In Vivo Study of the Antibacterial Chitosan/Polyvinyl Alcohol Loaded with Silver Nanoparticle Hydrogel for Wound Healing Applications

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Silver nanoparticles have attracted great interests widely in medicine due to its great characteristics of antibacterial activity. In this research, the antibacterial activity and biocompatibility of a topical gel synthesized from polyvinyl alcohol, chitosan, and silver nanoparticles were studied. Hydrogels with different concentrations of silver nanoparticles (15 ppm, 30 ppm, and 60 ppm) were evaluated to compare their antibacterial activity, nanoparticles’ sizes, and in vivo behaviors. The resulted silver nanoparticles in the hydrogel were characterized by TEM showing the nanoparticles’ sizes less than 22 nm. The in vitro results prove that the antibacterial effects of all of the samples are satisfied. However, the in vivo results demonstrate the significant difference among different hydrogels in wound healing, where hydrogel with 30 ppm shows the best healing rate.

1. Introduction

In recent decades, nanoparticles (NPs) have been investigated for various biomedical applications and are considered to be the “material of the 21st century” because of their unique designs and property combinations compared with conventional materials [1, 2]. There is a wide range of applications of NPs such as in human health appliances, industrial fields, medical applications, biomedical fields, engineering, electronics, and environmental studies [1–7].

Basically, many benefits of using nanoparticles are proved over other drug delivery systems such as enhancing the solubility of highly hydrophobic drugs, providing sustained and controlled release of encapsulated drugs, and intensifying the stability of therapeutic agents by chemical or physical means and targeted treatments when modified with cell-specific ligands [2]. Among all of the nanomaterials, a variety of metallic nanoparticles have been considered as the foremost attention due to their antibacterial application to human health. Antibiotic resistance has always been one of the most significant health threats due to continuous adaptation of microbes to our antibiotic. This problem has risen the attention for metallic drugs that were used to treat infections before the era of antibiotics’ total dominance. The most widely used delegate of metallic NPs is silver nanoparticles (AgNPs) because of their highly effective antibacterial activity both in solution and in components and their extremely large surface area, which provides better contact with...
microorganisms [3–11]. When we apply AgNPs on the wound, they get attached to the cell membrane and also penetrate inside the bacteria. The bacterial membrane contains sulfur-containing proteins, and the AgNPs interact with these proteins in the cell as well as with the phosphorus-containing compounds like DNA. When AgNPs enter the bacterial cell, it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerate, thus protecting the DNA from the silver ions. The AgNPs preferably attack the respiratory chain and cell division, finally leading to cell death [12, 13]. AgNPs (ranging in size from 1 to 100 nm) can be prepared with many methods: (i) chemical synthesis, (ii) physical dispersion, (iii) photochemical synthesis, and (iv) biological synthesis [8, 9].

Furthermore, to control the release rate of silver ion from AgNPs and increase the antibacterial effect, several studies have suggested combining AgNPs with other biocompatible polymers such as chitosan, polyvinyl alcohol, poly(vinyl alcohol), poly(vinyl pyrrolidone), and poly(lactic acid) to create wound healing application in the type of topical hydrogels, dressing, and mats [14–17]. For example, Zhou et al. created the matrix of gelatin/carboxymethyl chitosan loaded with silver nanoparticles. The results show that this matrix has good physical properties and long-time antibacterial activity [18]. The study of Gaafar et al. showed that AgNPs used singly or combined with chitosan NPs are promising drugs to eliminate the parasite [19]. However, the poor physical property of chitosan requires a combination of this natural polymer and other synthesized polymers [20, 21]. Recent studies showed that a promised alternative to enhance the benefits of chitosan properties is to blend chitosan with another water-soluble polymer such as poly(vinyl alcohol) (PVA) [3, 14, 15, 22]. Due to the high resistance to oil, grease, and solvents, high chemical stability, and excellent oxygen and aroma barrier properties, PVA presented itself as a factor in wound healing dressing to create a covered membrane-absorbing water, helping chitosan and AgNPs to easily access and kill bacteria.

