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Original article

Analysis of antibiofilm activity gold and silver nanoparticles against uropathogenic *Escherichia coli* using confocal laser scanning microscopy

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Abstract

Infectious diseases, including urinary tract infections (UTIs), significantly impact global health, diminishing the quality of life and increasing clinical and economic burdens. Concerns arise due to the potential development of antimicrobial resistance (AMR) facilitated by biofilm formation. Nanotechnology, specifically silver and silver nanoparticles (1–100 nm), proves effective against bacterial strains, making them valuable in the biomedical field. Advances in microscopic imaging techniques, such as Confocal Laser Scanning Microscopy (CLSM), enhance our understanding of biofilms. This study aims to assess the effectiveness and optimal concentration of gold and silver nanoparticles in combating biofilms formed by uropathogenic Escherichia coli (UPEC) through CLSM analysis. The research comprises the following stages: rejuvenation of bacterial isolates and inoculum preparation, testing the anti-biofilm activity of gold and silver nanoparticles at concentrations of 25, 50, 75, and 100 ppm against UPEC using CLSM analysis. The findings reveal that both gold and silver nanoparticles exhibit anti-biofilm activity against UPEC at concentrations of 25, 50, 75, and 100 ppm, as confirmed by CLSM analysis. The optimal concentration for anti-biofilm activity of gold and silver nanoparticles against UPEC is identified as 100 ppm.

Keywords: CLSM, gold, silver, nanoparticle, biofilm, UPEC

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Introduction

Infectious diseases, as indicated by Frenkel (2021), stand as a primary contributor to global morbidity and mortality. Among these, urinary tract infections (UTIs) present a significant clinical and economic burden, being prevalent worldwide with more than half of adult women experiencing multiple occurrences (Yang et al., 2022). Research across American and European nations underscores the prevalence of nosocomial urinary tract infections at 42%, surgical site infections at 24%, and ventilator-associated pneumonia (VAP) at 11% (Khatoon et al., 2018).

While antibiotics have traditionally proven successful in treating bacterial infections, their use contributes to the alarming rise of antimicrobial resistance (AMR). Presently, over 700,000 individuals worldwide succumb to AMR annually (Mancuso et al., 2021). AMR mechanisms, such as inhibiting access or reducing antimicrobial permeability, including biofilm formation ability, pose significant challenges (Dutt et al., 2022). Bacterial biofilms, characterized by microorganism aggregates embedded in a self-produced matrix of extracellular polymeric substances (EPS), further complicate treatment (Funari & Shen, 2022).

Various strategies aim to control and counteract biofilms, including the use of antimicrobial peptides, biofilm-degrading enzymes, Quorum Sensing (QS) inhibitors, essential oils, and nanotechnology/nanoparticles (Vallet-Regí et al., 2019). Nanoparticles, defined by dimensions ranging from 1 to 100 nm, have exhibited toxic effects against numerous bacterial strains, making them promising in biomedical applications (Harish et al., 2022). Notably, gold and silver nanoparticles emerge as the most promising materials due to their broad spectrum, stability, and biocompatibility (Franco et al., 2022; Vallet-Regí et al., 2019).

The swift expansion of knowledge in the field of biofilms is currently propelled by advancements in microscopic imaging techniques, with Confocal Laser Scanning Microscopy (CLSM) standing out as the most commonly employed method for biofilm analysis (Gomes & Mergulhão, 2017). CLSM, a non-destructive and non-invasive approach, excels in providing three-dimensional images of biofilms, offering valuable insights into their complexity and dynamics (Frühauf et al., 2022). Various optical technologies, including CLSM, are instrumental in visualizing the 3D structure and confirming the presence of biofilms (Kırmusaoğlu, 2019).

CLSM enables multiple image acquisitions throughout the entire depth of biofilms, providing crucial information on their formation, development, morphology, structure, and architecture under different conditions. This is invaluable for clinical, environmental, and laboratory qualitative observations of biofilms (Mhade & Kaushik, 2023). A more comprehensive understanding gained through CLSM analysis can inform nuanced approaches to treating biofilm infections,

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including strategies to disrupt biofilms, rendering biofilm bacteria susceptible to antimicrobial treatment and host immune responses (Reichhardt & Parsek, 2019).

While nano-particles have been extensively studied as a remedy for biofilm resistance globally, research in Indonesia is still in its early stages. Testing of nanoparticles as anti-biofilm agents in the country primarily relies on classical quantification techniques such as colony forming unit (CFU) counting and crystal violet staining. The objective of this study is to evaluate the antibiofilm characteristics of gold and silver nanoparticles and determine the optimal antibiofilm concentration against UPEC using CLSM analysis. This research is pivotal in establishing the potential of gold and silver nanoparticles as effective anti-biofilm agents and assessing their efficacy against UPEC through advanced CLSM analysis.

