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Gold nanoparticles in an enhancement of antimicrobial activity

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Abstract: The effect of antimicrobial photodynamic therapy (aPDT) on Gram-positive bacterium *Staphylocccus aureus* was studied. Methylene blue (MB) at non-toxic concentration of 31.25μg/ml was used as a photosensitizer. LEDs diodes were used as a light source to study the effect of methylene blue alone and the MB-gold nanoparticle mixture on the viability of *S. aureus* cells. Biogenic gold nanoparticles (biolAuNPs, 10ppm) and chemically synthesized gold nanoparticles (chemAuNPs, 3ppm) were tested as enhancement agents. In the presence of MB alone as a photosensitizer, the killing effect was about 92% after 30min of irradiation. The aPDT therapy was enhanced by addition of biolAuNPs and chemAuNPs and killing rate of *S. aureus* was 95-96% after 30min of irradiation. The probable mechanism of enhancement of MB-mediated photodynamic bactericidal efficacy against *S. aureus* in the presence of gold nanoparticles is discussed leading to the conclusion that colloidal gold increases the accumulation of MB in bacterial cells.

***Keywords*:** gold nanoparticles, photodynamic therapy, methylene blue, staphylococcus aureus

1. Introduction

Gold (*lat. aurum, Au*), in its metallic form, is used for ages by humanity in various sectors of life, because of its unusual properties. This metal is characterized by shiny, unique yellow color and high density. Metallic gold is non-active and does not oxidize in water (it doesn't change a color). One of the most important advantages of gold is its corrosion resistance. Its chemical properties include non-reaction with strong bases and pure acids. The principal gold minerals are native gold, electrum, Au-Ag tellurides, aurostibite, maldonite, and auricupride. Calaverite or gold telluride is an uncommon telluride of gold mineral with chemical formula ($AuTe_2$) (Habashi, 2016; Yannopoulos, 1991).

Due to its exceptional properties gold and its alloys are readily used in the manufacture of jewelry. However, gold mining and processing is a problem. The main issues are related with high costs, long and tedious processes that contradict the rules of green chemistry, due to chemical extraction (the well-known is cyaniding leaching). Mining of this metal have negative impact on the environment through pollution of air and water. Thus, huge accumulation of toxic waste can have deleterious effect on quality of human health and life. Another disadvantage of mining of this metal is indirect effect on global warming (Abdul-Wahab and Marikar, 2012; Ogola et al., 2002). The gold mining process is responsible for the emission of gases such as carbon dioxide or methane. Diffusion of these gases into the atmosphere leads to the greenhouse effect, manifested by a lower atmosphere and warming of the planet's areas (Csillik and Asner, 2019). For example, the Amazon rainforest is the sixth largest location in gold mining, and this process result in the Amazon deforestation and destruction of important habitat. Moreover, cut down or burned plants unable absorb carbon dioxide, which is released into the atmosphere, thereby causing environmental pollution and consequently, the greenhouse effect (Csillik and Asner, 2019).

For the last decade, attention was paid to nanobiotechnology, as an emerging area of science which can improve human health and life. Biosynthesis of gold nanoparticles using widespread plants, bacteria and fungi is considered environmentally safe and uncomplicated (Soliman et al., 2020; Hassan

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et al., 2018). Mild conditions, such as low temperature and low pressure are the advantages of this green-production (Salem and Fouda, 2020; Hassan et al., 2019). Biological synthesis reduces toxic chemicals and labor-intensive processes (Fouda et al., 2019; Hassan et al., 2019). This process is based on the reduction of toxic metal ions by living organisms or their extracts, so it uses the natural components (proteins, sugar) to formation and stabilization of metallic nanoparticles (Lohße et al., 2016; Tikariha et al., 2017). It should be noticed, that the bio-production of metallic nanoparticles does not require the addition of reducing and stabilization agents, and the obtained colloidal nanoparticles show an excellent stability (Lee et al., 2020; Salem and Fouda, 2020). Biogenic gold nanoparticles are low-toxic, show catalytic activity (Ghodake et al., 2010; Vithiya and Sen, 2011), and can be produced on a large scale (Salem and Fouda, 2020). The common way of gold nanoparticles biosynthesis is the use of tetrachloroauric solutions (tetrachloroauric acid) (Ahmed et al, 2016).

