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Original Research Article

Effect of electrospun SF/CS composite fiber scaffold on cell proliferation and osteogenic differentiation of hBMSCs in vitro

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ABSTRACT: Objective: Using electrospinning to preparesilk fibroin/chitosan (SF/CS) nanofiber membrane scaffolds, and then evaluating its properties and effects on proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) . **Methods:** The regenerated silk fibroin (SF) and chitosan (CS) were dissolved in the mixed solvent system of trifluoroacetic acid and dichloromethane by mass ratio (1 : 0, 1 : 1) . The structure and properties of the electrospun films were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and thermal gravity/differential thermal gravity analysis (TG/DTG). Cells in the experimental group were inoculated on the surface of SF and SF/CS membrane respectively. Cells in the control group were directly inoculated in culture dish. hBMSCs were used in each group to induce osteogenesis. CCK-8 was used to study the growth and proliferation of cells. Energy dispersive spectrometer (EDS) and alizarin red staining (ARS) were used to detect the ability of osteogenesis and mineralization. **Results:** Compared with SF scaffolds, SF/CS scaffolds had more uniform fiber diameter (SEM) and more stable conformation (FTIR) ; TG/DTG results showed that SF scaffolds had more thermal stability. CCK-8 showed that compared with the control group, there was no significant difference in proliferation of hBMSCs between SF and SF/CS groups when co-cultured for 5 and 7 days (P>0.05) . After 21 days of culture, elemental analysis indicated that the SF/CS group had higher calcium content. Compared with the control group and SF group, calcified nodules of hBMSCs in SF/CS group were significantly increased and staining was deep. **Conclusions:** Electrospinning SF/CS nanofibers scaffolds have good biocompatibility and can promote osteogenic differentiation of hBMSCs.

KEYWORDS: Electrospun; Silk fibroin; Chitosan; HBMSCs; Proliferation; Osteogenic differentiation

Bone defects from various causes have always been a clinical challenge. The rapid development of bone tissue engineering in recent years has brought new hope for bone defect repair. Scaffold materials have been studied intensively, but ideal tissue engineering scaffolds with good biocompatibility and osteoinductive and osteogenic properties have not been prepared yet[1]. Electrostatic spinning is one of the techniques to prepare nano-scale to micron-scale fibrous scaffold materials with nanostructures similar to extracellular matrix

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(ECM)[2]. The ECM in the human body consists mainly of two types of extracellular polymers, proteoglycans and fibronectin with a fibril diameter of 50 ~ 150 nm[3]. Silk fibroin (SF) is a natural polymeric fibronectin formed by natural cocoon degumming. In recent years, SF has received attention in the field of tissue engineering because of its good biocompatibility, robust mechanical properties, and easy processing[4–5]. Chitosan (CS) is a product of chitin deacetylation, formed by glucosamine and N-acetylglucosamine units linked by (1–4) glycosidic bonds, with a molecular weight distribution between 300 ~ 1000 ku[6–8].CS has excellent antibacterial activity and biocompatibility. It has been reported in the literature that CS has a beneficial effect on osteoblast differentiation[6]. However, the use of CS alone suffers from high swelling rate and poor cell adhesion[8]. Therefore, in this study, we proposed to examine the structure and properties of SF and CS blended by electrostatic spinning technique, and evaluate the biocompatibility and effect of electrostatic spun SF/CS composite nanoscaffolds on the osteogenic differentiation ability of human bone marrow mesenchymal stem cells (hBMSCs) by in vitro cellular experiments.

1 Materials and Methods

1.1 Main materials and instruments

Alveolar bone fragments were taken from the Department of Oral Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China for implant surgery. The volunteers were physically fit, free of systemic diseases, and all were males of the same age. The donors themselves had signed an informed consent form, and the experimental protocol was approved by the medical ethics committee of the hospital.

