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Original Article

Characterization of synthesized carboxymethylnanochitosan loaded with streptomycin and testing its *in vitro* anticancer activity

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ABSTRACT

This study aims to synthesize carboxymethylnanochitosn (CMNC) from synthetic nanochitosan (NC) which was produced from chitosan and encapsulated streptomycin(S) then characterized and bioassayed. A quantity of 2 mg of chitosan was selected for nanochitosan synthesis. Maximum absorbance (λ*max*) was determined for streptomycin (S), CMNC and CMNC-S were 280, 265 and 270 nm, respectively. The loading efficiency of CMNC with S was 90%. Fourier transform-infrared (FTIR) spectroscopy for CMNC and CMNC-S showed that absorption peaks at the same frequencies 3000 -3800 cm^{-1} with the change in stretching of the absorption percent, which increased sharply. Scanning electron Microscope (SEM) of chitosan, NC, CMNC and CMNC-S showed aggregation of CMNC and entrapment of S nanoparticles within CMNC. Results of atomic force microscope (AFM) images and analysis illustrated that the concentration 2mg/ 100 ml of soluble chitosan recorded a diameter 54.64 nm and the insoluble chitosan at the same concentration resulted in 105.52 nm. According to the process of CMNC preparation, the nanoparticles size achieved 35 nm. After loading CMNC with S, the average of the nanoparticles increased to 37 nm. Results confirmed the loading process of CMNC with S. Particle size distribution showed median particle size of CMNC recording 770 nm and for CMNC-S was 526 nm. The concentration 50µg/ml of CMNC-S reached 37.5% growth inhibition (GI%) against rhabdomyosarcoma (RD) cell line in comparison with 38.5% GI for streptomycin (100µg/ml) after 24 hrs of incubation.

Keywords: Nanochitosan derivatives, Streptomycin, Encapsulation, Cell- line, Growth inhibition%

INTRODUCTION

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action due to various problems such as low lifetime, poor bioavailability, *in vivo* low stability, solubility, intestinal absorption problems, therapeutic effectiveness, side effects, and exceed the safe therapeutic concentrations. In most cases, only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biological properties [1]. Streptomycin (S) is bactericidal antibiotic drug under aminoglycosides group and produced from *Streptomyces griseus* [2].

It is known to have toxic effects causing nephrotoxicity and neuroparalysis [3]. Nanotechnology applications in drug delivery systems have opened up new possibilities in direct-targeted effect and release of drugs [4]. Continuous release of drug can be achieved by encapsulating the active ingredient in a polymer matrix, thus, the dose and frequency of administration would be reduced [5-6]. Chitosan is a polyaminosaccharide with many significant biological like biodegradable, biocompatible, bioactive and chemical properties. All of these properties make chitosan and its derivatives widely used in many biomedical fields [7]. Carboxymethylated chitosan has received more attention because of its good water solubility especially low molecular weight, and it is convenient to be applied in medicine and pharmaceutics because of their ability to fit the neutral environment of the human body [8,9]. Carboxymethylchitosan (CMC) is prepared by means of carboxymethylation, as some of the –OH groups of chitosan were substituted by $–CH_2COOH$ groups. Therefore, the reactive ligands such as –COOH and $–NH_2$ groups are still amenable to chemical modifications to improve its physical properties for metal chelation and dye binding [10].

