In Vitro Cytotoxicity and Cell Seeding Studies of a Chitosan-silver Composite for Potential Wound Management Applications

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Abstract: *There have been concerns raised over the treatment of acute burns with silver particles because of the potential toxicity caused by silver. As the wound heals, a membrane is formed between the silver particle and the blood, but this membrane is not impenetrable, which might lead to further silver absorption. This paper reports the in vitro cytotoxicity of silver-chitosan composites prepared as films and paste with various concentration of chitosan and a fixed concentration of 3 w/v % of silver nitrate, $AgNO_3$. UV photo reduction was used to convert the silver ions* (Ag^+) *to silver particles, and the effect of adding silver to chitosan was thoroughly studied. Cell attachment studies using Normal Human Dermal Fibroblasts (NHDFs) are being carried out, and more evidence for the reliability of using silver as an antimicrobial agent in chitosan composites for wound management applications could be gained.*

Keywords: chitosan, silver, cell seeding, cytotoxicity, wound management

1. INTRODUCTION

Chitosan [*β*-(1-4)-2-amino-2-deoxy-D-glucose] is a unique basic polysaccharide and is generally represented as a homopolymer. The deacetylation of chitin that yields chitosan is rarely complete, and most commercial and laboratory products tend to be copolymers of N-acetylglucosamine (NAG) and N-glucosamine.[1] The ratio of the two types of repeating units depends on the source and the method of preparation of chitosan, but glucosamine units predominate. The structure of chitosan is similar to that of cellulose except at carbon-2, where the hydroxyl group of cellulose is replaced by an amino group. Although the β-(1-4) anhydroglucosidic bond of chitosan is also present in cellulose, the characteristic properties are not shared by cellulose. Figure 1 presents the structural similarities of chitin, chitosan and cellulose.

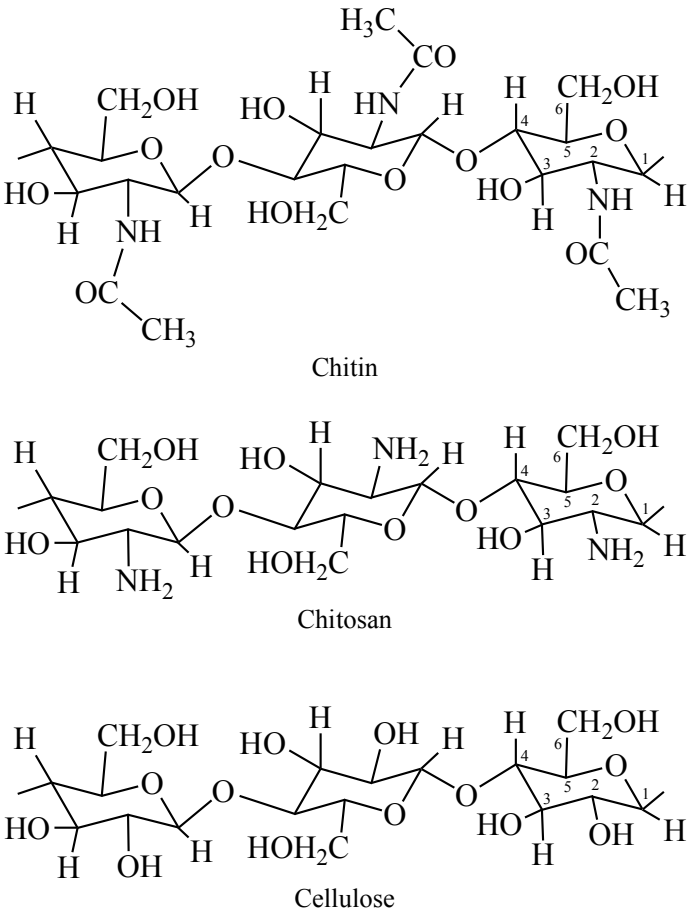

Figure 1: Chemical structures of chitin, chitosan and cellulose.[1]

Chitosan is a biopolymer that is generally known to accelerate the healing of wounds in humans.[2] It has been reported that chitosan stimulates the migration of polymorphonuclear and mononuclear cells and accelerates re-epithelisation and normal skin regeneration.[3] Some reports also reveal that chitosan possesses a considerable antibacterial activity against a broad spectrum of bacteria.[4,5] Chitosan is a positively charged molecule, and it interacts very well with the negatively charged microbial cell wall, leading to the leakage of the intercellular contents. This leakage results in binding between chitosan and DNA, and mRNA synthesis is inhibited by the penetration of chitosan into the nuclei of the microorganisms, thus resulting in interference with the synthesis of proteins in the microorganism.

