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Bio-Inspired, Water-Soluble to Insoluble Self-Conversion for Flexible, Biocompatible, Transparent, Catecholamine Polysaccharide Thin Films

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In nature, a variety of functional water-insoluble organic materials are biologically synthesized in aqueous conditions without chemical additives and organic solvents. Insect cuticle, crustacean shells, and many others are representative examples. The insoluble materials are prepared by enzyme reactions and programmed self-assembly in water from water-soluble precursors. If the water-basis could be adapted, environment-friendly strategy developed in nature, many problems caused by the vast consumption of petroleum-based olefin materials could be solved or significantly attenuated. Here, the spontaneous formation of water-insoluble, biocompatible films from a water-soluble polymer is demonstrated without using any chemical additives and organic solvents. It is found that a water-soluble chitosan–catechol polymeric precursor is spontaneously self-converted to flexible water-insoluble thin film by simple dehydration. The preparation of mechanically robust, water-insoluble, flexible, transparent chitosan–catechol film is a completely unexpected result because most water-soluble polymers exist as powders when dehydrated. The film can be used as a bag similar to polyvinyl one and is multifunctional and biocompatible for drug delivery depots and tissue engineering applications.

1. Introduction

Synthetic polymer films are widely used in numerous applications such as polyvinyl packaging bags, poly(ethylene terephthalate) (PET) bottles, polyester protective films for a liquid-crystal display (LCD), polyethylene window films for ultraviolet (UV) protection, polyurethane interlayer films in laminated glass, and biomedical devices such as blood bag, catheters, and angioplasty balloons. However, the formation of these olefin-based thin films are not ecofriendly materials that are poorly degradable in environment and are toxic to human.[1–3] It has been shown that the degradation of synthetic material films derived from petroleum such as polyethylene and polypropylene requires several decades.[4,5] Furthermore, to produce the synthetic polymer films, toxic organic solvents, for example, chloroform, ethyl acetate, or tetrahydrofuran (THF) are currently used and are evaporated generating the synthetic polymeric films.[6–8] Moreover, high-temperature melting-pressing or sintering has been traditionally employed to prepare polymeric films, which require significant energy consumption.[9,10] Recently, the use of naturally occurring polysaccharides such as cellulose and starch has been alternative methods,[11–13] but the processing of those materials need further improvement to avoid chemical additives, enzymes, or solvents.

Biologically synthesized polymeric films present in nature are environmentally friendly. Examples of the films include protection layers in plant, outer shells of fruits, insect cuticles, and crustacean shells under water.[14–20] These biologically synthesized natural films are degradable and non-toxic to human, which is suitable for a sustainable society. Furthermore, the water insoluble polymeric film formation strategy developed by nature is environment friendly and energy efficient as it does not require use of organic solvents or high-temperature process such as evaporation.[21–23] Nature utilizes abundant polymers such as cellulose, chitosan, and proteins, which in turn undergo unique dehydration process depending on the property of each polymer. Unlike the manmade process that utilizes organic solvents and their evaporation, nature has developed to a general strategy converting 'water-soluble' polymeric precursors before film formation into "water-insoluble" products during the film formation.

In general, flexible, polymeric thin films are not prepared simply through water-drying process (i.e., dehydration). Rather, in most cases, the water-soluble polymers become powders when dehydrated. Thus, for successful preparation of thin films from natural sources, an additional mechanism such as chemical crosslinking might be associated during the water-drying process. We chose catecholamine crosslinking chemistry that is found in insect cuticle and squid beak, and chitosan was selected an abundant source of polymeric material. The catechol groups react with amine groups to form covalent linkages

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that play an important role in mechanically robust polymeric films.[16–18,24,25] In detail, insect cuticle film is hardened by covalent bond formation in chitin-protein polymeric matrix by the catecholic small molecule agents such as *N*-acetyldopamine (NADA) and *N*-b-alanyldopamine (NBAD).[16–18] Similarly, the catechol is incorporated as a form of amino acid called 3,4-dihydroxy-L-phenylalanine (DOPA) in squid beak that form covalent linkages mostly with histidine exhibiting mechanically robust thin films.[24,25] Thus, we hypothesized that both dehydration process motivated by current film preparation process and the aforementioned catecholamine chemistry inspired by nature might result in formation of an ecofriendly, flexible, degradable, thin film. This new natural film formation would be meaningful in that no toxic, organic solvents are used, and the film is prepared by property changes of water solubility of a given polymer.

In this study, we report a biologically inspired strategy for the formation of multifunctional thin films from an abundant natural source, chitosan. The film is flexible, degradable that exhibits excellent tensile strength (≈10 MPa) superior to commercial A4 paper (≈3.5 MPa), which can be utilized similarly as poly(vinyl) bags. Also, the mechanical properties of the film are controllable in a biomimetic way in which the degree of catecholamine crosslinking is responsible for the mechanical properties of the film found in insect cuticles[16–18] and squid beaks[24,25] is responsible for the film mechanics. The applications of this film are not limited to ecofriendly degradable films, extending to drug-encapsulating/releasing depots and cell adhesive medical films. Furthermore, the film preparation method reported herein is scalable and solvent-free that can easily be adapted to existing industrial processes.

