Original Article

Nickel Nanoparticles/Recycled Polyethylene Terephthalate Nanofibers Reduce *AlgD* Expression in *Pseudomonas aeruginosa*

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ABSTRACT

Background and objectives: Recycled polyethylene terephthalate (RPET) nanofibers have become an important part of human life, with a continuous increase in their production and consumption. Herein, the antibacterial activity of nickel nanoparticles/recycled polyethylene terephthalate nanofibers (NiNP/RPET NF web) was evaluated by analyzing alginate expression in *Pseudomonas aeruginosa*, as an opportunistic microorganism.

Methods: NiNPs were synthesized and NiNP/RPET NF was produced by adding 25 µg/ml of NiNP to 10% solutions of RPET at a weight ratio of 3%. After exposing *P. aeruginosa* (PA01) to NiNP/RPET NF, the biofilm-forming capacity was determined and real-time PCR was performed to measure *algD* expression.

Results: Treatment with 25 µg/ml of NiNP/RPET NF reduced growth of *P. aeruginosa* on Mueller Hinton agar but did not result in complete inhibition. The biofilm optical density (550 nm) was 0.464 ± 0.021 after treatment with NiNP/RPET NF and 0.082 ± 0.011 in the absence of NiNP/RPET NF. This indicates the significant reduction of biofilm formation after exposure to NiNP/RPET NF ($p=0.01$). In addition, a 0.6-fold ($p=0.03$) reduction in alginate expression was detected by real-time quantitative real-time PCR.

Conclusion: Our results indicate the potential of NiNP/RPET NF for application in nano-based antibacterial medical systems.

Keywords: Nickel nanoparticles, polyethylene terephthalate, recycling, *Pseudomonas aeruginosa*, Alginate.

INTRODUCTION

Chemically recycled polyethylene terephthalate (PET) can be utilized in the production of polyurethane, which has various applications in important industrial processes. Much attention has been paid to metal oxide nanoparticles (NPs) in recent years owing to their capabilities, e.g. antibacterial activity, in a variety of biomedical applications (1, 2). Recycled nickel NPs/PET nanofibers (NiNP/RPET NF) have multifunctional applications, and thus the production of antibacterial nanofibers is of interest today (1).

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, non-glucose fermenting, and biofilm-forming bacterium that is widespread in natural environments. The bacterium is responsible for a wide range of opportunistic and life-threatening infectious diseases in humans (3, 4). The mucoid phenotype of *P. aeruginosa* produces large amounts of alginic hexachloride exopolysaccharide known as alginate, which is encoded by the *algD* gene. Resistance to antibiotics subsequent to the formation of *P. aeruginosa* biofilm in the airways of patients with cystic fibrosis, ventilator-associated pneumonia, and chronic lung disease may prolong therapy, exacerbate clinical symptoms, and even lead to death (5, 6). Thus, a combination of anti-biofilm agents with traditional antibiotics may be a promising approach for controlling biofilm-associated infections by inducing microbial transition to the planktonic growth state (7, 8).

Recently, growing attention has been paid to metal oxide NPs as antibacterial agents. NiNPs are a special class of metal oxide NPs with unique magnetic properties and high biocompatibility (9, 10). The small size (<100 nm) and the high surface-to-volume ratio of metal NPs mediate their antibacterial properties by increasing membrane permeability to cytoplasmic leakage and generating reactive oxygen species-mediated cell damage (11). Given the favorable antibacterial and antiviral properties of NiNPs, they are suggested as antibacterial candidates for fighting infectious diseases (10, 12, 13).

Given the importance of biofilms in developing diseases and drug resistance, researchers are looking for effective ways to inhibit and prevent biofilm formation in nano-based medical systems (14). Despite the wide range applications of NiNPs, little is known about the mechanisms through which they inhibit *P. aeruginosa* biofilm formation. Therefore, the present study evaluated the role of NiNPs in inhibiting alginate production and its mucoid phenotype by assessing *algD* expression using real-time quantitative PCR.

MATERIALS AND METHODS

In this study, NiNPs were synthesized by normal chemical reduction of an aqueous solution containing Ni (II) sulfate hexahydrate ($NiSO_4.6H_2O$) in addition to sodium hydroxide (NaOH) at room temperature and in ambient atmosphere as formerly described (1). For the synthesis of NiNP/RPET NF, the RPET solution at a concentration of 10% was prepared by solving recycled PET chips in trifluoroacetic acid and dichloromethane with a weight ratio of 3:7. Subsequently, the NiNPs were integrated in the 10% solutions of RPET at a weight ratio of 3% (1).

