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MICROBIOLOGICAL APPLICATIONS OF A NEW WATER DISPERSIBLE MAGNETIC NANOBIOCOMPOSITE

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Abstract

Our goal was to fabricate a novel water dispersible nanobiocomposite useful for the improvement of classical antimicrobial therapies. Water-soluble magnetite nanoparticles were protected by chitosan and polyvinyl alcohol to increase system's bioaccommodation. The presence of magnetite was confirmed by XRD. SEM results also indicate that the fabricated nanobiocomposite is composed of nanosized magnetite particles. FT-IR spectrum of the nanobiocomposite, revealed the presence of adsorption peaks specific for PVA, CS and Fe_3O_4. Our results demonstrate that the nanobiocomposite has the ability to modify and improve antimicrobial activity of gentamicin, ciprofloxacin and cefotaxime against S.aureus and P.aeruginosa. Fabricated nanobiocomposite exhibited a low cytotoxic effect on eukaryotic cells being thus a good candidate for developing new antimicrobial strategies aiming to potentate the antimicrobial effect of drugs and controlling their delivery.

Keywords *PVA, CS, magnetite, water soluble nanoparticles, eukaryotic cells, prokaryotic cells.*

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Introduction

Since the introduction of antibiotics in the clinical therapy, bacterial strains have increasingly developed more sophisticated resistance strategies, leading to the occurrence and spread of the so-called 'superbugs', resistant to practically all or most of the antimicrobial drugs available on the market [1]. *Staphylococcus aureus* is a leading cause of a large spectrum of diseases, such as skin and soft tissue infections, pneumonia, bloodstream infections, osteomyelitis and endocarditis, as well as toxin-mediated syndromes, like toxic shock and food poisoning [2]. This bacterium has developed resistance to a wide range of antimicrobial drugs, which complicates the treatment of the generated infections [3,4]. Only approximately 20% of the strains remain sensitive to penicillin, which is still the mainstay of treatment of staphylococcal infection [5]. The frequently isolated methicillin-resistant *S. aureus* (MRSA) is resistant to all beta-lactam antibiotics [6-8]. Glycopeptide antibiotics are effective against most MRSA strains but, in the last few years, isolates of MRSA that have reduced susceptibility to glycopeptides (glycopeptide-intermediate *S. aureus*) have been isolated [9]. Some recent isolates prove to exhibit frank resistance to glycopeptides (vancomycin-resistant *S. aureus*) [10]. *Pseudomonas aeruginosa* is a notoriously difficult organism to control with either antibiotics or disinfectants [11]. It has joined the rank of 'superbug', due to its enormous capacity to engender resistance to different classes of antibiotics [1]. This versatile microbe efficiently uses and combines three of the major mechanisms by which organisms resist the action of antimicrobial agents, i.e.: restricted uptake

and efflux; drug inactivation and changes in targets. As an opportunistic pathogen, *P.aeruginosa* causes persistent infections in immunocompromised and chronic patients, exhibiting enormous antibiotic resistance levels in cystic fibrosis individuals [12]. Persistent bacterial infections represent one of the major causes of mortality and morbidity in vulnerable individuals worldwide and their treatment has a huge economic impact [13]. Therefore, there is an urgent need for new avenues of therapeutic treatment, and a new era of prophylactic treatment has begun. The most plausible approaches include: bacterial interference [14], bacteriophage therapy [15], bacterial vaccines, cationic peptides and cyclic D,L-a-peptides [16]. Due to the complexity and instability of biological components, most of the ecological strategies are difficult to validate using the available knowledge. Therefore, the usage of antimicrobial drugs remain one of the effective options in treating infections and the major task is to optimize their efficiency, but also maintain lower concentrations of active compounds, which are usually toxic in high amounts. Nanotechnology has proven a surprising potential in fighting against microbial infections by offering drug controlled release systems and targeting mechanisms [17,18**].** Magnetite nanoparticles were incorporated into polymers, especially water soluble structures, such as polyvinyl alcohol) (PVA), polyethylene glycol (PEG), polyacrylic acid (PAA), DNA and polysaccharide matrix to improve the biocompatibility or bioactivity for biomedical applications [19-21]. Recent studies focus mainly on the magnetic polymer particles, because they exhibit wide applications in the fields of targeted delivery of drugs [22-25]. Chitosan (CS) is used in biomedical applications for its many significant biological and chemical properties [26]. PVA is a non-toxic, non carcinogenic, biodegradable, biocompatible, water soluble and inexpensive polymer. It is a target compound for novel technological applications, such as biomaterials, biosensors and drug delivery systems development [27]. Polymer coating of magnetite nanoparticles enhances the compatibility between nanoparticles and the aqueous medium, prevents oxidation of magnetite, and reduces toxicity and aggregation [28]. In our previous studies we have demonstrated that the incorporation of cephalosporins of second, third and fourth generation into magnetic CS microspheres improved the delivery of antibiotics in active forms [29,30]. Here, we report the synthesis and bio-evaluation of a new antibiotic carrier based on PVA, CS and magnetite with potential application for the improvement of antibiotic activity.

