Fabrication of Antimicrobial Wound Dressings Using Silver-Citrate Nanorods and Analysis of Their Wound-Healing Efficacy

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Abstract

*Staphylococcus epidermidis* is well-known not only as an innocuous normal flora species commonly isolated from human skin, but also as an important bacterial species to keep skin healthy, because this species can protect the human skin from pathogenic microorganisms. However, *S. epidermidis* turns into a potential pathogen in damaged skin, because these bacteria can easily form a biofilm on the wound area and provide antimicrobial resistance to other microorganisms embedded in the biofilm. Thus, it is important to kill *S. epidermidis* in the early stage of wound treatment and block the formation of biofilms in advance. In the present study, hydrogel wound dressings were fabricated using polyvinyl alcohol/polyethylene glycol containing silver citrate nanorods, which have been proven to have strong antimicrobial activity, especially against *S. epidermidis*, and their wound-healing efficacy was investigated *in vivo* using a rat experiment.

**Keywords**: Antimicrobial Activity, Burn, Hydrogel, Silver Citrate Nanorod, *Staphylococcus epidermidis*, Wound Dressing

1. Introduction

The antimicrobial activity of silver has been known since the 1800s and silver-based compounds (silver nitrate, silver-sulfadiazine, silver citrate, silver nanoparticles, etc.) have been used in clinical fields until the present day[1-6]. The antimicrobial effect of silver has long been known, but its action mechanism is still not well-understood. There are several controversial hypotheses about the mechanism of how silver actually exhibits antimicrobial properties, and more studies are needed[7-9]. Some hypotheses suggest that silver species (silver ions[10,11], silver complexes[12], or silver nanoparticles[13,14]) released from silver-based compounds kill microbes by contacting, penetrating microbe’s cell wall, and making microbe’s constituents (proteins, cell walls, or nuclei) lose their functions[8,11,15-19]. The antimicrobial effect of a silver-based compound depends not only on the type of compound (silver nitrate, silver-sulfadiazine, etc.) and released species (Ag+, Ag0, etc.), but also on the morphology (size[15] and shape[20]) of the compound. As the size of the compound decreases, the surface area increases. A large surface area leads to a high efflux rate of silver species, resulting in enhanced antimicrobial activity. The antimicrobial activity also depends on the shape of the compound. Even compounds of same size can exhibit different antimicrobial ability if their shape is different. The shape of the compound depends on the exposed lattice plane because the surface energy of the compound changes accordingly. Therefore, the release rate of the silver species depends on which crystal plane they are released from, and ultimately depends on the shape of the compound.

In a previous work[21], it has been reported that simple agitation can change the size and shape of silver-citrate compounds (Ag2Cit) from an unspecified shape of microstructures (bulk silver citrate) to rod-shaped nano-
structures (silver-citrate nanorods) without changing the chemical composition and found that silver-citrate nanorods exhibit improved antibacterial activity. Since silver-citrate nanorods are smaller in size than bulk silver-citrate, the portion of the surface area of silver-citrate nanorods is 3 to 24 times larger than that of bulk silver-citrate. The larger surface area of silver-citrate nanorods allows more antimicrobial species to be released per unit time. The antimicrobial activity of bulk silver citrate and silver-citrate nanorods was tested against three gram-positive (Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus) and three gram-negative bacteria (Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa), and their antibacterial ability was compared. The antimicrobial activity of the silver-citrate nanorods was 1.33.3 times higher than that of bulk silver citrate for most bacteria. Especially, silver-citrate nanorods showed the most enhanced antimicrobial activity against S. epidermidis. S. epidermidis is a representative coagulase-negative staphylococcus and has been regarded as an innocuous normal flora species commonly isolated from human skin and mucosa. S. epidermidis is an endogenous organism of human skin, and all human beings are colonized with this microorganism at birth. This human indigenous microflora species can play an important role in maintaining healthy skin because it protects the skin from pathogenic microorganisms\cite{22,23}. However, in damaged skin tissue, such as burn wounds, S. epidermidis can be a potential infection-causing pathogen\cite{24,25} and form a biofilm, providing antimicrobial resistance to other bacteria\cite{23}. Because microorganisms embedded in biofilm exhibit strong resistance to antibiotics\cite{26,27}, it is important to prevent biofilm formation by killing S. epidermidis in the early stage of burn wound treatment\cite{22}. Because silver citrate nanorods exhibit the most enhanced antimicrobial activity against S. epidermidis, we can expect that wound dressings containing silver citrate nanorods are especially adaptable for burn patients. Herein, we utilized silver citrate nanorods in an antimicrobial wound dressing. We investigated the toxicity of silver citrate nanorods for mammalian skin cells (HS68 fibroblasts) and made a wound dressing containing silver citrate nanorods using polyvinyl alcohol (PVA) and polyethylene glycol (PEG). We also applied this wound dressing to artificially created burn wounds on a rat skin.

