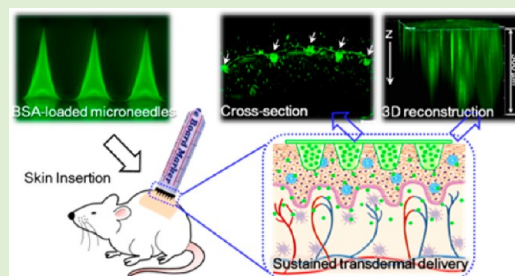


Chitosan Microneedle Patches for Sustained Transdermal Delivery of Macromolecules

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ABSTRACT: This paper introduces a chitosan microneedle patch for efficient and sustained transdermal delivery of hydrophilic macromolecules. Chitosan microneedles have sufficient mechanical strength to be inserted *in vitro* into porcine skin at approximately 250 μm in depth and *in vivo* into rat skin at approximately 200 μm in depth. Bovine serum albumin (BSA, MW = 66.5 kDa) was used as a model protein to explore the potential use of chitosan microneedles as a transdermal delivery device for protein drugs. *In vitro* drug release showed that chitosan microneedles can provide a sustained release of BSA for at least 8 days (approximately 95% of drugs released in 8 days). When the Alexa Fluor 488-labeled BSA (Alexa 488-BSA)-loaded microneedles were applied to the rat skin *in vivo*, confocal microscopic images showed that BSA can gradually diffuse from the puncture sites to the dermal layer and the fluorescence of Alexa 488-BSA can be observed at the depth of 300 μm . In addition, encapsulation of BSA within the microneedle matrix did not alter the secondary structure of BSA, indicating that the gentle nature of the fabrication process allowed for encapsulation of fragile biomolecules. These results suggested that the developed chitosan microneedles may serve as a promising device for transdermal delivery of macromolecules in a sustained manner.



INTRODUCTION

Transdermal drug delivery is successful in a number of applications, including hormone replacement therapy, smoking cessation, and pain management;¹ however, it is limited to a narrow range of compounds that easily pass through the skin. Several challenges have been encountered in expanding use of the technology to the delivery of hydrophilic macromolecules, such as peptides, proteins, and vaccines. These biopharmaceuticals cannot permeate the outermost layer of the skin, the stratum corneum, at sufficient levels to achieve a considerable therapeutic effect. Although chemical enhancers and mechanical abrasion can increase drug permeation, they may irritate or cause damage to the skin.¹ Therefore, the challenge of creating an effective transdermal delivery system involves breaking the skin barrier for drug transport without irritating the skin.

The use of needles with microscale dimensions in increasing skin permeability to drugs has effectively facilitated transdermal delivery.^{2–5} Microneedle technology offers a minimally invasive route of drug administration. This technology involves the creation of reversible microchannels in the skin,⁶ thereby enabling the delivery of a broad range of therapeutic macromolecules that cannot permeate intact skin. Because these needles have micrometer-sized dimensions, microneedles caused significantly less pain and tissue damage than a 26-gauge hypodermic needle. The pain sensation caused by microneedles is dependent on the insertion depth and the numbers of microneedles.⁷

Recently, polymeric microneedles made from biodegradable or dissolving polymers have received considerable attention.^{4,5,8–10} Microneedles made of water-dissolving polyvinyl pyrrolidone (PVP),^{4,5} polyvinyl alcohol (PVA),⁵ amylopectin, carboxymethyl cellulose (CMC),⁹ and maltose¹⁰ can efficiently deliver protein

drugs into the skin. However, these microneedles dissolve quickly upon contact with water in the skin and result in excessively rapid drug delivery.^{9,11} Biodegradable microneedles made of poly-L-lactic acid (PLA), poly glycolic acid (PGA), or copolymer (PLGA)⁸ can offer extended drug release. However, harsh fabrication methods (i.e., using high temperature or organic solvent) for these microneedle systems may damage temperature-sensitive drugs, particularly peptides and proteins.

This study developed a biodegradable microneedle patch made of chitosan for efficient and sustained transdermal delivery of macromolecular drugs (Figure 1). Specifically, human growth hormone therapy^{3,12} and vaccines which may require long exposure time¹³ will benefit from this delivery device.¹⁴ As a drug delivery system, chitosan has attracted increasing attention because of its excellent biocompatibility, degradability, and nontoxicity. Drugs loaded in chitosan carriers can be released through swelling and degradation of the chitosan matrix, leading to a clear sustained-release effect.¹⁵ Chitosan of suitable molecular weight can be cleared by the kidney *in vivo*, whereas that of excessive molecular weight can be degraded by proteases into fragments suitable for renal clearance.¹⁶ Its safety has been demonstrated in both animal models and humans for use as dietary supplements or drug carriers.^{17–21} Under weakly acidic conditions, an aqueous chitosan solution can be easily obtained by protonation of its amino groups (pK_a value of approximately 6.5) to confer positive charges, gelation, and membrane-forming properties.¹⁵ Therefore, chitosan-based delivery systems offer

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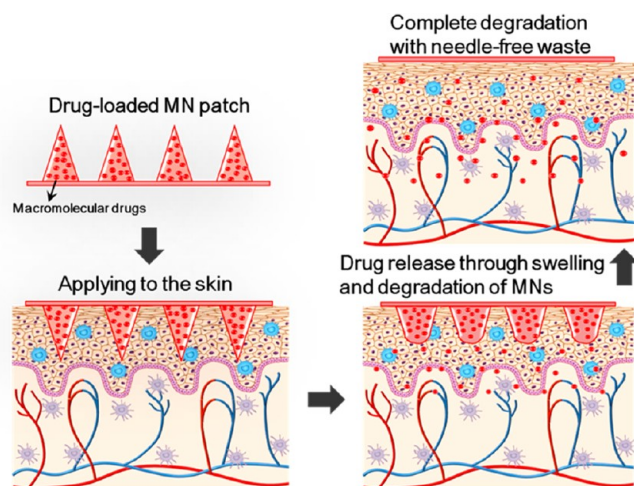


Figure 1. Schematic illustrations of transdermal delivery of macromolecules using chitosan microneedle (MN) patches.

