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Preparation and characterization of a chitosan/galactosylated hyaluronic acid/heparin scaffold for hepatic tissue engineering

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Preparation and characterization of a chitosan/galactosylated hyaluronic acid/heparin scaffold for hepatic tissue engineering

Abstract:

Cell culture microenvironment and hepatocyte-specific three-dimensional tissue-engineering scaffold play important roles for bioartificial liver devices. In the present study, highly porous sponge scaffolds composed of chitosan (CS) and galactosylated hyaluronic acid (GHA, galactose moieties were covalently coupled with hyaluronic acid through ethylenediamine), were prepared by freezing-drying technique. Because the growth factors specifically bind to heparin with a high affinity and biological stability of the growth factors are modulated by heparin. Heparin was added into CS/GHA scaffold under mild conditions. The effects of heparin on the morphology, structure, porosity, mechanical properties of the CS/GHA/heparin scaffold were studied. CS/GHA scaffold containing heparin maintains the porous structure and good mechanical properties. Furthermore, addition of heparin with the growth factors into the scaffold resulted in a significantly improved the microenvironment of cell growth and prolonged liver functions of the hepatocytes

such as albumin secretion, urea synthesis and ammonia elimination. These results indicate that this CS/GHA/heparin scaffold is a potential candidate for liver tissue engineering.

Keywords: chitosan; galactosylated hyaluronic acid; heparin; EGF; hepatocyte; scaffold

1. Introduction

Cells, scaffold materials and growth factors are the three basic elements of tissue engineering. In a typical tissue engineering approach, a highly porous three-dimensional (3D) scaffold is designed and constructed firstly. 3D structure provides better physical and mechanical microenvironmental surroundings for cell proliferation and differentiation similar to that of naturally occurring extracellular matrix (ECM) [1-5]. An ideal scaffold for liver tissue engineering need stable 3-D spatial microenvironment to mimic the organized architectures of native liver, and highly open porous structure for cell migration and nutrient diffusion besides good biocompatibility and biodegradability [6-8]. In addition, scaffolds require specific interaction with ECM components, growth factor, and the cell surface receptor to culture anchorage-dependent cells such as hepatocytes [9-11].

Biomaterials play an important role as scaffolds in the synthesis of ECM environments for tissue regeneration [12]. The ECM components of the target tissue should be mimicked by the scaffolds [13]. Hyaluronic acid is glycosaminoglycans (GAGs) in the matrix of embryonic and fetal tissues in the liver, and chitosan is similar to GAGs, the components of liver ECM, which makes them the ideal scaffold

materials for hepatic tissue engineering [14-19].

In our previous work, chitosan (CS)/ galactosylated hyaluronic acid (GHA) scaffolds were suitable for improving hepatocytes adhesion and maintaining liver functions [20]. In order to adjust the interaction of hepatocytes with the scaffold, we have modified materials such as chitosan, alginate and hyaluronic acid with galactose moieties as a specific adhesive ligand to asialoglycoprotein receptor (ASGP-R) of hepatocyte[21-24]. Previous work confirmed that CS/GHA scaffolds as a synthetic ECM can improve hepatocyte maintain better activities than chitosan and chitosan/ hyaluronic acid scaffolds.

Furthermore, the scaffold should provide the optimal microenvironment for the seeded cells [25]. The addition of growth factors is necessary for cell culture in vitro, which contribute to tissue regeneration at various stages of cell proliferation and differentiation. But one of the reasons for limiting use of growth factors is the very short half-life time in vitro to keep their biological activities [26, 27]. The growth factors, such as EGF, FGF, HGF, are known to be stored in the body, interacting with GAGs such as heparin. These growth factors specifically bind to heparin with a high affinity, and both the mitogenic activity and biological stability of the growth factors are modulated by heparin. Heparin also protects the growth factors from inactivation by acid and heat as well as from degradation by proteases [28-30]. In this study, heparin molecules were introduced into CS/GHA scaffolds, which is one of the GAGs.

In this study, we first prepared highly porous 3D sponges composed of CS, GHA

and heparin by freezing-drying technique. Then epidermal growth factor (EGF) was introduced into scaffolds through the high affinity of heparin with growth factors. Final we compared the liver-specific functions of hepatocytes between CS/GHA and CS/GHA/heparin sponges.

2. Materials and methods

2.1. Materials

Chitosan was obtained from 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). Heparin sodium was purchased from Sigma Chemical Co. (MO, USA). GHA was synthesized from HA and lactobionic acid according to the method described previousely [20]. The content of galactose moieties in the GHA was evaluated by NMR and element analysis and it showed that 15% of carboxylic acid in HA reacted with L-NH_2. Heparin Binding EGF was purchased from Cytolab Co. (Rehovot, Israel). ELISA kit for EGF was obtained from Jingmei Biotechnology Co. (Jiangsu, China). All other chemicals were purchased from Shenggong Biology Engineering Co. (Shanghai, China) unless otherwise stated.

