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WATER DISPERSIBLE METAL OXIDE NANOBIOCOMPOSITE AS A POTENTIATOR OF THE ANTIMICROBIAL ACTIVITY OF KANAMYCIN

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Abstract

This study reports the evaluation of a water dispersible metal oxide nanobiocomposite based CS and magnetite as a potential drug delivery system and its cytotoxicity. The results proved that loading kanamycin sulfate into the water dispersible metal oxide nanobiocomposite improve the delivery of this drug in active form reducing minimum inhibitory concentration of kanamycin by two (in case of S. aureus) to four folds (in case of E. coli), as compared with the kanamycin control. Furthermore, cytotoxicity test revealed that the nanobiocomposite has a very low toxic effect on eukaryotic cells. Our data suggest that water soluble metal oxide nanobiocomposite derivative is a good candidate for developing alternative strategies for enhancing the activity of antimicrobial drugs, without increasing the amount of the loaded active compound.

Keywords *Nanocomposite, drug delivery, kanamycin, metal oxide, flow cytometry*

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Introduction

Antibiotics have been used for the last 70 years to treat patients with infectious diseases. When prescribed and taken correctly, antibiotics value in patient care is enormous. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted, making the drugs less effective. Even though strategic priorities for combating antimicrobial resistance are periodically proposed [1] their efficiency cannot be immediately quantified. Since the development of new antimicrobials is an elaborate process, strategies aimed to improve natural and artificial antimicrobial compounds are one of the greatest expectations for fighting against resistant infections. Due to the high toxicity of antibiotics, intelligent antimicrobial approaches intend to use carrier systems to control drugs release and to maximize their target activity [2-5]. Ideal carriers should be soluble, biocompatible and biodegradable polymers with low cytotoxic activity [6,7]. Magnetite (Fe_3O_4) is an interesting cubic inverse spinel structured material widely used in biomedical applications, such as magnetic resonance imaging [8], bio-separation [9], drug targeting [10,11] and hyperthermia [12,13]. For biomedical applications magnetite nanoparticles are often treated with polymers meant to modify their surface, and consequently, increasing chemical stability and improving their biocompatibility. Polymeric magnetic nanoparticles used for drug transporting and releasing contain a magnetic nucleus and a biodegradable polymer shell [14]. Chitosan is a biopolymer exhibiting many useful features such as hydrophilicity, biocompatibility and biodegradability that makes it a good candidate for bio therapeutic approaches [15]. Recently published studies report the incorporation of cephalosporins of second, third and fourth generation into magnetic chitosan

microspheres with an improved delivery of antibiotics in active forms [16]. Kanamycin is a widely used natural aminoglycoside bactericidal antibiotic isolated from the bacterium *Streptomyces kanamyceticus* [17]. Despite its antimicrobial efficiency, kanamycin use can be restricted due to its side effects including high toxicity and allergenic potential when used in high doses [18]. The aim of this study was to use water dispersible metal oxide nanobioscompsite as a molecular carrier for controlled release of kanamycin sulfate in order to maximize its antimicrobial activity without increasing the amount of the active drug.

Experiment Details

Materials. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3$), ammonia (NH_3), methanol (CH_3OH), and chitosan (CS, 85 % deacetylated) were purchased from Sigma-Aldrich.

Fabrication. Magnetite is usually fabricated by wet chemical precipitation from an aqueous iron salts solution into the alkaline media [19-21]. Briefly, chitosan powder was added into acetic acid aqueous solution under vigorous stirring and then $Fe^{3+}/Fe^{2+}= 2$ was added into the chitosan containing solution. The mixture was stirred and dropped into the NH_3 solution leading to the formation of a black precipitate. In order to obtain the water soluble magnetic biocomposite, the product was grounded in the presence of ultrapure water and 1N acetic acid as mentioned recently [22]. The content of metal oxide existing in the fabricated nanobiocomposite was identified as magnetite. FT-IR identified the functional groups of chitosan in the nanobiocomposite and SEM reveals that magnetite was successfully integrated into the polymer matrix with no visible agglomerate formation at low particle amounts .

