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Designing vascular supportive albumen-rich composite bioink for organ 3D printing

Suihong Liu [a], Haiguang Zhang [a,b,*], Qingxi Hu [a,b], Zhipeng Shen [a], Deepti Rana [c], Murugan Ramalingam [d,**]

[a] Rapid Manufacturing Engineering Center, Shanghai University, Shanghai, 200444, China
[b] Shanghai Key Laboratory of Intelligent Manufacturing and Robotics, Shanghai University, Shanghai, 200072, China
[c] Department of Biomechanical Engineering, Technical Medical Centre, University of Twente, Enschede, 7522NB, the Netherlands
[d] Biomaterials and Organ Engineering Lab, Centre for Biomaterials, Cellular and Molecular Theranostics, School of Mechanical Engineering, Vellore Institute of Technology, Vellore, 632014, India

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ABSTRACT

Bioink plays a major role in 3D printing of tissues and organs. Alginate is a widely used component for bioinks but its cellular responses are limited, which limits its clinical translation. In this study, we demonstrate the printability and cellular compatibility of composite bioink consists of sodium alginate (NaAlg) and egg white, also called albumen. The experimental conditions necessary for 3D printing composite bioink were optimized by changing different concentration ratios of Albumen/NaAlg and their various physicochemical properties were studied. The structural characteristics of the 3D printed scaffold was also investigated. *In vitro* experiments showed that human umbilical vein endothelial cells can successfully attach to the printed scaffold and maintain high viability during the course of study. Interestingly, vascular sprouting and neovascular network formation was observed inbetween fibers within the printed scaffold. In conclusion, the results of this study demonstrate that 3D printed Albumen/NaAlg composite bioinks with favorable biological functionality hold a great potential in tissue and organ engineering.

1. Introduction

Tissue engineering is one of the promising technology to alleviate the organ shortage crisis. The majority of strategies currently used in tissue engineering employ a scaffold, which is used to accommodate cells in a three-dimensional (3D) structure in order to facilitate growth and formation of the desired tissue and organ (Huang et al., 2012; Ozbolat, 2015; Rana et al., 2014; Vijayavenkataraman et al., 2016). 3D printing is an additive manufacturing technique, which is capable of producing scaffolds or anatomical structures for tissue and organ engineering with patient-specific requirements where cells are presented in a desired location. It has the ability to make scaffolds with highly tunable compositional variations and reproducible architectures (Murphy and Atala, 2014; Skardal and Atala, 2015).

Bioink plays a crucial role in 3D printing, which provides temporary environment for the cells to adhere, infiltrate, proliferate, and differentiate into a specific tissue lineage (Kim et al., 2016; Shi et al., 2016). Various natural biomaterials such as gelatin, collagen, alginate, chitosan, starch, cellulose etc have been explored for 3D printing. Although, the natural biomaterial based bioinks shows greater biocompatibility when compared to synthetic biomaterials but often lacks printability, especially at lower concentrations. Therefore, the development of bioinks with high printability and biocompatibility has become a major challenge in current 3D printing research and organ development.

Protein-based composites are being considered as one of the desirable biomaterials as they can be fabricated into a wide range of tissue structures with intrinsic and tunable properties, including nontoxicity, modulation of mechanical properties, biocompatibility and biodegradability (Silva et al., 2014; You et al., 2017). Among different protein-based natural polymers, egg white proteins (also called as albumen) are extensively used in food products, especially in edible gels, owing to their high nutritional value and versatile techno-functional properties (Nyemb et al., 2016). Additionally, albumen is a natural source of proteins that is known to have wound healing, anti-bacterial,

* Corresponding author. Rapid Manufacturing Engineering Center, Shanghai University, Shanghai, 200444, China.
** Corresponding author.
E-mail addresses: haiguang_zhang@i.shu.edu.cn (H. Zhang), rmurug2000@gmail.com (M. Ramalingam).

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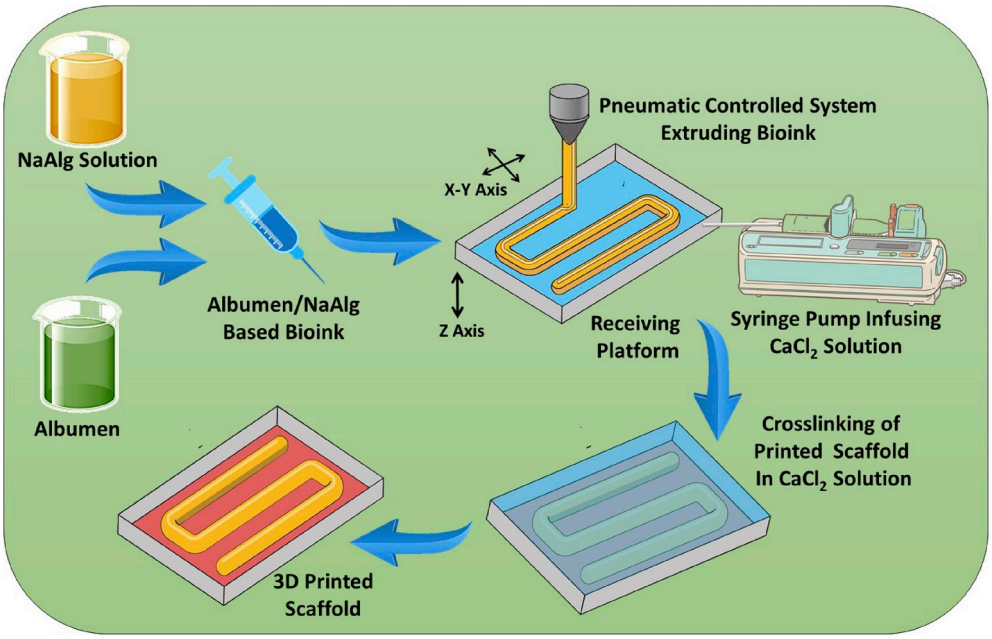

Fig. 1. The schematic diagram of albumen/NaAlg composite scaffold preparation process.

anti-hypertensive, anti-inflammatory and cell growth stimulatory properties (Geng et al., 2016; Miguel and Aleixandre, 2006; Mine et al., 2004). Albumen encompasses a variety of globular proteins, such as ovalbumin (54%), ovotransferrin (12%) and lysozyme (3.5%) (Weijers et al., 2006). The lysozyme and ovotransferrin both shows antimicrobial properties. Interestingly, it has been reported that ovalbumin or ovomucoid can stimulate the proliferation of myoblasts and growth of myotubes (Mizunoya et al., 2015). These key merits of albumen exhibit bioactive functionalities of interest for various biomedical applications. However, the use of albumen in biomedical applications remains limited. For instance, previous studies have reported that the albumen porous scaffolds were prepared either by using albumen or single proteins from albumen via different approaches, such as thermal treatment (Babaei et al., 2019), freezing-thawing (Shaabani et al., 2016), microfluidic (Liu et al., 2017) and crosslinking mechanism (Jalili-Firoozinezhad et al., 2015b). However, the lack of efficient method and the weak mechanical strength of albumen gel limit its application in biofabrication. On the other hand, composite of albumen with other polymers, to fabricate hybrid or composite scaffolds, can be conducive to overcome this issue. Some studies have shown that blending of albumen with various biopolymers, such as silk fibroin (You et al., 2017), polyvinyl alcohol (Jahani-Javanmardi et al., 2016), cellulose acetate (Wongsasulak et al., 2010), and poly (ethylene oxide) (Wongsasulak et al., 2007), can be used to modify mechanical and physicochemical properties of composite scaffolds.

