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Evaluation of novel alginate dialdehyde cross-linked chitosan/calcium polyphosphate composite scaffolds for meniscus tissue engineering

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ABSTRACT

Chitosan is a transformed deacetylated polysaccharide from chitin with a variety of applications in the biomedical field. However, chitosan scaffolds have poor mechanical properties. Calcium polyphosphate was added into the chitosan solution before cross-linking with alginate dialdehyde, in order to improve the properties of chitosan. With the increase of calcium polyphosphate, the compressive strength was improved and the degradation rate of composites decreased. The cytotoxicity of chitosan/calcium polyphosphate composite scaffolds was investigated by using meniscus cartilage cells. The adhesion and proliferation of meniscus cartilage cells were promoted by the novel composite scaffolds. All the results indicated that chitosan/calcium polyphosphate composite scaffold may be a potential candidate for meniscus tissue engineering.

1. Introduction

Tissue engineering applies methods from materials engineering and life sciences to create artificial constructs for regeneration of new tissues (Williams, 2004). The seeding cell, scaffold material, growth factor and tissue construction are the four factors in tissue engineering, but also the main scientific problems in tissue engineering research (Leor, Amsalem, & Cohen, 2005). There are three systems adopted by tissue engineering: one common approach is to grow specific cells on a three-dimensional (3D) scaffold which has good biocompatibility and biodegradation under controlled culture conditions. The scaffold with cells is implanted into the body subsequently. Another approach is the implanted cells grow to be some microstructures by bioprocess. The last one is to implant scaffolds directly in vivo and the cells grow in the scaffolds. After proliferation and differentiation, the tissue is formed and conformed to the surrounding tissues (Drotleff et al., 2004; Mano, Sousa, Boesel, & Neves, 2004). The advantages of this approach are the reduced number of needed operations, and shortened the recovery time for the patient (Rezwan, Chen, Blaker, & Boccaccini, 2006).

Meniscus is an important structural and functional part of knee, which is the biggest and most complicated joint in human body. It has the functions of absorbing shakes, transferring loads, stabilifying the joint and so on. It is attached to the transverse ligaments, the joint capsule, the medial collateral ligament (medially) and the meniscus–femoral ligament (laterally) (Sweigart & Athanasiou, 2001). In these years, meniscus injury is one common disease, and the traditional total or subtotal meniscectomy will cause many disadvantages of the structure and function of knee, such as large irreparable tears (Verdonk, Van Laer, & Verdonk, 2008), and the sewing repair is limited because of the characteristics of the blood supply and mechanics of meniscus. Although the meniscus allograft transplant is an effective method in clinic at present, it has the defects on the lack of donor, the faultiness of preservation, immune rejection and the side-effect dimness. So the researchers focus on the meniscus regeneration by tissue engineering principles and techniques. The ideal meniscus tissue engineering materials have good biocompatibility, controlled biodegradation, enough mechanical property, specifically 3D porous structure and good material–cell interface (Buma, Ramrattan, van Tienen, & Veth, 2004; Chung & Burdick, 2008).

Chitosan (CS), a co-polymer of N-acetyl-glucosamine and N-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer (Khor & Lim, 2003), is a transformed polysaccharide obtained by the deacetylation of natural chitin, which is one of the important natural polymers in the shells of crustaceans and the cytoderm of many fungi (Liu & Huang, 2008). It has a variety of applications in the biomedical field such as bone reconstruction, cell encapsulation, drug delivery, and tissue engineering (Park, Gabrielson, Pack, Jamison, & Wagoner Johnson, 2009). The electrostatic interactions between CS and anionic glycosaminoglycans (GAG), porteoglycans or other negatively charged molecules are due to

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the cationic nature of CS. And GAG link a large number of cytokines/growth factors, which make it become an important component of proteoglycan and typically presented in the cartilaginous ECM (Wendt, Jakob, & Martin, 2005). So CS has been used as a kind of materials in cartilage engineering due to these properties (Chen & Cheng, 2008; Rotter, Bücheler, Haisch, Wollenberg, & Lang, 2007; Xia et al., 2004).

