

Synthesis of a Ubiquitinated Histone Mimic Bearing a New Thioether Linkage

Qiaoqiao He^{a,c}

Yunkun Qi^b

Chao Liu^c

Jiabin Li^{*b}

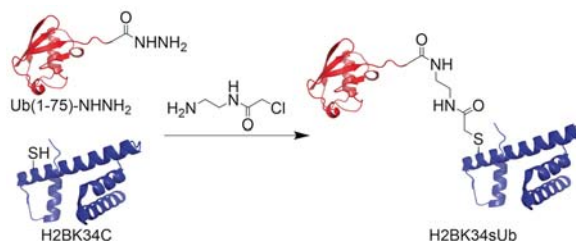
Honggang Hu^{*c}

^a Key Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Peking University, Shenzhen Graduate School, Shenzhen 518055, P. R. of China

^b School of Life Sciences, University of Science and Technology of China, Hefei 230027, P. R. of China
ljchem@163.com

^c Department of Organic Chemistry, School of Pharmacy, Second Military Medical University, Shanghai 200433, P. R. of China
huhonggang_fox@msn.com

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Abstract Current studies on histone ubiquitination require the development of more effective approaches for the preparation of ubiquitinated histones. Here, we report a new approach to obtain ubiquitinated histone mimics by using a thioether linkage. The synthetic ubiquitinated histone mimic is stable under reducing conditions and has the same atom number as the native structure. Biochemical experiments showed that the mimic is a good surrogate for natural ubiquitinated histone.

Key words ubiquitin, histone modification, thioether linkage, nucleosome, thermal stability

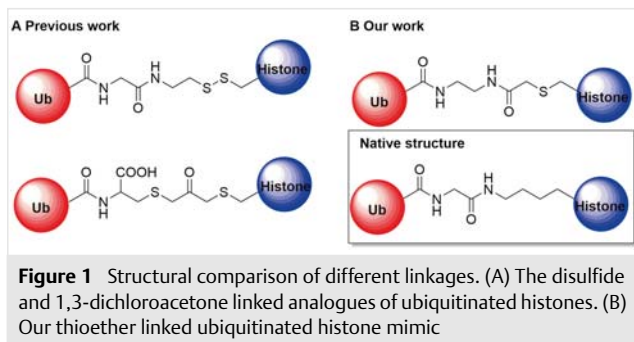
Histone ubiquitination is an important post-translational modification on multiple lysine sites of four histones (H2A, H2B, H3, H4).¹ This modification plays vital roles in DNA-related processes such as DNA replication, DNA repair, and assembly of nucleosomes.² In particular, ubiquitination can serve as an anchoring site for the recruitment of DNA-related proteins. For example, H2A Lys15 ubiquitination (H2AK15Ub) can recruit repair factor 53BP1 to enhance DNA damage repair.³ Ubiquitination can also directly affect the structure of nucleosomes and even chromosomes. For instance, H2BK120 ubiquitination can affect the compaction of the nucleosome or even high-order structures.⁴ Despite the progress that has been made on understanding the changes of structure and biochemical function induced by ubiquitination at certain sites,^{3b,5} it remains difficult to elucidate the molecular mechanism through which ubiquitination executes functions. Biochemical dissection of these processes is hampered by the difficulty of obtaining homogeneous ubiquitinated histones from reconstituted enzymatic systems.

Recent progress in chemical protein synthesis⁶ has led to the development of some total chemical synthetic^{5c,7} and semisynthetic^{8,9} strategies to prepare histones with ubiquitin modifications at specific sites. These methods employ native chemical ligation¹⁰ and solid-phase peptide synthesis (SPPS) technologies to obtain homogeneously modified histones. However, the main drawback of these methods is that they are usually time consuming and technically demanding. The ubiquitinated histone mimics, on the other hand, provide attractive alternatives for natural ubiquitinated histones because they are relatively easy to synthesize.

In 2010, Muir and co-workers developed a disulfide exchange method for preparing histone H2B ubiquitination at Lys120, and used this mimic (H2BK120ssUb) to explore how the histone methyltransferase hDot1L executed its function (Figure 1 A).¹¹ They revealed that H2B ubiquitination can directly disrupt the high-order structure of chromosome.⁴ In 2014, the Yao group synthesized another ubiquitinated histone mimic by taking advantage of a highly reactive bifunctional crosslinker, 1,3-dichloroacetone (Figure 1 A).¹² This method was applied to the synthesis of H2BK120Ub, which was used to solve the crystal structure of the DUB module of SAGA bound to H2BK120Ub containing nucleosomes.^{5a} Notwithstanding the above advances, the disulfide mimic is relatively unstable under reductive conditions, and the 1,3-dichloroacetone-crosslinked mimic shows a fairly large structural difference when compared with the natural structure. Therefore, the development of a structurally more similar and stable mimic is still needed for studies on the molecular mechanism of ubiquitinated histones.