The antibacterial activity of chitosan can be enhanced by incorporating silver compounds, which are known to have antimicrobial activity. Studies on the incorporation of silver sulfadiazine into chitosan have been widely carried out because the topical use of silver sulfadiazine is believed to induce considerable toxicity problems.[6] In this work, chitosan-silver nanocomposites were produced by a UV photo reduction technique in which silver ions (Ag^{+}) were converted into silver metal (Ag^{0}) in the chitosan solution in situ. The slow release of the silver nanoparticles in the chitosan samples is thought to be an effective method for the long-term inhibition of the growth of bacteria.

The metabolic fate of metallic silver in the human body is affected by the chemical nature of silver itself, which influences the absorption, distribution and metabolism of the silver. Therefore, it is vital to study the in vitro, ex vivo and in vivo toxicity of silver. This study concentrated on the cytotoxicity of silver nanoparticles incorporated in chitosan using Normal Human Dermal Fibroblasts (NHDFs). NHDFs were chosen mainly because they continuously secrete macromolecules into the extra-cellular matrix. This work aimed to unravel the effect on NHDF cells upon exposure to chitosan preparations containing silver nanoparticles.

2. EXPERIMENTAL

2.1 Materials

Non-water-soluble chitosan, with a deacetylation degree of 90% (molecular weight 100 kDa), was purchased from Hunza Nutriceuticals Sdn. Bhd. (Perak, Malaysia). Water-soluble chitosan sourced from China was purchased from Sigma Aldrich. Analytical-grade highly purified silver nitrate salt was purchased from Sigma Aldrich (USA), and 100% acetic acid, glycerol and sodium hydrogen bicarbonate of analytical grade were purchased from Fluka Chemical (Switzerland).

2.2 Sample Preparation

Chitosan solutions containing 3 wt.% silver nitrate, $AgNO_3$ were prepared from both water-soluble and non-water-soluble chitosan using a UV photo reduction process conducted for predetermined time periods. Silver nanoparticles formed in the chitosan samples were in the range of 5–10 nm. The optimum concentration for water-soluble chitosan was 3 wt.%, whereas 2.85 wt.% was used for non-water-soluble chitosan. Samples were prepared in film and paste form. Chitosan samples were first immersed in the medium (Dulbecco Modified Eagle's Medium, DMEM) at 24, 48 and 72 hours at 37°C without

agitation and were evaluated at various test solution concentrations (which are termed extract concentrations hereafter) of 2×10^5 ppm, 1×10^5 ppm, 0.5×10^5 ppm and 0.25×10^5 ppm. Chitosan powder without silver was used for comparison. Ion release studies of chitosan containing silver were conducted in our laboratory to determine the release profile of silver nanoparticles.

2.3 Cytotoxicity Tests with NHDF Cells

NHDF cells were cultured in DMEM (Invitrogen, Australia) containing penicillin/streptomycin (Invitrogen) and L-glutamine (200 mM; Invitrogen) and supplemented with 10% foetal bovine serum (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 MTT Test

Studies of the viability of the cells in the presence of chitosan samples were performed using the MTT test. The cells were resuspended in culture medium and plated in 24-well plates at a density of 5×10^4 cells/mL and then incubated for 24 hours in a humidified atmosphere of 5% CO_2. Thereafter, the medium was replaced with 1 mL of the test solution; culture medium was used as control. Following 24 hours of incubation, the test solutions were removed, and each well was treated with 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [5 mg/mL in phosphate buffer saline (PBS), Gibco, NY] and incubated for 4 hours at 37°C in a humidified atmosphere of 5% CO_2. The yellow MTT is reduced to blue-purple formazan in the presence of the mitochondrial dehydrogenase. This enzyme is present in intact living cells, hence the blue-purple colour produced should be proportional to the number of viable cells present.[7] The MTT solution was then replaced with 100 μL/well of dimethylsulfoxide (DMSO, AMRESCO, Ohio, USA) to dissolve the formazan salts, followed by 10 minutes of slow agitation, yielding a blue-purple solution. The absorbance of this solution was measured at 570 nm using a Universal Micro plate Reader (BioTek Instruments, USA).

