Erschienen in: Chemical communications ; 55 (2019), 87. - S. 13093-13095
https://dx.doi.org/10.1039/c9cc07303e

Assembly of branched ubiquitin oligomers by click chemistry

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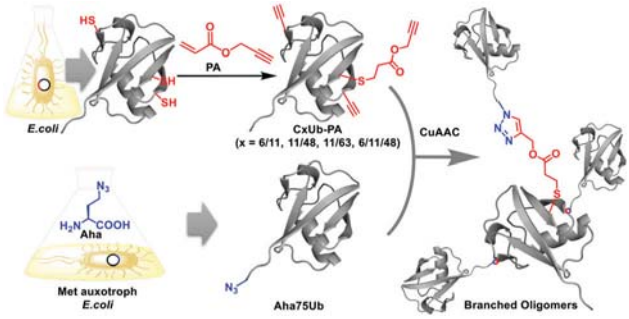
Ubiquitin monomers functionalized with an azide or multiple alkynes were utilized for the assembly of branched ubiquitin oligomers (K6/K11, K11/K48, K11/K63, K6/K11/K48) by click chemistry. The oligomers resist deubiquitylase-catalysed hydrolysis and exhibit stability in eukaryotic cell lysates.

Ubiquitylation is a complex and important post-translational modification process,[1,2] through which substrate proteins are tagged with a 76-amino acid polypeptide called ubiquitin (Ub) or with polymeric Ub chains.[3,4] One type of chain known as branched polyUb is characterized as two or more lysine residues (K6, K11, K27, K29, K33, K48 or K63) of one Ub unit being modified and prolonged by additional Ub molecules.[5–7] Proteomic studies have confirmed the existence of different branching sites, such as K6/K11, K27/K29, K29/K33.[8–10] It was reported that proteins modified with K11/K48- or K29/K48-branched chains enhance substrate recognition and degradation by the proteasome.[11–13] Yet, the physiological functions of most branched Ub chain types are still poorly understood. One of the obstacles is the limited availability of polyUb with defined branching sites.

Making use of *in vitro* enzymatic assembly of two Ub mutants (Ub ΔG76 and Ub K6R/K48R), Komander and colleagues succeeded in preparing a K6/K48-branched trimer.[14,15] Some chemical methods were also developed for the generation of defined branched Ub oligomers.[16–19] Fushman and colleagues explored an approach based on silver-catalysed chemical condensation to produce K11/K33-, K11/K63-, and K48/K63-branched trimers.[20,21] Strieter and colleagues were able to synthesize three Ub trimers branched at sites K6/K48, K11/K48, and K48/K63 *via* thiol–ene coupling.[22,23] Liu and colleagues reported the synthesis of K11/K48-branched oligomers[24] and the crystal structure of a K11/K63-branched trimer that were prepared by solid-phase peptide synthesis in combination with native chemical ligation.[25] Notably, all branched Ub trimers synthesized by the above mentioned methods are susceptible to deubiquitylase (DUB)-catalysed hydrolysis,[26,27] which restricts their application in studies employing eukaryotic cells or lysates thereof.[28–30] Here, we describe an approach based on click chemistry for the preparation of defined branched Ub oligomers that resist the action of DUB enzymes and, thus, remain stable in cell lysates (Scheme 1).



Scheme 1 Generation of branched ubiquitin (Ub) oligomers *via* mutagenesis (Lys to Cys) of Ub followed by modification with propargyl acrylate (PA) (upper panel), the incorporation of azidohomoalanine (Aha) at the C terminus of Ub in methionine (Met) auxotrophic *E.coli* (lower panel), and the bioconjugation of alkyne- and azide-functionalized Ub monomers *via* copper(I)-catalysed azide–alkyne cycloaddition (CuAAC).

To generate Ub chains with defined branching sites, we constructed Ub mutants, in which the respective lysine residues (*i.e.*, K6/K11, K11/K48, K11/K63, K6/K11/K48) were mutated to cysteines (note that wild-type Ub has no cysteine). Subsequent reaction with propargyl acrylate (PA) enabled the modification of the Ub mutants with multiple alkyne groups at the defined sites. We revealed the full conversion of cysteines to the PA-modified form in Ub monomers by a fluorescein-5-maleimide (F5M) labelling assay (Fig. S1, ESI†).

