Nanoscience and Nanotechnology: An Indian Journal

Research | Vol 13 Iss 1

The Release Property of Amoxicillin Nanoparticle and their Antibacterial Activity

Maysaa Ch AL-Mohammedawi[1*], Rana A Mohsien[2], Mokhtar J Kadhim[2], Enas S Bahjat[2], Duna Q Al Azawi[2], and Samra A Qaraghuli[3]

[1]Nanomedicine-Laboratory of Immunology and Biomedical Research, School of Medicine, Deakin University, 75 Pigdons Rd Geelong, Australia

[2] Biotechnology Department, College of Science, Al Nahrain University, Baghdad, Iraq

[3]College of Pharmacy, Al Mustansyria University, Baghdad, Iraq

[*]**Corresponding author:** Maysaa Ch AL-Mohammedawi, Nanomedicine-Laboratory of Immunology and Biomedical Research, School of Medicine, Deakin University, 75 Pigdons Rd Geelong, 3216, Australia, Tel: +61 478 795 442; E-Mail: cmaysaa@gmail.com

Received: March 08, 2019; **Accepted:** April 20, 2019; **Published:** April 29, 2019

Abstract

Early efforts have been made in an attempt to reduce the adverse side effect of multiple drug resistance organisms, and new classes of antimicrobial nanoparticles (NPs) and nanosized carriers for antibiotics delivery were developed. PURPOSE: This study was focused on the assessment of the physiochemical characterization, *In vitro* drug release, biofilm formation and antimicrobial properties of amoxicillin encapsulated within the Poly (ε-caprolactone) (PCL) nanoparticles. Methods: amoxicillin (AMX) nanoparticles were prepared using the emulsion solvent evaporation method with different concentrations of polycaprolactone (PCL) and Poly Vinyl Alcohol (PVA). These nanoparticles were subsequently characterized and evaluated for their antibacterial activity and biofilm inhibition activity using mean and standard error for Data analysis. Results: It was found that increased PCL concentration resulted in an increase in entrapment efficiency (EE%) to 83.3%. Meanwhile, an increase in the PVA concentration led to a decrease in the EE% and an increase in nanoparticle size. Enhancements in the percentage of practical yield to 80.2% as the polymer concentration rose. Fourier transform infrared spectra data for the MNPs, CS-coated AMX, and AMX-PCL-NPs nanoparticles were compared, which confirmed the PCL coating on the AMX and the AMX-PCL-NPs loaded nanoparticles. In addition, the antimicrobial activity of the nanoparticles e was determined using agar diffusion and growth inhibition assays against both gram-positive *Staphylococcus aureus*, gram-negative *Pseudomonas aeruginosa* and *Proteus mirabilis* bacteria. Furthermore, 10 µg/ml was the minimum inhibitory concentration of the AMX-PCL-NPs nanoparticle which inhibited biofilm formation in *Staphylococcus aureus* bacteria. Conclusion: Thus, this study presents a novel ß-lactam antibacterial-nanocarrier system that can reduce and inhibit bacterial growth showing it to be a promising tool for numerous medical applications.

Keywords: ***Poly (ε-caprolactone); Poly vinyl alcohol; Amoxicillin nanoparticle; Antimicrobial activity; Biofilm***

Introduction

There is a growing interest among material scientists to provide antimicrobial properties to implantable biomaterials in order to reduce the adverse side effects of the traditional oral antibiotic therapy and the associated Surgical Site Infections (SSIs)

Citation: Mohammedawi ALM, Mohsien AR, Khadim JM, et al. The Release Property of Amoxicillin Nanoparticle and their Antibacterial Activity. Nano Tech Nano Sci Ind J. 13(1):130.

© 2005 Trade Science Inc.

when bio-implants are involved. Therefore, in addition to serving its primary function, the implant will also help prevent the formation of bacterial biofilms and the released antimicrobial agent will kill or inhibit the growth of bacteria, thereby reducing the potential of SSIs [1]. This will help overcome the failure of a single antibiotic therapy that may contribute to poor stability of the drug in the acidic pH of the stomach, poor permeability of antibiotics across the mucus layer, or due to the availability of sub-therapeutic antibiotic concentrations at the infection site after oral administration in a conventional capsule or tablet dosage form. All these problems encourage scientific researchers through addition surface modifications to the nanoparticles by preparation an antibiotic delivery system which is able to localize the drug at the infection site and to achieve bactericidal concentrations that are desirable [2,3]. Optimal modifications to some physicochemical properties of polymeric nanoparticles, such as size and surface characteristics, makes it possible to modulate their bio distribution parameters. When designing nanoparticles for a drug delivery system, which can give a large bio distribution of the drug and can allow the reaching of particular sites that are different from reticuloendothelial cells, it is necessary to avoid the removal of drug-loaded nanoparticles from the blood by the endocytic uptake of Kupfer cells or other phagocytic cell populations within the Mononuclear Phagocyte System (MPS) [4].

Moreover, more effort was focused on Polymeric Nanoparticles (NPs) in order to increase their capacity for intracellular drug delivery and therapeutic effects by enhancing stability and sustaining release, especially for drugs that act via intracytoplasmic receptors. For example, Sahoo et al. [5] reported that NPs enhanced the paclitaxel efficacy on the breast cancer cell line via sustained intracellular delivery [6]. Another study also presented the improvement of the indomethacin cytotoxic effects on the glioma cell line when it was nano-encapsulated. Besides these examples, the nanoparticle-based delivery system can also be designed to target specific tissues, cells and/or intracellular compartments [7]. Biodegradable polymeric NPs are highlighted areas of drug delivery research; they are used to modify the release and distribution profile of antibiotics, allowing effective delivery to be improved and toxic effects to be lowered [7-9].

The biodegradable antimicrobial-based polymers in which antimicrobial drugs, namely ampicillin, and amoxicillin, were chemically incorporated into -anhydride and amide bonds backbone of poly (anhydride-amides) by solution polymerization-were designed to locally prevent infections associated, e.g., with medical devices or by controlled hydrolytic degradation. The *in vitro* degradation of the polymer into bioactive products were measured and the antibacterial properties examined using gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) bacteria [10]. The objective of this study was to focus on the testing the antibacterial activity of amoxicillin antibiotic encapsulated with PCL polymer, which is widely used as a biodegradable polymer for biomedical applications, such as surgical sutures and drug delivery devices, and polyvinyl alcohol as an emulsifier capping agent in the analysis of an *in vitro* drug release analysis.

