20

Paradigm Shift for Designing Oxygen Therapeutics: New Insights Emerging from Studies with Transgenic Mouse Models of Sickle Cell Disease

Synergy of Supra Plasma Expansion and High O_2 Affinity of Blood Substitutes

Seetharama Acharya, Craig Branch, Amy G. Tsai, and Marcos Intaglietta

Abbreviations

BOLD	Blood oxygen level dependent
CBF	Cerebral blood flow
EAF	Extension Arm Facilitated
FCD	Functional Capillary Density
Hb	Human hemoglobin
MAP	Mean arterial pressure
MP	Maleimide PEG modified Hb
MP4	Maleimide PEG modified Hb constituted as a 4 gm % solution,
MP8	Maleimide PEG modified Hb constituted as 8 gm % solution
MRI	Magnetic resonance imaging
PEG polyethylene glycol, PEGylation-conjugation of PEG-chains P5K2, P10K2, P5K4, P3K6, P5K6	The PEG-Hb conjugates studied here has been defined by the general formula PxKy, where 'P' represents PEG chain of mass 'x'in kilo daltons, and K represents number of copies represented by 'y' conjugated to a given Hb molecule
RBC	Red blood Cells

S. Acharya (✉) · C. Branch
Departments of Medicine, Physiology and Biophysics, and of Radiology Albert Einstein College of Medicine, Bronx, NY, USA

Department of Bioengineering, UCSD, San Diego, CA, USA
e-mail: seetharama.acharya@einsteinmed.org

A. G. Tsai · M. Intaglietta
Department of Bioengineering, UCSD, San Diego, CA, USA

Introduction

The RBC serves a dual role in oxygen delivery to tissues, it carries oxygen from lungs to tissues and modulates the blood flow/microcirculation through blood shear thinning to achieve a proper delivery of oxygen [1]. However, design of Hb derivatives as oxygen therapeutics with an oxygen affinity comparable to or lower than that of Hb in RBC has focused on increasing the oxygen carrying capacity of blood to compensate for blood loss or anemia. Multiple intrinsic properties of blood and Hb inside the RBC modulate the hyper/hypotensive activity of circulatory system to optimize oxygen delivery. In designing our PEG-Hbs as non-hypertensive solutions we departed from the original paradigm. We have combined the potential advantages of high oxygen affinity of Hb for targeted oxygen delivery to oxygen starved tissues with intrinsic improvements in perfusion properties induced by colloidal plasma expanders that afford in situations of anemia in designing our new oxygen therapeutics to mitigate the vasoconstriction mediated acellular Hb. This has necessitated the use of lower amounts of oxygen therapeutics, and this represents a complete paradigm shift.

In this chapter we focus the attempt to mimic the dual role of RBC in circulation with the designed molecules. Here, we review the development of new strategies to endow these dual RBC-like properties to the new oxygen therapeutics. This new class of oxygen therapeutics represents novel biomaterials, nano-oxygen pumps, that increase the efficacy of oxygenation of Hb (in flow) in RBCs in the lungs and oxygen delivery (out-flow) from RBCs in the tissues when the level of Hb (hematocrit) in the blood drops to the transfusion trigger levels or below. The new platform for treating anemia recommends the use of lower amounts (dosage) of these new semisynthetic biomaterials as opposed to the use of larger dose (compensate for blood loss or anemia) as strategized

H. Liu et al. (eds.), *Blood Substitutes and Oxygen Biotherapeutics*, https://doi.org/10.1007/978-3-030-95975-3_20

with conventional low oxygen affinity oxygen therapeutics. This paradigm shift is needed since the new high oxygen affinity biomaterials increase not only increase tissue oxygenation through better extraction of oxygen from the RBC in the tissues, and in doing so release NO, a vasodilator. As a consequence, increase in tissue oxygenation by these nano-oxygen pumps resembles the normal oxygenation through RBC with normal hematocrit levels but through improved oxygen extraction rather than as envisioned by blood substitutes. Accordingly, these nano oxygen pumps have a self-activated mechanism for improving perfusion/microcirculation coupled with improved oxygen extraction. Besides, they also increase the oxygen saturation in the lungs, thus becoming a mechanism for improving the oxygen carrying capacity of Hb within RBC as it gets attenuated by anemia, an approach very distinct from the old paradigm advocating increased the oxygen carrying capacity of circulatory system (to restore the lost oxygen carrying capacity either completely or partly) to increase oxygen delivery.

The delineation of these new concepts has been facilitated by the development of new PEGylated Hbs. A new platform for PEGylation of proteins has been developed at Einstein. referred to as extension arm facilitated (EAF) PEGylation (EAF PEGylation). This platform is essentially a protein thiolation facilitated PEGylation protocol [2, 3]. The first product of this platform is EAF P5K6 Hb with six copies (K) of PEG, denoted by letter P, of mass 5000. Sangart version of hexa-PEGylated Hb is referred as maleimide PEG Hb, (MP) with the tradename Hemospan (see below for full description of the platform). A 4 gm % solution of Hemospan is referred to as MP4, and an 8 gm % solution as MP8, but MP4 is the frequently used formulation. It may be noted the concentration of Hb in MP4 is at least threefold lower than in previously designed oxygen therapeutics. In EAF P5K6 Hb we have two PEG-5Kchains directly conjugated to Cys-93(β) and rest are conjugated on the extension arms built on ε-amino groups of Hb. We have a di-PEGylated Hb with two copies of PEG 5 K on Cys-93(β) conjugated directly, P5K2 Hb. P10K2 Hb is a version di-PEGylated Hb generated using maleimide PEG-10,000 instead of maleimide PEG-5000. EAF P3K6 Hb is a newer version of hexa-PEGylated Hb generated using maleimide PEG-3000 using extension arm chemistry, to have a smaller PEG-shell. These aspects are discussed in detail in the Chapter.

Development of EAF P5K6 Hb and Its Prototype Hemospan as an Oxygen Carrying Colloidal Plasma Expanders

In designing the PEG Hb as a new class of oxygen therapeutics, we at Einstein had the following structural and functional properties for Hb derivatives as guiding principles for our development. This has led to the design of Extension Arm Facilitated (EAF) PEGylation platform conjugating PEG-chains to proteins (Fig. 20.1).

(i) Design of a new PEGylation strategy to surface decorate Hb such that it will induce very limited perturbation of the hydration layer of Hb and does not weaken the inter-dimeric interactions of the tetramer. Besides, it should neither perturb the overall surface charges of the molecule nor weaken the intra or interdimeric interactions of Hb. Hexa-PEGylation of Hb using six copies of PEG 5K chains by a novel Extension Arm Facilitated (EAF) PEGylation, EAF P5K6 Hb platform appears to accomplish all these structural aspects [2, 3].

(ii) The PEGylated molecule exhibits a hydrodynamic volume comparable to a polymeric form of Hb with three to four copies of the tetrameric Hb unit and thus avoids extravasation in the circulation. Though the total mass of PEG conjugated Hb is only 30 K, the Hb surface decorated with six copies of PEG 5K chains by EAF PEGylation (EAF P5K6 Hb) exhibits a molecular radius of ~ 6.5 nm. The elution pattern of EAF P5K6 Hb on molecular sieve chromatography is sharper than polymerized bovine Hb even though the average hydrodynamic volume of the two is comparable.

(iii) Lowering of hematocrit due to blood loss has an intrinsic vaso-constrictive impact because of reduced shear thinning of RBC; however, this hematocrit reduction induced vaso-constriction can be partially compensated or attenuated by conventional colloidal plasma expanders with viscosities around 2.8 cp and compensated even better with colloidal plasma expanders with viscosities around that of blood (5 cp) or higher than 5.0 (Supra Plasma Expanders).

(iv) Previously designed oxygen therapeutics are low-oxygen affinity Hbs with affinities comparable to that of the RBC or lower. These will release their oxygen early at the arteriolar level causing the vessel to vaso-constrict thereby limiting microcirculatory perfusion. In contrast, PEG Hbs are generally high oxygen affinity Hbs and thus were chosen as they will be applied to target oxygen delivery to hypoxic regions in the body. PEGylation of proteins is viscogenic and thus can compensate for the vasoconstrictive activity of Hb to some degree. PEGylated Hb and its prototype PEG Alb are novel semisynthetic hybrid biopolymers with ordered and disordered regions and with novel molecular properties yielding non-hypertensive colloidal plasma expanders. These represent a novel class of oxygen therapeutics that mimic some of the properties of erythrocytes.

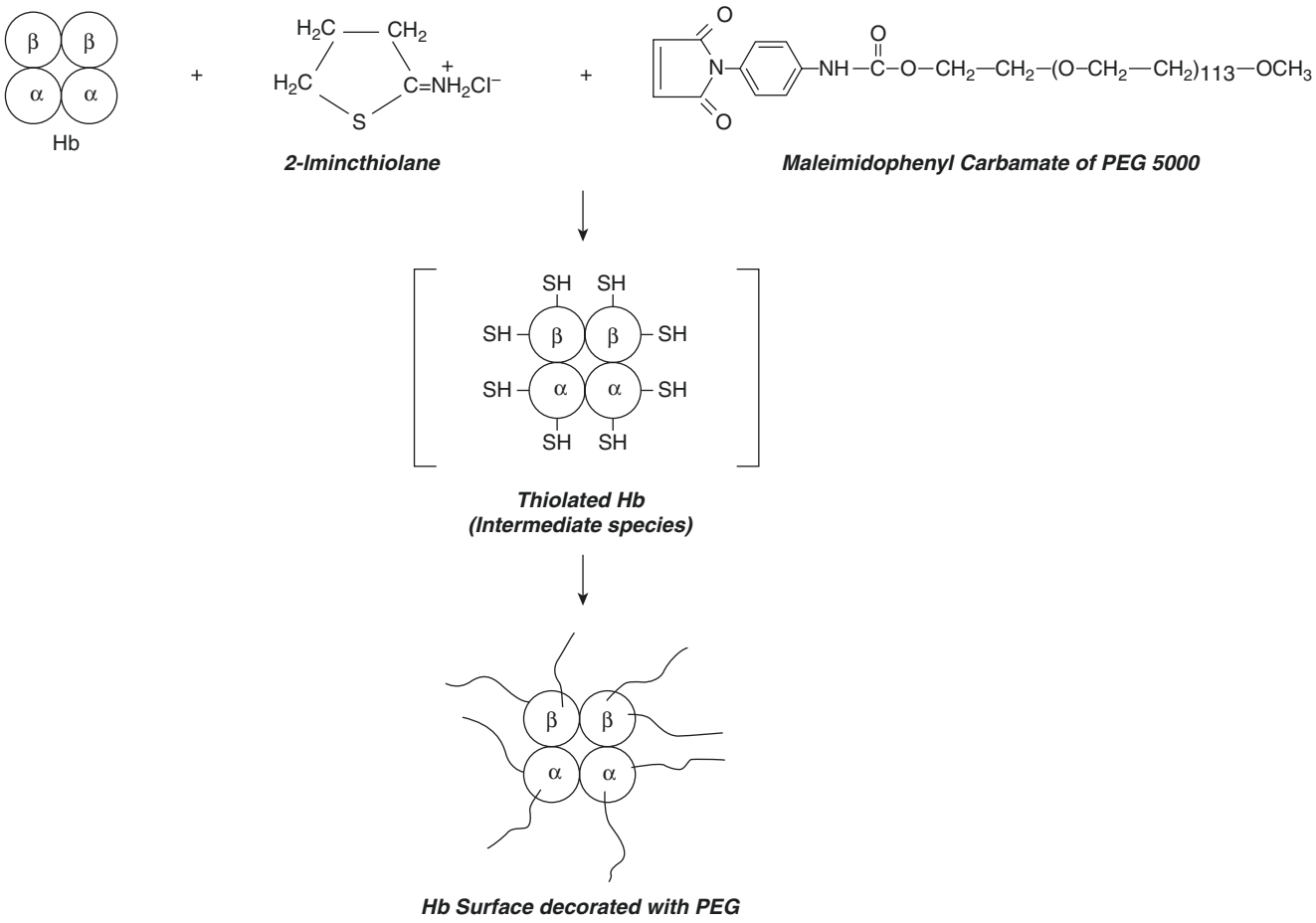

Fig. 20.1 Schematic representation of Extension Arm Chemistry Facilitated PEGylation

Interdimeric Interactions of Hb PEGylated Using Extension Arm Chemistry

Earlier versions oxygen therapeutics are essentially intramolecularly crosslinked Hbs with the crosslinking between like chains, i.e., between αβ dimers of the tetramers or polymeric Hbs to avoid nephrotoxicity. Direct PEGylation of un-crosslinked Hb induces a weakening of interdimeric interactions of tetrameric Hb as seen with Enzon PEG-Hb, a PEGylated bovine Hb with ten copies of PEG-5K chains. However, very little nephrotoxicity is seen with Enzon PEG-Hb, showing PEGylated αβ-dimers are not readily filtered through kidneys. We have compared the influence of PEGylation on hexaPEGylated Hb (PEGylated with PEG-5K) on the inter-dimeric interactions of human Hb. HexaPEGylation of Hb with PEG-5K weakens the interdimeric interactions in a PEGylation chemistry dependent fashion [4–6]. The hydrodynamic volume of PEGylated αβ-dimers with an average of three PEG-5K chains (molecular mass of αβ-dimers is 48K and lower than tetrameric Hb) present in a sample of hexaPEGylated Hb is significantly higher than intramolecularly crosslinked Hb, i.e., PEG-chains conjugated onto Hb are essentially disordered. Accordingly, there is very little nephrotoxicity with PEGylated Hbs with six or more PEG 5K chain. Concomitant with this weakening of inter-dimeric interaction on PEGylation, direct PEGylation increases the oxygen affinity and abolishes cooperativity.

