

Silver Nanoparticle Synthesis Using Ultrasound and Halloysite to Create a Nanocomposite with Antibacterial Properties

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Abstract—The antibacterial nanocomposites creation is a current trend against microbial contamination and microorganism's biofilm formation. Existing methods for producing nanocomposites based on silver nanoparticles are difficult, expensive, and not environment friendly; therefore it has become necessary to develop a new method for their synthesis that didn't have these minus. The paper discusses the possibility of silver nanoparticles synthesizing and obtains a new nanocomposite using halloysite nanotubes and ultrasound. Transmission electron microscopy revealed the silver nanoparticles presence on the inner and outer surface of halloysite nanotubes, and sample mapping showed a uniform distribution of silver nanoparticles in the nanocomposite. The antibacterial activity of the obtained nanocomposite against the strain *Serratia marcescens* (*S. marcescens*) was more than twice higher than that of the control. The swarming motility method showed that the diameter of migration of *S. marcescens* was 2.05 ± 0.05 cm, and in the presence of the nanocomposite, it was 1.63 ± 0.04 cm, indicating the ability of the nanocomposite to inhibit biofilm formation in these bacteria. In the future, the obtained nanocomposite can be used as an additive to various materials or as a coating protecting against bacterial contamination of various surfaces and materials.

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INTRODUCTION

Recently, there has been wide development of new functional materials that release active components and antibacterial substances [1, 2]. This is because bacteria have become resistant to most existing antibiotics. The formation of microbial biofilms contributes to bacterial resistance, allowing subpopulations of bacteria tolerant to antibiotics to transfer this property to other microorganisms [3]. Currently, the following strategies exist to prevent bacterial contamination: the creation of antiadhesive surfaces and antibacterial materials [4–7]. Therefore, the development of new nanocomposites and their preparation methods in order to improve existing materials with antibacterial properties is very significant in science and in practice.

One problem in creating of antimicrobial materials is the search for an effective antimicrobial agent and a way of binding it with various materials [8, 9]. Silver nanoparticles were chosen as an antibacterial agent due to their ability to damage the cytoplasmic membrane and respiratory enzymes of microorganisms and prevent growth of Gram-positive and Gram-negative bacteria, fungi, and viruses, and also due to microorganisms' lack of resistance to silver [10–13]. However, the direct addition of silver nanoparticles has some

drawbacks: a coating or substance acquires a yellow-brown color over time and its decomposition process is accelerated [14]. Therefore, to eliminate these drawbacks, it was proposed to use halloysite nanotubes as a carrier, the inner cavities of which can contain various compounds. Halloysite nanotubes are a natural nanosized material with proven biocompatibility and wide availability [15, 16]. A unique feature of halloysite is the difference in chemical composition of the outer and inner surfaces of the nanotubes. The inner surface is contain the alumina and has a positive surface charge, while the outer surface is contain the silica, which has a negative charge [17]. Owing to these physical-chemical properties, it was possible to demonstrate the possibility of halloysite nanotubes as a reaction catalyst for producing silver nanoparticles from silver nitrate.

As a rule, silver nanoparticles are synthesized by such methods as laser radiation, chemical reduction, electrochemical reduction, thermal decomposition, and lithography [18]. Although these are effective, most are quite expensive and involve toxic reducing agents that have a negative impact on humans, living organisms, and the environment. Recently, the development of simple, economical, and environment friendly methods for synthesizing nanomaterials is one

of the most important research areas in nanotechnology. Therefore, synthesis of nanoparticles using natural materials, plant components, or microorganisms is becoming increasingly popular [19–22].

Our objective was to develop and refine a method for producing a halloysite-based silver-containing nanocomposite and to study its antibacterial activity and ability to inhibit biofilms.

EXPERIMENTAL

As a test culture, we used the Gram-negative strain of *Serratia marcescens* ATCC 9986® (*American type culture collection*, United States). This culture was chosen due to ability of these microorganisms to adhere to various biotic and abiotic surfaces (polystyrene and glass), the presence of pathogenicity factors (enterotoxigenic, hemolytic, DNAase and lecithinase activity), their environmental persistence and resistance to certain antibiotics and disinfectants [23]. For research, a culture of microorganisms was grown in a *Nutrient broth* (Oxoid LTD., England) with a volume fraction of 1% glycerol at a temperature of 30°C for 18 h. The halloysite nanotubes (Lab Safety Supply Inc., United States) and silver nitrate with a purity of at least 99.9% were used (Sigma-Aldrich/Fluka, Switzerland).

