The Synergistic Effects of Chitosan-Alginate Nanoparticles Loaded with Doxycycline Antibiotic Against Multidrug Resistant Proteus Mirabilis, Escherichia Coli and Enterococcus Faecalis

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Abstract
This study focused on the synthesis of chitosan-alginate (CH-ALg) nanoparticles by ionotropic gelation technique using sodium alginate and calcium chloride. The particle size of the synthesized nanoparticles was confirmed by atomic force microscope (AFM) and it was 61.9 nm. While the nature of functional groups present in chitosan nanoparticles was determined by FT-IR analysis. The antibacterial activity of chitosan-alginate was tested against multidrug resistance (MDR) gram-positive (Enterococcus faecalis) and gram-negative (Proteus mirabilis) bacteria. The results showed a significant effect against MDR isolates. The nanoparticles were loaded with the antibiotic doxycycline in order to improve the antibacterial activity and drug delivery efficiency. The synergistic effects of the biosynthesized chitosan-alginate and the loaded doxycycline at different concentrations against MDR bacteria were also investigated. The results showed that doxycycline-loaded nanoparticles have superior effectiveness compared to native doxycycline against gram negative and gram positive bacteria.

Keywords: chitosan-alginate, antibacterial, Enterobacteriaceae, synergistic effect, Enterococcus faecalis, Proteus mirabilis, doxycycline, Enterococcaceae.

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1. Introduction

Gastrointestinal infections are among the world's major causes of global mortality and morbidity [1]. The most severe clinical presentations of bacterial gastroenteritis occur at various levels of severity such as diarrhea, fatigue, vomiting, anorexia, abdominal pain, malaise and dehydration [2]. Enterobacteriaceae and Enterococccaceae are the most dominant bacterial agents correlated with gastrointestinal infections, especially those causing diarrhea [3]. These enteric bacteria are transmitted in food and water contaminated via the fecal-oral route [4]. This family is responsible for opportunistic infections, including pneumonia, septicemia, meningitis and urinary tract infections [5]. Most of these isolates are resistant to various antimicrobials, such as carbapenems, which are often believed to be the last line of antibiotic defense against resistant micro-organisms [6]. Although many of the modern antibacterial agents have been produced in recent decades, none of them has enhanced their efficacy against multidrug-resistant bacteria [7, 8]. Nanotechnology has recently become an important alternative antibacterial strategy in the pharmaceutical and biomedical fields due to the re-emergence of infectious diseases of antibacterial-resistant strains, especially gram-negative bacteria [9]. Biodegradable nanoparticles are of great importance nowadays because they can break down into their natural raw materials safely and relatively quickly and disappear into the environment after they have served their function [10]. They may also be classified as ecofriendly, such as chitosan, alginate, dextran, starch and cellulose [11].

Chitosan is a modified biopolymer, derived from partial chitine deacetylation. It consists of alternating units of (1 - 4) connected N-acetyl glucosamine and glucosamine [12]. The main sources of raw materials for chitin extraction are cuticles of different crustaceans, mainly crabs, shrimps and insect exoskeletons [13]. The chitosan's most important biological activities include their roles as antimicrobials, antivirals, antitumors and antioxidants [14]. Moreover, they have antihypertensive, anticoagulant, anti-allergic, anti-inflammatory, anticancer, anti-inflammatory, and mucoadhesive activities [15, 16]. Those properties are particularly appropriate for a broad range of biomedical and pharmaceutical applications, including wound healing [17], gene delivery [18], tissue engineering [19], and drug delivery [20]. Chitosan has a wide spectrum of antimicrobial activities against gram-positive and gram-negative bacteria, with a high rate of destroying through the interaction of chitosan and its derivative products with the bacterial cell wall [21]. Doxycycline antibiotic encapsulation by chitosan-alginate nanoparticles aids to avoid adverse health effects by protecting sensitive tissues from rapid exposure to drugs, while also helping to improve drug efficacy by gaining slow and sustained release directly at the infection site [22]. This study aimed to synthesize chitosan-alginate nanoparticles by the ionotropic gelation method, characterize these synthesized NPs, and evaluate their antimicrobial activities against multi-drug resistance bacteria isolated from diarrheal cases. In addition, the synergistic effect of chitosan-alginate nanoparticle loaded with different concentrations of doxycycline was investigated against multidrug resistance bacteria like Proteus mirabilis and Enterococcus faecalis.

