Guanidinylated Poly(allyl amine) as a Gene Carrier

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ABSTRACT: Guanidinylated poly(allyl amine) (GA) was synthesized and used as a gene carrier. The degree of guanidinylation in GA increased linearly when the feed ratio of guanidino groups in 1*H*-pyrazole-1-carboxamidine to amino groups in poly(allyl amine) (PA) was below 0.7, and the amino groups of poly(allyl amine) with a weight-average molecular weight of 15,000 (PA15) were almost replaced with guanidino groups when the ratio reached 2. GA showed good plasmid condensation and protection ability. Nanoparticles with a narrow size distribution, good dispersity, and spherical shape could be assembled between GA and DNA. With an increase in the N/P ratio [where N is the amount of nitrogen in the polycation (for GA, three nitrogens per guanidino group) and P is the amount of plasmid phosphate in the DNA as moles] or the degree of guanidinylation, the ζ-potential of GA/DNA nanoparticles increased, whereas the sizes of GA/DNA nanoparticles decreased sharply with increasing N/P ratios. Compared with polyethylenimine with a weight-average molecular weight of 25,000 and PA15, GA essentially showed decreased cytotoxicity to HeLa, 293T, and HepG2 cell lines, and guanidinylated PA15 exhibited the lowest cytotoxicity. Guanidinylation of PA enhanced its gene transfection. This enhancement was dependent on the degree of guanidinylation and the cell lines. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 112: 926–933, 2009

Key words: biomaterials; drug delivery systems; guanidinylation; poly(allyl amine)

INTRODUCTION

Because clinical safety is a major consideration in the application of gene therapy, the use of polymer-mediated nonviral gene vectors has recently emerged as a viable alternative to viral gene vectors because of their lesser immune reaction, ability to deliver high-molecular-weight DNA molecules, and lower production costs.[1] Commonly used polymer gene vectors include cationic polymers such as chitosan, polyethylenimine (PEI), poly(amido amine) dendrimer, poly(L-lysine), and polyphosphate.[2–6] PEI is one of the most efficient gene vectors because of its good buffering capacity below the physiological pH.[3] On the other hand, cationic polymers with only primary amino groups such as poly(L-lysine)and chitosan are inefficient gene vectors.[7–9] In our research group, rather than doing our best to synthesize some new types of cationic polymers as efficient gene vectors, we have taken the approach of studying whether appropriate chemical modification of a commercially available polymer can enhance its gene transfection efficiency and reduce its cytotoxicity. Therefore, chitosan has been modified with urocanic acid, mannose, galactose, and PEI.[9–13] The gene transfection efficiency of modified chitosan is much higher than that of chitosan, and modified chitosans have also shown low cytotoxicity.

Poly(allyl amine) (PA) is also a commercially available and inexpensive polycation. As a gene carrier, its low transfection efficiency and high toxicity limit its efficient applications. Boussif et al.[14] synthesized derivatives of PA by transamidification with methyl glycolate, which led to decreased toxicity and a drastic increase in their gene transfection efficiency. In comparison with amino groups, guanidino groups are highly basic ($pK_a = 12.5$) and can be fully protonated under the physiological pH; this leads to a highly positive charge density and strong condensation ability of DNA.[15] Guanidino groups can form

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bifurcated hydrogen bonds with nucleic acid phosphates, and this results in additional affinity with DNA.[16] Moreover, guanidine has been reported to form hydrogen bonds with the phospholipids present in the lipid bilayers and to play a great role in facilitating cellular uptake and protein transduction.[17–19] In this study, to enhance gene transfection efficiency and reduce cytotoxicity, amino groups of PAs with molecular weights of 15,000 (PA15) and 70,000 (PA70) were transformed into guanidino groups by the guanidinylation reaction between PA and 1*H*-pyrazole-1-carboxamidine hydrochloride (PC) under a weakly basic condition. The structure of the guanidinylated poly(-allyl amine) (GA) was confirmed by ^{1}H-NMR and ^{13}C-NMR. Also, the effect of the degree of guanidinylation on the biological performance of GA as a gene vector, including its plasmid condensation and protection ability, cytotoxicity, and transfection efficiency in HeLa (human cervix epithelial carcinoma cells), 293T (human kidney cells), and HepG2 (human hepatoblastoma cells) cell lines, was evaluated.

EXPERIMENTAL

Materials

PA15, PA70, PC, branched polyethylenimine with a weight-average molecular weight of 25,000 (PEI25K), agarose, ethidium bromide (EtBr), and calf thymus DNA were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The Cell Titer 96 Aqueous One solution cell proliferation kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)] was purchased from Promega (Madison, WI). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were acquired from Gibco BRL Life Technologies (Paris, France). The Luciferase Reporter 1000 assay system for *in vitro* transfection assay and the pGL3 control vector with the SV-40 promoter and enhancer encoding firefly (*Photinus pyralis*) luciferase were obtained from Promega. Plasmid pEGFP-N2, which has the early promoter of cytomegalovirus and the enhanced green fluorescent protein gene, was obtained from Clontech (Palo Alto, CA).

