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Preprint *in* International Journal of Pharmaceutics · November 2020

DOI: 10.1016/j.ijpharm.2020.119928

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Microneedles with dual release pattern for improved immunological efficacy of Hepatitis B vaccine

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ARTICLE INFO

Keywords:
Microneedles
Dual release pattern
Improved immunological efficacy
Hepatitis B vaccine

ABSTRACT

In this study, dissolving microneedles (DMNs) with dual-release pattern, capable of both bolus release and slow release, were prepared. These DMNs were used with a hepatitis B vaccine that requires multiple shots to achieve immunological efficacy comparable to that obtained when two separate shots are administered.

Dissolving microneedles with HBsAg in PLA tips and CMC coating formulation together (HBsAg-PLA/CMC-DMNs) consist of polylactic acid (PLA) tips for slow release, a carboxy-methyl cellulose (CMC) coating formulation for bolus release, and a dissolving base of polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) for dissolution in the skin. The *in vitro* release pattern of HBsAg from the CMC coating formulation and PLA tips was observed. Through an *in vivo* test, 1) the delivery efficiency of HBsAg-PLA/CMC-DMNs was observed, and 2) the immunological efficacy of this method was compared with the efficacy of two shots delivered by conventional intramuscular (IM) administration and two shots delivered by HBsAg-coated microneedle (CMNs) administration. HBsAg-PLA/CMC-DMNs punctured the skin successfully. The PVA/PVP base was completely dissolved within 10 min of insertion, resulting in the delivery of all microneedle tips into the skin. In the *in vitro* release experiment, all of the HBsAg in the CMC coating formulation was released within 20 min, and the HBsAg present in the PLA tips was gradually released over more than 55 days. The antibody titer of one shot of HBsAg-PLA/CMC-DMNs was the same as or higher than two shots delivered by conventional IM and CMN methods. DMNs with dual-release pattern can deliver two formulations simultaneously with a single shot, resulting in improved immunological efficacy of HBsAg that requires multiple doses. In addition, this dual-release MN system can be used for the delivery of other drugs that require multiple administrations.

1. Introduction

Microneedles (MNs) are attached to the skin employing a transdermal patch, and studies show that they provide immunological efficacy comparable to that achieved with needle-syringe-based administration (Suh et al., 2014; Sullivan et al., 2010). MNs can deliver chemical and biological drugs into the skin layer through the stratum corneum, the outermost resistance layer regardless of the molecular weight or polarity of the drug (Teo et al., 2006). The administration of MNs is effective for immunogenicity because the vaccine is administered to the skin layer having Langerhans cells and dendritic cells (Pasparakis et al., 2014). In addition, MNs cause little pain, improve patient compliance, can be self-administered, require minimal medical expertise, and improve thermo-stability (Al-Qallaf and Das, 2008; Arya et al., 2017; Ita, 2015; Mistilis et al., 2015; Norman et al., 2014).

Currently, microneedles are manufactured by various

Abbreviations: HBsAg, Hepatitis B surface antigen; HBV, Hepatitis B virus; IM, Intramuscular; ID, Intradermal; MNs, Microneedles; Alum, Aluminum hydroxide; PLA, Polylactic acid; CMC, Carboxy-methyl cellulose; DMNs, Dissolving microneedles; CMNs, Coated microneedles; HBsAg-AL-IM, Intramuscular administration of HBsAg with Alum; HBsAg-CMNs, Coated microneedles with HBsAg; HBsAg-PLA-DMNs, Dissolving microneedles with HBsAg in PLA tip; HBsAg-CMC-DMNs, Dissolving microneedles with HBsAg in CMC coating formulation; PLA/CMC-DMNs, Dissolving microneedles with two formulation of PLA and CMC; HBsAg-PLA/CMC-DMNs, Dissolving microneedles with HBsAg in PLA tips and CMC coating formulation together.

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https://doi.org/10.1016/j.ijpharm.2020.119928
Received 30 July 2020; Received in revised form 6 September 2020; Accepted 26 September 2020
Available online 16 October 2020

micromanufacturing processes, including 3D printing (Elkasabgy et al., 2020). There are four types of MNs: solid, coating, dissolving and swellable. The most commonly used MN types for medical applications are dissolving MNs (DMNs) and coating MNs (CMNs). DMNs are made of water-soluble substances, and the drug is encapsulated inside the DMNs. The drug is released into the skin by dissolution of the DMN matrix (Sullivan et al., 2008). CMNs are prepared by coating a drug-containing formulation onto solid MNs composed of a non-soluble polymer. As noted, the tips of DMNs dissolve in the skin, resulting in low risk of biological contamination (Matsuo et al., 2012). However, despite this and other advantages, DMNs provide only one drug release rate because of the rapid dissolution of the water-soluble polymer of which the DMN matrix consists. To overcome this limitation, it has recently been proposed to develop DMNs that contain nanoparticles or that consist of separable MNs (Lee et al., 2008; Lee et al., 2019). However, other studies have not shown that MNs can provide a multiple release pattern, nor have previously developed DMNs been able to deliver drugs by both bolus release and sustained release simultaneously. If DMNs can be developed to provide both bolus and slow release of drugs together, various medical applications are possible.

Hepatitis B virus (HBV) infection can be effectively prevented by conventional intramuscular administration of the vaccine, but successful antibody formation by conventional administration needs three vaccinations given over two months duration. Multiple administrations are inconvenient for users and not efficient for achieving effective immunogenicity due to poor patient compliance (Van Herck et al., 2007).

In this paper, HBsAg-PLA/CMC-DMNs with dual-release rate pattern was developed and applied to a vaccine that needs to be administered multiple times, so that the vaccine can have high immunogenicity with a single administration. As shown in Fig. 1, HBsAg-PLA/CMC-DMNs consist of polylactic acid (PLA) tips for slow release, a carboxy-methyl cellulose (CMC) coating formulation for bolus release, and a dissolving base consisting of polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) for dissolution in skin. As shown in Fig. 2, when HBsAg-PLA/CMC-DMNs are administered to the skin, the PVA/PVP base dissolves quickly to separate the tips and the two formulations can be delivered into the skin, allowing both bolus and sustained release of HBsAg. In this study, the resulting antibody titer of DMNs with dual-release pattern was compared to that achieved with two shots delivered by IM administration and two shots delivered by CMNs with a bolus release pattern.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS), fluorescein isothiocyanate dextran (FITC-dextran), polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) were obtained from Sigma-Aldrich (St. Louis, MO). Polylactic acid (PLA) was purchased from Lactel (Birmingham, AL). Carboxymethyl cellulose (CMC) was obtained from Whawon (Gyeonggi-do, South Korea).

