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Paper

Design of Thermosensitive Bioglass/Agarose-Alginate Composite Hydrogel for Chronic Wound Healing

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Chronic wounds are a major health problem around the world, and there is a need to develop new types of dressing materials to enhance chronic wound healing. Humidity and the angiogenic conditions are two important factors that may significantly affect the healing process. Therefore, a new wound dressing system based on Bioglass (BG) and agarose-alginate (AA) has been designed, which can create moisture environment and improve the angiogenic condition of wound area at the same time. The obtained BG/AA hydrogel has thermosensitivity that gels at physiological temperature through the interactions between agarose and alginate polymer chains, and the chains can be further cross-linked by ions released from BG. The BG/AA hydrogel can promote migration of fibroblast and endothelial cells, it can also enhance the angiogenesis of endothelial cells in fibroblast-endothelial cell co-culture model *in vitro*. The potential of the BG/AA hydrogel as wound dressing has been further evaluated by using the rabbit ear ischemic wound model. The results demonstrate that the BG/AA hydrogel can enhance the blood vessel and epithelium formation, which contribute to the wound healing. The present study suggests that this new BG/AA hydrogel system may be used as a bioactive dressing for chronic wound healing.

1. Introduction

Chronic wounds are a major health problem around the world.[1,2] Unlike acute wounds, chronic wounds experience abnormal healing process and usually take excessive time to heal, which produce a great burden to patients and their family.[3] Chronic wounds can be classified into three major groups: venous leg ulcers, diabetic foot ulcers, and pressure ulcers.[4,5] Many local factors may cause impair healing process of chronic wounds includes ischemia, infection, tissue maceration and presence of foreign body.[6,7] The lack of sufficient blood flow (ischemia) is among the biggest problems, especially for diabetic foot ulcers.[8] Thus, improving angiogenic condition of chronic wound area is essential to enhance wound healing.[9]

Bioglass (BG) is a silicate-based inorganic material, which have been widely reported as a biomaterial for hard tissue repair and regeneration in past four decades.[10,11] From the end of last century, BG's ability to bond soft tissues and promote angiogenesis in vitro and in vivo has drawn more attention.[12,13] These features made BG a possible reagent to enhance chronic wound healing. Some publications have revealed BG's ability to promote wound healing in animal models.[14,15] However, when used as a stimulator for chronic wound healing, BG still has some disadvantages. BG powders may be easily removed from target place without a secondary dressing. Also, BG powders will result in a high local pH in wound area when directly applied,[16] which may cause uncomfortable for patients. Besides, previous reports advocated that a moisture environment is crucial for the healing of chronic wounds, which can speed epithelialization and collagen synthesis.[4,17,18] Therefore, it is meaningful to develop a proper BG dressing for chronic wound healing, which can maintain the bioactivity of BG to enhance wound healing, promote the adhesion of BG to wound bed, and create a favorable moisture environment in wound area.

Hydrogel dressing is a good candidate to provide moisture environment due to its high water-content.[19] Among them, natural-sourced alginate hydrogel has a long history as a wound dressing itself or with other therapies (such as drugs and proteins).[19,20] Our previous study has demonstrated that BG could be incorporated in alginate hydrogel, which showed bioactivity in stimulating angiogenesis and osteogenesis.[21] Therefore, our first hypothesis is that the combination of BG and alginate hydrogel may result in a bioactive hydrogel system that can stimulate angiogenesis, create a moisture environment, and then enhance chronic wound healing.

However, alginate hydrogels are usually formed by cross-linking using calcium ions,[22] and lack of the environmental sensitivity. For clinical applications, environmental sensitivity, especially thermosensitivity, can bring many conveniences,

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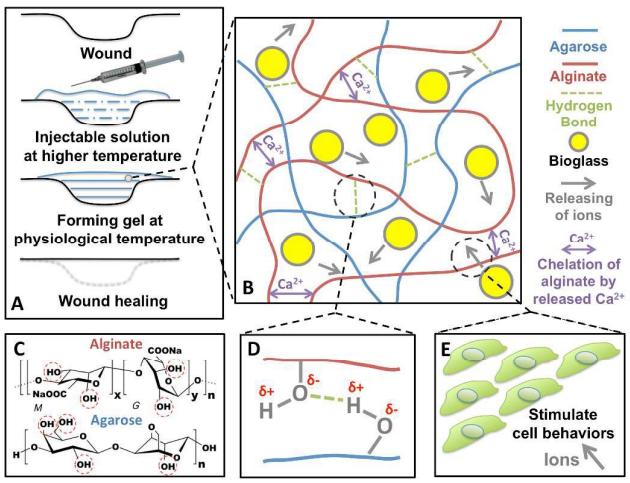

Fig. 1 The design of thermosensitive BG/AA hydrogel for wound healing. (A) An optimal thermosensitive hydrogel wound dressing; (B) Structure of the BG/AA hydrogel networks; (C) Molecular structures of alginate and agarose, which contains a lot hydroxyl group; (D) Hydrogen bonding between hydroxyl groups of different polymer chain; (E) Releasing ions can stimulate cell behaviors.

