



GELATIN-ALGINATE SPONGE: A POTENTIAL SCAFFOLD FOR ADIPOSE TISSUE ENGINEERING

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ABSTRACT

Gelatin-alginate sponge (GA sponge) is one of the ideal materials to be studied and applied in soft tissue regeneration, especially in adipose tissue engineering. This sponge is made from the combination of gelatin and alginate with crosslinking activity of 1-Ethyl-(3-3-dimethylaminopropyl) carbodiimide. Gelatin and alginate were mixed at ratio 8:2 with 0.3% EDC and freeze-dried to form the GA sponge. After that, GA sponge was evaluated the structure by Hematoxylin and Eosin staining method and scanning electron microscopy imaging, the components by Fourier Transform Infra Red, the water absorption, *in vitro* biodegradation by using collagenase; *in vitro* cytotoxicity towards human fibroblasts. Finally, adipogenic differentiation potential of human adipose-derived stem cells inside the GA sponge was studied. The results show that the GA sponge with spongy and stable properties has high water absorption capacity, *in vitro* biodegradability, non-cytotoxicity. Simultaneously, human adipose-derived stem cells can adhere and differentiate into fat cells within the GA sponge. With these results, the GA sponge has properties suitable for applications in adipose tissue engineering.

KEYWORDS: Gelatin, Alginate, sponge, scaffold, tissue engineering, adipose derived stem cells.

INTRODUCTION

In recent years, adipose tissue engineering has been investigated in many researches with the goal is replacing the traditional method of tissue regeneration.^[5,17] This is a modern method, a potential development in aesthetic medicine as well as to heal defects in natural tissue regeneration. Three important factors in tissue engineering are cells, scaffolds and growth factors. In adipose tissue engineering, scaffolds can be made from many different materials and they need to have the properties required to ensure the stem cells can survive, adhere, proliferate and differentiate into fat cells and then regenerate tissue. Gelatin and alginate occupy a large part in the field of regenerative medicine.^[7, 23] Gelatin is derived from collagen so it is biocompatibility and biodegradability. In addition, gelatin also contains arginine-glycine-aspartic molecule segment (RGD) which forms the ligands for binding to receptors on the cell membrane in order to promote adhesion, migration, proliferation and differentiation of cells.^[10] Alginate polysaccharide does not cause an immune response and have biodegradability.^[15, 20] Although GA sponge has been extensively studied in different ratio of mixing, the role of this sponge as scaffold for tissue engineering has not been studied.^[5]

We made GA sponge and evaluated the properties of GA sponge to use it as a scaffold for adipose tissue engineering.

MATERIALS AND METHODS

Samples

The GA sponge was made from the mixing of gelatin (Sigma) and alginate (Sigma) with crosslinking of 1-Ethyl-(3-3-dimethylaminopropyl) carbodiimide (EDC) (Sigma). Human adipose derived stem cells (hADSCs) were supplied from the Laboratory of Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science, Vietnam National University at Ho Chi Minh City.

Making of the GA sponge

Gelatin and alginate is completely dissolved in distilled water at 50°C to create 1% gelatin and 1% alginate solutions. These solutions were then mixed with the ratio of 8 gelatin : 2 alginate in volume and incubated at -80°C in 24 hours. Next, these frozen blocks were incubated in 0.3% EDC for 24 hours at 4°C in dark condition and freeze-dried. Finally, GA sponges were sterilized by irradiation in 25kGy and cut into small samples of size 3x3x3mm³ for all experiments.

Structure of the GA sponge

The structure of GA sponge was studied by scanning electron microscopy (SEM) and H&E staining.

The composition of the GA sponge

The composition of GA82 sponge was analyzed by fourier transform infra red (FTIR) for determination of the existence of gelatin, alginate and the links between them in forming the GA sponge.

