

The Preparation of ZnO@Chitosan@Gelatin Composite Nanofibers for Tissue Engineering Applications

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Abstract. Chitosan is a novel biocompatible, biodegradable polymer for potential use in tissue engineering. In this work, ZnO@chitosan (CS)@gelatin (GEL) composite scaffolds were prepared by combining coaxial electrospinning methods and RF magnetron sputtering. And polyethylene oxide (PEO) was added into CS solution to improve spinnability. The prepared CS-PEO@GEL nanofibers were characterized using SEM and TEM. The prepared ZnO@CS-PEO@GEL composite scaffolds were characterized using SEM, XRD and LSCM studies. The composite scaffolds were three-dimensional network structured. In addition, cytotoxicity of the composite scaffolds were studied by CCK-8 assay. The scaffolds showed good biocompatibility and cytocompatibility, according to this study.

1. Introduction

Tissue engineering is considered to be a very promising approach for treating bone injury. The major challenge in cell therapy is to design ideal carriers that are able to deliver therapeutic cells efficiently and specifically to the target site, avoiding the drawbacks of traditional cell therapy, such as limited cell retention, inferior cell survival, and so forth^[1,2]. Over the past few decades, chitosan(CS) three-dimensional network structure have received much attention as cell carriers. Chitosan(CS) is the derivative of Chitin and its structure is similar to polysaccharide, so it has some excellent properties, such as biocompatibility, nontoxicity, biodegradability, so chitosan have been widely researched in many fields^[3]. In tissue engineering, cells can be cultured on the chitosan three-dimensional network structure within the desired tissue to provide a local therapeutic effect, which have the potential to mimic the extracellular matrix (ECM) and can serve to organize the cells into a three-dimensional architecture.

As is known to all, electrospinning is a simple and multifunction technique and can produce micro-nanometer-scale fibers and has been applied in the fabrication of tissue-engineering scaffolds^[4]. But a simple electrospinning usually cannot meet many demands. Compared with the traditional electrospinning, the coaxial electrospinning is an efficient way to fabricate the core-shell structure nanofibers^[5]. Usually, two materials were used in the coaxial electrospun, and one should has highly spinnable to enhanced the spinnability of another material which maybe with poor spinnability. In this study, we used the chitosan as shell and the gelatin as core. But because of its polyelectrolyte nature, chitosan shows the poor spinnability^[6]. A few attempts have been made to prepare chitosan nanofibers by electrospinning, it has been demonstrated that when chitosan was dissolved in trifluoroacetic acid^[7] or acetic acid^[8] can be electrospun. Recently, a lot of reports show that can improve the spinnability of chitosan by using other synthetic polymers such as poly(lactide-co-glycolide) (PLGA)^[9], polyethylene oxide (PEO)^[10,11].etc, the results show that those



synthetic polymers can improve the spinnability of chitosan. In order to improve the biological performance of the chitosan-based nanofibers, some researchers have attempted other natural polymers such as gelatin with chitosan by electrospinning and show better bioactivity^[12]. Because both chitosan and gelatin are hydrophilic materials, when the nanofibers were kept in water, their structure will be damaged. So some cross-linking agents such as glutaraldehyde^[13] have been studied to solve this problem.

Another problem is how to improve the osteoinduction of the nanofibers. An alternative strategy is to dope bioactive trace elements into these materials. The therapeutic ions including Zn^{2+} , Mg^{2+} , Cu^{2+} , Sr^{2+} , and Si^{4+} ions can accelerate angiogenesis and simulate the osteogenic differentiation of mesenchymal stem cells (MSCs)^[14-16]. Among the bioactive metal ions, zincite (Zn) ions have been widely investigated in bone repair materials, because they are able to increase new bone formation and inhibit bone resorption. In this work, we induced Zn by RF method. A layer of ZnO nanofilms was used as the modified materials that was grown on the surface of the nanofibers by magnetron sputtering using ZnO target.

2. Experimental apparatus and medicinal materials

2.1. Experimental Materials

Chitosan(CS, deacetylation of the chitosan is 95%); polyethylene oxide(PEO, average molecular weight of polyethylene oxide is about 300000); gelatin(GEL, Type A, AR grade); glutaraldehyde(GA, ARgrade,25%, V/V); acetic acid(AR grade); Humanosteoblast like cell line (MG-63, from National Centre for Cell Science, Pune) ; Alpha minimum essential medium (α -MEM); fetal bovine serum (FBS); Cell Counting Kit8(CCK-8);

ZnO target(the diameter 6cm, purity is 2N5); Dimethyl sulfoxide(DMSO), Calcein acetoxymethyl(Calcein AM); ethidium homodimer-1(EthD-1); phosphate buffered saline (PBS) and 0.25% trypsin-EDTA phenol red were purchased from Shanghai Beyotime Biotechnology.

