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Study on the mechanisms of chitosan and its derivatives used as transdermal penetration enhancers

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ABSTRACT

The efficacy of chitosan (CS) and its derivatives used as transdermal penetration enhancers has been confirmed in our previous research. This study investigated the mechanisms of penetration enhancement by CS and its derivatives, i.e., N-trimethyl chitosan (TMC) with different degree of quaternization (DQ) and mono-N-carboxylmethyl chitosan (MCC). After treatment with CS, TMCs or MCC, the secondary structure changes of keratin in stratum corneum (SC) from mice were examined by an Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) combined with the application of the second-order derivative, deconvolution and curve-fitting. The water content in the SC was also studied by ATR-FTIR. HaCaT cell lines were employed as the cell models in the study. HaCaT cells were first treated with blank D-Hanks solution, CS or its derivatives, and were then fluorescent labeled with $DiBAC_4$ (3). The change of membrane potential was measured by a flow cytometer (FCM). Alternatively, the treated HaCaT cells were labeled with NBD-C_6-HPC and the change of membrane fluidity was examined under a Confocal Laser Scanning Microscope (CLSM). It was found that CS, TMCs and MCC could significantly affect the secondary structure of keratin in SC in different ways. Although the amide II absorption peak of keratin moved to a lower wave number following treatment with CS, TMCs, or MCC, the β-turning structure of keratin was converted to β-sheeting and random coiling after treatment with TMCs and was converted to β-sheeting and α-helix following treatment with MCC and CS. At the same time, CS and its derivatives all could increase the water content of SC, decrease HaCaT cells membrane potentials and enhance HaCaT cells membrane fluidity significantly. The effect of TMCs appeared to be independent of their DQ. The results suggest that the mechanisms of transdermal enhancement of CS, TMCs and MCC are closely related to their effects on the secondary structure of keratin and water content in SC, cell membrane potential and fluidity.

1. Introduction

Chitosan (CS) is the only polycationic polysaccharide in nature which attracts much interest in pharmaceutical applications. Due to its poor solubility at physiological pH (pH above 6.5), several derivatives of CS have been synthesized which are soluble in a wider pH range. Among them, N-trimethyl chitosan (TMC) and mono-N-carboxymethyl chitosan (MCC) are most frequently studied and used because of their well-defined structures, improved solubility and easy preparation.

Many studies have shown that CS, TMC and MCC could significantly enhance drug absorption across mucosa epithelia (Thanou et al., 2001; Hamman et al., 2002; Jonker et al., 2002; Sinswat and Tengamnuay, 2003; Di Colo et al., 2004; Giuseppina et al., 2005) and the mechanisms have also been clearly elucidated (Junginger and Verhoef, 1998; Kotze et al., 1999; Thanou et al., 2000a,b). However, few studies have been done on the transdermal enhancement of CS and its derivatives. Stratum corneum (SC) of skin is the main barrier against drug transdermal penetration. Although the composition of SC is very different from that of epithelial cells, which is composed of dead keratinized cells and fibrosis protein, SC also has fixed negative charges in the tight junction between cells which are similar to those found in epithelial cells (Hamman et al., 2002). It is therefore reasonable to speculate that CS and its derivatives could be potential transdermal penetration enhancers effective in Transdermal Drug Delivery Systems (TDDS). Recently, it was reported that CS appeared to interact with negative charges in the skin to improve drug diffusion to deeper skin layers (Taveira et al., 2009). Our previous study had also shown that TMC could prominently increase the drug transdermal penetration *in vitro* and *in vivo* (Wen et al., 2008). Despite the above studies, the mechanisms of the transdermal enhancement of CS and its derivatives have not been elucidated.

In this study, after treatment with CS, TMCs or MCC, the secondary structure changes of keratin in stratum corneum (SC) from mice were examined by an Attenuated Total Reflection-Fourier

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Transform Infrared (ATR-FTIR) combined with the application of the second-order derivative, deconvolution and curve-fitting. The water content in the SC was also studied by ATR-FTIR. Also, the water content in SC was studied after treatment by CS and its derivatives using ATR-FTIR determination. HaCaT cells were attempted to be employed as a cell model to study the effect of CS and its derivatives on cell membrane potential and fluidity. Through those studies, the mechanisms of CS and its derivatives used as transdermal penetration enhancers were tried to be explained somewhat.

2. Materials and methods

2.1. General chemicals and polymers

CS (Mw 210 kDa, DD > 95%) from a shrimp shell was bought from Haipu Biotechnology Co. Ltd. (Qingdao, China). Fluorescent probes used were $DiBAC_4$ (3) ([bis-(1,3-dibutylbarbituric acid) trimethine oxonol]) and NBD-C6-HPC ([2-6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino hexanoyl-1-hxadecanoyl-sn-glyxero-3-phosphochline]). All probes were purchased from Invitrogen Incorporation (USA), received in solid form and solubilized in absolute ethanol. All the other chemicals were of analytical grade and used without further purification.