such as flexibility and injectability.[23] (Fig. 1A) Similar to alginate, agarose is also a natural-sourced polymer. It has achieved various biomedical applications including cell culturing,[24] cartilage regeneration[25] and nerve repair,[26] although there are few reports on its application in wound caring. Thermosensitivity is an attractive feature of agarose: its polymer chains present as random coil conformation at high temperature (>65~95°C, according to agarose type), and starts form double helices aggregates near physiological temperature.[23] It is also known that both alginate and agarose contain many hydroxyl groups (Fig. 1C), which may lead to interactions between these two type of molecules through hydrogen bonds.[27] (Fig. 1D) Also, the presence of sufficient hydroxyl groups in both polymers offers enough water affinity, which can provide a moisture environment. Therefore, our second hypothesis is that the combination of alginate and agarose may result in the formation of a thermosensitive agarose-alginate (AA) system with high water content. Aqueous environment is crucial for BG to release bioactive ions,[28] and these ions are the key factors to promote angiogenesis according to previous reports.[29,30] Thus, we assume that by combining BG and AA hydrogel, the BG can retain its bioactivity in this high water content environment. Furthermore, the bioactivity of BG will regulate cell behaviors and angiogenesis (Fig. 1E), and finally enhance chronic wound healing.

Based on the hypotheses mentioned above, the aim of the present study was to design and prepare BG/AA composite hydrogels with both thermosensitivity and bioactivity (Fig. 1B). The physical and chemical properties of hydrogels were investigated, the effect of composite hydrogels on cell proliferation, migration, and in vitro angiogenesis were then analyzed. Furthermore, the function of BG/AA composite hydrogels as wound dressing was evaluated in a rabbit ear ischemic chronic wound model.

2. Experimental section

2.1. Preparation of the hydrogel

Agarose solution (BIOWEST, 2% wv in deionized water) was prepared by autoclaving, and the solution was then transferred to 50 °C water bath for further use. To generate AA hydrogel, alginate solution (LV, Sigma, 2% wv in deionized water) was mixed thoroughly with agarose solution at 50 °C. When preparing the BG/AA hydrogel, 45S5 BG powders (Provided by Shanghai Institute of Ceramics, Chinese Academy of Science, and the average particle size is 20 μm) was mixed thoroughly with AA solution at 50 °C. AA or BG/AA hydrogel can be achieved by cooling the mixture down to physiological or room temperature. AA hydrogel was designated as x/y-AA, where x/y presents the agarose/alginate volume ratio (9/1, 7/3, 5/5, 3/7 and 1/9). Each BG/AA hydrogel was designated as zBG, where z presents the BG content (0, 1, 5, 10 and 20% w/v).

2.2. Gelling time

In order to evaluate the retention of the thermosensitivity in composite hydrogel, gelling time was measured according to previous report with some modification.[31] One milliliter of AA or BG/AA solution at 50 °C was injected into mold (15 mm diameter) at room temperature, and then the mold was titled every 5s and inverted every 10s. The hydrogel lost the ability to run away from the mold after t_1 seconds, and then totally fixed its shape in the mold after t_2 seconds. Gelling time was recorded as the time range between t_1 and t_2.

2.3. Water content, swelling ratio and mass loss

Cylindrical hydrogels (1 mL, diameter = 15 mm) were used to determine the water content, swelling ratio and mass loss. The wet weight (W_{wet}) and dry weight (W_{dry}) were measured after gelling and freeze-drying respectively, water content of the hydrogel was calculated as: Water content = $(W_{wet}-W_{dry}) / W_{wet}\times 100\%$. The dried hydrogel was then soaked in excess deionized water for 48 hours, and the weight after swelling (W_{swell}) was measured. Swelling ratio of the hydrogel was calculated as: Swelling ratio = $(W_{swell}-W_{dry}) / W_{dry}\times 100\%$. The dry weight of the swelled hydrogel (W_{sdry}) was measured, and the mass loss was calculated as: Mass loss = $(W_{dry}-W_{sdry}) / W_{dry}\times 100\%$.

2.4. Mechanical properties

In order to determine the interactions between alginate and agarose polymer chains and the effect of BG powders on the polymer networks, mechanical properties of different hydrogels were tested. Cylindrical hydrogels (height = 10 mm, diameter = 15 mm) were used in the mechanical test (Electronic Universal Testing Machine, Zwick T1-FR020.A50, compressive speed = 1 mm min^{-1} and no preload). The compressive stress-strain curves of hydrogels were measured after gelling for 1 hour or 2 days in a specific environment

(0.01M Ca^{2+} for AA hydrogel, distilled water for BG/AA hydrogel). Compress strength and elastic modulus of the hydrogel were calculated based on the stress-strain curve.[32]

2.5. Cell isolation and culture

Fibroblast and endothelial cells are two important cells that involved in the wound healing process. Thus, human dermal fibroblasts (HDF) and human umbilical vein endothelial cells (HUVECs) were used for cell experiments in this study. HDFs were isolated from the superficial layer of adult human skin according to previous report,[33] and cultured in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and 100 U/ml penicillin–streptomycin (P/S). HUVECs were isolated from human umbilical cord vein according to previous report,[34] and cultured in endothelial cell medium (ECM, Sciencell) supplemented with 5% (v/v) FBS, 1% (v/v) endothelial cell growth supplement (ECGS, Sciencell) and 100 U/ml P/S. All cells were cultured in 10 cm Petri dishes with 10 ml medium in the humidified incubator at 37 °C with 5% CO_2, media were changed every 2 days.

