Regular Article*Highlighted Paper selected by Editor-in-Chief***Hepatic and Intrahepatic Targeting of Hydrogen Sulfide Prodrug by Bioconjugation**Kosuke Sakai,^{a,#} Hidemasa Katsumi,^{*,a,#} Kentaro Kamano,^a Kiyo Yamauchi,^a Ayuko Hajima,^a Masaki Morishita,^a Toshiyasu Sakane,^{a,b} and Akira Yamamoto^a^aDepartment of Biopharmaceutics, Kyoto Pharmaceutical University; Yamashina-ku, Kyoto 607–8414, Japan; and^bDepartment of Pharmaceutical Technology, Kobe Pharmaceutical University; Higashinada-ku, Kobe 658–8558, Japan.

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Hydrogen sulfide (H₂S) is an endogenous gaseous transmitter known to play an important role in biological functions. For the hepatic and intrahepatic targeting of H₂S prodrug at the cellular level, we developed two types of sulfo-albumins, in which five sulfide groups (source of H₂S) were covalently bound to succinylated (Suc) or galactosylated (Gal) bovine serum albumin (BSA). Sulfo-BSA-Suc and polyethylene glycol (PEG)-Sulfo-BSA-Gal, both released H₂S in the 5mM glutathione solution, but not in the plasma. Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal were taken up by RAW264.7 cells (mouse macrophage-like cells) and Hep G2 cells (human hepatocellular carcinoma cells), respectively, and H₂S was released. These results indicate that Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal selectively released H₂S intracellularly. In a biodistribution study, up to 80% of ¹¹¹In-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal rapidly accumulated in the liver, 30 min after intravenous injection in mice. Furthermore, ¹¹¹In-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal predominantly accumulated in liver nonparenchymal (endothelial cells and Kupffer cells) and parenchymal cells (hepatocytes), respectively. These findings suggest that targeted delivery of H₂S prodrug to a specific type of liver cells was successfully achieved by bioconjugation.

Key words drug delivery; hydrogen sulfide; prodrug; bioconjugation; hepatic targeting; gaseous signaling molecule

INTRODUCTION

Hydrogen sulfide (H₂S) has been considered as a gaseous transmitter that exhibits important biological functions, including anti-oxidative stress and anti-inflammatory effects.^{1,2)} H₂S is generated by enzymes such as cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) in the liver.^{3,4)} H₂S modulates hepatic lipid and glucose metabolism, reduces oxidative stress, and inhibits fibrosis.^{5–8)} Hepatic diseases are associated with the deficiency of H₂S levels in the liver.^{9,10)} Therefore, the hepatic targeting of H₂S is expected as a promising approach for the treatment of hepatic diseases.

We have recently developed a conjugate of H₂S prodrug with macromolecule in order to control the biodistribution of H₂S for the treatment of reactive oxygen species-mediated diseases.¹¹⁾ We demonstrated that macromolecular H₂S prodrug (sulfo-albumin), in which multiple sulfide groups (source of H₂S) were chemically linked to serum albumin, effectively prevented the carbon-tetrachloride-induced hepatic injury after intravenous injection. To obtain the maximum therapeutic potentials of H₂S, however, H₂S should be selectively delivered to the site where H₂S was lacked. Therefore, the hepatic and intrahepatic targeting of H₂S is required for a more efficient treatment for hepatic diseases.

It is well known that the liver is composed of two types of the cells. Hepatic cells contain parenchymal cells (PC) including hepatocyte and nonparenchymal cells (NPC) including

Kupffer cells and endothelial cells.^{12,13)} To date, it was reported that hepatic ischemia/reperfusion injury was effectively prevented by the NPC targeting of catalase and superoxide dismutase (reactive oxygen species scavengers) with mannosylation or succinylation because of the expression of mannose and scavenger receptors that recognize mannosylated and negatively charged macromolecules in NPC.^{14,15)} Furthermore, PC targeting of prostaglandin E₁ (PGE₁) and prevention of fulminant hepatitis were successfully achieved by using galactosylated polymers, because galactosylated macromolecules is recognized by asialoglycoprotein receptors in liver PC.¹⁶⁾ Therefore, the hepatic targeting and controlling intrahepatic distribution of sulfo-albumin may be achieved by chemical modification for the hepatic and intrahepatic targeting of H₂S and the efficient treatment of hepatic diseases.

