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Acids ‘generally recognized as safe’ affect morphology and biocompatibility of electrospun chitosan/polyethylene nanofibers

Mai Bay Stie¹, Megan Jones², Henning Osholm Sørensen³, Jette Jacobsen¹, Ioannis S. Chronakis⁴ and Hanne Mørck Nielsen¹*

¹ Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
² Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom
³ Department of Chemistry, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark
⁴ DTU food, Technical University of Denmark, Kemitorvet, B202, 2800 Kgs. Lyngby, Denmark
⁵ Present address: Department of Physics, Technical University of Denmark, Fysikvej, 2800 Kgs. Lyngby, Denmark

* Corresponding author: e-mail hanne.morck@sund.ku.dk, Tel.: +45 35336346

Highlights

- Electrospun chitosan nanofibers produced by use of minute amounts of GRAS acid
- Nanofiber morphology and disintegration depend on the type of GRAS acid used
- Residual acid is present in fibers after electrospinning, except for acetic acid
- Compatibility with cell monolayers relates to residual acid, but not a pH effect

Abstract

Electrospinning of neat chitosan is currently achieved by using strong acids or organic solvents, which limits the use of chitosan nanofibers as biocompatible scaffolds for drug delivery and tissue engineering. The aim was to elucidate the effect of specific acids generally recognized as safe (GRAS) on the properties of electrospun chitosan-based nanofibers. Electrospinning chitosan in dilute acetic acid or succinic acid with polyethylene oxide resulted in white and separated nanofibers, whereas nanofibers electrospun in dilute citric acid were transparent and interconnected. Including succinic or citric acid in the spinning process induced disintegration of the fiber mat after four hrs in water, and a concentration-dependent effect on epithelial cell viability. Chitosan nanofibers electrospun in acetic acid maintained their shape and fibrous structure after four hrs in water, and showed no effect on cell viability. This study demonstrates that the choice of GRAS acid highly determines the properties of electrospun chitosan nanofibers.
Keywords
Chitosan, electrospinning, nanofiber properties, solvent, generally recognized as safe
1. Introduction

Electrospinning is a simple and scalable technique used to produce nanofibers of proteins, synthetic and natural polymers (Pakravan, Heuzey, & Ajji, 2011; Stephansen, García-Díaz, Jessen, Chronakis, & Nielsen, 2015). Chitosan, a biopolymer consisting of D-glucosamine and N-acetyl-D-glucosamine, and its derivatives possess excellent mucoadhesive properties (Ways, Lau, & Khutoryanskiy, 2018) and have been shown to inhibit the growth of both bacteria and fungi (Goy, Morais, & Assis, 2016; Guo et al., 2006). Electrospun chitosan nanofibers are therefore promising candidates for a variety of applications in tissue engineering, as wound dressings and as drug delivery systems for mucosal and cutaneous applications (Ding et al., Deng, Du, Shi, & Wang, 2014).

However, chitosan has limited spinability as a result of its polycationic structure and restricted chain flexibility (Desai, Kit, Li, & Zivanovic, 2008; Duan, Dong, Yuan, & Yao, 2004; Pakravan et al., 2011), due to the fact that sufficient intermolecular entanglement is crucial for the formation of nanofibers by electrospinning. Thus, neat chitosan has so far only been electrospun in highly acidic solvents such as 90% (w/w) acetic acid (Geng, Kwon, & Jang, 2005) or trifluoro acetic acid (TFA) (Ohkawa, Cha, Kim, Nishida, & Yamamoto, 2004), which lower the surface tension of the dispersion and forms ionic interactions with the amino groups of chitosan hereby shielding the positive charges, as well as in combination with volatile organic solvents such as dichloromethane (DCM) (Ohkawa et al., 2004). Although chitosan is well-known to be biocompatible and biodegradable, the high concentration of acidic excipients and the inclusion of toxic organic solvents used to aid the electrospinning of chitosan limit the applicability of the nanofibers as biocompatible and safe tissue engineering scaffolds, wound dressings or drug delivery systems. An alternative strategy to improve the spinability of chitosan is to blend in a co-spinning polymer such as polyethylene oxide (PEO), which has excellent spinability and therefore can facilitate the electrospinning of chitosan (Duan et al., 2004). The delivery of small molecule drugs and proteins has been explored using chitosan/PEO nanofibers as vehicles. For example, chitosan/PEO nanofibers with graphene oxide-bound doxorubicin electrospun in 2% (w/w) acetic acid, induced stronger cytotoxicity against human lung epithelial carcinoma A549 cells, compared to free doxorubicin (Ardeshirzadeh, Anaraki, Irani, Rad, & Shamshiri, 2015). In addition, chitosan/PEO nanofibers electrospun in HFP (1,1,1,3,3,3-hexafluoro-2-propanol) were able to encapsulate, release and facilitate permeation of insulin across buccal mucosa ex vivo (Lancina, Shankar, & Yang, 2017). Chitosan/PEO nanofibers are also promising wound dressings with antibacterial properties (Kurtz & Schiffman, 2018). For example, chitosan/PEO nanofibers induced 50% inhibition of P. aeruginosa after only 180 min of exposure of bacteria to the nanofibers (Rieger & Schiffman, 2014). For tissue engineering, Asadian et al., 2018 have shown that non-thermal plasma treatment of chitosan/PEO nanofibers electrospun in 9:1 acetic acid:water significantly improved cellular responses such
as cell interaction and proliferation due to a change in the surface hydrophilicity and surface free energy, which is related to the adsorption capacity of the nanofibers. Thus, the tunability and applicability of chitosan-based nanofibers are widespread. Importantly, promoting these biomaterials into clinically applicable systems requires use of safe excipients.

