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Safety and efficacy of self-assembling bubble carriers stabilized with sodium dodecyl sulfate for oral delivery of therapeutic proteins

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ABSTRACT

Sodium dodecyl sulfate (SDS) is generally regarded as a potent permeability enhancer in oral formulations; however, one concern related to the use of any permeation enhancer is its possible absorption of unwanted toxins during the period of epithelial permeability enhancement. In this work, the safety and efficacy of an SDS-containing bubble carrier system that is developed from an orally administered enteric-coated capsule are evaluated. The bubble carriers comprise diethylene triamine pentaacetic acid (DTPA) dianhydride, sodium bicarbonate (SBC), SDS, and insulin. Upon exposure to the intestinal fluid, DTPA dianhydride hydrolyzes to yield acids, and SBC rapidly reacts with these acids to generate CO_2, producing bubble carriers, each containing a self-assembling water film. The hydrophilic insulin is entrapped in the self-assembled water film, which is stabilized by SDS. The SDS in the bubble carrier system can act as a dissolution enhancer in the dispersion of insulin molecules, as a surfactant that stabilizes the bubble carriers, as a protease inhibitor that protects the protein drug, and as a permeation enhancer that augments its oral bioavailability. Hence, a significant increase in the plasma insulin level and an excellent blood glucose-lowering response in diabetic rats are effectively achieved. Moreover, the enhancement of epithelial permeation by this SDS-containing formulation does not promote the absorption of intestinal endotoxins. The above facts indicate that the bubble carrier system that is stabilized by SDS can be used as a safe and potent carrier in the oral delivery of therapeutic proteins.

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1. Introduction

The oral administration of therapeutic proteins such as insulin has been an elusive goal of the drug delivery community. However, increasing the oral bioavailability of protein drugs to a therapeutically effective level remains a major challenge, owing to their presystematic enzymatic degradation and poor intestinal absorption. To address these concerns, our group developed a bubble carrier system to treat diabetic rats by loading an acid initiator (diethylene triamine pentaacetic acid, DTPA, dianhydride), a foaming agent (sodium bicarbonate, SBC; $NaHCO_3$), a surfactant (sodium dodecyl sulfate, SDS), and a protein drug (insulin) into an enteric-coated gelatin capsule [1]. Following oral administration, the intestinal fluid that passed through the gelatin capsule saturated its loaded contents; concurrently, DTPA dianhydride formed an acidic environment, while SBC decomposed to generate CO_2 bubbles in the acid environment. The gas bubbles grew among the surfactant molecules (SDS) owing to the generation of CO_2. The walls of these bubbles comprised a self-assembled film of water, which contained insulin and was stabilized by two layers of SDS molecules. The bubble carriers bumped into the intestinal walls and burst, facilitating the transport of insulin molecules across the epithelium and enabling their eventual absorption into systemic circulation, where they had blood glucose-lowering effects (Fig. 1).

Surfactants are some of the most frequently used adjuvants in pharmaceutical formulations [2]. SDS, an anionic surfactant, is an FDA-approved excipient in oral formulations, primarily functioning as a dissolution agent and a stabilizer [3]; it may also serve as a protease inhibitor [4]. Furthermore, it can be utilized as a permeation enhancer for the absorption of protein drugs across the intestinal epithelium *via* both paracellular and transcellular routes, causing temporary and reversible

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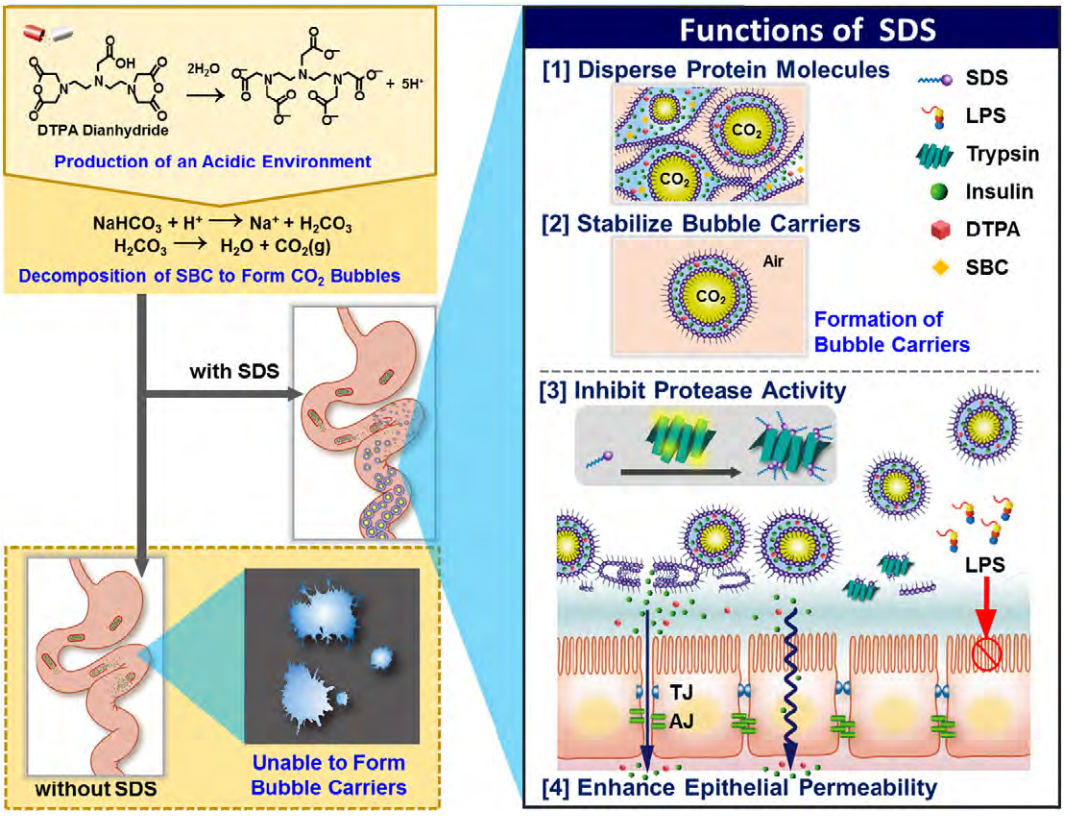

Fig. 1. Mechanism of formation of SDS-containing bubble carriers that are developed from an orally ingested test capsule, and enhancement of oral bioavailability of insulin by SDS.

alteration of the properties of the mucosal/epithelial barrier [5]. To enhance the intestinal permeability *in vivo*, the protein drug and a sufficient concentration of the permeation enhancer must be simultaneously delivered to the absorption site [6]. However, an overdose of a permeation enhancer may cause irreversible damage to local intestinal walls, resulting in the absorption of toxic substances, and especially endogenous endotoxins [7].

