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Pharmaceutical Integrity of Lyophilized Methemoglobin-Albumin Clusters after Reconstitution

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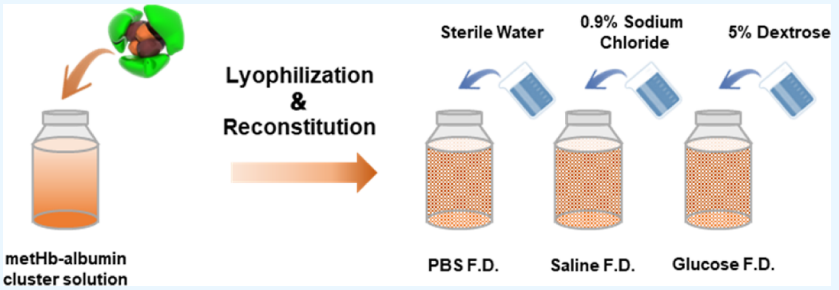

ABSTRACT: Covalent attachment of a ferric hemoglobin (metHb) core to three human serum albumin molecules to form metHb-albumin clusters has previously been used to develop an antidote for hydrogen sulfide poisoning. Lyophilization is one of the most effective approaches to preserve protein pharmaceuticals with minimum contamination and decomposition. However, there is concern that lyophilized proteins may undergo pharmaceutical alteration on reconstitution. This study investigated the pharmaceutical integrity of metHb-albumin clusters on lyophilization and reconstitution with three clinically available reconstitution fluids, (i) sterile water for injection, (ii) 0.9% sodium chloride injection, and (iii) 5% dextrose injection. The metHb-albumin clusters retained their physicochemical properties and structural integrity on lyophilization and reconstitution with sterile water for injection or 0.9% sodium chloride injection, along with comparable hydrogen sulfide scavenging ability compared to non-lyophilized metHb-albumin clusters. The reconstituted protein completely rescued lethal hydrogen sulfide poisoning in mice. On the other hand, lyophilized metHb-albumin clusters reconstituted with 5% dextrose injection showed physicochemical changes and a higher mortality rate in mice subjected to lethal hydrogen sulfide poisoning. In conclusion, lyophilization represents a potent preservation method for metHb-albumin clusters if either sterile water for injection or 0.9% sodium chloride injection is used for reconstitution.

1. INTRODUCTION

Lyophilization is one of the most effective approaches to preserve protein pharmaceuticals for long periods with minimum contamination and decomposition.[1−4] Accordingly, several biopharmaceuticals that require strict storage are marketed worldwide in a powder form. However, freeze-drying and reconstitution can introduce significant pharmaceutical alterations in biopharmaceuticals, such as changes in physicochemical properties, denaturation or degradation of proteins, and aggregation, leading to a loss of therapeutic efficacy and safety problems[5,6] In addition, it is important to choose a suitable fluid for reconstitution, such as sterile water for injection, 0.9% sodium chloride injection, or 5% dextrose injection, as in some cases lyophilized biopharmaceuticals can be unstable to particular reconstitution fluids. For example, HERCEPTIN, an antibody preparation, aggregates in 5% dextrose injection.[7] In addition, in protein pharmaceuticals that use a cross-linker, such as antibody−drug conjugates, it is necessary to consider the cross-link stability[8,9] when formulating the product. Investigations of structure and pharmaceutical activity after reconstitution thus play an important role in the development process for lyophilized protein pharmaceuticals.

metHb-albumin clusters have recently been developed as a promising antidote for hydrogen sulfide (H_2S) poisoning.[10] metHb-albumin clusters are composed of a metHb core covalently bound to human serum albumin (HSA) molecules via a bifunctional cross-linker (Figure 1). In order to use metHb-albumin clusters as a feasible and practical antidote for H_2S poisoning, lyophilization to a practical product form is desirable as it allows stable storage at sites such as health clinics and adjoining factories where poisoning can potentially occur, or in ambulances where first-line treatment can be performed.

