UC Irvine

UC Irvine Previously Published Works

Title

Mixing-sequence-dependent nucleic acid complexation and gene transfer efficiency by polyethylenimine.

Permalink

<https://escholarship.org/uc/item/2q63080j>

Journal

Biomaterials science, 3(7)

ISSN

2047-4830

Authors

Cho, SK
Dang, C
Wang, X
et al.

Publication Date

2015-07-01

DOI

10.1039/c5bm00041f

Peer reviewed

Cite this: *Biomater. Sci.*, 2015, **3**, 1124

Mixing-sequence-dependent nucleic acid complexation and gene transfer efficiency by polyethylenimine

S. K. Cho,^a C. Dang,^b X. Wang,^c R. Ragan^c and Y. J. Kwon^{*c,d,e,f}

Polyplexes, complexed nucleic acids by cationic polymers, are the most common forms of nonviral gene delivery vectors. In contrast to a great deal of efforts in synthesizing novel cationic polymers and exploring their extracellular and intracellular delivery pathways, polyplex preparation methods of mixing nucleic acids and cationic polymers are often overlooked. In this study, the mixing sequence, that is adding nucleic acids to polymers or *vice versa*, was found to greatly affect complexation of both plasmid DNA and siRNA, polyplexes' size, and polyplexes' surface charge, which all collaboratively affected the transfection efficiency and cytotoxicity. Adding polyethylenimine (PEI), the most conventionally used standard in nonviral gene delivery, to plasmid DNA and siRNA resulted in larger polyplexes, higher gene expression and silencing, but higher cytotoxicity than polyplexes prepared in the reverse order. Based on the experimental results, the authors developed a model that gradual addition of cationic polymers (*e.g.*, PEI) to nucleic acids (*e.g.*, plasmid DNA and siRNA) incorporates more copies of nucleic acids in larger polyplexes in a smaller number, results in higher gene expression and silencing levels in transfected cells, and generates higher cytotoxicity by leaving more free polymers upon complete mixing than the other mixing sequence. The proposed model can be explored using a broad range of cationic polymers and nucleic acids, and provide insightful information about how to prepare polyplexed nonviral vectors for efficient and safe gene delivery.

Received 6th February 2015,

Accepted 6th March 2015

DOI: 10.1039/c5bm00041f

www.rsc.org/biomaterialsscience

Introduction

Gene therapy is a promising tool in treating challenging human diseases of a broad range, including Leber's congenital amaurosis, X-linked severe combined immunodeficiency (SCID), adenosine deaminase deficiency (ADA)-SCID, adrenoleukodystrophy, chronic myelogenous leukemia (CML), and Parkinson's disease.^{1–4} Gene therapy started from a concept of correcting an abnormal gene by delivering a desirable therapeutic transgene (*i.e.*, DNA),⁵ and has recently evolved to interfere with target biological activities at a translational level by delivering small nucleic acids (*e.g.*, siRNA and antisense ODN).^{6,7} In order to address the clinical challenges associated

with using viral vectors (*e.g.*, immunogenicity, onco/tumorigenicity, and inefficient and cumbersome preparation),^{8,9} developing nonviral vectors that are as efficient as viral counterparts with improved safety measures has been of great interest.^{10,11} Among numerous forms, complexes of nucleic acids and cationic polymers, often termed polyplexes, are the most representative nonviral vectors.^{12,13} Polyethylenimine (PEI) is one of the earliest employed and most widely used cationic polymers in complexing nucleic acids owing to its desirably high cationic density and strong proton-buffering capacity in a broad range of pH.^{14–18}

Many factors determine the nucleic acid complexation by polymers, and thereby the transfection efficiency: molecular weight, charge density, and morphology of polymers, nucleic acid size, ionic strength of complexation solvents, nucleic acid and polymer concentration, and molecular ratios of amines of the polymer to phosphates of nucleic acids (*i.e.*, N/P ratios).^{14,19–21} One fundamental, under-investigated factor in polyplex preparation is how to initiate molecular interactions between polymers and nucleic acids. Boussif *et al.* briefly noted 10-fold increased transfection when cationic polymers (PEI) were added to plasmid DNA,¹⁴ yet Kircheis *et al.* did not observe such differences in transfection efficiencies of

^aDepartment of Chemistry, Dong-A University, Busan, South Korea

^bSchool of Biological Sciences, University of California, Irvine, CA 92697, USA

^cDepartment of Chemical Engineering and Materials Science, University of California, Irvine, CA 92697, USA. E-mail: kwonjy@uci.edu

^dDepartment of Pharmaceutical Sciences, University of California, Irvine, CA 92697, USA

^eDepartment of Biomedical Engineering, University of California, Irvine, CA 92697, USA

^fDepartment of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697, USA

polyplexes prepared with different mixing sequences.²² These discrepancies require more detailed investigation since PEI is regarded as the gold standard when comparing efficiencies of nonviral vectors.