This work optimizes the dose of SDS that is used in the bubble carrier system to facilitate the crossing of its encapsulated insulin molecules through the epithelial barrier, while eliminating the possibility of damage to the intestinal walls and absorption of endogenous endotoxins.

2. Materials and methods

2.1. Materials

SDS, DTPA dianhydride, SBC, bovine insulin (Catalogue No. I1882), and fluorescein isothiocyanate (FITC)-lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while cyanine 5 (Cy5) and FITC-insulin (bovine) were obtained from Lumiprobe Corp. (Broward, FL, USA) and Invitrogen Corp. (Carlsbad, CA, USA), respectively. All used chemicals and reagents were of analytical grade.

2.2. Preparation of enteric-coated test capsules

To prepare enteric-coated capsules for use in the animal studies, hard gelatin capsules (size 9; Torpac Inc., Fairfield, NJ, USA) were manually filled with a powder mixture of DTPA dianhydride (8.0 mg), SBC (8.0 mg), insulin (0.4 mg), and a pre-determined dose of SDS [0 mg (w/o SDS), 1.6 mg (low dose; LD SDS), 3.2 mg (medium dose; MD SDS), or 4.8 mg (high dose; HD SDS)] as per the manufacturer's instructions. The FDA Inactive Ingredients List allows up to 95 mg SDS to be used in oral capsules for humans [2]; this dose is equivalent to a dose of 3.4 mg for a rat model of 300 g, based on the animal's surface area [8]. The as-prepared capsules herein were dipped into a methanol solution that contained Eudragit® L100-55 (15% w/v, Evonik Industries, Parsippany, NJ, USA) and subsequently dried using an air-blower; this process was performed three times.

2.3. Characterization of bubble carriers

FITC-insulin was used in this investigation to observe the formation and development of bubble carriers. The contents of each test capsule [w/o SDS, LD SDS, MD SDS, or HD SDS] were firstly exposed to deionized (DI) water. The formation of the bubble carriers and changes in their sizes and number in DI water were then monitored using a fluorescence microscope (Axio Observer; Carl Zeiss, Jena, Germany) and analyzed using the "Analyzing Particles" tool in ImageJ. The loading efficiency of the bubble carriers was calculated by subtracting the fluorescence intensity of free FITC-insulin from the total fluorescence intensity [9]. The fluorescence intensity of FITC-insulin was determined using a microplate spectrophotometer (SpectraMax M5, Molecular Devices, CA, USA).

The formed bubble carriers were also characterized by small-angle X-ray scattering (SAXS) using the BL23A1 beamline at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. The procedure and conditions that were used in the SAXS experiment have been described in our previous study [1]. The SAXS experiment yielded the scattering intensity profile as a plot of the scattering intensity $I(q)$ as a function of scattering vector, $q = (4\pi/\lambda)\sin(\theta/2)$ with a scattering angle of θ.

2.4. In vitro enzymatic degradation

Insulin is very sensitive to trypsin, which is a proteolytic enzyme in the small intestine [10]. The ability of SDS to protect insulin against proteolytic attack by trypsin was investigated *in vitro*. Briefly, free-form insulin and SDS at various doses were diluted in Tris buffer that contained

0.1 mM EDTA [11]. Degradation studies were performed by adding 100 μL of each insulin sample to 100 μL of the enzyme solution that contained 0.5 mg trypsin, yielding an insulin-to-trypsin molar ratio of 10:1 at 37 °C with shaking [11]. Two hours later, 200 μL of 2% trifluoroacetic acid (TFA) was added to terminate the enzymatic reaction. The amount of intact insulin in each test sample was then analyzed by high-performance liquid chromatography (HPLC).

2.5. Epithelial permeability enhancement

The evaluation of the capacity of SDS to enhance the epithelial permeability of insulin (25 μg/mL, 1 mL) was investigated in Caco-2 cell monolayers that were grown on a tissue-culture-treated polycarbonate filter in a Costar Transwell with 12 wells/plate (Corning Costar Corp., NY, USA). Monolayers of Caco-2 cells, which are derived from human colorectal adenocarcinoma, are widely regarded as a potent experimental model of intestinal epithelial cells for predicting drug permeability [12]. Following treatments with different doses of SDS, changes in transepithelial electrical resistance (TEER, which was originally in the range of 600–800 $\Omega \cdot cm^2$) or the tightness of the cell monolayers were measured using a Millicell®-Electrical Resistance System (Millipore Corp., Bedford, MA, USA). Concurrently, samples (50 μL) were collected from the receiver compartment of the Transwell at distinct time points, and their insulin concentrations were measured using ELISA (Iso-Insulin ELISA, Mercodia AB, Uppsala, Sweden). The apparent permeability (P_{app}, cm/s) of insulin was calculated as described in the literature [13].

The medium that contained FITC-insulin (25 μg/mL, 1 mL) was introduced into the donor compartment of the Caco-2 cell monolayers. Following incubation with SDS for 15 min, test samples were aspirated. Cells were then washed three times with pre-warmed phosphate buffered saline (PBS) before they were fixed in 4% paraformaldehyde. Next, the Z-stack images of the fixed cells in 1.0 μm optical sections were obtained by an inverted confocal laser scanning microscope (CLSM; Zeiss LSM780, Carl Zeiss, Jena GmbH, Germany) and used to construct a 3D image for visualization using the ZEN 2011 Light Edition software.