Adsorbtion of antibiotic on the surface of water soluble nanobiocomposite. The nano-support and the antibiotic to be adsorbed (kanamycin sulfate) were mixed in the presence of 2 mL of ultrapure water until the latter completely evaporated at 40°C. The amount of the antibiotic adsorbed on the nano-support was selected at 10 %.

Antimicrobial activity assessment. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 bacterial strains were used in the study. Quantitative testing of the antimicrobial activity of nanobioscomposite and the establishment of minimum inhibitory concentration (MIC) were determined by microdilution technique in standardized Mueller Hinton broth, using 96 multiwell plates [23,24]. Briefly, twofold serial microdilutions were achieved in 200 µL medium, the dilution range varying, depending on the bacterial strain, in accordance with CLSI breakpoints. Subsequently, the wells were seeded with 50 µL of bacterial suspension, with a density adjusted to an optical density corresponding to 0.5 MacFarland standards. After incubating the plates at 37°C for 24 hours, OD600 nM was achieved using an ELISA reader machine and MIC values were calculated.

Cytotoxicity assay

Cells: HCT8 (CCL-244TM) and Hep2 (CCL-23TM) lines were used for assessing cytotoxicity of CS Fe_3O_4. Cells were cultivated in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated foetal bovine serum (Sigma) and maintained at 37°C, 5% CO_2, in a humid atmosphere.

Apoptosis detection: 3.5 x 10^5 HCT8 or HEp2 cells were seeded in 3.5 cm diameter Petri dishes and treated with 100 µg/mL CS Fe_3O_4 for 24 hour. The total cells were resuspended in 100 µL binding buffer, and stained with 5µL Annexin V-FITC and 5µL propidium iodide, and maintained in dark. At least 10,000 events from each sample were acquired using EPICS XL Beckman Coulter flow cytometer. The percentage of treatment affected cells was determined by subtracting the percentage of apoptotic/necrotic cells in the untreated population from the percentage of the apoptotic cells in the treated population.

Cell cycle distribution: 3.5 x 10^5 cells were plated in 3.5 cm diameter Petri dishes and treated with 100 µg/mL CS Fe_3O_4, for 24 hours. After the treatment period, the cells were taken from the substrate, fixed in 70% cold ethanol for at least 30 minutes at -20°C, washed twice in phosphate buffered saline (PBS), and then incubated for 15 min, at 37°C, with RNase A (100 µg/mL), and 1 h with 100 µg/mL propidium iodide.

After staining, the acquisition was done using Epics XL Beckman Coulter flowcytometer. Data were analyzed using FlowJo software and expressed as fractions of cells in different cycle phases.

Results and Discussions

Although aminoglycosides are largely used in the treatment of serious systemic infections, their use is limited because of their side- effects, mainly oto- and nephrotoxicity occurred in 15%–17% of patients, hearing loss (8%), vestibular (3%) and retina toxicity [25]. Taking into account these limitations, controlling aminoglycosides concentration is critical in order to reduce their toxic effects. Recent studies report the use of nanocarriers to increase stability and delivery of aminoglycosides in active forms [26-28].

In this paper we report the results concerning the influence of a nanocarrier based on CS, PVA and Fe_3O_4 on the activity of kanamycin used in conventional doses, against Gram-positive and Gram-negative bacterial strains.

The data obtained after assessing kanamycin adsorbed on the surface of water dispersible metal oxide nanobiocomposite antimicrobial effect revealed that the nanobiocomposite has greatly improved the antibiotic activity on both *S. aureus* and *E. coli* tested strains. The MICs of the samples treated with kanamycin loaded on the surface of nanobiocomposite were significantly reduced by two (in case of *S. aureus*) to four folds (in case of *E. coli*), as compared with kanamycin only control (figure 1). This result reveals that water soluble nanobiocomposite potentiates the antimicrobial effect of kanamycin sulfate against *S. aureus* and *E. coli* strains and can be used as an efficient molecular carrier for antimicrobial drugs. Furthermore, our data suggest that nanobiocomposite can be further considered as alternative strategies for enhancing the activity of antimicrobial drugs without increasing the amount of active compound. In order to test if the fabricated water soluble nanobiocomposite has any deleterious effect against eukaryotic cells, we assessed the *in vitro* impact on HCT8 and HEp2 cultures. Cell cycle assay revealed that 10 μg/mL water soluble nanobiocomposite has no effect on either HCT8 or HEp2, and starts to alter cell cycle only when used in higher amounts (figure 2). 100 μg/mL of water soluble nanobiocomposite induced a cytotoxicity of about 10% in both tested eukaryotic cell lines as revealed by the appearance of an additional peak (under G0) on the histogram (figure 3). Under G0 additional peaks usually reveal the presence of dead apoptotic and necrotic cells. Our results demonstrate that the water dispersible nanobiocomposite can be safely used in low concentrations, not being toxic for the host cells, while it becomes cytotoxic if used in very high concentrations, usually unlikely to achieve within the host.