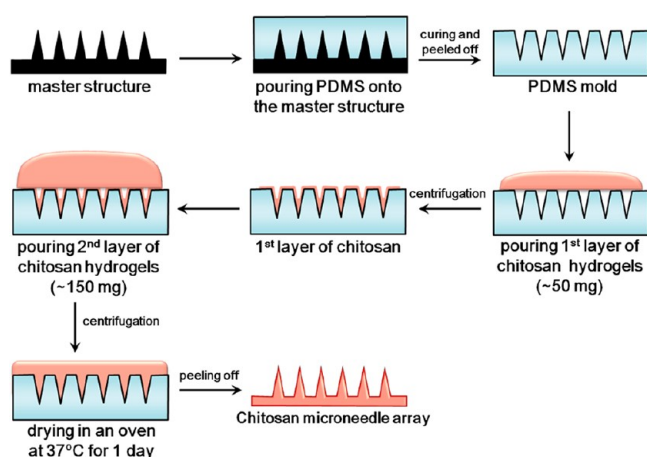


Figure 2. Schematic illustrations of chitosan microneedle fabrication process.

advantages over other hydrophobic polymers (such PLA, PGA, or PLGA) by avoiding organic solvents or harsh conditions of

heat for encapsulation of biopharmaceuticals. These remarkable properties make chitosan a potential candidate as a needle material.

A double casting process was used to produce mechanically robust chitosan microneedles. This fabrication process was modified from previous studies^{9,14} and performed by casting concentrated chitosan hydrogels into a mold during centrifugation. Additionally, the mechanical properties, skin insertion abilities, and in vitro drug delivery properties of the fabricated microneedles were investigated. To assess the feasibility of using chitosan microneedles for transdermal delivery of macromolecules, Alexa Fluor 488-labeled bovine serum albumin (Alexa 488-BSA) was encapsulated within microneedles and its in vivo transdermal transport behavior was investigated in a rat model. Finally, changes in BSA secondary structure before and after encapsulation in chitosan microneedle patches were measured using circular dichroism (CD) spectropolarimetry.

EXPERIMENTAL SECTION

Materials. Chitosan (approximately 80% deacetylated, viscosity ≥ 400 mPa·s, 1% in 1% acetic acid at 20 °C), Rhodamine 6G (MW = 479 Da) and BSA (MW = 66.5 kDa) were obtained from Sigma-Aldrich (St. Louis, MO). Polydimethylsiloxane (PDMS; Sylgard 184), Alexa 488-BSA, and Optimum Cutting Temperature compound (OCT) were purchased from Dow Corning (Midland, MI), Invitrogen (Eugene, OR), and Tissue-Tek (Sakura Finetek, Torrance, CA), respectively. All chemicals were used as received without additional treatment.

Fabrication of Chitosan Microneedles. Three pyramidal microneedle master structures of various sizes were created using an electro-discharge machining process (Micropoint Technologies Pte, Ltd., Singapore). Each master structure consisted of 225 (15 × 15) pyramidal needles and the tip-to-tip distance of all samples was 500 μ m. Microneedle molds were made from PDMS to inverse-replicate these master structures precisely. To prevent adhesion with PDMS molds, the master structures were sputter-coated with platinum at 15 mA for 120 s (JFC-1600 Auto Fine Coater, JEOL, Japan). PDMS microneedle molds were made by pouring PDMS solutions over the microneedle master structure and allowing the polymer to cure overnight at room temperature. The cured PDMS molds were subsequently peeled from the master structures and repeatedly used to make chitosan microneedles (Figure 2).

To serve as the microneedle matrix, chitosan powder was first dissolved in a 1% (v/v) aqueous solution of acetic acid to obtain a 2% (w/v) chitosan solution. The obtained viscous chitosan solution was subsequently dialyzed (MWCO: 14 kDa, BioDes, Inc., Hauppauge, NY) at room temperature against deionized (DI) water for 48 h with several

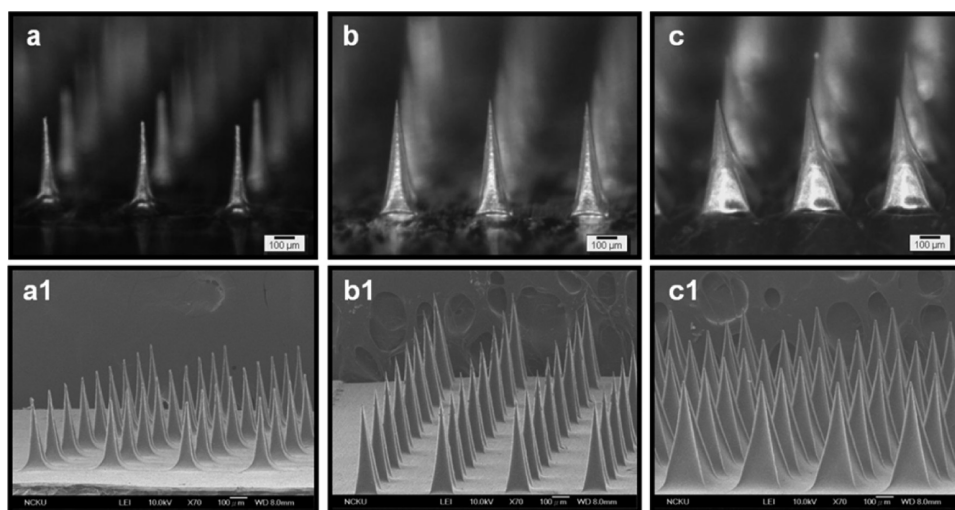


Figure 3. Chitosan microneedle arrays molded from three different master structures. Bright-field (a–c) and scanning electron (a1–c1) micrographs of P1 (a and a1), P2 (b and b1), and P3 (c and c1) microneedles.

water exchanges to remove excess acetic acid (final pH of approximately 6.0). This near-neutral chitosan solution was purified by filtration, and water was subsequently evaporated until the concentration of the chitosan solution was approximately 10 wt %, which resulted in a viscous hydrogel. The solute concentration in the solution was determined by weighing the solution mass before and after evaporation. This concentrated chitosan hydrogel was used for the casting process.

A double casting process was used to mold microneedles from concentrated chitosan hydrogels (Figure 2). A first layer consisting of approximately 50 mg of chitosan hydrogel was placed on a PDMS mold in a centrifuge tube and centrifuged in a swinging bucket rotor (221.12 V03, Hermle Labortechnik GmbH, Wehingen, Germany) at 4500 rpm (3350g) at 30 °C for up to 1 h to fill the microneedle mold cavities. This horizontal centrifugation helped to push the solution into the mold holes. A second layer of chitosan hydrogels (approximately 150 mg) was subsequently placed on the centrifuged first layer, followed by further centrifugation at 4500 rpm at 30 °C for up to 2 h. This double casting process was required to fill the mold cavities with more chitosan to produce robust chitosan microneedles. The filled mold was dried in an oven at 37 °C. During the oven-drying process, the weight of the samples was monitored using an accurate microbalance at different time intervals until a constant weight was obtained. The water content of a dry sample was less than 0.1 wt %. Finally, the chitosan microneedle array was gently peeled out of the mold. The resulting chitosan microneedles were examined using a stereomicroscope (SZ-61, Olympus, Olympus Corporation, Tokyo, Japan) and a scanning electron microscope (SEM, Hitachi S-4000, Tokyo, Japan).

