Genetically Encoded Dihydroxyphenylalanine Coupled with Tyrosinase for Strain Promoted Labeling

Augustine George

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Mohan Indhu

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Sundarapandian Ashokraj

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Ganesh Shanmugam

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Ponesakki Ganesan

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Numbi Ramudu Kamini

CSIR-Central Leather Research Institute: Central Leather Research Institute CSIR

Ayyadurai Niraikulam (✉ ayyadurai@clri.res.in)

Central Leather Research Institute CSIR <https://orcid.org/0000-0002-7333-6344>

Research Article

Keywords: Protein modifications, dihydroxyphenylalanine (DOPA), bicyclonon[6.1.0]-4-yne (BCN), dibenzocyclooctyne-PEG-Fluor 545 (DBCO), cell labeling, apoptosis

Posted Date: May 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-296659/v2>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The newly developed molecular biology approach expanding the genetic code was used to incorporate the non-canonical amino acid dihydroxyphenylalanine for fine-tuning of proteins. Further, the congener protein was enzymatically converted to form quinone for strain promoted click chemistry. The reaction yields a single product with defined stereochemistry and temporally controlled conjugation with bicyclonon[6.1.0]-4-yne (BCN) as well as dibenzocyclooctyne-PEG-Fluor 545. The promising bioconjugation of congener protein with dibenzocyclooctyne-PEG-Fluor 545 was used as a fluorescent marker for selective cell imaging and detection of programmed cell death in EAhy926 cells.

Introduction

Protein modifications through chemical and genetic methods have a remarkable impact on cell labeling, bio-conjugation studies, protein translocation, protein-protein interaction [1–2]. The chemo-selective modification of canonical amino acids on proteins had initially been executed via amide coupling of lysine [3], sulfhydryl reaction of cysteine [4], N-terminal transamination [5] and, oxidative coupling of proline without alteration in the protein structure and function [6]. However, most of the chemical modifications have limitations in biocompatibility, regioselectivity, and homogeneity [7]. Despite these challenges, in recent decades' genetic incorporation of non-canonical amino acids [8] for protein modification has extended the possibilities of proteins for novel applications. Later, combinations of genetic code engineering and chemical methods have been employed to prepare the protein conjugates for various purposes [9–12]. Of which, metal (Cu-I) catalyzed azide-alkyne [13] and biocompatible strain promoted oxidation-controlled click reactions were developed and used for *in-vitro* protein labeling, cell imaging, and immobilization. Chemo-selective modifications of proteins were also performed with specificity via an enzymatic approach for conjugating various small molecules to the proteins. Mostly, enzyme-mediated modifications using sortase [14], subtiligase [15], biotin ligase-formyl-generating enzyme (FGE) [16], and tubulin tyrosine ligase were achieved on N and C-terminal tags, and internal motif sequence was appended or inserted onto the proteins [17]. The unprecedented specificity of these enzymes has enabled modification of the full-length proteins with fluorophores through expanding the genetic code.

Here, we selected tyrosine (Tyr) as a suitable chemical moiety for cell-selective labeling due to its hydrophobicity and propensity to form the π - π stacking interaction, which causes restricted exposure of Tyr on protein surface [18–19]. Consequently, any reactive small molecule can be conjugated to the Tyr either through enzymatic or chemical catalyst-based methods. For instance, tyrosinase-mediated modification of Tyr residues in the native protein is a well-established method that has been attempted for cell labeling, antibody-drug conjugates, and tagging the protein and peptides [20–22]. Enzymatic oxidation of Tyr is a well-known multistep reaction, which results in the formation of a by-product and inhibits the enzyme activity as well as generates toxicity. Besides, it is very difficult to maintain specificity, monitor product formation, and remove the enzyme after the reaction. Typically, many of the biological oxidases such as peroxidase, laccase, and polyphenol oxidase can catalyze the oxidation of the catechol

group to *o*-quinones but not the phenol group. Among all oxidases, only tyrosinase can act upon both Tyr and DOPA due to phenol monooxygenase and diphenoxidase activity, respectively [23]. Hence, we aimed to combine the genetic code engineering for the residue-specific incorporation of DOPA into the proteins and subsequent mild treatment of tyrosinase to tag the genetically modified protein with a fluorophore. This quinone-mediated metal-free click chemistry is a useful aspect for protein labeling which readily generates the ortho-quinone from a catechol group through the enzymatic oxidation approach [21].

In principle, orthoquinone is generated from Tyr residue by the catalytic action of tyrosinase through an intermediate-DOPA formation. However, DOPA is also a suitable substrate similar to Tyr for enzyme recognition. Initially, a small molecule DOPA was selected to demonstrate the feasibility of enzyme-mediated quinone reaction. To evaluate the tyrosinase-mediated modification in DOPA incorporated proteins [Annexin A5 (anX) and Green Fluorescent Protein (GFP)], we selected highly reactive strained cyclic ring containing alkynes namely BCN and DBCO, which was already used in life and material science applications via Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC) and Strain-Promoted Oxidation-Controlled Cyclooctyne-1,2-quinone Cycloaddition (SPOCQ) [7, 24, 25, 26]. Initially, the reaction was optimized with BCN to understand the one-step SPOCQ. Then, the identical conjugation reaction was performed with DBCO to explore the selective labeling of cells and apoptosis detection (Scheme 1).

