Materials Science and Engineering C 74 (2017) 167–176

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

TPGS-chitosan cross-linked targeted nanoparticles for effective brain cancer therapy

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ARTICLE INFO

Article history:
Received 29 October 2016
Received in revised form 24 November 2016
Accepted 3 February 2017
Available online 4 February 2017

Keywords:
Brain cancer
Transferrin receptor
Targeted nanomedicines
Synergistic effect
Chitosan
TPGS

ABSTRACT

Brain cancer, up-regulated with transferrin receptor led to concept of transferrin receptor targeted anticancer therapeutics. Docetaxel loaded D-α-tocopherol polyethylene glycol 1000 succinate conjugated chitosan (TPGS-chitosan) nanoparticles were prepared with or without transferrin decoration. In vitro experiments using C6 glioma cells showed that docetaxel loaded chitosan nanoparticles, non-targeted and transferrin receptor targeted TPGS-chitosan nanoparticles have enhanced the cellular uptake and cytotoxicity. The IC_{50} values of non-targeted and transferrin receptor targeted nanoparticles from cytotoxic assay were found to be 27 and 148 folds, respectively higher than Docel™. In vivo pharmacokinetic study showed 3.23 and 4.10 folds enhancement in relative bioavailability of docetaxel for non-targeted and transferrin receptor targeted nanoparticles, respectively than Docel™. The results have demonstrated that transferrin receptor targeted nanoparticles could enhance the cellular internalization and cytotoxicity of docetaxel via transferrin receptor with improved pharmacokinetics for clinical applications.

1. Introduction

Brain cancer is a tough task in oncology which affects many people around the globe. In one US report, the incidence of primary brain tumors is 7–8 per 100,000, and estimated new cases of brain and other nervous system is approximately 23,380 [1]. Among these, glioma is the most common and aggressive type with a survival time of 12–15 months and a 5-year survival rate of <5% [2]. Also, the 1 year survival span of brain cancer patients has not significantly improved. The reduced efficacy of chemotherapy is mainly attributed to the existence of the blood brain barrier (BBB), which inhibits the penetration of drug molecules into brain given systemically [3–5]. Another hindrance is the blood-tumor barrier (BTB), which limits drug accumulation and uptake, results in reduced drug accumulation in brain tumor [6]. Furthermore, occurrence of multidrug resistance (MDR) is one of the major reasons for treatment failure. MDR occurs through various mechanisms, among which, p-glycoprotein (p-gp), a member of ATP-binding cassette (ABC) transporter, acts as an efflux pump for most hydrophobic anti-cancer agents [7].

Further, targeting anti-cancer drugs into the brain is a challenging area for drug delivery scientists [8–10]. Since the BBB does not transport the anti-cancer drug, higher doses are required to achieve desired therapeutic effect, which produces unwanted side-effects [11]. To solve these problems, many drug targeting and delivery strategies to the brain cancer have been discovered and developed in order to manipulate the endogenous transport systems and overcome the BBB without physically affecting brain tissues [12–15]. Many nanocarriers are under investigation such as albumin nanoparticles, gelatin nanoparticles, nanoconjugates, polymeric nanoparticles, solid-lipid nanoparticles, carbon nanotubes, nanoparticles, and liposomes etc. [16].

Nanomedicines are products of innovative strategies which are developed to overcome the limitations of conventional chemotherapeutics by entrapping the anticancer agents in biocompatible and biodegradable nanocarrier systems resulting in its controlled and specific release to the target cancer cells. Nanoparticles can passively target tumors by the enhanced permeability and retention (EPR) effect or actively target by conjugating the ligand on the surface of nanoparticles.

