A TUBULAR POLYCAPROLACTONE/ HYALURONIC ACID SCAFFOLDS FOR NASAL CARTILAGE TISSUE ENGINEERING

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Abstract

In this preliminary study, 3D nanofibrous porous scaffolds in the form of spiral tubes for future application as nasal cartilages implants were fabricated by combining polycaprolactone electrospun fibers with drug modified hyaluronic acid gel. It is expected that the spiral form of the scaffold with open geometries, large surface area, and distance between the scaffold walls will be helpful for improving future cell penetration into the scaffolds, nutrient transport and metabolic waste removal, which are otherwise limited in conventional electrospun tissue-engineered scaffolds. The tubular scaffolds structure, its porosity and fibers' diameter were assessed via scanning electron microscopy, and biological properties of the scaffolds were evaluated in an in vitro study using Simulated Body Fluid (SBF). SEM results showed that apatite formed within a short period on tubular scaffolds after its immersion in SBF, demonstrating high in vitro bioactivity of the scaffolds.

Keywords: *electrospinning, tissue engineering scaffolds, nasal cartilages, hyaluronic acid, PCL*

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Introduction

A cartilage tissue is a sort of connective tissues which can be flexible and elastic, but still resistant. It can be found in the surface of articulations between the bones, in rib cage, ear, bronchus, or nose. Cartilage is the support and connection tissue for different organs and it is mostly composed of chondrocyte cells and collagen fibers which are immersed in the extracellular matrix [1]. Cartilage lesions have generally mechanical origins: naturally with aging or by trauma, which is the most frequent lesion origin for nasal cartilage [2]. Thus it is important to be able to fix it using rhinoplasty for correcting and constructing the form of the nose and for restoring the respiratory functions. The main nasal cartilages are: the greater alar cartilage, the lateral nasal cartilage, the lesser alar cartilages, and the cartilage of the septum (FIG. 1).

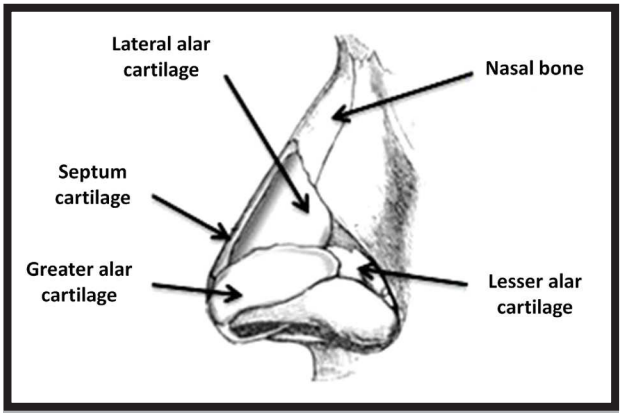

FIG. 1. Nasal cartilages.

Septum is the cartilage which separates the nasal cavities in front of the nose, making it crucial for the respiratory system [3] and exposed to trauma or congenital diseases [4,5]. Even small chondral defects may necessitate surgical intervention. The requirements for scaffolding materials for use in cartilage tissue engineering are well-defined; however the ability to produce such materials has been limited.

Electrospinning is a process that can generate three dimensional nanofibrous or microfibrous scaffolds with high porosities, large surface-to-volume ratios, and variable fiber diameters [6]. However, it has been reported that electrospun nonwovens have limited cellular penetration depth because of the increased thickness of the nanofiber layers and the reduced pore size [7]. Pore size below cellular diameter cannot allow cell migration within the structure.

Porosity and thereby cell penetration could be enhanced in scaffolds by using the strategy of creating a spiral tubes where additional porosity is created between the walls of the tubes. Therefore the purpose of this preliminary work was to create an easy method to small nasal cartilage implant production. By using post-processes after electrospinning of fibrous nonwovens, such as surface modification and rolling up, we were able to produce three-dimensional fibrous scaffolds of desired morphology for further application. It is expected that the spiral form of the scaffold with open geometries, large surface areas, and distance between the scaffold walls will be helpful for improving cell penetration into the scaffolds, nutrient transport and metabolic waste removal, which are otherwise limited in conventional electrospun tissue-engineered scaffolds [8].