2.2. Mice

Healthy male Kunming species mice weighing 20 ± 2 g were supplied by the Experimental Animal Breading Center of Medical College of Wuhan University. All of the procedures for animal experimentation were performed according to approved protocols and in accordance with recommendations of the NIH guideline for the proper use and care of laboratory animals.

2.3. Cell line

HaCaT cell lines were gifted from the Laboratory of Molecular Biology of Dermatology department (Renmin Hospital of Wuhan University, China). The cells were grown in DMEM, supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Cells were maintained within their exponential growth phase.

2.4. Synthesis and characterization of TMCs

TMCs with degree of quaternization (DQ) of 20% (TMC20), 40%(TMC40), and 60% (TMC60) were synthesized by varying the number of times and durations of reaction steps as reported by Snyman et al. (2001). Briefly, TMCs were synthesized by reductive methylation of CS through a chemical reaction between CS and iodomethane in the presence of sodium hydroxide. The reaction step was repeated several times with the product obtained from each step to increase the DQ of the TMCs.

All TMCs were characterized by ^{1}H NMR. The products were measured in D_2O at 80 °C, using a 600-MHz spectrometer (Varian unity Inova, USA). The DQ of the synthesized TMCs was calculated with the following equation (Hamman and Kotze, 2001): $DQ(\%) = [(\int TM/\int H) \times (1/9)] \times 100$, where $\int TM$ is the integral of the trimethyl amino group (quaternary amino) peak at 3.3 ppm and $\int H$ is the integral of the ^{1}H peaks from 5.0 to 6.0 ppm.

2.5. Synthesis and characterization of MCC

MCC was synthesized as reported by Di Colo et al. (2004). Briefly, CS was dissolved in 0.7% (v/v) acetic acid solution. Following filtration to remove traces of non-dissolved material, glyoxalic acid was added and the mixture was stirred at room temperature for 90 min. The reaction pH was then brought to 4.5 by addition of 1 M NaOH over 30 min, after which stirring was continued for another 90 min to yield a polymer of imine. Subsequently, the imine was reduced by adding dropwise 5% (w/v) aqueous solution of sodium borohydride and leaving the mixture under stirring at room temperature for 1 h. The polymer was then precipitated by an excess of ethanol and collected by filtration under vacuum. After washing with ethanol and air drying, the product was pulverized by a ball-mill, and then subjected to a second carboxymethylation step using the same procedures as described above. The product was converted into its sodium salt and the solution (pH 10.5) was lyophilized.

MCC was characterized by IR spectroscopy and alkalimetry (Riccardo et al., 1982). 0.2 g MCC was dissolved in 60 ml 0.1 M HCl solution followed by adding pure water to 100 ml. 20 ml sample solution was then taken out to adjust pH to 2.0 with 0.1 M HCl solution and titrated with 0.1 M NaOH solution. Thus the titration curve was constructed.

2.6. Preparation of SC

Mice skin was obtained promptly after post-mortem and carefully shaved with a razor after the removal of hair by electric clippers. Epidermis was gently peeled off by trimming off subcutaneous fat and immersing full thickness skin samples in water. SC sheets were obtained by floating freshly prepared epidermis on an aqueous solution of 0.0001% trypsinase and 0.5% sodium bicarbonate for 24 h. Digested material was removed from the underside of the SC with tissue paper and the isolated sheets were rinsed in acetone for 30 s to remove any sebaceous or subcutaneous fat contamination, cryodesiccated and stored over silica gel under vacuum (Daughety and Mrsny, 1999).

2.7. ATR-FTIR investigation of SC samples

6 mm × 6 mm pieces of dry SC were incubated for 24 h in 3 ml of the respective formulation at room temperature, where the formulation was 2.5% TMC60, 2.5% TMC40, 2.5% TMC20, 2.5% MCC, 2.5% CS and the blank solvent (used as control), respectively. The CS, CMCs and MCC were solubilized in solvent of 100 ml 1% (v/v) acetic acid and then adjusted pH to 6.0 with 0.1 M NaOH solution. Thereafter the SC sheets were cleaned carefully with distilled water to remove all the residual solvent on the SC surface. After cryodesiccation, spectral measurements were made with a Nicolet FTIR 5700 Fourier Transform Infrared Spectrometer (Thermo Nicolet, USA) equipped with a ATR attachment under the following conditions: IR reflection crystal of a diamond monocrystal, scanning temperature range of 18–20 °C, scanning times of 64, resolution of 4 cm^{-1}, and scanning range of 800–4000 cm^{-1}. The curves in each group were recorded and analyzed by the second-order derivative, deconvolution and curve-fitting.

2.8. Determination of water content in SC by ATR-FTIR

6 mm × 6 mm pieces of dry SC were incubated at room temperature in 3 ml of the respective formulation same as described above. At predetermined time intervals (0, 2, 4, 6, 8, 12 h), SC samples were taken out and cleaned carefully with distilled water to remove the residual solvent on the surface. After removing the residual water on the surface of SC with filtering paper, the water content in SC was examined by ATR-FTIR under the conditions described above. The ratio of amide I absorption peak and amide II absorption peak was calculated to define the water content in SC.