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Targeted delivery and controlled release of Paclitaxel for the treatment of lung cancer using single-walled carbon nanotubes

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ABSTRACT

A new type of drug delivery system (DDS) based on single-walled carbon nanotubes (SWNTs) for controlled-release of the anti-cancer drug Paclitaxel (PTX) was constructed in this study. Chitosan (CHI) was non-covalently attached to the SWNTs to improve biocompatibility. Biocompatible hyaluronan was also combined to the outer CHI layer to realise the specific targeting property. The results showed that the release of PTX was pH-triggered and was better at lower pH (pH 5.5). The modified SWNTs showed a significant improvement in intracellular reactive oxygen species (ROS), which may have enhanced mitogen-activated protein kinase activation and further promoted cell apoptosis. The results of western blotting indicated that the apoptosis-related proteins were abundantly expressed in A549 cells. Lactate dehydrogenase (LDH) release assay and cell viability assay demonstrated that PTX-loaded SWNTs could destroy cell membrane integrity, thus inducing lower cell viability of the A549 cells. Thus, this targeting DDS could effectively inhibit cell proliferation and kill A549 cells, is a promising system for cancer therapy.

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1. Introduction

Paclitaxel, a natural tetracyclic diterpene compound, was first isolated from plants of the genus Taxus in 1963 [1]. It is one of the most efficient chemotherapy drugs for urothelial, breast, lung and ovarian cancers [2]. The drug mechanism of Paclitaxel involves promoting microtubule protein polymerisation, inhibiting the composition of cell inner frames and interference of cell mitosis [3]. However, PTX has poor water solubility. Moreover, it can lead to various chemotherapy side effects, such as myelosuppression, neurovirulence, cardiovascular toxicity, hepatotoxicity, and anaphylaxis. PTX is formulated for clinical use as a solution of Cremophor® EL in anhydrous ethanol (50:50 v/v), a combination known as Taxol® [4], which can induce hypersensitivity and other acute adverse effects. Drug resistance also limits clinical use. Therefore, an efficient carrier for the delivery of Paclitaxel is urgently needed.

Single-walled carbon nanotubes (SWNTs) offer many advantages as drug delivery vehicles. Many SWNTs-based drug delivery systems (DDSs) have been designed and prepared. SWNTs have a hollow construction, a large surface area for carrying cargoes, stable physical and chemical properties [5–9], and the bilayer graphene construction enables them to combine with aromatic drugs through π–π stacking interactions, hydrophobic interactions, and van der Waals interactions [6,10–12]. Additionally, SWNTs can protect the drug from enzymatic degradation before it enters cancer cells [9]. However, SWNTs also have limitations, including poor water solubility, potential toxicity [13], and, in many cases, SWNTs require modification, which greatly improves the therapeutic efficiency of the drug while reducing its toxicity. Some groups have found that short (<1 μm) and well-functionalized SWNTs are rarely retained in the reticuloendothelial systems (RES) and could be cleared *via* renal pathways, thus inducing little toxicity *in vivo* [10,14–16]. Therefore, researchers have modified SWNTs with biological species such as poly(ethylene glycol) [17,18], polysaccharides [19,20] and proteins [21].

Chitosan (CHI) is a polysaccharide consisting of repeating D-glucosamine and *N*-acetyl-D-glucosamine units, linked *via* 1–4 glycosidic bonds [22]. CHI has several favourable characteristics including biocompatibility, biodegradability, and non-toxicity [23]. These properties make CHI a good candidate for drug delivery. Another typical hydrophilic polysaccharide, hyaluronan (HA), is also widely used due to its excellent biocompatibility and low cytotoxicity. CD44 is an important HA receptor commonly found on cancer cell surfaces. It is responsible for the proliferation, transfer, invasion and tumour-associated angiogenesis of cancers. The overexpression of the HA receptor CD44 on cancer cells results in greatly enhanced binding and endocytotic uptake of such compounds [24–26]. Therefore, HA is often used for targeted drug delivery to tumour cells [27,28].

