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Short communication

Analysis of the surface structure of DNA/polycation/hyaluronic acid ternary complex by Raman microscopy

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ABSTRACT

We have developed a new type of ternary gene transfection system comprising plasmid, polyethyleneimine, and hyaluronic acid (HA). HA recharged the positively charged DNA complexes into negative, and diminished the adverse interactions with bio-components. HA also improved the transcription efficiency, stabilized the dispersion, and protected the particles against lyophilizing induced inactivation. It enabled the preparation of very small complex particles, which showed high *in vivo* gene expression. In spite of the many attractive properties of the ternary complex systems, their detailed structure is still not clear. In this study, surface structures of the complexes were analyzed by non-confocal laser Raman microscopy. The correlation between the ζ-potential and the Raman spectrum is discussed. The DNA complex that showed the highest gene-expression level was prepared at the mixing ratio of COOH/NH = 0.5. At this ratio, all the amino groups were just protonated, and the surface was completely covered by HA attributed to the reduced nonspecific interaction of the complex in which all the amino groups have already interacted with carboxyls of HA, but not too much HA on the surface taken up by the cells.

1. Introduction

Various viral vectors have been explored for gene carrier systems. These vectors have strong gene transfection ability on mammalian cells; however, risks such as a random recombination or immunogenicity have been reported [1]. Thus, great effort has been focused on developing safer alternatives using non-viral vectors. A variety of polycations and cationic lipids that can electrostatically associate with DNA have been examined as transfection mediators [2]. Several DNA/polycation (or cationic lipid) complexes have already realized high gene expression on the cultured cells. But high efficiency in *in vivo* gene expression by non-viral vectors has not been achieved [3]. Non-viral vectors should overcome many barriers such as low efficiency in delivery to target cells, escape from the endosome, internalization into the nucleus, transcription, and translation.

DNA/polycation (or cationic lipid) complexes are usually positively charged on their surfaces. The cationic complex surface causes adverse interaction with bio-components containing activation of complements, coagulation of blood cells, or aggregation with serum proteins [4–6].

In order to diminish these side effects, we coated the cationic complexes with hyaluronic acid (HA) to negative [7]. HA is a low toxicity mucopolysaccharide distributed widely in the extracellular matrix and the joint liquid of mammals, and has been approved by the FDA to be used for injection.

HA coatings could efficiently reduce nonspecific interactions, and stable dispersion of DNA complexes was obtained even in the presence of serum proteins or red blood cells. It would work not only as a protective coating, but also as a ligand for tumor cell targeting, because a receptor for HA, CD44, is overexpressed on the majority of malignant cell surfaces [8–10]. The transcription efficiency of the DNA complex could also be improved by the loosening effect of HA on the tightly compacted DNA molecule [11].

Recently, we found that the DNA/polycation complex coated by HA could be freeze-dried without the loss of gene transfection activity, owing to the protective effect of HA against the aggregation of DNA complex particles. Moreover, it realized the preparation of small DNA complex particles (<70 nm) with high concentration enough to be applied to an *in vivo* gene transfection. These finely dispersed DNA/polycation complexes coated by HA showed very high *in vivo* gene transfection efficiency on the tumor tissues after intravenous or intratumor injections [12,13].

Although many attractive properties of these ternary complexes have been revealed, their detailed surface structure is still not clear in spite of the great importance for efficient gene expression. Since methods to measure the detailed surface structure or composition

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of DNA complexes have not been established, the function of these complexes has usually been discussed on the basis of the feed ratio of each component [14–16]. The only characteristic parameter that has been used to describe the surface structure of DNA polyion complex is ζ-potential [17]. The structure and physical property of the complex surface are the essential factors to determine the function of the particles, such as binding to target cells, interaction with blood components, induction of immune response, and deliverability to the tissues in living organisms.

Our interest has been focused on analyzing the complex particles by Raman microscopy, which is recognized as an effective technique to measure the surfaces structure [18]. It is difficult to measure the correct Raman spectrum in a very narrow area by a conventional confocal laser Raman microscopy, because of the high temperature induced by the focused laser beam [19]. Here we used the non-confocal laser Raman microscopy, which irradiates a relatively low power unfocused laser beam, and, thus, would not cause a heat transformation of the samples. It has a high numerical aperture, which makes the observation area wider, and remarkably enhances the Raman intensity [20]. Then, the detailed surface structure of the nanoparticles can be analyzed.

