Accelerating the remodeling of collagen in cutaneous full-thickness wound using FIR soldering technology with bio-targeting nanocomposites hydrogel

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Abstract

A novel composite wound dressing hydrogel by incorporating single-walled carbon nanotubes and indocyanine green into a dual-crosslinked hydrogel through Schiff base reaction was developed. The objective was to prevent wound infection and enhance the thermal effect induced by laser energy. The hydrogel matrix was constructed using oxidized gelatin, pre-crosslinked with calcium ions, along with carboxymethyl chitosan, crosslinked via Schiff base reaction. Optimization of the blank hydrogel's gelation time, swelling index, degradation rate, and mechanical properties was achieved by adding 0.1% SWCNT and 0.1% ICG. Among them, the SWCNT-loaded hydrogel BCG-SWCNT exhibited superior performance overall: a gelation time of 102 seconds; a swelling index above 30 after equilibrium swelling; a degradation rate of 100.5% on the seventh day; and a compressive modulus of 8.8 KPa. It displayed significant inhibition against methicillin-resistant Staphylococcus aureus infection in wounds. When combined with laser energy usage, the composite hydrogel demonstrated excellent pro-healing activity in rats.

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Abstract: A novel composite wound dressing hydrogel by incorporating single-walled carbon nanotubes and indocyanine green into a dual-crosslinked hydrogel through Schiff base reaction was developed. The objective was to prevent wound infection and enhance the thermal effect induced by laser energy. The hydrogel matrix was constructed using oxidized gelatin, pre-crosslinked with calcium ions, along with carboxymethyl chitosan, crosslinked via Schiff base reaction. Optimization of the blank hydrogel's gelation time, swelling index, degradation rate, and mechanical properties was achieved by adding 0.1% SWCNT and 0.1% ICG. Among them, the SWCNT-loaded hydrogel BCG-SWCNT exhibited superior performance overall: a gelation time of 102 seconds; a swelling index above 30 after equilibrium swelling; a degradation rate of 100.5% on the

seventh day; and a compressive modulus of 8.8 KPa. It displayed significant inhibition against methicillin-resistant Staphylococcus aureus infection in wounds. When combined with laser energy usage, the composite hydrogel demonstrated excellent pro-healing activity in rats.

Keywords: Adhesive hydrogel, Antibacterial ability, Wound healing, Carboxymethyl chitosan, SWCNTs

Introduction

Collagen makes up 70-80% of the skin's dry weight, serving as its main component and giving it strength. During the healing process of skin wounds, various types of collagens (I, III, V, VII, and XVII) play important roles. Type I collagen can promote re-epithelialization by stimulating keratinocyte migration and enhance matrix remodeling by increasing the expression of matrix metalloproteinases in keratinocytes. Additionally, type I collagen has immunomodulatory properties that contribute positively to wound healing. A study published in Nature by Karin suggests that a three-dimensional environment rich in high-density fibers of type I collagen can induce immune suppressive functions in M2 macrophages, which may have beneficial effects on diabetic foot ulcer (DFU) wound healing. However, insufficient deposition of type I collagen within hypertrophic scar tissue fails to provide adequate immunomodulation for proper regulation of wound healing. Previous studies have shown that low-level laser therapy effectively stimulates fibroblast proliferation within specific energy ranges and promotes production of both type I and III collagens; however, laser treatment often fails to regulate the ratio between these two types of collagens leading to tissue fibrosis directly associated with excessive secretion of collagen.

In the realm of nanomaterials, there has been a considerable amount of attention directed towards carbon nanotubes (CNTs), owing to their extensive potential in the field of biomedicine and biotechnology. These remarkable structures have shown promise in delivering bioactive substances such as medications, proteins, and nucleic acids; facilitating targeted therapy for tumors; and enabling biological imaging. The immense potential of carbon nanotubes stems from their inherent mechanical, optical, and electrical properties due to their small size, large surface area, low density, and high stability. One of the most appealing advantages of carbon nanotubes is their ability to effectively penetrate biological barriers and even enter the cell nucleus. However, the application of carbon nanotubes has been severely limited by their super hydrophobicity and tendency to aggregate in aqueous media. Previous studies have shown that collagen/single-walled carbon nanotube composites hold great practical value as scaffolds in tissue engineering. Collagen has also been used to stabilize silver nanoparticles in water. Furthermore, functionalizing type I collagen with single-walled carbon nanotubes (SWCNTs) allows for good dispersion of SWCNTs in aqueous solutions, making collagen-functionalized SWCNTs suitable for biomedical and biotechnological applications. Additionally, research has indicated that under 808 nm laser irradiation, indocyanine green (ICG) generates a photothermal effect that enables the destruction of tumor cells by damaging bacterial cells and neighboring cells.

