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Chitin based biomaterial for antimicrobial therapy: fabrication, characterization and *in vitro* profile based interaction with eukaryotic and prokaryotic cells

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ABSTRACT

The aim of the present study was to develop new drug delivery systems based on natural, biodegradable polymers. The authors report the successful fabrication and characterization of a chitin/alginate biomaterial, as well as *in vitro* biological assays, aimed to evaluate its biocompatibility and efficiency concerning the release of antibiotics in active forms. The tested biomaterial improved the antimicrobial activity of the antibiotics recommended by CLSI to be tested for *Escherichia coli* and *Pseudomonas aeruginosa* infections, results that together with the clinically appealing nature of the proposed material and its simple preparation procedure, show a promising potential for the use of this biomaterial in drug delivery in a safe and effective manner.

Keywords: *biomaterials, chitin, alginate, drug delivery, antimicrobial therapy*

1. INTRODUCTION

In the last years, biocomposites based polymers from natural sources are of considerable interest due to the functionalities unavailable to bulk materials [1]. Chitin is a co-polymer of N-acetyl-glucosamine and N-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer [2]. This biopolymer is the most abundant polymer after cellulose [3] and is synthesized by a broad variety of organisms of different taxonomic groups, including insects, arachnids and crustaceans, but also in lower invertebrates such as sponges, coelenterates, nematodes and mollusks [4]. Chitin is a biocompatible, biodegradable and non-toxic polymer. These properties lead to several biomedical applications in tissue engineering and wound healing [5]. Chitin can be easily processed into gels [6], beads [7], nanoparticles [8] or scaffolds [9]. Sodium alginate is composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues and is used to formulate various drugs [10]. Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed, and has been extensively investigated and used for many biomedical applications, due to its biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [11]. Alginate gel cross-linked divalent metal ion has been largely employed in biomaterial encapsulation for its superior biocompatibility and low cost [12]. With the advancement of macromolecular chemistry,

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the use of polysaccharides has been extended to newer applications in pharmaceutical, biomedical and agriculturalfields [13]. The biodegradability and biocompatibility of these materials would reduce or eliminate side effects in biomedical applications [14]. In the recent years, there is a growing interest to incorporate antimicrobial drugs into polysaccharides [15] or to use them in combination with iron oxide [16], zinc oxide [17] or silica network [18] to enhance the overall antimicrobial activity. In the present study, chitin gel was entrapped into an alginate matrix cross-linked by calcium ions. The structure and properties of the newly fabricated material was investigated by Scanning Electron Microscopy (SEM), Fourier Transform InfraRed Spectrometry (FT-IR), Thermogravimetric analysis (TGA), and *in vitro* profile based interaction with eukaryotic and prokaryotic cells.

2. EXPERIMENTAL SECTION

2.1. Materials. Sodium alginate, chitin, $CaCl_2$ and methanol were purchased from Sigma-Aldrich and used without any further purification.

2.2. Fabrication of biocomposite. The biocomposite was prepared as follows: two grams of chitin was dispersed with 100 mL $CaCl_2$ saturated methanol solution and the mixture was keep at -5^{o}C 12 hours. After this step, two grams of sodium alginate were dissolved in 100 mL ultrapure water and then the solution was dropped into chitin/$CaCl_2$ solution under vigorous stirring leading to the formation of a white hydrogel. The product was filtered and repeatedly washed with ultrapure water and subsequently dried at room temperature.

2.3. Characterization of the fabricated biocomposite.

2.3.1. FT-IR. A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI) connected to the software of the OMNIC operating system (Version 7.0 Thermo Nicolet) was used to obtain FT-IR spectra of hybrid materials. The samples were placed in contact with attenuated total reflectance (ATR) on a multibounce plate of ZnSe crystal at controlled ambient temperature (25^{o}C). FT-IR spectra were 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. All spectra were rationed 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.

2.3.2. SEM. SEM analysis was performed on a HITACHI S2600N electron microscope, at 15 keV, in primary electrons fascicle, on samples covered with a thin silver layer.

