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Preparation and characterization of chitosan/galactosylated hyaluronic acid scaffolds for primary hepatocytes culture

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Abstract Hepatocyte-specific three-dimensional tissue-engineeringed scaffold plays an important role for developing bioartificial liver devices. In the present study, galactose moieties were covalently coupled with hyaluronic acid through ethylenediamine. Highly porous sponge composed of chitosan (CS) and galactosylated hyaluronic acid (GHA) was prepared by freezing-drying technique. The morphology of the scaffolds was observed via scanning electron microscopy. Porosity and pore size of the sponge were greatly dependent on the content of GHA and freezing temperature. The addition of GHA not only improved the wettability and changed their mechanical properties, but also significantly influenced the cell attachment ratio. Moreover, liver functions of the hepatocytes such as albumin secretion, urea synthesis and ammonia elimination in the CS/GHA scaffolds were improved in comparison with those in the chitosan scaffolds.

1 Introduction

In a typical tissue engineering approach, to control tissue formation in three-dimensions (3D), a highly porous scaffold is critical. It has been demonstrated that a 3D structure, which biomimicked architecture for promoting cell growth and maintaining cell functions similar to that of naturally occurring extracellular matrix (ECM), provides better physical and mechanical microenvironmental surroundings for cell proliferation and differentiation [1–5]. To design an ideal scaffold, a highly open porous structure with well interconnected pores is required, not only to achieve sufficient cell seeding density within the scaffold, but also to facilitate the free transport of nutrients and oxygen for subsequent cell proliferation and differentiation [6, 7].

The scaffolds for liver need stable 3-D spatial microenvironment to mimic the organized architectures of native liver and highly open porous structures besides good biocompatibility and biodegradability [8]. In addition, scaffolds require specific interaction with extracellular matrix components, growth factor, and the cell surface receptor to culture anchorage-dependent cells such as hepatocytes [9–11]. In order to adjust the interaction of hepatocytes with the scaffold and promote hepatocytes to organize into a three dimensional architecture while enhancing cellular functions in scaffolds, we have modified materials such as chitosan and alginate with galactose moieties as a specific adhesive ligand to asialoglycoprotein receptor (ASGP-R) of hepatocyte [12–16].

Biomaterials play a pivotal role as scaffolds to provide three-dimensional templates and synthetic extracellular matrix environments for tissue regeneration [5]. The extracellullar matrix (ECM) components of the target tissue should be mimicked by the scaffolds [17]. The structure of chitosan is similar to glycosaminoglycans, the components of liver ECM, which makes it an ideal scaffold material for hepatic tissue engineering [18–22]. Hyaluronic acid is glycosaminoglycans (GAGs) in the matrix of embryonic and fetal tissues in the liver. So HA is candidate matrix component as 3-D scaffolds for ex vivo cultures of hepatic cells [11, 23].

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In this study, we first synthesized galactosylated hyaluronic acid (GHA) prepared through the covalent coupling of lactobionic acid with HA. Then we prepared highly porous three-dimensional sponges composed of chitosan and GHA through the electrostatic interaction of carboxylic groups of GHA with amine ones of chitosan. Final we compared the liver-specific functions of hepatocytes between chitosan and chitosan/GHA sponges.

2 Materials and methods

2.1 Materials

Chitosan was supplied by Haihui Bioengineering Co. (Qingdao, China). The degree of deacetylation was 90%, viscous average molecular weight was 2×10^5. Hyaluronic acid (HA) with viscous average molecular weight of 8×10^5 was purchased from Freda Biochem Co. (Shandong, China). Lactobionic acid (LA) was purchased from Sigma Chemical Co. (MO, USA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and Nhydroxy-sulfosuccinimide (sulfo-NHS) were purchased from Medpep Co. (Shanghai, China). All other chemicals were purchased from Shenggong Biology Engineering Co. (Shanghai, China) unless otherwise stated.

2.2 Synthesis of GHA

Firstly, primary amine was introduced into lactobionic acid [13]. Briefly, lactobionic lactone prepared by dehydration of lactobionic acid was refluxed with excess ethylenediamine dissolved in anhydrous dimethyl sulfoxide (DMSO) for 2 h. The monoamine terminated lactobionic lactone (L-NH_2) was precipitated with chloroform and the obtained precipitate was vacuum-dried. Then, 1 g of the L-NH_2 was reacted with 1 g of HA dissolved in 100 ml of 20 mM TEMED (*N,N,N*-tetramethylethylenediamine, pH 4.7) for 24 h at room temperature using 1 g EDC and a co-reactant 0.1 g sulfo-NHS as the activation agents. The obtained GHA was purified by dialysis against Milli Q for 1-week and lyophilized. The reaction scheme is shown in Fig. 1. Synthesis of GHA was confirmed through NMR and elemental analyses.

