Terminal-Specific PEGylation of Polypeptides in a Dilute Solution

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ABSTRACT: PEGylations of polypeptides with a four-arm PEG (polyethylene glycol) in a dilute DMSO solution resulted in a successful conjugation with terminal specificity, which supports an intriguing PEGylation mechanism via conformational and kinetic control. © Crown in the Right of Canada. J Appl Polym Sci 118: 3269–3273, 2010

Key words: peptides; modification; star polymers; PEGylation

INTRODUCTION

The modification of peptides (or proteins) with poly(ethylene glycol) (PEG) has received considerable attention since Abuchowski et al. discovered, in 1977, that PEGylated bovine liver catalase was nonimmunogenic and nonantigenic but significantly increased the *in vivo* circulation half-life with persisting enzyme activity.[1,2] The efficacy of using PEGylated peptide as a pharmaceutical agent often relies on the uniformity of its chemical structure, i.e., the site and number of PEG adducts on a peptide chain. As peptides usually have multiple reactive groups (OH, NH_2, SH), being able to target PEGylation at specific sites is very appealing, but at the same time, a daunting task.

In the past, PEGylation specificity was achieved mainly by applying exclusive reactions between PEG and a unique group on the peptide, usually introduced either chemically[3-9] or biologically.[10-20] For example, a cysteine residue was covalently linked to a peptide via mutagenesis. The SH group of the cysteine then reacted with thiol-selective maleimide-PEG. As the post-added groups are normally separate from the active peptide segments, PEGylations in this case do not appear to impose negative effects on the peptide activities. The advantage of this approach, however, is usually dampened by very costly and time-consuming challenges to meet regulatory safety approval for newly mutated or chemically altered peptides.[21]

Solid-phase peptide synthesis was also applied to achieve site-specific PEGylation.[22] A peptide sequence was tethered to a solid, followed by the addition of amino acids, one at a time, with a PEG-amino acid at each specific step. The process is also time-consuming and costly due to the complications of protection and deprotection.

Solution properties, which can influence the reactivity of different groups, may also be applied to control the site of PEGylation. For instance, pH adjustments render amino groups to differ in reactivity, allowing the most reactive amino to be PEGylated.[5,23,24] Recently, a growth-hormone releasing factor was mono-PEGylated either at the terminal or on the backbone by adjusting solvent compositions. PEGylation specificity was demonstrated but complicated by the presence of other by-products.[25]

"On-column" PEGylation has been used to influence both the site and the extent of conjugation. By immobilizing peptides onto a column, at least a partial separation between reaction-components can be achieved. Another adopted strategy was to bury nontargeted reactive groups in a column (e.g., an ion-exchanger) allowing the targeted reactive groups to face the mobile phase, but only with very limited success in controlling the site of PEGylation.[23,26,27]

A recent approach to PEGylation involved using an activated PEG, such as NHS (*N*-hydroxysuccinimide) ester.[28-30] The high reactivity of PEG-NHS makes it a more universal PEGylation agent than its previous counterparts. However, this agent works efficiently only when PEGylation site specificity is not required. On the other hand, the activated PEG

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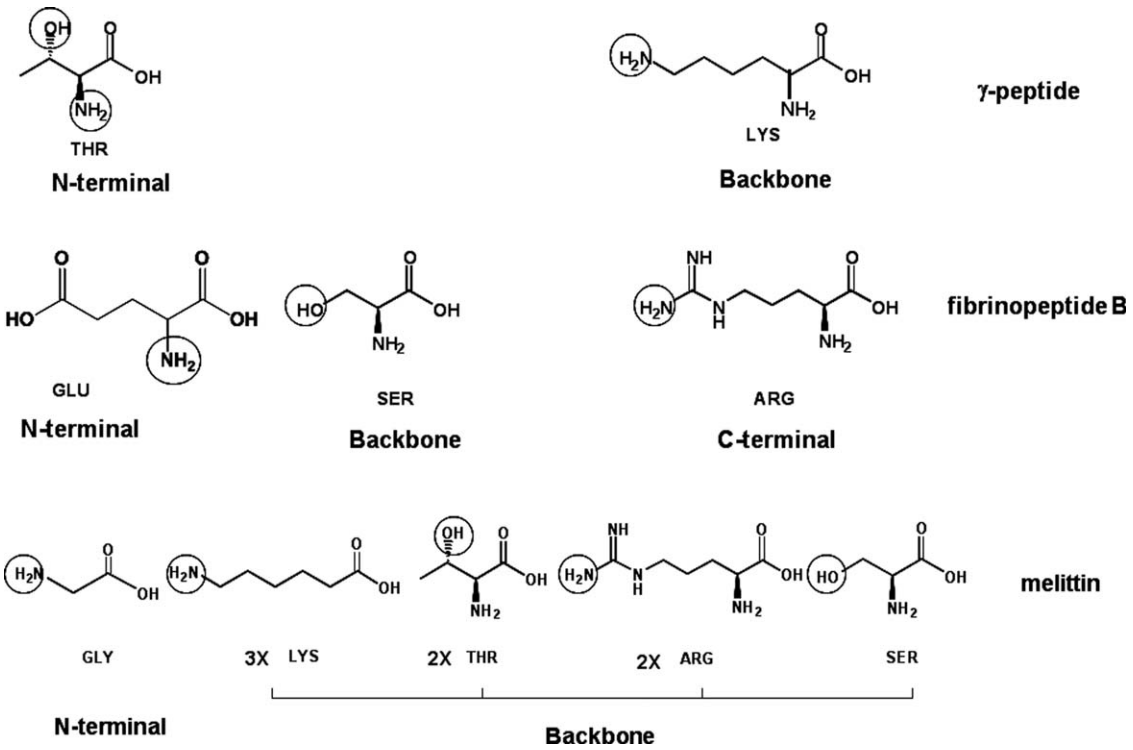

