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Original Article

DEVELOPMENT OF JACALIN CONJUGATED NANOSTRUCTURED LIPID CARRIERS FORMULA FOR TRANSCUTANEOUS VACCINE DELIVERY

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ABSTRACT

Objective: Most of transcutaneous vaccines consist of protein or peptide antigens having poor immunogenicity, high molecular weight, and hydrophilic properties. These features become the major challenge in developing a non-invasive vehicle for targeting to skin antigen presenting cells (APCs). This research aimed to develop a transcutaneous vaccine formula in the form of nanostrucured lipid carriers (NLCs).

Methods: NLCs were prepared by emulsification and solidification method. Bovine serum albumin (BSA) was used as antigen protein model, which was incorporated into lipophilic phase as solid dispersion form in PEG 20000. NLCs were constructed from a mixture of Suppocire® NA and oleic acid as lipophilic phase and stabilized by Lutrol® F 68 as surfactant and ethanol as cosurfactant. Jacalin, a lectin isolated from jackfruit (*Artocarpus integrifolia)* seed used as an adjuvant, was conjugated on the surface of BSA-loaded NLCs under acidic condition by utilizing electrostatic interaction.

Results: The average particle size of the resulting dispersion was 382.3±10.80 nm with a polydispersity index of 0.159±0.02 and zeta potential of -2.96±0.71 mV. BSA entrapment efficiency in NLCs was higher than 80%. TEM observation revealed the NLCs spherical particles with un-aggregated BSA dispersed homogenously. Hemagglutination test indicated that jacalin was adsorbed onto the surface of BSA-loaded NLCs as active form. The physical stability evaluations including determination of average particle size, size distribution, and zeta potential showed relatively stable nanoparticles during 4-week storage.

Conclusion: The NLCs obtained having high payload of protein and surface anchored jacalin is promising as a transcutaneous vaccine platform.

Keywords: NLCs, Transcutaneous vaccine, Jacalin, Adjuvant, Microemulsion, Co-lyophilization, Protein.

INTRODUCTION

Nowadays, most vaccines are administrated via injection route that has many shortcomings, such as invasive use of needles, requirement for trained personnel, and problems related to storage and transportation. Skin is a promising route for vaccination due to abundance of antigen presenting cells (APCs) such as Langerhans cells (LCs) in the epidermis and dendritic cells (DCs) in the dermis that can deliver the antigens and initiate transcutaneous immunization [1]. Most of transcutaneous vaccines consist of protein or peptide antigens. However, the high molecular weight and its hydrophyllicity can be the major obstacle for transcutaneous administration [2]. Various transcutaneous vaccine delivery systems such as PLA nanoparticles [3], hydrogel patch [4], liposome [5], transfersome, ethosome and cubosome [6] have been developed.

In recent years, nanostructured lipid carriers (NLCs), the second generation of solid lipid nanoparticles (SLNs), have received increasing attention because of their favorable features, such as feasible incorporation of lipophilic and hydrophilic drugs, possibility of controlled drug release and drug targeting, increased drug stability, and high drug payload [7,8]. It has been reported that coenzyme Q10-loaded SLNs and NLCs increased the skin penetration compared to nanoemulsion [9]. Another research showed that NLCs increased skin permeation, sustained the release and optimized antifungal effect of miconazole nitrate [8]. Owing to these characteristics NLCs will be potential for enhancing skin permeation of protein antigens via transcutaneous application.

Another problem for the use of subunit protein antigens is the fact that they show poor immunogenic effect. Black et al reported that many peptide subunit antigens need delivery with immunestimulatory adjuvant to prove that they are potential as vaccine candidates [10]. The use of adjuvant to enhance, prolong and accelerate immune response has been reported by several researchers [11]. This research was proposed to provide an effective delivery system for antigen and adjuvant simultaneously. One of potential adjuvant for transcutaneous vaccine is jacalin. Jacalin is a lectin isolated from jackfruit *(Artocarpus integrifolia)* seed, which showed adjuvant effect on humoral immune response against the trinitrophenyl (TNP) and *Trypanosoma cruzi* [12]. Furthermore, jacalin having specificity for Gal/GalNac [13, 14] can act as a potential candidate for DC targeting due to the presence of GalNAc on DCs' surface [15]. In this research, bovine serum albumin (BSA) was used as protein model and incorporated into lipid phase as solid dispersion form in PEG 20000 to increase its dispersibility in order to provide high entrapment efficiency. NLCs were further conjugated with jacalin on the surface by utilizing electrostatic interaction for optimizing APCs' targeting.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) was purchased from Sigma Aldrich. Jacalin was isolated from jackfruit seed using guar gum column as described by Laija et al. [16]. Suppocire ® NA was a generous gift from Kalbe Farma (Indonesia). Oleic acid was purchased from Bratachem (Indonesia). Lutrol® F 68 Poloxamer 188 was provided by PT. BASF Indonesia. PEG 20000 was purchased from Fluka. All other reagents were analytical grade.

