Preparing low-toxicity silver nanoparticles using chitosan derivatives and halide ions

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In our past work, we presented a green and easy-to-perform method for preparing silver nanoparticles, using carboxymethyl chitosan and chlorides. The objective of this study was to investigate the cytotoxicity of the silver nanoparticles that were prepared in our previous work. Further, we intended to determine whether this method was suitable for synthesising silver nanoparticles using other similar polymers and halide compounds. The results indicated that the silver nanoparticles produced through this approach exhibited low cytotoxicity. In addition to carboxymethyl chitosan and chlorides, we also observed that other similar polysaccharides and halides could be used in this preparation.

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1. Introduction

Chitosan (CS) is produced through deacetylation of chitin—a natural polysaccharide in the exoskeleton of crustaceans (crabs, shrimp) and the second most abundant biopolymer in nature. CS is composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit; Fig. 1, a) and N-acetyl-D-glucosamine (acetylated unit). Due to reactive hydroxyl groups and primary amino groups, which are distributed in almost all units, CS can be chemically modified easily. Similar to other natural polymers that are usually chemically inert, nontoxic, less expensive, biodegradable, eco-friendly, and widely available [1], CS and its derivatives have been used in many studies and applications [2–6].

In our past work, we presented a "green" and easy-to-perform method for preparing silver nanoparticles, using commercially available carboxymethyl chitosan (CMCS; Fig. 1, b) [7-9]. Since the carboxymethyl derivative is obtained by carboxylation of the hydroxyl and amine groups of chitosan using monochloroacetic acid, commercially available CMCS contains a small amount of chlorine. The suspension of silver chloride would be produced in the mixture of this polysaccharide derivative and silver nitrate, owing to this small amount of chlorine in commercially available CMCS. In our past work, we reported that the photolysis of silver chloride is the key to producing silver nanoparticles. AgCl is reduced to silver nanoparticles in the CMCS matrix, after being irradiated by sunlight.

Nevertheless, we are not the first ones to use chitosan or its derivatives to prepare metal nanoparticles [10–14]. However, other methods do not completely avoid using toxic reductants or high power. Comparatively, our method of solar photolysis is more suited for physiological applications, given that it does not involve the use of any toxic chemical reagents and consumes low energy. To the best of our knowledge, this is the first time that silver nanoparticles have been synthesized using such a simple method.

However, the cellular reactivity of silver nanoparticles prepared using our method is still uncertain. In addition, screening tests are necessary for other halide ions and natural or synthetic polymers with a similar molecular structure to that of CMCS.

In the present work, the cytotoxicity of the silver nanoparticles prepared using solar photolysis of silver chloride in the CMCS aqua solution will be evaluated. In addition, four types of polymers (*N*-trimethyl chitosan chloride [TMCC; Fig. 1, c], *N*-maleoyl chitosan [*N*-MACH; Fig. 1, d], sodium alginate [SA; Fig. 1, e], and sodium polyacrylate [SP; Fig. 1, f]) and two types of halide ions (sodium bromide and trichloroacetic acid) will be tested using this method.

