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Effect of shear stress on structure and function of polyplex micelles from poly(ethylene glycol)-poly(L-lysine) block copolymers as systemic gene delivery carrier

Kaori M. Takeda [a], Yuichi Yamasaki [a, e, **], Anjaneyulu Dirisala [a, b, e], Sorato Ikeda [a], Theofilus A. Tockary [a, e], Kazuko Toh [e], Kensuke Osada [b, d, e, ***], Kazunori Kataoka [a, b, c, e, *]

[a] Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan
[b] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan
[c] Division of Clinical Biotechnology, 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
[d] Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan
[e] Innovation Center of NanoMedicine (iCONM), Kawasaki Institute of Industrial Promotion, 3-25-14 Tonomachi, Kawasaki-ku, Kawasaki, 210-0821, Japan

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ABSTRACT

Structural stability of polyplex micelles (PMs), prepared from plasmid DNA (pDNA) and poly(ethylene glycol)-*b*-poly(L-lysine) block catiomer (PEG–PLys), was evaluated in terms of their resistance against shear stress. When exposed to shear stress at magnitudes typically present in the blood stream, structural deterioration was observed in PMs owing to the partial removal of PEG–PLys strands. Eventually, impaired PEG coverage of the polyplex core led to accelerated degradation by nucleases, implying that structural deterioration by shear stress in blood stream may be a major cause of rapid clearance of PMs from blood circulation. To address this issue, introduction of disulfide crosslinking into the PM core was shown to be an efficient strategy, which successfully mitigated unfavorable effects of shear stress. Furthermore, improved *in vivo* blood retention profile and subsequently enhanced antitumor efficacy in systemic treatment of pancreatic adenocarcinoma were confirmed for the crosslinked PMs loaded with pDNA encoding an anti-angiogenic protein, suggesting that high stability under the shear stress during blood circulation may be a critical factor in systemically applicable gene delivery systems.

1. Introduction

Polymer-based gene carriers applicable via systemic route have been extensively studied as alternatives to viral vectors used for gene therapy [1–3]. The most prospective system among them is a polyplex, which is a polyion complex formed by electrostatic interaction between negatively charged plasmid DNA (pDNA) and positively charged polyelectrolyte (polycation) [4–6]. Nevertheless, longevity in blood circulation, which is required for systemic gene delivery, remains a challenge in polyplex research [7,8], despite many ideas proposed for improving the stability of polyplexes in biological milieu [9–11]. One attractive approach is the use of poly(ethylene glycol) (PEG)-polycation block copolymers to construct polyplex micelles (PMs) with a core-shell architecture, wherein a single pDNA molecule is packaged as a core surrounded by a PEG shell [12–15]. A typical example of PMs studied to date is the one formed from pDNA and block catiomer of PEG and poly(L-lysine) (PEG–PLys) [6,12,16]. The steric repulsive effect of the PEG shell provides a high shielding effect against nucleases [17]. Furthermore, an increased PEG density by modulation of the number of the associated polymer chains in PMs significantly increased their retention time in blood circulation [18].

* Corresponding author. Innovation Center of NanoMedicine (iCONM), Kawasaki Institute of Industrial Promotion, 3-25-14 Tonomachi, Kawasaki-ku, Kawasaki, 210-0821, Japan.
** Corresponding author. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan.
*** Corresponding author. Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan.
E-mail addresses: yamasaki@bmw.t.u-tokyo.ac.jp (Y. Yamasaki), osada@bmw.t.u-tokyo.ac.jp (K. Osada), kataoka@bmw.t.u-tokyo.ac.jp, kataoka@pari.u-tokyo.ac.jp (K. Kataoka).

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Nonetheless, even for PEG–PLys PMs with fully packed PEG chains, longevity in blood circulation was limited to 60 min [18]. Thus, for proper PM design available for systemic application, further detailed research is required to gain insight into the factors crucial to the stability of PMs in blood circulation.

Here, we newly focused on shear stress as a crucial factor affecting the integrity of polyplexes in the blood stream. Shear stress in the blood stream varies primarily according to the diameter of the blood vessels [19] and thus depends on the species of animal and circulation site [20]. For example, in humans, shear stress values are reported to be approximately 1 dyne/cm^2 in the vena cava, approximately 10 dyne/cm^2 in the aorta, 20–40 dyne/cm^2 in venules, and 60–80 dyne/cm^2 in arterioles [19], while in mice, shear stress is more than 100 dyne/cm^2, even in relatively large vessels [20]. It is plausible that structural deterioration of PMs may be induced in such high-shear stress conditions in the blood stream. Actually, structural change of liposomes with size similar to PMs (approximately 100 nm) induced by shear stress at a magnitude comparable to that of the blood stream has been reported [21], however to our knowledge there has been no report of the effects of shear stress on the structure of polyplexes. In the present study, we confirmed that introduction of disulfide crosslinking into the PM core improves stability of PMs against shear stress, achieving their longevity in blood circulation. This was sufficient to exert significant suppression of intractable pancreatic tumor growth by systemically delivering a gene encoding soluble fms-like tyrosine kinase 1 (sFlt-1), a splice variant of vascular endothelial growth factor (VEGF) receptor 1.

