JOC The Journal of Organic Chemistry

JOCSynopsis

pubs.acs.org/joc

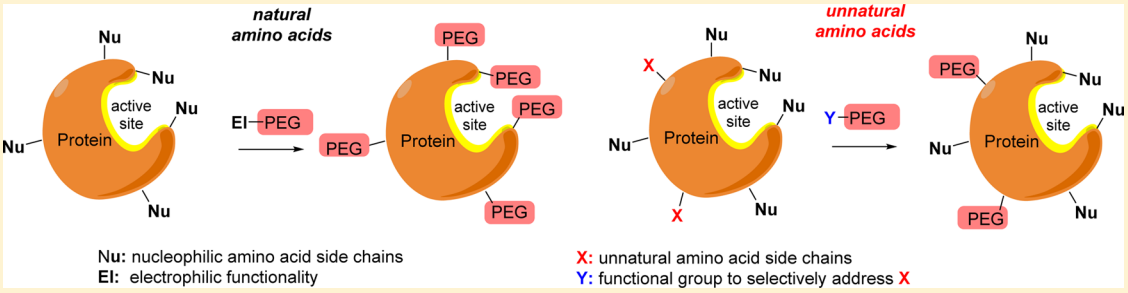
Site-specific PEGylation of Proteins: Recent Developments

Nicole Nischan[†,‡] and Christian P. R. Hackenberger*[,†,§]

[†]Leibniz-Institut für Molekulare Pharmakologie (FMP), Robert-Rössle-Strasse 10, 13125 Berlin, Germany

[‡]Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany

[§]Department Chemie, Humboldt Universität zu Berlin, Brook-Taylor-Strasse 2, 12489 Berlin, Germany



ABSTRACT: The attachment of linear polyethylene glycol (PEG) to peptides and proteins for their stabilization for *in vivo* applications is a milestone in pharmaceutical research and protein−drug development. However, conventional methods often lead to heterogeneous PEGylation mixtures with reduced protein activity. Current synthetic efforts aim to provide site-specific approaches by chemoselective targeting of canonical and noncanonical amino acids and to improve the PEG architecture. This synopsis highlights recent work in this area, which also resulted in improved pharmacokinetics of peptide and protein therapeutics.

Peptides and proteins are attractive targets and probes for biological and pharmaceutical research;[1] however, their benefits for *in vivo* applications are limited because of fast degradation by proteases, glomerular filtration in the kidney, and antigenic response.[2] These problems can be overcome by the attachment of macromolecules in order to (i) increase the molecular weight to decrease renal clearance and (ii) shield the surface of the peptide or protein that represent epitopes or sequences prone to proteolysis.[3,4] By far the most established strategy is the attachment of linear poly(ethylene glycol) (PEG) chains, referred to as PEGylation, which improve the stability, solubility, biocompatibility, and applicability of biomolecules. Other polymers can also be employed, among them synthetic poly(*N*-(2-hydroxypropyl)methacrylamide (HMPA),[5] polysialic acids (Lipoxen),[6] or biodegradable polypeptidic random coils consisting of proline, alanine, and serine residues (PASylation).[7] One of the major challenges for any of these approaches is finding the balance between maximal shielding of the protein while keeping its active site free. Early strategies for protein conjugation have been heuristic due to limited knowledge about the proteins' structure; however, structure determination methods like X-ray will allow for a more rational design. In this context, site-selective conjugation techniques are crucial for a modified protein that is both stable and active.

PEGYLATION IN PROTEIN DRUG DESIGN

PEGylation has been used for commercial protein drugs since the 1990s.[8] A famous example is PEGylated interferon α-2a, an antiviral cytokine for the treatment of hepatitis C and B that increases antigen presentation of the host and activates immune cells (Pegasys, Hoffmann-La Roche, Inc.).[9] PEG itself is chemically inert and has a very low immunogenicity. Upon attachment to a protein, PEG chains increase the solubility of a protein but also stabilize and shield its surface, which can be explained by the so-called hydrocloud model: while the PEG chains maintain full flexibility, they are entangled around the conjugated protein via hydrophobic interactions and at the same time build up hydrogen bonds to the surrounding water molecules (Figure 1). Consequently, PEGylation increases the

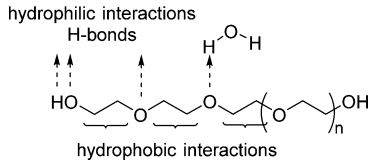


Figure 1. Hydrophilic and hydrophobic interactions of PEG.

effective mass of a PEGylated protein and reduces its accessible surface area.[10−12] The addition of linear PEG with a mass of 30 kDa or more decreases renal clearance dramatically. Furthermore, PEGylation leads to an increase in the circulation half-life of proteins from 5- to over 100-fold;[13] e.g., the conjugation of a 40 kDa PEG chain to IFN-α2b led to a 330 fold increase of in serum half-life in Sprague Dawley rats.[14] In

