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Novel Nanofibers Made of Chitosan/Hyaluronan Electrostatic Complex

Christian Enrique Garcia Garcia[1,2*], Frédéric Bossard[2], and Marguerite Rinaudo[3*]

[1]*Department of Chemical Engineering, University of Guadalajara, México*

[2]*CNRS, Institute of Engineering, Grenoble-Alpes University, France*

[3]*Biomaterials Applications, Grenoble, France*

***Correspondence to:**
Christian Enrique Garcia Garcia
Department of Chemical Engineering
University of Guadalajara
Blvd. M. García Barragán #1451
C.P. 44430, Guadalajara
Jalisco, México.
E-mail: christian-enrique.garcia-garcia@univ-grenoble-alpes.fr
Tel: +33 768854970

Dr. Marguerite Rinaudo
Biomaterials Applications, Grenoble, France.
Email: marguerite.rinaudo38@gmail.com
Tel: +33 611434806

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Abstract

After preparation of pure chitosan nanofibers, development of electrospinning in our laboratory allowed to prepare new nanofibers made of hyaluronan (HA) and chitosan (CS) polyelectrolyte complex (PEC). It is the first time that such materials are produced taking advantage of the properties of these two important biological polymers. PEC fibers need to be stabilized by thermal treatment to enable their use as materials for biological applications. Spinnability of the blends and some physicochemical properties were evaluated as well as their mechanical properties under different fiber arrangement. Finally, the fibers were applied for chondrocyte development in view of tissue engineering.

Keywords

Chitosan, Hyaluronan, Electrospinning, Tissue engineering, Biomaterial

Introduction

PECs are a type of composite produced by mixing two polymers with opposing charges, stabilized by strong electrostatic interactions between the polycation and polyanion [1-5]. The materials based on PEC are characterized by a degree of swelling and solubility depending on pH and external ionic concentration [6]. Often, CS is selected as polycation due to its specific properties (biocompatibility, biodegradability, and antimicrobial) and associated with synthetic or natural polyanions. HA a natural polysaccharide which is a critical component of natural extra-cellular matrix is widely used in tissue engineering and regenerative medicine [7]. In order to prepare PEC systems, CS/Polyethylene oxide (PEO) and Alginate/PEO solutions have been mixed for electrospinning with different volume ratios [8]. Ma and co-workers successfully electrospun CS/HA solutions by mixing different volumes of 1% wt. HA solubilized in water (W)/ Formic acid (FA) (25/75 w/w) with positively charged 7% wt. CS in W/FA (20/80 w/w). The weight and mole ratios of $-NH_2$ groups on the CS backbone to –COOH groups of HA are able to be determined easily. Under those previous electrospinning conditions, smooth and thin fibers were obtained with a mole ratio $-NH_2$/-COOH less than 1 but no test on stability in aqueous medium was performed [1]. Core-shell fibers were obtained by mixing 1% wt. HA solution (W/FA/Dimethylformamide = 5.0/2.5/2.5, w/w, as a blend solvent) and 7% wt. CS solution (W/FA 1/9, w/w, as a blend solvent) at the different blending volume ratio HA/CS from 9/1 to 5/5, i.e., $-NH_2$/-COOH charge ratios from 1.64 to 14.73, respectively. However, HA remains soluble depending on the weight ratio and CS makes the shell [2]. Another method allowed the production of

CS-HA-PEO bilayer material by sequential electrospinning of HA-PEO onto a freshly formed CS-PEO layer; the ratio of the thickness of the CS:HA layers was 2:1. A fraction of HA remains soluble but it was demonstrated a significantly higher number of living cells on the surface of the CS–HA compared with CS with a better biocompatibility [9].

In our work, PEC made of CS and HA was prepared from CS and HA solutions in the same solvent (FA/W 1/1 v/v) at controlled -NH_2/-COONa charge ratios. The stability of the materials obtained was studied on the nanofibers obtained by electrospinning after a thermal treatment. Mechanical properties in dried state, degree of swelling, and solubility in phosphate buffer (pH = 7.4) were determined. Then, the application for biological development of chondrocytes for tissue engineering was introduced.