2.6. Cytotoxicity test

Cytotoxicity of the hydrogel toward HDFs and HUVECs were evaluated according to ISO 10993-5. Briefly, hydrogels (1 mL; 0BG = 0.98 ± 0.01 g; 1BG = 1.01 ± 0.01 g; 5BG = 1.02 ± 0.01 g; 10BG = 1.04 ± 0.01 g; 20BG = 1.15 ± 0.01 g.) were immersed in serum-free DMEM or ECM (10 mL) at 37 °C with 5% CO_2 for 24 hours. Then the supernatant was collected and sterilized with a filter (0.22 μm, Millipore). Supernatants derived from DMEM were supplemented with 10% (v/v) FBS + 100 U/ml P/S, and supernatants derived ECM were supplemented with 5% (v/v) FBS, 1% (v/v) ECGS and 100 U/ml P/S for cell culturing. Cells were seeded into 96-well plates (3000 cells/well for HDFs, 4000 cells/well for HUVECs) and cultured in DMEM or ECM extracts of different hydrogels. Cells cultured with DMEM + 10% (v/v) FBS + 100 U/ml P/S or ECM + 5% (v/v) FBS + 1% (v/v) ECGS + 100 U/ml P/S were used as the control group. CCK-8 assay (Beyotime) was used to determine the relative cell number at day 0, 1, 3, 7 according to the manufacture's instruction. Cell growth ratio was calculated as A_t/A_0, where A_t stands for the relative number at day t and A_0 stands for the initial relative cell number.

2.7. *In vitro* scratch assay

In vitro scratch assay was undertaken to evaluate the effect of hydrogels on in vitro cell migration according to the previous reports.[35,36] HDFs or HUVECs were seeded into 24-well plates and cultured to achieve a confluent monolayer. Then, a scratch was created in each well by scraping the cell monolayer with a p200 pipet tip. Cells were gently washed with culture medium, and cultured with low-serum medium (1 mL) on each well. Low-serum medium is same as complete medium expect a low FBS content of 1% (v/v). Transwell loaded with hydrogel (100 μl) was then added to each well. Cells cultured without hydrogels were used as the control group. Images of each sample were captured at 0 h and after 1 d culture by a microscope (Leica DMI3000), and analyzed quantitatively by Image J (NIH) to determine the area of initial scratch (A_0) and healing scratch (A_t). Migration ratio was calculated as: Migration ratio = $(1-A_t/A_0)\times 100\%$.

2.8. *In vitro* angiogenesis

Angiogenesis is an important process in wound healing to support tissue repairing,[9] and many different cell types such as fibroblast and endothelial cells are involved in this process. In this study, the HDFs-HUVECs co-culture system was chosen to evaluate the in vitro angiogenesis according to our previous report.[37] HDFs were seeded into 6-well plates and cultured to achieve confluent monolayers, then 10^6 HUVECs were seeded on confluent HDFs in each well, and cultured with co-culture medium (2.5 mL, complete DMEM: complete ECM = 1:1) for 4 hours to allow HUVECs adhesion. Then, transwell loaded with hydrogels (250 μL) was added to each well. Cells cultured without hydrogels were used the as the control group. After culturing for one day, the in vitro angiogenesis process of the co-culture system was analyzed quantitatively by immunofluorescence staining and gene expression measurement according to previous report.[37]

For immunofluorescence staining, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). After permeated with ice methanol, cells were then blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 1 hour at 37 °C. Following an overnight incubation in a primary antibody solution containing rabbit anti-von Willebrand Factor (vWF, Life), cells were incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Life) for 2 hours. After the vWF staining, nuclei were stained by 4-6-diamidino-2-phenylindole (DAPI, FluoProbes) according to manufacture's protocol. PBS washing was performed three times after each step. Fluorescence stained cells were observed under an inverted fluorescence microscope (Leica DMI3000). At least 20 images were taken from each sample, and the formation of tubes and circles of HUVECs in each image was analyzed by Image J.

For gene expression measurement, HUVECs and HDFs in the co-culture system were first separated using CD31 magnetic beads (Invitrogen) according to previous reports.[37,38] TRIzol reagent (Invitrogen) was used to extract RNA from cells, and cDNA was synthesized from RNA using ReverTra Ace-a kit (Toyobo) according to the instructions. Then, Q-rtPCR was performed in triplicate by using SYBR Green PCR Master Mix Kit (Toyobo) and 7900 Real-Time PCR system (Applied Biosystems), and VEGF (F:5'TATGCGGATCAAACCTCACCA3' R:5'CACAGGGATTTTTCTTGTCTTGCT3') and GAPDH (F:5'ACGGATTTGGTCGTATTGGGCG3' R:5'CTCCTGGAAGATGGTGATGG3') primers were used. mRNA expression of VEGF was normalized to GAPDH and compared by the $\Delta\Delta C_t$ method.