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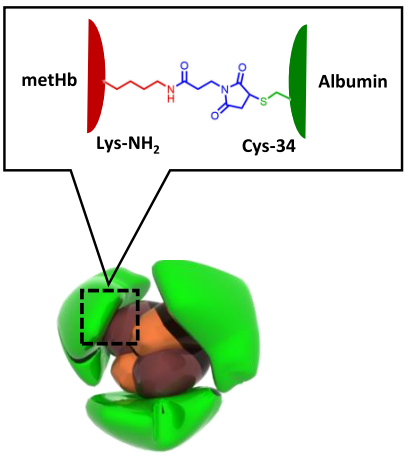

Figure 1. Structure of metHb-albumin clusters.

While the structure and pharmaceutical activity of metHb-albumin clusters in phosphate-buffered saline (PBS) have been reported previously,[10] it is not known whether lyophilized metHb-albumin clusters maintain physicochemical characteristics, structural integrity, and detoxification activity after reconstitution. Furthermore, the stability of metHb-albumin clusters in reconstitution fluids such as 0.9% sodium chloride injection and 5% dextrose injection has not been confirmed.

In this study, we aimed to investigate whether metHb-albumin clusters can be stored in a powder form and to determine optimal reconstitution conditions to broaden the options for prompt use of metHb-albumin clusters as an antidote. For this purpose, lyophilized metHb-albumin clusters were stored in the powder form for 1 week at 4 °C followed by reconstitution with either sterile water for injection, 0.9% sodium chloride injection, or 5% dextrose injection. The structural integrity and H_2S-binding capacity of reconstituted metHb-albumin clusters were analyzed in vitro. Furthermore, H_2S detoxification by reconstituted metHb-albumin clusters was investigated using lethal H_2S poisoning mice.

MetHb-albumin clusters are composed of a core metHb with an average of three HSA molecules bound. MetHb was

