pH and glucose dual-responsive injectable hydrogels with insulin and fibroblasts as bioactive dressings for diabetic wound healing

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KEYWORDS

Injectable hydrogel, wound dressing, drug delivery system, diabetic foot ulcers, wound healing, pH responsive, glucose responsive

ABSTRACT

pH and glucose dual-responsive injectable hydrogels were prepared through the crosslinking of Schiff's base and phenylboronate ester using phenylboronic modified chitosan, polyvinyl alcohol and benzaldehyde-capped poly(ethylene glycol). Protein drugs and live cells could be incorporated into the hydrogels during the *in situ* crosslinking, displaying sustained and pH/glucose triggered drug release from the hydrogels, and cell viability and proliferation in the three-dimensional hydrogel matrix as well. Hence, the hydrogels with insulin and fibroblasts were considered as bioactive dressings for diabetic wound healing. A streptozotocin-induced diabetic rat model was used to evaluate the efficacy of hydrogel dressings in wound repair. The results revealed that the incorporation of insulin and L929 in the hydrogels could promote neovascularization and collagen deposition, and enhance the wound healing process of diabetic wounds. Thus, the drug- and cell-loaded hydrogels have promising potential in wound healing as a medicated system for various therapeutic proteins and live cells.

Introduction

Diabetes mellitus is a complex metabolic disorder and has severely affected thousands of people in the world.[1,2] Diabetic foot ulcer (DFU) is a kind of lesion formed on the feet that cannot proceed through the normal wound healing. It is a chronic, non-healing complication of diabetes, leading to high hospital costs and even amputation.[3-5] The medical treatment of DFU remains a challenge. Historically, wound dressings were first considered to take a passive and protective effect in the wound-healing process. The development of novel and efficient wound dressings is very important for wound repair, and an ideal wound dressing should provide a moist wound environment to the wound area, offer protection from secondary infections, remove wound exudate and promote tissue regeneration.[3,6,7] It is widely believed that DFU therapy

should actively promote wound healing by correcting the expression of biological factors that are important in the healing process.[8] The development of next-generation DFU therapies include three technologies, topical drug treatment (e.g. insulin and growth factors), cellular therapies (e.g. stem cells and fibroblasts) and scaffolds as well, and most current treatments employ one of these approaches.[9-12] However, DFU is a complex clinical problem, and necessary combination of technologies for diabetic foot ulcer therapy should be taken into account to address effectively the complex underlying pathology and enable reliable DFU repair. Thus, wound dressings could be considered as medicated systems, combining drug treatment, cellular therapy and scaffold for DFU treatment.

Hydrogels have attracted increasing attentions in the past decades for biomedical application,[13-17] and the utility of hydrogels as wound dressing became focused in recent years.[18,19] Hydrogels contain a large amount of water due to their three-dimensional hydrophilic polymeric networks, and hence provide a moist environment to the wound area, helping wounds to heal faster.[3] There are many reports on wound dressing with bioactive substances (protein drugs, growth factors, live cells and so on) for diabetic wound healing,[2,3,10,18] while very a few works have considered the special physiological environment of diabetic ulcer area. High level of glucose is an important factor for diabetes mellitus,[4,20] and glucose control is likely of great importance among metabolic factors.[5] For the distinct physiological microenvironment between diabetic ulcer and normal tissues, such as acidic pH and high levels of glucose,[21,22] smart hydrogels containing 'sensor' moieties which can respond to environmental pH and glucose concentration,[17,23,24] are ideal choices of wound dressings as medicated systems through the smart drug delivery for diabetic wound healing. Moreover, hydrogels are ideal materials as extracellular matrix (ECM) of various cells which would benefit the growth of skin tissue,[25,26]

because live cells could secrete appropriate combinations and ratios of necessary factors for enhanced tissue regeneration.[9] In addition, injectable hydrogel is another attractive feature due to its nonsurgical treatment to the patients for the purpose of mini-invasive medicine, especially for the irregular trauma in the deeper parts.[27,28] Thus, environmentally sensitive and injectable hydrogels could provide a promising therapy for DFU treatment by combining these therapies (smart drug delivery treatment, cell/tissue repair and extracellular matrix scaffold) to maximize their DFU repair potential. Herein, in this work, considering the acidic pH and high glucose level condition at diabetic ulcer area, smart hydrogels with pH and glucose responsibility are developed to incorporate drug and cells as bioactive dressings for diabetic wound healing, coupling drug treatment, cellular therapy and extracellular matrix scaffold into a whole.

In our previous work, pH and glucose sensitive injectable hydrogels were prepared through the covalent crosslinking of imine bond and phenylboronate ester using phenylboronic modified chitosan and oxidized dextran.[29] Drugs and cells can be incorporated into the hydrogels, allowing responsive drug release and cell proliferation. In this work, phenylboronic modified chitosan (CSPBA), polyvinyl alcohol (PVA) and benzaldehyde-capped poly (ethylene glycol) (OHC-PEG-CHO) were employed as starting materials. Benzoic-imine bonds instead of imine bonds were used to design the dually responsive hydrogels for benzoic-imine bond formed from aliphatic amine and aromatic aldehyde group was more stable at physiological pH due to the π-π conjugation.[30,31] Studies showed that insulin could accelerate wound healing and skin repair in diabetes by regulating some important factors in the skin's healing response, such as the function of epidermal and dermal skin cells, vascular and immune cells,[32-34] and fibroblasts loaded in the hydrogel dressing could significantly improve the skin regeneration by favorable growth of microvessels, hair follicles and faster formation of epidermis.[19] Herein, insulin and fibroblasts

were selected as model drug and cells, to exploit insulin and fibroblasts dual-loaded smart hydrogels as bioactive wound dressings for diabetic ulcer. The physicochemical properties of the CSPBA/PVA/OHC-PEG-CHO hydrogels were characterized, and the diabetic ulcer repair efficacy of the bioactive dressings was evaluated *in vivo* through SD diabetic wound rat model.

Experimental Section

Materials

CSPBA (the PBA content in CSPBA is 0.32) and benzaldehyde capped poly (ethylene glycol) (OHC-PEG-CHO) (MW~2000 Da) were synthesized according to our previous work.[29,35] Polyvinyl alcohol (PVA) (MW~4000 Da) and bovine insulin were purchased from Sigma Aldrich (St. Louis, US). All the reagents were used as received without further purification.

Preparation of CSPBA/PVA/OHC-PEG-CHO hydrogels

CSPBA and OHC-PEO-CHO solutions were prepared by dissolving the desired amount of polymer in distilled and deionized water. PVA solution was obtained by dissolving the desired amount of polymer in distilled and deionized water at 90 °C and then cooled down to room temperature. The pH of the solution was adjusted to 7.4 using 2 M HCl/NaOH. As a typical hydrogel preparation, 0.25 mL of PVA solution was added to 0.25 mL of OHC-PEO-CHO solution to get a mixed solution, and then 0.5 mL of CSPBA solution was added. The gelation occurred within ~10-20 s of stirring.

Characterization of CSPBA/PVA/OHC-PEG-CHO hydrogels

Rheological analysis

The rheological behavior of the hydrogels was performed on a rheometer with a 20 mm-diameter parallel plate (Rheo Stress I, KAAKE) at 37±0.1 °C. Both G' (elastic moduli) and G'' (loss moduli) of the CSPBA/PVA/OHC-PEO-CHO aqueous at different pH and polymer content were tested to characterize the viscoelasticity. The desired amount of CSPBA, PVA and OHC-PEO-CHO were dissolved in water to form sample solutions respectively, and the pH of the solution was adjusted to the required value using 2 M HCl/NaOH. The solutions were firstly mixed under vigorous stirring for 10 s to form a mixture, and then the mixture was quickly poured onto the plate. A gap of 1 mm and a frequency of 1 rad/s were selected in the measurement. To ensuring a linear viscoelastic region, strain amplitude of 2% was used in the time sweeping tests. The frequency sweep was performed over the frequency range from 0.1 to 100 rad/s at a strain of 2%. Strain sweep analyses were carried out with pre-made hydrogels, G' and G'' of the hydrogels were monitored under continuous strain sweep with alternate small oscillation force (γ = 2% strain) and a large one (γ =200% strain).

