INTRODUCTION

Liver tissue engineering is a promising scientific approach that attempts to create functional cellular constructs by utilizing artificial extracellular matrix (ECM) [1]. The artificial ECM should be designed to establish an appropriate microenvironment, which could promote to form liver-like tissues and maintain their differentiated functions [2-5]. It is becoming increasingly clear that the microenvironment around cells plays an important role in regulating the cellular behaviors, including proliferation and growth, survival, morphology, migration, and differentiation [6,7].

Among different ECM used in liver tissue engineering, alginate hydrogels have been extensively used in regenerative medicine applications [3,4]. However, the drawback of alginate hydrogels is a low cell attachment rate as a result of the formation of a hydrated and electronegative surface layer [8]. It should be noted that cell anchorage is a strict requirement for survival of most cell types [9]. Hepatocytes are attachment-dependent cells...
and lose their liver-specific functions without anchoring on a proper ECM [2]. Thus, it is necessary to modify the alginites by immobilization of cell recognition motives [8,10,11].

Recently, it has been realized that even in the condition of absenting modification of cell-adhesive proteins, cells can adhere to the surface of materials bearing charges [12-14]. For example, neurite outgrowth occurred to a greater extent on positively charged fluorinated ethylene propylene (PEP) films than on negatively charged or uncharged films [15]. The osteoblast and fibroblast showed an enhanced attachment to films with incorporation of positively charged monomers [16]. In contrast, Chen et al. reported that the endothelial cells exhibited a large spreading area when the zeta potential was lower than -14 mV [17]. It was found that the dynamic cell behavior was related with the charge densities on the surface of materials [18,19]. However, few studies about the effect of surface charges on the hepatocytes have been conducted. In the present study, the dynamic behavior of hepatocytes affected by the surface charge density was investigated.

We hypothesized that alginate hydrogels could be copolymerized with poly-L-lysine (PLL) to produce a positively charged hydrogel as a substrate to enhance hepatocytes attachment and differentiation. In this study, the composite hydrogels composed of alginate and PLL were firstly fabricated, and then the surface charges were determined by zeta potential measurements. Furthermore, the effects of surface characteristics on the protein adsorption and cellular behavior were evaluated. The results showed that both the protein adsorption and cellular behaviors were influenced by the surface charges. It suggested that the surface charges might modulate the protein adsorption, which further influence the behavior of cells.

**MATERIALS AND METHODS**

**Materials**

Alginates (ALG) with a low content of guluronic acid were purchased from Qingdao Crystal Salt Bioscience and Technology Corporation (Qingdao, China). Poly-L-lysine (PLL, Mw 29,000), Fibrinogen (Bovine Plasma) was purchased from Sigma. Fibrinogen at 0.5 and 1.0 mg/mL in 0.9 % w/v NaCl solution was prepared in sterile conditions (by 0.22 μm filtration) before use. Fibrinogen at 0.5 and 1.0 mg/mL in 0.9 % w/v NaCl solution was prepared in sterile conditions (by 0.22 μm filtration) before use. Fibrinogen holds a negative net charge at pH=7.4. In all solutions, pH was adjusted to 7.4 by using acetic acid and sodium hydroxide solutions.

**Preparation of alginate-based hydrogels**

The alginate solution was cast onto a dry glass slide. The glass slides with sodium alginate solution were then immersed in a 50 mmol/L CaCl₂ solution. After being gelled for 30 min, the alginate hydrogels (control group) were fabricated. In order to fabricate the ALG/PLL (AP) films, the slides with gel were incubated in PLL solution (1mg/ml in 0.9 % w/v NaCl solution) to form membrane on the gel surface. The slides with membrane were subsequently rinsed with 0.9% NaCl solution. Furthermore, the ALG/PLL/ALG (APA) films were formed through counteracting excessive charges of AP films by a 0.15% alginate solution. The films were then washed with a large amount of double distilled water.

