Title: The antimicrobial effect of copper nanoparticles

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The antimicrobial effect of copper nanoparticles

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Introduction

Nanoparticles (NPs) are small particles less than 100 nanometers in size. NPs range in size and the original martial from which they are made. The distinguishing feature of these particles is their ability to gain improved properties when compared to their bulk counterparts (the original material from which they were made)(Song & Kim, 2009). Commonly, NPs are synthesized using chemical methods, which are hazardous, however, biological methods that utilize enzymes(Willner, Baron, & Willner, 2006), microorganisms(Nair & Pradeep, 2002) or plants(Shankar, Rai, Ahmad, & Sastry, 2004) are being developed to replace chemical methods(Song & Kim, 2009). NPs offer a wide variety of applications in the medical field(Ghosh, Han, De, Kim, & Rotello, 2008), the textile industry(Sawhney et al., 2008), data storage(Hamann, O'Boyle, Martin, Rooks, & Wickramasinghe, 2006), and many others. The oil industry is one of the industries that might be greatly improved with the introduction and development of NP technology. The pipelines underwater seem to undergo biocorrosion, which is the deterioration of carbon steel pipelines as a result on biofilm formation. The key players that contribute the most to the corrosion of pipelines are sulfur reducing bacteria(SRB) (Akpan & Imuk). NPs present a plausible solution to prevent the biofilm formation.

Objectives

This experiment is designed to test the effectiveness of copper NPs as anti-biofilm agents. If these NPs prove effective, they will present a great alternative to coat carbon steel pipes that are used to transport oil underwater.

Materials

E. coli culture in lysogeny broth

6-well plate

Ethanol

Distilled water

Nanoparticle coated coupons (1)

Non-coated nickel coupons (2)

0.02% acridine orange

Spectrophotometer

Olympus BX51 Fluorescence microscopy, Japan

Methods

Bacterial subculture (*E. coli*) was prepared by inoculating a colony from a culture plate into a tube containing lysogeny broth (LB) overnight. Next day the bacterial culture was used to prepare 3 dilutions through serial dilution. Each dilution was measured using a spectrophotometer. The dilution that measured 1 OD (optical density) - which approximately harbors $1x10^9$ bacterial cells- was then diluted to achieve a bacterial concentration of $1x10^7$ bacterial cells per 4 mL liquid broth. The OD was calculated from the absorbance using the formula: (absorbance of sample- absorbance of blank)/path length. 3 nickel coupons were then cleaned with ethanol (to ensure sterility) and allowed to dry. The first 2 coupons were not coated with NPs, while one was coated with copper NPs. Once the coupons dried, 2

coupons were incubated with bacterial culture, one was coated with NPs while the other was not. The third coupon was incubated in distilled water to be used as a control for the experiment. The total incubation period was 4 hours. After 4 hours, the nickel coupons were washed with distilled water 3 times. The washing procedure was accomplished by dipping the coupons into a beaker of distilled water using forceps to ensure that only attached bacteria remain on the coupons. Following the washing procedure, the coupons were incubated with 0.02% acridine orange (AO) for 4 minutes and covered with aluminum foil to prevent the interference of the light with the dye's ability to stain the cells. After 4 minutes the coupons were washed 3 times with distilled water to ensure the removal of residual stain. The coupons were then viewed using upright Olympus BX51 fluorescence microscope. This microscope permits the use of magnifications of 10X, 20X, 40X, and 100X. A blue filter was used and five fields for each coupon were viewed at 100X and photos were captured. Bacterial cells in each field were counted for both the NP coated and non-coated nickel coupons.

Results

The results of this experiment were consistent with other experiments and support the proposed antibacterial attachment property of NPs(Ghasemian, Naghoni, Rahvar, Kialha, & Tabaraie, 2015). Figure 1 demonstrates the 3 nickel coupons viewed at 100X under the florescence microscope. Fig 1C represent a field from the non-coated control coupon. This coupon exhibits no bacterial growth. Fig 1A and 1B represent a selected field from the non-coated nickel coupon and the NP coated nickel coupon respectively. Both coupons seem to have bacterial growth. However, it is clear that there is a significant reduction in the number of bacterial cells in the NP coated coupons (B) compared to the non-coated coupons (A). It is worth to mention that the bacterial cells in the NP coated coupons had a consistent orange color (B). However, bacteria on non-coated coupons demonstrated a variety of colors ranging

from green to yellow to orange although both coupons were incubated under the same conditions.

