

Preparation of Fluorescently Labeled Bacteria via Biogenic Synthesis of Orange-Red Emitting Gold Nanoclusters

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Abstract. Whole-cell bacteria-based luminescent probes are crucial for monitoring bacterial interactions with biotic surfaces. The primary approach to developing these probes involves genetic engineering, which is labor-intensive and sophisticated technology. In this study, we created fluorescent probes derived from gram-negative and gram-positive bacteria. This was achieved through the chemical modification of their surfaces with fluorescent glutathione-stabilized gold nanoclusters (bio-GSH-AuNCs) that were synthesized using biogenic approaches. On the other hand, similar nanoclusters produced via traditional chemical reduction approach (ch-GSH-AuNCs) were shown to be ineffective in modifying the bacterial surface. We developed and characterized biofilms of the rhizobacteria *Azospirillum baldaniorum* Sp245 and *Bacillus subtilis* 26D. Notably, this study marks the first report of generating fluorescently labeled bacterial biofilms through the biogenic synthesis of fluorescent gold nanoclusters. The resulting fluorescent bacterial probes, doped with bio-GSH-AuNCs, show significant potential for visualizing the interactions between rhizobacteria and plant root systems. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: fluorescence; gold nanoclusters; whole-cell probes; biofilms; biogenic synthesis.

Paper #9158 received 30 Aug 2024; revised manuscript received 21 Oct 2024; accepted for publication 30 Oct 2024; published online 25 Nov 2024. [doi: 10.18287/JBPE24.10.040309](https://doi.org/10.18287/JBPE24.10.040309).

1 Introduction

Luminescent technologies hold immense practical significance in analytical chemistry, cell biology, and biomedicine. Luminescent sensors are valued for their simple analytical procedures, high sensitivity, and low background signal. The equipment required to detect luminescent signals is widely available and accessible [1]. Typically, luminescent labels refer to molecular organic dyes or emitting nanomaterials [2, 3]. However, luminescent sensors can also be developed using living cells, particularly promising bacterial cells. One of the most notable advantages of luminescent bacterial probes is their ability to colonize diverse substrates, including the tissues of larger organisms [4]. For instance, intestinal microflora performs essential functions within the host organism [5]. Similarly, plant growth-promoting rhizobacteria (PGPR) form biofilms

on root surfaces [6]. Luminescently labeled bacteria offer a means to visualize interactions with biological tissues, providing valuable insights into symbiotic relationships between bacteria and their host organisms. Moreover, luminescent bacterial probes have potential applications in disease diagnostics. Numerous bacteria play a critical role in the microenvironment of malignant tumors. Most of these bacteria are located within the tumor cells [7]. Bacterial biofilms complicate the treatment of chronic wounds [8].

Three main strategies for creating luminescent probes using bacteria are genetic engineering, loading fluorescent tags into bacterial cells, and chemical modification of the bacterial surface [9]. The genetic engineering approach introduces genes encoding fluorescent reporter proteins into the bacterial genome. This can be accomplished by inserting the gene, coding for the fluorescent protein, into a plasmid, which is

subsequently delivered into the bacterial cell. Alternatively, transposons can directly integrate the marker gene into the bacterial chromosome [10]. Cao et al. [11] utilized the probiotic strain *Escherichia coli* Nissle 1917, labeled with the yellow photoactive fluorescent protein FAST, to visualize microflora within the intestines of mice. Similarly, another study employed the *Bacillus subtilis* NCIB 361 strain to express the recombinant red fluorescent protein mKate and track interactions between bacteria and the roots of *Arabidopsis thaliana* [12]. The use of fluorescent proteins in scientific research comes with several limitations. Firstly, they often exhibit a low quantum yield of fluorescence. Additionally, some commonly used fluorescent proteins require dissolved oxygen to fluoresce [13]. In contrast, luciferases are also employed as reporter proteins in developing luminescent whole-cell bacterial probes. However, many luciferase systems necessitate the addition of an external substrate, luciferin, to produce a chemiluminescent signal, typically in the spectral range of 550–570 nm. An exception to this is the LuxCDABE enzymatic system (luxCDABE operon of *Vibrio fischeri*), which is advantageous because it enables the synthesis of luciferin directly within bacterial cells [14]. For example, Matsumoto et al. [15] successfully integrated the luxCDABE operon into the genome of *Pseudomonas syringae* pv. tomato DC3000 using a Tn7 transposon. This genetic modification resulted in luminescent bacterial probes that were used to track bacterial colonization in plant tissues across several plant species. Similarly, Jutras et al. [16] utilized luminescent probes based on *Agrobacterium tumefaciens* to study bacterial interactions with *Nicotiana benthamiana* leaves. Despite these advancements, the genetic engineering approach has its drawbacks. Creating the necessary genetic constructs and transferring them into bacterial cells (a process known as transformation) is labor-intensive. Moreover, transformation is not practical for all bacterial species.