Preparation and characterization of GA

PA15 and PA70 were guanidinylated by methods similar to those described by Choi et al.[20] Briefly, to 1.27 g (13.4 mmol of amino groups) of PA dissolved in 40 mL of a phosphate buffer (pH 7.4), different molar ratios of PC were added (Table I). The solutions were adjusted to pH 9.5 with a 4*M* NaOH solution. The guanidinylation reaction was performed at room temperature for 60 h. After dialysis (molecular weight cutoff = 3500) against deionized water for 3 days, the lyophilized products were collected. The guanidinylated PA15 and PA70, coded as GA15-1, GA15-2, GA15-4, and GA70-2 according to the degree of guanidinylation (11.9, 33.5, 97.1, and 33.7 mol %, respectively), were used as gene vectors in this study (Table I). The NMR spectra of GA were recorded with a Bruker Avance 600 NMR spectrometer (Bruker, Germany) in a D_2O solution. The degree of guanidinylation of GA was calculated from the integral area ratio of the methylene peak at 3.21 ppm [$-CH_2NHC(NH)NH_2$] to that at 2.62 ppm (CH_2NH_2).

TABLE I
Characteristics of GA

Sample	M_w of PA·HCl (kDa)	Feed ratio of guanidio groups to amino groups	Degree of guanidinylation (mol %)[a]	Yield of GA (%)
GA15-1	15	0.15	11.9	95
GA15-2	15	0.35	33.5	92
GA15-3	15	0.65	61.5	90
GA15-4	15	2.00	97.1	86
GA70-1	70	0.15	10.7	92
GA70-2	70	0.35	33.7	87
GA70-3	70	0.65	62.5	91
GA70-4	70	1.00	75.6	79

M_w = weight-average molecular weight.
[a] Determined by ^{1}H-NMR. The means of three experiments are shown.

DNA condensation ability assay

To confirm the DNA condensation ability of GA, agarose gel electrophoresis was performed. Polymer/DNA complexes with different N/P ratios [where N is the amount of nitrogen in the polycation (for GA, three nitrogens per guanidino group) and P is the amount of plasmid phosphate in the DNA as moles], which ranged from 0.5 to 10, were freshly prepared before use by the gentle vortexing of a mixture of the pGL3 control (0.1 μg/well) and polymer solution (10 μL) at room temperature for 20 min. The complexes were incubated at room temperature for 20 min, and this was followed by the addition of 2 μL of a 6× agarose loading dye mixture (Biosesang, Seongnam, Korea). After further incubation for 10 min, the mixture solutions were loaded onto 0.8% agarose gel with EtBr (0.1 μg/mL) and run with a trisacetate buffer at 100 V for 40 min. The gel was analyzed on an ultraviolet illuminator to determine the location of the DNA.

Nuclease resistance of the GA/DNA complexes

The nuclease resistance of the GA/DNA complexes was evaluated by agarose gel electrophoresis with a method similar to that previously described by Gebhart et al.[21] Briefly, 2 μL of polymer/DNA

complexes with an N/P ratio of 5 and naked plasmid DNA (pGL3 control, 0.1 μg) were separately incubated with 1 μL of DNase-I (1 unit) in a DNase/Mg^{2+} digestion buffer (50 m*M* Tris-Cl, pH 7.6, and 10 m*M* $MgCl_2$) at 37°C with shaking at 100 rpm for 30 min. For DNase inactivation and DNA release, all samples were treated with 4 μL of 250 m*M* ethylene diamine tetraacetic acid for 10 min and mixed with 8 μL of sodium dodecyl sulfate dissolved in 0.1*M* NaOH (pH 7.2). Finally, samples were incubated at room temperature for 2 h, and this was followed by the addition of 3 μL of a 6× agarose loading dye mixture (Biosesang). After further incubation for 10 min, the mixture solutions were analyzed with agarose gel with the same DNA release assay protocol.

Particle size and ζ-potential measurement

The particle sizes and ζ-potential of the polymer/DNA complexes were measured with an electrophoretic light scattering spectrophotometer (ELS8000, Otsuka Electronics, Osaka, Japan), with 90 and 20° scattering angles, respectively. Polymer/DNA complexes were prepared in water with N/P ratios of 1, 5, 10, 20, and 30, respectively. The volume of each sample was 2 mL with 80 μg of calf thymus DNA.

Morphology observation with energy-filtering transmission electron microscopy (EF-TEM)

For EF-TEM measurements, the final concentration of DNA in the complex solution was 20 μg/mL. Ten microliters of GA15-2/DNA complexes with an N/P ratio of 10 was carefully dropped onto clean copper grids and negatively stained with 1.5 wt % phosphotungstic acid (pH7.4) for 5 s. The copper grid surface was dried at room temperature for 5 min before observation by EF-TEM (Libra 120, Carl Zeiss, Germany).