Methods

Rejuvenation of isolates and preparation of bacterial suspensions

The UPEC bacterial isolate (S58) was obtained by isolating it from a urine sample received at the microbiology laboratory of RSUD in Surabaya. This particular isolate is known for its strong biofilm-producing characteristics and was selected as the test bacteria for this study. The isolation process involved the rejuvenation of the isolate on new slanted Nutrient Agar (NA) media. Subsequently, for the culture inoculum, the isolate was cultured by planting it in RPMI media, and the culture was then incubated for 24 h at room temperature. Following the overnight incubation, the culture in RPMI was subjected to centrifugation at 5000 rpm for 5 min. The resulting supernatant was carefully removed, and the pellet formed was resuspended in physiological water. The suspension was then adjusted to achieve an optical density at 490 nm (OD_{490}) of 0.5

Formation of biofilm *in vitro*

The biofilm creation stage on a glass coverslip involves cultivating a biofilm on the coverslip submerged in RPMI media. To initiate this process, a 10 µL volume of UPEC suspension with a turbidity of 0.5 at λ=490 nm was inoculated into RPMI media. The inoculated setup was then incubated at 37 ºC for 24 h under aerobic conditions. Following this incubation period, the glass coverslip, now containing the developed biofilm, was carefully transferred into fresh RPMI media within a 24-well microplate

The addition of gold nanoparticles and silver nanoparticles

A 5 mL culture was prepared in a 24-well microplate for the specified treatment, comprising 3 mL of liquid RPMI media, a glass coverslip enveloped with biofilm, 1 mL of gold nanoparticle solution (E), and 1 mL of silver nanoparticle solution (P) at concentrations of 25, 50, 75, and 100 ppm. The chosen concentrations were based on findings from prior research by Purbowati et al. (2022), highlighting varied antibiofilm activity via the crystal violet method. This culture was incubated at 37 ºC for 24 h under aerobic conditions. Controls were incorporated for comparison, with the positive control lacking gold nanoparticles, and the negative control excluding the test bacteria. Following the 24-h incubation, the biofilm on the coverslip underwent continuous staining with Syto 9 and Con A. The staining procedure involved sequential steps, including washing and blocking, staining with Syto 9, re-blocking, and staining with Con A. These steps were crucial for a detailed analysis of the biofilm using advanced imaging techniques, ensuring a comprehensive understanding of the effects of gold and silver nanoparticles at various concentrations on biofilm formation.

Staining and CLSM analysis (modification of the method from Jurcisek et al., 2011)

For staining UPEC cells (Syto) with the matrix (Con A), the biofilm on the glass coverslip was positioned in a 24-well plate. Subsequently, the preparation underwent a series of steps: it was washed with PBST for 10 min, blocked with 2% BSA in PBST at room temperature for 30 min, and washed again with PBST for 10 min. The preparations were then treated with 50 µL of Syto 9, incubated for 1 h, and washed with PBST for 10 min. This process was repeated, involving a second blocking step with 2% BSA at room temperature for 30 min and another wash with PBST for 10 min. The final stage comprised incubation with 50 µL of Con A for 20 min, followed by a wash with PBST for 10 min. The prepared slides were then observed using Confocal Laser Scanning Microscopy (CLSM).

Results

The impact of introducing gold nanoparticles at different concentrations on the structure and matrix of UPEC biofilms.

The investigation into biofilm morphology and matrix involved a dual staining procedure using CLSM Olympus type FV1000. Syto9, a green fluorescent nucleic acid marker at a 1:500 dilution, was used to color UPEC bacterial cells, while Concanavalin A stained the biofilm matrix in red. The images were captured and quantified using Olympus Fluoview Software version 1.7a at 400x magnification.

Figures and Tables 1 illustrate that UPEC biofilms treated with varying concentrations of gold nanoparticles appeared noticeably smaller than the negative control. As the concentration of gold nanoparticles increased, there was a corresponding decrease in UPEC biofilm density, bacterial cell density, and biofilm matrix density. The lowest densities were observed at a concentration of 100 ppm. Morphological observations indicated significant differences between biofilm structures without gold nanoparticles (K-) and those treated with different concentrations of gold nanoparticles (E25, E50, E75, and E100). The UPEC biofilm in the negative control (K-) exhibited denser, more even, and wider characteristics, while biofilms in treated samples and K+ (with antibiotic addition) displayed a more tenuous, uneven structure with distinctive small groups or aggregates. Quantification of Syto9 and ConA in arbitrary units

revealed a decrease in intensity with increasing gold nanoparticle concentration (E25, E50, and E100), although E75 did not follow this pattern. However, when compared to quantification without gold nanoparticles (K-), all treatments still exhibited higher values. The carrier substance of gold nanoparticles is suspected to influence the color absorption of Syto9 and ConA

