

## Evaluation of bicinchoninic acid as a ligand for copper(i)-catalyzed azide–alkyne bioconjugations†

Erik H. Christen,<sup>‡a,b,c</sup> Raphael J. Gübeli,<sup>‡a,b,d</sup> Beate Kaufmann,<sup>a</sup> Lars Merkel,<sup>e</sup> Ronald Schoenmakers,<sup>a,b</sup> Nediljko Budisa,<sup>e</sup> Martin Fussenegger,<sup>c</sup> Wilfried Weber<sup>\*,a,b,c,d</sup> and Birgit Wiltschi<sup>f</sup>

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The Cu(i)-catalyzed cycloaddition of terminal azides and alkynes (click chemistry) represents a highly specific reaction for the functionalization of biomolecules with chemical moieties such as dyes or polymer matrices. In this study we evaluate the use of bicinchoninic acid (BCA) as a ligand for Cu(i) under physiological reaction conditions. We demonstrate that the BCA–Cu(i)-complex represents an efficient catalyst for the conjugation of fluorophores or biotin to alkyne- or azide-functionalized proteins resulting in increased or at least equal reaction yields compared to commonly used catalysts like Cu(i) in complex with TBTA (tris-[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine) or BPAA (bathophenanthroline disulfonic acid). The stabilization of Cu(i) with BCA represents a new strategy for achieving highly efficient bioconjugation reactions under physiological conditions in many application fields.

The cycloaddition of terminal azides and alkynes represents a highly specific conjugation reaction in organic chemistry.<sup>1</sup> Originally this reaction required elevated temperatures and organic solvents. It was adapted to physiological conditions (aqueous environment, pH 7.2, 37 °C) by the introduction of Cu(i) as a catalyst by Meldal *et al.*<sup>2</sup> and Sharpless *et al.*<sup>3</sup> This protocol facilitated the efficient modification of proteins, lipids or nucleic acids in a highly selective manner as the reaction partners, azides and alkynes, are absent in biomolecules.<sup>4</sup> Usually, copper is added to the reaction as Cu(II), which is then reduced *in situ* to Cu(i) by reductants, such as ascorbic acid<sup>3</sup> or tris(2-carboxy-

ethyl)phosphine (TCEP).<sup>1</sup> For an efficient cycloaddition reaction, Cu(i) must be present at high levels.<sup>1</sup> However, Cu(i) is thermodynamically unstable, it is easily oxidized to Cu(II) and/or disproportionates to Cu(0) and Cu(II).<sup>5</sup> In order to prevent Cu(i) oxidation or disproportionation, it is therefore necessary to add a suitable ligand stabilizing Cu(i) in aqueous solution under aerobic conditions.<sup>6</sup> The ligand must still allow enough flexibility for catalysis of the cycloaddition by Cu(i) to take place. Recently, Cu(i) stabilizers such as tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) or bathophenanthroline disulfonic acid (BPAA) have been reported to improve bioconjugations (Scheme 1).<sup>1,5</sup> While these Cu(i) complexes have successfully been used in different application fields, the coupling efficacies were dependent on the kind of protein and ligand used.<sup>7</sup> The poor water solubility of the TBTA ligand represented a further restriction in the reaction setup. In order to overcome this limitation and add a new degree of freedom when optimizing click chemistry-based bioconjugation reactions, we explored the suitability of the bicinchoninic acid (BCA)–Cu(i) complex as a cycloaddition catalyst. The sodium salt of the 2,2'-bipyridine derivative BCA is water-soluble and coordinates Cu(i) in a 2 : 1 stoichiometry.<sup>8</sup> The resulting BCA–Cu(i) complex has a strong violet color with an absorption maximum at 562 nm (Scheme 1).<sup>9</sup>

As a model protein for the coupling reactions we used a variant of the barnase inhibitor barstar (b\*)<sup>10</sup> in which the N-terminal methionine had quantitatively been replaced by either azidohomoalanine (b\*[Aha]) or homopropargylglycine (b\*[Hpg]) (see ESI and Fig. S1 and S2† for the production, purification and characterization of the proteins). In order to evaluate whether the ligand BCA can enhance the Cu(i)-catalyzed cycloaddition, we reacted 1.4 μM of b\*[Aha] with 20 μM of the alkyne-functionalized fluorescent dye LM155 (Scheme 1 for the dye structure) in the presence of increasing BCA concentrations (the other coupling reagents CuSO<sub>4</sub>, TCEP and Cu-wire were added at standard concentrations<sup>11</sup>). After 60 min, the reaction products were resolved on SDS-PAGE and visualized by fluorescence imaging or Coomassie staining (Fig. 1a). In the presence of BCA at concentrations of 0.5–8 mM, a new band (labeled \*\*) appeared that migrated slightly slower than the unmodified protein (labeled \*). Comparison with fluorescence images (excitation: 254 nm) revealed that this new band corresponded

