Antibacterial hydrogel microparticles with drug loading for wound healing

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Abstract
Wound healing and regeneration are critical in medical care and pose a huge challenge for healthcare systems. It has great significance to develop functional materials for promoting wound repair. Herein, we presented novel antibacterial hydrogel microparticles with drug loading to treat the wound. The chitosan (CS) droplets were generated from a microfluidic electrospray system and solidified by sodium hydroxide solution. With further drying and drug uploading process, the drug-loaded CS microparticles (CSMPs) were achieved. The CSMPs exhibited excellent biocompatibility and antibacterial property, and displayed excellent capability in promoting wound healing in multiple stages. Hence, it is expected that the presented CSMPs can serve as multifunctional dressings for wound healing applications.

Introduction
Skin plays an important role in protecting internal organs and preventing body dehydration [1–7]. When skin is damaged, its protected mechanism will be lost and cause severe health problems. Therefore, various strategies have been developed for promoting wound healing, and wound dressings are the most common method. Many kinds of wound dressings, such as electrospun nanofiber, porous foams, sponge, hydrogels, and microparticles have been reported in recent years [8–12]. These dressings allow oxygen to permeate, absorb excess tissue exudates as well as maintain the moist wound environment. However, the whole wound healing process of the skin involves four overlapping stages: hemostasis, inflammation, proliferation, and remodeling [13–15]. Although the dressings have displayed excellent functions, most of them can only play roles in only one or two stages. This may affect the wound healing effect because the four stages are highly orchestrated, and the insufficient development of any stage can impact the whole process. Hence, a novel wound healing dressing is still anticipated.

In this paper, we proposed a kind of novel chitosan microparticles (CSMPs) with drug encapsulation as wound dressing for promoting wound healing. Chitosan (CS) is derived from chitin, a natural polysaccharide, and has a large number of active amino groups. As the only type of cationic polymer in nature, CS possesses excellent biocompatibility, biodegradation, hemostatic performance, and antibacterial property [16–19]. Hence, the CS and its derivatives are potential in building antibacterial materials. Among these materials, the CSMPs are fascinating because they are controllable, flexible, and convenient for mass transfer [20–22]. Nevertheless, the current reported CSMPs also suffered the weakness that the simple component can not meet the requirement to be effective in multiple stages of wound healing. Therefore, CSMPs with more functions still need to be studied and developed.
In this work, the CSMPs were prepared using a microfluidic electrospray system (figure 1(a)). The CS solution was injected into the microfluidic chip and cut into droplets by the added electric field, and the droplets were collected by a container containing sodium hydroxide solution [23–25]. With the further drying process, the CSMPs were prepared (figure 1(b)). It’s worth noting that the CSMPs had porous microstructure, by which they could load drugs [26–32]. Herein, CSMPs were employed to load basic fibroblast growth factor (bFGF) (figure 1(c)). bFGF is a kind of growth factor that can strongly promote the angiogenic effect [33, 34]. When the obtained drug-loaded CSMPs were used for wound treatment, they displayed great capability in hemostasis, antibiosis, and angiogenesis, which meant that they could promote wound healing in multiple stages (figure 1(d)). These results indicate that the prepared drug-loaded CSMPs are ideal wound dressing for promoting wound healing.

Experimental section

Materials
Basic fibroblast growth factor (bFGF), chitosan (CS), bovine serum albumin (BSA) and calcein-AM were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Sodium hydroxide and paraformaldehyde were obtained from Sinopharm Chemical Reagent Co., Ltd L929 cell lines, CCK-8 kit, and staining kits for H&E staining, Masson’s trichrome staining, IL-6 and TNF-α staining were obtained from Keygen technology development Co. Ltd, Nanjing, China. All other reagents were of analytical grade or higher and used as received.

