OPEN ACCESS IOURNAL

BIOINTERFACE RESEARCH IN APPLIED CHEMISTRY

ORIGINAL ARTICLE www.BiointerfaceResearch.com ISSN 2069-5837

Volume 3, Issue 5, 2013, 643-652

Received: 29.08.2013 / Accepted: 22.08.2013 / Published on-line: 27.08.2013

Fabrication and characterization of bacterial exopolysaccharides microcapsules for antibiotics drug delivery

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ABSTRACT

A great number of bacterial extracellular polysaccharides (EPS) have been isolated and characterized in the last decades, but only a few have reached the industrial development stage for food, pharmaceutical and other industries applications. The purpose of this study was to characterize and formulate at microscale level the bacterial EPS extracted from the Gram-negative *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains cultures, in order to used them as carriers for different antibiotics. Prepared EPS was characterized by XRD, FT-IR and SEM. The low cytoxicity on HeLa cell line, as well as the results of the qualitative and quantitative antimicrobial activity assay are proving the potential of the polysaccharidic extracts for the encapsulation and for the slow and prolonged release of antibiotics in active form. The results demonstrate the effectiveness of bacterial EPS polymers for the development of new carriers of antibiotics. However, further studies are required to optimize the obtained systems by characterizing the specific interactions between the EPS matrix and the active substances, as well as by establishing their *in vitro* and *in vivo* bioavailability.

Keywords: *bacterial exopolysaccharides, antibiotics, drug delivery, XRD, SEM, FT-IR.*

1. INTRODUCTION

Biopolymers can find applications in any field, especially in the medical one, for the design of biomaterials used to fabricate different medical devices, from external equipments to intrathecally implanted ones [1]. The biopolymers used for medical applications must have good biocompatibility and meet the required physic-chemical properties [2]. Polysaccharide polymers have a high degree of biocompatibility and biodegradability, which are two of the basic qualities that biomaterials should possess [3, 4]. Recent studies have revealed other features, such as antimicrobial and anti-tumor properties as well as gene expression modulation. Effective release of drug substances involves an optimum balance between removal from the bloodstream by the kidneys and other organs and its mobility within blood vessels (extravasation). Therefore, the properties of a good delivery system lie in their molecular architecture: molecular weight and conformation, flexibility and chain branching, the location of the active substance in the polymer conformation. Renal filtration can be achieved for polymers with a molecular weight between 30 and 50 kDa, depending on the structure, molecular conformation and flexibility. Hardly degradable polymers with a molecular weight too large to be excreted, may be retained in the body for a while longer [5]. The most important condition for a

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carrier system is to protect the drug from degradation till it reaches the area of interest where it should be released in an active form [6].

Delivery systems based on polysaccharides have been designed to deliver the drug substance to the site of action for a long period of time, so that therapeutic levels can be reduced considerably [7]. On the other hand, different types of delivery polysaccharidic systems are used to reduce fluctuations in plasma concentrations of drug substance in order to achieve an effective pharmacological response [8]. From the pharmacokinetic point of view, development of polysaccharide drug delivery systems depends, *in vivo*, on the molecular distribution of electrical charges, various modifications of the chemical structure and the degree of polydispersity [9, 10].

Polysaccharides as alginates, chitin, dextrans, that hold similar chemical structure, possess the advantage of simple extraction processes and poor immunogenicity. Their chemical structure offers the possibility of non-covalent binding of other compounds and multipath degradation. With a molecular weight of 10-1000 kDa, alginate is hardly degradable by enzymatic equipment of mammals and it is not proper for cell adhesion in unmodified form [11]. Recent studies have investigated the reduction of molecular weight in the presence of gamma radiation or treatment with periodate oxygen in order to obtain optimum *clearance* [12].

Chitosan is a biodegradable polymer with a wide range of applications in the pharmaceutical field, because its electric charge density, low toxicity and adherence to mucous membranes. It was demonstrated that the polymer favors dissolution of poorly soluble substances and has an important role in fat metabolism. Chitosan interaction with polyphosphates, sulfates and glutaraldehyde will result in the formation of gels. Gelation of chitosan allows the use of a wide range of applications in the food and pharmaceutical industry. The influence of micro-delivery systems based on chitosan was studied [13]. Chitosan is used as an excipient in the formulation of oral pharmaceuticals [14]. A vast number of bacterial extracellular polysaccharides (EPSs) have been isolated and characterized in the last decades for their composition, structure, biosynthesis and functional properties but only a few have been industrially developed by now for the food, pharmaceutical and other industries [15, 16].