Acetic acid is a commonly used solvent for chitosan, yet chitosan-based nanofibers have also been electrospun by the use of citric acid (in the presence of the surfactant Triton™ X-100) by the Nanospider™ electrospinning technology (Martinová & Lubasová, 2008). Martinová et al. compared the effect of thermal cross-linking of chitosan/PEO nanofibers in the presence of either acetic acid or citric acid, and demonstrated that including citric acid significantly improved the aqueous stability of the nanofibers over a period of at least two months (Martinová & Lubasová, 2008). However, a systematic study on the effect of the type of acid on other fiber properties relevant for the use of chitosan-based nanofibers in the context of e.g. drug delivery or tissue engineering has not yet been reported to the best of our knowledge.

In this work, we report on the successful production of biocompatible chitosan nanofibers by electrospinning utilizing dilute acids that are generally recognized as safe (GRAS) by the U. S. Food and Drug Administration (FDA). A systematic and comparative study was conducted applying acetic acid, succinic acid, and citric acid with one, two and three carboxylic acid groups, respectively, as solvents for chitosan prior to electrospinning. We hypothesized that the properties of the electrospun chitosan nanofibers would depend on the polymer composition and, in particular, the acid used to disperse the chitosan. The aim of the study was to obtain high-content chitosan nanofibers using only minute amounts of the aforementioned biocompatible acids as solvents. We show that the morphology, disintegration rate and compatibility of the nanofibers to cell monolayers are highly dependent on the acids used to disperse the biopolymer chitosan.

2. Materials and methods

2.1 Materials

Chitoeuticals Chitosan 95/100 (Mw 100-250 kDa, pharmaceutical grade, deacetylation degree 93.3%) was obtained from Heppe Medical Chitosan (Halle, Germany). PEO (Mw 900 kDa), ninhydrin reagent (2% solution in DMSO and lithium acetate buffer pH 5.2), succinic acid, acetic acid, Hanks’ Balanced Salt Solution (HBSS), Dulbecco’s Modified Eagle’s Medium (DMEM), glutamine, penicillin, streptomycin and phenazine methosulfate (PMS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Citric acid was obtained from Amresco (Solon, OH, USA). Fetal bovine serum (FBS) was purchased from PAA laboratories (Brøndby, Denmark). N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) was from PanReac AppliChem (Damstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
sulphophenyl)-2H-tetrazolium) (MTS) was obtained from Promega (Madison, WI, USA). The TR146 cell line was kindly provided by the Imperial Cancer Research Technology (London, United Kingdom).

2.2 Preparation of polymer dispersions

Dispersions of chitosan and PEO were prepared in ultrapure water (purified by a PURELAB flex (ELGA LabWater, High Wycombe, United Kingdom) to concentrations of 2% (w/w) and 4% (w/w), respectively. The minimum amounts of acetic acid (0.7% (w/w)), succinic acid (2.1% (w/w)) or citric acid (4.6% (w/w)) that were required to disperse chitosan was used. The minimum amount of each of the acids were determined by dispersing chitosan (final concentration of 2% (w/w)) in an increasing amount of each of the respective acids, followed by visually assessment to confirm if a clear dispersion of chitosan was obtained. The dispersions were stirred for two days to ensure complete hydration of the polymers. Blend dispersions for electrospinning were prepared immediately before electrospinning by mixing the aforementioned chitosan and PEO dispersions in various ratios followed by stirring for 30 min at room temperature.

2.3 Characterization of polymer dispersions

The pH and conductivity of the polymer dispersions were determined by using a Mettler InLab Viscous pH-electrode (Mettler Toledo, Greifensee, Switzerland), and a Metrohm 912 conductivity meter at 25°C (Herisau, Switzerland). The surface tension was determined at room temperature on a Force Tensiometer K-100 (Krüss, Hamburg, Germany). In short, >2 mL of polymer dispersion was transferred to a SV01 – PTFE measuring vessel (Krüss, Hamburg, Germany) and a PL03 rod (Krüss, Hamburg, Germany) was used for the measurements. The surface tension was determined according to the Wilhelmy method based on the wetting of rod. The maximum measuring time was 120 s with a minimum standard deviation of 0.01 mN. The data was processed using Krüss Laboratory Desktop 3.2 software.