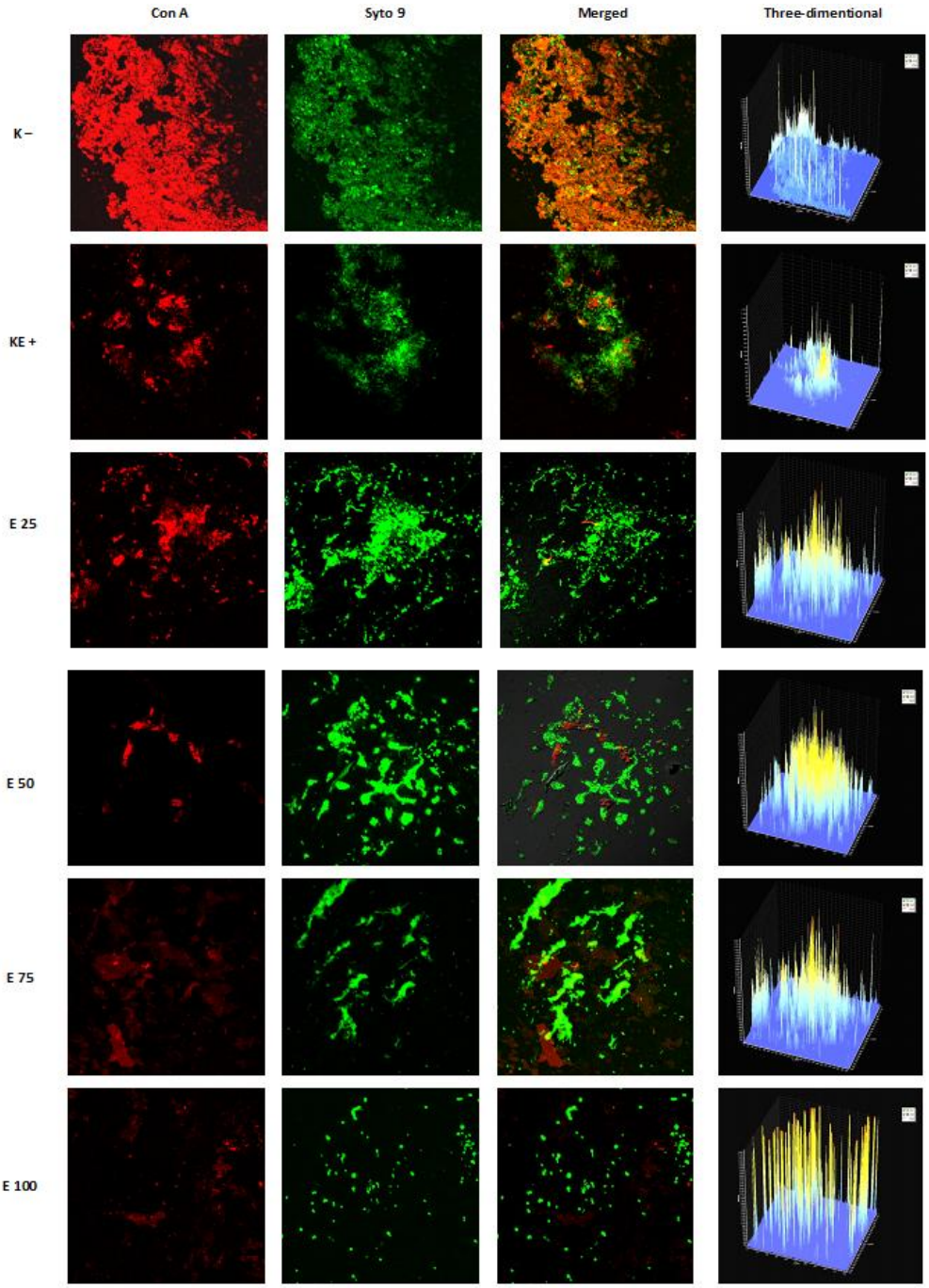

Figure 1. Effect of adding gold nanoparticles at various concentrations on the morphology and matrix of UPEC biofilms. Description: The presence of a biofilm matrix is indicated by the intensity of Con A expression. The density of UPEC bacterial cells in the biofilm is indicated by the expression intensity of Syto 9. The effect of gold nanoparticles on UPEC biofilm morphology is demonstrated by the expression intensity of both color/merged and 3D images. K-: Negative control (UPEC biofilm culture), KP+: Positive control (UPEC biofilm culture with the addition of antibiotics), E25: addition of gold nanoparticles at 25 ppm, E50: addition of gold nanoparticles at 50 ppm, E75: addition of gold nanoparticles at 75 ppm, and E100: addition of gold nanoparticles at 100 ppm, quantification using Olympus Fluoview Software version 1.7a. Syto9 and ConA with units of au.

