3D Printing of Diatomite Incorporated Composite Scaffolds for Skin Repair of Deep Burn Wounds

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Abstract: Deep burn injury always causes severe damage of vascular network and collagen matrix followed by delayed wound healing process. In this study, natural diatomite (DE) microparticles with porous nanostructure were separated based on the particles size through a dry sieving method and combined with gelatin methacryloyl (GelMA) hydrogel to form a bioactive composite ink. The DE-containing inorganic/organic composite scaffolds, which were successfully prepared through three-dimensional (3D) printing technology, were used as functional burn wound dressings. The scaffolds incorporated with DE are of great benefit to several cellular activities, including cell spreading, proliferation, and angiogenesis-related gene expression in vitro, which can mainly be attributed to the positive effect of bioactive silicon (Si) ions released from the embedded DE. Moreover, due to establishment of bioactive ionic environment, the deep burn wounds treated with 3D-printed DE incorporated scaffolds exhibited rapid wound healing rate, enhanced collagen deposition, and dense blood vessel formation in vivo. Therefore, the present study demonstrates that the cost-effective DE can be used as biocompatible Si source to significantly promote the bioactivities of wound dressings for effective tissue regeneration.

Keywords: Diatomite; 3D printing; Burn wound; Skin regeneration; Tissue engineering

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1. Introduction

As the largest surface barrier of human body, the skin plays a crucial role in the maintenance of body temperature and fluid, regulation of metabolic process, and defense of outside invasion[1,2]. However, severe burns caused by thermal exposure, chemical injury, and electricity always lead to serious damage of fibrous matrix and blood vessels network in skin tissue[3]. From the perspective, the wound dressings that have capacity of promoting gas exchange, blood vessel formation, collagen synthesis, and skin tissue regeneration are urgently needed for efficient treatment of skin burn wound[4].

Research on bioactive scaffolds for the treatment of burn wound has generated sustained interest during the past few decades. It should not be ignored that the physical structure and chemical composition have become the key factors which are closely related to the realization of the multiple functions of the scaffolds[5]. Notably, three-dimensional (3D) printing technology has emerged as one of the optimal methods for the preparation of tissue-engineered scaffolds. The additive manufacturing of layer-by-layer deposition allows fabricating sophisticated porous constructs combing biomaterials and bioactive molecules[6,7]. The previous studies have suggested that 3D scaffolds with accessible porous architecture...
can promote cell migration, oxygen permeability, and ingrowth of surrounding tissue[8-10]. Thus, 3D-printed scaffolds have great potential to satisfy the requirements of ideal wound dressings[7].

With the aim to endow wound dressings with multiple biofunctions, the novel approach of incorporating various inorganic biomaterials into soft polymers has been explored. In the past few years, silica has aroused great attention in the field of tissue engineering[11-13] due to the enhanced collagen deposition and blood vessel formation induced by silicon (Si) element during the wound healing process[14,15]. However, traditional methods for preparing silica with nanostructure are cumbersome procedures and may introduce organic impurities[16], which limit its application to a certain extent[17].

Encouragingly, diatomite (DE, SiO₂·nH₂O) is siliceous skeleton deposited by natural diatom. With uniform porous architecture, DE possesses several superior properties, including good mechanical strength, excellent absorption performance, high specific surface area, and hydrophilicity[18-20]. As a natural occurring mineral compound, the applications of DE in biomedical engineering such as reinforcement, sensing, drug delivery, and hemostasis have been investigated[21-23]. More importantly, DE exhibits great potential to serve as a stable Si source to release bioactive Si ion which can improve tissue regeneration[24]. On this ground, it is reasonable to speculate that this natural cost-effective filler can be used to construct a composite wound dressing with desirable bioactive properties.

Herein, we successfully prepared a bioactive DE incorporated composite scaffold through 3D printing technology for the treatment of burn damaged skin. The biocompatible DE microparticles were sieved and embedded in gelatin methacryloyl (GelMA) hydrogel to form inorganic/organic composite ink for the development of 3D-printed scaffold. Taking advantage of DE, the 3D-printed composite scaffolds exhibited prominent abilities to support cell spreading, promote cell proliferation, and enhance vascularization in vitro. In addition, the effective influences of 3D-printed DE incorporated scaffolds on blood vessel formation, collagen deposition, and tissue regeneration of skin wounds were confirmed in the deep second-degree burn wound model. Therefore, the DE incorporated scaffolds can be considered as promising candidates to facilitate the regeneration of vascularized tissue in a convenient and efficient manner.

