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Effect of Different Crosslinking Strategies on Physical Properties and Biocompatibility of Freestanding Multilayer Films Made of Alginate and Chitosan

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Freestanding multilayer films prepared by layer-by-layer technique have attracted interest as promising materials for wound dressings. The goal is to fabricate freestanding films using chitosan (CHI) and alginate (ALG) including subsequent crosslinking to improve the mechanical properties of films while maintaining their biocompatibility. Three crosslinking strategies are investigated, namely use of calcium ions for crosslinking ALG, 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide combined with *N*-hydroxysuccinimide for crosslinking ALG with CHI, and Genipin for crosslinking chitosan inside the films. Different characteristics, such as surface morphology, wettability, swelling, roughness, and mechanical properties are investigated showing that films became thinner, exhibited rougher surfaces, had lower water uptake, and increased mechanical strength after crosslinking. Changes of wettability are moderate and dependent on the crosslinking method. In vitro cytotoxicity and cell attachment studies with human dermal fibroblasts show that freestanding CHI-ALG films represent a poorly adhesive substratum for fibroblasts, while studies using incubation of plastic-adherent fibroblast beneath floating films show no signs of cytotoxicity in a time frame of 7 days. Results from cell experiments combined with film characteristics after crosslinking, indicate that crosslinked freestanding films made of ALG and CHI may be interesting candidates for wound dressings.

1. Introduction

Chronic wounds develop because of a defective regulation of processes involved in wound healing. They can be the result of severe injuries (e.g., burns, trauma, surgery) as well as main or side effects of chronic diseases (e.g., diabetes, venous disease, neurological conditions).[1] Chronic wounds have a strong impact on patients as they experience pain, reduced mobility and work ability, and often social isolation. They furthermore represent a significant burden for the health care systems. The major challenge with chronic wounds is to reestablish function and anatomical integrity of the damaged tissues. Hemostasis, inflammation, cell proliferation, and tissue remodeling are four processes that are related to normal wound healing.[2] Different types of cells play significant roles in the aforementioned steps, including blood cells (e.g., platelets, granulocytes, macrophages, lymphocytes) and tissue cells (fibroblasts, endothelial cells, keratinocytes). In addition, extracellular matrix proteins, proteoglycans, and other biomolecules are essential to orchestrate the wound healing process into success.[3]

A variety of wound dressing strategies can be employed to cope with chronic wounds, aiming to maintain a moist environment with materials like films, foams, and hydrogels that contain water-adsorbing materials (e.g., hyaluronan, alginate), to reduce infections (e.g., dressings containing silver ions, chitosan, or

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disinfectants) and to support healing and structural reconstruction (e.g., collagens, glycosaminoglycans, growth factors).[1,4] Nevertheless, the majority of wound dressings today tend to focus only on some of the aforementioned issues without addressing all aspects in a concerted manner.

Wound dressings can be produced by a variety of techniques that result in fibrous fabrics or non-woven materials using textile technologies, but also membranes and films prepared by thermal or solvent-based techniques.[5] The layer-by-layer (LbL) technique, which is based on the alternating deposition of polyanions and polycations on liquid/solid or liquid/liquid interfaces, can form surface coatings of nanometer thickness, but also freestanding films of micrometer thickness.[6,7] LbL has been acknowledged as a feasible method to produce novel biomaterials due to its ease of use, versatility, and ability to incorporate different molecules, as well as the ability to control topography, microarchitecture, and biomechanics of coatings, films, capsules, nano and micro particles.[8–10] An important advantage of LbL technique is that the layer-wise formation of films also permits the loading of bioactive molecules, such as pharmaceuticals and growth factors with the final aim of providing a biomimetic microenvironment for control of cell behavior.[11]

Different types of biomaterials of natural origin have been used for biomedical applications.[12,13] One large family of natural polymers are polysaccharides, which exhibit high hydration rates, are abundant, biocompatible, and very often biodegradable.[14] They are typically extracted from microorganisms, plants and animals, and exhibit a wide spectrum of biological effects.[15] Alginate (ALG) is extracted from cell walls of brown algae and consists of alternating blocks of mannuronic and guluronic acid.[16] It is a biocompatible, biodegradable, and non-immunogenic polyanion.[17] Polymeric formulations of ALG have been investigated as wound dressings and tissue engineering products, proving to accelerate the healing mechanism compared to the traditional gauze dressings.[18] Chitosan (CHI), which is derived from the exoskeleton of arthropods, is obtained after deacetylation of chitin and comprises β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units.[19] It represents a cationic polymer, widely used in biomedical industry because of its biocompatibility, low toxicity, and antibacterial properties.[20] Since, many polysaccharides represent highly hydrophilic molecules of good water solubility, they can be used to form coatings, films, and hydrogels of low stability and mechanical strength.[10] Hence, different physical and chemical crosslinking strategies have been developed to enhance structural and mechanical stability of such formulations. For example, calcium ions are used for crosslinking ALG by ionic crosslinking pendant carboxylic groups of guluronate blocks that lead to formation of "crystalline" segments, a strategy that has been used to prepare micro capsules or particles and hydrogels.[16] A chemical strategy is the combination of 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), previously used to crosslink multilayers from CHI and hyaluronic acid.[21] Carbodiimide conjugation works via the activation of carboxyl groups for direct reaction with primary amines to achieve amide bond formation. Finally, Genipin has been used recently to stabilize thin multilayer films made of ALG and CHI,[22] which is based on its ability to crosslink amino groups of CHI.[23]

The group of Mano et al. introduced the fabrication of freestanding multilayer films with LbL technique for different tissue engineering applications, focusing on preparing crosslinked adhesive films mainly for bone and cardiac tissue engineering.[6,8,24] Here, we focus on the effect of different crosslinking strategies using calcium ions, EDC-NHS, and Genipin in a comparative manner on the physical properties and biocompatibility of freestanding multilayer films made of ALG and CHI to explore the potential of these systems to be used as wound dressings, which should present low cell attachment, the ability to take up wound fluid, and protect against infections. Results are presented herein.

