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Site-selective installation of an electrophilic handle on proteins for bioconjugation

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ABSTRACT

Site-selective protein modification strategies can be used to insert non-natural functional groups into protein structures. Herein, we report on the use of the bis-electrophile 3-bromo-2-bromomethyl-1-propene as a reagent to introduce an electrophilic handle at cysteine residues under mild conditions. This method is demonstrated on a variety of proteins containing a solvent-exposed cysteine residue, including an anti-HER2 nanobody. Chemically distinct protein conjugates are then efficiently formed through further reaction of the electrophilic site with various nucleophiles, including thiols and amines. The resulting chemically-defined conjugates are highly stable in the presence of glutathione or human plasma and retain both the structure and function of the native protein.

1. Introduction

Site-selective methods for protein modification[1] are important for conjugating useful synthetic molecules such as fluorescent probes[2–4] and drugs[5–7] to proteins in a well-defined manner. Techniques to achieve selective protein labelling include both the modification of natural amino acids and the introduction of unnatural amino acids via genetic expansion to install bioorthogonal chemical handles.[8–10] *In vitro*, cysteine is often targeted for protein bioconjugation due to its low natural abundance and high nucleophilicity.[11–14] A high degree of chemoselectivity can be achieved through careful control of the reaction conditions to enable cysteine to react preferentially over other nucleophilic residues such as histidine and lysine.[15–17] For proteins lacking a cysteine residue, site-directed mutagenesis can be used to introduce a single cysteine at a user-defined site within the protein sequence,[18,19] allowing this method to be applicable to a wide range of proteins.

Cysteines can be converted to suitable orthogonal handles for further derivatization to provide access to a diverse range of proteins functionalized at specific sites.[9,20,21] An electrophilic motif, which is not found naturally in proteins, may be incorporated and subsequently conjugated using various nucleophiles.[22,23] For example, a cysteine can be selectively converted to dehydroalanine,[24] which then serves as a handle for further functionalization via thiol-[25,26] and aza-Michael addition[27] or carbon-based radical mechanisms.[28] An alternative method to incorporate an electrophilic handle is by using bis-electrophilic reagents such as dibromomaleimide[29] or dibromopyridazinedione.[30] However, these linkers have been shown to be unstable in the presence of excess thiols, making them only useful for the temporary modification of cysteine residues. Moreover, these linkers could be used to form ubiquitin-protein conjugates.[31]

With this in mind, our group has previously shown that it is possible to introduce an electrophilic handle at a cysteine residue via the homobifunctional electrophile 3,3-bis(bromomethyl)oxetane (Fig. 1a)[32] under relatively harsh reaction conditions, up to 37 °C and pH 11.0. However, unlike the dibromomaleimide and dibromopyridazinedione reagents, the conjugates formed from the site-selective bis-alkylation using the oxetane reagent are stable in the presence of biological thiols such as glutathione (GSH). In order to take advantage of the increased S_N2 reactivity exhibited by allylic systems, in this work we investigated the use of 3-bromo-2-bromomethyl-1-propene **1** for alkylating proteins at cysteine residues (Fig. 1b). This electrophilic handle was then further modified by nucleophiles to yield chemically-defined protein conjugates. The high reactivity of this alkylation reagent for

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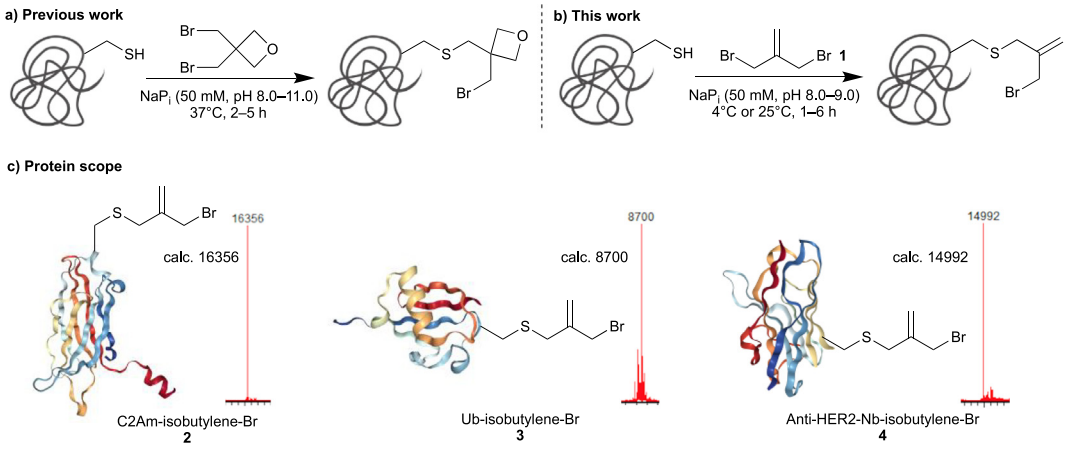