Calcium polyphosphate (CPP) is a novel inorganic polymer biomaterial for bone substitution in recent years owing to its excellent biocompatibility and osteoconductive property, with low Ca:P ratio of 0.5 and a linear P—O—P linkage structure (Kandel et al., 2006; Pilliar, Filiaggi, Wells, Grynpas, & Kandel, 2001; Qiu, Zhao, Wan, Zhao, & Chen, 2006). With different sintered temperatures, CPP shows varied degradation rates which could be used to control the degradation of this material (Wang, Chen, Liu, & Rüssel, 2008). It was demonstrated that CPP showed good property in vitro along with attached marrow-derived mesenchymal cells for transplantation into a site for bone regeneration in vivo (Lee et al., 2001). And in the recent years, some researchers have found that the chondrocytes would not get calcification in the porous material, but showed good appearance and cellularity (Gan, Tse, Pilliar, & Kandel, 2007).

However, the CS scaffold materials are poor in mechanical properties, especially the compressive strength, and CPP has good strength but fragile. So in this study, we investigated the structure, the mechanical, degradation properties and the biocompatibility of CS blended with different contents of CPP to form 3D composite scaffolds. We assumed that the addition of CPP can improve the properties of CS scaffold and give us a novel method to synthesize a new kind material of cartilaginous engineering.

2. Experiment

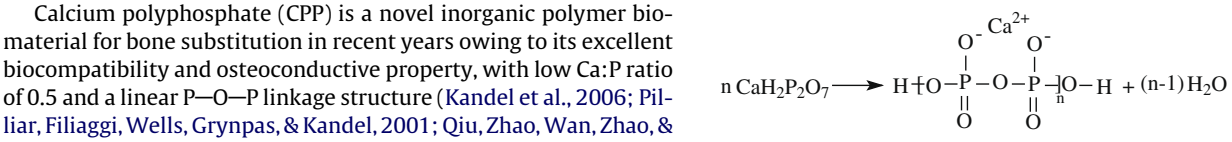
2.1. Materials

CS (with a MW of about 4.2×10^5 and a degree of deacetylation of 85%). Sodium alginate (low viscosity grade, 1000 cps at 25 °C). Diphenyl tetrazolium bromide (MTT) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS, pH 7.2, 0.1 mol/L) was prepared by dissolving 15.6 g of disodium hydrogen phosphate dodecahydrate, 2.4 g of potassium dihydrogen phosphate, 2 g of potassium chloride and 80 g of sodium chloride in 1 L distilled water. All other chemicals of the analytical reagent were used and were obtained from Kelong Co. (Chengdu, China).

2.2. Synthesis CPP

The process of producing CPP powders was followed as Kai Qiu made. Calcium carbonate was slowly added into the phosphoric acid (15%) with Ca/P 1:2. After being evaporated in vacuum and washed by ethanol, the powders were calcined at 500 °C for 10 h and heated to 1200 °C resulting to melt. The following equations were the condensation reaction:

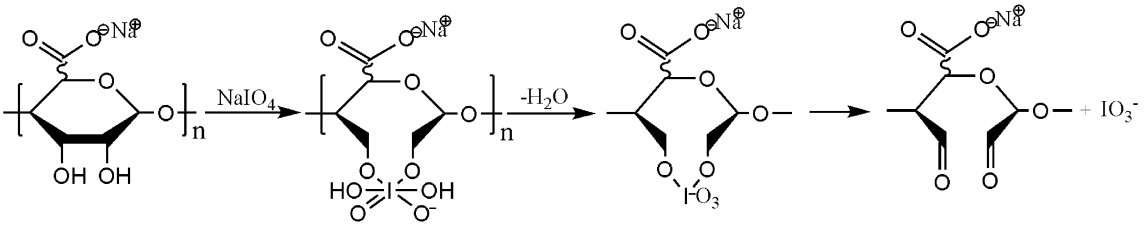
$$Ca(H_2PO_4)_2 \cdot xH_2O \rightarrow Ca(H_2PO_4)_2 + xH_2O$$
$$Ca(H_2PO_4)_2 \rightarrow CaH_2P_2O_7 + H_2O$$



The molten CPP was poured directly into the ice to avoid crystallization during cooling. The CPP powders were produced by ball milling and followed by screening.