2.3. Biological assay

2.3.1. Antimicrobial profile. An adapted diffusion method was used in order to assess the potentiating effect of the biocomposite on the antimicrobial activity of piperacillin-tazobactam (TZP), cefepime (FEP), piperacillin (PIP), imipenem (IPM), gentamicin (CN), ceftazidime (CAZ) against *P. aeruginosa* ATCC 27853 and cefazolin (KZ), cefaclor (CEC), cefuroxime (CXM), ceftriaxone (CRO), cefoxitin (FOX), trimethoprim/sulfamethoxazole (SXT) against *E. coli* ATCC 29522 reference strains. 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 the 0.5 McFarland standard. Five µL of the stock solutions of the dispersed biocomposite were spotted over the antibiotic disks. The plates were incubated 24h at 37°C, and the inhibition zones diameters for each antibiotic, after the addition of the tested biomaterial suspensions

were quantified and compared with the growth inhibition zones obtained for the respective antibiotics [19, 20, 21, 22].

2.3.2. Eukaryotic cell cycle assay. In order to obtain a fine powder each compound was mortared and was weighed to obtain 100 mg/mL stock concentration. 3.5 x 10^5 HEp cells were seeded for 24h in 3.5 cm diameter Petri dish, and thereafter treated with 1mg/ml compound (final concentration). After 24 hours, the cells were harvested, washed in phosphate saline buffer (pH = 7.5), fixed in 70% cold ethanol and maintained at -20^{0}C, over night. Each sample was washed in PBS, treated with 100µg/mL RNAse A for 15 minutes and coloured with 10µg/ml propidium iodide by incubation at 37^{0}C, for 1 hour. The acquisition of events was done using Epics Beckman Coulter flow cytometer. Data were analysed using FlowJo software and expressed as fractions of cells in the different cell cycle phases.

2.3.3. Assessment of eukaryotic cell viability. 3.5x10^5 HEp cells were seeded in each of the 24 wells of a multi-well plate. After 24 hours, the cells were treated with 1 mg/mL compound. The effect of compounds were evaluated after 24 hours by adding 100 µl PI (0.1mg/mL) and 100 µL fluorescein diacetate (FdA). In order to evaluate dead cells (red) and viable ones (green), fluorescence was quantified using Observer.D1 Carl Zeiss microscope.

3. RESULTS SECTION

FT-IR spectroscopy was used to examine the interactions between the chitin and AlgNa, as shown in Figure 1. Each spectrum is the average of three tests and all spectra are shifted upwards to prevent overlap. For the AlgNa, characteristic bands are at 1027 and 1077 cm^{-1} of the C-O-C (cyclic ether) stretching vibration, the band at 2920 cm^{-1} of C-H stretching, and a broad band due to the hydrogen bound OH group appeared between 3200 and 3400 cm^{-1} attributed to the complex vibrational stretching, associated with free, inter and intra molecular bound hydroxyl groups.