Due to its unique properties gold nanoparticles turned out to be great enhancement agents in antimicrobial photodynamic therapy (aPDT) against drug resistance pathogens (Maliszewska et al., 2019; Maliszewska et al., 2020). This form of treatment uses non-toxic photosensitizers (general dyes) that generate reactive oxygen species (ROS) in the presence of oxygen and light of appropriate wavelength (Huang et al., 2011; Mesquita et al., 2018). Nanotechnology creates a new generation of photosensitizers as conjugates of a photosensitizer bound to the surface of gold nanoparticles (Calavia et al., 2018; Lkhagvadulam et al., 2013)

In this study biogenic (biolAuNPs) and chemically synthesized nanoparticles (chemAuNPs) were tested as enhancement agents in photodynamic therapy against *Staphylococcus aureus* using methylene blue as photosensitizer.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

All chemicals used in this study was obtained Sigma Aldrich (Poland) and POCH (Poland). Methylene blue (MB) was prepared by dissolving 10mg of dry powered dye in 10ml of deionized water. This initial solution (1mg/ml) was stored in room temperature in dark.

Colloidal gold nanoparticles used in this work were synthesized by two method. The biogenic gold nanoparticles (biolAuNPs) were obtained by procedure described previously with a slight modification (Maliszewska et al., 2014). The basal medium used in experiments consisted of (%): saccharose 2.0; $NaNO_3$ 0.1; $Na_2HPO_4x2H_2O$ 0.0534; $MgSO_4x7H_2O$ 0.05; KH_2PO_4 0.0272; yeast extract 0.6. The Erlenmeyer flasks were inoculated with conidia (10^5/ml) and incubated at 22°C with shaking (120rpm) for 4 days. Then the biomass was filtered (Whatman filter paper No. 1) and washed with distilled water to remove any medium component. Fresh and clean biomass (5g) was taken into the Erlenmeyer flasks, containing 50ml of Milli-Q deionised water (UV Ultrapure Water System, Burnstead, USA) and $HAuCl_4x4H_2O$ (1mM). The mixture was incubated with shaking at 50°C for 8h (in dark). Control (the gold ions without microbial biomass) was also run along with the experimental flasks. To isolate the gold nanoparticles, biomass was washed twice with deionized water. Ultrasonic disruption of cells was carried out with an ultrasonic processor (TURBO 36800) over four-five 20s periods and with an interval of 60s between periods. The sonicated samples were centrifuged at 3500rpm for 15min at 4°C to remove cell-debris. The gold nanoparticles were separated by the sucrose density gradient technique described by Maliszewska (2013) and spheres concentrated in the 30% fraction were studied. The chemical synthesis of gold nanoparticles (chemAuNPs) was previously described by Maliszewska et al. (2014). This type of colloidal gold nanoparticles was formed by chemical reduction of $AuCl_4^-$ using 0.25mmol chloroauric acid trihydrate disolved in 150ml of water, 4.3% polyethyleneimine as stabilization agent and 10% Tween 80 aquous solutions. The 235ml of mixture was shaking for 4h, then reduction agent 50mM aqueous ascrobic acic was dropped for 20min with shaking. The solution was stored by night.

2.1.2. Microorganism

The one colony of bacterium *Staphylococcus aureus* (ATCC 11632) was taken from agar plate and was inoculated in 2ml of Mueller-Hinton liquid medium. Thus, prepared suspension was incubated for

24h at 37°C in dark. After 24h of incubation this suspension was centrifuged at 5min/6000rpm. The supernatant was rejected, the bacterial pellet was suspended in deionized water, mixed and diluted. This suspension contained approximately 1×10^6 of colony-forming units (CFU/mL).

2.2. Methods

2.2.1. Characterization of gold nanoparticles

Gold nanoparticles were characterized by color and by UV-vis, TEM microscopy and FTIR technique. Shimadzu UV-1650PC spectrophotometer and UV-vis probe program was used to determine UV spectrum in range 200-800nm. Size and shape of gold nanoparticles was verified using Zeiss EM 900 transmission electron microscope, by placing the probes on grid made of copper and covered by carbon and dried on air. The studied gold nanoparticles and biomass were lyophilized and set on a KBr pellet to determine the spectrum probes and the existing bonds using Perkin Elmer 1600 Fourier transform infrared spectrophotometer.

