Hemoglobin bis-tetramers *via* cooperative azide–alkyne coupling†

Jonathan S. Foot, Francine E. Lui and Ronald Kluger*

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Cross-linked hemoglobin-azides react with a bis-alkyne to form a bis-tetramer through sequential "click" reactions where the second step is promoted by the first.

The possibility of producing a circulating oxygen-carrier based on altered hemoglobin (Hb) has been the subject of great interest for its potential applications in transfusion medicine.[1] Outside the red cell, the Hb tetramer ($\alpha_2\beta_2$) dissociates into non-functional αβ dimers, which are rapidly excreted. Appropriate chemical cross-linking of the Hb tetramer maintains its structural and functional integrity. However, clinical evaluations of stabilized tetramers led to the conclusion that species larger than the tetramers are needed in order to avoid harmful side effects associated with an induced increase in blood pressure due to scavenging of nitric oxide near the endothelia of blood vessels.[2] One way to increase the size of the species whilst maintaining oxygenation capacity would be to connect pairs of cross-linked tetramers to give cross-linked bis-tetramers. Our laboratory has previously developed methods for such a process based on the formation of inter-tetrameric amide linkages.[3,4] However, the reactions used for producing the connection, aminolysis of activated esters, compete with hydrolysis, thereby limiting efficiency. We therefore sought an alternative approach to making bis-tetramers of Hb that would not involve competing reactions in the coupling process.

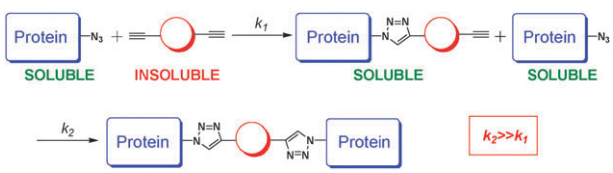
In order to avoid competitive hydrolysis, we designed reagents that make use of copper-catalyzed azide–alkyne coupling reactions ("click chemistry").[5] Although this reaction has been used successfully in bioconjugation (attachment of polymers, small molecules, fluorophores, *etc.*), its extension to protein–protein coupling applications has been limited, requiring differential modification of the proteins (one as an azide, the other as an alkyne), typically with one of the proteins in excess to ensure conversion.[6]

For coupling Hb, we considered that it would be more efficient to synthesize a single azide-functionalized, cross-linked tetramer that would then react with a bis-alkyne to link the proteins. Thus, we synthesized and characterized a modified Hb containing a cross-linker with an azide (HbN_3) and developed a route to coupling two of these to a single bis-alkyne. Synthesis of homodimers *via* sequential reactions of a bis-alkyne with a protein azide appears to be an unlikely prospect, requiring that both ends of the alkyne react with different HbN_3 molecules rather having reactions occur at only one end. However, the desired coupling was readily achieved by applying a principle of phase-transfer catalysis: The central bis-alkyne is sufficiently insoluble that reactions are likely to be limited to the interface between phases, assuring that the first coupling reaction of the bis-alkyne with one HbN_3 will occur at a relatively slow rate. The second coupling reaction of the resulting soluble Hb–alkyne intermediate with HbN_3 has all species in a common phase and therefore proceeds at a faster rate, ensuring that a second protein reacts (Scheme 1). Thus, the two steps of the coupling process are cooperative. This approach permits the use of greater than stoichiometric amounts of the bis-alkyne linker with assured coupling of proteins.

Synthesis of the azide-functionalized cross-linker **1** was achieved in 36% yield in four steps from 4-bromomethylbenzoic acid. The two dibromosalicylate groups in **1** direct acylation to amino groups in the cationic site that normally binds the effector 2,3-bisphosphoglycerate.[7] Reaction of 2.0 eq. of this reagent with human adult Hb (Scheme 2) gave complete conversion to cross-linked azide HbN_3 as confirmed by HPLC analysis (G200 size-exclusion column).