In this study, we delved into the effects of the mixing sequence of PEI and nucleic acids (plasmid DNA and siRNA) on the polyplex size, nucleic acid condensation, polyplex morphology, transfection efficiency, and cell viability. The results of this study led us to a model explaining mixing-sequence-dependent molecular interactions, which can possibly be applied to the polyplex formation behaviors of other polymers and nucleic acids. This study is designed to provide insightful information on developing polymeric nonviral carriers for efficient and safe delivery of plasmid DNA as well as oligonucleotides such as siRNA.

Experimental

Materials

Polyethylenimine (branched, 25 kDa) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Aldrich (Milwaukee, WI) and used without further purification. Plasmid DNA encoding enhanced green fluorescent protein (eGFP) (5.0 kbp) was a gift from Dr. Pamela Davis (Case Western Reserve University, Cleveland, OH). Silencer® GFP siRNA was purchased from Ambion (Austin, TX, USA) and ethidium bromide was purchased from Fisher Scientific (Pittsburgh, PA). NIH 3T3 cells (ATCC, Rockville, MD) and NIH 3T3 cells stably expressing GFP²³ were cultured in Dulbecco's modified Eagle's medium (DMEM) (MediaTech, Herndon, VA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% antibiotics (100 units mL⁻¹ penicillin; 100 µg mL⁻¹ streptomycin) (MediaTech). Nuclease-free water was supplied from Fisher Scientific (Pittsburgh, PA). V-1 quality mica was purchased from Ted Pella (Redding, CA).

Preparation and characterization of nucleic acid/PEI polyplexes

A plasmid DNA stock solution was prepared at a concentration of 40 µg mL⁻¹ in nuclease-free water. 20 µg of plasmid DNA-containing stock solution (500 µL) was added dropwise with a pipette to a PEI-containing solution in an eppendorf tube prepared at various concentrations (500 µL) in nuclease-free water while vortexing at the lowest speed to achieve predetermined N/P ratios (0, 1, 2.5, 5, 7.5, 10, 12.5, and 15) and this was denoted as DtoP polyplexes. In addition, a PEI-containing solution at various concentrations (500 µL) was added dropwise to a plasmid DNA-containing stock solution (500 µL) to prepare PtoD polyplexes. Freshly prepared polyplexes were incubated at room temperature for 30 min prior to characterization and transfection studies. siRNA/PEI polyplexes were prepared similarly by mixing siRNA (20 µg of siRNA in 500 µL nuclease-free water) with PEI (in 500 µL nuclease-free water) at the same varying N/P ratios in both reverse mixing sequences

(i.e., the siRNA solution to the PEI solution [RtoP] and the PEI solution to the siRNA solution [PtoR]).

The size and surface charge of the resulting plasmid DNA/PEI and siRNA/PEI polyplexes in 1 mL DI water were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) at 25 °C. The morphology of the polyplexes was visualized by atomic force microscopy (AFM) and transmission electron microscopy (TEM). Briefly, samples for AFM were prepared by depositing 25 µL of polyplex solution (containing 0.5 µg of nucleic acids) onto freshly cleaved mica, incubated for 10 min. AFM images were acquired in solution to avoid morphological changes of polyplexes using a MFP-3D-Bio AFM (Asylum Research, Santa Barbara, CA). Commercial silicon nitride AFM probes (OMCL-TR 400 PSA, Olympus, Center Valley, PA) with pyramid-shaped tips and Au coating on the reflective side of the cantilever were used for all AFM measurements. The tip has a radius of curvature of less than 20 nm and a nominal spring constant of 0.08 N m⁻¹ as provided by the manufacturer. Samples for TEM were prepared by dropping 10 µL of a polyplex-containing solution onto the carbon-coated TEM grid (Electron Microscopy Sciences, Hatfield, PA), followed by drying overnight under vacuum. Samples were then negatively stained with 2% uranyl acetate in DI water. Samples were examined under a Philips CM20 transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ) at 80 kV.

Nucleic acid complexation assays

The efficiency of nucleic acid complexation by PEI was evaluated by shielded fluorescence by the ethidium bromide (EtBr) exclusion assay and retarded migration during electrophoresis. For the EtBr exclusion assay, 0.6 µg of EtBr was mixed with 6 µg of nucleic acids (plasmid DNA or siRNA) in 150 µL DI water. The resulting EtBr-labeled nucleic acids were complexed by PEI in 150 µL DI water at varying N/P ratios. After 30 min at room temperature, the fluorescence intensity was measured using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 320 nm and an emission wavelength of 600 nm. The condensation efficiency was quantified by the reduced EtBr fluorescence of the resulting polyplexes, and compared with the fluorescence of free EtBr-labeled nucleic acids at the same concentration. In order to compare nucleic acid complexation indicated by retarded electrophoretic migration, plasmid DNA/PEI and siRNA/PEI polyplexes were loaded in 1% agarose gel containing 1 µg mL⁻¹ EtBr. Plasmid DNA/PEI polyplexes were run in the gel in Tris-borate-EDTA (TBE) buffer at 110 V for 60 min, while siRNA polyplexes were run in the gel in Tris-acetate-EDTA (TAE) buffer at 60 V for 15 min. Nucleic acid bands in agarose gels were visualized using a UV transilluminator (FluorChem, Alpha Innotech, Santa Clara, CA).