Cell viability assays

In vitro cytotoxicity tests were investigated with the Cell Titer 96 Aqueous One solution cell proliferation kit (MTS; Promega). Cells were seeded in a 96-well plate at an initial density of 1×10^4 (HeLa and 293T) or 2×10^4 (HepG2) cells/well in 200 μL of completed DMEM and incubated for 18–20 h to reach 80% confluence at the time of treatment. Completed DMEM was replaced by 100 μL of fresh, serum-free DMEM containing various amounts of polymers (1, 5, 10, 20, and 30 μg/mL). Cells were incubated for 24 h, and this was followed by the addition of 20 μL of the Cell Titer 96 Aqueous One solution reagent. After further incubation for 1–2 h, the absorbance was measured at 570 nm with an enzyme-linked immunosorbent assay plate reader (GLR 1000, Genelabs Diagnostics, Singapore) to evaluate the metabolic activity of the cells:

$$\text{Cell viability}(\%) = (\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100$$

where $\text{OD}_{\text{sample}}$ is the optical density from a well treated with the polymer and $\text{OD}_{\text{control}}$ is the optical density from a well treated with phosphate-buffered saline (PBS) buffer only.

In vitro luciferase activity assay

Cells were seeded in a 24-well plate at an initial density of 1×10^5 (HeLa and 293T) or 2×10^5 (HepG2) cells/well in 1 mL of completed DMEM and incubated for 18–20 h to reach 70–80% confluence at the time of transfection. The medium was replaced with 200 μL of serum-free DMEM with polymer/pGL3 control complexes (1 μg) at various N/P ratios (5, 10, 20, and 30) and additionally incubated for 6 h. Then, the media were changed with freshly completed DMEM and allowed to incubate for 24 h. After removal of the growth medium, each well was washed gently with PBS twice, and 200 μL of a reporter lysis buffer (Promega) was added; then, the cells were frozen at −80°C before luciferase analysis according to the manufacturer's protocols. Relative light units were measured with a chemiluminometer (Autolumat LB953, EG&G, Berthold, Germany). Protein quantification was determined by the bicinchoninic acid method, and relative light units were normalized to the protein concentration in the cell extracts.[22] Each transfection experiment was carried out in triplicate, and transfection activity was expressed as relative light units.

RESULTS AND DISCUSSION

Preparation and characterization of GA

The amino groups of PA were easily substituted by guanidino groups in a mildly basic buffer solution with PC as a guanidinylation reagent (Fig. 1). The appearance of the new peaks at 3.21 ppm assigned to $-CH_2NHC(NH)NH_2$ in ^{1}H-NMR and at 158.3 ppm in ^{13}C-NMR clearly indicated the successful incorporation of guanidino groups into PA [Fig. 2(A,B)].[20] The degree of guanidinylation of GA was calculated from the integral area ratio of the methylene peak at 3.21 [$-CH_2NHC(NH)NH_2$] to that at 2.62 ppm (CH_2NH_2). The results showed that the degree of guanidinylation increased linearly when the feed ratio of guanidino groups to amino groups was below 0.7 (Fig. 3 and Table I). The equation is as follows:

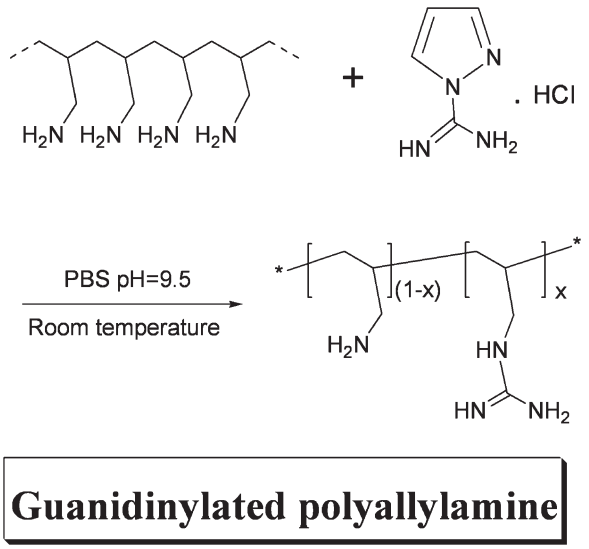

Figure 1 Guanidinylation reaction of PA.

$$DG = 0.97 \times FR \quad (1)$$

where DG is the degree of guanidinylation of GA and FR is the molar feed ratio of guanidino groups to amino groups.

Because of the hindered effect of neighbor groups, the increase in the degree of guanidinylation of GA slowed when its value was above 68 mol %. The amino groups of PA15 were almost replaced with guanidino groups when the molar feed ratio of guanidino groups to amino groups reached 2.

Characterization of GA/DNA complexes

Naked DNA can be condensed into nanoparticles via the electrostatic interactions between the polycations and DNA, and this results in enhanced cellular uptake efficiency. The condensation ability of GA to DNA was evaluated with agarose gel electrophoresis. As shown in Figure 4, the migration of DNA was completely retarded when the N/P ratios in the GA15-2/DNA, GA15-4/DNA, and GA70-2/DNA complexes were around 3, whereas migration was

