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Research Article

Effect of Lipophilic Bismuth Nanoparticles on Erythrocytes

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Lipophilic bismuth dimercaptopropanol nanoparticles (BisBAL NPs) have a very important antimicrobial activity; however their effect on human cells or tissues has not been completely studied. Undesirable effects of bismuth include anemia which could result from suicidal erythrocyte death or eryptosis. The objective of this research was to determine the effect of bismuth dimercaptopropanol nanoparticles on blood cells. The nanoparticles are composed of 53 nm crystallites on average and have a spherical structure, agglomerating into clusters of small nanoparticles. Based on cell viability assays and optical microscopy, cytotoxicity on erythrocytes was observed after growing with 500 and 1000 μM of BisBAL NPs for 24 h. AM Calcein was retained inside erythrocytes when they were exposed to 100 μM (or lower concentrations) of BisBAL NPs for 24 h, suggesting the absence of damage in plasmatic membrane. Genotoxic assays revealed no damage to genomic DNA of blood cells after 24 h of exposition to BisBAL NPs. Finally, 100–1000 μM of bismuth nanoparticles promotes apoptosis between blood cells after 24 h of incubation. Hence BisBAL NPs at concentrations lower than 100 μM do not cause damage on blood cells; they could potentially be used by humans without affecting erythrocytes and leukocytes.

1. Introduction

Bismuth is used in industry and for treatment of gastrointestinal diseases [1, 2]. Previous reports described the very interesting antimicrobial and antibiofilm properties of bismuth nanoparticles with significant potential to be used in biomedical sciences [3–5]. Since elemental bismuth has very low water solubility, it has been chelated with compounds containing hydroxyl and sulfhydryl groups to produce water soluble and biocompatible complexes that are employed in pharmaceutical and personal care products (e.g., Pepto Bismol, De-Nol, and Tirtec (Pylorid), catalysts, alloys, and pigments) [6].

Early, the antimicrobial activity of nanostructures of several metals like silver, gold, zinc, titanium, and bismuth has been described with very good results [7–11]. However, most of them present high toxicity on human cells, limiting their use [12–15]. Several approaches were made to reduce the undesired effects of metal nanoparticles; the most followed one is adding a biocompatible cover of cellulose, chitosan, or polymers like poly(lactic-co-glycolic acid) PLGA [16].

The toxicological aspects of bismuth compounds are well established and the reported side effects may include nephropathy, hepatitis, and encephalopathy [17]. Several studies described the absence of cytotoxicity of bismuth and their compounds on human cells [18–20], and our group has reported no cytotoxicity of 2 mM bismuth oxide nanoparticles on monkey kidney cells, after 24 hours of exposition [4]. The untoward effects of bismuth include anemia [21], which could at least in theory result from stimulation of suicidal erythrocyte death or eryptosis.

Eryptosis, the suicidal death of erythrocytes, is caused by several anemia-inducing endogenous substances, diabetes, chronic renal failure, hemolytic uremic syndrome, sepsis,

malaria, iron deficiency, and exogenous substances [22]. Several drugs have been described to trigger eryptosis, like alantolactone, gramicidin, naphthazarin, nelfinavir, hemolysin, listeriolysin, paclitaxel, chlorpromazine, cyclosporine, methylglyoxal, amyloid peptides, anandamide, Bay-5884, curcumin, valinomycin, aluminium, mercury, lead, and copper [22–26]. Eryptosis is characterized by phosphatidylserine exposure at the erythrocyte surface [27]. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and result from phospholipid scrambling of the cell membrane [28].

The objective of this work was to determine whether BisBAL NPs trigger eryptosis in a cell culture. The effect of BisBAL nanoparticles with an average diameter of 53 nm and spherical shape was evaluated on blood cells. Based on cell viability assays and optical microscopy, cytotoxicity on erythrocytes was observed after growing with high concentrations (500–1000 μM) of BisBAL NPs for 24 h. This data correlates with hemoglobin level of erythrocytes exposed to the same concentrations of bismuth nanoparticles. Calcein AM was retained inside erythrocytes when they were exposed to 100 μM (or lower concentrations) of BisBAL NPs for 24 h, suggesting the absence of damage in plasmatic membrane of host cells. Genotoxic assays revealed no damage to genomic DNA of blood cells after 24 h of exposition to BisBAL NPs and finally 500–1000 μM of bismuth nanoparticles promotes apoptosis between blood cells after 24 h of incubation. All together, these results suggest that ≤100 μM of BisBAL NPs does not present side effects on blood cells and could be used in humans.

