Anti-tumor activity of paclitaxel-loaded chitosan nanoparticles: An in vitro study

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ABSTRACT

Chitosan nanoparticles containing the anticancer drug paclitaxel were prepared by a solvent evaporation and emulsification crosslinking method. The physicochemical properties of the nanoparticles were characterized by various techniques, and uniform nanoparticles with an average particle size of 116 ± 15 nm with high encapsulation efficiencies (EE) were obtained. Additionally, a sustained release of paclitaxel from paclitaxel-loaded chitosan nanoparticles was successful. Using different ratios of paclitaxel-to-chitosan, the EE ranged from 32.2 ± 8.21% to 94.0 ± 16.73%. The drug release rates of paclitaxel from the nanoparticles were approximately, 26.55 ± 2.11% and 93.44 ± 10.96% after 1 day and 13 days, respectively, suggesting the potential of the chitosan nanoparticles as a sustained drug delivery system. Cytotoxicity tests showed that the paclitaxel-loaded chitosan had higher cell toxicity than the individual paclitaxel and confocal microscopy analysis confirmed excellent cellular uptake efficiency. TEM images showed the ultrastructure changes of A2780 cells incubated with paclitaxel-loaded nanoparticles. Flow cytometric analysis revealed two subdiploid peaks for the cells in the paclitaxel-loaded nanoparticles and paclitaxel treated groups, respectively, with the intensity of the former higher than that of the latter. Moreover, the cell cycle was arrested in the G2-M phase, which was consistent with the action mechanism of the direct administration of paclitaxel. These results indicate that chitosan nanoparticles have potential uses as anticancer drug carriers and also have an enhanced anticancer effect.

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1. Introduction

Paclitaxel is a natural hydrophobic diterpenoid product extracted from the bark of the Pacific yew tree. Because it promotes tubulin polymerization, stabilizes microtubules, blocks cells at the G2-M interface, and induces cell apoptosis [1], paclitaxel is effective for treating a large spectrum of cancers, including ovarian cancer, breast cancer, and non-small cell lung cancer [2,3]. Because paclitaxel is water-insoluble, it is normally used together with adjuvants of polyethylated castor oil and polyvinyl alcohol (PVA) via intravenous administration. However, there are possible issues with this drug and its respective adjuvants. First, normal cells are likely to be affected, leading to prolonged, sometimes irreversible, peripheral neuropathy. Second, the hypersensitivity reactions, caused by its adjuvant can, under certain circumstances, be fatal. Third, the rapid metabolism of paclitaxel after intravenous injection can reduce its therapeutic efficiency.

Given these serious drawbacks, alternative methods for administering paclitaxel may be ideal, including drug-loaded liposomes, nanoparticles, and microspheres [4]. Degradable polymers have also been tested as potential carriers in various drug delivery systems because of their properties of low toxicity, long-circulation, and tumor localization that are associated with such polymer carriers [5]. Of these carriers, degradable polymeric nanoparticles are perhaps the most optimal choice for use as anticancer agents due to their unusual beneficial properties, most notably enhanced drug availability for prolonging the drug effects in tumor tissues [6]. However, with respect to free drugs, these drug loaded nanoparticles have been shown to decrease the permeability of the drugs across the cell membrane [7].

Chitosan, a deacetylated derivative of chitin, is a positively charged polymer carrier. The cell adhesion and potential uptake of chitosan particles is also most favorable due to their attraction to negatively charged cell membranes, an attractive feature for the treatment of solid tumors [8–11] and an alternative mode of paclitaxel functioning. Moreover, chitosan has shown favorable biocompatibility [12] as well as the ability to increase cell membrane permeability both in vitro [13] and in vivo [14]. Chitosan can also be degraded by lysozyme in the body.
In this study, chitosan nanoparticles were used as carriers for the mitotic inhibitor paclitaxel. Chitosan nanoparticles were synthesized by a solvent evaporation and emulsification crosslinking method, with trisodium citrate as the crosslinking agent. The Chitosan nanoparticles were characterized by Fourier Transform Infrared (FT-IR) spectroscopy, Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). The experimental data on in vitro anti-tumor effects against A2780 cells indicated, for the first time, an enhanced anti-tumor effect of chitosan nanoparticles with chemotherapy drug paclitaxel. Our results also showed that paclitaxel-loaded chitosan nanoparticle could neutralize the surface negative charge of A2780 cells so as to damage the membrane of the cell.