2. Materials and methods

2.1. Materials

Diatomaceous silica (DE), gelatin, and lithium phenyl-2,4,6-trimethyl-benzoylphosphinate (LAP) were purchased from Sigma-Aldrich Co., Ltd. (USA), methacrylic anhydride (MA) was purchased from Shanghai Titan Scientific Co., Ltd. (China).

2.2. Synthesis of GelMA hydrogel

A 20 g of gelatin was weighed and dissolved in 200 mL of deionized water at 50°C, and then, 12 mL of MA was added to react for 3 h. After the reaction completed, centrifugation was performed at 3500 rpm/min for 3 min. Then, the supernatant was collected and diluted for 3 – 5 times. Next, the product was put into a dialysis bag and dialyzed at 40°C for 7 days. Finally, GelMA was obtained by freeze-drying the product.

2.3. Synthesis of DE-GelMA composite inks

DE microparticles were sieved using a 500 mesh screen and sterilized under ultraviolet light for 1 h. Next, sterile phosphate-buffered saline (PBS) was added and followed by sonication for at least 1 h to form DE dispersion. Besides, the same volume of PBS was added to dissolve the weighed GelMA and LAP powders at 65°C to obtain a 12% (v/v) GelMA solution. After that, the DE dispersion and GelMA solution were thoroughly mixed to obtain a DE-containing ink with a GelMA concentration of 6% for the following 3D printing.

2.4. 3D printing of DE-Gel scaffolds

The fabrication of 3D-printed scaffolds in the study relied on a bioprinting platform with a cooling channel (BioScaffolder 3.2, GeSiM, Germany). The composite inks with gradient DE content (Gel, 5DE-Gel, 10DE-Gel, 20DE-Gel, and 30DE-Gel) were stored in 4°C refrigerator to form pre-gel and then used for extrusion 3D printing, respectively. During the printing process, the pre-gelled ink was extruded out through a 27 G needle (250 μm) under proper air pressure (40 – 60 kPa) at 10°C. After that, the printed scaffold was exposed to blue light about 45 s for cross-linking.

2.5. Characterization of DE microparticles, DE-GelMA inks, and the 3D-printed scaffolds

The morphologies, structure, and elemental distribution of the freeze-dried DE-containing 3D-printed scaffolds were detected by applying a scanning electron microscopy (SEM, SU8220, Hitachi, Japan) with an equipment of energy-dispersive spectroscopy (EDS). The SEM (SU9000, Hitachi, Japan) was used to determine the morphology of DE microparticles. The phase identification of DE microparticles was conducted rely on X-ray diffraction (Rigaku D/Max-2550 V, Geiger-Flex, Japan). Besides, a MCR301 rotational rheometer (Anton Paar GmbH, Austria) was used to test the viscosity of DE-GelMA composite inks at changing shear rate (0.1
– 10 s⁻¹), and the rheological properties of GelMA and 30DE-GelMA inks were measured through varying the temperature from 40°C to 4°C.

2.6. Cell culture

Two types of cells, human umbilical vein endothelial cell (HUVEC) and human dermal fibroblast (HDF), were used in this study. HDFs were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, USA) with accessory fetal bovine serum (FBS, 10% v/v) and penicillin/streptomycin (P/S, 1% v/v). HUVECs were cultured in the endothelial cell culture medium (ECM, Sciencell, USA) containing 5% (v/v) FBS, 1% (v/v) endothelial cell growth factor/heparin kit (ECGS/H), and 1% (v/v) P/S. Cells were all cultured in an incubator with a temperature of 37°C and atmosphere of 5% CO₂.

2.7. Proliferation assay and cell morphology

The proliferation of HDFs and HUVECs seeded on the 3D-printed scaffolds was determined by cell counting kit-8 (CCK-8) (Beyotime, China). ECM/DMEM medium containing 10% CCK-8 solution was used to culture the cell-laden 3D-printed scaffolds at 37°C for 3 h, and then, the supernatant was transferred to the wells in a 96-well plate. Next, the microplate reader (Tecan, Germany) was applied to measure the absorbance of the supernatant of CCK8-containing medium at 450 nm.

