Synthesis of Novel Chitosan-Grafted-Derivatives Nano Cationic Polymers as Antitumor Agents

Synthesis of Novel Chitosan-Grafted-Derivatives Nano Cationic Polymers as Antitumor Agents

H S. Al-Lami[1] M H Zaboon[1] and A A. Saleh[2]

[1]Chemistry Department, College of Science, Basra University/Basra -Iraq
[2]Pathological Analyses Department, Science College, Basra University/Basra-Iraq

hadisalman54@yahoo.com

Abstract. Chitosan obtained from isolated chitin of shrimp shells by a modified chemical method with a high degree of deacetylation to improve its solubility. This was undergoing a graft copolymerization process to prepare acetyl chitosan (NACS), acetyl chitosan grafted polylactide (NACS-g-PLA), chitosan grafted polylactide (CS-PLA), and polylactide chitosan grafted poly(ethylene glycol) (CS- PLA-g-PEG) nanoparticles. They characterized by FT-IR and 1HNMR. Chitosan and its derivative nanoparticles morphology examined using SEM and average nanoparticle size counted by ImageJ program. After treatment with chitosan derivative nanoparticles (1 mg/mL) at various time intervals (24, 48 and 72 hour) for three different models of human breast cancer cell lines which are BT, MCF-7 and SKBR3 cell lines, the cell proliferation, cell viability percentage, and genotoxicity as a DNA fragmentation index (%DFI) were analysed by MTT assay, and flow cytometry techniques. The results displayed that CSNPs and its derivatives NACS-g-PLA, CS-g-PLA NPs, and CS- PLA-g-PEG, have strong antitumor activities by inducing *in vitro* treated BT, MCF-7, and SKBR3 cell lines as a highly significant effect, ($p < 0.001$) on cell proliferation growth as observed with untreated control cells in a different pattern. Furthermore, it can be said since genotoxicity results that prepared polymers NPs were considered as slight /or no effect on the nucleic material of the BT cell lines, as demonstrated as %DFI in compare with positive and negative control samples.

1. Introduction

The main motivation for the development of bio-based polymers is their biodegradability, and this becomes important due to strong public concern about waste reflecting recent changes in the polymer industry. The sustainability of bio-based polymers allows them to use for general and engineering applications [1].

Ongoing increases in the design of *in vitro* and *in vivo* applications for gene delivery based on polymers are the chemical modification of the cationic polymers in such a way that they can overcome all the barriers in the body before reaching the site of action. Today, non-viral systems such as DNA transposons being actively developed as biomedical tools. DNA-based non-viral vectors and discusses the toxicity related to the utilization of these vectors during gene therapy [2].
Gene therapy is of great interest, and polymer delivery systems considered as a safer and more effective pathway for therapeutic genes. Cationic polymers have been were improved to increase the

efficiency of DNA transfer. The systems will provide an effective tool for gene therapy in the future [3].

Polymeric nanoparticle systems are useful in biomedical applications for many important scientific reasons; they are easy to prepare from well-understood polymers [4] and have high stability in biological fluids as well as during storage [5-7]. They are also the most used in the field of tumors as an anticancer treatment. In recent times, the nanoparticle's gene delivery system becomes more effective and target specifically that more surface-specific research is going on about extensively. In this context, cationic polymers have shown a new light because of their significant property to form aggregates [8, 9]. The future trends of polymers have enormous potential for new important applications.

This study describes the preparation of a series of chitosan nanoparticle derivatives and their characterization and concentrate on their *in vitro* cytotoxicity against three models of breast cancer cell lines using MTT assay and genotoxicity test as a DNA fragmentation method using flow cytometry technique. It is also focused on the therapeutic potential in cancer treatment using chitosan-based nanoparticles.

2. Materials and Methods

2.1 Materials

Pure chitosan with a high degree of deacetylation (DD=92.1) is achieved by alteration of chitin extracted from the shrimp shells as reported in the literature [10].

Different companies provided all the chemicals used, The solvents, methanol, ethanol, acetic acid, and other chemicals like acetyl anhydride, L-lactide, and poly(ethylene glycol) with 1000 g/mole in molecular weight are utilized as obtained. Toluene was dried by sodium wire for 24 hours, and then it was filtered and distilled.

2.2. Methods

2.2.1. Synthesis of N-acetyl chitosan (NACS)

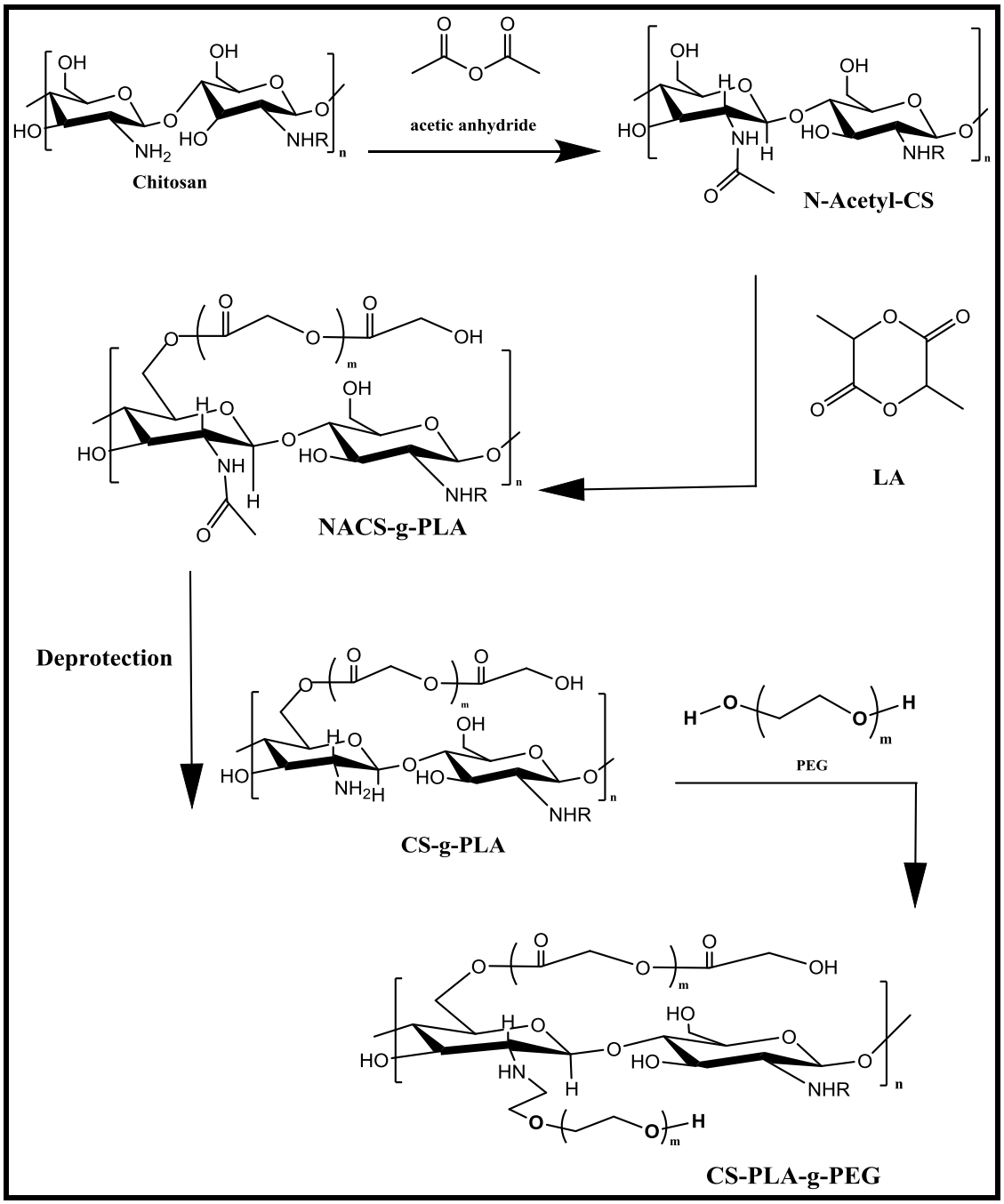
Two grams of Chitosan were dissolved in (50 mL) 2% aqueous acetic acid, and then it was diluted by adding 250 ml methanol. Acetic anhydride was added to the solution with strong stirring for 2 h, and then the mixture poured into dilute aqueous 10% NaOH solution. The precipitated polymer was filtered and washed well with ethanol, then with acetone, and after that, it was dried in the vacuum desiccator [11,12]. The white powder product of (NACS) obtained with an 85% yield. Scheme (1) represents the chemical reaction.