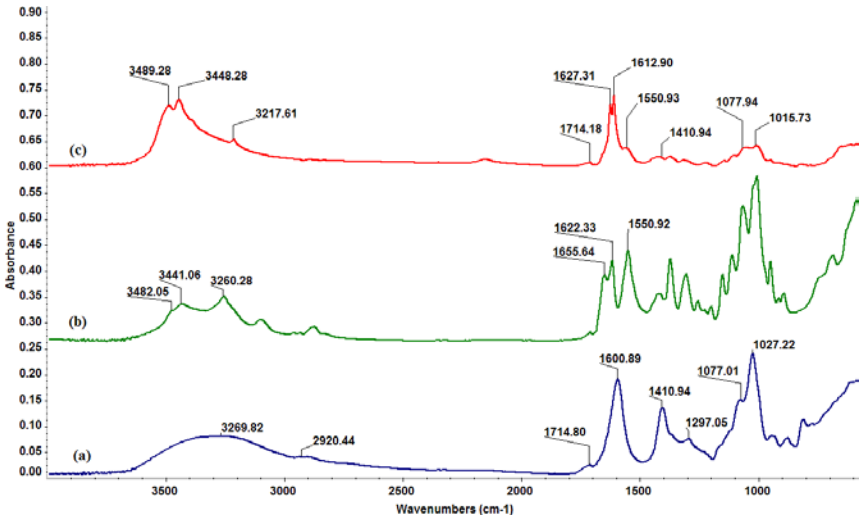

Figure 1: FT-IR spectra of bulk materials and biocomposite

In the alginate spectrum there are two specific strong absorption bands at 1601 cm^{-1} and 1410 cm^{-1} attributed to asymmetric and symmetric stretching vibrations of COO^- groups on the polymeric backbone (figure 3a) [23]. The spectrum of α-chitin (figure 3b) shows the typical features of α-chitin, such as a doublet amide I band at 1655 and 1661 cm^{-1}, a singlet amide II band at 1550 cm^{-1}, and three defined bands in the high wave number region assigned as N-H stretching at 3260 cm^{-1}, O(3)-H stretching at 3441 cm^{-1}, and O(6)-H stretching vibrations at 3482 cm^{-1}, respectively [24]. The FT-IR spectrum of the biocomposite is shown in figure 1c. Most of the above mentioned

absorption bands were observed, apparently due to the integrated components of chitin and AlgNa. No new peaks were observed in the spectrum of the biocomposite.

Scanning Electron Microscopy (figure 2) revealed that the biocomposite exhibit a rough surface. Generally, the surface of the beads is pelicular, being characteristic to alginate based materials. On the surface there can be identified different spherical and elongated micropores. Also, could be observed on the surface a wrinkling structure.

