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Research Paper

Redox sensitive cationic pullulan for efficient gene transfection and drug retention in C6 glioma cells

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ABSTRACT

Thiolated cationic pullulan was synthesized by conjugating pullulan with polyethyleneimine (PEI) and mercaptosuccinic acid (MSA). The formed conjugate was oxidized to obtain disulfide linked cationic pullulan (PPMSS). PPMSS exhibited good buffering capacity and nanoplexes formulated were of size less than 150 nm. Nanoplexes formed with PPMSS are redox sensitive and susceptible to reductive cleavage by dithiothreitol (DTT) ensuring the intracellular release of DNA. *In vitro*, cytotoxic evaluation studies of polymers in C6 cell lines established its non-toxic nature. The studies using endocytosis inhibitors revealed the uptake pathways of nanoplexes. Further, the plasmid and polymer tracking studies indicated the successful unpacking of DNA from the nanoplexes and its nuclear localization. The gene transfection efficiency was established by the p53 gene expression studies. Furthermore, the ability of the polymer to inhibit efflux pumping in cancer cells has also been elucidated in terms of P-gp inhibition studies and drug retention kinetics using the anticancer drug, doxorubicin (DOX). Our results also suggest that greater retention of DOX was accompanied by the reduction of disulfide linkage by a ubiquitous intracellular stimulus, glutathione. Thus simultaneous gene and drug delivery using redox sensitive cationic polymers may have a promising potential in cancer therapy.

1. Introduction

The advancement in nano-biotechnology in the recent past have steered the development of multifunctional nanoparticles for use in biomedical applications (Bao et al., 2013). There has been an enormous influx of research in the development of multifunctional nanoparticles for multimodal imaging and diagnostics as well as treatment of cancer (Baetke et al., 2015). Despite intense ongoing research, many cancers are still incurable, as reflected by the continuing increase in mortality rate (Lammers et al., 2011). Chemotherapy is one of the potential treatment options for cancer, but drug resistance exhibited by the cancer cells limits the efficacy of this treatment resulting in recurrence of cancer and its survival. (Fletcher et al., 2010). The major protein that is involved in the phenomenon of multidrug resistance is P-glycoprotein (P-gp). P-gp is a membranous molecular pump that actively effluxes the anticancer drugs from within the cells. Current pharmacological strategies such as the use of P-gp inhibitors have given limited success owing to poor specificity, toxicity and unwanted pharmacokinetics resulting in adverse side effects (Gergely et al., 2006). To date, many nano based strategies have been found useful for overcoming this barrier with desirable therapeutic benefits (Frank et al., 2014). It is reported that the chemotherapeutic activity of many drugs will be further enhanced if the polymer or the nanomaterial itself has the potential to inhibit the P-gp activity or reduce its expression (Hugger et al., 2003). In this line, great efforts have been made towards the development of functional polymeric nanomaterials intended for P-gp inhibition. Previous studies showed that the PEGylated derivatives such as lysine- linked di-tocopherol PEG 2000 succinate (PLV2k) (Jinling et al., 2000) and thiolated PEG-g-PEI can inhibit the efflux pump and enhance the chemotherapeutic effect. The inhibitory activity of thiomers on the efflux pump is mediated by the presence of thiol groups (Werle et al., 2008; Grabovac et al., 2015; Netsomboon et al., 2016). It has been observed that the thiolated PEG-g-PEI displayed more pronounced efflux pump inhibition in comparison with well-known P-gp inhibitors such as 6-mercaptopurine, vitamin E-TPGS as well as myrj® and brij® (Iqbal et al., 2010). However, there are only limited reports available on the potential benefit of polymer based systems in terms of the simultaneous effect on P-gp

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inhibition and cancer therapeutic gene delivery efficacy. Combining these two functions together in a single system can greatly enhance the cancer therapeutic potential. In this line, thiolated pullulan based cationic polymers have been synthesized with the purpose of simultaneous P-gp inhibition and therapeutic gene delivery. Pullulan is a non-ionic polysaccharide and is non-toxic, non-immunogenic and non-carcinogenic in nature. Recently it has been widely explored for various biomedical applications including drug and gene delivery. Cationicity can be inserted to pullulan by incorporating PEI, a known gene transfecting agent. Pullulan contains a flexible hydrophilic group which reduces the heavy surface positive charge and hence systemic side effects can be minimized. Previously, our lab has reported a pullulan-PEI-cysteine based system with both gene delivery and efflux pump inhibition properties (Priya and Rekha, 2016). However, there is always an increased need for improved colloidal stability, easy release of DNA, targetability and simultaneous retention of the anticancer drugs in cancer cells. To this end, functional thiolated cationic polymers have been synthesized with the incorporation of mercaptosuccinic acid (MSA) on to the back bone of a pullulan-polyethylenimine conjugate. Mercaptosuccinic acid provides thiol groups to pullulan-PEI. It has been reported previously that mercaptosuccinic acid acts as a ligand to target cancer cells effectively via EPR effect (Lin et al., 2016). Apart from this, the free carboxyl group in mercaptosuccinic acid may offer the electrostatic repulsion required to maintain the colloidal stability of NP in circulation and with the easy intracellular release of DNA. The strategy of forming reversible disulfide bonds via oxidation of thiol groups ensures the stability of the nanocomplex in the oxidative extracellular milieu while also causing loosening of the complex and liberation of the therapeutic gene at the reductive intracellular environment (Ou et al., 2008). This is mainly due to elevated concentrations of glutathione (50–1000 times normal level) inside the cytoplasm of every cell and more importantly in the tumor cell (Balendiran et al., 2004; Cheng et al., 2011). In addition, the thiomers are also shown to demonstrate high transfection efficiency and low cytotoxicity. It was, therefore aimed to synthesize a redox sensitive cationic polymer, which can perform the dual functions of gene delivery and efflux pump inhibition, as a potential therapeutic strategy to combat cancer.