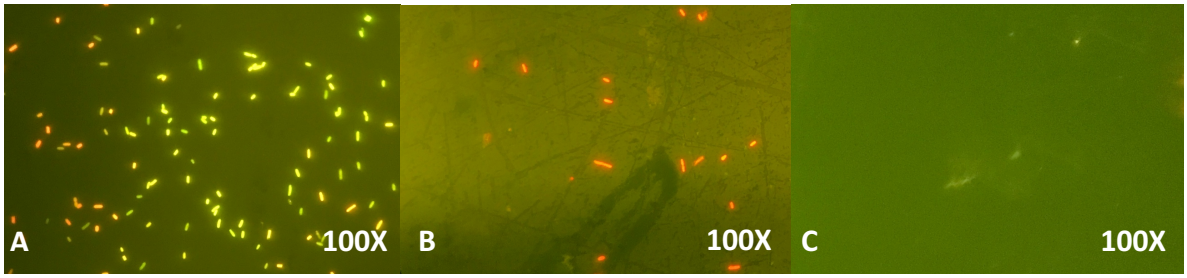

Figure 1: Bacterial cells attached to nickel coupons stained with acridine orange and viewed at 100X using fluorescence microscopy. The staining procedure used is reported in the methodology section. Considerable number of bacteria is present. **A**) Nickle coupon not coated with nanoparticles exhibited high bacterial attachment. **B**) Nickle coupon coated with nanoparticles exhibited a significantly reduced bacterial attachment. **C**) Nickle coupon used as a control. This coupon was not coated with nanoparticles and was not exposed to bacterial culture. Hence, no bacterial cells are visible. This figure clearly exhibits the effectiveness of nanoparticle to inhibit bacterial attachment.

Table 1 below presents the number of bacterial cells found in 10 randomly selected fields in the non-coated and the NP coated coupons. There's a large variation in the number of bacterial cells in each field in the non-coated coupons, however, in the NP coated coupons the number of bacterial cells are more coordinated. Moreover, there is a clear reduction in the number of bacterial cells in the NP coated coupons compared to the non-coated coupons. This difference in growth and attachment is demonstrated in figure 2.

Non-coated coupon	**Nanoparticles Coated coupon**
693	120
391	87
450	66
350	181
610	110
SD = 147	SD = 43
Average = 499	Average = 113

Table 1: The number of bacterial cells in 5 different fields from the non-coated (left) and NP coated (right) nickel coupons. The SD and average number were calculated for each coupon and were used to create figure 2. The cells were counted at a magnification of 100X using manual methods.

SD; standard deviation

Figure 2 below represents a graph that demonstrates the quantitative difference in bacterial growth between the non-coated coupons and the NP coated nickel coupons. The Non-coated nickel coupons demonstrate large bacterial population with a large standard deviation. NP coated nickel coupons on the other hand demonstrated lower bacterial growth and a lower standard deviation.

