Triple protection delivery system for retinal: Preparation, skin penetration and controlled release at the hair follicles.

P. Supmuang* and S. Wanichwecharungruang**

*Program in Biotechnology, **Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand. E-mail: psupason@chula.ac.th

ABSTRACT

To continuously supply skin with vitamin A, nanoparticles which can penetrate into the skin via hair follicles and slowly release un-degraded retinal, were designed and fabricated. The nanoparticles were prepared by covalently linking retinal to succinylchitosan and then allowing the obtained retinilidenesuccinylchitosan (RSC) to self-assemble into nanoparticles in water. Loading of the antioxidants, butylated hydroxytoluene (BHT) and vitamin E (vitE), individually into the RSC particles was also carried out to further improve the stability of the grafted retinilidene moieties in the particles. At the same loading level, vitE was more effective than BHT in protecting the retinilidene moieties from being degraded by a combination of light, heat and oxygen exposure. The vitE-loaded RSC particles possessed three potential protection barriers for the retinilidene moieties, that is (i) a chemical barrier via the imine bond, (ii) a physical barrier from the chitosan matrix around the retinilidene core, and (iii) an antioxidative barrier of the vitE which was confined within the grafted vitamin A derivatives. Covalently labeling the RSC particles with the fluorophore rhodamine B was carried out and used with confocal fluorescent microscopic analysis to reveal the ex-vivo penetration of the particles into the epidermis and dermis of porcine ear skin via hair follicles, followed by the clear release and diffusion of retinal from the particles within the tissue.

Keywords: retinal, chitosan, imine, skin penetration

1. INTRODUCTION

Vitamin A is essential for normal skin-cell proliferation and regulation [1]. Both retinol and retinal can be converted to retinoic acid, an active form of vitamin A, in the human body. Vitamin A and its derivatives have been used for the treatment of acne, rosacea and seborrheic dermatitis [2, 3]. The compounds can increase the epidermal thickness, decrease wrinkling, roughness, and uneven melanin synthesis in human skin, and so they are popularly used in cosmetics [4-6]. Since UVB and UVA radiation can reduce the vitamin A content in the human epidermis [7, 8], and so a continuous supply of un-degraded vitamin A to the skin would be beneficial. However, retinal and other forms of vitamin A possess a serious instability problem. Reported attempts to improve their stability have included both chemical derivatization [9] and physical encapsulation [10-13] strategies. Nevertheless, up until now, no system offers a suitable stability for the cosmetic use of vitamin A derivatives, where a long shelf-life is typically required.

Chitosan (CS) is a naturally derived non-toxic, biocompatible and biodegradable polymer that possesses an anti-bacterial activity on its own, and its applications in drug delivery are well documented. Here, we demonstrate the use of CS as a polymer base for the preparation of a controlled release system for retinal with triple protection barrier. Stability of retinal in the new delivery system was evaluated, along with the ex-vivo ability to permeate into porcine ear skin and subsequently release the retinal into the dermis.

2. MATERIAL AND METHODS

2.1 Synthesis of retinilidene-succinylchitosan (RSC)

N-Succinylchitosan (SC) was synthesized from chitosan (CS, degree of deacetylation of 85%, Mw = 30 kDa, Seafresh Chitosan Lab Co., Ltd., Thailand) based on the method of Tree-Udom [14]. The synthesis of RSC was carried out at a 3:1 (mol) ratio of the monomeric unit of SC to retinal, and is schematically shown in Scheme 1.

Retinilidene-succinylchitosan (RSC): Yellow powder.

Degree of retinal substitution: 0.20. ATR-FTIR (cm⁻¹): 3289 (N-H str and O-H str), 2872 (C-H str), 1645 (C=O str), 1565 (N-H bend/O-H Deff), 1147 (C-N str), 1023 (C-O str).

2.2 VitE and BHT encapsulation

The encapsulation was carried out by dropping the solution of the mixture of retinal (Sigma-Aldrich, 15.86 mg) and α-tocopherol (Sigma-Aldrich, vitE; 10.57 mg) (in 1.0 ml ethanol) into the aqueous SC particle suspension (46.28 mg, 19 ml water) under ultrasonic (40 kHz at 5 °C), light-proof and N2 atmospheric conditions. The mixture was then continuously sonicated for another 4 h under the same conditions. Dry particles were obtained by freeze drying the aqueous suspension. The product was then subjected to ATR-FTIR and UV-Vis spectrophotometric analysis. BHT encapsulation was carried out similarly except that the vitE was replaced with BHT.

