Terplex DNA Delivery System As a Gene Carrier

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Purpose. To characterize the physical and biochemical properties of the DNA terplex delivery system, which has previously been shown to deliver and express pSV-β-gal plasmid efficiently in cultured smooth muscle cells (SMC) (1).

Methods. Atomic force microscopy (AFM), zeta-potential measurement (ZP), gel electrophoresis (GE), circular dichroism (CD), fluorescence quenching and ^{1}H-NMR spectrometry were used.

Results. AFM showed that the plasmid DNA of about 600 nm long in its extended state was condensed to the size of about 100 nm by terplex formation. The DNA condensing effect of the terplex system was as good as unmodified PLL, as shown by an ethidium bromide displacement assay. Zeta-potential measurement showed that the terplex system exerts a slightly positive surface charge (+2 mV) at a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL, which showed the best transfection efficiency on SMC. GE indicated that electrophoretic mobility of the terplex system decreased with increasing amounts of stearyl-PLL, indicating that the surface charge of the terplex system became more positive as more stearyl-PLL was added. Results from CD showed that there was no significant changes in tertiary structure of plasmid DNA from the terplex formation. Presence of strong hydrophobic interaction between stearyl-PLL and LDL was confirmed by ^{1}H-NMR, where about a 30% decrease in ε-methylene peak of PLL backbone was observed when stearyl-PLL was mixed with LDL, but this phenomenon was not observed when unmodified PLL was used.

Conclusions. Our results indicate that the plasmid DNA, when formulated with the stearyl-PLL and LDL, forms a stable and hydrophobicity / charge balanced terplex system of optimal size for efficient cellular uptake and the DNA is still intact after the terplex formation. This information is expected to be utilized for the development of much improved transfection vectors for *in vivo* gene therapy.

KEY WORDS: terplex system; gene delivery; non-viral gene carrier.

INTRODUCTION

Although human gene therapy shows great promises, its clinical efficacy has not been definitely established yet (2–5). Major difficulties lie in the lack of efficient and safe vectors to deliver genes to the target cells. Until now, the majority of human gene therapy protocols have used vectors derived from retroviruses or adenoviruses (6,7). Retroviral vectors in particular have been successful by introducing new genes into the genome of actively dividing cells, resulting in a stable gene transduction (8). However, this insertion of genes into the host's genome is a random process, and its long-term risk due to the possibility of inserted genes combining with endogenous viruses or activation of oncogenes is a major concern, limiting the use of such vectors in human gene therapy (9). On the other hand, vectors based on non-viruses, such as cationic liposomes (10,11) or lipopolyamine (12), have drawbacks of low transfection efficiency or precipitation problems, even though they seem to be safe for human clinical use (13).

Modifications of poly(L-lysine) to improve the delivery and expression efficiency of transfected genes have been successfully exploited in various ways, most notable among these being ligand conjugation for tissue targeting (14,15) and lipophilic poly(L-lysine) (16). We have developed a novel gene delivery system based on stearyl-poly(L-lysine) (stearyl-PLL) and low density lipoprotein (LDL). This supramolecular gene carrier, or terplex system, is unique because the main driving force for the terplex formation is a balance between hydrophobic and electrostatic interactions between stearyl-PLL, LDL and DNA. In this manuscript, we report the physicochemical characterization of the terplex system using analytical techniques and instrumentation to design a better DNA delivery system with an ultimate goal of human use.

MATERIALS AND METHODS

Materials

Ethidium bromide, poly(L-lysine) (PLL, mol. wt. 50,000), stearyl bromide, and low density lipoprotein (LDL) were purchased from Sigma (St. Louis, MO). SeaKem GTG agarose was purchased from FMC (Rockland, ME), 2× YT Broth from Bio 101 (Vista, CA), *E. coli* (HB 101 strain) and pSV-β-gal plasmid vector (6821 bp) from Promega (Madison, WI). Restriction enzymes (SalI and EcoR1) were obtained from Boehringer Mannheim GmbH (Germany) and molecular weight ladder (λDNA/HindIII) from Gibco BRL (Grand Island, NY). Rudy mica with 107 μm thickness and 12.7 mm in diameter was purchased from S & J Trading Inc. (Glen Oaks, NY). All other reagents were of analytical grade and used without further purification.

