Journal of Controlled Release 144 (2010) 75–81

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Pegylated immuno-lipopolyplexes: A novel non-viral gene delivery system for liver cancer therapy

Yurong Hu, Kun Li, Lei Wang, Shasha Yin, Zhenzhong Zhang, Yun Zhang *

School of Pharmacy, Zhengzhou University, 100 Science Road, Zhengzhou 450001, PR China

ARTICLE INFO

Article history:
Received 16 October 2009
Accepted 1 February 2010
Available online 6 February 2010

Keywords:
Receptor-mediated endocytosis
Polyethylenimine
Pegylated immnuo-lipopolyplexes
Gene therapy
Liver cancer therapy

ABSTRACT

In this study, pegylated immuno-lipopolyplexes (PILP), a novel and efficient gene delivery system was developed by employing DNA/polyethylenimine (PEI 25 kDa) polyplexes, as well as anionic liposomes composed of POPC, (DSPE)-PEG2000 and (DSPE)-PEG2000-biotin, at five different lipid/DNA molar ratios (50/1, 90/1, 130/1, 170/1 and 210/1), and by using streptavidin-monoclonal antibody conjugating through the biotin group located at the distal end of the PEG spacer as targeting antibody. This vector was highly effective in protecting DNA from enzyme digestion, and stable in particle size and zeta potential even after 20 day-storage at 4 °C. At the lipid/DNA molar ratio 170/1, the PILP were found to have the highest *in vitro* transfection efficiency with an average particle size of 132 nm and an average zeta potential of +9.5 mV. These complexes showed high efficiency in gene delivery to liver cancer cells with no significant cytotoxicity. Interestingly, the *in vitro* transfection efficiency did not decrease significantly up to 10 days of storage of PILP at 4 °C. Intravenous administration of the PILP resulted in tumor and liver targeted gene expression of the reporter genes EGFP and luciferase as opposed to the lung targeted gene expression obtained with PEI/DNA complexes, causing no cytokine production and liver injury. We conclude that the PILP are promising gene delivery systems which may be used to target the liver cancer.

1. Introduction

Gene therapy focuses on the therapeutic use of genes delivered to cells and promises considerable advances in the treatment of several important diseases. In order to succeed in cancer gene therapy, the efficient delivery of therapeutic genes to a target site is a major challenge. Viral vectors are very effective gene delivery systems because of their high transfection efficiency, however, immunogenicity and toxicity issues have arisen and few clinical results have been translated into a successful commercial product [1].

Non-viral vectors have several advantages in terms of safety and pharmaceutical issues such as lack of immunogenicity, easy synthesis and large-scale production; however, they tend to show deficiency for active tumor targeting and poor transfection efficiency, especially in the presence of serum *in vivo*. Cationic polymers and lipids are by far the most widely used vectors in non-viral gene and oligonucleotide delivery. Cationic liposomes transfect cells based on the phagocytosis, not the endocytosis, of the complex [2,3]. Some liposome formulations are not satisfactory due to their low transfection efficiency and cytotoxicity [4,5]. Polycationic polymers like polylysine (PLL), polyethylenimine (PEI) or polyamidoamine (PAMAM) dendrimers [6–9] are able to compact DNA, which is an advantage in gene transfer [10,11]. However, following intravenous injection of cationic lipid/DNA complexes (lipoplexes) or polycationic polymers/DNA complexes (polyplexes), there is rapid sequestration of the complex by lung, with fewer uptakes by liver or tumors [12]. If gene therapy is to be used for liver cancer, then alternative forms of gene targeting technology need to be developed. Gene targeting technology should enable the delivery to liver cancer of a non-viral formulation of the therapeutic gene via a simple intravenous injection. Such a gene targeting technology should have the following characteristics: (i) stability in the blood with a prolonged residence time; (ii) transport to liver cancer cells *in vivo*; (iii) endocytosis across the tumor cell membrane; and (iv) gene expression in the tumor cells with the desired biologic end-point [13].

