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Preparation of silver nanoparticles with antimicrobial activities and the researches of their biocompatibilities

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Abstract Silver nanoparticles were prepared by chemical reduction method using chitosan as stabilizer and ascorbic acid as reducing agent in this work. The silver/chitosan nanocomposites were characterized in terms of their particle sizes and morphology by using UV spectrophotometer, nano-grainsize analyzer, and transmission electron microscopy. Antibacterial activities of these nanocomposites were carried out for *Staphylococcus aureus* and *Escherichia coli*. The silver nanoparticles exhibited significantly inhibition capacity towards these bacteria. Detailed studies on the biocompatibility of the silver/chitosan nanocomposites were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and cell adhesion test. The results indicated that these silver/chitosan nanocomposites were benefit for the proliferation and adhesion of L-929 cells, and the biocompatibilities between the nanocomposites and the cells would become better with the culturing days. We anticipated that these silver/chitosan nanocomposites could be a promising candidate as coating material in biomedical engineering and food packing fields wherein antibacterial properties and biocompatibilities are crucial.

1 Introduction

Due to the excellent antibacterial activities, silver nanoparticles are widely used in biomedical fields to control infections [1–8]. However, silver nanoparticles as antimicrobial agent are now gaining increasing appeal for medical applications because of their negative impact to the biocompatibility, for example, causing thrombogenic effect [9–11]. However, no sufficient information is available on the detailed molecular mechanics of silver nanoparticles toxicity [12]. Thus, the investigation of antibacterial silver nanoparticles with good biocompatibilities is now coming into our consideration.

To prepare silver nanoparticles, chemical reduction is the most extensively applied and the most promising industrial application method due to its simpleness and high yield [13]. Based on the principle of "green synthesis" [14], we reviewed the chemical methods in previous researches, and chose chitosan as stabilizer and ascorbic acid (VC) as reducing agent to prepare silver nanoparticles in this work. Chitosan, which exhibits excellent biocompatibility, biodegradability, antibacterial and antifungal activities, was used to prepare silver nanopartilces in many researches [15–18]. Besides, VC is considered to be one of the best reducing agents candidates in many pharmacological reactions with its mild reducing capability and non-hazardous performance [19, 20]. However, of all the previous research to prepare silver nanoparticles, only few works focused on the toxicity and the biomedical performance of these silver nanoparticles [5, 21, 22].

In this work, by using chitosan and VC, silver/chitosan nanocomposites were prepared. The best preparation conditions were systematically investigated and the antibacterial activities of these silver/chitosan nanocomposites were presented by using *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). In addition, the biocompatibility of these silver/chitosan nanocomposites was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell adhesion test. The results indicated that these silver/chitosan nanocomposites

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showed good cell compatibility, as the nanocomposites were benefit for the growth and proliferation of L-929 cells.

Therefore, we anticipate that by preparing silver nanocomposites with chitosan and VC, effective, non-toxic and inexpensive antibacterial material within good biocompatibility may be obtained, and our consideration about the biomedical performance of the silver nanoparticles would give some guidance for the extension of silver nanoparticles in biomedical fields.

2 Materials and methods

2.1 Materials

Chitosan (Mw, 10,000; deacetylation degree of 90%) was purchased from Boao Technology Co. Ltd (Shanghai, China). Silver nitrate ($AgNO_3$) and vitamin C (VC) were obtained from Country medicine group (ChengDu, China). Beef extract and peptone were provided by RongHai biological reagent company (ChengDu, China). Calf serum—clarified and DMEM culture fluid were purchased from YunHong biological reagent company (ChengDu, China). All the other reagents were obtained from Chemical Reagent Factory of Kelong (ChengDu, China), which were of analytical grade, and used without further purification.

2.2 Preparation of silver/chitosan nanocomposites

Silver/chitosan nanocomposites were prepared by the direct reduction of $AgNO_3$ with VC. Firstly, 1 ml $AgNO_3$ aqueous solution of determined concentration was added to 10 ml chitosan acetic acid solution (1%, w/w) to obtain homogeneous solution. After magnetic stirring for 30 min, 1 ml VC solution (the concentration was the same as $AgNO_3$ solution, pH value was adjusted to 6) was added dropwise into the mixture, then the reaction continued for another 60 min while stirring under the determined temperature. After that, the silver/chitosan nanocomposites were prepared.

