Facile Preparation of $\beta$-Cyclodextrin-grafted Chitosan Electrospun Nanofibrous Scaffolds as a Hydrophobic Drug Delivery Vehicle for Tissue Engineering Applications

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ABSTRACT: Despite advances in the bio-tissue engineering area, the technical basis to directly load hydrophobic drugs on chitosan (CTS) electrospun nanofibers (ENs) has not yet been fully established. In this study, we fabricated CTS ENs by using an electrospinning (ELSP) system, followed by surface modification using succinyl-beta-cyclodextrin ($\beta$-CD) under mild conditions. The $\beta$-CD-modified CTS ($\beta$-CTS) ENs had slightly increased hydrophobicity compared to pristine CTS ENs as well as decreased residual amine content on the surface. Through FTIR spectroscopy and thermogravimetric analysis (TGA), we characterized the surface treatment physiochemically. In the drug release test, we demonstrated the stable and sustained release of a hydrophobic drug (e.g., dexamethasone) loaded on $\beta$-CD ENs. During in vitro biocompatibility assessments, the grafting of $\beta$-CD was shown to not reduce cell viability compared to pristine CTS ENs. Additionally, cells proliferated well on $\beta$-CD ENs, and this was confirmed by F-actin fluorescence staining. Overall, the material and strategies developed in this study have the potential to load a wide array of hydrophobic drugs. This could be applied as a drug carrier for a broad range of tissue engineering applications.

1. INTRODUCTION

Natural polymers have been explored as biomedical materials for use in treating diseases of patients suffering from trauma or diseases such as cancer in a wide array of clinical areas. An ideal bioscaffold would be able to deliver therapeutic drugs and growth factors to provide rapid treatment. The therapeutic activity of encapsulated compounds can be optimized by providing appropriate drug concentrations using controlled drug delivery systems (DDSs). For certain disease states or tissue conditions, hydrophobic drugs are employed to provide maximum therapeutic efficacy. For example, in our previous report, Heo et al. developed $\beta$-cyclodextrin ($\beta$-CD) conjugated gold nanoparticles (GNPs) in order to load hydrophobic curcumin as the pristine GNPs do not have any capacity for loading hydrophobic drugs. The functionalized GNPs showed dual-synergistic effects for bone tissue regeneration. This demonstrated the ability of $\beta$-CD to provide hydrophobic drug delivery. $\beta$-CD has a unique property in that it creates an inclusion complex with hydrophobic drugs even when mounted on a hydrophilic scaffold. The cyclic oligosaccharides retain the hollow shape of the molecule, which has an interior hydrophobic cavity and a hydrophilic outer surface. This molecular structure serves as a bridge to deliver hydrophobic drugs from a wide array of biomaterials. In the tissue engineering field, many researchers have sought to use natural polymers as suitable tissue reconstructive materials. Among these, functionalized chitosan (CTS)

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electrospun nanofibers (ENs) have been used in the development of innovative drug delivery systems and progressive implantable scaffolds for tissue regenerative medicine. CTS is the second most abundant polysaccharide derived from deacetylation of chitin and provides reactive hydroxyl and amino groups. CTS is also biocompatible, nontoxic, and biodegradable. For these reasons, CTS ENs have been widely used as a component of biocompatible, nontoxic, and biodegradable. Inorganic nanoparticles has broadly used CTS ENs for antibacterial wound dressing and dental barrier membrane applications by encapsulating inorganic nanoparticles. Despite its success as a biofunctional material, unmodified CTS does not have the capability to load hydrophobic drugs to promote dual-functional tissue regeneration and other therapeutic applications. Therefore, a facile β-CD grafting technique on pristine CTS ENs for hydrophobic drug loading should be established to aid in the development of future functional bioscaffold designs.

In order to solve the abovementioned issues, herein, we prepared β-CD-grafted CTS ENs under a mild surface modification process. We hypothesized that grafting of β-CD did not impair the structure of CTS ENs if we use a mild surface modification approach. This approach allows for a facile gateway to load hydrophobic compounds on the functionalized CTS scaffolds. In this study, we fabricated CTS ENs first by using an electrospinning (ELSP) system. Then, we produced scaffolds treated with succinyl-β-CD via succinimide-ester bonding reaction. In this experiment, we assumed that the carboxyl group of succinyl-β-CD could react with the amine group of the CTS ENs without collapsing the structure. The objective of this study was to prepare and characterize β-CD-grafted CTS ENs as well as compare them against a control of unmodified CTS ENs to verify the feasibility of hydrophobic drug loading, both for release kinetics and biocompatibility for the future tissue engineering application. The physicochemical properties of the developed scaffolds were analyzed by SEM, water contact angle, residual amine content after/before modification of β-CD, thermogravimetric analysis (TGA), and FTIR spectroscopy. Finally, in vitro biological cell viability and proliferation were evaluated using mouse fibroblast NIH3T3 cells to characterize their biocompatibility for further applications in the tissue engineering field.

