Antimicrobial potential of bismuth lipophilic nanoparticles embedded into chitosan-based membrane

Marco Antonio MARTÍNEZ-MARTÍNEZ1, Rene HERNANDEZ-DELGADILLO1, Bilal Saad ABADA2, Nayely PINEDA-AGUILAR3, Juan Manuel SOLIS-SOTO3, María Argelia Akemi NAKAGOSHI-CEPEDA1, Sergio Eduardo NAKAGOSHI-CEPEDA1, Shankararaman CHILLAM2, Rosa Isela SÁNCHEZ-NÁJERA1 and Claudio CABRAL-ROMERO1

1 Dental School, Autonomous University of Nuevo León, UANL, Monterrey, Nuevo León, México
2 Department of Civil Engineering, Texas A&M University, College Station, TX, USA
3 Advanced Materials Research Center, CIMAV Unidad Monterrey, Nuevo León, México

Corresponding author, Claudio CABRAL-ROMERO; E-mail: claudio.cabralrm@uanl.edu.mx

The objective of this work was to analyze the antimicrobial and antibiofilm activities of bismuth lipophilic nanoparticles (BisBAL NPs) incorporated into chitosan-based membranes. Chitosan-based membranes were homogeneously embedded with BisBAL NPs, confirming the bismuth presence by scanning electron microscopy. The tensile strength of chitosan-based membrane alone or with BisBAL NPs showed similar results as elongation, suggesting that BisBAL NP addition did not affect membrane mechanical properties. Chitosan-based membranes complemented with 100 µM of BisBAL NPs caused a complete inhibition of biofilm formation and a 90–98% growth inhibition of six different oral pathogens. Cytotoxicity studies revealed that 80% of human gingival fibroblasts were viable after a 24-h exposure to the chitosan-based membrane with 100 µM of BisBAL NPs and collagen. Altogether, we conclude that the biological properties of chitosan-based membranes supplemented with BisBAL NPs could be a very interesting option for tissue regeneration.

Keywords: Antimicrobial activity, Bismuth lipophilic nanoparticles, Chitosan-based membranes, Biofilm, Cytotoxicity

INTRODUCTION

The concept of guided tissue regeneration (GTR) was introduced by Lindhe group in the 1980s and has been used for bone and periodontal tissue regeneration1. According to the GTR principle, a membrane could exclude epithelial and soft connective tissue cells from the wound space to facilitate the infiltration of regenerative cells types, such as osteoblasts, to promote tissue regeneration2. The main objectives of a membrane are to promote tissue regeneration developing an adequate environment and to exclude fast proliferating cells that interfere with tissue regeneration3. The main objectives of a membrane are to promote tissue regeneration developing an adequate environment and to exclude fast proliferating cells that interfere with tissue regeneration. Several non-resorbable and resorbable membranes have been developed and extensively studied with very interesting results. However, there is a continuous innovation effort to develop improved membranes that intend to comply with important criteria for clinical use, such as bioactivity, biocompatibility, and biodegradability, chitosan has extensively been employed as scaffold material4. Recently, GTR has become more popular as an efficient technique for the reconstruction of damaged tissue5. In early stages of regeneration, GTR should support blood coagulation and provide a mechanical barrier to impede filtration of cells of connective tissue6. The first material employed as a GTR membrane was cellulose acetate, which also promoted periodontal tissue formation7. According to their degradation characteristics, GTR membranes can be classified into polytetrafluoroethylene (PTFE)-based nonresorbable membranes and resorbable membranes made of synthetic polyesters and collagen-based membranes8. PTFE-based membranes have the disadvantage of an increased risk of bacterial colonization, while resorbable membranes are less strong. Recently, a chitosan-based membrane has attracted attention because of its easy synthesis, biodegradability, and stability9. Furthermore, it is an excellent mucoadhesive material due to its functional groups, which facilitate hydrogen bonding and electrostatic interactions with mucin10. Chitosan membranes can be prepared in acetic acid solution by the solution casting-evaporation method11. Chemical cross-linkers, like glutaraldehyde, can fix the membranes for increased stability and mechanical strength12. To confer antimicrobial properties, chitosan membranes have been supplemented with several compounds with antimicrobial properties, such as antibiotics, silver.
sulfasalazine, chlorhexidine, and silver nanoparticles\textsuperscript{[15-17]}. However, chlorhexidine and silver nanoparticles have the disadvantage of being highly cytotoxic, which limits their usage in clinical practice\textsuperscript{[18,19]}. It will be interesting to supply chitosan-based membranes with non-cytotoxic compounds that provide antimicrobial and antibiofilm properties to prevent microbial infections.

