Research Article

Evaluation of New Film Based on Chitosan/Gold Nanocomposites on Antibacterial Property and Wound-Healing Efficacy

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Received 8 May 2020; Revised 4 July 2020; Accepted 14 July 2020; Published 29 July 2020

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Chitosan (CS) is a natural polymer with applications in wound restorations and coatings for artificial implants. However, the lack of antimicrobial activity restricts its further application. Thus, the purpose of this study is to prepare CS/AuNPs film by adding gold nanoparticles (AuNPs) to modify chitosan. Through transmission electron microscopy, we found that the presence of CS can effectively prevent the agglomeration of nanoparticles. Furthermore, through the CCK experiment, we found that AuNPs at a certain concentration can improve the adhesion and proliferation of cells, which has also been proved in animal experiments, including gross appearance, histological observations, and immunofluorescence staining. Such findings support the feasibility of using the film as a promising candidate for tissue engineering of skin in a near future.

1. Introduction

The skin is the largest organ of the human body and plays an important role in protecting the body from external injuries. However, it is also the most vulnerable, which usually occurs in trauma, burns, and certain specific endocrine diseases, such as diabetes. Patients with full-thickness skin damage not only have to undergo physical and mental torture but also bear huge economic pressure. When the integrity of the skin is destroyed, there are distinct but closely related physiological repair processes, including coagulation, inflammation, hyperplasia, and tissue remodeling [1]. Despite the self-healing nature of the skin, anti-infective treatment is essential for full-thickness wounds. With the rapid development of tissue engineering, many breakthroughs have been made in wound dressings, providing new possibilities for solving skin defects. According to varieties of literatures, one innovative approach is to combine polymer matrix with inorganic nanoparticles to form the inorganic/organic hybrid system, which possess superior chemical and physical properties than those of the parent polymer or inorganic species [2, 3].

The selection of proper biopolymer matrix should be the prime consideration in preparation of wound dressings [4, 5]. Moreover, the ideal wound dressing should have good biocompatibility, hydrophilicity, mechanical strength, and appropriate antibacterial property to promote cell adhesion, proliferation, and differentiation [6, 7]. Chitosan (CS), as a widely used natural polymer, has obtained great attention in skin tissue engineering [8]. It can promote the proliferation and differentiation of a broad spectrum of cells associated with wound healing, including keratinocytes, vascular endothelial cells, and fibroblasts. Furthermore, it has been reported that CS can accelerate wound healing in both animal models and clinical studies [9, 10]. Compared with other types of natural polymers, positively charged CS has a higher affinity for cells and other growth factors, which is likely due to the charge-charge interactions. Moreover, CS can be directly used to synthesize a great variety of metal nanoparticles as a reducing and stabilizing agent [8]. However, despite its advantages, many studies have shown that the poor mechanical strength of CS has a negative impact on the support and protection of wound surface in...
these problems. Despite the specific mechanism of this antimicrobial activity not being yet fully understood, several generally accepted hypothesis include surface charge, inhibition of mRNA, and blocking the oxygen path. However, this antibacterial activity is limited by a number of factors, especially environmental PH. Chitosan can only play an antibacterial role in the acidic medium, since it possesses poor solubility at high pH; in addition, the PH value of the film should be neutral in the preparation process. Therefore, interesting phenomena can be found in many studies that chitosan solution has antibacterial property while chitosan film does not [13]. Fortunately, modification with the incorporation of antibacterial material can effectively solve these problems.

The application of metal nanomaterials has attracted much attention and provided an innovative approach for the repair of skin defects in recent years following the rapid development of nanotechnology [14]. With the small size and large surface area, nanoparticles can be desirable delivery vehicles. In addition, they carry intrinsic properties beneficial for wound healing, including excellent antioxidant and antimicrobial activities as well as anti-inflammatory and antiangiogenic activities [15, 16].