2.3 Preparation of scaffolds and membranes

Chitosan and CS/GHA sponges were fabricated using the freeze-drying technique. Briefly, Chitosan solution (2 wt%) was prepared by dissolving chitosan in 0.5 M acetic acid. Two percent chitosan acetate solution and different concentrations of GHA aqueous solution (0%, 0.02 wt %, 0.1 wt %, 0.2 wt %) were mixed, to be in a volumn ratio (r) of 1:1 and stirred with a magnetic bar at 37°C for 60 min. 0.5 ml solution was poured into each well of 24-well polystyrene culture plates (Costar Co., Ltd). The plates were frozen at −20°C, −80°C and liquid nitrogen, respectively, then lyophilized in a freeze-dryer to form porous structure. Lyophilized scaffolds were treated by gradient ethanol process. Before cell seeding, the scaffolds were sterilized by ultraviolet light for 12 h.

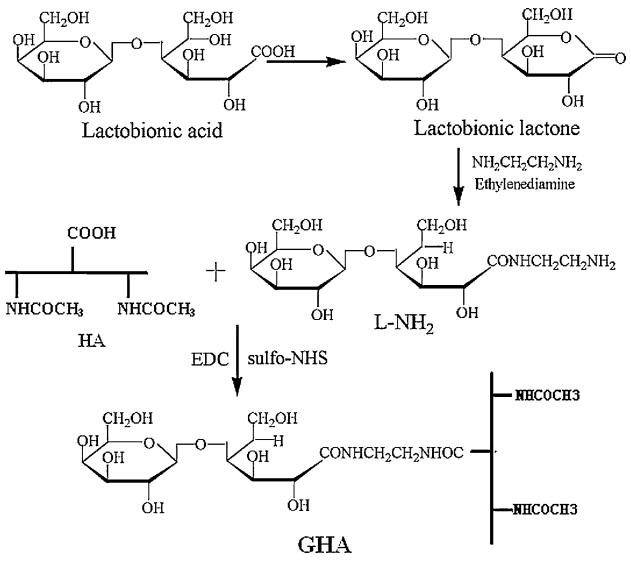

Fig. 1 Synthesis scheme of galactosylated hyaluronic acid (GHA)

Chitosan, CS/HA, CS/GHA and collagen membranes were prepared by pouring 1 ml mixing solutions in each well of 6-well culture plate. Then the solutions were decanted after 60 min and the surfaces were rinsed 3 times with PBS. Before cell culture, it was sterilized.

2.4 Morphology of the scaffolds

The morphology of CS and CS/GHA scaffolds were observed on a Hitachi (Japan) X-650 scanning electron microscope (SEM). Mean pore diameters were estimated by analysis of digital SEM images. Average pore sizes were determined based on the sizes of 5 pores. Porosity was determined by liquid displacement method.

2.5 X-Ray photoelectron spectroscopy (XPS) analysis of the scaffolds

XPS analysis of the scaffolds of various proportions was performed using a PerkinElmer 5600 electron spectroscopy for chemical analysis (ESCA).

2.6 Wettability of the membranes

The static contact angles of chitosan, CS/GHA, and GHA membranes were measured using a contact angle goniometer

(Harke-Spca, Baoding, China). Deionized distilled water was dropped onto the surface of the membranes before measuring. Every data presented were the mean values of at least five independent measurements.

2.7 Mechanical property of the sponges

Deflection-Force curves and the Young's modulus of the sponges were measured using an M500-10AX testing machine (Testometric Universal Tester, England). The Young's modulus was calculated from five specimens (thickness, 12 mm; diameter, 15 mm; compress rate, 0.5 mm/min) in the dry state.

2.8 Rat primary hepatocyte isolation and culture

Hepatocytes were isolated from male Wistar rat by perfusion the liver with a two-step collagenase perfusion technique of Seglen. The collagenase-perfused liver was dissected, suspended in Hanks solution, and filtered through 100 μm nylon mesh. Hepatocytes were purified by a density-gradient centrifugation (50 g force, 10 min) using 45% Percoll solution (Pharmacia, Piscataway, NJ) at 4°C. Cell viability measured by trypan blue exclusion was more than 90%.