For the observation of cell morphology, scaffolds were put in a 48-well plate, and then, cells were seeded on the 3D-printed scaffolds with a density of 2 × 10⁴ per well. After cultured for 1 day and 5 days, the scaffolds were fixed in 4% paraformaldehyde solution for 12 h and rinsed with PBS solution. The DAPI (Sigma-Aldrich, USA) and Alex Fluor 647-conjugated phalloidin (Molecular Probes, USA) were applied to stain the nuclei and cytoskeleton of cells on the scaffolds separately. After that, confocal laser scanning microscopy (CLSM, TCS SP8, Leica, Germany) was employed to take the fluorescence images of cell distribution. The cell number and spreading area of HUVECs on the scaffolds on day 1 were determined in four different areas of CLSM images using ImageJ (NIH, USA), an image processing software.

2.8. Ionic release behavior of the scaffolds

With the aim to determine the cumulative Si ions released from the 3D-printed scaffolds with different concentrations of DE during the cell-cultured process, the supernatant medium was collected after the cell-laden scaffolds culturing for 1, 2, 3, and 5 days. After filtration, the concentrations of Si element in ECM were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) with model number of 715-ES (Varian, USA). The concentrations of the Si ion released from the 3D-printed scaffolds were calculated by adding the differential concentrations at each time point together.

2.9. Gene expression of the HUVECs in the scaffolds

The expression of genes related to angiogenesis in HUVECs seeding on the 3D-printed scaffolds was detected by real-time quantitative polymerase chain reaction (RT-qPCR). After culturing for 5 days, Trizol reagent (Invitrogen, USA) was used to extract the ribonucleic acid (RNA) of HUVEC cells on scaffolds, then, the obtained RNA was transcribed into complementary DNA (cDNA) by applying cDNA synthesis kit (TOYOBO, Japan). Next, RT-qPCR was conducted by applying the SYBR Green QPCR Master Mix (Takara, Japan), and the angiogenic genes such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor-1α (HIF-1α), vascular endothelial cadherin (VE-cad), and kinase domain receptor (KDR) were studied. Meanwhile, GAPDH served as the housekeeping gene for endogenous control. The procedure was performed using StepOnePlus Real-Time PCR Systems (Applied Biosystems, Thermo Fisher, USA). First, the pre-denaturation was conducted at 95°C for 30 s followed by 5 s and 30 s of the PCR reactions at 95°C and 60°C in turn. The process was looped 40 times in total. After that, the relative expression levels of these angiogenic genes were normalized by the 2⁻ΔΔCt method. For RT-qPCR, the primer sequences were listed in Table 1.

2.10. In vivo burn wound-healing study

The wound-healing animal experiment was proceeded according to the guidelines sanctified by the Institutional Animal Care and Utilization Committee of Nanjing First Hospital, Nanjing Medical University. BALB/c mice (8 weeks old, male, SPF grade) which purchased from the Charles River Laboratories Research Model Technology Co. Ltd. (Beijing, China) were used in this study. In this study, the animal models with second-degree burn skin wounds were established. First, mice were anesthetized by intraperitoneal injection and the hair on the murine back was removed. After disinfection, a metal rod (10 mm in diameter of section) immersed in boiling water at 100°C was pressed on the back of the mice for 5 s to create a deep burn wound with circular shape (10 mm in diameter). After that, the mice were split into three groups randomly: Blank, Gel, and 5DE-Gel. The 3D-printed scaffolds (5 mm of radius and 1 mm of thickness) were transplanted onto the burn wound site on murine back and then medical dressings (3M, USA) were used to fix the scaffolds. Next, the wounds were recorded by phone camera on days 0, 2,
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5, 8, 11, and 24. The scaffolds were changed at each time point. The area of wound sites was measured using ImageJ (NIH, USA), an image processing software. The formula to calculate the relative wound area is as follows:

Relative wound area (%) = A/Ar × 100%

Where, Ar indicates the value of the wound bed area at some point in time and Ao indicates the value of initial wound area (0 d).

After 14 days, the skin samples were obtained after sacrificing all of the mice and then fixed in 4% paraformaldehyde.