2.6. Animal studies

Wistar rats with a mass of approximately 300 g were used in the animal studies, which were carried out in compliance with the "Guide for the Care and Use of Laboratory Animals", which was prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press in 1996. All studies were approved by the Institutional Animal Care and Use Committee of National Tsing Hua University.

2.7. In vivo safety study

Safety has always been an issue when applying permeability enhancers in oral protein formulations. The local action of a permeability enhancer may disrupt the intestinal mucosal epithelium [14]. Lipopolysaccharide (LPS), which is a negatively-charged amphiphilic component of the outer membrane of Gram-negative bacteria, is the most potent endotoxin in the intestinal tract [15]. Damage to the intestinal wall may permit LPS to leak rapidly into the portal circulation, causing toxic shock [16].

The safety of the bubble carriers that were developed from the orally administered test capsules was examined *in vivo* using an intestinal closed-loop method [17]. To avoid contamination, all experimental procedures were carried out using sterilized equipment under a sterile hood. Briefly, rats that had fasted overnight (n = 3) were anaesthetized by an intramuscular injection of 50 mg/kg Zoletil® (Virbac Laboratories, Carros, France). Following a midline incision, the small intestine was exposed, and a closed loop was formed from a 10 cm-long segment close to the distal end of the stomach, as follows. First, the proximal end of the intestinal loop was ligated, and FITC-LPS (2 mg/mL, 0.5 mL) was introduced along with the test capsule that contained one of various doses of SDS *via* a syringe; in this work, Cy5-insulin, which was synthesized following a method that has been described in the literature, was used [18]. The distal end was subsequently ligated to form a closed loop.

Two hours later, the rats were sacrificed, and the intestinal loops were retrieved and washed three times with isotonic PBS. The retrieved tissues were fixed in 4% paraformaldehyde, washed with s-Collidine buffer, and then processed for paraffin-embedding. The embedded sections were dewaxed, hydrated, stained with Alexa-555-labeled wheat-germ-agglutinin (WGA) and DAPI (Invitrogen, Carlsbad, CA, USA), and examined by CLSM. Additionally, specimens of the loops were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned with a thickness of 5 μm, and then stained with hematoxylin and eosin (H&E). Tissue-embedded blocks were also prepared for transmission electron microscopic (TEM) inspection (JEOL 2010F, Tokyo, Japan) [19].

After the rats had been sacrificed, the internal organs of each animal were harvested and examined using an *in vivo* imaging system (IVIS; Xenogen, Alameda, CA, USA) to observe their accumulation of Cy5-insulin and FITC-LPS. The fluorescent intensities from regions of interest were quantified using Living Image 3.1 software.

2.8. In vivo efficacy and bioavailability

Diabetes was induced in test rats *via* the intraperitoneal injection of streptozotocin, as described previously [20]. Rats with fasting blood glucose levels above 300 mg/dL were regarded as diabetic and included in the study [20]. The efficacy and bioavailability of the insulin that was orally delivered by the test capsules were examined by randomly dividing the diabetic rats into four groups (n = 6 for each studied group). The following formulations were individually administered to the diabetic rats: a subcutaneously (SC) injected free-form insulin solution (5.0 IU/kg), an orally ingested free-form insulin solution (30.0 IU/kg), and an orally administered enteric-coated capsule that was filled with DTPA dianhydride (8 mg), SBC (8 mg), insulin (30.0 IU/kg), with or without MD SDS.

Blood samples were collected from the tail vein, and the blood glucose levels were determined using a hand-held glucose meter (LifeScan Inc., Milpitas, CA, USA). Then, plasma insulin levels were analyzed by centrifuging the blood samples (10,000 rpm for 15 min) with subsequent quantification using the aforementioned bovine insulin ELISA kit. The relative bioavailability (BA_R) of the absorbed insulin was calculated using the following formula [13].

$$BA_R = \frac{AUC_{(oral)} \times Dose_{(sc)}}{AUC_{(sc)} \times Dose_{(oral)}} \times 100\%$$

where AUC denotes the total area under the plasma insulin concentration *vs.* time curve.

2.9. Statistical analysis

All data are presented as a mean ± standard deviation (SD). The one-tailed Student's *t*-test was performed to compare pairs of groups, and one-way analysis of variation (ANOVA) was followed by the Bonferroni *post hoc* test to assess variations among three or more groups. Differences were considered statistically significant when the *P* values were <0.05.

3. Results and discussion

The inclusion of a permeability enhancer in a carrier system that can safely assist the oral absorption of therapeutic proteins has always been a major focus of drug delivery research. SDS is normally regarded as a potent permeability enhancer in oral formulations; however, its effects on the absorption of unwanted toxins are yet to be understood. In this

work, the safety and efficacy of a self-assembling bubble carrier system that was stabilized by various doses of SDS were evaluated. To optimize the therapeutic effects, the bubble carrier system that was formulated with SDS must effectively enhance the bioavailability of the therapeutics, while eliminating the possibility of the absorption of intestinal endotoxins.

3.1. Characteristics of bubble carriers

The bubble carriers comprise DTPA dianhydride, SBC, SDS, and insulin. Theoretically, upon exposure to water, DTPA dianhydride hydrolyzes to yield acids, releasing protons [21], and thereby forming an acid solution. In an acidic environment, SBC reacts with the acid rapidly to generate CO_2 [22], producing bubble carriers, each of which contains a self-assembling water film. As a surfactant, SDS can reduce the surface tension of the gas-water interface. The hydrophilic insulin is entrapped in the self-assembled water film that is stabilized by SDS (Fig. 1). The surface tension of an aqueous surfactant affects its ability to form bubbles, as well as the structure and lifetime (stability) of those bubbles [23].