Table 1. Quantification of adding effect gold nanoparticles at various concentrations on the morphology and matrix of UPEC biofilm

	A	B	C	D	E	F
Dosage AuNps	Control (-)	25 ppm	50 ppm	75 ppm	100 ppm	Control (+)
Syto9 (au)	851,47	303,814	187,036	313,586	165,631	8,215

Con A (au)	45,939	83,747	28,203	33,388	27,644	12,542

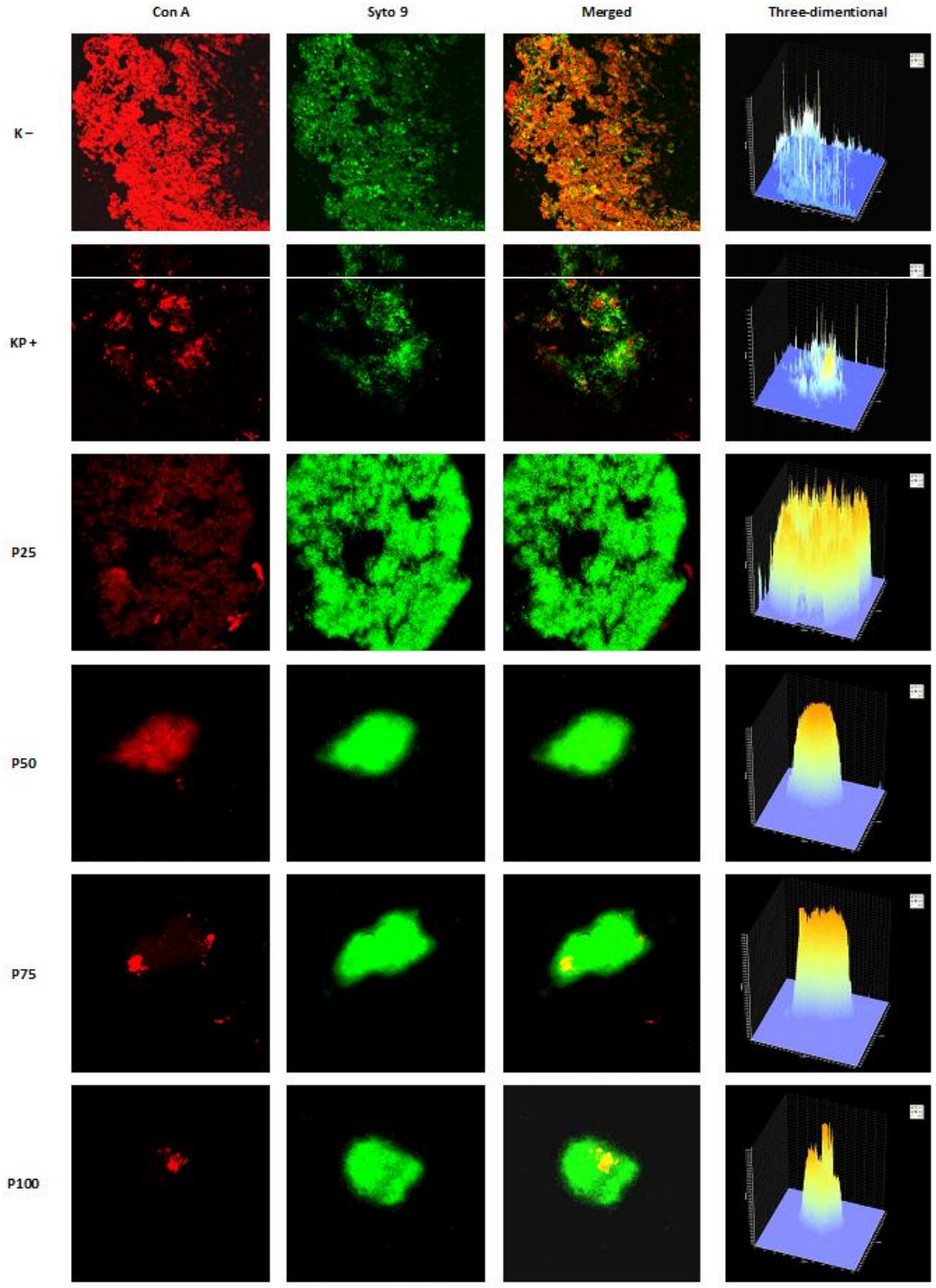

Figure 2. Effect of adding silver nanoparticles at various concentrations on the morphology and matrix of UPEC biofilm. Description: The presence of a biofilm matrix is indicated by the intensity of Con A expression. The density of UPEC bacterial cells in the biofilm is indicated by the expression intensity of Syto 9. The effect of silver nanoparticles on UPEC biofilm morphology is demonstrated by the expression intensity of both color/merged and 3D images. K-: Negative control (UPEC biofilm culture), KP+: Positive control (UPEC biofilm culture with the addition of antibiotics), P25: addition of silver nanoparticles at 25 ppm, P50: addition of silver nanoparticles at 50 ppm, P75: addition of silver nanoparticles at 75 ppm , and P100: addition of silver nanoparticles at 100 ppm, Quantification using Olympus Fluoview Software version 1.7a. Syto9 and ConA with units of au.

