Anionic surfactants enhance click reaction-mediated protein conjugation with ubiquitin

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ABSTRACT

The Cu(I) catalyzed alkynyl azide cycloaddition (CuAAC) has become increasingly important in the conjugation chemistry of biomolecules. For example, it is an efficient and convenient method to generate defined ubiquitin protein conjugates. Here, we investigate the effect of surfactants on the efficiency of CuAAC for chemical protein ubiquitylation. We found that anionic surfactants enhance conjugate formation by up to 10 fold resulting in high yields even at low (i.e., micromolar) concentrations of the reactants. Notably, the herein investigated conjugates are functional and thus properly folded.

1. Introduction

Click chemistry represents a powerful synthetic method inspired by nature’s strategy to use simple building blocks for generating large oligomers with remarkable modularity and diversity. Click chemistry employs functional groups that are easy to synthesize and enable highly reliable, rapid and selective reactions.

The prime example of click chemistry is the Cu(I) catalyzed cycloaddition of azides and alkynes (CuAAC). CuAAC relies on a 1,3 dipolar cycloaddition to yield 1,2,3 triazoles and proceeds significantly accelerated and with almost complete regioselectivity for 1,4 disubstituted triazoles under Cu(I) catalysis. The reaction requires no protection groups since the reactive moieties, azides and terminal alkynes, are orthogonal to a wide variety of functional groups and have great kinetic stability. Referred to as ‘the’ click reaction, CuAAC has proven itself as extremely versatile and promoted efforts in key areas like bioconjugation, materials science, and drug discovery.

The chemical properties of 1,2,3 triazoles render the click reaction applicable in the context of biomolecules. The heterocycle is water soluble and mimics certain aspects of the natural peptide bond by offering similar electronic properties and configuration of atoms. Notably, it is not susceptible to hydrolytic cleavage. Indeed, several members of the 1,2,3 triazole family have been shown to be biologically active in terms of cytokine inhibition, antiviral activity, or anti bacterial activity highlighting their cellular stability.

Together with the aforementioned favorable characteristics of 1,2,3 triazoles, the bioorthogonality of the click reaction renders it a very valuable strategy for bioconjugation. Indeed, CuAAC has been employed to attach various small compounds to biomolecules like peptides, oligosaccharides, virus particles and DNA, and the respective conjugates have been very useful for studying biological processes. Also, CuAAC contributed to novel applications in immobilization of carbohydrates, DNA and proteins onto surfaces as well as in activity based protein profiling.

Until recently, biomolecules were chemically modified with the respective reactive moiety to allow tagging via click reaction. Nowadays, engineering of natural building blocks like amino acids or nucleotides enables the insertion of a bioorthogonal reactive group via biosynthesis. Along these lines, unnatural amino acids have been used to generate proteins containing azide or alkyne functionalities by expansion of the genetic code. Using such functionalized proteins, it was shown that CuAAC cannot only be used to tag biomolecules with small entities but also to generate protein protein conjugates. We developed a straightforward
method for the generation of defined mono ubiquitylated conjugates as well as site specifically linked ubiquitin dimers by combining incorporation of unnatural amino acids and CuAAC. The resulting bioorthogonally linked proteins were shown to be functional indicating that the method developed is suited to resolve a major bottleneck in ubiquitin research, the lack of availability of homogenous populations of defined ubiquitin conjugates for functional characterization. However, product yields were rather low limiting its applicability. In attempts to improve the approach, we realized that addition of the surfactant sodium dodecyl sulfate (SDS) results in a significant increase of the yield of CuAAC. This improvement was crucial, for example, for the generation of site specifically linked poly ubiquitin chains that were used successfully in biochemical assays. In order to gain more insights into this effect, we investigated a variety of surfactants for their potential to promote CuAAC for the generation of ubiquitin protein conjugates. We observed that a negative charge combined with an alkane chain of certain length is crucial to enhance product formation. Positively charged surfactants showed no impact indicating that partial unfolding of proteins hence, better access to the reactive groups might play role, but is not sufficient for promoting the reaction or explaining the observed effects.

2. Results and discussion

Low, likely sub denaturing concentrations of SDS (i.e., 1 mM) were found to significantly increase the yield of CuAAC between two suitably functionalized proteins. To gain further insight into this effect, we studied other additives (Table 1) with respect to their potential to promote product formation. As model systems for bioorthogonal ubiquitylation via CuAAC, modification of human DNA polymerase beta (Pol beta) at position K61 and di ubiquitin formation at position K6 and K48, respectively, were investigated.