Synthesis of Stearyl-PLL

Stearyl-PLL was synthesized by N-alkylation of PLL with stearyl bromide. Typically, 30 mg of stearyl bromide in a solution of a mixture of 2 ml dioxane and 200 μl 1N NaOH was added to 100 mg of PLL-HBr in 2 ml dimethylsulfoxide (DMSO). The mixture was allowed to react for 24 hr at room temperature. The solution was then poured into a large excess of diethyl ether. The precipitated polymer was dissolved in DMSO and reprecipitated in diethyl ether. The formed stearyl-PLL was finally purified by dialysis against deionized water, followed by lyophilization. The resulting polymer was characterized by ^{1}H-NMR, and the stearyl group content was calculated by comparing the proton signals of the ε-methylene in PLL to those of the methyl group in stearyl chain in the solution of D_2O/Dioxane-d6 mixture.

Plasmid DNA (pSV-β-gal)

E. coli (strain: HB101) were transfected with a pSV-β-gal control vector using the Electro Cell Manipulator™ 600 (BTX

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Electroporation System, San Diego, CA) at 1.41kV for 7.5 msec, and grown in an ampicillin rich environment in a petri dish at 37°C overnight. Clones of transfected *E. coli* were further grown in 2× YT Broth (with 200 μg ampicillin/ml) in a 37°C water bath with vigorous agitation and aeration (American Scientific Products Model YB-521, IL) for 18 hr. The *E. coli* culture of OD_{600} over 3.0 was used for isolation and purification of pSV-β-gal plasmid DNA using the Biggerprep™ Plasmid DNA Preparation Kit (5 Prime → 3 Prime Inc., Boulder, CO) according to the manufacture's protocols. Purity of isolated plasmid DNA was determined spectrophotometrically by the OD_{260}/OD_{280} ratio and the plasmid used here proved to be pure as the OD_{260}/OD_{280} ratio was 1.81. Purity of the plasmid DNA was also proved by gel electrophoresis after SalI and EcoR1 treatment with λDNA/HindIII fragments as a molecular weight marker and one band corresponding to 6.8 kb was visible. Concentration of plasmid DNA was determined by spectrophotometer (one unit of OD_{260} = 50 μg of DNA). Plasmid DNA was stored at −20°C until use.

AFM Imaging

Red mice is freshly cleaved, and then soaked in 3 mM $MgCl_2$ overnight to favor the replacement of potassium ions by divalent magnesium ions for stronger DNA binding (17). As soon as the substrate is brought up to air, 7μl of 0.6 mg/ml DNA in deionized water was deposited on the mica surface and allowed to absorb for 2 min in the air. Sample was rinsed gently with deionized water (3 min) and blown dried with nitrogen (N_2). Nanoscope II SFM (Digital Instruments, Santa Barbara, CA) was used to image in air at room temp, in the attractive force regime and under 30–60% relative humidity. The microscope was operated using cantilever oscillation frequencies between 12 and 24 kHz. Force minimization was maintained by reducing the set point voltage (withdrawing the piezo from the tip) to minimize sample damage.

Zeta-Potential Measurement

Zeta-potential of the DNA terplex system was determined at room temperature with the Malvern Zeta-Sizer™ 3 (Malvern Instrument Inc., Southborough, MA). Terplex samples were prepared by mixing plasmid DNA and LDL with various amounts of stearyl-PLL in PBS 30 min before measurement in a clean hood.

Gel Electrophoresis

Electrophoretic mobility of the DNA terplex system was determined using a gel electrophoresis system. Varying amounts of stearyl-PLL were mixed with 3 μg of LDL, and 3 μg of DNA in PBS buffer was then added to the above mixture with gentle mixing. The terplex system was incubated for 30 min at room temperature prior to loading onto a 1% agarose gel slab. Gel electrophoresis was carried out at room temperature in TBE (45 mM Tris-Borate, 1 mM EDTA) buffer using the Easy-Cast ™ Electrophoresis System (Owl Scientific Inc., Woburn, MA) at 110V, 22mA for 2hr using an Electrophoresis Constant Power Supply (Pharmacia Fine Chemical Model ECPS 3000 / 150). Ethidium bromide was used for DNA staining after gel running according to the standard method (18), and DNA bands were visualized by an UV illuminator.

