

Anti-tumor Effects of Chitosan-grafted Cationic Polymer Nanoparticles against Human Breast Cancer Cell Lines

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ABSTRACT

Nanoparticles made from natural and synthetic polymers (biodegradable and nonbiodegradable) witness ongoing an interesting area of research and a techno-economic sector with full expansion in many application domains. Therefore, Chitosan was the target in this work. It is obtained with a 98.3% yield, with a high degree of deacetylation (92.1%) from the local shrimp cortex.

Chitosan-poly (PEG, PVA, and PVP) derivatives were synthesized by grafting copolymerization of chitosan with PVA, PVP, and PEG polymers, with yield, reached 77%, 78.3, and 87.5% respectively. FT-IR spectra of the chitosan and its derivatives verified the expected copolymers structures desired to be synthesized. All of the chitosan and its grafted polymers were converted to nanoparticles size by subjecting them to 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 micrographs revealed that the nanoparticles with spherical shapes and with different sizes were gained, but in general, they are less than 100nm in diameters.

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 comparing with the non-treated cell lines, especially with chitosan-grafted-poly (ethylene glycol) nanoparticles (CS-g-PEG), the cell viability reduced to $23.33\% \pm 1.528$, $25.67\% \pm 1.155$ and $45.67\% \pm 5.508$ against BT cells, MCF-7 cells, and SKBR3 cells, respectively.

Keywords

Nanoparticles, Chitosan, ImageJ program, Breast cancer, Cell viability.

Introduction

Biopolymer nanoparticles are small colloidal particles which are made of non-biodegradable and biodegradable polymers with a diameter generally around 200 nm [1]. These structures can enhance dermal uptake or improve the tolerability of active substances and allow drug targeting to the different layers of skin. In the preparation stage, the choice of the polymeric material is a crucial step in developing a pharmaceutical strategy [2].

They have many benefits over the other nanoparticle systems know so far, this is mightier due to their ease the preparation from well-

understood polymers and have high stability in biological fluids as well as during storage. The fabrication of these systems is greatly dependent on their morphology and composition of the periphery; they are characterized by their physicochemical structures. They include polymeric nanoparticles (NPs), dendrimers, polymeric micelles, polymersomes, polymer conjugate, polymer-lipid hybrid, and polyplex [3].

Nanoparticle innovation is being incorporated into numerous regions of sub-atomic science and biomedicine. Nanoparticles are magnificent tumor-focusing vehicles because of its distinctive property of solid tumors [4]. They can bind DNA fragments, drugs, and proteins, thus exerting the function of transportation and targeted therapy, and enhance increasing targeting efficiency of drug molecules. At present, numerous surface-modified compounds

are available, including 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 [5,6]. The most important applications are in the delivery of medicine and the gene and in the field of tumors [7]. Here in, we aimed to develop antitumor system based on chitosan-some polymers nanoparticle derivatives to improve their bioavailability and studying their cytotoxicity and Genotoxicity against human breast cancer cell lines as antitumor agents.

Materials and Methods

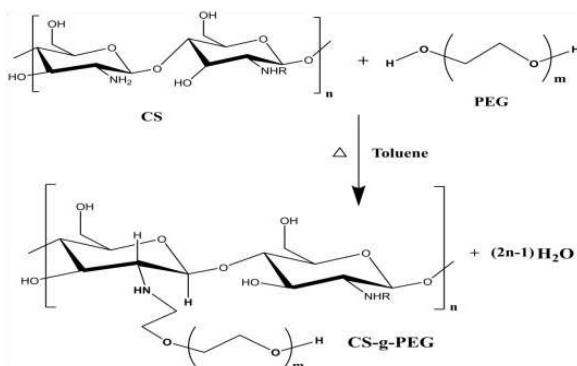
The high purity white powder product of chitosan with a 98.3% yield with a high degree of deacetylation was obtained from the shrimp cortex as described in the literature [8]. It was used as a base material to prepare the different polymeric chitosan nanoparticle derivatives. The solvents used, like toluene was sodium wire dried, and the others mentioned in the preparation methods were used as received.