<sup>a</sup>Faculty of Biology, University of Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany. E-mail: wilfried.weber@biologie.uni-freiburg.de; Fax: +49 761 203 97660; Tel: +49 761 203 97654

<sup>b</sup>BIOSS Centre for Biological Signalling Studies, University of Freiburg, Hebelstrasse 25, 79104 Freiburg, Germany

<sup>c</sup>Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland

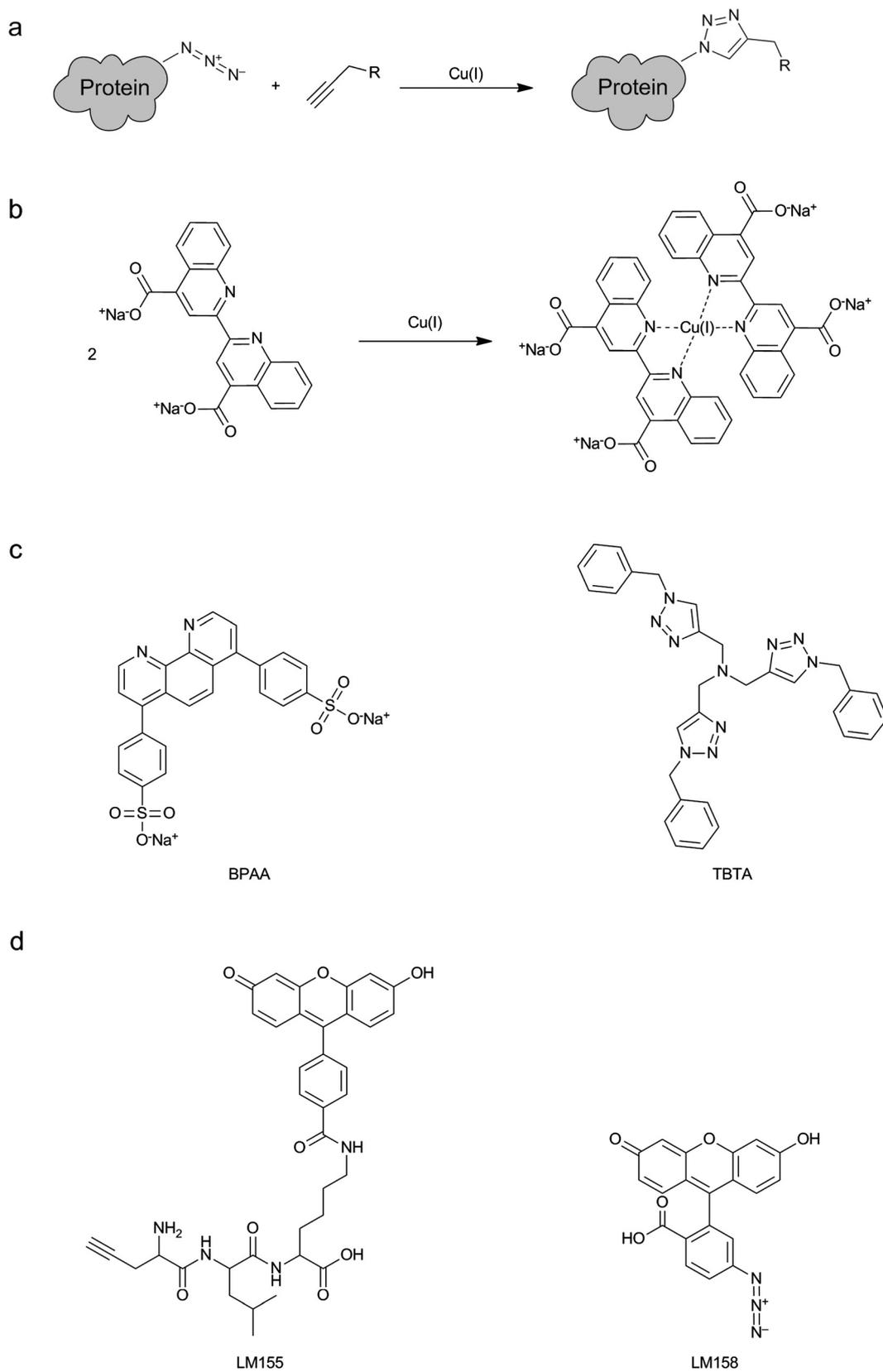
<sup>d</sup>Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg, Albertstrasse 19a, 79104 Freiburg, Germany

