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Synthesis of polymeric chitosan derivative Nanoparticles and their MTT and flow cytometry evaluation against breast carcinoma cell

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Abstract: Chitosan extracted from shrimp cortex was grafted with some different anhydride compounds like; acetic anhydride, propionic anhydride, succinic anhydride, and phthalic anhydride. The chitosan-grafted-anhydrides were copolymerized with L-lactide in ring opening polymerization. All grafted chitosan derivatives were obtained in very good yield, and they were characterized by FTIR and the resulted spectra confirmed the right structures of chitosan and its different synthesized derivatives, and then they were converted to nanoparticles in size by subjecting them to sonication method.In vitro cytotoxicity detection of different chitosan anhydride derivative nanoparticles and their grafted-polylactide were concerning three different types of human breast cancer cell lines as MTT assay, the results exhibit the highly significant cell growth inhibition of these tumor cells compared with a positive control; furthermore, the chitosan anhydride derivatives grafted polylactide demonstrated increasing in reducing cell viability in comparison with their non-grafted form. The DNA fragmentation index percentage was evaluated for some of the studied polymer nanoparticles, using acridine orange dye and the results were shown no or less effective against BT breast carcinoma cell lines DNA

1 Introduction

Applications of polymers were extendedto medical devices, electronic devices, packaging and manufacturing of textiles, artificial fiber, and composites, etc. [1], especially cationic polymers are inherent bioactive and intrinsic therapeutic potential, as base biomaterials. It is covering the fields of drug and gene delivery as well as tissue engineering. Gene therapy is of great interest, and polymer delivery systems are considered as a safer and more effective pathway for therapeutic genes. Cationic polymers have been improved to increase the efficiency of DNA transfer. The systems will provide an effective tool for gene therapy in the future [2].

Polymeric nanoparticles systems are useful in biomedical applications, for many important scientific reasons, which are: they're easy to prepare from well-understood polymers and have high stability in biological fluids as well as during storage. And the most used in the field of tumors as an anticancer treatment [3].

Natural cationic polymers are among the materials that have been studied. They possess active groups, in which they can be chemically altered to improve their physicochemical properties, have potential as drug or gene delivery systems, they are well suited for new advances in the treatment of diseases.

The natural, biodegradable chitosan polymer has got special attention due to their effective complexation between chitosan and DNA, which can be synthesized easily and the stability of the resulting chitosan-DNA complex.

In previous work, chitosan was grafted with different polymers, namely; PEG, PVA, and PVP. They were obtained with high yield, and the FT-IR spectra of them verified the expected chitosan-grafted structures desired to be synthesized. The cytotoxicity of the studied chitosan-g-polymers were examined against differentiated three types of breast cancer cell lines, and the results revealed the highly significant ($p<0.001$), the effect of these polymers in comparison with the non-treated cell lines, especially with chitosan-grafted-poly (ethylene glycol) nanoparticles (CS-g-PEG), the cell viability reduced to 23.33% ± 1.528, 25.67% ± 1.155 and 45.67% ± 5.508 against BT cells, MCF-7 cells, and SKBR3 cells, respectively [4].

Chitosan has three types of reactive functional groups, an amino group on its backbone as well as both primary and secondary free hydroxyl groups at the C-2, C-3, and C-6 positions respectively [28,29]. Herein, we extended our research work to prepare different chitosan grafted anhydrides and their polylactideanalogue derivatives, and examine their MTT and flow cytometry evaluation against breast carcinoma cells.

2 Materials and methods

2.1 Materials

Chitosan was obtained by deacetylation process of chitin extracted from local shrimp shells as described in the literature [5]. Acetic anhydride, propionic anhydride, succinic anhydride, and phthalic anhydride, were purchased from BHD, and L-lactide from Sigma-Aldrich. Solvents used were dried by normal procedures [6].