In the second strategy, fluorescent nanomaterials are incorporated into bacterial cells to enable *in vivo* tracking. Tang et al. [17] demonstrated this approach using 3 nm fluorescent green-emitting silicon nanoparticles to track bacteria in animal wound models. The nanoparticles were internalized by the bacterial cells through an ATP-binding transport protein. However, a significant limitation of this strategy is the restricted size range of nanoparticles that bacteria can effectively internalize. Besides, it is essential to note that nanoparticle size is not the sole determinant of penetration through the bacterial cell wall; it is a complex, multifactorial process.

A promising approach for developing luminescent bacterial sensors involves the chemical modification of bacterial surfaces. The bacterial cell wall is composed of many molecules, including peptidoglycan, teichoic acids, proteins, and polysaccharides. These components are rich in various functional groups, such as amino, carboxyl, thiol, and phosphoric acid residues [18]. The functional groups can be utilized to conjugate fluorescent labels

onto the bacterial surface. This binding can occur through electrostatic, hydrophobic, or covalent interactions. Studies have demonstrated that bacterial surfaces can be effectively doped with various luminescent materials, including blue-emitting carbon dots [19], luminophores with aggregation-induced emission enhancement (AIE luminophores) [20], persistent luminescent nanoparticles [21], and metallic nanoclusters [22].

Fluorescent gold nanoclusters (AuNCs) are small supramolecular complexes, typically 2–3 nm in size, composed of gold atoms and organic molecules [23]. These nanoclusters exhibit unique optical properties, making them valuable for developing luminescent probes for bacterial detection. The synthesis of AuNCs is straightforward and does not require organic solvents. Key advantages of AuNCs include high photostability, the ability to tune fluorescence emission across a wide spectral range, and a large Stokes shift [24]. Several studies have used AuNCs to chemically modify bacterial surfaces, giving them luminescent properties [25, 26]. However, a limitation of this approach is that chemically synthesized AuNCs cannot bind to individual bacterial cells without specific recognition molecules. Goswami et al. [27, 28] developed fluorescent bacterial probes using *Bacillus cereus* and *E. coli* through bacteria-mediated biogenic synthesis of AuNCs to address this issue. The biosynthesized AuNCs (bio-AuNCs), stabilized by mercaptopropionic acid (MPA), were localized on the bacterial cell surfaces. This “green” synthetic approach eliminates recognition molecules needing to modify bacterial surfaces with fluorescent labels. Despite these advancements, the literature has limited information regarding the biogenic synthesis of AuNCs using bacteria. There are opportunities to develop this technology further. For instance, bio-AuNCs could be synthesized using less toxic ligands than MPA. Additionally, the potential for creating luminescent bacterial biofilms through the biogenic synthesis of AuNCs remains unexplored.

In this study, we developed fluorescent bacterial probes using the rhizobacteria *Azospirillum baldaniorum* and *Bacillus subtilis*. The bacteria were doped with biogenic gold nanoclusters (bio-AuNCs) stabilized by non-toxic glutathione residues (bio-GSH-AuNCs). To the best of our knowledge, we demonstrated the potential for biogenic synthesis of gold nanoclusters by bacterial biofilms for the first time.

2 Materials and Methods

2.1 Bacterial Cultures and Growth Conditions

A. baldaniorum Sp245 and *B. subtilis* 26D strains were obtained from the Collection of Rhizosphere Microorganisms at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (<http://collection.ibppm.ru/catalogue.html>). Bacterial cultures were grown to the late exponential phase. Cultivation was conducted with constant agitation on a vibration

rotator at 30 °C. *A. baldaniorum* was cultured in a liquid selective synthetic medium containing sodium malate [29], while *B. subtilis* was grown in LB medium [30]. Light microscopy was employed using a DM2500 microscope (Leica Microsystems, Germany) to ensure the bacterial inoculum's purity. The "crushed drop" method was applied for this purpose ($\times 480$ magnification).