Experiment Details

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

Synthesis. In the present paper, a nanobiocomposite based on CS, PVA and magnetite was prepared by a modified precipitation method [31]. One gram of PVA was solubilized in a known volume of distilled-deionized water, corresponding to a 1.00% (w/w) solution, under stirring at room temperature. 2 mL of a basic aqueous solution consisting of 28% NH_3 were added to PVA solution. 200 mL solution containing one gram of CS, 5 mL of 1N acetic acid solution, $FeCl_3$ and $FeSO_4 x 7H_2O$ (2/1 molar ratio) were dropped under permanent stirring up to pH = 8, leading to the formation of a fibrous black precipitate. After washing with ultrapure water for several times and adding 10 μL of acid acetic solution 1N the fibrous precipitate become water soluble (figure 1).

Fabrication of antibiotic (ATB) bound magnetic nanobiocomposite. The magnetic nanobiocomposite and the antibiotics to be adsorbed (gentamicin, ciprofloxacin and cefotaxime) were mixed in the presence of 2 mL of ultrapure water until the latter completely evaporated at 40^{o}C. The amount of the antibiotic adsorbed on the nanobiocomposite was selected at 10 %.

Characterization.

FT-IR. A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI) connected to software of the OMNIC operating system (Version 8.0 Thermo

Nicolet) was used to obtain FT-IR spectrum. The sample was placed in contact with attenuated total reflectance (ATR) on a multibounce plate of ZnSe crystal at controlled ambient temperature (25^{o}C). FT-IR spectrum was collected in the frequency range of 4,000–650 cm^{-1} by co-adding 32 scans and at a resolution of 4 cm^{-1} with strong apodization. The spectrum was ratioed against a background of an air spectrum. After every scan, a new reference air background spectrum was taken. The plate was carefully cleaned by wiping with hexane twice followed by acetone and dried with soft tissue before filling in with the next sample. The spectra were recorded as absorbance values at each data point in triplicate.

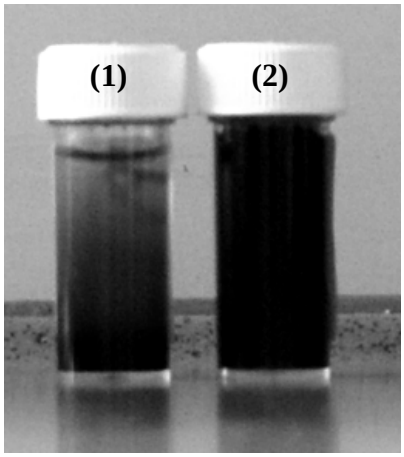

Figure 1: *Water insoluble nanobiocomposite in basic aqueous solution (1) and water soluble nanobiocomposite (2)*

XRD. X-ray diffraction analysis was performed on a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu Kα radiation from a Cu X-ray tube (run at 15 mA and 30 kV) was used. The sample was scanned in the Bragg angle 2θ range of 10-80.

SEM. SEM analysis was performed on a HITACHI S2600N electron microscope, at 25 keV, in primary electrons fascicle, on a sample covered with a thin silver layer.

Cells. HEp2 (CCL-23^{TM}) cell line was used in our experiments. Cell were cultivated in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated foetal bovine serum (Sigma) and maintained at 37^{0}C, 5% CO_2, in a humid atmosphere.

