Research article

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Fabrication of silver nanocomposites by thiol-end capped ABC triblock copolymer and investigation of their Inhibitory effect against bacteria and yeast *in vitro*

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Abstract

Background and objective: Silver nanoparticles are interested in antimicrobial studies due to their significant inhibitory effect. Despite the widespread research in this regard, preparation of water-dispersible and stable silver nanoparticles has not been solved sufficiently. At this work, fabrication, characterization, and antimicrobial activity of silver-decorated polymeric nanocomposite based on a pH-responsive thiol-end capped ABC triblock copolymer is presented.

Materials and methods: PAA-*b*-PDMAEMAQ-*b*-PCL-SH triblock copolymer was firstly synthesized by combination of ring-opening polymerization and reversible addition fragmentation chain transfer polymerization techniques. For preparation of silver-decorated nanocomposite, the triblock copolymer was self-assembled into polymer micelles with 20 nm diameter. Then, Ag nanoparticles were incorporated into the core of micelles when reducing agent of sodium borohydride was added. The Ag-doped nanocomposite was characterized by Fourier transform infrared, ^{1}H nuclear magnetic resonance, UV-VIS spectroscopy, laser scattering technique, and transmission electron microscopy. Antimicrobial activity of the silver-doped nanocomposite was examined against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* at three concentrations of 10, 25, and 50 mg/ml.

Results and conclusion: The least and highest antagonisms were observed against *E. coli* by inhibition diameters of 4, 28 and 32 mm and *C. albicans* by inhibition diameters of 58, 101 and 119 mm for three concentrations of 10, 25, and 50 mg/ml, respectively. Among bacteria, the highest inhibition was observed for *S. aureus* by 45, 51 and 59 mm inhibition diameter induced by the three tested concentrations of the nanocomposite. It was accounted as 29, 34 and 40 mm inhibition diameter for *B. cereus*. Other than antimicrobial property, the synthesized silver nanocomposite could be introduced for de novo drug delivery system in cancer therapy by using both hydrophobic and hydrophilic anticancer drugs due to its physicochemical properties.

Keywords: Antimicrobial activity, polymeric micelle, reversible addition fragmentation chain transfer polymerization, ring-opening polymerization, silver nanocomposite

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1. Introduction

In the last decade, silver nanoparticles have been largely studied owing to their application in several fields of adsorption, catalysis, biotechnology, bio-sensing, microelectronics, optics, and biomedicine [1-3]. Although its mode of action is not well understood, silver is active agent against bacteria, viruses and fungi. What is important is that silver nanoparticles have well-developed surfaces providing maximum contact with the environment which leads to high antimicrobial activity [4-7]. To develop silver nanoparticles for antimicrobial purposes, their shape, size and stability should be optimized [8-10]. In this regard, interaction of the nanoparticles with amphiphilic block copolymers are developed [11-13]. By selection of appropriate solvents, copolymers self-assemble into polymeric micelles and can be applied as nano-reactor for incorporation of metals (such as Ag, Au, Pd) in nanoparticles synthesis. Therefore, they are prevented from aggregation [14-16]. Several synthesized copolymers as nano-system for preparation of silver nanocomposite have been reported [17-19]. However, use of organic solvents in those studies is dangerous which restricts their biomedical application.

To solve this issue, we added colloidal silver nanoparticles to the prepared thiol end-capped copolymers to increase dispersity and stability of silver nanoparticles in aqueous solutions. In fact, the thiol groups bind covalently to the surface of silver nanoparticles via Ag-S bond. Therefore, it is expected that the synthesized thiol-ended copolymers strongly interact with silver nanoparticles which leads to its extensive use as antimicrobial agent in numerous fields. After experimental preparation, antimicrobial activity of the synthesized nanocomposites containing silver nanoparticles was studied against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.

