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Antimicrobial and Antibiofilm Activities of a Bismuth Lipophilic Nanoparticles Hydrogel against Methicillin-resistant Staphylococcus aureus biofilm

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Outline

Introduction

Despite of continuous effort by pharmaceutical industry and medicine, the multidrug resistance among pathogen microorganisms against most common antibiotics has increased drastically. Multidrug resistance has become one of most important health problems worldwide. Nosocomial infections caused by multidrug resistant microorganisms are hard to treat, since 70% of these microbes are resistant against most common drugs. *Staphylococcus aureus* is a pathogen identifies as the etiological agent of health-care-associated infections and community acquired ones[1] (Figure 1.1). The clinical relevance of *S. aureus* infections is due to their resistance to common antibiotics specifically to methicillin (methicillin-resistant *S. aureus*, MRSA)[2]. The infections caused by *S. aureus* community acquired have been increased in the last two years with 50% of these infections caused by MRSA. The strain USA-300 has been recognized as an etiology of osteomyelitis[3]. Their therapeutic management is a cost driver in healthcare, specifically in hospitals. Among the different factors as the major cost drivers are: prolonged hospital length to stay, cost of patient isolation and complications[4]. The treatment of MRSA infectious is complicated not only to their resistance to beta-lactam antibiotics, but also by their growing as biofilm. Microorganisms into biofilms become 1000 times more resistant than planktonic bacteria to physical and chemical attacks[5]. The tolerance of biofilms to antimicrobials increase with biofilm maturation and it is attributed mainly to restricted penetration of antibiotics, slow growth of pathogen microorganisms. Despite of their effectiveness, antibiotics more commonly employed on infectious diseases treatment are very expensive for most of patients, constituting a huge disadvantage during clinical practice in developing countries. It is urgent to develop new alternative drugs with antimicrobial and antibiofilm properties, non-antibiotic type, low cost and safe for treating infectious diseases. To attend this challenge of biofilms and their increased tolerance to antimicrobial agents, topical administration arises as an interesting alternative since it provides high local concentrations by delivering drugs directly to the site of infection and avoiding systemic side effects.

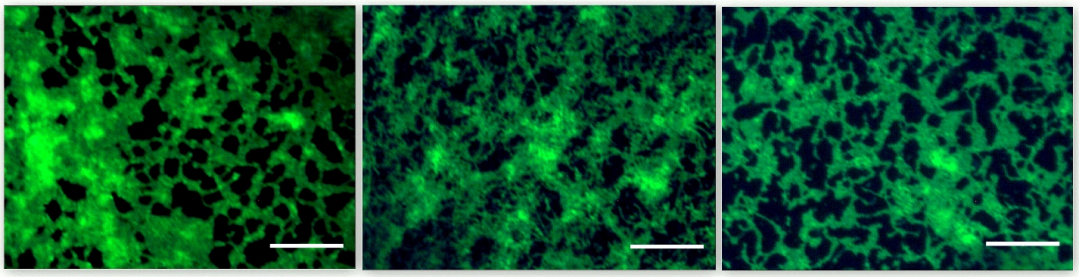


FIGURE 1.1
Staphylococcus aureus methicillin-resistant (MRSA) stained with SYTO9 green observed by fluorescent microscopy. Bar indicates 10 μM.

Nanotechnology holds the promise of revolutionize modern medicine developing smart drugs with the ability to overcome biological barriers to efficiently get the target sites of diseases[6,7]. The increase of multidrug resistance among pathogen microorganisms to the common antibiotics force to use higher doses of antibiotics to effectively inhibit the bacterial growth. Nanocomposites have been shown antimicrobial activity against gram positive and gram negative bacteria. Nanostructures of several metals like; silver, gold, zinc, and titanium and bismuth have been described with very good results[8-12]. However, most of them present high toxicity on human cells,

limiting their used[13-16]. Bismuth is considered as “green metal”, non-carcinogenic, and less bioaccumulative than other heavy metals like lead and antimony[17]. It is used in industry and for treatment of gastrointestinal diseases[18,19]. Early reports of our group described antimicrobial and antibiofilm properties of bismuth lipophilic nanoparticles (BisBAL NPs; Figure 1.2) against oral pathogens including bacteria, fungus and parasites. The BisABL nanoparticles did not present cytotoxicity on epithelial and blood human cells[20-24]. These studies have been described that bismuth nanoparticles inhibit *Streptococcus mutans* growth at concentrations lower than 1 mM and bismuth oxide nanoparticles exhibited antifungal activity on *Candida albicans* since 2 mM, showing better results than commercial antifungals.

