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Primary Arylamine-Based Tyrosine-Targeted Protein Modification

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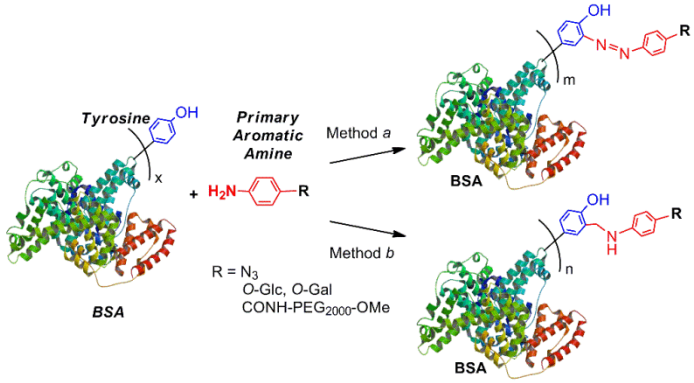


Primary arylamines were demonstrated to site-specifically incorporate bioorthogonal azide functionality, glycans for glyco-engineering, and PEG chains for PEGylation to protein *via* tyrosine-targeted modification.

Abstract. Tyrosine-targeted modification is of great interest in the site-specific protein modification applications. Aniline derivatives are attractive molecules for tyrosine-targeted protein modifications through either diazonium coupling or three-component Mannich type reactions. In this report, with BSA as a model protein, primary arylamines were demonstrated to incorporate bioorthogonal azide functionality for further site-specific protein modification *via* click chemistry, glycans for glyco-engineering, and PEG chains for PEGylation to the protein *via* tyrosine-targeted modification. The successful primary arylamine-based BSA modifications were confirmed by SDS-PAGE, Western blot, and MALDI-TOF mass spectrometry. In comparison, three-component Mannich type reaction affords much higher reaction yields than diazonium coupling reaction in all modifications. Further, this study confirmed the importance of the electron withdrawing substituent on the para position of phenyl ring for tyrosine-targeted diazonium coupling reaction.

***Keywords*:** Primary arylamine, tyrosine, diazoanium coupling, three-component Mannich type reaction, glycan

Introduction

Chemical modification of proteins is a very important and necessary approach for understanding protein structure and function and the mechanisms of biological pathways that proteins are involved in. For example, protein labelling can facilitate protein localization, binding partner identification, and purification as well.[1] Furthermore, chemical modification can expand protein's functional capacity, especially for therapeutic proteins for enhanced pharmacodynamic and pharmacokinetic properties.[2] The key point for protein modification is to carry out a well-defined site-specific chemistry without reducing the protein's original activity. A diversity of chemically reactive functionalities exists on proteins and their random modifications often diminish the protein activity. Recent advances facilitate site-specific modification by targeting to either a specific endogenous amino acid or an unnatural amino acid introduced through recombinant techniques in the protein of interest.[3,4]

For modification of specific endogenous amino acids, it can be targeting to thiols of cysteine residues, amines of lysine residues and the *N*-terminus, carboxylates of aspartate and glutamate residues and the *C*-terminus of the protein.[5] However, most of these conjugation protocols suffer from either harsh reaction conditions that often induce protein denaturation or lower reaction yields. Recently, significant attention has been paid to the bioorthogonal modification of the aromatic amino acid side chains. In particular, tyrosine is an attractive target for protein modification because of its relatively infrequent occurrence (e.g., versus other reactive endogenous amino acids such as lysine) and ability to be modified without altering charge. One tyrosine modification method is a palladium-catalyzed allylic alkylation of tyrosine residues, which employs electrophilic π-allyl intermediates derived from allylic acetate and carbamate precursors to modify proteins in aqueous solution at room temperature.[6] Other methods were also developed for tyrosine-targeted protein modification with aniline derivatives, in which the phenol group present on tyrosine can react as nucleophile through addition to multiple activated bonds. Diazonium coupling reaction has been developed, in which a diazonium salt derived from the aniline derivatives couples to the phenol of tyrosine residues in proteins.[7] The reaction occurs on the ortho position to tyrosine phenol group. This diazonium-mediated targeting of tyrosine residues has been applied to polymer conjugation of medically relevant proteins and peptides.[8] Another approach is the three-component Mannich type reaction, which uses aniline derivatives and an aldehyde allowing for highly selective tyrosine

residue modification at the ortho position on phenol group as well.[9] The three-component Mannich type reaction occurs in mild conditions and low millimolar concentration of reagents at pH 5.5 - 6.5.[10] Overall, both methods use aniline derivatives and are tyrosine-targeted in mild, biocompatible, and metal-free conditions and have been explored for specific protein modification applications.[11-14] To date, no report has described a comparison study between these two tyrosine-targeted modifications with common primary arylamine derivatives.