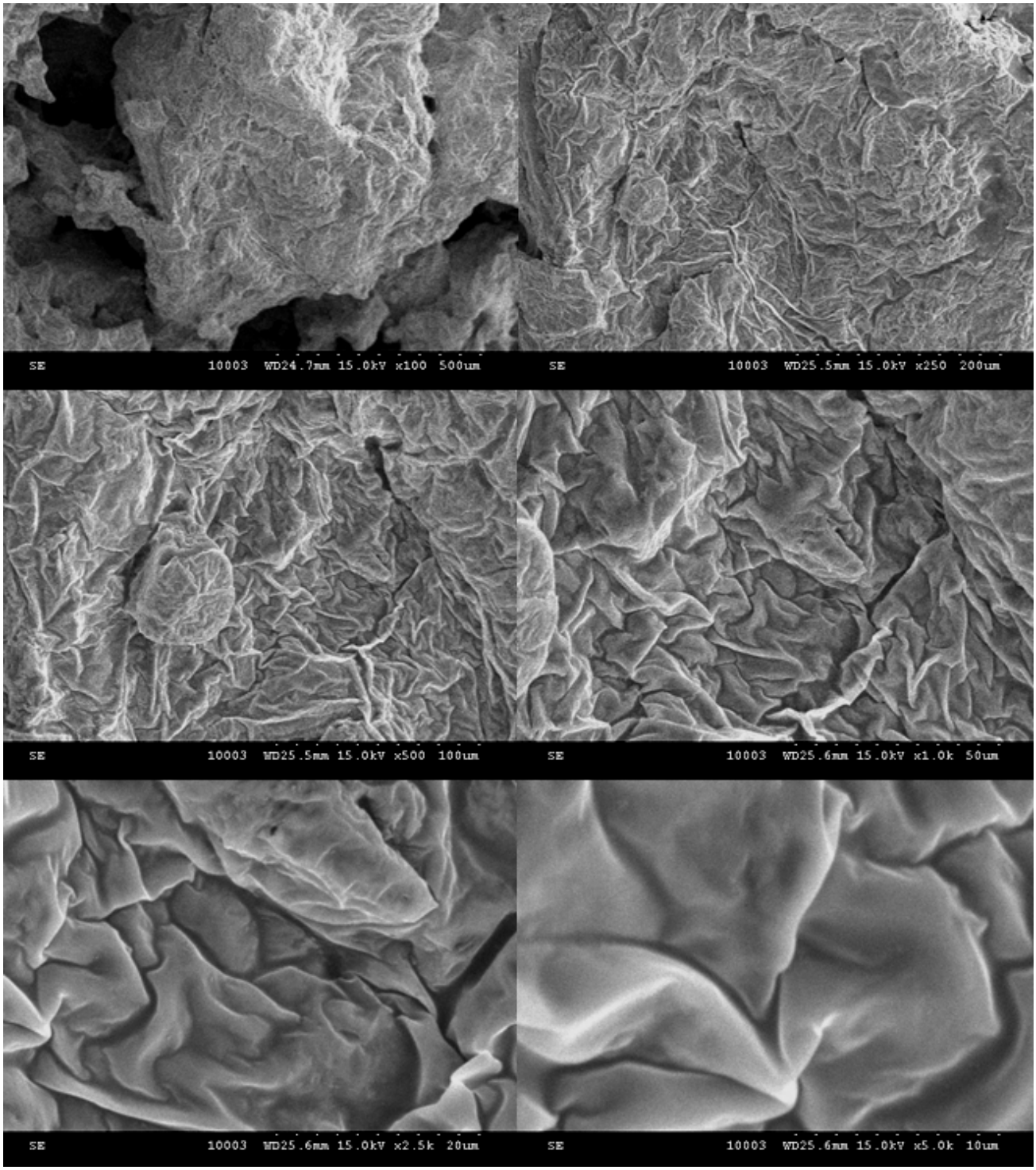

Figure 2: SEM micrograph of CHT/ALG composite

Most of the naturally occurring polysaccharides, e.g. cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, are neutral or acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides. Their unique properties include polyoxy salt formation, ability to form films, to chelate

metal ions and optical structural characteristics [25, 26, 27]. Considering the fact that the human body tissues have a negative charge, it was proposed that the use of cationic mucoadhesive polymers, such is chitin, which may interact intimately with these structures, would increase the concentration and residence time of the associated drug [28]. Chitin exhibits a lot of advantages due to its unique properties including acceptable biodegradability, biocompatibility, as well as the ability to increase membrane permeability, both *in vitro* and *in vivo* and be degraded by lysozyme in serum [14,29]. It has been demonstrated that multifunctionalized chitin nanogels could be useful for protein and drug delivery with simultaneous imaging and biosensing [30,31]. A prodrug was found to be released slowly into blood following the subcutaneous injection the prodrug either pendanted through a covalent bond to carboxymethyl-chitin or entrapped within carboxymethyl-chitin matrix in the presence of Fe^{3+}. The prodrug was then hydrolyzed, to become the active form, by enzymes in the blood [32]. The limited ability of chitin for controlling drug release in acid medium [33], could be overcome by incorporating chitin gel into an acid-resistant polymer, such as sodium alginate [34, 35]. During the present study the alginate/chitin biomaterial improved the anti-*Pseudomonas* activity of beta-lactam antibiotics, i.e. penicillins (PIP, TZP) and fourth generation cephalosporins (FEP) (figure 3).

