# Novel nanocarriers with unique ginsenoside "shell" for regulating circadian rhythm skin care: Ginseng Loves Your Skin Day and Night

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## Abstract

Ginsenosides are the main functional components of ginseng extracts. Extensive studies have confirmed that many types of ginsenosides have advanced effects on skin care. However, current research on ginsenosides barely consider the different needs of skin during daytime and nighttime, which is regulated by circadian rhythm. During daytime, the primary role of skin is to defend against the reactive oxygen species (ROS) caused by external UV and pollutants; While during nighttime, skin needs a strong self-repairing mechanism to promote the proliferation and rejuvenation of the cells, as well as to regulate the melatonin synthesis and restore the skin circadian rhythm. Ginsenosides are mainly divided into two categories: protopanaxadiol-type (PPD) and protopanaxatriol-type (PPT). They have completely different pharmacological effects and cosmetic efficacy, so they must be well separated and enriched when use as active ingredients. This study reports the exclusive applications of PPD and PPT ginsenosides in skin care during daytime and nighttime, respectively, to achieve a synergistically regulation of the skin circadian rhythm. The specially transformed PPD and PPT ginsenosides used in this study have pure constituent structures that can provide better bioactivities. Furthermore, these transformed PPD and PPT ginsenosides are amphiphilic molecules, which are natural alternatives for replacing the chemically synthesized encapsulation materials such as cholesterol. It can be served as a stable “shell” layer for nanocarriers (e.g., micelles, liposomes, nanoparticles) to encapsulate and release both oil-soluble and water-soluble active substances, which can effectively reduce irritation and improve the transdermal permeation efficiency and bioactivities.

**Keywords:** Rare Ginsenosides, circadian rhythm skin care, nano-carriers

**Introduction**.

Panax ginseng C.A.Meyer, a traditional and precious Chinese medicine, *a.k.a.* the "king of herbs" since ancient times, has attracted attention for thousands of years. Dried roots of Panax ginseng can be used as medicine, and its stems, leaves, flowers and fruits can also be used as medicine. "Panax" in its Latin scientific name is a combination of "pan" (meaning "total") and "Axos" (meaning "medicine"), meaning that ginseng is an effective treatment for all diseases. Ginseng has complicated chemical components, variety of bioactivities, unique pharmacological effects and high application value. It is widely used in medicine, pharmacy, health food, cosmetics and other fields [1-5].

Ginsenosides are the key functional component of ginseng extract [3]. As a class of glycosides formed by connecting sugars and aglycones, ginsenosides belong to triterpenoid saponins and are the main effective substances of ginseng extracts, accounting for about 3% ~ 6%. It can play key roles in anti-tumor, anti-aging, cell apoptosis inhibition, reducing blood sugar/fat reduction, learning and memory improvement, immunity enhancement and skin care, etc. Proto-ginsenosides are ginsenosides that can be directly extracted from Panax ginseng and other Araliaceae plants including Ra, Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, etc. These ginsenosides mainly belong to two categories, namely Protopanaxadiol-type (PPD) saponins and protopanaxatriol-type (PPT) saponins. Due to their structural differences, PPD and PPT have disparate pharmacological effects and cosmetic efficacy, so they must be well separated and enriched for use [1-5]. Recent pharmaceutical research has focused on the derivatives of proto-ginsenosides, namely rare ginsenosides (e.g. Rg2, Rg3, Rg5, Rh2, etc.) due to their stronger bioactivity and bioavailability relative to proto-ginsenosides [2]. Rare ginsenosides are quite expensive since they can hardly be extracted directly from plants, and can only be obtained by transforming or metabolizing proto-ginsenosides.

At present, ginseng for cosmetic use are mainly comprised of total extracts (ginseng roots, stems, leaves, fruits and flowers), high-purity ginsenosides are barely added to cosmetics and almost no rare ginsenosides are added directly. Korean ginseng in cosmetics are mainly red ginseng or bio-transformed ginseng extracts. Most of the ginseng cosmetics in the market are made of ginseng extracts, which have uncertain functional components, inconsistent standards, poor stability and skin absorption.

In this study, the protopanaxadiol-type and protopanaxatriol-type ginsenosides were enriched, separated and transformed efficiently by natural extraction process. Mass production of rare ginsenosides protopanaxadiol-type (Exp-PPD) and protopanaxatriol-type (Exp-PPT) was realized, resulting in improved bioactivity, clearer components, more unified standards and stable quality of the final product, as opposite to conventional ginseng extracts.

We further explored and proved that exclusive applications of Exp-PPD and Exp-PPT can well meet the specific requirements of skin care during daytime and nighttime, respectively . Skin biological clock is a regulatory response of skin to environmental pressure, which protects skin from various environmental damage. There are significant circadian rhythms in DNA replication, DNA repair mechanism and cell division of epidermal stem cells. Epidermal barrier functions, such as transepidermal water loss, cuticular hydration and sebum secretion, are also regulated by circadian rhythm. The immune system, such as the expression of antigens, is also controlled by the biological clock. The secretion of melatonin is also affected by the biological clock. Stress, pollution, ultraviolet and blue light damage will interfere with the secretion of melatonin, thus affecting the skin biological rhythm [6-9]. In this study, the precise and rare ginsenoside combination Exp-PPD and Exp-PPT are specially designed for circadian skin care, aiming at the regulation of melatonin's key synthesis enzymes and regulating the skin's biological clock. AANAT/ASMT are two proteases involved in melatonin synthesis, which convert the precursor molecule (serotonin, 5-HT) in the body into melatonin. Melatonin significantly increased the expression of clock, BMAL1, Per1, per2, cry1 and CRY2, and had a feedback regulation effect on these essential clock genes [10-13]. According to the molecular activity and action mechanism, Exp-PPD and Exp-PPT can be accurately used in daily and night skin care products to jointly regulate the AANAT/ASMT’s expression and therefore melatonin production for skin circadian rhythm. This interesting finding provides a unique way of using traditional plant extracts for the precise skin care.

