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Research Article

Synthesis of Thermal Polymerizable Alginate-GMA Hydrogel for Cell Encapsulation

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Alginate is a negative ionic polysaccharide that is found abundantly in nature. Calcium is usually used as a cross-linker for alginate. However, calcium cross-linked alginate is used only for *in vitro* culture. In the present work, alginate was modified with glycidyl methacrylate (GMA) to produce a thermal polymerizable alginate-GMA (AA-GMA) macromonomer. The molecular structure and methacrylation (%DM) of the macromonomer were determined by ^{1}H NMR. After mixing with the correct amount of initiator, the AA-GMA aqueous solution can be polymerized at physiological temperature. The AA-GMA hydrogels exhibited a three-dimensional porous structure with an average pore size ranging from 50 to 200 μm, directly depending on the macromonomer concentration. Biocompatibility of the AA-GMA hydrogel was determined by *in vivo* muscle injection and cell encapsulation. Muscle injection *in vivo* showed that the AA-GMA solution mixed with initiator could form a hydrogel *in situ* and had a mild inflammatory effect. Human umbilical vein endothelial cells (HUVECs) were encapsulated in the AA-GMA hydrogels *in situ* at 37°C. Cell viability and proliferation were unaffected by macromonomer concentrations, which suggests that AA-GMA has a potential application in the field of tissue engineering, especially for myocardial repair.

1. Introduction

Hydrogels derived from organic materials such as polysaccharides are promising materials for tissue-engineered scaffolds and have attracted many interests in the field of tissue engineering. Natural materials have many desirable characteristics such as their three-dimensional porous structure, biodegradability, good water absorption, and tissue-like elastic properties [1–7]. These properties make hydrogels ideal candidates for creating environments where cells and tissues can grow [8]. Alginate is an anionic polysaccharide produced by brown algae that is widely used in pharmaceutical and biomedical applications. More recently, *in situ* alginate hydrogel has been used in growth factor delivery [9] and ventricular remodeling for myocardial infarction [10] and showed promising results. Ionic cross-linked alginate such as calcium alginate is highly characterized and has been used for multiple tissue engineering applications [11–14]. While this ionic cross-linked alginate cannot gelate *in situ*, the alginate solution required treatment by calcium solution *in vitro* before the cross-linked hydrogel applied *in vivo*.

Recently, synthesis of a polysaccharide modified with thermal cross-linkable or photo-cross-linkable groups, such as an acrylic or a methacrylic group, may provide an alternative method for hydrogel formation [15–22]. Most of these works focused on photo-cross-linking, which utilizes ultraviolet light to initiate a free radical polymerization reaction, forming covalent cross-links through the acrylate or methacrylate groups [23, 24]. These free radical-cross-linked hydrogels allow macromonomers to gelate *in situ* and enables the hydrogels to form different shapes to better match the implantation site [25]. Although these photo-cross-linked hydrogels have many useful properties and have been used as cell carriers for several tissue engineering applications including cartilage, bone, nerve, and liver [26–36], one concern with the photo-cross-linking process is the potential

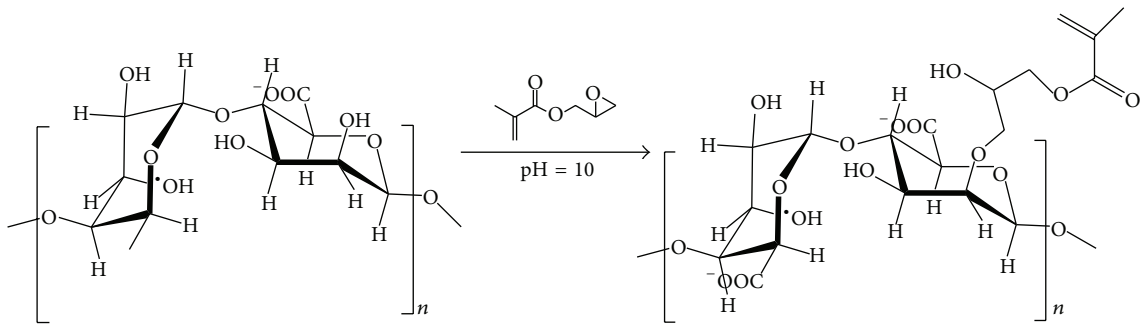

Figure 1: Reaction scheme of the macromonomer AA-GMA.

damage to the cells encapsulated in the macromonomers. More importantly, since we cannot use the UV photo-cross-linking process *in vivo*, this limits the use of those hydrogels as injectable hydrogels and therefore limits their therapeutic application.

