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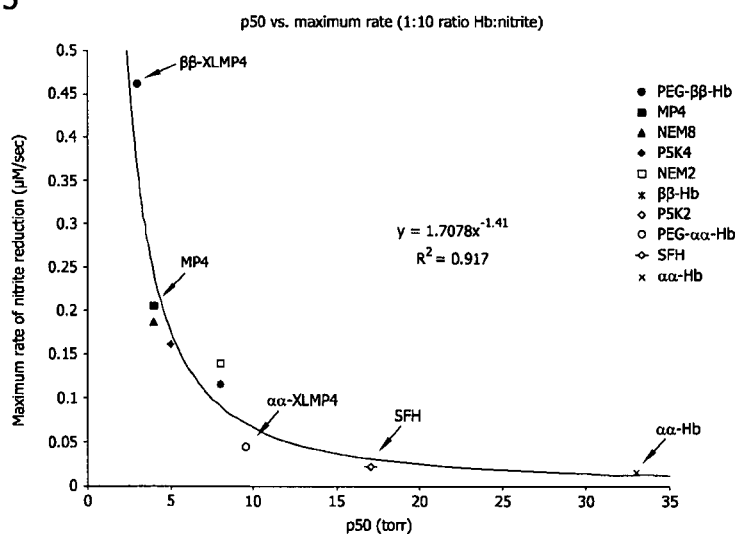
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(54) Title: DIASPIRIN CROSSLINKED PEGYLATED HEMOGLOBIN

FIG. 5



(57) Abstract: PEGylated diaspirin-crosslinked hemoglobin conjugates having high oxygen affinity are described which have enhanced nitrite reductase activity to deliver oxygen, carbon monoxide, nitric oxide or mixtures thereof to tissues to treat various diseases and conditions.

DIASPIRIN CROSSLINKED PEGYLATED HEMOGLOBIN

FIELD OF THE INVENTION

[0001] The present invention generally relates to crosslinked pegylated hemoglobin and hemoglobin compositions for reducing nitrite to nitric oxide at an enhanced rate in the microvasculature, compared to native hemoglobin. Specifically, the present invention is directed towards using a high oxygen affinity intramolecularly-crosslinked pegylated hemoglobin conjugates having enhanced nitrite reductase activity that can also deliver oxygen, carbon monoxide, nitric oxide, or mixtures thereof to tissues.

BACKGROUND OF THE INVENTION

[0002] Hemoglobin-based oxygen carriers (“HBOC”) have long been associated with vasoconstriction that has been attributed to nitric oxide (NO) scavenging by heme. Oxygen carriers that are useful as oxygen therapeutics (sometimes referred to as “oxygen-carrying plasma expanders”), such as stabilized hemoglobin (Hb), have been shown to have limited efficacy because they scavenge nitric oxide, causing vasoconstriction and hypertension. The propensity of these oxygen carrying solutions to cause vasoconstriction can manifest as hypertension in animals and man. Although the mechanisms underlying the vasoconstrictive effects of HBOCs are not well understood, it has been suggested that the heme iron may combine rapidly and irreversibly with endogenous NO, a powerful vasodilator, thereby causing vasoconstriction.

[0003] In part because of these vasoconstrictive effects, no oxygen carrier to date has been entirely successful as an oxygen therapeutic agent (OTA), although products comprising modified cell-free Hb have been the most promising. Human Hb cross-linked between α -chains with bis-dibromosalicyl-fumarate ($\alpha\alpha$ Hb) was developed by the U.S. Army as a model red cell substitute, but was abandoned after it exhibited severe increases in pulmonary and systemic vascular resistance (Hess, J. et al., 1991, Blood 78:356A). A commercial version of this product was also abandoned after a disappointing Phase III clinical trial (Winslow, R. M., 2000, Vox Sang 79:1-20).

[0004] Two molecular approaches have been advanced in attempting to overcome the NO binding activity of Hb. The first approach used site-directed mutagenesis of the distal heme pocket in an attempt to create a recombinant hemoglobin with reduced NO-binding affinity (Eich, R.F. et al., 1996, Biochem. 35:6976–83). The second approach used a chemical modification

approach wherein the size of the Hb was enhanced through oligomerization in an attempt to reduce or possibly completely inhibit the extravasation of Hb from the vascular space into the interstitial space (Hess, J.R. *et al.*, 1978, J. Appl. Physiol. 74:1769-78; Muldoon, S.M. *et al.*, 1996, J. Lab. Clin. Med. 128:579-83; Macdonald, V.W. *et al.*, 1994, Biotechnology 22:565-75; Furchgott, R., 1984, Ann. Rev. Pharmacol. 24:175-97; and Kilbourne, R. *et al.*, 1994, Biochem. Biophys. Res. Commun. 199:155-62).

[0005] In fact, recombinant Hbs with reduced association binding rates for NO have been produced that are less hypertensive in top-load rat experiments (Doherty, D.H. *et al.* 1998, Nature Biotechnology 16:672-676 and Lemon, D.D. *et al.* 1996, Biotech 24:378). However, studies suggest that NO binding may not be the only explanation for the vasoactivity of Hb. It has been found that certain large Hb molecules, such as those modified with polyethylene glycol (PEG), were virtually free of vasoconstriction, even though their NO association rates were identical to those of the severely hypertensive $\alpha\alpha$ Hb (Rohlf's, R.J. *et al.* 1998, J Biol. Chem. 273:12128-12134). Furthermore, it was found that PEG-Hb was extraordinarily effective in preventing the consequences of hemorrhage when given as an exchange transfusion prior to hemorrhage (Winslow, R.M. *et al.* 1998, J. Appl. Physiol. 85:993-1003).

[0006] The conjugation of PEG to Hb reduces its antigenicity and extends its circulation half-life. However, the PEG conjugation reaction has been reported to result in dissociation of Hb tetramers into $\alpha\beta$ -dimer subunits causing gross hemoglobinuria in exchange-transfused rats receiving PEG-conjugates of Hb monomeric units below 40,000 Daltons ("Da") (Iwashita and Ajisaka Organ-Directed Toxicity: Chem. Indices Mech., Proc. Symp., Brown *et al.* 1981, Eds. Pergamon, Oxford, England pgs 97-101). A polyalkylene oxide ("PAO") conjugated Hb having a molecular weight greater than 84,000 Daltons was prepared by Enzon, Inc. (U.S. Pat. No. 5,650,388) that carried about 10 copies of PEG-5,000 chains linked to Hb at its α and ε -amino groups. This degree of substitution was described as avoiding clinically significant nephrotoxicity associated with hemoglobinuria in mammals. However, the conjugation reaction resulted in a heterogeneous conjugate population and contained other undesirable reactants that had to be removed by column chromatography.

[0007] PEG conjugation is typically carried out through the reaction of an activated PEG moiety with a functional group on the surface of biomolecules. The most common functional groups are the amino groups of lysine, imidazole groups of histidine residues, and the N-terminus of proteins; thiol groups of cysteine residues; and the hydroxyl groups of serine, threonine and

tyrosine residues and the C-terminus of the protein. PEG is usually activated by converting the hydroxyl terminus to a reactive moiety capable of reacting with these functional groups in a mild aqueous environment. One of the most common monofunctional PEGs used for conjugation of therapeutic biopharmaceuticals is methoxy-PEG ("mPEG-OH"), which has only one functional group (*i.e.* hydroxyl), thus minimizing cross-linking and aggregation problems that are associated with bifunctional PEG. However, mPEG-OH is often contaminated with high molecular weight bifunctional PEG (*i.e.* "PEG diol"), which can range as high as 10 to 15% (Dust J.M. *et al.* 1990, *Macromolecule* 23:3742-3746) due to its production process. This bifunctional PEG diol has roughly twice the size of the desired monofunctional PEG. The contamination problem is further aggravated as the molecular weight of PEG increases. The purity of mPEG-OH is especially critical for the production of PEGylated biotherapeutics, because the FDA requires a high level of reproducibility in the production processes and quality of the final drug product.

[0008] Conjugation of Hb to PAOs has been performed in both the oxygenated and deoxygenated states. U.S. Pat. No. 6,844,317 describes conjugating Hb in the oxygenated, or "R" state by equilibrating Hb with the atmosphere prior to conjugation to enhance the oxygen affinity of the resultant PEG-Hb conjugate. Others describe a deoxygenation step prior to conjugation to diminish the oxygen affinity and increase structural stability, enabling the Hb to withstand the physical stresses of chemical modification, diafiltration and/or sterile filtration and pasteurization (U.S. Pat. No. 5,234,903). For intramolecular cross-linking of Hb, it is suggested that deoxygenating Hb prior to modification may be required to expose lysine 99 of the α -chain to the cross-linking reagent (U.S. Pat. No. 5,234,903).

[0009] The kinetics of Hb thiolation with 2-iminothiolane prior to conjugation with PEG was investigated by Acharya *et al.* (U.S. Pat. No. 7,501,499). It was observed that increasing the concentration of iminothiolane from 10-fold, which introduced an average of five extrinsic thiols per tetramer, to 30-fold nearly doubled the number of extrinsic thiols on Hb. However, the size enhancement seen after PEG conjugation was only marginal, even with double the number of thiols. This suggested that the conjugation reaction in the presence of 20-fold molar excess of maleimidyl PEG-5000 covered the surface of the Hb with less reactive thiols, resulting in steric interference that resisted further modification of Hb with more reactive thiols. Consequently, to achieve the desired degree of conjugation of modified Hb (*i.e.* 6 $\pm$ 1 PEG per Hb molecule), Acharya *et al.* thiolated Hb with an 8-15 fold molar excess of iminothiolane, and then reacted the thiolated Hb with a 16-30 fold molar excess of maleimidyl PEG-5000. However, these high

molar excess reactant concentrations in large-scale production significantly increase the cost for preparing the HBOC and increase the heterogeneity of the final product. Moreover, such high molar excess of the maleimidyl PEG-5000 also results in a more heterogeneous product with the production of a greater number of unwanted side reactants.

[0010] In previous studies, it was observed that the molecular size of surface modified hemoglobin has to be large enough to avoid being cleared by the kidneys and to achieve the desired circulation half-life. Blumenstein, J. *et al.*, determined that this could be achieved at, or above, a molecular weight of 84,000 Daltons ("Da") ("Blood Substitutes and Plasma Expanders," Alan R. Liss, editors, New York, N.Y., pages 205-212 (1978)). In that study, the authors conjugated dextran of varying molecular weight to Hb. They reported that a conjugate of Hb (with a molecular weight of 64,000 Da) and dextran (having a molecular weight of 20,000 Da) "was cleared slowly from the circulation and negligibly through the kidneys." Further, it was observed that increasing the molecular weight above 84,000 Da did not significantly alter these clearance curves. Intramolecular cross-linking chemically binds together subunits of the tetrameric hemoglobin unit to prevent the formation of dimers which are prematurely excreted by the kidney. (See, *e.g.*, U.S. Pat. No. 5,296,465.

[0011] Nitrite reacts with oxyhemoglobin to form methemoglobin, and reacts with deoxyhemoglobin to form methemoglobin and nitric oxide. The vasodilatory effect of nitrite differs from that of traditional NO donors in the presence of hemoglobin and can in part be explained by the nitrite reductase activity of hemoglobin. See Crawford *et al.* 2006 Blood 107:566-574; Huang *et al.* 2005 J Biol Chem 280:31126-31131; Huang *et al.* 2005 J Clin Invest 115:2099-2107. Studies have shown that nitrite is converted to NO only through reaction with deoxy hemes with the hemoglobin tetramer (Cosby, K. *et al.* 2003, Nat. Med. 9:1498), and further, that faster reduction of nitrite occurs where the protein heme is in the relaxed or R-state conformation. It is believed that this nitrite reductase activity of hemoglobin is under allosteric control and produces NO at a maximal rate when deoxygenated hemes are in an R-state conformation. R-state stabilizing effects can occur, for example, through modifications at β Cys93 sites, such as maleimide PEG conjugation results in increased nitrite reductase activity. Further, it has been shown that while cell-free Hbs caused vasoconstriction and reduced perfusion, MalPEG-Hbs maintained blood flow and microvascular perfusion pressure, which is thought to be related to the lack of vasoconstriction (Tsai, A. G. *et al.* 2006, Blood 108:3603). Other studies also suggest that the modification of cell-free hemoglobin derivatives with multiple

chains of PEG may suppress vasoactivity. Experiments utilizing R-State stabilized Hbs with five to six PEG chains demonstrated 10-fold faster nitrite reductase activity as compared to native Hb (Lui, F. E. *et al.* 2008, *Biochemistry* 47(40), 10773-10780). However, it was concluded that any further PEG conjugation at accessible lysine residues did not contribute to increased nitrite reductase activity.

[0012] Consequently, there is a need for a method of delivering oxygen, carbon monoxide, nitric oxide, or mixtures thereof to tissue and reducing nitrite to nitric oxide at an enhanced rate in the microvasculature through the use of a high oxygen affinity hemoglobin having increased nitrite reductase properties as compared to existing Hbs.

SUMMARY OF THE INVENTION

[0013] In one aspect, the present invention is directed to a β,β -intramolecularly-crosslinked polyoxyalkylene oxide (PAO) hemoglobin conjugate having a P50 ranging from about 2 to 5 mmHg as measured at 37 °C and pH 7.4. The hemoglobin conjugate when fully deoxygenated at 25°C exhibits a maximal nitrite reductase activity that is at least 10-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.

[0014] Another aspect of the invention is directed to a β,β -intramolecularly-crosslinked PAO hemoglobin conjugate having a P50 ranging from about 2.0 to 5.0 mmHg as measured at 37 °C and pH 7.4 wherein the hemoglobin conjugate has a maximal nitrite reductase activity of at least 0.25 $\mu\text{M}/\text{sec}$ when fully deoxygenated at 25°C.

[0015] Yet another aspect of the invention is directed to pharmaceutical compositions comprising the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate and a pharmaceutically acceptable carrier. The compositions can be for use in the treatment of acute liver failure, beta thalassemia, a burn, chronic critical limb ischemia, carbon dioxide or cyanide poisoning, chronic obstructive pulmonary disease (COPD), congestive heart failure, hypoxia, malaria, organ ischemia, peripheral vascular disease, porphyria, pre-eclampsia in pregnancy, sepsis, sickle cell disease, retinal disease, an intra-ocular condition, testicular torsion, trauma, shock, traumatic brain injury, ulcers, vasospasm, or a combination thereof. The compositions can also be for use as an adjunct to angioplasty, as an adjunct for plastic surgery, or as an adjunct in implanting a ventricular assist device; as a blood substitute, a cardioprotectant, a cryopreservative, a hemodialysis adjunct, an oncology agent, an organ preservative, a performance enhancement agent, a surgery adjunct, or a wound healing agent; in

imaging; to improve lung function; or a combination thereof. The compositions can also be for veterinary treatment of loss of blood due to injury, hemolytic anemia, infectious anemia, bacterial infection, Factor IV fragmentation, hypersplenation and splenomegaly, hemorrhagic syndrome in poultry, hypoplastic anemia, aplastic anemia, idiopathic immune hemolytic conditions, iron deficiency, isoimmune hemolytic anemia, microangiopathic hemolytic anemia, parasitism, or surgical-anesthesia induced brain damage, or a combination thereof.

[0016] Still another aspect of the invention is directed to a method of treatment comprising administering such a hemoglobin conjugate or pharmaceutical composition to a subject in need thereof. The method is for the treatment of any one or more of the conditions described above.

[0017] Another aspect of the invention is directed to a method of delivering oxygen, nitric oxide, carbon monoxide or mixtures thereof to tissue and reducing nitrite to nitric oxide (NO) in the microvasculature. The method comprises administering the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate or the pharmaceutical composition to a subject in need thereof, wherein following administration, the hemoglobin becomes unliganded and converts nitrite to nitric oxide in the microvasculature.

[0018] Another aspect of the invention is directed to a method of making the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate as described above. The method comprises the steps of mixing β,β -intramolecularly-crosslinked hemoglobin with 2-iminothiolane (2-IT) in an aqueous diluent to form a thiolated hemoglobin; and adding PAO to the thiolated hemoglobin in the aqueous diluent to form the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate.

[0019] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] Figure 1 is a size exclusion chromatogram of stroma free Hb (SFH) (- - -) and $\beta\beta$ -Hb (—), respectively, in non-dissociation condition (PBS), demonstrating confirmation of DBBF crosslinking of $\beta\beta$ -Hb.

[0021] Figure 2 is a size exclusion chromatogram a size exclusion chromatogram of stroma free Hb (SFH) (- - -) and $\beta\beta$ -Hb (—), respectively, in dissociation condition (PBS), demonstrating confirmation of DBBF crosslinking of $\beta\beta$ -Hb.

[0022] Figure 3 is a reverse-phase high performance liquid chromatogram demonstrating the confirmation of DBBF crosslinking of the β -subunits wherein (SFH) (---) and $\beta\beta$ -Hb (—), respectively.

[0023] Figure 4 a size exclusion chromatogram of PEG- $\beta\beta$ -Hb (---) and $\beta\beta$ -Hb (—), respectively, in non-dissociation condition, demonstrating PEGylation of PEG- $\beta\beta$ -Hb.

[0024] Figure 5 is a graphical depiction of the nitrite reductive properties of various hemoglobins.

[0025] Corresponding reference characters indicate corresponding parts throughout the drawings.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0026] β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugates of high oxygen affinity have been discovered which have enhanced therapeutic properties as compared to conventional hemoglobin therapeutics. These hemoglobin conjugates, when deoxygenated, exhibit a maximal nitrite reductase activity that is at least 10 fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions. The hemoglobin conjugates have a maximal nitrite reductase activity of at least 0.25 $\mu\text{M/s}$ when in fully deoxygenated form at 25°C. The hemoglobin can be delivered in liganded form to deliver oxygen, carbon monoxide or nitric oxide to a subject, and also converts nitrite to nitric oxide in the microvasculature once unliganded.

[0027] Without being bound by any particular theory, it is believed that by crosslinking the hemoglobin in the oxygenated, R-state conformation that deoxygenated hemes in the non-dissociable tetramer are locked in a non-flexible R-state that promotes the reduction of nitrite at those deoxy hemes. The unexpectedly much greater nitrite reductase activity of the hemoglobin conjugates of the present invention provides more NO which escapes heme capture and is released from the Hb to the tissue, thereby leading to greater vasodilatory effects compared to other hemoglobin therapeutics. This is believed to occur because the cross-linking of the high-affinity, R-state structure promotes properties within the heme pocket to enhance the reduction of nitrite. Without cross-linking, there is more hemoglobin allosteric conformational change, alternating between T- and R-state heme conformation structures. The cross-linked, fully stabilized R-state structure, even in a deoxygenated heme pocket within the tetramer, is believed to enhance nitrite reduction to NO within the heme pocket.

[0028] The present invention is directed to a β,β -intramolecularly-crosslinked polyoxyalkylene oxide (PAO) hemoglobin conjugate having a P50 ranging from about 2 to 5 mmHg as measured at 37 °C and pH 7.4. The hemoglobin conjugate when fully deoxygenated at 25°C exhibits a maximal nitrite reductase activity that is at least 10-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.

[0029] The hemoglobin conjugate when fully deoxygenated at 25°C can exhibit a maximal nitrite reductase activity that is at least 15-fold or at least 20-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions. Preferably, the hemoglobin conjugate when fully deoxygenated at 25°C exhibits a maximal nitrite reductase activity that is 10-fold to about 25-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions, more preferably about 15-fold to about 25-fold greater, 10-fold to about 21-fold greater, or about 15-fold to about 21-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.

[0030] A β,β -intramolecularly-crosslinked PAO hemoglobin conjugate of the invention can have a P50 ranging from about 2.0 to 5.0 mmHg as measured at 37 °C and pH 7.4 and a maximal nitrite reductase activity of at least 0.25 $\mu\text{M}/\text{sec}$ when fully deoxygenated at 25°C. Preferably, the maximal nitrite reductase activity of the hemoglobin conjugate is at least 0.30 $\mu\text{M}/\text{sec}$ when fully deoxygenated at 25°C, and more preferably 0.35, 0.40 or 0.45 $\mu\text{M}/\text{sec}$. The maximal nitrite reductase activity of the hemoglobin conjugate can range from 0.25 to about 0.50 $\mu\text{M}/\text{sec}$, or from about 0.30 to about 0.47 $\mu\text{M}/\text{sec}$.

[0031] A variety of Hbs may be utilized with the present invention. The Hb may be obtained from animal sources, such as human, bovine, porcine, or equine hemoglobin. Human Hb is preferred. The Hb can be obtained from natural sources or can be produced by known recombinant methods.

[0032] The hemoglobin conjugates of the present invention have a high oxygen affinity greater than that of stroma-free hemoglobin. This means that the hemoglobins will have a P50 less than 15 mmHg as measured at 37°C and pH 7.4, preferably from about 2 to about 5 mmHg, and most preferably from about 2 to about 4 mmHg, or 3 mmHg.

[0033] The hemoglobin conjugates can have a colloid osmotic pressure (COP) of at least about 50 mmHg, preferably at least about 60, 65, 70 or 75 mmHg.

[0034] The hemoglobins are β,β -intramolecularly crosslinked to prevent dissociation into dimers and to avoid being cleared by the kidneys, extending circulation half-life. Bis(2,5-dibromosalicyl) fumarate (DBBF) crosslinker is used to crosslink two $\beta 82$ lysine residues of the

hemoglobin molecule. Any of the known methods of DBBF crosslinking can be used, such as that described by Walder et al, Biochemistry, 1979; 18(20): 4265-70.

[0035] Polyethylene oxides for use in conjugating hemoglobins of the invention include, but are not limited to, polyethylene oxide, polypropylene oxide and a polyethylene/polypropylene oxide copolymer. The PAO has a molecular weight of about 2,000 to about 20,000 Daltons, preferably from about 3,000 to about 10,000 Daltons, more preferably from 4,000 to about 6,000 Daltons, and most preferably about 5,000 Daltons. The most common PAO presently used to modify the surface of Hb is PEG because of its pharmaceutical acceptability and commercial availability. PEG is available in a variety of molecular weights based on the number of repeating subunits of ethylene oxide (*i.e.* -CH₂CH₂O-) within the molecule, to achieve a desired molecular weight based on the number and size of the PEG molecules conjugated to Hb.

[0036] The hemoglobins can be conjugated with on average about 7 to about 11 PAO molecules per hemoglobin tetramer. Preferably, the hemoglobins are conjugated to on average about 9 to about 10 PAO molecules per tetramer.

[0037] One or both of the terminal end groups of the PAO polymer are converted into a reactive functional group ("activated"). For example, PEG-OH has been used to prepare PEG-halide, mesylate or tosylate, which is then converted to PEG-amine ("PEG-NH₂") by performing a nucleophilic displacement reaction with aqueous ammonia (Zalipsky, S. *et al.*, 1983, Eur. Polym. J. 19:1177-1183), sodium azide or potassium phthalimide. The activated PEG can then be conjugated to a heme protein through the interaction of the PEG amine group ("NH₂") with a carboxyl group ("COOH") of the heme protein.

[0038] In addition to functionalizing PEG with an amine group and converting it to a maleimide group, PEGs that are activated therewith, are known to be used in the art. For example, PEG may be activated with p-nitrophenyl carbonate, aldehyde, aminopropyl, aminoethyl, thiol, aminoxy, hydrazide, and iodoacetamide, to name a few. Such functional PEG can be conjugated to the surface amino acid side chains of proteins using known methods.

[0039] PEG-NH₂ can be further functionalized to conjugate with groups other than carboxyl. For example, U.S. Pat. No. 6,828,401 discloses the reaction of PEG-NH₂ with maleimide to form mPEG-maleimide. In this reaction, mPEG-OH is reacted with a tosylating reagent (p-toluenesulfonyl chloride) and a base catalyst (triethyleneamine) in the presence of an

organic solvent (dichloromethane) to produce mPEG-tosylate. The mPEG-tosylate is then reacted with 28% ammonia water and maleic acid anhydride in an organic solvent mixture of N, N-dimethylacetamide (“DMAc”) and N-cyclohexylpyrrolidinone (“CHP”) to produce a maleamic acid compound. This compound is then reacted with pentafluorophenyltrifluoroacetate in the presence of dichloromethane to produce the mPEG-maleimide.

[0040] Alternatively, mPEG-maleimide can be made by reacting mPEG-OH with a tosylating reagent (p-toluenesulfonyl chloride) and a base catalyst (triethylamine) in the presence of an organic solvent (dichloromethane) to produce mPEG-tosylate. The mPEG-tosylate is then reacted with 28% ammonia to prepare mPEG-NH₂. The mPEG-NH₂ is then reacted with N-methoxy carbonyl maleimide (MCM) in the presence of saturated sodium hydrocarbonate (NaHCO₃) to produce mPEG-maleimide.

[0041] Non-limiting examples of amino acid residue side chains of human Hb that can be modified using amine reactive chemistry for conjugation to PAO are presented in Table 1 below:

Table 1 - Amine Reactive Chemistry and Potential Sites of Modification

α-chain		
Residues	Positions	Reacts With
Lys	7, 11, 16, 40, 56, 60, 61, 90, 99, 127 and 139	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.
His	20, 45, 50, 58, 72, 87, 112 and 122	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.
Val	1	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.
β-chain		
Residues	Positions	Reacts With
Lys	8, 17, 59, 61, 65, 66, 82, 95, 120, 132 and 144	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.
His	2, 63, 77, 92, 97, 116, 117, 143 and 146	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.
Val	1	Succinimide; NPC (p-nitrophenyl carbonate); isocyanate; aldehyde; isothiocyanate; epoxides.