Moreover, many skin care actives are either water-solvable or with high molecular weight (>500 Da), which are hard to penetrate through the stratum corneum, epidermis to dermis layer. To solve this problem, plenty of methods and formulations have been developed to boost the efficacy of active delivery [14]. In this study, according to the characteristics of molecular polarity, we further explored the unique use of Exp-PPD and Exp-PPT as a

replacement of traditional stabilizer such as cholesterol to encapsulate actives, forming various nanocarriers including micelles, liposomes, and nanoparticles etc. This innovative nanocarrier "shell" can largely improve the performance of both oil-soluble and water-soluble active substances, achieving excellent skin care effects, soothing and less irritation, improving transdermal permeation and bioactivities.

**Materials and Methods**.

*Rare Exp-PPD and Exp-PPT extraction and condensation*

All experimental used PPD-type ginsenosides (Exp-PPD) and PPT-type ginsenosides (Exp-PPT) materials were kindly provided by Macau University of Science and Technology. Exp-PPT and Exp-PPD ginsenosides are consistently extracted and prepared using in-house methods. Exp-PPD and Exp-PPT were extracted from ginseng root using ethanol and separated by a specific lab process. The separated ginsenosides were further converted to rare ginsenosides by hydrolyzing with acids and solvent extraction for three times prior to freeze-drying. UPLC-MS were used to characterize the exact ginsenoside structures and compositions.

*Circadian rhythm skin care by EXP PPD and EXP PPT Ginsenosides*

**In-vitro test:** Various in-vitro cell models including keratinocytes, melanocytes, and dermal fibroblasts have been established to demonstrate the efficacy of Exp-PPD ginsenosides in day skin care and Exp-PPT ginsenosides in night skin care, respectively. The evaluation spanning anti-oxidation, anti-inflammation, skin barrier protection and anti-aging effects are carefully carried out. Furthermore, the melatonin related proteins AANAT and ASMT which further relates to many circadian rhythm clock genes are measured to demonstrate the uniqueness Exp-PPD and Exp-PPT combination.

1. ROS scavenging caused by UV damage or $H_2O_2$: 3T3 cell, after UV or $H_2O_2$ damage, apply the drug and cultured for 12h. Intracellular ROS was stained with DCFH-DA dye and the ROS scavenging was analyzed by fluorescence microscope. The experimental sample is with 0.003% Exp-PPD, and the positive control was 0.008% tocopherol acetate.

2. SOD enzyme activity: HSF cell, after UV damage, apply the drug and cultured for 6h, and SOD enzyme activity was tested. The experimental sample is with 0.003% Exp-PPD, and the positive control was 50μM Coenzyme Q10 (CoQ10).
3. B16 cell tyrosinase inhibition and melanin content model: with or without addition α-MSH, B16 cells, apply the drug and cultured for 48h to test tyrosinase activity and melanin content. The experimental sample is with 0.0015% Exp-PPD, and the positive control is 0.05% 4-Butylresorcinol.
4. MMP-1 expression: HaCaT cells, after UV damage, the concentration of MMP-1 was detected by ELISA after 24 hours of culture. The experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM CoQ10.
5. col1 and col III expression: HSF fibroblasts, after UV damage, the concentration of col1 and col III were detected by ELISA after culture for 6h. The experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM CoQ10.
6. ROS scavenging of HSF: HSF cell, after UVB damage (100mJ/$cm^2$), HSF cells were co-cultured with samples for 24h, the changes of intracellular ROS were detected. The experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM Coenzyme Q10 (CoQ10).
7. Anti-inflammation: After HaCaT cells were stimulated by UV, the inflammatory factor TNF-α and PEG2 were detected by ELISA after culture for 24h, the experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM Coenzyme Q10 (CoQ10).
8. Soothing: HaCaT, 24h, CLSM detects intracellular fluorescent calcium flux after Capsaicin activates TRPV1, the experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM Coenzyme Q10 (CoQ10).
9. Skin barrier by filaggrin expression: HaCaT, 24h, CLSM detects the effect of drugs on filaggrin protein in cells, and the experimental sample is with 0.003% Exp-PPT, and the positive control was 50μM Coenzyme Q10 (CoQ10).
10. Franz cell transdermal permeation: In vitro skin preparation: take 3 healthy SPF KM mice, shave the back hair with a razor, wash the skin with warm water, and peel the back skin with a surgical scissors. Rinse with 0.9% sodium chloride solution and soak in normal saline for treatment. In vitro transdermal diffusion test: take mouse skin, cut it into a circle of appropriate size, and bathe it in constant temperature water at (32.0 ±

0.5) ℃ for 20min. Take 1ml of each of the three drug solutions and place them in the drug supply pool, seal the opening of the drug supply pool with a sealing film, and turn on the Franz cell. The diffusion meter shall be kept at a constant temperature (32.0 ± 0.5 ℃), the magneton speed shall be 200r/min, and samples shall be taken at 0.5, 1, 2 and 12h after the test. 0.5ml of samples shall be taken each time (and the receiving solution with the same temperature and volume shall be supplemented). The samples shall be stored in a brown sample bottle and stored at 4 ℃ for testing. Drug concentration detection: use Agilent HPLC 1100 to determine the sample concentration and calculate the transmittance.