Silkworm cocoons (Huzhou Xintiansi Biotechnology Co., Ltd.), CS, dichloromethane, trifluoroacetic acid (Shanghai Aladdin Reagent Co., Ltd.), α-MEM medium, fetal bovine serum, 0.25% trypsin-EDTA Cyan/chain double antibodies (Gibco, USA), osteogenic induction medium (Cyagen, USA), CCK-8 kit (Shanghai Biyuntian Biotechnology Co., Ltd.), phosphate buffer (PBS) (Hyclone, USA), alizarin red staining (ARS) solution (Cyagen, USA), dialysis bags (Union Carbide Corporation, USA). High voltage DC power supply (Tianjin Dongwen High Voltage Power Supply Co., Ltd.), Micro syringe pump (Zhejiang Smith Medical Instruments Co., Ltd.), Ultra clean bench, constant temperature incubator, centrifuge (MD Spectra Max, USA), Inverted microscope (Leica, Germany), Spectra Max 190 enzyme marker (Molecular Device. USA), S-4800 field emission scanning electron microscopy (SEM) (Hitachi, Japan), Discovery TGA 55 thermogravimetric analyzer (TA, USA), Frontier FT-IR/NIR/FIR spectrometer (Bruker, Germany).

1.2 Experimental method

1.2.1 Preparation of regenerated SF

The cocoons were boiled in aqueous Na_2CO_3 solution with a mass concentration of 0.5% for 30 min and rinsed in deionized water. Repeat 3 times, vacuum dry for 24 h and then dissolve in $CaCl_2$/CH_3 CH_2 OH/H_2O molar ratio of 1 : 2 : 8 solvent, dissolve in a bath with a mass ratio of 1: 10 at a constant temperature of 70°C for 1 h. Place in a dialysis bag (MW 3500), dialyze with deionized water, change water every 8 h, and freeze dry after 3 d to form regenerated SF.

1.2.2 Preparation of electrostatic spun wire scaffolds

The solvent of electrospinning solution was trifluoroacetic acid/dichloromethane (mass ratio 7 : 3). In this solvent mixture, 10% SF electrospinning solution by mass concentration and 4% CS solution by mass concentration were prepared first, and then the blended solution was formed with SF/CS mass ratio 1 : 1 to obtain electrospinning SF solution and SF/CS solution, respectively. The two groups of solutions were electrospun with the following spinning parameters: voltage 20 kV, distance from needle tip to collection plate 18 cm, syringe pump pushing speed 0.8 mL/h. The collected two groups of electrospun films were immersed in a mixture of 7% ammonia and 75%

ethanol for 30 min and dried at room temperature.

1.2.3 Stent measurement and characterization

(1) The fiber morphology of SF and SF/CS electrospun membrane scaffolds were observed using SEM, and Image J software randomly measured at least 100 fibers from 10 images to calculate the diameter. (2) Fourier transform infrared spectrometer (FTIR) measurements were performed on two groups of electrospun membrane scaffolds with a resolution of 2 cm-1 and a wave number range of 800 ~ 1800 cm-1. (3) Thermal gravity/differential thermal gravity anal-ysis (TG/DTG) analysis was performed on two groups of electrospun membrane scaffolds at 25 ~ 800 °C with a temperature rise rate of 10 °C/min.

1.2.4 Isolation and culture of hBMSCs

Immediately after extraction of osteopontic bone from implant surgery under aseptic conditions, the jaws were rinsed 3 times with PBS on the ultra-clean bench, centrifuged at 1000 r/min for 5 min, resuspended in α-MEM medium with 10% FBS, inoculated in a medium dish and then cultured at 37°C in a 5% volume fraction CO2 incubator. When the primary cells grew to 80%~90% of the bottom of the bottle, they were passaged at a ratio of 1 : 3, and the 2nd ~ 3rd generation was used for subsequent experiments[9].