Bismuth compounds are non-cytotoxic and less bioaccumulative than other metals including arsenic, antimony, and lead\textsuperscript{[20-22]}. Bismuth has been used in catalysts, alloys, pigments, and in medicine \textit{e.g.} Pepto-Bismol\textsuperscript{[23-25]}. Recently, our group reported on the excellent antimicrobial properties of bismuth lipophilic nanoparticles (BisBAL NPs); the minimal inhibitory concentration (MIC) of BisBAL NPs against oral pathogenic microorganisms was 5–10 µM\textsuperscript{[26]}. Cytotoxicity studies revealed that BisBAL NPs were not cytotoxic for human epithelial and blood cells in contrast with silver or gold nanoparticles\textsuperscript{[27,28]}. When mineral trioxide aggregate (MTA), which is one of the most commonly used biomaterials in endodontic treatment, was supplemented with BisBAL NPs, the latter did not significantly modify the physical properties of MTA, nor was it cytotoxic for human gingival fibroblasts (HGFs), but it did provide antimicrobial activity as it inhibited the growth of \textit{Enterococcus faecalis}, \textit{Escherichia coli}, and \textit{Candida albicans}, and it also detached the biofilm of fluorescent \textit{E. faecalis} after 24 h of treatment\textsuperscript{[29]}.

Here, we describe the production of chitosan-based membranes supplemented with BisBAL NPs to confer antimicrobial properties without modifying the excellent biological characteristics of chitosan-based membranes.

**MATERIALS AND METHODS**

**Synthesis and characterization of BisBAL NPs**

The colloidal method according to Badireddy \textit{et al.}\textsuperscript{[30]} was applied. Briefly, a stock solution of 50 mM Bi\textsuperscript{3+} solution was prepared by dissolving Bi(NO\textsubscript{3})\textsubscript{3}·5H\textsubscript{2}O in 20 mL of propylene glycol heated to 80°C and well-mixed for 2 h. A 2:1 molar ratio of Bi (Bis) to 2,3-dimercapto-1-propanol (BAL) was prepared by adding 25 µL of 10 M BAL to 10 mL of 50 mM Bi\textsuperscript{3+} solution. A stock suspension of 25 mM BisBAL NPs was prepared by diluting 5 mL of 50 mM BisBAL in 4.25 mL of ice-cold ultrapure water followed by the addition of a freshly prepared ice-cold solution of 0.75 mL of 75 mM NaBH\textsubscript{4} while mixing thoroughly. During this reaction, the pink color of soluble BisBAL instantly transformed to a black colored suspension composed of BisBAL NPs. Information on the shape, size, and distribution of BisBAL NPs was obtained using scanning electron microscopy (SEM; FEI Tecnai G2 Twin, Hillsboro, OR, USA; 160 kV accelerating voltage). The specific presence of bismuth was corroborated by EDS spectrum by SEM.