The method of synthesizing silver nanoparticles using ultrasound is based on the techniques described by (Abdullayev et al., 2011; Yakoot and Salem, 2016; Jiang LP et al., 2004) with minor modifications and additions. To obtain silver nanoparticles, a 0.1% aqueous silver nitrate solution was mixed with 50 mg of halloysite nanotubes and left for 30 min at 3000 rpm in V-32 multivortex (BioSan, Latvia). Then, the resulting suspension was placed in a BANDELIN SONOPULS HD 2070 ultrasonic homogenizer (BANDELIN Electronic GmbH, Germany, 20 kHz) at a power of 35.7 V in pulsed mode for 30 min. After completion of the ultrasound process, the sample was washed three times at 6500 rpm, dried at 40°C for 2–3 h, and ground to a powder. The hydrodynamic diameter and zeta potential of the obtained silver nanoparticles with halloysite nanotubes were assayed by dynamic light scattering and Doppler laser velocimetry using a Zetasizer Nano ZS device (Malvern, UK) and standard U-shaped cuvettes. The halloysite with silver nanoparticles was visualized by dark field microscopy using an Olympus BX51 microscope equipped with a CytoViva condenser (CytoViva Inc., United States), transmission electron microscopy (TEM) using an HT7700 Exalens microscope (Hitachi, Japan), and atomic force microscopy (AFM) using a Dimension Icon microscope (Bruker, United States). The qualitative elemental composition of the test sample was determined by energy dispersive X-ray spectroscopy using an Oxford Instruments X-Max™ 80T silicon-drift detector.

Antibacterial activity of the obtained nanocomposite in relation to bacterial strain *S. marcescens* ATCC 9986 was determined using semiliquid agar prepared according to the procedure [26] (1% peptone, 0.5% NaCl, and 0.3% agar). For this, a night culture with a 0.4 optical density (OD) concentration at 595 nm was added to the semiliquid agar and evenly distributed. Then, 50 µL of the suspension of halloysite with silver nanoparticles at a concentration of 20 mg/mL was added to the center and cultured at 28°C for 24 h. Native halloysite nanotubes and a pure *S. marcescens* culture were used as the control. The study was repeated three times, and the antibacterial effect was determined by the presence of a zone of growth inhibition of the test culture.

Quantitative determination of antimicrobial activity in relation to planktonic strains was carried out on a Multiscan FC microplate photometer (Thermo Scientific, United States), followed by plotting of the growth curve. Cultivation was carried out in a 96-well nonadhesive tablet with continuous vigorous shaking and OD measurement every hour for 48 h. A culture of *S. marcescens* was added to the experimental wells at a concentration of 0.1 OD at a wavelength of 595 nm in the nutrient medium, followed by 1.0 mg/mL of halloysite nanotubes containing silver nanoparticles. As a control, we used a culture with the addition of native halloysite nanotubes at a similar concentration, as well as a culture with addition of deionized water in an equal volume. The growth curve was plotted, and the data were statistically processed in Microsoft Excel 2016.

The ability to suppress biofilm growth was studied by swarming motility assay according to the procedure [27] on agar medium (1% peptone, 0.5% NaCl, 0.3% agar, and 0.5% sterile D-glucose). Halloysite nanotubes with silver nanoparticles at a concentration of 1.0 mg/mL were added to the experimental Petri dishes. As a positive control, sterile halloysite at a similar concentration and a medium with addition of deionized water in an equal volume were used. Then *S. marcescens* culture (0.4 OD at 595 nm) in a volume of 5 µL was applied to the center of the medium. The plates were incubated at 28°C for 24 h, and the migration distance was measured.