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The properties strictly influenced by experimental conditions of temperature and reactant concentrations. The presence of Carboxyl group in CMC chain enables its solubility in the neutral, acidic and basic medium. It is a highly soluble polymer. Another property of CMC was its moisture adsorption and retention. This was very prominent characteristic, which makes its importance high in the cosmetic industry and drug formation [11]. Among the colloidal systems, there has been increasing interest in self-aggregates or nanoparticles of amphiphilic polymers for biotechnological and pharmaceutical applications, due to their formation of nanosized vehicles with a hydrophobic core and hydrophilic shell [12].There are two methods followed up for the substitution. These are reductive alkylation method and direct alkylation method. The second method was the direct alkylation. In this method, the chitosan reacted with monochloroacetic acid. The isopropanol in water was used as a solvent. In this method, the O-Carboxymethyl and N-O Carboxymethyl were formed by varying the pH. At mild pH 8 to 8.5, the amino group active took part in reaction and formed N-Carboxymethyl [13]. Rhabdomyosarcoma (RD) is a malignancy that arises from skeletal muscle precursors [14]. It is the most common type of soft tissue sarcoma in children and adolescents less than 20 years old, with an incidence of 4.5 cases per million children/adolescents per year [15]. The current study aims to encapsulate the (CMNC) with streptomycin and examination their effect as antitumor against RD cell line.

MATERIALS AND METHODS

Preparation of nanochitosan (NC) **[16]:** Chitosan nanoparticles were prepared by ionic gelation method. Chitosan (0.2, 2 or 20mg) were weighted and dissolved in 100ml (2% glacial acetic acid) and mixed for 30 min at 4000 rpm using heat magnetic stirrer. A quantity of 17.8mg of Tripolyphosphate (TPP) was added in the ratio 5:2 (Chitosan: TPP). Instead of filtration, the mixture was centrifuged at 8000 rpm for 15 min in a high-speed cooling centrifuge. The supernatant was discarded and the deposit was washed twice with deionized water. The precipitate was lyophilized.

Preparation of CMNC [17]**:** Nanochitosan was used instead of the chitosan in the synthesis of CMNC and centrifuged at 14000 rpm instead of the filtration steps. The supernatant was kept in a deep freeze for subsequent steps.

Preparation of CMNCS**:** During the preparation of CMNC, streptomycin was supplemented to the reactants in a ratio of 1:5 (CMNC: S) and mixed well for 24 hrs at room temperature. Subsequent steps were followed as in the preparation of CMNC mentioned above. The supernatant was kept in a deep freeze for subsequent steps.

Loading efficiency% **[18]:** It was calculated according to the following formula:

Loading efficiency % = (Total amount of S added (mg) – Unbound S (mg) X 100Total amount of streptomycin (mg)

The amount of streptomycin encapsulated was determined after centrifuging the solution then the supernatant was analyzed for streptomycin content spectrophotometrically by measuring the absorbance at 280 nm using UV Spectrophotometer (BUCK, USA). Lambda maximum (λ_{max}) was determined for S, CMNC and CMNC-S by using 500 µg/ml in deionized water for each compound. Scanning wavelength was extended from 100 nm to 850 nm. Standard curves were determined for S, CMNC and CMNC-S by preparation of serial concentrations 50- 1000 µg/ml. Absorbency (α) for each compound was determined by calculating the slope (tan θ) from the standard curve**.**

Characterization

Fourier transform-infrared (FTIR) spectroscopy**:** FTIR spectra was implemented under dry air at room temperature within the wave number range of 4,000–500 cm^{-1} for analysis of CMNC.

Scanning electron microscope (SEM)**:** It was accomplished using INSPECT S50 device (UK).

Atomic force microscope (AFM)**:** The surface morphology of the nanoparticles was observed by using atomic force microscopy (AFM).

Particle size distribution**:** Dynamic light scattering (DLS) was used to measure average particle size and size distribution (polydispersity index) of formulated nanoparticles. The mean particle size of CMNC and CMNC-S loaded nanoparticles were determined at 25 °C using the 90Plus Particle Sizing Software Ver. 5.35, Brookhaven Instruments Corp.