Preparation of chitosan-grafted-poly(ethylene glycol) (CS-g-PEG)

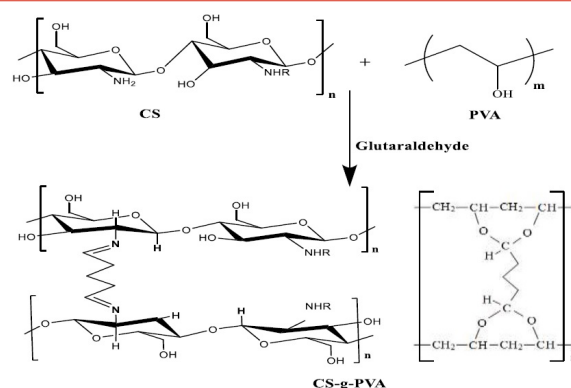
Two grams of poly(ethylene glycol), MWT 10000 g/mole, were dissolved in 100ml dry toluene, then the volume reduced to 50ml by distillation. It was allowed to interact with 1g chitosan, in refluxing apparatus for 3hr at boiling temperature of toluene under a dry nitrogen atmosphere. Afterward, it was left to cool down to ambient temperature, then the product was filtered and washed with diethyl ether several times, and the white solid powder of grafted chitosan (CS-g-PEG) was obtained with an 87.5% yield after drying [9]. Scheme (1) represents the grafting reaction.

Preparation of chitosan-grafted-poly(vinyl alcohol) (CS-g-PVA)

CS-g-PVA was prepared by reaction between 2 wt% chitosan dissolved in 1% aqueous acetic acid and 10wt% poly(vinyl alcohol) dissolved in deionized water. The homogeneous mixture of the two solutions was obtained by stirring for 3h, and then, glutaraldehyde, used as a crosslinking agent was added to the reaction mixture and stirred for an extra one minute. The product dried overnight in a hot air oven at 40°C [10,11]. The white powder product of (CS-g-PVA) was obtained with a 78.3% yield. Scheme (2) represents the chemical reaction.



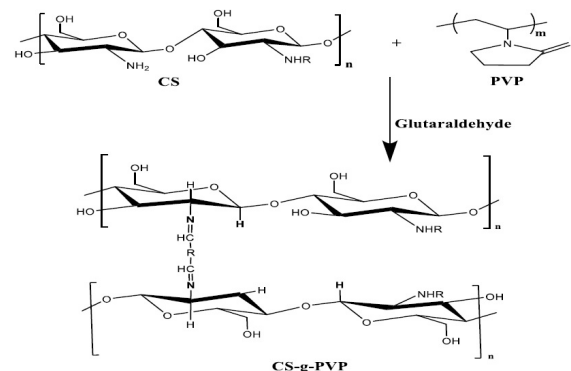
Scheme 1: Chemical grafting reaction equation of the CS-g-PEG.



Scheme 2: Chemical Grafting reaction equation of the CS-g-PVA.

Preparation of chitosan-grafted-poly(vinyl pyrrolidone) (CS-g-PVP)

2wt% of chitosan dissolved in 1% aqueous acetic acid was allowed to interact with 10wt% of polyvinyl pyrrolidone deionized water solution. The two solutions were mixed by stirring for 6h to reach homogeneity. Then, glutaraldehyde, used as a crosslinking agent was added to the reaction mixture and stirred for an extra one minute. The product, dried overnight in a hot air oven at 40°C [12,13]. The yellow powder product of (CS-g-PVP) was obtained with a 77% yield. Scheme (3) represents the chemical reaction.



Scheme 3: Chemical Grafting reaction equation of the CS-g-PVP.

Preparation of chitosan and its derivatives Nanoparticles

Chitosan and its cationic grafted polymers nanoparticles were prepared by dissolving 25mg of the material in 25mL of 2% acetic acid solution with pH \approx 3.0, with overnight stirring and heating at 50°C, then the mixture was dispersed by subjection to the ultrasonic instrument for 5min at 50W with a time of 5sec and 10sec between pulses, to perform the nanoparticles of the polymer [14]. The produced nanoparticles then 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.

