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Original paper

Synthesis and characterization of zein-chromium nanoparticles with antibacterial effects

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Abstract

Antibiotic resistance is a major problem of modern medicine. Due to the ability of Gram-negative bacteria to develop resistance against antibiotics, novel therapeutic strategies are emerging based on nanomaterials that perform a controlled release of antibacterial agents that don't promote resistance. Protein nanoparticles are good candidates for this approach because they are biocompatible, biodegradable and non-toxic. Zein is a protein from corn that is able to assemble into nanoparticles. Zein nanoparticles loaded with essential oils, plant extracts or with silver nanoparticles presented a good stability and superior antibacterial effects. Taking into account that trivalent chromium ions (Cr^{3+}) conjugated with amino acids have antibacterial and antifungal activity, here we have synthesized and tested the antibacterial effect of zein nanoparticles loaded with Cr^{3+} against two *E. coli* strains with different antibiotic resistance profiles. The nanoparticles that we synthesized are 20-50 nm in size. While zein nanoparticles without Cr^{3+} have no antibacterial effect, chromium-zein nanoparticles inhibit the growth of the two strains and the biofilm formation. Given the effectiveness of chromium-zein nanoparticles against bacterial strains with resistance to different classes of antibiotics and the advantages offered by zein, these are good candidates for diverse biomedical applications.

Keywords Zein nanoparticles, chromium, antibiotic resistance, nanoparticles with chromium, antibacterial nanomaterial.

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Introduction

Antibiotic resistance is a major problem of modern medicine, being a serious threat to human health, especially in the case of hospital patients, nursing homes residents, people in rehabilitation clinics or long-term care facilities, people travelling to the south-eastern Asian countries or medical tourists [1]. The irrational usage of antibiotics in both humans and farm animals selected resistant bacterial strains. A recent survey of the World World Health Organization revealed that antibiotic resistance is widespread in both low and high-income countries [2]. Different strategies should be considered in order to overcome this problem, including the prevention of transmission and reservoir build-up [1] and the development of antibacterial compounds that don't promote resistance, such as peptides [3] or nanoparticles [4].

Zein is an alcohol-soluble prolamine found in the endosperm of corn (*Zea mays* L.) kernels [5]. Comprising mostly non-polar residues, zein is insoluble in water and naturally occurs as disulfide-linked aggregates of high molecular mass [6]. Zein extraction by different methods leads to the formation of fragments with different molecular weights called α, β, δ and γ zein [7], α and β zein being the major components [6]. The amino acids composition of zein accounts to its poor nutritional value and its limited usefulness in food industry. Nevertheless, zein has a wealth of industrial applications [6]. Being recognized by US FDA as a safe biomaterial [8, 9], different applications in the biomedical field are under development, including controlled-release drug delivery systems [10, 11] and nanoparticles (NPs) [12-15].

NPs are popular drug delivery systems due to their controllable size, surface properties or drug release dynamics [16, 17]. Protein NPs have gained increasing attention because they meet these criteria and additionally are biocompatible, biodegradable and non-toxic [18]. The hydrophobic regions of zein easily assemble to form colloidal NPs with a polar surface that have the ability to retain both hydrophobic and hydrophilic compounds [19]. Zein NPs don't have a good colloidal stability and are prone to aggregation and precipitation [20], these shortcomings being enhanced by pH values larger than 5 or in the presence of salts [21]. The improvement of zein NPs properties was achieved by coating them with emulsifiers such as lecithin [21] or other compounds [22]. In a recent study, A. GAGLIARDI & al [23] tested the influence of different surfactants on zein NPs stability and identified that sodium deoxycholate monohydrate (SD) produces the most stable formulation, resulting NPs being also similar in size and efficient in retaining both hydrophobic and hydrophilic compounds [23].

Previous studies reported a good antibacterial effect of zein NPs loaded with natural compounds such as thymol [15] or carvacrol [24]. A different direction of improving the thermal stability and antibacterial performance of zein NPs involved the introduction of silver (Ag) NPs into the zein NPs matrix [25]. The resulting nanocomposite material presented a good release of Ag^+ that stabilizes at 10 hours of incubation. While zein NPs excerpt a certain antibacterial effect, but cannot fully prevent bacterial growth, the zein/Ag NPs significantly decrease the growth of bacteria until sterilizing the culture medium. This property is independent on the amount of Ag used in the preparation of zein/Ag NPs showing that just a small amount of Ag NPs significantly changes the effect of zein NPs [25].