**In-vivo test:** The skin care performance of Exp-PPD serum for day and Exp-PPT serum for night skin care has been evaluated by an in-vivo study (N=8) for 28 consecutive days. The subjects were all female, the minimum age was 46 years, the maximum age was 53 years, and the average age was 48.8 ± 2.8 years. After cleaning and drying the face, during the day, apply day essence (contains Exp-PPD) to the skin on the application side and blank essence on the control side; and similarly, apply night essence (contains Exp-PPT) to the skin on the application side and blank essence on the control side. Repeat this day and night application for consecutive 28 days. VISIA-CR and PRIMOS-CR are used to collect the wrinkle images of the subject's product application side and the control side, respectively, and use the analysis software to obtain the proportion of wrinkle areas on both sides; CL400, GL200 and elasticity test probes are used to detect the ITA° value, gloss and skin elasticity R2 and F4 values. The above measured values are taken as the baseline value (D0), and then the product is distributed and the use method is guided. 28 days after using the product (D28), the subjects need to pay a return visit and carry out the same test.

*Nanocarrier with Ginsenoside "shell":*

Ginsenosides nanocarriers preparation: The ginsenosides and benchmark cholesterol nanocarriers are prepared using flash nanoprecipitation method. The free model actives are 0.05wt% 4-Butylresorcinol for simulating the daytime skin care components, and 50μM of CoQ10 for nighttime component. All the nanocarriers are prepared to give the same active concentration at the dosage level of 5%. The comparison was all made at an equal-molar

concentrations of the cholesterol which concentration has been optimized from previous studies. All nanocarriers are well characterized prior to use.

Transdermal permeation efficacy: The Franz diffusion cell device using rat skin in vitro is used for measuring transdermal permeation of model active encapsulated by ginsenosides and by cholesterol nanocarrier.

Improved bioactivities: Various in-vitro cell models have been established to elucidate the advantages of ginsenosides nanocarriers versus traditional cholesterol stabilized nanocarriers in terms of active efficacy.

**Results**.

*Circadian rhythm skin care by Exp-PPD and Exp-PPT Ginsenosides*

In-vitro cell models confirm that Exp-PPD is excellent for the day skin care requirements (e.g., UV protection, ROS clearance, SOD enzyme activity, melanin reduction etc.). These in-vitro cell model methods were described previously in the method session. As shown in Figure 1 (a) and (b), the Exp-PPD can effectively scavenge ROS caused by UV damage as well as by $H_2O_2$ damage. Figure (c) indicates that Exp-PPD can recover SOD enzyme activity after UV damage. Figure (d) and (e) show that Exp-PPD has skin whitening ability by effectively inhibiting tyrosinase activity and reducing the melanin content in cell models with and without $\alpha$-MSH. All these performances suggest that Exp-PPD meets the requirements of daytime skin care products.

Likewise, in-vitro cell models also confirm that the Exp-PPT is more suitable for night skin care (COL production, MMP reduction, anti-oxidation and anti-inflammation). These in-vitro cell model methods were described previously in the method session.

As shown in Figure 2 (a), the Exp-PPT can effectively reduce the MMP-1 concentration increase caused by UV damage. Figure (b) and (c) indicates that Exp-PPT can promote both Human COL-I and COL-III production after UV damage. Figure (d) shows that Exp-PPT has anti-oxidation ability especially for dermis layer where ROS caused by UV in HSF cells can be scavenged. Furthermore, anti-inflammation including reducing the TNF-$\alpha$ and PGE2 after UV damage was seen by of Exp-PPT. All these performances suggest that Exp-PPT is quite suitable for the requirements of nighttime skin care products.