The purpose of this study is to develop the hepatic and intrahepatic targeting of H₂S prodrug at the cellular level with chemical modifications. We prepared two types of sulfo-albumin derivatives for PC and NPC targeting of H₂S. For targeting of H₂S to NPC and PC, succinic acid (Suc) and galactose (Gal) were chemically conjugated with sulfo-bovine serum albumin (BSA) to obtain Sulfo-BSA-Suc and polyethylene glycol (PEG)-Sulfo-BSA-Gal, respectively. Next, H₂S release properties in the plasma and live cells, hepatic and intrahepatic distribution of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal were examined.

MATERIALS AND METHODS

Chemicals BSA was purchased from Sigma-Aldrich

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(St. Louis, MO, U.S.A.). Diethylenetriaminepentaacetic acid (DTPA) anhydride, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and HSip-1 were purchased from Dojindo Laboratory (Kumamoto, Japan). Thioacetic acid, dimethyl sulfoxide (DMSO), succinic anhydride and trinitrobenzenesulfonic acid (TNBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and 4-aminophenyl β -D-galactopyranoside were purchased from Tokyo Kasei Industry (Tokyo, Japan). α -Succinimidylsuccinyl- ω -methoxy-polyoxyethylene (PEG₂₀₀₀-NHS, average molecular mass, 2000Da) was purchased from NOF Corporation (Tokyo, Japan). ¹¹¹InCl₃ was kindly gifted from Nihon Medi-Physics (Takarazuka, Japan). Fetal bovine serum (FBS) was obtained from Biosera (Ringmer, U.K.). Dulbecco's modified Eagle's medium (DMEM) and antibiotic-antimycotic mixed stock solution were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were commercial products of reagent grade.

Animals Male ddY mice (5-week-old) were obtained from Japan SLC Inc. (Shizuoka, Japan), and housed in a temperature controlled room. All experiments were carried out in accordance with principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory. The Animal Experimentation Committee of the Kyoto Pharmaceutical University approved all protocols for animal experiments.

Synthesis of Sulfo-BSA-Suc BSA (30mg) was added in 6mL 0.1M phosphate buffer (pH 7.4), and then 1mg SPDP dissolved in 60 μ L DMSO was added. The mixture was stirred at room temperature for 2h. Then, the mixture was washed 5 times with ultrapure water by ultrafiltration (vivaspin MWCO: 10 kDa, Sartorius, Göttingen, Germany) to remove unreacted agents and then lyophilized (PDP-BSA). Next, succinylation was conducted as previously reported with slight modification.¹⁷⁾ PDP-BSA (30mg) was added in 3mL 0.1M borate buffer (pH 8.5), and then 3.1mg succinic anhydride dissolved in 30 μ L DMSO was added. The mixture was stirred for 1h. Then, the mixture was washed 5 times with ultrapure water by ultrafiltration and then lyophilized (PDP-BSA-Suc). Next, PDP-BSA-Suc (30mg) was added in 6mL phosphate buffer. 1.1 μ L thioacetic acid was added to PDP-BSA-Suc solution and stirred for 1.5h. Then, the mixture was washed 5 times with ultrapure water by ultrafiltration and then lyophilized (Sulfo-BSA-Suc). Sulfo-BSA without succinylation was also prepared as a control using the same method.

Synthesis of PEG-Sulfo-BSA-Gal To avoid aggregation of BSA during coupling reaction, we first introduced PEG to BSA. Briefly, BSA (30mg) was added in 3mL 0.1M phosphate buffer (pH 7.4). PEG₂₀₀₀-NHS (10.6mg) was added to the solution and stirred for 2h at room temperature. Then, the mixture was washed 5 times with ultrapure water by ultrafiltration and then lyophilized (PEG-BSA). Next, PEG-BSA (30mg) was added in 3mL 0.1M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.0), and EDC (14.1mg) and 4-aminophenyl β -D-galactopyranoside (19.9mg) were added to obtain PEG-BSA-Gal. The reaction mixture was stirred for 2h and washed 5 times with ultrapure water by ultrafiltration and then lyophilized. Next, PEG-BSA-Gal (30mg) was added in 6mL 0.1M phosphate buffer (pH 7.4). SPDP (1.1mg) in 60 μ L DMSO was added to PEG-BSA-Gal solution and stirred

for 2h. Then, the mixture was washed 5 times with ultrapure water by ultrafiltration and lyophilized (PEG-PDP-BSA-Gal). Next, PEG-PDP-BSA-Gal (30mg) was added in 6mL phosphate buffer and 1.2 μ L thioacetic acid was added and stirred for 1.5h. The mixture was washed 5 times with ultrapure water by ultrafiltration and then lyophilized (PEG-Sulfo-BSA-Gal). PEG-Sulfo-BSA without galactosylation was also prepared as a control using the same method.