To generate ubiquitin with a C-terminal azide (Ub G76Aha), selective pressure incorporation was employed for introduction of the unnatural amino acid azidohomoalanine (Aha) during biosynthesis. To introduce the alkyne functionality in either Pol beta or ubiquitin, we site specifically replaced the aforementioned tiodene lysine residues with the propargyl derivatized pyrrolysine analog Pik by amber codon suppression.

Initially, two cholesterol derivatives, sodium cholate and 3-(3-cholamidopropyl)-dimethylammonio) 1-propanesulfonate (CHAPS), were tested for their influence on the generation of ubiquitin conjugated Pol beta (Pol beta Ub; Fig. 1). For both surfactants no significant effects on reaction yield were observed up to concentrations of 10 mM.

As opposed to SDS, cholesterol derivatives do not denature proteins. Thus, a plausible reason for the beneficial effect of cholesterol derivatives could be that weakening of the conformational rigidity of the protein is required, for example, to increase the accessibility and thus reactivity of the alkyne and azide groups. To address this hypothesis, we tested the detergents cetyltrimethylammonium bromide (CTAB) and dodecyltrimethylammonium bromide (DTAB) that like SDS, are known to be able to denature proteins. Similar to cholesterol derivatives, both substances did not promote product formation at all concentrations tested.

Next, sodium dodecyl sulfate, which is structurally closely related to SDS, was tested for its effect on Pol beta Ub conjugate formation (Fig. 1A). Compared to SDS, sodium dodecyl sulfate only lacks one oxygen atom and thus exhibits a sulfur carbon linkage instead of an oxygen carbon linkage between the charged head group and the alkane tail. With this surfactant, we observed a positive effect on Pol beta Ub conjugate formation, comparable to that of SDS (note that due to limited solubility, sodium dodecyl sulfate could only be added up to a concentration of 3 mM, whereas we tested concentrations as high as 10 mM for all other surfactants).

Table 1

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Known characteristics</th>
<th>Effect on yield of click reaction between Ub G76Aha and Pol beta K61Plk</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(3-Cholamidopropyl)-dimethylammonio) 1-propanesulfonate (CHAPS)</td>
<td>Cholesterol derivative, non-denaturing, zwitterionic</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>Cholesterol derivative, non-denaturing, negatively charged</td>
<td>No effect</td>
</tr>
<tr>
<td>Cetyltrimethylammonium bromide (CTAB)</td>
<td>Denaturing, positively charged</td>
<td>No effect</td>
</tr>
<tr>
<td>Dodecyltrimethylammonium bromide (DTAB)</td>
<td>Denaturing, positively charged</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium hexyl sulfonate</td>
<td>Negatively charged</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium octyl sulfonate</td>
<td>Denaturing, negatively charged</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium dodecyl sulfonate</td>
<td>Denaturing, negatively charged</td>
<td>Slight increase of yield</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Denaturing, negatively charged</td>
<td>Increase of yield up to ~70%</td>
</tr>
<tr>
<td>N-Lauroylsarcosine</td>
<td>Denaturing, negatively charged</td>
<td>Increase of yield up to ~70-100%</td>
</tr>
</tbody>
</table>

Figure 1. Effect of cholesterol derivatives on click reaction between Pol beta and ubiquitin. (A) Upon addition of the zwitterionic surfactant 3-(3-Cholamidopropyl)-dimethylammonio) 1-propanesulfonate (CHAPS) or (B) the anionic sodium cholate to the click reaction, no increase in product yield was observed compared to the reactions without surfactant. In each case, addition of 1 mM SDS (+SDS) served as positive control for increased conjugate formation. Positions of Pol beta, ubiquitylated Pol beta, and molecular mass markers are indicated.
To analyze the influence of the length of the alkane chain on conjugation efficiency, we tested several different, commercially available alkane sulfonates (Fig. 3A). We found that with decreasing length of the alkane chain, the stimulating effect of the alkyl sulfonate on product formation decreased as well. In more detail, while upon addition of dodecyl sulfonate a high product yield was already observed at a concentration of 0.25 mM, with decyl sulfonate increased yields were only observed at a concentration of 2 mM or higher. For octyl and hexyl sulfonates, only little effects on the click reaction were detectable within the concentration range tested. By using Na2SO4, we also checked, if a negatively charged molecule without any alkane chain attached is a useful additive for CuAAC between the proteins tested. However, no effect on product formation was observed (data not shown).

