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Comparative Cytotoxicity and Genotoxicity Assessments of Chitosan Amino Acid Derivative Nanoparticles toward Human Breast Cancer Cell Lines

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CHITOSAN, a natural macromolecule, is widely used in medical and pharmaceutical fields because of its distinctive properties such as a bactericide, fungicide and above all its antitumour effects. In this study; we aimed to develop an antitumour system based on Chitosan (Cs)and its some amino acid derivatives, namely Chitosan-Arginine (Cs-Arg) and Chitosan-Clycine-Aspartic acid (Cs-Gly-Asp)derivatives nanoparticles to improve their bioavailability and anticancer activity in antitumour treatments. The derivatives were obtained in a very good yield, and they were characterized by FTIR and some of them characterized by 1H-NMR, and the resulted spectra confirmed the right structures of synthesized chitosan derivatives. All the chitosan and its grafted amino acids were converted to nanoparticles in size by subjecting them to the sonication method. The scanning electron microscope (SEM) was used to determine the shape and size of the prepared polymeric nanoparticles, and they developed using the ImageJ program.

The MTT assay and the flow cytometry technique for all prepared polymeric nanoparticles were determined against three different types of human breast cancer cell lines, and the results revealed the highly significant ($p< 0.001$), in the reducing of breast carcinoma viability in comparison with untreated cell lines, but the cytotoxicity effect of Cs-Arg nanoparticles were larger than Cs-Gly-Asp nanoparticles, whereas there was no genotoxicity effect against BT cell lines for the Cs-Gly-Asp nanoparticles and slight effect for Cs-Arg nanoparticles.

Keywords: Nanoparticles, Chitosan, Arginine, Glycine, Cytotoxicity, Genotoxicity, Cell lines.

Introduction

History of Chitosan on the planet was at first done by Rouget in 1859 [1]. Chitosan was for quite a while the subject of central research in a manner of speaking. In any case, this circumstance has changed since the mid-1970s, when its tremendous potential in different applications has been noticed in the therapeutic field [2].

In addition to the chitosan applications for the improvement, separation, and concentration of metal particles, the properties of polyfunctionality nature of chitosan helps in making a wide scope of utilitarian materials, and opening another window of utilization for the natural materials. Chitosan and other natural polymers have received more attention [3] because they can be customized for targeted delivery of drugs, gene and improve bioavailability [4].

They were used within the biological application as they hold a high degree of molecular weight and structure; they could be subjected to different modifications dependent on the chemical rationale of design [5]. Gene and drugs delivery and antitumor represented as the important applications of biomedical nanomaterials [6]. They have been used as a promising tool for nanoscale drug carrier systems, especially in oral administration of poorly absorbed therapeutic

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drugs [7,8], the advances in nanotechnology and bioengineering are supporting tremendous efforts in optimizing the methods for genomic, epigenome and proteomic profiling [9].

In the field of nanomedicine and nanotechnology, methods are focus on expressing therapeutic biocompatible agents such as nanoparticles, nanocapsules, micellar systems, and conjugates. To achieve the suitable carrier system, the nanoparticles can be considered as a good candidate for therapeutic applications [10] such as they exist in the same size domain as proteins. They have large surface areas and ability to bind to numerous surface functional groups, and they may possess controllable absorption and release properties and particle size and surface characteristics.

Both natural (e.g. dextran and gelatin) and synthetic polymers (polylactide, polyethylenimine, and poly-L-lysine) were utilized to prepare polymeric nanoparticles. Many of these nanoparticles have been employed for oral and peptide drug delivery [11]. The most important applications are in the delivery of medicine and the gene and in the field of tumors [12].

A cell line is a permanently established cell culture that will proliferate indefinitely inappropriate fresh medium and space. Cell lines are used extensively in the biomedical field of cardiology and neuroscience[13,14]. It is a permanently established cell culture which proliferates indefinitely under given appropriate conditions. The oldest and commonly used human cell line, HeLa was the first cultured cancer line in 1951. The significant difficulties and challenges are in the production of stable cell lines which have low transfection adequacy as well as integration frequency. Stable expression can be influenced by the transfection method used [15]. Therefore, we tried, in this research, to prepare some chitosan-grafted-amino acids and asses their cytotoxicity and genotoxicity toward human breast cancer cell lines in a comparative mode.

Experimental

Material

Materials used in this work were purchased from different companies; Arginine, and aspartic acid with purity 98% and glycine (purity 99%) from BHD, glutaraldehyde and dimethyl sulfoxide (DMSO) with 99% purity from Sigma-Aldrich.

Methods

Synthesis of Chitosan - grafted - Arginine Nanoparticles (Cs-g-Arg)

Chitosan extracted from shrimp cortex as described in the literature was used as a base material to prepare the amino acid chitosan nanoparticle derivatives[16].

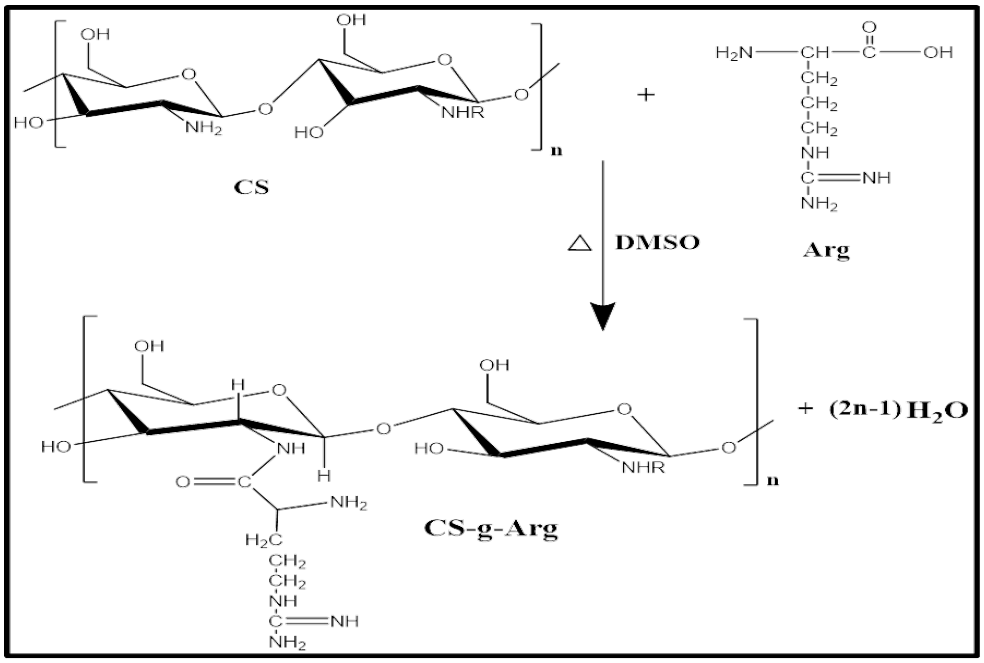
Cs-g-Arg was prepared by reaction between 1g of chitosan with 2g arginine dissolved in 50 ml dry DMSO, the mixture was heated at 145°C for 24h under a dry nitrogen atmosphere, the reaction mixture was cooled down to ambient temperature, the product filtered and washed with methanol several times. It was dried in the vacuum desiccator [17]. The white powder product of (Cs-g-Arg) was obtained with 88.5% yield. Scheme 1 represents the chemical reaction.