2. Materials and methods

2.1. Materials

Thiol-end capped triblock copolymer [poly (acrylic acid)-*b*-(dimethylaminoethyl methacrylate quaternary ammonium alkyl halide)-*b*-(ε-caprolactone)-SH] abbreviated as [P(AA-*b*-DMAEMAQ-*b*-CL)-SH] or ABC triblock copolymer was synthesized in our previous study [20]. For incorporation of silver nanoparticles into the previously prepared ABC triblock copolymer, dimethylsulfoxide (Merck, Germany), silver nitrate (AcrosOrganics), and sodium borohydride (Sigma, USA) were directly used without further preparation. *B. cereus* (ATCC 6633), *S. aureus (*ATCC 25923), *E. coli* (ATCC 25922), and *C. albicans* (ATCC 20032) were obtained from Hangzhou Microbe Reagent Co., Ltd. (China). Bis(2-hydroxyethyl) and $(CTA\text{-}PCL\text{-}S)_2$ were synthesized as described previously [20].

2.2. Method

2.2.1. Synthesis of $(PDMAEMAQ\text{-}b\text{-}PCL\text{-}S)_2$ copolymer

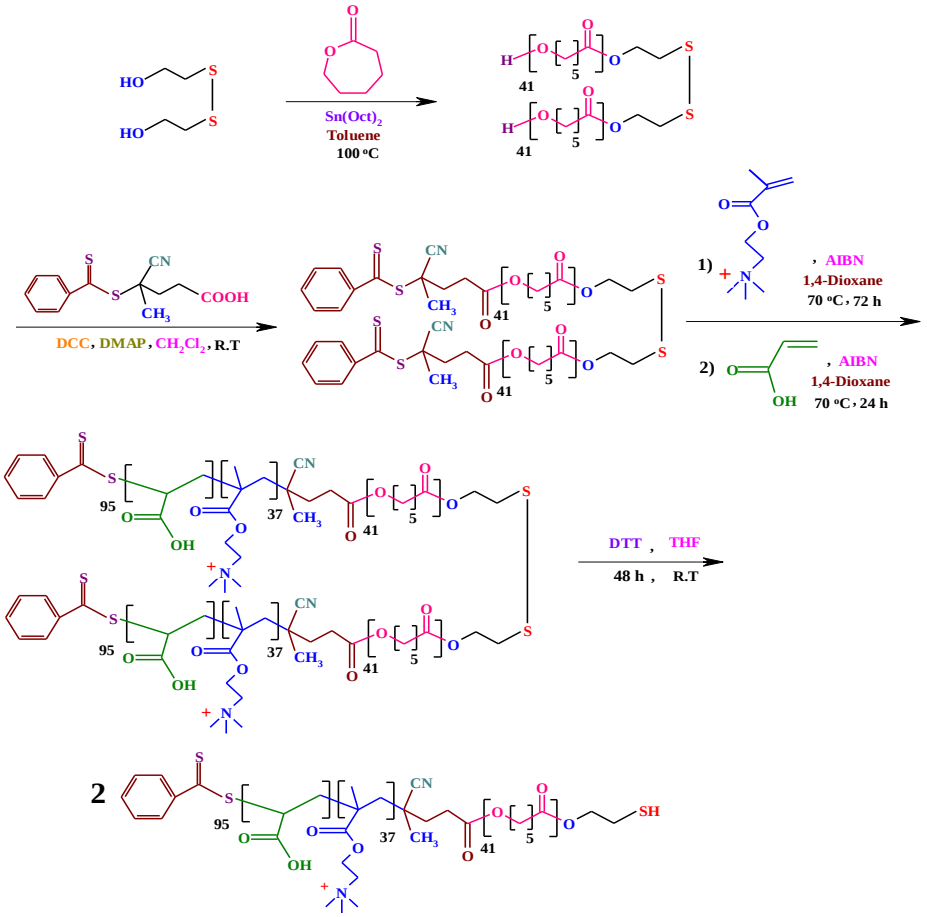
Macro-RAFT agents of $[(CTA\text{-}PCL\text{-}S)_2]$ (1 g, 0.12 mmol), DMAEMAQ monomer (1.6 g, 15 mmol), and AIBN (2 mg, 12 µmol) were dissolved in 1,4-dioxane (8 ml). The mixture was degassed via three freeze-evacuate-thaw cycles, and moved to oil bath at 80 ±3 ºC for about 48 h. Finally, the product was precipitated by addition of 200 ml diethyl ether and dried under reduced pressure at room temperature [20].

