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Enhanced Antimicrobial Activities of Silver Nanoparticles using Polyrhodanine Nanotubes

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Abstract

In the field of wastewater purification and reclamation, nanotechnology offers effective treatment options for maximum riddance of bacteria and fungi. Polyrhodanine (PRD) nanofibers impregnated with silver nanoparticles were synthesized by a simultaneous chemical oxidation polymerization process in which the silver ions were reduced to silver nanoparticles, the rhodanine monomer was oxidized to form silver impregnated polyrhodanine. The antimicrobial efficacy was tested through the disc diffusion method, broth dilution assay, and flow cytometry analysis in order to gain valuable information on the mechanisms of the nanoparticles and polymer complex. This complex yielded excellent antimicrobial properties against bacteria as well as fungi. Strong inhibitory effects against Gram-positive and Gram-negative bacteria were recorded and the disc diffusion method showed that the polyrhodanine-silver complex had better antibacterial efficacy than pristine silver nanoparticles. *Aspergillus spp.* were more susceptible to the complex whereas *Penicillium chrysogenum* had stronger resistance. The contact time of nanoparticles with the various microorganisms also supported the notion that the decrease of cell viability is more pronounced when exposed to the synthesised PRD-Ag complex. As a rapid and sensitive method, flow cytometry analysis confirmed that Gram-positive bacteria were the most susceptible microorganisms with a viability reduction as low as 5.33 %. Gram-negative *S. typhi* was more susceptible compared to *E. coli* with viability reductions down to a range of 35.95-23.71 %.

Keywords: Antimicrobial compounds, polyrhodanine-silver complex, silver nanoparticles, bacteria, fungi, wastewater

1 INTRODUCTION

Rapid growth in the demand of high quality water combined with the increasing shortage and restrictions in water supply have accelerated the requirement for alternative resources. According to the United Nations, the demand for clean water has increased up to sevenfold in the past century as the world is facing daunting challenges in meeting the growing water demands as the available water supply is constantly decreasing as the global population is increasing, along with urbanization, industrialization and economic fluctuations, with the strongest effect in developing countries (EPA, 1993; Hanjra & Qureshi, 2010; UNDP, 2011; Akpor & Munchie, 2011). An adequate supply of water is one of the most important conditions for a healthy life, however, contaminated water is one of the most prominent challenges due to waterborne diseases causing death in many parts of the world, and especially affecting young children, elderly generations and immune compromised individuals. Even though water is a unique environment with low nutrient concentrations, a diversity microbial communities live and proliferate in untreated water system. Wastewater sources, occasionally the only available water, are contaminated with microbes, including pathogenic bacteria, fungi and viruses that can cause severe health implications, even lead to death. Water treatment systems have multiple processes, such as chlorination, ozonation, membrane filtration, and UV disinfection, but despite these efforts, the regrowth of microorganisms after distribution pose potential health threats. Recently, attention has been directed towards the synthesis and production of nano-sized antimicrobial agents that have increased potency and additional antimicrobial properties as they have the potential to contribute to long-term water quality remediation and recycling. A wide variety of nanomaterials are in various research stages, each possessing unique functions that can potentially influence the viability of contaminated water sources (Tiwari et al., 2008).

Silver nanoparticles have already shown strong antimicrobial capabilities and have been successfully implemented in various fields (Sondi & Salopek-Sondi, 2004; Morones et al., 2005) and can be effectively used as antimicrobial agents against bacteria, fungi, as well as viruses (Beyth et al., 2015). Consequently, silver nanoparticle investigations have shifted to biocidal applications (Sondi & Salopek-Sondi, 2004; Baker et al., 2005). The particle size of the materials are an efficient tool to improve the biocompatibility and can be altered to facilitate applications in various environments (Franci et al., 2015; Rai et al., 2009; Kim et al.,2008; Singh & Tiwari, 2015). Silver nanoparticles and silver impregnated polymers are on the forefront of wastewater treatment as they have the potential to develop into sustainable purification methods that can adhere to more stringent health regulations. The high surface area to mass ratio of the metal nanoparticles provides the advantage of different properties than that of the bulk material (Tiwari et al., 2008; Franci et al., 2015). This study reports the synthesis of a polyrhodanine-silver complex in the form of nanofibers and the antimicrobial effectiveness against

commonly found wastewater bacteria and fungi. The polyrhodanine polymer was used and has been consequential from the rhodanine monomer (2-thioxo-1,3-thiazolidin-4-one). 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 includes it in the engineering and nanotechnology field (Baryshnikov et al., 2012; Kong & Jang, 2008). The complex was formed by means of chemical oxidation polymerization of the rhodanine monomer with silver ions as oxidant. The ions were simultaneously reduced to silver nanoparticles that attached to the polyrhodanine fibre.

The bacteria investigated included Gram-negative *Escherichia coli* and *Salmonella 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 spectrum of ailments from minor to severe conditions (Rice et al., 2010; Cortezzo et al., 2004). Two *Aspergillus* fungal strains were evaluated (*A. fumigatus* and *A. ochraceus*), both capable of producing cytotoxic mycotoxins and they bear the most significant clinical relevance for humans as they may cause acute and chronic infections of the respiratory tract, digestive system, and nervous system (Gniadek, 2012). Opportunistic fungi, *Penicillium chrysogenum* was also used as clinical infections can occur in immune deficient individuals, organ transplantation, or HIV infections (Swoboda-Kopec et al., 2002).