Figure 1 Structures of residues on a γ-peptide, fibronopeptide-B, and melittin with potential reactive groups for PEGylation; the indicated number preceding each individual amino acid represents the repeats on the peptide.

is subject to extensive hydrolysis (due to its high reactivity) in aqueous solutions, where the majority of PEGylations were carried out. The hydrolysis of PEG-NHS is often overwhelming over the PEGylation itself. Thus, a large excess of PEG, e.g., 50 : 1 mol/mol, has to be applied to ensure enough amount of PEG-NHS for PEGylation. A mixture of PEGylated species, including hydrolyzed PEG and PEGylated peptides differing in the site and number of PEG adduct, became the end result.[31]

In an attempt to find a convenient approach to PEGylation with site-specificity, we studied the PEGylations of three peptides, namely, fibrinopeptide-B, melittin, and a γ-peptide with a four-arm bulky PEG in dilute solutions, with DMSO as the solvent. Our results suggest that conjugations under these conditions are quantitative and terminal-specific, which supports a very intriguing PEGylation mechanism via conformational and kinetic control in dilute solutions.

EXPERIMENTAL PART

The fibrinopeptide-B (H_2N-GLU-GLY-VAL-ASN-ASP-ASN-GLU-GLU-GLY-PHE-PHE-SER-ALA-ARG; M_w = 1553 Da), melittin (H_2N-GLY-ILE-GLY-ALA-VAL-LEU-LYS-VAL-LEU-THR-THR-GLY-LEU-PRO-ALA-LEU-ILE-SER-TRP-ILE-LYS-ARG-LYS-ARG-GLN-GLN-$CONH_2$; M_w = 2846 Da) were purchased from Sigma–Aldrich (Mississauga, ON, Canada). The γ-peptide (H_2N-THR-ILE-GLY-GLU-GLY-GLN-GLN-HIS-HIS-LEU-GLY-GLY-ALA-LYS-GLN-ALA-GLY-ASP-VAL-COOH, M_w = 1900 Da) was previously custom made (Advanced Protein Technology Centre Peptide Synthesis Facility, The Hospital for Sick Children, Toronto, Ontario, Canada). The structures of the amino acid units on the three peptides, which have potentially reactive groups for PEGylation, are illustrated in Figure 1. The four-arm mPEG-NHS (NHS = *N*-hydroxysuccinimide) (M_w = 10,684 Da) was obtained from Nektar Therapeutics (Huntsville, AL, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Nepea, ON, Canada). The dialysis membranes with a molar mass cut-off of about 1200 Da (benzoylated cellulose) were from Sigma–Aldrich. For FTIR scanning, a ThermoNicolet IR 100 spectrometer (Thermo Electron Corporation, PA, USA) was used. Each sample solution was deposited onto a polyethylene (PE) film in the middle of a paperboard-based sample holder. The solvent was allowed to evaporate until the samples were completely dry. The sample was then scanned for FTIR spectrum. A Waters 2690 Separations Module, equipped with Symmetry® C_{18} 5 μm 3.9 × 150 mm HPLC column, was used to characterize the reaction components. Phosphate buffer (0.05*M*, pH = 2.5) was used as the mobile phase at a flow rate of 1 mL/min.