<sup>e</sup>Department of Chemistry – Biocatalysis, TU Berlin, Franklinstrasse 29/OE1, 10587 Berlin, Germany

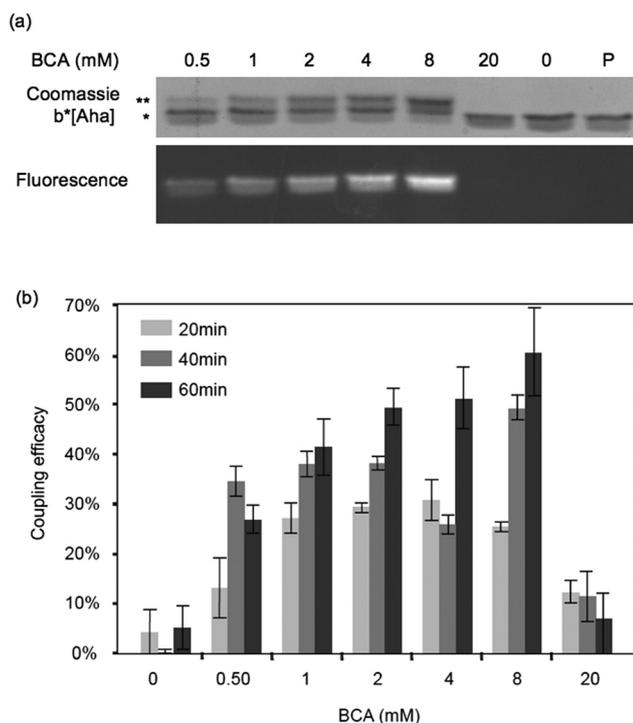
<sup>f</sup>ACIB, Petersgasse 14, 8010 Graz, Austria

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‡These authors contributed equally.



**Scheme 1** Schematic representation of the cycloaddition of azides and alkynes catalyzed by BCA-chelated Cu(I). (a) Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with an azide-labeled protein (grey). The 1,4-regioisomer of the triazole is preferentially formed. (b) Structure of the BCA-Cu(I) complex. (c) Structure of the Cu(I) ligands bathophenanthroline disulfonic acid (BPAAs) and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA). (d) Structure of the alkyne-functionalized dye LM155 and the azide-functionalized dye LM158.