In this study, thermal polymerizable alginate-GMA macromonomers were synthesized. Thermal initiator was used to avoid UV irradiation, which is harmful to the cells and human, and allows the macromonomers to polymerize at physiological temperature. The mild reaction conditions enable cell and protein encapsulation as well as *in vivo* gelation. The properties of alginate-GMA were investigated extensively, including gelation time, swelling ratio, and porosity. Cell viability and proliferation were assessed using the human endothelial cell lines HUVEC and L929 in preliminary tests for the potential application of alginate-GMA as an injectable hydrogel for use in tissue engineering, especially for myocardial repair.

2. Materials and Methods

2.1. Materials. Sodium alginic acid (Sigma Aldrich), glycidyl methacrylate (GMA) (Fluka), NaOH (Beijing Chemical Works), ammonium persulfate (APS), and N′,N′,N′,N′-tetramethylethylenediamine (TEMED) (Sigma Aldrich) were used. H-DMEM (GIBCO), FBS (MEDgenics Inc.), penicillin, streptomycin, haematoxylin-eosin, propidium iodide, and calcein-AM and MTT (Sigma Aldrich) were also used.

2.2. Synthesis of AA-GMA Macromonomers. Briefly, 2 g of sodium alginic acid was dissolved in distilled water to make a 0.02 g/mL solution of alginate. 5.68 g glycidyl methacrylate (GMA) was added to the alginate solution and thoroughly mixed with stirring. 5 M NaOH was added to adjust the pH to 10. The reaction was conducted at 60°C under N_2 protection for 4 hours. The solution was precipitated with an excess of ethanol. The product was thoroughly washed by ethanol 5-6 times and freeze-dried under vacuum (Figure 1).

2.3. Thermal Polymerization of AA-GMA. To prepare the hydrogels, AA-GMA macromonomers were dissolved in distilled water at concentrations of 0.03 g/mL to 0.07 g/mL; 5% (w/w of AA-GMA) of APS and TEMED were added to the solution. The solution formed hydrogels in a water bath at 37°C in 5 to 20 minutes.

2.4. ^1H NMR. Sodium alginic acid and AA-GMA samples were dissolved in D_2O at a concentration of 0.005 g/mL. ^1H NMR spectra were recorded with a 400 MHz Bruker AV400 NMR spectrometer. The percentage of double bonds was calculated using the formula below (HAA represents H atoms in the alginate backbone; Ha represents H atoms in the methyl group of GMA):

$$\mathrm{DM\%} = \frac{\text{Average}\,[\mathrm{Ha}/3 + (\mathrm{Hb} + \mathrm{HC})/2 + \mathrm{Hd}/6]}{(\mathrm{HAA} - 1)/6}. \quad (1)$$

See [22].

2.5. Surface Morphology. The surface morphology of freeze-dried thermal cross-linked AA-GMA hydrogels was characterized with a Quanta 200 FEG scanning electron microscope operated at 20 kV.

2.6. Swelling Assay. A weighed amount of dry thermal cross-linked AA-GMA hydrogel was placed in an excess volume of 1X PBS for 24–120 hours. The surface of the hydrogel was blotted with tissue paper before the wet weight was measured. The equilibrium swelling ratio (q) was calculated as the ratio of the wet weight to the dry weight:

$$q = \frac{W_w}{W_d}. \quad (2)$$

2.7. In Vivo Inflammatory Effects. Male Sprague-Dawley (SD) rats weighing about 250 g were used. One hundred microliters of 0.05 g/mL macromonomer solution with a premeasured amount of APS and TEMED was injected into inner side of each leg. Each week after injection, animals were euthanized and surrounding tissues were histologically processed using haematoxylin-eosin stains [37].