2. Materials and methods

2.1. Materials

Pullulan (Fluka), mercaptosuccinic acid, polyethylenimine (PEI; MW 25,000 Da, Aldrich US), 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride (EDC), carbonyl diimidazole (CDI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's Reagent), sodium borohydride ($NaBH_4$), hydrogen peroxide (H_2O_2), DNase I, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMEM, trypsin, EDTA, dimethyl sulphoxide (DMSO), doxorubicin hydrochloride, were purchased from Sigma-Aldrich Chemicals Co, USA. p53 Dominant-Negative Vector was from Clontech, USA, YOYO iodide, Hoechst 33342 from Invitrogen and FBS was purchased from GIBCO, USA, Calf thymus DNA (ctDNA), was purchased from Worthington Biochemical Corp. All other reagents were of analytical grade from Merck, India.

2.2. Preparation of PPMSS (disulfide cross linked pullulan-PEI-mercaptosuccinic acid)

The introduction of thiol groups (mercaptosuccinic acid) to the amino groups of pullulan −PEI (PP) was carried out using a water soluble carbodiimide crosslinker, EDC. The whole approach was made in two steps. A known quantity of pullulan was initially dissolved in DMSO, followed by the addition of a cross-linker CDI to pullulan under stirring. The reaction was done at 37 °C for 2 h to form reactive carbamate with the hydroxyl group of pullulan. To this, PEI (at 1:0.8 w/w) dissolved in 20 mM borax was added, and was kept under stirring at room temperature for overnight. The PP conjugate was then precipitated out using acetone, redissolved in water and centrifuged using a filter of MWCO 30000 Da to remove the unreacted PEI and was then dialyzed using a membrane of MWCO 100 kDa in deionized water. Pullulan-PEI (PP) was then conjugated with different amounts *i.e.* 30, 40, and 50 mg of mercaptosuccinic acid (MSA). Mercaptosuccinic acid, as mentioned, was added to 100 mg of PP conjugates in the presence of a cross-linker EDC (0.1 M) and the reaction was carried out overnight at pH 6.0 under stirring at room temperature. Later, dialysis was performed with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4 containing 1 mM EDTA and subsequently with deionized water. A known amount of the resultant polymer (Pullulan-PEI-MSA) was taken, mixed with 100 mM of PBS (pH 3.5) and 100 μl of H_2O_2 was added to enable the oxidation of −SH group. The oxidation reaction was carried out for 2 h at room temperature under stirring. The reaction was stopped by adding 0.1 M PBS, pH 4.5 and then overnight dialysis was done to remove unreacted molecules, leaving behind the disulfide-linked cationized pullulan product (PPMSS). Similarly, control groups were prepared by conjugating PEI (200 mg) with different amounts of MSA alone (PEI-MSA) using EDC and later carried out oxidation and dialysis as mentioned above.

2.3. Characterization techniques

The IR spectra of pullulan-PEI, PPMSS, and PEI-MSA were recorded using a Fourier Transform Infrared spectrometer (Nicolet Impact 410) over an ATR scan range of 500–4000 cm^{-1}.

^1H NMR spectra of PPMSS and PEI-MSA were measured in D_2O using a 500 MHz spectrometer (Bruker Avance).

The disulfide/thiol contents of PPMSS and PEI-MSA were measured using a previously reported protocol (Damodaran, 1985). The disulfide content of the polymers prepared at a concentration of 1 mg/ml was assessed using 2-nitro 5-thiosulfobenzoate (NTSB) in a microplate reader (Synergy H1, USA). The quantification was done using cystamine as standard. Similarly, the free thiol content of the polymer was determined by Ellman's assay after reduction of the disulfide linkages by sodium borohydride (Winther and Thorpe, 2014). The measurements were recorded using a UV spectrophotometer (Varian Cary, USA) at a wavelength of 412 nm. The amino content of the polymers was simultaneously quantified using $CuSO_4$ assay.

2.4. Determination of particle size and zeta potential

Nanoplexes of different weight ratios ranging from 1:1 to 5:1 were prepared by mixing, and vortexing varying quantity of PPMSS or PEI-MSA with a fixed amount of ctDNA (10 μg) with the final volume, made up to 1 ml with distilled water. The vortexing was done for 30 s, and it was then incubated at RT for 20 min. The hydrodynamic size and zeta potential of the nanoplexes were measured using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C.

2.5. TEM analysis

The particle size and morphology of the PPMSS and PEI-MSA nanoplexes were observed using TEM. Here, the nanoplexes of desired ratios (PPMSS/ctDNA 4:1 & PEI-MSA/ctDNA 4:1) were formed as above. Using a fine micro tip, a small drop of the nanoplex was added onto a copper grid and air dried. The dried