

Bioactive glass fiber mat delivering Zn ions for full-thickness wound regeneration

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ABSTRACT

Full-thickness wounds are associated with high morbidity and mortality. Here, biodegradable bioactive glass (BG) fiber mats with glass composition $70\text{SiO}_2\text{-(30-x)CaO-xZnO}$ (mol%), where $x = 1, 3$ or 5 mol%, were synthesized for wound healing applications, facilitating therapeutic zinc ion release along with silica species and calcium ions. Cell attachment (fibroblasts and endothelial cells) and controlled ion release from the fibres is expected to promote angiogenesis. BG fibers ($0.19\text{--}0.32\text{ }\mu\text{m}$ diameter) were synthesized through sol-gel electrospinning into a 3D extracellular matrix (ECM)-mimicking mat, which favors the attachment and migration of cells compared to BG microparticles. Human dermal fibroblast (HDF) cell culture demonstrated no cytotoxicity when exposed to Zn-containing BG fibers (Zn-BG). Dissolution products of Zn-BG fibers containing $5\text{ mol\% ZnO}$ promoted angiogenic factors (VEGF and bFGF) expression compared to Zn-free BG fibers. Zn-BG-HDF media (supernatant from the co-culture of Zn-BG dissolution products and HDFs 1:1 mixed with fresh endothelial cell medium) promoted the metabolic activity of human umbilical vein endothelial cells (HUVECs) compared to 70S30C -HDF media. All BG-HDF media increased cell migration compared to untreated DMEM-HDF media, with the highest migration observed in response to BG with $5\text{ mol\% ZnO}$. When immersed in DMEM for 24 h , Zn-BG released $0.5\text{--}6\text{ }\mu\text{g mL}^{-1}$ of Zn, which is a concentration range with potential to elicit other beneficial effects in wound healing, such as antibacterial activity, positive inflammatory response, and follicle regeneration. Results suggest that Zn-BG fiber mats could help the regeneration of full-thickness wounds.

1. Introduction

Wounds less than about 1 mm deep regenerate spontaneously after injury through an ordered process—hemostasis, inflammation, proliferation, and remodeling [1,2]. However, wounds that fully penetrate the dermis to the hypodermis, or even to the muscle and bone underneath, heal slowly and cause permanent function loss to tissues [3]. These wounds, categorized as full-thickness wounds, carry a significant socio-economic burden and are associated with a mortality rate of nearly 20% [4,5].

Patients afflicted by severe full-thickness injuries face an elevated risk of infection, often accompanied by necrosis [6,7] and follicle loss, resulting in functional impairment and reduced stem cell recruitment [8]. The compromised ECM and impaired angiogenesis in the dermis are

the major reason for the slow wound contraction, due to the lack of nutrient and oxygen supply [9]. The conventional healing approach, autografting, proves suboptimal due to the limited availability of suitable grafts and associated donor site morbidity. [10,11]. 3D wound matrices offer a potential alternative as they provide sites for cell attachment (therefore avoiding anoikis), give mechanical support, and can promote the release of biological cues to cells [12]. Advanced therapeutic approaches for wound healing include the delivery of biomolecules and proteins via hydrogels [13–16], but there are limitations in terms of reproducibility, high cost, and storage longevity. Recently, silicate and borate-based materials have been reported as strategies for wound regeneration [17–23]. Bioactive glasses (BGs) are a commercially successful product [24] and release therapeutic ions and bond to hard and soft tissues [25–30] with most clinical applications in

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orthopaedics and trauma. In addition to osteogenesis, dissolution products of BGs have been found to elicit other beneficial effects, including the stimulation of blood vessel formation [31,32] through the up-regulation of VEGF and bFGF expressions from fibroblasts [33,34]; promotion of ECM deposition through the up-regulation of collagen, fibronectin, PDGF, and TGF- β from fibroblasts [35,36]; prevention of infection through the release of antibacterial ions (e.g., Ag, Cu, Zn, Ce, and Ga) [37–45]; and induction of anti-inflammatory effects through the stimulation of macrophage polarization [46,47].

Here, we focus on Zn incorporation into sol-gel glasses, which can be challenging due to its role as a network intermediate. Zn is an important trace element in the skin, interacting with more than 300 proteins [48]. Zn is antibacterial even at very low concentrations, as $0.05 \mu\text{g mL}^{-1}$ Zn released from BGs is reported to have antibacterial effects [49,50]. A higher concentration of $5 \mu\text{g mL}^{-1}$ Zn has been reported to kill >99 % of most bacteria while remaining non-cytotoxic to eukaryotic cells [51–56]. The antibacterial mechanism involves the stimulation of reactive oxygen species (ROS), up-regulation of protease expression and interference with bacterial membrane activities through thiol-group binding [57,58]. In terms of anti-inflammatory activities, Zn operates in conjunction with specific Zn-transporter mRNA to maintain hydroxyl radical concentrations by promoting the expression of superoxide dismutase. It also upregulates anti-inflammatory cytokines until a critical Zn level is reached [59,60]. In macrophage cultures, $1 \mu\text{g mL}^{-1}$ of Zn released from BG was reported to stimulate the up-regulation of Interleukin-10 (IL-10) and the down-regulation of Interleukin-1 β (IL-1 β), promoting a shift of macrophage polarization from inflammatory (M1) to anti-inflammatory populations (M2) [61]. Similar effects were observed in vivo [40], although anti-inflammation responses were reduced when the released Zn exceeded $15 \mu\text{g mL}^{-1}$ [62]. Furthermore, Zn is essential for the development and regeneration of hair follicles [63–65]. Zinc chloride was found to stimulate the migration and proliferation of human hair follicle dermal papilla cells and upregulated their expressions of genes (VEGF, BMP-6, KGF, HGF, PDGF- α , PDGF- β , and C-Myc) [17]. While the exact range of Zn dose for promoting follicle regeneration remains unconfirmed, a range of $2 \mu\text{g mL}^{-1}$ [66–68] to $13 \mu\text{g mL}^{-1}$ is suggested [17,65].

Here, the focus is to investigate the effect of Zn delivery from novel glass fibers and its effect on angiogenesis. Zn has been reported to be pro-angiogenic but its mechanism remains uncertain. Zn has been reported to stimulate paracrine signaling from macrophages and fibroblasts to encourage the expression of angiogenic factors [69,70], promotion of desirable endothelial cell behavior [71,72], and stimulation of blood vessel formation in vivo [73,74]. For example, Aina et al. found that bioactive glass containing Zn (Zn-BG) particles promoted the proliferation and migration of endothelial cells [75]. In recent studies, Zhu et al. showed the paracrine angiogenic effects of Zn-BG by culturing macrophages with alginate-chitosan hydrogels containing Zn-BG. The hydrogel composite was antibacterial in vitro and stimulated wound closure and angiogenesis in vivo [40]. Another BG composition, known as 1605 (20 CaO- 4 P₂O₅- 6 Na₂O- 51.6 B₂O₃- 12 K₂O- 5 MgO- 1 CuO- 0.4 ZnO, wt%), was proposed as an anti-biofilm wound matrix against *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus*, and *Acinetobacter baumannii* [76,77]. It was also reported to stimulate fibroblast migration [78,79], VEGF expression from fibroblasts [77], and in vivo tissue granulation and wound healing [80,81].

Within wound sites, non-soluble ECM proteins allow attachment, proliferation, and migration of fibroblasts and endothelial cells [1,2,82]. Existing literature on BG-based wound matrices primarily incorporates BG particles in a polymeric matrix [83–88]. Recently, Mo-Sci corporation (Rolla, MO) developed a melt-blowing approach to fabricate borate-based BG fibers within various compositions [89–91]. These fibers have demonstrated clinical success in chronic wounds, e.g. diabetic venous ulcers that had not healed after many months of conventional treatment. While the precise mechanism of action is not yet fully understood, it is believed to result from a combination of ion release, fiber

morphology, and biodegradation rate. As the fibers were melt-derived, they are short and heterogeneous, contain up to 40 wt% of Na and K, and have up to 75 % of beading [92–97]. To further advance wound dressing technology, our objective is to produce homogenous, ultrafine fibrous scaffolds that are more flexible in composition with controlled ion release profiles using the sol-gel approach [98,99].