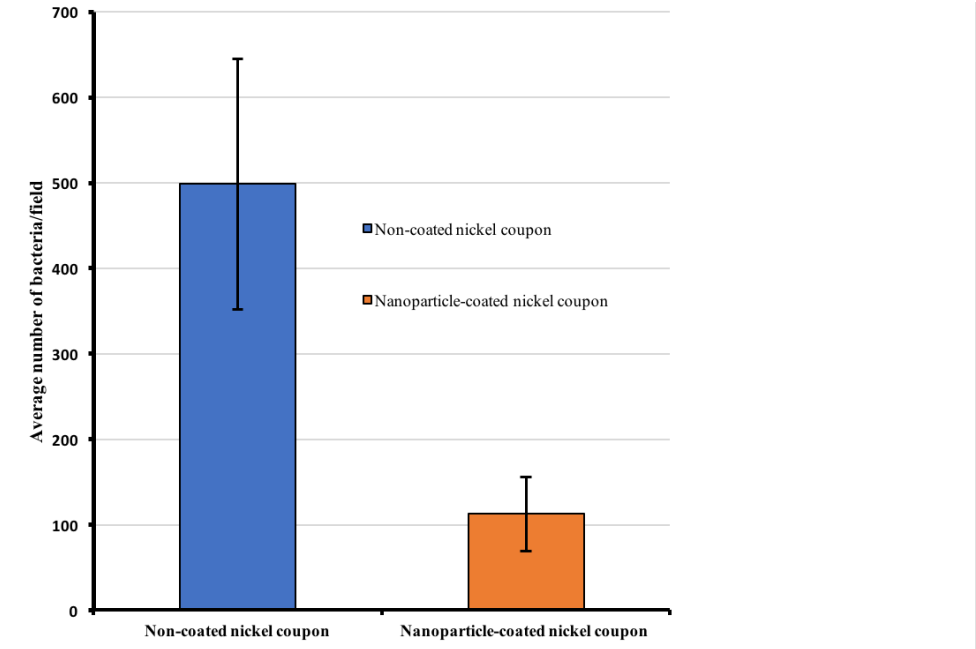

Figure 2: A graph exhibiting the average number of bacterial cell in nanoparticle coated nickel coupons and non-coated nickel coupons. The non-coated coupons (blue) exhibited significant bacterial growth and attachment. The nanoparticle coated coupon (orange) on the other hand exhibited a significantly reduced bacterial growth and attachment.

Discussion

The difference in bacterial burden between the non-coated nickel coupon and the NP coated nickel coupon is apparent qualitatively through figure 1, and quantitatively through figure 2. The bacterial number is greatly reduced in the NP coated coupon compared to the non-coated

coupon. This can be attributed to the effective properties of NPs as antimicrobial agents. The exact mechanism is not clearly understood for how NPs interfere with bacterial growth. However, hypotheses suggest that NPs cause their antibacterial effect by directly damaging the bacterial cell (mechanical damage), creating ROS groups which damages the bacterial cells, or the toxic effects of metal ions on the bacterial cells(Stankic, Suman, Haque, & Vidic, 2016). The image in this experiment were taken by a mobile phone, hence, manual quantification of the bacterial cells was mandatory. However, it is recommended to use a software like ImageJ to obtain better images and to be able to quantitate the bacterial cells more accurately. In addition, bacterial cells in the non-coated coupon exhibited two populations, a green/yellow population and an orange population. The NP coated coupon however have a homogenous population of orange bacterial cells. This difference in the color is a result of AO's staining properties. AO stains double stranded DNA green/yellow (excitation at 502nm and emission at 530nm), whereas it stains single stranded DNA & RNA orange (excitation at 460 and emission at 640). Orange color can indicate viable cells, as living cells are rich in RNA, or cells that senescent and their DNA has degraded to produce single stranded DNA. Green/yellow color on the other hand indicates that cells are intact and rich in DNA(Mason & Lloyd, 1997). Acridine Orange is also affected by the intracellular pH, it is orange in low pH, and green/yellow in high pH(Kasibhatla et al., 2006). The variability of AO's staining properties makes it difficult to interpret the exact cause of the different populations observed in this experiment, therefore a confirmatory procedure is required. However, it is likely that the orange cells seen in the NP coated coupon represent inactive bacterial cells that are being degraded.

It is worth to mention that the standard deviation of the fields counted in the non-coated coupon is very high compared to that of the NPs coated fields counted. This indicates uneven

bacterial distribution in the non-coated coupon which can be a result of improper washing technique.

This experiment is suggestive for the effectiveness of NPs as antimicrobial agents. However, improvements can be made to ensure that the antibacterial activity is only caused by NPs. Testing NPs effect on carbon steel coupons is also recommended to ensure that these particles are effective in the protection of oil pipelines. Moreover, different types of bacterial cultures and species should be testes with focus on SRB which are major contributors to biocorrosion. Also, incubation conditions should be manipulated to mimic the adverse conditions in which underwater pipeline are exposed to determine whether NPs can withstand and remain effective under such conditions.

Conclusion

The results of this experiment demonstrate the antibacterial attachment properties of copper NPs. NPs provide an effective solution for the biofilm dilemma in underwater oil pipes. However, further testing is required to determine the safety of these NPs on marine life and their effectiveness in preventing the attachment of other bacterial types than *E. coli*.

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