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Combining the single-walled carbon nanotubes with low voltage electrical stimulation to improve accumulation of nanomedicines in tumor for effective cancer therapy

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ABSTRACT

Effective delivery of biomolecules or functional nanoparticles into target sites has always been the primary objective for cancer therapy. We demonstrated that by combining single-walled carbon nanotubes (SWNTs) with low-voltage (LV) electrical stimulation, biomolecule delivery can be effectively enhanced through reversible electroporation (EP). Clear pore formation in the cell membrane is observed due to LV (50 V) pulse electrical stimulation amplified by SWNTs. The cell morphology remains intact and high cell viability is retained. This modality of SWNT + LV pulses can effectively transfer both small molecules and macromolecules into cells through reversible EP. The results of animal studies also suggest that treatment with LV pulses alone cannot increase vascular permeability in tumors unless after the injection of SWNTs. The nanoparticles can cross the permeable vasculature, which enhances their accumulation in the tumor tissue. Therefore, in cancer treatment, both SWNT + LV pulse treatment followed by the injection of LIPO-DOX® and SWNT/DOX + LV pulse treatment can increase tumor inhibition and delay tumor growth. This novel treatment modality applied in a human cancer xenograft model can provide a safe and effective therapy using various nanomedicines in cancer treatment.
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1. Introduction

Nanoparticles were introduced to biomedical engineering more than a decade ago, particularly for tumor drug delivery [1–4]. Owing to the enhanced permeability and retention (EPR) effect, nanovectors could carry the antitumor reagent to the blood vessels in the tumor and possibly leak into the tumor tissue through the intercellular space between the endothelial cells [3–5]. Although the antitumor drug could reach the target region because of the accumulation of nanoparticles in the tumor, the concentration of the drug in the tumor tissue remains poor because of the size of nanoparticles and their circulation time [6]. In this study, we propose an improvement in the EPR effect by electrical stimulation.

Electroporation (EP) is an efficient physical method of transporting biomolecules or nanoparticles into the cells because of its local electric field at the target tissue [7–9]. Application of a strong electrical stimulation results in an increased transmembrane potential that could in turn result in the formation of pores in the cell membrane. These reversible pores could last for a specific time, during which the cells could be repaired [10], and the biomolecules could diffuse or drift by the strong electrophoretic force into the target region [11,12]. Due to its effective transfection ability in the treatment of various types of tumors, this method is commonly used for cancer therapies including gene therapy and targeted electrochemotherapy (ECT) [13–22].

Clinical trials using ECT have been approved since the 1990s [23]. During the past few decades, this technology was greatly improved owing to its high efficiency to deliver chemical therapeutics or small molecules into the tumors. Among these anticancer drugs, bleomycin, cisplatin, and doxorubicin are promising drugs that can be applied in combination with EP [24]. These anticancer agents can be administered by intravenous (IV) or intratumoral injections and usually have a dosage limitation because of the high biotoxicity. Therefore, it is very important to note the adverse effects caused by these drugs, including pulmonary fibrosis, nephrotoxicity, and neurotoxicity [25,26]. Using EP could effectively lower the dosage and decrease the side effects. Moreover, the incorporation of a nanovector could overcome the limitation of the anticancer agent's poor solubility in aqueous solutions. However, effective EP usually requires high voltages (HVs), which could result in irreversible EP and tissue ablation [27,28]. Here, we report the application of a single-walled carbon nanotube (SWNT) pulsing buffer, which can be combined with low external static electric fields. Using

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this method, the delivery of nanoparticles could be effectively enhanced by reversible EP. Moreover, the conventional ECT limitation could be overcome.

Considering their unique physical properties, carbon nanotubes (CNTs) have various applications in biomedical nanotechnology including photothermal therapy, near infrared imaging, and photoacoustic imaging [29–35]. However, there remain few applications for the field emission capability of CNTs that can be applied in biomedical research, particularly in tumor models. Due to the high aspect ratio of CNTs, they are capable of enhancing the electric field at their tips and creating localized high-field regions [36–38]. This well-known phenomenon has been broadly used in field emission applications in which CNTs act as an electron field emitter [39]. Moreover, in vitro electropermeabilization studies, which are based on CNTs, have revealed that they could amplify the external electric field, thereby inducing pore formation in the cell membrane [40–43]. When the cells were exposed to electric field inside the microfluid chip embedded with CNTs, it led to leakage of the intracellular components, and a low voltage (LV) was sufficient for the electrical stimulation [42]. Wang et al. demonstrated that the MWNT-enhanced EP effect via significantly lower electric fields had the potential for tumor cell ablation in vitro [43].

In the present study, we employed SWNTs as nanoelectrodes dispersed in the pulsing buffer around the cellular environment during electro-stimulation in order to lower the EP voltages and reduce cell mortality. Using an animal model, we also demonstrated that this method enhanced the EPR effect, thereby improving the delivery of nanoparticles that permeate from the blood vessel to the tumor tissue. Moreover, we presented evidence that SWNTs and liposomal nanovector with doxorubicin (SWNT/DOX and LIPO-DOX®) can be used as nanomedicines with SWNT + EP, which showed improvement in tumor inhibition as compared with nanomedicines alone. These results suggest that the new modality can be applied to various types of nanomedicines to improve antitumor therapy efficacy.