Hepatocytes at a cell density of 2×10^5 cells pre one sponge were absorbed into the dry sponges placed in a well of the 24-well polystyrene (PS) plate filled with 1 ml of William's medium E (WE). The cells were cultured with serum free WE containing antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin and 100 μg/ml neomycin), HEPES (18 mM), epidermal growth factor (EGF; 10 ng/ml), insulin (100 nM), and incubated in a humidified atmosphere of 5% CO_2 and 95% air, at 37°C, with medium changes every 48 h.

2.9 Hepatocyte attachment

The isolated hepatocytes were suspended in different membranes coated 6-well PS dish at 5×10^4 cells/ml in Williams' E medium without serum. The cultures were incubated in a humidified air/CO_2 incubator (95/5, v/v). After a prescribed time, the medium including free non-adhered cells was thoroughly washed with PBS solution. The MTT assay was used as a measure of relative cell viability.

2.10 Observation of hepatocytes in sponges by SEM

The morphologies of hepatocytes within CS and CS/GHA sponges were observated by SEM. The cell-seeded scaffolds were rinsed with PBS solution and fixed with 2.5% glutaraldehyde in PBS at 4°C overnight, and then washed with PBS three times each for 10 min. The sponges were dehydrated in a graded ethanol solution (20%, 30%, 50%, 70%, 80%, 90% ethanol) each for 10 min, and finally in pure ethanol twice for 10 min each. Samples were then dried and sections were coated with ultrathin layer of gold and examined by SEM (Hitachi X-650, Japan).

2.11 Measurement of hepatocytes functions

During the hepatocytes culture period, medium samples were collected every 48 h and stored at −20°C until assayed by using commercial kits.

Urea synthesis was determined by the method described as follow: Briefly, 96-well plates were added with 20 μl standard pure urea (7.14 μmol/ml) and individual sample, followed by the addition of 200 μl A liquid (containing H_2SO_4, H_3PO_4, benzaldehyde, ammonium metavanadate) and 20 μl B liquid (iodopyrine). After reaction at 37°C for 15 min, the color change was monitored spectrophotometrically at 600 nm.

The amount of albumin in the medium was examined by the method of spectrophotography. Briefly 24-well plates were added with 5 μl physiological salt solution, standard pure albumin (4 mg/ml), and individual samples and then shaken evenly, followed by the addition of 1.5 ml succinate buffer (pH 4.2) containing bromocresol green (0.15 mmol/l) and polyxyethylene lauroyl ether Brij-35 (30%). After reaction at 25°C for 5 min, the color change was monitored spectrophotometrically at 630 nm. By establishing a standard curve using the standard pure albumin and analyzing the result, albumin secretion of hepatocytes in the culture medium was determined.

Ammonia elimination was estimated as follows: hepatocytes in the sponges were cultured with 1 mM NH_4Cl in Hanks solusion for 2 h, and then the ammonia concentration was measured with an ammonia test kit according to the manufacturer's instructions.

2.12 Statistical analysis

All data were presented as mean ± standard deviation (SD). The statistical significance of the data obtained was analyzed by Student's *t*-test.

3 Results and discussion

3.1 Synthesis of GHA

Galactose moieties were coupled to HA according to the reaction scheme shown in Fig. 1. The first step was the introduction of ethylenediamine into lactobionic acid by reaction of lactobionic lactone dehydrated from lactobionic

Table 1 Elemental analyses of GHA and HA

Sample	N (%)	C (%)	H (%)
HA	3.18	36.20	5.19
GHA	3.55	40.90	6.47

acid with 30-fold excess of ethylenediamine. The purity of the product was checked by mass spectrum and showed no detectable free ethylenediamine in it. The second step was the coupling of the L-NH_2 into HA using EDC and sulfo-NHS as activation agents. The GHA was analyzed with NMR; however, the peaks of galactose residue in the GHA were difficult to be assigned due to the overlapping with those of HA. The content of galactose moieties in the GHA was evaluated by element analysis of carbon, hydrogen and nitrogen content shown in Table 1 and it showed that 15% of carboxylic acid in HA reacted with L-NH_2.

