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Bactericidal Activity of Various Antibiotics versus Tetracycline-loaded Chitosan Microspheres against *Pseudomonas aeruginosa* Biofilms

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Abstract

The discovery of biofilms in 1980's has brought much interest to the study of the contribution of bacterial biofilms with many recurrent and chronic infectious diseases. In this study, we evaluated the utility of chitosan microspheres in delivering antibiotic in dosage form that could be effective against *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilms. *P. aeruginosa* isolates were collected and identified using standard methods. A modified microtiter plate test was used to determine the biofilm-forming capacity of the isolates. Moreover, bactericidal activity of various antibiotics vs. tetracycline-loaded chitosan microspheres against *P. aeruginosa* sessile and planktonic cells was tested. Results showed that, most *P. aeruginosa* strains (92.9%) were efficient biofilm producer-strains. There were differences in the antibiotic susceptibility of planktonic and sessile cell populations. Fluoroquinolones, aminoglycoside and tetracycline showed more potent activity (MIC_{50} was 0.8, 4.88 and 34.19 µg/ml, respectively) than penicillin, cephalosporin, clarithromycin and macrolides. Biofilm growth was inhibited after 3 h treatment with 2x and 4x MICs and after 24 h treatment with MIC of tetracycline-loaded chitosan microspheres prepared by coacervation method than that prepared by water in oil emulsion method. This was correlated to the cumulative amount of tetracycline that was released from tetracycline-loaded chitosan microspheres prepared by coacervation method which released about 60% of tetracycline in the first 6 h and continued for 24 h. This in the clinical field may be translated into maintaining constant drug concentration for a prolonged period and maximize the therapeutic effect of antibiotics while minimizing antibiotic resistance and improved patient compliance. So, the use of tetracycline-chitosan microspheres may be a new strategy for the development of a specific drug delivery system to increase the efficacy of tetracycline against biofilm-associated *P. aeruginosa* infections. However, it would be appropriate to conduct clinical studies to confirm this.

Keywords: Biofilm formation of *P. aeruginosa*; Tetracycline; Antimicrobial susceptibility; Microtiter plate method; Drug-loaded chitosan microsphere

Introduction

P. aeruginosa is one of the most important opportunistic human pathogens. It has emerged as a dominant pulmonary pathogen with biofilm-forming capability, resulting in progressive chronic pulmonary infections, cystic fibrosis [1]. There is also an increasing awareness of the important role of *P. aeruginosa* biofilms in the contamination of medical biomaterials such as catheters and prostheses. Biofilm infections are difficult to eradicate with antimicrobial treatment, and *in vitro* susceptibility tests show considerable resistance of biofilm cells to killing [2].

Biofilms are defined as microbial-derived sessile communities attached to a surface and embedded in a self-produced polymeric matrix. They play a central role in the pathogenesis of serious infections caused by *P. aeruginosa*. Bacteria grown in biofilms are more resistant to antimicrobial agents than their planktonic counterparts [3]. Susceptibility testing of planktonic bacteria may fail to predict *in vivo* resistance of device-related infections to antimicrobial agents. Standardized laboratory models to test antimicrobial agents in biofilms are still lacking, although a broad range of models for quantifying treated vs. untreated biofilms have been described. In most of these models, the quantification of biofilm is done by conventional plating after disruption of the biofilm. These methods are labour-intensive and slow, and the process of disrupting the biofilm can be incomplete or kill cells so that, the number of colonies does not necessarily reflect the number of viable bacteria in the biofilm. Indirect methods are based on quantification of biomass (both living and dead cells), viability assays (living cells) and matrix quantification [4].

Many studies have now demonstrated that biofilm-producer microorganisms have an inherent lack of susceptibility to antibiotics, whereas planktonic cultures of this same organism do not. This resistance is lost once the biofilm is reverted to conditions that permit planktonic growth [5]. The innate resistance of microbial biofilms to antibiotic therapy has led to problems in their eradication and in the management of patients with device related infections. Biofilms may also interfere with the immune clearance of infectious agents. This difference in antibiotic susceptibility between planktonic and biofilm populations of the same organism may result from differences in the diffusion of antibiotics or much more complex changes in the microbial physiology of the biofilm. The concentrations of antibiotics needed to inhibit bacterial growth in the sessile phase are often much higher than those required for bacteria in the planktonic phase [6].

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Tetracyclines are broad-spectrum antibiotics with a wide range of activity against both Gram-positive and Gram-negative bacteria. *P. aeruginosa* is less sensitive but is generally susceptible to tetracycline. The tetracycline's act by blocking the binding of aminoacyl tRNA to the A site on the ribosome. Tetracycline inhibits protein synthesis on isolated 70S or 80S (Eukaryotic) ribosome's, bacteriostatic, and in both cases, their effect is on the small ribosomal subunit. However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. The combination of their broad spectrum and low toxicity has led to their overuse and misuse by the medical community and the wide-spread development of resistance has reduced their effectiveness [7].

Chitosan is a natural organic material which is obtained by the deacetylation of chitin from the exoskeleton of animal sources particularly in crustacean, mollusks, insects and certain fungus. It is biocompatible and biodegradable. Various applications of chitosan polymers ranging from water treatment, pulp and paper industry, to pharmaceutical, cosmetics, agriculture, food, membrane are proposed [8]. Antimicrobial activity is one of the attractive features of chitosan. The antimicrobial activity of chitosan varies depending on their physical properties (degree of deacetylation and molecular weight), solvent, microorganism species and source [9]. Isolated reports are available on the use of combinations of antibiotics and chitosan and its derivates as antimicrobials. Decker et al. [10] proposed on a synergistic chlorhexidine/chitosan combination for improved antiplaque strategies. Tobramycin is one of the antibiotics which are reported to show synergistic action with chitosan in planktonic culture of *P. aeruginosa* [11]. Bioadhesive and antimicrobial properties of chitosan and its derivatives are effective in antimicrobial drug delivery control-release of chlorhexidine and nystatin oral preparation [12], release of ampicillin [13] and drug delivery system for ofloxacin [14] in ophthalmic preparation. Tobramycin sulfate gastrointestinal release preparations make chitosan attractive for combination with antimicrobial drugs [15].