The materials chosen to make spiral scaffolds tubes are poly(ε-caprolactone) (PCL) and hyaluronic acid modified with Osteogenon. PCL is a synthetic polymer with good mechanical properties and easy to process. Implants produced from PCL are in clinical use for fracture fixation in regions of limited mechanical load and are well suited for the fabrication of scaffold for the regeneration of cartilage and bone tissue. Compared with other polymers such as poly(lactic acid) (PLA), poly(glycolic acid)(PGA) or poly(lactic-co-glycolic acid) (PLGA), PCL has a much longer degradation time, lasting up to three years. In this aspect, PCL may be a suitable scaffold material for cartilage tissue engineering in facial reconstructive surgery [9,10]. PCL is characterized also by good formability into the fibres. However PCL has also some disadvantages, as limited bioregulatory activity and hydrophobicity [11]. Hyaluronic acid (HA) is a glycosaminoglycan, distributed widely throughout connective, epithelial, and neural tissues. In particular, it is one of the main components of the cartilage's extracellular matrix, providing it with good biocompatibility, biodegradability and non-toxicity [12].

Osteogenon (Osteo) is an ossein-hydroxyapatite complex (OHC) also containing osteocalcin and type I collagen [13]. Osteo was added to the HA solution as a modifier to improve the bioactivity of the final tubes.

We hypothesize that producing tubes by combining PCL nonwoven manufactured by electrospinning with the HA hydrogel layer modified with Osteo, and then rolling up the obtained microfibrous sheets, we could provide a spiral form of the scaffold with dual and designed porosity that would be impossible to obtain by using only an electrospinning method. Furthermore, the application of Osteo modified HA would improve the bioactive behaviour of the scaffold. The aim of our preliminary research was to determine the parameters of scaffolds production and to assess the bioactivity of the scaffolds produced in the form of spiral tubes.

Materials and methods

Materials

Poly(ε-caprolactone) (PCL) was purchased from Sigma Aldrich (average molecular weight: 80 kDA). Sodium hyaluronate (HA) was obtained from Contipro Company (Dolni Dobrouć, Czech Republic) with a molecular weight of approximately 1320 kDa. The properties of HA are presented in TABLE 1. Osteogenon (Osteo) was purchased from PIERRE FABRE. Dimethylformamide (DMF, POCH, Poland) and deionized water were used as solvents for HA solution preparation. The final solution was 0.085 wt% of HA in a 1:1 mixture of DMF:H_2O. Stable dispersion of Osteo suspension was achieved by sonication of the solution. A similar solution was also made with an addition of 0.500 g of Osteo. A 20 wt% of PCL was dissolved in equal parts of chloroform and methanol (POCH, Poland).

The PCL solutions were electrospun using a TIC 1092012 electrospinning machine (Bielsko-Biala, Poland). There are a variety of parameters that influence the morphology and diameter of the electrospun fibers, including the intrinsic properties of the solution, the operational conditions and ambient parameters. Operational conditions (controlled variables) include the flow rate, electric field strength, distance between tip and collector, needle tip design, and collector composition and geometry. Evaluation of process parameters for drug modified electrospun nonwovens scaffolds before setting the final parameters of the experiments was conducted. The prepared PCL solution was put in a syringe of 10 mL topped with a needle the diameter of which was 0.22 mm. The needle was connected to 25 kV voltage, and the distance between needle and the collector was 200 mm. The rotary collector was wrapped with silica-coated paper. All parameters used during electrospinning are shown in TABLE 2.

Spiral tubes fabrication

In order to make three-dimensional tubular scaffold, electrospun PCL nonwoven (FIG. 2a) was cut into strips of 3 cm x 15 cm. A 0.1 mL of HA was added on the nonwoven surface. By pressing the metallic shaft into the surface of the PCL nonwoven, a uniform distribution of HA/Osteo was achieved. Then a metal spin was used for rolling up the nonwoven (FIG. 2b), to finally get the spiral tube (FIG. 2c). To investigate the effect of tubular fibrous structure and hyaluronic acid coating on the properties of the scaffolds, three groups of scaffolds have been prepared: pure PCL spiral tubes, PCL spiral tubes coated with HA, and PCL spiral tubes coated with Osteo modified HA.

TABLE 1. Properties of sodium hyaluronate used in the study.

Appearance	White or almost white powder, granules or fibrous aggregate
Molecular weight [MDa]	1.32
pH	6.6
Intrinsic viscosity [m^3/kg]	1.96
Sodium hyaluronate [%]	97.6
Residual isopropanol [%]	<0.5
Loss on drying [%]	3.4
Protein [%]	0.029
Chlorides [%]	<0.05

TABLE 2. Parameters used during electrospinning process.