In EAF PEGylation described above, two of the six PEG-chains are directly conjugated to Hb through the side chain functions, thiol group of Cys-93(β) and remaining four PEG-chains are conjugated on the thiol group of the extensions introduced on the surface amino groups of Hb [7]. We can protect reversibly thiol group of the Cys-93(β) as mixed disulfide during EAF PEGylation of Hb and this will generate EAF PEG-Hb wherein all PEG-chains are conjugated only through thiol group on the extension arm [5, 6] or by carrying out the reaction in deoxy conformation [2, 7].

On the contrary, in EAF P5K6 Hb, the strength of interdimeric interactions of Hb remains essentially unperturbed (Fig. 20.2). The absence of PEGylation on Cys-93(β) does

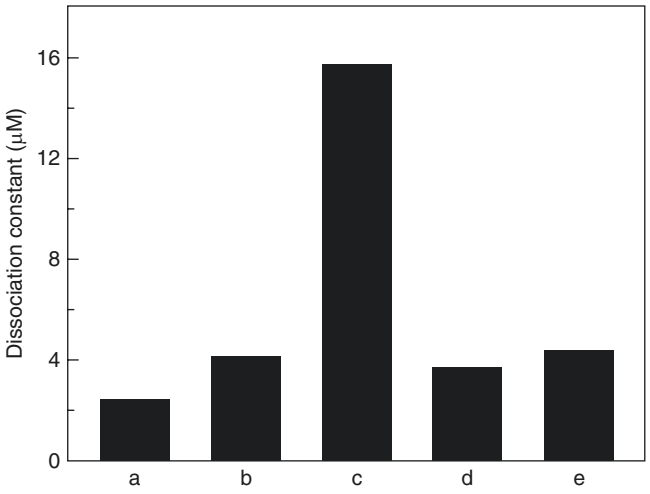

Fig. 20.2 The tetramer-dimer dissociation constant of the EAF-PEG-Hb. HbA (*a*), the PEG-Hb with free Cys-93(β) (*b*), the PEG-Hb without free Cys-93(β) (*c*), Hb- NEM modified in the presence of iminothiolane with free Cys-93(β) (*d*), and Hb NEM modified in the presence of iminothiolane without free Cys-93(β) (*e*) were loaded on the Superose 12 column for the dissociation constant measurement

not increase the stability of PEGylated Hb to any noticeable level. The factors influencing the choice of EAF P5K6 Hb as the best molecule for our studies are: (i) Overall stability of the molecule (ii) High oxygen affinity (iii) significant level of cooperativity, and (iv) Absence of the Bohr effect. Many of these molecular and functional properties of EAF P5K6 Hb are counter intuitive to the desired properties for a blood substitute as per old paradigm for the design of oxygen therapeutics.

The potential advantages of PEGylation induced plasma expander like properties of PEG Hb and the high oxygen affinity in an oxygen therapeutic has been discussed in detail by Winslow [8]. Based on observed viscosity of solutions of EAF P5K6 Hb as a function of protein concentration, it has been constituted as a 4 gm % solution and at this concentration, a solution of EAF P5K6 Hb has a viscosity comparable to that of conventional colloidal plasma expanders. At 4 gm %, EAF P5K6 Hb has a viscosity around 2 cp, slightly lower than the viscosity of conventional colloidal plasma expanders.

It may be noted here that a 4 gm % solution of EAF P5K6 Hb represents significantly a lower concentration of Hb as compared to the previous blood substitutes designed as per old paradigms and which are constituted as 12 gm % (or more) solutions. Given the high oxygen affinity of this PEG Hb and the lower concentration of EAF P5K6 Hb in the designed product solution, there has been significant skepticism in the scientific community in the ability of PEG-Hbs to deliver oxygen.

Hemospan (MP4) Is Non-toxic in Phase III Clinical Trial, However Not Superior to Conventional Colloidal Plasma Expander

Sangart licensed the platform for EAF hexa-PEGylation of Hb from Einstein and developed its blood substitute candidate under the trade name Hemospan [9].

However, Sangart introduced two significant changes into the platform licensed from Einstein, in preparing their molecule, Hemospan. First, instead of using chromatographically purified Hb, Sangart used stroma free Hb prepared from outdated blood from blood banks. Second, Sangart used a two-step procedure for EAF PEGylation instead of the one step platform considered to be better. Stroma free Hb was first reacted with iminothiolane at a protein concentration of 1 mM (in the one step platform practiced at Einstein Hb is reacted at 0.5 mM Hb concentration) and then mixed with PEG-5K maleimide. The major limitations of the two-step approach are: (i) a higher level of thiolation of Hb due to the higher protein concentration, and (ii) the thiolation reaction is done under oxy conditions in the absence of PEG maleimide. Accordingly some degree of air oxidation of thiol groups generated on Hb is expected in the two-step PEGylation process and also to generate intra and/or intra molecular crosslinks before the PEG maleimide is added to the reaction mixture as the second step of EAF PEGylation. In the one step platform, thiolation is done in the presence of PEG maleimide and thiol groups react with PEG maleimide as they are generated *in situ*.

Hemospan generated from stroma free Hb carried nearly 8 copies of PEG 5K chains compared to six PEG-5K chains in EAF P5K6 Hb [9] and exhibited molecular radius around 10 nm. Hemospan exhibits a higher oxygen affinity as compared to EAF P5K6 Hb and does not exhibit any cooperativity. The viscosity at 4 gm/dl (MP4) is around 2.2 cp and noticeably higher compared to 4 gm % EAF P5K6 Hb. At 8 gm/dl (MP8) of hemospan, the viscosity is around 5.2 cp and comparable to that of blood. The molecular radius of Hemospan was calculated from the colloidal osmotic pressure of the solution which is a colligative property. The molecular radius value for EAF P5K6 Hb is calculated from dynamic light scattering, a molecular property. Given the fact that interaction of EAF P5K6 Hb is comparable to native Hb, the higher molecular radius calculated for Hemospan suggests the presence of some PEGylated dimers due to the two-step preparation of EAF PEGylated Hb.

On the positive side, Hemospan was essentially free of Hb dependent toxicity in clinical trials. In phase III clinical trial with orthopedic surgery patients, Hemospan behaved essentially as colloidal plasma expanders in terms of mortality; subsequently Sangart did not pursue regulatory approval as a drug in view of the prohibitive cost of Hemospan as a colloi-

dal plasma expander. However, the clinical trial data indicated that Hemospan was superior to colloidal plasma expander hetastarch by showing (i) A lower number of hypotensive episodes (ii) A shorter length of hypotensive episodes. The data suggest that hemospan provides a better protection presumably either through targeted oxygen delivery to hypoxic areas, or through supra plasma expander activity of MP4 (see below), the primary objective of the designed molecule.

Accordingly, Sangart was moving forward with the clinical trial albeit with a different end point; reduction in ischemia mediated effects by following changes in lactic acid production. Though phase II trials provided positive results, Sangart could not raise the funds to pursue phase III clinical trial, returned the patents to Einstein and closed its operation. Therefore, a remaining unanswered question is whether a 4 gm % solution MP4 (a low level of Hb or very low level of high oxygen carrying capacity) with its high oxygen affinity Hb has contributed to the absence of therapeutic benefits of Hemospan over hetastarch in the Phase III clinical trials or the design of EAF P5K6 Hb is tuned properly to achieve improved oxygen delivery.

EAF P5K6 Hb Is a Semisynthetic, Colloidal Supra Plasma Expander

As noted above vaso-constrictive effect induced by a decrease in hematocrit level (or anemia) could be off-set to some level by conventional colloidal plasma expanders like dextran 70. Surprisingly, when EAF P5K6 Hb (or MP4) tested in hamster extreme hemodilution models (hematocrit levels 11%; severe anemia), the functional capillary density and blood flow seen is noticeably better than seen with conventional colloidal plasma expanders like dextran-70 at 6 gm % (2.8 cp) (Table 20.1). On the other hand, it is not as good as with the supra plasma expander dextran 500 at 6 gm % (5.2 cp). The increased functional capillary density and high blood flow arising from the vaso-dilatory influence of supra plasma expanders. This vaso-dilatory influence seen with high density colloidal plasma expander has been attributed to the increased shear thinning of blood and the resultant increased intrinsic NO production by the endothelium. Given the fact the viscosity of a 4 gm % MP4 (or EAF P5K6 Hb) is lower than dextran 70, it is clear that the supra plasma expansion activity of EAF P5K6 Hb is, apparently, a consequence of some unique structural aspect of this semisynthetic conjugated protein. A schematic representation of this semisynthetic supra plasma expander is shown in the Fig. 20.3. The packing density of central protein core, which is a compactly folded protein, is higher than the PEG-shell engineered outside and the PEG shell is placed outside the hydration layer of protein through a zone of extension arms. The essentially disordered PEG chains give a degree of flexibility (ability to change shape) as a function of hydrostatic pressure, i.e., these semisynthetic molecules are endowed with a degree of

Table 20.1 Influence of pattern of PEGylation of Hb on microcirculation parameters

Sample	FCD	Flow (normalized to baseline)	Tissue PO2 (mm Hg)
Dextran 70	0.38	0.80	1.1
Dextran 500	0.71	1.20	1.6
P5K2 Hb	0.77	0.93	7.0
P10K2 Hb	0.79	0.95	6.4
P5K4 canine Hb	0.71	0.79	2.5
P5K4 rHb (αH20C)	0.62	0.69	6.1
MP4	0.68	0.62	1.8
MP8	0.71	0.55	4.1

FCD functional capillary density, *rHb* recombinant Hb, *αH20C* histidine at position 20 in α-globin mutated to Cysteine. MP4 EAF P5K6 Hb of Sangart constituted as 4 gm % solution, MP8 EAF P5K6 Hb of Sangart constituted as 8 gm % solution

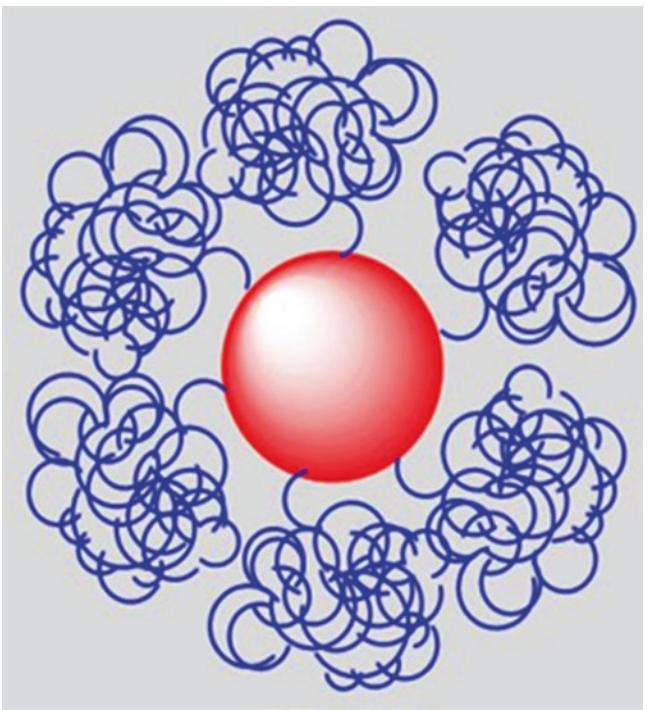

Fig. 20.3 Schematic representation of the six PEG-5K PEG-shell engineered onto Hb or Alb. The central core represents the protein (either Hb or Alb) where the polypeptide chains are packed compactly, ie., the atoms are packed densely. In the outer PEG-shell the six PEG chains are more disordered compared to central protein core. The outer PEG-shell contributes a mass of only 30 K of the total mass of 94 K, even though they occupy more space as compared to the protein core contributing to a mass of 64 K. Accordingly, the packing density of atom in PEG-shell is lower compared to that in protein core. The differential packing densities of the two regions of the molecule gives a degree of pseudoplasticity to these PEGylated molecules when they are in blood flow

pseudoplasticity. It may be noted that pseudoplasticity is an intrinsic property of RBC. Accordingly, these semisynthetic nanomaterials placed in plasma are "mini-RBCs", i.e., oxygen carrying nanoparticles (with a molecular radius around 6–7 nM). The pseudoplasticity and the high oxygen affinity of EAF P5K6 Hb distinguishes it from polymeric Hbs designed as oxygen therapeutics even though seems to have nearly comparable hydrodynamic volume.