1.2.5 Cell inoculation

SF and SF/CS electrospun membranes were cut into 1.5 cm diameter circles and sterilized overnight in 75% ethanol. The samples were placed in 24-well plates with 5 sub-wells per group, and each well was pre-cultured with 1 mL of complete medium (90% α-MEM medium, 10% FBS, 1% double antibody) for 1 d. The 2nd ~ 3rd generation hBMSCs were inoculated on SF and SF/CS electrospun membranes in 24-well plates at a density of 5 × 103 cells/cm^2[10]. The control group was not placed on electrospun membrane only equal amount of cells were added and the fluid was changed every 3 d.

1.2.6 CCK-8 detection

After cell inoculation, three samples were selected from each group on days 1, 3, 5 and 7. The samples were placed in a new 24-well plate, 50 μL of CCK-8 detection reagent and 500 μL of complete medium were added to each well, incubated at 37℃ for 4 h, and 100 μL of each well was pipetted into a 96-well plate, and the wavelength of the enzyme standard was 450 The absorbance (OD) values were measured at 450 nm. The experiment was repeated three times.

1.2.7 Observation of cell adhesion morphology

After 3 d of inoculation and culture according to method 1.2.5, the original culture medium was changed into osteoinductive medium, and the medium was changed once every 2 d. When the culture reached day 21, 3 samples were selected from each of SF and SF/CS group samples, rinsed 3 times with PBS, fixed with 2.5% glutaraldehyde for 2.5 h, dehydrated with alcohol gradient, and completely dried, the cell morphology on the surface of each group specimen was observed under SEM, and the atomic percentages of elements on the surface of membrane fibers were determined using the SEM self-contained energy spectrometer.

1.2.8 ARS staining

Cells from each group on the 7th, 14th, 21st and 28th days of osteogenic induction were taken, stained with alizarin red and observed under an inverted microscope for mineralized nodule formation.

1.3 Statistical analysis

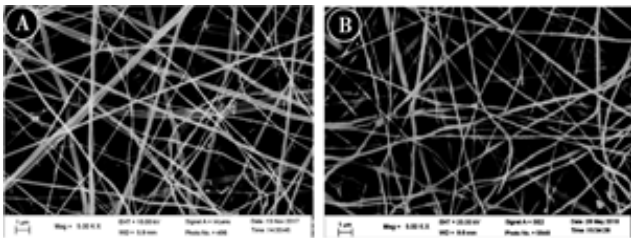
SPSS 17.0 software was applied for statistical analysis, and LSD test after one-way ANOVA was used, and $P<0.05$ was considered a statistically significant difference.

2 Results

2.1 Microscopic morphology of electrospun membrane support

SEM results showed that both SF and SF/CS electrospun membrane scaffolds could form porous structures with flat surfaces and scattered dis-

ordered fibers. Compared with SF scaffolds, SF/CS scaffolds were smoother without beads and had more uniform diameters. image J software showed that the average diameter of SF scaffolds was (348 ± 125) nm, and the fiber diameter of SF/CS group was (317 ± 96) nm (**Figure 1**).

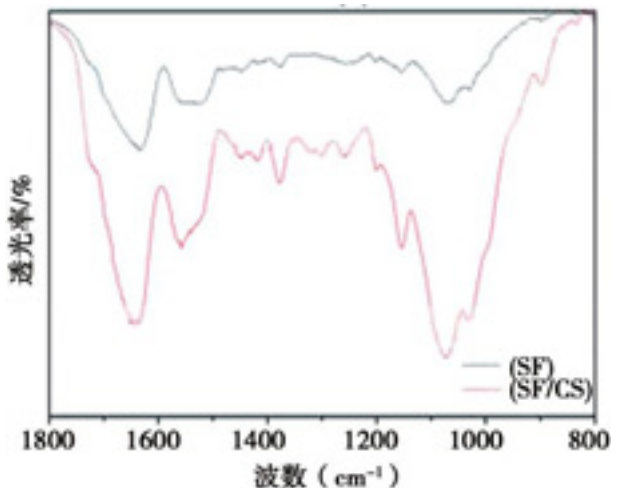


A: SF film; B: SF/CS film.