2. Materials and methods

2.1. Materials

2-Iminothiolane (Traut's reagent) and Dithiothreitol (DTT) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ethylenediaminetetraacetic acid (EDTA) and sodium dextran sulfate (M_w = 5000) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dextran (M_w = 70000) and fluorescamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DNaseI, *Hin*dIII, and plasmid DNA pBR322 were purchased from Takara Bio Inc. (Kusatsu, Japan). Other pDNAs (pCAG-Luc1, pCAG-Luc2, and pCAG-sFlt-1) were obtained by amplification with *Escherichia coli*, followed by extraction and purification with NucleoBond Xtra Maxi Plus Endotoxin Free kit (Qiagen Science Co., Inc., Hilden, Germany). pBR322 was used for transmission electron microscopy (TEM) analysis. pCAG-Luc1 was used to quantify the associated polymer number in PMs. pCAG-Luc2 was used for evaluation of resistance against DNaseI, *in vitro* transfection efficacy, and *in vivo* blood circulation of PMs. For blood circulation analysis, pCAG-Luc2 was labeled with Cy5 using Label IT® Tracker Nucleic Acid Localization Kits (Mirus Bio Co., Madison, WI). pCAG-sFlt-1, a plasmid encoding sFlt-1, was used for the *in vivo* tumor growth suppression study.

2.2. Synthesis of PEG–PLys block cationer

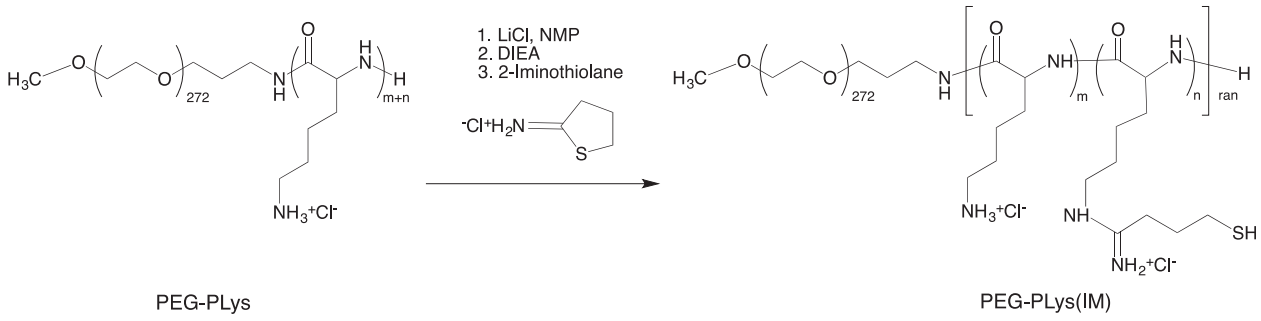
PEG–PLys block cationer with PEG molecular weight (M_w) 12 k and PLys degree of polymerization (DP) 37 was synthesized as previously reported [22]. In brief, *N*-ε-trifluoroacetyl-L-lysine-*N*-carboxy anhydride (Lys(TFA)-NCA) was conjugated onto the ω-NH_2 terminal group of MeO-PEG-NH_2 via ring-opening polymerization in *N*,*N*-dimethylformamide containing 1 M thiourea. The molecular weight distribution (M_w/M_n) of the polymer was determined to be 1.06 by using gel permeation chromatography (GPC) (TOSOH HLC-8220, Tokyo, Japan). Next, MeO-PEG–PLys(TFA) was dissolved in methanol containing 1 N NaOH and stirred at 35 °C for 12 h to remove protective TFA groups. PLys DP was determined to be 37 by comparing ^{1}H NMR integration ratios between methylene protons of the PEG chain (CH_2CH_2O) and those of the lysine unit ($(CH_2)_3CH_2NH_3$) measured by a JEOL AL 300 spectrometer (JEOL Ltd., Tokyo, Japan).

2.3. Synthesis of thiolated PEG–PLys with 2-iminothiolane (PEG–PLys(IM))

PEG–PLys(IM) was prepared by conjugating cyclic thioimidate, 2-iminothiolane, to the side chain of the PLys segment of the PEG–PLys (Scheme 1), according to a previously reported procedure [23]. In brief, PEG–PLys (112 mg, 6.19 μmol) was dissolved in *N*-methylpyrrolidone (NMP, 7.1 mL) containing 5 wt% LiCl. After addition of 217 μL of *N*,*N*-diisopropylethylamine (DIEA), 29.2 mg of Traut's reagent in NMP (2.9 mL) was added, and stirred at room temperature for 24 h. Next, the reaction was terminated by precipitation into an excess volume of diethyl ether. The precipitate was washed twice with diethyl ether, and dissolved in 0.01 N HCl for dialysis against distilled water. Introduction rate of 1-imino-4-mercaptobutyl (IM) was determined to be 49% of Lys residues from ^{1}H NMR spectra by comparing the peak intensity ratio of the methylene protons of the lysine unit and those of IM unit.

2.4. Preparation of polyplex micelles

Non-crosslinked polyplex micelles (PMs) were prepared by mixing pDNA with PEG–PLys in 10 mM HEPES at a fixed residual molar ratio of amino groups in PEG–PLys (N) to phosphate groups in pDNA (P) of 2.0 (N/P_{feed} = 2.0). Crosslinked polyplex micelles (CPMs) were prepared with PEG–PLys(IM) following a previously reported procedure [23]. In brief, PEG–PLys(IM) was dissolved in 10 mM HEPES containing 100 mM DTT and left for 90 min at room



Scheme 1. Thiolation scheme of PEG–PLys with 2-iminothiolane.