Domestic Wastewater Treatment: The Influence of Silver Impregnated Polyrhodanine Nanofibers on Gram-Positive and Gram-Negative Bacteria

Corli de Klerk, Elvis Fosso-Kankeu*, Lissinda du Plessis and Frans Waanders

***Abstract*—**Polyrhodanine nanofibers impregnated with silver nanoparticles were produced by a simultaneous chemical oxidation polymerization process in which the silver ions were reduced to silver nanoparticles onto the rhodanine monomer, which were then oxidized to construct the silver impregnated polyrhodanine nanofibers. The antimicrobial efficacy was tested by the disc diffusion method, broth dilution assay, as well as flow cytometry analysis to acquire significant information on the mechanisms of the nanoparticles and polymer complex. This complex resulted in excellent antimicrobial properties and inhibitory effects against both Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Salmonella typhi*). Disc diffusion analyses proved that the polyrhodanine-silver complex had superior antibacterial efficacy compared to the pristine silver nanoparticles. Gram-positive bacteria were found to be the most susceptible to the PRD-Ag complex with minimum inhibitory concentrations (MIC) of 12.5 µg/disc. The MIC of Gram-positive bacteria was slightly higher at 25 µg/disc. The investigation of the contact time of the nanomaterial with the various bacteria also supported the notion that a reduction in cell viability is more prominent in the presence of the synthesized PRD-Ag nanofibers compared to pristine silver nanoparticles. This study also demonstrated that the determination of total cell concentration by means of flow cytometry analysis is a rapid and sensitive method that could be widely applied in wastewater treatment technology. The flow cytometry analysis supported the initial findings and confirmed that the Gram-positive bacterial strains are more susceptible to the antibacterial complex with cell viability decreased to 51.10% for *B. subtilis* and a significant decrease to 5.33% for *S. aureus.*

***Keywords*—**Antibacterial mechanism, flow cytometry, silver nanoparticles, polyrhodanine, wastewater treatment

I. Introduction

The imbalance between water supply and demand is a global concern. Restrictions in the water supply have placed emphasis on the desperate need for alternative and clean water resources [1-12]. Growing water demands are a result of the ever-growing population, increased urbanization, as well as significant economic fluctuations experienced in developing countries [13-16]. It is a well-known fact that access to adequate water is one of the most important requirements for a healthy well-being; however, the use of contaminated water causes a significant number of deaths all over the world. Children and the elderly are the primary sufferers of water-related deaths, as they are more susceptible to poor hygiene conditions and infections [14,17,18]. A diversity of microbial communities thrives in wastewater sources, even though it has been proven low in nutrients [19-22].

Frequently, wastewater sources are the only water available to communities, but are pervaded with pathogenic bacteria, fungi and viruses that can lead to severe diseases and in some cases, result in death [23,24]. Extensive research has been done in the field of water treatment and system development. Common treatment options include chlorination, ozonation, UV disinfection, and membrane filtration; however, the regrow of harmful organisms after distribution remains a health risk to society [25-28].

Attention has been directed to the development of point-of-use treatment systems with focus on the synthesis and production and nano-sized antimicrobial agents to improve the overall potency of the treatment technique [29-33]. This development will also contribute to the long-term remediation of overall water quality and facilitate recycling options. Silver nanoparticles are one of the most popular nano-materials that have been developed to exhibit strong antimicrobial abilities [34,35]. These nanoparticles have already been tested and successfully implemented in various fields and have shown to be effective against both Gram-positive and Gram-negative bacteria [16]. As a result, silver nanoparticles and silver impregnated polymers are on the forefront of wastewater as it has the potential to develop into sustainable water purification systems [36,37].

Polyrhodanine, the rhodanine polymer, a product of the 2-thioxo-1,3-thiazolidin-4-one polymer, has also received a great deal of attention to develop it into an antimicrobial polymer [38]. Rhodanine and rhodanine derivatives have been widely used for a variety of applications, including antiviral, antibacterial, and anticorrosion agents and it has been widely used in the pharmaceutical field; however, new technologies are arising that include it in the engineering and nanotechnology field [38-40]. This study describes the fabrication of a polyrhodanine-silver complex in the form of nanofibers and the

