Novel Biologically Inspired Collagen Nanofibers Reconstituted by Electrospinning Method

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Summary: The possibility to prepare bioinspired collagen nanofibers by electrospinning from aqueous suspension of telopeptide-free collagen molecules avoiding both organic solvents and blends with any synthetic and natural polymers has been investigated. The results have highlighted the need for a basic atmosphere between the needle and the ground collector in order to increase the environmental pH during the collagen molecules self-assembly along the electrostatic force lines. Morphological, spectroscopic and calorimetric analyses carried out on the electrospun collagen nanofibers have opened the possibility to take advantage of this new approach in order to prepare an ideal biomimetic reinforcing component of new biomedical and surgical biomaterials.

Keywords: biomimetic; collagen; electrospinning; nanofibers; self-assembly

Introduction

Electrospinning technique is an efficient processing method to manufacture micro- and nano-sized fibrous structures by electrostatic force for different applications.[1–5] In the biomaterials field, electrospinning technique has been successfully utilized to prepare new drug delivery materials[6,7] and tissue engineering scaffolds.[8–10] Fiber mats of biodegradable polymers having a diameter in the nano- to submicro-scale can be considered to mimic the nanofibrous structure of native extracellular matrix (ECM). Native extracellular matrix, constituted of proteins and polysaccharides improving cell growth in its nanofibrous porous structure, controls not only the cell phenotype, but the whole structure of the biological tissues.[11] Collagen, for its natural origin, biocompatibility, non-immunogenicity and bio-resorbility, is a particularly suitable material to prepare tissue-engineering scaffolds. Tailored ultra thin collageneous membranes have been electrospun to prepare synthetic nanoscale fibrous extracellular matrix for tissue-engineering scaffolding.[12,13] Electrospun gelatin and collagen nanofibers have been crosslinked to improve their thermomechanical performance following different methods.[14,15] Gelatin and collagen blends with glycosaminoglycans, elastin and biodegradable polymers have been electrospun in order to improve nanofibrous scaffolds bioactivity and mechanical properties.[16–21] However, gelatin and collagen nanofibers electrospun alone or in combination with glycosaminoglycans and synthetic polymers have been always obtained from collagen solution in organic solvents such as fluorinated alcohols. In electrospinning experiments, the solvent performs a crucial function: in fact, not only it dissolves the polymer, but also allows to form the electrified jet, carrying the dissolved polymer towards the collector and then vaporizing rapidly during the polymer fibers formation. Trifluoroethanol, dichloromethane, and mainly 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP), used first by Matthew et al. (2002),[22] have been utilized for collagen

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dissolution, successfully obtaining electrospun collagen nanofibers. Huang et al. failed to prepare pure electrospun collagen fibers by acid solution and only collagen-polyethylene oxide composite fibers were electrospun in acid solution.[23]

In this study the possibility to prepare biomimetic collagen nanofibers by electrospinning aqueous suspensions of collagen molecules, free from non-helical region and glycosidic portions, avoiding both organic solvents and blends with synthetic or natural polymers was investigated. Morphological, spectroscopical and calorimetric analyses carried out on the electrospun biomimetic collagen nanofibers, by applying a basic atmosphere between the needle and the ground collector, have suggested the possibility to take advantage of this new bioinspired approach for potential innovative biomedical and surgical applications.

Materials and Methods

Materials

All the chemicals used were of high chemical grade. Type I collagen was extracted from equine Achilles tendon using the standardized manufacturing method of OPOCRIN SpA described here. The inner part of the equine tendons, from a certified slaughterhouse, rigorously controlled by the veterinary authorities, was dissected ensuring the complete removal of the synovial membrane. This tissue was finely ground and suspended in HCl water solution until pH 2.5, then pepsin (1% w/w with respect to the tissue) was added overnight for the removal of the non-helical regions of the molecules.[24] After the enzymatic treatment, the fibers were precipitated by raising the pH up to 5.5 by adding a NaOH solution. Then these fibers were repeatedly washed with distilled water and successively treated with 1 M NaOH solution for 1 h for the removal of the glycosidic portions and for virus inactivation. Finally, the collagen fibers were treated with HCl solution of pH 5.5. A homogeneous suspension, 1% (w/w), of nonhelical region-free collagen molecules was obtained by dissolution by stirring the fibers in 0.3% (w/v) acetic acid.