In this study, we analyzed surface structures of the DNA/polycation ternary complex coated with hyaluronic acid using the non-confocal laser Raman microscopy. The correlation between the ζ-potential and the Raman spectrum was discussed, and the structures required for high gene expression are examined.

2. Methods

2.1. Materials

Hyaluronic acid sodium salt (from microorganism), and Linear polyethyleneimine (MW 25,000) were purchased from Nacalai Tesque, Inc., and Polyscience, Inc., respectively. DNA Sodium Salt (from Salmon) (sDNA), and 4′,6-diamidino-2-phenyl-indole (DAPI) were obtained from Wako Pure Chemical Industries, Ltd. YOYO-1 iodide was purchased from Invitrogen Corp. Plasmid DNA with cytomegalovirus promoter coding firefly luciferase gene (pDNA) was amplified in *Escherichia coli*, and purified with a QIAGEN Plasmid Mega Kit. Cell culture lysis reagent and luciferase assay substrate were purchased from Promega Corp. Protein assay kit was obtained from Bio-Rad Laboratories. Fluorescence labeled polymers, FITC-PEI and Texas Red-HA, were synthesized as described previously [7].

2.2. Fluorescence microscopic observation

Fluorescence microscopic observation was carried out by an IX70 microscope (Olympus) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera connected to a DVD recorder. pDNA was fluorescently labeled by 4′,6-diamidino-2-phenyl-indole (DAPI), and was mixed with FITC-PEI and Texas Red-HA, in this order. Mercaptoethanol was added as an antioxidant. Final concentrations of pDNA, DAPI, FITC-PEI, TR-HA, and mercaptoethanol were 10 μM, 6.25 μM, 120 μM, 120 μM, and 6.25% (v/v), respectively. Each fluorophore was individually excited by the light transmitted though the dichroic filters with band pass of 330–385, 470–490, or 510–550 nm, respectively. Wavelength range of the emission filter is above 420, 520, or 590, respectively.

2.3. Preparation of freeze-dried DNA complex

An aqueous solution of DNA was mixed with solution of linear PEI and then with HA solution. The DNA/PEI/HA complexes were prepared at 1:*x*:12 or 1:12:*x* (*x* = 1–24) (in charge) in pure water. After standing at room temperature for 30 min, they were freeze-dried.

2.4. ζ-Potential and size measurement

The freeze-dried complex containing 16 μg of sDNA was rehydrated with water (80 μL). After 30 min, it was diluted with 920 μL of water and supplied to a particle analyzer (MALVERN Zetasizer Nano ZS).

2.5. Raman microscopy

Raman spectra of the freeze-dried sDNA complex were obtained by a non-confocal laser Raman Microscope (EX7001, EX control Japan) equipped with a thermoelectrically cooled CCD array detector. The samples were illuminated with a 633-nm laser (He–Ne laser) excitation powered at 21 mW through the 100× microscope objective lens. All Raman spectra were recorded by summing 30 scans with exposure time of 5 s.

2.6. In vitro transfection

B16 cells, a mouse melanoma cell line, were seeded onto 24-well plates at 3.2×10^4 cells per well, and cultured for 2 days in MEM media supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 unit/mL), and streptomycin sulfate (0.1 mg/mL). The primary growth medium was then replaced with 500 μL of fresh MEM with FBS and the antibiotics. Freeze-dried pDNA complex was rehydrated in pure water ([DNA] = 200 μg/mL), and diluted with the same volume of double-concentrated PBS. After 30 min, it was added to the cells (1.25 μg of plasmid per well), and incubated for 4 h at 37 °C. Five hundred microliters of fresh medium was then added to the wells. After an additional 20-h incubation at 37 °C, the cells were lysed, and transgene expression and protein content in the lysate were assessed with the corresponding assay kit.

The amount of DNA complex that remained in the supernatant medium after the transfection period was measured as follows; DNA was pre-labeled by YOYO-1 (YOYO/DNA = 1/5 in mole on the basis of nucleotide) prior to the transfection. After transfection, the supernatant in each well was collected, and the same volume of 30% NaCl was added to it. The DNA complex was completely dissociated after incubation for 3 days at 37 °C, and concentration of the released DNA was determined by the fluorescence intensity at 510 nm (Ex = 490 nm).