Further analysis of the distribution of cross-linked subunits under fully dissociating conditions was then carried out on a C4 column (Fig. 1). The chromatogram showed that the β-subunits and small amounts of the α-subunits of the tetramer were modified by the reagent. The two major peaks corresponding to the modified α and β-subunits were assigned as β-lys82/β′-lys82 (BPG site) cross-linked (59 min) and α-lys99/α′-lys99 cross-linked (78 min). The remaining minor peaks between 55 and 75 min are from a mixture of unsymmetrical cross-linked tetramer and singly modified β-subunits. Comparison with authentic αα and ββ cross-linked materials and mass analysis (ESI-MS) of the isolated peaks confirmed these assignments. Integration of the peak areas suggests that the HbN_3 material contains approximately a 40 : 30 : 30 ββ (sym) : ββ (unsym) : αα (sym) mixture. This is also apparent by SDS-PAGE analysis, with two bands appearing around the 31 kDa marker (see Fig. 2, lane 2), corresponding to the αα and ββ cross-linked materials.

Before commencing our bis-tetramer study, we investigated the reactivity of HbN_3 towards bioconjugation with the 5 kDa



Scheme 1 Solubility directed approach to homodimers.

Davenport Laboratories, Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 3H6.
E-mail: rkluger@chem.utoronto.ca
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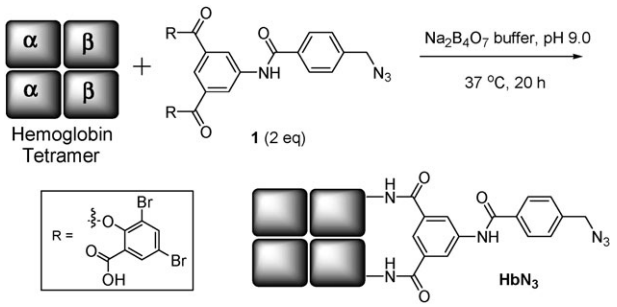


Scheme 2 Reaction of Hb with reagent 1. Linkages to the protein are undefined due to the ensuing mixture of αα and ββ linking.

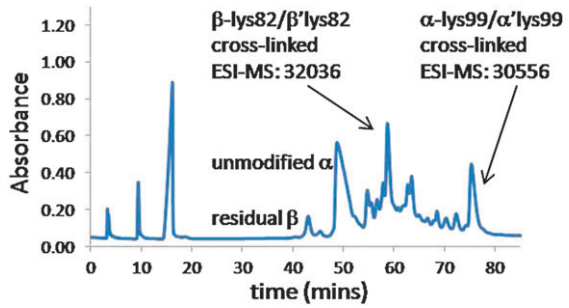


Fig. 1 C4 HPLC analysis of HbN_3.

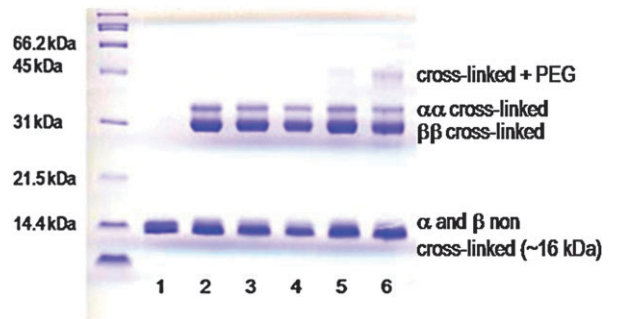


Fig. 2 SDS-PAGE (12%) analysis under reducing conditions; lane 1: native Hb—α subunit band appears below β, lane 2: HbN_3, lane 3: 50 mol% $Cu(MeCN)_4PF_6$, 20 h, lane 4: 50 mol% $CuSO_4$–TCEP, 20 h, lane 5: 50 mol% $CuSO_4$–Na ascorbate, 20 h—faint band at ~45 kDa,‡ lane 6: 50 mol% $CuSO_4$–copper powder (1–2 mg), 20 h—band at 45 kDa.‡