2.2. Preparation of scaffolds and membranes

CS/GHA/heparin sponges were fabricated using the freeze-drying technique. Briefly, CS acetate solution (2 wt%), GHA aqueous solution (0.4 wt %) and heparin aqueous solution (0.2wt%, 0.4 wt %) were mixed, to a fixed volumn ratio of 2:1:1 and stirred with a homogenizer at a speed of 24000 rpm at room temperature for 5 min.

0.5mL mixed solution was poured into 24-well polystyrene culture plates (Costar Co., Ltd). The plates were frozen at -20°C for 12 h and 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.

CS, CS/GHA and CS/GHA/heparin monolayer membranes were prepared by pouring 1 mL mixing solutions in 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.

2.3 Morphology of the scaffolds

Scaffolds were fractured in liquid nitrogen, and then samples were sputter coated with gold. The morphology of CS, CS/GHA, and CS/GHA/heparin 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 30 pores. Porosity was determined by liquid displacement method.

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

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

2.5 Wettability of the membranes

The static contact angles of CS, CS/GHA, and CS/GHA/heparin membranes were measured using a contact angle goniometer (Harke-Spca, Baoding, China). Every data presented were the mean values of at least five independent measurements.

2.6 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 three specimens (thickness, 10 mm; diameter, 15 mm; compress rate, 0.5mm/min) in the dry state.

2.7 Binding and release of EGF from CS/GHA/heparin scaffolds

The CS/GHA/heparin scaffolds were immersed in an EGF solution (100ng/mL, 2mL) for 4 h at 4 °C in the 24-well polystyrene (PS) plate. Then the scaffolds were rinsed 3 times with PBS. The scaffolds binding EGF were frozen at -20°C for 12 h and then lyophilized in a freeze-dryer. The CS/GHA scaffolds were added 0.5 mL EGF solution (400ng/mL) as control group, then frozen and lyophilized in a freeze-dryer.

The amount of EGF released from the scaffolds was determined by ELISA. Each scaffold was immersed in 2 mL of William's E (WE) medium in the 24-well plate. The medium was changed every 48 h, and then the amount of EGF in the medium was analyzed by ELISA.

2.8 Hepatocyte isolation and culture

Hepatocytes were isolated from the liver of a male Wistar rat by perfusion the liver with a two-step collagenase perfusion technique of Seglen. Then hepatocytes were purified by a density-gradient centrifugation 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 plate filled with 1ml of WE medium. The cells were cultured with serum free WE containing antibiotics (50μg/ml penicillin, 50μg/ml streptomycin and 100μg/ml neomycin), HEPES (18mM), EGF (10 ng/ml), insulin (100nM), and incubated in a humidified atmosphere of 5% CO_2 and 95% air, at 37 °C, with medium changes every 48 h.

Hepatocytes were seeded onto different membranes coated 6-well PS dish at 5×10^4 cells/ml in Williams' E medium without serum. After the prescribed time, the plate was washed with PBS solution to remove free non-adhered cells. The MTT assay was used as a measure of relative cell viability.

The morphologies of hepatocytes within sponges were observated by SEM (Hitachi X-650, Japan).

2.9 Measurement of liver- specific functions of hepatocytes in the sponges

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

Liver functions of the hepatocytes such as albumin secretion, urea synthesis and ammonia elimination in the scaffolds were determined by the method described in our previous work [20].

3. Results and discussion

3.1 Morphology of porous 3D scaffolds

The polymer scaffolds for use in liver tissue engineering should be highly porous with large surface/volume ratios. The highly porous 3D sponges were fabricated using

the procedure of freezing and lyophilizing the frozen mixed solution (1wt% CS, 0.1wt% GHA, 0.1wt% heparin). The freezing and lyophilization techniques created openpore microstructures with a high degree of interconnectivity.

Fig. 1 shows the cross-sectional structures of the CS (A), CS/GHA (B), and CS/GHA/heparin (C) sponges. These three types of sponges exhibited 3D and highly porous honey-like microstructure with interconnected pores and pore sizes ranging from 100–200 μm in diameter, as estimated by SEM analysis. The pore sizes of liver tissue engineering scaffolds were proved that the pore size large than 100μm provided optimum viability and function, with no mass transfer limitations [31, 32].

Table 1 shows the porosity and pore size of sponges subjected to varying heparin content. All porosity values are above 90%, and the addition of heparin does not affect significantly the porosity of the scaffolds. The average pore sizes have a little amount of increase after the addition of heparin in the CS/GHA sponges. Polyelectrolyte complex was thought to have formed between CS and GHA/heparin. The treatment of CS with GHA/heparin changed the morphology of sponge to have larger pores and more connected structure compared to the CS sponge. The 0.1% heparin content in CS/GHA/heparin scaffold was selected as test scaffold, which porosity was 91% and average pore size was 162μm.

