Graphical Abstract

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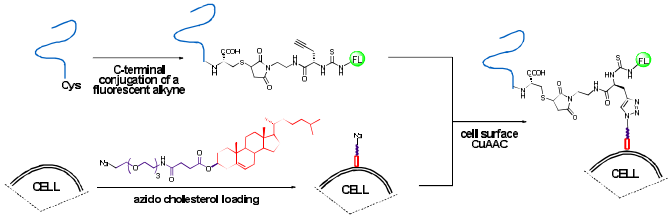

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Semisynthesis of membrane-anchored cholesteryl lipoproteins on live cell surface by azide – alkyne click reaction

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ABSTRACT

Cholesteryl lipoprotein semisynthesis was accomplished *via* a copper-catalyzed azide-alkyne cycloaddition on the surface of live cells. In this convergent synthesis an azido-cholesterol was introduced into the cell membrane without the application of detergents followed by conjugation of the *C*-terminal alkyne modified protein. This cytocompatible method resulted in a folded membrane-anchored protein containing a small molecule fluorophore in the lipid headgroup.

Membrane-anchored proteins have essential roles in living systems as enzymes, inhibitors, scaffolding proteins, signalling proteins, complement regulatory proteins or antigens. Covalent modifications of these proteins include the addition of a myristoyl, palmitoyl, prenyl or glycosylphosphatidylinositol (GPI) moiety,[1] and the resulting lipoproteins are anchored to biological membranes *via* hydrophobic interactions between these lipid chains and the lipid bilayers of the cell. GPI-anchored proteins (GPI-APs) are among the most-investigated membrane-associated proteins, because of their unexplored functions which might be related to several diseases such as paroxysmal nocturnal hemoglobinuria, prion diseases, carcinogenesis or sleeping sickness.[2] The complex structure of the GPI glycolipids has inspired the preparation of several simplified membrane anchors that were applied in biophysical studies.[3-5] In order to introduce functional proteins into membranes, isolated GPI-APs were investigated and were found to retain the biological function after re-insertion into membranes.[6] Moreover, proteins fused with GPI signal sequences were found to be membrane-anchored and functional after *in vivo* posttranslational replacement of the GPI signal sequence with a GPI moiety.[7] Based on these features, GPI-APs, engineered GPI-APs and their simplified semisynthetic analogues are potential candidates for cell surface presentation of proteins.

If semisynthetic lipoproteins are to be anchored to the plasma membrane for the investigation of their physical or biological properties, their exogenous introduction requires both pure lipoproteins lacking surplus lipids over the stoichiometric anchor lipid and a mild membrane delivery method compatible with live cell applications.[4,8] The amphiphilic nature of lipoproteins, however, denotes an inherent difficulty. Preventing their denaturation often requires the application of detergents[5,9] or lipid species that solubilize lipoproteins *via* the formation of mixed micelles. During cell membrane delivery the lipoprotein and these additional amphiphiles co-associate with the plasma membrane, and thus, contaminate it. Recently we have shown that cholesterol can be applied as a protein membrane anchor and in the form of β-cyclodextrin inclusion complex, the cholesteryl lipoprotein can be purified and delivered to the plasma membrane of live cells without membrane perturbing agents.[8] This finding inspired us to use cholesterol derivatives for introducing a bioorthogonal functionality to the cell membrane. In this way biomolecules containing complementary functionality can be directly conjugated to the headgroup of the cholesterol moiety that is pre-incorporated into the outer leaflet of the cell membrane. In our method an amphiphilic cholesterol containing an azide in the headgroup was delivered to the plasma membrane and then a protein equipped with a fluorescent alkyne tag was conjugated *via* a copper-catalyzed azide-alkyne cycloaddition (CuAAC).[10,11] The ligand-accelerated CuAAC reaction is rapid and chemoselective between azides and alkynes, however, the *in situ* preparation of the effective Cu(I) catalyst[11] generates reactive oxygen species that provides oxidative stress and induces protein degradation.[12] Accelerator ligands (e.g. tris(triazolylmethyl)-amines, His) were developed to decrease this drawback as they were reported to maintain the effectiveness of the catalytic copper complex and the reducing agent at low concentrations, and their application could shorten the reaction time to 10 min.[13,14] In the presence of His, longer reaction times were necessary, but its lower toxicity makes the slower reaction feasible and finally resulted in higher conversion. In another approach, copper-free click reactions were utilized to eliminate