2.2 Methods

2.2.1 Preparation of N-acetyl chitosan (NACS)

One gram of chitosan was dissolved in 50 ml of a 2% acetic acid solution, and after that, 250ml methanol was added in parallel with the addition of 6g acetic anhydride. The mixture was stirred vigorously for 2 h, followed by pouring the mixture into an excess of 10% NaOH solution to precipitate the grafted Chitosan, Scheme (1). The product was filtered, washed with ethanol and acetone alternatively [7,8]. The yield of resulted dried white powder of (NACS) was gotten in an 85%.

2.2.2 Preparation of N-propionyl chitosan (NPCS)

NPCS was prepared by the reaction between 1g chitosan dissolved in 50 ml 2% aqueous acetic acid, and then the solution was added to 250 ml dry methanol followed by the addition of 6g propionic anhydride with vigorous stirring for 2h in a dry nitrogen atmosphere, scheme (1). The dry precipitated product, with a yield of 83% was obtained by pouring the mixture into 10% of sodium hydroxide solution and filtration accompanying with washing with dry ethanol and then acetone [9,10].

2.2.3 Preparation of N-succinyl chitosan (NSCS)

NSCS was obtained from the reaction of 2g chitosan with 6g succinic anhydride in dry dimethyl sulfoxide, 100ml. The mixture was heated gradually up to 160°C and kept at this temperature for 6h under the nitrogen atmosphere, Scheme (1). The white precipitate was starting to come off the solution after cooling to ambient temperature. Then, it was filtered, washed several times with dry ethanol, and dried in the vacuum desiccator [11,12]. The white powder result of (NSCS) was acquired with a 68% yield.

2.2.4 Preparation of N-phthaloyl chitosan (NPHCS)

Two grams of chitosan and 6g phthalic anhydride were heated up to 130°C in 100ml of dry N,N-dimethylformamideunder a dry nitrogen atmosphere, Scheme (1). After cooling at room temperature, the whole mixture was poured into an ice bath to precipitate the desired product. The yellow product was attained after filtration, washing with dry methanol, and drying in vacuum desiccator [13,14].

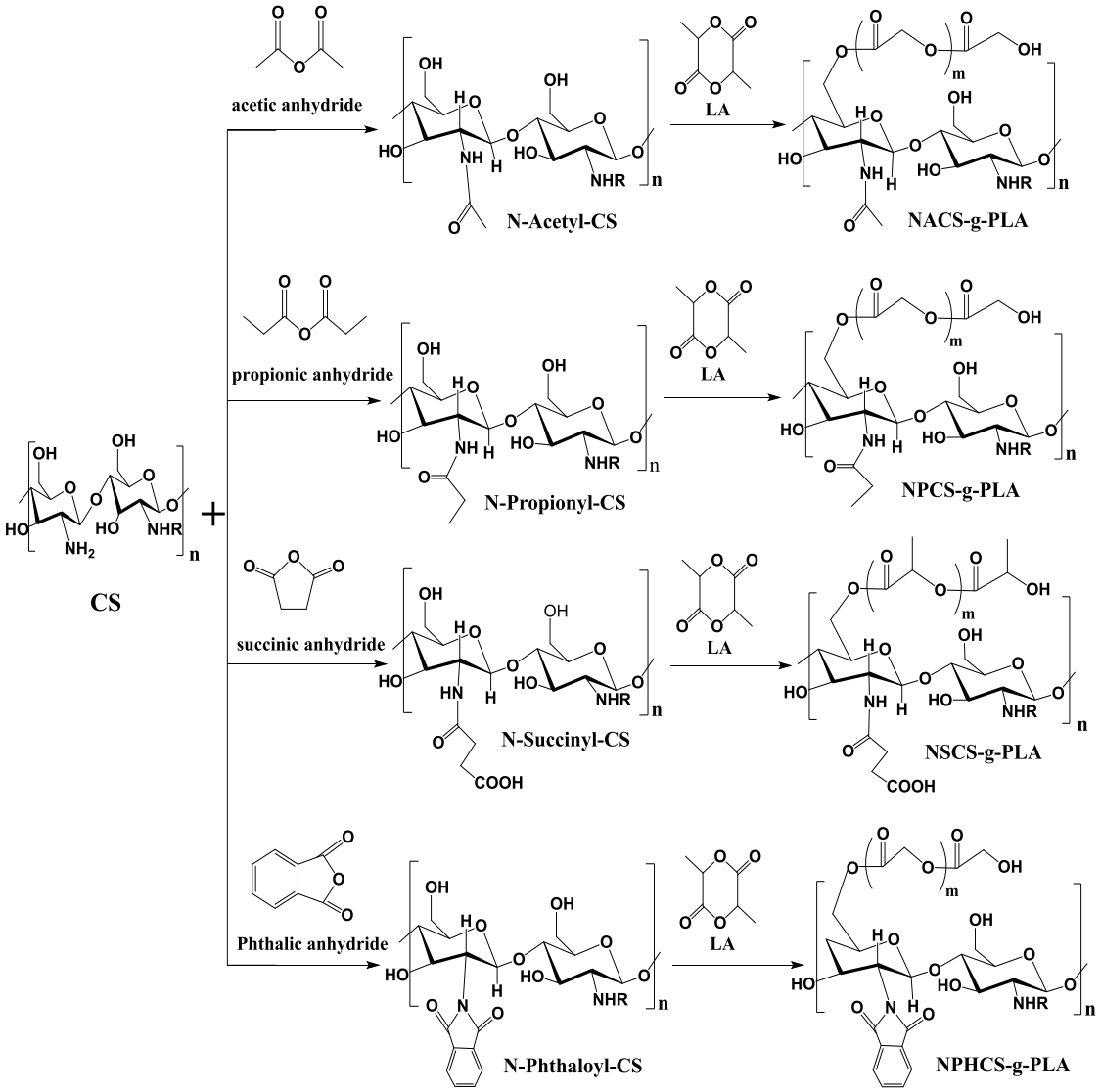
2.2.5 Preparation of chitosan N-anhydrides grafted polylactide derivatives