The disc diffusion method was applied and correlated with other antimicrobial procedures. The choice of the disc diffusion method is based on the fact that it is an easy and economical assay to perform. It is also commonly used to test the sensitivity of microorganisms to antibiotics and antimicrobial compounds. From this assay the organisms can be classified as either sensitive or resistant based on the inhibition zone that forms around the impregnated disc. Compared to commercial agents, the synthesized complexes show more variations and are not as consistent. Furthermore, the materials are also commonly insoluble in aqueous solutions and cluster together in the media (Sheehy et al., 2014). Since there are more microbial cells in wastewater which can be cultured on growth media, quantitative flow cytometry was used to analyse the total cell concentrations in water samples. Flow cytometry was also used to differentiate between viable, intermediate, and non-viable cells, since a single sample can be accurately analysed in 5 minutes with an accurate quantification bound of approximately 200 cells/mL (Hammes et al., 2008).

The impregnated fibres have been proven to exhibit strong antimicrobial abilities against Gram-positive and Gram-negative bacteria as well as fungi. Furthermore, it has been stated that tubular nanocomposites demonstrates stronger antimicrobial abilities when compared to other nanostructures as the tubes can easily penetrate the cell wall, leaking the cell contents and leading to cell lysis. It is however, to the best of our knowledge the first time that PRD-Ag nanoparticles will be applied for the inhibition of microorganisms in water.

2 MATERIALS AND METHODS

2.1 Materials

Rhodanine, buffer tablets, Luria-Bertani (LB) broth and Sabouraud dextrose (SD) broth were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), silver nitrate ($AgNO_3$) and ethyl alcohol 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. Bacteria were supplied from the North-West University (South Africa) and fungi sources were supplied by the University of Johannesburg (South Africa). The LIVE/DEAD BacLight bacterial viability kit was purchased from ThermoFisher Scientific (Massachusetts, United States) that was used for the flow cytometry analysis. Deionized water was used to make up the volumes and respective solutions.

2.2 Synthesis of the antimicrobial compounds

2.2.1 Silver nanoparticle synthesis

The nanoparticle reaction time was varied to obtain various particle shapes and sizes since the morphology can have a significant influence on the antimicrobial activity. 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. 20 mL of 0.001 M sodium was then added and the solution was mixed for 5 minutes and aged for up to 24 hours at 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 progressed to yellow and then cooled to 25°C. The silver nanoparticles were retrieved by centrifugation and purified with deionized water.

2.2.2 Polyrhodanine-silver complex synthesis

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

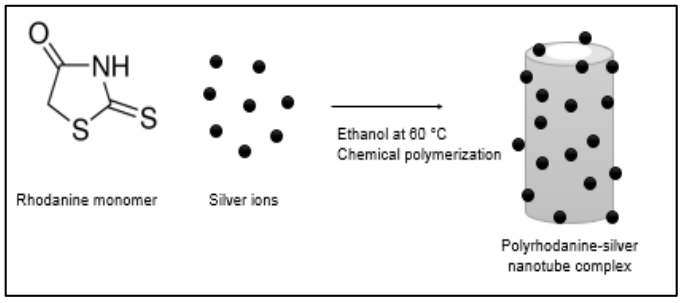

Figure 1: The schematic synthesis of the polyrhodanine-silver complex from rhodanine and silver ions

2.3 Characterization

To determine the morphology of the synthesized compound and to verify the attachment of the nanoparticles to the fibres, 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.

2.4 Evaluation of antimicrobial action

Pathogenic or opportunistic bacteria were selected for antibacterial screening of the produced compounds. For the antibacterial assays, Gram-positive *S. aureus* and *B. subtilis,* and Gram-negative *E. coli* and *S. typhi* were used, which have varying cell membrane characteristics.

2.4.1 Antibacterial screening

Bacteria were inoculated in sterilized LB nutrient broth and incubated for 12 hours at 36°C in a shaking incubator at 120 rpm. The bacterial suspensions were used when the samples indicated an optical density of 0.1 at 600 nm, indicating colony-forming units (CFUs) between 10^7 and 10^8 cells.

Disc diffusion assay

In order to determine the antibacterial ability of silver NPs, the rhodanine monomer and the polyrhodanine silver composite, to restrict the growth of the selected bacteria, a preliminary phase of antibacterial tests were undertaken. 6 mm sterile blank diffusion discs (Davies Diagnostics (Pty) Ltd, South Africa) were impregnated with 15 µL of antibacterial complexes to obtain discs with concentrations of 800, 400, 200, 100, 50, 25, and 12.5 µg/disc of each compound. A sterile swab was dipped in the microbial suspension and spread across the agar plate and the impregnated discs were dried and 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 had identical antimicrobial compound concentrations) and the susceptibility diameter zone was recorded as the average value of four 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.

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 concentrations of 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µg/mL. Then 2 mL 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} and plated onto an agar plate and incubated for 24 h at 36°C to determine the Minimum Bactericidal Concentration (MBC). The MBC is defined as the highest dilution of the compound that killed >99.9% (3log) of the bacteria.

