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Research paper

A novel *S*-sulfhydrated human serum albumin preparation suppresses melanin synthesis

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ABSTRACT

Products of ultraviolet (UV) irradiation such as reactive oxygen species (ROS) and nitric oxide (NO) stimulate melanin synthesis. Reactive sulfur species (RSS) have been shown to have strong ROS and NO scavenging effects. However, the instability and low retention of RSS limit their use as inhibitors of melanin synthesis. The free thiol at Cys34 on human serum albumin (HSA) is highly stable, has a long retention and possess a high reactivity for RSS. We report herein on the development of an HSA based RSS delivery system. Sulfane sulfur derivatives released from sodium polysulfides (Na_2S_n) react readily with HSA. An assay for estimating the elimination of sulfide from polysulfide showed that almost all of the sulfur released from Na_2S_n bound to HSA. The Na_2S_n-treated HSA was found to efficiently scavenge ROS and NO produced from chemical reagents. The Na_2S_n-treated HSA was also found to inhibit melanin synthesis in B16 melanoma cells and this inhibition was independent of the number of added sulfur atoms. In B16 melanoma cells, the Na_2S_n-treated HSA also inhibited the levels of ROS and NO induced by UV radiation. Finally, the Na_2S_n-treated HSA inhibited melanin synthesis from L-DOPA and mushroom tyrosinase and suppressed the extent of aggregation of melanin pigments. These data suggest that Na_2S_n-treated HSA inhibits tyrosinase activity for melanin synthesis via two pathways; by directly inhibiting ROS signaling and by scavenging NO. These findings indicate that Na_2S_n-treated HSA has potential to be an attractive and effective candidate for use as a skin whitening agent.

1. Introduction

Ultraviolet (UV) irradiation produces reactive oxygen species (ROS) that ultimately cause cell death [1]. To protect the skin from UV damage, melanin, a dark colored pigment, is produced by melanocytes [2]. While melanin is essential for skin health, a demand for melanin scavenging preparations exists. Chloasma (melasma) is a condition in which the skin develops discolored areas that are caused by overproduction of melanin and are sometimes regarded as a metaphor of aging. In addition, inhibitors of melanin synthesis are popular cosmetics for brightening the skin, especially in Asian countries [3].

Tyrosinase catalyzes the production of melanin from tyrosine via DOPA and dopaquinone in melanocytes [4]. Its activity is regulated by a variety of factors such as ERK1/2 and Akt signaling [5]. ROS such as hydrogen peroxide produced by UV irradiation, activates tyrosinase and promotes melanin synthesis in melanocytes [2]. UV also causes the production of nitric oxide (NO) and stimulates tyrosinase activity via cGMP [6], a second messenger of NO.

On the other hand, thiol compounds with anti-oxidant effect has been widely used as supplements, radioprotection agents and perm agents [7]. Thiol-containing compounds undergo self-oxidation to form sulfonic acid, sulfenic acid and sulfonic acid [8]. Thiol also scavenges NO via *S*-nitrosation [8]. Because of these effects, thiol-containing compounds are often used in treating chloasma [7,9]. However, the skin whitening effect of thiols is very weak, a demand for more effective ROS and NO scavenging agents exists.

Reactive sulfur species (RSS) including cysteine persulfide have recently been reported to have stronger anti-oxidant effects than thiols.

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RSS contain a reactive thiol group [10] and the p*K*a of most RSS are much lower than that for thiols [11]. Therefore, RSS can react effectively with both ROS and NO and predicted to reduce the extent of melanin production. Sodium polysulfides (Na_2S_n), diallyltrisulfide (DATS) and dimethyltrisulfides (DMTS) are commonly used as RSS donors [12]. However, Na_2S_n has a low retention at neutral pH and has an offensive smell. In addition, DATS and DMTS, which are produced by garlic and onions are also odorous and their potential for such treatments is limited [13]. Furthermore, the half-life of Na_2S_n is very short in serum and, based on in vivo models, multiple injections are needed for them to be effective. Thus, the development of novel RSS-delivery-systems would be highly desirable.

Human serum albumin (HSA) is the most abundant protein in serum and is widely used as a drug carrier because of its biocompatibility and long plasma retention properties [14,15]. HSA contains a total of 35 Cys residues and one of them, Cys^{34}, is present in the form of a free thiol group [16]. Cys^{34} is sometimes a target for a drug binding site, because of its reactive thiol group [17,18]. For example, in the presence of nitric oxide (NO) the Cys^{34} thiol group is *S*-nitrosated. We previously demonstrated that *S*-nitrosated HSA (SNO-HSA) allows NO to be retained for long periods in serum [19]. SNO-HSA has various biological functions, including a liver protective effect against ischemia/reperfusion [20] and tumor suppressing effects [21].