Methods

Preparation of CSMPs
CS was dissolved in the acetic acid solution (2% v/v) to form a CS solution. A microfluidic electrospray platform and a home-made microfluidic chip were employed for the preparation of CS droplets. The microfluidic chip was connected with a peristaltic pump, by which the CS solution was pumped into the chip. When a high voltage DC supply was employed and running, the electrostatic field was formed. Under the added electrostatic field, the CS solution at the end microfluidic chip formed Taylor Cone. With the synthetical effect of the driving force of
the pump and the electrostatic field, the CS solution was continuously injected into the chip and cut into droplets, and the droplets were collected with a container containing sodium hydroxide solution. Because the sodium hydroxide could react with the acetic acid in the droplets, the CS was solidified and formed the hydrogel CSMPs. With further gently washing with water and drying process, the dried CSMPs were obtained. For drug-loading, the CSMPs were incubated in the bFGF solution for about 2 h. Then the CSMPs were washed gently with PBS solution at least 3 times.

**Characterization of CSMPs**

A stereomicroscope was employed to provide the optical characterization of the morphology of the CSMPs. The microstructure of the dried CSMPs was observed by field emission scanning electron microscopy (FE-SEM).

The biocompatibility of CSMPs was evaluated by co-culturing CSMPs with L929 cells, and the group without CSMPs was set as control. The cells of the two groups were supplied with DMEM containing fetal bovine serum (10% v/v) and penicillin-streptomycin (1% v/v). To study the cell viability, the cells were dying by calcein-AM and observed by a fluorescence microscope. Besides, a CCK-8 kit was employed to assay the cell activities of the two groups.

The drug release property of the CSMPs was studied using BSA as the model drug. Three kinds of CSMPs were prepared by CS solution with concentrations of 1, 2, and 3%, respectively. Then the three types of CSMPs were incubated in the drug solution (1 mg mL⁻¹) for drug uploading. Then the CSMPs were incubated in PBS solution at 37 °C for 48 h. The released drugs amount were measured at 1, 2, 4, 6, 12, 24, 36, 48 h. The released ratios of the drugs were calculated by comparing the released amount with the initial uploaded amount of the CSMPs.

**In Vitro** antibacterial test. *S. aureus* and *E. coli* were employed as the typical Gram-positive and Gram-negative bacteria for the test. Bacterial suspension of *S. aureus* or *E. coli* was prepared by adding the corresponding bacterial isolates until the turbidity was up to 0.5 based on McFarland standards. Then the isolates of bacteria were coculture with the CSMPs in 24-well plates for 24 h. To dyeing the bacteria, the equal volumes of Component A (SYTO 9 dye) and Component B (Propidium iodide) were combined in a microfuge tube and mixed thoroughly. Then the dye mixture was added into the 24-well plates (3 μl of the dye mixture for each mL of the bacterial suspension) and mixed thoroughly. Subsequently, the plates were incubated at room temperature in the dark for 15 min. Finally, 5 μl of the stained bacterial suspension was trapped and put between a slide and an 18 mm² square coverslip and observed by a fluorescence microscope.

**In vivo experiment and analysis**

The 8 to 12 week old male SD rats were employed as the model animals, and the experiment was carried out under the guidance of the Laboratory Animal Care and Use Guidelines. The SD rats were fasted overnight before use. For constructing the skin wound model, all the rats were anesthetized, and round wounds of the backs with a diameter of 1 cm were caused by a 10-mm punch biopsy.[35] All rats were put back into cages and kept alone.

The rats were divided into 4 groups of 5 animals each randomly, and the wounds were treated with PBS solution (PBS group, 120 μl), CSMPs without bFGF (CSMPs group, 120 μl), bFGF solution (bFGF group, 120 μl), and the CSMPs with bFGF (CS-bFGF, 120 μl), respectively. To demonstrate typical and specific results of the CSMPs for wound healing in vivo, the wounds were photoed after 3, 6, and 9 days. 9 days later, the rats were sacrificed, and the granulation tissues of the wounds were excised. The tissues of each group were divided into two pieces. One piece was immersed in the 4% paraformaldehyde solution for histology and immunohistochemistry analysis, and the other was stored in liquid nitrogen for immunofluorescent staining.

