THERANOSTIC TOOL FOR TARGETING OF BACTERIAL CELLS BASED ON SILVER NANOPARTICLES

[1]Michael GARGULÁK, [1]Matouš ČÍŽEK, [2,5]Michaela DOČEKALOVÁ, [2]Marta KEPINSKA, [1,3]Andre FERREIRA DA SILVA, [1]Aneta SUROVÍKOVÁ, [2]Dagmar UHLÍŘOVÁ, [2]Martina STAŇKOVÁ, [1,5]Branislav RUTTKAY-NEDECKÝ, [5]Aleš VAVŘINÍK, [5]Jiří SOCHOR, [6]Hoai Viet NGUYEN, [1,2,4,5]René KIZEK

[1]University of Veterinary and Pharmaceutical Sciences Brno, Pharmaceutical Faculty, Brno, Czech Republic, EU, kizek@sci.muni.cz
[2]Prevention Medicals, Studenka-Butovice, Czech Republic, EU, uhlirova@preventionmedicals.cz
[3]University of Lisboa, Faculty of Pharmacy Avenida Professor Gama Pinto, Lisboa - Portugal, EU, andre_ferreira.silva@hotmail.com
[4] Department of Biomedical and Environmental Analyses, Faculty of Pharmacy, Wroclaw Medical University, Wroclaw, Poland, EU, zalewska.m@gmail.com
[5]Mendel University in Brno, Faculty of Horticulture, Department of Viticulture and Enology, Lednice, Czech Republic, EU, sochor.jirik@seznam.cz
[6] Research Center for Environmental Monitoring and Modeling, VNU University of Science, Hanoi, Vietnam, nguyenviethoai@hus.edu.vn

Abstract

Infectious diseases are known to be a constant threat to human health also in the beginning of the 21st century. The rapid growth of the human population has led to the agricultural intensification, which has caused an increase in resistant bacterial species in the environment. The aim of the project was to design and verify a theranostic tool combining the diagnostic and therapeutic parts for targeting the bacterial cells. The construct was composed of: A) silver nanoparticles prepared by green synthesis (AgNPsGS); B) antibiotic encapsulated in apoferritin (APO); C) modified magnetic gold nanoparticles (AuNPs). The effect of theranostic nanotransporter on prokaryotic cell culture was model-monitored by altering their growth characteristics (OD monitoring). During the project, 20 various types of AgNPsGS were prepared. The best antibacterial effect (85 % growth inhibition of *S. aureus*) with the minimal inhibitory concentration (MIC50) of 0.4 μg/mL was observed by AgNPsGS4 (*Thymus serpyllum* L.). SPIONs (20 mg/mL, magnetic AuNPs) were modified with antibody (1 mg/mL IgY) and oxidized graphene sheets (1 mg/mL). The EDC/NHS polymer (2.2 mM EDC, 4 mM NHS, 3 h, 37 °C) was used for binding. The apoferritin (SPIONs/Au/APO) was attached to the SPION/AuNPs. In the SPIONs/Au/APO cavity (12 nm), the antibiotic doxorubicin (2 μM DOXO, the DOXO encapsulation efficiency was around 13 % of the applied concentration) was model-sealed. The created SPIONs/Au/AgNPsGS4/APO/DOXO construct was applied to the *S. aureus* model culture. In the presence of 70 μg of SPIONs/Au/AgNPsGS4/APO/DOXO, a dramatic growth inhibition of *S. aureus* (95 % growth inhibition and 13 mm wide inhibition zone on agar medium) was observed. The biological effect of the nanotransporter lies in ROS (reactive oxygen species) formation, DNA destruction and cell membrane damage.

Keywords: Nanomedicine, silver nanoparticles, nanoconstructs, targeted therapy

1. INTRODUCTION

Tumor diseases are one of the most common diseases worldwide [1]. Secondary infections are serious complications in immunocompromised patients who have been treated with cytostatics [2]. According to

statistical analyzes, patients with neutropenia due to tumor diseases and their treatment have a 15-60 % higher mortality rate for this infectious disease [3]. The effectiveness of the fight against bacterial infectious disease is lower every year due to the phenomenon of bacterial resistance to the antibiotics used and antibacterial chemotherapeutics. The main problem is their huge consumption in livestock farming as well as in human medicine, or poorly chosen therapies for mistakenly determined FAO diagnoses [4]. In the Czech republic, almost 70 tons of antibiotics and antibacterial chemotherapeutics were consumed in 2010 [5].