To discuss the affecting factors of the reducing reaction, including concentration of $AgNO_3$ solution, concentration of chitosan and the temperature, samples with different conditions were prepared by using the same procedure mentioned above, as shown in Table 1.

2.3 Characterization of the silver/chitosan nanocomposites

The colors of the obtained silver/chitosan nanocomposites were observed by Kodak digital camera in this study. UV spectrophotometer (Shanghai Spectrum Instrument Factory), with matching analysis software of spwin-uvs was employed to detect the adsorption peaks of the nano-silver. 2 ml solution was aspirated into the quartz cuvette, and detected at the scanning range of 350–600 nm. The particle size of the silver nanoparticles was measured by a nano-grainsize analyzer (NPA 150, analysis range: 0.8–6.5 nm), and the results were analyzed by a Nano analysis software. Transmission electron microscopy (TEM) observations were carried out on a JEM 100C × II instrument.

2.4 Antibacterial activity tests

2.4.1 Qualitative methods: inhibition zone method

40 μl-silver/chitosan nanocomposites was smeared onto nylon membranes (diameter = 8.0 mm), and were dried in a vacuum oven. Then the membranes were immersed into NaOH solution (2 mol/l) to neutralize the unreacted acetic acid. After that the membranes were washed with plenty of deionized water and dried at room temperature before use. The resulted silver nanoparticles-coated-nylon membranes were placed in the middle of the culture dishes with 200 μl

Table 1 Samples of silver/chitosan nanocomposites prepared under different conditions

Affecting factor	Concentration of $AgNO_3$ (mM/ml)	Concentration of chitosan (g/ml) (%)	Temperature (°C)	Code
Concentration of $AgNO_3$	0.0035	1	100	$(Ag^0)_{0.0035}$–CS
	0.0050	1	100	$(Ag^0)_{0.0050}$–CS
	0.0075	1	100	$(Ag^0)_{0.0075}$–CS
Concentration of chitosan	0.0050	0.2	100	Ag^0–$(CS)_{0.2\%}$
	0.0050	0.5	100	Ag^0–$(CS)_{0.5\%}$
	0.0050	1.0	100	Ag^0–$(CS)_{1.0\%}$
	0.0050	2.0	100	Ag^0–$(CS)_{2.0\%}$
Temperature	0.0050	1	20	Ag^0–CS-20
	0.0050	1	40	Ag^0–CS-40
	0.0050	1	80	Ag^0–CS-80
	0.0050	1	100	Ag^0–CS-100

suspension of *S. aureus* and *E. coli* bacteria, respectively. Then the samples were incubated at 37°C for 24 h. The presence of any clear zone that formed around the film disk was observed and recorded by a digital camera. The culture dishes with chitosan coated membrane and without membrane were also prepared as a control.

2.4.2 *Quantitative methods: the minimum inhibitory concentration (MIC)*

The culture dishes with agars were prepared as mentioned in inhibition zone method. The silver/chitosan nanocomposites solution was diluted with chitosan solution, and the diluted ratios were settled as follows: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32 (v/v). Then, 1 ml of the diluted solution was smeared on the agar, and the samples were incubated at 37°C for 24 h. The culture dishes without the diluted solutions were also prepared as a control.