Bovine serum albumin (BSA) is the most abundant plasma protein and is widely used as carrier or supporter protein for vaccine and affinity ligand engineering. BSA also is often used in many immunochemical experiments such as ELISA, immunoblotting and immunohistochemical studies to block nonspecific binding sites. In this study, we examined tyrosine-targeted modifications of BSA with primary arylamine derivatives of azide, glycan, and PEG molecules *via* diazonium coupling reaction and three-component Mannich type reaction, respectively (Figure 1). Tyrosine modification with an azide provides bioorthogonal functionality for site-specific and biocompatible protein modification *via* copper-free click chemistry,[10] and tyrosine modification with glycans provides a novel approach for protein glyco-engineering applications, which could enhance protein stabilities and pharmacokinetics. In addition, tyrosine-targeted PEGylation prolongs the half-life and enhance the pharmacokinetics of therapeutic proteins too.

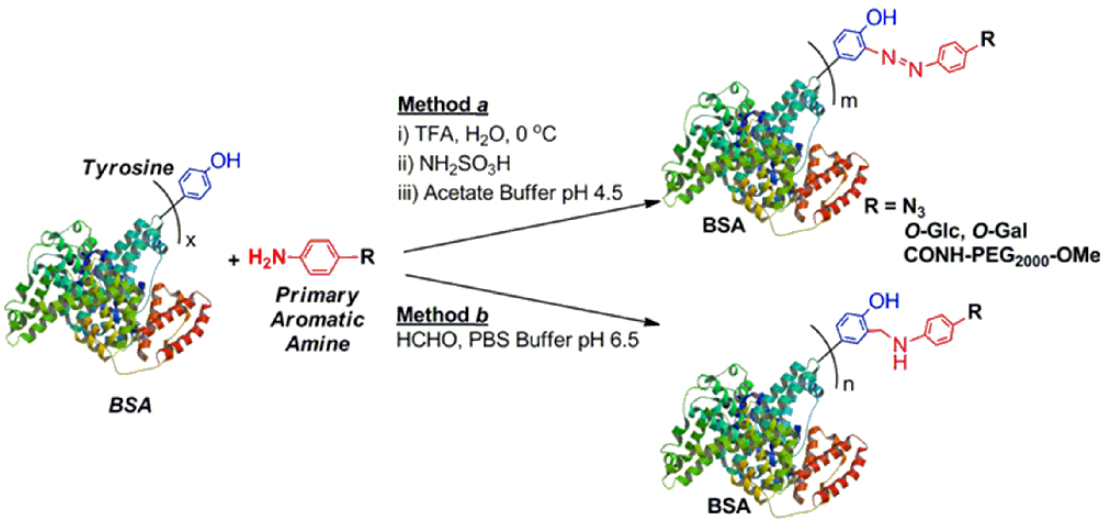


Figure 1. Primary arylamine-based tyrosine-targeted modification of BSA *via* diazonium coupling reaction (Method *a*) and three-component Mannich type reaction (Method *b*).

Results and Discussion

Tyrosine-targeted azide modification of protein

The azide functional group has been a versatile bioorthogonal chemical reporter in the biomolecules for highly selective and biocompatible bioconjugation since it is small, inert, and stable in physiological settings.[3] It has been introduced to diverse classes of biomolecules, such as proteins, lipids and glycans for bioorthogonal ligation with high reactivity, selectivity, and excellent stability under physiological conditions.[15] Various chemical, enzymatic, and genetic methods have been explored to introduce azide functional group into the biomolecules so far. In this study, we investigated a novel tyrosine-targeted azide modification of BSA with 4-azidoaniline *via* diazonium coupling reaction and three-component Mannich type reaction, respectively.

