Hyaluronic Acid and its Derivative as a Multi-functional Gene Expression Enhancer: Protection from Non-specific Interactions, Adhesion to Targeted Cells, and Transcriptional Activation

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Abstract

Hyaluronic acid (HA), a natural anionic mucopolysaccharide, can be deposited onto the cationic surface of DNA/polyethyleneimine (PEI) complexes to recharge the surface potential and reduce nonspecific interactions with proteins. HA can also be used as a ligand to target specific cell receptors. Furthermore, HA-coating enhanced the transcriptional activity of the plasmid/PEI complexes, probably by loosening the tight binding between DNA and PEI, which facilitated the approach of transcription factors. Amphoteric HA derivative having spermine side chains (Spn-HA) with a structure similar to HMG protein showed higher transcription-enhancing activity than HA. Plasmid/PEI/Spn-HA ternary complex exhibited 29-fold higher transgene expression efficiency than naked plasmid/PEI complexes in CHO cells.

Keywords: Transfection; Hyaluronic acid; Transcription; HMG

1. Introduction

A variety of cationic polymers or lipids, which can electrostatically bind to DNA,

have been explored as synthetic non-viral vectors [1,2]. However, the surface of the DNA/polycation complex is usually positively charged. The activation of complements [3], coagulation of blood cells [4], and self-aggregation with serum proteins [5] have been major problems, especially for *in vivo* gene therapy. The positive charge on complexes should be shielded to obtain stable dispersion in blood. Ogris et al. reported that DNA complexes coated and shielded with a neutral polymer having active ester side chains showed enhanced systemic duration [6] and higher gene expression in the tumor [7]. We have developed a novel poly(ethylene glycol) derivative with carboxylic acid side chains (PEG-C) as a self-assembling protective coat on DNA/polycation complexes [5], which can reduce nonspecific interactions with blood cells or serum proteins. Introduction of a ligand to PEG-C can also increase higher transgene expression in target cells [4,8].

PEG-C can be deposited onto the surface of plasmid/polycation complexes to form ternary structures without destroying them, while common polyanions such as heparin and polyacrylic acid will disrupt DNA/polycation condensation [8-10]. We have explored various kinds of anionic polymers in the search for other polyanions which can form ternary complexes, and found that natural hyaluronic acid (HA) is one of the few polyanions which can coat DNA/polycation complexes without disrupting their structures.

HA is an acidic mucopolysaccharide distributed widely in the extracellular matrix and found in the liquid portion of mammalian joints. It is a relatively low toxic polymer approved by the FDA for injections. Moreover, a receptor for HA, CD44, is known to be overexpressed on various tumor cell surfaces [11,12]. HA would thus work both as a protecting coat against blood components and as a ligand for targeted cells.

For efficient *in vivo* gene transfection, non-viral vectors need to overcome many barriers such as internalization into cells, escape from endosomes and trafficking into the nucleus. Low efficiency in transcription of DNA/polycation complexes in the nucleus also represents an obstacle for high gene expression. The polycation may interfere with transcription, since access of transcription factors to a DNA molecule can be hindered when DNA tightly binds to the polymer. As mentioned above, addition of HA to the DNA/polycation complexes will not decompose them nor release free DNA molecule. However, HA might loosen the DNA-polycation binding to some extent by interacting with the polycation, and the approach of transcription-factors to the swollen complex would be facilitated. In this study, we examined the possibility of HA for loosening DNA/polycation complexes and its effect on enhancing transcriptional activity.

In the mammalian cell nucleus, tightly condensed chromosomal DNA is uncoiled by an HMG (High Mobility Group) protein before transcription begins. HMG protein is a non-histone DNA-binding protein having both cationic DNA-binding domains and an anionic c-tail. The acidic region has been reported to be essential for transcriptional activation [13]. A specific interaction between the acidic region of HMGB1 and histone was reported [14], but such ampholytes having both basic and acidic groups may also have non-specific physical properties for loosening the tight interaction between DNA and cationic polymers. In order to examine the transcriptional activation potential of polyampholytes, an amphoteric HA derivative, Spn-HA, was synthesized to mimic the structure of HMG protein and investigated for its transcription-enhancing activity potential.

2. Materials and method

2.1. Materials

Linear PEI (MW 25,000) was obtained from Polyscience, Inc. Hyaluronic acid sodium salt (from Microorganism), spermine, and bovine serum albumin (BSA) were purchased from Nacalai Tesque, Inc., Wako Pure Chemical Industries, Ltd., and SIGMA Chemical Co, respectively. YOYO-1 iodide and uridine 5'-triphosphate P^3-(5-sulfo-1-naphthylamide) tetra(triethylammonium) salt (γ-AmNS-UTP) were purchased from Molecular Probes, Inc. Plasmid DNA containing firefly luciferase gene and cytomegavirus promoter was amplified in *Escherichia coli*, and purified with a QIAGEN Plasmid Mega Kit. The *E. coli* RNA polymerase holoenzyme was purchased from EPICENTRE Technologies. ATP, CTP and GTP were obtained as lithium salts from Roche Diagnostics, Co.

