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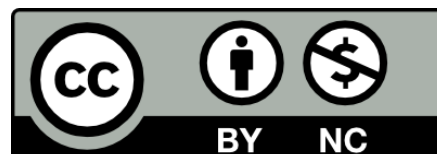
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Biophysical analysis of silver nanoparticles prepared by green synthesis and their use for 3D printing of antibacterial material for health care

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Abstract—The resistance of microorganisms to antibiotics is growing steadily. The development of new antibacterial agents is highly topical. Metal nanoparticles have shown significant antibacterial activity similar to the plant/animal materials used in traditional medicine. The study focuses on the synthesis of silver nanoparticles (AgNPs) modified with biomolecules from used plant extracts (*T. serpyllum*, *S. officinalis*, *T. pratense*). The obtained nanoparticles were studied in detail by physicochemical methods. In addition, they were deposited on acrylonitrile butadiene styrene (ABS). We created unique antibacterial material using 3D printing. 20–40% inhibition of *S. aureus* and *E. coli* was observed in the evaluation of their efficacy.

Keywords—3D printing, silver nanoparticles, green synthesis, antibacterial material, nosocomial infection

I. INTRODUCTION

At the beginning of the 21st century, infections (especially nosocomial) (Fig. 1 A) were developed rapidly due to their correct use of antibiotics and disinfection in hospital facilities. Poorly understood mechanisms have remained a major problem (Fig. 1 B). Prevention of these infections is not easy [1]. According to the National Institutes of Health (NIH), nearly 100 000 people die in the United States as a result of these infections which translates to an average of about 300 deaths per day, accounting for more loss of human lives than from HIV, car accidents and breast cancer put together. The observed rapid increase in bacterial resistance requires the search for new strategies [2]. One possible solution could, therefore, be the use of nanotechnologies (Fig. 2 A). Certain types of nanoparticles, including silver nanoparticles, show antimicrobial, antiviral and antifungal effects. In addition, green synthesis of AgNPs has been reported in literature using different routes including enzymes, plant and animal extracts. These synthesis methods are cost-effective, environmentally friendly and do not require the use of high pressure and temperature [3]. 3D printing is widely applied across different fields and in various kinds of industries including chemical laboratories [4]. This technology uses plastic filaments with different physicochemical properties to produce metallic, ceramic or biological materials. Due to the unique and versatile nature of 3D printing, its application in the field of biomedicine is extensive. The aim of this work is therefore to propose a new 3D printed material made from AgNPs having antimicrobial activities.

II. MATERIAL AND METHODS

A. Chemicals

Silver nitrate, methanol, NaCl and other chemicals were purchased from Merck (USA) at a purity > 99%. Tryptone and yeast extract from Duchefa Biochemie (Germany) were purchased for the cultivation of microorganisms.

B. Instruments

Photometry: BS-300 chemical analyzer from Mindray (China). Spectrophotometry: A UV/VIS UV-3100PC from VWR (USA) single-beam spectrophotometer was used to record the UV-VIS spectra. The VIS spectrum was measured every 2 nm in the range of 350–700 nm. The Infinite F50 (Tecan) (Tecan, Switzerland) was used for measurement on a polystyrene microtiter plate (Gama Group a.s., Czech Republic). Electrochemical analysis of silver was performed by DPV method, 0.2 M acetate buffer (pH 5), scan from -0.1 to 0.6 V, polarization rate of 25 mV/s (working carbon electrode from Metrohm, Switzerland). 3D printer (Profi3DMaker from 3Dfactories, technology – FDM). The extruder was heated to 230 ° C and the plate temperature was 80 ° C, the jet 0.5 mm. At 70 ° C, a thin layer of 3D adhesive was applied to the plate.

C. Preparation of AgNPs by green synthesis

Photometry: Biological material – Breckland thyme (*T. serpyllum*; T), garden sage (*S.officinalis*; S), red clover (*T. pratense*; J) – was collected in the locality of Boritov (CZ, Czech Republic) from June to August 2018. Plants materials were dried at 60 ° C for 48 hours, homogenized by grinding between 1–2 mm particle size. Preparation of the plant extract: The mixture was stirred in water (80 ° C) for 60 min in a ration of 1:10 followed by centrifugation (15 min, 4000 g). The extract was mixed with 0.1 M AgNO₃ (1:1) (Fig. 3 A), and the prepared particles were purified with methanol (1:1). After precipitation, methanol was removed and the AgNPs were dried at 24 h, 60 ° C (Fig. 3 B) in VWR dryer (USA). The percentage yield (%; Y) was calculated according to the following formula (a – amount): $Y = a_{\text{actual}}/a_{\text{theoretical}} \times 100$ (1)

Materials were characterized by elemental analysis of Xray diffraction (XRD) and direct electrochemical detection of silver in nanoparticles.

