Cytotoxicity of AgNPs/CS composite films: AgNPs immobilized in chitosan matrix contributes a higher inhibition rate to cell proliferation

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ABSTRACT
In this study, the cytotoxicity of silver nanoparticle-doped chitosan composite films (AgNPs/CS) was investigated in vitro. In this slow-release system, the doped silver nanoparticles (AgNPs) might modify both the surface properties of the matrix and the ion environment of the surrounding fluid via slow-release, determining the dominant mechanism is of interest. Here, AgNPs (average size is 25 nm) were doped into chitosan (CS) films by mechanical mixing to form a slow-release system. The surface properties and stabilities of the films and the Ag-releasing behavior were studied by means of X-ray diffraction (XRD), scanning electron microscopy (SEM), UV-visible spectrophotometry, and a weight loss method. The morphology of adhered cells and the survival rate (obtained by both MTT and CCK-8 assays) of human umbilical vein endothelial cells (HUVEC) were employed to describe the cytotoxicity. Using statistical analysis, the following conclusions can be made: the doped AgNPs dispersed in the CS matrix with a polycrystalline structure. During the early erosion, a small amount of debris peeled off and became suspended in the fluid. After that erosion, the composite film became relatively stable, and the doped Ag was slowly released into the fluid. In comparison with the released Ag (either in the peeled debris or dissolved in the fluid), Ag immobilized in the AgNPs/CS films shows a more significant influence on cell adhesion and subsequent proliferation. Film thickness and AgNP content show a synergistic effect on the survival rate of the cell, with the AgNPs content being the key factor.

KEYWORDS
adhesion morphology; agnps; chitosan; cell proliferation; human umbilical vein endothelial cell

Introduction
Silver nanoparticles (AgNPs) are characterized as an efficient broad-spectrum antibacterial agent, and these particles are widely used in many different medical (such as burn dressing, wound care products, and gynecological anti-inflammatory) and consumer products. However, the cytotoxicity and genotoxicity of AgNPs raise risk for patient exposure. The cytotoxicity of AgNPs is directly proportional to the silver concentration, and 5–50 μg/ml AgNPs cause acute toxicity in rat liver cells (BRL 3A). Different types of cells show a different cytotoxic response. However, maintaining the lowest possible concentration of AgNPs would be beneficial for safety. Doping AgNPs into the biomaterial matrix may mitigate the toxic effects via controlling the release rate of Ag ions and retaining the local antibacterial effect. Recent reports show that diverse biomaterials that incorporated AgNPs such as PVA/silver nanocomposite hydrogel, AgNPs-decorated silica on polyaacryamide film, chitosan silver nana composites, yttria-stabilized-zirconia bio-ceramic, dopamine-modified alginate/chitosan (DAL/CHI) polyelectrolyte, AgNPs-hydroxyapatite (HAP) composites and so on, exhibit good antibacterial properties with low or no cytotoxicity. For example, AgNPs/hyperbranched polylysine (HPL) nanocomposites coated on poly(glycolic acid)-based surgical sutures reduced (>99.5%) the adhesion of living Staphylococcus aureus cells for 30 d and was non-cytotoxic to L929 mouse fibroblast cells.

In this study, AgNPs were incorporated into chitosan to improve the biocompatibility and antibacterial ability of an implantable heart pump made of a titanium alloy. In addition to an antibacterial effect, AgNPs in chitosan films will also interact with tissues. Because the doped AgNPs might modify both the surface properties of chitosan and the ion environment of the surrounding fluid via slow-release of ions, determining which of these modifications dominates
the interaction between cells and the film is of interest. In this work, the stability of chitosan in enzyme-free fluid and the release of AgNPs were studied. The cell adhesion state and survival of human umbilical veins endothelial cells (HUVEC) were employed to evaluate the cytotoxicity of both the film and the released AgNPs.

**Experimental results**

**The state of AgNPs**

Before doping, AgNPs are well dispersed in the water solution, and the average particle size is approximately 25 nm (Fig. 1A). The zeta potential of the AgNPs in solution was measured by a Zeta potential analyzer (Zeta SIZER NANO-ZS, Malvern, UK), and this potential is concentration dependent, decreasing from $-1.91 \text{ mV}$ to $-12.5 \text{ mV}$ as the concentration of AgNPs decreases from 0.5 mg/mL to 0.016 mg/mL. When the absolute value of zeta potential is smaller than 30 mV, the nanoparticles in solution are not stable, indicating that when the AgNPs solution is mixed with a chitosan solution, the AgNPs tend to aggregate. These clusters are evenly distributed in the CS film (Fig. 1B), exhibiting a polycrystalline-structured metallic phase (Fig. 1C).