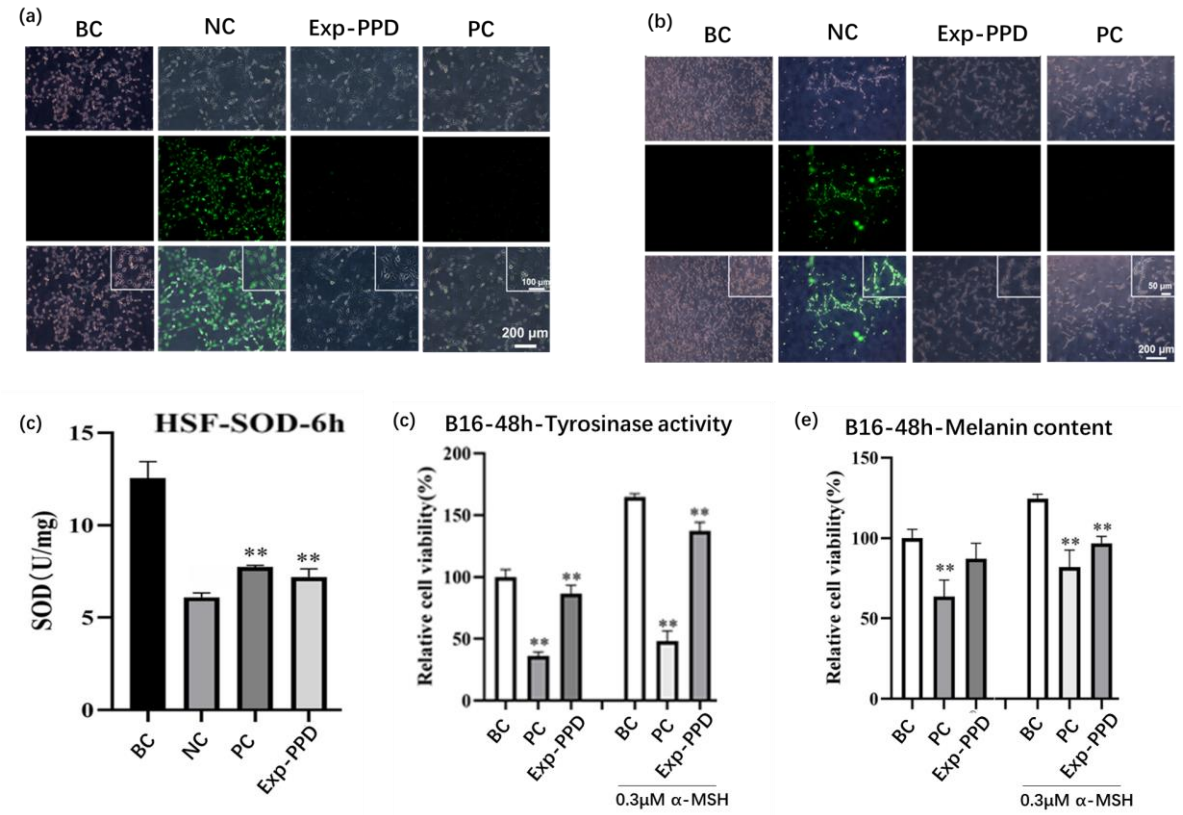


Figure 1. In-vitro models to demonstrate the Exp-PPD performances that meets the day skin care requirements: (a) ROS scavenging caused by UV damage (b) ROS scavenging caused by $H_2O_2$ damage (c) SOD enzyme activity recovering (d) Tyrosinase activity inhibition and (e) Melanin content reduction

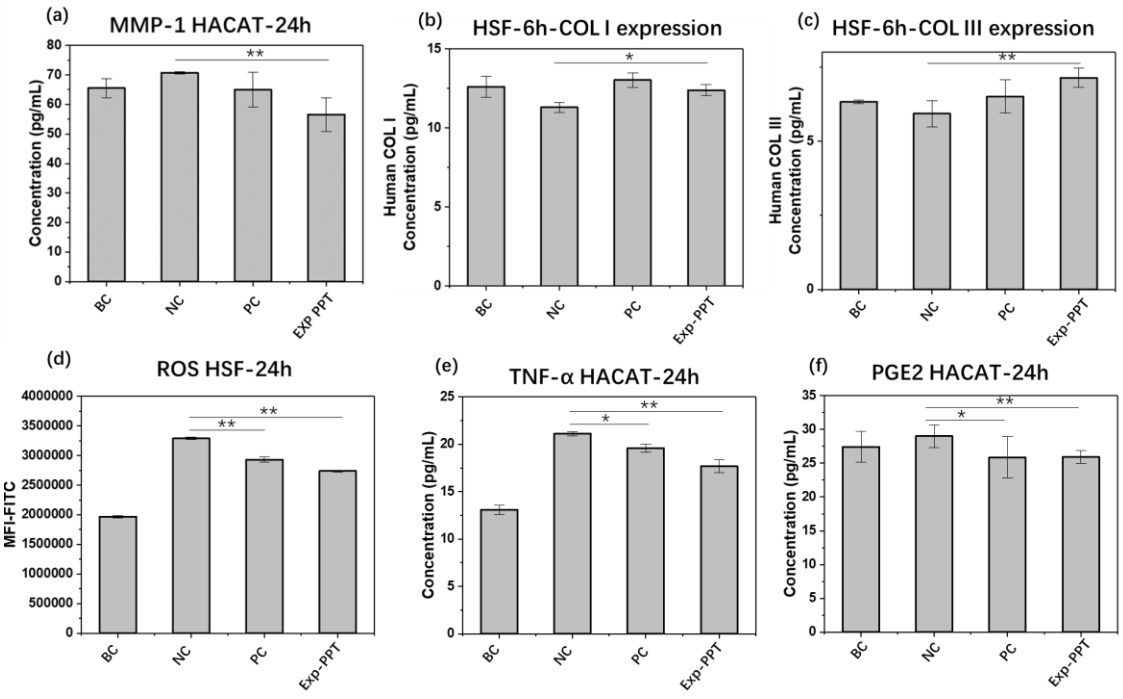


Figure 2. In-vitro models to demonstrate the Exp-PPT performances that meets the night skin care requirements: (a) MMP-1 inhibition after UV damage (b) COL I production (c) COLIII production (d) ROS scavenging (e) and (f) anti-inflammation of TNF-α, PGE2 reduction