Nowadays there is an increasing interest in the use of polymeric carriers for the controlled delivery of drugs including antibacterial agents. Microspheres are drug delivery system that can be designed to give different release profiles thus allowing a controlled nature of therapy. The use of chitosan microspheres for antibiotic delivery can be used to deliver the intended therapeutic concentrations to the infection site to elicit its activity without having to use larger doses of drugs [13]. Many factors affect the entrapment efficiency of the drugs in the chitosan microspheres e.g. nature of the drug, chitosan concentration, drug polymer ratio and stirring speed. Also the drug release behavior from chitosan microspheres is very important and is affected by molecular weight and concentration of the chitosan, the cross linking agent used and its concentration, and process variables like stirring speed, type of oil, additives as well as the amount of drug. Several kinetic models have been proposed for the release of drugs from chitosan microspheres but in most cases the best fit is the Higuchi equation [16].

To investigate the potential use and benefits of using chitosan in drug delivery of antibiotics for inhibiting growth of *P. aeruginosa* biofilms, several research aspects were achieved through the following steps; (i) we investigated the antimicrobial activity of clinically relevant antibiotics, including amikacin (aminoglycoside), tetracyclin, amoxycilin (penicillin), cephalexin (cephalosporin), clarithromycin and erythromycin (macrolides) and levofloxacin (fluoroquinolones) against *P. aeruginosa* in the biofilm and planktonic phase of growth *in vitro*; (ii) we determined the activity of chitosan alone and in combination with tetracycline in the chitosan microsphere formulation; (iii) we used and evaluated a simple screening method to identify *P. aeruginosa* biofilms and (iv) we evaluated the pharmacokinetic behavior of tetracycline-loaded chitosan microspheres prepared by two different methods (coacervation method and water in oil emulsion method).

Material and Methods

Bacterial strains

Forty-two isolates of *P. aeruginosa* were collected from November 2010 to June 2011 from various clinical specimens including burns and wound swabs, tracheostomy and endotracheal aspirations, urinary catheters and blood from patients of different inpatient departments of King Khalid University hospital, Saudi Arabia. The identification of isolates was done according to standard method described elsewhere [17]. One strong biofilm producer and multidrug resistant strain isolate was selected for further study. *P aeruginosa* ATCC 27853 was used as control strain.

Antimicrobial agents and chitosans

Amikacin (AMK) (Zhejiang Younging, China), tetracycline (TET) (Seed Pharmaceuticals, Egypt), amoxycilin (AMX) (Sigma Aldrich), cephalexin (CEX) (Al kahira Pharmaceuticals, Egypt), clarithromycin (CLA) (Dainabot Co., Ltd., Tokyo, Japan), erythromycin (E) (Sandoz, Spain) and levofloxacin (LVX) (Taisyo Pharma, Tokyo, Japan) were all purchased commercially from the manufacturers. Stock solutions of each antibiotic were freshly prepared at the beginning of each week and kept at –4°C. High (> 75% deacetylated), medium (75–85% deacetylated) and low (75-85% deacetylated) molecular weight chitosans were Sigma-Aldrich products.

Susceptibility testing of antibiotics and chitosans

Antimicrobial susceptibility of antibiotics was determined by agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) (2007) [18]. All antibiotics were incorporated into serial two-fold concentrations of Mueller-Hinton agar (Oxoid). The antibiotic concentrations used in this experiment ranged from 0.025-2000 µg/ml depending on the MIC of the antibiotics. Inoculated plates were incubated at 35°C for 24 h. The plates were then visually inspected for any visible growth. The least antimicrobial concentration showing no growth was considered as the MIC of this antimicrobial.

MIC values of chitosans were determined by the microbroth dilution method using 0.6-5,000 µg/ml of chitosan solution. The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The chitosan were first dissolved in 10% dimethyl sulfoxide (DMSO) (DMSO concentration does not offer inhibition to microorganism growth) and then diluted to the highest concentration (5,000 µg/ml) to be tested, and the serial two-fold dilutions were made based on a microwell dilution method. The 96-well plates were prepared by dispensing into each well 95 µl of Muller Hinton Broth (MHB) and 5 µl of the inocula. A 100 µl of aliquot from the stock solutions of the

chitosan prepared at the concentrations of 5,000 µg/ml was added into the first wells. Then, 100 µl from their serial dilution were transferred into consecutive wells. The last well containing 195 µl of MHB without compound and 5 µl of the inocula on each strip were used as negative control. The final volume in each well was 200 µl. The plate was covered with a sterile plate sealer and incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of micro-organisms, after incubation. All MICs were performed in triplicate.

MIC_{50} and MIC_{90}

The concentrations that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were calculated for each antimicrobial agent. The formula of geometric means was used as follows:

$$MIC_{50} = (M<50) + \frac{(n-x)\times[(M>50)-(M<50)]}{y}$$

where M < 50 is the MIC of the highest cumulative percentage below 50%, M > 50 is the MIC of the lowest cumulative percentage above 50%; n is 50% of the number of organisms tested, x is the number of organisms in the group at M < 50, and y is the number of organisms in the group at M > 50. MIC_{90} was calculated by substituting 90% for 50% in the MIC_{50} formula [19].