2.2.2. BacTiter-Glo™ Microbial Cell Viability Assay

BacTiter-Glo™ Microbial Cell Viability Assay method was prepared according to manufacturer's recommendations (Promega, 2018). Luminescent signal value enabled determine changes of viability of *S. aureus* cells.

2.2.3. Effect of methylene blue on the viability of *S. aureus* (dark cytotoxity)

The dark cytotoxicity of methylene blue was studied using MB at final concentrations range of 1.953-1000µg/ml. All samples containing MB and bacterial cells were incubated for 2h in dark at 37°C. Then, BacTiter-Glo™ Microbial Cell Viability Assay was used to determine viability of *S. aureus* (CFU/ml). The BacTiter-Glo™ reagent volume of 100µl was added to all samples. The luminescence values were measured at 500nm using SpectraMax Gemini dual-monochromator spectrofluorometer.

2.2.4. Effect of gold nanoparticles on the viability of *S. aureus* (dark cytotoxity)

The colloidal gold particles at final concentrations of 10, 20, 30ppm (biolAuNPs) and 3, 10, 20, 30, 40ppm (chemAuNPs) were added to bacterial suspension and incubated in dark at 37°C. CFU/ml was determined by serial dilution method.

2.2.5. Effect of LEDs diode light on the viability of *S. aureus*

The bacterial suspension was irradiated by diode LEDs light with wavelength of 630nm (10mW cm^{-2}) for 10, 20 and 30min. CFU/ml was determined by series of dilution method.

2.2.6. Enhancement of photodynamic therapy by gold nanoparticles

The MB at a final concentration of 31.25µg/ml and biolAuNPs/chemAuNPs at final concentrations of 10ppm/3ppm were added to bacterial suspension. Then probes were incubated in dark at 37°C. After incubation the probes were irradiated by diode LEDs light for 10, 20 and 30min. CFU/ml was determined by serial dilution method.

2.2.7. Membrane fluidity measurement by fluorescence polarization spectroscopy

The measurement of membrane fluidity was tested using method previously described with a slight modification (Wang et al., 2018). Bacterial suspension was centrifuged (5min/6000rpm), the supernatant was rejected and biomass of *S. aureus* was washed twice in PBS (phosphate buffered saline) buffer. Then, bacterial biomass was re-suspended in PBS buffer to obtain an optical density (OD_{600}) of 0.5 and mixed with: a) MB solution to give a final concentration of 31.25µg/ml (this concentration of MB was used in all studied samples); b) MB solution and biolAuNPs to give a final concentration of 10ppm; c) MB and chemAuNPs to give a final concentration of 3ppm; d) biolAuNPs to give a final concentration of 10ppm; e) chemAuNPs to give a final concentration of 3ppm.

All samples were incubated at 37°C in dark. After incubation, samples were centrifuged (5min/6000rpm) supernatant was rejected and sediment was suspended in mixture of PBS buffer and fluorescent membrane probe (1,6 diphenyl-1,3,5-hexatriene (DPH) dissolved in tetrahydrofuran at final concentration 2.0µg/ml). These mixtures were incubated for 1h at 37°C in dark. After incubation all samples were centrifuged, supernatant was rejected and sediment was suspended in PBS buffer. Fluorescence polarization, a measure of membrane fluidity, were measure using excitation 360nm and emission 430nm (5.0/5.0nm slit widths) (Jasco FP-8300 Spectrofluorimeter). The values of grating factor (G) and anisotropy of all samples were designated to determine of membrane fluidity (Wang et al., 2018).

2.2.8. The effect of gold nanoparticles on efficiency of accumulation of methylene blue by *S. aureus* cells

The MB at a final concentration of 31.25µg/ml and biolAuNPs/chemAuNPs at final concentrations of 10ppm/3ppm were added to bacterial suspension. All samples were incubated in dark at 37°C with shaking (125rpm) for 5min, 10min, 15min, 30min, 45min, 60min, 75min, 90min, 105min, 120min and 150min. Each sample was centrifuged (5min, 6000rpm) and the concentrations of methylene blue in supernatant were measured using spectrophotometric method.