2.3 Stability of the retinilidene moieties

The stability of the grafted retinilidene moiety in the vitE-loaded RSC, BHT-loaded RSC and unloaded RSC nanoparticles was evaluated in comparison with the free
retinal, under in vitro accelerated degradation conditions. A freshly prepared aqueous suspension (50 ml) of vitE-loaded RSC, BHT-loaded RSC or unloaded RSC particles (46.28 mg of SC which corresponds to 15.86 mg of retinal), as well as free retinal were incubated for 5 h at 40 °C in sunlight (UVA and UVB intensity of ~ 3 – 5 and ~ 0.10 – 0.15 mW/cm², respectively) and with continuous air exposure through stirring at 300 rpm. The degradation of the retinal and retinilidene moiety was quantitatively monitored through the decrease in the absorbance at 350 and 372 nm, respectively, by taking a 5 ml aliquot after 0, 30, 60 and 120 min. In the case of free retinal, ethanol was added to aid the solubility. The experiments were carried out in triplicate.

![Scheme 1. Synthesis of retinilidenesuccinylchitosan (RSC).](image)

2.4 Fluorescence labeled RSC particles

RhoB was added at 0.01% by weight into the aqueous SC particle suspension (50 ml) under a light-proof and N₂ atmosphere and the mixture was left to stand overnight at 25 °C. The RhoB-labeled SC particles were then purified from free RhoB by dialysis against water under light-proof condition. Then, the RhoB-labeled SC was grafted with retinal as described.

2.5 Morphology of the nanoparticles

The aqueous suspensions of the SC, RSC, vitE-loaded RSC, BHT-loaded RSC, RhoB-RSC and vitE-loaded RhoB-RSC nanoparticles were evaluated for their hydrodynamic diameter and zeta potential by dynamic light scattering (DLS) analysis (Zetasizer nanoseries S4700, Malvern Instruments, UK). The dry particles were also subjected to scanning electron microscopy (SEM, JSM-6400, JEOL, Japan), and transmission electron microscopy (TEM, JEM-2100, JEOL, Japan).

2.6 Skin permeation

Fresh porcine ear skin from a one month old White Large piglet was purchased from a local slaughterhouse and was used within 6 h of execution of the donor.

Confocal laser scanning fluorescence microscopy (CLFM) was performed on a Nikon Digital Eclipse C1-Si equipped with Plan Apochromat VC 100x, Diode Laser and 85 YCA-series Laser (405 nm and 561 nm, respectively, Melles Griot, USA), a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software.

To evaluate the ability of the vitE-loaded RhoB-RSC nanoparticles to penetrate into the skin, 15 µL of the aqueous suspension of vitE-loaded RhoB-RSC nanoparticles (3085 ppm RhoB-RSC; equivalent to 1057 ppm retinal) were applied onto a 1.5 x 1.5 cm piece of the pig ear skin. The final coverage of RhoB-RSC, retinilidene (retinal) and vitE on the skin was approximately 20.7 and 4.7 µg cm⁻², respectively. The skin piece was massaged for 2 min and then left at room temperature for another 30 min before being subjected to CLFM analysis. The obtained spectra of each pixel were then unmixed into RhoB, retinilidene plus retinal, vitE, skin auto-fluorescence and hair auto-fluorescence components using chemometric analysis (image algorithms), and images indicating the locations of RhoB-RSC, retinilidene/retinal moieties and vitE in the skin tissue were then constructed using the obtained resolved signals.