In this work, an innovative SWNTs-based DDS was built for the controlled-release of the anti-cancer agent PTX. The SWNTs were non-covalently 'encapsulated' by chitosan to improve their biocompatibility. The conjugation of HA imparted the nanotubes with the capability to

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target tumour cells. Subsequently, PTX in aqueous solution was attached to the modified nanotubes. The pharmaceutical efficacy was further examined by measuring the viability of A549 cells *in vitro*, and reactive oxygen species (ROS) and LDH release assays were conducted to evaluate the cellular injury. Western blotting was used to monitor the expression of tumour cell apoptosis-related protein.

2. Materials and methods

2.1. Materials

The various materials used in this work were as follows: Carboxyl SWNTs (purity >90%, carboxyl content = 2.73%, length = 5–30 μm, diameter = 1–2 nm, Chengdu Organic Chemistry Institution), CHI (MW < 5000 Da, Jinan Haidebei Co., Ltd.), HA (MW = 6000 Da, Shandong Furuida Group Co., Ltd., Zibo, China), PTX (Beijing Huafeng United Technology Co., Ltd., Beijing, China), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), dichlorofluorescein diacetate (DCFH–DA), lysis buffer and TOX7 kit (Sigma–Aldrich, St. Louis, MO, USA), WST–1 reagent (Beyotime Biotechnology Institution, Jiangsu, China), RPMI 1640, foetal bovine serum, penicillin, streptomycin and trypsin (Gibco, Grand Island, NY, USA).

2.2. Synthesis of SWNTs–CHI

SWNTs (20 mg) were sonicated in CHI solution (60 mg in 0.1 M NaCl and 0.02 M acetic acid, 40 mL) for 30 min and stirred at room temperature for 24 h. The SWNTs–CHI was dialysed (dialysis bags, MWCO = 10 kDa) for 3 days in ultrapure water. After drying at 37 °C under vacuum, the modified SWNTs–CHI was collected.

2.3. Synthesis of SWNTs–CHI–HA

SWNTs–CHI (20 mg) was sonicated in HA solution (20 mg HA activated by EDC·HCl and NHS in 20 mL, pH 7.4, 0.01 M PBS) for 30 min and stirred at room temperature for 24 h. SWNTs–CHI–HA was dialysed (dialysis bags, MWCO = 10 kDa) for 3 days in ultrapure water. After drying at 37 °C under vacuum, the modified SWNTs–CHI–HA was collected.

2.4. Loading of PTX onto nanotubes

SWNTs, SWNTs–CHI, and SWNTs–CHI–HA (10 mg) were sonicated in PTX solution (15 mg in 15 mL pH 7.4 PBS) for 30 min and stirred at room temperature for 24 h. The products were washed with pH 7.4 PBS buffer and centrifuged several times until the supernatant became colourless. The nanotubes, loaded with PTX, were collected after drying at 37 °C *in vacuo*. Then, the nanotubes (1 mg; SWNTs, SWNTs–CHI, SWNTs–CHI–HA, and SWNTs–CHI–HA/PTX) were dispersed in 5 mL of ethanol. Next, the dispersal solution was dropped onto a copper mesh. The morphology of the nanotubes was then observed by transmission electron microscopy (TEM; JEM–2100F, JEOL, Peabody, MA, USA).