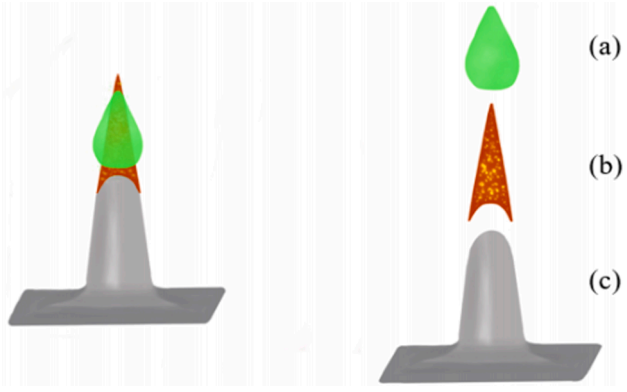

Fig. 1. DMNs with dual-release pattern for prime and boost immunization of HBsAg. (a) CMC coating formulation for bolus release (HBsAg 0.15 μg), (b) PLA tip for slow release (HBsAg 0.15 μg), (c) PVA/PVP base for delivery of two formulations after insertion into skin.

2.2. Fabrication of HBsAg-PLA/CMC-DMNs and model drug microneedles

A microneedle master-mold was manufactured by QuadMedicine (Seongnam, Korea) using a micromilling process. Microneedles were pyramidal shaped, with a height of 800 μm and a square base width of 350 μm; each array contained 145 microneedles. Then the mold was prepared from a master structure using micromolding. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, MI, USA) was mixed with a curing agent in a 10:1 ratio, poured into a master structure, and cured at 70 °C to produce a PDMS mold.

Microneedles containing HBsAg were prepared as shown in Fig. 3. HBsAg was supplied kindly by LG Chem/Reserarch Park (Seoul, Korea). Polylactic acid (PLA) (MW: 40 K; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dichloromethane to obtain a PLA solution with a concentration of 0.5% (w/v). An HBsAg solution (6.5 μL) in PBS with a concentration of 461 μg/mL was added to 6 mL of the PLA solution, and the HBsAg solution was dispersed in the PLA solution by ultrasonic treatment with Sonication (Vibra-Cell Processors, Sonics & Materials, Inc. Canbury, CT) for 10 s (amplitude of 10%). Then 300 μL of the emulsified solution was loaded into the PDMS mold, centrifuged for 20 min at a rotation speed of 1000 rpm and 4 °C to fill the PLA and HBsAg into the mold, and then centrifuged again for 2 h at a rotation speed of 3500 rpm. The residual solvent of water and dichloromethane was removed by an additional drying process at 4 °C for 6 h to obtain the dried PLA tips containing HBsAg.

To prepare the dissolving base on the PLA tips, the particles of PVA and PVP (Sigma-Aldrich) were mixed in a 1:1 ratio and the mixture was dissolved in distilled water to prepare a 20% (w/w) of PVA/PVP solution. A 0.12 g PVA/PVP solution was loaded into a mold with dried PLA tips and centrifuged at 4 °C for 10 min. Thereafter, drying was further performed at 4 °C for 1 day. The dried microneedles (HBsAg-PLA-DMNs) were then removed from the mold.

The coating of the formulation for bolus release was applied to the PLA tips of HBsAg-PLA-DMNs. CMC was dissolved in distilled water and the HBsAg solution was added to the prepared CMC aqueous solution so that the final concentration of CMC was 6% (w/w). After loading the HBsAg coating solution into a 500 μm dip-coating well, the HBsAg-PLA-DMNs were dip-coated in the CMC solution with HBsAg twice for 1 s and dried at 4 °C for 30 min. Finally, HBsAg-PLA/CMC-DMNs containing 0.15 μg of HBsAg in the PLA tips and 0.15 μg of HBsAg in the CMC coating formulation were prepared.

In order to observe the distribution of the formulation in the skin after administration of PLA/CMC-DMNs, PLA/CMC-DMNs containing fluorescence were prepared instead of HBsAg. A 0.1% (w/v) concentration of Rhodamine B isothiocyanate-Dextran (RITC-dextran, Mw: 70 kDa) (Sigma-Aldrich, St. Louis, MO, USA) was added in the PLA tips, and 0.1% (w/v) of fluorescein isothiocyanate dextran (FITC-dextran, Mw: 10 kDa) (Sigma-Aldrich) was embedded in the coated CMC formulation by the same preparation process. The manufactured microneedles containing fluorescence were observed using an optical microscope (Eclipse 80i, Nikon, Tokyo, Japan) and a confocal microscope (ELIPSE TE2000-E, Nikon, Osaka, Japan).

2.3. Antigenicity maintenance of HBsAg during the preparation process

The maintenance in antigenicity of HBsAg in the PLA tips and the CMC-coated formulation of DMNs was measured with an ELISA kit ($n = 5$; Thermo Fisher Scientific, Rockford, IL). To measure the HBsAg content in the PLA tips, uncoated HBsAg-PLA-DMNs was added in 1 mL of

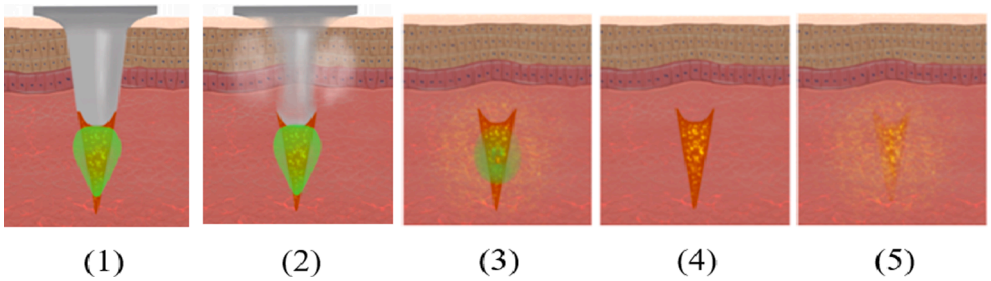

Fig. 2. Schematic image of dissolving microneedles with dual-release pattern. (1) Insertion of HBsAg-PLA/CMC-DMNs into skin, (2) dissolution of PVA/PVP base, (3) bolus release of HBsAg from coating part, (4) remaining PLA tip, (5) slow release of HBsAg from PLA tip.

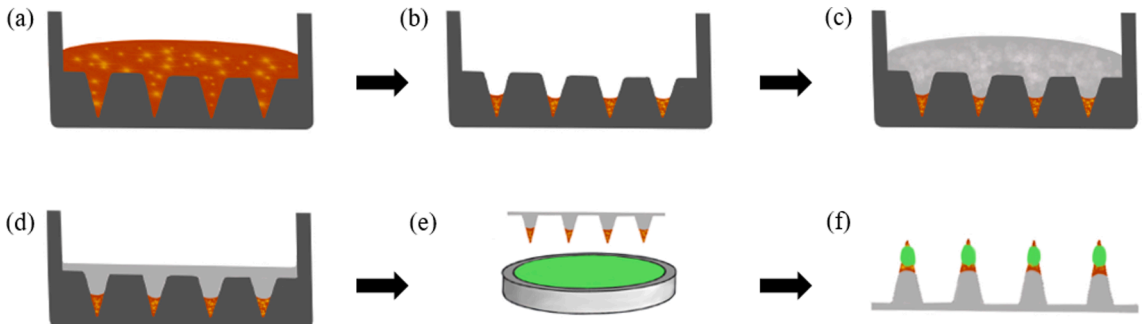

Fig. 3. Schematic description of the manufacturing process of HBsAg-PLA/CMC-DMNs using micromolding and dip-coating processes. (a) Cast HBsAg distributed PLA dichloromethane solution, (b) centrifuging to fill the mold and drying to remove dichloromethane, (c) cast PVA/PVP solution for dissolving base, (d) centrifuging to fill mold and drying to remove the water, (e) dip-coating HBsAg CMC aqueous solution with HBsAg, (f) dissolving microneedles consisting of PLA tips and CMC coating formulation (HBsAg-PLA/CMC-DMNs).

