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Nano-Poly Chitosan-Ampicillin Drug: Synthesis, Characterization and Cytotoxicity

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Abstract

Natural Nano chitosan polymers were analysed and characterized by FT-IR, ^{1}HNMR, TEM, AFM, and XRD techniques. The Nano-chitosan was then combined with Ampicillin drug, which was synthesized by FT-IR, and 1HNMR. Cytotoxic activity of new Nano-Chitosan-Ampicillin drug was evaluated.

Keywords: Chitosan, Ampicillin, Nano co-polymer-drugs, cytotoxic activity

Introduction

Cancer is a leading global cause of millions of deaths annually [1]. However, the medical treatment of malignant tumors is not sufficient enough. So, the development of novel agents with reasonable therapeutics is the key topic with much concern [2]. Targeting the anticancer drugs including tumor-specific cell signaling, cell division, energy metabolism, gene expression, and drug resistance are essential to enhance the efficacy of antitumor drugs with lower toxicity. Heterocyclic compounds are considered as major components in chemical therapeutics. Thereafter, it is recommended to reduce the use of chemicals as much as possible to synthesize the targeted heterocyclic compounds. Natural sources are preferred to afford biocatalysts for the synthesis reactions [3-6].

In cancer chemotherapy, nano-medicines for administration have become highly relevant since the use of nano-particulated drug delivery systems has offered several advantages over conventional drug administration [7-9].

Metallic nanoparticles comprising nanoparticles have roles in biological and medical implications [10]. They comprise drug and targeted gene delivery and anticancer activity [11]. They supply many ways to control the release profile of encapsulated moieties. NPs can be prepared by many methods including laser ablation, microwave-assisted, chemical reduction, electro-deposition, solvo-thermal and green synthesis. Natural polymers such as chitosan possess advantages over their synthetic counterparts regarding inherent bioactivity. They are able to possess receptor binding ligands to cells with greater susceptibility to degradation due to cellular enzymatic action [12-14].

Chitosan is found in the cell walls of parasites, yeast, and the exoskeletons of arthropods such as insects, crabs, and shrimp. Chitosan is pH-dependent, non-toxic, anti-bacterial, easily bio absorbable, biodegradable, high molecular weight and biocompatible.[15, 16]

The biocompatibility and biodegradability of these polymers, accompanied by the ease of chemical modification and blending, allowed them to be essential platforms for the development of an impressive amount of research.[17] Chitosan showed promising results in the delivery of anticancer chemotherapeutics to the target tumor cells. Nano-chitosan loaded with therapeutics appears to be more stable, permeability, and bioactivity [18].

The main objective of this work is to synthesize Natural Nano chitosan polymers using ecofriendly methods to act as selective anticancer agents. In this approach, we are focusing on employing sono-synthesized Ampicillin nano-chitosan to contribute in this application.

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Materials and Methods

Materials

Low molecular weight chitosan (Mwt <100 kDa) was provided from Bio Basic Inc. (Canada). Sodium tripolyphosphate (TPP), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, USA. Acetic acid was of analytical grade.

Synthesis of Nano Chitosan Solution

Chitosan is slowly dissolved in 4% acetic acid solution via sonication. Deionized water added and a filter (0.22 mm) is used to get a final stock solution of 1% (w/v, 1 mg/ml). Sodium tripolyphosphate (TPP) dissolved in deionized water at 0.5 mg/ml concentration to get nano chitosan solution.[19-21]

Synthesis of Nano co-polymer-drug

Few drops HCl added to 30 mL THF to dissolve 0.03 moles polymer and drug then this mixture was subjected to reflection for 24h to afford a precipitate. This precipitate was washed by diethyl ether then 2.0 M NaOH and leave to dray for 16 h to be ready as Nano co-polymer-drug.[21]

Cytotoxicity Activity

Cell cultures Maintenance:

During passage, AMJ13 cells were maintained in RPMI-1640 containing 10% Fetal bovine serum, 100 units'/mL penicillin, and 100 g/mL streptomycin. A passage was performed with Trypsin-EDTA, the cells were weekly twice reseeded at 80% confluence, and at 37°C they were incubated .