The previous four anhydride chitosan's achieved from the above reactions, N-acetyl chitosan, N-propionyl chitosan, N-succinyl chitosan, andN-phthaloyl chitosan were grafted with lactide to prepare N-acetyl-chitosan-grafted-polylactide(NACS-g-PLA), N-propionyl-chitosan-grafted-polylactide (NPCS-g-PLA), N-succinyl-chitosan-grafted-polylactide (NSCS-g-PLA), and N-phthaloyl-chitosan-grafted-polylactide (NPHCS-g-PLA) respectively, in the following typical procedure.

Two grams of lactide were interacted with 1g of the prepared chitosan derivatives(N-acetyl chitosan, N-propionyl-CS, N-succinyl-CS, and N-phthaloyl-CS) in 50ml dry toluene and refluxed for 6 hours under a dry nitrogen atmosphere. Afterward, the hot mixture was left to cool down to ambient temperature, Scheme (1). The powder products, namely, NPHCS-g-PLA, NSCS-g-PLA, NACS-g-PLA, and NPCS-g-PLA were obtained with yields reached 78% by precipitation in 40 ml of acetone and to wash away the untreated LA monomer and the PLA homopolymer may form, and by filtration and drying [15].

2.2.6 Preparation of Chitosan Derivatives Nanoparticles

The derivatives based on chitosan prepared;N-acetyl chitosan, N-propionyl chitosan, N-succinyl chitosan, and N-phthaloyl chitosan and their polylactide derivatives, N-acetyl-chitosan-grafted-polylactide, N-propionyl-chitosan-grafted-polylactide, N-succinyl-chitosan-grafted-polylactide, and N-phthaloyl-chitosan-grafted-polylactide, they were converted to nanoparticles by the same procedure described in the literature (4,16).