As the results above indicated a correlation between the efficiency of the click reaction and the charge and the length of the alkyl chain of the additive, we finally tested N-lauroylsarcosine (NLS, Fig. 3B). Remarkably, nearly complete conversion of Pol beta to the respective ubiquitin conjugate at concentrations of 1 mM or 2 mM of NLS was found. The effect of NLS was even superior to that of 1 mM SDS. At concentrations of NLS and SDS of 5 mM and higher, product formation was hampered (Fig. 3B). Furthermore, the results obtained with NLS and SDS indicate that while the positive effect of alkyl chains on product formation requires the presence of a negatively charge head group, its actual identity is of no or little concern.

To obtain evidence that the effect of negatively charged surfactants on chemical ubiquitylation is not restricted to Pol beta but can be generalized, we determined the effect of the two most effective surfactants identified, SDS and NLS, on click reaction mediated formation of ubiquitin dimers (Ub Ub). To do so, we used two ubiquitin variants containing Plk either at position K6 or K48 for conjugation with Ub G76Aha (Fig. 4). In both cases, the surfactants increased di ubiquitin formation upon click reaction with similar efficiency. Interestingly, the concentration of NLS required for optimal product yield was slightly higher than for Pol beta Ub formation ranging between 2 mM and 5 mM, whereas for SDS, efficient product formation was observed between 0.5 mM and 2 mM. The results obtained for the conjugation of ubiquitin K6Plk are particularly remarkable since for this position Ub Ub formation was initially found to be rather inefficient55 (Fig. 4A).

To investigate whether the employed surfactant concentrations interfere with protein folding, we tested the stability of the proteins used in presence of different concentrations of SDS and NLS by CD spectroscopy (Fig. 5A and B). For both, ubiquitin and Pol beta, a slight increase of the minimum at 207 nm was observed upon addition of SDS, indicating a slight increase of α helical secondary structure. Stabilization of α helical secondary structures by SDS was described for ubiquitin61 and other proteins62 before. As the changes are only minor compared to the changes observed for the fully denatured proteins, we conclude that the SDS concentrations used to enhance CuAAC efficiencies (0.25 1 mM) do not induce significant protein unfolding. This is in line with previous findings that ubiquitin adopts a native structure in presence of SDS at concentrations of up to 2 mM.51,61 Similarly, for NLS concentrations of up to 2 mM, no changes in the CD spectra of both proteins were observed demonstrating intact secondary structure upon addition of this surfactant (higher concentrations of NLS could not be tested due to increased absorbance of the sample below 220 nm).

To further prove that the concentrations of surfactant used to enhance CuAAC do not influence their functionality, we tested a ubiquitin dimer generated in the presence of SDS for its recognition by the ubiquitylation cascade. For this purpose, an E6AP autoubiquitylation assay was carried out (Fig. 5C), which we have previously used to demonstrate proper folding and recognition of ubiquitin dimers generated by click reaction (note that in previous work, click reactions were performed without SDS).25 Accordingly, we generated ubiquitin dimers linked at position K6 by click
reaction in presence of 0.5 mM SDS and subsequently purified these by size exclusion chromatography. For both substrates, mono ubiquitin and di ubiquitin, poly ubiquitylation of E6AP was detected (the higher reaction efficiency observed with mono ubiquitin is in line with our previous findings indicating that di ubiquitin is less efficiently used by the ubiquitylation cascade\(^5\)). Additionally, di ubiquitylated E6AP, but not mono ubiquitylated E6AP was observed in the reaction with di ubiquitin as substrate. This demonstrates that indeed, di ubiquitin was used for E6AP ubiquitylation. Thus, in line with previously published data\(^6\),\(^7\) the concentration of SDS needed to enhance the click reaction does not significantly interfere with protein folding and function.

3. Conclusion

In this study, we investigated the previously reported positive impact of SDS on bioorthogonal protein conjugation by CuAAC. We found that this effect is specifically observed with surfactants that exhibit both, a negatively charged head group and an alkane chain of sufficient length. While providing increased conformational flexibility and thereby accessibility of the reactive groups might play a role in promoting CuAAC, it is presumably not the predominant determinant causing this effect. For all proteins tested both, NLS and SDS, robustly and efficiently increased product formation indicating a general effect of these compounds on the click reactions for chemical ubiquitylation. Interestingly, we found that the optimal concentrations to enhance CuAAC were different for SDS and NLS and were also dependent on the proteins targeted for ubiquitylation. This indicates that optimal reaction conditions have to be determined for each protein pair studied by titration of the respective surfactant. Importantly, for the proteins tested, the concentrations of detergent that efficiently enhance conjugation are non denaturing and do not interfere with protein folding and function.