Consequently, we hypothesized that HSA could be used as a RSS carrier (such as SNO-HSA) via the *S*-sulfhydration of Cys^{34}-SH. As a source of polysulfur, DATS and DMTS are limited because of their lipophilicity and volatility. Hence, commercially available Na_2S_n (Na_2S, Na_2S_2, Na_2S_3 and Na_2S_4) was used in this study. Ogasawara et al. previously prepared sulfur-bound serum albumin reacted with sodium sulfide (NaHS) by a simple mixing of the reagents [22]. The sulfur from NaHS was added to Cys^{34} and the resulting preparation protected liver damage caused by lipid peroxide. We adopted this method for preparing RSS-added-HSA using Na_2S_n for RSS delivery.

In this work, we reported the preparation of Na_2S_n-treated HSA and its use as a novel delivery system of RSS. The added sulfur was analyzed by means of a sulfane sulfur probe [23] and the elimination of sulfide from polysulfide [24]. To evaluate the effect of Na_2S_n-treated HSA on skin whitening, the effect of the Na_2S_n-treated HSA on melanin synthesis was studied using a B16 melanoma cell line.

2. Material and methods

2.1. Materials

Human serum albumin (HSA) was purchased from KAKETSUKEN (Kumamoto, Japan) and all HSA samples were defatted by a charcoal treatment. Sodium sulfide and sodium tetrasulfide were purchased from DOJINDO Laboratory (Kumamoto, Japan). Sulfane sulfur probe 4 (SSP4) was prepared as previously described [23]. L-DOPA, glutathione, (DTNB), ascorbic acid and sodium satiric Griess reagent (sulfanilamide, naphthylethylenediamine-HCl) were purchased from Nakarai Chemicals (Kyoto, Japan). Sephadex G-25 desalting column (φ 1.6 × 2.5 cm) was purchased from GE Healthcare (Kyoto, Japan). Dulbecco's modified Eagle medium (DMEM) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical (Osaka, Japan). Mushroom tyrosinase was purchased from Sigma-Aldrich. All other chemicals were of the best grade that was commercially available, and all solutions were prepared in deionized and distilled water.

2.2. BCA protein assay

Protein concentrations were measured using a BCA protein assay. 10 μL aliquots of samples and bovine serum albumin (BSA) standards were incubated in 100 μL of reaction buffer at 25 °C for 30 min. After the reaction, micro-plate reader was used to measure the absorbance of 540 nm. BSA was used to construct a standard curve.

2.3. Synthesis of Na_2S_n treated-HSA

HSA (300 μM) was incubated with 1 mM of sodium polysulfides (Na_2S_n) in PBS (pH 7.4) for 1 h at 37 °C. After the reaction, excess sodium polysulfides were removed by gel filtration with a Sephadex G-25 column.

2.4. Determination of sulfur binding rate by elimination method for sulfide from polysulfide (EMSP)

EMSP was prepared as previously described (3 × EMSP by addition of 792 mg of L-ascorbic acid to 5 mL of 3 N of NaOH) [24]. Samples (7.5 μM, 133 μL) were incubated with 66.7 μL of 3 × EMSP for 3 h at 37 °C. A 1% zinc acetate solution (600 μL) was then added to the reaction solution, followed by vortexing immediately. The samples were centrifuged at 8,000 × *g* for 5 min and washed with phosphate buffered saline (PBS) twice. After removing the supernatants, deionized and distilled water (200 μL) was added to the precipitates. After adding 1% zinc acetate (300 μL), 50 μL of 20 mM *N,N*-dimethyl-*p*-phenylenediamine and 20 mM $FeCl_3$ in 7.2 N HCl, the solution was incubated for 30 min at 25 °C. Samples were centrifuged at 8000 × *g* for 1 min and transferred into 96-well plates and the OD at 665 nm measured. Na_2S was used to construct a standard curve.

2.5. Detection of sulfane sulfur with SSP4

Each sample (20 μM) was incubated with 5 μM of SSP4 in 1 mM Cetyltrimethylammonium Bromide / PBS (pH 7.4) for 10 min at 25 °C. After incubation, the fluorescence measured by a spectrophotometer (JASCO Corporation) with excitation at 457 nm, emission at 490–535 nm.

