Mechanical properties of hyaluronic acid modifying chitosan/collagen/nano-hydroxyapatite composite scaffold and its effect on osteoblast proliferation*☆

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Abstract

BACKGROUND: Seed cell exerting its function is required to depend on the extracellular matrix in tissue engineering, so that biocompatible material is important to be selected.

OBJECTIVE: To prepare a novel composite scaffolds of chitosan/collagen/nano-hydroxyapatite (HA-CS/Col/nHAP) and to optimize the technology of tissue engineered-stents according to the circumstances of cell adhesion.

METHODS: Chitosan was modified by hyaluronate acid. The structure was observed by differential scanning calorimetry and the Fourier transformed infrared spectroscopy. Three composites of HA-CS/Col/nHAP according to different ratio of chitosan and collagen solution (1: 2; 1: 1 and 2: 1) were prepared. The composite scaffolds were co-cultured with osteoblast MC3T3-E1, and the proliferation and cell growth curve were measured by CCK-8 method.

RESULTS AND CONCLUSION: Hyaluronic acid and chitosan were crosslinked with amide linkage. Pore size was on the range from 50 μm to 250 μm. Porosity was increased with increased collagen level and elastic modulus, but density was reduced. Increased collagen content was beneficial for cell adhesion and proliferation on stent in the primary phase of cell co-culture. However, from day 10, no significant difference was determined among three samples. At the beginning of cell culture, cells adhered to the airspace insides the composite scaffolds. In the following days, cells grew in a colony manner, and cell-cell junction could be easily observed. These indicate that HA-CS/Col /nHAP composite scaffolds can improve the adhesion and proliferation of osteoblast. The ratio of chitosan to collagen volume at 1: 1 was optimal.

INTRODUCTION

Many biomaterials have been developed for tissue engineering such as gel, macroporous sponge and bioceramic over the past decade. These biomaterials can provide space for cellular proliferation and metabolism, help transmit chemical or mechanical signals and regulate cellular phenotype[1-3]. As for bone scaffolds, good biocompatibility, mechanical properties and biodegradability are beneficial to the adhesion and proliferation of seed cells[4-6]. Chitosan, a natural biodegradable polysaccharide with good characteristics of biocompatibility and moulding, therefore it has been widely used in the field of bone tissue engineering[7-8]. Chitosan application is limited due to poor hydrophilia, because the hydrophilia of chitosan is not good. In view of cellular adhesion, the surface of hydrophilic material has better capability of adhesion and proliferation than that of hydrophobic material[9-10]. Chemically modified chitosan, by means of inducting hydrophobic group as hyaluronic acid and polyglycol can enhance surface invasion to increase cellular adhesion ability. Both collagen and hydroxyapatite ceramic (HAP) are major constituent of bone, offering a microenvironment which is similar to natural extracellular matrix[11-12]. Collagen has very good biocompatibility and degradation, but the disadvantage is the low biological activity[13-14]. HAP is an important inorganic composition in bone. Nano-HAP, besides of normal characteristic of HAP, has minute particle size, which enlarge specific surface area, resulting in larger contact area with cells[15-18].

This study prepared chitosan/collagen/nano-hydroxyapatite (HA-CS/Col/nHAP) composite scaffold, and measured its physical and chemical properties, especially mechanical properties. Furthermore, we three-dimensionally cultured pre-osteoblast MC3T3-E1 with the prepared scaffold, and observed cellular morphology and proliferation. The aim was to find the relationship between biomaterial properties and cellular capability, so as to provide optimized scaffold platform for bone tissue engineering.

MATERIALS AND METHODS

Design

A cellular observation.

Time and setting

The experiment was conducted in October 2010 at the Institute of Medical Equipment of the Academy of Military Medical Sciences.

Materials

Sodium hyaluronate, carbodiimide, and chitosan were bought from Sigma. MC3T3-E1 was provided by Institute of Basic Medicine of Peking Union Medical College.

Methods

Scaffold preparation

Preparation of hyaluronic acid modifying chitosan: Dissolved a certain amount of sodium hyaluronate with water, then added the same mole of carbodiimide

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as a crosslinking agent, and N-hydroxy succinimide, stirred for 15 minutes, and blended with chitosan, stirring until the solution was stable and steady. Adjusted pH with NaOH solution to 4–5, and continuously stirred for 6 hours.