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antimicrobial effectiveness against Gram-positive and Gram-negative bacteria, which are frequently found in domestic wastewater sources. The complex was formed by means of a chemical oxidation polymerization process of the rhodanine monomer using the silver ions as oxidant. Concurrently, the silver ions were reduced to silver nanoparticles that attached to the polyrhodanine fibre. The bacterial strains used to investigate the effectiveness of the complexes included Gram-negative *Escherichia coli (E.coli)* and *Salmonella typhi (S. typhi)*, both known to cause severe gastroenteritis that can have severe consequences when left untreated. Gram-positive bacteria included *staphylococcus aureus* and *Bacillus subtilis* that can cause a wide range of ailments from minor to severe conditions [41,42]. Disc diffusion and broth dilution assays were used to determine the antimicrobial influence of the synthesized compounds and the results were compared with commercial antibacterial agents. Additionally, quantitative flow cytometry analyses were used to assess the total cell concentrations in the wastewater samples and effectively distinguish between viable, intermediate, and non-viable cells.

II. MATERIALS AND METHODS

A. Materials

Rhodanine powder, phosphate buffer tablets, and Luria-Bertani (LB) broth were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Silver nitrate ($AgNO_3$) and ethyl alcohol were obtained from Minema Chemicals (Johannesburg, South Africa) and Associated Chemicals Enterprises (ACE, Johannesburg, South Africa), respectively. All chemicals were of analytical grade and used without further purification. All the bacterial strains were supplied by the North-West University (South Africa). The LIVE/DEAD BacLight bacterial viability kit was purchased from ThermoFisher Scientific (Massachusetts, United States), which was used for the flow cytometry analysis. Deionized water was used to make up the volumes and respective solutions.

B. Synthesis of the Antimicrobial Compounds

To evaluate the antimicrobial consequence of the polyrhodanine-silver complex, pristine rhodanine monomers and silver nanoparticles (Ag NPs) were prepared as well as a silver nanoparticle impregnated-polyrhodanine complex (Ag-PRD).

C. Silver Nanoparticle Synthesis

The reaction time of the Ag NP synthesis process was varied to obtain different particle shapes and sizes since the morphology of the particles can have a substantial effect on the antimicrobial activity of the particle. Silver seeds were prepared by the rapid injection of 0.5 mL 10 mM $NaBH_4$ into 0.5 mL 0.01 M $AgNO_3$ solution with continuous stirring. A volume of 20 mL 0.01 M sodium solution was then added and the solution and mixed for 5 minutes. The solution was aged for up to 24 hours at a temperature of 25°C. Spherical silver hydrosols were synthesized by reducing silver nitrate (100 mL, 0.001 M) at boiling temperature with 3 mL seed solution and sodium citrate with a concentration of 0.001 M. The solution was heated until the colour evolved to a yellow colour and then cooled down to 25°C. The silver nanoparticles were retrieved by centrifugation and purified with deionized water.

D. Polyrhodanine-Silver Complex Synthesis

The polyrhodanine-silver complex was synthesized from the addition of 1 g/L $AgNO_3$ to 200 mL ethanol while stirring to obtain complete dissolution. A mass of 1 g/L of the rhodanine monomer was then added to the solution at a temperature of 60°C, while stirring the solution vigorously. The solution was kept for 24 h at 60°C to ensure complete particle formation and then centrifuged to remove the product. The complex was washed with ethyl alcohol to remove any impurities and dried for 3 days at 60°C, prior to characterization. Fig. 1 represents the polymerization process, which occurs between the rhodanine monomer and silver ions to form cylindrical nanotubes of polyrhodanine-silver.

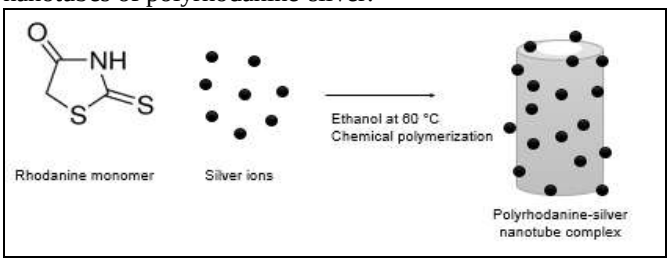

Fig 1: The schematic synthesis of the polyrhodanine-silver complex from rhodanine and silver ions

E. Characterization

The morphology of the synthesized compound was determined by means of scanning electron microscopy (SEM, FEI Quanta 250 FEG ESEM with an integrated Oxford Inca X-Max EDS system, Czech Republic) micrographs were acquired. The samples were coated with a layer of a gold-platinum composite prior to analysis. The characterization was also used to determine the successful impregnation of the nanoparticles on the polyrhodanine polymer.