2. Results and Discussion

Catechol-conjugated chitosan (denoted as "chitosan–catechol") was synthesized via standard EDC coupling by forming the amide bond between a carboxylic acid group of hydrocaffeic acid and an amine group along chitosan backbone. In general, the conjugated catechol groups in chitosan are oxidized being quinone under the alkaline condition in the presence of dissolved oxygen, and the transformed quinone can reactive with amine, thiol, and other catechol groups via Michael-type addition reactions and/or Schiff-base formations.[26–28] Thus, we hypothesized that the catechol-quinone mediated crosslinking chemistry can dramatically convert the solubility of the polymer from being water-soluble to insoluble. It might be analogous to the process often found in nature such as the formation of exoskeletal cuticle in insects and chitin shell in crustaceans in that the organisms utilize water-soluble precursors to form water-insoluble, water-protective outer layers. However, the true biomimicry in cuticle sclerotization requires far more complicated chemistry than the one shown in this study. The hardening (i.e., sclerotization) is also related in part to the impregnation of hydrophobic cuticular proteins into the chitin scaffolds, acting as polymeric fillers. At the same time, the co-secretion of low-molecular-weight catecholamines such as *N*-acetyl-dopamine (NADA) and *N*-*β*-alanyl-dopamine (NBAD) occurs. NADA and NBAD are incorporated into the cuticular matrix and oxidatively crosslink between cuticular components.[29] **Figure 1** shows the water soluble-to-insoluble conversion of chitosan–catechol and the comparative study using unmodified chitosan. General procedures to prepare the films are simple dissolution of the natural polymers followed by drying the water. The first noticeable observation is that both chitosan and chitosan–catechol are able to form free-standing organic thin films (Figure 1A,B). However, a large difference exists in water solubility of the chitosan-based films. In detail, the film prepared using chitosan rapidly dissolves within 10 s when immersed into water (the right panel photos, (A) (Figure S1, Supporting Information), but the chitosan–catechol film is completely insoluble remaining intact for 7 days (B) (Figure S2, Supporting Information). In fact, the film remains insoluble for more than a month by observation. The dramatic solubility conversion of chitosan–catechol from highly soluble to be completely insoluble originates from the catechol conjugation. In contrast, chitosan–catechol films from drying acidic (HCl) solutions were completely dissolved in DDW probably due to the uncrosslinked structures

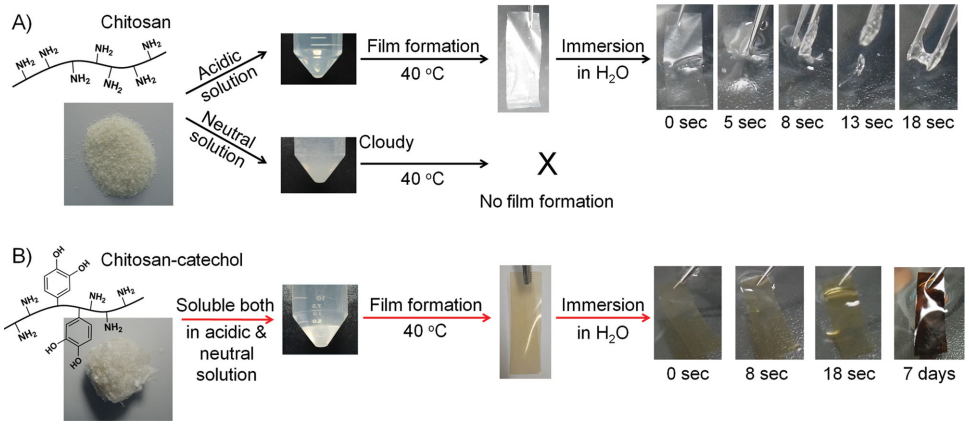

Figure 1. Preparation of chitosan and chitosan–catechol films. A) Unmodified chitosan (first, left) was dissolved in acidic solution (upper second) and drying water in an incubator at 40 °C (upper third). The photographic images of the rapid dissolution of the chitosan films in DDW (upper fourth). No film formation by the chitosan that was directly dissolved in DDW (lower, second). B) Chitosan–catechol (first, left) was dissolved in DDW (second) and incubated at 40 °C (third) for dehydration. The photographic images of the insolubility in water of the chitosan–catechol films as a function of times.

