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# Cytocompatibility of Human-like Collagen/nano-hydroxyapatite Porous Scaffolds Using Cartilages

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**Abstract:** Human-like Collagen (HLC)/Nano-hydroxyapatite (nHA) porous scaffold was constructed for the culture of cartilages. The cellular structure of scaffold and pore size were examined through Scanning Electron Microscopy (SEM). The pores with diameter ranging from 100 to 300 μm distributed evenly in the scaffolds and these pores interpenetrated and interconnected with each other. Rabbit ear cartilages were separated using digestion method and then cultured on the HLC/nHA scaffolds to evaluate the compatibility and cytocompatibility of scaffolds. After cultured for 14 days, cartilages maintained chondrocytic spherical morphology and infiltrated into porous scaffolds. This demonstrated excellent cytocompatibility and compatibility of HLC/nHA scaffolds. HLC/nHA porous scaffolds manifested great promising prospect in cartilage tissue engineering.

**Key words:** Human-like collagen, porous scaffold, cytocompatibility

## INTRODUCTION

The repair of articular cartilage defect is still one of the difficult and not completely resolved problems owing to impossible spontaneous recovery in clinical. At present, injecting hyaluronic acid or implantation became two main therapeutical methods. Injecting hyaluronic acid could construct cartilage and retard aging to some extent, but that is not an ideal way after all. Implantation includes osteoarticular autografts with inherent donor site limitations and new damaging generation and fresh osteochondral allograft with tissue exclusion and disease transfer (Zhao *et al*., 2002). In recent years, tissue engineering approach which is the construction of a scaffold to promote tissue regeneration brought novel prospect to articular cartilage defect repair and had been studied widely.

HLC is a water-soluble protein produced through technique for gene engineering. This protein possesses several advantages which are different from animal collagen, such as virus-free, low immunogenicity and processability. Currently, human-like collagen is used in many fields extensively as one kind of excellent tissue engineering material, such as hemostatic material, skin tissues and blood vessel tissues (Guo *et al*., 2010). Nano-hydroxyapatite (nHA) has been proved to be a ceramic material with good biocompatibility, even osteoconductivity and osteoinductivity (Wang *et al*., 2010).

In this study, HLC/nHA porous scaffolds were constructed by crosslinking of cobalt 60 for the culture of cartilages. And then the characteristics of HLC/nHA scaffolds were verified through scanning electron microscopy observation and several cell tests.

## METERIALS AND METHODS

**Materials:** Human-like collagen (Mw = 97000) was provided by JuZi biogene technology Ltd. Co. (Xi'an, China), which was produced by recombinant *Escherichia coli* BL21 (Hua *et al*., 2006). Nano-hydroxyapatite (20 nm) was received from Epri nano materials Ltd. Co. (Nanjing, China). Collagenase type II (351 U $mg^{-1}$) was purchased from Gibco. Trypsin (250 U $mg^{-1}$) was purchased from Amresco. Dulbecco's Modified Eagle's Media (DMEM, low glucose) and Fetal Bovine Serum (FBS) were provided by Hyclone. All other reagents and solvents are high purity grade or analytical grade.

**Preparation of HLC/nHA composite scaffolds:** HLC was dissolved in distilled water at a concentration of 8% (w/v)

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at room temperature. Then nHA power was slowly dropped into human-like collagen solution according to a HLC/nHA ratio of 1:6 (w/w). They were homogenized and adjusted to pH 7.0 to obtain HLC/nHA blend. After removing entrapped air bubbles, the HLC/nHA blend was injected into a mould with 10 mm diameter and 50 mm height, lyophilized in the vacuum freeze dryer for 24 h to gain a cellular HLC/nHA scaffold. Then the cellular scaffolds were cross-linked with cobalt 60 for 100 min to obtain a three-dimensional composite porous scaffold possessing certain strength and hardness.

**Cell separation and culture:** Cartilages used in this study were isolated from two-week rabbit ears by collagenase type? digestion. Specifically, the rabbit ears were immersed in the iodophor and washed with phosphate buffer saline (PBS, pH7.4) supplemented with penicillin (100 U $mL^{-1}$) and streptomycin (100 U $mL^{-1}$) till the color of iodophor faded away in aseptic room. After removed two sides skin, cartilage tissue was washed with PBS for 3 times again. Afterwards, cartilaginous membrane was eliminated lightly. The residual part was diced into 1 $mm^3$ sized tissues and placed in 50 mL centrifuge tube containing 10 mL collagenase type II (0.15%) prepared with DMEM involved 5% FBS. After digested in a controlled crystal oscillator (37°C) for 50 min, the digesting solution was filtered through a copper mesh (cell strainer, 200 meshes) and centrifuged at 1200 rpm for 10 min. The indigested cartilages were digested again. When this procedure was repeated for 4 times as described previously. Tissue pieces vanished and digestion was over. The gathered cells at a density of $2\times10^5$ $mL^{-1}$ were inoculated in 25 $cm^2$ tissue culture flask and were cultured at 37°C, 5% $CO_2$ and 95% humidity in DMEM. The culture medium was changed every 2 days. Cartilages could be passaged when chondrocytes bespreaded 80% of culture flask.