2.2. Synthesis of Spn-HA

HA sodium salt (10 mg) was dissolved in 4 mL of water and mixed with spermine (50.9 mg). Water-soluble carbodiimide (WSC) (77.1 mg) was then added to the solution, which was adjusted to pH 4.9. After standing at RT for 5 h, the solution was dialyzed for 4 days against running water and 3 more days in distilled deionized water. The solution was freeze-dried and a white spongy Spn-HA was obtained, with a yield of 10.7 mg. Spn-HA tested positive with the ninhydrin test, while a mixture of HA and spermine

similarly dialyzed gave a negative result. Elemental analysis revealed that 27.4% of carboxyl groups had reacted with spermine. This was in good accordance with ^{1}H-NMR of the product taken in D_2O. A 27.3% value was estimated from the integration of the resonance peaks at 1.1 ppm (-N-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-N- in spermine residue) and 1.9 ppm (-NCOC$\underline{H}_3$). IR spectrum of the product showed more absorption at 1650 cm^{-1} arising from the amide linkage compared to the original HA.

2.3. Fluorescence microscopic observation

Fluorescence microscopic observation was performed using an IX70 microscope (Olympus) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera. DNA was visualized with the fluorescent dye, 4',6-diamidino-2-phenyl-indole (DAPI). Mercaptoethanol was added as an antioxidant. Final concentrations of DNA, DAPI and mercaptoethanol were 3 μM (in base), 3 μM, and 3% (v/v), respectively. Bovine serum albumin (BSA) was added to the DNA complex suspension as a 20 mg/mL aqueous solution (final concentration of 10 mg/mL).

2.4. Distribution of DNA complex in the mouse peritoneal cavity

The fluorescent dye YOYO-1 was used instead of DAPI to visualize DNA. DNA/PEI/HA (or Spn-HA) complex was prepared at 1:1:9.7 (w/w/w) in saline. One hundred μL was injected into the peritoneal cavity of each mouse. The final concentration of YOYO-1 and DNA in the injected solution was 50 μM and 250 μM, respectively. After 30 min, the mice were sacrificed by ether asphyxiation and the abdominal cavity was exposed through a midline incision. The internal organs were illuminated with a UV lamp to observe DNA distribution in tissues.

2.5. ζ-potential and size measurement

ζ-potential and size of the complexes were measured with a particle analyzer (MALVERN Zetasizer Nano ZS). Typically, to an aqueous solution of plasmid DNA (1.25 μg in 12.5 μL) was added an equal volume of linear PEI (1.25 μg in 12.5 μL in quadruple condensed PBS) followed by addition of HA (12.25 μg in 25 μL in H_2O), in this order. The mixture was then diluted with 950 μL of PBS and its ζ-potential and diameter were analyzed.

2.6. Transcription procedure

The complex suspension was prepared by mixing the aqueous solutions of plasmid (0.33 μg in 3.3 μL), linear PEI (0.33 μg in 3.3 μL), HA or Spn-HA (1.6-6.5 μg in 6.6 μL), and 9.95 μL of water. A mixture of 100 mM Tris-HCl (pH7.5; 5 μl), 500 mM KCl (10 μL), 1 M MgCl (0.25 μL), 10 mM ATP, CTP, and GTP (each 0.5 μL), 5 mM γ-AmNS-UTP (0.1 μL) and 100 mM DTT (1 μL) was then added to the suspension. After incubation for 20 min at 37 °C, *E. coli* RNA polymerase (0.1 U/μL in 10 μL) was added and the samples were kept at 37 °C for a set time. The reaction was quenched by adding 50 mM EDTA (1 mL). The amount of RNA transcripts produced was estimated by monitoring the increase in fluorescence intensity at 465 nm (Ex = 330 nm) from the AmNS-pyrophosphoric acid byproduct.

2.7. Evaluation of degree of DNA complex-loosening

The degree of DNA condensation in the complex was evaluated by measuring the fluorescence intensity of YOYO-1 probe intercalated to DNA complexes. A mixture of YOYO-1 (240 μM in 12.5 μL H_2O), 12.5 μL of 50% 2-mercaptoethanol (ME), and 12.5 μL of quadruple condensed PBS were added to an aqueous solution of plasmid DNA (5 μg in 12.5 μL H_2O), for a final concentration of 1212 μM in base. The solution was diluted with 800 μL of PBS, and the fluorescence intensity of the solution was measured with a JASCO Spectrofluorometer FP-777 at 512 nm (Ex = 492 nm). PEI (5.2 μg in 50 μL PBS) was then added to the plasmid solution at a 1:1 weight ratio (N/P = 8) of DNA to PEI, and the fluorescence intensity was again measured. After 2 min, HA or Spn-HA (49 μg in 100 μL PBS) was added, and recovery of the fluorescence intensity in the suspension was monitored. The mixed solution of plasmid/YOYO-1 and HA (or Spn-HA) without PEI was also measured to examine the influence of HA (or Spn-HA) on the fluorescence intensity.