3. Results and discussion

3.1. Fluorescence microscopic observation of DNA/PEI/HA ternary complex

The fluorescence microscopic observation was carried out to confirm the formation of ternary complex by mixing three components of pDNA, PEI, and HA. The fluorescence microscopic images of the complex observed by exciting light of specific length corresponding to each component, DAPI in DNA, FITC-PEI, or Texas Red-HA, are shown in Fig. 1. The brightened dots were observed at the same positions in all the pictures, confirming that DNA/PEI/HA ternary complexes were certainly formed.

3.2. Analysis of surface structure of the DNA complexes

ζ-Potentials of HA was −74 mV. The initial surface charge of the DNA/PEI binary complex was +29 mV, and as adding HA, it decreased relative to the amount of HA. A small amount of HA could effectively recharge the DNA/PEI complex, and addition of HA at

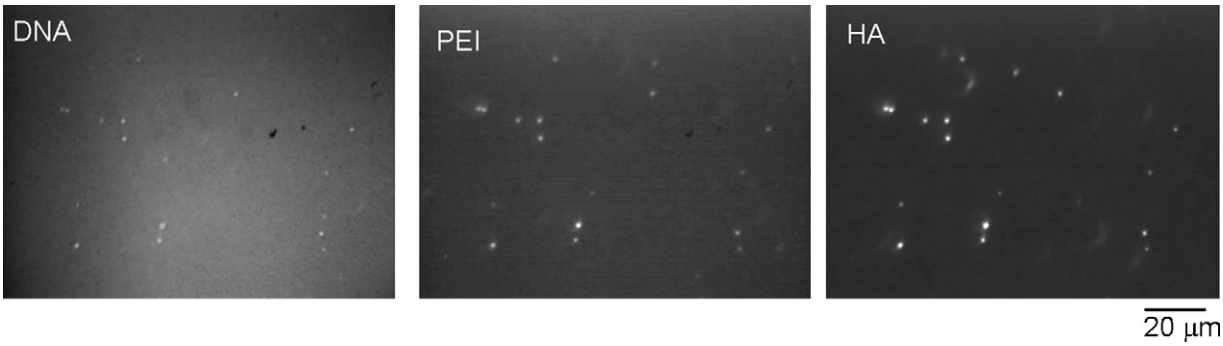

Fig. 1. Fluorescence images of the DNA/PEI/HA ternary complexes excited by the light for (left) DAPI in DNA, (center) FITC-PEI, (right) Texas Red-HA (final concentrations of DNA, DAPI, FITC-PEI, TR-HA, were 10 μM, 6.25 μM, 120 μM, and 120 μM, respectively).

the ratio of COOH/NH > 0.1 (in charge) resulted in a complex with negative surface charge (Fig. 3a).

Fig. 2 shows the Raman spectrum of the DNA, PEI, HA and their mixtures. The intensity ratio of the peaks at 1458 cm^{-1} and 1127 cm^{-1} varied with the mixing ratio of PEI and HA. The peak at 1458 cm^{-1} is attributed to the CH_2 in PEI, and that at 1127 cm^{-1} is to the COC in HA. The relative intensity of the peaks at 1458 cm^{-1} and 1127 cm^{-1} in the Raman spectrum of the DNA complexes normalized by the whole peak area plotted in Fig. 3b and c. The horizontal axis of the graph represents the COOH/NH ratio. Increase in the amount of HA added to the DNA/PEI complex resulted in a decrease in the relative intensity of the peak at 1458 cm^{-1}. It is known that the cationic charge in polyamine molecules such as ethylenediamine delocalized more than one amino atom [21]. The same is expected for the diamine structures in the PEI molecule. The peak at 1458 cm^{-1} would be assigned to the CH_2 groups between the partially protonated ammonium ions in PEI, and would be diminished by the higher protonation level induced by multi-anions of HA. The intensity of the peak at 1458 cm^{-1} plateaued at COOH/NH = 0.5, where all the amines seem to be protonated (Fig. 4).