2. Materials and methods

2.1. Synthesis of paclitaxel-loaded chitosan nanoparticles

Paclitaxel-loaded nanoparticles were prepared by a solvent evaporation and emulsification crosslinking method [15]. Briefly, 5 mg paclitaxel was dissolved in 2 ml dehydrated alcohol and ultrasonicated for 15 min. This organic phase was then slowly poured into the aqueous solution of sodium dodecyl sulfate (SDS) and polyvinyl alcohol (PVA). The solution of 1.1 ml SDS and 0.4 ml PVA in 10 ml deionized water was maintained at 35.5 °C for 2 h under stirring rate of 500 rpm. The oil-in-water emulsion was formed after removal of alcohol under reduced pressure. The resulting aqueous suspension containing paclitaxel was denoted as solution 1, which was then added into the acetic acid solution of chitosan. By varying the ratio of solution 1 and chitosan solution, the paclitaxel-loading amount may be finely tuned. The resulting mixture was then sonicated at an energy output of 50 watts for 30 min to obtain a uniform solution, denoted as solution 2, which was then added dropwise to 30 ml liquid paraffin under stirring for 12 h. The water-in-oil emulsion was formed without extra emulsifier. Finally, 1 ml of 20 wt.% sodium citrate was added to crosslink chitosan particles for 1 h, and the resulting nanoparticles were purified and stored after being dried. Fluorescein isothiocyanate (FITC) was conjugated with the amine group of chitosan nanoparticle for confocal microscopy analysis [16]. For the control group, chitosan nanoparticles without paclitaxel were prepared using a similar method like those described above except without any paclitaxel-loading.

2.2. Characterization of paclitaxel-loaded chitosan nanoparticles

The chemical composition of chitosan nanoparticles loaded both with and without paclitaxel was characterized with Fourier transform infrared spectroscopy (FT-IR, AVATAR 370, Thermo Nicolet, USA) after a thorough mixing of the nanoparticles with potassium bromide. The morphology of the as-prepared nanoparticles was visualized under TEM (H-600, Hitachi, Japan) and AFM (Nano Scope IIIa, Veeco, USA). Zeta potential was determined with dynamic laser scattering (DLS, Zetasizer Nano ZS, Malvern, UK).

2.3. The paclitaxel-loading ratio and encapsulation efficiency

The paclitaxel-loading efficiency of chitosan nanoparticles was determined by extracting paclitaxel out of the nanoparticles. Briefly, 10 mg of the paclitaxel-loaded nanoparticles were placed in 50 mL of dehydrated alcohol and stirred vigorously for 48 h to extract the requisite paclitaxel from the nanoparticles. The resulting mixture was then centrifuged for 10 min at 8000 rpm. The supernatant was then assayed by UV spectrophotometer (UV-VIS, 8500 spectrophotometer, Techcomp, China) at the fixed λmax value of 227 nm. Nanoparticles without paclitaxel were also assayed using the same procedure. The results also showed that paclitaxel-loaded chitosan nanoparticles could neutralize the surface negative charge of A2780 cells so as to damage the membrane of the cell.

2.4. Drug release studies

To obtain the paclitaxel release profile from chitosan nanoparticles, 10 mg of prepared paclitaxel-loaded nanoparticles (n = 7) were suspended in each 3 ml phosphate buffer solution (pH 7.4), and shaken at 100 strokes/min at 37 °C. At each time interval, the samples were withdrawn and centrifuged 10 min at 8000 rpm. The supernatants were detected with a UV spectrophotometer by measuring the absorbance (227 nm).

2.5. Cell uptake efficiency of nanoparticles

A2780 cells (from ATCC) were maintained in a 5% CO2 humidified atmosphere at 37 °C in RPMI 1640 medium (Gibco, Carlsbad, CA, USA).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Drug loading contents and encapsulation efficiency of paclitaxel-loaded chitosan nanoparticles (PTX-CNPs).</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX:chitosan ratio</td>
<td>5:10</td>
</tr>
<tr>
<td>Rotating speed (rpm)</td>
<td>500</td>
</tr>
<tr>
<td>Drug loading efficiency (%)</td>
<td>16.7±10.86</td>
</tr>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>32.2±8.21</td>
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supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. 1×10^5 cells were incubated overnight in this media at 37 °C in each well of 24-well plates (Nunc International, Aperville, IL, USA). Cells were washed twice with 0.1 M PBS before adding FITC-labeled nanoparticles. At different intervals, fluorescence was monitored in the green channel for FITC (excitation 490 nm, emission 520 nm), and the cells were imaged with a confocal fluorescence microscope (LSM5 PASCAL, ZEISS, Germany).