To a 20 mL vial, mPEG-NHS (13 mg, 1.2 μmol) and γ-peptide (12 mg, 6.3 μmol) were added before they were dissolved in DMSO (2.2 mL). The mixture

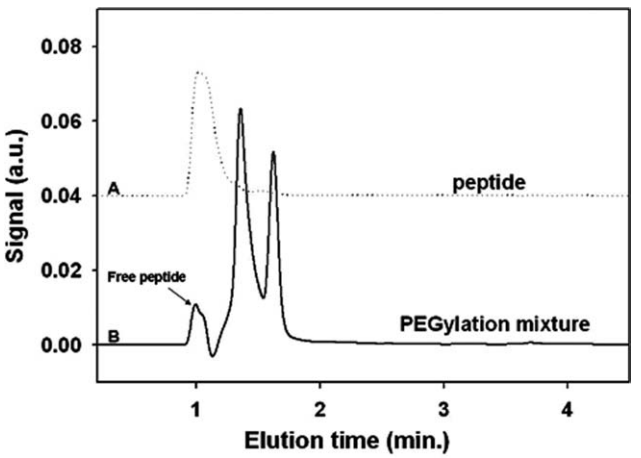

Figure 2 HPLC profiles of (A) γ-peptide, 0.48 mg/mL (dotted line; shifted upward by 0.04 unit of Y-axis for clarity); (B) PEGylation mixture, 1 mg/mL (solid line); the concentration of peptide in A corresponds to that in B before PEGylation.

was stirred at room temperature for 2 h (or over night, the results of which was the same). The reaction was then quenched into a fourfold volume of water compared to that of the original organic solution, before it was dialyzed against deionized water. The dialysis was conducted three times, each for 12 h. The resultant solution was freeze-dried to yield a white powder. The lyophilized solids were then used for FTIR and HPLC measurements. Similar conditions were applied for the respective PEGylations of fibrinopeptide-B and melittin.

RESULTS AND DISCUSSION

Before PEGylation, we studied the effect of the presence of water (0.03% in DMSO) on mPEG-NHS. A solution of mPEG-NHS in DMSO was prepared and the NHS ester peak at 1740 cm^{-1} was followed by FTIR (spectrum not shown). The persistence of this peak from the sample after sitting at room temperature for 2 h supports the absence of hydrolysis of mPEG-NHS in DMSO [H_2O:NHS $\approx$ 7 : 1 mol/mol; half-life of mPEG-NHS = 44 min at 25°C in pH = 8 water (from the company technical note)]. The absence of hydrolysis was attributed to the hydrogen bonding between water and PEG so that water molecules were not free for hydrolysis. It has been shown that water molecules associate with ethylene oxide (CH_2CH_2O) unit with a molar ratio of 3 : 1.[32,33] Under the conditions of this study, the molar ratio of water to ethylene oxide unit was only about 0.11 : 1 (<< 3 : 1). In addition, a fraction of water might be confined in the peptide coil during PEGylation, making the hydrolysis of the activated PEG more unlikely. It should be noted that the survival of NHS-PEG is critical for subsequent successful PEGylations.

Figure 2 shows the HPLC profiles of γ-peptide (corresponding to the original amount before PEGylation) and the reaction mixture, respectively. The peak at 1.0 min of the lower HPLC trace corresponds to the unreacted γ-peptide. The percentage of unreacted γ-peptide was estimated, based on the areas under the HPLC traces, to be about 15%, which was slightly lower than the theoretical percentage leftover (20%), if the PEGylation went to completion (with the amount of γ-peptide being in excess). The two peaks at 1.4 and 1.6 min correspond to the PEGylated peptide, while the original PEG after complete hydrolysis also has two peaks at about 1.3 min and 1.5 min, corresponding to a 3-arm and 4-arm PEG, respectively.

No other peaks, due to the presence of other by-products, were detected by HPLC. Based on the above information, we conclude that the yield of PEGylation under our current experimental conditions is quantitative for the NHS-PEG.

