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Hemoglobin Dendrimers: Functional Protein Clusters

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The reactions of bifunctional electrophilic reagents with nucleophilic groups in multisubunit proteins create covalent cross-links between peptide chains, preventing dissociation of subunits. Reagents with functional group specificity and regiospecificity have been used to produce homogeneous, stabilized entities with defined properties.[1−5] In one important application, cross-linking of the tetrameric form of human adult hemoglobin (HbA) creates stabilized oxygen carriers that may serve as short-term alternatives to transfusion of red cells.[6,7] While the cross-linked tetramers are effective oxygen carriers, their physiological side effects have limited their general utility.[8−11] Hemoglobin in red cells is present in very high local concentrations, whereas cross-linked hemoglobin tetramers in circulation are dispersed and this may lead to interactions with other dispersed species, such as NO.

We reasoned that by linking several HbA tetramers into a defined cluster, we could mimic the intracellular concentration and monitor the effects of proximity on the protein's function. We have shown that two cross-linked tetramers with conjugation sites can be connected to a rigid diamine to produce a cross-linked bis-tetramer.[12] We now extend the approach to produce a *cluster of cross-linked hemoglobins* with a polyamine dendrimeric core (Figure 1).[13,14] The cluster provides a structurally defined oligomer (in contrast to the widely used heterogeneous materials from nonspecific reactions[15]).

HbA was reacted with trimesoyl tris(3,5-dibromosalicylate) TTDS[3], acquiring a bis-amide cross-link between the ϵ-amino groups of Lys-82 of the β subunits. The third ester does not react with the protein in the major product, 3,5-dibromosalicyl-trimesyl-(Lys-β-82)−(Lys-β-82)-hemoglobin (DBST-Hb)[16] and is available to react with amines (Scheme 1).

TTDS was added as a solid (0.003 g, 3×10^{-6} mol) to 1.0 mL of 1.5×10^{-6} M deoxyHb in 0.050 M pH 9.0 sodium borate. The solution was kept under a stream of nitrogen for 40 min followed by a stream of carbon monoxide for 15 min. The resulting solution of cross-linked carbonmonoxyHb was passed through a column of Sephadex G-25, which had been equilibrated with 0.1 M MOPS (pH 8), to remove TTDS and its hydrolysis products. The protein-containing effluent was transferred into 1 mL vials and frozen. At pH 8.0 addition of the amino groups of the dendrimer to DBST-Hb competes most effectively with hydrolysis of the ester.[17] Frozen solutions of DBST-Hb were thawed and added slowly to solutions of the dendrimer in order to maintain an excess of dendrimer.

Parallel reactions were conducted in order to optimize conditions for the production of conjugates containing multiple equivalents of cross-linked hemoglobin with respect to dendrimer (nHb-Den). We used generation 4.0 polyamidoamine dendrimer (PAMAM; 64 terminal amino groups, molecular mass 14,215 kDa). The solutions of DBST-Hb were added slowly to scaled amounts of dendrimer (10 wt % solution in methanol): 0.21 mL (1 equiv of dendrimer:1 equiv of DBST-Hb), 0.11 mL (1:2), 0.055 mL (1:4), 0.027 mL (1:8), 0.014 mL (1:16), 0.007 mL (1:32 equiv), and 0.004 mL (1:
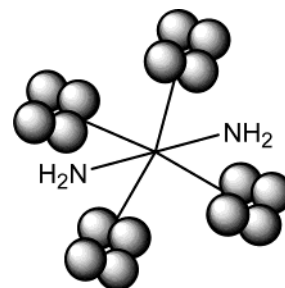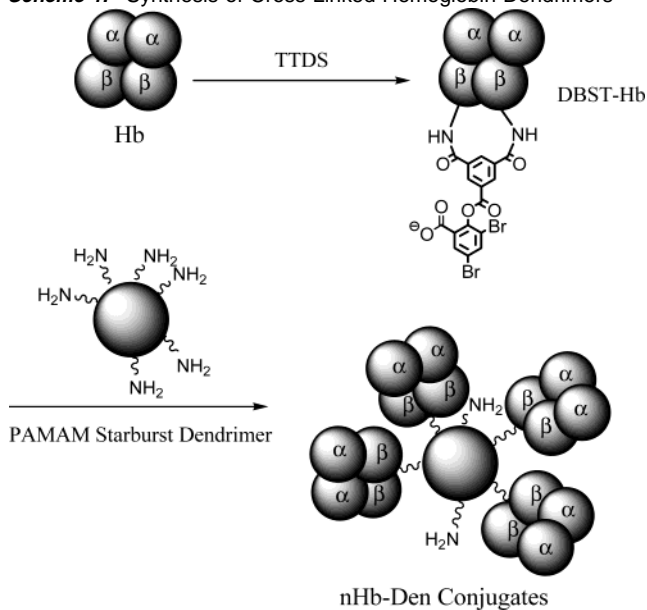
Figure 1. Hemoglobin clusters with a dendrimeric common core.

Scheme 1. Synthesis of Cross-Linked Hemoglobin Dendrimers

64). The molecular weight distributions of the reaction mixtures were analyzed by SDS-PAGE. With a larger amount of dendrimer, the second-order addition process competes more effectively with hydrolysis, producing more conjugate in total. The maximum yield of nHb-Den was achieved with 1.2×10^{-5} mol of DBST-Hb added to 0.21 mL of dendrimer solution.