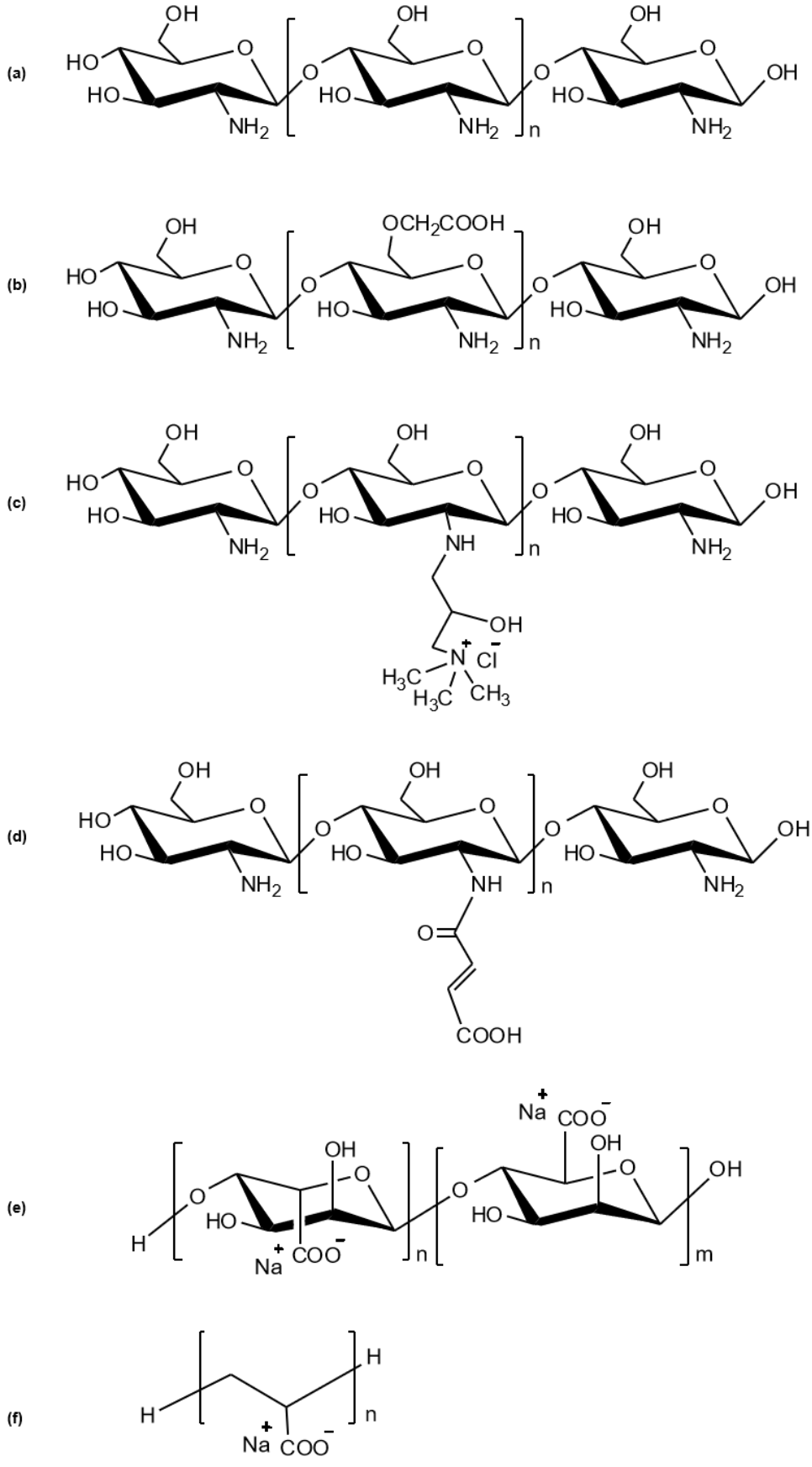

Fig. 1. Chemical structures of the polymers involved in this study: a. chitosan; b. carboxymethyl chitosan; c. N-trimethyl chitosan chloride; d. N-maleoyl chitosan; e. sodium alginate; f. sodium polyacrylate

2. Experimental

2.1. Materials

All compounds were used as received. Pharmaceutical grade CMCS (95.1% amino-group substituted ratio) was obtained from Honghai Biotechnology Co., Ltd. (Qingdao, China). *N*-trimethyl chitosan chloride and *N*-maleoyl chitosan were obtained from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). Food grade sodium alginate was obtained from Bright Moon Seaweed Group Co., Ltd. (Qingdao, China). Silver nitrate (analytical reagent), sodium chloride (analytical reagent), sodium bromide (analytical reagent), trichloroacetic acid (analytical reagent), and sodium polyacrylate were obtained from Kelong Chemical Reagent Factory (Chengdu, China). Ultrapure water was obtained from a Millipore Milli-Q Plus filtration system. All cell lines were purchased from ATCC (Manassas, VA, USA).