Materials and Methods

The polymer, PCL (Mwt=50 KDa) (Sigma-Aldrich Chemical USA), the solvents, Di-Chloro-Methane (DCM) (Fisher Chemical), and the antimicrobial agent amoxicillin (Mediatech Inc.), were all purchased from the Al-Harthia company office, Baghdad, Iraq. Amoxicillin (AMX) is a broad-spectrum antimicrobial agent with high selectivity and is in the beta-lactam antibiotics class, which inhibits bacterial cell wall synthesis by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall.

Preparation of the standard calibration curve of amoxicillin

Aliquots of 2, 4, 6, 8 and 10 ml were pipetted out from the stock standard solution of amoxicillin and the volume was made up to 10 ml with PBS pH 7.4 to obtain concentrations in the range of 20 µg/ml to 100 µg/ml. The absorbance of these solutions was measured at 290 nm by UV-Visible spectrophotometer, using a phosphate buffer of pH 7.4 as blank. The absorbance values were plotted against the concentration to obtain the standard graph.

Formulation of amoxicillin nanoparticles

Four amoxicillin Nano-formulation particles were prepared using the double emulsion solvent evaporation method with different concentrations of Polycaprolactone (PCL) and Poly Vinyl Alcohol (PVA), **TABLE 1** [10].

TABLE 1. **Formulation plan for amoxicillin nanoparticles.**

Nanoformulation	Amoxicillin mg/ml	PCL polymer W/V (%)	PVA W/V (%)
AMX-NP-1	5	1	2
AMX-NP-2	5	2	2
AMX-NP-3	5	1	3
AMX-NP-4	5	2	3

Five mg of amoxicillin was dissolved in 1 mL of phosphate buffered saline (0.01M, pH 7.4) to form an aqueous solution. Then it was emulsified in an organic phase consisting of different concentrations of PCL polymer (2.5 mg to 5 mg) in 2.5 ml of the organic solvent di-cholo methane (DCM) to form a primary water in oil emulsion produced using a micro tip probe sonicator (VC 505, Vibracell Sonic, Newton, MA, USA) set as a 55 W for 2 min. over an ice bath. After that, the emulsion was further emulsified in an aqueous solution made up of 12.5 ml of polyvinyl alcohol PVA stock solutions (100 ml 2% to 3% w/v), **FIG. 1**, to form a water-in-oil-in-water emulsion.

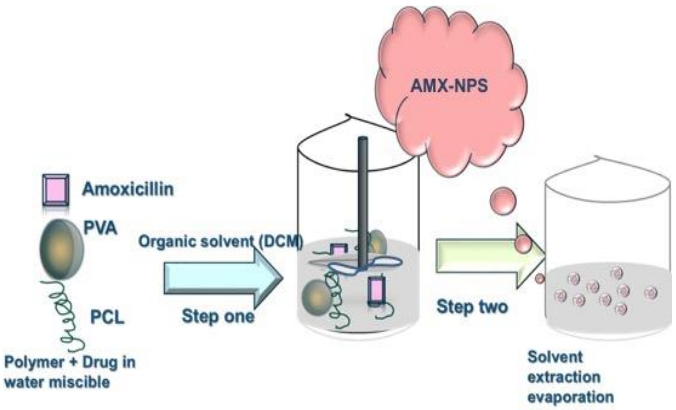

FIG. 1. **Schematic representation of the double emulsion-solvent evaporation method for nano-formulated amoxicillin nanoparticles preparation using double-emulsification the W/O1/O2 method Steps.**

The emulsification was carried out using a micro tip probe sonicator (VC 505, Vibracell Sonic, Newton, MA, USA) set as a 55 W of energy output for 5 min. over an ice bath by adding the primary emulsion drop to the 20 ml of phosphate buffer (0.01 M, pH 7.4). The emulsion was stirred at room temperature for 2 hrs on a magnetic stir plate to allow evaporation of the organic solvent. One hour of vacuum drying was subsequently performed to remove any residual organic solvent present.

Any excess amount of PVA was removed by ultracentrifugation at 16,000 rpm at 40°C for 20 min. (Remi, India), followed by washing with double distilled water. The supernatant was collected and kept to estimate the amount of the drug which was not encapsulated. The recovered Nano-particulate suspension was lyophilized for two days (-800°C and<10 mm mercury pressure, LYPHILOCK 12, Labconco, Kansas City, MO, USA) to provide the lyophilized powder for further use.

Percentage yield

The practical yield was calculated as the weight of the dry nanoparticles recovered from each batch in relation to the sum of the starting materials. This practical yield is essential in order to know about percent yield or efficiency of any method, which would be helpful in selecting the appropriate method of production [10,11].

$$\%\text{Practical Yield (PY)} = (\text{Practical Yield/Theoretical Yield}) \times 100$$

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopic analysis (Shimadzu-FTIR, Model-8000 provided by Chemical Dept. /College of Science/Al-Nahrain University/Baghdad/Iraq). The analysis was conducted to verify the possibility of chemical bonds between drug and polymer. Samples of pure amoxicillin, pure PCL and amoxicillin -PCL physical mixture 1:1, were scanned in the IR range from 400 cm to 4000 cm^{-1} with carbon black as a reference **FIG. 2**. The detector was purged carefully with clean dry helium gas to increase the signal level and reduce moisture.