The γ-peptide is an acidic (3 carboxylic acid groups) polypeptide composed of 19 amino acid units. Its reactive groups (for PEGylation) include one LYS NH_2 on the backbone, one OH and one NH_2 on the N terminal. For the PEGylation of this peptide, an ester was formed peaking at 1740 cm^{-1} (Fig. 3, spectrum C). The reduction in amide peak at about 1650 cm^{-1} was not clearly understood. This ester is newly formed from PEGylation, rather than from the initial mPEG-NHS, because the resultant reaction mixture was quenched and dialyzed with water. NHS ester would not have survived these treatments. As the OH group that can form an ester with PEG-NHS, is present only on the N-terminal, we conclude that this PEGylation is N-terminal-

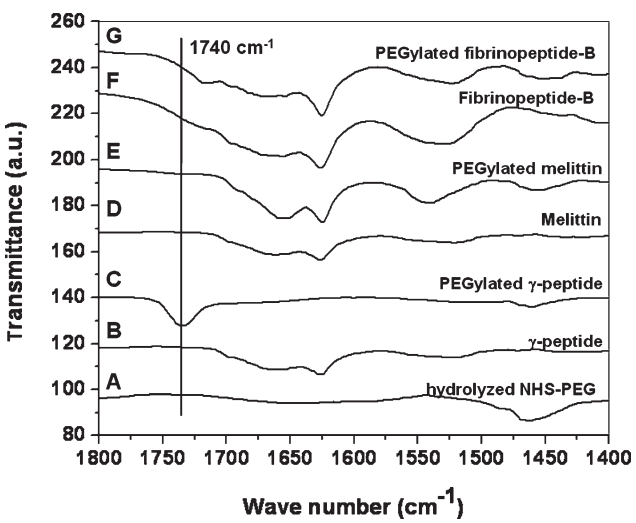

Figure 3 FTIR Spectra of (A) mPEG-NHS after dialysis; (B) γ-peptide only; (C) PEGylated γ-peptide; (D) melittin only; (E) PEGylated melittin; (F) fibrinopeptide-B; (G) PEGylated fibrinopeptide-B. All PEGylated peptides were scanned after dialysis.

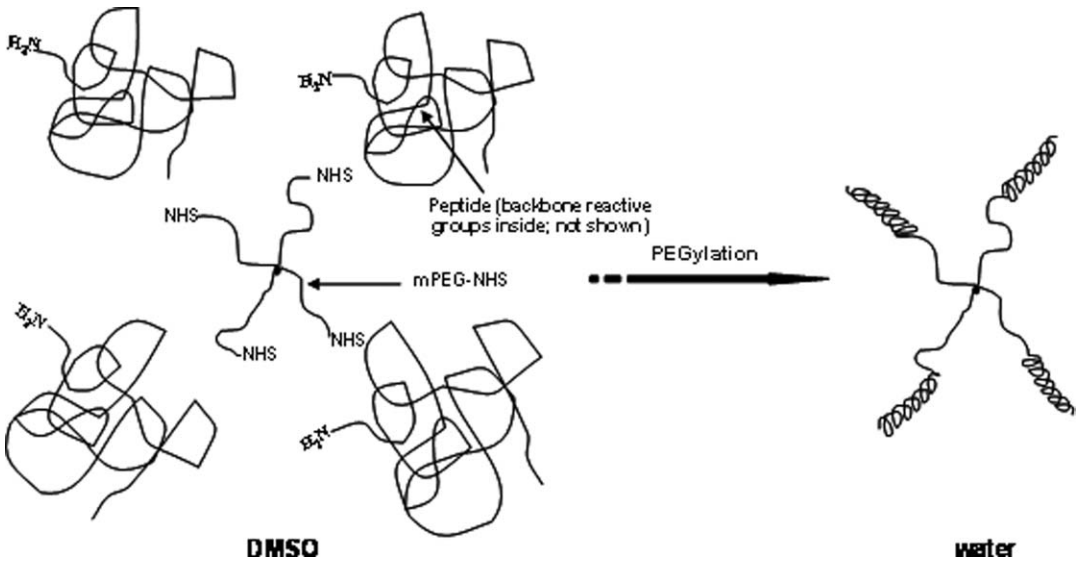

Figure 4 A proposed illustrative mechanism of the PEGylation of polypeptide via conformational and kinetic control. In dilute solution [The radius of gyration R_g for γ-peptide was estimated to be about 0.18 nm[34†]; γ-peptide accounts for only 4% of the total volume.], only the terminal groups of peptides collide with PEG. The natural peptide conformation (e.g., helix) could be restored in water after PEGylation in DMSO.[35]

specific. As can be seen later, this terminal specific PEGylation is exclusive with an absence of PEGylation on the backbone.

