2.7 mEq/L for the 1/2 mixture and 115.9± 2.4 mEq/L with Dm of −0.3± 1.3 mEq/L for the 1/4 mixture. With the D method, maximum Dm of −1.6± 0.8 mEq/L was noted in the 1/8 mixture and the other Dms did not exceed it.

Contrary with the W method, Cl^- concentration was measured as 105.3± 1.2 mEq/L for the HbV. And also Cl^- concentration was measured as 104.5± 0.9 mEq/L for the 1/2 mixture and 104.8± 1.1 mEq/L for the 1/4 mixture. These values differed −20.1 ± 0.4 and −8.8± 0.5 mEq/L from those each estimated value, respectively. However actually measured Cl^- concentration of 104.9± 1.6 for the 1/16 mixture and 104.8± 1.5 mEq/L for the 1/32 differed −1.1 ± 0.7 and −0.2± 0.6 mEq/L from each corresponded estimated value, respectively. Therefore those actually measured values were evaluated as the acceptably determined value.

As shown in Table 3, Na^+ concentration for medium separated form the HbV was determined as 154.5± 2.1 mEq/L with the D method and as 151.5± 0.8 mEq/L with the W method. Cl^- concentration was determined as 156.2± 6.9 mEq/L for the medium with the D method and 151.3± 0.8 mEq/L with the W method. K^+ in the medium was not detected with the D method but was determined as 0.40± 0.02 mEq/L with the W method.

Hematocrit and hemoglobin value for blood donated by the volunteers was 43.4± 3.7 % and 15.1 ± 1.5 g/dl, respectively.

Table 3. Electrolyte Concentrations in Medium of HbV

	Na^+	K	Cl
dry method	154.5±2.1	ND	156.2±6.9
wet method	151.5±0.8	0.40±0.02	151.3±0.8

mean ± standard deviations mEq/L
ND : not detectable

4. Discussion

Several reports have pointed out that contamination of HBOCs in blood specimen interferes clinical laboratory examinations[6,7,8], particularly spectrophotometry used. Further Miyake et al[9] have reported that exact determination of blood type, such as A, B, O, and AB, with an automated blood type analyzer could not be guaranteed until the HbV contamination would become less than 5 %. In addition it has been reported by Ali et al that contamination of HBOC in the circulating blood does interfere with pulse oxymetry for oxygen saturation monitoring.[10]. Murata et al[11], therefore, eliminated the HbV vesicles from patients plasma by ultracentrifugation for a number of clinically laboratory tests. Alternatively Takaori et al[12], Murata et al[13], Sou et al[14] mixed the blood with high molecular weight dextran, such as 200 ~ 600 kD, and separated the HbV vesicles entrapped into aggregated red cells and obtained HbV free plasma. These procedures separating the HbV vesicles, however, would not be applicable for clinical laboratory practice, particularly in emergency medicine. Cameron et al[15] has reported, nevertheless, that electrolyte concentrations in blood containing Hemospan™ (HBOC) could be determined using with Rcohe/Hitachi 902 ISE Modular Analytics (Mannheim, Germany) without any HBOC separation. Their blood samples, however, had been diluted 8 times in the analyzer. In practice where HBOC would be used for treatment for massive hemorrhage, the HBOC might be contaminated up to 40 % in blood as documented in a guidance for clinical application of the HBOC[16] and thus their corroboration above would not be guaranteed.

Fortunately in this study, we could measure the electrolyte concentrations in the 1/2 mixture with HbV and even in the original HbV with the D method while a few values for K^+ were exceeded a little beyond the acceptable range. In contrast with the W method the electrolyte concentrations could not be measured while the mixture rate was higher than 1/8 but could be less than 1/16. Reason why definite determination could be most done with the D method but not with the W method until the HbV was diluted less than the 1/16 remained obscure. It also remained to reveal that the liposome vesicle per se or liposome enclosed hemoglobin would affect on measurement with the W method.

In processing the HbV formation, namely encapsulation of hemoglobin solution which obtained from hemolysed red cells, K^+ should be enclosed into liposome vesicle. The vesicles were rinsed several times with physiological saline after the encapsulation. In fact, Na^+ in the HbV was found at concentration of 154.5± 2.1 mEq/L with the D method and 151.5± 0.8 mEq/L with the W method. However K^+ concentration for the HbV per se and the medium separated was determined as 0.35± 0.01 mEq/L and 0.40± 0.02 mEq/L with the W method, respectively. Liposome membrane is defined as semipermeable by Chang[17]. Therefore possibility that K^+ might diffuse out through the liposome membrane during storage could be anticipated. This possibility remained also to be revealed in the future.

Incidentally any abnormal findings were noted on neither hematocrit nor hemoglobin value for the blood donated by volunteers.

5. Summary

It was confirmed that the dry method mounted on Vitros200™ (Orho Clinical Diagnostics) was most adaptable to determine electrolyte concentrations, such as Na^+, K^+, and Cl^-, in serum containing the HbV. On the other hand, the wet method mounted on TBA200FRNEO™ (Toshiba Medical System) was limited to determine them until the HbV would be mixed less than 1/16. Reason for the limited capacity for the wet method remained to be explained in the future. Reason for presence of trace amount of K^+ in the HbV and for possible permeation of ions through liposome membrane also remained to be studied.

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人工酸素運搬体（HbV）を含む血清での電解質測定

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要約　オーソ・クリニカル・ダイアグノステイック社製ビトロス250™を用いたdry法はリポソーム膜に包埋されたヘモグロビン粒子である人工酸素運搬体（HbV）が共存しても血清中の血清電解質（Na^{+}，K^{+}，Cl^{+}）濃度の測定で臨床的に十分耐えら得るものと評価された．一方，東芝メディカルシステムズ（TBA200FRNEO™）を使用したwet法での測定ではHbVが1/8までの混入では予想濃度値との間に差を生じた．しかし1/16以下の混入においては臨床検査値として認容される精度で測定可能であった．

wet法において一定濃度以下のHbV混入にならないと上記血清中電解質の濃度測定ができなかった理由，さらにwet法で僅かながら検出されたHbV，およびHbV浮遊液中のK^{+}の由来については今後に研究する課題として残された．

original article

Measurement of electrolyte concentrations in serum containing liposome vesicles

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Abstract

We reported in previous study that wet method using conventional electrode was not satisfactory for measurement of electrolyte (Na and Cl) ion concentration when one of artificial oxygen carrier, HbV (liposome encapsulated hemoglobin), was contained more than 1/8 volume ratio in serum. In present study we attempted to clarify the above reason using liposome in which distilled water was encapsulated and which was emulsified in distilled water. The liposome emulsion was mixed with 1.68% saline at volume ratio of 2.5 : 1.2 and then the mixture was diluted at 1/2, 1/4, 1/8, 1/16, 1/32 with 0.83% saline. Human albumin* was added at 4% in those mixtures adapting to a clinical specimen. Subsequently Na and Cl ion concentration in the above mixtures were measured by the wet method (W method) using TBA-200FRNEO™ and dry chemistry method (D method) using VITROS250™, respectively. As results, Na and Cl ion concentration differences in the mixtures measured by either wet or dry method and either Na ion or Cl ion concentration differences between D and W method were obtained in most similar ranges. Therefore it was concluded that measurement of Na and Cl ion concentration by D method is not interfered with coexisted liposome vesicles at maximally 22% volume ratio in serum. And it was assumed that coexistence of both hemoglobin and liposome vesicle could limit the capacity with W method for measurement of the electrolyte ion concentration in serum containing the HbV. Consequently we evaluated the D method as it can be used for measurement of electrolyte ion concentration in serum containing liposome vesicles while some correction factors might be provided necessarily. Accuracy in determination of liposomecrit, volume ratio of liposome mass in the emulsion, with ultracentrifugation and diffusion of water molecule through the liposome membrane by osmosis were also discussed in this paper.

* Human albumin powder supplied from Sigma-Aldrich contains a trace amount of Na.

Keywords

liposome encapsulation, electrolyte concentration measurement, liposome, polarographic method, phospholipid membrane permeability, artificial oxygen carrier

Introduction

Many studies[1-7] have been reported on interference with hemoglobin based artificial oxygen carrier (HBOC) to laboratory examinations in clinical practice. The interference with HBOC in those studies attributed most to light absorbance of hemoglobin molecule with spectrophotometry. We expected, however, that polarographic measurement for electrolyte (Na, K, Cl) ion concentrations in serum would never be interfered even if the serum had contained HBOC. Thus we tried to measure them in serum containing HBOC, hemoglobin encapsulated liposome vesicle emulsified in the physiological saline (HbV). The measurement could performe most satisfactorily by dry chemistry method but surprisingly was limited partially by conventional wet method[8]. Namely

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clinically acceptable value could not be obtained, if volume of the HbV in the serum exceeded over 1/8. We wondered whether liposome per se or liposome encapsulating hemoglobin would have limited the measurement.

In this study to elucidate mechanism for the above limited ability with the wet method, we prepared liposome vesicles encapsulating distilled water instead of hemoglobin solution and attempted to demonstrate sole effect of those liposome vesicles attributed to the limitation with the wet method comparing with the dry chemistry method.