2.11. Histological analysis of the wound healing study

The skin samples harvested from in vivo experiment were immersed in 4% paraformaldehyde for at least 24 h, then dehydrated, embedded in paraffin, and cut into sections with thickness of 6 μm. After drying, the sections were baked at 60°C for 1 h, followed by deparaffinizing to water and staining with hematoxylin and eosin (H&E) (Sigma-Aldrich, USA), Masson’s Trichrome (Beyotime, China), and immunofluorescence for histological analysis. For immunofluorescent CD31 protein staining, the sections were immersed in 0.01 M citrate buffer solution with pH of 6.0 at 99°C for 20 min for antigen retrieval. After cooling, the non-specific binding was blocked by dropping 5% (v/v) bovine serum albumin on the sections. Next, sliced tissues were incubated with a diluted (1:200) CD31 primary antibody (ab28364, Abcam) solution overnight at 4°C, and then, solution of green fluorescent secondary antibody was dropped and incubated for 1 h in the dark. The observation of stained sections was performed using a microscope (Model DMi8 S, Leica, Germany) and a confocal laser scanning microscope (Model TCS SP8, Leica, Germany). Moreover, the statistics of collagen area and vessel number were conducted using ImageJ (NIH, USA).

2.12. Statistical analysis

The numerical data in this study are expressed as mean ± standard deviation and analyzed by one-way analysis of variance using Origin 2017 software (OriginLab, USA). Significant differences were denoted with *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results and discussion

3.1. Characterization of DE microparticles

DE microparticles with small particle size were segregated through a dry sieving method using a 500 mesh sieve. As observed by the SEM, the obtained DE microparticles were <20 μm in length with highly regular nanoscale pore structure (Figure 1A and B). According to the X-ray diffraction (XRD) pattern, the peaks of 22.0° and 26.6° of DE could be indexed into SiO2, with the cristobalite (PDF No. 39-1425) and quartz (PDF No. 46-1045) structure (Figure 1C). The peaks of cristobalite and quartz between 20° and 40° are typical for DE[27,28]. In addition, the microparticles with the concentrations of dispersion ≤50 μg/ml exhibited low cytotoxicity to HDFs and human umbilical vascular endothelial cells (HUVECs) culturing for 5 days (Figure 1D and E), which confirmed the good biocompatibility of DE.

3.2. Characterization of DE-containing biomaterial inks and 3D-printed DE-containing composite scaffolds

With the aim to develop an inorganic/organic composite wound dressing for skin repair, GelMA pre-gel solution was selected to serve as the matrix of the biomaterial ink for 3D printing. After that, DE microparticles were incorporated into the 6% (v/v) GelMA solution in different ratios of 0, 5%, 10%, 20%, and 30% to obtain GelMA, 5%DE-GelMA, 10%DE-GelMA, 20%DE-GelMA, and 30%DE-GelMA composite inks. Based on the previous studies, GelMA was defined as a temperature-sensitive hydrogel material, which could form reversible physical cross-linking at low temperature[29,30]. From this perspective, modulus-temperature test was performed to determine the appropriate temperature for the fabrication of 3D-printed scaffolds with structure stability. The results showed that GelMA inks with/without DE changed from liquid to gelatinous status when the temperature decreasing below 15°C (Figure 2A). Therefore, the inks with ideal formability were anticipated to allow for extrusion 3D printing at near 10°C. Besides, according to the result of flow behavior test, the decrease in viscosity with shear rate varying from 0.1 to 10 s⁻¹ indicated the obvious shear thinning performance of the inorganic/organic composite inks, which made them suitable for extrusion 3D printing (Figure 2B).

Next, the five inks were utilized to print 3D porous scaffolds with different concentrations of DE, which were denoted as Gel, 5DE-Gel, 10DE-Gel, 20DE-Gel, and 30DE-Gel, respectively (Figure 2C). With the increasing of DE content, the transparency of the scaffold gradually decreased. Then, the inside morphology of these scaffolds was characterized by SEM. As shown in Figure 2D, the DE microparticles were embedded in the scaffolds uniformly without obvious aggregation. The incorporation of DE led to the change of the inner walls of pore structure from smooth to rough. In addition, the elemental mapping observed
by EDS confirmed the distribution of DE in the freeze-dried composite scaffolds (Figure 2E). According to the previous studies, the swelling ratio of 6% GelMA hydrogel was in the range of 10 – 15%. Notably, increasing DE concentrations enhanced the water absorption capacity of composite hydrogel due to the hydrophilic nature of DE particles. Thus, the swelling rate of DE-containing composite hydrogels should be higher than that of GelMA hydrogel. Moreover, the compressive modulus of 5 – 7% (v/v) GelMA was in the...
range of 3 – 8 kPa[31-34]. It should be emphasized that the photoinitiator concentration, degree of methacrylation, UV intensity, and exposure time can affect the mechanical properties of GelMA. Zhang et al. summarized the effects of different additives on the mechanical properties of GelMA hydrogel in their review article. They claimed that most of the additives could improve the mechanical properties of the GelMA hydrogel[35]. It has been reported that the DE microparticles could serve as reinforcing filler in hydrogel networks[20,21]. Based on above, the mechanical properties of DE-Gel will be improved by the incorporated DE microparticles.