3.2 ESCA survey scan spectra analysis

The changes in surface chemical composition of the scaffolds were further investigated by ESCA spectra. Fig. 2 shows that all scaffolds surface have carbon (binding energy, 284 eV), oxygen (binding energy, 530 eV), and nitrogen (binding

energy, 397 eV) characteristic peaks, and composite scaffolds containing heparin have sulfur (binding energy, 166 eV) characteristic peak. Table 2 shows the surface elemental composition of different scaffolds. The content of oxygen and nitrogen in the CS/GHA/heparin scaffold decreased, and the content of sulfur increased with the addition of heparin. These changes correspond to the increased heparin contents. Furthermore, with the increase of the addition amount of heparin, the sulfur content also increased, fully proved that the scaffold prepared by freezing-drying technique, the surface exists a considerable amount of heparin. For the next step the sponge can provide sufficient binding sites for growth factors.

3.3 Mechanical properties of the matrices

The mechanical properties of the scaffolds in tissue engineering 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 [33]. It is still a great challenge to porous scaffolds prepared with natural polymers to maintain excellent mechanical properties. The Young's modulus of the sponges is shown in table 3. The data showed that the mechanical strength of the CS is higher than that of the CS/GHA and CS/GHA/heparin. The addition of GHA/heparin in CS networks can reduce mechanical strength, while increase the flexibility of the sponges. After equilibration in water, the hydrated sponges appeared to be transparent and displayed a soft consistency. There were slight differences of mechanical properties between Cs/GHA/heparin sponge and CS/GHA sponge. Under

in vitro cell culture conditions, the chitosan-based sponges were sufficiently stable to hold the cells for more than 45 d, and did not contract as some collagen-based materials do, and did not as the alginate-based sponges lost part of their mechanical strength once wet. In this research, the CS/GHA/heparin scaffolds maintain the suitable mechanical properties for soft liver tissue engineering.

3.4 Wettability

The water contact angle on the surface of the materials can reflect their average hydrophilic property from the macro. And the physical and chemical properties of the material microscopic area that can be detected by contact with water from the micro. 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 CS membranes and they became smaller with the increase in contents of GHA in previous research. The hydrophilicity of the scaffolds was further improved with the addition of heparin in CS/GHA sponges. Fig. 3 shows the static contact angles of CS, CS/GHA, and CS/GHA/heparin membranes. The contact angle of the membrane was reduced from 62° to 55° and 49° with the more binding of heparin. The results suggested that the hydrophilicity of Cs/GHA/heparin membranes were much improved by addition of heparin.

3.5 The release of EGF from CS/GHA/heparin/EGF composite scaffold

The main objective of CS/GHA scaffold added heparin is to be effective in combination with growth factors. Because heparin can bind to growth factor specifically, protect growth factors from the external environment, and prevent the

degradation of the activity. Growth factor binding to heparin composite scaffold can also realize the controllable release of time and space, and a certain concentration in a long time, so as to stimulate cell growth and differentiation [34, 35].

The experimental group scaffolds (CS/GHA/heparin/EGF) and the control group scaffolds (CS/GHA/EGF) were immersed in 2 mL of William's medium E (WE) in the 24-well plate. The medium was changed every 48 h, and then the amount of EGF in the medium was analyzed by ELISA. Fig. 4 shows the release curve of EGF from scaffolds. On the first day there was a clear burst release behavior in the control group, EGF release amount reached 136 ng, followed by a rapid decline, third days for 8 ng, fifth days to 3ng, seventh days to 0.7 ng, ninth days to 0.06 ng. The behavior of the EGF release in the experimental group containing heparin was different from that of th former. The CS/GHA/heparin sponge maintained stable EGF release amount about 13ng in the first eleven days, thirteenth days to 4 ng, fifteenth days to 0.9 ng. The results show that the scaffolds with heparin can maintain the activity of growth factors for a long time, which can help to improve the microenvironment of cell culture in vitro.

3.6 Hepatocyte culture

In order to investigate the effect of adhesion between hepatocytes and materials, we add the freshly isolated rat hepatocytes into different materials coated PS dishes. The MTT test is an indirect method of assaying cell growth and proliferation since the A_{490} values can be correlated to the cell number. Fig. 5 shows the result of MTT assay as a measure of cell viability of hepatocytes adhesion on different materials coated

surface. We define the hepatocytes adhesion to chitosan-coated PS dish was 100%, and compare the other materials coated PS dish with the former. The cell attachment to CS-coated PS dish was low, while the cell attachment significantly increased with the addition of GHA to CS. It was already reported that hepatocytes adhesion to galactosylated material-coated dish was galactose-specific recognition between material molecules and the ASGPR of hepatocytes[20-23]. The addition of heparin has slightly effect on hepatocytes adhesion because GHA play a major role on the adhesion of hepatocytes.

Fig. 6 shows the scanning electron micrographs of hepatocytes attached into the sponges after culturing 3 days. Cell morphology was observed to understand behavior of the hepatocytes in the sponges. The results showed that the number of cells within CS/GHA/heparin scaffold was more than others. In addition, more hepatocytes aggregated to form multicellular spheroids in CS/GHA/heparin sponge. The formation of hepatocyte spheroids in CS/GHA/heparin scaffold is attributed to introduction of GHA into the chitosan system. In addition heparin can maintain the activity of growth factors in medium for a long time, which can help to improve the microenvironment of cell culture in sponges.