Synthesis of Chitosan-Glycine-Aspartic acid nanoparticle (Cs-Gly-Asp)

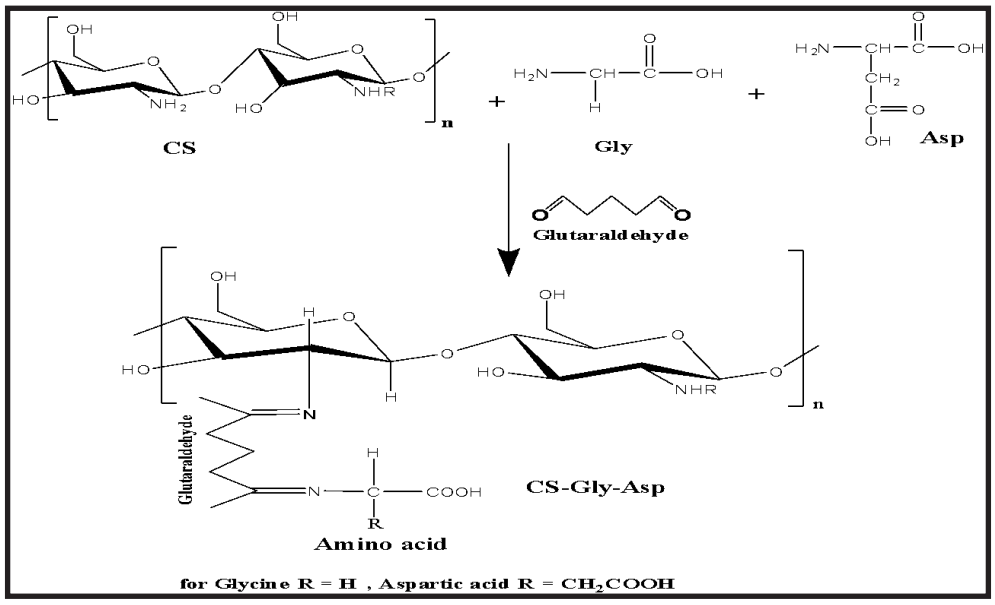
Cs-g-Asp was prepared by the reaction between 1g chitosan and amino acid was (0.5 g Glycine, 0.5 g Aspartic acids) dissolved in 50 ml of 1% acetic acid by weight and stirred for 3 h using a magnetic stirrer at room temperature. The homogeneous mixture was extruded in the form of droplets using a syringe into NaOH-methanol solution (1:20 w/w) under a stirring condition at 400 rpm. Then, it was allowed to react with a 6.25%glutaraldehyde solution at 50˚C for about 10 minutes after washing with water. Finally, the products were washed with hot and cold water, followed by drying in the vacuum desiccator [18,19]. The yellow powder of (Cs-Gly-Asp) was obtained with a 77% yield. Scheme 2 represents the chemical reaction.

Nanoparticles preparation

Chitosan and chitosan-amino acid derivatives were dissolved with 2% acetic acid solution pH=3. The solution was then subjected to sonication technique for 5min at 50W with a pulse of 5sec and 10sec between pulses[20]. The produced nanoparticles then were characterized by Scanning Electron Microscope, SEM, (ZEISS, Germany) adjusted at 100 Kv. ImageJ program was used to estimating the mean size and total account of each polymer NPs.



Scheme 1. Chemical grafting reaction equation of the Cs-g-Arg.



Scheme 2. Chemical grafting reaction equation of the Cs-Gly-Asp.

Cell toxicity assays

MCF-7, BT and SKBR-3 cells were grown in appropriate media MCF-7: DMEM + %10 FBS; BT: RPMI + %20 FBS+ insulin; SKBR3: DMEM + %15 FBS. Cells were cultured in a 95% CO_2 incubator at 37 ºC. Cells were cultivated for 24 h prior to transfection, one hundred µl of cells (7500 total cells) were seeded into 96 well microtiter plates and left to adhere for 24 h by incubation overnight on the CO_2 incubator at 37ºC.

The following day, the medium was expelled from the wells and supplanted with filter sterilized complete medium containing the polymer at concentrations 1 mg/ml, (100 µl/ well). The plates were then incubated with polymer solutions for 24, 48 and 72 h, separately, and cell viability was compared to the untreated cells. In the case of the 24 h incubation media containing polymer was isolated at 24 h and switched with complete media (and so on with other maturation times). 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 3.5 h. and secured with tinfoil, agitate cells were done on an orbital shaker for 15 min. Then the medium was removed and DMSO (100 µl) was added before a further incubation of 30 min at 37 ºC.

Lastly, the absorbance at 550 nm with a reference filter of 620 nm of the plates was read with the Tecan plate reader. Absorbance values were blanked against DMSO only, and the absorbance of cells exposed to medium only (nor chitosan neither its amino acid derivatives were added) was taken as 100 % cell viability (the control) [21].

DNA fragmentation assay

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

BT breast cancer cell lines (2×10^5 cell/ml) were cultured in RPMI media containing 20% FBS+insulin, at 10 ml per Petri dish. Upon formation of monolayer cells, 100 µl of a concentration (1 mg\ ml) for each chitosan or chitosan amino acid derivatives nanoparticles were added. After 24 hours of incubation, cells were collected by the addition of trypsin, centrifuged for 5 min at 1000×, and lastly washed with PBS. Cells were stained according to the kit's 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.

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, highly significant if $P < 0.001$.

Results and Discussion

Characterization of the prepared chitosan nanoparticle derivatives

FTIR characterization

FTIR spectra of the Chitosan and its amino acid derivatives were recorded from the SHIMADZU 8400S FTIR spectrometer/Japan. The FTIR of pure chitosan exhibits broadband with a peak at 3429 cm^{-1} due to (-OH) stretching and a (-NH) stretching, a weak band with a peak at 1639 cm^{-1} assigned for amide I which was left from the deacetylation process, verified that chitosan with a high degree of deacetylation was obtained, (Fig. 1). Also, the appearance of a strong band at 1454 cm^{-1} due to (C-N) stretching bond confirms the deacetylation process [22,23].