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2. Materials and Methods

2.1 Synthesis of Antimicrobial Reagents (Silver Citrate Compounds)

Silver citrate compounds (bulk silver citrate and silver citrate nanorods) were prepared according to the previously reported recipe (21). Briefly, aqueous solutions of silver nitrate (0.10-0.25 M) and trisodium citrate dihydrate (0.10-0.25 M) were prepared in different beakers using silver nitrate (AgNO$_3$ ≥ 99.0%, Aldrich) and trisodium citrate dihydrate (TSC, C$_6$H$_{14}$Na$_3$O$_7$·2H$_2$O, 99.0%, OCI Ltd.), respectively. For the synthesis of silver citrate nanorods, the two solutions were mixed and stirred at 100–900 rpm for 15-120 min. The stirring process is very important to change the morphology (size and shape) of silver citrate compounds into rod-shaped ones with diameters of tens of nanometers. If the two solutions are mixed without stirring, silver citrate nanorods do not form.

2.2 Preparation of Wound Dressing Containing Silver-Citrate Compounds

To prepare the hydrogel wound dressing, polyvinyl alcohol (PVA, M.W. 89,000-98,000, Aldrich) and polyethylene glycol (PEG400, Alfa Aesar) were used as received. Five milliliters of aqueous PVA solution (5 or 10%(w/v)) was mixed with 5 mL of aqueous PEG solutions (10, 20, or 40%(w/v)), respectively, to make six solutions with different PVA:PEG ratios: 1) 5%:10%, 2) 5%:20%, 3) 5%:40%, 4) 10%:10%, 5) 10%:20%, 6) 10%:40%. All mixed solutions were stirred at 85°C for 30 min until the color of the solution changed from...
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After cooling the solutions under ambient conditions (~25°C), they were poured into six different chalets, respectively. All chalets containing six solutions were stored at ~70°C for 12 hours for gelation. The frozen hydrogels were allowed to completely thaw at room temperature (~25°C), and this process (freezing and thawing) was repeated at least twice. Gel content ratio, swelling ratio, and mechanical properties of the prepared six hydrogels were measured, and optimal ratio of PVA to PEG was selected for making hydrogel wound dressings. For the in vivo rat experiment, 14, 140, or 1400 μg of bulk silver citrate or the same amount of silver citrate nanorods were added to 10 mL of the hydrogel solution prepared by optimal ratio of PVA to PEG, respectively. Finally, a total of nine hydrogel wound dressings were prepared, each containing three different materials (bulk silver citrate, silver citrate nanorods, or sterilized water) in three different masses (14, 140, or 1400 μg).

2.3 Measuring the Mechanical Properties of Hydrogel Wound Dressings

The physical properties of the hydrogels prepared with different ratio of PVA to PEG were measured using a Texture Analyzer (TA-XTi, Stable Micro Systems, England). Since the distance between the crossheads of the Texture Analyzer was fixed at 3 cm, we cut the hydrogels longer than 3 cm to stably fix them to the crosshead. We measured the thickness and width of each hydrogel to calculate cross-sectional area that was taken into account when calculating the tensile stress. The crosshead was stretched at a rate of 30 mm/min while measuring the tensile stress and strain.