Materials And Methods

Chemicals

PCR reagents, T4 DNA ligase, and restriction endonucleases were obtained from Promega (Madison, WI, USA). Isopropyl- β -D-thiogalactopyranoside (IPTG), lysozyme, DOPA, mushroom tyrosinase (m-tyrosinase), BCN, DBCO, Doxorubicin hydrochloride (Doxo), Dulbecco's Modified Eagle Medium (DMEM) high glucose, Fetal Bovine Serum (FBS), Antibiotic Antimycotic solution (100X), Trypsin-EDTA solution and most of the chemicals required for these experiments were procured from Sigma (St. Louis, USA). The host bacterium *Escherichia coli* (*E. coli*) strain XL1-blue (Strata gene, USA) was used for plasmid DNA preparation. The nickel nitrilotriacetic acid (Ni-NTA) affinity column was purchased from clone tech (Takara Bio, USA). *E. coli* strain JW2581 Tyr auxotroph was obtained from *E. coli* Genetic Stock Centre (Yale University, USA) and plasmid pQE-80L was obtained from Qiagen (Valencia, USA).

Residue-specific incorporation of DOPA

Residue-specific incorporation of DOPA was carried out in *E. coli* JW2581 containing pQE80L-anX and GFP vector as described by George et al [27]. The cells harboring pQE80L-anX and GFP plasmids for residue-specific incorporation were grown in LB broth containing ampicillin and incubated overnight at 37 °C. The cultures were collected after centrifugation at 5000 rpm, 4 °C, 15 mins and 1 % inoculum were added into 20% glucose minimal media (MM) supplemented with 20 amino acids (40 mg/L), 1 mM CaCl₂, 1 mM MgSO₄, thiamine HCl (1 mg/mL) and ampicillin (100 µg/mL). Subsequently, cells were

centrifuged at 5000 rpm for 15 min and washed thoroughly in 1X phosphate buffer saline (1X PBS, pH = 7.4), and further cells were inoculated into the same 20% glucose MM supplemented with 19 amino acids (40 mg/L) except Tyr, 0.03 mM Tyr, and ampicillin (100 µg/mL). The cells were grown to mid-log phase ($OD_{600} = 0.6-0.8$) at 37 °C and 180 rpm. At this point, 1 mM of DOPA was added to the culture flask before IPTG induction. The optimum expression of DOPA incorporated anX and GFP was achieved upon 1 mM IPTG induction for 6 h at 37 °C. As a result, dihydroxyphenylalanine incorporated Annexin A5 (anX-DOPA) and dihydroxyphenylalanine incorporated Green Fluorescent protein (GFP-DOPA) were obtained from residue-specific incorporation methods. Then, the cells were harvested and frozen at -80 °C for further experiments.

Cycloaddition of BCN with DOPA

50 µL of DOPA stock solution (10 mM) was treated with 60 µL of tyrosinase (60 U) in Tris buffer, pH 6.8, and mixtures were incubated at room temperature (RT) in dark condition for 5 min. Subsequently, 100 µL of 2.5 molar excess of BCN stock solution (10 mM) in a 1:1 ratio of methanol and MilliQ (MQ) water was added to the tyrosinase treated DOPA and the reaction was carried out in dark condition at RT for 2 h. Then the reaction mixture was filtered through a 0.22 µm Millipore filter device (Millipore, USA). The tyrosinase-treated DOPA with BCN and tyrosinase-treated DOPA without BCN was subjected to *Ultraviolet-Visible* (UV/Vis) spectroscopy.

Mass Spectrometric Analysis (HR-MS)

Tyrosinase catalyzed dihydroxyphenylalanine conjugated with bicycle [6.1.0] non-4-yne (DOPA-BCN) reaction mixture was lyophilized and dissolved in 1 mL of HPLC grade methanol and was thoroughly filtered using Millex-syringe driven filter unit. Then, 10 µL of samples (DOPA and DOPA-BCN) were injected into the High-resolution mass spectrometry (HR-MS) (Finnigan LCQ advantage max, Thermo Fisher Scientific) equipped with a syringe pump.

Preparation of protein conjugates

The conjugation of fluorescein isothiocyanate with recombinant Annexin A5 (anX-FITC) was prepared by incubation of protein with Fluorescein isothiocyanate (FITC) at 37 °C in the dark for 1 h as described earlier [27]. Initially, 100 µg of FITC was dissolved in Dimethyl sulfoxide (DMSO), and it was sequentially added to 1 mg of protein in 1X PBS at pH 7.4. Eventually, an tyrosinase catalyzed dihydroxyphenylalanine incorporated Annexin A5 conjugated with Dibenzocyclooctyne-PEG4-Fluor545 (anX-DOPA-DBCO) conjugate was prepared by the treating 100 µl of anX-DOPA (0.5 mg) with 120 U of tyrosinase in Tris buffer pH 6.8 and incubated at RT for 5 min. At the same time, similar reactions were performed by mixing the tyrosinase treated anX-DOPA (quinone formation in protein) with DBCO (200 µM) in 0.1% DMSO for 2 h at RT. After labeling, anX-FITC and anX-DOPA-DBCO conjugate were separated from free FITC and DBCO by a 10 kDa cut-off filter (Millipore, USA). Both protein conjugates were excited using fluorescence spectrometric analysis.