In Vitro **anticancer activity:** The anticancer efficacy of S, CMNC and CMNC-S against (RD) cell line was evaluated. The colorimetric cell viability MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used [19,20]. A liquates of 100 µl of RD cells (10^6 cell/ml) were dispensed using 96-well tissue culture plates. Two concentrations of S, CMNC, CMNC-S (50 and 100µg/ml) were prepared by dissolving in sterile distilled water (DW). A liquates of 100 µl of various concentrations were added to each well and incubated at 37◦C for 24 hrs. After the incubation, 10 µl of MTT solution (5mg/ml) was added to each well and incubated at 37◦C for 4 hrs. Finally, 50µl of DMSO (dimethyl

sulfoxide) was added to each well and incubated for 10 min. RD cells were cultured in complete medium without S, CMNC and CMNC-S as a positive control (AC), and in a complete medium without cells and test solutions as a blank. The absorbance was measured for each well at 620nm using an ELISA reader. Percentage of inhibition ratio was calculated according to the formula:

$$\text{Inhibition } (\%) = \left(\text{AC - } AS \text{ / AC} \right) \times 100$$

Where AC and AS are the optical density for positive control and test samples, respectively.

RESULTS AND DISCUSSIONS

A quantity of 2mg of soluble chitosan was selected for synthesis NC based on the quantity produced, the speed of the separation, proportion to the amount of TPP used and to the results of AFM.

Loading efficiency % of CMNC with streptomycin: Figures (1, 2) illustrate the λ*max* and standard curves for S, CMNC and CMNC-S which were used to determine the loading efficiency %. Figure (1a, b, c) shows λmax of the S, CMNC and CMNC-S. Streptomycin recorded 280 nm while the least absorbance was recorded in CMNC 265 nm. The highest absorbance occurred in CMNC-S 270 nm. The high absorption value of the S is due to the presence of a large number of interrelated rings and active groups. These active groups were manifested in the CMNC alone. When the S was loaded in CMNC, this led to the disappearance of several active groups on the S and thus low absorption appeared. As a result of loading, CMNC-S stabilization was less than the S and higher than the CMNC. The absorbency (α) values for S, CMNC and CMNC-S were calculated from standard curves which were 0.002, 0.004 and 0.006 (Abs.ml/ µg) respectively. The loading efficiency% of S was 90% according to the equation of Chopra *et al.*[18].

Characterization

***Fourier transform-infrared (FT-IR) spectroscopic studies*:** No noticeable differences between results of analyses of CMNC and CMNC-S and the absorption peaks located at the same frequencies 3000 -3800 cm $^{-1}$ with the change in stretching of the absorption percent that increased sharply (figure 3, B). FTIR spectra of CMNC displayed a weak peak at 1431.1 cm^{-1}which could be assigned to the symmetrical COO− group stretching vibration. The asymmetrical stretching vibration of the COO− group near 1550 cm^{-1} overlapped with the deforming NH_2 vibration at 1600 cm^{-1} exhibiting a very strong peak. The broad peak in CMNC at 3200–3700 cm^{-1}may due to both O–H and N–H stretching vibrations. FTIR spectral scanning of the CMNC-S showed a wide strong absorption peak at 3000–3800 cm^{-1} which associated with the O–H stretching from the inter and intramolecular hydrogen bonds as approved by Gilbert [21]. These results confirm the encapsulation of S within CMNC

***Scanning electron microscope (SEM) morphology*:** Figure (4a, b, c, d) shows SEM three-dimensional morphological structures of chitosan(C), NC, CMNC and CMNC-S were shown in (figure 4). Fig. (4a) showed the fibril structure of chitosan and Fig. (4b) showed the nano-spherical structure of nanochitosan which were reported by Ahmed [22]. Fig.(4c) displays an aggregation of CMNC because of their gelation properties. Fig. (4d) illustrates engulfment of streptomycin (S) by CMNC and it was embedded as nanoparticles. The low percent of antibiotic was loaded within CMNC.