Protein lyophilization

The formation of protein dispersion in amphiphilic polymer was proposed to form fine spherical particles of protein and avoid its aggregation during subsequent emulsification [17]. Solid dispersion of BSA in PEG 20000 was prepared by dissolving BSA in deionized water and mixed with aqueous solution of PEG 20000 in 1:2, 1:3, and 1:4 mass ratios. The mixture was then rapidly frozen in ethanol at -85 ^{o}C and lyophilized using Teltstar LyoQuest at a condenser temperature of -65 ^{o}C and a pressure of 0.211 mBar for 24 h. The optimum ratio of BSA to PEG 20000 was selected based on the success of particle size reduction of BSA. This optimum ratio was indicated by the formation of a clear co-lyophilisate dispersion

observed visually in oleic acid prior to emulsification process for encapsulation in NLCs.

Preparation of NLCs

NLCs were prepared by emulsification and solidification as reported by Kwon (2010) with modifications [18]. Aqueous solution of Lutrol® F 68 was warmed up at 38 °C and mixed with ethanol. Suppocire® NA and oleic acid containing dispersed BSA, used as lipid phase, were melted together at the same temperature with water phase. Then the aqueous phase was gently poured into the melted lipid phase and homogenized using Ultra Turrax T-25 (Ika Labor-Janke) for 20 minutes. The pre-emulsion obtained was probe-sonicated (Virtis VirSonic 300, A=30 %) for 5 minutes to decrease the globule size. Finally, the warm microemulsion was diluted ten times in cold deionized water (5 ± 2 °C) in order to obtain NLCs. The NLCs particles were collected by centrifugation at 12000 rpm.

Jacalin conjugation onto NLCs particles

Jacalin was conjugated on the NLCs particles by utilizing electrostatic interaction. Jacalin was dissolved in buffer pH 5.0 and NLCs suspension was also adjusted at the same pH. In order to adsorb jacalin onto the surface of NLCs, jacalin solution was added to NLCs suspension with gentle stirring for 6 h. After collecting by centrifugation and rinsing using 3% Lutrol® F 68, NLCs were then subjected to lyophilization for 24 h. Diagram of jacalin conjugated BSA-loaded NLCs (JCBN) preparation conducted in this research is depicted in Fig. 1.

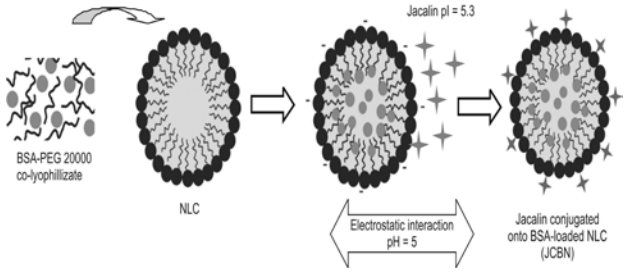

Fig. 1: Schematic of step by step preparation of jacalin conjugated BSA-loaded NLCs (JCBN)

Particle size measurement of NLCs

The average diameter and the polydispersity index of NLCs were measured by photon correlation spectroscopy (PCS) using a Delsa™ Nano C Particle Analyzer (Beckman Coulter) at a fixed angle of 90° and temperature of 25 °C.

Measurement of NLC zeta potential

The zeta potential was measured by electrophoretic light scattering using a Delsa™ Nano C Particle Analyzer (Beckman Coulter).

