Biomaterials 32 (2011) 652–663

Contents lists available at ScienceDirect

Biomaterials

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

Polyplex micelles prepared from ω-cholesteryl PEG-polycation block copolymers for systemic gene delivery

Makoto Oba [a], Kanjiro Miyata [b], Kensuke Osada [c], R. James Christie [c], Mai Sanjoh [d], Weidong Li [c], Shigeto Fukushima [c], Takehiko Ishii [d], Mitsunobu R. Kano [b,e], Nobuhiro Nishiyama [b], Hiroyuki Koyama [a], Kazunori Kataoka [b,c,d,*]

[a] Department of Clinical Vascular Regeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[d] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[e] Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hong, Bunkyo-ku, Tokyo 113-8655, Japan

ARTICLE INFO

Article history:
Received 11 August 2010
Accepted 24 August 2010
Available online 6 October 2010

Keywords:
Non-viral gene vector
Polyplex micelle
Cholesterol
Pancreatic tumor
Anti-angiogenic therapy

ABSTRACT

Polyplex micelles formed with plasmid DNA (pDNA) and poly(ethylene glycol) (PEG)-*block*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] exhibit effective endosomal escaping properties based on di-protonation of diamine side chains with decreasing pH, which improves their transfection efficiency and thus are promising candidates for local *in vivo* gene transfer. Here, PEG-PAsp(DET) polyplex micelles were further improved as *in vivo* systemic vectors by introduction of cholesterol (Chole) into the ω-terminus of PEG-PAsp(DET) to obtain PEG-PAsp(DET)-Chole. Introduction of the cholesterol resulted in enhanced association of block copolymers with pDNA, which led to increased stability in proteinous medium and also in the blood stream after systemic injection compared to PEG-PAsp(DET) micelles. The synergistic effect between enhanced polymer association with pDNA and increased micelle stability of PEG-PAsp(DET)-Chole polyplex micelles led to high *in vitro* gene transfer even at relatively low concentrations, due to efficient cellular uptake and effective endosomal escape of block copolymers and pDNA. Finally, PEG-PAsp(DET)-Chole micelles achieved significant suppression of tumor growth following intravenous injection into mice bearing a subcutaneous pancreatic tumor using therapeutic pDNA encoding an anti-angiogenic protein. These results suggest that PEG-PAsp(DET)-Chole micelles can be effective systemic gene vectors for treatment of solid tumors.

1. Introduction

As expectations for gene therapy increase, so have efforts to develop non-viral vectors with high transfection ability and low toxicity [1,2]. Polyplexes, which are composed of polycations and plasmid DNA (pDNA), are expected as alternatives to viral vectors due to the fine-tuned properties for specific applications by altering the structure of the polycation used for polyplex formation [3–5]. Polyplex micelles formed with poly(ethylene glycol) (PEG)-*block*-polycation block copolymers and pDNA are particularly promising candidates [6–8], due to their excellent characteristics as *in vivo* gene vectors [9,10]. The biocompatible PEG shell layer surrounding the polyplex core contributes to high colloidal stability, allows micelles to maintain their initial size of approximately 100 nm, and reduces non-specific interactions with blood components, which are all desirable properties for systemic administration.

Recently, we reported that polyplex micelles prepared with pDNA and PEG-*block*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide} [PEG-PAsp(DET)] [11] achieved successful *in vitro* transfection of primary cells due to effective endosomal escape of pDNA contained in the micelle core. The PAsp(DET), polycationic segment of the block copolymer is characterized by a distinctive two-step protonation behavior in response to pH and possessed endosomal membrane-selective destabilizing capacity upon acidification [12]. Furthermore, PEG-PAsp(DET) polyplex micelles have shown successful *in vivo* gene transfer by local administration in

* Corresponding author. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Tel.: +81 3 5841 7138; fax: +81 3 5841 7139.
E-mail address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.09.022

several animal models including: a clamped rabbit carotid artery with neointima without vessel occlusion by thrombus [13], a mouse skull by regulated release from a calcium phosphate cement scaffold to induce bone regeneration through the osteogenic factors [14], and a rat lung pulmonary arterial hypertension model via intratracheal administration [15]. In these cases, however, excess block copolymers relative to pDNA (high N/P ratio) were required to achieve high transfection efficiency, suggesting the existence of free polymer. If free polymer plays a significant role for gene transfer with polyplex micelles prepared from PEG-PAsp (DET) and pDNA, the transfection efficiency under highly diluted conditions, such as systemic application, could be drastically decreased.