Received: September 17, 2014
Published: October 21, 2014

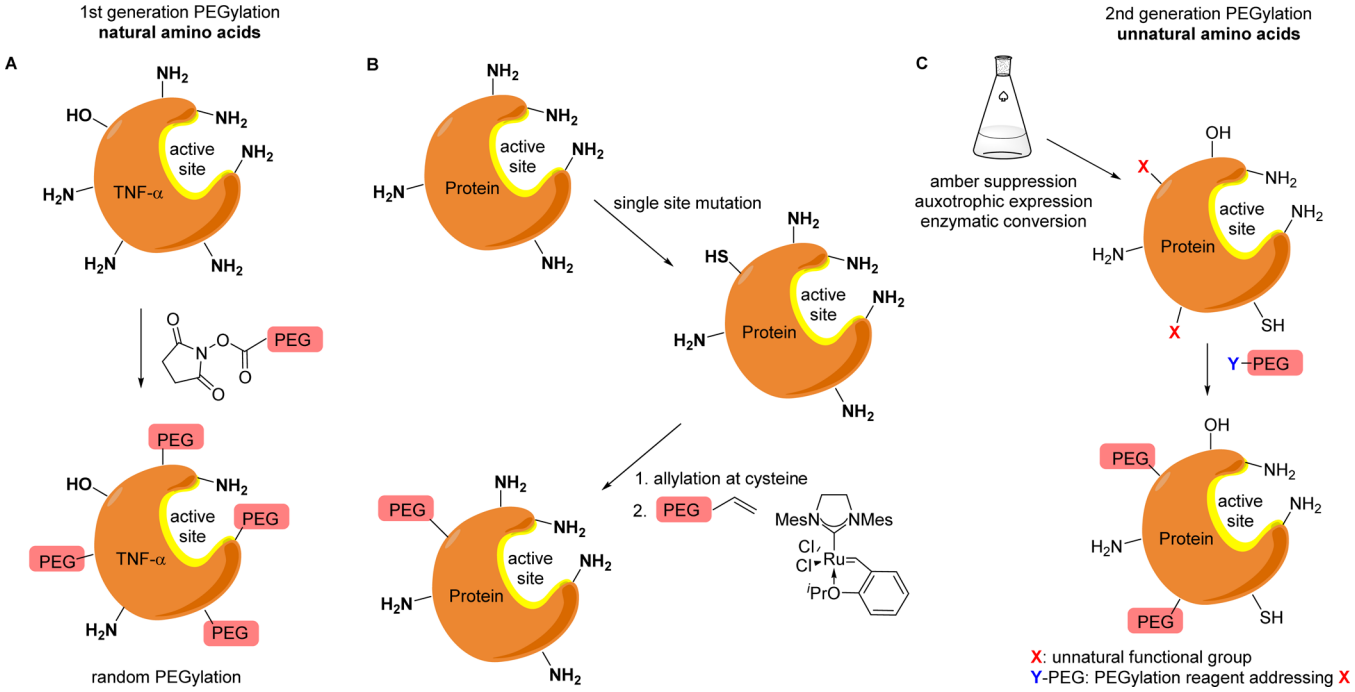


Figure 2. Examples for different protein PEGylation strategies. (A) Reaction of nucleophilic canonical amino acids with PEG succinimide esters.[25] (B) Two-step modification of naturally occurring functional groups by sequential allylation and cross metathesis.[30] (C) Chemoselective PEGylation of noncanonical amino acids.

addition, there is a correlation between the size and number of attached PEG chains and the *in vivo* half-life; e.g., conjugation of Fab antibody with one 25 kDa PEG, one 40 kDa PEG, or two 25 kDa PEG chains leads to a 15-, 17-, or 27-fold increase of serum half-life in rats.[15] This can even be further improved by designing the shape of the hydrocloud: branching points increase the probability of the PEG to be present near the PEGylation site and the surface of the protein, thus improving the shielding of the surface compared to single linear PEGs of the same molecular mass due to the so-called "umbrella effect."[16−19] While noteworthy drawbacks of PEG are its polydispersity, which creates an analytical challenge,[20,21] and its nondegradability, taking these disadvantages together with all its advantageous effects, PEGylation can be considered not only a useful modification element for the stabilization of proteins in biological experiments but also a very powerful tool for various pharmaceutical applications.[22,23]

FIRST-GENERATION OF PEGYLATION TECHNOLOGIES: ONE-STEP MODIFICATION OF NUCLEOPHILIC CANONICAL AMINO ACIDS

The first and most straightforward strategy for the covalent attachment of PEG chains on proteins employs naturally occurring nucleophiles, amine or thiol groups, in the side chains of the amino acids lysine or cysteine. These were addressed using electrophilic PEG derivatives carrying a succinimide[24−26] or maleimide,[27] an aldehyde for reductive amination,[28] or a tosyl group.[29] Because of the presence of several nucleophiles in a protein and the often observed need to use an excess of the PEGylation reagent to achieve reasonable conversions, usually several residues in a protein are modified resulting in a heterogeneous mixture of protein−PEG conjugates. These protein mixtures vary in PEGylation sites as well as in PEGylation extent (Figure 2A), whereas their analysis and identification are difficult and their purification is highly challenging.[20] Most importantly, by unwanted PEGylation near or at the active site of the protein its activity can be considerably decreased, clearly limiting these approaches. Success of these technologies depended on the structure of the protein and tuning the ratio of protein and PEGylating reagent. PEGylated protein drugs made in this manner were administered as protein mixtures with decreased biological activity. Nevertheless, their strongly improved *in vivo* residence half-life in serum led to an overall significant superiority in the pharmacological potency as compared to non-PEGylated drugs, resulting in successful FDA approval of several PEGylated protein drugs.[8]

TWO-STEP PEGYLATION STRATEGIES OF CANONICAL AMINO ACIDS

In order to enhance the PEGylation selectivity, canonical amino acids with a unique reactivity can be placed at the surface of the protein by site-directed mutagenesis. These amino acids can then be either modified via strategies discussed before or further used for the incorporation of an unnatural functional group for a chemoselective final PEGylation step (Figure 2B). Frequently, cysteine residues are used in the latter two-step protocol, which can, for instance, be allylated followed by metathesis with an appropriate allyl-PEG[30] or transformed directly with PEG-phenyl-oxadiazolyl methylsulfone reagents.[31] Also, tyrosine can be functionalized with diazo acetophenone, which subsequently reacts with an aminoxy-PEG reagent to form an oxime−PEG linkage.[32] Finally, disulfide bridges can be reduced and transformed to an artificial bridge by dibromomaleimide PEGs.[33,34]