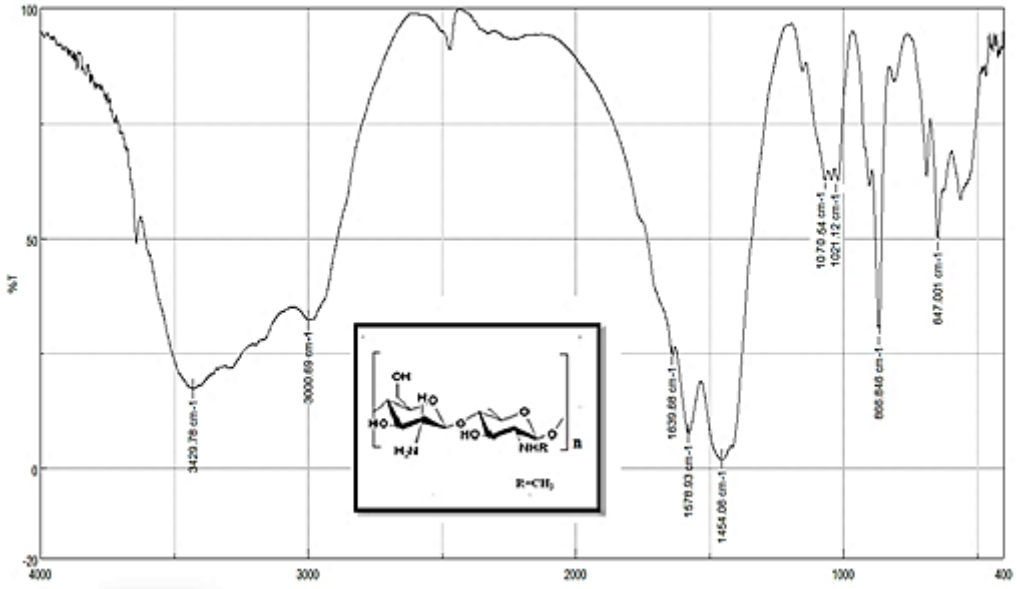


Fig. 1. FTIR spectrum of Chitosan.

Grafting arginine amino acid to chitosan gave the characteristic absorption band in FTIR. For example, the absorption of (O-H) and (N-H) appeared at 3390–3348 cm^{-1} and peaks at 2924-2854 cm^{-1} represented the stretching vibration of a methylene group ($–CH_2–$), (Fig. 2). The characteristic band at 1797 cm^{-1} corresponded to –C=O stretching vibrations of the ester carbonyl group and 1678 cm^{-1} C=O and C=N stretching vibrations, 1408 cm^{-1} C–N stretching vibrations and 1134-1076 cm^{-1} C–O–C stretching vibrations are assignable to chitosan. New absorption peaks for Arginine appeared at 1797 cm^{-1} and 1643 cm^{-1} C=O and C=N stretching vibrations, which confirm the synthesis of Cs-g-Arg [24].

FTIR spectrum obtained for CS-Gly-Asp derivative shows an absorption band at 1753 cm^{-1} characteristic for the C=O, the band at 2972 and 2953 cm^{-1} due to aliphatic C-H stretching. The band at 1564 cm^{-1} due to C=N stretching vibration. The band appeared at 1438 $cm^{-1}$is assigned to the aromatic C=C stretching vibration. A new peak appears at about 1564 cm^{-1} due to imine bond (-C=N-) which was formed as a result of the cross-linking reaction between the amino group in chitosan and aldehydes group in glutaraldehyde (Fig. 3). This was due to the overlapping of peaks corresponding to –NH-stretching vibrations in $-NH-COCH_3$ of the original chitosan with that of imino (-C=N-) stretching at 1564 cm^{-1} of the newly formed structure between the amino group of chitosan and an aldehyde group of glutaraldehyde. A reaction takes place in the formation of cross-link as follows [19]:

$---NH_2 + O=HC --- \rightarrow ---N=CH---$

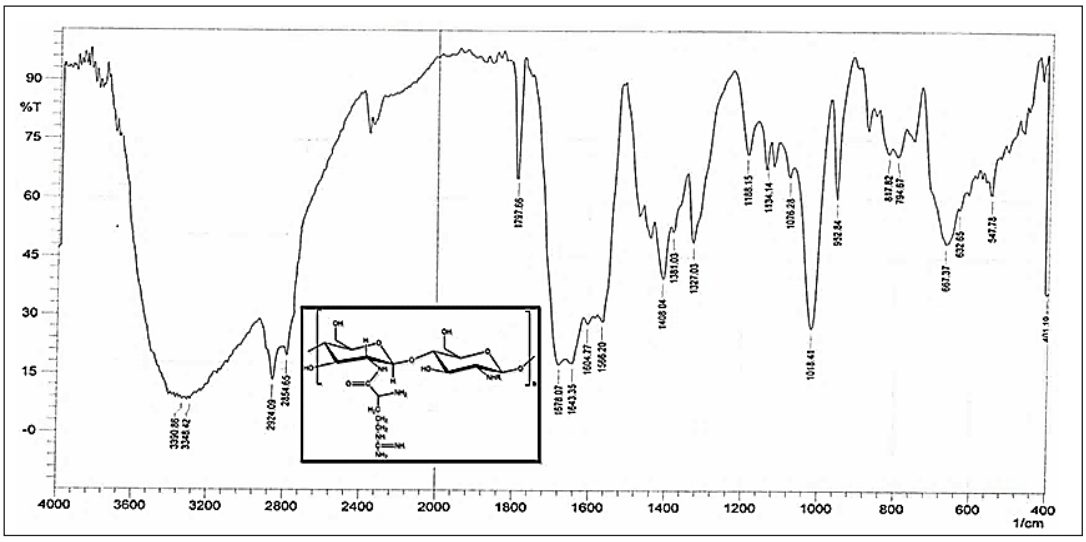


Fig. 2. FTIR spectrum of Chitosan-grafted-Arginine (Cs-g-Arg).

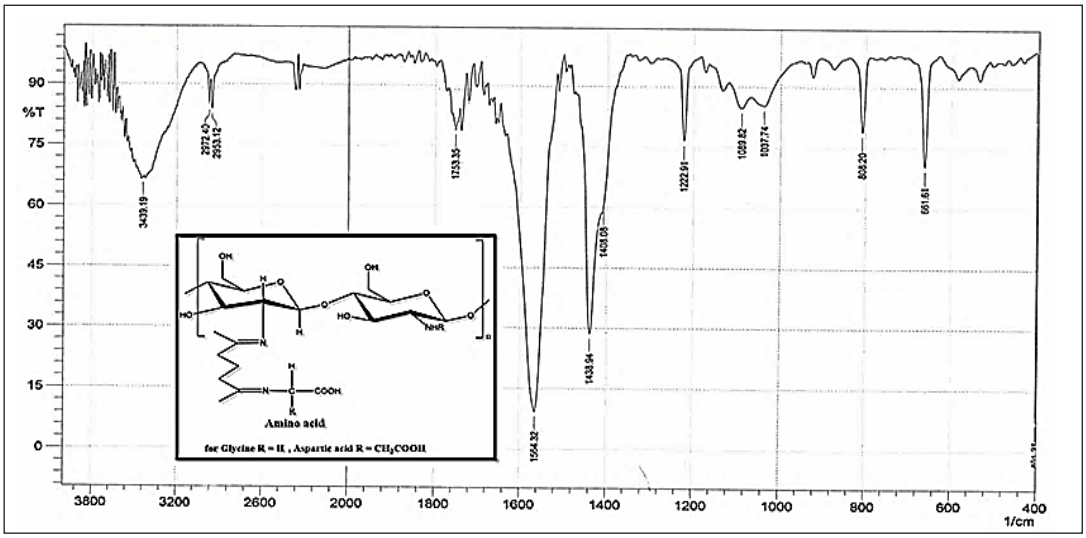


Fig. 3. FTIR spectrum of chitosan-glycine-aspartic acid (Cs-Gly-Asp).