2.2 Biofilm Formation

Bacterial biofilms were formed by cultivating overnight bacterial cultures in liquid medium. Bacterial cultures were diluted with sterile media to achieve a 1×10^5 CFU/mL concentration. Following dilution, 2 mL of the bacterial suspensions were dispensed into sterile Petri dishes. The Petri dishes were then incubated under specific temperature conditions. *B. subtilis* 26D strain was incubated for 72 h, whereas *A. baldaniorum* Sp245 strain required an incubation period of 120 h.

2.3 Quantitative Analysis of Biofilm Thickness

Biofilm thickness was assessed using phase-contrast microscopy on a Leica LMD7000 laser microdissector. The equipment was provided by the Simbioz Center for the Collective Use of Research Equipment (IBPPM RAS, Saratov). The sample preparation and result analysis procedure followed the methodology outlined in Ref. [31]. Before microscopy, coverslips with biofilms were inverted so the biofilm faced downward and placed onto a glass slide with a well. To measure the biofilm thickness, the microscope was first focused on the bottom surface of the sample at the glass interface, recording this focal distance as Z_1 in micrometers. Then, the focal distance to the top surface of the biofilm was determined and recorded as Z_2 . The biofilm thickness was calculated using the formula: $Z = (Z_2 - Z_1) \times (n / n_1)$, where n is the refractive index of glass (1.5), and n_1 is the refractive index of air (1.0).

2.4 Quantification of Biofilm Biomass

Initially, the biofilms were rinsed three times with phosphate buffer (pH 7.2) to eliminate any planktonic cells. Subsequently, a 1% aqueous solution of crystal violet dye was applied to the biofilms and allowed to incubate at room temperature for 20–30 min. After incubation, excess dye was removed by washing the biofilms three times with phosphate buffer. The adsorbed dye was then desorbed by adding ethanol, followed by gentle shaking and a brief standing period at room temperature, not exceeding 10 min. The optical density of the desorbed dye solution was measured at 590 nm. The optical density values obtained are directly proportional to the biofilm biomass present.

2.5 Scanning Electron Microscopy of Biofilms

The biofilm samples were prepared for scanning electron microscopy (SEM) following the protocol outlined in

reference [32]. Initially, the samples were fixed with 3% glutaraldehyde. They were then dehydrated using a graded series of acetone concentrations (50%, 70%, 90%, and 100%) and subsequently dried with hexamethyldisilazane. Once dried, the samples were sputter-coated with gold under vacuum conditions. SEM was performed at the Educational and Scientific Institute of Nanostructures and Biosystems at Saratov State University, utilizing a Mira II LMU field emission scanning electron microscope (TESCAN, Czech Republic). The biofilms were examined at an operating voltage of 30 kV in secondary electron mode, with magnifications ranging from $1000\times$ to $10000\times$.

2.6 Chemical Synthesis of Fluorescent Gold Nanoclusters

All glassware was first cleaned with aqua regia (HCl/HNO_3 in a 3:1 ratio) and subsequently rinsed with ethanol and water to ensure purity. Following the method described by Luo et al. [33], 1.5 mL of a 100 mM reduced glutathione solution was mixed with 43.5 mL of milliQ H_2O in a glass vial and stirred thoroughly. Subsequently, 5 mL of 20 mM HAuCl_4 solution was added to the mixture and stirred for an additional 2 min. The reaction mixture was then incubated at 70 °C without stirring for 24 h.

2.7 Staining of Bacterial Cultures with Chemically Synthesized Fluorescent Gold Nanoclusters

In order to stain bacterial cultures with chemically synthesized fluorescent gold nanoclusters (ch-GSH-AuNCs), overnight cultures were first grown with constant shaking at 100–120 rpm in a shaker-incubator. The cultures were then washed once with phosphate buffer (pH 7.2) and diluted to 1×10^8 CFU/mL. Subsequently, ch-GSH-AuNCs were added to the bacterial suspension to achieve a final concentration of 25 μM . The mixture was incubated at 37 °C for 48 h. Following incubation, the bacterial suspensions were centrifuged at 4000 rpm for 10 min. Finally, the fluorescence of both the cells and the supernatant was evaluated using a transilluminator.