Nitroblue Tetrazolium (NBT) Staining

The formation of dopaquinone and BCN conjugation with the tyrosinase-treated DOPA incorporated proteins (GFP-DOPA and anX-DOPA) was confirmed by NBT staining. The reaction mixtures were loaded into the Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred onto the methanol-activated polyvinylidene difluoride (PVDF) membrane. Protein bands on the membrane were incubated in NBT staining solution (2 M sodium glycinate, 0.24 mM NBT, pH 10) in a dark room for 3 h. Subsequently, the membrane was washed with a sodium borate solution overnight for protein band visualization.

Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Analysis

The protein samples were subjected to MALDI-TOF analysis as described earlier [28] and were desalted by a C18 zip-tip (Millipore, USA). After desalting, protein samples were examined by MALDI-TOF with a sinapinic acid matrix on a Micro flex LT instrument (Bruker Daltonik, Germany). The MALDI-TOF was operated in a linear model to provide optimal resolution with a 25 kV extraction voltage and 13 kV reflectron potential.

Cell line and culture conditions

The Endothelial cell type (EA.hy926) cells were obtained from ATCC, Washington DC, USA. The cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic antimycotic solution in humidified air with 5% CO₂ and 37 °C.

Cell viability assay

The EA.hy926 cells were grown till 80-90% confluence and trypsinized using Trypsin-EDTA solution for 2 min [29]. After trypsinization, 2×10^4 cells from the parent flask were cultured in a 96-well plate for 24 h and different concentrations of Doxo were diluted using serum-free DMEM high glucose-containing 1X antibiotic antimycotic solution and added with the cells for 24 h. After the indicated treatment period, the cells were incubated for 3 h at 37 °C in a culture medium containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 1X PBS at pH 7.4. The blue MTT formazan precipitate was then dissolved in 100 µL of DMSO, and the absorbance was measured at 570 nm with a multi-well plate reader. The cell viability was expressed as the percentage of the absorption values in the treated cells relative to the non-treated (control) cells.

Fluorescence microscopy analysis

As described, the above cells (2×10^4) were grown and Doxo (50 µg/mL) was used to induce apoptosis in a 100 µL medium. After the treatment, the cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 10 to 15 min at RT, and washed with 1X anX binding buffer (0.2 µm sterile-filtered 0.1 M HEPES (pH 7.4), 1.4 M NaCl, and 25 mM CaCl₂ solution). Then, the cells were incubated with 0.5

µg/mL concentrations of anX-FITC and anX-DOPA-DBCO with the addition of 1 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) and were diluted by 1X anX binding buffer at pH 7.4 and incubated for 15 min at RT (in dark). Thereafter, cells were washed with 1X anX binding buffer and sealed with a coverslip using glycerol mounting medium. The cells were then viewed by a 10 X objective lens under an inverted fluorescence microscope (Leica DMI8, Germany) using a three-color filter and the image was analyzed by Leica Application suite 4.7 (Leica, Munich, Germany).

Flow Cytometric Analysis

EA.hy926 cells (1×10^5) were grown as described above and were collected by centrifugation at approximately 500 x g for 5-10 mins at RT (18-24°C) and washed by resuspending in 500 µL ice-cold 1X PBS and pelleted by centrifugation. Again, the cells were washed once with 1X anX binding buffer and were stained with anX-FITC, anX-DOPA-DBCO, anX-FITC+Propidium iodide (PI), and anX-FITC+anX-DOPA-DBCO. The staining solution was prepared as follows; working concentration of anX-FITC and anX-DOPA-DBCO (0.5 µg/mL), PI (5 µg/mL), and were diluted in 1X anX binding buffer at pH 7.4. Cells were resuspended in 100 µL of the staining solution and stained for 15 min at RT in the dark. Then, the cells were diluted in 1X binding buffer to a final volume of 400 µL and analysed by flow cytometric analysis (FCM) in BD FACS Cantoll (BD Biosciences, CA) having three color filters. The labeled cells were excited according to the reported excitation wavelength and suitable emission filters were used to detect fluorescence signals. Relative change in the fluorescence was determined by FACS-DiVa software.

Results

Characterization of enzyme-mediated quinone reaction

The cycloaddition reaction was carried out using dopaquinone to confirm the catalytic action of tyrosinase towards the substrate DOPA (Fig. 1a). Initially, DOPA was treated with 60 U of tyrosinase to produce dopaquinone intermediate in tris buffer pH 6.8, which is further converted into dopachrome within a second ($t_{1/2} > 1s$) by an intramolecular cyclization (Michael- 1, 4 additions) and forms stable melanin pigment [30, 31]. As reported by Dukler et al, we also observed a similar absorption spectrum peak corresponding to dopachrome at 475 nm as a consequence of tyrosinase catalysis (Fig. 1b). The conversion of DOPA to dopachrome does not lead to a reduction in the absorbance but leads to a gradual shift in the absorption spectrum [32]. In contrast, a sharp absorbance reduction represents a straight line in the UV/Vis spectral region (200-600 nm) which was obtained after the tyrosinase-treated DOPA conjugated with BCN (Fig. 1b), and this indicates that the reaction is stabilized after the addition of BCN. The absorbance of tyrosinase-treated DOPA was compared with BCN conjugated dopaquinone that provided the relative amount of DOPA conjugated with BCN. The conjugation efficiency between dopaquinone and BCN was found to be around >90%, which was determined by measuring the difference in the intensity of absorbance maxima at 475 nm before and after BCN conjugation. DOPA conjugation was also confirmed by HR-MS analysis with expected molecular mass for DOPA (198.07 m/z) (Fig. 1c) and BCN functionalized dopaquinone (355.18 m/z) (Fig. 1d). As a positive control, Tyr was also