**Stability and release of Ag from the AgNPs/CS films**

Figure 2 shows the variation of the residual mass ratio vs. soaking time in the F12-K cell culture medium. A significant mass loss is found after soaking for 2 d, and the early mass loss of 5% AgNPs/CS is much higher than that of 0.5% AgNPs/CS and CS films. During the subsequent 12 d, the residual mass of each kind of films fluctuates around a certain value (marked as the dashed lines in Fig. 2), and no significant difference is found among different time point by one-way ANOVA analysis. This result implies that Ag doping has significant influence on the early erosion behavior and cause a different mass loss, but after that, the AgNPs/CS films become relatively stable. The paired t-test of the individual samples also gives the same result. For the other 3 types of enzyme-free solution (SBF, distilled water, and serum-free F-12K medium), we found the same result shown in Fig. 2.

The AgNPs/CS film is a slow release system. In a liquid environment, AgNPs may release in conjunction with the swelling or degradation of CS. In the enzyme-free solution, swelling of CS can be observed, while degradation of CS is not found. Early erosion occurs during the swelling process, and then the film becomes stable. The doped AgNPs are released from the AgNPs/CS film in 2 ways: buried in Chitosan erosion debris, or directly released into the medium through a water channel that formed because of swelling. Table 1 and Table 2 provide the evidence. The detected Ag content in the debris-involved solution (Table 1) is much higher than that in the debris-removed solution (Table 2, take sample CS3 as an instance). Excluding the contribution of Ag that is buried in debris, the release of Ag via swelling is progressive and steady.

**Morphology of adhered cells**

Generally speaking, cells will undergo morphological changes to stabilize at the cell–material interface. The whole process progresses in a sequential fashion and consists of cell attachment, filopodial growth, cytoplasmic webbing, flattening of the cell mass and ruffling of the peripheral cytoplasm. Cell adhesion

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**Figure 1.** (A) TEM image of AgNPs before doping; (B) the distribution of AgNPs in the CS film that was obtained by EDX, the white dots represent Ag; (C) the XRD pattern of the AgNPs/CS film.
morphology has been often used to describe the interaction between cells and materials.

The morphologies of adhered HUVEC seeded on the stabilized AgNPs/CS films are recorded in Fig. 3. These images include some rather large changes. Both the Ag content and film thickness affect the morphology of adhered cells. To describe this parameter quantitatively and with statistical rigor, the cell spreading area was measured and was used as an index to describe cell spreading (see Fig. 4, A1-A4). Moreover, the cell was modeled as an ellipse, and the ellipticity (long/short axial ratio) was used to describe the shape of the cells (see Fig. 4, B1-B4). In Fig. 4, the detailed data are plotted and fitted with Gaussian and Extreme Value Distributions. Here, CS films that are not doped with Ag have similar distributions to the control (cells seeded in culture plate), which indicates that the cells’ adhesion state on these CS films is normal; the CS film itself has little influence on cell adhesion.

However, the distribution curves of cells on the AgNPs/CS films deviate from that of the control film. When the AgNPs doped content and film thickness is increasing, the peak values of both the area and ellipticity distribution curves shift leftward. Especially when the content of Ag exceeds 5% (v/v), the spreading area for most cells is less than 2000 μm² and the ellipticity is approximately 1. This result illustrates that most of the seeded cells are not spreading well and that the shape of the cells changes from spindle to round, which means that the cells are poorly adhered on the AgNPs/CS films. When the doping content of Ag is less than 0.5%, the shapes of the cells remain normal, but the average spreading area of the cells becomes smaller than that of the control as the film thicknesses increase. Furthermore, one-way ANOVA results show that films with thicknesses of 20 μm and 44 μm have a significant impact on cell morphology, which can be explained by surface energy.16,17 Because a lower film thickness contributes to higher surface energy, this higher surface energy enhances the adhesion of HUVEC.