Apoptosis detection. 3.5 x 10^5 cells were seeded in 3.5 cm diameter Petri dish and treated with 100 µg/mL nanobiocomposite for 24 h. The total cells were suspended in 100 µl of binding buffer, and stained with 5µL Annexin V-FITC and 5µL propidium iodide, 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 percentage of apoptotic cells in the treated population.

Cell cycle distribution. 3.5 x 10^5 cells were plated in 3.5 cm diameter Petri dish and treated with 100 µg/mL nanobiocomposite, for 24 h. After treatment cells were taken from the substrate, fixed in 70% cold ethanol for at least 30 minutes at -20^{0}C, washed twice in PBS, and incubated 15 min at 37^{0}C with RNAse A (100 µg/mL), and 1 h with propidium iodide (100 µg/mL). After staining the acquisition was done using Epics XL Beckman Coulter flowcytometer. Data were analysed using FlowJo software and expressed as fractions of cells in the different cycle phases.

Bacterial strains. *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 references bacterial strains were used in the study**.**

Antimicrobial susceptibility testing: qualitative assay. An adapted diffusion method was used in order to assess the potentiating effect of the polymeric composite on the antimicrobial activity of cefotaxime, ciprofloxacin and gentamicin. The tested antibiotics have been chosen according to CLSI recommendations. Standardized antibiotic discs have been placed on the Mueller Hinton agar medium distributed in Petri dishes previously seeded with a bacterial inoculum with a density corresponding to a density of 0.5 McFarland. Five µL of the stock solutions of the water soluble magnetic nanobiocomposite were spotted over the antibiotic disks. The plates were incubated 24h at 37°C, and the growth inhibition zone diameters for each antibiotic, after the addition of the tested material suspensions were quantified and

compared with the growth inhibition zones obtained for the respective antibiotics [32].

Antimicrobial susceptibility testing: quantitative assay. Quantitative testing of antimicrobial activity of nanobioscomposite and the establishment of minimum inhibitory concentration (MIC) was determined by the microdilution technique in liquid medium (Mueller Hinton broth), using 96 multiwell plates [33]. Twofold serial microdillutions were achieved in 200 µL medium, the dilution range varying, depending on the tested antibiotic and the bacterial strain, in accordance with the CLSI breakpoints. Subsequently, the wells were seeded with 50 µL of each bacterial suspension, adjusted to a density corresponding to0.5 McFarland. Positive and negative controls were used. After incubating the plates at 37^{o}C for 24h, the results were macroscopically assessed for bacterial growth, the MIC value corresponding to the well with clear content, thus without no visible microbial growth.

Results and Discussions

In the recent years, magnetic scaffolds (i.e. Fe_3O_4 micro-and nanospheres) coated with polymeric layers have showed attractive support for many biomedical applications such drug delivery, drug targeting, hyperthermia, antimicrobial activity, biofilm inhibition and magnetic resonance imaging [34-36]. In this paper, we report the successful fabrication of a newly magnetic material, in order to increase microbicidal properties of cefotaxime, gentamicin and ciprofloxacin and, thus, combat the opportunistic infections. FT-IR spectroscopy was used to identify the functional groups of CS, PVA and Fe_3O_4, as shown in Figure 2. Characteristic peaks assignment of chitosan are 3353 cm^{-1} (O-H stretch overlapped with N-H stretch), 2929 and 2858 cm^{-1} (C-H stretch), 1644 cm^{-1} (amide II band, C-O stretch of acetyl group), 1485–1370 cm^{-1} (asymmetric C-H bending of CH_2 group) and C–O–C bonds at 1027 and 1056 cm^{-1} [31]. The absorption peaks of PVA at about 3286 cm^{-1} (–OH stretching) and 1413 cm^{-1} for the –C–O group [37]. Most of the above mentioned bands were observed in the FT-IR spectrum of nanobiocomposite, due to the integrated components of the PVA, CS and Fe_3O_4. Also, magnetite characteristic vibrations can be observed at ~ 527 cm^{-1}. The SEM micrographs of water dispersible biocomposite reveal a microporous structure. Magnetite was successfully integrated in the PVA/CS matrix with no visible agglomerate formation at low magnification. At higher magnification, SEM image reveals visible agglomerates with the average diameter of 100-200 nm. Although there are a number of reports regarding the toxicity evaluation of inorganic nanoparticles, knowledge on biodegradable nanomaterials, including CS, which have always been considered nontoxic, is still limited [38]. However, previous studies show that coating of inorganic particles with polymers reduces their cytotoxicity [39].