2.8. Cell Encapsulation and Cell Viability Assay. The human endothelial cell line HUVEC was used. Cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO_2 at 100% humidity. Different amounts of AA-GMA with APS and TEMED were

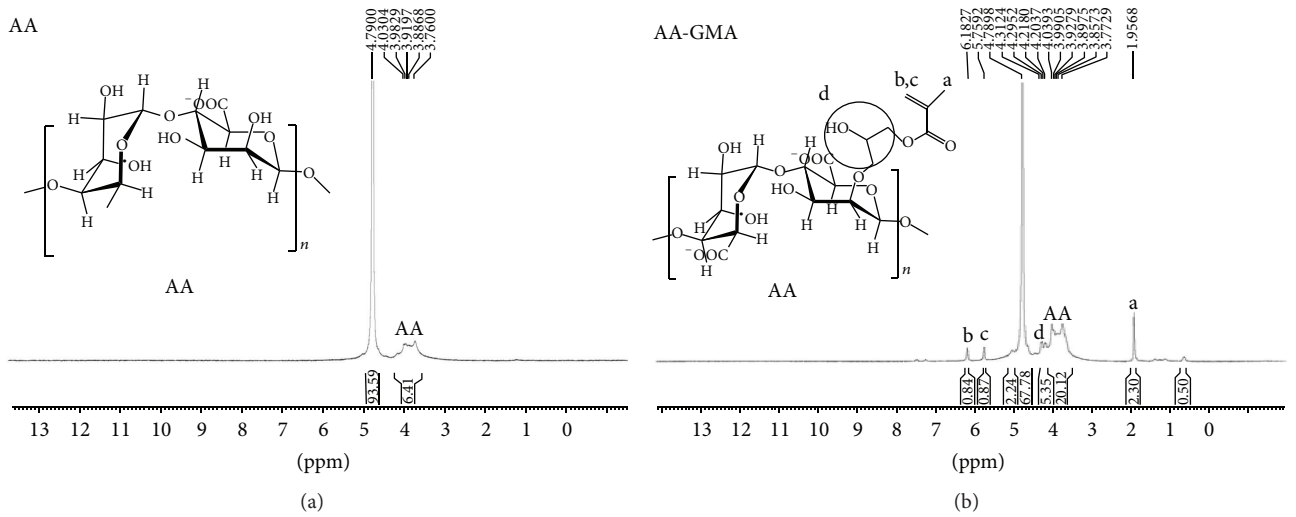

Figure 2: ^{1}H NMR spectra of (a) sodium alginic acid and (b) the AA-GMA macromonomer.

Table 1: Thermal polymerized AA-GMA hydrogels investigated (0.5 mL aqueous solution).

Sample number	AA-GMA (g)	AA-GMA concentration (g/mL)	APS (g)	TEMED (μL)	Gelation time (min)
1	0.015	0.03	0.0015	1	22
2	0.025	0.05	0.0025	2	12
3	0.030	0.06	0.0030	3	8
4	0.035	0.07	0.0035	3	5

dissolved in 1.5 mL growth media. 1×10^6 cells in growth media were added to the solution and mixed evenly. 0.5 mL of this mixture was then added in 24-well cell culture plates and placed in incubator to let the polymerization occur. After 24 hours, hydrogels with cells were transferred into 6-well plates and immersed in culture media.

Cell viability and morphology were characterized at 24, 48, 72, and 96 hours after seeding. For the cell viability assay, cell-loaded gels were stained with calcein-AM and propidium iodide (PI) for 20 minutes at 37°C. All live cells were labeled with calcein-AM and dead cells with PI. Cells were imaged using a fluorescence microscope (OLYMPUS IX71). To analyze cell growth, the number of cells in an 8 mm^2 area was counted in 5 images from each sample. Due to the 3D structure, it was necessary to exclude cells outside of the focal plane, so the brightness threshold was set to eliminate the background light.

3. Results and Discussion

3.1. Synthesis and Characterization of AA-GMA Macromonomers and Hydrogels. The thermal polymerized AA-GMA macromonomers were synthesized through reaction of the epoxy group of GMA with the hydroxyl group of alginate under an alkaline condition. The structure of the AA-GMA macromonomer, shown in Figure 1, was confirmed by ^{1}H NMR spectroscopy in D_2O (Figure 2). The methacryloyl group of GMA was effectively attached to the available –OH groups of alginate via open-ring reaction of epoxy group [37]. Compared with the spectrum of alginate (Figure 2(a)), six new resonance peaks from the AA-GMA macromonomer appeared. This indicates that six different species of H appeared after the reaction, and this illustrates the mechanism of reaction as shown in Figure 1. From the ^{1}H NMR spectra of the AA-GMA macromonomers, the %DM is determined by using formula (1) shown before, and under these reaction conditions, the %DM is about 25.8%.