Kim et al. pioneered the BG sol-gel electrospinning technique [100], with sol-gel precursors blended with a polymer and delivered from a syringe, where they were then attracted toward a collector by electric potential. The diameters of the BG fibers can be adjusted by altering the precursor concentration and the electrospinning parameters. The voltage applied had a significant impact on fiber morphology (fiber thickness and level of beading) [101]. Poolagasundarampillai et al. introduced the first binder-free sol-gel electrospinning approach to produce 3D structured mats (0.5–2 mm in thickness) compared to conventional 2D mats (< 0.5 mm) with BG fibers with a diameter of $1.5 \mu\text{m}$ [102]. The unique architecture of this fiber mat, also referred to as cotton-wool-like fiber mats, allows bone cell adhesion and promotes their proliferation and migration [103]. Silicate-based BG fibers have been produced in different compositions (13–93 [104], 58S [105]) and textures (co-axial MBG fibers [106]) for bone and tissue engineering. Incorporation of small amounts of therapeutic ions can result in substantial changes in biological properties of the glasses. For example, BG fibers with 1–2 mol% B incorporation induced much faster wound closure and less exudate formation in rabbits [107] and adding another 0.01 mol% Ag resulted in additional antibacterial properties similar to that of the gentamicin, a common antibiotic [108,109]. However, for soft tissue repair, a polymer was reintroduced into the spinning process to reduce the thickness of 3D fibers, which resulted in fiber diameters 0.21–0.34 μm , similar the thickness of collagen fibrils [110]. Fibers with up to 30 mol% CaO were found to be non-cytotoxic to Human Dermal Fibroblasts (HDFs) and stimulated higher VEGF expression from the cells, compared to control media, indicating their potential angiogenic activities.

In this study, novel 3D biodegradable Zn-containing BG fiber mats were synthesized to deliver therapeutic Zn ions for wound regeneration, with the intention that the fiber mats could be used underneath conventional dressings. Zn incorporation into electrospun fibers is complicated by the electric field involved and the need to optimize the variables in the electrospinning process, such as humidity and electric field strength. The aim was to produce homogeneous fibers with diameters ~200 nm, mimicking the extracellular matrix (ECM) of the skin because its 200 nm diameter aligns with the scale of collagen fibrils naturally present in the ECM. BG fibers with a nominal composition of 70 mol% SiO₂-(30-x) mol% CaO- x mol% ZnO (x = 0, 1, 3, and 5, labeled as 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn respectively) were prepared through the sol-gel electrospinning method [75,111–113]. The effects of Zn incorporation on the physical and chemical structures of BG fibers were assessed through XRD, FTIR, and SEM. The dissolution profiles of the resultant fiber mats were accessed in the TRIS buffer solution and DMEM. This is the first study of the effect of Zn-BG on cell proliferation, protein expression, and angiogenesis evaluated by both Human Dermal Fibroblasts (HDF) and Human Umbilical Vein Endothelial Cells (HUVECs). This included investigation of any synergistic effect of HDF conditioned media with the glass dissolution products on the angiogenic response of HUVECs.

2. Methods

2.1. Preparation and characterization of Zn-BG fiber mats

Tetraethyl orthosilicate (TEOS), calcium nitrate tetrahydrate (Ca(NO₃)₂•4H₂O), and polyvinyl butyral (PVB) were obtained from Sigma Aldrich (UK) while zinc nitrate hexahydrate (Zn(NO₃)₂•6H₂O) was obtained from Thermofisher (UK). The sol-gel precursors of the Zn-BG fiber mats were prepared through the step-wise addition of TEOS, Ca(NO₃)₂•4H₂O, Zn(NO₃)₂•6H₂O to a mixture of ethanol, water, and 2 N

nitric acid, as illustrated in Table 1 [110,114]. After being stirred at room temperature for 24 h and then rested for 48 h, the precursor was mixed with a PVB binder (1 g of PVB in 11.4 mL of ethanol) to acquire the ideal viscosity (0.13 Pa s) for electrospinning [102]. A 10 mL syringe with a gauge 22 stainless steel needle was filled with the sol and used for electrospinning (Spraybase, Dublin), under the following parameters: electric field strength: 10 kV; flow rate: 0.4 mL h⁻¹; air gap distance: 12 cm; relative humidity: 25 %–35 %; inner diameter of spinneret: 0.7 mm; room temperature. Then, the resultant fiber mats were heated to 600 °C at a rate of 1 °C min⁻¹ with a dwell at 600 °C for 3 h before oven cooling, to burn out the PVB and thermally stabilize the sol-gel glass.

The X-ray diffraction (XRD) patterns of the fiber mats were collected from an XRD analyzer (Bruker D2 PHASER) using Cu-K α radiation (λ = 1.542 Å), with the following parameters: applied voltage: 30 kV; applied current: 10 mA; scanning interval: 10° < 2 θ < 70°; step size: 0.035°; time per step: 12 s. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR Nicolet iS10) was used to assess the chemical functional groups in the BG fiber mats under the wavelength of 500–4000 cm⁻¹. The microstructures of the fiber mats were coated with 10 nm of gold and characterized under the Inlens mode of the LEO-1525 scanning electron microscope (SEM), with 5 kV accelerating voltage and 8 mm working distance. The diameters of the fibers were measured with ImageJ and the mean diameters were derived from 10 measurements.

2.2. Dissolution study

50 mg of the BG fiber mats were immersed in 75 mL of 5 mM tris (hydroxymethyl) aminomethane buffer solution (TRIS) or Dulbecco's Modified Eagle Medium (DMEM) and placed in an incubator shaker at 37 °C and 120 rpm [110,115]. 1 mL of the extracts were collected at each time point (4, 24, 48, 72, 96, and 168 h for TRIS and 4, 24, 72, and 168 h for DMEM), filtered with 0.2 μ m surfactant-free cellulose acetate syringe filters (Corning, USA) and diluted with 9 mL of 1 N nitric acid. The elemental concentration was determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) at the wavelengths of 317.9 nm (Ca), 251.6 nm (Si), and 213.8 nm (Zn). The pH change of the media was measured at each time point with a pH meter (Oakton pH 11 m with Oakton general-purpose glass pH probe, Ag/AgCl electrode, and Oakton ATC probe).

2.3. In vitro study on fibroblasts and endothelial cells

DMEM supplemented with 10 % (v/v) fetal bovine serum and 1 % penicillin/streptomycin (FBS, Thermo Trace Ltd., Melbourne, Australia) was used as culture media for human dermal fibroblasts (HDF, Sciencell, USA, passage number 6). BG-conditioned media were filtered dissolution products of 6 mg mL⁻¹ of BG fiber mats in the supplemented media. HDFs seeded in 96-well plates at a density of 1 $\times$ 10³ cells cm² were first cultured in fresh DMEM for 24 h and then cultured with undiluted and diluted (1/2 and 1/4 respectively) BG-conditioned media for 7 d. HDFs

Table 1

Sol-gel compositions 70 SiO₂-30-x CaO-x ZnO, in mol% (x = 0, 1, 3, 5) and the quantities of TEOS, nitric acid, ethanol, calcium nitrate tetrahydrate, and zinc nitrate hexahydrate used.

