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Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated

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Abstract

Colloid osmotic pressures of hemoglobin solutions containing unmodified, intramolecularly cross-linked, intermolecularly polymerized, or polyethylene glycol (PEG) surface-conjugated hemoglobin have been measured to determine their macromolecular solution properties. Tetrameric and polymeric hemoglobins show nearly ideal solution behavior; whereas, hemoglobins conjugated to PEG have significantly higher colloid osmotic activity and exhibit solution non-ideality. From these studies, the average calculated molecular weights are 65,300 ± 3500 for unmodified and intramolecularly cross-linked hemoglobin tetramers, 156,000 for ring-opened raffinose polymerized human hemoglobin, 97,000 for pyridoxalated human hemoglobin conjugated to a carboxy-PEG polymer, and 117,000 for bovine hemoglobin conjugated to a methoxy-PEG polymer. The calculated radius of gyration for tetrameric hemoglobins is 2.9 ± 0.2 nm compared to 4.9 nm for the polymerized hemoglobin, and 7.2 and 14.1 nm for the human and bovine PEG-conjugated hemoglobins, respectively. Exclusion volumes are calculated to be 823 ± 148 nm^3 for tetramers, 4000 nm^3 for polymers, and 13,000 nm^3 and 94,000 nm^3 for human and bovine PEG-conjugated hemoglobins, respectively. These studies show that polyethylene glycol conjugated to surface amino groups greatly increases the effective macromolecular size of hemoglobin in solution. © 1997 Elsevier Science B.V.

Keywords: Modified hemoglobin; Acellular oxygen carrier; Colloid osmotic activity; Polyethylene glycol; Exclusion volume; Radius of gyration

1. Introduction

Modified hemoglobin solutions are being considered for use as acellular oxygen carriers to sustain oxygen-carrying capacity and restore intravascular volume during blood loss or hemodilution. An important consideration in their utility is the large volume of acellular hemoglobin that will be infused. Large influxes of intravenous protein solutions alter the solution properties of the blood, one being the colloid osmotic (oncotic) pressure (COP). Oncotic pressure acts in opposition to hydrostatic pressure to balance the distribution of fluid between blood and

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interstitial compartments [1]. COP is a colligative property, depending proportionally on the concentration of protein exerting the force and specifically on the macromolecular properties of that protein.

The studies reported here were carried out to determine macromolecular solution properties of some modified hemoglobins. Polymeric and surface-conjugated hemoglobin solutions are compared to unmodified or intramolecularly cross-linked hemoglobin tetramers. Intermolecular cross-linking to form hemoglobin polymers lowers COP as a result of the smaller number of oncotically active macromolecules/heme. In contrast, synthetic polymers of polyoxyethylene [also referred to as polyethylene glycol (PEG)] conjugated to the surface of hemoglobin markedly increases COP due to the interaction between the PEG polymers and solvent water. Solutions with high COP may be expected to cause significant transcapillary filtration of water in the direction from the interstitial space into the vascular compartment [1]. This is consistent with previously reported physiological effects showing that PEG-modified hemoglobin increases blood volume and cardiac output [2]. The physical phenomena underlying these effects have not been elucidated and provide the basis for this study.

2. Materials and methods

2.1. Protein solutions

The colloid osmotic pressures of several proteins were measured as a function of their concentration. The proteins included in this study were: human serum albumin (HSA) (Baxter Healthcare), purified human hemoglobin A_0 (HbA_0) (preparation described in Ref. [3]) (a gift of Hemosol), human hemoglobin cross-linked with bis(3,5-dibromosalicyl)fumarate between α subunits ($\alpha\alpha$Hb) [4] (a gift of the U.S. Army), human hemoglobin cross-linked with trimesoyl tris(methyl phosphate) between β subunits (TmHb) [5] (a gift of Hemosol), human hemoglobin intra- and intermolecularly polymerized by reaction with ring-opened o-raffinose (o-R-polyHb) (preparation described in Ref. [3]) (a gift of Hemosol), and finally, hemoglobins conjugated to surface amino residues using a synthetic polymer of polyethylene glycol. One surface modification reaction is with human hemoglobin that was first reacted with pyridoxal 5′-phosphate [6]; it was then conjugated to carboxypolyoxyethylene to produce Hb–$(OCH_2CH_2)_n$–COO^- [7,8], where $n \approx 70$ with an approximate molecular weight for each PEG unit of 3000 (Talarico, personal communication) (PHP) (a gift of Apex BioScience). The second surface modification reaction is to unmodified bovine hemoglobin conjugated to methoxypolyethylene glycol to produce Hb–$(OCH_2CH_2)_n$–O–CH_3 [9], where $n \approx 110$ with an approximate molecular weight for each PEG unit of 5000 (Shorr, personal communication) (PEG-Hb) (a gift of Enzon).

