e.g., proteins and synthetic polymers).

Branched polypeptides developed in our laboratory with the general formula poly[Lys(X_i-DL-Ala$_m$)] (XAK), where $i < 1$, m approx 3, and X represents the side-chain terminal residue ***(6–8)*** will be used (**Fig. 1**) to illustrate the methods utilized for different conjugation procedures. Depending on the nature of the amino acid X, polypeptides exhibit polycationic (e.g., poly[Lys(DL-Ala$_m$)], poly[Lys(Ser$_i$-DL-Ala$_m$)], amphoteric (e.g., poly[Lys(Glu$_i$-DL-Ala$_m$), or polyanionic (e.g., poly[Lys(Ac-Glu$_i$-DL-Ala$_m$)]) character under physiological conditions (pH 7.3 in 0.15 *M* NaCl).

These macromolecules were applied for the synthesis of B-cell epitope peptide conjugates to be used as target antigens for the specific and sensitive detection of mucin 1 glycoprotein-specific antibodies ***(9)***, of T-cell epitope conjugates with peptides derived from 16/38-kDa proteins from *M. tuberculosis* ***(10–13)*** and of peptides containing HSV-neutralizing antibody epitope(s) ***(14,15)***. In these conjugates the oligopeptide component(s) was/were coupled in multiple copies in a uniformly oriented manner. By these constructs we have shown that (1) peptides attached to carriers preserved their biological function (e.g., antibody binding, T-cell recognition, specific immunogenicity, interaction with phospholipid mono- or bilayers), and (2) the physicochemical properties of the polypeptide component have a marked influence on the attached epitope-related activities ***(1,13,16)***.

2. Materials

1. Amino acid derivatives, reagents for coupling and analysis, organic solvents, buffers, and HPLC eluents used for the preparation of oligo- and polypeptides are identical with those described in Chapter 4.
2. Branched-chain polypeptides poly[Lys-(X_i-DL-Ala$_m$)], where X = Glu, Ser, or Boc-Cys(Npys)Opfp, were produced in our laboratory as described.
3. Cross-linking compound]3-(2-pyridyldithio) propionic acid *N*-hydroxy-succinimide ester (SPDP) (Sigma, Poole, UK).
4. Benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Fluka, Buchs, Switzerland).
5. Chloroacetic acid pentachlorophenyl ester (ClAc-Opcp) (Fluka).
6. DL-dithiothreitol (DTT) (Fluka).

3. Methods

Abbreviations used in this chapter follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature ***(17)*** in accordance with the recommended nomenclature of graft polymers ***(18)***.

3.1. Synthesis of Branched-Chain Polymer Polypeptides

The synthesis of branched polypeptides used for the preparation of all conjugates described in this account was published in detail earlier ***(6–8)***. Briefly, poly[Lys] was prepared by the polymerization of N^{α}-carboxy-N^{ε}-benzyloxycarbonyl-lysine anhydride. After cleavage of the protecting groups, poly[Lys (DL-Ala$_m$)] (AK) was produced by grafting of short oligomeric DL-Ala side chains onto the ε-amino groups of poly[Lys]. Benzyloxycarbonyl-protected amino acid derivatives (Z-Ser-OPcp and Z-Glu(OBzl)-OPcp) were coupled to the end of the side chains of AK by the HOBt-catalyzed active ester method. Blocking groups were removed completely with HBr in glacial acetic acid, as

confirmed by UV spectroscopy at 254 nm, resulting in poly[Lys(X_i-DL-Ala$_m$)] (XAK) polymers (where i ≤ 1 and m ≈ 3, *X* = Ser or Glu). The amino acid composition of polypeptides as well as peptide conjugates were determined by amino acid analysis using a Beckman (Fullerton, CA) model 6300 amino acid analyzer. Prior to the analysis, samples were hydrolyzed in 6 *M* HCl in sealed and evacuated tubes at 110°C for 24 h. The size and molecular weight of polymers were calculated from the amino acid composition and the sedimentation analysis of poly[Lys] ***(19)***.