Chromium (Cr) is a transitional metal that can exist in different oxidation states. Cr^{3+} is an essential nutrient necessary for the normal metabolism of carbohydrates; the deficiency of Cr^{3+} leading to glucose intolerance [26]. Cr^{3+} complexes with different ligands were shown to have antimicrobial activity [27-30], therefore we hypothesized that zein NPs loaded with Cr^{3+} (Cr-zein NPs) could also have antibacterial effects. The aim of this study was to synthesize zein NPs loaded with Cr^{3+} and to test their efficiency against two *Escherichia coli* (*E. coli*) strains that have different antibiotic resistance profiles. Taking into that zein NPs present a degree of hydrophobicity, we also investigated if Cr-zein NPs could bind LPS molecules from the outer membrane of bacteria in order to understand the interaction of these NPs with bacterial cells.

Material and Methods

Materials

All reagents used in the present study, including zein, ethanol, sodium deoxycholate monohydrate (SD), potassium dichromate ($K_2Cr_2O_7$), sodium hydroxide (NaOH), constituents for sodium buffered saline (PBS) pH 7.4 or constituents of Luria Bertani medium were purchased from Sigma Aldrich. In all experiments we used Ultrapure Mili-Q water.

NPs synthesis protocol

Cr-zein NPs were synthesized using $K_2Cr_2O_7$ as a source of Cr ions. Zein NPs were prepared with SD based on the nanoprecitation technique developed by A. GAGLIARDI & al [23]. The later reported that 1.25% w/v SD mixed with 2 mg/mL zein produced the best colloidal drug delivery system [23]. The corresponding quantities of zein and SD were solvated in 2:1 v/v ethanol – water mixture at room temperature and the solution was placed in the sonication bath. One half of the solution was used exactly as described by A. GAGLIARDI & al [23] in order to obtain the zein-SD nanoparticles (zein NPs) that were used for control tests. In the other half of the solution we added drop by drop a 3% aqueous solution of $K_2Cr_2O_7$. The mixing was performed during 30 minutes. The solution was shacked overnight in order to allow ethanol to evaporate. Afterwards, the Cr-zein NP solution was dialyzed (cut-off 50 kDa) against water for 12 hours in order to remove leftover compounds and then lyophilized.

Cr-zein NPs characterization

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) were performed using an Inspect F50 High Resolution Scanning Electron Microscope that operated at 30 kV. The hydrodynamic size and zeta potential of Cr-zein NPs in water was determined using a Malvern Nano-ZS instrument (Malvern Instruments, Malvern, UK), at 25°C and a refractive index of 1.52.

Antibacterial effect of Cr-zein NPs

The antibacterial effect of Cr-zein NPs was investigated on two *E. coli* strains with different antibiotic resistance profiles (Table 1). The first strain was the antibiotic susceptibility reference strain *E. coli 335-235-2*, ATCC 25922, Serotype O6, Biotype 1. The second strain was a *E. coli* 109 isolated from clinics.

Table 1. Antibiotic resistance/sensibility of the bacterial strains used in this study

Bacterial strain	Tested antibiotics						
	Amoxicillin + Clavulanic Acid	**Cefazoline**	**Cefuroxime**	**Gentamicin**	**Nitrofurantoin**	**Norfloxacin**	**Trimethoprim+ Sulfametho-xazole**
E. coli 335-235-2	R	I	S	R	S	S	S
E. coli 109	S	S	R	I	I	I	R

Legend: R, resistant; I, intermediate resistant; S, sensitive (according to the Clinical and Laboratory Standards M02-A12, M07-A10, and M11-A8).

NPs (Cr-zein NPs and zein-NPs) were dispersed in sterile distilled water and used for the treatment of microorganisms. The disk diffusion tests involving both strains were performed by adding 5 µg, 10 µg, 15 µg and respectively, 20 µg NPs/spot to bacterial cultures in the exponential growth phase on CLED growth medium (cystine-lactose-electrolyte-deficient). After adding the NPs, the Petri plates were incubated over 24 hours at 37°C and results were read immediately.