2.6. DPPH radical tests

DPPH (250 μM) in ethanol was mixed with the same amount of MES buffer (50 mM, pH 7.4). Na_2S_n-treated HSA (40 μM) was the added to this DPPH solution, which was then incubated for 30 min at 25 °C and the absorbance of the DPPH radicals was measured at 540 nm. Scavenged radical rates were converted using the following formula;

$$\text{Scavenged radical (\%)} = (Abs_{sample}\text{-}Abs_{pbs})/\ Abs_{pbs} \times 100$$

2.7. NO and SNO analysis

Na_2S_n-treated HSA (50 μM) was incubated with an NO donor, NOC7 (200 μM), for 30 min at 25 °C. After the reaction, the concentration of NO and SNO were measured by a Griess assay with minor modifications [25]. The Griess reagent solution was prepared by mixing 0.1% N-1-Naphtylethylene-diamide dihydrochloride and 1% sulfanilamide in 2% phosphoric acid. The reaction buffer was composed of 0.1 M NaCl, 0.5 mM DTPA and 10 mM AcONa·AcOH (pH 5.5). Samples (20 μM) were reacted with the Griess reagent solution (60 μL) in reaction buffer (110 μL) with 3 mM $HgCl_2$ in 10 mM Na Acetate (pH 5.5). After a 15 min incubation, the absorbance of 540 nm was measured by means of a microplate reader. The remaining NO/SNO ratio (%) was calculated and compared to PBS values for the samples.

2.8. Cell culture

B16 melanoma cells were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan), and were cultured in DMEM containing 10% fetal bovine serum and an antibiotics solution. Cells were grown with maintained at 37 °C in humidified air containing 5% CO_2 in incubator (passage number 10–20).

2.9. Melanin production

B16 melanoma cells were seeded in 24 well plates at a concentration of 2.5×10^4 cells/well and cultured under 5% CO_2 at 37 °C for 24 h. Samples were treated with 0.4 mM tyrosine and 10 mM NH_4Cl in DMEM containing 10% FBS and then incubated under 5% CO_2 at 37 °C for 72 h. After the incubation, the cells were washed twice with PBS and dissolved in 1 N NaOH (200 μL). After a 2 h incubation on 60 °C, the absorption (405 nm) was measured by means of a micro-plate reader.

2.10. UV radiations

A hand held UV lamp was used to irradiate the samples at a distance of 5 cm distance from the well plate. This UV lamp provides a UV intensity of 614 or 743 μW/cm^2 respectively with 254 nm or 365 nm radiation from a distance of 5 cm.

2.11. Scavenging activity of Na_2S_4-treated HSA against intracellular ROS, NO, RSS

ROS and NO in B16 melanoma cells were measured by each of the fluorescence probes, CM-H_2DCF-DA and DAF-FM-DA, respectively. B16 melanoma cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well and cultured in 37 °C, 5% CO_2 for 24 h. After culturing, the media was removed and replaced with CM-H_2DCF-DA (5 μM) or DAF-FM-DA (10 μM) in PBS. The probes were taken up by the cells by incubating them at 37 °C for 30 min. After the reaction, the supernatants were removed, the samples diluted in PBS and the fluorescence measured immediately. Cells were radiated by a UV lamp for 15 min. After the irradiation, the fluorescence intensity (Ex. 485 nm, Em. 535 nm) was measured by means of a fluorescence micro-plate reader.

2.12. Mushroom tyrosinase activity and melanin aggregation

Tyrosinase and L-DOPA solutions were prepared in PBS (pH 7.4) immediately before the assay. Tyrosinase, isolated from mushrooms, was used for examining the inhibitory activity of Na_2S_n-treated HSA. A 20 μL portion of mushroom tyrosinase (537 U/mL) and 100 μL of Na_2S_n-treated HSA (40 μM) were mixed well with PBS (60 μL) in 96 well plates and 20 μL of L-DOPA (5 mM) was then added. After a 30 min incubation, the level of synthesized melanin was analyzed by measuring the OD 490 nm. For assaying melanin aggregation, the mixture was centrifuged at 20,000 *g*, 15 min for 3 h. The white arrow shows the aggregated material. Non-aggregated melanin in the supernatant was measured at an OD of 490 nm.

2.13. Safety tests

The topical cream used in this study was prepared by mixing water (30 mL) Jojoba Oil (15 mL) and 5 g of emulsifying wax at 60 °C. After cooling, the Na_2S_4-treated HSA (20 μM) and the resulting suspension were mixed well. The Skin Irritation Test was done following the OECD Test Guideline 439 using the LabCyte Epi-Model (a 3D cultured human skin model).

2.14. Statistical analysis

The statistical significance of the collected data was evaluated by ANOVA analysis followed by Newman-Keuls method for more than 2 means. Differences between the groups were evaluated by Student's *t*-test. $P < 0.05$ was regarded as being statistically significant.