2. Materials and methods

Samples collection

Two hundred thirty stool samples including 83 isolates of E. coli, 29 isolates of Proteus mirabilis and 10 isolates of Enterococcus faecalis were collected randomly from adults and children suffering from intestinal infection. Samples were collected from participants of both sexes from different hospitals in Baghdad, including the Medical City, Baghdad Hospital, Children Protection Hospital, and the Medical City Hospital.
and Al-Aelweia Hospital for Children, during the period from September 2018 to December 2018. All samples were taken under sterile conditions then transferred to the laboratory within 1-2 hrs.

**Isolation and identification of bacteria**

The initial identification of all bacterial isolates was confirmed by Gram stain. After that the samples were inoculated in different culture media, including EMB agar, blood agar, and MacConkey agar and incubated at 37 °C for 24 hrs under aerobic conditions. The appearance, morphology, and color of the colonies were examined, as well as the positive cultures. Several biochemical tests were performed on these isolates, such as those of catalase, sugar fermentation, oxidase, and IMViC. Other biochemical tests, using the API 20 E kit (BioMérieux, France), were employed for further identification. The identification of the isolates was also confirmed by Vitrek 2 compact auto-analyzing system manufactured by BioMérieux.

**Antibiotic susceptibility test**

Susceptibility tests of bacterial isolates were carried out by a modified Kirby-Bauer’s disk diffusion method [23], based on the Clinical and Laboratory Standards Institute’s (CLSI) guidelines [24]. One to three colonies of Enterobacteriaceae isolates were grown overnight on Müller-Hinton agar with an optimal incubation temperature of 37°C for 24 h. Cultures of Enterobacteriaceae were adjusted to 0.5 McFarland standards and the streaking method was used to plate the bacterial suspension on Müller-Hinton agar by a sterile swab. The isolates were classified as sensitive (S) or resistant (R) as indicated by the criteria prescribed by the CLSI (2018). The following antibiotics were tested: Amikacin (AK), Clindamycin (DA), Erythromycin (E), Tobramycin (TOB), Cefotaine (CTX), Rifampin (Ra), Ciprofloxacain (CIP), Trimethoprim (TMP), Metronidazole (MET), Nitrofurantoin (F), Imipenem (IPM), Doxycycline (DOX), and Colistin (CT). All the antibiotics used in this study were purchased from Himedia, India.

**Synthesis of chitosan-alginate nanoparticles**

Chitosan/alginate nanoparticles were prepared in two steps based on the ionotropic pre-gelation method [25] with a minor modification according to the ideal preparation. Calcium chloride solution (7.5 ml) with a final concentration of 0.147 g/ml was added slowly to sodium alginate solution (117.5 ml of 0.0063% w/v) to induce gelation. Subsequently, the mixture was stirred for 60 minutes, then 25 ml of chitosan solution with final concentrations of 3 mg/ml and 0.15 mg/ml was added drop-wise with regular stirring for 90 minutes. Thereafter, ultrasonication for 90 minutes was applied as the final step.

**Preparation of chitosan–alginate loaded with doxycycline**

Doxycycline antibiotic at a concentration of 16 µg/ml, according to CLSI resistance rate, was added to calcium chloride solution, followed by stirring for 60 minutes. Then, sodium alginate and chitosan, respectively, were added drop-wise with gentle stirring for 90 minute. The final step was ultrasonication for 90 minutes. The prepared nanoparticles with doxycycline were stored in a refrigerator until use.

**Characterization of the prepared nanoparticles**

3D surface topography was provided by Atomic Force Microscopy (AFM), the measures of which relying on van der Waals or other attractive and repulsive forces [26]. Five drops of chitosan-alginate nanoparticle were added on a glass slide and left until drying and precipitating. X-Ray Diffraction was employed as another characterization tool for obtaining critical features, such as crystal structure and size. This technique has been widely applied in the characterization of nanoparticles, including those synthesized using biological agents [27].

**Antibacterial activity of chitosan-alginate nanoparticles**

The bacterial inoculum was prepared according to CLSI instructions; A loop full of a single bacteria isolate was inoculated into 10 ml tube containing Muller Hinton broth and incubated overnight for activation. After 24 hours, the bacterial suspension was compared with McFarland tube to obtain a culture with 1.5*108 CFU/ml, which was confirmed by spectrophotometer (600 nm), where an absorbance of 0.08-0.1 was considered as acceptable. 1 ml of the bacterial inoculation was mixed with 1 ml of the prepared chitosan-alginate nanoparticles at a ratio of 1:1 (v/v) [28]. For the negative control sample, 1 ml of normal saline was added to 1 ml of bacterial suspension in another tube. The mixtures where then incubated with shaking overnight. To evaluate the available cell count, 0.1 ml was dispersed for 3 times by a loop over the surface of Muller Hinton agar plates, while rotating the plate at an angle of 60 after each application.
3. Results and discussion