More recently bismuth nanoparticles synthesis was modified adding the reaction with 2,3-dimercapto-1-propanol (BAL), developing the BisBAL nanoparticles with lipophilic property. The antimicrobial activity of these nanostructures increased 1000 times in comparison with our previous synthesis and may due their lipophilic attribute. When the antimicrobial activity was studied, the results showed MICs values of 5-10 µM to inhibit oral bacterial and fungal growth. BisBAL NPs compete in efficacy against most common antibiotics. Despite of antimicrobial and antibiofilm properties of BisBAL NPs seems to be interesting, their effect of BisBAL nanoparticles against multidrug resistant pathogens (like MRSA) has not been explored. Will be interesting to develop a pharmaceutic presentation with BisBAL NPs as active ingredient and demonstrate their clinical application. Since infectious diseases initiate in specific sites on human body, they can be treating locally. Therefore we developed a hydrogel loaded with BisBAL nanoparticles and evaluated their potential as antimicrobial agent. MRSA was used as target to determine their possible clinical application as alternative treatment of infectious diseases.

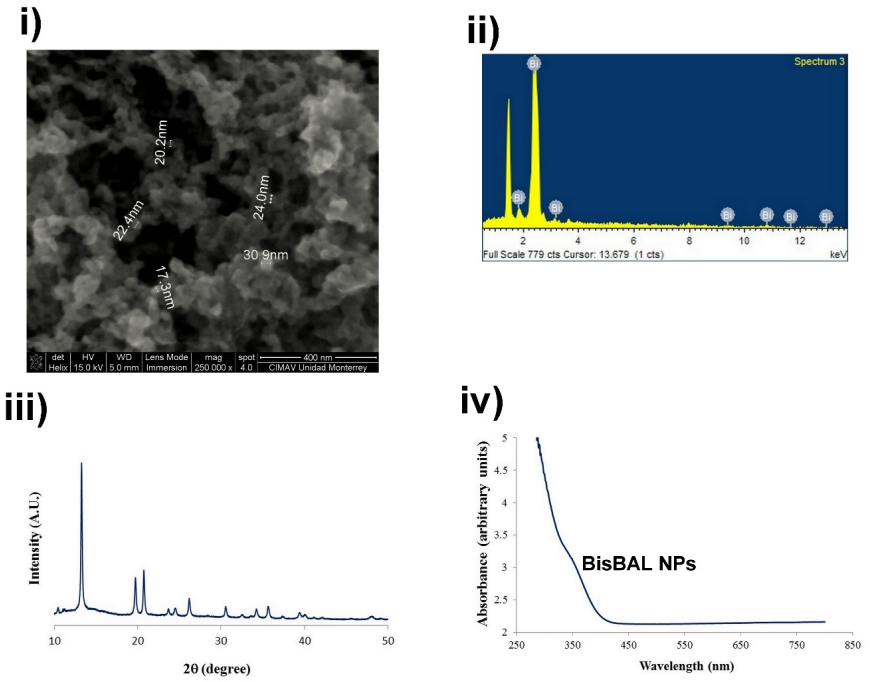


FIGURE 1.2

i) Bismuth lipophilic nanoparticles (BisBAL NPs) observed by Scanning Electron Microscopy (SEM). ii) EDS spectrum showed the element composition in the sample observed by SEM. iii) The bismuth presence in the sample of BisBAL nanoparticles was identified by X-ray diffraction pattern. iv) The UV-Bis absorbance.

In this work is described the effectiveness of a BisBAL NPs-Gel to detach Methicillin-resistant *Staphylococcusaureus* biofilm. 6µM was the MIC of BisBAL nanoparticles necessary to inhibit the MRSA growth competing in efficacy with most common antibiotics. A 24h MRSA biofilm was detached from 96-well plate and bone surface after exposition to BisBAL NPs-Gel for 24h supporting the bactericidal findings. Finally, bismuth lipophilic nanoparticles showed not cytotoxicity on human gingival fibroblasts, suggesting the lack of non-desired effects. Altogether these results suggest BisBAL nanoparticles are a low cost and safe alternative to fight against MRSA infections.