***Atomic Force Microscopy (AFM)*:** In the processes of producing nanochitosan particles, the concentration of chitosan was variable and the concentration of TTP was constant due to productivity. Analysis results of AFM illustrated that the concentration 2mg/100 ml of soluble chitosan gave 54.64nm average diameter and 90% of nanoparticles with a diameter of 80 nm. Insoluble chitosan at concentration 2mg/100 ml recorded a mean diameter of 105.52 nm and 90% of nanoparticles with a diameter of 170 nm. Concentrations of soluble chitosan 20 mg/100 produced nanoparticles almost similar to the concentration 2mg/100ml but the process of producing NC was incomplete because of high concentration of chitosan and constant concentration of TPP that precipitated at the beginning (Figure 5a,b,c,d).

Figure (6a, b) shows 3D AFM images and analysis of CMNC and CMNC-S. According to the process of CMNC preparation, nanoparticle size with mean diameter 35 nm and 90% of 56 nm diameter was synthesized. After loading with antibiotic(S), the average nanoparticles size increased up to 37 nm and 90% of particles diameter reached 62 nm. These results confirmed the loading process of CMNC with S.

***Particle size distribution*:** Figure (7a, b) illustrates the particle size analysis of CMNC and CMNC-S. The natural texture of the products is gelled, so there was a pool of produced nanoparticles that had a significant direct effect on the measurement of nanoparticles.The median particle sized of CMNC was 770 nm and for CMNC-S was 526 nm. This result is not compatible with results of AFM analysis which indicated lower diameters and

considered more reliable because of the precision 3D scanning of samples. Approximate sizes were obtained by Chopra *et al*. [18]. The particle diameter (z-average) for the streptomycin loaded chitosan-alginate nanoparticles was 328.4 nm (Fig. 3). It is noteworthy that the hydrodynamic diameter of the particles measured by light scattering is higher than the size estimated from microscopy particularly because of the high swelling capacity of chitosan-alginate nanoparticles. Hence, the actual diameter of these particles can be assumed to be significantly smaller than this.

***In vitro* bioassay:** The best treatment was CMNC-S of 50 µg/ml where the growth inhibition (GI) of 37%, compared with 23% of the S at the same concentration and approaching to the concentration 100mg/ml of S (table 1). The loading efficiency of CMNC with S was 90%, indicating that the real concentration 45mg/ml of S was loaded. GI% for CMNC-S (100mg/ml) was close to 50 mg/ml and this may due to the mechanism of releasing S which almost at the same rate within only 24 hrs of culture incubation. Therefore, increasing the incubation period will increase the rate of inhibition due to the slow release of the S, that referred by Ahmed and Hussein [22, 23]. Figure (8a,b) illustrated the growth inhibition of RD cell line treated with CMNC-S after 24hrs of incubation (b) in comparison with the control treatment (a).

CONCLUCTIONS

Production of nanomaterials from biological sources that do not have any side effects on human health and used as drug delivery was achieved. Production of nanochitosan was used to synthesize the CMNC and the best concentration was 2mg/100ml to obtain nanoparticles. The results of the characterization showed the encapsulation of streptomycin in the CMNC. The CMNC succeeded in loading the streptomycin by 90% and achieved a growth inhibition of RD cell-line more than the streptomycin with the same concentration of 50µg/ml. Results of inhibition were recorded after 24 hrs of incubation. It is known that the release of the drug entrapped in the technique of encapsulation is slow so it is expected to increase the efficiency of inhibition as the time of incubation increase.

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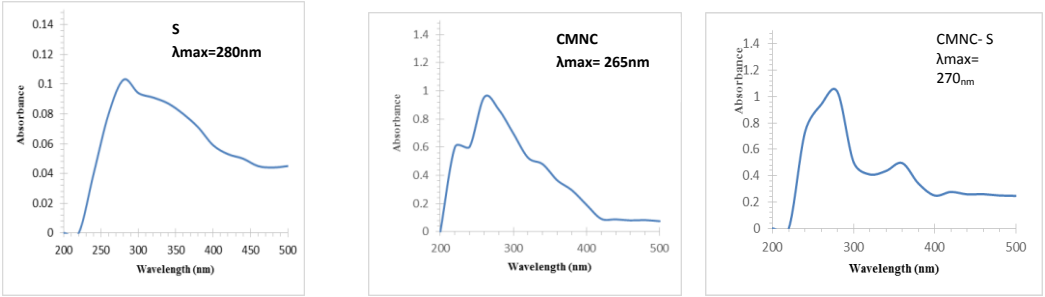
***Table 1: In vitro bioassay of CMNC and CMNC-S against RD cell line*.**