However, the use of this chitosan/polyvinyl alcohol/AgNPs (PCA) gels carries some unpredictable risks regarding their interaction with biological systems [23, 24]. Several studies have suspected the negative effects of the strong oxidative activity of AgNPs releasing silver ions with biological systems by inducing cytotoxicity, genotoxicity, immunological responses, and even cell death [25–29]. Therefore, the profuse applications of AgNPs raise concerns about human exposure, because they can easily pass through the blood-brain barrier by transcytosis of capillary endothelial cells or into other critical areas or tissues [30]. Obviously, human became at risks induced by exposure to nanoparticles (NPs; diameter < 100 nm) either from ambient air or therapeutic uses as drug delivery [31]. According to Aueviriayvit et al., Ag products in colloidal form for medicinal or other purposes have activated Ag+, which might have a direct effect on human health [32]. Moreover, it is hypothesized that Ag+ possesses an enhanced toxicity potential than elemental Ag and AgNPs [28]. The interaction processes of nanomaterials with biological systems are unknown and consequently might be of great concern [24, 33]. The toxicity of other NPs in different organisms has been reported in various studies, whereas the toxicity of AgNPs has not been extensively explored. For example, titanium dioxide (TiO2) NPs induce reactive oxygen species (ROS), which further initiate lipid peroxidation, protein dysfunction, and DNA degradation, finally triggering oxidative damage in the mouse brain [33]. Little is known about the diversified mechanisms of action of the cytotoxicity of AgNPs, as well as their short- or long-term exposure outcomes, on human physiology [34, 35]. Therefore, the toxicological studies on AgNPs have become a raising topic over the past few decades due to their unique properties on the nanoscale and being widespread in many commercial products that were launched into the market recently [36]. On the other hand, several studies of PCA system used cross-linkers such as glutaraldehyde, which causes risks to human health [37, 38].

In Vietnam, recently, medical products using the technology of silver nanoparticles have been developed. In 2016, Hiep et al. synthesized the PCA gels using microwave irradiation method [3]. Compared with other rays, for example, γ-ray and UV ray can induce the formation of AgNPs [39–41]; however, γ-ray causes structural changes in organic compounds [42] and the UV ray method is complicated and requires additional substances [40, 41]; the major impact of microwave is heating. Heating using microwave irradiation is simple, economical, and fast, which can be employed to form AgNPs. PVA in the PCA system under microwave exposure plays a role as a reducing agent, which leads to the reduction of Ag+. The work also successfully cross-links PVA and chitosan without the need of any cross-linkers used in other studies [3, 38].

The aim of this study was to investigate the antibacterial activity and effects of hydrogel, containing polyvinyl alcohol, chitosan, and silver nanoparticles in our previous study, on wound healing to determine the optimal formulation of it [3]. In that research, Hiep et al. loaded AgNPs with two concentrations (5000 ppm and 10000 ppm) with the release rate of 40% and positive antibacterial effect. As mentioned above, the improper concentration of AgNPs causes harmful effects.
Figure 2: TEM images and particle size histograms of PCA15 (a), PCA30 (b), and PCA60 (c) fitted by log-normal distribution function; scale bar: 200 nm.
2. Experimental Procedure

2.1. Materials. Chitosan powder (shrimp shells—low viscosity), Biebrich scarlet-acid fuchsin, phosphotungstic acid solution, phosphomolybdic acid solution, and aniline blue solution were purchased from Sigma-Aldrich, USA. Absolute alcohol, ethanol, xylene, hydrochloric acid, ferric chloride, silver nitrate (AgNO3 ≥ 99%), and acetic acid (CH3COOH ≥ 99.5%) were purchased from Xilong, China. Albino mice were supplied by the School of Biotechnology, International University, HCMC. The pathogens, such as Staphylococcus aureus ATCC 25913 and Pseudomonas aeruginosa ATCC 9028, were obtained from the American Type Culture Collection. ProGel® is a commercial gel containing 50 ppm AgNPs produced by C&D Company of Production and Application of New Materials, Hue City, Vietnam. All other chemicals used were of analytical grade.