2. Experimental Section

2.1. Materials

Chitosan (medium viscosity, 85/500) was obtained from Heppe Medical Chitosan GmbH (Halle, Germany). Alginic acid sodium salt low viscosity (ALG) was received from Alfa Aesar, (Karlsruhe, Germany). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Alfa Aesar, (Karlsruhe, Germany). N-Hydroxysuccinimide (NHS) was purchased from Sigma-Aldrich (Steinheim, Germany). Genipin was purchased from Wako Chemicals GmbH (Neuss Üdesheim, Germany). $CaCl_2$, NaCl, HCl, and acetic acid were purchased from Merck (Darmstadt, Germany).

2.2. Polymeric Solutions and Substrate Preparation

150 mM NaCl washing solution was prepared and pH adjusted to 4 using 1 M solution of HCl. CHI solution (2 mg mL^{-1}) was prepared by dissolving it in 150 mM NaCl solution adding 30% acetic acid to adjust the pH to 4. For the preparation of a 5 mg mL^{-1} ALG solution, alginate was dissolved in 0.15 M NaCl solution as well and pH adjusted to 4 using HCl. Afterward, all solutions were sterilized by filtration using 0.22 µm pore size filters and stored at 4 °C.

For atomic force microscopy (AFM) experiments, silicon wafers were cleaned for 10 min in a solution made of a 5:1:1 ratio of milli-Q H_2O, NH_4OH (25%), and H_2O_2, respectively. The same method was applied for cleaning of glass slides used as fort preparation of freestanding multilayer films. Subsequently, the wafers were removed and washed six times with milliQ water for 5 min. Finally, the wafers were dried with N_2 gas and placed in a desiccator for 24 h. They were sterilized by soaking them in 70% ethanol followed by rinsing with milli-Q water prior to dip coating.

2.3. Dip Coating

The LbL method was applied to fabricate CHI-ALG freestanding multilayer films. Films containing 100 bilayers of CHI and ALG were fabricated using an automated dip coating
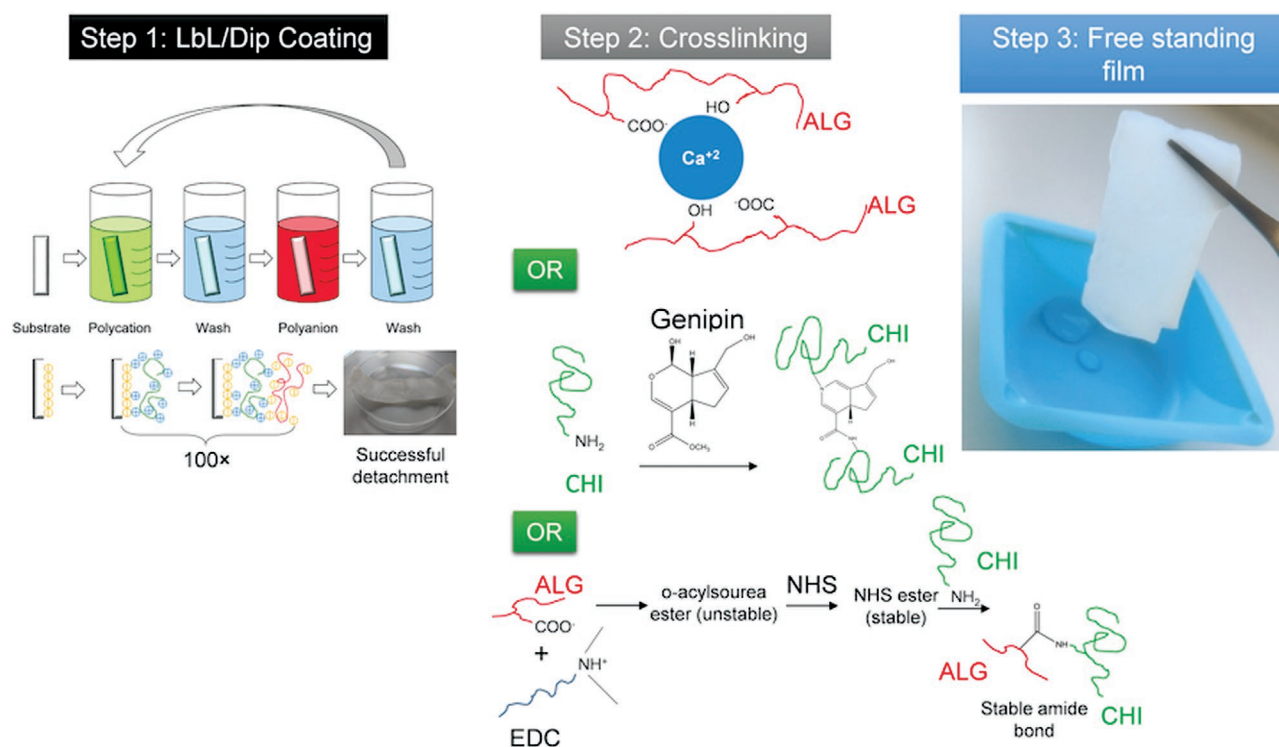
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Figure 1. Scheme of film preparation, crosslinking mechanisms and photograph of resulting films.

device (DR01, Riegler & Kirstein, Berlin, Germany). The first layer was CHI and the last ALG. The substrates used for LbL were glass slides ($76 \times 26mm^2$, Thermo Scientific, Hungary), because preliminary experiments revealed that glass was an appropriate substrate for the fabrication and proper detachment of films. The coating duration for each polyelectrolyte was 5 min with a 2.5 min washing step in-between each coating. Dip coating was performed at room temperature with a dipping speed of 0.8 cm s^{-1}. After the dip coating process was finished the samples were washed with milli-Q water and placed inside an oven for 45 min at 120 °C. This thermal treatment further enhanced the films detachability from the substrates without any problems and was not done to dry the films. The detached films were then stored at 4 °C in 150 mM NaCl solution to avoid growth of microorganism, while keeping them in a hydrated state.