2.2.2. Synthesis of N-acetyl-chitosan grafted Polylactide

Two grams of lactide are dissolved in (100 ml) dry toluene, and then the volume was reduced to (50 mL) by distillation, followed by adding (1 g) of N-acetyl-CS, and the mixture was refluxed for 6 h under a dry nitrogen atmosphere. After cooling to room temperature, the mixture poured into a beaker containing (40 mL) of acetone to wash away the unreacted LA monomer and the PLA homopolymer may form [13]. The product filtered and dried in the vacuum desiccator. The powder result of (NACS-g-PLA) was acquired with an almost 78% yield. Scheme (1) is showing the chemical equation of the grafting process.

2.2.3 Synthesis of chitosan grafted polylactide (CS-g-PLA)

One gram of N-acetyl-chitosan-g-polylactide is dissolved in (20 mL) of hydrazine, and then the solution heated up at 100°C for 2 h to remove the protection given by the acetyl groups. The collected product washed and filtered thoroughly with water and ethanol respectively [13]. The dried yellow powder product of (CS-g-PLA) was obtained with a 75% yield, Scheme (1).



Scheme (1): Chemical grafting reactions of the synthesized Chitosan derivatives.

2.2.4 Synthesis of chitosan-polylactide grafted poly(ethylene glycol) (CS-PLA-g-PEG)

Two grams of poly(ethylene glycol) (MWT 10000 g/mole) were dissolved in 100mL dry toluene, after that the volume was reduced to 50 mL by distillation. This solution was added to (1 g) of chitosan-g-polylactide and the stirred mixture refluxed at 110°C for 3 h under a dry nitrogen atmosphere [10]. The chemical reaction showed in Scheme (1). The mixture was left to cool down to ambient temperature, and then the product was filtered and washed with diethyl ether several times. Afterward, it was dried in the vacuum desiccator. The white powder product of (CS-PLA-g-PEG) was obtained with an 86% yield.

2.3 Nanoparticles preparation of chitosan and its derivatives

Chitosan and Chitosan derivatives set up by dissolving 25mg of each in 25 ml of 2% acetic acid solution in a pH≈3.0. The solution left stirring under heat at 50 ºC for an overnight, then the mixture is scattered by subjection to the ultrasonic instrument for 5 min at 50 W with a pulse of 5sec and 10sec between pulses, to perform the nanoparticles of the polymer [14].

2.4 SEM measurements

The morphology and size of polymer NPs are defined using a ZEISS Scanning Electron Microscope, SEM, by transferring an aliquot of a watery suspension of polymer NPs onto a carbon covered copper matrix and permitted to be air-dried. The grid then scanned and adjusted at 100 kV. ImageJ program used to estimate the mean size of the total account of each polymer NPs.

2.5 The cytotoxicity assay

The cytotoxicity assay was evaluated in three different types of human breast carcinoma cell lines which are MCF-7, BT and SKBR-3, protected in an appropriate vehicle for each sort, at 37ºC in a moist 5% CO_2 environment. The cells cultured in a 96-well plate (7500 cells/well) and permitted to grow for 24 hours. Then 1mg/mL of each polymer nanoparticle solution applied to the already designated wells and further incubation carried out in 24, 48, and 72 hours respectively. Afterwards, the viability of the tested cells estimated using MTT. Absorbance at 550 nm measured with the Tecan plate reader, and reference at 620 nm. The cell viability of untreated cells (positive control) took as 100% cell viability. All transfection and cytotoxicity assays performed in triplicate [15].

2.6 Genotoxicity assay

Chitosan and its derivative nanoparticles induced apoptosis was affirmed by flow cytometry utilizing a commercially accessible Acridine orange apoptosis detection kit (Bio-Vision), on BT breast cancer cell lines, as a % DNA fragmentation index (% DFI).

The BT breast cancer cell lines (2×105 cells/ml) was cultured in RPMI medium containing 20% Fetal bovine serum FBS+ insulin, at 10 ml per Petri dish. Upon the formation of a monolayer of cells, 100 µl of a concentration (1mg\ ml) for each chitosan or chitosan derivatives nanoparticles added. After 24 hours of incubation, cells gathered by the addition of trypsin, then centrifuged at 1000x for 5 min, and lastly washed with Phosphate buffer saline PBS. Cells stained according to the kit's protocol and analyzed. The samples incubated and analyzed by Calibur Flow Cytometer. The Cell Quest software and MOdFit software were used to determine (% DFI). In this study, the negative controls also maintained against the positive controls [16]. The determinations performed in duplicates. Following 24 hours of brooding, centrifuged for 5 min at 1000x.

2.7 Statistical analysis

All samples were assessed in triplicates and were presented as (means ± SD). For statistical analysis, the variance (ANOVA) test was used to assess the significance between the groups. Differences were regarded as statistically highly significant if $P < 0.001$.

3. Results and Discussion

3.1 Characterization of chitosan and its derivatives

3.1.1 FTIR characterization

Chitosan used as a base material to prepare some distinctive polymeric derivatives in this work. The FTIR spectra of Chitosan and all their prepared grafted derivatives were recorded using Shimadzu spectrometer. Chitosan exhibits broadband in the FTIR with a peak at 3429 cm^{-1} due to (-OH) stretching and a (-NH) stretching, Figure (1). Nearly the disappearance of the band at 1639 cm^{-1} belong to amide I, and at the same time, the appearance of a strong band at 1454 cm^{-1} attributed to (C-N) stretching bond validated that chitosan with a high degree of deacetylation was obtained from the deacetylation process [17,18].