3.3. Biological activities of the 3D-printed DE-containing scaffolds in vitro

The biological effects of DE incorporated scaffolds on HDFs and HUVECs were evaluated separately. CLSM images demonstrated the stained nuclei and cytoskeleton of HDFs and HUVECs seeded on the 3D-printed scaffolds. Although HDFs presented uneven distribution as aggregated clumps on the scaffolds on day 1 (Figure 3A), after 5 days of culture, HDFs rapidly proliferated, migrated, and covered the entire surface of the scaffolds (Figure 3B). Besides, the cell proliferation assay was also performed after 1, 3, and 5 days. The HDFs adhered on the 3D-printed scaffolds in each group performed great proliferation independent of DE concentrations (Figure 4A).

Interestingly, the HUVECs seeded on DE-containing scaffolds exhibited better cell spreading with more obvious presence of filopodia than those on GelMA scaffolds on the 1st day (Figure 5A). Compared with HDFs, HUVECs appeared more sensitive that the cell viability was significantly affected by the changes of DE content. According to Figure 5C and D, the cell numbers of HUVECs adhering on the 5DE-Gel and 10DE-Gel scaffolds were higher than that in Gel group, and the incorporation of DE greatly increased the cell spreading area of HUVECs. Among the five groups, the Gel, 5DE-Gel, 10DE-Gel, and 20DE-Gel scaffolds were able to support the cell survival and spreading during culture for 5 days (Figure 5B). It is worth noting that the Gel, 5DE-Gel, and 10DE-Gel scaffolds presented good performance in network formation of HUVECs. Especially, the 5DE-Gel scaffolds significantly promoted HUVECs proliferation as shown in Figure 4B. In contrast, the 30DE-Gel group negatively affected cell viability, resulted in poor cell state. The reason might be that high contents of DE could release excess amount of ion which triggered adverse side effects on cell activities. As known, the cell attachment and spreading always strongly influenced by the surface topography[36,37]. Fortunately, the incorporation of proper amount of DE microparticles increased the roughness of scaffolds, thereby providing binding sites for orientation and movement of cells adhering on the surface[38]. Therefore, the satisfactory attachment, migration, and proliferation of skin cells laid a solid foundation for the application of scaffolds in wound therapy.

Subsequently, the angiogenesis activity of 3D-printed DE-containing scaffolds was detected by RT-qPCR. It is
well-known that efficient vascularization plays a crucial role during the wound-healing process\cite{39}. Herein, the enhanced expression of several angiogenesis-related genes, including VEGF, HIF-1α, VE-cad, and KDR, was detected in HUVECs on the 5DE-Gel and 10DE-Gel scaffolds (Figure 4C). In addition, according to the results of ICP-AES, the concentrations of released Si ion increased from 0 to 38.4 mg/L with the upgrading of DE content in 3D-printed scaffolds after 5 days of culture (Figure 4D). It could be realized that the high expression levels of angiogenesis-related genes were strongly associated with the bioactive ionic environment with appropriate concentration of Si element\cite{40}. Several previous studies indicated that the secretion of VEGF in cells could be promoted with ionic stimulation\cite{41-43}. Specifically, Si ions have been indicated to guarantee the stability of HIF-1α through hindering the degradation effect from PHD2 protein\cite{41}. As a result, the activation of VEGF gene correlated with the upregulation of HIF-1α gene induced by hypoxia\cite{44-46} followed by the positive expression of VE-cadherin\cite{46}. Meanwhile, KDR protein, as a universal regulator of endothelial cell, was also stimulated because its role in mediating diverse bioactivities of VEGF\cite{47,48}.