RESULTS AND DISCUSSION

Among the developments of silver nanocomposites, the combination of silver nanoparticles with halloysite nanotubes mainly requires additional modification using various stabilizers, specialized equipment, and various chemical reactions. Preparation of silver nanoparticles by thermal decomposition of silver acetate in the cavities of halloysite nanotubes is described in [14]; the method of silver nitrate reduction with sodium borohydride on the surface of silane- and chitosan-modified halloysite nanotubes is presented in [28]. Compared to analogous techniques,

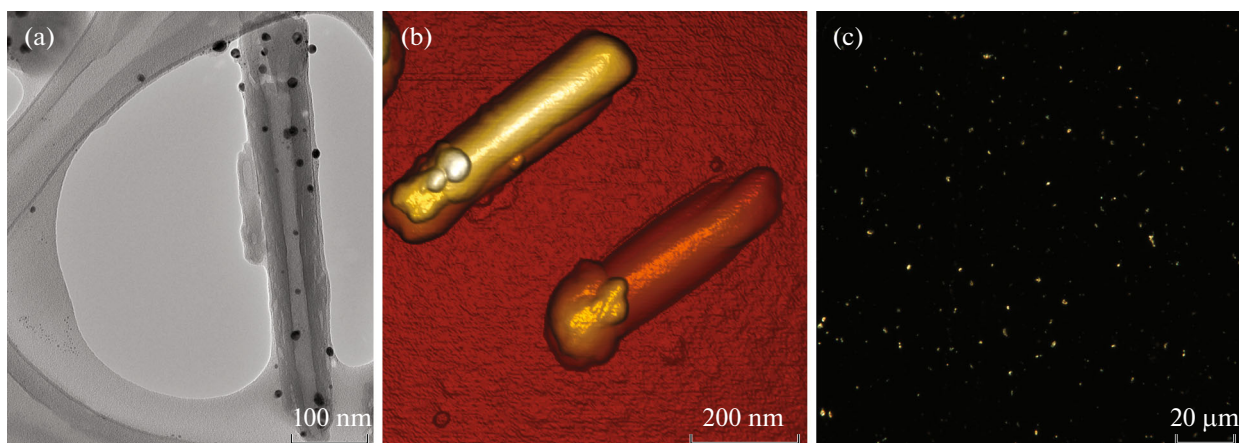


Fig. 1. (Color online) (a, b) TEM and AFM images of halloysite nanotubes and silver nanoparticles; (c) dark-field microscopy image of halloysite nanotubes and silver nanoparticles.