Three groups of hydrogels were made with three concentrations of macromonomer solutions and are listed in Table 1. The polymerization time was determined by a rheometer. The AA-GMA macromonomer underwent a free radical polymerization under thermal condition with the addition of initiator. According to Table 1, AA-GMA solutions of lower concentration have longer polymerization times, while AA-GMA solution of higher concentration has shorter polymerization times. This shows that, through controlling the macromonomer concentration, we can control the polymerization time to meet different needs.

SEM was used to characterize the morphology of the freeze-dried cross-linked AA-GMA hydrogel. As shown in

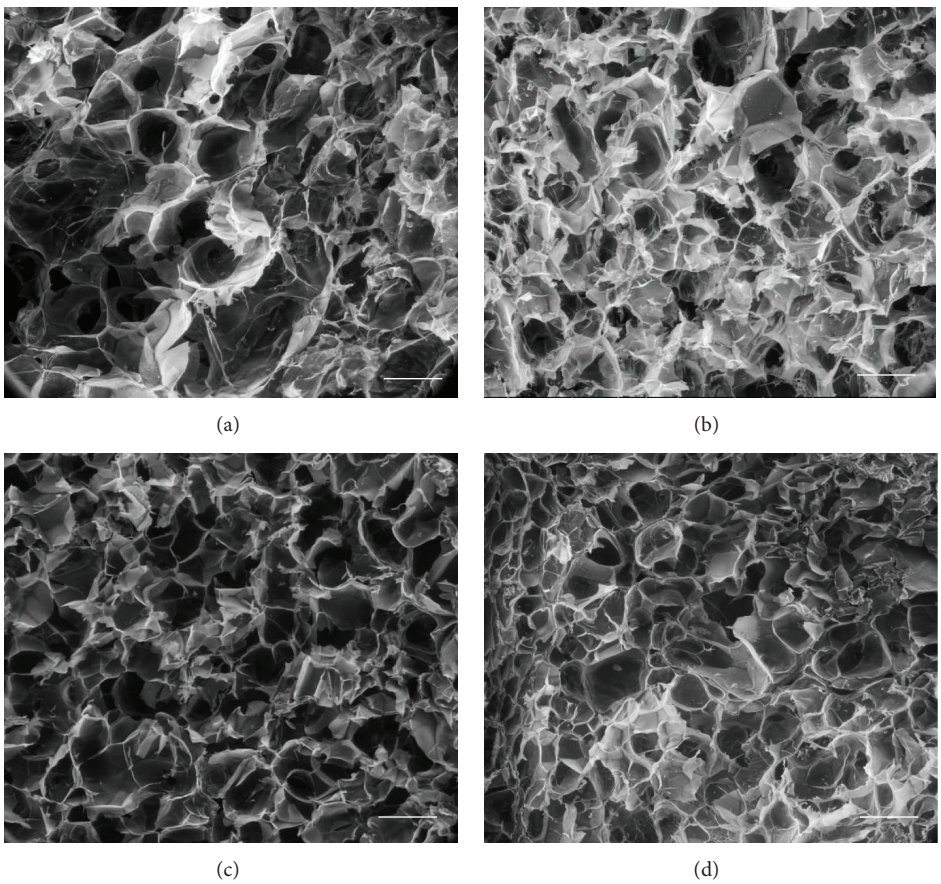

Figure 3: SEM images of the (a) 0.03 g/mL AA-GMA, (b) 0.05 g/mL AA-GMA, (c) 0.06 g/mL AA-GMA, and (d) 0.07 g/mL AA-GMA freeze-dried hydrogen. The bar is 100 μm.

Figure 3, the freeze-dried hydrogel has porous structure with pore size about 50–150 μm, which increased with deceasing gel content.

Swelling property influences the solute diffusion rate, the surface properties of the hydrogel, and the mass transport properties through the scaffold. Figure 4 shows the swelling property of AA-GMA hydrogels with different gel concentrations. Basically, the swelling ratio increased with decreasing macromonomer content in the gel. This results mainly because hydrogels with lower macromonomer concentrations have larger pore sizes, resulting in faster transport of water through the gel. Moreover, hydrogels with larger pores can retain more water compared to hydrogels with smaller pores.