dichloromethane and shaken for 10 min to dissolve the PLA tips and to obtain the amount of HBsAg in the tips. PBS (0.3 mL) was added to dichloromethane and mixed, and another 0.3 mL of PBS was added and mixed again. Finally, 0.4 mL of PBS was added and mixed, and then the two immiscible solutions were each centrifuged for 3 min at 3000 rpm (Shi et al., 2002). The supernatant of PBS was obtained and the content of HBsAg was measured using an ELISA kit. The antigenicity of HBsAg in the PLA tips, which was known in quantity, was compared with the antigenicity of the stock HBsAg solution at 4 °C. To measure the content of HBsAg in the coating formulation of CMC, HBsAg-CMC-DMNs were prepared by the same coating method, and the PLA tip portion was cut by a Rotary microtome (RMC- Boeckeler, Tucson, Az) and put in PBS for 1 h, and an ELISA kit was used to measure HBsAg antigenicity. The antigenicity of HBsAg was compared with the antigenicity of the stock HBsAg solution at 4 °C (Nguyen et al., 2019).

2.4. Mechanical properties of HBsAg-PLA/CMC microneedles.

In order to observe the mechanical properties of HBsAg-PLA-DMNs before coating them with the CMC formulation, the number of microneedles (HBsAg-PLA-DMNs) was adjusted to four (2 × 2) in an array. Force-displacement patterns were measured by applying a vertical force up to 10 N on a microneedle array at a rate of 1 mm/min using a force displacement machine ($n = 10$ arrays; 500 N Zwicki, Zwick GmbH & Co. KG, Ulm, Germany). The change in shape of the HBsAg-PLA-DMNs was recorded through measurement using an optical microscope (Eclipse 80i, Nikon, Tokyo, Japan) and compared with a force–displacement curve.

2.5. In-vitro skin puncture performance of HBsAg-PLA/CMC-DMNs

Puncture performance, defined as the ratio of the number of holes generated to the number of microneedles, was measured. HBsAg-PLA/CMC-DMNs were placed on a full porcine skin (CRONEX, Seoul, Korea) on a digital weight scale and pressure was applied for 10 *s* with a hand press while measuring with a digital balance to maintain 30 N of force. Then the HBsAg-PLA/CMC-DMNs were removed before detachment and the skin surface was stained for 5 min using 0.25% (v/v) of trypan blue solution (Sigma-Aldrich). The trypan blue solution on the skin surface was cleaned-off with PBS. The number of holes stained with trypan blue was then counted using an optical microscope (Sv-35, Sometech, Seoul, Korea) and puncture performance was calculated.

2.6. Dissolution of PVA/PVP component in HBsAg-PLA/CMC-DMNs

The dissolution of the tip base of the HBsAg-PLA/CMC-DMNs and delivery of two formulations was investigated using *in vitro* insertion into the porcine skin. HBsAg-PLA/CMC-DMNs were placed on a full porcine skin (CRONEX, Seoul, Korea), and 30 N of force was applied vertically for 10 s with a hand press. Then microneedles were removed from the skin after 3, 7 and 10 min of insertion, respectively. The recovered microneedle samples were observed using a scanning electron microscope (ScanMing Electron Microscope [SEM], JSM-7001F, JEOL Ltd, Tokyo, Japan) to observe the change in morphology of the HBsAg-PLA/CMC-DMNs.

2.7. In vitro release test

Since the PVA/PVP dissolving base interfered with the ELISA for measurement of HBsAg, PLA tips of HBsAg-PLA-MNs without the water-soluble part were produced with the same preparation method and the PLA tips were cut by a Rotary microtome (RMC, Boeckeler) to obtain only the tips. The tips were then placed in 1 mL of PBS and stirred at 37 °C. A 500-μL solution was sampled and 500 μL of new PBS was added at 1, 7, 14, 21, 28, 35, 42, 49 and 56 days. The amount of HBsAg in the sampled solution was measured by ELISA kit ($n = 5$; Thermo Fisher Scientific, Rockford, IL). In addition, the PLA tips were collected during the *in vitro* experiment period, and the change in tip morphology was

observed using SEM.

2.8. Animal

Five-week-old female BALB/c mice (Koatech, Pyungtek, Korea) were purchased and housed under standard laboratory conditions in the animal research facility, International Vaccine Institute (IVI). All animal studies were approved by Institutional Animal Care and Use Committees (IACUC) at the International Vaccine Institute (2017-001).

2.9. In vivo vaccination study

Animal experiments were performed to examine the immunogenicity of HBsAg-PLA/CMC-DMNs. Before the experiments, the dorsal hair of the mice was removed using a hair clipper (Thrive, Japan), the remaining hair was removed using a hair removal cream (Nair, NJ, USA), and then the backs of the mice were washed using 70% (v/v) ethanol. The experiments were conducted a day after hair removal. A solution of 100 mg/kg ketamine (Yuhan, Seoul, Korea) and 12.5 mg/kg rompun (Bayer, Leverkusen, Germany) was administered to the mice to anesthetize them, and the microneedles was administered with a clip exerting 20 N of force on the backs of the mice for 30 min. Information about the animal experiments is shown in Table 1. There were two control groups (groups 1 and 2) and five experimental groups (groups 3–7). Group 7 was administered with HBsAg-PLA/CMC-DMNs. After administration, blood was collected every 2 weeks for 8 weeks to measure immunogenicity. The microneedles used for the experiments were collected and the amount of remaining HBsAg on them was measured by ELISA kit ($n = 5$; Thermo Fisher Scientific, Rockford, IL).