2. MATERIALS AND METHODS

2.1. Materials. CTS (average MW, 370 kDa; deacetylation degree 85%), succinyl-β-CD, trifluoroacetic acid (TFA, ReagentPlus, 99%), fluorescamine (F9015, ≥98%), hexylamine (99%), and dexamethasone [DEX, ≥98% (HPLC), powder] were purchased from Sigma-Aldrich (St. Louis, MO). 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM) was obtained from Wako Pure Chemicals (Osaka, Japan). Ethyl alcohol anhydrous (EtOH, 99.9%) and methyl alcohol (MeOH) were purchased from Daejung (Chemical & Metals Co. Ltd., Korea). DCM (Extra Pure, 99.0%+) was purchased from Junsei (Junsei Chemical Co. Ltd, Japan). Sodium hydroxide (NaOH) was purchased from YPC (Yakuri Pure Chemicals Co., Ltd. Japan). Fibroblast NIH3T3 (CRL-1658) was obtained from ATCC. DMEM, FBS, trypsin–EDTA, antibiotics (PS, penicillin–streptomycin), and Dulbecco’s PBS were purchased from Gibco (Rockville, MD, USA). Deionized-distilled water (DW) was produced by an ultrapure water system (Puris-Ro800; Bio Lab Tech., Korea). The dialysis membrane tube was purchased from Spectrum Spectra (Spectra/Por 4 dialysis tubing, 12–14 K MWCO, 45 mm flat width, 100 ft length). All other reagents and solvents were of analytical grade and used without further purification.

2.2. Preparation of the CTS Composite and Fabrication of CTS ENs via the ELSP System. The preparation details of the CTS composite and ELSP procedures for CTS ENs have been previously reported. Briefly, the CTS ENs were fabricated via the ELSP system using a 5 wt % CTS composite dissolved in mixed TFA/DCM (7:3) solvent. For the ELSP process, the CTS polymer solution was loaded into a Luer-lock syringe attached to a metal blunt needle (22 G, Kovax-needle, Korea Vaccine Co., Ltd., Korea), and generated ENs were deposited onto an aluminum-foil-covered rotating mandrel at 23 kV using a high-voltage DC power supply (Nano NC, Korea) with a 1 mL/h feed rate (KDS-200, KD Scientific Inc.) and a 15 cm needle tip-to-collector distance. The manufactured CTS ENs were dried overnight under vacuum in order to remove any residual solvent. For pH neutralization, the dried CTS ENs were immerged in 50 mL of 3.2 M NaOH/MeOH (neutralization solution) for ten min, followed by rinsing with DW carefully until the pH reached 7. After this, the formed nanofibers were frozen and lyophilized.

2.3. Preparation of β-CD-Grated CTS ENs under the Mild Surface Modification Process. In order to graft β-CD on the CTS ENs, the CTS ENs were cut into a circle using an 8 mm punch. A reaction solution was made by dissolving 1.14 mg of the β-CD powder and 0.55 mg of DMTMM in 1 mL of DW. The solution was protected from light and mixed using a vortex to dissolve completely, and then, 1 mg of CTS ENs was added to the solution. The CTS ENs were incubated at RT in the dark overnight with gentle shaking. Finally, β-CD-grafted CTS ENs were obtained after washing with DW and drying at RT.

2.4. Quantification of the Amine Content on the Scaffolds. 1 mg of both CTS ENs and fluorescamine was dissolved in an acetone/propanol (2:1) mixture. Afterward, the supernatant was harvested after vortex mixing. The same method was carried out on β-CD-grafted CTS ENs to compare with unmodified CTS ENs. This was done to determine the amount of amine on the surface that was reduced by β-CD treatment. The amine content was quantified by calibration against a series of hexylamine standard solutions (0–100 μM).

