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Dendronylation: Residue-specific chemoselective attachment of oligoglycerol dendrimers on proteins with noncanonical amino acids

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ABSTRACT

Polyglycerol dendrimers as an important class of polymeric materials especially attractive for covalent attachment to therapeutic proteins as a useful alternative to traditional PEGylation procedures. Herein, we combine in vivo noncanonical amino acid (ncAA) incorporation and chemoselective conjugation in vitro to produce novel hybrid protein–dendrimer conjugates with the defined architectures. We incorporated Azidohomoalanine (Aha) as methionine substitute in vivo into various protein scaffolds to allow non-invasive dendrimer conjugations (dendronylation). Our approach makes recombinant proteins accessible for the design of multivalent dendrimer conjugates since it enables the preparation of many sequences with various positions for regioselective dendronylation.

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Polyglycerol dendrimers as nontoxic, non-immunogenic and highly soluble in water are especially attractive polymers for covalent attachment to therapeutic proteins as useful alternative to traditional PEGylation procedures.[1–3] Traditional approaches for chemical posttranslational modification by derivatization of solvent-accessible reactive side chains such as those of canonical amino acids lysine or cysteine often lead to heterogeneously labeled mixtures of modified proteins.[4] Thus, selective methods that allow absolute control of the type and position of polymer attachment within a protein are required. To achieve this goal we use reprogrammed ribosomal synthesis to install a bioorthogonal azide functional group at a pre-selected site, which then can be chemoselectively modified in a way that homogeneous, structurally defined conjugates are generated.[5] In particular, we use efficient auxotrophy-based residue-specific methods to introduce azidohomoalanine (Aha) at methionine (Met) positions into engineered proteins[6] which are subsequently subjected to Cu-catalyzed azide-alkyne cycloaddition (CuAAC) reactions[7] with alkyne-functionalized oligoglycerol dendron propargyl-generation 2 (dOG) to obtain homogeneous protein conjugates.

We have chosen cysteine-free barstar[8] with only one methionine exclusively at N-terminal (Nt) position (Met1; denoted as B*). In addition, we have engineered double mutant with two methionine side chains (Met1 + Glu47Met; denoted as B**). Barstar is well suited as a model protein due to its high stability and small and simple structure of 90 amino acids. Barstar is the intercellular inhibition of barnase and is widely used to study protein structure, stability and folding.[9] Its overall protein structure consists of a βαβ motif which is characteristic for some nucleic acid binding proteins. In the present study, we have introduced Aha in both protein variants. Other target protein used to attach more than two dendron molecules to one scaffold is green fluorescent protein (GFP) engineered to contain three solvent-exposed Met residues at positions 50, 134 and 143 engineered by site directed mutagenesis as described elsewhere.[10] The structural and functional integrity of all resulting protein-conjugates is checked by far-UV CD spectroscopy (B*/B**) activity assay. Autofluorescence is the main feature of structural and functional integrity of GFP as a chromophore in denatured form is efficiently quenched by water as solvent.[11] Therefore, GFP with its high stability and intrinsic fluorescence properties[12,13] of the β-barrel structure makes it an almost ideal protein scaffold for the rational decoration with multiple dOGs.

Protected propargylated [G2] dendron (Fig. 1A) was synthesized as previously reported in the literature.[14] The dendron was deprotected by heating a methanolic solution with Dowex-H (2 eq. by weight) at 65 °C for 5–6 h. The reaction mixture was filtered and purified by reverse phase HPLC using 40% water/methanol, which resulted pure compound as colorless viscous liquid (yield-90%). For a high-expression of the target proteins, we choose methionine auxotrophic *Escherichia coli* B834 (DE3) as the expression host.

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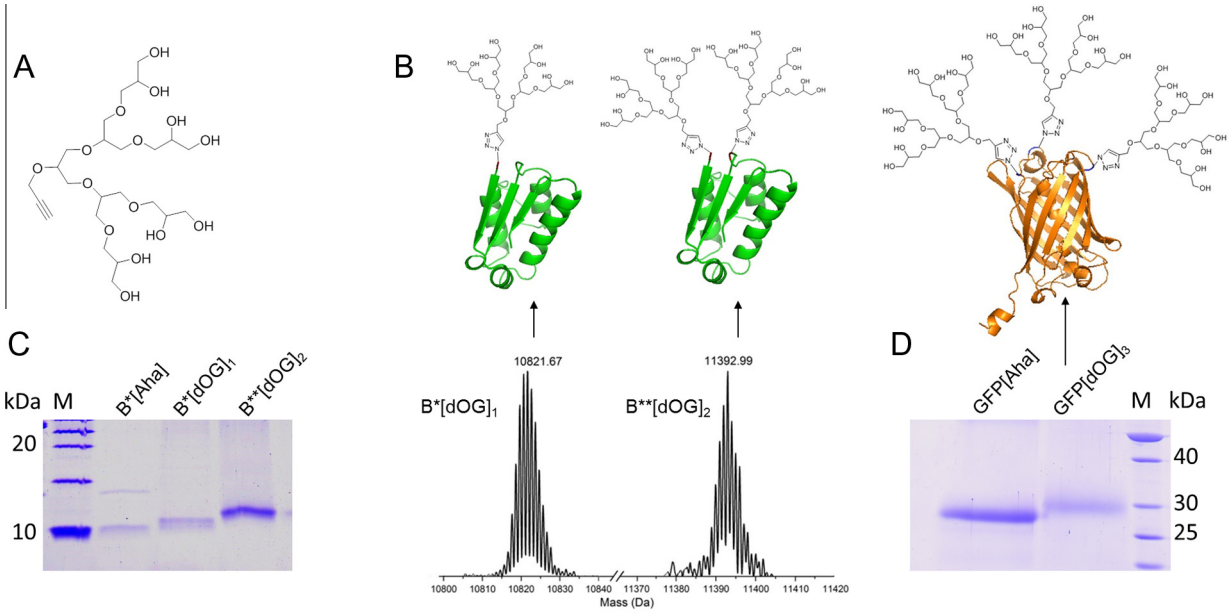