2.2. Preparation of silver nanoparticles using CMCS

An aqueous solution of commercially available CMCS (0.1%, w/v) was prepared and stirred overnight. As determined using ion chromatography (ICS-3000, Dionex, USA), the concentration of chloride ions in the stock solution of commercially available CMCS was 14 mg/L (approximately 0.4 mM). Different amounts of a 0.6 M aqueous solution of $AgNO_3$ (200 μL and 67 μL) were added dropwise under magnetic stirring into 100 mL of CMCS stock solution. The solutions were then irradiated via sunlight or fluorescent lamp (30 W, λ = 400-750 nm, illumination distance 0.5 m) for 7 h. The mixtures were stirred with a magnetic bar during irradiation.

2.3. Cell culture and cytotoxicity evaluation

Human lung cancer (A549) and human skin fibroblast cells were used in this study. All cell lines were cultured in ATCC-recommended media, with 10% foetal bovine serum and 1% penicillin/streptomycin, in a humidified incubator at 37°C with 5% CO_2.

Cells were plated in a 96-well plate at the rate of 5×10^3 cells/well in a 100 mL medium, grown for 24 h, and exposed to multiple concentrations of silver nanoparticle suspensions for 48 h. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay, as previously described [15]. Precisely 10 μL of the CCK-8 solution was added to each well. We incubated the plate for 1-4 h in the incubator. The absorbance was measured at 450 nm using a microplate reader.

2.4. Preparation of silver nanoparticles using other polymers or halide ions

An aqueous solution of 0.1% (w/v) *N*-trimethyl chitosan chloride, N-maleoyl chitosan, sodium alginate, and sodium polyacrylate, was prepared and stirred overnight.

After mixing all reactants, the reaction mixtures were irradiated under sunlight for 7 h. The solutions were stirred during the irradiation.

2.4.1. AgNPs preparation using N-trimethyl chitosan chloride

Precisely, 200 μL aqueous solution of $AgNO_3$ (0.6 M) was added dropwise into 100 mL of *N*-trimethyl chitosan chloride stock solution.

2.4.2. AgNPs preparation using N-maleoyl chitosan and bromine or organochlorine ions from sodium bromide or trichloroacetic acid

Precisely, 200 μL of $AgNO_3$ (0.6 M) aqueous solution and 10 μL of sodium bromide or trichloroacetic acid (4 M) aqueous solution were added dropwise to 100 mL of *N*-maleoyl chitosan stock solution.

2.4.3. AgNPs preparation using sodium alginate or sodium polyacrylateand chlorine ion

Precisely, 200 μL of $AgNO_3$ (0.6 M) aqueous solution and 10 μL of sodium chloride (4 M) aqueous solution were added dropwise into 100 mL of sodium alginate or sodium polyacrylate stock solution.

2.5. Characterization of silver nanoparticles

Before characterization, the nanoparticles were purified by centrifugation (Thermo Sorvall ST16R, 6000 g, 10 min). The precipitates were rinsed three times and re-suspended in ultrapure water.

The reactions were monitored using a UV-3600 UV-Vis spectrophotometer (Shimadzu, Japan).

Transmission electron microscopy (TEM) was conducted on a LIBRA 200 TEM (ZEISS, Germany) at an accelerating voltage of 200 kV. The TEM samples were prepared by slowly evaporating a drop of the nanoparticle solution on a copper grid covered by a carbon-supported film at 25℃.

X-ray diffraction (XRD) experiments were performed on a D/MAX-2500PC X-ray diffractometer (Rigaku, Japan), using Cu Kα radiation. The tube voltage and current were 40 kV and 150 mA, respectively. The purified nanoparticle suspensions were coated on glass plates and

air-dried naturally. The obtained films were used for XRD measurements.

3. Results

3.1. Characterization and cytotoxicity evaluation of silver nanoparticles prepared using CMCS

XRD patterns of the nanoparticles purified using centrifugation showed a total of ten peaks (Fig. 2, c). In addition to the peaks corresponding to Ag^0 (2θ=38.1°, 44.3°, 64.5°, 77.4°, and 81.4°), five more peaks (2θ=27.8°, 32.3°, 46.3°, 54.8°, and 57.5°) corresponding to the diffraction lines of AgCl were also observed. There was an obvious enhancement in the intensity of the peaks corresponding to AgCl on decreasing the molar ratio of silver and chloride ions (Fig. 2, c; 1:1). According to the XRD quantitative method [16, 17], the relative content of AgCl in the latter (molar ratio of silver and chloride ions is 1:1) is higher.