PEG–alkyne **2** (Fig. 3). Stability tests on HbN_3 showed that the protein was highly sensitive to high levels of Cu(II)/Cu(I), presumably due to redox reactions at the ferrous heme centres. Thus, for our initial screening reactions, we used only 50 mol% $CuSO_4$, and 10 eq. of **2**. Reactions were followed by SDS-PAGE and HPLC. Fig. 2 shows that reactions carried out in the presence of soluble Cu(I) or Cu(II) with TCEP as a reductant (lanes 3 and 4) failed to proceed. With sodium ascorbate as reductant, trace amounts of the $HbPEG_1$ product (lane 5) resulted. Replacing the reductant with Cu(0) powder, to allow for disproportionation to occur, afforded a 20% yield after twenty hours (lane 6). Continuation of this reaction for a period of three weeks led to a 70–75% yield suggesting that the three different types of cross-linked HbN_3 react at different rates with the PEG–alkyne reagent.

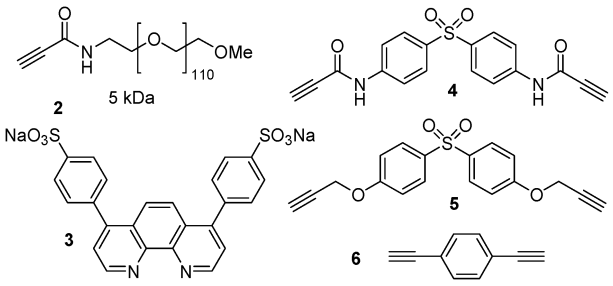
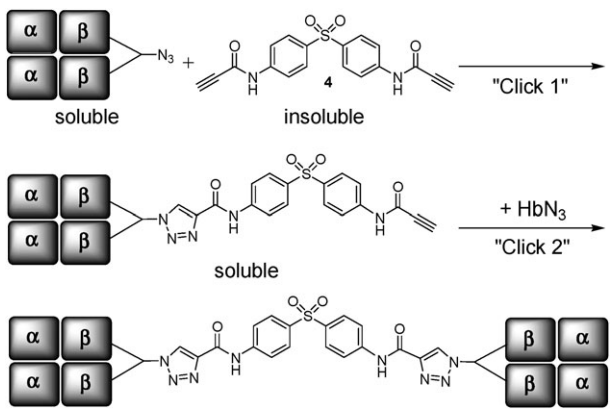


Fig. 3 Reagents used in this study.

Further optimization gave highest yields of $HbPEG_1$ with 2.0 eq. of cupric sulfate and 1–2 mg of copper powder in the presence of 6 eq. of an activating ligand (reagent **3**), typically for four hours, giving 55–60% formation of coupled product without degradation of the protein. As a control, both native and ββ cross-linked bis-trimesyl-Hb (no azide group)[8] were exposed to these conditions for 18 hours. As expected, no $HbPEG_1$ was formed in either case.

Reaction of HbN_3 with 0.5 eq. (1 : 1 azide–alkyne) of the bis-alkynes **4–6** gave only trace amounts of the bis-tetramers (HPLC analysis). However, when we increased the amount of the bis-alkyne to 10 eq., in order to take advantage of the increased solubility of the Hb–triazole–alkyne intermediate, we obtained the bis-tetramers in two cases after eight hours. As expected, the more electron-deficient bis-alkyne **4** was most reactive toward HbN_3 (Scheme 3),[5a,9] producing the highest yield of bis-tetramer (20–25% composition; Fig. 4), whereas bis-alkyne **5** gave a lower yield (10% composition). The third linker (**6**) was either too small or not reactive, forming only a trace amount of bis-tetramer with HbN_3. Comparison of the product with a structurally defined amide-linked bis-tetramer[3] by both HPLC and SDS-PAGE confirmed the proposed structure (Fig. 4 and ESI†).

Further attempts to improve yield by increasing time, copper loadings, bis-alkyne loading, temperature, *etc.* were not effective. Given our observations that our HbN_3 material is a mixture of components, this is not surprising. The apparent yield of 20–25% corresponds to a 33–40% conversion of starting material (2 eq. HbN_3 give one Hb–Hb), which correlates well



Scheme 3 Reaction of HbN_3 with bis-alkyne **4** gives a soluble Hb–alkyne which reacts to form a bis-tetramer *via* sequential coupling reactions.