**Zeta potential measurements**

Zeta potential is an important and useful indicator of surface charge. The zeta potentials of membrane were determined using the SurPASS Electrophoretic Analyzer (Anton Paar GmbH, Austria) equipped with a slit-type channel. The streaming potential was measured by the Ag/AgCl electrodes. Membrane material served as the top and bottom of the channel. Before starting the measurement, the membranes were rinsed with double distilled water to remove NaCl. A background electrolyte of 1 mmol/L KCl solution was used. The zeta potential was obtained from the streaming potential measurements based on the Smoluchowski equation [20,23]. Since the surface conductivity of the membranes cannot be determined directly, the zeta potential obtained from the streaming potential measurement is considered an apparent value.

**Measurement of protein adsorption onto films**

Fibrinogen was used for the protein adsorption assay. The adsorption experiments were performed in 10 mmol/L phosphate buffer (KH₂PO₄/Na₂HPO₄) in order to keep a constant value during the adsorption process. The membranes were immersed in buffer solution containing different amounts of protein at 37 °C for 24 h. Protein concentration of the centrifuged supernatant was measured by the Bradford method. The protein adsorbed on membranes was calculated using the following equation:

\[ q = \frac{(C_f - C_i) V}{m} \]

Where \( \frac{(C_f - C_i) V}{m} \) is the initial protein concentration and \( C_f \) is the protein concentration in the supernatant after adsorption studies, respectively. \( V \) is the volume of the solution and \( m \) is the surface area of membranes.

**Cell culture and microscopy**

Human hepatocarcinoma HepG2 cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) were cultured in MEM medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. Cells were seeded with 2.0×10⁵ cells/well of the 24-well plate. They were incubated in a humidified incubator at 37 °C in 5% CO₂ and 95% air atmosphere. Half of the medium was refreshed every day, and the morphology of the entrapped HepG2 cells was observed under an optical microscopy (Olympus CK40, Tokyo, Japan).

**Cell proliferation and viability**

After detaching cells, cell number assessed using a hemocytometer with trypan blue. In order to evaluate the cell viability, 100 µL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] solution (5 mg/mL Sigma) was added into each well of a 24-well culture plate and incubated at 37°C for 4 h. The medium was removed and replaced with dimethyl sulfoxide (DMSO, Sigma) to solubilize the MTT tetrazolium dye. The absorbance was determined at 570 nm using a plate reader (Infinite F50, Tecan Co., Switzerland). The cell viability was calculated by the absorbance per 2×10⁶ cells. Results were expressed as mean±SD for three replicates.

**Realtime RT-PCR**

Two-step quantitative real-time reverse transcription
polymerase chain reaction (qRT-PCR) was performed on RNA according to the manufacturer’s instructions (PrimeScript<sup>TM</sup> RT reagent Kit, SYBR Premix Ex Taq<sup>TM</sup>, TaKaRa). After cultured for 5 days, total cellular RNA in different groups was extracted with Trizol agent. Total RNA was quantified by UV spectrophotometry. RNA was reverse-transcribed with oligo (dT) primers and then amplified. The thermal profile was 37 for 15min, then 85 °C for 5s, followed by 40 amplification cycles consisting of 95 °C for 30s, 60 °C for 30s, and 72 °C for 1 min. All primers were synthesized by Takara.

**Albumin enzyme-linked immuno-sorbent assay**

Cell culture supernatants were collected and albumin concentration determined by a human albumin enzyme-linked immuno-sorbent assay (ELISA) kit as the manufacturer’s instructions (Bethyl, Montgomery, TX, USA). The absorbance was determined using a plate reader (Infinite F50, Tecan Co., Switzerland).

**Statistical analysis**

All experiments were performed at least three times. All values were expressed as means±standard deviations. Statistical analyses were performed with Student’s t-test, p<0.05 being regarded as statistically significant.

**RESULTS**

**Zeta potential analyses**

Charged films are characterized by their zeta potentials, which are the potentials at the hydrodynamic slipping plane adjacent to the phase boundary. Figure 1 displayed the different zeta potential of alginate-based films including alginate hydrogels (control group), ALG/PLL (AP) and ALG/PLL/ALG (APA). All of the alginate-based films showed negative zeta potentials at pH 7.4, indicating that these films had a net negative charge at neutral pH. The zeta potential of alginate hydrogels was -10.6 mV, followed in turn of -3.5 mV for AP and -6.4 mV for APA. The results indicated a varying degree of net negatively charged groups on different surface of alginate-based films.