Wound coverings are a form of biomaterial utilized for the purpose of concealing wounds, ulcers, or other forms of injuries. There exist primarily three categories of wound dressings developed to facilitate wound healing [1-3]. Conventional passive dressings serve the purpose of passively enveloping the wound, absorbing exudate, and offering limited safeguarding to the injury. On the other hand, active dressings engage with the wound in an interactive manner by effectively absorbing exudate and harmful substances while establishing an optimal therapeutic environment for gas exchange. The outer layer structure of the barrier serves as a protective shield, safeguarding the wound against microbial invasion from its surroundings. This effectively prevents any contamination and transmission of harmful microorganisms to the wound. Occlusive dressings not only act as a physical barrier shielding the wound from external elements but also create an optimal moist environment that promotes continuous tissue regeneration throughout the healing process. Moreover, it is crucial for dressings to adhere appropriately to the wound without causing any secondary harm or damage [4]. Recently, there has been extensive research on the utilization of biodegradable hydrogel molecules in the pharmaceutical and biomedical fields due to their remarkable properties including high swelling capacity, biocompatibility, and small volume[5-9]. Hydrogels derived from aqueous gels offer several advantages such as creating a moist environment, exhibiting excellent biocompatibility, effectively absorbing wound exudate while minimizing adhesion to injured tissues [10]. In addition, the utilization of hydrogel dressings containing

antimicrobial medications can effectively prevent infection and secondary damage during dressing changes by achieving sustained release effects. It is important to emphasize that careful design is necessary for stable hydrogels equipped with outstanding compressive characteristics so as to reduce potential damage coming from external forces[11-13]. Gels are typically formed through physical or chemical processes that rapidly construct a three-dimensional network using precursor or monomer materials[14-15]. The mechanism of crosslinking solidification directly affects both the stability of hydrogels and the state of active substances incorporated within them[16].

Extracellular polysaccharide produced through aerobic fermentation of Sphingomonas/ Pseudomonas bacteria facilitates covalent crosslinking. It comprises four distinct monosaccharides as repeating units, including two d-glucose carbohydrates, one l-rhamnose, and one d-glucuronic acid[26-28]. GG has found extensive application in the formulation of wound dressings, prevention of scar formation, and inhibition of postoperative adhesions. For example, when GG was combined with cinnamic acid ester, it resulted in the creation of a photo-crosslinked polymer known as GG/cinnamate. This novel material exhibited promising potential for preventing adhesion formation when tested on rats [20,29,30]. Numerous investigations have highlighted the exceptional mechanical properties and wound dressing capabilities of GG hydrogels. Hydrogel formulations can be prepared by crosslinking GG using calcium or magnesium ions[31-33]. However, the limited stability of gellan gum in physiological fluids due to monovalent cations hampers its potential biomedical applications like cell encapsulation. This is because the exchange of divalent cations leads to a loss of mechanical stability [26,34]. To address these issues such as inadequate mechanical strength, poor physiological stability, and high gelation temperature, one possible solution is to utilize the oxidation conversion of cis-diols on GG chains into reactive aldehydes. These aldehydes can then react with amino groups to form Schiff's base linkages or ionic crosslinks. For instance, chitosan-GG (OG) crosslinking can be achieved by reacting the abundant amine groups on chitosan chains with oxidized aldehyde groups on oxidized gellan gum (OG), resulting in stable hydrogel formation. Carboxymethyl chitosan (CMCS), which has improved solubility compared to chitosan[15], is commonly used for constructing biomedically applicable hydrogels.

In this study, we present the synthesis of biodegradable composite hydrogels containing microspheres. These hydrogels are formed through the crosslinking of carboxymethyl chitosan (CMCS) and oxidized cold-set gelatin (OG) using Schiff's base as a mediator. To enhance their long-term antibacterial properties, we incorporated single-walled carbon nanotubes (SWCNTs) and indocyanine green (ICG) nanoparticles, which possess photothermal effects with antibacterial activity. We conducted comprehensive investigations to optimize the mechanical properties, tissue adhesion, cytotoxicity, skin irritation, and in vivo wound healing performance of these nanoparticle-composite hydrogels for potential use as wound dressings. Furthermore, we examined the healing efficacy of these hydrogels on skin tissue wounds under low-energy laser stimulation.

Materials and methods

Materials

The Gellen adhesive (GG) was obtained from Shanghai Macklin Biochemical Co., Ltd. It has a high molecular weight of 500 kDa and an acetylation degree exceeding 90%. Carboxymethyl chitosan (CMCS), with a carboxymethylation degree surpassing 80% and exhibiting a viscosity range of 60-1000 mPa·s, was sourced from Shanghai Traditional Chinese Medicine Chemical Reagent Co., Ltd. Bovine serum albumin (BSA) was acquired from Nanjing Sunshine Biotechnology Co., Ltd. The single-walled carbon nanotubes referred to as SWCNTs were directly obtained for this study from Sigma-Aldrich Company in the United States. These SWCNTs have diameters ranging between 0.7 to 1.3 nm and boast a purity level above 90%. Indocyanine green (ICG), originating from Shanghai Macklin Biochemical Co., Ltd., has an approximate molecular weight of 774.96 Daltons.