Method

Liposome vesicles encapsulating distilled water were prepared in Waseda University. 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl choline, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate which were supplied from Nippon Fine Chemical Co. Ltd. Osaka, Japan, and 1,2-distearoyl-*sn*-glycerol-3-phosphatidyl ethanolamine-*N*-PEG5000 from NOF Corp. Tokyo, Japan were mixed at mole composition of 5/5/1/0.033. The mixture of 10 g was dissolved in 140 ml of distilled water and stirred with a stirring machine model SFS710-AA (Advance Corp. Tokyo, Japan) overnight. After liposome vesicle formation, the vesicles were extruded out through 220 nm pore sized FM filter (Millipore Corp. Billerica, Ma. U.S.A.) under 30 kgf/cm^2 and their size was adjusted approximately 240 nm which was confirmed with Zeta-Sizer Nano ZS (Malvern Instruments Ltd. Worestershire, U.K.). Volume of vesicles (liposomecrit) emulsified in distilled water was determined to be 52% after 100,000 G ultracentrifugation with Optima LX-80 (Beckman Coulter Co. Brea, Ca. U.S.A.). The emulsion was sealed with air and stored at 4 ℃ in a refrigerator. Human albumin powder (A1653-5G Sigma-Aldrich St Louis, Mo. U.S.A.) was dissolved in the emulsion, 1.68% and 0.84% saline at 4% for each, simulating human serum. Then 2.5 ml of the emulsion, 1.2 ml of the 1.68% saline and 2.5 ml of 0.84% saline were mixed. This mixture (L_1) was diluted by the 0.84% saline with 4% albumin at 1:1 volume rate and prepared 1/2 mixture ($L_{1/2}$). Subsequently 1/4, 1/8, 1/16, and 1/32 mixture ($L_{1/4}$, $L_{1/8}$, $L_{1/16}$, $L_{1/32}$) were prepared in the same diluting fashion as for the 1/2 mixture. It spent 30 ~ 40 minutes for preparation of those mixtures. Na and Cl ion concentration in those mixtures were measured duplicated with the dry (D) and the wet (W) method, respectively, and whole measurement was performed within approximately 80 ~ 100 minutes. For measurement with D method, VITROS250™ (Ortho Clinical Diagnostics, Rochester, N.Y. U.S.A.) was used and TBA-200FRNEO™ (Toshiba Medical System Corp. Ohtawara, Tochigi) was used for W method. On every study day, the 0.83% saline with and without the albumin were prepared and used for reference measurement of electrolyte ion concentration by D and W method, respectively. Estimated concentrations of electrolyte ion in the L_1 and serially diluted mixtures were calculated by simple, mathematical dilution equation. Incidentally (1 − 0.52) was used as ratio of outer water volume of liposome vesicles in original, undiluted liposome emulsion for the equation, since liposomecrit was determined as 52% with the ultracentrifugation.

The human albumin powder supplied from Sigma-Aldrich company contains a few amount of Na not accompanied with Cl ion[9]. We measured Na ion concentration in the 0.84% saline with 4% albumin and obtained 150.0 ± 1.5 mEq/L for it by D method and 143.9 ± 1.3 mEq/L by W method.

These experimental procedures were performed in Osaka Prefecture Saiseikai Noe Hospital on five separate days. Mean and standard deviations for actually measured value (Am ± SD), its corresponding estimated value (Em ± SD) and difference (Dm ± SD) between the actually measured value and the estimated value for each measurement of the mixtures were listed in Table 1. Difference between ion concentration measured by D method and by W method for each mixture, was listed in Table 2. In Table 3, difference between Na and Cl ion concentration in the same mixture measured by D method and W method was listed, respectively. Student t-test was used for statistical analysis and $p < 0.05$ was evaluated as significant difference.

Results

Regarding to Na ion concentration, in general, actually measured values were lower than their estimated values for each using either D method or W method. This trend was noted markedly and significantly in L_1 and L_2. As shown in Table 1., actually measured Na ion concentration was 24.8 ± 0.8 mEq/L in L_1 and 2.9 ± 0.6 mEq/L in $L_{1/2}$ less than each corresponded estimated value by D method, respectively. The similar trend that the actually measured value was 27.9 ± 1.4 less in L_1 and 2.0 ± 1.0 mEq/L less in $L_{1/2}$ than its corresponded estimated value was noted with W method. Every actually measured value of Na ion concentration in $L_{1/8}$ ~ $L_{1/32}$, however, did not differ significantly from each estimated value not regarding to the methods used for measurement.

Actually measured Cl ion concentration in L_1 by D method was 109.5 ± 0.7 mEq/L and differed 34.2 ± 0.7 mEq/L from its estimated value. This difference was significantly greater compared with other differences for $L_{1/2}$ ~ $L_{1/32}$ ranged within 7.2 ± 0.7 ~ 4.4 ± 4.4 mEq/L. The similar trend

Table 1. Na and Cl ion concentration actually measured by D and W method, its estimated value and difference between actually measured and estimated value

			L_1	$L_{1/2}$	$L_{1/4}$	$L_{1/8}$	$L_{1/16}$	$L_{1/32}$
Na	D	Am±SD	123.7±0.8	135.9±0.8	142.2±1.0	145.5±1.0	147.1±1.2	147.8±1.1
		Em±SD	148.5	138.9±0.3	143.5±0.4	146.2±0.5	147.8±0.5	148.6±0.6
		Dm±SD	−24.8±0.8	−2.9±0.6	−1.3±0.9	−0.8±0.8	−0.7±0.8	−0.8±0.6
	W	Am±SD	116.0±1.4	130.1±1.2	137.1±1.0	140.6±1.3	142.2±1.1	143.1±1.3
		Em±SD	143.9	132.1±0.6	137.6±0.5	140.6±0.5	142.3±0.6	143.1±0.5
		Dm±SD	−27.9±1.4	−2.0±1.0	−0.5±0.5	0.0±0.9	0.0±0.7	0.0±0.8
Cl	D	Am±SD	109.5±0.7	122.1±0.9	127.9±0.6	131.5±0.5	132.6±0.7	133.4±0.8
		Em±SD	143.7	129.3±0.3	133.7±0.4	136.1±0.3	137.8±0.3	138.2±0.3
		Dm±SD	−34.2±0.7	−7.2±0.7	−5.8±0.5	−4.5±0.4	−5.2±0.6	−4.8±0.7
	W	Am±SD	108.7±0.9	123.3±0.7	130.7±0.7	134.1±0.6	136.1±0.7	136.8±1.0
		Em±SD	148.5	128.9±0.4	134.3±0.3	137.4±0.3	139.0±0.3	139.9±0.4
		Dm±SD	−39.8±0.9	−5.6±0.8	−3.6±0.4	−3.3±0.5	−2.9±0.4	−3.1±0.8

Am ± SD : mean of actully measured value ± standard deviations
Em ± SD : mean of estiamted value ± standard deviations
Dm ± SD : mean of difference between acutally measured and its estimated value ± standard deviations
n = 10 unit : mEq/L
D : D method W : W method

was noted in Cl ion concentration differences measured by W method, namely difference of 39.8± 0.9 mEq/L for L_1 which was significantly greater than those for $L_{1/2}$ ~ $L_{1/32}$ ranged within 5.6± 0.4 ~ 2.9± 0.4 mEq/L.

As shown in Table 2, every Na ion concentration measured by D method was higher than that by W method. Difference between the both methods ranged within 7.7± 1.9 ~ 4.7± 1.8 mEq/L for Na ion concentration. Difference of 7.7± 1.9 mEq/L in L_1, however, was much greater compared with differences for other mixtures. On the other hand, no significant difference for Cl ion concentration was observed in the L_1. The differences for Cl ion concentration in $L_{1/2}$~$L_{1/32}$ measured by D method ranged within −1.2± 0.7 ~ −3.5± 0.8 mEq/L and were significantly less than those by W method.

We added human albumin powder in the liposome

Table 2. Comparison of ion concentrations measured by D and W method
Difference between Na ion concentrations measured by by D and W method
Difference between Cl ion concentrations measured by by D and W method

	L_1	$L_{1/2}$	$L_{1/4}$	$L_{1/8}$	$L_{/16}$	$L_{1/32}$
Na_D — Na_W	7.7± 1.9	5.8± 1.9	5.1.± 1.6	4.9± 1.9	4.8± 1.8	4.7± 1.8
Cl_D — Cl_W	0.8± 1.2	−1.2±0.7	−2.8±0.7	−2.6±0.8	−3.5±0.8	−3.4±0.7

Na_D : Na ion concentration measured by D method
Na_W : Na ion concentration measured by W method
Cl_D : Cl ion concentration measured by D method
Cl_W : Cl ion concentration measured by W method

n = 10 mean ± standard deviations unit : mEq/L

emulsion and the serial mixtures. This procedure increased Na ion concentration in every mixture. Therefore Na ion concentration was higher than Cl ion concentration in the same mixture as shown in Table 1. Differences between Na ion and Cl ion concentration in each mixture as shown in Table 3 were ranged within 13.8± 1.2 ~ 14.5± 0.9 mEq/L with D method and 6.1 ± 0.6 ~ 7.4± 0.9 with W method. The latter values were less than former significantly. Those differences dispersed in relatively narrow range with each method and no significant difference was noted between each values, respectively.

Table 3. Difference between Na and Cl ion concentration for serial mixtures measured by D and W method, respectively

	L_1	$L_{1/2}$	$L_{1/4}$	$L_{1/8}$	$L_{1/16}$	$L_{1/32}$
D	14.2± 0.8	13.8± 1.2	14.3± 1.0	14.0± 0.8	14.5± 0.9	14.4± 0.9
W	7.4± 0.8	6.9± 0.7	6.4± 0.6	6.5± 0.9	6.1± 0.6	6.3± 0.8

n = 10 mean ± standard deviations unit : mEq/L
D : D method W : W method

Discussion

Actually measured values for Na ion concentration measured by the D method, in general, were higher than those by the W method. On the other hand, values measured for Cl ion by the D method were lower than those by the W method. And actually measured values for L_1 mixture differed considerably greater from its corresponded estimated value. These differences for L_1 were greater than those for other mixtures not regarding to either ions or methods used. The similar trend was observed in the previous study[8]. Those differences might be explained as interference with liposome vesicles contaminated in serum to polarographic measurement. Difference between the actually measured value and its estimated value became smaller corresponding to dilution of the liposome emulsion from the 1/2 to 1/32 mixture as shown in Table 1. It might be interpreted that less mixing of the liposome vesicle interfered less to electrolyte ion measurement with the polarographic method. Those changes in the differences, however, appeared in most similar fashion with either the D or W method. In addition differences between Na and Cl ion concentration in same mixtures were limited in relatively narrow range with either D or W method as shown in Table 3. Differences between the actually measured and its estimated value either by D or W method were most similar for every mixture. Therefore reliability upon the both methods could be evaluated likely most equivalent.