F. Evaluation of Antimicrobial Action

Pathogenic bacteria were chosen for antibacterial screening of the produced materials. Gram-positive *S. aureus* and *B. subtilis*, and Gram-negative *E.coli* and *S. typhi* bacteria were used. Gram-positive bacteria and Gram-negative bacteria each have different cell membrane characteristics, thus both groups were tested and analysed.

G. Antibacterial Screening

Bacteria were inoculated in sterilized LB nutrient broth and incubated for 12 hours at 36°C in a shaking incubator. The bacterial suspensions were used when the samples indicated an optical density of 0.1 at 600 nm, representing colony-forming units (CFUs) between 10^7 and 10^8 cells.

H. Disc Diffusion Assay

6 mm sterile blank diffusion discs (Davies Diagnostics (Pty) Ltd, South Africa) were impregnated with 15 µL of antibacterial complexes to prepare discs with concentrations of 800, 400, 200, 100, 50, 25, and 12.5 µg/disc of the individual antimicrobial compounds. A sterile swab was dipped in the microbial suspension and spread across the agar plate and the impregnated discs were carefully placed on the freshly

inoculated agar surface with sterile forceps. The plates were incubated in an inverted position for 24 h at 36°C. Commercial antibacterial compounds were used as the positive controls, Vancomycin (30 µg) for Gram-positive bacteria and Carbenicillin (100 µg) for Gram-negative bacteria. All experiments were conducted in quadruplicate (four diffusion discs that have identical antimicrobial compound concentrations) and the susceptibility diameter zone was recorded as the average value of three replicate measurements. This procedure was used to determine the Minimum Inhibitory Concentration (MIC) that is defined as the lowest concentration of antimicrobial compound that inhibits the growth of a microorganism after incubation.

I. Broth Dilution Method

Bacterial solutions of the tested complexes were twofold serially diluted in LB nutrient broth in sterile test tubes in order to obtain final concentrations of 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µg/mL. 2mL of the culture was added to each test tube and incubated for 12 h. The solution in each tube was serially diluted up to 10^{-8}, plated onto an agar plate, and incubated for 24 h at a temperature of 36°C to establish the Minimum Bactericidal Concentration (MBC) of each complex for all tested bacteria. The MBC is defined as the highest dilution of the compound that lead to a reduction of >99.9% of the bacteria. The bacterial survival percentage was determined by (1) below:

$$Survival\ \% = \frac{Colony\ number\ in\ test\ solution}{Colony\ in\ control\ solution} \times 100 \qquad (1)$$

J. Determination of Minimum Exposure Time for Effective Bactericidal Activity

The flow cytometry analysis was done with the use of a LIVE/DEAD BactLight bacteria viability kit. All of the samples were collected in micro-centrifuge tubes and centrifuged for 1-3 minutes to pellet the cells. For each strain of bacteria, a cell-killed sample was prepared by using the pellet of the centrifuge culture that was added to 1 mL isopropyl-alcohol, incubated at room temperature for 60 minutes and mixed thoroughly every 15 minutes. An untreated control sample and the experimental sample were also collected in micro-centrifuge tubes, centrifuged to pellets and the supernatants removed. All the samples, including the cell-killed sample, were then washed with 1 mL buffer solution, followed by 1-3 min centrifuge. The supernatant were decanted slowly and each sample pellet was suspended in 1 mL buffer solution. The cells were stained with 10 µL SYTO 9 and propidium iodide (PI) and inoculated in the dark for at least 15 minutes before flow cytometry measurements were taken. To test the efficacy of the polyrhodanine-silver nanoparticles, serially diluted samples of each bacterial strain was set up to determine the mechanism of inhibition with complex concentrations ranging from 800 µg/mL to 6.25 µg/disc. A real wastewater sample was also inoculated with various concentrations and tested to determine if the real-life application is possible. Cells were analysed with a FACSVerse (BD Biosciences, San Jose, CA, USA) flow cytometer. After incubation, the stained cells were analysed at 480/500 nm as the excitation/emission wavelengths for Syto-9 and 490/635 nm excitation/emission wavelengths for PI.