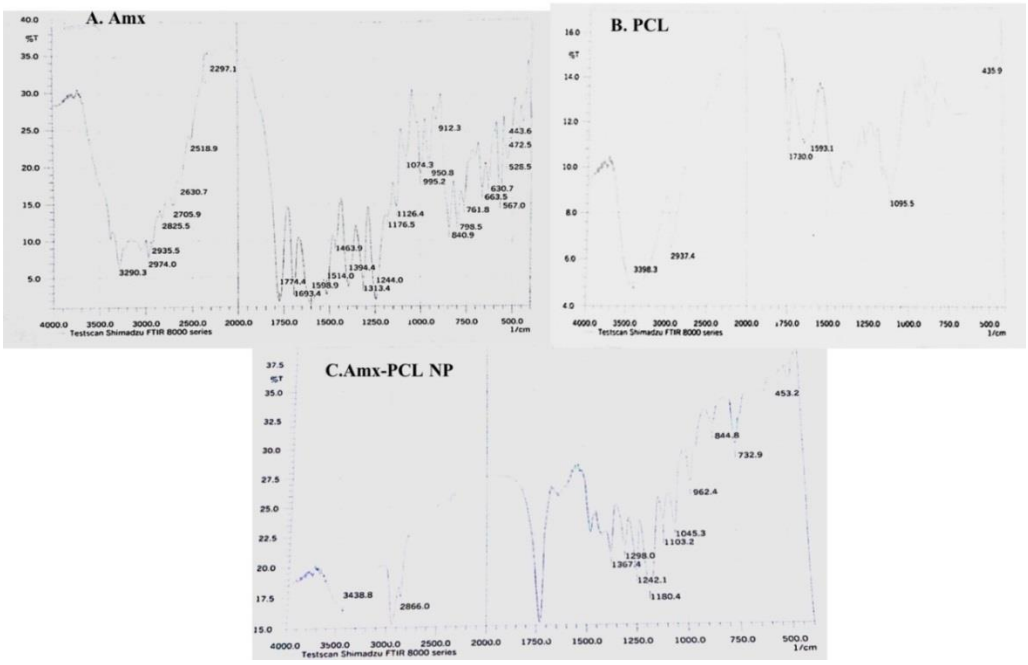

FIG. 2. **Infrared spectra of (A) amoxicillin (AMX); (B) Polycaprolactone (PCL); and (C) AMX-PCL-NPs nanoparticles.**

Encapsulation efficiency (EE%)

The Entrapment Efficiency (EE%) of amoxicillin nanoparticle was measured at a wavelength of 290 nm by a UV spectrometer (Spectronic Genesys 10 Bio, Thermo Electron Cooperation, WI, USA). The standard curve was prepared using drug concentrations ranging from 20 mcg/ml to 100 mcg/ml and had a regression equation of y=0.0174x with R2=0.9919. In each sample, EE% was measured by separating the aqueous phase with the colloidal one after centrifuging at 4300 rpm for 60 min (VWR micro 18R, VWR Inc., West Chester, USA). The entrapment efficiency of the drug loading was calculated using the equation below [11].

$$\text{Entrapment efficiency (\%)} = (AT\text{-}AF)/AT \times 100$$

In which AT is the total drug amount and AF is the Nano encapsulated drug amount.

Determination of drug release profile

One milliliter of the above AMX-NP nanoparticles were centrifuged for 45 min at 20,000 rpm, and the precipitates were re-dispersed in 1 mL of buffer solutions at pH values of 1.5, 6.0, and 7.0. The dispersed particles were incubated for different time intervals (2-24 h.) at 37°C, which was conducted in triplicate [12]. The amount of released drug was measured at 290 nm by UV-spectrometry. In addition, drug loading efficiency was measured in the same manner. Briefly, the weight of amoxicillin was measured after lyophilization and then dissolved in 1 mL of distilled water. The loaded amount of drug was measured by UV-spectrometry, using the following formula [13,14].

$$\text{Drug loading efficiency} = \text{Wt. of drug in NPs}/\text{Wt. of (PCL-PVA)} \times 100$$

Detection of the antibacterial activity of amoxicillin nanoparticles

A standard agar well diffusion method was used to test the evaluation of the antibacterial activity of amoxicillin nanoparticles. *Staphylococcus aureus, Proteus mirabilis,* and *Pseudomonas aeruginosa* clinical isolates were used as the bacterial model. Pure cultures of each of the bacterial isolates were subcultured in a nutrient broth for 24 h at 37°C. After that, the inoculums (having a turbidity of 0.5 MacFarland standard 10^8 CFU per ml) were spread with sterile cotton swabs on Mueller Hinton agar plates. Wells 6 mm in diameter were made using a sterile cork borer and 50µl of each amoxicillin-nanoparticle; amoxicillin free drug and PCL-PVA nanoparticle suspension was poured into each well of the plates. The plates were incubated overnight at 37°C and results were recorded by measuring the diameter of inhibition zone (mm) [15,16].

Determination of MIC which defined as minimum inhibitory concentration required to inhibit the growth of 90% of *S. aureus* isolate by Colony Forming Unit (CFU) upon applying different concentrations (10 µg/mL to 320 µg/mL) of AMX alone, AMX-NP2 by plate counting method. Antibacterial test pathogen was grown in LB liquid medium at 37°C for 12 h before being diluted in fresh LB liquid medium to reach OD_{600}=0.003 (optical density) and then was added to the culture medium. the *S. aureus* isolate was incubated after mixing with either AMX free drug, PCL-PVA NP or AMX-NP2 at 37 µg/mL C for 24 h. Serial dilutions were then made and after incubation, the Cell Forming Units (CFUs) was determined using the conventional plate count method [17]. Amoxicillin free *S. aureus* isolate was used as a negative control.

Developing a biofilm

An overnight culture of *Staphylococcus aureus* (biofilm producer isolate) was inoculated into LB broth medium. After 24 hrs, the bacterial growth culture was diluted 1:100 in to fresh LB containing 1% glucose. 100 µL of the dilution was placed in

each per well in a 96 well plate. 100 µL of sub- MIC AMX, PCL-PVA (100 µg/mL) and AMX-NP2 were added at different concentrations (1.25-100 µg/mL) and incubated for 18-24 hrs, at 37°C. OD (600 nm) was measured. The data did not show any effect on plankton growth.

Staining the biofilm

The medium was removed, and the plates were rinsed three times with PBS. The dried plate with remaining biofilm was stained with 100 µL of a 0.1% crystal violet solution and incubated at room temperature for 45 min. After that, the crystal violet was removed, and the plate was rinsed again with PBS. The plate was shaken to remove any free solution and then dried by turning it upside down on a stack of paper towels for 2 hrs to rid the plate of all leftover cells and dye.

Quantifying the biofilm

200 µL of 30% acetic acid in water was placed into each well of the microtiter plate to solubilize the unwashed stain that colored the biofilm on the inside of the wells. The microtiter plates were incubated for 30 min at room temperature. 125 µL of the solubilized stain was transferred to a new flat-bottomed microtiter plate. The biofilm was quantified by reading absorbance in a plate reader at 550 nm using 30% acetic acid in water as the blank.

Results and Discussion

The absorbance measurement of amoxicillin standard solutions containing 20 mcg/ml to 100 mcg/ml of drug in a pH 7.4 phosphate buffer saline **FIG. 3**, which presents the standard calibration curve. The curve was found to be linear in the range of 20 mcg/ml to 100 mcg/ml at λ max 290 nm. The regression value was found to be 0.9919.

