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Grafting PNIPAAm from β-barrel shaped transmembrane nanopores

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ABSTRACT

The research on protein-polymer conjugates by grafting from the surface of proteins has gained significant interest in the last decade. While there are many studies with globular proteins, membrane proteins have remained untouched to the best of our knowledge. In this study, we established the conjugate formation with a class of transmembrane proteins and grow polymer chains from the ferric hydroxamate uptake protein component A (FhuA; a β-barrel transmembrane protein of *Escherichia coli*). As the lysine residues of naturally occurring FhuA are distributed over the whole protein, FhuA was reengineered to have up to 11 lysines, distributed symmetrically in a rim on the membrane exposed side (outside) of the protein channel and exclusively above the hydrophobic region. Reengineering of FhuA ensures a polymer growth only on the outside of the β-barrel and prevents blockage of the channel as a result of the polymerization. A water-soluble initiator for controlled radical polymerization (CRP) was consecutively linked to the lysine residues of FhuA and *N*-isopropylacrylamide (NIPAAm) polymerized under copper-mediated CRP conditions. The conjugate formation was analyzed by using MALDI-ToF mass spectrometry, SDS-PAGE, circular dichroism spectroscopy, analytical ultracentrifugation, dynamic light scattering, transmission electron microscopy and size exclusion chromatography. Such conjugates combine the specific functions of the transmembrane proteins, like maintaining membrane potential gradients or translocation of substrates with the unique properties of synthetic polymers such as temperature and pH stimuli handles. FhuA-PNIPAAm conjugates will serve as functional nanosized building blocks for applications in targeted drug delivery, self-assembly systems, functional membranes and transmembrane protein gated nanoreactors.

1. Introduction

Protein-polymer conjugates represent an active research field that has been growing in prominence over the last ten years [1–12]. Linkage of polymers can prepare proteins for specific applications and confer them with properties they cannot offer on their own. The effect of covalently attached polymer chains to the protein ranges from improved solubility, enhanced biocompatibility and stability to tunable enzyme activity [13–16]. Protein-polymer conjugates find versatile applications in biomedicine as nanocarrier systems for drug delivery, especially in cancer therapy [17,18]. Similar to the uses of physically entrapped tissues in hydrogels for regenerative medicine [19–21], hydrogels synthesized from protein-polymer conjugates have been successfully used in tissue engineering [22]. There are multiple uses in bio-sensing and diagnostics and they have been successfully used as biomimetic protocells [18,23]. They have been employed in electronic devices as functional materials and in ultra-thin membranes with the protein acting as a sacrificial template [13,18,24].

Two methods have been well established for the synthesis of protein-polymer conjugates. Using the grafting-to technique, presynthesized polymers with protein-reactive end groups are

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attached to the protein. However, steric hindrance around the protein often results in low attachment yields. Moreover, the isolation of conjugates from unreacted polymers and proteins is challenging. The second strategy, the grafting-from technique, focusses on polymerizing monomers directly from a protein. Here, a higher yield of attached polymer chains can be reached as the steric hindrance around the protein is lower for small monomers. Furthermore, the purification is easier as only small molecular components need to be removed. These advantages favor the grafting-from strategy; however, there have not yet been as many reports as for the more traditional grafting-to approach.

Controlled radical polymerizations (CRP), particularly atom-transfer radical polymerization (ATRP) [25–33] and reversible addition-fragmentation chain transfer (RAFT) polymerization [34–38] have been commonly used to synthesize protein-polymer conjugates via grafting-from strategy. Performing the CRP in pure aqueous environment (without addition of an organic co-solvent) is a challenging task because the reaction in water is highly accelerated, instability of the catalyst complex is a major issue, and additionally, the loss of terminal bromine can occur [39]. Nonetheless, the reaction conditions of ATRP and related techniques, namely activators generated by electron transfer (AGET) ATRP, activator regenerated by electron transfer (ARGET) ATRP, and single electron transfer living radical polymerization (SET-LRP) were recently optimized to develop procedures for CRP under biologically relevant conditions [40–43].

Diverse polymeric architectures have been synthesized on the surface of proteins ranging from a variety of stimuli-responsive polymers [16,28,29,34,35,44] to block-copolymers synthesized by sequential polymerization steps [16,29,35,45]. Moving on to more sophisticated protein structures, the groups of Finn and Douglas used virus-like particles as scaffold to independently modify the inside and the outside of a viral capsid [46–49]. The hence generated conjugates can be specifically tailored for desired applications like targeted drug delivery. Although many globular proteins like bovine serum albumin, ferritin, lysozyme or chymotrypsin have been extensively studied for conjugation [28,31,35,37], transmembrane proteins remain virtually untouched by the protein-polymer conjugation community. The latter can likely be attributed to challenges in purification (incl. e.g. extraction from membrane fractions) and handling of purified samples.

