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Potential Electron Mediators to Extract Electron Energies of RBC Glycolysis for Prolonged in Vivo Functional Lifetime of Hemoglobin Vesicles

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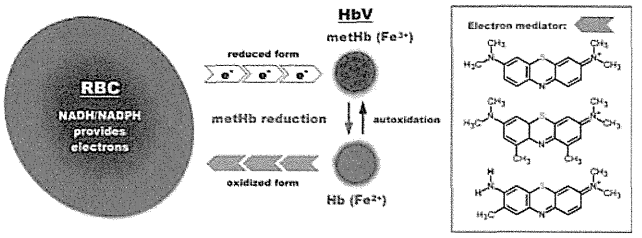
Supporting Information

ABSTRACT: Developing a functional blood substitute as an alternative to donated blood for clinical use is believed to relieve present and future blood shortages, and to reduce the risks of infection and blood type mismatching. Hemoglobin vesicle (HbV) encapsulates a purified and concentrated human-derived Hb solution in a phospholipid vesicle (liposome). The in vivo safety and efficacy of HbV as a transfusion alternative have been clarified. Auto-oxidation of ferrous Hb in HbV gradually increases the level of ferric methemoglobin (metHb) and impairs the oxygen transport capabilities. The extension of the functional half-life of HbV has recently been proposed using an electron mediator, methylene blue (MB), which acts as a shuttle between red blood cells (RBC) and HbV. MB transfers electron energies of NAD(P)H, produced by RBC glycolysis, to metHb in HbV. Work presented here focuses on screening of 15 potential electron mediators, with appropriate redox potential and water solubility, for electron transfer from RBC to HbV. The results are assessed with regard to the chemical properties of the candidates. The compounds examined in this study were dimethyl methylene blue (DMB), methylene green, azure A, azure B, azure C, toluidine blue (TDB), thionin acetate, phenazine methosulfate, brilliant cresyl blue, cresyl violet, gallocyanine, toluylene blue, indigo carmine, indigotetrasulfonate, and MB. Six candidates were found to be unsuitable because of their insufficient diffusion across membranes, or overly high or nonexistent reactivity with relevant biomolecules. However, 9 displayed favorable metHb reduction. Among the suitable candidates, phenothiazines DMB and TDB exhibited effectiveness like MB did. In comparison to MB, they showed faster reduction by electron-donating NAD(P)H, coupled with showing a lower rate of reoxidation in the presence of molecular oxygen. Ascertaining the best electron mediator can provide a pathway for extending the lifetime and efficiency of potential blood substitutes.



INTRODUCTION

Donated human blood is necessary for modern medicine. Actually, the growing demand for blood necessitates the development of a safe and functional blood substitute that can relieve the persistent need for donors and risks of contaminated blood. A blood substitute can also be designed to possess desired qualities such as long shelf life and to be universally applicable irrespective of blood type. Various approaches to find a viable blood substitute are being undertaken using hemoglobin.[1−3] One candidate is Hb-vesicle (HbV) encapsulating a purified and concentrated human Hb in a phospholipid vesicle (liposome), shielding the vasculature from the toxic effects of free Hb.[4,5] The in vivo safety and efficacy of HbV as a transfusion alternative and oxygen therapeutic have been studied extensively. All Hb-based oxygen carriers (HBOC) suffer from oxidation of the iron-containing heme group. The ferrous Hb (Fe^{2+}) autoxidizes to ferric methemoglobin (metHb, Fe^{3+}) and loses the ability to bind oxygen properly. The ferric metHb can be reduced back to the functional ferrous form by donation of electrons from a reducing compound.[6] In a healthy human being, enzymatic systems keep the fraction of metHb at low levels. Such systems include nicotinamide adenine dinucleotide (NADH)-dependent methemoglobin reductase, and nicotinamide adenine dinucleotide phosphate (NAPDH)-dependent methemoglobin reductase, which respectively use cytochrome b_5 and flavin for electron transport.[7] Superoxide dismutase and catalase remove active oxygen species.[8] Glutathione (GSH) and ascorbic acid (AsA) can reduce metHb directly.[9] However, the HbV does not contain corresponding enzymatic systems because pasteurized and purified Hb solution is used to provide utmost safety from infection.

We previously clarified that the phenothiazine dye methylene blue (MB) acts as an electron mediator between electron energy-rich RBCs and HbV to increase the functional lifetime of HbV in blood circulation.[10] Effective reduction of metHb

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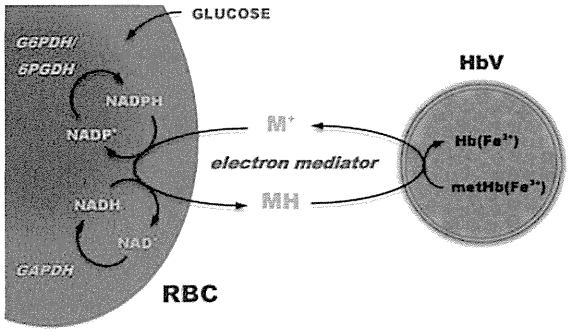
was achieved using this method. An electron shuttle providing electrons for metHb reduction in HbV from an energy-producing external source such as the RBC can thereby seemingly stall the autoxidation of the Hb inside the phospholipid vesicle and extend the functional half-life of the blood substitute.[10] Apparently, NADH and NADPH in RBCs are the main donating biomolecules for reducing MB; then MBH (leucomethylene blue) is able to reduce metHb directly back to functional Hb[10,11] (Scheme 1). Also, NADH and NADPH are regenerated continuously from the oxidized states ($NAD^+/NADP^+$) during glycolysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the Embden–Meyerhof pathway produces NADH,[12] whereas glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose-phosphate pathway produce NADPH.[13]

Scheme 1. Schematic Illustration of How Electron Mediators Shuttle Electron Energies to Hb Secluded inside HbV[a]



[a]The electron mediator enables electron transfer from energy-rich molecules within RBCs to ferric metHb (Fe^{3+}), which is reduced to functional ferrous Hb (Fe^{2+}). Glucose supports regeneration of NAD(P)H through glycolytic enzymatic systems within the RBC.

This work examines 15 potential mediators, usually recognized as dye compounds, for electron mediating abilities in comparison to MB.[10] The success or failure in this regard is then assessed by correlation between chemical properties of the compounds and the metHb reducing ability in HbV. The following dyes were investigated in this study: the phenothiazines MB, dimethyl methylene blue (DMB), methylene green (MG), azure A (AA), azure B (AB), azure C (AC), toluidine blue (TDB), and thionin acetate (TH); the phenazine, phenazine methosulfate (PM); the phenoxazines brilliant cresyl blue (BCB), cresyl violet (CV), and gallocyanine (GC); the indamine toluylene blue (TLB); and the indols indigo carmine (IC) and indigotetrasulfonate (ITS) (Scheme 2). They were selected for their similarity in chemical structure to MB, water solubility, or the reported redox potential ($E^{0\prime}$) between Hb and NAD(P)H. The chemical properties examined were incorporation into phospholipid vesicles and RBCs, reduction rates with physiologically relevant reducing agents (NADH, NAPDH, AsA, and GSH), reoxidation with oxygen, and $E^{0\prime}$.

RESULTS

MetHb Reduction in HbV in the Presence of RBCs and a Dye. Figure 1 presents the change of the metHb levels in HbV in the presence of RBCs and a dye for 80 min. Each experiment was performed under aerobic conditions. A metHb level limit of 25% from the initial approximately 50% was used as indication to divide the dyes into those mediating a promising metHb reduction inside the HbV, and those not deemed suitable for the purpose. Nine dyes (MB, DMB, AA, AB, AC, TDB, BCB, CV, and TLB) reached metHb levels below 25% and were able to sustain a metHb level under this limit throughout the course of the experiment. AA, AB, AC, and TDB all produced similar metHb reduction curves with quick reduction of metHb. Compared to the other dyes with positive results, MB, DMB, and TLB appeared to produce a slower initial metHb reduction in the HbV. Both BCB and CV displayed rapid, efficient reduction, but after the 5 min sample, more prominent reformation of metHb was observed with these dyes. GC displayed a slow reduction, but it was insufficient to reduce the metHb level to 25%. For PM, IC, and ITS, the metHb increased from the start of the assay, whereas MG and TH showed initial reduction of metHb in the HbV, but then failed to reach or stay below the 25% limit within the 80 min time span.

Incorporation of the Dye Molecule into Vesicles and RBCs. Three different categories can be used to describe the results of the dyes in the empty vesicle incorporation experiments. No incorporation (<0.5%) was observed with GC, IC, and ITS; low incorporation observed with MB and TLB (8–9%); and high incorporation observed with the others (>59%). The respective values are presented in Table 1.

Similarly to the empty vesicles experiment, low incorporation in the RBCs was visible not only with IC and ITS, but also with PM (<0.5%). Together, MB, GC, CV, and TLB indicated higher incorporation, whereas MG, AA, AB, AC, TDB, and BCB displayed lower incorporation in comparison to the empty vesicles. DMB showed similar values in both assays.

Reduction Rates of the Dyes by Biological Reductants. Some examples of reduction curves are displayed in Figure 2. The calculated rates are presented in Table 1. NADH and NADPH produced the highest reduction rates with all the dyes, except with IC and ITS. These dyes behaved differently and were not apparently reduced to any significant extent by the dinucleotides or other reducing agents. Rates for direct reduction for the other dyes with AsA were notably lower than those of NADH/NADPH, but the reducing agent producing the lowest rates was GSH. Some reduction curves with AsA and GSH displayed fluctuating values. Overall, MG and TH exhibited the highest rates with all the reducing agents, but PM also showed indications of producing high reduction rates. However, because of overlapping absorbance spectra, the reduction of PM with NADH and NADPH could not be followed accurately.