Table 2. Quantification of adding effect gold nanoparticles at various concentrations on the morphology and matrix of UPEC biofilm

	A	B	C	D	E	F
Dosage AuNps	Control (-)	25 ppm	50 ppm	75 ppm	100 ppm	Control (+)
Syto9 (au)	851,47	1303,814	287,036	313,586	165,631	8,215
Con A (au)	45,939	83,747	28,203	33,388	27,644	12,542

Effect of adding silver nanoparticles at various concentrations on the morphology and matrix of UPEC biofilms

Figures and Table 2 illustrate a significant reduction in the size of UPEC biofilms treated with silver nanoparticles compared to the negative control. As the concentration of silver nanoparticles increased, there was a corresponding decline in UPEC biofilm density. Similarly, an increase in the concentration of gold nanoparticles led to a decrease in the density of both UPEC bacterial cells and the biofilm matrix, with the lowest density observed at a concentration of 100 ppm.

Morphological observations revealed marked differences between biofilm structures without gold nanoparticles (K-) and those treated with various concentrations of gold nanoparticles (P25, P50, P75, and P100). The negative control (K-) displayed a denser, more even, and wider appearance, while treated samples and K+ (with antibiotic addition) exhibited a more delicate, uneven structure with numerous empty spaces between colonies. Moreover, there was a distinctive formation, where bacterial cells and their matrix tended to aggregate into small groups.

Quantification results for Syto9 and ConA in arbitrary units further indicated a decrease in intensity with increasing gold nanoparticle concentration (P25, P50, and P100), although this trend did not apply to P75. Nevertheless, when compared to quantification without gold nanoparticles (K-), all treatments still exhibited higher values. There is a suspicion that the carrier substance of gold nanoparticles may influence the color absorption of Syto9 and ConA.

Discussion

The CLSM system is instrumental in generating precise multidimensional data from fluorescently labeled targets over time. Its advantage lies in capturing optical slices from specimens, producing three-dimensional (3D) images with volumetric parameters (X, Y, Z) and temporal data (Gupta et al., 2022). Molecular imaging techniques, such as CLSM, contribute significantly to in-depth biofilm analysis, providing insights into composition, structure, cell-cell communication, metabolism, drug response, and reactions to environmental stress (van Hoogstraten et al., 2023).

Nanotechnology has emerged as a promising approach for bacterial biofilm treatment. Metallic nanomaterials, including gold (Au) and silver (Ag), act as effective antimicrobial agents by inhibiting and disrupting biofilms. These nanoparticles serve as antibacterial agents, anti-biofouling agents, or carriers for delivering antimicrobial agents within the biofilm matrix (Mukherjee et al., 2023).

Visualization of metabolic pathways aids in assessing drug absorption and efficiency. Common biofilm visualization methods include scanning electron microscopy (SEM) and atomic force microscopy (AFM), while staining techniques like crystal violet and confocal laser scanning microscopy (CLSM) provide detailed three-dimensional images of biofilm structures and bacterial cell viability (van Hoogstraten et al., 2023).

Nanomaterials, with diameters ranging from 1 to 100 nm, exhibit unique physicochemical and biological characteristics, making them valuable in medical imaging, drug delivery, and disease diagnostics. The antibacterial properties of nanoparticles are manifested through mechanisms like membrane disruption, reactive oxygen species (ROS) production, ATP depletion, and DNA synthesis inhibition (Piktel et al., 2021).

The interaction of metal nanoparticles with bacterial walls and membranes, causing membrane potential changes and increased permeability, plays a crucial role in antibacterial action. Metal nanoparticles can deplete ATP and destabilize the outer membrane, leading to bacterial death. Nanomaterials exert antibacterial effects through various mechanisms, including direct contact with bacterial cell walls, biofilm formation inhibition, immune response triggering, ROS production, and intracellular effects initiation (Okkeh et al., 2021).

Gold nanoparticles (AuNPs) exhibit strong antibacterial potential by damaging bacterial DNA, interacting with proteins and lipopolysaccharides, and enhancing antibiotic delivery to gram-negative bacteria. Metal nanoparticles, like gold, can disrupt metabolic pathways, affecting the respiratory chain, ATP production, and replication processes, ultimately inhibiting bacterial homeostasis (Kaur et al., 2023).

