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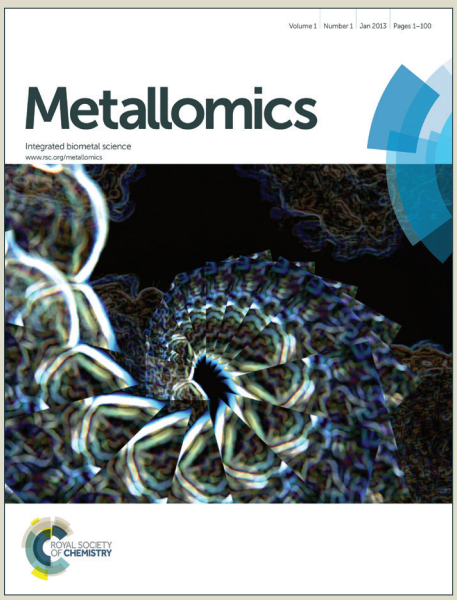

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Title

Inclusion bodies and pH lowering: as an effect of gold nanoparticles in *Streptococcus pneumoniae*.

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Abstract

Streptococcus pneumoniae is a human pathogen whose principal virulence factor is its capsule. This structure allows the bacteria to evade the human immune system. The treatment of infections caused by this bacterium is based on antibiotics, however the emergence of antibiotic resistant strains makes this task increasingly difficult. Therefore, is necessary to investigate new therapies such as those based on gold nanoparticles; unfortunately the mechanisms involved have not been investigated yet. As far as we know, this study is the first approach, which attempt to explain how gold nanoparticles destroy the bacterium *Streptococcus pneumoniae*. We found that the particle mean size was an important issue, and the effect on the bacteria was dose-dependent. The cellular growth was inhibited by the presence of the nanoparticles, and also the viability. The pH of the media was acidified, but interestingly the reactive species were not affected. The analysis of transmission electron microscopy, revealed a inclusion bodies of gold

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nanoparticles the bacteria composed by nanoparticles. We presented the first findings that attempt to explain how gold nanoparticles lyse the Gram positive bacteria.

Keywords: *Streptococcus pneumoniae*; AuNPs; Antibacterial properties; Capsule; Reactive oxygen species; inclusion bodies of gold nanoparticles.

Introduction

Streptococcus pneumoniae is an encapsulated Gram positive coccus bacterium, that is spherical in shape with diameters between 1-2 μm termed coccus.[1] *S. pneumoniae* is common in the respiratory tract of human.[2-4] This bacterium is an opportunist pathogen which infects nasopharynx and lung, causing sinusitis or pneumonia. This coccus can reach the blood system and could produce septicaemia that is lethal whether is not treated properly.[5] The successful invasiveness of this bacterium is mainly due to the capsule.[6-9] In fact this structure, mainly composed by carbohydrates, is considered one of the most important virulence factors developed by this pathogen because it allows *S. pneumoniae* to evade the immune system of the host.[10-12] Antibiotics such as penicillin, erythromycin, cephalosporins, rifampin, vancomycin and trimethoprim-sulfamethoxazole are the mainstay treatment for infections caused by *S. pneumonia*e.[13-15] Until the 1970s all the isolates were sensitive to antibiotics, however, in the 1990s the sensitivity diminished by a considerable amount. Therefore, is imperative to find new therapies for the treatment of infections caused