Reoxidation Rate of the Dyes by Molecular Oxygen. Sodium hydrosulfite effectively reduced all dyes, but because of interference with the spectrum of PM, L-cysteine was used to produce the reduced leuco form of this dye. The reoxidation experiments produced results that can approximately divide the dyes into two categories: dyes with low reoxidation rates, and dyes with high reoxidation rates, as shown in Figure 3. Calculated rate constants are based on at least two repeats and displayed in Table 1. Low rates, listed in increasing rate order, were obtained with MG, TLB, AB, DMB, TH, TDB, MB, AC, and AA. High rates, also listed in increasing order, were produced by GC, PM, BCB, CV, ITS, and IC. Apparently, phenothiazines and the indamine gave lower rates, whereas the phenazine, the phenoxazines, and the indols gave higher rates.

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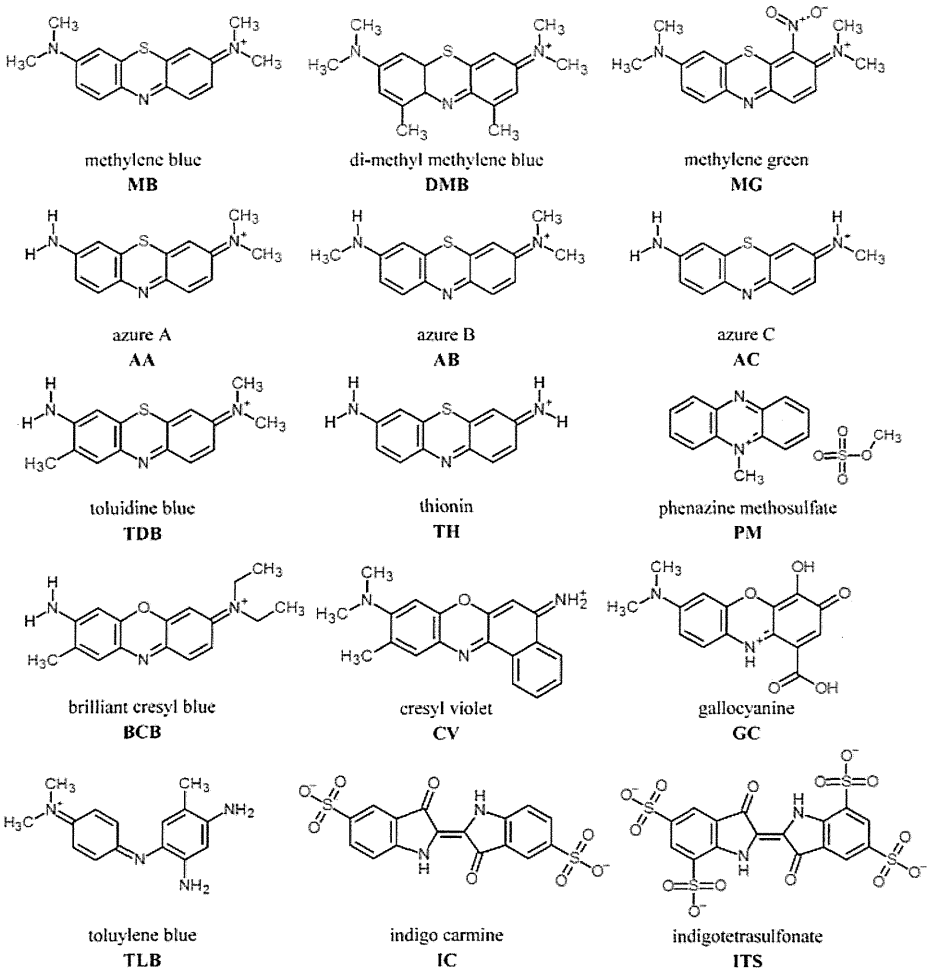
Scheme 2. Structures of the 15 Dye Molecules Screened As Potential Electron Mediators in This Work



DISCUSSION

The results of this study indicate that reduction of metHb secluded inside HbV appears to be possible with numerous potential electron mediators. Of 15 dyes, 9 displayed a favorable metHb reduction. In relation to the examined properties of the compounds, the 6 dyes which were found to be unsuitable possibly suffered from insufficient diffusion across membranes and excessive reaction rates with relevant biomolecules. Using MB as a guidepost, DMB and TDB show promise because of their slightly higher reaction rates with NADH/NADPH and lower reaction rates with AsA, while simultaneously also having lower reoxidation rates in the presence of oxygen. Comparisons with the incorporation into lipid membrane, reduction by biological reductants, and reoxidation by oxygen, as well as the $E^{0\prime}$s, might explain the results of the metHb reduction experiment and elucidate the desired properties of an electron mediator that can perform extraction of energies for devices such as HbV.

From the experiments examining the incorporation of the dyes into the empty vesicles, results showed that GC, IC, and ITS exhibited extremely low incorporation (Table 1). The same low incorporation was observed for RBC with PM, IC, and ITS. Consequently, IC and ITS were not incorporated in either entity. One component of the lipids in the vesicles, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG), possesses carboxylic acid group and provides the vesicles surface negative charges. Biomembranes, including those of RBCs, are known to be charged negatively. Negatively charged IC and ITS might be incapable of accessing the negatively charged membranes. This lack of ability might provide further explanation to the IC and ITS showing no metHb reduction at all (Figure 1). GC displayed low incorporation into the RBC and none in the vesicles. The minor diffusion into the HbV is inhibiting possible mediating abilities, allowing only slow and insufficient reduction. PM shows very low incorporation into RBCs, and should therefore be unable to extract energies. The PM metHb reduction curve appeared to support this assumption because no initial metHb reduction was observed. In the end of the PM experiment, a minor metHb reduction was visible. If PM managed eventually to extract some electron energies, this delayed reduction might derive from the minor incorporation. The other dyes, MB, DMB, AA, AB, AC, TDB, BCB, CV, and TLB, displayed metHb reduction to levels less than 25% metHb in HbV. Apparently, dye incorporation values over 8% in the vesicles, and 17.1% in the RBC, are sufficient to enable electron mediation. Both MB and TLB have low incorporation into both vesicles and RBCs, but DMB had high incorporation, which might indicate that incorporation is not the main contributing factor to the slower initial metHb reduction that was observed with these dyes.

All dyes except the indols IC and ITS were reduced by the dinucleotides NADH and NADPH. This result for IC and ITS was consistent with those for the other two reducing agents:

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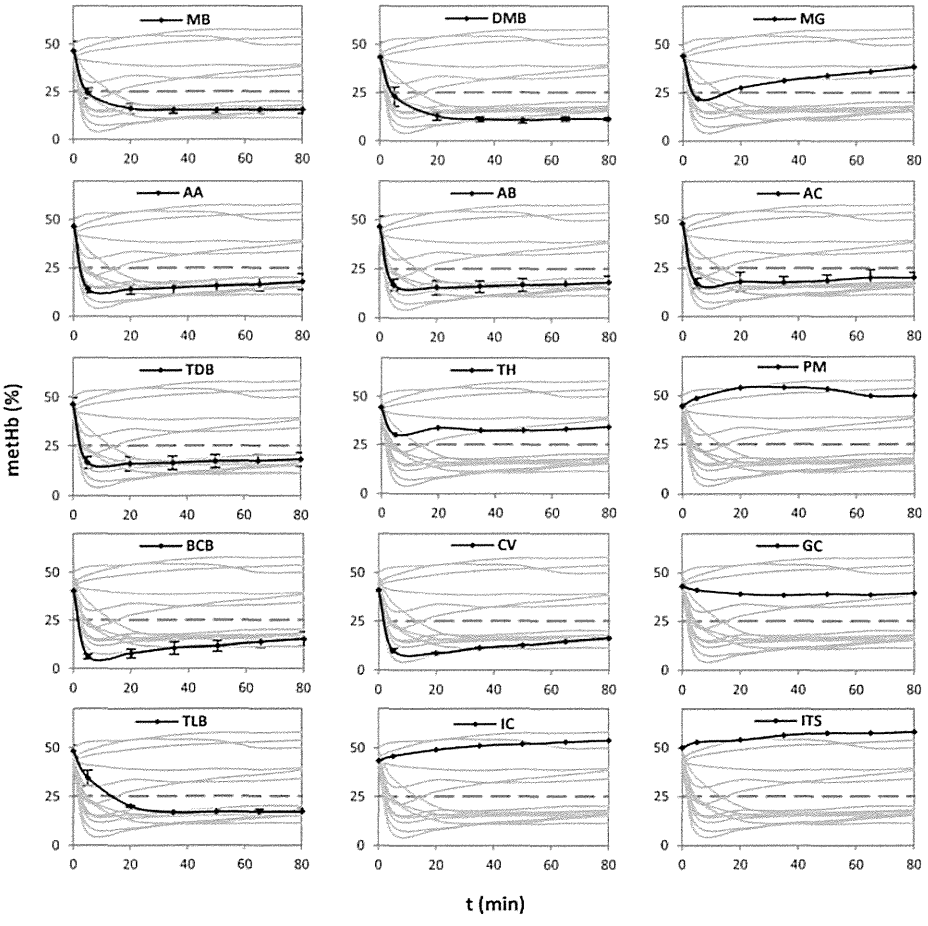

Figure 1. Reduction of metHb in HbV in the presence of RBCs and each dye. Each experiment was performed at 37 °C for 80 min under an aerobic condition. RBC suspension (12 g Hb/dL), HbV suspension of 50% metHb level, 0.3 mM dye solution, and 100 mM α-D-glucose solution were mixed in a 2:1:1:1 volume ratio. MB, DMB, AA, AB, AC, TDB, BCB, CV, and TLB assays were performed multiple times and are therefore also displayed with error bars (mean ± standard deviation). The dashed red line denotes the 25% border used to separate the dyes into suitable/unsuitable reduction. Each dye is displayed separately with the other dyes in the background for comparison.