A new and innovative option in the fight against resistant pathogens is the use of nanomaterials with antibacterial effect. The nanotechnology approach is an important strategy for obtaining new tools and procedures for overcoming bacterial infections and resistence [6]. Green synthesis of nanoparticles reduces chemical consumption and improves bioavailability, and consequently the surface modification of nanoparticles dramatically increases already known effects on target cells [7-10]. Green synthesis of nanoparticles uses the introduction of biological molecules from living organisms. Numerous studies have shown no significant antibacterial effects of silver nanoparticles (AgNPs) [7]. Surface modification of AgNPs provides further significant improvement of their antibacterial properties. In our concept we are coming with the use of modified gold nanoparticles, silver nanoparticles obtained by green synthesis in combination with a suitably chosen antibiotic enclosed in a nanoprotein construct [11]. Apoferritin is a protein that is known for its ability to self-organize in dependence on pH change [12]. This outstanding feature has been used for our experiments. The cavity of apoferritin is about the order of several nanometers, and therefore low-molecular compounds can be enclosed here in non-negligible concentrations.

2. MATERIAL AND METHODS

All used reagents were purchased in Merck-Sigma-Aldrich (USA) in analytical grade. Distilled water was prepared by the Aqual system (Tišnov, Czech Republic) and ultra-pure water was prepared by the ELGA (USA) system to the sterile 18 MΩ quality.

For the purpose of this project, 10 types of AgNPsGS from Vietnamese plants were prepared. Four of them were selected to have the greatest antimicrobial effect. All samples were transferred to a Petri dish and placed in a drier (HN101, Lang-Shan, China) for 24 hours at 60 °C. Then the samples were homogenized to a fine powder using a mixer (Mixer Grinder Mixer HL1643 / 06, Philips, The Netherlands). The material thus prepared was used to prepare the plant extract. 15 g of the prepared plant material was mixed with 500 ml of ultra-pure water at 80 ° C. Extraction was carried out for 1 hour with constant stirring (IKA RH basic, Malaysia) at 80 °C and 300 rpm. Each plant extract was filtered and allowed to cool to room temperature. After filtration, the plant extracts were mixed in a 1: 1 ratio with 0.1 M $AgNO_3$ and allowed to stir with a magnetic stirrer (IKA RH basic, Malaysia) at room temperature (23 °C) for 18 hours [13] By the green synthesis the following plants were prepared: AgNPsGS1 (*Lagerstroemia indica*), AgNPsGS2 (*Carica papaya*), AgNPsGS3 (*Polyalthia longifolia*), AgNPsGS4 (*Ficus bengalensis*).

Doxorubicin fluorescence (DOXO) measurements were performed by pipetting into the microtiter plate (Brand, Germany) 100 µl of sample with a concentration of 10 µM DOXO. The weight concentration of apoferritin in acid form in the solution was 3.0 mg / ml. For measurement, the Multimode microplate reader M200 PRO (Tecan, Japan) was used, fluorescence of doxorubicin (Ex 480 nm, Em 650 nm) with a typical peak at 560 nm.

For MIC testing in microtiter plates, the cultured sample of the selected microorganism was first diluted to OD 0.1 at 600 nm using a pure sterile LB medium. 250 µl of the prepared diluted micro-organism was pipetted into the well of the microtiter plate and 50 µl of the test sample was added to each well. Measurement conditions were set to 18 hours, absorbance reading for 30 min, shaking for 30 min, shaking at 50 Hz for 5 s, temperature 35 °C, wavelength 600 nm. The Multimode microplate reader M200 PRO (Tecan, Japan) was used for measurement.

3. RESULTS

3.1. Preparation of the theranostic SGA nanotransporter

In the experiment, a teranous nanotransporter was designed and studied by biophysical and microbiological methods for specific targeting of S. aureus. The proposed construct is composed of three separate parts (**Figure 1**). Part A of nanoconstruct is made of silver nanoparticle prepared by green synthesis with significant antibacterial effects. Part B of the nanocomposite consists of apoferritin encapsulated with an appropriate antibiotic.