2.5 Biocompatibility test

2.5.1 *Cytotoxicity test: MTT method*

After cell culture for 1, 3, 5, 7 days, the L-929 cells was determined by MTT assay. 40 μl silver/chitosan nanocomposties solution was smeared on the slide (8 × 8 mm) and dried in a vacuum oven for 12 h. Then the slides were immersed into NaOH solution (2%, wt%) for 1 h to neutralize the unreacted acetic acid. After that the membranes were washed with plenty of distilled water and dried at room temperature before use. The resulted membranes were disinfected with ozone and UV. The L-929 cells were seeded onto the membranes at a density of approximately 5×10^3 cells/well. After various time intervals, 20 μl MTT solution (1 mg/ml in the test medium) was added to each well and incubated for 5 h at 37°C. Mitochodrial dehydrogenases of viable cells cleave selectively to the tetrazolium ring, yielding blue/purple formazan crystals. Dimethyl sulfoxide (DMSO) of 200 μl was added to dissolve the formanzan crystals. The solution of each sample was moved into 96 wells cell cultures for examination by Microplate reader (Model50, Bio Rad Corp.) at 570 nm. All the experiments were repeated five times, and the value of the RGR (relative growth rate) can be calculated by the following formula:

$$\text{RGR} = \text{absorbance values of the experimental group} / \text{absorbance values of the negative control group} \times 100\%$$

2.5.2 *Cell adhesion experiment*

The samples were prepared and cultured as mentioned in the MTT test. After determined days, the membranes were immediately rinsed with PBS and fixed in 3 wt% glutaraldehyde in PBS at 4°C for 1 h. The fixed samples were dehydrated through exposure to a gradient of alcohol (30, 50, 70, 80, 90, 95, and 100%). The critical point drying of specimens was undertaken with liquid CO_2. The specimens were sputter-coated with gold and examined by an S-2500C microscope (Hitachi, Japan).

3 Results and discussion

3.1 Preparation of the silver/chitosan nanocomposites

In this work, several silver/chitosan nanocomposites had been prepared under different conditions, as shown in Table 1. The growth and the size of the nanoparticles were discussed, and the best preparation condition of the chitosan–silver nanoparticles composites was summarized.

3.1.1 *Effect of the concentration of $AgNO_3$ solution*

Presentation color of the silver nanoparticles has a relationship with the size and the shape of the particles. According to a previous research, the phenonenon that the solution colour changed into red, green and brown, could indicate the growth of the particles. In this study, with the increase of the $AgNO_3$ concentration, the colour of the silver nanoparticle solution changed from light yellow to golden yellow, and finally to red yellow, which indicated the growth of the nano-sliver particles. TEM was employed to observe the morphology of the nano-slivers, as shown in Fig. 1a. The appearances of the nano-silvers were round and with a regular shape. The particle size of the chitosan–silver nanoparticles composits were investigated by UV adsorption. As can be seen in Fig. 1b, the adsorption peak of the silver nanoparticles particles was between 400 and 420 nm. With the increase of the silver concentration, the adsorption peak shifted to right and gradually widened. It has been reported that the adsorption peak sites were basically related to the particle size [23]. The smaller the particle was, the greater the adsorption peak tended to the left. Besides, the more extensive size distribution was, the wider the adsorption peak would be. Thus, from the results of the UV adsorption, it can be conclued that, with the increase of the $AgNO_3$ concentration, the particle size of the silver nanoparticles increased and the particle size distribution became wider. This could also be proved by using a nano-grainsize analyzer. As shown in Fig. 1c, with the increasing of the silver concentration from 0.0035 to 0.0075 mmol/ml, the average diameter of the nano particles increased from 21.0 to 110 nm and the distribution of the nano-silver particles became wider.