Cytotoxicity inspection:

A 96-well plate was used for the MTT assay [22, 23]to measure the cytotoxicity of (X-SUBSTANCES). The cell line was seeded at 1 × 104 per well. The cells were treated with X-SUBSTANCES at a variety of concentrations after 24 hours or after a monolayer had been established. In order to determine cell viability, medium was removed after 48 hours of treatment, and 28 micro liters of MTT solution was added. Cells were incubated for 2.5 hours at 37°C with a 2 mg/mL MTT solution. To dissolve the crystals in the wells after removing the MTT solution, 130 l of DMSO (dimethyl sulfoxide) was added which made the incubation temperature increased for 15 minutes to 37 °C with vigorous shaking. Microplate reader at 492 nm was utilized to determine the absorbency. Triplicates of the assay were performed. The growth rate inhibition (cytotoxicity percentage) was calculated by utilization the equation below:

Rate of Inhibition =(A-B)/A*100

The optical density of the control is denoted by A and the optical density of the samples denoted by B. An inverted microscope was used to observe the shape of the cells, which were seeded into 24-well micro titration plates at a density of 1×105 cells mL−1 and incubated for 24 h at 37°C . X-SUBSTANCES were then applied to the cells for 24 hours. Staining the plates with crystal violet stain and incubating them for 10 to 15 minutes at 37°C followed the exposure period. We removed the dye stain gently with tap water after it was thoroughly washed off. Using an inverted microscope and a digital camera, images of the cells were captured under a magnifying glass at 100x magnification. An inverted microscope was used to observe the cells at 100x magnification, and a digital camera was attached to the microscope to capture images

Statistical analysis

A t-test was performed using Graph Pad Prism 6 to analyze the collected data statically. The results were expressed as the mean + SD of three measurements.

Results and Discussion

Characterization of the synthesized nanoparticles

To assess the reactive functional groups of the encapsulated nano-chitosan; Fourier transform infrared (FTIR) analysis was used within the range (400 - 4000 cm^{-1}) using Nicolet Avatar FTIR 370 CSI. In Nano Chitosan's, IR spectrum revealed absorption band at 1402.47 cm^{-1} of aromatic C=C, weak band at 3069.93 cm^{-1} and at 3049.95 cm^{-1} that related to alcoholic OH at 2970.22 cm^{-1} that assigned to aliphatic C-H, at 1671.7 cm^{-1} of ester C=O, at 1277.68 cm^{-1} of C-N and an absorption bond at 736.49 cm^{-1} that related to aromatic ring (Figure 1).

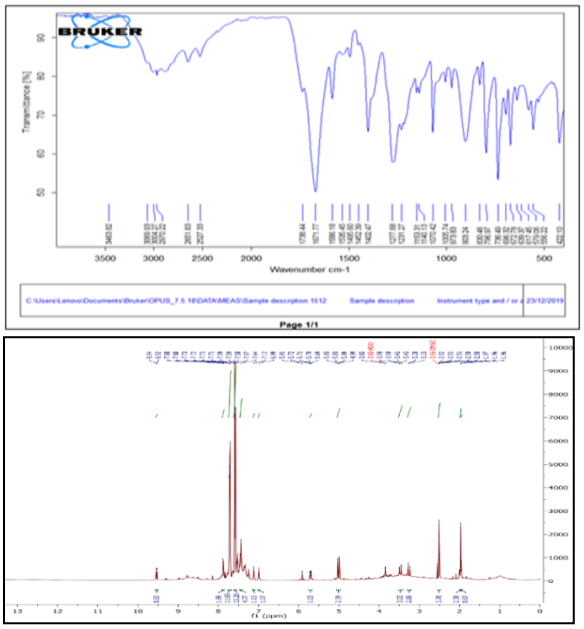


Figure 1. FT-IR and ^{1}HNMR of Nano Chitosan

While 1H NMR of Nano chitosan confirmed the structure; it exhibits a signal at 5.70 ppm for C=C-H and no signals of acidic OH at 9.5-13ppm. Also, it has signals at 7.58 ppm (aromatic C-H), and at 9.52 ppm (CHO), together with appearance of signal at 3 ppm (CH_3) (Figure 1).

Atomic force microscope AFM is used to measure particles size of Nano Chitosan (Figure 2), the particles outside surface has a roughness coefficient of 0.827 nm, with square root is 0.955 nm. According to figure 3A, the average particle height also was 3.30 nm. As a result of the test, Chitosan has a molecular size of 69.42 nm.