2.10. Enzyme-linked Immunosorbent Assay (ELISA)

The IgG titer in serum was measured by ELISA. For antigen coating, 2 μg/mL of HBsAg in coating buffer (50 mM sodium bicarbonate buffer (Sigma-aldrich, St. Louis, MO, USA, pH 9.6) was incubated on the 96-well plates (Nunc, Roskilde, Denmark) at 4 °C overnight. After washing with washing buffer (PBS (Gibco, Grand Island, NY, USA) containing 0.05% tween-20 (Sigma-aldrich, St. Louis, MO, USA)), the coated plates were incubated with blocking buffer [PBS containing 2% bovine serum albumin (Sigma-aldrich, St. Louis, MO, USA) and 0.05% tween-20)] for 1 h. For the antibody reaction, the serum was diluted into 1:30 with antibody buffer (PBS containing 0.5% BSA and 0.05% tween-20), and serial diluted (5-fold) on the plate. Plates were incubated at 37 °C for 1 h and diluted serum was washed out with washing buffer. After three washing steps, 1:3000 diluted HRP-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) in antibody buffer was added to each well, and incubated at 37 °C for 1 h. After washing with washing buffer, TMB (3,3′5,5′-tetramethylbenzidine) solution (Millipore, Billerica, MA, USA) was added to each well and the reaction was stopped by 0.5 N HCl (Sigma-aldrich, St. Louis, MO, USA). The absorbance was measured at wavelength of 450 nm by an ELISA Reader Spectra Max 190 (Molecular Devices, San Jose, CA, USA) and the IgG titer was calculated using Softmax program (Molecular Devices, San Jose, CA, USA). The results were transformed into log2 values.

2.11. Statistical method

The arithmetic mean and standard error of the mean were calculated. A two-tailed Student's t test ($\alpha = 0.05$) was performed when comparing two different conditions, and ANOVA was used when comparing multiple groups. A value $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characteristics and antigenicity maintenance of HBsAg-PLA/CMC-DMNs

As shown in Fig. 4, the fabricated microneedles have a pyramidal shape with a total length of 800 μm and a base width of 350 μm square, with 145 microneedles per array. Fig. 4(a) shows the size of a 26-gauge needle compared to HBsAg-PLA/CMC-DMNs. As shown in the image of HBsAg-PLA-DMNs in Fig. 4(b), the PLA tips and PVA/PVP dissolving

Table 1
Information on groups for animal tests.

	Control group		Experimental group				
Group number	1	2	3	4	5	6	7
Sample Info.	PBS	PLA/CMC-DMNs	HBsAg-AL-IM	HBsAg-AL-IM	HBsAg-CMNs	HBsAg-PLA-DMNs	HBsAg-PLA/CMC-DMNs
	Mock	Mock	Bolus injection	Bolus injection	Bolus release	Sustained release	Bolus and sustained release
Administration Route	IM	MN	IM	IM	MN	MN	MN
Alum	-	-	+	+	-	-	-
dose (μg) at 0 day	0	0	0.15	0.3	0.15	0.3	0.3 (0.15+0.15)
dose (μg) at 14 day	0	0	0.15	No	0.15	No	No
Graphic description							

−: without, +: with, No: no administration, AL: alum, IM: intramuscular administration, MN: microneedle administration, CMNs: coated microneedles, DMNs: dissolving microneedles.

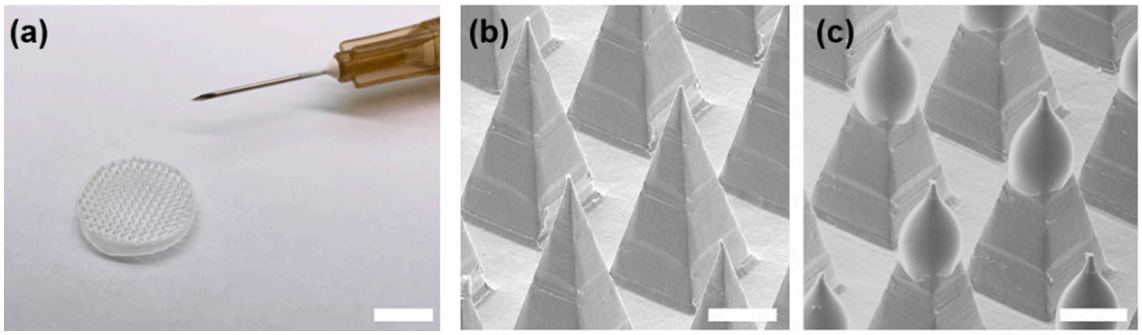

Fig. 4. (a) Comparison images of HBsAg-PLA/CMC-DMNs adjacent to a 26-gauge needle to illustrate their relative sizes (scale bars, 5 mm). (b) SEM image of HBsAg-PLA-DMNs: dissolving microneedles with HBsAg in PLA tips. (c) SEM image of HBsAg-PLA/CMC-DMNs: dissolving microneedles with HBsAg in PLA tips and CMC coating formulation (scale bars, 200 μm).

components were attached well. The PLA tips were well-bonded to the PVA/PVP base, and when removed from the PDMS mold, the PVA/PVP base had sufficient adhesion to the PLA tip so that the PLA-DMNs could be successfully removed from the mold. The CMC formulation on the PLA tips shown in Fig. 4(c) was located on the surface of the tips because the PVA/PVP base could dissolve in the aqueous CMC coating solution during the coating process. If centrifugation was performed for over an hour, the aqueous solution of PVA/PVP could run down onto the solidified PLA tip. Thus, centrifugation was performed for only 10 min to fill the PVA/PVP solution in the mold and then the solution was allowed to evaporate without centrifugation.

When fluorescence distribution in PLA-DMNs as shown in Fig. 5(a) was observed using RITC-dextran, the shape of the arch was formed by the surface tension of the PLA-dichloromethane solution in the cavity of the PDMS (Fig. 5(b)). Fig. 5(d) shows that the FITC-dextran-CMC formulation was uniformly distributed on the PLA tips of the PLA/CMC-DMNs [Fig. 5(c))]. If the coating formulation ran over the PLA tips onto the PVA/PVP base, the PVA/PVP base was dissolved by the coating solution during the coating process. Thus, the depth of the coating solution was set to 500 μm in order not to exceed the height of the PLA tips.

The denaturation of HBsAg was observed during the process of loading HBsAg into HBsAg-PLA/CMC-DMNs. After encapsulating 0.15 μg of HBsAg in the PLA tips, the concentration of HBsAg was measured using ELISA, which determined that 91% of initial antigenicity was maintained after the molding and drying of the tips. For HBsAg in the CMC formulation, 98% of initial antigenicity was maintained after the coating and drying of the HBsAg-CMC formulation. In the process of preparation of the PLA tips containing HBsAg, antigenicity was reduced slightly because of contact with the organic solvent during the dispersal of HBsAg into the DCM/PLA solution. Also, consistent antigenicity was shown during the coating process (Stivaktakis et al., 2004). The antigenicity of HBsAg in HBsAg-PLA/CMC-DMNs was maintained during the molding and coating processes (Stivaktakis et al., 2004). Solidified CMC-HBsAg used as a bolus formulation in this study was stable at 40 °C for 2 months (Na et al., 2020). This solidified HBsAg has improved storage stability at high temperatures.