Fabrication of Drug-Loaded Microneedles. A modified casting process was developed to encapsulate model drugs within the microneedles. BSA or Alexa 488-BSA was mixed with the dialyzed chitosan solution (2 wt %, pH of approximately 6.0) and the mixture was further concentrated at 37 °C to form a viscous hydrogel containing 0.15 wt % of drugs. The drug-loaded hydrogel was applied onto the PDMS mold as the first layer only, and the same centrifugation process as presented above was used to fill the mold cavities. The second layer of chitosan hydrogels without drugs was also applied to the mold to produce a complete microneedle array. Finally, the sample was dried in an oven at 37 °C for 24 h. The same fabrication process was also used to encapsulate Rhodamine 6G in the microneedles.

Mechanical Property Test. Mechanical compression tests were performed using a universal testing machine (AGS-500NX, Shimadzu Corporation, Kyoto, Japan). A microneedle array was placed on the flat rigid surface of a stainless steel base plate. An axial force was applied by a mount of a moving sensor, perpendicular to the axis of the microneedle array, at a constant speed of 66 mm/min.⁹ The initial distance from the tips of the microneedle arrays to the mount was set at 1 cm. The force was measured when the moving sensor touched the uppermost point of the microneedle array. The testing machine subsequently recorded the force required to move the mount as a function of microneedle displacement.

Imaging of Microneedle Insertion: In Vitro and in Vivo. To compare the skin insertion capability of three pyramidal microneedles, Rhodamine 6G-loaded chitosan microneedles were inserted into porcine cadaver skin using a constant force. These microneedles were inserted by pressing against their backing layer with the universal testing machine, starting at an initial distance of 1 cm from the skin using a force of 0.4 N/needle for 10 min; the microneedles were removed after insertion. The skin was subsequently washed under running water and wiped to ensure that no red dye (Rhodamine 6G) was deposited on the skin surface. Finally, the skin was imaged under a stereomicroscope to calculate the insertion ratio and subsequently excised for histological sections to determine the insertion depth. The insertion ratio for each test sample was calculated by dividing the number of red spots on the skin after insertion by the number of needles in the arrays. The insertion depth was defined as the average penetration of needles which can be successfully inserted into the skin of five microneedle patches.

To evaluate the possibility of insertion of chitosan microneedles by a homemade applicator with an application force of ~9.5 N/patch, microneedle patches were fixed on the applicator first and then pressed manually against the skin for 5 min. The microneedle insertion site on

Table 1. Detailed Dimensions of Fabricated P1, P2, and P3 Chitosan Microneedles and Their Insertion Ratio and Depth Evaluated in a Porcine Cadaver Skin under a Constant Force

samples (<i>n</i> = 5)	P1 microneedles	P2 microneedles	P3 microneedles
Base width (μm)	150	200	300
Height (μm)	450	600	600
Tip radius (μm)	10	5	5
Aspect ratio	3	3	2
Insertion ratio (%)	67.0 ± 9.6	90.1 ± 6.9	98.0 ± 3.5
Insertion depth (μm)	55.0 ± 7.9	104.6 ± 6.9	150.8 ± 5.5

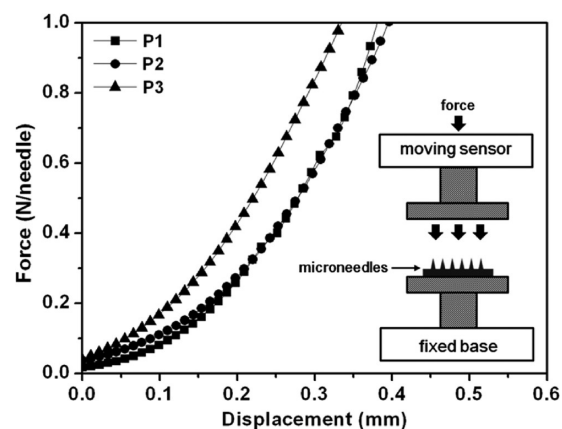


Figure 4. Results obtained in the compression test for P1, P2 and P3 chitosan microneedles. Inset shows the schematic illustration of the apparatus used.

the skin surface was exposed to blue tissue-marking dye (Shandon, Richard-Allan Scientific, Kalamazoo, MI) for 1 min to identify the sites of stratum corneum perforation. After washing the skin and wiping residual dye from the skin surface, skin sections were processed for histological evaluation.

To prepare histological specimens, microneedle insertion sites were excised from bulk skin using a scalpel. Each isolated skin section was embedded in OCT compound in a cryostat mold and frozen in liquid nitrogen. The frozen OCT-skin samples were subsequently sliced into 5-μm thick sections using a cryotome (Shandon Cryotome E, Thermo Electron Corporation) and placed on silane-coated glass slides (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan). The skin sections were finally viewed using an inverted fluorescence microscope (IX-71, Olympus, Tokyo, Japan).

To visualize the holes made by microneedles in living rat skin, microneedles were inserted into the skin of a Sprague–Dawley (SD) rat by the homemade applicator, and a liquid bandage (Kobayashi Pharmaceutical Co., Ltd., Japan) was subsequently applied to the puncture sites for 5 min to create an inverse replica of the holes by in situ molding of the skin surface. The resulting films were gold-coated for SEM imaging.

In Vitro Transdermal Delivery of BSA to Porcine Cadaver Skins. BSA-loaded P3 microneedle patches were inserted into pig cadaver skins and secured to the skin using a dermal tape. The skin was subsequently placed in a sealed Petri dish with phosphate buffered saline under the skin to prevent skin dehydration for 8 days at 37 °C. At a specified time interval, the microneedle patches were removed from the skin and the insertion site was then tape-stripped three times consecutively with 3M Transpore tapes to remove the residual BSA on the skin surface. The amount of proteins delivered into the skin was determined based on a mass balance of three parameters: the total amount of BSA encapsulated in microneedles before insertion into skin, the amount of BSA remaining in microneedles after insertion, and the residual BSA left on the skin surface.

