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Nano-Coupling of Cephalosporin Antibiotics with Fe_3O_4 Nanoparticles: Trojan Horse Approach in Antimicrobial Chemotherapy of Infections Caused by *Klebsiella spp.*

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Abstract

In the present study we had an aim to develop the methods of functionalizing the surface of magnetite nanoparticles with cefotaxime and ceftriaxone antibiotics. The quantitative analysis of the nanostructured cephalosporins was determined by Atom Absorbance Spectroscopy (AAS) and based on the Lambert-Beer law. The engineered nanostructures were tested on gram-negative microorganisms *Klebsiella* spp., of Enterobacteriaceae, and gram-positive bacteria *Staphylococcus aureus*, each having multi-drug resistance properties.

Keywords

Magnetite Nanoparticles, Cephalosporin, Cefotaxime, Ceftriaxone Klebsiella, *Staphylococcus aureus*

1. Introduction

In past years, the number of nosocomial infections, caused by gram-negative microorganisms, has increased. The microorganisms belonging to the family of *Enterobacteriaceae* and *Pseudomonas* have the greatest clinical significance. The bacteria of genera *Escherichia*, *Klebsiella*, *Proteus*, *Citrobacter*, *Enterobacter*, *and Serratia*

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from the *Enterobacteriaceae* family are often referred to as causes of postoperative complications, sepsis, and meningitis. Most of *Enterobacteriaceae* are opportunistic pathogens, since these bacteria are normally (except genus *Serratia*) obligate or transient representatives of the intestinal microflora and cause infections in immune-compromised patients only under certain conditions [1] [2].

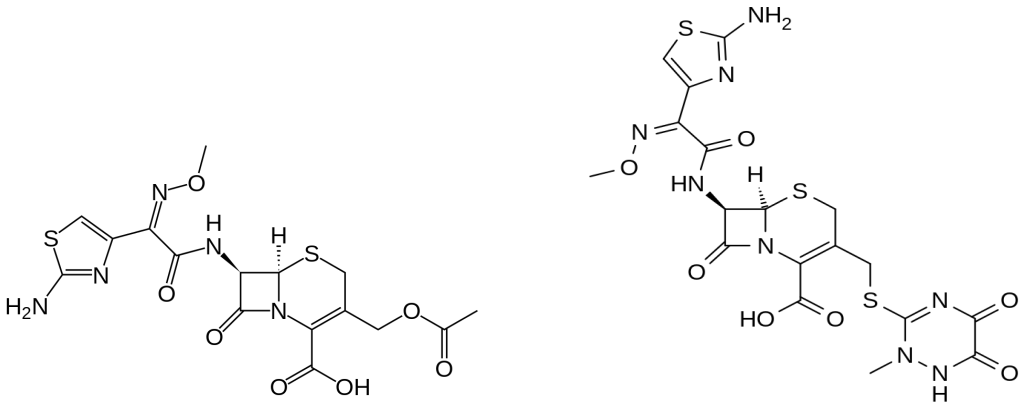
The fundamental requirements for an effective antimicrobial therapy are: high sensitivity of the pathogens towards the antibacterial medicine and the maximum concentration of the drug in the infected zone with minimal side effects. Today the traditional therapy is ineffective due to the great resistance of many bacterial pathogens towards antimicrobial medicines and it makes the treatment of infections intricate. From day to day the number of reports about microorganisms' cultures, which are resistant to antibiotics, is increasing. The fastness to antibiotics is connected with their ability to produce extended spectrum beta-lactamases [3] [4].

Life threatening infections, such as *Klebsiella*, *Escherichia coli* or *Enterobacter*, which belong to the *Enterobacteriaceae* family and also *Staphylococcus aureus*, all have a common mechanism of resistance—they are extended spectrum beta-lactamases producers and have developed resistance to the commonly prescribed antibiotics. Intestinal gram-negative bacilli with resistance to cephalosporins were first identified in the mid-80s in Western Europe. Most of these strains (*Klebsiella pneumoniae*, *Escherichia coli*) were resistant to all beta-lactam antibiotics, except for carbapenem and cephamycin [5]. Genes, in which information about beta-lactamase spread spectrum synthesis is encoded, localize in plasmids that facilitate the dissemination of beta-lactamases spread spectrum among Gram-negative bacteria [6]. The study of the nosocomial epidemic of infections, caused by *Enterobacteriaceae* that produce beta-lactamase spread spectrum, indicates that these strains emerged in response to the intensive use of cephalosporins [7] [8]. Therefore, solution of the problem with antibiotic resistance is an urgent global healthcare priority and can potentially prevent thousands of needless deaths around the world.

In this regard, the nanotechnology-based approach will be able to overcome the complications associated with the traditional antimicrobial therapy. The application of magnetite nanoparticles in medicine has attracted a great deal of attention not only for their extraordinary properties, owing to their ultra small size, such as high surface area, enhanced reactivity, biocompatibility, but also because their surface can be functionalized by drugs and these nanostructures can be delivered to targeted zones by the application of an external magnetic field [9]-[11]. There are facts that such kind of nanostructure is capable of transporting drugs across cell membranes before release, ensuring that the treatment will only damage bacterial cells.

In this study, we report a simple, one-pot preparation of dispersions of Fe_3O_4 nanoparticles that bind with cephalosporin antibiotics cefotaxime and ceftriaxone (**Figure 1**) through a self-assembly process using the method of wet chemical co-precipitation. Carboxylate functional groups of cefotaxime and ceftriaxone as well as amine functionalities are able to bind to the surface of the magnetite nanoparticles and thus stabilize their surface, preventing from further agglomeration.