The purpose of this study was to prepare at microscale level the bacterial EPS extracted from Gram-negative *Klebsiella pneumoniae* and *P. aeruginosa* cultures, in order to assay their cytotoxicity and potential to use them as carriers for different antibiotics.

2. EXPERIMENTAL SECTION

The exopolysaccharidic fractions were extracted from two *Klebsiella pneumoniae* (K742, K800) and one *Pseudomonas aeruginosa* (P568) strains using a method modified from Cerantola *et al*. [17]. After extraction, the polysaccharic material was air-dried and characterized by FT-IR spectroscopy, X-ray diffraction and SEM.

The FT-IR spectra of air-dried EPS were compared with two important drug delivery polysaccharides FT-IR spectra, i.e. chitosan with medium molecular mass and sodium alginate (Sigma).

To highlight the main functional groups of the EPS material there were used a FT-IR SPECTRUM BXII spectrometer with library spectra, "Pharmaceuticals, Drug & Antibiotics-1338 Spectra" and Pike Miracle ATR-reflection ATR-Diamond / ZnSe accesory. Samples were analyzed in ATR mode (attenuated Total Reflectance) using a ZnSe crystal at room temperature (25 ° C). FT-IR spectra were collected between 4000 and 600 cm^{-1}.

X-ray diffractometer analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. For all the tests, there has been used for the Kα radiation with a Cu X-ray tube (15 mA and 30 kV). The samples were scanned in the Bragg angle 2θ between 10 and 80.

SEM analysis was performed on a HITACHI S2600N electron microscope, at 20 keV, in secondary electrons fascicle, on samples covered with a thin silver layer.

In vitro cytotoxicity assay of bacterial EPS fraction on eukaryotic cells has been studied using double fluorescent staining with acridin orange (coloring dead cells in red) and FITC (coloring live cells in green). An inoculum of 5 x 10^5 cells were seeded in 6 multi-well plates in DMEM medium (*Dulbecco's Modified Eagle Medium*) (*Sigma*) supplemented with 10% fetal calf serum (Sigma) and incubated at 37 ° C in a humidified atmosphere with 5% CO_2. The eukaryotic HeLa cells monolayer treated for 24 hours with different concentrations of the EPS stock solution prepared in 0.5M acetic acid was observed with an inverted microscope, the ratio between red and dead cells being established.

The antibiotics were adsorbed onto the EPS support at a final concentration of 10 %, i.e.100 mg mixture of EPS-ATB - antibiotics containing 90 mg polysaccharide and 10 mg antibiotics). The polysaccharides (K742, K800 and P568) and the antibiotics (cefotaxime -CEF, ceftriaxone - CTX, colistin - COL, neomycin - NEO, norfloxacin - NOR) to be adsorbed were mixed in the presence of 2 mL of deionized water until the latter completely evaporated at 40^{o}C. The obtained hybrid materials were kept in cool place until use. The structure of prepared EPS-ATB materials was investigated by FT-IR.

A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI), connected to the OMNIC operating system software (Version 8.2 Thermo Nicolet) was used to obtain FT-IR spectra of EPS-ATB. 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 ratioed against a background of an air spectrum.

Qualitative testing of the microbicidal activity of the EPS-ATB was performed by disk diffusion against standardized strains of *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *Salmonella enteritidis* ATCC 14028 and *Bacillus subtilis* ATCC 6633 strains. The growth inhibition zone of antibiotic solution *versus* the hybrid system, was measured with a Mahr digital calipers after distributing 10μL of each stock solution (of antibiotic and hybrid system respectively) containing the same antibiotic concentration (1 mg/mL) on the solid Muller Hinton medium followed by incubation for 24 hours at 37^{o}C.

Quantitative testing of the microbicidal activity of the EPS-ATB was performed using serial binary microdilution method in liquid medium (Mueller-Hinton broth) in 96-well plates (18). Serial two-fold dilutions (333.33-0.26μg/mL) were performed for the neomycin - NEO, ceftriaxone - CTX, colistin - COL, norfloxacin - NOR and cefotaxime - CEF antibiotics in solution as well as loaded in EPS microspheres. MIC value was established by macroscopic examination of the wells, as the last concentration at which the inhibition of microbial growth was noticed, namely the absence of turbidity in the liquid medium as well as by the spectrophotometric reading of the obtained bacterial cultures absorbance at 620nm.

3. RESULTS SECTION

FT-IR spectra of microbial specimens provided a number of absorbtion bands that could describe the molecular composition of the cells. Many of these bands are also sensitive to structural changes,

various intra- and intermolecular interactions including H-bonding pattern, membrane constitution, lipid-protein interaction and conformational states like different secondary structures of proteins.