3.2. *Mechanical properties and puncture performance of HBsAg-PLA-DMNs*

When vertical force was applied to HBsAg-PLA-DMNs, the deformation proceeded in two stages. The initial bending of the PLA tips by the vertical force generated at the interface of the PVA/PVP dissolving base and the tips was followed by compression deformation of the PVA/PVP base by the continuous vertical force. As shown in Fig. 6(a), the inflection point could be found at 0.27 mm (region I of Fig. 6(a)), which was due to the mechanical failure at the interface of the PLA tip and the PVA/PVP base. Also as shown in Fig. 6(a), a compressive fracture of the PVA/PVP tip base was observed at 0.6 mm (region II of Fig. 6(a)). The initial deformation of the PLA tip was not compressive but bending because of the excellent mechanical strength of the tip (region I). The deformation at the interface was caused by the difference in the mechanical strength of the tip and the base. The force increased exponentially as the initial deformation in the region I increased. When vertical force was applied to the HBsAg-PLA/CMC-DMNs after the initial deformation, compression deformation occurred at the base of the PVA/PVP, and the force showed a linear relationship with the deformation (region II).

Fig. 6(b) shows that all HBsAg-PLA/CMC-DMNs penetrated the skin successfully and that generated holes were stained. HBsAg-PLA/CMC-DMNs had sufficient mechanical strength to penetrate the skin and successfully deliver vaccines into the skin layer.

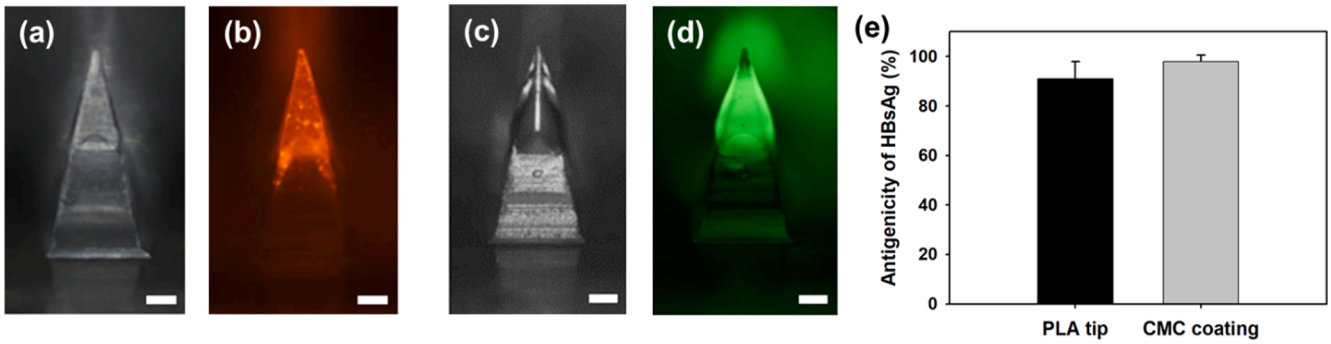

Fig. 5. (a) Optical image of HBsAg-PLA-DMNs, (b) fluorescence image of RITC-dextran-PLA-DMNs, (c) optical image of HBsAg-CMC/PLA-DMNs, (d) fluorescence image of FITC-dextran-CMC/PLA-DMNs (scale bars, 100 μm). (e) Antigenicity maintenance of HBsAg in HBsAg-PLA/CMC-DMNs (n = 5).

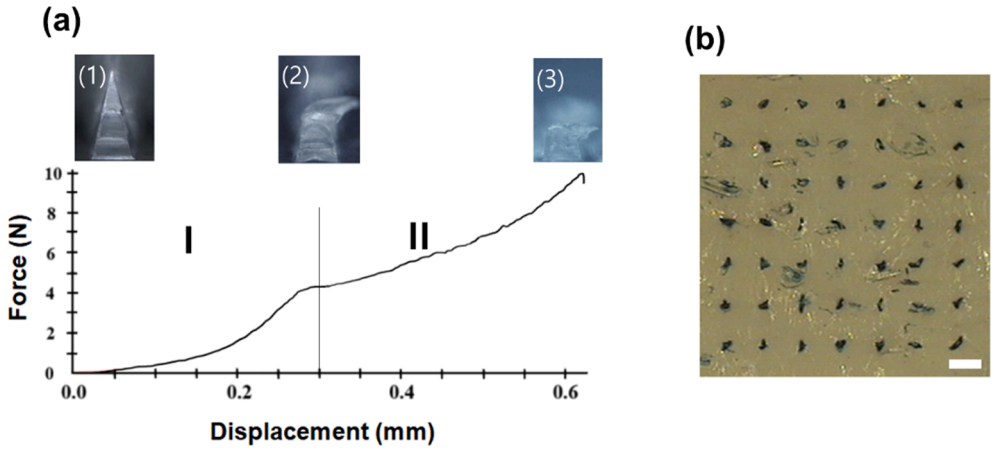

Fig. 6. (a) Force-displacement graph of HBsAg-PLA-DMNs consisting of region I and region II: (1) initial stage, (2) at 0.3 mm of displacement, (3) at 0.6 mm of displacement. (b) Optical image of porcine skin stained with trypan blue solution after insertion and removal of HBsAg-PLA/CMC-DMNs (scale bars, 300 μm) (n = 5).

3.3. Dissolution of PVA/PVP base of HBsAg-PLA/CMC-DMNs

Fig. 7 shows SEM images after insertion of HBsAg-PLA/CMC-DMNs into porcine skin for 3 min, 7 min and 10 min to observe the morphological changes in the HBsAg-PLA/CMC-DMNs. Fig. 7(a) is a SEM image of HBsAg-PLA/CMC-DMNs before administration. As shown in Fig. 7(b), the PVA/PVP base began to dissolve after insertion into the skin, and most of the coating formulation was dissolved in the skin at 3 min post-insertion. After 7 min post-insertion, partial separation of the PLA tips occurred at the junction of the PLA tips, and the dissolving base (Fig. 7 (c)). Finally, after 10 min post-insertion, the PVA/PVP base dissolved completely, resulting in the successful delivery of the PLA tip and the CMC coating formulation with HBsAg into the skin (Fig. 7(d)). Fig. 7(e) is a graph showing the amount of PLA tip delivered into the skin according to insertion duration. Ten minutes is the minimum required insertion time for the successful delivery of HBsAg-PLA/CMC-DMNs as a result of the complete dissolution of the PVA/PVP base. A pyrogen test and an acute dermal toxicity test were conducted with HBsAg-coated MNs made with PLA and CMC, which are used to make HBsAg-PLA/CMC-DMNs. In these tests, the HBsAg-coated MNs did not induce dermal toxicity and irritation. Also, there was no inflammatory and allergic reaction at the application site of the HBsAg-coated MNs (Na et al., 2020). In regard to the toxicity of PVA in the PVA/PVP base, aqueous PVA gel did not cause a skin reaction, but PVA can cause minor skin irritation with repeated exposure (Nair, 1998). PVP with relatively high solubility in water has been reported to have high biodegradability and extremely low cytotoxicity (Teodorescu et al., 2019). Therefore, PVA and PVP are safe materials for single or multiple administrations into the skin, but the toxicity of PVA should be considered when microneedles are administered frequently.