The aim of this study was to further develop PEG-PAsp(DET) polyplex micelles towards *in vivo* systemic pDNA delivery vectors. In order to enhance the association of PEG-PAsp(DET) polymers with pDNA and thus increase the efficiency of cellular internalization of polymer necessary for improved endosome escaping, we utilized both electrostatic interaction between polycations and pDNA and hydrophobic interaction by cholesterol to form micelles with improved stability. Specifically, cholesterol was introduced onto the ω-terminus of the PAsp(DET) segment in PEG-PAsp(DET) block copolymer. Cholesterol introduction significantly increased the number of block copolymers associating with a pDNA. *In vitro* experiments were done to demonstrate improved transfection efficiency of PEG-PAsp(DET)-Chole polyplex micelles at low N/P ratios and under the diluted conditions compared to the control micelles formed without cholesterol modified block copolymer. Then, the enhanced stability by the cholesterol introduction in blood was shown, thus allowing successful treatment of a subcutaneous tumor by systemic administration of micelles prepared with PEG-PAsp(DET)-Chole and therapeutic pDNA encoding for an anti-angiogenic protein.

2. Materials and methods

2.1. Materials

Dichloromethane (CH_2Cl_2), *N,N*-dimethylformamide (DMF), triethylamine (TEA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Wako Pure Chem. Co. Ltd. (Osaka, Japan). Cholesterol chloroformate was purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). Diethylenetriamine (DET) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled over CaH_2 under reduced pressure. DMF was dehydrated using activated molecular sieves (4A) and distilled under reduced pressure. PEG-PAsp(DET) block copolymer (PEG: 12,000 g/mol, polymerization degree of PAsp(DET) segment: 68) was synthesized as previously reported [11]. Alexa Fluor 680 (Alexa680) succinimidyl ester was a product of Invitrogen (Carlsbad, CA). A Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). The Luciferase assay kit was a product of Promega (Madison, WI). Plasmid pCAcc+Luc coding for firefly luciferase under the control of the CAG promoter was provided by RIKEN Gene Bank (Tsukuba, Japan), amplified in competent DH5a *Escherichia coli*, and then purified using a HiSpeed Plasmid MaxiKit purchased from Qiagen Sciences (Germantown, MD). pDNA encoding for a soluble form of VEGF receptor-1 (sFlt-1) was prepared as previously reported [16].

2.2. Animals

Balb/c mice (female, 8 weeks old) and balb/c nude mice (female, 5 weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animals were treated in accordance with the guideline of the Animal Ethics Committee of The University of Tokyo.

2.3. Synthesis of α-methoxy-ω-cholesteryl carbamate poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PEG-PAsp(DET)]

PEG-*b*-poly(β-benzyl L-aspartate) (PEG-PBLA) (PEG: 12,000 g/mol, polymerization degree of PBLA segment: 68) was prepared as previously reported [11]. PEG-PBLA (210 mg) was dissolved in CH_2Cl_2 (4 mL), followed by the addition of 11 v/v% TEA/CH_2Cl_2 (200 μL) and cholesterol chloroformate (344 mg) in CH_2Cl_2 (1 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The reactant polymer was isolated by precipitation into diethylether and lyophilized from benzene, to obtain PEG-PBLA-Chole (197 mg). PEG-PBLA-Chole (100 mg) was dissolved in DMF (4 mL), followed by reaction with DET (50 equiv. to benzyl group of PBLA segment, 1.43 g) at 40 °C. After 1 h, the reactant mixture was slowly added to a 20% acetic acid (13.8 mL) solution, and subsequently dialyzed against 0.01 N HCl and finally distilled water. The final solution was lyophilized to obtain PEG-PAsp (DET)-Chole (98 mg).