Cell toxicity assays

MCF-7, BT and SKBR-3 human breast cancer cell lines were grown in appropriate media MCF-7: DMEM + %10 FBS; BT: RPMI + 20% FBS+ insulin and SKBR3: DMEM + %15 FBS. Cells were cultured in a 95% CO₂ incubator at 37°C. Cells were cultivated for 24 h prior transfection, One hundred μ l of cells (7500 total cells)

were seeded into 96 well microtitre plates and left to adhere for 24 h by incubation overnight on the CO₂ incubator at 37°C.

On the following day, after removing the medium from the wells and, they were replaced with complete sterilized filter medium consisting the chitosan or its derivatives nanoparticles at concentrations of 1mg/ml (100 µl/well). The plates were transfer to be incubated with polymer solutions for 24, 48 and 72 h, individually, and cell viability compared to cells treated with media only. In the case of the 24 h incubation media containing polymer was removed at 24 h and replaced with complete media (and so one with other incubation 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 covered with tinfoil, agitate cells was done on orbital shaker for 15 min. Then the medium was removed and DMSO (100 µl) added before a further incubation of 30 min at 37°C. Finally 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 (no polymer added) was taken as 100% cell viability for the control [15].

Results and Discussion

Infrared spectrum of chitosan-grafted-poly(ethylene glycol) (CS-g-PEG)

Grafting chitosan with poly(ethylene glycol) produce an interesting FTIR spectrum, Figure 1. It shows at 3421-3298 cm⁻¹ the absorption of (O-H) and (N-H), 2916 and 2850 cm⁻¹ to (CH). The stretching of carbonyl group C=O appears as a sharp peak at 1669 cm⁻¹ and a peak at 1558 cm⁻¹ related to NH₂, 1188 and 1253 cm⁻¹ was assigned to the characteristic of C-O-C stretches vibrations of repeated -OCH₂CH₂ units of PEG block and the -COO- band stretching vibrations, respectively [16].

Infrared spectrum of chitosan-grafted-poly(vinyl alcohol) (CS-g-PVA)

FTIR spectrum of CS-g-PVA is also shown in Figure 2. It shows that peaks around 3466 cm⁻¹ which represents strong and broad H-bonded O-H stretch due to the presence of alcohol group. The peaks around 2922-2856 cm⁻¹ indicate medium C-H stretch in alkanes. Another peak around 1674 cm⁻¹ are indicative of medium N-H bend due to the presence of amine group corresponding to that of chitosan. Peaks around 1440 m⁻¹ are due to the C-H stretching of CH₂ and CH₃ groups. Peaks around 1290 cm⁻¹ are due to the strong C-N stretch present in amines. A single peak is obtained at 1066 cm⁻¹, around 1153 and 1026 cm⁻¹ which are due to strong C-O-C stretching [10].

Infrared of chitosan-grafted-poly(vinyl pyrrolidone) (CS-g-PVP)

Figure 3 exhibits the FTIR spectrum of CS-g-PVP derivative. The absorption of (O-H) and (N-H) at 3448 cm⁻¹ and a peak at 2920-2870 cm⁻¹ assigned for aliphatic bond stretching (C-H) at 1651 and 1535 cm⁻¹ (C=N NH₂) assigned for related to N-H amide I-II bond,

1427 cm⁻¹ are due to the C-H stretching of CH₂ and CH₃ groups and at 1269 cm⁻¹ to amines C-N stretching. Peaks at 1153 and 1041 cm⁻¹ are due to the presence of strong (C-O-C) stretching [17].

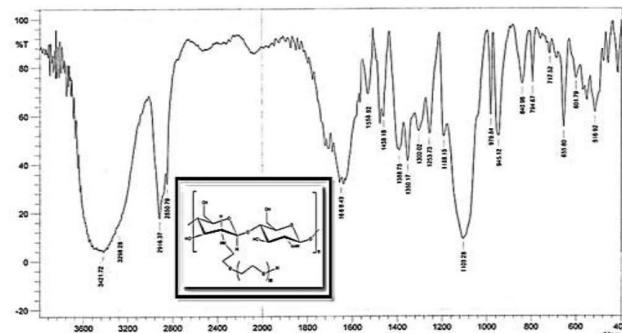


Figure 1: FTIR spectrum of chitosan-g-poly(ethylene glycol) derivative.