Physicochemical Characteristics of Sulfo-BSAs The molecular weight of each sample was measured by MALDI TOF-MS (AXIMA performance, Shimadzu Corporation, Kyoto, Japan). The number of sulfide groups (source of H₂S) was calculated by measuring the number of PDP per BSA as previously reported.¹⁸⁾ The number of succinylated amino groups was measured by the TNBS method.¹⁹⁾ The number of PEG molecules per BSA were estimated based on the molecular weight. The number of Gal in BSA was measured by the anthrone method.²⁰⁾ The diameters and zeta potentials were measured by dynamic light scattering and laser doppler methods as previously reported.¹¹⁾

Biodistribution of Sulfo-BSAs For the biodistribution experiments, each Sulfo-BSAs was radiolabeled with ¹¹¹In by using DTPA anhydride as previously described.²¹⁾ Then, ¹¹¹In-Sulfo-BSAs were injected into the tail vein of mice at a dose of 1mg/kg. At predetermined times after injection, blood and major tissues were collected under isoflurane anesthesia. The radioactivity in each sample was measured in a gamma counter as previously described.¹¹⁾ For competition of hepatic uptake experiment, just after intravenous injection of unlabeled BSA-Suc or BSA-Gal (20mg/kg), ¹¹¹In-Sulfo-BSA-Suc and ¹¹¹In-PEG-Sulfo-BSA-Gal were injected into the tail vein of mice at a dose of 1mg/kg, respectively. Radioactivity in each sample was measured 30min after injection as described above.

Intrahepatic Distribution of Sulfo-BSAs For the intrahepatic distribution experiments, ¹¹¹In-Sulfo-BSA-Suc and ¹¹¹In-PEG-Sulfo-BSA-Gal were injected into the tail vein of mice at a dose of 1mg/kg. After 30min, collagenase perfusion was conducted under anesthesia, and the amount of radioactivities in PC and NPC was calculated using the method as previously described.^{11,14)}

H₂S Release Ability of Sulfo-BSAs To give a final concentration of 150 μ M sulfide group, Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal were added in 0.1M phosphate buffer containing 5mM glutathione (GSH) (pH 7.4) in sampling tube. H₂S concentration-time profile was evaluated using methylene blue method as previously described.¹¹⁾ H₂S release in phosphate buffered saline (PBS) and PBS containing 10% mouse plasma was also measured after 20min-incubation according to the same method.

Cell Culture RAW264.7 (mouse macrophage-like cells) and Hep G2 cells (human hepatocellular carcinoma cells) were grown in DMEM supplemented with 10% FBS and 1% of an antibiotic-antimycotic mixed stock solution at 37°C in humidified air containing 5% CO₂.

Cellular Uptake of Sulfo-BSAs and H₂S Release Ability of Sulfo-BSAs in RAW264.7 and Hep G2 Cells Cellular uptake of fluorescein isothiocyanate (FITC)-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in RAW264.7 and Hep G2 cells was evaluated according to previous reports.¹¹⁾ H₂S release ability of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in

RAW264.7 and Hep G2 cells was assessed using fluorescent probe (HSip-1) methods, as reported previously.^{11,22)}

Statistical Analysis Statistical significance between two groups was evaluated by Student's *t*-test. $p < 0.05$ was considered to be significant. All results are expressed as the mean $\pm$ standard deviation (S.D.) of 3 experiments.

RESULTS

Physicochemical Characteristics of Sulfo-BSAs Table 1 shows the physicochemical characteristics of various Sulfo-BSAs. The number of sulfide groups on each Sulfo-BSA was approximately 5–6 mol/mol. The number of Suc on Sulfo-BSA-Suc was approximately 20.7 mol/mol. The number of Gal and PEG on PEG-Sulfo-BSA-Gal was approximately 19.1 and 6.9 mol/mol, respectively. The diameter of Sulfo-BSAs ranged from 7.3 to 10.7 nm. All Sulfo-BSAs had a negative charge and Sulfo-BSA-Suc had maximally negative charge (-38.6 mV).