Preparation of composite scaffolds: added collagen (extracted from bovine achilles tendon with collagen enzyme, made in our laboratory) solution (swelling with 1% acetic acid, mass concentration 0.5%) into hyaluronic acid modifying chitosan in accordance with the volume ratio of 1: 2 (A), 1: 1 (B) and 2: 1 (C). Whereafter, added nHAP (equal quality to chitosan powder) into the composite, stirred for 2 hours. After dissolving completely, the composite was dumped quickly into frozen petri dish at −70 ℃, and lyophilized to create macroporous scaffold. The product was neutralized with a 1% NaOH solution, washed with deionized distilled water and molded into small cylindrical with a radius of 5 mm, thickness of 2 mm. Lyophilized again. Finally, the three-dimensional HA-CS/Col/nHAP scaffolds were sterilized by gamma irradiation at a dose of 25 kGy.

Fourier transformed infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analysis

Hyaluronic acid modifying chitosan structure was analyzed with FT-IR (FTS3000, DIGILAB, USA) and DSC (PE-DS-2C, USA).

Characteristics of physical and chemical properties of the scaffolds

Under a scanning electron microscope, microstructure analysis determination of pore size: the scaffolds were placed in liquid nitrogen, and quickly removed with a sharp knife to cut toward cross section and longitudinal section. Samples were fixed in the aluminum plate coated with gold, and then observed under scanning electron microscope.

Determination of porosity and density: using liquid displacement method[18], measured a volume of dehydrated alcohol (V_1) with a graduated cylinder, took a certain quality (W) of scaffolds to immerse in it, repeatedly vacuumed until no bubbles escape. Read V_2 using cylinder, then removed scaffolds, readings V_3. Calculated porosity (P) and density (d) according to the following formula:

$P = (V_1–V_3)/(V_2–V_3)$, $d=W/(V_2–V_3)$.

Determination of mechanical properties: using Instron Model 5865 mechanical testing machine (100 N sensor, USA). Three samples A, B and C were detected the elastic modulus of compression strain in wet manner. Parameters were: 0.5 Hz, sawtooth waveform, 0.1 N pre-load, 50% increments, and 100.0% / min velocity; each sample was measured five times. Mean and standard deviation were calculated.

Three-dimensional culture with pre-osteoblast MC3T3-E1

Prior to seeding cells, the scaffolds were soaked in sterile phosphate buffered saline (PBS) for 2 hours, then drawn off water and were placed in a Petri dish. MC3T3-E1 cell suspension digested by trypsin, were seeded in scaffolds at a density of $2×10^9$/L. After 30-minute incubation, the scaffolds were turned over. After 2 hours, 10 mL of medium (α-MEM with 10% fetal bovine serum plus 1% antibiotics and 1% hydroxyethyl piperazine ethanesulfonic acid) was added to each cell-seeded scaffold. Co-cultured for 14 days, all incubations were at 37 ℃ in a humidified atmosphere of 5% CO_2, with changing the medium every 2 days.

Determination of proliferation by CCK-8 kit

Three pieces of cell-seeded scaffolds were selected at 1, 3, 5, 7, 10 and 14 days. Scaffolds were put into 24-well plate, adding 600 μL/well culture medium and 60 μL CCK-8 reagent, incubated for 4 hours at 37 ℃ shading from ambient light. After the incubation, 150 μL solutions were suck out from 24-well plate into 96-well plate, and measured its absorbance (TECAN, Switzerland) at 450 nm. A proliferation curve was generated based on absorbance value.

Hematoxylin-eosin staining

At days 7 and 14, the cell-seeded scaffolds were fixed by 10% formaldehyde. After dehydration, the scaffolds were embedded in paraffin and sliced. Cell morphology and the distribution were observed under light microscope (Olympus, Japan).

Main outcome measures

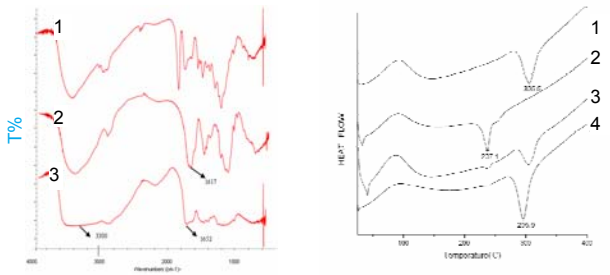
The structure of hyaluronate acid-chitosan crosslinking is observed by DSC and FTIR. The pore size, porosity, density and mechanical strength of the composite scaffolds were observed as well as cell adhesion ratio, growth curve and hematoxylin-eosin staining with the MC3T3-E1 and scaffolds.

