

RESEARCH ARTICLE



Mechanism of enhanced oral absorption of akebia saponin D by a self-nanoemulsifying drug delivery system loaded with phospholipid complex

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ABSTRACT

Akebia saponin D (ASD) exhibits a variety of pharmacological activities, such as anti-osteoporosis, neuro-protection, hepatoprotection, but has poor oral bioavailability. A self-nanoemulsifying drug delivery system loaded with akebia saponin D - phospholipid complex (APC-SNEDDS) (composition: Peceol: Cremophor[®] EL: Transcutol HP: ASD: phospholipid; ratio: 10:45:45:51:12.3, w:w:w:w) was first developed to improve the oral absorption of saponins and it was found to significantly enhance ASD's oral bioavailability by 4.3-fold ($p < .01$). This study was conducted to elucidate the mechanism of enhanced oral absorption of ASD by the drug delivery system of APC-SNEDDS. The aggregation morphology and particle size of ASD and APC-SNEDDS prepared in aqueous solutions were determined by transmission electron microscope and particle size analyzer, respectively. Stability of ASD and APC-SNEDDS in gastrointestinal luminal contents and mucosa homogenates were also explored. The differences of *in situ* intestinal permeability of ASD and APC-SNEDDS were compared. APC-SNEDDS reduced the aggregation size from 389 ± 7 nm (ASD) to 148 ± 3 nm (APC-SNEDDS). APC-SNEDDS increased the remaining drug in large intestine luminal contents from $47 \pm 1\%$ (ASD) to $83 \pm 1\%$ (APC-SNEDDS) during 4 h incubation. APC-SNEDDS provided an 11-fold increase in K_a value and an 11-fold increase in P_{eff} value compared to ASD. In summary, APC-SNEDDS improved ASD's oral bioavailability mainly by increasing membrane permeability, destroying self-micelles and inhibiting the intestinal metabolism.

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Introduction

Saponins, a major class of natural phytochemicals, hold great promises for being developed as therapeutic and chemopreventive drugs [1]. However, the generally low oral bioavailability and the side effects, observed when used at high concentrations, have limited their pharmacological impact *in vivo* [1]. Many biocompatible and biodegradable nanoparticles, such as nanoliposomes, nanoemulsions, self-nanoemulsifying drug delivery systems (SNEDDS), lipid nanocarriers, micelles, and polymeric nanoparticles, have been used to enhance oral absorption of bioactive phytochemicals [2–5]. Among these alternatives, SNEDDS offers several advantages, including maintaining the drug in small droplets of oil, improving stability of drug against enzymatic degradation and first-pass hepatic metabolism, enhancing absorption afforded by

surfactant - induced permeability changes, and increasing dosage versatility in the form of either in liquid or solid dosage [3,6]. Thus, SNEDDS represents a very attractive drug delivery system for poorly absorbed saponins.

However, a portion of the saponin not having sufficient liposolubility may result in a failure of the SNEDDS formulation. Some measures can be taken to improve the liposolubility of drugs, including phospholipid complexation [7,8], hydrophobic ion pairing [9–11], and chemical modification ion [12]. Compared to the other approaches the use of phospholipid complex (PC) is a more universally applicable and an easier technique for formulating various saponins [13–15]. PC exhibits a cell membrane like structure with the agents interacting with the polar portions of phospholipid while the nonpolar parts of phospholipid warping over the complex, resulting in higher lipophilicity of the entire complex.

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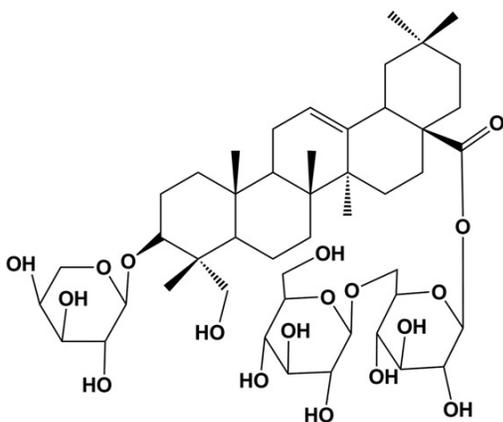


Figure 1. Chemical structure of ASD.

The combined utilization of PC and SNEDDS (PC-SNEDDS) may represent a promising oral drug delivery system for poorly lipophilic saponins.