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by this pathogen, a good alternative for this purpose, could be the usage of gold nanoparticles (AuNPs). AuNPs have shown a good antibacterial effect when they are tested in growth media.[16,17] *Escherichia coli*,[18] *Staphylococcus aureus, Citrobacter koseri, Bacillus cereus*, *Pseudomonas aeruginosa*,[19] *Bacillus subtilis*, *Klebsiella pneumoniae*,[20] *Salmonella typhi*,[21] all of them are bacteria, which are sensible to the presence of AuNPs in the cellular growth. The average diameter of the AuNPs, which has been tested is between 5-50 nm.[22-24] Nevertheless, the major effectiveness was observed with sizes around 20 nm.[25,26] The minimal inhibitory concentration (MIC) is 2,500 μg/ml with a size of 13 nm Fe_3O_4@Au for *Pseudomonas aeruginosa*[19] and 5,000 μg /ml with a size of 22 nm AuNPs for *E. coli* showing better antibacterial activity for Gram positive than Gram negative bacteria.[27] One advantage of using AuNPs is the fine-tuning antimicrobial profile because the surface of AuNPs can be functionalized sequentially using polyoxometalates and lysine.[28] In fact the nanoparticles can be functionalized with antibodies against a specific target in order to diminished the effective antibacterial concentration. The mechanism by which the AuNPs destroy the bacteria is not clear yet, however, several mechanisms have been proposed, for instance, in *E. coli* the AuNPs could bind to the bacterial membrane, causing a collapse in its potential, then, the ATPase activity is inhibited and therefore the ATP levels decrease. Interestingly, in these experiments reactive oxygen species (ROS) were not detected.[16,29,30] Another mechanism proposed is the alteration of pH. It has been suggested that the NPs could modify the pH of bacterial environment in such a way that when the pH is acidified the environment becomes toxic for bacteria.[31] Additionally the intracellular pH could be modified as well.[32] All these mechanisms

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have been proposed for *E. coli,* a bacilli Gram negative without capsule. Nonetheless, for *S. pneumoniae,* a Gram positive encapsulated pathogen, the mechanism has not been investigated yet. In the present study we attempt to investigate whether the antibacterial properties of the AuNPs inhibit the cellular growth of a bacterium whose principal virulence factor is the capsule. Therefore, the AuNPs effect is analysed in a mutant bacterium (non-capsulated), which is compared with the wild type bacterium (capsulated).

Experimental

Cellular cultures

S. pneumoniae R6 (ATCC BAA-255) and TIGR4 (ATCC BAA-334) strains were cultivated for 18 h, at 37ºC and 10% CO_2 on Todd Hewitt agar. There after, bacteria were inoculated in Todd Hewitt broth supplemented with 0.5% of yeast extract and incubated for 18 h 10% CO_2 at 37º C.

Gold nanoparticles synthesis (AuNPs)

Gold nanoparticles were synthetized using 10 ml of 1 mM chloroaudric acid ($HAuCl_4$) were boiled. When the ebullition temperature was reached, 18 ml of 38.88 M sodium citrate dehydrated ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$) were added and the mix was boiled for 15 min. Thus, the solution was maintained at room temperature for 2 h. Three washes with water milliQ (centrifuging at 23,143 x g) were performed in order to eliminate traces of $HAuCl_4$ and citrate. After that, gold nanoparticles were covered with polyethylene glycol (PEG, Molecular weight

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3,400). 0.5 mg/ml were mixed with in a shaking grate. The mix was maintained in agitation for 2 h at room temperature and washed with water (milliQ) and centrifuged (23,143 x g)[33]. In order to corroborate the results all the experiments were performed using nanoparticles (20 nm) purchased from Sigma (catalog number 765716) obtaining also similar results.

Determination of the weight

In order to determine the yield after synthesis, empty tubes were weighed. Then 1 ml of AuNPs pegylated was added and centrifuged at 23,143 x g, the supernatant was eliminated and tubes were dried for 3 days at room temperature, and they were weighed again to obtain the dry weight. All the experiments were performed by triplicate in three occasions.

Inhibition of cellular growth

In order to investigate the inhibition of cellular growth, the bacteria (R6, avirulent)[34] and TIGR 4 (virulent serotype 4)[35] were cultivated in Mueller-hinton media supplemented with 5% sheep blood, then the optical density was adjust to 0.5. After that, 100 µl of AuNPs were added. The concentration tested (20 nm ± 0.9) were 1024, 512, 256 and 128 µg/ml. Thereafter, the bacteria cultures were incubated at 37º C and 10% CO_2. Cellular growth was determined each hour by spectrophotometry at 600 nm.