Materials and methods. All chemicals were used as received. $FeCl_36H_2O$, $FeSO_47H_2O$, NH_4OH (25%), were purchased from Sigma-Aldrich (Taufkirchen, Germany); Acetic Acid solution (33%), phosphate buffer PREP I



(a) (b)

Figure 1. The chemical structure of (a) cefotaxime (6*R*,7*R*,*Z*)-3-(Acetoxymethyl)-7-(2-(2-aminothiazol-4-yl)2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid; (b) Ceftriaxone (6*R*,7*R*)-7-{[(2*Z*)-2-(2-amino-1,3-thiazol-4-yl)->2-(methoxyimino)acetyl]amino}-3-{[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)thio]methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

and II, Crystal Violet Solution were purchased from Merck (Germany) and Nutrient Broth from Biolife (Milano, Italia).

Synthesis of Fe_3O_4 coated by cephalosporin (cefotaxime and ceftriaxone) Fe_3O_4@cefotaxime (Fe_3O_4@CTAX) and Fe_3O_4@ceftriaxone (Fe_3O_4@CTRIX) NPs. Magnetic iron oxide nanoparticles are usually prepared by wet chemical precipitation from aqueous iron salt solutions in alkaline milieu created by using NH_4OH [12]. The formed Fe_3O_4 nanoparticles (NPs) were stabilized by cefotaxime and ceftriaxone molecules. Originally, $FeCl_3 \cdot 6H_2O$ (0.01 M) and $FeSO_4 \cdot 7H_2O$ (0.006 M) were dissolved in 200 mL of deionized water under the bubbling of gaseous N_2. Then, 25% aqueous ammonia solution (1.5 M) was dropped into solution with vigorous stirring until the pH of the solution raised to 9.5. When the color of reaction mixture turned black, the aqueous solutions of cephalosporines, taken in excess, were added to reaction mixture. The magnetite NPs coated by corresponding cephalosporines during reaction was separated by strong NdFeB permanent magnet and repeatedly was washed with distilled water. The obtained NPs were dried at ambient conditions and the iron content in the sample was analyzed by atom absorption spectroscopy and performed on Varian SpectrAA 220FS Atomic absorption spectrometer. Samples were prepared by Milestone ETHOS 1 Microwave extraction unit. The UV spectra have been recorded on Spectrophotometer Specord 250 Plus. UV spectra were recorded at 238 nm for standard solutions of cefotaxime with different concentrations in the range of 0.01 - 0.1 $\mu g \cdot mL^{-1}$

Characterization of structure. X-ray diffraction analysis was performed on Rigaku Mini Flex 600 XRD diffractometer, equipped with CuKα radiation, at ambient. The samples were scanned in the Bragg angle range of 20˚ - 70˚ for 2 hours.

The functional groups, present in the powder samples of Fe_3O_4@CTRIX and Fe_3O_4@CTAX NPs, were identified by Fourier Transform Infrared (FTIR) spectroscopy. FTIR spectra were recorded on a Varian 3600 FTIR spectrophotometer in KBr tablets. The spectrum was taken in the range of 4000 - 400 cm^{-1} at room temperature.

Scanning Electron Microscope (SEM) and Energy-Dispersive Spectrum (EDS) analysis. SEM and EDS analysis of Fe_3O_4@CTRIX and Fe_3O_4@CTAX nanoparticles were taken on Field Emission Scanning Electron Microscope JEOL JSM-7600F at an accelerating voltage of 15.0 kV, SEI regime. EDS of the same samples was performed with an EDAX X-ray energy-dispersive analysis system, attached to the JEOL JSM-7600F transmission electron microscope

Determination of antibacterial activity. Antibacterial activity of cephalosporins and Fe_3O_4@CTAX, Fe_3O_4@CTRIX was tested by diffusion methods on *Staphylococcus aureus* and *Klebsiella* spp. [13]. For this purpose cephalosporins were taken in amount 30 μkg (indicator disks were purchased by Research-and-Development Center of Pharmacotherapy, 192236 St. Petersburg). *Klebsiella* spp. was cultivated on Sabouraud medium and *Staphylococcus aureus* on selective saline agar (cultures were kindly provided by one of the clinical laboratories of Baku). Microbiological tests were performed on Petri dishes. In order to identify the MIC of Fe_3O_4@CTAX and Fe_3O_4@CTRIX, microdilution method was used. The stock solutions of the substances prepared in distillated water were distributed in multi-well plates. Each well was inoculated with 0.1 mL of microbial suspensions of 0.5 Mc Farland turbidity, prepared from 24h fresh culture. Sterility control wells (nutrient broth) and microbial growth controls (inoculated nutrient broth) were used. The plates were incubated for 24 h at 37˚C [13] [14]. In order to measure antibacterial effect on biofilm development, the nutrient broth was deleted; wells were washed with PBS three times and stained by crystal violet 1% for 15 minutes. The biofilm, formed onto the plastic wells, was re-suspended in 30% acetic acid and the intensity of the colored suspension was assayed by measuring the absorbance at 590 nm [15]-[17]. All microbiological studies were performed 4 times in order to exclude mistakes and to prove reproducibility.

2. Results of Experiments

The functionalizing of magnetite nanoparticles surface with cefotaxime and ceftriaxone antibiotics was carried out by wet co-precipitation method. The purity and crystalline properties of the Fe_3O_4@cefotaxim (Fe_3O_4@CTAX) and Fe_3O_4@ceftriaxone (Fe_3O_4@CTRIX) were investigated by powder X-ray diffraction (XRD). The XRD patterns are shown in **Figure 2**. All the reflections can be indexed to Fe_3O_4 nanoparticles with cubic structure. In XRD peak broadening testifies for the formation of nanocrystals.