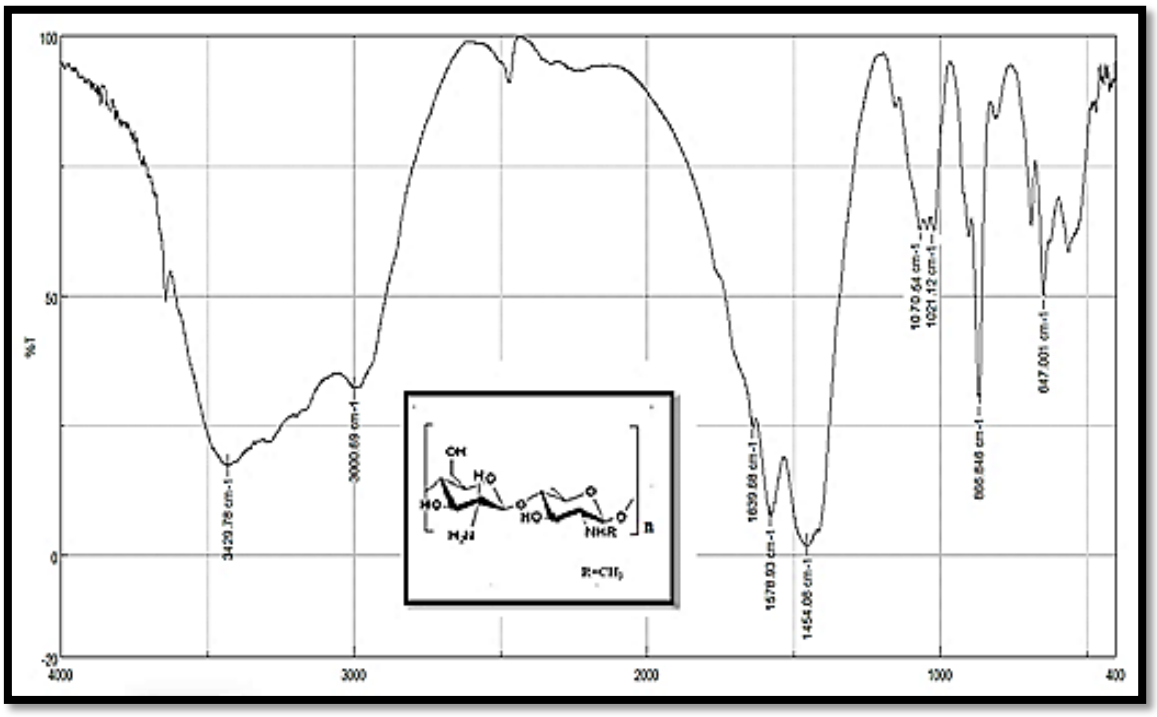


Figure (1): FTIR spectrum of chitosan.

Examining NACS FTIR spectrum revealed that the presence of a new peak at 1733 cm^{-1} assigned for C=O stretching band of ester formed from the reaction of acetic anhydride with chitosan, Figure (2).The other two peaks at 1674 cm^{-1} and 1589 cm^{-1} are the characteristic absorptions of the amide –C=O and $–NH_2$ groups (amide I and amide II), respectively. They are apparently stronger than those peaks of original CS. The peak at 1491 cm^{-1} designated for aliphatic C-H bond stretching of methylene group's.

Grafting the polylactide to NACS to prepare NACS-g-PLA displays an extra peak at 1456 cm^{-1} in FTIR, credited to the methyl asymmetric deformation of polylactide and its related peaks of 1134 cm^{-1} and 1089 cm^{-1} which are due to strong C-O-C stretching, Figure (3). Freeing the $–NH_2$ groups of the original chitosan by removing the acetyl groups leads to the presence of two bands at 1570 cm^{-1} and 1658 cm^{-1} that belong to $C-NH_2$ vibration. This is considered as an attractive group for another grafting, which was made by poly(ethylene glycol) with a molecular (10000 g/mole). The indication of the successful grafting occurred comes from the appearance of the strong peaks at 1199 and 1296 cm^{-1} assigned to the characteristic of C–O–C stretches vibrations of the polyethylene glycol repeated units [7].

3.1.2 ^{1}H-NMR characterization

INOVA 500 MHz. ™NMR spectrometer was used to record the 1HNMR. Figure (4) shows the 1H-NMR spectrum of chitosan. The proton peaks area of CS appeared at 4.6 ppm (H-1 of glucosamine ring), 3.1 ppm (H-2 of glucosamine ring), and 3.1-3.8 ppm (H-3, H-4, H-5, and H-6). Moreover, the presence of the peak at displacement (2.5) ppm belongs to the protons of the DMSO solvent used for measuring the NMR spectrum.

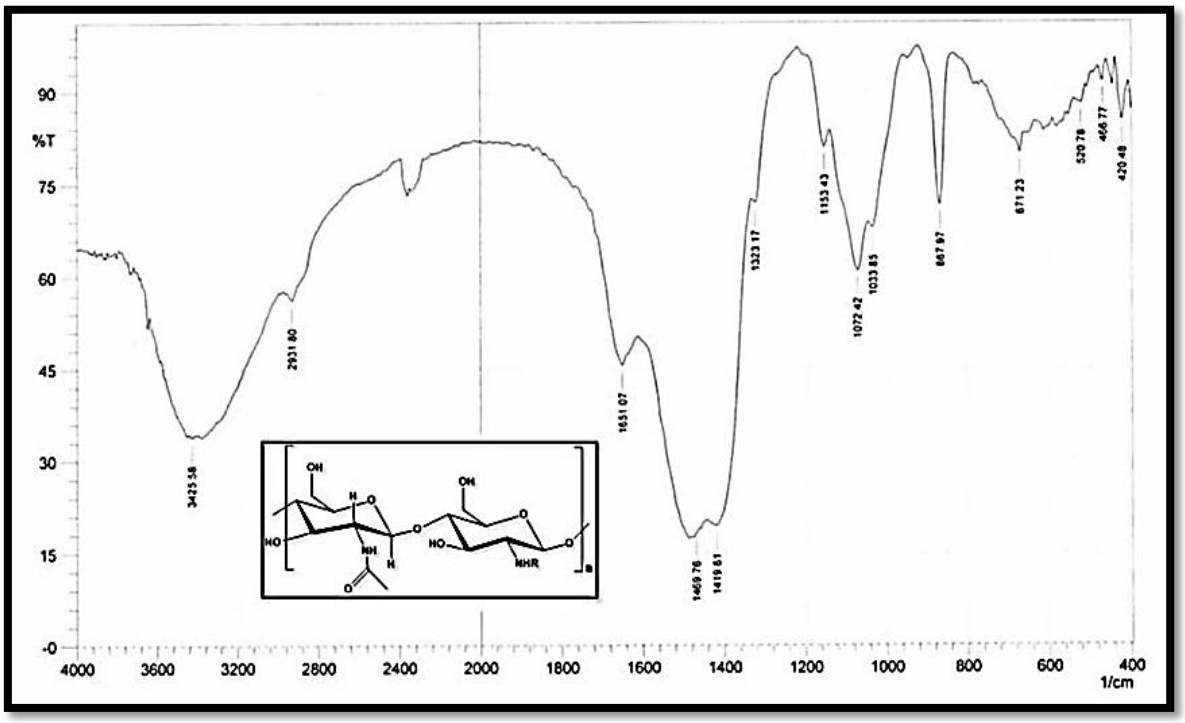


Figure (2): FTIR spectrum of N-acetyl chitosan.