2.8 Biogenic Synthesis of Fluorescent Gold Nanoclusters

The following protocol achieved the biogenic synthesis of fluorescent gold nanoclusters (bio-GSH-AuNCs) with bacterial cells. The cultures were washed with distilled water and diluted to a concentration of 1×10^8 colony CFU/mL. To bacterial suspension, 30 μL of 100 mM glutathione and 100 μL of 20 mM HAuCl_4 were added. The mixture was then incubated at 37 °C for 48 h.

In the biofilm-mediated synthesis, mature biofilms grown on cover glasses were transferred to Petri dishes and washed three times with phosphate buffer (pH 7.2)

to eliminate non-adherent planktonic cells. Each dish was supplemented with 2 mL of distilled water, followed by the addition of 60 μL of 100 mM GSH and 200 μL of 20 mM HAuCl_4 . The dishes were incubated at 37 °C for 48 h. After incubation, the cover glasses were thoroughly washed three times to remove any excess unreacted materials. The bio-GSH-AuNCs were visualized using a DMI6000 B inverted fluorescent microscope (Leica, Germany). Images were captured with a Leica DFC 450 camera, with an excitation light source ranging from 340 to 380 nm.

2.9 Characterization of Fluorescent Gold Nanoclusters

Extinction spectra were recorded using a Specord S600 spectrophotometer (Analytic Jena, Germany) over 300–800 nm wavelengths. Fluorescent properties were assessed with a Cary Eclipse spectrofluorimeter (Agilent Technologies, USA), with a slit width of 10 nm for measurements. Transmission electron microscopy (TEM) analysis was performed using a Libra 120 microscope (Carl Zeiss, Germany) at an accelerating voltage of 120 kV. For TEM sample preparation, the samples were applied to nickel grids coated with formvar and subsequently dried.

2.10 Assessment of Bacterial Viability

The viability of individual bacteria and cells within biofilms was evaluated using a culture-based technique. This assessment followed the biogenic synthesis of bio-

AuNCs. The biofilms were detached from cover glass surfaces using 1 mL of sterile phosphate buffer (pH 7.2). Similarly, planktonic cultures were resuspended in 1 mL of the same buffer after the biogenic synthesis of bio-AuNCs. The optical density of bacterial suspensions was adjusted to 0.5 optical units at $\lambda = 600$ nm. Subsequently, the suspensions were serially diluted to 10^4 , 10^5 , and 10^6 in phosphate buffer and plated onto LB agar nutrient media (100 μL per plate). After incubating at 37 °C for 24–48 h, colony counts were conducted, considering the dilutions.

3 Results and Discussion

Gold nanostructures can be synthesized using various methods, including traditional chemical reduction techniques and biogenic “green” approaches that utilize whole cells or cell extracts for gold reduction [34, 35]. Fig. 1 shows the characterization of ch-GSH-AuNCs. These nanoclusters were obtained through chemical reduction.

The absorption spectrum of these nanoclusters did not exhibit a distinct peak (Fig. 1b), indicating that the synthesized nanoclusters were smaller than 5 nm and that plasmon-resonant nanoparticles were not formed. This was corroborated by electron microscopy, which showed that the ch-GSH-AuNCs had an average size of 1.5 ± 0.5 nm (Fig. 1a). The fluorescence properties of the ch-GSH-AuNCs revealed an excitation maximum at $\lambda = 410$ nm and an emission maximum at $\lambda = 613$ nm (Fig. 1c).