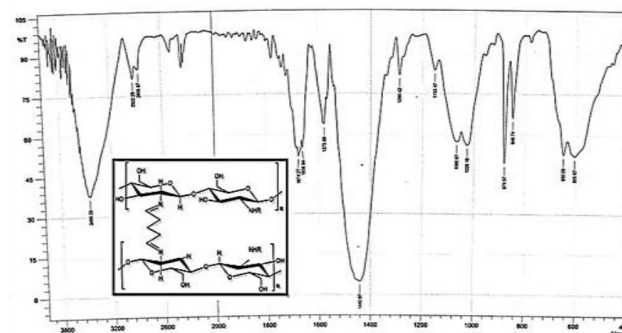


Figure 2: FTIR spectra of chitosan-g-poly(vinyl alcohol).

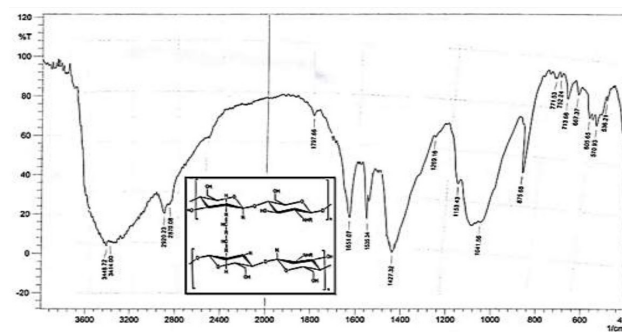


Figure 3: FTIR spectrum of chitosan-g-poly(vinyl pyrrolidone).

Scanning Electron Microscopy, (SEM), of the Polymeric Nanoparticles

SEM is used to characterize morphology, and to determine particle size or shape. The technique is extremely widespread (it was the first real nanoscale method), and it has become a standard procedure for a huge range of samples. It offers ultimate resolution down to the very low nm range and to single atoms [18]. It is one of the most multipurpose tools for the analysis of micro- and nanostructures with a wide range of applications, and image recording on the specimen surface [19].

The small size of NPs is the main factor that affects the properties, toxicity, and cytotoxicity of a material. The size and shape are influences bio distribution of the nanoparticles in an organism, as well as the process of recognition of them as an extracorporeal agent and the elimination of them through the immunological

response of that organism [20], by which the size of nanoparticles provides the best therapeutic efficacy direct testing.

The size and morphology of nanoparticles were analyzed using the examination of the morphology and the nanoparticles geometry of the prepared polymers SEM studies. According to the SEM images of chitosan and its derivatives was performed by using SEM at different amplifications nm [21]. In Figures (4) to (5), the size of the polymeric nanoparticles was found to range from 38.3 to 90.9 nm, with spherical in their shapes.

Figures 4 and 5 show the SEM image and size of chitosan nanoparticles as representative for prepared nanoparticles, respectively. Purified chitosan is appearing to be in the shape of small spherical nanoparticles with a minimum and a maximum size ranging mainly between 17-100 nm.

Chitosan is an 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 [22].

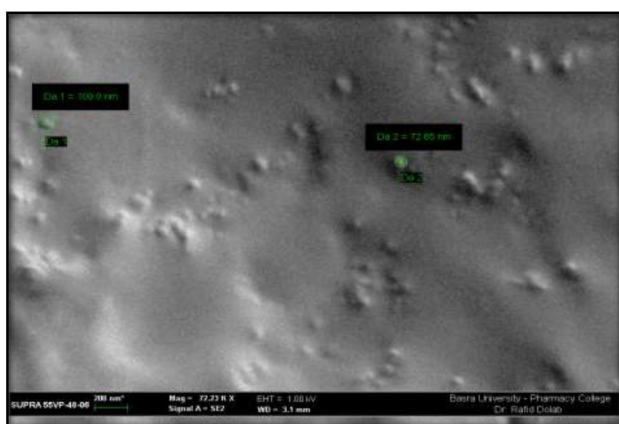


Figure 4: SEM micrograph of prepared chitosan nanoparticles.