Sodium alginate (NaAlg) is an environment-friendly natural polysaccharide carbohydrate extracted from brown algae and has been used in 3D printing for several years. Owing to its properties such as non-toxicity, biodegradability, and biocompatibility, NaAlg has been widely used in the fields of regenerative medicine, tissue engineering and drug delivery applications. However, pure form of alginate-based bioinks are inert hydrogels that inhibits cell adhesion, mainly due to its repelling anionic surface and lacking cell adhesion sites (Yang et al., 2018). To overcome these limitations and improve the printability, bioactive materials may be incorporated within the alginate bioinks (Rana and Ramalingam, 2017; Rana et al., 2016; Xiao et al., 2017). The bioactive materials such as albumen can be considered as a unique admixture function for tissue scaffolds (Morgan et al., 2013). Albumen containing ovalbumin proteins have the potential to bind and transport a number of endogenous and exogenous compounds including proteins and growth factors (Fasano et al., 2010; Oettl and Stauber, 2010). Therefore, it could be speculated that the albumen/alginate composite bioink-based scaffolds may even attract angiogenic factors from the surrounding microenvironment. Also, the albumen and fibrous protein composite bioink have been reported to show adequate mechanical and biological properties (You et al., 2017). Therefore, albumen/alginate composite bioinks are of great importance for tissue engineering applications. However, to the best of our knowledge, there has been no report in literature, about the application of the albumen/NaAlg composite bioinks for the fabrication of scaffold via 3D printing technique.

Keeping the above points in view, in this study, authors have made an attempt to optimize Albumen/NaAlg composite bioink composition, from different concentration ratios, such that it could elicit balanced physicochemical as well as biological properties suitable for 3D printing of tissues and organs.

2. Materials and methods

2.1. Materials

Fresh chicken eggs were purchased from the local market. Egg white or albumen was removed from the shell and separated from the egg yolk and used in the production of composite scaffold without further treatments. Sodium alginate (NaAlg) and calcium chloride ($CaCl_2$) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). NaAlg was dissolved in deionized water and placed in a shaker for 10 h at 120 rpm at room temperature to prepare a 5% w/v NaAlg solution. Similarly, 4% $CaCl_2$ solution was prepared with deionized water and used as cross-linking agents.

2.2. Preparation of Albumen/NaAlg composite bioink

To prepare Albumen/NaAlg composite bioink, the 5% (w/v) NaAlg were mixed with different volume ratios of albumen, namely, 1:1, 2:1, 3:1, 4:1, 5:1 and 6:1. The viscosity measurement of each composite solution was carried out by using a rotational viscometer (NDJ-5S, Shanghai Changji Geological Instrument Co. LTD, China) and the values were recorded at room temperature. In brief, the viscometer measures the torque required to rotate the spindle within the sample test tube at a constant speed of 20 rpm. Based on the torque value, the viscometer

determines the viscosity of the test sample i.e., Albumen/NaAlg composite bioinks. Prior to test, the solutions were stirred and homogenized using a magnetic stirrer for 2 h at room temperature. At least five measurements were taken per sample and the values were averaged for further process. Additionally, to study the morphology and flowability of the mixed Albumen/NaAlg bioink at different volume ratios, the freshly prepared albumen/NaAlg bioink mix was poured into a 2 mL cryogenic vial (Corning, NY) and kept at room temperature for 30 min. Subsequently, each vial was inverted to observe the flowability of the Albumen/NaAlg composite bioink.

2.3. Rheological assessment of Albumen/NaAlg composite bioink

To analyse the printability of the composite bioink, the composite albumen/NaAlg bioink with Albumen/NaAlg at a volume ratio of 1:5 was prepared and measured using a rotational rheometer (DHR-3, Waters China Limited, USA) with 25 mm diameter plate geometry according to the manufacturer's protocol. The steady shear sweep analysis was conducted at 37 °C on the bioink, the curves of viscosity of Albumen/NaAlg (1:5) composite bioink was captured. Moreover, the storage modulus and loss modulus of the fully crosslinked composite bioink gel bulk was measured.

2.4. 3D printing of Albumen/NaAlg composite scaffold

To develop Albumen/NaAlg composite scaffold, the authors have custom-made the extrusion-based 3D printing system and it was used in the rest of the printing process. The scheme of the printing process is given in Fig. 1. The main components of the system contain an automated X–Y–Z stage along with the controller where the positioning precision is 0.1 mm, a digital display pneumatic controller (TENSUN industrial equipment co. LTD, Shenzhen, China), an air compressor (OTS-1100x2-60L 3P, Guangzhou tiguan trading co. LTD, China), syringe needle, receiving platform, and a computer. The receiving platform mounted to a solo motorized linear Z stage, and it can be controlled to only move up and down to ensure stable receiving platforms that contain cross-linking reagent solution. Moreover, the syringe nozzle mounted in the X–Y plane that is capable of printing with high precision. The syringe nozzle was connected with digital display pneumatic controller by using the air tube for convenience of controlling and displaying pressure. Air compressor was used to provide air pressure for extruding materials. According to section 2.2, the mixed solution with Albumen/NaAlg at a volume ratio of 1:5 was used as the solution for printing the scaffold. First, 10 mL Albumen/NaAlg was added to a syringe and a 160 μm internal diameter needle was used for extrusion printing. Next, $CaCl_2$ solution was added to the Petri dish (100 mm, Corning, NY) and the entire bottom surface is submerged as receiving bath to cross-link the printed Albumen/NaAlg scaffold. Finally, the prepared Albumen/NaAlg bioink was extruded from the syringe needle to generate continuous filament at room temperature. The filaments were cross-linked in $CaCl_2$ solution bath and deposited layer by layer to form a designed grid structure. During the printing process, an air pressure value of 2.5 ± 0.1 Psi was applied to extrude the Albumen/NaAlg bioink. The distance between the needle and substrate is 2 mm. Integral control programs were developed by the authors using G-code commands based on the geometry of the designed printing structure. The dimension of a designed grid structure scaffold was 30 mm × 30 mm × 1 mm. In short, the interval of parallel-arranged filaments was 1 mm in each layer, and adjacent layers were perpendicularly stacked to construct the porous structure. After printing, another $CaCl_2$ solution poured into a Petri dish to make the whole scaffold immersed into cross-linking solution for 2 h to further cross-link the albumen/NaAlg scaffold. Subsequently, it was removed from the cross-linking solution and placed in an oven for 3 h to dehydrate and dry at 37 °C.