Realistically, the plate count method is not very accurate with standard errors of more than 30%, while the flow cytometry analysis has an error of less than 5 %. The plate count method also require substantial dilution quantities for quantitative analysis compared to flow cytometry that is able to detect up to 20 000 events without prior dilution [43].

III. Results and Discussion

A. Characterization

Silver Nanoparticle Formation

The effect of varied reaction times for the formation of silver nanotubes showed that short reaction times resulted in insignificant particle formation and a clear colour transition occurs in which a darker yellow indicated improved reaction with better particle formation. As the colour proceeded to dark brown, the particle formation was satisfactory. As the reaction proceeded further, the colour changed to a dark grey, indicative of larger particle formation. A study done by Raza and co-workers established the shape of nanoparticles from the colour differences [44]. The yellow and grey solutions are characterized by spherical particles, whereas the green solution is dominated by triangular formations.

Polyrhodanine-Silver Complex

The SEM micrographs (Fig. 2) showed the PRD-Ag complex morphology was tubular with spherical silver nanoparticles attached on the outside of the cylinders. The average tube diameter and length was approximately 0.3 µm and 3.0 µm, respectively. The Ag NPs covering the PRD tubes had an average size of 40 nm.

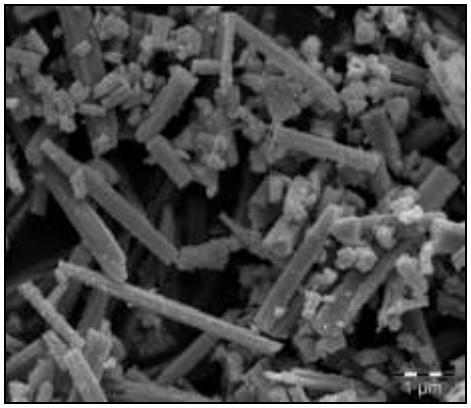

Fig 2: SEM micrograph of the synthesized polyrhodanine tubes coated with silver nanoparticles (reaction time=24 h)

B. Antibacterial Activity

Antibacterial Activity of the Silver Nanoparticles

The total antibacterial activity of the Ag NPs exhibited increased action against Gram-negative bacteria compared to the Gram-positive bacteria. This is due to the structural differences between the two variations of investigated bacteria. Gram-positive cells have a thicker peptidoglycan cell wall which hinders the silver ions from easily entering the cell or damaging the cytoplasm [45]. The findings of the MIC

determinations indicated that smaller particles had a stronger antibacterial effect towards *S. aureus* and *B. subtilis* and were more resistant towards *E.coli* and *S. typhi* (Table 1). The increased antimicrobial activity could be described by the mechanism in which the smaller particles can easily penetrate the cell membrane and larger particles only harm the outside membrane. This effect correlated with similar studies from Morones and co-workers [46], Pal *et al.* [47], and Zarei and colleagues [48]. Therefore, it can be reported that the bactericidal properties of Ag-NPs are reliant on the size and concentration, as well as the bacterial species, which are investigated.

Ag-NPs that have been aged for 8 hours showed the strongest bactericidal effect towards Gram-positive bacteria and 12 hour aged Ag-NPs was found to be more effective towards Gram-negative bacterial strains.

TABLE 1: MIC (µG/DISC) VALUES OF THE SYNTHESIZED COMPLEXES. THE DIFFERENT NANO-SILVER PARTICLES WERE ALSO EVALUATED AND COMPARED TO OTHER ANTIMICROBIAL COMPOUNDS. A REPRESENTS E.COLI, B S.TYPHI, C B. SUBTILIS, AND D S. AUREUS TIME (T) IS REPRESENTED IN HOURS.

	Ag t=0	Ag t=1.5	Ag t=3	Ag t=8	Ag t=12	Ag t=24	Rh	PRD-Ag
A	800	600	300	200	100	200	400	25
B	800	600	500	200	100	100	200	25
C	400	200	200	200	100	200	200	12.5
D	600	300	200	100	50	400	100	12.5

TABLE 2: MBC (µG/ML) VALUES OF THE SYNTHESIZED COMPLEXES. THE DIFFERENT NANO-SILVER PARTICLES WERE ALSO EVALUATED AND COMPARED TO OTHER ANTIMICROBIAL COMPOUNDS. A REPRESENTS E.COLI, B S.TYPHI, C B. SUBTILIS, AND D S. AUREUS. TIME (T) IS REPRESENTED IN HOURS.