Transmission electronic microscopy (TEM)

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Bacteria were cultivated in Todd Hewitt broth and were tested with (20 ± 0.9 nm) 512 µg/ml of AuNPs. Media growth was eliminated by centrifugation at 7000 x g for 10 min. Bacteria were fixed with 2% formaldehyde for 1 h. A washed was performed with PBS buffer (10 mM). Then, samples were fixed with a pH 7.3 buffer solution containing 2.5% glutaraldehyde and 4% formaldehyde. Three washes were performed with PBS solution at room temperature for 5 min. After that, the contrast was performed for 1.5 h using a mix of 2% osmium tetroxide and 4% uranyl acetate (1:1). Thus three washes were performed with PBS. Samples were dehydrated with ethanol using concentrations of 30, 50, 70, 80 and 96% changing each 15 min and 2 changes with ethanol absolute for 30 min were performed. These samples were tested with propylen oxide in two occasions for 30 min. The slides were performed in a propylen oxide-resin (1:1) for 5 h (1:2) overnight, thereafter epoxy resin was added at 60º C for 24 h. The TEM observation was performed at 60 and 80 KV.

Determination of pH in the bacterial culture

To determine the pH during the incubation of *S. pneumoniae* with AuNPs, the bacteria was cultivated in 10 ml of Todd Hewitt broth until reach 0.5 of optical density at 600 nm. Then, 1024, 512 and 256 µg/ml AuNPs were added, creating different batches and the pH was determined each hour using a pH meter. As a control: Todd Hewitt broth with or without AuNPs, bacteria without AuNPs. All the conditions tested were incubated at 37º C, 10% CO_2. The pH was determined each h.

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Reactive oxygen species

Free radicals were determined using the Red hydrogen peroxide assay kit for microplates ENZO**.** A standard curve was prepared using 3.33, 1.11, 0.37, 0.12, 0.04, 0.01 μM H_2O_2, which were performed by serial dilutions of a stock solution 10 μM H_2O_2**.** Samples were tested in the same way and all were determined by spectrophotometry at 576 nm.

Results

Gold nanoparticles synthetized (AuNPs) are spherical

In order to obtain the gold nanoparticles system used throughout this study, we performed several synthesis based on the well-known Turkevich method.[33] This protocol allows us to obtain near spherical AuNPs with good control over their average diameters. Sets of different sizes were tested in a previous experiment, where the best results were obtained with nanoparticles of 20 ± 0.9 nm. For that reason all the subsequent experiments were performed using nanoparticles of this size (Fig. 1).

AuNPs has antibacterial properties over capsulated bacteria

AuNPs have shown a good antibacterial activity when they are tested in Gram negative bacteria however, the mechanism responsible for that behaviour is poorly understood. In fact, for pathogen bacteria such as *S. pneumoniae* (an encapsulated pathogen) the antibacterial properties of AuNPs remain unknown. In order to investigate the antibacterial properties of this nanoparticles, the bacterium *S. pneumoniae* was growth in Todd Hewitt broth. When the bacterial culture

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reached an optical density (at 595 nm) of 0.15, different concentrations of AuNPs ranging from 128 up to 1024 µg/ml were added. All the bacterial cultures were followed for 22 h taking samples each 2 h. The inhibitory effect observed was dose-dependent, because the major effect is observed with concentrations of 1024 (●) or 512 µg of AuNPs (■) (Fig. 2). The concentrations of 256 (▲), or 128 (▼) showed a result close to that of the control (without AuNPs) (♦) (Fig. 2). Surprisingly, the antibacterial effect was similar for both *S. pneumoniae* TIGR4 strain (wild type) (Fig. 2B) and *S. pneumoniae* R6 strain (a mutant non-capsulated) (Fig. 2A**).** This result shows that the AuNPs have antibacterial properties over bacteria regardless they are capsulated or not.

AuNPs inhibits the viability of *S. pneumoniae*

The fact that AuNPs inhibited the cellular growth of *S. pneumoniae* suggests that AuNPs could affect the viability. In order to explore this assumption, *S. pneumoniae* was growth in Todd Hewitt broth and concentrations of 256, 512 and 1024 µg/ml were added. Samples were collected each 2 h and the viability was determined. The results show that the viability was affected when the Todd Hewitt broth was supplemented with 512 and 1024 µg/ml of AuNPs (Fig. 3). These results clearly show that the AuNPs inhibit the cellular growth and the viability of *S. pneumoniae*.