The previous studies indicated that the increased pH value contributed to the enhancement rate of natural silica degradation\cite{49}. There is general agreement that dissolution of silica at near-neutral pH is due to the breaking of Si-O-Si siloxane bonds on the surface of particles that attacked by water molecules\cite{50}. The increase of pH value led to the deprotonation of surface silanol groups, which further facilitated the breaking of the bridging Si-O-Si bonds. Therefore, the degradation behavior of DE will be obviously promoted under alkaline conditions, and the concentration of Si ion released from the scaffolds will increase probably.

Based on above, the incorporation of 5% DE microparticles endowed 3D-printed composite scaffolds with excellent abilities to promote proliferation and vascularization of HUVECs significantly. Therefore, the 3D-printed 5DE-Gel scaffold was selected as an optimal composite wound dressing with high bioactivity for subsequent experiments.

3.4. Skin repair of deep burn wounds in vivo

Encouraged by the satisfactory bioactivities of the 3D-printed 5DE-Gel composite scaffolds, animal models with deep second-degree burn wounds were established to further explore the therapeutic effect of the scaffolds in vivo. Mice were divided into three groups randomly and received different treatments: Blank, Gel, and 5DE-Gel. After burn occurring, the area around the wound site, which was named as stasis zone, was subjected to tissue necrosis within 48 h because of hypoxia and ischemia, leading to further expansion of the burn wound area on day 2\cite{4}. Judging from the gross photos of wound sites, the wound closure rate in 5DE-Gel group was significantly higher than that in the other two groups (Figure 6A). Besides, as shown by the quantitative statistics of wound area, the burn wound beds that treated with 5DE-Gel scaffolds exhibited the fastest healing rate among the three groups (Figure 6B). On day 14, compared with the Blank (25.40 ± 7.05%) and Gel groups (32.48 ± 1.79%), the relative wound area of the 5DE-Gel group reduced to 6.22 ± 2.04% without burn eschar, indicating the complete wound-healing procedure. Besides, no significant difference was found between the wound healing rates of control and Gel groups. The thick wound beds in Gel group could be attributed to the attachment and bonding of the Gel scaffold on the wound surface, which accompanied by inflammatory reaction. In this study, the raw material used for GelMA preparation was type A-gelatin, which was isolated from bovine skin by an acidic method and widely applied to GelMA synthesis\cite{51,52}. It has been reported that type A-based GelMA might elicit pro-inflammatory activity in the animal models\cite{51}.

Figure 5. Evaluation of viability of HUVECs in the scaffolds. The distribution of HUVECs seeded on 3D-printed scaffolds with various concentrations of DE microparticles for (A) 1 and (B) 5 days using CLSM observation. Scale bar: 500 μm. Quantitative analysis of the (C) cell density and (D) cell spreading of HUVECs seeded on the scaffolds for 1 day (n = 4). *P < 0.05, **P < 0.01, and ***P < 0.001.
Although GelMA is well known for its good degradability and biocompatibility, there are still endotoxins inside the material that could initiate inflammatory response\textsuperscript{52}. Fortunately, the microenvironment of enriched Si ions established by silica particles might inhibit the expression of pro-inflammatory-related genes including TNF-\textgreek{a}, IL-6, IL-1\textbeta, and IL-12b in macrophages\textsuperscript{53}. Therefore, the inflammation of burn wound was prevented in 5DE-Gel group probably due to the inflammation reduced effect of silica. In addition, DE microparticles endowed the composite scaffolds enhanced protein adsorption, water absorption, and enzymatic degradation\textsuperscript{20}, leading to satisfactory wound healing.

H&E staining was performed on the skin samples for subsequent histological analysis. Obviously, continuous dermal tissue, accomplished epithelialization, and hair follicles morphogenesis (red arrows) could be revealed in the regenerated skin of 5DE-Gel group (Figure 6C). In contrast, the burn wounds in Blank and Gel groups were covered by scars without intact structure of dermis and epidermis. The previous studies have presented that the re-epithelialization strongly associated to the formation of granulation tissue\textsuperscript{54,55}. Therefore, rapid filling of granulation tissue in the wound sites probably contributed to timely tissue regeneration in the 5DE-Gel group, while the wound-healing progress was postponed without positive intervention in the other two groups.