2.5. Hydrophobic Drug Loading on ENs and Its Release Test. To demonstrate the hydrophobic drug loading capacity of βCTS ENs, we used DEX as a model drug. The experimental details have been described in our previous report. Briefly, CTS and βCTS ENs were immersed in DEX/EtOH solution (1 mg/mL) with shaking for 2 days with protection from light. Finally, the DEX-loaded CTS and βCTS ENs were washed with fresh DW and dried at RT with protection from light. After that, the DEX-loaded CTS and βCTS ENs were incubated in a DW/EtOH mixture (50:50) with shaking at 37 °C for 14 days to quantify the release kinetics of DEX from the scaffolds. The release kinetics of DEX, the supernatant solution was collected from each sample after incubation at predetermined time points. It was diluted to be within the calibrated range, and the ultraviolet–visible absorption was measured using an UV-1650PC spectrophotometer (Shimadzu, Japan). The actual
loading amount was calculated from a standard curve of DEX of various concentrations (0, 10, 50, 100, 250, 500, and 750 µg/mL). Quantification was based on the solution absorption at 260 nm and then normalized as a “%.”

2.6. Evaluation of Biocompatibility of Unmodified and Developed CTS ENs. The biocompatibility of the produced scaffolds was analyzed by direct cell cultivation on the scaffolds. Before the in vitro assessment, scaffolds were sterilized via UV irradiation in the cell culture hood overnight. Each scaffold was placed in a 48-well culture plate with a piece of Teflon tubing to hold them in the well. Then, NIH3T3 cells were seeded onto the scaffolds at a density of 2 × 10^4 cells per scaffold. They were cultured in DMEM supplemented with 10% FBS and 1% PS in a 5% CO2 incubator at 37 °C. After 24 and 48 h of cell seeding, samples were washed with PBS and treated with the LIVE/DEAD Viability/Cytotoxicity Kit (L3224, Invitrogen, USA) according to the manufacturer’s instructions. Cell proliferation was determined after 1, 3, and 7 days of culture. NIH3T3-seeded ENs were evaluated by using the cell counting kit (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) (∼4) after washing with PBS. The absorbance of the medium containing CCK solution (1:10 dilution) was measured at 450 nm using a microplate reader (ELISA, Bio-Rad, Hercules, CA, USA). For F-actin staining, cells seeded on the ENs were washed with PBS and fixed in 3.7% formaldehyde solution for 20 min. After fixation, samples were washed 3 times with PBS and treated with 0.1% Tween 20. Then, they were washed with PBS three times again. The samples were soaked with rhodamine phalloidin (R415, Invitrogen, USA) for 40 min and treated with DAPI (R37605, Invitrogen, USA) for 10 min under dark conditions. These experiments were repeated in triplicate.

2.7. Analysis Equipment. The morphology of CTS and βCTS ENs was observed with a scanning electron microscope (Hitachi S-4700, Japan) at an acceleration voltage of 15 kV. All of the samples were sputter-coated with platinum for 10 min prior to SEM analysis. The water contact angles were measured using the drop method and a video camera (Phoenix 150, 82 SEO, Korea). Then, the measured angle was converted to a graph. In order to characterize the thermal decomposition profiles of both CTS and βCTS ENs, TGA was performed using TGA-50 (Shimadzu, Japan). Dry specimens (2 mg) of either scaffold were measured under a nitrogen flow rate of 50 mL/min and at a heating rate of 10 °C min⁻¹ in the temperature range from 25 to 600 °C. The surface molecular structure of scaffolds was characterized using FTIR spectroscopy with a resolution of 4 cm⁻¹ between 4000 and 500 cm⁻¹ (Spectrum One System, PerkinElmer).

2.8. Statistical Analyses. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., USA). The residual amine content on the scaffolds was compared with an unpaired t-test (n = 3). The cell proliferation was analyzed using two-way analysis of variance (n = 4). P values for statistical significance were presented as an asterisk (***p ≤ 0.001) on each graph.

3. RESULTS AND DISCUSSION

3.1. Surface Morphological Characterization of CTS and βCTS ENs Using SEM. The framework of our strategy is depicted in Figure 1. After fabrication of CTS ENs using the ELSP system, they were neutralized using NaOH to prevent redissolution in water as chitosan solubility requires an acidic condition.9–11 Then, we directly applied β-CD onto the CTS ENs via a mild surface modification approach. In order to characterize their morphological changes, we carried out SEM analysis (Figure 2a). Fortunately, there was no change, and the morphological structure did not present any signs of collapse or damage. Both groups showed structurally uniform shapes with an appearance like woven fabric. The present results coincided with our previous data.12 In that study, the mild surface treatment technique did not affect the physical properties either. From this analysis, we determined that facile surface treatment with β-CD against CTS ENs did not impose any structural change on the natural polymer-based ENs.