In this work, we report a novel method for obtaining ubiquitinated histone mimics. The ubiquitin derivative was ligated to histones through a thioether linkage (Figure 1 B). This mimic shares the same number of atoms as the natural

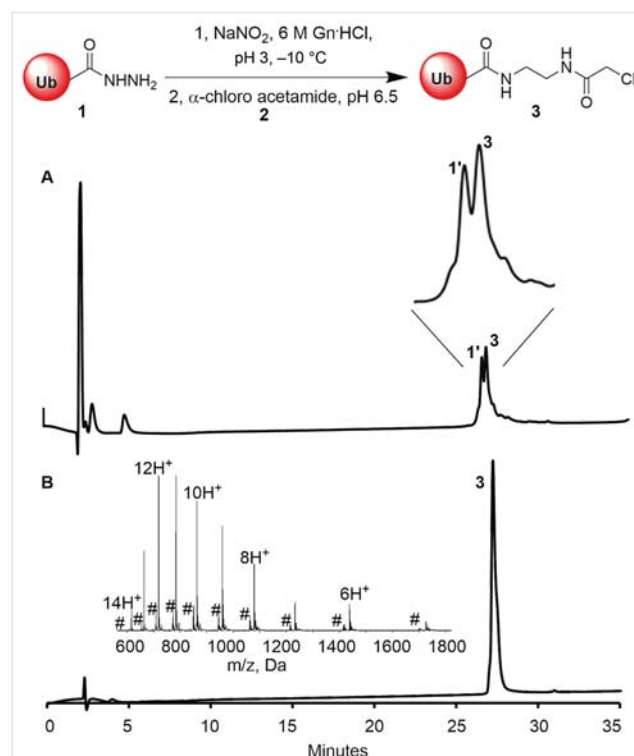
isopeptide, and is stable under reductive conditions. By using this method, histone H2B ubiquitination at Lys34 (H2BK34sUb) was prepared efficiently. Biochemical experiments showed that the mimic is a good surrogate for natural ubiquitinated histone. Thus, this work provides a simple and rapid method for preparing ubiquitinated histones, which is the basis for further structural and functional investigations on ubiquitination of histones and even other proteins.



The ubiquitinated histone is a branched protein in topology, and the C-terminal glycine (Gly) of ubiquitin forms an isopeptide linkage with the amino group on the side chain of specific Lys on histones. To prepare ubiquitinated histones in vitro, a convenient method is to conjugate recombinant ubiquitin and histones through a bifunctional crosslinker. Therefore, we used the substitution reaction between the sulfhydryl group of cysteine (Cys) and halogenated compounds, which has been successfully applied to the synthesis of proteins with methylation and other modifications,^{2c,13} to achieve site-specific histone ubiquitination. Fortunately, in four histones, there is only one cysteine (Cys110 in H3) and H3C110 mutation has been demonstrated to have little influence on nucleosome function,^{11a} which can provide good reaction selectivity by point mutation of lysine with cysteine at specific sites. Meanwhile, ubiquitin hydrazide obtained through hydrazinolysis of ubiquitin-intein fusion protein,^{8a,14} could be transformed into active derivatives easily. Therefore, we designed α -chloro acetamide as a linker to ligate Ub(1-75) hydrazide and histones. In fact, in 2014, the Brik group synthesized a thioether-linked ubiquitinated α -globin mimic by the reaction of α -bromo acetamide with Ub(1-75) hydrazide terminal and Cys104 in α -globin, which indicated that this method was feasible.¹⁵ It should be noted that thioether-linked α -globin would not be degraded by deubiquitinase USP2,¹⁵ thus we anticipated that our mimic should have similar properties. Given that native isopeptide is cleavable by deubiquitinases (DUBs), a nonhydrolyzable mimic should be of great significance in many studies, such as structural analysis and detailed affinity measurements of DUBs related complexes.

Histone H2B Lys34 ubiquitination (H2BK34Ub) was recently found to modulate gene transcription and to enhance H3 related methyltransferase activity.¹⁶ The only reported method for its preparation relied on protein total chemical synthesis, which required many steps.^{7a} Thus, H2BK34Ub was an attractive target to be synthesized by using the new method.

