Electrospinning pectin-based nanofibers: a parametric and cross-linker study

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Abstract

Pectin, a natural biopolymer mainly derived from citrus fruits and apple peels, shows excellent biodegradable and biocompatible properties. This study investigated the electrospinning of pectin-based nanofibers. The parameters, pectin:PEO (polyethylene oxide) ratio, surfactant concentration, voltage, and flow rate, were studied to optimize the electrospinning process for generating the pectin-based nanofibers. Oligochitosan, as a novel and nonionic cross-linker of pectin, was also researched. Nanofibers were characterized by using AFM, SEM, and FTIR spectroscopy. The results showed that oligochitosan was preferred over Ca^{2+} because it cross-linked pectin molecules without negatively affecting the nanofiber morphology. Moreover, oligochitosan treatment produced a positive surface charge of nanofibers, determined by zeta potential measurement, which is desired for tissue engineering applications.

Keywords Pectin · Oligochitosan · Electrospinning · Nanofiber · Tissue engineering

Introduction

Tissue engineering shows promise for closing the gap between the number of people waiting for an organ transplant and the number of people receiving one by restoring

the function of damaged tissue or replacing it altogether. In tissue engineering, the use of scaffolds allows cells to have the support necessary to grow and regenerate the extracellular matrix (ECM) (Hinderer et al. 2016; Naahidi et al. 2017). ECM is a complex 3D environment where tissue cells can proliferate and function. The matrix is composed of fiber forming proteins as well as collagen fibers and soluble factors (Hinderer et al. 2016). There is a great interest in fabricating scaffolds using nanofibers mimicking ECM for tissue engineering, considering the majority of the ECM biomolecules are at nanoscale and fibrous (Ao et al. 2017; Ingavle and Leach 2014; Limongi et al. 2016; Mortimer and Wright 2017; Ngadiman et al. 2017). Scaffolds with nanoscale architectures provide many more binding sites to cell membrane receptors, compared with micropore or microfiber scaffolds (Stevens and George 2005). Electrospinning is one of the most commonly used methods for fabricating nanofibers. The electrospinning process involves pumping a polymer solution through a syringe to form a droplet on the needle tip. When high voltage is applied, a cone shape (Taylor cone) is formed, followed by a thin stream of polymer solution. During the fly, the solvent evaporates leaving a solid fiber at the grounded collector (Agarwal et al. 2008; Babitha et al. 2017).

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Pectin is an anionic heteropolysaccharide found in the cell wall of all land plants (Noreen et al. 2017). Due to its biocompatibility and biodegradability, pectin has been used for various biological applications: including cell encapsulation, creating artificial red blood cells, bioprinting, and drug delivery (Banks et al. 2016; Crouse et al. 2015; Harvestine et al. 2014; Zhang et al. 2016a; Zhang et al. 2016b). Very recently, pectin has been explored for fabricating nanofibers. When electrospinning pectin alone, forming nanofibers remains challenging. A synthetic polymer such as poly(ethylene oxide) (PEO) or poly(vinyl alcohol) (PVA) is blended in with the pectin to improve its ability to be electrospun into fibers (2016, Cui et al. 2017). Moreover, cross-linking of pectin molecules within the nanofibers is essential to increase their stability in aqueous solutions. Since pectin is composed of galacturonic acid with carboxyl groups, divalent cations, such as Ca^{2+} , can be used as cross-linkers (Cui et al. 2017). This is very similar to the polysaccharide alginate (Bonino et al. 2011; Feng et al. 2017). However, the divalent cations of the cross-linking agents can negatively influence cell viability and function (Harvestine et al. 2014). These cations also have tendencies to leak out of the target material, leading to loss of stability and functionality. In our previous studies, oligochitosan, a natural cationic oligosaccharide, was proven to be an effective nonionic cross-linker of pectin (Crouse et al. 2015; Harvestine et al. 2014; Zhang et al. 2016a; Zhang et al. 2016b). Oligochitosan and pectin have complementary charges leading to the formation of polyelectrolytes. Additionally, oligochitosan can potentially generate a positive surface charge of the nanofibers, due to its protonated amino groups (Zhang et al. 2013). This is critical for tissue engineering applications, since a positively charged surface is preferred for cell adhesion (Ko et al. 2015; Suga et al. 2015; Zhang et al. 2017). Overall, the goal of this research is to optimize the parameters for electrospinning of pectin-based nanofibers and explore the potential of using oligochitosan as a cross-linker.