The effects of SDS at different doses on the formation/development of bubble carriers and their characteristics – including their structure, lifetime, and drug loading efficiency – was investigated by exposing a powdered mixture of DTPA dianhydride, SBC, SDS, and insulin to DI water. To visualize its distribution within the formed bubble carriers, insulin was labeled with a green fluorescent dye (FITC). According to Fig. 2A, in the absence of SDS (w/o SDS), the gas bubbles of CO_2 that were generated in water burst rapidly, and the insulin molecules tended to aggregate when they encountered water. Water sorption causes the aggregation of proteins [24]. Conversely, in the presence of SDS, the gas-water mixtures promptly produced bubble carriers, each of which was surrounded by a green fluorescent film of insulin. Here, SDS was used as a surfactant to stabilize the generated CO_2 bubbles and as a dissolution enhancer to disperse insulin molecules.

Both the size of the bubbles and their lifetime were affected by the dose of SDS that was applied in the generation of bubble carriers. Bubbles burst when they were under too much internal pressure owing to the generation of CO_2, and this bursting was followed by crushing their fluorescent films. Increasing the dose of SDS in the bubble carriers reduced their surface tension [25] and, therefore, their size (Fig. 2B), while increasing their lifetime (or the number of bubbles that remained for particular time) (Fig. 2C). In the determination of drug loading efficiency, the amount of drug that was loaded in the bubble carrier system increased with the dose of SDS (Fig. 2D).

Fig. 2E presents the SAXS profiles of the formed bubble carriers, which revealed structural changes of the SDS surfactant molecules upon bubble formation. When the SDS in powdered form came into contact with water, its SAXS profile exhibited a strong sharp peak that was associated with the multi-lamellar structure that was formed by the self-assembly of the SDS molecules in the dehydrated state. Upon the formation of bubble carriers, this sharp peak became much less intense and was transformed into a broad hump (indicated by the black arrow), which was not observable in the absence of SDS. In the presence of SDS, the surfactant molecules formed the layers that surrounded the bubbles, of which each consisted of hydrophobic sub-layers of the surfactant tails and hydrophilic sub-layers of the head groups of SDS, along with insulin, DTPA, and water molecules (Fig. 1).

3.2. Inhibition of enzymatic degradation

The ability of SDS to inhibit the degradation of insulin by trypsin, which is crucial for increasing its oral bioavailability, was evaluated *in vitro*. The amount of intact insulin that remained following enzymatic degradation was assessed by HPLC. According to Fig. 3A, in the absence of SDS, free-form insulin was rapidly degraded by trypsin, whereas adding SDS at various doses to insulin had a strong protective effect against trypsin ($P < 0.05$). A previous study indicated that in the presence of SDS, trypsin has negligible activity as it interacts directly with trypsin, triggering conformational changes of the enzyme [26].

3.3. Enhancement of epithelial permeability

The effects of SDS at several different doses on epithelial permeability were investigated *in vitro* using Caco-2 cell monolayers by measuring their reduction in TEER and the subsequent transport of the drug (insulin). As shown in Fig. 3B, SDS rapidly reduced TEER of the Caco-2 monolayers; thus, the action of SDS in the enhancement of drug transport was rapid (Fig. 3C). The P_{app} of insulin for the groups that had been treated with LD SDS, MD SDS, or HD SDS ($10.1 \pm 0.8 \times 10^{-6}$ cm/s, $13.8 \pm 0.8 \times 10^{-6}$ cm/s, or $18.8 \pm 1.3 \times 10^{-6}$ cm/s, respectively) significantly exceeded that of the group that received no SDS ($3.8 \pm 1.4 \times 10^{-6}$ cm/s; $P < 0.05$). Nevertheless, no further decrease in TEER over time was observed, suggesting that treatment with SDS at the doses used in this study did not damage the Caco-2 cell monolayers. Both the extent of TEER reduction and the amount of the drug that was transported across

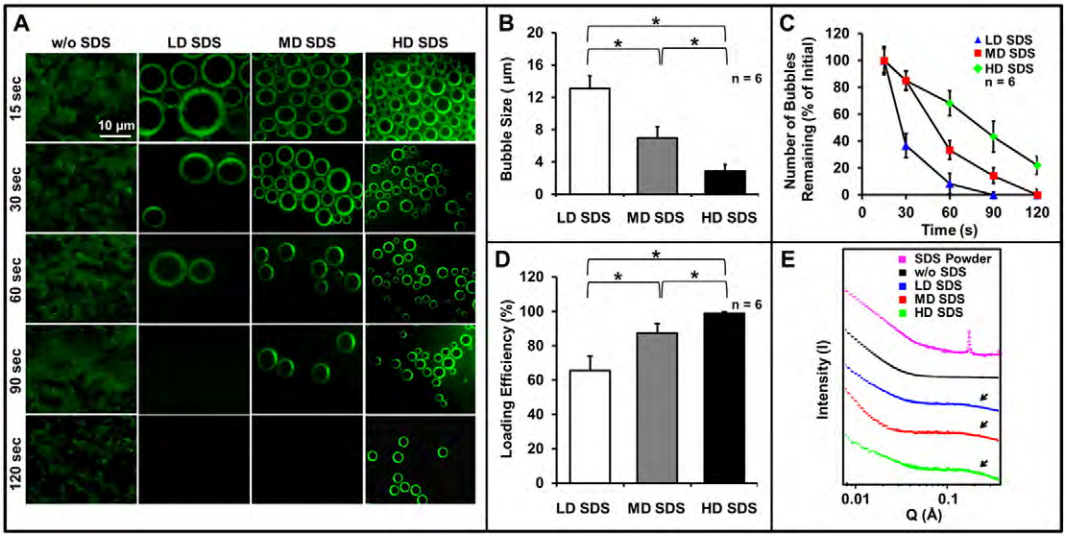

Fig. 2. (A) Fluorescence images of development of bubble carriers using various doses of SDS, and changes in (B) bubble sizes, (C) number of bubbles that remain as a function of time, and (D) drug loading efficiency. (E) SAXS profiles of structural changes of SDS surfactant molecules at various doses upon bubble formation. *: statistically significant ($P < 0.05$).