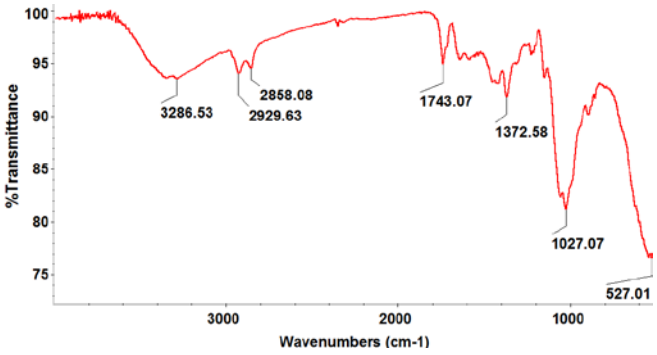

Figure 2: *FT-IR spectrum of nanobiocomposite*

The diffraction peaks (figure 3) centered at 2θ = 30.32, 35.72, 43.42, 57.44 and 63.08 indicate the formation of well-crystallized Fe_3O_4 [40].

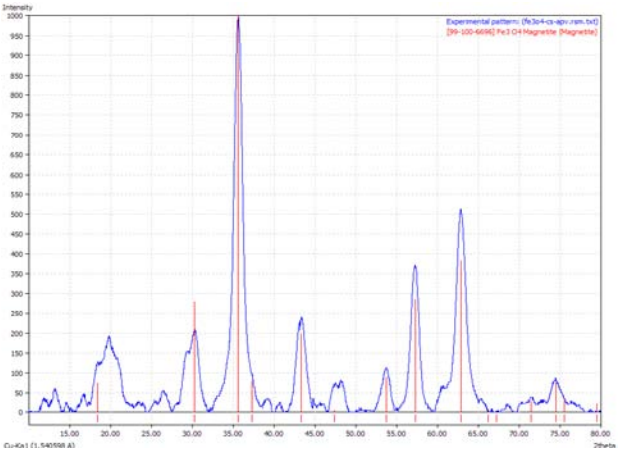

Figure 3: *XRD pattern of nanobiocomposite*

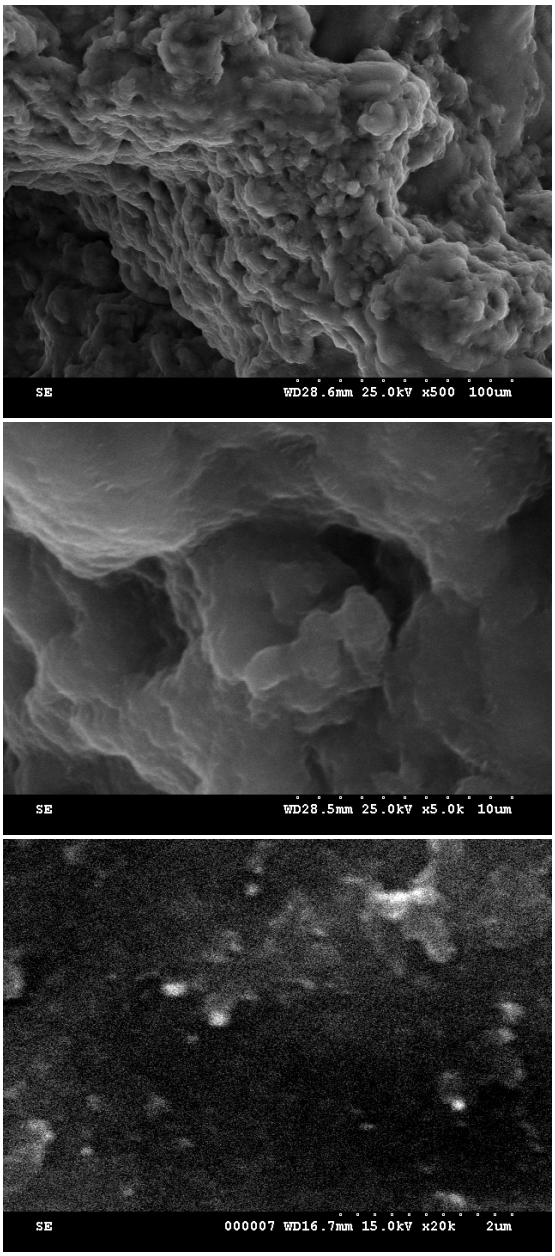

Figure 4: *SEM micrographs of fabricated nanobiocomposite*

In our study, the cellular cycle analysis in the presence of 100µg/mL of the tested nanobiocomposite showed an increased percentage of apoptotic cells (Table 1), demonstrated by the occurrence of an additional peak to the left of G1 (under G0), which usually highlights the presence of apoptotic cells (labeled M1 in figure 4). The other phases of the cellular cycle were not significantly changed, as compared to the untreated cells, therefore the percentage of viable cells remained acceptable, reaching almost 90%.

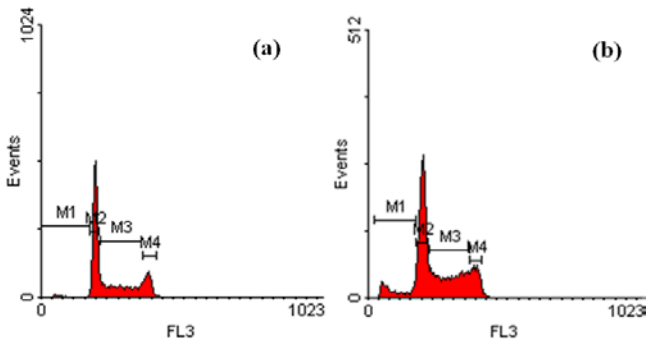

Figure 4: *Cellular cycle analysis histograms of HCT8 cells treated with 100µg/mL nanobiocomposite: (a) control; (b) nanobiocomposite*

Tab1e l: *The percentage of cells found in different cell cycle phases*

	SubG0 (M1)	G0/G1 (M2)	S (M3)	G2/M (M4)
HEp2 Control	2.89	46.13	34.12	16.99
CS Fe3O4 APV	10.7	41.95	34.56	13.15

The increased level of apoptotic cells could be explained by the cell membrane damage and resultant enzyme leakage induced by the tested nanoparticles. Other researchers demonstrated that CS nanoparticles induced the occurrence of necrotic or autophagic liver cell death, in the presence of 0.5% w/v of CS nanoparticles. Uptake of CS nanoparticles into the cell nucleus was observed by confocal microscopic analysis after 4h exposure with 1% w/v of chitosan nanoparticles [41]. On the other side, PVA coated iron oxide nanoparticles exposure ($20–80 \times 10^{-3}$ M) have been proved to lead to variations in both apoptosis and cell cycle of mouse fibroblasts, possibly due to irreversible DNA damage and repair of oxidative DNA lesions, respectively. Additionally, the formation of vacuoles within the cells and granular cells indicates autophagy cell death rather than either apoptosis or necrosis [42]. The results of the qualitative assay showed that the nanobiocomposite increased the activity of non-betalactam antibiotics, i.e. quinolones (ciprofloxacin) and aminoglycosides (gentamicin) against *S aureus* strain, while in case of *P. aeruginosa*, it stimulated only the activity of beta-lactam antibiotic cefotaxime (Figure 5). The most significant potentiating effect was obtained for gentamycin in case of *S. aureus.* These results clearly show that the