Nominal compositions [oxide mol%]	TEOS [mL]	2 N Nitric acid [mL]	Ethanol [mL]	Ca (NO ₃) ₂ •4H ₂ O [g]	Zn (NO ₃) ₂ •6H ₂ O [g]
70Si-30Ca	15.6	4.2	8	7.12	0
70Si-29Ca- 1Zn	15.6	4.2	8	6.84	0.3
70Si-27Ca- 3Zn	15.6	4.2	8	6.37	0.9
70Si-25Ca- 5Zn	15.6	4.2	8	5.9	1.49

in the control group were cultured in DMEM only. The metabolic activity of fibroblasts was determined on days 1, 3, and 7 through a Cell Counting Kit (CCK)-8 assay (Beyotime) according to the manufacturer's instructions. The metabolic activity was represented as units of optical density (OD) absorbance at 450 nm from an enzyme-linked immunosorbent assay microplate reader (Synergy 2, Bio-TEK). The supernatants of the HDFs were extracted on day 3 for the characterization of VEGF, bFGF, PDGF, and TGF- β through an ELISA assay (Beyotime).

Human umbilical vein endothelial cells (HUVECs, Sciencell, USA, passage number 6) were seeded in 96-well plates at a density of 3 $\times$ 10³ cells cm⁻² and cultured with the endothelial cell culture medium (Sciencell, USA) with 5 % (v/v) fetal bovine serum (FBS, Thermo Trace Ltd., Melbourne, Australia) and 1 % (v/v) endothelial cell growth supplement/heparin kit (ECGS/H, Promocell) for 24 h. HDFs were cultured in BG-conditioned media (6 mg mL⁻¹ in supplemented DMEM, while the positive control was HDF cultured in ordinary DMEM) for 2 d and the resultant media (BG-HDF conditioned media and DMEM-HDF media) was mixed with the endothelial cell culture medium by 1:1. 100 μ L of the BG-HDF conditioned media was applied to HUVECs. The mitochondrial activity of HUVECs was assessed through the CCK-8 assay and the expressions of VEGF, bFGF, and eNOS were determined through ELISA assays on day 3.

The migration of the HUVECs was assessed through a Transwell assay and scratch assay. In the Transwell assay, HUVECs were seeded in the upper layer of the Transwell plate at 3 $\times$ 10³ cells per well. Then, 250 μ L of the DMEM-HDF or BG-HDF conditioned media were added to the upper layer of the well, while 300 μ L of endothelial cell culture medium was added to the bottom. Cells will migrate from the upper layer to the bottom layer and the number of cells that pass through the filter was quantified. The cells were cultured for 16 h and the non-migrated cells at the upper layer were wiped off using a cotton swab. In the scratch assay, HUVEC were cultured until confluent in a 24-well plate. Then, scratches of $\sim$ 150 μ m was generated using pipette tips and 250 μ L of the DMEM-HDF or BG-HDF conditioned media were added. After 8 h, the leftover cells were stained with crystal violet for 30 min. Cells in the filter were fixed with 4 % Paraformaldehyde and stained with crystal violet for 30 min. The migrated cells were imaged using an inverted microscope (Leica Microsystems, Germany), and cell population was counted from the image using ImageJ and an open source cell counter (bitbucket), according to the manufacturer's instruction [116].

The statistical significance of the results was compared using a one-way ANOVA test followed by Dunn's Multiple Comparison. The significance was taken between individual components in the in vitro assessment and marked as (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

3. Results and discussion

3.1. Optimization of electrospinning variables to produce 3D fiber mats

The sol-gel electrospinning parameters were optimized to prepare long, uniform, and straight BG fibers without the presence of beads (Fig. 1(a–f)). The viscosity of the sol for fiber mat production was chosen to be 0.13 $\pm$ 0.025 Pa s, higher than the previously reported 0.069 Pa s which yields shorter fibers and some beads formation (Fig. 1(b)) [110]. Viscosity control was achieved by adding a PVB binder to the sol-gel precursor. The PVB binder does not incorporate into the BG structure and is burnt out during glass sintering, leaving thin mesoporous BG fibers.

The spinning distance was set at 12 cm to enable drying and bending instability of the gel during its propagation towards the collector. 10 kV was found to be the optimal voltage between the needle and the collector, as a lower voltage (6 kV) yielded 2D fiber mats rather than 3D ones, and a higher voltage (15 kV) yielded thicker BG fibers. Humidity also played a crucial role in determining fiber morphology. Fibers

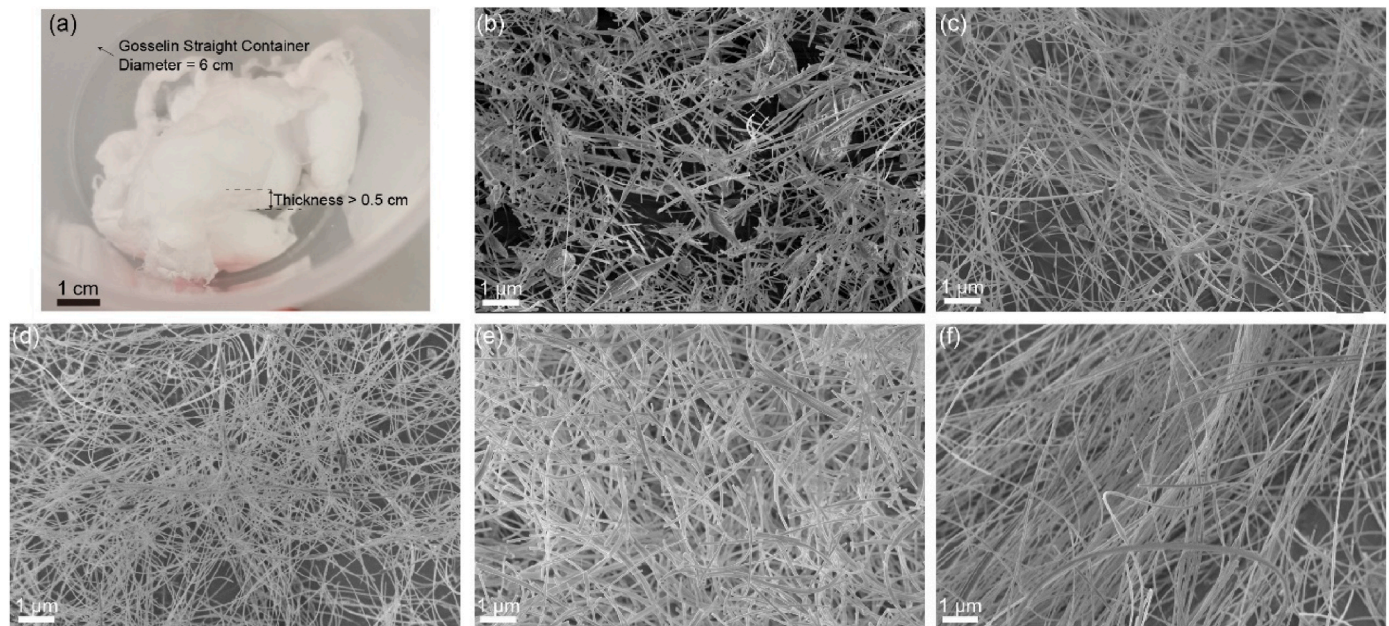


Fig. 1. (a) Photograph of an electrospun fibrous bioactive glass scaffold; (b) SEM image of fiber mats electrospun from sol with 0.069 Pa s viscosity (c–f) SEM images of the fiber mats with composition 70 SiO₂-30-x CaO-x ZnO, in mol% where x = 0–5: (c) 0; (d) 1; (e) 3; and (f) 5.

prepared under 25–35 % relative humidity exhibited long, thin, and homogeneous features, but those prepared under a relative humidity above 35 % are short-branched, thick, non-uniform, and with an increased yield of beads.

With the optimized condition, 3D BG fiber mats with a total thickness greater than 0.5 cm were produced (Fig. 1 (a)). The 3D morphology of the fibers was induced through the branching effect of the electrospun jet, probably caused by the repulsion of the Ca²⁺ and Zn²⁺ ions. All BG fibers had a thickness ranging from 160 to 320 nm (Fig. 1(c–f)), which mimicked the collagen and fibronectin fibrils in the extracellular matrix [117,118]. The incorporation of Zn did not affect the morphology and thickness of the fibers.