Biodistribution of Sulfo-BSAs Figure 1 shows biodistribution of various ^{111}In -Sulfo-BSAs after an intravenous injection in mice. ^{111}In -Sulfo-BSA and ^{111}In -PEG-Sulfo-BSA

slowly disappeared from plasma and gradually accumulated in the liver. In contrast, ^{111}In -Sulfo-BSA-Suc and ^{111}In -PEG-Sulfo-BSA-Gal rapidly disappeared from the blood stream and predominantly accumulated in the liver. To confirm whether ^{111}In -Sulfo-BSA-Suc and ^{111}In -PEG-Sulfo-BSA-Gal accumulate in liver *via* scavenger receptors and asialoglycoprotein receptors respectively, we performed competition experiments using BSA-Suc and BSA-Gal. Figure 2 and Fig. S1 show bio-distribution of ^{111}In -Sulfo-BSA-Suc and ^{111}In -PEG-Sulfo-BSA-Gal with or without an excess amount of unlabeled BSA-Suc or BSA-Gal. Hepatic accumulation of ^{111}In -Sulfo-BSA-Suc and ^{111}In -PEG-Sulfo-BSA-Gal was hardly prevented by coadministration of BSA-Gal and BSA-Suc, respectively (Fig. S1). In contrast, hepatic distribution of ^{111}In -Sulfo-BSA-Suc and ^{111}In -PEG-Sulfo-BSA-Gal was significantly inhibited by coadministration of BSA-Suc and BSA-Gal, respectively (Fig. 2).

Intrahepatic Distribution of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal Figure 3 shows liver PC and NPC accumulation of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal. Sulfo-BSA-Suc predominantly accumulated in NPC. In contrast, PEG-Sulfo-BSA-Gal significantly accumulated in PC.

Table 1. Physicochemical Characteristics of Various Sulfo-BSAs

Compound	Molecular weight	Modification degree (mol/mol)				Diameter (nm)	Zeta potential (mV)
		Sulfide groups	Suc	Gal	PEG		
BSA	66900	—	—	—	—	5.7 ± 0.5	-9.5 ± 1.5
Sulfo-BSA	67100	5.7	—	—	—	7.3 ± 0.3	-16.6 ± 0.3
Sulfo-BSA-Suc	70100	6.1	20.7	—	—	8.4 ± 1.9	-38.6 ± 1.5
PEG-Sulfo-BSA	79900	5.3	—	—	6.0	10.3 ± 0.9	-18.4 ± 0.7
PEG-Sulfo-BSA-Gal	87200	6.3	—	19.1	6.9	10.7 ± 1.3	-11.5 ± 0.5

BSA, bovine serum albumin; Suc, succinylated; Gal, galactosylated; PEG, polyethylene glycol.

Fig. 1. Biodistribution of (A) ^{111}In -Sulfo-BSA, (B) ^{111}In -Sulfo-BSA-Suc, (C) ^{111}In -PEG-Sulfo-BSA and (D) ^{111}In -PEG-Sulfo-BSA-Gal (1 mg/kg) after Intravenous Injection in Mice

●, plasma; ○, liver; ▲, kidney; △, spleen; ■, heart; □, and lung.

H₂S Release Ability of Sulfo-BSAs Figure 4 shows H₂S release ability of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in PBS, PBS containing 10% mouse plasma and phosphate buffer containing 5mM GSH after 20min-incubation. H₂S derived from Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal was clearly observed in the presence of GSH, whereas H₂S was hardly detected from Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in PBS and PBS containing 10% plasma. Figure 5 shows H₂S release rate from Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in the presence of 5mM GSH. The H₂S was gradually released from both Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in the presence of GSH.

Cellular Uptake of Sulfo-BSAs Figure 6 shows cellular uptake of FITC-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in RAW264.7 cells (scavenger receptors-positive cell lines) and Hep G2 cells (asialoglycoprotein receptors-positive

cell lines), respectively. Fluorescence intensities of FITC-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal gradually increased in each cell.

H₂S Release Ability of Sulfo-BSAs in RAW264.7 and Hep G2 Cells Figure 7 shows fluorescence intensity of a H₂S fluorescence probe (HSip-1) in RAW264.7 cells or Hep G2 cells after treatment of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal, respectively. Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal gradually increased the fluorescence intensities of HSip-1.