The relatively large difference between the actually measured value and its estimated value noted in the L_1 and $L_{1/2}$ mixture has never clarified in the previous and this study. However it was explained hardly for interference with liposome vesicles on polarographic measurement alone. It could not be excluded that the dilution equation to calculate estimated value was not fully adjusted. In the equation we used (1 − 0.52) as water volume rate in outer phase of vesicles of the liposome emulsion. We wonder, however, whether or not the ratio of (1 − 0.52) was exact value of the water volume in outer phase of liposome vesicles in the emulsion. The liposomecrit of 52% was determined by ultracentrifugation with 100,000 G. Regarding to this procedure, it was not warranted that the centrifugal force would drive out sufficiently water in void space among the vesicles and it would be suspected that much water volume more than (1 − 0.52) ratio would remain in sedimented liposome mass. We might assume that such nano sized vesicles had little deformability against extremely strong compressive force, such as 100,000 G.

Other possibility can be postulated that the vesicle would be very elastic and compressible. If so, centrifugating power was sufficient to compress vesicles and formed liposome layer on bottom of vessel showing absolute liposomecrit during the centrifugation. However, the layer would reexpand by membrane elasticity of vesicle during centrifugating power was reducing and was fixed at 52% after the power was released. Therefore liposomecrit measured after the centrifugation would be recognized higher than that during the centrifugation. HbVcrit, vesicle mass rate of the HbV which was manufactured by the same preparing process as that for the liposome used in this study, has been defined approximately 30%[10]. This value was slightly lower than 36% of the liposomecrit estimated from the actually measured value in this study. It is presumed that liposome membrane of the HbV posses lower lamellarity. Since hemoglobin as electrolyte shields the electric repulsion between lipid bilayer membranes when hemoglobin solution was encapsulated in the vesicle. Based on the HbVcrit, we estimated values of Na and Cl ion concentration in serum containing the HbV and fit them to actually measured values relatively better, respectively, in the previous study[8]. In addition, data[8] that the HbV has been composed with 9.74 g/dL of hemoglobin and 6.65 g/dL of phospholipid may support the above value of HbVcrit.

Regarding to the difference between the actually measured value and its estimated value, which was noted particularly greater in the L_1 and $L_{1/2}$, we could assume that distilled water encapsulated in the vesicle permeated through liposome membrane by osmosis after the mixing the liposome emulsion with mixture of the 0.84% saline of 2.0 ml and 1.68% saline of 1.2 ml. Liposome membrane was documented as a semipermeable membrane by Chang[11]. Diffusion coefficient varies certainly depending upon its membrane composition and upon physicochemical properties of solutions in inside and outside of the membrane. In this study, measurement of Na and Cl ion concentration was carried out at 30 ~ 40 minutes after preparation of the mixtures. Such interval is considered sufficient to move water through the semipermeable membrane of liposome.

In the previous study[8] we reported that a trace amount of potassium ion was noted in the HbV. In preparation of the HbV, hemoglobin solution which obtained from hemolysed red cells was encapsulated in liposome vesicle accompanying with intracelluar potassium (K) of red cells. The vesicles were rinsed several times with physiological saline after the encapsulation and excess hemoglobin and K outside of the vesicle were removed. The K encapsulated inside of the vesicle would partially diffuse out during the rinsing but not all. Therefore we anticipated that K remained inside might diffuse out through the liposome membrane during storage. In this study, however, we did not attempted to observe the above K ion diffusion through the liposome membrane. Either analyzer mounted D or W method is provided for measurement of electrolyte ion concentration in serum in practice and can be adopted for measurement of K ion concentration between 1.0 ~ 10.0 mEq/L with the W method and 1.0 ~ 15.0 mEq/L with the D method. Therefore accuracy with K ion concentration measurement is less than 1/10 ~ 1/20 that with Na ion. For this reason we used a single saline solution without K ion in this study. However either Na or K ion is monovalent ion. Therefore K ion is assumed to move most likely through a semipermeable membrane as same as Na does.

There were some limitations in this study. Analyzer, VITROS250™ for D method and TBA-200FRNEO™ for W method, both are essentially provided for measurement of samples obtained in clinical practice but not for saline or unphysiological solution, such as liposome emulsion. While we added human albumin powder in the mixtures simulating human serum, it might be not fully adjusted to their proper use. Actually measured Cl ion concentrations in 0.84% saline, such as 134.5 ± 0.6 by D method or 137.5 ± 0.2 mEq/L by W method, was considerably less than estimated value of 143.7 mEq/L respectively. Therefore apprehension that those analyzers had been essentially inadequate for this study remained.

In the second, any data for an accurate amount of Na

contained in the human albumin powder used in this study have not been informed from Sigma Adrich company. We dissolved the albumin powder at 4% in 0.84% saline and obtained Na ion of 150.0 ± 1.5 mEq/L by D method and 143.9 ± 1.3 mEq/L by the W method. Subsequently we subtracted 143.7 mEq/L (essential Na ion concentration for 0.84 % saline) from the measured values and we anticipated Na ion concentration associated with 4% albumin alone as 6.3 mEq/L by D method and 0.2 mEq/L by W method.

Conclusion

It was concluded that liposome vesicles contained in serum per se does not interfere essentially to measurement of Na and Cl ion concentration by either the dry method with VITROS250™ or the wet method with TBA-200FRNEO™. Therefore the limited capacity associated with the wet method noted in the previous study[8] was not clearly elucidated. It was suggested, however, possibly that coexistence of hemoglobin and liposome vesicle could limit the capacity with W method for measurement of electrolyte ion concentration in the serum.

The marked differences between actually measured value and its corresponded estimated value, noted particularly for the L_1, might attribute to the accuracy of determination of liposomecrit or to the water movement across the liposome membrane.

Acknowledgement

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リポソーム粒子を含む血清での電解質イオン濃度測定

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和文要約　以前発表した論文に臨床検査の対象となる血清に人工酸素運搬体の一つであるliposome encapsulating hemoglobin vesicle（HbV）が一定濃度以上含まれると従来の電極法，wet法（W法）を用いての血清電解質測定が不可能になることが認められると発表した．今回，その機序を解明するために蒸留水を包埋したliposome微粒子分散液を1.16％食塩液と容量比において38.5％に混合，そしてこの混合液，さらにその1/2，1/4，1/8，1/16，1/32の希釈液，すべてにその濃度が４％となるように人アルブミンを添加した．そしてこれら混合液についてそのNa，Cl濃度をW法，ならびにドライ法（dry chemistry method）を用いて測定した．その結果ではそれぞれの測定法にて測定した各混合液でのNaイオン濃度とClイオン濃度との差，また測定した各混合液でのNa，あるいはClイオン濃度の測定法にともなう差はすべてそれぞれ一定の範囲内にあった．それゆえにそれぞれの測定法に適合した補正法の必要性は認められるが，それぞれの測定法での血清電解質（Na，Clイオン）測定は可能であると認められた．すなわち少なくともliposomeそれ自身による測定法への干渉はないと認められた．そのためすでに発表した論文にて示された一定濃度以上のHbVの混入血清でのW法によって生じた測定上の誤差はliposome単独の影響に包埋されたhemoglobinの影響が加わることで生じたものと推定した．また今回の実験結果をふまえて超遠心法にて測定されたliposomeのエマルジョン内での容量比（liposomecrit），実験中におけるliposome膜を介しての水分子の拡散性についても考察した．

Original article

Effects of crystalloid and colloid osmotic pressure on HbV (liposome encapsulated hemoglobin) membrane

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Abstract

To clarify the unexpected decrease in electrolyte concentrations of saline mixed with liposome vesicle emulsion observed in previous studies, the same liposome vesicles were emulsified into 0.625 g% saline containing 0.4 g% albumin. Then Na and Cl ion concentrations were measured serially at 0, 15, 120 min, 24 and 168 hr, respectively, in Exp. 1. The measured values were within 107.5 ± 0.7 ~ 108.4 ± 1.0 mEq/L for Na ion and 106.0 ± 0.7 ~ 106.5 ± 0.8 mEq/L for Cl ion. These values were most comparable with the predict-1 value, which was calculated on the assumption that water permeates the liposome membrane freely. Results show that water permeation had been completed within at least 3.07 ± 0.03 min after the vesicles contacted with the saline. In Exp. 2, the liposome vesicles emulsified in 0.8 g% saline were mixed with 4 g% albumin solution. Then Na and Cl ion concentrations in the mixture were measured serially at 0 (detected time of 2.61 ± 0.03 min), 5, 15, 180 min after mixing, respectively. The Na and Cl ion concentrations were within 149.1 ± 1.2 ~ 149.4 ± 1.2 mEq/L and 138.9 ± 1.2 ~ 139.9 ± 1.0 mEq/L, respectively. No significant change was observed in either Na or Cl ion concentrations within 3 hr after mixing. Those results showed that colloid osmotic pressure associated with albumin in the saline did not contribute to the water permeation observed in Exp. 1. Therefore, results show that the unexpected decrease in electrolyte concentration of saline mixed with liposome emulsion observed in the previous study resulted from efflux of inside water of liposome vesicles induced by crystalloid osmotic pressure.