2.4. Crosslinking of PEMs

Freestanding Chi-ALG films were further crosslinked using three different methods. Non-crosslinked films were also investigated and used as control. After films were detached from the substrates, they were punched into circular disks of 12 mm diameter and placed inside 24-well plates. The protocol for Genipin crosslinking was following a previous work.[23] Films were immersed overnight in a Genipin solution (1 mg mL^{-1} in H_2O) at 37 °C. The next day, absolute ethanol was used to stop crosslinking, followed by three times washing (15 min each) with milli-Q water. Chemical

crosslinking using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) was carried out according to a protocol established previously.[19] EDC was used at a concentration of 50 mg mL^{-1} and NHS at 11 mg mL^{-1}. Both solutions were mixed and the films were incubated at 4 °C for 18 h and subsequently washed three times for 1 h in 150 mM NaCl solution at pH 8.0. The crosslinking with calcium ions was done using a solution of $CaCl_2$ (10 mg mL^{-1} in H_2O) with incubation for 30 min and a subsequent rinse with milli-Q water following a recently published protocol.[25] **Figure 1** summarizes the freestanding film preparation procedure along with the different crosslinking methods employed.

2.5. Wet Thickness Measurements

A total of 100 bilayer films were deposited on glass slides and peeled off the substrate as described above. The film thickness was measured using a micrometer (Mitutoyo no. 293-240, Mitutoyo Europe GmbH, Neuss, Germany).

2.6. Swelling Experiments

The swelling behavior of freestanding films was studied by calculating swelling ratio. These calculations were performed by first drying freshly prepared films for 48 h followed by rehydrating these films by immersing them in milli-Q water at pH 7 for 48 h.

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The swelling ratio is defined as the fractional increase in the weight of the film due to the absorption of water. The swelling ratio of films can be determined by the following equation.

$$\text{Swelling Ratio} = W_s / W_d \qquad (1)$$

where W_s and W_d are the weights of hydrated and dry free-standing films, respectively.

2.7. Dynamic Mechanical Analysis

Dynamic Mechanical Analysis (DMA) was used to characterize the mechanical properties of freestanding films where stress was applied in a sinusoidal waveform and the responses were measured as a function of frequency. All viscoelastic measurements were done on an Anton Paar MCR 502 (Anton Paar Germany GmbH, Ostfildern-Scharnhausen, Germany) device in stress control mode using plate-plate geometry with a plate diameter of 25 mm. The operating frequency range was from 0.1 to 200 rad s^{-1} under strain amplitude of 0.5%. All CHI-ALG freestanding films were subjected to a frequency sweep at 25 °C and 50% relative humidity. Experiments were conducted in triplicates and the data are presented as mean ± SD.

2.8. Atomic Force Microscopy

AFM measurements were done on ten bilayer films of CHI-ALG. Surface topography and roughness of the films coated on Si-wafers (10×10 mm^2) were determined using AFM (Nano-R, Pacific Nanotechnology, Santa Clara, CA, USA) under ambient (air) laboratory conditions of temperature and humidity in the close-contact mode. For topography pictures, scans of 5×5 μm^2 were recorded at 512×512 pixel resolution. For the determination of the average roughness R_a and the mean square root roughness R_q (RMS), scans of 3×3 μm^2 were recorded at 512×512 pixel resolution. Pictures were taken at five different locations of every sample. R_a and R_q were determined using "Gwyddion 2.49" software (Czech Metrology Institute, Brno, Czech Republic).

2.9. Static Water Contact Angle

Water contact angle of 100 bilayer films of CHI-ALG was measured using OCA 15+ system (Dataphysics GmbH, Filderstadt, Germany) by captive bubble method. In this method, a bubble of air is injected beneath a solid substrate to which the film is attached. The parameters like dosing volume, dosing rate, and fitting method were the following: 3 μL dispense volume was dispensed with a flow rate of 1 μL s^{-1}, while the Ellipse-fitting method was chosen as the preferred fitting approximation. Freshly prepared milli-Q water (0.055 μS cm^{-1}) was selected as the ambient phase. Each type of sample was studied by probing the surface with five air bubbles at different positions on the film. Calculation of contact angles was done by OCA 15+ software.

2.10. Culture of NHDF (Normal Human Dermal Fibroblasts Cell Line) Cells

Cryopreserved normal human dermal fibroblasts (NHDF) were obtained from ATCC (Manassas, USA). Cells were seeded in T75 culture flasks in Dulbecco's Modified Eagle Medium (DMEM) from Lonza GmbH (Cologne, Germany) containing 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), and 1% (v/v) penicillin/streptomycin/amphotericin (AAS, Sigma-Aldrich, Steinheim, Germany), without sodium pyruvate at 37 °C in a humidified 5% CO$_2$/95% air atmosphere using a NUAIRE DH Autoflow incubator (NuAire Corp, Plymouth, MN, USA). Cells were harvested at around 90% confluence with 0.25% trypsin/ 0.02% ethylenediaminetetraacetic acid (EDTA) without Ca^{2+} and Mg^{2+} (Biochrom, Germany). Trypsin was neutralized with DMEM containing 10% FBS and the cell suspension was transferred to 15 mL tubes and centrifuged for 5 min at 250 rpm speed at room temperature. After centrifugation, the cell-free supernatant was aspirated and the cell pellet was re-suspended in serum-free medium. NHDF cells were used to investigate cytotoxicity and biocompatibility of freestanding ALG-CHI films.

2.11. Studies on Cytotoxicity of Freestanding Films

The metabolic activity of NHDF cells cultured beneath floating freestanding films was analyzed using QBlue kit (Biochain, Heidelberg, Germany), which is based on the conversion of non-fluorescent redox dye resazurin to a highly fluorescent product (resorufin) by living cells. Films were built according to the aforementioned protocols and sterilized by immersion in 70% ethanol with subsequent washings using sterile NaCl solution (pH 7.4). 1 mL cell suspension with a quantity of 2.5×10^4 cells per well (DMEM, 10% FBS) was seeded onto the bottom of a 24-well plate for 72 h. Films were added on top of the confluent cell layer. After 24 h the films were transferred to a new well plate. QBlue reagent solution was prepared at a ratio of 1:10 with colorless DMEM and added to each well and incubated at 37 °C for 3 h. After incubation, duplicates of 100 μL of the supernatant were transferred to a black 96-well-plate. The converted product was fluorometrically quantified at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a plate reader (FLUOstar, BMG LabTech, Offenburg, Germany). Cells without films were used as negative control, while films without cells and culture medium were used as blanks. After the measurements were finished, QBlue medium was aspirated from the wells and replaced by fresh DMEM supplemented with 10% FBS and 1% AB pen/strep/amp. The films, which were stored in a different well plate, were added back to the respective wells. The protocol was repeated on the 4th and 6th day.