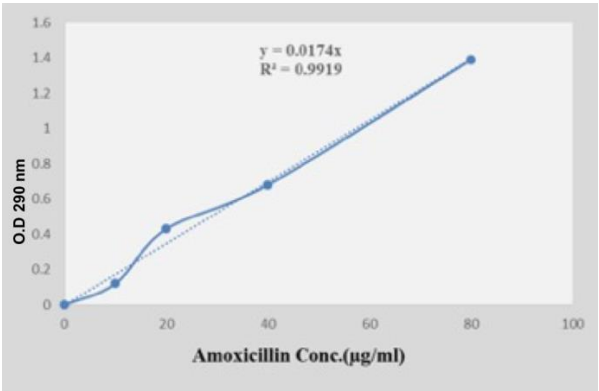

FIG. 3. **Represent the standard calibration curve of amoxicillin in phosphate buffer saline (7.4) at 290 nm.**

The nanoparticles were formulated with different concentrations of PCL and PVA as given in **TABLE 2** by varying weights (1,2)% of PCL dissolved in dichloromethane and PVA (2,3)% dissolved in water to investigate the corresponding modifications of particle size and entrapment efficiency **FIG. 4**. it was found that increased PCL concentration resulted in an increase in entrapment efficiency. The maximum entrapments were 83.31% and 60.9% found in AMX-NP2, AMX-NP1, and

AMX-NP3, respectively. The entrapment efficiency was 49.4% for AMX-NP4. Increasing PVA led to a decrease in EE% and an increase in nanoparticle size. Our result showed that rising PCL concentration was able to produce a higher amount of smaller particles.

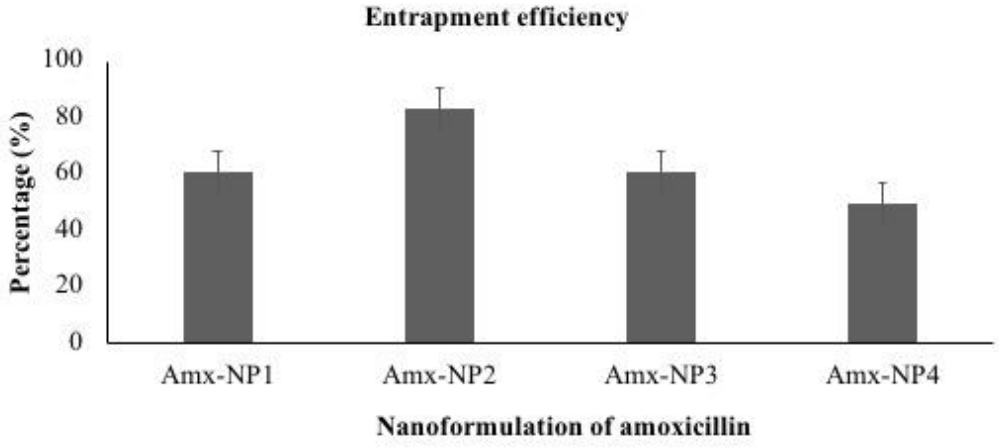

FIG. 4. **The drug entrapment efficiency of amoxicillin nanoparticles.**

In our study, PVA was used as a stabilizer for the preparation of PCL nanoparticles. This polymer is one of the most frequently used nanoparticle stabilizers since it enhances the production of stable particles that have a small size and narrow size distribution. Many papers have mentioned that a fraction of the PVA used in the formulation remains associated with the surface of the nanoparticles despite repeated washing [18].

Percentage of particle yield

The results of the percentage of practical yield studies are shown in **TABLE 2**. The percentage of practical yield increased as the amount of polymer added to each formulation increased, although it may not be dependent upon the PVA concentration in the formulation. Maximum yield was found to be 80.2% for AMX-NP4 and 70.4% for AMX-NP1.

TABLE 2. **Percentage of particle yield.**

Nanoformulation	Practical yield (mg)	The total amount of ingredients (mg)	Percentage yield (%)
AMX-NP1	43	61	70.4
AMX-NP2	52	84	61.9
AMX-NP3	39	73	53.4
AMX-NP4	57	71	80.2

Some formulations have quiet percentage yield whereas some have a higher yield this could be contributed to the variation in PCL concentration. In this study, we found that increasing in EE percentage led to a diquat in the percentage yield the reason

could be an increase in solvent/water ratio during nanoparticle preparation using double emulsion solvent evaporation method caused decreased in the drug loading and percentage yield. In addition, some of the AMX-NPs were not collected due to electrostatic adhesion of particles to particle collector during the dryness process. Therefore, AMX-NP2 was chosen as the optimized formulation based on high EE% and percentage yield.

FTIR

The IR spectrum of amoxicillin nanoparticles was scanned over a range of 400 cm^{-1} to 4500 cm^{-1} using an FTIR instrument (FTIR-1700, Shimadzu 8000 series, Kyoto, Japan) and compared with the polycaprolactone and pure amoxicillin spectra. Results showed the presence of IR bands of (AMX) in nanoparticles at the same wavelength: 1367.4 cm^{-1} for the N-C aromatic bond, 1730 cm^{-1} and 2866 cm^{-1} for C=O and O-H vibrations of carboxylic acid, and 3438 cm^{-1} for the vibration of alcohol functions. We also found the characteristic bands of PCL: at 1730 cm^{-1} there was the ester function; at 2937.4 we found the O-H of carboxylic acid, and the fine band of the external chain was observed at 1460 cm^{-1} **FIG. 5**. This indicates that functional groups in nanoparticles of AMX-PCL-NP are a combination of constituent components. In addition, the spectra of nanoparticles had no new peaks. Therefore, it can be concluded that the nanoparticles produced were the result of the blend of AMX and that PCL is formed only by the physical interaction between them. This can also be seen from the FTIR spectrum, which shows both components of amoxicillin and PCL. Thus, the FTIR spectra of amoxicillin and PCL are merely a mixture of amoxicillin and PCL.

These results were similar to those of other researchers who found IR bands of amoxicillin in microparticles using ethylcellulose (EC) and poly (ε-caprolactone) at the same wavelength: 1370 cm^{-1} for the N-C aromatic bond, 1730 cm^{-1} and 2950 cm^{-1} for the C=O and O-H vibration of carboxylic acid, 2090 cm^{-1} for the bending of S-C, at 3500 cm^{-1} of amine function and 3400 cm^{-1} vibration of alcohol functions. And in the same manner, it was found that the characteristic bands of PCL were identified at 1725 cm^{-1} for the ester function, at 2940 the O-H of carboxylic acid, and the fine band of the external chain was observed at 1470 cm^{-1} [19,20].