PEGYLATION OF THE N- OR C-TERMINUS OF A PROTEIN

Another approach to enhance the selectivity is to employ the reactivity of the protein's N- or C-terminus. The N-terminus

dx.doi.org/10.1021/jo502136n | J. Org. Chem. 2014, 79, 10727−10733

can be transformed selectively using native chemical ligation with a PEG thioester if an N-terminal cysteine is present.[35] In addition, the N-terminal amine itself can selectively be transformed at pH5 at which lysines are protonated; however, cysteine residues should not be present for this approach.[28] To modify the C-terminus, a thioacid can be incorporated using an intein-fusion protein and processed into a sulfonamide via reaction with an electron deficient sulfone-azide PEG reagent.[36] Alternatively, following work by Waldmann and Goody for the C-terminal modification of proteins with oxyamines,[37] the rearranged intein-fusion protein can be cleaved by hydrazine to functionalize the resulting C-terminal hydrazide with a pyruvoyl-PEG reagent.[38]

SECOND-GENERATION PEGYLATION: ADDRESSING NONCANONICAL AMINO ACIDS WITH CHEMOSELECTIVE REACTIONS

The challenge of getting complete control over the PEGylation site is met by the application of chemoselective reactions to address noncanonical amino acids (Figure 2C). Direct incorporation of PEGylated amino acid using amber suppression is possible but limited to very short linear PEGs.[39] A general selective incorporation of PEGs regardless of size and shape starts with the biochemical incorporation of a noncanonical amino acid that carries an orthogonal reaction handle into the protein. This amino acid can be addressed in a second step by chemoselective reaction in the presence of all other canonical functional groups. This approach could be demonstrated for a variety of non-natural functional groups that have been introduced to proteins in different ways, most importantly by selective pressure incorporation and genetic code expansion.[40,41] Very popular in this context are azides, which have been reacted in copper-catalyzed azide alkyne cycloadditions (CuAAC) to give a stable triazole linkage after incorporation as azidohomoalanine[42,43] by auxotrophic expression or azidophenylalanine[44] (Figure 3A) via amber

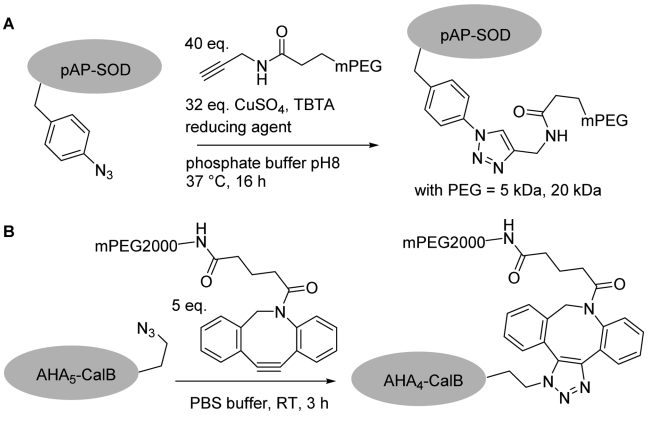


Figure 3. (A) CuAAC PEGylation of human superoxide dismutase-1.[44] (B) SPAAC PEGylation of lipase CalB.[51]

suppression. Azides can also be addressed using Staudinger ligations with a triarylphosphine–PEG reagent.[45] Alternatively, ketones can be incorporated as *p*-acetylphenylalanine via amber suppression and reacted with a PEG-oxyamine to form an oxime linkage,[46] as demonstrated by Cho et al. for developing a PEGylated human growth hormone (hGH) drug candidate. PEGylation of six hGH mutants and subsequent *in vivo* evaluation of pharmacokinetic parameters revealed the dramatic influence of the PEGylation site on drug efficacy and enabled identification of the mutant with highest drug exposure. Incorporation of acetophenone for subsequent oxime ligation can also be facilitated via enzymatic transpeptidation[47] or by native chemical ligation of synthetic peptides containing *N*-levulinyllysine, which enabled the successful total synthesis of a synthetic Erythropoietin that showed superior hematopoietic activity compared to the native protein.[48] Moreover, pyrroline–carboxylysine, incorporated via amber suppression with the pyrrolysine–synthetase, can be PEGylated with a 2-aminobenzaldehyde or 2-aminobenzophenone reagent using a ring-formation–condensation reaction.[49]

Similarly, alkene containing pyrrolysines can be addressed with thiol-PEGs in a thiol–ene reaction.[50]

RECENT ADVANCES FOR PROTEIN AND PEPTIDE PEGYLATION

Current research aims to provide novel chemoselective approaches by the incorporation of new unnatural amino acids and identifying innovative PEGylation reagents. Furthermore, researchers strive to improve the architecture of the PEG–protein conjugates according to pharmacological needs, and finally, they intend to explore new functions and applications of PEGylated proteins. Some success stories are highlighted in more detail in this synopsis.

STRAIN-PROMOTED AZIDE ALKYNE CYCLOADDITION

A metal-free chemoselective reaction for the conversion of azides is the strain-promoted azide alkyne cycloaddition (SPAAC) using strained cyclic alkynes.[52] The group of van Delft was able to develop a cyclooctyne derivative[51] that combines improved hydrophilicity and reaction kinetics based on studies from the Bertozzi[53] and the Boons laboratories,[54] respectively. For demonstration, they used the enzyme *Candida antarctica* lipase B (CalB), which was expressed in an auxotrophic strain of *E. coli* to introduce up to five azido homo alanines of which only one is solvent exposed. Remarkably, although it is generally challenging to get access to purified PEGylation reagents of high molecular weight, they were able to synthesize two dibenzocyclooctyne PEG derivatives with a molecular weight of 2 kDa in high yields of 41% over nine steps. Full conversion of the azido-CalB was reached in only 3 h in PBS buffer at room temperature using only 5 equiv of dibenzocyclooctyne or azadibenzocyclooctyne PEG (Figure 3B). Notably, the resulting protein–PEG conjugates also contained a double-modified protein, which can be attributed to the functionalization of the less exposed azides; however, later works demonstrate that thiol–yne addition of cysteins is a common side reaction of strained alkynes that can be circumvented by preventive alkylation of cysteins with iodoacetamide.[55] Nevertheless, this strategy is very attractive, especially if the azido-containing amino acids are in less reactive positions.