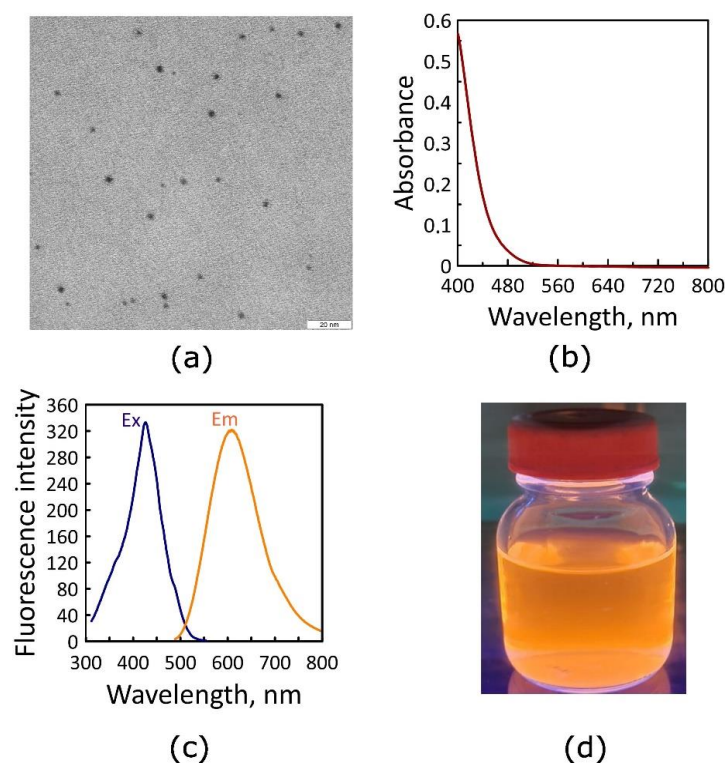


Fig. 1 Characteristics of the ch-GSH-AuNCs: (a) TEM-image of the ch-GSH-AuNCs (scale bar is 20 nm); (b) Absorption spectrum of the ch-GSH-AuNCs; (c) Fluorescence spectra of the ch-GSH-AuNCs; (d) A photograph of the ch-GSH-AuNCs suspension under UV-lamp irradiation $\lambda = 365$ nm).

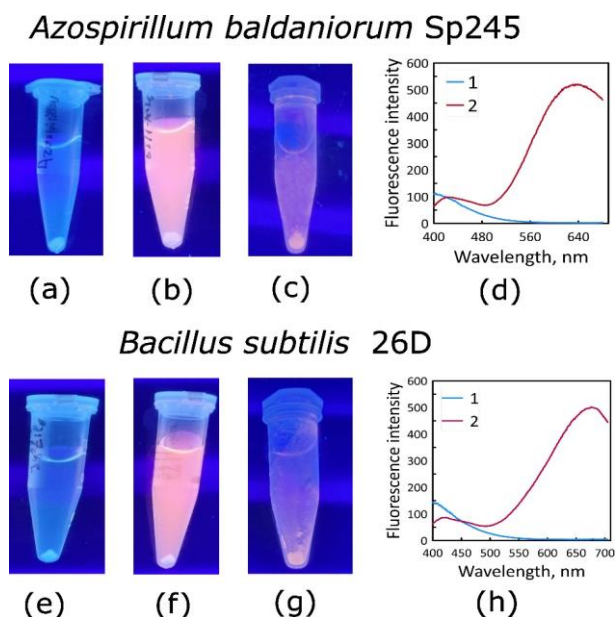


Fig. 2 Characteristics of the bio-GSH-AuNCs: (a) Photograph of the tube with *A. baldaniorum* culture; (b) Photograph obtained after incubation of *A. baldaniorum* bacteria with the ch-GSH-AuNCs for 1 h followed by centrifugation; (c) Photograph obtained after biogenic synthesis of bio-GSH-AuNCs by *A. baldaniorum* bacteria followed by centrifugation; (d) Fluorescence spectra (1 – *A. baldaniorum* culture without AuNCs, 2 – bio-GSH-AuNCs produced by *A. baldaniorum*); (e) Photograph of the tube with *B. subtilis* culture; (f) Photograph obtained after incubation of *B. subtilis* bacteria with the ch-GSH-AuNCs for 1 h followed by centrifugation; (g) Photograph obtained after biogenic synthesis of bio-GSH-AuNCs by *B. subtilis* bacteria followed by centrifugation; (h) Fluorescence spectra (1 – *B. subtilis* culture without AuNCs, 2 – bio-GSH-AuNCs produced by *B. subtilis*).

Fig. 2 presents the characterization of the bio-GSH-AuNCs synthesized biogenically by the bacterial cells of *A. baldaniorum* and *B. subtilis*.

