Original Article

Investigating the Anticancer Potential of Chitosan Nanoparticles: *In Vitro* Assessment on TP-53 and PC-3 Cell Lines

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Abstract

Background: The increased threat of cancer is a critical challenge due to the absence of absolute treatment that eradicates this disease. Chitosan nanoparticles (CS NPs) offer promising opportunities for the development of anticancer medicines, particularly due to their high degree of adaptability and flexibility. **Objectives:** The purpose of the study was to investigate the effect of CS NPs on the viability of cancer cells *in vitro*. **Materials and Methods:** CS NPs were synthesized using concentrated acetic acid and sodium tripolyphosphate as cross-linker. CS NPs were described using atomic ultraviolet-visible spectrophotometer (UV-Vis), Fourier Transform Infrared Spectroscopy (FT-IR), and High-Resolution Scanning Electron Microscope (HRSEM). Then, the cytotoxic effect of CS NPs was estimated on TP-53 and PC-3 cancer cells in comparison with HdFn normal cell line using 3-[4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide (MTT) assay. **Results:** The formation of CS NPs was confirmed by UV/Vis and FT-IR with mean size of the CS NPs of 299.2 ± 12.72 nm, and they had a spherical form uniform distribution. Cytotoxic activity of CS PNs showed that treatment of TP-53 and PC-3 with low concentrations of CS NPs showed no significant reduction in cell viability. On the contrary, increasing CS NPs concentrations markedly correlated with significant ($P < 0.01$) inhibition of TP-53 and PC-3 cell viability. **Conclusions:** The method of concentrated acetic acid and sodium tripolyphosphate was employed to synthesize CS NPs within the nanoscale range. CS NPs can induce a reduction in cancer cell viability in a dose-dependent pattern.

Keywords: Chitosan, cytotoxicity, MTT, nanoparticles, PC-3 cells, TP-53 cells

Introduction

One of the largest health concerns facing humanity is cancer. Cancer is a life-threatening condition that necessitates an effective treatment strategy.[1] Radiation therapy, chemotherapy, and surgery are now the cornerstones of cancer treatment.[2,3] Problems include metastasis, resistance to medications, toxicity, and undesirable adverse reactions, and cancer relapses are still frequently seen as a result of lingering tumor cells and the presence of tumor stem cells.[4,5]

In addition to increasing the solubility of drugs, extending the medication residence period, and enhancing stability, polymer nanoparticles have considerable potential for hydrophobic drug delivery.[6] Numerous advantages include low production costs, harmonious appearance, reduced toxicity, the capacity to protect medications and other medically active chemicals from the environment, enhanced bioavailability, and therapeutic efficacy.[7,8] Polymeric NPs are among the most thoroughly studied materials.

Chitosan is a cationic polymer found in nature, which is a linear polymer that is, deacetylated from chitin is chitosan.[9,10] Chitosan's use is encouraged by its

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availability, affordability, and abundance in a wide range of applications. Chitosan contains two kinds of reactant functional groups (a hydroxyl group and an amino group) that can be exploited chemically to change the material's properties and create various compounds under mild circumstances.[11,12] Such compounds can be used effectively in a variety of biomedical settings, such as improving nucleic acid transfection in gene therapy and a number of other applications aimed at maximizing medication delivery and supporting tissue engineering.[13] One of the key elements affecting chitosan's properties is the degree of deacetylation.[14] A stable chitosan-DNA complex can be created by effectively complexing the natural, biodegradable chitosan polymer with DNA.[15]

Chitosan has drawn more attention recently due to its industrial uses in the biomedical, food sectors, and chemical.[16] Chitosan has been shown in research to have several inherent qualities, including the ability to heal wounds and have antibacterial and antifungal effects.[17] Due to its availability, extremely versatile chemical compositions, simplicity of chemical modification, and low cost, chitosan has a variety of intriguing qualities.[18] As a result, it is frequently employed in biomedical applications. Because of a predominance of diffusion into tumors and a slow release of drug-free substances from the combines, chitosan and its derivatives exhibit excellent anticancer benefits with a reduction in the adverse effects of chemotherapy.[19] As a result, they are effective in treating tumor and lowering the negative nonspecific adverse effects of chemotherapy. Additionally, the combination of imaging contrast aids in the formation of these nanoparticles results in a very effective method for cancer diagnosis.[20]