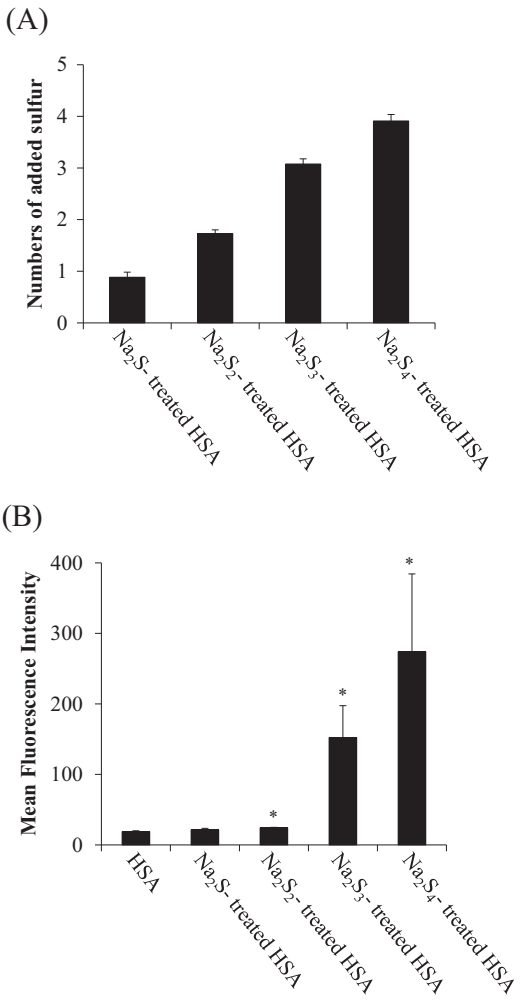

Fig. 1. Polysulfur binding to HSA by incubation with sodium polysulfide. (A) Valance dependency for adding sulfur to HSA with Na_2S_n. Sulfane sulfur in Na_2S_n-treated HSA samples were measured by EMSP. The values were subtracted from the untreated HSA. (B) SSP2 fluorescence intensity of Na_2S_n-treated HSA samples was analyzed by SSP2. Each value represents the mean ± S.E. (n = 3). *p < 0.05 as compared with HSA.

3. Results

3.1. Preparation of S-sulfydrated HSA

Na_2S_n-treated HSA was prepared from HSA that had been incubated with Na_2S_n and subjected to gel filtration after the reaction. To assess the amount of sulfane sulfur in the sample, EMSP, a novel quantitative method we previously developed, was employed [24]. Hence, Na_2S_4-treated HSA was prepared from HSA and Na_2S_n by allowing the reagents to react for 1 h at 37 °C. Different amounts of sodium polysulfides were allowed to react with HSA. Then, the HSA samples were incubated with EMSP solution, which was prepared at time of use, for 3 h at 37 °C. Based on the EMSP analyses, the level of *S*-sulfydration increased independently of the amount of sulfur (Fig. 1A). On the other hand, as shown in Fig. 1B, the Na_2S_3- or Na_2S_4-treatment enhanced the SSP4 (a fluorescence probe for sulfane sulfur) fluorescence intensity compared with the Na_2S- or Na_2S_2-treatments, suggesting that SSP4 possibly reacted with the polysulfide of the protein in a non-linear manner (Fig. 1B).

3.2. Antioxidant and NO suppressive effect of Na_2S-treated HSA

We postulated that Na_2S_n-treated HSA would suppress melanin production because of its antioxidant activity. Hence, a DPPH radical