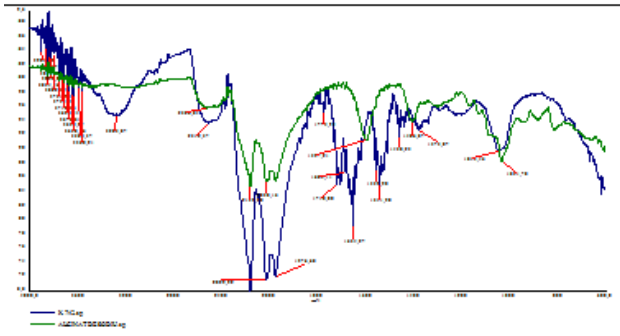

Figure 1: Comparative FT-IR spectra of K742 polysaccharide and sodium alginate

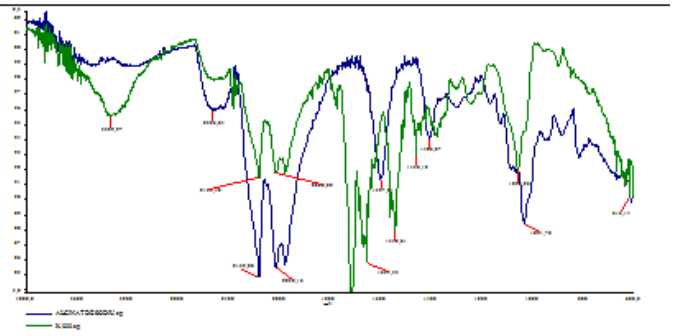

Figure 2: Comparative FT-IR spectra of K800 polysaccharide and sodium alginate

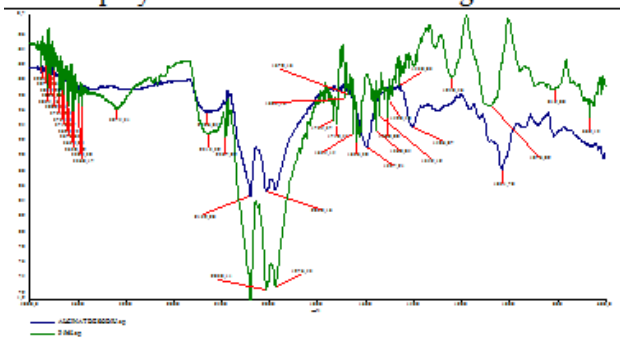

Figure 3: Comparative FT-IR spectra of P568 polysaccharide and sodium alginate

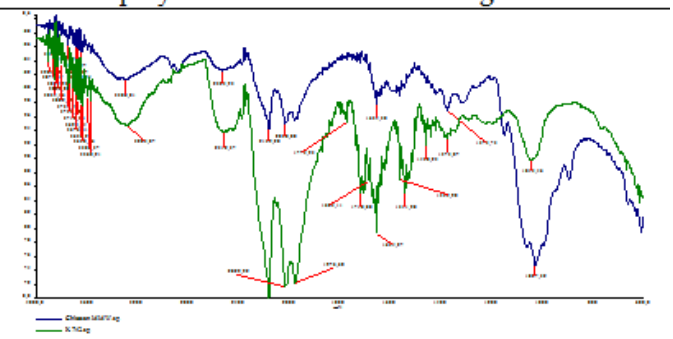

Figure 4: Comparative FT-IR spectra of K742 polysaccharide and chitosan

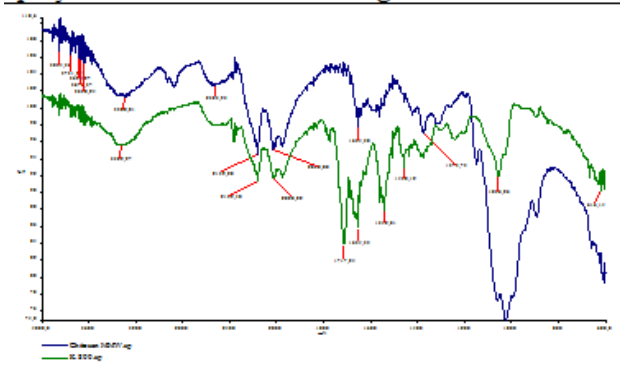

Figure 5: Comparative FT-IR spectra of K800 polysaccharide and chitosan

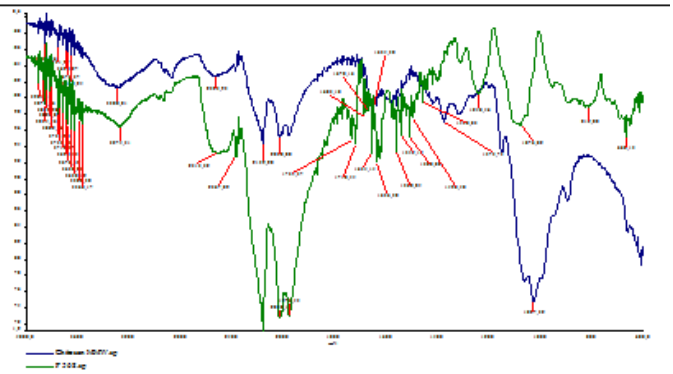

Figure 6: Comparative FT-IR spectra of K800 polysaccharide and chitosan

The literature recommends a range of 800-4000 cm^{-1} for the characterization of biological molecules [19]. Fingerprint regions of the reference (chitosan) are similar to each of the tested samples. Spectral bands