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FABRICATION AND EVALUATION OF CHLORHEXIDINE GLUCONATE-INCORPORATED CHITOSAN-POLY (VINYL ALCOHOL) HYDROGEL FILMS FOR INFECTED WOUNDS

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ABSTRACT

Antibacterial dressings play an essential role in wound repair and infection control. This study aimed to fabricate chlorhexidine gluconate (CHX)-loaded chitosan-based hydrogel films via a physical cross-linking approach. Chitosan (2 %w/v) and polyvinyl alcohol (10 %w/v) (2:3 w/w) hydrogels were prepared by repeated freeze-thaw cycles. The drug loading was performed by direct incorporation of the CHX into the polymer solution during the gelation process. The content of CHX loaded in the hydrogel matrix was observed to be 4.45 mg per gram of hydrogel, which is 89.01 ± 7.05 % of the initial amount added. The interconnected and porous structure of hydrogels was achieved with a high water content and a good swelling index. The physicochemical properties, drug loading, drug release profile, and antimicrobial activity of the hydrogels were investigated. The hydrogels with excellent physical and mechanical properties were obtained. The release of CHX from the hydrogels was biphasic, with an initial rapid release followed by a gradual release that reached approximately 85 % at 24 h. Furthermore, the CHX-loaded hydrogel films displayed effective antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with an inhibition zone of 18 mm and 12 mm, respectively. Therefore, this drug-loaded chitosan-based hydrogel may be a promising antibacterial dressing for wound care.

Keywords: chitosan, hydrogel film, chlorhexidine gluconate, wound healing, poly (vinyl alcohol)

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Introduction

A wound is an injury that disrupts the integrity of the skin by disordering its anatomical structure and normal physiology. Generally, it heals within 8-12 weeks through the normal healing phases, such as homeostasis, inflammation, tissue proliferation, and tissue remodeling.[1,2] However, microbial infections could delay the repair processes in a timely and orderly manner.[3] Wound infections are one of the most serious problems that may lead to life-threatening sepsis and eventually to a patient's death.[4] Several antimicrobial agents, especially antibiotics, can be used against a wound infection; however, they may be able to be resisted by microbes.[5,6]

Chlorhexidine gluconate (CHX) was chosen as a model drug in this study because not only is it a potent antiseptic against a wide range of gram-positive and gram-negative bacteria, but it also has a beneficial effect on granulation tissue formation in wound areas.[7] The dicationic group of CHX can interact with the anionic cell wall of various microbes causing it to rupture leading to cytoplasm leakage and cell death.[8] Gauze dressing containing 0.5% CHX (Bactigras®) is a well-known marketed product with superficial antimicrobial activity for burns, lacerations, and leg ulcers. But, it can absorb very limited excess exudate and is less likely to offer a moist environment to the wound site to accelerate healing.[9] To overcome this issue, CHX-incorporated hydrogels were developed for the wound dressing application.

Hydrogel-based materials are the most suitable candidates due to their three-dimensional (3D) structure with high water content.[10] Moreover, hydrogels are capable of fluid absorption, and turntable physical and mechanical performances provide a cooling and calming effect; subsequently, the release of therapeutic agents, growth factors, and biomacromolecules can be achieved.[1,10,11] In addition, natural and synthetic polymers are used for the fabrication of hydrogels to obtain a porous structure with good biocompatibility due to the naturally derived polymers and tailored characteristics of synthetic polymers.[12,13] Natural polymers, e.g., chitosan (CS), play a very important role in the healing process since they possess marvelous beneficial properties such as swelling ability, elasticity, biocompatibility, non-toxicity, hemostatic, and wound contraction.[14] Polyvinyl alcohol (PVA) is a synthetic biocompatible polymer that has been employed as the main component of several hydrogel dressings, owing to its relatively high mechanical strength, water retention, and good transparency.[12,15] Also, the hydroxyl group of the alcohol moiety makes PVA a suitable polymer to be used to crosslink in the freeze-thawing (F-T) hydrogel preparation method.[16] Therefore, this work intended to fabricate CHX-loaded CS/PVA hydrogels using the F-T method and investigate the feasibility of loading CHX to provide a simple and effective dressing for infected wounds. To exemplify the knowledge of hydrogels of CS/PVA, the F-T method was used for the preparation of the hydrogel to find an optimal polymer composition for wound application. Whereas, CHX, an antimicrobial agent, was loaded and the antibacterial effect was investigated to demonstrate the efficacy of the prepared hydrogel using the optimal polymer composition.