Finally, rheological analysis was conducted on an AR-G2 rheometer (TA Instruments, Waters, New Castle, DE, USA) fitted with a 40 mm stainless steel cone with a cone angle of 1° and a plate geometry. Shear rates of 0.1–5623 s⁻¹ was applied. To ensure uniform conditioning, a pre-shear of 100 Pa for 5 s was employed followed by a 5 min conditioning step. Measurements were conducted at 25°C, and to prevent evaporation of sample material, a protection cap lined with Vaseline® was used. A tolerance level of 5% was pre-set. Only measurements that reached equilibrium within 60 s were included.

To allow direct comparison, the theoretical concentration of $H_3O^+$ was estimated based on the Henderson-Hasselbalch equation and that the $K_{a1} >> K_{a2} >> K_{a3}$ for succinic acid and citric acid, assuming a total volume of 8 mL of water for all samples and no contribution from chitosan as a base.
2.4 Electrospinning of chitosan/PEO nanofibers

Nanofibers were produced by electrospinning while applying 15-18 kV by a ES50P-10 W high voltage source (Gamma High Voltage Research, Ormond Beach, FL, USA) to the polymer blend at a flowrate of 0.4 mL/hr (New Era Pump Systems, Farmingdale, NY, USA) ejected from a 1 mL syringe with a 20 G blunt needle (Photo-Advantage, Ancaster, ON, Canada). The experimental setup was placed in a semi-open custom-made acrylic box with a continuous flow of dry air that allowed for electrospinning at low humidity (<15% relative humidity). Increasing the voltage slightly (in the range 15-18 kV) could compensate for small changes in ambient parameters caused by an occasionally high relative humidity of the surroundings, and hereby facilitate continues electrospinning of nanofibers with that high chitosan content. No changes in fiber morphology or diameter were observed as a consequence of the slight voltage adjustments. The electrospun nanofibers were collected on a stainless steel plate covered with aluminum foil placed 15 cm from the needle tip.

2.5 Characterization of electrospun chitosan/PEO nanofibers

The morphology of the dry nanofibers was assessed by scanning electron microscopy (SEM). The nanofibers were mounted on aluminum stubs with carbon tape, sputter-coated with gold and visualized using a Phenome Pro X scanning electron microscope (Pheno-World, Eindhoven, The Netherlands). The diameters of the nanofibers were measured using ImageJ version 1.51j8 software (National Institute of Health, Bethesda, MD, USA).

Composition analysis was carried out using a Bomem MB-Series spectrophotometer (Bomem, Québec, QC, Canada) with an Arid-Zone™ light source spectrometer based on a universal attenuated total reflectance FTIR sensor equipped with DuraScope diamond ATR (SensIR Technologies, Danbury, CT, USA). Absorbance spectra (an average of 16 scans) were reported in the range of 1800-800 cm⁻¹.

The chitosan content was determined using a colorimetric assay based on the reaction of ninhydrin with the amino groups of chitosan. A known amount (1-1.5 mg nanofibers per mL) were dispersed in 0.5% (v/v) acetic acid and stirred over night at room temperature. 500 µL of sample were mixed with 200 µL 2% ninhydrin reagent. To allow the reaction to proceed, the samples were heated to 80-90°C for 10 min. Hereafter, the samples were cooled to room temperature and 100 µL in quadruplicates of each sample were transferred to a 96-well plate and the absorbance was measured at wavelength of 570 nm on a plate reader (FLUOstar OPTIMA, BMG LABTECH, Ortenberg, Germany). A new standard curve was prepared for each experiment. It was furthermore confirmed that PEO, succinic acid and citric acid in the concentration range used in the fibers had no influence on the ninhydrin reaction.
Powder X-ray diffraction (XRD) was performed in reflection mode with Cu Kα radiation (λ = 1.54184 Å) on a Bruker D8 Advance diffractometer (Bruker, Billerica, MA, USA) equipped with a LynxEye position sensitive detector. A slit opening of 0.3° was used on the incident side of the sample and had 2.5° axial Soller slits on both the incident and the diffracted side. The Cu Kβ₁ radiation was removed by a 0.02 mm thick Ni filter. Diffraction patterns were recorded from 5 to 50° in 2θ with a step size of 0.02° and 5 s exposures per step. The samples were rotated with 30 rpm during the measurements. Electrospun nanofibers were measured in low-background sample holders, and commercial powder samples i.e. chitosan, PEO, succinic acid and citric acid were measured in standard sample holders.