SDS-PAGE Electrophoresis

SDS-PAGE electrophoresis was carried out with a Phast System using PhastGel gradient 4–15 (both from Amersham-Pharmacia-Biotech). Each sample was submitted to reducing treatment with Laemmly buffer and 2 M urea (1 g collagen gel/mL solution 2 M Laemmly + urea) at 50 °C for 60 minutes. After brief centrifugation, 3 μL of every supernatant was placed on the SDS-PAGE slide and submitted to an electrophoretic run (parameters: 250 V, 10.0 mA, 3.0 W, 15 °C, 63 Vh) using BIORAD protein coded 161-0318 as a standard. At the end of the run, SDS-PAGE slide was treated with 10% (w/v) trichloroacetic acid for protein fixing and then colored with Coomassie Brilliant Blue R250.[25–28]

Electrospinning

The nanofibers were electrospun using the electrospinning setup shown in Figure 1.

For the process of electrospinning, a homogeneous suspension, 1% (w/w), of telopeptide-free type I collagen molecules dissolved in weak aqueous acetic acid solution was placed in a 5 mL syringe connected to a stainless steel needle using a teflon tube having an inner diameter of around 1.0 mm. When the needle was charged to a high DC voltage (Spellman, SL150, Spellman High Voltage Electronics Corp.) of positive polarity from the power supply, the collagen solution was ejected from the tip of the needle to generate ultra fine fibers, and the resulting nanofibers were collected on the grounded collector (negative polarity) that was placed 18 cm away from the needle tip. The applied high voltage was 18 kV. The syringe pump (KD Scientific 200) was used to feed the collagen solution to the needle tip at a constant mass flow rate of 0.01 mL/min. Furthermore, the electrospinning process was repeated by maintaining the above experimental processing variables, but applying NaOH vapour between the needle and the ground collector in order

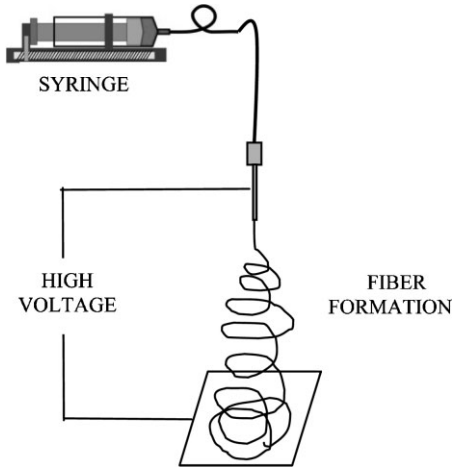

Figure 1.
Schematic diagram of the electrospinning apparatus.

to increase the environmental pH during the self-assembly of the collagen molecules along electrostatic forces lines.

Infrared Spectroscopy

Spectral data were recorded by a Thermo Nicolet 380 ATR-FTIR spectrometer. The spectra were the result of 100 scans. Background scans were obtained from a region of no sample and rationed against the simple spectrum.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) observations were carried out using a Philips 515. The samples were mounted on carbon tape on aluminium stubs and gold coated with an acceleration voltage of 30 mV for a time ranging from 30 to 180 s, prior to investigation.

Calorimetric Analysis

Differential scanning calorimetry (DSC) was performed using a TA-DSC Q100 in the temperature range $-20\,^{\circ}C$ to $180\,^{\circ}C$ under nitrogen flow at a heating rate of $10\,^{\circ}C/min$.

Experiments were performed in triplicate, and results are plotted as mean values $\pm$ SD. Differences were considered statistically significant at a significance level of 90%.

Results

Type I collagen molecules (300 nm long) having the stoichiometry $\alpha_1(I)_2\,\alpha_2(I)$, where $\alpha_1(I)$ and $\alpha_2(I)$ are two different types of polypeptide chains,[29] were obtained from equine tendon using the standardized manufacturing method of OPOCRIN SpA.[24] The telopeptide-free type I collagen molecules were obtained by a specific enzymatic treatment carried out at low pH and successively treated with 1 M NaOH solution for 1 h for the removal of the glycosidic portion and virus inactivation. A homogeneous suspension, 1% (w/w), of nonhelical region-free collagen molecules was obtained by dissolution by stirring of the fibers in 0.3% (w/v) acetic acid.