Characterization of Amoxicillin-Polycaprolactone Nanoparticles by Atomic Force Microscopy (AFM)

In this study, AFM was used to measure the topography of amoxicillin-PCL nanoparticles with surface roughness. The surface roughness of these nanoparticles is one of the most important parameters that typically have been used to reduce bacterial adhesion to implant materials [21].

The size of four Nano formulation nanoparticles was determined (Data not shown). The average roughness and topography of the high encapsulation efficiency Nano formula amoxicillin nanoparticles (AMX-NP2) were evaluated and the results of analyzing two-dimensional AFM images showed that the average roughness was 3.53 nm with a core roughness depth of 11.3 nm **(FIG. 5A and 5B)**. As well, the average diameter size was found to be 100 ± 11.5 nm, as shown in **FIG. 5C**. The top view of synthesized nanoparticles in a 3D structure indicates linear trends in roughness and particles in homogeneity of AMX-NP, which indicates the formation of smother layers. We found that the nanoparticles distributed uniformly were dispersed densely and had both smooth and rough surfaces. Some reports described a positive correlation between bacterial adhesion and surface roughness. Due to the higher contact surface for the attachment, the protection from shear forces and the increase in convection mass transport [22]. Surface roughness may influence surface properties like water contact angle [23]. Measured zeta potential may also depend on surface roughness.

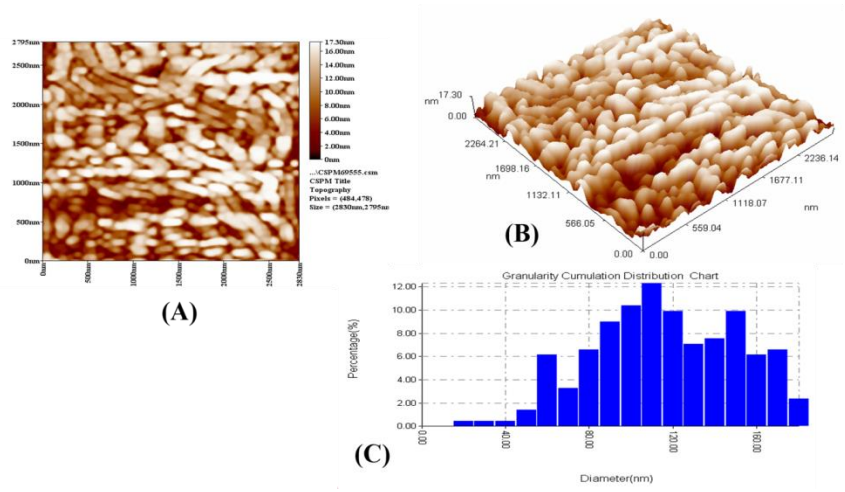

FIG. 5. **Represents (A) 2D micrograph of the atomic force microscopy of the AMX-NP2 nanoparticle; (B) 3D micrograph of AMX-NP2 with surface roughness; (C) Granularity cumulation distribution chart.**

The appearance of all four Nano formula nanoparticles showed that they were morphologically spherical to ovate in structure, having an average diameter in the range of 74 nm to 100 nm.

The above findings indicated a unique ratio of PCL (2%) showed a particle size below 200 nm with an optimal EE% and over 50% percentage yield.

Our result was similar to those obtained by a pilot study of Patel et al. [2] who found that the PCL nanoparticles prepared by the emulsion-diffusion method produces Nano capsules with a mean size of 294 nm to 401 nm in a reproducible and efficient way.

Drug release analysis

The advantage of a sustainable release of the local delivery of drugs for many days upon systemic administration is that it improves the effectiveness of the drug, selective targeting, decreasing the side effects, and reducing the frequency of administration. Therefore, it is of the utmost importance to investigate the release profiles, depending on the many parameters that it is possible to modulate, for the specific applications.

The *in vitro* release of amoxicillin from the PCL nanoparticle was investigated by measuring the concentration of amoxicillin released in the Phosphate Buffer Solution (PBS) (with a pH of 4.5, 6.8 and 7.4). The result of the release profiles for the amoxicillin from the prepared nanoparticles (AMX-NP2) at pH 4.5, 6.8, and 7.4, respectively. At pH 4.5, the proportion of amoxicillin released from the nanoparticles in the first two hours was 8% to 21%, while pH values of 6.8 and 7.4 were 27 and 19% respectively **FIG. 6**. When amoxicillin is released after 2 hrs from a PCL nanoparticle, this is explained by the high porosity of the PCL surface for nanoparticles. Amoxicillin was abruptly released from AMX-NPs under acidic conditions (pH 4.5) with about 45% of the drug released within 12 hrs, whereas at pH value of 7.0 and 6.0 it was 38% and 56% respectively. During the remaining 24 hrs period, the release profiles showed sustained release patterns which reached 75% at the acidic pH (4.5) and to 84% and 67% at values of the 7.4 and 6.8. The low release pertains to the strong chemical

adsorption of amoxicillin molecules into the polycaprolactone polymer. The remaining 25%, which is maintained inside the whole of nanoparticles, needs much more time to release.

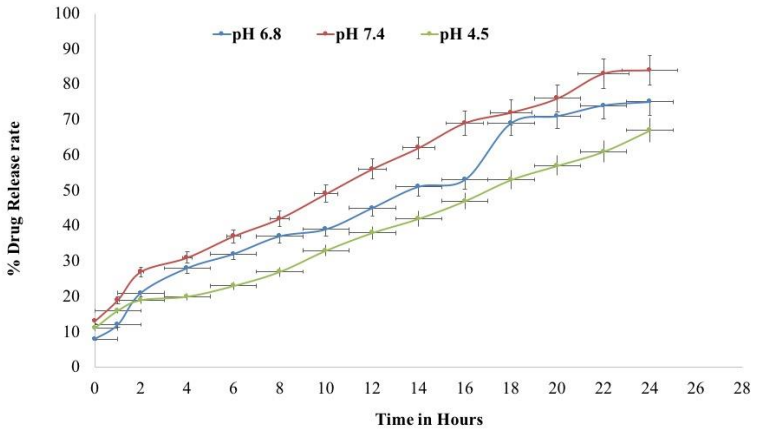

FIG. 6. **Release behavior of AMX-NP2 nanoparticle at different pH conditions.**

The most important features of the drug/polymer formulations are the release characteristics of polymeric nanoparticles because of the potential application in sustained drug delivery. One of the most important factors that affect the release rate of the entrapped drug is the size of nanoparticles. Larger particles have a smaller initial burst release and a longer sustained release than smaller particles. In addition, the greater the drug loading, the greater the burst and the faster the release rate [24].