3.2. In Vivo Inflammatory Assay. Male SD rats were injected with 0.1 mL of 0.04 g/mL AA-GMA macromonomer PBS solution with initiator for each leg. Each week the rats were euthanized and we found the AA-GMA macromonomer solution had formed a hydrogel in the muscle and the gel was clearly distinguishable. Figure 5 shows the inflammatory function of AA-GMA hydrogels *in vivo* in two weeks. All stained sections of the smears of AA-GMA hydrogels showed no dense collection of neutrophils and macrophages in two weeks. These observations show the AA-GMA hydrogel has a mild inflammatory effect, indicating good biocompatibility of the AA-GMA hydrogel.

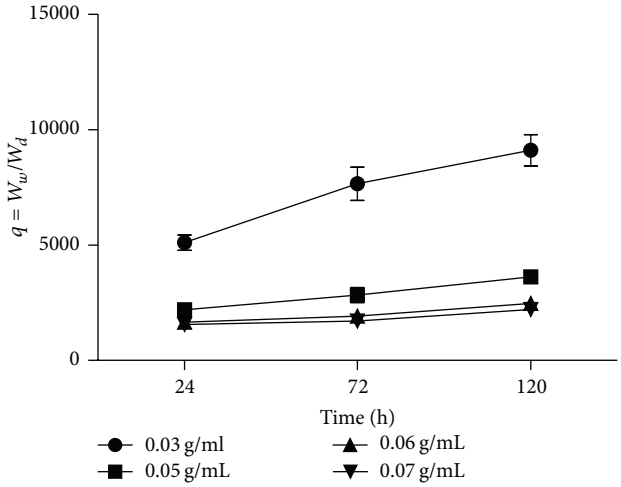

Figure 4: Swelling ratio of AA-GMA hydrogel of different concentration.

3.3. Cell Encapsulation. To evaluate the potential use of AA-GMA hydrogel as *in situ* cell matrices for tissue engineering

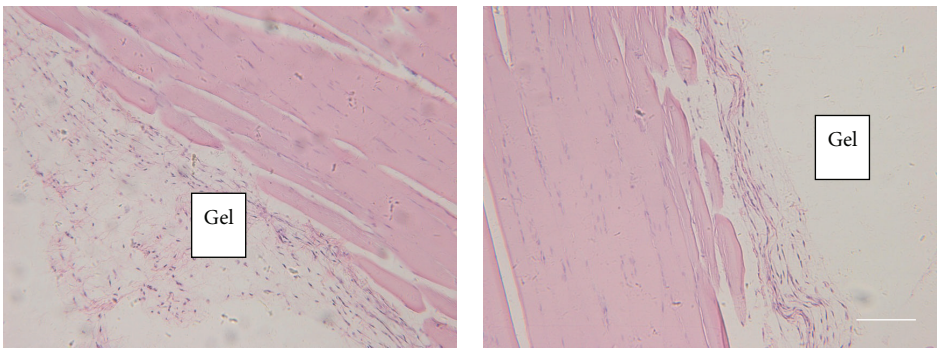

Figure 5: Representative hematoxylin-eosin stained sample of poly-AA-GMA hydrogel with subjacent muscle. Hydrogels removed (a) 1 week after injection and (b) 2 weeks after injection. The bar is 100 μm.