3.2. Synthesis and Characterization of Epitope Peptides

Peptides used in these studies for conjugate synthesis were prepared by Boc/Bzl strategy on MBHA resin or by Fmoc-based chemistry on Rink-amide resin as described in Chapter 4. For incorporation of SH group at the N-terminal (C^{91}SEFAYGSFVRTVSLPVGADE^{110}) or C-terminal position (H-^{9}LKNleADPNRFRGKDL^{22}C-NH_2, [Nle^{11}]-9-22-Cys) or in a central position (H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH_2), a Cys derivative, Boc-Cys(Meb)-OH, was added to the native sequence. After the removal of the peptides from the resin the crude products were purified by RP-HPLC and the purified samples were characterized by analytical HPLC, amino acid analysis and mass spectrometry as detailed in Chapter 4.

3.3. Two-Component Conjugate Containing Multiple Copies of Uniformly Oriented Antibody Peptide Epitope

Synthetic oligopeptides comprising linear or continuous topographic B-cell epitope sequences might be considered as specific and small-size antigens. It has been demonstrated that the avidity and specificity of antibody binding could be altered by conjugation to macromolecules or by modification in the flanking regions. However, no systematic studies have been reported to describe the effect of different carrier macromolecules in epitope conjugates. To this end the influence of carrier structure and topology on the antibody recognition of covalently attached herpes simplex virus type 1 glycoprotein D (HSV-1 gD)-related epitopes have been studied by comparing the monoclonal antibody binding properties of a new set of conjugates ***(20)*** with branched-chain polypeptide, poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK), tetratuftsin analog (H-[Thr-Lys-Pro-Lys-Gly]$_4$-NH_2) sequential oligopeptide carrier (SOC$_n$), multiple antigenic peptide (MAP), and keyhole limpet hemocyanine (KLH). In these novel constructs peptide ^{9}LKNleADPNRFRGKDL^{22} ([Nle^{11}]-9-22) representing an immunodominant B-cell epitope ***(21,22)*** was conjugated to polypeptides through thioether bonds (**Fig. 2**).

First, we prepared a derivative of polypeptide SAK possessing chloroacetyl groups in multiple copies at the α-amino groups of the branches. This compound

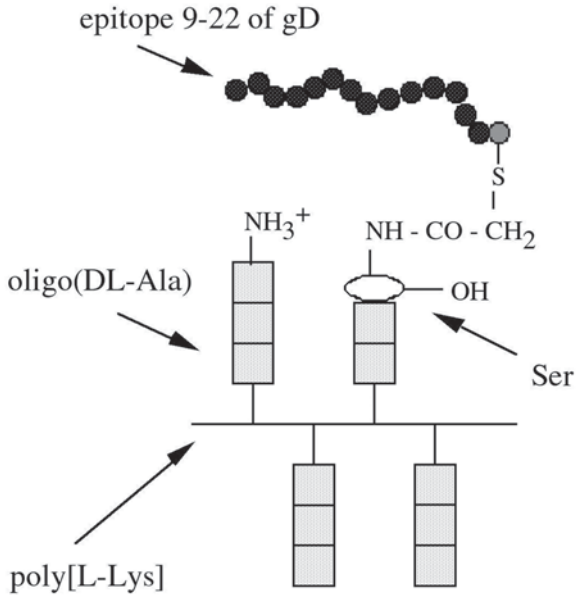

Fig. 2. Schematic structure of branched polypeptide (SAK) conjugate with multiple copies of peptide epitope corresponding to the 9-22 region of glycoprotein D of HSV-1.

was conjugated with epitope peptides possessing Cys at C-terminal position as outlined in **Fig. 3**. Binding data suggest that the chemical nature of the carrier and the degree of substitution have marked influence on the avidity of antibody binding.