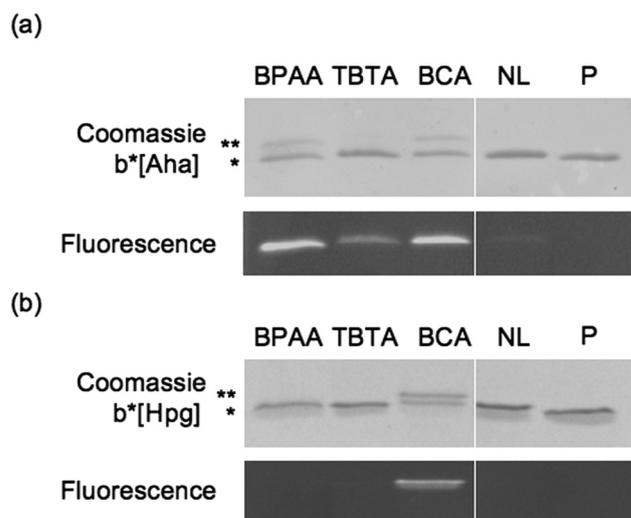


**Fig. 1** Characterization of the Cu(I)-catalyzed azide–alkyne cycloaddition using BCA as a Cu(I) chelator. (a) Effect of BCA on the Cu(I)-catalyzed azide–alkyne cycloaddition. b\*[Aha] was subjected to Cu(I)-catalyzed azide–alkyne cycloaddition with the alkyne-functionalized dye LM155 in the presence of increasing BCA concentrations. After 60 min the reaction products were resolved by SDS-PAGE and the proteins were visualized by Coomassie staining or fluorescence imaging. (\*), unlabeled b\*[Aha]; (\*\*), LM155-labeled b\*[Aha]. Lane P contains b\*[Aha] protein only in the absence of LM155 and coupling reagents. The reactions were performed in triplicate, one representative gel image is shown. (b) Kinetics of Cu(I)-catalyzed azide–alkyne cycloaddition between b\*[Aha] and LM155 at different BCA concentrations. b\*[Aha] and LM155 were coupled as described in Fig. 1a using different reaction times and BCA concentrations. The coupling efficacy was determined by quantifying the bands corresponding to native and LM155-functionalized b\*[Aha] on SDS-PAGE gels. The error bars represent the standard deviation from three coupling reactions.

to LM155-coupled b\*. The cycloaddition between LM155 and b\* was further confirmed by MALDI-TOF mass spectrometry yielding the  $m/z$  ratio of 11 182 Da corresponding to the calculated value (11 181 Da) of the reaction product (Fig. S3†).

In order to determine optimal reaction conditions, we varied the reaction times as well as the BCA concentrations and quantified the coupled and uncoupled protein bands on the Coomassie gel by densitometry analysis (Fig. 1b). At BCA concentrations of 4 or 8 mM and a reaction time of 60 min, maximum conjugation yields in the range of 60% were observed. At higher BCA concentrations (20 mM) almost no coupling was observed. This observation is in agreement with literature data on Cu(I)-catalyzed cycloaddition reactions indicating that a large excess of the ligand results in slower reaction kinetics (Fig. 1b).<sup>12</sup>

In order to compare the ligand BCA to the frequently used ligands BPAA and TBTA, we determined the optimal



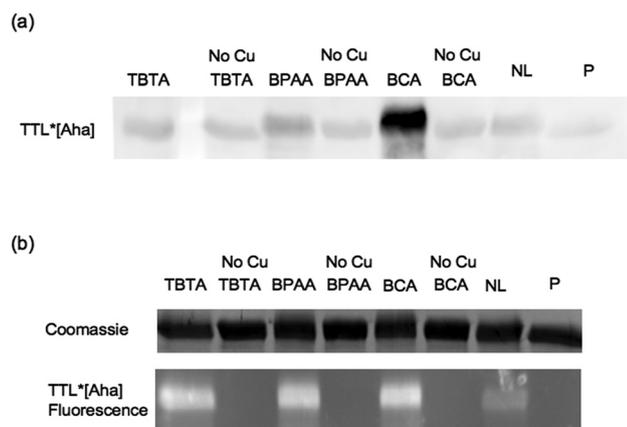
**Fig. 2** Comparison of different Cu(I) chelators for the Cu(I)-catalyzed azide–alkyne cycloaddition. Cu(I)-catalyzed azide–alkyne cycloaddition between (a) b\*[Aha] and the alkyne-functionalized dye LM155 or (b) b\*[Hpg] and the azide-functionalized dye LM158. The coupling reaction was performed in the presence of BPAA (6 mM), TBTA (2 mM) or BCA (4 mM) for 60 min prior to resolving the reaction products on SDS-PAGE. The proteins were visualized by fluorescence imaging and Coomassie staining. The coupling efficacy was determined by quantifying the bands of native (\*) and dye-functionalized (\*\*) protein. The reactions were performed in triplicate, one representative gel image is shown. Lane NL, no Cu(I) ligand added; lane P, unmodified protein only without dye and coupling reagents.

concentration of these ligands by a similar titration experiment (Fig. S4 and S5†). For BPAA, the highest coupling efficacy (55%) was observed for 2 and 6 mM ligand at a reaction time of 60 min (Fig. S4†). In contrast, TBTA resulted only in very poor coupling efficacies, which made it impossible to perform a quantitative analysis (Fig. S5†). We therefore used 2 mM TBTA for the subsequent coupling reactions as this value has consistently been described in the literature.<sup>11</sup>

We first directly compared the coupling efficacy of b\*[Aha] with the dye LM155 catalyzed by BCA-, BPAA- and TBTA-Cu(I) complexes under the above-determined optimized reaction conditions. While BCA and BPAA resulted in comparable yields of protein–dye conjugates, TBTA was the least-efficient ligand (Fig. 2a).