D. Spectrophotometric analysis of biological modification of AgNPs

Characterization of nanoparticle surface was performed by methods previously optimized [1-4]. Ferric reducing antioxidant power (FRAP) is based on the reduction of 2,4,6-tripyridyl-s-triazine complex (TPTZ) with FeCl₃ · 6H₂O and the absorbance was measured at 605 nm. The radical of 2,2'- azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS, 7 mM) and potassium peroxydisulfate (4.95 mM) were mixed in water. The absorbance was measured at 660 nm. The DPPH (1,1-diphenyl-2-picrylhydrazyl) method is based on quenching the color of the radical whose absorbance is measured at 450 nm. Total phenols were determined by the Folin-Ciocalteu reagent (1.5 mL of reagent was mixed with 200 mL of the sample), in which the mixture was left at 25 ° C for 5 min followed by the addition of 1.5 mL of 6% (w/v) Na₂CO₃. The sample was left for 90 min at 25 ° C and the absorbance was measured at 725 nm (TABLE 1). The total flavons content was determined by the following procedure: 0.5 mL of the sample was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of water. The resulting solution was left at 25 ° C for 30 min and the absorbance was measured at 415 nm. Total protein was determined by biuret (510 nm) and pyrogalllic method (520 nm).

E. Antibacterial activity of AgNPs

Escherichia coli and *Staphylococcus aureus* were

obtained from the Collection of Microorganisms of the Masaryk University, Brno (Czech Republic). Cultivation was carried out for 24 hours at 25 ° C in a sterile microtiter plate (0.3 mL). The measurement was performed on the Infinite F50 (Tecan) (Tecan, Switzerland) at 620 nm. The absorbance readings were recorded every 15 min.

F. Statistical analysis of data

Available experimental data have been processed and evaluated mathematically and statistically directly in the Qinslab database. The data presented in this work are expressed as average values.

III. RESULTS AND DISCUSSION

Cultures around the world have been using traditional herbal medicines for the treatment of countless diseases for centuries. Traditional medicine has employed, sage and thyme for the treatment of infections [5]. Similarly, clover is used to alleviate women menstrual symptoms [6]. In this study, the plant material was processed from one locality (Boritov, Czech Republic, 2018) and dried at 60 ° C immediately after collection. Subsequently, the plant material was milled to between 1–5 mm size. The herbal drug was weighed (0.5 g) and transferred to 10 mL of ultrapure water, the infusion was performed for 60 min at 20, 40, 60, and 80 ° C. Higher temperatures were not employed due to potential changes of biologically significant molecules. The extracts were subsequently centrifuged (400 g, 5 min) before filtration. The reaction of parameters for radicals, the amount of protein, total phenols, and flavonoids were obtained in the extracts. The extract thus prepared was mixed with silver nitrate (500 rpm, 25 ° C) in a 1:1 ratio. The course of AgNPs formation was monitored at 0–120 min during their preparation using VIS spectra. Evidence of formation of AgNPs was indicated by the presence of peak maxima of about 400 nm. Spherical AgNPs showed only one peak. The more the absorption maximum was shifted to higher wavelengths, the larger the AgNPs were. The appearance of peaks with a smaller area under the curve at lower wavelengths confirmed the presence of monodispersion. On the other hand, peaks with a larger area under the curve at higher wavelengths confirmed the presence of polydispersion. Characterization of AgNPs: The size of nanoparticles ranged from 20–60 nm, which is more than the average size (27 nm) of those prepared by Nicolescu et al [7]. The absorption spectra reached a peak at 440–470 nm (Fig. 2 B; TABLE 1), which is similar to study of Bumbac et al. [7] that found the maximum peak at 400–440 nm. On the contrary, some researchers reported the maximum peak in the area of 340–620 nm [8]. We prepared polyhedron-shaped AgNPs using green synthesis. The velocity constants of the AgNPsT, AgNPsS, and AgNPsJ were determined by integration method and experimentally ranged around $3 \cdot 10^{-7} \text{ M} \cdot \text{s}^{-1} \cdot \text{AU}^{-1}$. The effect of the biological extract on AgNPs generation was optimized in the experiment. The ideal time for nanoparticles formation was found to produce the largest amount of AgNPs in solution between 24–48 h. The yield of green synthesis of AgNPs for T, S, and J was in the range of 40–80% (TABLE 1). For basic characterization of the surface properties of AgNPs, simple reactions such as total phenols, flavones, radical quenching ability, total protein were employed.