2.2. Preparation of the CS/PVA Loaded with Silver Nanoparticle Gel. The silver nitrate solution that has silver ion concentrations of 15, 30, and 60 ppm was loaded into the PVA solution which has the concentration of 10 wt%. The mixture was put in a microwave oven with the setting wave time of 90 seconds and a power level of 800 W. This wave time was reported to be able to create silver nanoparticles with the size of 10–20 nm [3]. Each mixture solution including PVA and silver ions with four diverse concentrations was immersed in 2 wt% chitosan solution by the volume ratio of 1:1 [3]. Finally, four mixture gels were obtained as PCA15 (10 wt%-2 wt%−15 ppm), PCA30 (10 wt%-2 wt%−30 ppm), and PCA60 (10 wt%-2 wt%−60 ppm).

2.3. Transmission Electron Microscopy (TEM). Particle size and shape of AgNPs in PCA hydrogels were examined using transmission electron microscope (JEM-1400 Plus, JEOL, USA). The samples were prepared by applying a drop of PCA hydrogels onto a carbon-coated copper grid and drying. The diameters of AgNPs were measured by using image analysis software (ImageJ, NIH, USA).

2.4. Agar Diffusion Test. The antibacterial activity of AgNPs was evaluated by using the agar diffusion method against the gram-negative (Pseudomonas aeruginosa) and the gram-positive (Staphylococcus aureus) bacteria. The antibacterial activity of PCA gels was measured using the agar diffusion method. Briefly, 100 μl of the bacterial suspension was added and spread out Mueller-Hinton agar surface. Then, the samples were added to the suspension layer. The dishes were incubated upside down at 37°C, overnight. Zones of inhibition were evaluated by measuring the diameter of the bacterial growth inhibition zone around the membrane (in millimeter). The samples were performed with three replications for each bacterial strain. The positive control was a commercial gel, ProGel®, which contains ingredients including 50 ppm silver nanoparticles, Carbopol, and triethanolamine (TEA) (used as the control). The negative sample was the hydrogel which was prepared by polyvinyl alcohol and chitosan without silver.

2.5. Animal Study. In order to evaluate the biocompatibility of five sample groups (PCA gels with four different concentrations and a commercial gel, ProGel®), prepared PCA gels were poured into a syringe and sterilized by autoclave before subcutaneously implanted at the dorsal region under general anesthesia and antiseptic conditions. The operation process was performed following the policy of Institutional Animal Care and Use Committee of International University, Vietnam National University-Ho Chi Minh City, Vietnam. Mice were anesthetized with anesthesia Zoletil®, their hair was shaved at their back, and they were fixed on a table. The implanted site was cleaned by povidone solution and PBS buffer before making the laceration (8 mm × 8 mm) for samples’ application. The experimental study used 15 male Swiss albino mice (3 mice for each group). Figure 1 illustrates the mouse model treated with samples.

Treatment with ProGel® and PCA gel was applied shortly after surgery. The control group was treated with ProGel®; PCA15, PCA30, and PCA60 animals were treated with gel containing the concentration of 15, 30, and 60 ppm silver ion, respectively. Once daily for 12 days, the test samples were applied topically and allowed to heal.

2.5.1. Wound Size Reduction. The wound area of each animal was measured on days 4, 8, and 12 postsurgery. The wound size measurements taken at the time of surgery and at the time of biopsy were used to calculate the percent wound contraction as follows:

\[
\% \text{wound contraction} = \frac{A_o - A_t}{A_o} \times 100
\]

where \(A_o\) is the original wound area and \(A_t\) is the area of the wound at the time of biopsy.

2.5.2. Histological Examination. After 11 days, mice were sacrificed, and then the regenerated areas (0.8 × 0.8 cm²) were extracted. The extracted samples were fixed by 10% formaldehyde, embedded in paraffin, and then sectioned (3-5 μm) using a microtome before staining with hematoxylin and eosin (H&E) stain. The stained samples were observed by a light microscope (Nikon Eclipse, Ti-U series, Japan).