3. Results and discussion

3.1. Characterization of gold nanoparticles

Gold nanoparticles (biolAuNPs and chemAuNPs) were characterized by a reddish color (Fig. 1), which is consistent with previous observations by other authors (Abdel-Raoufa et al., 2017; Pestovsky and Martinez-Antonio, 2018).

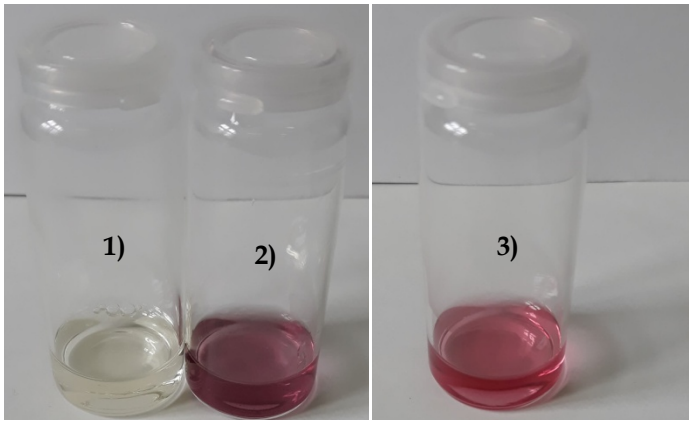

Fig. 1. Changes of color solution during the AuNPs synthesis. 1) control, (2) biogenic gold nanoparticles (biolAuNPs) and (3) chemically synthesiszed gold nanoparticles (chemAuNPs)

UV-vis spectrum (Fig. 2A) showed strong absorption peak with maximum at 514nm associated with surface plasmon resonance of biolAuNPs. The peak with maximum at 280nm is associated with presence of proteins-stabilization agents (Haiss et al., 2007; Aitken and Learmonth, 2002). In the case of chemAuNPs, the strong absorption peak with maximum at 524nm was noticeable (Fig. 2B). The obtained results coincide with those described by many authors. For example, Keijok et al. (2019) described biosynthesis of the spherical gold nanoparticles characterized by maximum absorption at 540nm. Pestovsky and Martinez-Antonio (2018) presented the synthesis of colloidal gold particles with an absorption maximum at 520nm.

The FTIR spectroscopy spectra of the biogenic gold nanoparticles, the fungal biomass and chemically synthesized gold nanoparticles are shown in Fig. 3. Both spectra of fungal biomass and biogenic gold nanoparticles (Fig. 3A and B) are characterized by the strong band between 3030 and 3690cm^{-1}, which is assigned to resonance of N-H stretching bindings (Alsharif et al., 2020). The bands with maxima at ~ 2977cm^{-1} are corresponding to CH_3- groups (Mohamed et al., 2019). The spectrum of the fungal biomass (Fig. 3A) exhibits peak with maximum at 1650cm^{-1} due to the presence of amide I

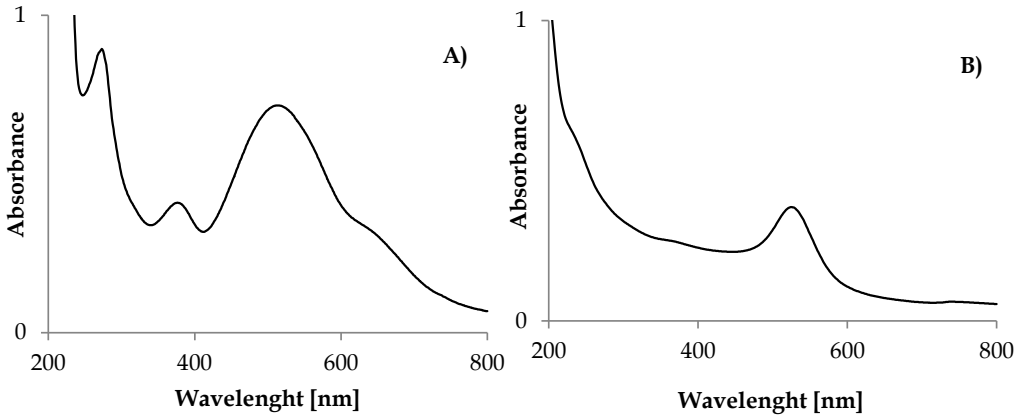

Fig. 2. UV-vis spectrum of biolAuNPs (A) and chemAuNPs (B)