In both patterns, all lines relate to magnetite and can be indexed using the ICDD (PDF-2/Release 2011 RDB) DB card number 01-075-0449 and card number 00-007-0322 for Fe_3O_4@CTRIX and Fe_3O_4@CTAX correspondingly. The patterns of Fe_3O_4@CTRIX and Fe_3O_4@CTAX NPs have characteristic peaks at 30.44˚ (220), 35.66˚

(311), 43.28˚ (400), 57.5˚ (511), 62.98˚ (440) (**Table 1**) and 30.58˚ (220), 35.74˚ (311), 43.06˚ (400), 57.33˚ (511), 62.79˚ (**Table 2**) correspondingly, which correlate with the standard pattern of Fe_3O_4 well. The intensity of the diffraction peak of (311) plane of both samples is stronger than the other peaks. The average crystal size, estimated from (311) peak, using the Scherrer formula, is 11.8 nm for Fe_3O_4@CTRIX and 8.5 nm for Fe_3O_4@CTAX pattern nanoparticles.

The analysis of IR spectra of nanostructures revealed that these compounds bind on the surface of the magnetite nanoparticles via carboxylate, amine, CONH and hydroxyl groups, by non-covalent interactions, depending upon steric necessity and the curvature of the surface.

Figure 3 and **Figure 4** present the FTIR spectra of Fe_3O_4@CTRIX and Fe_3O_4@CTAX correspondingly that have been compared with those of the free corresponding cephalosporins and Fe_3O_4 nanoparticles, in order to

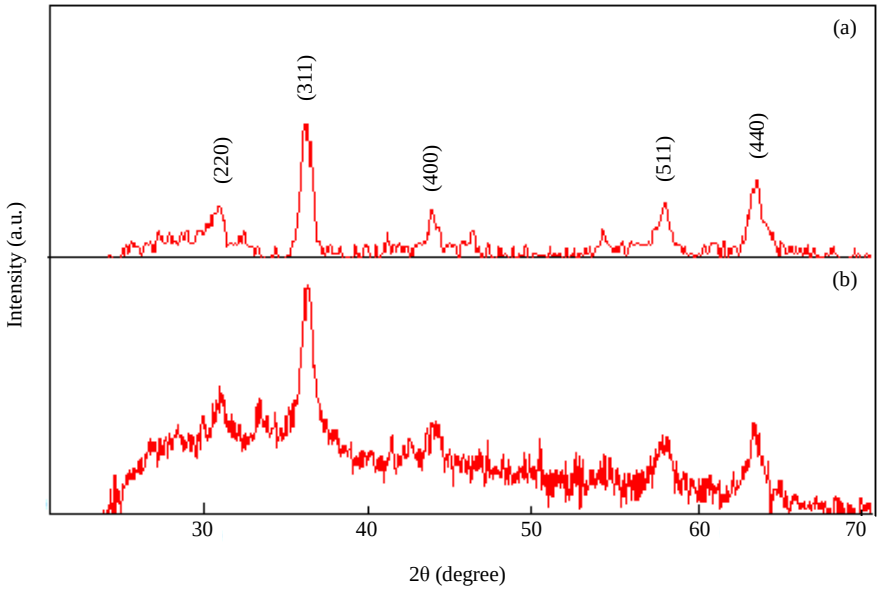


Figure 2. XRD pattern for the nanostructured Fe_3O_4@CTRIX (a) and Fe_3O_4@CTAX (b).

Table 1. Peak list for nanostructured Fe_3O_4@CTRIX.

No	2-theta(deg)	d(ang.)	Int. I(cps deg)	Phase name	Chemical formula
1	30.4452	2.93368	786.344	Magnetite (2,2,0)	Fe_3O_4
2	35.66 (3)	2.515 (2)	2106 (130)	Magnetite (3,1,1)	Fe_3O_4
3	43.2877	2.08845	658.227	Magnetite (4,0,0)	Fe_3O_4
4	57.5	1.60149	870.677	Magnetite (5,1,1)	Fe_3O_4
5	62.9795	1.47469	1173.09	Magnetite (4,4,0)	Fe_3O_4

Table 2. XRD Peak list for nanostructured Fe_3O_4@CTAX.

No.	2-theta(deg)	d(ang.)	Int. I(cps deg)	Phase name	Chemical formula
1	30.5822	2.92085	5636.93	Magnetite (2,2,0)	Fe_3O_4
2	35.74 (7)	2.510 (5)	22463 (883)	Magnetite (3,1,1)	Fe_3O_4
3	43.0 (6)	2.10 (3)	13463 (2210)	Magnetite (4,0,0)	Fe_3O_4
4	57.33 (15)	1.606 (4)	2693 (191)	Magnetite (5,1,1)	Fe_3O_4
5	62.78 (8)	1.4788 (17)	2976 (125)	Magnetite (4,4,0)	Fe_3O_4

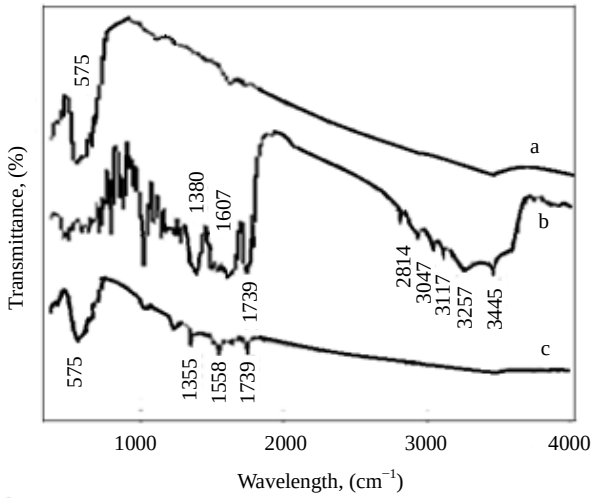


Figure 3. FTIR spectra (a) pristine Fe_3O_4, (b) pristine ceftriaxone, (c) Fe_3O_4@CTRIX.