AsA and GSH. Regarding the $E^{0\prime}$s of IC and ITS, it is theoretically possible for NADH, NADPH, and GSH to reduce these dyes, but no significant reduction was achieved. Consequently, this type of dye seems to be unable to react in a favored way with relevant donating compounds.[10,11] It can be concluded that IC and ITS are unable to extract electron energies for the intended purpose. Negatively charged IC and ITS might be unable to interact with negatively charged NADH, NADPH, and AsA.

The highest reduction rates were obtained with TH and MG with all reducing agents as shown in Table 1. The metHb reduction curves of MG and TH both display initial reduction, but are then unable to maintain low levels over time. Rapid depletion of intracellular energy-rich compounds might engender reduced cellular function for energy extraction. Actually, AsA shows important antioxidant effects in cells and tissue, counteracting free radicals that damage DNA, lipid membranes, and enzymes.[20] Experiments with AsA and GSH in several cases indicate minor reducing capabilities. Decrease in GSH has been reported in the presence of PM, in addition to overproduction of oxygen-reactive species (ROS) such as superoxide anion, ultimately leading to oxidative stress.[21] For PM no reduction rates with the dinucleotides are presented in this work, but a higher rate was visible with AsA, which can be expected to deplete GSH because GSH metabolism is necessary for AsA regeneration.[22] This might further support the conclusion that excessive rates are not desirable, although the low incorporation of PM in the RBC probably restricts the uptake and possible reaction with intracellular molecules. NADH and NADPH are probably the key biomolecules enabling reduction of the dyes, whereas minor or nonexistent reaction with AsA and GSH is desired to avoid promoting oxidative stress in the RBC. Because MB and TLB displayed the lowest reduction rates with NADH/NADPH, but DMB deviated by having a higher reduction rate, it appears that the lower reduction rate with NADH/NADPH is not solely responsible for the slower initial metHb reduction. Seven of the suitable dyes produced higher rates with NADH/NADPH than MB did, possibly indicating a more effective acquisition of cellular energies. Of the seven, three dyes produced lower rates with AsA. These were DMB, TDB, and BCB. Less affinity to react with AsA might result in less oxidative stress in the cells.

A chemical structure-related pattern emerged in the reoxidation experiments. The indols were fastest, followed by the phenoxazines and the phenazine, whereas the phenothiazines and indamine obtained lower rates than the others. Theoretically, a low reoxidation rate with oxygen might be desirable to retain the reduced form of the dye during diffusion from the RBC to the HbV. In addition, because the reoxidation of the reduced form leucomethylene blue (MBH) will generate

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Table 1. Summary of the Chemical Properties of 15 Dye Molecules to Be Selected As an Optimal Electron Mediator from RBC to metHb in HbV[a]

	second-order reduction rate constant (M^{-1} s^{-1})				incorporation (%)			
dye	NADH	NADPH	AsA	GSH	vesicle	RBC	reoxidation rate (M^{-1} s^{-1})	$E^{0\prime}$ (V)
TH	30.4	35.6	6.02	0.237	92.2	48.9	11.6	+0.060[14]
MG	21.3	23.0	15.6	0.112	86.4	40.4	5.80	+0.171[15]
GC	13.6	3.35	1.47	*n.r.*[b]	<0.5	9.4	58.3	+0.030[14]
AC	9.79	10.2	2.28	0.0893	97.4	44.5	18.0	+0.038[16]
CV	9.41	10.0	−0.0632	−0.245	78.1	78.1	451	−0.165[17]
AA	5.35	5.44	0.841	0.0523	92.6	39.6	19.9	+0.070[18]
TDB	**4.84**	**6.34**	**0.436**	**0.0291**	**84.2**	**40.0**	**11.7**	**+0.027[14]**
DMB	**4.47**	**6.96**	**0.0316**	***n.r.***	**87.2**	**86.1**	**10.7**	**+0.026[c]**
AB	3.88	4.02	0.674	0.0224	58.8	31.1	7.39	+0.070[18]
BCB	3.43	4.20	*n.r.*	0.0894	98.7	37.4	196	+0.035[17]
MB	**2.72**	**3.14**	**0.458**	**0.0155**	**8.0**	**19.4**	**16.5**	**+0.011[6]**
TLB	2.19	3.52	0.684	0.0634	9.1	17.1	5.84	+0.115[14]
ITS	*n.r.*	*n.r.*	*n.r.*	*n.r.*	0.5	<0.5	1030	−0.046[14]
IC	*n.r.*	*n.r.*	*n.r.*	*n.r.*	<0.5	<0.5	2750	−0.125[14]
PM	[d]	[d]	12.2	0.0404	71.3	<0.5	138	+0.080[19]

[a]The calculated second-order rate constants for each dye with respective reducing agent; incorporation into the vesicles or RBC fraction (dye concentration at 0.27 mM and lipid concentration at 0.73g/dL, while for RBC experiments dye concentration 0.21 mM and Hb concentration at 1.2 g/dL are displayed); reoxidation rate with O_2; and redox potential ($E^{0\prime}$). [b]*n.r.*: no reduction occurred or the reduction was negligibly small during the observed period. [c]Measured with an Ag/AgCl (3.33 M KCl) reference electrode from Horiba Ltd. [d]Unable to measure because of the overlapped absorption spectra of PM and NAD(P)H.

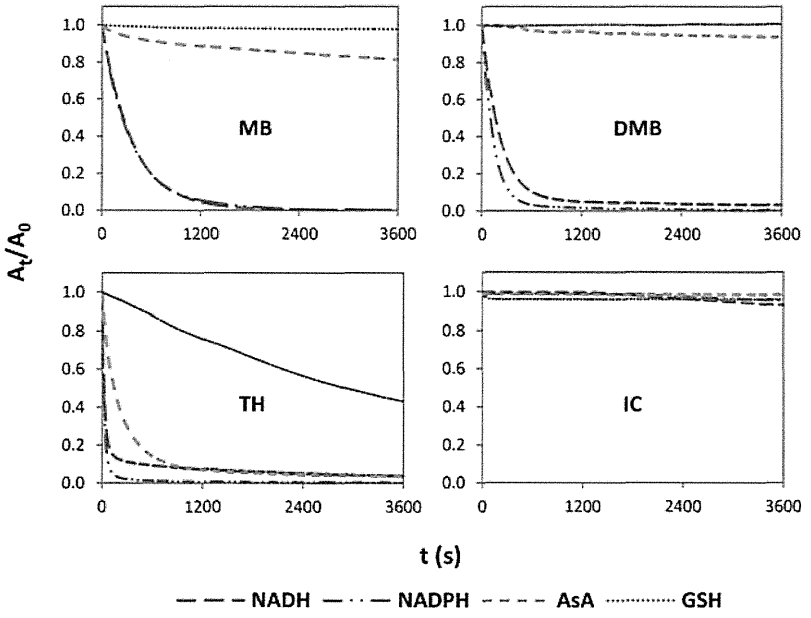

Figure 2. Reduction curves of four representing dyes (MB, DMB, TH, and IC) with reducing agents NADH, NADPH, AsA, and GSH. Deoxygenated dye (6 μM, 3 mL) and reducing agent (50 mM, 60 μL) solutions were mixed in a 1:167 molar ratio in a Thunberg cuvette and the reaction was monitored for 1 h.

ROS, e.g., superoxide,[23] other leuco dyes might also produce harmful species. According to the results of the metHb reduction experiments, BCB and CV displayed promising reduction, but compared to the other dyes with favorable metHb reductions, these dyes had a visibly more rapid reformation of metHb, possibly indicating that generation of ROS tended to enhance metHb formation. Actually, IC and ITS were ruled out as suitable dyes because of their lack of incorporation in either entity, and lack of reaction with the NADH/NADPH, but they slightly enhanced metHb formation as well. Of the dyes with suitable metHb reduction, DMB, AB, TDB, and TLB showed lower reoxidation rates than MB did, possibly pointing to less risk of unfavorable reaction in the presence of oxygen. However, the generation of ROS by the oxidation of these dyes should be the reason that the metHb level in HbV did not approach zero.