3.3.1. Synthesis of Chloroacetylated-Branched Polypeptide poly[Lys(ClAc$_j$-Ser$_i$-DL-Ala$_m$)] (SAK(ClAc)$_j$)

80 mg (0.145 mmol) poly[Lys(Ser$_{0.9}$-DL-Ala$_{3.5}$)] HBr salt (SAK, DP_n = 60, $MW_{monomer}$ = 550) was dissolved in 1 mL deionized water and the solution was diluted with 4 mL DMF. Chloroacetyl groups were introduced at the N^{α}-amino group of Ser residues by chloroacetic acid pentachlorophenyl ester. Six different SAKs—ClAc-OPcp ratio (1:1, 1:0.8, 1:0.6, 1:0.5, 1:0.4 and 1:0.3 mol/mol; 50 mg, 40 mg, 30 mg, 25 mg, 20 mg, 15 mg ClAc-OPcp respectively) were used and the active ester was added to the polymer containing solution dissolved in 5 mL DMF. The reaction mixtures were stirred overnight at room temperature. The solution was filled in Visking tubes (cutoff 8000–12,000) and dialyzed for 2 d against 0.1% acetic acid and freeze-dried. According to Cl analyses, 46.5%, 45.9%, 48.5%, 41.3%, 30.1%, and 21.7% of the side chains were blocked by the ClAc group, respectively.

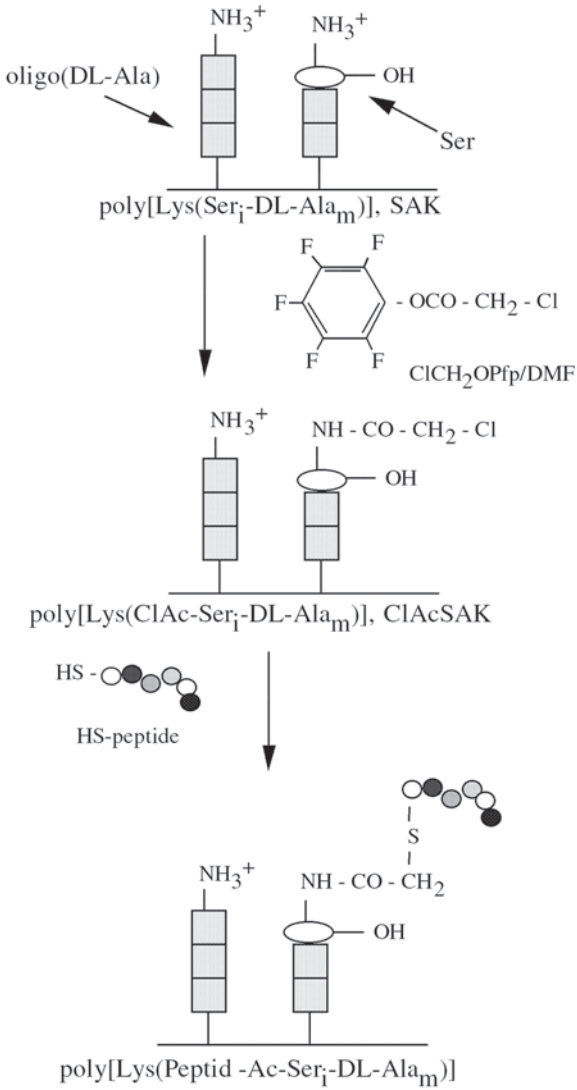

Fig. 3. Outline of the synthesis of ([Nle[11]]-9-22-Cys)-conjugate with chloroacetylated SAK by introduction thioether bond.

3.3.2. Conjugation of Peptide H-[9]LKNleADPNRFRGKDL[22]C-NH_2 ([Nle[11]]-9-22-Cys) With Chloroacetylated Branched Polypeptide poly[Lys($ClAc_j$-Ser_i-DL-Ala_m)] (SAK$(ClAc)_j$)

For the preparation of multivalent epitope conjugates, branched-chain polymeric polypeptide (SAK) was reacted with chloroacetic acid pentachlorophe-

nyl ester in DMF-water (9:1, v/v) solution. Four differently substituted carriers were applied. 15 mg of SAK$(ClAc)_{48.5}$, SAK$(ClAc)_{41.3}$, SAK$(ClAc)_{30.1}$, or SAK $(ClAc)_{21.7}$ (acetate salt form) were dissolved in 100 mL 0.1 *M* Tris-HCl buffer (pH 8.2). 1.2 eq (calculated for the chloroacetyl content of the polymers) of epitope peptide (23.4 mg, 20 mg, 14.8 mg, and 10.6 mg respectively) were added in two portions to the solution. The conjugation reaction continued for 24 h and was terminated by addition of an excess of Cys to block the unreacted chloroacetyl groups. Crude products were dialyzed against water to remove uncoupled peptide and Cys in Visking tubes (cutoff 8000–12,000) for 2 d. The average degree of substitution was calculated from the amino acid analyses. Depending on the input molar ratio, 44%, 22%, 9%, and 7% of the side chains was substituted by epitope peptide, respectively.