of chitosan–catechol networks. Previously, the method to prepare water-soluble, high molecular weight chitosan derivatives that can be dissolved in neutral pH solutions (up to 62.0 ± 18.1 mg/mL, Mw = 100 kDa, degree of catechol substitution: 7.2%) via conjugating hydrocaffeic acid was reported.[30] In this previous study, chitosan–catechol is not soluble in alkaline solutions due to rapid crosslinking reactions between chitosan–catechol chains, resulting in hydrogel formation rather than insoluble thin film.[30] We analyzed the processes why this phenomenon occurs. i) Immediately after the dissolution of chitosan–catechol (concentration = 1 mM), chitosan–catechol forms multi-cored nano-/micro-particulates via inter-molecular interactions showing two distinct ranges of 300–500 nm and 2–3.5 μm (**Figure 2**A). (ii) Water evaporation increases the concentration of the chitosan–catechol resulting in a hydrogel-like, intermediate material (Figure 2B) with the elastic modulus value of 11.3 ± 0.2 kPa determined by a rotational rheometer (Figure S3, Supporting Information). iii) Complete evaporation of the water produces chitosan-based solid films. As shown in Figure 2C, this film-producing method is scalable because the process only involves water-drying process, indicating that the final film size is dependent upon the size of molds. Thus, the process might be suitable for industry. This water-borne chitosan–catechol film is flexible, transparent that can be used as a bag analogous to the widely implemented poly(vinyl) bag (Figure 2D,E). The conventional film making process involves evaporation of toxic organic solvents, but the method described herein utilizes only water being environmental friendly. The insolubility of the film is due to the chemical crosslinking to form amine-catechol adducts, which has been used as a method to prepare hydrogels or to functionalize substrates.[26–28] With a little different perspective, a recent study by Miserez et al. for the preparation of insoluble chitosan–catechol thin films by adding iron oxide nanoparticles was reported.[31] The incorporation inorganic nanoparticles can provide several advantages including mechanical properties and others but might decrease reproducibility in preparation of the chitosan/iron oxide film due to the uncontrollable relative composition of the incorporated nanoparticles. This study focused on the self-conversion to be a purely organic chitosan–catechol film without contribution of inorganic particles. The phenomenon we found might be similar to catecholic conjugation mechanisms found in nature. Sclerotization or tanning, the process that stabilizes exoskeleton via quinone-mediated crosslinking, utilizes water-soluble polymeric reactants, which, in turn, becomes insoluble, rigid outer films by the oxidative catecholamine crosslinking in many insects.[16–18]

We prepared four different chitosan–catechol films with variable degree of crosslinking: uncrosslinked (UC), lightly crosslinked (LC), moderately crosslinked (MC), and heavily crosslinked (HC) (**Figure 3**A). The proposed oxidative crosslinking chemistry occurred within the chitosan–catechol films described in Figure 3B. Because of this crosslinking, exclusion of water molecules (i.e., deswelling) occurs being the

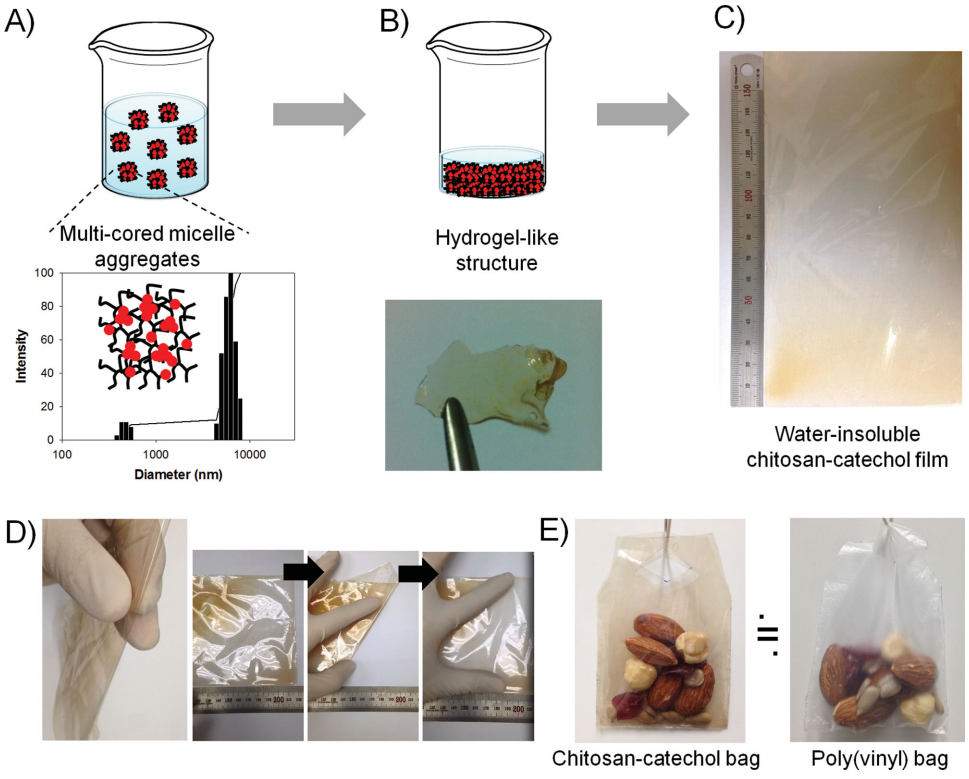

Figure 2. Formation of chitosan–catechol films. A) A schematic description and DLS data representing the initial state of the chitosan–catechol solutions: multi-cored micelle-like aggregates at an early stage of dehydration. B) The intermediate state of the formation of chitosan–catechol hydrogel-like structures during dehydration. C) The large area (17 cm × 10 cm) of the chitosan–catechol film, demonstrating facile scalability of the film preparation. D) Photos describing the flexibility and transparency of chitosan–catechol films. E) The transparent poly(vinyl)-like bag made from the chitosan–catechol film. It can be used similarly as commercial polyvinyl bags (E, left). The commercially available poly(vinly) bag containing nuts (E, right).