Treatment*(µg/ml)	**Mean**	**S.D**	**GI%**
S (50)	0.175	0.038	23
S (100)	0.140	0.066	38.5
CMNC (50)	0.212	0.045	6.8
CMNC (100)	0.181	0.008	20.6
CMNC-S (50)	0.142	0.012	37.5
CMNC-S (100)	0.147	0.014	35.5
Control	0.228		

** S: Streptomycin; CMNC: Carboxymethyl nanochitosan.*



a **b** **c**

Figure 1: UV- visible spectra of S, CMNC and CMNC-S compounds.

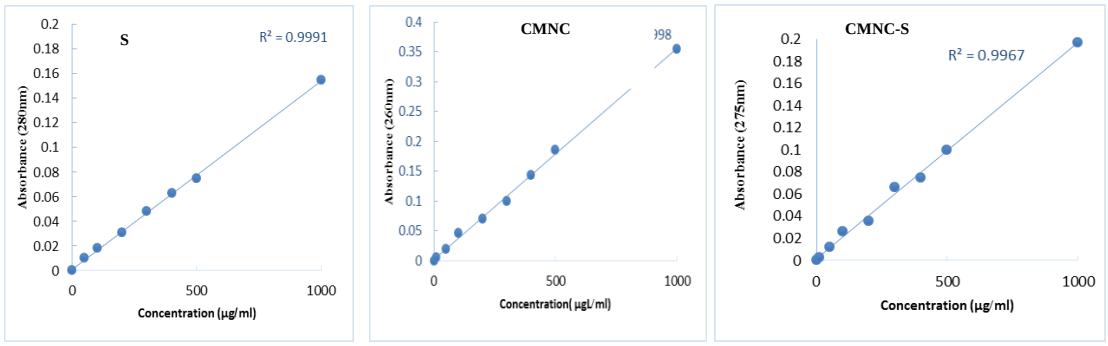


Figure 2: Standard curve of S, CMNC and CMNC-S.

