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ORIGINAL ARTICLE

Inhibitory Effect of Selenium Nanoparticles on the Biofilm Formation of Multidrug-Resistant *Acinetobacter Baumannii*

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Abstract

Background/Aim: Treatment of infections caused by biofilm-producing multidrug-resistant (MDR) pathogens represents a huge global problem due to primary antimicrobial multi-resistance enhanced by reduced penetration of antibiotics in the biofilm-embedded bacteria. The aim of this study was to determine the capacity of biofilm production among MDR *Acinetobacter baumannii* (*A. baumannii*) isolates obtained from different clinical specimens and to evaluate the inhibitory effect of selenium nanoparticles (SeNPs) coated with cationic polymer cetyltrimethylammonium bromide (CTAB) on the biofilm formation.

Methods: Antimicrobial effect of antibiotics (meropenem, imipenem, gentamicin, amikacin, ciprofloxacin, levofloxacin and trimethoprim-sulfamethoxazole) was determined by disk-diffusion assay, while sensitivity to colistin was determined with E test. All 60 isolates were tested on biofilm production in microtiter plates with crystal violet dye. Minimal biofilm inhibitory concentration (MBIC) of SeNPs was tested in order to prevent biofilm formation in microtiter plates.

Results: All tested clinical isolates were classified as MDR (n = 60) and extensively drug-resistant (XDR, n = 60). Out of the total 60 isolates, 55 isolates (92 %) showed the ability for biofilm formation, with the majority of them classified as strong (42 %) and moderate (42 %) biofilm producers. MBIC values of SeNPs for 55 biofilm-producing isolates ranged from 0.07 to 1.25 mg/mL. Strong biofilm producers had statistically higher MBIC (0.15 mg/mL) in correlation to other biofilm-producing isolates (0.07 mg/mL). There was no correlation between invasiveness of isolates with biofilm production and MBIC values.

Conclusion: Presented results are very promising and interesting especially in nanotechnology and medical fields, while SeNPs with the addition of cationic surfactant inhibit biofilm formation of MDR *A. baumannii* clinical isolates.

Key words: *Acinetobacter baumannii*; Selenium nanoparticles; Biofilm.

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Introduction

Acinetobacter baumannii (*A. baumannii*) is one of the most common human pathogens, associated predominantly with nosocomial infections such as ventilator-associated pneumonia and sepsis, urinary tract and skin and soft tissue infections, especially among critically ill patients

in intensive care units.[1] *A. baumannii* belongs to the ESKAPE group of microorganisms (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa, and Enterobacter spp*), which are globally notorious for human health due to their ability to acquire diverse resistance genes rapidly. Carbapenems and colistin are the most commonly used antibiotics for the treatment of multi-resistant bacterial isolates. Unfortunately, resistance to carbapenems is now significantly widespread, because of the genome plasticity of this bacterium, which easily acquires resistance genes, including genes for carbapenemases production. The high prevalence of carbapenem-resistant *A. baumannii* (CRAB) isolates carrying bla_{OXA-23}-like and bla_{OXA-24}-like genes has emerged as a serious problem in healthcare settings in Serbia.[2, 3] Also, bacteria can become resistant to other classes of antibiotics by regulation of antibiotic transportation through bacterial membranes (eg reduced expression of porins or enhanced activation of efflux pump), alteration of the target site (eg ribosomal protection protein) and enzymatic modifications and inactivation of antimicrobial substance (eg aminoglycosidases).[1] During the COVID-19 pandemic, the importance of this pathogen was extremely emphasised, because it was determined that co-infection between the SARS-CoV2 virus and this bacterium was very often associated with a fatal outcome.[4, 5] Most of the patients who died were in intensive care units with respiratory failure and were treated with the invasive mechanical ventilation. *A. baumannii* is capable of forming biofilm on artificial substrates, such as respiratory ventilators or vascular and urinary catheters. It is an excellent biofilm producer and biofilm production represents an additional factor contributing to the development of antimicrobial resistance. Taking all these facts together, it is obvious that the World Health Organization ranked multidrug-resistant (MDR) *A. baumannii* as the number one priority microorganism for antimicrobial substance research and development.

