Bioconjugation of L-3,4-Dihydroxyphenylalanine Containing Protein with a Polysaccharide

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ABSTRACT:

We describe the simple bioconjugation strategy in combination of periodate chemistry and unnatural amino acid incorporation. The residue specific incorporation of 3,4-dihydroxy-L-phenylalanine can alter the properties of protein to conjugate into the polymers. The homogeneously modified protein will yield quinone residues that are covalently conjugated to nucleophilic groups of the amino polysaccharide. This novel approach holds great promise for widespread use to prepare protein conjugates and synthetic biology applications.

Protein conjugation plays an important role in the development of effective vaccines, affinity matrices, drug delivery systems, and functional hydrogels. These conjugations occur commonly in nature, but experimentally it is difficult to prepare or adapt such biochemical approaches. Chemical activation of polysaccharide and proteins during the conjugation step is complex and also technically difficult. Generally, protein conjugation methods rely on chemoselective modification of canonical amino acids of the protein. To date, several approaches such as simple absorption and protein trapping with gel matrix or through covalent linkage have been used in preparing the bioconjugates. Among them, the most common and frequently employed approach is covalent linkage. Covalent linkages typically occur through amine coupling of lysines and sulfhydryl coupling of cysteine residues of protein. Cysteine is relatively a rare amino acid, and its thiol group can undergo disulfide exchange to form mixed disulfides, which is very important in preserving protein structure and function. Although amine coupling of lysine residues is considerably more prevalent in bioconjugation studies, it should be noted that reagents used for lysine modification can additionally modify the N termini of proteins. Therefore, it is a challenging goal to modify these residues. Furthermore, modification of these residues through chemical reactions is reversible, slow, and also inefficient. In general, approaches for the site-specific modification of canonical amino acid and finding ideal chemical candidate molecules that precisely modify the side chains for bioconjugation is highly challenging. Recently, protein—polysaccharide conjugates were achieved through tyrosinase mediated oxidation of tyrosine in the recombinant protein to form a reactive ortho-quinone. Further, bioconjugation was achieved through the nonenzymatic reaction of the nucleophilic amino groups of the polymer with

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electrophilic ortho-quinones of the protein. This method was successfully used for fabricating biological samples and in developing a novel microdevice which investigates how bacteria communicate with each other for enhancing their resistance toward drugs. However, in this approach it is very difficult to maintain specificity, monitor product formation, and remove the enzymes after the reaction.

Since preparing conjugates is a difficult process, a new bioconjugation reaction is currently of high interest to develop a systematic approach for coupling a specific protein of interest with polysaccharide targets. In an effort to establish a valuable protein–polysaccharide complex, we aimed to replace the tyrosine amino acid of recombinant protein with 3,4-dihydroxy-L-phenylalanine (L-DOPA). DOPA is an important neurotransmitter biosynthesized naturally in humans by tyrosinase-catalyzed oxidation of tyrosine in a melanin biosynthetic pathway, and by tyrosine hydroxylase in brain for catecholamine neurotransmitter biosynthesis. It also play an important role in adhesive and cross-linking property of mussel adhesive proteins. Recently, genetic incorporation of L-DOPA was achieved through a site specific suppression method, and it is significantly used in exploring protein–protein interaction. However, the practical importance of this method is still limited by low protein yields due to cellular toxicity. On the other hand, DOPA has also been incorporated into proteins by in vitro protein synthesis and solid-phase peptide synthesis. Here, we demonstrate the successful in vivo incorporation of L-DOPA in a residue specific manner, which also enables development of an efficient and improved bioconjugation strategy (Scheme 1).