2.2.2. Synthesis of $(PAA\text{-}b\text{-}PDMAEMAQ\text{-}b\text{-}PCL\text{-}S)_2$ copolymer

Macro-RAFT agents of $[(PDMAEMAQ\text{-}b\text{-}PCL\text{-}S)_2]$ (1 g, 0.068 mmol), AA monomer (1.5 g, 20 mmol), and AIBN (2 mg, 12 µmol) were dissolved in 1,4-dioxane (8 ml). As described in section 2.2, the mixture was degassed by three freeze-evacuate-thaw cycles, and moved to oil bath at 80 ±3 ºC for 48 h. At the end, the product was precipitated by addition of 200 ml diethyl ether and dried under reduced pressure at ambient temperature [20].

2.2.3. Synthesis of PAA-*b*-PDMAEMAQ-*b*-PCL-SH triblock copolymer

Triblock copolymer of (PAA-*b*-PDMAEMAQ-*b*-PCL-S)$_2$ and $NaBH_4$ were dissolved in dimethyl solfoxide (DMSO) and stirred for 24 h. Then, the product was freeze dried and stored until analysis. Preparation of the copolymer is illustrated in scheme 1.



Scheme 1- Synthesis of pH-responsive thiol-ended ABC triblock copolymer (PAA-*b*-PDMAEMAQ-*b*-PCL-SH) [20]

2.2.4. Preparation of silver loaded [PAA-*b*-PDMAEMAQ-*b*-PCL)-SH] nanocomposites

At first, 200 mg (12.5 mmol) of [PAA-*b*-PDMA EMAQ-*b*-PCL)-SH] copolymer and 0.02 g of silver nanoparticle were dissolved in 6 ml DMSO and stirred at 1200 rpm for 24 h. Then, the solution was added to a dialysis bag and stirred again at 1200 rpm for additional 48 h. The silver compounds were reduced by freshly prepared aqueous $NaBH_4$ solution (100 mg/ml) [20].

2.2.5. Antimicrobial activity test

Inhibition activity of the nanocomposites (silver loaded PAA-*b*-PDMAEMAQ-*b*-PCL-SH) was examined against *B. cereus*, *S. aureus*, *E. coli*, and *C. albicans* by Kirby Bauer disc diffusion method [17]. Culture medium of Muller Hinton agar (Merck, Germany) was used for the experiments. The silver nanocomposites at concentrations of 10, 25, and 50 mg/ml and 1 ml of the microbial suspension (1×10^5 CFU/ml) were transferred to the plates and incubated at 37 °C for 24 h. The results are expressed as diameter of inhibition zone.