Chemical properties: ABTS – a decrease of 40–80 % after 15 min of quenching radicals, DPPH – a decrease of 15–55 % after 15 min of quenching radicals, total phenols (extract): 1200–1800 mg/mL of GA equivalent. The SEM analysis showed that the particles were mostly polyhedron in shape and had a size of 50 nm. The SPR method determined the particle size at intervals of 20–60 nm and zeta potential in the range of -20 to -5 mV.

A. Electrochemical determination of silver concentration

Analysis of Ag(I) was performed in acetate buffer (0.2 M, pH 5.0) (carbon working electrode, Ag/AgCl 3M KCl reference electrode, platinum auxiliary electrode) according to the method previously published [9]. To increase the sensitivity of the method, accumulation potential at 400 mV for 240 s was performed. Scanning was done from 0–800 mV. The Ag(I) signal was recorded at a potential of 120 s.

The concentration dependence was linear with LOD = 1 nM, LOQ = 7 nM. AgNPs were mineralized by the following procedure: 200 μ L HNO₃ (65%) was added to the crushed printed material (1 mg). After 1 h, the mixture was decomposed by microwave decomposition (300 W, 3 min).

The mixture was then neutralized with 200 μ L of NaOH. The sample (10 μ L) was analysed by the optimized procedure described above. The amount of silver analysed in AgNPs was 40–65% of the amount deposited.

B. Modification of 3D filament

The possibilities of using 3D printing in chemical laboratories and for biotechnology applications have been intensively developed over the last ten years [10, 11]. 3D filament (fiber, ABS – acrylonitrile-butadiene-styrene, \varnothing 1.7 mm, white) of the required length (at least 40 cm for wheelshaped printing; $r = 14$ mm, force = 2 mm) was tensioned between two chemical stands. The purified silver nanoparticles were dispersed in 18 M Ω of water and acetone (Fig. 3 C) (1:1, $c = 3$ mg/mL). Silver nanoparticles in this form were applied to the fiber by means of a brush. The brush was cleaned between the use of different silver nanoparticles with a stream of flowing water, followed by 70% ethanol, washed with distilled water and dried. AgNPs were applied to the surface of a 3D filament of the ABS type, on which small notches were created for better attachment of AgNPs. AgNPs were dispersed in the mixture of acetone and water (1:1) at a concentration of 3 mg/mL before application. A 1 mL of such dispersion was applied to a 40 cm fiber (Fig. 3D). Between each printing of the material, the 3D printer jet was removed and cleaned with acetone. The Teflon tube was also cleaned with acetone. After the dispersion dried at the string, the string was printed (Fig. 3 E). In other procedures, PLA (polylactic acid) and PET-G (glycol-modified polyethylene terephthalate) filaments testing will be performed. Another direction of our work will be to use other printing methods that use lower temperatures.

C. Antibacterial activity by 3D printing of ABS-AgNPs-3D filament

Developing bactericidal surfaces using simple chemical methods can be a very promising way to a number of applications [12]. We describe an eco-friendly approach to the chemical synthesis of AgNPs. Antibacterial activity was determined in model organisms (*S. aureus*, *E. coli*). The growth curves (ABS, ABS-AgNPs material: 1 mg) were measured. The *differential vz/k (OD)* was performed as a difference between individual points of the growth curve of the bacterium and the AgNPs inhibition points. We found that AgNPsT, AgNPsS, and AgNPsJ showed an inhibitory activity of 20–40% of the control. Based on the IC₅₀ calculation, MICs were determined in AgNPsT (2.5 μg/mL), AgNPsS (3 μg/mL) and AgNPsJ (6 μg/mL). Individual types of AgNPs were deposited as described above on ABS material and tested platforms were printed by 3D printing. For all tested materials, at least 25% inhibition was detected compared to control (10¹⁰/mL cells). 3D printing-prepared platforms exhibited excellent antibacterial properties, which killed both Gram-negative and Gram-positive bacteria.