Bacterial infection is a common problem following skin wounds, which could lengthen inflammation, disturb epithelialization, and finally delay wound healing. Unfortunately, in clinical practice, misuse of antibiotics leads to the risk of emergence of resistant bacteria antibiotics. In order to solve this problem, numerous nanomaterials were incorporated to aliphatic polyesters to form antimicrobial nanocomposites [17, 18]. It has been reported that metal nanoparticles can destroy bacterial cells by releasing metal ions, which are not affected by the matrix of polymers. Accordingly, metal nanoparticles can optimize the bacteriostasis of CS matrix. Among various metal nanoparticles, cytotoxicity of AuNPs accounts to the significantly low extent compared with that of other nanoparticles like Ag, Cu, and ZnO [19]. However, the antibacterial mechanism of gold nanoparticles is not yet clear. But, controversy revolves around whether it is a direct contact or the release of gold ions. For a direct contact-killing mechanism, AuNPs adhere to the surface of bacterial cells by electrostatic interaction in the form of metallic gold which causes impaired cell membrane integrity, leakage of cell contents, and production of reactive oxygen species (ROS) [20]. For the gold-ion mediated mechanism, gold ions released tend to bind to thiol (sulfhydryl) groups of cellular lipoproteins to interfere with the respiratory chain and generate ROS. Therefore, the incorporation of AuNPs into CS matrix can optimally reduce the bacterial infection in wounds [21]. Moreover, spherical AuNPs of size 20–40 nm inhibit IL-6, IL-12, and TNF-a to a level to exert potent anti-inflammatory properties. Furthermore, studies have shown that AuNPs promote angiogenesis and alter collagen deposition within the extracellular matrix (ECM), which is extremely advantageous for the promotion of wound repair. Endogenous collagen matrix can support tissue regeneration through neovascularization, leading to more fibroblastic cell repopulation [22, 23].

However, AuNPs demonstrated a dose-dependent cytotoxicity, especially the smaller ones (<2 nm). Fortunately, the cytotoxicity can be significantly reduced when the AuNPs were coated with CS. The particle size of AuNPs increases under the coating of chitosan to avoid the absorption of cells, thus reducing the cytotoxicity to a certain extent [24]. In addition, colloidal gold nanoparticles tend to cluster together in the dispersions when they are being reduced, leading to a significant decrease in their bactericidal efficiency. Fortunately, the use of CS for stabilizing gold nanoparticles has gained attention recently, thus preventing AuNPs from agglomeration.

In this article, we will prepare CS/AuNPs film by blending gold colloid with CS solutions. Meanwhile, CS acts as a stabilizing agent in such processes. Measurement (TEM) was performed to observe the size distribution of AuNPs when CS is present. Also, the composite was investigated for its cytotoxic and antimicrobial activities. Furthermore, we assessed the positive effects of CS/AuNP composite on wound healing in a rat model of full-thickness skin defects.

### 2. Materials and Methods

#### 2.1. Materials

- Chitosan (deacetylation ≥95%), L-arginine, sodium citrate, and HAuCl₄·3H₂O (Au ≥47.5%, medical grade) were purchased from Aladdin, Shanghai, China; NaOH and acetic acid from Sigma-Aldrich, St. Louis, MO, USA; Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum from Life Technologies, Carlsbad, CA, USA; trypsin from Solarbio, Beijing, China; hematoxylin and eosin (H&E) and Masson trichrome staining kits from Solarbio, Beijing, China; Balb/c3T3 cells was from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

#### 2.2. Preparation of CS/AuNPs

Briefly, 1% (w/v) solution of CS was dissolved in 1% (v/v) acid acetic under stirring continuously overnight and centrifuged until most of the CS was fully dissolved. The deacetylation degree is really associated with antimicrobial properties, and a 30–40% degree of acetylation produced the highest antibacterial activity against S. aureus and E. coli. However, in the preparation of the film, we found that too low degree of acetyl would significantly reduce the mechanical strength of the film, thus affecting its practical application. Thus, the deacetylation (≥95%) was chosen. In addition, the chitosan was used medical grade after consulting relevant literatures. The HAuCl₄·3H₂O at the same molar as CS was added into the CS solution under magnetic stirring for half an hour. Then, the mixture solution was reduced by addition of sodium borohydride solution (0.5 ml and 0.1 M) in a dropwise fashion, and the stirring was continued until a transparent wine-red solution was obtained. The resulting solution was retained for preparing the CS/AuNP film.