2.8. Transfection procedure

The ternary plasmid complex suspension for transfection was prepared as follows: plasmid (1.25 μg in 12.5 μL in pure water) was mixed with linear PEI (1.25 μg in 12.5 μL in quadruple condensed PBS). HA or Spn-HA solution (6.12-24.3 μg in 25 μL H_2O) was diluted with 200 μL of F12 media and added to the plasmid/PEI suspension. CHO cells, a Chinese hamster ovary cell line, were seeded onto 24-well plates at 2.5×10^4 cells

per well and propagated for 2 days in F12 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. The primary growth medium was removed and replaced with 250 μL of F12 media supplemented with 20% FBS and 2% penicillin. The plasmid complex suspension (250 μL) was then added to the wells and allowed to incubate for 3 hours at 37 °C. The transfection medium was removed and replaced by fresh media. After 40 hours at 37 °C, transgene expression was assessed with a luciferase assay kit (Pica Gene) and protein content in the lysate was analyzed with a protein assay kit from Bio-Rad.

3. Results and discussion

3.1. ζ-potential of the plasmid complexes

ζ-potential of the plasmid/PEI binary complexes prepared at 1:0.65 or 1:1 (w/w) (N/P=5 or 8, respectively) gave potentials of +5.7 mV and +16.3 mV respectively, indicating that the particles were covered with excess PEI molecules. Addition of HA at a charge equivalent to PEI effectively modified the surface potential of the complexes. Plasmid/PEI/HA complexes prepared at 1:5:5 and 1:8:8 in equivalent charges (1:0.65:6.1 or 1:1:9.8, w/w/w) gave ζ-potentials of -18.5 and -18.6 mV respectively. This indicates that HA was deposited onto the plasmid/PEI complexes, leading to formation of plasmid/PEI/HA ternary complexes with net negative surface charges.

Spn-HA has similar numbers of amino to carboxyl groups, but it was expected that all the amino groups would not be protonated due to neighboring effects. The net charge on Spn-HA should therefore be anionic and was expected to potentially interact with the cationic surface of DNA/PEI binary complexes. Addition of Spn-HA prepared at plasmid:PEI:Spn-HA=1:1:10 (w/w/w) gave a negative ζ-potential of -6.1 mV. Spn-HA appears to electrostatically associate with excess cationic polymer on the complexes, imparting an anionic coat onto these particles.

3.2. Protection effect of HA and Spn-HA against BSA-induced aggregation

Anionic coating of the particles with HA or Spn-HA appears to protect the DNA/PEI complexes from undesirable interactions with serum proteins. Dispersion stability of the complexes prepared at plasmid:PEI:HA=1:1:9.7 (w/w/w) in the presence of albumin was examined by fluorescence microscopic observation. DNA was visualized with

DAPI and mixed with PEI. Globular DNA/PEI binary complexes (1:1, w/w) were observed as small fluctuating particles. Addition of HA to the solution did not cause visible changes in particle size or movement. When BSA was added to the plasmid/PEI binary complexes (final concentration of 10 mg/mL), the complexes aggregated and precipitated. On the other hand, the ternary complexes coated with HA or Spn-HA retained Brownian motion up to one-hour post-addition of BSA (Fig. 2).

Change in complex size before and after BSA addition was measured with a particle analyzer. The size of DNA/PEI binary complexes in PBS was approximately 230 nm. HA addition increased the diameter to 350 nm, most likely due to cross-linking of the complexes by this large polysaccharide. Spn-HA addition did not affect complex size, perhaps due to the small exclusion volume of this polyampholyte. BSA was added (final concentration of 10 mg/mL) to the DNA/PEI binary complexes and the measured diameters increased up to 500 nm. Aggregation gradually proceeded and within 30 min reached sizes of 600-900 nm (Fig. 3). When BSA was added to ternary complexes coated with HA or Spn-HA, they did not show apparent increases in size under similar conditions. This confirms that particle coating with HA or Spn-HA can minimize interactions with serum proteins.

3.3. Distribution in the peritoneal cavity.

Since DNA/PEI complexes coated with HA did not interact with serum proteins, they were expected to diffuse to target organs without aggregating or precipitating in body fluids. Fluorescently labeled DNA/PEI/HA ternary complexes (1:1:9.7, w/w/w) were injected into the peritoneal cavity of mice (ddY, male, 6-7 w) and their diffusing behavior in the cavity was examined. Since the binding of DAPI to DNA is reversible, a stable intercalating fluorescent dye YOYO-1 was used to visualize the DNA complex. Large amounts of YOYO-1 can extend the persistent length of the DNA molecules. YOYO-1 was therefore added at a ratio of YOYO-1/DNA = 1/5 or less (molar ratio based on nucleotides) to minimize effects on complex formation. DNA from salmon sperm was used for the experiment. The labeled DNA/PEI complexes with or without HA in 5% glucose (100 μL) were injected into the peritoneal cavity of mice (8.25 μg DNA/mouse). After 30 min, DNA/PEI/HA ternary complexes spread throughout the cavity whereas binary complexes without HA were retained in the injected area. Inertness of the ternary complexes coated with HA enhanced their diffusion and penetration through the intestines. This enhanced diffusion of HA-coated complexes

could be favorable for the delivery of DNA complexes to target diseased organs in gene therapy.