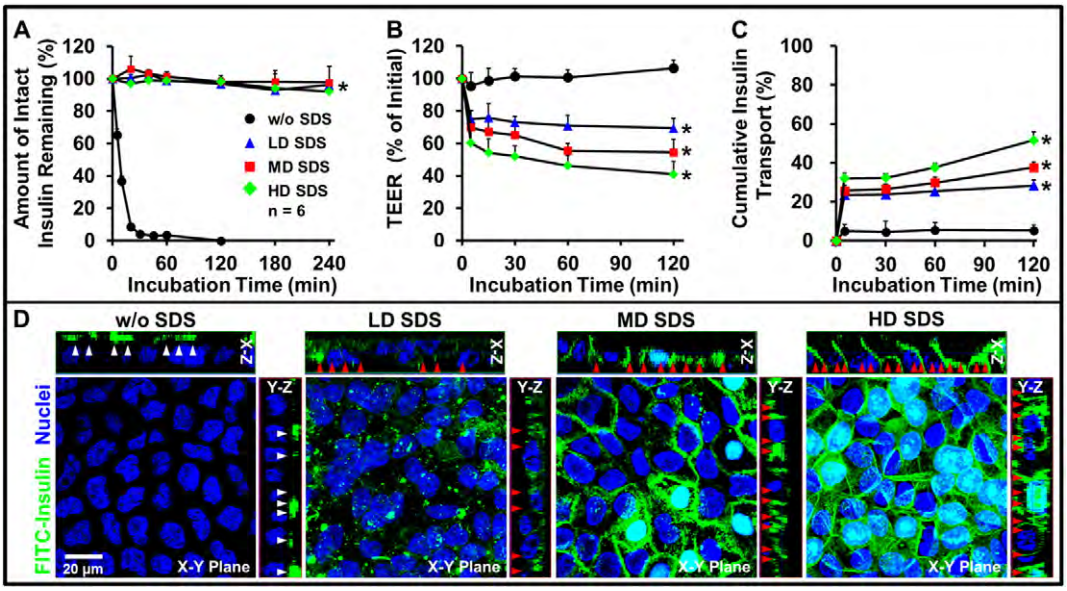

Fig. 3. (A) Extent of degradation of insulin by trypsin in presence of various doses of SDS as a function of incubation time. Effects of SDS at different doses on (B) reduction of TEER in Caco-2 cell monolayers and (C) cumulative transport of insulin as a function of incubation time. (D) Constructed 3D CLSM images that show paracellular/transcellular penetration of FITC-insulin across Caco-2 cell monolayers that had been treated with SDS at various doses for 15 min. *: statistically significant *vs.* w/o SDS group ($P < 0.05$).

cell monolayers depended on the dose of SDS. SDS affects the fluidity of cell membrane owing to its surface-active behavior and transiently depletes cellular ATP [27], causing the drug molecules to permeate the epithelial cells *via* the paracellular and transcellular routes.

CLSM was used to providing evidence of the mediation of the transport of FITC-insulin across the Caco-2 cell monolayers by SDS-containing formulations. According to Fig. 3D, in the absence of SDS, most the fluorescence signal of FITC-insulin (green color) was observed on top of the layer of Caco-2 cells (indicated by the white arrow heads in the X–Z and Y–Z planes), indicative of no drug permeation. Conversely, treatment with SDS caused FITC-insulin to permeate through monolayers of cells *via* both the paracellular and the transcellular pathways (represented by the red arrow heads); notably, the intensity of the fluorescence signals from the permeating FITC-insulin depended on the SDS dose. These findings reinforce the above TEER measurements and the insulin-transport experiments.

3.4. In vivo safety: structural and functional analyses

To be successfully absorbed into the systemic circulation, an orally administered protein drug must infiltrate through the mucus layer and travel across the barrier of the intestinal epithelium with the assistance of a permeation enhancer. Permeation enhancers such as SDS are able to cause changes in the barrier of the mucosal epithelium, so determining whether these changes could lead to toxicity *in vivo*, which is a critical safety concern, is important. An intestinal closed loop that was created in the proximal end of the small intestine in a rat model was used to study the safety of test capsules that contained various doses of SDS. The test capsules and LPS (which mimics the endogenous endotoxins) were administered together into the closed loop. In this investigation, LPS and insulin were labeled with FITC and Cy5, respectively. An advantage of using a closed loop is that all of the administered materials are readily available and accessible within the intestinal lumen, avoiding the problem of variation in the rate of gastric emptying among subjects [28].

Subsequent to treatment, histological changes in the mucus layer, intestinal epithelium, and the microvilli on the apical surfaces of epithelial cells were examined by WGA staining, H&E staining, and TEM, respectively. Histological examination yields visual evidence of morphological changes. Only treatment with the capsule that contained HD SDS made the mucus layer relatively thinner than that of the untreated control (normal) (Fig. 4A), owing to the cleansing action of the high concentration of the surfactant on the mucosa surface. Surfactants can reportedly reduce the viscosity and elasticity of mucus [29]. However, using SDS at the doses in this work did not cause any specific changes in the structural integrity of the epithelia (Fig. 4B), including their cellular microvilli (Fig. 4C). The local mucosal epithelium is expected to be exposed to a relatively low dose of permeation enhancer, owing to the fast formation and expansion of bubble carriers (Fig. 2A), regionally dispersing the SDS.

A concern in the use of a permeation enhancer is the possible absorption of endotoxins during the period of temporary epithelial permeability enhancement. To explore this possibility, *ex vivo* imaging of the major organs that were isolated from the test rats was performed using IVIS. *Ex vivo* fluorescence images reveal that Cy5-insulin was absorbed into the blood from the intestinal tract and accumulated in the heart, lung, liver, spleen, pancreas, and kidneys (Fig. 5A); the intensities of fluorescence in the organs increased significantly to an extent that depended on the SDS dose (Fig. 5B). Conversely, no absorption of FITC-LPS was detected, even at the highest dose of SDS (HD SDS) used (Fig. 5A and B). These analytical results agree with the findings in the literature that the physical barrier of the mucus layer prevents the absorption of the LPS into systemic circulation [30]. The above findings collectively support the safety of the bubble carrier system for oral protein drug delivery. However, since the mucus layer that was treated with HD SDS was relatively thinner than that in the normal control, the MD SDS was used for subsequent studies to ensure safety.