In order to analyse the biological effect of Cr-zein NPs, the selected strains were grown on Luria Bertani medium containing NaCl 1%, peptone 1%, yeast extract 0.5% and agar 2%. Before the experiments were carried out, the strains were activated by the passage of cells on solid medium and incubated for 18-24 hours at 37ºC. Growth curves were obtained using a liquid medium containing the same ingredients as the solid medium, except agar [31]. The bacterial cells from the solid medium were passed in the liquid medium and were incubated for 8 hours in order to reach an exponential grow phase. At this point, 0 µg/mL, 50 µg/mL and 250 µg/mL Cr-zein NP were added to the suspensions. The bacterial growth kinetics was addressed using a TECAN microplate spectrophotometer at a controlled temperature of 30°C. Prior to readings, the plates were placed in a vortex and mixed for 5 seconds. Bacterial growth was estimated as a result of the changes in absorbance ($OD_{600\ nm}$) in each well every 60 minutes for 12 hours. The effect of Cr-zein NPs was determined by comparing the growth curves of the two strains in the presence and absence of NPs.

At the end of the experiment we determined the viability of bacterial cells. Resazurin test is a metabolic assay that estimates the number of viable cells based on the amount of resazurin (blue, nonfluorescent) reduced to resorufin (pink, fluorescent) [32]. From each well we extracted 1.5 mL of bacteria suspension and incubated it with 50 µl of resazurin solution (0.15 mg/ml in distilled water) at 37°C for 1 hour with shaking. At the end on the incubation period, the samples were centrifuged for 5 minutes at 10^4 rpm and the optical density of the supernatant was read at 490 and 595 nm against distilled water [32]. The resazurin-based viability staining can be also used for quantifying viable biofilm cells grown in microtiter plates [33], therefore we used it for evaluating the biofilm formation inside the wells of the plates used for bacterial growth analysis. In each well, after throwing away all the liquid, we added 2 ml of distilled water and 50 µl of resazurine and the plate was placed on a shaker for 1 hour at 300 rpm. The optical density of samples was determined at 490 and 595 nm.

Results and Discussions

Characterization of Cr-zein NPs

The EM image of Cr-zein NPs is presented in Figure 1. It can be seen that the applied protocol led to the formation of 20-50 nm size NPs. EDS measurements (Figure 1) show that Cr^{3+} ions are loaded into the zein NPs (the normalized weight percentage of Cr is 1.53 and the normalized atomic percentage is 0.39). Additional to Cr, potassium (K) ions were also loaded into the NPs (0.84% in normalized weight, corresponding to 0.28% in normalized number of atoms). The Cr-zein NPs synthesis protocol involved adding a $K_2Cr_2O_7$ solution into the zein-SD-water-ethanol mixture. In this reaction media, $K_2Cr_2O_7$ dissociates into K^+ and Cr^{3+} ions [34]. These metal ions were loaded into the NPs. Moreover, resulting Cr-zein NPs are light green, confirming the loading of NPs with Cr^{3+} [35] which, as proven in the next sections, is the key component for the antibacterial activity of NPs.

DLS measurements showed that 65.3 % of Cr-zein NPs have a hydrodynamic radius of 226 nm, while the other 34.7 % have a hydrodynamic radius of 1247 nm. The polydispersity index of Cr-zein NP is 0.621, confirming the tendency of Cr-zein NPs to aggregate. In comparison to the work of A. GAGLIARDI & al. [23], EM measurements showed that Cr-zein NPs are smaller than the ~110 nm zein-SD NPs synthesized using the corresponding zein and SD amounts. Since they are prone to aggregation, we believe that Cr-zein NP aggregated during the lyophilisation process, explaining the occurrence of micrometer sized particles identified by DLS. Similar to zein-SD NPs [23], the Cr-zein NPs that we synthesized present negative surface charges, their zeta-potential being comprised between -27.4 and -32 mV.