2.2. Experimental instrument

Field Emission Scanning Electron Microscope (FESEM, Hitachi S-4800, 10KV); the transmission electron microscope (TEM, JEM 2100, 200kV, Japan); the Energy Dispersive Spectrometer (EDS, INCA, Oxford Instruments, England); the X-ray diffractometer (XRD, D8 Discover, Bruker); the enzyme linked immunosorbent assay reader (ELISA, BioTek Instruments,USA); the laser scanning confocal microscope (FV1000-IX81, Olympus, Japan); the thermogravimetric Analyzer (PYRIS 1, USA, PerkinElmer); the enzyme linked immunosorbent assay reader (ELISA, BioTek Instruments, USA).

3. Experimental part

3.1. Fabrication of CS-PEO@GEL core-shell nanofibers

Gelatin solution was prepared in 20%(V/V) acetic acid, and the concentration is 41% (W/V). Chitosan solution was dissolved in 20%(V/V) acetic acid and then polyethylene oxide powder was added to the above solution after stirred for 1 h. The concentration of chitosan and polyethylene oxide are 3%(W/V) and 3%(W/V). Afterwards, both solutions were transferred into 20 ml syringes which connected to the coaxial electrospinning machines. The flow rate of core-shell solutions is 0.5 ml/h and 0.3 ml/h. The voltage is 25 kV. The distance between tip and collector was 15 cm, and the rotate speed of collector was 300 rpm. Electrospinning was carried out at room temperature, and the humidity is about 40%.

And then the nanofibers were cross-linked by 3%(V/V) glutaraldehyde solution. 6 ml 50%(V/V) glutaraldehyde solution was dissolved in 94 ml ethyl alcohol and stirred. Then electrospun mats were cut into pieces of 1cm $\times$ 1cm and kept in it for 24 h. Finally the core-shell nanofibers were dried by freezer dryer.

3.2. Preparation of ZnO@CS-PEO@GEL nanofibers

A layer of ZnO nanofilms were used as the modified materials that were grown on the surface of the nanofibers by the RF magnetron sputtering method using ZnO target (the diameter 6cm). The back vacuum was 6×10^{-4} Pa and sputtering power was 150 w. The work pressure and the deposition time were 1.0 Pa and 10 min (or 30 min), respectively. The sputtering gas is Ar and the sputtering temperature is 100 °C.

3.3. Characterization Methods

The morphology of the materials was investigated using Field Emission Scanning Electron Microscope (FESEM, Hitachi S-4800, 10 kV). The core-shell structure of materials was investigated using a transmission electron microscope (TEM, JEM 2100, 200 kV, Japan). XRD patterns of materials were obtained by X-ray diffractometer (XRD, D8 Discover, Bruker). The viability of osteoblasts was investigated using an enzyme linked immunosorbent assay reader (ELISA, BioTek Instruments, USA). The MG-63 cells were visualized using a laser scanning confocal microscope (FV1000-IX81, Olympus, Japan), with excitation and emission of green (ex/em 494/517 nm for Calcein AM) and red (ex/em 528/617 nm for EthD-1) fluorescence.

3.4. Cell cytotoxicity assay

The cytotoxicity of the electrospun fibers was evaluated based on a procedure adapted from the ISO 10993-5:2009 standard test method. The viability of cells attached on the electrospun scaffold was quantitatively determined using the Cell Counting Kit8 (CCK-8). For the cytotoxicity assay, the samples which cross linked with glutaraldehyde were tailored and were incubated with complete medium at 37 °C for 24 h with an extraction ratio of 1 cm²/mL. After the incubation period, we collect extraction medium separately. The MG-63 cells were seeded in 96-well plate at a density of 5×10^3 cells per well. After 24 h of incubation, the culture medium was replaced with the prepared extraction medium as mentioned above and was incubated for another 24 h (or 48 h). Then the CCK-8 reagent was added to the wells and was incubated for another 1 h. The optical density (OD) of the samples was read at 450 nm in an enzyme linked immunosorbent assay reader (BioTek). The cell viability was calculated from the following equation:

$$\text{Cell viability} = \frac{OD(\text{sample}) - OD(\text{blank})}{OD(\text{control}) - OD(\text{blank})} \times 100$$

where $OD(\text{sample})$ is the optical density of the extraction medium mentioned above, $OD(\text{blank})$ is the optical density of the growth medium without cells, $OD(\text{control})$ is the optical density of the growth medium with cells.