Circular Dichroism (CD)

Circular dichroism spectra of the DNA terplex system were obtained using the Jasco J-720 Spectropolarimeter (JASCO, Tokyo, Japan). CD spectra of the DNA samples were obtained with a 1 cm path length cuvette in PBS (pH 7.4) buffer or in a high concentration NaCl solution. Settings for the measurement were 1.0 nm band width, 5 mdeg sensitivity, 4 sec response, and 3 accumulations.

Ethidium Bromide Displacement Assay

DNA condensing effect of the terplex system or stearyl-PLL itself was studied by monitoring the quenching of fluorescence intensity of the DNA-bound ethidium bromide using the ISS Photon Counting Spectrofluorometer (ISS Inc., Champaign, IL). Varying amounts of stearyl-PLL were added to the mixture of a fixed amount (1 μg) of plasmid DNA and ethidium bromide (1 μM). For the terplex system, LDL (1 μg) was also added to the DNA/ethidium bromide mixture before the addition of stearyl-PLL. Fluorescence intensity was measured 30 min after mixing and ethidium bromide was excited at 488 nm with emission at 594 nm.

^{1}H-NMR Analysis

Formation of the terplex system was examined by using the Varian Unity-Plus 400Mhz NMR Spectroscopy. Samples were prepared by mixing 1 mg of stearyl-PLL and 1 mg of LDL in 50 μl of D_2O. Trace amount of DMSO was added to the solution as an internal standard.

RESULTS AND DISCUSSION

^{1}H-NMR of Stearyl-PLL

Synthesized stearyl-PLL was characterized by ^{1}H-NMR and the substitution degree of the stearyl group in PLL was calculated from the ratio of the peak area of the ε-methylene group in PLL at 3.0 ppm to that of methyl group in stearyl substitution at 0.8 ppm. When 30 wt % of stearyl bromide was used in the reaction mixture, 18 mol % stearyl substituted PLL was typically synthesized, whereas 40 wt % of stearyl bromide in the reaction mixture resulted in 25 mol % stearyl substituted PLL (Fig. 1).

Formation of Terplex System

Fig. 2 shows that there is about a 30% decrease in the peak area of ϵ-methylene group in stearyl-PLL by addition of LDL. This phenomenon was not seen when unmodified PLL was mixed with LDL, suggesting that there is a strong hydrophobic interaction between fatty acyl chains in LDL and stearyl groups in stearyl-PLL. Balanced hydrophobic interaction between LDL and stearyl-PLL is found to be important in forming a soluble DNA conjugate.

AFM Imaging

As the atomic force microscope is such a powerful tool to scan the surface of sample in a nanometer scale, DNA became one of the most studied biological samples for the study of its supercoiling [19], bending [20], and other conformations [21].

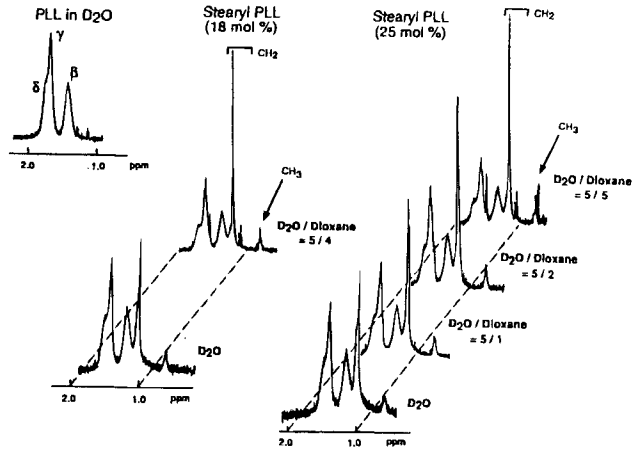

Fig. 1. Characterization of the stearyl-PLL by ^{1}H-NMR. Degrees of stearyl group substitution in PLL was calculated from the ratio of the peak area of epsilon methylene signal to that of methyl signal of stearyl group. Samples were dissolved in the mixture of D_2O and dioxane to minimize the formation of micelle-like structure, which makes the peak unclear.

