In vitro Anticancer Activity of Doxorubicin-Loading Pectin Nanoparticles

ABSTRACT

By using pectin (PEC) as carrier material and doxorubicin (DOX) as a model drug, the blank PEC nanoparticles (PEC-NPs) and the DOX-loading PEC nanoparticles (DOX-PEC-NPs) were prepared by microemulsification method and drug adsorption. The aim of this study is to investigate the anticancer activity of DOX-PEC-NPs in vitro to understand the advantages of PEC-NPs as an anticancer drug delivery system. The particle size, polydispersity index (PDI) and zeta potential of PEC-NPs were (278.80 ± 2.80) nm, (0.140 ± 0.014) and (−19.83 ± 0.21) mV, while those of DOX-PEC-NPs were (283.73 ± 3.26) nm, (0.157 ± 0.034) and (−18.00 ± 0.44) mV. The entrapment efficiency (EE%) and drug-loading rate (LR%) of DOX-PEC-NPs were (92.10 ± 0.60)%, and (18.72 ± 0.10)%, respectively. Using an MTT assay, the DOX-PEC-NPs proved to greatly inhibit the viability of MDA-MB-231 cells, A549 cells and NCI-H1299 cells, and the anticancer activity was higher than that of the DOX solution in these cells. The PEC-NPs had no cytotoxicity against the three tested cells. An inverted fluorescence microscope and flow cytometry were used to observe the intracellular uptake of DOX. The DOX-PEC-NPs resulted in faster and more DOX uptake than DOX solution in the tested cells. The results indicated that the PEC-NPs may be a potential anticancer drug delivery system which could reduce the dose and increase the activity of anticancer drugs.

KEYWORDS doxorubicin, pectin, nanoparticle, anticancer activity

INTRODUCTION

The cancer remains one of the leading factors of death in the world, caused by genetic instability and accumulation of multiple molecular alterations. Despite advances in our understanding of molecular and cancer biology, radiotherapy, chemotherapy and conventional surgical procedures, the overall survival rate of cancer patients have not improved significantly in the past two decades. So far, the chemotherapy is still one of the major therapeutic measures for cancer. However, the poor selectivity of chemotherapy drugs always keeps be a problem which is conflict with their anticancer efficacy.

Doxorubicin (DOX), a DNA intercalating agent, has been used as an effective chemotherapeutic for many types of solid tumours in breast, lung, ovarian, prostate and bladder. However, its use is severely limited by side-effects such as dizziness, lack of concentration and cognitive deficits characterised as ‘chemobrain’, and cardiotoxicity and heart failure as well.

The recent advances in nanoscience and nanotechnology have led to the development of nanosystems. As a drug delivery system, the nanoparticles have raised high expectations in cancer treatment and diagnosis, and are considered as one of the most promising research orientations for oncotherapy. Pectin (PEC), a kind of natural plant polysaccharide, has received considerable attention in biomedicine. The best features of the PEC, such as biodegradability, biocompatibility, controllable biologic activity and flexible chains that allow the modulation of the polysaccharide to a specific shape, enable its wide use as a popular carrier material in the field of micro and nanoparticle drug delivery system.

In this study, to reveal the advantages of PEC-based nanoparticles in anticancer drug delivery, the PEC was used as a carrier material to prepare PEC nanoparticles (PEC-NPs) based on microemulsification method, and the DOX was selected as a model drug to prepare the DOX-loading PEC.
norphosphates (DOX-PEC-NPs) by drug adsorption. The antancer activity and intracellular DOX uptake of DOX-PEC-NPs in vitro was evaluated using MTT assay, inverted fluorescence microscope and flow cytometry, respectively.

**MATERIALS AND METHODS**

**Materials**

The DOX was provided by Haizheng pharmaceutical industry (Zhejiang, China) and the PEC was provided by CP Kelco Company (Denmark). The oleic acid and sodium bis (2-ethylhexyl) sulfosuccinate (AOT) was provided by the Aladdin Company (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were provided by Sigma Company (St Louis, MO, USA). All other chemicals and reagents used were analytical grade.