3.4. Transcription study

HA was expected to loosen the plasmid/polycation complex through electrostatic interactions with the polymer, leading to an overall improvement in transcription factor access to the DNA. This was investigated with an *in vitro* system without using cells. Ternary complexes of plasmid:PEI:HA=1:1:4.9-19 (w/w/w) (or 1:8:4-16 in terms of charge) were prepared. Nucleotide triphosphates and RNA polymerase (from *E. coli*) were then added. Following incubation at 37°C for 90 min, the transcription reaction was stopped by addition of EDTA and the fluorescence intensity was measured. As shown in Fig. 4, plasmid/PEI/HA induced higher production of AmNS-pyrophosphoric acid compared to plasmid/PEI binary complexes. This indicates that HA can enhance the transcription of the plasmid.

The behavior of HA resembles that of HMG proteins, which can uncoil tightly compacted chromosomal DNA to facilitate its association with transcription factors [13]. An amphoteric derivative of HA was thus synthesized to mimic the HMG protein structure by grafting spermine onto HA with a water-soluble carbodiimide. Excess spermine was used to prevent cross-linking and the reaction was discontinued after 5 hours to yield water-soluble amphoteric HA derivatives (Spn-HA) having both amino- and carboxylic groups. The effect of Spn-HA on the transcription activity was then investigated simultaneously with HA and results are shown in Fig. 4. Spn-HA, as expected, had a superior enhancing effect to HA on the transcriptional activity. Both HA and Spn-HA displayed maximal transcriptional activation at a weight ratio of PEI:HA (or Spn-HA) = 1:10. The transcriptional rate of these ternary complexes was then measured and compared with those of plasmid/PEI binary complex (Fig. 5). The complex with HA or Spn-HA prepared at plasmid:PEI:HA (or Spn-HA) = 1:1:9.7 (w/w/w) showed 6.0- or 8.3-fold higher transcription rate, respectively, than those with plasmid/PEI binary complexes. Neither HA nor Spn-HA had any effect on the transcription efficiency of naked plasmid without PEI. These results suggest that amphoteric HA derivatives could possess HMG-like transcriptional enhancing properties.

3.5. Loosening behavior of the plasmid/PEI complex

DNA complex-loosening by HA and Spn-HA was evaluated by measuring the fluorescence intensity of the YOYO-intercalated DNA molecules. When YOYO-1 intercalates with DNA, it fluoresces with more intensity than when free. If the DNA is compacted in a globular state, its fluorescence intensity would be much lower than in the random coiled state. When the DNA complex becomes more loose due to presence of HA or Spn-HA, the fluorescence intensity of any intercalated YOYO-1 in the complex should cause a recovery. Therefore, change in the fluorescence intensity of YOYO-1-labeled DNA complexes before and after the addition of HA or Spn-HA was measured. YOYO-1-labeled naked plasmid yielded high fluorescence. This value decreased down to 61% of its original value upon addition of PEI (1:1, w/w). Addition of HA recovered the fluorescence to 68%. Spn-HA also recovered the fluorescence but to a smaller degree (Fig. 6). These results reveal that both HA and Spn-HA can loosen the plasmid/PEI complex to some extent, although they could not completely cause the complexes to fall apart. Recovery of the fluorescence intensity by Spn-HA was not as high as expected based on the transcription results. The influence of Spn-HA itself on the fluorescence intensity was examined. Spn-HA without PEI reduced the fluorescence intensity of YOYO-1/DNA to 80%, whereas HA addition did not have any effects. Since Spn-HA has a quenching effect, this amphoteric polysaccharide seems to effectively loosen the DNA/PEI complex, leading to overall higher transcription efficiency.

3.6. Transfection mediated by the DNA/PEI/HA (or Spn-HA) ternary complexes

Ternary complexes coated with HA or Spn-HA can easily disperse in serum-containing medium, favorably bind to CD44 positive-cells and can loosen DNA/PEI interactions favorable for enhanced transcription. Transfection efficiency of the HA- or Spn-HA-coated DNA/PEI complexes on CD44-expressing CHO cells was examined. Fig. 7 shows the results for plasmid/PEI/HA and plasmid/PEI/Spn-HA ternary complexes. Based on the total protein content, cell toxicity was not observed in either case. HA enhanced the transgene expression with a 6.4-fold higher luciferase activity than plasmid/PEI binary complexes. As was expected from the transcription study, Spn-HA had higher transcription enhancing effect than HA, and the ternary complex prepared at plasmid/PEI/Spn-HA=1:1:9.7 (w/w/w) (P:N:COOH=1:8:3.6), had 29-fold higher gene expression than the plasmid/PEI binary complex. This superior enhancing effect of Spn-HA could be due to its HMG-like transcriptional activation properties.

HA-receptors (CD44) present on CHO cells improve uptake of HA-coated ternary

complexes and lead to higher gene expression. This was examined by pre-addition of excess HA to cells prior to incubation with the plasmid complex. The complex was prepared with relatively small amount of HA (plasmid:PEI:HA=1:0.71:4.9 (w/w/w), (P:N:COOH=1:5:4)) in order to minimize un-complexed HA in the medium. Before the DNA complex was added, 200 μl of HA solution (2.4 mg/mL in PBS) was added to CHO cells to which 1 ml of F12 medium was added in advance. After incubation at 37°C for 90 minutes, the DNA/PEI/HA complexes were added to the wells, and the cells were treated and assayed as previously mentioned in the transfection study. The CHO cells pretreated by HA had diminished gene expression (less than 1/2 of the control cells treated with PBS alone) (Fig. 8). This reveals that the highly enhanced luciferase expression of HA-coated ternary complexes was also mediated by particle uptake through HA-specific receptors present on the surface of CHO cells.

