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Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity

Frederico Pittella [a], Mingzhen Zhang [b,d], Yan Lee [b,e], Hyun J. Kim [b], Theofilus Tockary [b], Kensuke Osada [b], Takehiko Ishii [a], Kanjiro Miyata [c], Nobuhiro Nishiyama [c], Kazunori Kataoka [a,b,c,*]

[a] Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[b] Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
[c] 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] School of Ophthalmology and Optometry, Wenzhou Medical College, Wenzhou, Zhejiang 325027, PR China
[e] Department of Chemistry, College of Natural Science, Seoul National University, Seoul 151-747, Republic of Korea

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ABSTRACT

Development of safe and efficient short interfering RNA (siRNA) delivery system for RNA interference (RNAi)-based therapeutics is a current critical challenge in drug delivery field. The major barriers in siRNA delivery into the target cytoplasm are the fragility of siRNA in the body, the inefficient cellular uptake, and the acidic endosomal entrapment. To overcome these barriers, this study is presenting a hybrid nanocarrier system composed of calcium phosphate comprising the block copolymer of poly(ethylene glycol) (PEG) and charge-conversional polymer (CCP) as a siRNA vehicle. In these nanoparticles, the calcium phosphate forms a stable core to incorporate polyanions, siRNA and PEG–CCP. The synthesized PEG–CCP is a non-toxic endosomal escaping unit, which induces endosomal membrane destabilization by the produced polycation through degradation of the flanking *cis*-aconitylamide of CCP in acidic endosomes. The nanoparticles prepared by mixing of each component was confirmed to possess excellent siRNA-loading efficiency (~80% of dose), and to present relatively homogenous spherical shape with small size. With negligible cytotoxicity, the nanoparticles efficiently induced vascular endothelial growth factor (VEGF) mRNA knockdown (~80%) in pancreatic cancer cells (PanC-1). Confocal laser scanning microscopic observation revealed rapid endosomal escape of siRNA with the nanoparticles for the excellent mRNA knockdown. The results obtained demonstrate our hybrid nanoparticle as a promising candidate to develop siRNA therapy.

1. Introduction

Since the finding of RNA interference (RNAi) in 1998 [1], the scientific community has experienced the excitement to develop a new research field. Short interfering RNA (siRNA), which allows the cleavage of the complementary mRNA for the reduced protein production in mammalian cells, provided new perspectives for potential treatment of intractable and genetic related diseases [2]. With the decoding of the human genome [3–5], it has become possible to aim a great variety of genes involved in key pathways of physiopathologies. However, a safe and efficient delivery of siRNA into the target cytoplasm has still been a major challenge. Naked siRNAs are susceptible to enzymatic degradation in the body and also possess large size (~13 kDa) and anionic charges suppressing the penetration into cellular membrane [6], thus requiring carrier systems to overcome these barriers.

Calcium phosphate (CaP) precipitates were used as transfection reagents of viral DNA for the first time in early 1970s [7], as they are believed to be non-toxic based on homology to natural inorganic materials such as teeth and bones. Notably, CaP precipitates can bind and encapsulate polyanions/nucleic acids by an easy and inexpensive method to protect the nucleic acids from enzymatic degradation and to deliver into cells. However, one of their major limitations is the uncontrollable rapid growth of calcium phosphate crystal after preparation, resulting in the formation of large agglomerates (>μm) to appreciably reduce the transfection efficiency [8–10]. In this regard, our previous studies have addressed poly(ethylene glycol) (PEG)-coating of CaP precipitates utilizing PEG-polyanion block copolymers [9,11–14]. Hydrophilic and neutral PEG is widely known

* 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 address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

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to provide a nanoparticle with excellent colloidal stability as well as reduced protein adsorption and immunogenicity [15–17]. Indeed, the integration of PEG-block polyanions, such as poly(aspartic acid) (PAsp) [9,12], poly(methacryl acid) [13], and siRNA [14], into CaP precipitates led to the formation of size-controllable hybrid nanoparticles with PEG palisade, which appreciably facilitated the internalization of nucleic acids by cells.

Herein, we considered the next challenge in the CaP carriers as the endosomal escape, since they are usually internalized by cells though endocytosis pathway to be delivered into acidic endosome or lysosome, resulting in enzymatic degradation of the payload nucleic acids [18]. Toward the endosomal escape with polymeric materials, our previous studies have reported a cationic polyaspartamide with a 1,2-diaminoethane side chain (poly{*N*-[*N*′-(2-aminoethyl)-2-aminoethyl] aspartamide}, PAsp(DET)) to exert strong membrane destabilization selectively in acidic endosomal compartments for efficient endosomal escape with low cytotoxicity [19–22]. Note that PAsp(DET) possesses two unique advantages for its excellent transfection: 1) the pH-selective membrane destabilization based on the distinctive two step protonation behavior in the side chain, i.e., mono-protonated form with minimal membrane damages at neutral pH and di-protonated form exerting strong membrane disruption at acidic pH [20]; 2) the spontaneous biodegradability based on the selective backbone cleavage even under physiological conditions [21].

