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A new bioorthogonal cross-linker with alkyne and hydrazide end groups for chemoselective ligation. Application to antibody labelling

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ABSTRACT

We describe here the synthesis of the first bioorthogonal cross-linking reagent based on aminocaproic acid core with a hydrazide function at one end to react with glycoproteins and an alkyne group at the other end for Cu(I)-catalyzed click chemistry to azide-derivatized probes. As an application, this cross-linker was used to orthogonally conjugate profluorescent 3-azido-7-hydroxycoumarin to immunoglobulin G (IgG). An immunoassay showed that IgG was mostly not affected by the Cu(I)-catalyzed click chemistry conditions. Successful conjugations and retained immunoreactivity demonstrate the potential of this new bioorthogonal cross-linker in chemoselective ligation.

1. Introduction

Biomolecules labelling is an important area of research with applications in the understanding of biological processes, the development of diagnostic tools[1] and new approaches for treatment of diseases.[2] Classical chemical modification of proteins relies on functional groups carried by amino acids side chains with lysine and cysteine being the most widely modified residues.[3] To covalently connect a probe or a biomolecule to a protein, the best strategy is to use a cross-linker that moves away the two molecules from each other, especially in the case of macromolecules, to decrease steric hindrance. Many bifunctional linkers, of the homobifunctional or heterobifunctional types (Fig. 1), are commercially available for this purpose. Most of these cross-linkers are designed to react with amino or sulfhydryl groups and are based on maleimide, *N*-hydroxysuccinimide (NHS) ester, pyridyl disulfide and α-haloacetyl reactive groups. The drawback of these reactive groups is their lack of selectivity and site-specificity since lysine residues are relatively abundant in proteins and cysteine residues are often involved in the structural integrity of the protein as disulfide bridges. Another possible side-reaction is protein homo-coupling. Although these cross-linkers still have widespread utility, recent developments in bioconjugation are directed towards alternative approaches exploiting chemoselective reactions between functional groups that are not naturally occurring in biomacromolecules and that only react with each other to ensure high selectivity and site-specificity. These reactions, that are termed bioorthogonal ligations, have found extensive applications in the selective derivatization of biological molecules during the past decade.[1,4–13]

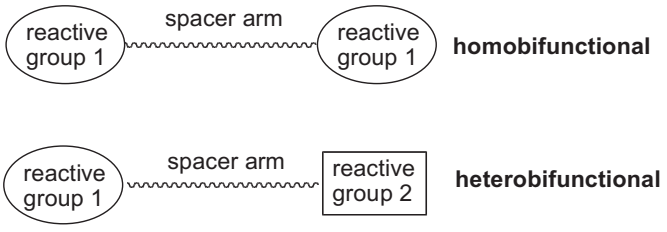

Fig. 1. Schematic design of homo and heterobifunctional cross-linkers.

One of the most successful approaches relies on the azide-alkyne [3+2] cycloaddition reaction, originally described by Huisgen.[14] This reaction leading to 1,2,3-triazole linkage, is the prototype of a set of selective reactions termed click chemistry reactions. It has enjoyed significant breakthroughs from Sharpless and Meldal groups who independently developed its copper(I)-catalyzed version (CuAAC),[15,16] and from Bertozzi group who developed its catalyst-free, strain-promoted (SPAAC) version.[17] The triazole ring is a highly stable linkage and has been described as an amide bond surrogate.[18,19] The growing interest for click chemistry reactions in

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the biological field is also attested by the recent commercial availability of azide or alkyne-tagged derivatives, such as biotin-based reagents, fluorescent dyes, nucleotides, nucleosides and non-canonical amino acids. Some spacers with azide or alkyne functionality at one end are also commercially available. Heterotrifunctional cross-linkers whose one of the reactive end groups is an azido group have been recently described for protein modification and immobilization[20] or to access sophisticated bioconjugates.[21]

We previously described the conjugation of decorated poly(amido) amine (PAMAM) dendrimer to immunoglobulin G (IgG) using a cross-linker derived from aminocaproic acid, with a hydrazide function in one end to react with oxidized IgG and form stable hydrazone, and a fluoro-nitroaromatic residue on the other end to react with nucleophiles, such as amines by aromatic nucleophilic substitution of the fluorine.[22] Furthermore the nitroaromatic moiety served as ^{1}H NMR and/or UV–vis probe.[23] As part of our current interest to explore new ways to site-selectively multi-label immunoglobulin G, we were interested in developing a new heterobifunctional and bioorthogonal linker to exploit the azide-alkyne [3+2] cycloaddition reaction to connect an azide-carrying probe to glycoproteins. In the literature, modification of intact IgG antibodies with alkyne or azide linkers for subsequent labelling by click reaction mostly relies on reagents carrying a *N*-succinimidyl ester function at the other end of the linker for ligation with primary amines of lysines residues.[24–27] Although a large number of lysine residues are located in the Fc region, favouring azide–alkyne functionalization of IgGs at their Fc region, these bifunctional linkers are not bioorthogonal.

Herein we describe the synthesis of a new bioorthogonal linker based on aminocaproic acid core with a hydrazide function at one end to react with sugar moieties of antibodies and an alkyne function at the other end for CuAAC reaction. As a proof of concept, this bioorthogonal linker was employed for the chemoselective ligation of a fluorescent dye to goat anti-rabbit secondary antibody (Fig. 2).