#### 2.3. Production of Films

The CS/AuNP solution from above and CS solution were evenly casts on the well, respectively, and were cross-linked with sodium hydroxide (NaOH) for
10 hours. 5% of NaOH solution was used to produce films. In practice, we found that the concentration of NaOH and the soaking time directly influence the physicochemical property of the film; high concentration and long soaking time can easily lead to film biological performance degradation, while low concentration and short soak time are easy to cause the film apart, so we choose the concentration of 5% and 10 hours. Next, films were washed with PBS after immersion in NaOH to reduce the pH. Prior to their use, the films were sterilized under UV overnight and placed into culture plates under aseptic conditions.

2.4. Characterization of CS/AuNPs. The morphology and structure of the AuNPs were observed by a JEOL-2000EX transmission electron microscope (TEM) operating at 200 kV. For TEM measurements, the sample solution was dropped on carbon-coated copper grids and deposited in a vacuum at room temperature before taking images. Furthermore, the size distribution was obtained by measuring the size of 100 individual nanoparticles using a statistical software (Image J).

2.5. Antibacterial Testing. Antimicrobial potential of the composite films was verified against Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli). Previous literature has shown that the higher the concentration of gold nanoparticles, the stronger the antimicrobial activity. Therefore, the experiment was conducted in four groups: CS, CS/AuNPs 1 (2 mg/mL), CS/AuNPs 2 (5 mg/mL), and CS/AuNPs 3 (10 mg/mL). For preparation of the inoculums, both strains were incubated at 37°C in Luria-Bertani (LB) broth for 12 hours. Then, prepared sterilized nutrient agar was spread evenly in Petri dishes.

After coagulation, the abovementioned bacterial suspension (0.3 mL) was added to culture media. Meantime, the sterilized circular material with a diameter of 20 mm (CS and CS/AuNPs) was placed on the dishes covered with bacteria for 12 hours at 37°C. After that, the surrounding bacteriostatic zone of the material was photographed and analyzed to evaluate the antibacterial activities of composite films.

Next, the antibacterial efficiency was analyzed in a quantitative manner by defining the absorbance reading of optical density. Both materials were placed in 2 mL of broth medium containing S. aureus and E. coli and cultured for 6 hours. Meanwhile, a blank control group was set. After that, 0.1 ml drops were sampled from each group in a 96-well plate, and the optical density at 600 nm was measured using a microplate reader.

2.6. Cell Proliferation Assay. In the cell experiment of this study, we aimed to explore the influence of the concentration of AuNPs on the cell survival rate and seek the most appropriate concentration range. In addition, we referred to similar relevant literature before constructing the experiment [25, 26]. CCK-8 assay was used to evaluate the effects of CS-AuNP composites on the survival and adhesion of Balb/c3T3 cells. According to related studies, although AuNPs is generally less toxic than other metal nanoparticles, it still possesses relatively high cytotoxicity with dose dependency. Therefore, The experiment was conducted in four groups: CS, CS/AuNPs 1 (2 mg/mL), CS/AuNPs 2 (5 mg/mL), and CS/AuNPs 3 (10 mg/mL) to determine the most appropriate concentration. After disinfection of surfaces, the materials were placed in 96-well plate. Balb/c3T3 cells were seeded on the material surfaces (10,000 cells/well) in the 96-well plate with DMEM supplemented with 10% FBS and incubated at 37°C for 3 days. During which time, the media were replaced every day. The rate of cell proliferation of each group was measured by CCK-8 assay at 1, 2, and 3 days of culture.