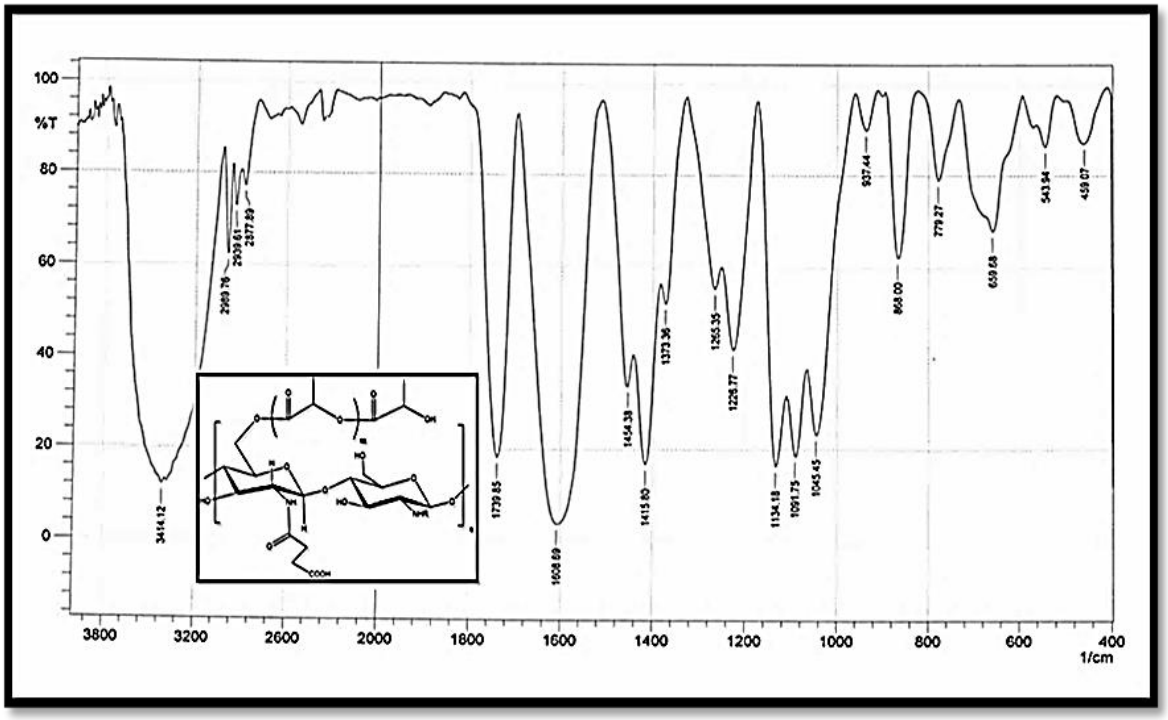


Figure (3): FTIR spectrum of N-acetyl-chitosan-grafted-polylactide.

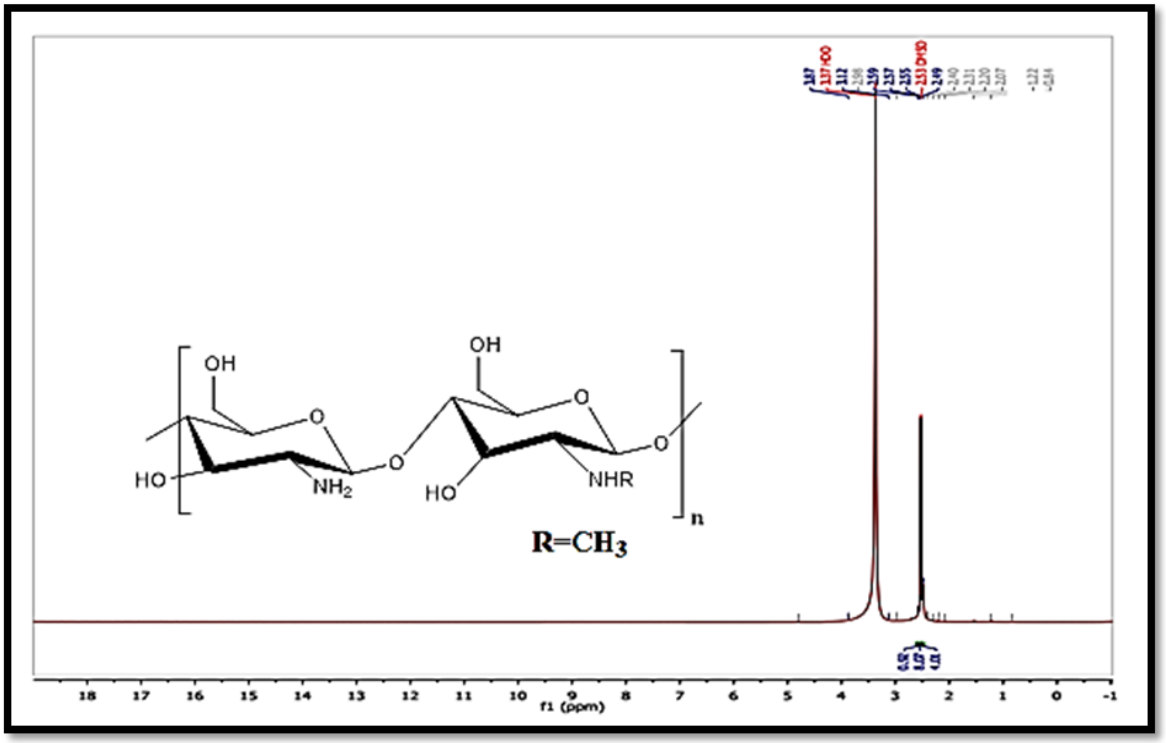


Figure (4): ^{1}H-NMR spectrum of chitosan.

N-acetyl-chitosan-grafted-polylactide delivered a 1H-NMR spectrum as shown in Figure (5). It has peaks at 1.29, 1.31 ppm and 1.32 ppm that belong to the methyl protons (CH_3) of PLA repeated unit, 4.32 ppm that belong to the methine protons (CH) of PLA repeated unit, 3.0-4.3 ppm (pyranose), and 7.4-7.5 ppm (NH-aromatic benzene ring), and a displacement at (11.0-12.0) ppm was ascribed to the acetic acid solvent protons.

^{1}H-NMR spectrum of CS-PLA is shown in Figure (6). The peaks at 1.45–1.50 ppm assigned for the methyl protons (CH_3) belong to PLA repeated unit, 4.4 ppm the methine protons (CH) of PLA repeated unit, 2.70 ppm (H-2), 2.79-4.40 ppm (H-3, H-4, H-5, and H-6) of pyranose, 4.5 ppm belongs to (H-1), 7.9 ppm (aromatic benzene ring), and at (11.5) ppm attributed to the solvent acetic acid protons.

