生物正交偶联的交联血红蛋白双四聚体不会引起循环中的血管收缩。

Cross-linked hemoglobin bis-tetramers from bioorthogonal coupling do not induce vasoconstriction in the circulation

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BACKGROUND: Hemoglobin-based oxygen carriers (HBOCs) are potential alternatives to red blood cells in transfusions. Clinical trials using early versions of HBOCs noted adverse effects that appeared to result from removal of the vasodilator nitric oxide (NO). Previous reports suggest that size-enlarged HBOCs may avoid NO-rich regions along the vasculature and therefore not cause vasoconstriction and hypertension.

STUDY DESIGN AND METHODS: Hemoglobin (Hb) bis-tetramers (bis-tetramers of hemoglobin that are prepared using CuAAC chemistry [BT-Hb] and bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry [BT-acHb]) can be reliably produced by a bio-orthogonal cyclo-addition approach. We considered that an HBOC derived from chemical coupling of two Hbs would be sufficiently large to avoid NO scavenging and related side effects. The ability of intravenously infused BT-Hb and BT-acHb to remain in the circulation without causing hypertension were determined in wild-type (WT) and diabetic (*db/db*) mouse models.

RESULTS: In WT mice, the coupled oxygen-carrying proteins retained their function over several hours after administration. No significant changes in systolic blood pressure from baseline were observed after intravenous infusion of BT-Hb or BT-acHb in awake WT and *db/db* mice. In contrast, infusion of native Hb or cross-linked Hb tetramers in both animal models induced systemic hypertension.

CONCLUSION: The results of this study indicate that bis-tetrameric HBOCs derived from the bio-orthogonal cyclo-addition process are likely to overcome clinical issues that arise from NO scavenging by Hb derivatives.

结论：本研究结果表明，
生物正交环加成法制备的双四聚体HBOCs
有可能克服NO清除引起的临床问题。

Worldwide blood shortages and the potential adverse effects of blood transfusions continue to stimulate interest in the development of hemoglobin-based oxygen carriers (HBOCs) as potential universal sterile alternatives to the use of red blood cells (RBCs) in transfusions.[1] Human adult hemoglobin (Hb) is

ABBREVIATIONS: BT = bis-tetramers of hemoglobin prepared using tetra-ester; BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry; COHb = carbomonoxy-hemoglobin; deoxyHb = deoxygenated hemoglobin; CuAAC = copper(I)-catalyzed azide alkyne cycloaddition; *db/db* = diabetic mice; HbA = hemoglobin A; HBOCs = hemoglobin-based oxygen carriers; Hp = haptoglobin; HR = heart rate; metHb = methemoglobin; NO = nitric oxide; oxyHb = oxygenated hemoglobin; PBS = phosphate buffered saline; SD = standard deviation; SBP = systolic arterial murine blood pressure; WT = wild-type; xl-acHb = hemoglobin tetramers that are specifically cross-linked and acetylated; xl-Hb = hemoglobin tetramers that are specifically cross-linked.

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a tetrameric protein composed of two αβ dimers. In the circulation, cell-free Hb spontaneously dissociates into the αβ subunits, which are small enough to be filtered by and trapped within the kidney, a condition that may induce renal toxicity.[2] Intra- or intermolecular chemical cross-links between subunits of the Hb tetramer prevent its dissociation into dimers.[3–6] The chemical linkages reduce HBOCs' renal toxicity and extend their duration of circulation.[7] Clinical evaluations of diverse HBOCs revealed that their use is associated with a multitude of side effects, such as hypertension, along with myocardial infarction, gastrointestinal complications with evidence of pancreatic injury and hepatocellular injury, acute renal failure, and mortality.[8–10] In general, the side effects have been ascribed to vasoconstriction caused by HBOCs produced from stabilized heme-containing proteins that enter sites where they scavenge endogenous nitric oxide (NO), a gaseous transmitter that induces relaxation of smooth muscles in blood vessels.[11–13] To produce effective HBOCs that avoid NO scavenging, enlarged HBOCs have been designed, prepared, and evaluated.[14–17] We note that alternative mechanisms by which HBOCs could induce adverse effects include hyperoxygenation[18] and heme-mediated oxidative damage.[19,20]

Previously, an Hb bis-tetramer (BT) (Fig. 1), was produced from Hb with a tetra-functional cross-linker that covalently joins two Hb molecules by creating amide linkages between the cross-linked tetramers.[15] The size of the BT, which consists of coupled tetramers, has an effective size that greatly exceeds that of the Hb tetramer (6.4 nm).[21] We expect that the enlarged size of the bis-tetramer would prevent the HBOC from readily entering endothelial cell junctions that are only somewhat larger than that of native Hb (approx. 7 nm).[22] According to this proposal, the size of bis-tetramers would thereby minimize or block their scavenging of endothelial NO. Studies using intravenous (IV) infusion of BT into mice reveal that the materials do not cause systemic hypertension that is seen with native Hb.[23] However, the route to production of the initially studied BT is relatively inefficient, with an overall yield of 40%. The tetra-functional cross-linkers used to produce BT are susceptible to competing hydrolysis, which renders the hydrolyzed sites unreactive.[15] Thus, we sought alternative chemical approaches to produce bis-tetramers without hydrolysis competing with protein modification.

We developed a set of bio-orthogonal chemical reactions, whose components can react with each other rapidly and selectively under physiological conditions.[24,25] In particular, copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) produces a triazole, which is a mimic of a peptide linkage.[26] The process has been successfully used to couple structurally defined proteins.[27–29] An initial attempt to apply CuAAC to producing a bis-tetramer led to our use of an azide-containing cross-linked Hb in a reaction with a bis-alkyne to create a triazole-containing interprotein link.[30] Two cross-linked Hb-azides undergo serial phase-directed cycloaddition to produce a bis-alkyne in the presence of copper(I), forming an Hb bis-tetramer efficiently.[30] However, we found that the reaction producing the azide-containing cross-link introduces cross-links between either α subunits or β subunits that are mutually exclusive. Although azides are present in cross-linked Hbs of both types, CuAAC giving the bis-tetramerT occurs only where the azide is located on the cross-link between the β subunits.[30] Because CuAAC is normally an effective process, we assume that the azide in the αα cross-linked Hb is blocked by the surrounding protein. Thus, a more efficient process requires selectively cross-linking only between β subunits.

To maximize the efficiency of the CuAAC process for the production of Hb bis-tetramer, we devised two alternative approaches to prepare the desired ββ cross-linked Hb-azide selectively. The first approach attains selectivity by using a trifunctional cross-linker that reacts to a greater extent at the β subunits (ε-amino groups at β-lys-82), trimesoyl tris(3,5-dibromosalicylate).[4,31] Sequential in situ aminolysis of the remaining activated ester on the cross-link with an amine-azide nucleophile results in an azide being appended to the cross-link (Fig. 2).[31] The second approach uses selective α-site acylation of amino groups to block reactions that introduce azides from occurring at amino groups on α subunits.[32] We followed the general approach of Walder and coworkers,[33] using reversible blocking of amino groups in the β subunits followed by acetylation of amino groups in the α subunits. The subsequent cross-linking reaction on the α-acetylated Hb is then highly specific for the desired amino groups in the β subunits.[32] The optimized bio-orthogonal processes result in formation of Hb BTs, designated BT-Hb and BT-acHb respectively (Fig. 1).[31,32]