To determine the loading amount and residual amount of BSA in microneedles, the patches before and after skin insertion were separately

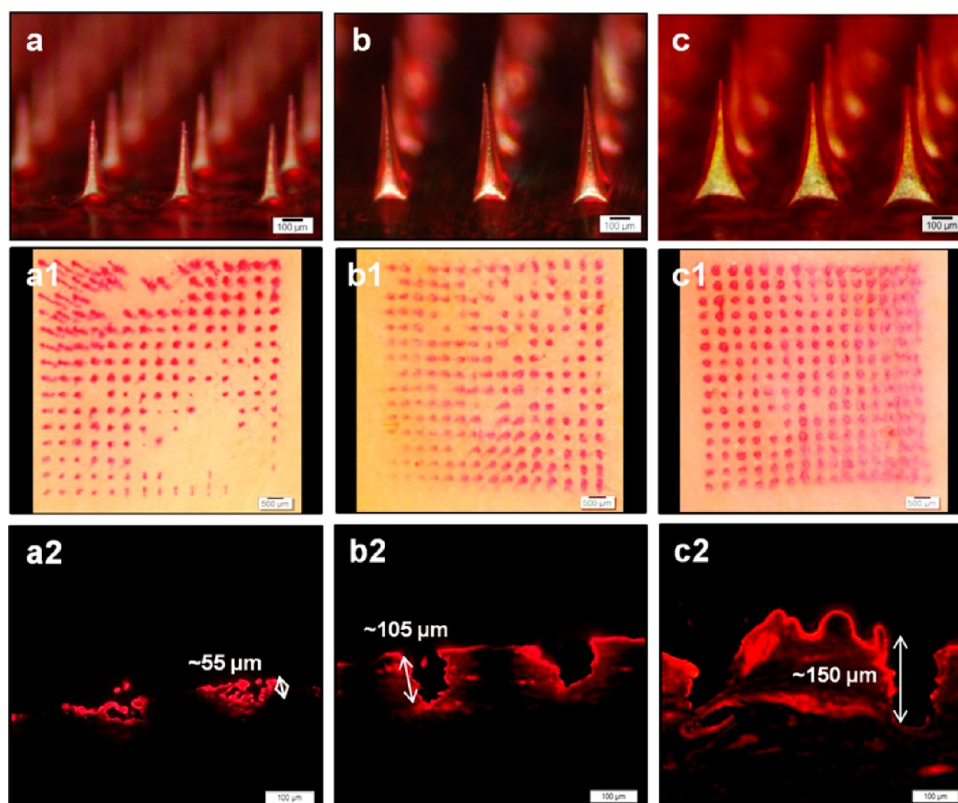


Figure 5. Skin insertion capability of Rhodamine 6G-loaded P1 (a–a2), P2 (b–b2), and P3 (c–c2) chitosan microneedles. Bright-field micrographs of Rhodamine 6G-loaded microneedles (a–c) and porcine cadaver skin after insertion of the microneedles (a1–c1). Fluorescence micrographs of histological sections of the skin pierced by the microneedles (a2–c2). The up down arrows in a2–c2 show the insertion depth of the microneedles.

dissolved in DI water with mixing at 4 °C for 2 days to release the BSA in the microneedles. The stripped tapes were also soaked in DI water for 1 h at 4 °C to recover the BSA on the tapes. The amount of BSA extracted from the microneedles or the stripped tapes was determined from the solution using the BCA Protein Assay Reagent Kit according to the instructions of the manufacturer (Pierce Chemicals, Rockford, IL). The amount of BSA delivered into the skin was calculated by subtracting the amount of BSA remaining in the microneedles after insertion and on the skin surface from the amount originally encapsulated in the microneedles at each sampling point.

In Vivo Transdermal Delivery of BSA to Rat Skins. All animal protocols were approved by the institutional animal care and use committee of National Cheng Kung University, and experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of National Cheng Kung University. Four-week-old male SD rats ($n = 5$ for each group), 200 ± 25 g, were used. The back hair was removed using an electric shaver under anesthesia with an intramuscular injection of a mixture of Zoletil 50 (35 mg/kg) and Rompun (2 mg/kg). Alexa 488-BSA-loaded P3 microneedle patches were administered to the back skin by the homemade applicator and fixed with dermal tape. The patches were removed from the skin after administration for 30 min, 1, 2, and 4 days. The microneedles and the skin surface after insertion were observed under a stereomicroscope.

To study the skin penetration of the Alexa 488-BSA model protein to the vertical direction of the rat skin, full-thickness skin was placed on a microscope slide and observed using a confocal laser scanning microscope (CLSM; FluoView FV1000, Olympus Corporation, Tokyo, Japan) at an excitation wavelength of 488 nm. Images were obtained in the xy -plane (parallel to the plane of the skin surface). The initially scanned skin surface ($z = 0 \mu\text{m}$) was defined as the imaging plane of the brightest fluorescence with a morphologic characteristic of the stratum corneum surface. Scanning was conducted once at the interval of $10 \mu\text{m}$ from the skin surface through the z -axis perpendicular to the xy -plane.²² The 3D confocal reconstruction images were obtained using the

xyz -stack to visualize the drug penetration into the skin. Subsequently, the skins were embedded in an OCT compound for cryosection, as described above, and examined histologically.

Stability of Encapsulated BSA. The secondary structure of BSA was examined using spectropolarimetry (JASCO, J-810, Tokyo, Japan) after encapsulation and release from chitosan microneedles. To extract BSA from the microneedles, BSA-loaded microneedle arrays were dissolved in 5 mL of DI water with mixing at 4 °C for 1 day. The concentration of BSA solution was determined using the BCA Protein Assay Reagent Kit. Subsequently, DI water was added to dilute BSA solution to $60 \mu\text{g/mL}$ for spectral measurements. CD spectra were obtained for untreated BSA, untreated BSA mixed with chitosan solution (pH of approximately 6.0), and BSA encapsulated in microneedles that were dissolved after 1 day of storage at 37 °C.