**Protein adsorption on the surface of alginate-based films**

Protein adsorption on the surface of alginate-based films with different terminal layer was studied at pH 7.4 (Figure 2). When the initial protein concentration was 1.0 mg/mL, the amount of adsorbed Fgn on AP films was higher than those on APA films and alginate hydrogels at pH 7.4. The amount of adsorbed Fgn on alginate hydrogels and APA films showed no statistically difference. When the initial protein concentration was 0.5 mg/mL, the amount of adsorbed Fgn was lower than that when the initial protein concentration of 1.0 mg/mL; however, the amount of adsorbed Fgn onto AP films was still highest. The results indicated that the terminal layer of films had great effects on the amount of adsorbed protein.

**Morphology of cells**

The morphology of cells was influenced by the surface charges on alginate-based films (Figure 3). In the control group, the morphology of cells was spherical and no cell adhered on the surface of pure alginate hydrogels during the whole culture time. Cells moved to form incompact cell aggregates, which were easily dispersed. However, on the AP films, most of cells firstly adhered on the surface and showed spread morphology. Then cells proliferated as two dimensional (2D) spread. Eventually they became a monolayer cultures with some multi-aggregates. In addition, the growth pattern and morphology of cells cultured on the APA films were distinct from those on the AP films. On the APA films, cells firstly formed multi-aggregates, which adhered on the surface. After that, the cells proliferated as 3D culture and the size of cell aggregates gradually increased.

**Proliferation and viability of cells**

The cell proliferation and cell viability during the culture time were shown in (Figure 4A and Figure 4B), respectively. In the culture system of alginate hydrogels, the number of cells almost remained unchanged during the whole culture time. It indicated that there was no cell proliferation. The growth of cells in the AP and APA culture systems was different than that in the alginate...

Figure 4 The proliferation and viability of HepG2 cells cultured on different films. A: The proliferation of cells. The (*) indicates that the cell proliferation on AP films is significantly higher than that on APA films and pure alginate hydrogels (control group) (*p<0.05). The (#) indicates statistical APA films significance relative to the control group (#p<0.05). B: The viability of cells. The (*) indicates that the cell viability on both the AP and APA films is significantly higher than that on pure alginate hydrogels (control group) (*p<0.05).
hydrogels culture system. Cells adhered on the AP films and showed proliferative tendency. After that, the number of cells increased dramatically and reached a maximum at about day 4. The number of cells was also increased on the APA films during the whole culture time, but its proliferative rate was lower than that in the AP culture system.

The viability of cells in each culture system was evaluated by using the MTT assay. Figure 4B shows the distinctive viability of the cells in each type of culture. During the whole culture time, the viability of cells in the control group was gradually decreased. However, the viability of cells in both the AP and APA film systems was increased at the beginning of culture. After culture for 2 days, the cellular viability in both groups was maintained at high level, which was about twice more than that in the control group. In addition, there was no significant difference in the viability between the AP film and APA film.

Gene expression analysis

Figure 5 shows the gene expression of HepG2 cells in different cultures. The expression products of these genes engage in xenobiotic and synthetic metabolism. In this study, the types of culture systems did not alter the expression the housekeeping gene, GAPDH. Most of gene expression in the APA group was higher than those in the AP group. The results show the xenobiotic metabolism-associated transcription factors in cells cultured on APA films versus AP films (CYP1A1 3.6-fold, CYP3A4 4.5-fold, EPHX1 5.5-fold, and UGT1A 2.9-fold). In addition, the gene expression of synthesis-associated transcription factors in cells cultured on APA films versus AP films is GSTA1 7.2-fold, NDUFA3 1.1-fold, and GCLM 4.0-fold, respectively. It suggests that the functional gene expression may be susceptible to the surface charges of films.