As shown in Fig. 3., plasmid DNA in its natural extended state in H_2O was about 600 nm in long axis (Fig. 3a). When the stearyl-PLL and LDL were added to the DNA to form a terplex system, the plasmid DNA was condensed to 100 nm in long axis (Fig. 3b) mainly due to the stearyl-PLL, even if heterogeneity was also seen in both shape and size of the terplex system. Sample preparation methods were also found to affect the results, especially the time for sample deposit on mica, blow-dry with nitrogen gas, and rinsing with double distilled water.

Particle size analysis revealed that the terplex system of a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL is typically about 100–400 nm in diameter, although big particles over 500 nm were formed when more amounts of stearyl-PLL than a 1:1:1 weight ratio was. Heterogeneity in the particle size distribution was quite commonly seen in each batch of terplex formulations. As the transfection experiments were performed without sizing

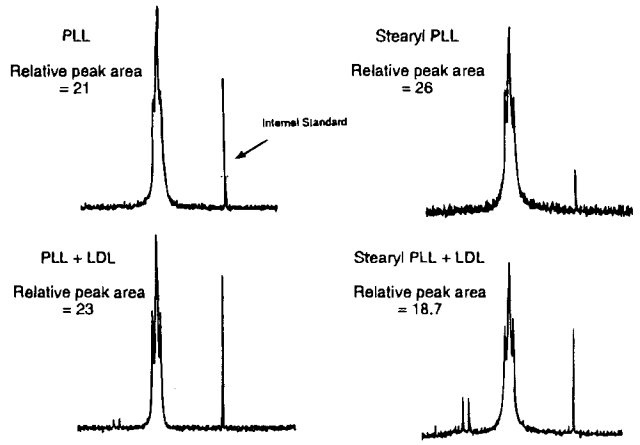

Fig. 2. Formation of the terplex system. Reduction in epsilon methylene signal of stearyl-PLL was registered when mixed with LDL, however, almost no change was observed when unmodified PLL was used. The reduction in the peak area is due to the strong interaction between the acyl chains in stearyl-PLL and LDL.

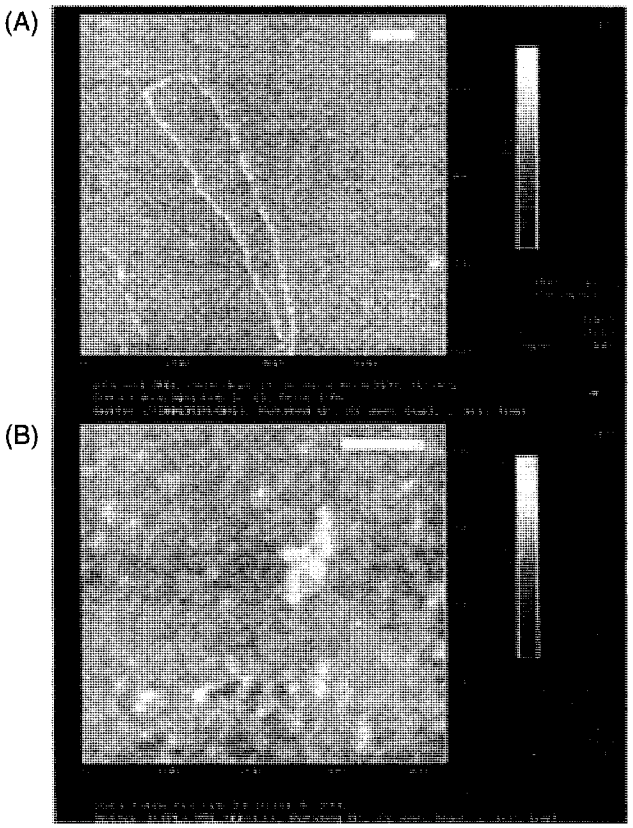

Fig. 3. Atomic force microscopy imaging of plasmid DNA in H_2O (A) and in the terplex formulation (B). DNA sample was prepared as explained in Experimental section. Condensation of plasmid DNA by the terplex system can be seen from the reduction of DNA size from 600 nm long to 100 nm long. (Scale bar is 100nm long).

the terplex systems, variations in transfection efficiency could have resulted from the size distribution of the terplex systems (unpublished data).