The SDS-PAGE analysis of the isolated collagen molecules (a) is reported in Figure 2 in comparison with the standard protein (b). Collagen molecules show high molecular weight bands described as $\alpha_1(I)$ and $\alpha_2(I)$ dimers and, at lower molecular weight, the typical bands of $\alpha_1(I)$ and $\alpha_2(I)$ chains of type I collagen.[30]

DSC thermogram of 1% (w/w) telopeptide-free type I collagen molecules air-dried

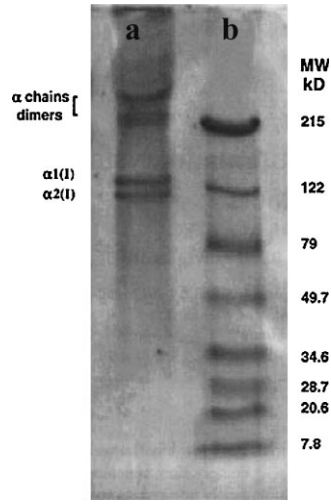

Figure 2.
SDS-PAGE analysis (a) HMW calibration kit, (b) telopeptide-free type I collagen molecules. α_1(I) and α_2(I) dimers appear as high molecular weight bands while α_1(I) and α_2(I) chains are ascribed to the two low molecular weight bands.

from aqueous suspension is reported in Figure 3a, revealing an endothermic transition at $T_D = (76 \pm 1)^\circ C$ due to the denaturation event. The calculated denaturation enthalpy value is $\Delta H_D = (35 \pm 1)$ J/g. These values are lower with respect to the $T_D = (112 \pm 1)^\circ C$ and $\Delta H_D = (41 \pm 1)$ J/g values obtained for air-dried equine tendon and those reported for natural collagen, even if the native collagen thermal transition enthalpy and denaturation temperature determined by DSC differ appreciably depending more strongly from the degree of hydration of the sample than from the source of collagen.[31,32]

The isolated and purified collagen molecules yield self-assembled collagen fibers when the aqueous collagen molecular suspension pH is raised up to 5.5. The morphology of the intertwined collagen fibers self-assembled from the aqueous suspension of telopeptide-free type I collagen molecules under pH variation is shown in the SEM micrograph reported in Figure 4.

Fibers are more than 100 μm in length and a few microns in thickness. They exhibit, by DSC, $T_D = (85 \pm 1)^\circ C$ and $\Delta H_D = (39 \pm 1)$ J/g values (Figure 3c), appreciably higher with respect to those obtained for air dried collagen telopeptide-free type I molecules, but lower than T_D and ΔH_D obtained for air-dried equine tendon.

FTIR spectroscopic analyses carried out on telopeptide-free type I collagen molecules air-dried from aqueous suspension (Figure 5a) and collagen fibers self-assembled from collageneous molecular suspension by raising pH (Figure 5b) reveal some appreciable differences concerning the characteristic absorption bands at 1650, 1560 and 1240 cm^{-1}, corresponding to the amide I, II and III region, respectively.

The observed differences in T_D and ΔH_D values determined by DSC for the natural and reconstituted collagen fibers can be ascribed to the different hierarchical structural organization of the collagen molecular assembling. Contrary to natural collagen fibers, which are constituted of microfibers assembled in sub-fibrils and fibrils crimped together,[33] collagen molecules self-assembled in reconstituted fibers do not have any hierarchical order. Probably, the crosssection of reconstituted fibers present the same homogeneous distribution of the molecules-molecules interactions.

Actually, if one obtains reconstituted nanofibrils from self-assembled collagen molecules, they could be mechanically brought together in micrometric fibers creating new inter fibrils interactions and crosslinks

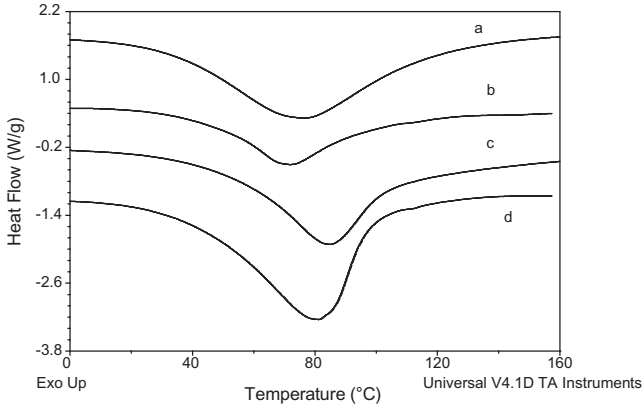

Figure 3.
DSC curves recorded from air dried telopeptide-free type I collagen molecules (a), air-dried electrospun nano-sized collagen fibers from aqueous suspension (b), air-dried micro-sized self-assembled collagen fibers from telopeptide-free type I collagen molecules aqueous suspension (c), air-dried electrospun nano-sized collagen fibers from aqueous suspension by applying a basic atmosphere between the needle and the ground collector (d).