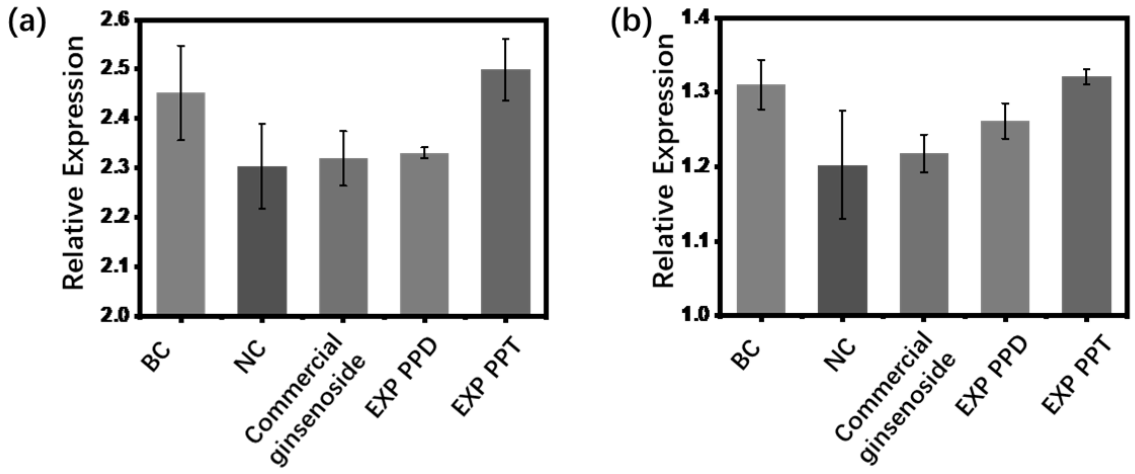


Figure 3. Relative expression of ASMT/AANAT: blank control (BC), UV damage negative control (NC), commercial ginsenoside, Exp-PPD and Exp-PPT

AANAT and ASMT are two essential proteases involved in the synthesis of melatonin, which has a feedback regulation on clock genes (CLOCK, BMAL1, PER, CRY). ROS resulted by UV light can significantly decrease the expression of AANAT as well as ASMT,

and therefore has quite negative impact on the melatonin synthesis and skin circadian rhythm. As shown in Figure 3, the NC control by UV damage shows much lower ASMT/AANAT expression compared to blank control (BC). The addition of a commercial ginsenoside sample has almost no impacts on restoring the ASMT/AANAT expression, whereas Exp-PPD and Exp-PPT ginsenosides, especially Exp-PPT can restore the expression of ASMT/AANAT, thereby promoting the production of melatonin.

In vivo study shows that after using Exp-PPD daytime serum and Exp-PPT night serum for 28 days, compared to the blank serum, the subject's eye wrinkle area was improved by 12.4%, skin elasticity was improved by 14%, the skin firmness was improved by 10.4%, skin color (ITA° value) was improved by 7.8% and skin radiance improved by 6.2%. These results suggest that using the right type of ginsenosides, specifically PPD-type at day and PPT-type at night, can improve the use of ginsenosides performance in the skin care application.

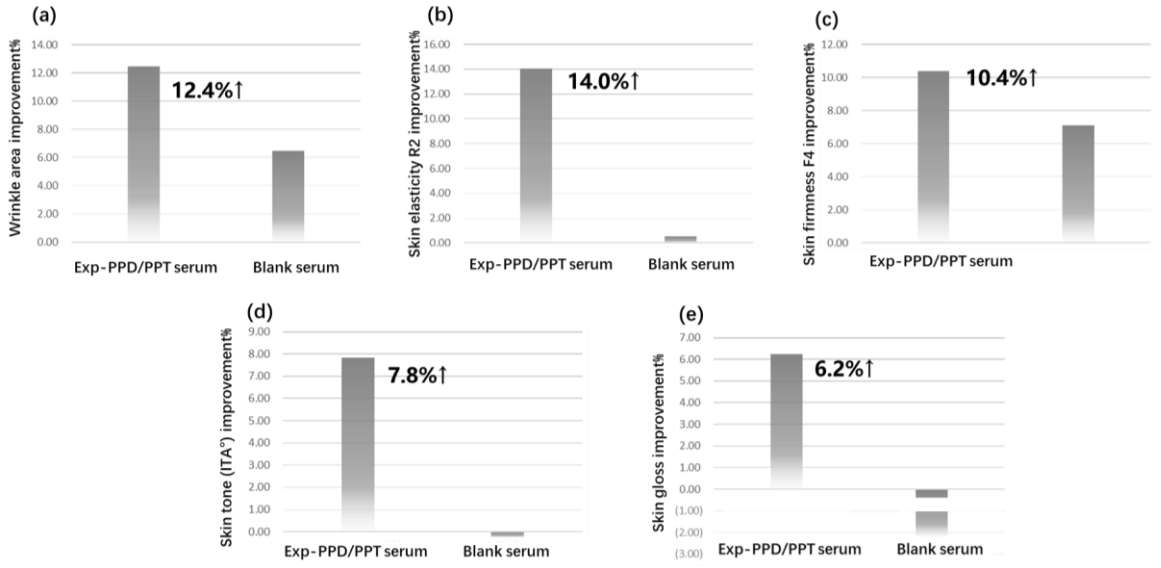


Figure 4. In-vivo skin care performance evaluation (N=8, 28 days) (a) wrinkle area improvement% (b) skin elasticity improvement% (c) skin firmness improvement% (d) skin tone (ITA°) improvement% (e) skin gloss improvement%