2.7. Animal Experiment. In total, 10 female Sprague Dawley rats (body weight about 300 g) were used in the study (5 rats in the CS group and 5 rats in the CS/AuNPs group). All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Jilin University and US National Institutes of Health guidelines. Three days before the experiments, the hair on the back were shaved followed by the application of hair removal cream. As in experiments above, films were disinfected before use. After placing them in a prone position, the back skin of anesthetized rats was disinfected with iodophor and then deiodized with 75% alcohol. Full-thickness skin defects were then made and photographed according to a plastic model (diameter 10 mm). After that, defects were covered with the corresponding film, which was changed every other day. The rats were individually fed and photographed every three days. Last, the wound healing rates in the two groups were measured and calculated using image analysis software (Image J). Wound-healing rate was calculated according to the following formula: wound healing rate = (S_t − S_0)/S_0 × 100%, where S_0 indicates the initial area of the wound and S_t indicates the residual wound area at the time of measurement.

2.8. Histopathologic Staining. For pathology evaluation, all rats were sacrificed under deep anesthesia on the day 12 after wounding. The skin samples containing the wound and surrounding skin were completely harvested and equally divided into two parts, one for histopathological staining and the other for immunofluorescence staining.

Specimens for histopathological staining were immersion fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration, embedding, and slicing (5 μm). After that, hematoxylin and eosin (H&E) and Masson staining were performed and observed under an optical microscope.

2.9. Tissue Immunofluorescence. Another part of the fresh tissue used for fluorescent staining was treated with frozen sections using a cryotome. After antigen retrieval and blocking, the sections were added with COL-1 (1:1000) and CD31 (1:1000) primary antibodies in a wet box at 4°C overnight.
Next, the corresponding secondary antibody was incubated at room temperature for two hours. After cleaning of PBST three times, the addition of DAPI, COL-1, and CD31 was observed and photographed under a fluorescence microscope and finally quantitatively analyzed by image processing software.

2.10. Statistical Analyses. The results obtained were subjected to a one-way analysis of variance using Origin 8.0 software. Data were presented as the mean ± standard deviation (SD). A P value < 0.05 was regarded as statistically significant.

3. Results

A flowchart of preparation process and main experimental design is shown in Figure 1.

3.1. Characterization of CS/AuNPs. In this study, we prepared a film composed of CS/AuNPs. Geometry, including shape and size, can mediate AuNP-cell interactions. Therefore, the cytotoxicity of AuNPs could be affected by the geometry. AuNPs possess size-dependent toxicity, and the Au NPs (D core <10 nm) exhibit higher cytotoxicity than that of large AuNPs (D core >15 nm). Other physical parameters include shape and dispersing state. The shapes of gold include gold nanocluster, nanorods, nanohexapods, and nanocages. Among which, the nanorods exhibit highest cytotoxicity while nanohexapods have negligible cell toxicity at relatively high concentration. In addition, the aggregation of Au NPs can cause significant cytotoxicity. For a better understanding of particle size distribution of the AuNPs, we used TEM. According to previous researches, the adjacent nanoparticles easily contact each other and form aggregations, which can induce a great cytotoxic effect, especially the larger ones [27]. Additionally, CS coating can prevent the agglomeration of particles by charge repulsion effect. As illustrated in Figure 2, it is evident that the AuNPs were uniformly distributed in the CS matrix without agglomeration. We next evaluated the particle size distribution of carriers, the peak of which lay in size from 6 to 10 nm. This size distribution was proved to be safe and devoid of toxicity in many literatures [20]. Moreover, the contacting surfaces became rough after the addition of AuNPs; the roughness has a positive effect on protein adsorption and cell adhesion.