To function as an effective electron mediator, the compound of interest should have an $E^{0\prime}$ between the $E^{0\prime}$s of the donating and accepting target molecules.[14] In this case, the main donating molecules are NADH/NADPH ($E^{0\prime}$ = −0.32 V) and the Hb/metHb-couple ($E^{0\prime}$ = 0.14 V) is the accepting target.[6] The $E^{0\prime}$s considered reliable are compiled in Table 1, with the

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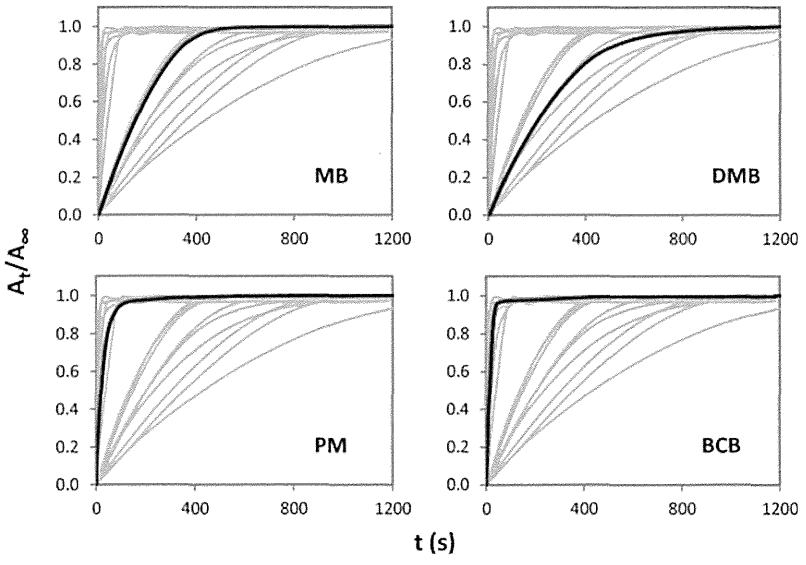

Figure 3. Reoxidation curves of four representing dyes (MB, DMB, PM, and BCB) with oxygen. 100 μL of 0.3 mM dye solution, 5−15 μL of 5 mM sodium hydrosulfite, and 2.49 mL deoxygenated PBS were mixed before addition of 500 μL air-saturated PBS. The Thunberg cuvette was monitored for 20−40 min until the reaction was finished. The other dyes are represented by the gray lines in the background for comparison.

values for MG, AA, and AB being converted from values obtained versus a standard calomel electrode (SCE).[15,18] Because no reliable $E^{0\prime}$ was found in the literature for DMB, an Ag/AgCl (3.33 M KCl) electrode was used to obtain the value for DMB. Actually, MG appears to have a potential that is higher than that of Hb, and should not be able to reduce metHb, but some reduction of metHb was observed in our metHb reduction experiments. Contamination of another dye compound such as MB might explain the slight reduction. The dyes TDB and GC have $E^{0\prime}$s that are approximately equal to that of MB. TDB has been proposed previously to act as an efficient mediator in the treatment of methemoglobinemia.[24] Therefore, GC was thought to share this trait. However, the metHb reduction experiment displayed a nondesirable reduction, which might be attributable to the minor incorporation of GC into the HbV.

As for the dyes' suitability in an in vivo setting, toxicity must be considered. In concentrations up to 2 mg/kg body weight, MB is considered nontoxic for use in methemoglobinemia treatment.[25] TDB has been proposed to have favorable metHb reducing abilities in methemoglobinemia with lower side effects than MB.[24] According to Wainwright, many phenothiazines have low mammalian toxicity,[26] although the details were not thoroughly described. Phenothiazines should be examined in greater detail. Consumption of NADH/NADPH by the dyes in the cells would affect the cell function. Binding and intercalation of the dyes in DNA should also be investigated.[27] The phenoxazines BCB and CV and the indamine TLB showed suitable metHb reduction curves. However, BCB reportedly indicates toxicity in porcine oocytes in some conditions, resulting in impaired fertilization and embryonic development.[28] Both CV and TLB were tested alongside MB for oxygen consumption in starfish eggs, based on the putative low toxicity of these dyes in this model.[17] Details of the toxic effects should be assessed to rule out potential precarious candidates.

Considering a clinical setting, the blood RBC concentration is reduced in cases of severe blood loss or very extensive exchange transfusion. It is required to clarify the minimal amount of RBC needed in the body to provide electron energies to metHb in HbV for sufficient regenerating functions. We also have to clarify the dose response for all the potential electron mediators and compare with that of MB, as reported in the previous report,[10] to optimize and possibly minimize the dosage of the mediators. We clarified effective metHb reduction with increasing MB dose (0.42−1.27 mg/kg) in rat experiments after injection of HbV ([Hb] = 10 g/dL, 10 mL/kg).[10] This corresponded to as much as 18% of whole blood volume (56 mL/kg). MetHb level decreased immediately from 30% to 10% within only 20 min. We also confirmed fast reduction of metHb in an experiment of rat hemorrhagic shock induced by 50% blood withdrawal followed by HbV resuscitation. Injection of MB immediately reduced metHb from 40% to 10% within 45 min. This will be reported elsewhere. Because the metHb reduction is achieved rapidly, short circulation half-life of the small dyes would not be so critical, although circulation half-life should be measured eventually.

In fact, MB is used clinically as an intravenous injection solution for the treatment of patients suffering from methemoglobinemia. Because MBH generation is dependent mainly on NADPH production, the efficiency of electron transport by MB is decreased in the instance of G6PDH deficiency.[25] However, according to studies conducted with normal cells, G6PDH-deficient cells, and MB, it was discovered that metHb reduction in the G6PDH-deficient cells were facilitated. Electron mediation between the normal and the G6PDH-deficient cells in the presence of MB appeared to be possible,[29] giving further proof of MB's mediating abilities. Our results indicate the potential of the compounds we selected for possible use for methemoglobinemia with better efficacy and safety than conventional MB.

CONCLUSION

Electron mediation from RBC into HbV appears to be effective for the reduction of metHb inside the vesicles with several potential compounds. Of 15 compounds screened in this work, 6 did not appear to be suitable for the purpose. Results show that PM, GC, IC, and ITS suffer from restricted diffusion across membranes. MG and TH stimulate high rates with physiolog-

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ically relevant reducing agents compared with other examined compounds, perhaps depleting antioxidant reserves of the cells and promoting oxidative stress. Including MB, 9 dyes exhibited effective metHb reduction. Summarizing the apparently suitable dyes in consideration of all the examined properties, DMB and TDB might be interesting for further study for metHb reduction inside HbV. Incorporation to the lipid membrane to some extent is essential. These dyes tended to show slower reduction rates by NAD(P)H compared to other dyes (but not the slowest). In comparison to MB, DMB and TDB indicate higher reduction rates in the presence of NAD(P)H, and lower rates with AsA. They also show slower oxidation of the leuco form in the presence of oxygen. Further research is necessary to ascertain the best candidate for extending the functional half-life of in vivo devices such as HbV.

MATERIALS AND METHODS

Hb Vesicles, RBCs, and Dyes. HbVs were prepared as described previously.[10] The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG, Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan) at the molar composition of 5/4/0.9/0.03. The Hb concentration of the suspension was adjusted to 10 g/dL. The suspension was exposed to air and incubated in a water bath at 37 °C until an internal metHb concentration of 50% was achieved.

Fresh blood was collected in heparinized tubes from healthy anesthetized Wistar rats. The blood was immediately washed by centrifugation at 2000 rpm (himac CF12RX; Hitachi Koki Co. Ltd., Tokyo, Japan), removing the plasma, and then adding phosphate buffered saline (PBS, pH 7.4 1×; Gibco Life Technologies, Paisley, Scotland). After three washing repetitions, the resulting RBC concentrate was diluted to 12 g Hb/dL with PBS and refrigerated until use within 36 h.

The dyes were purchased from various manufacturers: MB, GC, and ITS from Sigma-Aldrich Corp. (St. Louis, USA); MG, AA, AC, TLB, BCB, and CV from MP Biomedicals (Illkirch, France); AB, TH, IC, and PM from Wako Pure Chemical Industries Ltd. (Osaka, Japan); TDB from Waldeck GmbH & Co. KG (Münster, Germany); and DMB from Polysciences Inc. (Warrington, USA). We used the dyes without further purification. Stock solutions of 0.3 mM dye in PBS were stored in the dark at room temperature. The dye solutions were subjected to heating and sonication in a water bath before experiments to eliminate precipitate if formed during storage. All the dyes exhibited characteristic light absorption in the oxidized form and no absorption in the reduced leuco form, which was useful for determination of the dye concentration and the level of oxidation (see Supporting Information).

MetHb Reduction in HbV in the Presence of RBCs and Dye. The metHb reduction in HbV, in the presence of RBCs and an electron mediator, was examined using 80 min assays. An RBC suspension (12 g Hb/dL), a 50% metHb HbV suspension, a 0.3 mM dye solution, and 100 mM α-D-glucose solution were mixed in a 2:1:1:1 volume ratio. Glucose was added to maintain comparable glycolytic enzymatic conditions of RBCs in all experiments. The mixed solution was incubated in water bath at 37 °C. Samples were collected in hematocrit-glass capillaries (Hirschmann Laborgerate GmbH & Co., Germany) at 5 min, and thereafter every 15 min. The capillary samples were centrifuged for 5 min at 12 000 rpm (micro hematocrit centrifuge 3220; Kubota Corp., Tokyo, Japan), to separate the HbV from the RBCs. A small volume of HbV was then suspended in PBS in a Thunberg cuvette and deoxygenated for 10 min by N_2 bubbling before spectrophotometric analysis to ascertain the level of metHb (%) from the ratio of the absorbances at 405 and 430 nm, which respectively correspond to λ_{max} of metHb and deoxyHb.

Incorporation of a Dye Molecule into Vesicles and RBCs. An empty vesicle suspension (without Hb) was prepared similarly using the same lipids as in HbV. The vesicle suspension at a lipid concentration of 7.3 g/dL was mixed in a 1:9 volume ratio with 0.3 mM dye solution in ultracentrifuge tubes (Hitachi Koki Co. Ltd.). After incubation for 30 min at room temperature, the tubes were ultracentrifuged at 35 000 rpm for 1 h (himac CP80WX; Hitachi Koki Co. Ltd.). Spectrophotometric analysis of the resulting supernatant in a UV–vis spectrophotometer (V-650; Jasco Corp. Tokyo, Japan; with an integrated sphere (ISN-470) for light scattering correction) was used to determine the incorporated fraction of the dyes. Subsequently, removal of all supernatant and resuspension of the remaining pellet in PBS was done before dissolving the vesicles by mixing detergent (10% polyoxyethylene 10 lauryl ether) with the pellet resuspension in a 1:1 volume ratio. After 5 min in a 60 °C water bath, the dissolved pellet solution was examined using spectrophotometric analysis to verify the dye concentration. For some dyes with high incorporation, the supernatant spectra displayed shifting absorbance from the expected maximum peaks. In the same manner, dyes incorporated to a low degree showed some shifting spectra after pellet disruption and examination. Only results that were deemed reliable after verification of a correct spectrum for respective dye were accepted, leading to alternate use of the supernatant spectrum or the disrupted pellet spectrum for incorporation fraction calculation.