Scheme (1)

2.2.7 Cytotoxicity Study (MTT assay)

Briefly, BT, MCF-7, and SKBR3 (Michigan Cancer Foundation-7) cells were seeded in a 96-well plate (one hundred µl of cells containing 7500 total cells, and left to adhere for 24 h by incubation overnight on the CO_2 incubator at 37°C. MIF or MCNs were added to the well as predetermined drug concentration (1 mg/mL), and

incubated for 24h, 48 h and 72h at 37 °C with 5% CO=. The medium was removed and the cells were washed three times with PBS, MTT (20 µl of 5 mg/ml in PBS) was added to each well of the plates for all incubation times. Plates were incubated for a further 3.5h, and covered with tinfoil, agitate cells were done on an orbital shaker for 15 min. Then DMSO (100 µl) was added before a further incubation of 30 min at 37 ºC.

2.2.8 Genotoxicity

This method was run according to Zini and Agarwal [18]. BT breast cancer cell lines (2×10^5 cells/ml) were cultivated in RPMI media containing %20 FBS+ insulin, at 10 ml per Petri dish. Upon formation of a monolayer of cells, 100µl of a concentration (1mg\ ml) for each selected chitosan derivatives nanoparticles were added. After 24h of incubation, cells were harvested by the addition of trypsin, centrifuged for 5 min at 1000xg, and finally washed with PBS. Cells were stained according to the protocol and were analyzed. The sample was 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 were also maintained against the positive controls. The determinations were performed in duplicates.

2.2.9 Statistical analysis

All samples were assessed in triplicates and were presented as (means ± SD). For statistical analysis using Graph pad prism 5, one-way analysis of variance (ANOVA) test was used to test for significance between the groups. Differences were regarded as statistically highly significant if $P< 0.001$.

3 Results and Discussion

3.1 FTIR characterization of chitosan anhydride derivatives

All FTIR spectra were obtained from grafted chitosan samples in KBr pellets using a JASCO 4200 FT-IR spectrophotometer. The examined spectrum attained from NACS showed the common characteristic peak at 3600-3200 cm^{-1} for –OH and $–NH_2$, and2935-2858 cm^{-1} for C-H stretching. The major interesting observation was made by the existence of a new band at 1419 cm^{-1} corresponding to the symmetric stretching of the –COO group formed [12], Scheme (1). TheFTIR spectrumalsorevealedthe disappearance of the peak assigned for ($-NH_2$bending) in chitosan before grafting with all anhydrides at 1579 cm^{-1}, and no peaks appeared in the ester stretching region, i.e. 1725-1740 cm^{-1}, implies that the grafting reaction occurred on the N-position and –NH–CO– groups have been formed as shown in Scheme (1). This was also accompanied by the increasing the intensity of the peak assigned for the carbonyl amide group at 1651cm^{-1} than that peak of the original CS(1639 cm^{-1}), and these results agreed very well with that reported in the literature [19,20]. Figure (1) showed the FTIR spectra of chitosan (CS), acetyl chitosan (NACS), and acetyl chitosan-grafted-polylactide (NACS-g-PLA) as representative of the FTIR spectra obtained. The same was true for the FTIR spectra obtained from NPCS and NSCS [21,22]. In addition to that,the spectrum of NPHCS obtained showedthe presence of the strong absorption peak at 1778 cm^{-1} and 1716 cm^{-1} confirms the stretching vibration of (C=O anhydride) and 721 cm^{-1} to aromatic ring [22].

3.2 FTIR characterization of polylactide grafted into chitosan-anhydride derivatives

The grafting process of N-acetyl chitosan, N-propionyl chitosan, N-succinyl chitosan, and N-phthaloyl chitosan was extended with polylactide and they were also characterized by FTIR spectroscopy. The main new bands appeared in all the spectra obtained from the examining of the new grafting process are the stretching bands of the new carbonyl ester groups formed due to the esterification reaction of the –OH group with lactidesat 1737 cm^{-1}. The methyl asymmetric deformation of polylactide appears at 1456 cm^{-1}, the two peaks at 1134 cm^{-1} and 1089 cm^{-1} which are due to strong C-O-C stretching [23], and theseare very obvious in Figure (1), i.e FTIR spectrum of acetyl chitosan-grafted-polylactide (NACS-g-PLA).