Water absorption ability of the GA sponge

The GA sponges were weighed to determine initial weight - dry weight (W_d). Then, sponges were soaked in 2ml distilled water, incubated at 37°C and continuous shaken (100 rpm) for 1 hour, 2 hours, 6 hours, 12 hours, 18 hours and 24 hours. The excess water of sponges was removed on filter paper and weighed for wet weight (W_w). The experiment was repeated in 3 times. The increase of sample weigh is the volume of water

impregnated into the sponge, the degree of water absorption was calculated by the formula.

$$\text{Water uptake ability(\%)} = \frac{W_d - W_w}{W_d} \times 100$$

Cytotoxicity of the GA sponge

Toxicity of GA sponge was assessed by direct contact method according to ISO 10993-5: 2009 for assessing toxicity *in vitro*. Cultured hDASCs reached 80% confluence of dish. GA sponge was placed directly on the surface of cells in the culture dish. After 24 hours, we put out the sponge and observed the appearance of cell damage in the area around the sponge. The experimental results were compared with cell samples without contact to GA sponge. Latex rubber that caused cytotoxic towards cells was used as positive group. Toxicity was evaluated by cell damage under the table of ISO 10993-5: 2009.^[21]

Table 1. Reactivity grades for the direct contact test

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mid	Zone limited to the area under the specimen
3	Moderate	A zone extending specimen size up to 1.0 cm
4	Severe	The zone extends farther than 1.0 cm beyond specimen

*The achievement of a numerical grade greater than 2 is considered a cytotoxic effect.

In vitro biodegradation of the GA sponge

The GA sponges were weighed to determine original mass. Then, the sample was shaken in 1 ml 2U/ml collagenase with a speed of 80rpm at 37°C. After 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 36 hours, samples were dried and reweighed. The percent of the remaining weight was calculated versus the original mass.

The adipogenic differentiation of hADSCs inside the GA sponge

hADSCs were seeded into the GA sponge with 5×10^3 cells/sample of size $3 \times 3 \times 3 \text{mm}^3$ and cultured in adipogenic induced medium containing dexamethasone, indomethacin, insulin and isobutyl - methylxanthine at 37°C, 5% CO_2 in 21 days. Oil Red O staining was conducted to observe intracellular lipid droplets of hADSCs inside the GA sponge. hADSCs cultured in the plastic dish were used as control.

RESULTS

Structure of the GA sponge

Peptide bonds formed between gelatin and alginate with the EDC crosslinking substance had made the stable GA sponge structure (Fig. 1). There were multi-layer inserted random (Fig. 2B) along with fibers unevenly (Fig. 2A). The GA sponge structure with pore size in the range 50-100µm is suitable for adhesion and proliferation of cell.

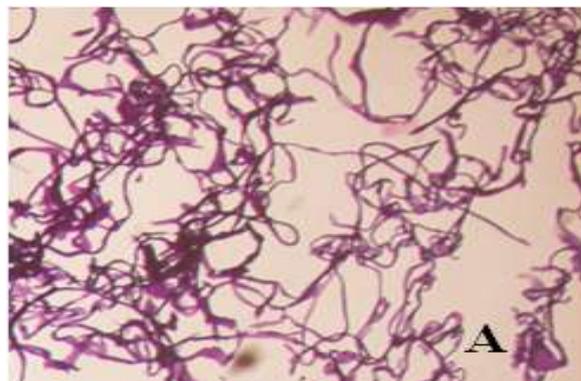


Figure 1. Structure of the GA sponge. (A) The GA sponge after freeze drying. (B) The GA sponge was cut into small samples of the size $3 \times 3 \times 3 \text{mm}^3$

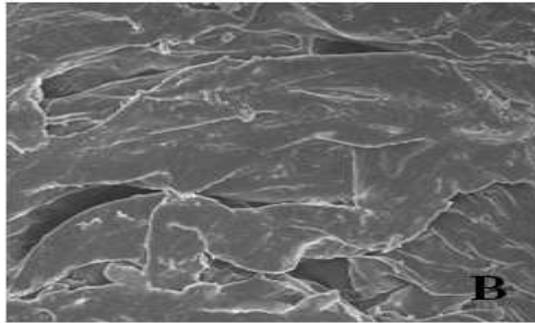


Figure 2. Morphology of the GA sponge. (A) The HE staining of the GA sponge (x100). (B) SEM image shows the surface of the GA sponge (x600)

