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Synthesis of Macrocycle (MC) – Mimics the Properties of Natural Siderophores and Preparation the Nanostructures on the Basis of MC and Magnetite Nanoparticles

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In this work we report of the synthesis of hydroxyl containing azacrown macrocycle (MC) that is able to mimic the properties of natural siderophores. On the basis of synthesized MC were prepared the supramolecular ensembles with magnetite nanoparticles, loaded by cephalosporin antibiotics. The synthesized MC was investigated by NMR, mass-, FTIR spectroscopy methods. The morphology of prepared nano-ensembles was analyzed by scanning electron microscopy SEM, FTIR, X-ray diffraction XRD analysis methods. The quantitative analysis of nanostructures was determined by atom absorbance spectroscopy (AAS) as well as on the basis of Lambert-Beer law by UV spectroscopy method. Prepared nanostructures were tested on gram-negative *Escherichia Coli* and gram-positive *Staphylococcus aureus,* having multi drug resistance properties. Has been found that the nanostructures significantly increase the antimicrobial effect of cephalsporins and decrease their MIC.

Introduction

Today's huge problem, faced to clinicians, is the problem of antibiotic resistance. Synthesis and application of new drugs, for which the microorganisms have not yet worked out the mechanism of resistance, is extremely long and laborious process that requires significant investment. As the samples of resistance can be provided the fact, revealed by Brin et al. (2005), that the quantity of the ESBLs producing strains of the *Escherichia Coli*, causing nosocomial and community-acquired infections, is increasing rapidly day by day. In turn, according to the research of Adamuet et al (2010), *Staphylococcus Aureus* also show the rise of the number of resistant strains to antibiotics, especially to β-lactames, particularly in developing countries. As a result, there is an urgent requirement of new modified drugs that are effective against antibiotic-resistant bacteria.
The one of the possible ways is nanostructuring of already used drugs that will enhance and improve their activity. In particular, as it was mentioned by Dorniani et al. (2013), attention is paid to the application of magnetite nanoparticles in targeted drug delivery, not only due to their biocompatibility, but also because of the possible targeting of these nanoparticles, loaded with drug, directly to the infected sites and organs of organism by means of the applied external magnetic field. And, in case with nanoantibiotics, as it was mentioned by Grumezescu et al., magnetite nanoparticles can reduce the toxicity and side effects of antimicrobial agents.
It is known that the development and functioning of most pathogenic microorganisms require a great concentration of iron ions, and as a result they have developed a special system of binding and transport of iron ions that are necessary for the functioning of their life cycle. This system, as described by Miethke and Marahiel (2007), is based on a shuttle mechanism that uses small-molecule compounds, called siderophores, that selectively bind to the iron ions. Many scientists, including Kline et al. (2000), point that antibiotics, coupled with siderophore-mimic compounds, can easily penetrate through the membrane of gram-negative bacteria.

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In accordance to above mentioned, it was interesting to synthesize the hydroxyl containing azacrown ether that is able to mimic the properties of natural siderophores and prepare the supramolecular ensembles of synthesized MC with magnetite nanoparticles, loaded by cephalosporin antibiotics. The investigation of their biological activity against gram-positive and gram-negative microorganisms is a matter of interest in terms of finding the synergistic effect of siderophore-mimic MC and magnetite NPs, loaded with cephalosporin antibiotics.

Materials and methods

All chemicals, used in the synthesis, were of analytical grade and used as received. Ethylenediamine, 1,3-dichloro-2-propanol, salysilaldehyde were purified by distillation under reduced pressure, created by water pump. $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, NH_4OH (25 %), were purchased from Sigma-Aldrich (Taufkirchen, Germany); Nutrient Broth was purchased from Biolife (Milano, Italia).