Laser soldering system

Based on extensive prior research, we have developed a comprehensive welding system that can meet all the necessary requirements during laser welding processes, as shown in Figure 1. The skin tissue connection machine used in this study consists of three components: a laser operational system, a thermograph, and

an overall control system. The laser operational system includes consecutive fiber lasers with a wavelength of 1064nm Nd:YAG, along with a workbench and relevant clamping devices. The thermograph comprises a Fortic near-infrared thermal imager and its corresponding control subsystem. At the same time, the overall control system encompasses the temperature output data processing subsystems of both the laser control system and the thermal imager. By referring to previous experiments, we have determined optimized fundamental parameters for the laser such as power level, scanning path, defocus amount, wavelength selection, and scanning speed [27-28]. Through thorough analysis of both macroscopic and microscopic aspects of skin tissue after performing laser welding procedures while considering factors like body temperature and thickness in rats' subjects; we further improved these parameters to achieve optimal results as presented in Table 1.

Fig.1 Laser soldering system and Ultra-clean operating table

Table.1 Experimental parameters

No.	Defocus amount	Power/w	Speed/mm. s^{-1}	Scanning mode [26]	Energy density/J.mm^{-2}	Incident angle/°
1	-1	7	100	Point	67.543	90
2						60
3						30

Preparation of BCG-SWCNT and BCG-ICG hydrogel

Initially, oxidized GG (OG) was synthesized by following a slightly modified procedure [28]. A solution of 3 g GG in 300 mL distilled water was heated to 90 °C. Subsequently, with continuous stirring at room temperature for 24 hours in the absence of light, 1.5 g NaIO4 was added. To conclude the oxidation reaction, 2 mL ethylene glycol was introduced after a duration of 30 minutes. The resulting solution underwent purification through dialysis (MWCO14,000) against distilled water for a period of three days to eliminate excess NaIO4 and subsequently lyophilized at -50 °C. The degree of oxidation determined via iodometric titration amounted to approximately 9.7%. In the subsequent step, hydrogel formation involved preparing an aqueous solution containing OG at a concentration of 35 mg/mL under constant stirring for half an hour.

The solution was supplemented with $CaCl_2$ at a concentration of 1mg/mL. Subsequently, a hydrogel was formed by thoroughly mixing a CMCS aqueous solution (40 mg/mL) and OG solution in varying volume ratios at 37 °C overnight. In order to achieve a homogeneous solution for the preparation of SWCNT and ICG embedded hydrogel, the pH was adjusted to 6.7. Following a waiting period of 15-30 minutes, the prepared gel received additions of SWCNT homogeneous dispersant (10mg/mL) and ICG (10mg/ml), resulting in what is referred to as BCG-SWCNT and BCG-ICG respectively. To ensure sterility during the fabrication process of the hydrogel, all materials were subjected to UV or autoclave sterilization procedures within an environment that adhered to sterile conditions (tissue culture hood).

Morphologies and mechanical properties

The structures of GMs, Gel and GMs/Gel scaffolds were analyzed through SEM imaging. The samples were subjected to freeze-drying at -50 °C for 24 hours before being coated with gold using a Cressington 108 Auto (Cressington, Watford UK) for a duration of 90 seconds. A Hitachi SU8010 SEM (Hitachi, Japan) was utilized to observe the morphologies under an accelerating voltage of 3 kV.

Mechanical properties/Compressive test

The hydrogels were prepared into dimensions of 40 mm ×10 mm × 1 mm and subjected to tensile testing using a universal testing machine (CMT-1104, SUST, China) at a crosshead speed of 100 mm/min until failure. Each group was tested with a minimum of three hydrogel samples. The Young's modulus was determined by analyzing the stress-strain curve within a strain range from 0 to 10%. To assess cyclic tensile properties, silicone oil was applied on the hydrogel samples to prevent water loss. The same testing conditions as

mentioned earlier were employed, but with a maximum strain of 100%. For compression testing, cylindrical-shaped hydrogel samples measuring 15 mm in diameter and 2 mm in thickness were compressed at room temperature using the universal testing machine at a rate of 10% strain per minute.

Swelling behaviors

For the swelling experiments, the BCG-SWCNT and BCG-ICG hydrogels were immersed in PBS (pH 7.4) at a temperature of 37 °C for a duration of 1 hour until they reached equilibrium swelling. The swollen samples were then periodically removed and weighed (W_t) after eliminating any surface adsorbed water using filter paper. To determine the hydrogel weight, lyophilization was performed at -50 °C followed by weighing (W_d). The swelling ratio was calculated using the following equation:

$$S_R = \frac{W_t - W_d}{W_d}$$

where S is the swelling ratio, and W_t and W_d are the weight of the hydrogel before and after swelling, respectively.

