Spontaneous Formation of Polyion Complex Micelles with Narrow Distribution from Antisense Oligonucleotide and Cationic Block Copolymer in Physiological Saline

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The association of block copolymers in aqueous medium has received considerable attention due to its high potential for biomedical and pharmaceutical applications. Indeed, hydrophobic drugs were shown to be successfully entrapped in the core of the polymeric micelles formed from amphiphilic block copolymers.[1] Impressive anticancer activity and longevity of circulation in the blood compartment were reported for the doxorubicin-incorporated micelle with a poly(ethylene glycol) (PEG) corona.[2] The concept of micelle stabilization in an aqueous medium by the hydrophilic corona surrounding the water-incompatible core can be extended to include macromolecular association through a force other than hydrophobic interaction. Recently, we have shown that a pair of oppositely charged block copolymers with PEG segments spontaneously forms a polyion complex micelle with a spherical shape and a considerably narrow size distribution.[3] Worth noting is that similar monodispersive associates were found to form when even one of the pair was changed from a block copolymer to charged oligomers with synthetic or natural origin. Just a simple mixing of the aqueous solutions of oppositely charged partners led to the spontaneous formation of monodispersive polyion complex micelles. As a typical example of these systems, we wish to communicate here the polyion complex micelle formation from a pair of cationic block copolymers and oligonucleotides with antisense activity to the oncogene (c-Ha-*ras*). This system is of interest from the standpoint of the development of a novel *in vivo* vector system for oligonucleotides as well as for constructing novel self-association systems of macromolecules at a nanoscopic level.

We used the poly(ethylene glycol)−poly(L-lysine) block copolymer (PEG-P(Lys)) as the counterpart of oligonucleotide to form polyion complexes through electrostatic interaction. As a detailed synthetic procedure of PEG-P(Lys) was previously reported,[3] only a brief description is given below: The poly(ethylene glycol)−poly(ϵ-benzyloxycarbonyl-L-lysine) block copolymer (PEG-P(Lys(Z))) was obtained by the ring-opening polymerization of the ϵ-benzyloxycarbonyl-L-lysine anhydride, which was synthesized from ϵ-benzyloxycarbonyl-L-lysine (Peptide Institute, Inc., Japan) by the Fuchs−Farthing method using triphosgene (Tokyo Kasei Kogyo Co., Ltd., Japan) in doubly distilled *N,N*-dimethylformamide (DMF) with α-methoxy-ω-aminopoly(ethylene glycol) (PEG, Nippon Oil & Fat Co., Ltd., Japan, M_w = 5050, functionality of amino group = 0.956) as an intiator. The PEG-P(Lys(Z)) was then deprotected using 30% HBr/AcOH (Tokyo Kasei Kogyo Co., Ltd., Japan) to obtain PEG-P(Lys). ^{1}H-NMR measurement in D_2O was carried out to determine the composition of PEG-P(Lys). The polymerization degree of the P(Lys) segment was calculated to be 18 from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2: δ = 3.7 ppm) and ϵ-methylene protons of P(Lys) ($(CH_2)_3$-CH_2NH_3: δ = 3.0 ppm). The oligonucleotide (15-mer, 5′-ATATTCCGTCATCGC-3′) used in this study was purchased from Toa Gosei Co., Ltd., Japan, and is reported to have a sequence that is complementary to part of the first four codons and the upstream sequence close to the ribosome-binding site of c-Has-*ras* mRNA.[4]

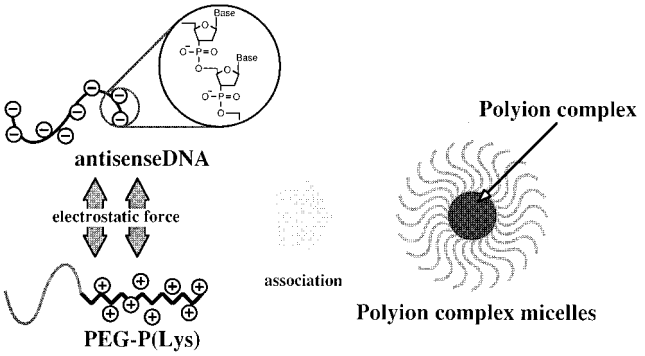

Figure 1. Concept of the formation of polyion complex micelles from a pair of cationic block copolymers and oligonucleotides.

The PEG-P(Lys) and oligonucleotide were separately dissolved in 10 mM Tris-HCl buffer (pH 7.4) at 1.0 mg/mL. The concentration of oligonucleotide was checked by the measurement of absorbance at 260 nm (V-550 UV/VIS spectrophotometer, JASCO). Both solutions were mixed under stoichiometric conditions where the unit ratio of L-lysine in PEG-P(Lys) and phosphate in the oligonucleotide is equal. It is to be noted that the stoichiometry for the formation of polyion complex micelles from a pair of oppositely charged block copolymers or from the combination of oppositely charged homopolymers and block copolymers has already been confirmed from viscosity measurements.[3] Then, 10 mM Tris-HCl including 0.3 M NaCl was added in the oligonucleotide/PEG-P(Lys) solution so as to change the ionic strength of the oligonucleotide/PEG-P(Lys) solution to physiological condition (0.15 M). The oligonucleotide/PEG-P(Lys) solution thus prepared showed no precipitate formation even after overnight storage at room temperature. This is in sharp contrast with a well-known phenomenon that the mixture of the poly(L-lysine) homopolymer and oligonucleotide readily forms precipitate under electrostatically neutralized conditions. It is likely to form the water-soluble associates with water-compatible poly(ethylene glycol) segments surrounding the core of polyion complexes formed between the poly(L-lysine) segments and oligonucleotides. Indeed, the associate formation in the size range of several tens of nanometers was confirmed through dynamic light scattering (DLS) measurements as described below.