[0042] One method to increase the number of available conjugation sites on Hb is to introduce sulfhydryl groups (also known as thiolation), which tend to be more reactive with MalPEG than free amines. A variety of methods are known for protein thiolation. In one method, protein free amines are reacted with succinimidyl 3-(2-pyridyldithio) propionate followed by reduction with dithiothreitol (“DTT”), or tris(2-carboxyethyl)phosphine (“TCEP”).

This reaction releases the 2-pyridinethione chromophore, which can be used to determine the degree of thiolation. Amines can also be indirectly thiolated by reaction with succinimidylacetylthioacetate, followed by 50 mM hydroxylamine, or hydrazine at near-neutral pH.

[0043] Another method described in U.S. Pat. No. 5,585,484 maintains the positive charge of the amino (α - or ϵ -) group of the Hb after conjugation. This method involves amidination of the ϵ -amino groups of Hb by 2-IT to introduce sulfhydryl groups onto the protein. This approach has at least two additional advantages over the previously used succinimidyl chemistry: 1) the high reactivity and selectivity of maleimide groups with sulfhydryl groups facilitates the near quantitative modification of the thiols, with a limited excess of reagents and 2) the thiol group of 2-IT is latent and is generated only *in situ* as a consequence of the reaction of the reagent with the protein amino groups. These advantages provide one additional benefit; they allow simultaneous incubation of Hb with both the thiolating and PEGylation reagent for surface decoration.

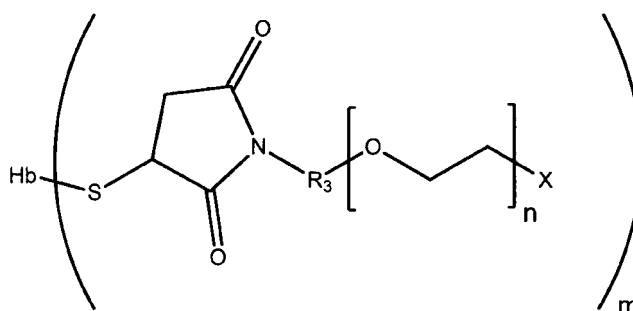
[0044] For example, MalPEG can be conjugated to Hb by thiolating an amine of the Hb to introduce thiol groups on the surface of the Hb. The two intrinsic thiol groups of Hb that are available for reaction are at β Cys93, and added thiol groups on the surface of the Hb can react with the maleimide of the maleimidyl PAO to form a pegylated Hb conjugate.

[0045] The polyalkylene oxide can be covalently attached via a thiol reactive moiety to a thiol group of an exposed amino acid side chain on the hemoglobin molecule while the hemoglobin is in the oxygenated state.

[0046] The maleimide-PEG can be conjugated to a thiol moiety of the hemoglobin selected from an intrinsic thiol moiety of a cysteine residue of the hemoglobin, a thiol moiety of a thiolated lysine residue of the hemoglobin, or a combination thereof.

[0047] The maleimide-PEG includes a linker to attach the maleimide to the PEG. Linkers can include, but are not limited to, alkylene such as ethylene, propylene, or isopropylene, phenylene, amide ($-\text{NH}-\text{C}(\text{O})-$), or phenyl carbamate (e.g., $-\text{Ph}-\text{NH}-\text{C}(\text{O})-$). Preferably, the polyalkylene oxide is linked to the thiol reactive moiety by a linker consisting of alkylene or phenylene, and more preferably alkylene such as ethylene.

[0048] The maleimide-PEG can have the structure



wherein Hb is hemoglobin, S is a thiol of the hemoglobin, R_3 is an alkylene or phenylene group, X is a terminal group, m is the average number of maleimidyl-activated PEG polymers conjugated to the hemoglobin, and n is the average number of oxyethylene units of a PEG having an average molecular weight of about 2,000 to about 20,000 Daltons. Preferably, R_3 is an alkylene such as ethylene; X is hydroxy, aryloxy such as benzyloxy, or C_1 - C_{20} alkoxy, more preferably C_1 - C_{10} alkoxy group, and still more preferably a C_1 - C_5 alkoxy group such as methoxy or ethoxy; n is from about 3,000 to about 10,000 Daltons, more preferably from 4,000 to about 6,000 Daltons, and most preferably about 5,000 Daltons; and m is about 7 to about 11, and more preferably about 9 or about 10.

[0049] Non-limiting examples of amino acid residue side chains that can be modified using thiol reactive chemistry are presented in Table 2 below:

Table 2 - Thiol Reactive Chemistry and Potential Sites of Modification

α-chain		
Residues	Positions	Reacts With
Cys	104	Maleimide; iodoacetamide; orthopyridyl-disulfide (OPSS); vinylsulfone.
β-chain		
Residues	Positions	Reacts With
Cys	93 and 112	Maleimide; iodoacetamide; orthopyridyl-disulfide (OPSS); vinylsulfone.

[0050] The two intrinsic thiols at the β Cys93 residues of hemoglobin can be pegylated or can be reacted with N-ethyl maleimide. Such modification of these cysteine residues decreases P50, and has R-state stabilizing effects.

[0051] The molecular weight of the PAO-Hb can be regulated by the conjugation reaction. Conventional thought suggested that increasing the molar ratios of the reactants would increase the number of PEG molecules bound to Hb. This included both the thiolation process of Hb (*i.e.* increasing the molar ratio of thiolating agent to Hb) and the conjugation process (*i.e.* increasing the molar ratio of thiol activated PEG to thiolated Hb). However, these excess molar ratios resulted in the binding of only 6 ± 1 PEG molecules per Hb (see U.S. Pat. No. 7,501,499).

[0052] Recently it was determined that a greater number of PAO molecules could be bound to Hb using lower molar ratios of reactants. The number of available thiol groups on Hb, before and after thiolation and after conjugation, was determined using the dithiopyridine colorimetric assay (Ampulski, R.S. *et al.*, 1969, Biochem. Biophys. Acta 32:163-169). Human Hb contains two intrinsic, reactive thiol groups at the $\beta 93$ cysteine residues, which was confirmed by the dithiopyridine reaction. After thiolation of SFH with 2-IT, the number of reactive thiol groups increased from two to over seven. In this example, an average of 8 PEG molecules was bound to Hb. This was achieved using a 7.5-fold molar excess of 2-IT over SFH in the thiolation reaction and a 12-fold molar excess of MalPEG over thiolated Hb in the conjugation reaction.

[0053] Hemoglobin is conjugated with polyalkylene oxide when it is in the oxygenated state to increase the oxygen affinity of the Hb-PAO conjugate.

[0054] Thus, another aspect of the invention is directed to a method of making the hemoglobin conjugate. The method comprises the steps of: mixing β, β -intramolecularly-crosslinked hemoglobin with 2-iminothiolane (2-IT) in an aqueous diluent to form a thiolated hemoglobin; and adding PAO to the thiolated hemoglobin in the aqueous diluent to form the β, β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate.

[0055] In this method, the 2-iminothiolane is present at a concentration of between about 8- and about 25-fold molar excess over the hemoglobin concentration, preferably about 15-fold molar excess.

[0056] The thiolation can be performed at a pH of about 7 to about 9.

[0057] The conjugation can be performed at a pH of about 7 to about 9.

[0058] In this method, the PAO-maleimide is present at a concentration of between about 10- to about 40-fold molar excess over the hemoglobin concentration based on 100% terminal activity, preferably about 28-fold molar excess.

[0059] The hemoglobin conjugates of the invention can be in oxygenated or deoxygenated form, can be liganded to CO or NO, or can be a mixture including two or more of

these four forms. HbO₂ is prepared by equilibrating non-oxygenated hemoglobin with air, pure O₂ gas or O₂ / nitrogen gas mixtures.

[0060] Deoxygenation can be performed by any method known in the art. One simple method is to expose the hemoglobin solution to an inert gas, such as nitrogen, argon or helium. To assure that deoxygenation is relatively homogeneous, the Hb solution is circulated in this process. Monitoring deoxygenation to attain desired levels may be performed by using a Co-oximeter 682 (Instrument Laboratories). If partial reoxygenation is desired, deoxygenated Hb may be exposed to oxygen or to a gas mixture containing oxygen, such as air.

[0061] Gas exchange to replace molecular oxygen with another gas may be accomplished through a gas-permeable membrane, such as a polypropylene or cellulose acetate membrane. See, for example, published U.S. Patent Application No. 2006/0234915. Commercially available gas-exchange devices utilizing these membranes include the CelgardTM polypropylene microporous hollow fiber device from Hoechst-Celanese (Dallas, TX) or the Cell-PharmTM hollow fiber oxygenator from American Laboratory (East Lyme, CT). In the Hoechst-Celanese CelgardTM device, oxygenated Hb is deoxygenated by passing an aqueous Hb solution through polypropylene microporous hollow filters at 10-100 ml/min/ft² while the system is purged with nitrogen at 5-20 psi. The Hb is generally circulated for about 5 to 30 minutes to achieve the desired percentage of deoxyHb. Another method for producing deoxygenated Hb comprises exposing a Hb solution to a chemical reducing agent such as sodium ascorbate, sodium dithionate and sodium bisulfite. Hb is partially deoxygenated by adjusting the reducing agent concentration, reaction time and temperature. Alternatively, a reducing agent may be used to substantially deoxygenate Hb, and then oxygen may be reintroduced to form a partially deoxygenated product. For example, Hb can be exposed to a 100 mM concentration of sodium bisulfite for about one hour before adding antioxidants.

[0062] Hb can be liganded to CO using any known methods for forming oxyhemoglobin, simply by substituting CO for O₂. This generally involves introducing a source of CO to a solution of hemoglobin such that the hemoglobin becomes liganded with CO instead of O₂ (K. D. Vandegriff, et al., Biochem. J. 382:183-189 (2004)). Since hemoglobin has a higher affinity for CO than it does for oxygen, it is not necessary to first deoxygenate the hemoglobin. Accordingly, the most convenient way of forming CO-Hb complexes is by introducing 100% gaseous CO to a solution of hemoglobin.

[0063] HbNO can be prepared by reacting deoxygenated hemoglobin with nitric oxide gas, or by exposing CO-Hb to NO gas such that the NO exchanges for CO. HbNO can also be made by reacting deoxygenated hemoglobin with a small NO-donor molecule like PROLI NONOate™ (i.e., 1-(hydroxy-NNO-azoxy)-L-proline, disodium salt; Cayman Chemical, Ann Arbor, Michigan).

[0064] It should be noted that hemoglobin to which NO, a free radical, is bound to the amino acid side groups in the globin chain are not NO-Hb complexes as defined herein, since such compounds do not contain diatomic (nonionic) NO as a ligand in the heme pocket instead of oxygen. For example, nitrosylhemoglobin is formed when native hemoglobin is exposed to a NO donor under conditions that cause it to bind to free sulfhydryl groups (U.S. Pat. No. 6,627,738). Such nitrosylhemoglobins still carry oxygen, whereas the NO-Hb complexes of the present invention do not. Furthermore, when the modified hemoglobin is formed by a reaction directed towards sulfhydryl moieties such as described above, these moieties are no longer available for NO binding.

[0065] The PAO-Hb conjugates of the present invention can be formulated as a pharmaceutical composition comprising the PAO-Hb conjugate in a pharmaceutically acceptable carrier for parenteral administration, such as an aqueous diluent. The concentration of the PAO-Hb conjugate in the carrier can vary according to the application. Preferably, the PAO-Hb conjugate concentration ranges from about 0.1 g/dl to about 10 g/dl, more preferably from about 2.0 g/dl to about 8.0 g/dl, and most preferably about 4.0 to about 6.0 g/dl. The selection of an appropriate concentration of hemoglobin depends on the colloidal osmotic (oncotic) properties of the final hemoglobin product. Preferably, the compositions of the invention can be normo-oncotic as compared to whole blood or hyperoncotic as compared to plasma. The hemoglobin concentration can be adjusted to obtain the desired oncotic pressure for each indication.

[0066] When the composition is formulated as a parenteral, the solution generally comprises a physiologically compatible electrolyte carrier isosmotic with whole blood and which maintains the reversible oxygen-, CO- or NO-carrying and delivery properties of the hemoglobin.

[0067] The pharmaceutically acceptable carrier can be an aqueous diluent. The aqueous diluent can comprise an aqueous solution of a colloid or an aqueous solution of a non-oxygen carrying component, such as an aqueous solution of proteins such as albumin, an aqueous

solution of glycoproteins, an aqueous solution of polysaccharides, or a combination thereof. The aqueous diluent can comprise an aqueous cell-free solution.

[0068] Suitable aqueous diluents include, but are not limited to, physiological saline, a saline-glucose mixture, Ringer's solution, lactated Ringer's solution, Locke-Ringer's solution, Krebs-Ringer's solution, Hartmann's balanced saline, heparinized sodium citrate-citric acid-dextrose solution, an acetate solution, a multiple electrolyte solution (e.g., Plasma Lyte® or Plasma Lyte-A® from Baxter International, Deerfield, IL), a lactobionate solution, and polymeric plasma substitutes, such as polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol, an ethylene oxide-propylene glycol condensate, or a combination thereof.

[0069] The composition can additionally comprise pharmaceutically-acceptable fillers, salts, and other materials well-known in the art, the selection of which depends on the dosage form, the condition being treated, the particular purpose to be achieved according to the determination of the ordinarily skilled artisan in the field and the properties of such additives. For example, the composition can include physiological buffers, carbohydrates (e.g. glucose, mannitol, or sorbitol), alcohols or poly alcohols, pharmaceutically acceptable salts (e.g., sodium or potassium chloride), surfactants (e.g., polysorbate 80), anti-oxidants, anti-bacterial agents, oncotic pressure agents (e.g. albumin or polyethylene glycols) or reducing agents (e.g., ascorbic acid, glutathione, or N-acetyl cysteine).

[0070] The pharmaceutical compositions have a viscosity of at least about 2 centipoise (cP). More specifically, the viscosity ranges from about 2 to about 5 cP, and particularly about 2.5 to about 4.5 cP.

[0071] In order to avoid complications in administration, the pharmaceutical composition is of high purity, i.e. free from stroma, phospholipids, and pyrogens, having an endotoxin level of no more than 0.25 EU/ml, as measured by the LAL (limulus amebocyte lysate) test, and having less than 8% methemoglobin.

[0072] Pharmaceutical compositions can be administered parenterally, such as by subcutaneous, intravenous, or intramuscular injection, or as large volume parenteral solutions. The compositions can also be administered by gavage.

[0073] A typical dose of hemoglobin conjugate as a therapeutic agent can be from about 1 to about 15,000 milligrams of hemoglobin per kilogram of patient body weight. For example,

when used as an oxygen therapeutic, the dosage will range between 100 to 7500 mg/kg patient body weight, more preferably 500 to 5000 mg/kg body weight, and most preferably 700 to 3000 mg/kg body weight. Thus, a typical dose for a human patient might be from a gram to over 1000 grams. It will be appreciated that the unit content of active ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount, as the necessary effective amount could be reached by administration of a number of individual doses. The selection of dosage depends upon the dosage form utilized, the condition being treated, and the particular purpose to be achieved according to the determination of those skilled in the art.

[0074] The PAO-Hb conjugates and pharmaceutical compositions can be used to deliver oxygen, CO and/or NO to a subject. A method of delivering oxygen, nitric oxide, carbon monoxide or mixtures thereof to tissue and reducing nitrite to produce further endogenous nitric oxide (NO) in the microvasculature includes administering the hemoglobin conjugate or the composition to a subject in need thereof, wherein following administration, the hemoglobin becomes unliganded and converts nitrite to nitric oxide in the microvasculature.

[0075] The hemoglobin conjugates and compositions thereof of the invention can be used: to treat acute liver failure, beta thalassemia, a burn, chronic critical limb ischemia, carbon dioxide or cyanide poisoning, chronic obstructive pulmonary disease (COPD) (e.g., acute exacerbations), congestive heart failure (e.g., acute heart failure, chronic heart failure), hypoxia (e.g., high altitude use including for pulmonary edema, decompression sickness), malaria (e.g., cerebral malaria (*Falciparum* occlusive events), organ ischemia (e.g., acute bowel ischemia (torsion), acute bowel ischemia (embolism), cardiogenic shock, acute vascular organ ischemia, stroke (before CAT scan), stroke (after CAT scan), myocardial infarction / severe cardiac ischemia), peripheral vascular disease, porphyria, pre-eclampsia in pregnancy, sepsis, sickle cell disease (e.g., stroke/transient ischemic attack, splenic sequestration, hepatic sequestration, priapism), retinal disease / intra-ocular condition (e.g., central retinal artery occlusion, central venous occlusion), testicular torsion, trauma / shock (e.g., traumatic hemorrhagic shock, non-traumatic hemorrhagic shock, pre-hospital / field use (military / emergency), traumatic brain injury / blast), ulcers, or vasospasm; as an adjunct to angioplasty, as an adjunct for plastic surgery (skin flaps) (e.g., acute treatment, chronic treatment), or as an adjunct in implanting a ventricular assist device; as a blood substitute (e.g., for acute blood loss, Jehovah's Witness, difficult to cross-match patient, rare blood group, sickle aplastic crisis, sickle cell anemia perioperative management, acute hemolytic anemia (autoimmune), acute hemolytic anemia (toxin), or other

refractory anemia), a cardioprotectant, a cryopreservative, a hemodialysis adjunct, an oncology agent (e.g., adjunct to radiotherapy or chemotherapy, solid tumors), an organ preservative (e.g., ex vivo, in donor, in recipient), a performance enhancement agent (e.g., civilian / athletic, military), a surgery adjunct (e.g., cardiopulmonary bypass (prime), cardiopulmonary bypass (adjustment), lung ischemia, pre-surgery conditioning, ruptured aortic aneurysm, replacement of thoracic aorta (dissection or aneurysm)), or a wound healing agent; in imaging (x-ray or magnetic resonance imaging (MRI)); to improve lung function (e.g., acute lung injury, chronic lung injury, transient viral pneumonia, neonatal distress syndrome); or a combination thereof. Such uses include administration of the conjugate or composition to a subject in need thereof.

[0076] Further, the hemoglobins and compositions of the invention can be used to treat non-traumatic hemorrhagic shock, pre-hospital setting trauma, traumatic hemorrhagic shock, acute lung injury, adult respiratory distress syndrome, traumatic brain injury, stroke, solid tumor cancer, organ degradation (ex-vivo), organ degradation (in recipient), severe sepsis / septic shock, myocardial infarction / cardiac ischemia, cardiogenic shock, acute heart failure, pulmonary embolism, various conditions by surgery (e.g., adjunct to angioplasty, adjunct to thoracic aortic repairs, adjunct to cardiopulmonary bypass, priming solution for cardiopulmonary bypass), or a combination thereof.

[0077] The numerous clinical settings in which the hemoglobins and compositions of the present invention are useful include the following:

[0078] Trauma. An acute loss of whole blood can result in a fluid shift from the interstitial and intracellular spaces to replace the lost volume of blood while shunting of blood away from the low priority organs including the skin and gut. Shunting of blood away from organs reduces and sometimes eliminates O₂ levels in these organs and results in progressive tissue death. The primary goal is to oxygenate affected tissues. This trauma can be in a pre-hospital setting or can result in traumatic hemorrhagic shock or traumatic brain injury.

[0079] Ischemia. The conjugates and compositions thereof can also be used to deliver oxygen, CO, and/or NO to areas that red blood cells or many other oxygen therapeutics cannot penetrate. These areas can include any tissue areas that are located downstream of obstructions to red blood cell flow, such as areas downstream of thrombi, sickle cell occlusions, arterial occlusions, angioplasty balloons, surgical instrumentation, and any tissues that are suffering from oxygen starvation or are hypoxic. All types of tissue ischemia can be treated including, for example, stroke, emerging stroke, transient ischemic attacks, myocardial stunning and

hibernation, acute or unstable angina, emerging angina, infarct, and the like. In particular, conditions resulting in ischemia include acute heart failure, cardiogenic shock, myocardial infarction / cardiac ischemia, stroke, pulmonary embolism, non-traumatic hemorrhagic shock, or cerebrovascular trauma.

[0080] Hemodilution. In this application, the therapeutic is administered to replace (or substitute for) the O₂ levels of the removed autologous blood. This permits the use of the removed autologous blood for necessary transfusions during and after surgery. One such surgery requiring pre-operative blood removal would be a cardiopulmonary bypass procedure.

[0081] Sepsis / Septic Shock. In sepsis, some patients may become hypertensive in spite of massive fluid therapy and treatment with vasoconstrictor agents. In this instance, the overproduction of nitric oxide (NO) results in lowered blood pressure. Therefore hemoglobin is a desirable agent for treatment of these patients because hemoglobin binds NO with a high avidity.

[0082] Hypoxemia. When a patient has acute lung injury caused by either pneumonia or pancreatitis, hypoxemia can be observed and can be alleviated by providing the hemoglobins or compositions of the invention to oxygenate the affected tissues.

[0083] Cancer. Delivery of O₂ to the hypoxic inner core of a solid tumor mass increases its sensitivity to radiotherapy and chemotherapy. Because the microvasculature of a tumor is unlike that of other tissues, sensitization through increasing O₂ levels requires O₂ be unloaded within the hypoxic core. In other words, the P50 should be very low to prevent early unloading of the O₂, increasing the O₂ levels, to insure optimal sensitization of the tumor to subsequent radiation and chemotherapy treatments.

[0084] Surgery. The hemoglobins and compositions of the invention can be used during various surgical procedures. For example, they can be used as an adjunct to angioplasty, thoracic aortic repairs, during a cardiopulmonary bypass procedure or as a cardiopulmonary priming solution.

[0085] Organ Perfusion. During the time an organ is maintained ex vivo or in an organ donation recipient, maintaining O₂ content helps preserve structural and cellular integrity and minimizes infarct formation. The hemoglobins and compositions can sustain the oxygen requirements for such an organ.

[0086] The hemoglobins and compositions thereof can also be used in non-humans, such as domestic animals (e.g., livestock and companion animals such as dogs, cats, horses, birds, reptiles). It is contemplated that the present invention finds utility in the emergency treatment of domestic and wild animals suffering a loss of blood due to injury, hemolytic anemias, etc.

Veterinary uses include treatment of loss of blood due to injury, hemolytic anemia, infectious anemia, bacterial infection, Factor IV fragmentation, hypersplenation and splenomegaly, hemorrhagic syndrome in poultry, hypoplastic anemia, aplastic anemia, idiopathic immune hemolytic conditions, iron deficiency, isoimmune hemolytic anemia, microangiopathic hemolytic anemia, parasitism, or surgical-anesthesia induced brain damage.

DEFINITIONS

[0087] When the terms “one,” “a” or “an” are used in this disclosure, they mean “at least one” or “one or more,” unless otherwise indicated.

[0088] “Activated polyalkylene oxide” or “activated PAO” as used herein refer to a PAO molecule that has at least one functional group. A functional group is a reactive moiety that interacts with free amines, sulfhydryls or carboxyl groups on a molecule to be conjugated with PAO. For example, one such functional group that reacts with free sulfhydryls is a maleimide group. A functional group that reacts with free amines is a succinimide group.

[0089] “Deoxyhemoglobin” or “unliganded hemoglobin” means any hemoglobin to which no exogenous ligand is bound to heme.

[0090] “Hemoglobin” or “Hb” refers generally to a heme protein that transports oxygen. In humans, each molecule of Hb has 4 subunits, 2 α -chain subunits and 2 β -chain subunits, which are arranged in a tetrameric structure. Each subunit also contains one heme group, which is the iron-containing center that in the ferrous (Fe^{2+}) binds the ligands O_2 , NO or CO. Thus, each Hb molecule can bind up to 4 ligand molecules, making HbO_2 , HbNO , or HbCO liganded compounds, respectively. Additionally, the hemoglobin may be liganded with mixtures of O_2 , NO and CO.

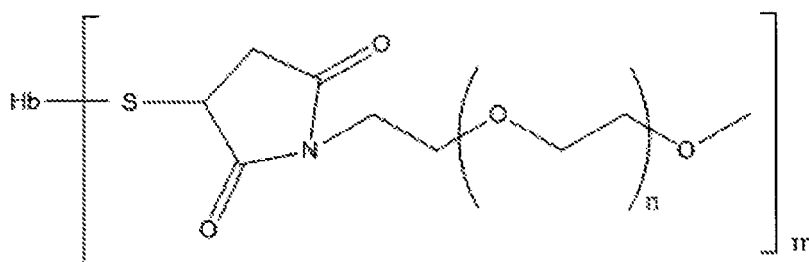
[0091] “Hemoglobin based oxygen carriers” (HBOCs) refers to hemoglobins that carry oxygen, but are also useful for carrying other molecular gases, such as carbon monoxide and nitric oxide.

[0092] “High oxygen affinity” refers to hemoglobin that has been modified to exhibit an oxygen affinity greater than that of stroma free-hemoglobin (SFH). Thus, a “high oxygen affinity” Hb has a P50 less than that of SFH, which has a P50 of 15 mmHg as measured at 37°C and pH 7.4.

[0093] "Liganded hemoglobin" means hemoglobin to which an exogenous ligand is bound to heme. Common preferred ligands include oxygen, carbon monoxide, and nitric oxide.

[0094] "MalPEG" refers to maleimidyl polyethylene glycol, and includes a maleimidyl moiety attached to polyethylene glycol via a linker.