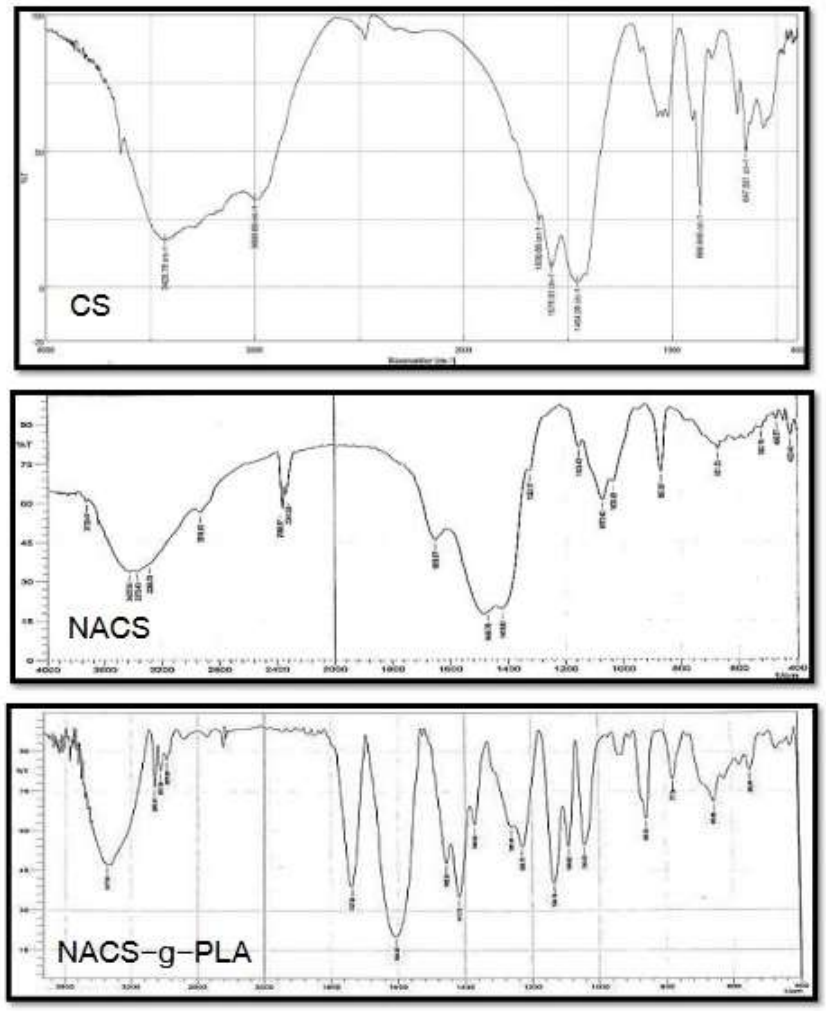


Figure 1: Representative FTIR spectra of the prepared grafted chitosan.

3.3 In vitro anticancer effects

The cytotoxicity of the different spherical in shape with a diameter less than 100 nm [24] of chitosan anhydride derivatives and their grafted-polylactide NPs was tested in three different human cancer cell lines as MTT assay, the results exhibit the highly significant cell growth inhibition of these tumor cells compared with a positive control; especially for NPHCS nanoparticles as anhydride chitosan derivatives at various time intervals; furthermore, in this experiment, we found that chitosan anhydride derivatives grafted polylactide enhanced anti-proliferative activity, indicating that the anticancer effects of NPHCS-g-PLA, NSCS-g-PLA, NACS-g-PLA and NPCS-g-PLA, as highly significant values, Table (1), could be due to the presence of PLA NPs that enhances the intracellular uptake behavior with tumor cell lines [25].

Figure (2) shows the percentage of cell viability with a significant reduction for NPHCS, NSCS, NACS, and NPCS NPs, respectively; considered the BT, MCF-7, and SKBR3 cell lines in comparison with control samples, meanwhile Figure (3) represented the cell viability percentage of treated cell lines with NPHCS-g-PLA, NSCS-g-PLA, NACS-g-PLA, and NPCS-g-PLA, respectively.