BSA contains 21 tyrosine residues,[16] which allow for multiple azide modifications. Initially, diazonium coupling reaction was conducted by first converting 4-azidoaniline to diazonium derivative with TFA and $NaNO_2$ at 0 °C, then reacting it with BSA in 0.5 M Acetate Buffer (pH 4.5) at 4 °C for 48 h. For the three-component Mannich type reaction, 4-azidoaniline, formaldehyde, and BSA were mixed in 0.1 M PBS buffer (pH 6.5) and were reacted at room temperature for 48 h. The successful conjugations *via* both reactions were confirmed by SDS-PAGE, the presence of protein was verified by typical Coomassie blue staining, in which BSA conjugates through diazonium coupling reaction show a slight molecular weight (MW) increase (Fig. 2A, Lane 2), while BSA conjugates *via* three-component Mannich type reaction show larger MW increase (Fig. 2A, Lane 3). In addition, the BSA-azide conjugates were confirmed by using MALDI-TOF mass spectrometry. Compared to BSA (Fig. 3A), BSA-azide conjugate displayed 740 D molecular weight increase (approximately 5 tyrosine residues were modified) *via* diazonium coupling reaction (Fig. 3B), while 2109 D molecular weight increase (approximately 14 tyrosine residues were modified) *via* three-component Mannich type reaction (Fig. 3C). The MALDI-TOF MS results correlate well with the SDS-PAGE results above. These results indicated that both types reaction with primary arylamine derivative of azide afforded tyrosine-targeted azide modification. However, three-component Mannich type reaction afforded three-fold higher yield of modification than diazonium coupling reaction.

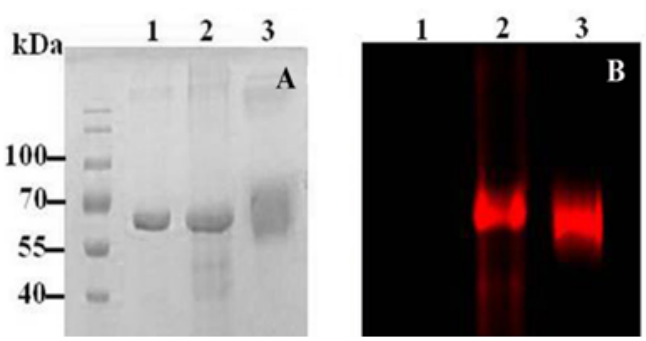


Figure 2. SDS-PAGE gel of tyrosine-targeted azide modification of BSA *via* diazonium coupling reaction (Method ***a***) and three-component Mannichtype reaction (Method ***b***) (**A**) and fluorescence image of SDS-PAGE gel after click labeling of BSA-azide conjugates obtained *via* Method ***a*** and ***b*** (**B**). Lane 1: BSA, Lane 2: 4-azidoaniline conjugation *via* Method ***a***, Lane 3: 4-azidoaniline conjugation *via* Method ***b***.

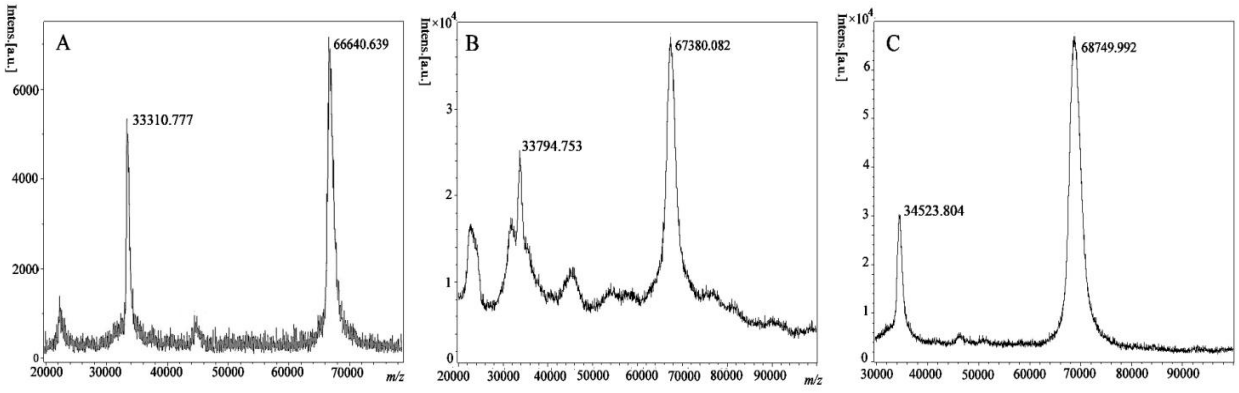


Figure 3. MALDI-TOF MS spectra of BSA (**A**) and tyrosine-targeted azide modification of BSA *via* Method ***a*** (**B**) and ***b*** (**C**). Matrix: saturated solution of sinapinic acid in 0.1% TFA/ACN (1:1, v/v).

Next, to test the reaction selectivity of the azide functional groups introduced, we explored regio- and chemoselective protein modification with cyclooctyne functionalized fluorescent dye Alexa Fluor® 647 DIBO (Life Technologies, USA) through copper-free click chemistry in PBS buffer (pH 7.4) at room temperature for 3 h. BSA was used as a negative control and subjected to the same reaction condition. The fluorescent images from the gel were detected by using a Typhoon 9410 fluorescent imaging system. BSA-azide conjugates were fluorescent (Fig.2B,

Lane 2 and 3), whereas no fluorescence was detected from control BSA (Fig.2B, Lane 1) upon the same copper-free click chemistry. These results indicated that the azide moiety in the protein was reactive toward the cyclooctyne and could be conjugated to cyclooctyne-containing molecules for site-specific protein modification applications.