Rheological properties

Rotational rheometer (MCR308, China) equipped with a 2.5cm flat plate and a 1 mm gap was used to evaluate rheological properties. A temporal-scan study took place in a consistent strain level at a magnitude of 1.0% with a frequency of 1 cycle per second under ambient temperature conditions of 25. The combination of the two precursor solutions occurred through the utilization of a dual-barreled syringe, followed by injection onto the parallel plate while simultaneously monitoring the evolution of storage modulus (G') and loss modulus (G") over a period

Ex vivo adhesion properties

The hydrogels underwent a transformation administered onto the exterior of pig hides, and rotational force was exerted at the boundary of the dermis. To assess the sticky substance properties of the hydrogels, a patch adhesion strength test was conducted. In summary, cuboidal shapes measuring 20 × 10 × 4 mm^3 were cut from porcine skins. Subsequently, a solution containing BCG (50 μL) was injected onto those back skins of pigs. Then, another back skin with either SWCNT or ICG dispersion liquid (50 μL) was put on top of the BCG-treated skin (adhering area: 1*1 cm^2). After a period of 30 minutes, a universal testing machine at a temperature of 25 pulled apart the overlapped skins using a cross-head speed set at 60mm/h until they separated.

In vitro biodegradation

Before conducting the research, the hydrogels had undergone certain preparations, weighed and then fully engrossed in a 5 mL solution of lysozyme (1000 U/mL) in PBS (pH = 7.4) at 37 with continuous stirring at a speed of 100 rpm for a period of 4 weeks. The solution used for incubation was refreshed biweekly. The hydrogels were rinsed with ddH2O and weighed on days 1, 3, 5, 7,14,21 and 28. Equation (3) was utilized to determine the percentage of degradation.

$Degradation = \frac{W_t}{W_0} \times 100\%$ (3)

where W_t represents the weight at each time interval after a certain period, and W_0 denotes the initial weight of the samples

Antibacterial evaluation

The bacterial strains S. aureus (ATCC25923), E. coli (ATCC25922), and MRSA (ATCC43300) were grown for an extended period of time at 37 in a Luria-Bertani medium under constant agitation. The optical density of the bacterial suspensions was determined at a wavelength of 600 nm using a microplate reader, with an OD600 value of 0.1 corresponding to a concentration of approximately 1 × 108 colony-forming units

per milliliter. Cylinder-shaped hydrogel samples measuring diameter=10 mm and height=2 mm was placed into individual wells within a multi-well plate, followed by incubation with diluted bacterial suspensions containing approximately106 colony-forming units per milliliter for a duration of12 hours at37 . Following the completion of the12-hour incubation period, the bacterial suspension underwent a thousand-fold dilution before spreading 100μL of this diluted solution onto agar plates. Subsequent cultivation for24 hoursat37 allowed for observation and analysis of result in bacterial colonies, in order to assess the antibacterial efficacy of the tested hydrogels.

In-vivo wound healing evaluation

The SD rats were obtained from Changzhou Covens Laboratory Animal co., LTD and acclimated to their new surroundings for a duration of one week. Only male rats weighing between 180-220g were selected for the study, and they were housed in a specific pathogen-free (SPF) animal facility with a light-dark cycle of 12 hours each. All surgical procedures adhered to the approved animal care protocols by the Ethics Committee of Nanjing Medical University.

The experimental groups consisted of 45 rats, which were assigned randomly and distributed among three test indexes at four different time points. Additionally, each time point (3, 7, 14, and 24 days) included three distinct groups (BCG-con with n = 5, BCG-SWCNT with n = 5, and BCG-ICG with n = 5), along with one control rat in each group.

After anaesthetization (1.5–3 vol.% Isoflurane), the dorsal surface hairs were shaved and full-thickness wounds measuring 12 mm in diameter were induced on the dorsal area of the diabetic mouse, and subsequently covered with hydrogel disks of varying dimensions (9 mm in diameter and 1 mm thick). Then we use the 1064nm laser to scanning the wounds in the 0 day and 3 days of wounds modeling respectively. For the control group, the wound site was left without any treatment. The wound healing rate was calculated using the following equation:

$$Healing\ Percentage(\%) = \left(\frac{\mathrm{WA_0} - \mathrm{WA_t}}{\mathrm{WA_0}}\right) \times 100\% \quad (1)$$

$\mathrm{W_{A0}}$ represents the initial wound area on day of injury (day 0), while $\mathrm{W_{At}}$ denotes the subsequent wound areas on corresponding days

Statical analysis

The data displays the averaged quantities along with their associated statistical uncertainty, represented as average ± variability. A p-value lower than 0.05 signifies a statistically meaningful differentiation.