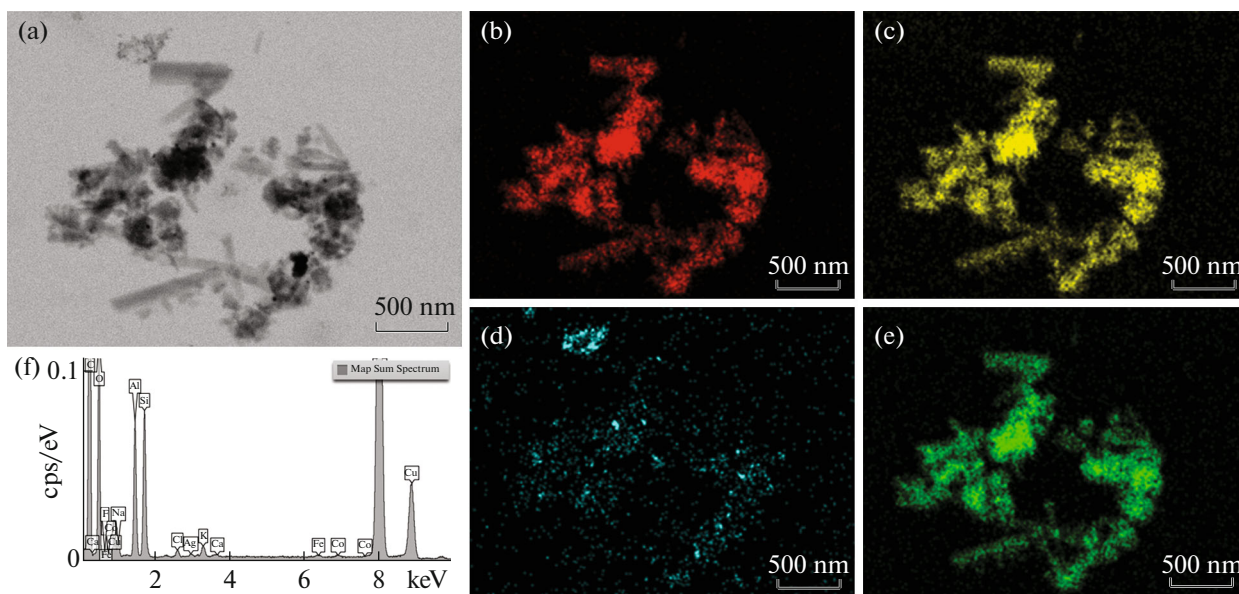


Fig. 2. (Color online) (a) TEM image of nanocomposite from which element distribution map was compiled: (b) aluminum, (c) oxygen, (d) silver, (e) silicon; (f) EDS of nanocomposite.

the main advantage of the technique proposed in this study for simultaneous production of silver nanoparticles and the nanocomposite based on it is simplicity of production and environmental friendliness, as well as the presence of nanoparticles both in the cavities of nanotubes and on their surfaces.

To synthesize nanoparticles and obtain the nanocomposite, studies were conducted to select the optimal concentration of silver nitrate and halloysite, the ultrasound power and exposure time. The effectiveness of selected parameters was assayed by preliminary studies, after which the obtained samples were visualized using AFM, TEM, and dark-field microscopy (Fig. 1). As a result, it was found that a silver nitrate concentration of 1 mg/mL, halloysite concentration

of 50 mg/mL, and exposure time of 30 min were optimal for nanoparticle synthesis.

As it can be seen in the TEM and AFM microphotographs in Fig. 1, the silver nanoparticles have different sizes and predominantly spherical shapes. The bulk of silver nanoparticles is attached to the surface or is located in the cavities of the nanotubes; however, there are single, individually located nanoparticles.

The hydrodynamic diameter and zeta potential of the obtained nanocomposite were 1038.40 ± 84.21 nm and -21.30 ± 1.40 mV; for native halloysite nanotubes, they are 569.40 ± 5.33 nm and -33.80 ± 0.87 mV, respectively. According to the results of a study of the zeta potential, the nanocomposite is unstable in aque-

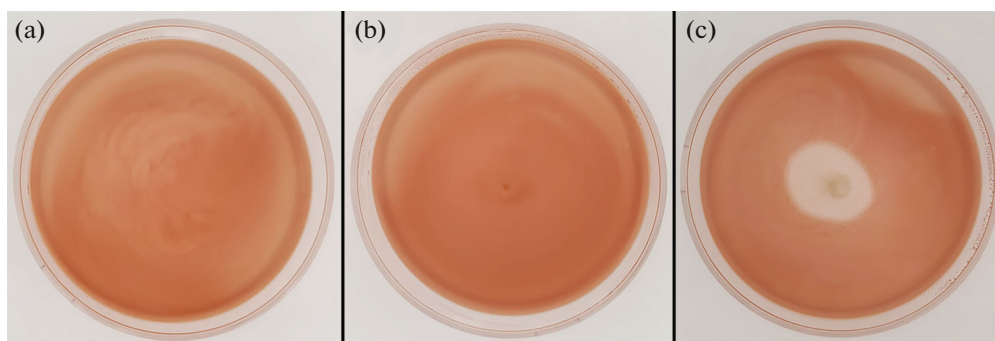


Fig. 3. (Color online) Antibacterial activity of nanocomposite against *S. marcescens*: (a) control culture of *S. marcescens*, culture of *S. marcescens* with addition of (b) native halloysite nanotubes and (c) obtained nanocomposite to center of medium.