Tissue Oxygenation with Supra Plasma Expanders in Situations of Low Hematocrit

In extreme hemodilution, the supra plasma expansion seen with dextran-500 is not accompanied by a noticeable increase in the level of tissues oxygenation. The inability of dextan-500 to improve tissue oxygenation reflects the level of anemia, i.e., overall poor (low) oxygen carrying capacity of the system (the hematocrit 11%, 4 gm % Hb), is too low to establish an oxygen gradient to facilitate the diffusion of oxygen from RBC through plasma to tissue even with the higher surface area and diminished blood flow.

The extreme hemodilution in hamsters essentially represents an experimentally induced severe anemia except for the fact the circulatory system has been partly compensated/stabilized by replacing the lost volume of blood with a conventional colloidal plasma expander with a viscosity around 2.8 cp. This model has served as a good system for mapping the oxygen therapeutics induced attenuation/improvement in mean arterial pressure, microcirculation, and improvement in tissue oxygenation, because the system is near to the critical oxygen carrying capacity.

MP4 (Hemospan at 4 gm %) is a supra plasma expander, superior to conventional colloidal plasma expanders (dextran-70). This is reflected by improved functional capillary density and microcirculatory blood flow. However, the tissue oxygenation seen with it is only marginally better than that seen with dextran 70 (Table 20.1). On the other hand, the microcirculatory response of MP8, (8 gm % solution of Hemospan) and with a viscosity comparable to dextran-500, did noticeably improve tissue oxygenation. It may be noted that the concentration of Hb in plasma with MP8 is only around 45% higher than that with MP4, not double. The colloidal osmotic pressure of MP8 is very high, and therefore a significant auto transfusion is induced by this molecule. This reflects the intrinsic potential of the high oxygen affinity EAF P5K6 Hb to deliver oxygen in situations of severe anemia.

EAF Hexa-PEGylation Induced Supra Plasma Expansion Activity of Alb

EAF P5K6 Alb has been generated as a prototype of EAF P5K6 Hb to map whether EAF hexa-PEGylation with maleimide-phenyl PEG-5K can induce supra plasma expansion. It may be noted that Alb and Hb have comparable molecular size, and both are globular proteins. Indeed, a 4 gm % solution of EAF P5K6 Alb gives a microcirculatory response comparable to MP4, but a flow rate higher than Hemospan. A 4 gm % just as EAF P5K6 Hb. Interestingly, it induces an increase in endothelial NO production in much the same way as high viscosity dextran-500 [10]. This is accomplished by increasing the shear thinning of blood. EAF hexa-PEGylation of Hb and Alb induces supra plasma expansion activity to these proteins. Engineering supra plasma expansion activity to Hb by EAF hexa-PEGylation coupled with the high oxygen affinity represents a major paradigm shift in the design of oxygen therapeutics discussed in this Chapter.

Hypertensive Activity and Tissue Oxygenation by Hb Modified at Cys-93(β) with Maleimide PEG and with High Oxygen Affinity

Intramolecularly crosslinked Hb, Bis succinimidyl PEG 10 K Hb through Cys-93(β) where the spacer arm of the crosslinker (PEG-chain) is located outside the central cavity of Hb [2] may be considered as an isomeric form of di-PEGylated Hb, PEGylated at its Cys-93(β) (P5K2 Hb). Both exhibit comparable high oxygen affinity Hb. Interestingly, the hydrodynamic volume of Bis succinimidyl-PEG 10,000 αα-fumaryl Hb as reflected by size exclusion chromatography is smaller than that of Bis succinimidyl PEG-10,000 Hb, and the latter is smaller than P5K2 Hb [11]. Two copies PEG-5K chains on Hb with its free distal end are more disordered than the spacer arm than in the intramolecularly crosslinked Hb, Bis Mal PEG-10 K.

The P5K2 Hb is non-hypertensive at 4 gm % and even though it has a viscosity of only 1.4 cp [11], and it maintains an excellent functional capillary density and blood flow in extreme hemodilution studies in the experimentally induced severe anemia hamster model [12]. When used at 4 gm/dl, P5K2 Hb with a viscosity just around 1.4 cp the functional capillary density and blood flow are in fact far better than dextran 70 control (with viscosity around 2.8 cp), and it may be noted that the high oxygen affinity Hb improves tissue oxygenation even though the amount of PEG Hb in plasma is only about 1 gm, and only about 20% of the total Hb in circulation (which is at nearly ~5 gm/dl). Nearly 4 gm/dl of the total Hb is coming from the RBC. Thus, a high oxygen affinity PEG Hb improves tissue oxygenation under conditions of severe anemia even though the level of the high oxygen affinity PEG Hb in plasma is only 20% of the total Hb in circulation. This represents about 10% of the total blood loss (about 8–10 gm) in hamster in extreme hemodilution studies that has been replaced as P5K2 Hb in the plasma. The microcirculation when P5K2 Hb is present in plasma, as represented by functional capillary density and blood flow, is also

better than with dextran 70 control. P10K2 Hb with a PEG mass conjugated to Hb double than that in P5K2 Hb also gives essentially identical results. When we can significantly improve the tissue oxygenation, the increase in the viscosity of the test solution of PEG Hb by itself contributes very little to improvement in microcirculation making dominant role of improved tissue oxygenation in dictating the microcirculation/perfusion readily apparent.

Pattern of PEGylation on PEG Hb Dictates the Efficiency of Tissue Oxygenation During Extreme Hemodilution

Direct PEGylation of Hb with maleimide on the reactive free thiols of Cys-93(β) in Hb (P5K2 Hb, P10K2 Hb or P20K2) was found to attenuate the intrinsic vasoconstrictive activity of Hb [11] as an inverse function of the PEG mass conjugated in top-load experiments with hamster. EAF P5K6 PEGylation of Hb provided the best non-hypertensive activity while P20K2 Hb provided the least attenuation of vasoconstrictive *activity*. Nonetheless, Hb with two PEG-5K chains, exhibited noticeable attenuation of the hypertensive activity of Hb, in comparison to control unmodified Hb [11]. Even though the oxygen affinity difference between EAF P5K6 Hb and P5K2 Hb is only marginal, the differences in tissue oxygenation level between P5K2 Hb and EAF P5K6 Hb is significant. Surprisingly, in extreme hemodilution studies we found that the functional capillary density and blood flow (i.e., microcirculation) does not correlate with the level of PEGylation or the oxygen affinity. We concluded that tissue oxygenation by the high oxygen affinity PEGylated Hb is impacted inversely by the amount of PEG in the PEG-shell of Hb, even though the overall effect of PEGylation on oxygen affinity appears to be minimal.

PEG-Shell of High Oxygen Affinity of EAF P5K6 Hb Attenuates the Efficiency of Tissue Oxygenation

In extreme hemodilution, tissue oxygenation obtained by P5K2 Hb is better in comparison to supra plasma expander, dextran 500 (Table 20.1). Though functional capillary density with these two preparations is comparable, the blood flow is noticeably better with dextran 500. When severe anemia is induced in hamster (4 gmHb/dl), a significant reduction in the oxygen carrying capacity (about 66 %) of the circulatory system, supra plasma expansion by itself is unable to significantly improve the oxygen delivery to tissues. Increasing the oxygen carrying capacity of the circulation in this extreme hemodilution model with 1 gm/dl of P5K2 Hb in plasma (20% the total overall oxygen carrying capacity) improves the tissue oxygenation, while the same amount of MP4 placed in plasma does not provide this therapeutic benefit. On the other hand, comparing MP8 to dextran 500 (with comparable viscosities), the MP8 is nearly as good at improving functional capillary density as MP4, but blood flow is noticeably lower compared to dextran 500. But MP8 improves tissue oxygenation better than dextran 500, although not as effectively as P5K2 Hb. Thus, at least in situations of severe anemia, the tissue oxygenation by high oxygen affinity PEG Hbs is not just a function of oxygen carrying capacity placed in plasma.

The increased oxygen affinity of P5K2 Hb is essentially a function of maleimide modification of Cys-93(β) while the contribution of the PEG-chains on the maleimide moiety in increasing the oxygen affinity appears to be marginal, if any. On the other hand, as multiple copies of the PEG-chains are conjugated to Hb and larger PEG-shell is engineered around Hb, further small increase in the oxygen affinity can be seen. But the influences of the maleimide modification of Cys-93(β) and the number of conjugated PEG-shells upon oxygen affinity do not seem to be additive or synergistic. The intrinsic oxygen affinity changes of PEG-Hbs is less than additive compared to the influence of maleimide modification of Cys-93(β) and of PEG-shell. Besides, as the number of copies of PEG-chains in the PEG-shell increase, the influence of allosteric effectors which to reduce the intrinsic oxygen affinity of Hb is significantly attenuated: increase in the destabilization (access to deoxy conformation) of oxy conformation by PEG shell occurs as the PEGylation increases. This is reflected as decrease in the delivery of oxygen. Tissue oxygenation by high oxygen affinity Hbs is not a direct correlate of the oxygen affinity.

Previous geminate recombination studies have shown that both maleimide modification of Cys-93(β) of Hb [13, 14] and the PEG-shell independently stabilize the oxy conformation and destabilize the deoxy conformation. Destabilization of the deoxy conformation is reflected by the observation that as the PEGylation increases allosteric effectors mediated lowering of the oxygen affinity of PEG Hb is [4] attenuated. Once Cys-93(β) of Hb is modified by a maleimide, the allosteric modification of oxygen affinity of Hb via modulation of geminate recombination by inositol hexa-phosphate (IHP) is essentially absent as compared to unmodified Hb.

These molecular aspects of reversible oxygen binding to PEG-Hbs could explain the lower efficacy of MP4 to improve tissue oxygenation in extreme hemodilution studies as compared to P5K2 Hb or P10K2 Hb. As noted earlier, the changes introduced by Sangart in the EAF PEGylation platform could have also contributed further to its lower efficacy. EAF P5K6 Hb prepared at Einstein provided a better tissue oxygenation as compared to MP4, however, its efficacy is noticeably lower than that of P5K2 Hb. The molecular dimensions of P5K2 Hb and EAF P5K6 Hb are significantly different, and we reason that the larger PEG-shell contributes to attenuation of the efficiency of oxygen release to tissues by PEG Hb, in spite of their contribution to induce supra plasma expansion activity to Hb.

High oxygen affinity of Hb is essentially a property of oxygen storage proteins like myoglobin. Increasing the oxygen affinity of Hb will have no influence on the oxygen carrying capacity of Hb since the oxygen tension at the lungs is very high due to high oxygen concentration in inspired air. While oxygen bound to Hb is unaltered, we anticipate that in the region where high oxygen affinity PEG-Hbs have to release their oxygen, tissue oxygenation will be a function of the oxygen affinity of the oxygen therapeutic placed in plasma and in particular stability of the deoxy conformation of PEG-Hbs, as well as the local tissue oxygen tension. The concentration of oxygen therapeutic in plasma represents the increased oxygen carrying capacity introduced to off-set lowered oxygen carrying capacity due to reduced hematocrit, in extreme hemodilution 11% instead of normal around 45%. The concentration of oxygen therapeutic in plasma should be high enough to build sufficient oxygen tension in the plasma to facilitate the diffusion mediated release of oxygen from plasma to tissues. Consideration of the structure of PEG-shell design, particularly the influence on- and off rates of oxygen from Hb is thus very important. Therefore, the molecular structure of engineered PEG-shell of PEG-Hb can contribute to attenuation of oxygen delivery by reducing the stability of deoxy conformation. This is apparent as evidenced by MP4 and the P4K2 design and the oxygen delivery by thesemolecules in extreme hemodilution. Even though MP4 did not improve tissue oxygenation, MP8 did improve the tissue oxygenation even though it is not as efficient as P5K2 Hb (at 4 gm %). The COP of MP8 is very high and this results in a significant auto transfusion and increased plasma volume, thereby the concentration of PEG Hb in plasma is not a direct correlate of Hb concentration in the test solution. The structural aspects of PEG-Hb coupled with increased plasma volume lowers the overall efficacy of tissue oxygenation when high oxygen affinity PEG Hb is used as oxygen therapeutics.