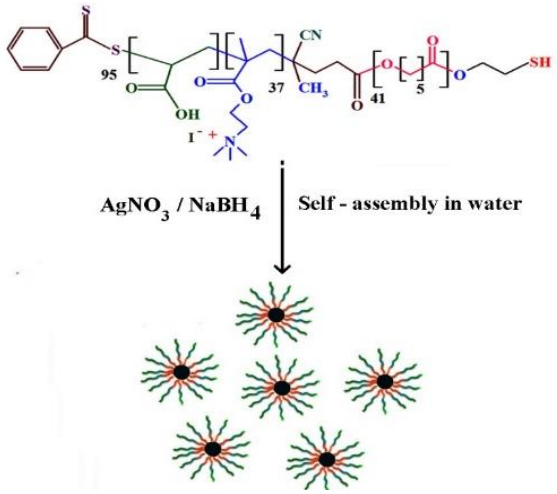
2.2.6. Characterization

Fourier transform infrared (FT-IR) spectra of the samples were obtained at wavenumber range of 4000 to 400 cm^{-1} by instrument Model 8101 M (Shimadzu, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained at 25 °C using a FT-NMR (400 mHz) Bruker spectrometer (Ettlingen, Germany). The samples were prepared in deuterated chloroform and DMSO-d6. Ultraviolet-Visible (UV-VIS) spectra were prepared by Shimadzu 1650 PC UV-VIS spectrophotometer (Shimadzu, Japan). Particle size was measured by laser scattering technique in dynamic light scattering (DLS) instrument (Zetasizer Nano ZS90, Malvern, UK) at 25 °C. Transmission electron microscopy (TEM) images was taken by CM10-TH microscope (Phillips, Netherlands) with 100 kV accelerating voltage.

3. Results and discussion

3.1. Characterization

Successful synthesis of ABC triblock copolymer of [PAA-*b*-PDMAEMAQ-*b*-PCL-SH] containing thiol end group by ring-opening polymerization (ROP) and reversible addition fragmentation (RAFT) polymerization techniques was confirmed in our previous work [20]. Fabrication of silver doped nanocomposites are graphically presented in Scheme 2.



Scheme 2- Preparation of silver loaded nanocomposites

As expected, silver cations strongly reacted with SH and OH groups of the triblock copolymer through *in situ* reduction. At this work, incorporation of silver nanoparticles into the polymeric micelles was done by silver nitrate reduction. This reaction was followed by color change from pale green to dark brown. Thus, silver nanoparticles were inserted into the polymeric shell and core of the micelles. After reduction, the silver particles were chelated in the structure which helped in their stabilization.

^{1}H NMR spectra of (PAA-*b*-PDMAEMAQ-*b*-PCL-S)$_2$ and [PAA-*b*-PDMAEMAQ-*b*-PCL-SH] copolymers are shown in Figure 1. Compared to ^{1}H NMR spectrum of (PDMAEMAQ-*b*-PCL-S)$_2$ copolymer, the most significant change in the spectrum was related to the new chemical shift of COOH group at 12.29 ppm. All other chemical resonances were labeled in ^{1}H NMR spectra of (PAA-*b*-PDMAEMAQ-*b*-PCL-S)$_2$ penta block copolymer.

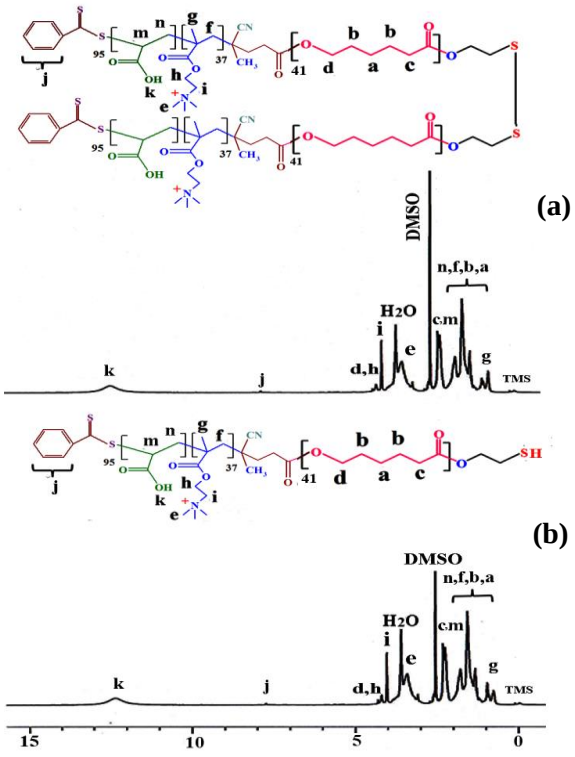


Figure 1- ^{1}H NMR spectra of a) (PAA-*b*-PDMAEMAQ-*b*-PCL-S)$_2$ copolymer, and b) PAA-*b*-PDMAEMAQ-*b*-PCL-SH copolymer

The silver nanocomposites were investigated using UV–VIS spectroscopy (Figure 2). The maximum absorbance at 420 nm also confirmed the inclusion of silver nanoparticle in the structure [21]. The symmetric absorption spectrum also shows a narrow size distribution without any aggregation.