Nanoparticles (NPs) are one of the most promising antibacterial agents. Due to their small size and high surface-to-volume ratio, NPs have physical and chemical properties that differ from their bulk material. Selenium is an essential mineral and micronutrient that is well known for its anticancer and antimicrobial activity, as well as being an important substance for improving reproductive capabilities.[6] Selenium nanoparticles (SeNPs), compared with selenium inorganic and organic compounds, emerged as a promising agent for antimicrobial and biomedical uses due to their low toxicity, degradability and high bioavailability.[7] When it comes to NPs, their ability for aggregation is quite challenging. In order to avoid this unfavourable occurrence, one uses surfactant-coated NPs methodology. Essentially, NPs are surrounded and covered by the surfactant which allows NPs to be more stable.

The aim of this study was to determine the capacity for biofilm production among MDR *A. baumannii* isolates obtained from different clinical specimens and to evaluate the antimicrobial effect of SeNPs coated with cationic polymer cetyltrimethylammonium bromide (CTAB) against planktonic isolates and biofilm formation of clinical isolates of MDR *A. baumannii*.

Methods

Bacterial isolates, species identification and antimicrobial resistance

The present experimental study included 60 non-redundant randomly selected MDR *A. baumannii* isolates from the bacterial collection of the Institute of Microbiology and Immunology in Belgrade. The isolates were recovered from patients admitted to hospitals in Serbia during January-June 2018. Identification of the *A calcoaceticus–baumannii* complex (Acb complex) was done using conventional bacteriological techniques employed in clinical microbiology. Species identification of isolates as *A. baumannii* was confirmed by Vitek 2 System (*Biomerieux*, France). Invasive isolates were those collected from normally sterile body sites (such as blood, peritoneal fluids and cerebrospinal fluids). Non-invasive isolates were those obtained from skin and soft tissue, urine and respiratory tract.

According to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the antimicrobial susceptibility of *A. baumannii* to meropenem, imipenem, gentamicin, amikacin, ciprofloxacin, levofloxacin and trimethoprim-sulfamethoxazole was determined by disk-diffusion assay (*Bio-Rad*, UK).[8] ComASP Colistin (*Liofilchem*, Italy) was used to determine the minimum inhibitory concentrations (MICs) for colistin, in accordance with EU-

CAST recommendations.[8] Based on antimicrobial resistance, all *A. baumannii* isolates were classified as follows: (i) MDR- resistant to at least one agent in three or more antimicrobial categories, (ii) extensively drug-resistant (XDR)- resistant to at least one agent in all, but two or fewer antimicrobial categories and (iii) pandrug-resistant (PDR)- resistant to all agents in all antimicrobial categories tested.[9]

Biofilm production assay

Quantification of biofilm production was done following the protocol by Stepanovic et al.[10] After the 37 °C overnight incubation in Trypticase Soy broth (TSB, *Biorad*, UK), strains were diluted in fresh TSB to achieve a final concentration of 10^6 CFU/mL. Aliquots of *A. baumannii* suspension (100 µL) were transported to each well of the 96-well microtiter plate and were incubated for 24 h at 37 °C. Microtiter plates were aspirated and washed three times with sterile phosphate-buffered saline (PBS). The plates were dyed with 100 µL of 2 % (w/v) crystal violet for 15 min, washed three times and dried overnight at room temperature. Thereafter, 100 µL of glacial acetic acid at 33 % (v/v) was used to dissolve the dye that had been bound to the biofilm matrix. Using an automated microtiter plate reader, the optical density (OD) of each well was determined spectrophotometrically at 570 nm (ICN Flow Titertek Multiskan Plus Reader, *Meckenheim*, Germany). TSB was the only suspension in the negative control wells. *A. baumannii* ATCC 19606 was used as positive control. Three standard deviations more than the mean OD of the negative control were designated as the cut-off OD (ODc).

The results were evaluated as follows:
OD ≤ ODc non-biofilm producers,
ODc < OD ≤ (2 × ODc) = weak biofilm producers,
(2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producers and
OD > (4 × ODc) = strong biofilm producers.