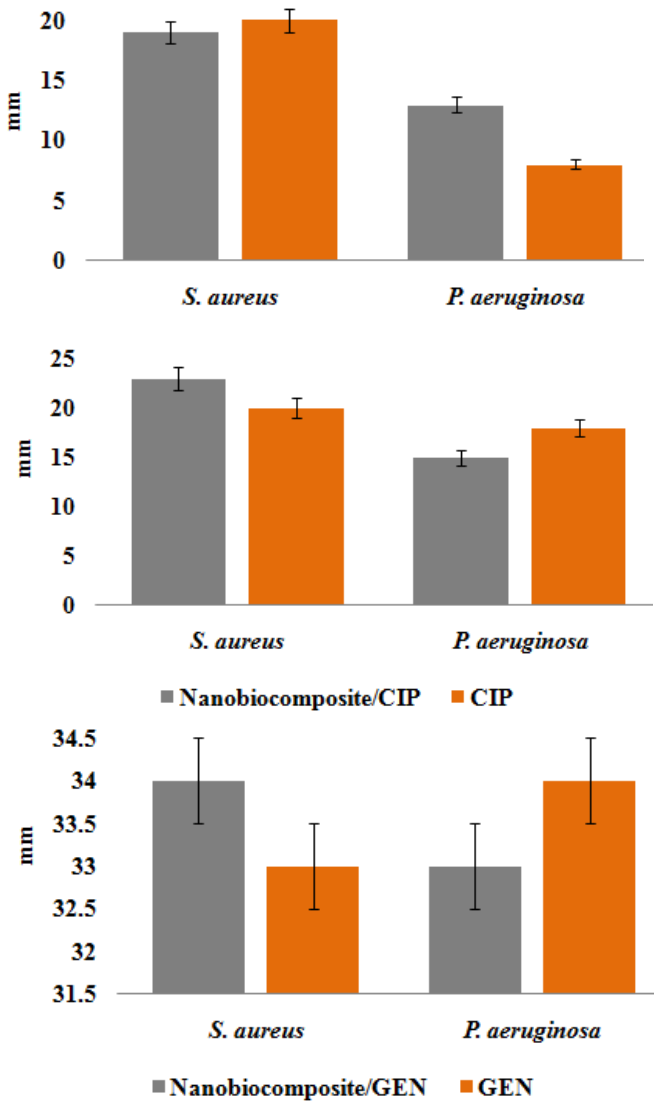

Figure 5: *The graphic representation of bacterial growth inhibition zones obtained for the nanosystem embedded antibiotic versus plain antibiotic control*

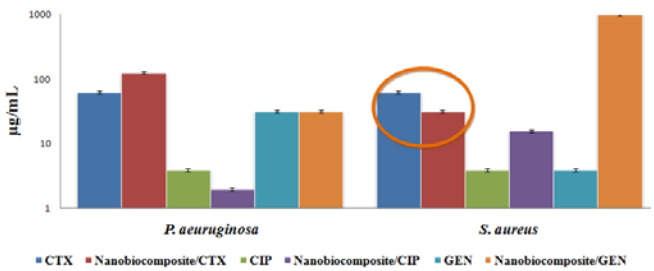

Figure 6: *The graphic representation of minimal inhibitory concentrations obtained for the nanosystem embedded antibiotic versus plain antibiotic control*

nanobiocomposite influence the antibiotic activity by modulating its uptake into the bacterial cell, facilitating the antibiotic to reach the bacterial target. The quantitative assay results were not always concordant with those obtained using the qualitative assay. Thus, in case of *P. aeruginosa*, lower MIC values were obtained only for ciprofloxacin embedded into the obtained nanosystem, while for *S. aureus*, only for cefotaxime (figure 6). These discordancies could be explained by the different interactions established between antibiotic and the obtained nanosystems on one side, and on the other side, between the antibiotic embedded into the nanosystem and bacterial target in liquid medium, as compared with the solid one.

The wide differences that occurred between Gram-positive *S.aureus* and Gram-negative *P.aeruginosa* strains can be also explained by the fact that due to their specific structural traits, the Gram positive species and Gram negative ones respond differently to environmental conditions, drugs and other chemicals.

Conclusions

Here we report that a newly synthetized water dispersible magnetic nanobiocomposite in able to improve the activity of some widely used antibiotics.. Our results revealed that fabricated composite has the ability to improve the activity of antibiotics belonging to different classes, providing different results towards Gram-positive and Gram-negative bacteria species. Combined with its low cytotoxicity, this finding suggests that the fabricated nanobiocomposite can be a useful tool for developing individualized controlled release antimicrobial strategies.

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