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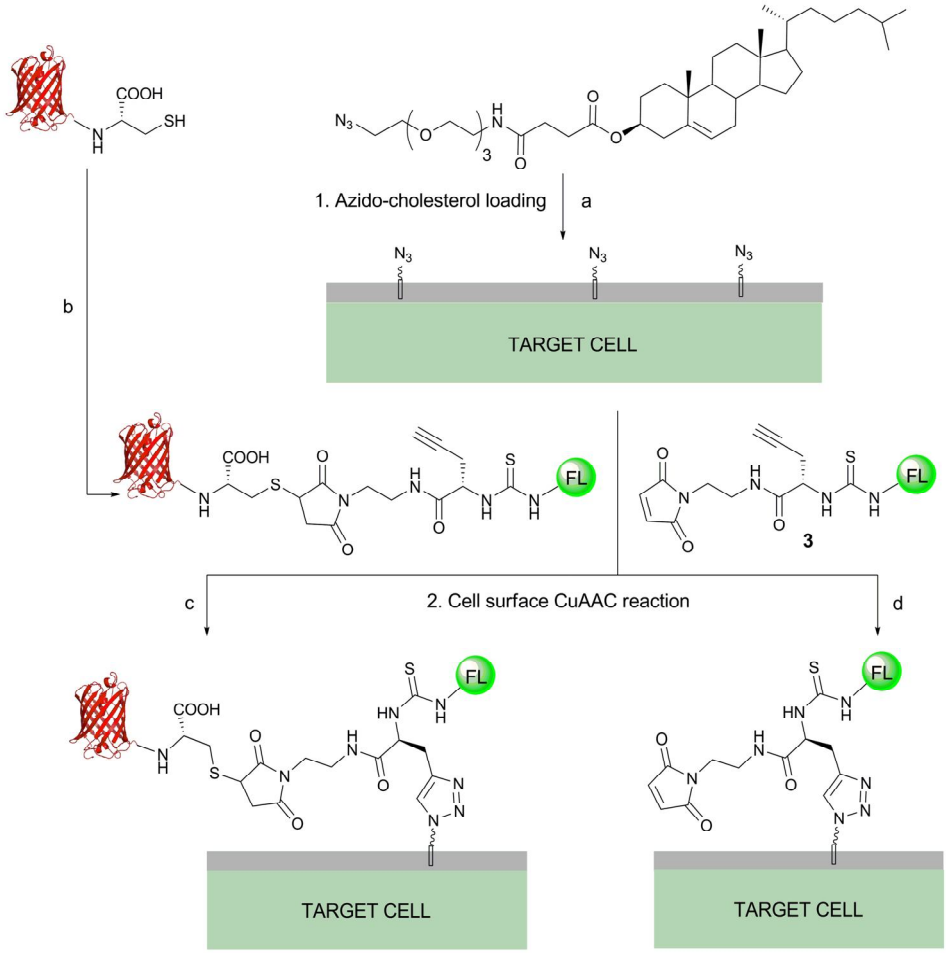