3.4. Three-Component Conjugate Containing Multiple Copies of Two Different, But Uniformly Oriented, T-Cell Peptide Epitopes

For the preparation of three-component conjugates containing multiple copies of two independently introduced and uniformly oriented T-cell peptide epitopes, an amphoteric branched chain polypeptide, poly[Lys-(Glu_i-DL-Ala_m)] (EAK), was used. This polypeptide, with free α-amino and γ-carboxyl groups at the end of the side chains, was conjugated with peptides representing two immunodominant regions of the 16-kDa and 38-kDa proteins of *Mycobacterium tuberculosis*, respectively ***(10–12)***. For conjugation, peptide ^{65}FNLWGPA FHERYPNVTITA^{83} (peptide 1) from the 38-kDa protein possessing free α-amino group at the N-terminal was used without modification, while the second epitope peptide originating from the 16-kDa protein was elongated with an *N*-terminal Cys [C^{91}SEFAYGSFVRTVSLPVGADE^{110}] (peptide 2). Consequently, an amide between the γ-COOH of EAK and α-NH_2 of peptide 1 and a disulfide bridge between the α-NH_2 of EAK and SH of peptide 2 were introduced (**Fig. 4**).

This synthetic strategy is outlined in **Fig. 5**. In the first step, the α-aminogroup of Glu in the side chain of EAK was modified by the heterobifunctional reagent SPDP to introduce protected SH groups into the polymer structure of [(SSP)EAK] ***(23)***. The extent of 2-pyridyl-disulfide group incorporation was determined spectrophotometrically from the amount of pyridine-2-thione released by reduction with DTT ***(10)***. The unmodified, free α-amino groups of Glu in the branches were blocked by acetylation using a mixture of acetic anhydride and imidazole. In the next step, peptide 1 was coupled to (Ac,SSP)EAK. This was achieved by the BOP reagent-based activation method in which the γ-carboxyl group of glutamic acid of (Ac,SSP)EAK was linked to the α-amino group of the phenylalanine residue in peptide 1 to provide isopeptide (γ,α type amide) bonding. After isolation of the product the presence of the 2-pyridyl-

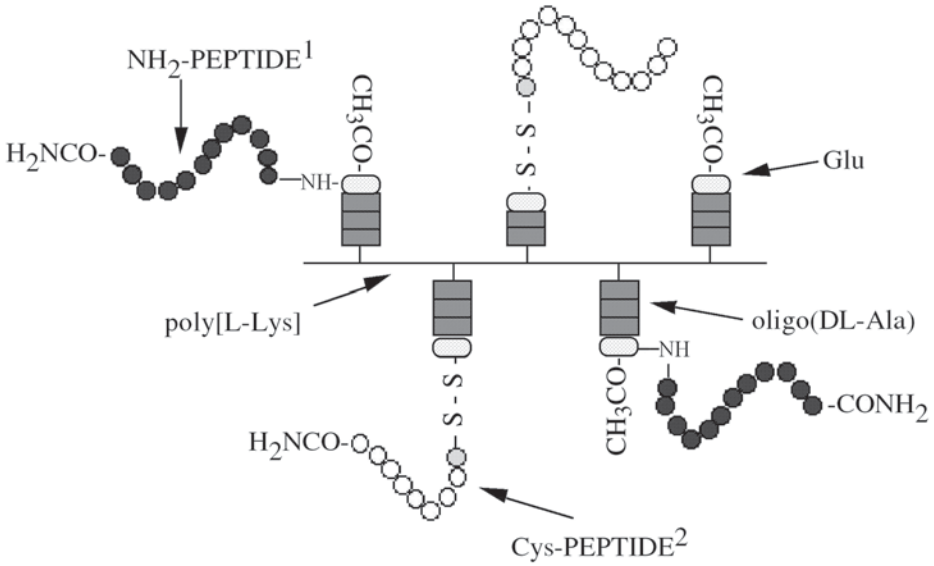

Fig. 4. Schematic structure of branched polypeptide (EAK) conjugate containing two different epitope peptides from *M. tuberculosis* proteins. ^{65}FNLWGPAFHERYPN VTITA^{83} (peptide 1) and C^{91}SEFAYGSFVRTVSLPV-GADE^{110} (peptide 2) are attached by amide and disulfide bonds, respectively.