subjected to tyrosinase treatment and subsequently conjugated with BCN to form a Tyr-BCN product which also yielded similar results as DOPA (Fig. S1). These results assured that the formation of dopachrome through intramolecular Michael- 1, 4 additions could be controlled within a short period (10 min) by the addition of BCN.

Biosynthesis of DOPA incorporated proteins and purification

To probe the *in vivo* expression of DOPA incorporated proteins, we selected GFP and anX having different number of surface-exposed Tyr residues. Before proceeding with the protein expression studies, Tyr residues that are located at the proteins' (anX and GFP) surface were perceived by extricating a 3D model structure from PDB (Fig. 2a, S3). GFP and anX were cloned and the expression was optimized in *E. coli* strain JW2581 using 1 mM IPTG [33]. Simultaneously, DOPA was introduced into the proteins (GFP and anX) through the mis-aminoacylation method after depleting the amino acid Tyr [34]. The expression efficiency of GFP-DOPA and anX-DOPA was compared with native proteins via 12% SDS-PAGE analysis (Fig. 2b, S3). The protein with the substitution of DOPA did not significantly alter the expression of proteins at 37 °C. Further, the protein purification was achieved by the Ni-NTA affinity column and was desalted using AKTA Explorer FPLC (Fig. S3). The incorporation efficiency of DOPA in anX was measured and compared with wild-type proteins by MALDI-TOF analysis (Fig. 2c), and the mass of anX was found to be increased from 35937 Da to 36129 Da which indicates the incorporation efficiency of > 98%. The mass of the GFP increased from 28267 Da to 28396 Da on DOPA incorporation (data not shown).

Structural and functional characterization of modified proteins

To resolve the secondary structure of proteins after DOPA incorporation, the protein samples (GFP and anX) were subjected to Circular Dichroism (CD) analysis. Typically, GFP is a beta-sheet protein, showing the positive and negative bands at 195 and 218 nm (Fig. S5), whereas, anX predominantly has alpha-helix with a negative deflection at around 222 nm and 208 nm (Fig. 2d). These two proteins have retained secondary structure with a characteristic beta-sheet and alpha-helix pattern after DOPA incorporation. From these studies, it signifies that GFP-DOPA could maintain its functional property by emitting the orange fluorescence at 525 nm due to the presence of DOPA at the Y66 position of chromophore forming peptide (Fig. S5) and anX-DOPA was able to bind to phosphatidylserine (PS) in the lipid membrane of apoptotic cells (Fig. 4c, d). The functional property of these modified proteins viz., GFP-DOPA and anX-DOPA was also evaluated through fluorescent spectroscopic analysis and further directed to cell labeling studies.

Coupling mechanism in protein for cell labeling

The tyrosinase-mediated modification was explored on DOPA incorporated proteins (GFP and anX) to establish a protein-based cell labeling method. Dopachrome product formation in proteins was optimized by incubating the DOPA incorporated protein (0.5 mg) with 120 U of tyrosinase in Tris buffer pH 6.8 (Fig. 3a). At this pH, the degree of polymerization and cross-linking mediated by a free amino group in the lysine residues are prevented [37]. To substantiate the dopachrome formation in proteins, a

redox staining assay was executed using NBT, for which tyrosinase treated samples were transferred onto PVDF membrane after SDS-PAGE. As a result, purple color owing to the redox cycling of dopaquinone and polymerization (shearing of proteins) was observed on the membrane, which corroborates the conversion of DOPA incorporated protein into quinoprotein by the action of tyrosinase [38] (Fig. S4). Further, residue-specific modification of anX-DOPA was supported by mass spectrometry (MALDI-TOF) with the expected mass shift (36129 Da) (Fig. 2c).

Our results reinforced the previous statement on DOPA modification to produce quinone in a single step and it was identified that 1, 2-quinones could quickly react with BCN [7]. Therefore, we aimed to functionalize BCN with DOPA incorporated proteins by mild stirring for 2 h at RT. After dialysis, bicycle [6.1.0] non-4-yne functionalized dihydroxyphenylalanine incorporated Annexin A5 (anX-DOPA-BCN) was confirmed by SDS-PAGE (Fig. 3b) and MALDI-TOF analysis with anticipated mass shift (37071 Da) (Fig. 3c), representing that five molecules of BCN have reacted with tyrosinase catalysed-anX-DOPA. The suitability of the reaction for residue-specific labeling was inspected and allowed to conduct a SPOCQ on quinoproteins. The anX-DOPA coupled with a strained alkyne fluorophore (DBCO) by the action of the tyrosinase were analyzed by SDS-PAGE (Fig. S6) and MALDI-TOF analysis with a mass shift (40808 Da) indicating that five molecules of DBCO are conjugated with surface-exposed DOPA residues of anX-DOPA (Fig. 3c). Moreover, tyrosinase catalyzed anX-DOPA-DBCO shows emission at 595 nm after dialysis (10 kDa) which signifies that anX-DOPA was tagged with the fluorophore (DBCO) (Fig. 3d). In contrast, anX-DOPA-DBCO was also prepared without tyrosinase as a control experiment, where the fluorescence was not exhibited when exposed to UV/Vis light after the dialysis (Fig. S6). It denotes the importance of tyrosinase for residue-specific protein modification and its key role in labeling experiments. The generality of this method can be applied to any proteins containing fewer numbers of surface-exposed Tyr residues. To test the application of fluorogenically labeled quinoprotein, we employed the single-step strain-promoted oxidation-controlled click reactions for cell labeling and imaging experiments. Hence, the fluorogenically labeled anX-DOPA was used for apoptosis recognition (Fig. 4c,d).