Both Ag content and film thickness influence cell adhesion, and these parameters have a cooperative effect. An interaction analysis between film thickness and AgNPs content using SPSS 18.0 software indicates that the Ag component is the key factor for the morphology of adhered HUVEC (see Fig. 5). This result means that the adhesion state of HUVEC is more sensitive to the content of AgNPs than film thicknesses in these AgNPs/CS films.

### Cell survival of HUVEC

The proliferation of HUVEC on stabilized AgNPs/CS films, in a debris-containing medium, and in a solution of AgNPs was studied by both the MTT and CCK8 assay to determine the main factor that inhibits the cells in the AgNPs/CS films. Figure 6 shows the MTT results of HUVEC survival rate after 24 h of seeding, which agree with the same law that was obtained by the CCK8 assay. Figure 6A exhibits the survival rate of HUVEC that were seeded on AgNPs/CS films. Before using the films, these films were soaked in distilled water for 4 d to allow them to stabilize and to avoid producing erosion debris during the cell culture process. All of the films show a significant inhibition effect on the proliferation of HUVEC, and this type of

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**Table 1. AgNPs total content in the debris-involved solution.**

| Sample name | Concentration(μg/mL) | Releasing rate |
|-------------|---------------------|----------------|
| CS1         | 0.1415 ± 0.133      | 37.14% ± 3.491%|
| CS2         | 0.1618 ± 0.085      | 4.247% ± 0.223%|
| CS3         | 0.4623 ± 0.061      | 1.214% ± 0.016%|

**Table 2. Concentration of AgNPs released into liquid from CS3 (debris-free).**

| Time   | 2D   | 14D  | 30D   |
|--------|------|------|-------|
| OD (10⁻³) | 3.77 ± 0.56 | 7.11 ± 0.24 | 12.71 ± 0.53 |
| released Ag (μg/mL) | 0.028 ± 0.004 | 0.052 ± 0.002 | 0.094 ± 0.004 |
| Ag release rate | 0.028% | 0.052% | 0.094% |
inhibition effect is strengthened by increasing either the Ag doped content or film thickness.

The survival rate of HUVEC cultured in AgNPs/CS erosion-F12K medium is shown in Fig. 6B. In this situation, the medium contains the debris that peeled early in the erosion of the corresponding films. A 2-way ANOVA analysis shows that both the AgNPs content and film thickness influence the cell survival rate, with the AgNPs content again playing a more important role.

Figure 3. Images of HUVEC after being seeded on AgNPs/CS films for 24 h with various amounts of AgNPs and film thicknesses. Pure chitosan films are labeled as CS, and films with AgNPs amounts of 0.05% (v/v), 0.5% (v/v) and 5% (v/v) are labeled as CS1, CS2 and CS3, respectively.

Figure 4. Area and ellipticity distributions of HUVEC seeded on AgNPs/CS films for 24 h. The fitted curves of the area distributions of CS, CS1, CS2, CS3 are shown in A1, A2, A3, A4, respectively. The fitted curves of the ellipticity distributions are shown in B1, B2, B3, and B4. AgNPs content and film thickness have a cooperative influence on the morphology of adhered HUVEC.

Figure 6C shows the relationship between the cell survival rate and the concentration of AgNPs that are released into the cell culture medium (without erosion debris). HUVEC are sensitive to Ag nano-particles, as illustrated by 10% of the cells being inhibited even when the concentration of Ag is as low as 0.0005 μg/mL. No significant difference is found when the concentration of AgNPs range from 0.0005 μg/mL to 5 μg/mL. According to our previous work, a significant impact
on the survival rate of HUVEC will be observed when the concentration of AgNPs exceeds 0.25 mg/mL.

Discussion

From the above experiments, we can describe the interaction process between cells and AgNPs/CS films as follows:

When the AgNP/CS film contacts the cells in the cell culture medium, the film will swell and a part of the film might peel off from the surface and become suspended in the solution. The quantity of film debris is related to the content of AgNPs, as well as the film thickness. Then, the swelled film becomes stable, and AgNPs are slowly released from the film through the water channel that forms during swelling. However, AgNPs are only slightly released to the solvent (70 ppm according to the reference), and the concentration of released Ag is not high (max. 0.094 μg/mL here in our experiment). Therefore, the proliferation of cells faces 3 inhibitory factors: the film itself, debris, and released Ag. Which of the 3 will be the key factor? Table 3 summarizes the whole experimental results of the 20 μm- and 40 μm- films on the cell survival rate. The results in this table imply that during the whole interaction process, the AgNPs/CS film itself has the most impact on the cells, followed by the debris. Because the release rate of AgNPs is rather low, the influence of released Ag is the weakest.