Materials and methods

Materials

Low methyl (LM) pectin was purchased from Willpower (Miami Beach, FL, USA). Pharmaceutical-grade oligochitosan (MW 2–3 kDa, > 90% deacetylation) was obtained from Zhejiang Golden-Shell Pharmaceutical Co. Ltd (Zhejiang, China). Polyethylene oxide (PEO, 600 kD), Pluronic® F-127, and CaCl_2 were purchased from Sigma Aldrich (St. Louis, MO, USA).

Parameters Optimization of the Electrospinning Process

The electrospinning setup (Linari Engineering, Valpiana, Italy) consisted of a syringe pump, 40 kV high voltage generator, and a stationary plastic collector plate covered by aluminum foil. The distance between the blunt syringe needle tip (25 Gauge) and the collector plate was kept constant at 20 cm. The solution preparation parameters studied include the pectin (4%, w/v): PEO (4%, w/v) ratio (from 70:30 to 40:60), Pluronic® F-127 concentration (from 2% (w/v) to 10% (w/v)), flow rate (from 0.2 to 1.2 mL/h), and voltage (from 10 to 25 kV). The influence of each parameter was investigated by varying one of the parameters while keeping the other parameters constant. Nanofibers were electrospun directly onto mica discs attached to the aluminum foil. The discs with nanofiber samples were then examined under a Bruker MultiMode atomic force microscope (AFM) with the Nanoscope IIIa controller using the contact mode.

Cross-linking of the pectin-based nanofibers

Collected nanofibers were first soaked in 95% ethanol for 2 min, followed either by a 150 mM calcium solution or a 5% (w/v) oligochitosan solution for 2 min. The nanofibers were then washed with DI water three times and oven dried before further analysis.

Characterization of the pectin-based nanofibers

Before scanning electron microscope (SEM) imaging was performed, the nanofiber samples were dried in a 35 °C oven, mounted onto an aluminum stub and sputter-coated with a 2 nm layer of iridium. Samples were then examined under a Hitachi S-4800 Ultra High Resolution Cold Cathode Field Emission Scanning Electron Microscope (FE-SEM). Dried nanofiber samples were also used for FTIR spectroscopy analysis using a Nicolet™ 6700 spectrometer (Thermo Fisher Scientific). To measure the surface charge (represented by the surface zeta potential), nanofibers were dissolved/suspended in de-ionized (DI) water and their surface charge was measured using a Brookhaven (Holtsville, NY) zeta potential analyzer.

Cell culture and cytotoxicity assay

Mouse MC3T3-E1 preosteoblasts (ATCC; Manassas, VT) were plated at a density of 2.5×10^5 cells/well in a 24-well plate and cultured in α -MEM (HyClone; Logan, UT) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37 °C in a humidified, 5%

CO₂ incubator. The cell culture medium was then replaced with fresh culture medium supplemented with various nanofibers (1 mg/mL) for 24 h after cell seeding. Cell toxicity was determined by measuring the release of lactate dehydrogenase (LDH) from membrane integrity-compromised cells into the culture medium at different time points using a Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions (Song et al. 2011). Briefly, 100 μ L of culture medium was added into a plate of 96 wells, mixed with 100 μ L working solution, and incubated at room temperature for 30 min in the dark. Then, the plate was read under the UVmax colorimeter (Molecular Devices, Sunnyvale, California) at optical density (OD) 490 nm. Blank culture medium was used as a control.