The suspensions were centrifuged after the biogenic synthesis procedure, resulting in fluorescent bacterial pellets (Figs. 2c, g). Notably, nanoclusters were almost absent in the supernatants, indicating that bio-GSH-AuNCs effectively modified the bacterial surface. This observation is supported by the fact that free nanoclusters cannot sediment under the applied centrifugation conditions (4000 rpm). Figs. 2d, h show the fluorescence spectra of both control bacterial suspensions and those containing bio-GSH-AuNCs. The emission peaks of bio-GSH-AuNCs shifted towards the red region compared to chemically synthesized GSH-AuNCs. Specifically, bio-GSH-AuNCs from *A. baldaniorum* had an emission peak at $\lambda = 638$ nm, while those from *B. subtilis* had an emission peak at $\lambda = 662$ nm. The fluorescence spectra of bio-GSH-AuNCs displayed two distinct peaks: one in the red region due to the nanoclusters and another in the blue region from the natural autofluorescence of the bacterial

cells. The biosynthesized nanoclusters showed maximum fluorescence when excited by ultraviolet light (365 nm), unlike ch-GSH-AuNCs, which were best excited by blue light. Additionally, when we tested the ability of ch-GSH-AuNCs to label bacterial cells (Figs. 2b, f), we found that the bacterial pellets did not fluoresce after incubation with ch-GSH-AuNCs. The nanoclusters remained in the supernatant, demonstrating that ch-GSH-AuNCs did not effectively bind to bacterial cells.

We have developed fluorescent probes using whole bacterial cells by synthesizing gold nanoclusters (AuNCs) through a biogenic process. This method utilizes rhizobacteria such as *A. baldaniorum* (gram-negative) and *B. subtilis* (gram-positive). These probes are particularly promising for studying how rhizobacteria interact with plant tissues. In contrast to ch-GSH-AuNCs, biogenic nanoclusters can chemically modify the surface of bacterial cells. The fluorescent properties and photostability of the bio-GSH-AuNCs are primarily attributed to glutathione residues, which stabilize the gold core through the formation of -S-Au- bonds. It is likely that the bacterial surface interacts with the functional carboxyl (-COOH) groups of glutathione residues, thereby anchoring the nanoclusters to the bacterial cell wall [28]. Importantly, glutathione, the ligand employed, is highly biocompatible and non-toxic to bacteria. The localization of biosynthesized nanoclusters within bacterial cells is an important area of inquiry. It is known that bacterial cell wall components can bind metal ions and subsequently reduce them to atoms. For example, Banerjee et al. demonstrated the use of *Bacillus anthracis* cell wall polysaccharides to produce copper nanoparticles [36]. Similarly, Goswami et al. showed that biogenic mercaptopropionic acid-stabilized AuNCs specifically formed on the surface of *E. coli* cells, as was confirmed by electron microscopy [28]. Drawing from research literature, we propose that the biogenic glutathione-stabilized bio-AuNCs are likely formed on the bacterial cell surface. This process is likely to be facilitated by cell wall biomolecules.

We conducted an investigation into the potential for biogenic synthesis of nanoclusters utilizing bacterial biofilms, specifically those formed by *A. baldaniorum* and *B. subtilis*. Both bacterial species demonstrated the ability to form biofilms on hydrophilic cover glass surfaces, although the extent of biofilm formation varied between the two species. Biofilms produced by *B. subtilis* were notably thicker, with measurements of $85.3 \pm 6.8 \mu\text{m}$, in contrast to those produced by *A. baldaniorum*, which measured $67.2 \pm 3.4 \mu\text{m}$. Correspondingly, the total biomass was observed to be greater in the biofilms of *B. subtilis* than those of *A. baldaniorum*.

We examined the ultrastructural features of biofilms formed by *A. baldaniorum* and *B. subtilis* using SEM (Fig. 3). The biofilms of *A. baldaniorum* displayed a complex spatial organization, characterized by a “mesh structure” with alternating dense and sparse clusters of cells. This matrix framework incorporated compact

fragments of cell debris and high-molecular-weight components, effectively embedding the bacteria within the biofilm. Additionally, fibril-like structures were observed to penetrate the cell layers, creating intercellular “bridges” (Fig. 3c). This structural arrangement likely plays a crucial role in adapting to biofilm formation on plant roots. Areas with higher cell density may be involved in synthesizing phytohormones and other compounds, while the sparse regions forming “channels” could facilitate their transport.

In the biofilms of *B. subtilis*, alternating regions of varying thickness and cell density were observed. Unlike *Azospirillum*, the biofilm matrix of *B. subtilis* did not form a structured framework. Instead, it consisted of a suspension containing macromolecular components and lysed cells with bacteria embedded within. The absence of fibril-like structures in the matrix is likely due to the denser packing of cells within the biofilm. Additionally, the cells of *B. subtilis* exhibited variations in size and shape, suggesting potential differentiation among the bacteria to perform specific functions (Fig. 3d).