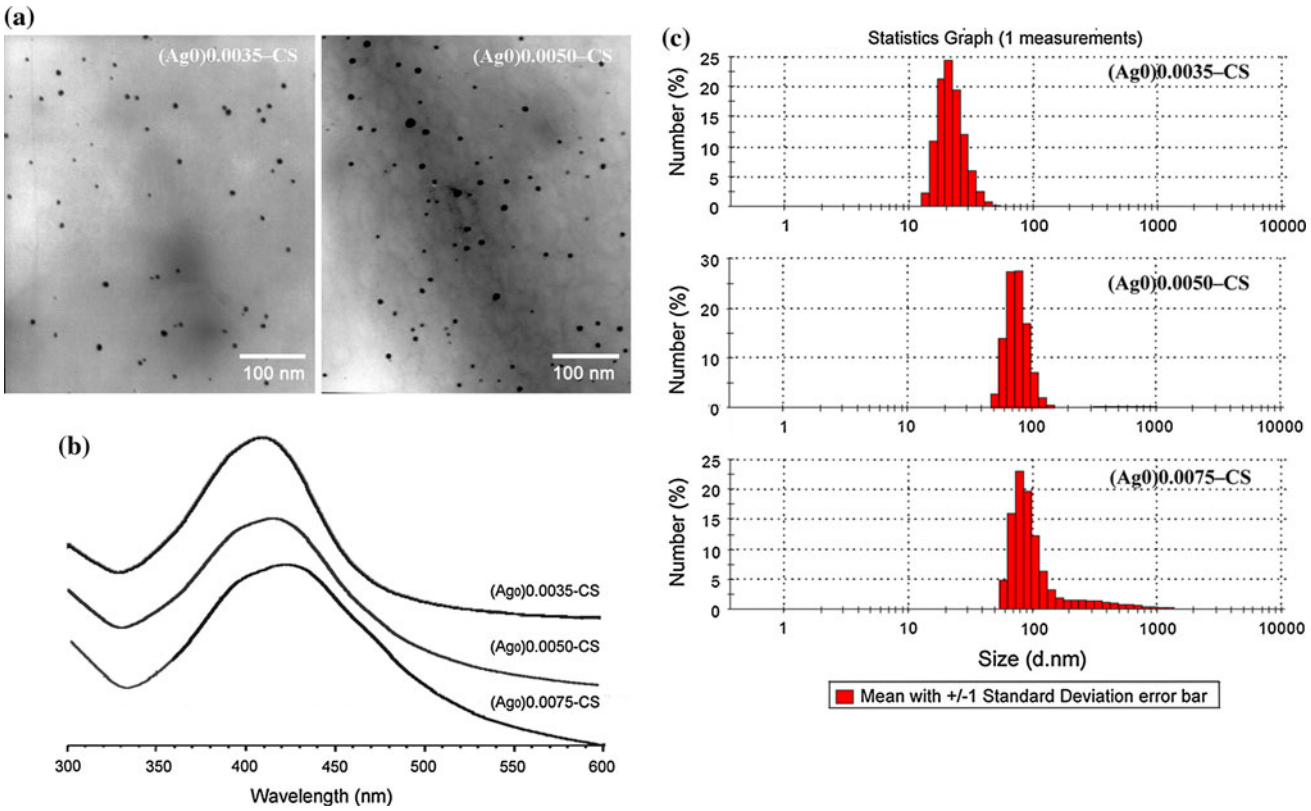

Fig. 1 **a** TEM images of the silver/chitosan nanocomposites. **b** UV absorbance of the silver/chitosan nanocomposites. **c** Distribution curves of the silver/chitosan nanocomposites

3.1.2 Effect of the concentration of chitosan

When the chitosan concentration varied from 0.2 to 2% (g/ml), the color of the solution changed from light green to brown. However, when the concentration was higher than 1% (g/ml), as for Ag^0–$(CS)_{2.0\%}$, the solution became very viscous, and the UV adsorption peaks were wide and random. This phenomenon may be related to the molecular weight, and the chelate state of the chitosan and the silver ions [15]. Therefore, the chitosan solution of 1% (m/m) was considered to be the best choice for preparing the silver/chitosan nanocomposites in this study.

3.1.3 Effect of the temperature

The boiling temperature played an important role in the process of the silver ion reduction using VC [24]. In this study, the temperature increased from 20 to 100°C. When the temperature increased to 100°C, the nanosliver particles with regular shape were obtained.

From the discussion mentioned above, the best preparation condition of the chitosan–silver nanoparticles composites could be generalized: chitosan solution (1%), silver nitrate solution (0.0035 to 0.0075 mmol/ml, same with the VC solution), and the reaction temperature (100°C).

