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Doxorubicin loaded folate-targeted carbon nanotubes: Preparation, cellular internalization, in vitro cytotoxicity and disposition kinetic study in the isolated perfused rat liver

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ABSTRACT

The objective of this study was to use the functionalized multi-wall carbon nanotubes (CNTs) for the delivery of doxorubicin (DOX). Polyethylene glycol (PEG) chains are attached to CNTs then folate-conjugation of PEGylated CNTs was prepared. The amount of drug loading was calculated by the Multivariate Calibration Method for simultaneous quantification of DOX and CNTs. Cytotoxicity was evaluated using the folate receptor-positive HeLa cell line. To assess distribution and elimination of free DOX and drug-loaded CNTs, a recirculating rat liver perfusion system was used and pharmacokinetic parameters were calculated using non-compartmental analysis. Loading efficiency of 84.3 ± 3.1% and 49.3 ± 5.4% was calculated for low-PEGylated and high-PEGylated CNTs respectively. A higher release rate of DOX was achieved at a higher amount of PEGylation. Folate-targeted CNTs expressed a 3.2-fold decrease in IC_{50} value compared with non-targeted CNTs. The result from liver perfusion experiments revealed that DOX accumulation in the liver was higher when PEGylation was lower. There was a 2.4-fold decrease in the elimination rate constant compared to free DOX, which was attributed to the redistribution of DOX from hepatocytes in a sustained release pattern that is consistent with an increase in the mean residence time and prolonged circulation. In conclusion, folate-targeted CNTs show great potential as a targeted anticancer delivery system.

1. Introduction

Doxorubicin (DOX) is a commonly used anthracycline for cancer chemotherapy that is rapidly cleared from the blood after intravenous injection [1]. Therefore many studies have been performed to improve the pharmacokinetics and biodistribution of DOX in order to enhance the efficacy while reducing the side effects of the drug. Following discovery of carbon nanotubes (CNTs), their biological attributes have attracted attention [2–4] and several in vitro studies have demonstrated that CNTs can effectively transport various biomolecules into the cells [5–10]. The application of CNTs in medicine and biology is subject to their surface chemical modification in order to introduce hydrophilic moieties onto the CNT hydrophobic surface to conquer a lack of solubility and to improve their biocompatibility [2,5,7–9]. Three main modifications including covalent attachment, non-covalent attachment and a hybrid approach have been used to create bio-modified CNTs [11]. The main disadvantage of non-covalent attachment and the hybrid approach is the lack of biomolecule specificity upon adsorption, which affects CNT dispersion stability by replacing the functional surface coating with all physiological fluids (cell culture media or blood), due to the presence of various proteins and other organic or inorganic molecules [12]. Since the stability under physiological conditions is crucial for CNT formulations, using covalent functionalization is rational and extremely promising approach for biomedical applications [12–14]. Liu et al. [15] showed that due to supramolecular chemistry characteristics, a high affinity for non-covalent binding exists between PEG-grafted phospholipid and CNTs via π–π stacking that causes creation of water-soluble CNTs. Another study, conducted by Heister et al. [12], emphasized that the covalent link between PEG and CNTs leads to stable dispersion and biocompatibility in various biological environments. Based on these concepts, creating a variety of CNT formulations bearing varying amounts of covalently attached oxygen-containing functional groups and PEG was considered as compatibility between CNTs with blood and host cells is required in this study for cell viability and liver perfusion [16,17]. As DOX delivery was previously investigated in different studies, non-covalent linkage of DOX through hydrophobic interactions was selected as the best strategy [15,18–20]. Covalent attachment

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as another approach was reported by Chaudhuri et al. [21], which resulted in low drug-loading efficiency (~28% by weight). In comparison, non-covalent attachment exhibited much higher drug-loading efficiency of ~400% by weight [15]. As DOX is mainly eliminated by the liver, and hepatic clearance is a primary determinant of DOX plasma concentration [22], evaluating the hepatic disposition of preparations by liver perfusion as a model to simulatein vivo conditions was investigated in this work, which has never been studied previously. Also, a multivariate calibration method for simultaneous determination of CNT and DOX was developed in this study. While a number of methods for individual determination of DOX and CNT have been reported before, a time-consuming separation step to determine each part is required and drug loading may be affected by the separation step. Besides solving these problems, the possibility of simultaneous quantification of both components indicates the superiority of the new method over the conventional one [15,20].

2. Materials and methods

2.1. Materials

Multi-walled carbon nanotubes (number of walls: 3–15, outer diameter: 5–20 nm, length: 1–10 μm, purity of over 95 wt.%) were purchased from Plasmachem, Germany. DOX hydrochloride from Sterling Biotech Ltd., India was kindly donated by Loghman Pharmaceutical Co., Iran. Diamine-terminated oligomeric poly(ethyleneglycol), O,O'-bis(2-aminopropyl)poly(ethylene glycol)3400, heterobifunctional polyethylene glycol or O-(2-aminoethyl)-O′-(2-carboxyethyl)polyethylene glycol hydrochloride 3000, dialysis bags with molecular weight cut-off 12 kDa, dialysis benzoylated tubing with molecular weight cut-off 2 kDa, bovine serum albumin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) powder were supplied from Sigma-Aldrich, UK. Transaminase kits for alanine transaminase (ALT) and aspartate transaminase (AST) measurement were purchased from MAN Co., Iran. N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), folic acid, thionyl chloride, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), HPLC-grade acetonitrile and methanol were obtained from Merck, Germany. The HeLa cell line (human, cervix, epithelial-like, carcinoma) was purchased from the Pasteur Institute of Iran, Tehran, Iran. Fetal bovine serum (FBS), RPMI 1640 and penicillin/streptomycin were obtained from Gibco Inc., UK. Other chemicals and solvents were of analytical grade and used without any further purification.