2. Material and Methods

2.1. Synthesis of BisBAL Nanoparticles. For the synthesis of bismuth nanostructures, the following chemical reagents were used: bismuth pentahydrate ($Bi(NO_3)_3{\cdot}5H_2O$), 2,3-dimercapto-1-propanol (BAL), sodium borohydride ($NaBH_4$), and propylene glycol, which were all analytical grade reagents purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water (Barnstead Nanopure Diamond) was used to prepare solutions and dilutions. BisBAL nanoparticles were synthesized using a method that was described in our recent publications. A stock solution of 2 : 1 molar ratio of Bi (Bis) to 2,3-dimercapto-1-propanol (BAL) (a.k.a. BisBAL) served as a cationic precursor for the BisBAL nanoparticles and the choice of molar ratio was based on the previous work, which showed BisBAL was stable over a wide pH range (4–11) and effective against microbial biofilm formation [29]. During the course of BisBAL reduction with $NaBH_4$, the pink color of soluble BisBAL instantly transformed to a black colored suspension composed of BisBAL nanoparticles. The stock suspensions of 25 mM of BisBAL nanoparticles in 10 mL batches were prepared and stored at 4°C until use.

2.2. Characterization of BisBAL Nanoparticles. The nanoparticle size distribution was measured by dynamic light scattering (DLS, ALV-GmbH, Germany, scattering angle set at 90°). Information on the shape and size of nanoparticles was obtained using scanning electron microscopy (SEM; FEI Tecnai G2 Twin, Hillsboro, OR; 160 kV accelerating voltage). Absorbance spectra of the nanoparticle suspensions were collected using the UV-Visible spectrophotometer (SpectraMax Plus384 Absorbance Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA). The rhombohedral crystallinity and crystallite size were determined using the X-Ray Diffractometer (XRD: Panalytical X'Pert PRO MRD) equipped with Cu Kα as X-ray source (λ = 1.541874 Å). Diffractograms were interpreted using the Debye-Scherrer formula (Panalytical X'Pert Data Viewer software) to estimate the rhombohedral structure and crystallite size. The lipophilicity of BisBAL nanoparticles was determined based on the affinity of nanoparticles to 1-octanol in 1-octanol-water phase mixture incubated for 30 minutes. The absorbance of nanoparticles in a phase was measured at 330 nm.

2.3. Blood Cells Culture and Their Quantification by MTT Assay. From a blood sample without anticoagulant erythrocytes, neutrophils and leucocytes were collected and separated by centrifugation at 14000 r.p.m. for 10 minutes [30]. After that, serum was retired from pellet of each fraction and cells were washed three times with cold PBS 1x. Erythrocytes, neutrophils, and leucocytes were cultured in minimum essential medium (MEM) supplemented with 10% of fetal bovine serum, respectively (FBS, Biowest, Nuaillé, France), onto 96-well plates (10^5/well for 24 h) in triplicate at 37°C in a humidified incubator containing 5% CO_2. The cell viability was evaluated by the amount of viable cells stained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Biotium, Hayward, CA) which was released with dimethyl sulfoxide (DMSO, Sigma-Aldrich Inc., St. Louis, MO, USA). The optical density was detected at 595 nm using a microplate absorbance reader (Biotek, Winooski, VT). The killing effect of bismuth nanoparticles on blood cells was calculated as cell viability, which was indicated as follows: (the percentage of viable cells) = (absorbance of treated wells − absorbance of blank wells)/(absorbance of the control wells − absorbance of blank wells) × 100%.

2.4. Influence of BisBAL NPs on Morphology of Erythrocytes Culture by Optic Microscopy. From a blood sample without anticoagulant, a monolayer of erythrocytes was obtained by seeding 1×10^5 cells in a 96-well microtiter plate (Thermo Fisher Scientific, MA, USA) in 100 μL of minimum essential medium (MEM) supplemented with 10% of fetal bovine serum (FBS, Biowest, Nuaillé, France) and incubated at 37°C with 5% of CO_2. 50, 100, 500, and 1000 μM of BisBAL NPs were added to cell culture and incubated for 1 hour at 37°C with 5% of CO_2 and their effect on cell morphology was observed by optical microscopy using an inverted microscope (Motic, Hong Kong). The presence of cytopathic effect was identified by amorphous shape, light refractive cells, and loss of confluent monolayer.

2.5. Effect of BisBAL NPs on Blood Cells. The possible cytotoxicity of BisBAL NPs against erythrocytes, leukocytes, and neutrophils was studied using the cell viability MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Biotium, Hayward, CA) [31]. Following the protocol

described above, 1×10^5 cells were incubated at 37°C and 5% of CO_2 overnight with 0, 5, 10, and 20 μM of BisBAL NPs and free drug cells with culture medium alone as untreated cells. After incubation, 10 μL of MTT was added to each well and incubated at 37°C and 5% of CO_2 for 2 hours in dark conditions, following which the medium was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the reduced MTT formazan product. The reduced MTT was then assayed at 595 nm using a microplate absorbance reader (Biotek, Winooski, VT) and DMSO was employed as blank. The assay was done in triplicate and the measured optical density was further analyzed by descriptive statistics.