Fluorescent microscopy and flow cytometry analysis of apoptotic EA.hy926 cells

During programmed cell death or apoptosis, the cell loses the phospholipid asymmetry of the plasma membrane, which reshuffles PS from its usual position [39]. In the presence of Ca^{2+} , the protein anX exclusively and sturdily binds with the PS molecule. Therefore, commercially available fluorescent tags such as FITC, Oregon-Green, or Alexa Fluor 488, succinimidyl esters were linked with anX by chemical coupling methods and they were extensively applied to probe apoptosis incidence. However, the classical coupling reactions cannot be controlled due to the presence of the free amino group in the lysine residues, which are mostly surface exposed and exist predominantly in or near the PS binding pocket. The consequent conjugation of anX with FITC would probably result in fluorescence quenching and yield a heterogeneous mixture [40, 41]. The added advantage of genetic code engineering in developing a highly controlled and homogeneously labeled fluorescent anX conjugate without altering its functional property. To evaluate the efficacy of anX-DOPA-DBCO for cell labeling, apoptotic studies were done in EA.hy926 cells. Apoptosis was induced in EA.hy926 cells by treating with different concentrations of

Doxo, and the morphological changes were observed using phase-contrast microscopy. Cells with flattened and disruptive membrane morphology were observed at the concentration of 50 $\mu\text{g/mL}$ Doxo (Fig. S7). The PS-binding activity of anX-DOPA-DBCO was analyzed on apoptotic EA.hy926 cells through flow cytometry. It was compared with the commonly accessible anX-FITC (Fig. 4d, S8). 0.5 $\mu\text{g/mL}$ concentration of anX-FITC and anX-DOPA-DBCO were used to stain the apoptotic cells (Fig. 4b, c) and it was compared to the cells without apoptosis induction (Fig. 4a). The cells treated with anX-DOPA-DBCO responded well for apoptosis and it was more or less similar to anX-FITC, which indicated that apoptotic labeling using anX-DOPA-DBCO resulted in PS binding even after protein modification (Fig. 4b-d).

Discussion

At first, the pertinence and hurdles of the reaction were examined at a small molecule level (DOPA). The *in situ* generated quinone through enzymatic catalysis rapidly reacts with the triple bond of the strained alkyne (BCN) via inverse electron demand Diels-Alder cycloaddition, which is sufficient for the generation of DOPA-BCN conjugate. In principle, the formation of a quinone from Tyr by enzymatic and chemical methods is one of the rapid reactions that non-specifically create covalent cross-linking. This is due to the electrophilic quinone that is readily attacked by the proximate nucleophiles especially lysine, histidine, and cysteine residues in the protein via Michael type additions [35]. Earlier FREMY'S SALT was tested for the direct conversion of Tyr to dopaquinone without the formation of any intermediate. The chemical oxidant-mediated conversion of Tyr to dopaquinone requires a longer incubation time (20 h) and a high concentration of FREMY'S salt (10M excess) for polymerization, intra cross-linking of proteins and peptides, and conjugation of fluorophore [26–36]. Secondly, FREMY'S SALT is a strong oxidant; hence it could polymerize, aggregate, and precipitate the protein. In such a condition, natural enzyme tyrosinase is necessary for reducing the limitations of oxidants like FREMY'S Salt [36]. Usually, chemoselective conjugation for proteins is primed on surface-exposed residues in a suitable reaction condition as it provides high selectivity and accessibility of enzymes towards the least distributed Tyr residues on the surface. The surface-exposed Tyr residues containing proteins (GFP and anX) were selected and through the energy minimization process in the protein modeling, it was predicted that anX has six Tyr residues (Y94, Y133, Y148, Y256, Y257, and Y297) on the surface, out of 11 residues. GFP has eight Tyr residues including chromophore Y66. Out of these 8 residues, 5 residues (Y92, Y151, Y182, Y200, and Y273) are surface exposed, Y143 is partially surface exposed and Y74 is buried internally. The proteins were genetically encoded with DOPA to develop the protein-based cell labeling method. Therefore, DOPA incorporated protein (anX-DOPA) were treated with tyrosinase and subsequently attached with BCN to study the one-step SPOCQ and identical bio-conjugation was performed with DBCO. The advantage of using DBCO (orange fluorophore) over FITC is that it has low quantum yield but high emission maxima, lower light scattering, and reduced autofluorescence [42]. FITC (shorter wavelength) with green spectral window is much more energetic than DBCO (longer wavelength) with red spectral window, as a result, shorter wavelength fluorophores including FITC, DAPI, Alexa-488 has potential photo-bleaching, lesser photo-stability and pH sensitivity in comparison with the light of longer wavelength orange and red fluorophores (DBCO) [43]. We postulate that tyrosinase-mediated modification in anX-DOPA did not alter

the function of proteins, especially for apoptosis detection. These preliminary results pave the way to extend the single-step strain promoted oxidation-controlled click reactions via expanding the genetic code to the other caspase pathway proteins such as Bax and Bcl-XL, which are involved in the early and late events of apoptosis.