Dye incorporation into RBCs was examined similarly, but with a RBC suspension (12 g Hb/dL), 0.3 mM dye solution, and 100 mM α-D-glucose solution (Sigma-Aldrich Corp., St. Louis, USA) present at a mixing volume ratio of 1:7:2 (blood:dye:glucose). The samples were incubated for 30 min in 37 °C water bath before centrifugation at 12 000 rpm for 1 min (micro hematocrit centrifuge 3220; Kubota Corp., Tokyo, Japan). The supernatant spectra were analyzed to ascertain the incorporated fraction of respective dye. No pellet examination was done with the RBCs.

Reduction Rate of the Dyes by Biological Reductants. Direct reduction of the dyes with four reducing agents was examined over the course of an hour. NADH was purchased from Sigma-Aldrich Corp., NADPH from Oriental Yeast Co. Ltd. (Tokyo, Japan), L-(+)-AsA and GSH from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Before the experiment, deoxygenation of the dye solution (6 μM, 3 mL) in a Thunberg cuvette was performed by attaching inlet and outlet needles through the rubber stopper and supplying nitrogen gas for 5 min. This procedure reduced partial oxygen tension to less than 0.10 Torr. The reducing agent stock solution (50 mM) was deoxygenated in a rubber-capped glass vial in the same manner. A gas-tight glass syringe with a needle was used to collect the reducing agent solution (60 μL) and inject it into the Thunberg cuvette. The molar ratio of a dye and a reducing agent was 1:167 to achieve sufficient excess. The reaction was monitored during 1 h using the spectrophotometer. Data of A_t/A_0 versus time were fit to an exponential curve using software

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(Microsoft Excel Solver; Microsoft Corp., Redmond, WA). The apparent reduction rate was calculated.

Reoxidation Rate of the Dyes by Molecular Oxygen. Oxygen was excluded from all solutions of the dye and reductant by complete deoxygenation with N_2 bubbling before the experiment. 100 μL of 0.3 mM dye solution was diluted with deoxygenated PBS to a volume of 2.49 mL in the Thunberg cuvette. A 5 mM solution of sodium hydrosulfite (Tokyo Chemical Industry Co., Ltd.) was injected into the cuvette to reduce the dyes to a reduced leuco form. Because of the quick deterioration of the reducing agent the injected volume varied between 5 and 15 μL. L-Cysteine (Wako Pure Chemical Industries Ltd.) was used for PM because of the absorption spectra overlap of PM and sodium hydrosulfite. 500 μL air-saturated (0.24 mM oxygen[30]) PBS was injected to start reoxidation and the change in absorbance was followed for 20–40 min until the reaction was complete. An exponential curve was fit using software (Microsoft Excel Solver; Microsoft Corp., Redmond, WA, USA) to the data $\Delta A_t/\Delta A_\infty$ versus time, enabling calculation of the apparent reoxidation rate.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Absorption spectra of all 15 dyes were measured at different concentrations and used for the measurement of concentrations and the level of oxidation/reduction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s). Of the authors, H.S. is an inventor holding some patents related to the production and utilization of Hb-vesicles.

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■ REFERENCES

(1) Winslow, R. M. (2006). *Blood Substitutes,* Elsevier Academic Press, London.

(2) Chang, T. M. (2012) From artificial red blood cells, oxygen carriers, and oxygen therapeutics to artificial cells, nanomedicine, and beyond. *Artif. Cells Blood Substit. Immobil. Biotechnol. 40,* 197–199.

(3) Alayash, A. I. (2014) Blood substitutes: why haven't we been more successful? *Trends Biotechnol. 32,* 177–185.

(4) Sakai, H., Sou, K., Horinouchi, H., Kobayashi, K., and Tsuchida, E. (2009) Review of hemoglobin-vesicles as artificial oxygen carriers. *Artif. Organs 33,* 139–145.

(5) Tsuchida, E., Sou, K., Nakagawa, A., Sakai, H., Komatsu, T., and Kobayashi, K. (2009) Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry. *Bioconjugate Chem. 20,* 1429–1440.

(6) Bunn, H. F., and Forget, B. G. (1986). *Hemoglobin: molecular, genetic and clinical aspects,* W. B Saunders Company, West Washington Square, PA, USA.

(7) Yubisui, T., Matsuki, T., Takeshita, M., and Yoneyama, Y. (1979) Characterization of the purified NADPH-flavin reductase of human erythrocytes. *J. Biochem. 85,* 719–728.

(8) Turrens, J. F., Crapo, J. D., and Freeman, B. A. (1984) Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase. *J. Clin. Invest. 73,* 87–95.

(9) Wright, R. O., Lewander, W. J., and Woolf, A. D. (1999) Methemoglobinemia: etiology, pharmacology, and clinical management. *Ann. Emerg. Med. 34,* 646–656.

(10) Sakai, H., Li, B., Lim, W. L., and Iga, Y. (2014) Red blood cells donate electrons to methylene-blue mediated chemical reduction of methemoglobin compartmentalized in liposomes in blood. *Bioconjugate Chem. 25,* 1301–1310.

(11) May, J. M., Qu, Z., and Cobb, C. E. (2004) Reduction and uptake of methylene blue by human erythrocytes. *Am. J. Physiol. Cell Physiol. 286,* C1390–C1398.

(12) Tomoda, A., Yubisui, T., Tsuji, A., and Yoneyama, Y. (1979) Kinetic studies of methemoglobin reduction by human red cell NADH cytochrome b_5 reductase. *J. Biol. Chem. 254,* 3119–3123.

(13) Nagai, M., Yubisui, T., and Yoneyama, Y. (1980) Enzymatic reduction of hemoglobins M Milwaukee-1 and M Saskatoon by NADH-cytochrome b_5 reductase and NADPH-flavin reductase purified from human erythrocytes. *J. Biol. Chem. 255,* 4599–4602.

(14) Fultz, M. L., and Durst, R. A. (1982) Mediator compounds for the electrochemical study of biological redox systems: a compilation. *Anal. Chim. Acta 140,* 1–18.

(15) Kubota, L. T., and Gorton, L. (1999) Electrochemical study of flavins, phenazines, phenoxazines and phenothiazines immobilized on zirconium phosphate. *Electroanalysis 11,* 719–728.

(16) Ruiz-Haas, P., and Ingle, J. D., Jr. (2007) Monitoring redox conditions with flow-based and fiber optic sensors based on redox indicators: application to reductive dehalogenation in a bioaugmented soil column. *Geomicrobiol. J. 24,* 365–378.

(17) Barron, E. S. G., and Hoffman, L. A. (1930) The catalytic effect of dyes on the oxygen consumption of living cells. *J. Gen. Physiol. 13,* 483–494.

(18) Strehlitz, B., Gründig, B., Vorlop, K.-D., Bartholmes, P., Kotte, H., and Stottmeister, U. (1994) Artificial electron donors for nitrate and nitrite reductases usable as mediators in amperometric biosensors. *Fresenius' J. Anal. Chem. 349,* 676–678.

(19) O'Neil, M. J. (2001) *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals,* 14th ed., Merck & Co., Inc., Whitehouse Station, NJ, USA.

(20) Bendich, A., Machlin, L. J., Scandurra, O., Burton, G. W., and Wayner, D. D. M. (1986) The antioxidant role of vitamin C. *Adv. Free Radic. Biol. Med. 2,* 419–444.

(21) Maridonneau-Parini, I., Mirabelli, F., Richelmi, P., and Bellomo, G. (1986) Cytotoxicity of phenazine methosulfate in isolated rat hepatocytes is associated with superoxide anion production, thiol oxidation and alterations in intracellular calcium ion homeostasis. *Toxicol. Lett. 31,* 175–181.

(22) May, J. M., Qu, Z. C., and Morrow, J. D. (2001) Mechanism of ascorbic acid recycling in human erythrocytes. *Biochim. Biophys. Acta 1528,* 159–166.

(23) McCord, J. M., and Fridovich, I. (1970) The utility of superoxide dismutase in studying free radical reactions II. The mechanism of the mediation of cytochrome c reduction by a variety of electron carriers. *J. Biol. Chem. 245,* 1374–1377.