[0095] "MalPEG-Hb" refers to Hb to which maleimidyl-activated PEG has been conjugated. The conjugation is performed by reacting MalPEG with thiol groups (and to a lesser extent, amino groups) on the Hb to form MalPEG-Hb. Thiol groups are found in cysteine residues present in the amino acid sequence of Hb, such as the two intrinsic thiols at β Cys 93, and can also be introduced by modifying surface amino groups to contain a thiol group. An exemplary MalPEG-Hb known as MP4 (Sangart, Inc.) has the following formula:



wherein Hb is hemoglobin; S is a thiol group on the hemoglobin; n is the number of oxyethylene units of the 5,000-Dalton polyalkylene oxide polymer; and m is the average number of maleimidyl-activated polyalkylene oxide polymers conjugated to the hemoglobin and is 7–8.

[0096] "Methemoglobin" or "metHb" refer to an oxidized form of Hb that contains iron in the ferric state. MetHb does not function as an oxygen or CO carrier. The term "methemoglobin %" as used herein refers to the percentage of oxidized Hb to total Hb.

[0097] "Methoxy-PEG" or "mPEG-OH" refer to PEG wherein the hydrogen of the hydroxyl terminus is replaced with a methyl ($-\text{CH}_3$) group.

[0098] "Modified hemoglobin" or "modified Hb" refers to Hb that has been altered by a chemical reaction, such as intra- and inter-molecular crosslinking, polymerization, conjugation, and/or recombinant techniques, such that the Hb is no longer in its "native" state. As used herein, the terms "hemoglobin" or "Hb" refer to both native unmodified Hb and modified Hb, unless otherwise indicated.

"Nitrite reductase activity" or "NRA" is the ability of hemoglobin or a hemoglobin-based protein to reduce nitrite to nitric oxide. "Maximal nitrite reductase activity" is the maximum rate that hemoglobin or a hemoglobin-based protein is able to reduce nitrite to nitric oxide. "Initial nitrite reductase activity" is the initial rate that hemoglobin or a hemoglobin-based protein reduces nitrite to nitric oxide when nitrite is added to the fully deoxygenated protein.

[0099] The term "non-oxygenated" means that the heme protein or hemoglobin is in the non-liganded, deoxygenated state, or it is liganded with a gas other than O₂, such as NO or CO.

[00100] "Oxygen affinity" refers to the avidity with which an oxygen carrier, such as Hb, binds molecular oxygen. This characteristic is defined by the oxygen equilibrium curve, which relates the degree of saturation of Hb molecules with oxygen (Y axis) with the partial pressure of oxygen (X axis). The position of this curve is denoted by the "P50" value, which is the partial pressure of oxygen at which the oxygen carrier is half-saturated with oxygen, and is inversely related to oxygen affinity. Hence, the lower the P50, the higher the oxygen affinity. The oxygen affinity of whole blood (and components of whole blood, such as red blood cells and Hb) can be measured by a variety of methods known in the art. (*see, e.g.*, Winslow, R.M. *et al.*, J. Biol. Chem. 1977, 252:2331-37). Oxygen affinity may also be determined using a commercially available HEMOXTM Analyzer (TCS Scientific Corporation, New Hope, PA). (*see, e.g.*, Vandegriff and Shrager in "Methods in Enzymology" (Everse *et al.*, eds.) 232:460 (1994)) ; and Vandegriff, et al., Anal. Biochem. 256(1): 107-116 (1998)).

[00101] The term "oxygen therapeutic agent" as used herein refers to a heme protein that is capable of binding to and carrying molecular oxygen to cells/tissues/organs in need thereof. When administered in the form of a CO- or NO-liganded heme protein, once the CO or NO is released from the heme moiety, the heme groups are then free to bind to and carry molecular oxygen.

[00102] "Polyethylene glycol" or "PEG" refer to a polymer of the general chemical formula H(OCH₂CH₂)_n OH where "n" is greater than or equal to 4, preferably about 45 to about 500, more preferably about 70 to about 250, and most preferably about 90 to about 140, or about 115.. The polymer can be substituted or unsubstituted, and the terminal hydroxy group can be replaced with a different conventional terminal group, such as methoxy or carboxy. PEGs are commercially available from many sources (*e.g.*, CarbowaxTM (Dow Chemical, Midland, MI), Poly-G® (Arch Chemicals, Norwalk, CT) and Solbase).

[00103] “Polyethylene glycol-conjugated hemoglobin,” “PEG-Hb conjugate” or “PEG-Hb” refer to Hb to which at least one PEG is covalently attached.

[00104] “Solution” refers to a liquid mixture and the term “aqueous solution” refers to a solution that contains some water and may also contain one or more other liquid substances with water to form a multi-component solution.

[00105] “Stroma-free hemoglobin” or “SFH” refer to Hb from which red blood cell membranes have been removed.

[00106] “Surface-modified hemoglobin” refers to hemoglobin to which chemical groups, usually polymers, have been attached, such as dextran or polyalkylene oxide. The term “surface-modified oxygenated hemoglobin” refers to Hb that is in the “R” state when it is surface modified.

[00107] “Terminal activity” is an indication of the percentage of PAO that is functionalized with a moiety capable of reacting with a reactive group of the heme protein or hemoglobin. “100% Terminal activity” indicates that the molar excess of the PAO used in the conjugation reaction is expressed on a basis that all of the PAO has a moiety capable of reacting with a reactive group of the heme protein or hemoglobin. For example, if an available Mal-PEG has 80% terminal activity such that 80% of the PEGs are functionalized with Mal, and the Mal-PEG is used in 20-fold molar excess over hemoglobin, then this molar ratio can be expressed as a 16-fold molar excess of Mal-PEG over hemoglobin based on 100% terminal activity.

[00108] “Thiolation” refers to a process that increases the number of sulfhydryl groups on a molecule. For example, reacting a protein with 2-iminothiolane (“2-IT”) converts free amines on the surface of the protein to sulfhydryl groups. These sulfhydryl groups are then available for reaction with a thiol reactive moiety, such as a maleimide.

[00109] “Unliganded hemoglobin” refers to any hemoglobin containing at least one heme moiety that is not liganded to a molecular gas such as oxygen, carbon monoxide or nitric oxide. As such, the hemoglobin is considered “unliganded” if only one of the heme moieties is not liganded to a molecular gas.

[00110] All publications, patents, and patent applications cited in this specification are incorporated herein by reference for the subject matter specifically referenced herein as if each

such publication, patent or patent application were specifically and individually indicated to be incorporated herein by reference.

[00111] Having described the invention in detail, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims.

EXAMPLES

[00112] The following non-limiting examples are provided to further illustrate the present invention.

Example 1: Preparation of PEGylated $\beta\beta$ -DBBF-crosslinked hemoglobin conjugate

[00113] Packed red blood cells ("RBCs") are procured from a commercial source, such as from a local Blood Bank, the New York Blood Center, or the American Red Cross. The material is obtained not more than 45 days from the time of collection. All units are screened for viral infection and subjected to nucleic acid testing prior to use. Non-leukodepleted pooled units are leukodepleted by membrane filtration to remove white blood cells. Packed RBCs are pooled into a sterile vessel and stored at 2-15°C until further processing. The volume is noted, and Hb concentration is determined using a commercially available co-oximeter, or other art-recognized method.

[00114] RBCs are washed with six volumes of 0.9% sodium chloride using a 0.45- μ m tangential flow filtration, followed by cell lysis by decreasing the concentration of salt. Hb extraction is performed using the same membrane. The cell wash is analyzed to verify removal of plasma components by a spectrophotometric assay for albumin. The lysate is processed through a 0.16- μ m membrane in the cold to purify Hb. The purified Hb is collected in a sterile depyrogenated and then ultrafiltered to remove virus. Additional viral-reduction steps, including solvent/detergent treatment, nanofiltration, and anion Q membrane purification may be performed. All steps in this process are carried out at 2-15°C.

[00115] Hb from lysate is exchanged into Ringer's lactate ("RL"), or phosphate-buffered saline ("PBS", pH 7.4), using a 30-kD membrane. The Hb is concentrated to 1.1-1.5 mM (in tetramer). Ten to 12 volumes of RL or PBS are used for solvent exchange. This process is carried out at 2-15°C. The pH of the solution prepared in RL or PBS is adjusted to 8.0 prior to thiolation.

The Hb is sterile-filtered through a 0.45 or 0.2- μ m disposable filter capsule and stored at $4\pm 2^{\circ}\text{C}$ before the chemical modification reaction is performed.

[00116] Crosslinking: $\beta\beta$ -DBBF crosslinked Hb was prepared by reaction of stroma-free hemoglobin (SFH) prepared from packed red blood cells with bis(3,5-dibromosalicyl)fumarate (DBBF) as described previously by Walder et al, *Biochemistry*, 1979; 18(20): 4265-70. Oxygenated SFH in borate buffer (pH ~ 8.5) was reacted with two fold molar excess of DBBF for about 16 hours at about $2-8^{\circ}\text{C}$.

[00117] Thiolation: Using the SFH prepared as described above, thiolation was carried out using a 15-fold molar excess of 2-iminothiolane (2-IT) over Hb. The ratio and reaction time were optimized to maximize the number of thiol groups for PEG conjugation and to minimize product heterogeneity. Approximately 1 mM Hb (tetramer) in RL (pH 7.0-8.5), PBS or any similar buffer, was combined with 15 mM 2-IT in the same buffer. This mixture was continuously stirred for about 6 hours at $10\pm 5^{\circ}\text{C}$.

[00118] The dithiopyridine colorimetric assay (Ampulski, R.S. *et al.*, *Biochem. Biophys. Acta* 1969, 32:163-169) was used to measure the number of available thiol groups on the surface of the Hb tetramer before and after thiolation, and then again after Hb-PEG conjugation. Human Hb contains two intrinsic reactive thiol groups at the $\beta 93$ cysteine residues, which was confirmed by the dithiopyridine reaction. After thiolation of SFH at a ratio of 1:<8 (SFH: 2-IT), the number of reactive thiol groups increased from two to greater than seven thiols.

[00119] PEG Conjugation: MalPEG was conjugated to the thiolated $\beta\beta$ -DBBF crosslinked Hb using a 28-fold molar excess of MalPEG based on 100% terminal activity over the starting tetrameric Hb concentration. The Hb was first allowed to equilibrate with the atmosphere to oxygenate the Hb. Approximately 1 mM thiolated Hb in RL (pH 7.0-8.5), PBS or any similar buffer was combined with 28 mM MalPEG in the same buffer. This mixture was continuously stirred for about 6 hours at $10\pm 5^{\circ}\text{C}$.

[00120] The resulting PEG-Hb conjugate was processed through a 70-kD membrane (*i.e.* <0 -volume filtration) to remove unreacted reagents. This process was monitored by size-exclusion liquid chromatography ("LC") at 540 nm and 217 nm. The concentration was adjusted to 4.4 g/dl Hb and the pH was adjusted to $6.0-7.8$.

[00121] The PEG-Hb conjugate was sterile filtered using a 0.2- μ m sterile disposable capsule and collected into a sterile depyrogenated vessel at $4\pm 2^{\circ}\text{C}$. The PEG-Hb conjugate was diluted to 4.4 g/dl RL and the pH adjusted to 7.4 ± 0.2 pH and then sterile-filtered (0.2 μ m) and aliquoted into endotoxin free sterile containers.

[00122] The final PEGylated $\beta\beta$ -DBBF crosslinked hemoglobin conjugate (“PEG- $\beta\beta$ -Hb”) had the properties shown in Table 3:

Table 3: Properties of PEG- $\beta\beta$ -Hb

Properties	Values
Hb Concentration (g/dL)	4.4
pH	7.4
Degree of PEGylation	7.6
COP (mm Hg)	85
P50 mm Hg	2.6
Hill number (n-value)	1.05

[00123] The structure of PEG- $\beta\beta$ -Hb was further confirmed via standard methodology. Size exclusion chromatography confirmed the presence of DBBF crosslinking (Figures 1 and 2). Reverse-phase high performance liquid chromatography confirmed the presence of DBBF crosslinking of the β globin subunits of the hemoglobin (Figure 3). The PEGylation of $\beta\beta$ -DBBF Hb was confirmed using size exclusion chromatography (Figure 4).

Example 2: Enhanced nitrite reductase activity exhibited by PEG- $\beta\beta$ -Hb

[00124] Deoxygenated PEGylated $\beta\beta$ -DBBF-crosslinked hemoglobin from Example 1 and other hemoglobin species for comparison purposes were reacted anaerobically with sodium nitrite in a sealed cuvette in the presence of sodium dithionite. A ten-fold excess of nitrite was used over heme, and the reaction was monitored spectrophotometrically. The resulting spectral data were deconvoluted using parent spectra for deoxyhemoglobin, iron-nitrosyl-hemoglobin, and methemoglobin. The rates were plotted as the derivative of the disappearance of deoxyhemoglobin and the maximum rates were used for comparison purposes.

[00125] SFH and PEGylated $\beta\beta$ -DBBF-crosslinked hemoglobin reduced nitrite to NO with maximum rates of 0.0215 $\mu\text{M/s}$ and 0.462 $\mu\text{M/s}$, respectively, showing a 21-fold higher maximum rate for PEGylated $\beta\beta$ -DBBF-crosslinked hemoglobin compared to SFH.

[00126] Referring to Figure 5, nitrite reductase activity of several types of modified Hbs were determined, including maleimide-conjugated non-crosslinked hemoglobin, which reacts at Hb $\beta 93\text{Cys}$ residues to decrease P50. Maleimides tested include the small molecule, *N*-ethylmaleimide (NEM), and MP4 (Sangart, Inc.) as well as the other hemoglobins specified in Table 4.

Table 4: Properties of Modified Hemoglobins

Hb	Conjugation Chemistry	Degree of Substitution per Tetramer	P50 (oxygen affinity)
SFH	N/A	-	16
MP4OX	Maleimide	8 (PEG 5K)	4
P5K2	Maleimide	2 (PEG 5K)	8
P5K4	Maleimide	4 (PEG 5K)	5
NEM2	Maleimide	2 (NEM)	9
NEM8	Maleimide	8 (NEM)	5
SVA-PEG-Hb	Succinimide	Not tested	7
$\alpha\alpha$ -Hb	DBBF	-	33
$\beta\beta$ -Hb	DBBF	-	8
PEG- $\alpha\alpha$ -Hb	DBBF/Maleimide	7 (PEG 5K)	9
PEG- $\beta\beta$ -Hb	DBBF/Maleimide	7.6 (PEG 5K)	3

[00127] Hbs cross-linked between α or β subunits exhibit varying P50. The P50 values ranged from 3 mmHg (for PEG- $\beta\beta$ -Hb) to 33 mmHg (for $\alpha\alpha$ -crosslinked Hb). It was observed that there is a direct correlation between P50 and nitrite reductase activity. Hbs with high oxygen affinity (low P50) reduced nitrite to NO at a higher rate than low affinity Hbs. With a 10-fold excess of nitrite, the maximum rate of nitrite reduction varied from 0.46 $\mu\text{M/s}$ (for PEG- $\beta\beta$ -Hb) to 0.015 $\mu\text{M/s}$ (for $\alpha\alpha$ -crosslinked Hb). NO produced by this reaction can escape heme capture and be released from Hb, and the rate of NO release is relative to the nitrite reductase activity. Together, these results suggest that oxygen affinity is important in cell-free Hb design, not only to enhance the delivery of hemoglobin ligands, but also to maximize nitrite reductase activity.

[00128] When introducing elements of the present invention or the preferred embodiments(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[00129] In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

[00130] As various changes could be made in the above compositions and methods without departing from the scope of the invention, it is intended that all matter contained in the

above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

WHAT IS CLAIMED IS:

1. A β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate having a P50 ranging from about 2 to 5 mmHg as measured at 37 °C and pH 7.4, wherein the hemoglobin conjugate when fully deoxygenated at 25 °C exhibits a maximal nitrite reductase activity that is at least 10-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
2. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is at least 15-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
3. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is at least 20-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
4. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is 10-fold to about 25-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
5. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is about 15-fold to about 25-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
6. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is 10-fold to about 21-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.
7. The hemoglobin conjugate of claim 1 wherein the hemoglobin conjugate when fully deoxygenated exhibits a maximal nitrite reductase activity that is about 15-fold to about

21-fold greater than that of deoxygenated stroma-free hemoglobin when measured under the same conditions.

8. A β,β -intramolecularly-crosslinked polyoxyalkylene oxide (PAO) hemoglobin conjugate having a P50 ranging from about 2.0 to 5.0 mmHg as measured at 37 °C and pH 7.4, the hemoglobin conjugate having a maximal nitrite reductase activity of at least 0.25 $\mu\text{M}/\text{sec}$ when fully deoxygenated at 25 °C.

9. The hemoglobin conjugate of claim 8 wherein the maximal nitrite reductase activity of at least about 0.30 $\mu\text{M}/\text{sec}$.

10. The hemoglobin conjugate of claim 8 wherein the maximal nitrite reductase activity of at least about 0.35 $\mu\text{M}/\text{sec}$.

11. The hemoglobin conjugate of claim 8 wherein the maximal nitrite reductase activity of at least about 0.40 $\mu\text{M}/\text{sec}$.

12. The hemoglobin conjugate of claim 8 wherein the maximal nitrite reductase activity ranges from 0.25 to about 0.50 $\mu\text{M}/\text{sec}$.

13. The hemoglobin conjugate of claim 8 wherein the maximal nitrite reductase activity ranges from 0.30 to about 0.47 $\mu\text{M}/\text{sec}$.

14. The hemoglobin conjugate of any one of claims 1–13 wherein the hemoglobin is conjugated to on average about 7 to about 11 PAO molecules per tetramer.

15. The hemoglobin conjugate of claim 14 wherein the hemoglobin is conjugated to on average about 9 to about 10 PAO molecules per tetramer.

16. The hemoglobin conjugate of any one of claims 1–15 wherein the polyalkylene oxide is covalently attached via a thiol reactive moiety to a thiol group of an exposed amino acid side chain on the hemoglobin molecule while the hemoglobin is in the oxygenated state.

17. The hemoglobin conjugate of claim 16 wherein the polyalkylene oxide is linked to the thiol reactive moiety by a linker consisting of alkylene or phenylene.

18. The hemoglobin conjugate of any one of claims 1–17 wherein the hemoglobin is bis(2,5-dibromosalicyl) fumarate crosslinked at two β 82 lysine residues of the hemoglobin molecule.

19. The hemoglobin conjugate of any one of claims 1–18 wherein colloid osmotic pressure is at least 75 mmHg.

20. The hemoglobin conjugate of any one of claims 1–19 wherein the P50 is about 2 to about 4 mmHg.

21. The hemoglobin conjugate of claim 20 wherein the P50 is about 3 mmHg.

22. The hemoglobin conjugate of any one of claims 1–21 wherein the hemoglobin conjugate is liganded to oxygen.

23. The hemoglobin conjugate of any one of claims 1–22 wherein the hemoglobin conjugate is liganded to carbon monoxide.

24. The hemoglobin conjugate of any one of claims 1–23 wherein the hemoglobin conjugate is liganded to nitric oxide.

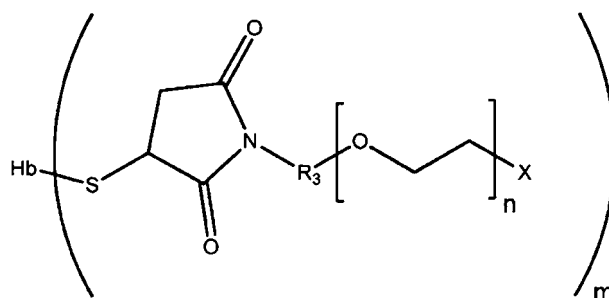
25. The hemoglobin conjugate of any one of claims 1–24 wherein the hemoglobin conjugate is deoxygenated.

26. The hemoglobin conjugate of any one of claims 1–25, wherein the PAO is a polyethylene glycol (PEG).

27. The hemoglobin conjugate of claim 26, wherein the PEG has an average molecular weight of about 2,000 to about 20,000 Daltons.

28. The hemoglobin conjugate of claim 26, wherein the PEG has an average molecular weight of about 3,000 to about 10,000 Daltons.
29. The hemoglobin conjugate of claim 26, wherein the PEG has an average molecular weight of about 4,000 to about 6,000 Daltons.
30. The hemoglobin conjugate of claim 29, wherein the PEG has an average molecular weight of about 5,000 Daltons.
31. The hemoglobin conjugate of any one of claims 26–30, wherein the PEG is a maleimide-PEG.
32. The hemoglobin conjugate of claim 31, wherein the maleimide is linked to the PEG via an alkylene or phenylene linker.
33. The hemoglobin conjugate of claim 32, wherein the alkylene linker is an ethylene linker.
34. The hemoglobin conjugate of any one of claims 31–33, wherein the maleimide-PEG is conjugated to a thiol moiety of the hemoglobin selected from an intrinsic thiol moiety of a cysteine residue of the hemoglobin, a thiol moiety of a thiolated lysine residue of the hemoglobin, or a combination thereof.
35. The hemoglobin conjugate of any one of claims 1–34, wherein N-ethyl maleimide is conjugated to β 93 cysteine residues of the hemoglobin.
36. The hemoglobin conjugate of any one of claims 31–34, wherein the maleimide-PEG has the structure

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wherein:

Hb is hemoglobin,

S is a thiol of the hemoglobin,

R₃ is an alkylene or phenylene group,

X is a terminal group,

m is the average number of maleimidyl-activated PEG polymers conjugated to the hemoglobin,
and

n is the average number of oxyethylene units of a PEG having an average molecular weight of about 2,000 to about 20,000 Daltons.

37. The hemoglobin conjugate of claim 36, wherein R₃ is ethylene.
38. The hemoglobin conjugate of claim 36 or 37, wherein X is methoxy or carboxy.
39. The hemoglobin conjugate of claim 38, wherein X is methoxy.
40. The hemoglobin conjugate of any one of claims 36–39 wherein n is from about 3,000 to about 10,000 Daltons.
41. The hemoglobin conjugate of any one of claims 36–39 wherein n is from 4,000 to about 6,000 Daltons.
42. The hemoglobin conjugate of any one of claims 36–39 wherein n is about 5,000 Daltons.

43. The hemoglobin conjugate of any one of claims 36–42 wherein m is about 7 to about 11.
44. The hemoglobin conjugate of any one of claims 36–42 wherein m is about 9 or about 10.
45. A pharmaceutical composition comprising the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate of any one of claims 1–44 and a pharmaceutically acceptable carrier.
46. The pharmaceutical composition of claim 45, wherein the composition is normo-oncotic with whole blood.
47. The pharmaceutical composition of claim 45 or 46, wherein the composition is hyperoncotic as compared to plasma.
48. The pharmaceutical composition of any one of claims 45–47, for use in the treatment of acute liver failure, beta thalassemia, a burn, chronic critical limb ischemia, carbon dioxide or cyanide poisoning, chronic obstructive pulmonary disease (COPD), congestive heart failure, hypoxia, malaria, organ ischemia, peripheral vascular disease, porphyria, pre-eclampsia in pregnancy, sepsis, sickle cell disease, retinal disease, an intra-ocular condition, testicular torsion, trauma, shock, traumatic brain injury, ulcers, vasospasm, or a combination thereof.
49. The pharmaceutical composition of claim 48, wherein the organ ischemia comprises acute bowel ischemia (torsion), acute bowel ischemia (embolism), cardiogenic shock, acute vascular organ ischemia, stroke, myocardial infarction, or severe cardiac ischemia.
50. The pharmaceutical composition of any one of claims 45–47, for use in the treatment of non-traumatic hemorrhagic shock, pre-hospital setting trauma, traumatic hemorrhagic shock, acute lung injury, adult respiratory distress syndrome, traumatic brain injury, stroke, solid tumor cancer, organ degradation (ex-vivo), organ degradation (in recipient), severe sepsis, septic shock, myocardial infarction, cardiac ischemia, cardiogenic shock, acute heart failure, pulmonary embolism, or a combination thereof.

51. The pharmaceutical composition of any one of claims 45–47, for use as an adjunct to angioplasty, as an adjunct for plastic surgery, or as an adjunct in implanting a ventricular assist device; as a blood substitute, a cardioprotectant, a cryopreservative, a hemodialysis adjunct, an oncology agent, an organ preservative, a performance enhancement agent, a surgery adjunct, or a wound healing agent; in imaging; to improve lung function; or a combination thereof.

52. The pharmaceutical composition of any one of claims 45–47, for veterinary treatment of loss of blood due to injury, hemolytic anemia, infectious anemia, bacterial infection, Factor IV fragmentation, hypersplenation and splenomegaly, hemorrhagic syndrome in poultry, hypoplastic anemia, aplastic anemia, idiopathic immune hemolytic conditions, iron deficiency, isoimmune hemolytic anemia, microangiopathic hemolytic anemia, parasitism, or surgical-anesthesia induced brain damage.

53. A method of treatment comprising administering a hemoglobin conjugate of any one of claims 1–44 or a pharmaceutical composition of any one of claims 45–47 to a subject in need thereof.

54. The method of treatment of claim 53, wherein the subject is an animal.

55. The method of treatment of claim 53, wherein the subject is a human.

56. The method of treatment of any one of claims 53–55, wherein the method is a method for the treatment of acute liver failure, beta thalassemia, a burn, chronic critical limb ischemia, carbon dioxide or cyanide poisoning, chronic obstructive pulmonary disease (COPD), congestive heart failure, hypoxia, malaria, organ ischemia, peripheral vascular disease, porphyria, pre-eclampsia in pregnancy, sepsis, sickle cell disease, retinal disease, an intra-ocular condition, testicular torsion, trauma, shock, traumatic brain injury, ulcers, vasospasm, or a combination thereof.