Scanning Electron Microscopy (SEM)

Hydrogels prepared with different polymer composition were freeze-dried, and the cross-section morphology of the hydrogels was performed on a Hitachi S-4800 scanning electron microscope.

Drug loading and Release

CSPBA, PVA and OHC-PEO-CHO solutions were prepared by dissolving the desired amount of polymer in water respectively, and the pH of the solution was adjusted to 7.4 using 2 M HCl/NaOH. Insulin was supplemented in the CSPBA solution under stirring to obtain a CSPBA/insulin solution. Equivalent volume (0.25 mL) of PVA and OHC-PEO-CHO solution

were firstly mixed and then added into the CSPBA/insulin solution (0.5 mL). The mixed solution was stirred vigorously to enable the hydrogel formation. After 30 min of incubation to ensure fully crosslinking of the hydrogels, the insulin-loaded hydrogels were immersed into 10 mL of media (0.01 M PBS containing the desired amount of glucose at different pH) in the vials at 37 °C. At desired time point, 2 mL of the release solution was collected from the vial and 2 mL of fresh PBS was added after each sampling to keep the volume of the total medium in the vial constant. The released drug was determined by RP-HPLC with a UV detector set at 227 nm. 20 μL of sample was injected into YMC-Pack Protein-RP column (150 mm × 4.6 mm, 5.0 μm, Agilent Corp., USA) and eluted with the mobile phase consisted of acetonitrile/methanol (32/68, v/v) containing 0.15% TFA at a flow rate of 1 mL/min. The mean value of three repeated tests was used to plot the release results.

***In vitro* Cell Culture in Hydrogels**

L929 cells were cultured using the complete DMEM medium at 37 °C in 5% CO_2, 95% humidified atmosphere in a cell incubator and were subcultured every 48 h. Before testing, the cells were harvested by trypsinization and re-dispersed in PBS at a desired cells density for use.

CSPBA, PVA and OHC-PEO-CHO were dissolved in DMEM medium at the required concentration respectively. The solution was irradiated with UV light for 15 min for sterilization. CSPBA solution (100 μL) was firstly mixed with the cells suspension (200 μL) in a 96-well. Then a mixture of PVA/OHC-PEO-CHO solution (100 μL, v/v, 1/1) was added and the hydrogel containing 1.2×10^6 cells/mL was formed under uniformly stirring. The cells-incorporated hydrogels were then transferred into a 24-well plate carefully and cultured in a cell incubator. 1

mL of DMEM medium (with or without glucose) was added to each well of the 24-well plate and replaced every day.

The viability of L929 cells in the hydrogels was determined using acridine orange (AO) staining. The hydrogels were cut into thin slices and rinsed twice with PBS after 1 or 3 days' cultivation. The hydrogel slices were stained using 5 μL of AO dissolved in PBS (0.1 mg/mL) for 30 s, and then the cells viability were performed using confocal laser scanning microscope (Leica TCSsp2, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The proliferation analysis of cells in the hydrogel was tested using cell counting assay (CCK). The cells-incorporated hydrogels in 24-well plates were rinsed twice with PBS after 1 or 3 days' cultivation, and then PBS (200 μL) and CCK solution (10 μL) was added to each well. The mixture was incubated at 37°C and 5% CO_2 for 2 h with gentle shaking, and then a 150 μL aliquot of the solution in each well was transferred into a 96-well plate individually. The absorbance at 450 nm was tested using an OPTI Max microplate reader.

In vivo diabetic wound healing study

Preparation of Streptozotocin (STZ)-induced diabetic animal model

Type 1 diabetes SD rats were induced by intraperitoneal injections of streptozotocin (STZ, Sigma-Aldrich) dissolved in citrate buffer (0.05 M, pH 4.5) at a dosage of 100 mg/kg body weight. Blood glucose levels were monitored by a glucose meter (Johnson, China) one week after induction of diabetes. A hyperglycemic phenotype was confirmed when blood glucose levels were over 300 mg/dL in the animals. The animal experiments were performed according to the guidelines in the Department of Medicine, Ningbo University, China.

Diabetic wound treatment with hydrogel dressings

STZ-induced diabetic SD rats (male, 9-week-old) were randomly divided into four groups (n=7). Under anesthetization, the back of each rat was shaved. Then a full-thickness skin wound (10 mm in diameter) was created on the back of each rat after sterilization using 70% ethanol. The wounds were immediately treated with 0.5 mL of PBS (group 1, control), 0.2 mL of CSPBA/PVA/OHC-PEO-CHO hydrogels consisted of 1.2 wt% CSPBA, 0.6 wt% OHC-PEG-CHO and 0.6 wt% PVA (group 2), insulin-loaded CSPBA/PVA/OHC-PEO-CHO hydrogels consisted of 1.2 wt% CSPBA, 0.6 wt% OHC-PEG-CHO, 0.6 wt% PVA and 0.3 wt% insulin (group 3), CSPBA/PVA/OHC-PEO-CHO hydrogels encapsulating both insulin and L929 cells (1.2 wt% CSPBA, 0.6 wt% OHC-PEG-CHO, 0.6 wt% PVA, 0.3 wt% insulin and 1.2×10^6 cells/mL, group 4), respectively. The administration of PBS was achieved by dropping it onto the wound area, and the hydrogel formulations were loaded by plastering to cover the entire defect. Within the experimental time, the wound areas were recorded using a camera and the blood glucose was measured at desired time points. On predetermined time points, two rats in each group were sacrificed for histopathological or immunohistochemical analysis.

Wound closure measurement

The wound area of each animal was photographed at desired time points using a digital camera. Image J software was used to measure the wound area, and the wound size and wound closure degree were evaluated as well. The wound area was calculated using the following equation: $Wound\ area\ (\%) = (A_0 - A_t)/A_0 \times 100,$ where A_0 is the wound area on the surgery day and A_t is the wound area at the indicated time point. The definition of wound repair completion was referred when the wound area was equivalent to the area at time point zero.

Measurement of blood glucose

For all animals, blood obtained from the tail vein at desired time point was tested using a blood glucose test meter and the blood glucose level was analyzed.

Histopathological and immunohistochemical analysis

The back skin around the wound site of the sacrificed rats was removed and fixed in 10% formaldehyde solution. The isolated tissues were embedded in paraffin and sectioned at a thickness of 4 μm. The sections were stained with hematoxylin/eosin (H&E), anti-CD31 antibody and Masson's trichrome（MT）to evaluate general aspects of the inflammatory infiltrate, neovascularization and collagen formation, under the standard procedures.