2.6. Analysis of Data. All data are presented as the mean ± standard deviation (S.D.). Data were analyzed by one-way analysis of variance (ANOVA) using the IBM SPSS 20®.
3. Results

3.1. TEM Analysis. The TEM images of PCA hydrogels providing the shape and size of AgNPs are presented in Figure 2. In Figure 2(a), AgNPs in PCA15 hydrogel are smooth and spherical, and the diameter ranges from 2 to 20 nm, showing PVA under microwave is an excellent reducing agent for AgNPs. Furthermore, the average size determined from the histogram is 7.34 nm (Table 1). However, when increasing the concentration of AgNO3 up to 30 ppm, Figure 2(b) shows that the rate of AgNPs over 10 nm enhances while the aggregation appears slightly, and the average diameter increases up to 8.31 nm (Table 1). On the other hand, the result of AgNPs in PCA60 hydrogel Figure 2(c) indicates instability of AgNPs when there are massive agglomerates of them, which caused incapability of exact measurement, and separate AgNPs are at least 17 nm.

3.2. Antibacterial Activity. The antibacterial properties of PCA gels with different concentrations of silver ions were evaluated using the agar diffusion method. Figures 3 and 4 show the inhibition zones of each sample for gram-negative strains (P. aeruginosa) and gram-positive strain (S. aureus). The overview of inhibition diameter proves that both strains were significantly inhibited for all samples, except the negative controls which were fabricated by polyvinyl alcohol and chitosan only. Among these, the gram-negative strain P. aeruginosa was more susceptible by a topical gel containing silver nanoparticles than the gram-positive strain S. aureus, especially with the positive control group which has the lowest inhibition area, except the PCA15 because they have a higher antimicrobial activity for gram-positive S. aureus than that for gram-negative P. aeruginosa. PCA30 and PCA60 show the predominant inhibition zones against P. aeruginosa compared to the ones with S. aureus. The group treated with ProGel® (50 ppm AgNPs) represents the average diameter of the inhibition zone as well as the group treated with gel PCA30.

Particularly, the inhibition diameter of each sample yields significantly statistical meaning with $p < 0.05$ with the control group. When doubling the concentration of silver ions from 15 ppm to 30 ppm, we observed the increase in inhibition diameter. Clearly, the gram-positive S. aureus is inhibited with $1.6 \pm 0.3$ mm diameter treated with the gel PCA30, 0.2 mm diameter more than the gel PCA15 and the gel PCA60 keep that inhibition zone against S. aureus. Meanwhile, the increase could be observed more clearly with the gel PCA30 against P. aeruginosa ($1.7 \pm 0.3$ mm diameter), more than 0.4 mm diameter compared with the gel PCA15. The gel PCA60 eventually increases the inhibition zone against P. aeruginosa ($1.9 \pm 0.3$ mm diameter) compared to S. aureus ($1.6 \pm 0.3$ mm diameter).
3.3. Wound Size Reduction. Figure 5 illustrates the critical changes in wound size during the wound healing progression of each sample, which is measured for the calculation of wound size reduction rate as shown in Figure 6. The wounds of the control group closed slower than those of other groups (Figure 5). However, the measurement was relative, which makes the difference of samples compared to the control group not significant. On day 4, all the wounds had started healing from 23 ± 2 to 30 ± 9 (%). Then, there is a scab formation that covered the defected area, which led to the limitation in the observation of the diameter of wound size. On day 8, all groups reveal partial wound closing, from 60 ± 3 to 75 ± 11 (%). On day 12, the mice were euthanized, and the scabs on wounds were removed. It showed that all wounds closed up to 98 ± 4 to 99 ± 1 (%). The subjects treated with PCA gel showed better wound size reduction than the control group; however, among the gels, the PCA30 and PCA60 were considered to be more effective. Specifically, wounds of PCA30 and PCA60 groups reduced (30 ± 9 (0-4), 75 ± 3 for PCA30, 75 ± 11 for PCA60 (5-8), and 99 ± 1 (9-12) (%)), while those of PCA15 groups healed slower (23 ± 3 (0-4), 69 ± 11 (5-8), and 98 ± 2 (9-12) (%)).