Antimicrobial and antibiofilm activities of BisBAL NPs-Gel on MRSA

BisBAL nanoparticles were synthesized and characterized as early was described in our recent publications[22]. To obtain the BisBAL NPs gel, 50 mL of sterile distilled water were heated until 70ºC and slowly 0.5 g of carbopol (Sigma Aldrich, MO, USA) were mixture with magnetic agitation. Following 2 mL of BisBAL nanoparticles were added to get a final concentration of 100 µM. Finally, 500 µL of triethanolamine (TEA; Sigma Aldrich, MO, USA) were added to the solution and employing more sterile water to get a final volume of 100 mL. Under these experimental conditions, the BisBAL NPs-Gel obtained a final concentration of 100 µM. The characterization of BisBAL NPs-Gel was made by Scanning Electron Microscopy (SEM), EDS spectrum, XRD and UV-visible absorption spectra to confirm the identity of bismuth (Figure 1.2 i-iv).

A general description of methodology followed to determine the antimicrobial and antibiofilm activities of BisBAL NPs-Gel is showed in Figure 1.3. The antimicrobial activity of BisBAL NPs-Gel on Methicillin-resistant *Staphylococcus aureus* growth (ATCC no 33592) was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Biotium, Hayward, CA)[25,26] according to the instructions of manufacturer. Briefly, $1X10^4$ MRSA cells were inoculated in 100 µl of trypticasesoybroth (TSB) medium in a 96-well polystyreneplate. Three wells with only TSB medium were used as MRSA growing control. 0.39-125 µM of BisBAL NPs-Gel were added to interfere with bacterial growth. As positive control 10 µM of Doxycycline was employed. The 96-well plate was incubated at 37ºC overnight. 10 µl of MTT was added to each well, the plate was protected against light and incubated at 37°C for 2h.200 µl of Dimethylsulfoxide (DMSO; Sigma Aldrich, MO, USA) was added to dissolve the reduced MTT. The number of live cells was determined by a Microplate Absorbance Reader (Biorad, Philadelphia, PA) at 595 nm. The experiment was repeated three times and the measured optical density were analyzed by descriptive statistics.

Based on the protocol described above, the antibiofilm property of BisBAL NPs-Gel was analyzed. A 24h MRSA biofilm on 96-well plate or bone surface was exposed to 100 µM BisBAL NPs-Gel or 10 µM of Doxycycline for 24h a 37°C. MRSA biofilm remains was washed three times with PBS and stained with FDA. The bacterial biofilm was observed under fluorescence microscopy at 495 nm (Thornwood, NY). The images were analyzed by using Axio Vision software (Thornwood, NY). The fluorescence intensity was measured using a 96-well scanning fluorometer Glomax® Multi + Microplate Multimode (Promega, Madison, WI) at wavelength of 525 nm.