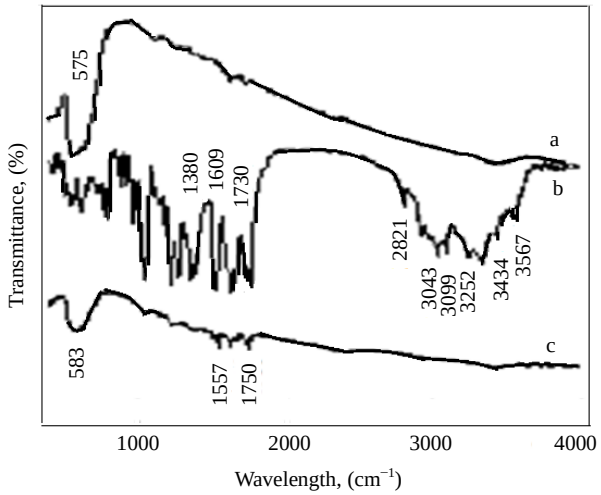
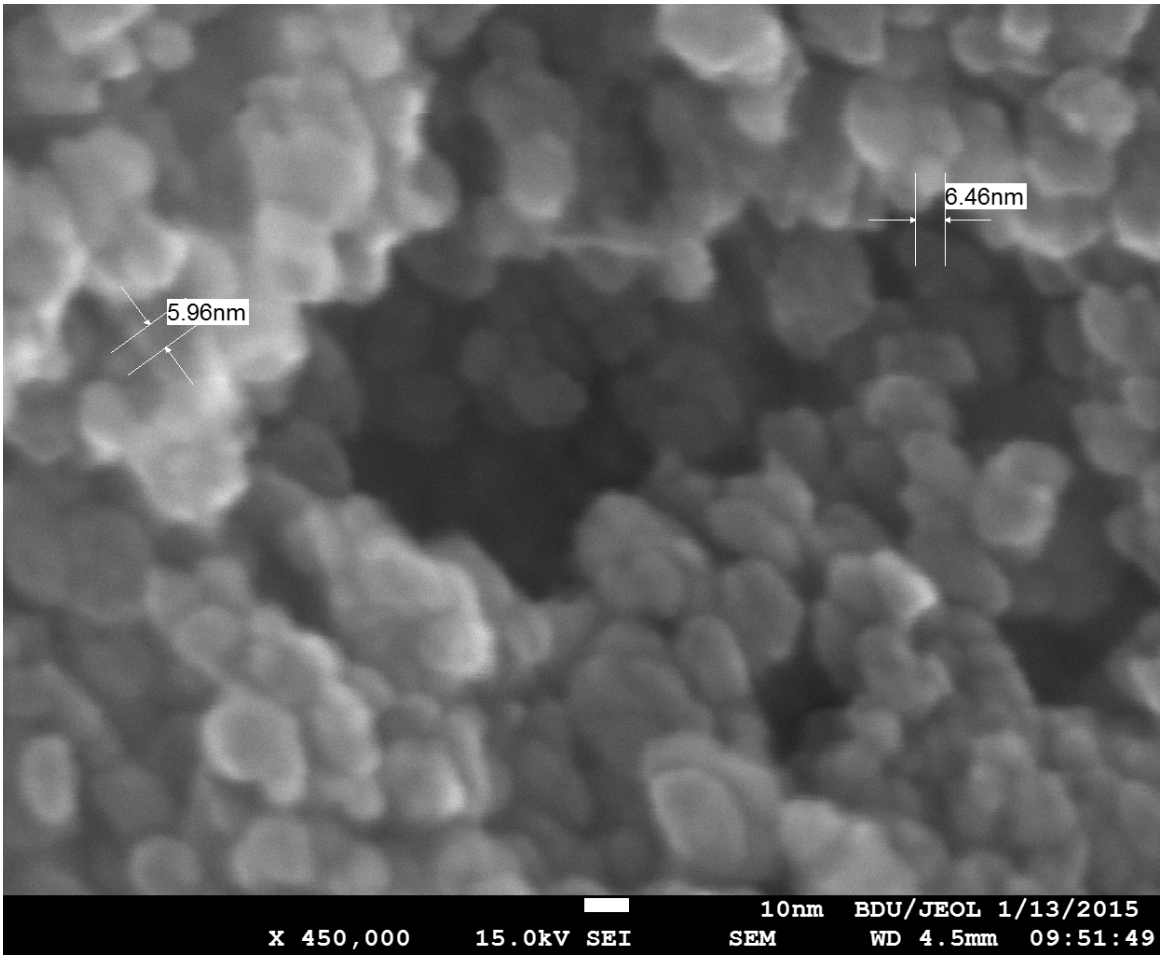


Figure 4. FTIR spectra for the (a) pristine Fe_3O_4, (b) pristine cefotaxime, (c) Fe_3O_4@CTAX.