2.4 Cell Culture

Mouse NIH3T3 and human HS68 cells were maintained according to the supplier’s recommendations. NIH3T3 cells were cultured in Dulbecco’s Modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HS68 cells were cultured in DMEM medium (Gibco) containing 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified incubator of 95% air and 5% CO2 at 37°C. Media were replaced every 3 days, and cells were subcultured when confluence reached approximately 80%.

2.5 Cytotoxicity Test

Cells were seeded in 96-well plates with conditioned media (9×10^5 NIH3T3 cells or 6×10^5 HS68 cells), and incubated until confluence reached to 7080%. Cells were then treated with 0-20 μg/ml of bulk silver citrates or silver citrate nanorods for 1 day. Cytotoxicity of the treated fibroblast cells was observed by 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO, USA). Data represent the mean ± standard deviation of more than three independent experiments. Statistical comparisons were made by an independent t-test.

2.6 Rat Clinical Experiment

A total of 18 male Sprague Dawley rats (350–400 g, 6–8 weeks old) were housed in individual cages at room temperature (standardized at 26°C) and acclimated for one week with standard rat feed. All rats were anesthetized with a mixture of isoflurane, USP (Abbott Labs, Chicago, Illinois, USA) and oxygen in an induction chamber before the experiment. Once properly anesthetized, the hair on the back of each rat was shaved and the shaved area was cleansed with 70% isopropyl alcohol (BD, Franklin Lakes, NJ, USA). The skin was allowed to dry and equilibrate to the ambient temperature for several minutes. An electric iron was heated to 100°C and placed on the back skin of each animal for 5 seconds to inflict a burn injury (dotted circle in Figure 6a). One hundred microliters of *S. epidermidis* suspension (4×10^6 CFU/mL in 1% peptone) was injected into the superficial wounds. All rats were divided into three groups, with six rats each, and the three groups were treated with different hydrogel wound dressings containing sterilized water, bulk silver-citrate, or silver citrate nanorods right after injection of *S. epidermidis* suspension. Sterilized gauzes (Tegaderm; 3M Health Care, USA) were placed on top of the hydrogel and fixed with a flexible band (Coban; 3M Health Care, USA). Rats were injected subcutaneously with 0.02 μg/kg fentanyl citrate (Abbott Labs, Chicago, Illinois, USA) twice daily to reduce pain from burns after burn injury. After 48 hours of applying the hydrogel to the burn wound, the wound site was examined visually and histologically. For histological examination, animals were euthanized via cervical dislocation under anesthe-
sia, and tissues containing the entire wound were collected and fixed in 10% neutral formalin solution for 24 hours. After fixation, tissues were dehydrated and embedded in a paraffin cube. The embedded tissue was cut using a microtome and attached to the surface of an adhesive slide coated with Polysine. After the paraffin was removed, the tissue was stained with hematoxylin and eosin Y to observe the sample under a microscope. This study was conducted in the Experimental Animal Raising and Research Laboratory of Chosun University, after approval of the local ethics committee. All experimental procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3. Results and Discussion

Typically, silver citrate nanorods are synthesized by mixing 0.2 M silver nitrate with 0.2 M sodium citrate at a stirring rate of 340 rpm for 30 min at room temperature. Under these experimental conditions (this is hereafter referred to as the reference condition), silver citrate nanorods with a length of 1.3 ± 0.4 μm and a diameter of 130 ± 30 nm (approximate aspect ratio of 10) were synthesized\(^2\). In this experimental procedure, the stirring process plays a crucial role for the formation of silver citrate nanorods as previously reported\(^2\). Without stirring, silver citrate nanorods are not formed as shown in Figure 1a, which is the electron micrograph of the precipitate in the mixed solution (0.2 M silver nitrate and 0.2 M sodium citrate) stored on a laboratory bench for 60 min after mixing without stirring. Even when the storage time was prolonged to 1440 min, silver citrate nanorods were not produced (Figure 1b). If the mixed solution was not stirred, the concentration of silver nitrate and sodium citrate also did not affect to the formation of silver citrate nanorods if stirring was not applied. At silver nitrate and sodium citrate concentrations of 0.15 M and 0.15 M, respectively, silver citrate nanorods were not synthesized (c). If the stirring process was performed, silver citrate nanorods were easily prepared by mixing 0.2 M silver nitrate aqueous solution with 0.2 M sodium citrate aqueous solution with stirring at 340 rpm for (d) 15, (e) 30, or (f) 120 min.