PALLADIUM-MEDIATED CONJUGATION

Other than copper, palladium catalysts have also been used for the bioorthogonal and chemoselective modification of genetically engineered proteins. The Lin laboratory developed a palladium catalyst for the copper-free Sonogashira C–C cross coupling to address alkyne groups on proteins in aqueous media. Specifically, they demonstrated this on a ubiquitin-

dx.doi.org/10.1021/jo502136n | J. Org. Chem. 2014, 79, 10727–10733

containing protein, in which homopropargyl glycine had been introduced via auxotrophic expression.[56] They synthesized an iodoaryl-functionalized PEG reagent via amide coupling of an amino PEG derivative of 5 kDa molecular weight with an iodophenyl group. They reached above 80% conversion of the protein using 50 equiv of PEG reagent and palladium catalyst at 37 °C in aqueous medium in only 30 min (Figure 4A).

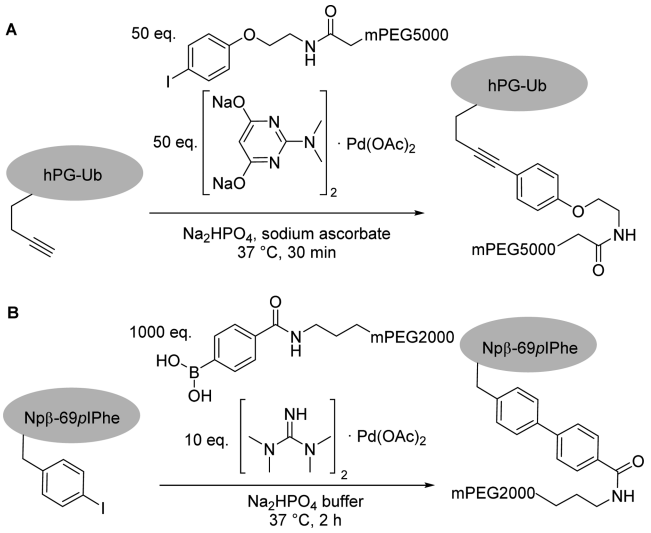


Figure 4. PEGylation via palladium-catalyzed C−C cross couplings. (A) Copper-free Sonogashira coupling.[56] (B) Suzuki−Miyaura PEG coupling.[57]

Another palladium C−C cross-coupling on proteins has been introdcued by the Davis laboratory. They demonstrated a Suzuki−Miyaura cross-coupling to react aryl iodides on proteins with PEG reagents of 2 kDa activated as boronic acids.[57] For this study, they incorporated *p*-iodophenylalanine into an all-β-helix protein from *Nostocpunctiforme* (Npβ) using amber suppression (Figure 4B). As a second protein target they chose subtilisin at which they chemically installed a *p*-iodobenzyl moiety on a cystein. The PEGylation of the proteins with the boronic acid PEG derivative led up to 70% conversion in 2 h at 37 °C using 1000 equiv of PEG reagent and 10−40 equiv of palladium. The reaction even works without addition of ligands to the palladium, which can be an advantage for reactions in a biological environment.

BRANCHED ALKYNE PEG REAGENTS

The Grabstein laboratory[58] transformed commercially available linear and branched succinimidyl PEG reagents of 10, 20, and 40 kDa via amide coupling into alkyne derivatives for CuAAC couplings. With these building blocks in hand, they PEGylated the pharmacologically highly relevant cytokine interferon β-1b at an azido homo alanine introduced via auxotrophic expression. As PEG reagents of this size react slower due to steric hindrance, an elaborate reaction optimization was devised which increased conversions above 90% with the use of 30 equiv of copper and 10 equiv of PEG reagents (Figure 5). Most interestingly, it was found that the addition of unreactive PEG-diol can improve the conversion of this reaction. Notably, the PEGylation was carried out under denaturing sodium dodecyl sulfate (SDS) conditions, which generally improves the accessibility of peptide side chains but makes it necessary to complete a challenging refolding step. The refolding of interferon β-1b was successful and the best product revealed a higher activity than interferon-derived drugs currently on the market.

BRANCHED PHOSPHITE PEG REAGENTS FOR CHEMOSELECTIVE STAUDINGER PHOSPHITE PEGYLATIONS

The synthesis of branched PEG derivatives is generally not straightforward and branched PEGylation reagents are rather expensive. A straightforward two-step protocol to branched phosphoramidate-linked PEG−peptide conjugates was accomplished by our laboratory. Symmetrical phosphites are synthesized neat from inexpensive commercial linear PEG-monomethyl ethers without the need of any purification step. PEG phosphites are then reacted with azides in a chemoselective Staudinger-type reaction that proceeds in high yields at physiological conditions, even in a cell lysate.[59−61] We demonstrated PEGylation with 660 equiv of symmetrical PEG phosphites derived from PEG alcohols of up to 2 kDa at pH 8 at 28 °C with the model protein SecB containing *p*-azidophenylalanine from a cell-free amber suppression expression system to yield branched PEG-phosphoramidate modified products[61] (Figure 6A left). In addition to stable phosphoramidate-branched PEGs, we also introduced UV-light cleavable PEGylation reagents based on a nitrobenzyl unit in order to remove the PEG-phosphoramidate at a moment of choice (Figure 6A, right).[62] Finally, we were able to use Staudinger phosphonite reactions for the conjugation of short PEG chains to proteins, in which aryl or triazole phosphonites are used in the reaction with aryl azides.[63,64]

Recently, we have studied the impact of PEG chain length at the phosphoramidate branching point on intracellular distribution and activity of proapoptotic peptides.[65] Interestingly, the intracellular application of PEGs for the stabilization of peptides had not been demonstrated before. Even with short branched PEG chains of a total mass of 1.5 and 4 kDa, the stabilization against proteolysis in cell lysate was significantly increased by a factor of 11 and 57, respectively. Most importantly, PEGylation with a weight increase of only 1.5 kDa was sufficient to increase the peptides' intracellular apoptosis inducing activity significantly in a concentration dependent manner. The attachment of longer PEG chains did not increase activity.