in the range 1200-900 cm^{-1} referred to in the literature as specific carbohydrates are found in all samples studied (figures 1-6) [20].

The XRD pattern of the obtained EPS is plotted in figure 7 and exhibits a broad peak in the range of 15–35° (2θ), which indicates an amorphous structure. Current peaks are characteristic for phosphate buffered saline (PBS) used in the preparation process of EPS.

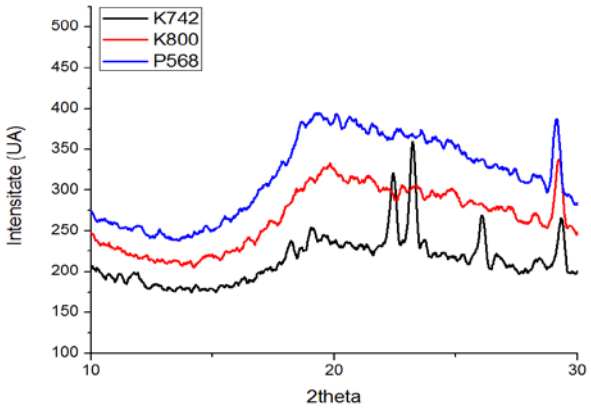

Figure 7: X-ray diffractograms recorded for purified polysaccharides of *Klebsiella pneumoniae* K742, K800 and *Pseudomonas aeruginosa* P568 strains

The SEM images exhibit characteristic sharp edges–or agglomerates composed by micrometric particles. The co-existence of the two types of structures can be explained by taking into account that K742, K800 and P568 polysaccharides have limited crystallinity, the sharp edged structures being resulted by grinding (Figures 8-10).