4. Conclusion

HA can be deposited onto the cationic surface of DNA/polyethyleneimine (PEI) complexes via electrostatic means, minimize nonspecific interactions with serum proteins, act as a ligand to specific cells and also behave like a transcriptional activator. An amphoteric HA derivative having spermine side chains (Spn-HA) exhibited higher transcription-enhancing activity and gene expression in cultured cells than HA. Water-soluble polyampholytes such as the one investigated having multifunctional properties can improve gene transfection and simultaneously also serve as artificial HMG protein models.

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Legends to figures

Fig. 1. Synthesis of Spermine-HA conjugate (Spn-HA)

Fig. 2. Fluorescence images of the plasmid/PEI binary complex (1:1 in weight) and plasmid/PEI/HA (or SPN-HA) ternary complexes (1:1:9.7 in weight) before and after the addition of albumin. Final concentration of BSA = 10 mg/ml.

Fig. 3. Averaged diameter of the complexes

Fig. 4. Relative fluorescence intensity of the AmNS-UTP solutions after the incubation for 90 min with RNA polymerase and plasmid complexes (n=3).

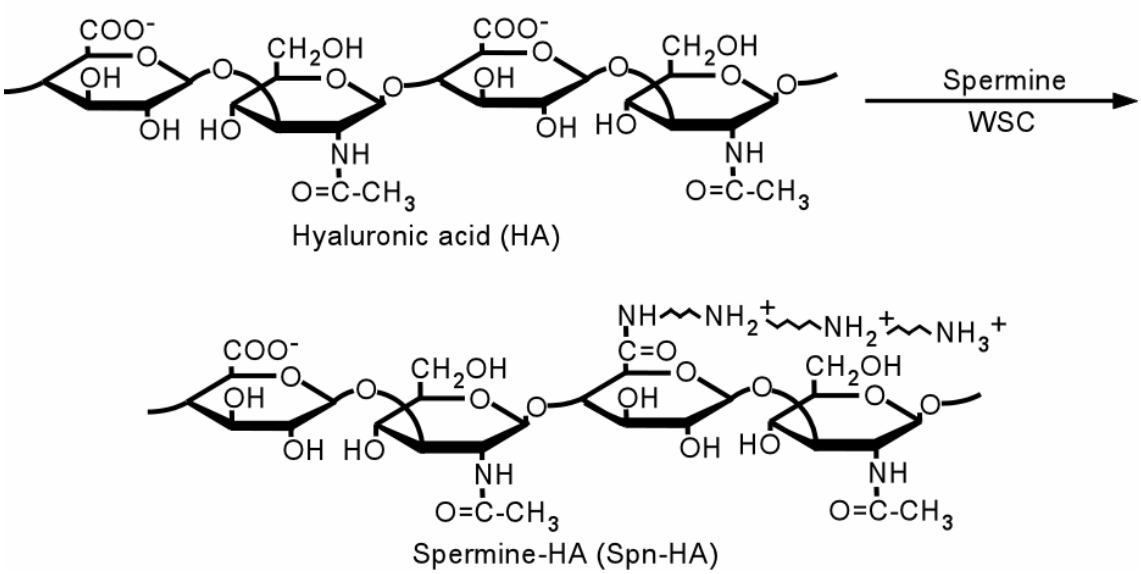
Fig. 5. Transcriptional activity of the plasmid complexes; ▲: Plasmid/PEI, ○: Plasmid/PEI/HA, ●: Plasmid/PEI/Spn-HA (n=3).

Fig. 6. Fluorescence recovery of the YOYO-1 in the plasmid/PEI complexes by □: HA, ○: Spn-HA, Δ: control.

Fig. 7. Transgene expression efficiency of HA- or Spn-HA-coated plasmid/PEI complexes on CHO cells (n=3).

Fig. 8. Effect of HA-preaddition on the transgene expression on CHO cells mediated by plasmid/PEI/HA (1:0.71:4.9 in weight) (n=3).