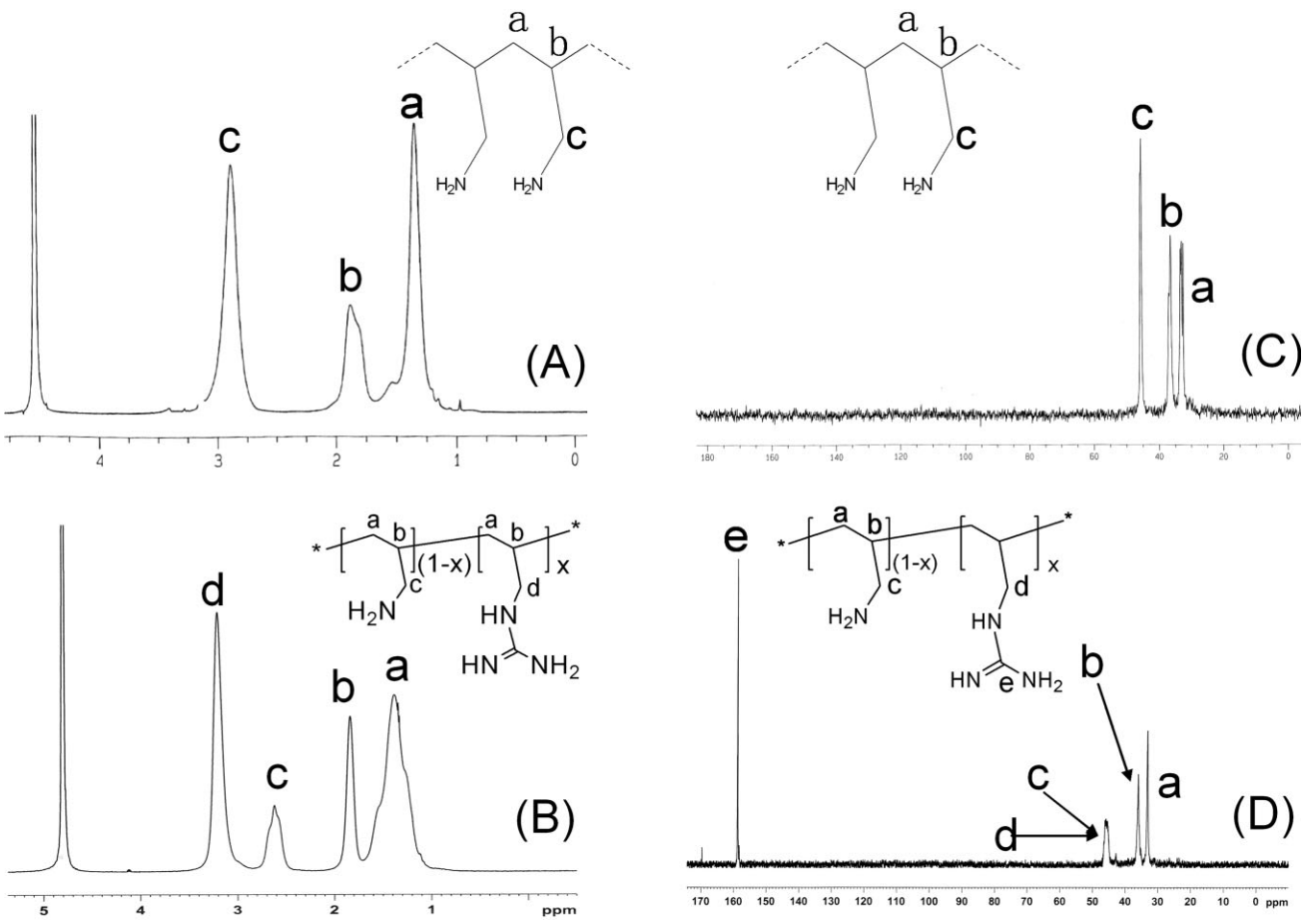

Figure 2 (A) ^{1}H-NMR spectrum of PA70 in D_2O: (a) 1.34, (b) 1.87, and (c) 2.70 ppm. (B) ^{1}H-NMR spectrum of GA70-4 in D_2O: (a) 1.33–1.38, (b) 1.84, (c) 2.62, and (d) 3.21 ppm. (C) ^{13}C-NMR spectrum of PA70 GA70-4 in D_2O: (a) 32.6–33.4, (b) 36.4, and (c) 45.5 ppm. (D) ^{13}C-NMR spectrum of GA70-4 in D_2O: (a) 33.2, (b) 35–36.7, (c,d) 42.9–46.3, and (e) 158.3 ppm.

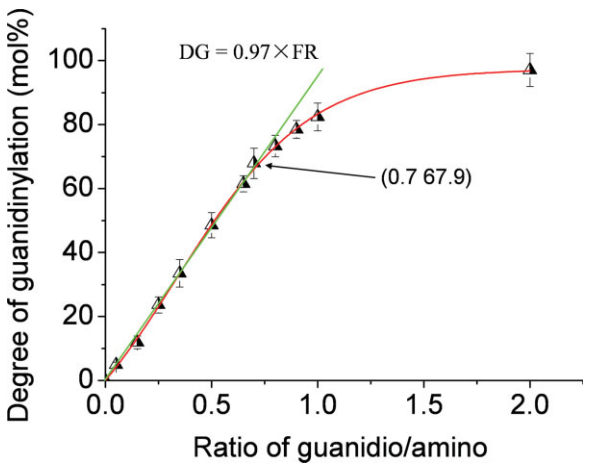

Figure 3 Feed ratios of guanidino groups in the PC to amino groups in the PA versus the guanidinylation degree of PA (mean ± standard deviation, n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

retarded when the N/P ratio reached 5 in the GA15-1/DNA complex; this suggested that GA15-2, GA15-4, and GA70-2 had better DNA condensation ability than GA15-1 because of the increased degree of guanidinylation (GA15-2 and GA15-4) or increased molecular weight (GA70-2).