Tyrosine-specific glyco-modification of protein

Glyco-engineering aimed adding carbohydrates to protein is an attractive approach to enhance therapeutic protein's efficacy, such as increasing its *in vivo* activity and prolonging the duration of action.[17] A successful example is the discovery of darbepoetinalfa, an erythropoietin analogue that contains additional carbohydrates resulting in a threefold increase in serum half-life and increased *in vivo* activity compared to recombinant human erythropoietin.[18] Several methods such as chemical and enzymatic methods have been developed for protein glyco-modification.[19] In this study, we examined a novel tyrosine-targeted glyco-modification of BSA with commercially available 4-aminophenyl β-D-glucopyranoside and 4-aminophenyl β-D-galactopyranoside (Sigma-Aldrich) *via* diazonium coupling reaction and three-component Mannich type reaction as above, respectively. The successful glyco-conjugations of BSA were confirmed by SDS-PAGE with the increased molecular weight of BSA conjugates observed. As shown in Fig. 4, the presence of protein was verified by typical Coomassie blue staining (Fig. 4A), while the presence of carbohydrates was confirmed by carbohydrate-specific staining (Fig. 4B). As a result, BSA-Glc and BSA-Gal conjugates were vaguely visualized for diazonium coupling reaction product (Fig. 4B, Lane 2 and 3) and clearly visualized for three-component Mannich type reaction product (Fig. 4B, Lane 4 and 5), while BSA alone was not stained by the carbohydrate staining (Fig. 4B, Lanes 1). In particular, BSA-Glc and BSA-Gal conjugates *via* three-component Mannich type reaction showed stronger glyco-staining and relative higher MW increase comparing to those *via* diazonium coupling reactions.

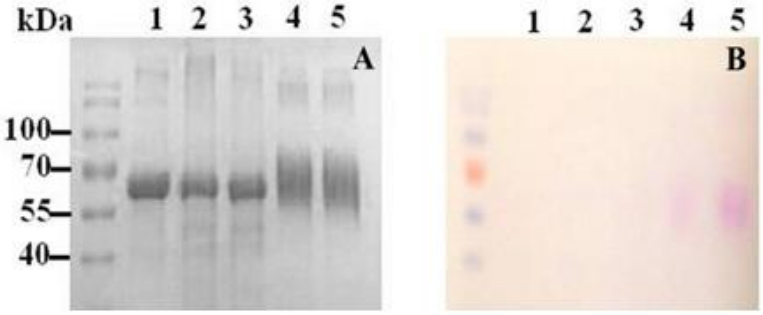


Figure 4. SDS-PAGE gel of tyrosine-targeted glyco-modification of BSA *via* Method ***a*** and ***b*** with Coomassie blue (**A**) and glyco-staining (**B**). Lane 1: BSA; Lane 2: 4-aminophenyl β-D-glucopyranoside modification of BSA *via* Method ***a***, Lane 3: 4-aminophenyl β-D-galactopyranoside modification of BSA *via* Method ***a***, Lane 4: 4-aminophenyl β-D-glucopyranoside modification of BSA *via* Method ***b***, Lane 5: 4-aminophenyl β-D-galactopyranoside modification of BSA *via* Method ***b***.

Further, BSA glycoconjugates were confirmed by using MALDI-TOF mass spectrometry. Compared to BSA, BSA-Glc conjugate showed 331 D molecular weight increase (approximately 1 or 2 tyrosine residues were modified) *via* diazonium coupling reaction (Fig. 5A), while 5363 D molecular weight increase (approximately 19 tyrosine residues were modified) *via* three-component Mannich type reaction (Fig. 5C). On the other hand, BSA-Gal conjugate displayed 913 D molecular weight increase (approximately 3 tyrosine residues were modified) *via* diazonium coupling reaction (Fig. 5B), while 5884 D molecular weight increase (approximately 21 tyrosine residues were modified) *via* three-component Mannich type reaction (Fig. 5D). These results indicated that both two types reaction with primary arylamine derivatives of glycans afforded tyrosine-targeted glyco-modification. Again, three-component Mannich type reaction afforded seven-fold higher yield of modification, which is similar to BSA azide modification above.