One may speculate that the selectiveness of PEGylating the γ-peptide is due to a decrease of nucleophilicity of the amino group (protonation) under acidic conditions. However, as indicated by the experiment described below, this is not the case. We conducted another PEGylation experiment on an acidic peptide fibrinopeptide-B, which ahs 14 amino acid units with one SER OH on the backbone, one lactam group on the N terminal, and one guanidine group on the C-terminal (Fig. 1). It is acidic because of the presence of 4 carboxylic acid groups (γ-peptide has 3). This peptide differs from the γ-peptide in that the single OH is on the backbone, rather than on the N-terminal. The completion of PEGylation was supported by the disappearance of NHS-PEG ester peak at 1740 cm^{-1} [from a reaction mixture without purification (room temperature, 2 h)]. We noted that in the control experiment, in which mPEG-NHS was dissolved in DMSO, the ester peak at 1740 cm^{-1} persisted. In the PEGylation of fibrinopeptide-B, the reaction yielded only an amide with the absence of any ester absorption in the FTIR spectra [Fig. 3(G)]. The minor peak at about 1710 cm^{-1} was attributable to COOH, which may span broadly over a big range between 1600 and 1800 cm^{-1}. In contrast, the absorption of an ester group is rather unlikely affected by its environment. Above all, the PEGylated fibrinopeptide-B has an almost identical IR spectrum to that of the original peptide, in the region between 1600 and 1800 cm^{-1}. For the PEGylation of fibrinopeptide-B, although OH under this condition was expected to be more reactive than the protonated NH_2, and its percentage ([OH]/([OH]+[NH_2]) = 33%) was the same as that of the γ-peptide, it was the NH_2 group that reacted with the PEG (with the source of NH_2 from either the N-terminal or the C-terminal). The absence of ester absorption in Figure 3(G) also supports the exclusiveness of the terminal PEGylation. Although under acidic conditions, the majority of the amino groups are expected to be protonated, the unprotonated portion (higher in DMSO than in H_2O) should be accountable for the PEGylation (driving the protonation to the left) without involving the OH group.

The PEGylation of melittin further supports the PEGylation specificity. Melittin is a basic peptide with 26 amino-acid units, including 3 LYS NH_2, 2 THR OH, 2 ARG NH_2, 1 SER OH on its backbone and 1 GLY N terminal (Fig. 1). In this PEGylation, although the OH percentage ([OH]/([OH]+[NH_2]) = 50%) was high, the reaction yielded only amide with the absence of any ester absorption in their FTIR spectra [Fig. 3(E)]; the reaction completion was supported by the disappearance of NHS ester FTIR peak at 1740 cm^{-1}, which persisted in the control experiment.

Based on the results of this study, we propose a mechanism to explain the terminal-specificity of

†For polyacrylamide in water (30°C), $r0/M_{1/2} = 0.01$ nm (r0: unperturbed end-to-end distance), the radius of gyration R_g is obtained through the relationship of $61/2R_g/M_{w1/2} = r0/M_{1/2}$. This R_g was very roughly treated as that of γ-peptide in DMSO. We expect that γ-peptide in DMSO is more compact than in water.

PEGylations described in this report, as illustrated in Figure 4. In organic solvents, the natural conformation of a peptide (in water) is disrupted so that a random coil (or partially random) is expected to form. The structure of the "coil" resembles that of a "pseudo reversed micelle" with a hydrophilic core surrounded by a hydrophobic corona, governed by thermodynamic functions, enthalpy H and entropy S. Because of the unfavorable interactions between the highly polar hydrophilic groups (OH, NH_2, and COOH) and the solvent, these groups withdraw, possibly associated with some water, into the peptide "coil" to minimize the enthalpy of the system. On the other hand, the "pseudo reverse micelles" could be stabilized by intramolecular attractions between oppositely charged moieties (e.g., protonated NH_2 and deprotonated COOH) on the side chains, and hydrogen bonding.[36] The end hydrophilic groups could retain in the medium due to the higher flexibility of the chain ends, which compensate for the enhanced enthalpy, caused by the unfavorable group-solvent interactions. Thus, the buried groups (and water) would not collide with NHS on the PEG. On the other hand, the PEG was such chosen that its size was large enough to avoid its penetration into and entanglement with the peptide coil in a dilute solution (peptide concentrations $<1\%$). As collision is an essential step of a reaction profile, PEGylation under these conditions takes place only via the peptide end groups.

CONCLUSION

PEGylations of three polypeptides, γ-peptide, fibrinopeptide-B, and melittin, were carried out in a dilute DMSO solution. The resultant conjugations are quantitative and terminal-specific, supporting a mechanism based on conformational and kinetic control.

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