2.6. Effect of BisBAL NPs on Hemoglobin Level. To analyze the hypothesis if the addition of BisBAL NPs to a cell culture could trigger erythrosis, the hemoglobin (Hb) level was determined by Coulter STKS [32] (Coulter Corporation, Hialeah, FL, USA), following the instructions of provider. After 24 h incubation, the nanoparticles solution was removed from cell culture and washed three times with PBS 1x and the level of Hb was measured. The assay was done in triplicate and the measured concentration was further analyzed by descriptive statistics.

2.7. Influence of Bismuth Nanoparticles on Plasmatic Membrane of Erythrocytes by AM Calcein Assay and Fluorescence Microscopy. Based on the protocol described above, the influence of BisBAL NPs on erythrocytes was evaluated by AM Calcein assay and fluorescence microscopy. After treatment with 50–1000 μM of BisBAL NPs over 24 hours, cells were washed three times with PBS and stained with AM Calcein (Biotium, Hayward, CA) [33, 34]. The cytotoxicity and integrity of cell membrane were interpreted as not Calcein AM retained in the interior of cells with FITC filter at 485 nm (Thornwood, NY).

2.8. BisBAL NPs on Genomic DNA of Erythrocytes by Comet Assay. To determine the possible damage in genomic DNA of erythrocytes after exposition to BisBAL NPs, Oxiselect Comet Assay was employed (Cell Biolabs, Inc., CA, USA), following the instructions of provider [35]. Briefly, erythrocytes were incubated at 37°C and 5% of CO_2 overnight with 20 μM of BisBAL NPs or 10% H_2O_2 (Sigma, SL., USA) as positive control and free drug cells with culture medium alone as untreated cells. After incubation, cells were collected by centrifugation at 700 ×g for 2 minutes and discarding the supernatant. Cells were washed with PBS and combined with comet agarose at ratio 1 : 10 and pipetted 75 μL/well onto the OxiSelect Comet Slide. The slide was maintained horizontally and it was transferred to 4°C in the dark for 15 minutes. Carefully, the slide was transferred to a container with lysis buffer (25 mL/slide) and was incubated for 30 minutes at 4°C in the dark. The lysis buffer was replaced with alkaline solution (25 mL/slide) and was again incubated for 30 minutes at 4°C in the dark. The slide was transferred to a horizontal electrophoresis chamber applying a current setting of 300 mA for 30 minutes. After that, the slide was washed with sterile milliQ water and water was replaced with cold 70% ethanol for 5 minutes. Ethanol was removed and slide was allowed to air dry. Once the agarose and slide were completely dry, it was added to 100 μL/well of diluted vista green DNA dye and incubated at room temperature for 15 minutes. Slides were viewed by epifluorescence microscopy using a FITC filter (Thornwood, NY).

2.9. Apoptotic Effect of BisBAL Nanoparticles on Erythrocytes. To analyze if BisBAL NPs could lead to apoptosis after incubation with erythrocytes, the CF 488A/7-AAD Apoptosis Assay kit was employed [36]. Following the instructions of provider (Biotum, Hayward, CA), a confluent monolayer of erythrocytes grown with 20 μM of BisBAL NPs for 24 hrs was harvested and washed with 1x of PBS. After that, cells were resuspended with 1x binding buffer and aliquots of 100 μL/tube were made. 5 μL of CF488A-Annexin V and 2 μL of 7-AAD working solution were added to the cells and incubated at room temperature for 30 minutes in the dark. Finally, 400 μL of binding buffer was added to each tube and cells were analyzed by flow cytometry at 488 nm in a flow cytometer BD Accuri C6 (BD Biosciences, San Jose, CA, USA).

3. Results

3.1. Characterization of BisBAL NPs. Bismuth nanoparticles obtained were spherical in shape with the number-weighted average hydrodynamic diameter of 53 nm (Figure 1). The nanoparticles are composed of rhombohedral crystallites (≈18 nm) with dithiols as lipophilic surface chemical groups and the lattice spacing of individual crystallite was 0.325, which is consistent with nanoscale bismuth nanoparticles [37]. UV-Vis absorbance measurements revealed that the nanoparticles had greater (≈70%) affinity towards 1-octanol rather than water, which further suggests that lipophilic property of the nanoparticles arises from the dithiols bounds to nanoparticle surface.