With the materials being readily prepared and purified, our present study assessed the in vivo effects of the triazole-linked materials in the circulation, specifically to determine if they would induce hypertension. We assessed the vasoconstrictive effects of BT-Hb and BT-acHb in both healthy wild-type (WT) mice and diabetic (*db/db*) mice using non-invasive measurements. A prominent feature of the *db/db* mice is the presence of endothelial dysfunction, which is associated with reduced vascular NO levels.[34] The *db/db* mice are more susceptible to HBOC-mediated vasoconstrictive effects, making them a more stringent model for detecting the potential adverse effects of HBOCs.[35]

MATERIALS AND METHODS

Animal studies

Animal studies were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital, Boston, Massachusetts. We studied the circulatory response of 8–10 week old male C57BL/6 J WT mice (25 ± 2 g) and B6.Cg-m$^{+/+}$Lepr^{db}/J (with C57BL/6 J

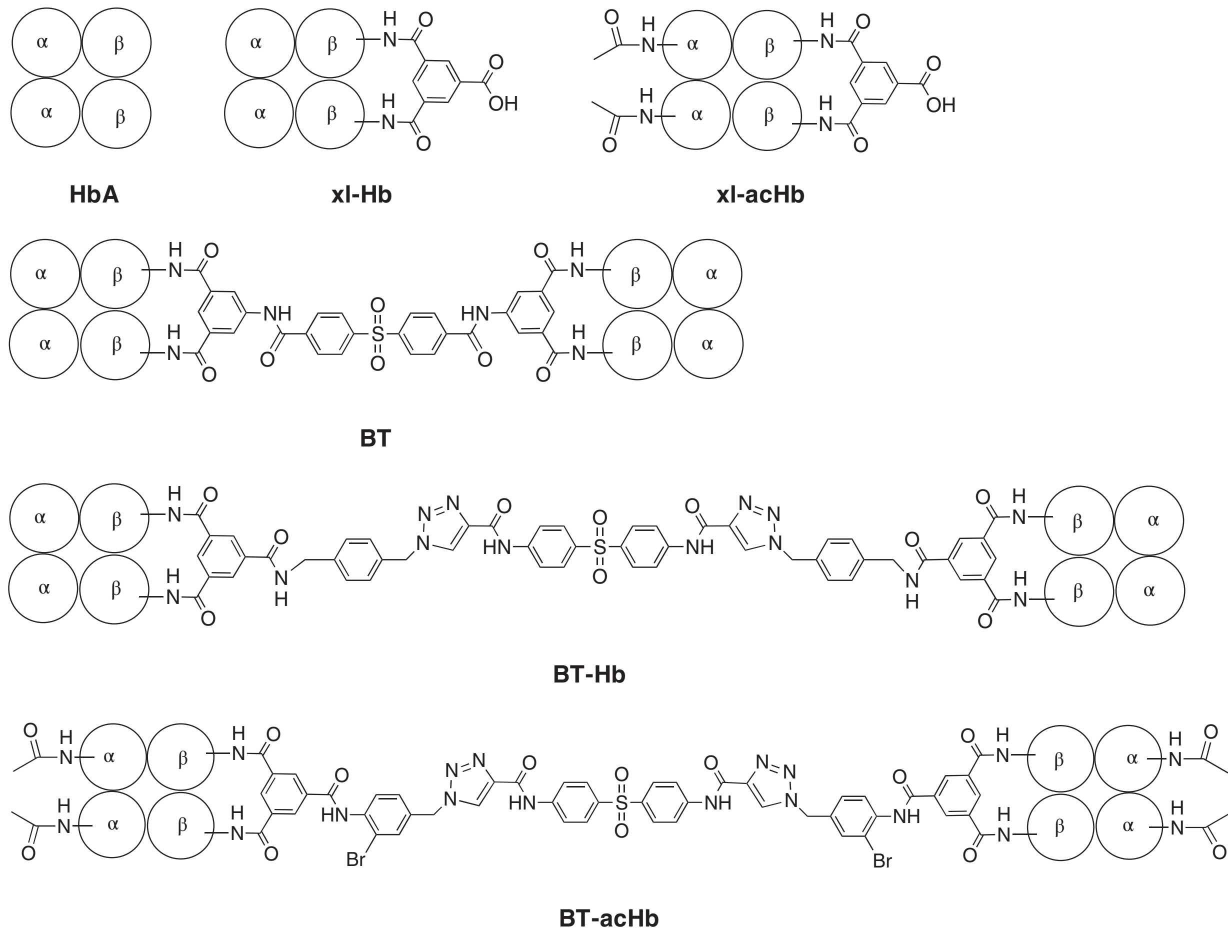


Fig. 1. Structures of hemoglobin-based oxygen carriers tested in the present study and previously tested bis-tetramer. HbA is native human hemoglobin A; xl-Hb is human Hb cross-linked between the β subunits; xl-acHb is human Hb acetylated in the α subunits and cross-linked between the β subunits; BT is a member of the first generation of Hb bis-tetramers prepared using a different chemical approach.[23] BT-Hb is a bis-tetramer formed by connecting two cross-linked Hb tetramers together using CuAAC chemistry; the interprotein linkage contains two bio-orthogonal triazole groups; BT-acHb is acetylated bis-tetramer prepared using similar CuAAC chemistry as BT-Hb did.

background) *db/db* mice (42 ± 3 g). All mice were obtained from Jackson Laboratory (Bar Harbor, Maine).

Preparations of BT-Hb, BT-acHb, and Hb-based control solutions

BT-Hb was prepared from human adult Hb and purified as previously described[31] with minor improvements (Fig. 2). Briefly, a 0.5 mM solution of deoxygenated hemoglobin (deoxyHb) (in sodium borate buffer, 50 mM pH 9.0) reacted with trimesoyl tris(3,5-dibromosalicylate) (2 eq.) for 12 minutes under nitrogen at 37 °C. Four-azidomethylbenzylamine (40 eq.) was then added. The mixture was flushed with carbon monoxide and stirred at room temperature for one hour. The resulting solution was purified using gel filtration, centrifugation, and heat treatment, to yield the cross-linked Hb-azide tetramer. The carbomonoxy-Hb-azide (in phosphate buffer, 0.02 M, pH 7.4) was mixed with bis-alkyne (10 eq.), bathophenanthroline (2 eq.), copper(II) sulfate ($CuSO_4$) (4 eq.), and ascorbic acid (40 eq.) under carbon monoxide. The ascorbic acid was added last to initiate the CuAAC reaction by reducing Cu(II) to Cu(I). The reaction mixture was stirred at room temperature for 4 hours. The modified Hbs were analyzed by gel-filtration column chromatography (Superdex G-200 HR, 10 × 300 m). Protein samples (0.5 mM) were eluted under partially dissociation conditions (37.5 mM Tris–HCl, pH 7.4, and 0.5 M magnesium chloride) and monitored at 280 nm. BT-Hb was purified using gel-filtration chromatography (Sephadex G-100, 1000 × 35 mm).[31] BT-acHb was prepared and purified according to the previously published method.[32]

We used Hb and cross-linked Hb (xl-Hb and xl-acHb) as controls in the infusion experiments (Fig. 1). Hb was obtained from Oxygenix, Inc. upon closure of their operations in Canada. The xl-Hb and xl-acHb are cross-linked tetrameric counterparts to BT-Hb and BT-acHb, respectively,