The urgent need for improvements in biomaterials-based nanoparticles has increased through the past few decades as alternatives for effective, efficient, affordable, and biocompatible alternative therapy and called for the eradication of resistant cancer cells. For this reason, the objective of this research is to utilize chitosan prepared within the nanoscale range to investigate its cytotoxic activity against cancer cells.

Materials and Methods

Materials

Low molecular weight chitosan was obtained from Sigma-Aldrich (Germany). Acetic acid and sodium tripolyphosphate were purchased from VWR (USA). All materials, media, and reagents used in tissue culture technique were purchased from Germany's Sigma-Aldrich.

Synthesis of chitosan nanoparticles (CS NPs)

Low molecular weight CS powder (2 g) was dissolved in 287.5 mL distilled water and 32.5 mL concentrated acetic acid to produce CS solution. The insoluble components were then removed from the CS solution using Whatman filter paper No. 7, and the solution was twice further filtered through a 0.22-μm filter to produce a highly homogeneous mixture free of any insoluble CS. Tween 80 (0.005%–0.02% v/v) was added to the CS solution as an emulsifier to stop the development of large-diameter particles and prevent NPs coagulation. Under steady magnetic stirring at room temperature, sodium tripolyphosphate (TPP 1 mg/mL) was added. Finally, ultracentrifugation (14,000 × *g*) was used to extract the nanoparticles from the suspension for 20 min at 14°C.[21]

Characterization of CS NPs

Ultra violate/visible (UV/Vis) spectrum

After melting in 2% (v/v) aqueous acetic acid, the produced CS NPs were subjected to UV-visible spectral scans. This was achieved at a wavelength spectrum of 200–600 nm utilizing a Shimadzu spectrometer (Model UV/1800, Japan) running at an accuracy of 1 nm.[22]

Fourier Transform Infrared Spectroscopy (FT-IR)

Using a Bruker Optik QCL system, the spectrum of FT-IR was captured in transmission mode in the 400–4000 cm^{-1} range.

High Resolution Scanning Electron Microscope (HRSEM)

The morphology and size of CS NPs were examined using HRSEM (ZEISS Gemini 300, Germany). Drops of CS NPs solution were cast onto glass slides to create the SEM samples, which were then allowed to air dry at ambient temperature. SEM pictures were captured at the scale of 4–5 μm. Using ImageJ software, SEM pictures were analyzed to determine the mean diameter of length and area (30 measurements).

Cell lines and cell line maintenance

A human lung adenocarcinoma cell line TP-53, prostatic adenocarcinoma PC-3, and normal HdFn cell lines were supplied from Al-Nahrain Biotechnology Center/Al-Nharin University. Cells were grown in RPMI-1640 media contained 10% fetal bovine serum, 10^3 IU of penicillin G, and 0.001 g of streptomycin per 100 mL of media. A humidified incubator with 5% CO_2 was used to incubate the cells at 37°C. Cells were seeded at a density of 2×10^5 cells/mL into tissue culture flasks. Cells were detached after a quick trypsinization (50 mg/mL) when they entered the exponential growth phase (between 36 and 48 h), and then they were seeded at the desired concentration.[23]

Cytotoxicity test (MTT assay)