2.5. Drug loading efficiency and embedding ratio measurement

After collecting the free PTX solution in Section 2.4, the amount of free (unbound) PTX on the nanotubes was measured using the characteristic absorption wavelength (227 nm) of PTX with a UV–visible (UV–vis) spectrophotometer (UV–2550; Shimadzu, Kyoto, Japan) as follows:

$$\text{Drug loading efficiency} = \frac{\text{Weight of PTX in nanotubes}}{\text{Weight of total nanotubes}} \times 100\%$$

$$\text{Embedding efficiency} = \frac{\text{Weight of PTX in nanotubes}}{\text{Weight of total PTX}} \times 100\%$$

2.6. In vitro drug release

Nanotubes (1 mg; SWNTs/PTX, SWNTs–CHI/PTX and SWNTs–CHI–HA/PTX) were dispersed in 0.5 mL of pH 7.4 and pH 5.5 PBS in dialysis bags (MWCO = 10 kDa) and shaken in 20 mL of pH 7.4 and pH 5.5 PBS at 37 °C at 60 rpm, respectively. After 2, 6, 12, 24, 48, and 72 h, 2 mL aliquots were taken and the amount of free PTX was measured at 227 nm using the UV–vis spectrophotometer as follows:

$$\text{Drug cimulative releasing amount} = \frac{\text{Weight of release PTX in PBS buffer solution}}{\text{Weight of PTX in nanotubes}} \times 100\%$$

2.7. In vitro cytotoxicity assay

Fibroblasts and A549 cells were maintained in RPMI1640 medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. Exponential-phase cells were collected and seeded in a 96-well plate. After reaching confluence, the cells were treated with SWNTs, SWNTs–CHI, SWNTs–CHI–HA, SWNTs/PTX, SWNTs–CHI/PTX, SWNTs–CHI–HA/PTX and PTX at the concentration of 80 μg/mL. Usually cells during exponential growth phase were used, since cells grow vigorously in this period. When cells attained relatively high confluence degree, the growth rate would slow down, thus we can make sure that cells number of the same groups in each well were almost the same, which would guarantee the accuracy of the experiments. Before adding WST–1, the background absorbance of each well was measured at 450 nm. Then, the cell viability was determined using a microplate reader (Multiskan MK3; Thermo Scientific, Waltham, MA, USA) at 450 nm after 2 h.

2.8. Estimation of intracellular reactive oxygen species (ROS)

Oxidation by ROS causes non-fluorescent dichlorofluorescein diacetate (DCFH–DA) to convert to a fluorescent molecule. This conversion was used to measure the level of intracellular ROS in A549 cells. Briefly, 5×10^3 cells were treated with 80 μg/mL of SWNTs/PTX, SWNTs–CHI/PTX and SWNTs–CHI–HA/PTX in serum-free media for 4 h, washed with 1 × PBS and then incubated with 100 μL of serum-free media supplemented with 50 μM of DCFH–DA for 3 h at 37 °C. The media was then aspirated, and the product was harvested in 150 μL lysis buffer. Fluorescence was recorded using a Perkin Elmer fluorescence spectrometer at $\lambda_{ex} = 505$ nm and $\lambda_{em} = 523$ nm. The final value of the intracellular ROS concentration was normalised and represented as a percentage with respect to the untreated control.

2.9. Lactate dehydrogenase (LDH) release

Released LDH was quantified using a TOX7 kit. Briefly, 5×10^4 A549 cells were seeded per well of a 24-well plate and were treated with SWNTs/PTX, SWNTs–CHI/PTX, SWNTs–CHI–HA/PTX and PTX. The assay was performed according to the manufacturer's protocol. Briefly, 100 μL of assay mixture was added to 50 μL of supernatant from each of the samples. The samples were incubated for 30 min at room temperature, and the reaction was terminated by the addition of 15 μL of 1 N HCl. The absorbance was then recorded using a MRX Microplate Reader at 490 nm with a reference wavelength of 650 nm.

2.10. Western blotting

A549 cells, grown to 70% confluence in a flask, were cultured with a medium containing 20 μg/mL of SWNTs/PTX, SWNTs–CHI/PTX, SWNTs–CHI–HA/PTX, and PTX. After 48 h, cells were washed with PBS, harvested with a scraper and centrifuged at 2000 × *g* for 10 min at 4 °C. After

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