Effective condensation is an important requirement for DNA stability against degradation by nucleases.[23] An evaluation of DNase-I protection was carried out via agarose gel electrophoresis. The results showed that naked DNA was completely degraded by DNase-I within 30 min, whereas the DNA condensed with GA at an N/P ratio of 5 was efficiently protected from enzymatic hydrolysis

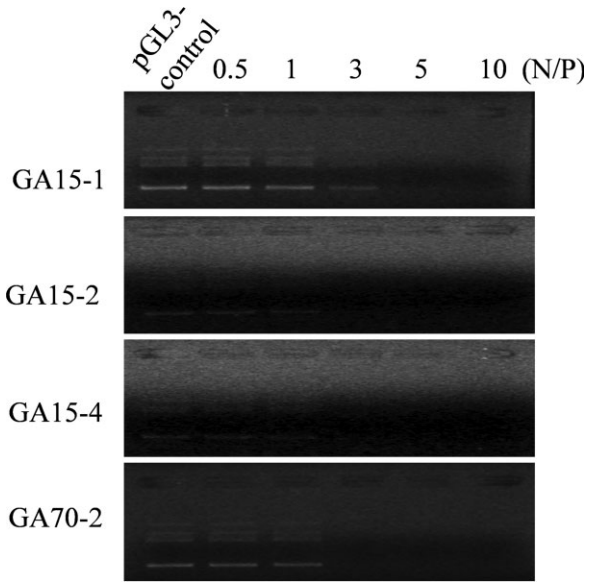

Figure 4 Agarose gel electrophoresis of polymer/DNA (pGL3 control) complexes at various N/P ratios.

(Fig. 5), which is one of the crucial factors for efficient gene delivery *in vitro* and *in vivo*.

The particle sizes of polymeric gene delivery complexes are known to dramatically affect the gene transfection efficiency. It has been reported that endocytosis by many types of cells is limited to sizes less than 250 nm.[24] As shown in Figure 6(a), the particle sizes of the complexes decreased sharply with increasing N/P ratios. At an N/P ratio of 1, the complexes were not formed completely, and the particles were very large. When the N/P ratio was above 10, all the particle sizes were less than 240 nm, and this suggested their endocytosis potential. According to the size values of the GA/DNA nanoparticles at the same N/P ratio above 10, GA70-2/DNA < GA15-2/DNA < GA15-1/DNA < PA15/DNA < GA15-4/DNA. The sizes of the GA70-2/DNA complexes were smaller than those of the GA15-2/DNA complexes because the high molecular weight of GA70-2 resulted in strong DNA condensation ability. The sizes of the PA15/DNA, GA15-1/DNA, and GA15-2/DNA complexes showed a tendency to decrease orderly with the increase in the degree of guanidinylation. Interestingly, among the GA15-4/DNA, GA15-2/DNA, GA15-1/DNA, and PA15/DNA complexes, the particle sizes of the GA15-4/DNA complexes were the largest. It is thought that the molecular chains of GA15-4 were not flexible enough because of the strong electrostatic repulsive interactions of too many guanidino groups. The rigid molecular chains of GA15-4 retarded its ability to wrap around DNA into small spherical particles. Details will be reported in another article.

The formation of GA/DNA complexes was also confirmed by the observation of the morphology. Representative EF-TEM images of GA15-2/DNA complexes at an N/P ratio of 10 demonstrated the relatively homogeneous nanoparticles with spherical

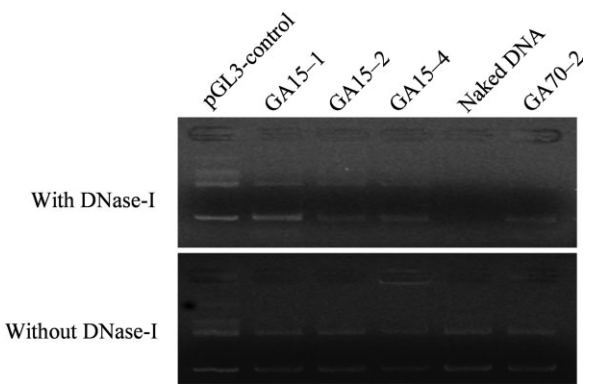

Figure 5 Nuclease resistance assay of DNA (pGL3 control). DNA was released by the addition of 1% sodium dodecyl sulfate to the polymer/DNA complexes at an N/P ratio of 5.