The bacterial survival percentage was determined by Equation [1]:

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

Determination of minimum exposure time for effective bactericidal activity

To evaluate the antimicrobial activity of the synthesized silver compounds, the rhodanine monomer and the polyrhodanine-silver complex as a function of contact time, the MBC determined from the broth dilution assay was considered and a culture volume of 50 µL inoculated at an optical density of 0.1 (OD_{600}) and grown for 24 hours at 36°C. The surviving colonies were counted as compared to the positive control.

2.4.2 Antifungal screening

Disc diffusion assay

Fungi sources were grown in sterilized SD nutrient broth (10 g/L peptone, 20 g/L dextrose) for 24 h at 37 °C in a shaking incubator. The fungi that were tested included *A. fumigatus, A. ochraceus*, and *P. chrysogenum.* Mature fungal strains were harvested from SD agar plates and incubated in SD nutrient broth for 24 h at 36°C. Then 1 mL conidial suspensions of each fungal isolate was spread on a 90-mm diameter petri dish containing 20 mL SD agar and left to dry. The synthesized complexes were dissolved in dimethyl sulfoxide (DMSO) and used to impregnate 6-mm sterile diffusion discs with 15 µL of each test compound to achieve concentrations of 800, 400, 200, 100, 50, 25, and 12.5 µg /disc. Amphotericin B (AmB) was used as the positive control at a concentration of 20 µg/disc, while DMSO was used as the negative control.

The plates were incubated at 36°C for 48 h and the radius of the inhibition zone was measured on two axes at right angles to each other. The Minimum Inhibition Zone (MIZ) was determined with equation [2]:

$$\%\ MIZ = \frac{\pi r^2}{\pi R^2} \times 100 \qquad [2]$$

Where r=r2-r1 (r2 representing the radius of the zone of inhibition of AmB and r1 the radius of the inhibition zone of the tested compound, and R is the radius of the petri dish (90 mm diameter)).

Radial growth reduction

SD nutrient agar plates were inoculated in the centre of the petri dish by a drop of fungal broth that contained varied concentration of the synthesized compounds (2000, 1000, and 500 µg/mL). The petri dishes were incubated at 37°C and the relative growth of the fungus colony was observed by measuring the radial growth for 48 hours. The inhibition rate (%) was calculated by comparing the control growth of the fungal mycelia with that of the *in vitro* assay compound growth.

2.5 Flow cytometry

The flow cytometry analysis was done with the LIVE/DEAD BacLight 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 bacteria strain a cell-killed sample was prepared by using the pellet of the centrifuge culture, added to 1 mL isopropyl-alcohol, incubated at room temperature for 60 minutes and mixed every 15 minutes. An untreated control sample and the experimental sample were

also collected in micro-centrifuge tubes, centrifuged to pellets and the supernatants were removed. All the samples, including the killed sample, were then washed with 1 mL buffer solution, followed by 1-3 min centrifuge and removal of the supernatants. Each sample pellet was suspended in 1 mL buffer solution. Bacterial cells were stained with 10 µL SYTO 9 and propidium iodide (PI) and inoculated in the dark for 15 minutes before flow cytometry measurements.

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 nanoparticle 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 by flow cytometry at 480/500 nm as the excitation/emission wavelengths for Syto-9 and 490/635 nm excitation/emission wavelengths for PI. Viable gates were set up on the dot plot FSC and SSC during analysis.

3 RESULTS AND DISCUSSION

3.1 Characterization

The SEM micrographs indicate that the polyrhodanine-silver complex was tubular with silver nanoparticles attached to the outside of the tubes. The average tube diameter and length was graphically determined to be approximately 0.3 µm and 3 µm, respectively. Additionally, the tubes were decorated with silver nanoparticles with an average size of 40 nm. This correlates with a similar study done by Kong et al. (2009) where tube diameters were found to be 0.2 µm, length was 8 µm, and nanoparticles were 20 nm.

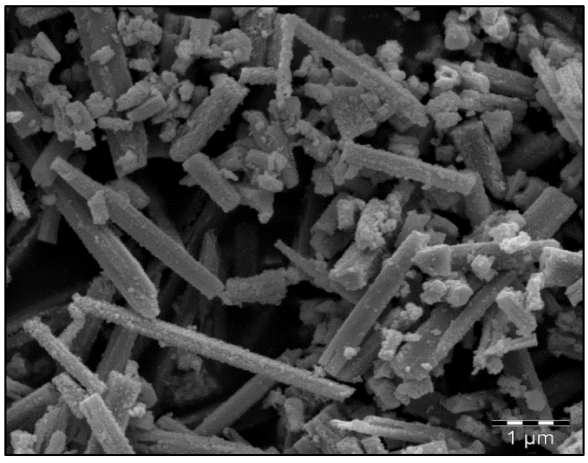

Figure 2: SEM micrograph of the synthesized polyrhodanine tubes coated with silver nanoparticles (reaction time=24 h)

The effect of varied reaction times for the formation of silver nanotubes can be seen from Figure 2. The shorter reaction time resulted in insignificant particle formation and the transition of colour can be seen from tube 1 to 3 where a darker yellow indicated improved reaction. The colour of tube 4 changed to darker brown with significant particle formation. The colour changed to a dark grey colour in tube 6, indicative of larger particle formation. A study done by Raza et al. (2016) and co-workers established the shape of nanoparticles from the colour differences. The yellow and grey solutions are characterized by spherical particles, whereas the green solution is dominated by triangular formations.