Gel Electrophoresis

A gel electrophoresis (1% agarose) was performed to examine the nature of interactions between the plasmid DNA, LDL, and stearyl-PLL. As shown in Fig. 4, decreased electrophoretic mobility of the DNA terplex was observed when 3 μg of plasmid DNA was complexed with 3 μg of LDL and as little as 1 μg of stearyl-PLL (3:3:1 weight ratio of DNA:LDL:stearyl-PLL, lane 7). Electrophoretic mobility of the DNA terplex system decreased gradually with increasing amounts of stearyl-PLL, and completely retarded at a weight ratio of 3:3:5 of DNA:LDL:stearyl-PLL (lane 9). For comparison, DNA (3 μg) was complexed with increasing amounts of stearyl-PLL without LDL (lanes 3–5 for 1–5 μg of stearyl-PLL), where gel retardation of the DNA complex was not affected significantly by the presence of LDL. The best transfection result on smooth muscle cells was achieved from the terplex system with a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL (lane 8).

Zeta-Potential

Among the critical factors affecting the transfection efficiency is the surface charge of the DNA delivery system. The

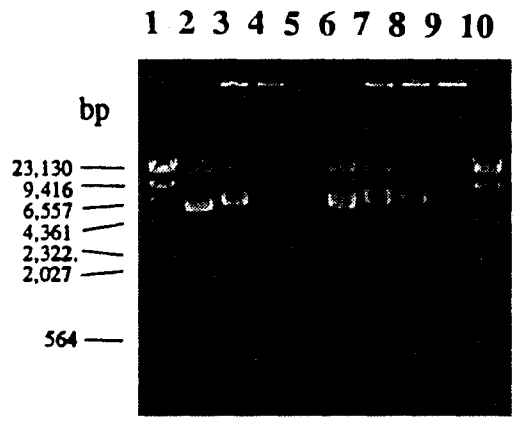

Fig. 4. Electrophoretic mobility of the terplex system on a 1% agarose gel electrophoresis. Lanes 1 and 10, molecular weight ladder; lane 2, plasmid DNA (3μg); lanes 3–5, DNA (3μg) mixed with 1μg, 3μg, and 5μg of stearyl-PLL; lane 6, DNA (3μg) mixed with LDL (3μg); lanes 7–9, DNA (3μg) and LDL (3μg) mixed with 1μg, 3μg, and 5μg of stearyl-PLL. DNA was visualized with ethidium bromide staining.

terplex system was examined for its surface charge as shown in Fig. 5. The plasmid DNA (30 μg) in PBS buffer (pH 7.4) showed a negative zeta-potential (−13 mV), and the charge went up to +5 mV with increasing amounts of stearyl-PLL. The zeta-potential was about +2 mV for the terplex system of a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL, which resulted in the best transfection efficiency on smooth muscle cells.

Circular Dichroism

Tertiary structure of plasmid DNA in the terplex system was examined using a circular dichroism (CD) spectropolarimeter. The CD spectrum of DNA:LDL mixture (Fig. 6,b) was similar to that of natural DNA (Fig. 6,a), in having both negative and positive CD bands of moderate magnitudes at wavelength above 220 nm and a crossover point between 248 and 262 nm, characteristic of the B-DNA conformation [22]. As more amount of stearyl-PLL was used in the terplex formulation, CD

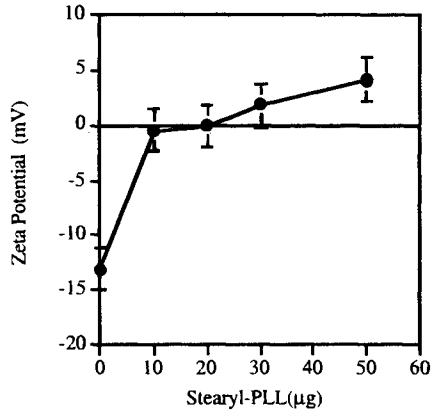

Fig. 5. Surface charge of the terplex system was determined by zeta-potential measurement. Fixed amount (30 μg) of plasmid DNA was mixed with 30 μg of LDL with increasing amounts of stearyl-PLL. The Zeta-potential showed about +2 mV at a 1:1:1 ratio of DNA:LDL:stearyl-PLL.