**Preparation of PEC-NPs and DOX-PEC-NPs**

The PEC-NPs were prepared by microemulsification method optimised in previous studies. Briefly, the AOT was dissolved in oleic acid and the PEC solution was added slowly while stirring. The coarse emulsion was ultrasonic, treated to form a clear transparent emulsion. The propyl alcohol solution of calcium chloride was dispersed in oleic acid well and mixed with the microemulsion to make PEC, gelated into NPs. The liquid mixture was then centrifuged to separate PEC-NPs. The precipitate was collected and dried to get white PEC-NPs powder. The PEC-NPs powder was suspended in the DOX solution for drug adsorption to prepare DOX-PEC-NPs. The particle size and drug-loading property of PEC-NPs was measured.

**Cell lines and cell culture**

All human cell lines used in this study, including MDA-MB-231 breast adenocarcinoma cells, A549 lung cancer cells and NCI-H1299 lung cancer cells (the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China), were cultured in DMEM, including 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, respectively. These cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were subcultured when they reached ~90% confluence, using a 0.25% trypsin solution.

**In vitro anticancer activity test by MTT assay**

The cell viability of MDA-MB-231 cells, A549 cells and NCI-H1299 cells was measured by MTT assay. Briefly, the cells were seeded on a 96-well microtiter plate at a density of 5 × 10⁴ cells/well in 100 µL of medium and incubated at 37°C under 5% CO₂ in a humidified incubator for 24 h. After treatment, the supernatant was discarded, and then 150 µL of DMSO was added to each well to dissolve the MTT formazan. The absorbance of each well was measured at a wavelength of 570 nm with a microplate reader (Synergy HT, BioTek, USA). The inhibition rate (IR%) was calculated as the following equation. The 50% inhibitory concentration (IC₅₀) was calculated as well.

\[ \text{IR} \% = \frac{\text{OD}_{\text{Control}} - \text{OD}_{\text{Drug}}}{\text{OD}_{\text{Control}}} \times 100\% \]

**Intracellular DOX uptake test**

The cells were seeded on a 6-well microtiter plate at a density of 3 × 10⁵ cells/well in 3 mL of medium and incubated at 37°C under 5% CO₂ in a humidified incubator for 24 h. To localise DOX in cells, the cells were treated with DOX solution or DOX-PEC-NPs for 4 h, wherein, the concentration of DOX was 5 µg·mL⁻¹. After treatment, the supernatant was removed and the cells were rinsed with cold phosphate-buffered saline (PBS, 4°C) for three times. The cells were then fixed with 4% paraformaldehyde solution for 30 min at a room temperature followed by observation under an inverted fluorescence microscope (TE2000-s, Nikon). To measure intracellular fluorescence intensity caused by DOX uptake, the cells were treated with DOX solution or DOX-PEC-NPs for 4 h (MDA-MB-231 cells and NCI-H1299 cells) or 20 h (A549 cells), wherein, the concentration of DOX was 1 µg·mL⁻¹. After treatment, the cells were harvested and washed with cold PBS for three times, and then the fluorescence intensity was measured using flow cytometer (Galios, Backman Coulter), with excitation and emission wavelength setting as 488 nm and 575 nm, respectively.

**Statistical analysis**

The data were expressed as mean ± standard deviation (SD). The statistical analysis was performed with SPSS19.0 software using the Student’s t-test. The difference with \( P < 0.05 \) (*), \( P < 0.01 \) (**) or \( P < 0.001 \) (***) was considered statistically significant.

**RESULTS AND DISCUSSION**

**Preparation of PEC-NPs and DOX-PEC-NPs**

The spherical PEC-NPs and DOX-PEC-NPs of narrow distribution and high drug-loading capacity were prepared using the methods in previous studies. The particle size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE%) and drug-loading rate (LR%) were shown in Table 1.
Table 1 The properties of PEC-NPs and DOX-PEC-NPs.