3.2 Antibacterial activity tests of chitosan–silver nanoparticles composites

3.2.1 Qualitative method: inhibition zone method

The antibacterial activity of the chitosan/Ag nanocomposites as coating material was tested toward *S. aureus* and *E. coli* by the inhibition zone method. As shown in Fig. 2, in spite of the antibacterial activity of chitosan [17], no inhibition zone was obtained around the chitosan coated nylon membrane. This phenomenon was due to the neutralization of acid groups by NaOH during the treating procedure, which made the chitosan lose its positive charges and its antibacterial properties. Compared to the chitosan, the silver/chitosan nanocomposite showed significant inhibition effect on both gram-negative bacteria (*E. coli*) and gram-positive bacteria (*S. aureus*), wherein, the size of its inhibition zone was 2.0 and 1.5 mm, respectively. The results demonstrated that the antibacterial activity of the composites came from the silver nanoparticles. Furthermore, images of the bacteria on the silver/chitosan nanocomposites by using SEM or AFM observation were not shown, since the morphology of the bacterial was not change in the anti-bacterial activity.

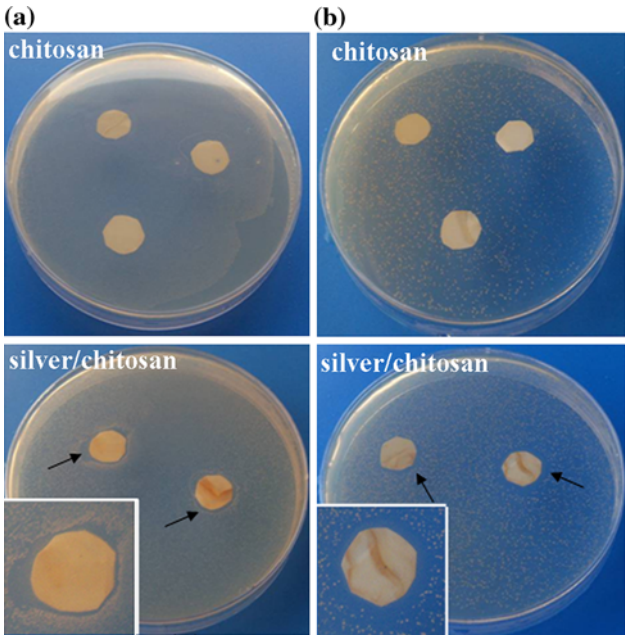

Fig. 2 Antimicrobial activities of chitosan coated nylon membrane and silver/chitosan nanocomposites coated nylon membrane against **a** *E. coli* and **b** *S. aureus*

3.2.2 *Quantitative method: the minimum inhibitory concentration*

The MIC was defined as the lowest silver/chitosan nanocomposites concentration resulting in the lack of visible microorganism growth. As shown in Table 2, the results indicated that both chitosan and silver/chitosan nanocomposites had inhibition effects to *E. coli* and *S.aureus*. Because of the good antibacterial property of the silver nanoparticles, the silver/chitosan nanocomposites expressed much stronger antibacterial capability than the chitosan solution.

Table 2 Results of the MIC test for the silver/chitosan nanocomposites against *E. coli* and *S. aureus*

Samples	Bacterial	Dilution ratio					
		1/1	1/2	1/4	1/8	1/16	1/32
$[(Ag^0)_{0.0035}–CS]$	*E. coli*	−	−	−	−	−	+
	S. aureus	−	−	−	−	+	+
$[(Ag^0)_{0.0050}–CS]$	*E. coli*	−	−	−	−	+	+
	S. aureus	−	−	−	+	+	+
$[(Ag^0)_{0.0075}–CS]$	*E. coli*	−	−	−	+	+	+
	S. aureus	−	−	+	+	+	+
CS	*E. coli*	−	−	+	+	+	+
	S. aureus	−	+	+	+	+	+

"−" there is no growth for the bacterial, "+" there is growth for the bacterial

For the silver/chitosan nanocomposites, when the concentration of the silver ions increased, the inhibition ability against both *E. coli* and *S. aureus* decreased, and the MIC of the composites increased. This could be explained by the discussion mentioned above. When the concentration of the silver ions increased, the size of the obtained nano-sliver particles would increase. However, the particles with larger size have smaller surface area and smaller infiltration rate, which could have worse antibacterial effect. Moreover, the shape of the silver nanoparticles ions also affected the antibacterial activity. If the atomic density of the polyhedron surface was higher, the antibacterial effects were more obviously [14].