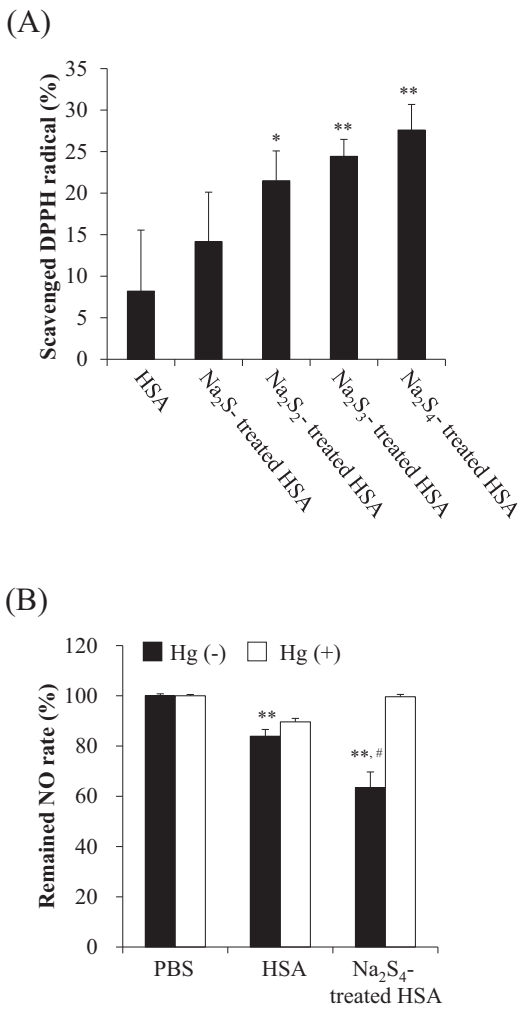

Fig. 2. Anti-oxidant properties of Na_2S_n-treated HSA. (A) DPPH radical scavenging activity of HSA and Na_2S_n-treated HSA. The concentration of DPPH radicals was measured by the oxidation of linoleic acid in the presence of HSA and Na_2S_4-treated HSA samples. (B) Scavenging of NO by Na_2S_n-treated HSA. NO concentration was measured by a Griess assay after the reaction with Na_2S_4-treated HSA (50 μM) and NOC7 (200 μM). Each value represents the mean ± S.E. n = 3. *p < 0.05, **p < 0.01 as compared with control. #p < 0.05 as compared with HSA.

test [26,27] was carried out to analyze the antioxidant activity in vitro. As a result, the Na_2S_n-treated HSA had a significantly higher concentration of added sulfur (Fig. 2A). To elucidate the effect of NO, NOC7 (a NO donor) was co-incubated with Na_2S_4-treated HSA at 25 °C. After a 30 min period of incubation, the remaining NO concentration was quantitated by a Griess assay. As seen in the closed bars of Fig. 2B, the Na_2S_4-treated HSA scavenged significantly more NO compared with the control and HSA (Fig. 2B). Elemental mercury (Hg) is known to reduce SNO and release NO_2^-. When Hg was added to a solution of Na_2S_4-treated HSA, NO_2^- was released, suggesting that the Na_2S_4-treated HSA was scavenged via *S*-nitrosation.

3.3. Melanin suppress effect of Na_2S_n-treated HSA

B16 mice melanoma cells were cultured and melanin synthesis was promoted by adding tyrosine to the media. As shown in Fig. 3, the Na_2S_n-treated HSA inhibited melanin synthesis and the inhibition was dependent on the sulfur content. Cell images of B16 melanoma cells after the application of the Na_2S_n-treated HSA also demonstrated that Na_2S_n-treated HSA decreased the ratio of production of melanin positive cells (Fig. 3).

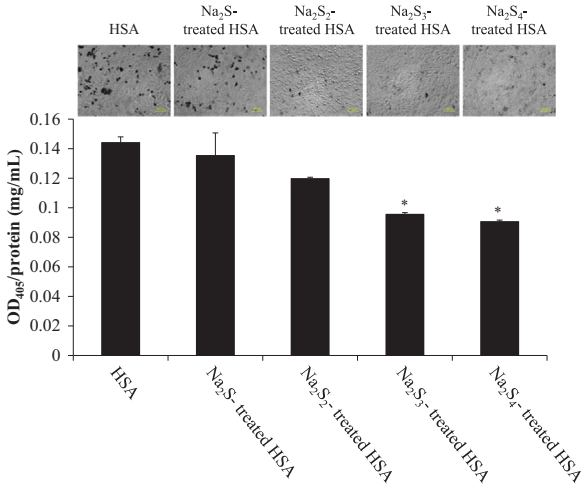

Fig. 3. Effect of Na_2S_n-treated HSA on melanin synthesis in B16 melanoma cells. Melanin content was measured by the absorption of at 405 nm after incubating Na_2S_n-treated HSA with 0.4 mM tyrosine and 10 mM NH_4Cl for 72 h. Protein contents were analyzed by BCA protein Assay. Each value represents the mean ± S.E. n = 3. *p < 0.05 as compared with HSA. Cell image after the treatment with Na_2S_n-treated HSA in B16 melanoma cells. The photos were taken after a 72 h treatment with Na_2S_n-treated HSA in the presence of 0.4 mM tyrosine and 10 mM NH_4Cl.

3.4. Antioxidant effect of Na_2S_4-treated HSA with irradiation of UV

To examine whether the Na_2S_n-treated HSA suppressed the formation of UV-induced ROS or NO, an oxidative stress test was performed using B16 melanoma cells as models. ROS production by Na_2S_4-treated HSA in B16 melanoma cells by irradiation with 2 different UV devices for 15 min was measured by CMH_2-DCF-DA. The findings indicate that the Na_2S_4-treated HSA caused a significant decrease in the fluorescence of CMH_2-DCF-DA to PBS and HSA by irradiation at 254 nm and 365 nm (Fig. 4AB). Conversely, the Na_2S_4-treated HSA also suppressed the production of NO in B16 melanoma cells by irradiation with 254 nm UV (Fig. 4CD). These results indicate that Na_2S_n-treated HSA suppresses melanin synthesis by inhibiting ROS and NO produced by UV irradiation.