Akebia saponin D (ASD, Figure 1), a poorly lipophilic triterpenoid saponin, isolated from the rhizome of *Dipsacus asper* Wall, possesses many pharmacological activities including anti-osteoporosis [16], hepatoprotection [17], cardioprotection [18–20], and neuroprotection [21–23] activities. However, ASD is poorly absorbed when orally administered and a high dose is needed to achieve its therapeutic effect. Our team employed the PC-SNEDDS approach to deliver the poor lipophilic ASD to improve its bioavailability [24]. PC-SNEDDS-loaded ASD (APC-SNEDDS) showed a 4.3-fold increase of oral bioavailability, thus, exhibiting greatly improved potential for further clinical application. *Ex vivo* and *in vivo* studies are all important means to reveal the mechanism for promoting oral absorption of drug delivery system [25]. In the study, multiple methods were adopted to elucidate the possible mechanisms involved in the oral absorption of PC-SNEDDS, as a promising oral drug delivery system, since these results might provide compelling evidence for the use of PC-SNEDDS for poorly lipophilic saponins.

Materials and methods

Materials

Akebia saponin D (ASD, 95.23% purity) was a pilot product prepared by our laboratory. The purity of ASD was calibrated based on a standard purchased from the National Institutes for Food and Drug Control (Beijing, People's Republic of China). Akebia saponin D phospholipid complex (A-PC, the content of ASD was 26.8%) and Akebia saponin D phospholipid complex loaded self-nanoemulsifying delivery system (APC-SNEDDS; composition: Peceol: Cremophor® EL: Transcutol HP: ASD: phospholipid; ratio: 10:45:45:51:12.3, w:w:w:w) were prepared and characterized according to our previous work [24]. In brief, oil and cosurfactant were selected according to their ability to dissolve A-PC, while surfactant was chosen based on its emulsification efficiency in SNEDDS. All the other chemicals were of either analytical or chromatography grade.

Aggregation morphologies and particle size of ASD and APC-SNEDDS in aqueous solution

The aggregation morphologies of ASD and APC-SNEDDS aqueous solutions (all containing 9 mg/mL of ASD) were determined using

transmission electron microscope (TEM, Hitachi H-600, Hitachi Co, Tokyo, Japan). The particle sizes of ASD and APC-SNEDDS aqueous solutions (all containing 9 mg/mL of ASD) were measured by dynamic light scattering using a BI-90 Plus particle size analyzer (Brookhaven, US).

Stability of APC-SNEDDS in gastrointestinal luminal contents and mucosa homogenates

The procedures were conducted according to a reported study [26]. Male Sprague Dawley rats were fasted for 12 h and sacrificed by decapitation under anesthesia. The stomach, small intestine, and large intestine were isolated and flushed with cold saline to collect the gastrointestinal luminal contents for each segment. The mucosa of each segment was scraped with a glass plate and dispersed in cold saline. Both the luminal contents and mucosa obtained from three rats were homogenized and then centrifuged to collect the supernatants. The final protein concentration in each supernatant was determined using a BCA protein assay kit and then adjusted to 1 mg/mL by diluting with cold saline. Thereafter, ASD, and APC-SNEDDS were added to each diluent to make a final concentration of ASD of 0.5 mg/mL and then incubated at 37 °C with continuous shaking. At predetermined time points, 0.1 ml sample was taken out and mixed with 0.2 ml acetonitrile. The mixture was vortex-mixed for 3 min and then centrifuged, at $10,012 \times g$ for 10 min. An aliquot of 10 μ L supernatant was used for HPLC analysis.

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the Ethics Committee of China Pharmaceutical University.

In situ evaluation of ASD in APC-SNEDDS across intestinal barrier using single-pass intestinal perfusion model (SPIP)

Previously, our group found that ASD had the highest absorption constant and intestinal permeability in the jejunum [27]. Moreover, multidrug resistance-associated proteins (MRPs) are largely expressed in the jejunum [28]. The jejunum segment was selected for the SPIP studies to study the influence of MRP inhibitor on the intestinal absorption of ASD.

The SPIP study of ASD was performed according to previously published reports [29,30]. Briefly, rats were anesthetized with 20% urethane (1.3 g/kg, *i.p.*). After the abdomen was opened, the jejunal segment (10 cm in length) was cannulated at both ends with glass tubes and gently rinsed with physiological saline. Afterward, Krebs-Ringer's buffer solution containing samples (ASD, A-PC, APC-SNEDDS) were perfused through the lumen at a flow rate of 0.2 ml/min for about 30 min to reach steady-state conditions. ASD, A-PC, and APC-SNEDDS dissolved in Krebs-Ringer's buffer solution (all equivalent to 0.5 mg/mL of ASD) were perfused through the loop and collected in weighted tubes at intervals of 15 min for 90 min. At the end of the experiment, the inlet and outlet tubes were weighed and the luminal length and radius were measured. The samples were filtered and analyzed by HPLC.