	Ag t=0	Ag t=1.5	Ag t=3	Ag t=8	Ag t=12	Ag t=24	Rh	PRD-Ag
A	2000	1500	1000	400	200	800	800	200
B	2000	1000	600	200	200	600	800	200
C	1500	800	800	100	100	400	400	50
D	1500	800	800	50	100	800	200	100

Antibacterial Effect of the Rhodanine Monomer

The results further indicated that all investigated bacteria were susceptible to the rhodanine monomer. Gram-positive bacteria were found to be more vulnerable than Gram-negative. *S. aureus* and *B. subtilis* showed inhibition zones of up to 13.75 ± 0.17 mm and 15.83 ± 0.51 mm, respectively. The largest clear zones for *E.coli* and *S.typhi* were determined to be 21.13 ± 0.35 mm and 15.17 ± 2.12 mm diameters, respectively.

It was also found that the MIC for Gram-positive bacteria was significantly lower than Gram-negative bacteria. *S. aureus* was also found to be the most susceptible to the rhodanine monomer with an MIC value of 100 µg/mL and MBC of 200 µg/mL. The Gram-negative bacteria were more resistant towards the compound with MBC values of 800 µg/mL.

Antibacterial Effect of the Silver Coated Polyrhodanine Polymer

The antibacterial consequence of the PRD-Ag nanotubes had a stronger effect compared to Ag-NPs or rhodanine monomers and the formidable antibacterial activity can be explained by the synergistic action of both the silver and rhodanine. Furthermore, the tubular structure of the PRD-Ag complex is also able to enhance the antibacterial action, since it ruptures the cell wall to form a synthetic pore. This leads to extensive cell damage and outflow of the cell constituents. In addition, the contact time of the complexes on the bacteria also plays an important role in the antibacterial efficacy with a sizeable reduction in cellular propagation detected after only 30 min of incubation in the presence of the PRD-Ag complex. A period of 24 hours of incubation caused nearly 100% inhibition for both Gram-positive and Gram-negative bacterial strains and it was evident that 8 hours contact time was necessary to enhance the antibacterial effect considerably. The mechanism of inhibition is believed to be similar to that of the silver NPs where the cell membrane and cytoplasm is damaged, leading to ATP (Adenosine triphosphate) level modification [47].

C. Flow Cytometry Analysis

Fig. 3 and Fig. 4 display the flow cytometry results of Gram-negative and Gram-positive bacteria, respectively. It can be clearly distinguished that the amount of viable cells decreased with an increase in nanoparticle concentration. This indicated that higher NP concentrations would effectively damage the cellular membranes of the bacterium, leading to cell lysis. These results were detected after only 1 hour of incubation; consequently, extended incubation periods will result in increased cell damage and decreased survival rates. Therefore, longer exposure times will effectively disinfect the water from bacterial contamination. In Fig. 3. *S. typhi* showed an increased resistance to the antimicrobial compound with a viability decrease from 35.95% at the minimum concentration to 23.7% at the maximum concentration. *E.coli* resulted in a similar trend with lowered cell viability decrease from 78.15% to 51.10%, indicating that *E.coli* is a more resistant bacterium than *S. typhi*, especially during short incubation stages. Observation of *B. subtilis* susceptibility indicated that the cellular viability over the examined spectrum reduced from 78.15% to 51.10% viability and *S. aureus* was reduced from 80.93 % to a staggering 5.33% at the MIC.

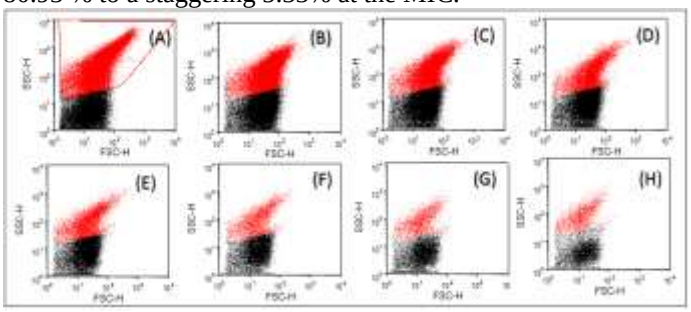

Fig 3: The PRD-Ag nanoparticle effect on Gram-negative S. typhi at increasing antimicrobial compound concentration serially diluted represented from 10-7 (A and B) to 10-1(H). (A) Indicates the gated region of viable cells.