In order to verify the integrity of the Albumen/NaAlg composite scaffold and the size scale of the filaments, the morphology of the fabricated scaffold was observed under an inverted fluorescent microscope (Eclipse Ti–U, Nikon Instruments Inc., Japan) with a bright field.

2.5. Fourier transform infrared (FTIR) analysis

In order to confirm the mixed albumen/NaAlg cross-link reaction and analyze the composition of the fabricated Albumen/NaAlg composite scaffold, FTIR measurements were performed by Fourier transform infrared spectrometer (AVATAR 370, Nicolay co., LTD, USA) in a range between 4000 and 800 cm^{-1}. The FTIR spectra of the Albumen/NaAlg composite sample, the NaAlg sample, the albumena sample were tested.

2.6. Wettability analysis

The static contact angle measurements were performed on an optical contact angle meter. The contact angle of the albumen/NaAlg composite sample and the NaAlg sample was measured at room temperature through the sessile drop method. Briefly, a drop (10 μl) was formed on a needle (diameter of about 500 μm) and deposited on the surface of the sample. The images were captured and auto-analysis by drop shape analysis was performed. The angle between the drop and the surface was measured on the picture and determined as the average of the angles measured to the left and right of the drop. At least three measurements were taken per sample.

2.7. Swelling analysis

For assessing the swelling property, the equilibrium swelling behavior of the Albumen/NaAlg composite scaffolds was evaluated at 37 °C in phosphate buffer saline (PBS, pH 7.4) and RPMI 1640 culture medium for 12 h, respectively. After removing excess water from the surface of the samples with filter paper, the swollen weight (W_a) of scaffolds was recorded. The dry weight (W_b) of samples were captured, swelling percentage of scaffolds was calculated by using the following equation:

$$\text{Swelling ratio}(\%) = ((W_a – W_b) / W_b) \times 100\%$$

The NaAlg scaffold was used as control groups. Each swelling experiment was performed on three samples and averaged. Moreover, the dimensional change in each of the printed Albumen/NaAlg composite filament was measured every 15 min using a microscope until no further dimensional change was recorded (150 min). The initial dimensional prior to soaking were subtracted from the measurements after immersion and the relative size increase (%) was reported.

2.8. Cell culture studies

The HUVECs purchased from Zhongqiaoxinzhou Biotech CO., Ltd., Shanghai, China, were cultured in RPMI 1640 culture medium, according to the manufacturer's protocol, which consisted of 10% fetal bovine serum, 0.292 mg/mL L-glutamine, 4.766 mg/mL HEPES, 0.85 mg/mL NaHCO3, 1% penicillin (100 units/mL), and streptomycin (100 μg/mL), in a humidified incubator at 37 °C and 5% CO_2. The cells were cultured for 3 days and then suspended in fresh medium. After reaching 80% confluence, cells were detached from a Petri dish with 0.25% trypsin to generate cell suspension with a density of 6×10^6 cells/ml. The printed Albumen/NaAlg composite scaffolds were immersed in 75% ethanol under UV light for 6 h for sterilization and then washed with PBS three times. Next, the composite scaffolds were washed with fresh medium for three times and then immersed in freshly prepared medium for 2 h before cell seeding. Finally, the prepared cell suspension was slowly seeded on the composite scaffold until the surface of the scaffold was fully covered. After allowing adherence for 4 h, the fresh medium was added into the Petri dish until the whole scaffold was immersed and medium changed every day thereafter. The cellular morphology was