Statistics analysis

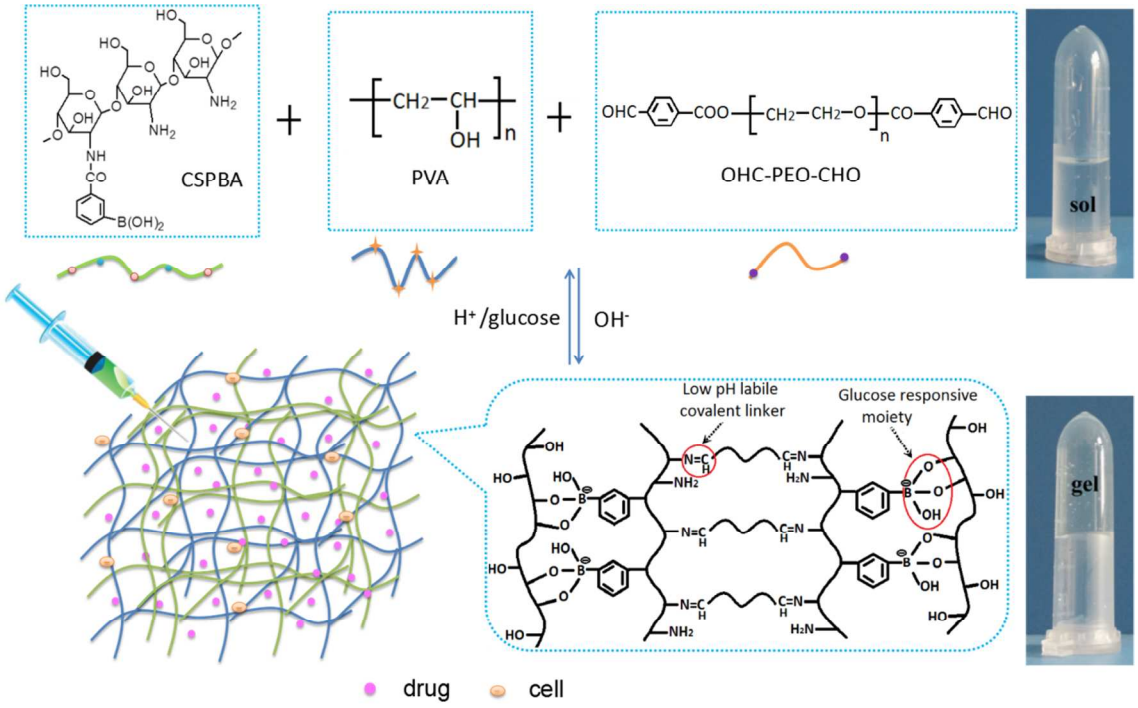
Statistical significance was assessed using one-way ANOVA method. The difference was considered to be statistically significant if the *p* value was less than 0.05.

Results and discussion

Hydrogel Formation

CSPBA/PVA/OHC-PEO-CHO hydrogels were formed through the crosslinking of pH responsive benzoic-imine (Schiff's base) and glucose responsive phenylboronate ester and allowed no inclusion of extraneous reagents except water. The friendly and *in situ* crosslinking process enabled the encapsulation of drug and cells into the hydrogel simultaneously. The formation of hydrogel was illustrated in Scheme 1. Sol-to-gel of the polymer solution can be observed by mixing the CSPBA, PVA and OHC-PEO-CHO solutions at pH 7.4 and the formed

hydrogel can transform to solution again by adding a tiny amount of HCl. The reversible sol-to-gel and gel-to-sol transitions can be repeated several times by adding a tiny amount of NaOH or HCl, indicating the repeatability of pH responsiveness. The gel-to-sol transition can also be observed by adding a tiny amount of glucose solution, showing the glucose responsiveness of the hydrogel. SEM images demonstrated that the lyophilized hydrogels were highly porous and had an interconnected polymer network due to the formation of ice crystal, which was in accordance with other reports.[29] The pore of the network became larger with lower crosslink density, as shown in Figure 1.



Scheme 1. Illustrative formation of the CSPBA/PVA/OHC-PEO-CHO hydrogel.

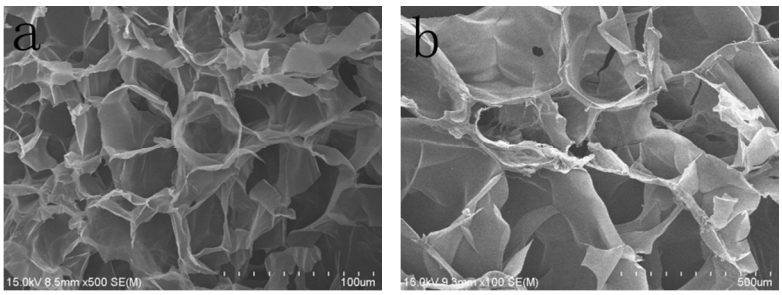
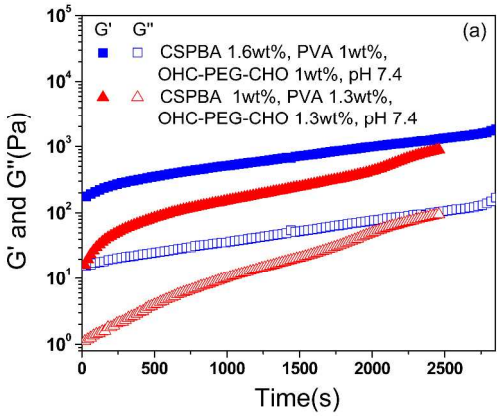


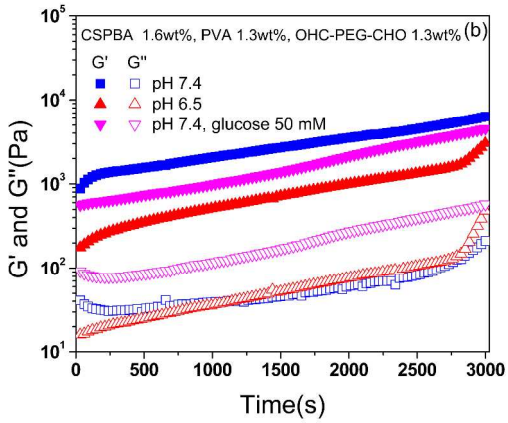
Figure 1. SEM images of CSPBA/PVA/OHC-PEO-CHO hydrogels formed at pH 7.4, consisting of 1.6 wt% CSPBA, 1wt% PVA and 1wt% OHC-PEO-CHO (a), and 1.6 wt% CSPBA, 0.5 wt% PVA and 0.5 wt% OHC-PEO-CHO (b).

Characterization of CSPBA/PVA/OHC-PEO-CHO hydrogels

The gelation behavior of the system and the dependence of the moduli of the hydrogels on the polymer content and environmental parameters (e.g. pH and glucose concentration) were demonstrated by rheological analysis. As plotted in Figure 2a and 2b, the G' surpassed G'' immediately after the addition of the PVA/OHC-PEO-CHO to CSPBA solution due to the quick formation of the hydrogel. This could provide the hydrogel formation from diffusion of the polymer solution to the surrounding tissues when it was injected into the body.[36] For the mixing process was slow on a parallel plate with a diameter of 20 mm, the G' value kept increasing due to the crosslinking reaction during the test process.[24] The moduli of hydrogels were also found to have a remarkable dependence on the polymer content, as shown in Figure 2a. The storage modulus G' of the hydrogel could reach to ~1 kPa at 1 wt% of CSPBA, 1.3 wt% of PVA and OHC-PEO-CHO. And the G' modulus was remarkably increased (>5k) with the increase of CSPBA concentration when the overall polymer concentration was kept constant in the hydrogel. For example, hydrogel consisted of 1.6 wt% of CSPBA, 1 wt% of OHC-PEO-CHO and 1 wt% of PVA in Figure 2a. In addition, the hydrogel formation was disparity when there was a small

change in the environmental parameters. As shown in Figure 2b, decreasing the pH from 7.4 to 6.8 could result in a modulus reduction and slowed gelation process of the hydrogel. Increasing the glucose concentration in the medium (50 mM) could also reduce the modulus of hydrogel remarkably when the pH was kept constant (Figure 2b). Thus the gelation process and hydrogel strength were adjustable by controlling the polymer composition and pH value, which was favorable for *in vivo* injection. However, once the hydrogels were stably formed, the G' was independent of the shear frequency from 0.1 to 100 rad/s because of the chemical crosslinking, i.e. the Schiff's base and phenylboronate ester, indicating that the gel was robust,[31] as shown in Figure 2c. The rheological property of the hydrogels was in agreement with our previous work.[29]





