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Prostate stem cell antigen antibody-conjugated multiwalled carbon nanotubes for targeted ultrasound imaging and drug delivery

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ABSTRACT

Multiwalled carbon nanotubes (MWCNTs) are cut short and grafted with polyethylenimine (PEI) for further covalent conjugation to fluorescein isothiocyanate (FITC) and prostate stem cell antigen (PSCA) monoclonal antibody (mAb). The *in vitro* and *in vivo* toxicity data reveal that the as-prepared CNT-PEI(FITC)-mAb has good biocompatibility. Combined flow cytometry and confocal luminescence imaging experiments confirm that the CNT-PEI(FITC)-mAb can specifically target the cancer cells which over-express PSCA. The results of *in vitro* and *in vivo* ultrasound (US) imaging indicate that CNT-PEI(FITC)-mAb has great potential to be used as a targeted US contrast agent. The *in vivo* anti-cancer efficacy testing using PC-3 tumor-bearing mice as animal models demonstrates that CNT-PEI(FITC)-mAb can targetedly deliver drug to the tumors and suppress tumor growth. Findings from this study suggest that the CNT-PEI(FITC)-mAb could be used as a multifunctional platform for simultaneous US imaging and drug delivery applications.

1. Introduction

With the development of nano-biotechnology, it becomes feasible to develop multifunctional nanostructured materials for simultaneous diagnoses and treatment of cancers by the integration of molecular imaging and therapy technologies [1]. For conventional molecular imaging technology, a variety of new contrast agents have been developed in the past decade to realize the visualization of tumor lesions [2], but the cancer therapy can not be achieved at the same time by only using the contrast agents. On the other hand, it is necessary to observe and track the lesion site in real time during cancer treatment, so that the treatment effect can be conveniently evaluated. Therefore, the development of multifunctional nanoplatforms that combine imaging capabilities and therapeutic functions into a single system becomes important [3,4].

In recent years, carbon nanotubes (CNTs) have emerged as promising candidates for highly efficient delivery of drugs and biomolecules due to their unique structure and properties [5,6]. CNTs can be conjugated with anti-cancer drugs either by covalent or noncovalent interaction methods [7]. Some cancer chemotherapy agents with extended π-structures larger than one aromatic ring, such as anthracycline antibiotic doxorubicin (DOX), can be easily bound to CNTs non-covalently by strong π–π interactions [8–12]. It would be much more desirable if CNT-anticancer drug conjugates were equipped with tumor-targeting ligands or antibodies. Such tumor-targeting formulations can not only improve their internalization efficiency, but also minimize the potential toxic side effects [9,13].

Ultrasound (US) imaging is a well-established clinical imaging modality with non-invasive, non-ionizing, real-time and cost-effective features [14,15]. For better visualization of specific tissues, microbubbles and perfluorocarbon emulsions of various formulations have been developed and applied clinically as US contrast agents, but they are suffered from instability to insonification pressures, broad size distribution, and poor circulating acoustic contrast properties [2,16]. Inorganic material-based US contrast agents have recently attracted great attention due to their tunable particle sizes, good biocompatibility and superior stability than traditional organic microbubbles under US exposure [17,18]. Very recently, Delogu et al. described the use of functionalized

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multiwalled carbon nanotubes (MWCNTs) as hyperechogenic material [18]. The US signal of CNTs was equal to that of sulfur hexafluoride, a commercially available contrast agent. Therefore, the excellent echogenic property of functionalized CNTs is highly beneficial for the development of CNT-based tumor-targeting US contrast agents.

Prostate stem cell antigen (PSCA), which belongs to the family of glycosyl phosphatidylinositol anchored cell surface antigens, has high prostate-specificity [19]. In normal prostate, only basal cells express low levels of PSCA, while exocrine cells and stromal cells show almost no PSCA expression. On the contrary, the high-grade prostatic intraepithelial neoplasia, hormone-dependent prostate cancers, and hormone-independent prostate cancers all show a high PSCA expression, and the PSCA is strongly expressed in 100% of metastatic prostate cancers [20,21]. PSCA has many important advantages, such as large molecular weight, being located on the cellular surface, and a comparatively higher level of expression at various stages of human prostate cancer development. Therefore, PSCA is a promising target for the diagnosis and treatment of prostate cancers [22,23].

In the present work, the shortened MWCNTs were covalently functionalized with polyethylenimine (PEI), followed by the conjugation of fluorescein isothiocyanate (FITC) and PSCA monoclonal antibody (mAb_{PSCA}) (Fig. 1). The formed nanomaterial CNT-PEI(FITC)-mAb combines receptor-specific targeting, luminescence imaging, US imaging, and drug delivery into one system. Such a MWCNT-based multifunctional platform is favorable for targeted diagnosis and treatment of prostate cancers. The *in vitro* and *in vivo* toxicity of the resulting nanomaterial CNT-PEI(FITC)-mAb was evaluated. Flow cytometry and confocal luminescence imaging were employed to research the specificity of CNT-PEI(FITC)-mAb to the PC-3 cells with overexpressed PSCA. The US imaging effects of CNT-PEI(FITC)-mAb were investigated, and this material was then used for *in vivo* targeted US imaging. Finally, DOX was used as a model drug to study the targeted drug delivery of CNT-PEI(FITC)-mAb and chemotherapy effects of PC-3 tumor-bearing mice intravenously injected with CNT-PEI(FITC)-mAb/DOX.