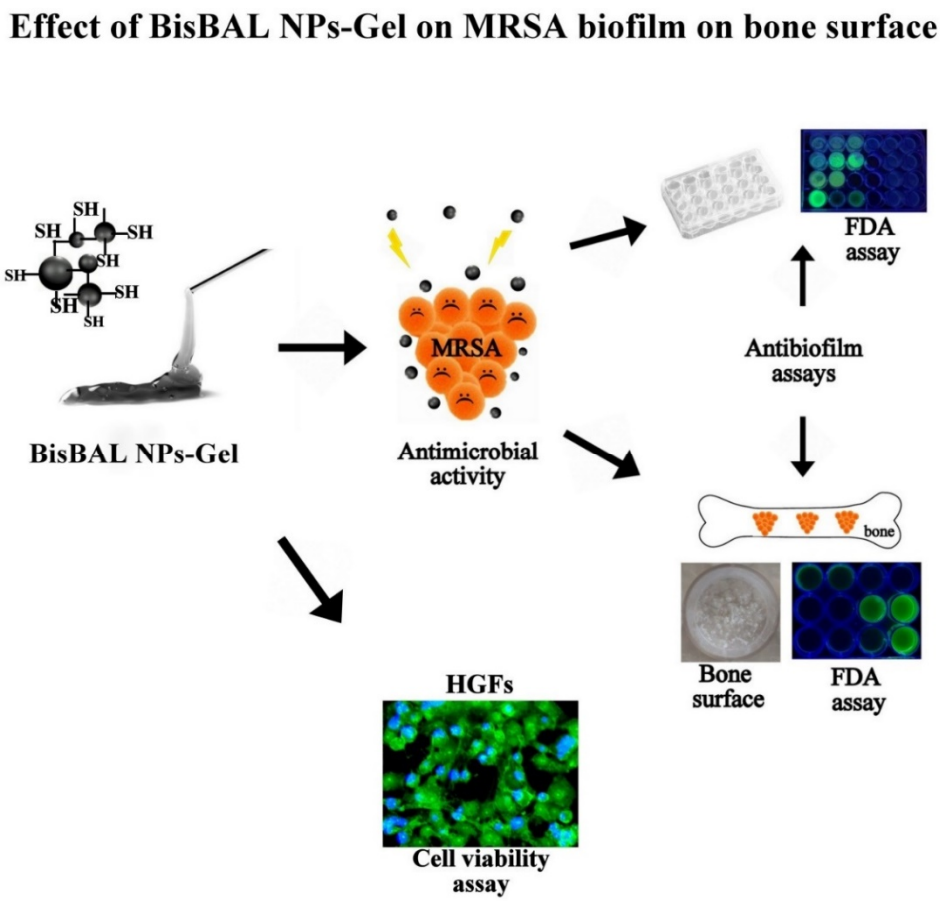


FIGURE 1.3
Graphical abstract to determine the antimicrobial and antibiofilm activities of a BisBAL-NPs-Gel on MRSA biofilm.

To explore the possible cytotoxic effect of BisBAL NPs-Gel on Human Gingival Fibroblasts (HGFs), cells were cultivated in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, California, USA) and 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma-Aldrich Corporation, St. Louis, MO) at 37 ºC and 5% CO_2[27]. After obtaining cell confluence, BisBAL nanoparticles were added at a final concentration of 1000-5 µM. Cells were maintained in growth medium for 24h. After that the medium was removed and cells were washed with phosphate buffered saline (PBS). The cell viability was measured by Fluorescein Diacetate assay (FDA, Sigma-Aldrich Corporation, St. Louis, MO). Data were analyzed to determine the number of viable cells.

Characterization of the BisBAL NPs-Gel by SEM

The BisBAL NPs-Gel showed a cluster of donut shape with an electro dense core of BisBAL nanoparticles visualized by SEM. The average size of each donut was of 145.6 nm in diameter

(Figure 1.4i, ii and iii). Bismuth composition was corroborated inside of SEM images by EDS spectrum (Figure 1.4iv). The BisBAL nanoparticles synthesized by colloidal method were used as active ingredient to develop a hydrogel and it was stable to room temperature for until 3 months. Since early it was described the antimicrobial activity of BisBAL nanoparticles we corroborate this property in the hydrogel composite BisBAL-NPs-Gel against MRSA growth. Following a deep characterization of the bactericidal activity of BisBAL-NPs-Gel against MRSA was made to compare their efficacy with most common antibiotics like Doxycycline. The bismuth NPs hydrogel will have the advantage of being non-antibiotic type with a lower cost of synthesis in comparison with antibiotics.