The present studies were designed to develop a novel non-viral gene delivery system, called pegylated immuno-lipopolyplexes (PILP), which is a ternary complex formed with anionic liposomes, cationic polymer and DNA. This is the second generation of non-viral gene delivery vectors that can improve gene transfer compared to the first generation of non-viral gene delivery vectors represented by lipoplexes and polyplexes. The plasmid DNA is compacted by PEI. PEI has the ability to enter the nucleus [14] and to accelerate gene entry into the nucleus from the cytosol [15]. The surface of the lipopolyplexes is decorated with strands of polyethylene glycol (PEG) to promote stabilization in the bloodstream and the tips of the PEG strands are conjugated with a targeting monoclonal antibody (MAb). This peptidomimetic MAb triggers receptor-mediated endocytosis

* Corresponding author. Tel.: +86 371 6778 1910; fax: +86 371 6778 1907.
E-mail address: zhang_yun@ymail.com (Y. Zhang).

0168-3659/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2010.02.005

into tumor cells. The pattern of gene expression *in vivo* is determined by the receptor specificity of the targeting MAb [16].

This study has explored the strategies for development of the pegylated immuno-lipopolyplexes as well as evaluated their *in vitro* and *in vivo* gene transfection efficiency, stability and cytotoxicity.

2. Materials and methods

2.1. Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), Distearoylphosphatidylethanolamine (DSPE)-PEG2000 and DSPE-PEG2000-biotin were obtained from Avanti-Polar Lipids, Inc. (Alabaster, AL, USA). Protein G-Sepharose and Sephacryl S-300HR were purchased from GE Healthcare (Piscataway, NJ, USA). Bicinchoninic acid (BCA) protein assay reagents were obtained from BioTeke Corporation (Beijing, China). Pancreatic DNase I with a specific activity of 2000 Kunitz units/mg, polyethylenimine (MW 25 kDa, branched), streptavidin and other molecular biology grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The transferrin receptor (TfR) monoclonal antibody (MAb) used in this study is the rat 8D3 MAb to the mouse TfR. The anti-insulin receptor MAb used for gene targeting to human hepatoblastoma cells is the murine 83–14 MAb to the human insulin receptor (HIR). The TfRMAb and HIRMAb were individually purified with protein G affinity chromatography from hydridoma generated ascites. The luciferase plasmid DNA is derived from the pGL2 luciferase expression plasmid (Promega). EGFP plasmid encoding the enhanced green fluorescent protein gene, the SMMC-7721 human liver cancer cells and the mouse hepatocellular carcinoma cell line (H22) were obtained from Henan Academy of Medical and Pharmaceutical Sciences.

2.2. Preparation of pegylated lipopolyplexes

Plasmids were transformed and amplified in *E. coli* DH5α competent cells (TIANGEN, Beijing, China) and purified by EndoFree Plasmid Maxi Kit (Qiagen). In order to prepare the lipopolyplexes, polyplexes and liposomes need to be prepared first and separately. Polyplexes were prepared with plasmid DNA and branched polyethylenimine (B-PEI, 25 kDa) at an N/P ratio of 10 based on the method of Kim WJ [17]. The N/P ratio of the nitrogen atoms of PEI to DNA phosphates describes the amount of polymer used for polyplex formation. The anionic liposomes were formulated as follows: a mix of three lipids of POPC, DSPE-PEG2000 and DSPE-PEG2000-biotin in chloroform at a molar ratio of 50:30:20 was dried under N_2 gas stream while vortexing to produce thin layer lipid film in a glass tube. The lipid film was then hydrated with 0.05 M HEPES buffer (pH 7.0) and vortexed for 1 min, followed by 2 min of bath sonication. After the PEI/DNA polyplexes are ready and sit at room temperature for 15 min, different amounts of preformed anionic liposomes were added to the freshly prepared polyplexes at different lipid/DNA molar ratios (50/1, 90/1, 130/1, 170/1 and 210/1). Consequently lipopolyplexes formed because of the electrostatic interactions between the positively charged polyplexes and the negatively charged liposomes decorated with PEG. The lipopolyplexes were then extruded through polycarbonate membranes with 100 nm pore diameter with a Liposofast device (Avestin, Toronto, ON, Canada) to obtain a uniform size distribution. These lipopolyplexes are called pegylated lipopolyplexes (PLP).