Results and discussion

Fabrication and characterization of hydrogels

The composite hydrogels were formed through the process of crosslinking CMCS with pre-crosslinked OG containing calcium ions, as illustrated in the schematic diagrams (Fig. 2(a)). The gelation primarily occurs through a Schiff-base reaction involving the amino groups in CMCS and aldehyde groups in OG (Fig. 2(b)). The aldehyde group was formed by oxidizing the cis-o-diol present in GG with sodium periodate, resulting in an estimated 9.7% degree of oxidation determined by iodometric titration. Throughout the entire reaction process, prior to that, OG underwent physical crosslinking treatment with Ca^{2+}. The temperature was maintained below $\mathrm{T_{gelation}}$ to facilitate the formation of a double helix conformation for OG[40]. After being combined with CMCS, GG undergoes ionic crosslinking and reinforcement of chemical crosslinking is achieved by the formation of Schiff base, resulting in the establishment of a dual network structure. [28]. The presence of -CHO facilitates the crosslinking between CMCS and OG, enabling gel formation. To fabricate drug-loaded composite gel scaffolds (Fig.2(c)), various drugs such as BSA, SWCNT, and ICG are dissolved or dispersed in the GG solution at specific concentrations. These three composite hydrogels contain different pigment groups that can regulate the laser's energy absorptivity. As a result, the laser treatment on wounds can be modified and varied under these hydrogels, as depicted in fig.2(d).

Figure.2. Preparation and characterization of the hydrogels. (a) Representation of the molecular structure of Gellan Gum (GG), Gellan gum with oxidation (OG), and chitosan carboxymethylated (CMCS). (b)(c)Schematic illustration of the preparation of BCG-Con/SWCNT/ICG hydrogels through Schiff base reaction. (d) Schematic illustration of BCG-Con/SWCNT/ICG hydrogels in the treatment of wounds in conjunction with the 1064nm consecutive low energy laser. (e)(f)(g) The hydrogel morphologies were examined using SEM, with scale bars measuring 250 μm, 50 μm, and 10 μm correspondingly. (h) Pore size, (i) swelling ratio, (j)density, and (k) Thermal Gravimetric Analysis of the hydrogels. Data are presented as mean ± SD ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

The SEM images in Fig. 2 e-g display the lyophilized hydrogels created using different types of nanoparticles derived from CMCS and OG composite solution. It is evident that the hydrogels formed by BSA, CMCS, and OG possess an uninterrupted porous structure with three dimensions, which is a result of ice crystals during freeze-drying. The existence of this three-dimensional porous structure enhances the uptake of interstitial fluid and blood, thereby encouraging beneficial hemostasis.[34]. It can be observed that the hydrogels created using CMCS and OG possessed a continuous porous structure in three dimensions, which was formed through the freezing process involving ice crystals. The microstructure of the three different hydrogels prepared with various core components did not exhibit any significant disparities. The walls of the pores exhibited a consistent texture, while the voids displayed a compact appearance. These porous formations promote cellular nourishment and oxygen provision during the process of wound healing. High-resolution SEM images demonstrated a slight reduction in pore size for the hydrogels in the following order: BCG-Con, BCG-ICG, and BCG-SWCNT (as depicted in Fig.2(h)). It is important to note that there was an increase in surface roughness observed for BCG-SWCNTs (inset of Fig.2.(g)), potentially enhancing RBC adhesion and blood clot formation[35].

The OG/CMCS composite gel was utilized to encapsulate the SWCNTs due to their negative surface charges, which enable them to form an electrostatic bond with the cationic OG polymer. As a result, the BCG-SWCNTs hydrogels exhibited reduced swelling ratios and increased density (as shown in Fig.2. (i), (j)).

Furthermore, the thermal stability of hydrogels was investigated using thermal gravimetric analysis (TGA), as depicted in Figure.2(h). The weight reduction observed in BCG-SWCNTs within the temperature range of 450-550°C can be attributed to the dihydroxylation process occurring in NHCH2-COOH groups present in its structure. Notably, when compared to pure BCG hydrogel, it was found that incorporating SWCNTs into the hydrogel resulted in a decreased final weight loss. This observation suggests that SWCNTs have a positive impact on enhancing the thermal stability of the hydrogel.

Gelation time, biocompatibility, and biodegradation of the hydrogels

The solidification time of hydrogels plays a crucial role in the biomedical field when it comes to hemostasis applications. The time at which gelation occurs can impact how well the hydrogel can be injected during clinical use. Rapid gelation is not ideal for removing air bubbles from the hydrogel, as these bubbles can create flaws in the scaffolds and affect overall performance. In our experiments with gels, we noticed a notable rise in the quantity of amine groups relative to aldehyde groups within OG as the proportionate mass of CMCS escalated.