BSA entrapment efficiency within NLCs

The efficiency of BSA entrapment within NLCs was determined indirectly by quantifying untrapped BSA. This evaluation was applied on BSA-loaded NLCs without jacalin conjugation. Protein was quantified from free BSA that might contained in the supernatant resulted from the last step of NLCs preparation. The unentrapped BSA was precipitated using concentrated TCA solution up to 2% TCA in the final solution. The precipitate was washed with 2% TCA solution three times then dissolved in 1 ml deionized water.

Subsequently, 800 µL of unentrapped BSA solution was reacted with 200 µL Bradford reagent and incubated for 5 minutes. The absorbance was measured using spectrophotometer (Beckman DU 7500i) at 595 nm. The absorbance of NLCs without BSA treated using the same method was used as blank. The BSA concentration was calculated by comparing to a standard curve of known concentrations of standard BSA.

Transmission electron microscopy

The surface morphology of NLCs was examined by transmission electron microscopy. NLCs suspensions were mixed with ammonium molybdate and ammonium acetate, and then dropped onto carbon-coated copper grid. The air-dried samples were then directly observed under the transmission electron microscope (Jeol JEM 1400).

Jacalin activity measurement using hemagglutination activity test

Hemagglutination activity test was used to verify the completion of jacalin adsorption onto the surface of NLCs (concentration directly proportional to activity). The activity of jacalin conjugated on the surface of NLCs was estimated by comparing the minimal amount of lectin resulting in the last visible erythro-agglutination of human erythrocytes (O group) against the positive control (jacalin solution) and negative control (BSA-loaded NLCs without NLCs). Since activity is inversely proportional to minimal amount of lectin resulting in the last visible erythro-agglutination, the activity of JCBN was estimated according to following equation [19]:

$$\text{Activity of JCBN (\%)} = \frac{C_{\text{jac sol}}}{C_{\text{NLCs jac}}} \times 100\%$$

Where:

$C_{\text{jac sol}}$ = minimal amount of lectin in jacalin solution resulting in the last visible erythro-agglutination (µg/mL)

$C_{\text{NLCs jac}}$ = minimal amount of lectin in JCBN resulting in the last visible erythro-agglutination (µg/mL)

Stability study

Lyophilized NLCs powder was kept in glass vials and stored at temperature of 4 °C for 4 weeks. The particle size, polydispersity index, and zeta potential were evaluated weekly.

Statistical analysis

Statistical significance of stability data were assessed using one-way analysis of variance (ANOVA) with Tukey post test. P-values <0.05 were considered as significant. All the calculations were performed using a statistical software program Graph Pad In Stat, version 3.0 (Graph Pad Software Inc, San Diego).

RESULTS AND DISCUSSION

Optimization of NLCs formulations

In this study, NLCs were prepared using different ratios of Suppocire ® NA and oleic acid in Lutrol® F 68 and ethanol solution. The total amount of surfactant-cosurfactant admixture was varied in the range of 20-60% and the optimum result indicated by the formation of clear dispersion was only reached by 60% admixture. At concentration higher than 60%, viscosity of the pre-emulsion was very high that caused the difficulties in homogenization process. Therefore, 60% was chosen as the optimum surfactant-cosurfactant admixture for the following formulation developed. The next step was adding oleic acid into the lipid phase of Suppocire® NA pre-emulsion at varying concentration of 0, 5, and 10% with the lipid phase admixture was maintained at 3% within all formulations (F1 to F3). In order to produce comparable NLCs particle size using 60% of surfactant-cosurfactant admixture, the amount of Suppocire® NA in formulation F2 and F3 was adjusted to compensate the amount of oleic acid added in the lipid phase admixture. As shown in Table 1, the size of NLCs decreased when oleic acid was added. The viscosity of the lipid phase was decreased and easily extruded into small globules by increasing proportion of oleic acid. Furthermore, the addition of liquid oil resulted in decreasing oil phase's melting point that can improve particles' fluidity, which is beneficial for skin permeation. However, addition of oleic acid more than 10% was not applicable. Preliminary study using several concentrations of oleic acid showed that addition of oleic acid more than 10% (w/w) to Suppocire® NA could not produce solid lipid matrix (data not shown). Based on data in Table 1, F3 was the optimum formula with particle size of 142.9±4.17 nm and used for following BSA encapsulation. The same result was also reported by Yuan and coworkers [20]. It was found that the addition of 10 wt% of oleic acid to the oil phase produced the smallest size of monostearin lipid nanoparticles.