Fig. 4 presents fluorescence microscopy images of bacterial biofilms after applying a biogenic synthesis protocol for AuNCs. The images reveal the formation of the bio-GSH-AuNCs, which subsequently modified the biofilm matrix of *A. baldaniorum* and *B. subtilis*. The bio-GSH-AuNCs exhibited fluorescence in the orange spectral range. In contrast, the control images only show the autofluorescence of the biofilm matrix.

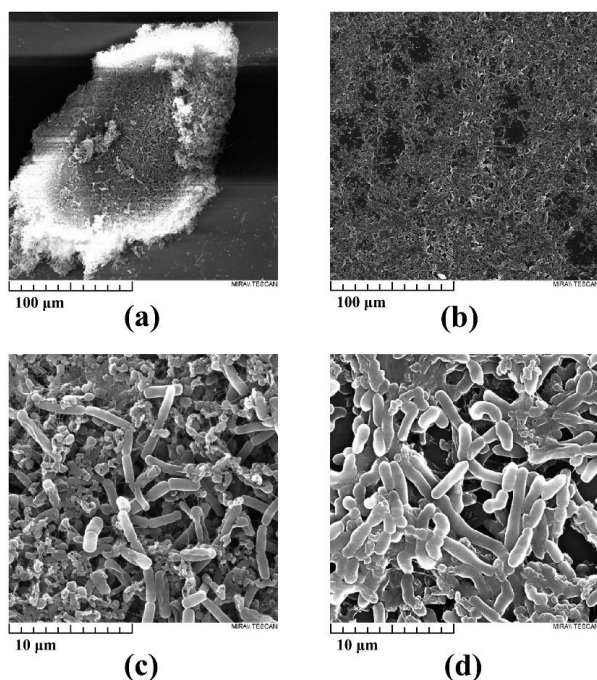


Fig. 3 SEM images of obtained bacterial biofilms: (a) SEM image of *A. baldaniorum* biofilm ($\times 1000$); (b) SEM image of *B. subtilis* biofilm ($\times 1000$); (c) SEM image of *A. baldaniorum* biofilm ($\times 10000$); (d) SEM image of *B. subtilis* biofilm ($\times 10000$).

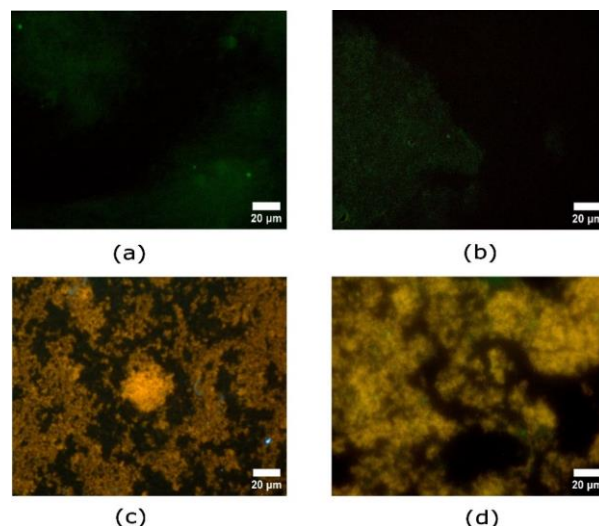


Fig. 4 Microscopic images of bacterial biofilms: (a) *A. baldaniorum* biofilm without AuNCs; (b) *B. subtilis* biofilm without AuNCs; (c) *A. baldaniorum* biofilm obtained after biogenic synthesis of bio-GSH-AuNCs; (d) *B. subtilis* biofilm obtained after biogenic synthesis of bio-GSH-AuNCs.

Preserving bacterial cell viability following the biogenic synthesis of bio-AuNCs is crucial. We conducted further studies to address this concern. We evaluated bacterial viability using *A. baldaniorum* Sp245, as it is more sensitive to environmental factors when compared to *Bacillus* species. Native planktonic bacterial cultures and biofilms that were not exposed to the chemical compounds, required for bio-AuNCs biosynthesis, served as control samples. Fig. 5 shows that both planktonic bacterial cells and biofilms retain viability during the AuNCs biosynthesis process. In planktonic bacterial cultures, the CFU/mL declined from 1.2×10^8 to 1.4×10^6 (Fig. 5a, b). In biofilm cultures, the CFU/mL declined from 5.2×10^8 to 3.1×10^7 (Fig. 5c, d). Biofilms exhibit a greater resistance to the toxic effects of gold ions compared to individual bacterial cells.