The inhibitory effect of the AgNPs/CS film is attributed to the cytotoxicity of the AgNPs, and the modification of physical properties such as electrostatics and hydrophilicity. The adhesion and spreading of cells are restrained, and the subsequent proliferation is thus inhibited.

Summary: A film composed of AgNPs doped in a CS coating by mechanical mixing exhibits metallic attributes. During the early erosion of these AgNPs/CS films in enzyme-free liquids, a part of the AgNPs separated from the film with the erosion debris, and the film became relatively stable.

Figure 5. Interaction between film thickness and AgNPs content on adhesion morphology of HUVEC seeded on AgNPs/CS films for 24h. (A) estimated marginal means of HUVEC area from AgNPs content. (B) estimated marginal means of HUVEC area from film thickness. (C) estimated marginal means of HUVEC ellipticity from AgNPs content. (D) estimated marginal means of HUVEC ellipticity from film thickness. The AgNPs content has a larger effect than film thickness.

Figure 6. Survival rate of HUVEC. (A) seeded on AgNPs/CS films, normal culture; (B) seeded on culture plate, cultured with erosion-F12K medium (erosion debris contained); (C) seeded on culture plate, cultured in AgNPs contained medium (simulate the Ag releasing environment). In comparison with the blank (cells seeded on a culture plate, normal culture), ** represents P < 0.01, * represents P < 0.05. The data are expressed as the means ± SD (n = 8).
Subsequently, tiny amounts of AgNPs were slowly released into the surrounding liquid through a swelling channel in the CS. Most of the doped AgNPs remained in CS films and restrained cell proliferation via inhibiting the stretching and adhesion behavior of the HUVEC.

Materials and methods

Materials and reagents

HUVEC were obtained from the ATCC cell bank. AgNPs solution (Silver nano-monomer solution, PH = 7.2, concentration of 10,000 ppm) was purchased from Shanghai Hu Zheng Nano Techco. Ltd. Chitosan (CS), (C6H11NO4)n, with a deacetylation degree over 90% and molecular weight (M) of 200 kDa, was purchased from the Zhejiang Aoxing Biotechnology Co. Ltd., China. F-12K (NUTRIENT MIXTURE F-12 HAM KAIGHN’S) medium, phosphate buffered saline (PBS) and 0.25% trypsin with 0.02% EDTA were purchased from Keno biomedical Technology Co., Ltd. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing biological engineering materials Co., Ltd. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma (No. M2128). CCK-8 reagents were purchased from Dojindo Molecular Technologies, Inc., Japan. DMSO was purchased from Enox. Penicillin-Streptomycin for Cell Culture (ST488) was purchased from Beytime. All other reagents were of analytical grade and purchased from commercial sources in China.

Preparation of AgNPs/CS films

A series of AgNPs/CS films were obtained by mechanically agitating the admixture of AgNPs (10,000 ppm) and 10 mg/mL Chitosan solution (dissolved in 2% (v/v) acetic acid) at room temperature with different volume percentages of AgNPs (0%, 0.05%, 0.5%, 5% separately), and subsequently applying these mixtures to glass substrates (24 × 24 mm²). After drying at 60°C for 6 h, the samples were deacidified by 0.1 mol/L NaOH and dried again. The thicknesses of the films were controlled by the volume of the mixture added to the substrate and measured by a Profilometer (Dektak 6M, Veeco, USA). Films with thicknesses of 44 μm, 20 μm, 8.0 μm, 4.2 μm and 1.4 μm were prepared. Films were labeled as CS, CS1, CS2 and CS3 according to the volume fractions of AgNPs, which were 0%, 0.05%, 0.5%, 5%, respectively.

Surface properties

The state of Ag

The state of AgNPs before doping was observed with a JEM-2100 transmission electron microscope (TEM). The phase structure of the composite film was analyzed by X-ray diffraction (XRD), and the distribution of AgNPs was qualitatively described by an Energy Dispersive X-ray Detector (EDX) that was installed in the JSM-5610 scanning electron microscope (SEM).