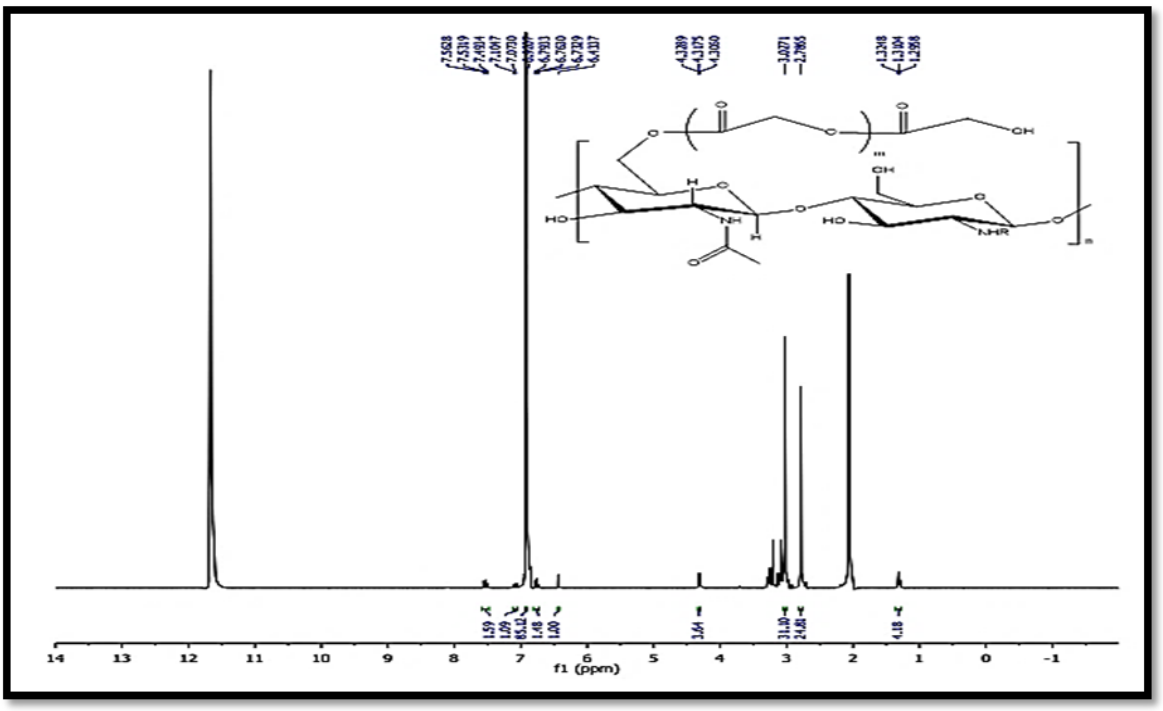


Figure (5): ^{1}H-NMR spectrum of (NACS-g-PLA).

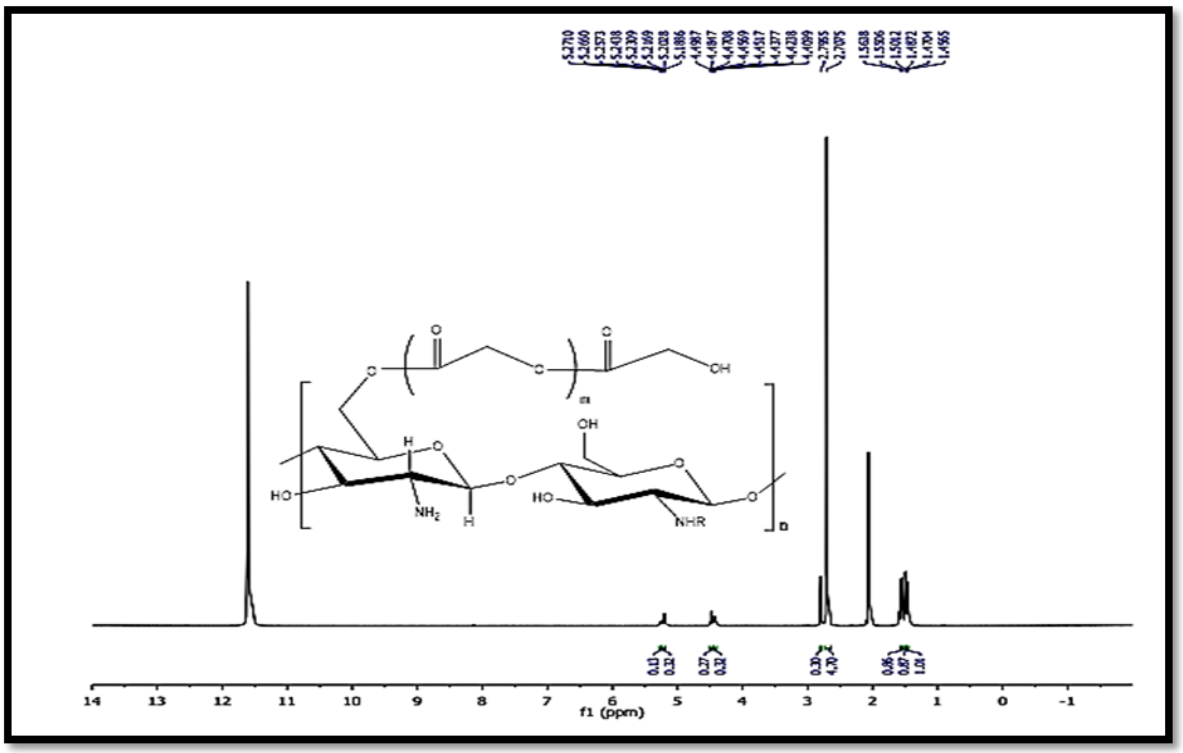


Figure (6): ^{1}H-NMR spectrum of (CS-g-PLA).

In Figure (7), the 1H-NMR spectrum of CS-PLA-PEG gave the following peaks: 1.2, 1.3 ppm and at1.9 ppm 6.2 ppm the methine protons (CH) of PLA repeated unit, 2.3-3.7 ppm (CH_2) of PEG, 3.0-3.8 ppm (pyranose), and 7.4-7.9 ppm (NH- aromatic benzene ring). Moreover, the presence of the peak at displacement (11.5) ppm assigned to the protons of the acetic acid solvent.