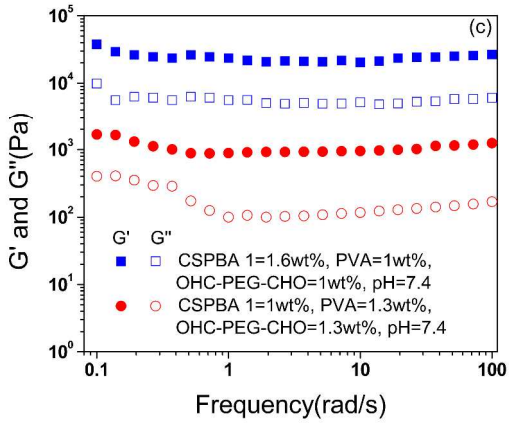
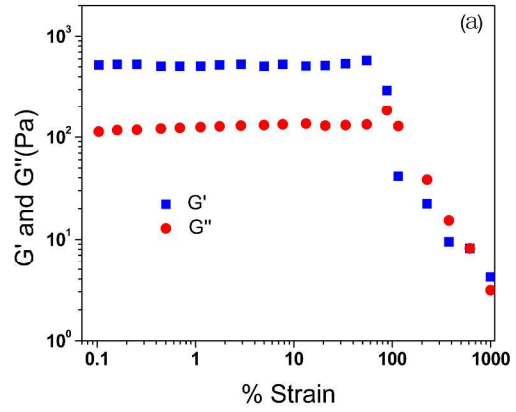


Figure 2. Time dependence (a, b) and frequency dependence (c) of elastic modulus (G', solid symbols) and loss modulus (G'', open symbols) of CSPBA/PVA/OHC-PEO-CHO mixtures at 37 °C with different polymer composition, glucose concentration and pH.

Since the hydrogel network is covalently cross-linked by dynamic Schiff's base and phenylboronate ester, it is expected to be injectable. Strain amplitude sweep was operated to demonstrate the elastic response of the hydrogel (CSPBA 1 wt%, PVA 1.3 wt% and OHC-PEO-CHO 1.3 wt%) with strain. The elastic modulus (G') decreased rapidly above the critical strain region (γ=100%), as shown in Figure 3a, suggesting the collapse of the hydrogel network. Herein the hydrogel was performed on a large amplitude oscillatory strain of 200%, resulting in a decrease of G' from ~800 to ~10 Pa and tan δ (G''/G') value at about 0.4 to 0.6 (Figure 3b). This means that the hydrogel has a loose network in large oscillatory force. It is noted that the G' recovered to the initial value quickly when the amplitude was decreased to a small strain of 2%, and the tan δ (G''/G') value was about 0.11 to 0.18 (Figure 3b). This means that the inner network was quickly recovered and the hydrogel returned to the original state.[37]

In vitro adhesive-healing test of the hydrogel was performed to further demonstrate the dynamic covalent cross-link in a macroscopic view. One piece of the hydrogel (CSPBA 1.6 wt%, PVA 1wt% and OHC-PEO-CHO 1 wt%, red hydrogel was obtained by adding a tiny amount of DOX for easy observation) was cut into two small pieces (Figure 3c), then the two pieces were placed closely together along the cut section in a container saturated with moisture in air. After incubation at room temperature for 30 min without any external stimulus, the two pieces of hydrogels merged together. As shown in Figure 3d, the merged hydrogel could be lifted up vertically by clamping at one side of the hydrogel using a tweezer without breaking into two pieces.



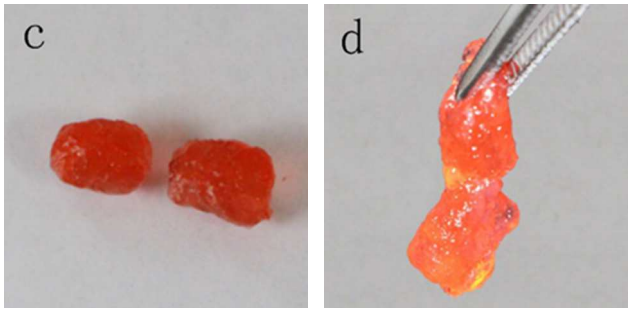


Figure 3. Rheology analyses of the dynamic and covalent crosslinked hydrogels. The dependence of elastic moduli (G') and loss moduli (G'') on strain amplitude (a); G' and G'' from continuous strain sweep with alternate small oscillation force (γ= 2% strain, solid dots) and large oscillation force (γ=200%, hollow dots) (b). The frequency was 1.0 Hz. Photos were taken during the adhesive-healing process of the hydrogel at 0 min (c) and 30 min (d).

In Vitro Drug Release

Insulin is the most popular drug used in blood glucose control for diabetes mellitus. Herein, bovine insulin was adopted as a model drug and was encapsulated into CSPBA/PVA/OHC-PEO-CHO hydrogels at pH 7.4. Sustained and triggered drug release from the hydrogels could be observed (Figure 4). Decreasing the media pH or increasing the glucose level in the medium resulted in the accelerated insulin release from the hydrogels due to the swollen or hydrolysis of the hydrogel matrix, because the Schiff's base is labile at acidic pH,[31] and phenylboronic group preferred to combine with glucose than hydroxyl group of PVA in the presence of glucose.[20] As shown in Figure 4a, at a release time of 34 h, the cumulative release of insulin was 9% at pH 7.4, and the value increased to 33 and 43% at pH 6.5 and pH 7.4 containing 3 mg/mL of glucose respectively. The drug release can be described using the followed formula: $M_t / M_\infty = kt^n$, where M_t / M_∞ is the fraction of drug released at time t varied from 0 to 1, and n is the diffusion related

component.[29,38] The diffusion component *n* can be obtained by fitting the cumulative drug release curve using Origin software and the calculated *n* values based on Figure 4a are 0.39, 0.32 and 0.33 at pH 7.4, pH 6.5 and pH 7.4 containing 3 mg/mL of glucose, respectively, indicating a diffusion-controlled mechanism. This means that the insulin molecules diffuse mainly through the swollen hydrogel matrix. Decreasing of CSPBA concentration and crosslinker content led to the increase of insulin cumulative release from 9% (Figure 4a) to 39% (Figure 4b) at pH 7.4 after releasing for 34 h. Similarly, decreasing the media pH or increasing the glucose level led to the accelerated release of insulin from hydrogels. As shown in Figure 4b, the value increased from 39% at pH 7.4 to 80% and 50% at pH 6.5 and pH 7.4 containing 3 mg/mL of glucose at 34 h, respectively. The calculated *n* values based on Figure 4b are 0.44, 0.62, and 0.57 at pH 7.4, pH 6.5 and pH 7.4 containing 3 mg/mL of glucose, respectively. This followed Fickian diffusion mechanism under physiological condition attributed to the hydrophilicity of the drug. While the kinetics deviated from the Fickian mechanism and followed non-Fickian behavior at acidic pH and high level of glucose, indicating the diffusion of insulin molecules through the swollen hydrogel matrix and the hydrolysis of hydrogels as well.[29,38] The pH and glucose triggered drug release indicates that the hydrogels should be positive for the application of the system in DFU treatment due to the acidic pH and high glucose level in diabetes ulcer area.