disulfide groups was verified. In the last step, a disulfide bridge was introduced by the substitution of the protecting group of SH of the polymer by peptide 2 [C^{91}SEFAYGSFVRTVSLPVGADE^{110}]. The completion of the reaction between (Ac,SSP)E(peptide 1)AK and peptide 2 was spectrophotometrically determined.

In vitro T-cell immunogenicity data obtained with this conjugate using T cell hybridomas, lymph node cells from immunized mice, and human PBMC cultures from PPD-positive individuals indicated that both epitopes were efficiently recognized ***(10)***.

3.4.1. Synthesis of poly[Lys-(Ac$_y$,SSP$_x$-Glu$_i$-DL-Ala$_m$)], (Ac,SSP)EAK

100 mg (215 µmol) EAK was dissolved in 7.5 mL 0.1 *M* PBS (pH 8.0, adjusted with 1 *M* NaOH). 20 mg (64.0 µmol) SPDP (Sigma, Poole, UK) was dissolved in 2 mL abs. methanol, and added dropwise to the solution of the polypeptide. The reaction mixture was stirred for 30 min at room temperature and dialyzed for 24 h against distilled water (*see* **Note 1**).

110 mg (235 µmol) (SSP)EAK was dissolved in 1 mL distilled water and diluted to 5 mL with DMF at 4°C. In the meantime, 312 µL (3.3 mmol) acetic anhydride and 224 mg (3.3 mmol) imidazole were mixed in 1 mL DMF at 4°C