3.5. Direct suppression of tyrosinase and melanin aggregation by Na_2S_n-treated HSA

Some commercial anti-melanin agents are known to directly inhibit tyrosinase activity. Thus, we tested whether the Na_2S_n-treated HSA altered the activity of tyrosinase. The findings indicated that the Na_2S_n-treated HSA inhibited mushroom tyrosinase to a greater extent than non-treated HSA (Fig. 5A). After being generated, melanin readily undergoes aggregation and induces medulla formation [28]. Therefore, we next addressed the issue of whether Na_2S_n-treated HSA inhibits the aggregation of melanin. Consequently, when tyrosinase and L-DOPA were incubated together for 3 h, melanin pigments became aggregated, but the Na_2S_n-treated HSA prevented the aggregation (Fig. 5B). On the one hand, HSA was also found to inhibit aggregation, indicating that HSA itself could prevent the binding of L-DOPA to tyrosinase.

3.6. Safety test of Na_2S_n-treated HSA using 3D cultured human skin

Skin irritation tests for the Na_2S_4-treated HSA was performed using 3D cultured human skin cells according to OECD guidelines. As a result, the numbers of surviving cells were not decreased by Na_2S_4-treated HSA with/without the use of a topical cream (Fig. 6A). The use of an LDH cytotoxicity detection kit also revealed that skin cells were not

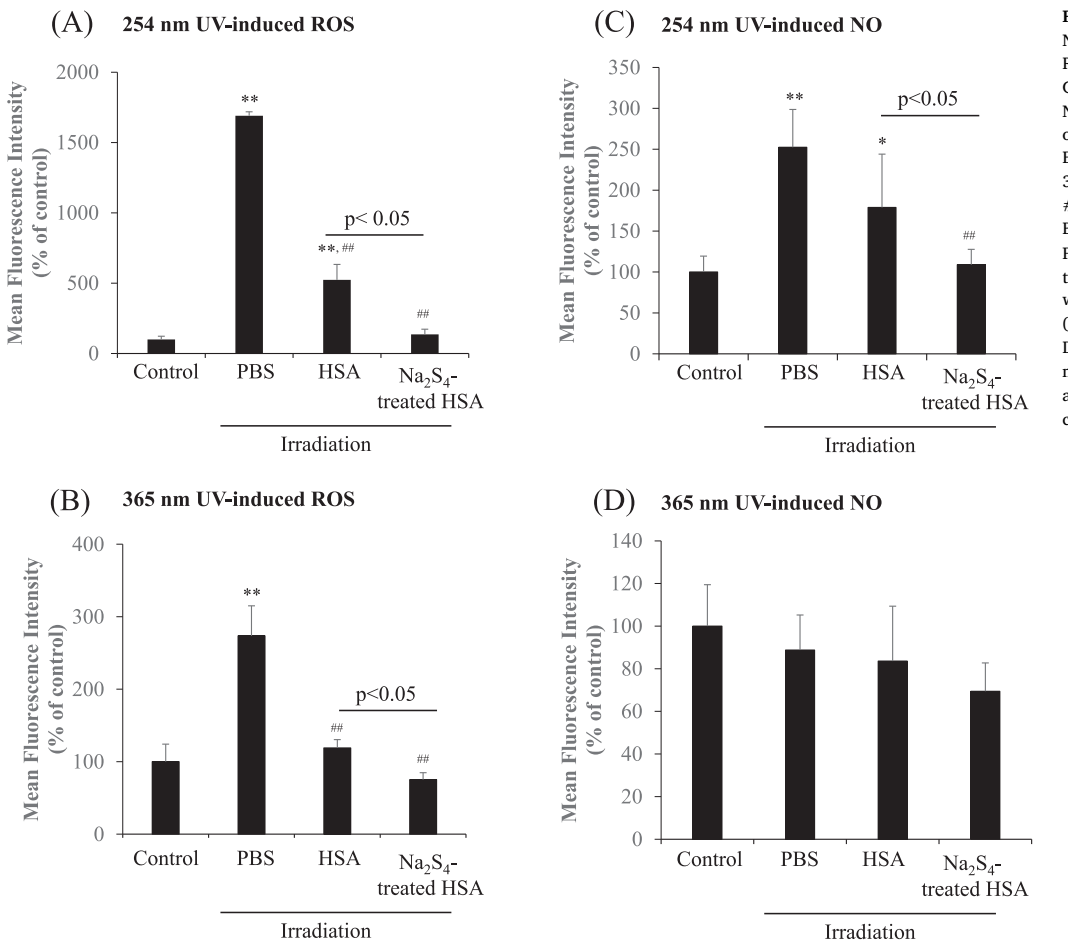

Fig. 4. ROS and NO scavenging effects of Na_2S_4-treated HSA under UV irradiation. ROS in B16 melanoma cells was detected by CM-H_2DCF-DA in the presence of HSA and Na_2S_4-treated HSA with 15 min irradiation of 2 different UV, (A) 254 nm, (B) 365 nm. Each value represents the mean ± S.E. n = 3. **p < 0.01 as compared with control. ##p < 0.01 as compared with HSA. NO in B16 melanoma cells was detected by DAF-FM-DA in the presence of HSA and Na_2S_4-treated HSA after irradiation for 15 min with 2 different wavelengths, (C) 254 nm, (D) 365 nm. NO synthesis was measured by DAF-FM-DA. Each value represents the mean ± S.E. n = 3. *p < 0.05, **p < 0.01 as compared with control. ##p < 0.01 as compared with PBS.