The nanoparticle AMX-NP2 was selected as the optimized formulation for further delivery release studies, with 83.31% EE for drug release after 24 hrs. The prepared nanoparticles proved to have a good sustained release after 24 hrs at pH 7.4.

Similar results were obtained when Ahymah Joshy et al. [25] found that the *in vitro* drug release percentage of amoxicillin released from lanthanum hydroxyapatite Nano rods at various time intervals was about 27% to 35% within 5 to 7 hrs followed by continued slow release.

As was mentioned earlier, using a double emulsion solvent evaporation method for preparing AMX-NPs was an effective method where the release kinetics of antibiotic was slow. The initial antibiotic release from the PCL NPs occurs by a slower release mediated through diffusion the amoxicillin from the polymeric matrix to the solution followed by subsequent degradation of the polymer matrix.

The slow release of antibiotic meaning that the more of the amoxicillin encapsulated into the nanoparticle that related to the suitable concentration of the polymeric matrix (2%). This has an advantage in the therapeutic effect of the antibacterial agents using PCL as an encapsulating polymer through the double emulsion-solvent protocol.

Microbial assay for amoxicillin nanoparticles

It is necessary to confirm the activity of the compounds released from nanoparticles for a medical application of these nanoparticles. This was realized by assessing the drug's effectiveness to inhibit microbial growth. A well diffusion test was considered an effective procedure to do this; the **FIG. 7** represents a table and a photo of a Petri dish showing the zones of inhibition of *S. aureus*, *P. aeruginosa*, and *P. mirabilis* growth upon the addition of free-amoxicillin and an amoxicillin-NP

mixture to agar wells. The antibiotic concentrations in each well were 100 µg/mL, 200 µg/mL, and 300 µg/mL for the AMX-NP2 nanoparticle and 100 µg/mL for both the amoxicillin free drug and the PCL-PVA nanoparticle. Results showed that the diameter of the zone of culture-growth inhibition was increased to the same extent (18 mm) in both the amoxicillin free drug and the amoxicillin encapsulation once in the *S. aureus* isolate. It decreased to 8 mm or less for both the *P. mirabilis* and *P. aeruginosa* isolates, as compared with amoxicillin free drug. The slightly smaller inhibition zones on the plates containing the *P. aeruginosa* and *P. mirabilis* isolates compared to those with *S. aureus* reflects the difficulty of the test compounds to diffuse across the extra outer membrane present in the gram-negative isolate (*P. aeruginosa, P. mirabilis*).

Samples	*S.aureus* Inhibition zone (mm)	*P.aeruginosa* Inhibition zone (mm)	*Proteus mirabilis* Inhibition zone (mm)
PCL-PVA NP	-	-	-
Amoxicillin free drug (100mg)	18	20	23
Amx-PCL NP2 (100 µg)	4	-	-
Amx-PCL NP2 (200 µg)	2	8	8
Amx-PCL NP2 (300 µg)	18	5	6
			

FIG. 7. **(A) Zones of inhibition (mm) of *S. aureus* isolate the first column with its plate image; (B) *P. aeruginosa* second column with the plate figure; (C) and the third is *Proteus mirabilis*.**

Our results were in agreement with those obtained by Ahymah Joshy [25], who found the antimicrobial activity of the amoxicillin lanthanum hydroxyapatite nano rods *against E. coli, S. aureus, Bacillus* and *Pseudomonas.* Our results were also in agreement with another study done by Sahoo et al. [18] using amoxicillin incorporated into the Poly(anhydride-amides) polymer, which showed that *S. aureu*s was the most susceptible organism to test compounds and showed more potent activity against gram-positive than gram-negative bacteria. The slightly smaller inhibition zones on the plates containing *E. coli* compared to those with *S. aureus* reflect the difficulty of the test compounds to diffuse across the extra outer membrane present in the gram-negative *E. coli*.

The MIC value was tested for the *S. aureus* clinical isolate at different concentrations (10 µg mL^{-1} to 320 µg mL^{-1}) of the amoxicillin free antibiotics, amoxicillin-PCL nanoparticle (AMX-NP2) and the void nanoparticle (PCL-PVA). Results in **FIG. 8** revealed a high rate of resistant towards amoxicillin free antibiotics at MIC_{320} µg mL^{-1}. While a significant inhibition in the growth of *S. aureus* when applying the AMX-NP2 nanoparticle at MIC_{160} µg mL^{-1}. Finally, no observed differences in the growth of *S. aureus* upon applying PCL-PVA void nanoparticle as compared to the control sample.

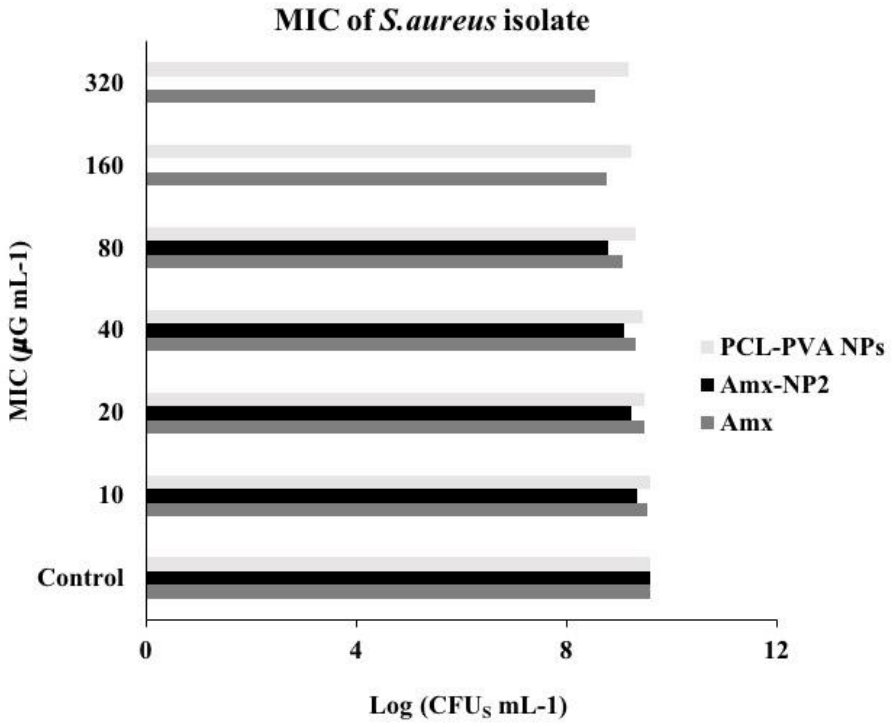

FIG. 8. **The MIC values of the amoxicillin free antibiotic, amoxicillin encapsulated NPs (AMX-NP2) and the void NPs (PCL-PVA) against cell forming units of *S. aureus* isolates at 37**°C **after 18 h of incubation.**