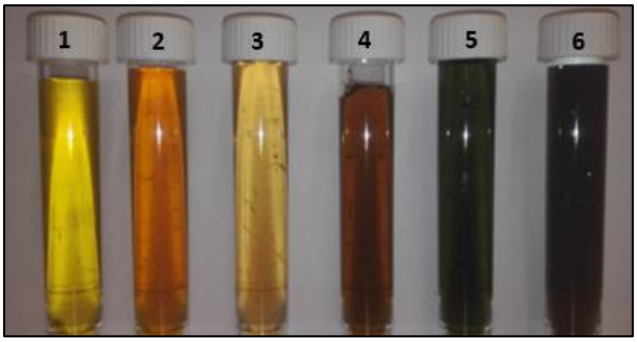

Figure 3: Varied reaction times for silver nanoparticle formation. (1) t=0, (2) t=1.5, (3) t=3, (4) t=8, (5) t=12, and (6) t=24 hours

3.2 Antimicrobial studies

3.2.1 Antibacterial activity

The overall antibacterial activity of the silver nanoparticles exhibited stronger action against Gram-negative bacteria than Gram-positive bacteria. This is possibly due to the cell wall structural differences as Gram-positive cells have a thicker peptidoglycan cell wall that prevents the silver ions from entering the cell or damaging the cytoplasm (Rai et al., 2009). Additionally, the effect is also dependent on the shape and size of the Nanosilver particles. The antibacterial efficacy of smaller nanoparticles was also stronger than that of larger particles and also showed large MIC reductions (Table 1). The MIC results indicate that smaller particles have an overall stronger effect towards *S. aureus* and *B. subtilis* and were more resistant towards Gram-negative *E.coli* and *S. typhi.* The increased 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 coworkers (2005), Pal et al. (2007) and Zarei et al. (2014). It can, therefore, be reported that the bactericidal properties of Nanosilver is dependent on the size and concentration, as well as the bacterial species that are investigated. Silver nanoparticles aged for 12 hours produced the strongest antibacterial effect (Table 1 and Table 2) and *B. subtilis* appeared to be the most vulnerable, closely followed by *S. aureus.*

The antimicrobial efficacy of the rhodanine monomer revealed that all bacteria were susceptible and clear inhibition zones were observed. Gram-positive bacteria seemed to be less susceptible than Gram-negative with *S. aureus* and *B. subtilis* exhibiting maximum inhibition zones of 13.75±0.17 mm and 15.83±0.51 mm respectively. *E.coli* and *S.typhi* inhibition zones were observed to be 21.13±0.35 mm and 15.17±2.12 mm. The rhodanine monomer MIC for Gram-positive bacteria is also considerably lower compared to Gram-negative bacteria (Table 2).

Table 1: MIC (µg/disc) values of the synthesized complexes. The different nanosilver particles were also evaluated and compared to other antimicrobial compounds. The data is a representation of three experimental analysis.

Complex	Ag (t=0)	Ag (t=1.5)	Ag (t=3)	Ag (t=8)	Ag (t=12)	Ag (t=24)	Rh	PRD-Ag
E.coli	800	600	300	200	100	200	400	25
S. typhi	800	600	500	200	100	100	200	25
B. subtilis	400	200	200	200	100	200	200	12.5
S. aureus	600	300	200	100	50	400	100	12.5
A. fumigatus	800	800	800	600	300	400	100	50
A. ochraceus	800	800	600	600	300	400	100	50
P. chrysogenum	600	600	600	600	500	800	100	50

The antimicrobial effect of the synthesized nanotubes has a stronger antibacterial effect than that of the silver nanoparticles or the rhodanine monomer. The strong antibacterial activity can be explained by the synergistic action of both the silver and rhodanine. Additionally, the tubular morphology is also able to increase the effect, since it disrupts the cell and forms a synthetic pore. This leads to extensive cell damage and leakage of the cell constituents. The contact time also plays a significant role in the antibacterial efficacy with a substantial growth reduction observed after only 30 min incubation in the presence of the PRD-Ag complex (Figure 4). 24 hour incubation also results in nearly 100 % inhibition for both Gram-positive and Gram-negative bacterial cells. It is also evident that after 8 hours contact time the effect is enhanced, resulting in large decreases of survival. 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 level modification.

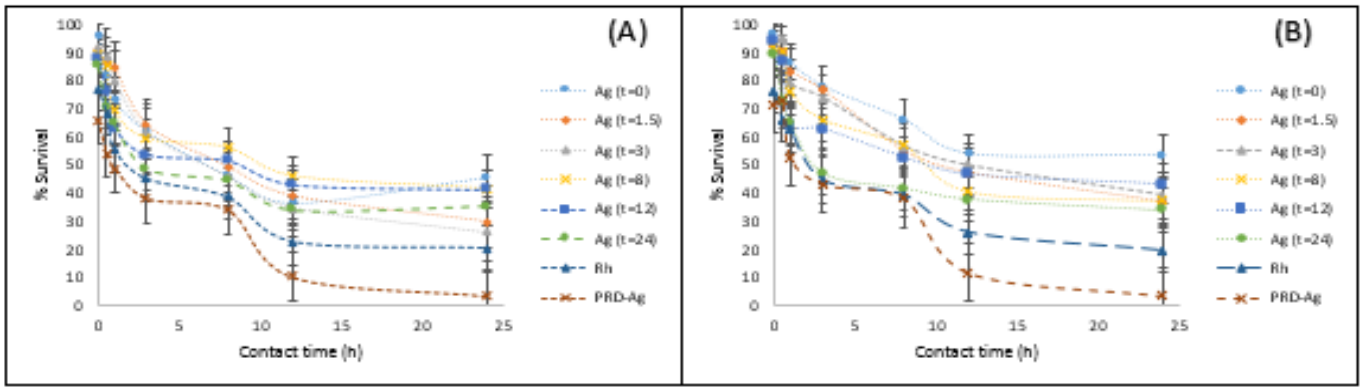

Figure 4: Effect of contact time on (A) E.coli (Gram-negative) and (B) Bacillus subtilis (Gram-positive). The data is a representation of three experimental analysis, mean±std error.