3.2. Morphology of Erythrocytes after Exposition to BisBAL NPs. In order to determine a possible alteration in the morphology of erythrocytes growing with lipophilic bismuth nanoparticles, a monolayer of erythrocytes was exposed to several concentrations of BisBAL NPs (5, 20, 50, 100, and 1000 μM) for 1 hour. After this time, cells were observed finding changes in cell morphology among cells growing with 500 and 1000 μM of BisBAL NPs (Figure 2). These cells were light refractive and become rounded out of monolayer. In contrast, cells growing with 5–100 μM of BisBAL NPs did not show significant changes in their appearance when they were compared with cells growing in culture media without any drug.

3.3. Impact of BisBAL Nanoparticles on Erythrocytes, Leukocytes, and Neutrophils Count and Hemoglobin Level. With the objective of characterizing in deep the effect of BisBAL NPs on blood cells, cell viability MTT assays and hemoglobin level were carried out. The results obtained described a little decrease in the count of blood cells and hemoglobin level when 50–1000 μM of BisBAL NPs was added to culture cells for 18 h (Figure 3). The addition of 1% SDS or bulk (1000 μM of bismuth nitrate) reduced cell growing in approximately

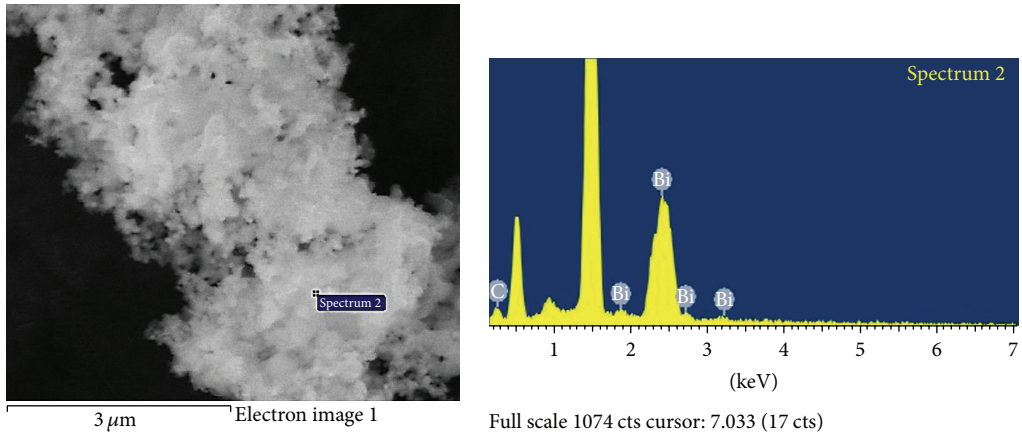

Figure 1: Lipophilic bismuth nanoparticles visualized by scanning electron microscopy. The dominant population of spherical shaped nanoparticles (<100 nm) showing the nanoparticle clusters interspersed among the lesser electron dense materials is shown in the TEM images (a). (b) Spectrum of elements in the sample observed by SEM.