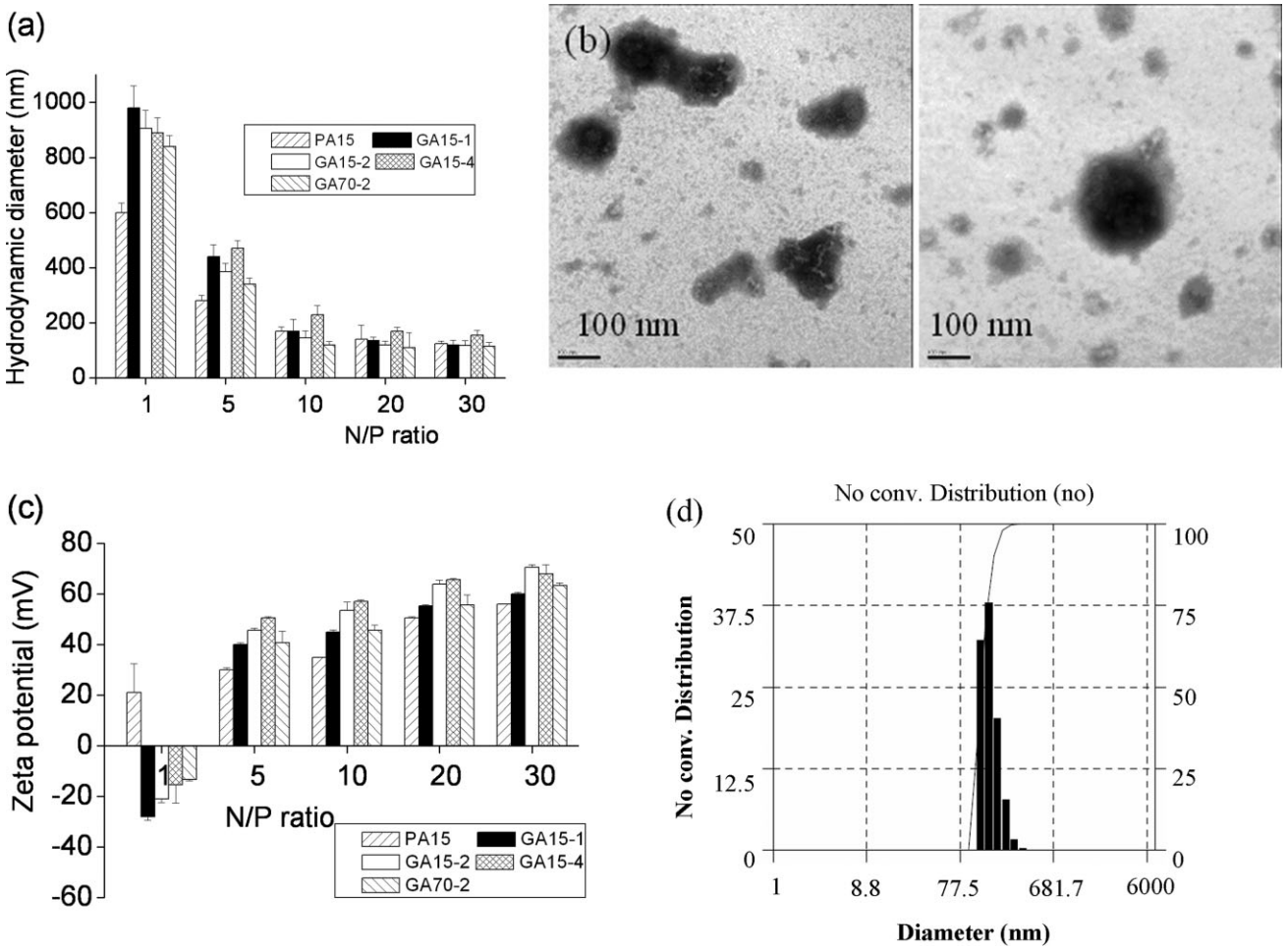

Figure 6 (a) Particle sizes of polymer/DNA complexes in distilled water at various N/P ratios (mean ± standard deviation, n = 3). (b) EF-TEM images of GA15-2/DNA complexes at an N/P ratio of 10 (ca. 140 nm). Phosphotungstic acid was used as a negative staining agent. (c) ζ-Potential of polymer/DNA complexes in distilled water at various N/P ratios (mean ± standard deviation, n = 3). (d) Size distribution of GA15-2/DNA complexes in distilled water at an N/P ratio of 10 (146 ± 25 nm).

shapes and compact structures [Fig. 6(b)]. Sizes observed from EF-TEM images were very similar to those measured by dynamic light scattering.

As mentioned previously, the polymer/DNA complexes were formed via the electrostatic interactions between the polycations and DNA. A positive ζ-potential of untargeted polyplexes is necessary for attachment to anionic cell surfaces, which consequently facilitates uptake by the cell.[25] Figure 6(c) shows the ζ-potential of the complexes with various N/P ratios. At an N/P ratio of 1, the ζ-potential of the GA/DNA complexes was negative, whereas the ζ-potential of the PA15/DNA complexes was positive. This is the reason that the amount of positive charge in the guanidino group is one third of that in the amino group with the same number of N atoms. The ζ-potentials increased rapidly from −20 to +40 mV with the N/P ratio increasing from 1 to 5 and then increased gradually from +40 to +70 mV with the N/P ratio increasing from 5 to 30. Meanwhile, at the same N/P ratio, with an increase in the degree of guanidinylation, the ζ-potential of the GA/DNA nanoparticles showed a tendency to increase. The high ζ-potential resulted in strong charge-repulsive forces, which prevented aggregation among the complexes. The unimodal particle size distribution further suggested good dispersion due to the high ζ-potential of the GA/DNA complexes [Fig. 6(d)].