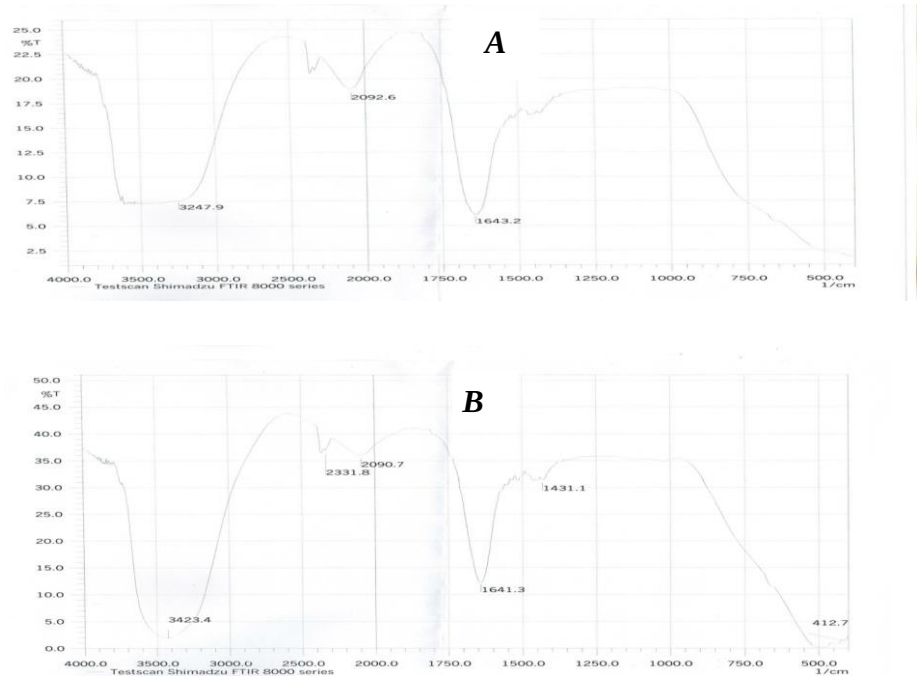
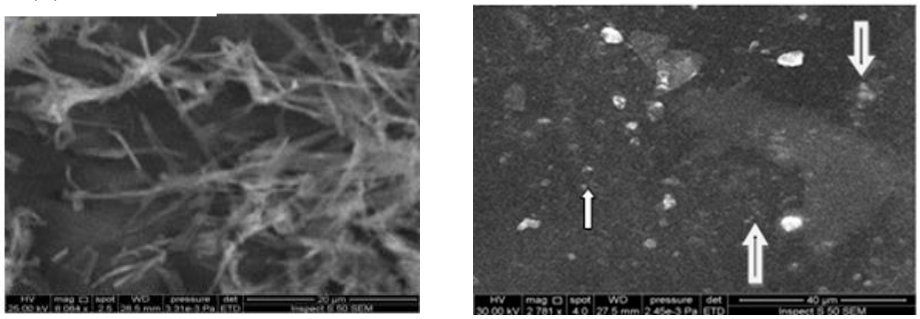
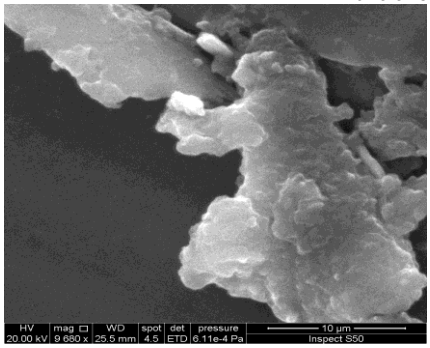


Figure 3: Fourier transforms infrared (FTIR) spectra. A: CMNC, B: CMNC-S.