2.12. Studies on Adhesion and Growth of NHDF Cells on Freestanding Films

Cells suspended in DMEM and 10% FBS were seeded on top of the films at the density of 2.5×10^4 cells per well. After 24 h, the medium was aspirated from the wells. Cells were washed

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once carefully using phosphate buffered saline (PBS). MTT solution was prepared in PBS (5 mg ml⁻¹). 50 µL of MTT solution was added along with 450 µL of colorless DMEM to each well and incubated for a period of 2.5 h. Following this step, the formazan crystal-stained cells were visualized under 2× and 5× optical zoom by Stereomicroscope SMZ-168 (Motic Microscopes, Xiamen, China) to detect adherent cells and cell colonies. Then, the MTT solution was carefully aspirated and 0.04N HCl in isopropanol (500 µL) was added to dissolve the formazan dye from stained cells for their quantification. The well plates were kept on a shaker for 1 h and afterward 100 µL of the solution was added to a new 96-well plate (in duplicates). The absorbance of the supernatant was quantified using a plate reader (FLUOstar, BMG LabTech, Offenburg, Germany) at a wavelength of 550 nm. Isopropanol solution without MTT was used as the blank and MTT stained cells seeded on TCPS wells without films were used as control.

2.13. Environmental Scanning Electron Microscopy

Surface topography of polyelectrolyte multilayers (PEMs) and the presence of cells were studied with a Philips environmental scanning electron microscopy (ESEM) XL 30 FEG microscope from Thermo Fisher (Kandel) GmbH (Karlsruhe, Germany) at a water vapor pressure of 1.2–1.7 mbar (in the sample chamber) in wet mode and with use of a GSE (gaseous secondary electron) detector. Cells were seeded on top of the films at density of 2.5×10^4 cells per well in DMEM containing 10% FBS. Then, the medium was aspirated and films with cells were taken out on the 3rd day. Cells were fixed with 4-formaldehyde on the film surface. The micrographs were taken at the voltage of 20 kV. The magnification used to obtain these micrographs varied from 100 to 5000 times.

2.14. Statistical Analysis

Statistical analysis of data was performed with Origin 8.5G Pro software (OriginLab Corp., Northampton, USA). Mean values, standard deviation, and one-way analysis of variance (ANOVA) (with post-hoc Tuckey) were calculated and indicated in the respective figures. A value of $p < 0.05$ or below was considered for significance testing.

3. Results and Discussion

3.1. Physical Characterization of Freestanding Multilayer Films

The effect of the crosslinking methods on the structural properties of the wet freestanding films was studied by different analytical methods. **Figure 2** shows the result of thickness measurements, which demonstrates that chemical crosslinking caused a decrease of thickness compared to the native film with lowest thickness for Genipin crosslinking, while crosslinking with CaCl₂ caused only a minor reduction. All differences were significant compared to the native film ($p \leq 0.05$). Such decrease in thickness after crosslinking is probably due to the formation of
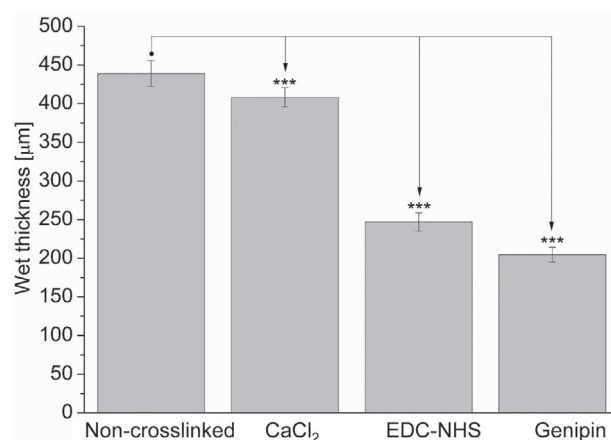

Figure 2. Wet thickness measurements for the PEMs with and without crosslinking treatment; $n = 15$, mean ± SD, *** $p \leq 0.001$.

additional bonds between molecules in the film thereby reducing their mobility and has been known to contract hydrogels.[26]

Swelling ratio, related to the water uptake of dry films, is an important parameter affecting crosslinked films since it determines their applicability as wound dressings where they must take up wound fluids. The experiments were done with distilled water to achieve maximum swelling due to the fact that no counterions are present to screen charged groups. In addition, a higher pH 7 was chosen, at which the amino groups of chitosan are deionized weakening the electrostatic interactions between CHI and ALG, which may also permit more swelling.[27] **Figure 3** show results of these measurements. It was apparent that the swelling ratios of native films are higher than that of the crosslinked films. This is obviously related to the fact that the interaction between both polyelectrolytes ALG and CHI is relatively low at a pH 7, when amino groups of CHI are not charged though other interactions (e.g., hydrogen bonding) will contribute to the stability of films while the other films are still crosslinked.[22] It should be emphasized that no dissolution of
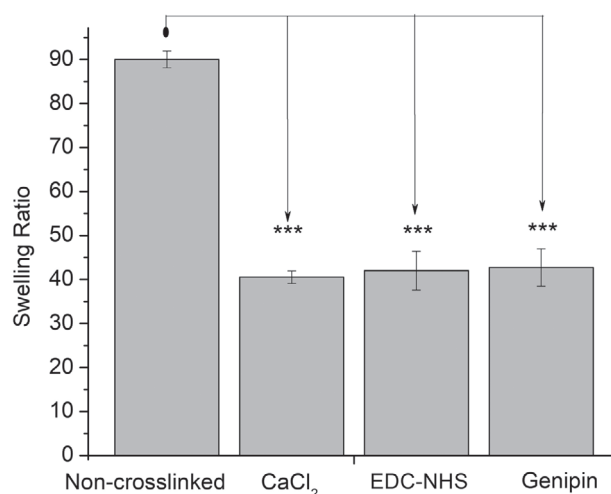