Statistical Analysis. A comparison between two groups was performed using the one-tailed Student's t -test using statistical software (SPSS, Chicago, IL). Data are presented as mean $\pm$ SD. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of Chitosan Microneedles. Micro-molding techniques have been developed to fabricate biomedical devices with microstructures for low cost, ease of processing, and mass production potential. However, simply filling microneedle PDMS molds with polymer solution and subsequently drying them does not produce solid and rigid needles. This may be attributed to a void structure formed in the microneedle matrix after water evaporation.⁹ To avoid this problem, we used a double casting method using a highly viscous chitosan hydrogel (approximately 10 wt %) to fill the mold twice. During the casting process, horizontal centrifugation was applied to push the hydrogel into the mold cavities and continuously compressed the

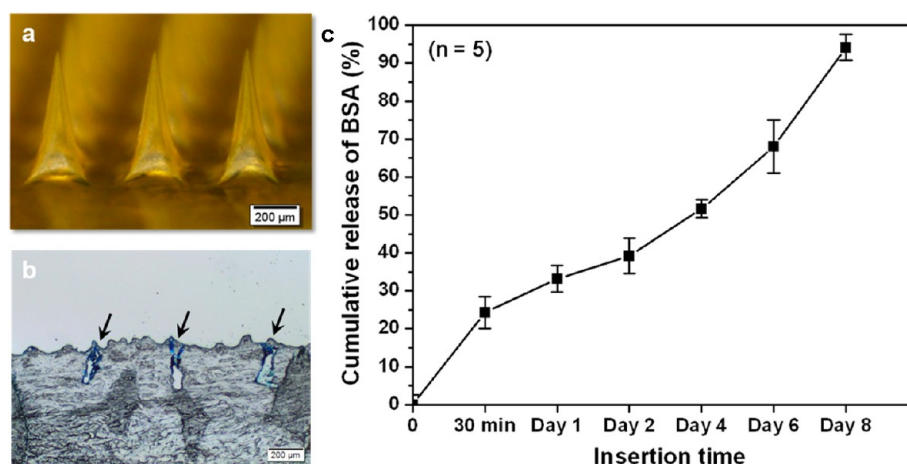


Figure 6. In vitro transdermal delivery of bovine serum albumin (BSA) to porcine cadaver skin using P3 microneedles. Bright-field micrograph of BSA-loaded microneedles (a). Histological section of skin puncture sites stained with blue tissue-marking dye (arrow) (b). In vitro drug release profiles (c): the BSA loading amount in the microneedles was $275 \pm 3 \mu\text{g}$ per patch ($n = 5$).

mold content to minimize void formation. Using this method, we successfully replicated solid chitosan microneedles from three pyramidal master structures (Figure 3), called P1, P2, and P3 microneedles. The detailed dimensions of each needle are shown in Table 1.

One of the main requirements for polymer microneedles is sufficient mechanical strength for skin insertion. Several factors affect the mechanical strength of microneedles, including the material composition, geometry, and aspect ratio.^{9,23,24} In this study, microneedles with a pyramidal shape were selected because of their superior mechanical strength as compared to conical microneedles. This may be attributed to their larger cross-sectional area at the same base width/diameter.⁹ Mechanical compression tests were performed using a universal testing machine to compare the mechanical properties of these three pyramidal microneedles. As shown in Figure 4, microneedles with a smaller aspect ratio (i.e., P3 microneedles) exhibited higher mechanical strength. P1 and P2 microneedles with the same aspect ratio and differing dimensions exhibited similar mechanical properties. These results indicated that the aspect ratio may be a crucial parameter in influencing the mechanical properties of microneedles.

Insertion Capability of Three Pyramidal Microneedles.

To compare the skin insertion ratio and insertion depth of fabricated pyramidal microneedles, Rhodamine 6G, a fluorescent red dye, was encapsulated within the P1, P2, and P3 chitosan microneedles for skin insertion (Figure 5a–c). After applying a constant force to press the Rhodamine 6G-loaded microneedles into a porcine cadaver skin and subsequently removing the microneedles, the skin surface exhibited an array of red spots corresponding to the sites of microneedle insertion (Figure 5a1–c1). These spots cannot be washed away or wiped off from the skin surface, indicating that the red dye was located within the skin. This was further confirmed by histological observations, which showed that the red dye released from the chitosan microneedles was deposited at the microneedle puncture sites (Figure 5a2–c2).

According to the resulting red spots (Figure 5a1–c1) and histological examination (Figure 5a2–c2), the insertion ratio and depth for each microneedle are shown in Table 1. As shown in the table, the P3 microneedle had the highest insertion ratio ($98.0 \pm 3.5\%$, $n = 5$) and the deepest insertion depth ($150.8 \pm 5.5 \mu\text{m}$, $n = 5$), which may be attributed to its superior mechanical

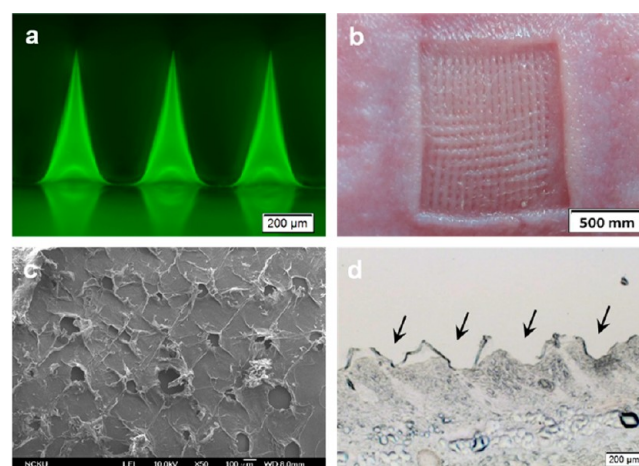


Figure 7. In vivo skin insertion capability of P3 microneedles evaluated in a rat model. Fluorescence micrographs of Alexa Fluor 488-labeled BSA (Alexa 488-BSA)-loaded microneedles (a). Bright-field micrograph of microneedle puncture marks on the skin (b). Scanning electron micrograph of an inverse replica of skin surface after insertion and removal of microneedles (c). Histological section of skin puncture sites (arrow) (d).

strength. However, although the results of the compression test showed that the other two microneedle designs exhibited similar mechanical strength (Figure 4), the P1 microneedle exhibited considerably inferior insertion capability to the P2 microneedle. This may be attributed to its relatively blunt tip (tip radius of $10 \mu\text{m}$) compared to that of the P2 microneedle ($5 \mu\text{m}$). This result was consistent with previous studies, which found that the skin can be punctured more easily when the microneedle tip sharpness is increased.^{7,23,25}

These results indicated that P3 microneedles had the highest mechanical strength and greatest insertion capability. Therefore, P3 microneedles were chosen for subsequent studies.