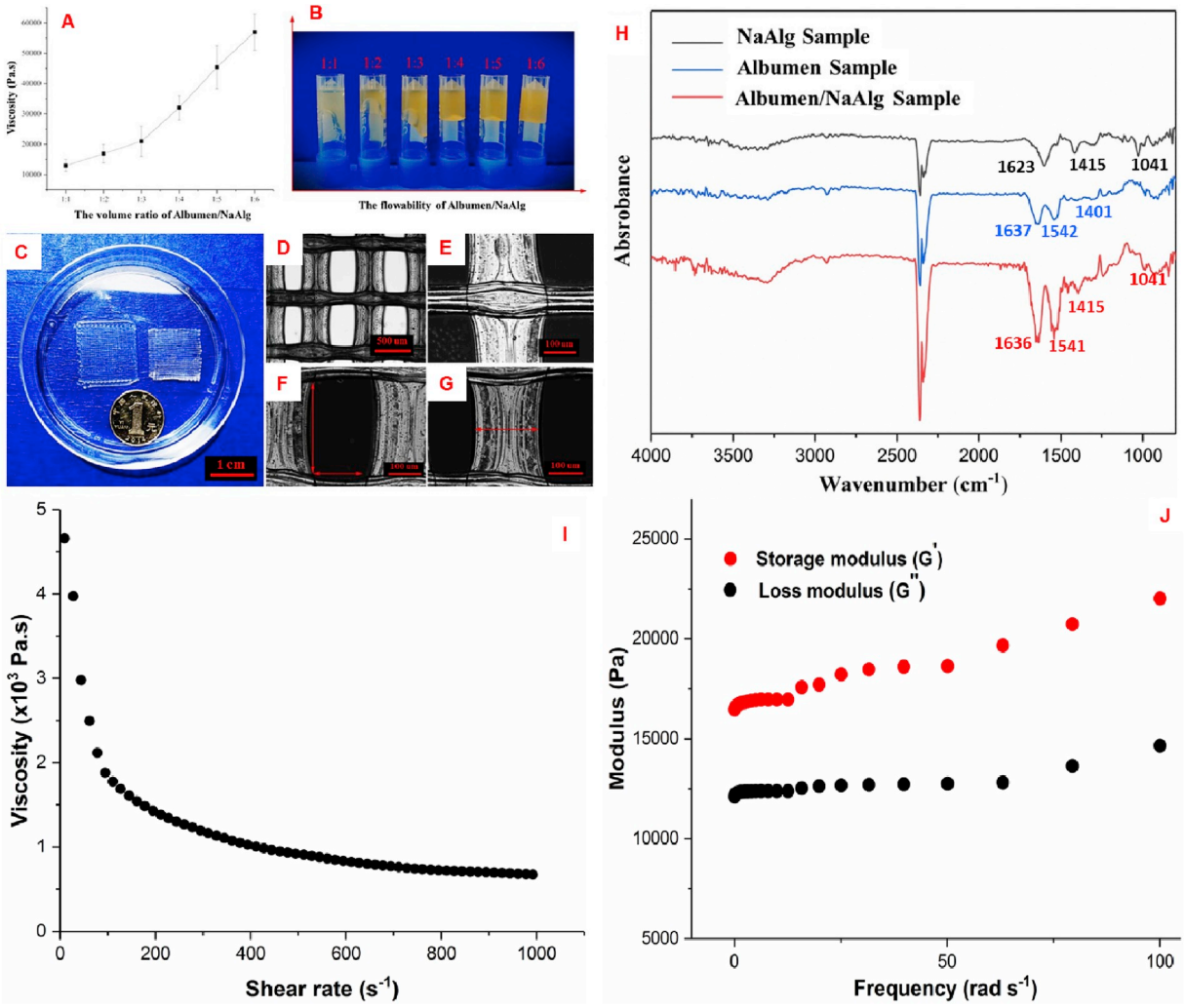

Fig. 2. Physicochemical properties of the composite albumen/NaAlg bioink. (A) Viscosity of Albumen/NaAlg composite materials at the volume ratio of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6; (B) Flowability of Albumen/NaAlg composite materials at the volume ratio of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6; (C)The printed two Albumen/NaAlg composite scaffolds: The dimension of a designed grid structure bone scaffold was 30 mm × 30 mm × 1 mm and 25 mm × 25 mm × 1 mm, respectively. (D) The grid structure, (E) the perpendicular stacked structure, (F) the pore and (G) the filament structural morphology under a bright field microscope; (H) FTIR spectra of the NaAlg sample, the Albumen sample, the Albumen/NaAlg sample; (I) The viscosity of the composite albumen/NaAlg bioinks that exhibited shear thinning behavior. (J) The modulus of crosslinked composite Albumen/NaAlg gel bulk indicated a higher storage modulus than loss modulus.

observed using an inverse fluorescence microscope (Eclipse Ti–U, Nikon Instruments Inc., Japan).

The cell viability of the fabricated Albumen/NaAlg composite scaffold was tested after 4 days of culture by Live/Dead assay. A Live-Dead Cell Staining Kit (Biovision, Inc., San Francisco, CA) was used for the visualization of live and dead cells on the scaffold. In this experiment, the staining solution was prepared using a mixture of 1 μL of Live-Dye and 1 μL of PI in 1 mL of staining buffer according to the manufacturer's protocol. On day 4, the cultured Albumen/NaAlg composite scaffold was removed from the culture medium and gently washed with PBS. Then, the staining solution was directly seeded on the surface of scaffold and then incubated for 15 min at 37 °C. Finally, the staining solution was removed, and the scaffold was mounted on microscope slides for imaging. In addition, another fluorescence study was performed to visualize the morphology of the attached cells on the surface of the composite scaffold. After culturing for 4 days, the samples were stained with tetramethylrhodamine (TRITC) - phalloidin and 4′, 6 - diamidino -2- phenylindole (DAPI) (Yeason, Shanghai, China) to visualize the cell cytoskeleton and nucleus, respectively. Briefly, the scaffold was rinsed 3 times with PBS, 5 min at a time. Then the scaffold was fixed (4% paraformaldehyde) and permeabilized (5 min, 0.5% Triton X-100 in PBS). Then 100 μL of TRITC-phalloidin was slightly injected on the surface of the scaffold and incubated in a dark environment for 30 min at room temperature to stain the cell cytoskeleton, after washing 3 times in PBS, 100 μL of DAPI solution was added the surface of scaffold to stain the cell nuclei for 30 s. Images were captured by using a fluorescence microscope to observe the characteristics of the cells.

2.9. CCK-8 assay

The cell proliferation and biocompatibility of scaffolds were analyzed using the Cell Counting Kit-8 (CCK-8; KeyGEN BioTECH). Briefly, previously sterilized albumen/NaAlg composite scaffolds were added to wells of the 96-well plates (no. 3524; Costar). The prepared cells suspension seeded in corresponding well at the density of 1×10^5 cells/mL, three rows of well of each plate were covered. After culturing for 1, 3 and 5 days, 10 μL of CCK-8 solution was added to each well and incubated for 2 h in a CO_2 incubator at 37 °C. The absorbance was measured at 450 nm using a microplate reader (Infinite 200Pro, Tecan Group Ltd., Switzerland). The NaAlg scaffold was also tested as control groups. Furthermore, a fresh medium was used instead of the immersion medium as a positive control group and only contain CCK-8 and medium