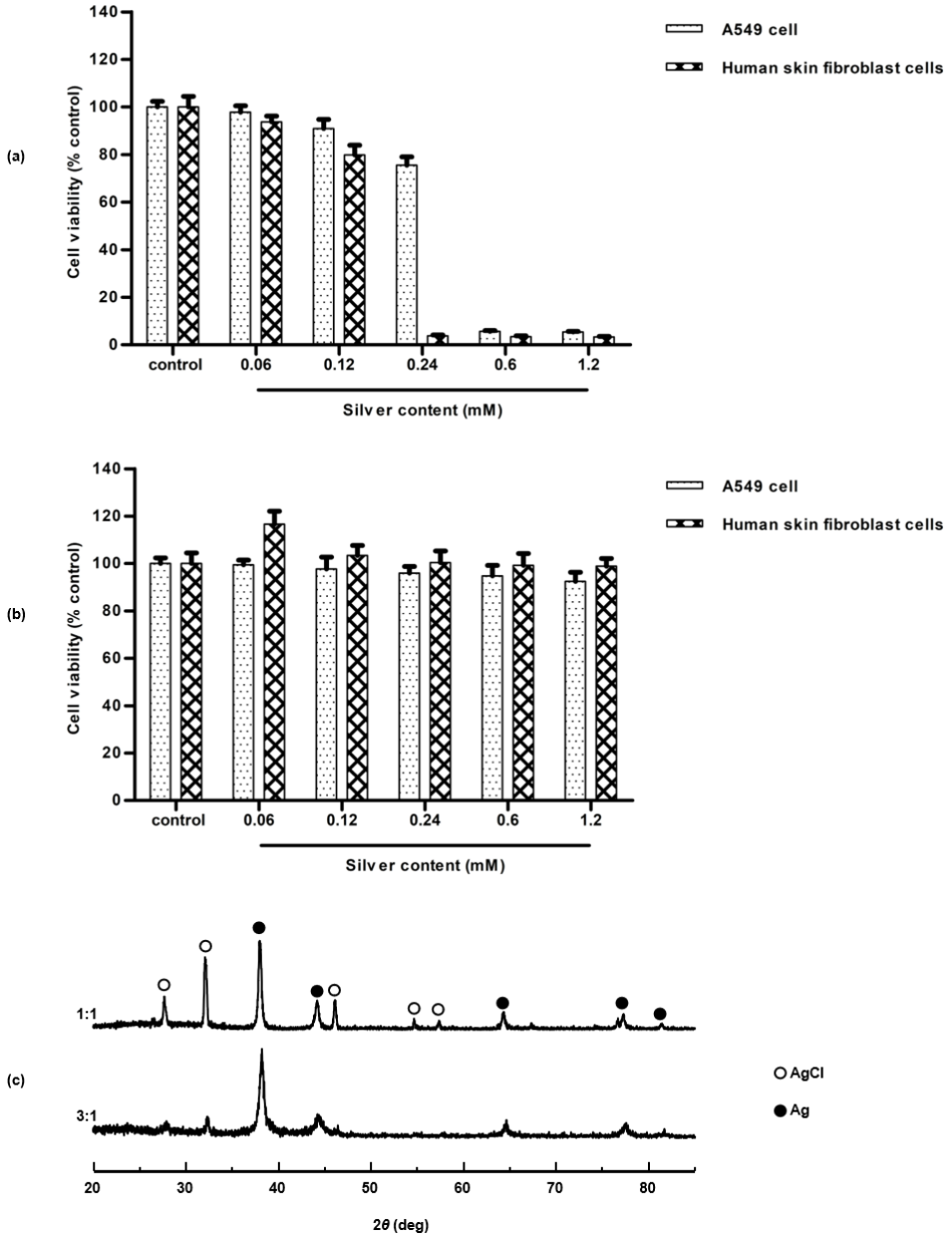

Fig. 2. Cytotoxicity evaluation and XRD spectra of silver nanoparticles with different AgCl content that are prepared using CMCS. (a) Effect of uncentrifuged reaction mixture, in which the molar ratio of silver and chloride ions is 3:1, on the cell viability of human lung cancer (A549) cells and human skin fibroblast cells (n=4). (b) Effect of uncentrifuged reaction mixture, in which the molar ratio of silver and chloride ions is 1:1, on the cell viability of human skin fibroblast cells (n=4); (c) XRD spectra of silver nanoparticles purified from the reaction mixtures. XRD, X-ray diffraction; CMCS, carboxymethyl chitosan