bound (Shi et al., 2015). Moreover, the spectrum of biomass exhibits band at 1264cm^{-1} assigned to amide III bound (Shi et al., 2015). The peaks at 1075 and 1083cm^{-1} are assigned to carbonyl group. After biosynthesis of gold nanoparticles (Fig. 3B), the spectrum shows, that the peak with maximum at 1650cm^{-1} was shifted to 1661cm^{-1}. The similar observation was reported by Honary et al. (2012), who described the presence of strong peak at 1620cm^{-1}. A peak observed at 1271cm^{-1} is attributed the amide III bound group. The peaks at 1075cm^{-1} and 1083cm^{-1} (assigned to presence of carbonyl groups) were shifted to strong band at 1045cm^{-1} and disappearing band at 1151cm^{-1}. The above observations may indicate the participation of proteins as stabilizers and reducing agents (Shi et al., 2015). The spectrum of chemically synthesized gold nanoparticles is shown in Fig. 3C. The peak with maximum at 3209cm^{-1} is corresponding to resonance of hydroxyl groups. The band with maximum at 2908cm^{-1} is assigned with methylene groups. The strong peak with maximum transmittance at 1621cm^{-1} proves the presence of carbonyl group, as a replacement of ascorbic acid's C-C double bond. Bands with the maxima at 1342cm^{-1} and 1026cm^{-1} was observed in relation to occurrence of C-H groups (Agudelo et al., 2018; Umer et al., 2014).

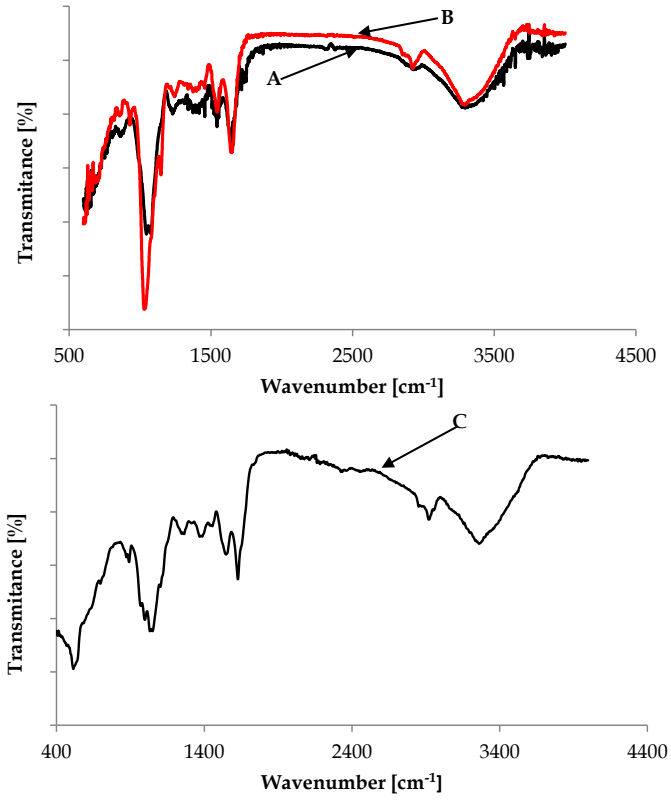

Fig. 3. FTIR spectra of: biomass before incubation with gold ions (A), biolAuNPs (B), chemAuNPs (C)

TEM image (Fig. 4A) showed that biosynthesized nanoparticles were small spherical structures. The average size of these gold nanoparticles was about 15±3nm. The chemically synthesized nanoparticles were also spherical in shape with an average size of 20±3nm (Fig. 4B).