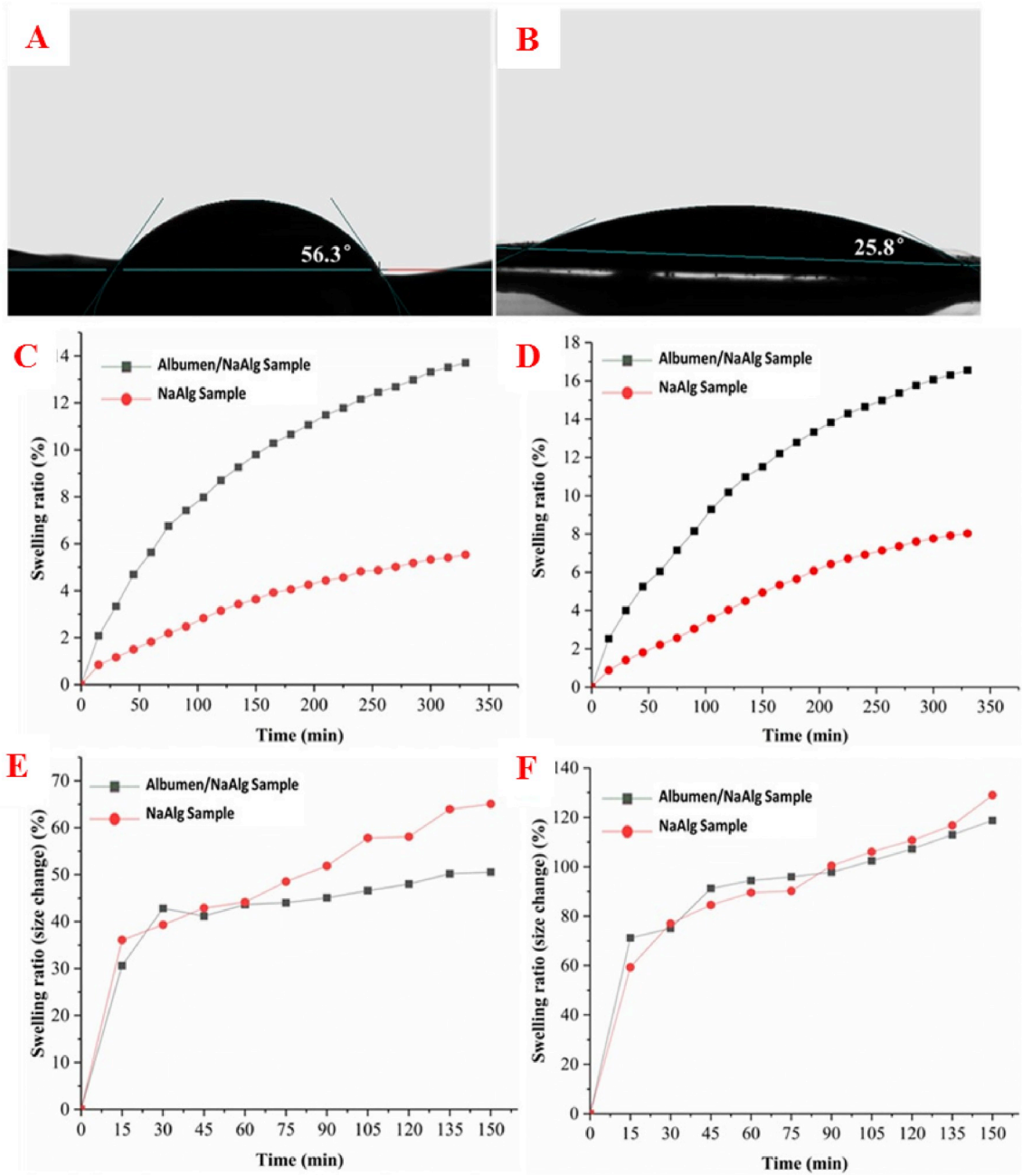

Fig. 3. Water contact angle of (A) NaAlg and (B) Albumen/NaAlg membranes. Swelling of NaAlg and Albumen/NaAlg sample in PBS and RPMI 1640 medium: the mass swelling ratio: (C) RPMI 1640 medium, (D) PBS; the swelling ratio of filament size: (E) RPMI 1640 medium, (F) PBS.

as a background group.

2.10. Statistical analysis

All data are presented as mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-test was performed. Statistical analysis was performed using Origin 2017 software.

3. Results and discussion

3.1. Fabrication of Albumen/NaAlg composite scaffold

The primary requisite for the fabrication of 3D printed Albumen/NaAlg composite scaffold is bioink's printability and shape fidelity of the printed scaffold. For this reason, the viscosity of different bioink compositions i.e., volume ratio of Albumen/NaAlg (1:1, 1:2, 1:3, 1:4, 1:5 and 1:6) was measured by a rotational viscometer. The values as shown in Fig. 2A had viscosity range from 13000 ± 1989 Pa s to 57000 ± 5906 Pa s. The viscosity of the composite bioinks increased exponentially with the increase of the volume ratio of NaAlg. Additionally, the visual inspection of Albumen/NaAlg composite bioink in cryogenic vials showed that the 1:4 vol ratio of Albumen/NaAlg is the transitional point; higher viscosity with decreased flowability and extrusion properties of composite bioinks with 1:4 to 1:6 compositions were observed (Fig. 2B). Based on these observations, composite bioink with 1:5 vol ratio was selected to be used for scaffold fabrication.

In addition, bioink should present appropriate viscoelastic properties for bioprinting tissue-engineering scaffolds that could carry cells and bio/therapeutic molecules. The viscoelastic properties of the composite

bioink exhibited shear thinning behavior (Fig. 2I), similar to the other bioinks, which favor the viability of the entrapped cells due to the alleviated shear stress when passing through printing nozzles at a certain flow rate (Gao et al., 2017; Hölzl et al., 2016). The shear stress sweep graphs showed higher storage modulus and lower loss modulus of the crosslinked composite bioink, which further confirms ability of composite bioink to retain its shape, and thus applicable for 3D bioprinting of tissue-engineering scaffold (Fig. 2J).

The Albumen/NaAlg composite scaffolds were fabricated by combining extrusion-based 3D printing and crosslinking mechanism. The scaffolds were fabricated with the pneumatic pressure of 2.5 ± 0.1 Psi and 4% $CaCl_2$ receiving solution. The integrity of the printed albumen/NaAlg composite scaffold was maintained. Moreover, in order to investigate the morphology of the fabricated scaffold and the size scale of the formed filaments, the micrograph of the printed scaffold was obtained (Fig. 2C–G) by an inverted fluorescent microscope with a bright field. The printed scaffold contained 2 layers; adjacent layers were perpendicularly stacked to construct the porous structure. Based on microscopic observations, the printed filament ranged in size from 120 ± 5 to 324 ± 8 μm, and the formed pore geometry of the scaffold was rectangular. However, it was notable that the formed filament size of lower layer was significantly smaller than the upper layer. This may be caused due to different crosslinking times and the different formed way. In brief, the printed filament of lower layer was immediately exposed to the $CaCl_2$ receiving solution to initiate crosslinking and it has been already reported that the diameter of the printed filament would slightly shrink upon $CaCl_2$ crosslinking(Li et al., 2016). However, for the upper layers, the printed filaments were first stacked on the lower layer, then moved down by the receiving device and cross-linked, which caused the filament of the upper layer to slightly crush and widen. In addition, the Albumen/NaAlg composite scaffold with uniform pore structure allows sufficient oxygen and nutrient mass transport within the scaffold. These results indicate that the Albumen/NaAlg composite hydrogel scaffolds were successfully built with appropriate printability and shape-fidelity.

3.2. FTIR analysis

FTIR spectroscopy was used to investigate the presence of different functional groups and the nature of chemical bonds present in the composite bioink. The FTIR spectra of the Albumen, NaAlg, and the Albumen/NaAlg composite bioink were shown in Fig. 2H. By comparison with the spectra of NaAlg and Albumen, the main characteristic peaks of the Albumen/NaAlg hydrogel could be assigned. The FTIR spectrum of the Albumen/NaAlg hydrogel combines the features of those of NaAlg and Albumen. The peaks of NaAlg appear at 1623, 1415, and 1041 cm^{-1}. The peaks of Albumen appear at 1637, 1542 and 1401 cm^{-1}. As for Albumen/NaAlg composite bioink, the most prominent peaks in the area of the spectrum are around 1636, 1541, and 1415 cm^{-1}. Amid I (1700–1600 cm^{-1}, related to C═O stretching vibrations), amide II (1575–1480 cm^{-1}, related to 60% N–H bending and 40% C–H stretching vibrations), and amide III (1400–1200 cm^{-1}, related to N–H bending and C–H stretching vibrations) regions of the FT-IR spectra can be used to study changes in the secondary structures of proteins (Kong and Yu, 2007). The bands at 1041 cm^{-1} could be attributed to C–O stretching vibration of NaAlg. In addition, the O–H stretching vibrations and the stretching vibrations of C–H bands can be observed in each spectrum. From the peaks of the spectrum, it could be interpreted that the mixture of Albumen and NaAlg did not cause any drastic alteration in the position of characteristic peaks associated with the secondary structure of the protein present in the individual components. Though, the Albumen/NaAlg composite bioink combines the characteristic features of Albumen and NaAlg. Similar findings have been reported elsewhere that no difference between FTIR spectra of silk fibroin hydrogel and blended silk fibroin-gelatin (Rattanamanee et al., 2013), egg white/silk fibroin composite films (You et al., 2017), and egg white and gelatin composite hydrogels were observed (Babaei et al., 2019). Altogether, these results indicates that no covalent intermolecular interactions took place between Albumen and NaAlg in the composite bioink.