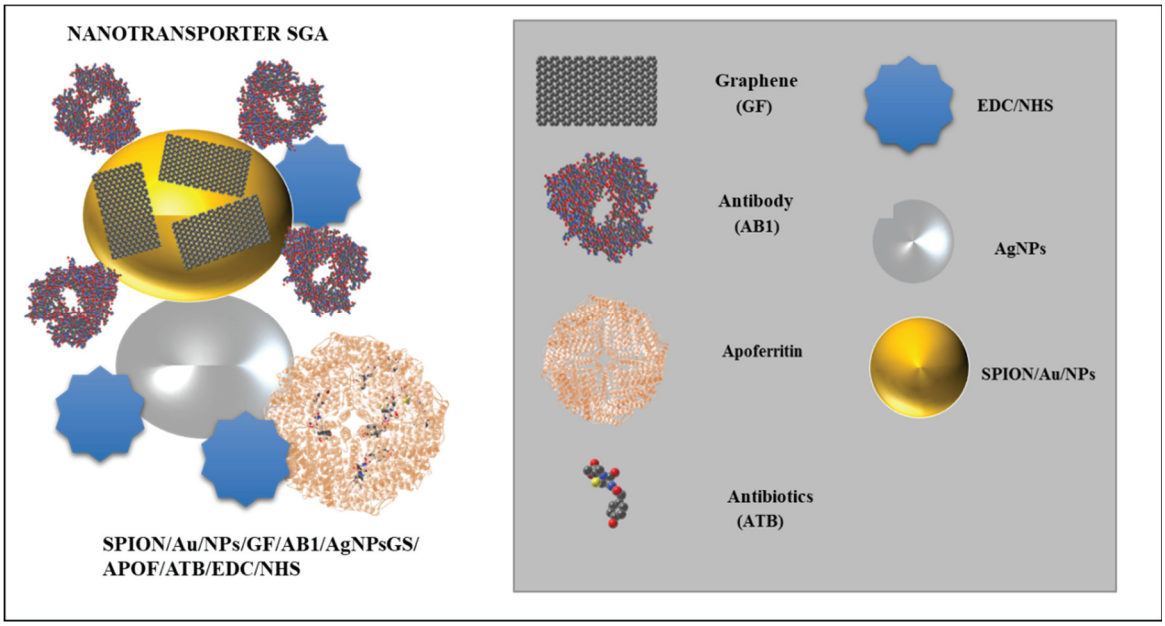

Figure 1 Proposed antibacterial nanotransporter scheme for sensitive and specific targeting on *S. aureus* bacterial cells. The nanotransporter consists of three separate units. Gold supraparamagnetic particles SPION modified by graphene sheet and antibody. (AND); silver nanoparticles formed by green synthesis of AgNPs (B); apoferritin cage with closed antibiotic (C). The individual parts are linked by the EDC / NHS binding polymer.

Part C of the nanoconstruct consists of a superparamagnetic gold nanoparticle (SPION/Au/NPs). On the surface of the nanoparticle, an anti-protein A antibody was then attached to target surface A protein *S. aureus*. Additionally, the SPION/Au/NPs surface was modified by graphene sheets to bind cytotoxic compounds produced by *S. aureus*. The individual parts of the construct are interconnected with the EDC / NHS binding polymer to form a complete SGA theranostic construct.

3.2. Monitoring of bacterial growth in the presence of AgNPs

Growth curves of S. aureus bacterial culture were recorded over 18 h. All AgNPsGS showed a high inhibitory activity (above 60 %). For all other experiments, AgNPsGS3 were selected because they exhibited the highest antibacterial effect on *S. aureus* culture (95.6 %).

3.3. Testing the SGA nanotransporter

The binding efficiency of AgNPsGS3 to the remaining parts of the construct was about 15 %. Various variants of the construct underwent biophysical and microbiological testing. DOT BLOT, as a biological immunoassay method, was used to demonstrate antibody binding to SPION / Au / NPs. DOT BLOT can detect anti-protein A at 40 ng / ml