3.2. Antibacterial Testing. In the process of wound healing, due to the loss of natural protective barrier, it is vulnerable to microbial invasion and infection, thus delaying the healing. Therefore, the excellent antibacterial property is essential for a qualified wound dressing. In fact, there are different opinions about the antibacterial properties of CS. Some studies believe that chitosan itself has no antibacterial property, while others believe that CS, as a positively-charged polymer, can destroy the cell membrane of bacteria by means of charge effect, thus exerting antibacterial property [28]. In conclusion, the antimicrobial properties of CS need to be optimized by modification. The antimicrobial potential of gold was first explored and verified by Robert Koch [29]. The property can be attributed to its unique chemical and structural features. The AuNPs is almost 250 times lesser than bacteria, making it easy to stick to the cell wall and disrupt their physiological processes leading to cellular death. Moreover, while killing bacteria, AuNPs yield ROS. These free radicals can destroy DNA and other metabolic components [30].

The antibacterial properties of the modified CS were evaluated by the inhibition zone method and optical density values against S. aureus and E. coli. As shown in Figure 3, the zone of inhibition of CS was significantly improved by the addition of AuNPs. Furthermore, the inhibition rate of composites against S. aureus is lower than that against E. coli, which is consistent with previous studies. Next, we quantitatively analyzed the antibacterial properties of the material by the change of absorbance value (Figure 4). Among results, lower absorbance was found in the CS/AuNPs group. At the same time, the lowest turbidity was observed in the CS/AuNPs group. Therefore, the antibacterial property of CS was significantly improved after the modification of gold nanoparticles.

3.3. Cell Proliferation Assay. Balb/c3T3 cells have been widely used to study epidermal defects for its unique function, including proliferation and differentiation in the healing wound and filling the wound. Fibroblasts secrete a large amount of collagen and cytokines to synthesize the extracellular matrix and promote the synthesis of new blood vessels, thus achieving wound healing [31]. Figure 5 shows the effects of different concentrations of AuNPs on cell viability. On the first day, there were no significant differences between the groups, whereas after 2 days, the survival rate was lowest in CS/AuNPs (10 mg/mL) (P < 0.05). Three days later, although the survival rate of CS/AuNPs (5 mg/mL) was lower than that of CS/AuNPs (10 mg/mL), the number of surviving cells in both CS/AuNPs (5 mg/mL) and CS/AuNPs (10 mg/mL) was higher than that of the pure CS group. On the one hand, AuNPs promote the expression of cell-related growth factors, including VEGF and COL-1. [32, 33], thus promoting the synthesis of extracellular matrix, which in turn provides a more suitable environment for cells. On the other hand, from the perspective of material structure, rough contact area is also conducive to cell adhesion and proliferation.
Figure 2: (a) TEM of CS/AuNPs. (b) Size distribution of CS/AuNPs.

Figure 3: Inhibition zone of CS, CS/AuNPs 1 (2 mg/mL), CS/AuNPs 2 (5 mg/mL), and CS/AuNPs 3 (10 mg/mL) against E. coli (a) and S. aureus (b).

Figure 4: Average OD at 600 nm (OD600) of CS, CS/AuNPs 1 (2 mg/mL), CS/AuNPs 2 (5 mg/mL), and CS/AuNPs 3 (10 mg/mL) against S. aureus (a) and E. coli (b). Error bars represent the mean ± SD (n = 3; * statistically significant difference, *P < 0.05).
Figure 5: CCK-8 assays of Balb/c3T3 cells cultured on CS, CS/AuNPs 1 (2 mg/mL), CS/AuNPs 2 (5 mg/mL), and CS/AuNPs 3 (10 mg/mL). Error bars represent the mean ± SD (n = 3; * statistically significant difference, * P < 0.05).

Figure 6: The differential effects of composites on wound healing. (a) Representative macroscopic appearance of wounds; CS (a1), CS/AuNPs (a2); (b) wound-healing curves. * Error bars represent the mean ± SD (n = 3; * statistically significant difference, * P < 0.05).
Figure 7: Differential effects of composites on histologic features of wounds at 12 days. (a) H&E staining; (b) MT staining; CS (1); CS/AuNPs (2).