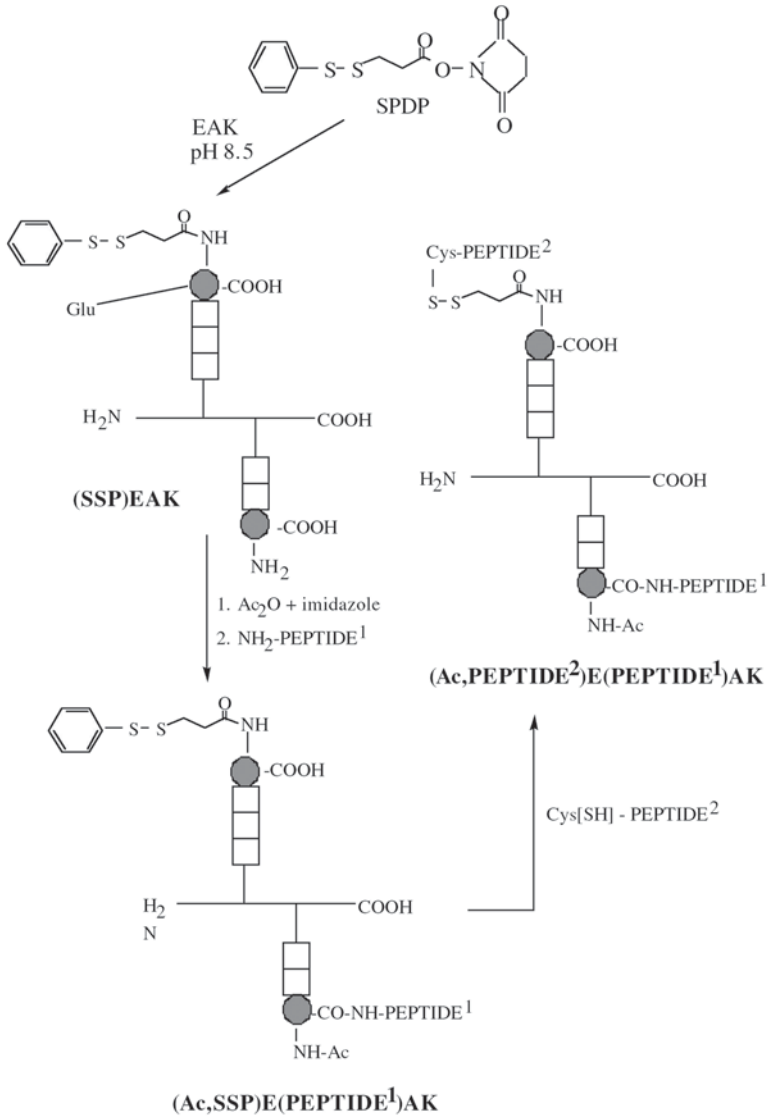

Fig. 5. Outline of the synthesis of conjugates containing two different epitope peptides, ^{65}FNLWGPAFHERYPNVTITA^{83} (peptide 1) and C^{91}SEFAYGSFVRTVSLPV GADE^{110} (peptide 2), and branched polypeptide EAK.

and stirred for 10 min. The two solutions were then mixed, and acetylation was continued for 2 h at room temperature. After dialysis against distilled water, the product was isolated by freeze drying (*see* **Note 2**).

3.4.2. Poly[Lys-(Ac_y,SSP_x)-Glu_i($Peptide_1$)-DL-Ala_m)], (Ac,SSP)E($Peptide_1$)AK

10 mg (19 μmol) (Ac,SSP)EAK (calculated on the basis of 19.3% 2-pyridyldisulfide group content, and 80.7% acetyl group content) was dissolved in 1 mL water and diluted five times with DMF. 8.9 mg (20 μmol) BOP reagent, 3.3 mg (25 μmol) HOBt, and 7 μL (40 μmol) DIEA were dissolved in 1 mL DMF. The activating mixture was added to the polymer solution and stirred for 20 min. 10 mg (4.5 μmol) peptide ^{65}FNLWGPAFHERYPNVTITA^{83} (peptide 1), representing 0.25 mol peptide/polymer side chain, was dissolved in 4 mL DMF at a concentration of 2.5 mg/mL DMF, and mixed with 4.5 μmol DIEA. The peptide solution was added to the preactivated polymer solution and allowed to couple for 24 h at room temperature under stirring. The solution was then dialyzed against distilled water for 48 h and the product was isolated by freeze drying.

3.4.3. Poly[Lys-(Ac_y,$Peptide_2$)-Glu_i($Peptide_1$)-DL-Ala_m)], (Ac,$Peptide_2$)E($Peptide_1$)AK

10 mg (Ac,SSP)E(Peptide1)AK conjugate (containing 3 μmol protected thiol groups) was dissolved in 1 mL distilled water. 10 mg (4.5 μmol) peptide C-^{91}SEFAYGSFVRTVSLPVGADE^{110} (peptide 2) dissolved in 1 mL PBS (pH 8.0, adjusted with 1 *M* NaOH) was added to the conjugate solution. The reaction mixture was stirred for 30 min at room temperature, followed by dialysis against distilled water for 48 h, and was freeze dried.

3.4.4. Analysis of Conjugates by RP-HPLC

The compositions of the conjugates were analysed by RP-HPLC with a Delta-Pak RP C_{18} column (7.8 mm × 30 cm) packed with spherical 15-μm silica of 300 Å pore size (Nixon Waters Ltd., Tokyo, Japan). We used an eluent system of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80/20 v/v (eluent B) using high-purity (Analar) solvents and distilled, deionized water. The elution gradient was 10–100% eluent B in 35 min. The injection volume was 100 μL containing 10 μg of conjugate, polymer, or peptide. Compounds were dissolved in eluent immediately before application and filtered through 0.45-μm Spartan 13 (Schleicher and Schuell, Dassel, Germany) filters. Free peptide or polymer polypeptide samples were run as standards and their retention times were determined. UV absorbance was monitored at a wavelength of 220 nm. All analyses were carried out at ambient temperature with a flow rate of 1.0 mL/min. As a control, in some cases, the HPLC profile of the peptide and conjugate mixture was also recorded.