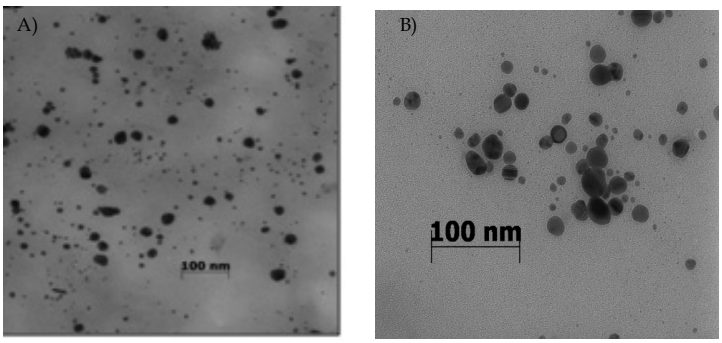

Fig. 4. TEM image of biolAuNPs (A) and chemAuNPs (B)

3.2. Effect of methylene blue on cell viability of *S. aureus* (dark cytotoxity)

Fig. 5 presents the effect of methylene blue on viability of *S. aureus.* As can be seen there, methylene blue at concentrations between 1.953-31.25µg/ml showed insignificant 0.03 logarithmic unit reduction (that is up to 20% killing of *S. aureus*). Methylene blue at concentrations of 62.5, 125, 250, 500, 1000µg/ml showed 0.09, 0.07, 0.16, 0.19, 0.39 logarithmic units reduction in *S.aureus* viability, respectively. On the basis of these results it was considered that MB at concentration of 31.25µg/ml would be used in all further experiments. The similar results were reported by Caires et al. (2017). These authors used methylene blue at a non-toxic concentration of 20µg/ml. Catao and Batista (2020) described studies on MB as a photosensitizer at a concentration of 50µg/ml.

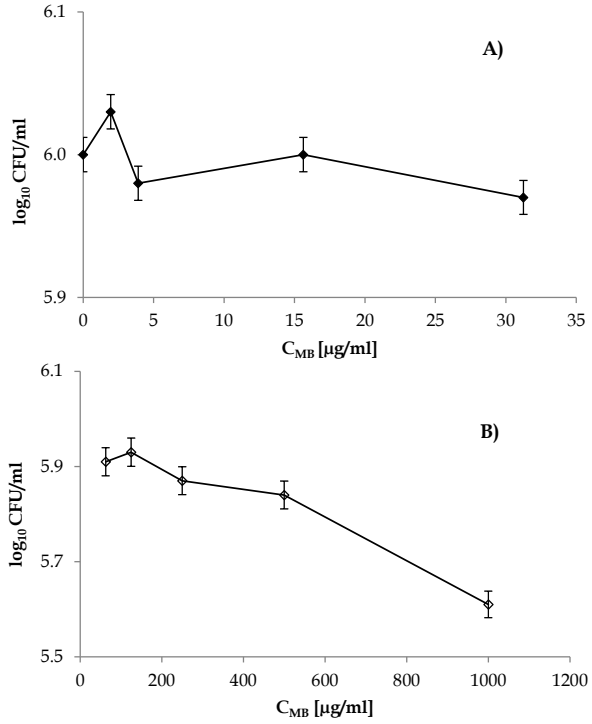

Fig. 5. The effect of methylene blue on viability of *S. aureus:* MB concentration ranged from 1.953µg/ml to 31.25µg/ml) (A), MB concentration ranged from 62.5µg/ml to 1000µg/ml (B)

3.3. Effect of colloidal gold nanoparticles on viability of *S. aureus* (dark cytotoxity)

The effect of biolAuNPs/chemAuNPs on viability of *S. aureus* is presented in Fig. 6. As can be seen in Fig. 6, biolAuNPs at concentrations in range of 10-20ppm showed insignificant 0.12 logarithmic unit killing of *S.aureus*. BiolAuNPs at concentration of 30ppm showed 1.92 logarithmic unit reduction in

S.aureus viability. BiolAuNPs at a concentration of 40ppm showed very high killing effect on *S.aureus* (under detection level- data not shown). ChemAuNPs at concentrations of 3, 10, 20, 30, 40ppm showed 0.28, 0.46, 0.66, 0.45, 0.65 logarithmic unit reduction in *S. aureus* viability, respectively. On the basis of the obtained results it was considered that biolAuNPs at a concentration of 10ppm and chemAuNPs at a concentration of 3ppm would be used in all further experiments. It should be noticed that chemAuNPs at a concentration of 10ppm showed a high toxicity to bacterial cells and 70% mortality was observed. Sanchez-Lopez et al. (2020) described an *in vitro* study using gold nanoparticles at a concentration of 10ppm that induced cell death below 40%.