3.3. Wettability analysis

Scaffold's wettability is a key factor to be considered in bioprinting as it determines its hydro-phobicity or -philicity that could affect its biological behavior (Di et al., 2017). It has been already stated that hydrophilic scaffolds could contribute to cells growth on its surface and within the pores. The results for water contact angle measurement of NaAlg and Albumen/NaAlg scaffold were shown in Fig. 3A–B. The water contact angle of the cross-linked NaAlg membrane is 56.3°, and for cross-linked Albumen/NaAlg composite membrane is 25.8°. These observations suggest that mixing od Albumen which is a common food ingredient, into the highly hydrophilic NaAlg could led to increased hydrophilicity of the composite scaffold. The increased hydrophilicity of the composite scaffold could retain high water content for a prolonged time to support cell growth *in vitro*.

3.4. Swelling analysis

The swelling capacity of the hydrogel is one of the most important factors to investigate its potential for tissue engineering applications. The swelling curves of 3D printed NaAlg scaffold and Albumen/NaAlg composite scaffold as a function of time in PBS and RPMI 1640 culture medium at 37 °C are presented in Fig. 3C–F. The mass swelling of NaAlg and Albumen/NaAlg in RPMI 1640 culture medium as shown in Fig. 3C, indicated that the swelling ratios of Albumen/NaAlg were obviously higher than that of NaAlg sample. Similarly, in PBS, the mass swelling of Albumen/NaAlg was also higher than NaAlg (Fig. 3D). The swelling ratio of Albumen/NaAlg was about twice as high as that of NaAlg. However, it was observed that the swelling ratio of the scaffold in PBS was greater than those in RPMI 1640 culture medium. The size swelling ratio of NaAlg hydrogel filament and Albumen/NaAlg composite hydrogel filament were measured in RPMI 1640 culture medium and PBS as shown in Fig. 3E and F. In RPMI 1640 culture medium, all filaments showed an obvious increase in size swelling ratio over the first 30 min, and the swelling ratio of Albumen/NaAlg was higher than that of NaAlg after 30 min. From 30 min to 150 min, the size swelling ratios of all filaments slowly increased and maintained equilibrium. In PBS, the higher swelling ratio of Albumen/NaAlg was observed than NaAlg after first 15 min, and then the size swelling ratios of all filaments slowly increased. The size swelling ratio of the scaffold in PBS was observed to be greater than those in RPMI 1640 culture medium. These results indicates that the water absorption ability of the Albumen/NaAlg composite scaffold filament are better than pure NaAlg scaffold, which is in accordance with water contact angle measurement results (Fig. 3A–B). Moreover, the swelling ratio of the Albumen/NaAlg composite scaffold was found to be increasing slowly and have no significant change after 300 min, and the value of maximum swelling was found to be 14.52 ± 0.65% and 17.12 ± 0.53% in culture medium and PBS, respectively (Fig. 3C–D). However, comparison of the volume and size of the scaffold before and after swelling revealed that the slight additional swelling of the scaffold did not have any significant impact on the feature resolution of the printed scaffold. Overall, these results imply that the Albumen/NaAlg composite 3D printed scaffold have high swelling properties and hydrophilicity than the control NaAlg scaffold.

3.5. In vitro cell studies

To evaluate the biocompatibility and vasculogenesis potential of the printed Albumen/NaAlg composite scaffold, HUVECs were seeded on the surface of the composite scaffolds, cultured for 4 days and stained for live/dead assay (Fig. 4). The HUVECs cultured on scaffold surface