1H NMR characterization

The ^{1}H-NMR spectroscopy technique was used to confirm the structure of Chitosan and its two amino acid derivatives. The spectra were recorded by INOVA 500 MHz. ™NMR Spectrometer/USA using deuterated acetic acid and DMSO solvents.

Figure 4 shows the ^{1}H-NMR spectrum of Cs, the proton peaks area of Cs appeared at 4.6 ppm (H-1 of glucosamine ring), 3.1 ppm (H-2of glucosamine ring), 3.1–3.8 ppm (H-3, H-4, H-5, H-6). Moreover, the figure shows the presence of the peaks at displacement (2.5) ppm,which areattributed to the protons of DMSO solvent [25].

The ^{1}H-NMR spectrum of Cs-g-Arg is displayed in Fig. 5. It shows that the presence of the following peaks: at 4.6 ppm (H-1), 3.3 ppm (H-2), 4.0–3.5 ppm (H-3, H-4, H-5, H-6). Compared with Cs, it is easy to observe the characteristic Arg 2.31–3.58 ppm chemical shifts of the methylene protons in (CH_2) groups, 1.6 ppm the amine protons in (NH_2)groups [26]. In additionto the protons of DMSO solvent appears at displacement (2.4) ppm.

Cs-Gly-Asp derivative gave a 1H-NMR spectrum shown in Fig. 6. One can observe easily the following peaks: at 4.44 ppm (H-1), 3.5–3.85 ppm (H-2, H-3, H-4, H-5, H-6), 1.47–1.6 ppm the methyl protons in (CH_3) groups, 2.0-2.3 ppm the methylene protons in (CH_2) groups, 2.2-3.5 ppm (COOH of Asp). It is also noticed that the presence of the peaks at displacement (11.5) ppm belong to the protons of acetic acid solvent [27].

Characterization of Chitosan nanoparticle derivative by SEM

Figures 7 and 8 show the SEMmicrograph and its Image-J size of chitosan nanoparticles, respectively. Purified chitosan is seen to be in the shape of small spherical nanoparticles with a minimum and a maximum size of nano polymeric ranging between 17-100 nm.

Chitosan is acetylated glucosamine that has gained interest due to its suitable properties to be used for nanoparticle preparation fabrication. Biocompatibility, pH sensitivity, mucoadhesivity, and low immunogenicity make this material to be used extensively [28].

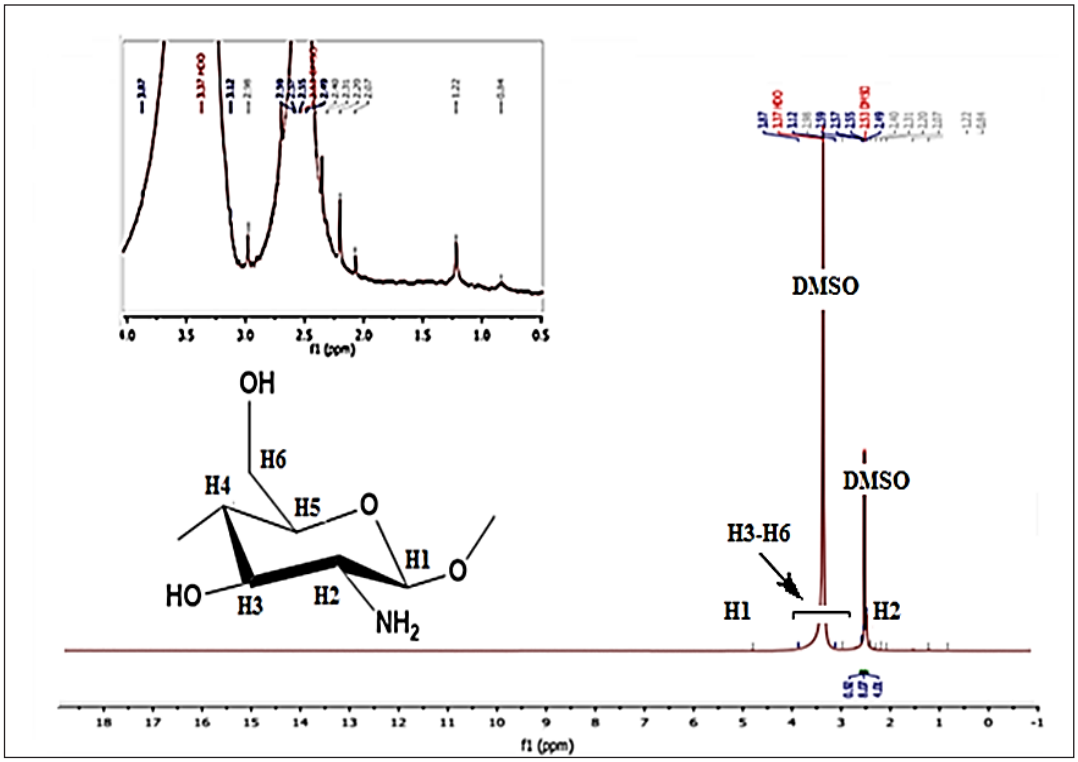


Fig. 4. ^{1}H-NMR spectrum of Chitosan.