In this work, in order to improve the endosomal escape as well as the colloidal stability of CaP precipitates, a block copolymer of PEG and an endosomal escaping polymer was synthesized and integrated into the CaP nanoparticles incorporating siRNA. Indeed, we modified the flanking primary amines of PEG-PAsp(DET) with *cis*-aconitic anhydride [23,24] to convert the cationic charges to net negative ones with two carboxylates of the *cis*-aconityl moiety (PEG-poly{*N*-[*N*′-(*N*″-*cis*-aconityl-2-aminoethyl)-2-aminoethyl]aspartamide}, PEG-PAsp(DET-Aco)) for effective binding to CaP nanoparticles. Noteworthy, the prepared *cis*-aconitylamide shows high stability at neutral and basic pHs but it becomes cleavable at acidic pH to reproduce cationic PAsp(DET) from anionic PAsp(DET-Aco) in endosome/lysosome, which is termed the charge-conversional polymer [23–25]. The hybrid nanoparticle prepared from PEG-PAsp (DET-Aco), siRNA, and CaP does not contain inherent toxic materials, such as polycations, thereby leading to potentially lower toxicity compared to conventional polyplex carriers from polycations and siRNA. Thus, the nanoparticles prepared by simple mixing of each component were physicochemically and biologically characterized by the comparison with non-charge-conversional control polyanions to demonstrate the utility of our hybrid system from the PEG–charge-conversional polymer for siRNA delivery.

2. Material and methods

2.1. Materials

cis-Aconitic anhydride, tricarballylic acid, and Dulbecco's modified eagle's medium (DMEM) were purchased from Sigma–Aldrich (St. Louis, MO). α-Methoxy-ω-amino-poly(ethylene glycol) (MeO–PEG–NH_2) (M_w: 12,000) and β-benzyl-L-aspartate *N*-carboxyanhydride (BLA–NCA) were obtained from NOF Co, Inc. (Tokyo, Japan) and Chuo Kaseihin Co., Inc. (Tokyo, Japan), respectively. *N*-Methyl-2-pyrrolidone (NMP), diethylenetriamine (DET), dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), and acetic anhydride were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) or Nacalai Tesque (Tokyo, Japan), and used after a conventional distillation. Acetic acid, acetonitrile, acetone, diethyl ether, and hydrochloric acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The primers for human actin and human VEGF were synthesized by Hokkaido System Science (Hokkaido, Japan) and the sequences are: CCAACCGCGAGAAGATGA (actin forward); CCAGAGGCGTACAGGGATAG (actin reverse); AGTGGTCCCAGGCTGCAC (VEGF forward); TCCATGAACTTCACCACTTCGT (VEGF reverse). All the siRNAs were synthesized by Hokkaido System Science (Hokkaido, Japan) and the sequences of VEGF siRNA (siVEGF) are: 5′-GGAGUACCCUGAUGAGAUCdTdT-3′ (sense); 5′-GAUCUCAUCAGGGUACUCCdTdT-3′ (antisense), and GL3 luciferase siRNA (siGL3) are: 5′-CUU ACG CUG AGU ACU UCG AdTdT-3′ (sense); 5′-UCG AAG UAC UCA GCG UAA GdTdT-3′ (antisense).