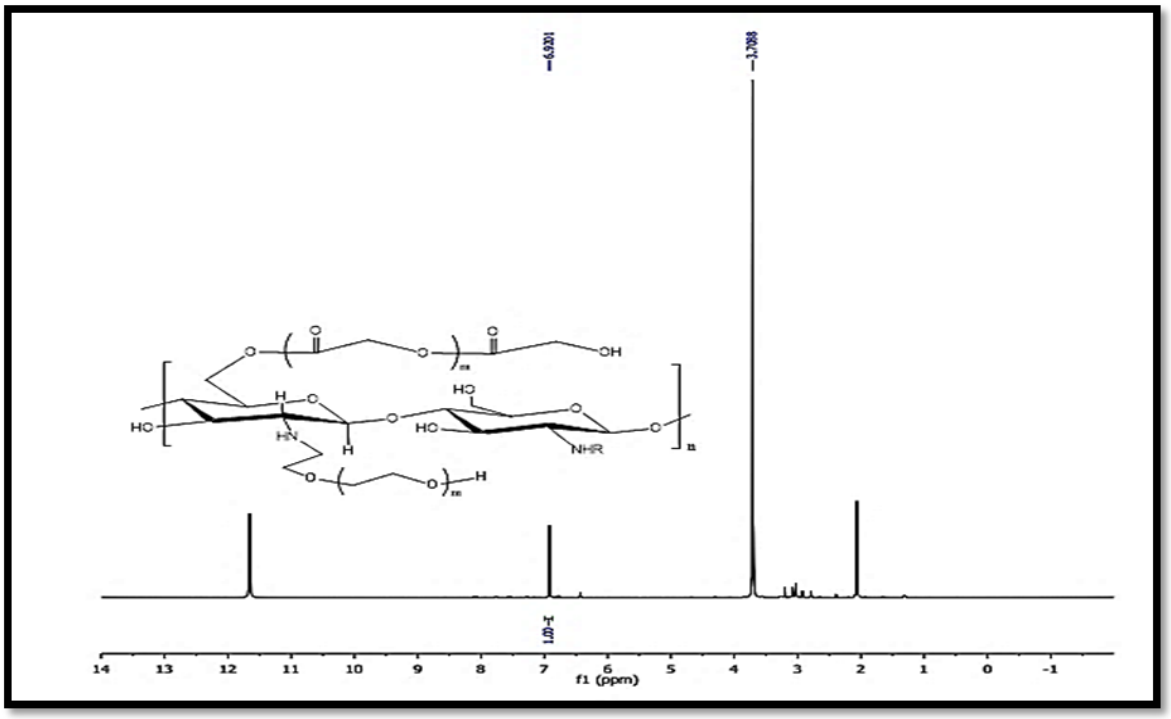


Figure (7): ^{1}H-NMR spectrum of (CS-PLA-g-PEG).

3.2 Scanning electron microscopy of chitosan nanoparticles

SEM micrographs and ImageJ results verified the formation of Nanosize and spherical shape of pure chitosan nanoparticles and its derivatives. Figure (8A) shows the SEM micrograph of the purified CS as small spherical nanoparticles with a minimum and a maximum size ranging from 17-100 nm; in the interim, the SEM images of NACS-g-PLA, Figure (8B) showed up various sizes with a spherical shape that appropriated between 38, 55 and 93 nm. The surface of the sample appeared as bead-like structures. In contrast, the SEM image shown in Figure (8C) which belongs to CS-g-PLA NPs appears to have a size running from 24 nm to 85 nm. The homogeneity appeared by CS-PLA-g-PEG nanoparticle surface structure and tiny-pores density exhibited the high volume proportion of nanoparticles dispersed of spherical shape with various sizes from 15 to 82 nm, Figure (8D).

3.3 In vitro cytotoxicity evaluation

A comparison *in vitro* cytotoxicity investigation of pure chitosan CS and its derivative NACS-g-PLA, CS-g-PLA NPs, and CS- PLA-g-PEG nanoparticles in the three types of the human breast carcinoma cell lines, MCF-7, BT, and SKBR-3 cells was accomplished individually utilizing MTT assay. The mean population triplicate time (PTT) of % cell viability between non-treated control samples of chitosan and chitosan derivative NPs treated cells for all the subjected carcinoma cell lines analyzed, and the results are shown in Table (1); $p < 0.001$. These pieces of information propose that the chitosan and chitosan derivative

NPs were cytotoxic and had a highly significant consequence of the cellular proliferation of human breast cancers cell lines.

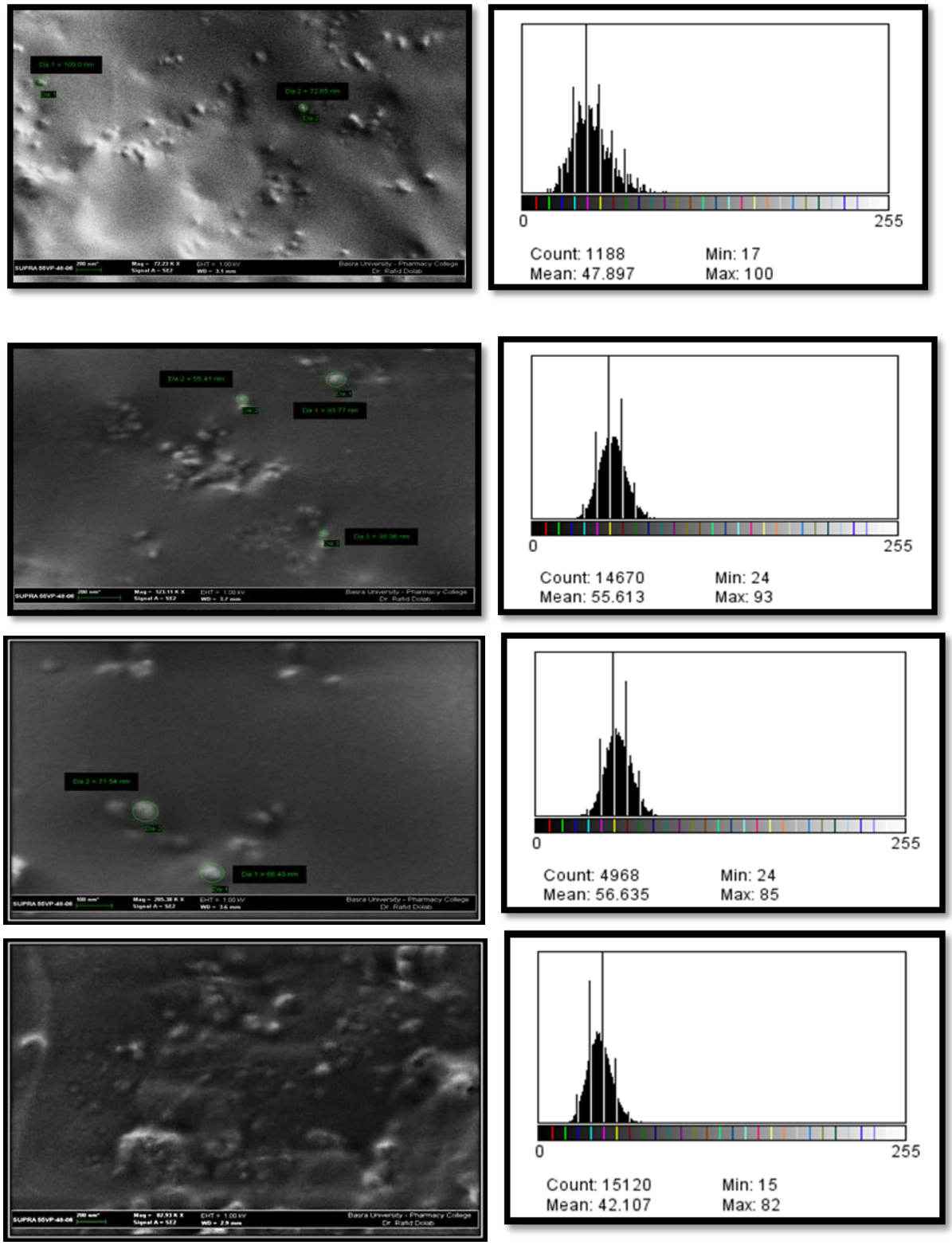


Figure (8): Scanning electron microscopy (SEM) images of pure chitosan CS (A), and its Derivatives: (B), NACS-g-PLA (C) CS-g-PLA and (D) CS-PLA-g-PEG NP.