Using the MTT colorimetric assay, the cytotoxic potential of CS NPs was evaluated against TP-53, PC-3, and HdFn cells. 96-flat plates were seeded with an aliquot of 200 μL of suspended cells (1×10^5 cells/mL) in culture media, and the

plates were then incubated for 24 h at 37°C with 5% CO_2. A variety of concentrations for each treatment (25–400 µg/mL) were introduced to the wells after the medium had been removed from the incubation period. Cells without any treatment are used as control (cells treated with serum-free media). For an additional 24 h, plates were incubated at 37°C and 5% CO_2. About 10 µL of MTT solution was then added to each well, and the plates were then incubated at 37°C with 5% CO_2 for another 4 h. After properly decanting the media, 100 µL of the solubilization solution was added after waiting 5 min. Using an ELISA reader, the final response (formazan formation) was revealed at 575 nm (Bio-Rad, USA).[24] The cytotoxicity was expressed as IC_{50} and the formula used to calculate viability (%) was as follows:

Viability (%) = OD control − OD sample/OD Control × 100

Statistical analysis

Graph Pad Prism version 9.0 (Graph Pad Software Inc., La Jolla, CA) was carried for all statistical analyses. To investigate the variations between the several groups, a one-way analysis of variance (ANOVA) (Tukey's *post hoc* test) was done. The statistical thresholds for significance were **P* 0.05 or ***P* 0.01 for all data given as mean standard deviation. Triplicates of each experiment were run independently ($n = 3$).

Ethical approval

The study adhered to ethical standards as outlined in the Helsinki Declaration and received approval from both the General Directorate of Health and the Hospital Laboratory, as well as the Scientific Committee of the College of Science for Women at the University of Babylon in Hilla City, Iraq. Prior to sample collection, verbal and written consent from patients was obtained. The study protocol was subjected to review and approval by a local ethics committee at the College of Science for Women, University of Babylon under Project No. 6 on October 4, 2023.

Results

Synthesis and characterization of CS NPs

The method of synthesizing CS NPs was first mentioned by Calvo *et al*.[25] who concentrated on the chitosan: TPP ratio. Using it to regulate particle size and dispersity. This extensively utilized technique, however, is vulnerable to unregulated intra- and intermolecular crosslinking between TPP and chitosan which causes accumulation, excessive growth, and significant polydispersity of CS NPs.[26] Another factor that highly affects the size of CS NPs is the primary concentration of CS, molecular weight, the ability of CS for acetylation in an acidic environment, and the presence of salts in the reaction medium.[27] The UV/Vis spectra of synthesized CS NPs were examined, the results in Figure 1 show a corresponding transition of CS NPs detected at a wavelength of 250 nm.[22]

Figure 2 displays the FT-IR spectra of synthesized CS NPs. At 3230 cm^{-1}, the OH bond stretching vibration was detected in CS NPs, whereas at 2800 cm^{-1}, the C–H asymmetrical stretching band was observed. The existence of the C=O bending of the amide I band, which bent the oscillations of the N–H (amide II band , N-acetylated residues), was linked to the absorption peaks at 1680 and 1475 cm^{-1}. The peak at 1170 cm^{-1} was attributed to the (C–O–C) bridge's antisymmetric stretching, whereas the vibration at 590 cm^{-1} was related to C–H stretching.[28] This suggests that to create CS NPs, the TPP anions and the ammonium groups of CS were cross-linked.[29]