Transmission electron microscopy (TEM) images show that E. coli cells treated with gold nanoparticles undergo severe morphological deformation, accompanied by a significant increase in intracellular ROS concentration (Tian et al., 2021).

Gold nanoparticles, even at low concentrations (3.54 µg/mL), exhibit inhibition of bacterial growth and induce cell death, resulting in pleomorphic changes in treated E. coli cells (Candreva et al., 2023). Considering that E. coli poses a global health threat due to multi-drug resistance, AuNPs demonstrate strong antibacterial activity against it. Complexes of AuNPs with substances like lysozyme or cinnamic acid enhance antibacterial effects, reaching inhibitory rates of up to 98%-99%. Combining antibiotics or antimicrobial peptides with AuNPs, as seen with polymyxin B sulfate coupled AuNPs (PMB-AuNPs), proves effective in improving antimicrobial efficiency (Tian et al., 2021).

The challenges in biofilm destruction include limited access of antimicrobial agents due to the protective extracellular polymer matrix, immune system interference, bacterial cell resistance, and heterogeneity among biofilm layers (Makhlouf et al., 2023). Silver nanoparticles (AgNPs) provide antibacterial effects against various bacteria, disrupting bacterial cell walls, damaging DNA, and inducing cell death. AgNPs can also release Ag+ ions, contributing to their antimicrobial effect. AgNPs exhibit antibiofilm activity by disrupting biofilm integrity, inducing oxidative stress, and inhibiting quorum sensing mechanisms (Adnan et al., 2023).

The use of AgNPs in biofilm treatment involves disrupting existing biofilm biomass and inhibiting biofilm formation. Higher concentrations are required for a significant reduction in existing biofilm compared to initial biofilm formation. AgNPs can penetrate biofilm matrices, disrupt quorum sensing, and enhance antibiotic efficacy. Analysis using Confocal Laser Scanning Microscopy (CLSM) allows non-destructive detection of biofilms at different depths, revealing 3D structures and aiding in understanding antimicrobial resistance properties (Mukherjee et al., 2023).

Understanding biofilm architecture is crucial, as extracellular polymeric substances (EPS) contribute to antimicrobial resistance by inhibiting antibiotic transport. CLSM, coupled with live/dead staining, provides a fluorescence assay of bacterial viability. The surface properties of implanted materials also influence biofilm formation, and investigations utilizing CLSM complement microbiological approaches to elucidate biofilm matrix stability and function (Mountcastle et al., 2021; LewisOscar et al., 2021).

In conclusion, gold and silver nanoparticles exhibit antibiofilm activity against UPEC at concentrations of 25, 50, 75, and 100 ppm, as evidenced by CLSM analysis. The optimal concentration for antibiofilm activity of gold and silver nanoparticles against UPEC, determined through CLSM analysis, is 100 ppm. These findings contribute to a deeper understanding of biofilm treatment approaches and failures (Reichhardt & Parsek, 2019; van Hoogstraten et al., 2023).

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References

Adnan, M., Siddiqui, A. J., Ashraf, S. A., Ashraf, M. S., Alomrani, S. O., Alreshidi, M., Tepe, B., Sachidanandan, M., Danciu, C., & Patel, M. (2023). Saponin-Derived Silver Nanoparticles from Phoenix dactylifera (Ajwa Dates) Exhibit Broad-Spectrum Bioactivities Combating Bacterial Infections. *Antibiotics*, *12*(9), Article 9. https://doi.org/10.3390/antibiotics12091415

Aflakian, F., Mirzavi, F., Aiyelabegan, H. T., Soleimani, A., Gholizadeh Navashenaq, J., Karimi-Sani, I., Rafati Zomorodi, A., & Vakili-Ghartavol, R. (2023). Nanoparticles-based therapeutics for the management of bacterial infections: A special emphasis on FDA approved products and clinical trials. *European Journal of Pharmaceutical Sciences*, *188*, 106515. https://doi.org/10.1016/j.ejps.2023.106515

Ali, S. G., Ansari, M. A., Alzohairy, M. A., Alomary, M. N., AlYahya, S., Jalal, M., Khan, H. M., Asiri, S. M. M., Ahmad, W., Mahdi, A. A., El-Sherbeeny, A. M., & El-Meligy, M. A. (2020). Biogenic Gold Nanoparticles as Potent Antibacterial and Antibiofilm Nano-Antibiotics against Pseudomonas aeruginosa. *Antibiotics*, *9*(3), 100. https://doi.org/10.3390/antibiotics9030100

Al-Momani, H., Almasri, M., Al Balawi, D., Hamed, S., Albiss, B. A., Aldabaibeh, N., Ibrahim, L., Albalawi, H., Al Haj Mahmoud, S., Khasawneh, A. I., Kilani, M., Aldhafeeri, M., Bani-Hani, M., Wilcox, M., Pearson, J., & Ward, C. (2023). The efficacy of biosynthesized silver nanoparticles against Pseudomonas aeruginosa isolates from cystic fibrosis patients. *Scientific Reports*, *13*, 8876. https://doi.org/10.1038/s41598-023-35919-6