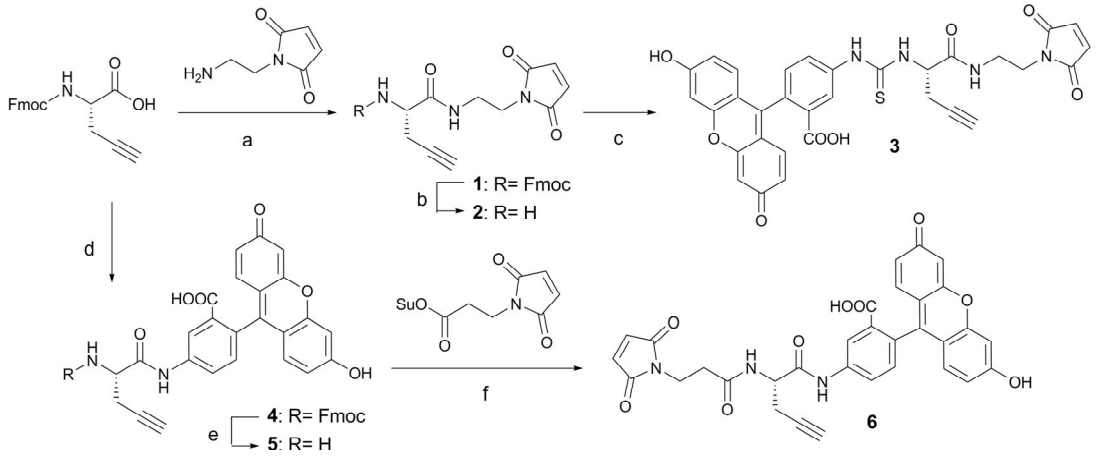
Figure 1. Cell surface preparation of cholesteryl-mCherry. (a) Cells were treated with azido-cholesterol – β-cyclodextrin inclusion complex (30 μM) in serum free medium, RT, 30 min; (b) 5 eq. **3**, 20 mM Tris, 10 mM TCEP, pH 7.5, RT, 1 h. Azido-cholesterol pre-treated cells were incubated with (c) 60 μM mCherry-**3** or with (d) 30 μM **3** in the presence of 50 μM $CuSO_4$, 500 μM NaAsc, 100 μM His and 500 μM aminoguanidine in PBS, pH 7.4, 30 min. FL represents fluoresceine.

the cytotoxic effects of copper.[15] The reactive compounds in these reactions, however, could participate in nonspecific conjugations with thiols in Cys containing proteins. Furthermore, the complicated synthesis of such reagents (e.g. tetrazines, *trans*-cyclooctynes) inhibits their widespread applications in bioconjugation.[16] Beyond the high reaction rate and chemoselectivity, the advantages of the CuAAC reaction for our purpose includes the hydrolytic and enzymatic stability of azides, alkynes, and the triazole product formed in the headgroup of the cholesterol anchor in live cell cultures.[14,17]

In our semisynthetic strategy *C*-terminal protein cholesterylation was achieved by modification of the *C*-terminus with an alkyne tag followed by a CuAAC reaction with a membrane-incorporated azido-cholesterol (Figure 1). The procedure was demonstrated by the cell surface conjugation of the red fluorescent protein mCherry. The fluorescence of this model protein is sensitive to structural changes, thus, is indicative about the retention of the native protein structure after lipidation, and further, can be directly imaged on the cell surface. A fluorescein labelled propargylglycine (Pra) derivative was applied as the *C*-terminal alkyne tag, and accordingly a dual fluorescent protein was obtained that was found to be advantageous to demonstrate the presence of both the protein and the linker moiety on the cell surface.

The azido-cholesterol derivative was prepared by *N*-acylation of 1-amino-11-azido-3,6,9-trioxaundecane with cholesterol hemisuccinate as earlier reported.[8] In this lipid, cholesterol is linked to the polar di(ethylene glycol) spacer *via* an ester group that was found to be resistant against hydrolysis under physiological conditions. The polar oligoether spacer provides hydrophilic character to the headgroup and assists in exposing the azide group toward the extracellular space which is required for the cell surface CuAAC reaction.

C-Terminal tagging of the model protein was achieved *via* the Michael addition of the Cys-extended mCherry-Cys protein and a maleimido alkyne. In order to prepare fluorescein labelled maleimido alkynes, Fmoc-Pra-OH was used as a starting material that was transformed in two ways (Scheme 1). In the first strategy, *N*-(2-aminoethyl)maleimide was *N*-acylated with Fmoc-Pra-OH in the presence of HOBt, EDC and DIEA. Next, the N^{α}-Fmoc deprotection of **1** was investigated under different conditions (For details see ESI Table S1). This was found to be a difficult step, because under basic conditions both the secondary amine deprotecting agents and the resulting amine **2** were sufficiently nucleophilic to give Michael adducts with the maleimido-alkyne.[18] The Fmoc protecting group could be cleaved under neutral condition using tetrabutylammonium fluoride (TBAF),[19] however, this resulted in **2** being obtained in low isolated yield. When piperidine was used (7.5 eq., DMF, 5 min) the concurrent Michael addition was found to be very fast and the piperidine adduct of **2** was obtained. Under milder conditions (1.5 eq. piperidine, 0 °C, 5 min) the Fmoc deprotection was



