International Journal of Pharmaceutics 500 (2016) 196–209

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical nanotechnology

AN *in vitro* evaluation of a carmustine-loaded Nano-co-Plex for potential magnetic-targeted intranasal delivery to the brain

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ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 14 January 2016
Accepted 16 January 2016
Available online 19 January 2016

Keywords:
Carmustine
Iron oxide nanoparticles
Magnetite
Superparamagnetic
Core-shell
Chemotherapeutics
Blood brain barrier
Brain tumor
Polyplex
Nano-co-Plex

ABSTRACT

Targeted delivery of carmustine (BCNU), an efficient brain tumor therapeutic, has been challenged with bioavailability issues due to the Blood Brain Barrier (BBB). The currently effective delivery approach is by implants at the site of the tumor, but this is highly invasive. The intranasal route, which is non-invasive and bypasses the BBB, may be alternative route for delivering BCNU to the brain. In this work, polyvinyl alcohol/polyethyleneimine/fluorecein isothiocyanate complex (Polyplex) coated iron-oxide nanoparticles (Magnetite) were synthesized employing co-precipitation, epoxidation and EDC/NHS coupling reactions. The Polyplex coated magnetite (Nano-co-Plex) was loaded with BCNU for potential magnetically targeted delivery to the brain following intranasal administration. The Nano-co-Plex was characterized employing Thermogravimetric analysis (TGA), Superconducting Quantum Interference Device (SQUID) magnetometry, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), X-ray Diffractometry (XRD), Transmission Electron Microscopy (TEM) and Zetasize analysis. Results revealed superparamagnetic hexagonally shaped "core-shell" nanoparticles with cell labeling attributes, of size ranging between 30–50 nm, and a zeta potential value of $+32 \pm 2$ mV. The Nano-co-Plex synthesized was found to possess high degree of crystallinity with 32% Polyplex coating. The loading and release studies indicated a time-dependent loading with maximum loading capacity of 176.82 μg BCNU/mg of the carrier and maximum release of 75.8% of the loaded BCNU. Cytotoxicity of the BCNU-loaded Nano-co-Plex displayed superiority over the conventional BCNU towards human glioblastoma (HG) cells. Cell studies revealed enhanced uptake and internalization of BCNU-loaded Nano-co-plex in HG cells in the presence of an external magnetic field. These Nano-co-Plexes may be ideal as an intranasal magnetic drug targeting device for BCNU delivery.

1. Introduction

Brain cancer is a life threatening disease that occurs when there is formation of abnormal cells within the brain, of which glioma is the most common (Killela et al., 2013). Despite the poor prognostic factors of malignant tumor, the quality of life of patients is seriously affected and may often lead to cognitive dysfunction (Omuro and DeAngelis, 2013). It was estimated that there would be about 23,000 new cases and over 15,000 death in 2015 in the United State alone due to brain cancer (Siegel et al., 2015). Several highly effective chemical and biological therapies have been researched to target brain tumors in order to improve its treatment. However, limited results have been achieved due to the difficulty of chemotherapeutics penetrating into the CNS due to the protective mechanism of the Blood Brain Barrier (BBB). Radiotherapy and surgery are the alternative options that have been used both of which only provide temporary life extension (Hainfeld et al., 2013). The surgical technique is highly invasive and is challenged with excising tumor cells which may in fact affect normal brain cells. It is estimated that the 2 year survival rate is approximately 25% (Sullivan et al., 2014).

Carmustine (BCNU) is known to be an effective chemotherapeutic for the treatment of brain tumors (Qian et al., 2013). It is a

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http://dx.doi.org/10.1016/j.ijpharm.2016.01.043
0378-5173/

nitrosourea compound which is highly lipophilic. It hydrolyzes *in vivo* to produce reactive metabolites which cause alkylation and crosslinking of DNA and RNA. It is known to inhibit repair and causes formation of high molecular complexes through *de-novo* purine synthesis (Dhakane and Ubale, 2012). Targeted delivery of BCNU to the brain has been challenged with bioavailability issues. The systemic route of drug administration is fraught with a short elimination half-life resulting in frequent administration leading to side effects such as hepatotoxicity and pulmonary fibrosis (Qian et al., 2013). Several drug delivery strategies have been developed to overcome these challenges and improve the treatment of brain tumors. These include the use of polymers in various embodiments such as nanoparticles, micelles, microcapsules, copolymers, bioconjugation, microspheres and implants (Fattahi et al., 2013a, b; Kim et al., 2009; Zhang et al., 2009a,b; Xu et al., 2006; Painbeni et al., 1998). However, most of these approaches lack the ability to specifically target the tumor cells and instill adequate local concentrations of chemotherapeutic agents. Alternative strategies include the development of microelectromechanical systems (MEMS) devices (Li et al., 2005, 2004). MEMS devices function by a micro-reservoir releasing a specific dose of chemotherapeutics at the site of the tumor thereby improving local concentration and targeting ability. Biodegradable polymeric microchips (Grayson et al., 2003) are similar to MEMS in terms of multi-dose controlled release at the target site. However, these approaches are characterized by invasiveness, which is of major concern, especially during implantation of the devices. The most effective and well known approach is via localized delivery employing implants at the site of the tumor. Gliadel Wafer® (MGI Pharma, Bloomington, MN, U.S.A.) implants are currently approved for tumor resurrection, but high risk factors such as infection post implantation and brain abscess limit its use (McGovern et al., 2003).