Biofilm formation

Biofilm formation was determined by using a modified microtitre plate test [20,21]. Bacteria were grown overnight on pseudomonas agar (Oxoid) plates and subcultured onto trypticase soy agar (TSA) (Oxoid) plus 5% glucose. Bacteria were resuspended in trypticase soy broth (TSB) plus 5% glucose. The optical density at 650 nm (OD_{650}) of the bacterial suspensions was determined and aliquots of 100 µl were inoculated in nine parallel wells of a 96-well polystyrene plate. After incubation for 48 h at 37°C, the plates were softly shaked to collect planktonic bacteria. The wells were rinsed with phosphate buffer saline (PBS) and fixed with 150 µl absolute methanol for 10 min. Attached bacterial material was stained by adding 150 µl crystal violet (1% w/v) for 20 min. The plates were rinsed with tap water (to remove excess crystal violet dye) and the amount of attached material was measured by solubilisation of the crystal violet dye in 150 µl of 33% glacial acetic acid. The A_{570} was measured using an enzyme-linked immunosorbent assay (ELISA) reader. Interpretation of biofilm production was according to the criteria of Stepanovic et al. [22].

Bactericidal activity of antibacterial agents against biofilm-forming sessile cells and planktonic cells

The antimicrobial activity of amikacin, levofloxacin and tetracycline were investigated against one strain of *P. aeruginosa* isolates which was selected from our collection based on their ability to form fully established, mature biofilm (quantified using crystal violet staining). As described by Ishida et al. [1] and Stepanovic et al. [23], bacteria were incubated in TSA with 5% glucose for 24 h at 37°C and re-suspended in saline adjusted to 0.5 McFarland turbidity. Then, 200 µl of this suspension and the catheter pieces of 1x 1 cm^2 surface area were added to 18.8 ml of TSB with 1 ml of 5% glucose and incubated for 6 days at 37°C. The catheter pieces incubated with the organisms were washed gently with PBS and transferred to MHB containing a given antibiotic for 3, 6 and 24 h at 37°C. The bacteria recovered from the catheter and PBS was designated biofilm and planktonic cells, respectively. The catheter pieces were transferred to 1 ml of PBS and sonicated for 10 min (Branson Ultrasonic Cleaner; Branson Cleaning Equipment Company). The suspensions were diluted and plated on nutrient agar plates containing 0.1% magnesium chloride, which inactivated any residual antibiotic [24], and viable cells were counted after incubation for 24 h at 37°C. For planktonic cells, 1 ml of the PBS that the catheter pieces had been soaked in was transferred to MHB containing the desired antibiotic. The number of surviving bacteria was determined in the same way as for the biofilm cells. The time kill curves of sessile cells were illustrated at different MICs (MIC/2, MIC, 2x MIC and 4x MIC) of each antibiotic. A bactericidal effect was defined as a ≥ 3 log cfu/ml reduction compared with the initial inoculum after 24 h of incubation [25]. The bactericidal effects of each antibiotic against sessile and planktonic cells of a selected *P. aeruginosa* strains was determined. The catheter pieces were preferred for easy collection of sessile and planktonic cells and for further scanning.

Preparation of antibiotic-loaded chitosan microspheres

We chose tetracycline to be loaded with chitosan as microsphere because it was the less effective studied antibiotics against our strains and chose low molecular weight chitosan as it had more antibacterial effect.

Preparation of tetracycline-loaded chitosan microspheres

Chitosan microspheres were prepared by two methods. The first one was coacervation method [26,27]; 20 ml of 2.5% w/v low molecular weight chitosan solution in 0.5 M acetic acid was added to 1% Tween 80. An overhead stirrer was used to mix the components with the speed set at 1000 rpm. Twenty milliliter of 20% w/v Na_2SO_4 solution was added drop wise while stirring; a precipitate of microspheres was formed. Stirring was continued for an hour to stabilize the microspheres. The microspheres were separated by centrifugation at 3000 rpm for 30 min and then air dried in an oven at 37°C for 48 h. The formed microspheres were stored in a desiccator at room temperature. The second method was the water in oil (w/o) emulsion method followed by ionotropic gelation method [28]; 25 ml of light liquid paraffin was mixed with 50 ml of dichloroethane and 1.5% v/v Span 80. This was mixed by an overhead stirrer. Then 20 ml of 2.5% w/v chitosan in 0.5 M acetic acid solution were added slowly while stirring. The mixture was further stirred for 30 min to stabilize the emulsion. Ten milliliter of 1.5 M potassium hydroxide (KOH) solution was added drop wise with a syringe at a rate of 2 ml/15 min while cooling. The mixture was further stirred for an additional 2 h to stabilize the microspheres. The formed microspheres were washed in petroleum ether, filtered and air dried in an oven at 37°C for 48 h [29]. Tetracycline-loaded chitosan microspheres were obtained by soaking 100 mg of empty microspheres in a batch in 20 ml of 2.5% w/v tetracycline HCL solution for 48 h which were washed once by distilled water and then filtered and air dried in a desiccators. The soaking and filtration were carried in the dark to avoid photodegradation of the tetracycline. The spheres were stored in the dark due to its high photosensitivity in desiccators [30].

Percentage yield

The percentage of practical yield microsphere was calculated as the weight of microspheres recovered from each batch in relation to the sum of chitosan and drug added by using this formula; Percentage yield = (Practical Yield) / (Theoretical Yield) ×100 [16].