Keywords

liposome, phospholipid membrane, permeability, osmotic pressure, crystalloid, colloid, water efflux, artificial oxygen carrier, hemoglobin based

1. Introduction

We reported interference with a hemoglobin-based oxygen carrier (HbV, prepared by encapsulation of hemoglobin solution with liposome membrane) on electrolyte ion measurements using polarography in our previous study[1]. In that study, the electrolyte concentrations were determined using a wet method (TBA-200FRNEOTM; Toshiba Medical Systems Corp., Ohtawara, Tochigi, Japan), which showed limited performance when the HbV was mixed at lower volume rates. To explain the limitation, we prepared emulsions of liposome vesicles in which distilled water was encapsulated. Then we observed the interference with liposome vesicles per se on measurements of electrolyte (Na and Cl) ion concentrations by comparing results obtained using wet and dry methods[2]. Results show that liposome vesicles alone never interfere with polarographic measurements of electrolyte ion concentrations when using the W method. In the study, however, we observed lower electrolyte ion concentrations in the mixture of the liposome emulsion than those predicted, particularly when the mixing rate of the emulsion in saline was at 1:1 but not less. Some possibilities were proposed. Among them, it was strongly presumed that water inside of liposome vesicles diffused out through the liposome membrane by osmosis because the liposome membrane had been documented as a semipermeable membrane by Chang[3]. Therefore it was

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thought that the saline mixed with the liposome emulsion would be diluted more excessively than when diluted with water outside of the vesicles. Therefore, assuming that water does not permeate the membrane, a lower electrolyte concentration might be observed than the calculated concentration.

To resolve the speculation presented above, we attempted first to measure Na and Cl ion concentrations serially until 168 hr after the liposome emulsion was mixed with saline. Second, we observed additional effects of colloid osmotic pressure on the liposome vesicles for 3 hr because, in the previous studies, human albumin had been added at 4 g% to all examined materials for adjustment to the clinical sample[1-2].

2. Materials and Methods

Liposome vesicles encapsulating distilled water were prepared at Waseda Bioscience Research Institute in Singapore. Preparation of the liposome vesicle encapsulating distilled water was described elsewhere[2]. Briefly, a mixture of cholesterol, phospholipid, and other lipids was dissolved in distilled water and stirred overnight. The liposome vesicle diameter was adjusted approximately 220 nm by extrusion through filters under 30 kgf/cm^2 pressure. Liposomecrit, the volume ratio of liposome vesicles emulsified in distilled water, was determined as 46.6% after 100,000 × g ultracentrifugation (Optima LX-80; Beckman Coulter Inc., Brea, Ca. U.S.A.). The emulsion was sealed with air and stored in a refrigerator at 4°C.

In the first experiment (Exp. 1), human albumin powder (A1653-5G; Sigma-Aldrich Corp., St Louis, Mo. U.S.A.) was dissolved in distilled water to 2.5 g%. This albumin solution of 0.1 ml was added to the liposome emulsion of 0.5 ml. Then this mixture was transferred to a sample analysis vessel for VITROS250TM (Ortho Clinical Diagnostics Inc., Rochester, N.Y. U.S.A.). To the mixture described above, 2.5 g% saline of 0.2 ml was added and mixed well within 3-4 s. The vessel was set into the analyzer quickly. Analyses of Na and Cl ion concentrations were started promptly. Subsequently, Na and Cl ion concentrations in the same mixture of the liposome emulsion, saline, and albumin as prepared above were measured concomitantly at 15 and 120 min, and at 24 and 168 hr using the same analyzer. These measurements were performed in triplicate for each sample. The procedure described above and measurements were repeated on four separate days.

In the second experiment (Exp. 2), the liposome emulsion of 0.4 ml was mixed with 2.0 g% saline of 0.4 ml in the sample vessel for VITROS250TM. Subsequently, 25 g% albumin solution of 0.2 ml was added to the mixture. Immediately after mixing, measurements of Na and Cl ion concentrations in the above mixture were conducted. Then the liposome emulsion, the saline, and albumin solution were mixed at the same mixing rate described above. The Na and Cl ion concentrations in the latter mixture were measured concomitantly using the same analyzer at 5, 15, and 180 min after mixing. Each measurement was conducted in triplicate. The experimental procedures and measurements were repeated on four separate days. Before and after measurements of the mixtures, Na and Cl ion concentrations in 0.96 g% saline of 1.0 ml mixed with 0.4 g% the albumin of 0.2 ml in Exp. 1 and with 4.0 g% the albumin in Exp. 2 were measured, respectively, as reference in triplicate.

The predicted concentrations of Na and Cl ions for the mixture were calculated mathematically. The predict-1 values in Exp. 1 and Exp. 2 were calculated on the assumption that water molecules permeate the liposome membrane freely as follows. For example, the concentration (g%) of Cl in the mixture was obtained dividing the Cl amount in the mixture by the water volume of the mixture as $0.2 \times 2.5 / 0.1 + 0.2 + 0.5$. Then the concentration of Cl (0.625 g%) was converted to 106.9 mEq/L through multiplication by 154/0.9.

However, the predict-2 value in Exp. 1 was calculated on the assumption that water molecules do not permeate the liposome membrane. In the latter calculation, liposomecrit of 46.6 vol% was used for subtraction of the water volume in the liposome emulsion. Consequently, the water volume of the mixture (0.1 + 0.2 + 0.5) used for the equation for calculating the predict-1 value was modified to 0.1 + 0.2 + 0.5 (1 - 0.466).

The increment in Na concentration associated with addition of albumin which fundamentally contains Na molecule[4] was calculated by averaging differences between Na and Cl ion concentrations found in Exp. 1 and Exp. 2, respectively. Consequently, 0.7 mEq/L was determined as the increment in Na ion concentration in Exp. 1 in which the albumin was added at 0.4 g%, and as 6.6 mEq/L in Exp. 2 added at 4.0 g%.

These experimental procedures including preparation of mixtures and measurement of Na and Cl concentrations were conducted at Osaka Prefecture Saiseikai Noe Hospital. All data were recorded and reported as means and standard deviations in Table 1 and Table 2. Comparison of means was done using Student's t test and $p < 0.05$ was inferred as statistically significant.

3. Results

3.1. Experiment 1

The interval between mixing 2.5 g% saline into the liposome emulsion containing albumin and display of Na and

Cl concentrations on the analyzer was 3.07 ± 0.03 min. As shown in Table 1, the actually measured values for Na ion concentrations were within 107.5 ± 0.7 ~ 108.4 ± 1.0 mEq/L during 0 min and 168 hr after mixing the liposome emulsion with 2.5 g% saline. These Na concentrations did not differ significantly from each other. The predict-2 value, which was calculated on the assumption that water molecules do not permeate through the liposome membrane, was 152.8 mEq/L. This value was markedly higher than the actually measured values. On the other hand, the predict-1 value, which was calculated on the assumption that water permeates freely through the liposome membrane, was 108.8 mEq/L. It differed by 1.3 mEq/L at the most from the actually measured values.

Actually measured Cl ion concentrations ranged at 106.0 ± 0.7 ~ 106.5 ± 0.8 mEq/L during 0 min and 168 hr after mixing the liposome emulsion with 2.5% saline. The predict-2 value of 150.9 mEq/L for the Cl ion concentration was markedly higher than the actually measured value of 106.0 ± 0.7. On the other hand, the predict-1 value of 106.9 mEq/L was very close to the actually measured values with the maximum difference of 0.9 ± 0.8 mEq/L.

The Na ion concentration for the reference solution was 137.0 ± 0.8 mEq/L for the actually measured value and 136.2 mEq/L for the predicted value. The Cl ion concentration of the reference solution was 136.3 ± 0.7 mEq/L for the actually measured value and 135.5 mEq/L was for the predicted value. Differences of the actually measured value and the

Table 1. (Predict-1): Calculated Na and Cl ion concentrations (mEq/L) in the mixture on the assumption that water molecules permeated the liposome membrane freely. (Predict-2): Calculated Na and Cl ion concentrations in the mixture on the assumption that water molecules could not permeate the liposome membrane. The Na ion concentrations in predict-1, -2, and the reference solution were adjusted, respectively, by adding Na ion contained in albumin powder (see text).

Time after mixing	0 min. n=15	15 min. n=15	120 min. n=15	24 h. n=15	168 h. n=15	Reference Solution n=30
Na ion concentration						
actually measured	107.5 ± 0.7	108.2 ± 0.6	108.2 ± 0.8	108.3 ± 0.7	108.4 ± 1.0	137.0 ± 0.8
predict-1	108.8					136.2 (predicted)
predict-2	152.8					
Ci ion concentration						
actually measured	106.0 ± 0.7	106.0 ± 0.8	106.1 ± 0.7	106.5 ± 0.8	106.3 ± 0.7	136.3 ± 0.7
predict-1	106.9					135.5 (predicted)
predict-2	150.9					

Table 2. (Predict-1): Calculated Na and Cl ion concentrations (mEq/L) in the mixture on the assumption that water molecules permeated the liposome membrane freely. The Na ion concentrations for predict-1 and reference solution were adjusted, respectively, by adding concentration of Na ion contained in albumin powder (see text).

Time after mixing	0 min. n=15	5 min. n=15	15 min. n=15	180 min. n=15	Reference Solution n=30
Na ion concentration					
actually measured	149.3 ± 1.3	149.4 ± 1.2	149.3 ± 1.1	149.1 ± 1.2	142.2 ± 0.8
predict-1	146.7				142.1 (predicted)
Cl ion concentration					
actually measured	139.9 ± 1.0	139.5 ± 1.5	139.3 ± 1.2	138.9 ± 1.2	135.6 ± 0.7
predict-1	136.9				135.5 (predicted)

predicted value were 0.8 mEq/L for each of Na ion and Cl ion concentrations.

3.2. Experiment 2

The interval between the mixing 25 g% albumin solution with liposome vesicles emulsified in the saline and display of Na and Cl concentrations on the analyzer was 2.61 ± 0.03 min. Actually measured Na ion concentrations were 149.4 ± 1.2 ~ 149.1 ± 1.2 mEq/L during 0 min and 180 min after the mixing of the liposome emulsion and the albumin solution, as shown in Table 2. These values did not differ significantly from each other. A maximal difference of 2.7 mEq/L was observed between those actually measured values and the predict-1 value of 146.7 mEq/L, which was calculated using the equation on the assumption that water permeates the liposome membrane freely.