2.3. Conjugation of monoclonal antibody to the pegylated lipopolyplexes

The conjugate of either 8D3 MAb/streptavidin (8D3/SA) or 8314 MAb/stretavidin (8314/SA) was synthesized as described previously [18]. Once the pegylated lipopolyplexes are ready, 8D3/SA or 8314/SA was added at the molar ratio 1.5:1 of SA to biotin group in the PLP. The 8D3 or 8314 MAb immediately conjugates to the PLP because of the high affinity between SA and biotin, which are called pegylated immuno-lipopolyplexes (PILP) (Fig. 1). For tissue culture experiments, the PILP solution was sterilized with a 0.22 mm filter (Millipore Co., Bedford, MA).

2.4. Particle size and charge measurement

The mean particle size and charge of the PILP were measured by dynamic light scattering (DLS) and Laser Doppler Velociometry (LDV) on a Malvern Nano ZS90 instrument running DTS software (Malvern Instruments, UK). To characterize and evaluate the influence of long-term storage at 4 °C on the PILP, the pegylated immuno-lipopolyplexes (PILP) prepared at a lipid/DNA molar ratio of 170/1 were stored at 4 °C. Particle size and zeta potential were measured on 0, 5, 10, 20 days, respectively. Size and zeta potential were reported as mean ± SE ($n = 3$). Each mean is the average of 15 measurements and n represents the number of separate batches prepared for the measurements.

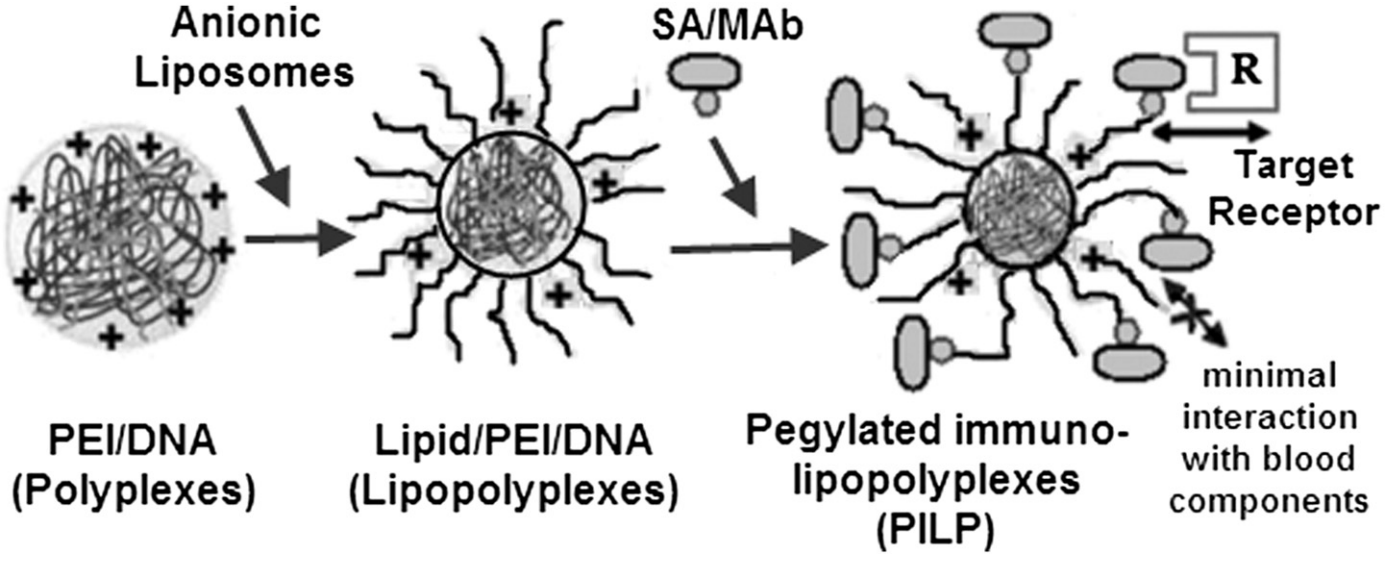

Fig. 1. Schematic of the designed pegylated immuno-lipopolyplexes (PILP) composed of PEI/DNA complex, streptavidin-monoclonal antibody (SA/MAb), anionic liposomes preformed by POPC, DSPE-PEG2000 and DSPE-PEG2000-biotin.