Isolation and identification of bacterial isolates
During this study, a total of two hundred thirty isolates were divided, according to the cultural and microscopical properties of Enterobacteriaceae and Enterococcaceae, into two groups; bacterial isolates that appeared in purple color represented gram positive bacteria (Enterococcus faecalis and Staphylococcus aureus), while those represented in red color represented gram negative bacteria (E.coli, Klebsiella pneumoniae ,Enterobacter cloacae, Proteus mirabilis, Pseusomonas aeroginosa, and Salmonella typhi). To confirm the diagnosis of Enterobacteriaceae isolates, after class diagnosis on culture media and biochemistry tests, the Api 20E system and VITEC2 compact system were used. Following all the identification steps for all the bacterial isolates, the results showed that the percentage of persistent E.coli was 36.2%, whereas the value was 28.8% for Klebsiella pneumoniae , 15.7% for Enterobacter cloacae, 12.7% for Proteus mirabilis, 2.6% for Pseusomonas aeroginosa, 1.7% for Enterococcus faecalis, 1.7% for Salmonella typhi, and 0.4% for Staphylococcus aureus.

Antibiotic susceptibility
The majority of the isolates showed multidrug resistance profiles; 100% of the isolates were resistant to metronidazole, erythromycin and clindamycin, with a high resistance rate to rifampin (99.6%). In addition, they showed approximately intermediate resistance for cefotaxime ,trimethoprim amikacin and nitrofurantoin, with resistance rates of 61.1%, 58.1%, 51.1% and 45%, respectively. However, they had low resistance rates for doxycycline (39.7%), colistin (354%), tobramycin (20.5%), ciprofloxacin (14%), and imipenem (3.1%), as shown in Figure-1. All bacterial isolates showed a high macrolide resistance rate. Generally, there are three mechanisms involved in the resistance to macrolides (29); (a) By modification of the target site through methylation or mutation which prevents the binding of the antibiotic to its ribosomal target, (b) through efflux of the antibiotic, and (c) drug-inactivated macrolides have low levels of activity against Enterobacteriaceae associated with poor membrane penetration of these antimicrobials, which prohibit their use in the treatment of Enterobacteriaceae [30]. Bacterial isolates that showed resistance to doxycycline, colistin, tobramycin, ciprofloxacin and imipenem represented a suitable choice for the part of this work that aims at restoring the activity of these antibiotics and reducing the microbial resistance through loading with chitosan-alginate nanoparticles.

Figure 1- Percentage results of antibiotics susceptibility test for all bacterial isolates. AK = Amikacin, DA = Clindamycin, E = Erythromycin, TOB = Tobramycin, CTX = Cefotaxime, RA = Refampin, CIP = Ciprofloxacin, TMP = Trimethoprim, MET = Metronidazole, F = Nitrofurantoin, IPM = Imipenem, DOX = Doxycycline, CT = Colistin. *R: Resistant, S: Sensitive.
The antibacterial activity of chitosan-alginate nanoparticle

The antimicrobial activity of chitosan nanoparticle was examined against the studied pathogenic bacteria, including the multi drug resistant gram negative (Proteus mirabilis, E.coli) and gram positive (Enterococcus faecalis) bacteria, which were selected based on their high resistance rate to many antibiotics. Chitosan was tested at two different concentrations (0.15 and 0.3 mg/ml) to report antibacterial activity. The results showed no significant differences as antibacterial agents between these two concentrations, as shown in Figure-2. The concentration of 0.3 mg/ml was fixed in the present study because it was more stable during the preparation in terms of separation layers. Chitosan and its derivatives were reported to be safe and having no significant toxicity [31]. Chitosan alginate nanoparticle, as demonstrated in Figure-3 and Figure-4, showed 100% inhibition rate to Proteus mirabilis and Enterococcus faecalis while they showed 80% inhibition rate against E.coli, as shown in Figure-5, in comparison with negative control and doxycycline. CH-ALg NPs were observed to be bactericidal against E.coli and bacteriostatic against P. mirabilis and E. faecalis, since the selected MDR isolate of E.coli was highly resistance toward 11 different types of antibiotics while E. faecalis and P. mirabilis showed intermediate resistance against 8 antibiotics out of the 13 used in the present study. Chitosan is non-toxic, biodegradable and biocompatible, and is among the most popular bacteriostatic and bactericidal natural polymers with inherent antimicrobial activity [32]. The explanation of chitosan's antibacterial effect on gram-positive bacteria is its non-covalent binding to teichoic acid embedded into the peptidoglycan layer [33]. Localized teichoic acid molecules on the surface are essential for cell division. Hence, chitosan interaction can affect this process and other processes that are essential for bacterial growth. [34] Teichoic acid has the role of protecting cells from environmental stress, through controlling the activity of the enzyme and ensuring a cationic concentration of the cell surface to promote cell binding to receptors. While its effect on gram-negative bacteria is associated with chitosan's chelation interaction with cations when the pH is above the pKa [35]. Another mechanism of action of chitosan is the electrostatic interaction with anionic parts of lipopolysaccharides from the outer membrane of gram negative bacteria [36]. Chitosan (at least low-molecular-weight polymers) may also pass through the membrane and interfere with DNA / RNA synthesis [37].