57. The method of treatment of claim 56, wherein the organ ischemia comprises acute bowel ischemia (torsion), acute bowel ischemia (embolism), cardiogenic shock, acute vascular organ ischemia, stroke, myocardial infarction, or severe cardiac ischemia.

58. The method of treatment of any one of claims 53–55, wherein the method is a method for the treatment of non-traumatic hemorrhagic shock, pre-hospital setting trauma, traumatic hemorrhagic shock, acute lung injury, adult respiratory distress syndrome, traumatic brain injury, stroke, solid tumor cancer, organ degradation (ex-vivo), organ degradation (in recipient), severe sepsis, septic shock, myocardial infarction, cardiac ischemia, cardiogenic shock, acute heart failure, pulmonary embolism, or a combination thereof.

59. The method of treatment of any one of claims 53–55, wherein the hemoglobin tetramer or pharmaceutical composition is administered as an adjunct to angioplasty, as an adjunct for plastic surgery, or as an adjunct in implanting a ventricular assist device; as a blood substitute, a cardioprotectant, a cryopreservative, a hemodialysis adjunct, an oncology agent, an organ preservative, a performance enhancement agent, a surgery adjunct, or a wound healing agent; in imaging; to improve lung function; or a combination thereof.

60. The method of treatment of any one of claims 53–55, wherein the hemoglobin tetramer or pharmaceutical composition is administered as an adjunct to angioplasty, as an adjunct to thoracic aortic repairs, as an adjunct to cardiopulmonary bypass, or as a priming solution for cardiopulmonary bypass.

61. The method of treatment of claim 53 or 54, wherein the subject is a non-human animal and the method is a method for veterinary treatment of loss of blood due to injury, hemolytic anemia, infectious anemia, bacterial infection, Factor IV fragmentation, hypersplenation and splenomegaly, hemorrhagic syndrome in poultry, hypoplastic anemia, aplastic anemia, idiopathic immune hemolytic conditions, iron deficiency, isoimmune hemolytic anemia, microangiopathic hemolytic anemia, parasitism, or surgical-anesthesia induced brain damage.

62. A method of delivering oxygen, nitric oxide, carbon monoxide or mixtures thereof to tissue and reducing nitrite to nitric oxide (NO) in the microvasculature, the method

comprising administering the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate of any one of claims 1–44 or the pharmaceutical composition of any one of claims 45–60 to a subject in need thereof, wherein following administration, unliganded hemes in the hemoglobin convert nitrite to nitric oxide in the microvasculature.

63. A method of making the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate of any one of claims 1–44, the method comprising the steps of:

mixing β,β -intramolecularly-crosslinked hemoglobin with 2-iminothiolane (2-IT) in an aqueous diluent to form a thiolated hemoglobin; and

adding PAO to the thiolated hemoglobin in the aqueous diluent to form the β,β -intramolecularly-crosslinked polyoxyalkylene oxide hemoglobin conjugate.

64. The method of claim 63, wherein the 2-iminothiolane is present at a concentration of between about 8- fold and about 25-fold molar excess over the protein concentration.

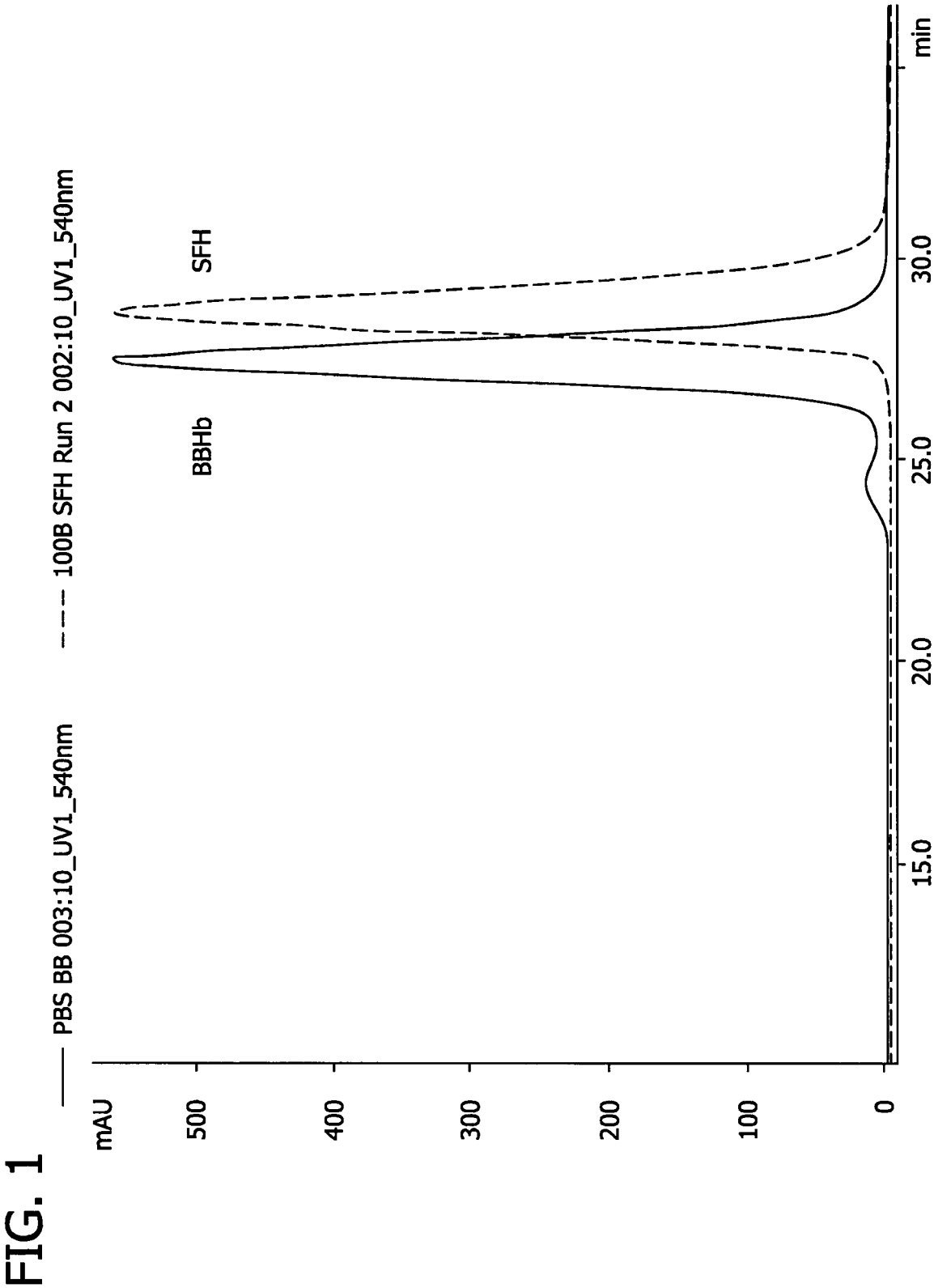
65. The method of claim 64, wherein the 2-iminothiolane is present at a concentration of about 15-fold molar excess over the hemoglobin concentration.

66. The method of any one of claims 63–65, wherein the PAO-maleimide is present at a concentration of between about 10-fold and about 40-fold molar excess over the protein concentration.

67. The method of claim 66, wherein the PAO-maleimide is present at a concentration of about 28-fold molar excess over the hemoglobin concentration.

68. The method of any one of claims 63–67, wherein the thiolation step is carried out at a pH of about 7 to about 9.

69. The method of any one of claims 63–68, wherein the step of adding the PAO-maleimide to the thiolated protein to form a PAO-maleimide conjugated protein is carried out at a pH of between about 7 and about 9.



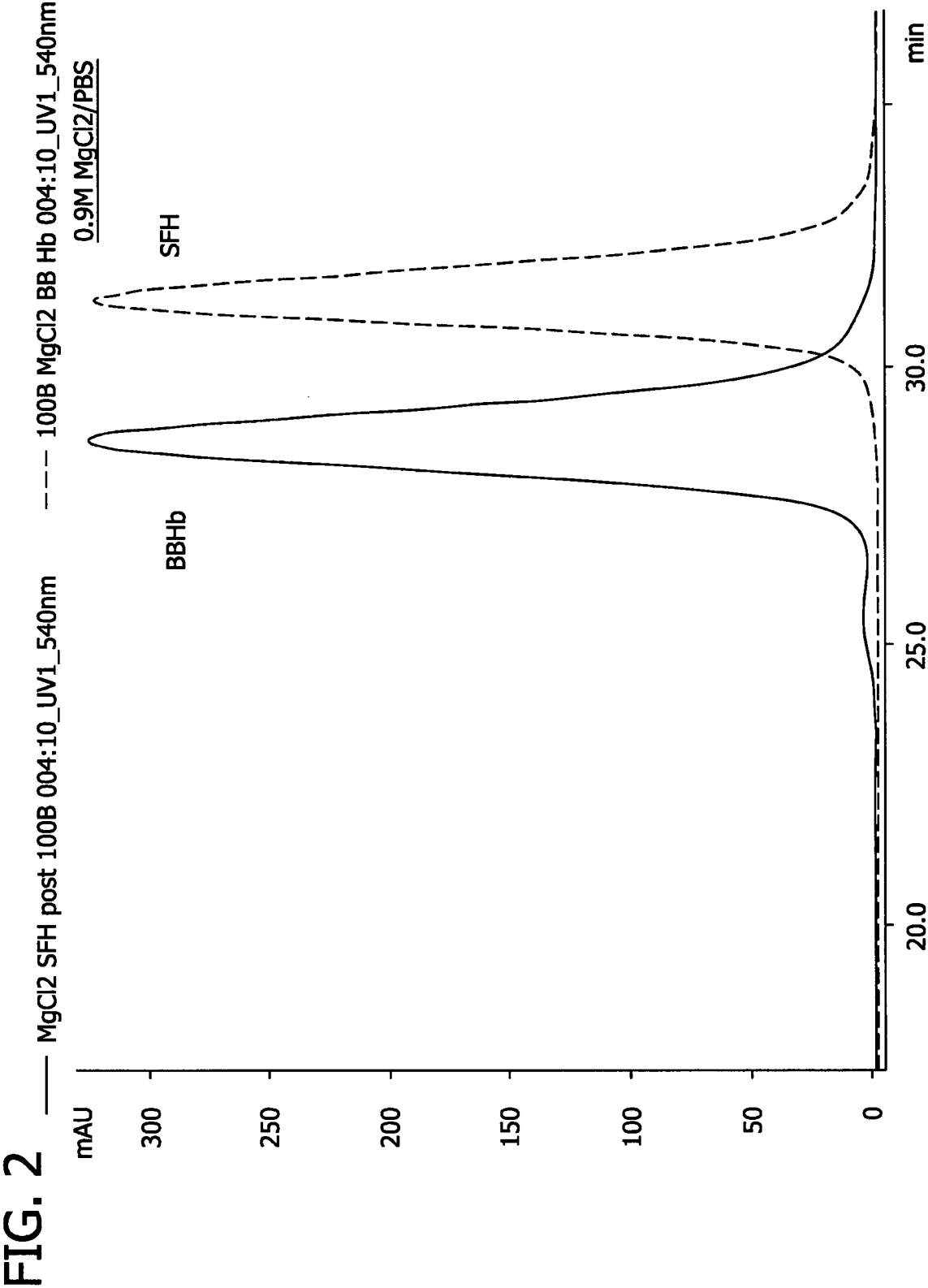


FIG. 3

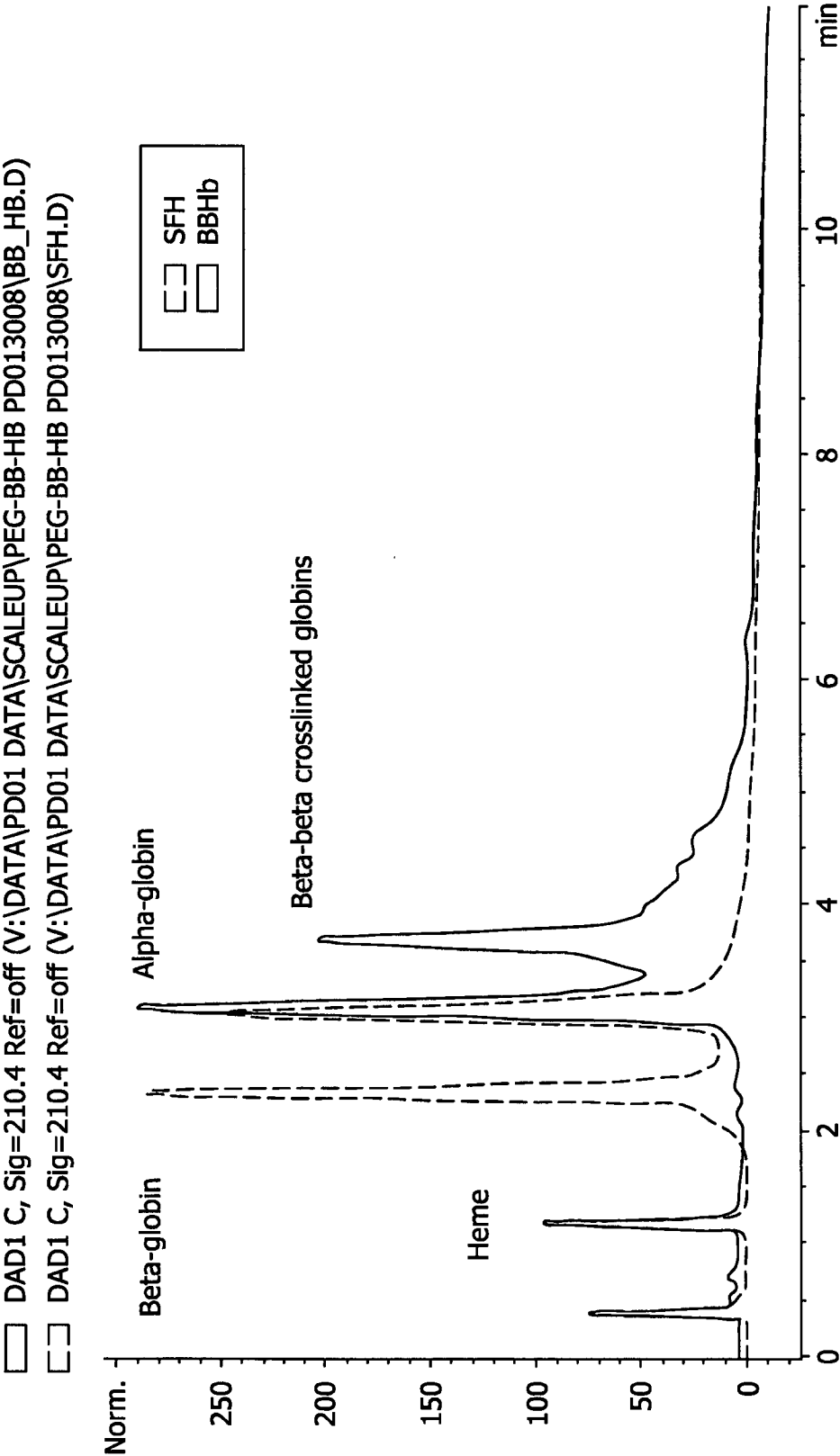


FIG. 4

VWD1 A, Wavelength=217 nm (V:\DATA\PD-02\SCALEUP\PEG-BB~1\PBSSUP~1\7BBHB2GD.D)
VWD1 A, Wavelength=217 nm (V:\DATA\PD-02\SCALEUP\PEG-BB~1\PBSSUP~1\9_15DV2G.D)

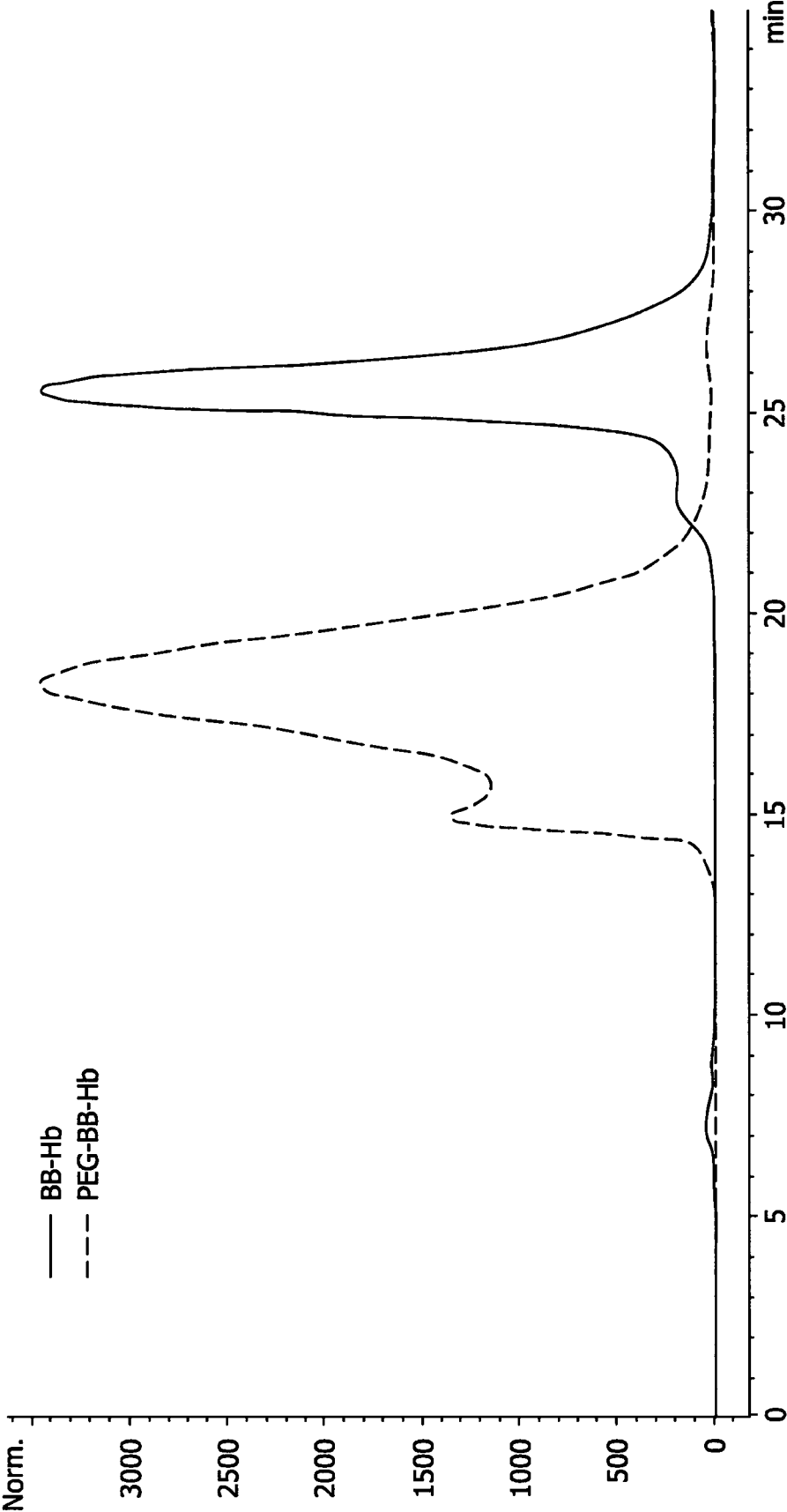
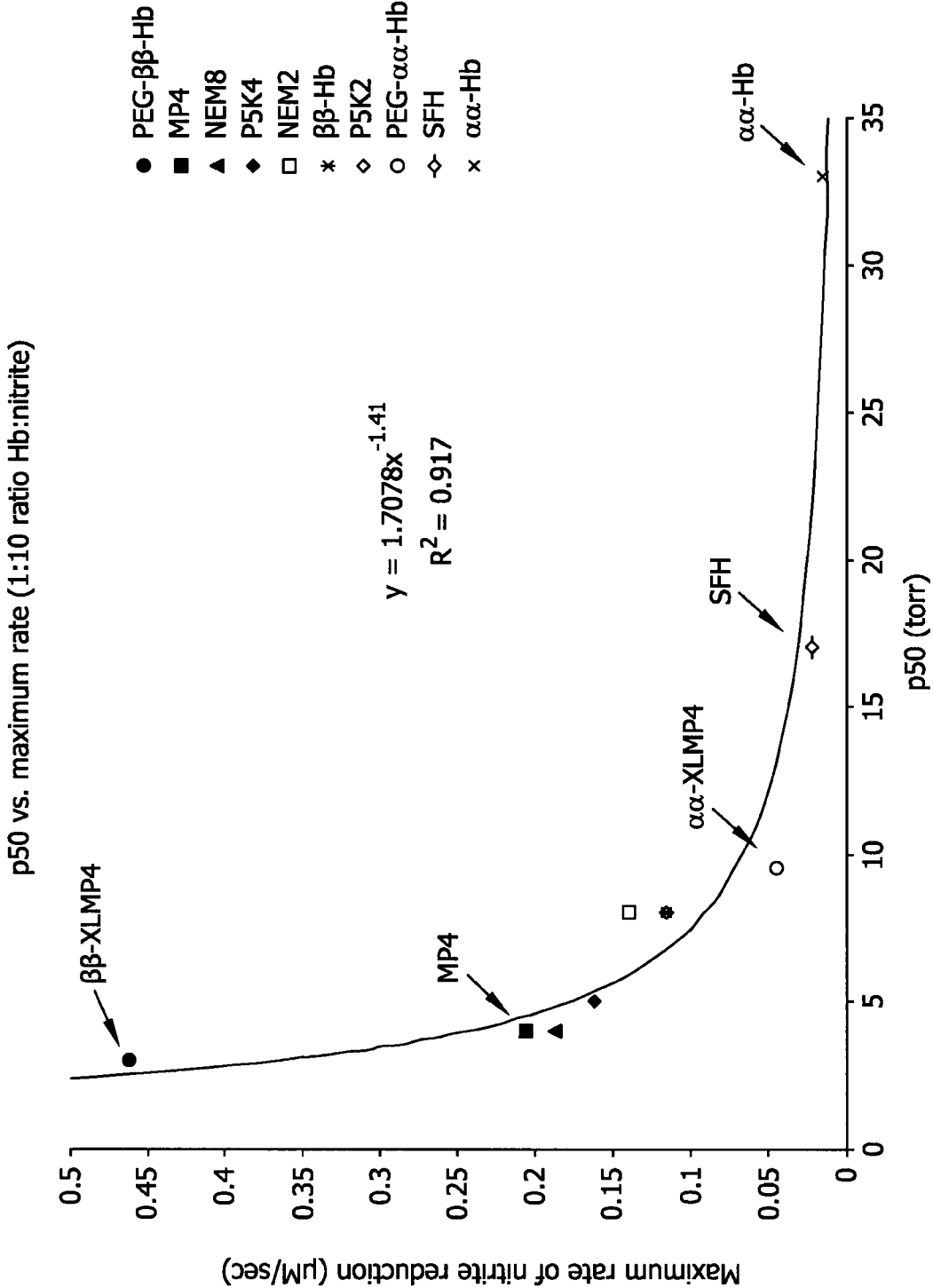


FIG. 5



INTERNATIONAL SEARCH REPORT

PCT/US 13/32694

International application No.

PCT/US 13/32694

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/14 (2013.01)

USPC - 530/380; 530/385; 424/93.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 530/380

IPC: A61K 35/14 (2013.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 530/385; 424/93.4 (See Search Words Below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATBASE: PGPB, USPT, USOC, EPAB, JPAB

Google: Scholar/Patents: intramolecular crosslink polyoxyalkylene polyoxyethylene oxide hemoglobin p50 enhanced nitrite reductase activity conjugated deoxygenated stroma-free dapsirin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0164915 A1 (WINSLOW et al) 28 July 2005 (28.07.2005) para [0014];[0018];[0020];[0021];[0022];[0059];[0135]-[0147]; Table 1;	1-15
Y	LUI et al. Enhancing Nitrite Reductase Activity of Modified Hemoglobin:Bis-tetramers and Their PEGylated Derivatives, in Biochemistry, 2009, Vol 48, pp 11912-11919. pg 11915, Scheme 1, Col 2, para 1; pg 11916, Table 1;pg 11917, Col 2, para 2 ?R-state?; pg 11918, Col 1, para 1, Figure 5a;	1-15

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 May 2013 (11.05.2013)

Date of mailing of the international search report

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Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

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INTERNATIONAL SEARCH REPORT

PCT/US 13/32694 10.00.2010
International application No.

PCT/US 13/32694

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 16-69
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.