Flow rate [mL/h]	3
Distance [cm]	20
Voltage [kV]	25
Collector speed [rpm]	415
Collector diameter [mm]	20
Temperature [°C]	19.7
Humidity [%]	40

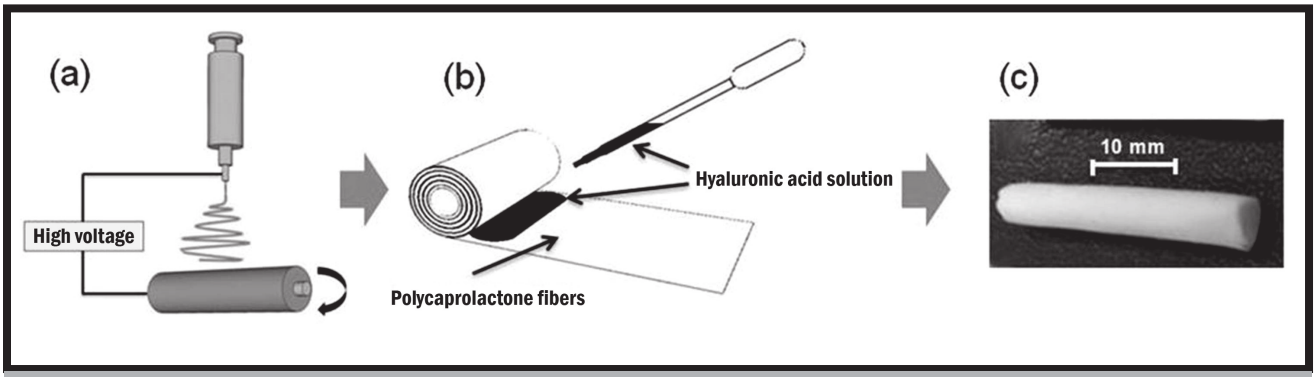

FIG.2. Scheme of samples preparation.

Scaffold Characterization

The thickness of the PCL fibrous samples was measured using a Mitutoyo 7316S Dial Thickness Gage. Each sample was measured five times in order to calculate the average value and the standard deviation. The sample microstructure was investigated with scanning electron microscopy (Nova NanoSEM 200, FEI). Before observation samples were coated with a thin carbon layer. Stereomicroscopy (SN from OPTA-TECH Company, equipped with CMOS 3 camera and OptaViewIS software) was also used to capture images of the scaffolds. Pure PCL fibrous nonwovens (PCL), PCL spiral tubes coated with HA (PCL/HA), and PCL spiral tubes coated with Osteo modified HA (PCL/HA+Osteo) before and after immersion in Simulated Body Fluid (SBF) were observed. The porosity and fiber diameter were evaluated by analysis of the SEM images of the scaffolds using Image-J software version 1.50. Two-dimensional (2D) images were converted into binary images. Surface area of pores in each image was separated from the whole area of the picture and reported as an average value for each scaffold. Average fiber diameter and the standard deviation were calculated from 100 measurements of different fibers.

***In vitro* tests in Simulated Body Fluid solution (SBF)**

The bone-bonding ability of a material is often evaluated by examining the ability of apatite to form on its surface in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. The bioactivity tests in SBF were performed as a first step of biological evaluation of tubes in order to check their osteoinductive behavior. Simulated Body Fluids (SBF) have the ability to form apatite calcium phosphates on immersed osteoinductive materials from a few days to 2 weeks. Therefore it is a good way to check if it is reasonable to perform more expensive biological tests (for instance: cell culture or animal *in vivo* studies). The purpose of the *in vitro* tests is to observe the formation of the apatite deposits in samples immersed in a SBF solution [14,15]. A common feature of bioactive materials is that their surfaces develop a biologically active apatite layer after implantation, which is essential for establishing bonding with natural tissue [16]. It is believed that if material has the ability to induce apatite formation on its surface after immersion in SBF solution, it will be bioactive *in vivo* as well.

To realize those tests, one litre of 1.5 x SBF was prepared according to the Kokubo's recipe [17]. It had ion concentrations that were nearly 1.5 x as those in human blood plasma. A solution with ion concentrations 1.5 times of SBF (1.5 SBF) was prepared, in order to accelerate the apatite formation. The application of 1.5 x SBF in order to enhance the kinetics of coating deposition is commonly used by other researches. Samples of PCL nonwoven and spiral tubes of PCL/HA and PCL/HA+Osteo were immersed in SBF at 37°C for 1, 3, and 7 days, respectively. In order to optimize the *in vitro* simulation, the SBF solution was changed every 2 or 3 days. Changes of the surface structure of immersed spiral tubes and nonwovens were analyzed after the specimens had been removed from SBF, washed with distilled water and dried.