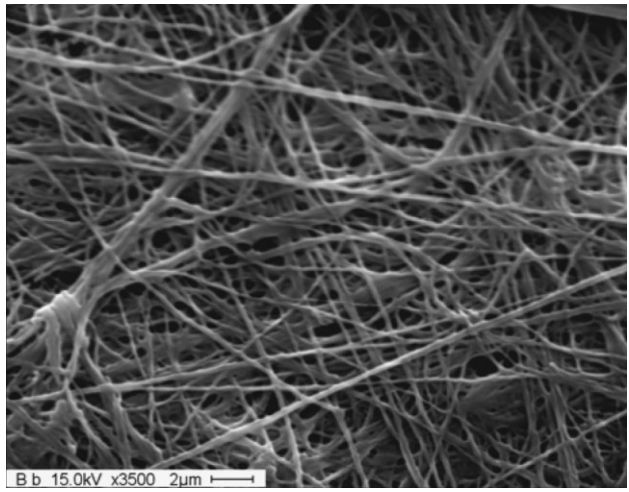

Figure 4.
SEM images of micro-sized self-assembled collagen fibers from aqueous suspension of telopeptide-free type I collagen molecules.

resembling partially the hierarchical structural organization of tendons. This represent a bioinspired approach for preparing innovative biomimetic reconstituted collageneous fibers as reinforcement component of new biomedical and surgical materials. With this in mind, the possibility to prepare collagen nanofibers by electrospinning aqueous suspensions of collagen molecules, free from non-helical regions,

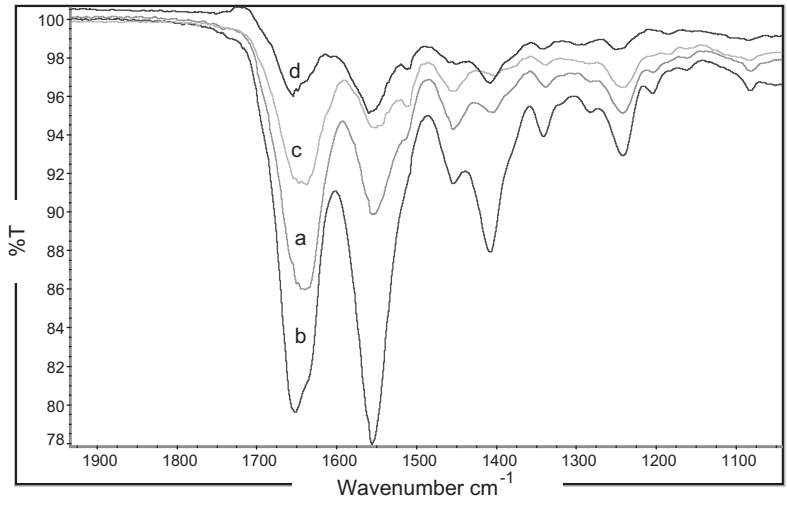

Figure 5.
FTIR spectra of air dried telopeptide-free type I collagen molecules (a), collagen fibers self-assembled from collageneous molecular suspension by raising pH (b), air-dried electrospun collagen nano-fibers (c) air-dried electrospun collagen nano-fibers from aqueous suspension applying a basic atmosphere between the needle and the ground collector (d).

avoiding both organic solvents to dissolve collagen molecules and blends with any synthetic and natural polymers was investigated.

In the electrospinning process, concentration of the polymer solution, applied voltage, air gap distance and delivery rate are critical experimental processing parameters which determine the size and morphology of electrospun fibers.[22] The solution delivery rate was changed between 0.005–0.02 mL/min while the applied voltage and air gap distance were varied in the range of 10–20 kV and 10–20 cm, respectively. At the beginning, the delivery of the homogeneous suspension, 1% (w/w), of telopeptide-free type I collagen molecules obtained by dissolution under stirring of the fibers in 0.3% (w/v) acetic acid was tried. The density of the molecular suspension appeared too high and the solution was diluted with acetic acid up to a value of pH = 2.5, obtaining after centrifugation a homogeneous solution easier to deliver. Fibers of some hundred nanometers in diameters have been electrospun using this diluted collagen molecular suspension, even if it was impossible to obtain homogeneous fibrous mats resembling those prepared with collagen solution in organic solvent and with synthetic and natural proteins (Figure 6a).