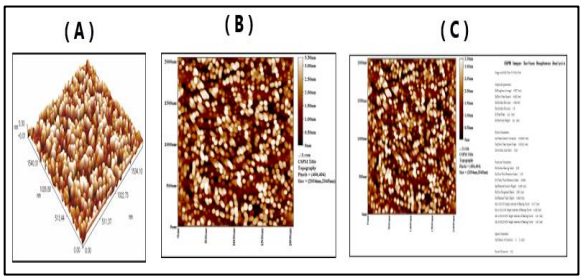


Figure 2. AFM technique (A) A photograph from a three dimensional point of view, (B) A photograph from a two dimensional point of view, and (C) A photograph from a two dimensional point of view with all detail

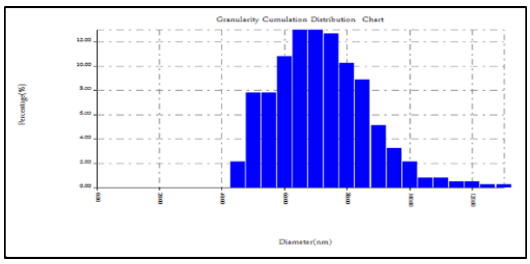


Figure (3): Nano chitosan particles size distribution

Avg. Diameter: 69.42 nm <=Diameter 10 %: 50.00 nm <=Diameter 50%: 65.00 nm <=Diameter 90 %: 85.00 nm

Table (1): AFM results report.

Volume (%)	Diameter (nm)<	Cumulation (%)	Volume (%)	Diameter (nm)<	Cumulation (%)	Volume (%)	Diameter (nm)<	Cumulation (%)
2.16	45.00	2.16	12.67	75.00	67.12	0.81	105.00	97.57
7.82	50.00	9.97	10.24	80.00	77.36	0.81	110.00	98.38
7.82	55.00	17.79	8.89	85.00	86.25	0.54	115.00	98.92
10.78	60.00	28.57	5.12	90.00	91.37	0.54	120.00	99.46
12.94	65.00	41.51	3.23	95.00	94.61	0.27	125.00	99.73
12.94	70.00	54.45	2.16	100.00	96.77	0.27	130.00	100.00

The XRD pattern of micro particle Chitosan appear the Peaks at values of 2θ (15.4°, 18.6°, 22.3°, 27.0°, 30.5° and 37.0°) in figure 4. Based on Bragg's Law, the average interplaner spacing between atoms (dhkl) was 0.385 nm using origin

$n\lambda = 2d\sin\theta$ ------- Bragg's Law software

According to Scherrer's equation, the total average crystallite size was 69.04 n

$D = k * \lambda / \beta cos\theta$ ----- Scherrer's equation

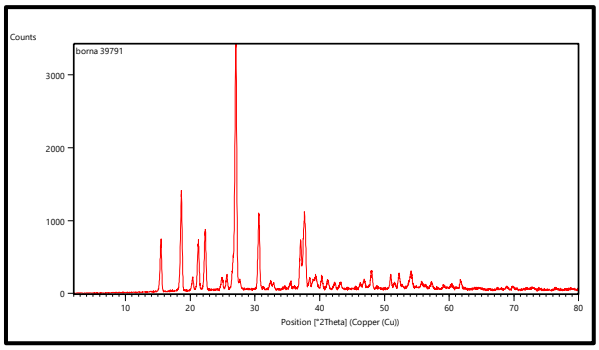


Figure 4. Chitosan nanoparticles x-ray diffraction

Table (2): Chitosan nanoparticles, crystallite sizes and atomic distances (d-spacing) within the nanoparticle

θ	2 θ	FWHM	d_{hkl} nm	D nm	d_{hkl} (Av.) nm	D(Av.) nm
7.733765	15.4675	0.1004039	0.5724151	79.849600	0.3851	69.0494
9.31007	18.6201	0.1136509	0.4761484	70.833825		
11.15343	22.3068	0.1176784	0.3982175	68.808052		
13.51585	27.0317	0.1302705	0.3295902	62.719995		
15.29801	30.5960	0.1189843	0.2919576	69.220243		
18.52677	37.0535	0.1332784	0.2424250	62.864653		

Figure 5 shows TEM images of the nanoparticles Chitosan, which illustrate different shapes such as the random arrangement of spherical shapes, rod shapes, annular disk shapes, and semi-spherical shapes in low and high resolution. Nano Chitosan particles have an average size of 69.81 nm, and table 3 demonstrates the proportions of angles, diameters, and standard deviations of the Nano Chitosan particles measured with image-j, while the histogram appear the proportions for the particle sizes distribution (Figure 6).