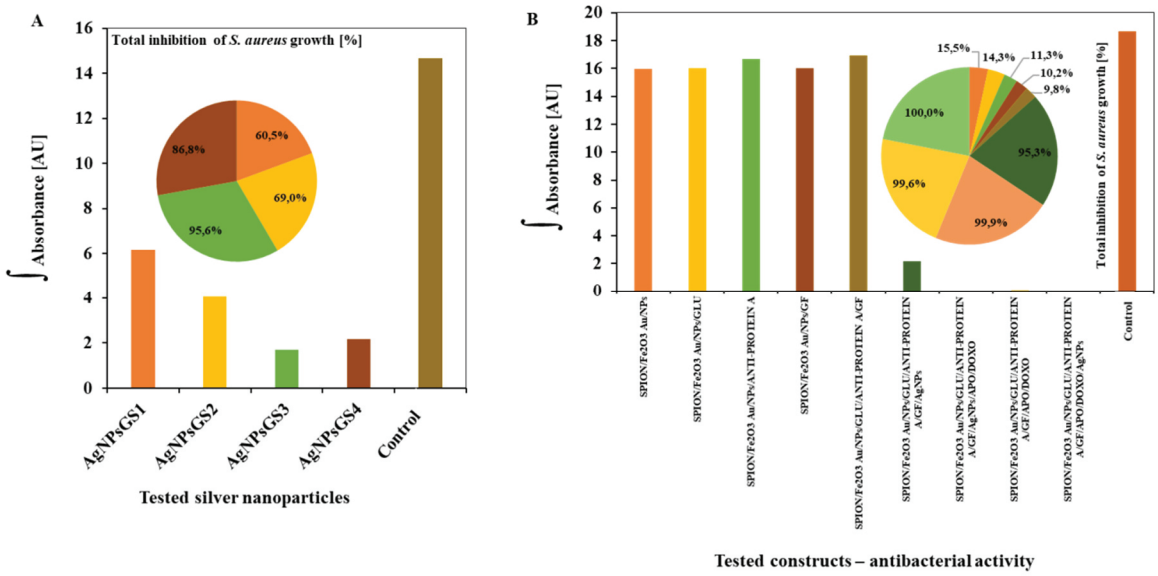

Figure 2 Integral absorbance of individual types of silver nanoparticles compared to control. In inset percentage inhibition of silver nanoparticles is given. The control is 100 %. Concentration 0.2 mg / ml, total volume 300 µl. Measured for 18 hours and readings were read at 30 ° C, at 37 ° C, shaking at 3 with 6 mm (A). Growth curves of S. aureus after application of various parts of the construct, total volume 300 µl. Measured for 18 hours and values were read at an interval of 30 minutes at 37 ° C (B).