In Vitro Transdermal Delivery of Model Protein to Porcine Cadaver Skins. To study the release profiles of encapsulated proteins from chitosan microneedles, we inserted BSA-loaded microneedle patches (Figure 6a) into the porcine cadaver skin by the homemade applicator and subsequently monitored drug release for 8 days. The histological section of the skin stained with tissue-marking dye shows the shape of needle

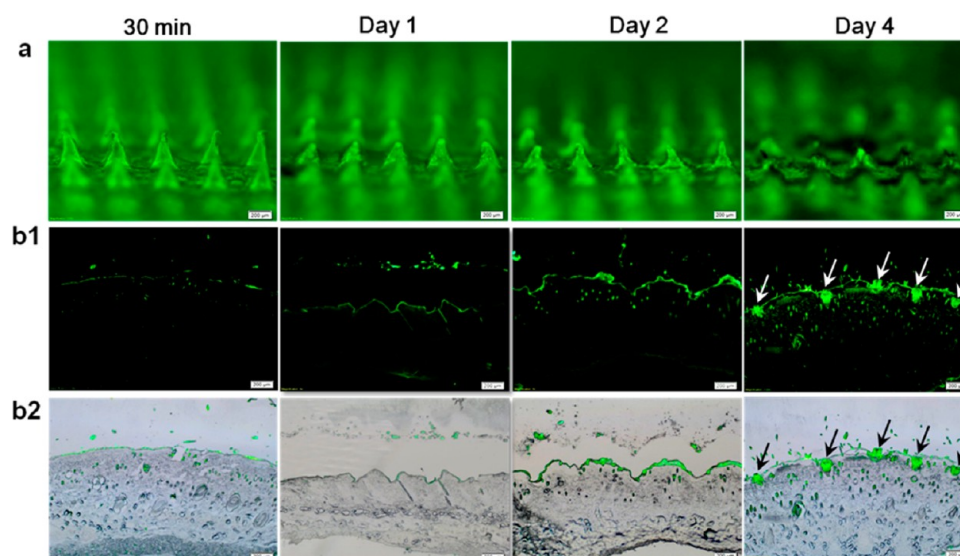


Figure 8. In vivo transdermal delivery of Alexa 488-BSA (green) to the back skin of Sprague–Dawley (SD) rats using P3 microneedles for 30 min, 1, 2, and 4 days. Fluorescence micrographs of Alexa 488-BSA-loaded microneedles after being removed from the skin (a). Fluorescence images (b1) and merged images of bright-field and fluorescence (b2) of histological sections of skin puncture sites. The arrows in b1 and b2 show the fragments of the microneedles left in the skin.

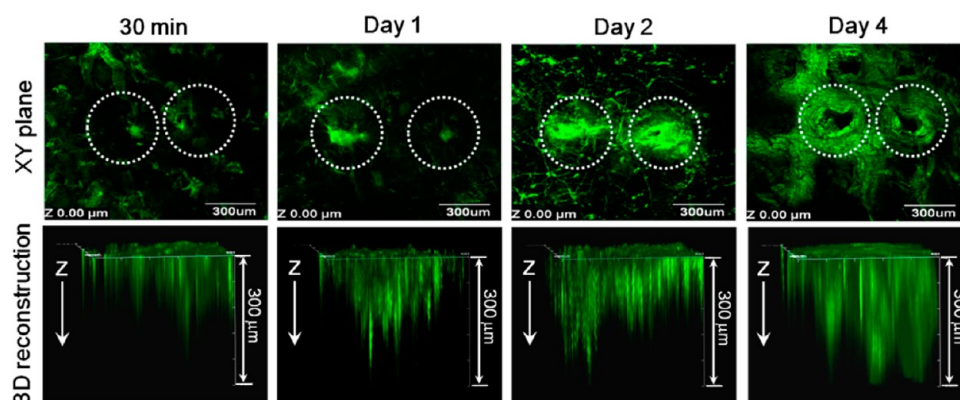


Figure 9. Penetration of Alexa 488-BSA (green) within the rat skin pierced by the P3 microneedles for 30 min, 1, 2, and 4 days. Confocal micrographs of skin surface (upper panel) showing a gradual diffusion of Alexa 488-BSA from the puncture sites (dash circle) to the surrounding skins. 3D confocal reconstruction images of skins (lower panel) showing penetration depth of Alexa 488-BSA in the skin.

penetration pathways and an insertion depth of approximately 250 μm (Figure 6b).

The release of BSA from microneedles exhibited an initial burst release within the first 30 min, followed by a slow and sustained release over time (Figure 6c). This release behavior can be explained by a set of interacting phenomena. First, when chitosan microneedles were inserted into the skin, the hydrophilic BSA located at or close to the surface of the microneedle may be rapidly dissolved by skin interstitial fluid, resulting in an initial burst release. Subsequently, the drug molecules may gradually diffuse out from the chitosan matrix because of hydration and swelling of the microneedles by the skin interstitial fluid. This was confirmed by microscopic examination, which showed that microneedle swelling occurred after skin insertion (image not shown). As shown in Figure 6c, chitosan microneedles provided sustained release of BSA for at least 8 days, with approximately 95% of the BSA released during this period.

In Vivo Transdermal Delivery of Model Protein to Rat Skins. To evaluate the insertion capability of chitosan microneedles in vivo, an Alexa 488-BSA-loaded microneedle patch

(Figure 7a) was inserted into the back skin of SD rats for 5 min. After removal of the microneedle patch, the skin surface exhibited the puncture marks caused by the microneedles (Figure 7b). A liquid bandage was subsequently applied to the punctured sites to create an inverse replica of the skin surface. Figure 7c shows the skin puncture holes with a diameter of approximately 100–200 μm made by the microneedles. A histological examination of the skin showed a microneedle penetration depth of approximately 200 μm , which corresponded to insertion across the stratum corneum and viable epidermis and into the superficial dermis (Figure 7d). These results demonstrated that chitosan microneedles are sharp and sufficiently strong to be inserted into living skin by a homemade applicator with an application force of ~ 9.5 N/patch.

To characterize the penetration kinetics of protein within the skin, Alexa 488-BSA-loaded microneedle patches were administered to the rat skin for 30 min, 1, 2, and 4 days. After peeling off the patch from the skin, the microneedles were examined using an inverted fluorescence microscope. As shown in Figure 8a, microneedles gradually became smaller and shorter as the insertion time increased. The long-term contact with skin interstitial

fluid can result in swelling and softening of chitosan micro-needles. Therefore, this decrease in the size of microneedles may be because some parts of the swollen chitosan matrix are broken and left in the skin when we remove the patch. A histological examination of the skin showed Alexa 488-BSA diffused from the

sites of skin puncture to deeper regions of the skin over time (Figure 8b1 and b2). Fragments of microneedles remaining in the skin (indicated by the arrow in Figure 8b1 and b2) were observed in the histological section of skin at Day 4 post-administration.