Besides, it can be seen from Table 2 that the *E. coli* was more sensitive to the composites than *S. aureus*, this phenomenon was associated to the structure of the cells [14]. The cell walls of the negative bacteria were much thinner, approximately 10–15 nm, compared with 20–80 nm of the positive bacteria's cell wall. Thus, the silver ions penetrate the thin wall of the negative bacteria more quickly and express its antibacterial capability more easily.

3.3 Biocompatibility of the silver/chitosan nanocomposites

3.3.1 *MTT test*

Cytotoxicity test is one of the most important indicators of the evaluation system for medical device. In this study, MTT method was used for testing the cytotoxicity grade of the silver/chitosan nanocomposites. According to the RGR mentioned above, the toxicity grade of material was divided into 5 levels: grade 0: RGR $\geq$ 100%; grade 1: 100% $>$ RGR $\geq$ 75%; grade 2: 75% $>$ RGR $\geq$ 50%; grade 3: 50% $>$ RGR $\geq$ 25%; grade 4: 25% $>$ RGR $\geq$ 1%; grade 5: RGR $=$ 1%, and only the grade 0 or 1 indicated the qualified level. As can be seen in Table 3, the cell toxicity of the silver/chitosan nanocomposites were in grade 1 or 0 during the culturing process. According to the standard toxicity rating, 0 and 1 grade were low toxicity in the framework of safety use. Moreover, the toxicity grade in the first and third days were grade 1, and with the culturing days the RGR decreased to grade 0. This was an expected result, which suggested that the silver/chitosan nanocomposites had little harm to the growth of the cells and the biocompatibilities between the nanocomposties and the cells would become better with the culturing time.

It can also be seen from Table 3 that, there was no significant difference in the toxicity grade between the silver/chitosan nanocomposites and the control group of chitosan solution. Recently, many studies concerned with the chitosan based on its good biocompatibility, non-toxicity and adsorption properties [25–29]. Therefore, it could be

Table 3 MTT test for the silver/chitosan nanocomposites

Samples	Culturing days	RGR (%)	Toxicity grade
$[(Ag^0)_{0.0035}–CS]$	1	80.1	1
	3	89.6	1
	5	103.4	0
	7	105.4	0
$[(Ag^0)_{0.0050}–CS]$	1	88.0	1
	3	93.8	1
	5	109.0	0
	7	118.3	0
$[(Ag^0)_{0.0075}–CS]$	1	93.3	1
	3	96.2	1
	5	107.0	0
	7	110.3	0
CS	1	94.0	1
	3	97.0	1
	5	104.7	0
	7	108.0	0

estimated that, the additional of chitosan into the silver/chitosan composites would somehow compensate the negative impact of the silver nanoparticles to the biocompatibilities.

3.3.2 *Cell adhesion test*

The toxicity of material can be also reflected by morphological changes of the cells which grew on the surface of material [30]. When the materials were non-toxic, the numbers and the morphology of the cells would keep unchanged. On the other hand, when the materials were toxic, the cells would be seriously distorted. In this study, the cells were grown on the surfaces of silver/chitosan nanocomposites coated membranes and the surfaces of chitosan covered membranes, respectively. By observing the growth of the cells, as shown in Fig. 3, it could be seen that the cell morphologies were triangle and spindle, and the cells extended pseudopodia to adhere onto the material and grew by monolayer. With the extension of culturing time, the cells proliferated quickly, with some differentiating fibroblasts showing a circular shape. It can also be seen in the Fig. 3 that there was no significant differences in the morphology and the quantity among the cells, which grew on the surfaces of silver/chitosan nanocomposites, chitosan and the control group. The good growth of the cells on the composite membrane proved that the implanted Ag had no negatively impact on the cell growth, and with the culturing days the cell proliferated well. These results demonstrated that, the antibacterial silver/chitosan nanocomposites prepared in this study had good compatibility