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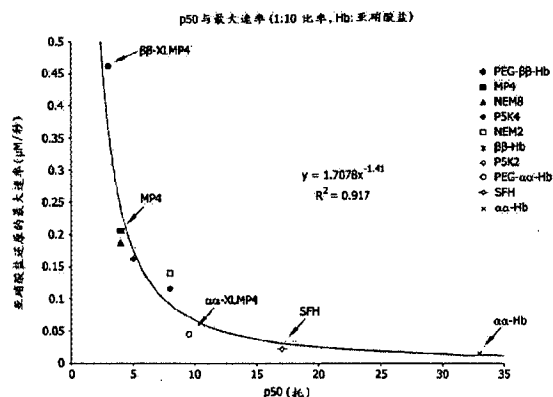
权利要求书5页 说明书17页 附图5页

(54) 发明名称

双阿司匹林交联聚乙二醇化血红蛋白

(57) 摘要

本发明描述了具有高氧亲和力的聚乙二醇化双阿司匹林交联血红蛋白缀合物,其具有增强的亚硝酸盐还原酶活性,用于将氧、一氧化碳、氧化氮或其混合物递送到组织中以治疗各种疾病和病状。



1. 一种 β , β - 分子内交联聚氧化烯氧化物血红蛋白缀合物, 其 P50 如在 37°C 和 pH 7.4 下所测量在约 2 至 5mmHg 范围内, 其中当在 25°C 下完全脱氧时所述血红蛋白缀合物展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为至少 10 倍。

2. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为至少 15 倍。

3. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为至少 20 倍。

4. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为 10 倍至约 25 倍。

5. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为约 15 倍至约 25 倍。

6. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为 10 倍至约 21 倍。

7. 如权利要求 1 所述的血红蛋白缀合物, 其中所述血红蛋白缀合物当完全脱氧时展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为约 15 倍至约 21 倍。

8. 一种 β , β - 分子内交联聚氧化烯氧化物 (PAO) 血红蛋白缀合物, 其 P50 如在 37°C 和 pH 7.4 下所测量在约 2.0 至 5.0mmHg 范围内, 当在 25°C 下完全脱氧时所述血红蛋白缀合物的最大亚硝酸盐还原酶活性是至少 0.25 μ M/ 秒。

9. 如权利要求 8 所述的血红蛋白缀合物, 其中最大亚硝酸盐还原酶活性是至少约 0.30 μ M/ 秒。

10. 如权利要求 8 所述的血红蛋白缀合物, 其中最大亚硝酸盐还原酶活性是至少约 0.35 μ M/ 秒。

11. 如权利要求 8 所述的血红蛋白缀合物, 其中最大亚硝酸盐还原酶活性是至少约 0.40 μ M/ 秒。

12. 如权利要求 8 所述的血红蛋白缀合物, 其中最大亚硝酸盐还原酶活性在 0.25 至约 0.50 μ M/ 秒范围内。

13. 如权利要求 8 所述的血红蛋白缀合物, 其中最大亚硝酸盐还原酶活性在 0.30 至约 0.47 μ M/ 秒范围内。

14. 如权利要求 1-13 中任一项所述的血红蛋白缀合物, 其中所述血红蛋白缀合至平均约 7 至约 11 个 PAO 分子 / 四聚体。

15. 如权利要求 14 所述的血红蛋白缀合物, 其中所述血红蛋白缀合至平均约 9 至约 10 个 PAO 分子 / 四聚体。

16. 如权利要求 1-15 中任一项所述的血红蛋白缀合物, 其中当所述血红蛋白呈氧合状态时所述聚环氧烷经由硫醇反应性部分共价连接至血红蛋白分子上暴露的氨基酸侧链的

硫醇基。

17. 如权利要求 16 所述的血红蛋白缀合物,其中所述聚环氧烷通过由亚烷基或亚苯基组成的接头键联至硫醇反应性部分。

18. 如权利要求 1-17 中任一项所述的血红蛋白缀合物,其中所述血红蛋白是在所述血红蛋白分子的两个 β 82 赖氨酸残基处双(2,5-二溴水杨基)富马酸酯交联的。

19. 如权利要求 1-18 中任一项所述的血红蛋白缀合物,其中胶体渗透压是至少 75mmHg。

20. 如权利要求 1-19 中任一项所述的血红蛋白缀合物,其中 P50 是约 2 至约 4mmHg。

21. 如权利要求 20 所述的血红蛋白缀合物,其中 P50 是约 3mmHg。

22. 如权利要求 1-21 中任一项所述的血红蛋白缀合物,其中所述血红蛋白缀合物与氧配位。

23. 如权利要求 1-22 中任一项所述的血红蛋白缀合物,其中所述血红蛋白缀合物与一氧化碳配位。

24. 如权利要求 1-23 中任一项所述的血红蛋白缀合物,其中所述血红蛋白缀合物与氧化氮配位。

25. 如权利要求 1-24 中任一项所述的血红蛋白缀合物,其中所述血红蛋白缀合物是脱氧的。

26. 如权利要求 1-25 中任一项所述的血红蛋白缀合物,其中所述 PAO 是聚乙二醇(PEG)。

27. 如权利要求 26 所述的血红蛋白缀合物,其中所述 PEG 的平均分子量是约 2,000 至约 20,000 道尔顿。

28. 如权利要求 26 所述的血红蛋白缀合物,其中所述 PEG 的平均分子量是约 3,000 至约 10,000 道尔顿。

29. 如权利要求 26 所述的血红蛋白缀合物,其中所述 PEG 的平均分子量是约 4,000 至约 6,000 道尔顿。

30. 如权利要求 29 所述的血红蛋白缀合物,其中所述 PEG 的平均分子量是约 5,000 道尔顿。

31. 如权利要求 26-30 中任一项所述的血红蛋白缀合物,其中所述 PEG 是马来酰亚胺-PEG。

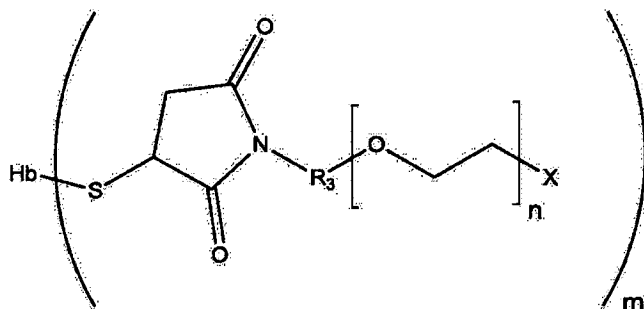
32. 如权利要求 31 所述的血红蛋白缀合物,其中所述马来酰亚胺经由亚烷基或亚苯基接头键联至所述 PEG。

33. 如权利要求 32 所述的血红蛋白缀合物,其中所述亚烷基接头是亚乙基接头。

34. 如权利要求 31-33 中任一项所述的血红蛋白缀合物,其中所述马来酰亚胺-PEG 是缀合至所述血红蛋白的硫醇部分,所述硫醇部分选自所述血红蛋白的半胱氨酸残基的固有硫醇部分、所述血红蛋白的硫醇化赖氨酸残基的硫醇部分或其组合。

35. 如权利要求 1-34 中任一项所述的血红蛋白缀合物,其中 N-乙基马来酰亚胺缀合至所述血红蛋白的 β 93 半胱氨酸残基。

36. 如权利要求 31-34 中任一项所述的血红蛋白缀合物,其中所述马来酰亚胺-PEG 具有以下结构



其中：

Hb 是血红蛋白，

S 是所述血红蛋白的硫醇，

R₃ 是亚烷基或亚苯基，

X 是末端基团，

m 是缀合至所述血红蛋白的马来酰亚胺基活化的 PEG 聚合物的平均数目，并且

n 是平均分子量为约 2,000 至约 20,000 道尔顿的 PEG 的氧化乙烯单元的平均数目。

37. 如权利要求 36 所述的血红蛋白缀合物，其中 R₃ 是亚乙基。

38. 如权利要求 36 或 37 所述的血红蛋白缀合物，其中 X 是甲氧基或羧基。

39. 如权利要求 38 所述的血红蛋白缀合物，其中 X 是甲氧基。

40. 如权利要求 36-39 中任一项所述的血红蛋白缀合物，其中 n 是约 3,000 至约 10,000 道尔顿。

41. 如权利要求 36-39 中任一项所述的血红蛋白缀合物，其中 n 是约 4,000 至约 6,000 道尔顿。

42. 如权利要求 36-39 中任一项所述的血红蛋白缀合物，其中 n 是约 5,000 道尔顿。

43. 如权利要求 36-42 中任一项所述的血红蛋白缀合物，其中 m 是约 7 至约 11。

44. 如权利要求 36-42 中任一项所述的血红蛋白缀合物，其中 m 是约 9 或约 10。

45. 一种药物组合物，其包含如权利要求 1-44 中任一项所述的 β，β-分子内交联聚氧化烯氧化物血红蛋白缀合物和药学上可接受的载体。

46. 如权利要求 45 所述的药物组合物，其中所述组合物在全血下处于正常胶体渗透压。

47. 如权利要求 45 或 46 所述的药物组合物，其中所述组合物与血浆相比处于高胶体渗透压。

48. 如权利要求 45-47 中任一项所述的药物组合物，其用于治疗急性肝衰竭、β 地中海贫血、烧伤、慢性严重肢体缺血、二氧化碳或氰化物中毒、慢性阻塞性肺病 (COPD)、充血性心脏衰竭、缺氧、疟疾、器官缺血、周围性血管疾病、卟啉症、妊娠中的先兆子痫、败血病、镰状细胞疾病、视网膜病、眼内病状、睾丸扭转、外伤、休克、外伤性脑损伤、溃疡、血管痉挛或其组合。

49. 如权利要求 48 所述的药物组合物，其中所述器官缺血包括急性肠缺血（扭转）、急性肠缺血（栓塞）、心源性休克、急性血管器官缺血、中风、心肌梗塞或严重心脏缺血。

50. 如权利要求 45-47 中任一项所述的药物组合物，其用于治疗非外伤性出血性休克、

入院前外伤、外伤性出血性休克、急性肺损伤、成人呼吸窘迫综合症、外伤性脑损伤、中风、实性肿瘤癌症、器官退化（离体）、器官退化（在接受者体内）、严重败血病、败血性休克、心肌梗塞、心脏缺血、心源性休克、急性心脏衰竭、肺栓塞或其组合。

51. 如权利要求 45-47 中任一项所述的药物组合物,其用作血管成形术的辅助物、作为整形外科的辅助物或作为植入心室辅助装置中的辅助物;作为血液替代物、心脏保护剂、冷冻保护剂、血液透析辅助物、肿瘤学试剂、器官防腐剂、性能增强剂、手术辅助剂或伤口愈合剂;用于成像;用于改善肺功能;或其组合。

52. 如权利要求中 45-47 任一项所述的药物组合物,其用于对由以下原因造成的失血进行兽医学治疗:损伤、溶血性贫血、感染性贫血、细菌感染、因子 IV 破碎、脾功能亢进和脾肿大、家禽的出血性综合症、发育不全性贫血、再生障碍性贫血、特发性免疫溶血性病状、铁缺乏、同族免疫溶血性贫血、微血管病性溶血性贫血、寄生或手术麻醉诱导的脑损伤。

53. 一种治疗方法,其包括向有需要的受试者施用如权利要求 1-44 中任一项所述的血红蛋白缀合物或如权利要求 45-47 中任一项所述的药物组合物。

54. 如权利要求 53 所述的治疗方法,其中所述受试者是动物。

55. 如权利要求 53 所述的治疗方法,其中所述受试者是人类。

56. 如权利要求 53-55 中任一项所述的治疗方法,其中所述方法是用于治疗以下疾病的方法:急性肝衰竭、 β 地中海贫血、烧伤、慢性严重肢体缺血、二氧化碳或氰化物中毒、慢性阻塞性肺病 (COPD)、充血性心脏衰竭、缺氧、疟疾、器官缺血、周围性血管疾病、卟啉症、妊娠先兆子痫、败血病、镰状细胞疾病、视网膜病、眼内病状、睾丸扭转、外伤、休克、外伤性脑损伤、溃疡、血管痉挛或其组合。

57. 如权利要求 56 所述的治疗方法,其中所述器官缺血包括急性肠缺血（扭转）、急性肠缺血（栓塞）、心源性休克、急性血管器官缺血、中风、心肌梗塞或严重心脏缺血。

58. 如权利要求 53-55 中任一项所述的治疗方法,其中所述方法是用于治疗以下疾病的方法:非外伤性出血性休克、入院前外伤、外伤性出血性休克、急性肺损伤、成人呼吸窘迫综合症、外伤性脑损伤、中风、实性肿瘤癌症、器官退化（离体）、器官退化（在接受者中）、严重败血病、败血性休克、心肌梗塞、心脏缺血、心源性休克、急性心脏衰竭、肺栓塞或其组合。

59. 如权利要求 53-55 中任一项所述的治疗方法,其中所述血红蛋白四聚体或药物组合物是作为血管成形术的辅助物、作为整形外科的辅助物或作为植入心室辅助装置中的辅助物;作为血液替代物、心脏保护剂、冷冻保护剂、血液透析辅助物、肿瘤学试剂、器官防腐剂、性能增强剂、手术辅助剂或伤口愈合剂;用于成像;用于改善肺功能;或其组合形式施用。

60. 如权利要求 53-55 中任一项所述的治疗方法,其中所述血红蛋白四聚体或药物组合物是作为血管成形术的辅助物、胸主动脉修复的辅助物、心肺分流术的辅助物或心肺分流术的预充溶液施用。

61. 如权利要求 53 或 54 所述的治疗方法,其中所述受试者是非人类动物并且所述方法是用于因以下原因造成的失血的兽医学治疗的方法:损伤、溶血性贫血、感染性贫血、细菌感染、因子 IV 破碎、脾功能亢进和脾肿大、家禽出血性综合症、发育不全性贫血、再生障碍性贫血、特发性免疫溶血病状、铁缺乏、同族免疫溶血性贫血、微血管病性溶血性贫血、寄生

或手术麻醉诱导的脑损伤。

62. 一种将氧、氧化氮、一氧化碳或其混合物递送到组织中并且在微血管系统中将亚硝酸盐还原为氧化氮 (NO) 的方法,所述方法包括向有需要的受试者施用如权利要求 1-44 中任一项所述的 β , β - 分子内交联聚氧化烯氧化物血红蛋白缀合物或如权利要求 45-60 中任一项所述的药物组合物,其中在施用之后,在所述微血管系统中所述血红蛋白中未配位的血红素将亚硝酸盐转化为氧化氮。

63. 一种制备如权利要求 1-44 中任一项所述的 β , β - 分子内交联聚氧化烯氧化物血红蛋白缀合物的方法,所述方法包括以下步骤:

将 β , β - 分子内交联血红蛋白与 2- 亚氨基硫杂环戊烷 (2-IT) 于水性稀释剂中混合以形成硫醇化血红蛋白;以及

添加 PAO 到于所述水性稀释剂中的所述硫醇化血红蛋白中以形成所述 β , β - 分子内交联聚氧化烯氧化物血红蛋白缀合物。

64. 如权利要求 63 所述的方法,其中所述 2- 亚氨基硫杂环戊烷以相对于蛋白质浓度约 8 倍与约 25 倍摩尔过量之间的浓度存在。

65. 如权利要求 64 所述的方法,其中所述 2- 亚氨基硫杂环戊烷以相对于血红蛋白浓度约 15 倍摩尔过量的浓度存在。

66. 如权利要求 63-65 中任一项所述的方法,其中 PAO- 马来酰亚胺以相对于蛋白质浓度约 10 倍与约 40 倍摩尔过量之间的浓度存在。

67. 如权利要求 66 所述的方法,其中所述 PAO- 马来酰亚胺以相对于血红蛋白浓度约 28 倍摩尔过量的浓度存在。

68. 如权利要求 63-67 中任一项所述的方法,其中所述硫醇化步骤是在约 7 至约 9 的 pH 值下进行。

69. 如权利要求 63-68 中任一项所述的方法,其中添加所述 PAO- 马来酰亚胺到所述硫醇化蛋白质中以形成 PAO- 马来酰亚胺缀合蛋白质的步骤是在约 7 与约 9 之间的 pH 值下进行。

双阿司匹林交联聚乙二醇化血红蛋白

发明领域

[0001] 本发明大体上涉及用于在微血管系统中与天然血红蛋白相比以提高的速率将亚硝酸盐还原为氧化氮的交联聚乙二醇化血红蛋白和血红蛋白组合物。具体地说,本发明是针对使用具有提高的亚硝酸盐还原酶活性并且还可以将氧、一氧化碳、氧化氮或其混合物递送到组织中的高氧亲和力分子内交联聚乙二醇化血红蛋白缀合物。

[0002] 发明背景

[0003] 基于血红蛋白的氧载体 (“HBOC”) 一直被与血管收缩相联系,所述血管收缩已被归因于血红素对氧化氮 (NO) 的清除作用。适合作为氧治疗剂的氧载体 (有时称为“携带氧的血浆容量扩张剂”) (诸如稳定化血红蛋白 (Hb)) 已显示具有有限的功效,因为其清除氧化氮,从而引起血管收缩和高血压。这些携带氧的解决方案引起血管收缩的倾向可在动物和人类中显现为高血压。尽管 HBOC 的血管收缩作用的潜在机制尚未充分了解,但已表明血红素铁可快速并且不可逆地与内源性 NO (一种强大的血管扩张剂) 组合,从而引起血管收缩。

[0004] 部分因为这些血管收缩作用,迄今为止尚无氧载体作为氧治疗剂 (OTA) 完全成功,不过包含修饰型无细胞 Hb 的产品已成为最有前景的产品。美国军队研发了用双二溴水杨基-富马酸酯的 α 链间交联的人 Hb ($\alpha\alpha$ Hb) 作为原型红细胞替代物,但在其展现肺及全身血管阻力剧烈增加之后被放弃 (Hess, J. 等, 1991, Blood 78 :356A)。此产品的商业形式在令人失望的 III 期临床试验之后也被放弃 (Winslow, R. M., 2000, Vox Sang 79 :1-20)。

[0005] 已提出两种分子途径,试图克服 Hb 的 NO 结合活性。第一种途径使用远端血红素口袋的定位诱变,以求形成 NO 结合亲和力降低的重组血红蛋白 (Eich, R. F. 等, 1996, 35 : 6976-83)。第二种途径使用化学修饰途径,其中通过寡聚化增加 Hb 的大小,以求降低或有可能完全抑制 Hb 从血管间隔中溢出进入胞间隙 (Hess, J. R. 等, 1978, J. Appl. Physiol. 74 : 1769-78 ; Muldoon, S. M. 等, 1996, J. Lab. Clin. Med. 128 :579-83 ; Macdonald, V. W. 等, 1994, Biotechnology 22 :565-75 ; Furchgott, R., 1984, Ann. Rev. Pharmacol. 24 :175-97 ; 以及 Kilbourne, R. 等, 1994, Biochem. Biophys. Res. Commun. 199 :155-62)。

[0006] 事实上,已产生对 NO 的关联结合速率降低的重组 Hb,其在最大负荷大鼠实验中更少产生高血压 (Doherty, D. H. 等 1998, Nature Biotechnology 16 :672-676 以及 Lemon, D. D. 等 1996, Biotech 24 :378)。然而,研究表明 NO 结合可能不是 Hb 的血管活性的唯一解释。已发现某些大 Hb 分子 (诸如用聚乙二醇 (PEG) 修饰的大 Hb 分子) 实际上无血管收缩,尽管其 NO 缔合速率与严重高血压 $\alpha\alpha$ Hb 的 NO 缔合速率目同 (Rohlf, R. J. 等 1998, J Biol. Chem. 273 :12128-12134)。此外,发现当在出血之前作为交换输血提供时 PEG-Hb 预防出血结果格外有效 (Winslow, R. M. 等 1998, J. Appl. Physiol. 85 :993-1003)。

[0007] PEG 缀合至 Hb 使其抗原性降低并且延长其循环半衰期。然而,已报导 PEG 缀合反应引起 Hb 四聚体解离成 $\alpha\beta$ -二聚体亚基,从而在接受低于 40,000 道尔顿 (“Da”) 的 Hb 单体单元的 PEG-缀合物的交换输血大鼠中引起严重的血红蛋白尿 (Iwashita 和 Ajisaka Organ-Directed Toxicity: Chem. Indicies Mech., Proc. Symp., Brown 等 1981,

编著 Pergamon, Oxford, England 第 97-101 页)。分子量大于 84,000 道尔顿的聚环氧乙烷 (“PAO”) 缀合 Hb 是由 Enzon, Inc. (美国专利号 5,650,388) 制备,其携带约 10 个拷贝的在 α 和 ϵ 氨基处键联至 Hb 的 PEG-5,000 链。此取代度描述为避免在哺乳动物中与血红蛋白尿有关的临床上显著的肾毒性。然而,缀合反应产生异质缀合群体并且含有其它不需要的反应物,其必须通过柱色谱法去除。

[0008] PEG 缀合典型地通过用生物分子表面上的官能团活化 PEG 部分的反应来进行。最常见的官能团是赖氨酸的氨基、组氨酸残基的咪唑基以及蛋白质的 N 端;半胱氨酸残基的硫醇基;以及丝氨酸、苏氨酸和酪氨酸残基的羟基以及蛋白质的 C 端。通常通过将羟基端转化成能够在温和水性环境中与这些官能团反应的反应性部分来活化 PEG。用于缀合治疗性生物药物的最常见单官能 PEG 之一是甲氧基-PEG (“mPEG-OH”),其仅具有一个官能团(即羟基),因此使与双官能 PEG 有关的交联和聚集问题减到最少。然而,mPEG-OH 往往被高分子量双官能 PEG(即“PEG 二醇”)污染,归因于其生产过程其可在高达 10 至 15% 范围内(Dust J.M. 等 1990,Macromolecule 23:3742-3746)。此双官能 PEG 二醇的大小是所需单官能 PEG 的大致两倍。随着 PEG 的分子量增加污染问题进一步加重。mPEG-OH 的纯度对于聚乙二醇化生物治疗剂的产生尤其关键,因为 FDA 要求最终药物产品的生产过程和质量的高水平的再现性。

[0009] Hb 已以氧合与脱氧状态两者缀合至 PAO。美国专利号 6,844,317 描述了通过在缀合之前将 Hb 与大气平衡来以氧合或“R”态对 Hb 进行缀合,以提高所得 PEG-Hb 缀合物的氧亲和力。其它描述了在缀合之前用于减小氧亲和力并且增加结构稳定性的脱氧步骤,从而使 Hb 能够承受化学修饰、透滤和/或无菌过滤以及巴氏灭菌法的物理应力(美国专利号 5,234,903)。关于 Hb 的分子内交联,表明可能需要在修饰之前使 Hb 脱氧以使 α 链的赖氨酸 99 暴露于交联试剂(美国专利号 5,234,903)。

[0010] Acharya 等研究了在与 PEG 缀合之前用 2-亚氨基硫杂环戊烷进行 Hb 硫醇化的动力学(美国专利号 7,501,499)。观察到使亚氨基硫杂环戊烷的浓度从 10 倍(其引入平均五个非固有硫醇/四聚体)增加到 30 倍使 Hb 上非固有硫醇的数目几乎加倍。然而,PEG 缀合之后所见的尺寸增加仅是微小的,甚至在硫醇数目加倍的情况下也是如此。这表明在 20 倍摩尔过量的马来酰亚胺基 PEG-5000 存在下的缀合反应以更少反应性硫醇覆盖 Hb 的表面,从而产生空间干扰,其抵抗更多反应性硫醇对 Hb 的进一步修饰。因此,为对修饰型 Hb 实现所要程度的缀合(即 6 ± 1 PEG/Hb 分子),Acharya 等用 8-15 倍摩尔过量的亚氨基硫杂环戊烷对 Hb 进行硫醇化,并且接着使硫醇化 Hb 与 16-30 倍摩尔过量的马来酰亚胺基 PEG-5000 反应。然而,在大规模生产中这些高摩尔过量反应物浓度使制备 HBOC 的成本显著增加并且使最终产品的异质性增加。此外,这种高摩尔过量的马来酰亚胺基 PEG-5000 还因产生更大数目的不需要的副反应物而产生更异质产品。

[0011] 在前述研究中,观察到表面修饰血红蛋白的分子大小必须足够大以避免被肾清除并且实现所要循环半衰期。Blumenstein, J. 等确定这可以 84,000 道尔顿 (“Da”) 或大于 84,000 道尔顿的分子量实现 (“Blood Substitutes and Plasma Expanders,” Alan R. Liss, 编著, New York, N. Y., 第 205-212 页 (1978))。在该研究中,作者将具有不同分子量的右旋糖酐缀合至 Hb。他们报导 Hb (分子量是 64,000Da) 与右旋糖酐 (分子量是 20,000Da) 的缀合物从循环中缓慢清除并且极少穿过肾。另外,观察到使分子量增加超过 84,000Da 不

显著改变这些清除曲线。分子内交联使四聚血红蛋白单元的亚基以化学方式结合在一起以防止形成通过肾过早排出的二聚体。(参见例如美国专利号 5, 296, 465)。

[0012] 亚硝酸盐与氧合血红蛋白反应以形成高铁血红蛋白, 并且与脱氧血红蛋白反应以形成高铁血红蛋白和氧化氮。在血红蛋白存在下亚硝酸盐的血管舒张作用不同于传统 NO 供体并且可部分通过血红蛋白的亚硝酸盐还原酶活性来解释。参见 Crawford 等 2006 Blood 107 :566-574 ;Huang 等 2005 J Biol Chem 280 :31126-31131 ;Huang 等 2005 J Clin Invest 115 :2099-2107。研究已显示亚硝酸盐仅通过与在血红蛋白四聚体情况下的脱氧血红素反应转化成 NO (Cosby, K. 等 2003, Nat. Med. 9 :1498), 并且另外, 在蛋白质血红素呈松弛或 R 态构象的情况下产生亚硝酸盐的更快还原。相信血红蛋白的这种亚硝酸盐还原酶活性是在变构控制下的并且当脱氧血红素呈 R 态构象时以最大速率产生 NO。R 态稳定化效应可例如通过在 β Cys93 位点处进行修饰来产生, 诸如马来酰亚胺 PEG 缀合产生增加的亚硝酸盐还原酶活性。另外, 已证实当无细胞 Hb 引起血管收缩并且减少灌注时, MalPEG-Hb 维持血液流动和微血管灌注压力, 这被认为与缺乏血管收缩有关 (Tsai, A. G. 等 2006, Blood 108 :3603)。其它研究也表明用 PEG 的多个链修饰无细胞血红蛋白衍生物可抑制血管活性。使用具有五至六个 PEG 链的 R 态稳定化 Hb 的实验展示与天然 Hb 相比快为 10 倍的亚硝酸盐还原酶活性 (Lui, F. E. 等 2008, Biochemistry 47 (40), 10773-10780)。然而, 推断出在可达的赖氨酸残基处的任何进一步 PEG 缀合不促成增加的亚硝酸盐还原酶活性。