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. XXXX, XXX, XXX–XXX

(24) Kiese, M., Lörcher, W., Weger, N., and Zierer, A. (1972) Comparative studies on the effects of toluidine blue and methylene blue on the reduction of ferrihaemoglobin in man and dog. *Eur. J. Clin. Pharmacol. 4*, 115–118.
(25) Bradberry, S. M. (2003) Occupational methaemoglobinaemia – mechanisms of production, features, diagnosis and management including the use of methylene blue. *Toxicol. Rev. 22*, 13–27.
(26) Wainwright, M. (2000) Methylene blue derivatives – suitable photoantimicrobials for blood product disinfection? *Int. J. Antimicrob. Agents 16*, 381–394.
(27) Atamna, H., Krugliak, M., Shalmiev, G., Deharo, E., Pescarmona, G., and Ginsburg, H. (1996) Mode of antimalarial effect of methylene blue and some of its analogues on *Plasmodium falciparum* in culture and their inhibition of *P. vinckei petteri* and *P. yoelii nigeriensis in vivo*. *Biochem. Pharmacol. 51*, 693–700.
(28) Wongsrikeao, P., Otoi, T., Yamasaki, H., Agung, B., Taniguchi, M., Nao, H., Shimizu, R., and Nagai, T. (2006) Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for in vitro production of embryos. *Theriogenology 66*, 366–372.
(29) Beutler, E., and Baluda, M. C. (1963) Methemoglobin reduction: studies of the interaction between cell populations and of the role of methylene blue. *Blood 22*, 323–333.
(30) Robinson, J., and Cooper, J. M. (1970) Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode. *Anal. Biochem. 33*, 390–399.

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Supporting Information

Potential electron mediators to extract electron energies of RBC glycolysis for prolonged *in vivo* functional lifetime of hemoglobin-vesicles

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Method to measure absorption spectra of all 15 dyes

Spectra were recorded in the range 300–700 nm using a UV–Vis spectrophotometer (V-650; Jasco Corp.) with an attachment of an integrated sphere (ISN-470). The optical path length was 1 cm. The following dyes were examined: the phenothiazines methylene blue (MB), di-methyl methylene blue (DMB), methylene green (MG), azure A (AA), azure B (AB), azure C (AC), toluidine blue (TDB), thionin acetate (TH); the phenazine, phenazine methosulfate (PM); the phenoxazines brilliant cresyl blue (BCB), cresyl violet (CV) and gallocyanine (GC); the indamine, toluylene blue (TLB); and the indols indigo carmine (IC) and indigotetrasulfonate (ITS). The spectra were obtained with dye solutions prepared with phosphate buffered saline (PBS, pH 7.4 1X; Gibco Life Technologies, Paisley, Scotland) at concentrations of 0.001, 0.01, 0.025, 0.05, and 0.075 mM for most dyes. Some dyes were checked at additional concentrations within this range. CV had low absorption. It was examined in the range of 0.006–0.1 mM. The spectra are displayed in **Figure S1**. The wavelengths of the maximum absorption (λ_{max}) and shoulder, and molar absorption coefficient (ε) of each dye are listed in **Table S1.**

To measure the spectra of the reduced forms, oxygen was excluded from all dye and reductant solutions by complete deoxygenation with N_2 bubbling. In the Thunberg cuvette, 250 µl 0.3 mM dye solution was diluted with deoxygenated PBS to a volume of 2.94 ml. 60 µl 50 mM sodium hydrosulfite solution (Tokyo Chemical Industry Co., Ltd.) was injected to achieve sufficient excess for reducing the dyes to leuco form to measure the spectra. The absorption spectra overlap of PM and sodium hydrosulfite interfered with the measurement and instead L-cysteine (Wako Pure Chemical Industries Ltd.) was used for producing the spectrum of the reduced form of PM.

Characteristic light absorption was observed in the oxidized form of all the dyes and no significant absorption at the established peak values in the reduced leuco form. This was useful for determination of the dye concentration and the level of oxidation. Twelve of fifteen dyes displayed shoulders in their spectra, but most dyes did not show linear relationship of absorption versus concentration at both observed wavelengths, especially at a higher concentration. This resulted in using one wavelength to determine the ε for the dyes at lower concentrations where a linear relationship was obtained. MB was an exception and both wavelengths were used to calculate ε.

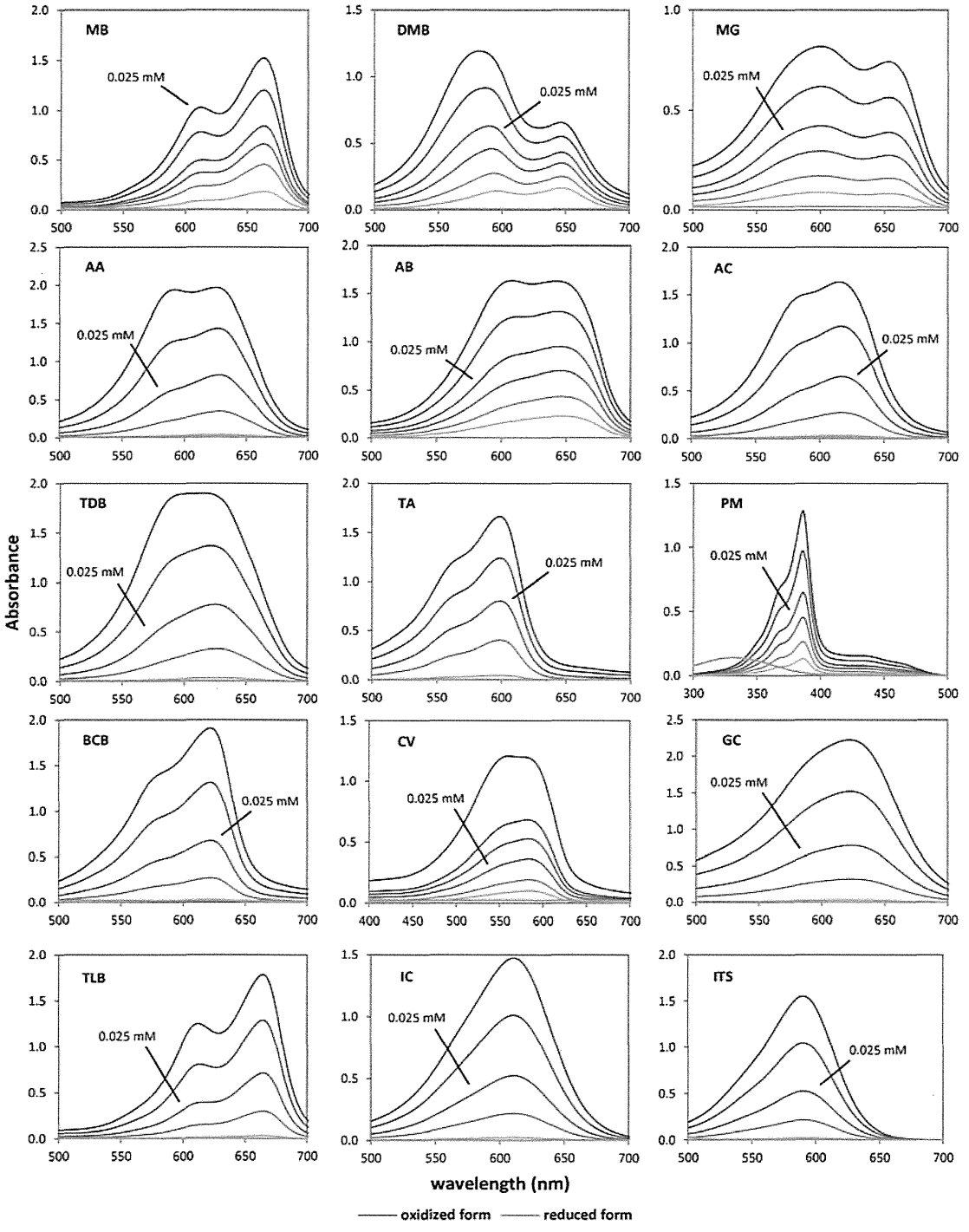

Figure S1. Absorption spectra of 15 dyes at different concentrations. The 0.025 mM line is marked in each spectrum for respective dye for comparison. The concentration of the reduced forms was 0.025 mM.

Table S1. The list of wavelength of the maximum absorption (λ_{max}) and shoulder with molar absorption coefficient (ε) of 15 dyes.

Dye	λ_{max} or shoulder (nm)	$\varepsilon \times 10^{-3}$ ($M^{-1}cm^{-1}$)
MB	664	67.8
	609	40.1
DMB	647	
	593	23.7
MG	654	
	599	16.5
AA	628	
	592	25.6
AB	646	
	604	32.8
AC	617	
	583	19.6
TDB	626	
	590	24.6
TH	599	
	563	21.0
PM	386	25.8
	370	
BCB	622	
	578	18.3
CV	583	14.0
	555	
GC	623	30.0
TLB	664	
	612	16.4
IC	610	19.9
ITS	591	20.7

1 腎性貧血治療の進歩

6．人工赤血球による生体組織への酸素輸送

はじめに

血液には実に多くの種類の蛋白質が存在しているが，そのうち最も濃度高く存在しているのが，血色素ヘモグロビン（Hb）である。その濃度は12～15 g/dL程度である。Hbの機能は酸素分圧に応じて酸素を可逆的に結合-解離することであり，生体組織への酸素輸送が生命の維持にとっていかに重要であるかを物語っている。

Hbは赤血球内に35 g/dL濃度で封入され，血液の流れに沿って体内を循環し，酸素運搬をつかさどっている。平常時において中心静脈血の酸素飽和度は75%程度であり，血液は一回の循環において酸素を全体の僅か25%しか放出せず，いざという時（酸素消費量の多い運動時，あるいは失血時）のために余力として残しているといえる。しかし，この重要な蛋白質Hbは最も多く存在しているにもかかわらず，赤血球からいったん遊離（溶血）すると，腎毒性（尿細管内での析出）や血管活性（NO〔一酸化窒素〕捕捉による血管収縮，血管損傷）など，様々な副作用（毒性）を呈する。もちろん，ハプトグロビンやヘモペキシンなどが溶出したHb，ヘムを排除しようとするが，その許容には限界がある。