Synthesis of MC 1,13-Diaza-5,9-dioxa-7-hydroxy-3,4:10,1-dibenzocyclopentadecan with further purification by recrystallization from benzene, yield 0.70 g (65 %), m.p. 148-149 °C. Found: C, 69.5; H, 7.4; N, 8.7. Calc. for $C_{19}H_{24}N_2O_3$: C, 69.5; H, 7.4; N. 8.5 %. *IR* (KBr): 3350 (OH); 3330, 1455 (NH); 1604, 1590, 1492 (Ar); 1251, 1035 (Ar—O—CH_2); 754 (1,2-Ar) cm^{-1} .^{1}H NMR (**δ**): 2.64 (s, 4H, NCH_2CH,N), 3.24 (br, 3H, NH and OH), 3.65 (s, 4H, $ArCH_2$), 4.14 (m, 5H, CH_2CHCH_2), 6.74-7.25 (m, 8H, ArH) ppm.

Synthesis of nanostructures, based on Fe_3O_4, coated by MC and cephalosporin antibiotics (cefotaxime and ceftriaxone): MC@Fe_3O_4@cefotaxime (MC@Fe_3O_4@CTAX) and MC@Fe_3O_4@ceftriaxone (MC@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, in the atmosphere of gaseous nitrogen, as described by Massart (1981).The formed Fe_3O_4 nanoparticles (NPs) were separated by strong NdFeB permanent magnet, repeatedly washed with distilled water and dispersed in ethanol. The ethanol solution of MC, taken in excess, was added to ethanol solution of Fe_3O_4 nanoparticles and vigorously stirred 45 minutes. Then nanoparticles were stabilized by cefotaxime and ceftriaxone molecules, by adding the water solution of corresponded antibiotics into reaction mixture. After stirring during 8 hours at ambient, the prepared nanostructures were separated by strong NdFeB permanent magnet and repeatedly were washed with distilled water. The obtained NPs were dried at ambient conditions, and the iron content in the samples 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 ceftriaxone with different concentrations in the range and 275 nm for standard solutions of MC.

Characterization of structure

XRD

X-ray diffraction analysis was performed on Rigaku Mini Flex 600 XRD diffractometer at ambient. In all the cases, Cu K **α** radiation from a Cu X-ray tube (run at 15 mA and 30 kV) was used. The samples were scanned in the Bragg angle 2 h range of 20–70 °.

FT-IR

The functional groups, present in the powder samples of MC@Fe_3O_4@CTRIX and MC@Fe_3O_4@CTAXNPs, 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 prepared samples of MC@Fe_3O_4@CTRIX and MC@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.