Determination of loading efficiency

Practical drug content was determined by taking a weighed quantity of chitosan microspheres (approximately 50 mg) in 100 ml volumetric flask. Sufficient quantity of water was added to make the

volume 100 ml. The suspension was shaken vigorously and left for 24 h at room temperature with intermittent shaking. The suspension was filtered and tetracycline content in supernatant was determined by UV spectrophotometer at 353 nm wavelength. The amount of drug loaded in microspheres was calculated by the following formula [31];

$$\text{Loading efficiency, } L = \frac{Qm}{Wm} \times 100$$

where: W_m is weight of microspheres in grams and Q_m is quantity of drug present in W_{mg} of microspheres

Entrapment efficiency

Efficiency of tetracycline entrapment for each batch was calculated in terms of percentage drug entrapment (PDE) as in the following formula;

$$PDE = \frac{W}{T} \times 100$$

where: W is weight of drug present in microspheres (practical drug content) and T is theoretical weight of drug [32].

Particle size analysis

The morphological features of tetracycline-loaded chitosan microspheres were assessed by light microscopy in a mount of isopropyl alcohol as a dispersing non-dissolving medium. Motic camera and Motic computer software were used for measuring the mean diameter of the particles (Moticam 2500, Motic Images Plus 2.0, Hong Kong). Microspheres of the various batches were characterized in terms of sphericity and clumping of microspheres.

In vitro release study

In vitro release studies of tetracycline-loaded chitosan microspheres were carried out using Franz diffusion model finite dosage apparatus (Franz). A jacketed franz diffusion cell of 12 ml capacity and 15 mm diameter was used. A semi permeable cellulose membrane (Cellophane membrane, Spectra Por® 12.000-14.000 molecular weight cut off) was fixed between the donor and receiver compartments while avoiding introduction of air bubbles. The reservoir solution contained synthetic serum electrolytic solution (SSES composed of 0.601 g of sodium chloride, 0.235 g of sodium bicarbonate, 0.0283 g of disodium hydrogen phosphate and 0.0284 g of sodium sulphate/100 ml) having a pH of 7.4 that correlate with pH of the *in vitro* antimicrobial tests was used as mentioned in Shanmuganathan et al. [33]. The solution was maintained at 37°C and stirred with a magnetic stirrer. Six hundred ul samples were withdrawn at regular time intervals (30 min, 1, 2, 3, 4, 5, 6 – 72 h) and analysed for percentage release of antibiotic spectrophotometrically. Values reported were the average of three determinations using the same technique. To determine the release profile of our microspheres, the following plots were made, cumulative percentage drug release vs. time (zero order kinetic model) [34]; log cumulative of percentage drug remaining vs. time (first order kinetic model) [35]; cumulative percentage drug release vs. square root of time (higuchi model) [36] and log cumulative percentage of drug release vs. log time (Korsmeyer-Peppas model) [37]. The r-square value was then calculated.

Assessment of antibacterial activity of tetracycline-loaded chitosan microspheres against biofilm-forming sessile cells

Antibacterial activity of tetracycline-loaded chitosan microspheres against biofilm-forming sessile cells was determined by using the same technique for testing tetracycline alone (mentioned before). The time kill curves of sessile cells of studied *P. aeruginosa* strain were illustrated at different MICs (MIC/2, MIC, 2x MIC and 4x MIC) of tetracycline.

Scanning electron microscopy

Bacteria (overnight bacterial culture diluted to obtain 1×10^7 cfu/ml) were cultured for 48 h in MHB containing pieces of catheter for micrographing bacterial biofilm. Primary fixation of samples was done by buffered Glutaraldehyde 2.5% for 1 h, then washed by phosphate buffer (pH = 7.2) and transferred to 1% (w/v) tannic acid in PBS (1 h), followed by washing in PBS. They were dehydrated by series concentration of ethanol, frozen in a freezer at -65°C and dried at the critical point of vacuum pressure under the following temperature conditions: condenser temperature -53°C and shelf temperature 15°C. Before examination under a scanning electron microscope (SEM) (JEOL, JSM-6060 LV), specimens were coated with 100 Å of a gold–palladium mix in an ion sputter (JEOL JFC 1100) using a voltage of 15–16 kV and a coating time of 30 seconds [20, 21].

Statistical analysis

The statistical package for social sciences (SPSS) version 15 (LEAD Technology Inc., Charlotte, NC, USA) was used to analyse the data. Data were statistically described in terms of range, mean, frequencies (number of cases) and relative frequencies (percentages) when appropriate. Statistical analysis was performed using different tests and the difference was considered to be statistically significant when the probability (P) value was < 0.05.

Results

Bactericidal activity of antibacterial agents and chitosans

Different antimicrobial agents (amikacin, tetracycline, amoxycilin, cephalexin, clarithromycin, erythromycin and levofloxacin) were chosen to test the susceptibility pattern of 42 *P. aeruginosa* isolates. Our study reported that, all strains were highly resistant to amoxycilin (MIC > 600 µg/ml), cephalexin (MIC > 600 µg/ml), clarithromycin (MIC > 2000 µg/ml) and erythromycin (MIC > 512 µg/ml) regarding to CLSI (2007). Amikacin, levofloxacin and tetracycline showed variable degrees of sensitivity against the studied isolates (Table 1). For each antimicrobial agent, the range of results, MIC_{50} and MIC_{90} were obtained for all strains. The MIC ranges of the isolates were ranged from 1.22-312.5 µg/ml for amikacin, from 0.15-39.06 µg/ml for levofloxacin and from 1.17-150 µg/ml for tetracycline. The MIC_{50} of amikacin and levofloxacin were shown to be within the breakpoints (4.88 and 0.8 µg/ml, respectively). While tetracycline MIC_{50} was more than its breakpoint (34.19 µg/ml). MIC_{90} for amikacin, levofloxacin and tetracycline were 54.87, 13.67 and 87 µg/ml, respectively. Low molecular weight chitosan showed the lowest MIC_{50} and MIC_{90} (156.25 and 312.5 µg/ml, respectively) than other molecular weights (312.5 and 625 µg/ml, respectively).