Albumin secretion

The specific rate of albumin secretion by the cells to the external medium, considered as an indicator of hepatocellular synthetic function, was tested by ELISA. The amount of albumin secretion in single cells was determined to assess the synthetic function of cells (Figure 6). At the beginning of the culture, the albumin secretion in different culture system was similar. As the culture time went on, the albumin secretion rate in the control group gradually decreased. However, both the albumin secretion rates in the AP and APA culture systems showed increasing tendency. The rate in the APA films was significantly higher than that in the AP films. After 6 days' culture, the albumin secretion per cells on the APA films was about twice more than that on the AP films. Therefore, the APA films might provide an ideal microenvironment, which was conducive to maintaining the liver-specific function for hepatocytes.

DISCUSSION

It is well known that the cell attachment depends on the properties of biomaterial surface. Adhesion of cells to the biomaterials plays an important role in influencing the dynamic behaviors of cells. The surface charge on the material surface is an important factor mediating the fates of cells. In this study, we fabricated a composite film composed of alginate and PLL. Through controlling the surface composite, the effect of surface charges on the adhesion, morphology, and phenotypic functions of hepatocytes was investigated.

Richert et al. have reported that pure electrostatic repulsion and attraction forces between cell membranes and the films determined the cellular attachment [19]. However, increasing evidence has shown that adsorption of proteins on the surface of biomaterials plays an important role in mediating the behavior of cells. When the biomaterials come into contact with the host environment in vivo or the cells in vitro, adsorption of proteins on the surface of biomaterials is generally regarded as the trigger event [6]. The protein adsorbing will define cellular adhesion and the subsequent biological event. Cells are highly dependent on specific proteins for anchorage and extracellular instructions and thus, the composition of the adsorbed layer is a key point of cell behavior. Thus, to better understand cell/biomaterial interaction,
the protein adsorption on the surface of materials should not be ignored [20, 21].

Various proteins may participate in the process of protein adsorption [24]. However, exploring the response of a single protein is a prerequisite to understand the mechanism of this process. The fibrinogen was generally considered as a model to study the protein adsorption on surface. A recognized viewpoint was that the positively charged groups on the surface of films increased the amount of adsorbed protein [22,23]. Our results further demonstrated that electrostatic interaction was a driving force for the adsorption of proteins on the surface of alginate-based films.

Fibrinogen can combine with the integrin on the cellular membrane. Thus, the adsorption of fibrinogen on the films may further promote the cell attachment. Our results showed that the attachment of HepG2 cells was correlated with the adsorption of fibrinogen. Furthermore, the morphology of cells is mainly determined by the force balance between cell-cell and cell-substrata. When cell contractile forces are greater than cell-substrata adhesion forces, spherical aggregates form; when cell contractile forces are weaker than cell-substrata adhesion forces, cells remain essentially spread [25]. In summary, the cells tend to attach, proliferate and form flat monolayer cultures, but exhibit limited function on APA films. In contrast, cells aggregate and exhibit improved function, but proliferate slowly on APA films. The cell-substrata interaction promotes the growth of cells and the cell-cell interaction maintains the function of cells [26]. One possibility is that the close cell-to-cell contact imposed on proliferating cell colonies may encourage formation of intercellular connections and maintains cell polarity.

CONCLUSION

In this work, we demonstrated that the behavior of HepG2 cells could be modulated via tuning the surface charges of alginate-based hydrogels. By cross-linking with the PLL, AP and APA films were fabricated, respectively. The surface of alginate-based hydrogels was endowed with different charges. Protein adsorption assay showed that fibrinogen deposition could be modulated via the terminal layer and the surface charges. Furthermore, HepG2 cells tend to attach, proliferate and form flat monolayer cultures, but exhibit limited function on AP films, while cells aggregate and exhibit improved function, but proliferate slowly on APA films. It suggests that the behavior of HepG2 cells is correlated with the adsorption of fibrinogen, which is influenced by the surface charges on the surface. The results have significant implications for understanding the effect of biomaterials’ surface charges on the protein adsorption and behavior of cells, and providing a platform for the future design of tissue engineering.

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REFERENCES