This improved bioconjugation method will facilitate the synthesis of homogeneously modified protein with reactive chemical groups that can be utilized to create novel protein–polysaccharide complexes. DOPA-enriched recombinant proteins are selectively oxidized by periodate to form an ortho-quinone intermediate which is then subsequently attacked by the amino groups of chitosan resulting in a nonenzymatic crosslinking reaction through Michael addition. To evaluate the scheme shown above, we chose green fluorescent protein (GFP) as a model protein since its functional and spectral properties were well-studied and characterized. Our current GFP variant contains eight tyrosine residues which will be replaced with L-DOPA through an in vivo incorporation method. The detailed sequence information about the GFP variant used in the study was mentioned in Table S1 (Supporting Information). Chitosan, a cationic polysaccharide, is selected as a target polysaccharide for the bioconjugation reaction. It is an attractive material in the biomedical field and has a wide range of applications in drug delivery, gene therapy, and tissue engineering due to its high biocompatibility and biodegradability properties. This polysaccharide molecule contains primary amino groups, which are nucleophilic and undergo reactions with a variety of electrophiles such as aldehyde and quinones. In this study, we expressed our recombinant protein by replacing tyrosine residues with unnatural amino acid L-DOPA by the selective pressure incorporation (SPI) method, which is a highly efficient method to express protein with high yield. It enables the simple residue specific incorporation of unnatural amino acids into different recombinant proteins. Cells containing recombinant GFP were expressed in minimal medium containing the 20 natural amino acids, 19 amino acid lacking tyrosine, and 19 amino acids plus L-DOPA and subjected to the SDS-PAGE analysis. Since oxidation of L-DOPA leads to the formation of highly toxic quinone moieties, we performed a series of optimization experiments with different reducing agents such as dithiothreitol (DTT) and L-ascorbic acid at various concentrations. To verify whether the reducing agent selectively plays any role in L-DOPA incorporation, crude bacterial lysates were subjected to gel electrophoresis. The band intensity of the DOPA protein expressed in the presence of the reducing agent was comparable with that of the band expressed in the absence of the reducing agent. This result suggests that the reducing agent played little or no effect in the expression and efficiency of L-DOPA incorporation into GFP (Figure 1a). Furthermore, GFP and GFPdopa were purified on a Ni-NTA affinity column and gel permeation chromatography (GPC) by AKTA Explorer FPLC system in the absence of reducing agent at 4 °C, respectively (Supporting Information Figure S1). The purification yield of GFPdopa was approximately 32 mg/L, which was similar to that of wild type GFP (34 mg/L). Interestingly, incorporation of L-DOPA into GFP altered the spectral properties; a distinct 19 nm red shift was observed in the emission peak (530 nm) when compared with the GFP protein (511 nm) and a distinct 11 nm shift was observed in the excitation peak (512 nm) compared to the GFP protein (501 nm) (Figure 1c). This shift might be due to the presence of additional hydroxyl group (electron donor) of L-DOPA when compared with L-tyrosine (Y66), which alters the chromophore chemistry. Further, to identify that L-DOPA was incorporated into the GFP protein, we employed a redox staining method using nitroblue tetrazolium (NBT) which detects proteins containing catechols, like DOPA, after their separation by SDS-PAGE and western transfer to the polyvinylidene membranes. This staining method confirmed the presence of L-DOPA only in the GFPdopa (Supporting Information Figure S2). Next, we investigated that this replacement occurred specifically in place of tyrosine, which is further confirmed by LC-MS/MS and ESI-MS.
Figure 1. (a) Polyacrylamide gel electrophoresis of protein labeled with \( \text{-DOPA} \). The labeled proteins are indicated by an arrow. Lane M: protein ladder (Promega prestained Ladder). Lane 1: positive control (expressed with all 20 canonical amino acid). Lane 2: test (expressed with 19 amino acid plus \( \text{-DOPA} \)). Lane 3: test 1 (expressed with 19 amino acid plus \( \text{-DOPA} \) with \( \text{-ascorbic acid} \)). Lane 4: test 2 (expressed with 19 amino acid plus \( \text{-DOPA} \) with DTT). Lane 5: negative control (expressed only with 19 amino acids). (b) Purified GFP shows the green native color (A) and GFPdopa shows changed color (B). (c) Normalized fluorescence excitation spectrum (A), emission spectrum (B) of GFP (~), and GFPdopa (~).

(Supporting Information Table S2 and Figure S3), and the efficiency is >90%.