2.9. *In vivo* chronic wound healing

Ischemic wound healing model in the rabbit ear was used to evaluate the function of the composite hydrogels as wound dressing to promote *in vivo* chronic wound healing. Ischemic wounds in the rabbit ear were created according to previous

reports.[39,40] Animal experiments' protocol was approved by the Ethics Committee of the Shanghai Jiao Tong University School of Medicine. New Zealand White rabbits (1000~2000g) were weighed and anesthetized with an intramuscular injection using sodium pentobarbital (30 mg kg^{-1}) and ketamine hydrochloride (30 mg kg^{-1}). To create ischemia, the central and the rostral artery of the rabbit ear were ligated, while the caudal artery and all veins were left intact. Following the ischemic surgery, 6 mm full-thickness wounds were created using punch biopsy, and perichondrium was removed as well. After being cleaned, hydrogels solution (100 µl) at 50 °C were added to the wounds, dressings were then formed over the wounds. Wounds treated without dressings were set as the control group. At least six wounds were evaluated for each group. Hydrogel dressings were replaced every 2~3 days, and the images of wounds were collected by a digital camera at different time intervals. On day 31, the wound tissues were harvested, formalin-fixed, paraffin-embedded and cut into 5-µm sections. Hematoxylin and eosin (H&E) staining was performed for histology analysis. The gap between newly formed epithelium in each group was analyzed by image J. Blood vessels were identified by defined lumens and the presence of red blood cells within their boundaries,[38] and at least 20 images at 200× magnitude were captured and blood vessels per square millimeters were analyzed by image J.

2.10. Statistical analysis

Results are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to determine statistical significance for all data with R (The R Foundation).

3. Results

3. 1. Characterization of the hydrogel

In this study, we prepared AA hydrogels with different agarose/alginate ratio. Then, considering higher mechanical property enhancement, we chose 5/5-AA group (with 1% wv agarose and 1% wv alginate) to prepare BG/AA hydrogels with different BG content. The results showed that with the decrease of agarose/alginate ratio, AA hydrogels became more transparent and less shape-stable (Fig. S1). In contrast, with the increase of the BG content, BG/AA hydrogels became less transparent but similar shape-stable (Fig. 2A).

3.1.1. Water content, swelling and mass lose For AA hydrogels, the water content, swelling ratio, and mass loss increased simultaneously when agarose/alginate ratio decreased (Fig. S1). For BG/AA hydrogels, both the water content and swelling ratio dropped when BG content increased (Fig. S2). The mass loss of 0BG hydrogel (i.e. 0% wv Bioglass, 1% wv agarose and 1% wv alginate) is 10.9%, and it decreased dramatically with the addition of a low amount of BG. Incorporation of 1% BG resulted in a low mass loss of less than 2.5%, and further increase of BG content show less affect on the mass loss. (Fig. S2)

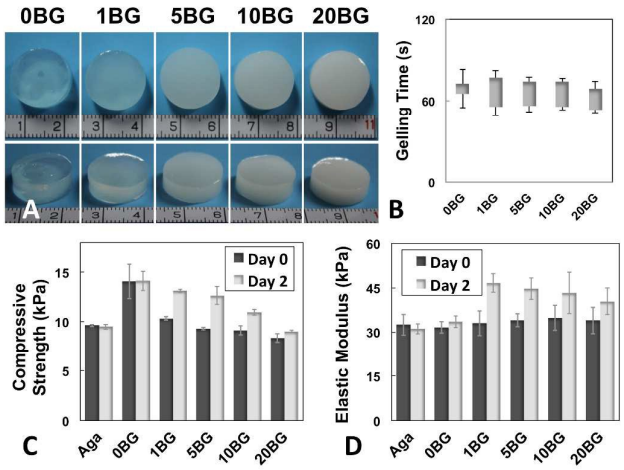

Fig. 2 Characterization of BG/AA hydrogel. (A) Appearance photographs; (B) Gelling time; (C) Compressive strength and (D) Elastic Modulus of BG/AA hydrogels after immersion in water for 0 or 2 days, Aga stands for pure 1% agarose hydrogel.

3.1.2. Gelling time The gelling time graph shows that all AA hydrogels can be gelled within certain time at room temperature, which means that they were able to remain as solution in higher temperature (more than 40 °C) and form gel at room temperature (Fig. S1). We observed that AA hydrogels with higher agarose/alginate ratio (9/1, 7/3, 5/5) showed similar gelling time range around 60s. When agarose/alginate ratio decreased to 3/7, the gelling time increased to 80~118s. Furthermore, hydrogel with the lowest agarose/alginate ratio of 1/9 showed a significant increase on gelling time that reached 188~317s. BG showed no significant effect on gelling time of AA hydrogels, since all BG/AA hydrogels experienced similar gelling time around 60s when compared with the 0BG group (Fig. 2B).