3.2 Morphology of CS/GHA sponge

The CS/GHA solution was prepared through the electrostatic interaction of the amine group of chitosan with carboxylic group of HA. The highly porous three dimensional sponges were fabricated using the procedure of freezing the solution of CS/GHA, and subsequently lyophilizing the frozen mixed solution. The freezing and lyophilization techniques created openpore microstructures with a high degree of interconnectivity. The diameter of scaffolds same as the well diameter of 24-well plate, and the thickness of scaffolds were 1.8 ± 0.1 mm when the sponges were fabricated using the method 2.3, which measured by analysis of digital SEM images. The morphologies of CS/GHA sponges were investigated based on different content of GHA, and freezing temperature [24].

Figure 2 shows cross-sectional structures of CS/GHA sponges fabricated by the freeze-drying technique with different freezing temperatures. The results indicated that freezing temperature had a significant effect on the porosity, pore size and distribution, which suggested that the different morphologies in these sponges were established by the growth rate of ice crystals formed in the process of freezing CS/GHA solution at different temperatures. The sponge prepared at −20°C was exhibited three-dimensional and highly porous microstructure with interconnected pores (Fig. 2c). Porosities and average pore sizes of the CS/GHA sponges were greatly influenced by the freezing temperatures (Table 2). The pore sizes of liver tissue engineering scaffolds were proved that the pore size

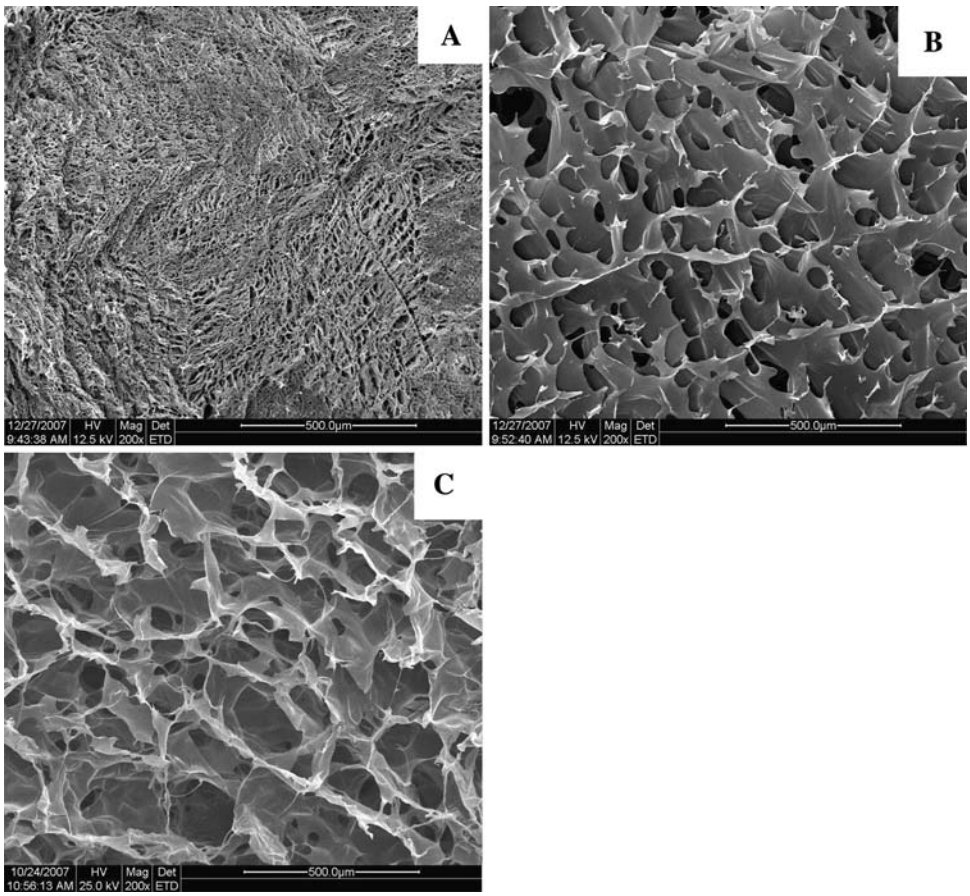

Fig. 2 Scanning electron micrographs of CS/GHA scaffolds as a function of the freezing temperature (×200). **a** liquid nitrogen; **b** −80°C; **c** −20°C

Table 2 Porosity and average pore size of CS/GHA scaffolds as a function of freezing temperature. Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

Freezing temperature (°C)	Porosity (%)	Average pore size (μm)
−20	90.1 ± 1.6	140.2 ± 62.3
−80	84.0 ± 1.3	82.1 ± 23.5
Liquid nitrogen	60.2 ± 1.4	15.6 ± 5.2

large than 100 μm provided optimum viability and function, with no mass transfer limitations [6, 25, 26]. So, −20°C was selected as freezing temperation.