Materials and Methods

Materials

CS (low molecular weight, ≥75% deacetylated), PVA (MW. 60 kDa with a degree of hydrolysis ≥98%), and CHX (20 %w/v) were procured from Sigma-Aldrich® (St. Louis, MO, USA). Acetic acid and other chemicals were of analytical reagent grades.

Preparation of CS-based hydrogels loaded with CHX

CS (2 %w/v) was dissolved in acetic acid solution (1 %v/v) by continuous magnetic stirring overnight. PVA solution (10 %w/w) was prepared by dissolving PVA in hot water at 80°C and stirred for 1 h. Then, CS and PVA solutions were mixed at varied weight ratios which were 3:2, 2:1, 1:1, 1:2, and 2:3

(Table 1) for 1 h under slow magnetic stirring to form a homogeneous mixture and to avoid air bubbles. Then, the 30 g of mixture solutions were gently poured into each dish mold (90 mm × 15 mm) and placed at –20°C for 18 h, and subsequently placed at 25°C for 6 h.[17] This freeze-thaw process was repeated for six cycles. CHX equivalent to 0.5 %w/v was incorporated into the most suitable hydrogel after the physical and mechanical characterizations by mixing the drug with the polymer solution during hydrogel preparation.

Characterizations of the hydrogels

1. Scanning electron microscope (SEM)

The structural morphology of hydrogel films was observed using an SEM (Camscan Mx2000, England). To make conductive hydrogels, the hydrogel samples were freeze-dried and cut into sections transversely before being coated with a thin layer of gold before SEM investigations. Images were captured using a 10.0kV acceleration voltage at 200× magnification.

2. Water content

Hydrogel samples with the size of 1.5 × 1.5 cm^2 were weighed (W_i) and dried in a hot air oven at 60°C until a constant weight was achieved (W_d). The amount of water in the hydrogels was calculated from the weight difference between the initial and the dried weight according to equation (1).[18]

$$Water\ content\,(\%) = \frac{Wi - Wd}{Wi} \times 100 \quad \text{.....(1)}$$

3. Swelling index

The fluid uptake capability of hydrogels was determined by the ability of the hydrogel to swell upon contact with water. Briefly, the hydrogel films were cut into 1.5 × 1.5 cm^2 pieces and heated at 60°C using a laboratory universal oven (Model: XU032, France-Etuves Asia Co., Ltd. Guangdong, China) until the weight was constant (W_d). Then, the films were soaked in 10 mL of phosphate buffer solution (PBS, pH 7.4) at 37°C. At specific time intervals up to 24 h, the swollen hydrogels were taken from the solution, and the excess liquid on the surface of the hydrogels was wiped off using filter papers before the hydrogels were weighed (W_s). Using the following equation (2), the percent swelling index of the hydrogels was calculated.[19]

$$Swelling index\,(\%) = \frac{Ws - Wd}{Wd} \times 100 \quad \text{.....(2)}$$

4. Gel fraction

The percentage of gel fraction shows the crosslink strength of the hydrogel matrices. The hydrogel samples (1.5 × 1.5 cm^2) were dried to a constant weight (W_d). The dried samples were soaked in PBS (pH 7.4) at room temperature for 24 h. Then, they were removed from the medium and dried at 60°C using a laboratory universal oven (Model: XU032, France-Etuves Asia Co., Ltd. Guangdong, China) until a constant weight (W_t) was reached. The gel fraction was computed using the following equation (3).[18]

$$Gel\ fraction\,(\%) = \frac{Wt}{Wd} \times 100 \quad \text{.....(3)}$$

5. Mechanical properties

The mechanical strength of the hydrogels was measured using a texture analyzer (TA. XT plus, Stable Micro Systems, UK). In brief, the hydrogel films (1.5 × 1.5 cm^2) were pressed by a 5-mm stainless steel ball probe attached to a texture analyzer. The ball probe was moved down at a speed of 5 mm/s and compressed on the hydrogels until the sample was torn. The force causing the hydrogels to break was then noted.[20]

