Bioconjugate Chemistry

Article

pubs.acs.org/bc

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

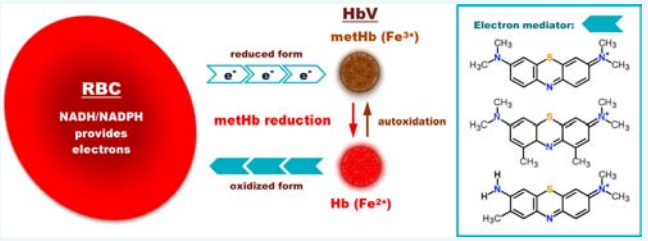
Karin Kettisen,[†,‡] Leif Bülow,[‡] and Hiromi Sakai*[,†]

[†]Department of Chemistry, Nara Medical University, Kashihara 634-8521, Japan

[‡]Department of Pure and Applied Biochemistry, Lund University, 221 00 Lund, Sweden

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.

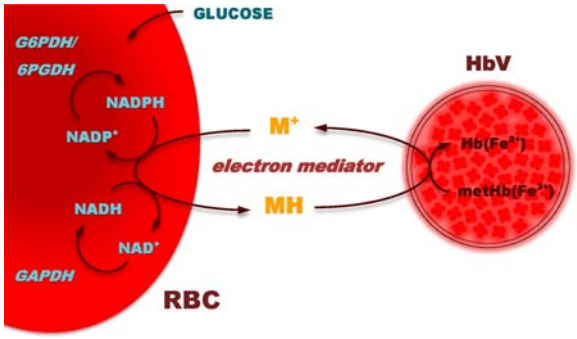
We previously clarified that the phenothiazine dye methylene blue (MB) acts as an electron mediator between electron energy-rich RBCs and HbV to increase the functional lifetime of HbV in blood circulation.[10] Effective reduction of metHb

Received: February 3, 2015
Revised: March 1, 2015
Published: March 3, 2015

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

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.

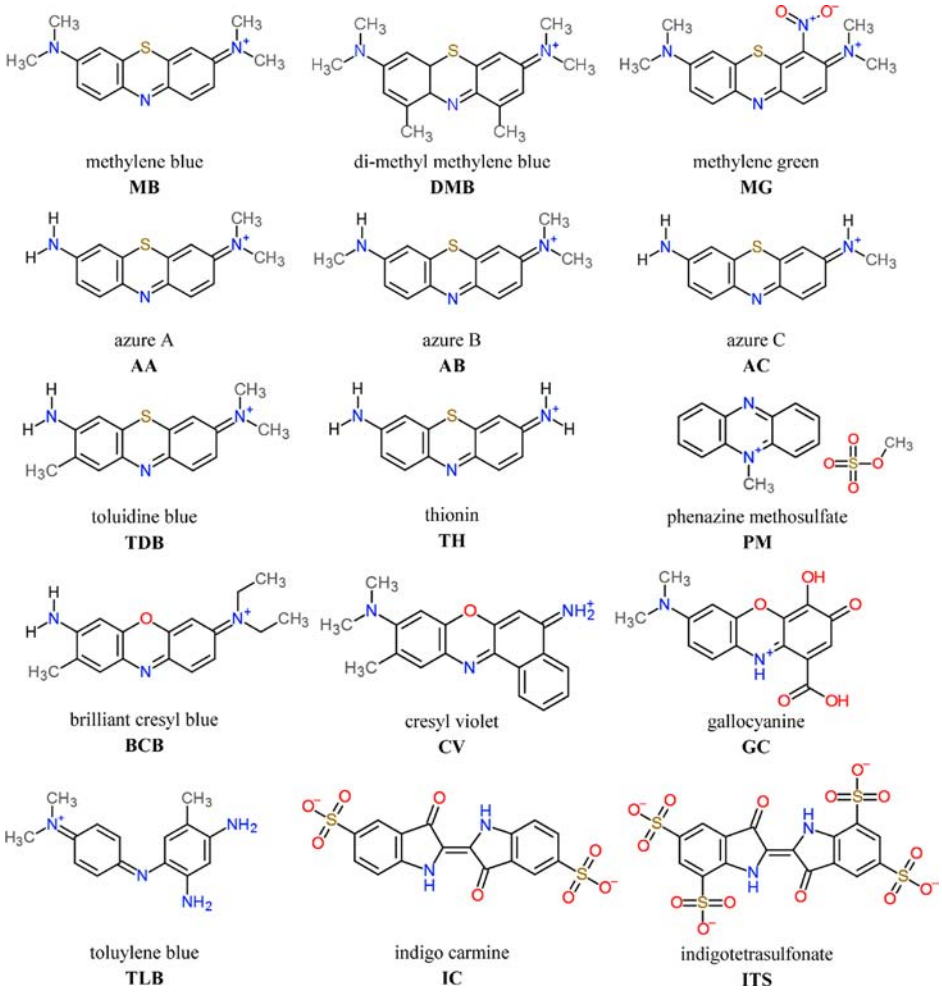
Similarly to the empty vesicles experiment, low incorporation in the RBCs was visible not only with IC and ITS, but also with PM (<0.5%). Together, MB, GC, CV, and TLB indicated higher incorporation, whereas MG, AA, AB, AC, TDB, and BCB displayed lower incorporation in comparison to the empty vesicles.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