IV. CONCLUSION

Silver nanoparticles have pronounced antibacterial properties. However, there is a concern about their persistence in the environment. An alternative is the surface treatment by biomolecules from plant extracts of such nanoparticles (these become better degradable in the environment). Our results show that the application of green chemistry techniques improves the synthesis of AgNPs (higher antibacterial effect and better environmental degradability). The thus prepared particles were deposited on an ABS material which, after its printing, exhibited an antibacterial effect.

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Figures:

Fig. 1 (A) A simplified representation of the spread of the infection. A patient is the source of infection, there is fecal contamination of medical equipment, clothing along with the hands of healthcare professionals and visitors. The infectious agent is deposited on door handles, toilets, furniture, beds, keyboards, etc. (B) Predicted probable targeting of antibiotics to a prokaryotic cell. The effect is directed to DNA/RNA synthesis, cell wall, cell membrane, protein synthesis and folate metabolism. The resistance of bacterial

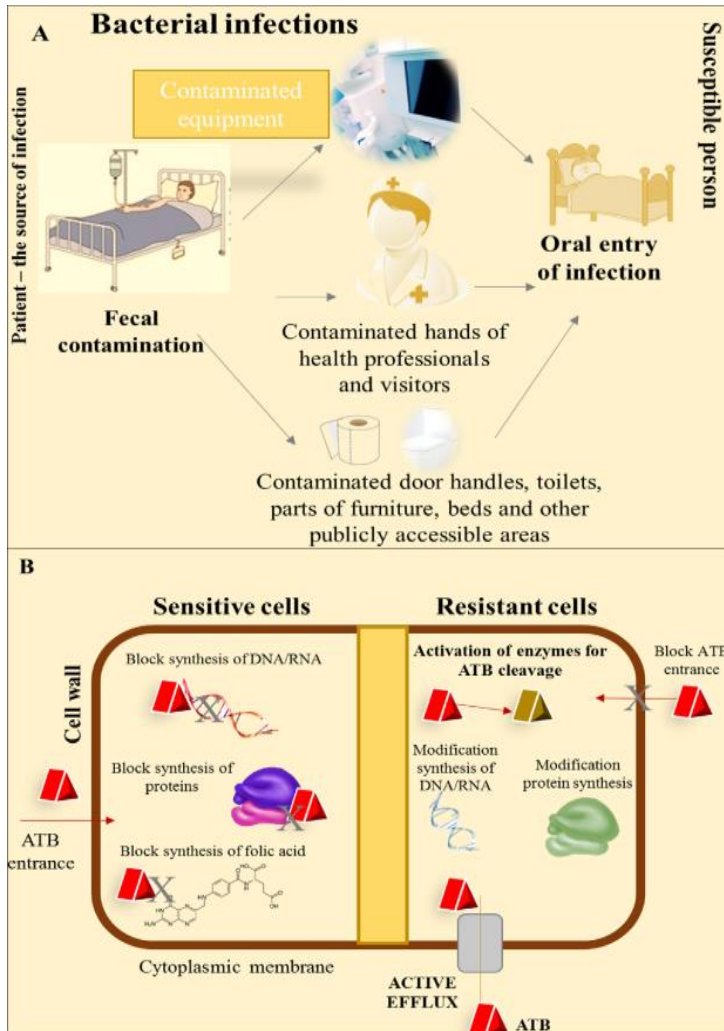


Fig. 2 (A) A frequency of occurrence of records on nanoparticles. 275,618 records from Web of Science based on the following keywords “nanoparticle, nanoparticle application, nanoparticle synthesis“ were evaluated. Individual records are expressed as the ratio of the number of searched records on the topic to the total searched records. (B) Typical VIS spectra of generated AgNPsT, AgNPsS, AgNPsJ.