Taking together, these results highlight that addition of non denaturing amounts of SDS or NLS is a straightforward and robust approach to enable efficient and fast click reactions between proteins with only micromolar concentrations of reactants. Therefore, two major limitations concerning click reaction between proteins have been overcome. Firstly, high concentrations of both, alkyne and azide, or large excess of one reactant, preferably the azide\(^2\) to accelerate the reaction rate are not required. This is of particularly importance because increasing the concentration of proteins functionalized with unnatural amino acids is often not possible as their preparation, purification, and/or solubility is limited. Secondly, rather long reaction times, which increase the risk of Cu induced protein oxidation and, thus, functional inactivation, are obsolete. Thus, addition of SDS or NLS represents a major advance for the applicability of Cu(I) catalyzed click reactions to generate protein-protein conjugates in high yields for further functional analysis.

4. Experimental section

4.1. Expression and purification of Ub G76Aha

The cDNA coding for human Ub G76Aha (synthesized by GeneArt; the initial ATG codon was omitted) was cloned into pGEX2TK and transformed into methionine auxotrophic Escherichia coli B834 (DE3) as described.\(^5\) For expression, cells were cultured in 1 L NMM (for composition see Ref. 67) containing 0.06 mM methionine overnight at 37°C. Subsequently, cells were harvested and resuspended in 100 ml NMM containing 0.5 mM azidohomoalanine (Iris Biotech). After incubation at 37°C for 30 min, 1 mM IPTG was added to induce expression for 6 h at 37°C. The cells were harvested by centrifugation and resuspended in 25 mL lysis buffer (1/2 PBS, 1% Triton X 100). Lysis was performed by sonication and the clarified lysate was loaded onto a GST TrapFFTM column (GE HEALTHCARE). After addition of 10 U thrombin, the column was incubated at room temperature overnight. Subsequently, Ub G76Aha was eluted with 1/2 PBS and purity of Ub G76Aha fractions was confirmed by SDS PAGE. The protein was stored at 4°C.

4.2. Expression and purification of Ub K6Plk and Ub K48Plk

Expression of Plk modified ubiquitin (Ub) was performed via amber codon suppression. The cDNA coding for Ub K6TAG or Ub K48TAG deriving from site directed mutagenesis was cloned...
into a pET11a plasmid which also contained an expression cassette for tRNAPyl.55,56 E. coli BL21 (DE3) was co-transformed with this plasmid and the pRSFDuet1 vector carrying the PylRS gene. Cells were cultured in LB medium supplemented with the appropriate antibiotics until an OD600 of 0.3 was reached. At this stage 1 mM Plk was added to the culture. At an OD600 of 0.8, expression was induced by addition of 1 mM IPTG. Expression proceeded for 14 h at 37°C and cells were harvested by centrifugation. After resuspension in lysis buffer (25 mM NaOAc, pH 4.5) cells were lysed by sonication and the clarified lysate was heated to 75°C for 30 min. After centrifugation, the supernatant was further purified via cation exchange chromatography using a HiTrap TM SP HP column (GE HEALTHCARE) with a linear gradient from 0 M to 1 M NaCl in 20 mM NaOAc pH 4.0. Purity of Plk modified Ub fractions was analyzed by SDS PAGE followed by Commassie blue staining and samples were stored after dialysis in 25 mM Tris, 100 mM NaCl, pH 7.5 at 4°C.

4.3. Expression and purification of Pol beta K61Plk

Expression of Plk modified DNA polymerase (Pol) beta was performed as previously described.56 E. coli BL21 (DE3) was co-transformed with a pET11a plasmid containing an expression cassette for tRNA55 and a codon optimized cDNA of N terminally His6 tagged Pol beta K61Plk and the pRSFDuet 1 vector carrying the PyIRS gene. Cells were cultured in LB medium supplemented with the appropriate antibiotics until an OD600 of 0.3. 1 mM Plk was added and cells were cultured until an OD600 of 0.8 before 1 mM IPTG was added and expression was allowed to proceed for 14 h at 37°C. Subsequently, cells were harvested, resuspended in freshly prepared lysis buffer (20 mM Tris HCl pH 7.9, 150 mM NaCl, 1 mM PMSF, 5 mM imidazole) and lysed by sonication. Clarified lysate was purified by affinity chromatography using a HisTrapTM column with a linear gradient from 5 mM imidazole to 500 mM imidazole. Pol beta containing fractions were pooled, diluted 1:6 with buffer (20 mM Tris HCl pH 7.0) and pH was corrected to 7.0. The solution was loaded onto a HiTrapTM SP HP column (GE HEALTHCARE) and further purified by ion exchange chromatography with a linear gradient from 50 mM NaCl to 1 M NaCl in 20 mM Tris HCl, pH 7.0 over 15 column volumes. Fractions containing pure Pol beta were pooled, dialyzed against 20 mM Tris, 100 mM NaCl, 1 mM TCEP, 1 mM EDTA, 10% (wt/v) glycerol, pH 7.5 and protein concentration was determined by absorption spectroscopy. Glycerol was added to a final concentration of 50% (wt/v) and samples were stored at 20°C.