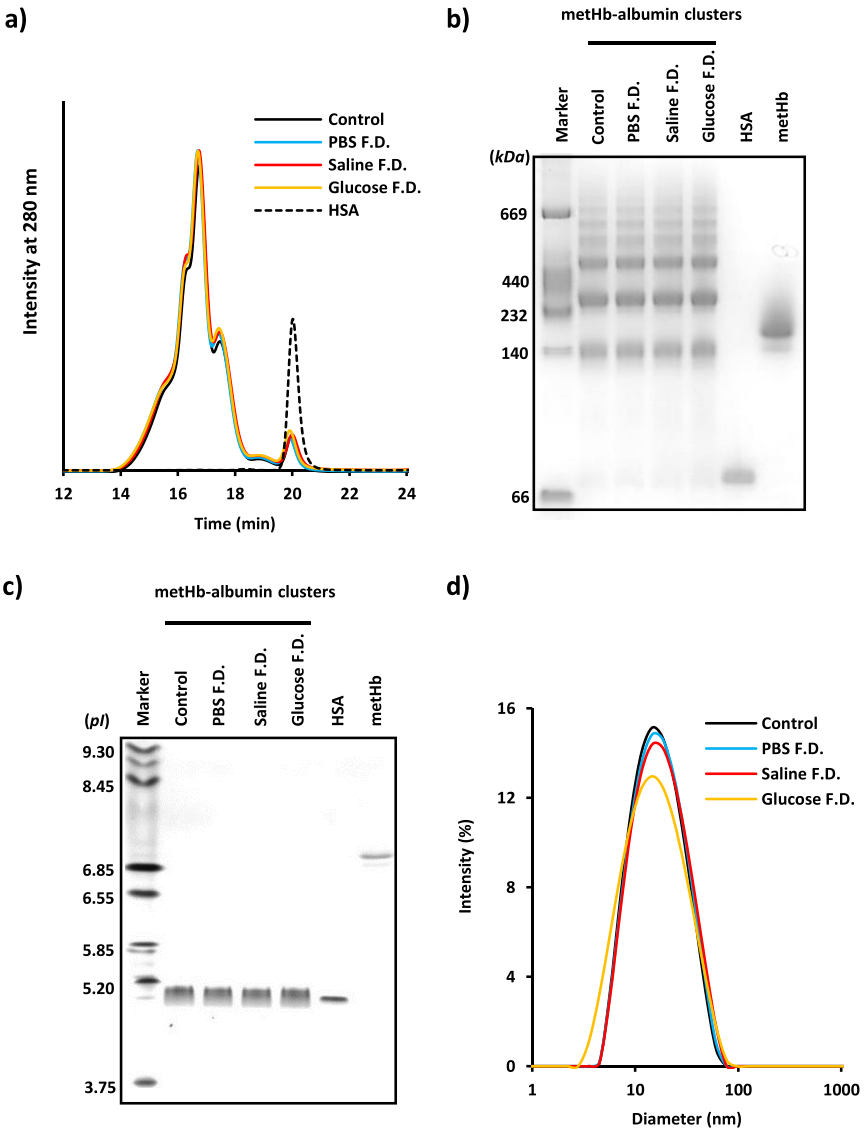

Figure 2. (a) SEC, (b) native-PAGE, and (c) isoelectric focusing (IEF) results, and (d) size distribution of lyophilized metHb-albumin clusters after reconstitution.

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covalently connected to HSA via lysine groups of metHb and Cys-34 of HSA using a bifunctional cross-linker.

2. RESULTS AND DISCUSSION

2.1. Structure of Lyophilized metHb-Albumin Clusters after Reconstitution. The stress of lyophilization and reconstitution may induce structural changes in proteins, such as aggregation or denaturation.[1,5] Therefore, the physicochemical characteristics of metHb-albumin clusters were evaluated following lyophilization and reconstitution. We tested sterile water for injection, 0.9% sodium chloride injection, and 5% dextrose injection as reconstitution fluids due to their clinical availability and wide use in clinics.

The size-exclusion chromatography (SEC) profiles of the lyophilized and reconstituted metHb-albumin clusters (PBS F.D., saline F.D., and glucose F.D.) were almost identical to that of control (non-lyophilized clusters) (Figure 2a). In addition, native-PAGE and isoelectric focusing (IEF) results after reconstitution showed similar electrophoretic patterns to control (Figure 2b,c). The calculated pI values after reconstitution matched that of HSA, not metHb (5.06, 5.06, 5.03, 5.01, 4.97, and 7.08 for control, PBS F.D., saline F.D., glucose F.D., HSA, and metHb, respectively). The results suggest that lyophilized metHb-albumin clusters maintain the cluster structure (one metHb surrounded by several HSA) following lyophilization and reconstitution. However, dynamic light scattering (DLS) data showed that while PBS F.D. and saline F.D. maintained similar physicochemical characteristics in the metHb-albumin clusters to those in the control (Figure 2d, Table 1), the size distribution of glucose F.D. shifted to slightly lower values, with an increased PD-index and ζ-potential compared to control (Figure 2d, Table 1). In our previous study, metHb-albumin clusters were found to be composed of one metHb with several molecules of HSA (n = 2−5) on the surface connected by lysine groups of metHb and Cys-34 of HSA by a heterobifunctional cross-linker.[10] The diameter of hemoglobin-albumin clusters is reported to decrease from 14.8 to 9.5 nm as the number of HSA molecules conjugated to hemoglobin decreases from 4 to 1.[11] This led us to speculate that reconstitution with 5% dextrose injection causes detachment of HSA from metHb-albumin clusters, resulting in a smaller particle size on average and a broader size distribution. It is unclear how 5% dextrose injection would promote cleavage of the cross-linker, but the following physical properties of 5% dextrose injection may be involved: (i) reactivity as a reducing sugar and/or (ii) a lower solution pH (pH 4.5, range: 3.2−6.5) than that in 0.9% sodium chloride injection (pH 5.6, range: 4.5−7.0) or sterile water for injection (pH 5.5, range: 5.0−7.0).[12−14] As the maleimide-thiol adduct can be degraded by some stimuli,[9] we speculate that 5% dextrose injection may affect the stability of the cross-link in metHb-albumin clusters.