Each protein was serially diluted in Ringer's lactate solution, and the pH was adjusted to 7.4 for COP measurements. Hemoglobin concentrations were determined using a Milton Roy 3000 diode array spectrophotometer (Urbana, IL) by the absorbance at 523 nm ($\epsilon_{523} = 7.12\ mM^{-1}$) [10].

2.2. Colloid osmotic pressure measurements

COP was measured using a Wescor 4420 colloid osmometer (Logan, UT) with a 30,000 molecular weight cut-off membrane. The osmometer was calibrated prior to measurement of each hemoglobin sample with 5% albumin as recommended by the manufacturer and as described previously [11]. Measurements were performed at room temperature, which ranged from 20–23°C. The temperature was recorded for each experiment.

2.3. Analysis

COP data were analyzed using thermodynamic equations for the reduction in chemical potential of solvent caused by the presence of solute. The osmotic force needed to balance the chemical potential across the membrane is given in Ref. [12],

$$\pi = RT(C/\mathrm{MW} + BC^2 + \dots) \quad (1)$$

where π is oncotic pressure, C is solute concentration, R is the gas constant, T is temperature in degrees Kelvin (K), MW is the first virial coefficient (the molecular weight of solute), and B is the second virial coefficient which provides a measure of solution ideality.

Eq. (1) can be rearranged into linear form by ignoring higher-order virial coefficient terms in Eq. (1) (see Section 4 and Fig. 2),

$$\pi/C = RT/\mathrm{MW} + RTBC \quad (2)$$

Using the gas constant $R = 62.364$ mm Hg M^{-1} K^{-1}, RT is in units of mm Hg/M. The number-average molecular weight of each protein was then determined by,

$$\mathrm{MW} = RT/(\pi/C)_{C=0} \quad (3)$$

where the value of $(\pi/C)_{C=0}$ is the intercept obtained from linear least-squares regression of π/C versus C, where C is in units of g/dl, and MW is in units of g/mol.

Values for the second virial coefficient (B in Eqs. (1) and (2)) were calculated from the slope of π/C versus C using linear least-squares regression where,

$$B = \Delta(\pi/C)/(\Delta CRT) \quad (4)$$

and the units of B were converted to cm^3 mol/g^2. Solution non-ideality can be estimated according to Ref. [13],

$$S_i = (1 + \mathrm{MW}CB) \quad (5)$$

such that when $S_i = 1$, the solution is perfectly ideal, and as the value of S_i increases above 1, the solution becomes more non-ideal. S_i was calculated using a protein concentration of 5 mg/ml for C to represent a typical value used for physiological studies during exchange transfusion [e.g., see Ref. [2]].

The exclusion volume is the effective volume occupied by a solute macromolecule (A), which is defined as the volume from the center of the molecule that is excluded from the volumes occupied by all other molecules in the solution. This value can be calculated from the first and second virial coefficients as [12],

$$A = 2(\mathrm{MW})^2 B/N \quad (6)$$

where N is Avogadro's number. For a compact spherical molecule in dilute solution, the center of mass of the macromolecule of radius R can approach another solute macromolecule only to within a distance of $2R$. Using the volume of a sphere, $(4/3)\pi R^3$, the radius of gyration (R_G) of the macromolecule provides an estimate of the volume occupied by the extended macromolecule in solution and can be calculated from the excluded volume.

$$A = (32/3)\pi R_G^3 \quad (7)$$

3. Results

COP as a function of concentration for each protein is shown in Fig. 1. The data plotted using Eq. (2) are shown in Fig. 2. Slopes and intercepts were calculated by linear regression of the data in Fig. 2 and are presented in Table 1.