XRD patterns (Fig. 2 (a)) for fiber mats with different Zn compositions displayed amorphous silica halos with wide SiO₂ peaks in the range of 20°–34° (2θ) at low intensity, without any prominent crystal peaks, similar to those obtained from mesoporous Zn-containing BG powders or scaffolds [119–121]. Some small peaks at 29°, 32°, 34°, 37°, and 48° were attributed to crystalline phases of calcium silicates, such as Wollastonite, or zinc oxide [110,122].

FTIR (Fig. 2 (b)) analyzed the bonds present in BG fiber mats with different Zn compositions. Bands at 1040 and 790 cm^{−1} corresponded to the Si-O stretching and the Si-OH peak, respectively. Bands at 470 cm^{−1} were identified as Si-O-Si rocking vibrations [123–125], while the band at 485 cm^{−1} represented the vibration of Zn-O bonding [126]. The absence of bands at 1500, 1420, 1638, and 1300–1500 cm^{−1} indicated the absence of CO₃^{2−} groups, NO₃[−] groups, water, or ethanol remaining in the fiber mats.

3.2. Dissolution profiles

BG fiber mats were biodegradable in both TRIS and DMEM and the release of Zn, Ca, and Si (expected to be in the form of SiO₄^{4−}) upon soaking in both solutions for 7 d was monitored. In TRIS, a high

proportion of Ca and Si was released after 24 h of immersion (Fig. 3). The concentration of Si released from all fiber mats was similar but the Zn-BG fibers released less Ca. This could be attributed to the reduced Ca content in BG fibers and the potential role of Zn within the glass network in reducing the ion release kinetics, as Zn functions as a glass intermediate that increases connectivity of the glass by binding with 6 adjacent non-bonding or non-bridging oxygens (Fig. 4) [75]. The pH changes during BG dissolution followed a pattern similar to that of the Ca release [124,127], confirming the ion exchange between the BG fiber mats and the soaking media. The release of Zn in TRIS peaked on day 2, followed by stabilization from day 4 to day 7, and generally agrees with the Zn content in BG composition.

In DMEM, the Si release was similar to that in TRIS, while the Ca release was significantly lower (Fig. 5). 70S30C fibers still released more Ca compared to fibers with Zn in their composition. By day 7, the Ca concentration reached approximately 60 μg mL^{−1}, a level similar to the Ca concentration reported to be beneficial for fibroblast proliferation (56–64 μg mL^{−1}) [128]. Zn-BGs still exhibited lower Ca release and pH in DMEM, but the difference was not as pronounced as that in TRIS. Zn release by day 7 increased from 0.5 to 4.5 μg mL^{−1} as Zn content increased from 1 to 5 mol%. The increase was not proportional to Zn content, consistent with observations by Neščáková et al. [121], due to zinc acting as a network intermediate, taking a forming role once the concentration exceeds the critical composition, strengthening the glass network. Maintaining the Zn release below 7 μg mL^{−1} is likely to prevent cytotoxicity, as Paasche et al. found that 7.41 μg mL^{−1} of Zn was toxic to fibroblasts [129]. The released Zn was within the therapeutic range expected to be anti-bactericidal (>5 μg mL^{−1}), modulate inflammation (1–15 μg mL^{−1}), and promote follicle regeneration (2–13 μg mL^{−1}) [51, 54,130–132].

3.3. Metabolic activity (MA)

Fibroblasts play a pivotal role in wound healing as they produce ECM proteins that express a number of growth factors for angiogenesis and tissue regeneration [2,133]. On day 1, all HDFs cultured with BG dissolution products exhibited higher MA relative to those cultured with basal DMEM (Fig. 6). This may be due to the role of Ca, which has been known to promote fibroblast proliferation [134–136]. An increase in the

Table 2

Fiber diameters of the electrospun bioactive glass scaffolds.

Sample	70S30C	70S29C1Zn	70S27C3Zn	70S25C5Zn
Fiber diameter [μm]	0.32 ± 0.07	0.19 ± 0.05	0.23 ± 0.05	0.24 ± 0.05

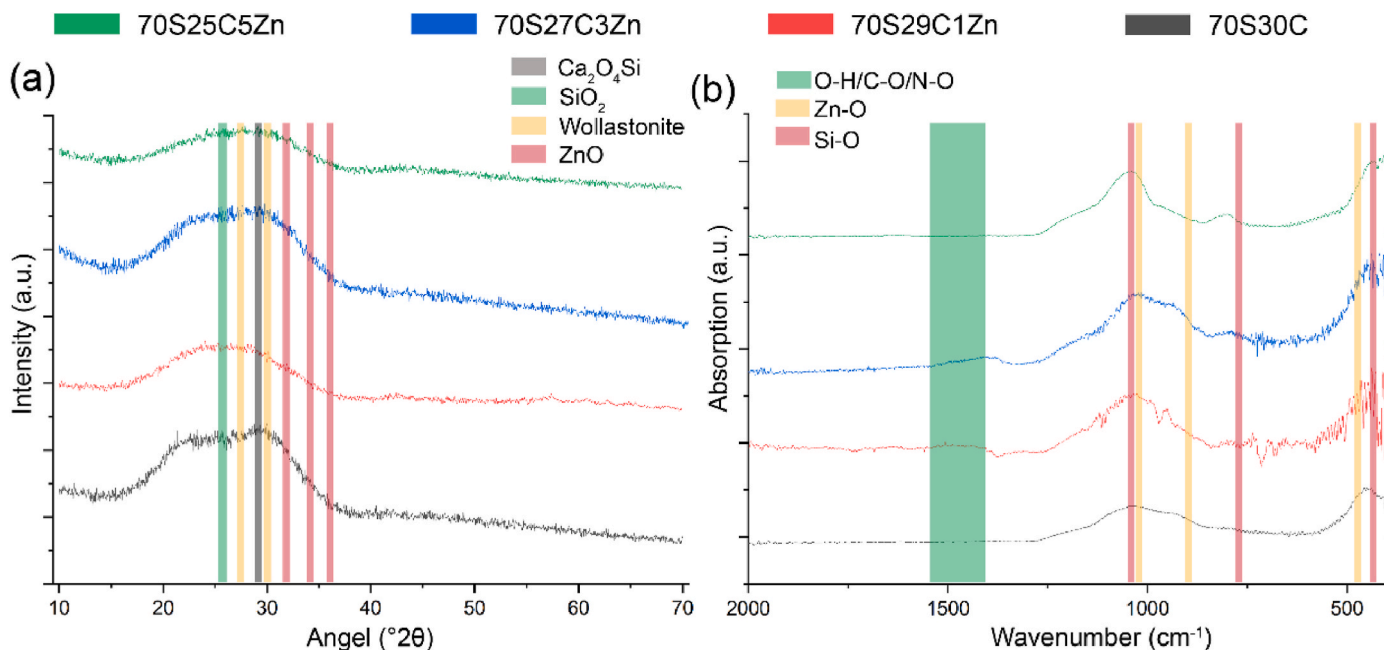


Fig. 2. Characterization of 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn bioactive glass scaffolds: (a) XRD patterns and (b) FTIR spectra.