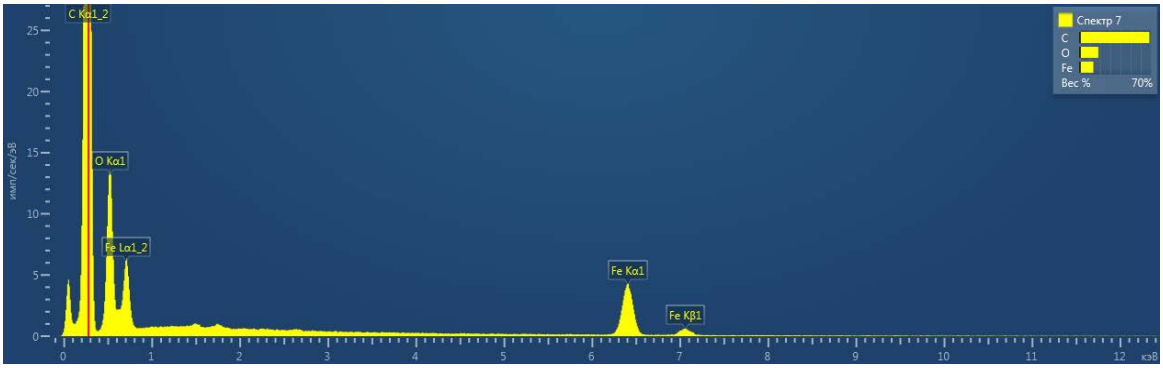
determine the coordination sites that may be involved in chelation with surface of magnetite nanoparticles. The both Fe_3O_4 nanostructures' samples spectra **Figure 3(a)** and **Figure 4(a)** exhibit a characteristic peak of Fe_3O_4 at about 575 - 583 cm^{-1} (Fe-O stretching). The IR spectra of cephalosporins [18] [19] exhibit the strong absorption band at 1730 - 1740 cm^{-1}, corresponding to β-lactam (C=O) stretching vibrations. This band is unshifted in the prepared nanostructures **Figure 3(c)**, **Figure 4(c)**, compared to the free cephalosporins **Figure 3(b)**, **Figure 4(b)**, indicating that this group is not involved in coordination. The band at 1600 - 1610 cm^{-1}, corresponding to the ν_{as} (COO) group of the free cephalosporins, is shifted (40 - 50 cm^{-1}) to lower wave numbers in the spectra of prepared nanostructures, indicating coordination through that group. The band at around 1380 - 1390 cm^{-1}, that correspond to symmetrical carboxylate group, ν_s(COO) also changes as a result of coordination. The bands in the wave number regions 3257 - 3099 and 3047 - 2814 cm^{-1}, corresponding to the ν(NH) and ν(CONH) groups of free cephalosporins, disappear in the spectra of nanostructures that provide the strong evidence that these groups are involved in chelation process with surface of magnetite NPs. The absence of band in the wave region 3445 - 3500 cm^{-1}, corresponding to OH group of free ceftriaxone in the Fe_3O_4@CTRIX, may indicate the ionization of this group during coordination. As it seen from FTIR spectra, the cephalosporins' molecules can provide several site of chelation with surface of magnetite NPs. We assume that absorption of cephalosporins' mo-

lecules occur trough carboxylic, amine, CONH and hydroxyl groups of ceftriaxone and cefotaxime by self-assembling onto surface of magnetic NPs. Thus, the IR spectral results provide strong evidences for the multiple chelation sites of drugs with surface of magnetite NPs.

Stabilization of magnetite NPs by ceftriaxone and cefotaxime monomeric coating led to preparing of Fe_3O_4@CTRIX and Fe_3O_4@CTAX, characterized with a very narrow size distribution (approximately 6 - 13 nm). The morphological characteristics of the Fe_3O_4@CTRIX and Fe_3O_4@CTAX NPs, determined by SEM, are shown in **Figure 5(a)** and **Figure 6(a)**. As it shown in **Figure 5(a)** and **Figure 6(a)**, the prepared nanostructures were