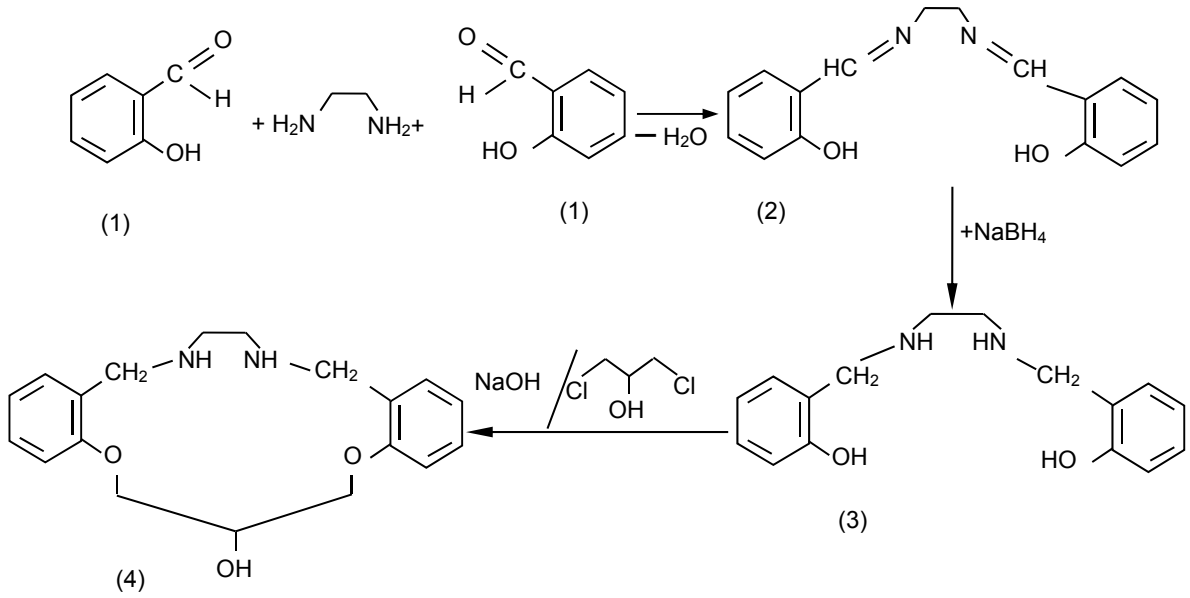
Determination of antibacterial activity

Antibacterial activity of pristine antibiotics and the prepared nanostructures were tested by diffusion method on *Staphylococcus aureus* and *Escherichia coli*, as described by Mayrhofer (2008). Cephalosporins were taken in amount 30 μg (indicator disks were purchased by Research-and-Development Center of Pharmacotherapy, 192236 St. Petersburg). The synthesized substances were also taken in amount equal to 30 μg. *Escherichia coli* was cultivated on Endo's medium and *Staphylococcus aureus* on Baird-Parker agar (cultures were kindly provided by one of the clinical laboratories of Baku). Microbiological tests were performed on Petri dishes. Due to the fact that this method provides only quality data, microdilution method was also performed, as it is written by Jorgensen and Lee (1975). By this method the MICs of the prepared nanostructures and usual antibiotics were identified and further compared to each other. To perform

microdilution method the stock solutions with different concentrations of the substances were prepared in distilled sterile water and were distributed in 96 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.

Results and discussions

Synthesis of MC1,13-Diaza-5,9-dioxa-7-hydroxy-3,4:10,1-dibenzocyclopentadecan(4) is shown on the scheme 1. N,N´ethylenebis(salysilimine) (2) were prepared by condensation of salysilaldehide (1) with ethylenediamine. The reduction of (2) was carried out by sodium borhydride. The ring closure step was carried out by reaction of corresponding saturated derivative (3) with1,3-dichloro-2-propanol.



Scheme 1. The synthesis of MC 1,13-Diaza-5,9-dioxa-7-hydroxy-3,4:10,1-dibenzocyclopentadecan

The purity and crystalline properties of the MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX were investigated by powder X-ray diffraction (XRD). The XRD patterns are shown in Figure 1 and 2 correspondingly. All the XRD peaks were well defined and corresponded 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 00-001-1111, for prepared nanostructures. The patterns of MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX NPs have characteristic peaks at 30.44° (220), 35.64° (311), 43.16° (400), 57.31° (511), 62.76° (440) Figure 1 and 30.58° (220), 35.66° (311), 43.30° (400), 57.30° (511), 62.89° (440) Figure 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 other peaks. The average crystal size, estimated from (311) peak, using the Scherrer formula, is 11.8 nm for MC@Fe_3O_4@CTAX and 8.5 nm for MC@Fe_3O_4@CTRIX pattern nanoparticles.