P. aeruginosa PAO1, the most commonly used strain for research, came from various types of non-cystic fibrosis infections and was consistently maintained at -80 °C at the Biobank of the Digestive Disease Research Institute (15). The bacterial strain was later used to study growth under normal conditions and under the influence of NiNP/RPET NF. *P. aeruginosa* was first grown at 37 °C on blood agar. A colony was sub-cultured in 10 ml of tryptone soybean broth (TSB), and then pure microbial counts were determined by measuring the turbidity of the TSB suspension via a spectrophotometer against the known McFarland turbidity standard. A bacterial suspension equivalent to 0.5 McFarland turbidity standard was used for antimicrobial sensitivity testing. The PET film was absorbed by low-concentration (25 µg/ml) NiNP, sterilized under ultraviolet light (15 min on each side), added to 2 ml of *P. aeruginosa* bacterial suspension, and finally incubated at 37 °C for nine hours. An untreated bacterial sample was used as negative control. Subsequently, 2 ml of the aliquots of the cultures were stored at -80 °C for RNA extraction. Then, a loop of grown bacteria was streaked on fresh Mueller Hinton agar, and the bacteria were incubated for 24 hours. The experiments were performed in triplicate with separately cultured bacteria. Biofilm assay was performed as described previously (16, 17). Briefly, *P. aeruginosa* bacterial suspension in the presence and absence of NiNP/RPET NF was diluted by 1:100 in fresh medium. Next,

200 µl of the dilution were added to wells of a 96-well microtiter plate. After 24 hours of incubation at 37 °C, the content of the wells were aspirated, and each well was washed slowly with 200 µl of dH_2O to avoid interrupting the biofilm. To stain the biomass, the microtiter plate was incubated at 37 °C for 15 min, air-dried for 15 min, and then stained with 1% crystal violet for 25 min. Next, the wells were washed with water and solubilized with 250 µl of 30% glacial acetic acid for 15 min.

The mean absorbance at 550 nm was measured for each well in triplicate compared with absorbance of 30% acetic acid in water as the blank.

Total RNA was extracted from the grown bacteria on Mueller Hinton agar in the presence and absence of NiNP/RPET NF using TRIzol LS Reagent (Invitrogen, UK) according to the manufacturer's protocol. The RNA elutes were stored at -80 °C until analysis. DNase I treatment was performed on RNA extracts to remove residual DNA. To ensure the absence of contaminating genomic DNA in the extracts, PCR reactions were carried out using 16S rDNA bacterial primers according to a previous study (18). Subsequently, 1 µg of RNA was use for cDNA synthesis using the miScript SYBR Green PCR Kit (Qiagen, Germany) according to the manufacturer's protocol. The quantity and quality of RNA were determined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The quantitative PCR reaction was performed in a LightCycler v 3.5 (Roche Applied Science, Mannheim, Germany) using the SYBR Green PCR kit (Qiagen, Germany). The reaction mixture contained 1X Q-PCR master mixer and 5 µM of primers for the alginate gene (18). The cycling conditions consisted of 5 min of initial denaturation at 97 °C, followed by 40 cycles of denaturation at 97 °C for 10 sec, annealing at 60 °C for 20 sec, extension at 60 °C with a transition speed of 3 °C for 10 sec, and final extension at 74 °C for 20 sec. Each sample was analyzed in triplicate and 16R rRNA was used as the reference gene. Fold changes in gene expression were calculated with cycle threshold values (Ct) and evaluated by the $2^{-\Delta Ct}$ method (19, 20).

All experiments were performed at the Digestive Disease Research Institute, Shariati Hospital, Tehran University of Medical Sciences between March 2019 and December 2019. The data are represented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for data analysis, and p-values less than 0.05 were considered statistically significant.

RESULTS

The formed NPs had a relatively uniform diameter (approximately 50 nm) with a spherical shape (Figure 1).

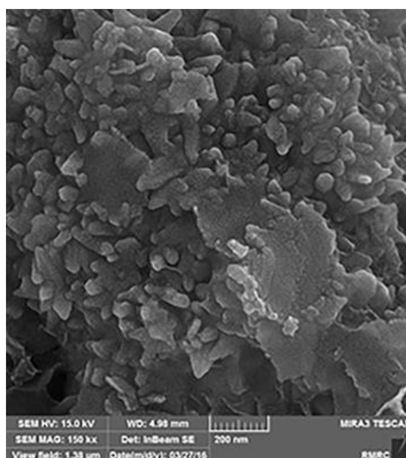

Figure 1- FESEM image of NiNPs prepared by the chemical reduction method

Growth of *P. aeruginosa* in TSB with and without exposure to 25 µg/ml of NiNP/RPET NF was evaluated by subculture on Mueller Hinton agar. Exposure to NiNP/RPET NF decreased the growth of *P. aeruginosa* but did not result in complete inhibition of bacterial growth. The biofilm optical density (550 nm) was 0.464 ± 0.021 after treatment with NiNP/RPET NF and 0.082± 0.011 in the absence of NiNP/RPET NF. This indicates the significant reduction of biofilm formation after exposure to NiNP/RPET NF (p=0.01). Figure 2 illustrates the antibacterial effect of NiNPs on the growth of *P. aeruginosa* after 48 hours.