2. Experimental section

2.1. Synthesis of CNT-PEI(FITC)

The preparation procedure of carboxyl-terminated MWCNTs (MWCNT-COOH) and the source of the chemicals and materials can be found in Supplementary File.

For synthesis of CNT-PEI(FITC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 0.2 g) and N-hydroxysuccinimide (NHS, 0.2 g) were introduced to a methanol suspension (150 mL) of MWCNT-COOH (30 mg) to activate the carboxylic acid groups of MWCNT-COOH, and the mixture was stirred gently at room temperature for 3 h. Then PEI (0.2 g) was added to the activated MWCNT-COOH solution, followed by another 24 h of stirring. The resulting MWCNT-PEI was separated by centrifugation, rinsed with methanol repeatedly to remove excess polymer, and then dried under vacuum.

For conjugation of FITC, MWCNT-PEI (10 mg) was dispersed in 10 mL of phosphate buffered saline (PBS), then FITC (5 mg) was introduced, and the mixture was stirred at room temperature in dark for 24 h. The resulting CNT-PEI(FITC) was recovered by centrifugation, washed in sequence with ethanol and PBS, and then dispersed in PBS.

2.2. Covalent immobilization of mAb_{PSCA}

The mAb_{PSCA} was directly immobilized on MWCNTs using the well-established glutaraldehyde method [24–26]. Briefly, 5 mg of MWCNT-PEI(FITC) dispersed in 1 mL of PBS (pH 7.4) was added dropwise to the PBS buffer (5 mL) containing 5% glutaraldehyde and the stirring was continued for about 2 h. Then the solid was separated by centrifugation and washed with PBS three times. After re-dispersed in PBS, the material was further incubated with mAb_{PSCA} (10 μL, 1 mg mL^{-1}) for 12 h at 4 °C. The antibody-conjugated material CNT-PEI(FITC)-mAb was washed with PBS several times to remove excess antibody and kept at 4 °C in PBS.

Characterization of the materials can be found in Supplementary File.

2.3. In vitro toxicity of CNT-PEI(FITC)-mAb

2.3.1. Cell culture

PC-3 cells overexpressing PSCA and MCF-7 cells low-expressing PSCA (as a control cell line), which were provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS (China), were cultured in Ham's F12 and Dulbecco's Modified Eagle Medium, respectively, at 37 °C under a 5% CO_2 atmosphere. Each cell culture medium was supplemented with 10% fetal bovine serum (FBS). The cells were routinely harvested by treating with a trypsin-ethylene diamine tetraacetic acid solution (0.25%).

2.3.2. Cytotoxicity

In vitro cytotoxicity was evaluated by performing 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) assays on PC-3 and MCF-7 cell lines. The cells, which were seeded in a 96-well cell culture plate at a density of 1×10^4 cells per well, were cultured under 5% CO_2 at 37 °C for 24 h. Then the cells were exposed to serial concentrations of the CNT-PEI(FITC)-mAb, and further incubated for 12 or 24 h. After removing the media, 100 μL of WST-1 solution (5 mg mL^{-1}, dissolved in PBS) was added and the cells were incubated for another 4 h. The absorbance was monitored using a microplate reader (Thermo scientific Multiskan MK3) at the wavelength of 450 nm. The cytotoxicity was expressed as the percentage of cell viability compared to that of untreated control cells.

2.3.3. Hemolysis assay [27]

Human red blood cells (HRBCs) were obtained by removing the serum from the blood (kindly provided by Shanghai Blood Center) using centrifugation and suction.

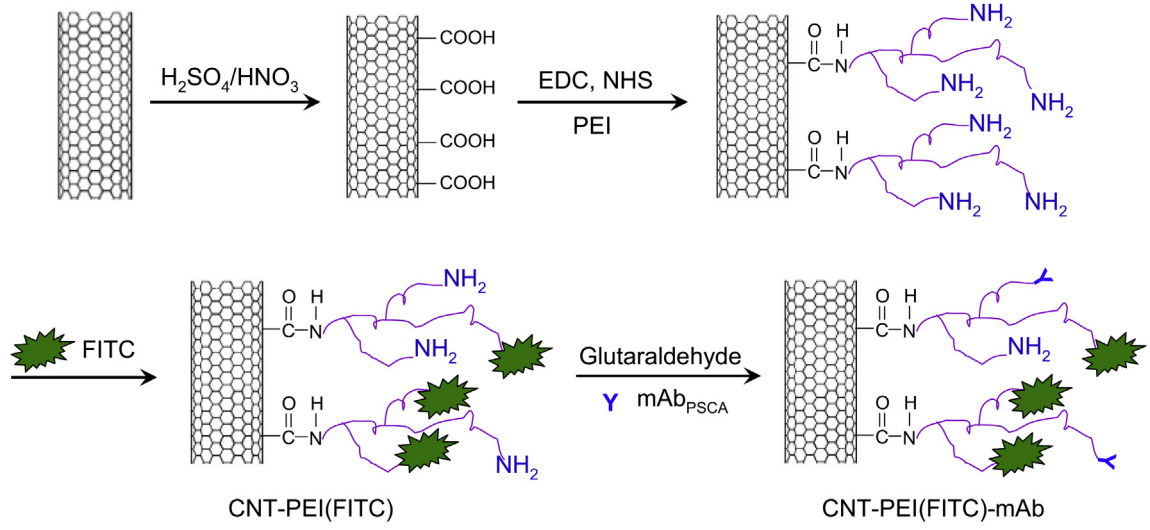

Fig. 1. Schematic diagram of the synthesis process for CNT-PEI(FITC)-mAb.