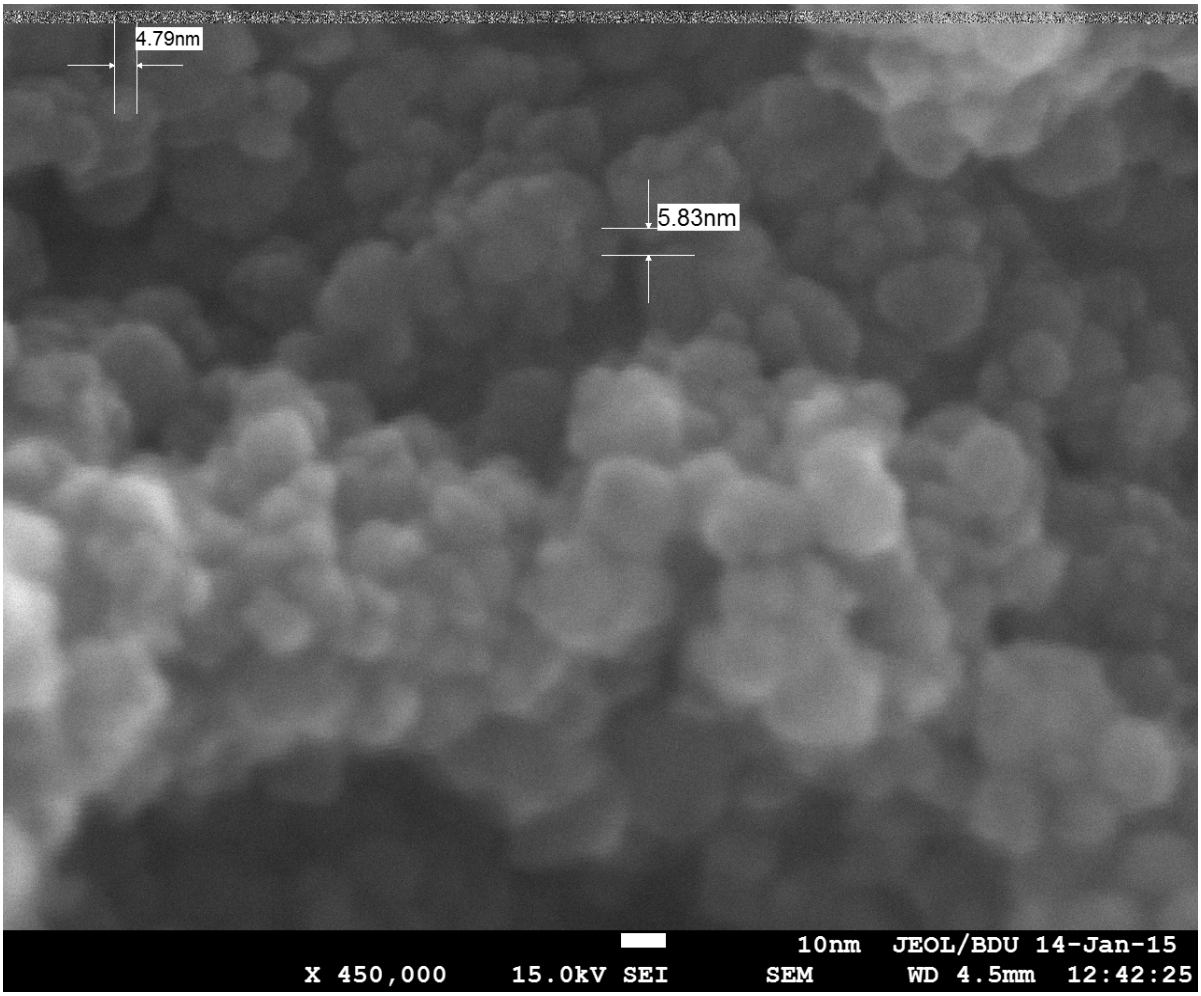


(a)

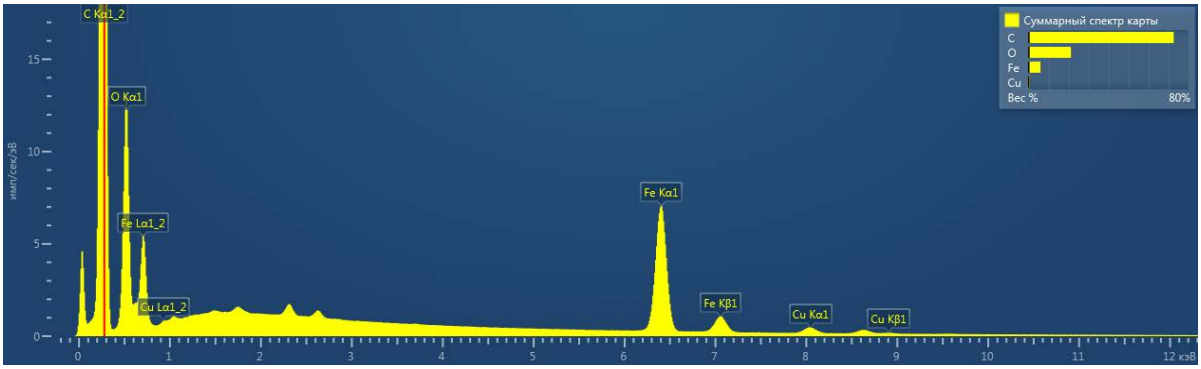


(b)

Figure 5. (a) SEM image of Fe_3O_4@CTRIX NPs; (b) ED pattern of Fe_3O_4@CTRIX NPs.



(a)



(a)

Figure 6. (a) SEM image of Fe_3O_4@CTAX NPs; (b) ED pattern of Fe_3O_4@CTAX NPs.

monodisperse with almost uniform size. The average sizes of formed nanoparticles correlate well with data obtained from XRD analysis. The data, obtained from SEM analysis of prepared samples of Fe_3O_4@CTRIX and Fe_3O_4@CTAX nanoparticles, are in good compliance with results of XRD analysis. These results revealed that the nanoparticles were homogeneously sized and uniformly shaped. The points, identified in the EDS spectrum **Figure 5(b)** and **Figure 6(b)**, demonstrate the presence of Fe and O as the main elements of the sample and support the data of magnetite nanoparticles formation (the other peaks are corresponding to Cu, C being charac-

teristic of the carbon-coated grid).

Quantitative analysis of cephalosporins' molecules, loaded on magnetic NPs, was performed by UV spectroscopy methods on the basis of Lambert-Beer law and the iron content in NPs samples was determined by AAS method. The UV spectrum of cephalosporin, as it is shown in **Figure 7**, can be divided into 3 main areas of absorption: intense main maximum absorption region at low wavelengths, consisting of either one, or more maxima ("left shoulder"), and a similar area of absorption at higher wavelengths ("right shoulder"), which is especially characteristic for the UV spectrum of cefotaxime.

The results of measurements through different methods and following calculations are correlating with each other very well and reveal that the concentrations of loaded cephalosporins' molecules in NPs are very close and make 0.18 gr and 0.21 gr of ceftriaxone and cefotaxime in 1 gr of Fe_3O_4@CTRIX and Fe_3O_4@CTAX NPs correspondingly.

By the diffusion method it reveals that Fe_3O_4@CTRIX and Fe_3O_4@CTAX, unlike usual cephalosporins, have no effect on *Staphylococcus aureus* (the data not shown). However, Fe_3O_4@CTRIX and Fe_3O_4@CTAX have significant antibacterial effect on *Klebsiella*, whereas pure cephalosporins have not (**Figures 8-10**).