Figure 3. Swelling ratio of (CHI-ALG)₁₀₀ PEMs in H₂O at pH 7.0 after 48 h; $n = 3$, mean ± SD; *** $p \leq 0.05$.
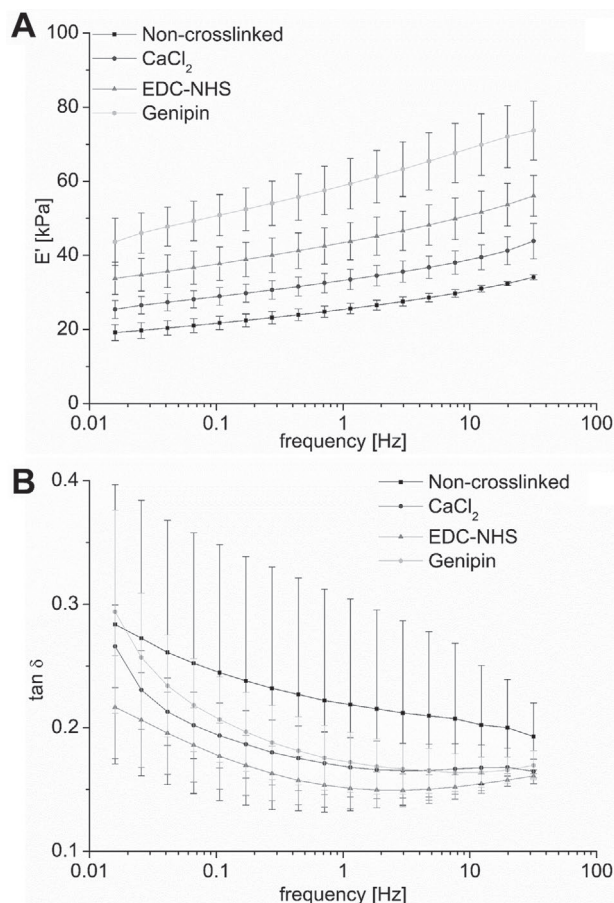
Figure 4. Dynamic mechanical analysis performed on CHI-ALG films before and after crosslinking treatment. (squares = non-crosslinked, circles = CaCl$_2$, triangles = EDC, hexagons = Genipin); The graphs represent A) Storage modulus (E'), B) Loss factor (tanδ); frequency ranged from 0.015 to 32 Hz at temperature of 25 °C; $n = 3$, mean ± SD.
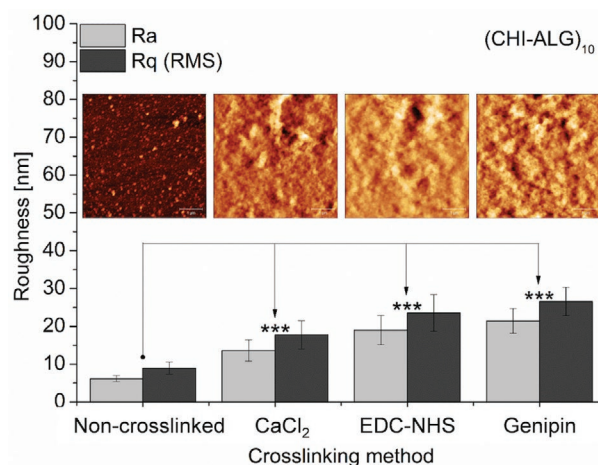

Figure 5. Surface topography and roughness (Ra = light gray, Rq = dark gray) of CHI-ALG PEMs before and after crosslinking treatment visualized by AFM; $n = 10$, mean ± SD; *** $p \leq 0.001$; scale bar: 1 µm.

films was seen under this condition. The swelling ratios of the crosslinked films were comparable to each other, but differed significantly from that of native film. The lack of diffrences in swelling among the crosslinked films was not expected because of the different crosslinking mechanisms since calcium ions affect only the ALG chains,[18,28] whereas Genipin covalently crosslinks the chitosan and EDC-NHS both polysaccharides[22,29]). However, as also visible in the next section mechanical properties were not much different, which goes along with relatively small changes in swelling properties of polymeric network.[30]

DMA was performed to investigate the effect of the different crosslinking methods on the mechanical properties of freestanding films. Mechanical properties of films are not only important for the potential application as flexible and mechanical resilient dressings, but are also important in affecting attachment of cells like fibroblasts that prefer stiff versus soft surfaces.[31] DMA was used to measure the storage modulus (E'), which reveals the elastic behavior of a material, while the loss modulus (E'') shows its viscous behavior. The E' values exhibited a consistent increase with frequency in all samples, indicating the elastic behavior of films (**Figure 4**A). It was evident that crosslinking increased E' of CHI-ALG

films as they exhibited a more elastic behavior than the native films. More specifically, E' values were 1.31× higher for CaCl$_2$ crosslinked films, 1.7× higher for EDC, and 2.29× higher for Genipin crosslinked films. The ratio of loss to storage modulus expressed as tanδ, provided information about the viscoelastic behavior and damping properties of the material.[32] tanδ values for all crosslinked films where similar, but always lower than the non-crosslinked samples in almost all frequencies (especially after 0.03 Hz) (Figure 4B). In all cases, a general decrease in frequency was observed. The decrease in tanδ indicates that the films acquired more elastic properties due to the crosslinking, which was expected.[22] The EDC-crosslinked samples showed the lowest values (on average) with different frequencies, an indication of lower dissipation properties and higher crosslinking degree.[8] tanδ values of the other two crosslinking methods were similar, especially in frequencies higher than 1 Hz (Figure 4B). In all cases, tanδ values ranged from 0.15 to 0.29, a clear indication of their viscoelastic behavior as natural polymers with high water absorption ability. Such inverse relationship between mechanical properties and hydration levels was previously observed by other authors.[8,33,34]

The surface roughness and topography of non-crosslinked and crosslinked PEMs were investigated by AFM in dry state of ten bilayer films prepared on silicon wafers. Thinner and dry films were selected due to difficulties in studying thicker freestanding films with AFM due to their highly swollen and soft nature. **Figure 5** shows that the surface roughness was significantly increased by all crosslinking methods. Compared to films previously prepared by other authors that were aiming for higher cell adhesion through increased roughness, our samples were relatively smooth, even after crosslinking.[8] Since these films are made to be used as potential wound dressings, reduced or no cell adhesion on them is a desirable property.