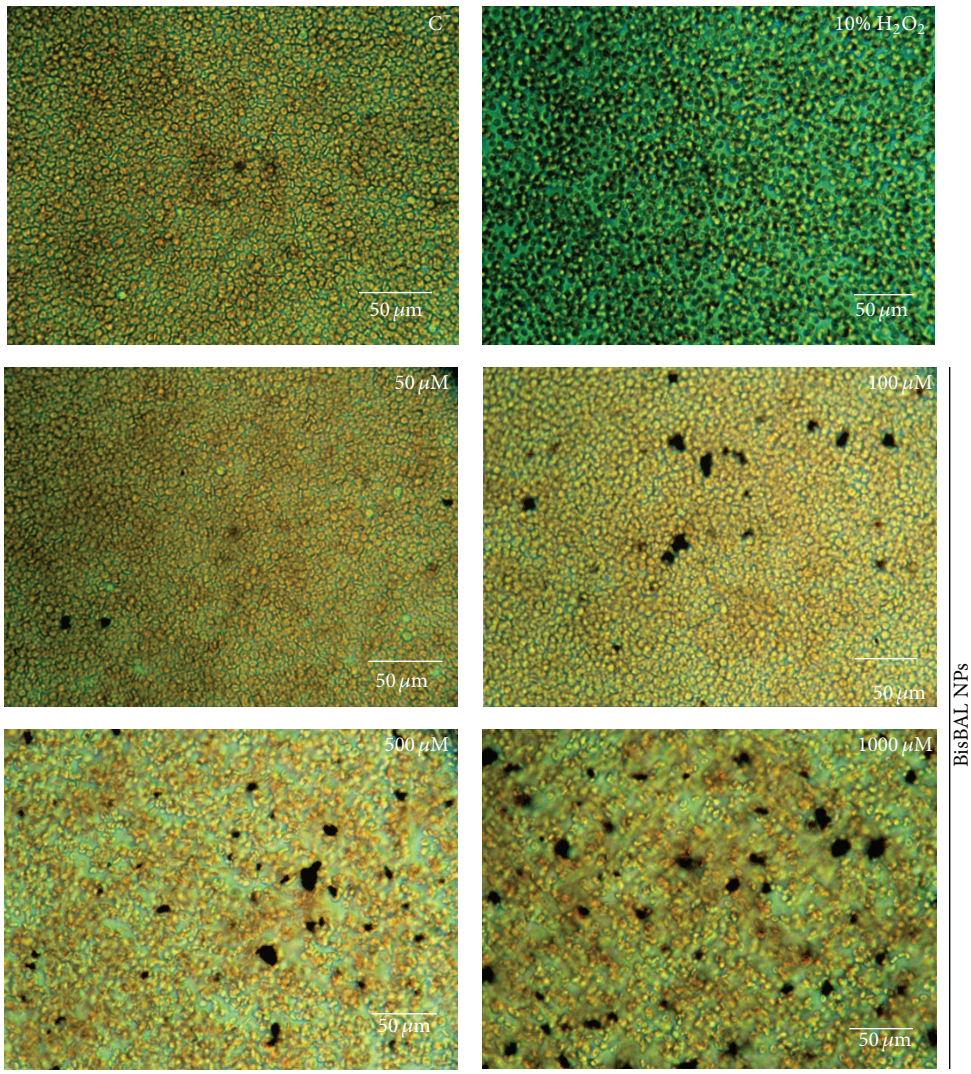

Figure 2: Effect of BisBAL nanoparticles on erythrocytes morphology. A monolayer of erythrocytes was obtained from a blood sample in 100 μL of minimum essential medium. 50, 100, 500, and 1000 μM of BisBAL NPs were added to cell culture, incubated for 1 hour at 37°C with 5% of CO_2 and their influence on erythrocyte culture was observed by optic microscopy using an inverter microscope (Motic, Hong Kong). The presence of cytopathic effect was identified by amorphous shape and light refractive cells. The bar indicates a size of 50 μm.