Zn content of BGs reduced the release rate of Ca and therefore the corresponding MA. On day 3, the 70S30C fibers caused a reduction in MA compared to basal DMEM, which agrees with the previously reported inhibition effect of calcium-silicate BGs on fibroblasts [33,34]. This is possibly due to high Ca level and corresponding high pH environment, as the pH values for undiluted, $\frac{1}{2}$ diluted, and $\frac{1}{4}$ diluted conditioned media were 9, 8.4, and 7.8 respectively, consistent with the data reported in Fig. 5 (d). A similar reduction in MA was observed when the cells were cultured with the $\frac{1}{2}$ diluted media (Fig. 6 (b)), while the $\frac{1}{4}$ diluted media (Fig. 6 (c)), with much lower Ca concentration and pH, did not reduce the HDF MA significantly. Zn-BGs did not exhibit any cytotoxic effects on fibroblasts, as expected, since the Zn level was below $7 \mu\text{g mL}^{-1}$ [129,137]. Several catabolic activities involved with apoptosis are catalyzed by Ca^{2+} , e.g. activated proteases (calpains), phospholipases, and endonucleases. While the Ca level in vivo is usually maintained at homeostasis, an abrupt increase of Ca may induce an adverse effect to cell metabolism. Presence of Zn may mitigate this by forming a Zn-Ca complex [138–140] or by promoting calcium metabolism in fibroblasts [141,142]. At day 7, there were no significant differences in MA between the cells cultured with basal DMEM and those exposed to BG dissolution products.

3.4. Growth factor expression from fibroblasts

Platelet-derived growth factor (PDGF) has been shown to stimulate the proliferation, migration of fibroblasts and their secretion of ECM proteins [2,143,144]. PDGF also contributes to increased angiogenesis and re-epithelialization by promoting the proliferation of keratinocytes and endothelial cells [133,145–147]. Here, media conditioned with dissolution products of all BG fibers stimulated PDGF expression compared to the basal DMEM, with 70S30C and 70S29C1Zn exhibiting the strongest effect, and 70S27C3Zn and 70S25C5Zn having a slightly lower impact (Fig. 7). Ca release may be the cause of the upregulation of the expression of PDGF (Fig. 7 (a)), in agreement with previous work [148,149], while the incorporation of Zn into the glass had no significant effect on PDGF expression. Conditioned media diluted by half (Fig. 7 (b)) reduced PDGF expression from HDFs.

TGF- β promotes the production of ECM proteins, including collagen, elastin, and fibronectin [150]. Its activation is an important signal for full-thickness wound regeneration and contributes to the reduction of

inflammation and the promotion of angiogenesis [151–153]. In the healing cascade, the TGF- β level in the epidermis should increase, and that in the dermis decrease, in order to promote wound closure and prevent fibrosis [154]. Undiluted 70S30C conditioned media reduced TGF- β expression (Fig. 7 (a)), which could be attributed to the reduced MA (Fig. 6) as TGF- β contributes to fibroblast chemotaxis in activation and proliferation [2,82]. Another possible cause can be Ca regulation in TGF- β expression as Ca was previously found to be the key regulator for factor IV of TGF- β chemotaxis [155,156]. The half diluted media with less Ca exhibited a slight stimulatory effect on TGF- β expressions (Fig. 7 (b)). The incorporation of Zn in the BG system restored TGF- β to the control level. This may be associated with the reduction of Ca release due to increased Zn content in BG or the influence of Zn on TGF- β through other pathways [157].

VEGF and bFGF are major proteins that ensure the survival of endothelial cells and stimulate their migration and proliferation to ultimately promote angiogenesis [158,159]. Up-regulated VEGF and bFGF expressions from HDFs were observed from HDFs treated with dissolution products of 70S25C5Zn (Fig. 7 (a)). 70S30C fibers did not show significant up-regulation of VEGF and bFGF compared to the control, possibly due to the reduced MA (Fig. 6). This agrees Norris et al., wherein SiO_2 -CaO based BG fibers up-regulated VEGF expression from HDF cell lines only when normalized to cell number [110]. Ca likely plays a role in promoting VEGF and bFGF expression from their interaction with HIF-1 α [160,161]. Ca signaling also stimulates the translation of HIF-1 α to α -subunits through the activation of classical protein kinase C- α and mTOR (activity of mammalian targets of rapamycin) pathway [162, 163]. 70S29C1Zn and 70S27C3Zn fibers induced VEGF and bFGF expression similar to that of the control, while 70S25C5Zn had significant up-regulation of both proteins. This could be attributed to that Zn started exhibiting angiogenic effects only beyond a certain critical concentration. Previous studies have reported that Zn-containing materials can stimulate the expression of VEGF and bFGF in vitro [65,69, 164,165], and angiogenesis in vivo [73,74]. Zn is reported to interact with HIF-1 and HIF-2 to stimulate VEGF expressions [70]. Yet, existing literature mainly focused on the paracrine angiogenic effect of Zn during anti-inflammation, the precise relationship between Zn concentration and its effects on fibroblasts and endothelial cells requires further analysis.

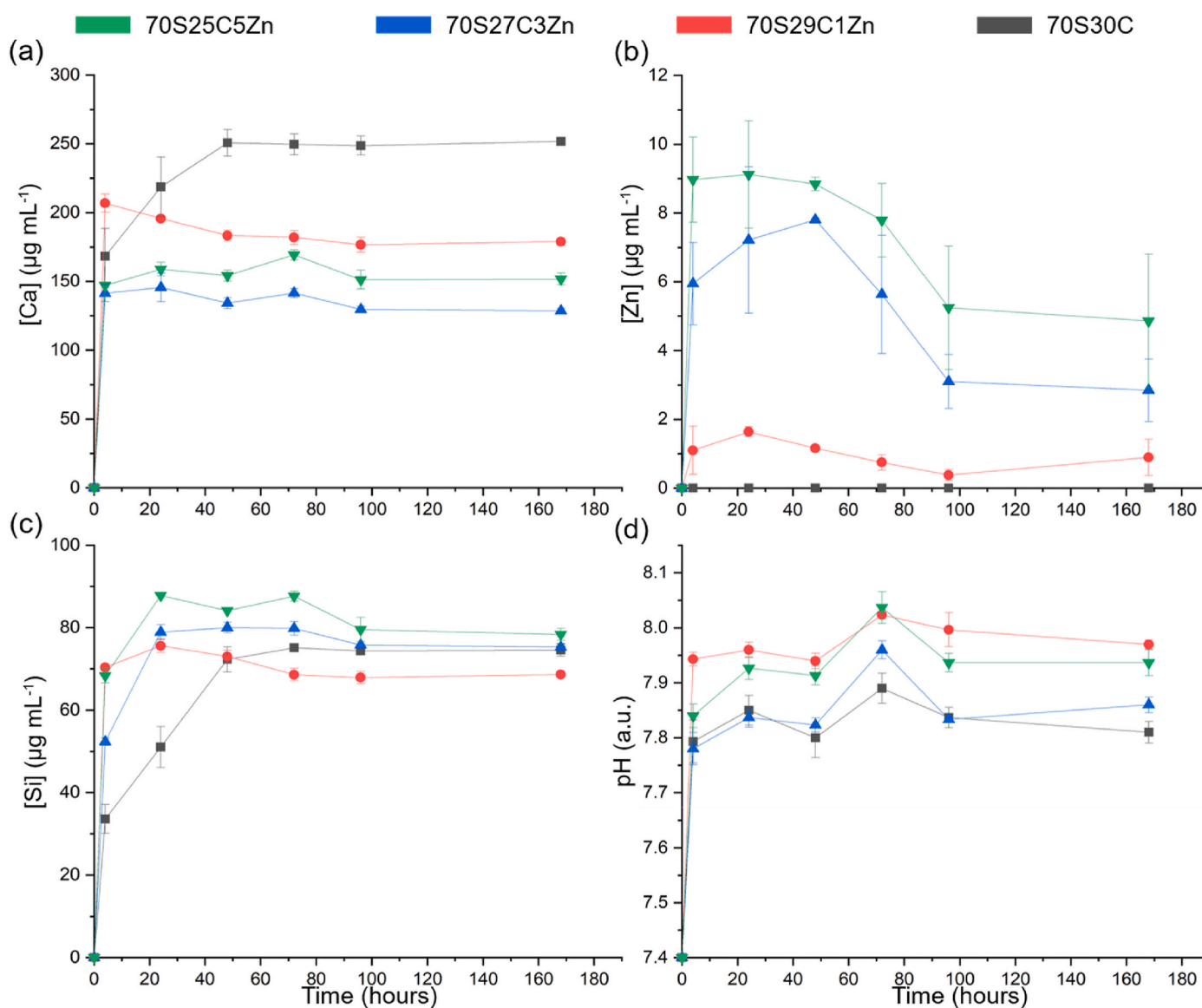


Fig. 3. Elemental concentration profiles (ICP) of TRIS buffer following immersion of bioactive glass fiber mats 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn over 7 d: (a) Ca, (b) Zn, and (c) Si; and (d) pH change.