AuNPs diminished the extracellular pH of *S. pneumoniae*

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Since AuNPs inhibited the cellular growth and the viability, we decided to investigate the molecular mechanism behind this effect. As it was previously mentioned, one of the mechanisms proposed in the literature for antibacterial effect of nanoparticles was the lowering in pH values. In order to explore this assumption, the pH of the culture media was determined during the cellular growth of *S. pneumoniae*. The results clearly show that the pH was acidified for more than 1 unit when AuNPs were added (Fig. 4). At the beginning of the experiment the pH was 8.8 and after 11 h of cultivation it dropped up to 7.2. The controls tested Todd Hewitt broth, inoculated or not, showed an acidification of only 0.4 units of pH. In order to investigate whether or not the acidification is due to bacterial lysis, we determined the pH during the cellular growth, previous to bacterial lysis induced by sonication without AuNPs (Fig. 4). This control showed a pH of 8.7 after 11 h of incubation without nanoparticles (Fig. 4). Our overall results, show that the acidification observed is due to the presence of AuNPs in the media and perhaps it helps to destroy the bacteria.

AuNPs did not modify the reactive oxygen species (ROS) yielded by *S. pneumoniae*

The acidification of the extracellular media was established as an effect related to the addition of AuNP's to the culture media. We also searched for a variation in other relevant parameters such us ROS. *S. pneumoniae* was cultivated in Todd Hewitt broth supplemented with AuNPs, samples were taken each hour, and the ROS were quantified by ENZO kit. The results did not show any important increase in the ROS production when *S. pneumoniae* was cultivated in presence of 512

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μg/ml AuNPs in comparison with the strains without nanoparticles (Fig. 5). These results suggest that the presence of AuNPs does not change the reactive oxygen species in the culture media.

AuNPs crosses the capsule of *S. pneumoniae*

The acidification of growth media caused by AuNPs, could be by direct contact of AuNPs with the bacterium. In order to explore more, the bacterium *S. pneumoniae* previously cultivated for 3 h in presence of AuNPs, was observed by TEM and several slices were performed. At the beginning the nanoparticles are observed surrounding the bacterium (Fig. 6B). Then the nanoparticles are grouped in a specific point of the bacterium (Fig. 6C), and later they are introduced into the cell, forming a structure termed by us inclusion bodies of gold nanoparticles (Fig. 6D), Finally, cells are lysed (Fig. 6E). Same results were obtained when the experiments were performed with TIRG 4 (a capsulated strain) (Fig. 6A´,B´, C´, D´ and E´). We believe that those inclusion bodies of gold nanoparticles are composed for a membrane because it has a spherical form whose size is close to 130 nm. Our overall results show that the AuNPs have an antibacterial effect, able to abate the viability of *S. pneumoniae*, in which, the nanoparticle can cross the capsule membrane, cellular wall, reaching the cytoplasm, in a structure composed perhaps by lipids. When this mechanism occurs, several cellular processes are modified, producing acidification of the extracellular environment and finally leading to cellular death.

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Discussion

AuNPs have a great potential as antibacterial agent, some of their properties have been studied in Gram negative bacteria such as *E. coli*. However, for many Gram positive bacteria, their effect is practically unknown. In fact, *S. pneumoniae* is a pathogen in which the antibacterial effect of AuNPs, have not been previously investigated. In this work, we study the antibacterial effect of AuNPs in *S. pneumoniae*. We present TEM images showing the ability of the nanovehicles to traverse the capsule in the TIGR 4 strain, this capsule helps *S. pneumoniae* to evade the immune system of the host. The methodology used to obtain AuNPs allows us to obtain a size of 20 nm, which showed an excellent antibacterial effect, this result was in agreement with some other studies found in the literature for *E. coli* where the optimum size of nanoparticles was the same diameter.[36,37] The antibacterial effect of AuNPs also was dose-dependent, because, when nanoparticles concentration was increased up to 512 or 1024 µg/ml, the cellular growth was abated. The cellular death was corroborated by determination of the viability, which decreased in presence of AuNPs, in opposition with the controls in which, the units forming colony (CFU) were 1 X 10^5. These results clearly showed that AuNPs are an excellent alternative to eliminate *S. pneumoniae.* The presence of nanoparticles no increased the ROS levels indicating that AuNPs kill to *S. pneumoniae* by a mechanism different from ROS production. The cellular death of *S. pneumoniae* could be related to a decrement in pH values, Perhaps, the increasing of H^+ destabilizes the membrane and the capsule of this pathogen, forming pores that allow AuNPs to enter. TEM images displayed different moments