damaged by the Na_2S_4-treated HSA (Fig. 6B). These data indicated that Na_2S_n-treated HSA is very safe for use against human skin under the concentrations examined in this study.

4. Discussion

Melanin is synthesized by the oxidation of tyrosine. Tyrosine is oxidized to L-DOPA, and then dopaquinone by the action of tyrosinase. Dopaquinone is spontaneously oxidized to melanin. Melanin induces the formation of black pigments and freckles, but also plays a role in protecting the skin from being damaged by UV radiation. In human skin, melanocytes produce ROS and NO when stimulated by UV radiation [2,29,30]. ROS promotes melanin synthesis by activating tyrosinase via the action of ATP synthase, phenylalanine hydroxylase, and the phosphorylation of MAPKs [31,32]. NO activates tyrosinase via increasing the cellular level of cGMP [6]. Therefore, ROS and NO scavengers are considered to be anti-melanogenesis agents. Here, we investigated the anti-melanin synthesis effect of Na_2S_n-treated HSA. Na_2S_n-treated HSA strongly suppressed the cellular levels of ROS and NO produced by UV radiation (Fig. 4). Furthermore, Na_2S_n-treated HSA had a direct effect on inhibiting the action of tyrosinase (Fig. 5A) and the aggregation of melanin (Fig. 5B). We were not able to clarify the mechanism for how sulfane sulfur was transferred from the Na_2S_n-treated HSA to a cell. Therefore, the nature of how the direct effects of Na_2S_n-treated HSA function remain unclear. Yamashita et al. demonstrated that dopaquinone binds to thiol proteins via cysteine residues [33]. Taken together, the inhibition of melanin aggregation by HSA and Na_2S_n-treated HSA may also involve the formation of disulfide bonds with dopaquinone or melanin. On the other hand, tyrosinase inhibition was dependent on the content of added sulfur (Fig. 5A). GSH is known to bind tyrosinase and decrease its activity [34]. Because *S*-sulfhydrated cysteine has a stronger reactivity than normal cysteine [11], glutathione persulfide (GSSH) may inhibit the action of tyrosinase more than GSH. Further studies regarding the issue of whether Na_2S_n-treated HSA increases intracellular GSSH is needed in the future.

ROS are produced by UV irradiation or external stress induce signs of aging, not only in the form of melanin synthesis but also by the appearance of wrinkles and sagging skin, caused by DNA damage and the formation of cross linked collagen. It is also known that ROS are an aggravating factor in various types of inflammation such as pimples and psoriasis. Various skin whitening agents, such as tranexamic acid [35] and arbutin [36], have been designed to address these issues. However, these compounds only inhibit melanin synthesis and have no effect on oxidative stress. Thus, the risks of ROS-induced toxicity remained. An advantage of using Na_2S_n-treated HSA is that it efficiently scavenges ROS (Figs. 2 and 4).

Hydrogen sulfide has been studied as a third essential molecule after nitric oxide and carbon monoxide. Therapeutic effects of hydrogen sulfide have been shown to be applicable to the treatment of ischemia/reperfusion [37], atherosclerosis [38], sepsis [39] and high fat diet-induced toxicity [40]. In addition, hydropersulfide has a higher activity than hydrogen sulfide. For example, Na_2S_4 effectively detoxifies methyl mercury and inhibits the differentiation of neuroblastoma cells, while Na_2S does not [12,41]. Therefore, not only a skin whitening effect but also other positive effects of Na_2S_n-treated HSA are possible.