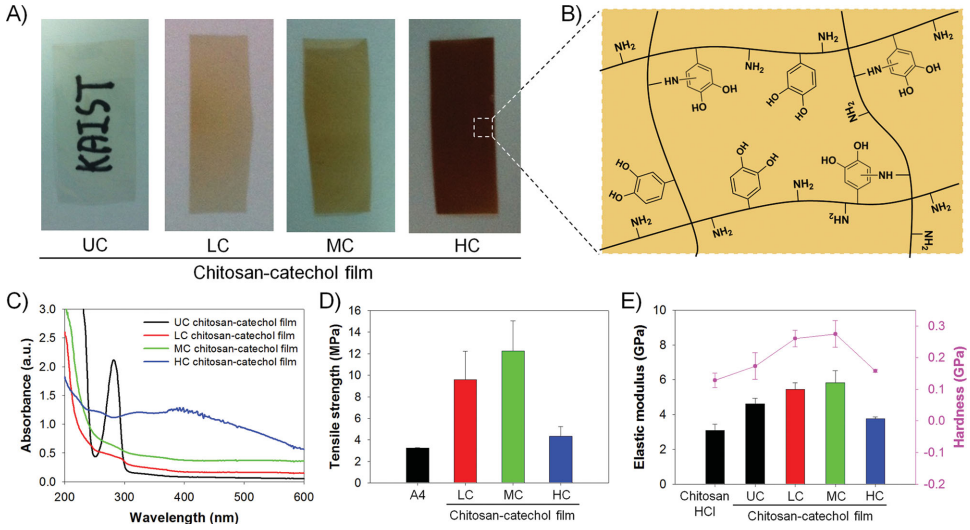

Figure 3. Characterization of chitosan–catechol films. A) Controlling the tanning of chitosan–catechol films. Photographic images of uncrosslinked (UC), lightly crosslinked (LC), moderately crosslinked (MC), heavily crosslinked (HC) chitosan–catechol films. B) Proposed mechanisms of quinone-involved crosslinking chemistry occurred in the chitosan–catechol. C) UV–Vis spectra of UC, LC, MC and HC chitosan–catechol films. D) The tensile strength of A4 paper, LC, MC, and HC chitosan–catechol films. E) Elastic modulus values (bar) and hardness (pink dot) of chitosan films, and UC, LC, MC and HC chitosan–catechol films.

hydrogel-like intermediate material described in Figure 2B.[32,33] The crosslinking results in broad up-shift in visible spectrum ranging from 350 to 750 nm due to the intrinsic chemical complexity of the crosslinking chemistry.[34] For the UC chitosan–catechol film, the typical absorption at 280 nm was detected (Figure 3C, black), that disappeared for the films that underwent crosslinking treatments (5.5 mM $NaIO_4$). Opaque, scattering properties exhibited overall upshift of the visible ranges, and the degree of upshift corresponds to the extent of crosslinking. Moreover, for the HC chitosan–catechol film, a broad peak appeared at 400 nm was detected which indicates quinone and quinone-mediated crosslinking products (Figure 3C, blue).[35,36] Tensile strength of the chitosan–catechol films was measured (Figure 3D). Commercial A4 paper was used as a control which exhibited the strength about ≈3.3 MPa. The LC chitosan–catechol film showed high tensile strength of ≈9.6 ± 1.7 MPa. The strength was further increased as the crosslinking was induced by the oxidant: 12.3 ± 2.6 MPa for the MC chitosan–catechol films. Unexpectedly, the tensile strength value was the lowest for the HC chitosan–catechol film (4.3 ± 0.9 MPa) probably because the over crosslinking reaction might induce the increase in film brittleness. The tensile strength of chitosan–catechol films (≈10 MPa) exhibited one-order magnitude lower than that shown in mcrofibrillated nanocellulose films (≈300 MPa), which were developed for construction, drillings, and pharmaceuticals.[11–13] However, its fabrication processes involved with high-pressure homogenization or use of costly enzyme require further research toward eco-friendly production, and moreover its long degradation time might not be suitable for biomedical applications. We also tested the nanomechanical properties to evaluate the elastic moduli and hardness of the chitosan–catechol films by using a nanoindentator (Figure 3E). The enhancement of elastic moduli and hardness by increase of crosslinking was clearly observed. The elastic modulus value of the crosslinked chitosan–catechol films (5.8 ± 0.7 GPa for MC and 5.5 ± 0.4 GPa for LC film) was much higher than that of chitosan (3.1 ± 0.4 GPa). Similarly, the hardness of the crosslinked chitosan–catechol films (0.27 ± 0.04 GPa for MC and 0.26 ± 0.03 GPa for LC) was also higher than that of chitosan (0.13 ± 0.02 GPa). Considering the biological materials in nature, the hardness of chitosan–catechol films is slightly lower than that of a squid beak (H_{dry} = 0.65 ± 0.14 GPa, H_{wet} = 0.43 ± 0.14 GPa) and is similar to the bovine hoof walls (H = 0.29 ± 0.02 GPa), the elytra cuticle of dung beetle (H = 0.32 ± 0.09 GPa), and the human finger nails (0.26 ± 0.01 GPa).[37–40] Also, the modulus (E = 5.96 ± 0.32 GPa) of the elytra cuticle of dung beetles is also similar to chitosan–catechol films.[40] We further tested the mechanical property of LC chitosan–catechol films in a hydrate state using a universal testing machine. Elastic modulus value of the hydrated LC chitosan–catechol films was 1.2 ± 0.4 GPa. Thus, considering the modulus obtained in a dry state (E_{dry} = 5.5 ± 0.4 GPa), the E_{wet} was decreased down to about twenty percent. Significant reduction (<10%) was expected before the experiments, but the modulus retaining might be due to the inter-chain chemical crosslinking. We found that the chitosan–catechol film undergoes slow, progressive oxidation within the film. In particular, the significant up-shift in UV–Vis spectrum after seven-day incubation of the LC chitosan–catechol film in PBS (pH = 7.4) was observed (Figure S4, Supporting Information). Also, as expected the tensile strength of the auto-crosslinked LC chitosan–catechol films was remarkably increased to 30.4 ± 1.2 MPa.