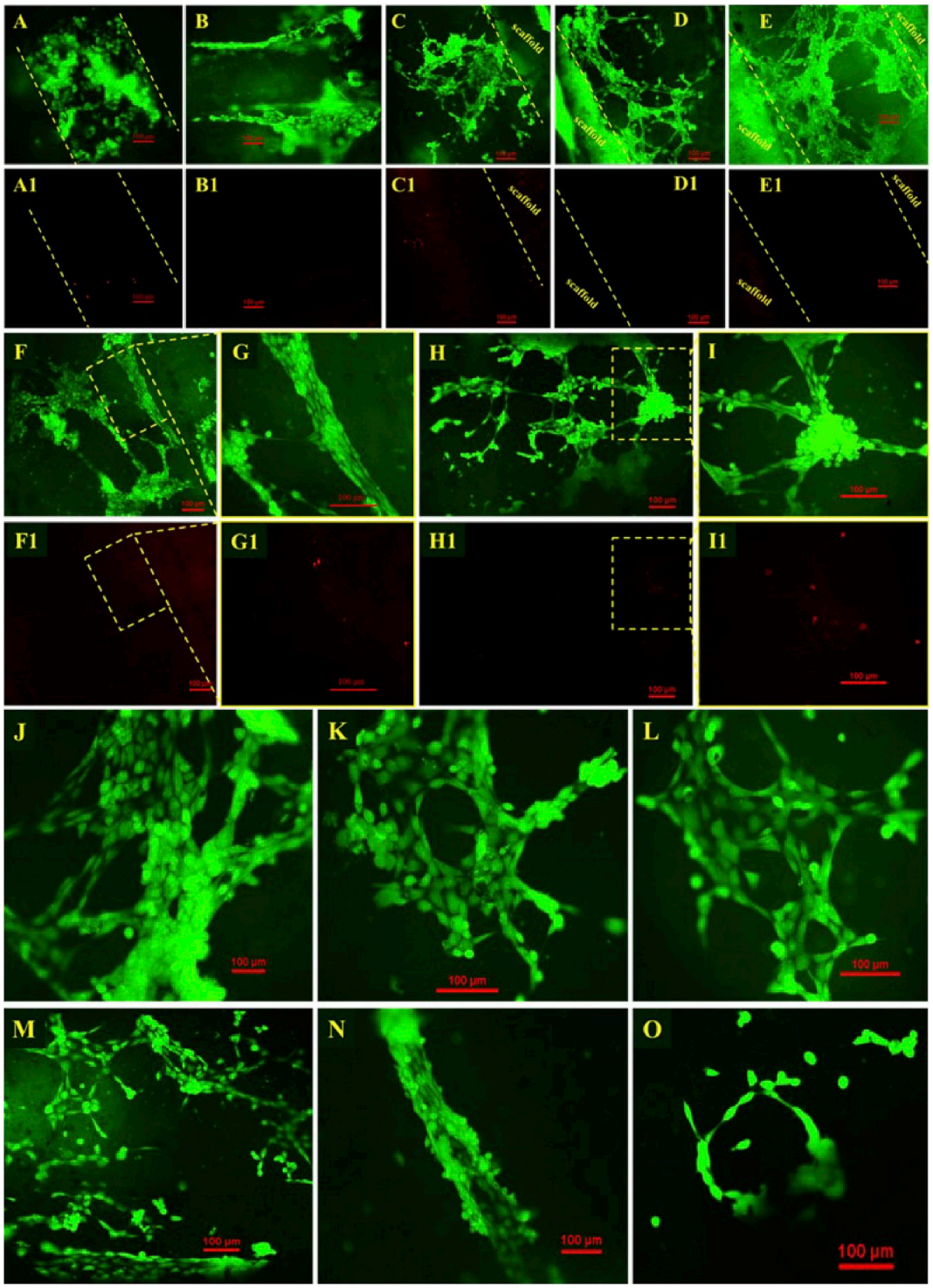

Fig. 4. Fluorescent microscope images of HUVECs cultured on the 3D printed Albumen/NaAlg composite scaffold after 4 days. (A–O) Live (Green) and (A1-I1) corresponding dead cells (Red). (A–B) HUVECs on the surface of the scaffold & (C–E) the endothelial cells sprouting between filaments of Albumen/NaAlg composite scaffolds. Yellow dashed lines indicate the edge of scaffold. (F–I) The vascular network formation within the 3D printed Albumen/NaAlg composite scaffold at different locations. Yellow dashed lines indicate the corresponding local magnification. (J–O) The higher microscopic images of endothelial sprouting and vascular networks within the Albumen/NaAlg composite scaffolds.

showed high cell viability (Fig. 4A and B). Moreover, the surface of the printed Albumen/NaAlg composite filament was fully wrapped by the cells to form a cell layer (Fig. 4B). Correspondingly, there were a few dead cells (Red) on the surface of the scaffold (Fig. 4A1 & B1). Interestingly, it was observed that sprout formation and primitive microvessel networks were formed between adjacent fibers (Fig. 4C–E). Robust endothelial sprouting into the surrounding fibers was also observed in Fig. 4C and D. A network of endothelial sprouting interconnection was built between adjacent fibers (Fig. 4E). Furthermore, several representative fluorescence images of endothelial sprouts, neovessels and vessel networks were captured at different scaffold areas as shown in Fig. 4F–I & Fig. 4J-O at higher magnification. Fig. 4H and I