Actually measured Cl ion concentrations were 139.9 ± 1.0 mEq/L at 0 min ~ 138.9 ± 1.2 at 180 min after mixing with the albumin solution. The Cl ion concentrations tended to decrease gradually over time, but no significant difference was found between the final measured value at 180 min and the initial value. Those values differed 3.0 ± 1.5 mEq/L at most from the predict-1 value of 136.9 mEq/L.

The Na ion concentration of the reference solution was 142.2 ± 0.8 mEq/L for the actually measured values and 142.1 mEq/L for the predicted value. The Cl ion concentration of the reference solution was 135.6 ± 0.7 mEq/L for the actually measured value and 135.5 mEq/L for the predicted value. Differences between the actually measured and predicted values were 0.1 mEq/L for each of Na ion and Cl ion.

4. Discussion

As observed in Exp. 1, the serially measured means of Na and Cl ion concentrations respectively showed a narrow range of less than 1.0 mEq/L. Furthermore, these values were closest to the predict-1 values calculated on the assumption that water would permeate the liposome membrane freely. On the other hand, they were so different from the predict-2 values calculated on the assumption that water would not permeate the liposome membrane. Results show that the added saline to the liposome emulsion should be diluted by water outside of the liposome vesicles alone. Paula et al.[5] reported that the water permeability coefficient for bilayer liposome membrane is $2.4 \cdot 10^{-2}$ - $5.0 \cdot 10^{-3}$ cm/s which was determined using a light scattering method. Carruthers et al.[6] and Jansen et al.[7] reported a similar but slightly smaller permeability coefficient. The Na or Cl ion concentrations were displayed on the analyzer within 3.07 ± 0.03 min after mixing the liposome emulsion with the saline in Exp. 1. Therefore it was presumed that the water efflux from the liposome vesicles was completed within the display period described above, which explains that the predict-1 value should become closer to the actually measured value for each electrolyte ion concentration. Hauser et al.[8] reported that the Na ion permeability coefficient through an egg lecithin membrane was 0.53-$2.2 \cdot 10^{-14}$ cm/s and less 1/1,000 times than that for Cl ion. Furthermore, Paula et al.[5] reported that the K ion permeability coefficient of $1.5 \cdot 10^{-10}$ - $1.7 \cdot 10^{-12}$ cm/s was markedly lower than the coefficient of water. However, those authors emphasize that those permeability coefficients varied depending on physicochemical property of phospholipid, thickness, and surface continuity of liposome membrane and the smooth sphericity of vesicles.

In the previous studies[1,2] we added albumin to the examined samples for simulating clinical samples and for accuracy in determination by analyzers. In Exp. 2, therefore, we examined the effect of colloid osmotic pressure influencing to the water efflux. We observed changes in Na and Cl ion concentrations of the liposome emulsified in the saline serially until 3 hr after mixing albumin. Results show that the Na and Cl ion concentrations did not change significantly within 3 hr. All experimental procedures were completed in the previous studies within 3 hr. Therefore, it was verified that colloid osmotic pressure associated with the added albumin did not affect the water efflux from the vesicles within 3 hr. However, a slight decreasing trend was observed in the Cl ion concentration from the initial to the final measurement time, although no statistical significance was found. We presumed that, if we would extend the observation period longer, we might find a statistically significant decrease in those changes, but such an investigation would be beyond the scope of this study. The above results demonstrated the possibility that the inside water of the liposome vesicles diffused out through the liposome membrane by osmosis and diluted the saline more than predicted. We can exclude the other possibility concerning with the measurement of liposomecrit[2].

One limitation of this study is that we were unable to measure the electrolyte concentrations immediately when the liposome vesicles contacted the saline in Exp. 1. We attempted to measure Na and Cl ion concentrations as soon as possible after the liposome emulsion was mixed with the saline. However, 3.07 ± 0.03 min was needed to display the ion concentrations on the analyzer. Therefore, we did not observe precise changes in concentrations of Na and Cl ion at the moment of mixing, namely, at 0 min after mixing. Detection and display by the analyzer were delayed extremely compared with water movement through the

liposome membrane, as reported previously by the authors[5-7].

Another limitation was that we used very low-concentration (0.4 g%) albumin for minimizing the contribution of colloid osmotic pressure in Exp. 1. Such a low concentration of albumin is never experienced in clinical practice. Therefore, it has not been warranted whether the analyzer can determine electrolyte concentrations accurately or not. Fortunately, the measured values of Na and Cl ion concentrations in the reference solution containing the low concentration albumin remained in the acceptable range.

5. Conclusion

It was concluded based on crystalloid osmotic pressure that the efflux of inside water of the liposome vesicles occurred promptly after mixing with the saline. Colloid osmotic pressure associated with 4 g% albumin did not influence the water efflux at least 3 hr. Therefore, the difference between actually measured and predicted values for Na and Cl ion concentrations — which was observed in the previous study[3] particularly when liposome emulsion was mixed to saline at 1:1 volume ratio — was explained as produced by the efflux of water from liposome vesicles.

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HbV（liposome encapsulated hemoglobin）膜への晶質，膠質浸透圧作用

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和文要約　前回の研究で 蒸留水内包 liposome emulsion を一定濃度の食塩液と混合した時，とくに1：1容量混合でみられた食塩液中のNaイオン，Clイオンの予想以上の低下原因を明らかにするため以下の研究を行なった．研究1では前回使用したものと同質，同サイズのliposome粒子を0.4 g%アルブミンを含む0.625 g%の食塩液に浮遊させ，0，15，120分，24，168時間後にその食塩液中のNa，Clイオン濃度を測定した．その結果，実測したNa，Clイオン濃度はそれぞれ107.5 ± 0.7～108.4 ± 1.0 mEq/L，106,0 ± 0.7～106.5 ± 0.08 mEq/Lにあり，時間経過による変化は認められなかった．そしてliposome粒子内部の水分によってNa，Clイオン濃度が希釈されると推定した予測値にほぼ一致した．したがって前回の研究で認められたliposome emulsionを一定濃度の食塩液と混合した時にみられた予想以上のNaイオン，Clイオンの希釈は上記liposome粒子内部の水分の浮遊液への移動によると結論された．またその水分移動はliposome粒子と食塩液との混合後測定器が表示するまでの3.07 ± 0.03分以内に生じていたことが推定された．研究2では 0.625 g% 食塩液に上記 liposome 粒子を浮遊させた後にアルブミンを 4.0% になるように添加し，0，5，15，180 分に浮遊液中のNa，Cl濃度を測定した．その結果，Na，Clイオン濃度はそれぞれ149.4 ± 1.2～149.1 ± 1.2 mEq/L，139.9 ± 1.0～138.9 ± 1.2 mEq/Lであって，多少，時間経過とともに低下傾向，すなわちコロイド浸透圧によると思われるliposome粒子の内部の水分の移動傾向がみとめられたが統計学的には有意性を立証するに至らなかった．すなわち初期測定時から3時間測定時までで変化は認められなかった．そのため前回の研究での観察時間，3時間以内では少なくとも測定系に共存した4 g%アルブミンの膠質浸透圧はこのliposome粒子内部の水分の粒子外移動に関与していなかったことが確認された．

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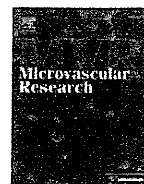

Regular Article

Increased viscosity of hemoglobin-based oxygen carriers retards NO-binding when perfused through narrow gas-permeable tubes

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ABSTRACT

Increased fluid viscosity of a solution of hemoglobin-based oxygen carriers (HBOCs) reduces vasoconstrictive effects because increased shear stress on the vascular wall enhances the production of vasorelaxation factors such as NO. Nevertheless, on a microcirculatory level, it remains unclear how viscosity affects the reaction of HBOCs and NO. In this study, different HBOCs were perfused through narrow gas-permeable tubes (25 μm inner diameter at 1 mm/s centerline velocity; hemoglobin concentration [Hb] = 5 g/dL). The reaction was examined microscopically based on the Hb visible-light absorption spectrum. When immersed in a NO atmosphere, the NO-binding of deoxygenated Hb solution (viscosity, 1.1 cP at 1000 s^{-1}) in the tube occurred about twice as rapidly as that of red blood cells (RBCs): 1.6 cP. Binding was reduced by PEGylation (PEG-Hb, 7.7 cP), by addition of a high molecular weight hydroxyethyl starch (HES) (2.8 cP), and by encapsulation to form Hb-vesicles (HbVs, 1.5 cP; particle size 279 nm). However, the reduction was not as great as that shown for RBCs. A mixture of HbVs and HES (6.2 cP) showed almost identical NO-binding to that of RBCs. Higher viscosity and particle size might reduce lateral diffusion when particles are flowing. The HbVs with HES showed the slowest NO-binding. Furthermore, Hb encapsulation and PEGylation, but not HES-addition, tended to retard CO-binding. Increased viscosity reportedly enhances production of endothelium NO. In addition, our results show that the increased viscosity also inhibits the reaction with NO. Each effect might mitigate vasoconstriction.