3.4. Histological Analysis of the Skin Lesions. Histological results are presented in Figures 7–10. On the 12th day of postwound, complete epidermal covering forms over the wound surface in all groups, except PCA15. In Figure 7, PCA15 shows large necrosis and zones of necrotic inflammatory cells in the top of the wound site, which was considered to be caused by infection. On the other hand, in the dermis, PCA15, PCA30, and PCA60 samples have granulation tissue and infiltrated inflammatory cells. Noticeably, wound of PCA30 was covered with necrotic tissue which is separated with the regenerative epidermis and prevents new tissue from the invasion of bacteria. This leads to stable growth of granule tissue, which
enhances the healing rate. Furthermore, there are lesions appearing in the epidermis of the control and the dermis of PCA60 sample. This may result from the agglomerates of AgNPs. Those lesions resulted in necrosis to control samples and hemorrhage to PCA60 samples.

4. Discussion

In this study, three different concentrations of silver nanoparticles, 15 ppm, 30 ppm, and 60 ppm, were selected to create three types of samples: PCA15, PCA30, and PCA60. The ratio of PVA and CS, 10 wt% and 2 wt%, respectively, was unchanged following the previous study. These synthesized samples were compared with a commercial topical gel, ProGel®, containing 50 ppm AgNPs to evaluate the possibility for synthesized gel in the market.

Agar diffusion results indicate that increase of the concentration of silver ions leads to more positive antibacterial effect. These observations also reconfirm the results from the previous study that as the number of released Ag ions
increased, the inhibition zone enlarged [3]. Those synthesized hydrogels were aimed at entrapping the silver nanoparticle biopolymer matrix including both natural polymer (chitosan) and synthetic polymer (PVA). Those products take advantages of both polymer sources and enhanced the antibacterial property of silver nanoparticle.

However, the histological results illustrate a different view comparing with in vitro results. The PCA15 group shows severe necrosis and inflammation caused by infection, although the agar diffusion test proves antibacterial properties of PCA15 and TEM image displays the proper size of AgNPs. This means that the amount of AgNPs should be higher to assure effective antibacterial activity. Nevertheless, the excessive concentration of AgNPs could bring negative effects on wound healing, which can be seen in Figures 7(b) and 10(b). The PCA60 and control groups (50 ppm) induce lesions caused by the agglomerates of AgNPs which were shown in TEM images. Furthermore, there is a higher level of inflammation around lesions in mice treated with PCA60 and control gel. Studies suggest that AgNPs possess anti-inflammatory properties [43]. However, inferring from the findings of this study, it indicates that AgNPs do induce inflammation, and they generate reactive oxygen species (ROS) inside the cell, which enhances inflammation [5, 24, 44]. As a result, a higher concentration of AgNPs produces better antibacterial effects; however, the stability of them plays a more important role in minimization of side effects of AgNP application. Overall, when the regenerative tissue of the PCA30 group was compared with the regenerative tissue of the PCA60 and control groups, PCA30 samples show a better healing status with no harmful signs to the tissue.

5. Conclusion

In summary, we successfully determine the optimal formula of hydrogel composed of polyvinyl alcohol, chitosan, and AgNPs in terms of antibacterial properties and biocompatibility. The formation of AgNPs was confirmed via TEM imaging. PCA30 hydrogel possesses excellent antibacterial activity to P. aeruginosa and S. aureus. Besides, in vivo experiment proves the ability to promote wound healing of the PCA30 sample. Therefore, the hydrogel polyvinyl alcohol/chitosan loaded with AgNPs is a potential application as an antibacterial topical gel.

Data Availability

The data supporting the conclusions of this article are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References