The disparity in the quantity of amino groups and aldehyde groups led to an increased gap, resulting in a longer duration for gel formation. To investigate the gelation time of BCG-Con/SWCNT/ICG hydrogels, we separately loaded CMCS/OG and BSA/SWCNT/ICG solutions into three cap tubes each (as shown in the inset of Fig. 2(a)). The time at which gelation occurs was then documented after mixing these solutions together in their respective cap tubes. Throughout this study, the gelation time for all three volume ratios remained consistent at approximately 90-120s. Figure .2(e) illustrates that BCG-Con exhibited the shortest gelation time, taking only 86s, while BCG-SWCNTs had the longest duration at 120s±15s.

In addition, we performed a study on the bonding capacity of the hydrogel utilizing swine dermis which exhibits physical attributes of living organisms akin to those seen within the dermal layer of humans.[36]. The BCG-SWCNTs/ICG hydrogels were applied onto the outer layer of the porcine dermis. Skin was sub-

jected to torsional strain; it was observed that the hydrogels adhered firmly and did not detach (inset of Fig. 2(b)(c)(d)). To investigate the adhesive characteristics, shear lap testing was conducted using a universal testing machine (inset of Fig. 2(f)(g)). The strength of adhesion values for the BCG-Con, BCG-SWCNTs, and BCG-ICG hydrogels were measured as 58.69±6.11KPa, 55.62±4.89KPa, and 49.78±5.61KPa respectively. The strong adherence between the skin surface and BCG-SWCNTs/ICG hydrogels can be ascribed to interactions involving covalent bonds and hydrogen bonding that occur due to chemical reactions between specific components in both materials[37]. Specifically, the hydrogels consist of ODEX that contains a high concentration of –CHO groups. These groups have the ability to react with $–NH_2$ groups in porcine skin and form a covalent bond known as –C=N. Additionally, there is hydrogen bonding between –CONH and –OH groups present in porcine skin, as well as -OH (from ICG) and -CHO (from OG) groups within the structure of the hydrogel. Therefore, by incorporating an appropriate amount of SWCNTs and ICG into hydrogels, their adhesive properties can be enhanced.

Good hydrogels should degrade once or before wound closure has been achieved. Our work analyzed the degradation properties of the hydrogels in vitro by observing their weight reduce when exposed to a solution containing lysozyme. (as shown in the inset of Figure 2(h)). It was observed that all the hydrogels experienced relatively rapid initial degradation within 7 to 8 days, the weights of these entities experience a gradual reduction. The BCG-SWCNTs hydrogel exhibited approximately 95% degradation after 4 weeks, while the BCG-ICG and BCG-Con hydrogels displayed a slower rate of degradation compared to the BCG-SWCNTs variant. An in vivo biodegradation study involving subcutaneous implantation into rat models demonstrated a similar trend: around 80%–90% of hydrogel degradation occurred within 2 weeks post-implantation (as depicted in the inset of Figure 2(i)). Our findings regarding the degradation behavior align with prior research, this is for the susceptibility of Schiff base bond to hydrolysis[38].

Figure.3. Characteristics related to gel formation, bonding ability, and breakdown properties were investigated for these hydrogel samples. (a)The capillary tubes were initially filled with solutions containing CMCS/OG and BSA/SWCNT/ICG before undergoing gelation processes. The adhesive behavior was evaluated by subjecting these hydrogels to torsional stress when applied onto porcine skin surfaces as shown in figures (b), (c), (d) respectively. The time required for complete gel formation was also measured as depicted in figure \euro. A schematic diagram illustrating how adhesion strength was tested is provided in figure f while figure g presents data on adhesion strength under torsional stress conditions on skin surfaces. Degradation studies were conducted both in vitro(h)and in vivo(i). Experimental results are expressed as mean values ± standard deviation based on three replicates(n=3).*Statistical significance at $p<0$ .05.

Mechanical properties

Angular frequency scanning experiments can be utilized in rheological measurements to investigate the network structure stability of hydrogels when subjected to cyclic shear forces. The G' values for BCG-Con, BCG-SWCNT, and BCG-ICG reach a stable state at approximately 1750 Pa, 2260 Pa, and 2110 Pa respectively. Both BCG-Con and BCG-ICG gels display fluctuations in G" at higher frequencies. As depicted in Figure .4.(a), the incorporation of SWCNT into the composite hydrogel enhances its G' value and ensures stability across a frequency range of 0.01 ˜ 100 Hz, suggesting that the composite hydrogel exhibits robust elasticity. Specifically, the G' value for BCG-SWCNT gel remains consistently high at 2630 Pa, while the G" value for BCG-ICG gel exhibits fluctuations that are not conducive to maintaining elasticity in the composite hydrogel. Moreover, throughout this study, G' consistently surpasses G" by one order of magnitude, indicating exceptional elastic performance of the composite hydrogel that plays a crucial role in promoting wound healing.

