Organic-Inorganic Hybrid-Nanocarrier of siRNA Constructing through the Self-Assembly of Calcium Phosphate and PEG-based Block Anionomer

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Short interfering RNA (siRNA) that mediates gene-silencing phenomena of RNA interference (RNAi) needs a carrier system to exert biological activity, especially, for *in vivo* application¹. Some lipid- and polymer-based systems are available for the *in vitro* application of siRNA, yet they often have drawbacks including a low stability in a serum-containing medium and significant cytotoxicity. From the standpoint of developing a safe and serum-tolerable carrier system, the calcium phosphate (CaP) co-precipitation method is of interest². To control the growth of CaP, that is the determining factor for the transfection efficacy, a novel concept has recently been introduced by our group based on the self-assembly of poly(ethylene glycol)-*block*-poly(aspartic acid) block copolymers (PEG-P(Asp)) with CaP to form the narrowly-distributed hybrid nanoparticles covered with PEG palisades³.

siRNA-entrapped PEG-P(Asp)/CaP hybrid nanoparticles indeed revealed an appreciable gene silencing in a cultured cell line⁴. Nevertheless, the requirement of chloroquin, a reagent facilitating endosomal-escape, impedes the wide applicability of this method. Herein, we overcome this critical issue using the newly designed system of siRNA-entrapped CaP nanoparticles. The strategy is to incorporate the molecular units facilitating endosomal-escape directly into the block copolymer structure. In this regard, poly(methacrylic acid) (PMA) was selected as the polyanion unit in the block copolymer. PMA undergoes a conformational transition at pH 4-6, which almost corresponds to the endosomal pH, to adopt a more hydrophobic conformation as compared to neutral

pH, thus expected to have an increased interaction with the endosomal membrane in order to perturb its structure to facilitate escape of the nanoparticles into the cytoplasm⁵. Here, two different compositions of the poly(ethylene glycol)-*block*-poly(methacrylic acid) (PEG-PMA) were used for the nanoparticle formation entrapping siRNA. PEG-PMA1 has a PEG segment with the molecular weight (Mw) of 7800 and the PMA segment with the Mw of 2000, while the Mw of the PEG and PMA segments of PEG-PMA2 are 7500 and 15500, respectively.

The diameters of the nanoparticles formed in the presence of the PEG-PMAs are determined by dynamic light scattering measurements^{6,7}. Figure 1A shows the change in diameters of nanoparticles with varying concentrations of PEG-PMAs at 3.0 mM phosphate. A similar trend was observed for PEG-PMA1 and PEG-PMA2, and the stable dispersion of nanoparticles was obtained over a certain concentrations; 350 μ g/mL for PEG-PMA1 and 450 μ g/mL for PEG-PMA2. Diameters above these critical concentrations of PEG-PMA were about 180-200 nm and 200-280 nm for PEG-PMA1 and PEG-PMA2, respectively. It is noteworthy that at those concentrations no precipitation of CaP was observed 24 h after the mixing of Ca/siRNA and phosphate/PEG-PMA solution. The polydispersity indices of the nanoparticles were below 0.1 for PEG-PMA1, suggesting a unimodal distribution, while the values were slightly higher (0.12-0.15) for PEG-PMA2. The zeta potential of nanoparticles composed of 450 μ g/mL of PEG-PMA1 or PEG-PMA2 was almost zero, -1.50 ± 0.36 and -0.439 ± 0.118 mV, respectively, indicating that the colloidal stability of the nanoparticles is caused by the PEG palisades with the steric repulsion effect. The incorporation efficacy of siRNA in the nanoparticles determined by the centrifugation method was around 80 % for PEG-PMA1 and 60-80 % for PEG-PMA2⁸ (Figure 1B).