2.5 Cell Seeding Test

NHDF cells (5×10^4 cells/mL) were seeded on the chitosan samples in 24-well culture plates and cultured for 24, 48 or 72 hours with a fresh medium every day. At selected time intervals, the cells were washed with PBS buffer and fixed with 2.5% glutaraldehyde at room temperature for 1 hour. After removal of the glutaraldehyde, the cells were dehydrated through a graded series of aqueous ethanol baths. Variable Pressure Scanning Electron Microscopy (VPSEM, Leo-Zeiss, Germany) was used to observe the cell morphology.

3. RESULTS AND DISCUSSION

Silver nanoparticles are being used with increasing frequency as wound dressings due to their antimicrobial activity. Studies have also shown that even a small dose of silver nanoparticles has the potential to cause toxicity, as determined by an array of cyto- and genotoxicity parameter.[8] The results shown in this paper describe the effect of incorporating silver into chitosan, especially with regard to chitosan's cytotoxicity.

3.1 Effect on Cell Morphology from Cell Seeding Studies

The first and most readily noticeable effect following the exposure of cells to toxic materials is the alteration in the cell shape or morphology in monolayer culture. The VPSEM of the film sample without silver [Fig. 2 (d), (e) and (f)] show well-interconnected NHDF cells, the number of which increased greatly with increasing incubation time. On the other hand, the images of the 2.85 wt.% chitosan samples [Fig. 2 (g), (h) and (i)] loaded with 3 wt.% A_gNO_3 showed a distinct morphological change, an indication of the shrinkage of cells after 48 hours of incubation. Slow propagation was observed after 72 hours. However, this microscopic observation of the silver-loaded chitosan showed no evidence of massive cell death, which was investigated further using a cell viability assay.

3.2 Cell Viability

Viability assays are vital steps in toxicity studies that determine the cellular response to a toxicant. Viability assays give information on cell death and metabolic activities. The MTT assay used in this work is a quick and effective method for testing mitochondrial activity, which correlates quite well with cell proliferation. It is quite surprising that the MTT assay results for chitosan powder (Fig. 3) showed a concentration-dependent decrease in the mitochondrial activity for cells incubated with the test solutions for 24 hours. With decreasing extract concentration, the percentage of viable cells decreased, with a minimum of 60% survival, which still correlates to the non-toxic category. The incubation for 72 hours yielded better cell proliferation behaviour, with more than 100% cell viability. This result agrees very well with the good biocompatibility properties of chitosan.

The toxicity of silver nanoparticles in chitosan samples was assessed using paste and film forms. Table 1 presents the results of the ion release study performed on both paste and film chitosan preparations containing silver nanoparticles in PBS. At 3 wt.% $AgNO_3$ and 2.85 w/v% chitosan, reduced substantially lower concentration of silver (0.07–0.2 ppm) diffused into the

medium for chitosan samples in the film form compared to samples in the paste form (0.4–4 ppm).