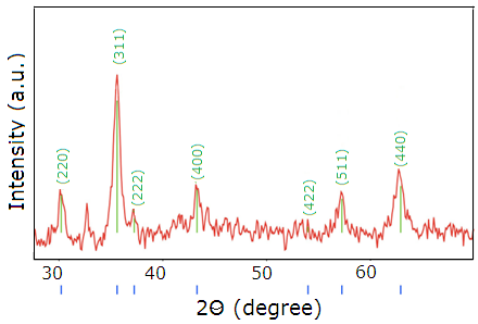


Figure 1. XRD pattern for the nanostructured MC@Fe_3O_4@CTAX

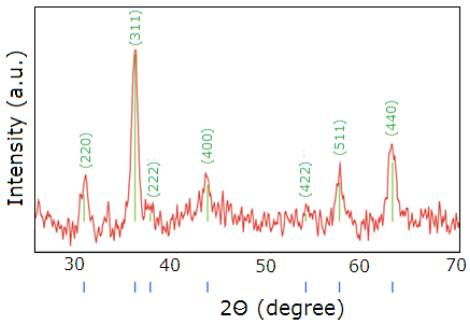


Figure 2. XRD pattern for the nanostructured MC@Fe_3O_4@CTRIX

Figure 3 (a) and 4 (a) present the FTIR spectra of MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX correspondingly. The spectra of prepared nanostructures were compared with spectra of pristine MC on the Figure 3 (b) and 4 (b); spectra of pristine cephalosporins Figure 3 (c) and 4 (c); and spectra of pristine Fe_3O_4 Figure 3 (d) and 4 (d); in order to determine the coordination sites that may be involved in chelation with surface of magnetite nanoparticles. The spectra of both nanostructures Figure 3 (a) and Figure 4 (a) exhibit a characteristic peak of Fe_3O_4 at about 574-580 cm^{-1} -(Fe–O stretching). The IR spectra of cephalosporins, described by Anacona and Lopez (2012), exhibit the strong absorption band at 1730–1740 cm^{-1}, corresponding to β-lactam (C=O) stretching vibrations. This band is not shifted in the prepared nanostructures Figure 3 (a) and Figure 4 (a), compared to the pure cephalosporins Figure 3 (c) and Figure (c), indicating that this group is not involved in coordination. In the both samples the band at 1600–1610 cm^{-1}, corresponding to the v_{as} (COO) group of the free cephalosporins, is shifted (20–50 cm^{-1}) to lower wavenumbers in the spectra of prepared nanostructures, indicating coordination through that group. The band at around 1370-1380 cm^{-1}, which corresponds to symmetrical carboxylate group v_s(COO), also changes that points to chelation via this group with the magnetite surface. The bands in the wavenumber regions 3250–3100 and 3050–2810 cm^{-1}, corresponding to the v(NH) and v(CONH) groups of pure cephalosporins, disappear in the spectra of nanostructures that provides the strong evidence that these groups are involved in chelation process. The absence of band in the wave region 3445-3500 cm^{-1}, corresponding to OH group of free ceftriaxone in the spectra of MC@Fe_3O_4@CTRIX, may indicate the ionization of this group during coordination. At the same time the comparison of spectra of pure MC Figure 3 (b) and 4 (b) with spectra of prepared nanostructures shows the shifting of strong band at 1455 cm^{-1}, corresponding to the NH groups of MC, to 1399 cm^{-1} region in the nanostructures. The wide band at 3330-3350 cm^{-1}, corresponding to v(OH) and v(NH) in pristine MC, disappears in the spectra of nanostructures Figure 3 (a) and 4 (a), and that is strong evidence of coordination of MC molecules with magnetite surface via OH and NH groups of MC. The intense band at 1251 cm^{-1} (Ar-O-CH2) of MC Figure 3 (b) and 4 (b) is shifted to 1180 cm^{-1} region in the spectra of MC@Fe3O4@CTAX and disappears in the spectra of MC@Fe3O4@CTRIX. These indicate the involving of macrocycle's oxygen atoms in coordination process.

As it seen from FTIR spectra the absorption occurs through carboxylic, amine, CONH, hydroxyl groups of ceftriaxone and cefotaxime and NH, OH and cyclic oxygen atoms of MC by self-assembling via non-covalent interaction with surface of magnetic NPs. Thus, the IR spectra results provide strong evidences for the multiple chelation sites of MC and drugs molecules with surface of magnetite NPs.