3.1. Calculations of molecular weights

Modified hemoglobin solutions are not homogeneous due to incomplete or random chemical modification reactions. Thus, COP measurements of a heterogeneous solution yield an average molecular weight, MW [13]. The measured values for MW given in Table 1 are compared in Fig. 3 with previously reported values for these proteins: HSA = 66,000 [14]; tetrameric human hemoglobin = 65,000 [15]; *o*-R-poly-Hb (average) = 128,000 (Adamson, personal communication); PHP (average) = 90,000 (Talarico, personal communication), and PEG-Hb = 125,000 ± 15,000 [16].

3.2. Calculations of second virial coefficients

The idealities of the hemoglobin solutions were estimated from the value of the second virial coefficient (B in Table 1; for all values of B given below, units are in cm^3 mol/g^2). The values for HSA and HbA_0 determined here (10.3×10^{-5} and 4.8×10^{-5}, respectively) are slightly higher than numbers reported earlier for bovine serum albumin and for bovine methemoglobin at 2.9×10^{-5} and 1.3×10^{-5}, respectively [17]. Nevertheless, both the earlier number for bovine methemoglobin and the number reported here for $oxyHbA_0$ agree well with the theoretical value of 4×10^{-5} for a compact spherical protein of molecular weight of $\sim 10^5$ [12]. Similarly, the average B value for the cross-linked hemoglobin tetramers ($\alpha\alpha$Hb and TmHb) is 5.7×10^{-5}, and the value for the polymerized hemoglobin is 4.7×10^{-5}. The tetrameric and polymerized hemoglobin solutions all exhibit nearly ideal solution behavior, giving S_i values near 1 (calculated at 5 g/dl) (Table 1).

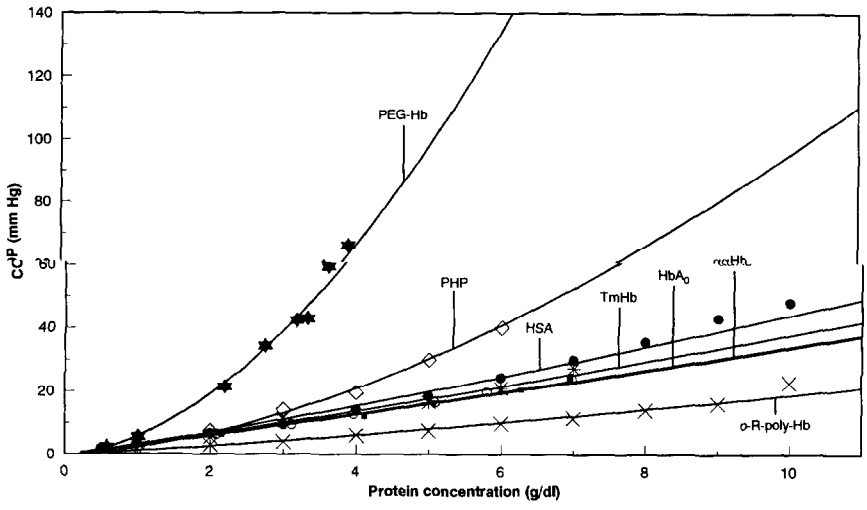

Fig. 1. Colloid osmotic pressure as a function of protein concentration. Abbreviations for the proteins are given in the text. The symbols are data points: HSA (●), HbA_0 (○), ααHb (■), TmHb (∗), o-R-poly-Hb (×), PHP (◇), PEG-Hb (★). The solid lines through the HSA, HbA_0, ααHb, TmHb, and o-R-poly-Hb data are linear regression fits; the solid lines through the PHP and PEG-Hb data are power series regression curve fits.

In contrast, the PEG-conjugated hemoglobins show very different behavior in solution. The second virial coefficient (B) for PHP (41×10^{-5}) is an order of magnitude larger than for the non-conjugated hemoglobins, and the value for PEG-Hb (207×10^{-5}) is two-orders of magnitude greater than for the non-conjugated hemoglobins. At 5 g/dl, these correspond to S_i values for PHP and PEG-Hb