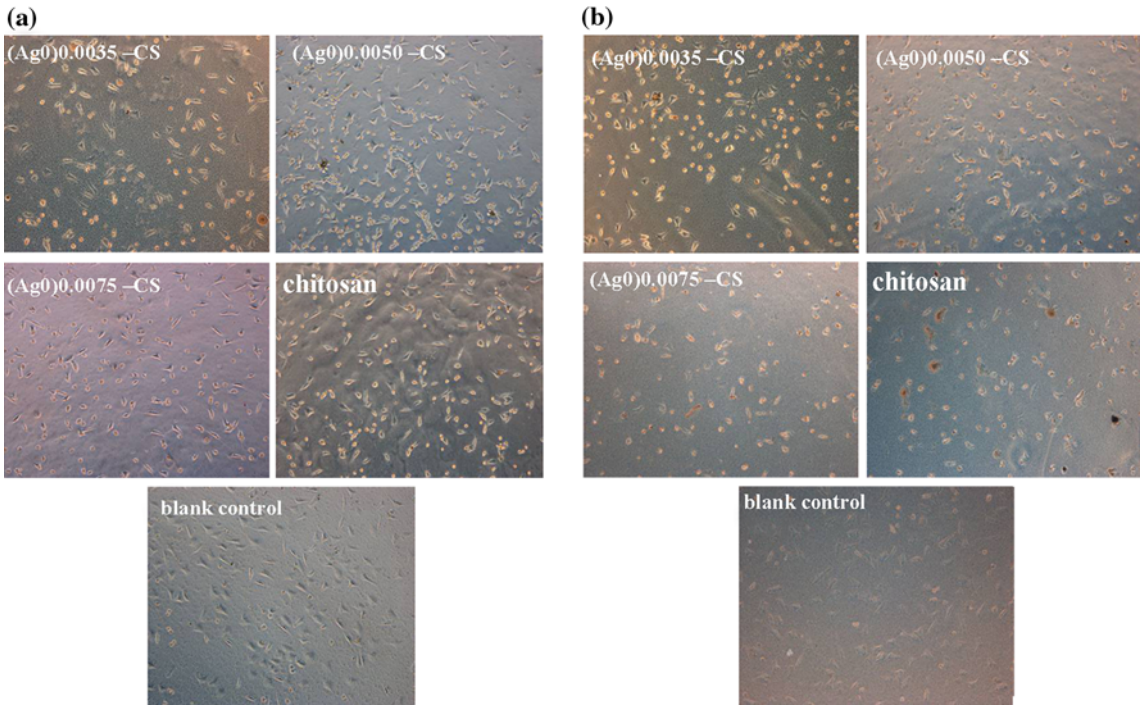

Fig. 3 Morphology of the L-929 cells cultured on the chitosan coated nylon membrane and silver/chitosan nanocomposites coated nylon membrane after **a** 1 day, and **b** 4 days

with the culturing cells and were within the good safety usage in bioengineering and food packing fields where innoxious was important.

4 Conclusions

In this study, silver/chitosan nanocomposites were prepared by adopting chitosan as stabilizer and VC as reducing agent. The best preparation conditions were obtained: chitosan solution (1%), silver nitrate solution (0.0035 to 0.0075 mmol/ml, same with the VC solution), and the reaction temperature (100°C). These composites showed good antibacterial activity in both inhibition zone test and MIC test, which suggested that the antibacterial activity of the composites mainly owed to the silver nanoparticless. Cell compatibilities of the Ag/chitosan nanocomposites were evaluated by the methods of MTT test and cell morphology. The MTT test indicated that the cytotoxicity level of the composites was within the safety range of the evaluation standard, and with the culturing days these composites would become more and more compatible with the L-929 cells. The cell morphology test also showed the good cell compatibility of the Ag/chitosan nanocomposites. This method of preparing silver nanoparticles was non-toxic, low-cost and convenient. We anticipated that these silver/chitosan nanocomposites might have some potential use in biomedical engineering, and the consideration about the biomedical performance of the silver nanoparticles would make some contributions to the usage of silver nanoparticles in biomedical fields.

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