In addition, we could verify the labelling of these alkyne-functionalized Ub monomers with azido-sulfo-Cy5 by click chemistry, which was evident through in-gel fluorescent visualization

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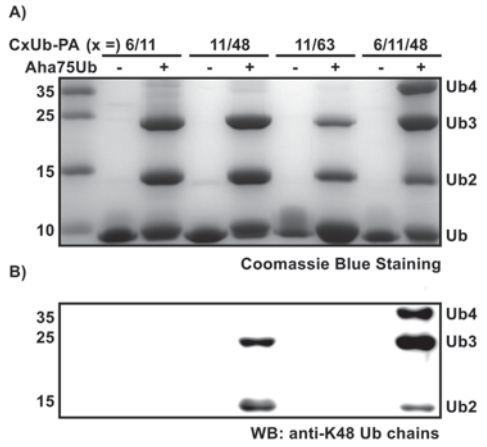

Fig. 1 Assembly of branched Ub oligomers by CuAAC. (A) SDS-PAGE analysis of the CuAAC-based protein coupling between the alkynes-functionalized Ub monomer CxUb-PA (x = 6/11, 11/48, 11/63, 6/11/48) and the azide-functionalized Aha75Ub. Proteins were visualised by Coomassie blue staining (B) Western blot analysis of the synthesized Ub oligomers using a K48 Ub chain-specific antibody.

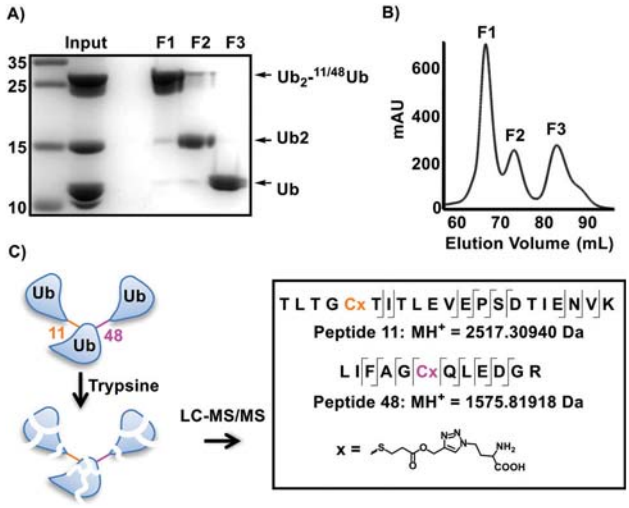

Fig. 2 (A) SDS-PAGE analysis of elution fractions from size exclusion chromatography for the isolation of branched Ub oligomers (Ub_2-$^{11/48}$Ub as an example). (B) Chromatogram indicates the separation of Ub trimer (F1), dimer (F2), and monomer (F3). Proteins were detected at 214 nm. (C) Trypsin digestion of Ub_2-$^{11/48}$Ub followed by LC-MS/MS analysis. Two peptides (11 and 48) containing the triazole-linkage (x = PA-Aha) were identified. The MS spectra are shown in Fig. S4 (ESI†).

of the dye-tagged proteins (Fig. S2, ESI†). For the preparation of azide-functionalized Ub, we introduced an unnatural amino acid (azidohomoalanine) at its C terminus *via* the selective pressure incorporation method as described before (Fig. S3, ESI†).[31,32]