First, recombinant Ub(1-75)-NHNH₂ was reacted with α -chloro acetamide for aminolysis. After two hours, the reaction was complete. HPLC analysis of the aminolysis reaction showed that the desired intermediate **3** was produced as the major component. Notably, in this reaction, a significant amount of side product **1'** was observed, which resulted from the hydrolysis of **1** (Figure 2). The hydrolysis reaction was also observed by Brik et al. when they carried out a similar aminolysis reaction.¹⁵ This might be due to the high activity of the azide intermediate after hydrazide oxidation.¹⁷ Nevertheless, the hydrolysate was inactive and was not expected to affect subsequent reactions. After purification by semipreparative HPLC, the desired product chloroacetamide Ub derivative **3** was obtained with an isolated yield of 28%.



Chloroacetamide Ub derivative **3** and Cys residue on H2BK34C underwent nucleophilic substitution reaction with high selectivity. The reaction was carried out in a concentration of 2 mM, which was optimal for the synthesis of H2BK34sUb. The HPLC trace (Figure 3 A) indicated that the reaction proceeded smoothly and achieved a maximum conversion of **3** in 12 hours. Moreover, no side product was observed. Subsequently, the final product H2BK34sUb **5** was obtained with a yield of 45% through purification by semipreparative HPLC. The homogeneity and molecular weight of **5** was confirmed by analytical HPLC and ESI-MS (Figure 3 B).

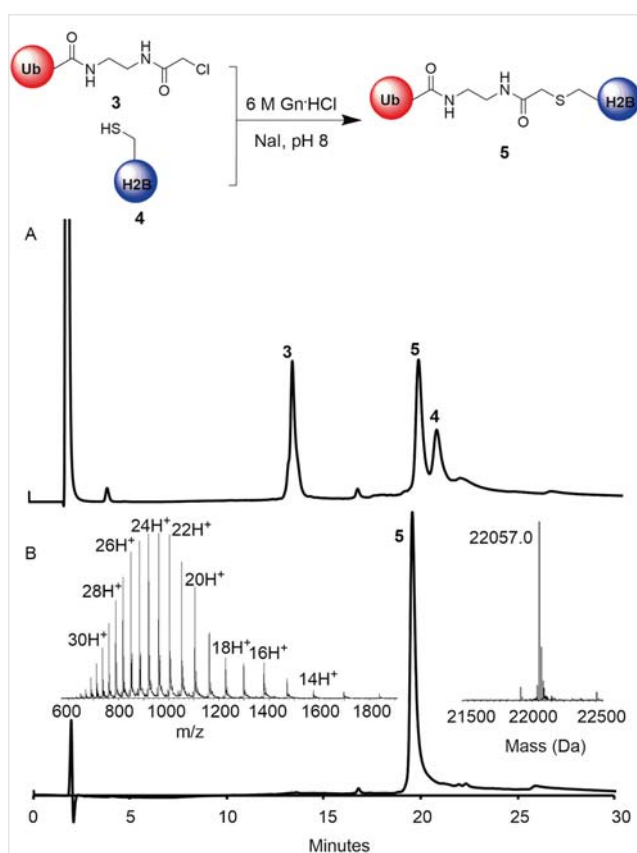


Figure 3 The synthesis of H2BK34sUb. (A) The HPLC trace (214 nm) of the substitution reaction after 12 hours. (B) The HPLC trace (214 nm) and ESI-MS characterization of purified H2BK34sUb. *m/z* calcd: 22058.6; found: 22057.0 [M + H]⁺.

To analyze the biophysical properties of the synthesized H2BK34sUb, *in vitro* reconstitution of histone octamer and nucleosome was carried out by using the standard protocols, and was compared with that of total chemically synthesized native H2BK34Ub.^{5c,18}

First, histone octamers were reconstructed with either H2BK34sUb or H2BK34Ub through dialysis. Either H2BK34sUb or H2BK34Ub, together with recombinant H2A, H3, and

H4, was dissolved in unfolding buffer (6 M Gn·HCl, 20 mM Tris, 1 mM EDTA, 5 mM DTT, pH 7.5), and then dialyzed against refolding buffer (2 M NaCl, 20 mM Tris, 1 mM EDTA, 1 mM DTT) for three rounds. When the dialysis process was complete, the octamer was analyzed and purified by size-exclusion chromatography (SEC 200). These results showed that the octamer containing the synthetic H2BK34sUb had similar retention volume to that of the octamer containing native H2BK34Ub (Figure 4 A), which indicated that the thioether linkage did not affect the formation of the octamer.