Table 1. Physicochemical Characteristics of metHb-Albumin Clusters[a]

	diameter (nm)	PD-index	ζ-potential (mV)
Control	14.3 ± 0.13	0.118 ± 0.021	−3.34 ± 0.31
PBS F.D.	14.7 ± 0.12	0.123 ± 0.019	−3.35 ± 0.52
Saline F.D.	14.7 ± 0.20	0.160 ± 0.026	−3.61 ± 0.07
Glucose F.D.	10.7 ± 0.10	0.262 ± 0.023	−2.60 ± 0.33

[a] n = 3, mean ± S.D.

2.2. Structural Integrity of metHb in metHb-albumin clusters following Lyophilization and Reconstitution. Maintenance of the structural integrity of the metHb part in metHb-albumin clusters is crucial to ensuring pharmacological activity given that the ferric heme in metHb is responsible for capture of H_2S.[15−17] We therefore assessed possible denaturation and fragmentation of metHb in metHb-albumin clusters.

Circular dichroism (CD) spectra of lyophilized metHb-albumin clusters under all reconstitution conditions were similar to that of control (Figure 3), indicating that not only HSA but also metHb retained its secondary structure (i.e., there was no denaturation). Furthermore, no more than 1% of released heme from metHb-albumin clusters was detected in any reconstitution fluid (Table 2). The results indicate that metHb in lyophilized metHb-albumin clusters retained an active ferric heme center after reconstitution. Ferric iron in metHb has a high binding affinity for H_2S, with the result that metHb-albumin clusters detoxify H_2S.[10] As heme released from heme proteins is a source of oxidative stress resulting in tissue damages,[18−20] it is important to avoid release of free heme, so the stability shown for metHb in the clusters represents an important safety observation. Based on these results, we anticipate that the lyophilized and reconstituted metHb-albumin cluster would retain its function as an antidote for H_2S poisoning without a decrease in efficacy or induction of toxicity from released heme.

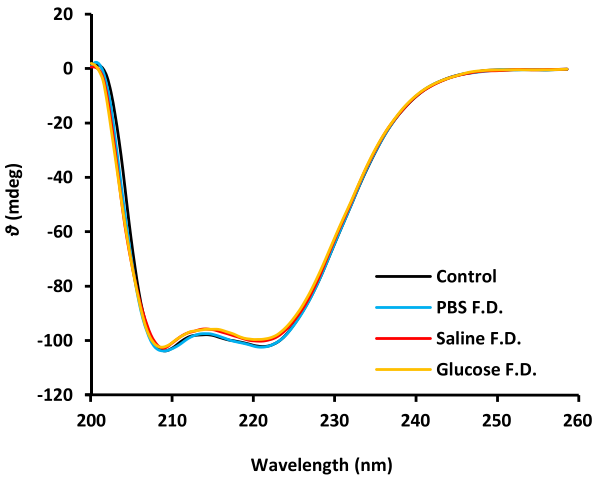

Figure 3. CD spectra of lyophilized metHb-albumin clusters after reconstitution. Spectra were recorded at 0.2 μM as metHb.

Table 2. Heme Release from Different Protein Preparations[a]

	released heme (%)
Control	0.26 ± 0.01
PBS F.D.	0.68 ± 0.54
Saline F.D.	0.48 ± 0.08
Glucose F.D.	0.49 ± 0.13
metHb	0.70 ± 0.19
metHb + 3HSA	0.35 ± 0.08

[a] n = 3, mean ± S.D.