Figure 3 shows SEM photographs of the cross sections of chitosan and CS/GHA sponges subjected to varying GHA content. The sponges exhibited porous, honey-like structures with interconnected pores. Porosities and pore size of chitosan sponges increased after the reaction of GHA and with an increase of GHA in the CS/GHA sponges as summarized in Table 3. Polyelectrolyte complex was thought to have formed between chitosan and GHA. The treatment of chitosan with GHA changed the morphology of sponge to have larger pores and more connected structure compared to the chitosan sponge. The 0.1% GHA content in CS/GHA scaffold was selected as test scaffold, which porosity was 90% and average pore size was 140 μm.

3.3 ESCA survey scan spectra analysis

The changes in surface chemical composition of the scaffolds were further investigated by examining ESCA spectra. The control chitosan surface shows carbon (binding energy, 284 eV), oxygen (binding energy, 530 eV), and nitrogen (binding energy, 397 eV) peaks (data not shown). For the CS/GHA surfaces, decreased nitrogen peak was observed. These changes correspond to the increased GHA contents. The chemical compositions of the CS/GHA sample and the controls, calculated from the ESCA survey scan spectra, are shown in Table 4. The nitrogen content (8.62%) of the chitosan was higher than that of the CS/

Table 3 Porosity and average pore size of CS/GHA scaffolds as a function of the content of GHA (chitosan, 1 wt%). Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

GHA (%)	Porosity (%)	Average pore size (μm)
0	89.4 ± 1.1	112 ± 43.5
0.01	89.5 ± 1.3	126 ± 33.5
0.05	89.9 ± 1.2	135 ± 50.2
0.1	90.1 ± 1.6	140 ± 62.3

Table 4 Elemental composition of chitosan, GHA and CS/GHA from XPS spectra

Sample	Composition (%)			
	O	N	C	S
CS	34.58	8.62	56.80	0
GHA	32.44	3.79	63.77	0
CS/GHA	39.29	6.36	54.35	0

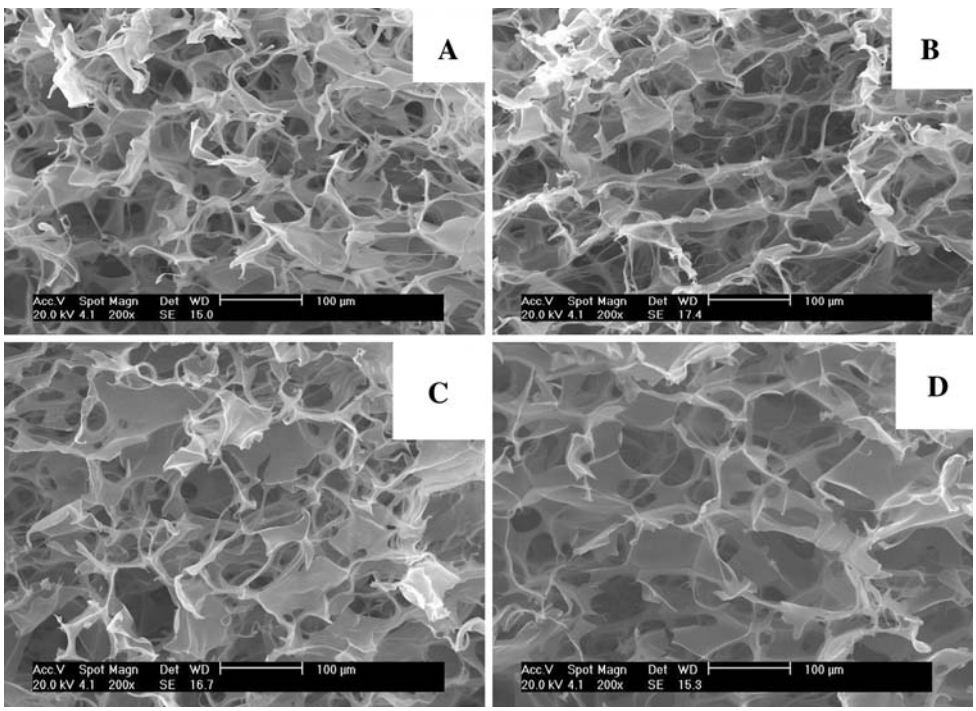

Fig. 3 Scanning electron micrographs of CS/GHA scaffolds as a function of the content of GHA (×200). **a** chitosan; **b** CS + 0.01% GHA; **c** CS + 0.05% GHA; **d** CS + 0.1% GHA (chitosan, 1 wt%)