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. A comparison of hepatocyte metabolic activities between the CS, CS/GHA and CS/GHA/heparin sponges is shown in Fig. 7. It is well known that multicellular

spheroids of hepatocytes with 3D structures maintain hepatic functions to a great extent [36-38]. The results indicated that the albumin secretion, urea synthesis and ammonia elimination of the CS sponge rapidly decreased without peak value in culture period. The albumin secretion and urea synthesis increased within CS/GHA scaffolds to a peak value during the first 3 days, whereas the peak value of the CS/GHA /heparin sponge at 5 day. As shown in Fig. 7, cells on CS/GHA/heparin scaffolds had much higher activity than those on chitosan scaffolds.

4. Conclusion

Hepatocyte specific porous scaffold of CS/GHA/heparin sponges were fabricated by freeze-drying technique. The scaffolds maintain a honey-like structure, good hydrophilicity and good mechanical properties. Addition of heparin in the scaffolds can maintain the activity of growth factors for a long time, which can help to improve the microenvironment of cell culture and function of cell in vitro. And the CS/GHA/heparin 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/heparin porous scaffolds would be a promising scaffold system for the artificial liver support system.

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Table 1 Porosity and average pore sizes of scaffolds

Table 2 Surface elemental composition of scaffolds from ESCA spectra

Table 3 The Young's modulus of scaffolds

Fig. 1 Scanning electron micrographs of the cross-sectional structures of the sponges (×200). A: 1wt% CS；B: 1wt% Cs+0.1% GHA；C：1wt% Cs+0.1% GHA+0.1% heparin.

Fig. 2 ESCA spectra of scaffolds. A: CS/GHA; B: heparin; C: CS/GHA/heparin (0.05wt%); D: CS/GHA/heparin (0.1wt%)

Fig. 3 Water contact angles of Cs/GHA/heparin membranes

Fig. 4 The release curve of EGF from scaffolds

Fig. 5 MTT assay as a measure of cell viability of hepatocytes adhesion on different materials coated surface.

Fig. 6 SEM of hepatocytes cultured in scaffolds. A: CS scaffold×800; B: CS/GHA scaffold×1000; C: CS/GHA/heparin scaffold×1000.

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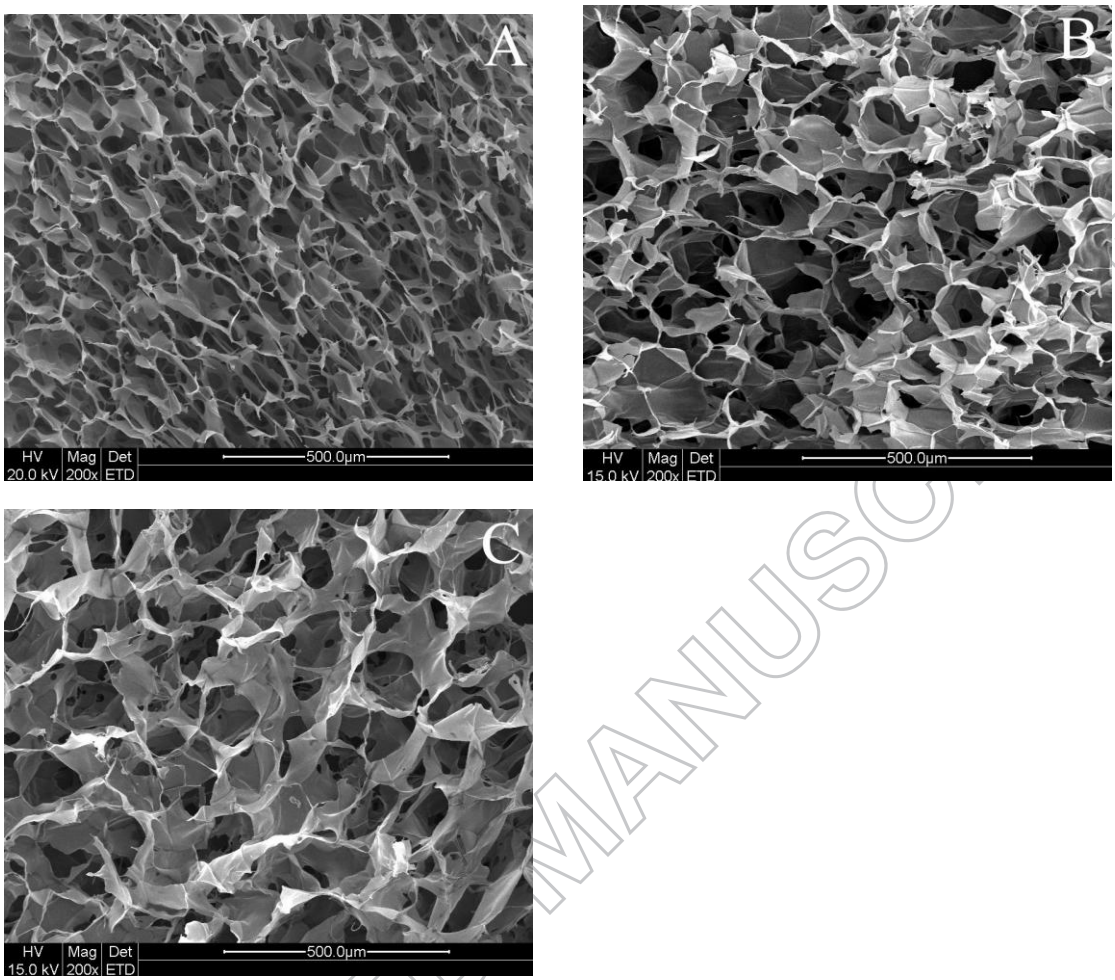