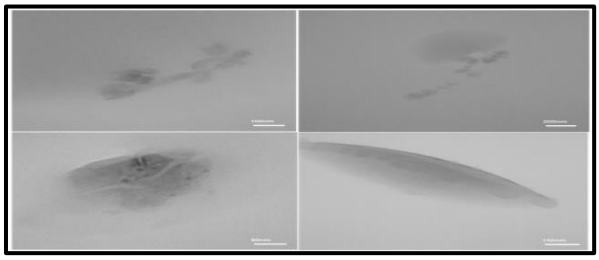


Figure 5. TEM images of Chitosan nanoparticles

Table (3): Diameters, standard deviations, and angles of Nano Chitosan

Area	StdDev	Angle	Diameter nm	D (av.) nm
34.921	33.674	-140.194	35.997	**69.819**
37.461	22.735	-43.958	37.419	
34.54	32.45	-124.061	37.671	
46.826	27.66	-62.403	47.898	
54.318	25.611	-93.013	55.544	
56.191	34.782	-33.147	56.906	
61.81	29.703	-41.269	63.12	
65.556	25.965	-17.904	66.511	
69.302	23.436	51.096	70.732	
72.112	21.01	-39.611	77.355	
80.096	22.558	-29.899	82.985	
89.905	32.591	-122.005	91.64	
89.905	39.161	-46.71	92.192	
102.08	47.598	-85.236	104.905	
109.572	28.902	4.436	112.62	
110.509	29.945	-54.405	112.798	

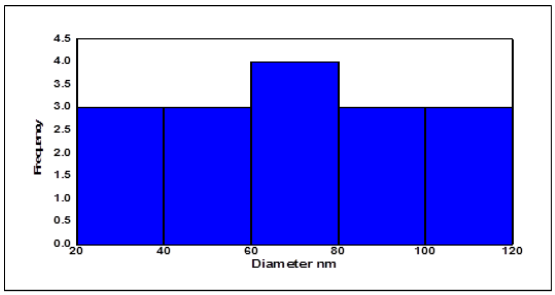
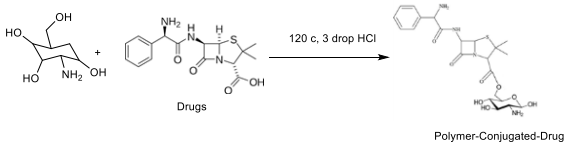


Figure 6. Histogram illustrating the size distribution of Nano chitosan particles

FT-IR and ^{1}HNMR were used to Characterized Nano Chitosan-Ampicillin Drug polymer. FT-IR showed absorption bands at 3350 cm^{-1} (NH_2), at 2922 cm^{-1} (aliphatic C-H), at 3074 cm^{-1} (aromatic C-H), at 1674 cm^{-1} (ester C=O), at 1555 cm^{-1} (CN), at 1150 cm^{-1} (C-O), at 1113 cm^{-1} (C-C) (Figure 7).

^{1}H NMR (600 MHz, DMSO,d_6) δ 8.24 (s, 2H, drug NH_2), 8.22 (s, 1H, NH), 6.42-6.37 (m, drug $CH_{arom.}$), 4.32-4.29 (d, 1H of CH^2), 4.14-4.11(d, 1H for CH^{22}), 3.11-3.10 (d, 1H, CH^{25}), 3.09 (s, 1H, CH^{18}), 3.08 (s, 1H, CH^{27}), 2.84-2.83 (d, 2H, CH^{11}), 2.81-2.60 (t, 1H, CH^6), 2.56 (d, 1H, CH^4) (Figure 8).