Biofilm profile (optical density)

Quantity of biofilm production was calculated according to the criteria of Stepanovic et al. [22] (Table 2). The strongly-biofilm producer isolates were represented by 42.9% of the isolates. While non-biofilm producers were represented by only 7.1% of the studied isolates. Weakly and moderately-biofilm producers were 21.4% and 28.6% respectively.

MICs (μg/ml)	Antibiotic			Chitosan (MW)		
	AMK	LEV	TET	High	Moderate	Low
[a] MIC_{50}	4.88	0.8	34.19	312.5	312.5	156.25
[b] MIC_{90}	54.87	13.67	87	625	625	312.5
MIC range	1.22-312.5	0.15-39.06	1.17-150	78.13-625	78.13-625	78.13-312.5
[c] Species-related breakpoints (S≤/R>)	16/64	2/8	4/16	-	-	-

[a]The minimal concentration at which 50% of the isolates was inhibited
[b]The minimal concentration at which 90% of the isolates was inhibited
[c]CLSI, 2007 [18].
AMK; Amikacin, LEV; Levofloxacin, TET; Tetracycline

Table 1: Comparative biostatic activity of the most effective antibiotics and different molecular weight chitosans against 42 *P. aeruginosa* isolates.

Biofilm production	*Average OD	Average OD results	Number of isolates (%)
Non- biofilm producer	≤ **ODc	OD ≤ 0.163	3 (7.1)
Weakly- biofilm producer	ODc < ~ ≤ 2× ODc	0.163 < OD ≤ 0.326	9 (21.4)
Moderately- biofilm producer	2× ODc < ~ ≤ 4× ODc	0.326 < OD ≤ 0.652	12 (28.6)
Strongly- biofilm producer	> 4× ODc	> 0.652	18 (42.9)

*Stepanovic et al. (2007) parameters [22]
**Optical density cut-off value (ODc) = average OD of negative control + 3SD of negative control.

Table 2: Classification of biofilm production of bacteria based on optical density measured at OD_{570} by ELISA reader.

Antibiotics	Sessile cells	Planktonic cells
AMK	64x MIC	8x MIC
LEV	64x MIC	4x MIC
TET	128x MIC	16x MIC
TET-CM by coacervation method	1x MIC	1x MIC
TET-CM by the w/o emulsion method	32x MIC	8x MIC

TET-CM; Tetracycline-loaded chitosan microsphere

Table 3: Antibacterial activities of studied-antibiotics alone and tetracycline-loaded chitosan microspheres on sessile and planktonic cells of a selected *P. aeruginosa* strain.

Bactericidal activity of antibacterial agents and tetracycline-loaded chitosan microspheres against biofilm-forming sessile cells and planktonic cells

Depending on the susceptibility testing and biofilm profile, we selected one strongly-biofilm producer strain for further studies. The selected strain was highly resistant to all antibiotics (amikacin; 93 μg/ml, levofloxacin; 19.53 μg/ml and tetracycline; 75 μg/ml). Table 3 shows the antibacterial ability of the tested antibiotics against sessile and planktonic cells of a selected *P. aeruginosa* strain. Moreover, it compares between the antibacterial effects of tetracycline-loaded chitosan microspheres prepared by coacervation method and by the w/o emulsion method. Regarding to selected strain, biofilm growth was inhibited by treatment with 64x MIC of amikacin and of levofloxacin and 128x MIC of tetracycline, whereas planktonic cells were inhibited with 8x MIC of amikacin, 4x MIC of levofloxacin and 16x MIC of tetracycline. Complete inhibition by MIC was achieved all over the sessile cells and planktonic cells by tetracycline-loaded chitosan microspheres prepared by coacervation method. While tetracycline-loaded chitosan microspheres prepared by the w/o emulsion method led to decrease MIC value needed for inhibiting growth (32x MIC) than using tetracycline alone (128x MIC), but to lesser effect than tetracycline-loaded chitosan microspheres prepared by coacervation method.

Time–kill kinetics

Time–kill kinetic was determined for each antibiotic and tetracycline-loaded chitosan microspheres at concentrations of 0.5x, 1x, 2x and 4x MICs for previously mentioned strongly-biofilm producer isolate. All three antibiotics failed to inhibit growth of a selected isolate at concentration of MIC/2 or MIC (Figure 1a-1c). At high MIC (4x MIC), levofloxacin was the most active agent compared to the other antibiotics tested (Figure 1b). Tetracycline-loaded chitosan microspheres prepared by coacervation technique showed more potent inhibitory effects against the studied strain at 1x, 2x, 4x MICs (Figure 1d) than that achieved with tetracycline-loaded chitosan microspheres prepared by the w/o emulsion method (Figure 1e). As shown in figure 1d, there was complete growth inhibition after 3 h treatment with 2x and 4x MICs and after 24h treatment with MIC of tetracycline-loaded chitosan microspheres prepared by coacervation method.

Scanning electron microscopy

Figure 2 shows scanning electron micrographs of sessile cells on the surface of a piece of catheter (Figure 2a) that had been incubated with *P. aeruginosa* for 48 h. The bacteria were covered with thick membranous and fibrous structure and cohered to each other through this fibrous structure unlike non-biofilm forming *P aeruginosa* ATCC 27853 (Figure 2b).