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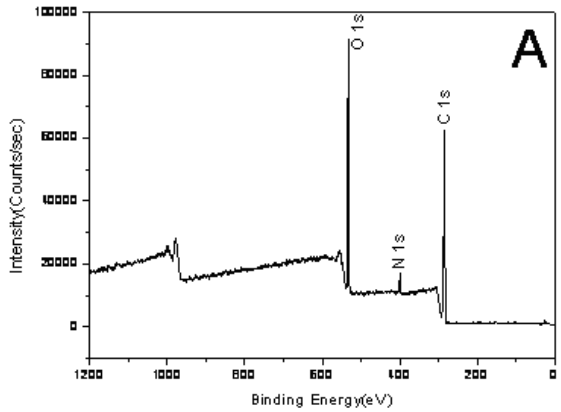
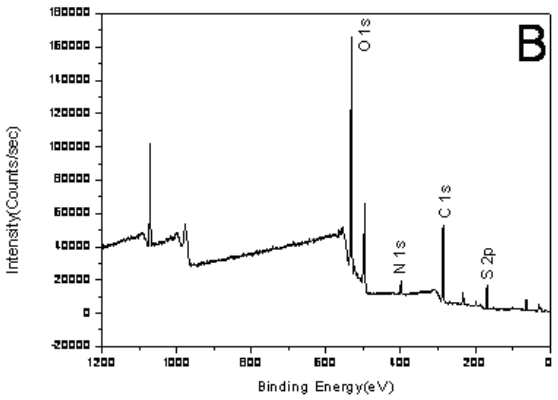
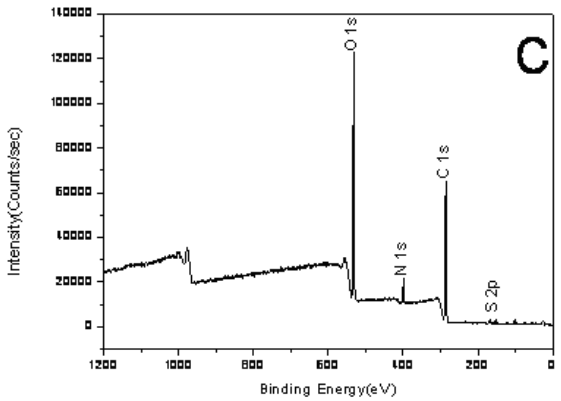






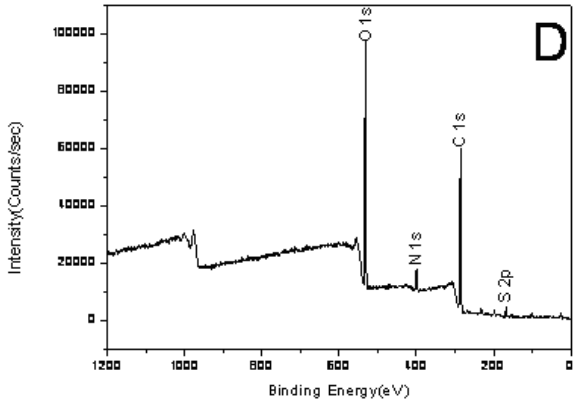


Fig. 2 ESCA spectra of scaffolds. A: CS/GHA; B: heparin; C: CS/GHA/heparin (0.05wt%); D: CS/GHA/heparin (0.1wt%)