**Synthesis of chitosan-based membrane supplemented with BisBAL NPs**

Chitosan-based membranes were synthesized according to Lieder \textit{et al.}\textsuperscript{[30]} with slight modifications. To develop a 1% chitosan-based membrane, 500 mg of chitosan (mol wt 50,000–190,000 Da based on viscosity, 75–85% deacetylated; Sigma Aldrich, St. Louis, MO, USA) was mixed with 49.75 mL of deionized water (ddH\textsubscript{2}O) and 250 µL of 100% acetic acid and allowed to settle in the dark for 1 h at room temperature. After centrifugation (5,000 rpm for 10 min; to remove air bubbles and non-dissolved particles), chitosan-membranes were supplemented with 500, 200, 100 or 0 µM of BisBAL NPs. The mixtures were poured into small (60×15 mm) Petri dishes and the membranes were dried at 37°C for 18 h without lid to facilitate the evaporation of the solvent. Next, the membranes were incubated with 2 mL 0.5 M NaOH at 37°C for 30 min to neutralize the solution. Excess of NaOH was eliminated by inverting the Petri dishes and the membranes were washed with ddH\textsubscript{2}O before being sterilized with 2 mL of 70% ethanol at 37°C for 30 min followed by exposure to ultraviolet light for 30 min. Finally, the membranes were allowed to dry at 37°C for 18 h and 10 mm circles were punched out to evaluate its possible antimicrobial properties.

**Characterization of chitosan-based membrane supplemented with BisBAL NPs by SEM-ED, FTIR, X-ray diffraction analysis and S and Optical microscopy**

In order to assess the presence and distribution of BisBAL NPs into the chitosan-based membrane, SEM was employed. The chemical elemental composition was determined by energy dispersive X-ray spectroscopy (EDS; Oxford INCA X-Sight, Tubney Woods, UK), several point-micro analyses (1 µm) were performed in all samples of chitosan-based membranes supplemented with BisBAL NPs. To further confirm the distribution, elemental mapping analysis was carried out. Each map was represented in a distinct color, which helps to identify an element in multi-constituent specimens. Membranes were also observed under optical microscope (Olympus BX53, Olympus, Waltham, MA, USA) using a 100× objective lens with oil. Membranes supplemented with BisBAL NPs were also characterized by FTIR to observe functional groups of membrane surfaces. The infrared spectra of membranes over the range of 500–4,000 cm\textsuperscript{-1} were collected at 4 cm\textsuperscript{-1} resolution using a Nicolet iS10 (Thermo Fisher Scientific, Waltham, MA, USA) and spectra were analyzed using omnic 9 software. 5 samples from each membrane were measured. Each sample was run for 128 scans, and all the scans from all of these 5 samples were averaged. The crystalline structure of chitosan-based membrane supplemented with BisBAL NPs samples were analyzed using an X-Ray diffractometer (model Empyrean, PANalytical, Malvern, UK) operated at a voltage of 45 kV and 40 mA with Cu k\textalpha radiations at λ=0.15418 nm between 20 angles of 10° and 70° with a scan step size 0.0334225 and a time per step of 120 s.

**Weight loss in phosphate buffered saline (PBS) of chitosan-based membrane plus BisBAL NPs**

Weight loss assays of chitosan-based membrane supplemented with BisBAL NPs were performed.
according to Cai et al. with slight modifications[31]. Chitosan-based membranes (50 mm of diameter in size) with 0, 100, 200 or 500 µM of BisBAL NPs were immersed in a Petri dish containing 20 mL of 10 mM PBS at pH 7.4 (Sigma Aldrich) at 37°C for 8 days. Next, the membranes were washed with distilled water and air-dried before being weighted. Residual weight (RW;%) was calculated according to the following equation:

\[ RW(\%) = \frac{W_c}{W_o} \times 100\% \]

**Mechanical properties of chitosan-based membranes supplemented with BisBAL NPs**

The mechanical properties of strips (7.62×0.635 cm) of chitosan-based membranes supplemented with 0, 100, 200 or 500 µM of BisBAL NP were determined with a tensile strength instrument (MTS, Eden Prairie, MN, USA). Pneumatic rubber sample holders were used. A laser extensometer was used to measure the strain and elongation. The mechanical analysis was performed at a stretching rate of 25 mm/min with a gap separation of 1 inch.