In order to obtain information on the shape as well as the size of the associates, angle-trace DLS measurements were carried at 30, 60, 90, 120, and 150° detection angles at 37.7 °C. A DLS-700 instrument (Otsuka Electronics Co., Ltd.) was used for these measurements. Details of the data analysis of the DLS measurements were reported elsewhere.[3] Figure 2 shows the relationship between the scaled average characteristic line width (Γ/K^2) and the magnitude of the scattering vector (K^2). The results presented in Figure 2 clearly indicate that the associates formed between PEG-P(Lys) and the

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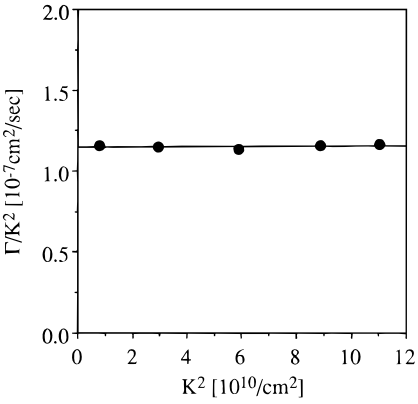

Figure 2. Relationship between the scaled average characteristic line width (Γ/K^2) and the magnitude of the scattering vector (K^2) for polyion complex micelles formed between the oligonucleotide and PEG-P(Lys) (detection angle: 30, 60, 90, 120, and 150°; temperature: 37.7 ± 0.1 °C; total concentration: 0.5 mg/mL; solvent: 10 mM Tris-HCl including 0.15 M NaCl).

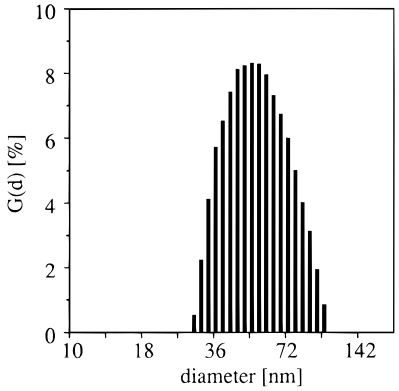

Figure 3. *z*-weighted size distributions analyzed by the histogram method for polyion complex micelles formed between the oligonucleotide and PEG-P(Lys) (detection angle: 90°; temperature: 37.6 °C; total concentration: 0.5 mg/mL; solvent: 10 mM Tris-HCl including 0.15 M NaCl).

oligonucleotide have a spherical shape, because the Γ/K^2 values are independent of the detection angle. As the dependence of the Γ/K^2 value on the detection angle was negligible, all the following DLS measurements were carried out at a 90° detection angle.

Figure 3 shows the *z*-weighted size distribution of polyion complex micelles obtained from the histogram analysis of the DLS data at 37.6 °C. Obviously, from the size distribution profiles, the formed micelles are confirmed to be unimodal. In line with the result of the histogram analysis, the polydispersity index (μ_2/Γ^2) by cumulant analysis was calculated to be as small as 0.012; the value is small enough to consider that the micelles have narrow size distribution.[5] Worthy of mention is that freeze storage has no effect on the size distribution of the micelles. Micelles in a thawing solution showed exactly the same results in both cumulant and histogram analysis of DLS. Since the polyion complex micelle formed between PEG-P(Lys) and the oligonucleotide is quite stable and has a significantly narrow size distribution with no secondary aggregates (the cluster of polyion complex micelles), it is reasonable to assume that the micelles may have a core−shell structure, in which poly(ethylene glycol) segments surround the core of the polyion complexes. The formation of a PEG corona surrounding the core was further suggested through laser-Doppler electrophoresis measurements (ELS-800, Otsuka Electronics Co., Ltd.), which allowed one to determine the electrophoretic mobility of the micelles to be 0.013. The average ζ-potential of the micelles was then calculated from this value to be 0.14 mV using the Smoluchouski equation.[3] This small absolute value of the ζ-potential of the micelles was consistent with the formation of a core−shell structure, where the PEG corona blocks the micelle aggregation through steric repulsion.

In conclusion, it was confirmed that the mixture of cationic PEG-P(Lys) and anionic oligonucleotides in aqueous medium led to the formation of polyion complex micelles. The formed micelles have a small size (ca. 60 nm) and a considerably narrow size distribution under physiological conditions (pH 7.4, ionic strength 0.15 M, temperature 37 °C). It was assumed that the micelles have a core−shell structure with the polyion complex core surrounded by the corona of poly(ethylene glycol). High water solubility and protection of the oligonucleotides in the micelle core provide this system with a potential utility in *in vivo* delivery of antisense oligonucleotides. Related to our work presented here, Kabanov *et al.* recently reported the complex formation of antisense oligonucleotides with poly(ethylene glycol)−polyspermine block copolymers which showed a relevant biological property toward a cultured cell line.[6] Worth noting is that the size of our micelle is in the same range with natural vector systems including viruses and lipoproteins, which may be one of the contributing factors in achieving an efficient tissue penetration at the target site in the body. Such a demonstration is now under investigation in our laboratory.

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