Design of EAF P3K6 Hb and Its Ability to Improve Tissue Oxygenation

One of the important differences between P5K2 Hb and EAF P5K6 Hb is the difference in molecular dimensions (hydrodynamic volume). Accordingly, we decided to evaluate the influence of the hydrodynamic volume of the PEG-shell on Hb generated by surface decoration of Hb with multiple copies of PEG-chains to improve tissue oxygenation. We decided to start with hexa-PEGylation to keep the number of PEG-chains conjugated to Hb same, six and reduce the molecular mass of PEG-chains to 3000 and generated EAF P3K6 Hb. This involves simply replacing maleimide PEG-5K with maleimide PEG-3K in Einstein EAF PEGylation platform.

Molecular and solution properties of EAF P3K6 Hb is compared with that of EAF P5K6 Hb in Table 20.2. The functional properties of EAF P3K6 Hb are essentially comparable to EAF P5K6 Hb except for the molecular and solution properties are distinct. This is anticipated because of the lower mass of PEG conjugated to Hb which is only 60% the mass of conjugated PEG in EAF P5K6 Hb. Compared to PEG-5K chains, the PEG-3K chains pack (organize/interact) much more compactly on the surface of Hb in the PEG-shell of EAF P3K6 Hb. The molecular shape of EAF P3K6 Hb as defined by molecular modelling appears to be more globular while EAF P5K6 Hb is more ellipsoidal.

Table 20.2 Molecular and functional properties of EAF hexa-PEGylated Hbs

Molecules	Radius (nm)	Molecular volume $(nm)^3$	Packing density of PEG shell	Oxygen affinity P50 mm Hg
HbA	3.0	116.6	–	14
EAF P3K6 Hb	4.9	493.0	47.7	8
EAF P5K6 Hb	6.5	1150.0	29.0	7–8

In view of the lower viscosity of 4 gm % solution of EAF P3K6 Hb compared to EAF P5K6 Hb, we used 6 gm % solution of EAF P3K6 Hb to evaluate the influence of PEG shell size on the tissue oxygenation in the extreme hemodilution in hamster. EAF P3K6 Hb increases the tissue oxygenation slightly better than that seen with P5K2 Hb or P10K2 Hb. This clearly reflects the influence of PEGylation pattern on the efficacy of PEG Hb to deliver oxygen. Even though the COP of a 6 gm % solution of EAF P3K6 Hb is higher than EAF P5K6 Hb and MP4, the concentration of EAF P3K6 Hb in plasma is just about 50% higher than that with MP4 or P5K2 Hb: increase in auto-infusion seems to be limited with EAF P3K6 Hb as this this increase plasma EAF P3K6 Hb is expected.

The functional capillary density seen with EAF P3K6 Hb is comparable to that seen with EAF P5K6 Hb, however flow is better with EAF P3K6 Hb compared to EAF P5K6 Hb. Both microcirculatory parameters are significantly better than with dextran 70. Therefore, we conclude that EAF P3K6 Hb is also a supra plasma expander just as EAF P5K6 Hb.

In extreme hemodilution studies, the amount of Hb placed in plasma in our studies by using a 4 gm % solution of PEG Hb represents only about 20% of total Hb in circulation. This will be slightly less than 30% in EAF P3K6 Hb experiments where we have used 6 gm % solution to maintain a viscosity comparable to that of 4 gm % of EAF P5K6 Hb. In severe anemia where hematocrit is very low and tissue oxygenation by the RBC (through direct diffusion) is essentially minimal, high oxygen affinity PEG-Hbs indeed improves oxygen delivery to tissues. In the presence of MP4 there is a significant oxygen debt in the venular blood as compared to EAF P3K6 Hb. In MP4 increase in tissue oxygenation is minimal, while with EAF P3K6 Hb it is slightly better than P5K2 Hb. However, concentration of EAF P3K6

Hb in plasma is nearly 50% more than with P5K2 Hb, i.e., overall efficacy may not reach the level seen with P5K2 Hb. The improved oxygen delivery in the presence of these high oxygen affinity Hbs could be a direct consequence of improved microcirculation, or increased oxygen carrying capacity of the system, or a combination of both increased oxygen carrying capacity and improvements in the oxygen transfer capability from the oxy-Hb in the RBC to the tissues (i.e., catalytic transfer). These aspects of high oxygen therapeutics contributing to improved tissues oxygenation are yet to be sorted out. We believe that oxygen transfer catalytic activity of PEG-Hbs (nano oxygen-pumps) plays a dominant role in situations of treating severe anemia, as more clearly reflected in the experiments with transgenic sickle mice discussed below.

It may be noted that we had generated P3K2 Hb also to see how this smaller molecule can compare with P5K2 Hb. However, this PEG-Hb was cleared from the circulatory system and P3K2 Hb was seen in the urine and thus could possibly damage the kidney (nephrotoxicity). Notably P5K2 Hb is not cleared through kidney, the shorter PEG 3K apparently binds tightly to the Hb molecule providing a compact molecule while the larger PEG 5K chains have more disorder in their structure. This apparently contributes significantly to the larger hydrodynamic volume and attenuation of kidney filtration (Fig. 20.4).

Therapeutic Application of High Oxygen Affinity PEG Hb in Treating the Pathophysiology of Sickle Cell Disease

Sickle cell disease is the first molecular disease to be identified and is a consequence of the presence of mutant Hb, namely HbS. HbS polymerizes when deoxygenated in a HbS concentration dependent fashion. The sickled erythrocytes are deformed to various degrees and clog the capillaries leading to regional vaso-occlusion. However, adult sickle cell anemia is essentially a co-morbidity disease. Accordingly, therapeutic benefits may be derived from approaches besides those focused on inhibiting deoxy HbS polymerization. Carbon monoxide has been advanced for modulation of the vaso occlusive crisis. Sangart's MP4CO, carbon monoxy Hb has been approved as orphan drug; however, Sangart ceased its operation before they could initiate a phase II clinical trial. Prolong's Sanguinate, a carbonmonoxy Enzon PEG Hb has also been advanced as an alternate therapeutic to inhibit polymerization, and phase II studies have provided positive results.

Alternatively, our studies evaluating EAF P3K6 Hb and EAF P5K6 Alb as a therapeutic agent to treat sickle cell disease using transgenic sickle mice have focused on the potential applications of supra plasma expansion activity with and without oxygen carrying capacity, respectively, to treat severe anemias and particularly with a goal of targeted oxygenation of hypoxic tissues.

(i) *Supra Plasma Expanders Attenuate Hypoxia Reoxygenation Mediated Vaso-Occlusion in Transgenic Sickle mice NY1DD, EAF P3K6 Hb is superior to EAF P5K6 Alb both in NY1DD and Berk***:** We used three models of transgenic mice in our lab at Einstein for study of SCD; each model differs from one another in terms of the degree of anemia and severity of disease. The mildest transgenic mouse model is NY1DD. The S + S Antilles models is also a mild model which express only mild anemia and very limited vaso-occlusion just as NY1DD but its tolerance for hypoxia is less than that of NY1DD. Severe vaso-occlusive episodes are induced into these mild models by subjecting the animals to hypoxia reoxygenation. The NY1DD model tolerates long exposure to hypoxia reoxygenation while the model S + S Antilles can tolerate only shorter durations of hypoxia and reoxygenation. In NY1DD and S + S Antilles, the cerebral blood flow is essentially comparable to the wild type of mouse, whereas the Berkley (Berk) mouse exhibits significantly higher CBF nearly 2.5 to three times higher than the wild type mouse and is accompanied by severe anemia with hematocrit levels around 30.

Intaglietta and his colleagues [15] have shown that MP4 oxy improves the functional capillary density in the Alabama transgenic mouse models (which are mild disease model just as with NY1DD) of sickle cell disease when the animals are exposed to hypoxia reoxygenation. This reflects the potential protective role of supra plasma expanders in mitigating the hypoxia induced influence on the microcirculation in mild sickle cell disease situations.

The therapeutic activity of supra plasma expanders with and without oxygen carrying capacity (EAF P3K6 Hb and EAF P5K6 Alb) to attenuate hypoxia-reoxygenation induced vaso-occlusion in the NY1DD models has been studied at Einstein. Both supra plasma expanders attenuated the hypoxia reoxygenation induced vaso-occlusion as reflected by the clearing of vaso-occlusion in veins of treated mice in comparison to untreated mice. EAF P3K6 Hb was better than EAF P5K6-Alb in this regard. The pattern of improvement obtained with EAF P3K6 Hb in the NY1DD was excellent – treated mice exhibited improvement as good as the wild type controls. Interestingly, pretreating NY1DD with EAF P3K6Hb protects the mice from developing hypoxia reoxygenation induced vaso-occlusion [16]. The amount of EAF P3K6 Hb in the plasma amounts to less than 10% of Hb in the RBC, which can hardly be considered as increasing the oxygen carrying capacity of blood. Rather, the protective effect against hypoxia-reoxygenation is likely like that obtained in experimentally induced extreme hemodilution studies that produce severe anemia discussed earlier. Though the level of anemia in NY1DD is mild, the hypoxia protocol induces a severe anemia as oxygen carried to tissues will be severely diminished. Accordingly, we conclude that the protection