The presence of the chitosan NPs (1mg/ml) strongly affects the growth and cell viability of human breast carcinoma cell lines in culture, as estimated by MTT assay, at various times ranging from 24 hours to 72 hours. Figure (9A) illustrates cell proliferation for each type of examined cell lines, and the results performed highly significant, ($p<0.001$), decreasing of the cell proliferation with increasing of the time exposure to chitosan nanoparticles in comparison with untreated cells (control samples). This effect could be attributing to the CSNPs that act at several levels to persuade the carcinoma cell demise, involving distracting the cell membrane, diminishing the negative surface charge and mitochondrial membrane potential, inducing lipid peroxidation, distressing the fatty acid composition of the membrane [19]. A new observation, developing from several recent studies [20, 21] recommended that CSNPs have strong antitumor activities by inducing in vitro apoptosis and growth suppression of human tumor cells. Subsequently, chitosan has shown to inhibit breast carcinoma in vitro, the chitosan derivatives NPs, NACS-g-PLA, CS-g-PLA NPs, and CS- PLA-g-PEG, treated cells also have a highly significant effect, ($p < 0.001$), on cell proliferation growth as observed with untreated control cells, (Figure 9B, C, and D). The mean population triplicate times (PTT) performance differs between non-treated control and chitosan derivative NPs subjected cells for all the tested times, Table (1). This revealed a significant reduction of the *in vitro* cytotoxicity rate of CS NPs derivatives against various types of breast cancers cell lines that increase with the increase in transfection time. The mode of action of CS derivatives nanoparticles suggests that every polymer nanoparticles was uptake by tumor cell lines in a different pattern regarding the type of polymer NPs and the tumor cell lines. This cellular uptake of polymers NPs has caused a significant diminution in cell proliferation and growth in comparison with untreated cells.

The proposed pattern of cytotoxicity of CS derivative nanoparticles may be assuming to the chemical composition of each one. Furthermore, the different response of breast cancer cells to CS and CS derivative nanoparticles demonstrated that the polymer nanoparticles affected the mitochondrial dysfunction, which possibly inhibits the proliferation of BT, MCF-7, and SKBR3 cell lines [22,23], in addition, to enhancing the apoptosis processes for human breast cancer cell lines.

Table (1): Demonstrate the mean population triplicate time (PTT) as an antitumor effect of pure chitosan CS, NACS-g-PLA, CS-g-PLA CS- PLA-g-PEG nanoparticles against the proliferation of human breast cancer cell lines BT cells, MCF-7 cells, and SKBR3, with highly significantly affected ($P<0.001$).

Sample	Cell Line		
	BT	MCF-7	SKBR 3
CS	62.00±13.528	22.00±5.000	49.33±3.055
NACS-g-PLA	50.33±28.746	26.33±10.017	40.33±3.786
CS-g-PLA	79.67±8.145	26.33±6.351	42.00±7.550
CS-PLA-g-PEG	52.33±1.528	85.67±1.528	35.33±1.528
Control	100.00±.000	100.00±.000	100.00±.000

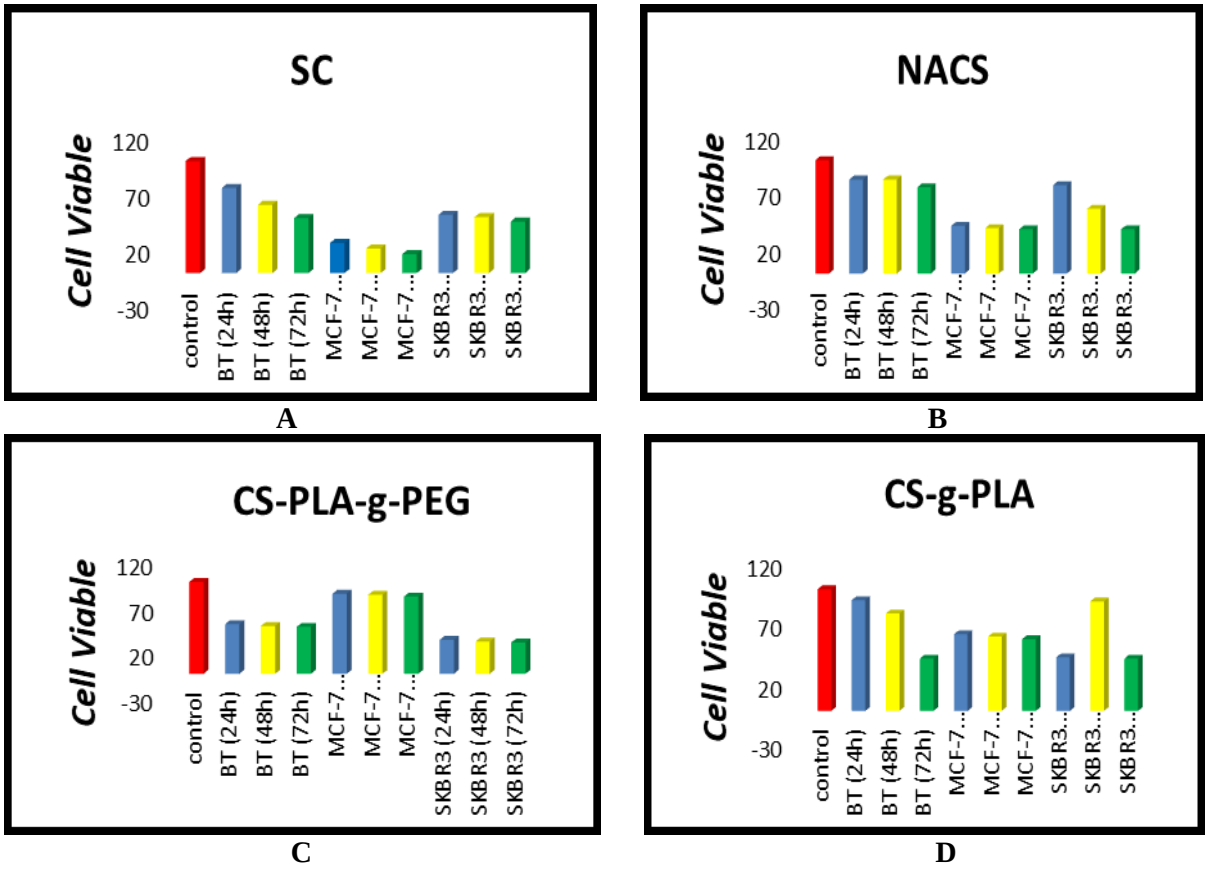


Figure (9): Cell viability percentages of human breast cancers cell lines (BT cells, MCF-7 cells and SKBR3 cells) affected with chitosan-chitosan derivatives nanoparticles at different times for 24h, 48h and 72h. (A) Pure chitosan CS, (B) NACS-g-PLA, (C) CS-g-PLA NPs and (D) CS- PLA-g-PEG.

3.4 Genotoxicity assay (DNA fragmentation)

In this study, the Flow Cytometry Technique with Acridine Orange (AO) staining was used to estimate the genotoxicity of the CSNPs and its derivatives on BT breast cancers cell line. Acridine orange took up by both viable and nonviable cells and emits green fluorescence if unified into a double-stranded nucleic acid (DNA) or red fluorescence if bound to single-stranded nucleic acid fragmented (DNA).

The effects of chitosan NPs and other derivatives were listed in Table (2) against BT breast cancer cell line, which demonstrated as a % DNA fragmentation index (% DFI), using flow cytometry assay in comparison with positive and negative control, Figures (10A, B, C, and D). Results revealed that CSNPs have no effect on the DNA of BT breast cancer cell lines; with a %DFI reach of 6.77%, and it appears less than the negative control (untreated cells) 13.6% and in comparison with positive control 36.0% (Figure 11). These investigations illustrate that the chitosan NPs have no negative effect on the nucleic material of the breast cancers cell lines and can be used as gene delivery. These results are in accord with the reported findings demonstrated the use of CSNPs as carriers to deliver various drugs and genes in cancer treatment [24-26].