2.3. H_2S-Binding Ability of Lyophilized metHb-Albumin Clusters after Reconstitution. The H_2S scavenging ability of lyophilized metHb-albumin clusters after

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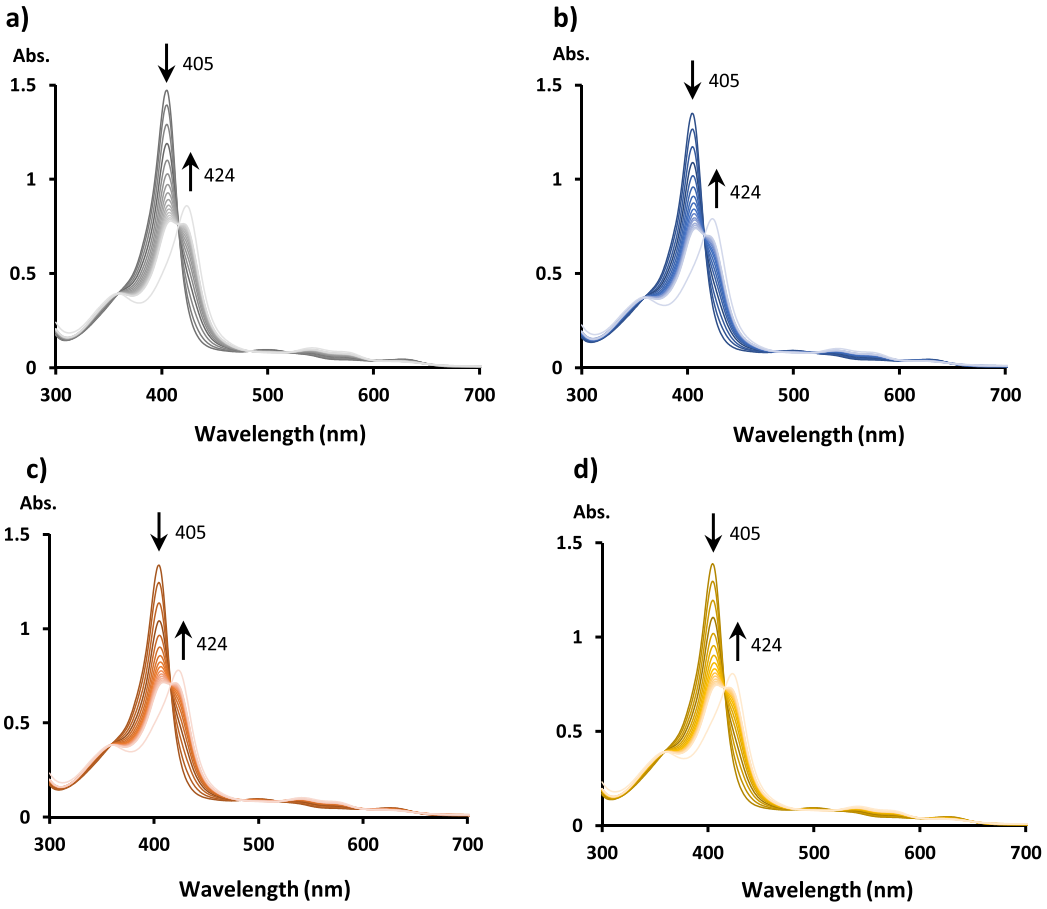

Figure 4. UV−vis spectral change of reconstituted metHb-albumin clusters in (a) control, (b) PBS F.D., (c) saline F.D., and (d) glucose F.D. on addition of NaHS. NaHS solution (a molar ratio of 0.2× heme in metHb) was gradually added to (a) control, (b) PBS F.D., (c) saline F.D., and (d) glucose F.D. All samples were adjusted to 2 μM as metHb.