2.3. Periodate oxidation of sodium alginate

Ten gram sodium alginate were dissolved in 400 ml distilled water, mixed with 10.8 g of sodium periodate and magnetic stirred in dark at room temperature for 24 h to obtain the product. All the processes were followed as in the following equation:



(1)

The solution was neutralized by 20 ml of ethylene glycol under dark for 15 min. The alginate dialdehyde (ADA) was purified by precipitation with the addition of 5 g sodium chloride and 600 ml ethanol. The polymer was dissolved in 200 ml distilled water again and re-precipitated by the addition of 400 ml ethanol. The process was repeated three times. The precipitates were pump filtrated and dried at room temperature under vacuum.

2.4. Synthesis of porous scaffolds

To prepare the porous CS scaffolds, CS powders were first added to form 5% (w/v) CS solution at 60 °C. Calcium polyphosphate with different amounts, were dispersed in ethanol, added into CS solution and stirred at 60 °C for 30 min to make the composite dispersed uniformly. To investigate the influence of different amounts of CPP on the structure and properties of the scaffolds, four ratios (CPP%: 0%, 30%, 50% and 70%) were chosen. Ten percentage (w/v) ADA in PBS was added into composite solution and stirred for 5 min. The sol was then poured into 24-well plate, frozen for 24 h at −20 °C and vacuum freeze-dried for 24 h.

2.5. FTIR spectroscopy

Approximately 1 mg powdered sample was commixed with 45 mg KBr powder and compressed into a flask. FTIR spectroscopy was performed on a Nicolet 560 spectroscopy (Wicolet Inc., USA).

2.6. Mechanical testing

Cylindrical specimens (diameter 15 mm, length 10 mm) were prepared for compress test. The compressive strength was tested at a strain speed of 2.0 mm/min on an electronic mechanical testing machine (AG-10TA, Shimazu, Japan). Because the specimen was an elastomer, the maximum was recorded before the 25% deformation of the specimen during compression.

2.7. Scaffolds degradation

In vitro degradation studies, materials were evaluated by weight-loss rate over time. Six degradation periods (1, 2, 3, 4, 5 and 6 week) were selected. The scaffolds were incubated in 15 ml Simulated Body Fluids (SBF) which were prepared as Ohtsuki did (Ohtsuki, Kushitani, Kokubo, Kotani, & Yamamuro, 1991) in sealed polyethene vials. At each time, the scaffolds were rinsed in distilled water and dried in vacuum at 50 °C before weighting. The percentage of weight loss was determined by using the following equation:

$$\text{Weight loss } (\%) = \frac{W_0 - W_t}{W_0} \times 100\% \quad (2)$$

where W_0 is the initial weight of the scaffold and W_t is the weight of the dried scaffold at time t

2.8. Meniscus cell isolation from rabbit knee meniscus

Meniscus cells were harvested from the 1-week New Zealand rabbits' knee meniscus of. After euthanizing rabbits, the tissues surrounded synovial membranes were removed. The meniscus was washed in PBS with 10% (v/v) gentamicin before cutting. After trypsinizing with 0.25% trypsin and 0.2% type II collagenase at 37 °C, cell suspensions were centrifuged at 1.2 kpm for 5 min. The cells were re-suspended in culture medium supplemented with 10% fetal bovine serum (FBS, Gibco), and plated in flasks at 37 °C in 5% CO_2.