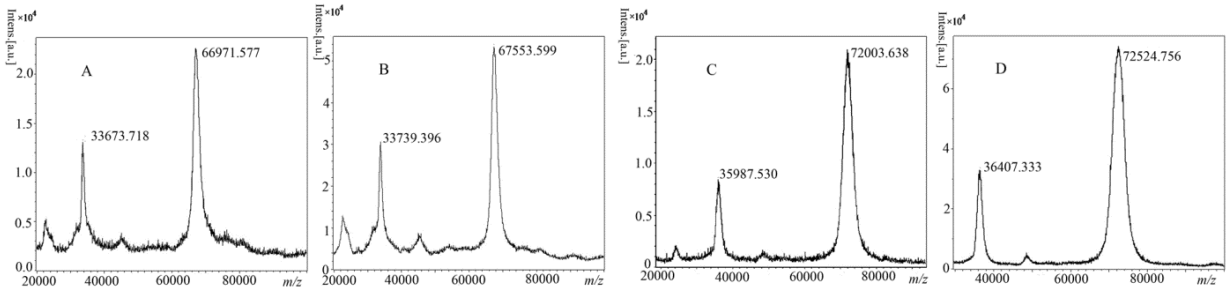


Figure 5. MALDI-TOF MS spectra of tyrosine-targeted glyco-modification of BSA with 4-aminophenyl β-D-glucopyranoside modification *via* Method ***a*** (**A**) and ***b*** (**C**) and 4-aminophenyl β-D-galactopyranoside modification *via* Method ***a*** (**B**) and ***b*** (**D**). Matrix: saturated solution of sinapinic acid in 0.1% TFA/ACN (1:1, v/v).

The low yield of diazonium coupling-based tyrosine-targeted glyco-modification may be due to low diazonium formation yield, low reactivity of the *O*-glycoside diazonium derivatives, and/or low stability of diazonium derivatives during relatively harsh acidic condition. Herein, we examined the diazonium formation and the stability of *O*-glycosides under the same acidic condition with and without BSA by monitoring UV spectrum over time, respectively. As a result, diazonium formations were observed (as $\lambda_{max.}$ = 303 nm) from both 4-aminophenyl-*O*-Glc and 4-aminophenyl-*O*-Gal after reacting with TFA and $NaNO_2$ at 0 °C, respectively (Fig. 6A and 6B). However, no apparent diazonium coupling products formation were observed when adding BSA in 0.5 M acetate buffer (pH 4.5) at 4 °C for 48 h for both reactions (Fig. 6A and 6B). This indicated the lower reactivity of the aryl diazonium salts bearing oxygen groups in the para position, which has both electron-withdrawing and electron-donating properties. This result is in agreement with the original report that substrate scope is limited to aryl diazonium salts bearing electron-withdrawing groups in the para position.[7] The diazonium formation was also confirmed by ^{13}C NMR (see Supporting Information, Fig. S1). In order to further confirm this postulation, we examined the diazonium formation and coupling reaction of 4-azidoaniline under the same acidic condition with and without BSA since it gives higher tyrosine-targeted modification yield above. As a result, both diazonium formation and diazonium coupling product formation were observed (Fig. 6C). Azide group is electron-withdrawing and thus gives relatively higher diazonium coupling reactivity. Taken together, the low yield of 4-aminophenyl-*O*-glycoside-based tyrosine-targeted glyco-modification is due to the low reactivity of the

diazonium *O*-glycoside derivatives. This study further confirmed the importance of the electron withdrawing substituent on the para position of phenyl ring of diazonium reagent for tyrosine-targeted diazonium coupling reaction.[7]