Fig. 1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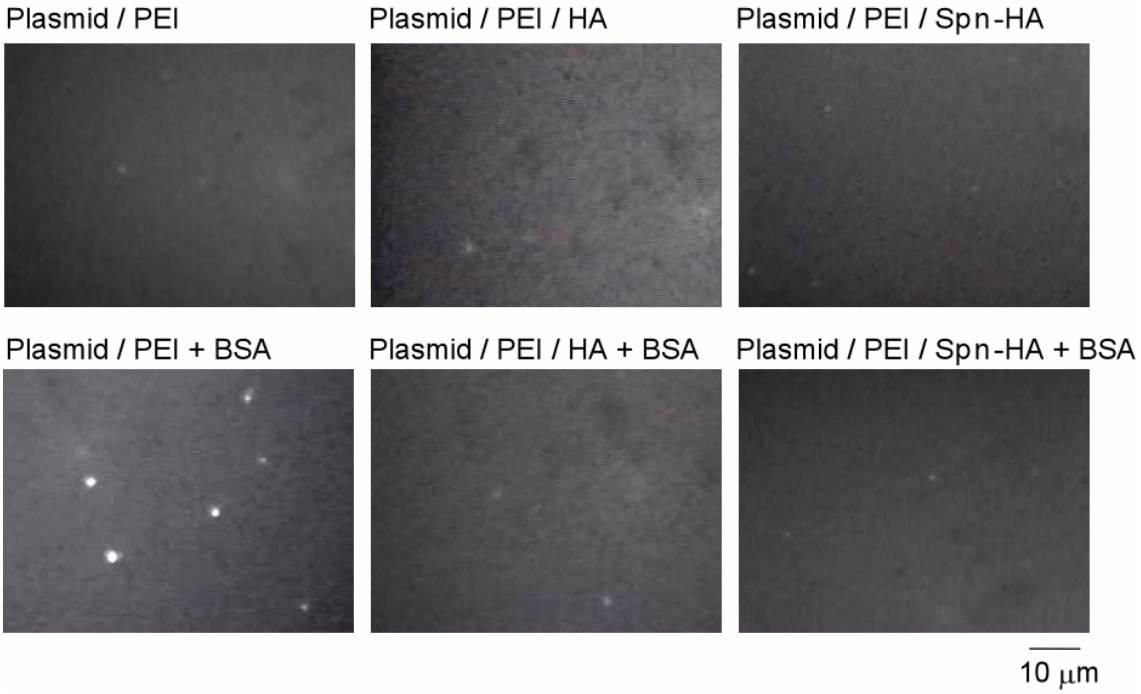
Hyaluronic acid (HA)
Spermine-HA (Spn-HA)

Fig. 2


Plasmid / PEI
Plasmid / PEI / HA
Plasmid / PEI / Spn-HA
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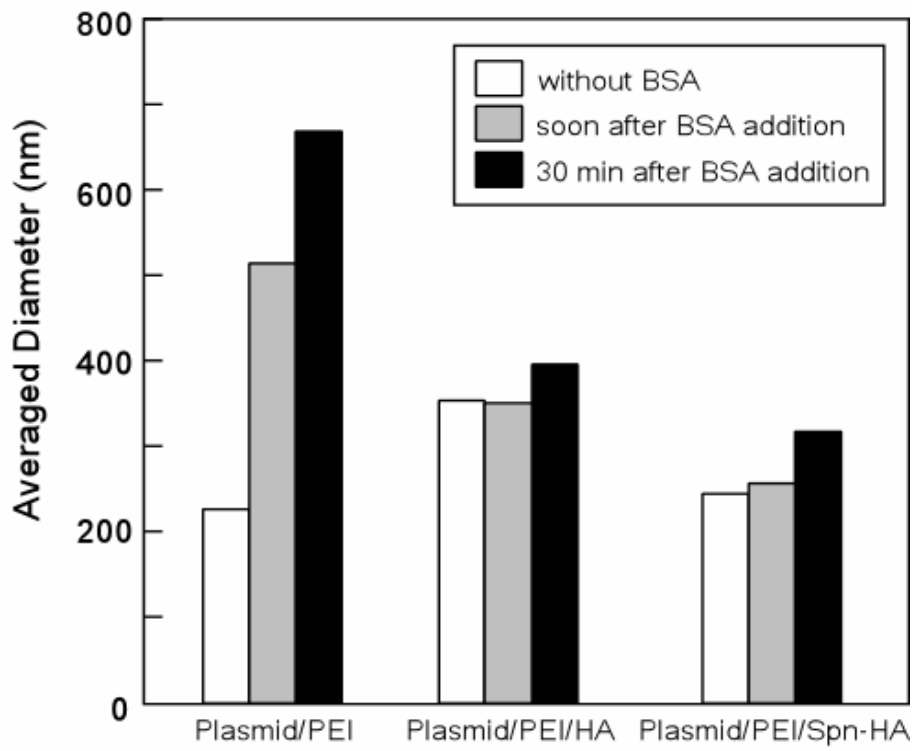
Plasmid / PEI / HA + BSA
Plasmid / PEI / Spn-HA + BSA
10 μm