6. Determination of drug content in the hydrogel films

Using the HPLC analysis, the total amounts of CHX in the hydrogel films were measured. Briefly, the hydrogel samples (1.5 × 1.5 cm^2) were accurately weighed, smashed, and placed in 10 mL of deionized water. The hydrogel fragments were constantly shaken at 120 rpm for 24 h using a GFL Shaking Incubator (Model: GFL 3031, ProfiLab24 GmbH, Berlin, Germany) to extract CHX from the hydrogels. Then, HPLC analysis was carried out using an Agilent Infinity Series 1260 HPLC (Agilent Technologies, Santa Clara, USA) with the detection at the wavelength 241

nm.[21] A C18 column (4.6 mm × 250 mm, 5 µm) was used as a stationary phase, while the mobile phase was the solvent mixture of acetonitrile and 1 % phosphoric acid (60:40, v/v) with a flow rate of 1 mL/min. The drug content was calculated as loading capacity (LC) and %recovery using equations (4) and (5), respectively.

$$LC = \frac{wt\ of\ drug\ loaded\ into\ the\ hydrogel}{total\ wt\ of\ the\ drug\text{-}loaded\ hydrogel} \quad \text{.....(4)}$$

$$\%Recovery = \frac{wt\ of\ drug\ loaded\ into\ the\ hydrogel}{initialwt\ of\ drugadded\ intothe\ hydrogel} \times 100 \quad \text{.....(5)}$$

7. *In vitro* drug release study

The release profile of CHX from the hydrogels was assessed by a vertical Franz diffusion cell system through a dialysis membrane (MWCO 6000-8000) with a slight modification of the previously described method.[22] The sample (1.5 × 1.5 cm^2) was weighted and placed in the donor compartment of the Franz cell and the receptor compartment was filled with PBS (pH 7.4). The temperature of the receiver chamber was maintained at 37°C by a circulating water jacket, with constant agitation of the release medium. At each time interval of 5, 10, 15, 30, 60, 120, 240, 480, 720, and 1440 min, 200-µL of the receptor medium was removed and replaced immediately with the same quantity of new medium to maintain the volume and sink condition. The amount of CHX released from the hydrogels was then analyzed by HPLC using the method mentioned above.

8. *In vitro* antibacterial study

The antibacterial activities of the prepared CHX-loaded hydrogels were determined by the disc diffusion method.[23] Gram-negative and gram-positive bacteria (*E. coli* and *S. aureus*) were used to evaluate the antimicrobial efficiency of the hydrogel dressings. At first, the bacterial strains were sub-cultured in tryptic soy broth (TSB) for 24 h at 37°C. Next, the bacterial cultures were diluted with TSB medium to make the optical density (OD) at 600 nm of 0.1 to obtain 10^8 CFU/mL of the bacterial suspension. Then, 100 µL of the bacterial suspension was spread on tryptic soy agar (TSA) plates using sterile cotton swabs. After that, a commercial sample and the sterile hydrogel samples with a diameter of 5 mm (sterilized under UV irradiation for 30 minutes on each side) were placed on the agar plates. The plates were kept at 37°C for 24 h before the diameters of the inhibition zone were determined. A blank hydrogel and a commercial antimicrobial dressing (Bactigras®) were used as a negative and positive control, respectively. The positive control was used to address whether the developed hydrogel film was able to show a comparable antibacterial effect per infected area.

Statistical analysis

All the experiments were carried out in triplicate. All the results were shown as mean ± standard deviation (SD). To identify significant differences between means comparisons, a one-way analysis of variance (ANOVA) was used which was computed using Microsoft® Excel for Mac 2021. The significance level of differences was determined at $p < 0.05$.

Results and Discussion

Preparation and characterizations of the hydrogels

The physical properties of blank hydrogels and CHX-loaded hydrogels were investigated, and the findings are listed in Table 1. The gel fractions of hydrogel samples were analyzed to determine the crosslink strength of hydrogel films. Increasing the PVA content to reach 60% (CS:PVA = 2:3) resulted in the highest gel fraction (about 90%). Further increase of PVA to more than 60 % did not improve the gel fraction (data not shown). Exudate absorbency and a moist environment are critical factors to heal wounds which were demonstrated using swelling index and water content analysis, respectively. The excellent swelling performance of the blank hydrogels ranged from 315 ± 15.42 % to 432 ± 10.1 %. Water contents of the hydrogels were discovered to be above 70 %, which could provide a proper moist environment for the wound area.