3.4. In vitro delivery of PLA/CMC-DMNs into porcine cadaver skin

Fig. 8(a) shows a 2-dimensional z-stack image of FITC-dextran and RITC in a full thickness of porcine skin during the initial attachment of PLA/CMC-DMNs. The fluorescence signals represent the PLA tips and coated CMC formulation on tips of PLA/CMC-DMNs in porcine skin. The dotted line represents the tip of PLA/CMC-DMNs in porcine skin, and the white solid line represents the porcine skin surface. Also as shown in Fig. 8(a), PLA/CMC-DMNs were successfully delivered into the skin. RITC in the PLA tips appeared as a red fluorescence 400 μm below the skin surface, and the FITC in the CMC coating formulation was green at the same location. Fig. 8(b) and 8(b′) show a 3-dimensional fluorescence image of RITC-PLA tips and FITC-dextran in CMC formulation in porcine skin at 0 min after insertion. Fluorescence was not observed in other regions except for the region near the tips of the PLA/CMC-DMNs after delivery into the skin, showing that the coating formulation was not peeled-off or left on the skin surface during insertion. Fig. 8(c) shows a 3-

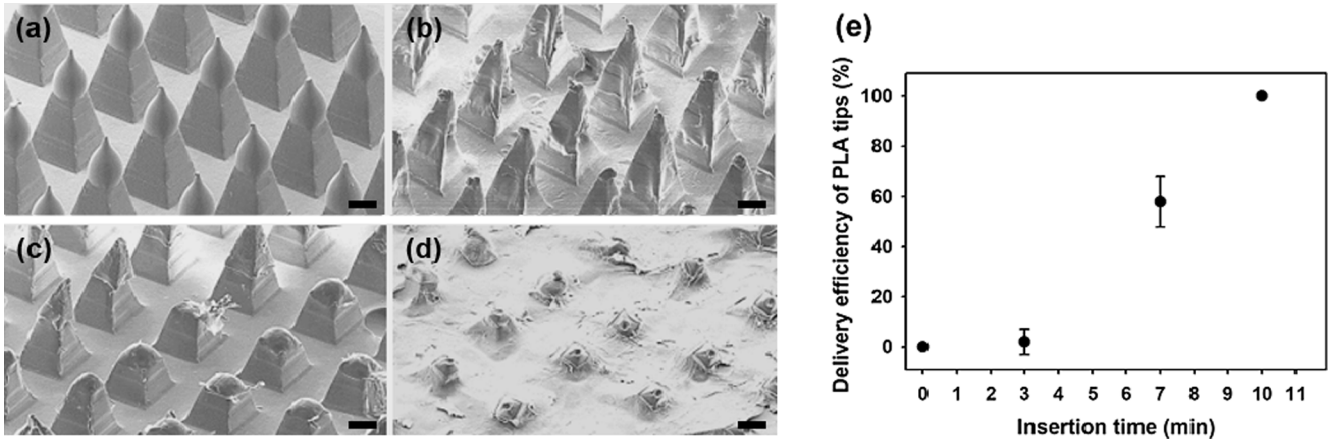

Fig. 7. SEM images of HBsAg-PLA/CMC-DMNs after insertion into porcine skin *in vitro* to observe gradual morphological changes of HBsAg-PLA/CMC-DMNs at different insertion durations. (a) HBsAg-PLA/CMC-DMNs before insertion, (b) at 3 min after insertion, (c) at 7 min after insertion, (d) at 10 min after insertion (scale bars, 200 μm). (e) Graph of delivery efficiency of PLA tips of HBsAg-PLA/CMC-DMNs (n = 5).

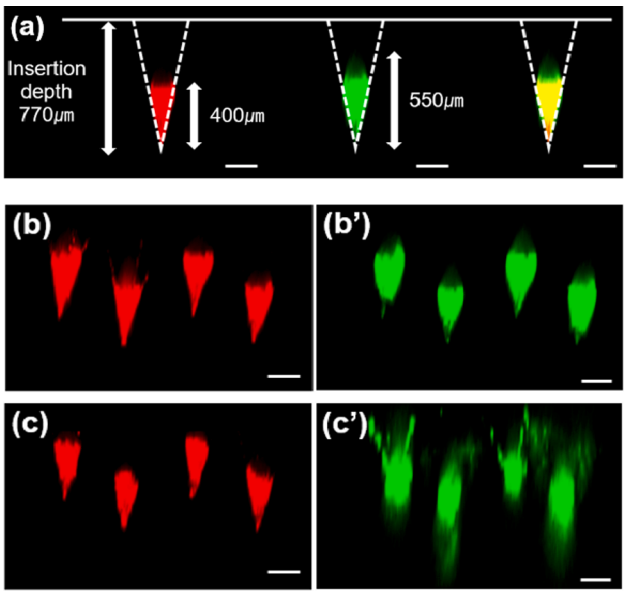

Fig. 8. (a) Two-dimensional z-stack image of RITC and FITC-dextran in a full thickness of porcine skin during attachment of PLA/CMC-DMNs. The dotted lines represent the tips of PLA/CMC-DMNs in porcine skin, and the white solid line represents the porcine skin surface. Three-dimensional fluorescence image of (b) RITC encapsulated in PLA tips and (b′) FITC in CMC coating formulation in porcine skin at 0 min after insertion. Three-dimensional fluorescence image of (c) RITC encapsulated PLA tips and (c′) FITC in CMC coating formulation in porcine skin at 10 min after insertion.

dimensional fluorescence image of RITC-PLA tips and FITC-dextran in CMC formulation in porcine skin at 10 min after insertion. There was no change in the fluorescence image of RITC in the PLA tips [(Fig. 8(c)], but FITC in the CMC formulation coated on the surface of the PLA tips diffused-out in porcine skin, as shown in Fig. 8(c′). The visualization of the different release rates of the two formulations shows the different diffusion patterns after insertion in the skin.

3.5. Characteristics of in vitro release of HBsAg from HBsAg-PLA/CMC-DMNs

HBsAg-CMC/PLA-DMNs contained 0.15 μg of HBsAg in the CMC coating formulation and 0.15 μg of HBsAg in the PLA tips. Thus, the total amount of vaccine used in the release experiment was 0.3 μg. As shown in Fig. 9(a), the HBsAg in the CMC- coating formulation released- out within 21 min as the CMC dissolved. As shown in Fig. 9(b), the PLA tips released an undetectable amount of HBsAg for 2 weeks. Subsequently, about 57% of the total HBsAg in the PLA tips was detected between 15 and 55 days and 2 ng of HBsAg per day got released during this period by the hydrolysis of the tips (Qi et al., 2017). The molecular weight of PLA was 40,000, and biodegradation proceeded slowly for several months (Beslikas et al., 2011). The release rate was controlled through the change in PLA molecular weight and the use of the co-polymers PLA and PGA (Li et al., 2019). Thus, the use of a single product featuring an array of HBsAg-PLA/CMC-DMNs accomplished both bolus release and slow release of the vaccine. As shown in Fig. 9(b), under the molding condition at 1000 rpm of centrifugation, HBsAg was encapsulated inside solidified PLA. However, if the rotation speed of centrifugation is increased, the aqueous HBsAg solution can cause phase separation from