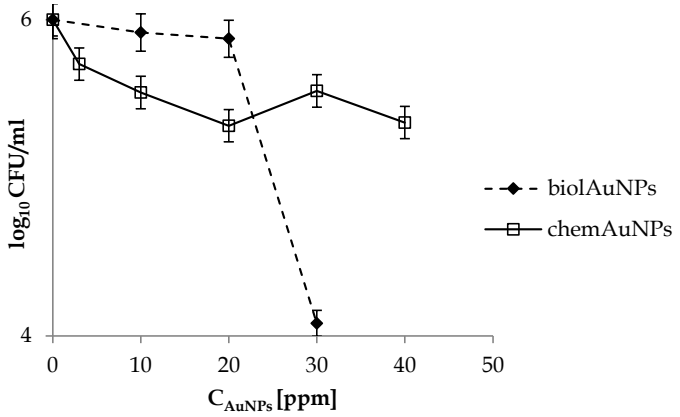

Fig. 6. The effect of biolAuNPs/chemAuNPs on viability of *S. aureus*

3.4. Enhancement of photodynamic therapy by gold nanoparticles

The enhancement of photodynamic therapy by biolAuNPs/chemAuNPs against *S. aureus* is presented in Fig. 7. As can be seen there, MB showed 0.17 $\log_{10}$ (23% kill), 0.17 $\log_{10}$ (23% kill) and 1.11 $\log_{10}$ (92% kill) reduction in *S. aureus* cells after 10, 20, 30min of irradiation, respectively. The mixture of MB and biolAuNPs had photobactericidal activity and a reduction of 0.48 $\log_{10}$ (67% kill), 0.52 $\log_{10}$ (70% kill) and 1.35 $\log_{10}$ (95% kill) after 10, 20, 30min of irradiation was observed. The mixture of MB and chemAuNPs showed a reduction in CFU of 0.46 $\log_{10}$ (65% kill), 1.36 $\log_{10}$ (95% kill) and 1.39 $\log_{10}$ (96% kill) after 10, 20, 30min of irradiation, respectively. Gueorgieva et al. (2010) reported photodynamic inactivation of *S. aureus* using methylene blue and diode laser light (630nm). The mortality of this coccus was found to be 99.9% after 20 min of irradiation. Tawfik et al. (2015) described a 97% mortality of *S. aureus* using mixture of MB and gold nanoparticles after irradiation with laser light (660nm).

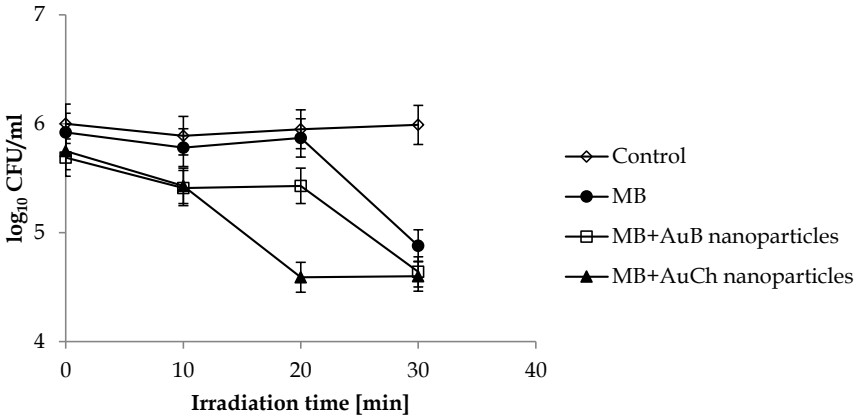

Fig. 7. The effect of MB, MB + biolAuNPs, MB + chemAuNPs on the viability of *S. aureus* after different exposure time