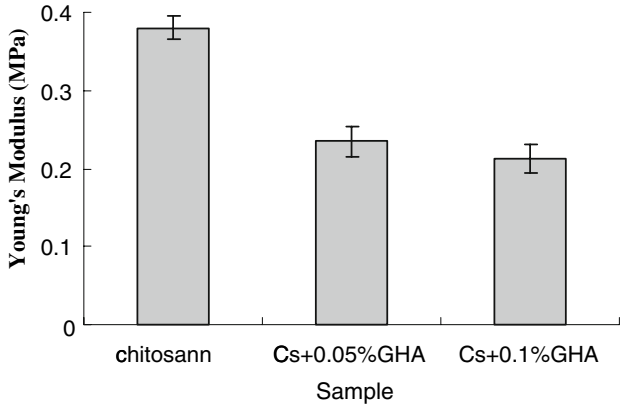

Fig. 4 The Young's modulus of CS/GHA scaffolds fabricated with different concentrations of GHA solutions. Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

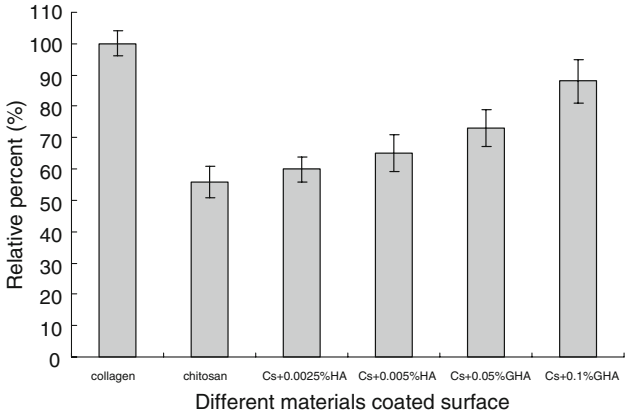

Fig. 6 MTT assay as a measure of viable hepatocytes attached on different materials coated surface. Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

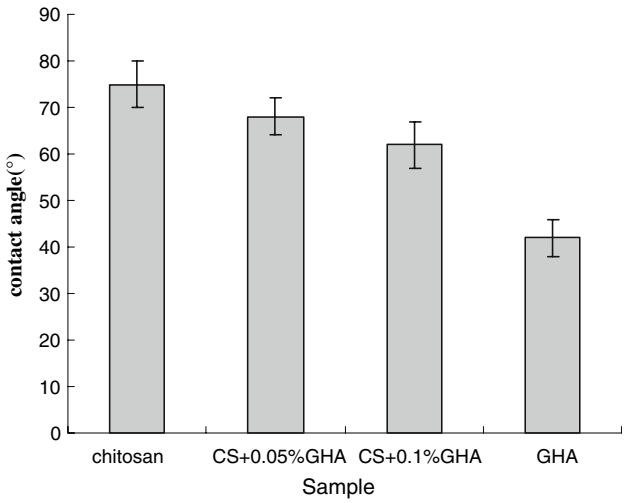

Fig. 5 Contact angles against water of CS/GHA membranes fabricated with different concentrations of GHA solutions. Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

GHA (6.36%). These results suggest that the scaffold prepared process can retain GHA in the CS/GHA scaffolds.

3.4 Mechanical properties of the matrices

The mechanical properties of the scaffolds in tissue engineering applications are of great importance due to the necessity of structural stability to withstand stresses incurred during culturing in vitro and implanting in vivo. The mechanical properties can also significantly affect the specific biological functions of cells within the engineered tissue. The Young's modulus of the sponges is shown in Fig. 4. The data showed that the mechanical strength of the chitosan is higher than that of the CS/GHA. The addition of GHA in chitosan networks can reduce mechanical strength, while increase the flexibility of the sponges. However, there were slight differences of mechanical properties between CS/GHA sponges fabricated with different concentrations of