3.1.3. Mechanical properties In this study, two important mechanical properties of the hydrogel, the compressive strength and elastic modulus were evaluated. For AA hydrogel, it is interesting to see that, after immersion in 0.01M Ca^{2+} solution for two days, hydrogels with highest and lowest agarose/alginate ratio (9/1 and 1/9) maintain similar mechanical properties (Fig. S3). At the meantime, other groups showed an increase of both compressive strength and elastic modulus with time, which suggests that calcium ions brought interactions inside polymer networks. Especially, the compressive strength of 7/3-AA and 5/5-AA group increased from 16.8±1.7 kPa and 14.0±1.1 kPa to 31.7±2.8 kPa and 33.7±0.8 kPa, respectively (Fig. S3). Based the mechanical properties enhancement, 5/5-AA group was chosen to prepare BG/AA hydrogel and perform further experiments.

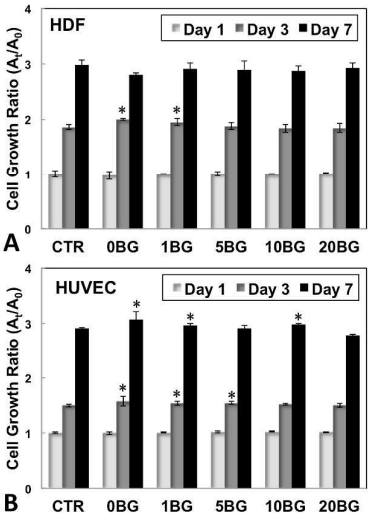

Fig. 3 Cell growth ratio of (A) HDFs and (B) HUVECs in the cytotoxicity tests of the BG/AA hydrogel. * denotes statistical significant higher than the control group ($p<0.05$).

For the mechanical strength of BG/AA hydrogel at Day 0, the 0BG group showed the highest compressive strength (14.07±1.72 kPa) while other groups revealed a similar value around 9 kPa (Fig. 2C). When 1% BG was added into the hydrogel, the compressive strength decreased to 10.13±0.16 kPa, which is only slightly higher than pure agarose (i.e., 1% wv agarose). Compressive strength slightly decreased when BG content increased from 1% to 20%. At the same time, all groups shared similar elastic moduli but increased slightly when BG content increased. Both pure agarose (without alginate and BG) and 0BG (without BG) had little change on mechanical properties after be kept in the moisture environment for 2 Days. However, all other groups that contain BG experienced a significant enhancement (Fig. 2C and 2D). This enhancing phenomenon is similar to that AA hydrogels has experienced after immersion in Ca^{2+} solution for two days, suggesting BG brought interactions inside polymer networks similar to that Ca^{2+} brought (Fig. S3). Ca^{2+} releasing was detected from BG/AA hydrogels (Supporting information).

3.2. Cytotoxicity of the hydrogel

For cytotoxicity evaluation, both HDFs and HUVECs were incubated with hydrogel extracts for 1, 3 and 7 days. All hydrogels extracts treated cells showed similar growth pattern as compared to control group (Fig. 3). On Day 3, the HDFs growth ratios of 0BG and 1BG are higher than the control group but return to the same after 1 week. For HUVECs, 0BG, 1BG, and 5BG show higher cell growth ratios than the control group on Day 3. After 1 week, 0BG, 1BG, and 10BG show higher cell growth ratios than the control group. In general, all hydrogel groups did not suppress the cell growth of HDFs and HUVECs.