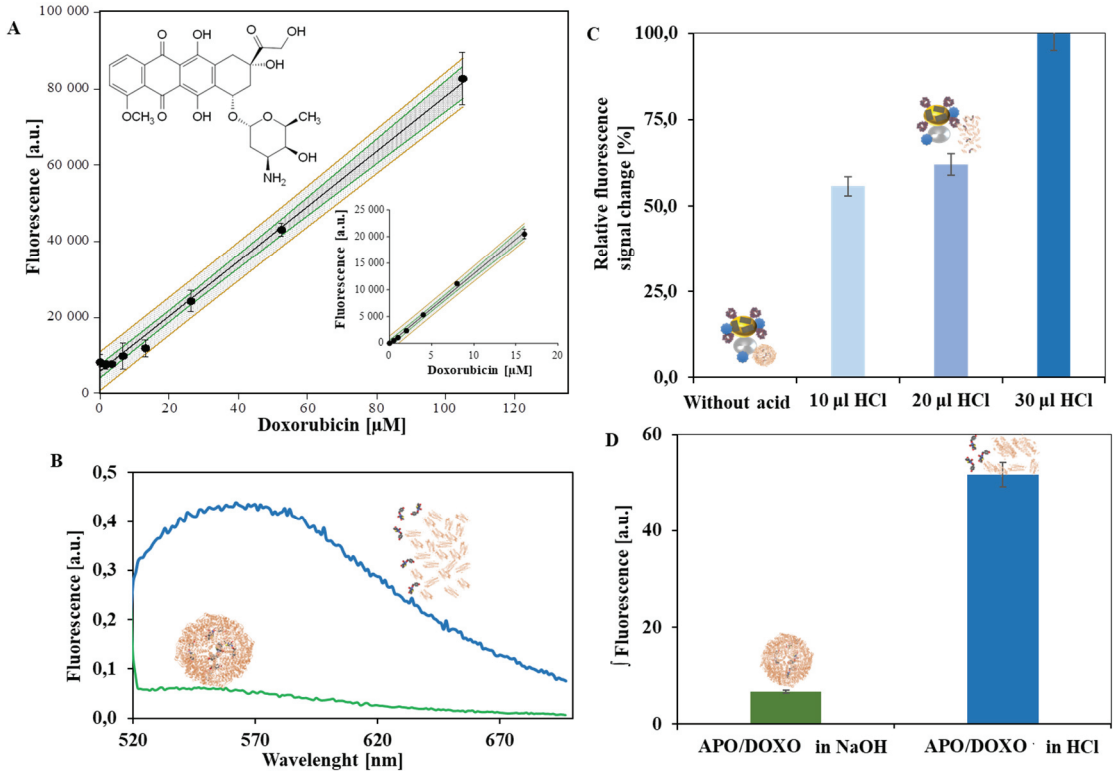

Figure 3 Doxorubicin Fluorescence Calibration Curve (Ex 480 nm, Em 650 nm) with typical peak at 560 nm. (A); Dependence of DOXO fluorescence on wavelength in open and closed apoferritin (B); Following the behavior of apoferritin in the acidic environment (different additions of 0.1 M HCl to a final volume of 30 µl), the release efficiency was determined as the fluorescence of doxorubicin. (C); Fluorescence integral of DOXO in apoferrin in acidic and basic media (D)

On the basis of all the experimental data obtained above, the nanoconstruct proposed by us was confirmed. The main aim of this work was to strengthen antibacterial properties. Therefore, the antibacterial effect of our nanoconstruct and its parts was monitored. (**Figure 2**). Part C composed of SPION / Au / NPs with bound antibody and graphene, coupling part C and A (AgNPs), linking part C and part B (apoferritin with encapsulated doxorubicin) and, of course, the entire final construct. **Figure 2** shows typical growth curves in the presence of the nanoconstructs tested. Part C showed no antibacterial effects, nor were they expected. In the presence of antibacterially active substances, however, dramatic inhibition has been observed.

Apoferritin is a protein that is known for its ability to self-regulate in dependence on pH change [12]. The apoferritin cavity is of the order of several nanometers, and thus low-molecular compounds can be enclosed at negligible concentrations at acidic pH, opening of the apoferrin structure, whereas basic environment for clustering. The anthracycline antibiotic doxorubicin at a concentration of 10 μM was stirred for 30 minutes while stirring. is sequentially closed (by changing the pH from acidic to alkaline) into the cavity of apoferritin. We found that doxorubicin (around 13 % of the DOXO concentration) was enclosed within the nanometric structure created (**Figure 3**). This fact can be used for the design of other nanoconstructions that will contain other antibiotics (penicillin G, vancomycin, etc.).

4. CONCLUSION

Advances in the treatment of bacterial infections are significant and, with the current antibiotic crisis, the importance of nanotechnology continues to grow. In recent years, researchers have been focusing on silver nanoparticles. In our work a nanoconstruct with multifunctional mechanisms of antibacterial activity was created - nanoantibiotic. The nanoconstruct binds to the bacterium by immunological binding via protein A with a specific antibody. Other parts of the SGA nanoconstruct are designed to damage the bacterial cell. The SGA nanoconstruct, designed by us, is functionalized by an antibiotic that is released from apoferithin at low pH. The antibiotic acts by a specific bacteriocidal mechanism. Another advantage of the prepared nanotransporter is the presence of graphene sheets. After bacterial lysis, bacterial toxins are released into the environment, from which are captured by graphene sheets with maximum sorption capacity to avoid damage to the host organism. Our SGA nanoconstruct showed very good overall stability in different environments. Its specific targeting to the bacterial cell was possible by binding to protein A. The synergistic effect of biogenic silver nanoparticles and antibiotic sealed in the apoferritin cage increased the inhibitory effect to more than 90 %. We expect the rapidly growing interest in theranostic applications based on metal nanoparticles that will not only encourage and stimulate research in this highly active region, but will also bring completely new fundamental knowledge.

ACKNOWLEDGEMENTS

This work has been supported by the project 3D ANTIMICROB (LC18002) and by the CA COST Action CA15114.

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