Results and discussion

Effect of preparation parameters on the electrospinning process

PEO was used as a synthetic carrier to improve the electrospinning ability of polymers (Bonino et al. 2011; Cui et al. 2016). First, different pectin:PEO ratios, 70:30, 60:40, 50:50, 40:60, and 30:70 (v/v), were tested to study the ratios' influence on the electrospinning process. It was found that no distinct nanofiber could be produced when the pectin's percentage was either too high (70:30) or too low (30:70) (Fig. 1a). When using the other ratios, 60:40, 50:50, and 40:60, nanofibers were produced, albeit with beads as shown in Fig. 1. Since the 60:40 ratio blend produced nanofibers

Fig. 1 AFM images of the nanofibers produced by various ratios of pectin: PEO. **a** 70:30, **b** 60:40, **c** 50:50, and **d** 40:60

with the least amount of beads, a 65:35 ratio, with a slightly decreased PEO percentage, was used for the duration of the study.

It has been demonstrated that Pluronic® F-127 can be used as a nonionic surfactant to aid in the electrospinning process (Bonino et al. 2011). To eliminate the bead formation during the process, various Pluronic® F-127 concentrations of 2, 5, 7.5, and 10% (w/v) were investigated. At lower concentrations of Pluronic® F-127, the solution surface tension was too high which caused bead formation in the fibers. When the concentration of Pluronic® F-127 was increased to 5%, uniform beadless nanofibers were successfully fabricated. However, no stable nanofibers were formed with further increase in the surfactant concentration. This is most likely due to the increasing concentration of the surfactant

which reduced the surface tension of the resulting material to the point where fibers could not hold their physical structure.

During optimization of the flow rate, it was noticed that the 0.4 and 0.7 mL/h settings led to the production of stable nanofibers with a smooth surface (Fig. 2b, c). When the flow rate was too low or too high, distinct nanofibers could either not be generated (Fig. 2a), or the surface morphology would not be optimized (Fig. 2d). Considering the production efficiency and general morphology, 0.7 mL/h was chosen for the rest of the study.

Finally, the voltage was not found to influence the electrospinning process significantly within the range of 10–18 kV (Fig. 3a–c). However, when the voltage exceeded 20 kV, nanofibers could not be detected (Fig. 3d). The

Fig. 2 AFM images of nanofibers produced under different flow rates. **a** 0.2, **b** 0.4, **c** 0.7, and **d** 1.2 mL/h

Fig. 3 AFM images of nanofibers produced under different voltages. **a** 10, **b** 14, **c** 18, and **d** 21 kV

electrospinning parameters for fabrication of the pectin-based nanofibers were optimized to a pectin:PEO ratio of 65:35 (v/v), Pluronic® F-127 concentration of 5% (w/v), flow rate of 0.7 mL/h, and voltage of 12 kV.

Effect of Ca^{2+} and oligochitosan on cross-linking of pectin-based nanofibers

As shown in Fig. 4, both Ca^{2+} and oligochitosan can be used in aqueous solutions as cross-linkers to improve the stability of the pectin-based nanofibers. The average diameters for untreated, Ca^{2+} -treated, and oligochitosan-treated nanofibers were 286 ± 38 , 285 ± 49 , and 219 ± 28 nm, respectively. The reduction in the nanofiber diameter when treated with oligochitosan might be caused by the

cross-linking of pectin molecules, leading to a more compact structure, while Ca^{2+} treatment did not significantly change the nanofiber size. When comparing the morphology of the Ca^{2+} -treated and oligochitosan-treated nanofibers, oligochitosan appeared to be the better choice. Ca^{2+} treatment caused the pectin nanofibers to aggregate and form flake-like structures (Fig. 4e), especially when the nanofiber density was high. This can be explained by different cross-linking mechanisms of Ca^{2+} and oligochitosan. Ca^{2+} cross-linking leads to the formation of macromolecular aggregates (egg-box structure) through the intermolecular chelating bonding, while oligochitosan cross-linking forms the pectin–oligochitosan electrolyte complexes (Feng et al. 2017; Zhang et al. 2016).