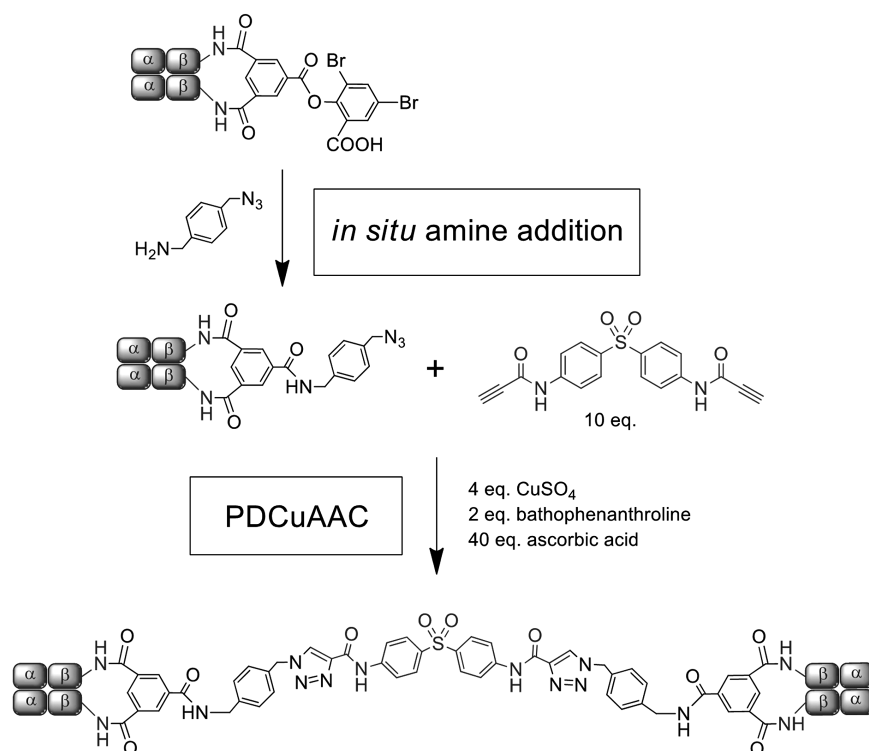


Fig. 2. Preparation of BT-Hb. The direct synthesis of ββ cross-linked Hb-azide enhances the conversion of Hb bis-tetramer using a phase-directed Cu(I)-catalyzed azide-alkyne Cycloaddition (PDCuAAC) process. BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry.

and were prepared according to literature methods with trimesoyl tris(3,5-dibromosalicylate) being the cross-linker.[31,32]

As in earlier studies,[23] all solutions to be used for infusion were prepared in phosphate-buffered saline (PBS), with 0.33 g/dL N-acetyl cysteine (pH 7.1) added to minimize autooxidation of the ferrous heme iron. The concentrations of Hb solutions were produced at 3.5 to 7 g/dL. The samples for study were subjected to sterile filtration through a series of a 0.45-μm filter and two 0.20-μm filters. The resulting solutions were stored in sealed vials under carbon monoxide at 4 °C. Before each infusion experiment, the Hb-containing solutions were oxygenated using photo-irradiation under oxygen for 1 hour at 4 °C. The solutions were warmed to 37 °C in a water bath immediately prior to infusion. The blood gas tensions of some of the oxyHb samples (HbA, xl-acHb, and BT-acHb) were measured using a blood gas analyzer (ABL800 Flex, Radiometer).

Blood sampling

Mice were anesthetized two hours after tail-vein IV infusion of a solution of the protein or the control solution of vehicle (buffer) with an intraperitoneal injection of ketamine (120 mg/kg) and fentanyl (0.09 mg/kg). Whole blood was collected into a heparinized syringe via an open-chest cardiac puncture. Blood plasma was separated after centrifuging samples of whole blood at 4000 rpm for 10 minutes at 4 °C. The collected plasma samples were stored at −80 °C. Animals were euthanized by deep anesthesia followed by cervical dislocation.

Measurement of concentrations of Hb and methemoglobin

The concentrations of Hb species in solutions and in plasma were measured with a Hb assay kit from QuantiChrom (BioAssay Systems). (Details concerning the use of this assay are available in the user manual at https://www.bioassaysys.com/Hemoglobin-Assay-Kit.html#tab1). The methemoglobin (metHb) content (including both oxidized HbA and oxidized Hb derivatives) in plasma samples was determined by least squares fitting of the data in visible spectra using the extinction coefficients of oxygenated hemoglobin (oxyHb), deoxyHb, metHb and carbomonoxy-hemoglobin (COHb) between 500 nm to 700 nm with Solver software (Excel 2011, Microsoft, Redmond, WA).

Measurement of systolic blood pressure and heart rate in awake mice

Systolic blood pressure (SBP) and heart rate (HR) were measured with a noninvasive tail-cuff blood pressure system in awake mice. To allow the mice to acclimate to the tail-cuff system, the mice were placed into restraining tubes for

10 minutes at least three times prior to taking any measurement. Stable and low SBP baselines indicated that mice became sufficiently comfortable during restraint. In WT mice, all native and modified Hb samples at a concentration of 3.5 g/dL were administered intravenously at a dosage of 0.4 g/kg body weight. Volumes of Hb-containing solutions or vehicle were injected via a tail vein of the mice over the course of 1 minute to arrive at a 15% top-load infusion (e.g., 0.3 mL in a 25 g mouse) as described previously.[23] The SBP and HR were measured at 10-minute intervals for an hour after infusing the test solutions.

In studies using *db/db* mice, the amount of protein (HbA, xl-acHb and BT-acHb, 3.5 g/dL) administered was maintained at 0.4 g/kg. This results in a larger top-load infusion (e.g., 0.6 mL in a 50 g mouse) as most of the additional weight of *db/db* mice relative to the weight of typical WT mice is due to the amount of additional fatty tissue, which is not highly vascular.[36] To avoid any potential adverse effects of the higher top-load infusion into the *db/db* mice, we used higher concentrations (7 g/dL) of xl-Hb and BT-Hb at the same dosage of 0.4 g/kg (e.g., 0.3 mL in a 50-g mouse).

Nitric oxide consumption assay

To determine whether the Hb derivatives react with exogenous NO (nitric oxide) before or after infusion, we measured the amount of NO scavenged by solutions of BT-Hb before infusion and plasma Hb after infusion, using a NO analyzer (280i NO Analyzer, Sievers).[37] The plasma samples were taken 2 hours after infusion. A glass reaction vessel connected to the analyzer was purged with nitrogen and filled with an NO donor (100 μM DETA NONOate, Cayman Chemical) in PBS. The slow decay of DETA NONOate releases NO, generating a steady signal. Samples (4 or 10 μL) of human HbA standards, BT-Hb, or plasma were injected into the reaction vessel (in duplicate) to produce decreases in signals. Consumption of NO was calculated from the area under the curve that records the decreasing concentration of NO. The analytical method was performed with task-specific software (Sievers). The standard curves of consumption of NO were constructed based on the assumption that purified standards for Hb (100% oxyHb) react stoichiometrically with NO. The ability of Hb solutions or plasma samples to bind NO was reported as the ratio of the NO consumption to the concentration of heme.

Statistical analysis

All data are shown as mean ± standard deviation (SD). A two-way analysis of variance (ANOVA) of group vs. time followed by the Bonferroni post-hoc test was used to compare the SBPs and HRs of awake mice before and after infusion of Hb-containing solutions. Plasma Hb concentration and consumption of NO by plasma Hb between groups were compared, respectively, by paired t-test and two-tailed P values were calculated. A P value of less than 0.05 was considered statistically significant. Analysis was performed using computer software (Excel 2011, Microsoft; StatPlus 6.1.7, AnalystSoft).