Materials and Methods

Materials

Chitosan sample from Northern cold-water shrimp, *Pandalus borealis,* was obtained from Primex Ehf (Batch TM4778, code 42010, Siglufjordur, Iceland), with a molecular weight (M_W) around 160 kg/mol and a degree of acetylation of 0.05, determined using ^{1}H NMR. The used Hyaluronan sample from Soliance (Pomacle,France) has a weight-average molecular weight (M_w) = 540 kg/mol. Formic acid (ACS reagent > 98%) from Sigma-Aldrich (Product of Finland, lot #STBJ3705) and Dulbecco's Phosphate Buffered Saline (DPBS) (ref. 14190-094, Lot 2118924) from Gibco (Made in UK) were utilized. Polyethylene oxide with a molecular weight of 1x10^3 kg/mol was used to prepare the fibrous mat. Deionized water was used as solvent for the solutions. All reagents and polymers were used as received without further purification.

For cell culture, the C20A4 human chondrocyte cell line [10] was selected and the samples were seeded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v of fetal bovine serum (FBS) and a 1% v/v, in proportion with the total volume, penicillin/streptomycin/glutamine solution. Phosphate Buffered Saline (PBS) solution has a pH = 7.4, measured in the laboratory, DMEM serum-free and 0.05% Trypsin-EDTA solution were also utilized in cell experiments. All biological reagents were purchased from Gibco Life Technologies (Paisley, UK).

Preparation of PEC solutions

CS and HA homogeneous solutions were prepared separately at 4% w/w in W/FA mixtures at ratio 1/1 (v/v) to obtain stable solutions. Under these conditions, the total functional groups contents are 0.233 [–NH_2]/L in CS and 0.1 [–COOH]/L in HA, respectively. Then, HA and CS solutions were mixed at different volume ratios corresponding to charge ratios Rc = 0.5, 1, 1.8, 2.35, and 3.0 under stirring getting a homogeneous blend.

Electrospinning of PEC

With the purpose of favor PEC spinnability, the addition of a 4% w/w PEO solution was needed. Using the same solvent as for the corresponding biopolymer mixture, a final content in PEC/PEO equal to 70/30 (w/w) was selected such as to preserve a high yield in polysaccharides.

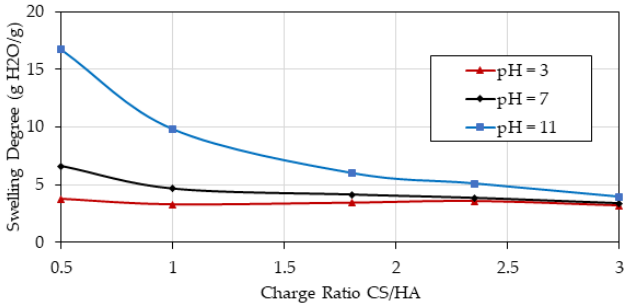

Figure 1: Swelling degree of PEC after thermal treatment immersed in aqueous medium at different pH.

Using a conventional vertical set up of electrospinning technique, the prepared solutions were placed in a 5 ml plastic syringe fitted with a 21-gauge stainless steel needle. Then, the ensemble syringe/needle was disposed in a syringe pump (model: KDS Legato 200, KD Scientific, Holliston, MA, USA), which delivers solutions at specified flow rate. Electrospinning was effectuated applying a voltage around 25 kV between the electrodes using a homemade dual high voltage power supplier (±20 kV, iseq GmbH, Radeberg, Germany).

The nanofibers were recovered on two different collectors: aluminum foil and rotatory cylinder. The experiments were performed placing the collector (metallic plate or rotatory cylinder) at 17 cm from the tip of the needle and the operating flow rates varied from 0.02 to 0.15 ml/h. In the case of rotatory collector, the distance was considered from the surface of the cylinder (diameter = 10 cm) to the needle tip. The cylinder rotation was set at 1500 rpm. Production of fibers was carried out at room temperature in closed Plexiglas® box with relative humidity ranging between 30% and 50%. The produced nanofibers matrices were left in ambient conditions to evaporate excess of FA and water and reserved for further analyses.