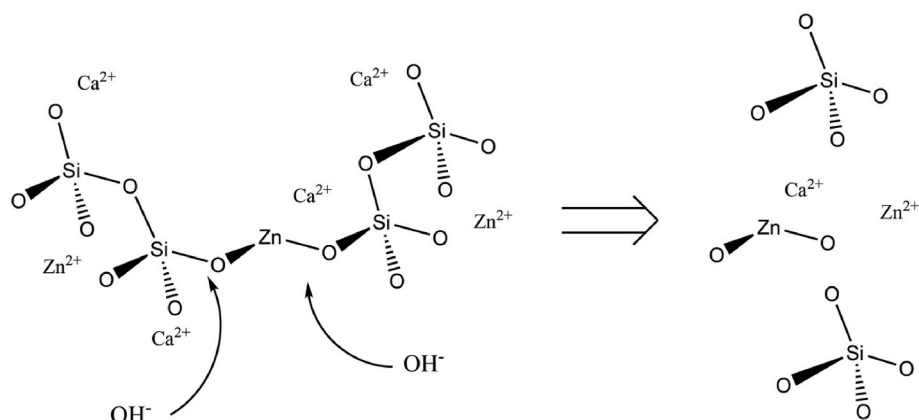


Fig. 4. Schematic of Zn being a glass intermediate, functioning both as a network former and modifier, and the formation of Zn-Ca complexes upon glass dissolution.

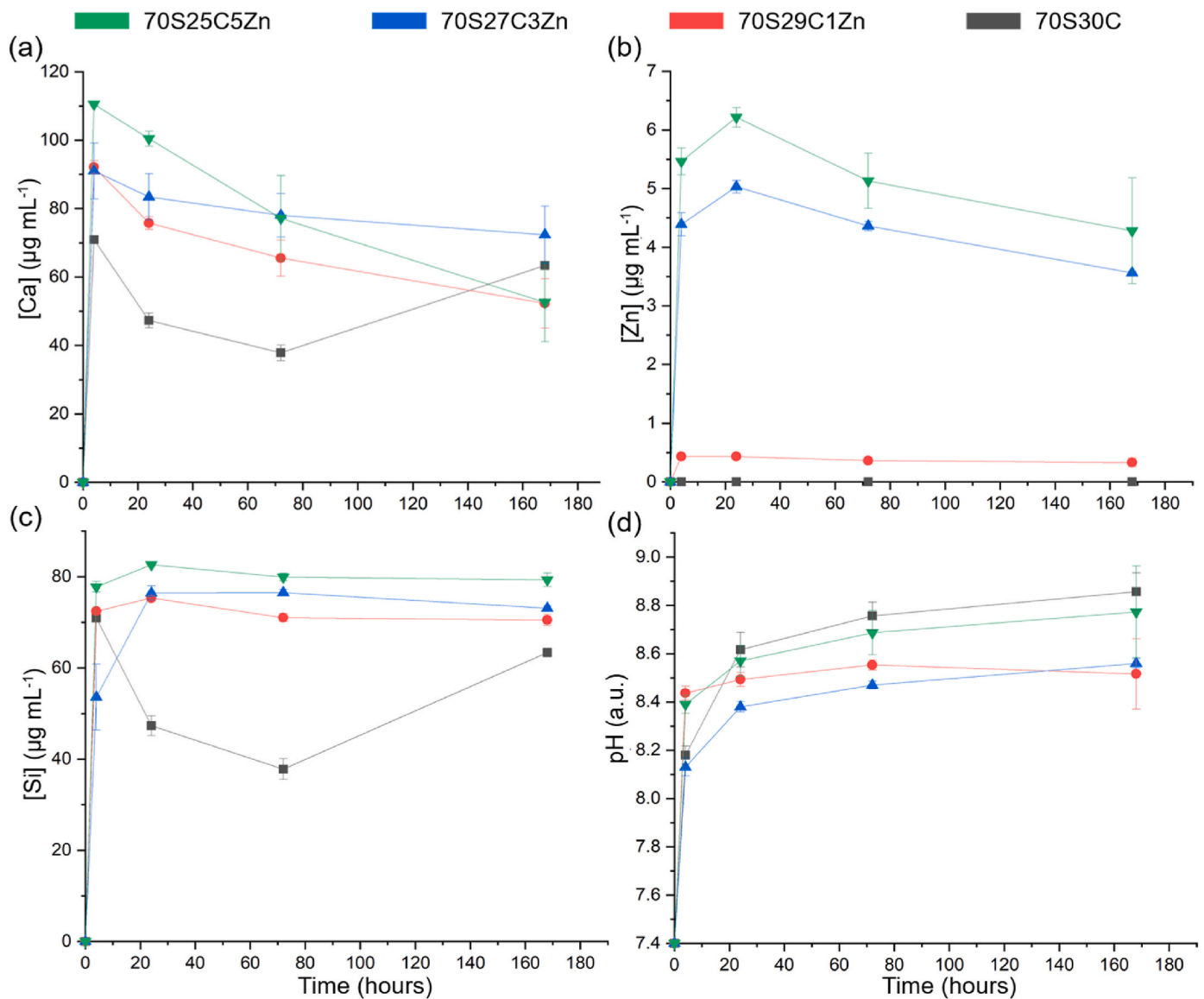


Fig. 5. Elemental concentration profiles (ICP-OES) of DMEM following immersion of bioactive glass fiber mats 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn over 7 days (a) Ca, (b) Zn, and (c) Si; and (d) pH change.

3.5. Metabolic activity and protein expression of HUVECs

The media conditioned the 70S30C glass and HDF cells (70S30C-HDF) significantly reduced the MA of HUVECs on days 1 and 3 (Fig. 8(a and b)), although by day 7, the MA had returned to a level similar to that observed with DMEM-HDF media. This finding contradicts previous studies that indicated 70S30C-HDF media promoted the MA of HUVECs [166]. Additionally, BGs of other compositions, such as the original 45S5 Bioglass, were also found to stimulate endothelial cell MA after pre-incubation with fibroblast-conditioned media [34,167]. One potential explanation could be the difference in surface area between BG fibers and particles, which leads to different ion release kinetics. According to Day et al., only small doses of 45S5 BG ($0.01\text{--}0.1\text{ mg mL}^{-1}$) stimulated HUVEC proliferation. In contrast, larger doses ($>0.1\text{ mg mL}^{-1}$) exhibited significant cytotoxicity, with up to a 90 % reduction in cell viability observed at a dose of 1 mg mL^{-1} [33,34,167]. The incorporation of Zn into BGs led to an increase in the MA, in agreement with previous research. Aina et al. reported that 45S5 BGs containing 5 wt% Zn incorporation significantly enhanced the proliferation of endothelial cells [75].