Static water contact angle (WCA) studies were performed to characterize the wettability of films, which is another important determinant of protein adsorption and cell adhesion. Hydrophilic, soft surfaces, and materials generally reduce protein adsorption and suppress cell adhesion.[35] All films were
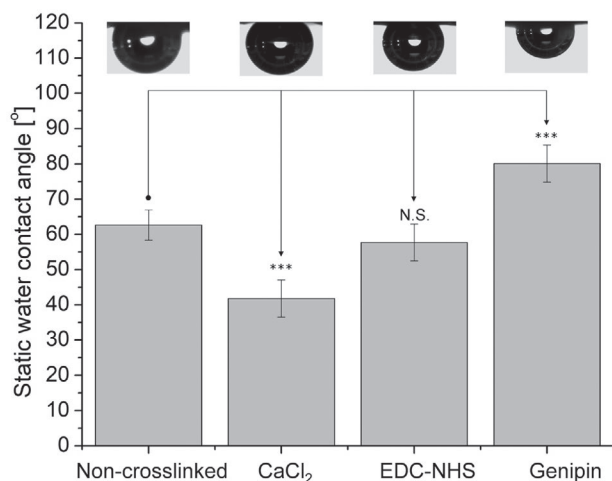
Figure 6. Static WCA values after PEM formation, with and without crosslinking treatment; $n = 100$, mean ± SD, N.S. $p > 0.05$, *** $p \leq 0.001$.
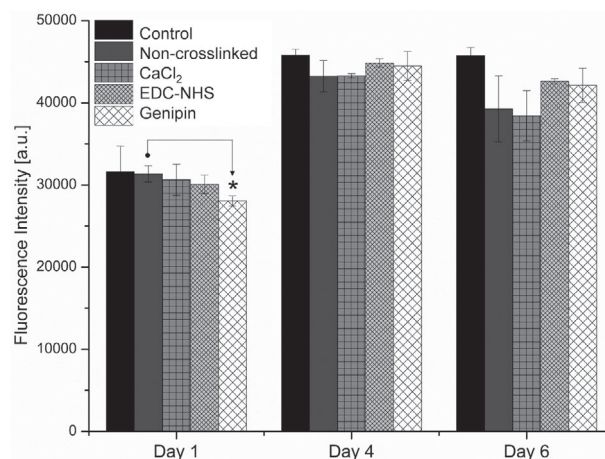

Figure 7. QBlue fluorescence intensity measurements of NHDF cells cultured in DMEM with 10% FBS, seeded on films with and without crosslinking. Measurements were done after 1, 4, and 6 days of incubation. Fluorescence intensity was determined at 544 nm excitation and 590 nm emission wavelength. Incubation time for QBlue was 3 h; $n = 6$, mean ± SD; * $p \leq 0.05$.

found to be hydrophilic with WCA below 90° (**Figure 6**). While there was no significant change after EDC-NHS treatment compared to native films (WCA ≈ 60°), the use of CaCl$_2$ and Genipin led to a significant change in the wetting properties with increased and decreased wettability, respectively. The films crosslinked with CaCl$_2$ were more hydrophilic compared to the native film, which might be related to an increased entrapment of water molecules in the films as found in another study[36,37] On the other hand, the average contact angle values increased to 80.12° ± 5.27° for films crosslinked with Genipin revealing an increase in hydrophobicity. The latter is probably due to the fact that Genipin will remain a part of the multilayers, providing additional aromatic heterocyclic ring systems, which are less hydrophilic. This result was in agreement with previous work from other investigators.[29] In case of samples treated with EDC-NHS crosslinking no change was observed though coupling of carboxylic groups to amino groups should reduce the quantity of polar or charged groups exposed to the aqueous environment, which we found also for smooth multilayer films made of hyaluronan (HA) and poly-L-lysine (PLL) in a recent work.[38] On the other hand, roughness of films increased, which will also decrease water contact angles according to Wenzel.[39] This could explain why the WCA remained similar compared to the values of the native films.

3.2. Biocompatibility Studies

Studies on cytotoxicity and biocompatibility were conducted to find out whether the crosslinker exert any cytotoxic effects that would promote undesirable inflammation when applied as wound dressing. In addition, cell adhesion and growth studies using human dermal fibroblasts were conducted to understand cell attachment, which is not desirable because any dressing material should not adhere to the wound area underneath.

Hence, a cytotoxicity assay was carried out to investigate whether the freestanding films display any toxic effect on NHDF cells, particularly when considering the use of chemical crosslinkers. **Figure 7** shows the results of Qblue assay, when

cells were cultured beneath floating films for a period of 6 d. Generally speaking, no profound decrease in fluorescence intensity (FI) was observed between the control, meaning fibroblasts cultured on TCPS as control and all cells cultured with freestanding films. Indeed, there was a non-significant reduction of FI for freestanding films crosslinked with Genipin after 1 day and for native and CaCl$_2$ crosslinked films after 6 days. Because the small decline in FI and a similar value for native and CaCl$_2$ crosslinked films, real cytotoxic effects could be excluded. Hence, it was concluded that none of the crosslinking strategies affects cell viability negatively.