Adding DBST-Hb to solutions that contain nHb-Dens leads to additional cross-linked Hbs combining with the conjugated dendrimer. Thus, DBST-Hb (1.2×10^{-5} mol, 8 equiv) was slowly added to 0.26 mL of dendrimer solution (1 equiv) at room temperature in the dark. Additional portions of DBST-Hb were thawed and reacted at 2-h intervals. The progress of the reactions was followed by anion exchange HPLC (AX-300), eluting with a pH gradient. The decrease of Hb-Den (1:1) and the increase of nHb-Den conjugate peaks were followed at 414 nm (Figure 2). The nHb-Dens were separated and isolated by preparative size-exclusion chromatography (Superdex G-200 HR) under conditions that
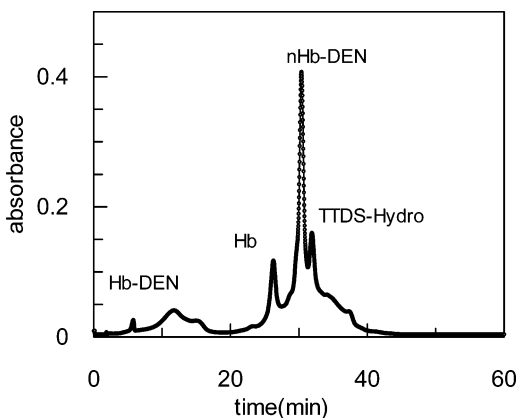
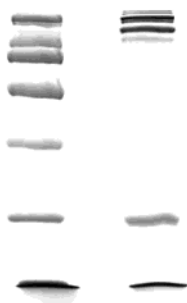**Figure 2.** AX-300 HPLC of conjugation reaction.

Figure 3. SDS-PAGE of conjugation reaction mixture: The lane on the left contains protein markers with molecular weights: 200,000, 116,000 94,000 70,000, 45,000, 30,000, and 14,000. The lane on the right is from the conjugation reaction mixture.

produce partial dissociation of native hemoglobin (2.5×10^{-2} M Tris-HCl, 0.5 M, magnesium chloride, pH 7.4). Native hemoglobin was used as a reference.

The molecular weight distribution of the reaction mixture and the number of hemoglobin molecules in a dendrimer conjugate were determined by SDS-PAGE (Figure 3). Bands at the top in the right lane are calculated to be 110 kDa, 142 kDa, and 174 kDa. We conclude that the principal conjugates are 3Hb-Den, 4Hb-Den, and 5Hb-Den.

Data for oxygen binding were collected in a computer interfaced to a UV–vis spectrophotometer and oxygen electrode with gas and temperature regulation.[18] Samples were prepared so that the final concentrations of added protein were between 40 and 60 μM (heme-based). Absorbance was followed at 560 nm with the sample maintained at 20 °C. Values of p_{50} and n_{50} were computed from the experimental points in the range 40–60% of oxygen saturation by regression analysis.

To assess the effects of the dendrimer on the protein, we also produced a material where only one hemoglobin tetramer is conjugated per dendrimer (Hb-Den) and its oxygen binding properties were evaluated. Oxygen affinities of the nHb-Dens are higher than that of the TTDS cross-linked tetramer while cooperativity is reduced (but not eliminated). In Table 1 the averaged properties of the proteins in the cluster are compared with those of the cross-linked tetramer and the Hb-Den conjugate. Conjugation to the dendrimer increases the oxygen affinity and decreases cooperativity. Bringing more tetramers into a clustered entity alters the oxygen binding behavior beyond that caused by the modification itself.

Table 1. Oxygen Affinities and Hill Coefficients of Modified Hemoglobin, Dendrimer Conjugate, and Dendrimer Cluster[a]

modified protein	p_{50} (Torr)	n_{50}
cross-linked Hb	4.36 ± 0.06	2.51 ± 0.08
Hb-Den	2.03 ± 0.06	1.88 ± 0.05
nHb-Den	1.56 ± 0.05	1.60 ± 0.05

[a] In 0.1 M pH 7.0 phosphate buffer at 20 °C.

Perutz proposed that in HbA repulsion between positive charges in the central cavity raises the free energy of the T state relative to the R state, thereby raising the oxygen affinity. Effector molecules such as chloride ions neutralize these positive charges and therefore lower the oxygen affinity.[19,20] By conjugating cross-linked tetramers to the dendrimer, the positively charged surface groups of the dendrimer are brought into proximity of the central cavity of HbA. Any further changes in oxygen-binding properties of the nHb-Den cluster should result from hemoglobin–hemoglobin interactions.

In conclusion, we have prepared a hemoglobin cluster by conjugating cross-linked tetramers to a defined core-starburst dendrimer. The altered oxygen-binding properties of the resulting cluster suggest that protein–protein interactions are manifested by their effect on oxygen-binding properties. This method of generating an assembly of interacting proteins should have applications in studying their interactions as well as in generating improved biomaterials of known structure (oligomerization is more likely to correlate with clinical success in red cell substitutes than are low oxygen affinity and high cooperativity[10]).

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