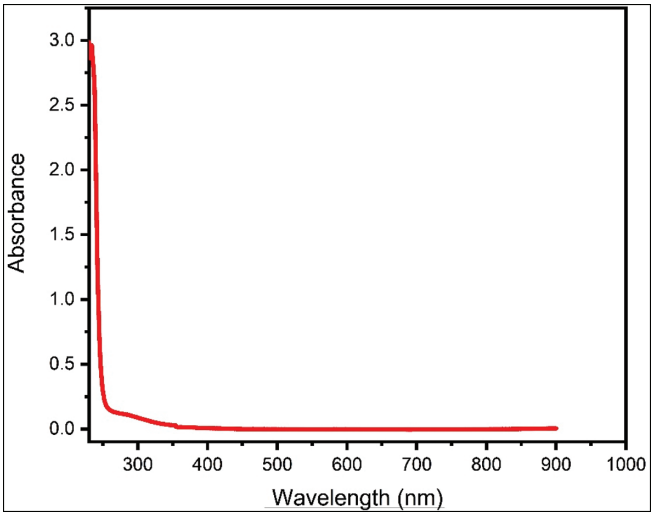

Figure 1: UV–visible spectrum of synthesized chitosan and chitosan nanoparticle

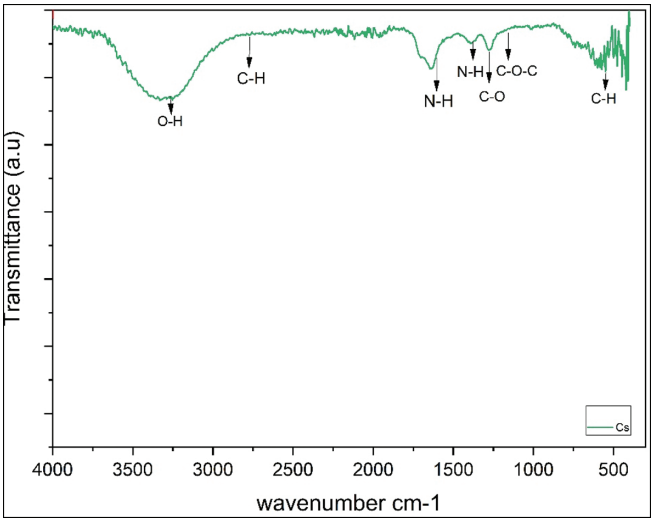

Figure 2: FT-IR spectrum of synthesized CS NPs

Using a scanning electron microscope, the shape and size of the CS NPs were examined. CS NPs. Images revealed that CS NPs composed of dispersed particles with approximately uniform spherical shapes. The mean particle size value was 299.2 ± 12.72 nm [Figure 3].

Cytotoxic activity of CS NPs on cancer cell viability

The cytotoxic potential of CS NPs was evaluated against TP-53 and PC-3 cancer cells compared with normal cells of HdFn cell line. Increasing concentrations of CS NPs (25, 50, 100, 200, and 400 µg/mL) were used in MTT experiment for each cell line. Results depicted in Figure 4a and Table 1 that at lower concentrations of CS NPs (25 and 50 µg/mL) no significant differences were observed in TP-53 cell viability reduction compared with HdFn cells. On the other hand, the survival rate of TP-53 cells was significantly ($P < 0.01$) reduced at higher concentrations of CS NPs with a maximum inhibition rate of 74.92 ± 1.8% at 400 µg/mL comparing with HdFn cells. IC_{50} values of CS NPs were 74.32 and 270.1 µg/mL in TP-53 and HdFn, respectively. In PC-3 cells, the cytotoxic activity of CS NPs was also determined in PC-3 cells [Figure 4b, Table 1]. At lower concentrations (25 and 50 µg/mL) no significant differences were noted in the pattern of cell inhibition; however, PC-3 cells exhibited a significant ($P < 0.01$) dose-dependent pattern of cell reduction compared with normal HdFn cells. The calculated IC_{50} of CS NPs in PC-3 cells was 109.5 µg/mL.