The tissues in paraformaldehyde solution were then taken out from the solution and dehydrated, and embedded in paraffin. The tissues were then sectioned into slides with a thickness of 5 μm with the standard protocols for H&E staining, Masson’s trichrome staining, and immunohistochemical evaluation by staining with IL-6 and TNF-α. The relative mRNA expression of IL-6 and TNF-α were also measured. The neovascularization evaluation was achieved using the primary antibodies CD31 and α-smooth muscle actin to react with the sections overnight at 4 °C.

**Statistical analysis**

SPSS 19.0 was performed for statistical analysis of data. The data were expressed as the mean ± SD. The results were assessed by the one-way analysis of variance (ANOVA) with Tukey’s post hoc test, in which p-value <0.05 was considered statistically significant.
Results and discussion

Preparation of CSMPs
In a typical experiment, the CSMPs were generated from a microfluidic electrospray system. The CS solution was pumped into a capillary microfluidic chip, and the solution at the end tip of the capillary formed Taylor Cone under an added electrostatic field. Because of the continuous injection of flow and the electrostatic field, the solution was cut into droplets and sprayed from the end tip of the chip. The droplets were collected by a container containing sodium hydroxide solution. Due to the basic environment, the CS droplets were solidified. Then the excess sodium hydroxide solution was removed, and the microparticles were washed with water gently to eliminate the residual sodium hydroxide. With the further drying process, the CSMPs with porous structures were prepared. To endow the CSMPs with more functions, the CSMPs were incubated in the bFGF solution for drug uploading. After the CSMPs were gently washed with PBS solution, the drug-loaded CMSPs were achieved.

Characterization of the CSMPs
The morphology of the CSMPs was characterized by optical microscope and scanning electron microscope. As shown in figure 2(a), the CS droplets were solidified in the container and displayed oval morphology. After washing and drying, the CSMP exhibited a macroporous structure (figure 2(b)). This was because that there was abundant water in the solidified CS droplets, and the drying process led to water loss and the structure collapse. This porous structure made it possible to upload drugs to promote wound healing. Since wound dressings contact tissues directly, they should have good biocompatibility. To investigate the biocompatibility of the CSMPs, we cocultured them with L929 cells, and the group without CSMPs was set as control. As shown in figure 2(c), the cells in the CSMPs group displayed a similar growth situation to that of the control group, and the assayed cell viability showed the same result (figure 2(d)). This indicated that the CSMPs had excellent biocompatibility.

To study the drug loading and release capability of the CSMPs, we employed bovine serum albumin (BSA) as the model drug and studied its uploading and release capability. As shown in figure 3, the CSMPs could release the model drugs for 48 h, which indicated that they could be reliable microcarriers for drug release. We also investigated the influence of the concentration of the CS solution. It was found that the CSMPs made by 1% CS solution exhibited the highest drug release efficiency, while that made by 3% CS solution showed the lowest release efficiency. This may be because the lower concentration of CS solution resulted in the larger pores of CSMPs for mass transfer, and vice versa. Hence, as a compromise strategy, the CS solution with a concentration of 2% was employed to fabricate CSMPs in the following experiments to acquire a moderate drug release speed.
As a kind of cationic polymer, CS can bind with the cell walls or cell membranes of bacteria with negative charges by its protonated amino groups, which causes the change of selective permeability and eventually leads to the death of the bacteria. To confirm the inherent antibacterial property of the CS component, we employed two types of bacteria (Staphylococcus aureus and Escherichia coli) as the model bacteria in the in vitro antibacterial experiment. As shown in figure 4(a), there were many dead bacteria (red) in the CSMPs groups, while it was seldom found in the control groups. The statistical result in figure 4(b) displayed it in a more intuitive view: the bacterial death rates of S. aureus and E. coli in CSMPs groups were over 90%, while they were lower than 10% in the control groups. This result indicated that the CSMPs had excellent antibacterial property, and could be used as an antibacterial dressing.