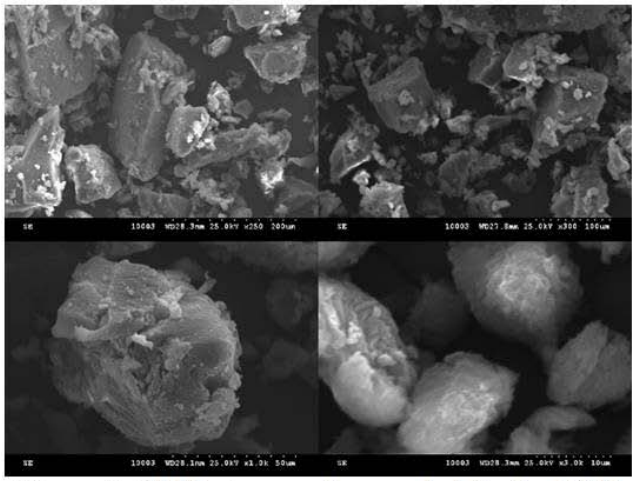

Figure 8: SEM micrographs recorded for the K742 polysaccharide

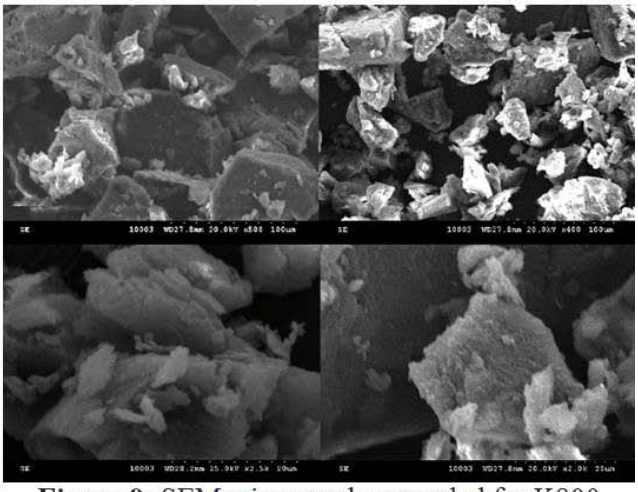

Figure 9: SEM micrographs recorded for K800 polysaccharide

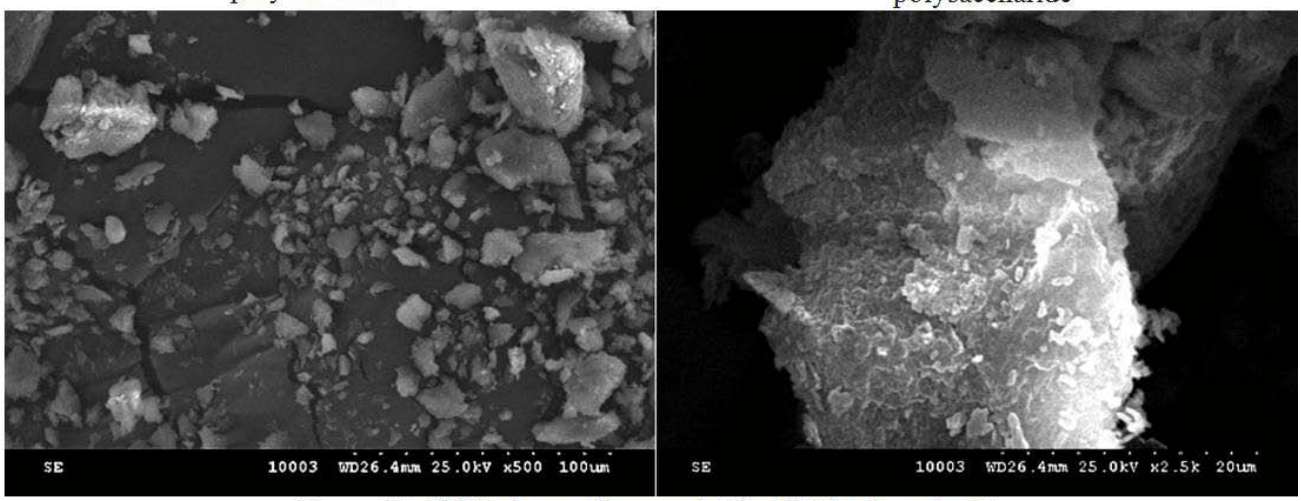

Figure 10: SEM micrographs recorded for P568 polysaccharide

The polysaccharides cytotoxicity was studied in HeLa cell lines (Figure 11), after solubilization in 0.5 M acetic acid.

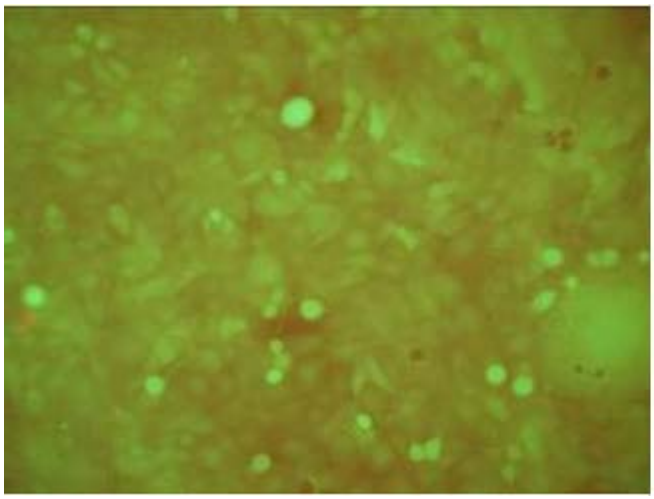

Figure 11: Fluorescence microscopic image of HeLa cell monolayer (control, 200x)

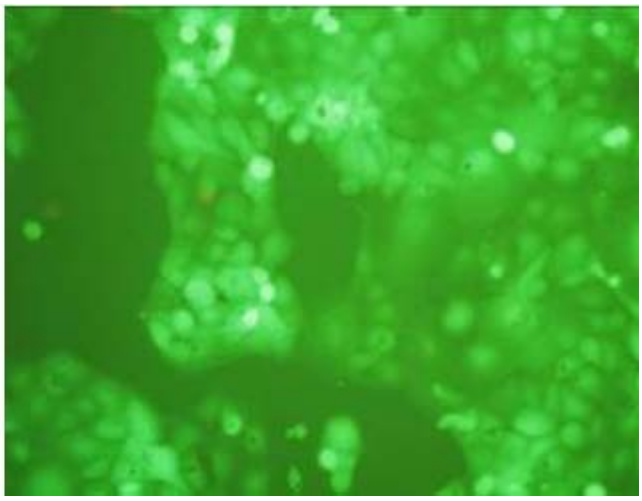

Figure 12: Fluorescence microscopy image of HeLa cell monolayer in the presence of K742 polysaccharide (200x, double staining with FITC and acridine orange)