The properties of the GA sponge to be a scaffold for tissue engineering

The chemical composition of the GA sponge

Gelatin is characterized by amide I group with C = O stretching peak at 1639 cm^{-1} , amide II with N-H bending peak in 1542 cm^{-1} . Alginate has significant absorption bands related to O-H hydroxyl group ($3490, 3433\text{ cm}^{-1}$), ether C-O-C ($1106, 1078, 1062, 1052\text{ cm}^{-1}$) and carboxyl - COO- ($2926, 1636, 1418\text{ cm}^{-1}$). FTIR result of the sponge has the peak 1637 cm^{-1} of the amide I group and amide II 1543 cm^{-1} , 1637 cm^{-1} of the carboxyl group, and 1028 cm^{-1} of ether (Fig. 3A). Thus, all the characteristics of gelatin and alginate are manifest in the spectrum of GA proved crosslinking substance - EDC had formed the bonds between two polymers in the GA sponge. The GA sponge after sterilized by irradiation in 25kGy was also analyzed by FTIR. The results showed that the absorption peak of GA sponge remains after irradiation (Fig. 3B). Thus, the method of sterilization by irradiation causes no change in functional groups of the GA sponge.

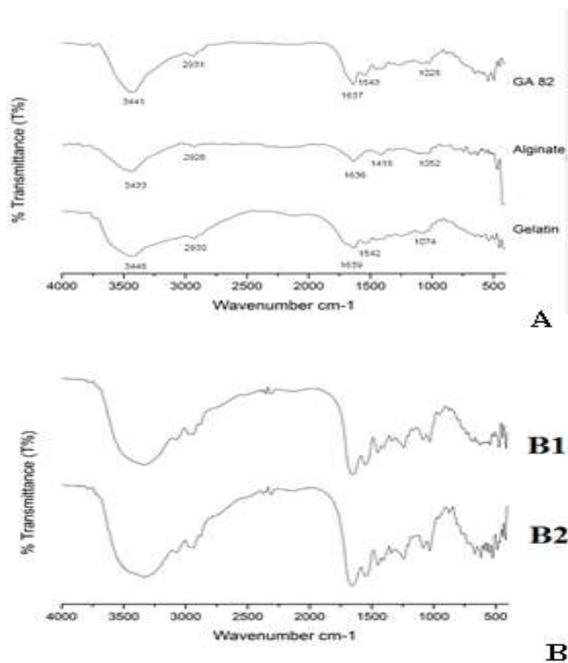


Figure 3. Infrared spectroscopy of the GA sponge. (A) FTIR analysis of gelatin, alginate and the GA sponge.

(B) FTIR analysis of the GA sponge before (B1) and after (B2) irradiation

The water absorption of the GA sponge.

The water absorption diagram of the GA sponge showed water absorption of this sponge during one hour. Water absorption speed of the samples was rapid and reached a water saturation point for 1 hour (Fig. 4). With this property, medium can easily go into this sponge and support for the proliferation as well as differentiation of cells that cultured inside of the sponge.

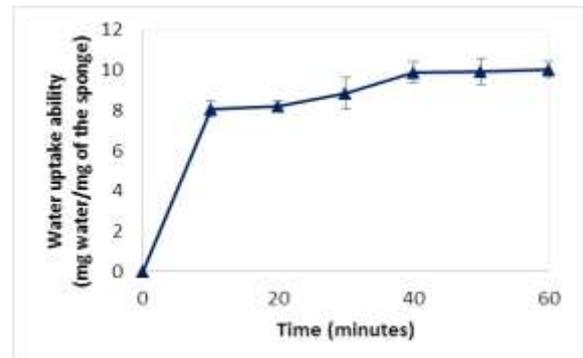


Figure 4. Graph of water absorption of the GA sponge at different periods of 1 hour

In vitro biodegradation of the GA sponge

The decomposition rate of the sponge in collagenase is slow in the initial period from 3 hours to 12 hours because of the closest links within the GA sponge (Fig 5). When gelatin is decomposed, the remaining alginate part lost the links that affects the mechanical force inside the sponge. Thereby, the sponge lost much weight in the later time points. Thus, the capable of biodegradation of the GA sponge with decomposition rate is suitable for the cells to proliferate and secrete the substrate to form a new extracellular matrix. Therefore, GA sponge is suitable to be a scaffold in tissue engineering.