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http://dx.doi.org/10.1016/j.msec.2017.02.008
0928-4931/

The present approaches for treating brain cancer are surgery, radiotherapy, chemotherapy and radiotherapy in combination with chemotherapy [17]. In the current scenario, a desired payload of anticancer drug at the brain tumor is a major obstacle due to the blood-brain barrier, efflux transport processes and endothelial cell metabolism that negatively affect the therapeutic efficacy of many drugs [2,18–20]. Recently, increase in the therapeutic index of chemotherapeutic drugs is achieved by developing different ligand mediated drug delivery systems. In some studies, the scientists have focused on macromolecular carriers such as monoclonal antibodies, proteins and peptides. Also, hybrid molecules were developed to bind with specific, overexpressed receptors such as transferrin, insulin, integrin etc. on tumor cells which are attractive avenues for selective targeting [21].

The biodegradability and non-toxicity of chitosan (as its breakdown product, glutamic acid, can enter normal cellular metabolism) makes it a good candidate for various biological applications including sustained release materials, drug carriers, biological adhesives, and biodegradable fibers, etc. [22]. This polycationic polysaccharide has been utilized in our study to form nanoparticles by the electrostatic interaction between anionic sodium tripolyphosphate and cationic chitosan [23]. In addition to biodegradability, chitosan solubility plays a key role in the use of chitosan derivatives for the development of bioadhesive nanocarrier systems [24]. The bioadhesiveness on the cancer cell surface can be achieved by strong electrostatic interaction between positive charge of chitosan and negative charge of the cell membrane [25]. Also, pH sensitive drug release in tumor tissues or cell microenvironment is achieved by chitosan polymer. Co-administration of p-gp inhibitor like D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) can also induce the synergistic cell apoptosis in glioma cells [7,26–28]. TPGS is a derivative of the natural vitamin E (α-tocopherol), which is conjugated with polyethylene glycol 1000. TPGS on acidification that is derivation to TPGS-COOH makes it available for conjugation to proteins and macromolecules. Herein, conjugation of chitosan with TPGS can firstly, increase the drug solubility at acidic pH environments. Secondly, zeta potential reversal can provide more stability to the nanoparticles, thirdly, cellular adhesion and retention of the delivery system at the cell surface can prolong the residence time, fourth, the drug release can be sustained at acidic pH and lastly, enhanced permeation can be achieved by reversible opening of tight junctions [7].

The transferrin receptor (TfR) is pervasively expressed at low levels in most normal human tissues. It acts as the main port of entry for iron bound Tf into cells. TfR1 is a type-II receptor that resides on the cell membrane and cycles into acidic endosomes within the cell in a clathrin/dynamin-dependent manner. The higher expression of the receptor on brain cancer cells, its ability to internalize, and the necessity of iron for cancer cell proliferation makes this receptor a long-established accessible portal for the delivery of drugs into brain cancer cells and thus, an alluring target for brain cancer therapy. Transferrin as well as its conjugates (with peptides, polymers, antibodies and antineoplastic drugs) have been thoroughly studied for glioma targeting and have produced remarkably effective therapeutic results [29–31]. Targeting cancer cells through use of the TfR can enhance drug delivery by increasing intracellular drug concentration resulting in enhanced tumor targeting, less non-specific toxicity, and therefore an overall increased therapeutic efficacy [32–33].

Our approach in this research was to develop TfR targeted bioadhesive TPGS conjugated chitosan (TPGS-chitosan) nanoparticles to deliver the potent cytotoxic agent DTX to brain cancer. Here, TPGS was functionalized to TPGS-COOH, then it was conjugated covalently to the amino ends of chitosan (i.e., TPGS-chitosan), as well as transferrin (i.e., TPGS-Tf). Secondly, conjugates (TPGS-chitosan and TPGS-Tf) were included in the nanoparticle preparations. The bioadhesive nanoparticles were then characterized for their size and size distribution, drug entrapment efficiency and drug release. A fluorescent marker coumarin-6 (CM6) was encapsulated to visualize the cellular uptake of the nanoparticles and the synergistic effects of chitosan and transferrin in cellular internalization of nanoparticles by qualitative cellular uptake studies in C6 glioma cells. In vitro cytotoxicity (IC_{50}) effect on C6 glioma cells for 24 h was used to evaluate the therapeutic effects of the nanoparticles with or without targeting function. Further, nanoparticles were also tested in vivo for pharmacokinetic parameters using Charles Foster rats, after intravenous (*i.v.*) administrations.