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of AuNP's interaction with the bacteria and could lead to a possible explanation of the underlying mechanism. We believe that the bacteria could attract the AuNPs, by electrostatic forces, thereafter, the NPs bind the bacterium and later they are internalised into the cytoplasm via a structure termed inclusion bodies of gold nanoparticles composed principally by lipids and/or carbohydrates, because, the structure is spherical. In the bacteria this structure induces damage and finally the bacterium is lysed. In conclusion our findings attempt to explain the mechanism by which the AuNPs inhibit the cellular growth and the viability of *S. pneumoniae.* This mechanism is based in pH lowering and formation of inclusion bodies. We believe that these studies are necessary because in the future nanotechnology is visualized as an alternative therapy to the use of antibiotics therefore we need known the mechanism of gold nanoparticles.

Acknowledgments

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Figure legends

Fig. 1 Transmission Electron Microscopy (TEM) of AuNPs.

The AuNPs reached an average size of 20 nm ± 0.9.

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Fig. 2 AuNPs inhibit the cellular growth at concentrations of 512 and 1024 µg/ml. R6 (A) and TIGR (B) strains were cultivated until reach an optical density of approximately 0.2. There after the AuNPs were added at concentrations of 128, 256, 512 and 1024 µg/ml, then the optical density was measured each 2 h in order to determine the cellular growth.

Fig. 3 AuNPs inhibit the viability at concentrations of 512 and 1024 µg/ml. R6 (A) and TIGR (B) strains were cultivated until reach an optical density approximately of 0.2. Thereafter the AuNPs were added at concentrations of 128, 256, 512 and 1024 µg/ml, then the CFU were calculated each 2 h in order to determine viability.

Fig. 4 AuNPs diminished the pH in the culture media. R6 (A) and TIGR (B) strains were cultivated until reach an optical density of approximately 0.2. There after the AuNPs were added at concentrations of 512 and 1024 µg/ml, then the pH was measured each hour.

Fig. 5 AuNPs did not modified the production of ROS of *S. pneumoniae* in the culture media. R6 (A) and TIGR (B) strains were cultivated until reach an optical density of approximately 0.2. Thereafter the AuNPs were added at a concentration of 1024 µg/ml, then samples were collected each hour, in order to determine ROS.

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Fig. 6 AuNPs cross the capsule in in a TIGR strain. R6 without capsule (A) and TIGR with capsule (B) strains were cultivated until reach an optical density of approximately 0.2. Thereafter the AuNPs were added at a concentration of 1024 µg/ml, samples were taken at 0.5, 1, 2 and 3 h in order to observe the effect by TEM.