reconstitution was quantitatively determined from the analysis of UV−vis absorption spectral changes. On progressive addition of an NaHS (H_2S donor) solution to each reconstituted metHb-albumin cluster solution, the Soret peak characteristic of metHb at 405 nm steadily shifted to a wavelength of 424 nm, characteristic of H_2S-bound metHb-albumin clusters (Figure 4). The H_2S-binding parameters (K and n) and fractional saturation were almost identical between the control and all reconstituted solutions (Table 3, Figure S1a,b). The results indicate that lyophilized metHb-albumin clusters retain a similar H_2S-binding affinity after reconstitution and thus would be expected to exert antidotal activity in vivo. Furthermore, the results are consistent with observations that the heme is retained in metHb within lyophilized metHb-albumin clusters after reconstitution (Figure 3 and Table 2).

Table 3. Equilibrium Constant (K) and Hill Coefficient for H_2S Binding by metHb-Albumin Clusters[a]

	K ($\times 10^6$ M^{-1})	Hill coeff. (n)
Control	0.20 ± 0.01	1.28 ± 0.05
PBS F.D.	0.22 ± 0.01	1.25 ± 0.01
Saline F.D.	0.23 ± 0.04	1.27 ± 0.03
Glucose F.D.	0.24 ± 0.01	1.29 ± 0.01

[a] n = 3, mean ± S.D.

2.4. In Vivo Pharmaceutical Activity of Lyophilized metHb-Albumin Clusters after Reconstitution. metHb-albumin clusters have been developed as an antidote for H_2S poisoning.[10] We evaluated the in vivo detoxification activity of lyophilized and reconstituted metHb-albumin clusters in lethal H_2S poisoning model mice. Intravenous administration of PBS F.D. or saline F.D. to lethal H_2S poisoning mice at a dose of 500 mg metHb/kg at 3 min after NaHS exposure led to rescue of all mice (Figure 5). The result is comparable to that for metHb-albumin clusters without lyophilization and reconstitution.[10] However, in the glucose F.D. group, one model mouse (out of eight) was not rescued (Figure 5). The results suggest that lyophilized metHb-albumin clusters in all reconstitution conditions possess pharmaceutical activity (antidotal efficacy) that is broadly comparable to the non-lyophilized product. However, considering the slight changes in physicochemical properties and the decrease in the survival rate in the glucose F.D. group (Figures 2 and 5), the possibility cannot be eliminated that there was a real true change in the efficacy for the glucose F.D. solution in vivo. One possibility is that the pharmacokinetic profile may have changed. It is known that hemoglobin and its derivatives, such as metHb, are scavenged by the mononuclear phagocyte system via formation of complexes with haptoglobin in vivo.[21,22] However, albumin wrapping around hemoglobin should prevent capture of haptoglobin, resulting in prolonged retention in the blood.[23,24] Thus, smaller metHb-albumin clusters (metHb/HSA = 1:1 and 1:2) should theoretically show shorter retention in the blood than larger ones (metHb/HSA = 1:3 and 1:4) due to the fact that smaller metHb-albumin clusters

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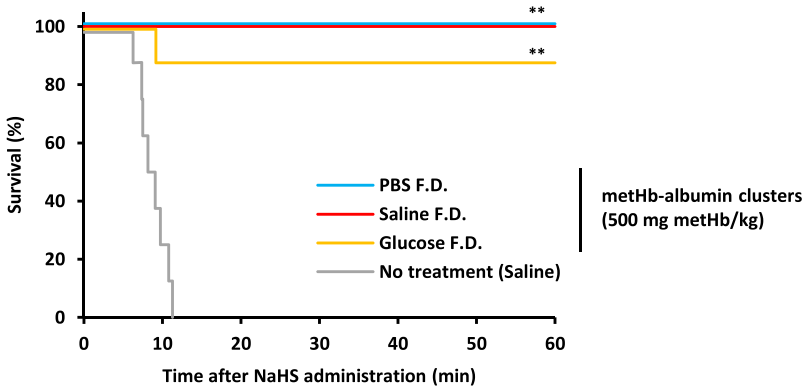