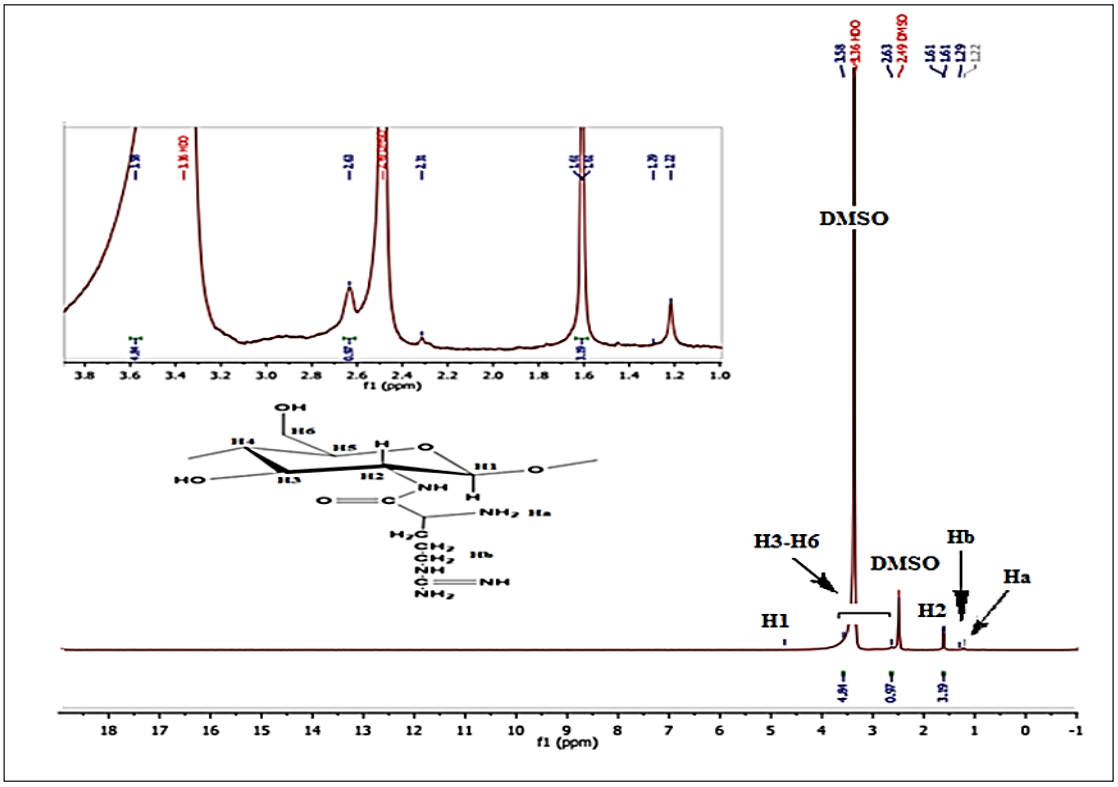


Fig. 5. 1H-NMR spectrum of (Cs-g-Arg).

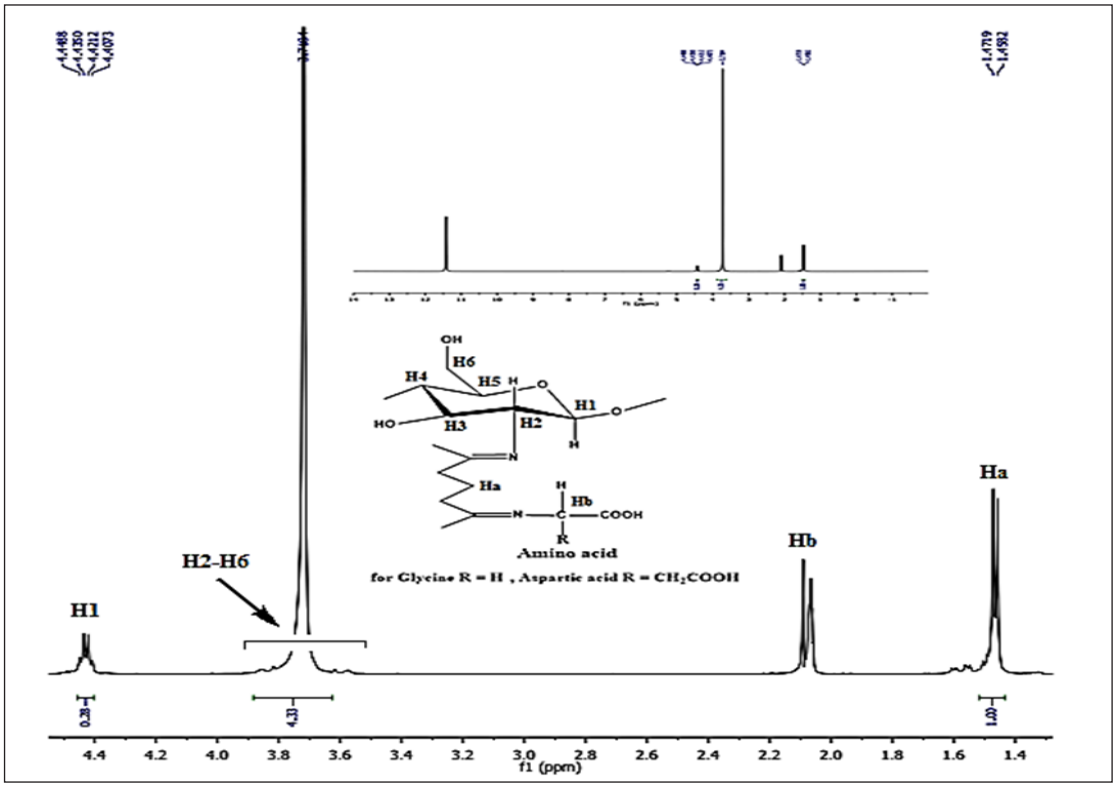


Fig. 6. ^{1}H-NMR spectrum of (Cs-Gly-Asp).

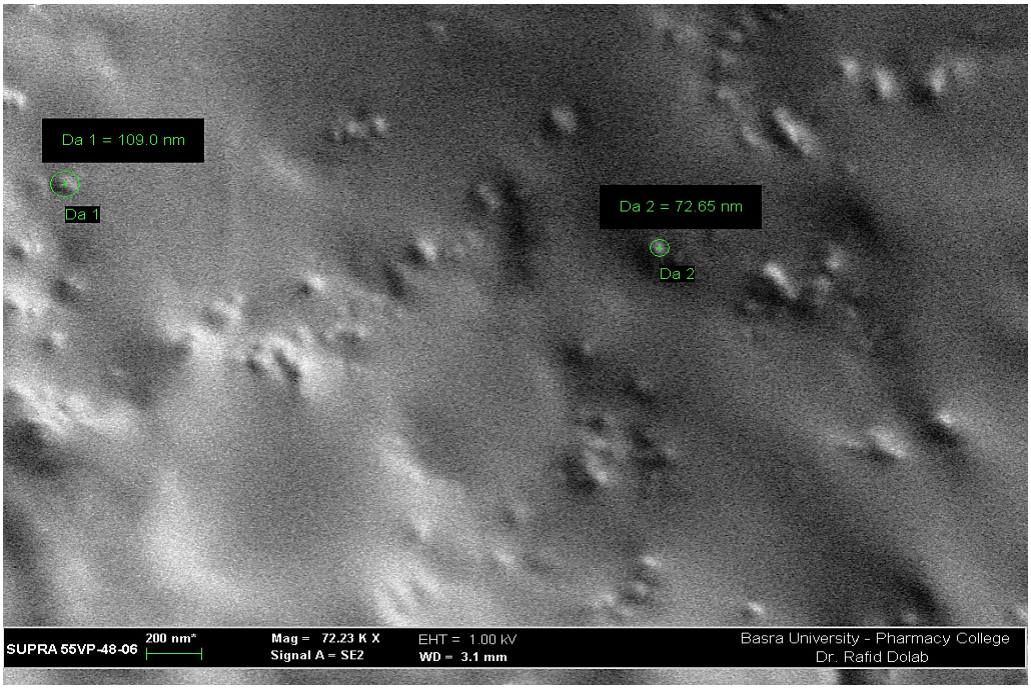


Fig. 7. SEM micrograph of prepared chitosan nanoparticles.