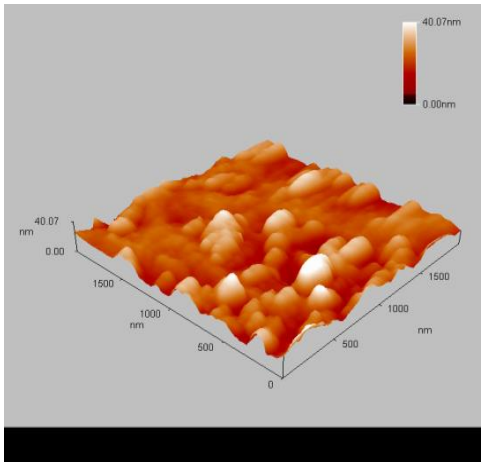
a (C) ***b (NC)***

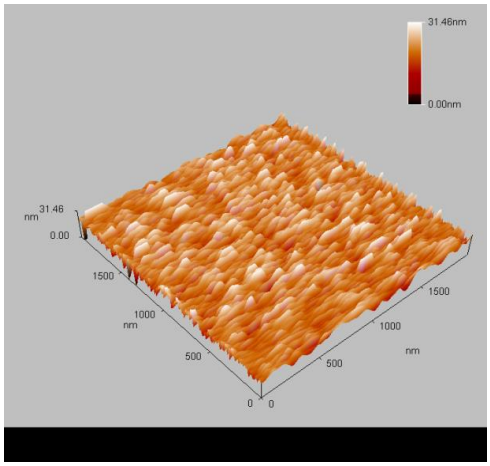




c (CMNC) ***d (CMNC-S)***

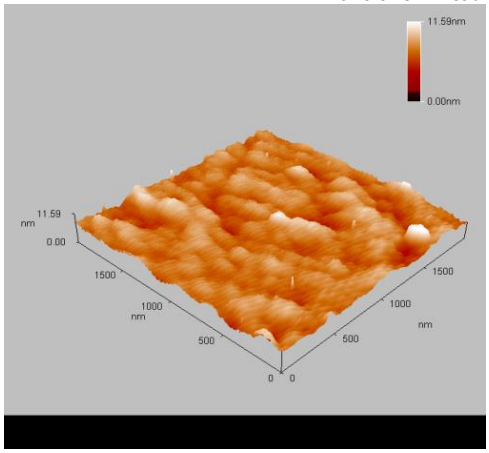
Figure 4: SEM image, [a: Chitosan structure with magnification (20µm) using SEM ; b: Nano-chitosan with magnification (40µm) Ali, et al., 2016)]; c: CMNC (10µm); d: CMNC-S.





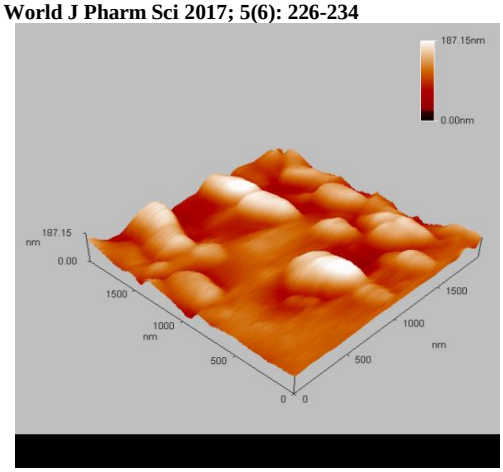
a (0.2mg soluble chitosan)
Avg. Diameter: 86.49 nm
<=10% Diameter: 35.00 nm
<=50% Diameter: 75.00 nm
<=90% Diameter: 140.00 nm

c (20 mg soluble chitosan)
Avg. Diameter: 59.18 nm
<=50% Diameter: 55.00 nm
<=50% Diameter: 55.00 nm
<=90% Diameter: 80.00 nm



b (2 mg soluble chitosan)

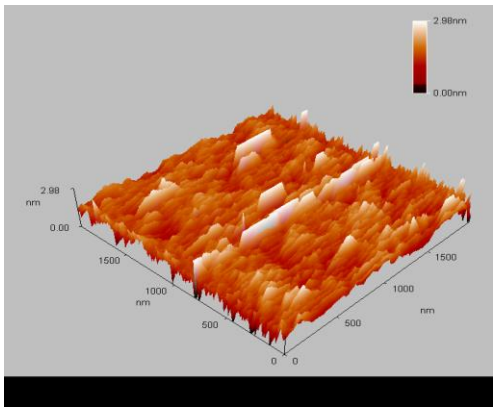
Avg. Diameter: 54.64 nm
<=10% Diameter: 25.00 nm
<=50% Diameter: 50.00 nm
<=90% Diameter: 80.00 nm



d (2 mg insoluble chitosan)

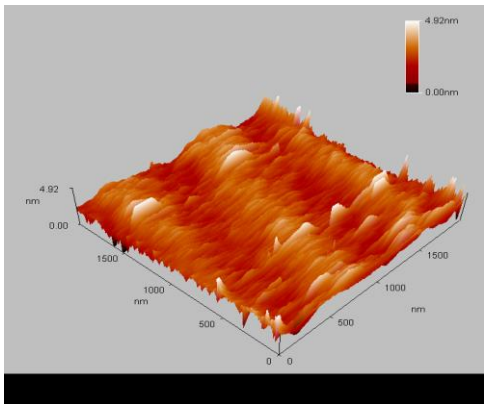
Avg. Diameter: 105.52 nm
<=10% Diameter: 40.00 nm
<=50% Diameter: 90.00 nm
<=90% Diameter: 170.00 nm