Membrane proteins have important functions in various biological processes, such as cell signaling-transduction pathways and in controlling a wide array of gradients such as chemical, electrical, and mechanical gradients. They can act as channels which enable highly selective transport of substrates or energy. Using these functions, although challenging, is a promising proposition.

Ferric hydroxamate uptake protein component A (FhuA), the largest of monomeric β-barrel transmembrane proteins, is located in the outer membrane of *Escherichia coli* (*E. coli*). FhuA functions as siderophore-mediated iron transporter, as receptor for bacteriophages like T1, T5 and the antibiotic albomycin [50]. FhuA has an elliptical cross section of 39–46 Å, a height of 69 Å[50] and has a highly hydrophobic region in the middle (2–3 nm) to enable the anchoring in the outer membrane [51]. It consists of 22 β-sheets forming a barrel (C-terminus) and the N-terminal cork domain which is blocking the channel [52]. FhuA, without this cork domain (FhuA Δ1-159), can function as a passive diffusion channel and has been used as a nanopore integrated in liposome/polymersome membranes for the translocation of compounds [53–55]. Because of its remarkable resistance towards high temperature, alkaline pH[56] and robustness in genetic modification [54,57–59], FhuA becomes an attractive scaffold for the development of smart protein-polymer conjugates. The resulting conjugates, or building blocks based on transmembrane proteins (BBTP), could be used to generate highly functional structures combining the aforementioned properties of transmembrane proteins with the unique properties afforded by synthetic polymers such as desired chain length and tunable stimuli handles like temperature and pH.

Here, we present the synthesis of BBTP from the transmembrane protein FhuA by grafting the thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAAm) from its surface. The grafting was performed from FhuA WT and three variants. In order to avoid growth of polymer chains inside the channel, two variants were reengineered to have up to 11 lysines, uniformly distributed in a rim exclusively on the outer surface. This distribution should facilitate the growth of polymer chains as well as avoid possible instability in the protein structure because of its modification. A water-soluble CRP initiator was linked to the amine groups of the lysine residues of FhuA and subsequently PNIPAAm chains were grafted from the protein surface. Although, non-specific grafting-to on the surface of cells (and hence membrane proteins) has been reported before [60], to the best of our knowledge, we present the first example of grafting a polymer from a transmembrane protein.

2. Results and discussion

The two amino acids commonly used for the chemical modification of proteins are lysines and cysteines [14]. For our purpose, targeting lysines was preferred as the total number of lysines is commonly higher in the amino acid sequence of proteins and more abundant on the protein surface [3,61]. Therefore, we exploited the lysines to link a water-soluble CRP initiator for the subsequent polymerization reaction (Fig. 1). The formation of BBTP was performed with FhuA WT (as found in nature) and three genetically engineered variants. Reengineering the surface residues of FhuA with the powerful protein engineering approach offers the possibility to introduce specific residues in desired positions for efficient grafting-from polymerization. Reengineered FhuA were generated to have up to 11 lysines exclusively located on the outer surface in the upper rim.

Starting from naturally occurring FhuA WT[52], the pore-blocking cork domain can be removed by deletion of the amino acids 1–159. The resulting variant FhuA Δ1-159 has already been used for stability studies [56], transport in artificial membranes [53–55] and formation of catalytically active channels (FhuA ΔCVF^{tev}) [62,63]. BBTP formation is successfully demonstrated for both variants here, but their use is not advantageous for any imaginable applications of BBTP. Although FhuA ΔCVF^{tev} has an open pore, the 28 lysine residues are non-uniformly distributed around the whole protein, including some exposed to the channel entrance or interior. Hence, their modification and polymer growth would probably block the channel. Furthermore, the modification of residues located in the hydrophobic region of FhuA could be hindered by the stabilizing agent, which ensures the proper folding and solubility of the transmembrane protein. Salt bridges are commonly formed between many positively charged lysine residues and negatively charged glutamic acid and aspartic acid residues. These salt bridges are a crucial factor for the stability of a protein. After linkage of chemical groups to a charged amino acid residue, the salt bridge would be destroyed and the stability decreased. Accommodating all mentioned factors, we designed two FhuA variants for predefined site-specific modification. Lysine residues (K) are located at defined positions, symmetrically distributed in a plane perpendicular to the protein channel, exclusively on the outer surface of the barrel and above the hydrophobic region (Table 1). FhuA $\Delta CVF^{tev}K_8^{mid}$ and FhuA $\Delta CVF^{tev}K_{11}^{up}$ contain lysine residues evenly distributed in a rim around FhuA and not directly involved in salt bridges. These lysines are expected to be easily accessible, and the resultant FhuA-polymer conjugates more stable.