Fig. 1. Incorporating an electrophilic tag into proteins at cysteine. a) Previous work to introduce an electrophilic handle containing an oxetane motif; b) This work, where an isobutylene-Br group was introduced under mild reaction conditions; c) Protein scope of the reaction with C2Am, Ub-K63C and anti-HER2 nanobody, containing an engineered solvent-exposed cysteine residue.

disulfide re-bridging has been demonstrated in previous work by our group.[33]

2. Results and discussion

2.1. Installing the electrophilic handle on proteins

We began investigating the viability of the cysteine alkylation reaction using proteins containing a single engineered cysteine residue. The engineered versions of the C2A domain of synaptotagmin-I (C2Am)[34] and ubiquitin (Ub-K63C)[35] were chosen to target the solvent-exposed cysteine residues that we envisioned would be highly reactive with **1**. Indeed, the reaction of C2Am with 100 equivalents of **1** at pH 9.0 proceeded efficiently at room temperature. Analysis of the reaction by Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry (LC-ESI-MS) after 1 h showed the complete conversion (>95%) of C2Am to the desired alkylation product **2** (Figs. 1c and S1). Similarly, incubation of Ub-K63C with 50 equivalents of **1** at pH 8.0 at 4 °C afforded the alkylation product **3** in good conversion after 6 h (Figs. 1c and S10). The chemoselectivity of the reaction was confirmed using Ellman's test: the protein conjugates remained unchanged while the unmodified proteins readily reacted with Ellman's reagent (Figs. S3 and S12), indicating that the cysteine residue had been modified selectively.

After obtaining these preliminary results, we proceeded to test **1** against an anti-HER2 nanobody containing both an engineered, unpaired cysteine residue and an internal disulfide. Based on previous studies demonstrating the ability to modify similar nanobodies without affecting the internal disulfide,[36] we were hopeful that under the same mild reaction conditions used for C2Am and Ub, site-selective modification of the anti-HER2 nanobody would be possible. While minimal reaction was observed using the same conditions that were used to achieve full conversion on C2Am (100 equivalents of **1**, 25 °C, pH 9.0), an increase to 1000 equivalents of **1** resulted in the single conjugate **4** after 1 h, as verified by LC-ESI-MS (Figs. 1c and S19).

Interestingly, during optimisation of the alkylation step for Ub-K63C, we observed that the alkylation product degraded to a product at 8618 Da (Supplementary Data S17). This reaction occurred relatively rapidly at room temperature. However, it was partially suppressed both by conducting the reaction at 4 °C and by keeping reaction times relatively short. We propose that this product forms from the reaction of a lysine[37] on Ub-K63C with the second S_N2 reactive site on **1** via an intramolecular "stapling" reaction. Due to the proximity effect, similar intramolecular reactions between an electrophilic, unnatural amino acid and nucleophilic residues such as cysteine and lysine have been reported.[38–40] Despite this, Ub-K63C conjugates with multiple linkers incorporated were not observed, indicating that the intermolecular reaction between **1** and Ub-K63C proceeds selectively at cysteine.

2.2. Conjugating with nucleophiles

Having determined that the alkylation step reliably introduces **1**, the isobutylene-Br handle, into proteins at cysteine residues, we proceeded to react the single conjugates **(2–4)** with a range of thiol nucleophiles.[32] Treatment of **2** with β-mercaptoethanol (BME), thiophenol (PhSH) and β-D-thioglucose sodium salt (βGluSNa) at room temperature afforded the desired products **2a-2c** after 30 min to 1 h (Figs. 2 and S4–6). These results indicated that the second electrophilic site on **1** was preserved following the alkylation step. Under similarly mild conditions, reacting **3** and **4** with BME, PhSH, and βGluSNa afforded the chemically-defined conjugates **3a–3c** (Figs. S13–15) and **4a–4c** (Figs. S22–24) further highlighting the high reactivity of the allylic system. However, in the reactions with **3** and **4**, analysis by LC-ESI-MS showed that a small amount of the single conjugate was converted to the unmodified protein.

Further tests using **2c** showed that the isobutylene linker in the conjugated proteins was highly stable when incubated with the endogenous thiol GSH[41] or human plasma for 24 h at 37 °C (Figs. S8 and S9). This demonstrates the possible use of this conjugation method to produce homogenous conjugates for *in vivo* applications.

We also investigated the reaction of **2–4** with amines, using benzylamine as a representative amine nucleophile capable of reacting under mildly basic conditions and in the presence of disulfides on proteins (pH 8.0–9.0). Owing to the reduced reactivity of amine groups as compared to the thiol nucleophiles, reaction times of up to 6 h were required to obtain the conversion to the conjugated products **2d–4d** (Figs. 2, S7, S16 and S25). Some hydrolysis of the bromide was observed in all cases but was least pronounced when using **2**. Some "stapling" was also observed with **3**. While the nucleophile scope can be expanded to encompass amines, these findings indicate that the inherent reactivity of the linker makes it prone to hydrolysis, which becomes more noticeable when less reactive nucleophiles are used. This result also lends support to