**Antimicrobial activity of chitosan-based membrane supplemented with BisBAL NPs by disc diffusion assay**

To verify whether the chitosan-based membrane supplemented with BisBAL NPs had stronger antimicrobial activity than the chitosan-based membrane alone, disc diffusion assays were employed[32]. *Porphyromonas gingivalis* strain W83 (ATCC no.s. BAA-308) was grown in Trypticase Soy Broth Agar (TSB; BD DIFCO, Sparks, MD, USA) at 37°C, overnight in aerobic conditions with standard inoculums (0.5 McFarland); 100 µL of bacteria culture was spread on TSB agar plate using a sterile 5-mm polystyrene ring. Chitosan-based membrane supplemented with 0, 100, 200 or 500 µM of BisBAL NPs were embedded into the agar plate. After an overnight incubation at 37°C, the halo diameter was measured with a Vernier. Antimicrobial assays were performed in triplicate.

**MIC of BisBAL NPs added to chitosan-based membrane to inhibit six different bacterial strains**

MIC was determined as described[33] with slight modifications. Chitosan-based membranes supplemented with 0, 10, 50, 100 or 200 µM of BisBAL NPs (5-mm in diameter) were located on the bottom of 96-wells plate and inoculated with 100 µL (1×10⁶ cells) of a microbial culture (Porphyromonas gingivalis strain W83, meticillin-resistant Staphylococcus aureus [MRSA], Candida albicans, Escherichia coli, Enterococcus faecalis, and Streptococcus gordonii), (ATCC, BAA-308, 33592, 90029, 25922, 11420, and 10558, respectively). As a negative control, microorganisms were grown in culture media without any chitosan-based membrane. Microorganisms growing with culture media without any chitosan-based membrane were used as growing control. After an overnight incubation at 37°C/5% CO₂, 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biotium, Hayward, CA, USA) was added, and the incubation was continued for another 2 h at 37°C/5% CO₂ in the dark. Next, the medium was removed and 100 µL dimethyl sulfoxide (DMSO) was added to dissolve the reduced MTT formazan product. To quantify the reduced MTT all surviving cells were transferred to a new 96-well plate before reading optical density at 570 nm (OD₅₇₀) using a microplate absorbance reader (Biotek, Winooski, VT, USA); DMSO was employed as blank. The assay was done in triplicate.

**Antibiofilm activity of chitosan-based membrane supplemented with BisBAL NPs by fluorescence microscopy**

Chitosan-based membranes supplemented with BisBAL NPs were placed into 96-well plates as described above to study their antibiofilm properties by fluorescence microscopy. Hereto, 24-h biofilms formed by *P. gingivalis* strain W83 or microorganisms isolated from patients with periodontal disease were incubated with the chitosan-based membrane alone or supplemented with 100 or 200 µM BisBAL NPs 37 ºC for 24 h. Next, the biofilm remnant was washed three times with PBS and stained with fluorescein diacetate (FDA) (Sigma-Aldrich). The cells that had remained inside the biofilm were quantified measuring the fluorescence intensity using a 96-well scanning fluorometer Glomax® Multi+Microplate Multimode (Promega, Madison, WI, USA) at emission and excitation wavelengths of 488 and 530 nm. Data were analyzed to determine the number of viable cells. The experiment was performed in triplicate.

**IC₅₀ determination of BisBAL NPs on HGFs**

The half maximal inhibitory concentration (IC₅₀) of BisBAL NPs was measured using a primary culture of HGFs. HGFs (1×10⁶ cells) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 (DMEM/F12) plus 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, CA, USA) and 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich) at 37°C in a humidified atmosphere with 5% CO₂. Cell viability MTT assay was employed (Biotium) to analyze the effect of 0, 50, 100, 150, 200, 250 and 500 µM of BisBAL NPs on HGFs for 24 to 48 h. After incubation, 10 µL of MTT was added to each well and incubated at 37°C and 5% CO₂ for 2 h in the dark. After removal of the medium, the reduced MTT formazan product was dissolved in 100 µL DMSO and the OD₅₇₀ was determined with DMSO as a blank using a microplate absorbance reader (Biotek) and DMSO was employed as blank.