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746–754

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:

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

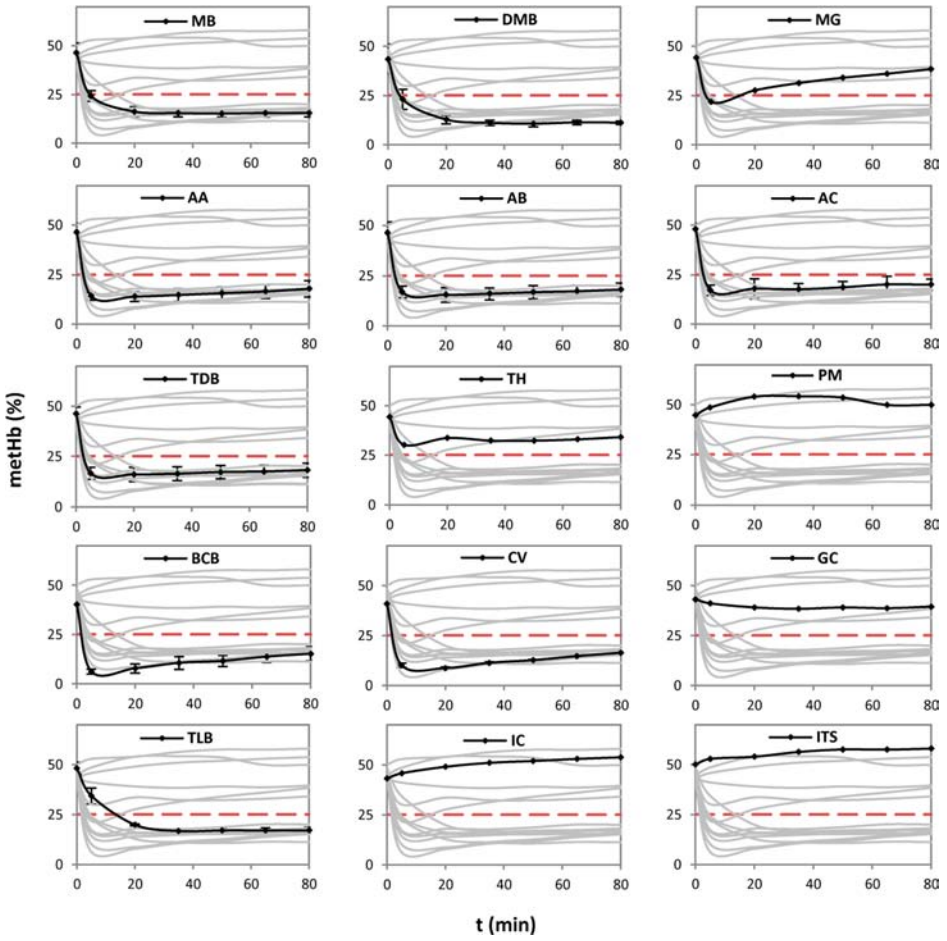


Figure 1. Reduction of metHb in HbV in the presence of RBCs and each dye. Each experiment was performed at 37 °C for 80 min under an aerobic condition. RBC suspension (12 g Hb/dL), HbV suspension of 50% metHb level, 0.3 mM dye solution, and 100 mM α-D-glucose solution were mixed in a 2:1:1:1 volume ratio. 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

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

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.