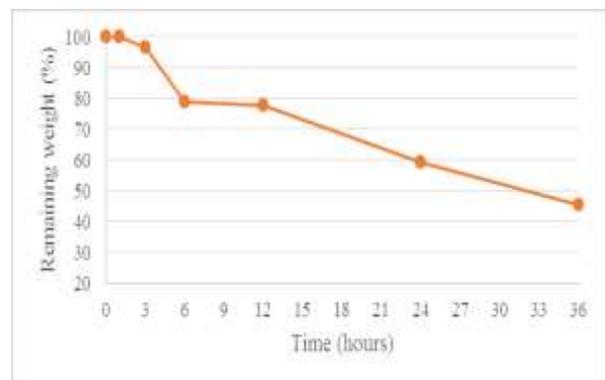


Figure 5. Diagram of *in vitro* biodegradation of the GA sponge in collagenase

In vitro cytotoxicity of the GA sponge

After 24 hours, the GA sponge is put out of the surface of ADSCs, the cells below the contact area with the GA sponge are normal as the control group, only a few cells die. There was no unusual phenomenon in the area

around the location of the GA sponge versus the control sample (Fig. 6). According to the ISO 10993-5: 2009, this sponge is not toxic to cells.

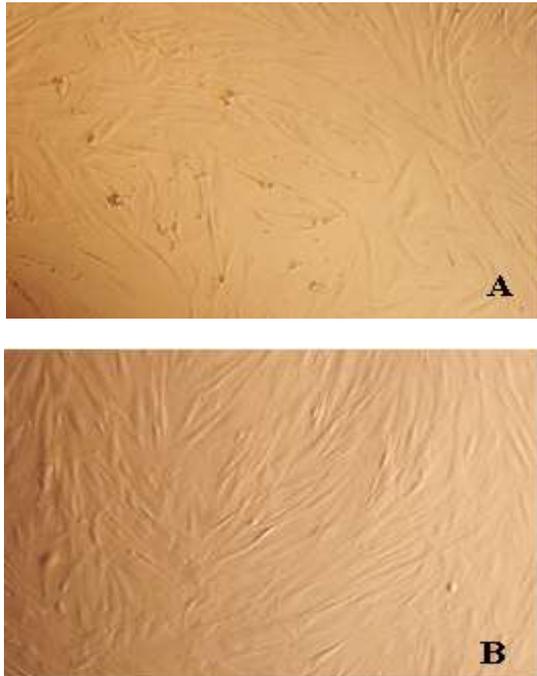


Figure 6. Morphology of ADSCs after 24 hours by *in vitro* toxic assessment of the GA sponge. (A) ADSCs after 24 hours of placing the GA sponge on the surface of them. (B) ADSCs after 24 hours without contact to the GA sponge (control)

In vitro adipogenic differentiation of hADSCs

hADSCs cultured in adipogenic medium have differentiated into fat cells with the big lipid droplets positive with Oil Red O dye inside cells after 21 days. In control cells, without induction, there was no differentiation (Fig. 7).

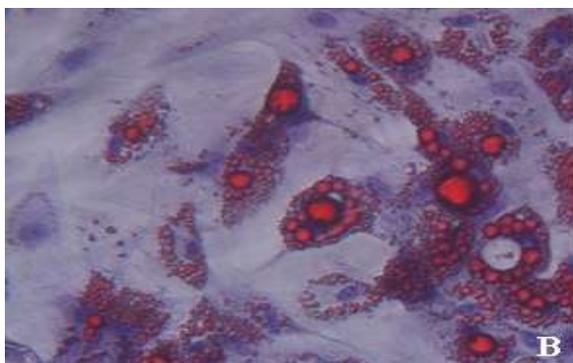


Figure 7. hADSCs were stained with Oil Red O dye after 21 days. (A) ADSCs in culture medium without induction were negative with dye (200X). (B) ADSCs in adipogenic medium were positive with dye (400X)