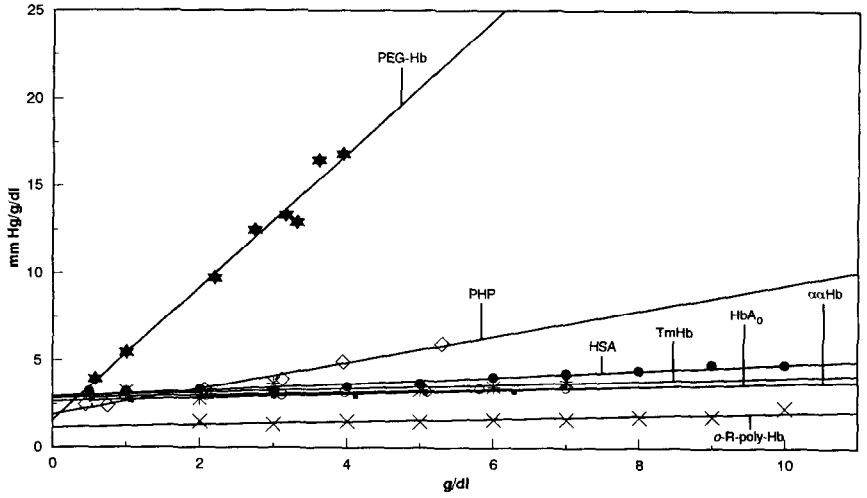

Fig. 2. Colloid osmotic pressure data plotted as π/C versus C, where π is colloid osmotic pressure in mmHg and C is protein concentration in g/dl. The symbols are data points and are designated in the legend to Fig. 1. The solid lines are linear regression fits to the data.

Table 1
Molecular weight (MW), solution ideality (B and S_i), exclusion volume (A), and macromolecular radius of gyration (R_G) of human serum albumin and unmodified or modified human or bovine hemoglobin. Calculations were determined from the slopes and intercepts from linear regression of the data in Fig. 2. Abbreviations are: HSA, human serum albumin; HbA_0, purified, unmodified human hemoglobin; $\alpha\alpha$Hb, human hemoglobin cross-linked between α subunits; TmHb, trimesoyl tris(methyl phosphate) human hemoglobin cross-linked between β subunits; o-R-poly-Hb, human hemoglobin polymerized with o-raffinose; PHP, human hemoglobin reacted with pyridoxal 5′-phosphate and surface-conjugated to polyethylene glycol; PEG-Hb, unmodified bovine hemoglobin surface-conjugated to polyethylene glycol

Sample	Slope	Intercept (mm Hg/g/dl)	MW (g/mol)	B (cm^3 mol)/g^2 $\times 10^5$	S_i^a	A (nm^3)	R_G (nm)
HSA	0.1895	2.908	63,000	10.3	1.03	1370	3.4
HbA_0	0.0881	2.828	65,000	4.8	1.02	660	2.7
$\alpha\alpha$Hb	0.1087	2.640	69,000	5.9	1.02	950	3.1
TmHb	0.1107	2.957	62,000	5.5	1.02	860	3.0
o-R-poly-Hb	0.0862	1.144	156,000	4.7	1.04	4000	4.9
PHP	0.7446	1.899	97,000	40.5	1.20	13,000	7.2
PEG-Hb	3.8227	1.564	117,000	207.0	2.21	94,000	14.1

[a]Assuming $C = 5$ g/dl.

of 1.2 and 2.2, respectively, indicating that these solutions are highly non-ideal, as reflected by the non-zero slopes for their regression lines in Fig. 2.

3.3. Calculations of excluded volumes and radii of gyration

Calculation of values for the radii of gyration (R_G) and for the exclusion volumes (A) for these proteins are given in Table 1 and are represented graphically in Figs. 4 and 5, respectively. Of all of the physical parameters determined here, these numbers most clearly illustrate the differences in solution properties of these hemoglobin solutions.

The molecular radius of native human hemoglobin determined by X-ray crystallography is approximately 3.2 nm [15]. This number agrees well with the values of R_G determined here for HbA_0, $\alpha\alpha$Hb, and TmHb (average value = 2.9 ± 0.2 nm) (Fig. 4). The R_G value for o-R-poly-Hb is 2-fold higher at 4.9 nm, as expected for a branched, polymerized hemoglobin. PHP and PEG-Hb are not intermolecularly polymerized. However, their R_G values are still higher at 8.4 and 14.1 nm, respectively, which are 3

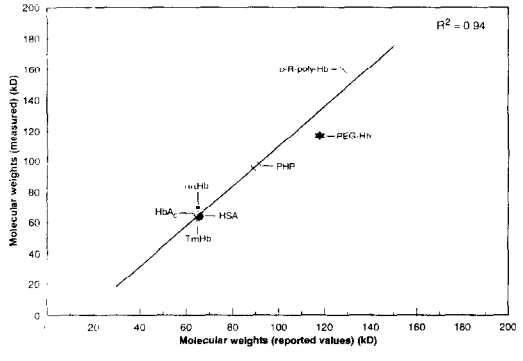

Fig. 3. Molecular weights in kDaltons (kDa) of proteins measured by COP (given in Table 1) compared to reported values for the molecular weights of these proteins. The symbols are data points and are designated in the legend to Fig. 1. The solid line is a linear regression fit through the data with a regression coefficient $R^2 = 0.94$.