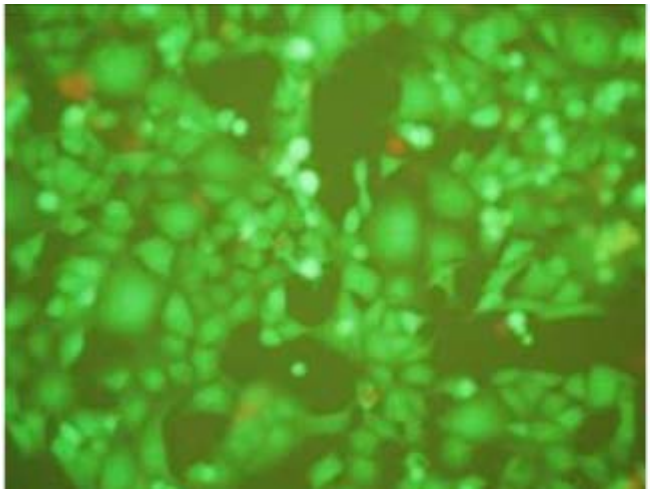

Figure 13: Fluorescence microscopy image of HeLa cell monolayer in the presence of P568 polysaccharide (200x double staining with FITC and acridine orange)

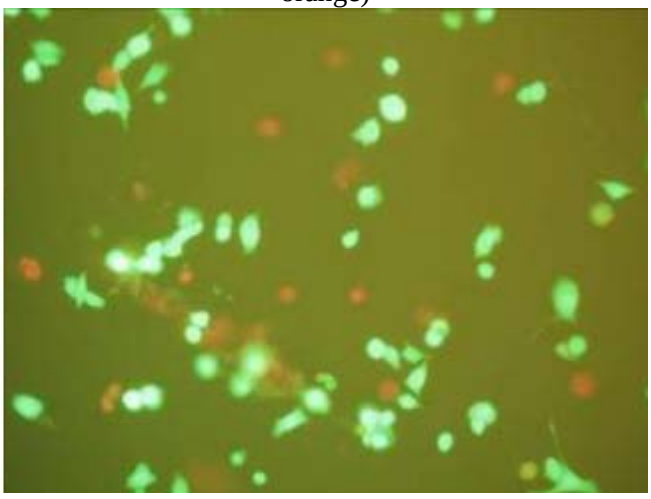

Figure 14: Microscopy image of HeLa cell monolayer in the presence of K800 polysaccharide (200x double staining with FITC and acridine orange)

At the used concentration, the cell monolayer was not affected by the solvent. The aspect of cell monolayer after 24 h in the presence of the EPS extracted from K742 and P568 (Figures 12 and 13) indicates the absence of cytotoxicity, dead cells being rarely observed (colored in red with acridine orange), without any disruption of the confluent monolayer. In the presence of EPS extracted from *K. pneumoniae* K 800 there was observed a cytotoxic effect, as revealed by both reducing the monolayer confluence and by the presence of a high proportion of round, dead cells, stained in red) (Figure 14). Other studies have shown that extracts of most marine bacterial strains harvested from the Red Sea exhibited an apoptotic effects on various cancer cell lines. *Halomonas* sp. and *Sulfitobacter* sp. EPS extracts had a potent antitumor effect, representing possible candidates for the isolation and structure elucidation of bioactive molecules [21]. The cytotoxic activity may be due to the presence of toxic compounds in the respective extract (K800). Other studies have shown that the rhamnolipid isolated from *Burckholderia pseudomallei* accumulated in the culture supernatant exhibited a high cytotoxic activity on various cell lines [22], while strains of *Pseudomonas*

aeruginosa are capable of producing a very structurally similar glycolipid with the rhamnolipid synthesized by *B. pseudomallei*, which also contains rhamnose and β-hydroxy acids, responsible for the haemolytic activity and macrophages inhibition [23,24]. Other authors demonstrated the plant cytotoxicity of EPS with different chemical structures, mediated by inducing increased levels of phenylalanine ammonia-lyase, an enzyme marker of stress response in plants. EPS action affecting the activity of phenylalanine ammonia-lyase also results in increased synthesis of hydrogen peroxide. Moreover, EPS alters the metabolism of ascorbate, another parameter indicative of the presence of stress conditions reported in hypersensitivity reactions [25].

