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How PEGylation influences protein conformational stability

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PEGylation is an important strategy for enhancing the pharmacokinetic properties of protein therapeutics. The development of chemoselective side-chain modification reactions has enabled researchers to PEGylate proteins with high selectivity at defined locations. However, aside from avoiding active sites and binding interfaces, there are few guidelines for the selection of optimal PEGylation sites. Because conformational stability is intimately related to the ability of a protein to avoid proteolysis, aggregation, and immune responses, it is possible that PEGylating a protein at sites where PEG enhances conformational stability will result in PEG-protein conjugates with enhanced pharmacokinetic properties. However, the impact of PEGylation on protein conformational stability is incompletely understood. This review describes recent advances toward understanding the impact of PEGylation on protein conformational stability, along with the development of structure-based guidelines for selecting stabilizing PEGylation sites.

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Introduction

Peptides and proteins are attractive targets for treatment of many human diseases [1,2]. However, their benefits are limited because of fast degradation by proteases, filtration through the kidneys, aggregation, and recognition/neutralization by antibodies [3,4]. In the late 1970s, Davis and Abuchowsky [5,6] demonstrated that non-specific covalent modification of a protein with a polyethylene glycol (PEG) electrophile resulted in a less immunogenic PEG-protein conjugate with extended serum half-life. Subsequently, PEGylation was found to reduce protein aggregation and proteolysis, and to increase protein shelf-life [4,7–9]. Accordingly, PEGylation is now a widely used strategy for enhancing the pharmacokinetic properties of therapeutically relevant proteins, and several PEGylated proteins are now clinically available, including bovine adenosine deaminase, L-asparaginase, interferon-α, granulocyte colony stimulating factor, growth hormone receptor antagonist, urate oxidase, and the Fab fragment of a monoclonal antibody against tumor necrosis factor-α [10].

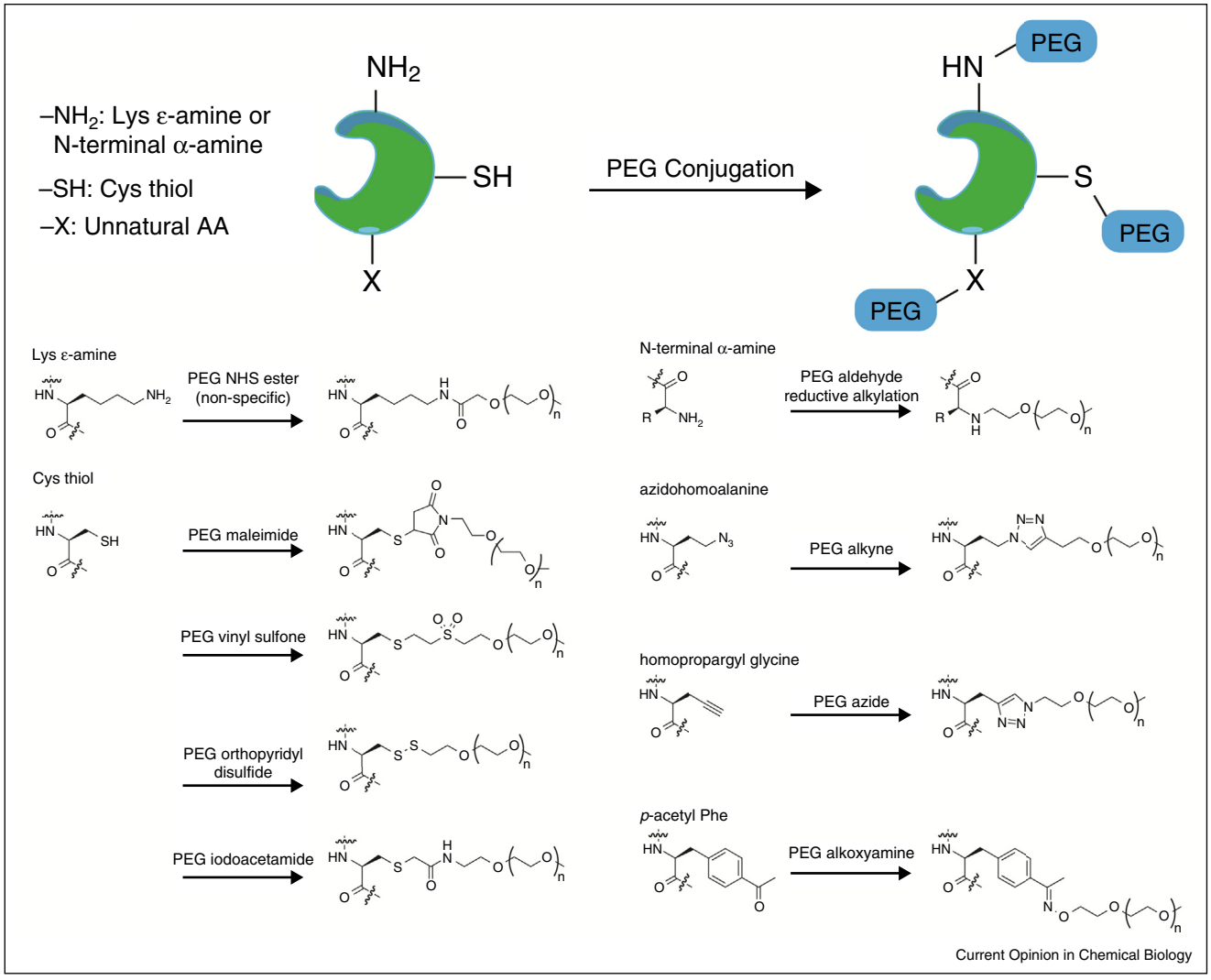
The extended serum half-life of PEGylated proteins is thought to derive from the large hydrodynamic radius of PEG, which decreases renal clearance by preventing large PEG-protein conjugates from being filtered through the pores of the glomerular wall. The large PEG is also thought to shield the protein from proteases and antibodies via simple steric occlusion. In many cases, a therapeutic PEG-protein conjugate is less active than its non-PEGylated counterpart in *in vitro* biochemical assays, presumably because the attached PEG also limits the access of substrates or binding partners to the protein surface/active site. However, in many cases, the extended serum half-life of the PEG-protein conjugate compensates for this modest loss of activity, resulting in a therapeutic protein that can be administered less frequently and with fewer side effects than its non-PEGylated counterpart.