Fig. 4 AFM (top panel) and SEM (bottom panel) images of the untreated (a, d), Ca^{2+} -treated nanofibers (b, e), and oligochitosan-treated nanofibers (c, f). Black arrows: flake-like structures. Scale bar (SEM images): 5 μm

Fig. 5 FTIR spectra of untreated (NF), Ca^{2+} -treated (NF- Ca^{2+}), and oligochitosan-treated nanofiber (NF-OC)

Chemistry and surface charge of pectin-based nanofibers

The interaction of Ca^{2+} and oligochitosan with the pectin-based nanofibers can be observed using Fourier transform infrared (FTIR) spectroscopy (Crouse et al. 2015; 2016a). As shown in Fig. 5, the FTIR spectra of non-treated nanofibers

and nanofibers treated with Ca^{2+} and oligochitosan, respectively, are shown. As described previously, the interaction of Ca^{2+} with the pectin-based nanofibers is demonstrated by the shift of the asymmetric stretch of the pectin carbonylate ion from 1606 to 1629 cm^{-1} . The interaction of oligochitosan with the nanofibers is evidenced by the significant loss of intensity of the carbonylate ion. This is visualized with a weakly resolved shoulder on the amide I peak at 1652 cm^{-1} . This interaction is also indicated by the relative increase in intensity of the peak of the pectin ester carbonyl group at 1745 cm^{-1} . As a positive surface is preferred for cell attachment, it is critical to produce nanofibers with positive surface charges for tissue engineering applications. As shown in Fig. 6, the untreated nanofiber solution produced a negative surface charge as pectin is a polyanionic polymer. The Ca^{2+} treatment generated a more positive surface, but the overall charge was still negative. This is most likely due to the free carboxyl groups of pectin (Zhang et al. 2013). A positive surface was generated when treated with oligochitosan because of the free amine groups.

Cytotoxicity of pectin-based nanofibers

To assess the potential application of pectin-based nanofibers in tissue engineering, it was important to show that the nanofibers were non-cytotoxic. Mouse MC3T3-E1

Fig. 6 Zeta potential of suspension/solution of untreated (NF), Ca²⁺-treated (NF-Ca²⁺), and oligochitosan-treated nanofiber (NF-OC)

Fig. 7 Lactate dehydrogenase (LDH) activity in culture medium of MC3T3-E1 cells after incubation with untreated (NF), Ca²⁺-treated (NF-Ca²⁺), and oligochitosan-treated nanofiber (NF-OC), respectively. LDH activity is expressed as absorbance (OD) per milligram protein

preosteoblasts were chosen for potential bone tissue engineering application considerations. Three days after incubation, MC3T3-E1 cells cultured under different conditions all became fully confluent (Figure S1). The LDH assay was used to determine cytotoxicity. Higher LDH enzymatic activity in the cell culture supernatant indicates higher cytotoxicity which corresponds to an increased number of dead and membrane-damaged cells (Shah et al. 2017). As shown in Fig. 7, pectin-based nanofibers did not show appreciable cytotoxicity in MC3T3-E1 cells at concentrations up to 1 mg/mL for a treatment period of 72 h, based on the LDH assay, especially for oligochitosan-treated nanofibers.

Conclusion

The parameters for producing pectin-based nanofibers were successfully optimized at a pectin:PEO ratio of 65:35 (v/v), Pluronic® F-127 concentration of 5% (w/v), flow rate of 0.7 mL/h, and voltage of 12 kV. Both Ca²⁺ and oligochitosan were proven to be able cross-linkers for pectin-based nanofibers to improve their stability in an aqueous solution environment. However, oligochitosan was shown to be superior when comparing the nanofiber morphology after the cross-linking treatment. Furthermore, the oligochitosan cross-linking treatment also led to a positive surface charge of nanofibers, without apparent cytotoxicity, which is preferable for tissue engineering purposes. In summary, pectin-PEO-oligochitosan nanofibers were successfully fabricated, which show great promises for biological applications.

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