These results were in agreement with those of previous studies [26,27], which showed an improvement in antibacterial activity of amoxicillin loaded chitosan nanoparticle with MIC_{90} values compared with those of amoxicillin alone. Another study done by Ayala-Nunez et al. [27] showed a significant improvement in the antibacterial activity of amoxicillin antibiotic in a dose-dependent manner using silver nanoparticles against MRSA and non-MRSA *S. aureus.*

Such an attitude could be due to the smaller particle size that permitted to perfect interaction with the bacterial surface membrane and enhance the penetration process through their cell wall. This study shows that the effectiveness of amoxicillin -PCL nanoparticles in prolonging the life of amoxicillin antibiotics to which multiple drug resistance bacteria have acquired resistance.

Antibiofilm activity

The antibiofilm effect of the amoxicillin free antibiotic, AMX-NP2 and PCL-PVA NP against multiple drug resistance *S. aureus* isolate was studied using *in vitro* polystyrene microtiter plate technique **FIG. 9**.

Results revealed that applying amoxicillin free antibiotic (100 µg/mL), 76% of *S. aureus* isolate was able to produce biofilm. In contrast to the AMX-NP2, which shows an obvious reduction in biofilm formation upon increasing the AMX-NP2 concentration up to 100 µg/mL to reach 22.06% inhibition in biofilm formation. Result of quantification for biofilm biomass indicated that AMX-PCL NP2 was more effective in the eradication of preformed biofilm built by *S. aureus.*

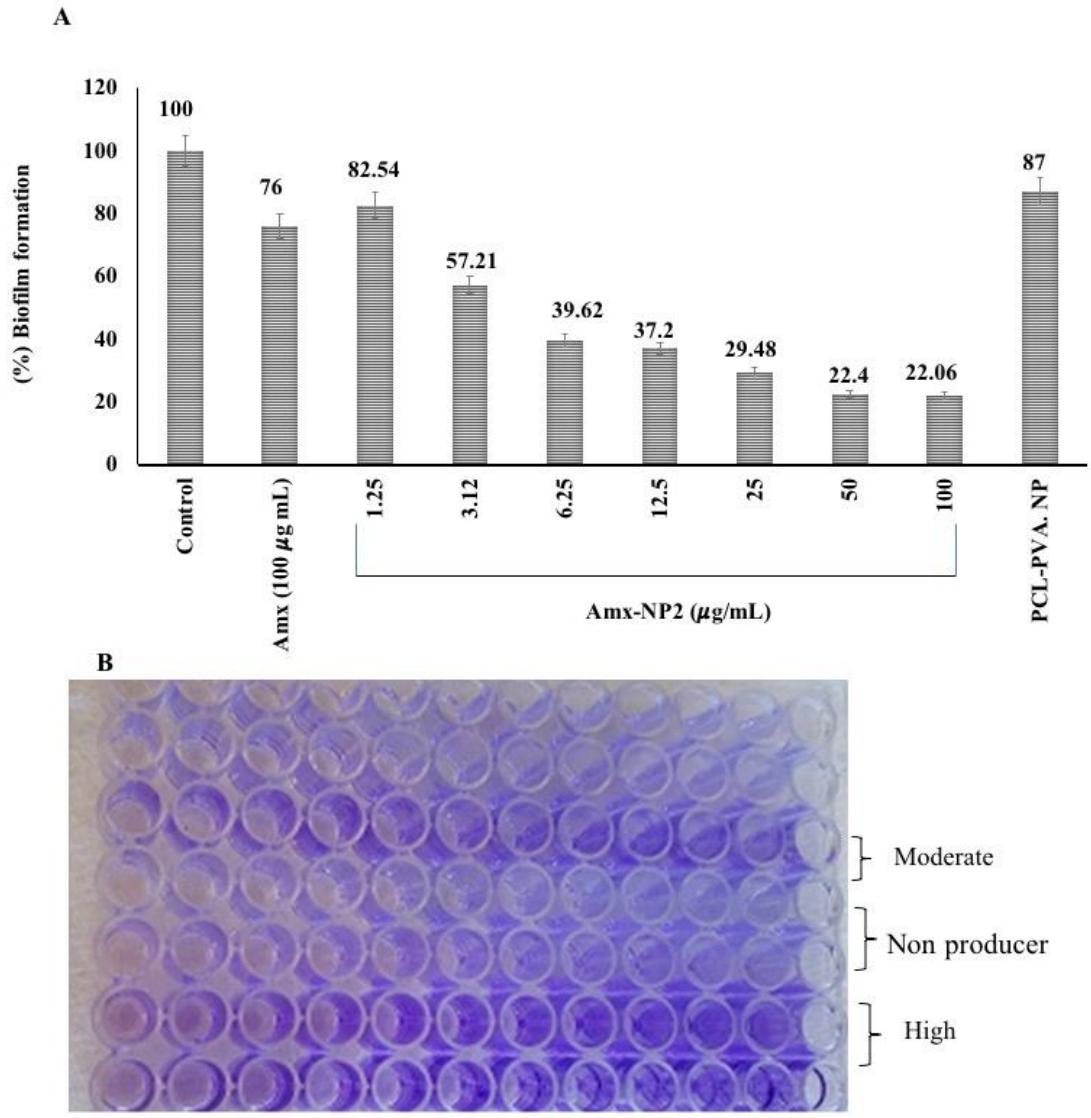

FIG. 9. **The biofilm formation (A) the inhibitory effects of AMX-PCL NP2 on *S. aureus* isolate; (B) Crystal violet assay to assess the antibiofilm activity of AMX-PCL NP2 *S. aureus.***

The effective of anti-biofilm activity attributed to the amoxicillin-nanoparticle combination, not to the effect of PCL-PVA NPs itself. These are promising outcome encouraging us for further studies to investigate an *in vivo* effect of the anti-biofilm activity at lower antibiotic concentrations. A similar study using amoxicillin with different Nps showed a synergistic antibiofilm affect against *S. aureus* at a lower concentration (40 µg/mL) than the antibiotic alone [28]. Another study by Rahim K et al. [29] reported that incorporation of the antibiotic with a nanoparticle led to an increase in the inhibitory effect of biofilm formation at certain degrees, yet, generally showed greater inhibitory activity than AMX-PCL NP2 or antibiotic alone.