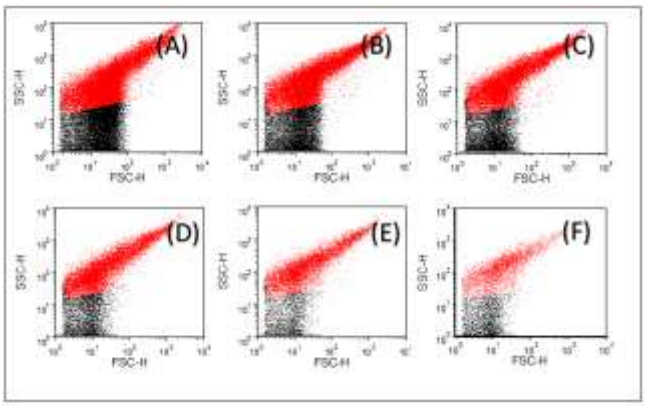

Fig 4: The PRD-Ag nanoparticle effect on Gram-positive B. subtilis at increasing antimicrobial compound concentration that has been serially diluted, represented from 10-7 (A) to 10-1 (F).

The susceptibility of microorganisms in domestic wastewater represented in Fig. 5 shows that the overall cellular viability decreased in the presence of the PRD-Ag nanoparticles. However, higher concentrations of the synthesized complex are required to perform in a similar and effective manner as pure bacterial strains. The actual wastewater viability decreased from 66.92% (12.5 µg/mL) to 52.46% (800 µg/mL). This effect is also ascribed to the considerably higher microbial concentration organisms in the wastewater compared to the pure culture samples that was controlled at specific concentrations at specific growth phases.

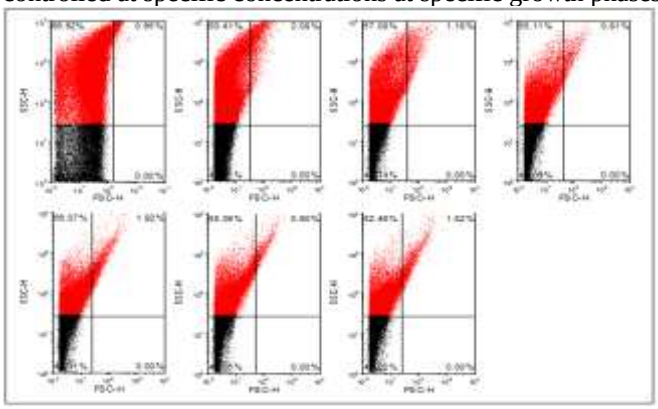

Fig 5: Real wastewater sample inoculated with increasing concentrations of PRD-Ag nanoparticles

D. Conclusion

Smaller silver nanoparticles exhibited a prominent antibacterial effect on the tested strains, while the overall effectiveness decreased with the increase of the particle reaction time. This shape-dependent antimicrobial interaction correlated with studies done by Pal et al, and Raza and co-workers [44, 49].

The nanostructured polyrhodanine polymer coated with silver nanoparticles was also found to be an appealing material for the inhibition and reduction of wastewater bacteria. The preliminary investigations of the silver-based nanoparticles provided valuable information on the inhibitory mechanism of silver and the size and morphology of the particle that possesses assorted inhibition mechanisms. It is suggested that the antimicrobial effect is potent because of the synergistic effect of silver NPs and Ag-PRD complex and the polyrhodanine nanofiber [40]. These nano-materials were found to have a strong impact in domestic wastewater microbial purification and the practical application is further supported from the findings of the flow cytometry analyses. Overall, the Gram-negative bacteria reacted to lower antimicrobial concentrations, however *S. aureus* was observed to be the most susceptible to the PRD-Ag complex. Additionally, the long-lasting effect of the complexes shows very strong promise for further development. Using the polyrhodanine-silver complex has shown that it would be able to provide acceptable reusable wastewater sources that can ease the demand for clean water of the ever-growing population.

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