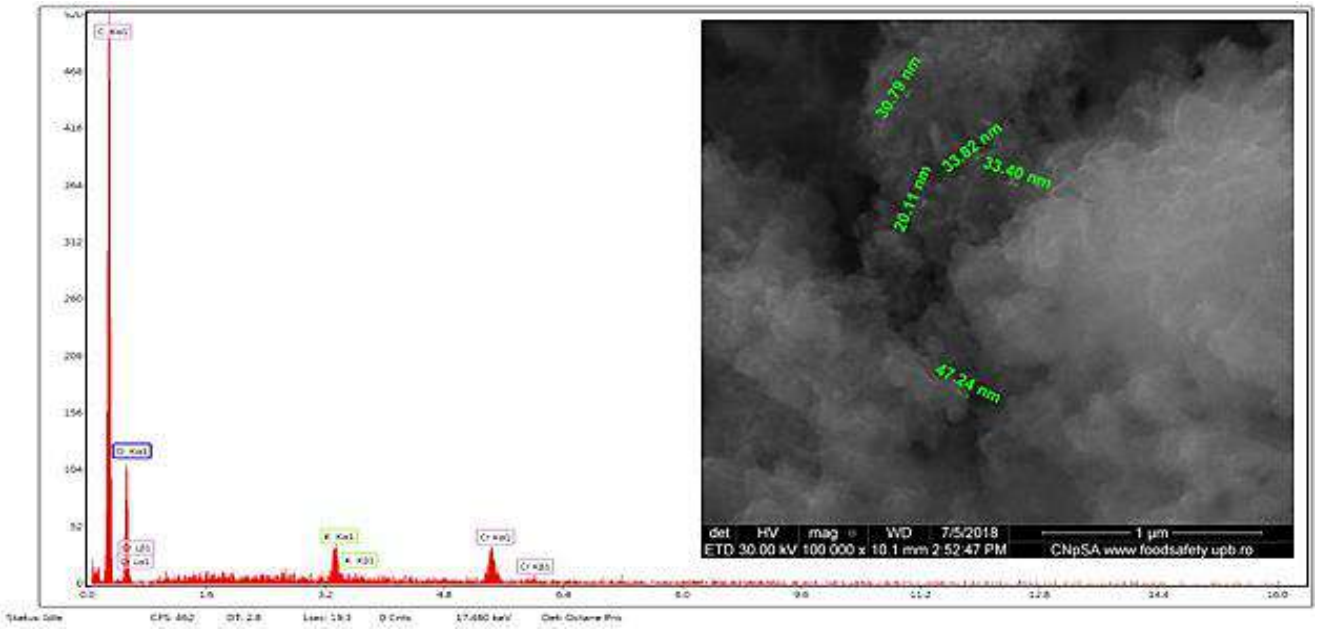

Figure 1. EDS measurement on Cr-zein NPs. The identified elements are labelled on the image. The electron microscopy image of Cr-zein NPs is presented in the insert.

Antibacterial effect of Cr-zein NPs

The susceptibility of the two *E. coli* strains analysed here to Cr-zein NPs and to zein-NPs was investigated by disc diffusion method. In Table 2 we present the diameters of inhibitions zones produced by NPs and in Figure 2 we present the images of the bacterial cells cultures treated with different concentrations of NPs. It can be seen that the inhibition zones of zein NPs are similar in size and are around the size of the deposited spots, suggesting that zein NPs don't have an inhibitory effect on the growth of either *E. coli* strain. On the other hand, when considering the spots with Cr-zein NPs, there is a constant increase of the inhibition zones with NPs concentration, supporting a clear inhibitory effect of Cr-zein NPs, *E. coli 335-235-2* being more sensitive than *E. coli 109*.

Table 2. The diameters of the inhibition zones produced by different concentrations of Cr-zein NP and zein NPs in *E. coli 335-235-2* and *E. coli* 109 cultures.

NPs quantity	**Inhibition zone diameter (mm)**			
	E. coli 335-235-2		*E. coli* 109	
	Zein NPs	Cr-zein NPs	Zein NPs	Cr-zein NPs
5 µg/spot	6	10	3	5
10 µg/spot	6	13	5	9
15 µg/spot	6	17	5	12
20 µg/spot	8	19	6	14

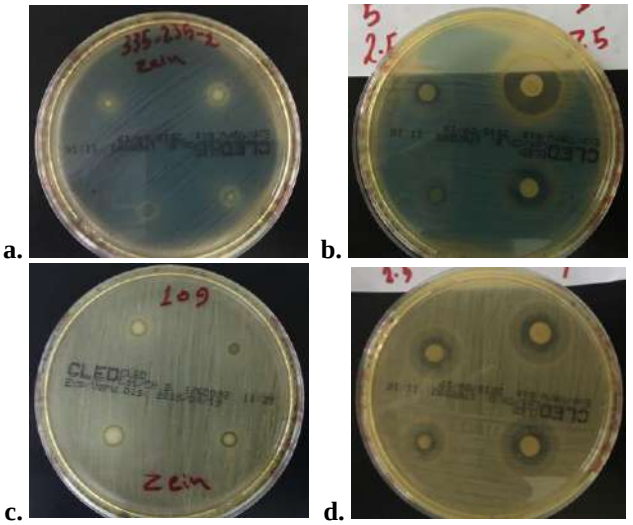

Figure 2. *E. coli 335-235-2* (**a, b**) and *E. coli* 109 (**c, d**) treatment with zein NPs (**a, c**) and Cr-zein NPs (**b, d**).