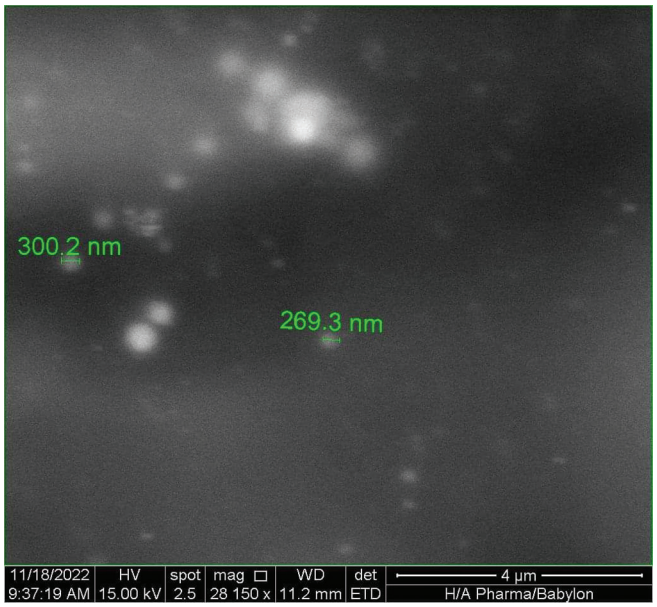

Figure 3: High-resolution scanning electron microscope image of CS NPs

Discussion

Chitosan nanoparticles were synthesized by ionic gelation using concentrated acetic acid to dissolve chitosan and tripolyphosphate (TPP) as a polyanionic cross-linker. Acetic acid protonates chitosan, making it soluble, while TPP initiates inter and intramolecular crosslinking between chitosan chains to form nanoparticles.[30] Parameters like chitosan and TPP concentration, mixing speed, temperature, and order affect nanoparticle size and properties. Ionic gelation is a simple, mild method to synthesize biocompatible chitosan nanoparticles with potential as drug delivery carriers.[31] The addition of TPP causes chitosan chains to form nanosized cross-linked structures by ionic interaction between the amine groups of chitosan and phosphates of TPP.[32] The nanoparticles can be optimized by tuning synthesis conditions for desired size, drug loading, and release characteristics.

Chitosan, a natural biopolymer derived from chitin, has emerged as a promising material for developing anticancer drug delivery systems. Chitosan can be fabricated into nanoparticles that have several favorable properties for cancer therapy.[33] The cationic nature of chitosan allows for electrostatic interaction with negatively charged DNA and RNA, enabling chitosan

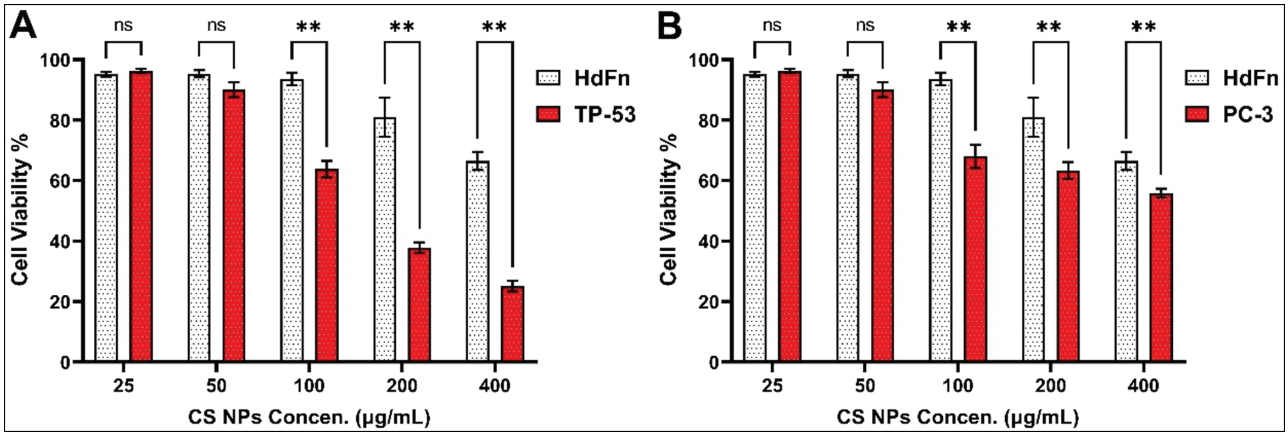

Figure 4: Mean ± SD cell viability of (a) TP-53 cells and (b) PC-3 cells compared with HdFn cells after treatment with CS NPs using MTT *in vitro* assay at 37°C, 5% CO_2 for 24 h. **: $P < 0.01$, NS: non-significant, SD: standard deviation, $n = 3$

Table 1: Multiple comparisons of mean ± SD cell inhibition of TP-53 and PC-3 with HdFn treated with CS NPs (25, 50, 100, 200, and 400 μg/mL) for 24 h