SeNPs synthesis

Five hundred mg of sodium selenite ($Na_2S_2O_3$) was dissolved in 50 mL of double distilled water, then it was sonicated for 10 min. Along with this solution, the CTAB solution was also prepared. Two hundred fifty mg of CTAB was dissolved in 50 mL of double distilled water and sonicated for 10 min. Afterward, both solutions were mixed and stirred for 10 min on a magnetic stirrer. After the addition of 1500 mg of ascorbic acid, the stirring was continued for 4 h and the temperature was set up to 80 °C. Finally, 6.25 mL of double distilled water and 1.876 mL of hydrazine were added. After 2 h, the solution was kept for 4 days at room temperature and it was filtered and washed with double distilled water and dried in an oven for 1 h at 50 °C. A similar procedure was reported previously.[11, 12]

Characterisation of SeNPs

Characterisation of SeNPs was performed using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) technique. Images were produced by TM3030 SEM Hitachi Japan SEM.

Testing of inhibitory effect of SeNPs on the biofilm formation of *A. baumannii* isolates

The minimum biofilm inhibitory concentration (MBIC) of SeNPs was analysed following the protocol of Bagheri-Josheghani et al.[13] Aliquots of 100 µL of bacterial isolates (in a final concentration of 10^6 CFU/mL) in double-strength MH broth were incubated with 100 µL of SeNPs (in final concentrations ranging from 1.25-0.0015 mg/mL). After overnight incubation at 37 ^{0}C, microtiter plates were treated as described above in section 2.2.

Statistical analyses

SPSS version 20.0 (*SPSS Inc*, Chicago, IL, USA) was used for statistical analysis. Statistical comparison of multiple groups on the occasion of deviation of normal data distribution was done using the Kruskal-Wallis rank sum test. To compare variances without normal distribution between the two groups, the Mann-Whitney U test was used. Fisher's exact test was used for comparison of the frequency of occurrence of the analysed categorical variables. A p value less than 0.05 was considered to be significant.

Results

Bacterial isolates and antimicrobial testing

All 60 isolates of *Acb complex* were identified as *A. baumannii.* Bacterial isolates were recovered from blood samples (18), cerebrospinal fluid (1), peritoneal fluid (1), lower respiratory specimens (20), urine (6) and from the wound and soft tissue specimens (14). Bacterial isolates were obtained from 19 female and 41 male patients. The median age of patients was 67.3 years, with a range of 18-88 years.

All isolates were resistant to carbapenems (imipenem, meropenem), while other antibiotics showed also high resistance rate: ciprofloxacin (93 %), levofloxacin (93 %), amikacin (95 %), gentamicin (98 %) and trimethoprim-sulfamethoxazole (93 %). All isolates were sensitive to colistin. According to antimicrobial resistance, all isolates were classified as MDR (n = 60) and XDR (n = 60) isolates. None PDR isolate was detected.

Biofilm production

Out of a total of 60 tested isolates, the majority of isolates were strong (n = 25, 42 %) and moderate (n = 25, 42 %) biofilm producers. Only 5 (8 %) isolates were weak producers, while 5 (8 %) isolates were not capable of biofilm production. Among non-biofilm producing isolates, 2 isolates were obtained from blood and 3 isolates were obtained from lower respiratory samples. Average biofilm mass according to O.D. for strong, moderate and weak biofilm producers were 0.720, 0.300 and 0.166, respectively. When the capacity of isolates to form biofilm with invasiveness of isolates or specimen type was compared, there was no statistical significance in both correlations ($p > 0.05$). There was no statistical correlation between antimicrobial resistance and biofilm production ($p > 0.05$). The distribution of biofilm production abilities of the tested isolates obtained from various clinical samples is displayed in Figure 1.

SeNPs characterisation

Figure 2A presents the SEM micrographs of the synthesised SeNPs which showed well-defined spherical morphology. The EDX spectrum (Figure 2B) contains a pronounced Se peak, along with the nitrogen (N), carbon (C) and oxygen (O) peaks. The last three originate from the cationic surfactant CTAB. Its role is to prevent agglomeration of SeNPs, as well as to make energetic SeNPs more stable.