**Chondrocytes cultured on HLC/nHA scaffold:** HLC/nHA composite porous scaffolds were cut into 10×3 mm cylindrical in super clean bench. Before seeding, cylindrical flaky scaffolds were dipped in DMEM for 24 h and then DMEM was discarded. Passage 2 chondrocytes digested by trypsin (0.25%) were counted and diluted to a concentration of $1.0\times10^5$ cells $mL^{-1}$. The 250 μL of cell suspensions was seeded onto the top of scaffolds which were placed in the wells of 48 well culture plates. Additional 1 mL of DMEM was added to each well after 4 h. Culture plates were placed in the incubator with 37°C, 5% $CO_2$ and 95% humidity. The culture medium was changed every day. Cartilages cultured on the scaffold were observed and analyzed after 14 days culture.

**Scanning electron microscopy (SEM):** After incubated on HLC/nHA porous scaffolds for 14 days, the morphology of cells was examined by SEM. The samples were washed with PBS buffer and then fixed in 3% glutaraldehyde at room temperature for overnight. After bathed three times with PBS, the samples were dehydrated with a series of graded ethanols and left to air dry. The samples were sputter-coated with a thin gold layer (Alves da Silva *et al*., 2010) and studied in Japan's Hitachi Company S-570 scanning electron microscope.

**Hematoxylin- eosin staining:** Samples were gathered after cultured for 14 days and fixed with 3% glutaraldehyde. After that, samples were dehydrated in a series of graded ethanols, then were made into paraffin block and cut to 5 μm thickness using a microtome. Sections were deparaffinized in xylene, hydrated to 70% alcohol and stained with standard hematoxylin/eosin staining (Donzelli *et al*., 2007). Specifically, sections were stained in hematoxylin for 10 min and then washed with tap running water. Afterwards, slices were immersed in 1% acid alcohol for 20 sec and then 1% aqueous ammonia for 30 sec. Sections were washed again in running water and stained in eosin for 1 min. After another wash with running water, sections were dehydrated in a series of graded ethanol, cleared in xylene and mounted in DPX.

**Fluorescent staining:** After chondrocytes-HLC/nHA scaffolds were cultured for 14 days, 10 μL of carboxyfluorescein diacetate (CFDA) solution was added into the wells of 48-well plates. Afterwards, the culture plates were placed in incubator for 30 min and then taken out. The samples were analyzed under the fluorescence microscope (TS100, Nikon, Japan).

## RESULTS AND DISCUSSION

**SEM imaging of scaffolds:** HLC/nHA composite porous scaffolds were fabricated through three main procedures including mixing, freeze-drying and cross-linking. Figure 1 represented the SEM images of the porous scaffolds structure. The pores with diameter ranging from 100 to 300 μm distributed evenly in the scaffolds and these pores of scaffolds interpenetrated and interconnected with each other.

**SEM imaging of cartilages cultured on HLC/nHA scaffolds:** The morphology of cartilages seeded on HLC/nHA scaffolds after incubated for 14 days was shown in Fig. 2. In the picture, it was shown that cartilages attached on the HLC/nHA scaffold and maintained chondrocytic spherical morphology. In