3.5. Three-Component Conjugate Containing Multiple Copies of Two Uniformly Oriented Antibody Peptide Epitopes

We have described a novel approach in which the carrier polypeptide is modified by 3-nitro-2-pyridinesulfenyl (Npys)-protected Cys ***(24)***; this derivative has been used for the conjugation of Cys-containing epitope peptides to poly [Lys]-based branched polypeptides ***(25)***. Considering the stability of the Npys group in the presence of pentafluorophenol, the Boc-Cys(Npys)-OPfp derivative was selected for the introduction of Npys groups to the N-terminal of the branches of polypeptide backbones. This new class of Cys(Npys)-derivatized branched polypeptides is stable for a couple of months and suitable for the effective preparation of epitope-peptide conjugates possessing increased water solubility. The incorporation of epitope peptides depends on the number of Npys groups in polymers as well as on the presence/absence of Boc-protecting group on the Cys residue. A conjugate was prepared in which two epitopes (^{9}LKNleA DPNRFRGKDL^{22} and ^{276}SALLEDPVG^{284}) ***(21,26)*** of HSV gD type 1 joined by a dipeptide Acp-Cys spacer (where Acp is e-amino caproic acid) were attached through the SH function of a single peptide to the Cys(Npys) modified branched polypeptide (**Fig. 6**).

3.5.1. Synthesis of Branched Polypeptides With Cys(Npys)Residues

3.5.1.1. Synthesis of Boc-Cys(Npys)-OPfp

500 mg (1.33 mmol) Boc-Cys(Npys)-OH (Mw: 375) and 245 mg (1.33 mmol) pentafluorophenol were dissolved in 5 mL DCM. Then 274 mg (1.33 mmol) DCC dissolved in 2 mL DCM was added to the solution and the reaction mixture was stirred in an ice bath for 15 min, then for 2 h at RT. The reaction was followed by t.l.c. DCU was filtered out after cooling the solution to 0°C and the solvent was evaporated. The crude product was purified by crystallization from methanol. Yield: 594 mg (83%) (Mw: 541). Melting point: 96–99°C. R_f: 0.92 (ethyl acetate), R_f: 0.65 (chloroform-methanol= 3:1, v/v). Optical rotation: $[\alpha]_D$ = −62.2 (c = 1, DMF).

3.5.1.2. The Synthesis of Poly[Lys(Cys(Npys)$_f$-DL-Ala$_M$)] (CAK)

20 mg (57 µmol) poly[Lys(DL-Ala$_{3.11}$)] (AK, DP_n = 66, $MW_{monomer}$ = 349) dissolved in 0.5 mL deionized water was diluted with 3 mL DMF. An equivalent amount of Boc-Cys(Npys)-OPfp (31 mg) dissolved in 2 mL DMF was added to the solution (*see* **Notes 3** and **4**). The reaction mixture was stirred overnight at RT. The solvent was removed *in vacuo* (30°C) and the remaining product was washed with ether (containing 10% DCM) repeatedly to remove unreacted ester and pentafluorophenol. Yield: 35 mg (*see* **Note 5**). The Boc-group was

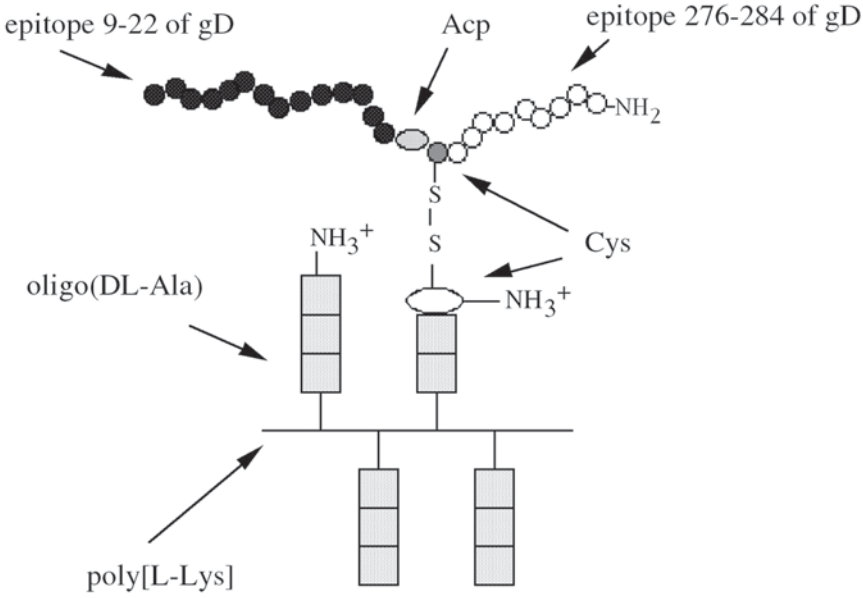

Fig. 6. Schematic structure of branched polypeptide (AK) conjugate with multiple copies of a single peptide containing epitopes corresponding to the 9–22 and to 276–284 regions of glycoprotein D of HSV-1. Two epitopes was joined first by an Acp-Cys dipeptide spacer and the SH function of the peptide was used for conjugation with Cys(Npys) with modified polypeptide.