FTIR study carried out on the air-dried electrospun collagen nanofibers (Figure 5c) reveals that their absorption spectrum does not change with respect to that recorded for air-dried collagen molecules. Also, calorimetric analyses carried out on these nanofibers (Figure 3b) do not reveal appreciable difference in T_D and ΔH_D values with respect to those obtained for air-dried collagen molecules. These results suggest that electrospun collagen nanofibers are assembled by electrostatic forces, which draw the positively charged molecular solution into a fine nano-sized filament, in a different asset with respect to the microfibers obtained by raising the pH in the same aqueous collagen molecular solution. In fact, the electrospun collagen nanofibers appear as a simple nano-sized collagen molecular assembly, without appreciable chemical bonds and interactions between the packed molecules.

The electrospinning process was repeated by maintaining the above experimental processing variables constant, but applying a basic atmosphere between the needle and the ground collector, in order to increase the environmental pH during the collagen molecules self-assembly along the electrostatic forces lines. Using this electrospinning process set-up, the formation of a three-dimensional network of nano-sized collageneous fibers that exhibit biomimetic morphology and dimension was achieved (Figure 6b). FTIR spectroscopy and DSC analyses carried out on these electrospun nano-sized collageneous fibers have shown

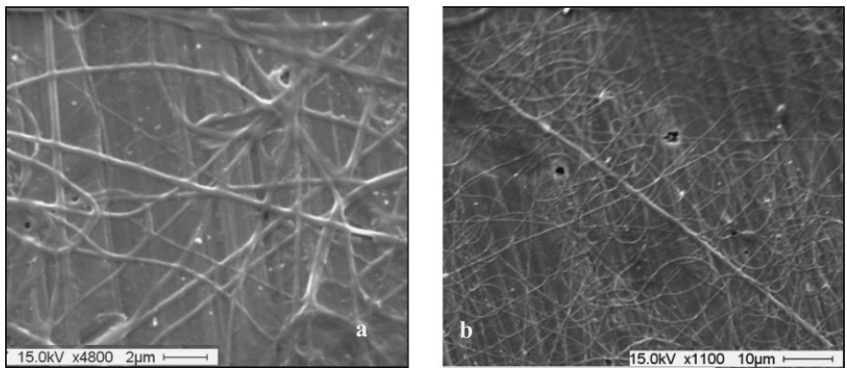

Figure 6.
SEM images of electrospun collagen nanofibers from telopeptide-free type I collagen molecule aqueous suspension, without, (a), and applying, (b), a basic atmosphere between the needle and the ground collector.

the formation of bonds and interactions between the assembled molecules, which proved to be insoluble without any further crosslinking treatment. In fact, the FTIR spectrum of air-dried electrospun nano-sized collagen fibers from aqueous suspension obtained in the presence of NaOH vapour, reported in Figure 5d, appears very close to that reported in Figure 5b for the air-dried micro-sized self-assembled collagen fibers. DSC analyses show that nano-sized collagen fibers electrospun in a basic atmosphere (Figure 3d) are more stable with respect to the nano-sized collagen fibers electrospun in the absence of it (Figure 3b).

Conclusion

The possibility to prepare bioinspired collagen nanofibers by electrospinning from telopeptide-free collagen molecule aqueous suspension avoiding both organic solvents and blend formation with any synthetic and natural polymers has been investigated.

A homogeneous suspension, 1% (w/w), of telopeptide-free type I collagen molecules, obtained from equine tendon by dissolution in 0.3% (w/v) acetic acid and a specific enzymatic treatment for removal of non helical region and glycosidic portions has been utilized to electrospin nano-sized collagen fibers which mimic native collagen fibers. Differential scanning calorimetry and infrared microscopy spectral analyses carried out on electrospun biomimetic collagen nanofibers have put in evidence the necessity to apply a basic atmosphere between the needle and the ground collector in order to increase the environmental pH during the collagen molecules self-assembly along the electrostatic forces lines. The present results not only define new electrospinning experimental condition suitable to obtain biomimetic collagen self-assembled nanofibers, but also open the possibility to take advantage of this new bioinspired approach for potential innovative biomedical and surgical applications.

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