The absorption rate constant (K_a) and apparent permeability coefficients (P_{eff}) were calculated using the following equations:

$$K_a = (1 - C_{\text{out}}Q_{\text{out}}/C_{\text{in}}Q_{\text{in}}) \times Q/\pi r^2 l$$

$$P_{\text{eff}} = -Q \times \ln(C_{\text{out}}Q_{\text{out}}/C_{\text{in}}Q_{\text{in}})/2\pi r l$$

where C_{in} (mg/mL) and C_{out} (mg/mL) are the inlet and outlet concentration of ASD in perfusion buffer, respectively. Q_{in} (mL) and

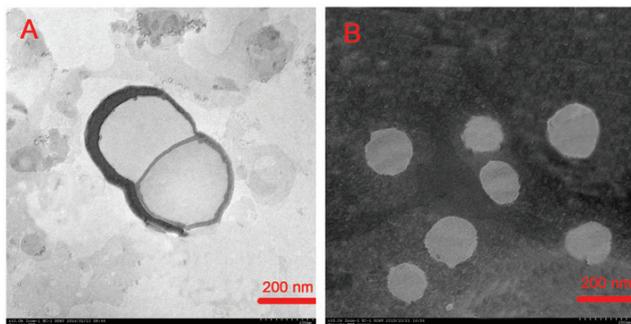


Figure 2. TEM image of (A) ASD ($\times 15,000$ magnification), (B) APC-SNEDDS ($\times 15,000$ magnification) aqueous solution (all contained 9 mg/mL ASD).

Q_{out} (mL) are the volumes of perfusion entering and exiting the intestine, respectively. Q (mL/min) is the flow rate, and r and l are the inner radius and length of perused segment, respectively.

Statistical analysis

Statistical analyses were performed using two-tailed Student's t test and a p value of less than .05 was considered significant.

Results

APC-SNEDDS changed the aggregation state of ASD in aqueous solution

Some saponins are natural surfactants that will form micelles in aqueous solutions when reaching their critical micelle concentration (CMC) [31,32]. The formation of micelles resulted in a large aggregate size and a very poor permeability in either the paracellular or transcellular pathways [31]. Therefore, preventing saponins from aggregation represents a strategy for improving the oral absorption of saponins. Some drug delivery systems, including lipid-based formulation and solid dispersion, have successfully increased the intestinal absorption and oral bioavailability of saponins by destroying self-micelles [29,31].

Previously, our team found that ASD was a natural surfactant with a CMC value of 66.07 $\mu\text{g/mL}$ [29]. ASD was orally administered to rats at a concentration of 9 mg/mL in a study on its bioavailability. At that concentration, ASD self-assembled as micelles of large particle size, greatly reducing its intestinal permeability and oral bioavailability [29]. We speculate that one mechanism of enhanced intestinal permeation and oral absorption by APC-SNEDDS was through changing the aggregation state of ASD in aqueous solution. The aggregation morphologies of ASD and APC-SNEDDS are displayed in Figure 2. ASD self-assembled as a spherical micelle with a particle size (number-weighted) of 389 ± 7 nm. After formation of APC-SNEDDS, the mean droplet size (number-weighted) was only 148 ± 3 nm. ASD is first encased in phospholipids to form complex and then completely embedded into the oil phase of the oil-in-water nanoemulsion, based on the understanding of the structure of APC-SNEDDS [24]. Therefore, the actual aggregation state of ASD in APC-SNEDDS is changing and complicated, and no aggregation or very small aggregation might occur. Molecular ASD and/or very small aggregates could be present when passing the intestinal barrier.

Compared to other oral drug delivery systems, which prevent saponins from aggregation, SNEDDS can more thoroughly destroy self-micelles.