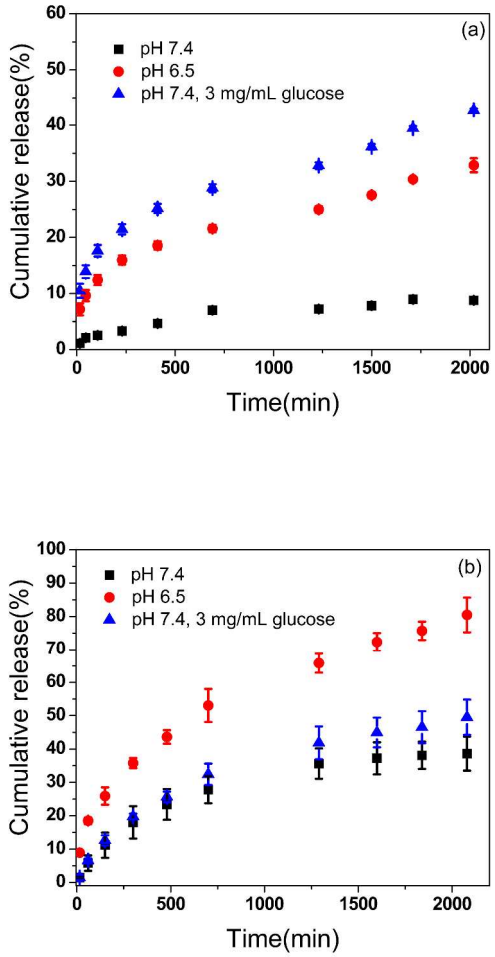


Figure 4. Cumulative drug release from the insulin incorporated hydrogels immersed in 10 mL of media with different pH and glucose concentration. (a) CSPBA 1.75 wt %, PVA 1 wt % and OHC-PEO-CHO 1wt %, (b) CSPBA 1.5 wt %, PVA 0.75wt % and OHC-PEO-CHO 0.75 wt %. The insulin loaded in each hydrogel was equivalent and was 3 mg/mL.

Cell Viability and Proliferation

Cell viability and proliferation were examined through three-dimensional cell cultivation in the hydrogel in order to demonstrate the biocompatibility and potential usage of hydrogels in drug/cell therapy. L929 fibroblasts were seeded and cultured in the hydrogels, and AO staining

showed uniform green fluorescence in both hydrogels with or without glucose on day 3, as shown in Figure 5, displaying good viability of the cells cultivated in the hydrogels matrix. Furthermore, the cell proliferation tested by CCK assay showed that L929 fibroblasts grew faster in the hydrogels with lower cross-linkage density and polymer concentration. As shown in Figure 6, the cell population in hydrogel-II (hydrogel with low polymer concentration and cross-linkage density) was 1.8 times at day 3 compared with day 1. While the cells encapsulated in Hydrogel-I (hydrogel with high polymer concentration and cross-linkage density) had no significant proliferation at day 3. This may be because that the better swelling property and looser network structure of the hydrogel with lower polymer concentration and cross-linkage density were favorable to the interchange of substances and cell spreading, leading to an accelerated proliferation of the cells. It is interesting to note that accelerated proliferation of the cells was also observed when a small amount of glucose (3 mg/mL) was added into the culture medium, and the number of cells in hydrogel-II with glucose medium was 2.2 times at day 3 compared with day 1 (Figure 6). The cell proliferation in the hydrogel matrix at high glucose concentration and pH/glucose-responsive insulin release could endow the hydrogel system with potential application in DFU treatment from both cell/tissue repair and drug therapy aspects.

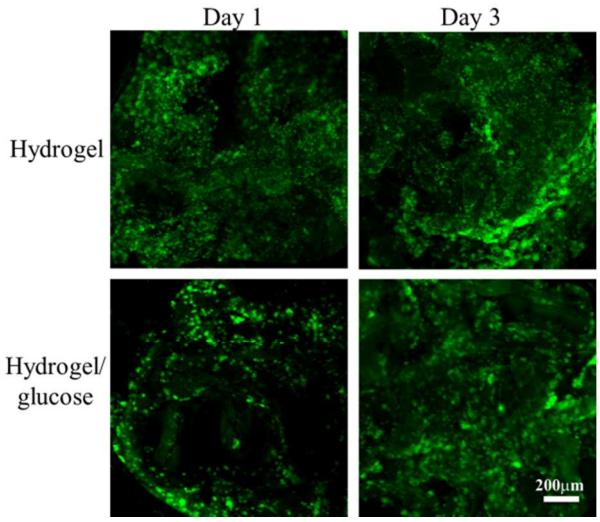


Figure 5. CLSM images of L929 fibroblasts cultivated in the CSPBA/PVA/OHC-PEG-CHO hydrogels (CSPBA 1 wt%, OHC-PEG-CHO 0.5 wt%, PVA 0.5 wt%) for one day and three days. The concentration of glucose in the medium was 3 mg/mL. Scale bar: 200 μm.

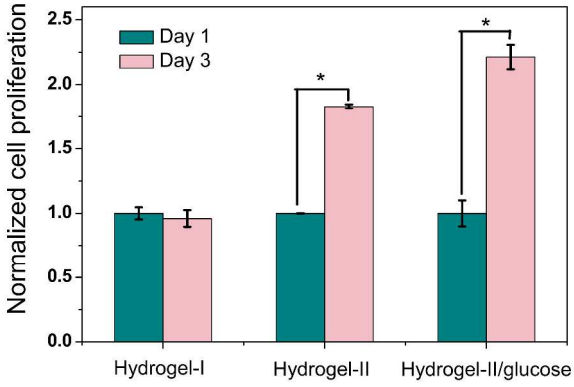


Figure 6. Population of cells cultivated in the CSPBA/PVA/OHC-PEO-CHO hydrogels for one day and three days (*p<0.05). Cell population was normalized against the population on day 1. The polymer composition for Hydrogel-I was CSPBA 1.5 wt %, PVA 1wt % and OHC-PEO-CHO 1wt %, and for Hydrogel-II was CSPBA 1 wt%, OHC-PEO-CHO 0.5 wt%, PVA 0.5 wt%. The concentration of glucose in the medium was 3 mg/mL.

Effect of hydrogel dressings on the blood glucose in STZ-induced diabetic rats

A STZ-induced diabetic rat model was used to evaluate the effect of the drug and cells dual-loaded hydrogels in diabetic wound repair. Blood glucose levels in normal SD rats were 89.5±6 mg/dL and elevated into a significantly high level of 325±46 mg/dL in SD rats receiving STZ after 1 wk. Thereafter, wounds were created on the back of STZ-induced diabetic rats, then neat hydrogel, insulin and insulin/L929 loaded hydrogels were treated on the wound areas respectively, with the administration of PBS as a control. Blood glucose was monitored during the experimental period. The results showed that the blood glucose levels in the control group elevated to 609±12 mg/dL within 6 days after wounding and then slightly dropped to 474±76 mg/dL in the next 12 days (Figure 7). The blood glucose levels in the other three groups received hydrogel dressing treatment were suppressed in a certain level, which was much lower than that of the control group, while the blood glucose levels were still above 300 mg/dL except insulin/L929 loaded hydrogel group (Figure 7). For the hydrogel group, the blood glucose levels kept relatively steady without significant elevation within the first 10 days after wound treatment, possibly due to the blood-glucose-lowering effect of chitosan,[39,40] but subsequently, the blood glucose levels increased again in the next 8 days. The blood glucose levels in insulin-loaded hydrogel group kept stable during the experimental period, which was significantly lower than that of the control group. While for the insulin/L929 loaded hydrogel group, the blood glucose levels dropped to below 300 mg/dL in the last 8 days. The relatively lower levels of blood glucose in insulin and insulin/L929 loaded hydrogels groups were possibly due to the sustained release of insulin from the hydrogels and the blood glucose management of chitosan. Diabetes has been shown to impair immune function and impede wound healing, as excessive glucose deposition in blood results in the peripheral neuropathy, which is critical to survival and recovery

from major wound injury.[41] Hence the management of blood glucose of the hydrogel dressings is favorable for wound healing in diabetes.