2.2. Synthesis of block copolymer with poly(ethylene glycol) and charge-conversional polymer (PEG–CCP) segments

2.2.1. Synthesis of poly(ethylene glycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide} (PEG-PAsp(DET))

PEG-PAsp(DET) was prepared as previously reported with slight modification [21]. Briefly, BLA–NCA (780 mg; 3.13 mmol) was dissolved in 0.7 mL of DMF, and then in 7.3 mL of DCM. The polymerization was initiated from the primary amino group of MeO–PEG–NH_2 (M_w = 12,000, 500 mg; 0.0417 mmol) to obtain PEG–PBLA (1100 mg) as a precursor. Size exclusion chromatography (SEC) was performed to determine the molecular weight distribution (M_w/M_n) of the obtained PEG–PBLA using a TOSOH HLC-8220 equipped with TSK gel columns (SuperAW4000 and SuperAW3000 × 2; eluent: NMP with 50 mM LiBr; flow rate: 0.3 mL min^{-1}; temperature: 40 °C) and an internal refractive index (RI) detector. The M_w/M_n was confirmed to be 1.07 from the SEC chart using PEG standards for the M_w calibration (data not shown). The degree of polymerization of PBLA in PEG–PBLA was determined to be 96 from the peak intensity ratio of the methylene protons of PEG (−OCH_2CH_2−, δ = 3.5 ppm) to the benzyl protons of PBLA ($C_6H_5CH_2$−, δ = 5.1 and 7.3 ppm) in the ^{1}H NMR measurement (data not shown). All of the NMR assays were performed using (3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid sodium salt d_4-TSPA) as an internal standard. Then, PEG–PBLA (100 mg) was dissolved in NMP (2 mL) and cooled at 5 °C. Diethylenetriamine (DET) (3 mL; 100 equiv to benzyl groups of PBLA segment) was diluted with the same volume of NMP, and then the first solution was added and stirred for 4 h at 0 °C (ice bath). The reaction was stopped adding the polymer solution to cold 20% acetic acid (30 mL) drop-by-drop. The neutralized solution was dialyzed against 0.01 M hydrochloric acid solution and then in de-ionized water at 4 °C. As a hydrochloride salt form, a white powder was obtained after lyophilization of the dialyzed solution (91.2 mg, 69.6% yield). The quantitative conversion of the BLA to Asp(DET) was confirmed from the peak intensity ratio of the methylene protons in PEG (−OCH_2CH_2−, δ = 3.7 ppm) to the ethylene protons in the 1,2-diaminoethane moiety ($H_2N(CH_2)_2NH(CH_2)_2NH$−, δ = 2.8–3.4 ppm) in the ^{1}H NMR spectrum in D_2O at 50 °C (Supporting Information).

2.2.2. Synthesis of poly(ethylene glycol)-b-poly{N-[N′-(N″-cis-aconityl-2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-PAsp(DET-Aco))

PEG-PAsp(DET) (17.5 mg, 0.0538 mmol of primary amine) was dissolved in 0.5 M $NaHCO_3$ at pH 9.1 (50 mL). *cis*-Aconitic anhydride powder (420 mg, 2.69 mmol) was added to the solution slowly and stirred at 0 °C for 2 h. The reaction mixture was purified by centrifugal ultrafiltration with Amicon Ultra (MWCO = 10,000; Millipore (Billerica, MA)) three times with de-ionized water at 4 °C. The final product was obtained as a white powder after lyophilization (14.9 mg, 64.7% yield). The quantitative conversion of primary amines in Asp(DET) side chain to *cis*-aconitylamide was confirmed from the peak intensity ratio of the methine protons in the main chain (−$COCH_2CH$(CO−)NH−, −$COCH(CH_2$−)NH−, δ = 4.8 ppm) to methine protons of the *cis*-aconityl moiety (−COC*H*:C(COONa)CH_2COONa, δ = 6.0 ppm) in ^{1}H NMR spectrum in D_2O at 50 °C (Fig. 1).

2.3. Synthesis of block copolymer with poly(ethylene glycol) and non-charge-conversional polymers (PEG–nCCP) segments

2.3.1. Synthesis of carballylic anhydride

Carballylic anhydride was prepared as previously reported [26] with slight modification. Briefly, tricarballylic acid (4.4 g, 0.025 mol) was reacted with acetic anhydride (4.73 mL, 0.05 mol) at 45 °C for 1 h. The excess of acetic anhydride was evaporated under reduced pressure. Further, the product was dissolved in the minimum amount of ethyl acetate at 80 °C and filtered. The solution was allowed to stand for 5 h at room temperature and then overnight at 4 °C. The obtained crystal was then vacuum-filtered, washed with excess of diethyl ether, and then dried in vacuum to yield a white crystal (760 mg, 19.2% yield). The reaction was confirmed by ^{1}H NMR spectrum in acetone at 25 °C (−$COCH_2CH$(CH_2COOH)CO−, δ = 2.94, 2.86 ppm), (CH_2COOH, δ = 2.44 ppm) (data not shown).

2.3.2. Synthesis of poly(ethylene glycol)-b-poly{N-[N′-(N″-carballylyl-2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-PAsp(DET-Car))

PEG-PAsp(DET) (15 mg, 0.046 mmol of primary amine) was dissolved in 0.5 M $NaHCO_3$ at pH 9.1 (50 mL). Carballylic anhydride powder (Car) (363 mg, 2.3 mmol) was added to the solution slowly and stirred at 0 °C for 2 h. The reaction mixture was purified by centrifugal ultrafiltration with Amicon Ultra (MWCO = 10,000; Millipore (Billerica, MA)) three times with de-ionized water at 4 °C. The final product was obtained as a white powder after lyophilization (13.5 mg, 68.3% yield). The quantitative conversion of the primary amines in the Asp(DET) side chain to carballylylamide was confirmed from the peak intensity ratio of the methine protons in the main chain (−$COCH_2CH$(CO−)NH−, −$COCH(CH_2$−)NH−, δ = 4.8 ppm) to the methylene protons of the carballylyl moiety (−CH_2CH(COONa)CH_2COONa, δ = 2.5) in the ^{1}H NMR spectrum in D_2O at 50 °C (Supporting Information).