Scheme 1. Preparation of fluorescent alkynes. (a) 1 eq. HOBt, 1 eq. DIC, 1 eq. DIEA, THF, RT, 8 h, 90%; (b) 1.7 eq. TBAF, DMF, RT, 1 h, 38%, or 3 eq. TBD-methyl polystyrene, DMF, RT, 24 h, 44%; (c) 1.5 eq. FITC, 5.5 eq. DIEA, THF, RT, 6 h, 71%; **3** was also prepared in one pot by combining (b) and (c): 3 eq. TBD-methyl polystyrene, 1.5 eq. FITC, DMF, RT, 24 h, 39%; (d) 1 eq. HOBt, 1 eq. DIC, THF, 1.2 eq. 5-aminofluorescein, RT, 18 h, 40%; (e) 2%(v/v) DBU, MeOH, RT, 20 min, 88%; (f) 1.25 eq. DIEA, 1.25 eq. maleimidopropionic acid-OSu, DMF, RT, 3 h, 85%.

incomplete and the piperidine adducts of **1** and **2** were formed. The application of sterically hindered amines, dicyclohexylamine and triethylamine, or bicyclic amidine bases required either a larger reagent excess or longer reaction time, but after complete Fmoc removal the intramolecular oligomeric adducts of **2** were obtained. The solid-supported amidine base TBD-methyl polystyrene was found to be the optimal cleavage agent, and amine **2** was obtained in higher isolated yield than in the case of TBAF. The formation of the intermolecular adducts of **2** was presumably reduced by the less basic suspension of the solid supported amidine as compared to the DBU solution. The filtrate of the reaction mixture was immediately used in the following step where fluorescein-isothiocyanate (FITC) was added to **2** resulting in the formation of the fluorescent alkyne tag **3**. In order to increase the overall yield of **3**, a one-pot approach was also investigated. The most efficient cleavage agents TBAF and TBD-methyl polystyrene were applied in the presence of FITC. The solid supported base was found to be more effective because of the simple work-up, and thus, the isolated yield of **3** was higher. The difficult preparation of the amino-maleimide intermediate was excluded in the second strategy. Fmoc-Pra-OH was used to *N*-acylate fluorescein amine in THF resulting in the formation of the protected alkyne **4**. Fmoc deprotection of **4** was found to be quantitative in the presence of 2% DBU, and the resulting amine **5** was *N*-acylated with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester giving the fluorescent maleimido alkyne **6**.

The structural differences between **3** and **6** is minimal, and it was found that such changes in the cholesterol anchor headgroup were tolerated with the retention of membrane association.[20] In order to demonstrate the usefulness of the prepared fluorescent maleimido alkynes, **3** was used to tag the *C*-terminus of mCherry-Cys. Since mCherry does not contain Cys residues, the *C*-terminal Cys extension makes the chemoselective *C*-terminal modification possible *via* Michael addition. The conjugation reaction was performed with a protein to tag ratio of 1:5, and the total amount of **3** was added in 5 portions over 1 h. The Michael addition was found to be fast and the resulting mCherry–**3** was separated from the excess of **3** by size exclusion chromatography. The purified conjugate was analyzed by SDS-PAGE and it was found that the protein alkyne did not contain surplus fluorescent tag **3** (Figure 2). Under denaturing conditions, the acylimine group of the mCherry fluorophore matured from the Met[71]-Tyr-Gly[73] sequence was hydrolyzed,[21] resulting in the appearance of additional bands with a M_w of 7.8 and 19.8 kDa. The bands corresponding to the full length mCherry-Cys and the *C*-terminal mCherry-Cys fragment were found to be fluorescent under UV light revealing the covalent attachment of the fluorescein labelled alkyne **3** to the *C*-terminus of mCherry-Cys. Excitation and emission spectra of the conjugate also evidenced the presence of both fluorophores.