3.5. In vivo efficacy: PK/PD profiles

To confirm the potential effectiveness of the bubble carrier system for the oral delivery of insulin, the PK and PD profiles of the diabetic rats that had received the SDS-containing capsule were analyzed. The control groups received free-form insulin solution by SC injection or oral ingestion; an additional control group was treated with the test capsule without SDS. Fig. 6 presents changes in plasma insulin and glucose levels following oral ingestion of the test samples. Upon oral administration, the free-form insulin solution exhibited no blood

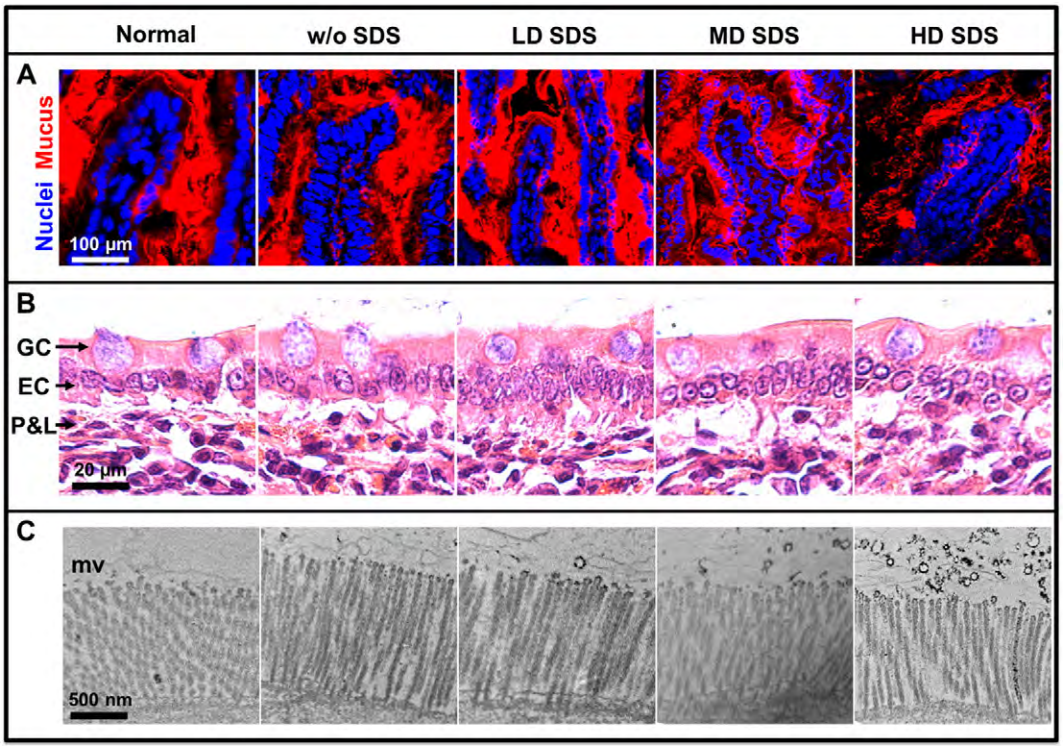

Fig. 4. Changes in (A) mucus layer (CLSM images, WGA/DAPI staining), (B) intestinal epithelium (histological micrographs, H&E staining), and (C) epithelial microvilli (TEM micrographs) that had been treated with test capsule with various doses of SDS, in an intestinal-closed loop study. GC: goblet cells; EC: epithelial cells; P&L: plasma cells and lymphocytes; mv: microvilli.

glucose-lowering activity, while SC injection of free-form insulin solution resulted in a rapid increase in plasma insulin concentration, which in turn caused a sharp decrease in the blood glucose level.

Notably, the group that was given the SDS-containing capsule had a significantly higher plasma insulin concentration than the group that received the capsule without SDS, so the former exhibited a more

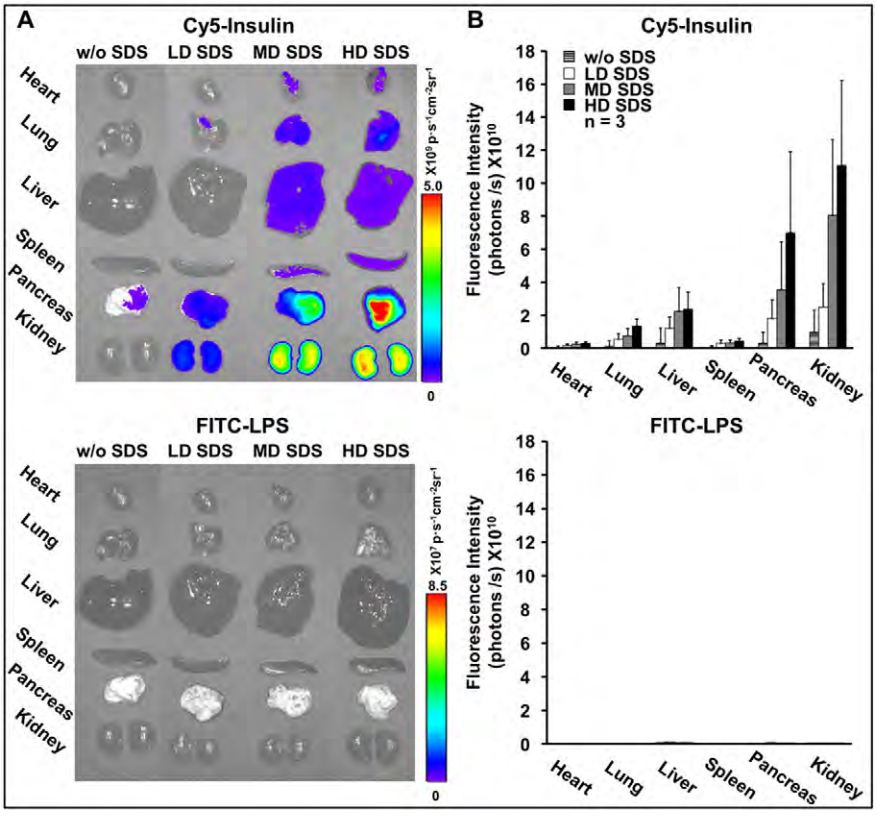

Fig. 5. (A) *Ex vivo* fluorescence images of accumulation of Cy5-insulin and FITC-LPS in isolated major organs and their (B) corresponding fluorescent intensities obtained by IVIS, following treatment with FITC-LPS and a test capsule that contained various doses of SDS, in an intestinal-closed loop study.