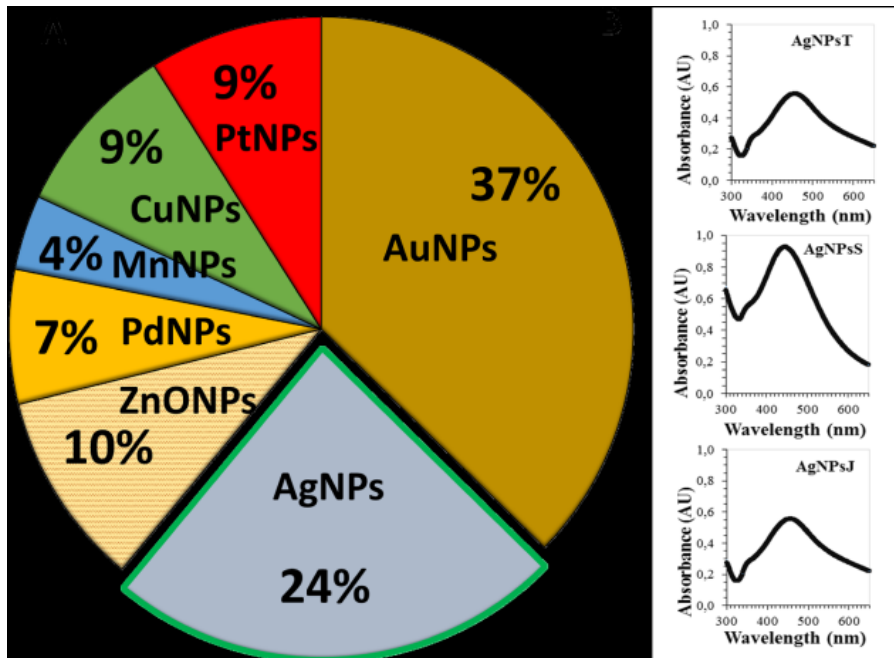


Fig. 3 (A) Typical appearance of *T. serpyllum*, *T. pratense*, *S. officinalis* extracts with addition of 0.1 M AgNO₃ for 60 min, 80 ° C, 560 rpm. (B) Visual appearance of precipitated (methanol 1 : 1) and dried AgNPsT, AgNPsS, AgNPsJ (24 h, 60 ° C). (C) Purified AgNPsT, AgNPsS, AgNPsJ dispersed in ultrapure water by ultrasound (3 mg/mL). (D) Visual appearance of ABS material after application of prepared AgNPsT, AgNPsS, AgNPsJ. (E) 3D printing-prepared platform with AgNPsT, AgNPsS, AgNPsJ (3 mg/mL). The extruder was heated to 240 ° C and the plate temperature was 80 ° C, the jet 0.5 mm.

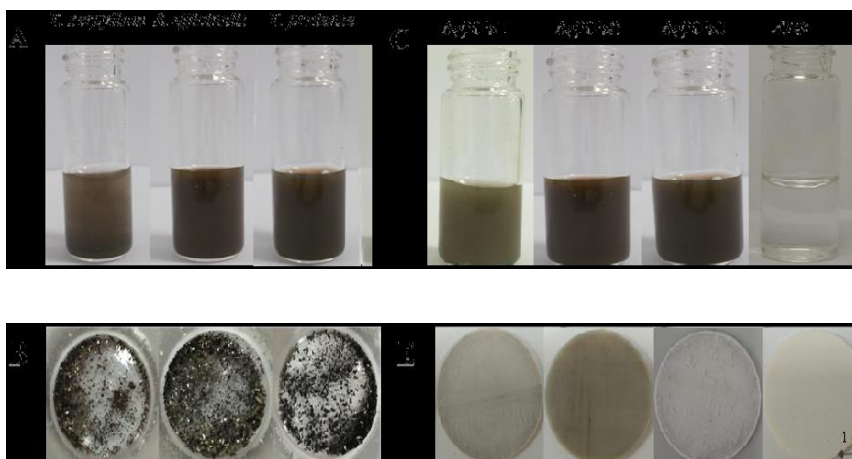


TABLE I. CHARACTERISTICS OF SYNTHESIZED SILVER NANOPARTICLES

| Nanoparticles | Parameters of prepared NPs | | |
|---------------|----------------------------|--------------------------|-----------------------|
| | Average yield (%) | Equivalent of phenol (%) | Maximum UV-VIS signal |
| AgNPsT | 65 | 0.304 | 420 |
| AgNPsS | 77 | 1.387 | 440 |
| AgNPsJ | 67 | 1.336 | 430 |

^a Plant extracts: T - thyme, S - sage, J - clover

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