CS NPs μg/mL	Mean inhibition ± SD (%)		*P* value	Mean inhibition ± SD (%)		*P* value
	HdFn	TP-53		HdFn	PC-3	
25	4.8 ± 0.8^{a}	3.8 ± 0.6^{a}	0.9957 NS	4.8 ± 0.8^{a}	3.8 ± 0.7^{a}	0.9968 NS
50	4.7 ± 1.2^{a}	9.9 ± 2.4^{a}	0.1413 NS	4.7 ± 1.2^{a}	9.7 ± 2.4^{a}	0.1910 NS
100	6.4 ± 2.1^{a}	36.2 ± 2.7^{b}	<0.0001 **	6.4 ± 2.1^{a}	31.9 ± 3.9^{b}	<0.0001 NS
200	19.1 ± 6.5^{b}	62.2 ± 1.7^{c}	<0.0001 **	19.1 ± 6.5^{b}	36.6 ± 2.8^{b}	<0.0001 NS
400	33.5 ± 3.0^{c}	74.9 ± 1.8^{d}	<0.0001 **	33.5 ± 3.0^{c}	44.1 ± 1.4^{c}	0.0014 **

**: $P < 0.01$, NS: non-significant. Different letters (a, b, c) are considered significant ($P < 0.05$) in column

nanoparticles to condense and deliver these genetic materials into cells.[34] Chitosan nanoparticles can also encapsulate chemotherapy drugs and release them in a controlled manner near tumor sites. A key advantage of chitosan is its low toxicity and high biocompatibility compared to other synthetic nanoparticles. ADditionally, chitosan nanoparticles can be further modified with targeting ligands to actively bind to receptors overexpressed on cancer cells.[35] Due to their low cost and high efficacy in controlled drug release, CS NPs are among the most researched nanomaterial as possible carriers for drugs. The mouse melanoma cell line and the human gastric cancer MGC803 cell line, on the other hand, have been shown to exhibit some degree of toxicity when treated with soluble CS and CS microspheres, suggesting their potential as antitumor medicines.[36] In the present study, TP-53 and PC-3 cells were used as cancer model cells. The results showed that treatment with low concentrations of CS NPs, and cancer cells revealed no morphological changes. Moreover, the inhibition rate increased when the concentration of CS NP in the culture media increased. As a result, it can be inferred that CS NPs' suppression of cell viability was distinctly dose-dependent. Size of CS NPs, surface characteristics and degree of deacetylation (46%–88%), all have an impact on how they are toxic to cancer cells.[37] Additionally, it has been found that CS NPs derived from CS with molecular weights of 10–213 kDa, a size of 110–390 nm, and a percentage of deacetylation of 46%–88% exhibit high toxic rates versus cancer cells.[38]

This study found that the TP-53 and PC-3 cell lines of human cancer are significantly inhibited in their ability to survive due to the treatment of CS NPs. The study was in line with Casettari *et al*. findings which showed that CS NPs had improved physicochemical characteristics, such as solubility, produced some intriguing beneficial properties for application in tissue engineering and delivery of drugs, with additional potentially beneficial medical properties like anticancer, antimicrobial, anticoagulant, and cholesterol-lowering actions.[39] Different forms of CS derivatives acetyl chitosan, acetyl chitosan grafted polylactide, and chitosan grafted polylactide were successfully synthesized and showed strong antitumor activities by inducing cell proliferation reduction.[40] Furthermore, CS NPs were widely used as nanocarriers for encapsulating antitumor drugs and sustain effective drug delivery to target cells.[41]

Conclusions

We can conclude that the method of using low molecular weight CS treatment with concentrated acetic acid was successfully employed to synthesize stable CS NPs. CS NPs were verified using UV/Vis spectrophotometer, FT-IR, and HRSEM. Significant cytotoxic action was observed by the CS NPs against TP-53 and PC-3 cells in dose-dependent pattern.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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