The hydrogels were made of PVA and CS which can form hydrogen bonds between the hydroxyl (–OH) groups of their core structures upon freezing and thawing. The F-T gelation process forms a crystalline zone in their microstructure which acts as the crosslinking points in the hydrogel network. Therefore, the number of F-T cycles would increase the degree of crystallinity and gel fraction, but the swelling capacity of the hydrogel may decrease. Crosslinked hydrogel matrices were obtained after repeated F-T cycles. The crosslinking degree of the polymer network is determined by the swelling capacity and gel fraction.[24] High water absorbency of CS/PVA films was observed suggesting the formation of strong intermolecular hydrogen bonds between the amino groups of CS and hydroxyl groups of PVA. However, the swelling index is inversely related to the degree of crosslink. The strength of hydrogel was improved by the level of crosslink. As evident, the increase of PVA content decreases the swell ability but increases the mechanical strength of the hydrogels. Higher CS composition resulted in higher water content and water absorption but lower toughness.[25]

The CS/PVA (2:3) hydrogel was selected for drug incorporation because this ratio provided the highest gel fraction with desirable water content and swelling capability compared to other hydrogel formulations. The water content, swelling index, and gel fraction of the drug-loaded hydrogels were comparable to the blank hydrogels. With the addition of CHX in the hydrogel structure, the gel fraction was found to be 92 ± 1.4 %. There was no statistically significant difference compared to the value of plain hydrogels which was 90 ± 1.9 %. In addition, a compression test was conducted to assess the toughness of the two hydrogel films under compression to assure the applicability of the optimal hydrogels. The hydrogel film was subjected to a compressive force until it was torn apart. The forces required to break the blank hydrogels and the CHX-loaded hydrogels were 1.76 ± 0.08 and 1.21 ± 0.09 N, respectively. A significant reduction ($p < 0.05$) in the hardness of CHX-loaded hydrogels compared to the blank hydrogels was observed which may have resulted from the additive of CHX solution added to the hydrogel, presumably glycerin that acts as a plasticizer.[26] This indicated that the CHX-loaded hydrogels had more flexibility to endure surrounding tissue and enhance their performance during the wound healing process.

Table 1 Equilibrium water content, swelling index, gel fraction, and toughness for CS/PVA hydrogels with and without the drug.

Sample (2% CS : 10% PVA)	Water content (%)	Swelling index (%)	Gel fraction (%)	Toughness (N/mm^2)
CS/PVA hydrogels 60:40 (3:2)	70 ± 0.5*	432 ± 10.1*	70 ± 0.0*	-
CS/PVA hydrogels 60:40 (2:1)	91 ± 0.4*	339 ± 4.6*	83 ± 1.3*	-
CS/PVA hydrogels 50:50 (1:1)	86 ± 0.2*	521 ± 5.5*	67 ± 0.2*	-
CS/PVA hydrogels 60:40 (1:2)	87 ± 0.2*	227 ± 0.4*	84 ± 2.7*	-
CS/PVA hydrogels 40:60 (2:3)	89 ± 0.3	315 ± 15.4	90 ± 1.9	1.76 ± 0.1
CHX-loaded hydrogels 40:60 (2:3)	87 ± 1.0	327 ± 6.2	92 ± 1.4	1.21 ± 0.1*

* Significant difference from CS/PVA hydrogels 40:60 (2:3) ($p < 0.05$)

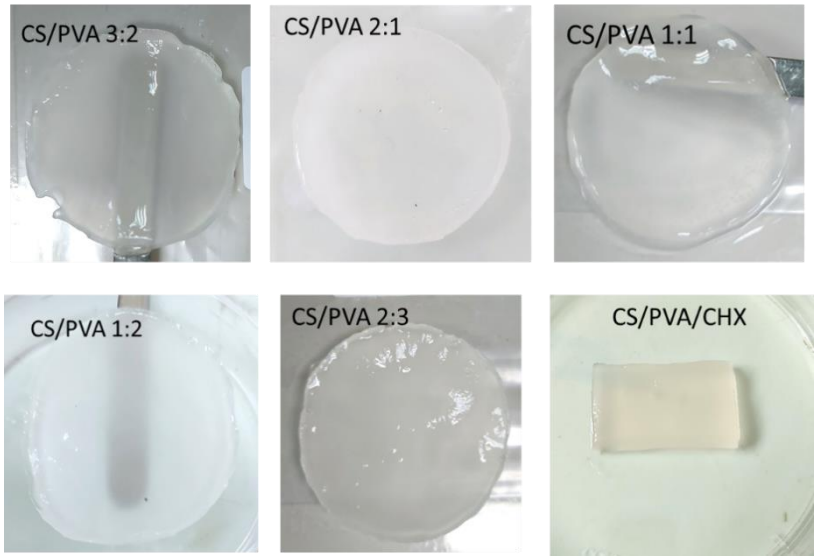

Figure 1 Images of the hydrogel film prepared using different polymer compositions.