2.2. Animals

Male Sprague–Dawley rats (250–300 g) were housed in a 12-h light–dark cycle and controlled temperature environment with free access to standard laboratory chow and water. The study was performed in accordance with ethical guidelines approved by the ethical committee of Pharmaceutical Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences, which are based on the NIH guide for the care and use of laboratory animals.

2.3. PEGylation of CNT

Briefly, 1 g of the CNT was suspended in 20 mL nitric acid (65%) and sulphuric acid (98%) in a ratio of 1:3 then refluxed for 21 h at 110 °C. The resultant mixture was diluted with distilled water and, after discarding the yellowish acidic part, the black suspension was centrifuged and the precipitate of oxidized CNTs (ox-CNT) containing carboxylic acid groups was collected by redispersing in a small amount of deionized water followed by the lyophilization step [23]. ox-CNT was then refluxed with 50 mL thionyl chloride to convert the carboxyl to an acyl group and the remaining solid residue was kept under nitrogen [24]. Then, 30 mg of acylated CNT was mixed well with dried diamine-terminated PEG_{3400} (300 mg) in a flask and refluxed at 100 °C under nitrogen protection for 6 days [25] (Fig. 1). The mixture was then cooled to ambient temperature and 10 mL of purified water was added and centrifuged at 40,000 *g* for 20 min to separate the black suspension containing untreated CNT from the black supernatant containing the excess amount of free PEG_{3400} and PEGylated CNTs. To obtain pure PEG-CNT, supernatant was collected and dialyzed (12 kDa) against fresh deionized water for 3 days. Dialyzed solution was then lyophilized and henceforth called “high-PEGylated carrier” with a yield of 58%. The same procedure with 30 mg of both precursors was conducted to prepare low-PEGylated CNT.

2.4. Folate conjugation of CNTs

Heterobifunctional PEGs, containing different terminal groups of amine chloride and carboxylic acid, were used to conjugate folic acid in a folate-hydrazide form. Folate-PEG-NH2 was then grafted to CNTs as described before. Folate-hydrazide was synthesized from folic acid and hydrazine hydrate as reported before [26,27]. To conjugate folate-hydrazide to PEG, the carboxylate group of heterobifunctional PEG was activated by DCC. Briefly, 125 mg DCC was added to 10 mL PEG solution of 2100 mg/mL in DMSO under nitrogen atmosphere at room temperature for 12 h (in molar ratio of 1:1). The activated PEG was reacted with 275 mg of folatehydrazide dissolved in 10 mL of DMSO. The reaction was performed under nitrogen atmosphere at room temperature for 24 h and after centrifugation, the resultant solution from the supernatant was dialyzed (2 kDa) against deionized water for 48 h and then freeze-dried and 300 mg of prepared folate-PEG-NH2 was used to react with 30 mg of acylated CNT via amide linkage under the same reaction described in Section 2.3. Pure folate-PEG-CNTs were obtained with a yield of 26% after dialysis and lyophilisation. The relevant chemical reaction and scheme are presented in Fig. 1.

2.5. Dispersion stability of carriers

To experimentally investigate the efficiency of functional groups on dispersion stability, a centrifugation step at 12,000 *g* for 30 min was performed to evaluate settling behavior in phosphate-buffered solution (PBS) and Krebs–Henseleit buffer solution (KHBS) while content of CNT in supernatant was considered as a function of stable part and freshly prepared ox-CNT in water dispersion serving as the 100% value.

2.6. DOX loading of CNTs

DOX was loaded through supramolecular interaction [15,19,20,28]. Different amounts of DOX were loaded onto the carrier in different ratios (0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1 and 4:1) in PBS medium at pH 5. After 24 h of stirring at room temperature, optimum drug loading was calculated in each preparation using the multivariate calibration method. The method for simultaneous determination of DOX and CNT was developed using UV–VIS spectroscopy double beam, Shimadzu (UV-1800) with 190–1100 nm spectral window and 1-nm interval. A specified amount of loaded carrier in simple and targeted forms was analyzed with the partial least square (PLS) method and drug loading was calculated by the following equations:

$$\text{Drug Loading} = [\text{mass of loaded DOX}/(\text{mass of carrier} + \text{mass of DOX})] \times 100$$

$$\text{Loading Efficiency} = [\text{mass of loaded DOX}/\text{total DOX added during the loading procedure}] \times 100.$$

The PLS algorithm was written in MATLAB (v. 7.10.0.499 for win 32) and the values of the root mean square error (RMSE) of cross-validation, which is an estimate of the absolute error of prediction by cross-validation for each component in the calibration matrix, demonstrated the accuracy of the proposed method.