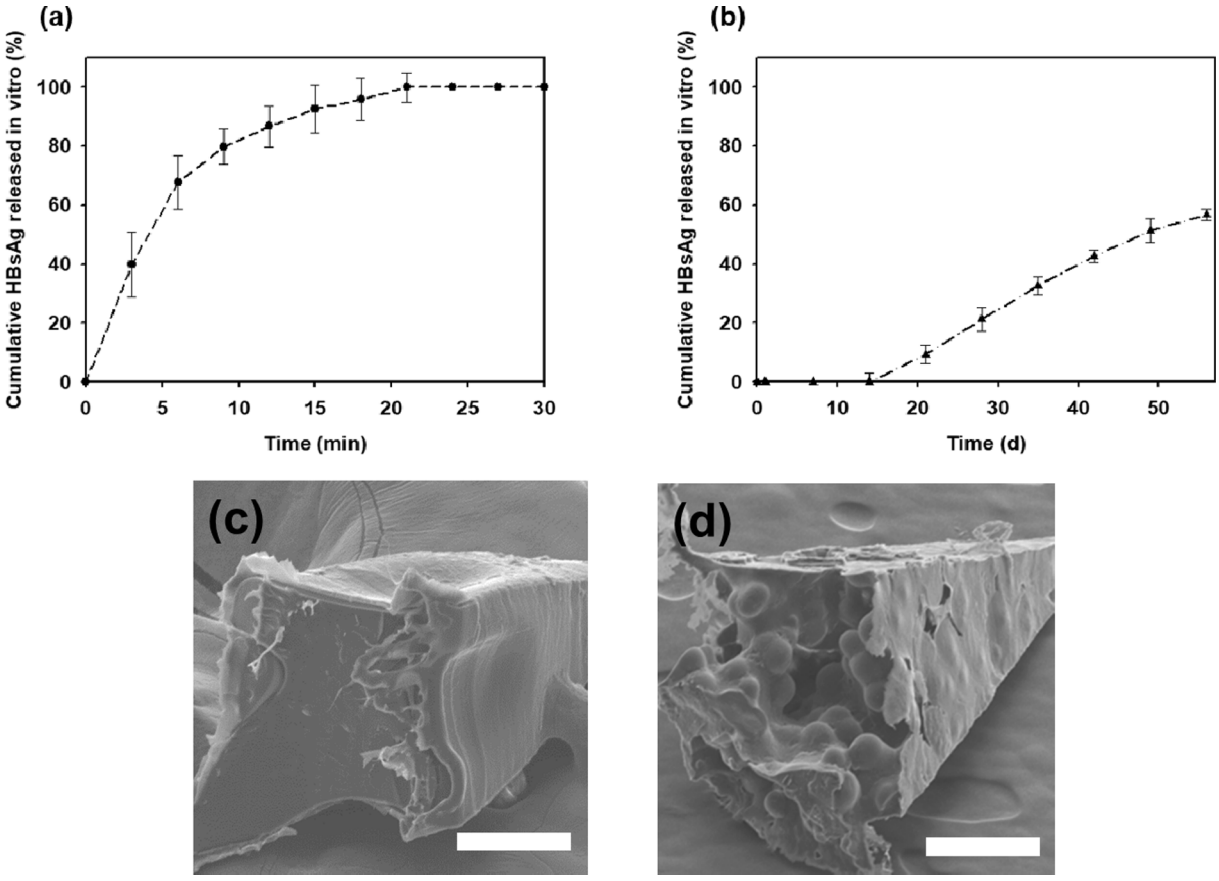

Fig. 9. Release profiles of (a) HBsAg from CMC coating formulation of HBsAg-CMC-DMNs and from (b) PLA tip (HBsAg-PLA -DMNs) (n = 5). SEM images of cross-section of PLA tip (c) before *in vitro* release test and (d) at 56 days (scale bars, 100 μm).

the PLA solution. A surfactant was not used in this study because it could affect the antigenicity of HBsAg.

A cross-section of sampled PLA tips was obtained by breaking the frozen sample to observe the morphology using SEM. Fig. 9(c) shows the bottom surface of a PLA tip at the beginning of the *in vitro* release test. Fig. 9(d) shows the cross-section of a PLA tip sampled at 8 weeks. After 8 weeks, the PLA tip retained its shape with holes outside and pores inside generated by the release of HBsAg solid formulation. The water-soluble particles with HBsAg were covered with PLA film, so unmeasurable HBsAg was released initially. The later release of inside HBsAg solid formulation was detected by the hydrolysis of PLA outside film and access of water inside tips.

3.6. *In vivo* vaccination study

As shown in Fig. 10, when 0.15 μg of HBsAg with aluminum hydroxide (Alum) was delivered twice by IM administration, the IgG titer was higher than that resulting from IM administration once with 0.3 μg of HBsAg with Alum. This result corresponds with previous studies of the boosting effect of HBsAg. Compared with a group that received two shots of 0.15 μg-HBsAg/Alum by IM administration, a group that received two shots of 0.15 μg-HBsAg-CMC with CMNs showed greater immunological response. The increased IgG titer that resulted from the use of CMNs was caused by immune cells located in the skin layer. However, the immune response did not occur after a single administration of 0.3 μg HBsAg-PLA-DMNs. As shown in the results of the *in vitro* release experiment, the initial amount released was unmeasurable, and 2 ng of HBsAg was released per day after 15 days. Thus, the low initial amount of HBsAg administered and the slow release rate were not sufficient to generate a primary immunological response. One shot of 0.3 μg-HBsAg-PLA/CMC-DMNs induced a higher immunological response than two shots of 0.15 μg-HBsAg/Al-IM and one shot of 0.3 μg-HBsAg/Al-IM. At 8 weeks, the antibody titer resulting from one shot of 0.3 μg-HBsAg-PLA/CMC-DMNs was compatible with that resulting from two shots of 0.15 μg-HBsAg-CMC-CMNs. The bolus release of HBsAg from the CMC-coating formulation produced an effect of immune priming, and the HBsAg released from the PLA tips gave a boosting effect even

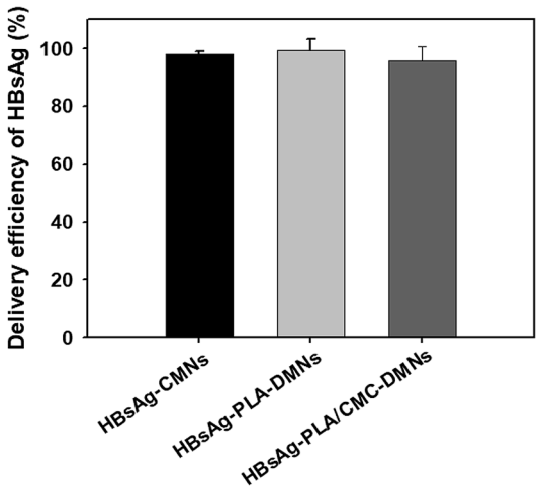

Fig. 11. Delivery efficiency of HBsAg of HBsAg-CMNs, HBsAg-PLA-DMNs, and HBsAg-PLA/CMC-DMNs by *in vivo* test (n = 5).

though small amounts of HBsAg were released slowly (Machluf et al., 2000; Pandit et al., 2007; Zheng et al., 2010).