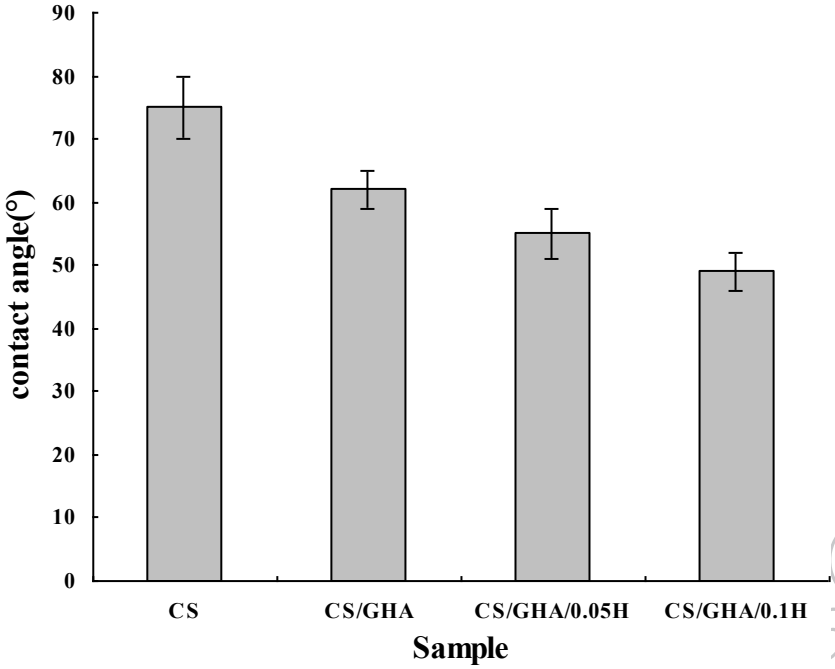


Fig.3 Water contact angles of Cs/GHA/heparin membranes

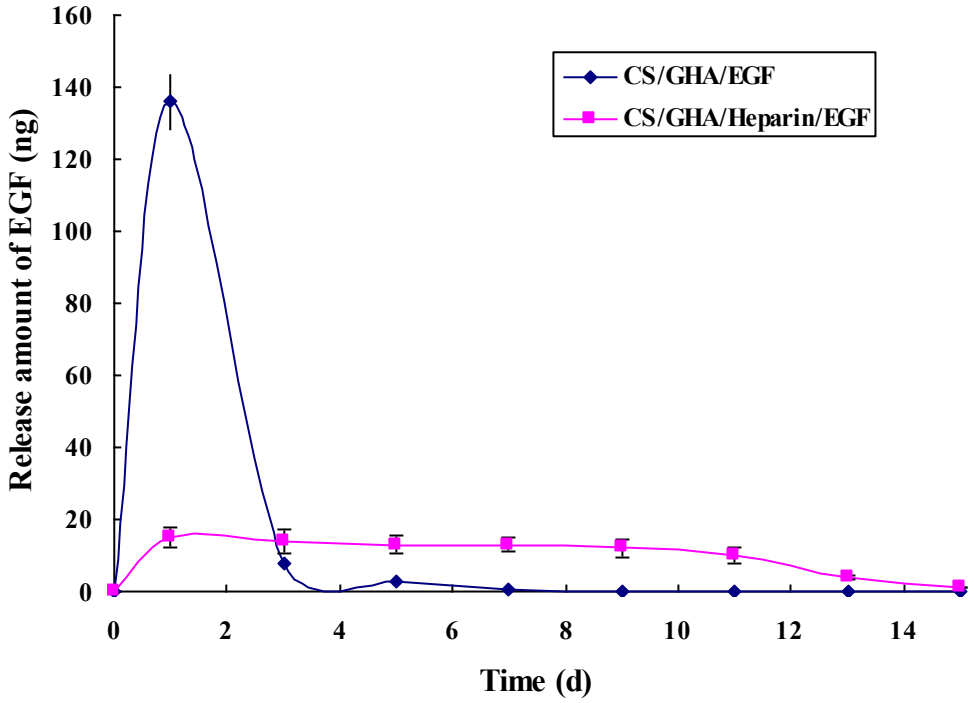


Fig. 4 The release curve of EGF from scaffolds

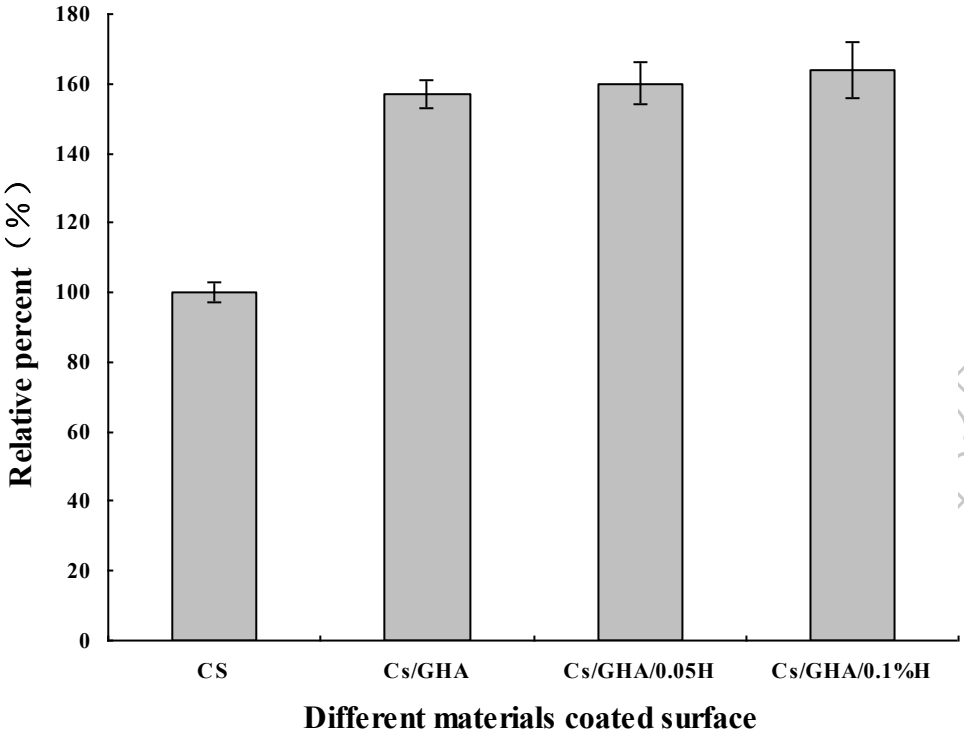