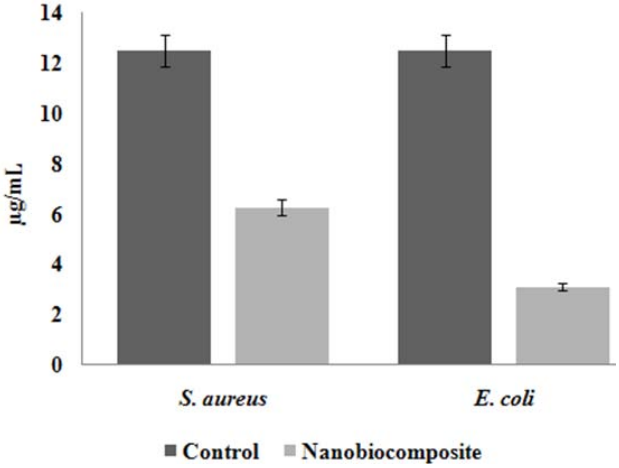

Figure 1: *Graphic representation of the antibacterial activity of plain kanamycin sulfate (control) and kanamycin sulfate adsorbed on the surface of the nanobiocomposite.*

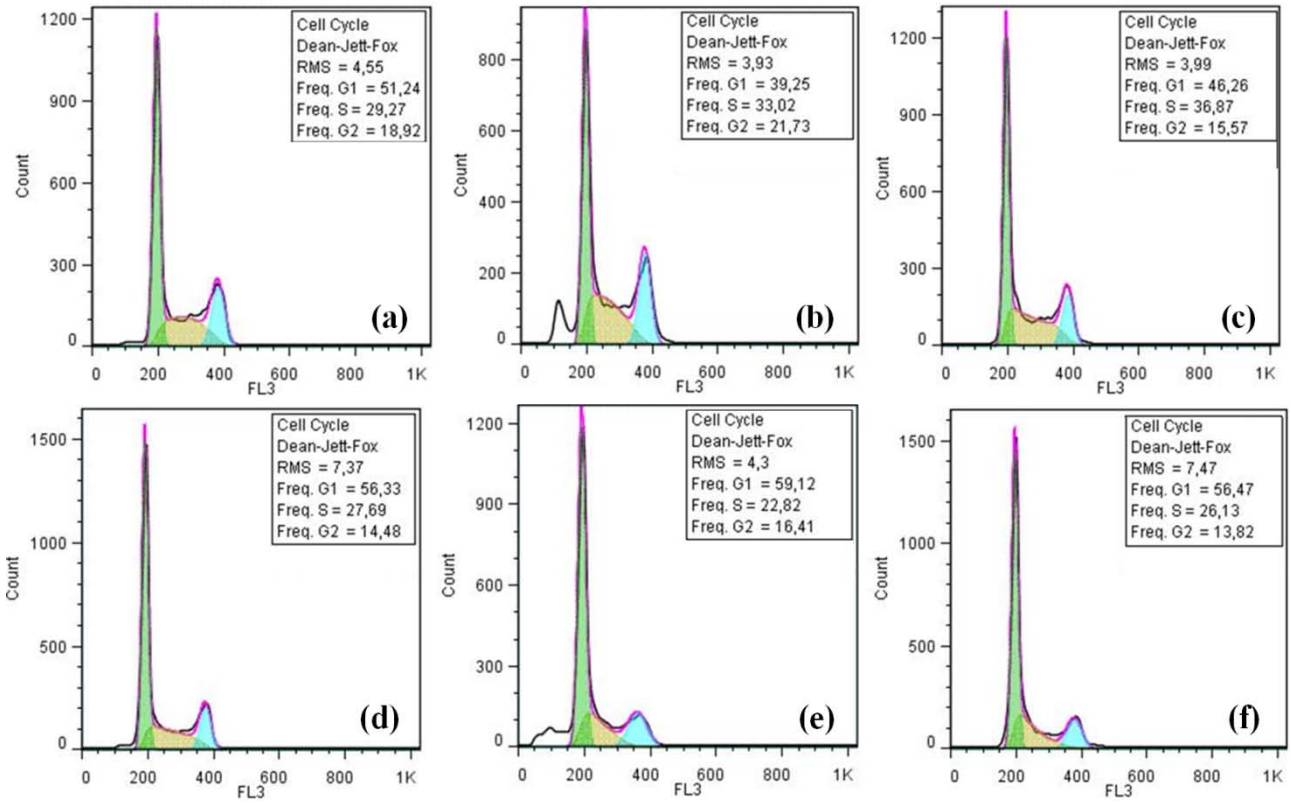

Figure 2: *Quantification of the cell cycle alterations by using 10 and 100µg/mL water dispersible nanobiocomposite in HCT8 (a,b,c) and HEp2 (d,e,f) cell lines after 24 h treatment. (b,e) concentration of 10µg/mL, water dispersible nanobiocomposite does not interfere with cell cycle on either HCT8 or HEp2, while (c,f) 100µg/mL of water dispersible nanobiocomposite induces a slight cytotoxicity, revealed by the occurrence of an additional under G0 peak; (a,d) controls.*

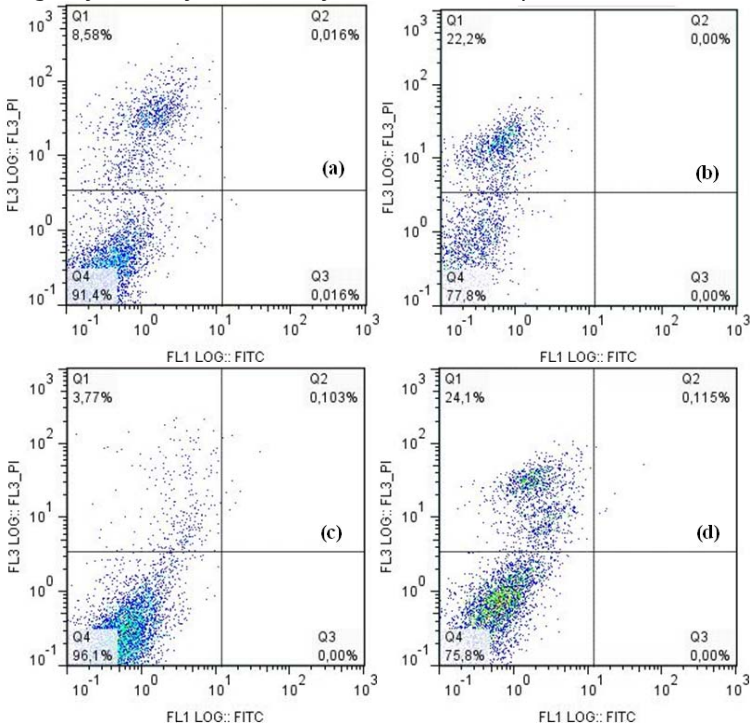

Figure 3: *Dot plots representing analysis of HCT8 (a,b) and HEp2 (c,d) cell death induced after 100g/mL nanobiocomposite treatment for 24h (b,d).*

Conclusions

Water dispersible metal oxide nanobiocomposite significantly enhances the antimicrobial effect of kanamycin sulfate against Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacterial strains. Furthermore, the fabricated nanobiocomposite revealed a low cytotoxic activity against eukaryotic cells at active concentrations. Taken all together, our results demonstrate that water dispersible metal oxide nanobiocomposite is a strong candidate for developing novel antimicrobial strategies aiming to potentiate the activity of antimicrobial drugs at therapeutic doses. This approach eliminates the toxic effects usually exhibited by high amounts of antibiotics against the human host.

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