3.5. Studies on mechanism of enhancing photodynamic therapy by gold nanoparticles

Fluorescence polarization (FP) is a membrane fluidity measurement method. The main mechanism of fluorescence polarization measurement is monitoring of moving membrane-bound fluorophore

connected with mobility of cell membrane. In this study, 1,6-diphenyl-1,3,5-hexatriene DPH was used as fluorophore. DPH was excited by polarized beam of light (360nm), then the emission (430nm) of light by fluorophore in two planes were observed- I_{vh} defines the intensity of light in horizontal direction, whereas I_{vv} defines the intensity of light in vertical direction to the excited light, FP is determined by the formula: $FP=I_{vh}-GI_{vh}/I_{vv}-GI_{vv}$, where G is grating factor of used PBS buffer. Significant values of fluorescence anisotropy changes, connected to the membrane fluidity, correspond with fluorescence polarization. Anisotropy defines only intensity of emitted light in horizontal direction (I_{vh}). Decrease in anisotropy values define more of the liquidity of cell membrane, whereas higher values define "packing" membrane. Anisotropy values changes were used in this study to observe membrane fluidity changes (Wang et al., 2016; Mhashal and Roy, 2016).

Table. 1. The effect of MB and gold nanoparticles on membrane fluidity of *S. aureus*

Samples	Anisotropy value
Control (bacterial suspension)	0.115
MB	0.111
MB+ biolAuNPs	0.0895
MB+chemAuNPs	0.0895
biolAuNPs	0.113
chemAuNPs	0.0830

As can be seen, the differences between the probes are insignificant, proving that MB and gold nanoparticles do not affect the fluidity of the membrane. Our results differ from those obtained by Mhashal and Roy (2016). These authors described that gold nanoparticles are able to change the fluidity of membrane, making it more liquid. The membrane fluidity depends on structural order of the lipids and a slight damage to the lipid bilayer may increase its fluidity. Interactions of a lipid with gold nanoparticles (adhesion) can cause undesirable angle of deviations between lipid's atom, which disrupt the order of the membrane.

Our hyphothesis speculate that gold nanoparticles have an efficiency of accumulation of methylene blue by *S. aureus* cells. The obtained results showed that MB was taken up by the cells during the first 15min of incubation. Extending the incubation time did not increase the accumulation of methylene blue in the bacterial cells (data not shown). When the MB + biolAuNPs mixture was used, it was observed that the accumulation of methylene blue in the cells slightly increased (by 15%). When the mixture of MB with chemAuNPs was used in the experiment, it turned out that the accumulation of MB in the cells increased by 52% (Fig. 8).

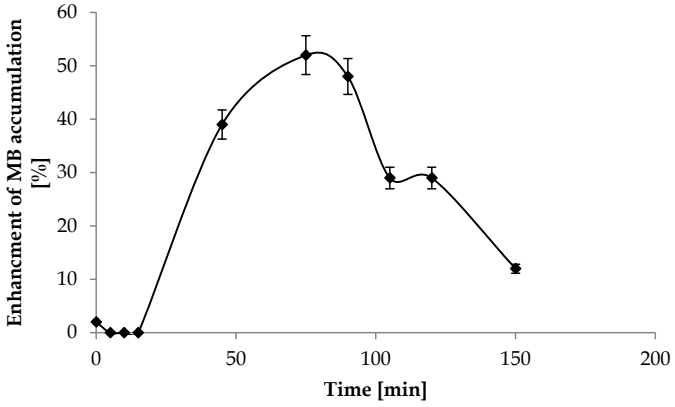

Fig. 8. The effect of chemAuNPs on accumulation of MB by *S. aureus*

4. Conclusions

Antimicrobial photodynamic therapy (aPDT) is a good approach to eradicate *Staphylococcus aureus.* The use of methylene blue as a photosensitizer reduce *S. aureus* up to 92%, while gold nanoparticles

enhance the efficiency of this treatment up to 96%. The mechanism of enhancement the effectiveness of aPDT by gold nanoparticles is still unknown, however an increase in the accumulation of methylene blue in the bacterial cells (by 52%) in presence of chemAuNPs was observed.

Acknowledgments

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