2. Materials and methods

2.1. Preparation of SWNT pulsing buffer

Raw SWNTs were purchased from Golden Innovation Business Co., Ltd. (Taipei, Taiwan). SWNTs were oxidized by exposure to a strong acid solution (H_2SO_4: HNO_3 = 3:1) for 24 h. After purification, oxSWNTs were refluxed in 4 M HNO_3 at 80 °C for 48 h to shorten the length. After washing, the resultant suspension was then diluted with 250 ml of water, and the oxSWNTs were collected on a 100-nm pore membrane filter (Millipore) and washed with deionized water. The obtained oxSWNTs were further resuspended in water and centrifuged at 4000 rpm for 30 min to remove the residuals. The collected oxSWNTs were lyophilized to dryness at room temperature. The resulting particles were dissolved in phosphate-buffered saline (PBS) (pH = 7.2) and sonicated for 30 min. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma-Aldrich) and sulfo-NHS (N-hydroxysulfosuccinimide) (Sigma-Aldrich) were added to the SWNTs and stirred at room temperature for 1 h. This was followed by treatment with poly (ethylene glycol) bis (3-aminopropyl) terminated (MW = 6000) (Sigma-Aldrich) for 72 h with stirring at room temperature. Unboned excess materials were removed by filter tubes (100 kDa AmiconYM-50, Millipore), centrifuged at 4000 rpm for 30 min, and washed thoroughly with water. Next, the SWNT-PEG_{6k} (20 μg/ml) was suspended in PBS with 0.01% Pluronic F127 (Sigma-Aldrich) as an SWNT pulsing buffer for cell electropermeabilization. The size distribution was deduced by TEM (Hitachi H-7650) images. High-magnification images of SWNTs were obtained from FEG-TEM (Philips Tecnai F30 Field Emission Gun Transmission Microscope).

2.2. DOX loading on PEGylated SWNTs

SWNT-PEG was loaded with doxorubicin non-covalently by π–π stacking and hydrophobic interactions according to the aromatic structure of DOX [44]. We mixed DOX with PEGylated SWNTs (weight ratio = 1:10) in PBS (pH = 7.4) overnight. Unabsorbed excess DOX was removed by filter tubes (100 kDa AmiconYM-50, Millipore), centrifuged at 4000 rpm for 30 min, and washed thoroughly with water until the filtrate was no longer red. The formed SWNT/DOX complex was characterized by a UV–vis spectrophotometer (SpectraMax Microplate Readers, Molecular Devices, USA). The particle size and zeta potential were analyzed by Zetasizer Nano-ZS90 (Malvern Instruments Ltd., UK).

2.3. EP systems

We used two types of commercial EP systems in our investigation: suspension and attachment types. Two tests were performed to investigate the repeatability combined with SWNT pulsing buffer. We used the Neon™ Transfection System (MP-100, Invitrogen™, Life Technology) and a Neon™ 100 μl kit (Invitrogen™, Life Technology) for the cell-suspended permeabilization. The attachment type used was a BEX electroporator (Tokiwa Science, Tokyo, Japan) with electrodes (LF650S7, LF647P2X5) for both the in vitro and in vivo experiments. For all the in vitro experiments, the Neon™ Transfection System was used, excluding the JC-1 and the real-time event of the EP experiments.

2.4. Propidium iodide dye delivery

HT-29 cells (human colon adenocarcinoma cell lines) were obtained from the American Type Culture Collection (ATCC). Cells were routinely cultured in flasks containing Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. For cell permeabilization in 100 μl of pulsing buffer, HT-29 cells were trypsinized, centrifuged for 5 min at 1500 rpm, and resuspended in buffer T® or SWNT buffer (10^6 cells/ml) mixed with propidium iodide (PI, 0.5 μg/ml). At 5, 15, and 60 min after EP, the cells were seeded into 24-well plates. Cells with pore formation were identified by the cellular uptake of the PI dye with the subsequent fluorescent signal immediately. The EP parameters were LV (100 pulses at 50 V, pulse duration = 40 ms) and HV (3 pulses at 1600 V, pulse duration = 10 ms). The PI signal expression in HT-29 cells was quantified by calculating the integrated optical density (IOD) using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

2.5. Real-time observation of cell electropermeabilization

To monitor the real-time event of EP, the cells were seeded into a 6-cm dish. After 24 h, the medium was changed with pulsing buffer mixed with PI. Next, the dish was placed under a fluorescence microscope (Olympus IX71) using the TRITC channel (Excitation filter: HQ535, Emission filter: HQ610). Before and after electro-stimulation, the images were captured (1.53–1.84 image/s) for 40 min by the camera (Olympus U-CMAD3). The results are demonstrated in Movies S1 and S2.

2.6. Cell viability

HT-29 cells were trypsinized, centrifuged for 5 min at 1500 rpm, and resuspended in buffer T® buffer or SWNT pulsing buffer (10^6 cells/ml) mixed with 0.5 μg/ml PI. At 60 min after EP, the cells were seeded into plates and changed with the fresh medium. After 24 h, the cells were stained with Calcien AM (Sigma-Aldrich) to observe the cell viability. Cells with pore formation were identified by the fluorescent signal of PI.

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