2.9. Biocompatibility of scaffolds in vitro

The biocompatibility of the composite was demonstrated by two categories in vitro cytotoxicity assays: extract and direct contact tests. The extract assay is based on the fact that metabolically active cells interact with a tetrasolium salt in a water soluble yellow dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) reagent to produce a soluble formazan dye with the wavelength of 490 nm. The intensity of the absorbance is proportional to the number of viable cells (Neamnark, Sanchavanakit, Pavasant, Rujiravanit, & Supaphol, 2008). The scaffolds were sterilized with a 75% ethanol solution. After washed in PBS, all the scaffolds were placed in the complete culture medium for 24 h at 37 °C in 5% CO_2. The meniscus cells were seeded into a 96-well culture plate with a cell density of 4×10^3 cells/well (1×10^5 cells cm^{-2}), and the plate was incubated at 37 °C in 5% CO_2 for 24 h. The extracts of scaffolds were placed in contact with the meniscus cells in 96-well plate for 24, 72, 120 and 168 h. As a control group, cells were treated with the same amount of medium. The medium were changed each 2 days. At each indicated time, 20 μl of MTT solution (5 mg/ml) was added into each well. After 4 h incubation, the medium was removed completely, and 100 μl per well of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured by an enzyme-immunoassay at 492 nm.

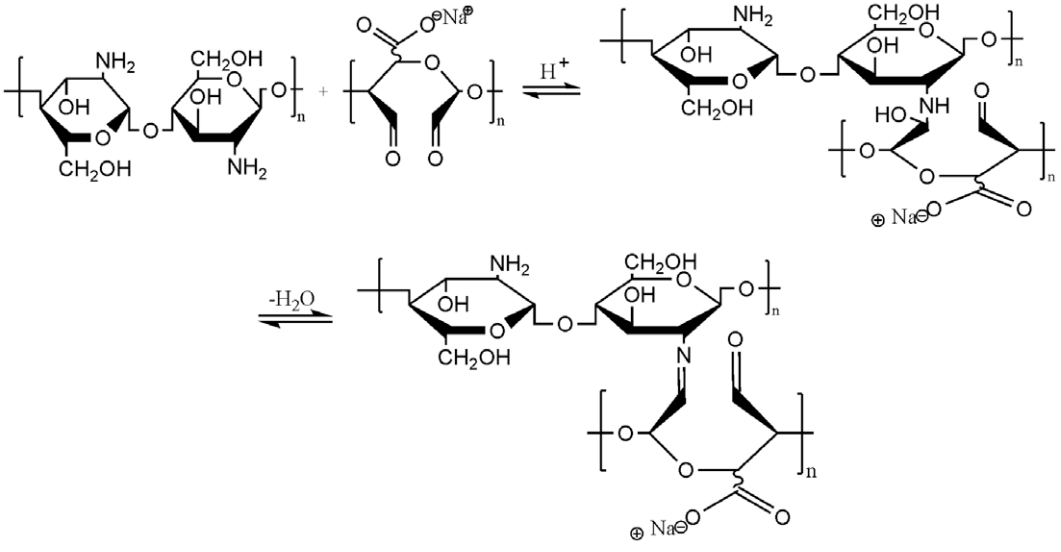
In the direct contact assay, the scaffolds were seeded with meniscus cells (1×10^5 cells cm^{-2}) for 7 days, and the culture medium were changed every 2 days. Prior to seeding, the scaffolds were immersed in culture medium for 7 days. After each indicated time, the scaffolds were fixed with glutaraldehyde, dehydrated with ethanol of various concentrations, critical point dried with isopentyl acetate and gold sputtered for SEM.

3. Results and discussion

3.1. Preparation of composite scaffolds

There are so many aldehydes in the chain of alginate dialdehyde, and the carbon in the carbonyl has the sp^2 hybrid orbit which makes it positive and sensitive to the nucleophile (Solomons, 1980). Moreover, there are some negative charges in the carbonyl oxygen. It is very easy for it to react with some polarity groups. On the other hand, there are some unshared electrons in the amido of chitosan which have nucleophilicity.

These amidos bared on the molecular chains reacted with the positive carbonyl carbon as the following equation, to form the Schiff's bonding which lead to the formation of gel network structure.