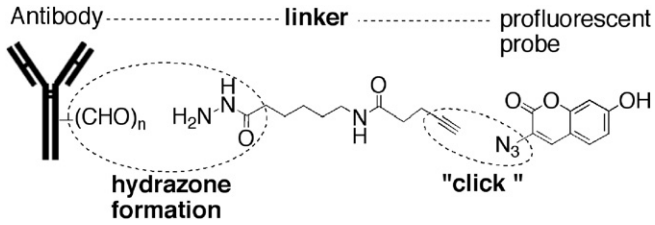

Fig. 2. Principle of the chemoselective ligation using the new bioorthogonal linker.

2. Results and discussion

2.1. Synthesis of the bioorthogonal cross-linker 4

The synthetic route leading to the bioorthogonal linker **4** is presented in Scheme 1. Commercial pentynoic acid was reacted with TSTU in DMF for 3 h at room temperature to afford *N*-succinimidyl ester **1** in 91% yield after chromatography. Compound **1** has been previously obtained by reaction of pentynoic acid and *N*-hydroxysuccinimide mediated by EDAC and DMAP or DCC with longer reaction times and lower yields.[28,29] Purification of the active ester intermediate **1** is recommended because the TLC of the crude activation step displayed byproducts with polarities close to that of compound **2** that did not allow a clean purification of the compound of interest. Compound **1** was then reacted with aminocaproic acid in aqueous $NaHCO_3$/dioxane for 5 h to afford compound **2** in 72% yield. The carboxylic acid function of the aminocaproic moiety was then activated with TSTU and allowed to react overnight with *tert*-butyl carbazate in DMF/dioxane/H_2O to yield the protected hydrazide **3** in 81% yield. Removal of the Boc protecting group was classically carried out with trifluoroacetic acid in chloroform. The hydrazide function is highly reactive towards aldehydes and ketones and some organic solvents contain traces of these compounds. Consequently, the crude trifluoroacetate salt was triturated in ice-cooled chloroform, to afford the hydrazide linker **4** in 68% yield. Depending on the way the bioconjugation is envisaged, i.e., performing the CuAAC reaction before the hydrazone formation or the other way round, the protected hydrazide **3** as well as the free hydrazide **4** can provide starting points for the following chemoselective ligation. Path **a**, the 1,3-dipolar cycloaddition is carried out with the protected compound **3** and Boc cleavage achieved in a second step before hydrazone formation. Path **b**, the sequence of reactions is reverse with the coupling between the hydrazide group of cross-linker **4** first and the aldehyde-containing biomolecule then the azide–alkyne [3+2] cycloaddition. Cross-linker **4** has an extended length of 16 Å estimated by the ChemDraw software, that increases to 17 Å after triazole formation (path **a**). This length is well suited for intermolecular conjugation.

To validate these two paths, a fluorescent probe was chosen to label goat anti-rabbit IgG as a model for bioconjugation using this new bioorthogonal cross-linker. This choice was motivated by the relevance of fluorophore-antibody conjugates in the field of immunoassays.[30] Interestingly, a series of non-fluorescent 3-azido coumarins have been described for use in CuAAC chemoselective ligation to yield coumarin-triazole. Among these, the 7-hydroxy derivative **5** (Fig. 3) displayed strong relative fluorescent intensity after the triazole linkage formation,[31] and was used as a probe for effective click reaction with alkynes,[32–35] and to monitor the progress of the CuAAC by measuring the increase in fluorescence intensity.[36–38]

2.2. Preparation of fluorophore-IgG conjugate following path a of Scheme 1

3-Azido-7-hydroxycoumarin **5**, prepared according to published procedure,[31] was reacted with the protected linker **3** using the $CuSO_4$-ascorbate catalytic system in ethanol/water (1:1)[31] for 24 h to provide the protected coumarin-triazole-linker **6** in 75% yield (Scheme 2). Removal of the Boc protecting group was then carried out with trifluoroacetic acid in chloroform to afford the free hydrazide **7** as the trifluoroacetate salt in 78% yield. Fluorescence analysis with variable excitation wavelength was performed to find out the optimum wavelength of excitation and emission to use with compound **7** and showed that a maximum of fluorescence at 476 nm was reached upon excitation at 390 nm in PBS pH 7.4 (Fig. 4a). Conversely, azido-coumarin **5** showed very weak fluorescence when excited at this wavelength (Fig. 4b). The last step was the orthogonal ligation between aldehyde groups of goat anti-rabbit IgG generated by mild oxidation of the carbohydrate moieties with sodium periodate[22,39,40] and a large excess of coumarin-triazole-hydrazide **7** (30 equiv) in acetate buffer pH 5.5. After reductive amination with ethanolamine and $NaBH_3CN$ to block the remaining free aldehyde functions, the immunoconjugate **8a** was purified by extensive dialysis in PBS pH 7.4, ultrafiltration and size exclusion chromatography. The conjugation ratio, defined as the average number of coumarins conjugated per IgG, was estimated to be 2.4. It was evaluated by UV–vis spectroscopy at 280 and 390 nm, which are the maximal absorbance wavelengths (λ_{max}) of IgG and coumarin-triazole derivative, respectively. In the chosen reaction conditions employed for IgG oxidation (10 mM $NaIO_4$, pH 3.8, 1 h), an average number of four labelling sites are expected to be generated according to Wolfe et al.[40] Conjugate **8a** showed strong fluorescence emission at 476 nm upon excitation at 390 nm in PBS