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Introduction

Cell-free hemoglobin-based oxygen carriers (HBOCs) have been developed for use as alternative materials for use in transfusion. Some examples are intramolecular crosslinked hemoglobin (Hb), polymerized Hb, and polyethylene glycol conjugated Hb (Chang, 2004; Natanson et al., 2008). The major remaining hurdle before clinical approval of this earliest generation of cell-free HBOCs is vasoconstriction and resulting hypertension, which are presumably attributable to the high reactivity of Hb with endothelium-derived nitric oxide (NO) (Olson et al., 2004; Palmer et al., 1987; Yu et al., 2008, 2010). As a small molecule, Hb permeates the endothelial cell layer to spaces near smooth muscle, and inactivates NO there. However, cellular Hb-vesicles (HbVs) that encapsulate concentrated Hb solution in phospholipid vesicles (Sakai et al., 2008c; Tsuchida et al., 2009) induce neither vasoconstriction nor hypertension (Sakai et al., 2000a,b). The absence of vasoconstriction in the case of intravenous HbV injection might be related to the lowered NO-binding (Sakai et al., 2008a,b, 2010) and lowered permeability across the endothelial cell layer in the vascular wall.

That the vascular wall responds to the shear stress induced by blood flow is well documented. The wall dilates and releases endothelium-derived relaxation factors such as NO and prostacyclin (Chen et al., 1989; de Wit et al., 1997; Bertuglia, 2001). To minimize the vasoconstrictive action of HBOCs, increasing the fluid viscosity would be appropriate to increase the release of vasorelaxation factors (Intaglietta, 1999; Tsai et al., 2005; Salazar Vázquez et al., 2008). Reportedly, a viscous fluid of PEG-modified Hb developed by Sangart Inc. (San Diego, CA) shows no vasoconstriction (Vandegriff and Winslow, 2009; Manjula et al., 2003). However, it remains unclear how the increased fluid viscosity affects the binding profiles of endothelial-cell-derived NO and HBOCs. On the other hand, HBOCs show a high affinity to carbon monoxide (CO), and it is important to examine the reaction profile, because CO is endogenously produced by constitutive hemeoxygenase-2 in hepatocytes; it serves as a vasorelaxation factor in hepatic microcirculation. It is reported that small molecular Hb permeates across the fenestrated endothelium,

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† Emeritus Professor Eishun Tsuchida passed away during the preparation of this manuscript.

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scavenges CO, and induces constriction of sinusoids and augments peripheral resistance (Goda et al., 1998).

Gas-permeable narrow tubes enable the measurement of the rates of O_2-release and CO-binding and NO-binding of HBOCs during their flow through the tubes at a practical Hb concentration (5–13 g/dL) (Sakai et al., 2003, 2010; Page et al., 1998). As described in this paper, we used gas-permeable narrow tubes made of perfluorinated polymer to study gas reaction profiles. We changed the viscosity of a Hb solution using PEGylation, by addition of water-soluble macromolecules, and by encapsulation in phospholipids, each of which is expected to be related to the mechanisms of vasoactive properties of HBOCs. The Hb concentration of PEG-Hb is difficult to increase through ultrafiltration because of its colloid osmotic pressure (Vandegriff et al., 1997). For that reason, we adjusted all HBOCs used for this study to 5 g/dL.

Materials and methods

Preparation of HbV, SFHb, Poly$_B$Hb, PEG-Hb, and human RBCs

With only slight modifications, HbVs were prepared using a method reported previously (Sakai et al., 1993, 2002; Sou et al., 2003). Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan) (Fig. 1). Then, Hb was stabilized by carbonylation (HbCO) and concentrated by ultrafiltration to 38 g/dL. Subsequently, pyridoxal 5′-phospate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 2.5. We use PLP instead of 2,3-diphosphoglyceric acid (2,3-DPG) because 2,3-DPG is chemically unstable. The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (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 5/5/1/0.033. The particle diameter was regulated using the extrusion method. The encapsulated HbCO was converted to HbO_2 by exposing the liquid membranes of HbVs to visible light under an O_2 atmosphere. Finally the Hb concentration of the suspension was adjusted to 5 g/dL or 10 g/dL. The particle size distribution was measured using a light-scattering method (Submicron Particle Size Analyzer, model N4 PLUS; Beckman Coulter Inc., Fullerton, CA).

Purified human Hb solution suspended in phosphate buffered saline (PBS) solution was prepared and mixed with PLP at molar ratios of PLP/Hb tetramer = 4 (Hb concentration = 5 or 10 g/dL).

To increase the viscosities of the HbV suspensions and Hb solutions, we used a high molecular weight hydroxyethyl starch, Hextend™, with Mw 670 kDa and 6 wt.% in a physiological Ringer lactate solution (HES solution) obtained from Hospira Inc. (Lake Forest, IL, USA). The volume ratios of HbV and Hb solutions ([Hb] = 10 g/dL) to HES were 50/50. Accordingly, the resulting Hb concentration became 5 g/dL.

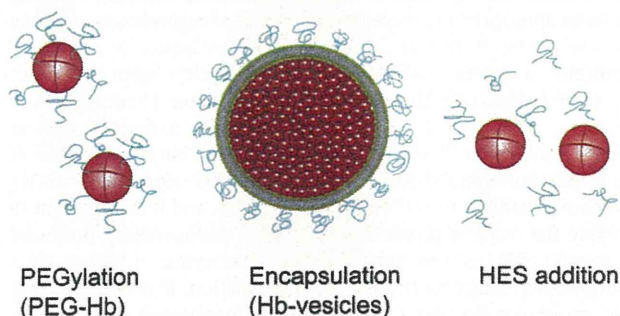

Fig. 1. Schematic representation of PEGylated Hb (PEG-Hb), encapsulated Hb (Hb-vesicles), and a simple mixture of HES and Hb to increase the viscosity of a Hb solution.

With some minor modifications, PEGylated human Hb (PEG-Hb) was prepared according to the method described in an earlier report (Vandegriff et al., 2003). The HbCO solution (5 g/dL, 50 mL, and 38.7 μM) in PBS was gently bubbled or flowed with nitrogen gas to remove O_2 in a sealed flask. Then, 15 times excess moles of 2-iminothiolane hydrochloride (IMT; Sigma Aldrich Corp.) were dissolved and stirred for 2.5 h in a dark anaerobic condition. Twenty times excess moles of α-[3-(3-maleimido-1-oxopropyl)amino]propyl-ω-methoxy PEG (Mal-PEG5000, Mr: 5552, Sunbright ME-050MA; NOF Corp.) were dissolved in the solution. The solution was kept anaerobic using a gentle N_2 and CO flow. After stirring for 3 h, the solution was diluted with 120 mL PBS and was ultrafiltrated using an appropriate system (cut off Mw. 50,000, polysulfone, 62 mm filter diameter; UHP-62 K; Advantec Co.). The dilution and ultrafiltration were repeated three times. Because of the high colloid osmotic pressure, ultrafiltration required high air pressure to permeate water. It was difficult to concentrate the Hb solution greater than 6 g/dL. Therefore, we selected Hb concentration of 5 g/dL for all the Hb-containing solutions. To convert HbCO to HbO_2, the PEG-HbCO solution was poured into a round flask and exposed to visible light of a halogen lamp. The flask was rotated and O_2 gas flowed into the flask. The photodissociated CO is emitted while O_2 binds to Hb.

For this study, we used fresh human blood specimens. The study was approved by Waseda University's Ethics Committee on Medical Research Involving Human Subjects (#2009-052), and performed according to the World Medical Association Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects (Revised Nov. 13, 2001). A blood specimen was withdrawn after obtaining written informed consent from donors. It was mixed immediately with an anticoagulant; then RBCs were pelleted at 800 × g for 30 min. They were resuspended and washed twice with PBS. The suspension was then filtrated through a leukocyte removal filter (Pall Corp., East Hills, NY). The RBC suspensions were prepared at a Hb concentration of 5 g/dL.

The P_{50} values and Hill numbers of HbVs, Hb solutions, and RBCs were obtained from the oxygen equilibrium curve measured using a Hemox Analyzer (TCS Scientific Corp., Philadelphia, PA) at 37 °C (Table 1). Steady-shear viscosity measurements were performed using a rheometer (Physica MCR 301; Anton Paar GmbH, Graz, Austria) at 25 °C.

Perfusion of Hb-containing fluids through narrow tubes

Narrow, gas-permeable tubes (25 μm inner diameter; 37.5 μm wall thickness; 150 mm length) were made of a fluorinated ethylene–propylene copolymer (Hirakawa Hewtech Corp., Ibaraki, Japan) as described in a previous report (Sakai et al., 2003, 2009a, 2010; Kubota et al., 1996) (Fig. 2). One end of the narrow tube was connected to a reservoir of the Hb-containing suspension. The narrow tube was immersed in a water bath (12 cm long × 3 cm width × 0.3 mm depth) made by two acrylic plates with a rubber supporting plate in between; it was placed horizontally on the stage of an inverted microscope (IX-71; Olympus Corp., Tokyo). The suspension in the reservoir was mixed gently and continuously using a magnetic stirrer (CC 301; AS One Corporation, Tokyo). It was then pressurized using a syringe connected to a syringe pump (FP-W-100; Toyo Sangyo Co. Ltd., Tokyo). The perfusion pressure was monitored using a digital pressure sensor (AP-C30; Keyence Co., Tokyo). The centerline flow velocity was analyzed using photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B; Vista Electronics Co., Ramona, CA) (Intaglietta et al., 1975). This method usually requires a significant change of contrasts because of the RBCs passing. However, stroma-free Hb, PEG-Hb, and HbVs are distributed homogeneously in the tube; no change of contrast is obtainable. Therefore, we added a small amount of RBCs (5 vol%) to enable centerline velocity measurements. This level of addition did not influence the reaction rate of the whole

Table 1
Physicochemical properties of HbVs, PEG-Hb, RBC, and Hb solutions, and mixtures of HES.