For qualitative assay, cytotoxicity was also assessed using live/dead viability/cytotoxicity kit. Briefly, the MG-63 cells with the density of 2×10^4 cells per well were seeded on a 48-well plate. After MG-63 cells were cultured for 24 h, the culture medium in each plate was replaced with extraction medium. The cells were cultured for another 24 h and 48 h, respectively. Then, the 48-well plate was washed by PBS and then incubated for 30 min with 250 μ L Calcein AM (2 μ M) and EthD-1 (4 μ M) in the dark. The MG-63 cells were observed by using a confocal microscope (Olympus), with excitation and emission of green (ex/em 494/517 nm for Calcein AM) and red (ex/em 528/617 nm for EthD-1) fluorescence.

4. Results and discussion

4.1. Morphology and microstructure

The images in Fig.1 revealed that electrospun fibers have uniform and smooth surfaces, with a diameter of 100-200nm. And the nano-morphology of electrospun fibrous scaffolds with a large surface-area-to-volume ratio and porous structure can mimic the native extracellular matrix (ECM) which can provide a good place to promote the cells adhesion and cells spreading. The TEM images in Fig.1(a3) showed that core-shell structure of nanofibers have been fabricated.

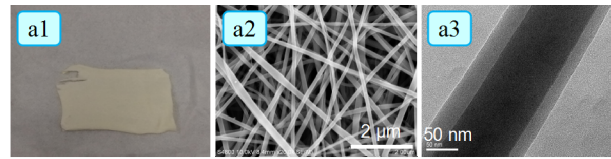


Fig. 1. The morphology and microstructure of CS-PEO@GEL nanofibers. (a1) the morphology of CS-PEO@GEL nanofibers; (a2) SEM image of nanofibers; (a3) TEM image of nanofibers.

The nano-ZnO that was grown on the electrospun fibers can be seen in the Fig.2, the sputtering time was 10 min and 30 min in b1 and b2, respectively. The ZnO particles became bigger when sputtering lasted longer.

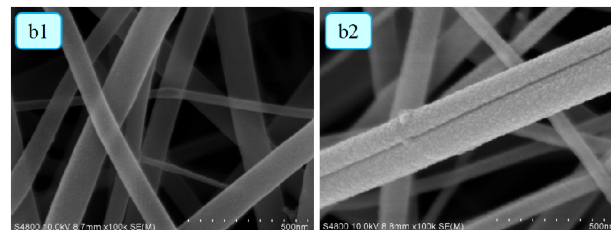


Fig.2. SEM images of ZnO@CS-PEO@GEL nanofibers in different time.(b1)10 min; (b2)30 min.

4.2. XRD analysis

The XRD pattern of ZnO@CS-PEO@GEL nanofibers was displayed in Fig.3. The characteristic peaks of the ZnO@CS-PEO@GEL nanofibers could be indexed to the (100), (002), (101), (102), (110), (103) planes of the zincite hexagonal structure (PDF#36-1451). The XRD pattern indicated that the nanoparticles on the CS-PEO@GEL nanofibers were ZnO and could be ascribed to P63/m space group.

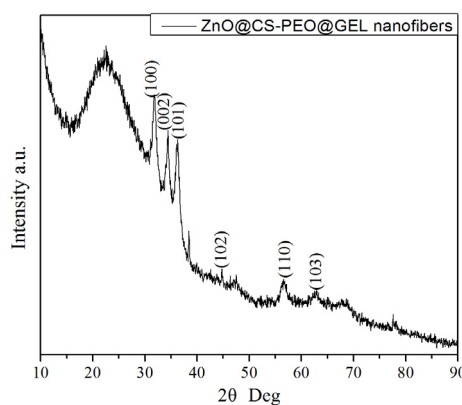


Fig. 3. XRD pattern of ZnO@CS-PEO@GEL nanofibers.