Figure 1 SEM observation of the microstructure of electrospun membrane scaffolds

2.2 FTIR analysis results

FTIR showed that the conformational changes from irregular nematic clusters and α-helical to β-fold after the addition of CS to the SF blend; the characteristic transmission peaks of the original SF group were located at 1640 cm-1, 1527 cm-1 and 1241 cm-1, and the transmission peaks moved to 1665 cm-1 after the addition of CS (**Figure 2**).

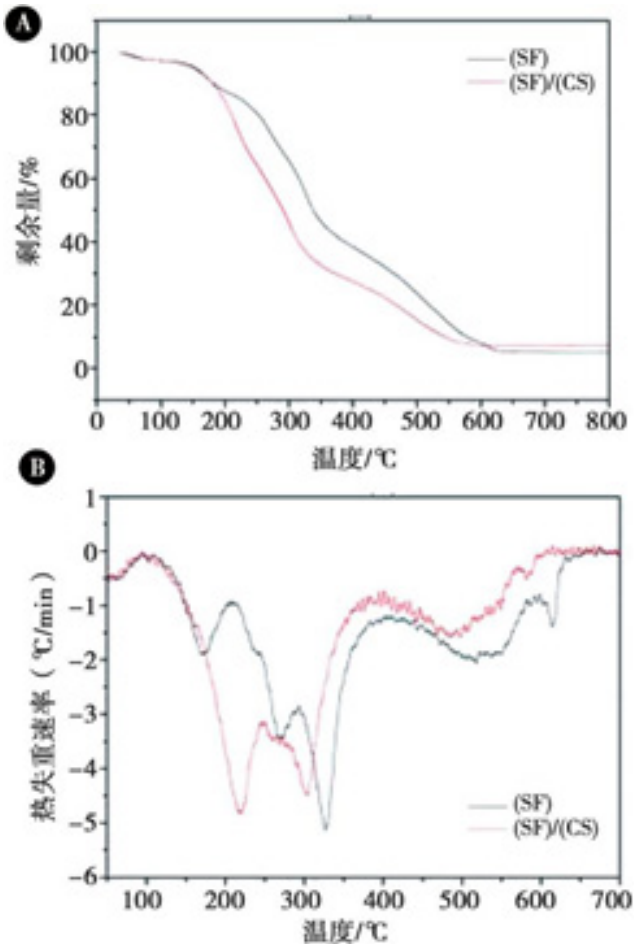


Light transmittance/%
Wave Number

Figure 2 FTIR analysis of electrospun membrane scaffolds

2.3 TG/DTG assay results

The TG results showed that the weight loss trends were basically the same for both groups of materials, but the SF stent had more residuals and better thermal stability at the same temperature. The DTG results showed that the peak weight loss started at 210°C for the SF/CS stent and at 330°C for the SF stent (**Figure 3**).



A: TG analysis profile; B: DTG analysis profile.

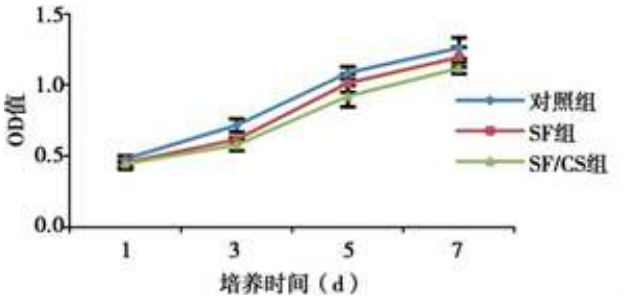
Remaining amount/%
Thermal weight loss rate
Temperature(°C)

Figure 3 TG/DTG analysis of electrospun membrane scaffolds

2.4 CCK-8 assay results

The hBMSCs were inoculated on two electrospun membrane scaffolds and subjected to CCK-8 assay, and the results showed that: the cells had significant proliferative activity on the electrospun membrane scaffolds in both groups, and the OD values increased with time, with the most significant proliferation at days 3 ~ 5, and the SF/CS group differed from the control group at day 3 ($P<0.05$). There was no statistically significant difference between the OD values of the two experi-

mental groups at days 5 and 7 compared with the control group (P>0.05) (**Figure 4**).