Inhibitory effect of SeNPs on biofilm formation of MDR *A. baumannii*

SeNPs exhibited an inhibitory effect on biofilm formation against 55 biofilm-forming isolates. One isolate from the wound swab had MBIC value of 1.25 mg/mL, while 4 isolates (2 respiratory, 1 urinary and 1 blood sample) had MBIC value of 0.625 mg/mL. All other 50 isolates have MBIC values of 0.3 mg/mL or lower. The median MBIC value for all 55 biofilm-producing isolates was 0.15 mg/mL, whereas $MBIC_{50}$ and $MBIC_{90}$ were 0.07 and 0.3, respectively. When SeNPs MBIC values with the invasiveness of isolates was compared, there was no statistically significant difference among tested isolates ($p > 0.05$). On the other hand, strong biofilm producers had higher MBIC in correlation to other isolates ($p < 0.001$), as represented in Table 1. There was no statistical correlation between antimicrobial resistance to antibiotics and MBIC SeNPs values ($p > 0.05$).

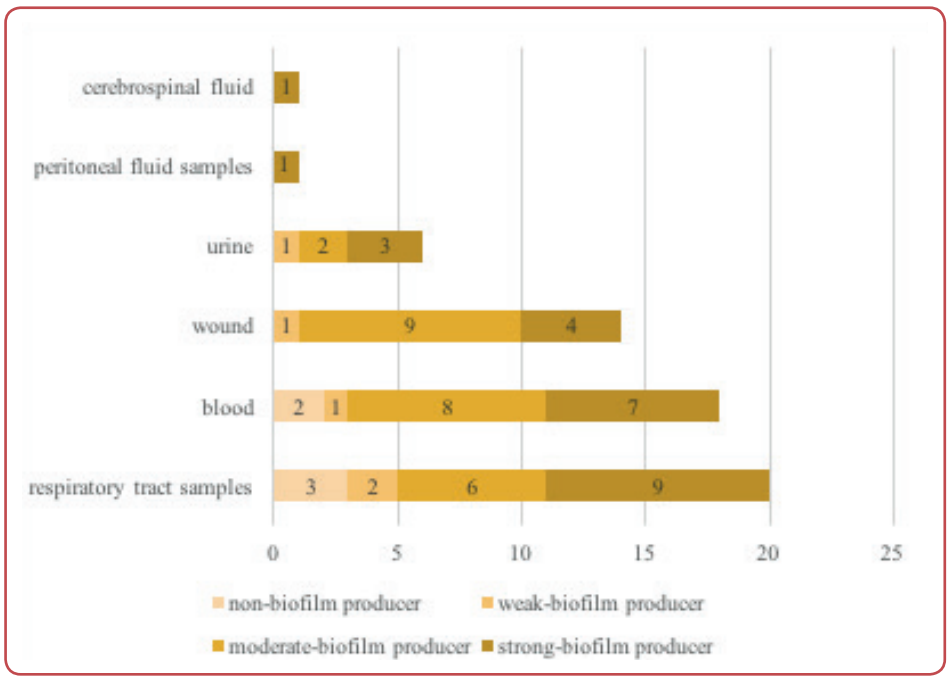

Figure 1: Biofilm formation abilities of 60 multidrug-resistant (MDR) isolates of Acinetobacter baumannii obtained from various clinical specimens