In this study, A549 and human skin fibroblast cells were used. Based on cell viability, no significant toxicity was exhibited by the diluted solutions towards the A549 cells, until the silver content was increased to 0.24 mM (Fig. 2, a). A similar result was demonstrated when human skin fibroblast cells were exposed to silver nanoparticles (Fig. 2, a). But, in this situation, the normal cells are more affected than cancer cells.

Surprisingly, the proliferation (Fig. 2, b) was observed when human skin fibroblast cells were exposed to silver nanoparticles containing more silver chloride (Fig. 2, c). Both normal cells (human skin fibroblast cells) and cancer cells (A549 cells) were not affected when using the molar ratio of silver and chloride ions of 1:1.

3.2. Preparation of silver nanoparticles using TMCC

We also tested the use of chitosan derivatives with high chloride content, i.e., TMCC, in the preparation of silver nanoparticles. The results are shown in Fig. 3.

The morphology of the nanoparticles could be easily observed *via* TEM. XRD patterns of the nanoparticles showed a total of ten peaks corresponding to Ag^0 and AgCl (Fig. 3, b). Silver nanomaterials were successfully prepared using TMCC, but precipitation was also produced (Fig. 3, a, inset). XRD results showed that the primary component of this sediment was silver chloride (Fig. 3, b).

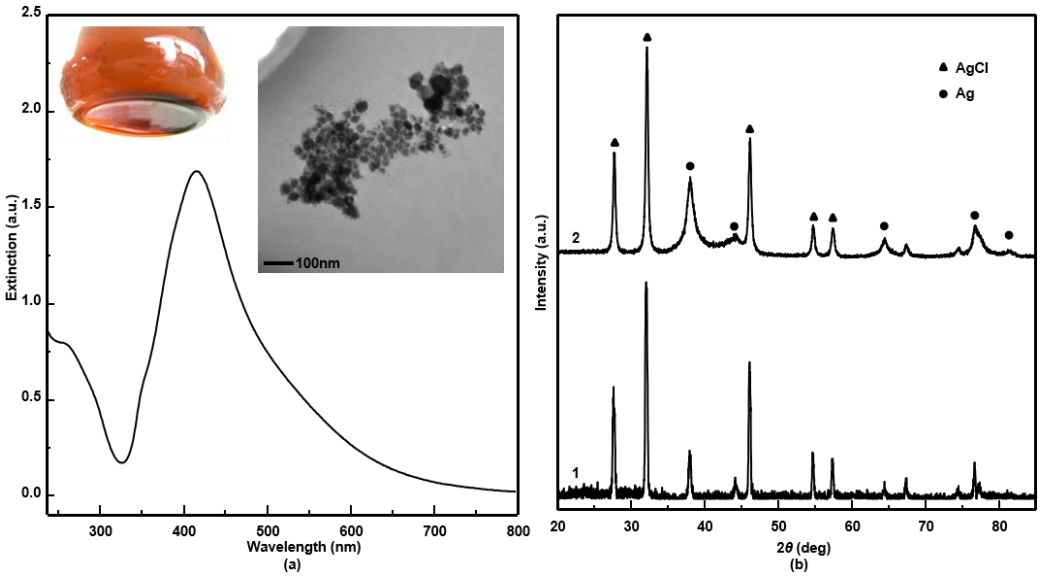

Fig. 3. Spectral and microscopic characterization of the silver nanoparticles prepared through the solar illumination of TMCC and silver nitrate. (a) UV-Vis absorption spectra, TEM image (right inset), and photograph (left inset). (b) XRD spectra (1, silver nanoparticles in the reaction solution; 2, the black precipitate of the reaction mixture). TMCC, N-trimethyl chitosan chloride; XRD, X-ray diffraction; TEM, transmission electron microscopy

3.3. Characterization of silver nanoparticles using sodium bromide or trichloroacetic acid

To expand the application scope of the use of chlorides, we tested the use of sodium bromide and trichloroacetic acid as alternatives to chlorides. To avoid potential interference by the halide ions contained in the reactants, a chloride-free chitosan derivative, *N*-MACH, was used as the polysaccharide. The result is shown in Fig. 4.