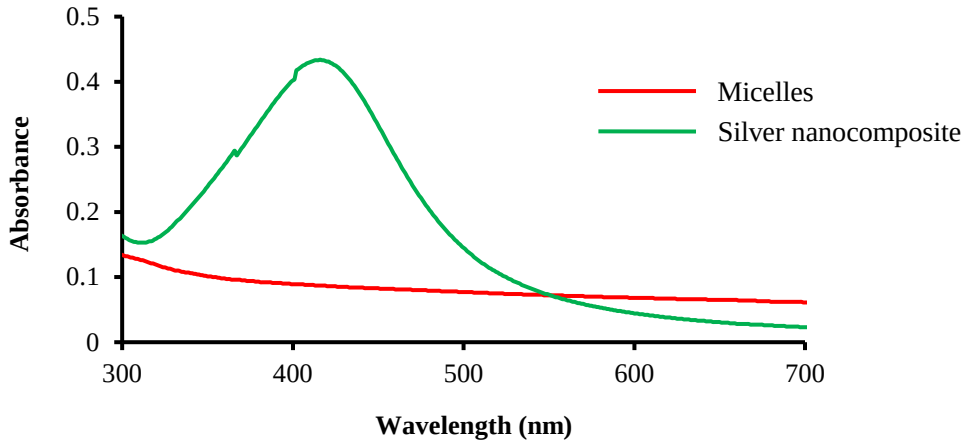


Figure 2- UV-VIS spectra of PAA-*b*-PDMAEMAQ-*b*-PCL-SH micelles and PAA-*b*-PDMAEMAQ-*b*-PCL-SH silver nanocomposite

TEM image showed a highly-dense silver nanocomposite formed by the process. The images clearly show formation of relatively homogenous nanocomposites by spherical shape with average diameter of 25 nm (Figure 3). The average size calculated by TEM was smaller than those obtained by DLS. It was due to the fact that TEM images shows the dried aggregates or the core of nanocomposite while DLS reflects the solved micelles. In addition, DLS shows an intensity-weighted diameter compared to TEM that exhibits a number-average diameter.

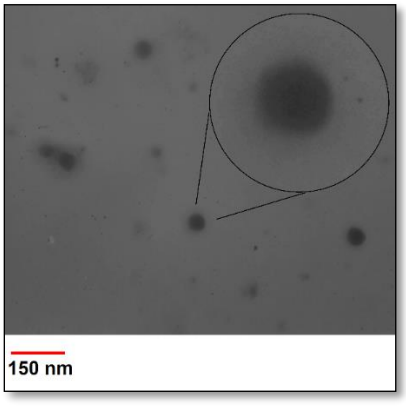


Figure 3- TEM image of PAA-*b*-PDMAEMAQ-*b*-PCL-SH silver nanocomposites

Average hydrodynamic diameter (D_h) and particles size distribution (PDI) of nanomicelles and silver nanocomposites were measured by DLS. Micelles with D_h of 70.19 nm and PDI of 0.15 were obtained. Average D_h of the silver nanocomposites and their PDI were 90 nm and 0.21, respectively. These results indicated that the nanocomposites were formed by different degree of core-shell chain in the presence of silver nanoparticles (Figure 4).