**Integrity of cell membrane after treatment with chitosan-based membranes supplemented with BisBAL NPs**

The effect of chitosan-based membranes supplemented with BisBAL NPs on cell morphology was analyzed on HGFs by Calcein AM assay and fluorescence microscopy. Calcein AM is a dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein, which is retained.
by cells with intact membranes. HGFs were exposed to chitosan-based membrane supplemented with 0 or 100 µM of BisBAL NPs and in some cases also with 0.5% of collagen for 24 h at 37°C and 5% CO₂. HGFs cultured in the medium was used as a growth control. After 24 h of treatment, cells were washed with PBS and stained with Calcein AM (Sigma-Aldrich). The cell morphology was observed with FITC filter at 496 nm employing an AE2000 inverter microscope (Motic, Carlsbad, CA, USA).

**Statistical analysis**
Statistical analysis was done to all related experiments among the groups as compared to the control using ANOVA followed Tukey’s HSD Test. For all statistical analyses, a significance level of α=0.05 was considered.

**RESULTS**

**Characterization of chitosan-based membrane supplemented with BisBAL NPs**
The synthesized chitosan-based membrane was completely transparent and the addition of BisBAL NPs did not change its appearance (Fig. 1A). The wet BisBAL NP-supplemented adapts to surfaces it is placed upon (Fig. 1B), showing high hydrophobicity a property that is interesting for clinical application. The results obtained from pH analysis showed that pH change from 7.4 to 6.9 after BisBAL NPs addition keep neutrality. Microscopic observations indicated that with higher concentrations, membranes look more homogeneous, suggesting that with adding more BisBAL NPs, there will be more crosslinking between membrane chains and therefore better mechanical strength (Fig. 1C). This finding is further confirmed in the next section, where tensile strength was improved with increasing the BisBAL NPs concentration in the membranes.

BisBAL NPs were spheres with an average diameter of 24 nm that aggregated into dense electronic clusters as can be seen in SEM images (Fig. 2Ai), which is consistent with previous publications. When BisBAL NPs were added to a chitosan-based membrane, their specific presence into the membrane was corroborated by SEM (Fig. 2Av). BisBAL NPs had the same shape and size as when they were synthesized (Fig. 2Ai), suggesting that they do not react with any chemical component during membrane developing. Chitosan-based membrane alone was used as negative control (Fig. 2Aiii) without nanoparticles. EDS spectrums revealed the presence of bismuth (Figs. 2Aii and Avi). The elemental map revealed a homogeneous distribution of carbon and oxygen in both pure and BisBAL NP-supplemented chitosan-based membranes (Figs. 2Bii, iii, vi and vii), whereas bismuth was detected only in the BisBAL NP-supplemented membranes with a highly homogeneous distribution (Fig. 2Bviii). Fourier-transform infrared spectroscopy with attenuated total reflection sampling was carried out in the range of 4,000–500 cm⁻¹, finding a chemical similarity in all membranes with or without BisBAL NPs. Specifically in the range of 823 cm⁻¹ to 834–836 cm⁻¹ was revealed that all BisBAL NPs-supplemented membranes presented slight band shifts. The tendency of this shift is suggestive of a Bi-O functional group (Bi-O groups peaks in the region 875 cm⁻¹, Fig. 2C). Altogether, the results suggest that BisBAL NPs is inertly and uniformly incorporated into chitosan-based membranes. The structural properties of the prepared samples were analyzed using the XRD technique. XRD diffraction patterns of the samples are shown in Fig. 2E. The XRD pattern of BisBAL clearly indicates the formation of bismuth in a single phase (Fig. 2Eiii). Peaks were obtained at 20 values of 27.2°, 38.01°, 39.66°, 48.76°, 56.09°, 62.27° and 64.58° which are in agreement with the JCPDS card No. 00-044-1246. The XRD pattern of chitosan exhibited a characteristic
Fig. 2 Characterization of chitosan-based membranes supplemented with BisBAL NPs by SEM, EDS, ATR-FTIR and X-ray diffraction analysis.