Chitosan–catechol films are able to encapsulate macromolecules such as polysaccharides and proteins. Thus, the chitosan–catechol films can be used as drug delivery depots. We chose dextran and bovine serum albumin (BSA) as model macromolecules.[41,42] Dextran has been known to exhibit a certain degree of anti-coagulant property.[42] Three samples were prepared: rhodamine-dextran conjugates (Rho-DEX) loaded, fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA)

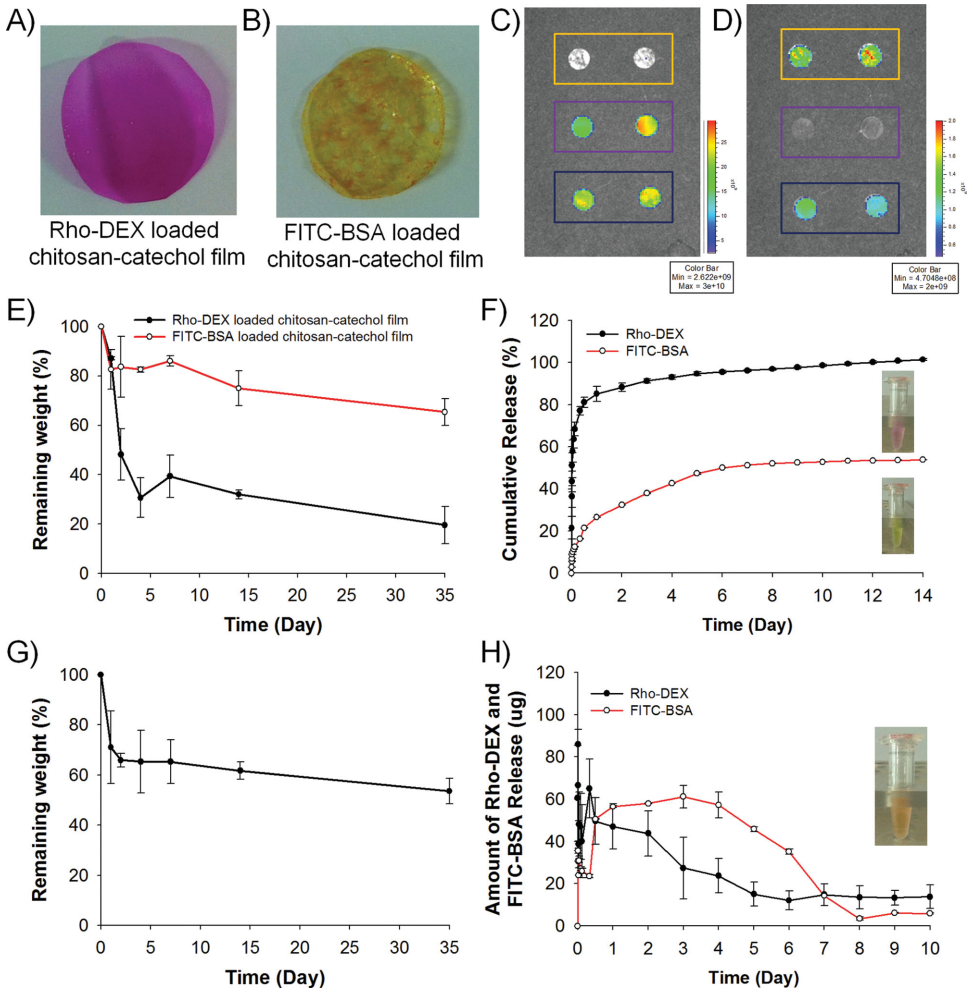

Figure 4. Chitosan–catechol films as drug delivery dopots. The photographic images of A) Rho-DEX loaded chitosan–catechol films and B) FITC-BSA loaded chitosan–catechol films. C) The fluorescence images (λ_{ex} = 540 nm and λ_{em} = 625 nm) of the FITC-BSA loaded films (top yellow rectangle). No fluorescence was detected. The fluorescence images of the Rho-DEX loaded films (middle purple rectangle). Strong fluorescent signals were measured. Dual FITC-BSA/Rho-DEX loaded films (bottom blue rectangle). Fluorescent signals were detected. D) The fluorescence images (λ_{ex} = 490 nm and λ_{em} = 520 nm) of the FITC-BSA (top yellow rectangle), Rho-DEX (middle purple rectangle), and dual FITC-BSA/Rho-DEX loaded films (bottom blue rectangle). E) Mass degradation kinetics of Rho-DEX loaded (black) and FITC-BSA loaded (red) films. F) Cumulative release of Rho-DEX (black) and FITC-BSA (red) from the drug-loaded films. G) Mass degradation kinetics of dual Rho-DEX and FITC-BSA loaded films. H) Amount of Rho-DEX (black) and FITC-BSA (red) release from dual-drug loaded films.

loaded, and both drug-loaded chitosan–catechol films. The photographic (**Figure 4**A,B) and fluorescent (Figure 4C,D) images demonstrate successful encapsulation of the macromolecules. The encapsulated molecules showed controlled release profiles from the chitosan–catechol films. When a single-type macromolecule is encapsulated, dextran release kinetics is fast compared to that of BSA release which was experimentally determined by film dry weight and cumulative release (Figure 4E,F). Irregular micropores were generated in the film after