2.6 Disintegration of electrospun chitosan/PEO nanofibers

2 mg (±10%) (m_initial) nanofibers were placed in metal baskets, submerged in 3 mL ultrapure water and incubated for 4 hrs at 37°C with mild shaking (50 rpm). Hereafter, the baskets were blotted gently on paper to remove excess water and the fibers were dried until constant weight (m_final), and the weight loss was determined (Equation 1):

\[ \text{Weight loss (\%) = } \frac{m_{\text{initial}} - m_{\text{final}}}{m_{\text{initial}}} \times 100\% \quad (\text{Eq. 1}) \]

2.7 Biocompatibility of electrospun chitosan/PEO nanofibers

TR146 cells were cultured in Corning Costar® polystyrene culture flasks (175 cm², Sigma Aldrich, St. Louis, MO, USA) in DMEM supplemented with FBS (10% (v/v)), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified environment at 37°C with 5% CO₂. Approximately 10⁵ TR146 cells/well were seeded in flat-bottom, transparent 12-well Nunclon™ delta cell culture-treated surface plates (Thermo Scientific, Roskilde, Denmark) and cultured for 48 hrs under the aforementioned conditions. The cells were washed twice in 2 mL 37°C hHBSS (HBSS with 10 mM HEPES and adjusted to pH 7.4) prior to exposure to the nanofibers. Nanofiber mats of 1 mg (±10%), 1.5 mg (±10%), and 2 mg (±10%) were submerged in 1.5 mL hHBSS at the surface of the cell monolayer and kept for 4 hrs at 37°C with gentle shaking (50 rpm). After the exposure, the remaining nanofibers were gently removed with tweezers, and the osmolality of buffer from each of the wells were evaluated (Osmomat 3000 Freezing point osmometer (Genotec, Berlin, Germany)). The cells were washed twice with hHBSS. The cell morphology was visualized under an Eclipse Ti-S bright-field microscope (Nikon, Tokyo, Japan). The viability of the cells after exposure to nanofibers was evaluated, as an indication of cytotoxicity, by the MTS/PMS colorimetric assay. Briefly, the cells were incubated at 37°C for up to 120 min with 1 mL reagent solution containing 240 µg/mL MTS and 2.4 mg/mL PMS in HBSS buffer with gentle shaking (50 rpm). Then, 100 µL samples in quintuplicate of the solution with metabolized MTS/PMS was transferred from each well to a transparent 96-well plate and
the cell viability was evaluated by measuring the absorbance at $\lambda 492$ nm in a plate reader (POLARstar OPTIMA, BMG LABTECH, Ortenberg, Germany). The absorbance of the unreacted MTS/PMS solution was defined as the blank ($\text{Abs}_{\text{blank}}$), while the control was defined from cells incubated with hHBSS ($\text{Abs}_{\text{control}}$, 100 % cell viability). The relative cell viability was determined (Equation 2):

$$\text{Relative cell viability (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}} \cdot 100\% \quad (\text{Eq. 2})$$

3. Results and discussion

3.1 Physiochemical characterization of chitosan/PEO dispersions

Although GRAS acids are used in the preparation process, a high concentration of acid may likely compromise the biocompatibility of the electrospun nanofibers, and furthermore restrict the encapsulation of drugs with limited chemical and physical stability such as biopharmaceuticals. In this study, chitosan was therefore dispersed in water with the minimum amount of either acetic acid (0.7% (w/w)), succinic acid (2.1% (w/w)) or citric acid (4.6% (w/w)) required to produce a 2.0% (w/w) clear dispersion of the polymer. These dispersions were then mixed with a 4.0% (w/w) PEO aqueous dispersion in different ratios prior to electrospinning. The theoretical contribution of hydronium ($\text{H}_3\text{O}^+$) from the aforementioned three acids was estimated (Table 1). The hydration of chitosan was mainly driven by the protonation of the amino groups of the polymer by the acids, but not exclusively since the concentration of $\text{H}_3\text{O}^+$ required to hydrate chitosan was lower for acetic acid compared to succinic acid and in particular in comparison to citric acid. Accordingly, the pH of the chitosan dispersion prepared in citric acid (pH 2.5) was the lowest of the three chitosan dispersions used (Table 1). This is in agreement with previous findings that acetic acid is a better solvent for chitosan compared to citric acid (Chen et al., 2007).
Table 1. Properties of the used GRAS acids and of the PEO and chitosan dispersions prepared in GRAS acids as used for electrospinning. Results are presented as the mean ± standard deviation. \(^a\) N=3; \(^b\) N=2, n=2.