The expression of angiogenic factors from HUVECs cultured with BG-

HDF media were also assessed. VEGF and bFGF expression from 70S30C-HDF media were significantly lower, while the eNOS expression was significantly higher (Fig. 8(b)) than those from DMEM-HDF media. As eNOS is a Ca/calmodulin-dependent enzyme [168], and the level of synthesized nitric oxide in vascular endothelium which influences the expressions of VEGF and bFGF [169], these expression changes can be largely attributed to Ca content. Zn-BG-HDF media further increased the VEGF and bFGF expressions and decreased eNOS expressions, compared to the 70S30C-HDF media, until reaching a similar level to the control. This could be due to lower Ca release from the glass caused by the higher Zn content in the glass composition (Fig. 5) but also the impact on Zn ions themselves, which requires further investigation.

3.6. Migration of endothelial cells

Endothelial cell migration is an important phase in the formation of new blood vessels and was assessed using a Transwell assay (Fig. 9(a–f)) and a scratch assay (Fig. 10(a–f)). In both assays, all BG-HDF media promoted HUVEC migration by 2–3 times compared to HDF media without bioactive glass dissolution products. This agrees with previous literature, where BGs in the form of nanoparticles or nanofibers can

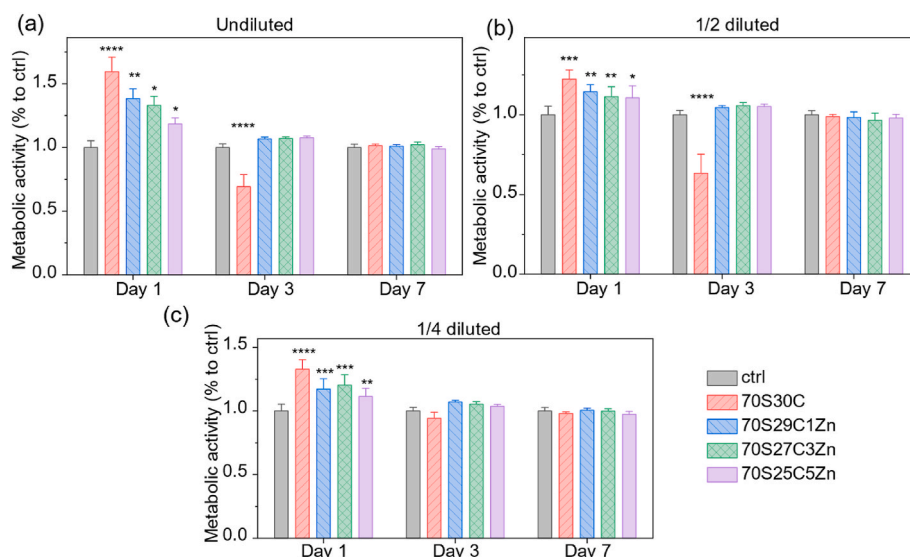


Fig. 6. The effect of conditioned media containing the dissolution products of 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn scaffolds on the metabolic activity (MA) of HDFs at days 1, 3, and 7, represented by normalized MA against the DMEM control. (a) undiluted media, (b) conditioned media $\frac{1}{2}$ diluted, and (c) conditioned media $\frac{1}{4}$ diluted. Represented by the intensity of absorbance at 450 nm. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, with respect to HDFs treated with the basal media).

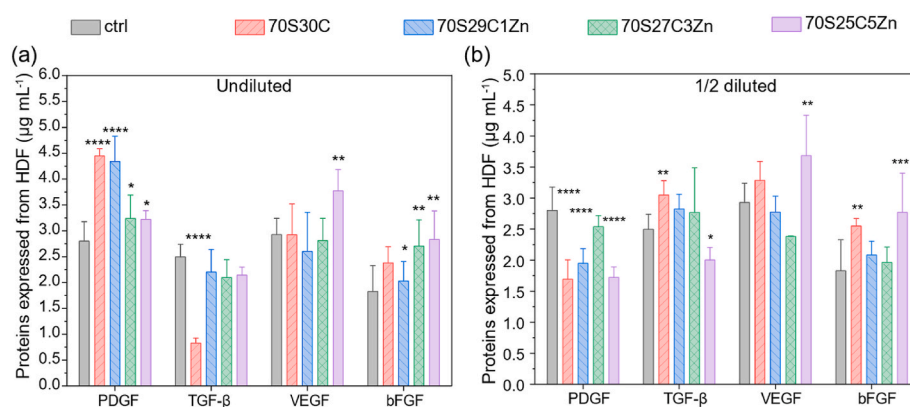


Fig. 7. PDGF, TGF- β , VEGF, and bFGF (FGF-2) expressed from the HDFs (ELISA assay) cultured with the media conditioned by the glass dissolution products of the 70S30C, 70S29C1Zn, 70S27C3Zn, and 70S25C5Zn scaffold, for 2 days: (a) undiluted media and (b) $\frac{1}{2}$ diluted. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, with respect to HDFs treated with the basal media).

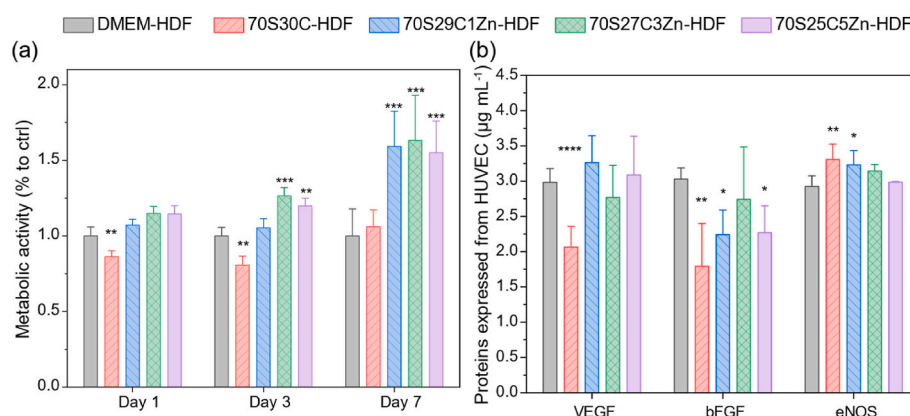


Fig. 8. (a) Metabolic activity of HUVECs after 1, 3, and 7 days cultured with 1:1 mixture of endothelial culture media: DMEM conditioned by HDFs and the glass dissolution products for 48 h (DMEM-HDF, 70S30C-HDF, 70S29C1Zn-HDF, 70S27C3Zn-HDF, and 70S25C5Zn-HDF), normalized against the DMEM-HDF control; (b) VEGF, bFGF (FGF-2), and eNOS expressed from HUVECs (ELISA assay) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, with respect to HUVECs treated with DMEM-HDF media).