The characteristic surface plasmon resonance (SPR) bands of nanomaterials were detected in sodium bromide and trichloroacetic acid reaction solutions. This indicated that other halides could also be used to prepare silver nanoparticles. Nonetheless, a comparison between the synthetic efficiency of these halides revealed that chlorides still had the highest efficiency.

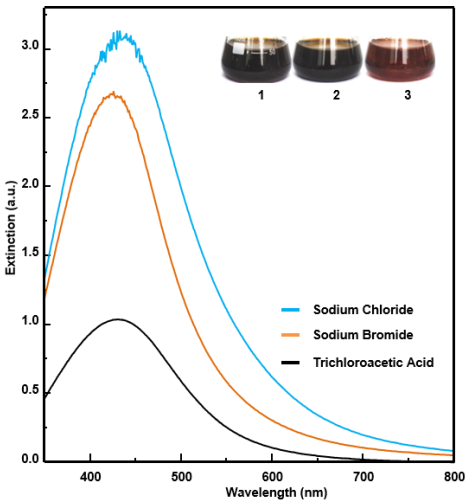

Fig. 4. UV-Vis absorption spectra and photographs (inset) of the silver nanoparticles prepared from the reaction mixtures containing N-maleoyl chitosan and sodium chloride (1), sodium bromide (2), and trichloroacetic acid (3) (color online)

3.4. Characterization of silver nanoparticles using natural (SA) or synthetic (SP) polysaccharides

Chitosan derivatives acted as stabilizers in the proposed silver nanoparticle preparation method; however, numerous polysaccharides and their derivatives, or even synthetic polymers, possess similar structures to that of chitosan. To compare the effects of other similar polymers on our preparation method, we also attempted to use other SA or SP. UV-Vis absorption spectra of these nanoparticles are shown in Fig. 5.