<table>
<thead>
<tr>
<th></th>
<th>PEO</th>
<th>CHITOSAN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acid properties</strong></td>
<td></td>
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</tr>
<tr>
<td>Mw, g/mol</td>
<td>H(_2)O</td>
<td>Acetic acid</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>60.05</td>
</tr>
<tr>
<td>pKa</td>
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<td>4.76</td>
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<tr>
<td></td>
<td>118.09</td>
<td>2.1</td>
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<tr>
<td></td>
<td>192.12</td>
<td>4.65</td>
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<tr>
<td>Boiling point, °C</td>
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<td>118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>235</td>
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<td></td>
<td></td>
<td>310</td>
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<tr>
<td><strong>Dispersion properties</strong></td>
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<tr>
<td>Polymer conc., % (w/w)</td>
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<td>2.0</td>
</tr>
<tr>
<td>Acid conc., % (w/w)</td>
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</tr>
<tr>
<td>pH(^a)</td>
<td>8.3±0.2</td>
<td>5.0±0.1</td>
</tr>
<tr>
<td>Theoretical [H(_3)O(^+)], mM</td>
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</tr>
<tr>
<td>Surface tension(^b), mN/m</td>
<td>60.5±0.6</td>
<td>60.1±1.9</td>
</tr>
<tr>
<td>Conductivity(^a), mS/cm</td>
<td>0.09±0.01</td>
<td>3.93±0.05</td>
</tr>
</tbody>
</table>

It is well-known that several parameters affect the electrospinning process and in particular the morphology of the produced nanofibers (Bhardwaj & Kundu, 2010). Some of the important properties that are known to influence the electrospinning process are listed in Table 1. Efficient solvent evaporation is essential for successful fiber production and depends on the boiling point of the solvent. It is worth noting that the boiling point of acetic acid (118°C) is the lowest of the three acids. The surface tension of the electrospun dispersion must be overcome by the electrical field for the fluid jet to be ejected from the needle to the collector. In this study, the specific acid in the amounts used did not influence the surface tension as no significant differences were found between the chitosan dispersions prepared with acetic acid, succinic acid or citric acid as solvents. The conductivity affects the charges at the surface of the dispersion, which is important for the stretching ability of the dispersion and the bending instability the electrospinning jet (Angammana & Jayaran, 2011). Expectedly, the conductivities of the chitosan dispersions were higher than the conductivity of the PEO dispersion, as chitosan is a cationic polyelectrolyte and PEO is neutral. The highest conductivity was found for chitosan dispersed in dilute acetic acid (Table 1).

Sufficient intermolecular entanglement of the polymer is crucial for the production of smooth fibers without artifacts such as beads. The viscosity of the dispersion is directly related to the intermolecular interactions of the polymer. The concentration of polymer must be high enough to facilitate entanglement; however, a too high viscosity can restrict the fluid flow and induce clogging of the needle, which will result in discontinuous electrospinning, and as a consequence, the production of nanofibers with artifacts. PEO
aids the electrospinning of chitosan through two mechanisms; i) by forming hydrogen bonds with the hydroxyl and amino groups of chitosan, which leads to favorable intermolecular interactions (Duan et al., 2004; Pakravan et al., 2011) and ii) by increasing the viscosity of the blend dispersion as shown in Figure 1. Flow curves of chitosan dispersed in the three acids tested, PEO and blends of the two polymers display that both PEO, chitosan and the blend dispersions have shear thinning properties (Figure 1) as reported previously by others (Pakravan et al., 2011). This property is indicative of inter-polymer entanglement, as the intermolecular interactions are broken under increased shear thus resulting in a decrease in viscosity (shear thinning).

For the highest concentrations of chitosan, the viscosities of the polymer dispersions were in general highest when prepared in acetic acid compared succinic acid or citric acid. The concentration of the chitosan dispersion (2% (w/w)) was lower than the concentration of the PEO dispersion (4% (w/w)) (Figure 1). Accordingly, increasing the chitosan:PEO ratio (w/w) resulted in a decrease in the total polymer concentration and thus a decrease in the viscosity of the blend dispersions (Figure 1).

![Figure 1. Viscosity of chitosan/PEO blend dispersions in GRAS acids used for electrospinning. Flow curves for dispersions prepared in the presence of acetic acid (black), succinic acid (grey), and citric acid (blue). Chitosan:PEO ratios (w/w): 0:100 (neat PEO), 15:85, 30:70, 45:55, 60:40 and 100:0 (neat chitosan). Neat PEO (red). N=2, n=2, presented as the mean ± standard deviation.](image-url)
3.2 Type of GRAS acid affects nanofiber morphology