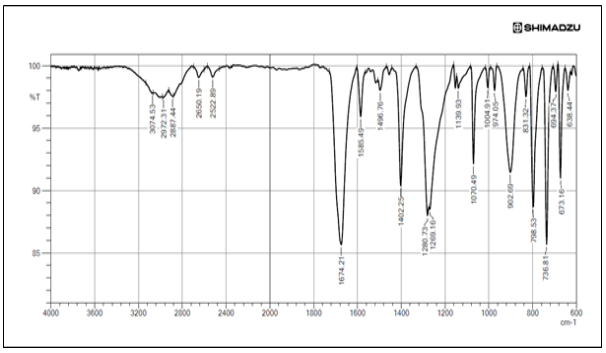


Figure 7. FT-IR spectra of the Nano Chitosan-Ampicillin drug

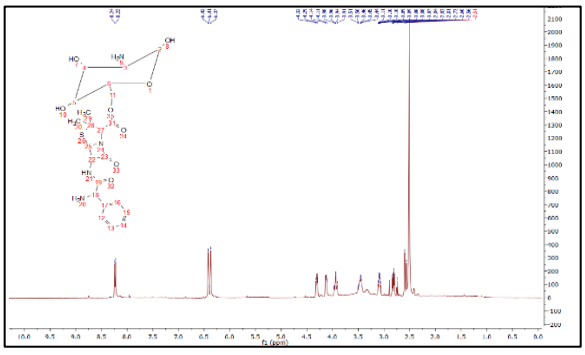


Figure 8. The ^{1}HNMR spectrum of the Nano Chitosan – Ampicillin

Cytotoxic Activity

The in vitro cytotoxicity of the synthesized nanocapsules was investigated towards human **AMJ13** (it is a new breast cancer cell line that has been established from a 70-year-old iraqi woman with a histological diagnosis of infiltrating ductal carcinoma) via standard established assays. The antiproliferative activities were expressed by median growth inhibitory concentration (IC_{50}). As shown in Figures 9 and 10, the in vitro antiprolefrative activity towards **AMJ13** cancer cell line was evaluated using SRB assay, the data indicated that ampicillin-linked chitosan nano polymer has a very strong biological activity that inhibits the proliferation of breast cancer cells.

The effectiveness of both compounds can be summarized as follows: Nano Chitosan with drug > Nano Chitosan without drug

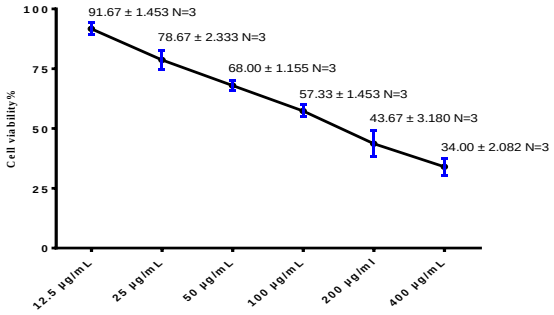


Figure 9. Cytotoxicity of Chitosan in AMJ13 cells IC50 =183.72 µg/ml

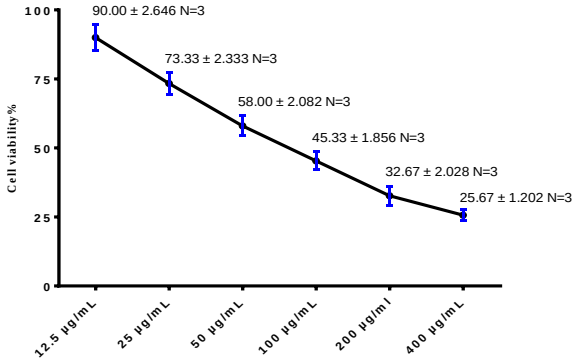


Figure 10. Cytotoxicity of co Nano Chitosan-Ampicillin in AMJ13 cells IC50=121.52 Mg/ ml.

Conclusion

The synthesis of ampicillin encapsulated nano-chitosan was successfully performed. Various techniques were employed to investigate the synthesized nanoparticles. They confirmed the formation of nanoparticles with spherical shape and good thermal stability. The efficacy of the synthesized nano-chitosan nanoparticles towards cancer cell line was investigated. The assessment of their cytotoxicity demonstrated their concentration-dependence through in vitro anti-proliferative activity in this cancer cell line. In conclusion, the tested compounds exerted an anti-proliferative activity on **AMJ13** cancer cell line

through reducing cell proliferation. It resulted in significant growth inhibitory effect. The data indicated that ampicillin-linked chitosan nano polymer has a very strong biological activity that inhibits the proliferation of breast cancer cells. This study has pointed out to the need of right selection in green organic compounds and methodologies for the synthesis of nanoparticles for different purposes with respect to the environment and human health.

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