RESULTS

Production and characterization of BT-Hb and BT-acHb

BT-Hb and BT-acHb were successfully prepared by reported methods.[31,32] The chemical and physical properties of the bis-tetramers are described and compared to BT[38] in Table 1. Both of the bis-tetramers used in this study are derived from human Hb. The nature of the cross-linking reaction and CuAAC reaction provides both an intramolecular cross-link between the β subunits as well as an interprotein linkage between two Hb tetramers via a set of triazole scaffolds. In addition, BT-acHb contains a set of α chain acetyl groups that have been added onto exposed amino groups. This leads to a more efficient formation of the bis-tetramer than in the absence of acetylation. The Hb derivatives were prepared in PBS that had been treated with the antioxidant N-acetyl cysteine to minimize auto-oxidation of the ferrous heme. The purity of samples of bis-tetramers is

TABLE 1. Chemical and physical characteristics of BT-Hb, BT-acHb, and previously reported BT

	BT-Hb	BT-acHb	BT[38]
Source	Human	Human	Human
Chemical changes	β cross-linked interprotein linked	Acetylated β cross-linked interprotein linked	β cross-linked interprotein linked
Buffer	PBS (with NAC)	PBS (with NAC)	PBS (with NAC)
pH	7.1	7.1	7.1
Size (kDa)	128	128	128
% Tetramer	0	0	0
% metHb	<1	<5	20.2
P_{50} (mm Hg)	6	4.7	9.3
n_{50}	2.2	2.0	2.7

BT = bis-tetramers of hemoglobin prepared using tetra-ester; BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry; metHb = methemoglobin; NAC = N-acetyl cysteine PBS = phosphate buffered saline.

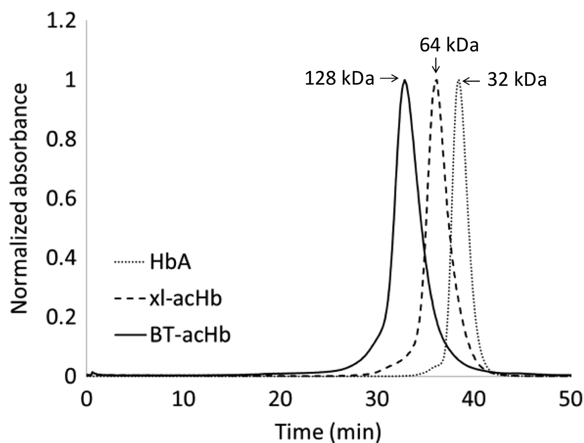


Fig. 3. Size exclusion chromatographic analysis of HbA, cross-linked Hb tetramer xl-acHb, and bis-tetramer BT-acHb. Sharp peaks and distinct retention times reflect the homogeneity of each derivative. The bis-tetramers (128 kDa) are roughly twice the size of cross-linked tetramers (64 kDa) and quadruple that of the dissociated Hb dimers (32 kDa), leading to their earlier elution as seen on the chromatogram. BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; HbA = hemoglobin A; xl-acHb = hemoglobin tetramers that are specifically cross-linked and acetylated.

critical as a solution of HBOC containing as little as 1% tetrameric Hb has been reported to induce effects of vasoconstriction in animal models.[35] The purification protocol we used to obtain solutions of BT-Hb and BT-acHb removes any detectable species smaller than bis-tetramers as confirmed by HPLC analysis (Fig. 3).

The auto-oxidation of heme iron to the met-heme state that does not bind oxygen is an important consideration in the use of HBOCs. After our successful production and purification of bis-tetramers, we found less than 1% of BT-Hb and 5% BT-acHb had been oxidized to the ferric state. The resulting BT-Hb and BT-acHb were stored under carbon monoxide. They were oxygenated immediately prior to infusion to minimize auto-oxidation of the heme iron. Less than 10% COHb was present in all heme-protein samples prior to infusion. The minor amount of COHb should not pose any significant influence in the present infusion experiment. Misra and coworkers[39] have also reported the safe use of 100% carbomonoxy-HBOC in preclinical studies.

Plasma levels of total Hb and metHb after infusion of BT-Hb or BT-acHb in WT mice

To compare the abilities of BT-Hb and BT-acHb to remain in the circulation to that of BT, we determined the total concentrations of Hb in plasma 2 hours after infusion (Fig. 4A). In WT mice, infusion of vehicle alone did not significantly alter the plasma level of cell-free Hb. In contrast, infusion of Hb-containing solutions produced significantly increased plasma Hb concentrations. Infusion of HbA increased plasma Hb levels from the baseline level observed in the vehicle group (0.04 $\pm$ 0.02 g/dL) to 0.21 $\pm$ 0.03 g/dL after 2 hours. Administration of xl-acHb, BT-acHb and BT-Hb increased plasma Hb levels to 0.50 $\pm$ 0.06 g/dL, 0.62 $\pm$ 0.06 g/dL and 0.62 $\pm$ 0.10 g/dL after 2 hours, respectively. These results show that BT-Hb and BT-acHb remain in the circulation in significant amounts 2 hours after infusion.

We compared the functional persistence of BT-Hb and BT-acHb in the circulation to that of BT (as reported from earlier studies) by measuring the concentration of metHb in plasma 2 hours after infusion (Fig. 4B). The results showed that less than 3% of Hb in plasma was present in the non-functional ferric form. In the case of addition of BT-acHb, although the metHb levels were approximately 13% to 14% prior to infusion (as a result of auto-oxidation of heme during the preparation process), low levels of metHb (2%) were detected in plasma 2 hours after infusion. Similar results were observed in an earlier study using BT,[23] in which plasma metHb level was 5% following infusion of BT solution containing 20% metHb. The low metHb levels in plasma after infusion of BT-Hb and BT-acHb indicate that the second-generation Hb bis-tetramers in the present studies remain in the circulation in a functional, oxygen-carrying state.

Hemodynamic effects of BT-Hb and BT-acHb in awake WT mice

To determine the hemodynamic effects of infusing BT-Hb and BT-acHb in awake WT mice, the SBP and HR were measured before and after IV infusion of the bis-tetramers (Fig. 5). The SBP and HR of awake mice were monitored for 1 hour following IV infusion of BT-Hb or BT-acHb (0.4 g/kg). Comparative measurements were performed using vehicle alone, HbA or the cross-linked tetrameric counterparts of the bis-tetramers (xl-Hb and xl-acHb). The baseline SBP of mice in all six groups was between 100 and 110 mm Hg. IV infusion of vehicle alone had no effects on SBP over the course of 1 hour after infusion (n = 11). IV infusion of HbA (n = 13), cross-linked xl-Hb (n = 4), or xl-acHb (n = 7) caused immediate and significant increases in SBP from baseline to 128 $\pm$ 2, 125 $\pm$ 4 and 129 $\pm$ 4 mm Hg, respectively, over 1 hour after infusion. In contrast, the SBP of WT mice receiving IV BT-Hb (n = 6) or BT-acHb (n = 7) remained at baseline levels of 104 $\pm$ 1 and 110 $\pm$ 2 mm Hg, respectively. No significant changes were observed in HR of mice after IV infusion of vehicle (n = 5), HbA (n = 7), xl-acHb (n = 7), or BT-acHb (n = 7), respectively. Our data show that infusion of BT-Hb or BT-acHb in awake WT mice does not induce systemic hypertension, suggesting that BT-Hb and BT-acHb may

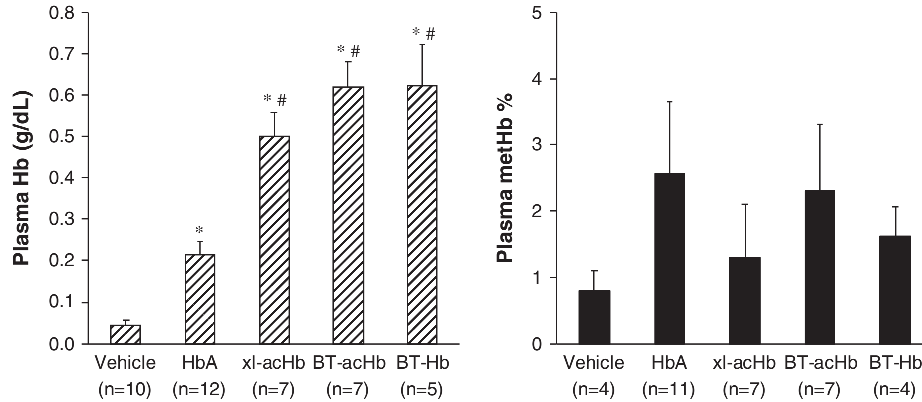


Fig. 4. Plasma levels of total Hb and metHb 2-hours after infusion in WT mice. *p < 0.05; value differs from plasma Hb concentration of the vehicle group. #p < 0.05; value differs from plasma Hb concentration of the HbA group. HbA = hemoglobin A; metHb = methemoglobin; WT = wild-type.