The therapeutic effect of the CSMPs
As mentioned above, the wound healing process consists of four overlapping stages, so wound dressings are anticipated to be endowed with many functions to play roles in multiple stages to enhance the therapeutic effect. It was confirmed that the CSMPs have biocompatibility, drug loading capacity, and antibacterial property. These features make them possible to be a multifunctional dressing for wound treatment. To study the in vivo therapeutic effect of the drug-loaded CSMPs, the rats with the same size of circle wounds were employed as the animal models and divided into four groups. PBS solution, CSMPs without bFGF (CSMPs), bFGF solution, and CSMPs with bFGF drug (CS–bFGF) were used to treat the wounds of the four groups, respectively. The wound recovery situation was observed and photoed on Day 0, 3, 6, and 9, shown in figure 5(a). It was found that the group treated with PBS solution exhibited the worst healing outcomes, while the group treated with CS–bFGF
showed the best curative effect. This could be attributed to the synthetical effect of the CS-bFGF. The CSMPs could not only form a physical dressing to resist secondary damage but also eliminate the bacteria from the wound to prevent infection. Furthermore, the loaded bFGF could strongly promote angiogenesis, meaning that the CS-bFGF could play roles in the whole wound healing process. In contrast, the PBS solution had no anoxoaction on wound healing. Since the CSMPs without bFGF (CSMPs) group and bFGF solution group could
only take part in only one or two stages of wound healing, the recovery situation of the two groups was situated between the PBS solution group and CS-bFGF group (figure 5(b)).

To further study the infection situation of the wounds, immunohistochemistry was employed to analyze interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). IL-6 and TNF-α are two kinds of typical proinflammatory factors distributed widely in the inflammatory sites. They play negative effects on wound regeneration. As illustrated in figure 6(a), a large amount of IL-6 and TNF-α were found on the wounds of the PBS solution group after 9 days of treatment, indicating the wounds occurred severe inflammation or infection formation. However, the wounds treated with CSMPs-bFGF displayed little positive expression of IL-6 and TNF-α, suggesting the wounds recovered well and had little inflammatory response. The relative mRNA expression of IL-6 and TNF-α in the groups also matched the immunohistochemistry results (figures 6(b) and (c)). Besides, we also investigated the collagen deposition in the wound sites. Collagen can promote the healing of wounds and the formation of tissue, so the deposited collagen in the wound sites indicates the status of tissue remodeling. The collagen of wounds was characterized using Masson’s trichrome staining. As shown in figure 6(a), the CSMPs-bFGF group exhibited the most collagen deposition, and the CSMPs and bFGF groups displayed moderate levels of collagen deposition, while the PBS group showed the least deposition. These results also indicated that the CSMPs-bFGF could play a key role in wound repair.

The deposited collagen could promote the differentiation of myofibroblasts, which are necessary for matrix formation and remodeling of wounds. Additionally, neovascularization is critical for tissue recovery and maturation. Herein, to investigate the myofibroblasts and neovascularization condition of the four groups, SpGreen and SpOrange were employed to carry out the immunofluorescence staining of CD31 and alpha-smooth muscle actin (α-SMA), which are markers of the vascular smooth muscle cells and the vascular endothelial cells, respectively, and the results were shown in figure 7. It was clear that the tissue of the CSMPs-bFGF group displayed the largest vascular area, while that of the PBS group was the smallest, and the CSMPs and bFGF groups exhibited medium levels. These results demonstrated that the drug-loaded CSMPs are the ideal dressing for wound treatment.

**Conclusion**

We have developed drug-loaded CSMPs for wound healing using the facile microfluidic electrospray technology. The CS solution was cut into droplets by the microfluidic electrospray platform and solidified with sodium hydroxide solution. After washing and drying, the porous CSMPs were obtained and could be employed for drug-loading. The in vitro experiments indicated the CSMPs had excellent biocompatibility, drug loading and release capacity, and antibacterial property, and the in vivo animal experiments displayed the synthetical functions in promoting wound healing over the multiple stages. These results indicate that the multifunctional drug-loaded CSMPs were the ideal material in wound treatment.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Declaration of conflicting interests

The authors declare that they have no competing interests.

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