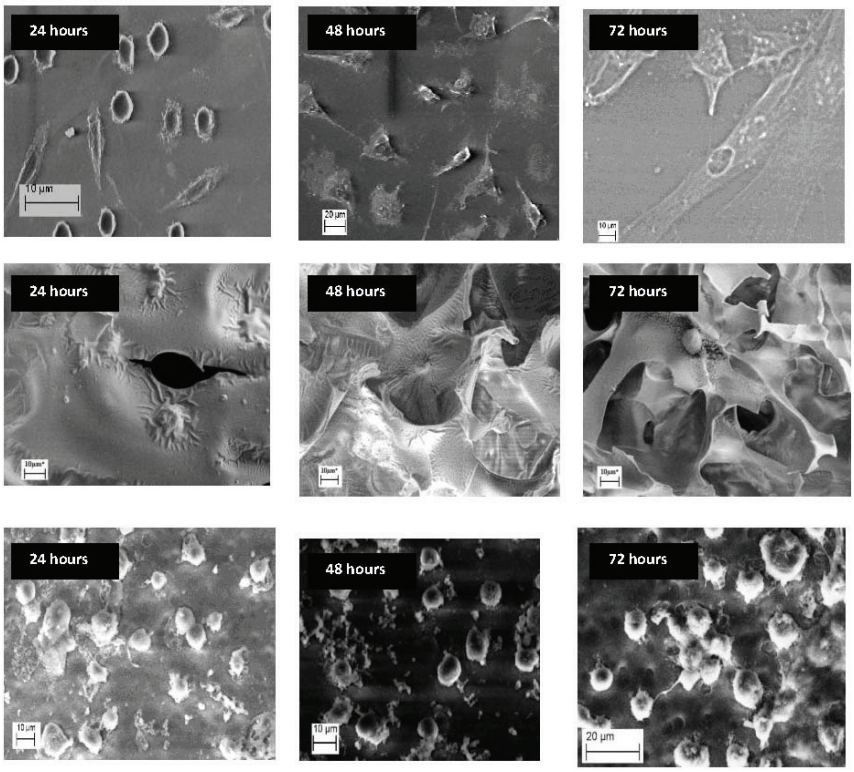

Figure 2: VPSEM micrographs of NHDF cells treated with: [i] the blank (a), (b) and (c); [ii] a chitosan film without silver (d), (e) and (f); and [iii] a chitosan film with silver (g), (h) and (i).

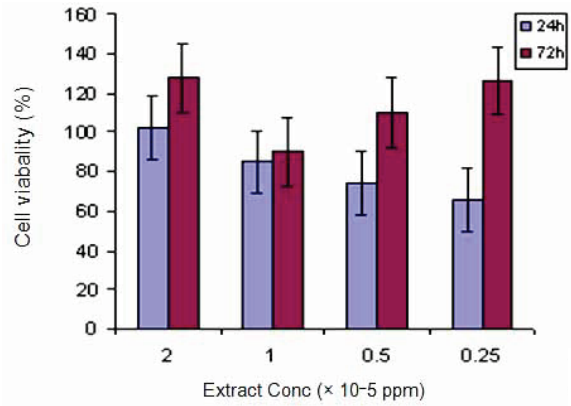

Figure 3: MTT assay results for chitosan powder.

This result is consistent with the fact that paste samples consist of loosely packed polymer chains with a greater polymer free volume and a higher water content. Upon immersion in the extraction medium, water molecules will quickly diffuse into the samples, and hence the higher diffusion of silver nanoparticles into the medium will be faster.

Table 1: Silver ion release from non-water-soluble chitosan film and paste.

Samples	Chitosan % (w/v)	Silver % (w/w)	Day	Concentration (ppm)
NWSC (film)	2.85	3.00	1	0.22
			2	0.11
			3	0.07
NWSC (paste)	2.85	3.00	1	1.93
			2	0.47
			3	4.05

Note: NWSC = non water soluble chitosan

This phenomenon is related to the percentage of viable cells for chitosan preparations containing silver. Figure 4 shows the lower cell viability for water-soluble paste samples (~ 60%) when compared with the non-water-soluble paste samples, which had cell viabilities of more than 80%. The non-water-soluble paste, however, resulted in significant toxicity at the high extract concentration of 2×10^5 ppm when incubated for 72 hours. More than 60% of cells died under these conditions. It is quite surprising that a similar concentration and incubation time gave a high cell viability for the water-soluble paste, which could be due to an experimental error.