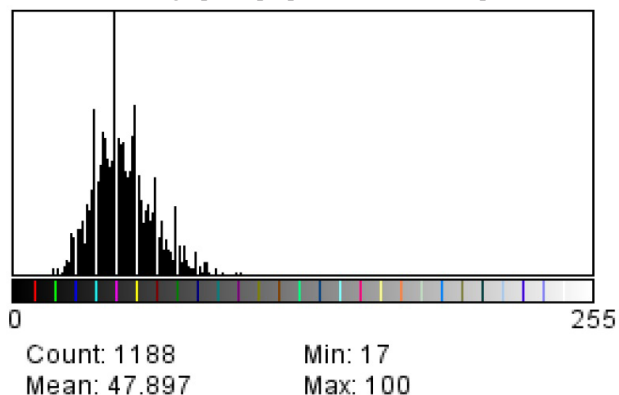


Figure 5: Size of chitosan nanoparticles using Image-J program.

Cytotoxicity effects of chitosan derivatives CS-g-PEG, CS-g-PVA, CS-g-PVP Nanoparticles

The *in vitro* cytotoxicity assay of the grafted PEG, PVA, and PVP were achieved on the subject of the MTT assay involving

three different types of human breast cancer cell lines, the results are demonstrated in Figure 6 and Table 1. They exhibited highly significant ($p < 0.001$), antitumor consequence beside proliferation of tumor cells regarding the treatment with these nanoparticles of polymeric chitosan derivatives, which represented with the decreasing in cell viability in comparison to control samples, especially CS-g-PEG NPs, which reach to $23.33\% \pm 1.528$, $25.67\% \pm 1.155$ and $45.67\% \pm 5.508$ for CS-g-PEG NPs against BT cells, MCF-7 cells, and SKBR3 cells, respectively; these graphic results may assume to their imine and free hydroxyl functional groups which are indicated as important in the function and biomedical application role for these polymers.

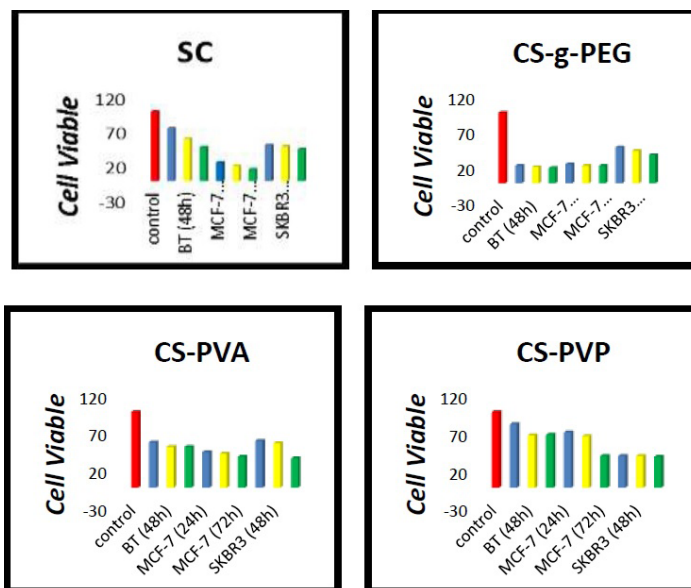


Figure 6: Cell viability percentages of human breast cancer cell lines (BT cells, MCF-7 cells, and SKBR3 cells) affected with CS-g-PEG, CS-g-PVA, CS-g-PVP NPs, at different times for 24h, 48h and 72h.

Sample	Cell lines		
	BT	MCF-7	SKBR 3
CS	62.00±13.528	22.00±5.000	49.33±3.055
CS-g-PEG	23.33±1.528	25.67±1.155	45.67±5.508
CS-PVA	61.33±11.846	44.33±3.055	53.33±12.503
CS-PVP	82.67±13.051	61.00±16.643	41.67±5.577
Control	100.00±0.000	100.00±0.000	100.00±0.000

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 ($P < 0.001$).

Conclusion

Chitosan and its cationic polymer derivatives nanoparticles demonstrated highly significant anti-tumor effect; ($P < 0.001$), against several types of human breast cancer cell lines at different times of treatment.

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