Incubation time(d)
OD value

Control group
SF Group
SF/CS group

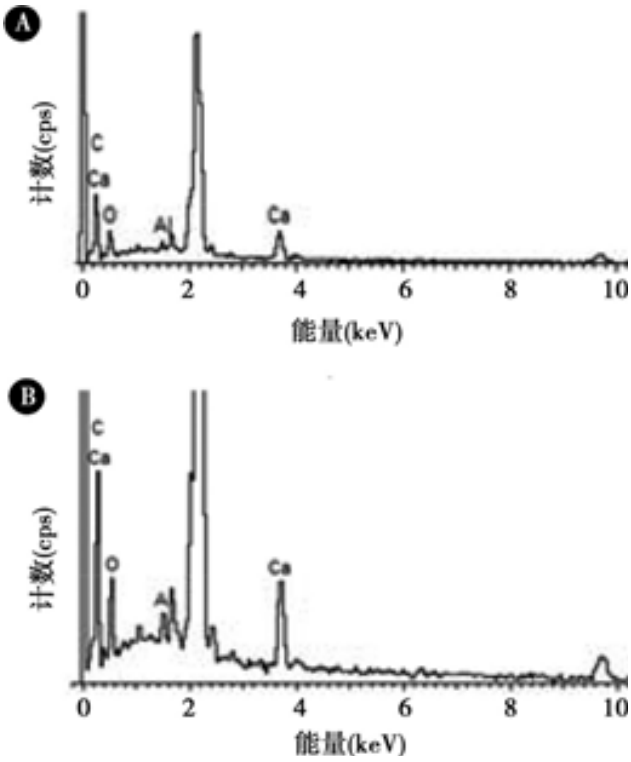
Figure 4 Effect of electrospun membrane scaffolds on proliferation of hBMSCs

2.5 Surface element analysis of hBMSCs-electrospun membrane scaffold after osteogenesis induction

The hBMSCs were inoculated in two groups of electrospun membrane scaffolds, and after 21 d of osteogenic induction culture, the elemental composition of the membrane fiber surface was determined by energy spectrometry, and **Figure 5** showed that the surface of both groups of membrane scaffolds contained carbon, oxygen, aluminum and calcium elements. The quantitative analysis of elements in Table 1 showed that the SF/CS group contained more calcium elements than the SF group, suggesting that CS may have a more positive effect on the promotion of osteogenic differentiation of hBMSCs.

2.6 Morphology of hBMSCs cultured on electrospun membrane membrane supports

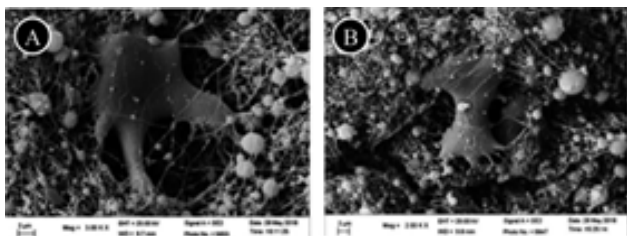
The fibrous membrane could be observed under SEM to provide a three-dimensional meshwork for the adhesion of hBMSCs, which facilitated the growth of cell pseudopods reaching in. At 21 d of osteogenic induction culture, mineralized granules were seen deposited around the cytosol of both groups of hBMSCs, with more fine tentacles and more dense mineralized granules in the SF/CS group compared to the SF group (Figure 6).



Counting (cps)
Energy

A: SF film; B: SF/CS film.

Figure 5 Surface energy spectra of cultured hBMSCs on membrane scaffolds



A: SF group; B: SF/CS group.