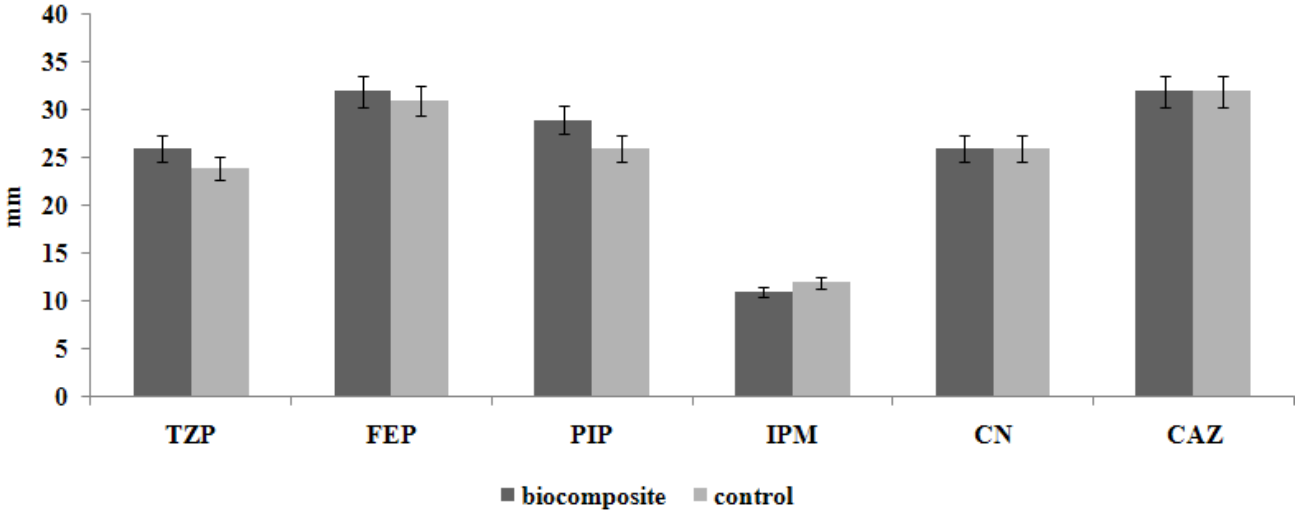

Figure 3: The growth inhibition zone diameters (mm) obtained for the tested antibiotics in the presence of biocomposite on the *P. aeruginosa* ATCC 27853 strain.

In case of *E. coli,* the improvement of the beta-lactam antibiotics activity was even more evident and larger, being noticed for first, second and third generations' cephalosporins (KZ, CEC, CRO and FOX) (figure 4).

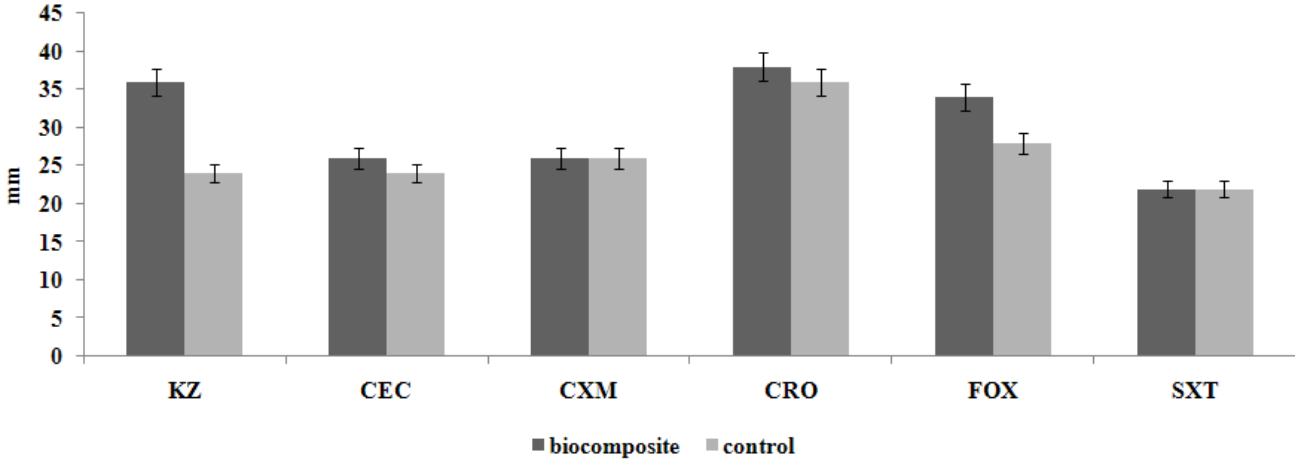

Figure 4: The growth inhibition zone diameters (mm) obtained for the tested antibiotics in the presence of biocomposite on the *E. coli* ATCC 25922 strain.

A slight cytotoxic effect of the obtained biocomposite was observed on HEp cells, after 24 hours of contact with the biomaterial in powder form, at 0.1 mg/mL concentration. The aspect of HEp cells examined in inverted microscopy after 24 hours of contact with the tested compound show the absence of dead cells occurred in red, while the viable ones are colored in green (figure 5).