Statistical analysis

SPSS 10.0 was used to test the significant differences among the three groups. Data were presented as means ± standard error. In any cases, the results were considered statistically difference at $P < 0.05$.

RESULTS

Structural characteristic of hyaluronic acid modifying chitosan (Figure 1)



a: FTIR curve
1: Chitosan; 2: hyaluronic acid; 3: hyaluronic acid/chitosan (Mass ratio 1: 10)

b: DSC curve
1: Chitosan; 2: hyaluronic acid;3: hyaluronic acid/chitosan physical mixture (Mass ratio 1: 10); 4: hyaluronic acid modifying chitosan (Mass ratio 1: 10)

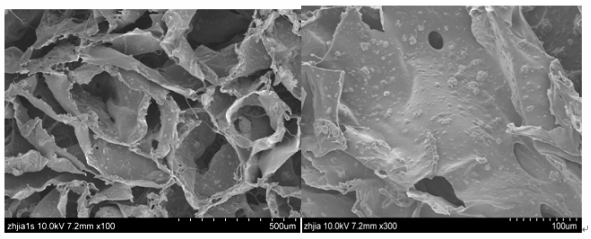
Figure 1 Fourier transformed infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) of chitosan, hyaluronic acid, hyaluronic acid/chitosan

An absorption at 1 617 cm^{-1} in hyaluronic acid (b) which was the absorption of carboxylic salt was disappeared in hyaluronic acid modifying chitosan (c), while strengthened at 1 652 cm^{-1} (c). It may be the result of the formation of amide linkage in the present of carbodiimide. Mechanism may be the condensation reaction occurred between carboxyl group in hyaluronic acid and amino group in chitosan: first, hyaluronic acid and

crosslinker carbodiimide react into O-isoureide, then O-N rearrange into N-ureide[19], finally nucleophilic substitution reaction occur between N-ureide and amino group in chitosan. An absorption close to 3 300 cm^{-1} in hyaluronic acid modifying chitosan (c) was broad and blunt, which suggested there was intermolecular association among hydroxides. This absorption peak overlapped with amino group which made it impossible to be speculated the changes of amino (Figure 1a). The endothermic peak of chitosan (a) appeared at 305.7 ℃, while hyaluronic acid (b) appeared at 237.02 ℃. The endothermic peaks of their physical mixture (c) both appeared at 305.7 ℃ and 237.02 ℃, whereas chemical composite appeared at 295.78 ℃ which showed a new compound appeared, namely cross-linked composite (Figure 1b).

Characteristics of physical and chemical properties of the scaffolds

Determination of pore size (Figure 2)



a: Longitudinally cut b: Partial enlargement

Figure 2 Scanning electron microscope micrographs of complex scaffold (B)

Figure 2 shows the scanning electron microscope photo of sample B. The longitudinally cut microstructure is depicted in (a). It can be seen that pore size of sample B was from 50 μm to 250 μm. Macropoles were in favor of accommodating more cells and promoting cell migration. Picture (2b) was partial enlargement of pore wall. It showed that hydroxyapatite particles were well-distributed and attached to the walls. Increasing material surface roughness favors cellular adhesion. But, nano-hydroxyapatite existed in the form of pellets on the pore walls. The reason may be that the thick substrate (chitosan, collagen, hyaluronic acid) hampered nHAP to disperse evenly. It will be expected to improve through technical optimization in preparation.

Scaffold porosity, density and mechanical modulus (Table 1)

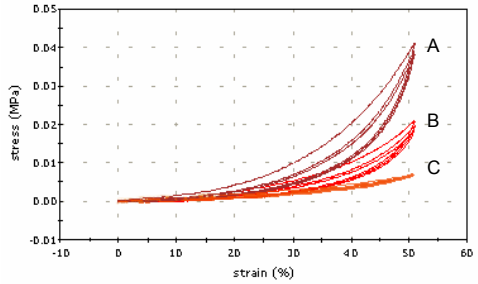
Table 1 Porosity, density and compressive Young's modulus of the scaffolds ($x \pm s$, n=5)

Sample	Porosity (%)	Density (kg/L)	Young's modulus (kPa)
A	46.20±1.88	0.10±0.24	1.51±0.19
B	50.40±5.14	0.09±8.01	29.31±0.38
C	62.50±4.53	0.05±5.34	36.94±0.25

As displayed Table 1, the porosity of the scaffold was higher with the increase of collagen content. However, the more collagen added, the lower density decreased because of low density of collagen. The result of mechanical test showed that Young's modulus in the three samples was increased with the increased collagen content, which indicated capability to stand strong elastic resistance in the scaffolds was enhanced when raising collagen content.