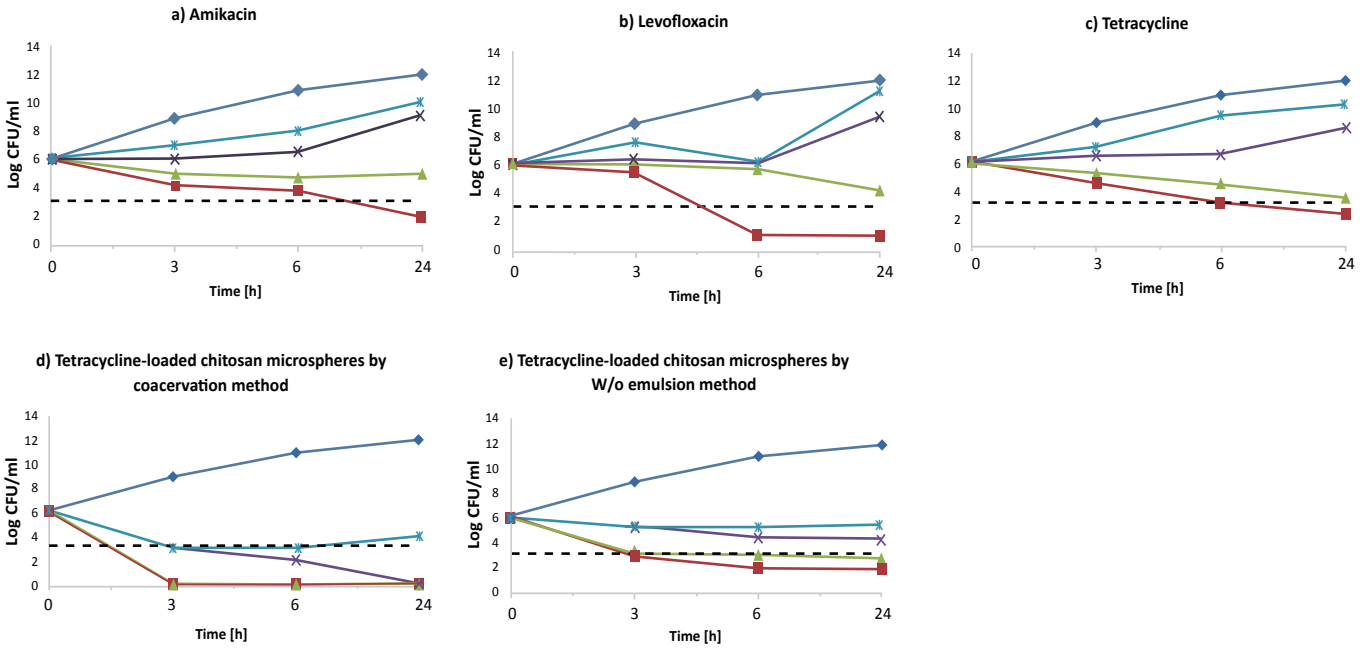

Figure 1: Time–kill kinetics of a selected strongly-biofilm forming *P. aeruginosa* isolate against a) amikacine, b) levofloxacin, c) tetracycline, d) tetracycline-loaded chitosan microspheres by coacervation and e) tetracycline-loaded chitosan microspheres by the w/o emulsion methods at 0.5x, 1x, 2x and 4x MIC. The dashed line represents 3 log reductions. ♦, antibiotic-free control; ■, antibiotic at 4x MIC; ▲, antibiotic at 2x MIC; x, antibiotic at MIC, ✱, antibiotic at MIC/2, CFU, colony-forming units.

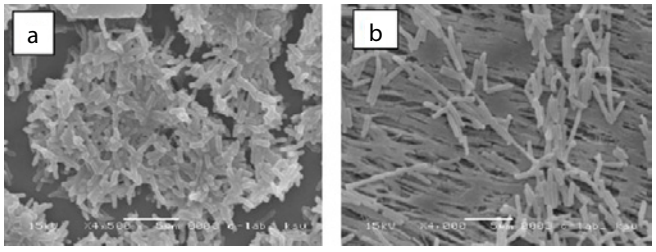

Figure 2: Scanning electron micrographs of (a) sessile cells on the surface of a piece of catheter and (b) non-biofilm forming *P. aeruginosa* ATCC 27853.

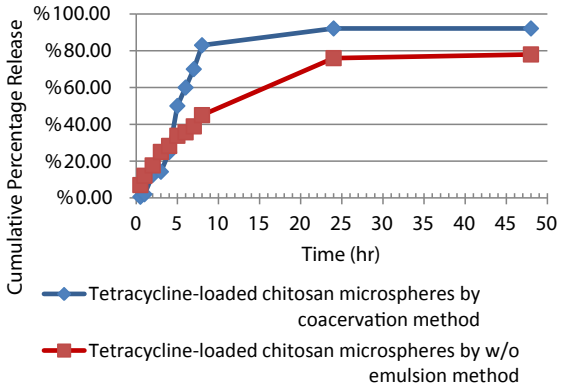

Figure 3: *In vitro* releases of tetracycline-loaded chitosan microspheres.