![Fig. 1. Morphology of silver citrate compounds prepared in different experimental conditions.](image)

Fig. 1. Morphology of silver citrate compounds prepared in different experimental conditions. Without stirring, silver citrate nanorods did not form (a, b, c). The morphology of bulk silver citrate precipitated in the mixed solution (0.2 M silver nitrate and 0.2 M sodium citrate) stored on a laboratory bench for (a) 60 min, and (b) 1440 min. The concentration of silver nitrate and sodium citrate did not also affect to the formation of silver citrate nanorods if stirring was not applied. At silver nitrate and sodium citrate concentrations of 0.15 M and 0.15 M, respectively, silver citrate nanorods were not synthesized (c). If the stirring process was performed, silver citrate nanorods were easily prepared by mixing 0.2 M silver nitrate aqueous solution with 0.2 M sodium citrate aqueous solution with stirring at 340 rpm for (d) 15, (e) 30, or (f) 120 min.

Silver citrate nanorods with a high aspect ratio are advantageous when they are used as antimicrobial agents or wound dressings. Since nanorod structures with a high aspect ratio resemble the extracellular matrix of the skin, cell adhesion (bacterial cells or human living cells) to the nanorod structure can be improved, resulting in enhanced antimicrobial or wound healing activity\(^2\). We surveyed many experimental conditions near the reference condition to find optimal conditions for producing silver citrate nanorods with a high aspect ratio. First, we investigated how the dimension of silver citrate nanorods was affected by the
stirring period (Figure 1d-f). Silver citrate nanorods were synthesized based on the reference condition for different stirring periods 15, 30, and 120 min. Within these stirring periods, the shape of silver citrate was maintained in the form of nanorods, but their sizes differed. When the stirring period was changed from 15 to 30 min, the length and diameter of silver citrate nanorods decreased from 1.8 ± 0.4 to 1.3 ± 0.3 µm in length and from 0.28 ± 0.05 to 0.11 ± 0.03 µm in diameter, and the aspect ratio also increased from approximately 6 to 12 (Supporting Figure 1a, b). However, when the stirring period was further prolonged to 120 min, both the length and diameter of silver citrate nanorods further decreased to 0.5 ± 0.2 µm in length and 0.06 ± 0.01 µm in diameter, and the aspect ratio was rather reduced from approximately 12 to 8 (Supporting Figure 1a, b). In addition to the stirring period, the stirring rate also affected the size of silver citrate nanorods (Figure 2a-c). When the stirring rate was increased from 100 (Figure 2a) to 340 rpm (Figure 2b), the length of silver citrate nanorods increased from 0.9 ± 0.2 to 1.1 ± 0.4 µm, but the diameter was decreased from 0.13 ± 0.05 to 0.08 ± 0.2 µm. Therefore, the aspect ratio was increased almost 2-fold from approximately 7 to 14 (Supporting Figure 2a, b). However, when the stirring rate was further increased to 900 rpm, the length reduced to 0.6 ± 0.2 µm and the diameter increased to 0.1 ± 0.2 µm, resulting in decrease of the aspect ratio from approximately 14 to 6 (Supporting Figure 2a, b). We also investigated concentration dependence on the dimension of silver citrate nanorods (Figure 2d-f). When the concentrations of silver nitrate and sodium citrate were 0.1 M and 0.1 M, respectively, mixtures of different diameters of silver citrate nanorods were produced as shown in Figure 2d. On the other hand, when the concentrations of silver nitrate and sodium citrate were as high as 0.25 M and 0.25 M, respectively, mixtures of different lengths of silver citrate nanorods were synthesized as shown in Figure 2f. The measured dimensions of silver citrate nanorods in each condition were not much different (0.7 ± 0.2 µm in length and 0.10 ± 0.03 µm in diameter in (d); 0.9 ± 0.3 and 0.12 ± 0.03 in (e); 0.8 ± 0.3 and 0.12 ± 0.02 in (f)), and the aspect ratios of three cases also had almost the same values (Supporting Figure 3a, b). Taking together all the experimental conditions mentioned above, silver citrate nanorods were produced under a wide range of experimental conditions, however, their dimensions depended on the experimental conditions. It was confirmed that the reference condition suggested in the previous paper is the optimal condition to prepare silver citrate nanorods with a high aspect ratio.