Stability in the enzyme-free fluid

The stability of the AgNPs/CS composite film in enzyme-free fluids was studied by the weight loss method. The residual mass ratio defined as follows was used to characterize the stability of the AgNPs/CS films:

\[ A = \frac{m}{m_0} \]  

Where, \( A \) represents the residual mass ratio after a certain soaking time, and \( m_0 \) and \( m \) respectively denote the initial and residual dry weight of the AgNPs/CS films before and after soaking. The enzyme-free fluids used in these experiments were simulated body fluid (SBF),\(^{18}\) distilled water, F-12K medium (containing 10% FBS, used for HUVEC culture), and serum-free F-12K medium (Sigma).

Release rate of AgNPs

After soaking in SBF for 2 d, the fluid was taken out and treated with a solution containing 500 μl HNO₃ (65%) to remove CS and to let Ag be released from the

| Table 3. Survival rate of HUVEC cultured in several media with a thickness of 44 μm. |
|---------------------------------|---|---|---|---|---|---|---|---|
|                                | CS | CS1 | CS2 | CS3 | CS | CS1 | CS2 | CS3 |
| **SR(%)**                      | 20 μm | 40 μm | 20 μm | 40 μm | 20 μm | 40 μm | 20 μm | 40 μm |
| Film                           | 0.85 ± 0.03 | 0.87 ± 0.06 | 0.86 ± 0.05 | 0.75 ± 0.10 | 0.17 ± 0.01 | 0.15 ± 0.02 | 0.14 ± 0.02 | 0.13 ± 0.02 |
| Debris                         | 0.98 ± 0.14 | 0.96 ± 0.14 | 0.90 ± 0.14 | 0.86 ± 0.14 | 0.55 ± 0.10 | 0.52 ± 0.09 | 0.40 ± 0.10 | 0.38 ± 0.10 |
| Released Ag                    | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 | ≥0.87 ± 0.12 |
erosion debris. Then, the content of Ag was detected with ICP IRIS intrepid II XSP (TJA, USA).

In order determine the actual amount of AgNPs that were released, a CS3 film was soaked in SBF for 2, 14, and 30 d, and the fluid was removed and centrifuged at 1200 rpm/min to separate the erosion debris. The absorbance of the supernatant was measured by a UV-spectrophotometer (Agilent 8453) at 410 nm, and the Ag concentration was subsequently determined by comparing the absorbance in the supernatants to a standard curve.

**Cytotoxicity**

HUVEC were cultured in a F-12K medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics mixture (100 U/ml penicillin and 100 µg/ml streptomycin). The harvested cells were cultured under 3 different conditions: For the first condition, the cells were seeded directly on the AgNPs/CS films at a density of $5 \times 10^3$ cells/cm² and cultured in a normal medium. Before using the films, these films were stabilized by soaking them in distilled water for 4 d and then were sterilized under ultraviolet light for 4 h. In the second condition, the cells were seeded in culture plate at a density of $1.56 \times 10^4$ cells/cm², and cultured in the “AgNPs/CS erosion-F12K medium.” Here, the “AgNPs/CS erosion-F12K medium” was obtained by soaking the AgNPs/CS in the F-12K medium for 48 h. During this period of time, fragments peeled from the film due to early erosion and existed in the medium. In the third condition, cells seeded on the culture plate ($1.56 \times 10^4$ cells/cm²) were cultured in the nanoAg-containing medium, which was obtained by adding a certain amount of AgNPs to the cell culture medium. All the cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. The cell morphology was observed by optical microscopy 24 h later, and the cell quantity was determined by both the CCK-8 (Dojindo Molecular Technologies, Inc., Japan) assay¹⁷ and MTT (Sigma) assay¹⁸. The relative survival rate (SR) of the cells was calculated by the following equation:

$$SR = \frac{E_{O.D.}}{C_{O.D.}} \times 100\%$$  (2)

Where, $E_{O.D.}$ and $C_{O.D.}$ respectively represent the O. D. value (proportional to cell number) of the experimental groups and control groups that were recorded from the MTT or CCK-8 assays.

**Statistical analysis**

SPSS18.0 was used to conduct statistical tests, including the t-test and one/2-way ANOVA analysis. Sig. <0.05 means a significant difference, and Sig. <0.01 means an extremely significant difference. Image J was used for statistical analysis of cell morphology. In this experiment, the cell spreading area and ellipticity (Long/short axial ratio) were surveyed.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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