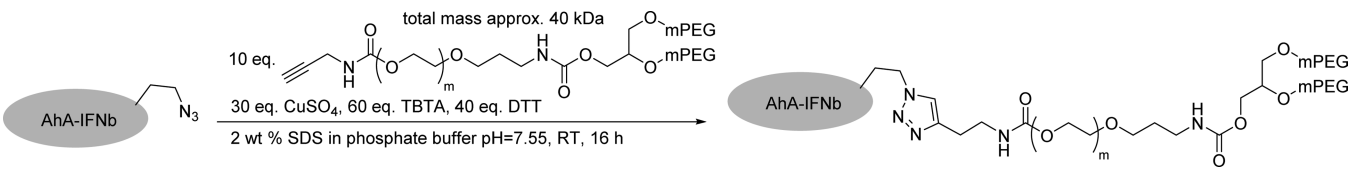


Figure 5. CuAAC with a branched alkyne PEG reagent.[58]

dx.doi.org/10.1021/jo502136n | J. Org. Chem. 2014, 79, 10727−10733

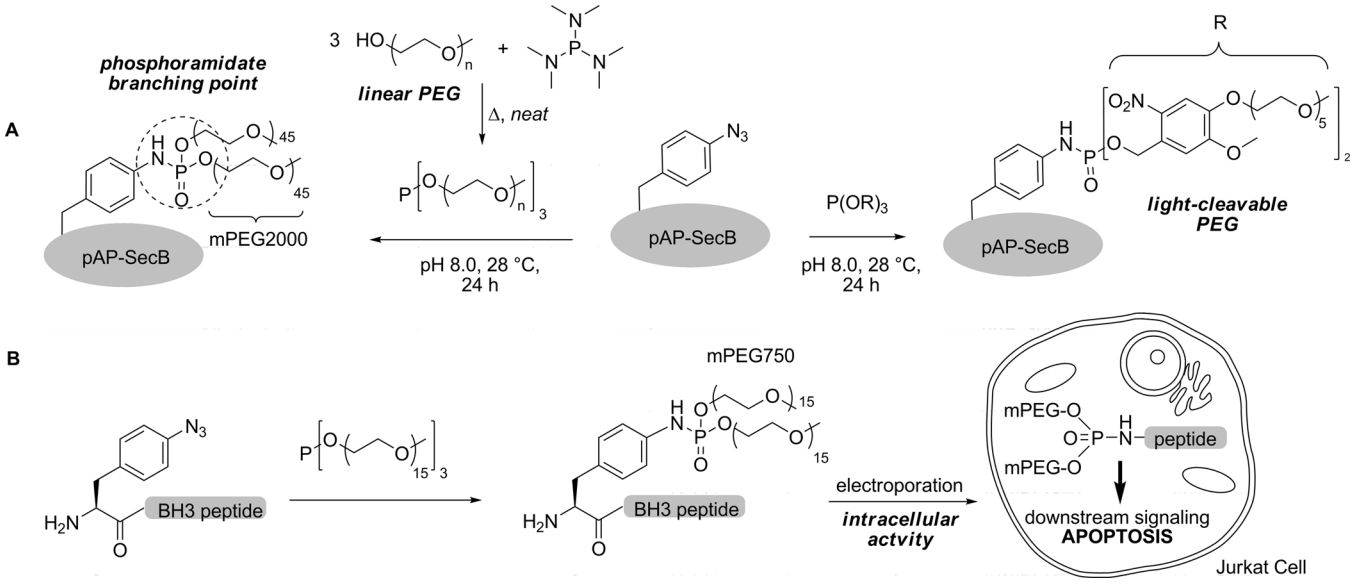


Figure 6. (A) Staudinger-phosphite PEGylation, stable (left)[61] and light cleavable (right)[62] phosphoramidate–PEG conjugate. (B) Intracellular activity of phosphoramidate PEGylated BH3-peptides.[65]

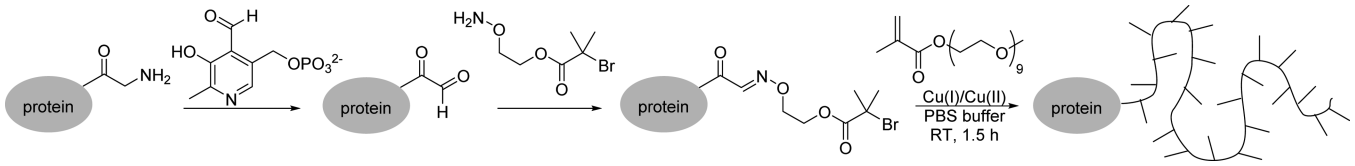


Figure 7. Construction of PEG brushes via ATRP.[66]

PEG BRUSHES VIA ATOM-TRANSFER RADICAL POLYMERIZATION (ATRP)

The Chilkoti laboratory established a two-step route to modify proteins site-selectively with a PEG brush.[66] After installation of a *tert*-butyl bromide that serves as ATRP initiator on the target protein, the PEG brush is constructed by living polymerization with (oligo PEG-methyl ether)methacrylate monomers in the presence of copper. Introduction of the initiator to myoglobin was demonstrated at the N-terminus by transforming glycine with pyridoxal-5-phosphate into an aldehyde followed by reaction with hydroxylamine-containing ATRP initiator (Figure 7).[66] C-Terminal introduction of a cysteine-modified initiator to GFP was possible via intein cleavage.[67] Alternatively, the C-terminus can be addressed via sortase transfer.[68] With this methodology, polymers of 100 kDa can be built up on a protein in only 1.5 h with yields of 70–90%. The conjugates displayed 5-fold increase of blood elimination time in mice.