Evaluation parameters of tetracycline-loaded chitosan microspheres

The percentage yield for tetracycline-loaded chitosan microspheres prepared by coacervation method was 93% ± 3 vs. 86% ± 2.9 for that prepared by the w/o emulsion method. The entrapment efficiency of tetracycline reached 70% ± 1.5 and 66% ± 2.1 for microspheres produced by coacervation and the w/o emulsion methods, respectively. The release of tetracycline was shown in figure 3. The microspheres prepared by coacervation method released about 60% of tetracycline in the first 6 h, while that produced by the w/o emulsion method released 60% of the tetracycline at 15 h. The speed of the used overhead stirrer was adjusted to 1600 rpm to produce uniformly sized spheres. The drug-loaded chitosan microspheres prepared by coacervation method were smaller than those prepared by the w/o emulsion method (59 μm ± 2.77 vs. 213 μm ± 7.31 in diameter, respectively). Both methods yielded spherical and almost regular smooth surface microspheres (Figure 4). The time release data of the tetracycline-loaded chitosan microspheres were fitted into the different kinetic models and was found that, the microspheres prepared by the w/o emulsion method followed the higushi model of release (r^2 = 0.997) and the microspheres prepared

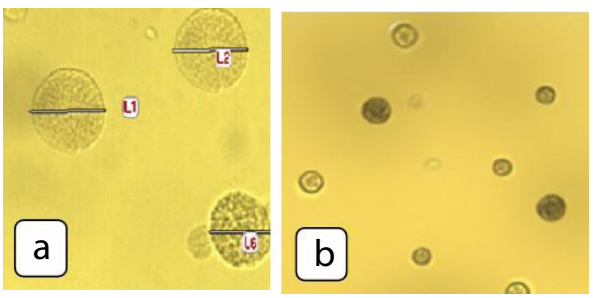

Figure 4: Drug-loaded chitosan microspheres photographed by light microscope and motic software (X1000 magnification), a) Microspheres prepared by the w/o emulsion method, b) Microspheres prepared by coacervation method.

TET-CM	Zero-order kinetics R2	First order kinetics R2	Higuchi model R2	Korsmeyer-Peppas model R2
TET-CM by w/o emulsion method followed by soaking in drug	0.8908	0.9880	0.9970	0.9852 (n > 0.5)
TET-CM by coacervation method followed by soaking in drug	0.5764	0.8706	0.7794	0.9719 (n > 1)

Table 4: Kinetic model fitting for the tetracycline-loaded chitosan microsphere release data.

by coacervation method followed the korsmeyer-peppas model (r^2 = 0.971, n > 1) (Table 4).

Discussion

P. aeruginosa biofilms exhibit increased antimicrobial resistance compared with planktonic isolates and are implicated in the pathogenesis of many acute and chronic infections. In our study, 42.9% of *P. aeruginosa* isolates were strong biofilm producers, 28.6% were moderate biofilm producers and 21.4% were weak biofilm producers. This quantity of biofilm production was approximately in agreement with the results of Zhao and Liu [38], who reported that, 35% were strong biofilm producers, 50% were moderate biofilm producers and 15% were weak biofilm producers according to the criteria of Stepanovic et al. [22].

Of the seven antibiotics examined, only three (amikacin, levofloxacin and tetracycline) showed antibacterial activity. The failure of antimicrobial agents to treat biofilms has been attributed to a variety of mechanisms. In general, organisms encapsulated in the biofilm grow more slowly than the planktonic organisms due to decreased nutrient and oxygen supply leading to a decreased metabolic rate and a decreased antimicrobial susceptibility. Furthermore, antimicrobial binding proteins are poorly expressed in these slow-growing biofilm bacteria. The biofilm matrix itself often delays or impedes the diffusion of antibiotic molecules into the deeper layer of the film (extrinsic resistance) [39]. Bacteria within the biofilm are phenotypically different from their planktonic form and activate many genes, which change their surfaces and other molecular targets, reducing the susceptibility to antimicrobial agents (intrinsic resistance). It is suggested that these phenotypic changes are more important for antimicrobial resistance than the external resistance mechanisms such as biofilm matrix or glycocalyx [20]. Also, bacteria within a biofilm can analyse the external environment, develop interbacterial communication and may transfer genetic information and plasmids within biofilms. As a consequence, bacteria in biofilms may survive the use of antibacterial agents at concentrations 100-1000 times higher than needed to inhibit planktonic bacteria of the same species [40]. This was reported in this study, tetracycline was needed to inhibit *P. aeruginosa* biofilm cells growth 112 times more than that needed to inhibit planktonic cells. The effect of antibiotics on biofilm inhibition is highly variable and unpredictable; make it increasingly difficult for clinicians to choose the most active antibiotic, particularly as biofilm susceptibility testing is not routinely performed as conventional clinical microbiology can detect only the planktonic cells.

Time–kill kinetics revealed that, all studied antibiotics (amikacin, levofloxacin and tetracycline) failed to inhibit growth of a selected strongly-biofilm producer isolate at a concentration of MIC/2 or MIC. At 2x MIC, three antibiotics have bacteriostatic activity but they reduced the bacterial growth to less than 3 $\log_{10}$. At high MIC (4x MIC), levofloxacin was the most potent agent compared to the other antibiotics tested against *P. aeruginosa* biofilms as single agent. This was in coordination with the results of Goto et al. [14]. Levofloxacin exhibited bactericidal effect before 6 h of incubation, while amikacin and tetracycline exhibited their inhibitory effects near to 24 h of incubation. This effect was probably accounted for the restriction of the empirical use of levofloxacin. It is effectively used for more serious infections [42]. The antibiotics displayed dose-dependent activity against studied isolate but no complete eradication was achieved. In clinical terms, the use of bactericidal agents for rapid clearance of a bacterial infection is essential in the treatment of severe life-threatening infections [43].

Chitosan has antibacterial properties against *P. aeruginosa* and may detach *P. aeruginosa* biofilms. In our study, low molecular weight chitosan exhibited antibacterial activity with MIC_{50} of 156.25 µg/ml. The antibacterial effect of chitosan was revealed by Lim and Hudson, [44]. They revealed that, the MIC of chitosan was influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium. The antibacterial activity of chitosan has been reported by many other investigators [45]. In our work, it was found that; low molecular weight chitosan had a higher antimicrobial activity against *P. aeruginosa* than the high or medium molecular weight one. This was not agreeing with the generally accepted concept that, the highest molecular weight chitosan should have the highest antimicrobial activity due to the greater number of reacting positive charges [46]. However, Zheng and Zhu [47] study was in accordance with our result explaining that, the low molecular weight chitosan had a higher antimicrobial activity especially against Gram-negative organisms because of low viscosity of its solution making it able to penetrate more into cells. Also, it had been mentioned in a recent study that, the mode of action of chitosan was not just involved altering a cell's permeability but also had been found to interfere with the expression of some genes in a bacterial cell [48].