Table 1: The size and polydispersity index of different NLCs formula

Component (%)	F1	F2	F3
Suppocire® NA	3	2.85	2.7
Oleic acid	0	0.15	0.30
Lutrol® F 68	30	30	30
Ethanol	30	30	30
Deionized water	37	37	37
Particle size (nm)	175.1±6.65	156.5±3.73	142.9±4.17
Polydispersity index	0.172±0.018	0.184±0.51	0.052±0.013

Poly dispersity index (PI) of all formulations developed in this research was in the range of 0.082-0.184. The low PI ≤ 0.5) represented the narrow particle size distribution indicating sufficient duration and speed of homogenization-sonication process, besides the right composition of formulation chosen. The rate of homogenization step had a high impact on NLCs particle size, especially during pre-emulsion preparation with the speed less than 8000 rpm resulting in higher NLCs particle size shown by more cloudy appearance of dispersion. Meanwhile, the rate of homogenization during solidification process had no influence on particle size, but it tends to determine the PI. In this research, the homogenization during solidification conducted by stirring using magnetic stirrer at scale 2 (Thermolyne plate magnetic stirrer, Nuova) was the optimum speed. The higher speed of stirring had no influence to the particle size, but it showed high impact on the PI indicated by the formation of a few bigger NLCs particles floated on the surface of solidification media. The trend of increasing PI due to the increasing speed of solidification stirring was also found in the preparation of Witepsol ® W35 and Dynasan ® 116 SLNs dispersion as reviewed by Mehnert and Mader [7].

BSA encapsulation within NLCs

In this research, BSA was incorporated in NLCs by dispersing in the lipophilic phase as solid in oil suspensions and followed by emulsification in water phase as S/O/W system for solidification of NLCs particles. Due to the fact that relatively large protein particles tend to be decanting from suspension and leaching out to aqueous phase, a low encapsulation of protein tends to occur. This phenomena was observed by King and Patrick when encapsulating vascular endothelial growth factor (VEGF) into poly(DL-lactic-*co*-glycolic acid)/poly(ethylene glycol) microspheres using a solid encapsulation/single emulsion/solvent extraction technique [21]. To overcome this problem BSA was co-lyophilized with PEG 20000 prior to encapsulation process. At appropriate ratio of PEG 20000 to BSA, fine spherical particles of BSA were produced after dispersing BSA-PEG 20000 co-lyophilisate in oleic acid [22]. The addition of fourfold PEG 20000 to BSA provided a clear dispersion in oleic acid. This ratio was applied for BSA encapsulations in which 0.005% BSA equivalent to 0.025% BSA-PEG 20000 (1:4) was loaded into F3 formula. The increase in particle size from 142.9±4.17 nm to 321±5.99 nm was observed after BSA loading.

Entrapment efficiency was determined indirectly. The NLCs showed a high entrapment efficiency of BSA, which was 85.3%±2.74%. The high entrapment efficiency achieved was indicative of the success of PEG 20000 addition as amphiphilic material in increasing the hydrophilic BSA dispersibility in lipophilic oil phase. The same result was also obatained in Castellanos's research, where addition of PEG increased protein encapsulation [23]. Furthermore, addition of oleic acid in lipid phase will decrease lipid nanoparticle crystallinity, hence increasing entrapment efficiency [8,24].

Conjugation of jacalin onto NLCs

In this research, jacalin with specificity binding to Gal/GalNac [13, 14] was proposed as adjuvant to target skin DCs that is known having GalNAc moieties on the cell surface [15]. Jacalin was adsorbed onto NLCs surface utilizing electrostatic interaction. The adsorption was conducted at pH 5 based on zeta potential measurement of jacalin solutions at various pH, where net jacalin charge became zero at pH around 5.3. At pH less than 5.3, jacalin will be positively charged due to protonation of basic amino acid. Furthermore, jacalin will remain active at pH 5 [25]. Meanwhile, at this pH NLCs were negatively charged with zeta potential of -11.31±1.56 mV as a result of fatty acid deprotonation at the end of oleic acid carboxyl group. This phenomenon was also observed when stearic acid/ceramide nanoparticles were adjusted in the range of pH 3.0 to pH 9.0 with decreasing zeta potential from -23 mV to -77 mV [18]. Both of charged NLCs and jacalin will undergo electrostatic interaction, hence jacalin was adsorbed onto the NLCs surface. The increase of NLCs particle size from 321±5.99 nm to 382.3±10.80 nm and an increase of zeta potential to -2.96±0.71 mV indicated the presence of adsorbed jacalin on the NLCs surface. Having particle size of 382.3±10.80 nm, the JCBN are expected to be able to penetrate through skin. Based on a research about the size of nanoparticles potential to be used as transcutaneous vaccine, only particles in the range of 50 to 500 nm were able to penetrate through hair follicles and sebaceous glands [26]. In addition, particles that penetrate through hair follicles will facilitate antigen presentation to skin APCs, high in population in these areas [6].