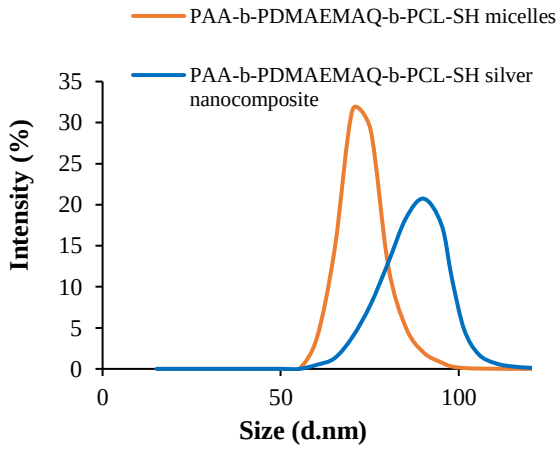


Figure 4- Results of DLS analysis of PAA-*b*-PDMAEMAQ-*b*-PCL-SH micelles and PAA-*b*-PDMAEMAQ-*b*-PCL-SH silver nanocomposite

3.2. Antimicrobial activity assay

Silver nanoparticles are of the most popular inorganic compounds that are used as anti-microbial agents. Inhibitory effect of PAA-*b*-PDMAEMAQ-*b*-PCL-SH silver nanocomposites was assessed by their contact to the viable cells of *S. aureus* and *B. cereus* as gram-positive bacteria, *E. coli* as gram-negative bacterium, and *C. albicans* as yeast. Silver nanocomposite at three concentrations of 10, 25, and 50 mg/ml were studied (Table 1). Different inhibition zones were observed for the tested microorganisms of which the highest diameter was related to *C. albicans* (58, 101, and 119 mm for 10, 25, and 50 mg/ml, respectively). It shows that the fabricated silver nanocomposites were greatly active against yeast. Moreover, gram-positive bacterium of *S. aureus* was more sensitive that the other bacteria in our study. In this regard, inhibition diameters of 45, 51, and 59 mm were measured at the three tested concentrations of nanocomposites (10, 25, and 50 mg/ml, respectively) for *S. aureus*. It was measured as 29, 34, and 40 mm for *B. cereus*. In comparison, *E. coli* by inhibition diameters of 24, 28, and 32 mm was the least affected microorganism. According to Punjabi et al., two mechanisms are supposed for antimicrobial activity of silver nanoparticles. On the one hand, the positively charged silver reacts with the negatively charged surface of microbial membrane and disturbs the integrity of cell membrane followed by changing the permeability of the cells. On the other hand, cationic nature of the silver particles leads to their interaction with some vital molecules in the microbial cells such as DNA or proteins, through which the cells lose their viability and die [22]. Considering the results reveals that the outer membrane of gram-negative bacteria has protective effect on the cells compared to gram-positive bacteria and yeasts which have no such protective envelopment [23].

Table 1- Antimicrobial activity of PAA-*b*-PDMA EMAQ-*b*-PCL-SH silver nanocomposites against four microorganisms

	Silver nanocomposite		
Microorganism	10 mg/ml	25 mg/ml	l50 mg/ml
S. aureus	45 mm	51 mm	59 mm
B. cereus	29 mm	34 mm	40 mm
C. albicans	58 mm	101 mm	119 mm
E. coli	24 mm	28 mm	32 mm

4. Conclusion

Novel amphiphilic triblock copolymer of PAA-*b*-PDMAEMAQ-*b*-PCL-SH was successfully incorporated with silver nanoparticles as a result of silver covalent bond with polar OH and SH groups in the copolymer. The polymeric micelles self-assembled in water by forming spherical shape consisting of PAA-*b*-PDMAEMAQ-*b*-PCL-SH shells and silver core. The cores were reduced to elemental silver nanoparticles. The nanocomposites of silver were then tested for their antimicrobial activity. All of the tested microorganisms were affected by the fabricated nanocomposites. The results showed that yeasts are the most sensitive and gram-negative bacteria are the most resistant microorganisms against our silver nanocomposites. According to our results, the silver nanocomposites could be used in biomedicine. Although, further toxicological studies are required to approve their beneficial impact.

5. Conflict of interest

There is no confilict of interest to be declared.

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