2. Material and methods

2.1. Materials

Docetaxel (DTX) of purity 99.56% used in this study was gifted from Neon Laboratories Ltd., Mumbai, India. Chitosan (extrapure) was purchased from Sisco Research Laboratories, Mumbai, India. D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) was gifted from Isochem, France. Dialysis membrane (Spectra/Por7®) of 1 kDa molecular weight cut off was purchased from Spectrum Laboratories Inc., Rancho Dominguez, CA, U.S.A. Clinical formulation Docel™ was purchased from RPG Life Sciences Limited, Mumbai, India. Transferrin (Molecular weight of 80 kDa), coumarin 6 (CM6), chloroform and sodium tripolyphosphate, were purchased from Sigma-Aldrich, St. Louis, MO, USA. C6 glioma cell line was provided by National Centre for Cell Science, Pune, India. T-25 culture flask and 96-well culture plates were purchased from Tarsons Products Pvt. Ltd., Kolkata, India. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, L-glutamine and trypsin-EDTA were purchased from Genetix Biotech Asia Pvt. Ltd., Mumbai, India. 3-(4,5-dimethyl thiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Himedia Laboratories, Mumbai, India. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Activation of TPGS, synthesis of conjugates and characterization

TPGS-chitosan and TPGS-transferrin conjugates were prepared after the activation of TPGS by acidification [34]. Carboxylic acid functional groups of TPGS were formed by ring-opening reaction in the presence of succinic anhydride and 4-dimethylaminopyridine. Briefly, TPGS (0.77 g, 0.5 mM), succinic anhydride (0.10 g, 1 mM) and DMAP (0.12 g, 1 mM) were mixed under nitrogen atmosphere and heated to 100 °C. This mixture was made to react for 24 h. It was then cooled to room temperature and 5 ml of cold dichloromethane was added and filtered to remove excessive succinic anhydride. Further, 100 ml of diethyl ether was added and stored at −10 °C for overnight. A white precipitate of TPGS-COOH was formed, it was filtered and dried in vacuum [35].

The carboxylic acid groups of TPGS were further conjugated to either chitosan or transferrin by carbodiimide chemistry in the presence of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and NHS (*N*-hydroxysuccinimide) in phosphate buffer saline (pH 5.5). For the conjugation of chitosan or transferrin to TPGS-COOH, EDC and NHS were added to TPGS-COOH solution to promote activation of –COOH groups by formation of reactive products with a molar ratio of 1:5 (TPGS-COOH: EDC/NHS). In short, TPGS-COOH (200 mg), EDC (96 mg) and NHS (74 mg) were mixed in 6 ml of phosphate buffer saline (pH 5.5) at 25 °C for 5 h and stored in a refrigerator at 4 °C over 24 h. Then it was mixed with solution of chitosan (124 mg) or transferrin (0.2% w/v) stirred at 4 °C for over 8 h. The resultant products i.e., TPGS-chitosan or TPGS-transferrin were then dialyzed using a dialyzing membrane (MWCO: 1 kDa) against phosphate buffer saline (pH 5.5) for 48 h in order to remove excess TPGS-COOH, NHS and EDC. The dialyzed products i.e., TPGS-chitosan or TPGS-transferrin were freeze dried [35–39]. Fourier transformed infra-red spectroscopy (FTIR) was conducted on Perkin Elmer Spectrum Two, Waltham, Massachusetts, U.S.A and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was conducted on JEOL FX Fourier Transform Multinuclear NMR Spectrometer, Japan. In addition, TPGS-chitosan was analysed by matrix-