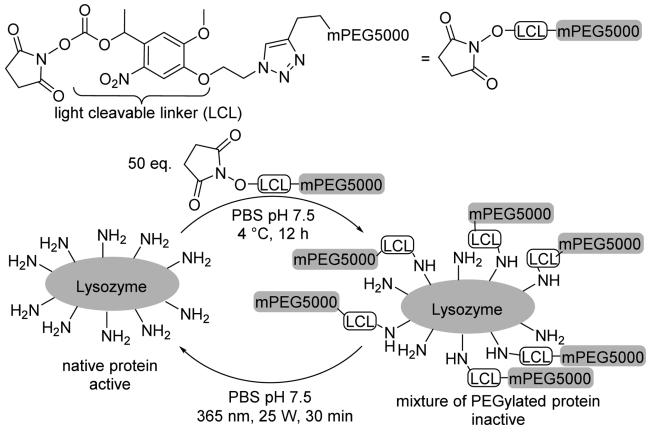


Figure 8. Deactivation and reactivation of proteins by UV-light-cleavable PEG reagents.[69]

TURN ON OF PROTEIN FUNCTION UPON LIGHT IRRADIATION

As mentioned before, the drawback of nonselective protein PEGylation is the loss of activity due to the blocking of the active site. The Deiters laboratory[69] took advantage of this observation by deactivating a protein upon the attachment of light-cleavable PEG chains and regaining its activity at a chosen time by irradiation (Figure 8). Specifically, they synthesized a succinimide-PEG reagent with a molar mass of 5 kDa in three steps that contains an *o*-nitrobenzyl linker to finally remove the PEG chains. With this reagent, they deactivated lysozyme, which contains six solvent exposed lysines in addition to the N-terminus, using 10–50 equiv of succinimide derivative; accordingly, the PEG chains covered most of the protein surface, thus deactivating it. At a moment of choice, these PEG chains were cleaved off within 30 min upon irradiation with light, exposing the native lysozyme surface and restoring its activity.

CONCLUSION

PEGylation is a state-of-the-art technology for the administration of peptides and proteins as biopharmaceuticals to increase their activity, stability, and circulation half-time. Currently, various efforts are being made to control the PEGylation site and thus maintain full activity of the protein. This controlled design is achieved by exploring the application

dx.doi.org/10.1021/jo502136n | J. Org. Chem. 2014, 79, 10727–10733

of new chemoselective reactions, which includes on one hand the development of new expression systems for the incorporation of new noncanonical amino acids and on the other hand getting access to innovative PEGylation reagents. In this process, the challenges are to increase the expression yields, but also the PEGylation reagent should be accessible in a straightforward fashion. Moreover, the conjugation itself should work with high conversion and the formed junction has to be stable. At the same time, pharmacological profiles of PEGylated proteins can be improved via changing the architecture of the PEGylation reagent via introducing branching points. Finally, it has been found that PEGylation can be employed to control of protein activity and even release function with temporal control. Taken together, the pharmacological relevance of PEGylation is continuously increasing due to the rewards for all of these contributions.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hackenbe@fmp-berlin.de.

Notes

The authors declare no competing financial interest.

Biographies

Nicole Nischan completed her Diplom in chemistry at the Technical University of Dresden before joining the lab of Christian P. R. Hackenberger for her doctoral work at Freie Universität Berlin in 2010, where she studies chemoselective reactions and their application to bioconjugation.

Christian P. R. Hackenberger completed his graduate studies with Prof. Sam Gellman (UW Madison) and his doctoral work with Prof. Carsten Bolm at the RWTH-Aachen. After his postdoctoral stay in the group of Prof. Barbara Imperiali at MIT he started his own lab at the Freie Universität Berlin as a FCI-Liebig-Scholar and Emmy Noether Fellow in 2005. In 2012, he accepted a call as the Leibniz–Humboldt Professor for Chemical Biology to the Leibniz Institut for Molecular Pharmacology and the Humboldt Universität zu Berlin. His lab is interested in studying the biology, function and pharmacological potency of naturally and unnaturally modified peptides and proteins.

ACKNOWLEDGMENTS

We thank the Deutsche Forschungsgemeinschaft (SPP1623, Teilprojekt Cardoso/Hackenberger/Leonhardt, SFB765 B5), the Fonds der Chemischen Industrie, the Einstein Foundation Berlin, and the Boehringer-Ingelheim Foundation (Plus 3 award).