Morphology of the hydrogel films

The morphology of the blank hydrogels and the CHX-loaded hydrogels was studied using SEM. The hydrogels with and without the drug had an interconnected 3D network structure. Figure 1 shows a highly porous structure of the hydrogels which favors the release of the drug. Adding CHX to the hydrogels resulted in a larger pore size (Figure 2b) compared with blank hydrogels (Figure 2a). The high porosity of the hydrogels provides a large volume for the absorption of exudates from the wound surface. Also, it supports the distribution of nutrients and the transmission of oxygen to the wound site to enhance healing. The difference in the pore size of the blank and CHX-loaded hydrogels could be due to the composition of the CHX used. CHX solution (20 %w/v) was incorporated into the polymer mixture for hydrogel preparation; whereas, the solution was composed of ethanol, PEG-esters, glycerin, and other additives. These excipients may interfere with or alter the interaction among the polymer chains at the microscopic level leading to different morphology under SEM.[27] Although, the pore size between the blank and CHX-loaded hydrogels was different. The change did not affect the swelling property of the hydrogels as previously mentioned. The finding was in concordance with Kapanya et. al., (2020) that reported insignificant differences between different concentrations of CHX in PVA hydrogels.[28]

Drug Content

The assayed content of CHX in the hydrogels was calculated as the LC which was 4.45 mg/g of the hydrogels with the %recovery of 89.01 ± 7.05 % as calculated from the initial amount of the drug added. A partial loss of CHX may be due to the loss of content during the hydrogel preparation. Furthermore, the incomplete crosslink of the hydrogels (about 90 % crosslink) may also be the cause of the drug loss.

Drug release

The cumulative drug release was studied in the PBS pH 7.4 as the wound pH is mildly basic during infections.[29] As shown in Figure 3, a rapid release was observed in the initial period and the release was gradual after 1 h. The release reached approximately 85 % at 24 h for some of the active compounds to be strained in the crosslinked polymer network. A sufficient concentration of CHX must be maintained in the wound area to achieve the antimicrobial effect. Thus, the amount of CHX released from the hydrogels was satisfactory to ensure the antibacterial effect. An initial burst release will provide a rapid

action and it should be complemented by a sustained release of CHX to ensure the desired MIC values (0.625 µg/mL for *S. aureus*) in the wound area.[30] Koburger (2010), reported that immediate effect and prolonged contact time of CHX are needed for the treatment of infections.[31]

Antibacterial activities

The antimicrobial activities and enhanced wound healing processes are of great concern to achieving an ideal wound dressing. Here, the antibacterial activities of hydrogels were explored against *E. coli* and *S. aureus,* which are the most common microorganisms found in infected wounds. The results revealed that the CHX-loaded hydrogels effectively inhibited both *E. coli* and *S. aureus* with the inhibition zone of 12 mm and 18 mm, respectively, as shown in Figure 4(a) and Figure 4(b). The antibacterial effect on gram-positive bacteria was better than the gram-negative bacteria due to the cell wall disruption differences as formerly discussed by the literature. Also, the satisfactory antibacterial effect may be due to the hydrophilic nature of the hydrogels along with high porosity which facilitates the release of the drug. The inhibition zone diameter of CHX-loaded hydrogels was twice of the positive control (Bactigras®). Although the clear zone diameter of the proposed

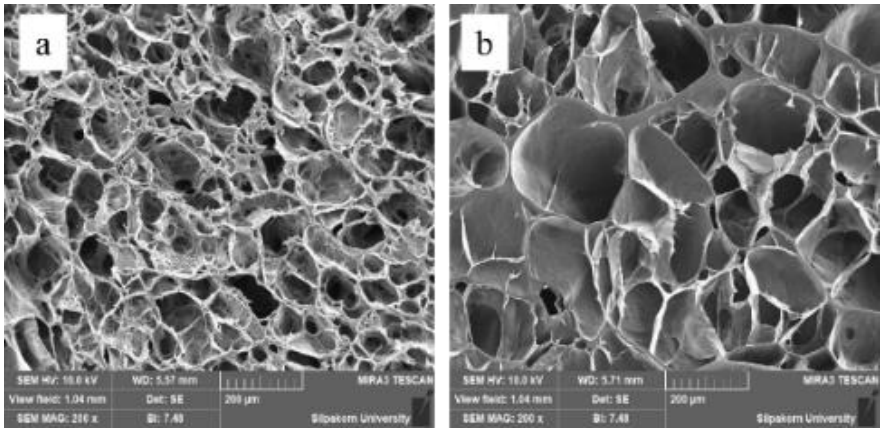

Figure 2 SEM images (200× magnification) of CS/PVA 2:3 hydrogels (a) blank hydrogel, (b) CHX-loaded hydrogel.