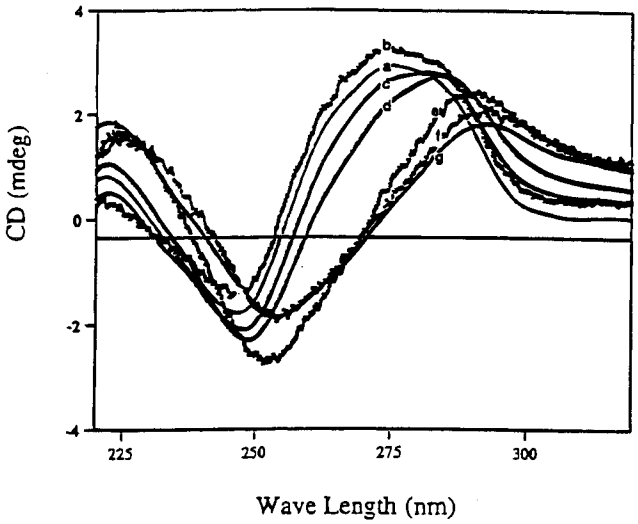

Fig. 6. Circular dichroism (CD) of the terplex system for the structure of the plasmid DNA (a, 10 μg), DNA with LDL (b, 10μg of each of DNA and LDL), and DNA in the terplex system with increasing amounts of stearyl-PLL (c–g). The amounts of DNA and LDL were fixed as 10 μg of each and the amounts of stearyl-PLL were varied as 5.0 μg (c), 10 μg (d), 15 μg (e), 25 μg (f), and 50 μg (g). The DNA structure remains almost unchanged up to the weight ratio of 1:1:1 of DNA:LDL:stearyl-PLL (d), however, the CD band shifted towards higher wavelength significantly at the 1:1:2 ratio and above (e–f).

spectra of the plasmid DNA changed accordingly from the natural B-DNA conformation (Fig. 6, c & d), and more shifts in maximum and minimum bands were also observed (Fig. 6, e, f, & g). The addition of an excess amount of stearyl-PLL to the DNA:LDL complex seems to cause the band shifts both at 275 nm and 245 nm. This pattern of red shift in CD spectra was different from when the natural DNA was placed in a high salt concentration of up to 5M NaCl. It is noteworthy that the terplex system of a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL (Fig. 6, d) was shown to result in the best transfection efficiency from the earlier report. It is not fully understood at this moment what causes the shift in bands for the terplex system with more amounts of stearyl-PLL (Fig. 6; e, f, and g) and a lower in vitro transfection efficiency from those formulations.

DNA Condensation

Poly(L-lysine) is known to condense the DNA and form a compact conjugate when mixed with DNA [23]. The terplex system with stearyl-PLL is examined for its DNA condensing ability using an ethidium bromide displacement assay. Quenching of ethidium bromide fluorescence intensity by the addition of increasing amounts of stearyl-PLL to either the ethidium bromide-DNA complex or ethidium bromide-DNA/terplex system was monitored using a spectrofluorometer. As shown in Fig. 7, a sharp decrease in fluorescence intensity was observed when 0.5 μg of stearyl-PLL was mixed with 2 μg of DNA, followed by a slow decrease at a ratio of 1:1:1 and reached 75% of their asymptotic values at a ratio of 1:1:2 of DNA:LDL:stearyl-PLL. The presence of LDL in the terplex

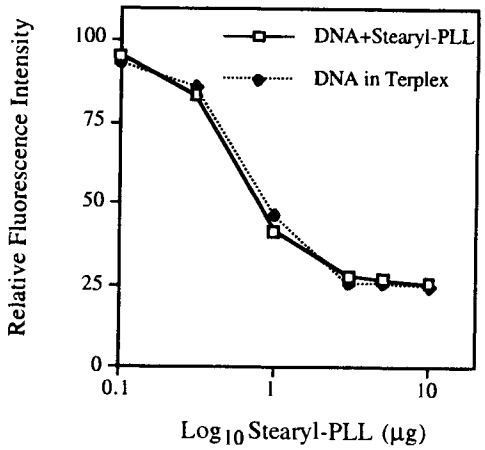

Fig. 7. Monitoring of fluorescence quenching of ethidium bromide by the condensation of DNA. Open rectangle is when the DNA was mixed with increasing amounts of stearyl-PLL only and closed diamond is when the DNA formed a terplex system with increasing amounts of stearyl-PLL. Ethidium bromide was excited at 488 nm with emission at 594 nm.

system was shown not to interfere with the DNA condensing ability of stearyl-PLL.