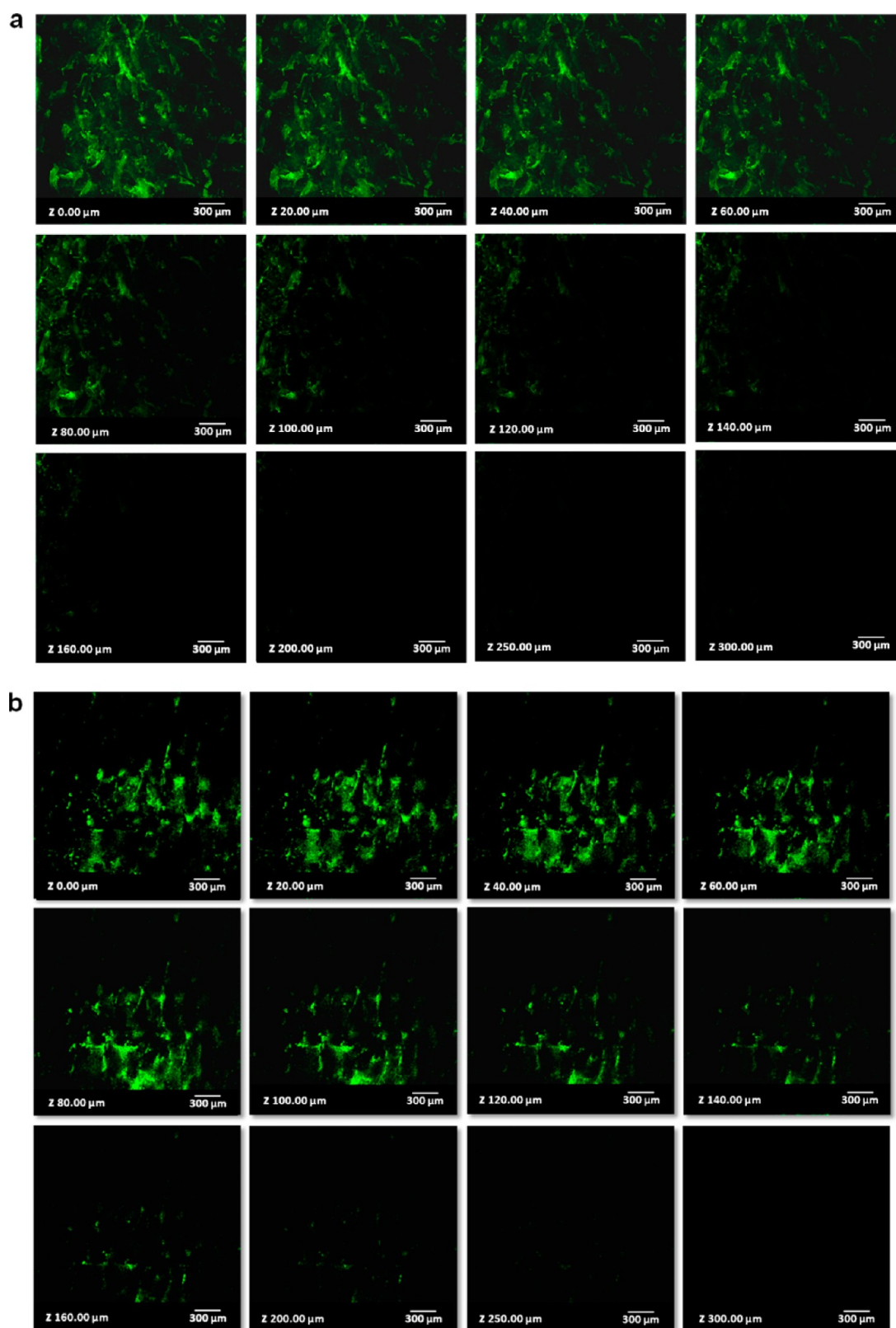


Figure 10. continued

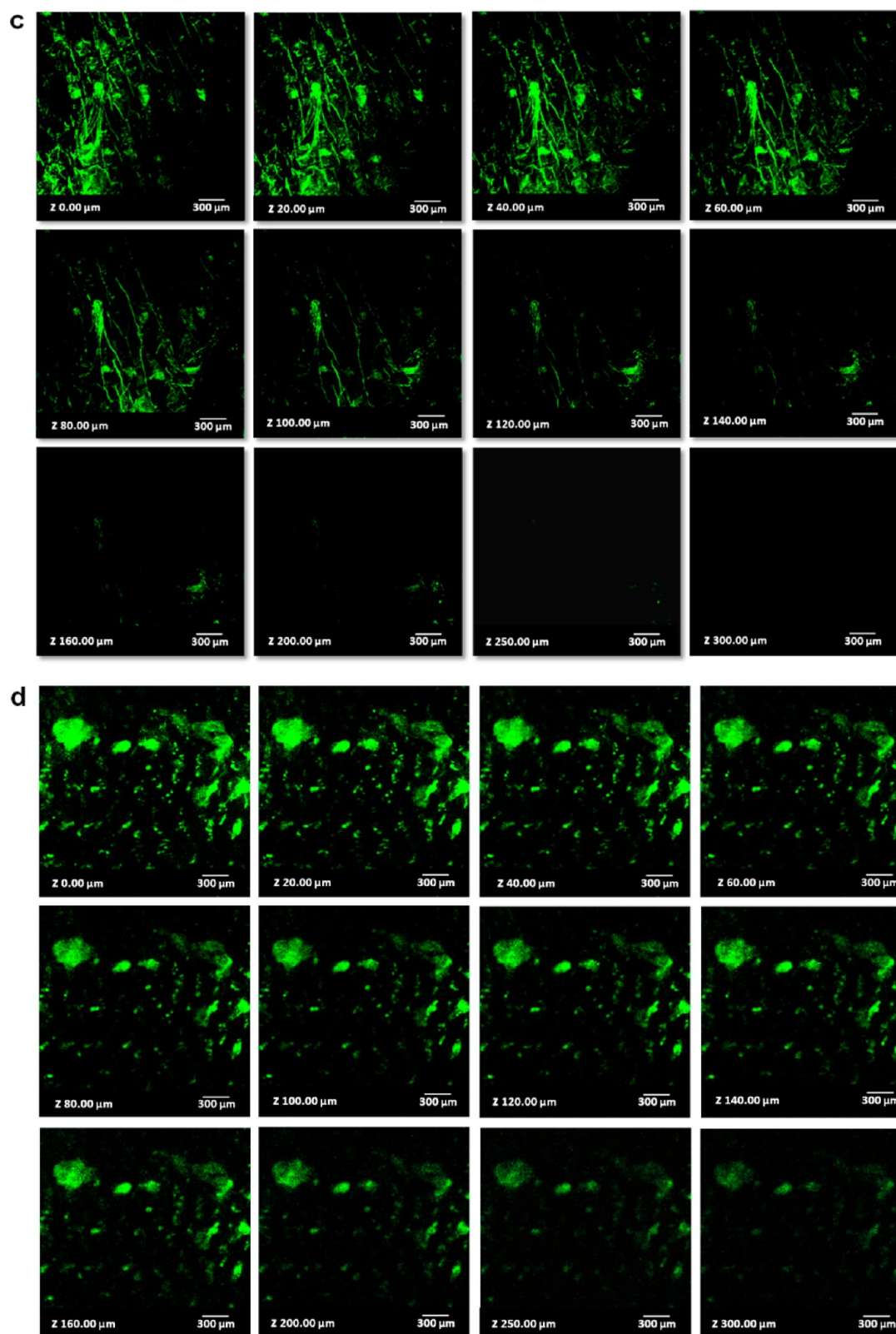


Figure 10. Confocal micrographs of penetration of Alexa 488-BSA (green) across the rat skin at varying depths after being pierced by P3 microneedles for 30 min (a), 1 (b), 2 (c), and 4 (d) days.