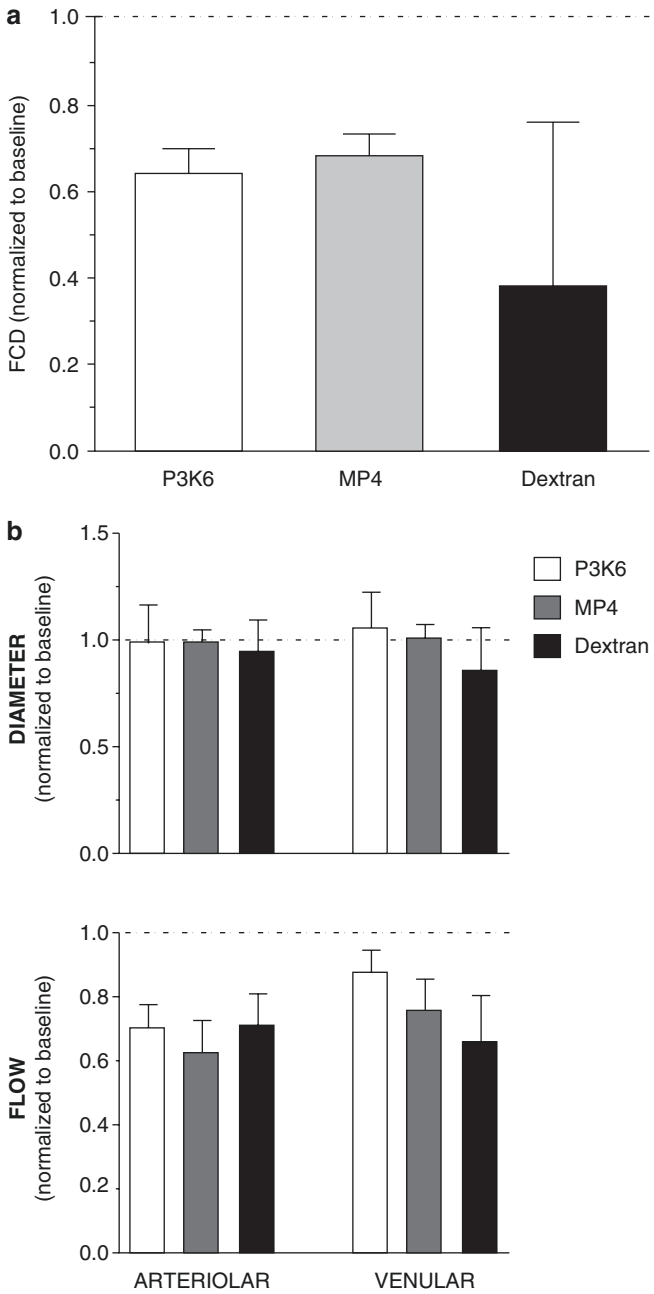

Fig. 20.4 (**a**) Influence of the mass of PEG-chain (size of PEG-shell) on Hb when it is surface decorated with EAF PXK6 pattern of PEGylation on microcirculation in extreme hemodilution studies in hamster as reflected by functional capillary density. X in PXK6 pattern, represents mass of the PEG-chains. (**b**) Influence of pattern of PEGylation of EAR PXK6 Hb, where as "X" represents the mass of PEG-chain used for surface decoration on blood vessel diameter and flow. The arterial diameter in all three materials. Venular diameter is essentially same as the base line but with dextran 70 it is slightly lower compared to PEGylated Hbs. The arterial and is nearly the same in all three samples, the venular flow seems to be best with EAF P3K6 Hb. (**c**) Influence of the mass of PEG-chain on Hb surface decorated in EAF PXKY pattern of PEGylation on oxygen distribution in arterioles, veins, and tissues in extreme hemodilution. Differences in Pattern of PEGylation on has essentially very limited influence on the PO2 in arterioles.as the P02 in the control dextran 70 samples is slightly less than the two Hb samples. Pattern of PEGylation on Hb impacts the venular PO2 levels. The PO2 level in EAF P3K6 Hb is comparable to that in control dextran sample are comparable while there is an oxygen debt in the MP4 sample. This suggests that as the blood has travelled from arterioles to veins, increased oxygen delivered to tissues has created an oxygen debt in the veins. Increased oxygen delivery (i.e., increased deoxygenation of oxy Hb as it travels through the capillaries to veins may be function of higher perfusion in the microcirculation due to the supra plasma expansion activity of MP4 or due to a combination of supra plasma expansion and increased oxygen extraction. Anyway, MP4 facilitates increased deoxygenation of oxy Hb. In dextran control there is not much decrease in oxy Hb in veins between EAF P3K6 Hb and dextran. Tissue oxygenation is best with EAF P3K6 Hb, and there is no difference in the tissue oxygen level in MP4 and dextran control. The difference in the tissue oxygenation level between that of dextran and MP4 is marginal. The decrease in venous blood should represent the metabolic consumption in MP4 treated animals

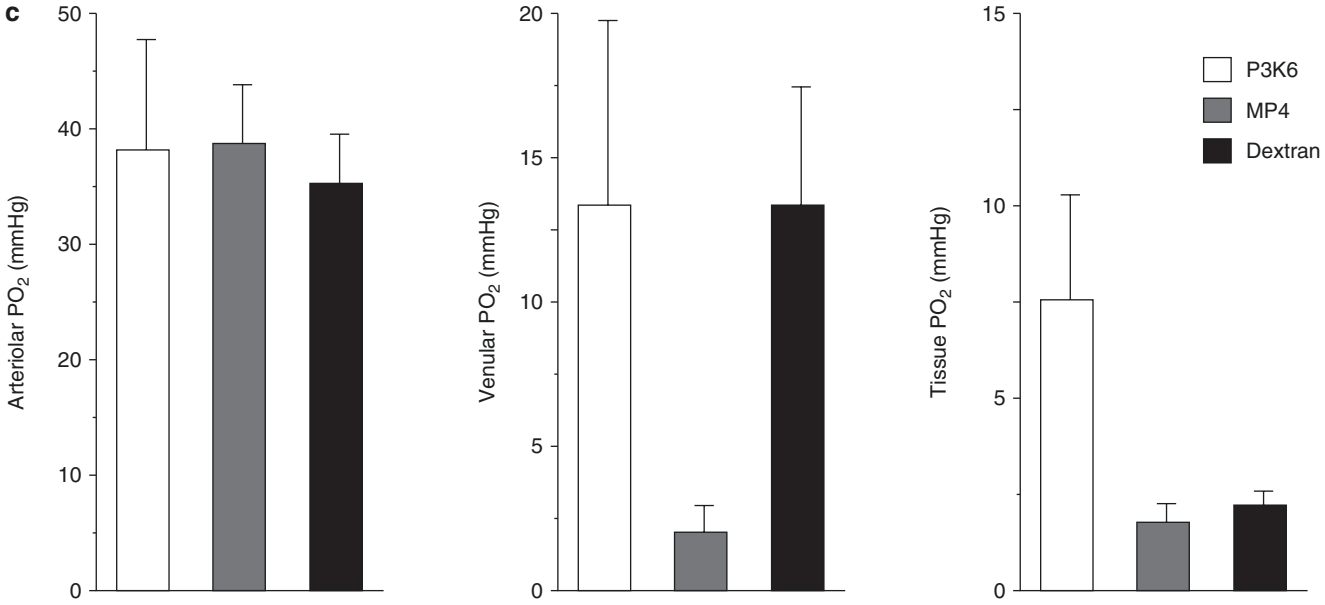

Fig. 20.4 (continued)

afforded by EAF P3K6 Hb represents essentially an improvement in the increase in the oxygen extraction from the RBC occurring during hypoxia. In the lungs, in a reverse way, EAF P3K6 Hb is expected to increase the oxygenation of the deoxy Hb in the RBC by facilitating the inflow of oxygen from alveolar space.

Berk is severe transgenic mouse model of sickle cell disease with severe anemia and intrinsic vaso-occlusion. Again, EAF P3K6 Hb is superior to EAF P5K6 Alb in attenuation of vaso-occlusion (Fig. 20.5). This reflects role oxygen delivery in attenuating the vaso-occlusion in these mice.

(ii) *Influence of Supra Plasma Expander EAF P5K6 Alb on Cerebral Blood Flow (CBF) in wild Type mice***:** We have demonstrated that the supra plasma expander EAF P5K6 Alb increases CBF after a 10% top load of the solution to the circulation. The increase in CBF was associated with a corresponding decrease in the level of deoxy Hb in the brain. This increase in CBF co-commitant with a decrease in de-oxy Hb is consistent with the supra plasma expansion activity seen in systemic microcirculation in extreme hemodilution studies (Fig. 20.6). It should be noted that in these studies, hematocrit was normal before and after administration of the compound and yet we still observed increased CBF. This finding is evidence of the supra plasma expansion activity obtained by top load of as little as 10% by volume of the test solution. Notably, the increase in CBF persisted for as long as 72 hours after the EAF P5K6 Alb infusion (Fig. 20.6). The 'luxury perfusion' induced by the supra plasma expander decreases the deoxy Hb content in blood.

(iii) *Influence of the severity of the sickle cell disease on the Cerebral Blood Flow (CBF) of transgenic sickle mice:* High CBF and increase in stroke represents a comorbidity of the adult sickle cell patients. We have compared the CBF in all three transgenic mouse models of sickle cell disease with that in wild type mice. In transgenic sickle mice there is an increase in CBF, (Fig. 20.7) and it corresponds to the severity of anemia just as the severity of the disease as represented by level of vaso-occlusion in steady state. The higher CBF shows a correspondence with levels of deoxy Hb in blood.

(iv) *EAF P5K6 Alb, Supra Plasma Expander without Oxygen Carrying Capacity, induces Oxygen Debt in the Brian in Transgenic sickle mice Berk:* Wild Type (WT) C57BL6 mice and three models of sickle cell disease (the mild NY1DD and Antilles models (not shown) and the severely anemic Berk model) underwent MRI assessment for CBF at 4 time points prior to and after administration of a 10% top load of EAF-P5K6-Alb, administered by tail vein. CBF was measured using arterial spin labeling methods, and brain tissue deoxy-Hb levels were assessed by blood oxygen level dependent changes observed before and after administration of 100% O2 [17] which is sensitive to the change in deoxy Hb in the venous effluent from the brain tissue. After the top load of EAF P5K6 Alb, CBF increased progressively, reaching maximal levels after 72 hours. In the NY1DD and Antilles animals, CBF remained

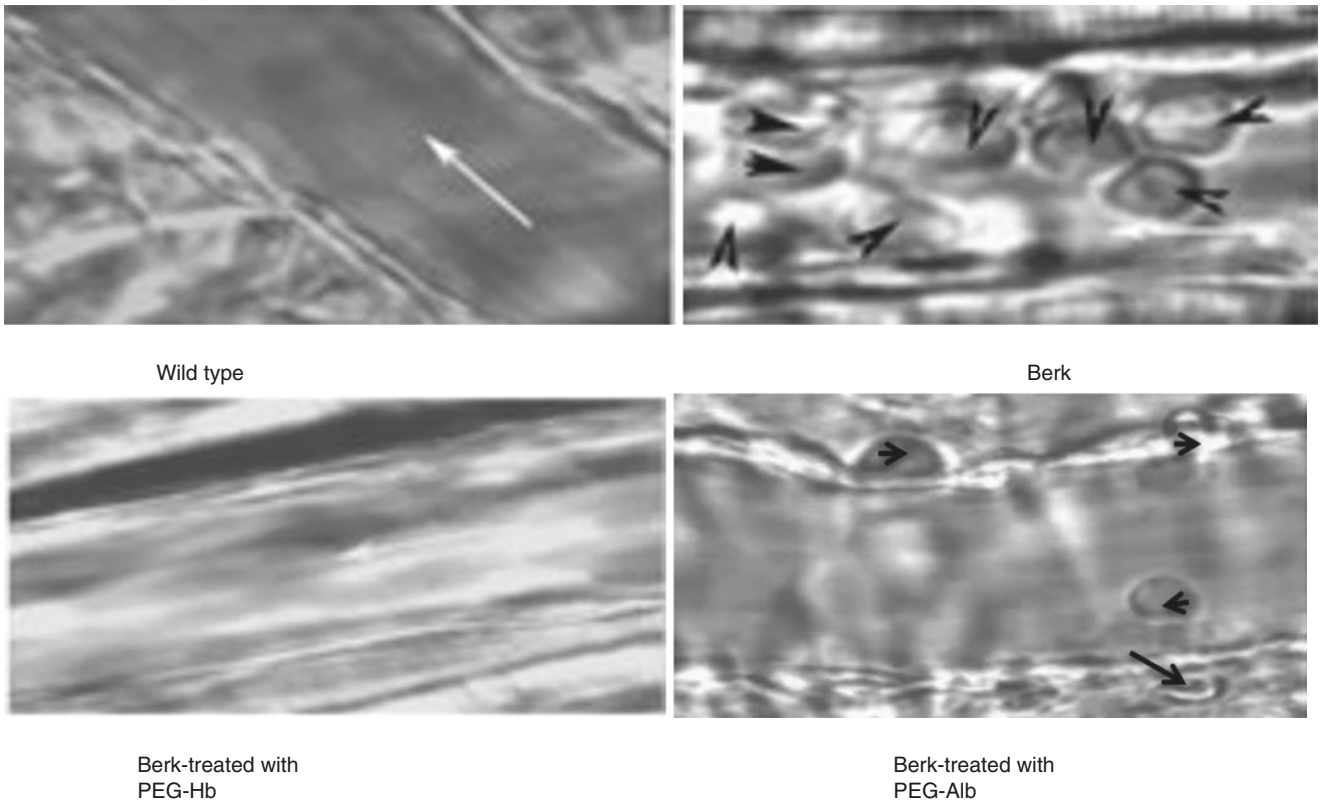

Fig. 20.5 Comparison of the video micrographs of veins of Berk mouse compared with wild type, before and after treating with EAF P3K6 Hb and EAF P5K6 Alb. Superiority of EAF P3K6 Hb can be seen from these studies