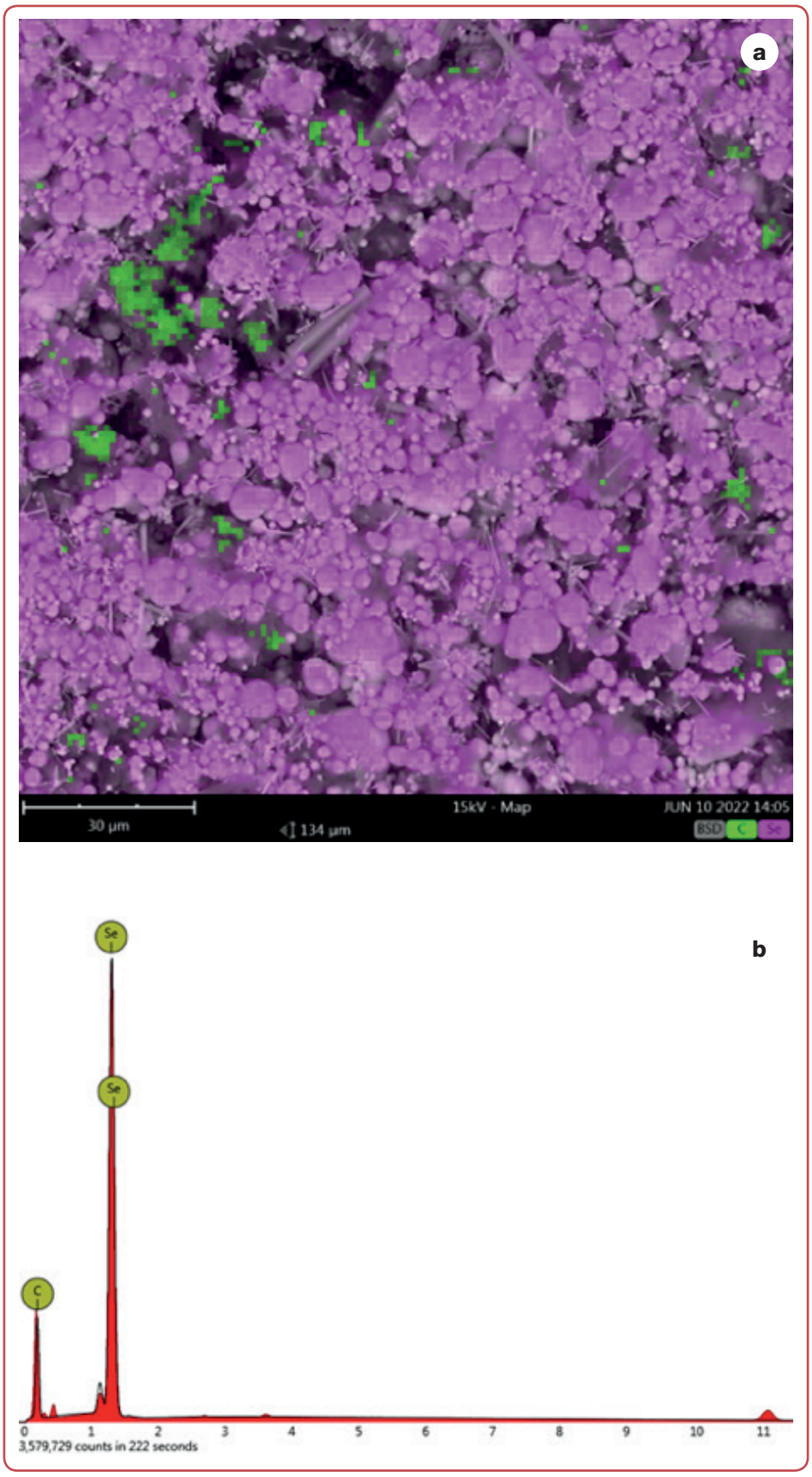

Figure 2: Characterisation of selenium nanoparticles (SeNPs) using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) technique

Table 1: Median minimal biofilm inhibitory concentration (MBICs) values of selenium nanoparticles (SeNPs) among non-producers, weak, moderate and strong biofilm producers

MBIC (mg/mL)	Non-producers (N = 5)	Weak producers (N = 5)	Moderate producers (N = 25)	Strong producers (N = 25)
Median (range)	0.00 (0.00-0.00)	0.07 (0.03-0.15)	0.07 (0.007-1.25)	0.15 (0.03-0.625)

Discussion

The emergence of MDR and XDR *A. baumannii* is becoming a critical health problem worldwide. Additionally, biofilm production of MDR and XDR *A. baumannii* isolates contributes both to antimicrobial resistance and to bacterial endurance in the hospital environment, leading to the increased prevalence of nosocomial infections.[14, 15] In presented study, all *A. baumannii* isolates were MDR and XDR, sensitive only to colistin, a last-resort antibiotic, which is now a first choice for the treatment of resistant isolates. Fortunately, resistance to colistin is still low and uncommon, but is worrisome and needs to be observed. Although presented resistance to other tested antibiotics is extremely high, similar frequencies of MDR and XDR isolates have been detected globally.[16, 17] Also, it is important to notice that all isolates were resistant to carbapenems, which, unfortunately, is becoming a common finding both in our region and globally.[18, 19]