3.3. Migration of HDFs and HUVECs cultured with the hydrogel

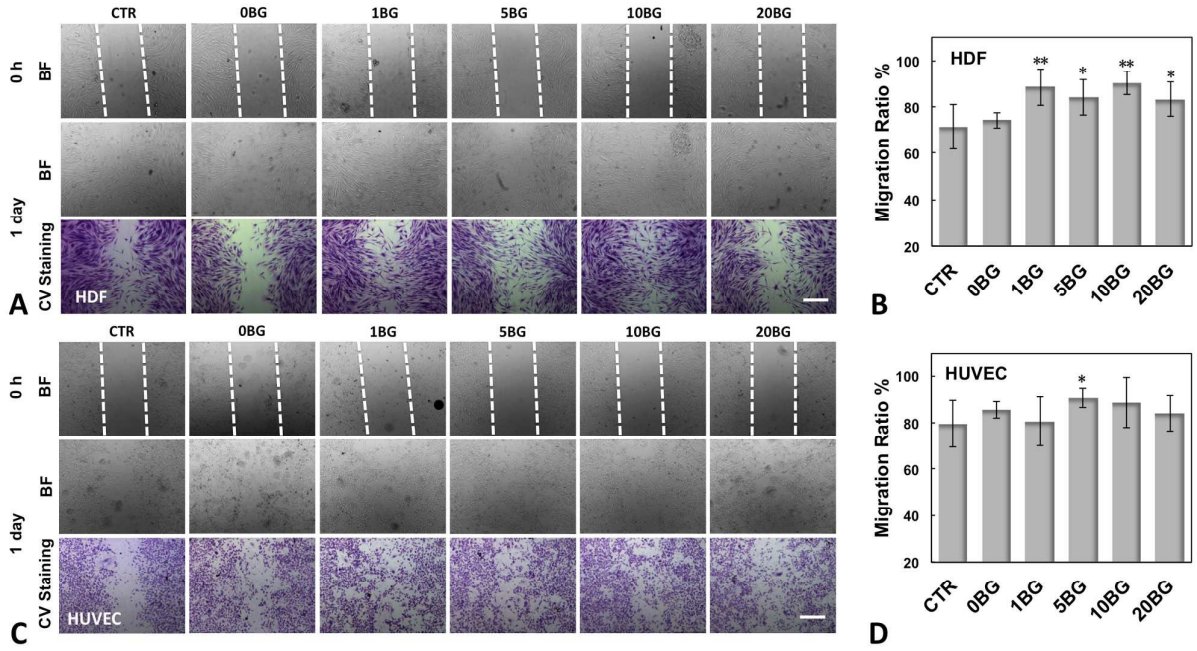

Fig. 4 Cell migration of HDFs and HUVECs with BG/AA hydrogels. (A) HDFs and (C) HUVECs after scratch (0h) and after one day migration; (B) HDF and (D) HUVECs migration ratio. * and ** denotes statistical significant higher than the control group (*, $p<0.05$; **, $p<0.01$), bar = 400 μm.

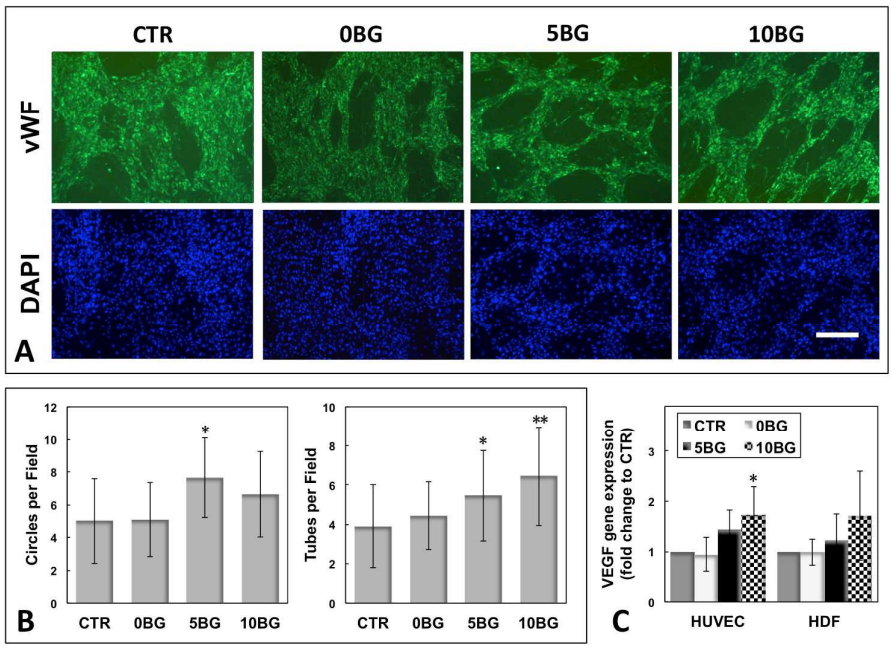

Fig. 5 *in vitro* angiogenesis in HDFs-HUVECs co-culture system cultured with the hydrogel. (A) vWF and DAPI fluorescent staining; (B) Circles and tubes formed by HUVECs; (C) VEGF gene expression of HUVECs and HDFs in the co-culture system, fold change to control group. * denotes statistical significant higher than control group ($p<0.05$), bar = 200 μm.

An *in vitro* scratch assay was used to evaluate hydrogel's affect on cell migration. After scratched by pipet tip, all cells shared a similar wound width (Fig. 4A and 4C). However, the cell migration behaviors were different after treated with different hydrogels. For HDFs, 0BG group showed no difference as compared with the control group, while groups containing BG enhanced the cell migration significantly (Fig. 4A and 4B). For HUVECs, groups containing BG also promoted cell migration (Fig. 4C), and the 5BG group showed a significant enhancement (Fig. 4D).

3.4. *In vitro* angiogenesis of HDFs-HUVECs co-culture system cultured with the hydrogel

3.4.1. Immunofluorescence staining After culturing on confluent HDFs for one day, HUVECs formed tubes and circles pattern spontaneously (Fig. 5A, vWF), while HDFs remained inside those circles since DAPI signals can be detected (Fig. 5A, DAPI). It is obvious that HUVECs cultured with 5BG or 10BG group developed more complicated networks, which was further confirmed by quantitative analysis of tubes and circles formed by HUVECs (Fig. 5B). The data shows that HUVECs cultured with 5BG group formed significantly more circles than the control group, and HUVECs cultured with 5BG and 10BG groups formed significantly more tubes than the control group.