	HbVs	PEG-Hb	RBC	Hb	HbV + HES	Hb + HES
[Hb](g/dl)	5	5	5	5	5	5
Suspension medium	PBS	PBS	PBS	PBS	PBS + HES	PBS + HES
Hb/PLP, molar	1/2.5	–	–	1/4	–	1/4
$k'^{(CO)}_{ON}$ ($10^5\ M^{-1}\ s^{-1}$)	2.1[a]	3.8[b]	0.65[a]	2.1[a]	2.1[a]	2.1[a]
$k'^{(NO)}_{ON}$ ($10^7\ M^{-1}\ s^{-1}$)	0.61[a]	3.0[c]	0.012[a]	2.4[a]	0.61[a]	2.4[a]
P_{50} (Torr)	25–31	10.2	27	26	25–31	26
Size	279 nm	14.1 nm	8 μm	65 kDa	279 nm	65 kDa
Viscosity (cP)						
at $10^3\ s^{-1}$	1.49	7.71	1.56	1.12	6.20	2.96
at $10\ s^{-1}$	1.65	9.48	2.03	1.12	11.9	5.09
Flow pressure (kPa)	10.5–11.2	18.2–19.1	8.1–9.6	6.2–7.7	24.2–24.7	11.4–11.9

[a] (Sakai et al., 2008b);
[b] (Vandegriff et al., 2008);
[c] (Rohlfs et al., 1998).

solution: we confirmed that perfusion of saline with 5 vol% RBCs (without Hb or HbVs) provided a negligibly small light absorption spectrum. The centerline velocity was adjusted to 1 mm/s by changing the pressure applied to the reservoir. We selected the velocity of 1 mm/s in the tube and the gas concentrations (see below) to obtain the absorption changes in the 12-cm-long tube, as in our previous studies (Sakai et al., 2003, 2010).

The water bath was filled with saline containing 10 mM sodium hydrosulfite ($Na_2S_2O_4$; Wako Pure Chemical Industries Ltd., Tokyo) bubbled with low-concentration NO (NO, 4.7%; N_2, 95.3%) or CO (CO, 14.14%; N_2, 85.86%). Actually, $Na_2S_2O_4$ is effective to eliminate trace amounts of remaining oxygen that might affect the reactions of Hb and either NO or CO. The narrow tube wall is made of perfluorinated polymer and is permeable only by gas molecules: not by $Na_2S_2O_4$. The entire perfusion experiment was performed at 25 °C. In our experiment, the inner volume of the narrow tube was 5.9×10^{-5} cm^3, which is much smaller (1/180,000) than the volume of the exterior water bath (11 cm^3). We assumed that NO or CO is abundant in the exterior area and that it was provided continuously into the tube in the binding experiment.

Equipment to monitor gas reactions in narrow tubes

The apparatus consisted of an inverted microscope with an objective lens of ×40 magnification (ULWD CDPlan 40PL; Olympus Corp.), a spectrophotometer (Photonic multi- channel analyzer Model PMA-11; Hamamatsu Photonics KK, Hamamatsu, Japan) connected through a C-mount, a thin optical guide, and a computer (FMV Biblio MG50R; Fujitsu Co. Ltd., Tokyo). The microscope's light source (a halogen lamp) intensity was controlled using a current stabilizer (TH4-100; Olympus Corp.). The scanned wavelength was 194–956 nm with a gate time of 100 ms/scan; data were obtained every 0.2 nm. One spectrum from a 25-μm-diameter spot over the centerline of the narrow tube was recorded and 100 scans were accumulated in 10 s. A measuring spot on the narrow tube within the visual field of the microscope was fixed on a monitor (PVM-14L2; Sony Corp., Tokyo) through a CCD camera (Model CS 230B; Olympus Corp.) by sliding the microscope stage.

Measurement of NO-binding rate of deoxygenated Hb-containing solutions

To measure the NO-binding rate, the tube was immersed in a saline solution containing 10 mM $Na_2S_2O_4$, which had been previously bubbled with a gas of 4.7% NO/N_2 balance (Takachiho Chemical Industrial Co. Ltd., Tokyo). The resultant NO concentration outside of the tube was approximately 88 μM. Spectroscopic measurements were performed at traveling distances of 10–90 mm. Three measurements were performed after a steady flow was attained. In the spectra of the 100%-deoxygenated and 100%-nitrosyl Hb-containing samples, two isosbestic points (449 and 592 nm) were connected by a straight line as the baseline (Sakai et al., 2010; Vandegriff et al., 1997). Absorbance data at 555 nm (A_{555}, λ_{max} of deoxyHb) and 575 nm (A_{575}, λ_{max} of HbNO) from the baseline were obtained to produce a calibration line showing the relation between the NO saturation (in %) and the ratio of the two absorbances ($R = A_{555}/A_{575}$) (Fig. 3). The NO saturation values of each sample were averaged. They are shown versus the traveling distance ($n = 3$, mean ± standard deviation).

Measurement of CO-binding rate of deoxygenated Hb-containing solutions

The method used to measure the CO-binding rate is fundamentally identical to that of the NO-binding rate. A gas of 14.14% CO/N_2 balance (Takachiho Chemical Industrial Co. Ltd.) was used to attain the CO

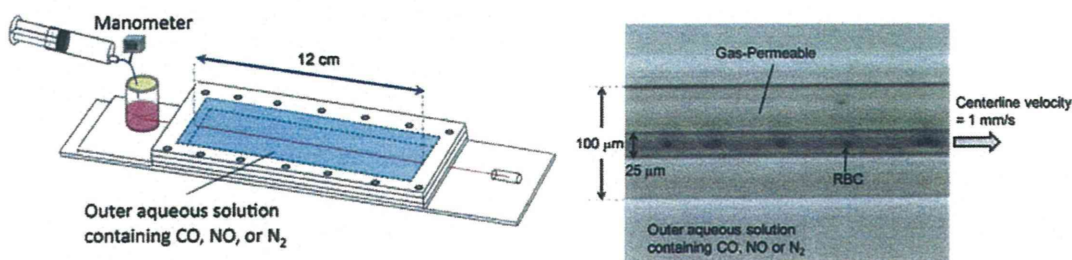

Fig. 2. (left) Schematic view of the experimental setup of a gas-permeable artificial narrow tube (red line) immersed in a water bath (light blue) produced by the gap separating two transparent acrylic plates with a rubber supporting plate. One end of the narrow tube was connected to a reservoir (pink) of the Hb-containing suspension. The reservoir is pressurized using N_2 gas for perfusion of a fluid through the tube. (right) Microscopic view of a gas-permeable artificial narrow tube. The tube, made of perfluorinated polymer, is gas-permeable. The tube is immersed in water equilibrated with low-concentration CO, or NO gases.

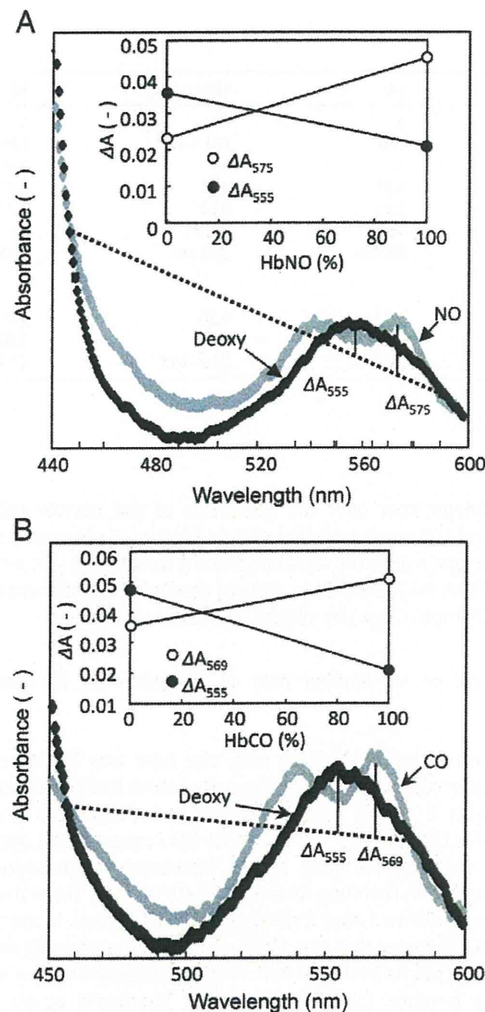

Fig. 3. Calculation of reaction levels (A) HbNO and (B) HbCO from the absorption changes. For the determination of HbNO level, for example, two isosbestic points of the spectra of deoxygenated and nitrosyl HbVs (at 449 and 592 nm) were connected by a straight line (baseline). Based on absorbances at 555 (ΔA_{555}, λ_{max} of deoxyHb) and 576 (ΔA_{575}, λ_{max} of HbNO) nm from the baseline, linear relations between the level of HbNO (%) and ΔA_{555} and ΔA_{575} are obtained individually as depicted in the inset. The ratio of the two absorbances ($R = A_{555}/A_{575}$) was used to obtain the level of HbNO. The levels of HbCO (B) were calculated similarly.

concentration: approximately 135 μM. Two isosbestic points (454 and 578 nm) of HbCO and deoxyHb were obtained (Sakai et al., 2010). The absorbance data obtained at 555 nm (A_{555}, λ_{max} of deoxyHb) and 575 nm (A_{569}, λ_{max} of HbCO) from the baseline were used to obtain the calibration curve of CO saturation (in %) versus $R = A_{555}/A_{569}$.

Results

NO-binding profile

All Hb-containing solutions showed a change of absorption spectroscopy in the Q band region (Fig. 4). Before NO-binding, they showed a single peak at 555 nm attributable to deoxyHb (top red lines). The absorption spectrum changes gradually with the traveling distance. Two new peaks tended to appear at 545 nm and 575 nm (blue and green lines), indicating the conversion of deoxyHb to HbNO (bottom red lines, 100% HbNO). Fig. 5 shows the change of the level of HbNO saturation of all the Hb-containing samples. The NO-binding profile of the stroma-free Hb solution is the fastest; that of RBCs is the slowest. The HbNO level of Hb solution was 94 ± 6% at 5 cm traveling distance, whereas RBC showed only 39 ± 1% at 5 cm traveling distance. Each of the Hb encapsulation (HbV, 71 ± 10%), PEGylation (PEG-Hb, 73 ± 2%), and HES-addition (Hb + HES, 66 ± 7%) showed retardation of NO-binding to some degree. However, they were much faster than RBCs. The HbVs suspended in HES (HbV + HES, 37 ± 3%) showed similar NO-binding profiles to those of RBCs.