Conclusion

In this study, we developed a biorthogonal accelerated tyrosinase-mediated strain-promoted oxidative approach for cell labeling, which needed an easily reactive quinone functional moiety. This was achieved through the incorporation of DOPA in proteins and ensue a single product formation. Most significantly, this method could be used for various applications apart from generating protein conjugates. It can also be applied for targeted drug delivery and proteins for tagging cellular functions. This facile conjugation approach is promising, in particular, for cell labeling and apoptosis detection.

Declarations

Acknowledgments

George A thankfully acknowledges the Council of Scientific and Industrial Research (CSIR) for the award of Senior Research Fellowship (SRF). Indhu Mohan (5/3/8/76/ITR-F/2020-ITR dated 09-03-2020) gratefully acknowledges the Indian Council of Medical Research (ICMR) for the award of Senior Research Fellowship (SRF). We thank the Director, CSIR-CLRI, for his support during the project and for allowing the work to be conducted at CSIR- Central Leather Research Institute (CLRI).

Funding

Not applicable

Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' contribution

All authors contributed to the study's conception and design. Among them NA, AG, and MI conceived the project and wrote the paper; AG, MI, and SA performed the experiments and analyzed the data; GS, PG, and NRK contributed to analyses and interpretation of data. All authors have read and agreed to the published version of the manuscript.

Ethical approval

This article does not contain any studies conducted on human or animal subjects. Hence, no ethics approval was required for this study.

References

1. Rabuka D, Rush JS, DeHart GW, Wu P, Bertozzi CR (2012) Site-specific chemical protein conjugation using genetically encoded aldehyde tags. *Nat Protoc* 7(6):1052-67. <https://doi.org/10.1038/nprot.2012.045>
2. Gao X, Wu H, Lee KC, Liu H, Zhao Y, Cai Z, Jiang Y (2012) Stable isotope N-phosphorylation labeling for Peptide de novo sequencing and protein quantification based on organic phosphorus chemistry. *Anal Chem* 84(23):10236-44. <https://doi.org/10.1021/ac301939v>
3. Kuo YH, Konopko AM, Borotto NB, Majmudar JD, Haynes SE, Martin BR (2017) Profiling Protein S-Sulfonation with Maleimide-Linked Probes. *ChemBioChem* 18(20):2028-2032. <https://doi.org/10.1002/cbic.201700137>
4. Chung HS, Wang SB, Venkatraman V, Murray CI, Van Eyk JE (2013) Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system. *Circ Res* 112(2):382-92. <https://doi.org/10.1161/CIRCRESAHA.112.268680>
5. Witus LS, Netirojjanakul C, Palla KS, Muehl EM, Weng CH, Iavarone AT, Francis MB (2013) Site-specific protein transamination using N-methyl pyridinium-4-carboxaldehyde. *J Am Chem Soc* 135(45):17223–17229. <https://doi.org/10.1021/ja408868a>
6. Spicer CD, Davis BG (2014) Selective chemical protein modification. *Nat Commun* 5:4740. <https://doi.org/10.1038/ncomms5740>
7. Borrmann A, Fatunsin O, Dommerholt J, et al (2015) Strain-promoted oxidation-controlled cyclooctyne-1,2-quinone cycloaddition (SPOCQ) for fast and activatable protein conjugation. *Bioconjugate Chem* 26(2):257-261. <https://doi.org/10.1021/bc500534d>
8. Nodling, AR, Spear LA, Williams TL, Luk L, Tsai YH (2019) Using genetically incorporated unnatural amino acids to control protein functions in mammalian cells. *Essays Biochem* 63(2):237–266. <https://doi.org/10.1042/EBC20180042>
9. Ayyadurai N, Prabhu NS, Deepankumar K, Jang YJ, Chitrapriya N, Song E, Lee N, Kim SK, Kim BG, Soundrarajan N, Lee S, Cha HJ, Budisa N, Yun H (2011) Bioconjugation of L-3,4-dihydroxyphenylalanine containing protein with a polysaccharide. *Bioconjugate Chem* 22(4):551–555. <https://doi.org/10.1021/bc2000066>
10. Saxon E, Armstrong JI, & Bertozzi CR (2000) A "traceless" Staudinger ligation for the chemoselective synthesis of amide bonds. *Org* 2(14): 2141–2143. <https://doi.org/10.1021/ol006054v>
11. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG (2003) Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J Am Chem Soc* 125(11):3192–3193. <https://doi.org/10.1021/ja021381e>
12. Agard NJ, Prescher JA, Bertozzi CR (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J Am Chem Soc* 126(46):15046–15047. <https://doi.org/10.1021/ja044996f>