Researchers are constantly looking for ideal strategies to overcome the above mentioned challenges. Magnetic targeting using magnetic nanoparticles (Yang et al., 2011a,b) and molecular targeting employing ligands (Ren et al., 2012) are the two most studied approaches to combat the challenges of side-effects, low deposition of drugs at the target site and the spreading of chemotherapeutics to healthy organs of the body (Yang et al., 2011a,b). Both strategies are quite efficient but are still fraught with low bioavailability issues since the mode of administration is still via the systemic pathway and will be limited by the BBB.

Intranasal drug delivery systems are more efficient in delivering therapeutic agents to the brain via the olfactory pathway. This method of administration has the advantages of circumventing the BBB, ease of administration, non-invasiveness, avoidance of the gastrointestinal tract and first pass metabolism, dose reduction and side-effect minimization (Mistri et al., 2012; Chalbot et al., 2010; Ozsoy et al., 2009; Afifi et al., 2005). The integration of magnetic targeting with an intranasal drug delivery system may be an enhanced strategy of delivering chemotherapeutics to the brain tumor cells.

In this study we have successfully synthesized BCNU-loaded magnetized nanoparticles coated with a complex of Polyvinyl alcohol (PVA) conjugated to Polyethyleneimine (PEI) and Fluorecein isothiocyanate (FITC) referred to as a 'Nano-co-Plex', intended for intranasal delivery to the brain via the olfactory pathway. The magnetic Nano-co-Plex (with the aid of an external magnet) would be projected and guided to the brain following intranasal administration. The choice of PVA is to facilitate and achieve good mechanical strength and chemical stability after conjugation. Essentially, branched PEI was selected due to the number of amine groups which are chemo-specific in forming amide bonds. It has also been widely used as a DNA and gene carrier as it possesses excellent ability to bind and release DNA (Zhang et al., 2014; Huth et al., 2004). FITC was selected due to its excellent fluorescent attributes, and it has been widely used as dye for cell labeling (Pinheiro et al., 2013; Xu et al., 2012; Ge et al., 2009). The conjugation of these compounds to form the Nano-co-Plex that has high BCNU loading and release capacity as well as having the attribute of high magnetization with a cell labeling property is a novel approach for synthesizing an intranasal targeted drug delivery system for the management of brain tumors.

2. Experimental

2.1. Materials

Polyvinyl alcohol (PVA) (30 kDa, 87–90% hydrolyzed), branched polyethylenimine (PEI) 1.8 kDa, iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), phosphate buffer solution (PBS) (pH = 7.2), hydrochloric acid (HCl), sulphuric acid (H_2SO_4), *N*-hydroxysuccinimide (NHS), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), FIuorescein isothiocyanate, epichlorohydrin, sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), dialysis tubing (mw 10,000), deionized water, glutaraldehyde (GA), carmustine (BCNU), chloroform, acetonitrile. These chemicals were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). All other solvents and reagents were of analytical grade and were used as received.

2.2. Preparation of the iron oxide nanoparticles

Iron oxide nanoparticles were synthesized by dissolving 0.3 mol of $FeCl_2{\cdot}4H_2O$ and 0.6 mol of $FeCl_3{\cdot}6H_2O$ in 60 mL aqueous medium to obtain 1:2 molar ratio of Fe^{2+} and Fe^{3+}. This mixture was added dropwise to 200 mL of NaOH (0.4 M, pH = 13) heated up at 75 °C. This was done under continuous stirring condition while the mixture was being purged with N_2 gas to eliminate oxygen. This condition was maintained until dark precipitates of iron oxide nanoparticles were produced. The iron oxide nanoparticles so formed were separated from the supernatant by centrifugation at 5,000 rpm for 30 min using an Eppendorf centrifuge 5804 (Hamburg, Germany). The precipitates were washed with deionized water purged with N_2 while under sonication (Sonics vibra cell, Newtown, CT, USA) for 30 min and subjected to centrifugation once again to separate the precipitates from the supernatant. The precipitates, which are magnetite nanoparticles, were then washed several times with deionized water and air dried.

2.3. Preparation of the Nano-co-Plex

In order to prepare the Nano-co-Plex for drug loading, it is necessary to first synthesize PVA chlorohydrin derivative (PVA-CH). PVA-CH was prepared by dissolving 500 mg of PVA in water (5 mL) at 80 °C. Dilute H_2SO_4 (1 M, 0.7 mmol) was added to the solution after the PVA has completely dissolved followed by drop wise addition of 22.8 mmol of epichlorohydrin. This mixture was stirred for 24 h after which 5% w/v $NaHCO_3$ was added to neutralize the mixture. Acetone was added to the mixture to precipitate out the chlorohydrins. The obtained PVA-CH was filtered, dissolved in 10 mL of deionized water and then dialyzed against water for 2 days while changing the water every 12 h. The product was subsequently freeze dried using Freezone 12 freeze drier (Labconco, KansasCity, USA).

FITC was included in order to investigate the cellular uptake and internalization characteristics of the Nano-co-Plex, and was conjugated by using the 1-ethyl-3(3-dimethylaminopropyl)carbodiimide/*N*-hydroxylsuccinimide (EDC/NHS) coupling method. NHS (115 mg) and 191.7 mg of EDC were added to 40 mL of PBS (pH = 6)