A) BisBAL NPs were analyzed by SEM and EDS (i and ii), Chitosan-based membrane alone by SEM and EDS (iii and iv), and Chitosan-based membrane plus 500 µM of BisBAL NPs by SEM and EDS (v and vi). B) An elemental mapping analysis of the elemental composition of Carbon, Oxygen, and Bismuth into chitosan-based membrane alone (i-iv) or with BisBAL NPs (v-viii) was determined. C) ATR-FTIR spectra for chitosan-based membranes with and without BisBAL. We can notice higher absorbance with higher BisBAL NPs, especially in the regions around 900–1,100 and 2,700–3,700 cm$^{-1}$. D) Stress-strain curve of chitosan-based membranes supplemented with 0, 100, 200 and 500 µM of BisBAL NPs. The mechanical analysis was performed at stretching rate of 25 mm/min. E) XRD patterns of i) chitosan-based membrane supplemented with BisBAL NPs, ii) chitosan-based membrane, iii) BisBAL NPs, iv) JCPDS card No. 00-044-1246 corresponding to elementary bismuth and v) XRD pattern of chitosan-based membranes supplemented with BisBAL NPs: enlargement in the range of 25–41°.
peak appeared at 2θ value of ~20° corresponding to the crystallographic plane (101) which match well with the literature values\(^3\) as shown in Fig. 2Eii. The broadening of the peak is due to the amorphous nature of chitosan\(^{38}\). The presence of chitosan as well as bismuth peaks is observed from the XRD pattern of chitosan-based membrane supplemented with BisBAL NPs sample (Fig. 2Ei) and enlargement of this (Fig. 2Ev) in the range of 25–41° corresponding to (012), (104) and (110) planes reflection of bismuth from BisBAL NPs.

**Mechanical properties of chitosan-based membrane supplemented with BisBAL NPs**

With the objective of analyze if BisBAL NPs addition to chitosan-based membranes could modify their mechanical properties, tensile strength and elongation were measured in membranes with 0, 100, 200 or 500 µM of BisBAL NPs. The incorporation of BisBAL NPs at 100 and 200 µM did not alter the tensile strength of chitosan-based membranes as both the pure and the supplemented had a tensile strength of 0.17 MPa. Even a higher concentration of BisBAL NPs (500 µM) the tensile strength measured was very similar (0.19 MPa) as can be observed in stress-strain curve at Fig. 2D. The tensile strength of chitosan-based membrane alone or with BisBAL NPs showed no differences suggesting that BisBAL NP addition did not affect membrane strength and elongation as is shown in summary in the Table 1.

The measure of tensile strength in wet conditions was not possible to do it due to technical difficulties; however, previous reports described a decreasing tendency of this property in wet chitosan membranes in comparison with dry membranes\(^{39,40}\).

**Weight loss in aqueous solution of chitosan-based membrane plus BisBAL nanoparticles**

BisBAL NP supplementation at 100, 200, and 500 µM did not change the RW of chitosan-based membranes after 8 days of agitation in PBS at 37°C (Fig. 3). These results suggest that BisBAL nanoparticles are tightly held into the chitosan membrane, preventing their loss and keeping good stability.

**Antimicrobial activity of chitosan-based membrane supplemented with BisBAL NPs**

BisBAL NP-supplemented chitosan-based membranes inhibited the growth of *Porphyromonas gingivalis* growth at all doses (100, 200, and 500 µM) after 24 h of exposure in disc diffusion assays, whereas pure chitosan membranes did not (Fig. 4A). Based on the diameter of inhibition halos, BisBAL NP inhibition of *P. gingivalis* growth was dose-dependent.