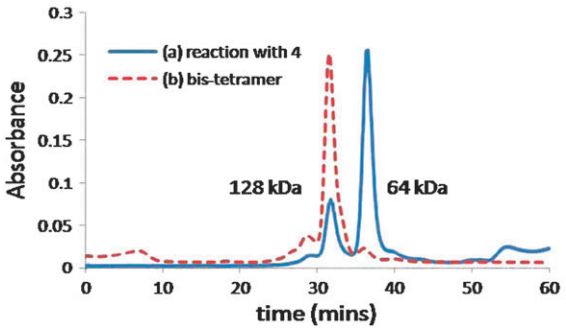


Fig. 4 (a) G200 HPLC analysis of reaction mixture at 280 nm—bis-tetramer appears at 32 min, (b) G200 HPLC analysis of authentic bis-tetramer.

with the amount of symmetrical ββ cross-linked HbN_3 present in the original mixture. Since β-lys82/β′-lys82 cross-linking occurs in the exposed BPG binding site, the azide is readily accessible. In contrast, α-lys99/α′-lys99 cross-linkers are submerged in the inner folds of the protein and are therefore much less available for reaction. The remaining non-specific ββ cross-linked material is also likely to be less accessible.

It is likely that in the case of the $HbPEG_1$, conjugation occurs first with the BPG–ββ-Hb-positioned azide cross-linker, followed by slower reaction with the various non-specific ββ-Hb–azides. The failure of the reaction to go to completion is a consequence of the active Cu(I) complex not reaching the submerged αα-Hb–azide. In the case of the bis-tetramer synthesis, steric hindrance between the functionalized proteins allows only for reaction between intermediate BPG–ββ-Hb–alkyne and unreacted BPG–ββ-Hb–N_3, with the remaining material either unable to react with the bis-alkyne (αα-modified) or reacting once (non-specific ββ modified) but becoming inaccessible for further reaction. Given this specific reactivity, the bis-tetramers thus produced are likely to be of higher homogeneity compared to that of the starting heterogeneous HbN_3 material.

Purification of the 3 : 1 reaction mixture (HbN_3–alkyne : Hb–Hb) to remove bis-tetramer and subsequent reintroduction to the reaction conditions failed to produce any further bis-tetramer, supporting our hypothesis that the reaction had gone to completion. HPLC analysis of the unreactive component confirms that no further BPG–ββ-HbN_3 remained (ESI†).

In summary, we have produced an azide-containing cross-linking reagent that reacts with Hb to form a mixture of cross-linked HbN_3 species. The major component of this reaction has been shown to be β-lys82/β′-lys82 cross-linked HbN_3. This material reacts rapidly and selectively with bivalent bis-alkyne reagent **4**, in sequential and cooperative coupling reactions, to provide almost quantitative yields of bis-tetramer, even in the presence of other constituents of the HbN_3 formation reaction. Due to the bioorthogonal[10] nature of the reactive groups, this method overcomes problems associated with the competing hydrolysis reactions observed in earlier approaches.

This is the first time click chemistry has been successfully applied to the derivatization of Hb, providing a new scaffold from which to develop future Hb-based oxygen-carriers and other materials.[11] The use of a bis-alkyne to connect two azide-functionalized proteins in sequential cooperative click reactions extends current protein coupling methods. This approach can produce a wide variety of novel homodimeric protein-coupled materials for medicinal and biological applications.

We thank NSERC Canada for support through a Strategic Project.

Notes and references

‡ Band appears ~10 kDa larger, rather than 5 kDa larger—this apparent higher mass is a typical observation when analyzing PEGylated proteins by SDS-PAGE.[12]

1 For a general overview of the current status in the field of hemoglobin-based blood substitutes, the authors refer you to the Proteins and Proteomics dedicated issue of *Biochim. Biophys. Acta*, 2008, **1784**(10), 1363–1480.

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