Thermal treatment of nanofiber mats

As proposed in literature, amide linkage was formed between -NH_2 and -COOH under controlled thermal treatment [9, 11-13]. In this work, CS and CS/HA complex under mat morphology were treated at 120 °C during 4 hours in air conditions for structural stabilization in presence of PEO.

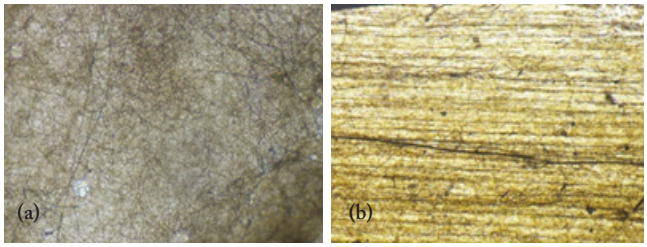

Figure 2: Morphology of electrospun nanofibers: (a) randomly and (b) aligned.

Mechanical properties of nanofiber mat

The measurements were carried out using an ARES-G2 rheometer (TA Instruments, New Castle, DE, USA) equipped with a rectangular geometry, used for axial tension consisting of two axial clamps that hold the material when the force was applied. Samples were taken from the nanofibrous electrospun matrices (randomly oriented and aligned fibers) maintaining a free length/width ratio around 2.69. The results were expressed as the Stress σ (Pa) = Force applied (N)/section area (m^2).

Tensile tests were performed starting from a zero-applied force until the material presented a breaking point, with a deformation rate of 0.01 mm/s. The experiments were carried out at constant temperature around 25 °C.

Cell culture and chondrocyte quantification

Chondrocytes C20A4 initial sample was disposed in a culture flask with 20 ml of complete DMEM. Cell sample was preserved into a cell incubator (inCu safe, Panasonic) at 37 °C and 5% CO_2 constant inlet flow during few days until confluence. The nanofiber mats having a regular shape with a surface of ~1 cm^2, were directly placed in a Petri dish and washed 2 times with the PBS solution to be subsequently hydrated in the DMEM culture solution during 2 days. After the DMEM solution being removed from the culture dish, 20 µl of a cell suspension at a concentration of 5.7 x 10^5 cell/ml, was disposed on the fiber mat followed by the addition of 2 ml of complete DMEM. The samples were maintained into a cell at 37 °C and 5% CO_2 constant inlet flow during few days before cell growth quantification, the culture solution was renewed every 2 days.

Cells were detached from the fibrous substrate and resuspended in DMEM in order to quantify the number of cells as a function of time. PEC supports were disposed in a 15 ml tube and carefully washed twice with 1 ml of PBS solution in order to remove remaining DMEM solution and unattached cells. Washing was followed by a detachment step consisting in the addition of 0.5 ml of Trypsin-EDTA 0.05% and vortex agitation at 1000 rpm during 60 seconds, four times. Further addition of DMEM and PBS washings helped to resuspend the extracted cells. Cell counting was carried out at times between 1 and 4 days after seeding and proliferation.

The final cell suspension was stained with Acridine Orange/Propidium Iodine fluorescent marker (F230001, Logos biosystems, Villeneuve d'Ascq, France) and cell quantification, in cell/ml, was performed on a dual brightfield and fluorescence cell counter (LUNA-FL, Logos biosystems, Villeneuve d'Ascq, France). This technique allows to identify and quantify the amount of total and living cells, for cell viability calculation. It gives also information about average cell size.

Results and Discussion

Nanofibers made of CS as reference [14-17] and CS/HA PEC have been processed by electrospinning in presence of PEO to favor spinnability. The samples were characterized as spun and after stabilization allowing to extract PEO and excess of salts and solvents in Ethanol (EtOH)/water 80/20 v/v. Due to the large solubility of HA in aqueous medium, a thermal treatment was firstly adopted and characterized by a weight loss at higher charge ratios (Table 1).

Table 1: Characterization of the PEC nanofibers obtained at different CS/HA ratios.