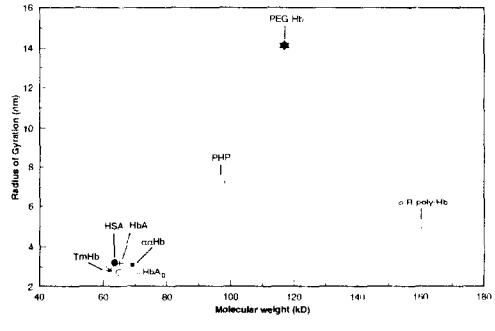

Fig. 4. Radius of gyration (R_G) as a function of protein molecular weight. R_G was calculated from Eq. (7) using the excluded volumes given in Table 1. R_G is in units of nm. Molecular weights are in units of kDaltons (kDa). The symbols are data points and are designated in the legend to Fig. 1. The known radius of native HbA based on its X-ray crystallographic structure (Dickerson and Geis, 1983 [15]) is shown by the symbol (+).

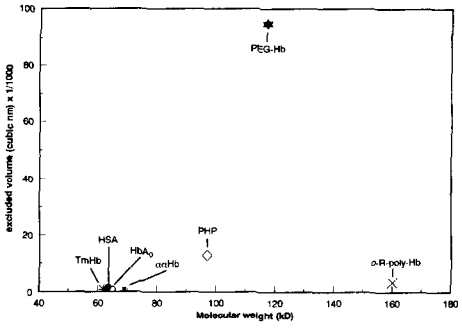

Fig. 5. Excluded volumes as a function of protein molecular weight. Excluded volumes were calculated from Eq. (6) using the values for MW and B given in Table 1. The symbols are data points and are designated in the legend to Fig. 1. Excluded volumes are in units of $nm^3 \times 10^{-3}$. MW is in units of kDaltons (kDa).

to 5-fold higher than the average R_G for the tetrameric hemoglobins and 2 to 3-fold higher than the R_G for the intermolecularly cross-linked o-R-poly-Hb, even though o-R-poly-Hb has a greater molecular weight (see Table 1).

Compared to the average value for the effective volume of hemoglobin tetramers (~ 800 nm^3), the polymerized hemoglobin (with a 2.5-fold higher molecular weight) has only a 5-fold higher excluded volume, suggesting that this polymerized hemoglobin is still fairly compact. In contrast, PHP and PEG-Hb (with molecular weights less than that of o-R-poly-Hb and only 1.5 to 2-fold higher than that of the tetrameric hemoglobins) have much higher excluded volumes: 15- and 120-fold higher, respectively, compared to the hemoglobin tetramers, and 3- and 24-fold higher, respectively, compared to o-R-poly-Hb (Fig. 5) (see Table 1). A schematic illustration of the differences in excluded volumes is presented in Fig. 6.

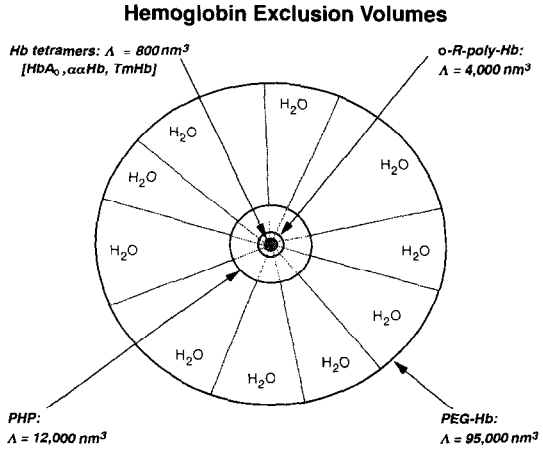

Fig. 6. Schematic diagram representing the excluded volumes of hemoglobin tetramers (HbA_0, $\alpha\alpha$ Hb, and TmHb), o-R-poly-Hb, PHP, and PEG-Hb.