In order to perform the CuAAC reaction on the surface of live cells, the azido-cholesterol was first introduced into the plasma membrane of cultured SH-SY5Y cells. β-Cyclodextrin was used as a delivery vector, and the corresponding inclusion complex was prepared by incubating 30 μM of azido-cholesterol and 75 μM of β-cyclodextrin overnight in DMEM cell culture medium. The cells were then treated with the resulting solution of the azidolipid complex for 30 min. It is important to note, that the normal cellular cholesterol level was not affected by the presence of 75 μM β-cyclodextrin, because cholesterol extraction requires much higher β-cyclodextrin concentration.[22] Parallel to the azido-cholesterol loading, the copper catalyst was prepared by reducing $CuSO_4$ (50 μM) with NaAsc (500 μM) in the presence of His (100 μM) over 15 min followed by the addition of the carbonyl capturing reagent aminoguanidine (500 μM). Finally, **3** or mCherry–**3** were added. Cells were then washed and treated with the resulting alkyne solutions. The ligand accelerated CuAAC reactions performed on live cell surface were monitored by fluorescence microscopy (Figure 3). In control experiments cells were not pretreated with azido-cholesterol, but incubated with **3** or with mCherry-**3** in the presence of the same catalyst and additives. These experiments resulted in no detectable fluorescent cell labelling even when **3** or mCherry–**3** was applied at 100 μM,

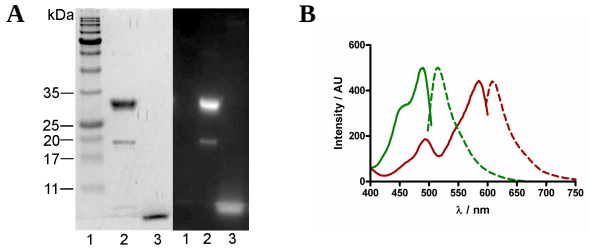


Figure 2. (A) Coomassie blue stained (left) and fluorescence detected (right) 15% SDS PAGE gel: lane 1, M_w marker; lane 2, mCherry–**3** conjugate; lane 3, **3**. mCherry does not produce fluorescence when excited at 365 nm. (B) Excitation (— λ_{em}= 518 nm; — λ_{em}= 610 nm) and emission (--- λ_{ex}= 488 nm; - - - λ_{ex}= 587 nm) spectra of 15 μM mCherry–**3** in H_2O at 25°C.

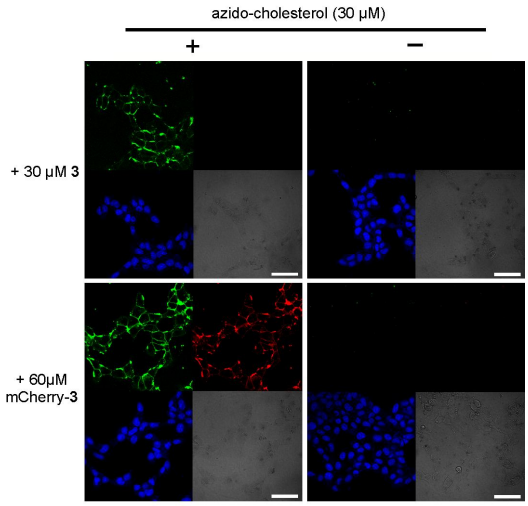


Figure 3. Confocal laser scanning and differential interference contrast (right bottom) images of SH-SY5Y cells. Azido-cholesterol pre-treated cells were incubated with **3** or with mCherry–**3** in DMEM in the presence of 50 μM $CuSO_4$, 500 μM NaAsc, 100 μM His and 500 μM aminoguanidine for 30 min. In control experiments SH-SY5Y cells were not pre-treated with azido-cholesterol. On the images fluorescein is green, mCherry is red and nuclei are blue, scale bars: 50 μm.