The successful design and confirmation of a protein with a novel \( \text{-DOPA} \) moiety further allowed us to prepare functional protein—polysaccharide complexes. To substantiate the reaction outlined in Scheme 1, purified GFP and GFPdopa was treated with sodium periodate (NaIO\(_4\)) and incubated with chitosan at room temperature. We could observe the slight fluorescence reduction in GFP and GFPdopa (less than 10%) during NaIO\(_4\) treatment; however, the amount of reduction is negligible (Supporting Information Figure S4—SS). Initially, we have evaluated the pH dependency of GFP and GFPdopa. The GFP-chitosan conjugation efficiency was estimated by using the intensity of the fluorescence at different conditions such as precipitation, washing, and resolubilization (Supporting Information). It indicates that GFP and GFPdopa fluorescence also depends on the pH of the environment (Supporting Information Figure S6) and also well correlated with the earlier report about the effect of pH on GFP.\(^{[15]}\) On the basis of the observation, pH 6.0 was selected for estimating fluorescence in the conjugation experiment. Initially, individual proteins were pretreated with periodate to convert DOPA into an ortho-quinone intermediate. After conjugation, chitosan was precipitated by raising the pH of solution to basic. Subsequently, the pellet and supernatant retrieved after centrifugation were subjected to fluorescence analysis and the relative amounts of proteins that conjugated successfully were assessed. As shown in Figure 2, fluorescence was almost exclusively observed (only 10% reduction was observed) in the supernatant of GFP, GFPdopa not treated with NaIO\(_4\), and NaIO\(_4\) treated GFP. Such a 10% reduction of fluorescence might be due to the NaIO\(_4\) effect on the protein. In contrast, a strong reduction in fluorescence was observed in NaIO\(_4\) treated GFPdopa. To measure the relative amount of protein conjugated with chitosan, we compared the initial fluorescence with the fluorescence retained in supernatants. On the basis of fluorescence intensity of the supernatant, efficiency of conjugation between GFPdopa and chitosan in the presence of NaIO\(_4\) was around 90%. GFP and GFPdopa pellets obtained by centrifugation were washed with PBS buffer to remove physically bound proteins and then resolubilized in aqueous acetic acid solution (Supporting Information). A strong fluorescence appeared in the resolubilized pellet of NaIO\(_4\) treated GFPdopa—chitosan conjugates. A modest level of fluorescence was observed in NaIO\(_4\) treated GFP reacted with chitosan; it may be due to unspecific binding of the protein rather than the GFP—chitosan bioconjugation (Figure 2a). Similarly, we observed a little fluorescence in the GFPdopa—chitosan conjugates, which are prepared in the absence of NaIO\(_4\). This little fluorescence may be due to the nonspecific binding of GFPdopa with chitosan during precipitation. This observation clearly indicates that cross-linking occurred specifically between the DOPA-containing protein and chitosan only in the presence of periodate. To be specific, the fluorescence results suggest that >90% of the initial fluorescence of GFPdopa was in the pellet after NaIO\(_4\) activation, whereas 10% when GFP treated with NaIO\(_4\) had occurred by physical entrapment. This demonstrates that conjugation of GFPdopa with chitosan occurs only through the quinone moiety rather than the canonical amino acids. Strong evidence that the quinone containing GFPdopa can be conjugated to chitosan is provided in Figure 2b. The cast chitosan films were incubated with GFP and GFPdopa in the presence and absence of NaIO\(_4\) (Supporting Information). These treated films were observed to have a relatively significant increase in absorbance at wavelengths above 350 nm. Control films incubated with or without protein show little absorbance above...
350 nm, and the films incubated with the GFP protein, NaIO₄ show no increased absorbance above 350 nm. Increased absorbance in the UV–vis range was identified in chitosan films incubated with NaIO₄ treated GFPdopa protein, which provide the evidence for quinone mediated reaction (Supporting Information Figure S7). It indicates that NaIO₄ selectively generates the quinone from the GFPdopa and facilitates the assembly onto the chitosan. To evaluate our currently developed bioconjugation method, proteins were further conjugated to chitosan hydrogels under heterogeneous conditions. Chitosan hydrogels were prepared as described earlier⁸ (see Scheme SI in Supporting Information). After the hydrogel preparation, NaIO₄ solution was spread onto the surface and treated with GFP and GFPdopa protein, respectively. After incubation, to remove nonspecifically bound proteins hydrogels were thoroughly washed with PBS buffer and imaged using a fluorescence microscope. Hydrogel samples were prepared for fluorescence analysis and each set of images obtained under identical condition. As expected, the hydrogel treated with the GFPdopa protein in the presence of NaIO₄ had a high level of fluorescence, whereas gels treated with chitosan and GFP in the presence of NaIO₄ has no significant fluorescence (Figure 2b). Interestingly, the initial bioconjugation study with chitosan solution shows around 10% physical entrapment of protein. However, in the bioconjugation study with hydrogel, we could not observe such physical entrapment of GFPdopa protein on chitosan.

This indicates that the nonspecific binding of GFPdopa protein is higher with chitosan solution when compared to the chitosan hydrogel. This is due to the interior physical entrapment of proteins by chitosan, which occurred when the pH was raised for precipitation. During the washing step, the nonspecifically bound protein onto chitosan surface could be easily removed, but the proteins which are trapped in the interior are very difficult to remove in the washing process. On the other hand, in the case of the bioconjugation reaction with hydrogel and spot assay, binding occurs only on the surface of the chitosan, and these nonspecifically bound GFP and GFPdopa proteins could be removed during the washing step. Next, we utilized this current strategy to develop the protein spot. For this purpose, we prepared chitosan on glass side, and further, quinone containing GFPdopa was covalently assembled onto the chitosan surface (Figure 2c) (Supporting Information Figure S8). Binding was quantified densitometrically using the Scion Image PC software package. Densitometric analysis revealed the high abundance binding of GFPdopa protein with chitosan coated in the glass slide (Supporting Information, Figure S8).

In conclusion, we developed a simple, efficient, and facile method to prepare bioconjugates by activating protein genetically encoded with L-DOPA, which allows it to be covalently coupled with chitosan. This is the first report that demonstrates the effective protein—polysaccharide bioconjugation through a combination of periodate mediated oxidation chemistry and genetic code engineering. The combination of biochemical conjugation with selective cross-linking chemistry will provide an alternative approach for the assembly of proteins onto the patterned surfaces. This approach can be used to explore the role of the adhesive and cross-linking nature of DOPA in mussel adhesive protein, which can be used in developing novel biosensor and in synthetic biology applications.

ASSOCIATED CONTENT

Supporting Information. Full experimental procedures, protein purification, LC MS/MS analysis, bioconjugation reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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