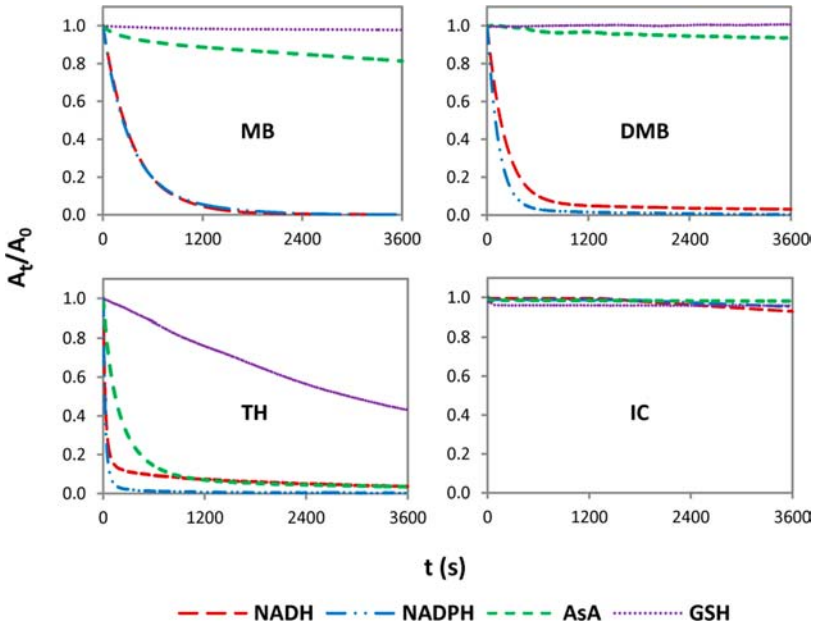


Figure 2. Reduction curves of four representing dyes (MB, DMB, TH, and IC) with reducing agents NADH, NADPH, AsA, and GSH. Deoxygenated dye (6 μM, 3 mL) and reducing agent (50 mM, 60 μL) solutions were mixed in a 1:167 molar ratio in a Thunberg cuvette and the reaction was monitored for 1 h.

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

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

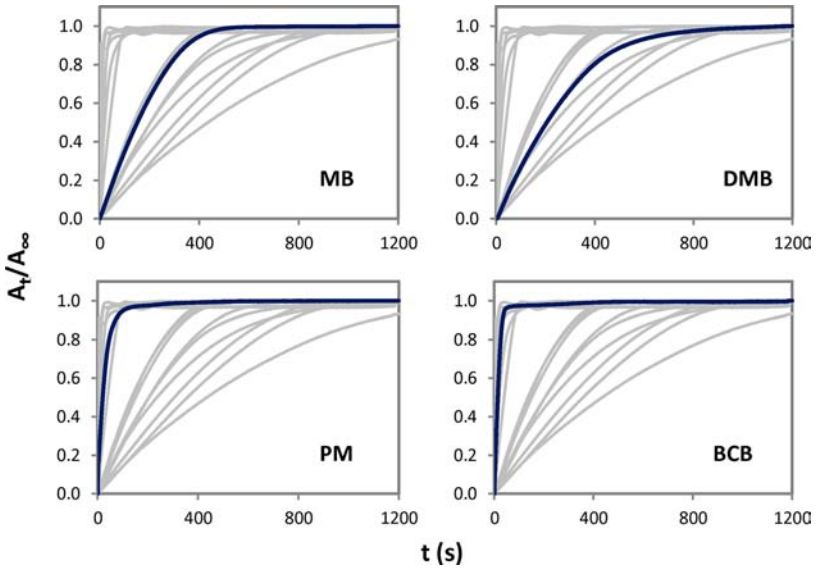


Figure 3. Reoxidation curves of four representing dyes (MB, DMB, PM, and BCB) with oxygen. 100 μL of 0.3 mM dye solution, 5−15 μL of 5 mM sodium hydrosulfite, and 2.49 mL deoxygenated PBS were mixed before addition of 500 μL air-saturated PBS. The Thunberg cuvette was monitored for 20−40 min until the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

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

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

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754

(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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: hirosakai@naramed-u.ac.jp. Tel and Fax: +81-(0)744-29-8810.

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.

■ ACKNOWLEDGMENTS

This research was supported by a Grant-in-Aid for Scientific Research (Kiban B, No. 25282136) from the Japan Society for the Promotion of Sciences (JSPS), and partly by Health and Labour Sciences Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan. Karin Kettisen was recipient of scholarships from the Scandinavia–Japan Sasakawa Foundation, Ångpanneföreningens Forskningsstiftelse, and Lundabygdens sparbanks stiftelse mm resestipendiefond för studerande. The authors acknowledge Ms. Tomoko Kure, Ms. Yumika Iga, Dr. Takashi Matsuhira, and Dr. Keizo Yamamoto (Nara Medical University) for their assistance in the experiments.

■ 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. 2015, 26, 746–754

(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.

DOI: 10.1021/acs.bioconjchem.5b00076
Bioconjugate Chem. 2015, 26, 746−754