Candreva, A., De Rose, R., Perrotta, I. D., Guglielmelli, A., & La Deda, M. (2023). Light-Induced Clusterization of Gold Nanoparticles: A New Photo-Triggered Antibacterial against E. coli Proliferation. *Nanomaterials*, *13*(4), 746. https://doi.org/10.3390/nano13040746

Dutt, Y., Dhiman, R., Singh, T., Vibhuti, A., Gupta, A., Pandey, R. P., Raj, V. S., Chang, C.-M., & Priyadarshini, A. (2022). The Association between Biofilm Formation and Antimicrobial Resistance with Possible Ingenious Bio-Remedial Approaches. *Antibiotics (Basel, Switzerland)*, *11*(7), 930. https://doi.org/10.3390/antibiotics11070930

Franco, D., Calabrese, G., Guglielmino, S. P. P., & Conoci, S. (2022). Metal-Based Nanoparticles: Antibacterial Mechanisms and Biomedical Application. *Microorganisms*, *10*(9), 1778. https://doi.org/10.3390/microorganisms10091778

Frenkel, L. D. (2021). The global burden of vaccine-preventable infectious diseases in children less than 5 years of age: Implications for COVID-19 vaccination. How can we do better? *Allergy and Asthma Proceedings*, *42*(5), 378–385. https://doi.org/10.2500/aap.2021.42.210065

Frühauf, H. M., Stöckl, M., & Holtmann, D. (2022). R-based method for quantitative analysis of biofilm thickness by using confocal laser scanning microscopy. *Engineering in Life Sciences*, *22*(6), 464–470. https://doi.org/10.1002/elsc.202200008

Funari, R., & Shen, A. Q. (2022). Detection and Characterization of Bacterial Biofilms and Biofilm-Based Sensors. *ACS Sensors*, *7*(2), 347–357. https://doi.org/10.1021/acssensors.1c02722

Gomes, L. C., & Mergulhão, F. J. (2017). SEM Analysis of Surface Impact on Biofilm Antibiotic Treatment. *Scanning*, *2017*, 2960194. https://doi.org/10.1155/2017/2960194

Gupta, Y., Costa, C., Pinho, E., A. Bastião Silva, L., & Heintzmann, R. (2022). IMAGE-IN: Interactive web-based multidimensional 3D visualizer for multi-modal microscopy images. *PLOS ONE*, *17*(12), e0279825. https://doi.org/10.1371/journal.pone.0279825

Harish, V., Tewari, D., Gaur, M., Yadav, A. B., Swaroop, S., Bechelany, M., & Barhoum, A. (2022). Review on Nanoparticles and Nanostructured Materials: Bioimaging, Biosensing, Drug Delivery, Tissue Engineering, Antimicrobial, and Agro-Food Applications. *Nanomaterials*, *12*(3), 457. https://doi.org/10.3390/nano12030457

Kaur, R., Kaur, K., Alyami, M. H., Lang, D. K., Saini, B., Bayan, M. F., & Chandrasekaran, B. (2023). Combating Microbial Infections Using Metal-Based Nanoparticles as Potential Therapeutic Alternatives. *Antibiotics*, *12*(5), 909. https://doi.org/10.3390/antibiotics12050909

Kędziora, A., Wieczorek, R., Speruda, M., Matolínová, I., Goszczyński, T. M., Litwin, I., Matolín, V., & Bugla-Płoskońska, G. (2021). Comparison of Antibacterial Mode of Action of Silver Ions and Silver Nanoformulations With Different Physico-Chemical Properties: Experimental and Computational Studies. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.659614

Khatoon, Z., McTiernan, C. D., Suuronen, E. J., Mah, T.-F., & Alarcon, E. I. (2018). Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, *4*(12), e01067. https://doi.org/10.1016/j.heliyon.2018.e01067

Kırmusaoğlu, S. (2019). The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents. In *Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods*. IntechOpen. https://doi.org/10.5772/intechopen.84411

LewisOscar, F., Nithya, C., Vismaya, S., Arunkumar, M., Pugazhendhi, A., Nguyen-Tri, P., Alharbi, S. A., Alharbi, N. S., & Thajuddin, N. (2021). In vitro analysis of green fabricated silver nanoparticles (AgNPs) against Pseudomonas aeruginosa PA14 biofilm formation, their application on urinary catheter. *Progress in Organic Coatings*, *151*, 106058. https://doi.org/10.1016/j.porgcoat.2020.106058

Makhlouf, Z., Ali, A. A., & Al-Sayah, M. H. (2023). Liposomes-Based Drug Delivery Systems of Anti-Biofilm Agents to Combat