Given that *A. baumannii* is most frequently associated with nosocomial infections, eg ventilator associated-pneumonia, sepsis, urinary, skin and soft tissue infections, the majority of presented isolates were recovered from lower respiratory samples (20/60, 33 %), blood (18/60, 30 %) and swabs obtained from skin and soft tissue infection (14/60, 23 %), as expected.[20] *A. baumannii* has excellent capacity to form biofilm in comparison to other bacteria, so it is not surprising that almost all of isolates (55/60, 92 %) were biofilm producers with an equal and significant percentage of moderate (25/55, 45 %) and strong (25/55, 45 %) biofilm producers. In the present study, equal numbers of non-invasive and invasive isolates were non-biofilm and strong biofilm producers, respectively, without a clear statistical correlation between bacterial invasiveness and capacity for biofilm production. This could be explained by the shedding of the exopolysaccharide biofilm envelope, enabling planktonic bacteria to be released from the biofilm community and enter the bloodstream causing bacteraemia. Although invasive bacteria are isolated from the blood, no one can claim with certainty and without additional sub-molecular testing that they do not come from the biofilms in tissue or on indwelling devices.[21] Also, the treatment of infections caused by biofilm-producing MDR *A. baumannii* isolates represents a huge challenge, while these isolates could be resistant to all antibiotics, regardless of the results of the antibiogram. This resistance stems from reduced or disabled penetration of antibiotics into the biofilm-embedded bacteria. Presented isolates represent a great example of a problematic situation for our healthcare system, similar to the results of Zeighami et al.[16] Better biofilm production among MDR *A. baumannii* isolates is explained by several mechanisms: lipid A modification, overexpression of efflux pumps and exposure to subminimal antibiotic concentration which creates positive feedback that allows a switch from planktonic to sessile growth in biofilm.[22]

Infections caused by biofilm-producing MDR *A. baumannii* are very demanding for treatment and there are worldwide efforts for the development of new promising and effective antibacterial substances. According to available literature data, this study represents one of the scarce investigations about the antimicrobial activity of SeNPs against MDR *A. baumannii* isolates. The results obtained in this study are very encouraging, because SeNPs prevented biofilm formation. Strong biofilm producers showed significantly higher MBIC values in relation to moderate and weak biofilm-producing isolates, as expected. This results, as well as the results of other authors, showed that SeNPs have great potential as medical devices coatings, because they efficiently prevented biofilm formation at low concentrations.[23-25] Hoseini Bafghi et al demonstrated that SeNPs reduced antifungal resistance due to diminished expression of resistance- related genes in resistant fungal isolates.[26] Biogenic SeNPs are capable of both the prevention of biofilm formation and degradation of mature biofilm matrix by degradation of the bacterial cell membrane and bacterial envelope called glycocalyx in biofilm producing isolates of *P. aeruginosa, S. aureus* and *S. Typhi*.[27] There are no precise data about mecha-

nism of action of SeNPs against biofilm producing *A. baumannii* isolates and according to available literature, it can only be assumed that SeNPs act on quorum sensing molecules, preventing the onset of biofilm formation by the switch interruption from individual planktonic isolates into collective, biofilm-associated existence.[28] The main goal of this work was to examine the possible antibiofilm effect of modified SeNPs. Due to technical limitations, authors' were not able to perform a complete characterisation of the NPs or determine the precise mechanism of action of these NPs during the process of biofilm formation. In future experimental work, it would be very valuable to obtain precise information about these two unanswered questions to highlight the importance of presented modification method for SeNPs synthesis.

Conclusion

In the present study, it was demonstrated that modified SeNPs successfully prevented biofilm formation in MDR *A. baumannii* isolates. Although stronger biofilm producers required higher concentrations of SeNPs, results suggest that SeNPs coated with CTAB should be considered as a potential coating for indwelling medical devices and further development in pharmaceutical nanotechnology.

Ethics

The study was approved by the Ethical Committee of the Medical Faculty, University of Belgrade (permission No 1550/II-4, dated 21 February 2019). The written informed consent was obtained from all patients.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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