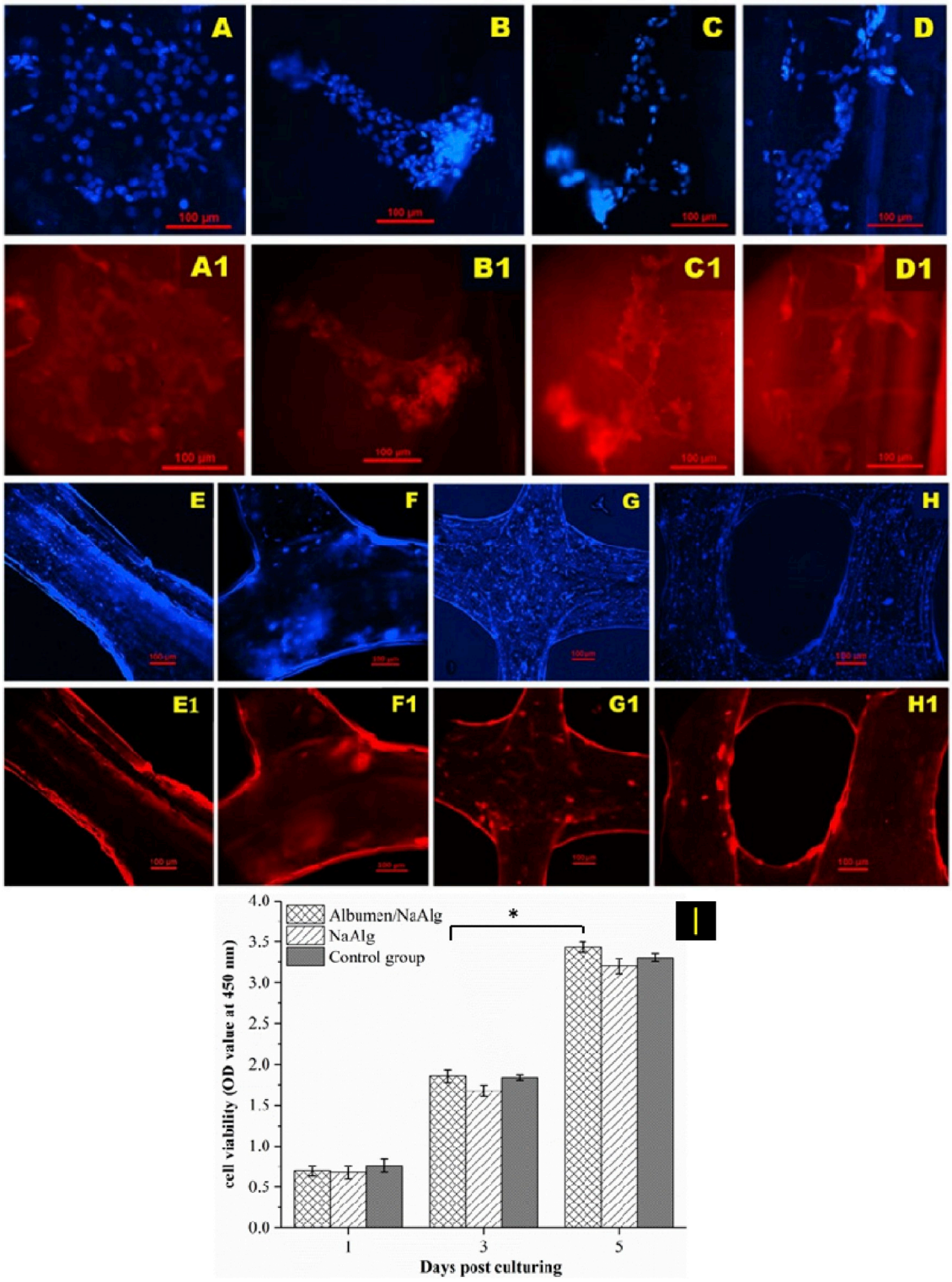

Fig. 5. Fluorescent microscopic images of HUVECs cultured after 4 days: (A) DAPI stained image showing nucleus of HUVECs cultured on the scaffold surface; (A1) corresponding TRITC Phalloidin stained image showing cell cytoskeleton on the scaffold surface; (B-D) DAPI stained images showing formation of vascular networks within 3D printed Albumen/NaAlg composite scaffold; (B1-D1) Corresponding Phalloidin stained cytoskeleton of the vascular networks. Fluorescent microscopic images of HUVECs stained with DAPI & Phalloidin showing scaffold structural boundaries after 4 days of culture: (E-E1) in single filament, (F–F1) T-type structure, (G-G1) the perpendicular stacked structure and (H–H1) the porous structure. (I) CCK -8 assay data analysis after culturing 1, 3, and 5 days. The single asterisk (*) indicates a statistically significant difference compared data for the Albumen/NaAlg group at 3 and 5 days time point ($n = 6$, $*p < 0.05$, error bars indicate SD).

showed that neovessel network was built within 3D printed Albumen/NaAlg composite scaffold with higher vessel sprouts and lacunarity (Fig. 4J-O). The average diameter of lacunae (holes present inbetween sprouts) varied between 50 and 100 μm. Fig. 4O presented a larger ring of endothelial cells, as a cross-section of a neovessel. Many of the initial sprouts were formed around the scaffold (Fig. 4M). Furthermore, a large neovessel with an obvious open lumen (Fig. 4N) was captured; the diameter is about 100 μm.

Additionally, fluorescent staining of the Albumen/NaAlg composite scaffold was carried out to visualize the cell nucleus and cytoskeleton after 4 days of culturing. Homogeneous and confluent distribution of cells on the surface of the scaffold was observed in the DAPI (cell nucleus) and TRITC-phalloidin (cytoskeleton) stained images (Fig. 5 A/A1). The representative fluorescence images of the neovessel and sprouts were also presented (Fig. 5B–D). Fig. 5E–H shows an endothelialized layer on the scaffold surface and exhibit the morphology of scaffold. Altogether, live/dead and cell nucleus/cytoskeleton staining images demonstrated that the cells were uniformly distributed, with high cell viability on the Albumen/NaAlg composite scaffold surface with endothelial cell sprouting that formed a prevascular network between adjacent fibers. Compared with previous studies on NaAlg (Khalil and Sun, 2009; Wang et al., 2003), the printed Albumen/NaAlg composite scaffold in the present study not only provides an adaptive 3D microenvironment for cell growth, but is also conducive enough to stimulate endothelial sprouting and formation of interconnected vessel networks *in vitro*. As stated in literature, the albumen (or egg white) have been already reported to use as ECM substitute that elicited prevascular network formation by endothelial and/or smooth muscle cell co-culture *in vitro* (Mousseau et al., 2014). Additionally, the extent of attachment, migration, and proliferation of different cell types have been also reported to be enhanced by proteins derived from albumen (Kaipparettu et al., 2008; Yan et al., 2006; Yoon et al., 2009). These results altogether proves that neither the preparation of Albumen/NaAlg composite bioink nor the fabrication process of 3D printing promoted any relevant change in the structure of albumen proteins, allowing to maintain their structural properties (Pal et al., 2011; Raikos et al., 2006), indicating that the fabricated Albumen/NaAlg composite scaffolds also have great potential to promote vascularization for tissue regeneration.

3.6. CCK-8 assay

In order to further quantify the cytoactivity of HUVECs and the influence of protein components in the fabricated Albumen/NaAlg composite scaffolds on cell proliferation, CCK-8 assay was performed. The CCK-8 assay data (Fig. 5I) show that, compared with day 1, the OD values of Albumen/NaAlg, NaAlg, and control group increased by about two to three folds after culturing for 3 days; the values in all groups was seen to be sharply improved (by approximately 5–6 times) on day 5. Moreover, some slight differences can be found that, after culturing 1 day, the OD values of Albumen/NaAlg and NaAlg samples were close and slightly lower than the control group. After 3 days of culture, the OD values of Albumen/NaAlg increased significantly, which was equal to that of the control group and higher than that of NaAlg sample. On day 5, the OD values of Albumen/NaAlg already exceeded that of NaAlg and control group. This indicates that cells can maintain their proliferation ability when cultured with the printed Albumen/NaAlg composite scaffold. These results imply that the scaffold containing albumen protein allows the growth of endothelial cells.

In addition, the previous study reported that albumen scaffolds supported active metabolism, proliferation, and migration of human dermal fibroblasts *in vitro* (Jalili-Firoozinezhad et al., 2015a). It has been already reported that both ovalbumin and ovomucoid can promote the proliferation of myoblasts and growth of myotubes (Mizunoya et al., 2015). Also, the fabricated Albumen/silk composite films significantly promoted initial adhesion of HUVECs and supported long-term cell proliferation (You et al., 2017). Therefore, we guess that the highest OD values of Albumen/NaAlg scaffold group achieved can be attributed to the following factors; firstly, the composite scaffold contained albumen protein which is conducive to cell attachment, proliferation, metabolism and migration; secondly, the 3D printed Albumen/NaAlg composite scaffold provides a favorable 3D condition for the even distribution and growth of cells; and the printed porous structure contribute to the mass transportation of sufficient nutrient and oxygen. These will be further research in the future.

4. Conclusion

In this paper, we reported a novel method for the preparation of the composite scaffold using Albumen/NaAlg composite bioink via combining extrusion-based 3D printing and crosslinking mechanism. We first proposed to use albumen mixed with NaAlg to prepare Albumen/NaAlg composite bioink. The different concentration ratio of Albumen/NaAlg composite bioink was studied. The composite scaffolds were fabricated using the selected concentration ratio of Albumen/NaAlg composite bioink and various characteristics of the composite bioink/3D printed scaffold was investigated. Furthermore, *in vitro* experiment with HUVECs showed that the cells not only maintained high cell viability on the composite scaffolds but also conduce to stimulate endothelial sprouting and interconnected vessel network formation after culturing for a few days. Altogether, the results of this study imply that the Albumen/NaAlg composite 3D printed scaffolds have great potential in tissue and organ engineering, especially for inducing vascularization within engineered tissues.

Declaration of competing interest

None.

CRediT authorship contribution statement

Suihong Liu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Haiguang Zhang:** Project administration, Writing - review & editing, Visualization. **Qingxi Hu:** Project administration, Writing - review & editing, Funding acquisition, Supervision. **Zhipeng Shen:** Formal analysis, Investigation, Data curation. **Deepti Rana:** Writing - review & editing, Visualization, Investigation. **Murugan Ramalingam:** Project administration, Writing - review & editing, Visualization, Supervision.

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