Nanoparticles morphology was determined using Transmission Electron Microscope. Based on the result presented in Fig. 2(A), the produced NLCs were spherical with particle size less than 500 nm. At 12,000 times magnification, dispersed BSA within NLCs could be seen, as shown in Fig. 2(B), as dark spots which were distributed homogenously throughout the inner space of NLCs.

Jacalin is a glycoprotein lectin that specifically binds to galactose moieties such as Gal/GalNAc [13,14], like general lectin plant, forming bi- or oligovalent bonding [27,28]. This feature can be used to verify its conformation stability where hemagglutination will be occurred upon addition to erythrocytes. As shown in Table 2, JCBN had hemagglutination activity that indicated the success of jacalin adsorption onto NLCs surface. However, the hemagglutination activity was lower than the jacalin solution (prepared by dissolving the same amount of jacalin initially added into NLCs dispersion for adsorption process) that could be resulted from incomplete adsorption process. The 78% remaining activity was the optimum result because the reaction could not occur below pH 5 due to the decrease in oleic acid carboxyl group deprotonation, while above pH 5 the positively charged jacalin would be decreased. On the other hand, NLCs without jacalin did not show any hemagglutination activity.

This suggests that NLCs base was safe without haemagglutination negative effect, while jacalin did show its activity to bind cell surface receptors of erythrocytes at concentration used in haemagglutination test. By considering much higher distribution volume and total amount of erythrocytes in the body compared to erythrocytes used in haemagglutination test, it is predicted that the haemag glutination will not occur during *in vivo* application of jacalin. Meanwhile, it is expected that jacalin will be effective as adjuvant to target APCs by specifically bind to cell surface receptors containing galactose moieties such as GalNac present on DCs surface [15]. For future *in vivo* study, it is required to adjust jacalin conjugation on NLCs at predetermined immunostimulatory concentration, but with no-side effect of haemaglutination.

Stablility of JCBN

Stability evaluation for 4-week storage showed that JCBN had a relatively stable particle size around 380-400 nm with polydispersity index remained very low in the range of 0.1-0.2 ($p<0.05$) as shown in Figure 3. This shows that Lutrol® F 68 was effective for maintaining the nanoparticles integrity during freeze drying, so that aggregation was prevented. Lutrol® F 68 formed a hydrophilic layer on the nanoparticles surface which plays an important role in nanoparticles redispersion after freeze drying [29].

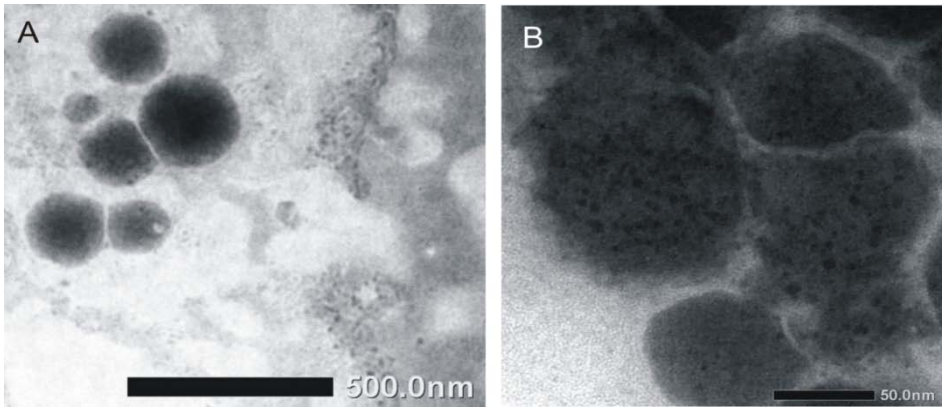

Fig. 2: Morphology of JCBN particles characterized by transmission electron micrograph. The NLCs were revealed as (A) spherical particles with (B) homogenous BSA distribution within the particles.