Further, fibroblast attachment and proliferation were studied using a formazan dye (MTT) to visualize cells on top of the films, as visualization by phase contrast microscopy was not possible due to film thickness and opacity. The formazan crystals formed inside viable cells were solubilized after taking micrographs for quantification of viable cells and hence cell attachment and growth. Both staining and assay were done on 1st and 7th day of culture. Micrographs shown in **Figure 8**A (upper row) demonstrated that the number of cells or cell clusters after 1 day of culture was much lower on all free-standing films compared to the control (TCPS). There were also no profound differences in the quantity of stained cells and clusters between the free-standing films whether native or crosslinked. The micrographs taken after 7 days of culture (Figure 8A, lower row) showed that the cell layer on the control TCPS became confluent. By contrast, cell clusters that could be visualized on the crosslinked films were sparsely distributed with a low surface coverage on samples. One observation was that clusters were slightly larger on the films crosslinked with Genipin. However, in general, no increase in the density of cells was visible compared to the results from day 1. The absorbance values were lower on day 1 for all types of films indicating a significantly lower quantity of cells on them, compared to the control (Figure 7). Micrographs taken after 7 days showed that the absorbance values were lower for all types of films indicating
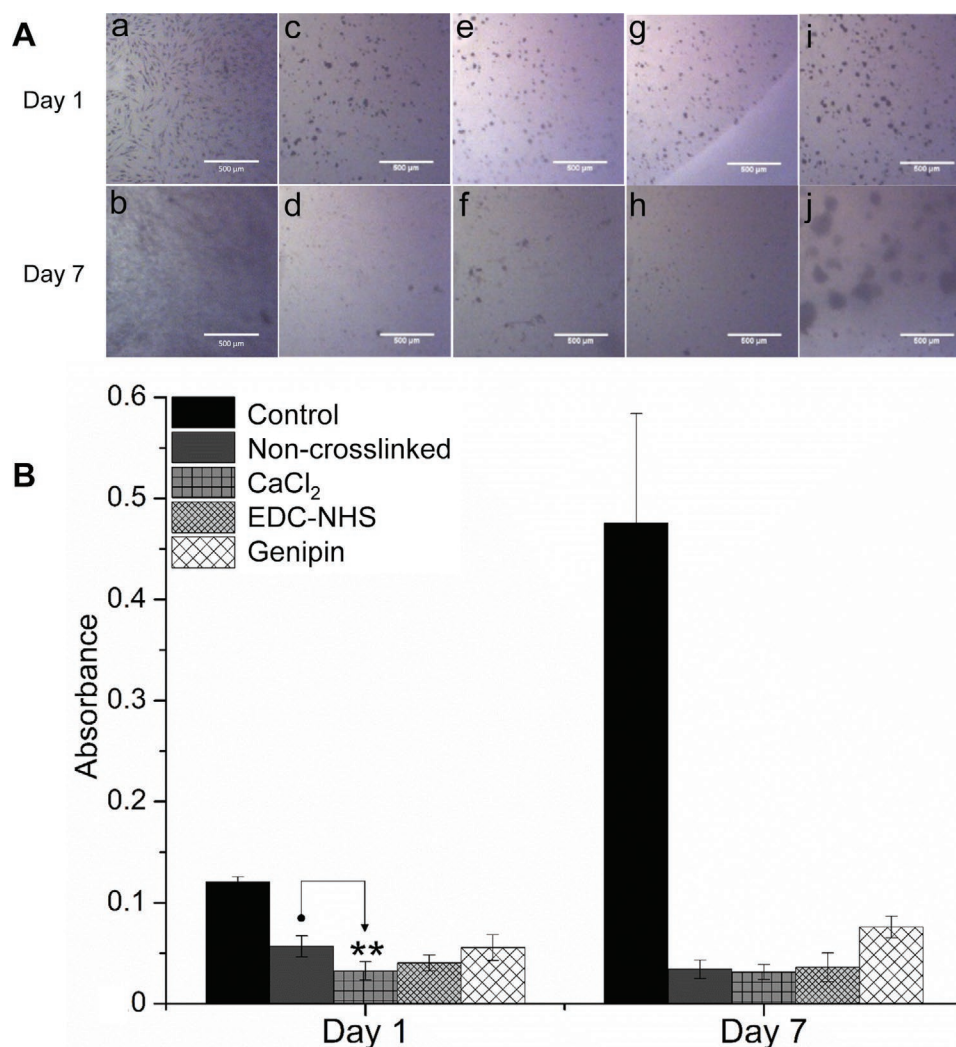
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Figure 8. A) Micrographs (a,b = Control; c,d = non-crosslinked; e,f = CaCl$_2$; g,h = EDC-NHS; i,j = Genipin) of fibroblast seeded in DMEM with 10% FBS on top of the films after being visualized by MTT staining after 1 day (upper row) and 7 days (lower row) for all crosslinking conditions; scale bars represent 500 μm; B) Metabolic activity of NHDF cells seeded on top of films measuring absorbance at 550 nm after 1 and 7 days of incubation; n = 6, mean ± SD; ** $p \leq 0.01$.

lower number of cells compared to the control ($p \leq 0.001$). There was no increase in values from day 1 except for fibroblasts plated on films crosslinked with Genipin. The absorbance values for all conditions except Genipin showed even a decline compared to the first day, indicating that films' surfaces are not favorable for cell attachment for all crosslinking conditions. However, as a sign of no toxicity, cells were able to form clusters, visible especially in Figure 7 (Genipin samples, micrographs i and j). No significant difference between the native samples and the crosslinked films was observed with the exception of CaCl$_2$ samples after day 1 ($p \leq 0.01$). In this case, the net charge of the films' surface is decreased due to the engagement of carboxylic groups of ALG with Ca ions. That leads to reduced cell attachment and growth on the surface.[40,41]

Based on both MTT and QBlue assay results we can conclude that films do not have any toxic effect on NHDF cells and at the same time do not favor their adhesion and proliferation on their surface. Both outcomes deem these films appropriate

candidates for wound dressings. On the other hand, no definite conclusion can be made on which of the crosslinking processes is more favorable, based on cell studies alone.