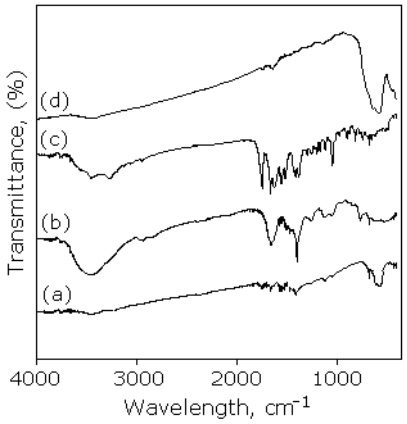


Figure 3. FTIR spectra (a) MC@Fe_3O_4@CTAX, (b) pristine MC, (c) pristine cefotaxime, (d) pristine Fe_3O_4

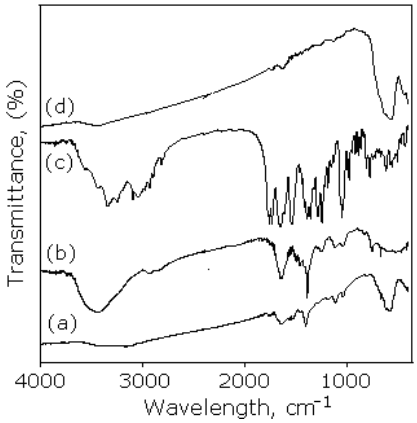


Figure 4.FTIR spectra (a) MC@Fe_3O_4@CTRIX (b) pristine MC, (c) pristine ceftriaxone, (d) pristine Fe_3O_4

The surface morphology of the MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX NPs, determined by SEM, are shown in Figure 5 and Figure 6 correspondingly. As it shown the prepared nanostructures are monodisperse with almost uniform size approximately 6-13 nm**.** The average sizes of formed nanoparticles correlate well with data, obtained from XRD analysis.

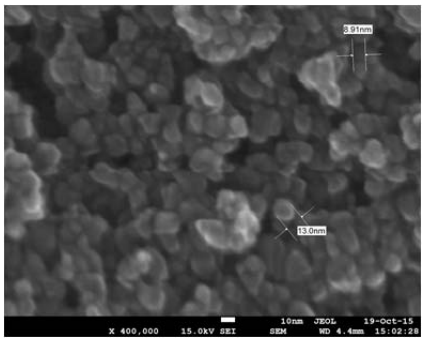


Figure 5. SEM image of MC@Fe_3O_4@CTAX NPs

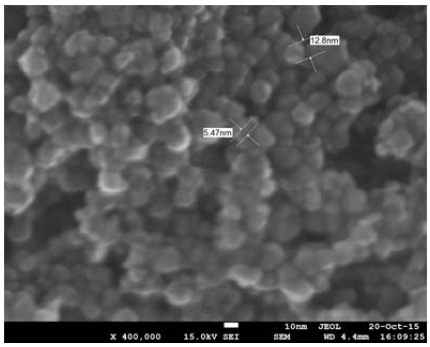


Figure 6. SEM image of MC@Fe_3O_4@CTRIX NPs

Quantitative analysis of cephalosporins' molecules, contained in MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX NPs, was analyzed by UV spectroscopy methods on the basis of Lambert-Beer law and the iron content in NPs samples was determined by AAS method. In accordance with results and following calculation of both methods, they correlate with each other very well and reveal very close value of loaded cephalosporins' molecules in NPs that makes 0.18 g and 0.21 g of ceftriaxone and cefotaxime in 1 gr of MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX NPs correspondingly.

The microbiological assay was performed on two different strains of bacteria: *Staphylococcus aureus* and *Escherichia coli*. The choice was based on the results of our previous research, performed by Hasanova et al. (2015), showing that gram-negative and gram-positive bacteria react differently to nanostructures, containing magnetite nanoparticles. We assumed that the presence of siderophoric system, which is responsible for iron uptake in the most of gram-negative bacteria, overcomes the destroying of β-lactam ring of cephalosporins via dragging drug loaded magnetite through the siderophoric channels in outer membrane of the bacteria. On the basis of this suppose we synthesized MC that is able to mimic the natural siderophores, in order to get the synergistic effect with antibiotics, coupled with magnetite NPs.