Table 2: Hemagglutination activity test results of jacalin adsorbed onto nanoparticles

Formula	Minimum protein concentration to induce haemagglutination (µg/mL)*)	Activity (%)
Jacalin solution	1.95	100**)
JCBN	2.5	78
BSA-loaded NLCs (without jacalin)	>10	0

*) hemagglutination activity was estimated from the minimal amount of lectin giving the last visible erythrocytes agglutination

**) jacalin solution was prepared by dissolving the same amount of jacalin initially added into NLCs dispersion for conjugation process

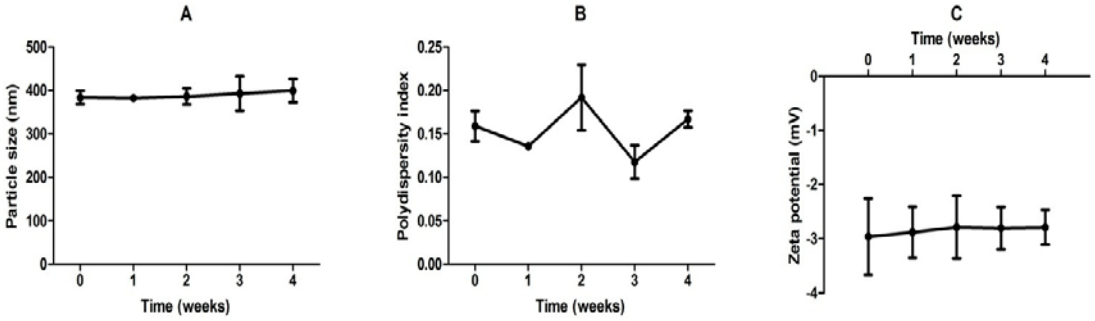

Fig. 3: Physical stability of JCBN particles during 4 weeks of storage at 4°C. The particles were stable with no significant different changes for all parameters tested (p>0.05), including: particle size (A), zeta potential (B) and polydispersity index (C).

The zeta potential of NLCs also did not show any tendency to change (p>0.05), albeit their low average of zeta potentials (in the range of – 2.79 to – 2.96). Generally, particles can be dispersed stably when the absolute value of zeta potential is above ±30 mV due to the electrical repulsion between particles [30]. However, the stable JCBN with no tendency of particle aggregation could be resulted from the combination of electrical repulsion and steric stabilization. Schwarz and Menhert were also found the influence of steric stabilization upon Poloxamer 188 (Pluronic ® F 68) addition on the stability of SLN comprised of cetylpalmitate and Compritol having zeta potentials between -18 mV to -27mV [31]. Furthermore, a study conducted by Zimmermann and Muller also supported this finding in which the combination of 8-9 mV zeta potential of Compritol ATO 888 SLNs and steric stabilization provided by Pluronic® F 68 produced stable dispersion [32].

CONCLUSION

The administration of subunit peptide vaccine via skin requires addition of adjuvant to optimize immune response induction. This research was aimed to develop an adjuvant in the form of NLCs as protein delivery system to improve transcutaneous permeation. Jacalin with specificity binding to Gal/GalNAc [13,14] was conjugated on the NLCs surface to target skin DCs population that is known having GalNAc moieties on the cells' surfaces [15]. The obtained particle size of NLCs constructed from Suppocire ® NA-oleic acid admixture with Lutrol® F 68 stabilizer was around 382.3±10.80 nm, which had spherical structure. BSA-loaded NLCs were successfully conjugated with jacalin at 78% of efficiency, as demonstrated by hemagglutination activity test. High entrapment efficiency of the NLCs was obtained due to addition of oleic acid and incorporation of BSA into lipid phase in solid dispersion form. The use of Lutrol® F 68 as sterical stabilizer can possibly compensate the small electrical repulsions to produce stable NLCs particle during storage at temperature of 4°C for 4 weeks. Hence, this formula is promising for effective delivery of protein antigen to target skin APCs.

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