Figure 2- The Antibacterial Activity of Chitosan-Alginate Nanoparticle against E. faecalis A: negative control, B: chitosan NP 0.15 mg/ml, C: chitosan NP 0.3 mg/ml.
The synergistic effect of chitosan alginate nanoparticle loaded with doxycycline antibiotic

Due to doxycycline’s antibacterial effects on a wide range of pathogens, it is currently one of the most commonly prescribed antibiotics worldwide for treating infectious diseases [38], therefore it was used in the present study and loaded with chitosan-alginate nanoparticles to improve drug delivery and treatment efficacy. Different concentrations were used in the synergistic effect for chitosan (0.3 and 0.15 mg/ml) and doxycycline (16 and 8 µg/ml). The results, as demonstrated in Figure 6, showed 100% inhibition of bacterial growth in negative control and about 98%, 80% and 60% inhibition of Proteus mirabilis, E.coli, Enterococcus faecalis, respectively, in the presence of doxycycline alone, with no growth of Proteus and Enterococcus. On the other hand, 20% of E.coli growth appeared upon treatment with chitosan alginate nanoparticles. However, the growth disappeared upon treatment with doxycycline loaded with chitosan alginate nanoparticles, which indicates the incidence of synergistic effects between the antibiotic and the prepared nanoparticles toward gram negative and gram positive bacteria as compared to native doxycycline alone, as shown in Figures 6 - 9. These results are in agreement with those reported by Yadav [39] who found an increase in the inhibition ability of chitosan-alginate microspheres loaded with doxycycline in comparison with doxycycline alone, which confirms the antimicrobial potency of the microspheres. Therefore, it is encouraging to use these doxycycline-loaded nanoparticles for the treatment of infections caused by enteric bacteria [40]. In
fact, nano-sized chitosan enables the uptake of drugs through the cell membrane. The absorption-enhancing effect and the nanosized particles together exhibited an ability to enhance drug bioavailability [41]. The second advantage of loading doxycycline with chitosan nanoparticles is to offer flexible routes of administration, particularly non-invasive routes, i.e. per oral, nasal, and ocular mucosa, which are preferred routes of administration [39].

Figure 6- The inhibition rates of CH-ALg nanoparticle against *Proteus mirabilis*, *E.coli* and *Enterococcus faecalis*.

Figure 7- Antibacterial Activity of Doxycycline Loaded with Chitosan Alginate Nanoparticle against *proteus mirabilis*. A: negative control, B: doxycycline alone, C: chitosan alginate nanoparticles, D: doxycycline (16 µg/ml) loaded on chitosan alginate nanoparticle (0.3 mg/ml), E: doxycycline (8 µg/ml) and chitosan (0.3 mg/ml), F: doxycycline (16 µg/ml) and chitosan (0.15 mg/ml).
Figure 8- Antibacterial activity of doxycycline loaded with chitosan alginate nanoparticle against Enterococcus faecalis. A: negative control, B: doxycycline alone, C: chitosan alginate nanoparticle, D: doxycycline (16 µg/ml) loaded on chitosan alginate nanoparticles (0.3 mg/ml), E: doxycycline (8 µg/ml) and chitosan (0.3 mg/ml), F: doxycycline (16 µg/ml) and chitosan (0.15 mg/ml).

Figure 9- Antibacterial Activity of Doxycycline Loaded with Chitosan Alginate Nanoparticle Against E.coli. A: negative control, B: doxycycline alone, C: chitosan alginate nanoparticles, D: doxycycline (16 µg/ml) loaded on chitosan alginate nanoparticles (0.3 mg/ml), E: doxycycline (8 µg/ml) and chitosan (0.3 mg/ml), F: doxycycline (16 µg/ml) and chitosan (0.15 mg/ml).