According to the previously published paper, silver citrate nanorods exhibited 1.5- or 1.8-fold greater antimicrobial activity than bulk silver citrate against the gram-negative or -positive bacteria tested, respectively[21]. Especially, the antimicrobial effect of silver citrate nanorods against *S. epidermidis* was enhanced 3.3-fold. *S. epidermidis* is not so harmful to healthy human and normal flora that reside in the skin or mucosa. However, *S. epidermidis* can form a biofilm at wound sites, and various bacteria (*S. aureus* and others) can exhibit resistance to antibiotics in the formed biofilm. Therefore, it is very important to remove *S. epidermidis* at early stages when treating burn injuries[22-25].
In order to take advantage of the strong antimicrobial effect of silver citrate nanorods against *S. epidermidis*, we fabricated hydrogel wound dressings containing silver citrate nanorods and applied them to burn injuries artificially contaminated by *S. epidermidis* on the back skin of rats.

In order for hydrogels to be used in the clinic, they must have adequate physical properties such as high swelling ratio, strong tensile strength, suitable Young’s modulus, and others. We synthesized six different hydrogels using polyvinyl alcohol (PVA) and polyethylene glycol (PEG) with different concentrations of PVA (5 and 10% (w/v)) and PEG (10, 20, 40% (v/v)), and tested their mechanical properties. Hydrogels made from a low concentration of PVA (5%) tore easily, even when the amount of PEG was increased to 40%. As shown in Figure 3a, b, and c, for hydrogels made of 5% PVA, the tensile strength was so weak that they tore easily even if they were stretched by hand. However, when 10% PVA was used, hydrogels exhibited relatively strong tensile strength especially in the case of 10 or 20% PEG (Figure 3d and e, respectively). When 10% PVA was used with 40% PEG, the hydrogel tensile strength was still strong but it was easily broken, probably due to its weak elasticity (Figure 3f). For quantitative measurement of the mechanical properties of the hydrogel, we used a texture analyzer and measured tensile strength, percent elongation, and Young’s modulus (Figure 3g). In Figure 3g, data of hydrogels made with 5% PVA were not included because the hydrogels made of 5% PVA with 10 or 20% PEG were so easily torn that it was impossible to measure their mechanical property using a texture analyzer. Additionally, because the tensile strength of hydrogels composed of 5% PVA and 40% PEG was about 10 times smaller than that of the hydrogel made of 10% PVA, only the data of hydrogels made with 10% PVA were included in Figure 3g. Young’s modulus is defined as the slope of the stress-strain curve as shown in Figure 3g. Therefore, a large Young’s modulus means that more force is needed to extend the hydrogel to the same length. If the Young’s modulus is too small, the shape of the hydrogel will not be maintained, and if it is too strong, a large amount of force is required to stretch it. Therefore, an ideal wound dressing would have a modest Young’s modulus. Figure 3g shows that Young’s modulus increased with the concentration of PEG, and hydrogels with a PEG concentration of 20% were considered to have the most moderate elasticity. The tensile strength and strain (percent elongation) also depended on the PEG concentration (Figure 4a). For example, when the concentration of PEG increased from 10 to 20%, there was little change in the strain, but from 20 to 40%, the strain decreased, as depicted by black squares in Figure 4a. The tensile strength trends were opposite to those of strain; tensile strength increased when the concentration of PEG increased from 10 to 20%, but the tensile
The strength of the hydrogel with 20% PEG was not significantly different from that of the hydrogel with 40% PEG (red triangles in Figure 4a). The hydrogels with 20% PEG showed good strain and tensile strength values.