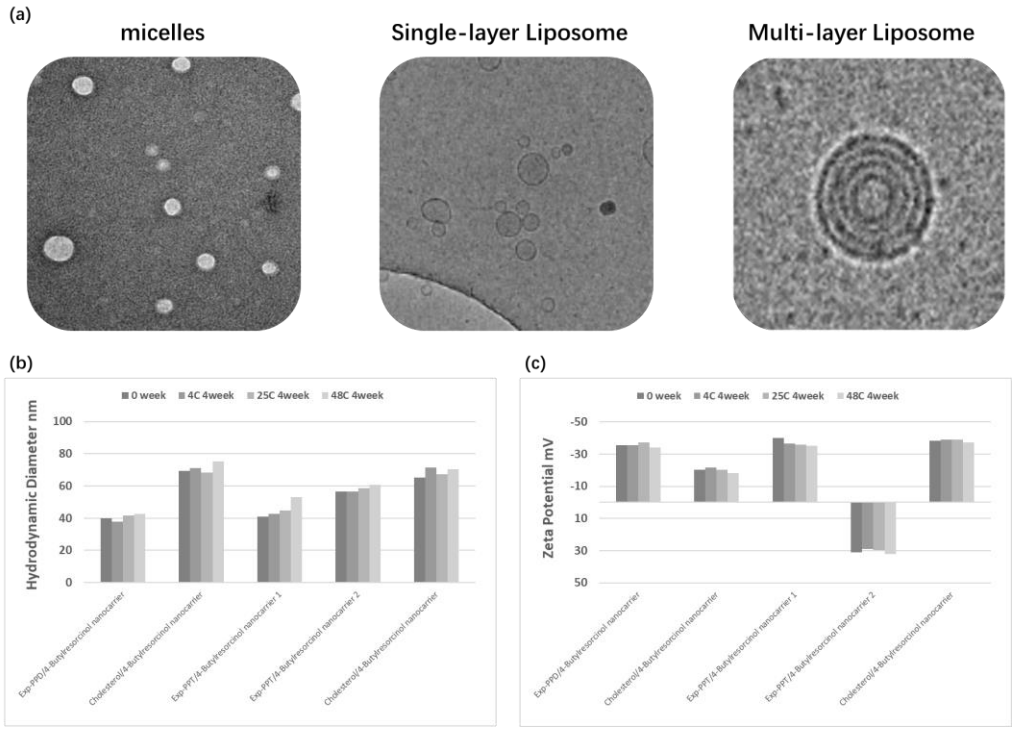


Figure 5. (a) Cryo-TEM images of representative nanocarriers with ginsenoside "shell" including micelles, single-layer liposome and multi-layer liposome. Stability challenge results of model nanocarriers discussed in this study (b) hydrodynamic size (c) zeta-potential

*Characterization of nanocarrier with Ginsenoside "shell":*

Ginsenoside was used as the "shell" materials to replace traditional substance such as cholesterol to form nanocarriers to encapsulate the model actives. The comparison was all made at an equal-molar concentrations of the cholesterol which concentration has been optimized from previous studies. The preparation follows the preparation methods described previously. The hydrodynamic size and zeta potentials were tracked at various challenge conditions (4°C, 25°C, 48°C, and 5 cycles of F/T) for a month to ensure the nanocarriers' stability and quality. As shown in Figure 5, the prepared model Exp-PPD/4-Butylresorcinol nanocarriers, Exp-PPT/CoQ10 nanocarriers 1, 2, and benchmarking cholesterol based nanocarriers are all with good stabilities. These results imply that Exp-PPT and Exp-PPD can be used as the "shell" material to effectively help stabilizing the nanocarriers and encapsulating water-insoluble and water-soluble actives.

*Advantages of Nanocarrier with Ginsenoside "shell":*

Transdermal permeation efficiency: The ginsenosides nanocarriers show significant advantages over cholesterol nanocarriers for transdermal permeation efficiency of encapsulated model actives. As shown in Figure 6, with the same 4-Butylresorcinol concentration, the Exp-PPD/4-Butylresorcinol nanocarrier has much larger accumulative transdermal permeation of 4-Butylresorcinol than "free" unencapsulated 4-Butylresorcinol as well as cholesterol/4-Butylresorcinol nanocarrier. This results clearly demonstrate the advantage of this novel ginsenoside "shell".