(3)

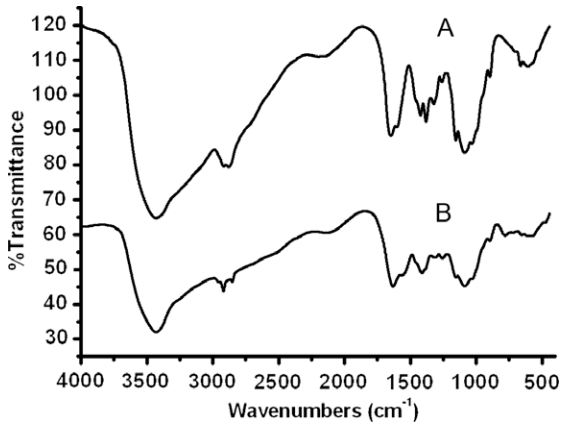


Fig. 1. FTIR spectra of (A) chitosan and (B) cross-linked chitosan.

In order to elucidate the structure of CS and the cross-linked CS, FTIR spectra were taken. Fig. 1 showed the FTIR spectra of CS before and after cross-linking with ADA. There was a strong absorption peak at 3432 cm^{-1} in each spectrum, which was due to the O—H stretching vibration of surface —OH groups in the materials, and the C—H stretch was appeared between 2990 and 2879 cm^{-1}. Compared these two spectrums, the intensity of the peak at 1410 cm^{-1} after cross-linking was increased substantially, which made the peaks at 1422 and 1381 cm^{-1} become one peak, due to the formation of Schiff's base (characteristic of the C=N). And the peak at 2920 cm^{-1} in spectrum B of cross-linked CS showed the C—H in Schiff's base stretching. All these changes between spectrum A and B represented that the aldehyde groups in ADA were combined with amine groups in CS by covalent bond, and formed a 3D cross-linked network structure.

3.2. Measurement of scaffolds' mechanical properties

Fig. 2 shows the compressive strength of the samples made at CPP% of 0%, 30%, 50%, and 70%. The compressive strengths of composites were significantly increased for the modifications of CPP (CPP%: 30%: 0.209 MPa, 50%: 0.310 MPa, 70%: 0.358 MPa), compared with the CS sample whose compressive strength was 0.181 MPa. It was indicated that the compressive strength was improved with the increase of CPP content. Zhang et al. have found (Zhang & Zhang, 2001) that the relationship between compressive strength and Young modulus of inorganic/organic composites like this sample followed the formula: E, $\sigma \propto \rho^n$, where n is the density coefficient which is greater than 1 generally. So with the density increased, the compressive strength of composite samples was improved. Moreover, according to the composite theory, the mechanical properties were better when the fillings in the composite system dispersed more equably. With the content of CPP increased, the probability of CPP existing in anywhere of CS fluid will increase, and the dispersion was more equable correspondingly. In the dispersion strengthening composite, the loads were mainly carried by base. The dispersion particles interrupted the dislocation movement, and the destructive cracks could not be formed to some extent. The yield strength of composite was shown as the following equation:

$$\sigma_y = \frac{G_m b}{\left(\frac{2d^2}{3V_p}\right)^{\frac{1}{2}}(1 - V_p)} \tag{4}$$

where G_m is the shear modulus, b is Burgers vector, d is the average diameter of particle, and V_p is the particle volume fraction in whole composite. So with content of CPP increased, V_p became bigger, σ_y of composite became bigger accordingly, and the mechanical property become better.

3.3. Degradation rate of scaffolds with different CPP contents

Fig. 3 displayed the weight variation of the CS/CPP composite scaffolds during the degradation period. These scaffolds' weight losses were increased slowly during the whole period. Moreover, the degradation rate was slowed when the CPP content increased, such as at the 35th day, the weight loss of the scaffold with 10% CPP was twice as the one with 50% CPP. The content of CPP was increased in the composite scaffolds lead to the content reduce of CS. For the composite which was made by inorganics and organic polymer, the polymer degraded first and it would play a leading role during the degradation period (Ding, 2007). So the degradation rate could be controlled to a fit one when the inorganics/organic polymer ratio was changed.