Fig. 3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400
200
0
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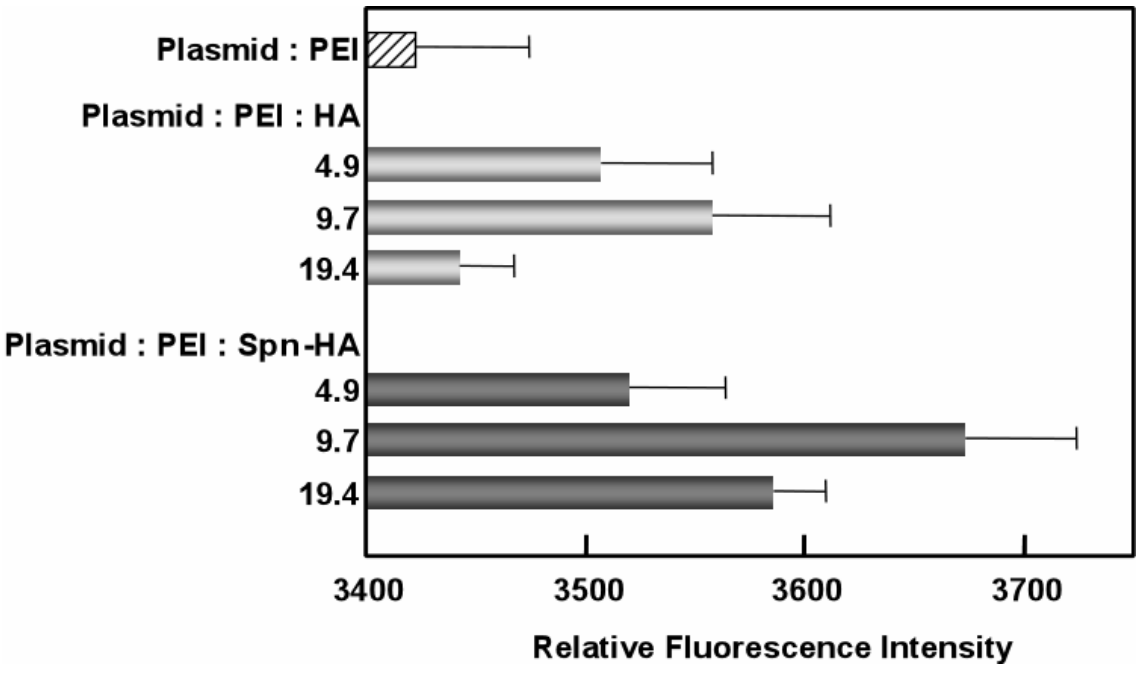
Plasmid/PEI/HA
Plasmid/PEI/Spn-HA

Fig. 4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Plasmid : PEI : HA
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9.7
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19.4
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3500
3600
3700
Relative Fluorescence Intensity

Fig. 5

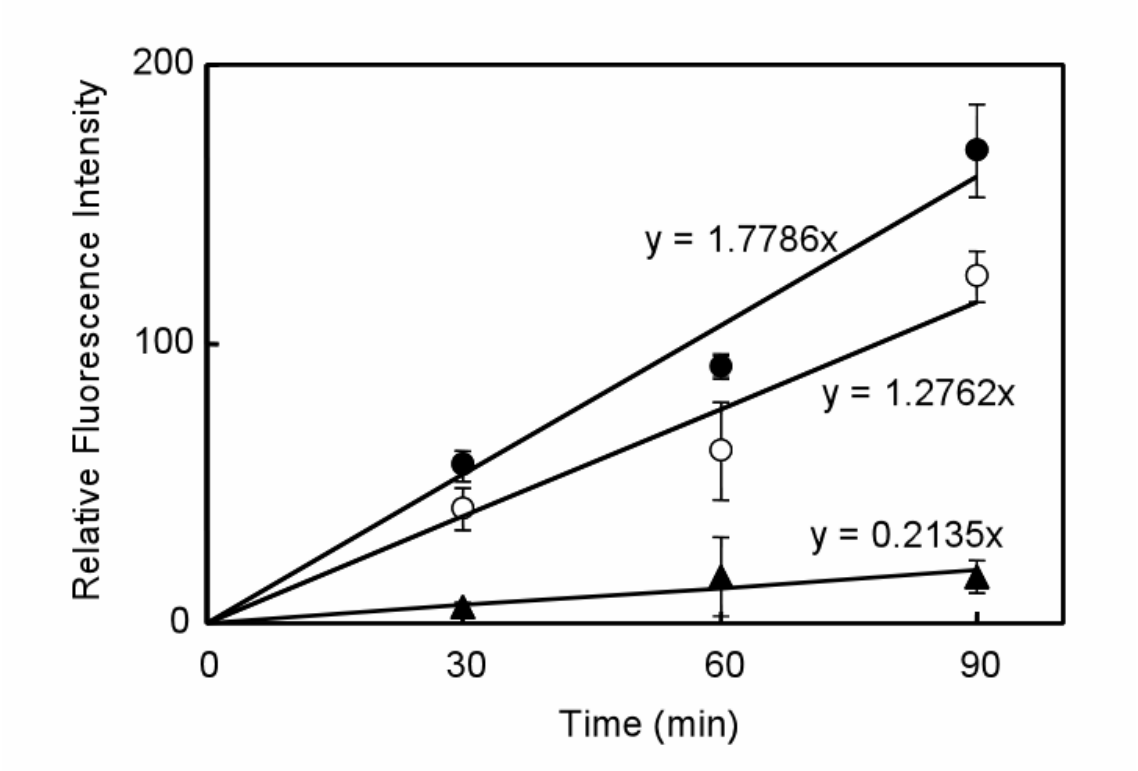
Relative Fluorescence Intensity
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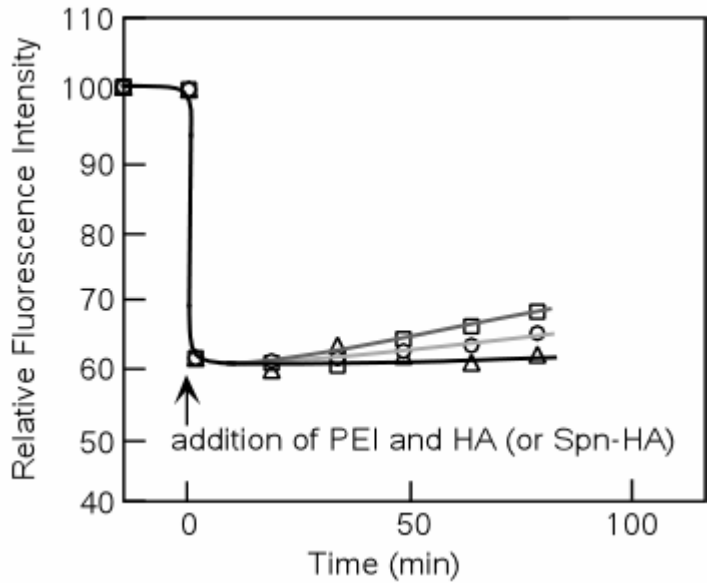
Fig. 6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100
90
80
70
60
50
40
0
50
100
Time (min)
addition of PEI and HA (or Spn-HA)