Table 1: The mean population triplicate time (PTT) ±standard deviation (SD) values as the antitumor effect of prepared polymer nanoparticles against the proliferation of human breast cancer cell lines BT cells, MCF-7 cells, and SKBR3, with $P< 0.001$

Sample	Cell Lines		
	BT	MCF-7	SKBR 3
NACS	80.67±4.041	40.33±1.528	58.00±19.519
NPCS	94.33±.577	70.00±19.079	45.67±9.815
NSCS	94.00±3.000	56.67±28.449	52.33±8.327
NPHCS	68.67±20.232	30.33±5.033	38.67±5.508
NACS-g-PLA	50.33±28.746	26.33±10.017	40.33±3.786
NPCS-g-PLA	46.33±29.366	17.33±8.963	39.67±5.033
NSCS-g-PLA	63.67±14.012	30.00±1.000	46.00±5.196
NPHCS-g-PLA	62.67±21.127	44.67±22.679	35.33±.577
Control	100.00±.000	100.00±.000	100.00±.000

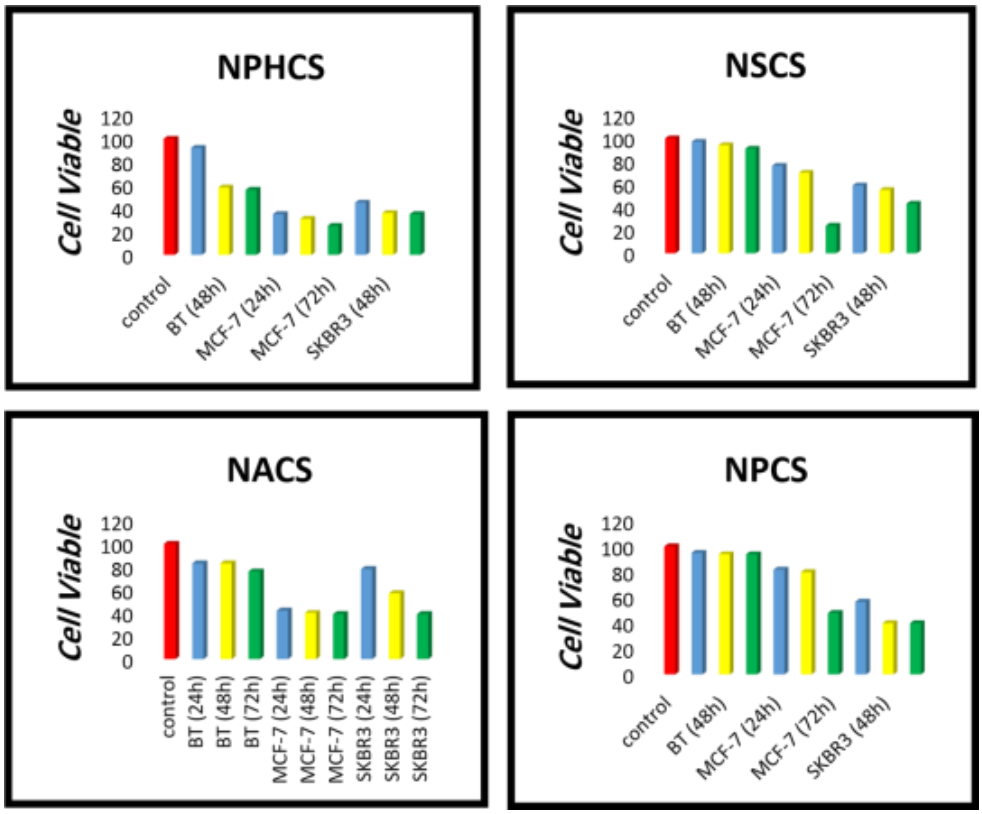


Figure 2: Cell viability percentages of human breast cancer cell lines (BT cells, MCF-7 cells and SKBR3 cells) affected with NPHCS, NSCS, NACS and NPCS NPs, at different times for 24h, 48h, and 72h.