3. RESULTS AND DISCUSSION

3.1 Retinilidenesuccinylchitosan (RSC)

SC was successfully synthesized by reacting CS with succinic anhydride and structural characterization was carried out as previously described [14]. The degree of succinyl grafting was approximated to be 0.15. Retinal was then grafted on to SC via imine linkage to obtain RSC (Scheme 1). The imine functionality was speculated through the appearance of the new absorption peak at 1620 cm⁻¹ (C=N str) and the dissapearance of the 1709 cm⁻¹ (C=O str of aldehyde) in the FTIR spectrum of the mixture of SC and retinal. The presence of the retinilidene moieties was further confirmed with the regeneration of aldehyde functional group (observed as absorption peak at 1709 cm⁻¹ in FTIR spectrum of the hydrolyzed RSC) upon the acid hydrolysis of the RSC. The UV absorption spectrum of the RSC preparation (Figure 1) showed the retinilidene absorption band at 352 nm, where the blue shift of absorption from a λmax of 375 nm to 352 nm indicates the change from retinal to retinilidene moieties. The degree of imine substitution of 0.20 was estimated by hydrolyzing the RSC with 0.1 M HCl, followed by partitioning the mixture with ethyl acetate and quantifying the released retinal in the ethyl acetate fraction using UV-vis spectroscopy with the aid of the calibration curve.

3.2 Particle formation

In water, RSC automatically self-assembled into water disperisible particulates. SEM and TEM images of the particles indicated a spherical morphology with an average dry particle diameter of 240 ± 28.81 nm (Figure 2b), while in water, the hydrodynamic diameter derived from DLS analysis was approximately 943 ± 5.6 nm (Table 1). This large size difference (>3.9-fold) between the hydrated and
anhydrous particle sizes indicates the severe swelling of the particles in water. It was expected that during the self-assembling process, the hydrophobicity of the retinilidene moieties would drive these moieties to be at the core of the forming spheres, away from the hydrophillic water medium, while the succinyl moieties and the hydroxyl moieties on the glucosamine rings would be at the surface of the spheres in full contact with water molecules. The obtained RSC particles were, therefore, dispersible in water.

**Figure 1.** UV-visible absorption profiles of succinylchitosan, retinal and retinilidenesuccinylchitosan.

![UV-visible absorption profiles](image)

**Figure 2.** SEM images of (a) SC, (c) RhoB-RSC and (d) vitE-loaded RSC particles, and TEM images of (b) RSC and (inset of d) vitE-loaded RSC particles.

3.3 VitE/BHT encapsulation and stability of retinilidene

To further protect the hydrophobic retinilidene moieties inside the RSC particles, two hydrophobic antioxidants, BHT and vitE, were encapsulated (separately) into the RSC particles. A loading of ~ 7.5% (by wt) could be obtained for both the BHT-loaded RSC and the vitE-loaded RSC particles. Whilst the zeta potential of the RSC particles was significantly greater (~2.4-fold) than the SC particles, and exceeded 20 mV, it was not significantly altered by the incorporation of either vitE or BHT (Table 1). Thus, these nanoparticles are likely to be relatively stable from agglomeration upon aqueous storage. Although the dry sizes of the BHT- and vitE-loaded RSC particles were much smaller (1.33- and 1.47- fold, respectively) than the unloaded RSC particles, the hydrodynamic diameters were only a little smaller (1.06- and 1.19- fold, respectively) (Table 1). The hydrophobicity of the loaded vitE or BHT thus likely made the particles smaller by increasing the hydrophobic interaction at the particles’ cores. As such, the confinement of the hydrophobic antioxidant molecules at the inside of the particles should help maximize their close proximity to the labile retinilidene moieties, and therefore should help protect the retinilidene moieties from being oxidized by adjacent reactive oxygen species (ROS).

**Table 1.** Spherical characters of the particles.

<table>
<thead>
<tr>
<th>Particles</th>
<th>Dry diameter (nm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>181.6 ± 8.7</td>
<td>572 ± 8.4</td>
<td>12.2 ± 1.8</td>
</tr>
<tr>
<td>RSC</td>
<td>240.1 ± 29</td>
<td>943 ± 8.4</td>
<td>29.7 ± 0.20</td>
</tr>
<tr>
<td>BHT-loaded RSC</td>
<td>180.4 ± 9.3</td>
<td>888 ± 1.6</td>
<td>28.8 ± 0.90</td>
</tr>
<tr>
<td>VitE-loaded RSC</td>
<td>162.4 ± 20</td>
<td>791 ± 10</td>
<td>25.5 ± 0.80</td>
</tr>
<tr>
<td>VitE-loaded RhoB-RSC</td>
<td>684.72 ± 50</td>
<td>883 ± 13</td>
<td>18.9 ± 0.50</td>
</tr>
</tbody>
</table>

The stability of retinilidene moieties in the particles suspended in water for up to 4 h under accelerated degradation conditions of combined heat, light and air (oxygen) exposure are shown in Figure 3. Under these conditions, the retinilidene moieties in the RSC were significantly more stable than the free retinal in the aqueous medium. In addition, a further improved stability was observed in the vitE-loaded RSC followed by that in the BHT-loaded RSC particles over that already afforded by the RSC particles (Figure 3). Thus, after 60 min under the acclererated degradation conditions, the vitE-loaded RSC showed an 80% retention level of intact retinilidene moieties while 90% of the free retinal had already been degraded. This improved retinilidene chemical stability implies the potential possibility for a better application of this vitamin A precursor form in various applications including cosmetics.