The antibody titers of most groups, including the HBsAg-CMC-CMNs group, increased for 6 weeks and then decreased, whereas the antibody titer of HBsAg-PLA/CMC-DMNs increased continuously and showed the highest average value at 8 weeks. Compared with the results of one shot of 0.3 μg-HBsAg/Al-IM, one shot of 0.3 μg-HBsAg-PLA/CMC-DMNs induced a significant increase in immunological response as a result of the sustained release of HBsAg from PLA tips. PLA carrier-based vaccines were developed because of the U.S. Food and Drug Administration (USFDA) approved biocompatibility and controlled rates of bioerosion and release (Peres et al., 2017). A continuous delivery for a long period to antigen presenting cells (APCs) induces improved immunological response through continuous stimulation of T cells (Alonso et al., 1993). Pulsatile poly-lactic-co-glycolic acid (PLGA) microspheres released in

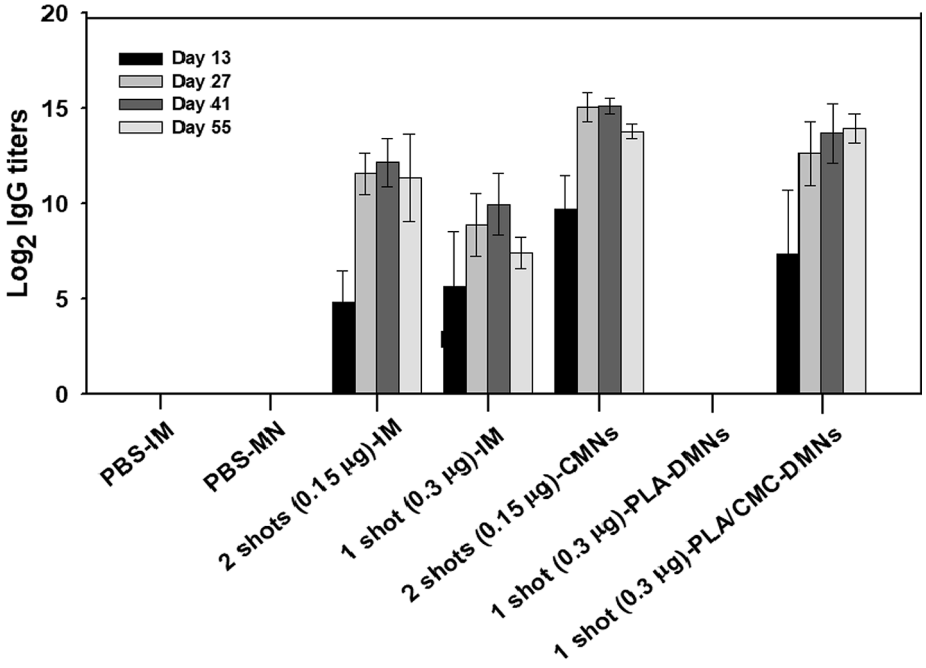

Fig. 10. IgG titer after single shot (Day 0) or primary (Day0) + boost (Day14) immunizations.

three pulses showed antibody titer similar to that of three bolus injections (Guarecuco et al., 2018). However, sustained or pulsatile vaccine delivery using a polymer carrier showed a continuous release of antigen. In this study, the combination of bolus release and continuous release of antigen was introduced to mimic two shots. As described above, delivery of the bolus release and sustained release formulations can be useful for vaccines that require multiple shots.

As shown in Fig. 11, when the HBsAg remaining on the MNs was recovered after the animal experiments and the amount of HBsAg was measured, more than 95% delivery efficiency was achieved by all MN groups. *Delivery efficiency* refers to the ratio of the amount of HBsAg delivered into the skin to the amount of HBsAg loaded on the microneedles. Thirty minutes of wear time for all MN groups was sufficient to make successful delivery.

4. Conclusion

In this study, 1) HBsAg was encapsulated in sustained-released PLA tips (HBsAg-PLA) and a bolus-release CMC-coating formulation (HBsAg-CMC) of HBsAg-PLA/CMC-DMNs, and 2) two formulations were delivered into the skin within 10 min of administration by rapid dissolution of the PVA/PVP base (DMNs) of HBsAg-PLA/CMC-DMNs. After intradermal delivery, the dual-release pattern of HBsAg—bolus delivery from HBsAg-CMC, and sustained delivery from HBsAg-PLA—were obtained. In order to prepare microneedles with dual-release pattern (PLA/CMC-DMNs), a micromolding method for dissolving microneedles (DMNs) and a dip-coating method for coated microneedles (PLA/CMC) were manufactured together. The CMC formulation enabled the HBsAg to be released within 20 min, and this bolus release showed an excellent effect of immune priming in animal experiments. The dissolving PLA tips released HBsAg more slowly by hydrolytic degradation of the tips, but the amount of HBsAg released was not sufficient for primary immunization. However, a small amount of HBsAg released over 55 days showed a boosting effect after inducing primary immunization. One shot of HBsAg-PLA/CMC-DMNs showed improved immunogenicity compared to two shots of conventional injection formulation containing Alum (HBsAg-Al-IM) and comparable immunogenicity to two shots of microneedle administration of HBsAg (HBsAg-CMNs). PLA/CMC-DMNs can deliver two formulations simultaneously during 10 min of insertion into the skin. Thus, the single product of PLA/CMC-DMNs can be used for the delivery of drugs requiring dual drug-delivery pattern. Therefore, in addition to vaccines, dual-release pattern microneedles can be used for drugs that require simultaneous and sustained release delivery, such as drugs used to treat diabetes and Alzheimer's disease.

Funding

This work was supported by the Gachon University research fund of 2019 (GCU-2019-0814), Korea Ministry of Trade, Industry & Energy, South Korea (MOTIE, 10067809 (Industrial Strategic Technology Development Program)) and Korea Ministry of Health and Welfare, South Korea (MoHW, HI15C2971 (Technology Development Program of Responding to Infectious Disease)).

CRediT authorship contribution statement

Ji Seok Kim: Resources, Formal analysis. **Jung-ah Choi:** Resources, Formal analysis. **Jong Chan Kim:** Resources, Validation. **Hayan Park:** Resources. **Eunji Yang:** Resources. **Ji Sun Park:** . **Manki Song:** Conceptualization, Funding. **Jung-Hwan Park:** Conceptualization, Funding.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [PJH is an inventor of patents that have been licensed to companies developing microneedle-based products and he is a shareholder of companies developing microneedle-based products.].

Acknowledgement

We appreciate LG Chem Ltd. to provide HBsAg vaccine and Quad-Medicine to provide a microneedle master structure.

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