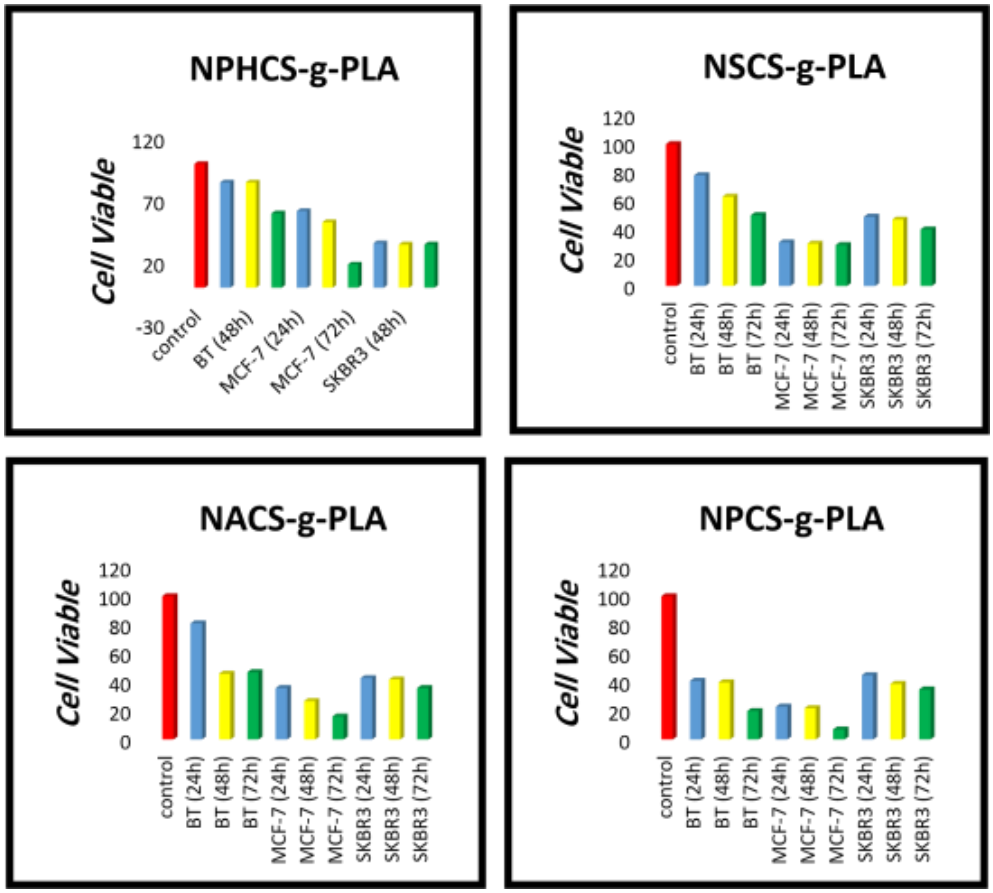


Figure 3: Cell viability percentages of human breast cancer cell lines (BT cells, MCF-7 cells, and SKBR3 cells) affected with NPHCS-g-PLA, NSCS-g-PLA, NACS-g-PLA, and NPCS-g-PLA NPs, at different times for 24h, 48h, and 72h.

3.4 Genotoxicity assay

For focusing on the therapeutic potential of NSCS and NACS-g-PLA for cancer treatment as their effect on the genetic material of BT breast cancer cell lines, the percentage DNA fragmentation index % DFI, were represented the good observed as genotoxicity assay. The observation comes from this study regarded with chitosan derivatives NPs, NSCS, which appears DFI % was reached at (27.2%), meanwhile the DFI% for NACS-g-PLA was decreasing and reaching (18.7%), otherwise, these values were represented no or slight effects on human nucleic acid and can be represented as well as for the nanoparticles pattern of the chitosan derivatives as gene delivery in cancer cell lines.Within comparison with (++) positive control, (++++) positive samples, and negative (non- treatment), Table (2) and Figures (4) and (5) once educated with the data regarding with the explanation of in vitro % DFI established, by which the percentage of DFI less than 15% DFI can be represented as an excellent pattern for the high integrity status of DNA [26], these results approved the using of these polymers as biomedical and nanomedicine applications and gene delivery systems.