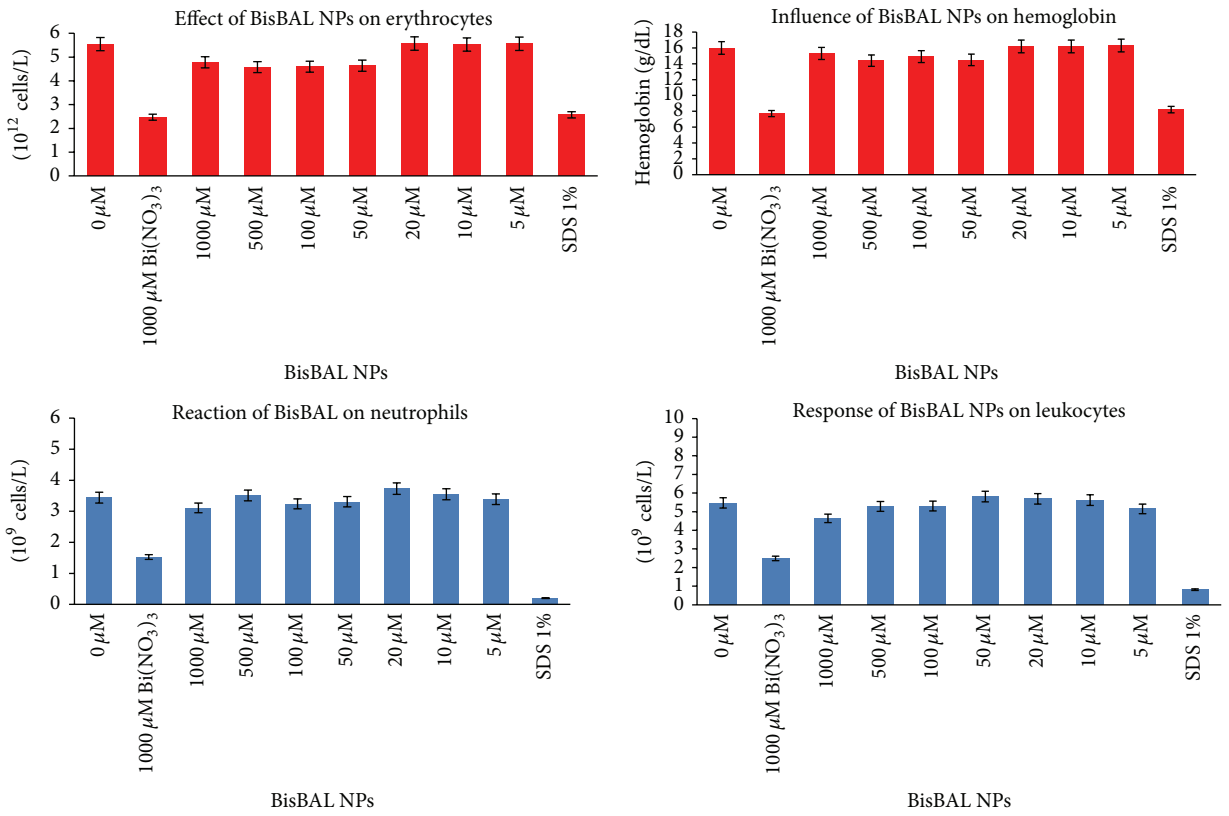

FIGURE 3: Impact of BisBAL nanoparticles on erythrocytes, leukocytes, and neutrophils count and hemoglobin level. Cell viability MTT assays were performed to determine the influence of 0, 5, 10, 20, 50, 100, 500, and 1000 μM of BisBAL NPs, added to an erythrocytes, leukocytes, and neutrophils monolayer and incubated for 24 hrs. 1% of SDS was used as positive control and 1000 μM of bismuth nitrate (bulk) was employed to compare with bismuth nanostructures. After that, the number of erythrocytes, leukocytes, and neutrophils and hemoglobin level were determined.

50% (Figure 3), showing a clear difference with same concentration of the bismuth nanocomposite. The viability did not change among cells growing with 0–20 μL of BisBAL NPs with the same results in the level of hemoglobin. All these results suggest that bismuth nanoparticles did not cause significant damage on human blood cells in the experimental conditions analyzed.

3.4. Influence of Bismuth Nanoparticles on Cell Membrane of Erythrocytes by AM Calcein Assay and Fluorescence Microscopy. Previous results indicated a similar influence of BisBAL NPs on blood cells, which could mean the same target on this kind of cells. To analyze if plasmatic membrane of erythrocytes was affected by the presence of bismuth nanoparticles, a monolayer of erythrocytes was incubated with 50, 100, 500, and 1000 μM of BisBAL NPs overnight.

After this, Calcein AM was added which is retained in the cytoplasm of living cells. When cells were observed by fluorescent microscopy, Calcein AM was retained only at 50 and 100 μM of BisBAL NPs (Figure 4). In contrast, erythrocytes growing with 500 or 1000 μM of BisBAL NPs did not retain the Calcein into their cytoplasm. The same result was found when red cells were growing with 10% of hydrogen peroxide (Figure 4). Altogether these results strongly suggest that plasmatic membrane is affected by interaction with bismuth nanoparticles at high concentrations.

3.5. BisBAL Nanoparticles on Genomic DNA of Erythrocytes by Comet Assay and Fluorescence Microscopy. To determine a possible damage on genomic DNA of erythrocytes by bismuth nanoparticles, genotoxic assays were developed, employing the Comet Assay and fluorescence microscopy. As can be seen in Figure 5, after incubation with 1–1000 μM of BisBAL NPs, the nuclei and indeed the entire cell appeared to be identical to nontreated cells, suggesting the absence of toxic effects on genomic DNA of erythrocytes by BisBAL NPs under our experimental conditions. In cells growing with 10% of H_2O_2 the classic stela of a comet was detected (Figure 5) indicating DNA damage. Based on these results, bismuth nanoparticles do not seem to affect DNA of host cells.

3.6. Apoptosis Performed by Erythrocytes Growing with Bismuth Nanoparticles. Based on last experiments, it was explored if bismuth nanoparticles could lead to apoptosis of host cells using the CF 488A/7-AAD Apoptosis Assay kit and