Polysaccharide polymers efficiency in delivering intracellular pharmacologically active compounds has been extensively studied in relation to anti-tumor drugs [26, 27] and non-viral transfection [28]. Maya *et al*. [29] demonstrated the effectiveness of chitosan in the intracellular release of tetracycline and its effectiveness against *S. aureus*. In our study, the entrapped of ATB onto the polysaccharides was proved by the FT-IR spectra (figures 15, 16, 17, 18, 19, 20), demonstrating the integrity of functional groups after loaded. Based on these arguments, we have concluded that our absorbtion processes were done successfully without changing the structure of antibiotics. The changes in area of the bands and many peaks in the "fingerprint" region between 1800 and 1200 cm^{-1} were observed. The "fingerprint" region of the spectra from the reference (K742; P568; K800) and EPS-ATB regions shows clear differences after deposition of ATB.

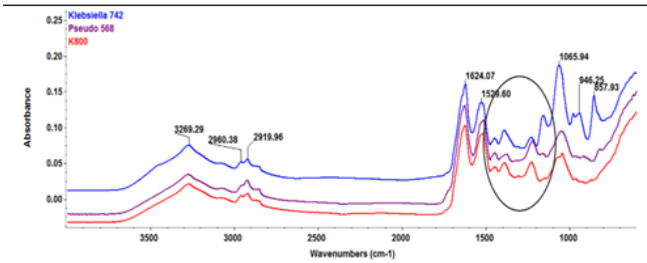

Figure 11: FT-IR spectra recorded for K742, K800 and P568 polysaccharides

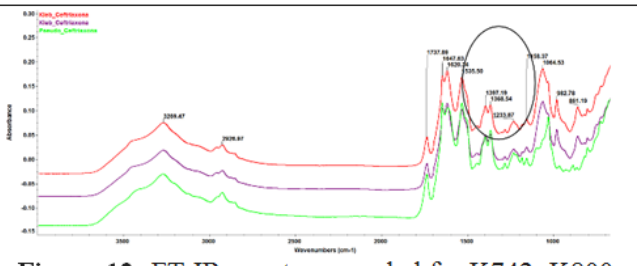

Figure 12: FT-IR spectra recorded for K742, K800 and P568 polysaccharides and ceftriaxone

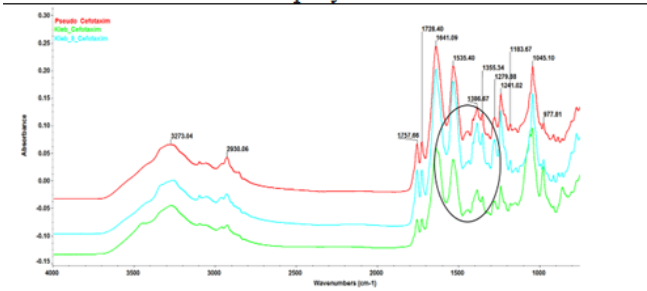

Figure 13: FT-IR spectra recorded for K742, K800 and P568 polysaccharides and cefotaxim

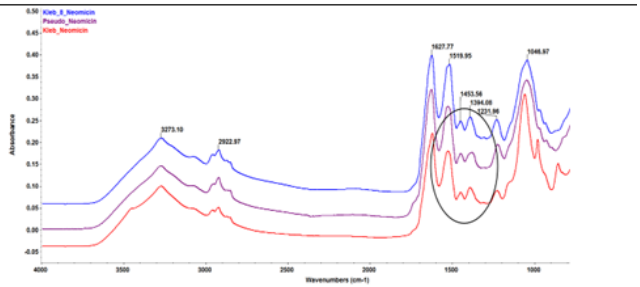

Figure 14: FT-IR spectra recorded for K742, K800 and P568 polysaccharides and neomycin

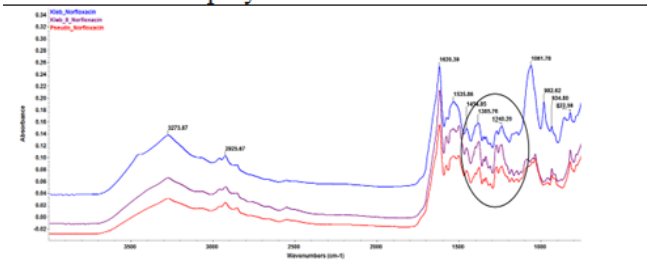

Figure 15: FT-IR spectra recorded for K742, K800 and P568 polysaccharides and norfloxacin