Figure 2- The growth of *P. aeruginosa* under normal growth conditions (left) and in the presence of NiNP/RPET NF (right) after 48 hours

The expression of *algD* in *P. aeruginosa* was measured with real-time quantitative RT-PCR in normal growth conditions and after treatment with NiNP/RPET NF.
The 16S rRNA gene was used as the internal control due to its persisting levels in normal growth conditions. A 0.6-fold reduction (p=0.03) in *algD* expression was observed after treatment with 25 µg/ml of NiNP (Figure 3).

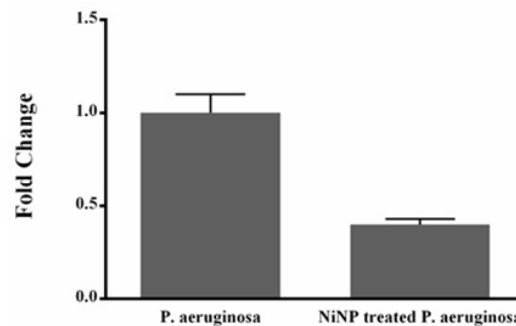

Figure 3- Fold change of alginate expression in *P. aeruginosa* under normal growth conditions and in the presence of NiNP / RPET NF

DISCUSSION

The present study evaluated the in vitro antibacterial activity of synthesized NiNPs against the Gram-negative bacterium *P. aeruginosa*. The results indicated that NiNPs decreased the biomass of *P. aeruginosa* biofilm and negatively affected alginate expression. This demonstrates the antimicrobial efficacy of NiNPs against *P. aeruginosa* by modulation of alginate and biofilm formation.

Given the importance of biofilm formation in several bacterial species including *P. aeruginosa*, researchers are seeking effective ways to inhibit biofilm formation. Current developments in nanotechnology enable the production of NPs that may help control drug-resistant microorganisms (21, 22). According to previous studies, NiNPs can be considered as suitable candidates for fighting infectious diseases (9, 23). However, little is known about the mechanism of effect of NiNPs on pathogenic bacteria. Preliminary data from the growth analysis suggest that NiNPs exert inhibitory effects on the growth of *P. aeruginosa*. Clinically, *P. aeruginosa* is a pathogen frequently isolated from infections related to cystic fibrosis, burns, and immunodeficiency disorders. The isolates of this bacterium are mucoid and express high levels of alginate, which is responsible for

biofilm formation. This arrangement is tolerant towards phagocytic cells and shows resistance to antibiotics (7).

In the present study, we observed a significant reduction in biofilm formation by *P. aeruginosa* after treatment with 25 µg/ml of NiNP on PET. Alginate is a large molecule that stimulates the architecture and progression of biofilm formation (24). Linker and Jones first reported that alginate is the polysaccharide secreted by the mucoid *P. aeruginosa* (25). Previous studies revealed the powerful antimicrobial effect of NiNPs compared to other metal NPs. Li et al. reported the potential antibacterial activities of NiNPs against *S. aureus*, *E. coli*, and *B. subtilis* (26). The antimicrobial efficacy of synthesized NiNPs against *P. aeruginosa* has been reported in recent studies (1, 27). It is recommended to investigate in vivo applications of NPs for the inhibition of antibiotic-resistant *P. aeruginosa* strains.

CONCLUSION

Herein, the growth and biofilm formation of *P. aeruginosa* in the presence of NiNP/RPET NF were evaluated. Our findings confirm that NiNP/RPET NF has an inhibitory effect on *P. aeruginosa* and alginate production, which may consequently reduce the mucoid status. Therefore, a combination of anti-biofilm agents with traditional antibiotics may be a promising approach for controlling biofilm-associated infections. Further studies are essential to confirm the inhibitory activity of nanofibers on *P. aeruginosa* in vivo.

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DECLARATIONS

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Ethics approvals and consent to participate

Not applicable.

Conflict of interest

The authors declare that there is no conflict of interest regarding publication of this article.

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