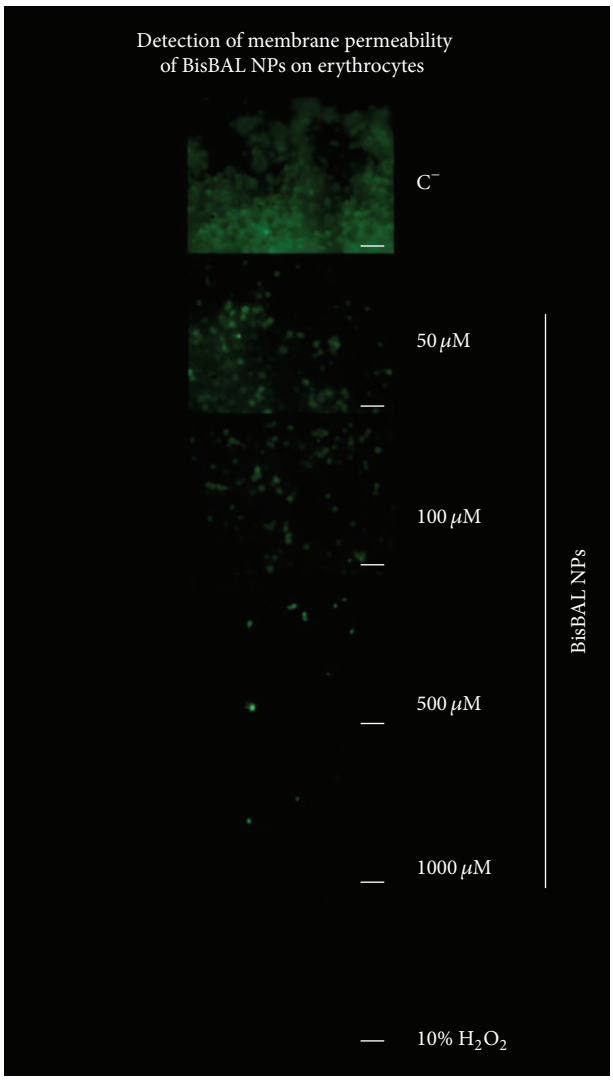

Figure 4: Influence of bismuth nanoparticles on membrane cell of erythrocytes by AM Calcein assay and fluorescence microscopy. Blood cells were treated with same techniques described above. After 24 h of incubation, the erythrocytes were stained with AM Calcein and observed under fluorescence microscopy at 485 and 358 nm (Thornwood, NY). The images were analyzed by using AxioVision software (Thornwood, NY). The bar indicates 5 μm.

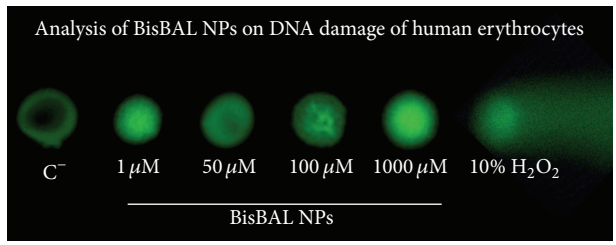

Figure 5: BisBAL nanoparticles on genomic DNA of erythrocytes by Comet Assay and fluorescence microscopy. To determine the possible damage in genomic DNA of erythrocytes after exposition to BisBAL NPs, Oxiselect Comet Assay was employed. Erythrocytes were incubated at 37°C and 5% of CO_2 overnight with 1–1000 μM of BisBAL NPs or 10% H_2O_2. Erythrocytes growing only in culture media were used as negative control (C^-). After treatment, blood cells were analyzed by fluorescence microscopy at 485 nm (Thornwood, NY). The presence of a stela is indicative of DNA damage.

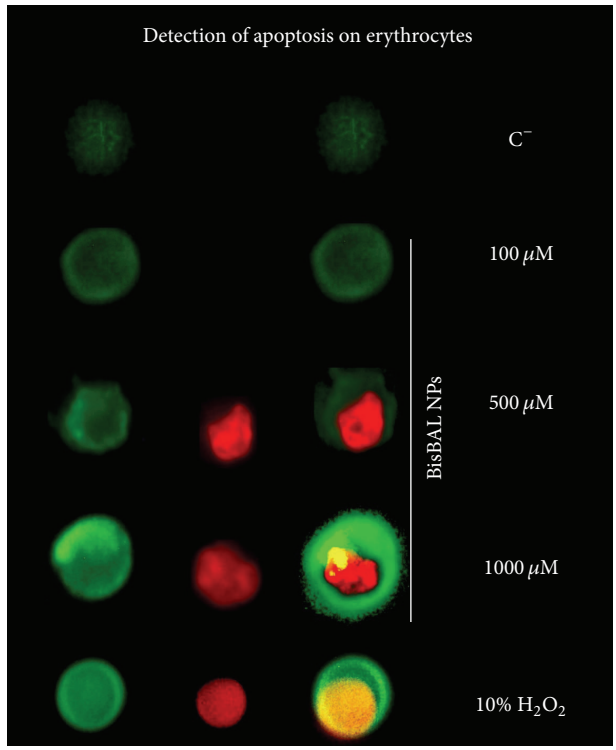

Figure 6: Apoptosis performed by erythrocytes growing with bismuth nanoparticles. The possible leading to apoptosis of blood cells by 100–1000 μM of BisBAL NPs was explored by Annexin V and 7-AAD assay using fluorescence microscopy at 485 nm (Thornwood, NY).