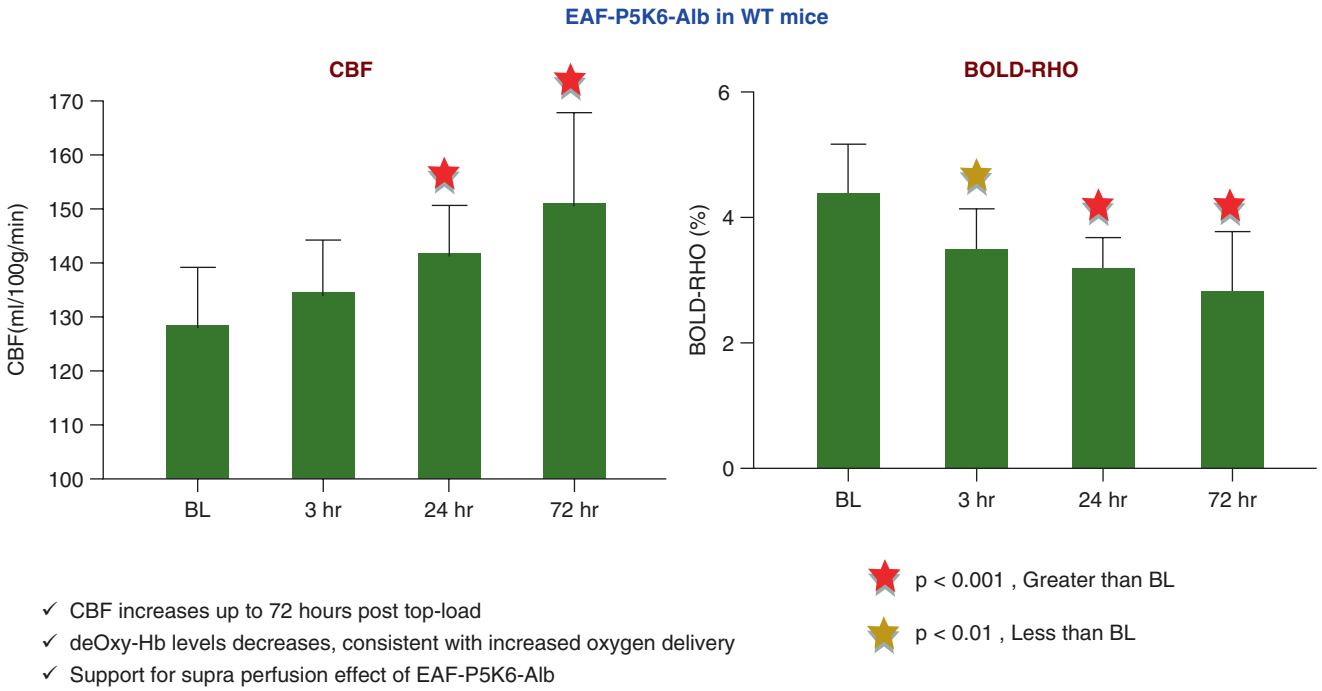

Fig. 20.6 Influence of top loading of EAF P5K6 Hb (10 %by volume) in wild type mice on cerebral blood flow (CBF) and deoxy Hb content in the blood as a function of time. CBF increases up to 72 hours after infusion of the test solution, in spite of the fact these mice of normal hematocrit. With the increase in blood flow, the amount of deoxy Hb in blood is reduced, consistent with a "luxury perfusion"

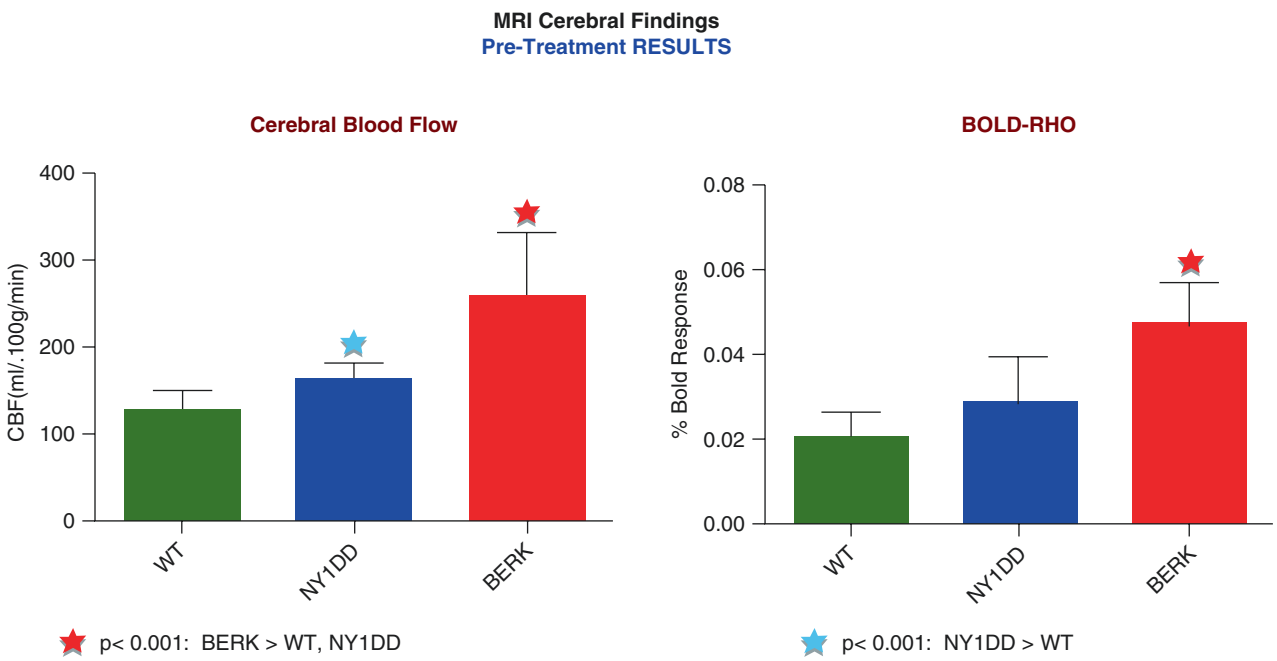

Fig. 20.7 Cerebral blood flow in transgenic sickle mice is a correlate of the severity of the disease. In transgenic mice, small but noticeable increase of CBF is seen. In S+ S Antilles, the CBF is nearly comparable to that of NY1DD. In Berk, severe disease model, CBF increased significantly. The increase in CBF is accompanied by corresponding decrease in deoxy Hb levels in blood

essentially unchanged at 24 hours or 72 hours. It may be noted that in these mice systemic circulation does not responds much to vaso-dilator, sodium nitroprusside, whereas the WT mice respond readily to vaso-dilators.

Surprisingly, Berk animals responded with decreases in CBF after top load of EAF P5K6 Alb. In Berk animals, anemia has driven the CBF significantly above WT levels, to facilitate an increase in oxygen delivery to meet cerebral metabolic demand in the face of inadequate oxygen carrying capacity. These CBF changes are confirmed in the deoxy-Hb measurements (sight side).

Increasing CBF in WT animals was associated with a concomitant increase in the efficacy of oxygen extraction in tissues and more efficient (frequent) reoxygenation of deoxy-Hb in lungs, resulting in decreased deoxy-Hb in the venous blood. In NY1DD animals, no change in CBF was reflected in no change in deoxy-Hb levels but the therapeutic benefit of EAF P5K6 Alb in attenuation of the hypoxia reoxygenation induced vaso-occlusion in systemic circulation has been demonstrated. The response of cerebral circulation in anemic BERK mice is very distinct as compared to WT or NY1DD. In BERK animals there is small drop in CBF on infusion of EAF P5K6 Alb. This reduction in CBF induced an increase in venous deoxy-Hb levels, suggesting further metabolic impairment. While the findings in the WT animals are interpreted to reflect the supra plasma expansion activity of the EAF P5K6 Alb, the decrease in Berk is likely resulting from an impaired CBF regulatory system in which nitric oxide responsiveness is impaired from NOS scavenging of free Hb from RBC lysis. It may be noted that Berk mice exhibit intrinsic high vaso-occlusion in systemic circulation, and it is cleared significantly cleared by EAF P5K6 Hb. Accordingly, this result suggests the need for SCD therapeutic compounds that improve the systemic circulation by attenuating the vaso-occlusion should be coupled with strategies that provide additional oxygen to hypoxic tissues like brain and kidney, particularly in disease situations with severe anemia.

(v) *EAF P3K6 Hb, a Supra Plasma Expander that Binds Oxygen Reversibly, Normalizes CBF in Berk without*

Creating an Oxygen Debt: Infusion of EAF P3K6 Hb to NYIDD prior to submitting the animals to hypoxia reoxygenation platform, protects these transgenic mice developing severe vaso-occlusion (Feng et al). Besides, EAF P3K6 Hb clears the systemic vaso-occlusion completely in Berk, and significantly better than EAF P5K6 Hb.

To map the influence of supra plasma expanders with and without oxygen carrying capacity on the cerebrovascular system, EAF P3K6 Hb was administered to WT and Sickle mice, and the response compared to the response after EAF P5K6 Alb, which does not carry Hb. In the WT and NY1DD mice there were no significant changes in either CBF or deoxy-Hb, except for a short-term slight reduction in CBF right after the top load was given for which no change in deoxy-Hb was observed. However, in severely anemic Berk mice the response was quite different, as illustrated here. While the top load of EAF P5K6 Alb induced a slight decrease in CBF which increased deoxy-Hb levels, the top load of EAF P3K6 Hb transiently decreased CBF but they rebounded above pre-top load levels by 72 hours. Importantly, the deoxy-Hb levels remained unchanged from pre-top load levels throughout these changes, suggesting that the EAF P3K6 Hb improved the oxygen extraction even when delivery was reduced by reduced CBF. This effect must be mediated by increased oxygen carrying capacity and a targeted delivery to the most hypoxic tissues. Thus, the EAF P5K6 Hb was protective against the decrease in CBF and maintained normal oxygen tension in the tissue.

Novel Aspects of EAF PEGylated Albumin and EAF PEG-Hb as Oxygen Therapeutics

Both EAF PEGylated Alb and EAF PEGylated Hb may be considered as novel oxygen therapeutics that help us to modulate oxygen delivery without significantly altering the oxygen delivering capacity of the circulatory system. These supra plasma expanders can be considered as novel nanoparticles that are endowed with RBC like biophysical property to modulate the microcirculation, and as such are unique molecular structures. These are nanostructures only from the perspective of their hydrodynamic volume and not by their mass. EAF PEG-Alb, one without any oxygen carrying capacity improves microcirculation in situations when the hematocrit levels are low (about 25% of the original) without significantly improving the tissue oxygen levels (extreme hemodilution). Even when hematocrit is normal, the supra plasma expansion properties increase the CBF and lowering the deoxy Hb levels in blood much like a vasodilator without apparent hypotensive effects. It may be noted that, we have previously suggested that EAF PEG Alb can be used in place of small amounts of blood for transfusion; this is possible since EAF PEG Alb improves oxygen extraction by improving functional capillary density and blood flow. It may be noted human body is designed to use just about 20% of the overall oxygen carrying capacity of the circulatory system. This redundancy in the oxygen carrying capacity vs the real need for the metabolic reaction allows us to minimize the use of blood transfusion when blood loss or anemia is marginal; if we can find a better way of improving the oxygen extraction when there is blood loss or anemia. This is a totally novel concept and practical application of these concepts requires further studies and undertaking of the clinical trials.

Improved oxygen extraction in the presence of supra plasma expander appears to be a function of the reversible oxygen binding of EAF P3K6 Hb in plasma in the circulatory system. The resolution of hypoxia reoxygenation induced vaso-occlusion in NY1DD (low anemia sickle cell model) is accomplished quicker in the presence of EAF PEG Hb than in its absence. On the other hand, EAF P3K6 Hb is better even when anemia is present and is in the range of transfusion trigger, i.e., severe as in Berk.

However, when severe anemia is present as in Berk (with Hb levels around 7– gm/dl), EAF P5K6 Alb creates an oxygen debt in the brain, even though we could see significant therapeutic benefit in terms of attenuating vaso-occlusion in systemic circulation. On the other hand, EAF P3K6 Hb normalizes CBF and provides protection against the by normalizing CBF in maintaining oxygen saturation in the blood. This is apparently accomplished by improved extraction from RBC, thus avoiding the creation of transient oxygen debt.

We believe that the therapeutic activity of EAF P3K6 Hb in BERK mice, particularly in normalizing the CBF without increasing the deoxy Hb content in the venous blood, cannot be attributed simply to increased oxygen carrying capacity afforded by EAF P3K6 Hb placed in plasma. The high oxygen affinity PEG Hb in the plasma accounts for less than 10% of total Hb in the circulatory system (Hb in RBC + EAF P3K6 Hb in the plasma). It may be noted that normalization of CBF in Berk by fetal Hb, requires the Hb content in blood to be around 11–12 gm/dl. This implies that the efficacy of EAF P3K6 Hb placed in plasma is nearly onefold higher than fetal Hb in the RBC. This is counter intuitive to our present understanding of delivery of oxygen by Hb, increase the oxygen carrying capacity to compensate for the reduction in hematocrit (oxygen carrying capacity).

Accordingly, we suggest the high oxygen affinity of EAF P3K6 Hb placed in plasma facilitates the transfer of oxygen from RBC by a "pull" mechanism to maintain a steady-state level of oxygen tension in plasma and this facilitates the diffusion of oxygen to the tissues. The supra plasma expansion activity of EAF P3K6 Hb, particularly, the increased functional capillary density facilitates a better diffusion of oxygen from plasma to tissues. PEG Hb in plasma acts as a nanomachine (catalysts) that facilitates more out-flow of oxygen from RBC to plasma (nano oxygen-pump). The increased oxygen tension in plasma due to the presence of EAF P3K6 Hb facilitates the diffusion (push) of oxygen to tissues thereby facilitating an increase in the delivery of oxygen to the tissues. The concepts of supra plasma expansion by low viscosity colloidal plasma expanders and catalysis of oxygen transfer by high oxygen affinity Hb are new concepts. The synergistic combination of these two in EAF PEG Hb provides a new opportunity to design new-found members of this novel class of molecules.