Due to their tunable properties, robustness and large-surface-to-volume ratio, electrospun nanofibers are excellent candidates for a variety of applications within health sciences. For the purpose of topical application to e.g. wounds or intact mucosal surfaces; flexible, smooth and homogenous nanofibers without artifacts are often preferred. Electrospinning of chitosan was found to be very sensitive to ambient factors such as temperature, airflow and humidity. Low humidity was especially crucial for electrospinning of fiber blends with the highest chitosan content (chitosan:PEO ratio (w/w) of 60:40). Noteworthy differences in the fiber morphology were observed depending on the acidic solvent used (Figure 2). Nanofibers electrospun in either dilute acetic acid or dilute succinic acid as solvents were thin and homogenous. Furthermore, the nanofibers produced with these two acids and a chitosan:PEO ratio (w/w) up to 45:55 were without artifacts (Figure 2A). The fiber diameter decreased slightly with increasing chitosan content. This was due to a decrease in the total polymer concentration and, consequently a decrease in the viscosity of the dispersions in blends with less PEO (Bhardwaj & Kundu, 2010). Interestingly, the nanofibers produced with dilute citric acid as a solvent for chitosan had a larger diameter compared to the nanofibers electrospun in dilute acidic acid or succinic acid as a solvent. In addition, these fibers did not appear as individual fibers, but were connected in a single swollen network, in contrast to fibers electrospun in either acetic acid or succinic acid (Figure 2A). The diameter of the nanofibers produced in the presence of citric acid increased with increasing chitosan content concurrent with an increase in the solvent i.e. citric acid (Figure 2B). Hence, the results indicate that the presence of citric acid causes the distinctive appearance of these nanofibers. Nanofiber mats produced with acetic acid or succinic acid as a solvent were white, airy and flexible. In contrast, nanofiber mats formed with dilute citric acid as a solvent were transparent, rigid and fragile (Figure 2C). This made it difficult to detach nanofibers electrospun in citric acid, especially those with high chitosan content, from the aluminum foil of the collector. Nanofibers with a chitosan:PEO ratio (w/w) of 30:70 were therefore chosen for further characterization to allow a comparison between the nanofibers electrospun in the three respective acids.
Figure 2. Effect of the chitosan:PEO ratio (w/w) and the acid used as solvent on the morphology of the electrospun nanofibers. 
A) Representative SEM images of nanofibers of increasing chitosan:PEO ratio (w/w) electrospun in acetic acid, succinic acid and citric acid, respectively. B) Diameter of nanofibers of increasing chitosan:PEO ratios (w/w) electrospun with acetic acid (black), succinic acid (grey), and citric acid (blue), respectively. N=3, n=100, presented as mean±standard deviation. C) 10 mm nanofiber mats of 30:70 (w/w) chitosan:PEO electrospun in either acetic acid, succinic acid, and citric acid.
3.3 Composition and solid state analysis of electrospun chitosan/PEO nanofibers

Fourier transformed infrared spectroscopy (FTIR) analysis was used for composition analysis and spectra of neat chitosan, PEO, the acids used as solvents, and the electrospun nanofibers are shown in Figure 3. Clearly, PEO can be identified in all the fibers by the characteristic vibration stretching of C–O–C at 1100 cm\(^{-1}\). Chitosan is identified by broad peaks centered around 1600 cm\(^{-1}\) and 1000 cm\(^{-1}\) indicative of primary amine N-H bending and C-N stretching, respectively. These peaks are more pronounced in fibers with higher chitosan content and absent in fibers of only PEO, as expected (Figure 3B). The FTIR spectra of acetic acid, succinic acid and citric acid show characteristic absorption bands around 1700-1720 cm\(^{-1}\) typical of the C-O stretching for carboxylic acids. It is expected that evaporation of the acetic acid occurred during electrospinning of the nanofibers due to the fact that the absorbance band indicative of the carboxylic acid C-O stretching is absent in the FTIR spectrum (Figure 3A). This is in accordance with that acetic acid has a lower boiling point and hence higher volatility compared to succinic acid and citric acid (Table 1). In contrast, succinic acid and citric acid can be detected in the nanofiber matrices produced with these acids (Figure 3A). The concentration of residual acid was expected to be highest in nanofibers electrospun in citric acid due to the higher initial concentration of acid used to disperse chitosan, the higher boiling point, and that the nanofibers electrospun in citric acid showed a very distinct morphology (Table 1, Figure 2A, 2C). Accordingly, the absorption peak centered around 1700 cm\(^{-1}\) was the most pronounced in the FTIR spectrum of nanofibers electrospun in citric acid. Assuming complete evaporation of the acids, the theoretical content of chitosan in these nanofibers is 30% (w/w). Thus, as some residual acid was detected by FTIR in nanofibers that were electrospun in either succinic acid or citric acid (Figure 3), the percentage of chitosan in these fibers would be expected to be lower than 30% (w/w). The chitosan content was determined using a colorimetric assay based on the reaction of the amino groups of chitosan with ninhydrin. In good correlation with the FTIR results, the chitosan content was measured to be 31±2% (w/w), 24±2% (w/w) and 19±2% (w/w) in nanofibers that were electrospun in acetic acid, succinic acid and citric acid respectively (N=2, n=2).
Figure 3. Composition analysis by FTIR of nanofibers electrospun in GRAS acids. A) Representative FTIR spectra of neat chitosan, PEO, acetic acid, succinic acid, citric acid, and nanofibers of 30:70 ratio (w/w) of chitosan/PEO electrospun in the aforementioned three acids, respectively. B) Representative spectra for nanofibers electrospun in succinic acid with an increasing content of chitosan. N=2.