In conclusion, we reported on the development of a novel RSS delivery system using serum albumin as a stable carrier. Reactive sulfur, when combined with HSA, had a stronger anti-oxidant effect than HSA and inhibited melanin synthesis in melanoma cells. The mechanism of anti-melanogenesis involves not only ROS and NO scavenging, but also

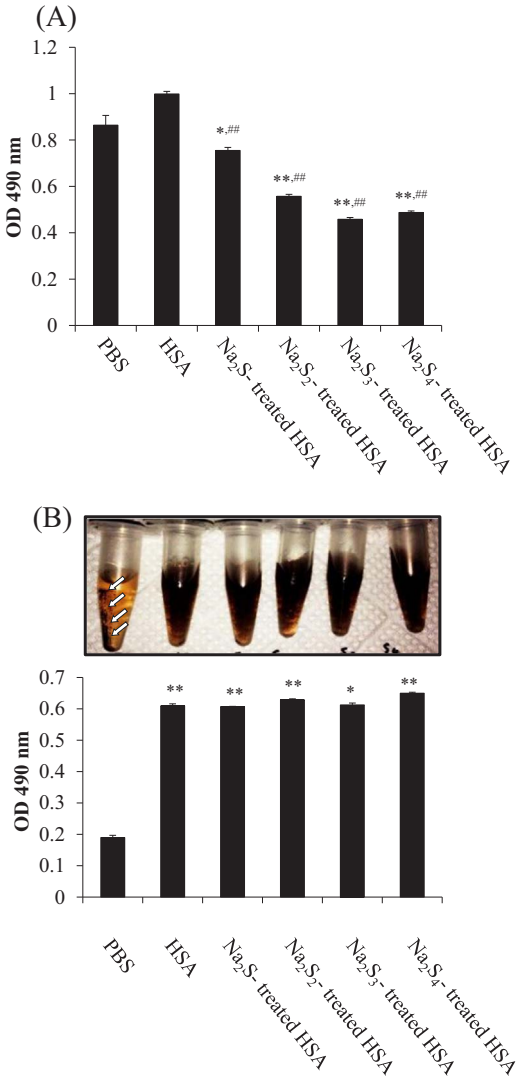

Fig. 5. Na_2S_n-treated HSA inhibits the oxidation of L-DOPA and melanin aggregation. (A) Melanin synthesis from tyrosinase and L-DOPA in the presence of HSA and Na_2S_n–treated HSA. Tyrosinase and samples were mixed and incubated at room temperature for 10 min. After the reaction, L-DOPA was added and the incubation continued for an additional 30 min (B) After 3 h, the mixture was centrifuged for 20,000 g, 15 min. Non-aggregated melanin in supernatant was measured using OD 490 nm. Tyrosinase and L-DOPA were co-incubated for 10 min and added HSA samples. White arrow showed the aggregation. Each value represents the mean ± S.E. n = 3. *p < 0.05, **p < 0.01 as compared with PBS. ##p < 0.01 as compared with HSA.

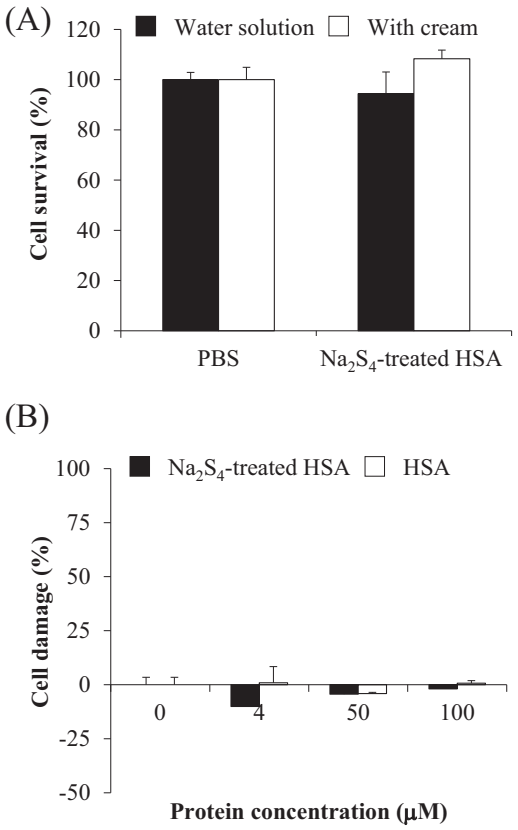

Fig. 6. Safety testing of Na_2S_4-treated HSA for 3D cultured human epidermis. (A) Cell survival tests were performed using Autologous Cultured Epidermis kit with in PBS solution (white column) and in cream (black). (B) Cell damage tests were performed using an LDH Cytotoxicity Detection Kit. Each value represents the mean ± S.E. n = 3.

suppression of tyrosinase activity and melanin aggregation. Hence, Na_2S_n-treated HSA has considerable potential for use as a safe skin whitening agent.

Competing financial interest

The authors declare that they have no competing interest.

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1. Study conception and design: M.I., Y.I., T.I., M.O., T.O.; 2. Acquisition, analysis and/or interpretation of data: M.I., Y.I., R.K., N.T.; 3. Drafting/revision of the work for intellectual content and context: M.I., Y.I., V.C., H.W., T.S. T.I., M.O., T.O.; 4. Final approval and overall responsibility for the published work; Y.I., V.C., T.I., M.O., T.O.

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