CO-binding profile

Figure 6 shows that deoxyHb with a single peak at 555 nm (top red lines) in the Q band changes gradually. Two new peaks tended to appear at 540 nm and 569 nm (blue and green lines), reflecting the conversion of deoxyHb to HbCO (bottom red lines, 100% HbCO). Figure 7 shows that the HbCO conversion rates of Hb and RBCs differed considerably. The Hb solution showed a much higher CO-binding rate than that of RBCs (90 ± 3% for Hb, 53 ± 2% for RBC at 5 cm traveling distance). The Hb encapsulation (HbV, 76 ± 6%) and PEGylation (73 ± 4%) showed retardation of CO-binding to some degree, but they are still faster than those of RBC. Addition of HES to both the Hb solution and HbVs did not seem to change the CO-binding rate.

Discussion

Our primary finding is that the NO-binding reaction of the Hb solution is retarded by encapsulation (HbVs), PEGylation (PEG-Hb), and simple HES-addition to the Hb solution when perfused through an artificial narrow plastic tube at Hb concentration of 5 g/dL. However, they showed much faster binding than RBCs did. The combination of HbVs and HES considerably retarded the NO-binding rate and showed a similar rate to that of RBCs. However, slight retardation of CO-binding was shown by encapsulation and PEGylation; the addition of HES showed no further retardation.

Conditions of hemolysis and studies related to the development of HBOCs have shown that entrapment of endothelium-derived NO induces vasoconstriction, hypertension, reduced blood flow, and vascular damage (Minneci et al., 2005; Natanson et al., 2008; Olson et al., 2004; Hess et al., 1993; Nakai et al., 1996, 1998; Rochon et al., 2004; Sloan et al., 1999). Physiological doses of CO are a vasorelaxation factor, especially in the hepatic microcirculation. Its entrapment by cell-free Hb solutions induces constriction of sinusoidal capillaries (Goda et al., 1998). These side effects caused by the presence of molecular Hb in plasma suggest that the cellular structure of RBCs plays a role in ensuring the bioavailability of NO and CO.

The suspension of HbVs has no colloid osmotic pressure. It is a particle dispersion that can be concentrated easily to [Hb] = 10 g/dL. In an earlier study, we measured the NO-binding rates of HbVs, stroma-free Hb, polymerized Hb, and RBC at 10 g/dL using the same experimental setup (Sakai et al., 2010). However, we were unable to measure PEG-Hb because it has a considerably high colloid osmotic pressure that hinders ultrafiltration. The maximum Hb concentration was as low as 6 g/dL (Sakai et al., 2000b; Vandegriff et al., 1997). One very promising material developed by Sangart Inc., PEG-Hb, is now in the final stages of clinical trials (Vandegriff and Winslow, 2009). Therefore, it is quite interesting to compare HbVs and PEG-Hb under identical conditions. In this study, we measured the NO-binding rates of these materials at [Hb] = 5 g/dL. Results show that PEG-Hb and HbVs exhibited similar NO-binding rates; they are slower than the stroma-free Hb solutions. This low rate is plausible because both PEG-Hb and HbVs reportedly show no vasoconstriction (Sakai et al., 2000a; Vandegriff et al., 2003). The retardation of NO-binding is induced by the lowered lateral diffusion and inefficient lateral fluid mixing effect when they flow through the narrow tube, according to our previous report (Sakai et al., 2010). The NO molecules that diffuse through the tube wall and enter the lumen would immediately react with Hb-

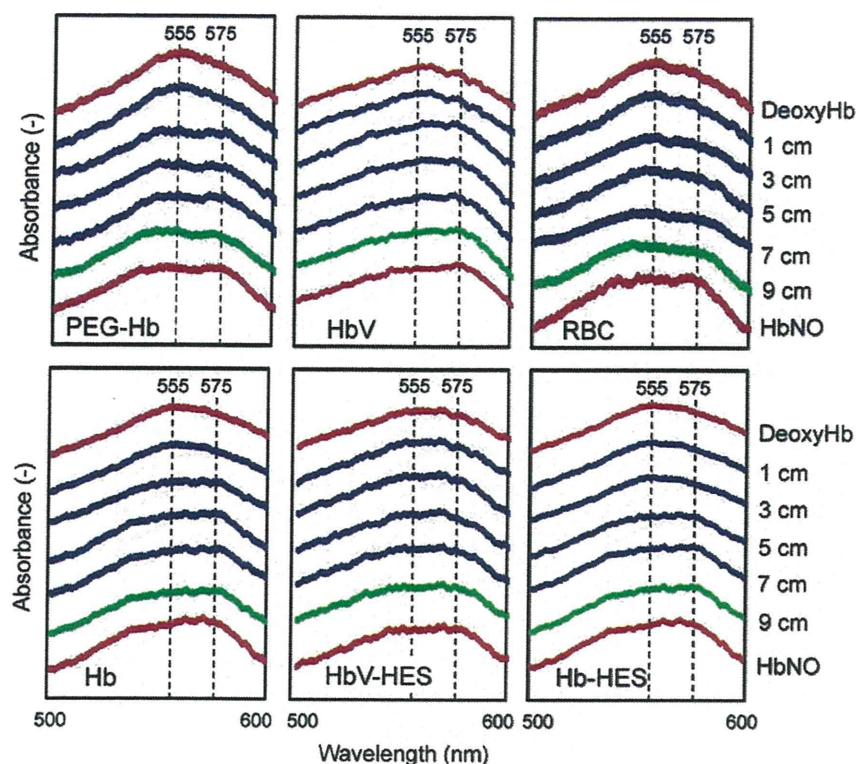

Fig. 4. Spectroscopic changes in Q bands of the Hb-containing fluids by NO-binding during perfusion through the narrow tube at traveling distances of 1–9 cm. Red plot lines show the absorption spectra of 0% and 100% reactions. Green lines show spectra at 9 cm traveling distance. Two characteristic new peaks attributed to HbNO (λ_{max} = 545 and 575 nm) increased with the traveling distance; deoxyHb (555 nm) decreased.

containing solutions at the interface. Therefore, fast mixing would be effective to create more NO-binding sites. Both viscosity and particle size influence such lateral diffusion. Actually, PEG-Hb shows a higher viscosity (7.7 cP at 10^3 s^{-1}) than that of the Hb solution. It is particularly interesting that the simple addition of HES to Hb increased the viscosity from 1.12 to 2.96 cP, and that it retarded NO-binding. Having a large diameter (279 nm), HbVs contributed to the retardation of the lateral diffusion. However, all these measures – encapsulation, PEGylation, and polymer-addition to Hb – were insufficient to retard the NO-binding to the level of RBCs.

It is quite noteworthy that all the gas reactions of RBCs are much slower than those of Hb solution and HBOCs. In our previous report of

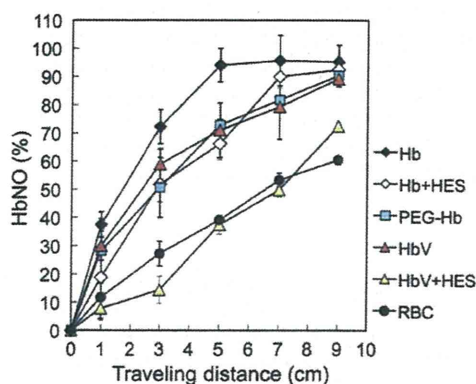

Fig. 5. Change of the level of NO-binding reactions of the Hb-containing fluids, Hb solution (black rhombi), PEG-Hb (blue squares), HbV (pink triangles), Hb + HES (white rhombi), HbV + HES (yellow triangles), and RBC (black circles) with traveling distance.

gas reactions in a diluted Hb-containing solution using the stopped flow rapid scan spectrophotometry and computer simulation, we clarified that the retardation of NO- and CO-bindings are due to the intracellular diffusion barrier created by the intracellular concentrated Hb solution (Sakai et al., 2008a). We suggested that the cell membrane does not seem to have a barrier function to gas diffusion and that the unstirred layer surrounding the cell surface would not be a major factor because of the rapid mixing at stopped flow method. In the present study we could measure the gas reactions at a practical Hb concentration in a flowing condition, which is more relevant to a physiological condition. RBCs tend to flow centerline and plasma layer (RBC-free layer) is created (Sakai et al., 2009a), which becomes a diffusion barrier for gas molecules (Vaughn et al., 1998; Butler et al., 1998; Tsoukias, 2008). This is in contrast to the molecules and particles of Hb, PEG-Hb, and HbV which are much smaller than those of RBCs. They are distributed homogeneously in the plasma phase in the tube. It is particularly interesting that addition of a high-molecular-weight HES to HbVs showed a considerably retarded NO-binding rate to the level of RBCs. The viscosity of the HbV suspension at [Hb] = 5 g/dL was 1.6 cP, which is nearly identical to that of RBC. Addition of HES to HbVs increased the viscosity to 6.2 cP because of the flocculation of HbVs through depletion interaction (Sakai et al., 2007, 2009b). The higher viscosity and larger size of flocculated HbVs are expected to contribute to the reduced lateral diffusion. Even though the flocculated HbVs dissociate at a higher shear rate, it is speculated that they would create a plasma layer of lowered HbV concentration just nearby the inner wall that might become a gas diffusion barrier, and retard the gas reactions significantly.

Even though the NO-binding rates differ considerably among samples, the CO-binding rates did not differ greatly because of the fundamentally lower CO-binding rate constants of the Hb-containing samples in comparison with the NO-binding rate constants, as shown