DISCUSSION

To date, various H₂S prodrugs have been developed for the treatment of reactive oxygen species-mediated disease. However, the controlled pharmacokinetics and H₂S release *in vivo* has not been examined for therapeutic use of H₂S prodrugs. As far as we know, this is the first report showing the hepatic and intrahepatic targeted delivery of H₂S prodrug using the chemical modification of targeting ligands.

Drug targeting using receptor mediated endocytosis is widely used in the drug delivery field. It was reported that negatively charged macromolecules accumulated in NPC including Kupffer cells and endothelial cells *via* scavenger receptor mediated endocytosis.^{17,23)} Furthermore, it was reported that galactosylated macromolecules selectively accumulated in PC through the asialoglycoprotein receptors-mediated endocytosis.^{24,25)} It was reported that the modification degree and density of Suc and Gal strongly affected the hepatic targeting efficiency of drug carriers.^{23,24)} Because the number of modification degree should be over approximately 20 Suc or 10 Gal molecules for the efficient hepatic targeting,^{23,24)} the Suc and Gal modification degree were designed as approximately 20 molecules/BSA in the present study. In the present study, Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal selectively accumulated in liver NPC and PC, respectively, which were in good agreement of the results of succinylated and galactosylated macromolecules.^{23,24)} These results indicate that sulfide groups and PEG on BSA hardly affect the affinity of Suc and Gal to the liver. Because BSA-Suc and BSA-Gal are well known to be recognized by scavenger receptors and asialoglycoprotein receptors,^{23,24)} the significant decrease in the hepatic uptake of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal after coadministration of BSA-Suc and BSA-Gal indicate that the negative charge of Sulfo-BSA-Suc and galactose of PEG-Sulfo-BSA-Gal were recognized by scavenger receptors and galactose

Fig. 2. Biodistribution of (A) ¹¹¹In-Sulfo-BSA-Suc and (B) ¹¹¹In-PEG-Sulfo-BSA-Gal (1 mg/kg) 30 min after Intravenous Administration in Mice

Just before ¹¹¹In-Sulfo-BSA-Suc and ¹¹¹In-PEG-Sulfo-BSA-Gal injection, 20 mg/kg of unlabeled BSA-Suc or BSA-Gal were intravenously injected into mice, respectively. Closed bar, without unlabeled BSA-Suc or BSA-Gal; Hatched bar, with unlabeled BSA-Suc; Open bar, with unlabeled BSA-Gal. ***p* < 0.01.

Fig. 3. Intrahepatic Distribution of (A) ¹¹¹In-Sulfo-BSA-Suc and (B) ¹¹¹In-PEG-Sulfo-BSA-Gal (1 mg/kg) 30 min after Intravenous Injection in Mice

***p* < 0.01.

Fig. 4. H₂S Release Ability of (A) Sulfo-BSA-Suc and (B) PEG-Sulfo-BSA-Gal in PBS, PBS Containing 10% Mouse Plasma and Phosphate Buffer Containing 5mM GSH after 20min-Incubation

N.D., not-detected.

receptors, respectively.

In general, the low-molecular H₂S prodrugs containing sulfide group show thiol-mediated H₂S release in the cytosol where approximately 5mM GSH (reduced thiol) is existed.²⁶⁾ The release studies of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in 5mM GSH indicate that Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal released H₂S in a similar manner to the existing low-molecular H₂S prodrugs containing of sulfide group.²⁷⁾ The release of H₂S from Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in the cell, but not in plasma indicates that Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal hardly released H₂S

Fig. 5. H₂S Release Rate from Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in Phosphate Buffer Containing 5mM GSH

●, Sulfo-BSA-Suc; ○, PEG-Sulfo-BSA-Gal.

Fig. 6. Fluorescence Images of (A) FITC-labeled Sulfo-BSA-Suc in RAW264.7 Cells and (B) PEG-Sulfo-BSA-Gal in Hep G2 Cells

Cells were treated with FITC-labeled Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal (0.5mg/mL) for 4h. Upper panels, fluorescence image; Lower panels, bright field image. Scale bar indicate 100 μm. (Color figure can be accessed in the online version.)