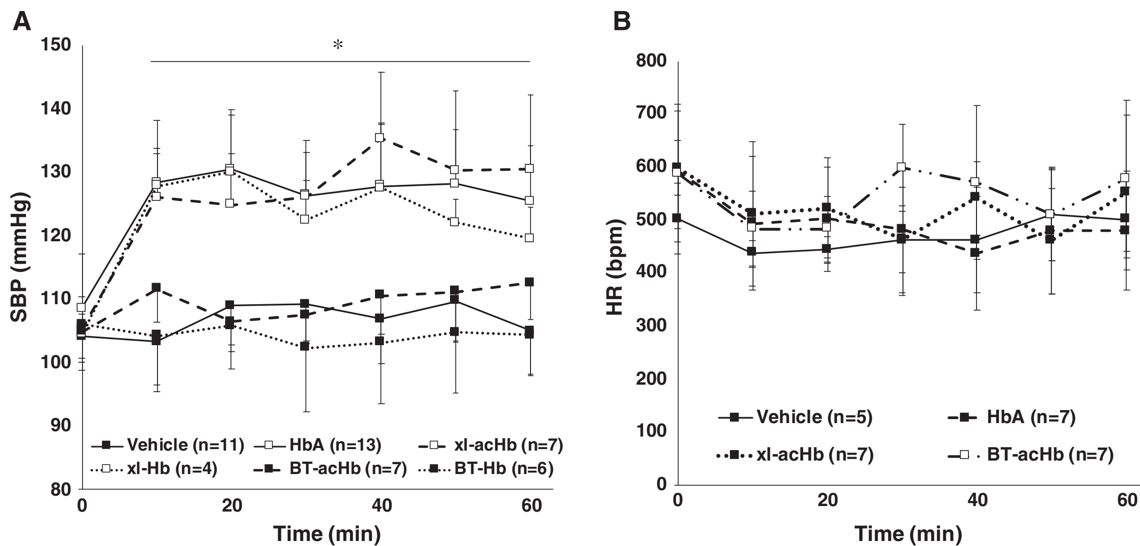


Fig. 5. Hemodynamic responses to IV Hb/vehicle infusions in awake WT mice. (A) Infusion of vehicle, BT-Hb, and BT-acHb did not cause significant changes in SBP compared to baseline (displayed as data point at 0 min), whereas the infusion of HbA, xl-Hb, and xl-acHb cause a significant and sustained increase in SBP compared to baseline. (B) The HRs of WT mice after infusion of vehicle, HbA, xl-acHb, and BT-acHb are not significantly different from baseline. Time 0 min is the time of injection and the average of each baseline. Values are displayed as mean ± SD. *p < 0.05; value differs from its own SBP baseline. BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry; HR = heart rate; SBP = systolic arterial murine blood pressure; SD = standard deviation; WT = wild-type; xl-acHb = hemoglobin tetramers that are specifically cross-linked and acetylated; xl-Hb = hemoglobin tetramers that are specifically cross-linked.

not be able to scavenge NO from the endogenous sites needed for signaling relaxation of blood vessels.

Hemodynamic effects of BT-Hb and BT-acHb in awake *db/db* mice

The *db/db* mouse, with reduced ability to produce vascular NO, is a more stringent model to assess Hb-mediated scavenging of NO.[35] We measured SBP and HR in awake *db/db* mice after infusion of BT-Hb and BT-acHb (Fig. 6). Baseline SBPs in all groups ranged between 100 and 110 mm Hg. The SBP in the group receiving vehicle (n = 5) remained at 108 ± 2 mm Hg during the first hour after infusion. Infusion of HbA, xl-Hb, or xl-acHb induced systemic hypertension, with a significant and sustained increase in SBP from baseline to 128 ± 3 mm Hg, 127 ± 2 mm Hg and 132 ± 4 mm Hg over the 1-hour duration of the measurements. In contrast, the SBP of *db/db* mice receiving BT-Hb remained at 105 ± 1 mm Hg after administration. Infusion of BT-acHb caused a small and transient increase in SBP to

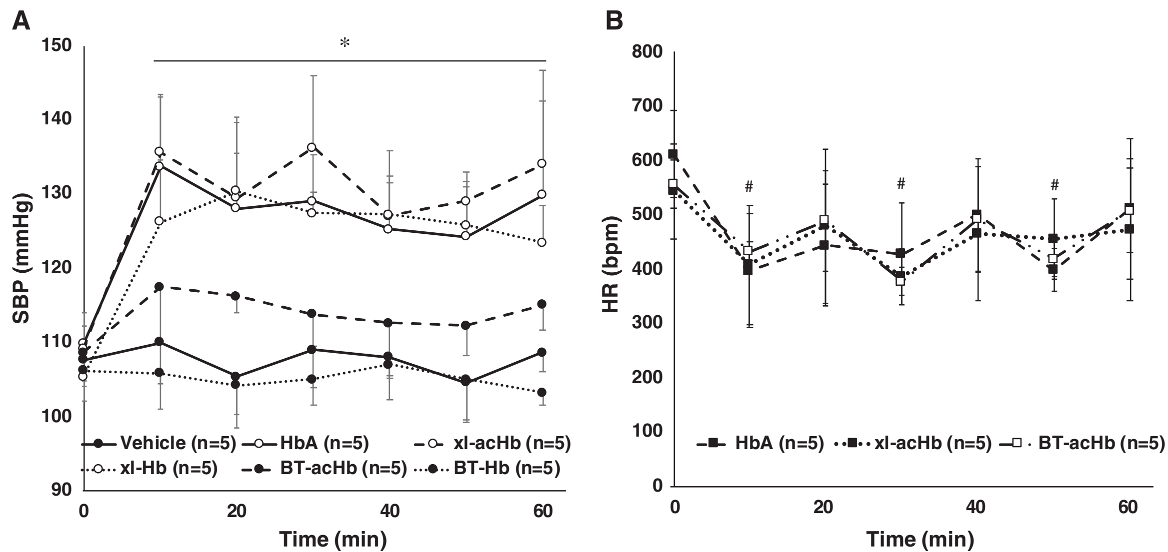


Fig. 6. Hemodynamic responses to IV Hb/vehicle infusions in awake *db/db* mice. (A) Infusion of vehicle, BT-Hb, and BT-acHb did not cause significant changes in SBP, while infusion of HbA and xl-acHb induced immediate increase in SBP. (B) The HRs of *db/db* mice after infusion of xl-acHb and BT-acHb are not significantly different from baseline. The HR of mice receiving HbA experienced significant drops at 10, 30, and 50 minutes. Time 0 min is at injection and the average BP of each baseline. Values are presented as mean ± SD. *p < 0.05; value differs from its own SBP baseline. #p < 0.05; value differs from its own HR baseline in the HbA group. BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry; *db/db* = diabetic mice; HbA = hemoglobin A; HR = heart rate; SBP = systolic arterial murine blood pressure; SD = standard deviation; WT = wild-type; xl-acHb = hemoglobin tetramers that are specifically cross-linked and acetylated.