fluorescence microscopy. The results indicate that BisBAL nanoparticles at concentrations 500 and 1000 μM promote apoptosis among erythrocytes (Figure 6). The fluorescent conjugate of Annexin V interacting with phosphatidylserine exposed by erythrocytes was clearly observed by fluorescence microscopy (Figure 6). 7-AAD (7-aminoactinomycin D) is a membrane-impermeant DNA-binding dye that is excluded by live cells; as can be seen in Figure 6, it was present in erythrocytes growing with 500 and 1000 μM of BisBAL NPs, suggesting their necrotic state. There was no difference between 100 μM of BisBAL NPs and control (cells growing with only culture media). These results suggest that high concentrations of bismuth nanoparticles inhibit erythrocytes growth by altering basic functions and promoting programmed cell death.

4. Discussion

In this paper we described the synthesis and characterization of lipophilic bismuth nanoparticles and their effect on blood cells. These kinds of nanoparticles have strong antimicrobial and antibiofilm activities described by our group and others [5]. However, the lack of strong experimental evidence of their potential cytotoxicity limits their use in humans. The toxicological aspects of bismuth compounds are well established and the reported side effects include nephropathy, hepatitis, encephalopathy, gastroenteritis, and osteoarthropathy [38–41]. It has been published that bismuth salts cause extensive cytotoxicity on epithelial cells at final concentrations of 2.5 mg/mL [42]. More recently, it was described that bismuth nanoparticles are nontoxic at concentration of 0.5 nM. Nanoparticles at higher concentrations (50 nM) kill 45, 52, 41, and 34% of HeLa cells for bare nanoparticles, amine terminated bismuth nanoparticles, silica coated bismuth nanoparticles, and polyethylene glycol (PEG) modified bismuth nanoparticles, respectively [43]. However, our group has reported no cytotoxic effects on monkey kidney cells at 2 mM of bismuth oxide nanoparticles after 24 h. In this work, the presented data shows the influence of lipophilic bismuth nanoparticles on blood cells. Side effect was not detected when BisBAL nanoparticles were added to human culture cells. Tight decreasing was observed in cells count when 50–1000 μM of BisBAL NPs was added (Figure 3). Hemoglobin level correlated with blood cells alteration, supporting the hypothesis of no toxic effects of BisBAL nanoparticles. Previously, Braun et al. have described that 48 h exposure to ≥500 μg/L bismuth stimulated eryptosis promoting apoptosis, increasing cytosolic Ca^{2+} activity and phosphatidylserine expression on cell surface, decreasing erythrocyte size, and leading to erythrocyte death [44]. This datum correlates with our finding using 1000 μM of bulk (bismuth nitrate) inhibiting 50% of cell growth (Figure 3). In our results, employing 500 and 1000 μM of BisBAL NPs, the phosphatidylserine expression on cell surface and decreasing in erythrocyte size were observed (Figures 2 and 6). Swy et al. described histopathological changes in rats treated with 20 mg kg^{-1} of bismuth nanoparticles PGLA encapsulated, including transient kidney injury and periportal inflammatory process in the liver [45]. However, ≤100 μM of bismuth nanoparticles did not cause erythrocytes death, keeping their normal morphology and membrane integrity without leading to apoptosis (Figures 2, 4, and 6). This datum is very important taking into account that early reports indicate that BisBAL NPs kill pathogenic microorganisms at 0.5–10 μM as final concentration [5] and compete in efficiency with antibiotics like vancomycin as broad-spectrum antimicrobial agents [3–5, 46]. Altogether these results suggest that lipophilic bismuth nanoparticles could be used in humans without altering blood cells provided that they were used at final concentrations lower than 100 μM.

The action mechanism of how bismuth or its compounds damage cells is unknown. Early report of Swy et al. using doses lower than 20 mg kg^{-1} of bismuth nanoparticles PLGA encapsulated were internalized into cells and remain into the cytoplasm without side effects [45]. Based on our experiments with Calcein AM and fluorescence microscopy, we can argue that in high doses (500–1000 μM) BisBAL nanoparticles enter the cell and stock into cytoplasm of host cells. The nanoparticles entry will alter the plasmatic membrane permeability of host cells, modifying their homeostasis and metabolism and finally leading to apoptosis.

5. Conclusions

In summary, lipophilic bismuth nanoparticles (BisBAL NPs) were not toxic to blood cells at final concentrations lower than 100 μM. Up to 500 μM, BisBAL NPs cause damage to plasmatic membrane of host cells, thereby leading to apoptosis or necrosis in the long term.

Conflict of Interests

The authors of this paper declare that there are not any potential competing interests regarding the publication of this work.

Acknowledgments

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