MIC determination of BisBAL NPs against 6 different microbial pathogens revealed that chitosan membranes supplemented with 10 µM BisBAL NPs interfered with the growth of the following pathogens: *Escherichia coli, Enterococcus faecalis, Candida albicans*, and MRSA (Fig. 4B). At 100–200 µM BisBAL NP supplementation, the growth inhibition was 83–99% for all microbial pathogens studied (Fig. 4B). The MIC of chitosan-based membranes supplemented with BisBAL NPs was defined at 100 µM. Altogether, these results suggest that BisBAL NPs provide broad bactericidal and antimycotical activity as BisBAL NPs caused growth inhibition of gram-positive, gram-negative, and multi-resistant pathogens.

**Antibiofilm property of chitosan-based membrane supplemented with BisBAL NPs**

Fluorescence microscopy of *P. gingivalis* and periodontal patient-derived biofilms exposed to pure or BisBAL NP-supplemented chitosan membranes revealed that either

![Weight loss in aqueous solution of chitosan-based membranes with BisBAL NPs](image)

**Table 1** Summary of mechanical properties of chitosan-based membrane supplemented with BisBAL NPs

| Chitosan-based membrane+BisBAL NPs | Stress (MPa) | Elongation (%) |
|-----------------------------------|-------------|---------------|
| 0                                 | 0.17±0.01   | 8.8±1.8       |
| 100                               | 0.17±0.01   | 13.0±6.2      |
| 200                               | 0.17±0.01   | 6.5±1.3       |
| 500                               | 0.19±0.01   | 16.0±8.5      |

Values indicate mean±SD (n=4).
100 or 200 µM BisBAL NP caused 89.4% less bacterial biofilm, whereas pure chitosan membranes did not (Fig. 5). No differences were found between *P. gingivalis* and a sample isolated from a patient with periodontal disease biofilms. These results suggest that BisBAL NPs provide antibiofilm effectiveness as of 100 µM supplementation to chitosan-based membranes.

**Cytotoxicity of chitosan-based membrane supplemented with BisBAL NP on HGFs**

Considering the antimicrobial and antibiofilm activities of BisBAL NP supplementation to chitosan-based membranes, its cytotoxic effect on HGFs was verified. IC50 value of BisBAL NPs on a primary culture of HGFs...
was determined. Cell survival was over 80% after 24 h exposure to 1–100 µM BisBAL NPs (Fig. 6A). Importantly, at higher concentration, HGFs survival dropped to 50% at 150 µM and 18% at 200 µM BisBAL NP. These results indicate that 100 µM BisBAL NPs provides antimicrobial and antibiofilm properties to chitosan-based membrane without being cytotoxic to HGFs. These results were supported by data obtained with Calcein AM assay and fluorescent microscopy. Chitosan membrane plus 100 µM of BisBAL NPs promotes cells rounded up suggesting a stress state but calcein AM was not released, indicating they were living, and observing cell plasmatic membrane integrity after 24 h of exposition (Fig. 6B). If chitosan membrane was also supplemented with 0.5% of collagen the cell proliferation increased significantly. Altogether these data suggest that chitosan-based membrane supplemented with 100 µM of BisBAL NPs has no side effects on HGFs under the experimental conditions analyzed.

**DISCUSSION**

In this work we presented evidence of antimicrobial and antibiofilm effectiveness of BisBAL NP-supplementation to chitosan-based membranes. The synthesized chitosan-based membrane was completely transparent and the addition of BisBAL NPs did not change its appearance nor tensile strength significantly, in concordance with previous reports of chitosan membranes. According to SEM images, BisBAL NPs spheres had an average diameter of 24 nm and aggregated into dense electronic clusters. The inclusion of BisBAL NPs into the chitosan matrix did not change their appearance, suggesting that BisBAL NPs did not react with any composite during membrane synthesis. The distribution of BisBAL NPs into the membrane was largely homogeneous. No bismuth was detectable in non-supplemented chitosan membranes. The absence of bismuth in chitosan matrices is important to prove that the antimicrobial and antibiofilm properties of the supplemented membranes are due to BisBAL NPs. Regarding X-ray diffraction patterns, the weaker diffraction peaks of chitosan-based membrane supplemented with BisBAL NPs compared with the BisBAL NPs alone indicate that the bismuth nanoparticles were covered by amorphous chitosan polymer. Moreover, the broad diffraction peak at 20 value of ~20° is related to the amorphous chitosan. Chitosan did not modify the crystal structure of BisBAL NPs.