In order to investigate RNAi activity of siRNA incorporated in the nanoparticles, we have established a 293 cell line stably expressing the GL3-luciferase gene and estimated the degree of gene silencing using the siRNA targeting GL3-luciferase gene⁹⁻¹¹. Figure 2A shows the effect of the PEG-PMA concentrations on the RNAi activity of the siRNA-loaded nanoparticles, expressed as the relative inhibition activity to the control (non-silencing) siRNA. PEG-PMA1 showed no remarkable RNAi activity (■), while the PEG-PMA2 system (●) achieved a gene silencing down to 20 % of the control (80% inhibition) in the polymer concentration range of 450 to 550 μ g/mL. The reason for the presence of the maximum transfection efficacy in terms of the PEG-PMA concentration is not yet clear, however, nanoparticles with smaller diameters seems to be more efficient than the larger CaP/PEG-PMA complex or precipitate formed at the lower PEG-PMA concentration. For example, at 250 μ g/mL PEG-PMA1, at which a large precipitate was formed, no silencing was recognized. The siRNA concentration dependency was evaluated using the sample solutions prepared by dilution of the stock nanoparticle solution formed at 450 μ g/mL of PEG-PMA2 and 2.5 μ M of siRNA. The significant inhibition was observed at a concentration as low as 6.7 nM (45% inhibition) and reached a plateau value around 25 nM (80% inhibition).

To clarify the advantages of this system, the transfection efficacy was further compared with other transfection reagents and the conventional calcium phosphate method (Figure 2B). Under the experimental conditions, the hybrid nanoparticles formed at 450 $\mu\text{g/mL}$ of PEG-PMA showed transfection efficacy similar to RNAiFect. Worth noting is that the nanoparticles are more efficient in silencing than RNAiFect at the more physiologically relevant conditions: Preincubation in the DMEM containing 10% FCS was performed for 30 min prior to the transfection (*-marked conditions in Figure 2B). Upon serum pre-incubation, the GL3 luciferase expression relative to the control increased from 36 to 58% in the case of RNAiFect, while the increase is only 29 to 35% for the hybrid nanoparticle system. An other type of lipid-based transfection reagent, lipofectAMINE, showed a non-specific effect in the serum-containing medium, which resulted in a significantly reduced luciferase activity even using the controlled non-silencing siRNA, presumably due to the toxicity. Tolerability to the serum is crucial when considering the *in vivo* application of the carrier system, especially, in the case of the systemic administration. Indeed, the *in vivo* application of a conventionally available cationic-lipid system is reported to be hampered in the serum-containing medium¹². Furthermore, no significant activity of siRNA was observed for the calcium phosphate crystal prepared in the absence of PEG-PMA, indicating the necessity of the block copolymer for the improved transfection activity.

In summary, we demonstrated a highly efficient transfection of siRNA to cultured mammalian cells using nano-sized calcium phosphate crystals with appreciable serum tolerability. This nano-particulate siRNA carrier possessing a biocompatible PEG palisade and a pH-responsive moiety of PMA to facilitate endosomal escape may have a promising utility in RNAi research directed toward disease treatment.

Figure legends.

Figure 1. Physicochemical characterization of nanoparticles composed of PEG-PMA1 (□) and PEG-PMA2 (○). (A) Size of the nanoparticles entrapping siRNA determined by DLS measurements. (B) Percentage of siRNA loaded in the nanoparticles formed at varying PEG-PMA concentrations.

Figure 2. Biological activity of siRNA incorporated in nanoparticles. (A) RNA interference by siRNA incorporated in nanoparticles formed at various concentrations of PEG-PMA. GL3 luciferase gene expression in 293 cells was inhibited by nanoparticles composed of PEG-PMA1 (■) and PEG-PMA2 (●). Results are shown as the percentage to the control expression of cells treated with nanoparticles entrapping non-silencing siRNA. (B) RNA interference by siRNA using various transfection reagents. Grey bars indicate GL3 luciferase gene expressions in 293 cells treated with non-silencing control siRNA, and white bars indicate those treated with GL3 siRNA. The nanoparticle samples are composed of calcium phosphate and PEG-PMA2 formed at 450 $\mu\text{g/mL}$.