Table (2): % DFI of chitosan NPs-chitosan derivatives NPs using BT breast cancers cell lines

Sample Name	DNA Fragmentation Percent
CS	6.77%
NACS-g-PLA	18.7%
CS-g-PLA	20.1%
CS-PLA-g-PEG	6.49%
Positive control	36.0%
Untreated sample	13.6%

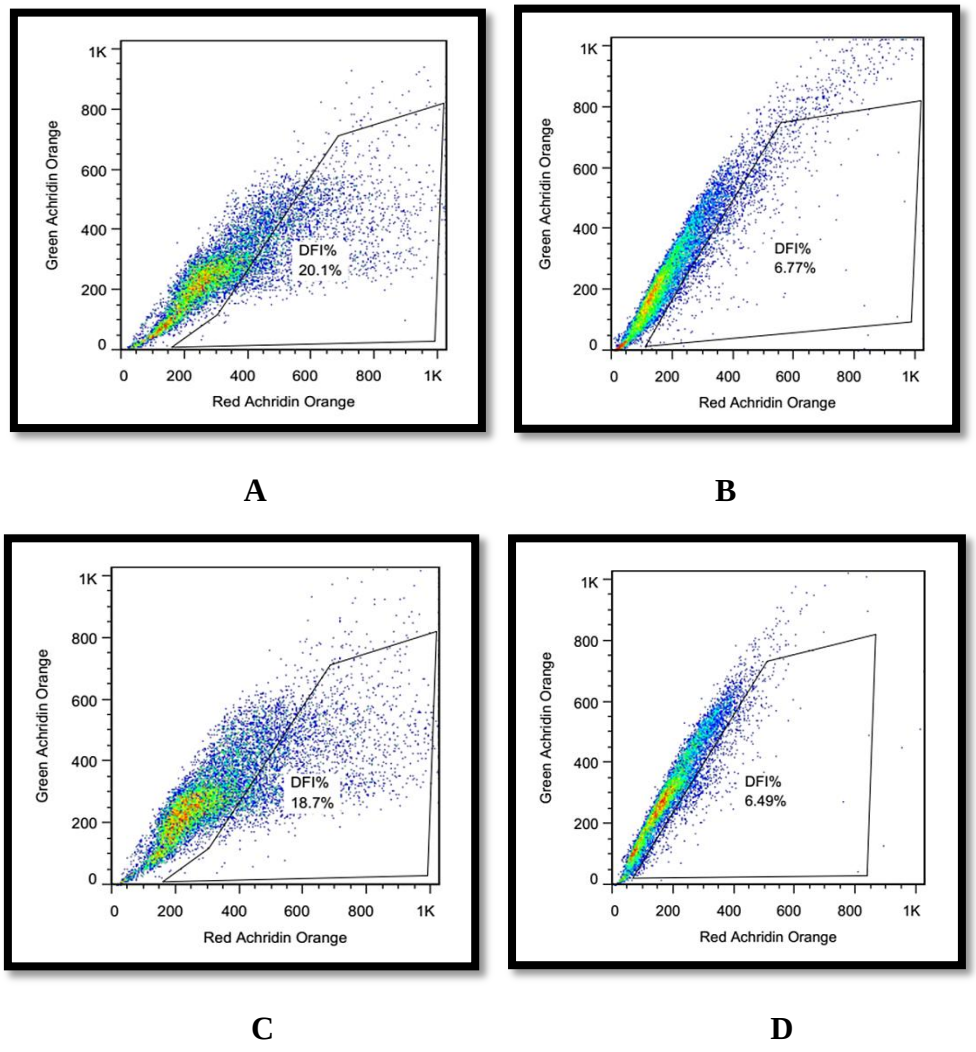


Figure (10): A: DNA Fragmentation of Chitosan. B: DNA Fragmentation of (NACS-g-PLA), C: DNA Fragmentation of (CS-g-PLA), and D: DNA Fragmentation of (CS-PLA-g-PEG).

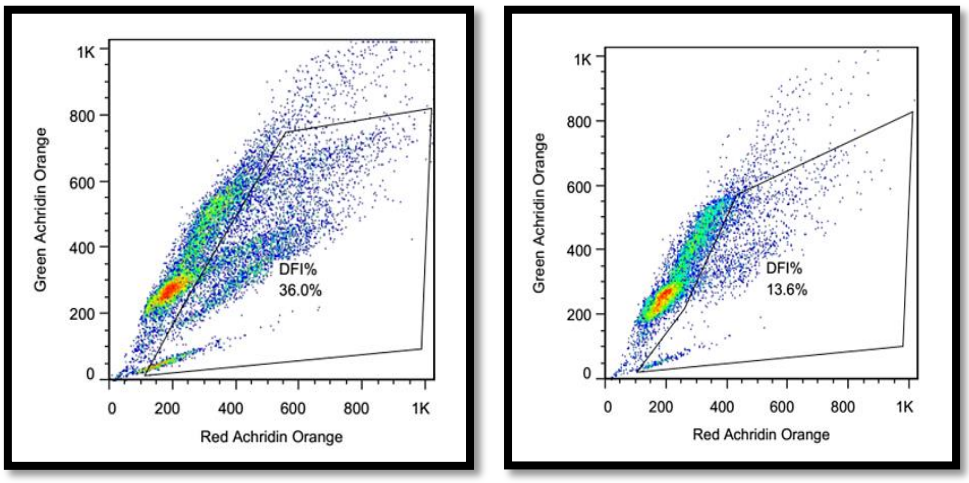


Figure (11): DNA Fragmentation of positive and negative control.

Another interesting achievement in comparison with positive control is that CS derivative nanoparticles acted multiple levels with %DFI for NACS-g-PLA (18.7%), CS-g-PLA (20.1%) and 6.49% for CS-PLA-g-PEG, compared to the positive control samples. However, these values imply that chitosan derivatives acted as a gene delivery treatment in cancer cell lines, and excellent for CS-PLA-g-PEG NPs, % DFI 6.49%. This result seems to be well accepted since the literature affirms that % DFI less than 15% DFI can be represented as an excellent pattern for the high integrity status of DNA [27]. These results approved the using of these polymers as biomedical and nanomedicine applications and gene delivery systems.

4. Conclusions

In vitro cytotoxicity of Chitosan and Chitosan derivatives prepared NPs against differentiated three types of human breast cancer cell line, namely BT cell lines, MCF-7 cell lines, and SKBR3 cell lines was performed. The cell proliferation of each type of breast cancer cell lines appeared highly significant decreasing ($p<0.001$) with all tested NPs polymers in comparison with the positive control samples through different periods of the experiment (24, 48 and 72 hours). Chitosan and its derivative NPs showed time-dependent growth inhibition of human breast cancers cell lines. The antitumor activity of CSNPs and CSNPs derivatives appears in a different pattern with no or less effect on the nucleic material of the BT cell lines, this may induced for antitumor therapy and mediated as gene delivery for human breast cancers.

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