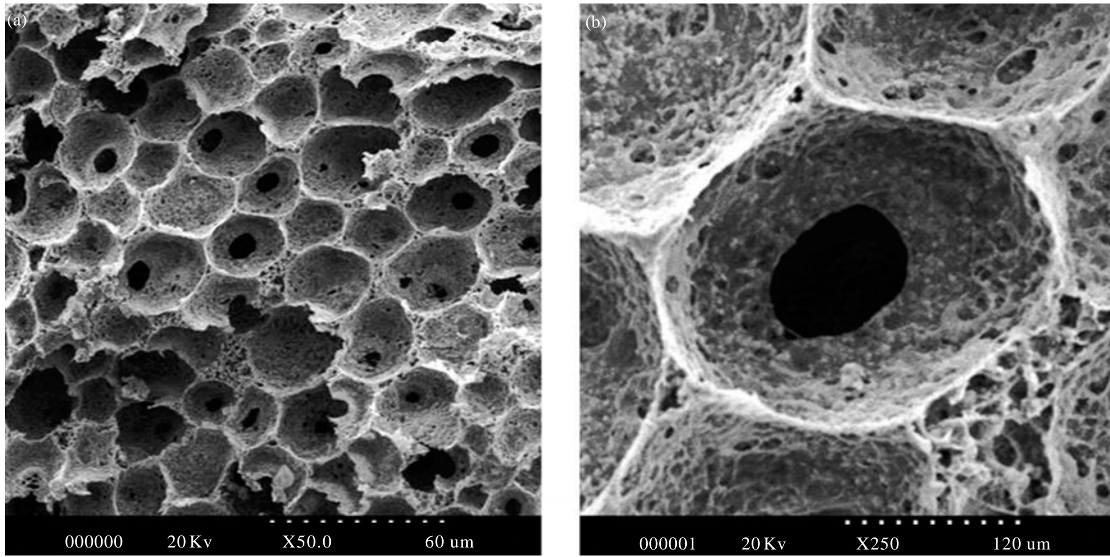


Fig. 1(a-b): SEM micrographs of HLC/nHA scaffold The scales bars in the left column and right column represent 0.6 mm and 120 μm, respectively

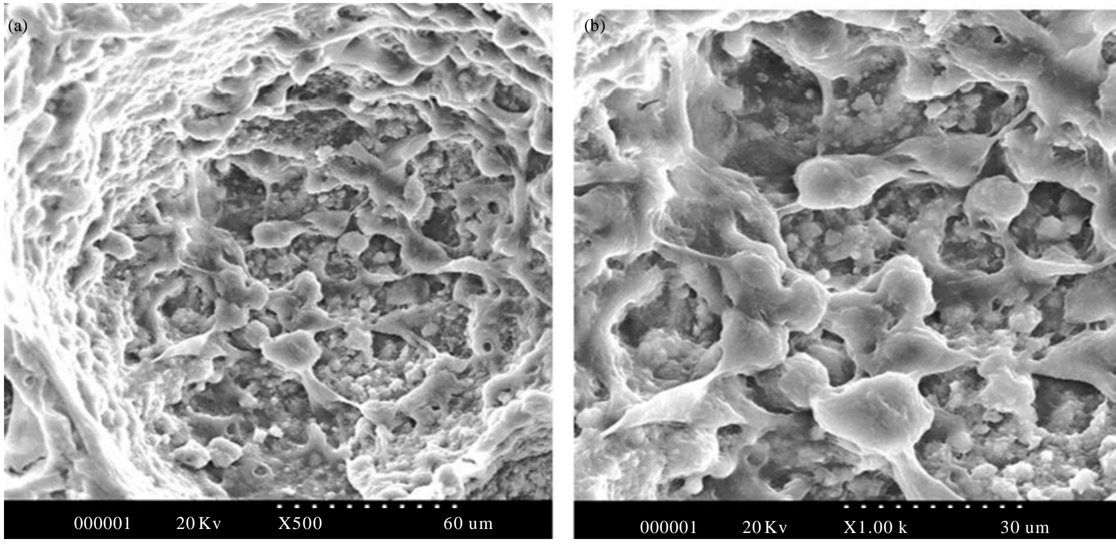


Fig. 2(a-b): SEM micrographs of cartilages cultured on HLC/nHA scaffold for 14 days The scales bars in the left column and right column represent 60 and 30 μm, respectively

addition, the surface of scaffolds was covered with extracellular matrix. This result showed good compatibility between chondrocytes and HLC/nHA scaffolds.

**Hematoxylin-eosin staining:** After 14 days of culture, composite scaffolds with cells were made into slides and then stained in hematoxylin-eosin. A mass of cells occupyed the whole scaffold and infiltrated into porous scaffold as shown in Fig. 3. Cells growed and proliferated well along the pores of porous scaffold. It demonstrated excellent compatibility existed in porous scaffold and cartilages.