For the preincubation study (*), DMEM containing 20% FCS was added to an equal amount of nanoparticle or lipoplex solutions and incubated at 37 ° C for 30 min prior to the transfection. In the calcium phosphate method, the transfection was performed under the same condition as the nanoparticle except in the absence of PEG-PMA. The concentrations of siRNA and calcium ion in all the experiments were 125 nM and 6.25 mM, respectively.

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- (6) The nanoparticles were prepared as follows: A solution of 2.5 M CaCl₂ was added to a solution of siRNA in a buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) to a final volume (Ca²⁺ 250 mM, siRNA 74 µg/mL). One volume of this 2x Ca/siRNA solution was quickly added to an equal volume of 2x Hepes/phosphate/PEG-PMA solution (140 mM NaCl, 50 mM HEPES, 6.0 mM Na₂HPO₄, PEG-PMA, pH 7.1). The mixed solutions were vigorously stirred by a vortex mixer for a few seconds and incubated at 37 °C for 24 h.
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- (8) siRNA content was determined as follows: The sample solutions (200µL) were centrifuged at 15000 g for 30 min to precipitate the nanoparticles. Supernatants (100 µL) were carefully removed to determine siRNA concentration by absorbance at 260 nm. The percentage of the loaded siRNA was calculated by subtracting the siRNA concentration in the supernatant from the total concentration.
- (9) siRNA targeting GL3-luciferase gene, antisense sequence; 5'-UCGAAGUACUCAG-CGUAA-GdTdT-3' , sense sequence; 5'-CUUACGCUGAGUACUUCGAdTdT-3', control (non-silencing) siRNA; antisense sequence; 5'-UUCUCCGAACGUGUCACGdTdT-3', sense sequence; 5'-

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(11) The 293 cell line stably expressing GL3-luciferase gene was established using Flp-In system (Invitrogen) according to the manufacturer's protocol. The cells were plated at the density of $\sim 8 \times 10^3$ cells/well in a 96 well plate and grown over night in medium containing 10% FCS. Before transfection, the medium was removed and 50 μ L of fresh medium containing 20% FCS was added to each well. The stock nanoparticle solutions (siRNA 2.5 μ M) were diluted with DMEM to desired concentrations. Fifty microliters of the diluted sample solutions were added to each well and the cells were incubated at 37°C for 42 h under 5% CO₂ atmosphere. Luciferase gene expression was measured using Luciferase assay kit from Promega. Protein concentration was determined by BCA protein assay kit from Pierce.

For the commercially available transfection reagents, RNAiFect and LipofectAMINE, transfection experiments were performed following a protocol provided by the manufacturer. The conventional calcium phosphate co-precipitation method was performed as Jordan et al. reported (Jordan, M.; Schallhorn, A.; Wurm, F. M. *Nucleic Acids Res.* 1996, 24, 596-601).

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Abstract

The development of siRNA delivery systems is a major key for practical RNA therapy that holds promise for the treatment of life-threatening human diseases, yet there still exists significant difficulties in their construction because of the various requirements including high transfection efficacy, tolerability in the biological medium, and low toxicity. Here we report the novel preparation route of organic-inorganic hybrid-nanocarriers entrapping siRNA based on the self-assembly of the block anioner, poly(ethylene glycol)-block-poly(methacrylic acid), with calcium phosphate crystals. The nanocarriers have diameters in the range of several hundreds of nanometers and revealed excellent colloidal stability due to the steric stabilization effect of the PEG palisade. The biological activity of siRNA loaded in nanocarriers was assessed using 293 cells stably expressing luciferase gene, showing the remarkably high gene silencing-efficacy without the use of any adjuvant molecules such as chloroquin. Further advantage of the system is the serum tolerability, which is of a critical issue in in vivo application.

Figure 1. Kakizawa et al.

Figure 2. Kakizawa et al.