The octamer and 147 base pair 601 nucleosomal targeted sequence were mixed together to refold into nucleosome by gradient dialysis. Native-page results (Figure 4 B) showed that both nucleosomes were reconstituted successfully and that there was no significant difference in the band position between H2BK34sUb and H2BK34Ub-derived nucleosomes. These results indicated that the synthetic H2BK34sUb showed similar biophysical activity to native H2BK34Ub.

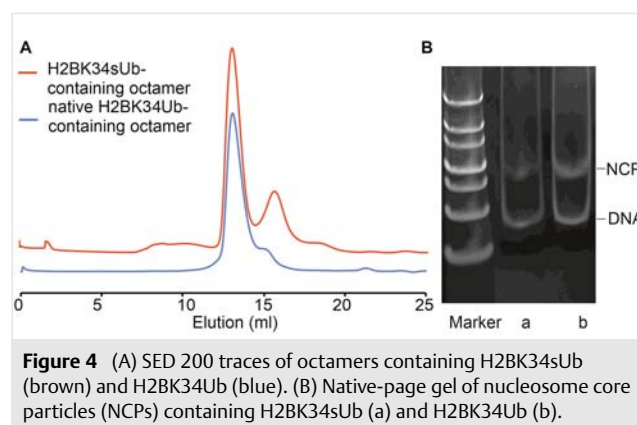


Figure 4 (A) SEC 200 traces of octamers containing H2BK34sUb (brown) and H2BK34Ub (blue). (B) Native-page gel of nucleosome core particles (NCPs) containing H2BK34sUb (a) and H2BK34Ub (b).

Thermal stability assays¹⁹ were conducted to further investigate the influence of the synthesized H2BK34sUb on the structure properties of the nucleosome. Histones would dissociate from DNA along with an increase in temperature. Subsequently, the direct contact between free histones with Sypro-Orange could induce the fluorescence emission. The curve of temperature and fluorescence intensity could reflect denaturation properties of the nucleosome.

According to Figure 5, both denaturation curves afforded two peaks, which represented H2A–H2B dimer dissociation (under lower temperature) and H3–H4 tetramer dissociation (under higher temperature), respectively. Our result indicated that the dissociation of H2A–H2BK34sUb dimers started at 61 °C and the dissociation of native H2A–H2BK34Ub dimers started at 60 °C. Along with the increase of temperature, the dissociation of the H3–H4 tetramer in NCP containing H2BK34sUb was observed, almost the same as the dissociation of the H3–H4 tetramer in NCP containing native H2BK34Ub.

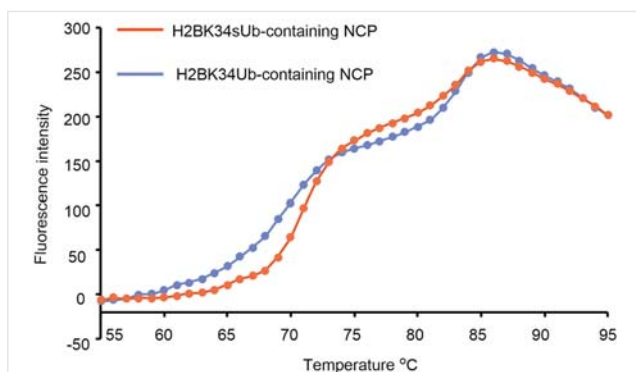


Figure 5 Thermal stability curves of NCPs containing native H2BK34Ub (blue) and H2BK34sUb (brown)

Compared with native H2BK34Ub, the synthetic H2BK34sUb had similar influence on nucleosome structure based on the above results. These results further indicate that the synthetic H2BK34sUb was a good surrogate for the native H2BK34Ub in biochemical and structural research.

In conclusion, we have developed a readily accessible approach for the preparation of ubiquitinated histone mimics, which are stable under reducing conditions and share the same atomic number as the native structure. We applied α -chloroacetamide in the ligation of recombinant ubiquitin hydrazide with mutated cysteine in histones, providing thioether linked histone ubiquitination mimics. The synthetic H2BK34sUb could be incorporated into the octamer and the nucleosome with almost equal reconstitution efficiency as native H2BK34Ub. Meanwhile, the thermal stability experiments indicated that synthetic H2BK34sUb had a similar influence on nucleosome stability as native H2BK34Ub. These results demonstrated that H2BK34sUb was a good surrogate for natural H2BK34Ub. We envision that our thioether-directed method will facilitate the preparation of ubiquitinated histones and promote the further structural and functional elucidation of this modification.

Acknowledgment

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Supporting Information

Supporting information for this article is available online at <https://doi.org/10.1055/s-0036-1588744>.

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