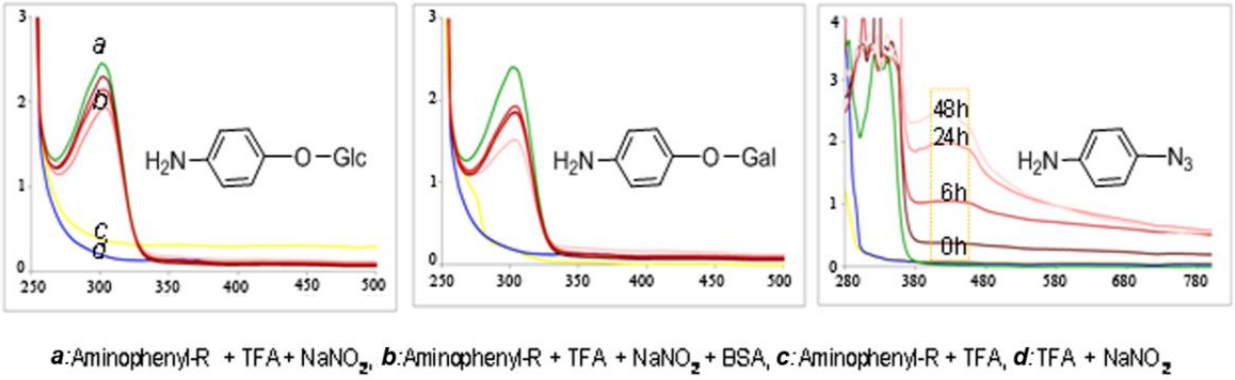
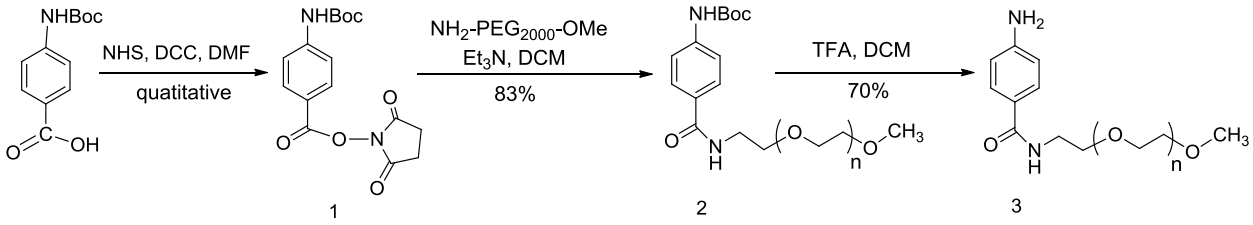


Figure 6. UV spectroscopy monitoring of tyrosine-targeted glyco- and azide modification of BSA *via* Method ***a*** with 4-aminophenyl β-D-glucopyranoside (**A**), 4-aminophenyl β-D-galactopyranoside (**B)**, and 4-azidoaniline (**C**).

Tyrosine-specific PEGylation of protein

PEGylation, covalent attachment of PEG polymer to proteins, has become a practical approach to prolong the half-life and enhance the pharmacokinetics of therapeutic proteins.[20] Jones et al reported a diazonium-based tyrosine PEGylation with aminobenzyl-*O*-PEG_n-OMe.[8] In the present study, we tested the feasibility of tyrosine-targeted PEGylation of BSA with 4-aminobenzoyl-*N*-PEG_{2000}-OMe through both diazonium coupling reaction and three-component Mannich type reaction. The 4-aminobenzoyl-*N*-PEG_{2000}-OMe was synthesized from commercially available amine-PEG_{2000}-OMe *via* amidation with Boc-4-Abz-NHS, followed by deprotection of Boc group, all in good yields (Scheme 1) (characterization see Supporting Information, Fig.S2 and S3). Next, tyrosine-targeted PEGylation was conducted *via* two types reaction as above, respectively. The PEGylated products were confirmed by SDS-PAGE and western blot using anti-PEG mAb, in which PEGylated products with relative higher MW increase in comparison to unmodified BSA were observed (Fig. 7A and 7B). In addition, the BSA-PEG conjugate displayed 7036 D molecular weight increase (approximately 3 tyrosine

residues were modified) *via* diazonium coupling reaction, while 9808 D molecular weight increase (approximately 5 tyrosine residues were modified) *via* three-component Mannich reaction (See Supporting Information Figure S4). These MALDI-TOF MS results are well correlated with the SDS-PAGE results above. These results indicated that both diazonium coupling reaction and three-component Mannich type reaction afforded tyrosine-targeted PEGylation.



Scheme 1. Synthesis of 4-aminobenzoyl-*N*-PEG_{2000}-OMe.

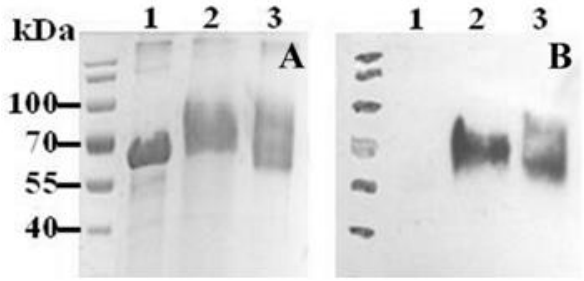


Figure 7. SDS-PAGE gel (**A**) and Western blot (**B**) of tyrosine-specific PEGylation of BSA *via* Method ***a*** and ***b***. Lane 1: BSA, Lane 2: PEGylation of BSA *via* Method *a*, Lane 3: PEGylation of BSA *via* Method *b*.