Fig. 7. H_2S Release Ability of Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal in RAW264.7 Cells and Hep G2 Cells, Respectively (100 μM Sulfide Group)

Cells were treated with HSip-1 (50 μM) and Sulfo-BSA-Suc (closed bar) or PEG-Sulfo-BSA-Gal (open bar).

in the blood stream and selectively released H_2S in the NPC and PC, respectively. Unfortunately, we could not directly detect H_2S release *in vivo* in the present study, because the specific measurement of H_2S *in vivo* is difficult using conventional analytical methods.²⁸⁾ However, these results, together with the results of cellular uptake and H_2S release studies in the RAW264.7 cells (scavenger receptors-positive cell lines) and Hep G2 cells (asialoglycoprotein receptors-positive cell lines)²⁹⁾ suggest that Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal selectively release H_2S in NPC and PC, respectively.

In conclusion, we successfully developed Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal for hepatic and intrahepatic targeting of H_2S prodrug. Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal selectively accumulated in liver NPC and PC, respectively. Sulfo-BSA-Suc and PEG-Sulfo-BSA-Gal hardly released H_2S in the plasma, but selectively released H_2S in RAW264.7 cells and Hep G2 cells, respectively. These findings provide useful information for targeted delivery of H_2S for the treatment of reactive oxygen species-mediated diseases.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

- Yonezawa D, Sekiguchi F, Miyamoto M, Taniguchi E, Honjo M, Masuko T, Nishikawa H, Kawabata A. A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. *Toxicology*, **241**, 11–18 (2007).
- Fan HN, Wang HJ, Yang-Dan CR, Ren L, Wang C, Li YF, Deng Y. Protective effects of hydrogen sulfide on oxidative stress and fibrosis in hepatic stellate cells. *Mol. Med. Rep.*, **7**, 247–253 (2013).
- Untereiner AA, Fu M, Módos K, Wang R, Ju Y, Wu L. Stimulatory effect of CSE-generated H_2S on hepatic mitochondrial biogenesis and the underlying mechanisms. *Nitric Oxide*, **58**, 67–76 (2016).
- Ahmad A, Gerő D, Olah G, Szabo C. Effect of endotoxemia in mice genetically deficient in cystathionine- γ -lyase, cystathionine- β -synthase or 3-mercaptopyruvate sulfurtransferase. *Int. J. Mol. Med.*, **38**, 1683–1692 (2016).
- Mani S, Li H, Untereiner A, Wu L, Yang G, Austin RC, Dickhout

- JG, Lhoták Š, Meng QH, Wang R. Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation*, **127**, 2523–2534 (2013).
- Mani S, Cao W, Wu L, Wang R. Hydrogen sulfide and the liver. *Nitric Oxide*, **41**, 62–71 (2014).
- Shimada S, Fukai M, Wakayama K, Ishikawa T, Kobayashi N, Kimura T, Yamashita K, Kamiyama T, Shimamura T, Taketomi A, Todo S. Hydrogen sulfide augments survival signals in warm ischemia and reperfusion of the mouse liver. *Surg. Today*, **45**, 892–903 (2015).
- Wei W, Wang C, Li D. The content of hydrogen sulfide in plasma of cirrhosis rats combined with portal hypertension and the correlation with indexes of liver function and liver fibrosis. *Exp. Ther. Med.*, **14**, 5022–5026 (2017).
- Manna P, Gungor N, McVie R, Jain SK. Decreased cystathionine- γ -lyase (CSE) activity in livers of type 1 diabetic rats and peripheral blood mononuclear cells (PBMC) of type 1 diabetic patients. *J. Biol. Chem.*, **289**, 11767–11778 (2014).
- Kang K, Zhao M, Jiang H, Tan G, Pan S, Sun X. Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats. *Liver Transpl.*, **15**, 1306–1314 (2009).
- Sakai K, Katsumi H, Sugiura M, Tamba A, Kamano K, Yamauchi K, Tamura Y, Sakane T, Yamamoto A. Pharmacokinetics and preventive effects of sulfo-albumin as a novel macromolecular hydrogen sulfide prodrug on carbon tetrachloride-induced hepatic injury. *J. Pharm. Sci.*, **107**, 2686–2693 (2018).
- Blouin A, Bolender RP, Weibel ER. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J. Cell Biol.*, **72**, 441–455 (1977).
- Tanaka T, Fujishima Y, Hanano S, Kaneo Y. Intracellular disposition of polysaccharides in rat liver parenchymal and nonparenchymal cells. *Int. J. Pharm.*, **286**, 9–17 (2004).
- Yabe Y, Nishikawa M, Tamada A, Takakura Y, Hashida M. Targeted delivery and improved therapeutic potential of catalase by chemical modification: combination with superoxide dismutase derivatives. *J. Pharmacol. Exp. Ther.*, **289**, 1176–1184 (1999).
- Yabe Y, Kobayashi N, Nishihashi T, Takahashi R, Nishikawa M, Takakura Y, Hashida M. Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. *J. Pharmacol. Exp. Ther.*, **298**, 894–899 (2001).
- Hashida M, Akamatsu K, Nishikawa M, Yamashita F, Takakura Y. Design of polymeric prodrugs of prostaglandin E(1) having galactose residue for hepatocyte targeting. *J. Control. Release*, **62**, 253–262 (1999).
- Takakura Y, Fujita T, Furitsu H, Nishikawa M, Sezaki H, Hashida M. Pharmacokinetics of succinylated proteins and dextran sulfate in mice: Implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors. *Int. J. Pharm.*, **105**, 19–29 (1994).
- Ming X, Carver K, Wu L. Albumin-based nanoconjugates for targeted delivery of therapeutic oligonucleotides. *Biomaterials*, **34**, 7939–7949 (2013).
- Habeeb AF. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.*, **14**, 328–336 (1966).
- Scott TA Jr, Melvin EH. Determination of dextran with anthrone. *Anal. Chem.*, **25**, 1656–1661 (1953).
- Hnatowich DJ, Layne WW, Childs RL. The preparation and labeling of DTPA-coupled albumin. *Int. J. Appl. Radiat. Isot.*, **33**, 327–332 (1982).
- Sasakura K, Hanaoka K, Shibuya N, Mikami Y, Kimura Y, Komatsu T, Ueno T, Terai T, Kimura H, Nagano T. Development of a highly selective fluorescence probe for hydrogen sulfide. *J. Am. Chem. Soc.*, **133**, 18003–18005 (2011).
- Yamasaki Y, Sumimoto K, Nishikawa M, Yamashita F, Yamaoka K, Hashida M, Takakura Y. Pharmacokinetic analysis of *in vivo*