Charge ratio	Weight ratio CS/HA	Electrospun products	Weight lost (%) during thermal treatment	Swelling degree at pH = 7.4	Solubility (%) at pH = 7.4
1.0	0.42	Fibers	20.24 ± 0.09	5.2 ± 0.7	21.2
2.35	1.0	Fibers	9.8 ± 2.5	3.3 ± 0.3	12.2
3.0	1.26	Fibers	10.9 ± 0.4	3.7 ± 0.5	13.9

Nanofiber stability and swelling

From the experimental results, it comes that, to get nanofibers, the stoichiometric charge ratio (-NH_2/-COOH) is necessary to control their physicochemical properties. From NMR, it was shown that the insoluble materials are made of pure complex. PEO was extracted in Ethanol (EtOH)/water mixture [18]. In phosphate buffer at pH = 7.4, the swelling degree is low 3 - 4% for higher charge ratios. In the same conditions, after ~8 hours, the solubility is around 13 - 14% allowing to use this biomaterial for applications in dried and wet states.

Swelling degree decreases when the charge ratio (as well as the weight ratio) increases. At pH = 3, due to the insolubility of HA (gel forming conditions), the swelling remains minimum whatever the composition. At pH = 7.4, in phosphate buffer the swelling trend was close to pH = 3 behavior. In basic conditions, due to the large solubility of HA, the swelling decreases rapidly when the HA content decreases (up to CS/HA~ 2).

Mechanical properties

Using scanning electron microscopy (SEM), the diameter of PEC and pure CS nanofibers was determined. The average diameter obtained for pure CS nanofibers was 180 nm for thin regular fibers forming a highly porous mat with a density around 0.037 ± 0.01 g/cm^3 [18]. The density of the PEC material was larger for aligned and randomly collected fibers (around 0.102 ± 0.022 and 0.097 ± 0.01 g/cm^3, respectively). Considering the morphology of the samples, mechanical test was performed to compare the performances of nanofibers with random distribution as it was done before [15, 18] with oriented nanofibers in the aim of increasing their mechanical properties.

On the PEC mats, the mechanical properties were determined and compared with that of pure CS obtained in the same experimental conditions (Figure 3).

Figure 3 reflects the role of alignment of nanofibers on their mechanical properties in the dried state as already observed with honeycomb structures based on Polycaprolactone [19]. It was necessary to consider that the density of the material was larger for aligned than for randomly collected PEC fibers and larger than that of pure CS as mentioned before.

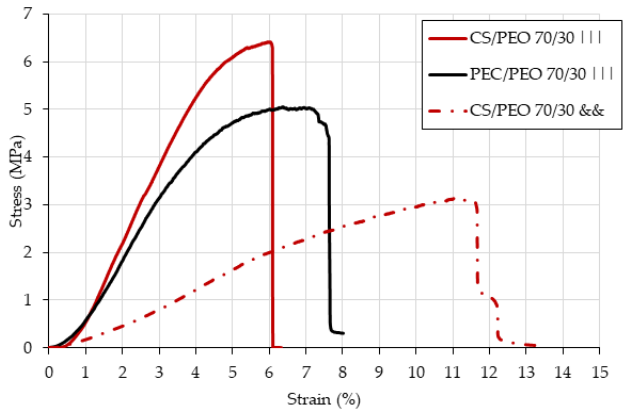

Figure 3: Comparison of mechanical behavior, under traction tests, of as-spun randomly collected (&&) and unidirectional oriented (|||) PEC and CS nanofiber mats. PEC NH_2/COOH = 2.35, composition polymer/PEO = 70/30. Solvent FA/W 1/1 v/v.

Stress under uniaxial tension was increased by fiber alignment and stress at break of aligned fibers was higher when CS/PEO and PEC/PEO systems were compared (i.e., 6.5 MPa compared with 5 MPa). The strain at break in aligned fibers was larger for PEC mat. In addition, it was clear that orientation increases the stress of CS fibers when contrasted to randomly orientated CS fibers (ratio = 6.5/3 MPa) (Figure 3).

In Figure 4, the anisotropic response of the materials was analyzed. The tensile properties were studied for the two orientations of aligned nanofibers made of CS/PEO and PEC / PEO. Itm was found that, in the main direction of CS/PEO fibers, stress at break was larger than the measured value in the transversal orientation (ratio = 1.5). Performances were decreasing while strain increases. The same trend was observed for PEC fibers (ratio = 2.5). These results were the mechanical signature of the anisotropic self-organization of fibers.