3.4.2. Gene expression We future evaluated the VEGF gene expression in cells from the co-culture system. The gene expression of the control group has been set as one, and every other group was compared to the control group. Thus, the control group has no error bar (Fig. 5C). Cells cultured with 0BG group showed a similar VEGF gene expression as compared to the control group, but those cultured with 5BG and 10BG groups experienced an up-regulation in VEGF expression, and 10BG group demonstrated the most significant enhancement.

3.5. *In vivo* chronic wound healing

For the animal experiment, hydrogel dressings were applied on the ischemic rabbit ear wounds. We chose 10BG group for this experiment, as its overall performance is better than 5BG group during *in vitro* angiogenesis experiment. The 0BG hydrogel dressing was transparent while 10BG dressing was translucent (Fig. 6A). The appearance of wounds was examined one month after surgery (Fig. 6B). All wounds were 6mm diameter in size at the beginning of the experiment (Fig. 6B, 0d), and no infection was observed during the one-month experiment for all groups. After surgery, wounds with dressing showed less bleed as compared with the control group. After two weeks of treatment, wounds with 10BG dressing showed a faster contraction than other groups (Fig. 6B, 2w and 3w, 1m). The scab fell off for all groups one month after the surgery, and the skin around wounds that treated with 10BG dressing healed better with a smaller remaining wound area.

After being harvested, the wound cross sections were analyzed by H&E staining, and Fig. 6C is the representative H&E staining micrograph of all groups. We observed that most wounds with 10BG dressing were closed and fully epithelialized while most 0BG and control groups still possessed a gap between newly formed epithelium. The H&E staining results also show that more blood vessels were appeared in repaired skin tissue from the wounds treated with 10BG dressing when compared with 0BG and control groups

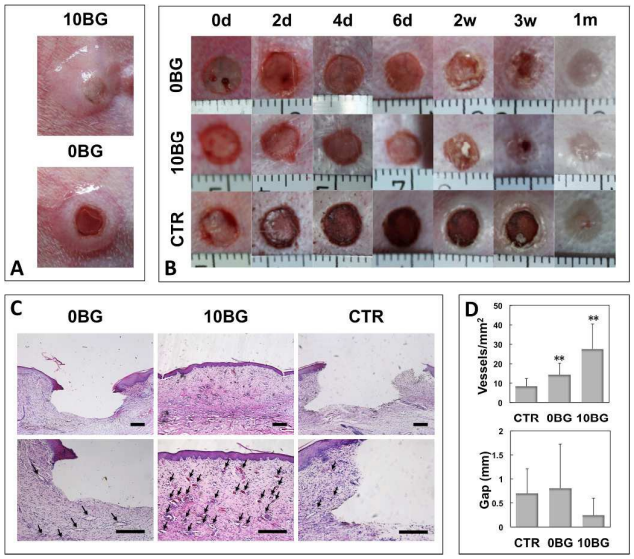

Fig. 6 Wound healing with hydrogel dressing in ischemic rabbit ear. (A) Hydrogel dressings on the wound; (B) Wound healing process; (C) H&E staining of the wounds sections after one month healing; (D) Blood vessels density and gap between newly formed epithelium in H&E staining images; ** denotes statistical significant higher than the control group ($p<0.01$), bar

(Fig. 6C). Quantitative analysis confirmed that vessels per millimeter square in 10BG groups were significantly higher than other groups, and 0BG group showed a higher number of vessels per millimeter square than the control group (Fig. 6D). In addition, red blood cells were more evident inside the blood vessels of the 10BG group than others (Fig. 6C). The average gap length between newly formed epithelium in 10BG group was lower than other groups while no significant difference can be detected due to the large variation in 0BG and control group (Fig. 6D).

4. Discussion

Alginate hydrogel can provide a moisture environment, which is important for wound healing. However, alginate hydrogels are usually prepared by calcium ions cross-linking, which have poor injectability due to the fast cross-linking reaction. BG is known to stimulate wound healing by enhancing angiogenesis, but the application of BG in dry powder form may not be optimal. In this study, we proposed a BG/AA hydrogel system, which is thermosensitive, injectable, and bioactive in stimulating angiogenesis.

The thermosensitive AA hydrogel system is based on composition of two natural polymers: agarose and alginate. One of our hypotheses is that the combination of agarose and alginate may form a thermosensitive hydrogel due to the interactions between two components and the thermosensitivity of agarose. When alginate is combined with agarose, the hydrogen bonds can be formed between hydroxyl groups of agarose and alginate molecules. As agarose will undergo transition from the solution to hydrogel when the temperature decreases, alginate chains can still retain within agarose networks, so the AA composites can still possess thermosensitivity as pure agarose. Indeed our results confirmed that the AA hydrogel is thermosensitive, and all AA composites with different ratio of agarose and alginate can form hydrogels within a given time at room temperature. We prepared two types of hydrogel: one is 1% wv pure agarose (Aga), and another one is 1% wv agarose with additional 1% wv alginate (5/5-AA, also known as 0BG). Results show that the mechanical strength of 5/5-AA (0BG) hydrogel is higher than the Aga hydrogel. Alginate as a solution has no mechanical strength, and there is no cross-linking agent for alginate in the AA composites. The enhancement of mechanical strength after addition of alginate into agarose suggests that agarose might interact with alginate during hydrogel formation. However, the enhancement may also be brought by the increased overall polymer concentration.