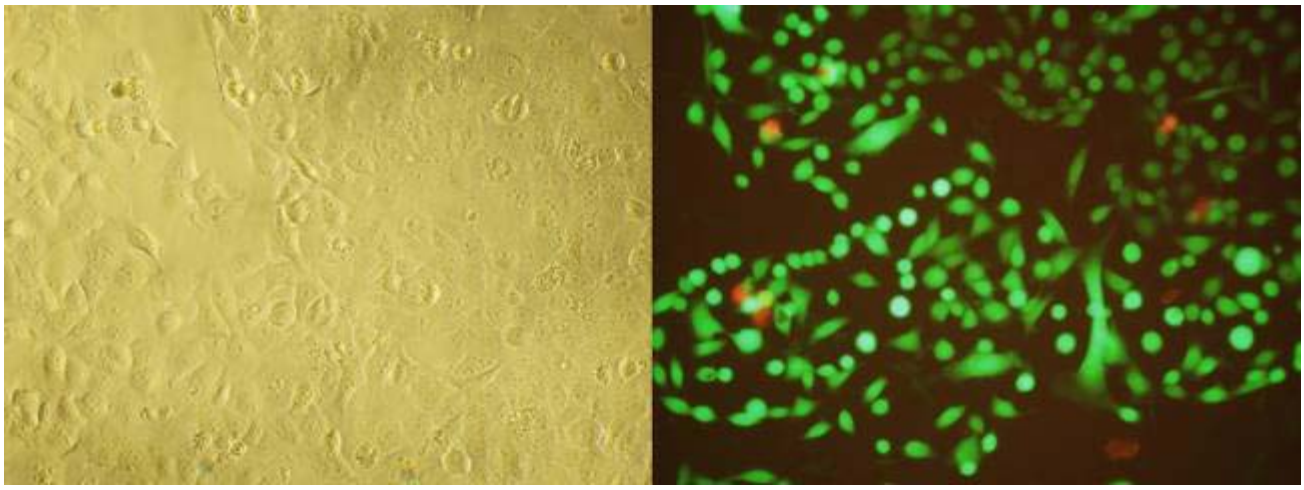

Figure 5: Interaction of biocomposite with HEp standardized cell line. Inverted microscopy images in visible and UV light (100x)

A slight inhibition of S phase of the HEp cells cycle was induced by the obtained biomaterial, tested at 0.1 mg/ml concentration (figure 6).

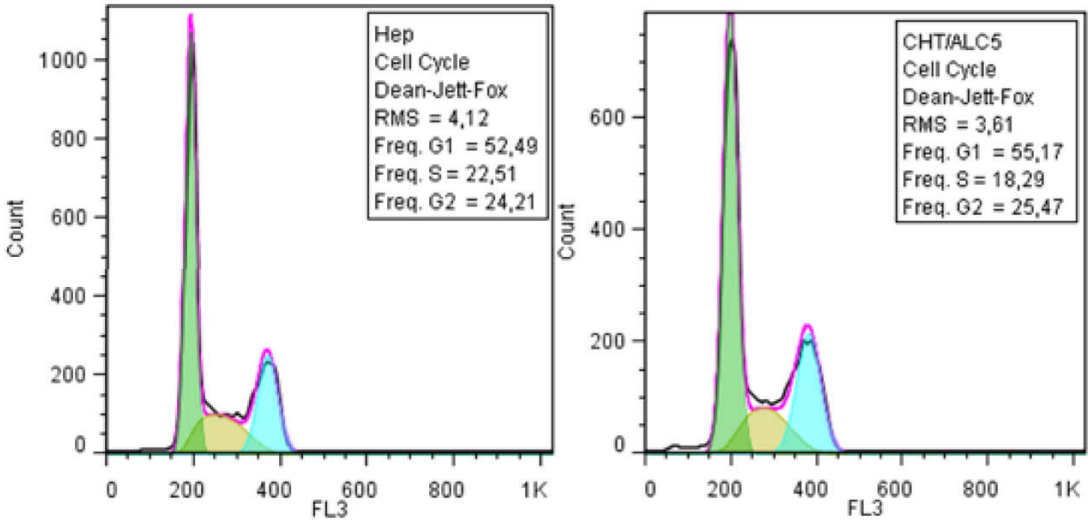

Figure 6: Analysis of cell cycle phases in HEp line treated with the tested compound at 100µg/mL concentration.

4. CONCLUSIONS

The authors report the successful fabrication and characterization of a chitin/alginate biomaterial, as well as *in vitro* biological assays. The tested biomaterial improved the antimicrobial activity of the currently used antibiotics in the treatment of *Escherichia coli* and *Pseudomonas aeruginosa* infections. Our results suggest that under *in vitro* conditions chitin/alginate biocomposite can act as promising carriers for different beta-lactam antibiotics, both penicillins and cephaloporins, and may be used as an alternative system in sustained delivery of these drugs.

5. ACKNOWLEDGMENT

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