REFERENCES

(1) Sood, A.; Panchagnula, R. *Chem. Rev. (Washington, D.C.)* **2001**, *101*, 3275.
(2) Werle, M.; Bernkop-Schnuerch, A. *Amino Acids* **2006**, *30*, 351.
(3) Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451.
(4) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003**, *2*, 214.
(5) Duncan, R. *Adv. Drug Delivery Rev.* **2009**, *61*, 1131.
(6) Gregoriadis, G.; Jain, S.; Papaioannou, I.; Laing, P. *Int. J. Pharm.* **2005**, *300*, 125.
(7) Skerra, A.; Theobald, I.; Schlapschy, M. (Technische Universitaet Muenchen, Germany) WO2008155134(A1), 2008.
(8) Ryan, S. M.; Mantovani, G.; Wang, X.; Haddleton, D. M.; Brayden, D. J. *Expert Opin. Drug Delivery* **2008**, *5*, 371.
(9) Nieforth, K. A.; Nadeau, R.; Patel, I. H.; Mould, D. *Clin. Pharmacol. Ther.* **1996**, *59*, 636.
(10) Mu, Q. M.; Hu, T.; Yu, J. K. *PLoS One* **2013**, 8.
(11) Yang, C.; Lu, D. N.; Liu, Z. *Biochemistry* **2011**, *50*, 2585.
(12) Hamed, E.; Xu, T.; Keten, S. *Biomacromolecules* **2013**, *14*, 4053.
(13) Hamidi, M.; Azadi, A.; Rafiei, P. *Drug Delivery* **2006**, *13*, 399.
(14) Ramon, J.; Saez, V.; Baez, R.; Aldana, R.; Hardy, E. *Pharm. Res.* **2005**, *22*, 1374.
(15) Chapman, A. P.; Antoniw, P.; Spitali, M.; West, S.; Stephens, S.; King, D. J. *Nat. Biotechnol.* **1999**, *17*, 780.
(16) Monfardini, C.; Schiavon, O.; Caliceti, P.; Morpurgo, M.; Harris, J. M.; Veronese, F. M. *Bioconjugate Chem.* **1995**, *6*, 62.
(17) Veronese, F. M.; Caliceti, P.; Schiavon, O. *J. Bioact. Compat. Polym.* **1997**, *12*, 196.
(18) Fee, C. J. *Biotechnol. Bioeng.* **2007**, *98*, 725.
(19) Pinholt, C.; Bukrinsky, J. T.; Hostrup, S.; Frokjaer, S.; Norde, W.; Jorgensen, L. *Eur. J. Pharm. Biopharm.* **2011**, *77*, 139.
(20) Veronese, F. M. *Biomaterials* **2001**, *22*, 405.
(21) Both PEG starting materials and PEGylated proteins are polidisperse, making the determination of the molar mass challenging, and estimations are usually based on MALDI or GPC analysis.[28] On top of this polydispersity, the yield of a protein per se cannot be determined directly by isolation and is commonly estimated by UV absorption. Given this double challenge to evaluate protein PEGylation reactions, yields are usually not discussed, but conversions are estimated based on LC–UV/MS or SDS gel electrophoresis.
(22) Cheng, Y.; Zhao, L.; Li, Y.; Xu, T. *Chem. Soc. Rev.* **2011**, *40*, 2673.
(23) Yu, H.; Nie, Y.; Dohmen, C.; Li, Y.; Wagner, E. *Biomacromolecules* **2011**, *12*, 2039.
(24) Basu, A.; Yang, K.; Wang, M.; Liu, S.; Chintala, R.; Palm, T.; Zhao, H.; Peng, P.; Wu, D.; Zhang, Z.; Hua, J.; Hsieh, M.-C.; Zhou, J.; Petti, G.; Li, X.; Janjua, A.; Mendez, M.; Liu, J.; Longley, C.; Zhang, Z.; Mehlig, M.; Borowski, V.; Viswanathan, M.; Filpula, D. *Bioconjugate Chem.* **2006**, *17*, 618.
(25) Tsutsumi, Y.; Kihira, T.; Tsunoda, S.; Kanamori, T.; Nakagawa, S.; Mayumi, T. *Br. J. Cancer.* **1995**, *71*, 963.
(26) Lecolley, F.; Tao, L.; Mantovani, G.; Durkin, I.; Lautru, S.; Haddleton, D. M. *Chem. Commun. (Cambridge, U.K.)* **2004**, 2026.
(27) Salmaso, S.; Semenzato, A.; Bersani, S.; Mastrotto, F.; Scomparin, A.; Caliceti, P. *Eur. Polym. J.* **2008**, *44*, 1378.