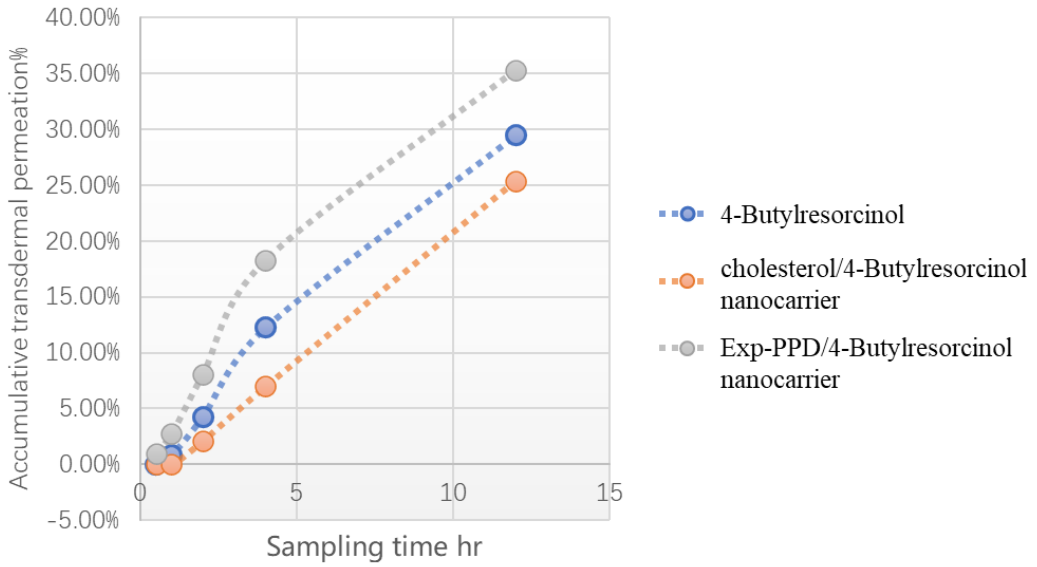


Figure 6. transdermal permeation efficiency by franz cell

Bioactivity enhancement: The ginsenosides nanocarriers also show significant advantages over cholesterol nanocarriers for bioactivities enhancement of encapsulated model actives. As shown in Figure 7 (a) and (b), the Exp-PPT/4-Butylresorcinol nanocarrier is effective for inhibiting tyrosinase activity and reducing the melanin content in cell models with and without α-MSH, better than unencapsulated 4-Butylresorcinol and cholesterol nanocarriers. Similarly, Exp-PPT/CoQ10 nanocarriers (1 and 2) show better anti-aging (reduction of MMP-1) and anti-inflammation (reduction of TNF-α, PEG2 etc.).

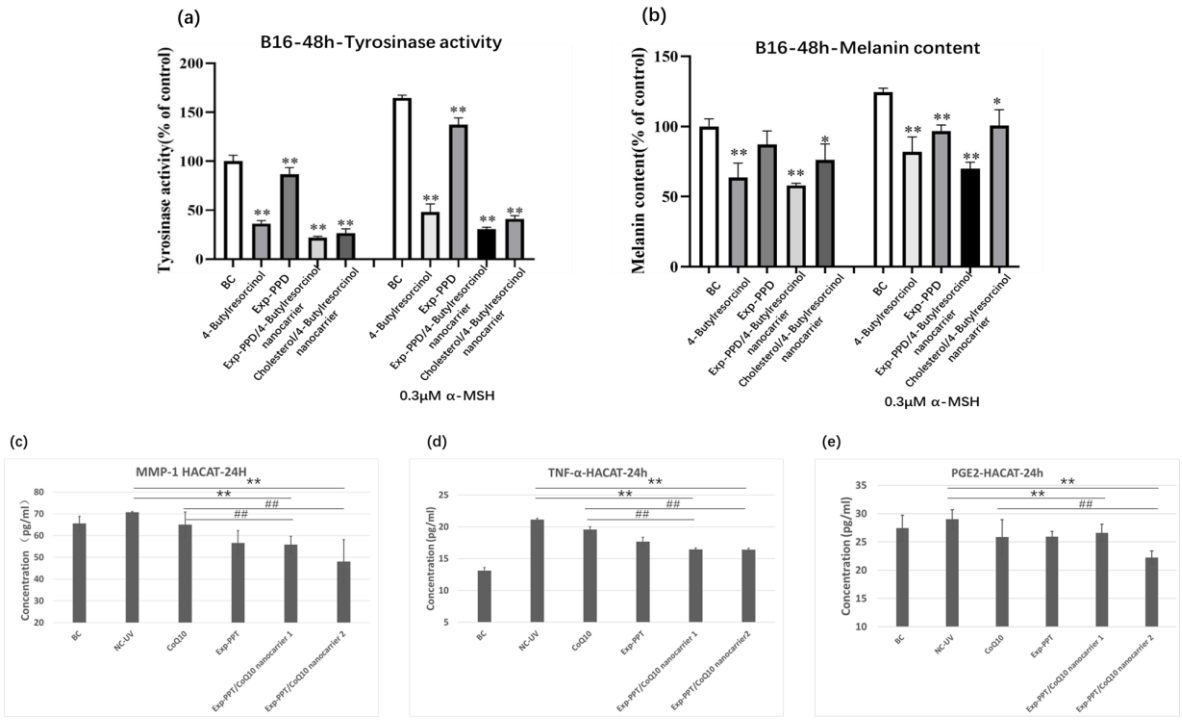


Figure 7. In-vitro models to demonstrate the Exp-PPD or Exp-PPT nanocarriers' improvements on active bioactivities than "free" actives and traditional cholesterol nanocarriers: (a) Tyrosinase activity inhibition and (b) Melanin content reduction (c) MMP-1 inhibition after UV damage (d) and (e) anti-inflammation of TNF-α, PGE2 reduction

Skin barrier & Soothing: Furthermore, as shown in Figure 8 (a), the Exp-PPT/CoQ10 nanocarriers show surprisingly performance on improving the skin barrier function such as promoting FLG production 140%, compared to free CoQ10 at the same concentration (5μM). Figure 8 (b) demonstrates the soothing benefits of the Exp-PPT/CoQ10 nanocarriers using a capsaicin model, compared to free CoQ10 and Exp-PPT alone. These results suggest that the Exp-PPT/CoQ10 nanocarriers can help improving the skin barrier function and soothing skin irritation.