Fig. 5 MTT assay as a measure of cell viability of hepatocytes adhesion on different materials coated surface.

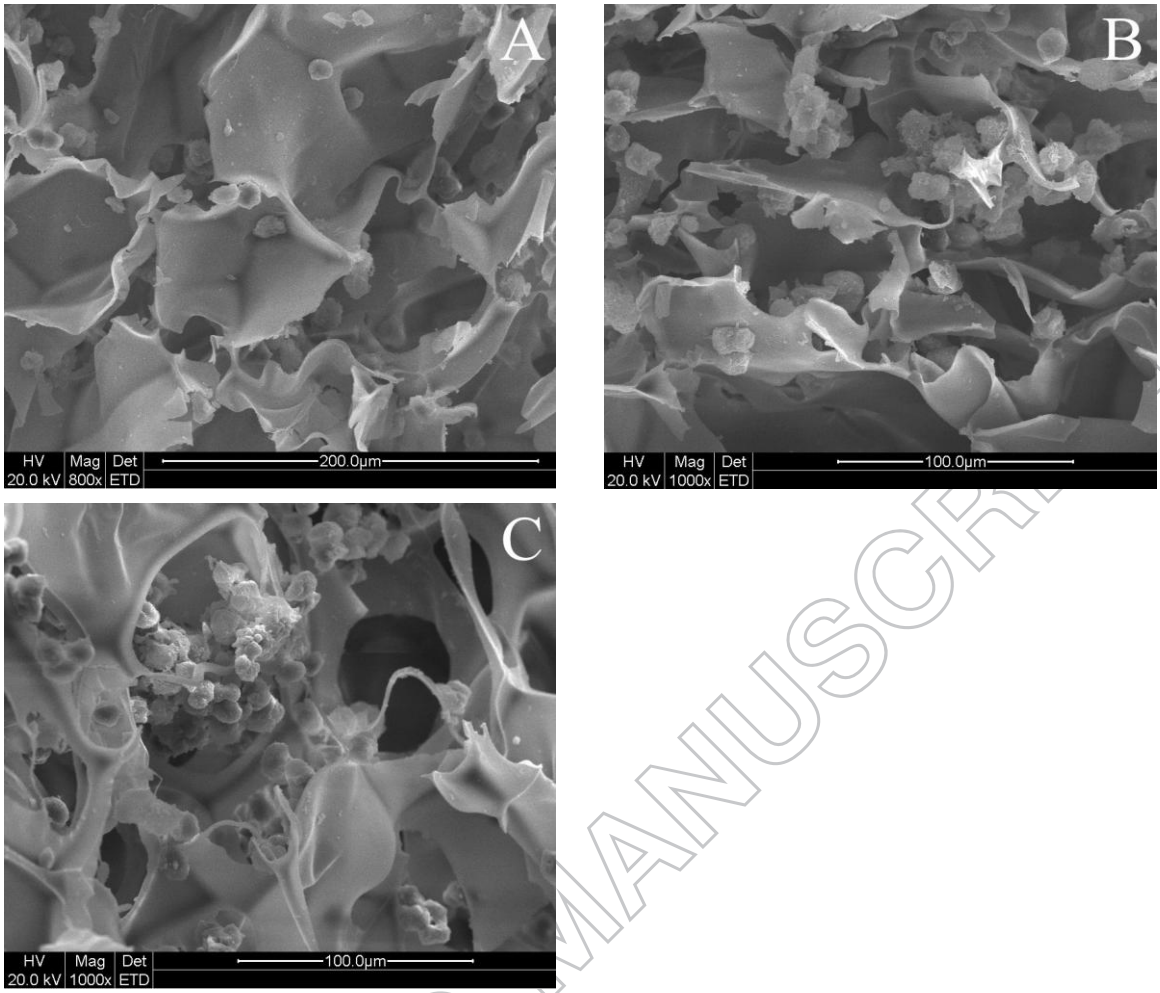
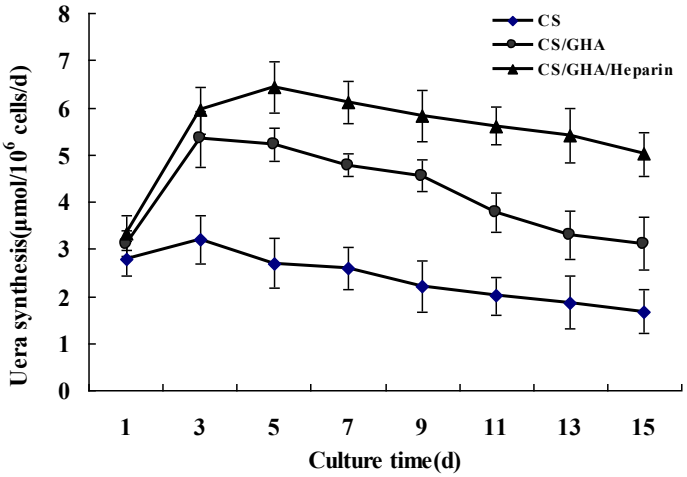
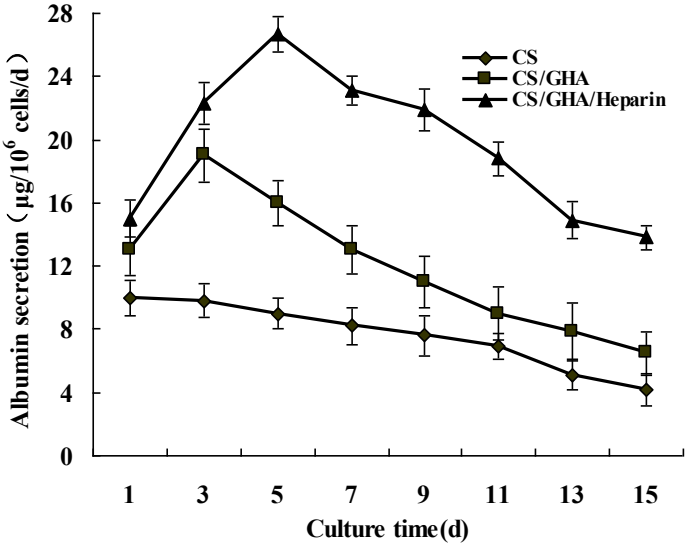