Table 2: MBC (µg/mL) values of the synthesized complexes. The different Nanosilver particles were also evaluated and compared to other antimicrobial compounds.

Complex	Ag (t=0)	Ag (t=1.5)	Ag (t=3)	Ag (t=8)	Ag (t=12)	Ag (t=24)	Rh	PRD-Ag
E.coli	2000	1500	1000	400	200	800	800	200
S. typhi	2000	1000	600	200	200	600	800	200
B. subtilis	1500	800	800	100	100	400	400	50
S. aureus	1500	800	800	50	100	800	200	100

The MBC's values indicate that bactericidal effects increase with time, however after 12 hours, the bacteria tend to recover in the presence of silver NPs. This effect is also observed from the MIC's and it correlates with the literature. It can be concluded that the optimal reaction time is between 8 and 12 hours.

3.2.2 Antifungal activity

The *in vitro* antifungal activity of nano-silver was tested against amphotericin B as the positive control. Nano-silver particles showed inhibition against all tested fungal strains, furthermore, it was observed that the higher the concentration of nano-silver, the stronger the reduction of fungal growth.

Table 3: Antifungal effects of complexes on the MIZ (Minimum Inhibitory zone) % of fungi after 48 hours of incubation. The data is presented as the mean±std error, n=3.

Complex	Ag (t=0)	Ag (t=1.5)	Ag (t=3)	Ag (t=8)	Ag (t=12)	Ag (t=24)	Rh	PRD-Ag
A. fumigatus	0.18±0.14	0.70±0.29	0.36±0.08	0.93±0.14	0.55±0.10	1.54±0.13	0.31±0.09	2.27±0.44
A. ochraceus	1.67±0.04	0.24±0.15	0.53±0.25	1.99±0.32	2.79±0.39	0.99±0.34	0.46±0.10	2.62±1.32
P. chrysogenum	0.12±0.08	1.59±0.07	1.02±0.69	1.12±0.47	0.36±0.08	1.08±0.20	0.12±0.05	2.01±1.29

These nanoparticles disrupt the fungal membrane and induce significant damage, leading to cell death (Kim et al., 2008). Silver nanoparticles aged for 8-12 hours produced the strongest antifungal effect (Table 3) and *A. ochraceus* appeared to be the most susceptible.

The effect of the rhodanine monomer on fungi requires higher concentrations than bacteria since fungi have a strong and rigid membrane. The antimicrobial effect is also considered as fungistatic rather than fungicidal, this is due to the ability of fungi to easily form spores and after damaged cells have recovered during incubation under suitable conditions. From Table 3 it can be observed that *A. ochraceus* was the most susceptible to the monomer, while *A. fumigatus* and *P. chrysogenum* were the most resistant.

The antifungal evaluation indicated that the PRD-Ag compound showed strong antifungal activities compared to the commercial AmB. The fungal strains treated with the PRD-Ag complex also showed a colour change from dark green to white. The fungi can be successfully inhibited at concentrations of 50 µg/mL. The most sensitive fungal strain was *A. ochraceus,* followed by *A. fumigatus. P. chrysogenum* was the most resistant with an MIZ of 2.01±1.29 mm. The MIZ of the *Aspergillus* species also increased up to 7 times in the presence of polyrhodanine while for *P. chrysogenum* the MIZ increase significantly up to 16 times. The MIC is also reduced in the PRD-Ag environment from 100 µg/disc to 50 µg/disc (Table 1). The effect was also clearly seen from day 3 and maintained until day 7, except for the case of *P. chrysogenum* where the clear zone decreased from day 4, indicating that the antifungal compounds have long-lasting effects mostly on *Aspergillus spp.*

3.3 Flow cytometry analysis

Figure 5 and Figure 6 show the flow cytometry results of Gram-negative and Gram-positive bacteria, respectively. It can be clearly distinguished that the amount of viable cells decreases as the concentration of nanoparticles increase, indicating sufficient membrane damage has occurred that will lead to cell death. These results were observed after only 1 hour of incubation, thus longer incubation times will lead to more substantial damage, leading to sufficient water disinfection as can be observed from Figure 4.

Flow cytometry analysis of Gram-negative *S. typhi* (Figure 5) showed increased resistance to the antimicrobial compound with a viability decrease of 35.95 % at the highest PRD-Ag concentration (800 µg/mL) to 23.71 % at the lowest concentration (12.5 µg/mL). *E. coli* (results not shown) showed increased viability compared to *S. typhi* with a reduction from 78.15 % to 51.10 %, indicating that *E. coli* is a more resistant Gram-negative bacteria than *S. typhi* at short incubation periods. Over the inspected spectrum, the viability of the Gram-positive *B. subtilis* decreased from 78.15 % to 51.10 %, while the viability of *S. aureus* (results not shown) was significantly reduced from 80.93 % to 5.33 %.