118 ± 8 mm Hg from 109 ± 4 mm Hg that returned quickly to the baseline and remained at 114 ± 6 mm Hg over the remaining duration of the experiment. The HR in the group receiving HbA (n = 5) decreased significantly at 10, 30, and 50 minutes when compared to its own baseline ($p < 0.05$). Infusion of BT-acHb did not change HR significantly in those mice. These results show that the IV infusion of BT-Hb or BT-acHb in *db/db* mice does not induce systemic hypertension.

Capability of BT-acHb and BT-Hb to scavenge NO in vitro

Infusion of BT-Hb and BT-acHb did not induce hypertension in WT or *db/db* mice. The ability of the Hb bis-tetramers to avoid inducing hypertension is likely to be a result of the bis-tetramers remaining in the vasculature. The bis-tetramers do not penetrate the endothelial cell layer and thereby avoid their scavenging of endogenously generated NO. In principle, it is possible but unlikely that the lack of vasoactivity is due to bis-tetramers not binding NO in general in the circulation as exemplified by materials that were produced from recombinant strategies.[40,41] Therefore, we examined the capability of BT-Hb and BT-acHb to scavenge NO before and after infusion into mice by studying whether the reaction between heme and NO would occur at a 1:1 molar ratio. Solutions of HbA and BT-Hb were prepared

因此，我们通过研究血红素和一氧化氮在1：1摩尔比下的反应，研究了bt-hb和bt-achb在小鼠输注前后清除NO的能力

and their NO removal capacities were measured (Fig. 7A). The slope of the fitted line to the NO consumption by BT-Hb is approximately unity (Fig. 7B), indicating that the BT-Hb can scavenge NO to the same extent as HbA. Analysis of plasma collected 2 hours after infusion of BT-acHb into mice (Fig. 7C and 7D) showed that the plasma was able to react with exogenous NO in one-to-one NO to heme ratio, similar to the reactions with HbA and xl-acHb. The results show that the bis-tetramers remain capable of binding NO to which they have access. This is consistent with the hypothesis that the lack of the vasoconstrictor effect from bis-tetramers is due to restricted access to endogenous NO.

双四聚体不穿透内皮细胞层，从而避免其清除内生生成的NO。进而不会引发高血压症状。

DISCUSSION

The bis-tetramers generated from efficient bio-orthogonal processes appear to overcome successfully the critical problems associated with induced hypertension that may have led to the failure of clinical trials of materials that contained smaller Hb species. In our study, the desirable property of steady levels of infused BT-Hb and BT-acHb in the circulation remain for the 2 hours after administration. The low levels of metHb in plasma indicate that the bis-tetramers remain in the useful form that is capable of carrying oxygen. IV administration of BT-Hb or BT-acHb did not induce systemic hypertension in awake WT or *db/db* mice. Yet BT-Hb

健康野生型小鼠和糖尿病小鼠（db/db）

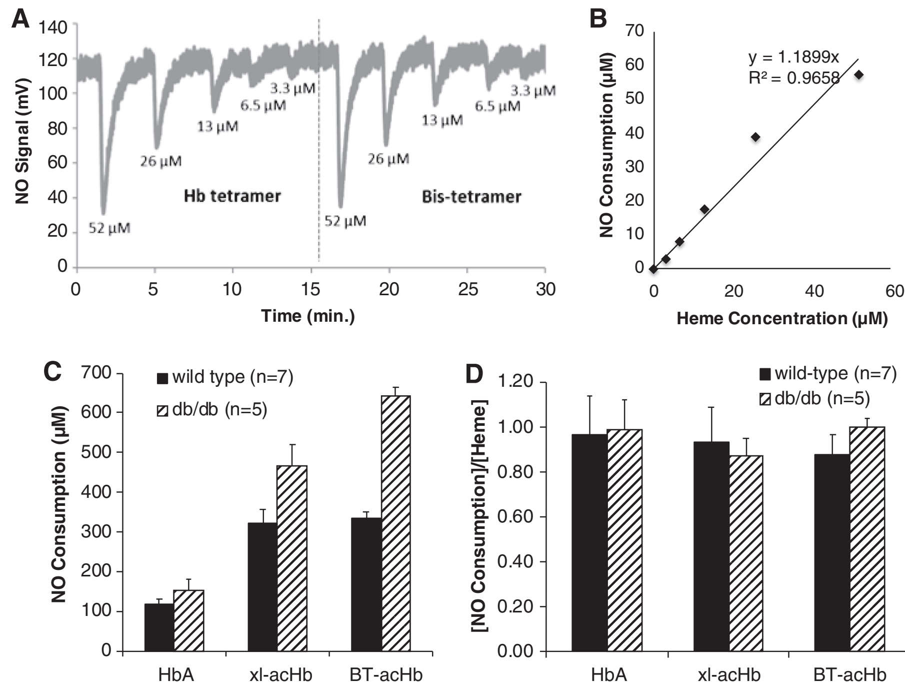


Fig. 7. NO consumption assay. (A) Signal readout of NO generated by a solution of a NO donor (DETA NONOate in Dulbecco's PBS). NO consumed by serial addition of differing amount of HbA and BT-Hb solution (before infusion) causes transient decreases in NO signal (mV). (B) NO consumption by BT-Hb (3.25, 6.5, 13, 26, and 52 μM heme). The slope of the fitted line indicated that each heme of BT-Hb before infusion can react with one NO molecule. (C) NO consumption by plasma samples collected from WT and *db/db* mice two hours after infusion of HbA, xl-acHb, or BT-acHb. (D) [NO consumption]/[heme] ratio. Hemes in different groups showed no discrimination in NO binding as they all bind NO in a 1:1 ratio. BT-acHb = bis-tetramers of hemoglobin that are specifically acetylated and prepared using CuAAC chemistry; BT-Hb = bis-tetramers of hemoglobin that are prepared using CuAAC chemistry; *db/db*diabetic mice; HbA = hemoglobin A; NO = nitric oxide; PBS = phosphate buffered saline; xl-acHb = hemoglobin tetramers that are specifically cross-linked and acetylated.

and BT-acHb bind exogenous NO in vitro and in vivo, showing that the mechanism by which these HBOCs avoid inducing hypertension is not a result of their inability to bind NO but is consistent with their size preventing their localization to the proximal source of endogenous NO that is needed to relax the muscles of blood vessels.

The first generation Hb bis-tetramer BT, with an amide-containing interprotein scaffold, was shown to remain in plasma in significant amounts after infusion in murine models.[23] BT-Hb and BT-acHb, with a triazole-containing interprotein scaffold, also withstand the murine clearance system and remain functional. The acetylated amino groups in BT-acHb do not compromise that bis-tetramer's ability to stay in the circulation and to carry oxygen.