X-ray diffraction patterns were obtained to investigate the solid-state crystalline properties of the electrospun chitosan/PEO nanofibers (Figure 4). The two Bragg peaks at 2θ=19.1° and 2θ=23.2° are characteristic of PEO. These peaks are also the dominating peaks in the XRD patterns of the electrospun nanofibers. The XRD pattern of neat chitosan shows a characteristic broad peak centered around 2θ=20.1°. The crystalline ordering of chitosan is lower than that of PEO in accordance with previous results (Pakravan, Heuzey, & Ajji, 2012). The peak intensity for chitosan was thus much lower than the signals recorded from PEO (normalized data presented in Figure 4). This combined with the smaller amount of chitosan to PEO explains why the broad peak of chitosan at 20.1° is invisible in the XRD pattern for the electrospun nanofibers. All nanofibers, like PEO, were found to have crystalline order, i.e. they display a diffraction pattern with Bragg peaks. Interestingly, the Bragg peak widths are narrower in the XRD patterns of the nanofibers compared to PEO itself. Especially, the XRD pattern of the nanofibers electrospun in citric acid shows sharp diffraction peaks, e.g. the overlapping peaks at around 23.3° in 20 of PEO are resolved in the pattern of the nanofibers material. The sharp diffraction peaks reflect that crystalline order is evident in the nanofibers. This is in good correspondence with XRD results reported by others, who showed that fibers of
high PEO content predominately show crystalline properties, in contrast to fibers with a high chitosan content (up to 90%), which are amorphous (Pakravan et al., 2012).

3.4 Disintegration of chitosan/PEO nanofibers is dependent on the choice of GRAS acid

Fast disintegration and low physical stability in aqueous media are well-known challenges for use of electrospun nanofibers. In addition, drug release kinetics may be very dependent on the disintegration profile of drug-loaded nanofibers. Nanofibers electrospun in succinic acid and citric acid disintegrated completely within 15 min of exposure to water (Figure 5A-B). In contrast, nanofibers electrospun with acetic acid as a solvent maintained their integrity (Figure 5A) and fibrous structure (Figure 5D) after four hrs in water, however, 55% of the initial weight of the fibers was lost (Figure 5B). PEO was included as a fast-disintegrating control (Figure 5A). This observed difference in disintegration properties may be due to the residual acid in the nanofibers electrospun in succinic acid and citric acid, in contrast to the nanofibers electrospun in acetic acid, which evaporated during electrospinning (Figure 3) or due to differences in the chitosan content depending on the GRAS acid. The weight loss from these nanofibers (55%) was lower than the theoretical PEO content (70%) of the nanofibers suggesting that any residual PEO in the nanofibers was below the limit of detection by FTIR. Composition analysis by FTIR showed that nanofibers electrospun in acetic acid primarily contained chitosan after exposure to water (Figure 5C). In accordance, using a water-soluble sheath of PEO around a core of chitosan as a strategy to form chitosan nanofibers has been demonstrated by others using coaxial electrospinning of chitosan in 90% (w/w) acetic acid and PEO in water.
Our results, however, demonstrate a strategy that can be employed to produce chitosan nanofibers under less harsh conditions.

Figure 5. Stability of chitosan/PEO nanofibers electrospun in GRAS acids in water. A) Images of nanofiber mats submerged in water over four hrs. B) Weight loss after four hrs in water of electrospun chitosan/PEO nanofibers of 30:70 ratio (w/w) of chitosan:PEO electrospun in acetic acid, succinic acid and citric acid, respectively. Mean±standard deviation. N=2, n=2. C) Composition analysis by FTIR and D) representative SEM images of chitosan/PEO nanofibers electrospun in acetic acid, before and after exposure to water for four hrs. N=2, n=2.

3.5 In vitro biocompatibility of nanofibers is dependent on the disintegration of chitosan/PEO nanofibers

Toxic solvents such as TFA or DCM are frequently used to aid the electrospinning of chitosan, but will unfortunately compromise the biocompatibility of the nanofibers and limit their potential use as a safe and patient-friendly platform for medical applications if not fully removed prior to use (ICH Harmonised Guideline, Impurities: Guideline for Residual Solvents Q3C). To assess the biocompatibility of the chitosan/PEO nanofibers electrospun in GRAS acids, monolayers of human buccal TR146 cells were exposed to the nanofibers for four hrs, and the relative cell viability compared to a control was evaluated (Figure 6A). Surprisingly, the cytotoxic effect of the nanofibers on the cells was highly dependent on the acid in...
which the nanofibers were electrospun (Figure 6A). Cell monolayers exposed to nanofibers electrospun in dilute acidic acid displayed no decrease in relative viability compared to the control. In contrast, cell monolayers exposed to nanofibers electrospun in either dilute succinic acid or dilute citric acid showed a concentration-dependent decrease in the cell viability relative to the control. Nanofibers electrospun in the presence of citric acid showed the most detrimental effect on the viability of the cell.