2.6. Changes in surface potential of cells

The change of zeta potential of A2780 cells treated with chitosan nanoparticles was determined by incubating the A2780 cells grown on glass cover slips with 25 μg/ml nanoparticles at intervals from 30 min to 4 h. The zeta potential of the treated cells was determined with a Zetasizer Nano-ZS90 (Malvern Instruments).

2.7. MTT (3-[4,5-dimethylthiazol-2-yl]2,5- diphenyltetrazolium bromide) assay

The effects of paclitaxel and paclitaxel-loaded nanoparticles on the viability of the cells were evaluated by MTT assay as described previously [17]. Briefly, the cells were cultured in 96-well plates with different concentration of paclitaxel (0.1, 1, and 10 μg/ml) and nanoparticles loaded with the same amount of paclitaxel for 72 h. In parallel with this experiment and to determine the cytotoxic effect of the chitosan, the cells were also incubated with different concentrations of the pure chitosan nanoparticles. Upon culturing with nanoparticles, the cells were washed twice with PBS (pH 7.4) and further incubated with MTT (Sigma Chemical Co.) for 4 h at 37 °C; the supernatants were aspirated, and 100 μl DMSO were added to each well. The plates were shaken for 5 min, and the absorbance data were obtained at 540 nm using a spectrophotometer. The percentage of the viable cells was obtained by comparing with the standard curved obtained with the known amount of cells.

Fig. 2. Drug release profile of paclitaxel-loaded chitosan nanoparticles.

Fig. 3. Microscope images of A2780 cells. (A) and (B): A2780 cells before incubated with FITC-labeled paclitaxel-loaded chitosan nanoparticles. (C) and (D) A2780 cells incubated with FITC-labeled paclitaxel-loaded chitosan nanoparticles for 12 h. (A) and (C) Green channel (excitation 490 nm, emission 520 nm). (B) and (D). Bright field. Scale bar = 30 um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.8. Flow cytometry

A2780 cells (1 x 10^5 cells/well) were seeded in 24-well plates and incubated at 37 °C in a 5% CO2 incubator overnight. After treatment with paclitaxel at different concentrations (0.1, 1, and 10 ug/ml) or the chitosan nanoparticles loaded with the same amount of paclitaxel (Untreated cells were used as controls), the cells were washed with 0.1 M PBS, trypsinized, and fixed in 75% ethanol, and stained with a solution containing 15 μg/ml propidium iodide (PI), 0.5% Tween 20, and 0.1% RNAse A, and incubated for 30 min at room temperature. Cells were sorted using a FACSScan (BD Biosciences, USA) and analyzed with CELLQuest Version 3.3 software.

2.9. Ultrastructure cell morphology

The A-2780 cells were cultured with paclitaxel (25 μg/ml) or the nanoparticles loaded with the same amount of paclitaxel and exposed at different times. Untreated cells were again used as control. After trypsinization, cells were fixed in 5% glutaraldehyde solution and dehydrated for TEM (JEM-1200EX, Japan) visualization.

2.10. Statistical analyses

Results are presented as mean ± standard deviation. The 2-way ANOVA and the Student’s t test were used to compare data from the different treatment groups. Statistical significance was accepted at p<0.05.

3. Results and discussion

3.1. Surface morphology and size distribution of paclitaxel-loaded nanoparticles

Paclitaxel-loaded chitosan nanoparticles were prepared by a solvent evaporation and emulsification crosslinking method. TEM and AFM were used to characterize the morphology of these paclitaxel-loaded chitosan nanoparticles, the images of which are shown in Fig. 1. These nanoparticles exhibit a fine spherical shape with smooth surfaces, without aggregation or adhesion and with an average size of 116 ± 15 nm (Fig. 1A). Fig. 1B shows the AFM image of a single paclitaxel-loaded nanoparticle, which also further confirms the spherical shape of the particle without adhesion and aggregation. The average size of the nanoparticles obtained from AFM is 110 ± 16 nm (Fig. 1B), which is consistent with that determined from the TEM observation.