The effects of chemical cross-linking and molecular size on HBOC clearance has been examined both in vitro and in rat models.[42–44] The primary metabolic pathway of HBOCs involves its binding to plasma haptoglobin (Hp) and endocytosis of the HBOC-Hp complex by the monocyte/macrophage scavenger receptor CD163.[45–47] CD163 also directly mediates the internalization of HBOC by macrophages in the absence of Hp.[48,49] Buehler et al.[43] studied the clearance of an array of cross-linked tetrameric and polymeric Hb in both pathways. They observed that the interactions between HBOCs and the two Hb-scavenger systems were influenced independently by the HBOC's unique cross-linking pattern and molecular size. In the Hp-CD163 pathway, cross-linking between the β subunits is associated with higher-affinity binding to Hp than αα cross-linking. In the Hp-independent CD163 pathway, the CD163 binding and macrophage uptake of HBOC are solely determined by molecular size. The cross-linked tetramers and bis-tetramers used in our studies as well as BT are all connected between the β subunits. The inverse dependence of CD163 uptake on the molecular size of HBOCs could explain the slightly higher, although statistically insignificant, plasma level of BT-Hb and BT-acHb compared to that of xl-acHb.

Our analysis reveals that the levels of metHb forms of the bis-tetramers remain low (<5%) regardless of the amounts of metHb introduced via infusion. The conversion of metHb to oxyHb in the plasma compartment can arise from reducing agents such as urate, ascorbate, and glutathione.[50,51] Addition of N-acetyl cysteine to our infusion solutions may also help to maintain the functional ferrous form

of iron in the bis-tetramers. Wang and coworkers[52] observed that canines receiving metHb infusions had some of the infused metHb convert to oxyHb. In addition, they showed that clearance of total Hb from plasma was faster after metHb infusion than after oxyHb infusion. Therefore, the observed low level of metHb in our studies may be due to the combined effects of active metHb reduction along with selective rapid hepatic removal.

The presence of the triazole-containing linker between tetramers in BT-Hb and BT-acHb does not induce an increase in SBP in WT mice. This compares well with outcomes from infusing BT that is linked with an amide-containing scaffold.[23] Although we did not conduct invasive hemodynamic measurements to study the systemic vasoconstrictor effects of BT-Hb and BT-acHb, in a previous study, we showed that infusions of BT had no significant effect on systemic vascular resistance.[23] The different interprotein cross-linking strategies (aminolysis vs. CuAAC) between the two distinct classes of bis-tetramer with alternative chemical constituents in the linkers (amides vs. triazoles; Fig. 1) do not affect the overall in vivo hemodynamic responses. Rather, the defined structural similarities of the two classes of bis-tetramers—the enlargement from 64 kDa to ca. 128 kDa—supports our hypothesis that the size and shape of Hb bis-tetramers is likely to be what keeps them in the circulation and prevents them from making contact with endogenous NO. Infusion of the triazole-coupled bis-tetramers in the *db/db* mouse model gave results that also indicate that it is as nonvasoactive as the amide-coupled comparator. We can therefore draw a general conclusion that bis-tetramers with other stable linkages will be nonvasoactive and that efficiency in production should be a key factor in selection of targets for preparation. The present report is an empirical guide to making chemically defined hemoglobin derivatives that avoid inducing vasoconstriction and hypertension. While the pattern of nonvasoactivity exhibited by Hb bis-tetramers is consistent with the increased size of these HBOCs, the exact mechanism by which these materials avoid inducing hypertension remains to be determined in future studies.

Previous HBOCs have been associated with induced hypertension as well as oxidative stress.[53] Our current study focuses on examining the vascular effects of BT-Hb and BT-acHb in an established rodent model of endothelial dysfunction. Rodents, unlike humans, can synthesize ascorbate through L-gulonolactone-γ-oxidase.[54] The presence of endogenously produced antioxidant in mice could potentially ameliorate HBOC-mediated oxidative stress. Future studies, using animals such as guinea pigs that lack the ability to produce this antioxidant,[19] may be helpful in evaluating the safety of HBOCs for use in humans.

CONCLUSION

Our study indicates that size and homogeneity of circulating Hb-derived entities are likely to be critical factors in avoiding hypertension-related side effects from HBOCs. The absence of systemic hypertension observed after IV administration of BT-acHb and BT-Hb in both healthy WT mice and *db/db* mice with endothelial dysfunction suggests a positive clinical potential for Hb bis-tetramers as RBC alternatives in transfusion medicine and in other applications that require oxygen transport. Future studies of in vivo responses of Hb bis-tetramers in large animal species, in complex disease models, including models of oxidative stress,[53] could lead to a well-rounded in vivo response profile for bis-tetramers in diverse clinical applications.

CONFLICTS OF INTEREST

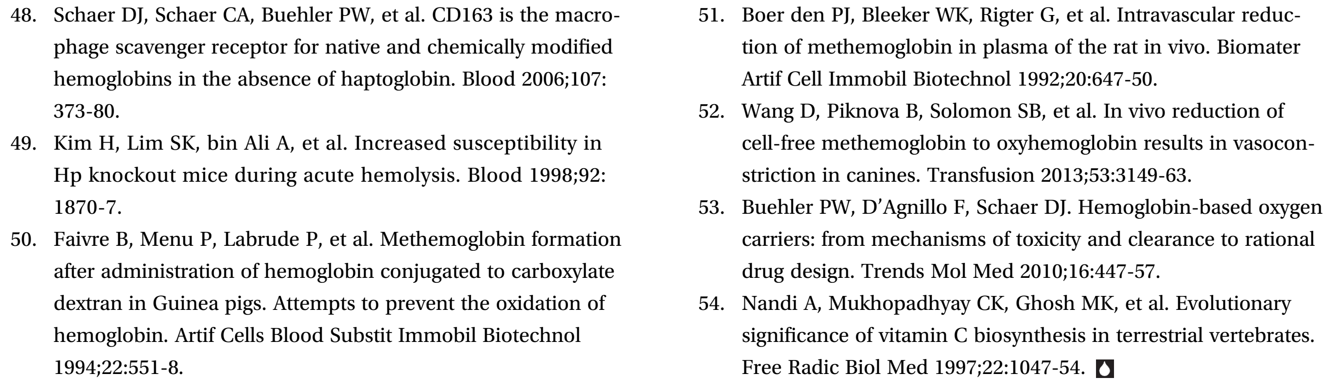
The authors have disclosed no conflicts of interest

REFERENCES

1. Jahr JS, Walker V, Manoochehri K. Blood substitutes as pharmacotherapies in clinical practice. Curr Opin Anaesthesiol 2007;20:325-30.
2. Bunn HF, Esham WT, Bull RW. The renal handling of hemoglobin: I. glomerular filtration. J Exp Med 1969;129:909-24.
3. Snyder SR, Welty EV, Walder RY, et al. HbXL99 alpha: a hemoglobin derivative that is cross-linked between the alpha subunits is useful as a blood substitute. Proc Natl Acad Sci USA 1987;84:7280-4.
4. Kluger R, Song Y, Wodzinska J, et al. Trimesoyltris (3, 5-dibromosalicylate): specificity of reactions of a trifunctional acylating agent with hemoglobin. J Am Chem Soc 1992;114:9275-9.
5. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert Opin Biol Ther 2008;8:1425-33.
6. Eike JH, Palmer AF. Oxidized mono-, di-, tri-, and polysaccharides as potential hemoglobin cross-linking reagents for the synthesis of high oxygen affinity artificial blood substitutes. Biotechnol Prog 2004;20:953-62.
7. Riess JG. Oxygen carriers ("blood substitutes")—raison d'etre, chemistry, and some physiology. Chem Rev 2001;101:2797-920.
8. Silverman TA, Weiskopf RB. Hemoglobin-based oxygen carriers: current status and future directions. Transfusion 2009;49: 2495-515.
9. Weiskopf RB, Silverman TA. Balancing potential risks and benefits of hemoglobin-based oxygen carriers. Transfusion 2013; 53:2327-33.
10. Natanson C, Kern SJ, Lurie P, et al. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA 2008;299:2304-12.
11. Yu B, Raher MJ, Volpato GP, et al. Inhaled nitric oxide enables artificial blood transfusion without hypertension. Circulation 2008;117:1982-90.
12. Minneci PC, Deans KJ, Zhi H, et al. Hemolysis-associated endothelial dysfunction mediated by accelerated NO

inactivation by decompartmentalized oxyhemoglobin. J Clin Invest 2005;115:3409-17.

13. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med 2002;8:1383-9.
14. Vandegriff KD, Malavalli A, Wooldridge J, et al. MP4, a new nonvasoactive PEG-Hb conjugate. Transfusion 2003;43:509-16.
15. Hu D, Kluger R. Functional cross-linked hemoglobin bis-tetramers: geometry and cooperativity. Biochemistry 2008;47:12551-61.
16. Hu D, Kluger R. Efficient generation of dendritic arrays of cross-linked hemoglobin: symmetry and redundancy. Org Biomol Chem 2008;6:151-6.
17. Gourianov N, Kluger R. Conjoined hemoglobins. Loss of cooperativity and protein–protein interactions. Biochemistry 2005; 44:14989-99.
18. Winslow RM. Oxygen: the poison is in the dose. Transfusion 2013;53:424-37.
19. Buehler PW, D'Agnillo F, Hoffman V, et al. Effects of endogenous ascorbate on oxidation, oxygenation, and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and guinea pig. J Pharmacol Exp Ther 2007;323:49-60.
20. Alayash AI. Haptoglobin: old protein with new functions. Clin Chim Acta 2011;412:493-8.
21. Sarin H. Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability. J Angiogenes Res 2010;2:14.
22. Palmer AF. Molecular volume and HBOC-induced vasoconstriction. Blood 2006;108:3231-2.
23. Lui FE, Yu B, Baron DM, et al. Hemodynamic responses to a hemoglobin bis-tetramer and its polyethylene glycol conjugate. Transfusion 2011;52:974-82.
24. Sletten EM, Bertozzi CR. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. Angew Chem Int Ed Engl 2009;48:6974-98.
25. Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed Engl 2001;40:2004-21.
26. Meldal M, Tornøe CW. Cu-catalyzed azide-alkyne cycloaddition. Chem Rev 2008;108:2952-3015.
27. Gupta Sen S, Kuzelka J, Singh P, et al. Accelerated bioorthogonal conjugation: a practical method for the ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjug Chem 2005;16:1572-9.
28. Natarajan A, Du W, Xiong C-Y, et al. Construction of di-scFv through a trivalent alkyne–azide 1,3-dipolar cycloaddition. Chem Commun 2007;252:695-7.
29. Bundy BC, Swartz JR. Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein-protein click conjugation. Bioconjug Chem 2010;21: 255-63.
30. Foot JS, Lui FE, Kluger R. Hemoglobin bis-tetramers via cooperative azide-alkyne coupling. Chem Commun 2009;299: 7315-7.
31. Yang Y, Kluger R. Efficient CuAAC click formation of functional hemoglobin bis-tetramers. Chem Commun 2010;46:7557-9.
32. Wang A, Kluger R. Increasing efficiency in protein-protein coupling: subunit-directed acetylation and phase-directed CuAAC ("click coupling") in the formation of hemoglobin bis-tetramers. Biochemistry 2014;53:6793-9.
33. Chatterjee R, Welty EV, Walder RY, et al. Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 alpha 1–lysine 99 alpha 2). J Biol Chem 1986;261:9929-37.
34. Pannirselvam M, Wiehler WB, Anderson T, et al. Enhanced vascular reactivity of small mesenteric arteries from diabetic mice is associated with enhanced oxidative stress and cyclooxygenase products. Br J Pharmacol 2005;144:953-60.
35. Yu B, Shahid M, Egorina EM, et al. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. Anesthesiology 2010;112: 586-94.
36. Yen TTT, Stienmetz J, Simpson PJ. Blood volume of obese (*ob/ob*) and diabetic (*db/db*) mice. Proc Soc Exp Biol Med 1970;133:307-8.
37. Wang X, Tanus-Santos JE, Reiter CD, et al. Biological activity of nitric oxide in the plasmatic compartment. Proc Natl Acad Sci U S A 2004;101:11477-82.
38. Lui FE, Kluger R. Enhancing nitrite reductase activity of modified hemoglobin: bis-tetramers and their PEGylated derivatives. Biochemistry 2009;48:11912-9.
39. Misra H, Kazo F, Newmark JA. Toxicology and safety determination for a novel therapeutic dual carbon monoxide and oxygen delivery agent. J Clinic Toxicol 2014;4:205.
40. Birukou I, Maillett DH, Birukova A, et al. Modulating distal cavities in the α and β subunits of human HbA reveals the primary ligand migration pathway. Biochemistry 2011;50: 7361-74.
41. Birukou I, Soman J, Olson JS. Blocking the gate to ligand entry in human hemoglobin. J Biol Chem 2011;286:10515-29.
42. Ship NJ, Toprak A, Lai RP, et al. Binding of acellular, native and cross-linked human hemoglobins to haptoglobin: enhanced distribution and clearance in the rat. Am J Physiol Gastrointest Liver Physiol 2005;288:G1301-9.
43. Buehler PW, Vallelian F, Mikolajczyk MG, et al. Structural stabilization in tetrameric or polymeric hemoglobin determines its interaction with endogenous antioxidant scavenger pathways. Antioxid Redox Signal 2008;10:1449-62.
44. Jia Y, Wood F, Buehler PW, et al. Haptoglobin preferentially binds β but not α subunits cross-linked hemoglobin tetramers with minimal effects on ligand and redox reactions. PLoS ONE 2013;8:e59841-9.
45. Levy AP, Asleh R, Blum S, et al. Haptoglobin: basic and clinical aspects. Antioxid Redox Signal 2010;12:293-304.
46. Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the haemoglobin scavenger receptor. Nature 2001;409:198-201.
47. Thomsen JH, Etzerodt A, Svendsen P, et al. The haptoglobin-CD163-heme oxygenase-1 pathway for hemoglobin scavenging. Oxid Med Cell Longev 2013;2013:11.



48. Schaer DJ, Schaer CA, Buehler PW, et al. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. Blood 2006;107: 373-80.
49. Kim H, Lim SK, bin Ali A, et al. Increased susceptibility in Hp knockout mice during acute hemolysis. Blood 1998;92: 1870-7.
50. Faivre B, Menu P, Labrude P, et al. Methemoglobin formation after administration of hemoglobin conjugated to carboxylate dextran in Guinea pigs. Attempts to prevent the oxidation of hemoglobin. Artif Cells Blood Substit Immobil Biotechnol 1994;22:551-8.
51. Boer den PJ, Bleeker WK, Rigter G, et al. Intravascular reduction of methemoglobin in plasma of the rat in vivo. Biomater Artif Cell Immobil Biotechnol 1992;20:647-50.
52. Wang D, Piknova B, Solomon SB, et al. In vivo reduction of cell-free methemoglobin to oxyhemoglobin results in vasoconstriction in canines. Transfusion 2013;53:3149-63.
53. Buehler PW, D'Agnillo F, Schaer DJ. Hemoglobin-based oxygen carriers: from mechanisms of toxicity and clearance to rational drug design. Trends Mol Med 2010;16:447-57.
54. Nandi A, Mukhopadhyay CK, Ghosh MK, et al. Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates. Free Radic Biol Med 1997;22:1047-54.