The gel's compressive performance was evaluated at 37°C using a universal mechanical testing system equipped with a 500 N load cell. The specimen underwent compression without any restrictions at a speed of 1 mm per minute until it reached the point of yielding. The compression characteristic curve of the hydrogel is illustrated in Figure 4. (b)(c). In BCG hydrogel, an elevated proportion of CMCS leads to a gradual reduction in the compression modulus, suggesting that hydrogels fabricated with a greater abundance of amine and aldehyde groups demonstrate enhanced resistance against compression. The incorporation of SW-

CNTs significantly enhances the mechanical characteristics of the composite hydrogel. However, excessive quantities may result in system-wide aggregation, leading to complete collapse under certain stress levels [42]. Notably, the BCG-ICG composite hydrogel exhibits remarkable antioxidant properties and achieves an optimal compressive performance with a compression modulus measuring 19.3 KPa.

As depicted in figure 4 (d), Fourier transform infrared spectroscopy was employed in this study to examine the alterations in functional groups within hydrogels. A peak is observed at 1732 cm^{-1}, indicating the occurrence of oxidation in the -CHO bond during gelation. The presence of peaks at 1630 cm^{-1} for BCG-SWCNT and BCG-ICG hydrogels confirms the Schiff base reaction between -CHO and -NH_2. Additionally, a peak at 3622 cm^{-1} corresponds to the -OH bond following CMCS and OG reactions. Moreover, there is a shift in the stretching vibration of -OH from 3622 cm^{-1} to 3615 cm^{-1}, which can be attributed to the conversion of -OH present in BCG into both -OH and -COOH within BCG-ICG/SWCNT samples.

In Figure 4 (e), X-ray diffraction patterns were acquired using a Rigaku X-ray diffractometer from Japan, covering the range of 10°-80°. The diffraction peaks observed at angles of 11.9°, 20°, and 25° corresponded to the crystallographic planes (001), (020,110), and (020) of HNTs respectively. These distinctive peaks exhibited a gradual increase in intensity when transitioning from BCG-ICG to BCG-SWCNT to BCG-Con samples, indicating uniform dispersion and loading of ICG and SWCNTs onto CMCS/OG hydrogels.

In vitro antibacterial ability of the hydrogels

Hydrogels are anticipated to facilitate wound repair following hemostasis. To assess the in vitro wound healing efficacy of the hydrogels, we conducted a wound scratch assay. The control groups consisted of BCG-Con dressings containing BSA and CMCS, as well as OG. As depicted in Figure 5(a), the BCG-SWCNT groups exhibited the highest rate of cell migration at 67.25% among all tested groups. Subsequently, RT-qPCR analysis was performed to investigate the expression levels of genes associated with wound healing in HUVEC cells. CD31 and VEGF represent typical angiogenesis genes involved in the process of wound healing [39].

As depicted in Figure. 5(b), the expression levels of CD31 and VEGF in HUVECs were significantly increased by BCG-SWCNT/ICG, particularly BCG-SWCNT, compared to BCG-ICG and BCG-Con groups. Likewise, both Cola1 and Col3a1 levels were notably elevated in HUVECs treated with BCG-ICG. Notably, among all groups, BCG-SWCNT exhibited the highest COL1a1 expression while displaying the lowest level of COL3a1. It is worth mentioning that although COL1 plays a crucial role in wound healing, the combination of high COL1a1 expression along with low COL3a1 expression may contribute to enhanced fibrosis around the wound area. These findings suggest that utilizing hydrogels composed of BCG-SWCNT/ICG could stimulate vessel fibrillar collagen formation and facilitate tissue regeneration through upregulation of genes related to vascularization and collagen production.

Figure.5. (a) Live/dead staining was used to visualize cell migration and healing rate of HUVECs treated with different hydrogels after 24 hours. Representative fluorescence images were captured, with a scale bar of 250 μm. (b) The levels of CD31, VEGF, Col1a1, and Col3a1 genes related to wound healing were analyzed in HUVECs cocultured with the hydrogels. (c) Hydrogel treatments were tested for their ability to inhibit the growth of S. aureus, E. coli, and MRSA over time using dynamic growth curves. A clinically used BCG-Con wound dressing served as a positive control group. (d) After 12 hours of treatment, bacterial colonies were photographed for analysis. Results are presented as mean ± SD (n = 3); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The hydrogels were tested for their antibacterial properties against S. aureus, E. coli, and MRSA. Initially, the number of bacteria was monitored by measuring the OD values at 600 nm in bacterial suspensions. Interestingly, all the hydrogels exhibited comparable antibacterial activity to the BCG group when it came to S. aureus and E. coli (see Fig. 5(c) inset). Additionally, we observed that BCG-SWCNT hydrogels displayed a more potent inhibitory effect on MRSA compared to BCG-ICG. To provide a visual representation of this observation, agar plates were used to culture bacterial suspensions after incubation for 12 hours (see Fig. 5(c) inset).