CONCLUSIONS

Among the major difficulties for successful and wide applications of gene therapy to human clinical use is the development of DNA delivery vectors which are safe, efficient, and well-characterized for routine gene transduction *in vivo* [24]. We have successfully designed a non-viral synthetic vector, the terplex system, based on a balanced hydrophobicity and net surface charge between the stearyl-PLL, low density lipoprotein, and plasmid DNA. Modification of PLL by stearyl chain was chosen for two reasons: 1) a hydrophobic interaction between stearyl groups in PLL and fatty acyl chains in LDL for stable complex formation, 2) an increased hydrophobicity for PLL for facilitated cellular uptake. Low density lipoprotein (LDL) is used for receptor-mediated endocytosis by smooth muscle cells as well as hydrophobic interaction. Not only the electrostatic interaction between the positive-charged stearyl-PLL and negative-charged plasmid DNA but the hydrophobic interaction between stearyl group in stearyl-PLL and fatty acyl chain in LDL is a main driving force for the formation of a soluble DNA terplex system.

Frequently, complexation between the plasmid DNA and cationic polypeptides (i.e. PLL) resulted in precipitation of DNA, limiting the DNA concentration that can be used. Once precipitated, plasmid DNA resulted in a decreased transfection efficiency than soluble plasmid DNA conjugates (unpublished data). In the case of terplex system, however, it formed a soluble DNA complex at a wide range of concentration of plasmid DNA, stearyl-PLL, and LDL.

^{1}H-NMR spectrum shows a strong hydrophobic interaction between stearyl-PLL and LDL, but not between unmodified PLL and LDL, leading to a soluble and compact DNA complex. This interaction is presumably due to the interaction between the stearyl group in modified PLL and hydrophobic acyl chain in LDL. Gel retardation data indicate that complete complexation was achieved at a 3:3:5 weight ratio of DNA:LDL:stearyl-PLL, even though the transfection efficiency of which was not as good as a 1:1:1 weight ratio one of DNA:LDL:stearyl-PLL. The DNA condensation assay monitored by fluorescence quenching of DNA intercalating dye (ethidium bromide) suggested that DNA condensation started at about a 1:1:1 weight ratio and above. It seems that fluorescence quenching data is more indicative of DNA condensation than gel retardation data, and more predictive of a better transfection result because the best transfection results came from the 1:1:1 weight ratio of terplex system rather than a 3:3:5 ratio one. DNA condensation by polymer was further appreciated when AFM images were taken to show that the size of DNA has shrunken from 600 nm to 100 nm in long axis by the terplex formation. The size reduction is thought to be mainly due to the interaction between plasmid DNA and stearyl-PLL. Generally, positive charge of cationic polymer-DNA complex has been known to give better transfection results, which was consistent with our terplex system as the zeta-potential of the best transfection efficiency terplex system at a 1:1:1 weight ratio of DNA:LDL:stearyl-PLL showed +2 mV.

In conclusion, we characterized the physico-chemical properties of the terplex system which has proven to effectively deliver and express the plasmid DNA in smooth muscle cells in culture. Even if we can't pinpoint a single factor at this stage which affects the transfection efficiency most, it can be rather regarded as a combination of those factors mentioned above, such as charge, size, and compactness of the system. Improvement in transfection efficiency is highly expected by characterizing the delivery system using the biophysical and chemical techniques mentioned above. There is a strong evidence from the data presented here that the terplex system has advantages as a potential non-viral gene delivery carrier, with good biocompatibility and high transfection efficiency. Application of the terplex system to the delivery of other genetic materials such as antisense oligonucleotide as well as the in vivo experiment is currently underway.

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