We next coupled the respective alkyne- and azide-functionalized Ub monomers by copper(I)-catalysed azide–alkyne cycloaddition (CuAAC). To examine the protein coupling efficiency, the reaction samples were resolved by SDS-PAGE (Fig. 1A) and the yield was quantified using ImageJ based on intensity of the gel bands. We observed the formation of all three branched Ub trimers (*i.e.*, Ub_2-$^{6/11}$Ub, Ub_2-$^{11/48}$Ub, Ub_2-$^{11/63}$Ub), as well as the branched tetramer Ub_3-$^{6/11/48}$Ub. The yield varied depending on the conjugation sites, from 11% for Ub_2-$^{11/63}$Ub to 35% for Ub_2-$^{11/48}$Ub, 36% for Ub_2-$^{6/11}$Ub and 20% for Ub_3-$^{6/11/48}$Ub. The formed oligomers were subsequently analysed by Western blot using a wild-type K48-polyUb specific antibody (Fig. 1B).[33] We only detected the Ub oligomers bearing the K48-linkage, *i.e.* Ub_2-^{11,48}Ub and Ub_3-^{6,11,48}Ub. This suggests that our synthesized branched Ub oligomers retain a conformation at the K48-branching site that is similar to that of native K48-linked Ub chains.[34] We then upscaled the protein coupling reaction and isolated the branched oligomer (Ub_2-$^{11/48}$Ub as an example) *via* size exclusion chromatography (Fig. 2A and B). Notably, we produced Ub_2-$^{11/48}$Ub in milligram quantities from one litre of bacterial cultures of each Ub monomer.

To validate the correct position of the branching sites of the synthesized Ub oligomer, we performed trypsin digestion of Ub_2-$^{11/48}$Ub and analysed the formed peptides by tandem mass spectrometry (LC-MS/MS) (Fig. 2C and Fig. S4, ESI†). Two peptides bearing the triazole linkage were identified, one at position 11 with a molecular mass of 2517.31 Da (calculated M_w = 2517.23 Da) and one at position 48 with a molecular mass of 1575.82 Da (calculated M_w = 1575.75 Da).

To evaluate the stability of the branched Ub oligomers prepared by click chemistry towards DUBs, we incubated Ub_2-$^{11/48}$Ub and a wild-type Ub trimer ($Ub_{3\text{-}wt}$^{48}) with two representative DUB enzymes,

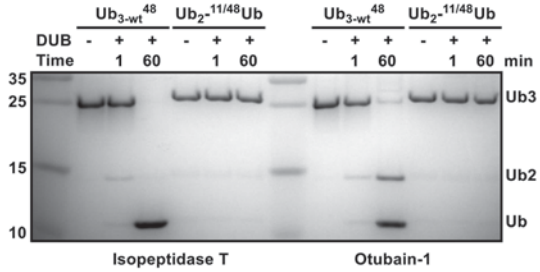

Fig. 3 SDS-PAGE analysis y of wild-type Ub trimer ($Ub_{3\text{-}wt}$^{48}) and the synthesized branched trimer (Ub_2-$^{11/48}$Ub) incubated with two representative DUB enzymes, Isopeptidase T and Otubain-1.

respectively (Fig. 3). Isopeptidase T (IsoT/USP5) of the Ub-specific protease family is primarily responsible for the disassembly of free Ub chains in cells,[35] and Otubain-1 (OTUB1) is a K48-polyUb specific DUB from the ovarian tumour (OTU) protein family.[36] We observed that both DUBs were able to disassemble the wild-type Ub trimer but not our synthesized branched trimer (Fig. 3). Moreover, we also demonstrated that Ub_2-$^{11/48}$Ub resisted DUB-catalysed hydrolysis in HEK293 cell lysates (Fig. S5, ESI†).

In summary, we describe here a robust and facile approach for the synthesis of linkage-defined, branched Ub oligomers. This approach integrates multiple chemical and biological methods, including the mutagenesis (Lys to Cys) of Ub followed by the modification with a small molecule linker (propargyl acylate), the incorporation of azidohomoalanine at the C-terminus of Ub, and protein coupling *via* CuAAC click chemistry. Notably, the synthesis of any kind of branched Ub oligomer (*i.e.* using any combination of lysine residues of Ub) should be possible by the described approach, simply *via* replacing the respective lysine residues by cysteines. Furthermore, we demonstrated that the

synthesized Ub oligomers resist DUB-catalysed hydrolysis in eukaryotic cell lysates. This feature will be valuable in identifying proteins that selectively interact with defined branched Ub oligomers in cells or cell lysates, contributing to the elucidation of the physiological function of differently branched Ub chains.

This work was supported by DFG within SFB969, project B3. XZ acknowledges a fellowship from DAAD. We also thank Dr Anna Śladewska-Marquardt and Dr Andreas Marquardt of the Proteomics Center, University of Konstanz, for MS measurement.

Conflicts of interest

There are no conflicts to declare.

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