Figure 5. Survival of lethal H_2S poisoning mice after administration of lyophilized and reconstituted metHb-albumin clusters. Mice received lethal doses of NaHS solution (30 mg/kg, s.c.) followed by intravenous administration of reconstituted metHb-albumin clusters with either sterile water or injection, 0.9% sodium chloride injection, or 5% dextrose injection at a dose of 500 mg metHb/kg at 3 min after H_2S administration, respectively. "No treatment" group received saline instead of reconstituted metHb-albumin clusters. (n = 8/group) $^{**}p < 0.01$ vs no treatment (saline).

do not completely coat metHb with albumin. As mentioned in Section 3.1, smaller metHb-albumin clusters (metHb/HSA = 1:1 and 1:2) were slightly increased in glucose F.D., and these may have a shorter blood retention time. Thus, glucose F.D. is likely to show lower antidote efficacy PBS F.D. or saline F.D.

3. CONCLUSIONS

This study showed that lyophilized metHb-albumin clusters maintained structural integrity and pharmaceutical activity in the form of H_2S scavenging ability following lyophilization and reconstitution with sterile water for injection or 0.9% sodium chloride injection but not with 5% dextrose injection. The results suggest that: (i) metHb-albumin clusters can be lyophilized for long-term storage, and that (ii) sterile water for injection and 0.9% sodium chloride injection are favorable reconstitution fluids for lyophilized metHb-albumin clusters. Pharmaceutical properties enabling lyophilization and reconstitution represent positive properties of metHb-albumin clusters as an antidote for H_2S poisoning from a viewpoint of allowing prompt use in emergency situations as well as easy preservation and transport while saving space and cost. Taking these factors into account, lyophilization allows preparation of a potent pharmaceutical form for metHb-albumin clusters developed as a H_2S antidote.

4. MATERIALS AND METHODS

4.1. Reagents. Sterile water for injection, 0.9% sodium chloride injection, and 5% dextrose injection were obtained from Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan). Sodium hydrosulfide (NaHS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). metHb-albumin clusters (5 g metHb/dL in PBS, pH 7.4) were prepared, as reported previously.[10]

4.2. Lyophilization and Reconstitution of metHb-Albumin Clusters. metHb-albumin cluster solution in PBS (5 g metHb/dL) was divided between 12 glass vials to form four groups (n = 3/group) of (i) control, (ii) freeze-dried and reconstituted with sterile water for injection [PBS F.D. (freeze-dried)], (iii) freeze-dried and reconstituted with 0.9% sodium chloride injection (saline F.D.), and (iv) freeze-dried and reconstituted with 5% dextrose injection (glucose F.D.). Before lyophilization, the original solvent (PBS) for the vials of two groups (saline F.D. and glucose F.D.) was replaced with purified water using centrifugal devices (Amicon Ultra-15, 10 kDa molecular-weight cutoff, St. Louis, MO, USA). On the other hand, the procedure for solvent replacement was skipped in the PBS F.D. group since the solvent was returned to PBS by reconstituting the salt derived from the PBS produced by the lyophilization process with sterile water for the injection. Then, all the metHb-albumin cluster solution (PBS F.D., saline F.D., and glucose F.D.) was diluted to 2 g metHb/dL with purified water and frozen using liquid nitrogen, followed by lyophilization under reduced pressure at −45 °C overnight using a freeze-dryer (FDU-1200; EYELA, Tokyo, Japan). Lyophilized samples were preserved in the dark in a desiccator at 4 °C. After 1 week, vials for the PBS F.D., saline F.D., and glucose F.D. groups were reconstituted with sterile water for the injection, 0.9% sodium chloride injection, and 5% dextrose injection, respectively, and adjusted to 5 g metHb/dL. Control samples (non-lyophilized) were held for 1 week in the dark in a refrigerator at 4 °C.