Figure 6 Morphology of hBMSCs cultured on membrane scaffolds

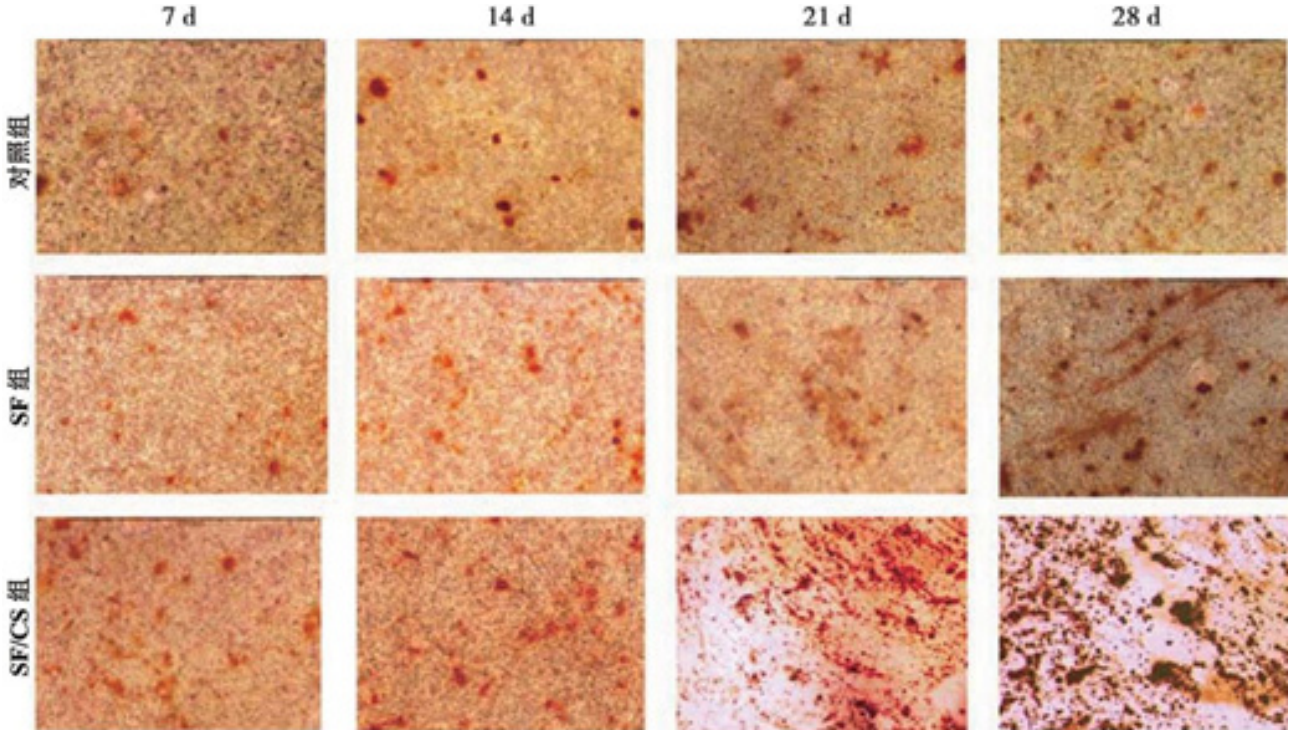
2.7 ARS experimental results

ARS staining showed that after osteogenic induction culture of hBMSCs, the staining of the experimental group inoculated with SF and SF/CS electrospun membrane scaffolds deepened with time; the differences between the experimental and control groups were not significant at days 7 and 14 of osteogenic induction; at day 21, more calcium nodules appeared in the SF/CS group,

significantly more than the SF and control groups; at day 28 At day 28, the SF/CS group showed more mineralized nodules and darker staining than before (**Figure 7**).