- disposition of succinylated proteins targeted to liver nonparenchymal cells *via* scavenger receptors: importance of molecular size and negative charge density for *in vivo* recognition by receptors. *J. Pharmacol. Exp. Ther.*, **301**, 467–477 (2002).
- 24) Nishikawa M, Miyazaki C, Yamashita F, Takakura Y, Hashida M. Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. *Am. J. Physiol.*, **268**, G849–G856 (1995).
- 25) Xiao Y, Zhang H, Zhang Z, Yan M, Lei M, Zeng K, Zhao C. Synthesis of novel tetravalent galactosylated DTPA-DSPE and study on hepatocyte-targeting efficiency *in vitro* and *in vivo*. *Int. J. Nanomedicine*, **8**, 3033–3050 (2013).
- 26) Hong R, Han G, Fernández JM, Kim BJ, Forbes NS, Rotello VM. Glutathione-mediated delivery and release using monolayer protected nanoparticle carriers. *J. Am. Chem. Soc.*, **128**, 1078–1079 (2006).
- 27) Zhao Y, Bhushan S, Yang C, Otsuka H, Stein JD, Pacheco A, Peng B, Devarie-Baez NO, Aguilar HC, Lefer DJ, Xian M. Controllable hydrogen sulfide donors and their activity against myocardial ischemia-reperfusion injury. *ACS Chem. Biol.*, **8**, 1283–1290 (2013).
- 28) Hanaoka K, Sasakura K, Suwanai Y, Toma-Fukai S, Shimamoto K, Takano Y, Shibuya N, Terai T, Komatsu T, Ueno T, Ogasawara Y, Tsuchiya Y, Watanabe Y, Kimura H, Wang C, Uchiyama M, Kojima H, Okabe T, Urano Y, Shimizu T, Nagano T. Discovery and mechanistic characterization of selective inhibitors of H₂S-producing enzyme: 3-mercaptopyruvate sulfurtransferase (3MST) targeting active-site cysteine persulfide. *Sci. Rep.*, **7**, 40227 (2017).
- 29) Shigeta K, Kawakami S, Higuchi Y, Okuda T, Yagi H, Yamashita F, Hashida M. Novel histidine-conjugated galactosylated cationic liposomes for efficient hepatocyte-selective gene transfer in human hepatoma HepG2 cells. *J. Control. Release*, **118**, 262–270 (2007).