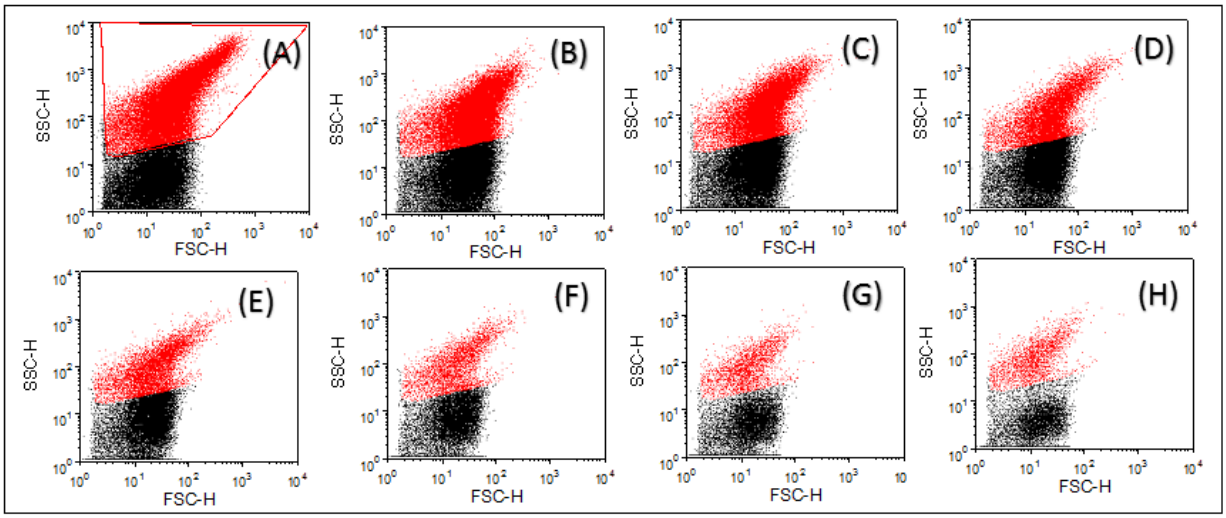

Figure 5: The PRD-Ag nanoparticle effect on Gram-negative S. typhi at increasing antimicrobial compound concentration serially diluted represented from 10^{-7} (A and B) to 10^{-1}(H). (A) indicates the gated region of viable cells.

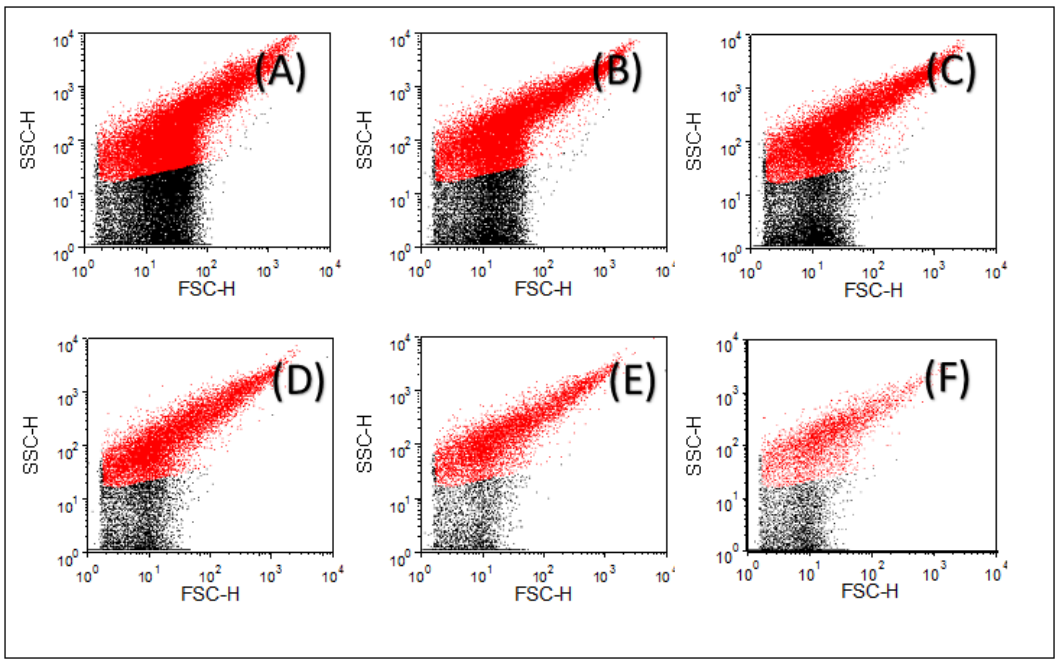

Figure 6: The PRD-Ag nanoparticle effect on Gram-positive B. subtilis at increasing antimicrobial compound concentration that has been serially diluted, represented from 10-7 (A) to 10-1 (F).