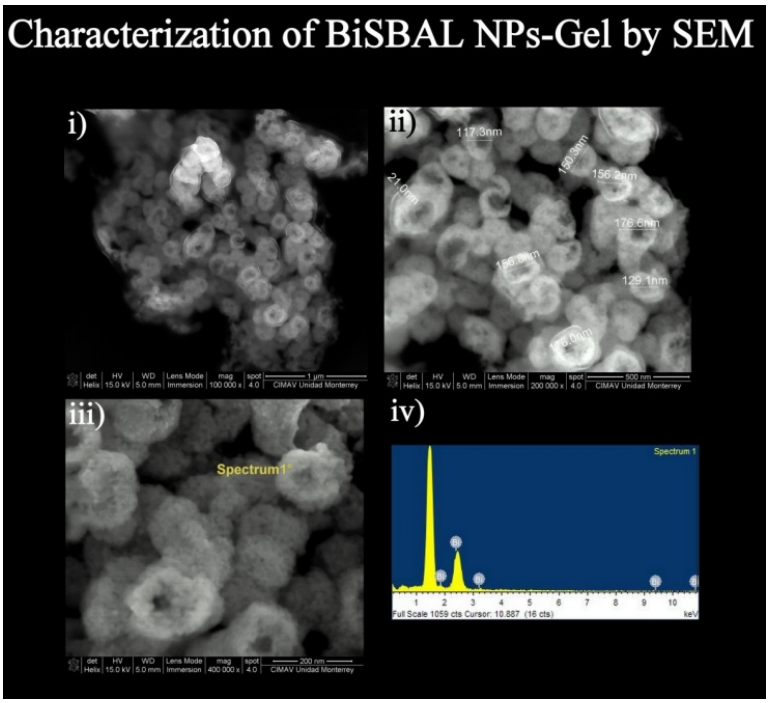


FIGURE 1.4
Characterization of the hydrogel of Bismuth Lipophilic (BisBAL) Nanoparticles by Scanning Electron Microscopy (SEM).

Determination of MIC of BisBAL NPs-Gel on MRSA growth

When Minimal Inhibitory Concentration (MIC) of BisBAL NPs-Gel was evaluated to interfere with MRSA growth, 125 µM of BisBAL NPs showed the higher inhibition of bacterial growth, leading to 20 µM of Doxycycline as can be seen in the Figure 1.5. The MIC was established in 6 µM of BisBAL NPs as the minimal amount of bismuth nanoparticles gel to block the MRSA growth.
Early reports have been described high antimicrobial activity against MRSA growth using gold and selenium nanoparticles with an apparent low cytotoxicity on mammalian cells[28,29]. Our findings are agreed with these reports supporting the hypothesis that nanotherapeutics is an innovative way to control MRSA infections.

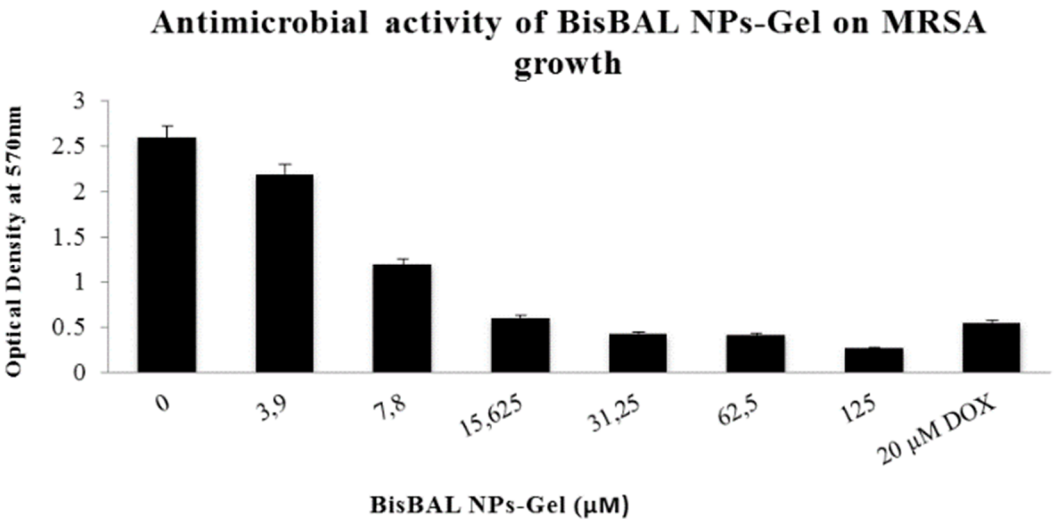


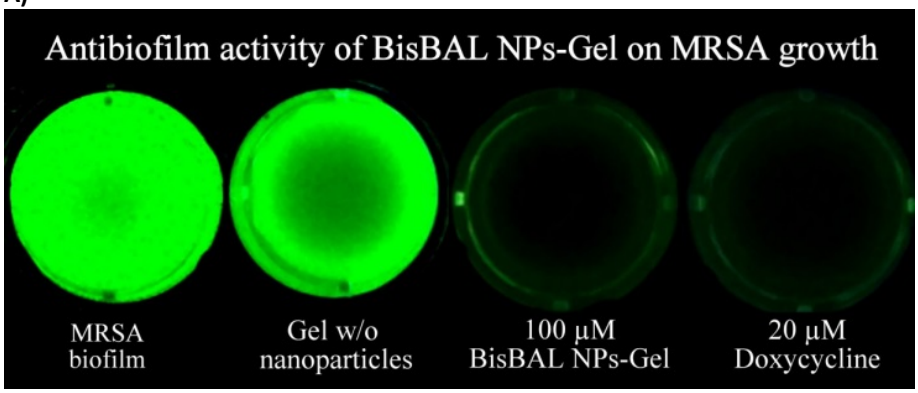
FIGURE 1.5
Minimal Inhibitory Concentration (MIC) of BisBAL nanoparticles against Methicillin-resistant *Staphylococcus aureus* growth.