So, we decided to identify the MIC of nanostructurized cephalosporins only on *Klebsiella* spp. The MIC of Fe_3O_4@CTRIX and Fe_3O_4@CTAX on *Klebsiella* was 2 μg/mL. According to the spectra, received by spectrophotometry (performed on *Spectrometer Specord* 250 *plus*), in case of *Klebsiella*, Fe_3O_4@CTAX exhibited an

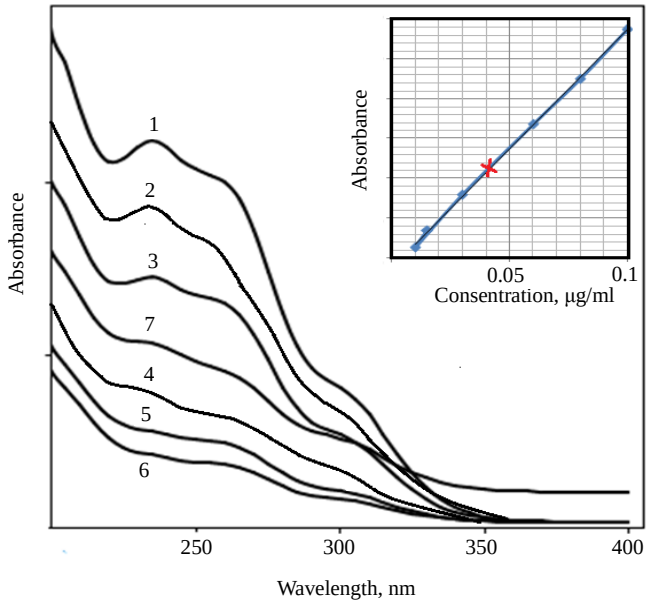
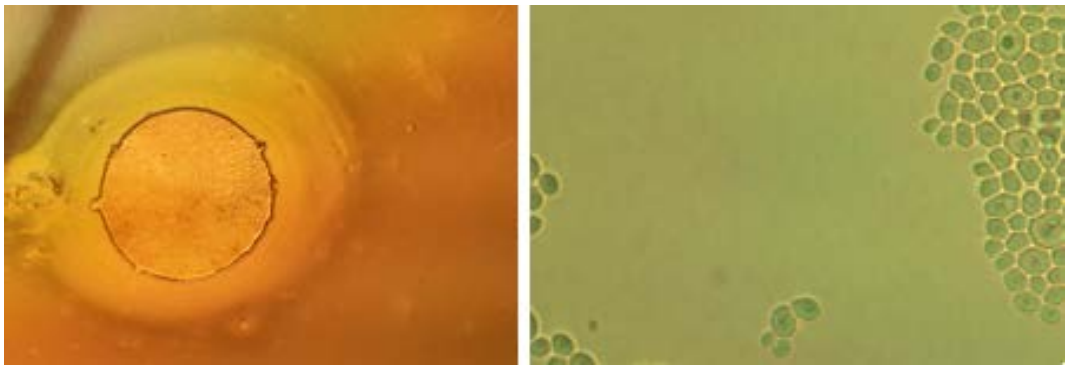


Figure 7. UV spectra of pure cefotaxime 1) 0.1 μg·mL^{-1}; 2) 0.08 μg·mL^{-1} 3) 0.06 μg·mL^{-1}; 4) 0.03 μg·mL^{-1} 4) 0.015 μg·mL^{-1}; 5) 0.01 μg·mL^{-1} and Fe_3O_4@CTAX 7) 0.043 μg·mL^{-1}.

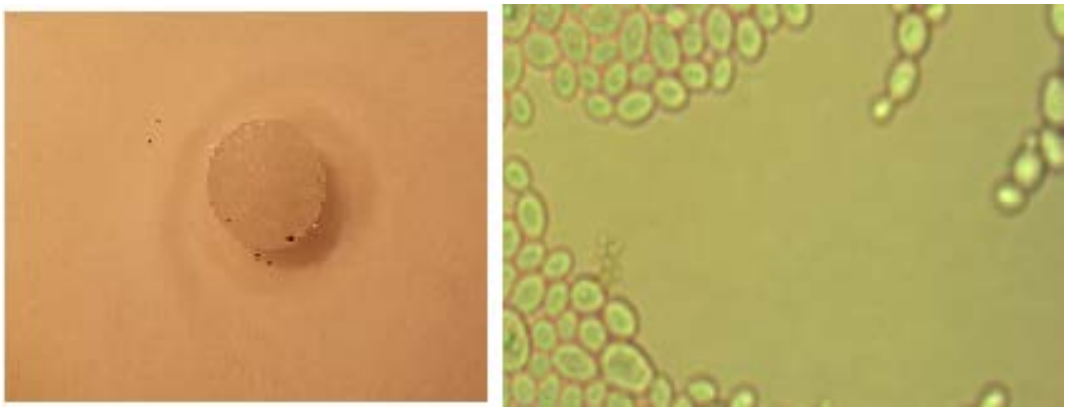
(a) (b) (c)

Figure 8. Usual cefalosporins (a)-ceftriaxone, (b)-cefotaxime against *Klebsiella spp.* and the view under microscope (c).



(a) (b)

Figure 9. Inhibition zones produced by Fe_3O_4@CTRIX against *Klebsiella spp.* (a) and its view under microscope (b).