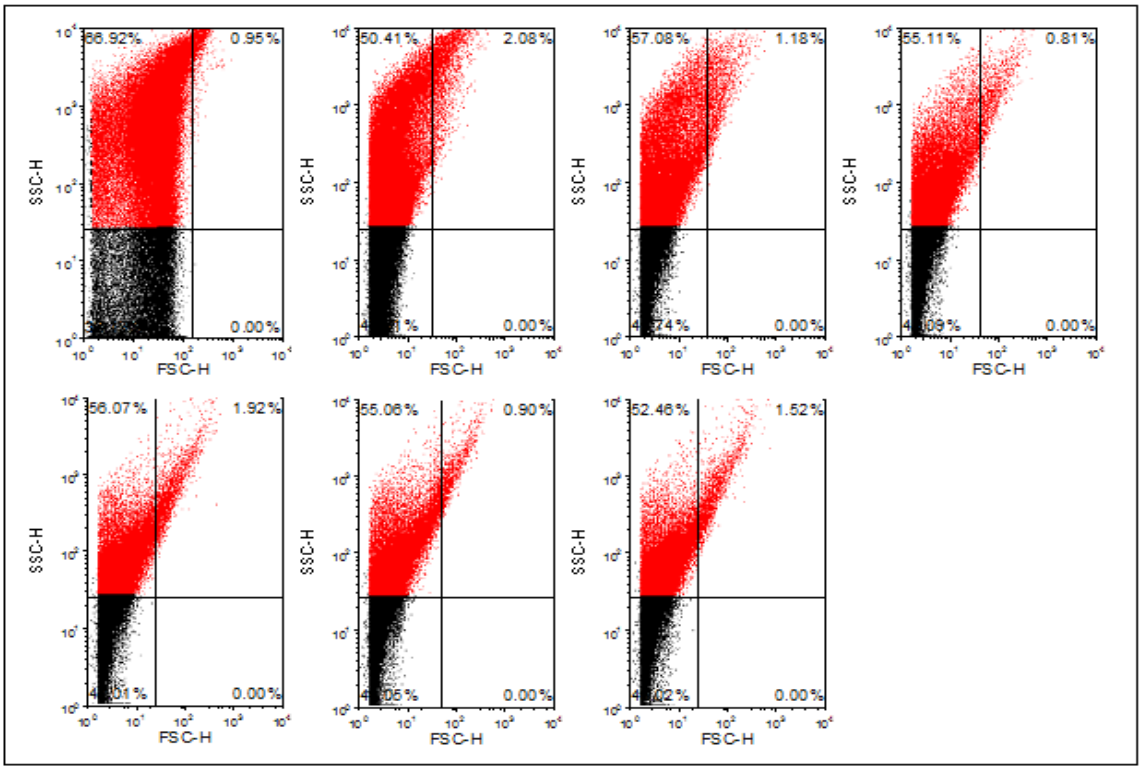

Figure 7: Real wastewater sample inoculated with increasing concentrations of PRD-Ag nanoparticles

From the real wastewater sample (Figure 7) it can be seen that the cell viability also decreased in the presence of the PRD-Ag nanoparticles. However, higher concentrations are required to perform in a similar effective manner as lower decrease of viable bacterial strains were recorded from 66.92% (12.5 µg/mL) to only 52.46% (800 µg/mL). This effect is also attributed to the adaptation capability developed by microorganisms in the harsh environment of wastewater compared to the commercial strains grown in the laboratory.

4 CONCLUSION

In conclusion, the smaller silver nanoparticles have exhibited potent effects against the tested bacteria and fungi. The efficacy decreased as the reaction time increased, since larger particles were formed leading to smaller accessible surface areas. This shape-dependent antimicrobial interaction correlated with studies done by Pal et al (2007) and Raza and co-workers (2016).

The nanostructured polymer coated with silver nanoparticles appeared to be an attractive material for the inhibition of bacteria as well as fungi. The preliminary investigations of the silver-based nanoparticles provided useful information on the inhibitory mechanism of silver and the size of the particle that possesses different levels of inhibition. It is suggested that the increased antimicrobial effect is due to enhanced potency because of the synergistic effect of silver NPs and Ag-PRD complex and the polyrhodanine nanofiber (Kong & Jang, 2009). The Ag NPs exhibited high antifungal properties, which could be very useful, especially against fungal strains resistant to conventional antimicrobial compounds.

Nanoparticles were found to have a major impact in wastewater microbial purification and the practical application is also supported from the findings of the flow cytometry analyses. Additionally, flow cytometry results are also typical of a real-time analysis as microbial detection times were decreased substantially. From all bacterial strains tested, Gram-negative bacteria reacted to lower antimicrobial concentrations, however *S. aureus* was overall the most susceptible to the PRD-Ag complex.

In this study the polyrhodanine-silver complex was the best antimicrobial compound achieving more than 95 % bacterial growth inhibition; this results shows a considerable improvement over other antimicrobial compounds previously tested. Commonly used NPs include modified titanium dioxide complexes that exhibit up to 60 % bacterial growth inhibition (Ungureanu et al., 2016), zinc oxide achieved approximately 80 % yeast growth inhibition (Kasemets et al., 2009) and 90-95% bacterial inhibition in the presence of gold nanoparticles. The latter is, however, extremely expensive and cannot be practically implemented or applied as an antimicrobial agent (Dijaz et al., 2014).

REFERENCES

[1] Akpor, O.B. & Munchie, M. 2011. Environmental and public health implications of wastewater quality. African Journal of Biotechnology, 10:2379-2387.