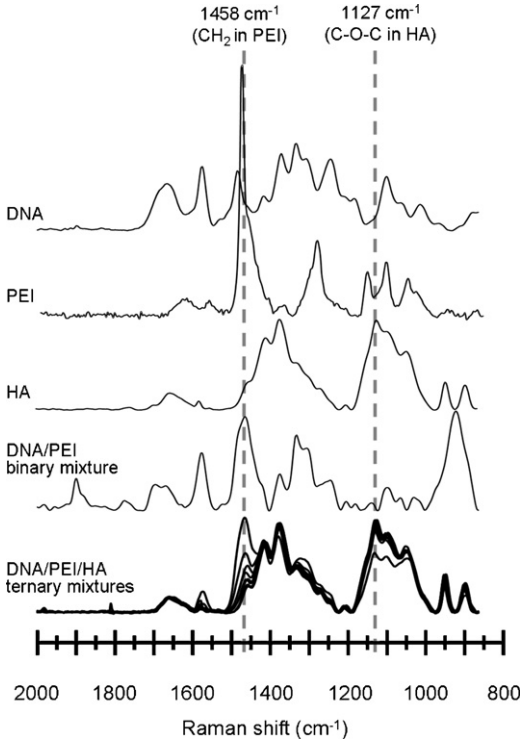

Fig. 2. Raman spectra of the DNA, PEI, HA, and their complexes.

The intensity of the peak at 1127 cm^{-1} from HA ether stretching enhanced with the amount of HA, and plateaued at the same point (half equivalent to the cation). The spectra beyond this point were almost the same as that of HA itself, probably due to the full coverage of the DNA/PEI complex surface with HA at those mixing ratios. However, the more HA was added, the more the ζ-potential decreased beyond this point, and was saturated to −75 mV

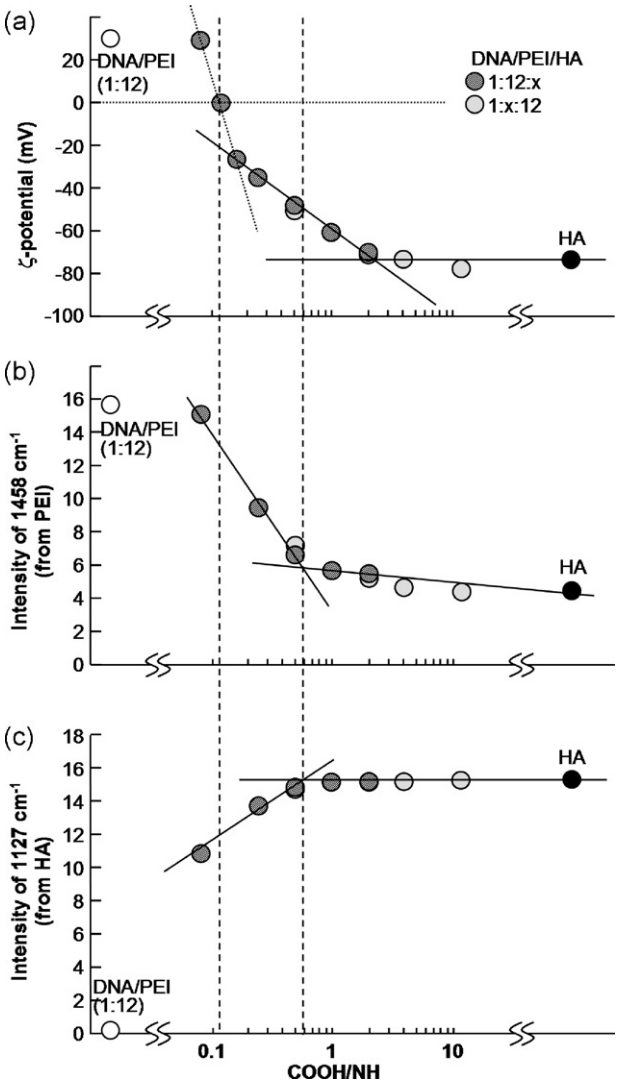

Fig. 3. (a) ζ-Potential of the DNA complex, and (b, c) intensity of the peaks at 1458 cm^{-1} and 1127 cm^{-1} in the Raman spectrum of the complexes.