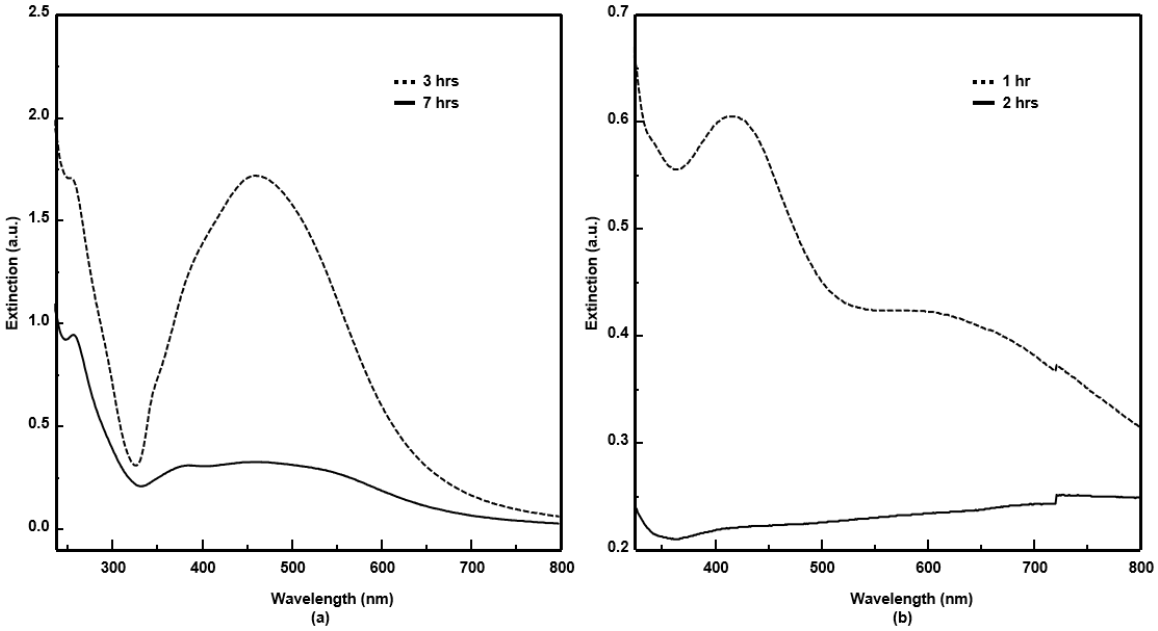

Fig. 5. UV-Vis absorption spectra of the reaction mixture containing sodium chloride and SA (a) or SP (b). SA (sodium alginate), natural polysaccharides; SP (sodium polyacrylate), synthetic polysaccharides

Regardless of the use of SA or SP sodium chloride solutions mixed with the silver nitrate solution, the UV-Vis absorption spectrum of silver nanoparticles reaction mixture exhibited a characteristic SPR band for AgNPs after a short exposure to sunlight. However, this peak completely disappeared after a slightly prolonged reaction time. This indicated that neither of these compounds could be used to prepare silver nanoparticles.

4. Discussion

In this work, we aimed to determine the toxic effect of silver nanoparticles prepared using CMCS on human lung cancer (A549) and skin fibroblast cell lines. Beyond that, finding new strategies to obtain AgNPs under sunlight illumination is another important purpose.

Based on cytotoxicity tests, it was revealed that these nanoparticles exhibited low toxicity. This feature is conducive to the wider application of the nanoparticles in the pharmaceutical field. In our previous work [7-9], we envisaged that silver nanoparticles prepared using CMCS can be used for downstream applications (such as wound dressing) without further purification. Therefore, it was necessary to test the cellular toxicity of the impure solutions containing nanoparticles.

Foldbjerg and colleagues [18] studied the toxicity of pure silver nanoparticles to A549 cells and found no significant cytotoxicity until the silver content was reduced below 0.5 μg/mL (about 0.0046 mM). Since the nanoparticles are prepared in an aqueous solution of CMCS, the unpurified nanoparticles adsorb CMCS molecules onto the surface. Because chitosan and its derivatives are known for their excellent biological properties, we hypothesized that silver nanoparticles adsorbed to CMCS on the surface (uncentrifuged samples) would have lower cytotoxicity. Our research confirms this hypothesis. Foldbjerg *et al*. used *N*-acetyl-L-cysteine to reduce the cytotoxicity of silver nanoparticles. Therefore, it appears that CMCS in our work functions as *N*-acetyl-L-cysteine in the Foldbjerg's mentioned study.

Interestingly, we observed that high silver chloride content in the silver nanomaterial, which was verified using XRD (Fig. 2, c), further reduced its cytotoxicity; this result is consistent with the findings of Zhang *et al.* [19]. However, the silver chloride content can only be increased up to a certain limit; excessive levels would lead to the precipitation of elemental silver instead of the nanoparticles.

With the exception of CMCS method, all other three procedures have significant drawbacks (easy to precipitate, slow reaction rate, nanoparticle instability and so on). This

is the reason we only studied cytotoxicity of CMCS-AgNPs. It is possible to overcome the drawbacks mentioned above by combining two of the preparation methods, but more research is still needed. We hope this article will lead to more people joining the study.

In addition, we found that other halides, apart from chloride, can also be used in this preparation, although the use of chlorides was the most optimal in terms of synthetic efficiency. However, only chitosan derivatives appeared to be suitable to prepare silver nanoparticles using this method.

5. Conclusions

This research has shown that the silver nanomaterials prepared in our previous work using CMCS and chloride ion exhibit low cytotoxicity. Apart from CMCS and chlorides, other similar polymers and halides (1. TMCC; 2. sodium bromide or trichloroacetic acid; 3. natural (SA) or synthetic (SP) polysaccharides) can also be used. However, the combination of CMCS and chlorides is the most optimal selection that exhibits high efficiency.

Acknowledgements

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