Gel fraction and swelling ratio are other important physical properties of hydrogels. Normally, hydrogels are composed of an insoluble network, which is not dispersed in water, and a soluble part dispersible in water. The ratio of the mass of the insoluble network (\(W_{\text{insoluble}}\)) to the mass of the entire hydrogel (insoluble network + soluble part; \(W_{\text{entire}}\)) is defined as a gel fraction (Equation 1), and swelling ratio is defined as the mass ratio of water contained in the dried insoluble hydrogel (Equation 2)\(^{[10]}\).

\[
\text{Gel Fraction (\%)} = \left(\frac{W_{\text{insoluble}}}{W_{\text{entire}}}\right) \times 100 \quad (1)
\]

\[
\text{Swelling Ratio (\%)} = \left(\frac{W_{\text{swollen}} - W_{\text{insoluble}}}{W_{\text{insoluble}}}\right) \times 100 \quad (2)
\]

Because the thawed hydrogels have a high water content, the water should be removed to measure the mass of the entire hydrogel (\(W_{\text{entire}}\)). Hydrogels were dried in an oven at 65°C, until the hydrogel mass was stable. After measuring the mass of the dried hydrogel (\(W_{\text{entire}}\)), we immersed the dried hydrogel into warm water (60°C) for over 48 hours to extract the soluble part. While the soluble part is extracted from the hydrogel, water is soaked into the hydrogel instead of soluble part. The swollen hydrogel weighed after wiping its surface (\(W_{\text{swollen}}\)). The swollen hydrogel was dried again in the oven at 65°C and weighed again (\(W_{\text{insoluble}}\)). The gel fraction or swelling ratio was calculated by substituting the measured three values (\(W_{\text{entire}}, W_{\text{swollen}}, W_{\text{insoluble}}\)) into Equation 1 or 2 as appropriate. Both gel fraction and swelling ratio were dependent on the concentration of PEG (Figure 4b). Since the gel fraction indicates how much network formed and the swelling ratio is the indicator how much liquid can be absorbed, hydrogels with...
a large gel fraction and high swelling ratio are more elastic and can absorb more amount of liquid, respectively. In general, however, the gel fraction is inversely proportional to the swelling ratio. As the gel fraction is large, the swelling ratio becomes smaller, and vice versa. Of the six hydrogels prepared at different concentrations, the hydrogel made of 10\% PVA with 20\% PEG showed optimal physical properties in terms of Young’s modulus, tensile strength, strain, gel fraction, and swelling ratio, and we chose this gel for in vivo rat experiment.

Prior to in vivo rat experiment, we tested the cytotoxicity of silver citrate compounds against epidermal fibroblast cells. If the silver citrate compounds exhibit the same toxicity toward epidermal fibroblast cells, they will not be useful as antimicrobial agents. To test cytotoxicity, MTT assays were performed with NIH3T3 (mouse embryonic fibroblast cell line) and HS68 (human skin fibroblast cells) cells. Cells were treated with various concentrations of bulk silver citrate or silver citrate nanorods (0, 0.1, 0.5, 1, 3, 5, 10, and 20 μg/ml), and subjected to MTT assay. No cytotoxicity was observed in NIH3T3 cells when treated up to 1 μg/ml, and then cytotoxicity gradually increased at higher concentrations. Ninety-five percent of cells for bulk silver citrate and 85\% for silver citrate nanorods survived upon treatment with 3 μg/ml material, and 67\% and 52\% survived at 5 μg/ml, respectively (Fig. 5a). In HS68 cells, toxicity was not observed up to 3 μg/ml, whereas 81\% and 64\% of cells were viable upon treatment with bulk silver citrate or silver citrate nanorods at 5 μg/ml (Fig. 5b). This is consistent with the result of the previous paper in which the minimal inhibitory concentration (the lowest concentration of antimicrobial reagents preventing bacterial growth) of bulk silver citrate and silver citrate nanorods against S. epidermidis was 2.0 and 0.6 μg/ml, respectively[21]. This indicates that the toxicity of silver citrate compounds toward human skin cells is high, but because of the relatively higher toxicity toward microorganisms, silver citrate compounds and especially silver citrate nanorods will be able to effectively remove harmful microorganisms without side effects to human skin when applied at a proper dose.