In severely anemic Berk mice, increasing the oxygen extraction from RBC is expected to increase the concentration deoxy HbS in the circulation without a compensation for increasing the oxygen carrying capacity of existing RBC, Increased oxygen extraction from RBC is a polymerization promoting process. However, we have not seen any evidence of that with Berk mice and deoxy HbS levels in the brain is essentially remains unchanged even when CBF has been pretty much normalized to wild type levels. Accordingly, we hypothesize that in the presence of EAF P3K6 Hb, there is an increase in the oxygen saturation of HbS in RBC in the lungs, i.e., an increase in the oxygen carrying capacity blood.

It is generally known that P50 of RBC in HbSS patients is around 35 mm Hg whereas the P50 of RBC from HbAA individuals is around 28 mm Hg. However, the intrinsic oxygen affinity of HbA *in vitro* is indistinguishable from that of HbS. 0xygen saturation levels in normal individuals is around 99% while that with SCD patients is around 95%. The lower oxygen affinity of RBC in SCD patients reflects the problem associated with inflammation in lungs and the presence of small deoxy HbS polymers in the RBC. The presence of high oxygen affinity EAF P3K6 Hb in the plasma, apparently, facilitates the in-flow of oxygen to RBC from the alveolar space in the lungs (a reverse of what happens in the tissues). The presence of EAF P3K6 Hb increases the oxygen tension in the plasma higher as compared to its absence, this facilitates the oxygenation of deoxy HbS in lungs better than in the control animals. This likely leads to the depolymerization of some deoxy HbS polymers in the RBC, thus adding to the increased oxygen carrying capacity of the blood. It may be noted here oxygen therapy is being advanced as a novel strategy to treat painful vaso-occlusive crisis in SCD patients and this is to increase the oxygen saturation in patients with sickle cell disease [16, 17].

The SCD therapeutic activity of EAF P3K6 Hb to attenuate hypoxia-reoxygenation induced vaso-occlusion in NY1DD as well as the normalization of CBF in Berk without any changes in deoxy HbS content in the veins, suggests a potential application of EAF P3K6 Hb as an alternate to oxygen therapy in emergency medicine and critical care medicine. Oxygen therapy is standard platform to treat pulmonary complications due to insufficient oxygenation of Hb in lungs, including but not limited to Covid-19. Low venous oxygenation levels are equivalent to hypoxia induced low saturation of Hb in blood. Our experience with EAF P3K6 Hb in transgenic sickle mice opens this approach for treating situation where there is a drop in oxygen saturation levels requiring oxygen therapy (Fig. 20.8).

Increasing oxygen affinity of Hb in RBC has been advanced as a therapy to treat Covid-19, hydroxymethyl furfural has been advanced as a potential therapeutic for this purpose [18]. GBT-440 [19], approved as an emergency medicine for sickle cell disease, is probably a better therapeutic to increase the oxygen affinity of Hb *in vivo*. Though there appears to be little doubt in terms of therapeutic benefit of GBT-440 in sickle cell disease, the mechanism of action of the therapy has been debated. Increasing the oxygen affinity of Hb has been suggested to increase the anemia effect in sickle cell anemia patients [20] just as increasing the oxygen extraction using supra plasma expanders. On the other hand, just as with high oxygen affinity oxygen therapeutic EAF P3K6 Hb, the high oxygen affinity RBC could also accomplish a targeted oxygenation of hypoxic areas of body in sickle cell patients. Similarly, high oxygen affinity RBCs can increase saturation of oxygen in lungs thereby contributing to overall therapeutic benefits. High oxygen affinity RBC generated *in situ* could also lead to increased oxygen saturation in lungs as well. High oxygen affinity fetal Hb has been demonstrated to accomplish peripheral oxygen saturation [20]. Increasing the oxygen affinity of RBC has also been shown to improve microcirculation and better tissue oxygenation [21]. An Interesting situation could be a combination of high oxygen affinity inducing GBT 440 along with EAF P3K6 Hb in treating severe sickle cell patients as well as patients with pulmonary complications with reduced oxygen saturation levels. In this respect, of particular interest, is covid-19 patients with severely low levels of oxygen saturation.

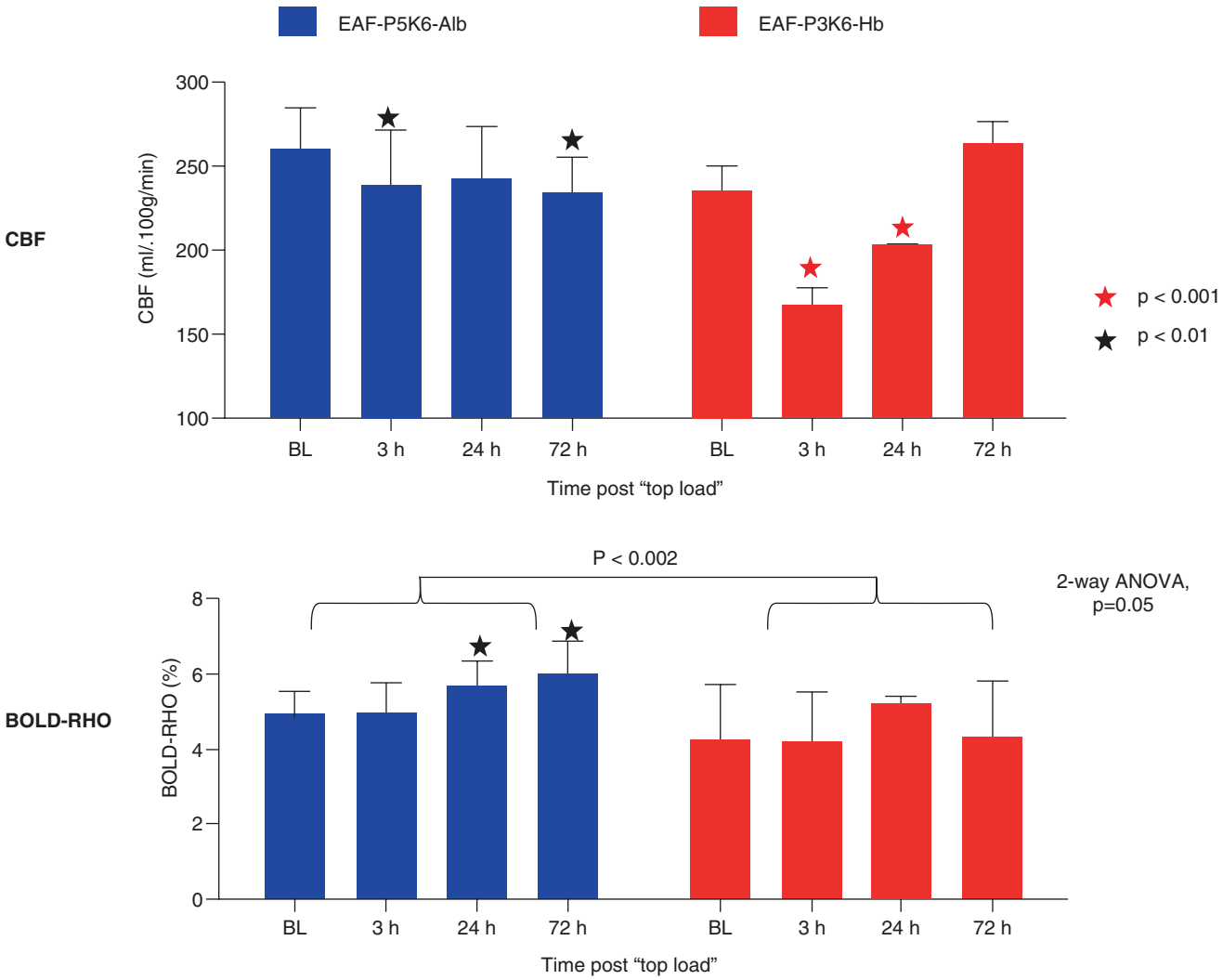

Fig. 20.8 Comparison of the influence of top loading transgenic sickle mice Berk with EAF P5K6 Alb and EAF P3K6 Hb on brain CBF and deoxy Hb levels. Hb levels in Berk mice is around 7–8 gm %, and hence are very severe. Berk mice do not show the supra perfusion seen with wild type, i.e., the system tuned up as much as possible. None the less we can see some lowering of the high CBF Berk mice, a consequence severe sickle cell disease with severe anemia. Even though EAF P5K6 Alb clears the systemic vaso-occlusion noticeably, it does very little to normalize the CBF and is accompanied by creating an oxygen debt in the brain. EAF P3K6 Hb, normalizes the CBF in 3 hours without creating an oxygen debt in the brain, and gets back to pre-infusion level by 72 hour

General Conclusions

In this chapter we have refocused the issues one needs to consider when designing high oxygen affinity Hb as a new class of oxygen therapeutics, particularly the influenced engineered modifications on on- and off-rates of oxygen form Hb and the role of the PEG shell in dictating the outcome when administered into animals. Our studies have established high oxygen affinity EAF P3K6 Hb is more efficient relative to EAF P5K6 Hb and MP4. The anti-anemia therapeutic efficacy as reflected from the studies with transgenic sickle mice is significantly higher than increasing the hematocrit level (transfusion). Surprisingly, this efficacy is superior to generating high oxygen affinity RBC *in vivo* either by chemical or genetic approaches as well; we calculate this efficacy as reflected by the normalization of CBF is nearly an order of magnitude better. Accordingly, a new concept has been advanced here: the efficacy of SCD therapeutic activity of high oxygen affinity EAF P3K6 Hb placed in plasma in transgenic sickle mice stems from a catalyst- like activity of EAF P3K6 Hb, a nano oxygen pump. This activity also facilitates an in-flow of oxygen to RBC in lungs besides the out-flow of oxygen from RBC to tissues in rest of the body. As such, the newly designed oxygen therapeutic is used in very small amounts as compared to Hb inside RBC (less than 10%).

These results are counter intuitive to current paradigm of designing blood substitutes which focus on increasing the oxygen carrying capacity of circulatory system. In the design of EAF P3K6 Hb and EAF P5K6 Hb, a new approach to design semisynthetic supra plasma expander has been exposed. The design of EAF P5K6 Alb, a non-oxygen carrying semisynthetic supra plasma expander confirms this activity is independent of oxygen binding/carrying capacity. However, we need to recognize that supra plasma expansion by EAF P5K6 Hb and EAF P5K6 Alb may be very distinct and may be a function of the efficacy of release of oxygen from RBC. It may be noted that release of oxygen from oxy Hb in RBC will also release NO, the vasodilator [21]. Thus the targeted oxygenation of tissues by high oxygen affinity PEG-Hb also accomplishes a targeted release of Nitric oxide, or targeted vasodilation. This is independent of the intrinsic supra plasma expansion activity engineered by EAF hexa-PEGylation. Thus, with EAF P3K6 Hb and EAF P5K6 Hb, a better perfusion could be expected with PEG Hbs relative to EAF P5K6 Alb through a synergy of improved shear thinning (supra plasma expansion) and release of NO through improved oxygen extraction of oxygen (deoxygenation) inside RBC. Supra plasma expansion by EAF P5K6 Alb operates primarily through increased shear thinning of blood and endothelial NO production. Increased deoxygenation of oxyHb inside RBC in the presence of high oxygen affinity PEG-Hb increases the flexibility of RBCs. Besides, with the newly designed PEG Hbs the low-level use of oxygen therapeutic is expected to attenuate the toxicity associated with the development of blood substituted as oxygen carrying materials on the one hand and the cost of the treating patients on the other.

Our initial thoughts in the design of high oxygen affinity EAF P5K6 Hb has been to target the oxygenation of hypoxic tissues, where the oxygen demand is high. EAF P3K6 Hb is essentially devoid of any Bohr effect. Accordingly, the in-flow and out-flow of oxygen to RBC in lungs and to tissues, respectively, is enhanced during acidosis essentially as a linear function of lowered pH induced by anemia/hypoxia. As the pH is lowered, the affinity oxygen to Hb inside RBC is lowered, i.e., the difference in the affinities of Hb inside RBC and EAF P3K6 Hb increases. The net oxygen-pull power of EAF P3K6 Hb increase in hypoxic tissues as a function of acidosis. Application of high oxygen affinity oxygen therapeutics as molecular pump placed in plasma to increase oxygen extraction from RBC that synergizes the high oxygen affinity PEG-Hbs with its supra plasma expansion activity induced by EAF PEGylation for treating anemia or blood loss, represents a clear paradigm shift for designing novel class of a oxygen therapeutics.