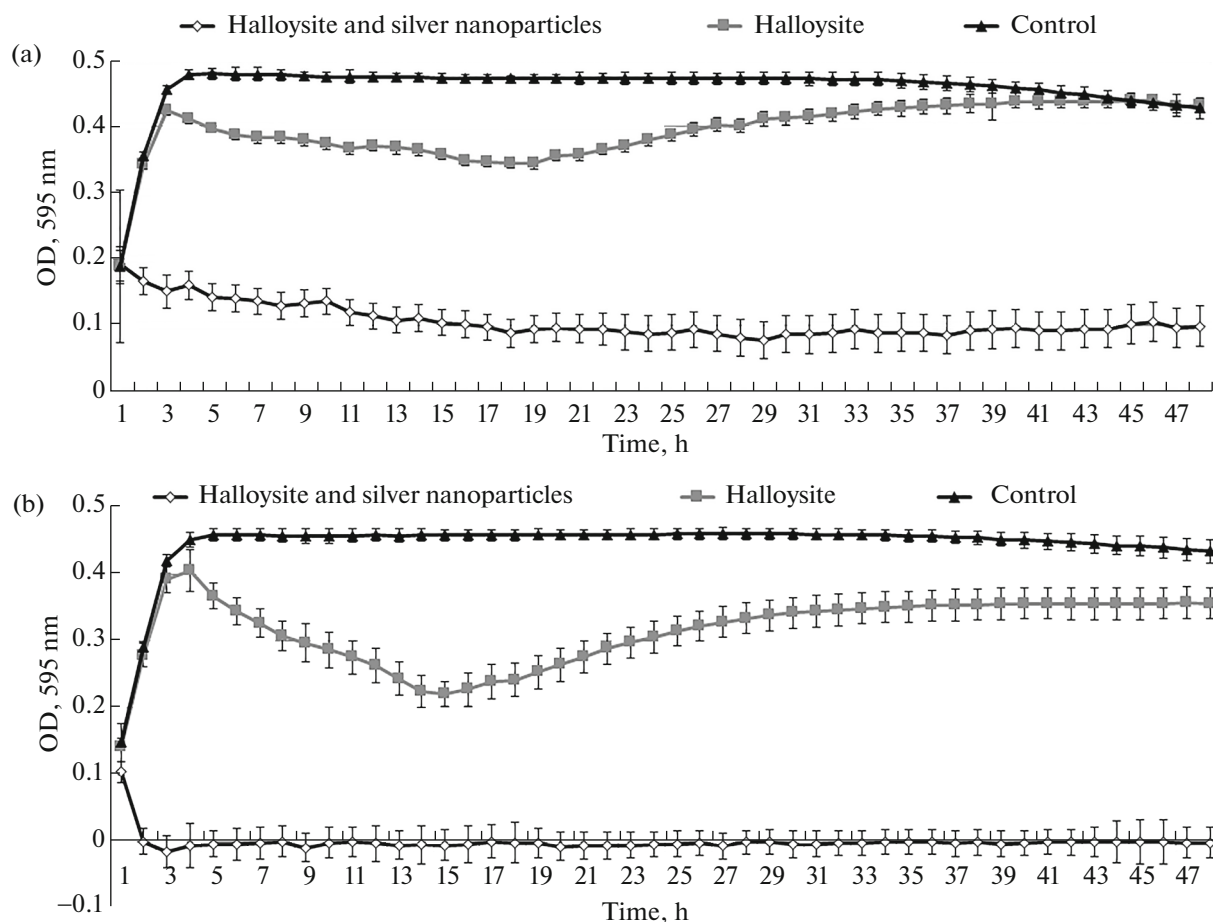


Fig. 4. Growth curve for *S. marcescens*; antibacterial effect of nanocomposite against *S. marcescens* at concentrations of (a) 1.0 and (b) 1.5 mg/mL.

ous solutions and is prone to coagulation or flocculation. Dark-field microscopy microphotographs (Fig. 1) indicate the absence of large aggregates of the obtained nanocomposite in an aqueous medium. It is likely that an increase in the hydrodynamic diameter and decrease in the negative zeta potential may be associated with the presence of silver nanoparticles on the surfaces of halloysite nanotubes.

Figure 2 shows the energy dispersion spectrum (EDS) and element distribution map of the resulting nanocomposite.

Mapping showed a uniform distribution of metallic silver throughout the sample. Based on the obtained TEM and AFM data and element distribution map, a nanocomposite consisting of silver nanoparticles and halloysite nanotubes was obtained.