4. Discussion

The HbA_0 data in Fig. 1 agree well with previous studies by Adair [18,19] who used sheep hemoglobin. Adair [18] found that the colloid osmotic pressures for purified hemoglobin were the same in distilled water as in the presence of 0.1 N NaCl and concluded that the measurements represented colloidal properties of the hemoglobin molecule rather than a Donnan effect from re-equilibration of electrolytes.

Fig. 1 shows that the COPs for HbA_0, the intramolecularly cross-linked hemoglobins, and the intermolecularly polymerized hemoglobin are all linear as a function of protein concentration within the range studied here (0–10 g/dl). For unmodified human hemoglobin, deviation from ideal solution behavior has been observed only at much higher concentrations (i.e., 29 g/dl) [20]. In contrast, the data for PEG-conjugated hemoglobins, PHP and PEG-Hb, show that COP is nonlinear as a function of protein concentration within the range of 0–6 g/dl. This suggests non-ideal solution behavior that is quantified by the data in Fig. 2.

All of the data in Fig. 2 were fit well using linear regression. This justifies using Eq. (2) for this analysis, because higher-order virial coefficients were not necessary to describe the data. This leads to the following conclusions: (1) The near-zero slopes for HSA and tetrameric and polymeric human hemoglobins in Fig. 2 show that within the range of protein concentrations studied here, these solutions are nearly ideal, representing a minimum number of fixed solvent interactions with these proteins. (2) The positive slopes for the PEG-conjugated hemoglobins in Fig. 2, on the other hand, demonstrate that within this range of protein concentration, these solutions are non-ideal, indicating a relatively larger fixed

solvent-accessible surface area. PEG polymers are highly hydrophilic: three molecules of water can associate with each PEG unit ($-OCH_2CH_2-$) [21,22]. (3) The absence of significant higher-order virial coefficients implies that there is no detectable protein aggregation over this range of protein concentrations.

PHP and PEG-Hb have similar molecular weights (97 versus 117 kDa). However, the estimated number of oxyethylene units ($-OCH_2CH_2-$) in each PEG chain conjugated to PHP is reported to be less than that for PEG-Hb ($n \approx 70$ versus ≈ 110; Talarico, personal communication and Shorr, personal communication, respectively). Assuming that both conjugation reactions are to tetrameric hemoglobins (which cannot be strictly correct since neither PHP nor PEG-Hb is intramolecularly cross-linked), the number of polymer conjugations to these hemoglobins can be estimated as follows: for PHP the average number of PEG strands is $(97{,}000 - 65{,}000)/3000 \approx 11$. This number is nearly 2-fold higher than what has been reported for an earlier PHP reaction product, (~ 6 PEG units per tetramer) [7,8]. For PEG-Hb, the average number of PEG strands is $(117{,}000 - 65{,}000)/5000 \approx 10$, which is consistent with the desired number of conjugations for this reaction product [23]. Thus, surface conjugations to either human or bovine hemoglobin appear to react with the same number of surface amino groups. However, the oncotic pressures, radii of gyration and exclusion volumes are significantly different. This may be due to the different lengths of the PEG polymers (3000 versus 5000 Da for PHP and PEG-Hb, respectively) and/or the different polymer termination groups. PEG chains on PHP terminate in a carboxylate group (the charge may reduce the osmotic pressure), whereas, PEG chains on PEG-Hb terminate in a methoxy group.

In summary, synthetic polymer surface modification of hemoglobin increases the molecular radius of the molecule by 3 to 4-fold. The hydration of the PEG units significantly increases the volume of the macromolecules in solution by solvent interaction, forming a fixed layer of associated water about the molecule. The result is a greater reduction in the chemical potential of the solvent and an increase in the colloid osmotic pressure exerted by PEG-conjugated hemoglobins when compared to the non-conjugated hemoglobins.

5. Conclusions

Blood replacement with cell-free hemoglobin solutions is being considered as a substitute for blood transfusions. Since these are cell-free solutions rather than particulate suspensions, they have solution properties different from that of blood, and their impact during transfusion cannot be predicted a priori based on whole blood or red blood cell transfusions. This ability to manipulate the COP of acellular hemoglobin solutions provides the opportunity to influence fluid balance in ways that may be therapeutically useful.

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