In summary, we have successfully produced fluorescent bacterial biofilms by biologically synthesizing gold nanoclusters (AuNCs). This innovative method can be compared with our previous approach [37]. In that earlier study, we demonstrated the selective fluorescent visualization of the biofilm matrix with the ch-GSH-AuNCs. However, a notable limitation of the previous technique was its potential difficulty in visualizing biofilms on biological tissues. Chemically synthesized nanoclusters often tend to aggregate when interacting with the complex structures of biological tissues [38]. In contrast, the biogenic synthesis of fluorescent nanoclusters likely mitigates such aggregation during the detection of biofilms on biological tissue surfaces due to the gradual formation of bio-GSH-AuNCs. Our newly developed approach shows significant promise for detecting rhizobacterial biofilms on root surfaces.

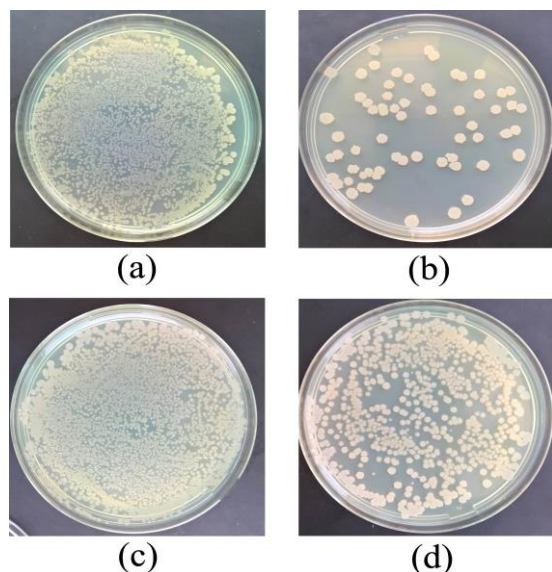


Fig. 5 Determining bacterial colony forming units: (a) native control planktonic cultures of *A. baldaniorum*, 1.2×10^8 CFU/mL; (b) planktonic cultures of *A. baldaniorum* after biosynthesis of bio-AuNCs, 1.4×10^6 CFU/mL; (c) biofilm control cultures of *A. baldaniorum*, 5.2×10^8 CFU/mL; (d) biofilm cultures of *A. baldaniorum* after biosynthesis of bio-AuNCs, 3.1×10^7 CFU/mL.

4 Conclusion

In this study, fluorescent whole-cell probes were developed using the rhizobacteria *A. baldaniorum* and *B. subtilis*. The bacterial surfaces were chemically modified with biogenically synthesized fluorescent glutathione-stabilized gold nanoclusters (bio-GSH-AuNCs). The fluorescently labeled bacteria had an excitation peak at a wavelength of 365 nm. For the bio-GSH-AuNCs-modified *A. baldaniorum*, the emission

peak was observed at $\lambda = 638$ nm, whereas for the bio-GSH-AuNCs-modified *B. subtilis*, the emission peak occurred at $\lambda = 662$ nm. Furthermore, fluorescent rhizobacterial biofilms doped with bio-GSH-AuNCs were successfully produced. Research also indicated that bacterial cells and biofilms remained viable during the biosynthesis of bio-AuNCs, although their viability was diminished. A notable distinction was observed between biogenic and chemically synthesized glutathione-stabilized AuNCs (ch-GSH-AuNCs) in their ability to modify bacterial cells. The ch-GSH-AuNCs were unable to bind to the bacterial cells. These findings suggest that biogenic synthesis is an effective method for creating bacteria-based luminescent probes. Such probes hold significant potential for future applications in visualizing interactions between rhizobacteria and plant root systems.

Acknowledgments

The authors thank Victor Galushka (SSU, Saratov, Russia) for scanning electron microscopy measurements using the “Educational and Scientific Institute of Nanostructures and Biosystems” equipment of the Saratov State University.

Funding

This research was supported by the Russian Science Foundation (Project № 23-24-00246).

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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