New Water-soluble Alkynylating Agent for Cell Surface Protein: 
Sulfo succinimidyl 4-Pentynoate

Choon Woo Lim, Hoa Thi Le, Ji Hye Han, Dong-Hwan Kim, Jae Gyu Jang, and Tae Woo Kim*

Graduate School of East-West Medical Science, Kyung Hee University, Gyeonggi-do 449-701, Korea
*E-mail: tw1275@khu.ac.kr

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Proteins embedded in membranes are critical for cell-cell communication, binding chemical messengers, and responding to environmental perturbation. Because cell surface proteins confer specific cellular functions and are easily accessible, they have been extensively targeted for drug design. In fact, plasma membrane proteins account for ~70% of all known drug targets. Profiling the cell surface protein expression of disease cells would provide protein targets for the design of therapeutic monoclonal antibodies or small-molecule drugs.

Analyses of cell surface proteome are commonly attempted by enriching plasma membrane proteins with sub-cellular fractionation, and then performing mass spectrometric analysis. Such studies reported relatively low cell surface protein identification percentages due to contamination with sub-cellular proteins from intracellular membranes.

To overcome the problems of sub-cellular fractionation, researchers have studied the topological characteristics of cell surface proteins. Plasma membranes are physical barriers against non-membrane permeable compounds, which only encounter the extracellular domains of cell surface proteins. For instance, Sulfo-NHS-Biotin (biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt) was used to selectively label cell surface proteins. Enrichment and ESI MS/MS protein profiling of biotinylated cell surface proteins from cell lysate resulted in a high cell surface protein identification percentage.

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Figure 1. (a) Module analysis of Sulfo-NHS-Biotin, (b) molecule design of Sulfo-NHS-E, a new crosslinker combining NHS/click chemistry, (c) schematic presentation of cell surface protein alkynylation by Sulfo-NHS-E and possible application of click-based cell surface capturing technology.
groups, copper(I) catalyzed azide-alkyne cycloadditions (described as “click reactions” in this paper) have been investigated to address biomolecule conjugation. Click bioconjugation is based on the orthogonality and efficiency of 1,2,3-triazole formation between azides and terminal alkynes in aqueous media. Recently, metabolic azide/alkyne labelings have been investigated for glycans, lipids, proteins, and nucleic acids. Commercially available, click-mediated protein labeling kits chemically transform reactive amino acid residues on proteins into azides or terminal alkynes, causing amide modifications of lysine amine groups or disulfide modifications of cysteine thiol groups (e.g. Acetylene-PEG$_4$-NHS ester and Acetylene-PEG$_4$-Maleimide from Click Chemistry Tools).

Heterobifunctional crosslinkers for bioconjugation can usually be disassembled into three components: a reactive group, functional group, and solubility control unit. For example, Sulfo-NHS-Biotin comprises an amine reactive NHS moiety (reactive group), biotin (functional group), and sulfonate (solubility control unit) (Fig. 1(a)). Due to the low pK$_a$ of sulfonic acid (−6.5), sulfonate groups are negatively charged in a broad pH range.

The negative charge has an important property in biological applications. Deduced from the logP of NHS acetate (−1.16) and the logD of sulfo-NHS acetate (−7.65), sulfonate introduction dramatically reduces the hydrophobicity of NHS by 10$^6$. In addition, sulfonate can suppress the membrane permeability of sulfo-NHS-ester reagent. Sulfo-NHS-

![Figure 2](image-url)

**Figure 2.** Time dependent amidation profile for Tyr(NO$_2$)-OMe and Sulfo-NHS-E.

![Figure 3](image-url)

**Figure 3.** HepG2 cell surface protein labeling with Sulfo-NHS-E. a, b, c, d: confocal fluorescence images; e, f, g, h: optical images; i: schematic representation of cell surface protein labeling. Cells were treated with Sulfo-NHS-E and reacted with Alexa Fluor® 488 azide under click reacti.
ester reagent is also water-soluble, enabling reactions in the absence of organic solvents such as DMSO or DMF.

The amine reactive N-hydroxysuccinimide ester of 4-pentynoic acid (succinimimidyl 4-pentynoate, NHS-E) has been widely utilized as an alkynylation agent of water-soluble small molecules, carbohydrates, nucleic acids, and proteins. However, its low water solubility can complicate some potential biological applications (e.g., selective labeling of cell surface protein). We designed a water-soluble version of NHS-E, sulfosuccinimidyl 4-pentynoate (Sulfo-NHS-E). Newly designed Sulfo-NHS-E will be ideal for labeling cell surface proteins and can be further functionalized by sequential click reactions (Fig. 1(b) and 1(c)).