Figure 8: Immunohistochemical staining of COL-1 (2) and CD31 (3) in the wound-healing region at 12 days after different treatments. CS (a); CS/AuNPs (b).
3.4. Animal Experiment. Next, we further explored the safety and efficacy of the CS/AuNPs complex film in animal experiments. In this study, a standard-sized (diameter 10 mm) circular skin wound was created on each rat’s back, and differences in healing between groups were then compared using external and data. Figure 6 shows macroscopic appearance of experimental wound healing at various times after surgery. The wound-healing rate of CS/AuNPs was faster than that of pure CS, and this trend began to be obvious on the day 6 and continued until sacrifice on day 12. On day 12, complete re-epithelialization was observed in group of CS/Au, while there were still hematoceles and effusion on the wound surface in the pure CS group. These results show that CS/AuNPs had good biocompatibility. In addition, we speculate that the antibacterial activity of AuNPs effectively prevent the invasion of microorganisms, so as to eliminate damage related factors and necrotic tissue and accelerate tissue healing. However, changes in microstructure, including collagen content, epithelial thickness, and related growth factors, cannot be determined by the external phase alone. Therefore, we performed relevant histopathologic staining and tissue immunostaining.

3.5. Histopathologic Staining. In view of re-epithelialization and collagen deposition playing important roles in the wound-healing process, we performed pathomorphological observations under H&E and Masson’s trichrome staining. Epithelial repair involves thickening to thinning of the epithelium, in which cells gradually evolve from proliferation to differentiation until skin thickness is normalized. In the pure CS group, the new epithelium was thicker with obvious subcutaneous clearance. By contrast, the epithelial thickness of the CS/AuNPs group was thinner. In addition, the CS/AuNPs group showed more pronounced angiogenesis and fewer inflammatory cells.

Collagen is the main structure of the dermis. In the process of injury recovery, fibroblasts secrete COL-1 in large quantities to form the extracellular matrix, which in turn provides a more suitable environment for the cells, thus accelerating wound healing. Therefore, collagen fibers are important for wound repair [34]. Untidy and loose collagen fibers were observed in the CS group, while the CS/AuNPs group displayed well-organized collagen fibers (Figure 7). These results indicate that the CS/AuNPs treated wound tissue has a large amount of collagen deposition and a tight arrangement of fibers. The results of the immunofluorescent staining of COL-1 also verified the findings of Masson’s trichrome staining (Figure 8).

Neovascularization is also a critical process for tissue regeneration, providing oxygen and nutrients for wound granulation tissue and new cells. Further, tissues were stained by CD31, which is one of the specific markers for neovascularization in callus tissues [35]. Results of immunofluorescence staining clearly demonstrated that AuNPs on the film promoted epidermal vascularization to a large extent. There are more vessels formed on the surface of the CS/AuNPs group than that of the pure CS group. Overall, CS/AuNP film can be used as a wound dressing with strong revascularization ability and collagen deposition capacity.

4. Conclusion

Here, CS/AuNP film was synthesized by physical blends of CS with AuNPs, which was characterized with TEM. Based on the further experiments, the addition of AuNPs strengthens the antibacterial properties of CS. Moreover, a certain concentration of AuNPs can improve the adhesion and proliferation of cells, which has also been proved in animal experiments, including gross appearance, histological observations, and immunofluorescence staining. Together, these results suggest the efficacy of CS/AuNPs for broad applications associated with wound healing. At present, in this study, we found that 5 mg/mL gold nanoparticles had the best biocompatibility, while 10 mg/mL gold nanoparticles had the best antibacterial property. Further exploration will be conducted in this concentration range. In addition, the cytotoxicity and antibacterial properties are of great significance for the application of wound dressings. Unluckily, the cytotoxicity and antibacterial properties of gold nanoparticles are obviously dose-dependent; the next research direction is aiming to reconcile this contradiction. Therefore, in the following research, further experiments on the preparation method, size, and shape of AuNPs will be conducted to construct a more suitable wound dressing.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The study was supported by the Jilin Province Science and Technology Plan Project (No. 18YJ012).

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