[0013] 因此, 需要一种通过使用与现有 Hb 相比具有增加的亚硝酸还原酶性质的高氧亲和血红蛋白将氧、一氧化碳、氧化氮或其混合物递送到组织中并且在微血管系统中以提高的速率将亚硝酸盐还原为氧化氮的方法。

[0014] 发明概述

[0015] 在一个方面中, 本发明是针对一种 β, β -分子内交联聚氧化烯氧化物 (PAO) 血红蛋白缀合物, 其 P50 如在 37°C 和 pH 7.4 下所测量在约 2 至 5 mmHg 范围内。当在 25°C 下完全脱氧时血红蛋白缀合物展现最大亚硝酸盐还原酶活性, 其比脱氧无基质血红蛋白在相同条件下测量时大为至少 10 倍。

[0016] 本发明的另一个方面是针对一种 β, β -分子内交联 PAO 血红蛋白缀合物, 其 P50 如在 37°C 和 pH 7.4 下所测量在约 2.0 到 5.0 mmHg 范围内, 其中当在 25°C 下完全脱氧时血红蛋白缀合物的最大亚硝酸盐还原酶活性是至少 0.25 μ M/秒。

[0017] 本发明的另一个方面是针对包含 β, β -分子内交联聚氧化烯氧化物血红蛋白缀合物和药学上可接受的载体的药物组合物。所述组合物可用于治疗急性肝衰竭、 β 地中海贫血、烧伤、慢性严重肢体缺血、二氧化碳或氰化物中毒、慢性阻塞性肺病 (COPD)、充血性心脏衰竭、缺氧、疟疾、器官缺血、周围性血管疾病、哮喘、妊娠先兆子痫、败血症、镰状细胞疾病、视网膜病、眼内病状、睾丸扭转、外伤、休克、外伤性脑损伤、溃疡、血管痉挛或其组合。所述药物组合物还可以用作血管成形术的辅助物、作为整形外科的辅助物或作为植入心室辅助装置中的辅助物; 作为血液替代物、心脏保护剂、冷冻保护剂、血液透析辅助物、肿瘤学试剂、器官防腐剂、性能增强剂、手术辅助剂或伤口愈合剂; 用于成像; 用于改善肺功能; 或其组合。所述组合物还可以用于兽医学治疗由以下原因造成的失血: 损伤、溶血性贫血、感染性贫血、细菌感染、因子 IV 破碎、脾功能亢进和脾肿大、家禽的出血性综合症、发育不全

性贫血、再生障碍性贫血、特发性免疫溶血性病状、铁缺乏、同族免疫溶血性贫血、微血管病性溶血性贫血、寄生虫病或手术麻醉诱导的脑损伤或其组合。

[0018] 本发明的另一个方面是针对一种治疗方法,其包括向有需要的受试者施用这种血红蛋白缀合物或药物组合物。所述方法用于治疗上文所描述的病状中的任何一种或更多种。

[0019] 本发明的另一个方面是针对一种将氧、氧化氮、一氧化碳或其混合物递送到组织中并且在微血管系统中将亚硝酸盐还原为氧化氮(NO)的方法。所述方法包括向有需要的受试者施用 β , β -分子内交联聚氧化烯氧化物血红蛋白缀合物或药物组合物,其中在施用之后,血红蛋白变得未配位并且在微血管系统中将亚硝酸盐转化成氧化氮。

[0020] 本发明的另一个方面是针对一种制备如上文所描述的 β , β -分子内交联聚氧化烯氧化物血红蛋白缀合物的方法。所述方法包括以下步骤:将 β , β -分子内交联血红蛋白与 2-亚氨基硫杂环戊烷(2-IT)于水性稀释剂中混合以形成硫醇化血红蛋白;以及添加 PAO 到于水性稀释剂中的硫醇化血红蛋白中以形成 β , β -分子内交联聚氧化烯氧化物血红蛋白缀合物。

[0021] 其它目标和特征将部分显而易见并且部分在下文中指出。

[0022] 附图简述

[0023] 图 1 是无基质 Hb(SFH)(---)和 $\beta\beta$ -Hb(-)分别在非解离条件(PBS)下的尺寸排阻色谱,表明对 $\beta\beta$ -Hb 的 DBBF 交联的证实。

[0024] 图 2 是无基质 Hb(SFH)(---)和 $\beta\beta$ -Hb(-)分别在解离条件(PBS)下的尺寸排阻色谱,表明对 $\beta\beta$ -Hb 的 DBBF 交联的证实。

[0025] 图 3 是表明对 β -亚基的 DBBF 交联的证实的逆相高效液相色谱,其中分别为(SFH)(---)和 $\beta\beta$ -Hb(-)。

[0026] 图 4 是 PEG- $\beta\beta$ -Hb(---)和 $\beta\beta$ -Hb(-)分别在非解离条件下的尺寸排阻色谱,表明 PEG- $\beta\beta$ -Hb 的聚乙二醇化。

[0027] 图 5 是各种血红蛋白的亚硝酸盐还原性质的图形化描述。

[0028] 相应参考字符指示在图式通篇中的对应部分。

[0029] 优选实施方案的描述

[0030] 已发现具有高氧亲和力 β , β -分子内交联聚氧化烯氧化物血红蛋白缀合物,其与常规血红蛋白治疗剂相比具有增强的治疗性质。这些血红蛋白缀合物当脱氧时展现最大亚硝酸盐还原酶活性,其比脱氧无基质血红蛋白在相同条件下测量时大为至少 10 倍。血红蛋白缀合物在 25°C 下呈完全脱氧形式时的最大亚硝酸盐还原酶活性是至少 0.25 μ M/s。血红蛋白可以配位形式递送以将氧、一氧化碳或氧化氮递送到受试者中并且一旦未配位就在微血管系统中将亚硝酸盐转化成氧化氮。

[0031] 在不受任何特定理论限制的情况下,人们相信,通过使血红蛋白交联成氧合 R 态构象,其使非可解离四聚体中的脱氧血红素被锁在非挠性的 R 态中,这促进那些脱氧血红素处亚硝酸盐的还原。本发明的血红蛋白缀合物的出乎意料地大得多的亚硝酸盐还原酶活性提供更多 NO,其逃脱血红素捕获并且从 Hb 释放到组织中,从而与其它血红蛋白治疗剂相比引起更大血管舒张效应。这种现象的产生被认为是因为高亲和力 R 态结构的交联促进血红素口袋内的性质以增强亚硝酸盐的还原。在没有交联的情况下,存在更多血红蛋白变构

构象变化,在 T 态和 R 态血红素构象结构之间交替变化。甚至在四聚体内的脱氧血红素口袋中,交联的完全稳定化 R 态结构也被认为使血红素口袋内亚硝酸盐还原成 NO 增强。

[0032] 本发明是针对一种 β, β -分子内交联聚氧化烯氧化物 (PAO) 血红蛋白缀合物,其 P50 如在 37°C 和 pH 7.4 下所测量在约 2 至 5mmHg 范围内。当在 25°C 下完全脱氧时血红蛋白缀合物展现最大亚硝酸盐还原酶活性,其比脱氧无基质血红蛋白在相同条件下测量时大为至少 10 倍。

[0033] 当在 25°C 下完全脱氧时血红蛋白缀合物可展现最大亚硝酸盐还原酶活性,其比脱氧无基质血红蛋白在相同条件下测量时大为至少 15 倍或至少 20 倍。优选地,当在 25°C 下完全脱氧时血红蛋白缀合物展现最大亚硝酸盐还原酶活性,其比脱氧无基质血红蛋白在相同条件下测量时大为 10 倍至约 25 倍,更优选地比脱氧无基质血红蛋白在相同条件下测量时大为约 15 倍至约 25 倍,大为 10 倍至约 21 倍,或大为约 15 倍至约 21 倍。

[0034] 本发明的 β, β -分子内交联 PAO 血红蛋白缀合物的 P50 如在 37°C 和 pH 7.4 下所测量可在约 2.0 至 5.0mmHg 范围内并且当在 25°C 下完全脱氧时最大亚硝酸盐还原酶活性是至少 0.25 $\mu\text{M}/\text{秒}$ 。优选地,当在 25°C 下完全脱氧时血红蛋白缀合物的最大亚硝酸盐还原酶活性是至少 0.30 $\mu\text{M}/\text{秒}$,并且更优选地是 0.35、0.40 或 0.45 $\mu\text{M}/\text{秒}$ 。血红蛋白缀合物的最大亚硝酸盐还原酶活性可在 0.25 至约 0.50 $\mu\text{M}/\text{秒}$ 或约 0.30 至约 0.47 $\mu\text{M}/\text{秒}$ 范围内。

[0035] 本发明中可使用各种 Hb。Hb 可获自动物来源,诸如人、牛、猪或马血红蛋白。人 Hb 是优选的。Hb 可获自天然来源或可通过已知的重组方法产生。

[0036] 本发明的血红蛋白缀合物具有大于无基质血红蛋白的高氧亲和力。这意味着血红蛋白的 P50 如在 37°C 和 pH 7.4 下所测量将小于 15mmHg,优选地是约 2 至约 5mmHg,并且最优选地是约 2 至约 4mmHg,或是 3mmHg。

[0037] 血红蛋白缀合物的胶体渗透压 (COP) 可以是至少约 50mmHg,优选地至少约 60、65、70 或 75mmHg。

[0038] 血红蛋白是 β, β -分子内交联的以防止解离成二聚体并且避免被肾清除,从而延长循环半衰期。双 (2,5-二溴水杨基) 富马酸酯 (DBBF) 交联剂用于交联血红蛋白分子的两个 β 82 赖氨酸残基。可使用已知 DBBF 交联方法中的任一种,诸如由 Walder 等, Biochemistry, 1979;18(20):4265-70 所描述的方法。

[0039] 用于缀合本发明的血红蛋白的聚环氧乙烷包括但不限于聚环氧乙烷、聚环氧丙烷以及聚环氧乙烷/聚环氧丙烷共聚物。PAO 的分子量是约 2,000 至约 20,000 道尔顿,优选地约 3,000 至约 10,000 道尔顿,更优选地 4,000 至约 6,000 道尔顿,并且最优选地约 5,000 道尔顿。目前最常见的用于修饰 Hb 的表面的 PAO 是 PEG,因为其药物可接受性和商业可利用性。基于分子内环氧乙烷的重复亚单元 (即 $-\text{CH}_2\text{CH}_2\text{O}-$) 的数目,PEG 可以各种分子量获得,以基于缀合至 Hb 的 PEG 分子的数目和大小实现所要分子量。

[0040] 血红蛋白可以平均约 7 至约 11 个 PAO 分子/血红蛋白四聚体进行缀合。优选地,血红蛋白缀合到平均约 9 至约 10 个 PAO 分子/四聚体。

[0041] PAO 聚合物的末端基团中的一者或两者转化成反应性官能团 (“活化”)。举例来说,PEG-OH 已用于制备 PEG-卤化物、甲磺酸酯或甲苯磺酸酯,其接着通过与氨水 (Zalipsky, S. 等, 1983, Eur. Polym. J. 19:1177-1183)、叠氮化钠或钾或邻苯二甲酰亚胺钾进行亲核置换反应转化成 PEG-胺 (“PEG-NH₂”)。活化的 PEG 可接着通过 PEG 胺基团 (“-NH₂”) 与血

红素蛋白的羧基 (“-COOH”) 的相互作用缀合至血红素蛋白。

[0042] 除用胺基团官能化 PEG 并且将其转化成马来酰亚胺基之外, 用其活化的 PEG 已知用于本领域中。举例来说, PEG 可用 (仅举几个例子) 碳酸对硝基苯酯、醛、氨基丙基、氨基乙基、硫醇、氨基氧基、酰肼以及碘乙酰胺活化。这种官能性 PEG 可使用已知方法缀合至蛋白质的表面氨基酸侧链。

[0043] PEG-NH₂ 可进一步官能化以与除羧基以外的基团缀合。举例来说, 美国专利号 6,828,401 公开了 PEG-NH₂ 与马来酰亚胺反应以形成 mPEG-马来酰亚胺。在此反应中, mPEG-OH 与甲苯磺酰化试剂 (对甲苯磺酰氯) 和碱催化剂 (三乙胺) 在有机溶剂 (二氯甲烷) 存在下反应以产生 mPEG-甲苯磺酸酯。mPEG-甲苯磺酸酯接着与 28% 氨水和马来酸酐在 N,N-二甲基乙酰胺 (“DMAc”) 与 N-环己基吡咯烷酮 (“CHP”) 的有机溶剂混合物中反应以产生马来酰胺酸化合物。此化合物接着与五氟苯基三氟乙酸酯在二氯甲烷存在下反应以产生 mPEG-马来酰亚胺。

[0044] 或者, 可通过使 mPEG-OH 与甲苯磺酰化试剂 (对甲苯磺酰氯) 和碱催化剂 (三乙胺) 在有机溶剂 (二氯甲烷) 存在下反应以产生 mPEG-甲苯磺酸酯来制备 mPEG-马来酰亚胺。mPEG-甲苯磺酸酯接着与 28% 氨水反应以制备 mPEG-NH₂。mPEG-NH₂ 接着与 N-甲氧基羰基马来酰亚胺 (MCM) 在饱和碳酸氢钠 (NaHCO₃) 存在下反应以产生 mPEG-马来酰亚胺。

[0045] 人 Hb 中可使用胺反应性化学进行修饰以便缀合至 PAO 的氨基酸残基侧链的非限制性实例呈现于下表 1 中:

[0046] 表 1- 胺反应性化学和潜在修饰位点

[0047]

α链		
残基	位置	与以下物质反应

[0048]

Lys	7、11、16、40、56、60、61、90、99、127以及139	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。
His	20、45、50、58、72、87、112以及122	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。
Val	1	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。
β链		
残基	位置	与以下物质反应
Lys	8、17、59、61、65、66、82、95、120、132以及144	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。
His	2、63、77、92、97、116、117、143以及146	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。
Val	1	琥珀酰亚胺; NPC (碳酸对硝基苯酯); 异氰酸酯; 醛; 异硫氰酸酯; 环氧化物。

[0049] 一种增加 Hb 上的可用缀合位点的数目的方法是引入巯基 (也称为硫醇化), 其倾向于与 MalPEG 比自由胺更具反应性。用于蛋白质硫醇化的各种方法是已知的。在一种方法

中,无蛋白胺与丙酸 3-(2-吡啶基二硫代)琥珀酰亚胺酯反应,随后用二硫苏糖醇(“DTT”)或三(2-羧乙基)膦(“TCEP”)还原。此反应释放 2-吡啶硫酮发色团,其可用于确定硫醇化的程度。胺还可以通过依次与琥珀酰亚胺基乙酰基硫代乙酸酯和 50mM 羟胺或胍在接近中性的 pH 值下反应来间接硫醇化。

[0050] 描述于美国专利号 5,585,484 中的另一种方法在缀合之后维持 Hb 的氨基(α -或 ϵ -)的正电荷。此方法涉及通过 2-IT 对 Hb 的 ϵ -氨基进行酰胺化以将巯基引至蛋白质上。此方法与先前所用的琥珀酰亚胺基化学相比具有至少两个额外的优势:1) 在巯基情况下马来酰亚胺基的高反应性和选择性促成在有限过量的试剂情况下对硫醇的几乎定量的修饰,以及 2) 2-IT 的硫醇基是潜伏性的并且仅因试剂与蛋白质氨基的反应而原位产生。这些优势提供一个额外的益处:其允许 Hb 与用于表面修饰的硫醇化和聚乙二醇化试剂同时孵育。

[0051] 举例来说, Ma1PEG 可通过硫醇化 Hb 的胺以在 Hb 表面上引入硫醇基来缀合至 Hb。Hb 中可用于反应的两个固有硫醇基位于 β Cys93 处,并且 Hb 表面上的外加硫醇基可与马来酰亚胺基 PAO 的马来酰亚胺反应以形成聚乙二醇化 Hb 缀合物。

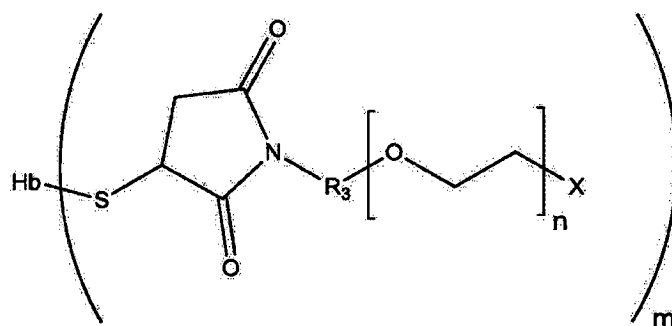
[0052] 当血红蛋白呈氧合状态时,聚环氧烷可经由硫醇反应性部分共价连接至血红蛋白分子上暴露的氨基酸侧链的硫醇基。

[0053] 马来酰亚胺-PEG 可缀合至选自以下的血红蛋白硫醇部分:血红蛋白的半胱氨酸残基的固有硫醇部分、血红蛋白的硫醇化赖氨酸残基的硫醇部分或其组合。

[0054] 马来酰亚胺-PEG 包括用于将马来酰亚胺连接至 PEG 的接头。接头可包括但不限于亚烷基(诸如亚乙基、亚丙基或亚异丙基)、亚苯基、酰胺($-\text{NH}-\text{C}(\text{o})-$)或氨基甲酸苯酯(例如 $-\text{Ph}-\text{NH}-\text{C}(\text{O})-$)。优选地,聚氧化烯通过由亚烷基或亚苯基并且更优选地亚烷基(诸如亚乙基)组成的接头连接到硫醇反应性部分。

[0055] 马来酰亚胺-PEG 可具有以下结构

[0056]



[0057] 其中 Hb 是血红蛋白, S 是血红蛋白的硫醇, R_3 是亚烷基或亚苯基, X 是末端基团, m 是缀合至血红蛋白的马来酰亚胺基活化的 PEG 聚合物的平均数目, 并且 n 是平均分子量是约 2,000 至约 20,000 道尔顿的 PEG 的氧化乙烯单元的平均数目。优选地, R_3 是亚烷基, 诸如亚乙基; X 是羟基、芳氧基(诸如苯甲氧基)或 C_1 - C_{20} 烷氧基, 更优选地是 C_1 - C_{10} 烷氧基, 并且更优选地是 C_1 - C_5 烷氧基, 诸如甲氧基或乙氧基; n 是约 3,000 至约 10,000 道尔顿, 更优选地 4,000 至约 6,000 道尔顿, 并且最优选地约 5,000 道尔顿; 并且 m 是约 7 至约 11, 并且更优选地约 9 或约 10。

[0058] 可使用硫醇反应性化学修饰的氨基酸残基侧链的非限制性实例呈现于下表 2 中:

[0059] 表 2- 硫醇反应性化学和潜在修饰位点

[0060]

α -链		
残基	位置	与以下物质反应
Cys	104	马来酰亚胺; 碘乙酰胺; 邻吡啶基-二硫化物(OPSS); 乙烯基砒。
β -链		
残基	位置	与以下物质反应
Cys	93和112	马来酰亚胺; 碘乙酰胺; 邻吡啶基-二硫化物(OPSS); 乙烯基砒。

[0061] 位于血红蛋白的 β Cys93 残基处的两个固有硫醇可聚乙二醇化或可与 N- 乙基马来酰亚胺反应。这些半胱氨酸残基的此类修饰使 P50 降低并且具有 R 态稳定化效应。

[0062] PAO-Hb 的分子量可通过缀合反应来调节。常规想法表明增加反应物的摩尔比将使结合于 Hb 的 PEG 分子的数目增加。这包括 Hb 的硫醇化过程 (即增加硫醇化剂与 Hb 的摩尔比) 与缀合过程 (即增加硫醇活化的 PEG 与硫醇化 Hb 的摩尔比) 两者。然而, 这些过量摩尔比仅使得 6 ± 1 个 PEG 分子 /Hb 结合 (参见美国专利号 7, 501, 499)。

[0063] 最近已确定使用更低的反应物摩尔比可使更大数目的 PAO 分子结合于 Hb。在硫醇化之前和之后以及缀合之后, Hb 上的可用硫醇基的数目使用二硫吡啶比色分析来确定 (Ampulski, R. S. 等, 1969, Biochem. Biophys. Acta 32 :163-169)。人 Hb 在 β 93 半胱氨酸残基处含有两个固有反应性硫醇基, 这通过二硫吡啶反应得以证实。在用 2-IT 硫醇化 SFH 之后, 反应性硫醇基的数目从两个增加到超过七个。在此实例中, 平均 8 个 PEG 分子结合于 Hb。这是使用在硫醇化反应中相对于 SFH 7.5 倍摩尔过量的 2-IT 以及在缀合反应中相对于硫醇化 Hb 12 倍摩尔过量的 MalPEG 来实现。

[0064] 当血红蛋白呈氧合状态时用聚环氧烷来缀合血红蛋白以增加 Hb-PAO 缀合物的氧亲和力。

[0065] 因此, 本发明的另一个方面是针对一种制造血红蛋白缀合物的方法。所述方法包括以下步骤: 将 β , β - 分子内交联血红蛋白与 2- 亚氨基硫杂环戊烷 (2-IT) 于水性稀释剂中混合以形成硫醇化血红蛋白; 以及添加 PAO 到于水性稀释剂中的硫醇化血红蛋白中以形成 β , β - 分子内交联聚氧化烯氧化物血红蛋白缀合物。

[0066] 在此方法中, 2- 亚氨基硫杂环戊烷以相对于血红蛋白浓度约 8 与约 25 倍摩尔过量之间、优选地约 15 倍摩尔过量的浓度存在。

[0067] 硫醇化可在约 7 至约 9 的 pH 值下进行。

[0068] 缀合可在约 7 至约 9 的 pH 值下进行。

[0069] 在此方法中, PAO- 马来酰亚胺基于 100% 末端活性以相对于血红蛋白浓度约 10 至约 40 倍摩尔过量之间、优选地约 28 倍摩尔过量的浓度存在。

[0070] 本发明的血红蛋白缀合物可呈氧合或脱氧形式, 可与 CO 或 NO 配位, 或可以是包括这四种形式中的两种或更多种的混合物。HbO₂ 是通过使非氧合血红蛋白与空气、纯 O₂ 气体或 O₂/ 氮气混合物平衡来制备。

[0071] 可通过本领域中已知的任何方法来进行脱氧。一个简单方法是使血红蛋白溶液暴露于惰性气体, 诸如氮气、氩气或氦气。为保证脱氧相对均匀, 在此过程中使 Hb 溶液循环。监测脱氧以达到所要水平可通过使用联合血氧计 682 (Instrument Laboratories) 来进行。如果需要部分再氧合, 那么可使脱氧 Hb 暴露于氧气或含有氧气的气体混合物, 诸如空气。

[0072] 用用另一种气体替换分子氧的气体交换可通过气体渗透膜（诸如聚丙烯或乙酸纤维素膜）来实现。参见例如已公布的美国专利申请号 2006/0234915。使用这些膜的市售气体交换装置包括来自 Hoechst-Celanese (Dallas, TX) 的 Celgard™ 聚丙烯微孔空心纤维装置或来自 American Laboratory (East Lyme, CT) 的 Cell-Pharm™ 空心纤维氧合器。在 Hoechst-Celanese Celgard™ 装置中, 通过在以 5-20psi 用氮气吹扫系统的同时使水性 Hb 溶液以 10-100ml/min/ft² 穿过聚丙烯微孔性中空过滤器来使氧合 Hb 脱氧。通常使 Hb 循环约 5 至 30 分钟以实现所要百分比的脱氧 Hb。用于产生脱氧 Hb 的另一种方法包括使 Hb 溶液暴露于化学还原剂, 诸如抗坏血酸钠、连二硫酸钠以及亚硫酸氢钠。通过调节还原剂浓度、反应时间以及温度使 Hb 部分脱氧。或者, 可使用还原剂使 Hb 基本上脱氧, 并且接着可再引入氧气以形成部分脱氧产物。举例来说, 在添加抗氧化剂之前可使 Hb 暴露于 100mM 浓度的亚硫酸氢钠约一小时。

[0073] 可使用任何已知用于形成氧合血红蛋白的方法、简单来说通过用 CO 取代 O₂ 来使 Hb 与 CO 配位。这通常涉及向血红蛋白的溶液中引入 CO 源以使得血红蛋白变得与 CO 而非 O₂ 配位。(K. D. Vandegriff 等, Biochem. J. 382 :183-189(2004))。因为血红蛋白对 CO 具有比对氧气更高的亲和力, 所以不必首先使血红蛋白脱氧。因此, 最方便的形成 CO-Hb 复合物的方式是通过向血红蛋白的溶液中引入 100% 气态 CO。

[0074] HbNO 可通过使脱氧血红蛋白与氧化氮气体反应或通过使 CO-Hb 暴露于 NO 气体以使得 NO 与 CO 交换来制备。HbNO 还可以通过使脱氧血红蛋白与小分子 NO 供体（如 PROLI NONOate™ (即 1-(羟基 -NNO- 氧化偶氮基)-L-脯氨酸二钠盐; Cayman Chemical, Ann Arbor, Michigan) 反应来制备。