Conclusion

Amoxicillin-polycaprolactone Nano formulations were prepared successfully by using the solvent evaporation method. The polymer-drug ratio influences the particle size as well as the drug release pattern of nanoparticles. The obtained nanoparticles are fine and free-flowing; the method followed is economical for the obtaining of reproducible nanoparticles, and the drug: polymer ratio has an impact on the drug encapsulation efficiency and *in vitro*. The yield was high and the encapsulation efficiency was good for all preparations but was highest for the AMX-NP2 Nano formulation.

Acknowledgments

The authors would like to thank the International Institute of Education- Scholar Rescue Fund (SRF-IIE) the USA and School of Medicine/Deakin University, Australia for hosting Dr. Maysaa as a postdoctoral fellow/academic visitor.

Conflict of Interest

The authors declare that they have no conflicts of interests.

Authors' Contributions

- Maysaa Chasib AL- Mohammedawi contributed for substantial designing, planning, data annotation, reporting and drafting the article. Tel: +61 478 795 442; E-mail: cmaysaa@gmail.com
- M Maysaa, AM Rana, JK Mokhtar, SB Enas, QA Duna, and AQ Samra contributing in antimicrobial susceptibility, reviewing project progress, revising the article and approving
- Samara Qaraghuli contributing to biofilm formation and drafting the article

REFERENCES

1. Liu H, Leonas KK, Zhao Y. Antimicrobial properties and release profile of ampicillin from electrospun poly (ε-caprolactone) nanofiber yarns. J Eng Fiber Fabr. 2010;5(4):0-19.
2. Patel VR, Amiji MM. Preparation and characterization of freeze-dried chitosan-poly (ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharm Res. 1996;13(4):588-93.
3. Chen C. Bioadhesive polymers for stomach targeted drug delivery. Proceedings of the 24th International Symposium on Controlled Release of Bioactive Materials. Stockholm. 1997.
4. Park H, Park k, Shalaby WS. Biodegradable hydrogels fBor drug delivery. 2011.
5. Sahoo SK, Labhasetwar V. Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. Mol Pharm. 2005;2(5):373-83.
6. Bernardi A. Selective cytotoxicity of indomethacin and indomethacin ethyl ester-loaded nanocapsules against glioma cell lines: an *in vitro* study. Eur J Pharmacol. 2008;586(1-3):24-34.

7. Hillaireau H, Couvreur P. Nanocarriers' entry into the cell: Relevance to drug delivery. Cell Mol Life Sci. 2009;66(17):2873-96.

8. Güncüm E, Işıklan N, Anlaş C, et al. Development and characterization of polymeric-based nanoparticles for sustained release of the amoxicillin-an antimicrobial drug. Artif Cells Nanomed Biotechnol. 2018;46:964-73.

9. Mora-Huertas CE, Fessi H, Elaissari A, Polymer-based nanocapsules for drug delivery. Int J Pharm. 2010;385(1-2):113-42.

10. Niwa T, Takeuchi H, Hino T, et al. *In vitro* drug release behavior of D, L-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate prepared by a novel spontaneous emulsification solvent diffusion method. J Pharm Sci. 1994;83(5):727-32.

11. Ramteke S, Maheshwari RB, Jain NK. Clarithromycin based oral sustained release nanoparticulate drug delivery system. Indian J Pharm Sci. 2006;68(4):479-84.

12. El-Shabouri MH. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. Int J Pharm. 2002;249(1-2):101-08.

13. Yeo Y, Park k. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res. 2004;27(1):1-12.

14. Atlas RM, Brown AE, Parks LC. Laboratory manual of experimental microbiology. 1995.

15. Jumaa T, Chasib M, Hamid MK, et al., Effect of the electric field on the antibacterial activity of Au nanoparticles on some Gram-positive and Gram-negative bacteria. Nanosci Nanotech Res. 2014;2(1):1-7.

16. Al-Moahmmedawi MC. Nanoformulation and antibiotic releasing property of cefotaxime nanoparticles.African J Biotech. 2016;15(14):539-48.

17. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163-75.

18. Sahoo SK, Panyam J, Prabha S, et al. Residual polyvinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release. 2002;82(1):105-14.

19. Ranjha NM, Khan IU, Naseem S. Encapsulation and characterization of flurbiprofen loaded poly (ϵ-caprolactone)-poly (vinylpyrrolidone) blend microspheres by a solvent evaporation method. J Sol-Gel Sci Tech. 2009;50(3):281-89.

20. Abdelmalek I, Svahn I, Mesli S, et al. Ormulation, evaluation and microbiological activity of ampicillin and amoxicillin microspheres. J Mater Environ Sci. 2014;5(6):1799-1807.

21. von Burkersroda F, Schedl L, Göpferich A. Why degradable polymers undergo surface erosion or bulk erosion. Biomaterials. 2002;23(21):4221-31.

22. Characklis WG. Bioengineering report: Fouling biofilm development: A process analysis. Biotechnol Bioeng. 1981;23(9):1923-60.

23. Bowen WR, Doneva TA. Atomic force microscopy studies of membranes: Effect of surface roughness on double-layer interactions and particle adhesion. J Colloid Interface Sci. 2000;229(2):544-49.

24. Hans ML, Lowman AM. Biodegradable nanoparticles for drug delivery and targeting. Curr Opin Solid St M. 2002;6(4):319-27.

25. Joshy MA, Elayaraja K, Suganthi RV, et al. in vitro sustained release of amoxicillin from lanthanum hydroxyapatite nano rods. Curr Appl Phys. 2011;11(4):1100-6.

26. Nguyen TV. Preparation of chitosan nanoparticles by TPP ionic gelation combined with spray drying, and the antibacterial activity of chitosan nanoparticles and a chitosan nanoparticle-amoxicillin complex. Res Chem Intermed. 2017;43(6):3527-37.

27. Ayala-Núñez NV, Villegas HH, Turrent L, et al. Silver nanoparticles toxicity and bactericidal effect against methicillin-resistant *Staphylococcus aureus:* Nanoscale does matter. Nano Biotechnol. 2009;5(1-4):2-9.

28. Srivastava N, Mukhopadhyay M. Green synthesis and structural characterization of selenium nanoparticles and assessment of their antimicrobial property. Bioproc Biosyst Eng. 2015;38(9):1723-30.

29. Rahim K, Mohamed AM. Bactericidal and antibiotic synergistic effect of nanosilver against methicillin-resistant *Staphylococcus aureus*. Jundishapur J Microbiol. 2015;8(11).