Future Directions

It may be noted here that microcirculatory parameters of P5K2 Hb are better than of EAF P3K6 Hb. Concomitant with this, the relative efficacies of tissue oxygenation of P5K2 Hb is better than with EAF P5K2 Hb (Table 20.3). The relative efficacies of some of the PEG Hbs studied by us is summarized in Table 20.3 and compared with that of MP4 and MP8, suggesting that tissue oxygenation is not a direct correlate oxygen affinity, but better tissue oxygenation is generally associated with better microcirculatory parameters. The data in Table 20.3 suggest di-PEGylated Hb is a better 'nano oxygen pump' compared to hexa-PEGylated Hb. We anticipate that the shear thinning is probably maximum with EAF P3K6 Hb and EAF P5K6 Hb, supra plasma expanders and hence expected to provide a better microcirculation. Therefore, higher efficacy of tissue oxygenation with P5K2 Hb is indeed surprising and suggest that as the efficacy of oxygen extraction from RBC increases, the concomitant release of NO plays a more important role in providing better microcirculatory parameters than the hexa-PEGylation induced supra plasma expansion.

Improving the efficacy of tissue oxygenation using small amounts of high oxygen affinity Hb placed in plasma through better oxygen extraction from RBC is a novel concept advanced here. The transfer of oxygen from oxy Hb in RBC to tissues is marginal even with supra plasma expander like EAF P5K6 Alb or dextran 500, i.e., diffusion mediated transfer of oxygen from through plasma when the hematocrit is low is negligible. The increase in the transfer of oxygen in the presence of high oxygen affinity Hb could be accomplished either through improved oxygen tension in the plasma (established through reversible equilibrium or through facilitated transport of oxygen by PEG-Hbs in the plasma. The release of vasodilator by the deoxygenation of oxy Hb inside RBC appears add significantly more improvement in microcirculation than that achieved through supra plasma expansion. The difference between EAF P3K6 Hb and EAF P5K6 Hb in tissue oxygenation certainly empha-

Table 20.3 Relative efficacies of tissue oxygenation by PEGylated Hbs

	Relative efficacy of tissue oxygenation
PEGylated sample	Tissue PO2/Conc of Hb in plasma
P5K2 Hb	7.8
P10K2 Hb	8.0
P5K4 canine Hb	4.0
P5K4 rHb(αH20C)	5.6
EAF P5K6 Hb (Einstein)	3.0
MP4 (EAF P5K8 Hb)	1.6
MP8 (EAF P5K8 Hb)	2.5
EAF P3K6 Hb	5.1

sizes the relative roles of on and rates of interaction of oxygen with PEG Hb.

Though Hb EAF hexa-PEGylated with maleimide PEG-5K was chosen initially because this represented the maximum number PEG-5K chains that can be accommodated on the molecular surface of Hb without perturbing interdimeric interactions of PEG-Hb. However, higher number of PEG-chains seems to attenuate the efficacy of oxygen extraction RBC and thus tissue oxygenation by high oxygen affinity Hbs. Lowering the mass of PEG-chains conjugated to Hb in hexa-PEGylated shows improvement in tissues oxygenation. In doing we have also reduced the molecular dimensions of hexa-PEGylated Hb. It may be noted that there is generally an increase in the efficacy of tissue oxygenation as the molecular dimensions, i.e., hydrodynamic volume of Hb is reduced. As the molecular dimensions are reduced, the viscosity of the plasma layer is also reduced and this impact the movement of PEG Hb in the plasma layer.

It has been noted earlier that oxygen therapeutics deliver oxygen more efficiently than the Hb inside RBC and this has been attributed to the facilitated transport of oxygen by Hb in plasma as compared to the diffusion of oxygen through plasma layer. We believe that this is the case with high oxygen affinity PEG-Hb as well and the inverse correlation of relative efficacy of PEG-Hb mediated improvements in tissue oxygenation as a function of the molecular dimension of PEG-Hb suggests that there may be room to further improve the efficacy of these 'nano oxygen pumps' either through the manipulation of the off rates of oxygen from oxy-PEG Hb or through manipulation of the packing density of PEG-shell.

We have generated P5K2 αα-Hb as way of stabilizing the deoxy conformation of P5K2 Hb molecule, which increases the P50 of the molecule. Preliminary studies show P5K2 αα-Hb retains better microcirculatory properties as compared to EAF P3K6 Hb, but not as effective as P5K2 Hb. The efficacy of tissue oxygenation is more comparable to that of EAF P3K6 Hb. The oxygen affinity of P5K2 αα-Hb is around 12 mm Hg, i.e., lower than that of P5K2 Hb. The lower P50 reflects the easy access of deoxy conformational state for this molecule compared to P5K2 Hb due the presence of αα-intramolecular crosslink. This presumably facilitates an early release of oxygen in plasma and thereby lowers the efficacy of this molecule as "nano oxygen-pump" even though the lower molecular mass and lower plasma viscosity with P5K2 αα-Hb as compared to EAF P3K6 Hb should increases its efficacy as 'nano oxygen pump'. Accordingly, both structural aspects contribute to the efficacy of high oxygen affinity PEG-Hbs to facilitate better tissue oxygenation. The slight increase in the oxygen affinity of Hb from that of P5K2 Hb to that of EAF P3K6 Hb may have contributed to the attenuation the microcirculatory benefits seen in extreme hemodilution with P5K2 Hb.

Accordingly, we need to ask a new question: (i) can we increase the packing density of the high oxygen affinity Hb by generating non-PEGylated high oxygen affinity Hb and (ii) then can we synergize the supra plasma expansion activity of EAF P5K6 Alb with a high oxygen affinity non-PEGylated Hb, for example αα-Hb modified at its Cys-93(β) with a maleimide or alternated oligomerized αα-Hb that has been generated using new oligomerization platform, extension arm facilitated short bis PEG-maleimide mediated oligomerization. The latter platform introduces only intermolecular crosslinks, on an average, one intermolecular crosslink per tetramer through Cys-93(β). This product has molecular dimensions comparable to EAF P5K6 Hb but has a compact packing density essentially same as Hb. The product is a high oxygen affinity polymeric Hb with an oxygen affinity around 12 mm Hg; the lower oxygen affinity stemming from the stabilization deoxy like structure of Hb by αα-fumaryl cross bridge and this could provide an insight into the lower efficacy of tissue oxygenation EAF P3K6 Hb as compared to P5K2 Hb. Similarly, αα-Hb could be dimerized using Bis Mal-PEG-600 and the second Cys-93(β) modified with N-ethyl maleimide, this will have molecular dimensions and oxygen affinity comparable to P5K2 Hb. A study of tissue oxygenation by these can help us to map the relative roles of facilitated transport of oxygen and diffusion mediated transport of oxygen.

Finally, though both EAF P5K6 Alb and EAF P3K6 Hb are supra plasma expanders, the response of cerebral circulation to these two supra plasma expander is very distinct as seen with wild type mice. EAF P3K6 Hb, does not show an increase in CBF, in fact a small decrease during early times and it reverses to higher values with longer times. In wild type mice the response to EAF P3K6 Hb is short lived as compared to EAF P5K6 Alb. We believe that this reflects the differential stability of these two semisynthetic proteins and coupled with high oxidative stress in transgenic sickle mice. Presumably, the subunit structure of Hb and the potential dissociation of the tetrameric structure into αβ-dimes contributes to this instability. Alb is a single polypeptide chain even though molecular mass of the two proteins is essentially the same. Intramolecularly crosslinked αα-Hb could be used to generate EAF P3K6 Hb to overcome the limitations and then the therapeutic efficacy can be considered with P5K2 αα-Hb to see when the normalization effect could last longer with these molecular species. Transgenic sickle mice, particularly Berk is a good system to evaluate/establish many new concepts delineated in this chapter for designing new class of oxygen therapeutics.

Key Points

High oxygen affinity oxygen therapeutic, EAF PEG-Hb, supra plasma expansion, Nano-oxygen pumps, on and off loading of oxygen to RBC, sickle cell disease, semisynthetic

supra plasma expanders, oxygen therapy, high oxygen affinity RBC.

References

1. Jansen FB. The dual role of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of blood flow. J Exp Biol. 2009;212:3387–93.
2. Acharya AS, Manjula BN, Smith P. 1996. Hemoglobin crosslinkers. US Patent 5585484.
3. Manjula BN, Tsai AG, Intaglietta M, et al. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated by thiolation: influence on hemoglobin structure and function. Protein J. 2005;24:433–63.
4. Li D, Manjula BN, Acharya AS. Extension arm facilitated PEGylation of hemoglobin: correlation of properties with the extent of PEGylation. Protein J. 2006;25(4):263–74.
5. Ananda K, Acharya AS. Role of extension arm in PEG-Hb conjugates on the Stability of Tetramer: Nonconservative EAF maleimide thiol-PEG mediated PEGylation. Artif Cells Blood Substit Biotechnol. 2008;36:499–512.
6. Li D, Hu T, Manjula BN, et al. Non-conservative surface decoration of hemoglobin: influence of neutralization of positive charges at PEGylation sites. Biochem Biophys Acta. 2008;1784:1395–401.
7. Li D, Hu T, Manjula BN, et al. Extension arm facilitated PEGylation of alpha-alpha hemoglobin with modifications exclusively targeted to amino groups: functional and structural advantages of free Cys-93(beta)in PEG-Hb adduct. Bioconjug Chem. 2009;20:2062–70.
8. Winslow RM. MP4, a nonvasoactive polyethylene glycol-hemoglobin conjugate. Artfi Organs. 2004;28:800–6.
9. Vandegriff KD, Malavalli K, Wooldridge J. MP4, a new nonvasoactive PEG-Hb conjugate. Transfusion. 2003;43:509–16.
10. Sriram K, Tsai TG, Cabrales P, et al. PEG-albumin supra plasma expansion is due to increased vessel wall shear stress induced blood viscosity shear thinning. Am J Physiol Heart Circ Physiol. 2012;302:H2489–97.
11. Manjula BN, Tsai AG, Upadhya R. Site-specific PEGylation of hemoglobin at Cys-93(β): correlation between the colligative properties of PEGylated protein and the length of the conjugated PEG-chain. Bioconjug Chem. 2003;14:464–72.
12. Cabrales P, Kanika N, Manjula BN, et al. Microvascular P02 during extreme hemodilution with hemoglobin site specifically PEGylated at Cys-93(β) in hamster window chamber. Am J Physiol Heart Circ Physiol. 2004;287:11609–17.
13. Juszazak LJ, Manjula BN, Bonaventura C. UV resonance Raman study of β-93-modified hemoglobin A: chemical modifier-specific effects and added influence of polyethylene glycol chains. Biochemistry. 2002;41:376–85.
14. Khan ID, Dansker U, Samuni A, et al. Cys-93(β) modified hemoglobin: kinetic and conformational consequences. Biochemistry. 2001;40:7581–75922.
15. Tsai AG, Cabrales P, Young MA, et al. Effect of oxygenated polyethylene glycol decorated hemoglobin on microvascular diameter and functional capillary density in transgenic mouse model of sickle cell disease. Artfi Nanomed Biotechnol. 2015;43:10–7.
16. Meng F, Kaul D, Thangswamy S, et al. Semisynthetic supra plasma expanders: a new class of therapeutics to improve microcirculation in sickle cell anemia. Artfi Cells Nanomed Biotechnol. 2019;47:73–82.
17. Branch C, Cui M, Thangaswamy M, et al. Therapeutic efficacy of semisynthetic supra perfusion resuscitation fluids EAF PEG-Alb and EAF PEG Hb are differentiated by their cerebral effects in animal models of sickle cell disease. Blood. 2015;126(23):773–3.
18. Li M, Wu L, Zhao T, et al. The protective role of 5-hydroxymethyl-2-furfural (5-HMF) against acute hypobaric hypoxia. Cell Stress Chaperones. 2011;16:529–37.
19. Metcalf B, Chung C, Dufu K, et al. Discovery of GBT 440, an orally Bioavailable R-state stabilizer of sickle cell Hb. ACS Med Che Lett. 2017;8:321–6.
20. Hebbel RP, Hedlund BE. Sickle hemoglobin oxygen shifting strategies have unequal cerebrovascular risks. Am J Hematol. 2018;93:321–5.
21. Helms CC, Gladwin MT, Kim-Shapiro DB. Erythrocytes and vascular function: oxygen and nitric oxide. Front Physiol. 2018;9:125, 1–9