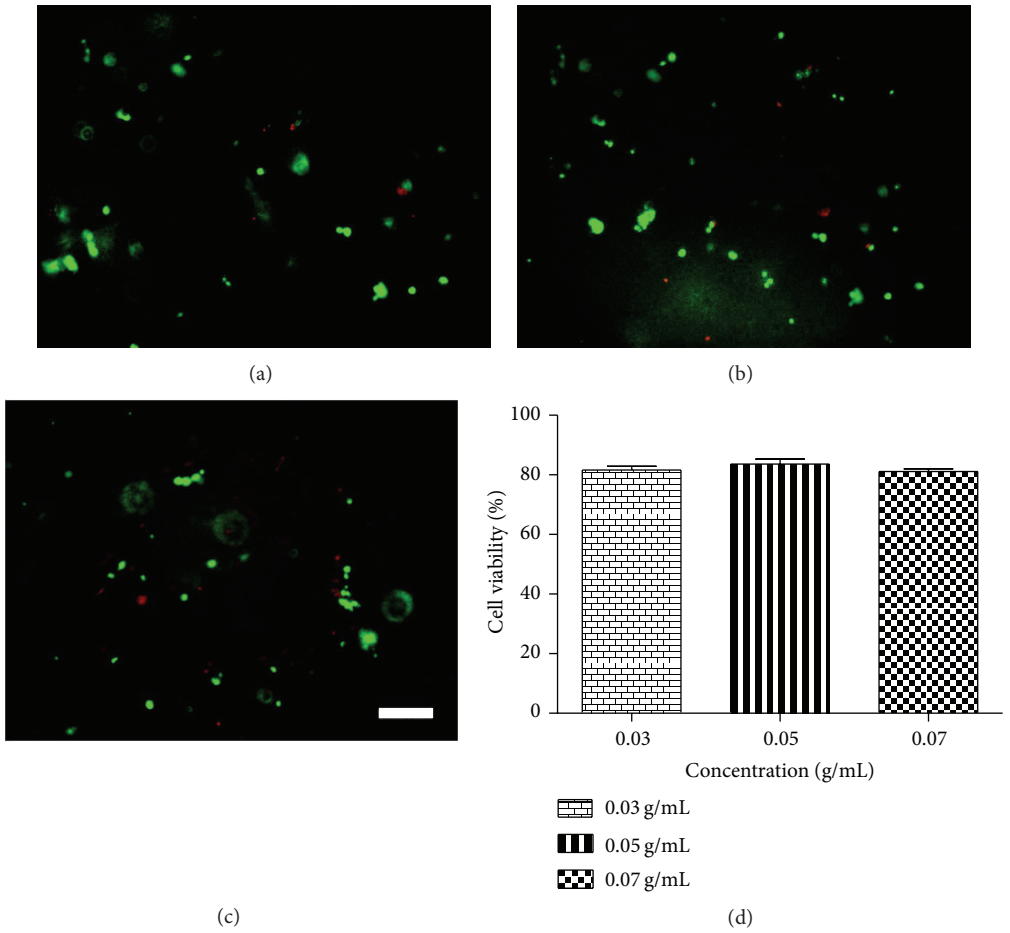

Figure 6: Cell viability of HUEVCs encapsulated in AA-GMA hydrogels at the (a) 0.03 g/mL for 24 h; (b) 0.05 g/mL for 24 h; (c) 0.07 g/mL for 24 h. The bar is 100 μm. (d) Live/dead assay; the histogram refers to the percentage of live cells in the different groups in 96 h.

applications, the human endothelial cell line (HUVEC) was used. This cell type may have a particular application in vascular grafting or promoting endothelialization of implants. Previous studies also indicated that methacrylated polysaccharide was cytocompatible [37]. For cell encapsulation, three different concentrations (0.03, 0.05, and 0.07 g/mL) of AA-GMA macromonomer were used. Cell viability was observed after thermal encapsulation. Figure 6 shows the cell viability of the three groups from 24 to 96 hours after seeding. Live cells were labeled by calcein-AM, and dead cells were labeled