Table 1 Quantitative analysis of surface elements in cultured hBMSCs on membrane scaffolds (%)

Element	SF Group		SF/CS Group	
	Weight percentage	Percentage of atoms	Weight percentage	Percentage of atoms
C	40.41	53.53	32.49	46.68
O	37.21	37.00	35.89	38.58
Al	3.03	1.79	5.51	3.51
Ca	19.34	7.68	26.11	11.23



Control group
SF Group
SF/CS group

Figure 7 Mineralization of hBMSCs cultured on membrane scaffolds

3 Discussion

Scaffold material is a central element of bone tissue engineering, not only as a carrier of seed cells and growth factors, but also to provide a suitable microenvironment for cell growth and adhesion as well as to provide support for new bone growth[11]. How to prepare the ideal scaffold has been a hot research problem in bone tissue engineering. Electrostatic spinning technology has received much attention as a method to fabricate tissue-engineered grafts structurally similar to ECM. The materials currently used in electrospinning are mostly synthetic polymers such as polycaprolactone (PCL), poly (lactic-coglycolic acid) (PLGA), and poly-L-lactide (PLLA), which have excellent physicochemical properties. However, due to the lack of cell recognition sites, synthetic polymer scaffolds have poor cell affinity and cell interactions.CS and SF are both natural polymer materials, and related studies have reported that CS has good biocompatibility, antibacterial effect,

and cell adhesion[7], but CS scaffolds still have poor mechanical properties However, CS scaffolds still have disadvantages such as poor mechanical properties, low bioresponsiveness, etc. In tissue engineering, SF/CS composite scaffolds have been studied because SF and CS can complement each other's advantages[11–12]. However, studies on osteogenic differentiation of electrospun SF/CS composite scaffolds are still relatively few.

The SEM of this experiment showed that both electrospun membranes were three-dimensional three-dimensional mesh structure with multiple pores, which not only provided space for cells to migrate inward and promoted cell pseudopods to adhere and grow, but also facilitated the entry of nutrients and the discharge of metabolites. CCK-8 showed that hBMSCs could proliferate stably in both experimental groups, with no toxic side effects on cells and good biocompatibility. This result is similar to the results of Chen et al[13].

The first peak is probably due to the decomposition of CS, which corresponds to the decomposition of CF3COO-ammonium salt, while the second peak is due to the decomposition of SF, which causes structural changes in the kinetics of thermal decomposition after the rate of weight loss increases significantly. The TG/DTG results showed that the weight loss trends of the two experimental groups were basically the same, and the SF group was more thermally stable. This thermal analysis technique also verifies the stable and plastic properties of SF, which is beneficial for its process development and performance optimization in more areas of tissue engineering in the future.

One of the evidences of the relatively high osteogenic capacity of hBMSCs[14] and their differentiation towards the osteogenic spectrum is their ability to mineralize inorganic phosphate at mid-differentiation, which can be monitored in the cell-secreted ECM for inorganic mineral deposition. When hBMSCs differentiate on nanofibers, anionic matrix molecules will take up Ca^{2+}, followed by PO^{3-}, leading to calcification through nucleation and growth. After 21 d of osteogenesis induction, cells adhering to the membrane surface and pseudopods penetrating deep into the fiber pores with deposition of surrounding mineralized particles were seen under SEM in both experimental groups. Surface elemental analysis showed higher calcium content in the SF/CS group compared to the SF group. arS results also showed that the staining intensity and the amount of calcium nodules in the SF/CS scaffolds were significantly higher than those in the SF group, which corroborated with the findings of Lai et al[16], further demonstrating that CS has a facilitating effect on the osteogenic differentiation process of hBMSCs.

In conclusion, the biocompatibility of the electrospun SF/CS scaffold prepared in this experiment is good, and CS has a significant effect on inducing osteogenic differentiation of hBMSCs, suggesting the possibility of inducing bone tissue regeneration by electrospun SF/CS membrane scaffold. However, the current experiments only suggest that CS can promote osteogenic differentiation from the cellular level, and further studies on the mechanism of how CS promotes osteogenic differentiation are needed.

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