(a) (b)

Figure 10. Inhibition zone produced by Fe_3O_4@CTAX against *Klebsiella spp.* (a) and its view under microscope (b).

inhibitory effect on biofilm development from 0.5 μg/mL and Fe_3O_4@CTRIX from 0.25 μg/mL, as it is shown in **Figure 11**. However, the amount of nanostructurized cephalosporins should not be very large for two reasons. Firstly because cephalosporins are concentration independent antibiotics [20] and the second reason is that NPs in big amount can aggregate and become inactive.

3. Discussion

The ability of NPs to penetrate through cell membrane of pathogenic microorganism may be forth by various factors, among them the nanoparticles size and engineering the surface of nanoparticles with special signal molecules that make possible for cell to recognize the nanostructure. It is known, microbes are adjusting and developing very fast multi-resistance against to known form of antibiotics, and the speed of working out of new classes of antimicrobial therapeutics is lower than the ability of microbes to develop resistance properties. In the 80 years of last century the majority of hospital strains of microorganisms exhibited high sensitivity to cephalosporins, which determined their high clinical efficacy in infections of different localization. However, in the last decade antimicrobial chemotherapy caused a significant increase in resistance of Gram-negative organisms to cephalosporins, mainly due to the emergence of the ability of production of beta-lactamases of various types and classes by microorganisms [21] [22]. *Klebsiella* genus is extended spectrum beta-lactamases producer, so it destroys the beta-lactam ring of cephalosporin, but at the same time it synthesizes and secrets siderophores—low molecular weight compounds, in order to improve the availability and absorption of iron [23]. Siderophore molecules represent the specific transport system in Gram-negative bacteria. Laden with iron, siderophores' molecules bind to the bacterial wall to move iron into microbial cells. Most probably the prepared nanostructures of Fe_3O_4@CTRIX

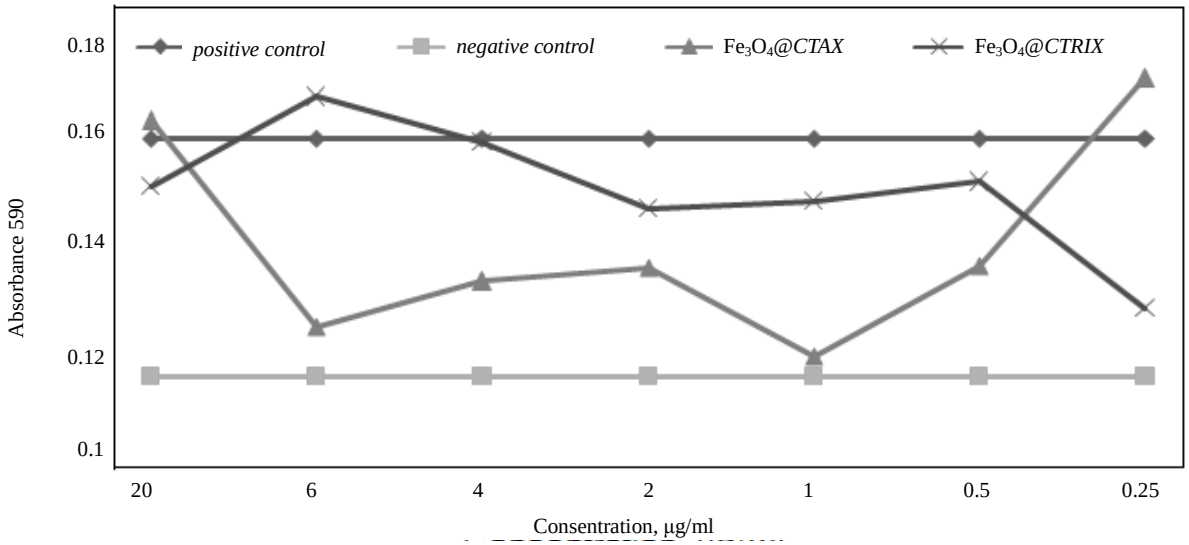


Figure 11. The effect of the Fe_3O_4@CTAX and Fe_3O_4@CTRIX on *Klebsiella's spp*. biofilm development after 24 h of incubation.

and Fe_3O_4@CTAX can avoid the destroying of beta-lactam ring of cephalosporins, due to siderophores which can bind the iron-containing nanoparticles, laden with antibiotics, and transport them through microbial cell. Thus prepared nanostructures play the role of so-called "Trojan horse", who deceives the security system of microbial cells and contributes to dragging the nano-antibiotic into the microbial cell. The absence of antibacterial effect on *Staphylococcus aureus* of prepared nanostructures Fe_3O_4@CTRIX and Fe_3O_4@CTAX is most likely explained by Fe_3O_4 NPs chelation with CONH group of beta-lactam ring, which is essential for pharmacological activity [24]. *Staphylococcus aureus* is referred to Gram-positive bacteria, which do not posses iron uptake system, based on syderophores. At the same time beta lactam ring of antibiotic is blocked by Fe_3O_4 NPs and cannot act in its usual way.

So, the expanding of antibacterial medicines' assortment by the modification of already existed antibiotics can help to fight against quick development of bacterial resistance. There is a clear advantage of nano-antibiotics that naturally occurring microbes have so far not developed resistance against them.

4. Conclusion

It can be assumed that the synthesized biocompatible Fe_3O_4-based nanostructures have practical importance, since they can be used for targeted drug delivery and controlled release of modified antibiotics. They may also be applied to decrease therapeutic doses of medicines, since the drugs with high dose have few serious side effects. In addition, Fe_3O_4@CTRIX and Fe_3O_4@CTAX nanostructures can serve as a model for the development of new alternative strategies by applying so called "Trojan horse" principle for improving the bactericidal effect of already used and new developed antibacterial medicines.

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