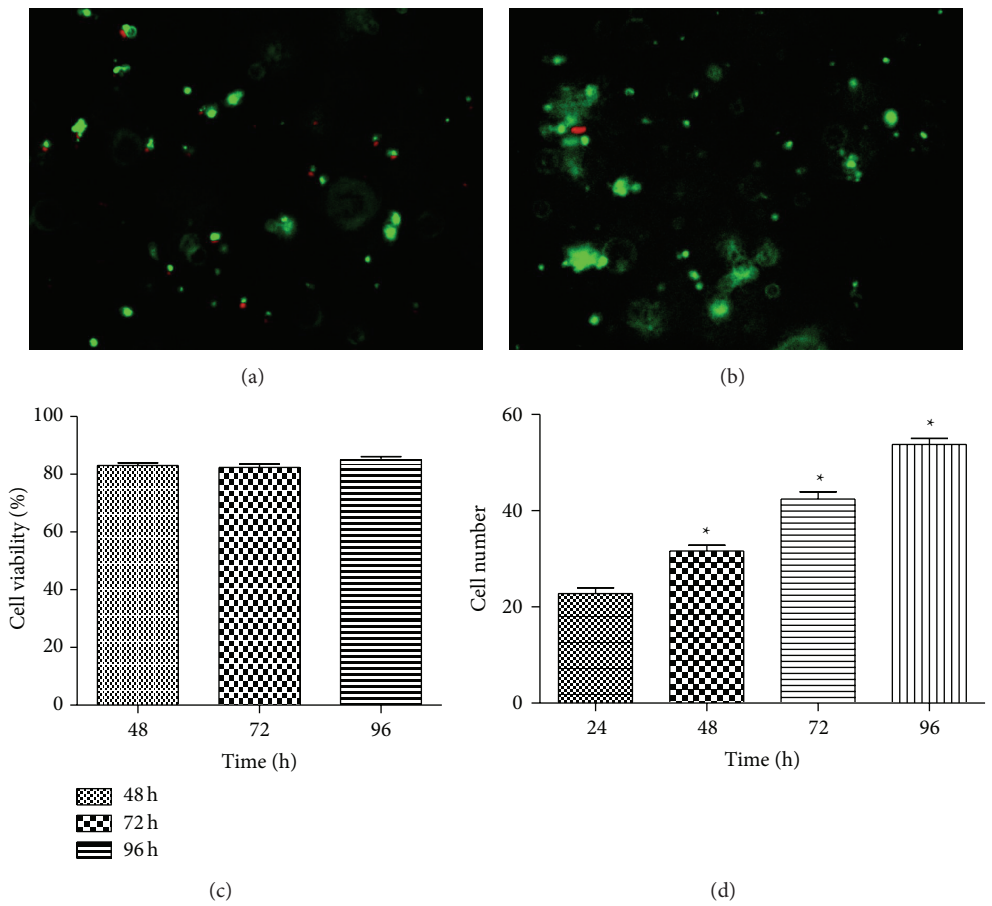

Figure 7: Cell growth of HUEVCs encapsulated in AA-GMA hydrogels at the (a) 0.05 g/mL for 24 h; (b) 0.05 g/mL for 96 h. (c) Cell viability in 0.05 g/mL hydrogels during 96 h. (d) Cell number in 0.05 g/mL hydrogels during 96 h. *Vs 24 h $P < 0.05$ using one-way ANOVA analysis.

by PI. After 24 to 96 hours in culture, three groups of encapsulated HUVECs all exhibited a high percentage of viability. There was no significant difference between the high concentration groups and low concentration groups while the higher concentration group had a little adverse effect in the first 24 hours (more red-labeled cells observed) (Figures 6(a), 6(b), and 6(c)); after 24 hours, the cells started to proliferate and the viability in 96 h showed no differences among the three groups (Figure 6(d)). Even when the higher concentration was used, few dead cells were observed, showing that AA-GMA hydrogels have good cytocompatibility. Since the cells were directly mixed with the reaction mixture during *in situ* gelation, it is possible that free radicals generated during the polymerization may have adverse effects on cell viability in the first 24 h [22]. This adverse effect may fade away with the disappearance of free radical. Figure 7 shows cell growth in the 0.05 g/mL AA-GMA group. Figures 7(a) and 7(b) show that the density of green fluorescence increases over time, which suggests an increase in cell proliferation in the hydrogels. Figure 7(c) shows that there are no significant changes in cell viability and Figure 7(d) shows that cell number increased during the 96-hour culture time. Both of the results illustrate that the 3D porous structure allows cell migration and nutrients transportation. Unlike the ionic hydrogels, which are usually premanufactured before application, such as alginate microbeads [4], this novel hydrogel can polymerize under physiological temperature and these observation demonstrate the hydrogel can not only be premanufactured to any structures but can also be a promising injectable hydrogel for *in situ* gelation for tissue engineering.

4. Conclusion

Alginate was modified with glycidyl methacrylate (GMA) to produce an alginate-GMA (AA-GMA) macromonomer, which polymerized at physiological temperature when using APS as an initiator and TEMED as a catalyst. AA-GMA hydrogels exhibited a three-dimensional porous structure with an average pore size ranging from 50 to 150 μm, and the pore size increased with deceasing macromonomer gel content. Muscle injection *in vivo* showed the AA-GMA solution mixed with initiator could form a hydrogel *in situ* and exhibited a mild inflammatory response. Human endothelial cells (HUVECs) were encapsulated in the AA-GMA hydrogels *in situ* at body temperature. Cell viability remained good at low and high macromonomer concentrations and cells

proliferated well in the hydrogels. These promising results show a potential use of AA-GMA hydrogels for *in situ* tissue engineering scaffold applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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