Overall, three-component Mannich type reaction afforded higher reaction yield than diazonium coupling reaction for tyrosine-targeted protein modification for all three modifications (Table 1). Compared to 4-azidoaniline and aminophenyl-*O*-Glc and aminophenyl-*O*-Gal, 4-aminobenzoyl-PEG afforded relative low yield of modification in three-component Mannich type reaction. This might be due to the steric hindrance of the polymer when conjugating to the tyrosine residues in the protein.

Table 1. Estimated average number of modified tyrosine residues in BSA *via* the two methods by using primary arylamine derivatives.*

R-Arylamine	*Diazonium coupling reaction*	*Three-component Mannich type reaction*
N_3	5	14
O-Glc	2	19
O-Gal	3	21
CONH-PEG	3	5

* *Based on MALDI-TOF MS data*

Conclusion

We have systematically investigated tyrosine-targeted protein modifications with primary arylamine derivative of azide, glycan and PEG molecules *via* diazonium coupling reaction and three-component Mannich type reaction, respectively. Both methods afforded tyrosine-targeted azide, glycan and PEG modifications. However, three-component Mannich type reaction afforded more than two to eight fold higher tyrosine-targeted modification yield compared to diazonium coupling reaction. In particular, almost 100% of tyrosine residues were modified through three-component Mannich type reaction with 4-aminophenyl-*O*-β-D-glucopyranoside and 4-aminophenyl-*O*-β-D-galactopyranoside. Our results further indicated that the electron withdrawing substituent on the para position of phenyl ring of diazonium reagent is necessary for tyrosine-targeted diazonium coupling reaction. In addition, diazonium coupling reaction depends on two-step reaction, in which the diazonium formation and its reactivity are the key points. Overall, combination of mild reaction conditions at pH 6.5 and a simple reaction procedure make the three-component Mannich type reaction a practical tyrosine-targeted protein modification with primary arylamine derivatives.

Experimental Section

Materials and Reagents. All reagents and solvents were purchased from commercial sources and were used as received unless otherwise noted. Albumin from bovine serum (BSA), 4-(Boc-amino)benzoic acid (Boc-4-Abz-OH), 4-azidoaniline, 4-aminophenyl β-D-glucopyranoside and 4-aminophenyl β-D-galactopyranoside were purchased from Sigma-Aldrich (St.Louis, USA).

Methoxy PEG amine HCl salt (MW 2000) was purchased from JenKem Technology USA Inc. (Allen, USA). Alexa Fluor® 647 DIBOwas purchased from Life Technologies USA. Mouse anti-PEG monoclonal antibody was purchased from Academia Sinica Taiwan.

General procedures

Tyrosine-specific modification of BSA-Method *a*. Aniline reagent (6.67 μmol) in 200 μL of H_2O was cooled to 0 °C in an ice bath. TFA (0.99 μL, 13.34 μmol) in 50 μL of H_2O was combined with $NaNO_2$ (0.69 mg, 10.01 μmol) in 50 μL of H_2O. The solution was cooled to 0 °C and added to aniline reagent solution and allowed to stir at 0 °C for 30 minutes. Then NH_2SO_3H (1.29 mg, 13.34 μmol) in 100 μL of H_2O was added. The solution was left to stir for another 20 minutes. BSA (0.44 mg, 6.67 nmol) in 200 μL of 0.5 M acetate Buffer (pH 4.5) was added. pH was measured to be 4.5. The solution was left to stir at 4 °C for 48 h. Finally, the reaction mixture was dialyzed (10 kDa cut-off) against deionized water for two days to remove all reagents and then lyophilized to afford the protein conjugates.

Tyrosine-specific modification of BSA-Method *b*. 200 μL of an aniline solution (6.67 μmol) in 0.1 M phosphate buffer (pH 6.5), 200 μL of a formaldehyde solution (0.55 μL, 6.67 μmol) in 0.1 M phosphate buffer (pH 6.5), and 200 μL of protein solution (0.44 mg, 6.67 nmol) in 0.1 M phosphate buffer (pH 6.5) were mixed to a microcentrifuge tube. pH was checked to be 6.5.The mixture was vortexed briefly to mix the reaction components, then allowed to stand at room temperature for 48 h. Finally, the reaction mixture was dialyzed (10 kDa cut-off) against deionized water for two days to remove all reagents and then lyophilized to afford the protein conjugates.

Fluorescence labelling of BSA-Azide Conjugates. For the azide modification, Alexa Fluor® 647 DIBO (50 mM) in 3 μL of PBS buffer (pH 7.4) solution was added to 30 μL of BSA-azide sample. The mixture was allowed to incubate in the dark at room temperature for 3 h. BSA without 4-azidoaniline modification was used as a negative control and subjected to the same reaction conditions. Protein samples were analyzed by SDS-PAGE and the fluorescent image from the gel was detected using an imaging system (Typhoon 9410 Variable Mode Imager, Amersham Biosciences, USA).