4.3. Cell cytotoxicity assay

As Fig.4 showed, after MG-63 cells were cultured in the extraction medium of CS-PEO@GEL nanofibers, ZnO@CS-PEO nanofibers(10 min)nanofibers, ZnO@CS-PEO nanofibers (30 min)nanofibers for 24 h, the cells viability were 81.24%, 84.11%, 75.62%, respectively. When MG-63 cells were cultured for 48 h, the cell viability were 83.75%, 86.99%, 78.71%, respectively. These were higher than the standard for cytotoxicity, which has been set at 70% according to the ISO standard(ISO 10993-5:2009). The results indicated that the materials with low toxicity were cytocompatible.

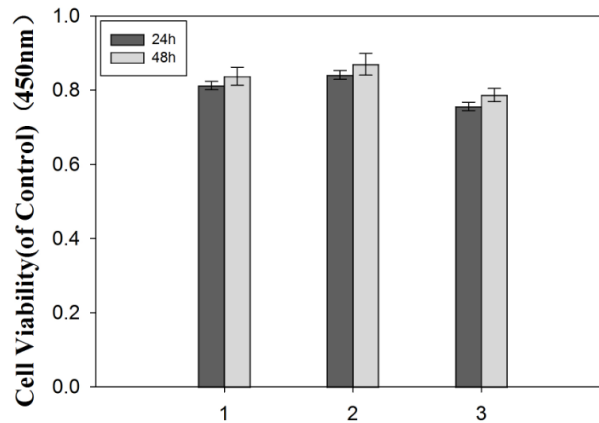


Fig.4. Cytotoxicity of (1)CS-PEO@GEL nanofibers (2)ZnO@CS-PEO@GEL nanofibers(10 min) (3)ZnO@CS-PEO@GEL nanofibers(30 min).

To evaluate cell viability after MG-63 cells were cultured in extraction medium for 24 h and 48 h, cell-seeded plates were viewed by fluorescent microscopy after staining with Calcein-AM/PI Double Stain Kit. As it shown in Fig. 5, 24 h after seeding, the cell number was equal in sample 1 and sample 2, but was lower in sample 3, consistent with the seeding efficacy results. After 48 h, cells were almost covered these images. In addition, the number of cells in samples 1 and 2 were higher than in sample 3, implying less cytocompatibility with higher ZnO. Those results were corresponded to the CCK-8 assay, and showed that all the samples exhibit excellent biocompatibility.

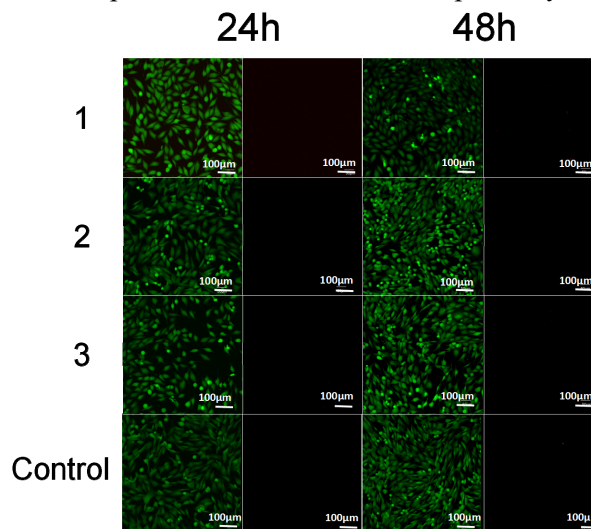


Fig. 5. Fluorescence images of live (green) and dead (red) MG-63 cells cultured on 48-well plates with the extraction medium of (1)CS-PEO@GEL nanofibers (2)ZnO@CS-PEO@GEL nanofibers (10 min) (3)ZnO@CS-PEO@GEL nanofibers(30 min) .

5. Conclusions

In this study, we successfully synthesized biomimetic highly porous ZnO@CS-PEO@GEL scaffolds by combining coaxial electrospinning and RF magnetron sputtering methods and characterized them by different analytical tests. All scaffolds fabricated by this method had cancellous bone like microstructure with open and interconnected pores both on the surface and within the scaffolds. The core-shell structure could be demonstrated in Fig.1. In the SEM images and XRD pattern, suggesting ZnO nano-particle was grown on the surface of CS-PEO@GEL nanofibers. The scaffolds also showed

suitable biocompatibility and cytocompatibility in cell cytotoxicity assay, which influenced significantly by induced ZnO. This study suggests that ZnO@CS-PEO@CEL scaffolds prepared by the combination of RF magnetron sputtering and coaxial electrospinning can be a promising substitute for tissue engineering applications.

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