Adipogenic differentiation of hADSCs inside the GA sponge

hADSCs were seeded inside the sponge and cultured. After 1-day incubation, samples were placed in

adipogenic medium. After the period of 7 days, 14 days, 21 days, the sponge can contain cells positive with Oil red O dye. The result showed that some small lipid droplets were formed within cells at 7th day (Fig. 8B). After 14 and 21 days, cells gradually accumulate larger lipid droplets (Fig. 8C, D, E, F). Based on the location of lipid droplets, we can determine the position of the cell inside the sponge. However, only a small number of cells may be able to be induced for adipogenic differentiation. There was no same phenomenon in the control group that the sponge and cells without induction after 21 days in culture medium (Fig. 8A).

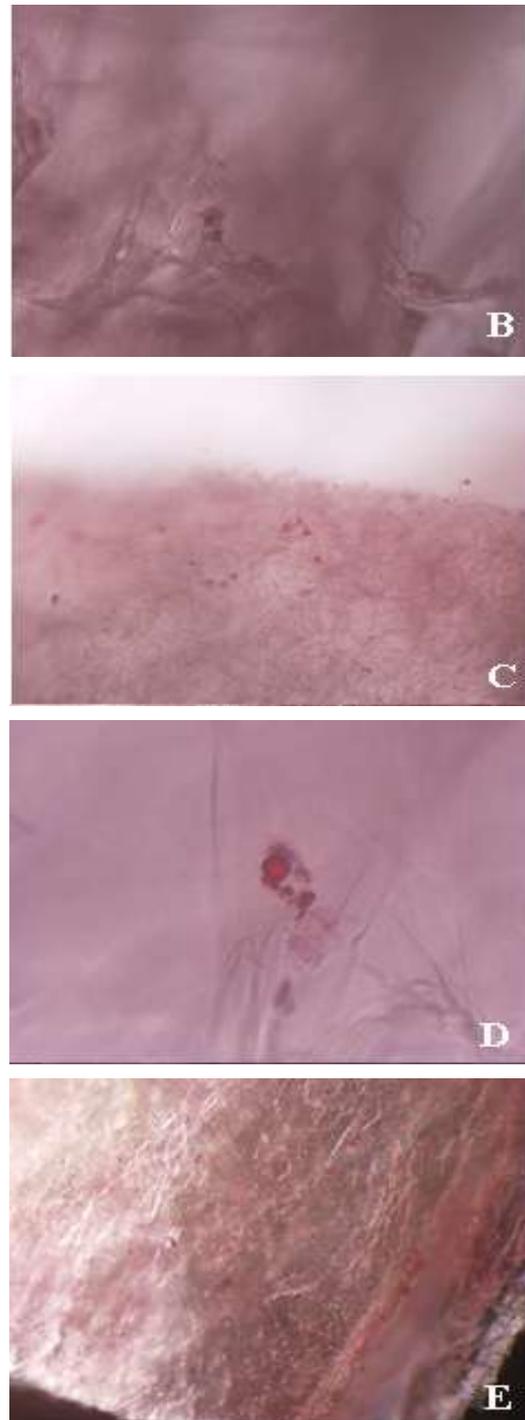




Figure 8: hADSCs within the GA sponge shows positive to the Oil Red O dye. (A) The GA sponge without hADSCs after 21 days in medium culture (x200). (B) The GA sponge with hADSCs after 7 days in adipogenic medium (x200). (C) The GA sponge with hADSCs after 14 days in adipogenic medium (x40). (D) The GA sponge with ADSCs after 14 days in adipogenic medium (x200). (E) The GA sponge with ADSCs after 21 days in adipogenic medium (x40). (F) The GA sponge with ADSCs after 21 days in adipogenic medium (x200)