Table 2: DNA fragmentation percent (% DFI) of chitosan NPs and chitosan derivatives NPs using BT breast cancer cell lines

Sample	DNA Fragmentation (%)
NSCS	27.2%
NACS-g-PLA	18.7%
++ control	36.0%
++++ control	64.2%
No treatment	13.6%

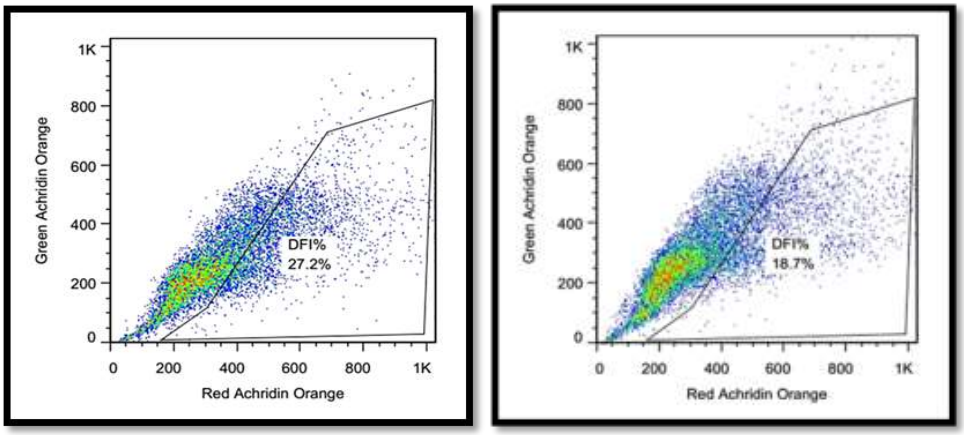


Figure 4: DNA fragmentation of(NSCS) and (NACS-g-PLA).

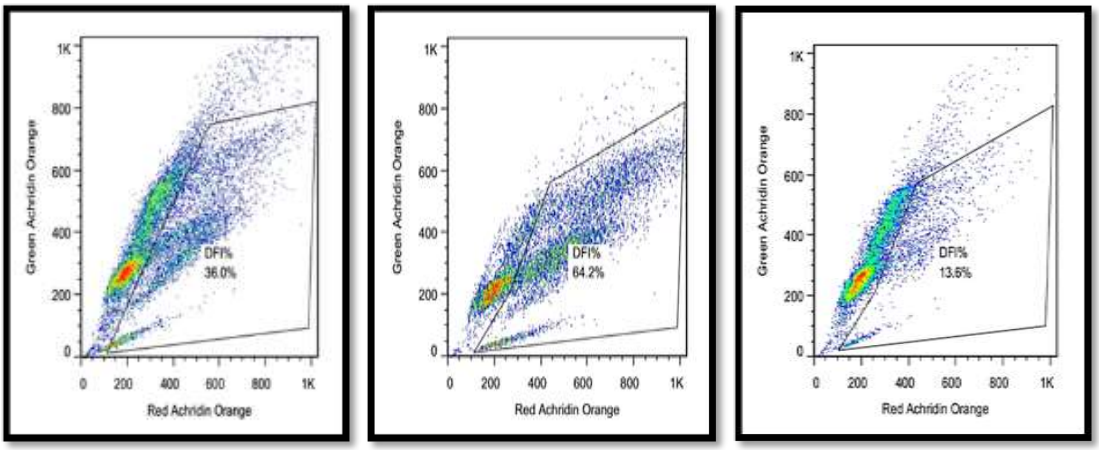


Figure 5: DNA fragmentation of (++) control and (++++) control and negative control.

4 Conclusion

These results suggested that these different chitosan anhydride derivatives and their grafted-polylactide NPs were shown good anticancer activity for cancer therapy in several human breast cancer cell lines, and with low or no genotoxicity effect, and they may be represented as a potential delivery system for gene therapy and \ or for cancer therapy.

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