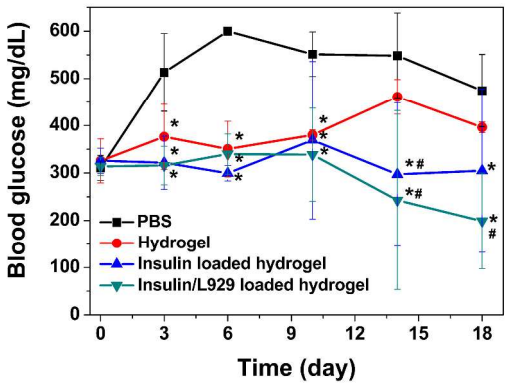
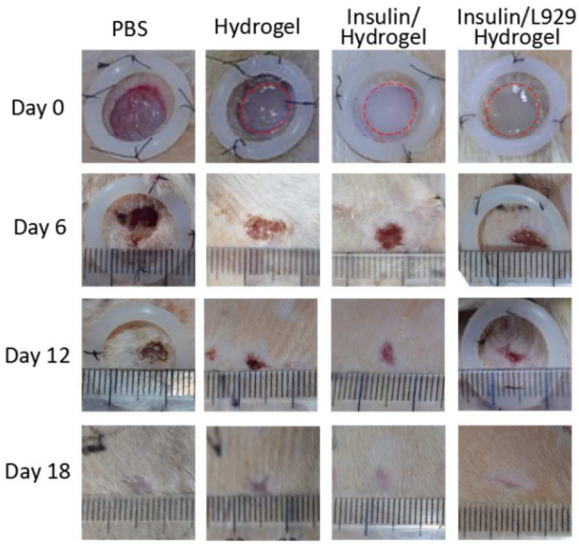


Figure 7. Changes in blood glucose in each diabetic rats group at varied time points. *p<0.05 compared with PBS control group; #p<0.05 compared with hydrogel group.

Effect of hydrogel dressings on wound healing in STZ-induced diabetic rats

To evaluate the wound healing capacity of the hydrogel dressings on diabetic skin wounds during the wound-repair process, images of the wound on the animals were taken at different time intervals, and wound areas and wound closure rate were calculated as well. As shown in Figure 8a, shrinkage of wound area was clearly seen for all groups with the growth of new epidermis extending to the center and recovering the whole lesion after 18 days post treatment. The wound closure rate was quantified and the results indicated that the groups treated with neat hydrogel, insulin and insulin/L929 loaded hydrogels displayed improved wound repair of the diabetic wounds after administration, compared with the control group (PBS). Specifically, the control group showed wound closure of 46±11% of the initial wound area at day 6, and the diabetic skin wounds dealt with neat hydrogels revealed enhanced recovery potential with wound

closure of 60±7% at day 6, as shown in Figure 8b. The facilitated healing process may be contributed to the moist environment in the wound areas provided by the hydrogel. A similar healing level of wound closure as the neat hydrogel group was observed in insulin-loaded hydrogel group at day 6, and the wound closure was 63±4%. Interestingly, the wound closure in insulin/L929 hydrogel group was significantly increased at day 6 (70±11%), and the wound area reached 92±8% of closure at day 12, which was significantly enhanced compared with the control and neat hydrogel groups. Studies showed that topical application of insulin could accelerate wound healing in diabetes via the activation of insulin signaling pathway,[32,42] thus the sustained release of insulin from the insulin-loaded hydrogel (Figure 4) could facilitate the wound healing process. Additionally, live cells (fibroblasts and stem cells) encapsulated in the hydrogel can secrete appropriate combinations and ratios of growth factors for enhanced tissue regeneration and accelerated wound healing.[9,43,44] It is reported that fibroblasts play a crucial role in the angiogenesis process due to their production of extracellular matrix molecules (e.g. collagen I) and their secretion of essential growth factors (e.g. VEGF).[45,46] These fibroblast-derived factors are necessary for endothelial cell sprouting and lumen formation. Research displayed that incorporation of fibroblast cells (e.g. human dermal fibroblasts and L929) in hydrogels could improve the expression of the growth factors (VEGF) and accelerate wound repair.[19,47] Thus, combining an appropriate dressing material (hydrogel) with drugs and cells (such as insulin and L929) could enhance the wound healing process, suggesting the insulin and fibroblasts dual-loaded hydrogels as bioactive wound dressings represent an attractive method for treating this devastating complication of diabetes.



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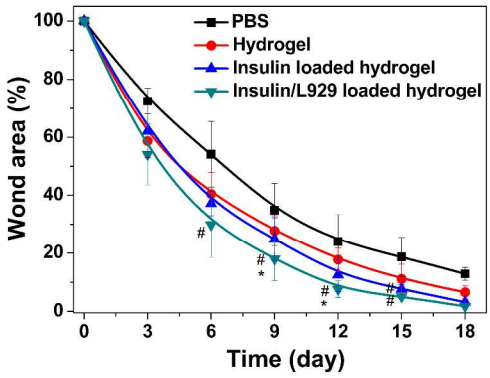
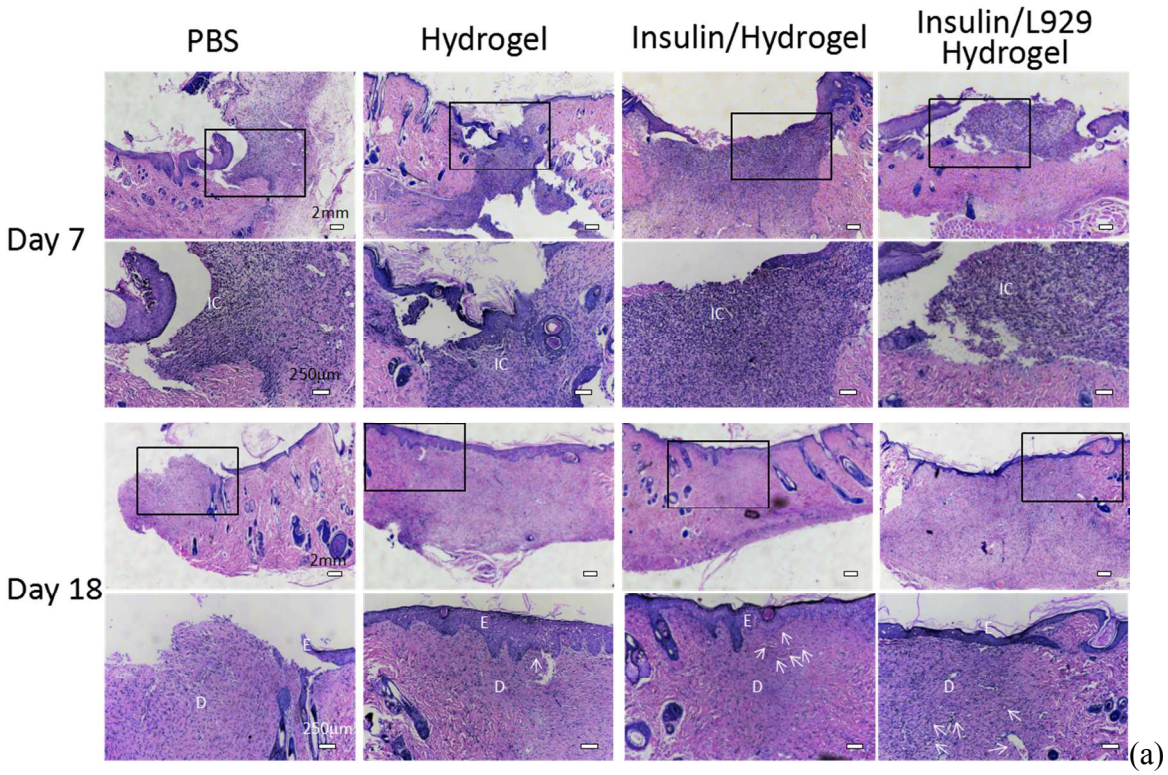


(b)

Figure 8. Wound healing by hydrogel dressings in STZ-induced diabetic rats. PBS, hydrogel, insulin-loaded hydrogel, and insulin/L929 loaded hydrogel were administrated on the diabetic skin wound. Representative images of the wound area were taken on day 0, 6, 12, and 18 (a). Wound closure was measured at varied time point using Image J (b). The wound closure was demonstrated as the percentage of wound area at the indicated day compared to the initial wound area. $^{\#}p< 0.05$ compared with PBS control group; $*p<0.05$ compared with hydrogel group.