Subsequently, further histological analysis was conducted through Masson’s Trichrome and immunofluorescence staining for skin samples. As shown in Figure 7A, the area occupied by collagen fibers (blue) in the dermis tissue of 5DE-Gel group was significantly larger than that of Blank and Gel groups. Accordingly, the statistics of collagen content visually displayed that the 3D-printed 5DE-Gel composite scaffolds were of great benefit to collagen deposition (Figure 7C). In addition, CD31, a specific protein involved in modulating angiogenesis\textsuperscript{56}, was stained to assess vascularization in the newly formed skin tissue (Figure 7B). The enhanced expression of CD31 protein in the immunofluorescent staining images of the 5DE-Gel group marked denser blood vessel formation in dermis, which was consistent with the statistical results of the number of blood vessels (Figure 7D). Based on above, the availability of DE microparticles in promoting collagen formation and angiogenesis was proved.

For the treatment of burn wounds, the high repair efficiency of the 3D-printed 5DE-Gel scaffolds was revealed. On the one hand, the bioactive Si ions...
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released from the scaffolds stimulated proliferation and migration of fibroblasts, which contributed to acceleration of granulation during the wound-healing process[57,58]. Meanwhile, the wound contraction that typically dominated by fibroblasts was promoted as well[59]. Moreover, the observation of enhanced collagen deposition could be documented by the previous studies, indicating that Si ions stimulate collagen fibril synthesis and alignment[60,61]. On the other hand, the incorporation of DE in the scaffolds was conducive to enhance blood vessel formation that dominated by vascular cells, as evidenced by both of the expression of angiogenesis-related genes in vitro and the study of burn wound healing in vivo. Due to the stimulation of Si ion to HUVECs, rapid sprouting and rebuilding of vasculature were activated[45,62,63], and then, the skin cells could be supplied with sufficient nutrients and oxygen[62,64], contributing to the high speed of wound closure in 5DE-Gel group. Therefore, the 3D-printed DE-containing composite scaffolds exerted prominent repair effects on burn wound healing and skin tissue regeneration.

4. Conclusions

In this study, we developed a 3D-printed composite scaffold with the incorporation of DE microparticles, which could serve as a functional dressing for the treatment of skin burn wound. Natural DE microparticles with porous morphology could be used as a stable Si source to enhance the bioactivity of biomaterials, due to its biocompatibility and degradability. Notably, by incorporating with DE microparticles, the 3D-printed composite scaffolds exhibited high biological activities such as supporting cell spreading, promoting cell proliferation, and upregulating expression of angiogenesis-related genes in vitro. Moreover, the DE-containing composite scaffolds possessed ideal capacity to create a bionic environment that efficaciously benefited the treatment of skin burn wounds, leading to enhanced angiogenesis and collagen deposition, which followed by rapid wound closure. In summary, the study proposed a referable strategy to apply DE to fabricate inorganic/organic composite wound dressing with favorable biofunctions for accelerating skin repair and tissue regeneration efficiently.

As known, the diatom species, source, morphology, and physical characteristics of DE could significantly influence its biological application[23,65,66]. Although DE exhibited great practicability in performance improvement for 3D-printed hydrogel scaffolds, the specific mechanism of biofunctions exerted by DE was not clear enough. Besides, the DE incorporated scaffolds proposed in this study can only release single Si ion, resulting in insufficient biological activities of the scaffold. To address these issues, some strategies can be considered for implementation to further enhance the properties of DE composites in the future. On the one hand, by taking advantage of the high surface area and ordered porous structure, natural DE microparticles can serve as vehicles to delivery bioactive molecules or drugs to target wound sites. On the other hand, surface modification and component doping for DE will lead to promotion of degradation performance and biological activity. Therefore, the application of DE in the field of tissue engineering needs to be further explored and expanded.
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Conflict of interest
The authors declare that they have no competing interests.

Author contributions
J.M. and C.W. conceived and designed the project. J.M. prepared and characterized materials, performed the 3D printing procedure, and conducted experiments in vitro. J.M., J.W., H.Zhang., L.D., H.Zhuang., and Z.Z. participated in animal surgeries for burn wound healing. J.M. collected and analyzed data in the study. J.M. wrote the manuscript and C.W. supervised the study. All authors discussed the manuscript.

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