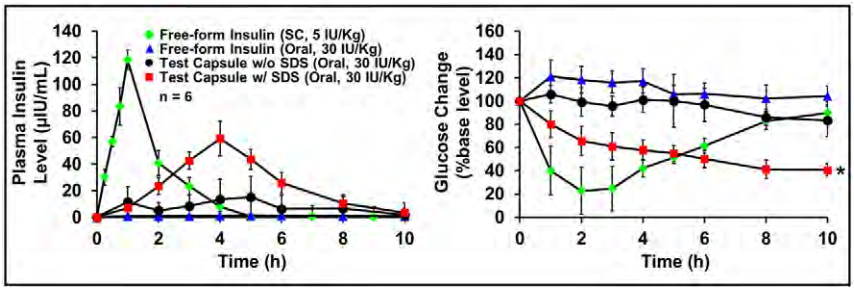

Fig. 6. Plasma insulin level and blood glucose change *vs.* time profiles of diabetic rats that had been treated with various insulin dosage formulations. Oral: oral administration; SC: subcutaneous injection. *: statistically significant *vs.* w/o SDS group ($P < 0.05$).

Table 1
Pharmacokinetic parameters of insulin in diabetic rats following subcutaneous (SC) injection of free-form insulin solution, oral ingestion of free-form insulin solution, or oral administration of an enteric-coated test capsule filled with or without SDS. C_{max}: maximum plasma concentration; T_{max}: time at which C_{max} is attained; $AUC_{(0-10\ h)}$: area under the plasma concentration *vs.* time curve; BA_R: relative bioavailability (n = 6).

	Free-form insulin (SC)	Free-form insulin (Oral)	Test capsule w/o SDS (Oral)	Test capsule w/ SDS (Oral)
Dose (IU/kg)	5.0	30.0	30.0	30.0
C_{max} (μIU/mL)	118.5 ± 7.4	N/A[a]	15.2 ± 15.7	59.2 ± 13.1
T_{max} (h)	1.0	N/A[a]	5.0	4.0
$AUC_{(0-10\ h)}$ (μIU h/mL)	190.9 ± 28.0	10.0 ± 3.5	76.9 ± 19.9	238.7 ± 31.8
BA_R (%)	100	0.9 ± 0.4	6.7 ± 1.7	20.8 ± 1.9

[a] N/A: not available.

pronounced reduction in blood glucose levels ($P < 0.05$). The absorption of insulin in the case without SDS was enhanced by DTPA, which can chelate Ca^{2+} ions, disrupting the epithelial tight junctions and thus enhancing paracellular permeability [31], while that in the case with SDS was enhanced by both SDS and DTPA. According to Table 1, in the cases with and without SDS, the $AUC_{(0-10\ h)}$ values were 238.7 ± 31.8 and 76.9 ± 19.9 μIU h/mL, and their corresponding BA_R values were 20.8 ± 1.9% and 6.7 ± 1.7%, respectively, suggesting that SDS plays a more significant role than DTPA in enhancing oral protein absorption.

4. Conclusions

This study demonstrates the efficacy of the SDS-containing bubble carrier system in enhancing the significant oral absorption of insulin, which has a substantial blood glucose-lowering effect in diabetic rats. However, using SDS at the doses in this study did not cause any specific changes in the structural integrity of epithelial cells; additionally, no absorption of LPS into systemic circulation was detected. These findings reveal that the bubble carrier system with SDS is a potent and safe delivery system for the oral administration of insulin.