Cytotoxicity of GA

The cytotoxicity of polymeric gene vectors is an important factor that affects the transfection efficiency. The cytotoxicity of polycations is probably caused by polymer aggregation on the cell surface due to the strong electrostatic interaction with the plasma membrane, which results in destabilization and ultimately impaired cell membrane functions. In particular, polycations with primary amino groups disrupt protein kinase C function through the disturbance of

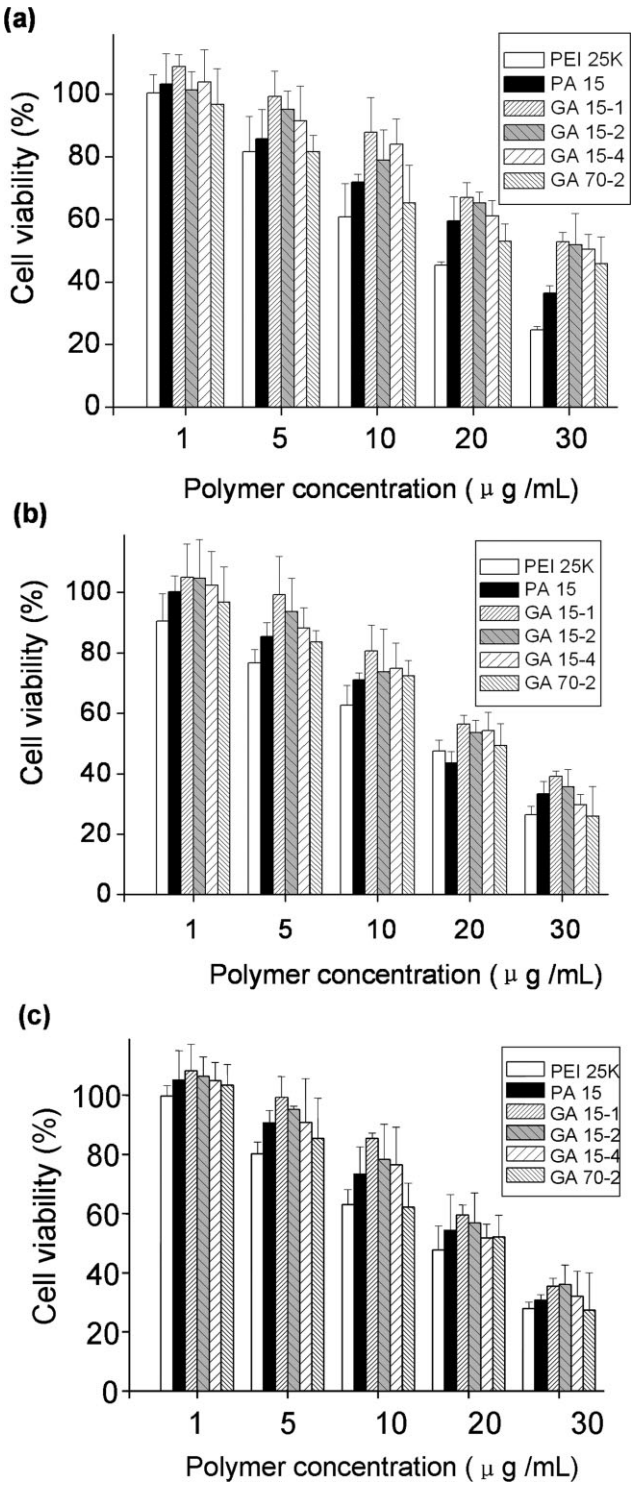

Figure 7 Cell viability of GA at various concentrations in different cell lines (mean ± standard deviation, n = 3): (a) 293T, (b) HeLa, and (c) HepG2.

protein kinase activity.[26] Figure 7 shows the cytotoxicity results for PEI25K, PA15, and GA according to the MTS method in three different cell lines. The cell viability decreased with increasing polymer dosage. The cytotoxicity of PEI25K was higher than that of PA15, GA15-1, GA15-2, GA15-4, and GA70-2. After amino groups of PA15 were transformed into guanidino groups, the cytotoxicity of GA15-1, GA15-2, and GA15-4 was lower than that of PA15. It is thought that guanidination would lead to delocalization of the cationic charge, and this would result in a reduction of toxicity.[27] It is also believed that the electrostatic force between the polycation and cell membrane was weakened because of the partial role