The ^{1}H NMR spectrum of each polymer was obtained with an EX300 spectrometer (JEOL, Tokyo, Japan). Chemical sifts were reported in ppm relative to the residual protonated solvent peak.

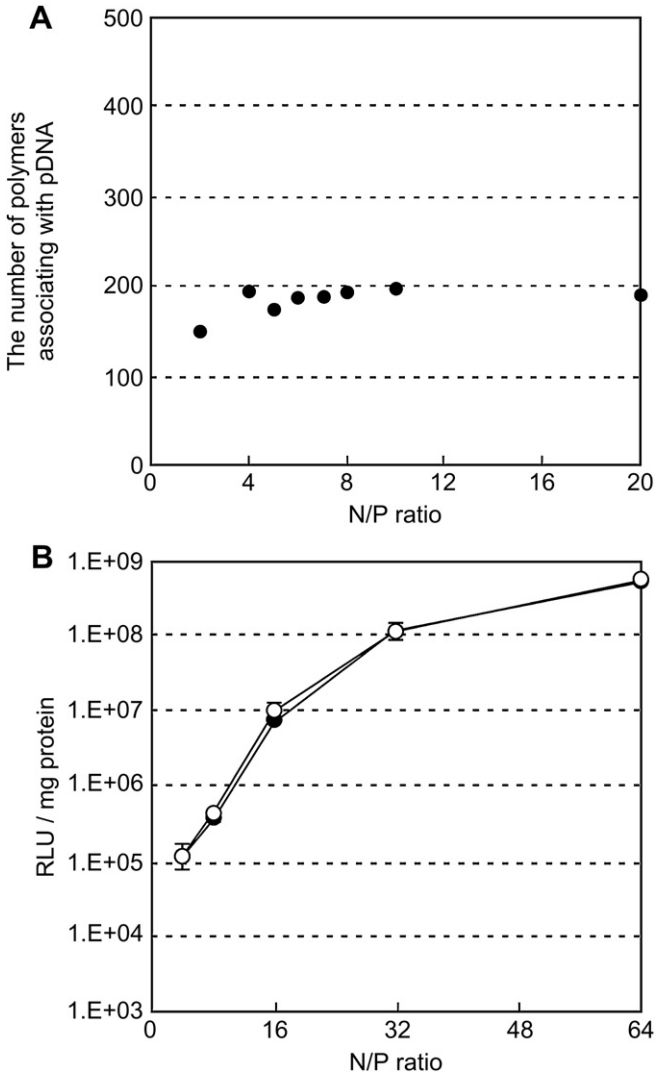

Fig. 1. (A) The number of PEG-PAsp(DET-Alexa680) block copolymers associating with a pDNA. (B) Transfection efficiency of PEG-PAsp(DET) polyplex micelles. Closed circles: addition of polyplex micelle solutions prepared at various N/P ratios into the cell culture medium. Open circles: addition of polyplex micelle solutions prepared at a constant N/P value of 4, with separate addition of PEG-PAsp(DET) free polymer solution into the cell culture medium to obtain the same N/P values shown in the experiment with open circles.

2.4. Introduction of Alexa680 into block copolymers

Alexa680 was introduced into the side chains of both PEG-PAsp(DET) and PEG-PAsp(DET)-Chole polymers. The typical synthetic procedure of PEG-PAsp(DET-Alexa680) is described as follows: Alexa680 succinimidyl ester (1 mg) in 100 μL of DMF was added to PEG-PAsp(DET) (30 mg) in 1.5 mL of 0.1 N $NaHCO_3$ (pH 9.3) and stirred at 4 °C for 1 h. The reacted polymer was purified by dialysis against distilled water and lyophilized to obtain PEG-PAsp(DET-Alexa680) (22 mg). Introduction of Alexa680 into PEG-PAsp(DET)-Chole was completed similarly using Alexa680 succinimidyl ester (1 mg) and PEG-PAsp(DET)-Chole (30 mg) to obtain PEG-PAsp(DET-Alexa680)-Chole (23 mg). The number of Alexa680 introduced into the strand of