Results and Discussion

The aim of this preliminary study was to fabricate three-dimensional composite scaffolds in shape of spiral tubes for cartilage tissue engineering. As a first step of our study electrospinning method was used to fabricate fibrous nonwovens. When considering the maintenance of the geometric structure (which is very important, regarding nasal cartilage replacement application) one obvious benefit of PCL application is its comparatively longer degradation time, which may help maintain the desired shape of the tissue until secreted ECM materials can supplement the scaffold with this function. The electrospinning process was successfully used to fabricate randomly oriented fibrous nonwoven of PCL (FIG. 3a). The thickness of electrospun PCL nonwoven was 0.44 ± 0.15 mm. The obtained nonwoven had open-pore geometry and porosity about 60% according to ImageJ software (TABLE 3).

TABLE 3. Porosity and average fiber diameter of obtained materials.

	Average fiber diameter [µm]	Porosity [%]
PCL	2.28 ± 0.76	59.8 ± 2.4
PCL/HA	2.73 ± 1.21	52.1 ± 3.7

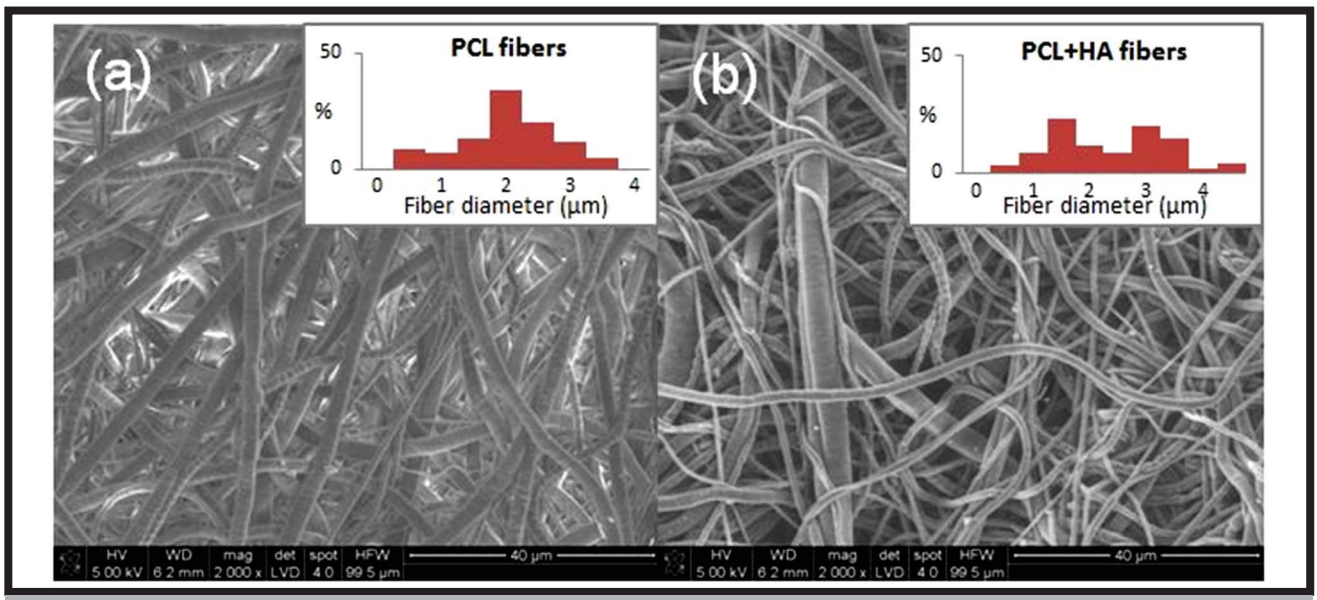

FIG. 3. SEM images of obtained samples together with fibers diameter distribution: (a) pure PCL nonwoven; (b) surface of PCL/HA tube.