Figure 5: Atomic Force Microscopic (AFM) 3D images of nanochitosan prepared by different concentrations and solubility, a: 0.2mg of soluble chitosan, b: 2 mg soluble chitosan, c: 20 mg soluble chitosan, d: 2 mg insoluble chitosan.



a (CMNC)

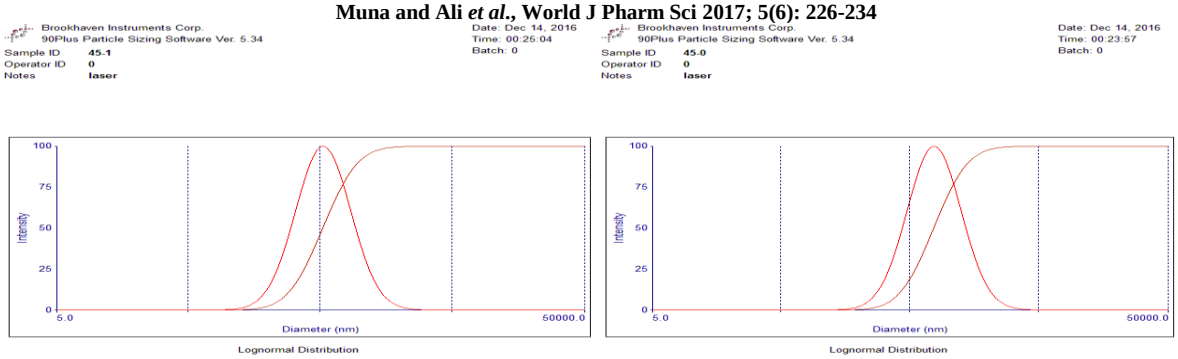
Avg. Diameter:35.76 nm;
<=10% Diameter: 18.00 nm;
<=50% Diameter: 32.00 nm;
<=90% Diameter: 56.00 nm



b (CMNC-S)

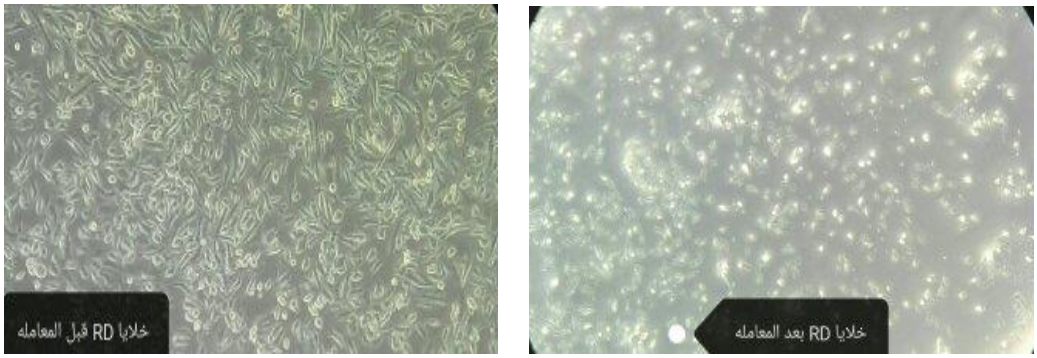
Avg. Diameter:37.39 nm
<=10% Diameter: 18.00 nm;
<=50% Diameter: 32.00 nm;
<=90% Diameter: 62.00 nm

Figure 6: Tapping-mode AFM image and three-dimensional height image of self-assembled nanoparticles formed by carboxymethylnanochitosan encapsulated streptomycin, a: CMNC; b: CMNC-S.



a: CMNC (Median diameter: 770nm) ***b: CMNC-S (Median diameter: 526nm)***

Figure 7: Particle size distribution of CMNC and CMNC-S.



a (Control) ***b (treatment)***

Figure 8: RD cell line treated with carboxymethynanochitosan- streptomycin after 24 hrs.

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