Relatively small differences contrasting the two directions could be in part due to connection between fibers occurring during their collection on the rotating cylinder before complete drying.

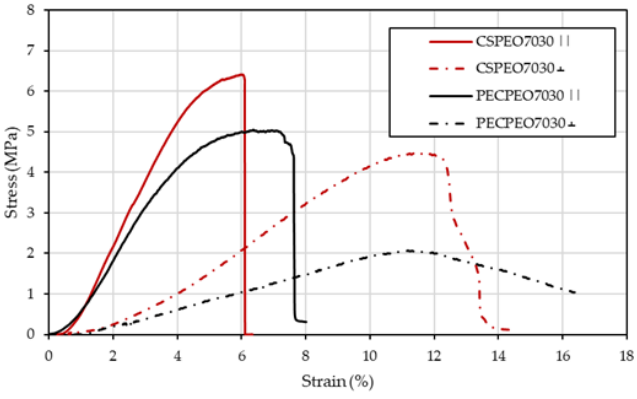

Figure 4: Comparison of mechanical response in dried state for aligned CS/PEO and PEC/PEO nanofibers (70/30 ratio). Tensile tests of the samples on the parallel direction (|||) and the transverse (⊥) direction of nanofibers.

Chondrocyte proliferation

Preliminary results for cell viability on PEC nanofiber mats (Rc = 2.35) were obtained by inversed fluorescence microscopy. Figure 5 shows cell proliferation on the PEC fibers at t = 10 days after seeding.

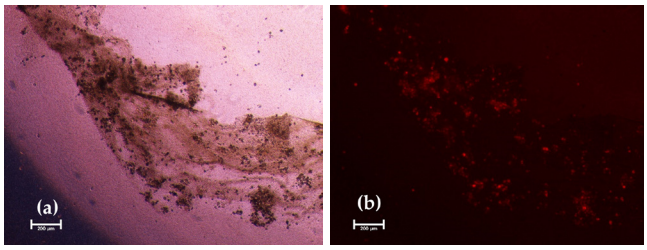

Figure 5: Chondrocyte C20A4 on PEC fibers at Rc = 2.35. (a) Direct observation, and (b) Staining by Red-FP transfections of cells. Filter excitation/emission 510 - 560 nm/590 nm. Scale bar = 200 µm.

In the same way, initial quantitative analysis of cell proliferation obtained on PEC nanofiber mats (Rc = 2.35) after thermal treatment and PEO extraction was performed. The results were compared with cell development on neutralized CS in Table 2.

Table 2: Influence of the substrate composition on development of chondrocytes. Comparison between CS and PEC (Rc = 2.35) fiber mats, cell average size was found ~ 20 µm.

Substrate		Seeding	Time = 2 days	Time = 4 days
CS/PEO 70/30	Cell density (cell/cm²)	1.4 x 10^4	2.0 x 10^4	3.14 x 10^4
	Cell viability	96.8%	100%	90%
PEC/PEO 70/30	Cell density (cell/cm²)	1.4 x 10^4	6.8 x 10^4	1.34 x 10^5
	Cell viability	96.8%	91.4%	100%

From these results, for substrates having the same dimensions, it was clear that PEC favors chondrocyte adhesion and development compared with CS support with a very high cell viability.

Conclusion

This paper described the main characteristics of new nanofibers made of CS/HA complex processed to get nanofibers. The role of the stoichiometric was examined and it was demonstrated that the -NH_2/-COOH ratio controls the degree of swelling and solubility of the material. The stress/strain for uniaxial tension show that CS was stronger than PEC with a lower strain. The influence of the orientation of fibers by using a rotating collector allows comparison of properties with randomly orientated fibers. Density of aligned PEC fibers was larger due to a lower porosity but the stress at break was larger allowing easier handling of the samples in the wet state. The stress in the two directions of orientation of the samples - in parallel and transversal directions of the tensile test specimen were also obtained. The relatively high stress in

the transversal direction seems to indicate connection between fibers occurring on the collector before complete drying of the electrospun solution.

At end, chondrocytes were developed on CS and PEC fibers showing the advantage of presence of HA on cell adhesion and proliferation. This new material should be convenient for tissue engineering application.

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