However, the accuracy of this method depends on the contrast and quality of the SEM images and the assumption that the region being imaged is characteristic of the whole material. The disadvantage is that it often underestimates the total porosity. The diameter of obtained PCL fibers was in the range from 400 nm to 3.6 μm, and an average fiber diameter was 2.28 ± 0.76 μm. Obtained electrospun nonwovens had high surface area and interconnected pore network. Achieved porosity of the scaffold should provide a facile transport of metabolic nutrients and waste through its pores. However the efficient cell implantation and blood vessel invasion would be impossible as the size of pores is too small. The porosity of obtained scaffold was improved by rolling up PCL nonwoven with a small amount of pure or modified HA solutions. Although PCL is not elastomeric, its electrospun nonwoven is fairly flexible and generally it was easy to roll it up. Spiral shape tubes (FIG. 4) were obtained with additional gaps between the spiral layers. Another notable limitation of the PCL nonwoven in cartilage tissue engineering field is its hydrophobic surface properties and lack of bioactive ligands. Therefore PCL has poor cellular adhesion properties on its own, without some form of functionalization [18]. In order to overcome this disadvantages, and to improve bioactivity of PCL scaffolds coating with HA with Osteo modified HA on electrospun PCL nonwovens was applied. HA is one of the main components of the cartilage's extracellular matrix, providing it with good biocompatibility. The measurements showed that the average diameter of fibers after addition of HA solutions is slightly higher than for pure PCL sample (TABLE 3). This suggests that a thin layer of hydrogel solutions covered the PCL fibers. The average porosity (space between the fibers) slightly decreased (about 13%) when HA solution was added on PCL nonwoven surface (TABLE 3). However novel porosity appeared, which represent space between scaffold walls in spiral tube (FIG. 4). Pores with elongated shapes could be observed on the cross section of obtained spiral tubes. The distance between walls was about 20 μm, however it can be regulated during fabrication process in order to obtain the pore size adequate for implantation side. As a first step of biological evaluation of tubes the bioactivity tests in 1.5 x SBF was performed in order to check their osteoinductive behavior of obtained materials. FIG. 5 shows the surface of the PCL, PCL/HA and PCL/HA+Osteo samples after 7 days of immersion in SBF solution. Apatite crystals were found on PCL/HA+Osteo samples after its immersion in SBF for 7 days, and covered most regions of the surface of the sample, whereas on PCL/HA samples, apatite growth was very slow and gentle apatite formation after 7 days was observed.

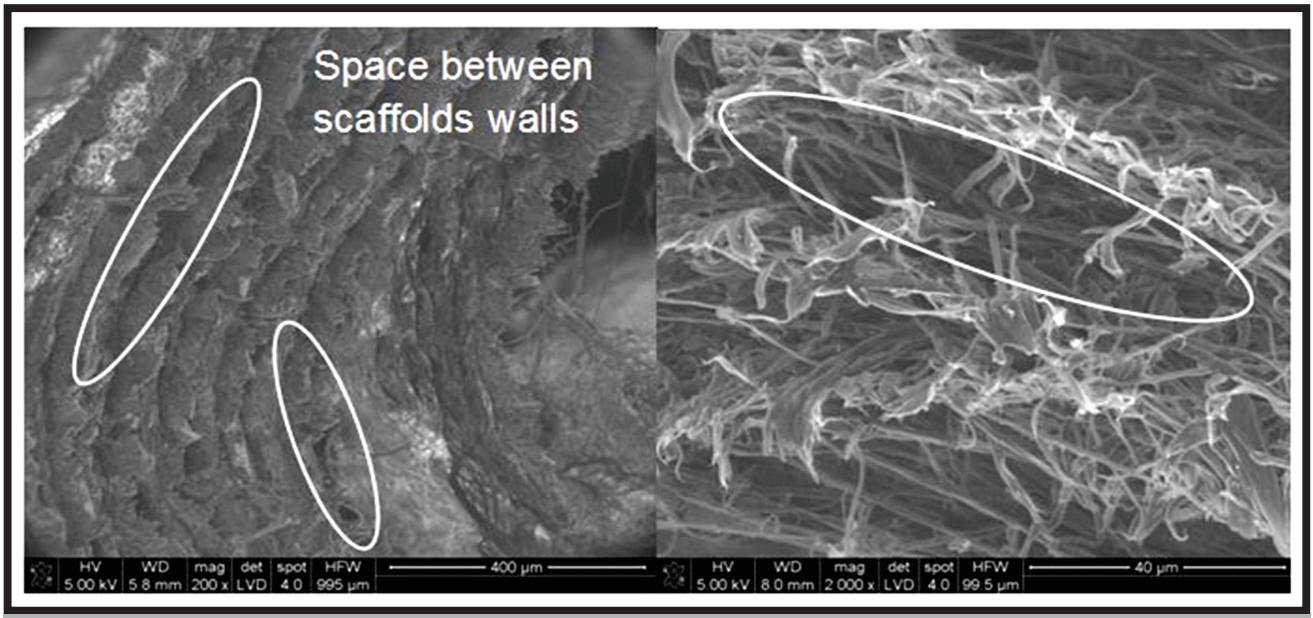

FIG. 4. Cross section of obtained spiral tubes (PCL/HA). Gaps between the walls.