4.4. Click reaction of Ub G76Aha and Pol beta K61Plk

5 µM Pol beta K61Plk was mixed with a 5 10 fold molar excess of Ub G76Aha and supplemented with 20 mM NaAc pH 5.0, 1 mM...
TCEP, 10 μM TBTB, 1 mM CuSO₄ and the respective detergent. Final concentrations of detergent ranging from 0 to 10 mM were tested. In order to prevent copper induced protein oxidation, reaction ves sels were flushed with argon multiple times. Reactions were allowed to proceed for 1 h at 4 °C. Then, reactions were stopped by addition of 10 mM EDTA. Product formation was analyzed via SDS PAGE followed by Coomassie blue staining.

4.5. Click reaction and purification of ubiquitin dimers
5 μM Ub K6P1K or Ub K4P1K were mixed with a 5 fold molar excess of Ub G76Aha and supplemented with 20 mL Tris pH 8.0, 5 mM Cu(II)Cl₂, 10 mM TPTA, and the respective detergent. Final concentrations of detergent ranging from 0 to 10 mM were tested. Reactions were allowed to proceed for 1 h at 4 °C. Then, reactions were stopped by addition of 50 mM EDTA. Product formation was analyzed via SDS PAGE and Coomassie blue staining. For preparative generation of ubiquitin dimers linked via K6, click reaction was performed with 0.5 mM SDS. Samples were applied on a Superdex 75 10/300 GL column with 10 mM Tris, pH 7.9, 300 mM NaCl. Fractions containing pure dimer were pooled, dialyzed against water and lyophilized. For further experiments, ubiquitin dimers were dissolved in 25 mM Tris, pH 7.5, 50 mM NaCl.

4.6. CD spectroscopy
Either 0.5 mg/mL wild type ubiquitin (Sigma) or 0.2 mg/mL Pol beta K61Plk were mixed in 25 mM Tris HCl, pH 7.5, 50 mM NaCl, concentrations of detergent ranging from 0 to 10 mM were tested. Reactions were allowed to proceed for 1 h at 4 °C before reactions were stopped by addition of 50 mM EDTA. Product for testing. Reactions were allowed to proceed for 1 h at 4 °C. Then, reactions were stopped by addition of 50 mM EDTA. Product formation was analyzed via SDS PAGE and Coomassie blue staining. For preparative generation of ubiquitin dimers linked via K6, click reaction was performed with 0.5 mM SDS. Samples were applied on a Superdex 75 10/300 GL column with 10 mM Tris, pH 7.9, 300 mM NaCl. Fractions containing pure dimer were pooled, dialyzed against water and lyophilized. For further experiments, ubiquitin dimers were dissolved in 25 mM Tris, pH 7.5, 50 mM NaCl.

4.7. Ubiquitylation assay
Ubiquitylation assays were performed as described. In brief, ubiquitin activating enzyme E1 (UBA1) and E2AP (E3 ligase) were expressed in the baculovirus system. The ubiquitin conjugation enzyme E2 (UbCH5b) was bacterially expressed. Ubiquitylation reactions contained 25 ng of UBA1, 25 ng of UbCH5b, 100 ng baculovirus expressed E2AP and 0.5 μL rabbit reticulocyte lysate translated labeled E2AP (to allow detection of E2AP by autoradiography) in a total reaction volume of 20 μL. Ubiquitin variants were supplied in a final concentration of 12.5 μg/mL (mono ubiquitin) and 25 μg/mL (K6 linked di ubiquitin). In addition, reactions contained 25 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM ATP and 4 mM MgCl₂. After incubation at 25 °C for 2 h, the reactions were quenched by addition of sample buffer (final concentra tion: 50 mM Tris HCl pH 6.8, 0.8% (w/v) SDS, 8% (w/v) glycerol, 2.5% (w/v) β-mercaptoethanol). Samples were boiled and separated by SDS PAGE. 35S labeled E2AP was detected via autoradiography.

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References and notes