Material stress-strain curve

As shown in Figure 3, the maximum stress of sample C was 40 kPa at Y direction. This suggested sample C had strongest capability for anti-compression. The next was B and A respectively. In addition, it can be found that this kind of materials also had stress strain hysteresis phenomenon, reflecting the viscoelastic character of the scaffolds.

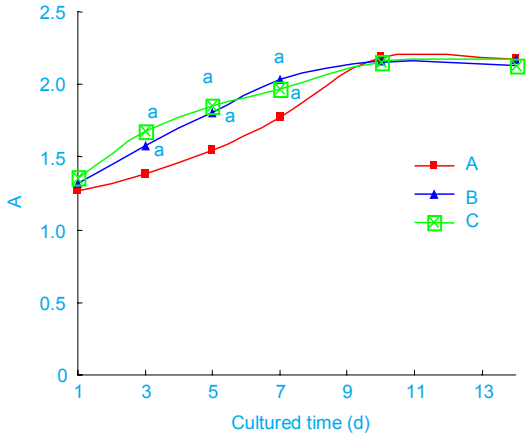


The ratio of chitosan to collagen is respectively 1: 2, 1: 1 and 2: 1 in samples A, B and C

Figure 3 Stress-strain curves of samples A, B and C

Cell proliferation curves

MC3T3-E1 cells were cultured for 14 days. As exhibited in Figure 5, during the first 7 days, the living cells of samples B and C were significantly higher than sample A ($P < 0.05$), suggesting that increased collagen content was favorable to adhesion and proliferation on the scaffold in the early period of three-dimensional-culture. Starting from day 10, the three samples had little differences in cell numbers. Instead, there occurred a plateau on the curve from day 10. Statistical results showed that data of day 14 groups had no significant difference (Figure 4).

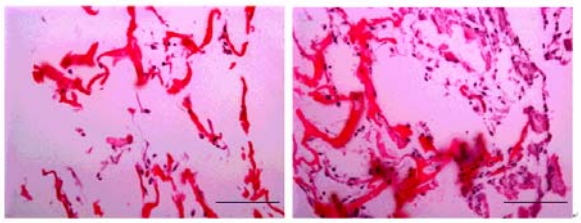


[a]$P < 0.05$, *vs*. sample A; the ratio of chitosan to collagen is respectively 1: 2, 1: 1 and 2: 1 in samples A, B and C

Figure 4 Cell proliferation curve of three-dimensional culture

Cell-scaffold construct morphology

As shown in Figure 5, at the beginning of culture (a), osteoblasts adhered to the pore wall, in irregular spindle manner, dispersed distribution. Nucleus and cytoplasm were clearly visible, but had a low quantity. With the days going on, cells grew in colony-like manner; cell-cell junction could be easily observed.



a: Day 7 b: Day 14

Figure 5 Three-dimensional-culture cell-scaffold complex (Hematoxylin-eosin staining, ×400)

DISCUSSION

Comparative analysis of mechanical properties

Elasticity modulus test results showed: C > B >A. Furthermore, stress-strain curve also manifested that the sample C had a high resistance to compression, which met the requirements of hardness of bone tissue engineering. Moreover, compared with porosity test results, the porosity was increased with the increased collagen content. Therefore, we speculated, due to the higher porosity caused by high collagen content, the ability of resisting external pressure was strengthened. It was lined with the conclusions that in terms of the same material, mechanical intensity of honeycomb one was higher than that of solid one at same area. Our previous study has found that materials with high porosity do favor for cells to enter into scaffolds. Thus, the sample C seemed to be the best choice. The sample C, however, contained a relatively more collagen, which had big bubbles during freeze-drying process, so that the appearance was uneven, and the yield was low. Hence, we choose the second mechanical intensity one, namely the sample B. That was why we chose the sample B to do scanning electron microscope micrograph. From the cell growth curve, during the first 7 days, cells in the samples B and C were higher than sample A, which suggested that the samples B and C were superior to sample A on cellular adhesion and proliferation. Overall, the sample B, which had the same volume ratio of collagen and chitosan, was the optimum scaffold.