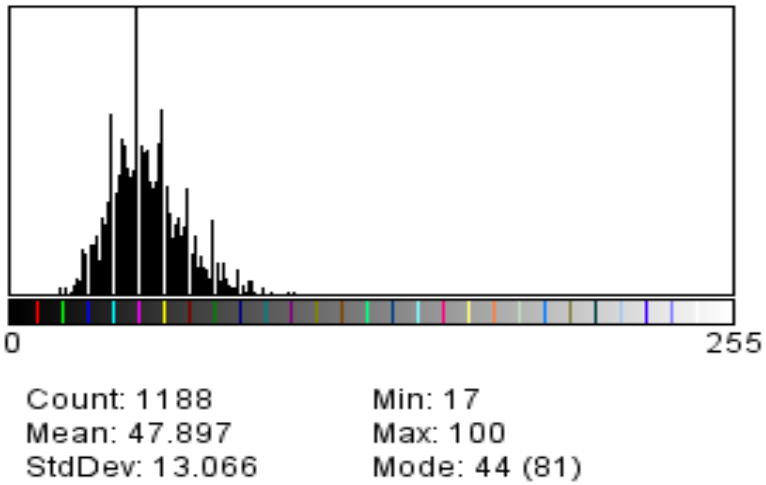


Fig. 8. Size of chitosan nanoparticles using Image-J program.

The successful preparation of Cs-Arg nanoparticles was proved by the SEM micrograph, and it showed the presence of spherical particles with diameters of 44-51nm (Fig. 9). The particle size represented an important parameter and one of the main goals of the delivery polymeric system. Figure 10 shows the minimum and maximum nano size values of Cs-g-Arg nanoparticles in the range of 14-79 nm. The small particle size and spherical shape were shown in Fig. 11 for Cs-Gly-Asp, which were determined using scanning electron microscopy (SEM). The results of SEM demonstrate clearer images of the homogenous dispersion of the nanoparticles with an approximate diameter of 59-77 nm in the polymer matrix. This is indicative of the use of the proper method in the preparation of nanocomposites. Figure 12 shows the minimum and maximum size values of Cs-Gly-Asp nanoparticles of 14-97 nm.

Fig. 9. SEM micrograph of (Cs-Arg) nanoparticles.

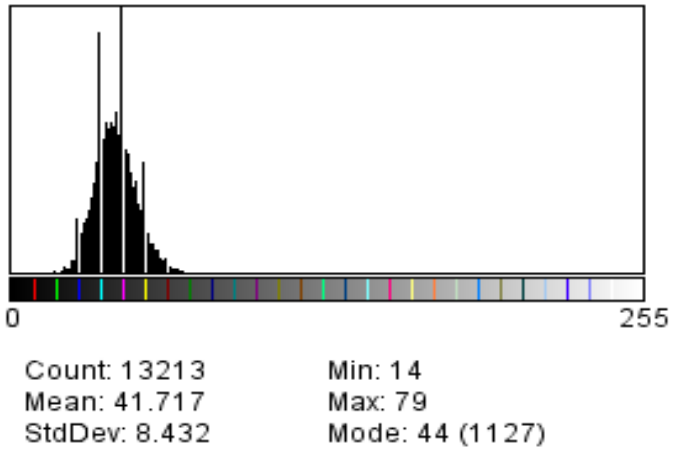


Fig. 10. Size of (CS-g-Arg) nanoparticles using Image-J program.

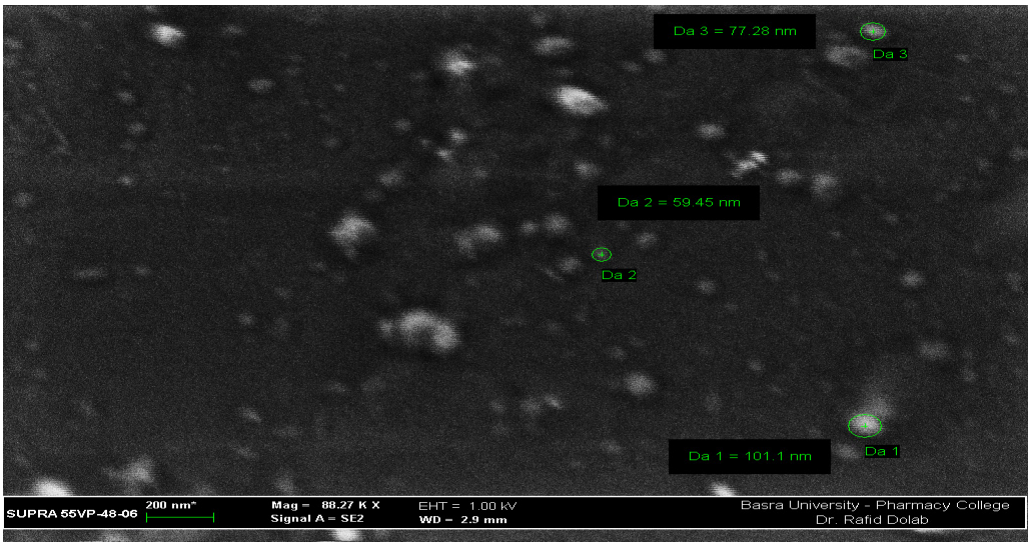


Fig. 11. SEM micrograph of (Cs-Gly-Asp) nanoparticles.

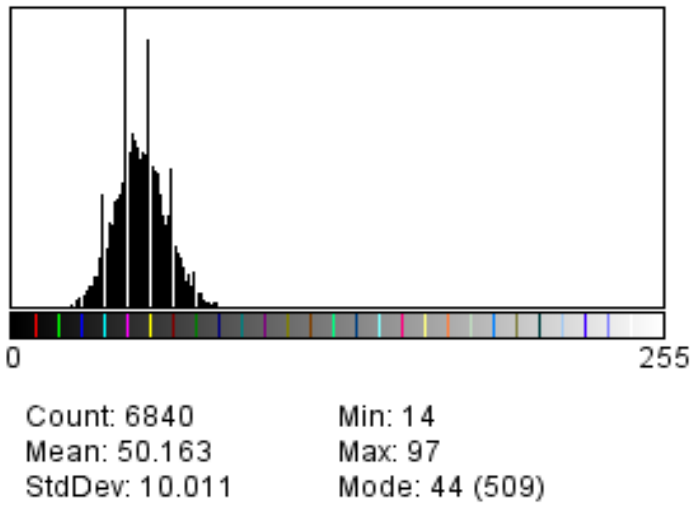


Fig. 12. Size of (CS-Gly-Asp) nanoparticles using Image-J program.