To visualize the BSA penetration in the vertical direction of the rat skin, the skin puncture sites were imaged and recorded at increasing depths from the skin surface using CLSM (Figures 9 and 10). The fluorescent of the Alexa 488-BSA was apparent at 30 min postadministration and gradually spread from the

locations of microneedle piercing to the surrounding tissue (Figure 9) and reached the dermal tissue from day 1 (Figure 10). Images of the skin at various depths showed that the maximal penetration depths observed in the skin at 30 min, 1, 2, and 4 day were approximately 160, 200, 250, and at least 300 μm (Figure 10).

Stability of Encapsulated BSA. The purpose of this study was to develop a polymer microneedle patch that can successfully encapsulate and deliver macromolecular drugs without denaturation. To assess the success of this device, we used BSA as a model protein and examined its change in a secondary structure after encapsulation and release from chitosan microneedles. Figure 11

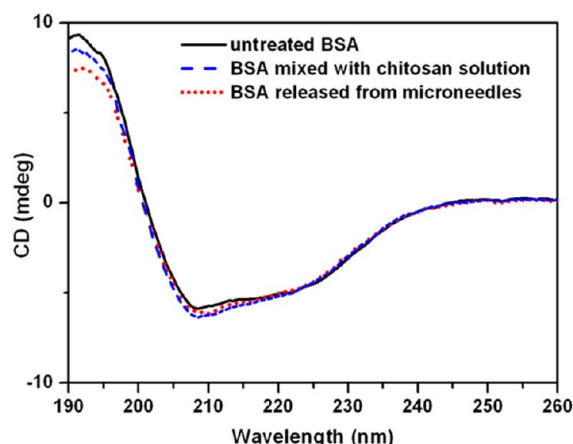


Figure 11. Circular dichroism spectrum of untreated BSA, BSA mixed with chitosan solution (pH ~6.0) and BSA encapsulated in chitosan microneedles that were dissolved after storage for 1 day at 37 °C.

shows the CD spectra of untreated BSA, untreated BSA mixed with chitosan solution (pH of approximately 6.0), and BSA encapsulated in a microneedle patch and subsequently released by dissolution in DI water after storage at 37 °C for 1 day. This result shows that no considerable change in protein secondary structure was detected after mixing with chitosan solution or encapsulation in microneedles, indicating that the fabrication and drug encapsulation processes of chitosan microneedles are sufficiently gentle to avoid denaturation of encapsulated biomolecules.

DISCUSSION

Microneedle technology has the potential to revolutionize therapeutics by enabling the delivery of various biomolecules.^{26–28} Regardless of the drug, a crucial challenge in manufacturing microneedles is to ensure the presence of a significant drug load in small needles. One of the most promising applications of microneedles is vaccinations,^{29,30} because of their limited drug-loading capacities. Prior studies have shown that intradermal influenza vaccination through microneedle patches can elicit the same immune responses at lower doses compared to traditional intramuscular injections, because the antigen-presenting cells found only in the skin develop an immunological response.^{3,30–32}

In this study, a chitosan microneedle patch was designed for efficient and sustained delivery of macromolecules. Chitosan was selected as the microneedle material because of its well-known biocompatible and biodegradable properties, and its immune stimulating activity, which can enhance both humoral and cellular responses.³³ In addition, chitosan can easily interact with negatively charged functional groups on proteins or other molecules for drug encapsulation because of its polymeric cationic characteristics. These unique characteristics make chitosan suitable as a carrier, especially for vaccine delivery.

This study demonstrated that chitosan microneedles can be inserted into living skin (Figure 7) by a homemade applicator and deliver encapsulated proteins (Figures 8–10). An in vitro transdermal delivery study showed that chitosan microneedles

exhibited a sustained release of BSA for at least 8 days (Figure 6c), indicating that this delivery system has potential to provide sustained immune stimulation through a prolonged release of antigens. A histological examination showed that P3 microneedles can penetrate through the viable epidermis and into the superficial dermis (approximately 200 μ m, Figure 7d) in vivo. Although one-third of the microneedle length penetrated the rat skin, partial microneedle insertion may be beneficial for delivering drugs to a specified depth within the skin, such as targeting epidermal Langerhans cells and dermal dendritic cells for vaccination purposes.

The success of a protein formulation depends on the stability of the delivery system and their ability to maintain the original structure and activity of the protein during preparation and delivery.³⁴ A gentle and well-controlled process for microneedle fabrication was required to prevent damaging the integrity of encapsulated biomolecules. The microneedle fabrication process used in this study is promising because it can be conducted under aqueous conditions at ambient temperatures, thereby avoiding damage to the fragile biomolecules during encapsulation (Figure 11).

These findings suggest that chitosan microneedles may offer an attractive opportunity for transdermal delivery of biomolecules. Among these biopharmaceuticals, vaccines are of particular interest because microneedles made of chitosan may serve as a device for antigen delivery and an adjuvant to stimulate the host immune system.³³ Additional studies to assess the feasibility and the performance of using chitosan microneedles for vaccination will be conducted in the future.

CONCLUSIONS

This study demonstrated the use of chitosan microneedles to achieve efficient and sustained delivery of macromolecules into the skin. An in vitro drug release study showed a sustained release of BSA from chitosan microneedles, with approximately 95% cumulative release observed in 8 days. An in vivo transdermal delivery study showed that the encapsulated BSA gradually diffused from the puncture sites to the dermal layer, reaching a penetration depth of 300 μ m. The gentle nature of the fabrication process enabled encapsulation of fragile biomolecules. The model protein retained its structural integrity after encapsulation and release from microneedles. These results suggest that the designed chitosan microneedles are promising and may serve as a useful device for transdermal delivery of diverse biomolecules.

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Notes

The authors declare no competing financial interest.

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