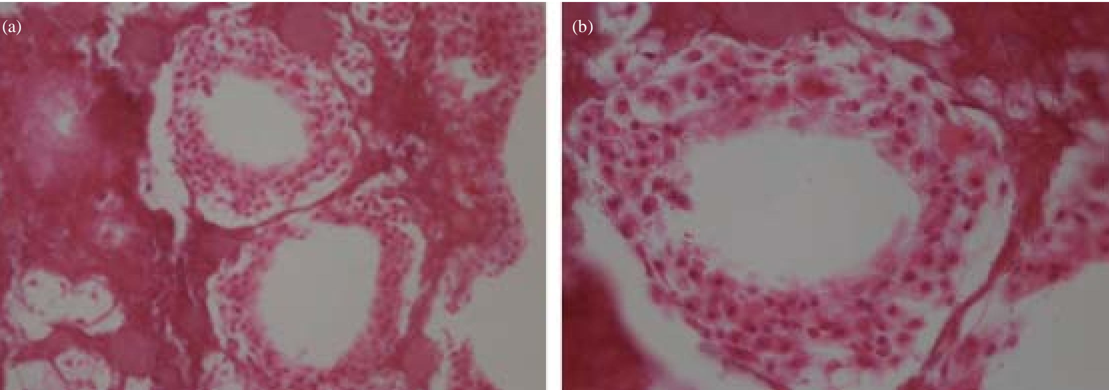


Fig. 3: Hematoxylin-eosin staining images of cartilages cultured on HLC/nHA scaffold for 14 days

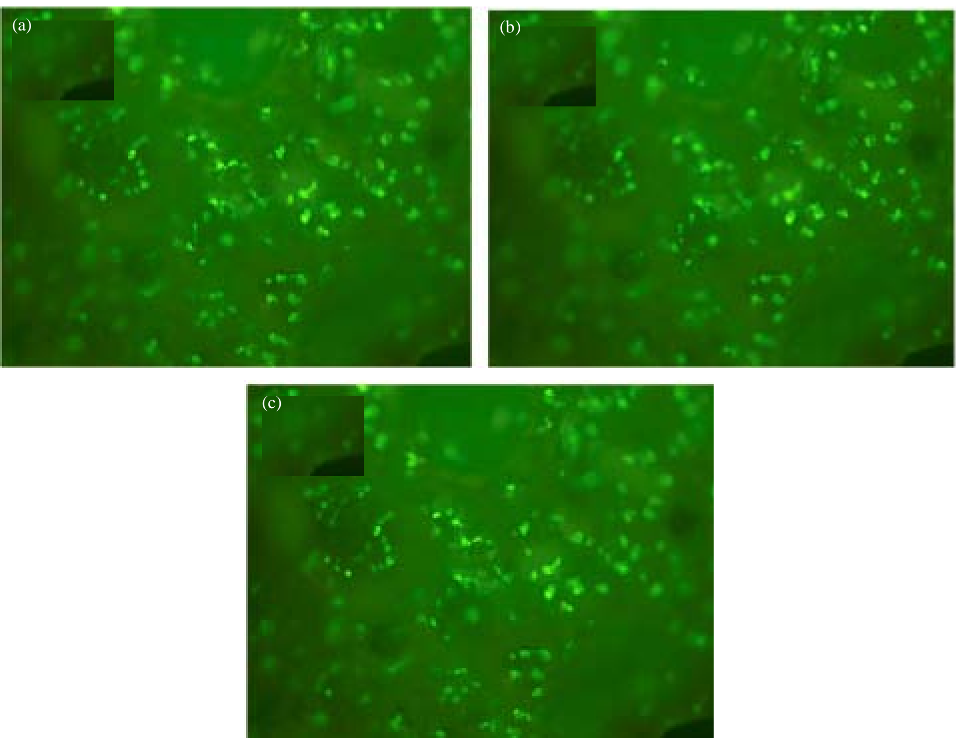


Fig. 4(a-c): Fluorescence staining images of cartilages cultured on HLC/nHA scaffold for 14 days, (a, b and c) Represent cartilage cells in different plane, respectively

**Fluorescence staining:** Cell infiltration and proliferation were important to a scaffold to support and guide tissue regeneration (Ma *et al*., 2003). Figure 4 showed the fluorescence staining images of cells cultured for 14 days on HLC/nHA scaffolds. Figure 4a, b and c showed cartilage cells in different plane, respectively. They showed that cells adhered on the walls of the HLC/nHA scaffolds and infiltrated into porous scaffolds. These demonstrated excellent cytocompatibility of HLC/nHA scaffolds. It ensured the further study of scaffold materials.

The size of pores in the scaffold was one important ingredient in tissue engineering. Small pores may have an influence on cell distribution and increase edge effect (Kang *et al*., 2009). In contrast, the structure and mechanical property of scaffold would be affected when pore size is too large. The pore of the HLC/nHA scaffold used in this study was managed by controlling the procedure of freeze-drying. As shown in Fig. 1, the pore size of the scaffold ranged from 100 to 300 μm which was appropriate for the proliferation of chondrocytes culture.

## CONCLUSION

The present study showed that HLC/nHA scaffolds possessed suitable three-dimensional porous microstructure and excellent cytocompatibility. This conclusion made a significant contribution to further study. HLC/nHA scaffolds manifested great promising prospect in cartilage tissue engineering.

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