APC-SNEDDS improved ASD stability in the gastrointestinal tract

ASD and APC-SNEDDS remained stable in the stomach contents, small intestine contents and all mucosa homogenates of each segment over a 4 h incubation time (Figure 3). After 4 h of incubation, the amount of drug remained by $47 \pm 1\%$ and $83 \pm 1\%$ for ASD and APC-SNEDDS, respectively. These results indicate that ASD might be hydrolyzed by large intestinal microflora, consistent with our previous research results [33], and APC-SNEDDS can effectively protect ASD from degradation within the large intestinal contents.

APC-SNEDDS increased *in situ* intestinal permeability of ASD

In this section, an *in situ* SPIP method was used to observe the influence of APC-SNEDDS on intestinal permeability of ASD. It is remarkable to note an 11-fold increase in K_a value and an 11-fold increase in P_{eff} value offered by APC-SNEDDS ($p < .001$) (Figure 4).

Discussion

Screening the new active molecules from plants is an important method for drug discovery. Saponins are believed to be responsible for the pharmacological activities of many Chinese medicinal herbs [34]. However, the oral bioavailability of most naturally occurring saponins is less than 10% [1,34] and their poor oral absorption has been the major challenge in clinically developing saponins useful drugs. Administration through injection represents one feasible strategy to overcome the poor absorption for part of saponins. Nevertheless, this route is not useful for most saponins because injection results in poor compliance and security risks. For some chronic diseases in which saponins hold great promise, oral administration is more suitable than administration through injection. Therefore, oral administration is the most appropriate drug-delivery approach for most saponins. Many drug delivery systems employ complexation by cyclodextrin to improve the oral absorption of saponins, [35], solid dispersion approaches [13], or the use of microparticles [36], micelles [14], and microemulsions [37]. Previously, our team developed a self-nanoemulsifying drug delivery system loaded with akebia saponin D - phospholipid complex (APC-SNEDDS), which greatly increased the oral bioavailability of ASD to 4.3-fold [24]. Elucidating the underlying mechanisms of enhanced oral absorption of ASD by APC-SNEDDS provides important information useful for other saponins.

The reasons for low bioavailability of ASD includes self-aggregation in gastrointestinal fluid [29], low intestinal permeability [27], metabolism in the intestine [38], decomposition in the liver, and excretion from the bile [39]. In this study, we demonstrate that APC-SNEDDS can significantly increase membrane permeability, destroy self-micelles and inhibit the intestinal metabolism. It was obvious that simultaneously inhibiting several limiting absorption factors can greatly improve the oral bioavailability of ASD.

Microflora hydrolysis and enzymatic metabolism are the key factors that limit the oral bioavailabilities of saponins [1]. Incorporating drugs into SNEDDS can offer protection against degradation in gastrointestinal tract [26,40]. The stability of ASD in gastrointestinal luminal contents and mucosa homogenates was tested to observe the effect of protection. The results showed that APC-SNEDDS could effectively protect ASD from degradation within the large intestinal contents.

Poor intestinal absorption is one factor that limits the oral bioavailability of most saponins [34]. Previous studies showed that