Cytotoxicity effects

To stimulate the *in vitro* cytotoxicity effect of chitosan and its CS-Arg and CS-Gly-Asp derivatives nanoparticles in human breast cancer cell lines, as cell viability assay, the comparison determined between these amino derivatives revealed the presence of different pattern of cytotoxicity of these derivatives but still highly significant, $p<0.001$, in decreasing of cell growth when compared with control groups, Table 1 and Fig. 13 represented the growth manner of the cell viability of all types of breast carcinoma as percentage of cell viability after treating with the two chitosan-amino acid derivatives, our investigation reported that chitosan-amino acid derivatives nanoparticles were significant against the growth progression of human breast cancer cell lines included in this study. These investigations were agreed with several new scientific publications, reported that the inclusion of amino acid molecules to the backbone of Cs has improved their physiochemical properties such as solubility and giving rise to some interesting synergistic characteristics for use in drug delivery and tissue engineering, as well as other potentially useful biomedical properties such as anticancer, anticoagulant, antimicrobial, and cholesterol-lowering activities [29,30] reported the synthesis of conjugate based on arginine Arg-grafted Cs and observed a great enhancement in the water solubility of Cs. As an amino acid with cationic surface charge, the Arginine has been reported to promote the internalization ability of Arg-grafted Cs NPs through the electrostatic interaction between positively surface-charged NPs and negatively charged cell membrane [1], and Park et al. [2] confirmed that the conjugation of Cs with Arginine enhanced small interfering RNA delivery to cancer cells.

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 highly significantly affected (p< 0.001).

Samples	Cell Lines		
	BT	MCF-7	SKBR 3
Cs	62.00±13.528	22.00±5.000	49.33±3.055
CS-Arg	28.67±9.074	26.33±5.508	44.67±4.163
No Cs, only Cs-Gly-Asp	76.67±13.650	22.00±1.000	32.00±2.000
Control	100.00±.000	100.00±.000	100.00±.000

Rlsd= 6.382

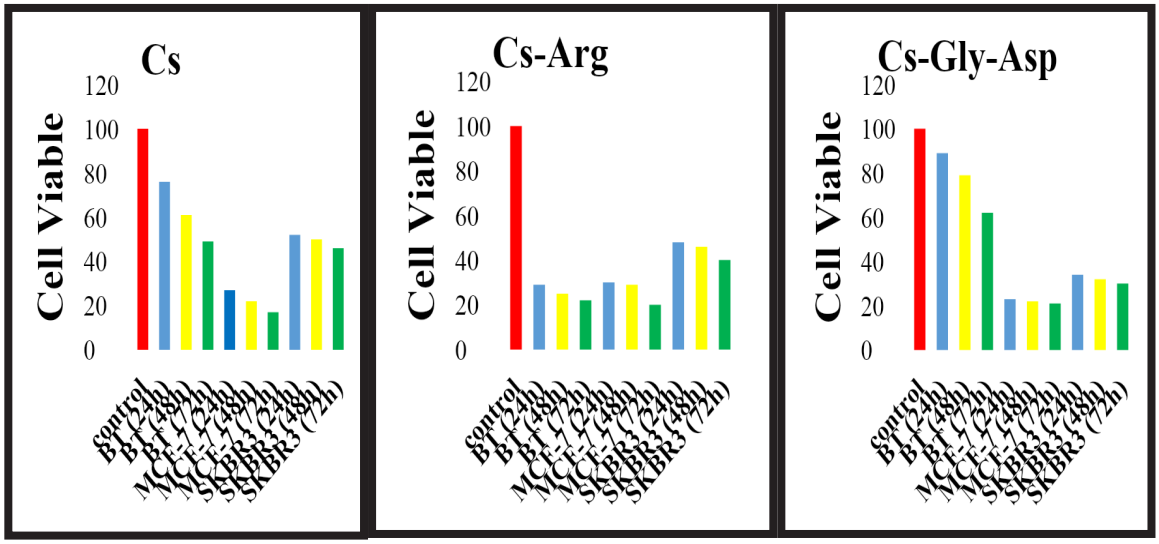


Fig. 13. Cell viability percentages of human breast cancer cell lines (BT cells, MCF-7 cells and SKBR3 cells) affected with Cs-Arg, and Cs-Gly-Asp NPs, at different times for 24 h, 48 h and 72h.

Genotoxicity assay (DNA fragmentation)

In this study, we used the Flow cytometry technique and acridine orange (AO) staining to estimate the genotoxicity of Cs NPs and its amino acid derivatives NPs, on BT breast cancer cell line. Acridine orange is taken up by both viable and non-viable cells and emits green fluorescence if combined into a double-stranded nucleic acid (DNA) or red fluorescence if bound to single-stranded nucleic acid fragmented (DNA). Today, the AO test is one of the most popular methods used to confirm the integrity of cell DNA. The effect of chitosan NPs and other derivatives on BT breast cancer cell lines, as a DNA fragmentation assay was listed in Table 2 and shown in Fig. 14, which established the genotoxicity as a % DNA fragmentation index (% DFI), using flow cytometry technique in comparison with (++) positive (media with cell lines and low concentration antitumor drug), (++++) positive (media with cell lines and high concentration antitumor drug) and negative controls (media only) (Fig. 15). The results showed that Cs NPs have no effect on the DNA of BT breast cancer cell lines; with % DFI reach to 6.77%, which appear less than negative control 13.6% and in comparison with (++) positive control 36.0% and (++++) control 64.2%. This data suggests that chitosan NPs have no negative effect on the nucleic material of the BT breast cancer cell lines and can be used as gene delivery uptake and that approved with several other recent reported results that have been intensively studied for use of Cs NPs as carriers to deliver various drugs and genes in cancer treatment [31-33], and that will be focused on the therapeutic potential of chitosan-based nanoparticles for cancer treatment.

A genuine observation comes from this study with regard to chitosan amino acid derivatives NPs, which act at multiple levels with % DFI, Cs-Arg (21.6%) and Cs-Gly-Asp (7.36%), respectively, in comparison with (++) positive control and positive (++++) samples. In turn, these values imply there is slight/or no effect on the human nucleic acid which can be represented as good as for nanoparticles pattern of the chitosan derivatives as gene delivery in cancer cell lines, 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 [34], these results approved the using of these polymers as biomedical and nanomedicine applications and gene delivery systems.

Conclusion

The low cytotoxicity with no genotoxicity of chitosan long chain amino acid derivatives of Cs-Gly-Asp in comparison with Cs-Arg enhances using of Cs-Gly-Asp in gene delivery for breast cancer cell lines and in gene therapy for both Cs-Arg and Cs-Gly-Asp.