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Fig 1

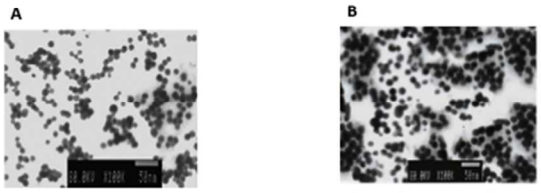

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Fig. 2

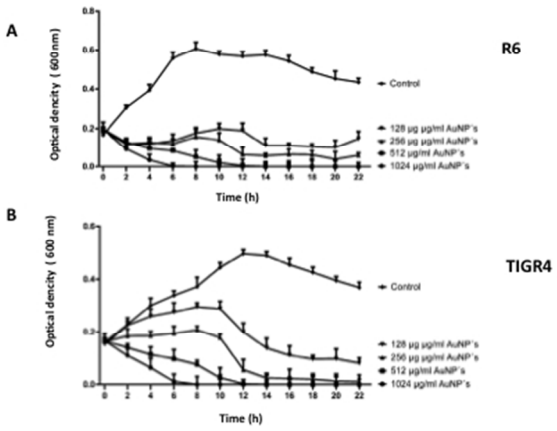

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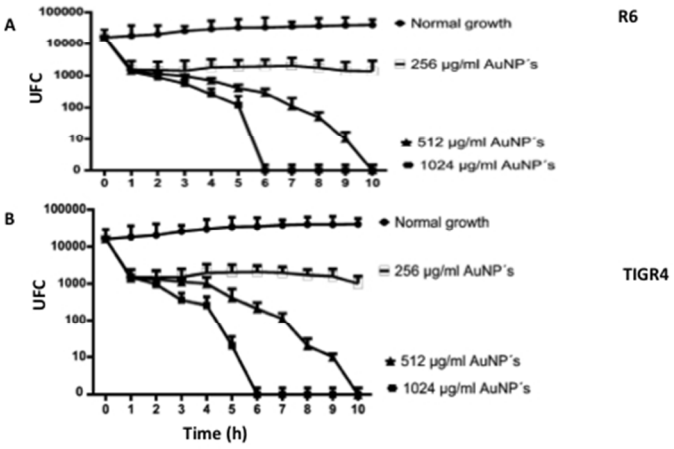
Fig.3



254x142mm (72 x 72 DPI)

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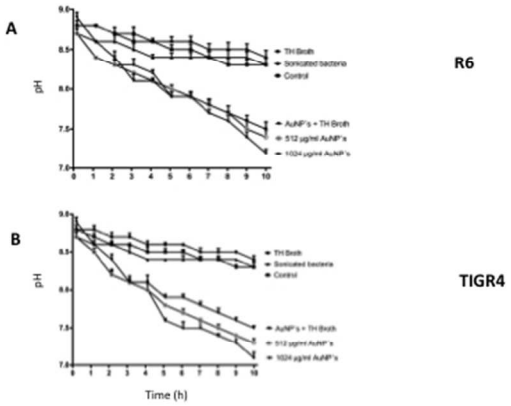
Fig.4



254x142mm (72 x 72 DPI)

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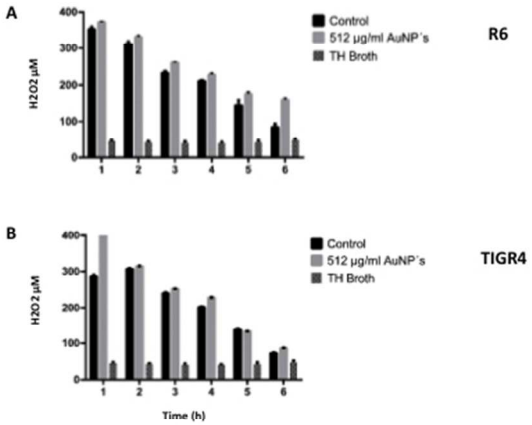
Fig. 5



254x142mm (72 x 72 DPI)

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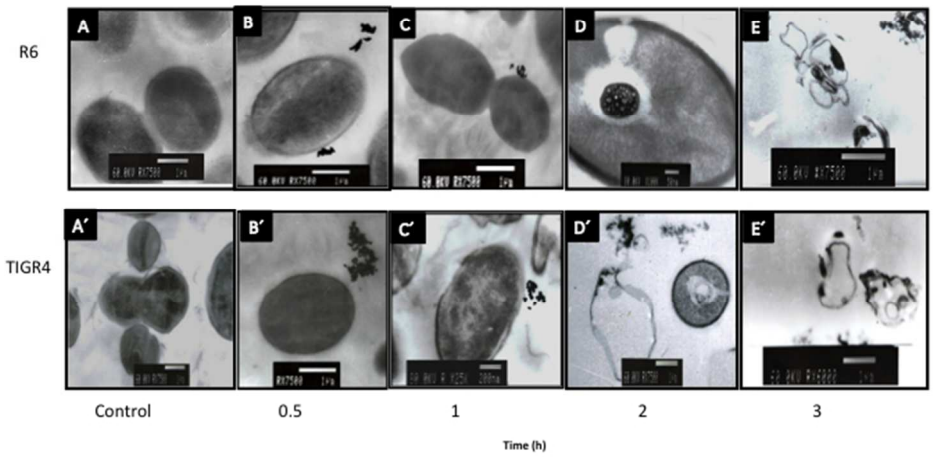
Fig. 6



254x142mm (72 x 72 DPI)

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