(28) Kinstler, O.; Molineux, G.; Treuheit, M.; Ladd, D.; Gegg, C. *Adv. Drug Delivery Rev.* **2002**, *54*, 477.
(29) Shaunak, S.; Godwin, A.; Choi, J.-W.; Balan, S.; Pedone, E.; Vijayarangam, D.; Heidelberger, S.; Teo, I.; Zloh, M.; Brocchini, S. *Nat. Chem. Biol.* **2006**, *2*, 312.
(30) Lin, Y. A.; Chalker, J. M.; Floyd, N.; Bernardes, G. J. L.; Davis, B. G. *J. Am. Chem. Soc.* **2008**, *130*, 9642.
(31) Toda, N.; Asano, S.; Barbas, C. F. *Angew. Chem., Int. Ed.* **2013**, *52*, 12592.
(32) Schlick, T. L.; Ding, Z.; Kovacs, E. W.; Francis, M. B. *J. Am. Chem. Soc.* **2005**, *127*, 3718.
(33) Jones, M. W.; Strickland, R. A.; Schumacher, F. F.; Caddick, S.; Baker, J. R.; Gibson, M. I.; Haddleton, D. M. *J. Am. Chem. Soc.* **2012**, *134*, 1847.
(34) Schumacher, F. F.; Nobles, M.; Ryan, C. P.; Smith, M. E. B.; Tinker, A.; Caddick, S.; Baker, J. R. *Bioconjugate Chem.* **2011**, *22*, 132.
(35) Marsac, Y.; Cramer, J.; Olschewski, D.; Alexandrov, K.; Becker, C. F. W. *Bioconjugate Chem.* **2006**, *17*, 1492.
(36) Zhang, X.; Li, F.; Lu, X.-W.; Liu, C.-F. *Bioconjugate Chem.* **2009**, *20*, 197.
(37) Yi, L.; Sun, H. Y.; Wu, Y. W.; Triola, G.; Waldmann, H.; Goody, R. S. *Angew. Chem., Int. Ed.* **2010**, *49*, 9417.
(38) Thom, J.; Anderson, D.; McGregor, J.; Cotton, G. *Bioconjugate Chem.* **2011**, *22*, 1017.
(39) Shozen, N.; Iijima, I.; Hohsaka, T. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4909.
(40) Lang, K.; Chin, J. W. *Chem. Rev.* **2014**, *114*, 4764.
(41) Kim, C. H.; Axup, J. Y.; Schultz, P. G. *Curr. Opin. Chem. Biol.* **2013**, *17*, 412.
(42) Schoffelen, S.; Lambermon, M. H. L.; van Eldijk, M. B.; van Hest, J. C. M. *Bioconjugate Chem.* **2008**, *19*, 1127.
(43) Nairn, N. W.; Graddis, T. J.; Wang, A.; Shanebeck, K.; Grabstein, K. 234th ACS National Meeting, 2007 BIOT 349.
(44) Deiters, A.; Cropp, T. A.; Summerer, D.; Mukherji, M.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743.
(45) Cazalis, C. S.; Haller, C. A.; Sease-Cargo, L.; Chaikof, E. L. *Bioconjugate Chem.* **2004**, *15*, 1005.
(46) Cho, H.; Daniel, T.; Buechler, Y. J.; Litzinger, D. C.; Maio, Z.; Putnam, A.-M. H.; Kraynov, V. S.; Sim, B.-C.; Bussell, S.; Javahishvili, T.; Kaphle, S.; Viramontes, G.; Ong, M.; Chu, S.; Becky, G. C.; Lieu, R.; Knudsen, N.; Castiglioni, P.; Norman, T. C.; Axelrod, D. W.; Hoffman, A. R.; Schultz, P. G.; DiMarchi, R. D.; Kimmel, B. E. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 9060.
(47) Peschke, B.; Zundel, M.; Bak, S.; Clausen, T. R.; Blume, N.; Pedersen, A.; Zaragoza, F.; Madsen, K. *Bioorg. Med. Chem.* **2007**, *15*, 4382.
(48) Kochendoerfer, G. G.; Chen, S. Y.; Mao, F.; Cressman, S.; Traviglia, S.; Shao, H. Y.; Hunter, C. L.; Low, D. W.; Cagle, E. N.; Carnevali, M.; Gueriguian, V.; Keogh, P. J.; Porter, H.; Stratton, S. M.; Wiedeke, M. C.; Wilken, J.; Tang, J.; Levy, J. J.; Miranda, L. P.; Crnogorac, M. M.; Kalbag, S.; Botti, P.; Schindler-Horvat, J.; Savatski, L.; Adamson, J. W.; Kung, A.; Kent, S. B. H.; Bradburne, J. A. *Science* **2003**, *299*, 884.
(49) Ou, W.; Uno, T.; Chiu, H.-P.; Grunewald, J.; Cellitti, S. E.; Crossgrove, T.; Hao, X.; Fan, Q.; Quinn, L. L.; Patterson, P.; Okach, L.; Jones, D. H.; Lesley, S. A.; Brock, A.; Geierstanger, B. H. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 10437.
(50) Li, Y. M.; Yang, M. Y.; Huang, Y. C.; Song, X. D.; Liu, L.; Chen, P. R. *Chem. Sci.* **2012**, *3*, 2766.
(51) Debets, M. F.; van Berkel, S. S.; Schoffelen, S.; Rutjes, F. P. J. T.; van Hest, J. C. M.; van Delft, F. L. *Chem. Commun. (Cambridge, U.K.)* **2010**, *46*, 97.
(52) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486.
(53) Sletten, E. M.; Bertozzi, C. R. *Org. Lett.* **2008**, *10*, 3097.
(54) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253.
(55) van Geel, R.; Pruijn, G. J. M.; van Delft, F. L.; Boelens, W. C. *Bioconjugate Chem.* **2012**, *23*, 392.
(56) Li, N.; Lim, R. K. V.; Edwardraja, S.; Lin, Q. *J. Am. Chem. Soc.* **2011**, *133*, 15316.
(57) Dumas, A.; Spicer, C. D.; Gao, Z.; Takehana, T.; Lin, Y. A.; Yasukohchi, T.; Davis, B. G. *Angew. Chem., Int. Ed.* **2013**, *52*, 3916.
(58) Nairn, N. W.; Shanebeck, K. D.; Wang, A.; Graddis, T. J.; VanBrunt, M. P.; Thornton, K. C.; Grabstein, K. *Bioconjugate Chem.* **2012**, *23*, 2087.
(59) Serwa, R. A.; Swiecicki, J.-M.; Homann, D.; Hackenberger, C. P. R. *J. Pept. Sci.* **2010**, *16*, 563.
(60) Majkut, P.; Boehrsch, V.; Serwa, R.; Gerrits, M.; Hackenberger, C. P. R. *Methods Mol. Biol.* **2012**, *794*, 241.
(61) Serwa, R.; Majkut, P.; Horstmann, B.; Swiecicki, J.-M.; Gerrits, M.; Krause, E.; Hackenberger, C. P. R. *Chem. Sci.* **2010**, *1*, 596.
(62) Serwa, R.; Wilkening, I.; Del, S. G.; Muehlberg, M.; Claussnitzer, I.; Weise, C.; Gerrits, M.; Hackenberger, C. P. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 8234.
(63) Vallee, M. R. J.; Artner, L. M.; Dernedde, J.; Hackenberger, C. P. R. *Angew. Chem., Int. Ed.* **2013**, *52*, 9504.
(64) Vallee, M. R. J.; Majkut, P.; Wilkening, I.; Weise, C.; Mueller, G.; Hackenberger, C. P. R. *Org. Lett.* **2011**, *13*, 5440.
(65) Nischan, N.; Chakrabarti, A.; Serwa, R. A.; Bovee-Geurts, P. H. M.; Brock, R.; Hackenberger, C. P. R. *Angew. Chem., Int. Ed.* **2013**, *52*, 11920.
(66) Gao, W.; Liu, W.; Mackay, J. A.; Zalutsky, M. R.; Toone, E. J.; Chilkoti, A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 15231.
(67) Gao, W.; Liu, W.; Christensen, T.; Zalutsky, M. R.; Chilkoti, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 16432.
(68) Qi, Y.; Amiram, M.; Gao, W.; McCafferty, D. G.; Chilkoti, A. *Macromol. Rapid Commun.* **2013**, *34*, 1256.
(69) Georgianna, W. E.; Lusic, H.; McIver, A. L.; Deiters, A. *Bioconjugate Chem.* **2010**, *21*, 1404.