Fig. 7

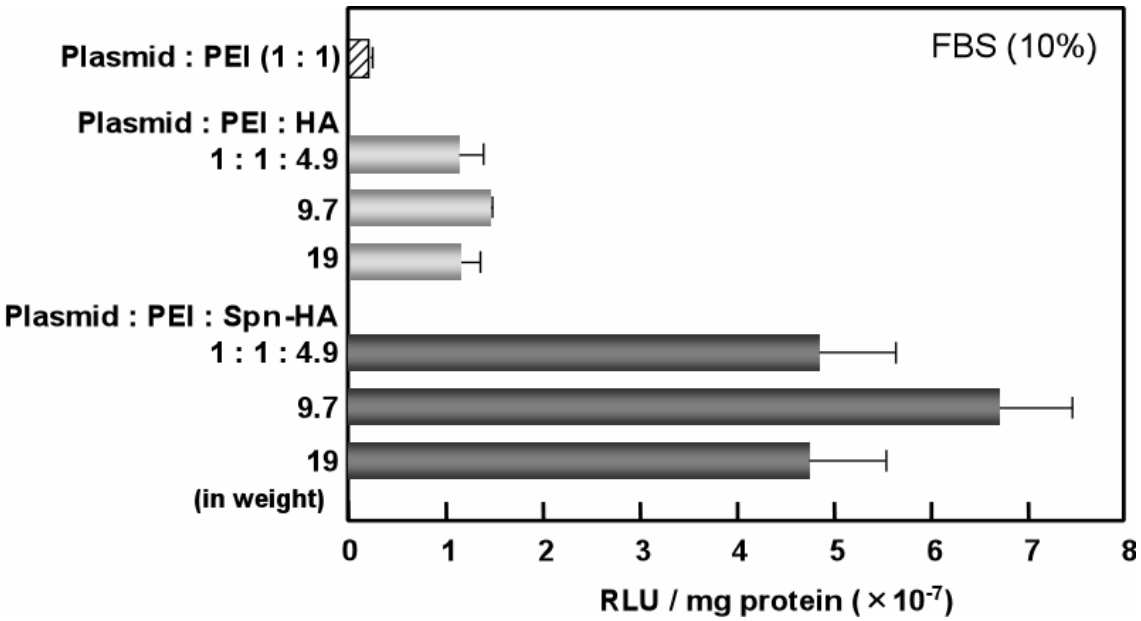
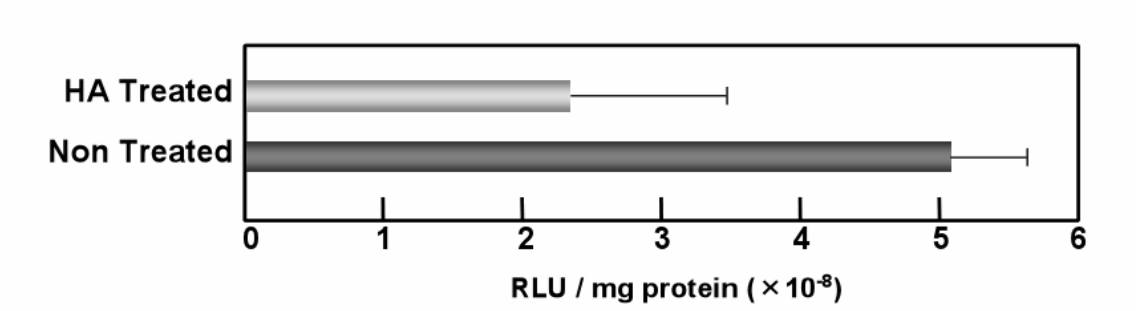

FBS (10%)
Plasmid : PEI (1 : 1)
Plasmid : PEI : HA
1 : 1 : 4.9
9.7
19
Plasmid : PEI : Spn-HA
1 : 1 : 4.9
9.7
19
(in weight)
0
1
2
3
4
5
6
7
8
RLU / mg protein (×10-7)

Fig. 8


HA Treated
Non Treated
0
1
2
3
4
5
6
RLU / mg protein (×10-8)