Site-specific PEGylation

Initial efforts to generate PEG-protein conjugates depended on reactions with limited chemoselectivity: protein surface nucleophiles (i.e., cysteines, lysines, etc.) were non-specifically modified with various PEG electrophiles, including chlorotriazines [6], succinimides [11], maleimides [12], and aldehydes [13], generally resulting in a heterogeneous mixture of PEG-protein conjugates that differed in the number, location, and occupancy of PEGylation sites. However, the discovery of new bioorthogonal reactions in parallel with the development of strategies for incorporating correspondingly functionalized unnatural amino acids into proteins now allows relatively good control of the site and degree of PEGylation.

For example (see Figure 1), under mildly acidic conditions, a PEG aldehyde can be appended selectively to the N-terminal α-amino group of a protein via reductive alkylation, owing to pK_a differences between the N-terminal α-ammonium and Lys ε-ammonium groups [14]. Cys residues can be targeted selectively via a number of approaches owing to the high nucleophilicity of the Cys thiol: these include conjugate addition to PEG maleimides and PEG vinyl sulfones; disulfide formation with PEG ortho-pyridyl disulfides; and nucleophilic

Figure 1



Examples of natural and unnatural amino acids used in protein-PEG conjugation.

substitution with PEG iodoacetamides [15]. Alternatively, bisalkylation of the Cys side-chain thiol, followed by spontaneous elimination gives dehydroalanine, which can be subsequently modified via conjugate addition with a PEG thiol, following the elegant approach of Davis and coworkers [16]. One potential complication of the conjugate addition approaches is that the products are generally diastereomeric mixtures that differ in the stereochemistry at one of the atoms of the original Michael acceptor; however the significance of this stereochemical hetereogeneity has not been explored experimentally.

In addition, unnatural amino acids bearing orthogonally reactive functional groups (i.e., azides, alkynes, ketones, and alkenes) can be incorporated into expressed proteins as methionine surrogates in auxotrophic bacterial strains (e.g., azidohomoalanine, homopropargylglycine, etc.) [17–19] or via amber suppression (e.g., *p*-azidophenylalanine, *p*-propargyloxyphenylalanine, *p*-acetylphenylalanine, etc.) [20–26]. These unnatural side chains can then be selectively modified with an appropriate PEG reagent to give a PEG-protein conjugate with PEGs installed at defined positions. For example (see Figure 1), an azide or alkyne-functionalized side-chain can be modified with an alkyne- or azide-PEG, respectively, via the copper-catalyzed azide/alkyne cycloaddition to give a triazole-linked PEG-protein conjugate [27]. Alternatively, a ketone-functionalized side-chain can be modified with a PEG alkoxyamine to give an oxime-linked PEG-protein conjugate [24].