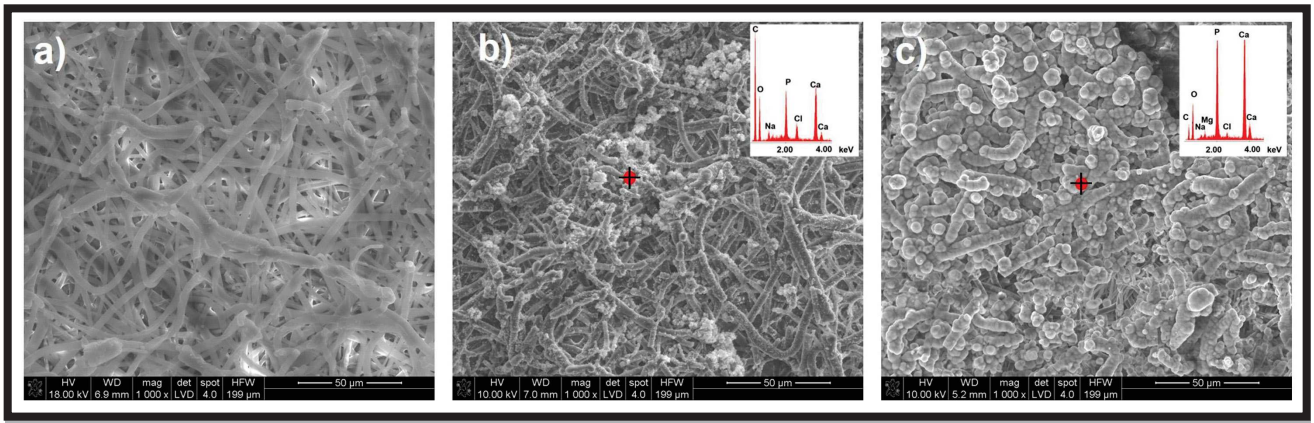

FIG. 5. PCL, PCL/HA and PCL/HA+osteo samples after immersion in SBF solution for 7 days.

Our study indicated that the apatite layer was formed continuously on the surface of Osteo modified PCL/HA samples, which means that such samples had greater ability to induce the formation of minerals *in vitro*. SEM demonstrated the changes in SBF that the scaffolds underwent after surface modification. Coating PCL with Osteo modified HA changes the surface properties of the pure PCL scaffold. It is expected that it would improve cell-scaffold interactions, however further biological study in cell cultures need to be performed.

Conclusions

Electrospinning has been widely used to produce fibrous scaffolds for tissue engineering applications. Although the method is very simple, easy, and effective for obtaining fibrous materials, the fabrication of three dimensional (3D) shapes has been a major obstacle for use in tissue engineering. In this study, a new fabrication method to produce controllable 3D spiral structures is developed. While there are still a number of challenges, 3D nanofibrous porous scaffolds in the form of spiral tubes hold a promising future for cartilage tissue engineering applications. The cartilage scaffold should require a 3D shape because a 2D structure is unable to replicate the behavior of cells *in vivo*. The structure of the tube proposed in our preliminary work consists of dual porous architecture containing fibrous walls and microsized pores formed between the walls of the rolled scaffold. The microscopic observation indicates that the obtained scaffolds in the form of tubes have an open pore geometry and additional porosity between the spiral walls which will be helpful for improving cell penetration into the scaffolds, nutrient transport and metabolic waste removal. The addition of Osteo into HA solution improves the bioactivity of obtained tubes. Osteo was incorporated into HA hydrogel to form a bioactive layer on fibrous PCL. This layer is essential for establishing bonding with natural tissue after implantation. By controlling appropriate processing conditions, we can successfully fabricate a highly porous 3D structure. Our spatially designed scaffold could provide more biomimetic micro-cellular environmental conditions than 2D electrospun scaffold, however further cellular study will be performed to confirm possible application in cartilage tissue engineering.

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