The weight loss assays indicated that neither the pure nor any of the BisBAL NP-supplemented chitosan-based membranes diminished weight after immersion in an aqueous solution. These results suggest that BisBAL NPs are strongly withheld into the chitosan membrane, preventing their loss and keeping good stability. Cai et al., described that hydrophilicity of pure cross-linked chitosan membranes did not change when weight loss was measured.

The disc diffusion assays revealed that BisBAL NP-supplementation provided antimicrobial activity against *Porphyromonas gingivalis* growth to the chitosan-based membrane in a dose-dependent way. Chlorhexidine and silver supplementation to chitosan membranes also provided antibacterial efficacy against *Staphylococcus aureus*. The MIC of chitosan-based membranes supplemented with BisBAL NPs was defined at 100 µM against six different types of pathogens. Chitosan-based membranes supplemented with 5, 10 or 15% w/w of silver nanoparticles showed good antimicrobial activity against gram-positive and gram-negative bacteria. A similar phenomenon has been reported for silver supplementation to chitosan membranes, which inhibited *Porphyromonas gingivalis* and *Fusobacterium nucleatum* growth in a dose-dependent way. However, silver nanoparticles have an important cytotoxicity on human cells limiting their application on clinical practice. In the other hand, BisBAL NPs showed absence of cytotoxic effect on human epithelial and blood cells after 24 h of exposition. Altogether these results suggest that BisBAL NPs contribute bactericidal and antymycotical activity to chitosan-based membranes being effective against a broad spectrum of pathogens, including gram-positive, gram-negative, and multiresistant species like MRSA. Furthermore, BisBAL NPs caused an inhibition of bacterial biofilm formation of 89% or more in comparison with chitosan membrane alone. No differences were found between *P. gingivalis* and a sample isolated from a patient with periodontal disease biofilms after treatment. Bacteria susceptibility against different antimicrobial agents can arise and depends on several factors. Among our results all microorganisms studied were sensitive to BisBAL NPs. Interestingly, *P. gingivalis* and *S. gordonii* were more resistant to BisBAL NPs, and 100 µM were needed to inhibit more than 90% of bacterial growth. In contrast, 80% of *E. faecalis* growth was decreased since the addition of 10 µM BisBAL NPs. We hypothesized that differences among gram-positive and gram-negative influences the effect of BisBAL NPs and also their growing speed. The incorporation of silver nanostructures into chitosan-polyethylene glycol membranes also reduced the biofilm formation of *Escherichia coli* and *Staphylococcus aureus*. Chitosan-based membranes supplemented with BisBAL NPs inhibited the early colonization of potentially pathogenic microorganisms preventing bacterial infection after surgery. Bacteremia after oral surgery is common and that increases the risk for bacterial endocarditis. Rahn et al., described the efficacy of povidone-iodine and chlorhexidine in the prevention of post-treatment bacteremia in 120 dental patients.

To verify that the antimicrobial BisBAL NPs were not cytotoxic for human cells, the IC₅₀ of BisBAL NPs for HGFs was determined. At 100 µM of BisBAL NPs, cell viability was 84%. These results indicate that at 100 µM, BisBAL NPs contribute antimicrobial and antibiofilm properties to chitosan-based membranes without having a cytotoxic effect on HGFs. Likewise, BisBAL NPs are not cytotoxic for human erythrocytes and epithelial cells.
CONCLUSION

BisBAL NPs provide antimicrobial and antibiofilm properties to chitosan-based membranes without adverse effects on HGFs. It constitutes an innovative alternative of GTR to prevent the risk of bacterial infections locally.

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