Considering that the results of growth-based viability assays might be influenced by the delay of the exponential growth phase in different Petri plates, we addressed the growth of *E. coli 335-235-2* and *E. coli* 109 strains in the presence of two different concentrations of Cr-Zein NPs in liquid medium. As shown in Figure 3, a pronounced extension of doubling time was observed for both *E. coli* strains when grown in liquid media with 50 µg/ml NPs. Their growth rates were dramatically affected by a 5 fold higher concentration of Cr-Zein NPs (250 µg/ml) when duplication time became infinite. Thus, it appears that both strains studied here have a similar behavior in the presence of Cr-zein NPs.

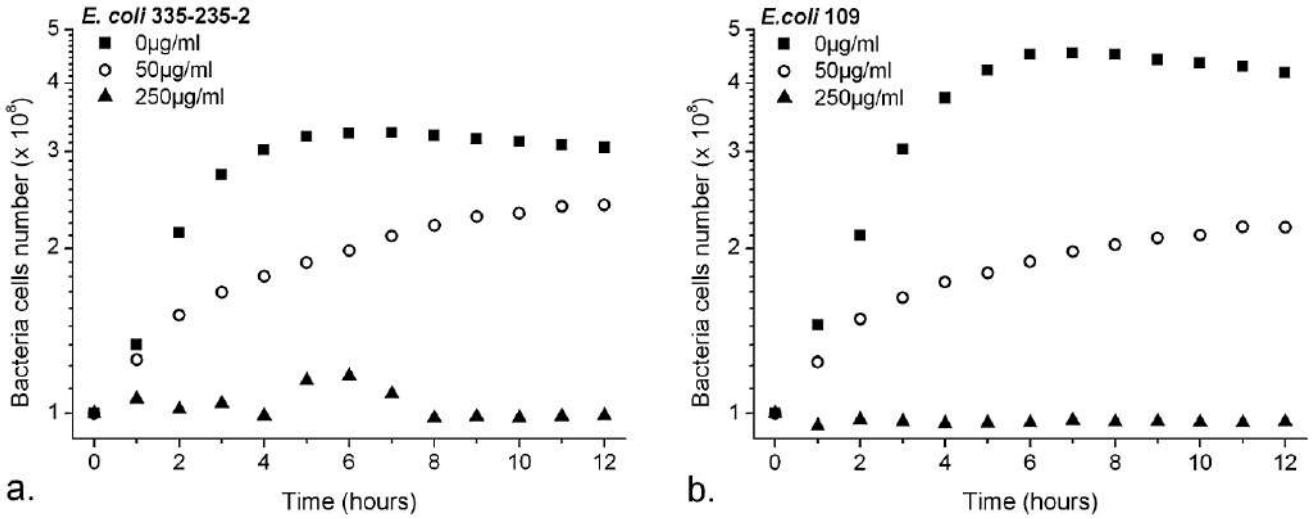

Figure 3. The growth curves of *E. coli* 335-235-2 (**a**) and *E. coli* 109 (**b**) in the absence and in the presence of two Cr-zein NPs concentrations normalized to the initial number of cells.

In order to quantify the effect of Cr-zein NPs after 12 hours of incubation, we performed the resazurin test for cell viability. As can be seen in Figure 4(a), 88.2% of *E. coli* 335-235-2 cells and 90.7% of *E coli* 109 cells are viable in the medium comprising 50 µg/mL Cr-zein NPs. The percents drastically decrease when adding 250 µg/mL Cr-zein NPs to 22.4% and 29.8% in the case of *E. coli* 335-235-2 and *E coli* 109 cultures. Results show that the degree of viability is dependent on the dose of NPs used in the treatment and strain *E coli* 109 appear to be more resistant to Cr-Zein NPs in comparation with *E. coli* 335-235-2 strain.