Inflammatory infiltrate in STZ-induced diabetic wound

Wound healing comprises several overlapping phases: hemostasis, inflammation, proliferation, re-epithelialization and remodeling.[9] Studies showed that a fibrin plug is formed within hours during hemostasis after injury, and inflammatory cells can be recruited to the wound site due to the cytokines and growth factor released by the aggregated platelets. The aggregation and activation of inflammatory cells are essential for the transition between inflammatory and repair phases.[34] H&E staining was performed on day 7 and day 18 after wounding to evaluate inflammatory infiltrate in the wound-healing process. As shown in Figure 9a, inflammatory cells with highly condensed chromatin can be observed in all groups at day 7 and day 18, and the density of inflammatory cells at day 7 was much higher than that of day 18. Quantitative analysis of the number of mononuclear leucocytes was done by counting the number of cell nuclei per field that had highly condensed chromatin.[33] The results revealed similar inflammatory cell infiltrate in the neat hydrogel group compared with the control group at day 7. A significant increase of inflammatory cell infiltrate in insulin and insulin/L929 hydrogel groups was observed at day 7, which was 1.6-fold higher than that of the control group (Figure 9b), possibly due to the topical insulin administration, as reported in previous work.[33] A sharp drop in the number of inflammatory cells in all experimental groups was observed at day 18 as the wound closure, and there was no fester at the wound site during the experimental period. Since some inflammatory cells (e.g. leucocytes) serve as immunologic effectors and sources of growth-promoting cytokines, the infiltration of inflammatory cells in wound areas may be conducive to wound repair.[34]



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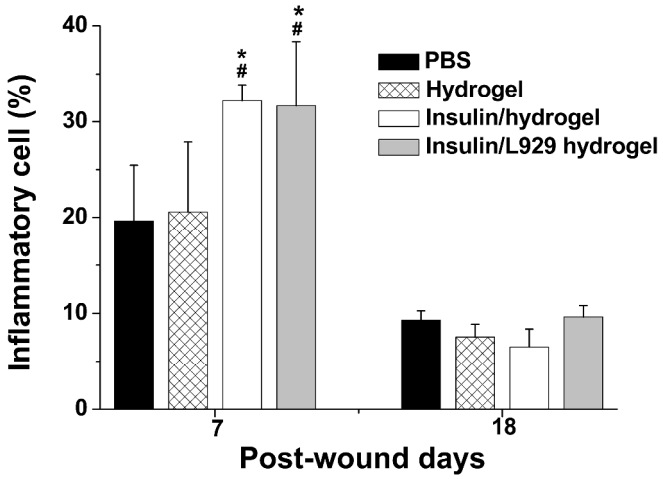


(b)

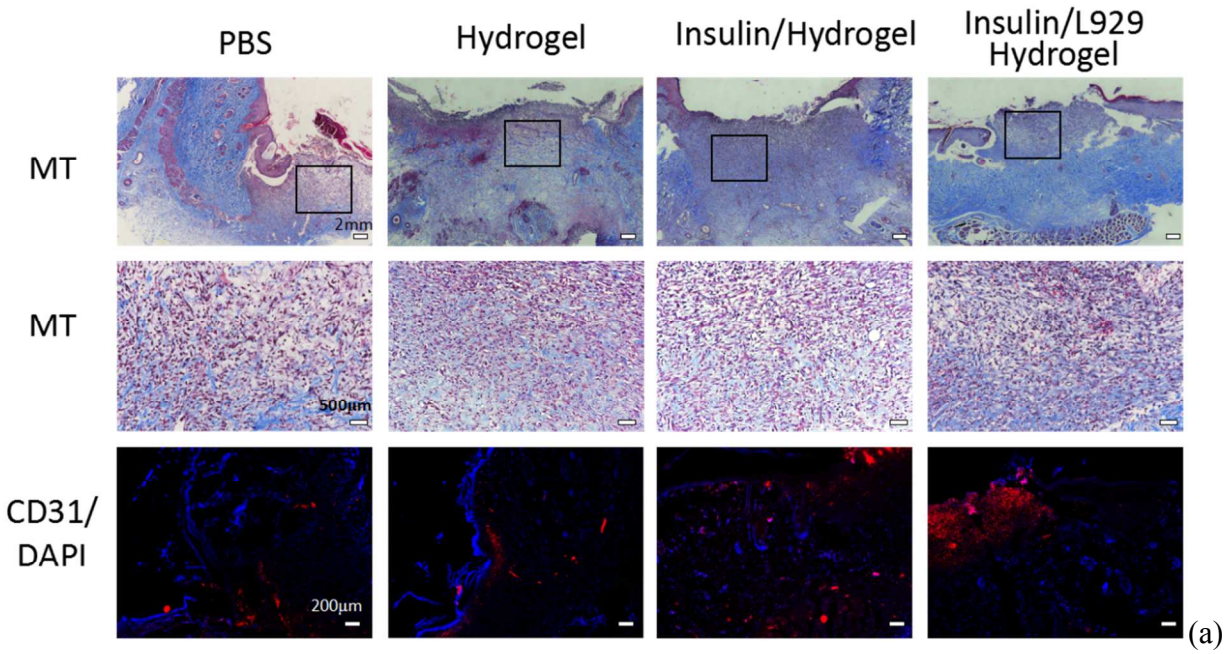
Figure 9 Images of H&E stained histopathological sections after 7 and 18 days of treatment (a). Graphical presentation of the number of inflammatory cells in wound tissue. Quantitative analysis of inflammatory cells was measured using Image J software (b). D, dermis; E, epidermis;

IC, inflammatory cell; Arrows, microvessels. *$p < 0.01$ compared with PBS control group, $^{\#}p < 0.01$ compared with neat hydrogel group.

Neovascularization in STZ-induced diabetic wounds

Neovascularization is very important in tissue remodeling process. Wounds may fail to heal because of inadequate local vascular supply, for many kinds of substances required for wound repair such as oxygen, nutrients, cells and growth factors are delivered and infiltrated into the wound sites through vasculature during the healing process.[48] Vascular formation in the wound areas occurs via chemotactic migration of endothelial cells, while this process is suppressed or even not occur in diabetes mellitus.[49] MT staining and immunocytochemistry were performed to confirm neovascularization in the wound-healing process. As shown in Figure 10, MT staining displayed a few spindle endothelial cells rather than red capillary vasculature in the four experimental groups at day 7. Immunocytochemistry results revealed a remarkably dense population of red CD31-positive cells in the insulin/L929 hydrogel group compared with the control and neat hydrogel groups. Moreover, quantified analysis of the immunochemical results showed a 1.7- and 2.4- fold increased population of CD31-positive cells in the insulin and insulin/L929 hydrogel group respectively, compared with the PBS control group (Figure 10b). As a wildly known marker of endothelial cells, CD31 positive-cells were considered to contribute to the neovascularization and enhance wound healing.[50-52] This was further confirmed by H&E and MT staining carried out on day 18 after wound treatment, and many more red capillary vasculatures were revealed in insulin and insulin/L929 groups than control and neat hydrogel groups (Figure 9a and 11a), being consistent with the immunohistochemistry result. It was reported that topical insulin, growth factors and genes (e.g. VEGF, IL-8 and PDGF-B) used in DFU treatment could enhance neovascularization,[33, 53-55] thus the insulin and live cells with

growth factors secretion encapsulated in the hydrogel dressing as bioactive agents are of great importance to neovascularization and wound repair.