1 人工赤血球の開発

虚血，貧血における有効な治療方法として赤血球輸血治療がある。わが国の献血・輸血システムは世界最高水準にあり，現行の医療に不可欠である。しかし，緊急時や大規模災害時の救命医療においては，血液型検査，感染の可能性（window period〔ウィンドウ・ピリオド〕によるHIV〔ヒト免疫不全ウイルス〕感染が話題になった），短い保存期限（日本では僅か3週間），少子高齢化（2027年には100万人分の輸血用血液が不足するとの日本赤十字社の試算）などが，危機管理体制の不安要素となりうる。そこで筆者らは，輸血代替として，赤血球の細胞型構造を模倣した人工赤血球（ヘモグロビン小胞体）製剤を開発してきた[1, 2]（図1）。

人工赤血球（粒子径約250 nm）は感染源を含まず，血液型がなく，長期間備蓄でき，いつでも何処でも使えるので，輸血治療を補完する技術として期待されている。さらに，脆弱な赤血球と比べ化学的に安定で，物性値の調節や機能分子の導入が可能なので，輸血では対応のできない疾患の治療や外科的治療への可能性も検討されている[3]。

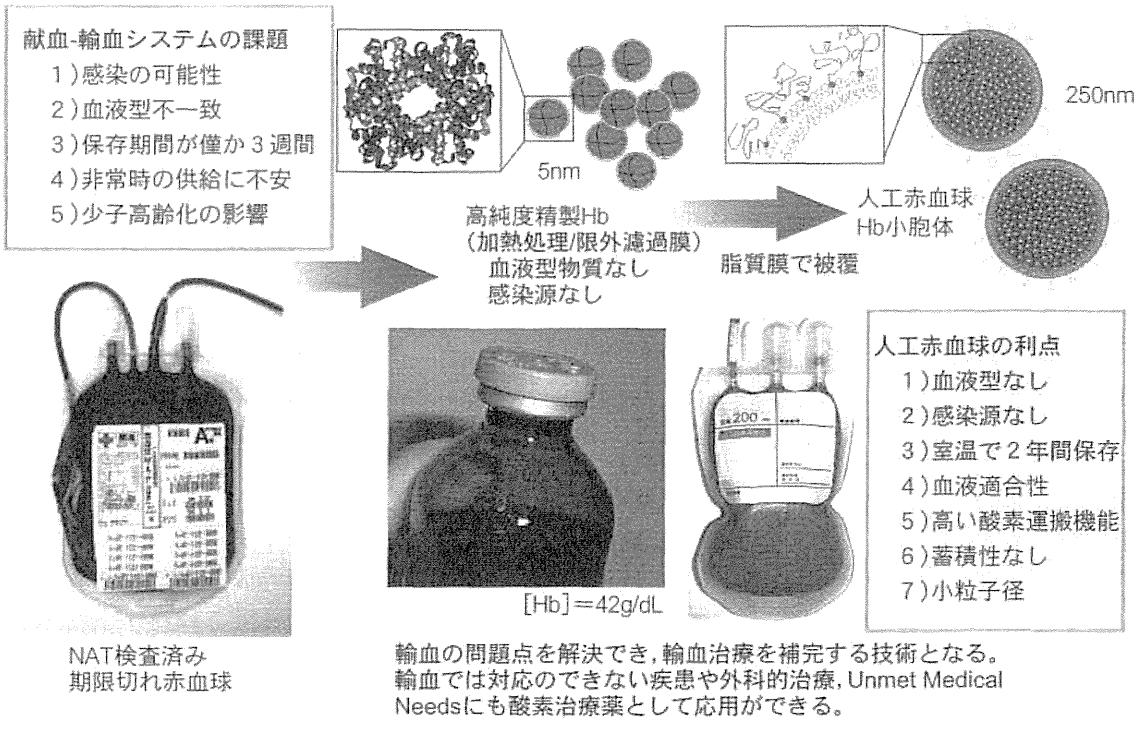

図 1　人工赤血球（ヘモグロビン〔Hb〕小胞体）製剤〕

期限切れ赤血球から精製した，高純度・高濃度ヘモグロビンをリン脂質小胞体に封入している。血液型なし，感染源なし，長期保存可能などの特徴を持っている。

（筆者作成）

2 ヘモグロビン（Hb）の人工酵素運搬体としての研究

実は，Hb を使った人工酸素運搬体の研究は随分と長い歴史がある。Hb の毒性は早くから知られていたので，欧米のグループが，二量体への解離を抑制するために分子内架橋（crosslink）をしたり，分子量を大きくするために重合（polymerize）したり，あるいは水溶性高分子を結合させて（polymer conjugation）大きくするなど，実に数多の cell-free Hb-based oxygen carriers（HBOCs）が試されたが，それでも副作用が残った[1]。血管内皮弛緩因子である一酸化窒素を Hb が捕捉することにより，血管収縮により血圧が亢進し，末梢血管抵抗が増大する状況に陥るのである（ウシ由来の Hb を架橋剤グルタルアルデヒドで重合した polyHb は，南アフリカとロシアのみのごく限られた地域で認可されていると聞くが，副作用が心配されている）。

また，米国 Sangart 社の PEG 修飾 Hb（Hemospan®）は臨床試験が進展し期待されていたものの，2013 年 11 月に開発を断念した。PEG 修飾によって分子サイズを大きくしたが，膠質浸透圧が生理条件をはるかに超え，Hb 濃度が僅か 4.8 g/dL と低いこと，また酸素親和度が高すぎる（P_{50} が低すぎる）ために，酸素輸送効果を明確に示すことができなかった（代用血漿剤としての効果に止まった）ことが原因と考えられる。

これらの結果から，筆者らは，やはり Hb は本来，赤血球あるいは赤血球構造に類似するカプセルの中にあるべき，という考えが正しいことを認識し「細胞型“cellular”」の人工赤血球（Hb 小胞体）を中心に研究している。

3 赤血球細胞型構造の生理的意義

赤血球の細胞型構造の生理的意義を理解すれば，上述の cell-free HBOCs に副作用が生じたことは容易に理解できる。赤血球は直径約 8 μm の中窪み円盤状粒子であり，蛋白質 Hb(分子量 64,500)の高濃度溶液を赤血球膜に内包した構造を持つ。Hb 溶液が赤血球膜で覆われている理由は，① 本来様々な毒性のある Hb の逸脱，血管壁との直接的な接触の抑制，② 腎臓の糸球体からの漏出など血管外漏出を防ぎ血中滞留時間を長くする，③ 35%濃厚 Hb 溶液の高い粘度と膠質浸透圧の抑制(赤血球は膠質浸透圧を示さない)，④ Hb 機能維持のための各種リン酸化合物などエネルギー分子，解糖- ならびに還元- 酵素系の保持，⑤ 血管弛緩因子（NO，CO）との反応性の制御などの役割もある。また，⑥ 血液（血球分散系）は非 Newton 流体で，体内循環とくに末梢血管内における特色ある流動形式と生理作用が特性である。

4 Hb 小胞体

いわゆる化学修飾 Hb の開発が北米で先行したが，NO 捕捉による副作用が問題となった。赤血球構造の生理的意義を考えれば当然の結果であり，これに対して，リン脂質小胞体（リポソーム）に高濃度 Hb を内包した Hb 小胞体 (250 nm) は，Hb の副作用を回避できる。

Hb 小胞体の特徴は，① 熱処理高純度 Hb を用い感染源なし・血液型なし，② 粒子表面の PEG 修飾と脱酸素化により 2 年間の備蓄が可能，③ 細胞型構造と最適粒子径により NO，CO との反応を抑制[5]，④ 血漿層に均一に分散して流動し，赤血球が通過できない狭窄血管なども通過できる，⑤ すぐれた血液適合性，RES に捕捉され分解・排泄される[6]，など。その効能として，⑥ 出血性ショック，あるいは制御不能出血に対する投与では赤血球輸血と同等の蘇生効果を示すこと[7]，⑦ 小粒径の特徴を活かすことにより，脳梗塞モデルにおいて梗塞巣を縮小すること，また皮弁モデルの虚血領域の酸素化と創傷治癒効果が得られること，⑧ 摘出臓器の灌流液としても有効であること，⑨ アロステリック因子を調節してヒト血液よりも P_{50} 値を小さくすると，低酸素領域に酸素を効率よく運ぶ，他方，⑩ CO 結合 Hb 小胞体の投与では，CO の徐放により細胞保護効果を示す[8]，などを報告している。

5 腎性貧血治療における人工赤血球の可能性

さて，第 6 章の主要テーマが「腎性貧血治療」ということなので，少しばかり腎臓を対象とした実験結果を紹介したい。筆者が大学院生だったときに慶應義塾大学医学部の小林紘一先生のグループと行っていた実験が，ラットの循環血液量の 90%を人工赤血球で置換するというものであった[9]（図 2）。全身への酸素供給の指標となる臓器が，腎臓であることから，腎皮質に polarographic 酸素電極（太さ 200 μm 程度）を穿刺し，組織酸素分圧の変動を観察した。麻酔したラットの頸動脈から 1 mL 脱血，頸静脈から 1 mL の人工赤血球を投与し，ヘマトクリット 5 %以下まで交換した。人工赤血球は赤血球と同様に膠質浸透圧を持たないので，5 %アルブミン溶液を併用した。アルブミンのみで血液稀釈をすると，代償機能により最初は心拍出量の 1.5 倍程度の増大がみられるがその後は低下し，また腎皮質酸素分圧も顕著に低下し，全例が 90%交換を待たずに死亡した。対して人工赤血球 / アルブミンで交換すると，腎皮質酸素分圧は高値を維持し，全例が生存した。