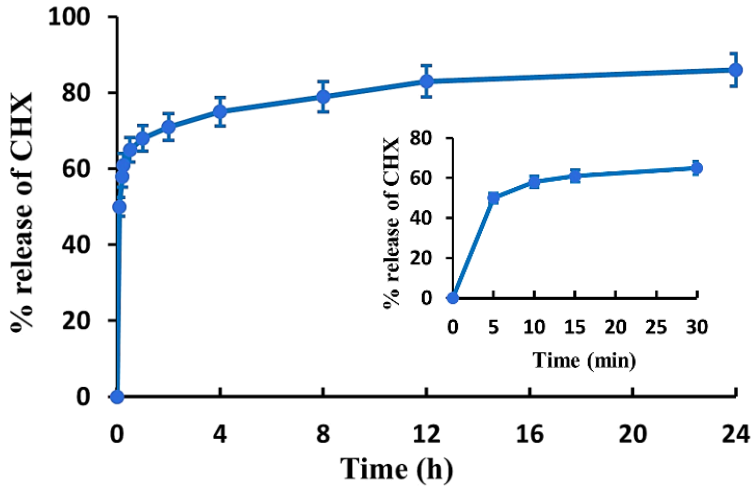

Figure 3 The release profile of CHX from the CS/PVA hydrogels.

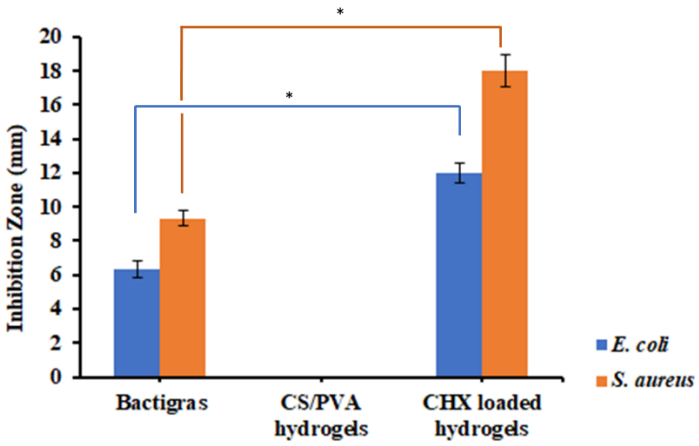

Figure 4 (a) Average diameter (mm) of inhibition zones against *E. coli,* and *S. aureus* of Bactigras®, blank CS/PVA hydrogels, and CHX-loaded hydrogels. (* Significant difference, $p < 0.05$) and (b) the pictures of the clear zone obtained by treating Bactigras® (top), blank hydrogel (left), and CHX-loaded hydrogel (right) on *S. aureus* and *E. coli*.

formulation and the commercial formulation cannot be precisely compared for the salt form and the amount of drug in the patch might be different, CHX-loaded hydrogel films showed to be a promising alternative to the commercial product for showing good antibacterial effect considered per infected area. Thus, the developed CHX-loaded hydrogel patch could be beneficial in wound care, especially infected wounds, for showing an enhanced antibacterial effect compared to the commercially available product.

Conclusion

In this work, CS/PVA hydrogels were prepared and the optimal ratio of the two components were the weight ratio of 2:3. The CHX-loaded wound dressing was further developed with the intention to treat wound infections. Highly porous hydrogels were received and desirable swelling performance was achieved. CHX was successfully incorporated into the selected CS/PVA hydrogel structure with an acceptable drug content. CHX-loaded hydrogel films kept high water content and improved swelling index without affecting cross-linking between polymer chains. In addition, they were presented with good flexibility to apply to the wounds. A biphasic release of CHX release from the hydrogels was observed. Moreover, a strong antibacterial activity against common wound-induced bacteria; *S. aureus* and *E. coli* were exhibited compared with a commercial antibacterial patch. Overall, a potential dressing loaded with CHX antimicrobial agents was revealed with desirable performances and the findings could further be developed and investigated to be used as a future candidate for the treatment of an infected wound.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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