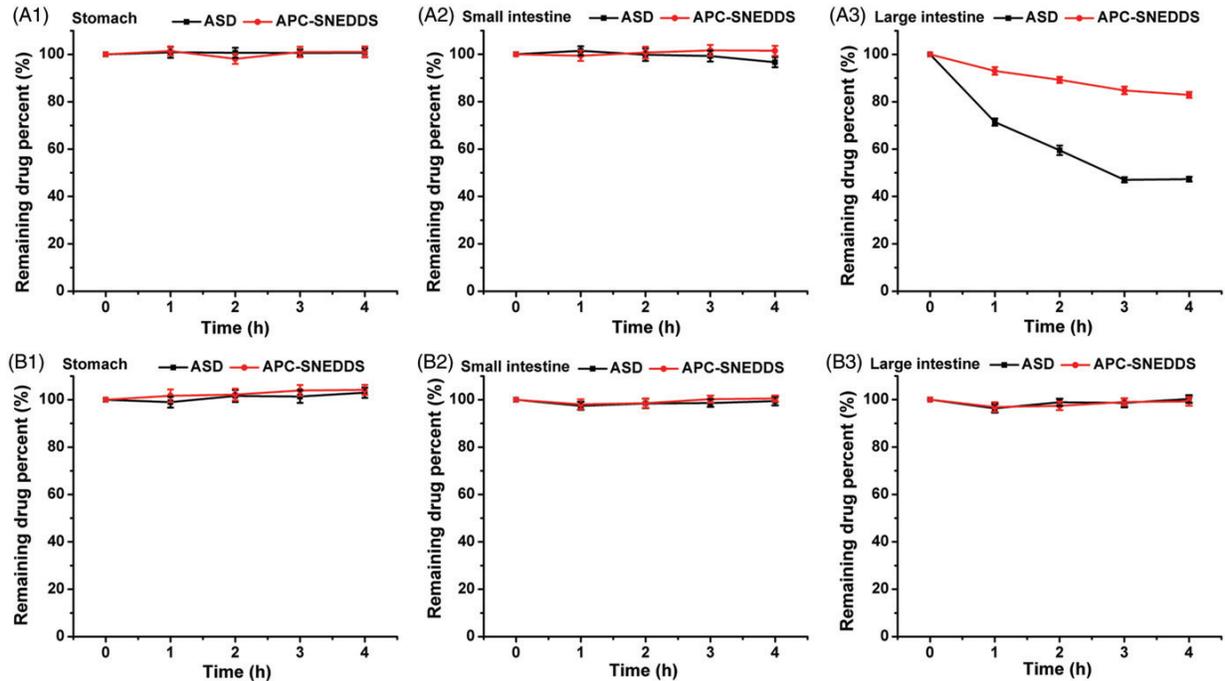


Figure 3. Stability of ASD and APC-SNEDDS in (A1~A3) intestinal luminal contents and (B1~B3) homogenates at a protein concentration of 1 mg/mL (mean \pm SD, $n = 6$).

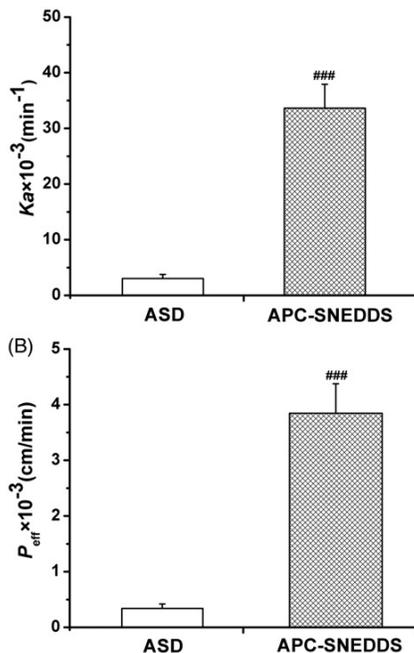


Figure 4. Comparison of (A) K_a and (B) P_{eff} of ASD and APC-SNEDDS in jejunum (mean \pm SD, $n = 6$). # $p < .05$, ## $p < .01$, ### $p < .001$ vs. ASD group.

the intestinal permeability of ASD was poor [27], mainly due to its unfavorable physicochemical properties (molecular weight >500 , >5 H-bond donors, >10 H-bond acceptors), self-micelle formation, and due to contributions from multi-drug resistance-associated protein (MRP) [29]. Increasing intestinal permeability might be an effective strategy to improve the oral bioavailability of ASD. In APC-SNEDDS, Cremophor[®] EL (Polyoxyyl 35 castor oil) was used as

a surfactant to form the drug delivery system and as an inhibitor to prevent MRP-mediated efflux. In addition, APC-SNEDDS could prevent ASD from aggregation and decrease the aggregation size from 389 ± 7 nm (ASD) to 148 ± 3 nm (APC-SNEDDS) in water. These two actions may promote the intestinal absorption of ASD. Our study showed that APC-SNEDDS provided an 11-fold increase in K_a value and an 11-fold increase in P_{eff} value compared to ASD. The data demonstrated that APC-SNEDDS significantly enhanced the intestinal permeability of ASD, probably owing to the absorption enhancers and the inhibition of aggregation.

Although APC-SNEDDS significantly increased the oral absorption to 4.3-fold, the absolute bioavailability is still low (about 1.78%) due to the poor bioavailability of ASD. This is a common problem for almost all published research that intends to improve the oral absorption of saponins through oral administration. The present study and other reports, adopt new drug delivery systems, more powerful oral absorption promoters, while simultaneously inhibiting several absorption limiting factors providing even greater absorption enhancement.

Conclusions

The present study demonstrated that APC-SNEDDS improves ASD's oral bioavailability mainly by increasing the membrane permeability, destroying self-micelles and inhibiting the intestinal metabolism. The results provide the strong evidence required for the rational design of PC-SNEDDS for use with poorly lipophilic saponins.

Disclosure statement

No potential conflict of interest was reported by the authors.

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