13. Meldal M, Diness F (2020) Recent Fascinating Aspects of the CuAAC Click Reaction. *Trends Chem* 2: 569-584. <https://doi.org/10.1016/j.trechm.2020.03.007>
14. Mazmanian SK, Liu G, Ton-That H, Schneewind O (1999) Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science* 285(5428):760–763. <https://doi.org/10.1126/science.285.5428.760>
15. Chang TK, Jackson DY, Burnier JP, Wells JA (1994) Subtiligase: a tool for semisynthesis of proteins. *Proc Natl Acad Sci* 91(26):12544–12548. <https://doi.org/10.1073/pnas.91.26.12544>
16. Miyao H, Ikeda Y, Shiraishi A, Kawakami Y, Sueda S (2015) Immobilization of immunoglobulin-G-binding domain of Protein A on a gold surface modified with biotin ligase. *Anal Biochem* 484:113–121. <https://doi.org/10.1016/j.ab.2015.05.010>
17. Row RD, Prescher JA (2018) Constructing New Bioorthogonal Reagents and Reactions. *NatRev Chem* 51(5):1073–1081.
18. McGaughey GB, Gagné M, Rappé AK (1998) pi-Stacking interactions. Alive and well in proteins. *J Biol Chem* 273(25):15458–15463. <https://doi.org/10.1074/jbc.273.25.15458>
19. Struck AW, Bennett MR, Shepherd SA, Law BJ, Zhuo Y, Wong LS, Micklefield J (2016) An Enzyme Cascade for Selective Modification of Tyrosine Residues in Structurally Diverse Peptides and Proteins. *J Am Chem Soc* 138(9):3038–3045. <https://doi.org/10.1021/jacs.5b10928>
20. Long MJ, Hedstrom L (2012) Mushroom tyrosinase oxidizes tyrosine-rich sequences to allow selective protein functionalization. *ChemBioChem* 13(12):1818–1825. <https://doi.org/10.1002/cbic.201100792>
21. Bruins JJ, Westphal AH, Albada B, Wagner K, Bartels L, Spits H, van Berkel W, van Delft FL (2017) Inducible, Site-Specific Protein Labeling by Tyrosine Oxidation-Strain-Promoted (4 + 2) Cycloaddition. *Bioconjugate Chem* 28(4):1189–1193. <https://doi.org/10.1021/acs.bioconjchem.7b00046>
22. Marmelstein AM, Lobba MJ, Mogilevsky CS, Maza JC, Brauer DD, Francis MB (2020) Tyrosinase-Mediated Oxidative Coupling of Tyrosine Tags on Peptides and Proteins. *J Am Chem Soc* 142(11):5078-5086. <https://doi.org/10.1021/jacs.9b12002>
23. Ito S, Sugumaran M, Wakamatsu K (2020) Chemical Reactivities of ortho-Quinones Produced in Living Organisms: Fate of Quinonoid Products Formed by Tyrosinase and Phenoloxidase Action on Phenols and Catechols. *Int J Mol Sci* 21(17):6080. <https://doi.org/10.3390/ijms21176080>
24. Miller LW, Cornish VW (2005) Selective chemical labeling of proteins in living cells. *Curr Opin Chem Biol* 9(1): 56–61. <https://doi.org/10.1016/j.cbpa.2004.12.007>
25. George A, Krishna Priya G, Ilamaran M, Kamini NR, Ganesh S, Easwaramoorthi S, Ayyadurai N (2017) Accelerated Strain-Promoted and Oxidation-Controlled Cyclooctyne-Quinone Cycloaddition for Cell Labeling. *ChemistrySelect* 2: 7117-7122. <https://doi.org/10.1002/slct.201700581>
26. Kim E, Koo H (2019) Biomedical applications of copper-free click chemistry: in vitro, in vivo, and ex vivo. **Chem Sci** 10(34):7835–7851. <https://doi.org/10.1039/c9sc03368h>
27. Boersma AW, Nooter K, Oostrum RG, Stoter G (1996) Quantification of apoptotic cells with fluorescein isothiocyanate-labeled annexin V in Chinese hamster ovary cell cultures treated with