Based on the growth curve of bacteria, both the BCG hydrogels and BCG-SWCNT/ICG groups effectively eliminated nearly all bacteria. However, the effectiveness of BCG-ICG in inhibiting MRSA growth was comparatively lower. The antibacterial properties of the hydrogels are attributed to CMCS and OG. CMCS has the ability to engage in interactions with teichoic acid and lipopolysaccharide present on the surface of bacteria [40]. Additionally, OG's -CHO groups have the potential to undergo a reaction with -NH_2 groups found in the wall of a bacterial cell[41], resulting in damage to the bacterial plasma membrane. These in vitro findings collectively suggest that BCG-SWCNT hydrogels not only facilitate wound healing but also impede bacterial proliferation.

In vivo wound healing and antibacterial properties

To assess the effectiveness of hydrogel dressings in promoting wound healing and combating bacterial infection, we created a model of infected wounds by removing full-thickness cutaneous wounds and exposing them to MRSA. Subsequently, hydrogel dressings were applied above the wound area along with laser scanning at 1064nm and 6.97J/cm2 on day 0 and day 3 after infection (as shown in the inset of Figure 6(a)). Images depicting the wounds observed during a 14-day period are presented in Figure 6(b). On day 3, serious infection and secretion were observed in both the BCG-Con group and BCG-SWCNTs group. Additionally, the BCG group exhibited only a wound contraction rate of 29.13%, while it was slightly higher at 32.02% for the BCG-ICG group (as indicated in the inset of Figure 6(c)(d)). Notably, compared to the control group and BCG-ICG group, significant acceleration in wound healing was observed with the use of BCG-SWCNTs hydrogels. These findings regarding in vivo wound healing outcomes aligned well with our earlier observations from an in vitro study.

On the 7^{th} day, the BCG-SWCNTs group exhibited the most effective therapeutic outcome with over 50% healing of wounds. Remarkably, after 13 days of treatment, these wounds showed nearly complete healing (99.28%). By day 14, the wounds treated with BCG-ICG hydrogel also demonstrated significant improvement in healing, while the BCG-Con group achieved a healing rate of 95.48%.

Figure.6. The therapeutic impact of hydrogels on in vivo MRSA-infected wounds was evaluated through an experimental setup illustrated schematically in Figure (a). Full-thickness skin injuries were inflicted and subsequently exposed to MRSA bacteria for a period of twenty-four hours before being treated with hydrogel injections followed by exposure to a laser emitting at a wavelength of 1064nm directly above the wounded area. Photographic evidence showcasing various treatment outcomes is displayed in Figure (b) across multiple time points including days zero through fourteen. The dynamic healing ratios for distinct treatment groups are depicted graphically in Figures (c) and (d) at intervals spanning from day four today fourteen post-treatment initiation

Histological examination of the skin samples involved staining with H&E and Masson's trichrome after treatment (inset of Fig. 7). The results from H&E staining revealed a significant presence of inflammatory cells infiltrating the control groups even after 14 days of infection, as indicated by the black dotted box. Additionally, both the control and BCG-ICG groups still exhibited skin defects, suggesting that bacterial infection hindered wound healing progress. In contrast, complete repair was observed in wounds treated with BCG-SWCNTs hydrogels without any signs of inflammation. This confirms the effective inhibition of bacterial infection and promotion of wound healing by the hydrogel. Furthermore, histological analysis demonstrated that wounds in the BCG-SWCNTs group displayed similar characteristics to adjacent normal tissues.

Masson's staining revealed that the BCG-SWCNTs hydrogel groups exhibited enhanced blue staining areas, indicating a significant increase in collagen deposition within treated wounds. This suggests that the hydrogels possess great potential as reliable and effective materials for promoting healing in infected wounds.

Figure.7. H&E and Masson's trichrome staining were performed on wound tissues 14 days after various treatments. Inflammatory tissues are indicated by blue dotted circles. The right panel displays magnified views of the areas outlined by black dotted boxes in the left panel. The scale bars represent measurements of 500μm and 200μm, respectively.

Conclusions

To conclude, we have achieved successful development of a versatile hydrogel consisting of BCG-SWCNTs that exhibits improved adhesion, antibacterial properties, and potential for wound healing. Our findings demonstrate that the hydrogels can undergo in situ crosslinking via the Schiff base reaction to rapidly cover wounds and prevent further infection. Additionally, these hydrogels exhibit excellent biodegradability both in laboratory settings and in living organisms. Moreover, when combined with 1064nm laser scanning, the BCG-SWCNTs hydrogels promote wound healing while effectively inhibiting bacterial infections caused by highly pathogenic MRSA. These results suggest that our hydrogel formulation holds promise as an emergency trauma management solution and as an efficient gel for accelerating wound healing with laser assistance.

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