3.2. Measurement of the Water Contact Angle and Amine Content. Even if there was no morphological change, we expected that there would be a change in physicochemical properties. To confirm this, we performed water contact angle analysis and measured the amine content and compared these for both β-CD-modified and pristine groups. In water contact angle analysis (Figure 2b), unmodified CTS ENs showed rapid water absorption as well as an initial contact angle around 40°. However, βCTS ENs looked like hydrophobic scaffolds in the initial time point. Interestingly, all water was absorbed within 20 s. We quantified the degree of the water contact angle at predetermined time points (Figure 2c). This phenomenon may be attributed to β-CD as it has a hydrophobic cavity in its chemical structure as well as a reduced content of hydrophilic
Although β-CTS ENs showed an initial hydrophobic tendency, the water absorption was all over within 20 s, indicating that these can serve well for tissue engineering applications. It is believed that suitable hydrophilicity provides an appropriate cellular environment on the various bio-substrates. Also, we measured the remaining amine contents on the scaffolds (Figure 2d). We predicted that the amount of amine would be relatively reduced after β-CD treatment. As expected, the amount of residual amine decreased by 3.6-fold, indicating that β-CD conjugation worked well. This result is consistent with our previous studies.

3.3. Physicochemical Characterization of CTS and βCTS ENs Using TGA and FTIR. We investigated the physicochemical properties of the developed ENs using TGA and FTIR analyses. TGA is able to verify the thermal stability of polymeric composites. As shown in Figure 3a, all samples displayed two distinct weight loss regions in the 25–600 °C range. The first weight loss was owing to the evaporation of water molecules in the EN substrates around 45 °C. The second weight loss was due to the degradation of the polymeric molecules at around 240 °C. The overall trend of each group was very similar. However, β-CTS ENs seemed to withstand thermal decomposition better than unmodified CTS ENs. According to the previous literature, Ma et al. demonstrated that the β-CD-loaded folic acid hydrogel was more thermally stable than a hydrogel without β-CD. Similarly, another study by Sharkir et al. revealed that the addition of β-CD, in a chitosan/nano-hydroxyapatite complex, increased the thermal stability as compared to an unmodified
These results indicate that the modification of β-CD on the CTS ENs can improve thermal stability due to chemical conjugation on the surface. In order to verify the surface chemistry, we performed FTIR analysis. As shown in Figure 3b, CTS ENs contained characteristic peaks around 1650, 1384, and 904 cm\(^{-1}\), corresponding to amide groups and saccharide structures. β-CD displayed an alpha-pyranyl vibration at a wavenumber of 942 cm\(^{-1}\). As expected, all these peaks appeared in β-CTS ENs. Based on these results, we confirmed that βCTS ENs were successfully prepared under this modification approach, which may allow for the loading of hydrophobic drugs.

### 3.4. Assessment of the Hydrophobic Drug Release Profile from CTS and βCTS ENs

As mentioned above, we anticipated that β-CD-grafted CTS ENs may have hydrophobic drug loading capacity. To verify this, we used DEX, as it is a lipophilic substance, and synthetic glucocorticoid, as it is used clinically as an anti-inflammatory drug. First, we quantified the drug loading amount on CTS and βCTS ENs. We confirmed that the βCTS ENs successfully loaded a larger amount of DEX compared to the bare CTS ENs, indicating a 2.3-fold increase in loading (data not shown). This phenomenon is due to the formation of an inclusion complex between β-CD and lipophilic soluble molecules such as DEX. As shown in Figure 4, we measured the amount of DEX released over the course of 14 days. In the CTS EN group, the DEX had nearly fully released by around 3 days of incubation. This phenomenon demonstrates the burst release of DEX physically adhered to the CTS ENs. However, the βCTS ENs exhibited a sustained release property. Even 14 days later, the drug was slowly coming out without being completely released. As expected, it is due to the drug holding ability within β-CD. In our previous publications, we grafted β-CD on a nanofibrous scaffold to load simvastatin (SIM), another hydrophobic drug. The result showed that grafting β-CD allowed for the sustained release of SIM. The release tendency is similar to our current results. Bioscaffolding research using β-CD has demonstrated its ability to deliver hydrophobic drugs into lesions and induce reconstruction of the desired tissue and/or treat cancer cells and bacteria. Techniques for loading hydrophobic drugs onto nanofiber membranes have received considerable attention in the biomedical field recently.

**Figure 3.** Characterization of thermal stability using the TGA tool (a) and surface chemical composition using FTIR analysis (b). The β-CD-grafted CTS nanofiber showed thermal stability and β-CD was chemically grafted well on the unmodified CTS nanofiber.