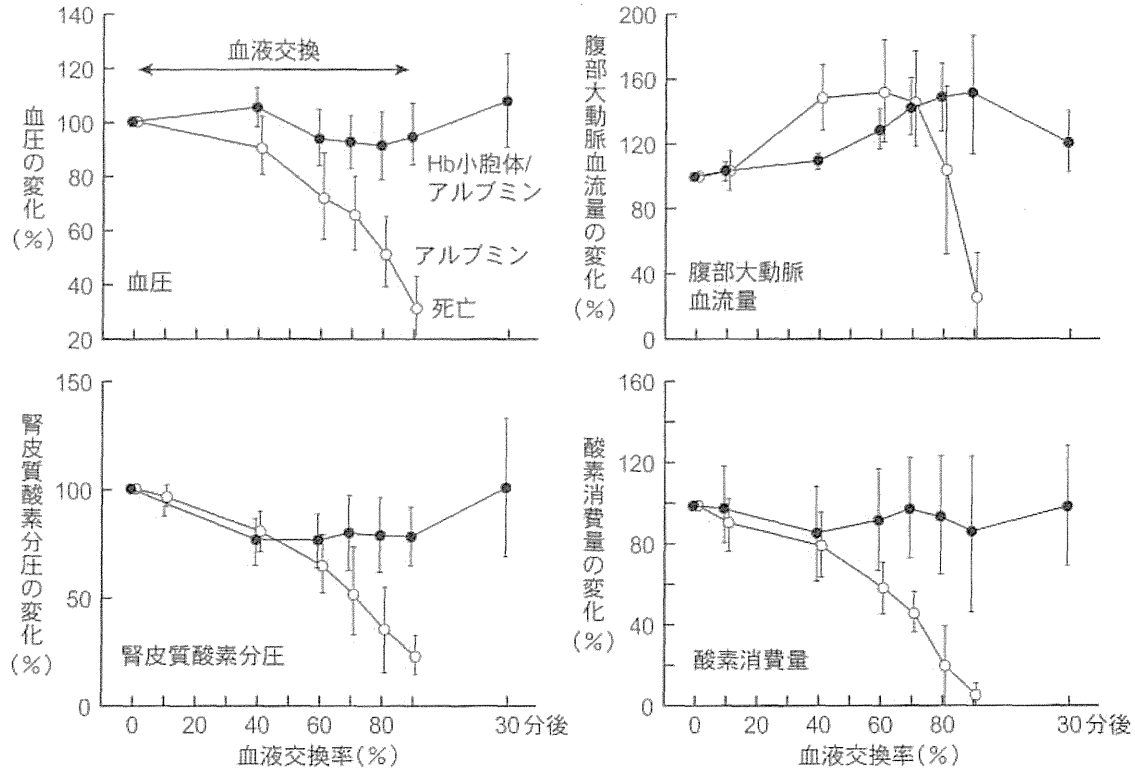

図2　ラット循環血液量の90%を人工赤血球で置換した場合の，血圧，腹部大動脈血流量，腎皮質酸素分圧，酸素消費量の変化

アルブミンによる血液交換では循環血液量が保たれ，最初は血流量が増えて酸素供給を補うが，さらに交換率が高くなると，各パラメータは悪化し，死に至る。人工赤血球があると安定に推移し，全例が生存する。

（筆者作成）

臨床現場を考えた場合，出血がひどく止血手段がない状況（uncontrolled hemorrhage）では，輸液を絶えず注入することになり，ヘマトクリットが低下する。この状況下で，人工赤血球を投与し続けることによる延命が期待できる。他方，循環血液量の40～50%を急速脱血してショック状態とした動物に，等量の人工赤血球を投与することにより蘇生でき，血行動態，血液ガス組成など対照群の脱血液を投与した場合と同等に推移し，全例が生存できることを確認している。このように，人工赤血球は，自然災害や有事の大量需要に対して，血液型にかかわらず，いつでも何処でも必要時に投与して延命ができる酸素輸液剤として期待できる。術前血液希釈，術中出血分の補充的投与の可能性も十分に考えられる。

おわりに

人工赤血球については上述のように，輸血代替物としてのみならず，酸素運搬体として様々な応用例が見出されている。是非とも皆様からのご支援をいただき，人工赤血球を実用化させたい。人工赤血球に興味のある方は，遠慮なくご一報頂きたい。

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（酒井　宏水）

文　献

1) 酒井宏水，土田英俊：人工赤血球の過去，現在，未来．ファルマシア 45：23-28, 2009.
2) 酒井宏水，堀之内宏久，東　寛 ほか：輸血代替としての人工赤血球(ヘモグロビン小胞体)製剤の安全性試験．人工血液 21：37-48, 2013.
3) Sakai H：Present situation of the development of cellular-type hemoglobin-based oxygen carrier (hemoglobin-vesicles). Curr Drug Discov Techonl 9：188-193, 2012.
4) Natanson C, Kern SJ, Lurie P, et al：Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death：a meta-analysis. JAMA 299：2304-2312, 2008.
5) Sakai H, Sato A, Masuda K, et al：Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. J Biol Chem 283 (3)：1508-1517, 2008.
6) Taguchi K, Urata Y, Anraku M, et al：Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. Drug Metab Dispos 37 (7)：1456-1463, 2009.
7) Sakai H, Seishi Y, Obata Y, et al：Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats：Profiles of Hb-vesicles degradation and hematopoiesis for 14 days. Shock 31：192-200, 2009.
8) Sakai H, Horinouchi H, Kobayashi K, et al：Hemoglobin-vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. Shock 31：507-514, 2009.
9) Sakai H, Takeoka S, Park SI, et al：Surface modification of hemoglobin vesicles with poly (ethyleneglycol)and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. Bioconjug Chem 8：23-30, 1997.

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特集：人間の体・五感に関連する造粒技術

人工赤血球（ヘモグロビン小胞体）微粒子分散液の特徴

Characteristics of Artificial Red Cells (Hemoglobin-vesicles) Dispersion as a Transfusion Alternative

奈良県立医科大学　酒井 宏水
Hiromi SAKAI

1. はじめに

血液には実に多くの種類の蛋白質が存在するが、最も沢山ある蛋白質は、血中濃度：12～15g/dLの血色素ヘモグロビン（Hb）である。Hbは酸素を可逆的に結合－解離する蛋白質である。ということは、酸素供給が生命活動を維持するために最も重要であることを意味するのかもしれない。Hbは赤血球（長径：8μm）の袋の中に極めて高濃度に（35g/dL）封じ込まれている。血液全体に対する赤血球の占有体積分率をヘマトクリットと呼ぶが、値はおおよそ40～50%であり、血液は赤血球という微粒子の高濃度分散液であることが解る。このため、血液は独特な溶液物性を示す。本稿では、この赤血球の役割（酸素運搬）を代替する人工物「人工赤血球」について概説する。血液に匹敵する酸素運搬機能を期待するには、人工赤血球についても高濃度微粒子分散液の構築が必要となる。

2. 人工赤血球の必要性

日本の献血－輸血システムの安全性は世界最高水準にあり、現行の医療に不可欠の治療行為である。しかし、感染の可能性や、保存期限が3週間と短く災害や有事の危機管理体制に不安を残している。受血者の血液型を確認する作業があり緊急時の対応に課題がある。少子高齢化により血液の需給バランスが崩れつつあることも事実で、2027年には101万人分の血液が不足するという試算が公表されている。人工赤血球は、これらの問題を改善する新しい製剤としてその実現が期待されている。人工赤血球の研究は、期限切れ血液に最も多く含まれるヘモグロビン（Hb）の有効利用の観点から政策的に始まった経緯がある。期限切れ赤血球は、我々が開発した精製/製造工程を経て、感染源を含まず、血液型がなく、長期保存に耐え、輸血治療を「補完」する人工赤血球製剤に「再生」される。また、輸血では対応のできない疾患や外科的処置、Unmet Medical Needsへの対応も期待されている。

Hbを使った人工酸素運搬体の研究は実は長い歴史がある。遊離Hbがもたらす腎毒性や神経毒性、あるいは血中滞留時間が短いことは早くから認識されていたので、分子内架橋（crosslink）をしたり、分子量を大きくするために重合（polymerize）したり、あるいは水溶性高分子を結合させて（polymer conjugation）分子量を大きくするなど、さまざまな構造のCell-free Hb based oxygen carriers（HBOCs）が試された。調製法が比較的簡単だからである。しかし、それでも副作用が残った[1]。赤血球の細胞型構造の生理的意義を理解すれば、前述のCell-free HBOCsに副作用が生じたことは容易に理解できる。赤血球は直径約8μmの中窪み円盤状粒子であり、蛋白質Hb（分子量64, 500）の高濃度溶液を赤血球膜に内包した構造を持つ。Hb溶液が赤血球膜で覆われている理由は、①本来さまざまな毒性のあるHbの逸脱、血管壁との直接的な接触の抑制、②腎臓の糸球体からの漏出など血管外漏出を防ぎ血中滞留時間を長くする、③35%濃厚Hb溶液の高い粘度と膠質浸透圧の抑制（赤血球は膠質浸透圧を示さない）、④Hb機能維持のための各種リン酸化合物などエネルギー分子、解糖－ならびに還元－酵素系の保持、⑤血管内皮弛緩因子（NO、CO）との反応性の制御などの役割もある。また、⑥血液（血球分散系）は非Newton流体で、体内循環とくに末梢血管内における特色ある流動形式と生理作用を示す。筆者らは、やはりHbは本来、赤血球あるいは赤血球構造に類似するカプセルの中にあるべき、という考えが正しいことを認識し「細胞型“cellular”」