Rat clinical experiments were performed on a total of 18 rats divided into three groups of 6 rats per group. As shown in Figure 6a, a burn injury was inflicted on the back skin of rats in each group by an electric iron, and bacteria (S. epidermidis) were injected through a microsyringe onto the burn wound. The wound area was immediately treated with hydrogel wound dressings containing sterilized water, bulk silver citrates, or silver citrate nanorods as shown in Figure 6b. After 48 hours, the wound area was examined visually (Figure 6c, e, and g) and histologically (Figure 6d, f, and h). The infected wounds can exhibit accelerated healing when

Figure 6. Clinical Rat Experiment. The back skin of the rats was exposed to a burn injury using a pharynx (dotted circle in (a)). The wound area was covered with three kinds of hydrogel wound dressings (b) containing sterilized water (c and d), bulk silver citrate (e and f), and silver citrate nanorods (g, h). After 48 hours, the wound surface was observed with the naked eye (c, e, and g), and histologically examined (d, f, and h).
Antimicrobial agents effectively inhibit the bacterial growth on the wound area [31]. The hydrogels containing silver citrate exhibited more effective antimicrobial activity than that containing only sterilized water. Therefore, we can expect that wounds treated with hydrogels containing silver citrates will be healed faster than those treated with hydrogels containing only sterilized water. As expected, wounds treated with hydrogels containing sterilized water had not yet healed (Figure 6c), but the wounds treated with hydrogels containing silver citrate compounds had almost healed (Figure 6e and g). Silver citrate nanorods exhibit stronger antibacterial activity than that of bulk silver citrate; however, there was no significant difference in the wound area upon treatment with these two types of hydrogel (Figure 6e and g). For more detailed observation, we performed a histological examination of each wound. As shown in Figure 6d, no epithelial layer was formed when no antimicrobial substance was used, and traces of pus were observed within the dermis. The wounds treated with hydrogels containing bulk silver citrates or silver citrate nanorods seemed similar in appearance, but histological examination showed a clear difference. In the case of wounds treated with hydrogels containing bulk silver citrates, which has relatively weak antibacterial ability, the epidermis was not formed sufficiently and its thickness was relatively thin, and traces of pus were still observed in the dermis. On the other hand, wounds treated with hydrogels containing silver citrate nanorods were fully recovered. Thick epithelial tissues were formed and traces of pus were not found at all. It should be noted that we tested three different concentrations (14, 140, 1400 µg) of bulk silver citrate and silver citrate nanorods and they exhibit the same healing tendency.

4. Conclusions

A wide range of experimental conditions (reagent concentration, stirring rate and reaction time) were investigated to synthesize silver citrate nanorods with a high aspect ratio. Silver citrate nanorods with an aspect ratio of 3.4-10.0 were prepared by mixing 0.2 M silver nitrate aqueous solution with 0.2 M sodium citrate aqueous solution at a stirring rate of 340 rpm for 30 min. We performed toxicity tests of silver citrate compounds on mammalian skin cells and found that the proper dose for killing bacteria without damaging skin cells is between 0.6 and 5 µg/mL. In order to synthesize hydrogels with suitable physical properties for clinical applications, hydrogels were synthesized with various concentrations of polyvinyl alcohol and polyethylene glycol, and their physical properties were investigated. Hydrogels containing bulk silver citrates or silver citrate nanorods were applied to artificially created burn injuries on the back skin of rats, and it was demonstrated that healing efficacy of wound dressing containing silver citrate nanorods were more effective than that containing bulk silver citrates.

In this study, we did not compare the antimicrobial activity of silver citrate compounds with that of commercialized ones (e.g., silver sulfadiazine etc.), because their chemical composition is different and their antimicrobial activity or healing efficacy cannot be compared directly. Since bulk silver citrates and silver citrate nanorods have the same chemical composition as silver citrate (Ag₃C₆H₅O₇), the silver contents of both compounds should be equal at the same mass. The same amount of silver contents in both compounds enables us to investigate shape- or size-dependent antimicrobial activity.

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