DISCUSSION

Adipose tissue engineering has been increasingly widespread developed as one of therapies for soft tissue regeneration. The study about suitable scaffolds for applications in tissue engineering is an important strategy for developing this new treatment. A suitable scaffold for tissue engineering must have the following characteristics: external geometry, surface properties, porosity and pore size, interface adherence and biocompatibility, degradation characterization, mechanical competence.^[7] Compared with many different scaffolds were applied in adipose tissue engineering, including synthetic polymers such as poly lactic acid (PLA), poly glycolic acid (PGA), poly lactic-co-glycolic acid (PLGA), poly ethylene glycol, fluoropolymers, silicones, ...; and natural polymers such as collagen, fibrin, gelatin, hyaluronan, matrigel, silk, ...; GA sponge made from gelatin- a biocompatible material and does not cause an immune response, supporting for adhesion cells but degrades rapidly in the body; combined with alginate- a polysaccharide does not cause an immune response and it is not being degraded by enzymes in the body. So this sponge can have accordant characteristics with the criteria of a scaffold in tissue engineering, especially in adipose tissue engineering. In this study, the GA sponge was created and evaluated the characteristics whether it is a potential scaffold for applications in adipose tissue engineering? We have conducted the initial experiments, the results showed that GA sponge had a porous structure with the high water absorption and biodegradation ability, non-toxicity to cells and it is the space for adhesion and differentiation into adipose cells *in vitro* of human adipose-derived stem cells. The GA sponge structure with a diameter of pores about 10-100 μ m is suitable for cell ingrowth. Because pore size is also a very important factor, it should not be

too small to prevent cellular penetration and extracellular matrix production, and it also not be too big that can affect the stability of its structure. High pore density within the GA sponge provides a large surface area to support the growth of cells inside. With such a pore size, the amount of water is absorbed into the GA sponge about 8-10 times the initial mass, this high water absorption capability demonstrate that the culture medium can be enough provided for cells inside the GA sponge. Thus, the porous structure of GA sponge will support the activities of the cell as well as ensuring the metabolism between cells and cells, between cells inside and outside the GA sponge. FTIR analysis results showed the presence of peaks characteristic of gelatin and alginate inside GA sponge with new links formed by EDC. Using EDC to support the formation of new links had studied in many researches.^[4] This will make the structure of GA sponge becomes more stable. When assessing the biodegradable ability of GA sponge in collagenase *in vitro*, it biodegraded slowly from the surface to the internal structure, the size become smaller but the bulk structure is maintained thanks to the new links between gelatin and alginate. The scaffold was gradually degraded to be replaced by newly grown tissue remodeling. Therefore, this type of degrading scaffolds provides longer mechanical stability for the tissue to regenerate. The applications of a scaffold in tissue engineering need the support of stem cells, so first of all, it is necessary to analyze the toxicity of GA sponge to the cells *in vitro*. Experimental results showed that GA sponge is not toxic to human adipose-derived stem cells according to the toxic levels of ISO 10993-5:2009. So, we seeded cells into the GA sponge and evaluated *in vitro* differentiation of hADSCs - a potential source of stem cells usually used in adipose tissue engineering. The appearance of the component of gelatin that is biocompatible and contains RGD peptides will enhance cell adhesion in GA sponge. Observed under inverted microscope, there were many differentiated cells inside the GA sponge with big lipid droplets in the cytoplasm which were positive for Oil Red O staining after 21 days in induction medium, similar to the results of the *in vitro* differentiation of hADSC in culture dishes. However, only partially differentiated fat cells were observed inside the GA sponge and positive with Oil Red O dye. Therefore, more studies should be performed to improve the GA scaffold to enhance adhesion and differentiation of cells within this 3D scaffold. Moreover, when traditional methods in soft tissue replacement had many disadvantages, applications of tissue engineering by an available scaffold which is made from nature with the easily graphic creating and these mentioned characteristics will be developed more and more.^[5]

CONCLUSION

Our study has evaluated many properties of the GA sponge *in vitro* and *in vivo*. The results showed that gelatin-alginate sponge could be a natural scaffold for soft tissue engineering with porous stable structure, good ability in water absorption, *in vitro* biodegradation, no

toxicity to cells and capable of supporting for cell adhesion and differentiation, suitable for many researches and applications in soft tissue engineering. This research will be a basis for many researches to evaluate the proliferation, differentiation *in vivo* of stem cells inside this sponge.

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