the release of the macromolecules, which is particularly significant for dextran (Figure S5B, Supporting Information). Figure S5A showed the SEM image of the Rho-DEX loaded chitosan–catechol films before the stability tests. No pore structures were found, indicating the micropores might be produced by the release of the encapsulated dextran. Moreover, after four weeks, weak fluorescent signals from the detector (Xenogen IVIS system) were remained, indicating the presence of un-released FITC-BSA. Due to the differential release kinetics, we tested abilities for dual drug release from the chitosan–catechol film (LC). Both Rho-DEX and FITC-BSA-loaded chitosan–catechol films showed fast release of dextran within first 2 days followed by slow release of BSA (Figure 4G). Correspondingly, the loss of dry weight of the film was measured (Figure 4H), showing intermediate kinetics of the loss compared to single-component loaded films.

The chitosan–catechol films can be a platform for immobilization of useful proteins on surfaces. Human growth factor (HGF) was covalently tethered with ease by adding HGF solution (100 ng in serum-free media, pH 7.2) to the chitosan–catechol (LC) film for 2 h in the presence of sodium periodate (93.5 μM), and the film was washed with serum-free media (**Figure 5**A). Michael addition reaction between the amine group of proteins and the catechol has been widely known surface chemistry for protein surface immobilization.[43,44] The

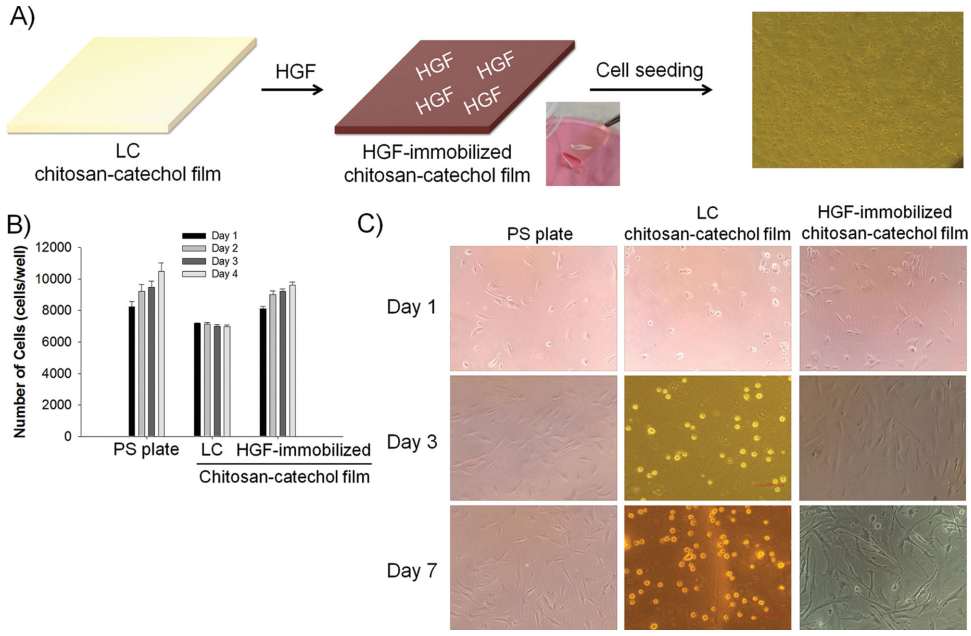

Figure 5. Cell culture chitosan–catechol films. A) Schematic illustrations of cell implantable, HGF-immobilized chitosan–catechol films. Preparation of LC chitosan–catechol film (left), HGF-immobilized chitosan–catechol film (middle) and cell culture on the film (right). B) Cell counting on the surfaces of polystyrene plate (positive control), LC chitosan–catechol films and HGF-immobilized chitosan–catechol films. C) The cell morphology on the surfaces of polystyrene dish (left), LC chitosan–catechol films (middle), and HGF-immobilized chitosan–catechol films (right) as a function of incubation time.