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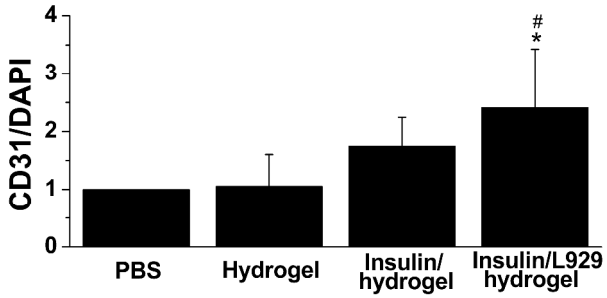
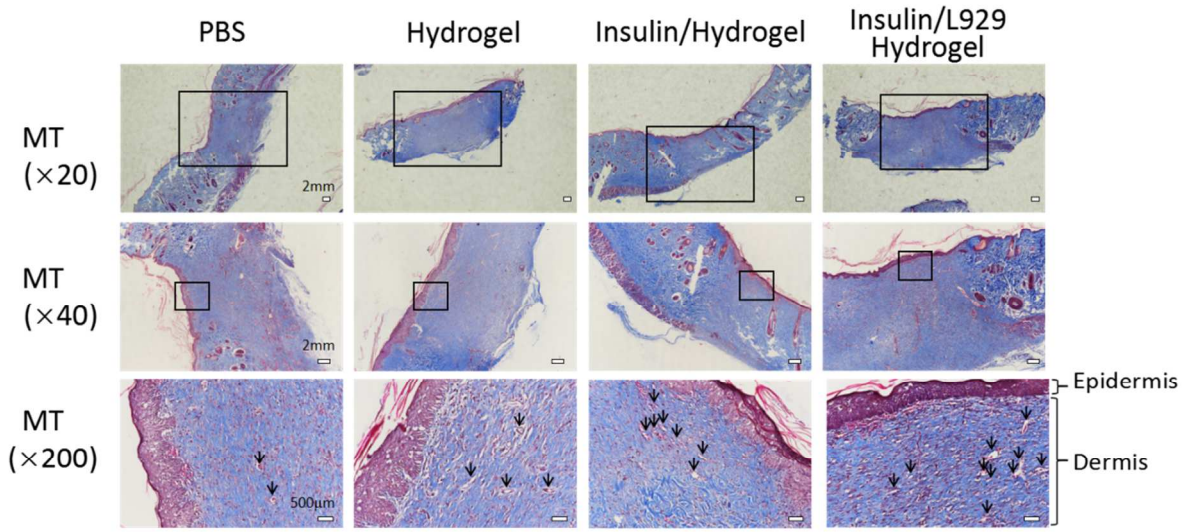


(b)

Figure 10. Vascular formation enhanced by insulin/L929 incorporated hydrogel dressings. Immunocytochemical fluorescence reveals CD31-positive cells (red) on day 7 after wounding. Images of MT stained and immunocytochemical sections after 7 days of treatment for the detection of endothelial cells (a), quantification analysis of CD31-positive cells using Image J (b). $^{\#}p< 0.05$ compared with PBS control group; $*p<0.05$ compared with hydrogel group.

Collagen deposition in STZ-induced diabetic wounds

Collagen deposition plays an essential role in the severity of scar formation during the maturation process of wound repair.[56] MT staining was performed on day 18 after wounding in order to demonstrate the synthesis and accumulation of newly formed collagen in the regenerated skin tissue. The results revealed more collagen deposition on the regenerated tissues in insulin/L929 hydrogel group compared with other groups (Figure 11a). Image J software was used to quantify the collagen deposition in the wound sites by measuring the intensity of the blue areas. As plotted in Figure 11b, wounds of the insulin/L929 hydrogel group displayed 58±2% of collagen deposition, which was 1.5-fold higher than that of the control group (40±1%). By comparison, the wounds dealt with neat hydrogel and insulin-loaded hydrogel showed 44±9% and 52±10% of collagen deposition, respectively (Figure 11b). At maturation stage of wound healing, wound contraction was resulted due to fibroblasts cross-linked with collagen. It is reported that hyperglycemia impairs wound healing by decreasing cell proliferation and affecting collagen synthesis,[33] hence the enhanced collagen deposition in insulin/L929 loaded hydrogel group may be due to the lower level of blood glucose (Figure 7). In addition, some growth factor (e.g. EFG and bFGF) can increase the accumulation of collagen and keratin so as to reduce scar formation.[57,58] Thus, the L929 cells may contribute to the effective collagen deposition in insulin/L929 hydrogel group by secreting such growth factors. These findings indicated that combining hydrogel with insulin and live cells promoted neovascularization and collagen deposition and enhanced the wound healing process of diabetic wounds.



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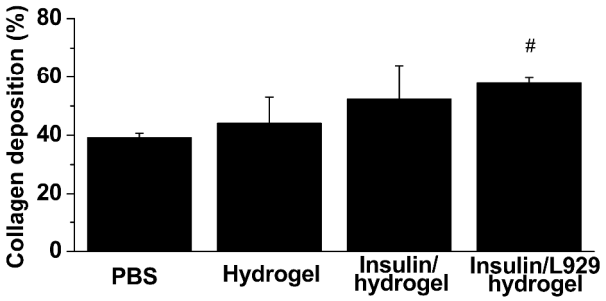

(b)

Figure 11. Collagen deposition improved with hydrogels containing insulin and L929 fibroblasts. The tissues of wound sites were performed by MT staining on day 18 after wounding. Blue areas showed newly synthesized collagen fibers and arrows indicate capillary vasculatures (a). The Image J software was used to quantify the collagen deposition in the wound sites (b). $^{\#}p< 0.05$ compared with PBS control group.

Conclusions

pH and glucose responsive injectable hydrogels were synthesized through the formation of Schiff's base and phenylboronate ester based on phenyboronic modified chitosan (CSPBA),

PVA and OHC-PEG-CHO. Protein drugs and live cells can be encapsulated in the hydrogels during the *in situ* crosslinking process. Sustained and pH/glucose triggered drug release from the hydrogels, and cell viability and proliferation in the three-dimensional hydrogel matrix were observed, allowing the hydrogels to serve as bioactive dressings for diabetic wound repair. *In vivo* evaluation of the hydrogel dressings for wound healing was performed using STZ-induced diabetic rat model from general aspects of the wound: the wound closure rate, inflammatory infiltrate, neovascularization, and collagen deposition as well. The results displayed enhanced wound healing of the insulin/L929 hydrogel dressings with increased neovascularization and collagen deposition, suggesting the hydrogels as a delivery platform for various therapeutic proteins and live cells for wound healing applications.

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Author Contributions

Lingling Zhao and Hui Tan conceived the project. Lingling Zhao and Lijing Niu carried out the preparation and evaluation of the hydrogel dressings. Feiyan Zhu participated in the data

analysis. Hongze Liang and Chaozong Liu directed the work. Lingling Zhao wrote the initial draft of the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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