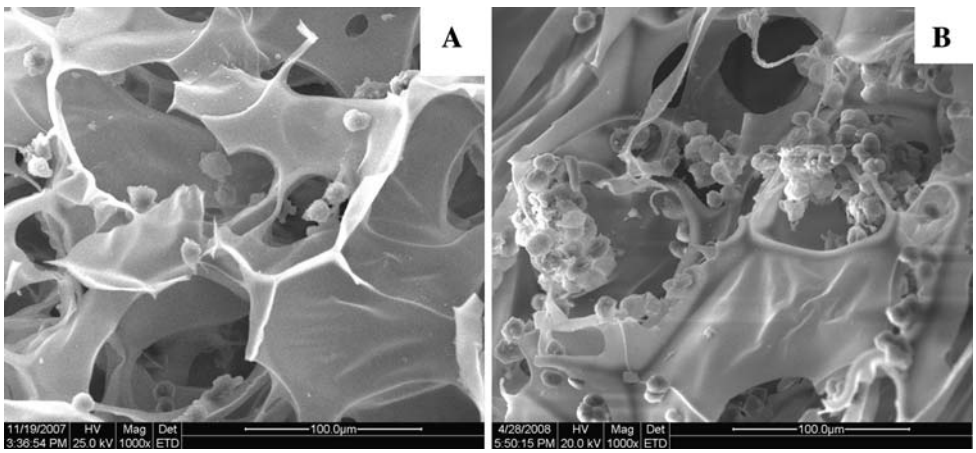

Fig. 7 SEM of hepatocytes cultured in **a** chitosan scaffold × 1,000; **b** CS/GHA scaffold × 1,000

GHA. The result implied that the degree of heterogeneity of CS/GHA is larger than that of chitosan, which led to decrease in mechanical strength.

3.5 Wettability

There are a number of carboxyl groups and hydroxyl groups in the GHA molecules which can combine much water by forming hydrogen bonds. Because of the hydrophilicity of GHA, it can be obviously found that the static contact angles against water of the CS/GHA membranes were much lower than that of the chitosan membranes and they became smaller with the increase in contents of GHA (Fig. 5). The results suggested that the hydrophilicity of CS/GHA membranes was much improved by addition of GHA.

3.6 Hepatocyte attachment

To understand the specific interaction between galactose ligands in GHA and ASGPRs on hepatocytes, we examined whether the CS/GHA material is adhesive to primary hepatocytes by an ASGPR-specific manner. The cell attachment to chitosan-coated PS dish was low, while the cell attachment slightly increased with the addition of HA to CS. When adding GHA to CS, the cell adhesion has a remarkable increase compared with CS-coated dishs. The MTT test is an indirect method of assaying cell attachment since the A_{490} values can be correlated to the cell number (Fig. 6). The hepatocytes adhesion to collagen-coated PS dish was defined as 100%. The results indicated that hepatocytes adhesion to the CS + 0.1% GHA-coated PS dish became as high as 88% after 4 h incubation, while hepatocytes adhesion to chitosan-coated PS dish was only 56%. It was already reported that hepatocytes adhesion to galactosylated material-coated dish was galactose-specific recognition between material molecules and the ASGPR of hepatocytes [13, 15, 16]. Therefore, it can be said that hepatocytes adhesion to CS/GHA-coated dish was galactose-specific recognition between GHA molecules and ASGPR of hepatocytes.

3.7 Observation of hepatocytes within CS/GHA sponge

Cell morphology was observed to understand behavior of the hepatocytes in the sponges. Figure 7 shows the scanning electron micrographs of hepatocytes attached into the CS/GHA and chitosan sponges after culturing 3 days. The results showed that the number of cells within CS/GHA scaffold was more than that of chitosan scaffold. In addition, more hepatocytes aggregated to form multicellular spheroids in CS/GHA sponge. The formation of hepatocyte spheroids in CS/GHA scaffolds is attributed to introduction of galactose ligands into the chitosan system, indicating that spheroids occurred in the GHA-containing sponges after ASGPR-mediated adhesion. It has already been reported that rat hepatocytes attached on galactose-derived substratum required the specific interaction between ASGPRs and galactose ligands to form anchored multicellular spheroids [27–29].