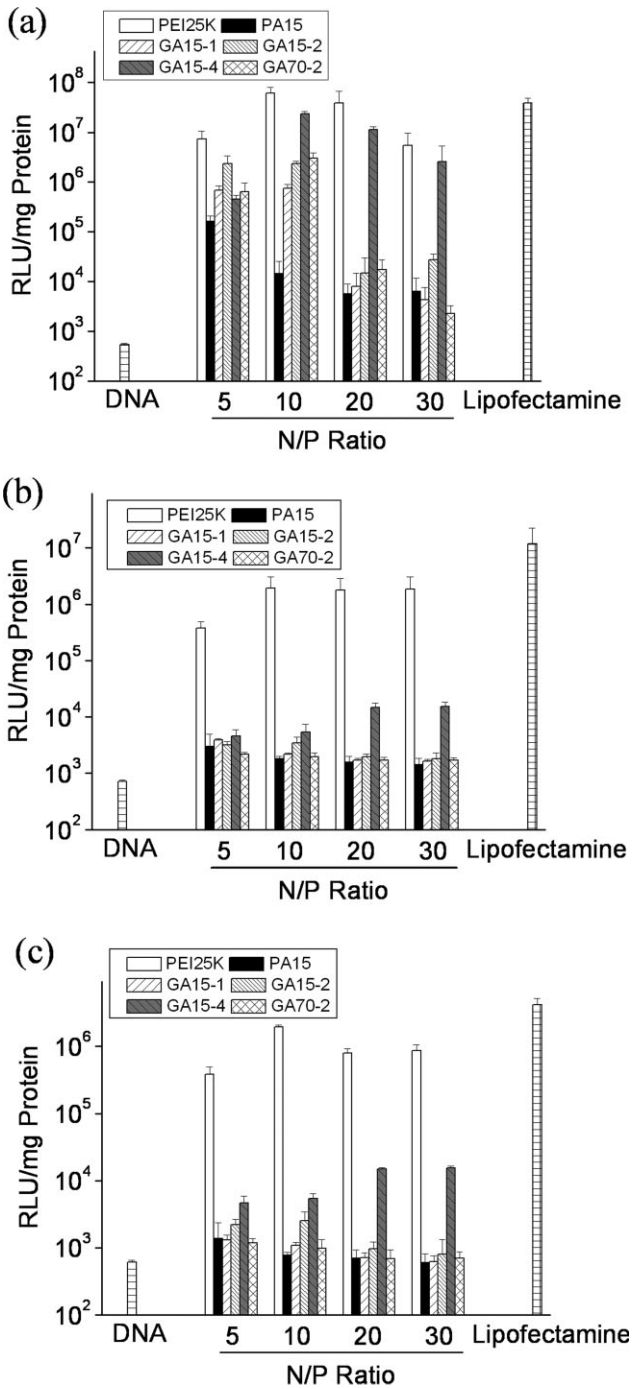

Figure 8 Transfection efficiency of the GA/pGL3 control at various N/P ratios and in different cell lines (mean ± standard deviation, n = 3): (a) 293T; (b) HeLa, and (c) HepG2.

of hydrogen bonding between guanidino groups in the GA and cell membrane, which also resulted in a reduction of toxicity.[28]

In vitro transfection efficiency assay of GA

In vitro transfection efficiencies in 293T, HeLa, and HepG2 cells with GA are shown in Figure 8. The best relative activity of the luciferase enzyme of GA15-4 in the 293T cell line reached 10^7 levels, which were about 1000 times higher than those in the Hela and HepG2 cell lines, and this suggested that the transfection efficiency of GA was strongly dependent on the cell lines. The transfection efficiency of PA15 in the 293T cell line showed a tendency to decrease monotonously, whereas the transfection efficiency of GA15-1, GA15-2, GA15-4, and GA70-2 in the 293T cell line showed a changeful character with an increase in the N/P ratio from 5 to 30. With the N/P ratio increasing from 5 to 10, the transfection efficiency of GA15-1, GA15-2, GA15-4, and GA70-2 increased, and this contributed to the enhanced cell uptake, which resulted from the increased surface charge and decreased particle sizes of the polymer/DNA complexes. The maximal activity of gene expression occurred at an N/P ratio of 10. When the N/P ratio exceeded 10, a gradual decline was observed because of the increased cytotoxicity that resulted from the superfluous polycations; this was similar to what was observed for PEI25K.

At the same N/P ratio, although the cytotoxicity of GA15-1 was the lowest, the transfection efficiency of the polymers increased with an increasing degree of guanidinylation, and it followed the order of PA15/DNA < GA15-1/DNA <GA15-2/DNA < GA15-4/DNA. Besides the cytotoxicity, the interaction of the cell and polymer/DNA complexes is a key issue for the gene expression of polymer/DNA complexes. As mentioned previously, with an increasing degree of guanidinylation in GA, the positive surface charge of GA/DNA nanoparticles showed a tendency to increase, and this facilitated the attachment of complexes to the negative cell surface. Additionally, the guanidine was able to form hydrogen bonds with the phospholipids present in the lipid bilayers, and this enhanced the cellular uptake.[17–19] Improved cellular uptake of polymer/DNA complexes is a requirement for good gene transfection efficiency.

CONCLUSIONS

GA was successfully prepared by the reaction between PA and PC. GA was capable of condensing and protecting DNA in the form of nanoparticles. The incorporation of guanidino groups into PA resulted in a reduction of the cytotoxicity along with an improvement of the gene transfection efficiency. The properties of GA as a gene vector, such as the particle size and ζ-potential of GA/DNA complexes, as well as the gene transfection efficiency were greatly dependent on the degree of guanidinylation.

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