Figure 1. (A) Chemical structure of propargylated oligoglycerol [G2] dendron (dOG). (B) Three-dimensional models (ribbon plots drawn by PyMOL of barstar (PDB code: 1B27) and GFP (PDB code: 4GF6) clarify with marked conjugation products (as a result of CuAAC reaction of azide-containing proteins with dOG). From left to right: B*[dOG]$_1$, B**[dOG]$_2$, GFP [dOG]$_3$ with deconvoluted ESI mass spectra after modification of both B*[Aha] and B**[Aha] with 1 and 2 dendrons, respectively (list of theoretical and experimentally found masses in SI). The absence of B*[Aha] signals in the spectra after CuAAC reaction confirms virtually quantitative conjugation. (C, D) Comparison of electrophoretic mobilities of B*[Aha] and GFP[Aha] with their conjugates B*[dOG]$_1$ and B**[dOG]$_2$ as well as GFP [dOG]$_3$ in Coomassie-stained 17% SDS–PAGE. Detectable shifts upon multiple dendronylation presents a reliable qualitative indication that the corresponding protein conjugates are formed.

Genes for both barstar variants (Met1; Met1 + Glu47Met) are carried by vector pQE80L with the inducible T5 promoter. Similarly, GFP is harbored by vector pET30b under the inducible T7 promoter.

The general approach of labeled B*, B**, GFP expression is a fermentation procedure based on the selective pressure incorporation (SPI) method.[15] First, the *E. coli* B834 cells have been cultured in full new minimum media (NMM)[16] containing 19 canonical amino acids and a limited Met concentration (0.06 mM). Cells are grown until Met is fully depleted from the growth medium, which usually takes place in mid-log growth phase with optical density values at 600 nm about 0.8–1.0. In the second phase, 100 mg L^{-1} L-Aha is added followed by induction of the target protein synthesis by Isopropyl β-D-1-thiogalactopyranoside (IPTG) which results in global Met → Aha substitution during translation, including the target protein. Under these conditions, azide-labeled B* (B*[Aha]) was expressed in yields of about 50% (7.36 mg L^{-1}) compared to native B* (14.1 mg L^{-1}). Similarly, azide-labeled double mutant B** (B**[Aha]) was expressed in yields of about 40% (5.2 mg L^{-1}) compared to native B** (12.8 mg L^{-1}). Moreover, this fermentation procedure yielded 2.58 mg L^{-1} GFP[Aha] which is about 30% compared to wild-type protein (9.25 mg L^{-1}). High Met replacement levels by Aha, containing no traces of the parent protein as contaminant, were further confirmed by electrospray mass-spectrometric analysis (ESI-MS) as shown in Figure 1 and Table S1.

CuAAC reactions of azide-containing proteins with dOG were carried out in an aqueous phosphate buffer with copper(I), aminoguanidine and L-ascorbic acid.[17] Gel electrophoresis mobilites and mass spectrometric analyses of the obtained dendron-conjugates revealed full agreement between expected and found masses as shown in Figure 1 and Table S2. In general, the respective Aha-containing species was not detectable by ESI-MS, indicating a high level of dendronylation after the CuAAC reaction.

The structural dependence of GFP fluorescence clearly indicates that the protein is not structurally compromised after conjugation with multiple dendrons. Moreover, t he level of structural perturbation in B* can be measured by circular dichroism and thermally induced unfolding.

In our previous studies, we found that incorporation of Aha in proteins generally does not affect their structure and function.[18] Far-UV CD analysis of B*[Aha] and related dendroproteins revealed almost superimposable spectra (Fig. 2). Importantly, the unchanged spectral shapes clearly indicate that the overall secondary structure is not significantly changed (within the limits of this spectroscopic method). Small variations in dichroic intensities between variants are most probably due to minor differences in protein concentration estimation. Although the measurement of thermal unfolding profiles revealed that the dendronylation leads to slightly less stable proteins variants in terms of T_m values that are lowered by 2–4 °C, it can be concluded that the overall protein scaffold is not dramatically influenced by the dOG attachments.