surface protein immobilization reaction was confirmed by improved cell adhesion and its proliferation. Normal human dermal fibroblast (NHDF) cells attached directly to the chitosan–catechol film exhibited non-proliferated behavior, indicating the cells were chemically immobilized onto the film surfaces by the catechol-mediated reaction (Figure 5A,B). However, the NHDF cells seeded onto the HGF-immobilized chitosan–catechol films adhered and proliferated showing a normal cellular activity.

3. Conclusion

In conclusion, we reported a biomimetic approach for the preparation of the water-borne yet water-insoluble chitosan films using chitosan–catechol polymeric precursors via the self-crosslinkable catecholamine chemistry. By simple drying of distilled water without adding any chemical additives and organic solvents, the chitosan–catechol solution spontaneously produced mechanically strong, flexible, biocompatible, and transparent freestanding films. The mechanical properties of the chitosan–catechol films were controllable by adjusting inter-molecular catechol-amine and/or catechol-catechol crosslinking density. As the process for film production is very simple (i.e., evaporation of water), the method is readily scalable for large-scale commercialization. These chitosan–catechol films can not only be used eco-friendly bags functioning similar to the widely used poly(vinyl) bags, but also be utilized as depots for controlled drug release and tissue engineering scaffolds.

4. Experimental Section

Materials: Chitosan (M.W. 100 kDa, 70% deacetylated) was purchased from Heppe Medical Chitosan GmbH (Halle (Saale), Germany). Hydrocaffeic acid (HCA, 3,4-dihydroxy hydrocinnamic acid) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), rhodamine-dextran conjugates (Rho-DEX) and fluorescein isothiocyanate-bovine serum albumin conjugates (FITC-BSA) were purchased from Sigma-Aldrich (Minnesota, USA). Ethanol was purchased from Merck Chemicals (Darmstadt, Germany). All other chemicals were of an analytical grade.

Synthesis of Self-Crosslinkable Catechol-Conjugated Chitosan (Chitosan–Catechol): Cchitosan–catechol was synthesized with slight modification via the standard EDC chemistry by forming an amide bond between the carboxylic acid group in hydrocaffeic acid and the primary amine groups in chitosan. Briefly, chitosan (1 g, 5.4 mmol for the monomer) was dissolved in deionized and distilled water (DDW, 100 mL) and the pH was adjusted to 5.5 to solubilize. Hydrocaffeic acid (1.18 g, 6.5 mmol) and EDC (1.24 g, 8.0 mmol) in 50 mL DDW and ethanol (1:1 v/v) were slowly added to the chitosan solution, and reacted for 12 hrs. Thus, the molar equivalent with respect to the glucosamine repeating unit is 1 : 1.2 : 1.15 (glucosamine unit : hydrocaffeic acid : EDC). The pH value of the reaction solution was maintained at 5.5. The product was purified by dialysis (MWCO = 1000 Da, SpectraPor, USA) against pH 5.0 HCl solution for 2 days and DDW for 4 h. The final chitosan–catechol conjugate was lyophilized and was kept in a moisture-free desiccator before use. ^{1}H-NMR spectroscopy (Bruker Avance, 400 MHz) was used to verify the conjugation reaction validity in a qualitative way. When the catechol moiety was successfully tethered, relatively small peaks appeared between 6.5 and 6.8 ppm (3H, in D_2O). For quantitative analysis for the catechol conjugation degree, UV–Vis spectrophotometer (Hewlett-Packard 8453, Groton, CT, USA) was used using dopamine standard solutions. The degree of catechol conjugation for the chitosan–catechol used in this study was 8.4%.

Preparation of Chitosan–Catechol Films: To fabricate lightly crosslinked chitosan–catechol films, chitosan (100 mg) was dissolved in DDW and then poured onto the polytetrafluoroethylene (PTFE) mold (diameter: 3.5 cm, thickness: 2 cm), which was cleaned with 70% ethanol before casting. Chitosan–catechol films were dried at 40 °C for one day, and the films were kept in a moisture-free desiccator before use. The dried chitosan–catechol films were peeled from the polytetrafluoroethylene mold and cut for further experiments. To fabricate the uncrosslinked films, chitosan–catechol was dissolved in 0.1 N HCl solutions (10 mL) and dried for one day. To utilize the self-crosslinking properties of catecholamine moieties, we control the oxidation reaction by using 0.1 molar $NaIO_4$ equivalents to the conjugated catechol for moderately crosslinking or 1 molar equivalent to the catechol for heavy crosslinking film preparation. In preparation of the crosslinked film, distilled and deionized water was used. The thickness of chitosan–catechol films was fixed from 10 μm to 20 μm measured by scanning electron microscope (SEM). Samples for tensile strength (TS) testing were cut into 1.5 cm by 2 cm rectangular strips. The samples were cut into 0.5 by 2 cm rectangular strips for UV–Vis spectroscopic analysis. Nano-indentation, SEM samples were prepared by using a biopsy punch (diameter: 6 mm).