Another hypothesis in this study is by combining BG with AA hydrogel, we can generate a bioactive hydrogel system that can not only create a moisture environment, but also promote chronic wound healing by regulating the ionic environment that may enhance cell migration and angiogenesis. Our results showed that BG/AA hydrogels with certain BG content promoted the cell migration of both fibroblast and endothelial cells in a 2D scratch wound model, which is in agreement with a previous study showing that Bioglass could stimulate the epithelial cell migration.[35]

Besides cell migration, angiogenesis plays an important role in wound healing: newly formed blood vessels are essential to bringing oxygen and nutrient to the wounds, which are important for tissue regeneration.[9,41] BG has shown stimulation on angiogenesis both *in vitro* and *in vivo* according to previous researches.[12,42–44] Therefore, we have evaluated the effect of BG/AA hydrogels on angiogenesis in order to confirm if the BG that incorporated within AA hydrogels is still bioactive. The results showed that BG/AA hydrogel promoted the in vitro angiogenesis of HUVEC in HUVEC-HDF co-culture system by stimulating VEGF gene expression in both HUVEC and HDF. VEGF is a well-known inducer of angiogenesis,[45] and our previous study showed that bioactive silicate materials can up-regulate the VEGF expression of cells in HUVEC-HDF co-culture system and subsequently enhanced the angiogenesis.[37]

As we discussed above, BG still retain its bioactivity after being combined with AA hydrogels, BG/AA hydrogels promoted both cell migration and in vitro angiogenesis. Previous reports suggest that the ions released from BG are key factors of the bioactivity of BG.[10,11,13] These ionic products that containing Si, Ca, P may regulate cell behaviors such as cell migration, proliferation, osteogenesis, and angiogenesis. In the present study, we excluded physical contact between hydrogels and cells by using transwell system. Thus, we proposed the regulation effect on cell behaviors in this study was due to the ionic environment created by BG/AA hydrogels.

In addition, the change of ionic environment also has effect on the hydrogel properties such as mechanical strength. Two days after preparation, the compressive strength and elastic modulus of BG/AA hydrogel increased. Calcium ions releasing from BG/AA hydrogels has been detected in this research and this can contribute to the enhancement of mechanical properties since alginate can be crosslinked by calcium ions. This phenomenon may help the dressing to keep its shape also keep alginate from leaking out.

After the *in vitro* analysis, rabbit ear ischemic model was chosen to further study the ability of our hydrogel system as a

chronic wound dressing. Rabbit ear ischemic model has been used widely as a chronic wound model.[39,46] Since the BG/AA system can transfer from solution into gel form at physiological temperature, we directly applied composite solution onto the wounds, and hydrogels were formed as soon as the temperature decreased to body temperature. Wounds that treated with the BG/AA hydrogel showed a better healing as compared to other groups, the epithelium regeneration is clearly better, and significantly more new blood vessels were formed than control groups. Cell migration and vascularization are two important processes in wound healing, impairing of these two processes could result in chronic wounds.[47] Now we demonstrated that the BG/AA hydrogel enhanced cell migration and angiogenesis both in vitro and in vivo, and confirmed our hypothesis that the combination of hydrogel moisture environment and the ionic microenvironment of BG promoted chronic wound healing.

Conclusions

We designed a novel thermosensitive BG/AA hydrogel by combining BG, alginate and agarose for chronic wound healing. The BG/AA composite hydrogel can regulate the microenvironment around the wounds by providing a favorable moisture environment and releasing bioactive ions to stimulate angiogenesis that promoted by the cell migration. Alginate, a widely reported wound dressing material, was combined with agarose, a thermosensitive component, resulting in the formation of a thermosensitive AA composite hydrogel. The interactions between these two polymers brought improvement of mechanical properties as compared to the pure agarose or alginate, and BG within the hydrogel could further strengthen the hydrogel networks by releasing cross-linking calcium ions. Furthermore, BG/AA hydrogel promoted *in vitro* migration of the fibroblast and endothelial cells, which are two important cell types in wound healing process, and the composite hydrogel showed bioactivity in stimulating the *in vitro* angiogenesis in the fibroblast-endothelial cell co-culture system. The animal study further confirmed the function of the composite hydrogel in stimulating blood vessel and epithelium formation and enhancing healing of chronic wound in a rabbit ear ischemic wound model. Above results suggest that this thermosensitive bioactive BG/AA hydrogel may have great potential to serve as a dressing material for chronic wound healing.

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