3.4. Bioactivity and toxicity

The MTT assay is used to evaluate the cytotoxicity of the materials. The relative absorbance of the meniscus cells in CS and CS/CPP composite scaffolds were shown in Fig. 4. It could be seen that all the relative absorbances were higher than 80%, which meant the scaffolds had no negative effect on the proliferation of meniscus cells. Moreover, the relative absorbance for 70% was signifi-

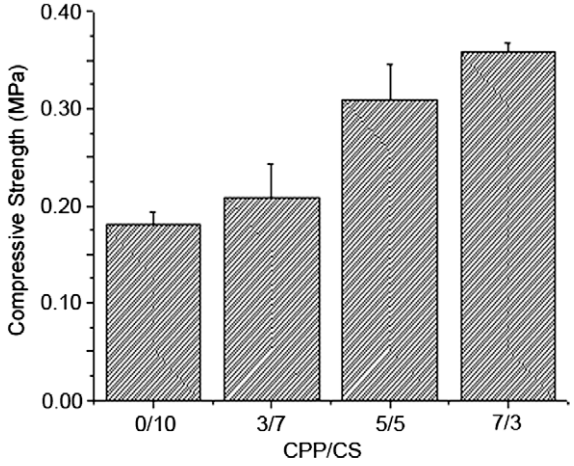


Fig. 2. Compressive strength of CPP/CS/ADA scaffolds with different CPP content.

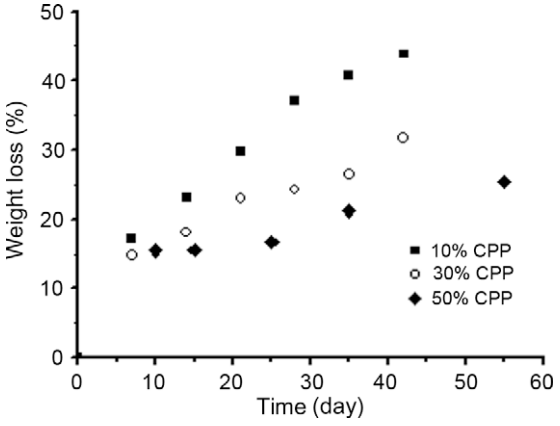


Fig. 3. Weight loss of CPP/CS/ADA scaffolds with different CPP content.

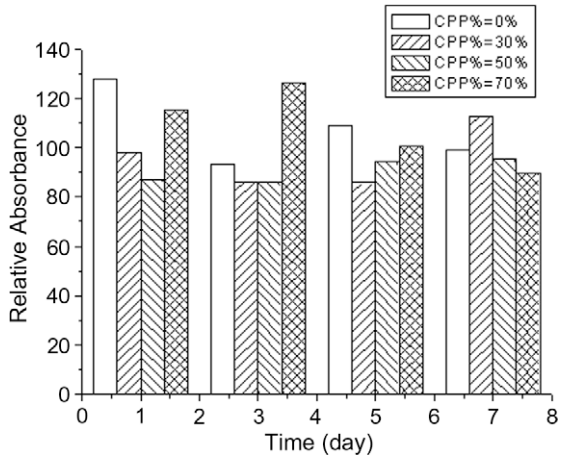


Fig. 4. MTT assay for proliferation of meniscus cells cultured on chitosan and CS/CPP composite scaffolds at different incubation periods.

cantly higher than that for CS after 3 days culture ($p < 0.05$), which indicated that as CPP degraded in the fluids and the chain disconnected, there were some energies released because the structure of CPP was similar to the structure of ATP (about 26 kcal/mol) (Van Wazer, 1962), and the energy release in the polymer hydrolysis could improve the cells growth. However as time went on, the ions concentration increased which restrained the growth of cells.

Cell adhesion and morphology investigated by SEM in the direct contact assay demonstrated that CS and CS/CPP composite scaffolds supported meniscus cell adhesion. After 7 days co-culture with the scaffolds, meniscus cells were showed an expanding morphology on the surfaces as Fig. 5 shown. It was indicated the scaffolds with various content of CPP were non-cytotoxic. More highly spread cells were visible on the material surfaces with the CPP content risen. It was considered that the energy release in the polymer hydrolysis accelerated the cell growth. More CPP, more energy release. On the other hand, the CS part on the scaffold surface gave cells a good adhesion and growth condition, because of its glycosaminoglycans (GAGs)-like chains. GAGs were important components of proteoglycan and typically presented in the cartilaginous extracellular matrix (ECM) (Barnewitz et al., 2006). The meniscus cell attachment and proliferation were determined by both of these two factors.