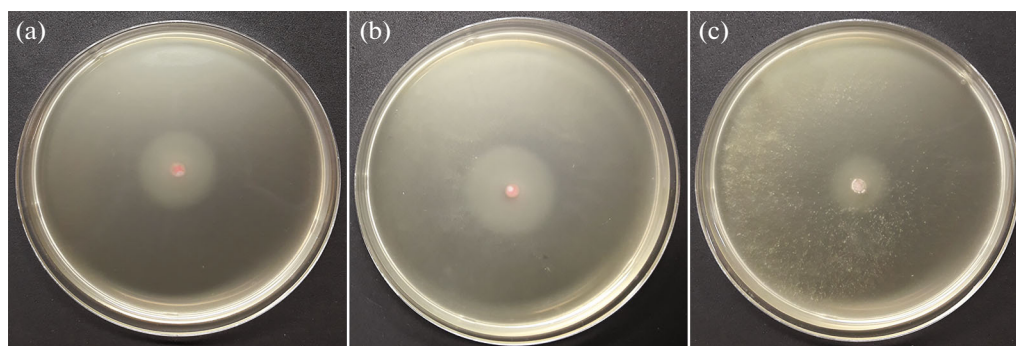


Fig. 5. (Color online) Swarming motility assay: (a) control-culture of *S. marcescens*; culture of *S. marcescens* on nutrient medium with addition of (b) native halloysite nanotubes and (c) resulting nanocomposite.

Study of the ability of the obtained nanocomposite to suppress the growth and development of opportunistic microorganisms makes it possible to use it as an antibacterial component for various materials. According to Fig. 3, the nanomaterial inhibits *S. marcescens* culture at a concentration of 1.0 mg/mL, which is indicated by the presence of a growth inhibition zone. When analyzing the antibacterial activity of the obtained nanocomposite in comparison with its counterparts, it was found that the inhibitory activity of nanocomposites based on halloysite nanotubes coated with silver nanoparticles against *E. coli* was 75% at a concentration of 1 mg/mL; in another study, the minimum inhibitory concentration of silver nanoparticles with halloysite nanotubes was 32 mg/mL towards Gram-negative *E. coli* [29, 30].

Further studies were conducted to determine the antibacterial activity using a microplate photometer, followed by construction of the growth curve, where the nanocomposite was studied at concentrations of 1.0 and 1.5 mg/mL against *S. marcescens* (Fig. 4).

According to the growth curve, a decrease in the concentration of bacteria by more than 2 times compared to the control was established 2 h after the start of the experiment with a nanocomposite concentration of 1.0 mg/mL. The antibacterial effect of the nanocomposite was observed throughout the entire experiment. With a nanocomposite concentration of 1.5 mg/mL, bacteria died 2 h after the study commenced. In samples with the addition of native halloysite nanotubes at concentrations of 1.0 and 1.5 mg/mL, a decrease in the bacteria concentration was observed in the first few hours, which may be due to their sorption ability. Study of the growth curve with silver nanoparticles at a concentration of 2 mg/mL, synthesized using an aqueous root extract of *V. Zizanioides*, showed that they exhibited no antibacterial activity against planktonic strains of *S. marcescens* [31].

Thus, analysis of the literature on existing antibacterial nanocomposites based on halloysite and silver nanoparticles and the study performed suggest that the

obtained nanocomposite exhibits high antibacterial efficiency against Gram-negative *S. marcescens*.

Next, experiments were conducted to determine the ability of the nanocomposite to inhibit the formation and development of biofilms (Fig. 5). Studies using the swarming motility assay can determine the quorum sensing of pathogenic and conditionally pathogenic microorganisms. Since this property is a key point in the bacterial formation of biofilms, its determination is an important stage in studying the ability of various substances to inhibit the development and growth of biofilms. Measurement of the diameter of the *S. marcescens* growth zone compared to the control shows the efficacy of this nanocomposite. Thus, the average migration diameter after 24 h in Petri dishes containing nutrient medium and the culture was 2.05 ± 0.05 cm; in dishes with halloysite and the culture, 2.41 ± 0.07 cm; and with nanocomposite and the culture, 1.63 ± 0.04 cm.

CONCLUSIONS

A new method for synthesizing silver nanoparticles using ultrasound and halloysite nanotubes is described. TEM images of the nanocomposite samples showed that silver nanoparticles were on the surfaces and in the internal cavities of halloysite nanotubes, due to which their antibacterial activity against *S. marcescens* was enhanced. In addition, the resulting nanocomposite has the ability to suppress the quorum sensing of *S. marcescens*, thereby hindering the ability of the bacteria to form biofilms.

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