Similar observations were made for the film samples (Fig. 5). The non-water-soluble film exhibited a relatively higher percentage of viable cells of 80%–100% at all extract concentrations, whereas approximately 60% cell viability was observed for the water-soluble film samples. Both water-soluble paste samples and film samples gave similar results in which the concentration dependence effect was not clearly observed. A more consistent non-toxic effect was seen for the non-water-soluble film, a result that might be due to the much lower concentration of silver released into the extraction medium by the film. The slightly higher silver concentration that diffused into the extraction medium was the most probable reason for the reduced cell viability for paste samples. It is very important to note that at concentrations as low as 1 ppm, silver can be very active against certain microorganisms. The tolerance level of silver in human body is between 50–200 ppm,[9] and one can safely assume that the silver released from our chitosan samples in paste form is both an effective antimicrobial and within the acceptance level of the human body.

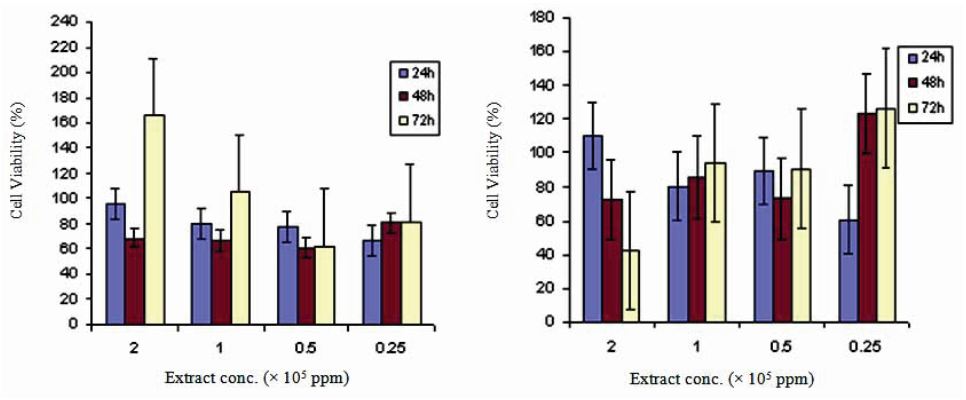

Figure 4: MTT assay results for the paste form of (a) 3 wt.% water-soluble chitosan loaded with 3 wt.% $AgNO_3$ and (b) 2.85% non-water-soluble chitosan loaded with 3 wt.% $AgNO_3$.

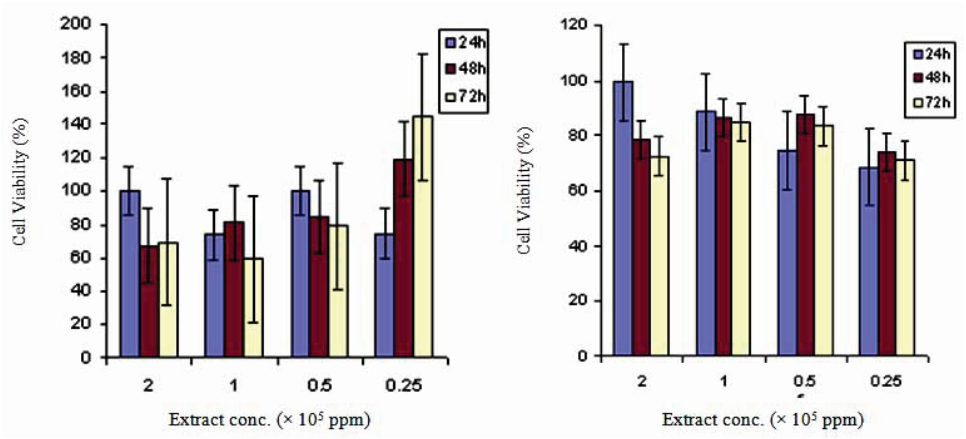

Figure 5: MTT assay results for the film form of (a) 3 wt.% water-soluble chitosan loaded with 3 wt.% $AgNO_3$ and (b) 2.85% non-water-soluble chitosan loaded with 3 wt.% $AgNO_3$.

4. CONCLUSION

The addition of silver nanoparticles to chitosan showed considerable cell cytotoxicity, mainly for water-soluble chitosan paste samples. With a slightly higher silver release rate into the extraction medium, the paste samples resulted in an unhealthy cell morphology. Given that the percentage of viable cells of all

samples was above 60%, the toxicity of the silver nanoparticles is still within the acceptable range for human use.

5. ACKNOWLEDGEMENT

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6. REFERENCES

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