The action mechanism of BisBAL nanoparticles to inhibit the bacterial growth is not well established. Our hypothesis is that bismuth nanoparticles alter the cellular membrane affecting their permeability leading to lysis. This hypothesis is based on early experiments using Calcein AM assays. Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of Calcein AM by intracellular esterases produces calcein, a hydrophilic strongly fluorescent compound that is well-retained in the cell cytoplasm. May be BisBAL NPs through their lipophilic property have affinity for cell membrane, penetrating to the cell cytoplasm and breaking down membrane structure. By the other side, we do not have evidence of damage on cell genome or inhibition of protein synthesis after exposition to bismuth nanoparticles.

Antibiofilm activity of BisBAL NPs-Gel on MRSA biofilm

When antibiofilm activity of BisBAL NPs-gel was explored, results showed that 100 µM BisBAL NPs-Gel detached 79% of MRSA biofilm, while Doxycycline removed 86% of cells in comparison with the growing control. Identical results were obtained on MRSA biofilm on boon surface. These results suggest that BisBAL NPs-Gel is as effective as Doxycycline to detach Methicillin-resistant *S. aureus* biofilm (Figure 1.6). Agarwala *et al* reported in 2014 that Copper oxide nanoparticles exhibited antibiofilm and bactericidal properties against MRSA in a dose dependent, however their cytotoxicity on mammalian cells was not explored[30].

A)



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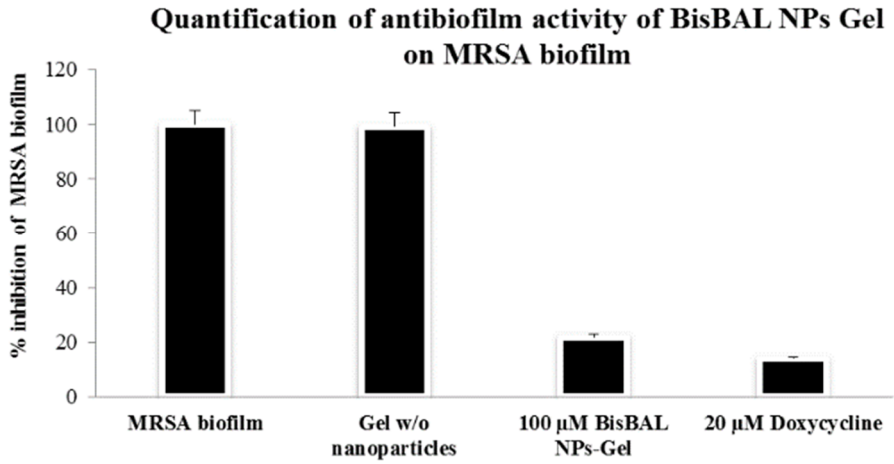

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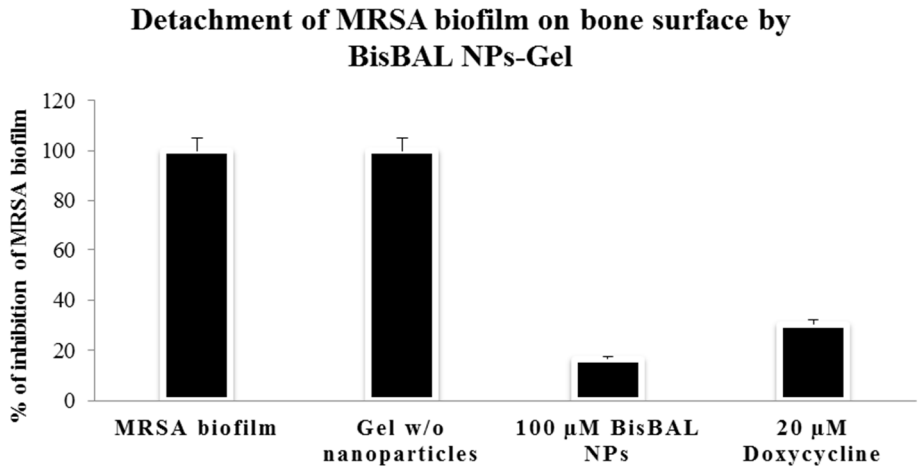

FIGURE 1.6
Antibiofilm activity of BisBAL-NPs-Gel against MRSA *S. aureus* biofilm by fluorescence microscopy.