[0075] 应注意, NO (自由基) 结合于球蛋白链中的氨基酸侧基的血红蛋白不是如本文所定义的 NO-Hb 复合物, 因为此类化合物不含替代氧作为血红素口袋中的配体的双原子 (非电离) NO。举例来说, 当天然血红蛋白在使得其结合于自由巯基的条件下暴露于 NO 供体时形成亚硝酰基血红蛋白 (美国专利号 6, 627, 738)。此类亚硝酰基血红蛋白仍携带氧, 然而本发明的 NO-Hb 复合物不携带氧。此外, 当通过针对巯基部分的反应 (诸如上文所描述) 形成修饰型血红蛋白时, 这些部分不再可用于 NO 结合。

[0076] 本发明的 PAO-Hb 缀合物可在用于非肠道施用的药学上可接受的载体 (诸如水性稀释剂) 中调配成包含 PAO-Hb 缀合物的药物组合物。载体中 PAO-Hb 缀合物的浓度可根据应用变化。优选地, PAO-Hb 缀合物浓度在约 0.1g/dl 至约 10g/dl、更优选地约 2.0g/dl 至约 8.0g/dl 并且最优选地约 4.0 至约 6.0g/dl 范围内。血红蛋白的适当浓度的选择取决于最终血红蛋白产物的胶体渗透压 (oncotic) 性质。优选地, 本发明的组合物与全血相比可以处于正常胶体渗透压或与血浆相比处于高胶体渗透压。可调节血红蛋白浓度以在各迹象下获得所要胶体渗透压。

[0077] 当组合物调配成非肠道药物时, 溶液通常包含与全血等渗并且维持血红蛋白的可逆的氧、CO 或 NO 携带和递送性质的生理学相容电解质载体。

[0078] 药学上可接受的载体可以是水性稀释剂。水性稀释剂可包括胶体的水溶液或非携氧组分的水溶液, 诸如蛋白质 (诸如白蛋白) 的水溶液、糖蛋白的水溶液、多糖的水溶液或其组合。水性稀释剂可包括水性无细胞溶液。

[0079] 合适的水性稀释剂包括但不限于生理盐水、生理盐水 - 葡萄糖混合物、林格氏溶

液 (Ringer' s solution)、乳酸化林格氏溶液、洛克 - 林格氏溶液 (Locke-Ringer' s solution)、克雷布斯 - 林格氏溶液 (Krebs-Ringer' s solution)、哈特曼氏平衡生理盐水 (Hartmann' s balanced saline)、肝素化柠檬酸钠 - 柠檬酸 - 葡萄糖溶液、乙酸溶液、多电解质溶液 (例如来自 Baxter International, Deerfield, IL 的 Plasma Lyte® 或 Plasma Lyte-A®)、乳糖酸盐溶液以及聚合血浆取代物, 诸如聚环氧乙烷、聚乙烯吡咯烷酮、聚乙烯醇、环氧乙烷 - 丙二醇缩合物或其组合。

[0080] 组合物可另外包含药学上可接受的填料、盐以及本领域中所熟知的其它物质, 其选择取决于剂型、所治疗的病状、根据本领域中的普通熟练技工的判断所要实现的特定目的以及此类添加剂的性质。举例来说, 组合物可包括生理缓冲剂、碳水化合物 (例如葡萄糖、甘露糖醇或山梨糖醇)、醇或聚醇、药学上可接受的盐 (例如氯化钠或氯化钾)、表面活性剂 (例如聚山梨醇酯 80)、抗氧化剂、抗菌剂、胶体渗透压剂 (例如白蛋白或聚乙二醇) 或还原剂 (抗坏血酸、谷胱甘肽或 N- 乙酰半胱氨酸)。

[0081] 药物组合物的粘度是至少约 2 厘泊 (cP)。更具体地说, 粘度在约 2 至约 5cP 并且特别地约 2.5 至约 4.5cP 范围内。

[0082] 为避免施用中的并发症, 药物组合物具有高纯度, 即不含基质、磷脂以及热原质, 内毒素水平如通过 LAL (鲎变形细胞溶解物) 测试所测量不超过 0.25EU/ml, 并且高铁血红蛋白不到 8%。

[0083] 药物组合物可诸如通过皮下、静脉内或肌肉内注射或以大体积非肠道溶液形式非肠道施用。组合物还可以通过灌胃来施用。

[0084] 血红蛋白缀合物作为治疗剂的典型剂量可以是约 1 至约 15,000 毫克血红蛋白 / 千克患者体重。举例来说, 当用作氧治疗剂时, 剂量将介于 100 至 7500mg/kg 患者体重、更优选地 500 至 5000mg/kg 体重并且最优选地 700 至 3000mg/kg 体重之间。因此, 对于人类患者来说典型剂量可以是 1 克至超过 1000 克。应了解含于各剂型的个别剂量中的活性成分的单位含量本身不需要构成有效量, 因为必要的有效量可通过施用许多个别剂量来达到。剂量的选择取决于所使用的剂型、所治疗的病状以及根据本领域技术人员的判断所要实现的特定目的。

[0085] PAO-Hb 缀合物和药物组合物可用于将氧、CO 和 / 或 NO 递送至受试者。将氧、氧化氮、一氧化碳或其混合物递送至组织中并且将亚硝酸盐还原以在微血管系统中进一步产生内源性氧化氮 (NO) 的方法包括向有需要的受试者施用血红蛋白缀合物或组合物, 其中在施用之后, 血红蛋白变得未配位并且在微血管系统中将亚硝酸盐转化成氧化氮。

[0086] 本发明的血红蛋白缀合物和其组合物可用于: 治疗急性肝衰竭、 β 地中海贫血、烧伤、慢性严重肢体缺血、二氧化碳或氰化物中毒、慢性阻塞性肺病 (COPD) (例如急性加重)、充血性心脏衰竭 (例如急性心脏衰竭、慢性心脏衰竭)、缺氧 (例如高海拔使用, 包括用于肺水肿、减压病)、疟疾 (例如脑型疟疾 (恶性阻塞事件)、器官缺血 (例如急性肠缺血 (扭转)、急性肠缺血 (栓塞)、心源性休克、急性血管器官缺血、中风 (CAT 扫描之前)、中风 (CAT 扫描之后)、心肌梗塞 / 严重心脏缺血)、周围性血管疾病、卟啉症、妊娠先兆子痫、败血病、镰状细胞疾病 (例如中风 / 暂时性缺血性发作、脾隔离、肝隔离、异常勃起)、视网膜病 / 眼内病状 (例如视网膜中央动脉阻塞、中央静脉阻塞)、睾丸扭转、外伤 / 休克 (例如外

伤性出血性休克、非外伤性出血性休克、入院前 / 现场使用（军事 / 紧急事件）、外伤性脑损伤 / 爆炸）、溃疡或血管痉挛；用作血管成形术的辅助物、用作整形外科（皮瓣）（例如急性治疗、慢性治疗）或用作植入心室辅助装置中的辅助物；用作血液替代物（例如用于急性失血、耶和华见证人（Jehovah's Witness）、难以交配的患者、稀有血型、镰刀状再生障碍危象、镰刀形红细胞贫血病围手术期管理、急性溶血性贫血（自体免疫）、急性溶血性贫血（毒素）或其它难治性贫血）、心脏保护剂、冷冻保护剂、血液透析辅助物、肿瘤学试剂（例如放射线疗法或化学疗法的辅助物，实性肿瘤）、器官防腐剂（例如离体、在供体内、在接受者内）、性能增强剂（例如平民 / 运动员、军队）手术辅助物（例如心肺分流术（预充）、心肺分流术（调节）、肺缺血、手术前适应、破裂性主动脉瘤、胸主动脉（解剖或动脉瘤）的替换）或伤口愈合剂；用于成像（x 射线或磁共振成像（MRI））；用于改善肺功能（例如急性肺损伤、慢性肺损伤、暂时性病毒性肺炎、新生儿窘迫综合症）；或其组合。此类用途包括向有需要的受试者施用缀合物或组合物。

[0087] 另外，本发明的血红蛋白和组合物可用于治疗非外伤性出血性休克、入院前外伤、外伤性出血性休克、急性肺损伤、成人呼吸窘迫综合症、外伤性脑损伤、中风、实性肿瘤癌症、器官退化（离体）、器官退化（在接受者中）、严重败血病 / 败血性休克、心肌梗塞 / 心脏缺血、心源性休克、急性心脏衰竭、肺栓塞、通过手术治疗的各种病状（例如血管成形术的辅助物、胸廓主动脉修复的辅助物、心肺分流术的辅助物、心肺分流术的预充溶液（priming solution））或其组合。

[0088] 本发明的血红蛋白和组合物适用的许多临床环境包括以下环境：

[0089] 外伤。全血的急剧损失可使得流体从胞间隙和细胞内间隙转移以替换丢失体积的血液，同时使血液分流远离所述皮肤和内脏的低优先级器官。使血液分流远离器官并且有时消除这些器官中的 O_2 水平并且产生进行性组织死亡。主要目标是氧合受影响的组织。此外伤可以是入院前的或可产生外伤性出血性休克或外伤性脑损伤。

[0090] 缺血。缀合物和其组合物还可以用于将氧、CO 和 / 或 NO 递送至红血细胞或许多其它氧治疗剂不能穿透的区域。这些区域可包括位于红血球流的阻塞的下游的任何组织区域，诸如血栓、镰刀形红细胞阻塞、动脉阻塞、血管成形术球囊、手术仪器以及遭受氧饥饿或缺氧的任何组织的下游区域。可治疗所有类型的组织缺血，包括例如中风、正在显现的中风、暂时性缺血性发作、心肌顿抑和冬眠、急性或非稳定性绞痛、正在显现的绞痛、梗塞等。具体地说，产生缺血的病状包括急性心脏衰竭、心源性休克、心肌梗塞 / 心脏缺血、中风、肺栓塞、非外伤性出血性休克或脑血管损伤。

[0091] 血液稀释。在此应用中，施用治疗剂以替换（或取代）所去除的自体血液的 O_2 水平。这允许在手术期间和之后使用所去除的自体血液来进行必要的输血。需要操作前血液去除的一种此类手术是心肺分流术程序。

[0092] 败血病 / 败血性休克。在败血病中，尽管大量流体治疗和用血管收缩剂治疗，一些患者仍可变得具有高血压。在此情况下，过量产生氧化氮（NO）产生更低血压。因此，血红蛋白是用于治疗这些患者的理想药剂，因为血红蛋白以高亲和力结合 NO。

[0093] 缺氧症。当患者具有由肺炎或胰腺炎引起的急性肺损伤时，可观察到低氧血并且可通过提供本发明的血红蛋白或组合物以氧合所影响组织来减轻。

[0094] 癌症。将 O_2 递送至实性肿瘤团块的缺氧内核使其对放射线疗法和化学疗法的敏

感性增加。因为肿瘤的微血管系统不同于其它组织,所以通过增加 O_2 水平进行敏化需要缺氧核心内不加载 O_2 。换言之,P50 应非常低以防止 O_2 的早期加载,从而增加 O_2 水平,以确保肿瘤对后续放射线和化学疗法治的最佳敏化。

[0095] **手术。**本发明的血红蛋白和组合物可在各种手术程序期间使用。举例来说,其可在心肺分流术程序期间用作血管成形术、胸廓主动脉修复的辅助物或作为心肺预充溶液。

[0096] **器官灌注。**在器官离体或在器官捐赠接受者中维持的时间期间,维持 O_2 含量有助于保存结构和细胞完整性并且使梗塞形成最大化。血红蛋白和组合物可持续这种器官的需氧量。

[0097] 血红蛋白和其组合物还可以用于非人类,诸如家庭动物(例如家畜和伴侣动物,诸如犬、猫、马、鸟、爬行动物)。预期本发明可用于罹患归因于损伤、溶血性贫血等的失血的家庭动物和野生动物的紧急治疗。兽医学用途包括治疗由以下原因造成的失血:损伤、溶血性贫血、感染性贫血、细菌感染、因子 IV 破碎、脾功能亢进和脾肿大、家禽的出血性综合症、发育不全性贫血、再生障碍性贫血、突发性免疫溶血性病状、铁缺乏、同族免疫溶血性贫血、微血管病性溶血性贫血、寄生或手术麻醉诱导的脑损伤。

[0098] **定义**

[0099] 除非另外指出,否则当本公开中使用术语“一个(种)”、“一(a或an)”时,其意指“至少一个(种)”或“一个或多个(一种或多种)”。

[0100] 如本文所用的“活化的聚环氧烷”或“活化的 PAO”是指具有至少一个官能团的 PAO 分子。官能团是与所要与 PAO 缀合的分子上的自由胺、巯基或羧基相互作用的反应性部分。举例来说,一种与自由巯基反应的此类官能团是马来酰亚胺基。与自由胺反应的官能团是琥珀酰亚胺基。

[0101] “脱氧血红蛋白”或“未配位血红蛋白”意指无外源性配体与血红素结合的任何血红蛋白。

[0102] “血红蛋白”或“Hb”通常是指转运氧的血红素蛋白。在人类中,各 Hb 分子具有 4 个亚基,2 个 α 链亚基和 2 个 β 链亚基,其以四聚体结构排列。各亚基还含有一个血红素基团,其是以亚铁(Fe^{2+})形式结合配体 O_2 、NO 或 CO 的含铁中心。因此,各 Hb 分子可结合到 4 种配体分子上,分别形成 HbO_2 、 $HbNO$ 或 $HbCO$ 配位化合物。另外,血红蛋白可与 O_2 、NO 以及 CO 的混合物配位。

[0103] “基于血红蛋白的氧载体”(HBOC)是指携带氧但还适用于携带其它分子气体(诸如一氧化碳和氧化氮)的血红蛋白。

[0104] “高氧亲和力”是指血红蛋白已被修饰以展现大于无基质血红蛋白(SFH)的氧亲和力。因此,“高氧亲和力”Hb 的 P50 小于 SFH 的 P50,SFH 的 P50 如在 37°C 和 pH 7.4 下所测量是 15mmHg。

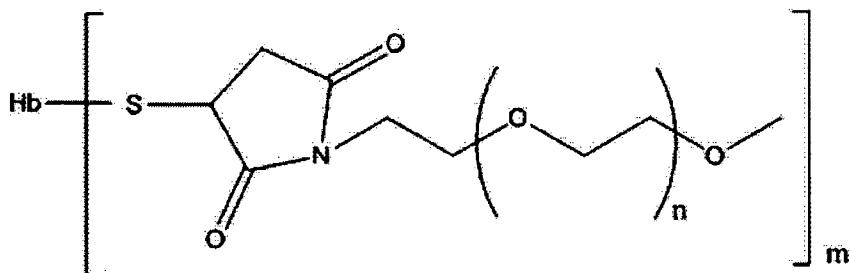
[0105] “配位血红蛋白”意指外源性配体结合于血红素的血红蛋白。常见优选配体包括氧、一氧化碳以及氧化氮。

[0106] “MalPEG”是指马来酰亚胺基聚乙二醇,并且包括经由接头与聚乙二醇连接的马来酰亚胺基部分。

[0107] “MalPEG-Hb”是指已缀合马来酰亚胺基活化的 PEG 的 Hb。缀合是通过使 MalPEG 与 Hb 上的硫醇基(并且在更低程度上与氨基)反应以形成 MalPEG-Hb 来进行。硫醇基可

见于存在于 Hb 的氨基酸序列中的半胱氨酸残基中,诸如在 β Cys 93 处的两个固有硫醇,并且还可以通过修饰表面氨基以含有硫醇基来引入。称为 MP4(Sangart, Inc.) 的示例性 MalPEG-Hb 具有以下分子式:

[0108]



[0109] 其中 Hb 是血红蛋白;S 是血红蛋白上的硫醇基;n 是 5,000 道尔顿聚环氧烷聚合物的氧化乙烯单元的数目;并且 m 是缀合至血红蛋白的马来酰亚胺基活化的聚环氧烷聚合物的平均数目并且是 7-8。

[0110] “高铁血红蛋白”或“metHb”是指含有呈三价铁状态的铁的 Hb 的氧化形式。MetHb 不充当氧或 CO 载体。如本文所用的术语“高铁血红蛋白%”是指氧化 Hb 与总 Hb 的百分比。

[0111] “甲氧基-PEG”或“mPEG-OH”是指羟基端的氢用甲基(-CH₃)置换的 PEG。

[0112] “修饰型血红蛋白”或“修饰型 Hb”是指已通过化学反应(诸如分子内和分子间交联、聚合、缀合和/或重组技术)改变以使得 Hb 不再呈其“天然”状态的 Hb。除非另外指出,否则如本文所用,术语“血红蛋白”或“Hb”是指天然未修饰 Hb 和修饰型 Hb。

[0113] “亚硝酸盐还原酶活性”或“NRA”是血红蛋白或基于血红蛋白的蛋白质将亚硝酸盐还原为氧化氮的能力。“最大亚硝酸盐还原酶活性”是血红蛋白或基于血红蛋白的蛋白质能够将亚硝酸盐还原为氧化氮的最大速率。“初始亚硝酸盐还原酶活性”是当添加亚硝酸盐到完全脱氧的蛋白质中时,血红蛋白或基于血红蛋白的蛋白质将亚硝酸盐还原为氧化氮的初始速率。

[0114] 术语“非氧合”意指血红素蛋白或血红蛋白呈非配位脱氧状态,或其与除 O₂ 以外的气体(诸如 NO 或 CO)配位。

[0115] “氧亲和力”是指氧载体(诸如 Hb)结合分子氧的亲合力。此特征通过氧平衡曲线来确定,所述氧平衡曲线将 Hb 分子的氧饱和度(Y 轴)与氧分压(X 轴)相联系。此曲线的位置由“P50”值表示,P50 是使氧载体用氧半饱和时的氧分压,并且与氧亲和力逆相关。因此,P50 越低,氧亲和力越高。全血(以及全血的组分,诸如红血球和 Hb)的氧亲和力可通过本领域中已知的各种方法来测量。(参见例如 Winslow, R. M. 等, J. Biol. Chem. 1977, 252: 2331-37)。氧亲和力还可以使用市售 HEMOX™ 分析器(TCS Scientific Corporation, New Hope, PA)来确定。(参见例如 Vandegriff 和 Shrager, “Methods in Enzymology”(Elsevier 等, 编著)232:460(1994));以及 Vandegriff 等, Anal. Biochem. 256(1):107-116(1998))。

[0116] 如本文所用的术语“氧治疗剂”是指能够结合于分子氧并且将其携带至有需要的细胞/组织/器官中的血红素蛋白。当以 CO 配位或 NO 配位血红素蛋白形式施用,一旦 CO 或 NO 从血红素部分释放,血红素基团就自由结合于分子氧并且携带分子氧。

[0117] “聚乙二醇”或“PEG”是指具有化学通式 H(OCH₂CH₂)_nOH 的聚合物,其中“n”大于或等于 4,优选地是约 45 至约 500,更优选地约 70 至约 250,并且最优选地约 90 至约 140,或

是约 115。聚合物可以是取代或未取代的,并且末端羟基可用不同常规末端基团(诸如甲氧基或羧基)置换。PEG 可商购自许多来源(例如 Carbowax™(Dow Chemical, Midland, MI)、Poly-G® (Arch Chemicals, Norwalk, CT) 以及 Solbase)。

[0118] “聚乙二醇缀合血红蛋白”、“PEG-Hb 缀合物”或“PEG-Hb”是指共价连接至少一种 PEG 的 Hb。

[0119] “溶液”是指液体混合物并且术语“水溶液”是指含有一些水并且还可以与水一起含有一种或多种其它液体物质以形成多组分溶液的溶液。

[0120] “无基质血红蛋白”或“SFH”是指已去除红血球膜的 Hb。

[0121] “表面修饰血红蛋白”是指已连接化学基团(通常是聚合物)(诸如右旋糖酐或聚环氧烷)的血红蛋白。术语“表面修饰氧合血红蛋白”是指当表面被修饰时呈“R”态的 Hb。

[0122] “末端活性”是用能够与血红素蛋白或血红蛋白的活性基团反应的部分官能化的 PAO 的百分比的指示。“100%末端活性”指示用于缀合反应的 PAO 的摩尔过量是以所有 PAO 均具有能够与血红素蛋白或血红蛋白的活性基团反应的部分为基础来表示的。举例来说,如果可用的 Mal-PEG 具有 80%末端活性以使得 80%的 PEG 用 Mal 官能化,并且以相对于血红蛋白 20 倍摩尔过量使用 Mal-PEG,那么基于 100%末端活性此摩尔比可表示为 Mal-PEG 相对于血红蛋白 16 倍摩尔过量。

[0123] “硫醇化”是指使分子上巯基的数目增加的方法。举例来说,使蛋白质与 2-亚氨基硫杂环戊烷(“2-IT”)反应使得蛋白质表面上的自由胺转化为巯基。这些巯基接着可用于与硫醇反应性部分(诸如马来酰亚胺)反应。

[0124] “未配位血红蛋白”是指含有至少一个未与分子气体(诸如氧、一氧化碳或氧化氮)配位的血红素部分的任何血红蛋白。因此,只要血红素部分中的一者未与分子气体配位,就认为血红蛋白“未配位”。

[0125] 本说明书中所引用的所有出版物、专利以及专利申请以引用的方式并入本文中用于本文所具体引用的主题,如同各个这些出版物、专利或专利申请具体并且个别地被指示以引用的方式并入本文中一般。

[0126] 已详细描述本发明,将显而易见的是改进和改变是可能的,而不会脱离本发明在随附权利要求中所界定的范围。

实施例

[0127] 提供以下非限制性实施例以进一步说明本发明。

[0128] 实施例 1:聚乙二醇化 $\beta\beta$ -DBBF 交联血红蛋白缀合物的制备

[0129] 压积红血球(“RBC”)获自商业来源,诸如当地血库、纽约血液中心(New York Blood Center)或美国红十字会(American Red Cross)。从采集时起获得材料不超过 45 天。在使用之前对所有单元关于病毒感染进行筛检并且进行核酸测试。通过膜滤法对非白细胞耗尽汇集单位进行白细胞耗尽以去除白血球。使压积 RBC 汇集到无菌容器中并且在 2-15°C 下储存直到进一步处理。记录体积,并且使用市售联合血氧计(co-oximeter)或其它领域认可的方法测定 Hb 浓度。

[0130] 使用 0.45 μ m 切向流过滤用六体积的 0.9%氯化钠洗涤 RBC,随后通过降低盐的浓度进行细胞溶解。使用相同膜进行 Hb 提取。通过用于白蛋白的分光光度分析对细胞洗涤

物进行分析,以检验血浆组分的去除。在寒冷条件下通过 $0.16\ \mu\text{m}$ 膜处理溶解产物以纯化 Hb。在无菌去热原条件下收集纯化 Hb 并且接着进行超滤以去除病毒。可进行其它减少病毒步骤,包括溶剂/去垢剂处理、纳米过滤以及阴离子 Q 膜纯化。此过程中的所有步骤均在 $2\text{--}15^\circ\text{C}$ 下进行。

[0131] 使用 30-kD 膜将 Hb 从溶解产物交换进入林格氏乳酸盐 (Ringer's lactate, RL) 或磷酸盐缓冲盐水 ("PBS", pH 7.4)。将 Hb 浓缩至 $1.1\text{--}1.5\text{mM}$ (按四聚体)。使用 10 到 12 体积的 RL 或 PBS 进行溶剂交换。此过程在 $2\text{--}15^\circ\text{C}$ 下进行。在硫醇化之前将在 RL 或 PBS 中制备的溶液的 pH 值调节至 8.0。将 Hb 通过 0.45 或 $0.2\ \mu\text{m}$ 一次性过滤囊进行无菌过滤并且在进行化学修饰反应之前在 $4\pm 2^\circ\text{C}$ 下储存。

[0132] **交联**:如先前由 Walder 等, Biochemistry, 1979; 18(20):4265-70 所描述通过由压积红细胞制备的无基质血红蛋白 (SFH) 与双 (3,5-二溴水杨基) 富马酸酯 (DBBF) 的反应来制备 $\beta\beta$ -DBBF 交联 Hb。在约 $2\text{--}8^\circ\text{C}$ 下,使于硼酸盐缓冲剂 (pH 值 ~ 8.5) 中的氧合 SFH 与两倍摩尔过量的 DBBF 反应约 16 小时。

[0133] **硫醇化**:使用如上文所描述制备的 SFH,使用相对于 Hb 15 倍摩尔过量的 2-亚氨基硫杂环戊烷 (2-IT) 进行硫醇化。将比率和反应时间最佳化,以使用于 PEG 缀合的硫醇基的数目最大化并且使产物异质性减到最低。将于 RL (pH 7.0-8.5)、PBS 或任何类似缓冲剂中的约 1mM Hb (四聚体) 与于相同缓冲剂中的 15mM 2-IT 组合。在 $10\pm 5^\circ\text{C}$ 下连续搅拌此混合物约 6 小时。

[0134] 在硫醇化之前和之后及然后再在 Hb-PEG 缀合之后,使用二硫吡啶比色分析 (Ampulski, R. S. 等, Biochem. Biophys. Acta 1969, 32:163-169) 测量在 Hb 四聚体表面上可用的硫醇基的数目。人 Hb 在 $\beta 93$ 半胱氨酸残基处含有两个固有反应性硫醇基,这通过二硫吡啶反应得以证实。SFH 以 $1:< 8$ (SFH:2-IT) 硫醇化之后,反应性硫醇基的数目从两个增加到大于七个硫醇。