In conclusion, our SPI approach for incorporation of Met analogue Aha in combination with site-directed mutagenesis made it possible to introduce or eliminate Met residues and subsequently engineer protein scaffolds with various positions for regioselective

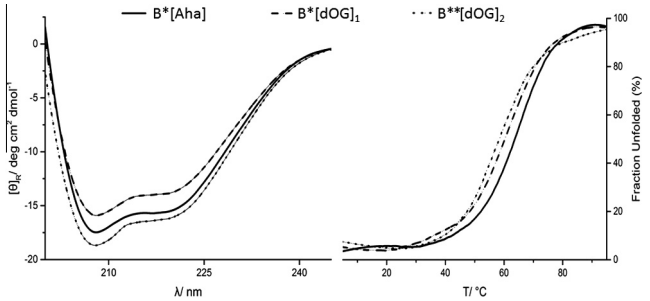

Figure 2. Spectral profiles and melting curves of barstar variants as determined by circular dichroism (CD). Secondary structure signals (left) and thermal unfolding profiles (right) of native and decorated barstar variants.

dendronylation. Our approach also presents a promising alternative to the widely used suppression-based methodology which is generally limited by low production yields and a technically complicated experimental set-up.[9,19] In this context, we present here efficient dendronylation of recombinantly expressed proteins by using suitably functionalized noncanonical amino acids for chemoselective conjugation in order to produce a novel protein conjugates with single and multiple dendrons. Non-proteinogenic amino acids with bioorthogonal handles such as Aha and its alkyne-containing counterpart Homopropargyl-glycine (Hpg) are attractive tags having small size and being genetically encoded. They are especially suitable for chemoselective reactions even in living cells since they are expected not to interfere with the protein's innate functions and structural integrity.[20] In this context, we were able to demonstrate that via CuAAC, different numbers of dendrons can be ligated to various azide-containing model proteins in almost quantitative yields giving access to active homogeneous dendroforms. Our systematic and modular approach for the generation of multivalent dendronylated systems by using engineered proteins as structured scaffolds offers an attractive alternative to the generally applied PEGylation of proteins.

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Supplementary data

Supplementary data (experimental details and supplementary information) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.09.055.

References and notes

1. Haag, R.; Sunder, A.; Stumbé, J. F. *J. Am. Chem. Soc.* **2000**, *122*, 2954.
2. Meng, F.; Manjula, B. N.; Smith, P. K.; Acharya, S. A. *Bioconjugate Chem.* **2008**, *19*, 1352.
3. Gupta, S.; Pfeil, J.; Kumar, S.; Poulsen, C.; Lauer, U.; Hamann, A.; Hoffmann, U.; Haag, R. *Bioconjugate Chem.* **2015**, *26*, 669.
4. Prabakaran, S.; Lippens, G.; Steen, H.; Gunawardena, J. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **2012**, *4*, 567.
5. Lang, K.; Chin, J. W. *ACS Chem. Biol.* **2014**, *9*, 16.
6. Budisa, N. *Angew. Chem., Int. Ed.* **2004**, *43*, 6426.
7. Meldal, M.; Tornøe, C. W. *Chem. Rev.* **2008**, *108*, 2952.
8. Nölting, B.; Golbik, R.; Fersht, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10668.
9. Lepthien, S.; Merkel, L.; Budisa, N. *Angew. Chem., Int. Ed.* **2010**, *49*, 5446.
10. Nischan, N.; Herce, H. D.; Natale, F.; Bohlke, N.; Budisa, N.; Cardoso, M. C.; Hackenberger, C. P. R. *Angew. Chem., Int. Ed.* **2015**, *54*, 1950.
11. Bae, J. H.; Rubini, M.; Jung, G.; Wiegand, G.; Seifert, M. H.; Azim, M. K.; Kim, J. S.; Zumbusch, A.; Holak, T. A.; Moroder, L.; Huber, R.; Budisa, N. *J. Mol. Biol.* **2003**, *328*, 1071.
12. Yang, F.; Moss, L. G.; Phillips, G. N. *Nat. Biotechnol.* **1996**, *14*, 1246.
13. Phillips, G. N. *Curr. Opin. Struct. Biol.* **1997**, *7*, 821.
14. Thota, B. N. S.; Berlepsch, H. V.; Boettcher, C.; Haag, R. *Chem. Commun.* **2015**, 8648.
15. Minks, C.; Alefelder, S.; Moroder, L.; Huber, R.; Budisa, N. *Tetrahedron* **2000**, *56*, 9431.
16. Budisa, N.; Steipe, B.; Demange, P.; Demange, P.; Eckerskorn, C.; Kellermann, J.; Huber, R. *Eur. J. Biochem.* **1995**, *230*, 788.
17. Ma, Y.; Biava, H.; Contestabile, R.; Budisa, N.; di Salvo, M. L. *Molecules* **2014**, *19*, 1004.
18. Merkel, L.; Beckmann, H. S. G.; Wittmann, V.; Budisa, N. *ChemBioChem* **2008**, *9*, 1220.
19. Hoesl, M. G.; Budisa, N. *ChemBioChem* **2011**, *12*, 552.
20. Dieterich, D. C.; Link, A. J.; Graumann, J.; Tirrell, D. A.; Schuman, E. M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9482.