3.2. The drug loading efficiency and encapsulation efficiency

The amount of paclitaxel-loaded in nanoparticles and the encapsulation efficiency are shown in Table 1. The EE of the formulations tested in this study range from 32.2 ± 8.21% to 94.0 ± 16.73%, and the encapsulation efficiency increases with the increase of the paclitaxel-to-chitosan ratio. When the paclitaxel-to-chitosan ratio is constant, the drug loading efficiency and the encapsulation efficiency decrease with the increase in the agitation intensity.

3.3. In vitro drug release kinetics

The in vitro cumulative paclitaxel release profile from the chitosan nanoparticles, shown in Fig. 2, indicates a significant initial burst release burst in the first three days, accounting for more than 60% of total release amount. The release rate gradually decreased to a constant profile from day 4 through day 13. The release test was performed in triplet to obtain the mean release value and the standard deviation. Approximately 28.55 ± 2.11% of the paclitaxel released in the first 24 h and approximately 65.34 ± 2.3% of the paclitaxel...
released in the first 3 days, which was then followed by a constant release for the subsequent 13 days. The approximately 93.44 ± 10.96% of the paclitaxel released in the first 13 days, suggests the potential of using chitosan nanoparticles for sustained drug release.

3.4. Changes in surface potential of A2780 cells

Zeta potential is defined as the difference of electrical potential between the surface of the cells and the bulk-surrounding medium. The change of the zeta potential of the A2780 cells treated with chitosan nanoparticles loaded with 1 μg/ml of paclitaxel for different times was determined. A2780 cells grown on glass coverslips were incubated with 25 μg/ml nanoparticles at intervals from 30 min to 4 h. The cells were detached from culture plates by adding 0.25% trypsin solution to prepare a cell suspension. The surface potential of the cell suspension was then determined using dynamic laser scattering (Zetasizer Nano-ZS90, Malvern Instruments), and analysis was performed at a scattering angle of 90° at 25 °C. After treatment of 4 h, the zeta potential of cells fell to −1.96 mV from the −6.57 mV of untreated cells. This decline clearly indicates that chitosan nanoparticle can neutralise the negative surface charge of A2780 cells and in turn increase the paclitaxel uptake by cells.

3.5. Cell uptake efficiency of nanoparticles

The cell uptake efficiency of chitosan nanoparticles by cancer cells was visualized using Confocal Fluorescent Microscopy. Fig. 3 shows the representative confocal microscopic images of A2780 cells after incubation with paclitaxel-loaded chitosan nanoparticles at an equivalent drug concentration of 1 μg/ml at different times. Cellular internalization of chitosan nanoparticles was observed after incubation. Specifically, when A2780 cells were incubated with paclitaxel-loaded chitosan nanoparticles for only 1 h, only weak green fluorescence was observed inside the cells. However, after 12 h of incubation, an intensive green fluorescence was observed inside the cells (Fig. 3B), suggesting that the cellular uptake of the paclitaxel-loaded chitosan nanoparticles is mediated by nonspecific absorptive endocytosis, with the paclitaxel subsequently being released from the nanoparticles.

3.6. MTT assay

The cytotoxicity of the paclitaxel and the paclitaxel-loaded chitosan nanoparticles was evaluated through an MTT assay. Here, a significant reduction in the A2780 cells viability was observed when the cells containing the same concentration of the direct administration of the paclitaxel were exposed to the paclitaxel-loaded chitosan nanoparticles. The viability of the A2780 cells after 72 h of incubation with the paclitaxel-loaded chitosan nanoparticles was significantly lower than for those nanoparticles that were directly administered paclitaxel for the same culture period. As shown in Fig. 4, approximately 44.0 ± 5.62% of the viable A2780 ovarian cancer cells were incubated with 0.1 μg/ml of paclitaxel. The viability of the A2780 cells was decreased to 24.6 ± 2.83% and 8.2 ± 1.91% at 1 μg/ml and 10 μg/ml of paclitaxel, respectively. These results demonstrated a concentration dependent response of paclitaxel-loaded chitosan nanoparticles. In contrast, the viability of the A2780 cells were 65.0 ± 10.64%, 46.2 ± 5.27%, and 27.5 ± 4.29% for cells directly administered with paclitaxel at 0.1 μg/ml, 1 μg/ml, and 10 μg/ml, respectively. Paclitaxel-loaded chitosan nanoparticles were found a higher cell toxicity than the free paclitaxel (p < 0.01, Fig. 4). The same amount of blank chitosan nanoparticles and dehydrated alcohol were also tested as the control groups. Alcohol (5% v/v) showed nearly no cytotoxicity and approximately 95 ± 6.88% of the A2780 cells were viable. Of particular interest was the observation that chitosan nanoparticles exhibited considerable anticancer effects. Approximately 85 ± 6.98%, 75 ± 5.86%, and 67 ± 5.37% of the A2780 cells were viable when incubated with blank chitosan nanoparticles at the concentration of 25, 50, and 75 μg/ml respectively. Therefore, because a nanoparticle formulation...
is expected to eliminate the side effects caused by Cremophor EL as well as to improve the cellular uptake of the drug, paclitaxel-loaded chitosan nanoparticles could be an ideal anticancer medicine for the targeted therapy.