TABLE 2. DNA Fragmentation Percent (% DFI) of chitosan NPs and chitosan derivatives NPs using BT breast cancer cell lines.

Sample	DNA Fragmentation (%)
Cs	6.77%
Cs-Arg	21.6%
Cs-Gly-Asp	7.36%
++ control	36.0%
++++ control	64.2%
No treatment	13.6%

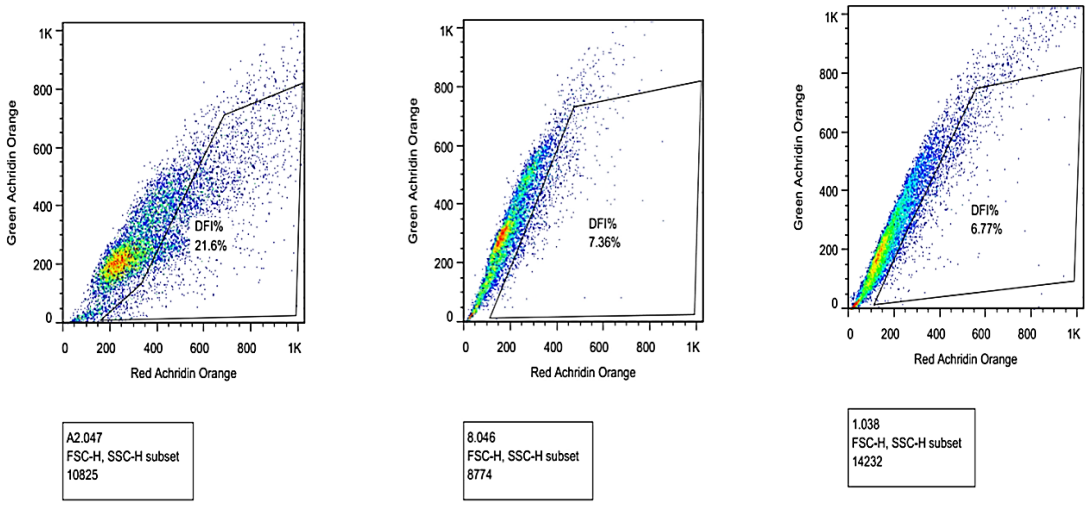


Fig. 14. DNA fragmentation of chitosan, Cs-Arg and Cs-Gly-Asp.

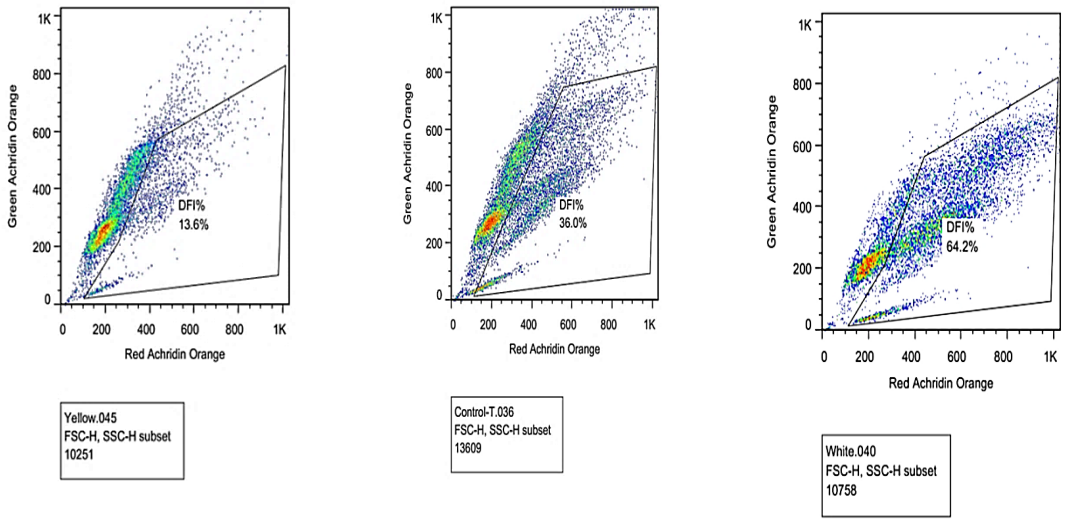


Fig. 15. DNA fragmentation of ++control, ++++ control and negative control

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تقييم مقارنللسمية الخلوية والجينية لجسيمات مشتقات الأحماض الأمينية النانوية للكيتوسان نحو خطوط خلايا سرطان الثدي البشرية

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يستخدم الكيتوسان، وهو بوليمر طبيعي، على نطاق واسع في المجالات الطبية والصيدلانية بسبب خصائصه المميزة كمبيدات للبكتريا والفطريات، وفوق كل ذلك تأثيرة كمضاد للأورام. نهدف في هذه الدراسة إلى تطوير نظام مضاد للأورام يعتمد على الكيتوسان (Cs) وبعض مشتقاته من الأحماض الأمينية، وهي الكيتوسان-الأرجينين (CS-Arg) والكيتوسان-أرجينين-الأسبارتيك (CS-Gly-Asp) النانوية ذات التوافق البيولوجي ونشاط مضاد للسرطان في علاجات مضاد الأورام. تم الحصول على المشتقات بحصيلة جيدة جدا، وشخصت بالتقنيات الطيفية وهي تقنية FTIR وتقنية 1H-NMR، وأكدت الأطياف الناتجة التراكيب الصحيحة لكل من الكيتوسان والكيتوسان المطعم بالأحماض الامينية، والتي حولت جميعها إلى جسيمات متناهية الصغر في الحجم من خلال تعريضهم لموجات فوق الصوتية. تم استخدام مجهر الإلكتروني الماسح (SEM) لتحديد شكل وحجم الجسيمات النانوية البوليميرية المحضرة، وتم تاكيدها باستخدام برنامج ImageJ.

تم تحديد السمية الخلوية للبوليمرات المحضرة باستخدام صبغة MTT، وكذلك تقنية التدفق الخلوي لجميع الجسيمات النانوية البوليميرية المدروسة ، ضد ثلاثة أنواع مختلفة من خطوط خلايا سرطان الثدي البشرية، واظهرت النتائج انخفاض عالي المعنوية ($P<0.001$)، في نمو خطوط خلايا سرطان الثدي مقارنة مع خطوط الخلايا غير المعالجة ، ولكن تأثير السمية الخلوية للجسيمات النانوية البوليميرية(CS-Arg) كان أكبر من الجسيمات النانوية البوليميرية (CS-Gly-Asp)، في حين لم يكن هناك تأثير سام ضد المادة الوراثية لخطوط الخلايا السرطانية نوع BT للجسيمات النانوية CS-Gly-Asp وكان التأثير طفيف بالنسبة للجسيمات النانوية CS-Arg.