Fig. 6 SEM of hepatocytes cultured in scaffolds. A: 1wt% CS scaffold×800; B: 1wt% Cs+0.1% GHA scaffold×1000; C： 1wt% Cs+0.1% GHA+0.1% heparin scaffold×1000.





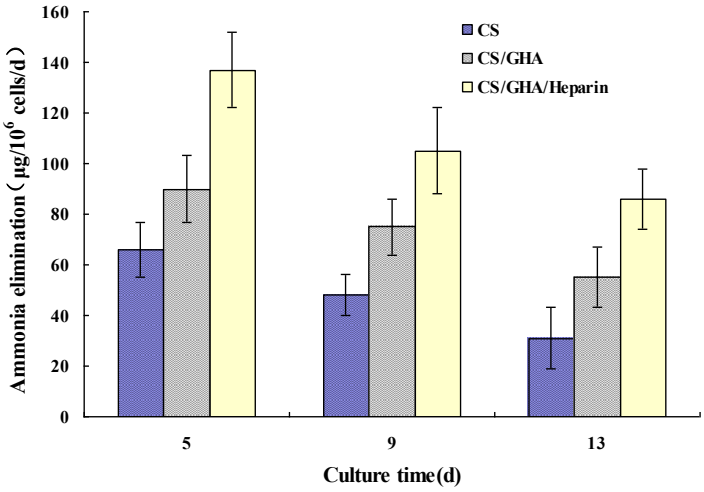


Fig. 7 Comparision of liver-specific functions: uera synthesis, albumin secretion, and

ammonia elimination in sponges.

Table 1 Porosity and average pore sizes of scaffolds

Table 2 Surface elemental composition of scaffolds from ESCA spectra

Table 3 The Young's modulus of scaffolds

Table 1 Porosity and average pore sizes of scaffolds

Scaffold	Porosity（%）	Average pore size（μm）
CS	89.2±1.2	112±43.5
CS/GHA	90.1±1.5	140±62.3
CS/GHA/heparin(0.05wt%)	90.4±1.4	153±64.2
CS/GHA/heparin(0.1wt%)	91.2±1.3	162±59.2

Table 2 Surface elemental composition of scaffolds from ESCA spectra

Sample	Composition (%)			
	O	N	C	S
Cs/GHA	39.29	6.36	54.35	0
Cs/GHA/hep(0.05)	36.23	5.51	56.89	1.38
Cs/GHA/hep(0.1)	33.81	5.89	57.64	2.66

Table 3 Mechanical properties of the scaffolds The of scaffolds

Scaffold	Young's modulus (MPa)
CS	0.38±0.015
CS/GHA	0.23±0.020
CS/GHA/heparin(0.05wt%)	0.21±0.018
CS/GHA/heparin(0.1wt%)	0.20±0.019