The viability of *E. coli* cells in the biofilms formed during the 12 hours of incubation with NPs is presented in Figure 4 (b). The viability of cells in the biofilm formed in the presence of 50 µg/mL Cr-zein NPs is 93% in the case of *E. coli* 335-235-2 and 94.7% in the case of *E. coli* 109 strain. In the presence of 250 µg/mL Cr-zein NPs, the biofilm viability of *E. coli* 335-235-2 cells is 36.7%, while that of *E. coli* 109 cells is 43.1%. It can be seen that E. coli 335-235-2 biofilm cells are slightly more sensitive than *E. coli* 109 cells. Also, the biofilm cells from both strains are slightly less sensitive than the cells from the liquid medium.

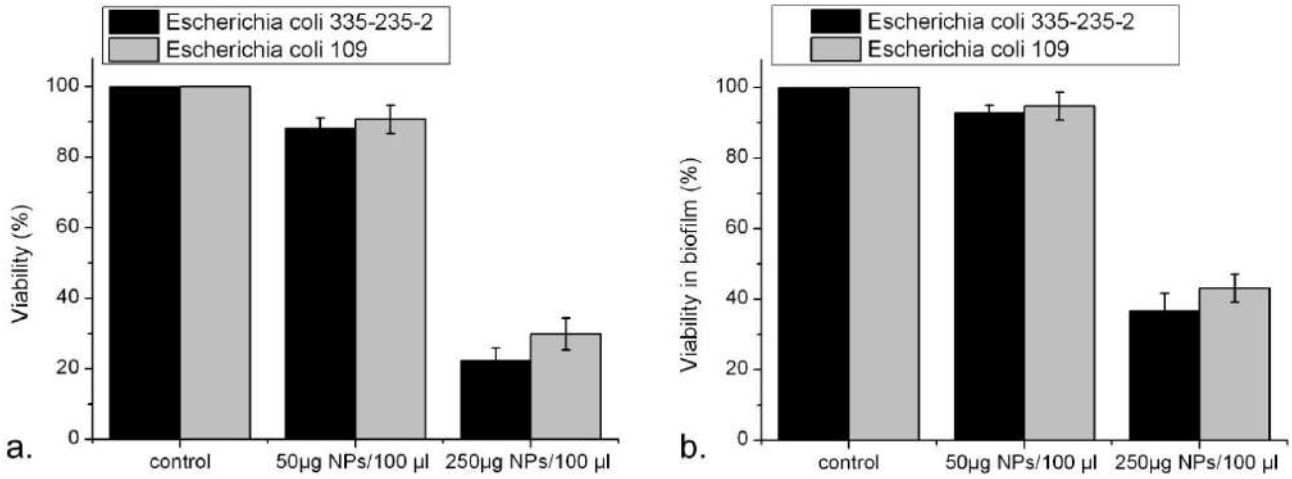

Figure 4. Resazurin viability test of *E. coli* 335-235-2 and *E. coli* 109 (**a**) and biofilm inhibition assay (**b**).

It is now common knowledge that a variety of metal ions are toxic to bacteria [36] as they are able to interfere with multiple bacterial cellular processes [37]. The toxicity of chromium ions for bacterial cells might be mediated by mechanisms as binding the phosphate groups in DNA in ternary complexes together with tyrosine and cysteine [38] or binding carboxyl and sulfhydryl groups in proteins [39]. Chromate was proved to cross bacterial membranes using the sulfate uptake pathway [40], but the crossing is inefficient due to its low solubility [41]. Considering these aspects, we considered an interesting approach to load Cr^{3+} ions into zein NPs and to test their antibacterial activity against antibiotic resistant *E. coli* strains. Results presented here support the efficiency of Cr-zein NPs against the considered strains and indicate their possible usage in practical applications.

Conclusions

Here we synthesized zein NPs stabilized with SD and loaded with Cr^{3+} ions. The antibacterial activity of these NPs was tested in comparison with zein NPs without ions against two *E. coli* strains resistant to different classes of antibiotics. While zein NPs appear inefficient against the considered strains, Cr-zein NPs appear to inhibit the growth of bacterial cells in liquid medium and the formation of biofilms. *E. coli* 335-235-2 strain, that is resistant to amoxicillin with clavulanic acid and to gentamicin, is slightly more sensitive to the action of Cr-zein NPs than *E. coli* 109 strain that is resistant to cefuroxime and trimethoprim with sulfamethoxazole. The Cr-zein NPs synthesized here present antibacterial activity. At the same time, being protein NPs, Cr-zein NPs should be non-toxic and biocompatible. These qualities should recommend them for various practical applications in the biomedical field.

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