Sulfo-NHS-E was prepared from 4-pentynoic acid using oxalic acid and N-hydroxysulfo succinimide (250 mg scale, yield, 70%). Sulfo-NHS-E was characterized by 1H-NMR, 13C-NMR, and HR MS (Supporting Information I). Since Sulfo-NHS-E has good water solubility, Sulfo-NHS-E stock solution (~100 mM) can be easily prepared without any additional organic solvents.

Sulfo-NHS-E is a new compound with different physical properties than NHS-E. The amidation reactivity of Sulfo-NHS-E in aqueous buffer was investigated. To monitor reactivity, 3-nitro-L-tyrosine methyl ester hydrochloride (Tyr(NO2)-OMe) was prepared from 3-nitro-L-tyrosine (Tyr(NO2)) by acid catalyzed esterification. Time dependent HPLC analysis shows that amidation between Tyr(NO2)-OMe and Sulfo-NHS-E was completed within 1 h, and 20% of the initial Sulfo-NHS-E was consumed by competitive water hydrolysis (Fig. 2 & Supporting Information II). The kinetics corresponded with the reported reaction time of Sulfo-NHS-Biotin.

Click reactions are reliable and efficient, but should meet specific criteria for biological applications. For selective cell surface protein labeling, the employed reaction must be nontoxic and non-interfering with the surrounding cellular milieu. Wu et al. reported that appropriate copper ligands reduce the cellular toxicity of copper(I) and enhance reaction rate. This study used a water-soluble ligand developed by the Finn group, tris(3-hydroxypropyltriazolymethyl)amine (THPTA). Click reaction optimization was reported and used with minor modifications; CuSO4/THPTA (1:5 ratio, 1 mM), sodium ascorbate (5 mM), aminoguanidine hydrochloride (5 mM) in phosphate buffer (0.1 M, pH 7.4).

The labeling efficiency of cell surface protein was first examined using HepG2 cell line as substrate. Cells were treated with Sulfo-NHS-E and subsequently labeled with Alexa Fluor® 488 azide to visualize the degree of alkynylation and localization of the added alkynyl groups. Cells were then visualized using confocal microscopy (Supporting Information III). The distribution of Alexa Fluor® 488 in the central cellular stack section of labeled cells confirms that Sulfo-NHS-E labeling was specific for the cell surface and that Sulfo-NHS-E was unable to penetrate cell membranes (Fig. 3). The red circle of Figure 3(d) indicates that Sulfo-NHS selectively alkynylated cell surface proteins.

The efficiency of click reactions in cell lysate is also important to further study the proteomics of alkylated cell endogenous metal chelaters, and different redox environments depending on cell type. In addition, cell lysis buffer contains unusual detergents, such as anionic SDS (sodium dodecyl sulfate). To investigate the influence of cell lysis on click efficiency, C6 (rat glioma) and HepG2 (human hepatocellular carcinoma) cell lines was used.

To investigate protein ethynylation by Sulfo-NHS-E and click reactions under cell lystate, the protein mixture (SDS-PAGE molecular weight standards, 9 protein cocktail) was treated with Sulfo-NHS-E and reacted with Alexa Fluor® 488 azide with or without cell lyses. The SDS-PAGE of click products is shown in Figure 4 (Supporting Information IV). Comparable levels of protein loading were demonstrated by Coomassie blue staining of the gels (Fig. 4(c)). Successful alkynylation of the protein mixture and fluorescence labeling through click reactions were confirmed by comparing lanes 1 and 2 in Figure 4(b). The cell lysate addition did not detectably influence Alexa 488 labeling (lane 2, 3, 4 in Fig. 4(b)). Notably, HepG2 originated proteins (line 4 in Fig. 4(c)) were not detected in in-gel fluorescence scanning (Fig. 4(b) line 4). This result supports the bioorthogonality of click reactions; selective fluorescence labeling between azido fluorophore and alkylated proteins.

In conclusion, we synthesized a new water-soluble alkynylation agent of cell surface proteins, Sulfosuccinimidyl 4-pentynoate (Sulfo-NHS-E). Sulfo-NHS-E has a sulfonate group at the terminal ending of the alkynylating agent, which can be used in sequential reaction for cell surface proteins labeling.
group that is negatively charged in a broad pH range and suppresses membrane permeability. Thus, Sulfo-NHS-E is expected to be an ideal alkynylation agent for cell surface protein labeling and further click applications. The feasibility of Sulfo-NHS-E was confirmed by confocal fluorescence microscopy and SDS-PAGE. Sulfo-NHS-E labeling was specific for cell surface proteins, as demonstrated by ring-shape fluorescence in the central cellular stack section of labeled cells under confocal fluorescence microscopy. The click conjugation between alkynylated proteins and azido fluorophore was efficient and selective with lysate spike conditions.

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Supporting Information. Supplementary data associated with this article can be found, in the online version, at http://

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