4. Conclusions

This work demonstrated the feasibility to synthesize the CS/CPP composite scaffold by means of commixture and freeze-drying technique. The novel organic/inorganic composite scaffold represented good mechanic characteristic compared with CS scaffold, and the degradation rate could be controlled by the change of CPP content, which lead to the study on gradient degradation biomaterials. Moreover, in the vitro cell culture studies, the results showed neither CS nor CS/CPP scaffolds were cytotoxic. SEM exhibited a good cell adhesion and morphology on the scaffold surfaces. Different contents of CPP induced different features and different interactions between cells and materials. Further studies will pay attention to the different content of CPP effects on meniscus cell factor expression in vitro and the scaffolds cultured in vivo.

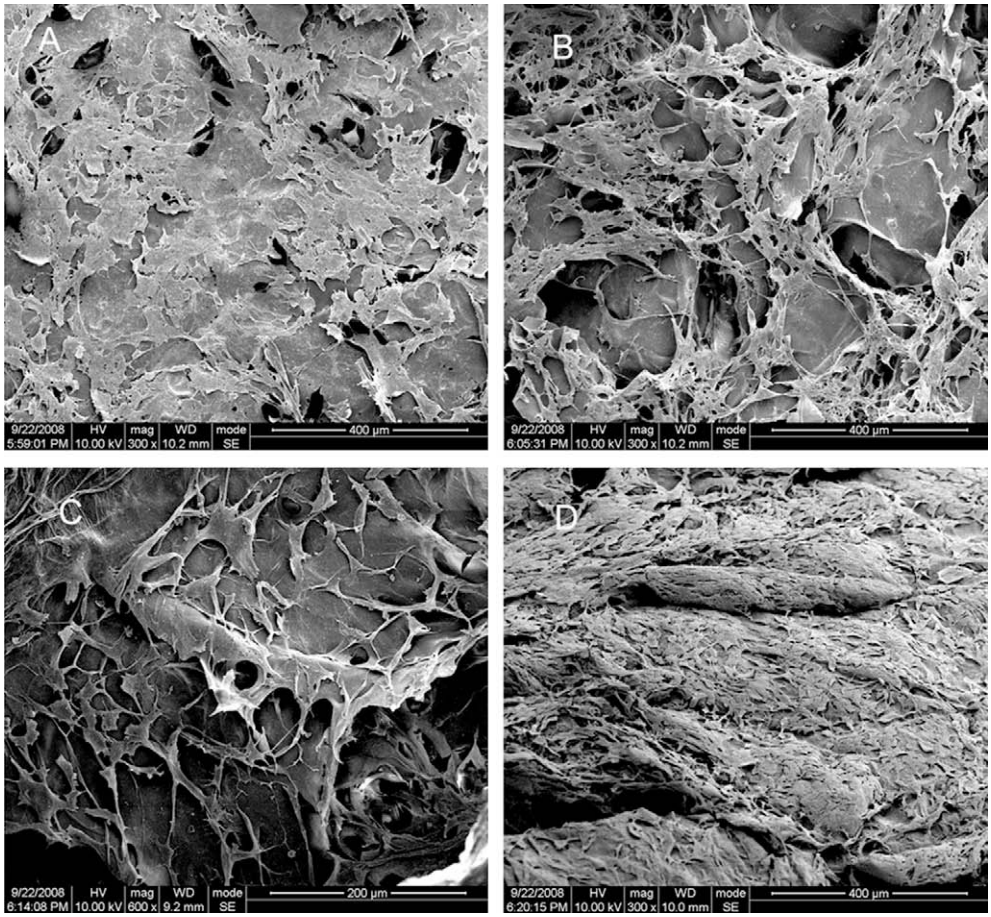


Fig. 5. SEM micrographs of the composite scaffolds with various contents of CPP seeded with meniscus cells cultured for 7 days: 0% (A), 30% (B), 50% (C), 70% (D).

Acknowledgements

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