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References

[1] E.Y. Chuang, K.J. Lin, P.Y. Lin, H.L. Chen, S.P. Wey, F.L. Mi, H.C. Hsiao, C.T. Chen, H.W. Sung, Self-assembling bubble carriers for oral protein delivery, Biomaterials 64 (2015) 115–124.
[2] S. Maher, R.J. Mrsny, D.J. Brayden, Intestinal permeation enhancers for oral peptide delivery, Adv. Drug Deliv. Rev. (2016)http://dx.doi.org/10.1016/j.addr.2016.06.005.
[3] J.Y. Hong, J.K. Kim, Y.K. Song, J.S. Park, C.K. Kim, A new self-emulsifying formulation of itraconazole with improved dissolution and oral absorption, J. Control. Release 110 (2006) 332–338.
[4] S.M. Deane, F.T. Robb, D.R. Woods, Production and activation of an SDS-resistant alkaline serine exoprotease of vibrio-alginolyticus, J. Gen. Microbiol. 133 (1987) 391–398.
[5] Y. Narkar, R. Burnette, R. Bleher, R. Albrecht, A. Kandela, J.R. Robinson, Evaluation of mucosal damage and recovery in the gastrointestinal tract of rats by a penetration enhancer, Pharm. Res. 25 (2008) 25–38.
[6] A. Makhlof, M. Werle, Y. Tozuka, H. Takeuch, A mucoadhesive nanoparticulate system for the simultaneous delivery of macromolecules and permeation enhancers to the intestinal mucosa, J. Control. Release 149 (2011) 81–88.
[7] F. McCartney, J.P. Gleeson, D.J. Brayden, Safety concerns over the use of intestinal permeation enhancers: a mini-review, Tissue Barriers 4 (2016) e1176822.
[8] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 22 (2008) 659–661.
[9] A.H. Liao, Y.K. Li, W.J. Lee, M.F. Wu, H.L. Liu, M.L. Kuo, Estimating the delivery efficiency of drug-loaded microbubbles in cancer cells with ultrasound and bioluminescence imaging, Ultrasound Med. Biol. 38 (2012) 1938–1948.
[10] R.J. Schilling, A.K. Mitra, Degradation of insulin by trypsin and alpha-chymotrypsin, Pharm. Res. 8 (1991) 721–727.
[11] T. Karamanidou, K. Karidi, V. Bourganis, K. Kontonikola, O. Kammona, C. Kiparissides, Effective incorporation of insulin in mucus permeating self-nanoemulsifying drug delivery systems, Eur. J. Pharm. Biopharm. 97 (2015) 223–229.
[12] Y.H. Lin, K. Sonaje, K.M. Lin, J.H. Juang, F.L. Mi, H.W. Yang, H.W. Sung, Multi-ion-crosslinked nanoparticles with pH-responsive characteristics for oral delivery of protein drugs, J. Control. Release 132 (2008) 141–149.
[13] K. Sonaje, Y.J. Chen, H.L. Chen, S.P. Wey, J.H. Juang, H.N. Nguyen, C.W. Hsu, H.W. Sung, Enteric-coated capsules filled with freeze-dried chitosan/poly(gamma-glutamic acid) nanoparticles for oral insulin delivery, Biomaterials 31 (2010) 3384–3394.
[14] T. Uchiyama, T. Sugiyama, Y.S. Quan, A. Kotani, N. Okada, T. Fujita, S. Muranishi, A. Yamamoto, Enhanced permeability of insulin across the rat intestinal membrane by various absorption enhancers: their intestinal mucosal toxicity and absorption-enhancing mechanism of n-lauryl-beta-D-maltopyranoside, J. Pharm. Pharmacol. 51 (1999) 1241–1250.
[15] H. Inagawa, C. Kohchi, G.I. Soma, Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness, Anticancer Res. 31 (2011) 2431–2436.
[16] W. Li, H. Zhang, X. Niu, X. Wang, Y. Wang, Z. He, H. Yao, Effects and mechanisms of cavidine protecting mice against LPS-induced endotoxic shock, Toxicol. Appl. Pharmacol. 305 (2016) 46–54.
[17] E.Y. Chuang, K.J. Lin, F.Y. Su, F.L. Mi, B. Maiti, C.T. Chen, S.P. Wey, T.C. Yen, J.H. Juang, H.W. Sung, Noninvasive imaging oral absorption of insulin delivered by nanoparticles and its stimulated glucose utilization in controlling postprandial hyperglycemia during OGTT in diabetic rats, J. Control. Release 172 (2013) 513–522.
[18] Y.P. Ho, H.H. Chen, K.W. Leong, T.H. Wang, Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-FRET, J. Control. Release 116 (2006) 83–89.
[19] K. Sonaje, E.Y. Chuang, K.J. Lin, T.C. Yen, F.Y. Su, M.T. Tseng, H.W. Sung, Opening of epithelial tight junctions and enhancement of paracellular permeation by chitosan: microscopic, ultrastructural, and computed-tomographic observations, Mol. Pharm. 9 (2012) 1271–1279.
[20] F.Y. Su, K.J. Lin, K. Sonaje, S.P. Wey, T.C. Yen, Y.C. Ho, N. Panda, E.Y. Chuang, B. Maiti, H.W. Sung, Protease inhibition and absorption enhancement by functional nanoparticles for effective oral insulin delivery, Biomaterials 33 (2012) 2801–2811.

[21] K. Wada, W. Yu, M. Elazizi, S. Barakat, M.A. Ouimet, R. Rosario-Melendez, J.P. Fiorellini, D.T. Graves, K.E. Uhrich, Locally delivered salicylic acid from a poly(anhydride-ester): impact on diabetic bone regeneration, J. Control. Release 171 (2013) 33–37.
[22] M.F. Chung, W.T. Chia, W.L. Wan, Y.J. Lin, H.W. Sung, Controlled release of an anti-inflammatory drug using an ultrasensitive ROS-responsive gas-generating carrier for localized inflammation inhibition, J. Am. Chem. Soc. 137 (2015) 12462–12465.
[23] S.G. Oh, S.P. Klein, D.O. Shah, Effect of micellar life-time on the bubble dynamics in sodium dodecyl sulfate solutions, AICHE J. 38 (1992) 149–152.
[24] I.J. Castellanos, R. Crespo, K. Griebenow, Poly(ethylene glycol) as stabilizer and emulsifying agent: a novel stabilization approach preventing aggregation and inactivation of proteins upon encapsulation in bioerodible polyester microspheres, J. Control. Release 88 (2003) 135–145.
[25] H. Kim, S. Lee, Y. Han, J. Park, Control of pore size in ceramic foams: influence of surfactant concentration, Mater. Chem. Phys. 113 (2009) 441–444.
[26] K.J. Sweadner, Trypsin inhibitor paradoxically stabilizes trypsin activity in sodium dodecyl sulfate, facilitating proteolytic fingerprinting, Anal. Biochem. 194 (1991) 130–135.
[27] E. Gundogdu, V. Mangas-Sanjuan, I. Gonzalez-Alvarez, M. Bermejo, E. Karasulu, *In vitro-in situ* permeability and dissolution of fexofenadine with kinetic modeling in the presence of sodium dodecyl sulfate, Eur. J. Drug Metab. Pharmacokinet. 37 (2012) 65–75.
[28] B.J. Aungst, Intestinal permeation enhancers, J. Pharm. Sci. 89 (2000) 429–442.
[29] G.P. Martin, C. Marriott, I.W. Kellaway, Direct effect of bile salts and phospholipids on the physical properties of mucus, Gut 19 (1978) 103–107.
[30] K. Sonaje, K.J. Lin, M.T. Tseng, S.P. Wey, F.Y. Su, E.Y. Chuang, C.W. Hsu, C.T. Chen, H.W. Sung, Effects of chitosan-nanoparticle-mediated tight junction opening on the oral absorption of endotoxins, Biomaterials 32 (2011) 8712–8721.
[31] M.C. Chen, F.L. Mi, Z.X. Liao, C.W. Hsiao, K. Sonaje, M.F. Chung, L.W. Hsu, H.W. Sung, Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules, Adv. Drug Deliv. Rev. 65 (2013) 865–879.