removed by 5 mL TFA-water (95:5, v/v) at 0°C for 1 h in the presence of 5% *p*-cresol. The solution was concentrated *in vacuo*, then dissolved in water and dialyzed against 0.1% acetic acid solution for 2 d and freeze dried (yield: 27 mg). The average degree of substitution was calculated from the UV absorption of the Npys group at λ = 350 nm (ε = 3930/M/cm in DMF) (***27***).

3.5.2. Conjugation of Epitope Peptides to Branched Polypeptides With Cys(Npys) Residues

Boc-deprotected branched polypeptide with the Cys(Npys) moiety, poly [Lys($Cys_{0.27}$-DL-$Ala_{3.1}$)] trifluoroacetate salt (10 mg, 16.5 µmol), was dissolved in 10 mL 0.06 *M* phosphate buffer (pH 5.5) (*see* **Note 6**) 1.35 amount of H-LKNleADPNRFRGKDL-Acp-CSALLEDPVG-NH_2 (14.8 mg, 5.4 µmol), calculated from the Npys-content of polymer, was added to the reaction mixture. The final concentration was 1 mg/mL for the polymer component. The solution was stirred for 4 h at RT, transferred to a Visking tube (cutoff 8000–12,000), and dialyzed for 2 d against 0.1% acetic acid. No Npys content was detected by UV spectroscopy at λ = 350 nm in the conjugate after freeze drying (*see* **Note 7**).

The yield was 15 mg (86%). According to the amino acid analysis, 26% of side chains of branched polypeptide contained the bifunctional epitope peptide.

4. Notes

1. To determine the degree of 2-pyridyl-disulfide group incorporation, 400 μL of (SSP) EAK or (Ac,SSP)E(peptide1)AK solution was reacted with 200 μL DL-dithiothreitol (DTT) (2.5 mg/mL PBS), and after 5 min the absorbance of the released pyridine-2-thione was measured at λ = 343 nm, ε = 8080/M/cm ***(27)***.
2. The blocking of free amino groups by acetylation was verified by the ninhydrin assay ***(28)***.
3. Prior to the incorporation of protected Cys residue into polymers, the stability of the Npys group was studied in the presence of pentafluorophenol. This was caused by the formation of this compound as byproduct during the reaction between Boc-Cys(Npys)-OPfp and the α-amino groups of the N-terminal amino acids of branched polypeptides. In a model experiment, Boc-Cys(Npys)-OH was kept in DMF-water (9:1, v/v) mixture in the absence or presence of pentafluorophenol. Based on HPLC analysis we found that the Npys group is stable under these conditions.
4. The input Boc-Cys(Npys)-OPfp/polymer ratio should be optimized in each case. Depending on the macromolecular partner we found optimal incorporation in the range of 0.75–1.5:1 molar ratio.
5. Branched polypeptides containing Boc-Cys(Npys) can be dissolved in DMF (A) or in DMF-0.06 *M* phosphate buffer (pH 5.5) = 75:25 (v/v) mixture (B).
6. We observed a low level of SH-peptide substitution when Boc-protected branched polypeptides were used. This might be due to the steric hindrance caused by the presence of the large number of Boc-Cys(Npys) groups at the end of the side chains. Therefore, we selected the unprotected form of modified polymers containing 27% Cys(Npys). In these cases the average degree of substitution of branched polymers with the 25-mer peptide having a Cys residue in central position was 26%.
7. In case of the presence of UV band at λ = 350 nm, Npys groups were reacted with the 1.2 eq excess of Cys in 0.06 *M* phosphate buffer (pH 5.5) followed by dialysis and freeze-drying.

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