<table>
<thead>
<tr>
<th></th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE%</th>
<th>LR%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEC-NPs</td>
<td>276.80 ± 2.80</td>
<td>0.140 ± 0.014</td>
<td>−19.83 ± 0.21</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DOX-PEC-NPs</td>
<td>283.73 ± 3.26</td>
<td>0.157 ± 0.034</td>
<td>−18.00 ± 0.44</td>
<td>92.10 ± 0.60</td>
<td>18.72 ± 0.10</td>
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</tbody>
</table>

Anticancer activity of DOX-PEC-NPs in vitro

The cytotoxicity of PEC-NPs is shown in Fig. 1. The results indicated PEC-NPs had no cytotoxicity in MDA-MB-231 cells, A549 cells and NCI-H1299 cells after the treatment for 24, 48 and 72 h. The inhibitory rate of DOX-PEC-NPs in the three tested cells is shown in Fig. 2 and the \( IC_{50} \) values are listed in Table 2. The results demonstrated that the anticancer activity of both DOX-PEC-NPs and DOX solution was in both time- and concentration-dependent manner. The viability of the three tested cancer cells decreased distinctly with the increase of drug concentrations.

Fig. 1 The cell viability of MDA-MB-231 cells, A549 cells and NCI-H1299 cells after 24, 48 and 72 h treatment with 16 μg·mL\(^{-1}\) of PEC-NPs (\( n = 5 \)).

Fig. 2 The inhibitory rate of DOX-PEC-NPs and DOX solution in MDA-MB-231 cells, A549 cells and NCI-H1299 cells after 24 h, 48 h and 72 h treatment at different drug concentrations (\( n = 5 \). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) vs. DOX solution).
In vitro anticancer activity of DOX-PEC-NPs

Table 2 The IC\textsubscript{50} values of DOX-PEC-NPs and DOX solution in different cancer cell lines (\(n = 3\)).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Treatment period</th>
<th>IC\textsubscript{50} ((\mu\text{g} \cdot \text{mL}^{-1}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DOX solution</td>
</tr>
<tr>
<td>MDA-MB-231 cells</td>
<td>24 h</td>
<td>9.71</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.59</td>
</tr>
<tr>
<td>A549 cells</td>
<td>24 h</td>
<td>14.30</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.46</td>
</tr>
<tr>
<td>NCI-H1299 cells</td>
<td>24 h</td>
<td>11.88</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>1.52</td>
</tr>
</tbody>
</table>

As shown in Table 2, the IC\textsubscript{50} values of DOX-PEC-NPs ranged from 0.24 to 8.36 \(\mu\text{g} \cdot \text{mL}^{-1}\), which was much less than those of DOX solution (0.46–14.30 \(\mu\text{g} \cdot \text{mL}^{-1}\)) in same cancer cells and treatment period.

Intracellular DOX uptake

The anticancer activity of DOX is likely due to their intercalation into DNA, which may disrupt replication and transcription of genomic DNA and lead to the death of cancer cells\textsuperscript{18,19}. In this test, the intracellular DOX uptake was measured to elucidate the higher anticancer activity of DOX-PEC-NPs. The DOX produced red fluorescence under the testing condition. The deeper the red colour in cells, the higher the intracellular concentration of DOX. After treatment with DOX solution and DOX-PEC-NPs for 4 h, the intracellular fluorescence increased when compared with negative control cells. As shown in Fig. 3, a stronger red fluorescence was observed in MDA-MB-231 cells, A549 cells and NCI-H1299 cells treated with DOX-PEC-NPs using fluorescence inverted microscope. The flow cytometry results (Fig. 4) were consistent with the observation under fluorescence microscope. The relative fluorescence intensity in cancer cells treated with DOX solution was significantly lower than that treated with DOX-PEC-NPs of same drug concentration (\(P < 0.01\) or 0.001). The highest difference was observed in A549 cells.

CONCLUSION

In this study, DOX-PEC-NPs were prepared in good shape, small size, narrow distribution and high drug-loading capacity. The blank NPs showed no cytotoxicity to the
tested cancer cells, and the DOX-PEC-NPs demonstrated stronger inhibitory rate in the test cancer cells than the DOX solution. It suggested that the PEC-NPs promoted the anticancer activity of DOX by enhancing the intracellular drug uptake in the cancer cells. The PEC-NPs may be a promising nanosize drug delivery system for anticancer chemotherapeutics, which may achieve the increased efficacy with decreased dose, thus to reduce the side effects.

REFERENCES