[2] Baryshnikov, G.V., Minaev, B.F., Minaeva, V.A. & Podgornaya, A.T. 2012. Theoretical study of the dimerization of rhodanine in various tautomeric forms. Chemistry of Heterocyclic Compounds, 10:1532-1547.

[3] Beyth, N., Houri-Haddad, Y., Domb, A., Khan, W. & Hazan, R. 2015. Alternative antimicrobial approach: nano-antimicrobial materials. Evidence-based complementary and alternative medicine, 2015:1-16.

[4] Cortezzo, D.E., Koziol-Dube, K., Setlow, B. & Setlow, P. 2004. Treatment with oxidizing agents damage their inner membrane of spores of Bacillus subtilis and sensitizes spores to subsequent stress. Journal of Applied Microbilogy, 97:838-852.

[5] Dijaz, S.M., Lotfipour, F., Jalali, M.B., Zarrintan, M.H. & Adibka, K. 2014. Antimicrobial activity of the metals and metal oxide nanoparticles, 44:278-284.

[6] Franci, G., Falanga, A., Galdeiro, S., Palomba, L., Rai, M., Morelli, G. & Galdiero, M. 2015. Silver nanoparticles as potential antibacterial agents. *Molecules*. 20:8856-8874.

[7] Gniadek, A. 2012. Cytotoxicity of Aspergillus fungi as a potential infectious threat. Department of Medical and Environmental Nursing, Faculty of Health Sciences, Jagielloniqan University Medical College, Krakow, Poland.

[8] Hammes, F., Berney, M., Wang, Y., Vital, M., Koster, O. & Egli, T. 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research, 42:269-277.

[9] Hanjra, M.A. & Qureshi, M.E. 2010. Global water crisis and future food security in an era of climate change. Food Policy, 35:365-377.

[10] Kasemets, K., Ivask, A., Dubourguier, H.C. & Kahru, A. 2009. Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast Saccharomyces cerevisiae. Toxicity in Vitro, 23:1116-1122.

[11] Kim, K.J., Sung, W.S., Moon, S.K., Choi, J.S., Kim, J.G. & Lee, D.G. 2008. Antifungal effect of silver nanoparticles on dermatophytes. Journal of Microbial Biotechnology, 18:1482-1484.

[12] Kong, H. & Sang, J. 2008. Synthesis and antimicrobial properties of novel silver/polyrhodanine nanofibers. Biomacromolecules, 9:2677-2681.

[13] Kong, H., Song, J. & Jang, J. 2009. One-step preparation of antimicrobial polyrhodanine nanotubes with silver nanoparticles. Macromolecular Rapid Communications, 30:1350-1355.

[14] Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramirez, J.T. & Yacaman, M.J. 2005. The bacterial effect of silver nanoparticles. Nanotechnology, 16:2346-2353.

[15] Pal, S., Tak, Y.K., Song, J.M. 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium Escherichia coli. Applied Environmental Microbiology, 73:1712-1720.

[16] Rai, M., Yadav, A. & Gade, A. 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances, 27: 76-83.

[17] Raza, M.A., Kanwal, Z., Rauf, A., Sabri, A.N., Riaz, S. & Naseem, S. 2016. Size- and shape-dependent studies of silver nanoparticles synthesized by wet chemical routes. Nanomaterials, 6:1-15.

[18] Rice, L.B. 2010. Progress and Challenges in Implementing the Research on ESKAPE Pathogens. *Infection Control & Hospital Epidemiology*, 31:7-10.

[19] Singh, V. & Tiwari, A. 2015. Evaluating the antimicrobial efficacy of chemically synthesized silver nanoparticles. International Journal of Current Microbiology and Applied Sciences, 4:5-10.

[20] Sheehy, K., Casey, A., Murphy, A. & Chambers, G. 2014. Antimicrobial properties of Nanosilver: a cautionary approach to ionic interference. Journal of Colloid and Interface Science, 443:56-64.

[21] Sondi, I. & Salopek-Sondi, B. 2004. Silver nanoparticles as antimicrobial agent: a case study on E.coli as a model for Gram-negative bacteria. Journal of Colloid and Interface Science, 275:177-182.

[22] Song, J., Kong, H. & Jang, J. 2011. Adsorption of heavy metal ions from aqueous solution by Polyrhodanine-encapsulated magnetic nanoparticles. Journal of colloid and interface science, 359:505-511.

[23] Swoboda-Kopec, E., Wroblewska, M.M., Rokosz, A. & Luczak, M. 2002. Mixed bloodstream infection with Staphylococcus aureus and Penicillium chrysogenum in an immunocompromised patient: case report and review of the literature. Clinical Microbiology of Infections, 9:1116-1117.

[24] Tiwari, D.K., Behari, J. & Sen, P. 2008. Application of nanoparticles in waste water treatment. World Applied Sciences Journal, 3:417-433.

[25] UNDP. 2011. Human development report 2011, UNDP, New York.

[26] Ungureanu, C., Dumitriu, C., Popescu, S., Enculescu, M., Tofan, V., Popescu, M. & Pirvu, C. 2016. Enhancing antimicrobial activity of TiO2/Ti by torularhodin bioinspired surface modification. Bioelectrochemistry, 107:14-24.

[27] Zarei, M., Jannejad, A. & Khajehali, E. 2014. Antibacterial effect of silver nanoparticles against four foodborne pathogens. Jundishapur Journal of Microbiology, 7:1-4.