- cisplatin. *Cytometry* 24(2):123–130.
28. Shevchenko A, Tomas H, Havli J., et al (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1: 2856–2860. <https://doi.org/10.1038/nprot.2006.468>
  29. Li D, Zhang R Z, Shi HX, Yang YH, Tian T, Wang L. (2019) Melanocyte spheroids are formed by repetitive long-term trypsinization. *Indian J Dermatol Venereol Leprol* 85(3):258–265. https://doi.org/10.4103/ijdv.IJDVL_275_17
  30. Zecca L, Bellei C, Costi P, Albertini A, Monzani E, Casella L, Gallorini M, Bergamaschi L, Moscatelli A, Turro NJ, Eisner M, Crippa PR, Ito S, Wakamatsu K, Bush WD, Ward WC, Simon JD, Zucca FA (2008) New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals. *Proc Natl Acad Sci* 105(45):17567–17572. <https://doi.org/10.1073/pnas.0808768105>
  31. Dukler S, Wilchek M, Lavie D (1971) Oxidation of tyrosine and its peptides with potassium nitrosodisulphonate *Tetrahedron* 27(3):607-614. [https://doi.org/10.1016/S0040-4020\(01\)90729-X](https://doi.org/10.1016/S0040-4020(01)90729-X)
  32. Kim JH, Jang DH, Lee KW, Kim KD, Shah AB, Zhumanova K, Park KH (2020) Tyrosinase Inhibition and Kinetic Details of Puerol A Having But-2-Enolide Structure from *Amorpha fruticosa*. *Molecules* 25(10):2344. <https://doi.org/10.3390/molecules25102344>
  33. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, New York
  34. Moor N, Klipcan L, Saftro MG (2011) Bacterial and eukaryotic phenylalanyl-tRNA synthetases catalyze misaminoacylation of tRNA (Phe) with 3,4-dihydroxy-L-phenylalanine. *Chem Biol* 18(10):1221–1229. <https://doi.org/10.1016/j.chembiol.2011.08.008>
  35. Lee BP, Messersmith PB, Israelachvili JN, Waite JH (2011) Mussel-Inspired Adhesives and Coatings. *Annu Rev Mater Res* 41: 99–132. <https://doi.org/10.1146/annurev-matsci-062910-100429>
  36. Wilchek M, Miron T (2015) Mussel-inspired new approach for polymerization and cross-linking of peptides and proteins containing tyrosines by Fremy's salt oxidation. *Bioconjugate Chem* 26(3): 502–510. <https://doi.org/10.1021/bc5006152>
  37. Burzio LA, Waite JH (2000) Cross-linking in adhesive quinoproteins: studies with model decapeptides. *Biochemistry* 39(36):11147–11153. <https://doi.org/10.1021/bi0002434>
  38. Paz MA, Flückiger R, Boak A, Kagan HM, Gallop PM (1991) Specific detection of quinoproteins by redox-cycling staining. *J Biol Chem* 266(2):689–692. [https://doi.org/10.1016/S0021-9258\(17\)35225-0](https://doi.org/10.1016/S0021-9258(17)35225-0)
  39. Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM (1997) Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem* 272(42):26159–26165. <https://doi.org/10.1074/jbc.272.42.26159>
  40. Ernst JD, Mall A, Chew G (1994) Annexins possess functionally distinguishable Ca²⁺ and phospholipid-binding domains. *Biochem Biophys Res Commun* 200(2):867–876. <https://doi.org/10.1006/bbrc.1994.1531>

41. Ernst JD, Yang L, Rosales JL, Broaddus VC (1998) Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells. *Anal Biochem* 260(1):8–23. <https://doi.org/10.1006/abio.1998.2677>
42. Shcherbakova DM, Subach OM, Verkhusha VV (2012) Red fluorescent proteins: advanced imaging applications and future design. *Angew Chem Int Ed* 51(43):10724–10738. <https://doi.org/10.1002/anie.201200408>
43. Jacobson K, Derzko Z, Wu ES, Hou Y, Poste G (1976) Measurement of the lateral mobility of cell surface components in single, living cells by fluorescence recovery after photobleaching. *J Supramol Struct* 5(4): 565(417)–576(428). <https://doi.org/10.1002/jss.400050411>

Figures

Figure 1

Characterization of enzyme-mediated quinone reaction using UV/Vis spectroscopy and HR-MS analysis. a Schematic representation of quinone formation from DOPA and click reaction with BCN. b The tyrosinase treated DOPA shows a absorbance spectrum of dopaquinone at 475 nm and reduction of absorbance spectra was observed after DOPA-BCN product formation. c and d The HR-MS analysis of DOPA and DOPA-BCN.

Figure 2

a Three-dimensional structure of anX which shows the surface-exposed Tyr residues. b The protein expression profile of anX and anX-DOPA; M: Protein Marker, 1: *E. coli* Tyr auxotroph, 2: anX without IPTG (-Tyr), 3: anX with IPTG (-Tyr), 4: anX without IPTG (+Tyr), 5: anX with IPTG (+Tyr), 6: anX without IPTG (+DOPA), 7: anX with IPTG (+DOPA). c MALDI-TOF analysis of anX and anX-DOPA. d CD analysis of anX and anX-DOPA.

Figure 3

Coupling mechanism in protein for cell labeling. a Schematic representation of protein encoded with DOPA treated with enzyme and subsequently attached with BCN. b SDS-PAGE analysis, M: Protein Marker, 1: anX-DOPA, 2: tyrosinase treated anX-DOPA, 3: anX-DOPA functionalized by BCN via tyrosinase. c Confirmation analysis of anX-DOPA-BCN (37071 Da) and DBCO functionalized anX-DOPA (40808 Da) by MALDI-TOF analysis. d Fluorescent spectroscopic analysis of tyrosinase catalyzed anX-DOPA-DBCO (red color), anX-DOPA-DBCO without tyrosinase (blue color-straight line), DBCO alone (black color).

Figure 4

Fluorescent microscopy and flow cytometry analysis of apoptotic EA.hy926 cells after 24 h. a Cells without treatment (control). b anX-FITC stained apoptosis induced cells. c anX-DOPA-DBCO stained apoptosis induced cells. d Dot plot of Doxo treated cells shows viable cells (anX-FITC-/PI-), late apoptosis (anX-FITC+/PI+) and late apoptosis (anX-FITC+/anX-DOPA-DBCO+).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [schema1.png](#)
- [MolebioSupportinginformationRevised.doc](#)