Choice of biomaterial substrate

In this study, the organic and inorganic composite materials made up the scaffolds. Chitosan, and collagen constituted of the organic phase. nHAP for the inorganic phase was used to simulate the ECM of natural bone[20]. The aim of chitosan modification was to solve the problem of cell adhesion. It depends on adhesive power that cells could adhere and spread on the surface of the scaffold. Our previous work has found that pure chitosan sponge had certain adsorbability in the early period of three-dimensional-sulture, but cells located in the sponge pores, only part of the cells adhered along the pore wall. As the cultured time went on, cells began to fall off, indicating that the adsorption capacity of material did not well. This experiment found that cross-linking of hyaluronic acid and chitosan could enhance the hydrophilicity and moisture of chitosan, which was in favor of cellular adhesion and proliferation. Furthermore, integrin αv and β3 subunits of osteoblast on the surface of hydrophilic scaffold was up-regulated, so did vinculin[21]. In addition, under the cross-linked action of carbodiimide, hyaluronic acid conducted a viscous sol-gel conversion, which further enhanced the mechanical strength and stability of chitosan. The speed of degradation was also decreased at the same time. In the clinic, there is a “cartilage bone” theory during bone reconstruction after bone fracture[22], that is, between inner and external callus, firstly form cartilage, later a large number of fiber and calcium appear in the cartilage, which form bone trabecula, becoming intermediate callus, and the last form whole bone tissue. Hyaluronic acid is an important component of cartilage ECM. It is beneficial to the formation of cartilage, and thus promoting bone formation.

Collagen, chitosan and hydroxyapatite are widely used in the scaffold material of bone tissue engineering. Chitosan acetate solution is changed into sponge during a progress of freeze drying and soaking with alkaline solution. This kind of sponge has three-dimensional porous structure, high porosity and specific surface area, providing more space for cells to grow and connect, which shows a better biological properties. Macropore (more than 100 μm) fully met the inoculated cell adhesion, spreading and reproduction, while micropore (less than 50 μm) played a more important role in the exchange process of cell metabolism[23]. Our previous work found that the higher chitosan concentration, the smaller the pore size and porosity were. This study has found that the appropriate initial concentration of chitosan was 3%, due to blended with collagen at the same volume; the final concentration was 1.5%, and the pore size was 50−250 μm.

Cells attached to scaffold are mainly mediated by integrin receptors on cell membrane. The molecular structure of collagen contains Arg-Gly-Asp (RGD) sequence that can specifically bind to integrin, thereby mediate cell adhesion[24-25]. The experimental results showed that more collagen content had advantage of cell adhesion. Whether it enables high expression of integrins is needed to be verified in the future. Hydroxyapatite can not only afford the living environment of osteoblasts, but also increase material roughness. The surface of nano-material has more particle boundaries compared to micron one, thus can promote the adhesion of osteoblasts. However, the experiment found that as a high viscosity of chitosan and collagen solution, so hydroxyapatite was difficult to disperse evenly in viscous liquid, which resulted in uneven distribution of the product. If nano-hydroxyapatite was added into the scaffold material in the form of self-assembled[26-27], maybe it would avoid this problem.

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透明质酸改性壳聚糖-胶原-羟基磷灰石复合支架力学特性及成骨细胞的增殖*☆

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摘要

背景：组织工程中，种子细胞需依赖于细胞外基质的存在才能发挥功能。因此支架材料的选择具有重大意义。

目的：制备一种新型改性壳聚糖-胶原-羟基磷灰石复合支架，优化易于细胞黏附的组织工程支架材料工艺。

方法：壳聚糖与透明质酸进行交联，红外和差示扫描量热图谱检测其结构；改性壳聚糖与胶原按1∶2，1∶1和2∶1制备3种改性壳聚糖-胶原-羟基磷灰石复合支架，将复合支架与成骨细胞 MC3T3-E1 联合培养，CCK-8法检测增殖，绘制生长曲线。

结果与结论：透明质酸和壳聚糖以酰胺键形成交联的新化合物，孔径在50~250 μm之间，孔隙率随着胶原水平、弹性模量的增加而增加，而密度则减少；增加胶原的含量在细胞联合培养初期有利于细胞对支架的黏附和增殖，但从第10天开始，3种样品中细胞数量相差不大，均出现平台期；苏木精-伊红染色发现成骨细胞在培养初期沿着支架材料内部空隙贴壁生长，随着培养天数的增加，贴壁细胞呈集落样生长，可明显看到细胞间连接。说明透明质酸改性壳聚糖/胶原/纳米羟基磷灰石复合材料可以作为骨支架材料供成骨细胞黏附、增殖，其中胶原与壳聚糖的体积比为1∶1为较优配比。

关键词：成骨细胞；复合支架；弹性模量；细胞增殖；骨组织工程

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