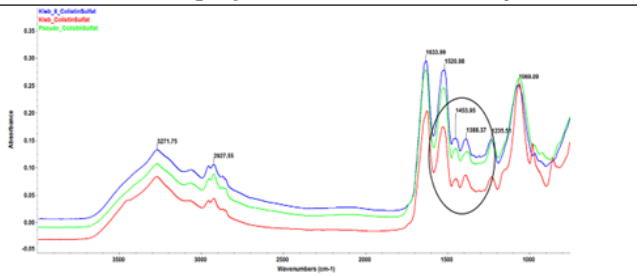

Figure 16: FT-IR spectra recorded for K742, K800 and P568 polysaccharides and colistin

The assay of the antimicrobial activity of the obtained hybrid systems was performed, in a first stage, by qualitative testing. Qualitative assessment of the microbicidal activity of antibiotic loaded in the

obtained EPS microspheres showed that the growth inhibition zones obtained for the encapsulated substances were lower than that obtained for the same, non-encapsulated, soluble antibiotic solutions at 24 hours (table no. 1). Instead, the diameter of the growth inhibition zones observed after 48 hours of exposure was increased for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 strains. K742 EPS matrix increased the diameter of the growth inhibition zones after 48 hours of exposure in 11, K800 in 9, and P568 in 14 working variants (table no. 1).

Table 1. Growth inhibition zone induced by the encapsulated antibiotics versus soluble antibiotic

	***S. aureus* ATCC 25923**		***B. subtilis* ATCC 6633**		***Ps. aeruginosa* ATCC 27853**		***S. enteritidis* ATCC 14028**		***E. coli* ATCC 25922**	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
K742-CEF	22,11	**28,51**	29,77	27,88	18,67	15,94	36,06	35,28	36,09	**36,51**
K800-CEF	24,84	**30,16**	31,22	**32,56**	17,82	14,21	34,66	33,46	36,45	**37,29**
P568-CEF	20,12	**23,24**	35,99	34,64	16,96	16,34	34,77	34,48	35,72	**36,4**
CEF	31,99		40,99		29,91		40,89		41,35	
K742-CTX	22,99	**24,09**	26,87	26,51	14,14	11,86	33,05	32,77	29,23	27,92
K800-CTX	18,58	**21,5**	27,26	25,57	17,53	14,23	33,9	34,58	27,16	**27,62**
P568-CTX	18,61	**23,33**	27,28	26,36	14,58	11,89	27,41	**27,89**	31,07	**31,95**
CTX	32,31		31,11		28,36		30,07		35,14	
K742-COL	-	-	13,68	**14,15**	17,58	16,55	15,25	15,27	17,38	17,18
K800-COL	-	-	14,09	13,37	18,05	17,27	18,08	18,04	16,59	**17,73**
P568-COL	8,68	**9,07**	12,94	12,48	15,94	**16,05**	16,15	14,68	15,62	**16,64**
COL	18,03		19,23		24,33		23,14		20,2	
K742-NEO	25,71	**26,07**	27,93	**29,43**	16,61	14,12	14,69	**15,29**	25,21	22,38
K800-NEO	24,55	**26,09**	29,37	28,85	16,91	12,98	22,57	22,05	21,84	21,83
P568-NEO	24,47	23,78	21,36	21,69	13,82	11,5	22,08	22,02	20,36	**20,87**
NEO	29,73		27,37		24,44		28,61		21,01	
K742-NOR	28,87	**29,01**	36,12	**38,47**	36,68	**38,73**	42,66	39,75	35,76	**38,76**
K800-NOR	27,58	**28,07**	36,66	35,99	16,96	16,34	34,77	34,48	35,72	**36,4**
P568-NOR	25,22	**29,14**	37,48	**37,8**	36,04	**38,95**	38,05	**39,42**	35,71	**36,53**
NOR	33,84		44,17		43,65		44,43		41,85	

These results demonstrate the gradual release of the active substances from the EPS, without a strong initial burst, with specificity for the used polysaccharide matrix, suggesting the need for further studies to optimize the prepared systems by the characterization of specific interactions between the matrix and the active substance, as well as their *in vitro* and *in vivo* bioavailability.

In all test variants, the MIC of the antibiotics solubilized in acetic acid were lower than those obtained with the encapsulated ones, correlated with the quality assay results, demonstrating the slow and gradual release of the antibiotic from the EPS matrix.

4. CONCLUSIONS

The low cytoxicity on HeLa cell line, as well as the results of the qualitative and quantitative antimicrobial activity assay are proving the potential of the obtained bacterial exopolysaccharidic extracts for the encapsulation and for the slow and prolonged release of antibiotics in active form. However, further studies are required to optimize the obtained systems by characterizing the specific

interactions between the EPS matrix and the active substance as well as by establishing its *in vitro* and *in vivo* bioavailability.

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