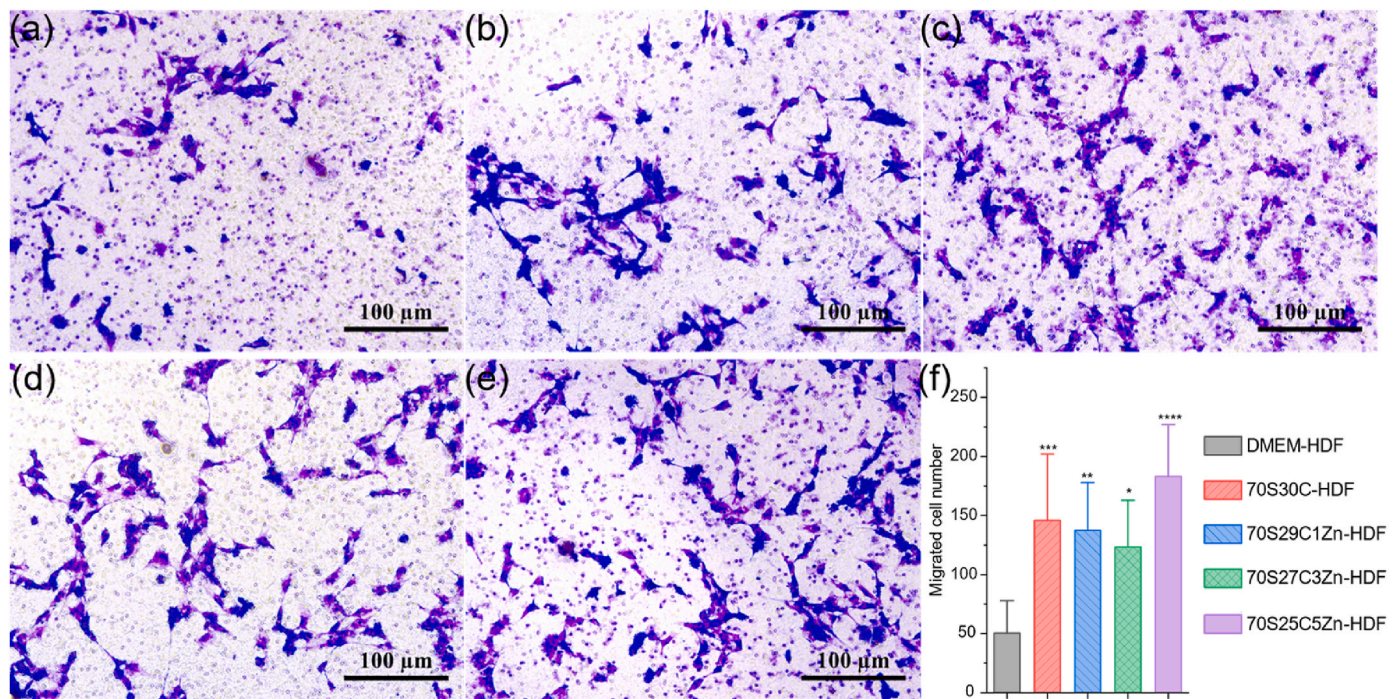


Fig. 9. (a–e) Optical images, and (f) cell number, of migrated HUVECs after being cultured for 16 h in a Transwell assay with (a) the DMEM-HDF medium, (b) 70S30C-HDF conditioned medium, (c) 70S29C1Zn-HDF conditioned medium, (d) 70S27C3Zn-HDF conditioned medium, and (e) 70S25C5Zn-HDF conditioned medium. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, with respect to HUVECs treated with DMEM-HDF media).

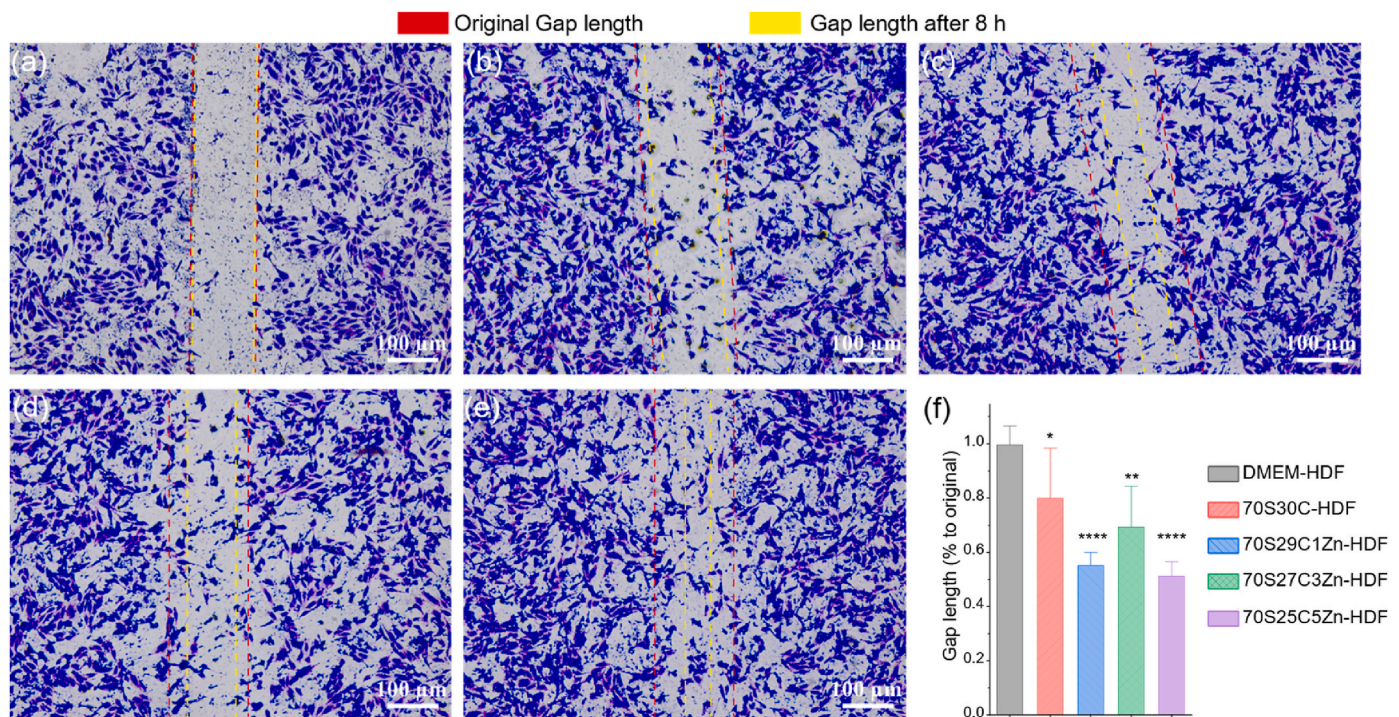


Fig. 10. (a–e) Optical images, and (f) gap length, of migrated HUVECs after being cultured for 8 h in a scratch assay with (a) the DMEM-HDF medium, (b) 70S30C-HDF conditioned medium, (c) 70S29C1Zn-HDF conditioned medium, (d) 70S27C3Zn-HDF conditioned medium, and (e) 70S25C5Zn-HDF conditioned medium. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, with respect to HUVECs treated with DMEM-HDF media).

promote endothelial cell migration in vitro [170–172] and hence vessel formation in vivo [40,78]. This can be due to the role of Ca, as Ca levels have been reported to impact various aspects of endothelial cell activities, such as the ligand-receptor interaction leading to inositol triphosphate production [173], modulation of the protein kinase C

expressions [174], inducing permeability and NO expressions [175], generation of intracellular second messengers [176], etc. The incorporation of Zn into BG further enhanced the migration, with 70S25C5Zn-HDF showing the greatest upregulation, consistent with previous findings [177]. This also agrees with the upregulation of Zn to

the expressions of VEGF and bFGF from fibroblasts (Fig. 7), which may denote the impact of Zn on angiogenesis in wounds.

BG fibers signal fibroblasts to release growth factors for the activation, proliferation, and migration of endothelial cells. Endothelial cells also have their own pathway of growth factor expression to support their activities, which may also be enhanced by the ion released from BG. Instead of applying BG dissolution products directly to HUVECS, this in vitro analysis using BG-HDF media considered both mechanisms and may better mimic the real-life situation that endothelial cells undergo during wound healing.

4. Conclusion

Zn-bioactive glass fiber mats were developed as scaffolds with fibers of similar dimensions to ECM (100–300 nm). These fibers demonstrated controlled release of Zn within the therapeutic range ($0.5\text{--}6\text{ }\mu\text{g mL}^{-1}$), which promotes angiogenic behavior. In HDF culture, Zn-BGs stimulated higher metabolic activity compared to Zn-free BG. Increasing Zn content to 70S25C5Zn significantly increased the expression of VEGF and bFGF by HDFs. Zn-BGs BGs stimulated higher metabolic activity and migration of HUVECs than Zn-free BGs, with 70S25C5Zn exhibiting the highest effect. This study indicates that BG fiber mats with 5 mol% of Zn incorporation have therapeutic potential for wound healing.

CRediT authorship contribution statement

Xingchen Zhao: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dong Zhai:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Joel Turner:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Chengtie Wu:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Gavin Jell:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Julian R. Jones:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Julian Jones reports financial support was provided by EPSRC. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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