In order to analyze the effect of the ligands on the reverse coupling configuration (protein–alkyne, dye–azide), we performed a cycloaddition reaction between b\*[Hpg] and the azide-functionalized fluorescent dye LM158 (see Scheme 1 for the dye structure). In this configuration, only BCA resulted in an efficient coupling (52% yield) while both other ligands, TBTA and BPAA, did not result in a detectable reaction product (Fig. 2b).

We next evaluated the effect of the three Cu(I) ligands on the coupling of azido-functionalized *Thermoanaerobacter thermo-hydrosulfuricus* lipase (TTL[Aha]). Such lipases are an industrially important class of enzymes as they are common in laundry detergents and in the food industry. Their targeted modification therefore represents an important engineering tool in designing efficient biocatalytic processes.<sup>13</sup>



**Fig. 3** Cu(I)-catalyzed azide-alkyne cycloaddition between Aha-substituted *T. thermohydrosulfuricus* lipase (TTL[Aha]) and (a) alkyne-functionalized biotin or (b) the alkyne-containing dye LM155. The coupling reaction was performed for 60 min in the presence of TBTA (2 mM), BPAA (6 mM) or BCA (0.5 mM) prior to resolving the reaction products on SDS-PAGE. Biotinylated proteins were analyzed by Western blotting. The biotin-containing bands were detected with horseradish peroxidase-functionalized streptavidin and subsequent chemiluminescent imaging. LM155-coupled proteins were analyzed as described in Fig. 2. No Cu, no copper was added to the reaction; NL, no Cu(I) ligand added; P, unmodified protein without dye and coupling reagents.

As conjugation partners for TTL[Aha] we chose alkyne-functionalized biotin *e.g.* as an anchor for enzyme immobilization *via* a streptavidin matrix, or LM155 for dye-labeling of the enzyme. We performed the coupling reaction using the optimized conditions for the three ligands (4 mM BCA; 6 mM BPAA; 2 mM TBTA) and visualized the reaction products on SDS-PAGE (Fig. 3). For the coupling reaction between TTL[Aha] and alkyne-biotin, the ligand BCA resulted in the best coupling efficacy while BPAA and TBTA resulted in close-to-background signals only (Fig. 3a). For the conjugation of TTL[Aha] with LM155 similar reaction efficacies were obtained for all three ligands used (Fig. 3b). This heterogeneous coupling efficacy is in agreement with previous reports indicating that the yield of Cu(I)-catalyzed cycloaddition reactions is profoundly influenced by the reaction partners, by the nature of the small-molecule ligand or whether the protein is alkyne- or azido-functionalized.<sup>7,14</sup> In agreement with literature data our results show that the concentration and the choice of the copper ligand strongly influence the efficiency of the cycloaddition reaction.<sup>14–16</sup>

The robust performance of the BCA-Cu(I) complex under different reaction conditions might be explained by the very strong chelating behavior of BCA that is relatively inert to interferences with other molecules<sup>9</sup> while still being flexible enough to expose Cu(I) for catalyzing the conjugation reaction. Especially the superior performance of the BCA ligand in comparison to the rigid phenanthroline derivative BPAA supports the flexibility argument: in BCA, one pyridyl-residue may reversibly

dissociate from the Cu(I) ion providing the empty coordination site necessary for the catalysis. Probably this coordination site is less accessible if the BPAA ligand chelates the Cu(I) ion, thus accounting for its inferior performance. In contrast the flexible chelating TBTA ligand provides similar performance to BCA. However, since TBTA is a neutral molecule with little H-bonding capacity, the BCA-complex carrying carboxylate groups obviously is better compatible with the local environment of some of the chosen model reactions and likely accounts for the superior performance of the BCA-Cu(I) system.

In this study we have introduced the BCA-Cu(I) complex to catalyze the cycloaddition between terminal azides and alkynes in bioconjugation reactions. We have demonstrated that in the presence of BCA significantly improved conjugation yields were obtained for certain combinations of a target protein and a small molecule binding partner while in all other combinations BCA performed at least equivalent to the previously described ligands TBTA or BPAA. We therefore expect that the findings described in this study will gain significant impact in many different areas of fundamental and applied research and development.

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