**Figure 3.** A plot showing the stability of retinal or retinilidene in the four samples in an aqueous environment at 40 °C under sunlight and continuous air exposure.
3.4 Skin penetration

The ability of the vitE-loaded RSC particles to penetrate the epidermis and enter the dermis and to release the retinal from the penetrated particles was verified ex-vivo using pig ear skin. In order to locate the particles in skin tissue by CLFM, the RSC particles were covalently labelled with the RhoB fluorophore and then the obtained RhoB-RSC particles were loaded with vitE. The vitE-loaded-RhoB-RSC particles showed a broadly similar hydrodynamic size to that of the vitE-loaded RSC particles (Table 1), and so should be a reasonable model for the penetration of the actual vitE-loaded RSC particles given the hydrated environment. Likewise the reduced zeta potential of the vitE-loaded-RhoB-RSC particles is not expected to be problematic in the short time course of this ex-vivo assay.

Using CLFM, the fluorescent signals from the RhoB, vitE and the retinal/retinilidene components were easily located in the pig skin tissue and indicated that the vitE-loaded-RhoB-RSC particles could penetrate into the pig ear skin tissue via the hair follicles (Figure 4). Since the fluorescent signals from retinal, vitE and RhoB did not always show up at the same location, it was concluded that the retinal and vitE were released from the RhoB-labelled particles. In fact, by measuring the ratio of fluorescent intensity of retinal to that of RhoB and of vitE to that of RhoB, along the depth of the skin around the hair follicles, it was observed that the ratios increased with the depth of the skin. This clearly indicated the dissociation of retinal and vitE from the RhoB-labelled particles and subsequent diffusion through the tissue. The small hydrophobic retinal molecules seem to move faster into the deeper tissue compared to the larger RhoB-labelled RSC particles. Fluorescent signal of retinal/retinilidene could be detected even at the depth of 200 µm from the stratum corneum, thus implying penetration of such moieties into the dermis.

**Figure 4.** CLFM images showing the accumulation of vitE-loaded RhoB-RSC particles in the hair follicle and subsequent diffusion of the retinal (and vitE) into the dermis. Images show the (a) unresolved fluorescent image, and the separate images from fluorescent signal of (b) retinal/retinilidene moieties, (c) RhoB and (d) vitE.

4. CONCLUSION

Here the preparation, characterization and stability testing of a retinal delivery system is reported. The prepared vitamin A derivative (RSC) in the form of sub-micron diameter sized particles could be loaded with vitE or BHT and the vitE-loaded RSC particles showed a greater stability of the grafted retinilidene moieties to a combined treatment of heat, light and air exposure. This is likely to be due to the three protection barriers of (i) a chemical barrier *via* acid labile imine linkages between the aldehyde functionality of retinal and the amino group of CS, (ii) a physical barrier of the SC matrix around the retinilidene moieties and (iii) an antioxidative barrier of vitE (or BHT) around the retinilidene moieties. The stability of the retinilidene moieties in the vitE-loaded RSC particles was significantly improved compared to free retinal even when tested under a 5 h exposure to high heat (40 °C), sunlight and oxygen (as ambient air). The ex-vivo skin penetration of the vitE-loaded RSC particles using porcine ear skin indicated that the particles could permeate into the skin tissue *via* hair follicles and both the loaded vitE and the grafted retinal could be released from the particles. Thus, application of these stable vitamin A derivative particles in non-acidic cosmetics and in dermatological applications are potentially promising.

**Acknowledgements**

The authors thank the Commission of Higher Education-Thailand Research Fund; the STAR, centenary academic development project, Chulalongkorn University; and the Thai Government Stimulus Package 2 (TKK2555).

**References**