[0135] **PEG 缀合**:使用基于 100% 末端活性相对于起始四聚体 Hb 浓度 28 倍摩尔过量的 MalPEG 将 MalPEG 缀合至硫醇化 $\beta\beta$ -DBBF 交联 Hb。首先使 Hb 与大气平衡以氧合 Hb。将于 RL (pH 7.0-8.5)、PBS 或任何类似缓冲剂中的约 1mM 硫醇化 Hb 与于相同缓冲剂中的 28mM MalPEG 组合。在 $10\pm 5^\circ\text{C}$ 下连续搅拌此混合物约 6 小时。

[0136] 将所得 PEG-Hb 缀合物通过 70-kD 膜 (即 < 0 体积过滤) 进行处理以去除未反应试剂。通过尺寸排阻液相色谱 ("LC") 在 540nm 和 217nm 下对此过程进行监测。将浓度调节到 4.4g/dl Hb 并且将 pH 值调节到 $6.0\text{--}7.8$ 。

[0137] 将 PEG-Hb 缀合物使用 $0.2\ \mu\text{m}$ 一次性无菌囊进行无菌过滤并且在 $4\pm 2^\circ\text{C}$ 下收集到无菌去热原容器中。将 PEG-Hb 缀合物稀释到 4.4g/dl RL 并且将 pH 值调节到 7.4 ± 0.2 pH 值并且接着无菌过滤 ($0.2\ \mu\text{m}$) 并且等分到无内毒素无菌容器中。

[0138] 最终聚乙二醇化 $\beta\beta$ -DBBF 交联血红蛋白缀合物 ("PEG- $\beta\beta$ -Hb") 的性质显示于表 3 中:

[0139] 表 3:PEG- $\beta\beta$ -Hb 的性质

[0140]

性质	值

Hb 浓度 (g/dL)	4.4
pH	7.4
聚乙二醇化的程度	7.6
COP(mm Hg)	85
P50mm Hg	2.6
希尔数 (n 值)	1.05

[0141] 经由标准方法论进一步证实 PEG- $\beta\beta$ -Hb 的结构。尺寸排阻色谱法证实 DBBF 交联的存在 (图 1 和 2)。逆相高效液相色谱法证实血红蛋白的 β 球蛋白亚基的 DBBF 交联的存在 (图 3)。 $\beta\beta$ -DBBF Hb 的聚乙二醇化是使用尺寸排阻色谱法证实 (图 4)。

[0142] 实施例 2:由 PEG- $\beta\beta$ -Hb 展现增强的亚硝酸盐还原酶活性

[0143] 出于比较的目的,使来自实施例 1 的脱氧聚乙二醇化 $\beta\beta$ -DBBF 交联血红蛋白和其它血红蛋白物质于密封透明小容器中在连二亚硫酸钠存在下厌氧地与亚硝酸钠反应。使用相对于血红素十倍过量的亚硝酸盐,并且以分光光度法监测反应。使用脱氧血红蛋白、铁-亚硝酰基-血红蛋白以及高铁血红蛋白的母体光谱对所得光谱数据进行去卷积。所述速率是曲线作为所述衍生物的所述失踪的脱氧血红蛋白以及所述最大速率用于比较目的。

[0144] SFH 和聚乙二醇化 $\beta\beta$ -DBBF 交联血红蛋白分别以 0.0215 μ M/s 和 0.462 μ M/s 的最大速率将亚硝酸盐还原为 NO,表明与 SFH 相比聚乙二醇化 $\beta\beta$ -DBBF 交联血红蛋白的最大速率为 21 倍。

[0145] 参考图 5,测定了若干类型的修饰型 Hb 的亚硝酸盐还原酶活性,包括马来酰亚胺缀合非交联血红蛋白,其在 Hb β 93Cys 处反应以降低 P50。所测试的马来酰亚胺包括小分子 N-乙基马来酰亚胺 (NEM) 和 MP4 (Sangart, Inc.) 以及表 4 中指定的其它血红蛋白。

[0146] 表 4:修饰型血红蛋白的性质

[0147]

Hb	缀合化学	每四聚体的取代度	P50 (氧亲和力)
SFH	N/A	—	16
MP40X	马来酰亚胺	8 (PEG 5K)	4
P5K2	马来酰亚胺	2 (PEG 5K)	8
P5K4	马来酰亚胺	4 (PEG 5K)	5
NEM2	马来酰亚胺	2 (NEM)	9
NEM8	马来酰亚胺	8 (NEM)	5

SVA-PEG-Hb	琥珀酰亚胺	未测试	7
$\alpha\alpha$ -Hb	DBBF	-	33
即-Hb	DBBF	-	8
PEG- $\alpha\alpha$ -Hb	DBBF/ 马来酰亚胺	7 (PEG 5K)	9
PEG-即-Hb	DBBF/ 马来酰亚胺	7.6 (PEG 5K)	3

[0148] α 或 β 亚基之间交联的 Hb 展现不同 P50。P50 值在 3mmHg (PEG- $\beta\beta$ -Hb) 至 33mmHg ($\alpha\alpha$ 交联 Hb) 范围内。观察到 P50 与亚硝酸盐还原酶活性之间存在直接相关性。具有高氧亲和力 (低 P50) 的 Hb 以与低亲和力 Hb 相比更高的速率将亚硝酸盐还原为 NO。在 10 倍过量的亚硝酸盐情况下, 亚硝酸盐还原的最大速率从 0.46 μ M/s (PEG- $\beta\beta$ -Hb) 变化到 0.015 μ M/s ($\alpha\alpha$ 交联 Hb)。通过此反应产生的 NO 可逃脱血红素捕获并且从 Hb 释放, 并且 NO 释放速率与亚硝酸盐还原酶活性有关。总而言之, 这些结果表明氧亲和力在无细胞 Hb 设计中是重要的, 不仅增强血红蛋白配体的递送, 而且使亚硝酸盐还原酶活性最大化。

[0149] 当引入本发明的元素或其优选实施方案时, 冠词“一 (a/an)”、“该”以及“所述”旨在指存在所述元素中的一者或多者。术语“包含”、“包括”以及“具有”旨在是包括性的并且意味着可能存在除所列元素以外的其它元素。

[0150] 鉴于以上内容, 将可见的是本发明的若干目标得以实现并且其它有利结果得以达成。

[0151] 因为在以上组合物和方法中可作出各种变化而不会脱离本发明的范围, 所以以上描述中所含以及随附图式中所显示的所有内容应视为说明性的并且不具限制意义。

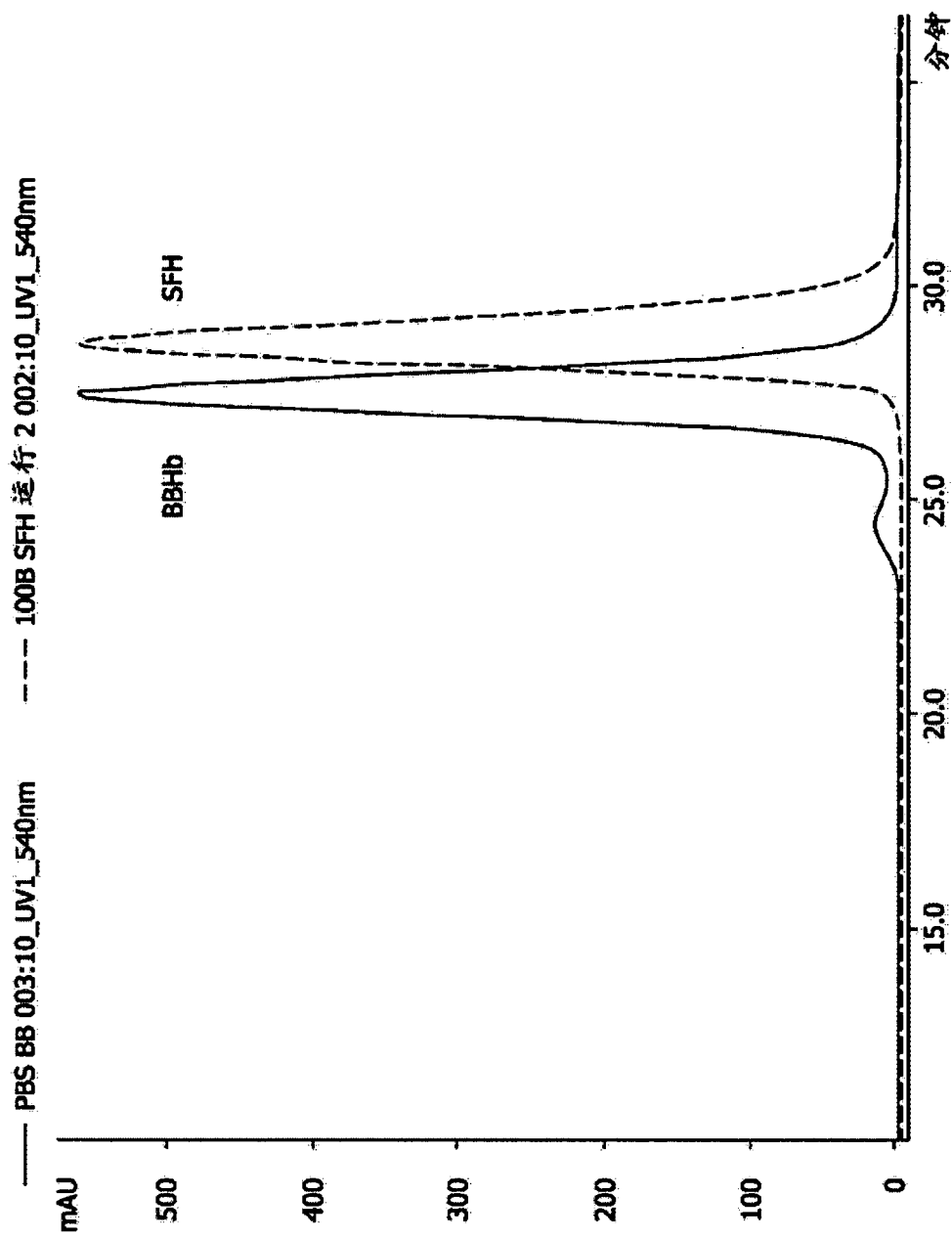


图 1

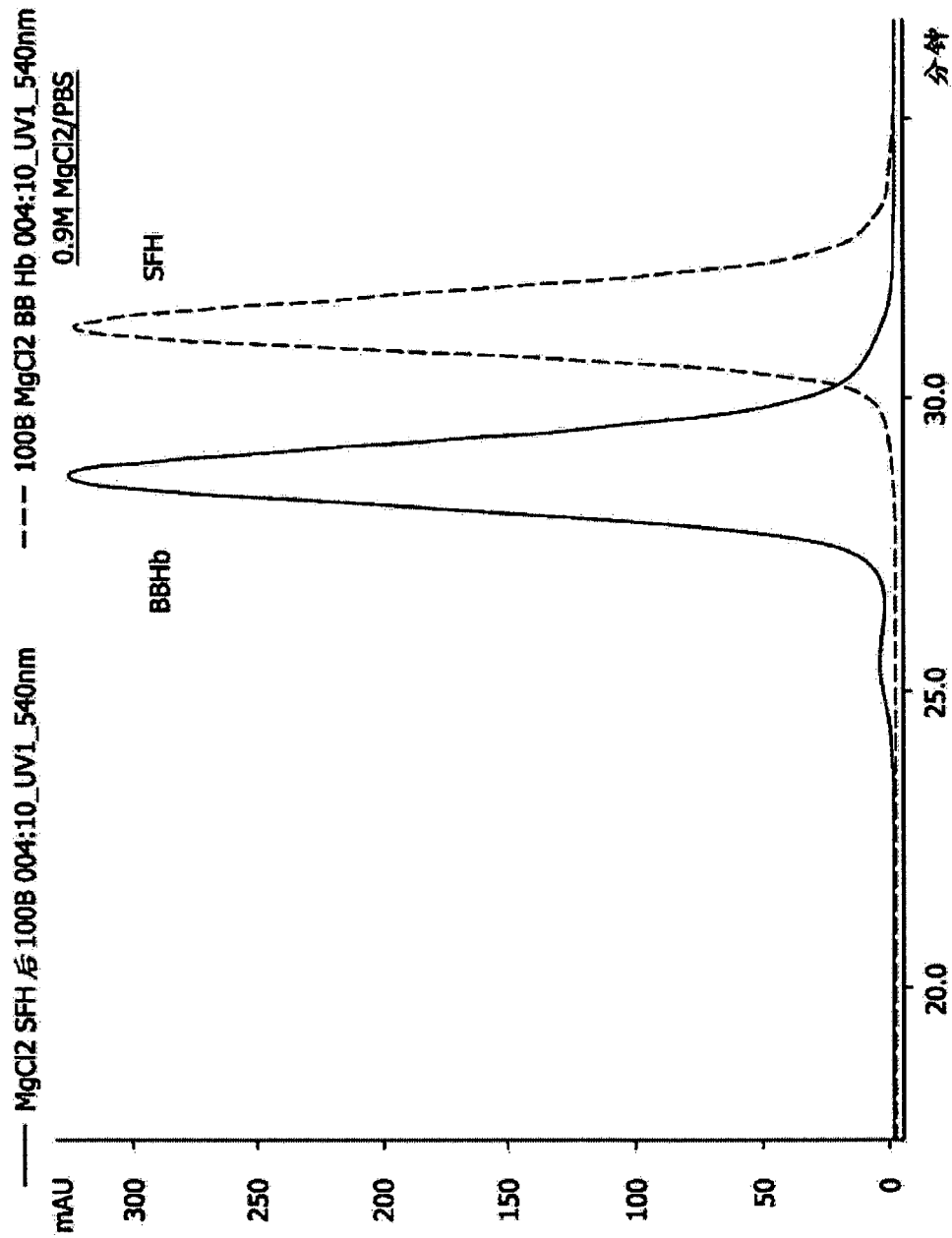


图 2

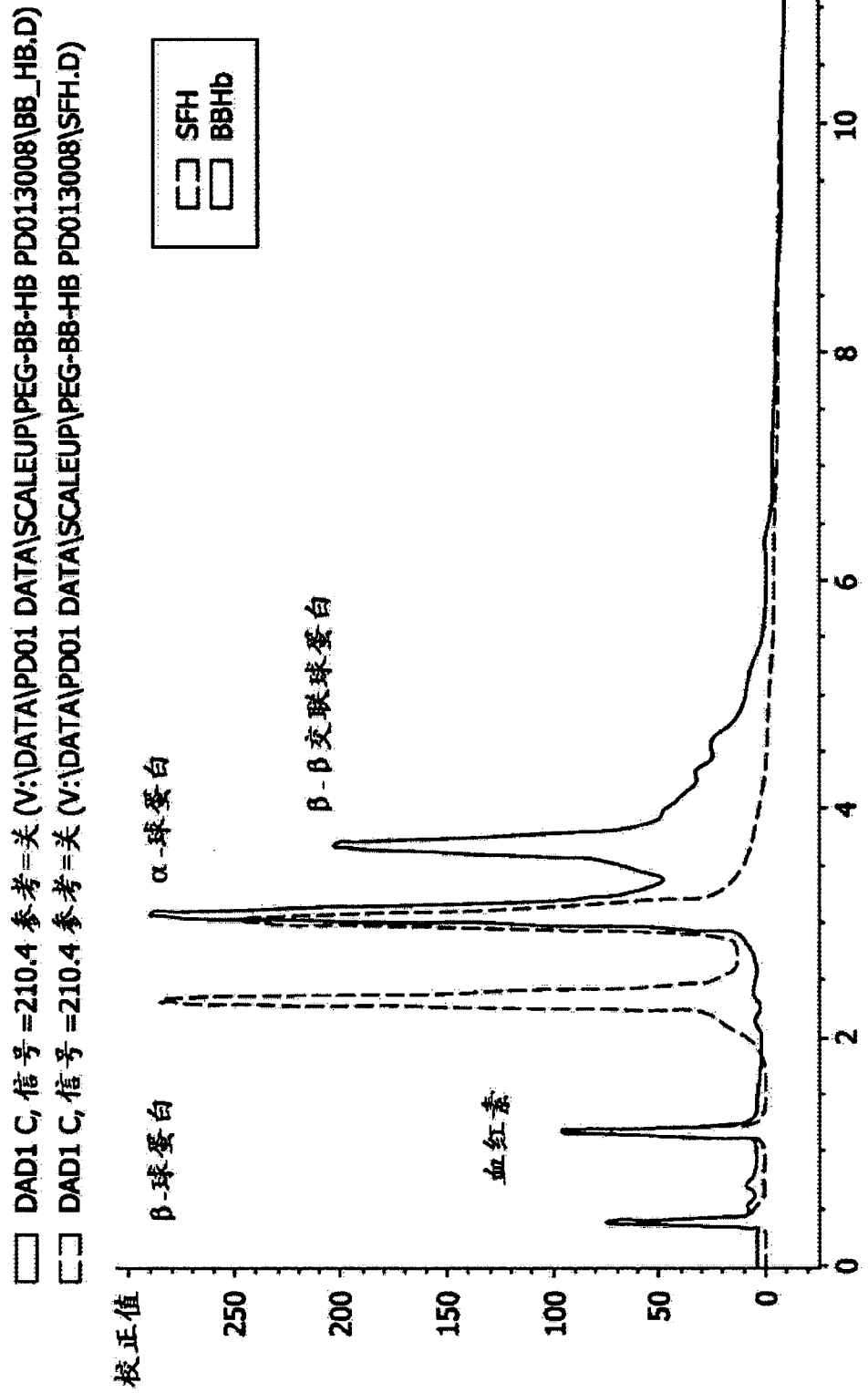


图 3

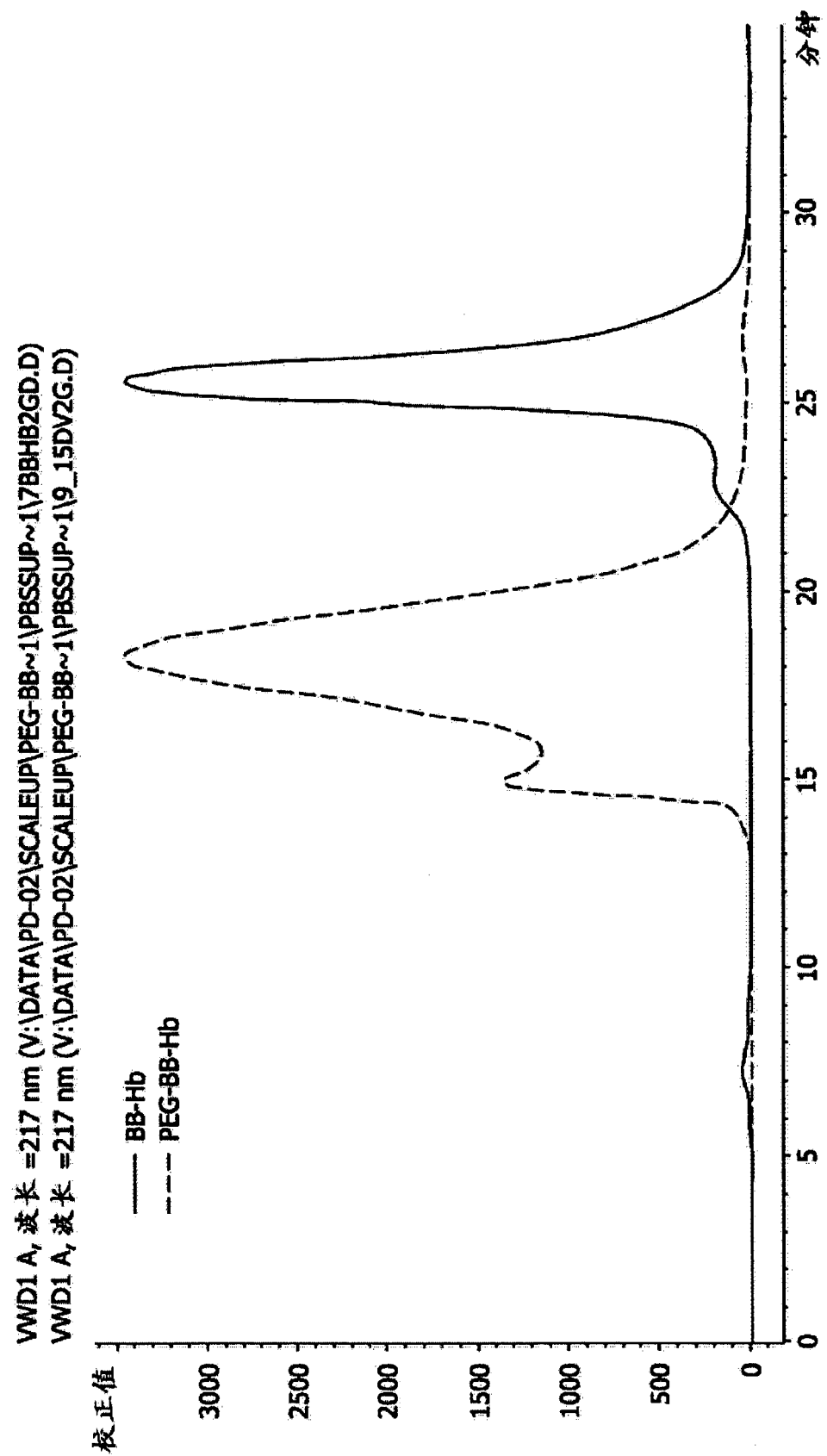


图 4

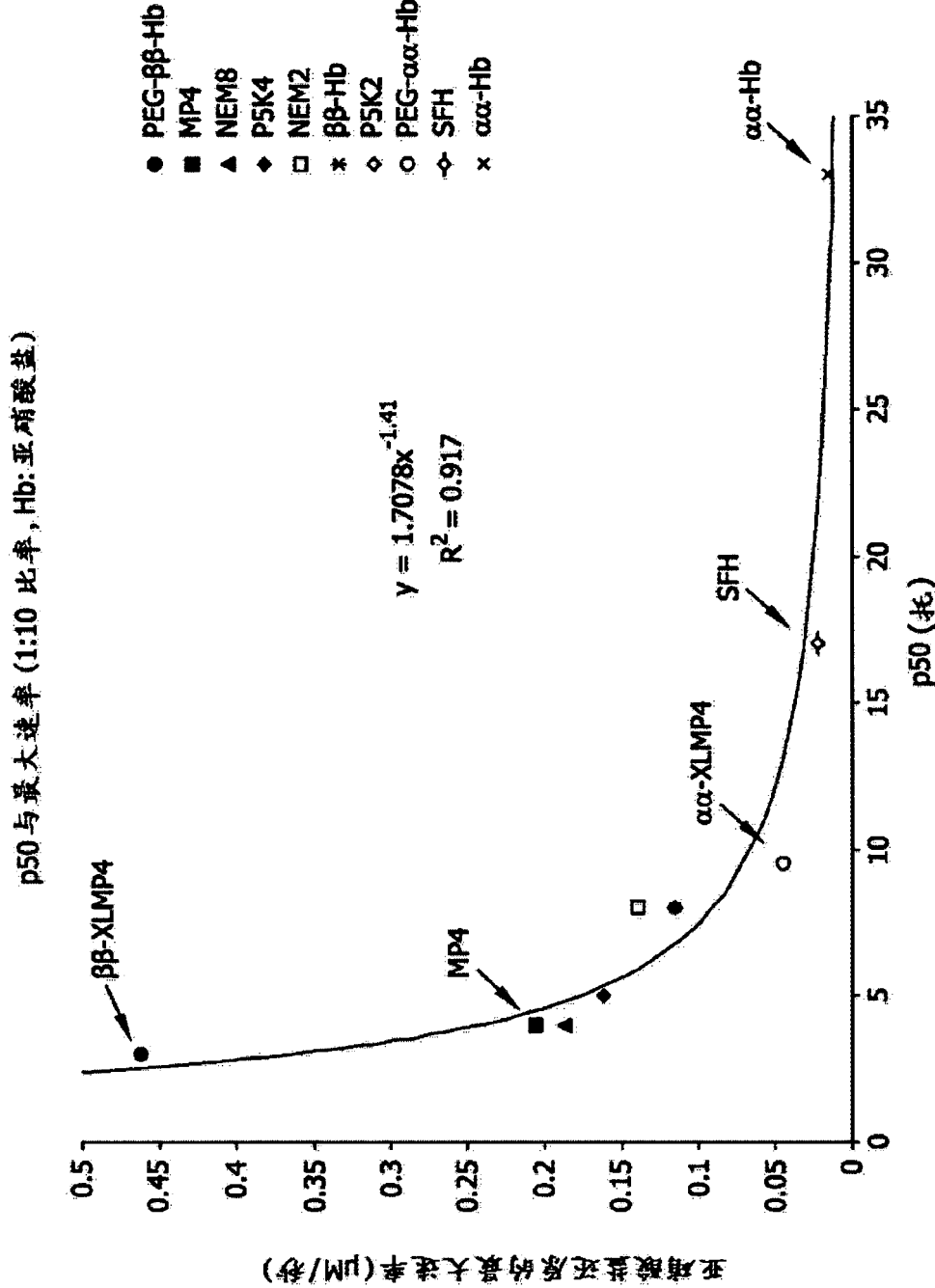


图 5

Abstract

PEGylated diaspirin-crosslinked hemoglobin conjugates having high oxygen affinity are described which have enhanced nitrite reductase activity to deliver oxygen, carbon monoxide, nitric oxide or mixtures thereof to tissues to treat various diseases and conditions.