4.3. In Vitro Evaluation. *4.3.1. Size-Exclusion Chromatography.* SEC was performed using a YMC-Pack Diol-300 column (8.0 mm × 300 mm, 5 μm, YMC Co. Ltd., Kyoto, Japan) with 50 mM phosphate buffer (pH 7.4) as the mobile phase.

4.3.2. Measurement of Physicochemical Characteristics. Diameter and polydispersity index (PD-index) were measured using a DLS instrument (Mobius, Wyatt Technology Corp., Santa Barbara, CA, USA). The ζ-potential was determined using a zeta-potential analyzer (ELSZ2KOP, Otsuka Electronics, Osaka, Japan).

4.3.3. Circular Dichroism. CD spectra were measured at 0.2 μM (as metHb) from 260 to 200 nm using a spectropolarimeter (J-1100, JASCO Corp., Tokyo, Japan).

4.3.4. Electrophoresis. Native-PAGE and IEF were performed by loading samples onto a 6% native polyacrylamide gel (SuperSep Ace; FUJIFILM Wako Pure Chemical, Osaka, Japan) and a pH 3−10 IEF gradient gel (Novex pH 3−10; Thermo Fischer Scientific, Waltham, MA, USA), respectively. Gels were stained with Coomassie Blue R-250 (CBB Stain One Super, Nacalai Tesque, Kyoto, Japan), and images were recorded by an Amersham Imager 600 (Cytiva, Marlborough, MA, USA).

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4.3.5. Determination of Released Heme. Reconstituted metHb-albumin clusters were diluted to 0.2 g metHb/dL in each reconstitution fluid followed by ultrafiltration (Nanosep 10 K Centrifugal Device, Pall Corp., USA). The free heme concentrations in the ultrafiltrate were determined using a heme assay kit (Sigma-Aldrich, St. Louis, MO, USA) by following the manufacturer's instructions.

4.3.6. H_2S Scavenging Ability. Sample solutions were diluted to 2 μM metHb with 100 mM phosphate buffer (pH 7.4), and NaHS solution dissolved in water (pH 12) was added dropwise. The changes in the UV−vis absorption spectra were recorded from 700 to 300 nm using a spectrophotometer (UV-1800; Shimadzu Corp., Kyoto, Japan). The binding affinities to H_2S were determined from the changes in absorbance at 424 nm to calculate an equilibrium constant (K) and Hill coefficient (n) based on the previous report.[25]

4.3.7. In Vivo Evaluation. 8 week old female ddY mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed under a 12 h light/dark cycle with food and water ad libitum. All animal experimental procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Keio University (approval number: A2021-025, Tokyo, Japan) and complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals at Keio University. Antidote activity in lethal H_2S poisoning mice was evaluated as mentioned in our previous report.[10] Briefly, ddY mice were subcutaneously administered 30 mg/kg of NaHS solution dissolved in saline, followed by intravenous administration of saline (n = 8) or each metHb-albumin cluster solution (PBS F.D., saline F.D., or glucose F.D., n = 8/group) at a dose of 500 mg metHb/kg (10 mL/kg) under anesthetized conditions at 3 min after NaHS exposure. Survival was monitored for 60 min after H_2S administration. Survival rates were analyzed using Kaplan−Meier curves and compared using the log-rank test. The data were considered significant when the probability value was <0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01054.

Equilibrium curves and Hill plots of reconstituted metHb-albumin clusters on addition of NaHS (PDF)

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Author Contributions

Y.S., K.T., and T.K. conceptualized and designed this study. Y.S., W.O., and Y.E. performed the experiments and analyzed the data. Y.S. acquired funding of the study. Y.S. and K.T. wrote the first draft of the manuscript, and T.K. and K.M. critically revised. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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