The buffer (hHBSS pH 7.4), in which the fibers were submerged, was withdrawn from the cells after four hrs of incubation, and the osmolality and pH of the buffer were determined (Figure 6B-C). All samples were isoosmotic (300 mOsmol/kg) (Figure 6B), but pH of the buffer decreased in the presence of an increasing amount of fibers electrospun in either succinic acid or dilute citric acid. This indicates that residual acid was released from the nanofiber mats into the surrounding buffer. In contrast, the pH of the buffer did not decrease after four hrs of exposure to nanofibers electrospun in the presence of acetic acid. In good correlation with the FTIR results (Figure 3), this suggests that the acetic acid evaporated during electrospinning, and that succinic acid and citric acid remained, at least in part, in the nanofibers. To rule out cytotoxicity caused by exposure of the cells to the acids, cell monolayers were incubated with the maximum theoretical concentration of each of the three acids found in nanofiber mats of 2 mg (assuming no loss of acid by evaporation during electrospinning) as a control (Figure 6, neat acid). Exposure of the cell monolayers to neat acid had no significant effect on the relative viability of the cells compared to the negative control (hHBSS). Hence, the observed reduction in cell viability did not result from a decrease in pH because of acid released from the fibers. Nanofibers electrospun in dilute succinic acid and citric acid as solvents disintegrated after submersion in aqueous buffer and released polymer aggregates (Figure 6D). In contrast, nanofibers electrospun in acetic acid maintained their shape and no aggregates were visible in the microscope. The observed decrease in cell viability in the presence of nanofibers electrospun in succinic acid and citric acid were most likely a consequence of insoluble aggregates of polymer covering the surface of the cell monolayer (Figure 6D). It should be noted, though, that cell monolayers, especially those cultivated for short periods, are not as resilient as e.g. the intact skin or buccal mucosa, and the severity of the effect might not be directly translated to an in vivo situation. Chitosan and PEO are in general regarded as nontoxic and biocompatible polymers: PEO has been approved as an ‘Inactive Ingredient’ by the FDA (FDA UNII 16P9295IIL). Chitosans has been approved by the FDA for specific applications e.g. in hemostatic wound dressings (e.g. FDA 510(K) no. K172324), and highly purified chitosans intended for use as food have received GRAS status (FDA GRN no. 443). Standardization of chitosan composition, polymer processing and supporting clinical data is, however, needed for further exploitation of chitosan as a GRAS excipient. As the observed effect on viability is not due to neither of the dispersed polymers nor the GRAS solvents, the observed loss of cell viability after exposure to the nanofibers is most likely caused by stress from the
friction between the cells and visible polymeric aggregates derived from disintegrated nanofibers (Figure 6D). These aggregates formed a thin layer on top of the cells; a layer that was visible by the naked eye.

Figure 6. Evaluation of the in vitro biocompatibility of chitosan/PEO nanofibers electrospun in GRAS acids. A) Relative viability compared to negative control (hHBSS) of TR146 cells exposed for four hrs to 1.0 mg (black), 1.5 mg (grey), 2.0 mg (light grey) nanofibers with a chitosan/PEO ratio (w/w) of 30:70 electrospun in either acetic acid, succinic acid, or citric acid. Cell monolayers were incubated with the maximum theoretical concentration of each of the three acids found in nanofiber mats of 2 mg (assuming no loss of acid by evaporation during electrospinning) as a control (neat acids, blue). B) Osmolality and C) pH of the buffer removed from the cells after four hrs of exposure to nanofibers. Mean values are presented, error bars represent standard error of mean for A) and standard deviations for B) and C). N=3-4. D) Representative microscopy images of TR146 cells after four hrs of exposure to nanofibers with a 30:70 ratio (w/w) of chitosan/PEO electrospun in citric acid and control. White arrows indicate aggregates of polymers.

4. Conclusion
This study demonstrates a protocol for the preparation of biocompatible chitosan/PEO nanofibers with high chitosan content by using only GRAS acids as solvents for chitosan prior to electrospinning, and thus avoiding the use of strong acids or toxic organic solvents. Interestingly, it was clearly demonstrated that the morphology and disintegration rate of the chitosan/PEO nanofibers were highly dependent on the specific acid used to disperse chitosan. Further, the effect of the nanofibers on the viability of epithelial cell monolayers was shown to be highly dependent on the degree of disintegration of the nanofiber mats related to residual content of acid in the fibers. The findings are important for the applicability of chitosan-based fibers in e.g. drug delivery and tissue engineering.
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6. Conflicts of interests
The authors declare no conflict of interest.

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