Glycostaining for BSA-Sugar conjugation. Glycoprotein staining kit (G-Biosciences, USA) was used to detect glyco-modified BSA in gel. It was performed according to the manufacturer's protocol. UV Analyses of BSA-Azide and BSA-Glycoconjugate formation *via* Method *a*. Aniline reagent combined with TFA and $NaNO_2$ (with and without BSA, maintaining the same concentration) was monitored at different time intervals 0, 6, 24, 48 h under UV spectroscopy. Aniline reagents combined with TFA and $NaNO_2$ were used as blank controls. UV analyses were performed on the Varian Cary 50 Bio UV/ Visible Spectrophotometer (Varian Medical Systems, Inc. Palo Alto, USA).

Synthesis of 4-aminobenzoyl-*N*-PEG$_{2000}$-OMe derivative. 4-(Boc-amino)benzoic acid (25.1 mg, 100 µmol) and *N*-hydroxysuccinimide (NHS, 11.5 mg, 100 µmol) were dissolved in 1 mL of DMF. The mixture was cooled to 4 ℃ and *N*,*N*′-dicyclohexylcarbodiimide (24.7 mg, 120 µmol) was added. The reaction was stirred at 25℃ for 24h, then the *N*,*N*′-dicyclohexylurea precipitate was removed by filtration to afford the crude Boc-4-Abz-NHS after concentration under reduced pressure. Then, the crude Boc-4-Abz-NHS and Et_3N (100 µL) were added to a solution of Amine-PEG-OMe HCl salt (MW 2000) (75.4 mg, 37.7 µmol) in 2mL of DCM under argon. The reaction was stirred at room temperature overnight and then concentrated under reduced pressure to afford the crude product, which was purified by silica gel column chromatography with chloroform/methanol (10:1) to give Boc-aminobenzoyl-PEG$_{2000}$-OMe (69.88 mg，83% yield). Finally, Boc-aminobenzoyl-PEG$_{2000}$-OMe was dissolved in a solution of DCM: TFA (1 : 1, v/v) and left to stir at room temperature for 3 hand then concentrated under reduced pressure to afford the crude product, which was dissolved in chloroform (2 mL) and precipitated with cold diethyl ether, followed by filtration and washing with diethyl ether to afford the 4-aminobenzoyl-*N*-PEG$_{2000}$-OMe derivative (56.2 mg, 70% yield).^{1}H NMR (400MHz, CD_3OD): δ, 7.64 (br.d, 2H, *J* = 8.9 Hz，Ar-H), 6.71 (br.d, 2H, *J* = 8.9 Hz, Ar-H), 3.62-3.69(m, 190H, $(OCH_2CH_2O)_n$), 3.38 (s, 3H，CH_3). ^{13}C NMR (400 MHz, CD_3OD): δ, 39.4, 57.7, 70.1, 71.6, 113.6, 122.2, 128.6, 151.2, 168.9.

Western blot. SDS-PAGE gel was transferred onto polyvinylidene fluoride (PVDF) membranes and incubated for 1 h inblocking buffer at room temperature. BSA-PEG conjugate was detected by1:1000 dilution of mouse anti-PEG monoclonal antibody (Academia Sinica, Taiwan) for 1h at room temperature followed by 1 h incubation with1:3000 dilution of goat anti-mouse polyclonal

secondary antibody (GenScript USA Inc). Bands were detected by enhanced chemiluminescence (ECL) and developed using an automated chemiluminescence detector.

MALDI-TOF MS Analysis. MALDI-TOF-MS spectra were performed on a BrukerautoflexⅢsmartbeam instrument (Bruker, Germany) operating in positive linear mode. 10μLMillipore® C4Ziptip pipette tips were used for the protein sample preparation. A saturated solution of sinapinic acid in 0.1% TFA/ACN (1:1, v/v) was used as a matrix and mixed with the samples dissolved in 0.1% TFA aqueous solution at a v/v ratio 1:1.

Abbreviations

BSA Bovine serum albumin

Gal Galactopyranoside

Glc Glucopyranoside

PVDF Polyvinylidene fluoride

TFA Trifluoroacetic acid

Competing interests

The authors declare no competing interests.

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Notes and references

†Electronic Supplementary Information (ESI) available: ^{13}C NMR spectra monitoring of tyrosine-specific glyco-modification of BSA via Method a and ^{1}H and ^{13}C NMR spectra of 4-Aminbenzyl-functionalized PEG derivative.

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