Bacterial Biofilm Formation. *Antibiotics*, *12*(5), 875. https://doi.org/10.3390/antibiotics12050875

Mancuso, G., Midiri, A., Gerace, E., & Biondo, C. (2021). Bacterial Antibiotic Resistance: The Most Critical Pathogens. *Pathogens*, *10*(10), 1310. https://doi.org/10.3390/pathogens10101310

Mhade, S., & Kaushik, K. S. (2023). Tools of the Trade: Image Analysis Programs for Confocal Laser-Scanning Microscopy Studies of Biofilms and Considerations for Their Use by Experimental Researchers. *ACS Omega*, *8*(23), 20163–20177. https://doi.org/10.1021/acsomega.2c07255

More, P. R., Pandit, S., Filippis, A. D., Franci, G., Mijakovic, I., & Galdiero, M. (2023). Silver Nanoparticles: Bactericidal and Mechanistic Approach against Drug Resistant Pathogens. *Microorganisms*, *11*(2). https://doi.org/10.3390/microorganisms11020369

Mountcastle, S. E., Vyas, N., Villapun, V. M., Cox, S. C., Jabbari, S., Sammons, R. L., Shelton, R. M., Walmsley, A. D., & Kuehne, S. A. (2021). Biofilm viability checker: An open-source tool for automated biofilm viability analysis from confocal microscopy images. *Npj Biofilms and Microbiomes*, *7*(1), Article 1. https://doi.org/10.1038/s41522-021-00214-7

Mukherjee, A., Bose, S., Shaoo, A., & K. Das, S. (2023). Nanotechnology based therapeutic approaches: An advanced strategy to target the biofilm of ESKAPE pathogens. *Materials Advances*, *4*(12), 2544–2572. https://doi.org/10.1039/D2MA00846G

Mutalik, C., Saukani, M., Khafid, M., Krisnawati, D. I., Widodo, Darmayanti, R., Puspitasari, B., Cheng, T.-M., & Kuo, T.-R. (2023). Gold-Based Nanostructures for Antibacterial Application. *International Journal of Molecular Sciences*, *24*(12), 10006. https://doi.org/10.3390/ijms241210006

Okkeh, M., Bloise, N., Restivo, E., De Vita, L., Pallavicini, P., & Visai, L. (2021). Gold Nanoparticles: Can They Be the Next Magic Bullet for Multidrug-Resistant Bacteria? *Nanomaterials*, *11*(2), 312. https://doi.org/10.3390/nano11020312

Piktel, E., Suprewicz, Ł., Depciuch, J., Chmielewska, S., Skłodowski, K., Daniluk, T., Król, G., Kołat-Brodecka, P., Bijak, P., Pajor-Świerzy, A., Fiedoruk, K., Parlinska-Wojtan, M., & Bucki, R. (2021). Varied-shaped gold nanoparticles with nanogram killing efficiency as potential antimicrobial surface coatings for the medical devices. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-91847-3

Purbowati, R., Sugiharto, Listyawati, A. F., Masfufatun, Tjandra, L., & Indahsari, N. K. (2022). Antibacterial and antibiofilm effect of silver and gold nanoparticles in Uropathogenic Escherichia coli : *Berkala Penelitian Hayati*, *27*(2), Article 2. https://doi.org/10.23869/bphjbr.27.2.20222

Reichhardt, C., & Parsek, M. R. (2019). Confocal Laser Scanning Microscopy for Analysis of Pseudomonas aeruginosa Biofilm Architecture and Matrix Localization. *Frontiers in Microbiology*, *10*, 677. https://doi.org/10.3389/fmicb.2019.00677

Tian, E.-K., Wang, Y., Ren, R., Zheng, W., & Liao, W. (2021). Gold Nanoparticle: Recent Progress on Its Antibacterial Applications and Mechanisms. *Journal of Nanomaterials*, *2021*. https://doi.org/10.1155/2021/2501345

Vallet-Regí, M., González, B., & Izquierdo-Barba, I. (2019). Nanomaterials as Promising Alternative in the Infection Treatment. *International Journal of Molecular Sciences*, *20*(15), 3806. https://doi.org/10.3390/ijms20153806

van Hoogstraten, S. W. G., Kuik, C., Arts, J. J. C., & Cillero-Pastor, B. (2023). Molecular imaging of bacterial biofilms—A systematic review. *Critical Reviews in Microbiology*, *0*(0), 1–22. https://doi.org/10.1080/1040841X.2023.2223704

Yang, X., Chen, H., Zheng, Y., Qu, S., Wang, H., & Yi, F. (2022). Disease burden and long-term trends of urinary tract infections: A worldwide report. *Frontiers in Public Health*, *10*. https://www.frontiersin.org/articles/10.3389/fpubh.2022.888205