and thus, evidenced that neither **3** nor mCherry-**3** had non-specific adsorption on the surface of SH-SY5Y cells. When cells pretreated with the β-cyclodextrin inclusion complex of azido-cholesterol were incubated with **3**, intensive green membrane fluorescence was observed indicating successful cell surface CuAAC between the cholesterol azide and **3**. When the azido-cholesterol-loaded cells were incubated with the dual fluorescent mCherry–**3** conjugate, intensive red and green fluorescence were detected on the cell surface. This indicated that the mCherry protein *C*-terminally tagged with the fluorescent alkyne **3** was covalently attached to the headgroup of the azido-cholesterol, and that the cholesterol moiety of the resulting cholesteryl lipoprotein was able to anchor the attached protein to the plasma membrane. The red fluorescence of the anchored mCherry protein was also indicative that the cell surface cholesteryl lipoprotein formation did not denature the protein.

In order to evaluate the practical usefulness of the cell surface semisynthesis of cholesteryl lipoproteins, the *in vitro* cytotoxic effects of the reactants were investigated in an XTT colorimetric viability assay 24 h and 48 h after the treatment of SH-SY5Y cells. It was found that the azido-cholesterol pretreatment did not influence cell viability, but the catalyst system alone was found

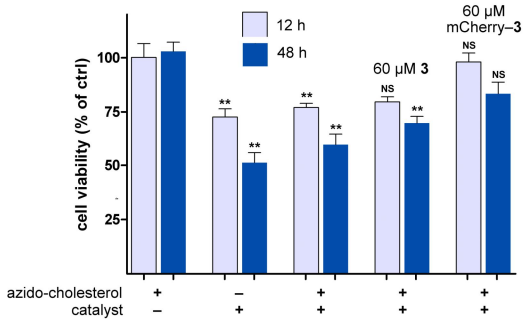


Figure 4. Effects of the cell surface protein conjugation on the viability of SH-SY5Y cells. The XTT colorimetric assay was performed 24 h and 48 h after the treatments; control cells were incubated in medium only. The data are mean ± s.d. (n= 3–6); **P<0.01, NS, not significant P>0.05 according to Dunnett's multiple comparison test, one-way ANOVA.

to be moderately cytotoxic (Figures 4 and S1). However, both **3** and mCherry-**3** dose-dependently rescued cells from the catalyst induced cell death, probably *via* partially chelating the toxic copper ions. The cell surface CuAAC reaction of mCherry-**3** and azido-cholesterol-loaded SH-SY5Y cells resulted in a viability loss of 2% and 17% 24 h and 48 h after the cell surface modification, respectively. This negligible cytotoxicity of the reported procedure proved that the method is practically usable.

Conclusion

A semisynthetic method was developed for the cell surface introduction of lipoproteins. Our convergent strategy includes the *C*-terminal tagging of recombinant proteins with fluorescent alkynes, and the simple preparation and cell membrane loading of amphiphilic azido-cholesterols. The ligand accelerated CuAAC reaction performed on the surface of live cells resulted in a cholesteryl lipoprotein that contained a small molecule fluorophore in the headgroup. Since surplus detergents or unreacted fluorescent lipids were not introduced into the plasma membrane, the fluorescent signal is unambiguously associated with the membrane-anchored protein. This feature makes the semisynthetic lipoprotein appropriate for immediate imaging studies. The method was demonstrated with the membrane-anchoring of the red fluorescent protein mCherry *C*-terminally tagged with a green fluorophore containing Pra derivative. The application of His and aminoguanidine in the CuAAC reaction, and the presence of a protein alkyne in a concentration comparable with the copper concentration protected the SH-SY5Y cells from the cytotoxic effects of the catalyst system, and under these conditions the mCherry protein was not significantly denatured. The reported fast and live cell-compatible CuAAC reaction is a mild, non-genetic method for introducing proteins or other molecules to the surface of cells or other membrane species *via* a cholesterol anchor. Since cholesterol is a major constituent of lipid rafts, that is the accumulation platform of GPI-APs,[23] the reported strategy is adequate for mimicking the protein anchoring function of GPIs in further applications.

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Supplementary Material

Experimental details.