3.7. Cell apoptosis

Cell apoptosis was evaluated by flow cytometry. Specifically, the A2780 cells were treated for 72 h with free paclitaxel or paclitaxel-loaded chitosan nanoparticles, containing the same concentration of the free paclitaxel. The number of apoptotic cells was then counted with a FACSCalibur flow cytometer. As shown in Fig. 5, the apoptotic cells incubated with free PTX were 7.83 ± 0.24%, 10.67 ± 0.56%, and 69.77 ± 7.32% when the concentration of paclitaxel was 0.1, 1 and 10 μg/ml, respectively. In contrast, the number of apoptotic cells increased to 22.3 ± 1.67%, 56.4 ± 6.97%, and 97.6 ± 10.58% when the A2780 cells were incubated with 0.1, 1 and 10 μg/ml of paclitaxel-loaded into chitosan nanoparticles, respectively (p<0.01). Therefore paclitaxel-loading in chitosan nanoparticles can induce more cancer cell apoptosis when compared with the direct administration of paclitaxel.

Since the cytotoxicity of paclitaxel is attributed to its stabilizing effect on microtubules that are necessary for the spindle formation and cell division, paclitaxel can cause cell cycle arrest in the G2/M phase and finally cell death through apoptotic mechanisms [18]. Paclitaxel-loaded chitosan nanoparticles can significantly enhance the efficiency of the intracellular delivery and the apoptosis-inducing effect of paclitaxel. Furthermore, paclitaxel-loaded chitosan nanoparticles can neutralize the surface negative charge of the A2780 cells that increase the paclitaxel dose to the attached cells.

Representative TEM images (Fig. 6) show the ultrastructural changes of the A2780 cells incubated with paclitaxel-loaded chitosan nanoparticles with 1 μg/ml of paclitaxel for different time periods. Fig. 6A shows the nuclei of untreated A2780 cells which were large and polymorphic and which show clear cell nuclei with a nucleus-to-plasma of about 1:1. The structure of the mitochondria in the cytoplasm appeared integrated with many rough endoplasmic reticula and ribosomes being observed (Fig. 6A). The membrane of the treated cells was damaged and the chromatin was concentrated into multiple aggregates at the boundary of the membrane (Fig. 6B). The chromatin concentrated into a crescent (Fig. 6C), and the formation of the apoptotic body (Fig. 6D) was also observed. Cells treated with paclitaxel-loaded chitosan nanoparticles for 24 h were shrunk the cytoplasm was condensed. Chromosome aggregation was observed for cells treated with paclitaxel-loaded chitosan nanoparticles for 24 h. Apoptotic bodies in were found in the nuclei of those cells treated with paclitaxel-loaded chitosan nanoparticles for 72 h. It is clear that from these observations, paclitaxel-loaded chitosan nanoparticles can be internalized into cells and paclitaxel can be released into the cytoplasm, resulting in cancer cell apoptosis.

4. Conclusion

It was determined in this work that chitosan nanoparticles are not only potential anticancer drug carriers but also have anticancer effects. Specifically, to eliminate the side effects of Cremophor EL, the paclitaxel-loaded degradable chitosan nanoparticles were developed in this study. Plain chitosan nanoparticles were shown to induce A2780 cancer cell apoptosis which was further enhanced by the cytotoxicity of paclitaxel. Paclitaxel-loaded chitosan nanoparticles were prepared by a solvent evaporation and emulsification cross-linking method. As a result uniform nanoparticles with tunable paclitaxel-loading were achieved. Sustained release of paclitaxel and higher cell toxicity than free paclitaxel were obtained from paclitaxel-loaded nanoparticles.

References