As it is known from our previous study, the Fe_3O_4@CTAX and Fe_3O_4@CTRIX have no antimicrobial effect on *S. aureus.* However, the results of agar diffusion test on *Staphylococcus aureus* show that the inhibition zones of pure cefotaxime and MC@Fe_3O_4@CTAX were almost the same, equal to 13 and 11 mm correspondingly; MC@Fe_3O_4@CTRIX and pure ceftriaxone have absolutely the same inhibition zone, as shown on the Table 1. Thus, the binding of MC to the Fe_3O_4@CTAX and Fe_3O_4@CTRIX NPs restores the antimicrobial properties of initial drugs in the MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX NPS correspondingly.

However, the effect of the MC@Fe_3O_4@CTAX on *E.coli* was significant. From the Table 2 it is seen that the diameter of inhibition zone of MC@Fe_3O_4@CTAX was 29 mm, whereas pristine cefotaxime produced 9 mm of inhibition zone's diameter. The Fe_3O_4@CTAX NPs' inhibition zone diameter is 22 mm, that is also noticeably greater than that made by pure cefotaxime. MC@Fe_3O_4@CTRIX and Fe_3O_4@CTRIX produced the same inhibition zone with diameter 34 mm; however, pure ceftriaxone produced the zone with diameter equal only to 7 mm. According to obtained results, we can suggest that MC mimics the properties of natural siderophores, bound with iron, helps antibiotic to avoid the mechanism of the resistance, elaborated by bacteria.

The microdilution method provided us information about MICs of the studied substances. As it shown on the Table 1 MICs of cefotaxime, ceftriaxone, MC@Fe_3O_4@CTAX and MC@Fe_3O_4@CTRIX on *S.aureus* were the same and equal to 6 μg/mL. In turn, as it shown on Table 2 MIC of Fe_3O_4@CTAX on *E.coli* was 6 times and MC@Fe_3O_4@CTAX 12 times lower than MIC of pristine cefotaxime. The results of ceftriaxone's nanostructures are even better. The MIC of Fe_3O_4@CTRIX was 12 times and MC@Fe_3O_4@CTRIX 24 times lower than MIC of pure ceftriaxone. The improvement of MIC on *Escherichia coli* correlates well with the increase of the antimicrobial effect, seen from diffusion method and also evidences that MC mimics siderophore and significantly improves the effectiveness of antibiotics.

Table 1. The diameter of inhibition zones, produced by synthesized nanostructures and pristine antibiotics, and their MICs on Staphylococcus aureus

	Cefotaxime	Ceftriaxone	Fe_3O_4@CTAX	Fe_3O_4@CTRIX	MC@Fe_3O_4 @CTAX	MC@Fe_3O_4 @CTRIX
Diameter (mm)	13	11	0	0	11	11
MIC (μg/mL)	6	6	-	-	6	6

Table 2. The diameter of inhibition zones, produced by synthesized nanostructures and pristine antibiotics, and their MICs on Escherichia coli

	Cefotaxime	Ceftriaxone	Fe_3O_4@CTAX	Fe_3O_4@CTRIX	MC@Fe_3O_4 @CTAX	MC@Fe_3O_4 @CTRIX
Diameter (mm)	9	7	22	34	29	34
MIC (μg/mL)	6	6	1	0.5	0.5	0.25

Conclusion

We reported of successfully synthesis of hydroxyl containing diazacrown ether (MC) that is able to mimic the properties of natural siderophores. The binding of MC to Fe_3O_4@CTAX and Fe_3O_4@CTRIX NPs overcomes the destroying of β-lactam ring of cephalosporins, due to their synergistic effect. It was shown that nanostructuring of cefotaxime and ceftriaxone significantly improves the antimicrobial effect of above-mentioned drugs, even on the resistant strains of *Escherichia coli*. We can also conclude that the impressive synergistic effect of synthesized MC and cephalosporin antibiotics, united in magnetite based nanostructures, impressively decreases the MIC of antimicrobial agents. As consequence, the nanotechnological approach creates a possibility to reduce the dosage of taken medicines with keeping therapeutic effect high and side effects low.

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