UV–Vis Spectroscopic Studies of Chitosan–Catechol Films: UV–Vis spectroscopy was used to monitor the transmittance with various degrees of crosslinking samples of chitosan–catechol films. The regular increase in the absorbance spectra is due to the accelerated crosslinking process catalyzed by $NaIO_4$. It was also confirmed the negligible attenuation level of chitosan–catechol films with various crosslinking density in the visible range.

Mechanical Characterization: Tensile strength of chitosan–catechol films was evaluated using a Universal Testing Machine (Instron 5583, Instron Engineering Co., Canton, MA, USA) equipped with a 150 N load cell. Tensile strength was collected by pulling the probe with a crosshead speed of 1 mm/min. The data rate of tensile strength was fixed to 5 pts/sec. Tensile strength was calculated by dividing the maximum (peak) load for breaking film by the cross-sectional area of the film. Nanomechanical properties of chitosan–catechol films were evaluated with a Nano-indentation System (Nano Indenter XP, MTS, USA). Nanomechanical values of the elastic modulus and hardness of the chitosan–catechol films were obtained by Oliver-Pharr methods. Fused silica was used as a reference material for tip calibration, and a pyramidal diamond tip (50 μm) was used for the measurements. The chitosan–catechol films were attached onto the aluminum holder before analysis. An indentation strain was fixed to 0.05 s^{-1}. The maximum excursion is 500 nm, with the approach rate of 2 nm/s and withdraws rate of 10 nm/s and a hold time of tip of 10 s. A total of 7–9 indentations were performed for each sample, and its average value was reported. All samples were measured in dried conditions.

Preparation of Model Drug-Loaded Chitosan–Catechol Films: Chitosan–catechol (100 mg) was dissolved in 10 mL DDW and then 1 mL of rhodamine-dextran (Rho-DEX) (10 mg/mL) was added. After mixing for 2 h, the mixture was poured onto the PTFE mold (diameter: 3.5 cm, thickness: 2 cm) and Rho-DEX loaded chitosan–catechol films were dried at 40 °C for one day. To fabricate the fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) loaded chitosan–catechol films, chitosan–catechol (100 mg) was dissolved in 10 mL DDW and then 1 mL of FITC-BSA (10 mg/mL) was added and stirred for 2 h.

Stability Tests: To test in vitro stability of chitosan–catechol films and drug-loaded chitosan–catechol films in pH 7.4 PBS solutions, weight losses were evaluated under physiological conditions. Briefly, lightly crosslinked (LC) films were placed in 2 mL test tubes and pH 7.4 PBS solutions were added onto the individual films. The films in test tubes were incubated at 37 °C. After pre-determined time intervals, the supernatants were collected and the weights of dried solid films were measured. All the samples were triplicate.

Release Kinetics of Model Drug from Chitosan–Catechol Films: Release rates of Rho-DEX from chitosan–catechol films were evaluated using a spectrofluorophotometer (SynergyTM Mx, BioTek, VT, USA) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Briefly, each film was placed in a 2 mL test tube, and then pH 7.4 PBS solutions (1 mL) were added to the test tube. After pre-determined time intervals, the supernatants were collected. The fluorescence of the collected solution was measured. Release rates of FITC-BSA from chitosan–catechol films were evaluated using the same method described above at excitation wavelength of 540 nm and an emission wavelength of 620 nm. All the samples were triplicate.

Cell Culture on Chitosan–Catechol Films: For cell culture on chitosan–catechol films, we prepared two chitosan–catechols films: lightly crosslinked and human growth factor-immobilized chitosan–catechol films. Moderately and heavily crosslinked chitosan–catechol films having a strong oxidant, sodium periodate which is substantially cytotoxic for biomedical applications were not used in this experiment. To immobilize the human growth factor (HGF), slightly crosslinked chitosan–catechol films were immersed in a medium containing HGF (100 ng) and $NaIO_4$ (0.1 mg). HGF-immobilized Sc-chitosan-cat film was cut into a rectangular sheet (0.5 cm × 2 cm) and was immersed with 70% ethanol for 2 h for sterilization and washed with media to remove all residual alcohol. HGF-immobilized Sc-chitosan-cat film was placed on a well of 12 well-plates and normal human dermal fibroblast (NHDF) cells (1.0×10^4 cells) were seeded on a film. The seeded chitosan–catechol film was incubated with serum-supplemented media in 5% CO_2 incubator at 37 °C with 99% humidity for 7 days, and the media were replaced for every 3 days.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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