Chitosan is a biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. In this study, tetracycline-loaded chitosan microspheres were prepared by two methods (the coacervation and the w/o emulsion) and was subjected to measurement of morphology, mean particle size, particle size distribution, percentage drug entrapment (PDE), drug loading, and *in vitro* drug release. The loading efficiency reached 70% and 66% for microspheres prepared by coacervation and the w/o emulsion method, respectively. Regarding to previous studies, it was sited that, the release of the drug from chitosan microspheres was strongly affected by the pH of the medium due to its action on the ionization of the glucosamine residues of chitosan. At lower pH, the D-glucosamine residues were ionized resulting in extensive swelling and faster release of the drug. In the study of Hejazi and Amiji [49], it was reported that, 70% tetracycline was released after 3 h, while 90% of the drug was released after 8 h at pH 3.0 and 5.0, respectively. In our study, we chose fixed pH at 7.4 resembling neutral pH and to be suitable for antimicrobial activity testing. As a result, the release of tetracycline was slow, the microspheres prepared by coacervation release about 60% of tetracycline in the first 6 h as

compared to the ones produced by the w/o emulsion method that released 60% of the tetracycline at 15 h. The slower release exhibited in the microspheres prepared by the w/o emulsion method over the coacervation method might be due to the viscous oily layer adhered to the surface of the former, which thus erode less easily. Also the oily layer may act as a slight barrier to the diffusion of water into the microsphere and thus producing swelling and drug release. This was in agreement with the previously cited study by Khare and Jain [50], they had studied the effect of the viscosity of the used oil on the drug release profile of microspheres. This was also seen when the release data were fitted into the different models where the release from the tetracycline microsphere by the w/o emulsion method followed the higushi model that resembled fickian diffusion of a drug from a polymeric matrix. In the case of microspheres prepared by the coacervation method, the data resembled a super case II transport (n > 1 in korsmeyer-Peppas model) where the drug release involved a mixture of diffusion and matrix erosion.

Regarding to our results, the cumulative amount of tetracycline that was released from tetracycline-loaded chitosan microspheres prepared by coacervation method led to extended effect from the start of treatment of studied strain and continue for 24 h of the application. Complete growth inhibition of our studied strain was achieved after 3 h treatment with 2x and 4x MICs and continued for 24 h treatment by tetracycline-loaded chitosan microspheres prepared by coacervation method. This in the clinical field may be translated into maintaining constant drug concentration for a prolonged period and maximize the therapeutic effect of antibiotics while minimizing antibiotic resistance and improved patient compliance. Moreover, constant drug level prevents acute exacerbations of infections due to release of planktonic bacteria from biofilm colonies [51]. The findings suggest that, chitosans act as enhancing agent to antibiotics in pharmaceutical preparations.

The exact mechanism of the antimicrobial action of chitosan is still unknown, but different mechanisms have been proposed. The possible mechanism of chitosan can be hypothesized to be due to three important characteristics of chitosan. It has a positive charge by binding to cell walls and to negatively charged matrix components of the biofilm that adsorbed a lot of antimicrobials and thence imparted resistance [52]. It also has a basic nature which can neutralize the acidic microenvironments of the biofilm and thereby reducing resistance to antibiotics that are affected by the acidic environments [53]. In addition, its surfactant and permeability enhancing properties that may help to dissolve into the glycocalyx allowing a greater penetration of the antibiotics and interrupting the microenvironment osmotic and oxidative stress and thence again are increasing sensitivity to the antibiotics [54].

In the w/o emulsion method, the optimum proportions of the components of the oil phase was a mixture of 25 ml of liquid paraffin and 50 ml of dichloroethane that produced rigid uniformly spherical particles that withstood washing, filtration and drying (higher proportion of liquid paraffin led to sticking microspheres that were not separated easily from the preparation mixture and leached oil during the washing and drying steps). This can be attributed to the potentially much higher viscosity of the liquid paraffin that impacted the morphology of the spheres. Crosslinking of microspheres was carried out using potassium hydroxide (KOH) as the ionotropic gelating agent. Cooling during gelation led to the better crosslinking. The time of crosslinking was also very important factor. Crosslinking time less than 90 min led to fragile microspheres and leaching occurred. Three hours were found to be optimal for forming non-fragile microspheres. Washing 3 times or more had been carried out with petroleum ether to remove any residual oils and produce clean non-sticky discrete rigid spherical microspheres that can be easily filtered and dried. Very low concentration of KOH produced no spheres and very high concentration produced aggregation of the spheres to form a jelly mass that sticks to the paddle of the stirrer. Freeze drying produced more porous microspheres with a quicker release profile than air dried ones. In the coacervation method, the microspheres formed quickly within 1h to form a turbid colloid. The microspheres were then separated by centrifugation. It produced much smaller microspheres than that produced by the w/o emulsion method. In both methods, the speed of the overhead stirrer was found best to be 1000 rpm; at higher rotations it distorted the morphology of the fragile microspheres.

In conclusion, our results suggested that tetracycline-loaded chitosan microspheres prepared by coacervation technique has promising antibacterial properties against *P. aeruginosa* biofilms. It may be a new strategy for the treatment of biofilm-associated acute and chronic infections, although it would be appropriate to conduct *in vivo* animal models and clinical studies to confirm this.

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