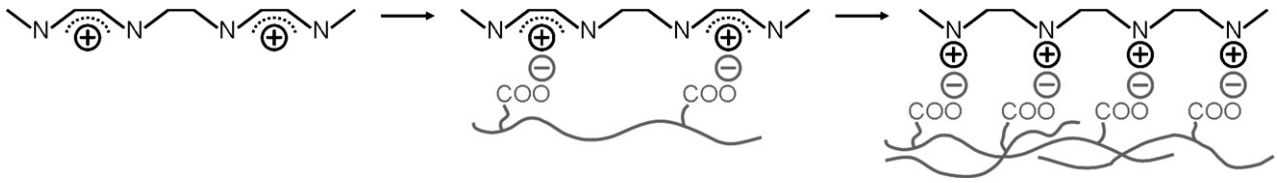

Fig. 4. Protonation of the amino atoms in PEI induced by anions of HA.

Fig. 5. Surface structure of the ternary complex coated by HA.

at HA/PEI ratio = 2 (in charge). The complex would have the outer corona of HA segment loops around the particle between HA/PEI ratio of 0.5 and 2 (Fig. 5).

3.3. Correlation between the gene-expression efficiency and surface structure of the ternary DNA complexes

The gene-expression efficiency of the DNA complexes (pDNA/PEI/HA = 1:*x*:12 and 1:12:*x* (*x* = 1–24) (in charge)) was examined with cultured B16 cells. DNA/PEI/HA complexes prepared at 1:1:12, 1:3:12, and 1:6:12 showed no reporter gene expression. In order to evaluate the amount of the DNA complexes that precipitated and had contact with the cells, the amount of DNA still suspended in the supernatant medium after the transfection was investigated. As shown in Fig. 6, at those ratio, most complexes remained in the medium, not attaching to the cells. On the other hand, all the DNA/PEI/HA complex prepared at 1:24:12 had precipitated, and were not present in medium. This could be the reason for the very high gene-expression efficiency of the complex. Although most binary complexes also precipitated during the transfection, the gene-expression efficiency of the DNA/PEI binary complex was lower. This would be owing to the low efficiency in the cell uptake of the aggregated binary complex or its low transcription activity [11,22].

Among DNA/PEI/HA complexes prepared at 1:12:*x*, that prepared at DNA:PEI:HA = 1:12:6 (in charge) showed the highest gene-expression efficiency. At this mixing ratio, all the amino groups were just protonated, and the surface was completely covered by HA, as mentioned above. The complex would thus not interact with serum in medium because all the amino groups have already interacted with carboxylate anions of HA, and would be taken up to the cells by the ligand effect of HA. However, it seems that DNA complex with further HA could not easily adhere to the cells by strong electrical repulsion.

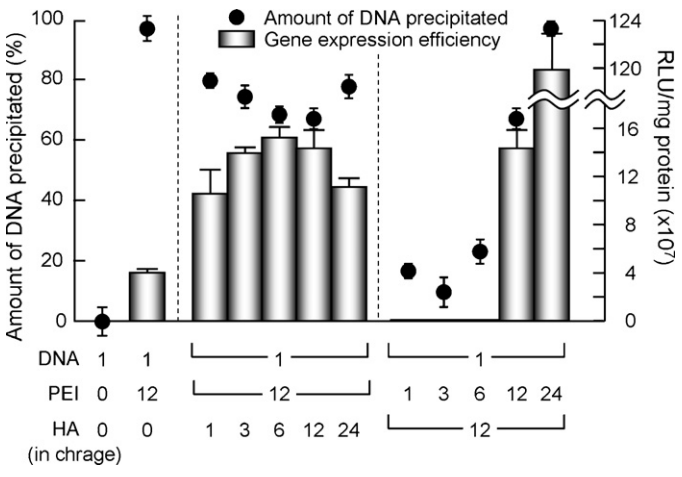

Fig. 6. *In vitro* transfection efficiency of the DNA complexes and amount of DNA precipitated during the transfection.

4. Conclusion

The surface structures of DNA/PEI/HA ternary complexes were analyzed by a non-confocal Raman microscopy. HA addition induced a higher level of protonation of the amino groups of PEI, and all the amines were protonated by the addition of the half equivalent of carboxyl groups. At this composition ratio, HA molecules completely covered the surface of the DNA/PEI complex, and the resulting ternary complex showed the highest gene-expression efficiency.

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