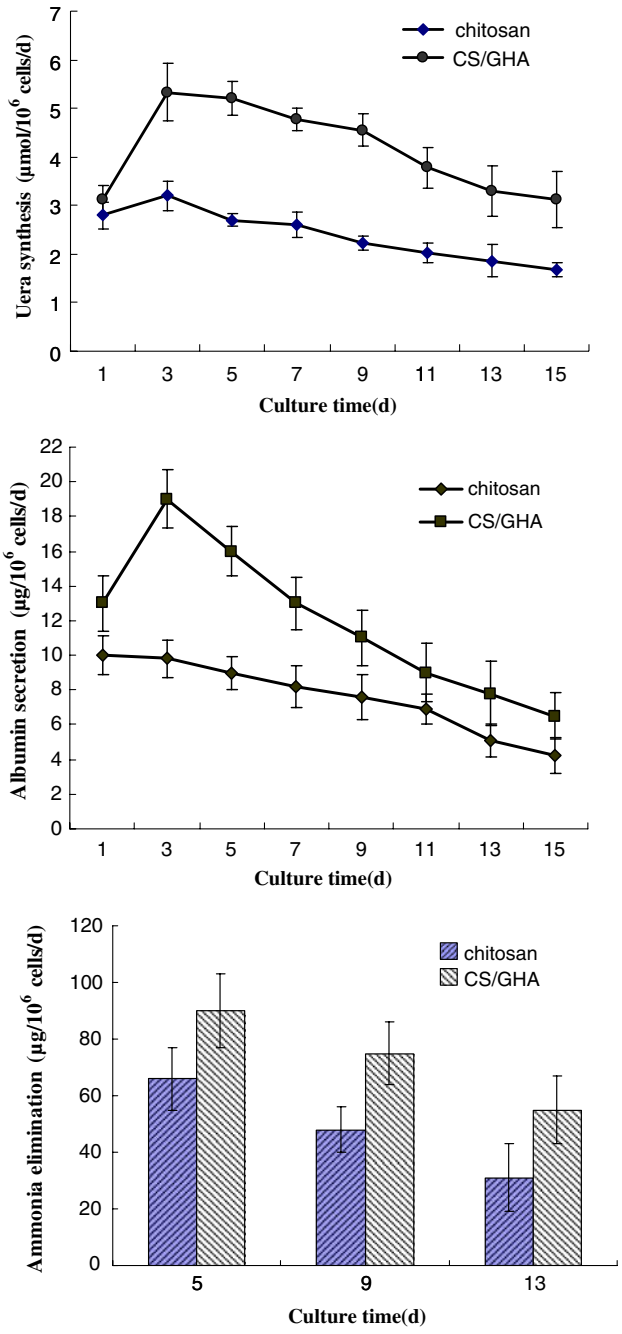

Fig. 8 Comparision of liver-specific functions: urea synthesis, albumin secretion, and ammonia elimination in chitosan and CS/GHA sponges. Date are represented as mean ± $SD_{(n-1)}$ ($n = 5$)

3.8 Metabolic activities

Metabolic activities were investigated in terms of albumin secretion which is specific to liver, urea synthesis which represents the function of detoxification and ammonia elimination which supposes the ability to cope with the microenvironment. It is well known that multicellular spheroids of hepatocytes with 3D structures maintain hepatic functions to a great extent [30–32]. The hepatocyte spheroids were observed within CS/GHA sponges (Fig. 7b) while not in chitosan sponges in same culture conditions. A comparison of hepatocyte metabolic activities between the chitosan sponges and the CS/GHA ones is shown in Fig. 8. The results indicated that the albumin secretion and urea synthesis of the chitosan sponge rapidly decreased with culture time whereas those of the CS/GHA sponge slowly decreased and maintained higher levels than those of the chitosan. During the first 3 days, the albumin secretion increased within CS/GHA scaffolds to a peak value, following the secretion slowly decreased with time. While the albumin secretion decreased in chitosan sponges without peak value in culture period. As shown in Fig. 8, cells on CS/GHA scaffolds had much higher activity for urea synthesis and ammonia elimination than those on chitosan scaffolds.

4 Conclusion

Galactose moieties were covalently coupled with hyaluronic acid in order to enhance the interaction of hepatocyte with scaffold materials. Hepatocyte specific porous scaffold of CS/GHA sponges were fabricated by freeze-drying technique. Morphology of CS/GHA sponge could be controlled by content of GHA and freezing temperature. The addition of GHA significantly improved the wettability and flexibility of chitosan scaffold system. And the CS/GHA scaffold induced the formation of cellular aggregates with enhancing liver specific metabolic activities and improved cell density to a higher level. So the CS/GHA porous scaffolds would be a promising scaffold system for the artificial liver support system.

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