Characterization of the prepared chitosan-alginate nanoparticle

Atomic force microscope

AFM was used to determine the surface morphology and the topography of the NPs. In addition, it was chosen as an imaging method which provides nanometer resolution and three-dimensional surface imaging. Also, this method requires minimal sample preparation and allows imaging in ambient and liquid conditions. The AFM gives a two and three-dimensional image of the surface of nanoparticles at an atomic level, as shown in Figure-10.
Figure 10- Atomic Force Microscopy images of chitosan-alginate nanoparticles illustrating 2D and 3D topologies.

The size of chitosan-alginate nanoparticles was estimated by using AFM, as shown in Table-1. The results showed that the average size of chitosan-alginate was 61.91 nm, which agrees with those of a previous study [42] which showed homogeneous particles with an average size of 60±20 nm.

Table 1- Results of Chitosan Average Size as analyzed by AFM.

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Fourier transforms infrared characterization

FTIR technique was used to identify the functional groups and the formation of possible interactions between the selected alginate and chitosan. The characteristic peaks of sodium alginate are described in Figure-10. The result showed a peak at 806 cm\(^{-1}\) that is characteristic of mannuronic acid residues [43], while that at 1,641 cm\(^{-1}\) corresponded to the COO- asymmetric stretching peak, and that at 3,452 cm\(^{-1}\) was the H\(_2\)O absorption peak [44]. The C–O stretching was found at 1313 cm\(^{-1}\) [45]. Similarly, the absorption bands of OH were identified at 3261 cm\(^{-1}\) with strong intra and/or inter hydrogen bonding [46]. In addition, Figure-9 shows the results of chitosan FTIR analysis. Characteristic peaks of chitosan were observed at 1564 and 1644 cm\(^{-1}\) which represent the amino group (NH\(^3\)) and amide I, respectively [47]. Three bands of primary amine were stretching between 3400 cm\(^{-1}\) and 3200 cm\(^{-1}\) [48]. Peaks observed at 2941, 1313, and 1228 cm\(^{-1}\) were due to symmetric or asymmetric CH\(_2\) stretching vibrations of pyranose ring. This was confirmed by results from a previous report [49]. The N-H deformation band of chitosan was found at 1,564 cm\(^{-1}\) [50]. Stretching vibrations of C-H bond at 2715 cm\(^{-1}\) indicated the presence of aliphatic groups [51]. The bands at 1100-1000 cm\(^{-1}\) is due to the saccharide structure of the chitosan [52], as illustrated in Figure-11. The results of the FTIR analysis of these mixed and oppositely charged polysaccharides (chitosan and alginate)
demonstrated changes of some bands’ placement and disappearance or appearance of new peaks in comparison to those of single alginate or chitosan. This was associated with possible electrostatic interactions between the mixed polymers. A broad band around 3500-3100 cm\(^{-1}\) was observed, indicating enhanced hydrogen bonding compared to that of chitosan or sodium alginate alone [53]. The amide-I peak of chitosan shifted from 1627 cm\(^{-1}\) to 1731 cm\(^{-1}\). On the other hand, in sodium alginate, the absorption bands of OH at 3261 cm\(^{-1}\) disappeared when it interacted with chitosan, suggesting an electrostatic interaction between alginate and chitosan [54].

Figure 11- Fourier transforms infrared spectroscopy measurements of sodium alginate.

Figure 12- Fourier transforms infrared spectroscopy measurements of chitosan and chitosan-alginate.
4. Conclusions

This study reported the synthesis of chitosan-alginate nanoparticles by ionotropic gelation method. The AFM results indicated that the synthesized NPs were in the range of nanometer with an average size of 61.91 nm. The FTIR spectrum indicated the involvement of functional activities of chitosan when it interacts with sodium alginate and calcium chloride. The study also showed high antibacterial activity of the prepared nanoparticles against gram negative (Proteus mirabilis) and gram positive (Enterococcus faecalis) bacteria. To increase the antibacterial activity and drug delivery efficiency, chitosan-alginate NPs were loaded with doxycycline. The results showed that the doxycycline-loaded NPs have superior effectiveness, as compared to the native doxycycline, against gram negative and gram positive bacteria. A main conclusion of this study is that the chitosan-alginate NPs are suitable for treatment of Enterobacteriaceae infections at low concentrations of loaded doxycycline.

References