Cytotoxicity of BisBAL NPs-Gel on Human Gingival Fibroblasts (HGFs)

When cytotoxic effect of BisBAL NPs-Gel was analyze on HGFs results show that 100 µM of BisBAL NPs-Gel decrease only 16% of cell viability after 24h of treatment in comparison with the growing control (Figure 1.7). Previous studies have been described antimicrobial properties of several metals like; silver, gold, zinc, and titanium with very good results[8-12]. However, most of them present high toxicity on human cells, limiting their use in clinical practice[13-16]. Our result is very important because mean that BisBAL NPs-Gel can be used at a final concentration of 100 µM as antimicrobial and antibiofilm drug against MRSA without affect mammalian cells. It is important to mention that bacterial and eukaryotic cells have important differences that may explain why BisBAL nanoparticles inhibit bacterial growth without affect human cells. First of all bacteria is around 10 times smaller than human cells. Following the hypothesis describe above, if BisBAL NPs attach to the cell membrane to penetrate to the cytoplasm altering their permeability, a human cell will require a higher quantity of BisBAL nanoparticles than a bacterial one to get the same effect. This phenomenon will explain the obtained data using 250-1000 µM of BisBAL NPs killing the human gingival fibroblast but remaining cell viability when 5-100 µM of BisBAL NPs were added to cell cultures. This hypothesis is supported by early experiments adding BisBAL nanoparticles to *Trichomonas vaginalis* culture. These parasites have a size similar to eukaryotic cells. 500 µM of BisBAL NPs were required to inhibit with parasitic growth[31] in comparison with 60-100 µM to block the growing of MRSA.

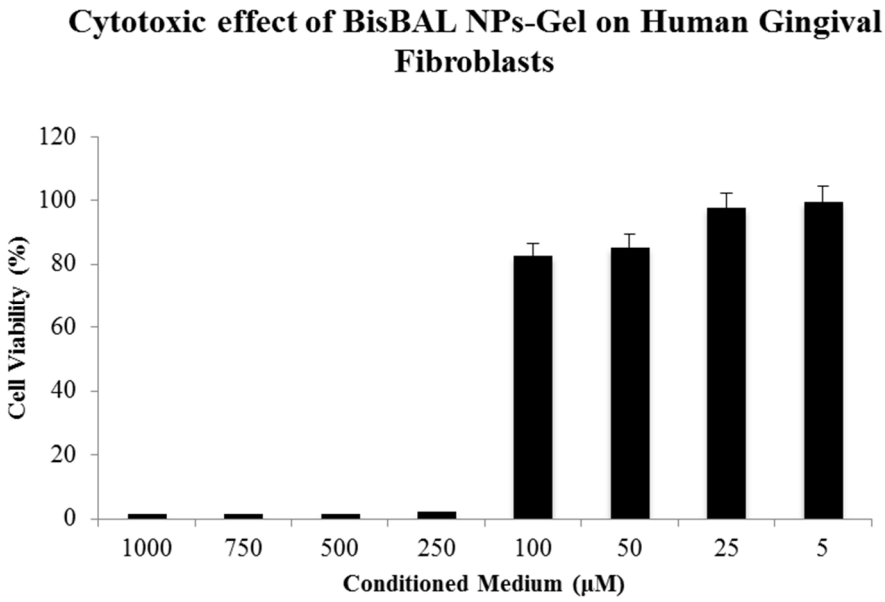

FIGURE 1.7
Cytotoxicity of the hydrogel of BisBAL Nanoparticles on Human Gingival Fibroblasts (HGFs).

Conclusion

Bismuth lipophilic nanoparticles as active ingredient of a hydrogel inhibit the MRSA growth since a

final concentration of 6 µM. 100 µM of BisBAL NPs-Gel detached the bacterial biofilm on dentin model suggesting that BisBAL nanoparticles hydrogel is a non-antibiotic, low cost and safe alternative to fight against MRSA infections. Applied in a topic manner, the BisBAL NPs-Gel provides high local concentrations by delivering bismuth nanoparticles directly to the site of infection avoiding side effects.

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