**Figure 4.** Release profile of DEX for 14 days from unmodified CTS and β-CD-grafted CTS nanofibers. The β-CD-grafted nanofiber enabled the sustained release of loaded DEX but the unmodified CTS nanofiber exhibited a burst release phenomenon.
For example, biodegradable nanofibrous masks are being developed to prevent COVID-19. In this case, the hydrophobic drug was loaded onto fabric membranes which could trap a virus for filtration. Thus, we anticipate that β-CD-grafted CTS ENs would be applicable in various biomedical and tissue engineering areas.

3.5. Characterization of Biocompatibility of CTS and βCTS ENs Using CCK-8 and F-Actin Staining. In order to evaluate the biocompatibility of the developed ENs, we cultivated fibroblast cells on both scaffolds and compared their cellular cytotoxicity through live/dead assay and quantification of cell proliferation using CCK-8 assay. The CCK-8 assay is often used to quantify cell viability for cells growing either on or inside bioengineered scaffolds. To verify the initial cell viability of ENs, the live/dead assay was conducted (Figure 5a). As shown in Figure 5a, live NIH3T3 cells displayed green fluorescence by calcein AM treatment. Both CTS and βCTS ENs showed almost no dead cells. After seeding onto the ENs, the cells were both living and well attached on the CTS and βCTS ENs at 24 h. Furthermore, cells adhered to both ENs had a stretched morphology after 48 h. It is believed that CTS-based scaffolds offer high cellular affinity. Next, we confirmed cell proliferation on each scaffold after 7 days of culture. As shown in Figure 5b, the cell proliferation rate was compared to the 1 day CTS EN group as a control group for quantification. There was no significant difference between the proliferation rates of CTS and βCTS at day 1 of culture with only a 1.1% difference between the two groups. This tendency was found to continue up to 7 days. During this test, no statistical difference was found between the two groups. This indicated that although modification of CTS ENs with β-CD can change the physiochemical properties slightly, there was no cytotoxic effect. In order to visualize cellular adherence and morphology, we performed F-actin staining (Figure 5c). The cells were greatly adhered to and observed to cluster on both ENs. These results indicate that both scaffolds provide an excellent environment for cell growth, clearly indicating they are nontoxic. This revealed that the β-CD-modified CTS ENs will provide suitable biocompatibility to tissue-engineering scaffolds designed to deliver hydrophobic drugs.

4. CONCLUSIONS AND FUTURE DIRECTION BASED ON OUTCOMES

There are many situations of accident, disease, or trauma that can lead to unexpected tissue loss. The goal of bioengineering in this field is to find and provide advantageous materials for the successful regeneration of damaged or missing tissue. To meet this goal, in this study, we prepared βCTS ENs under the mild surface modification approach. Even though we grafted β-CD onto the bare CTS ENs, there was no surface morphological change. The β-CD modification slightly affected the hydrophilicity and water contact angle, but the thermal stability was improved. Through amine quantification and FTIR analysis on the scaffolds, the success of the stable surface modification was verified. In the release profile of hydrophobic drugs, βCTS ENs showed excellent drug loading and sustained release capacity compared to CTS ENs. Finally, cell viability and proliferation were confirmed to demonstrate that both modified and unmodified scaffolds had no cellular cytotoxicity. In addition, cells were uniformly attached to the β-CD ENs. This suggests that our approach can be used for tissue and biomedical engineering applications by loading hydrophobic drugs. Based on this strategy, we will develop multifunctional hybrid wound dressing material by conjugating synthetic polymers as a poly-blend strategy in order to improve mechanical properties. Additionally, curcumin, which has antioxidant and anti-inflammatory properties, will be incorpo-

Figure 5. In vitro live/dead assay (a), cell proliferation test via CCK-8 (b), and F-actin with DAPI staining after 7 days of cultivation on the CTS ENs and βCTS ENs (c). Almost all cells were alive on each scaffold, and the NIH3T3 fibroblast proliferated very well. Interestingly, individual cells made accumulated clumps on the fibrous scaffolds. The cell proliferation rate was slightly reduced on βCTS ENs. However, there is no significant difference as compared to CTS ENs at each date.
rated.51 Because CTS ENs themselves have a hemostatic potential,52 a multifunctional wound dressing could be obtained if we use drug-loadable scaffolds like β-CD ENs. In this case, our outcome will pave the way for future developments in the bioengineering area.

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Notes
The authors declare no competing financial interest.

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