ESEM pictures taken at the top of the alginate ending side were in line with the previously obtained results from the AFM experiments (**Figure 9**). However, cell shape even after 72 h appeared rounded, which contradicts the nature of NHDF cells to stay elongated. We could not observe any major difference in cell numbers and/ or a specific trend regarding their spreading patterns. However, it was obvious that surface of the films was not suitable for cells to attach and elongate, creating colonies. The latter can be achieved via adjacent cells through ligand engagement by receptor-mediated cell mechanosensing (e.g., integrins and cadherins).[42] Judging from pictures a–d (Figure 9), it was evident that there were no organized colonies, while pictures e–h (Figure 9) clearly revealed round shaped cells. The crosslinking method did not seem to make any difference in this case, and there was no obvious difference
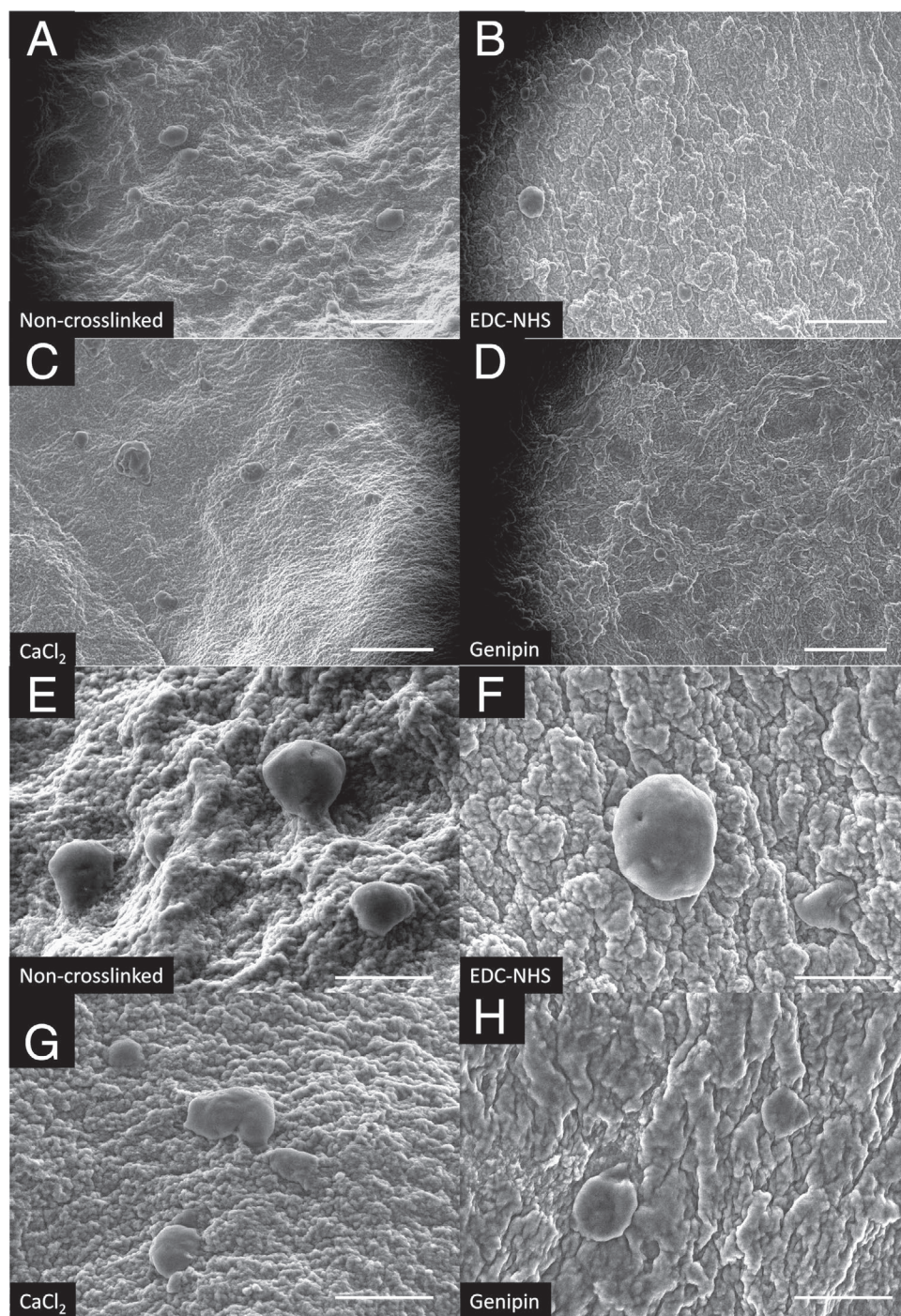
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Figure 9. ESEM pictures taken for both non-crosslinked and crosslinked conditions; scale bars for micrographs A–D) represent 100 μm and for micrographs E–H) 30 μm.

compared to the untreated films as well. When a cell adheres to a substrate it responds to the substrate stiffness through the rearrangement of the cytoskeletal components such as actin filament and microtubules.[43,44] Cells have more organized cytoskeletons on stiffer substrates.[45] They tend to remain rounded with few focal adhesions on top of soft surfaces.[46] Even after the films were crosslinked, the surface remained

relatively soft for this type of cells to be elongated. The latter could also explain the higher fluorescence intensity and absorbance values (long-term) observed for Genipin samples that also exhibited the highest E' after crosslinking, compared to native samples. (Figures 5–7). ESEM observations supported our previous MTT findings regarding cells' preference not to attach and proliferate on the surface of freestanding films, being once

again an indicator that these films might be promising for application wound dressings.

4. Conclusions

An ideal wound dressing should not only protect wounds from secondary infection but also provide the physiological environment where wounds can properly heal.[47,48] Swelling properties of alginate would help to keep the wound area moist for proper healing while chitosan can stimulate rapid mobilization, adhesion, and aggregation of platelets to the wound site, thereby assisting and later accelerating granulation and matrix formation.[49] In addition, chitosan's well-known antimicrobial properties are very favorable for wound healing applications.[50] Cell experiments and ESEM micrographs showed that CHI-ALG films did not promote growth and proliferation of NHDF cells. In addition, observations from the cytotoxicity assay revealed no toxic effects when cells were in contact with films. These results combined with the aforementioned properties of alginate and chitosan create a strong case for CHI-ALG freestanding films to be used in wound dressing. In terms of cytotoxicity, no crosslinking method appeared to make a difference (positive or negative) compared to the native samples. In terms of applicability to a wound site, EDC treated samples could have an advantage due to their improved mechanical properties. On the other hand, $CaCl_2$ crosslinked samples were the thickest, smoothest, most hydrophilic compared with the other crosslinked samples. The combination of these characteristics could be further used to create a wound dressing that also incorporates biomolecules for local, controlled release such as growth factors like fibroblast growth factor 2 (FGF2) that activate certain cellular functions and thereby expedite wound healing.

Conflict of Interest

The authors declare no conflict of interest.

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