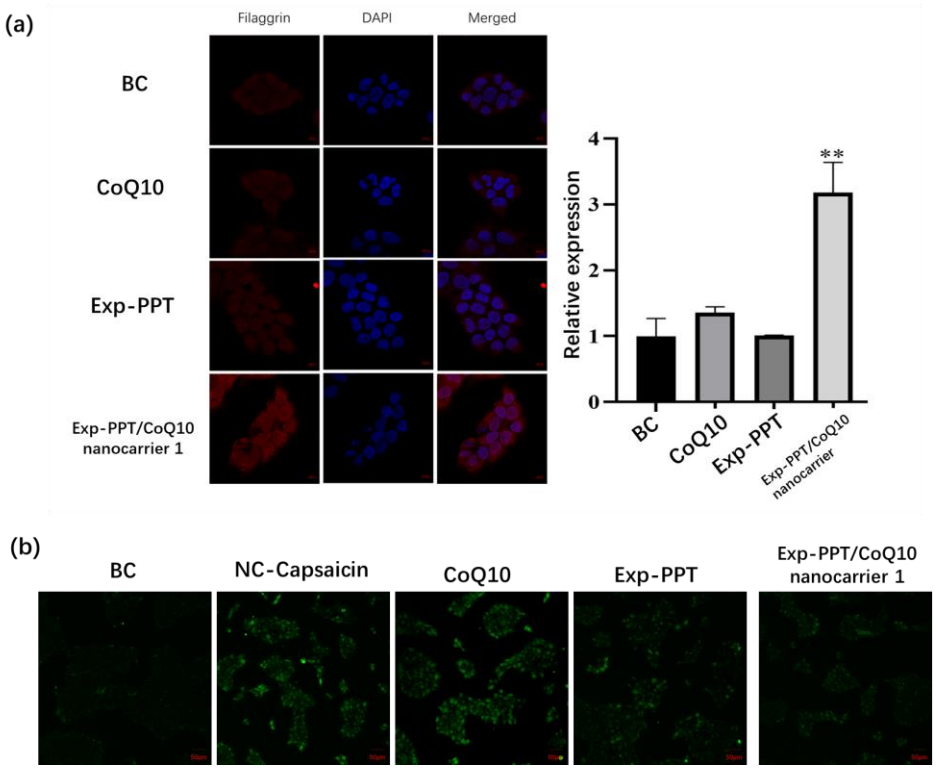


Figure 8. In-vitro models to demonstrate the Exp-PPT/CoQ10 nanocarriers' improvements on (a) skin barrier and (b) soothing of Capsaicin

**Conclusion.**

In summary, we report a novel nanocarriers comprised of a unique ginsenoside "shell" for regulating circadian rhythm skin care: Exp-PPD ginsenoside nanocarriers for daytime skin care and Exp-PPT ginsenoside nanocarriers for night skin care, exclusively. As demonstrated by Figure 9, these nanocarriers show excellent properties and performance for skin care applications. This study provides an interesting pathway of better utilizing ginsenosides for advanced skin care applications that Ginseng Loves Your Skin Day and Night, and ginsenosides "shell" protect and improve the functional actives.

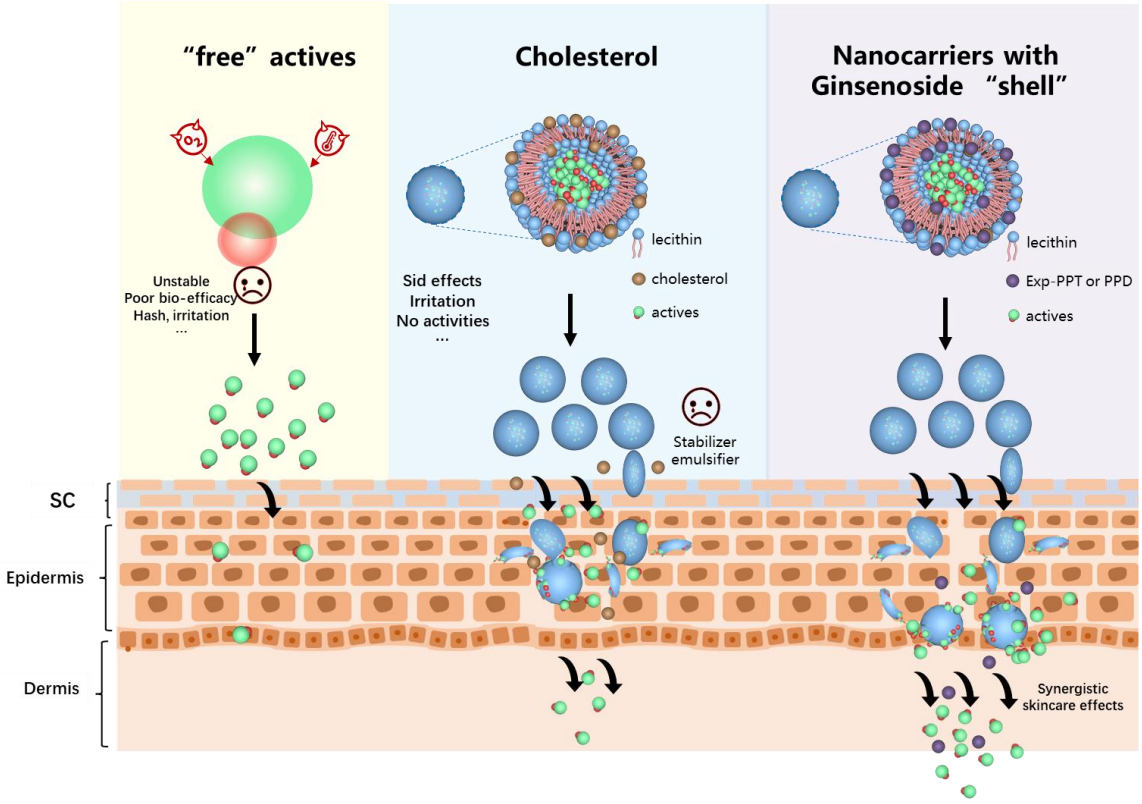


Figure 9. A schematic cartoon illustrating the advantages of nanocarriers with a ginsenoside "shell"

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**Conflict of Interest Statement**. The authors declare no competing financial interest.

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