In vitro transfection and cytotoxicity

NIH 3T3 cells were plated at a density of 4 × 10³ cells per well in a 96-well plate, 24 h prior to incubation with polyplexes. GFP plasmid DNA/PEI polyplexes prepared at an N/P ratio of

10 in serum-free media were added to cells at a concentration of $0.6 \mu\text{g mL}^{-1}$. After 4 h of incubation at 37°C , polyplex-containing media were replaced with polyplex-free, FBS (10% v/v)-containing media. The cells were further incubated for additional 20 h before assessing transfection and cytotoxicity, using cytometry and the MTT assay, respectively. The cells were harvested *via* trypsinization and their GFP expression was analyzed using a Guava EasyCyte Plus cytometer (Guava Technologies, Inc., Hayward, CA). NIH 3T3 cells stably expressing GFP (NIH 3T3/GFP) were plated at a density of 2×10^4 cells per well in a 24-well plate, 24 h prior to polyplex incubation. The cells were then incubated with GFP siRNA/PEI polyplexes prepared at an N/P ratio of 10 (at a siRNA concentration of $0.6 \mu\text{g mL}^{-1}$) as described earlier. Three days after incubation with the GFP siRNA/PEI polyplexes, the GFP silencing in NIH 3T3/GFP cells was quantified by the mean fluorescence intensities (MFI) using a flow cytometer. For the cytotoxicity assay, 100 μg of MTT in 10 μL PBS was mixed with 90 μL media and the resulting mixture was added to the cells for incubation at 37°C . After 2 h, MTT-containing media were aspirated and purple formazan crystals formed on the cells were dissolved by adding 200 μL of DMSO containing 10% glycine buffer (0.1 M glycine and 0.1 M NaCl). The absorbance at 560 nm wavelength was used to calculate the relative viabilities of the cells incubated with polyplexes by comparing the absorbance obtained from the cells incubated without polyplexes.

Statistics

Triplicate experimental data were analyzed using one-way ANalysis Of VAriance (ANOVA) at the significance level of $p < 0.01$ and presented as the mean with standard deviation, otherwise noted.

Results and discussion

Mixing sequence-controlled nucleic acid complexation

The key characteristics of polyplexes are how efficiently nucleic acids are condensed in size, sterically shielded, and electrostatically compensated by cationic polymers. Plasmid DNA and siRNA were mixed with PEI at varying N/P ratios of 0, 1, 2.5, 5, 7.5, 10, 12.5, and 15, where the N/P ratio refers to the molecular ratio of amines (N; cationic groups) in PEI to phosphates (P; anionic groups) in nucleic acids. An N/P ratio of 10 is commonly used to complex plasmid DNA with PEI as complete complexation, high transfection, and acceptable cell viability are observed.^{24,25} Four kinds of PEI polyplexes were prepared by two mixing sequences and two nucleic acids: adding nucleic acid-containing solutions to PEI-containing solutions (plasmid DNA to PEI [DtoP] and siRNA to PEI [RtoP]) or adding PEI-containing solutions to nucleic acid-containing solutions (PtoD and PtoR) (Fig. 1 and 2). As shown in Fig. 1A, adding plasmid DNA to PEI (DtoP) resulted in smaller polyplexes (~ 60 nm in diameter at an N/P ratio of 7.5 or higher) than those prepared by adding PEI to plasmid DNA (PtoD)

Fig. 1 Plasmid DNA complexation by PEI when plasmid DNA was added to PEI (DtoP) vs. PEI was added to plasmid DNA (PtoD), represented by (A) size and zeta-potential, (B) EtBr exclusion, and (C and D) retarded migration during agarose gel electrophoresis.

Fig. 2 siRNA complexation by PEI when siRNA was added to PEI (RtoP) vs. PEI was added to plasmid DNA (PtoR), represented by (A) size and zeta-potential, (B) EtBr exclusion, and (C and D) retarded migration during agarose gel electrophoresis.

(~ 200 nm at an N/P ratio of 7.5 or higher), indicating more efficient plasmid DNA condensation by DtoP than PtoD. The surface charge of the polyplexes prepared by DtoP ($\sim +27$ mV) was lower than that of the polyplexes prepared by PtoD ($\sim +43$ mV), at an N/P ratio of 7.5 or higher (Fig. 1A). The EtBr exclusion assay and migration retardation in agarose gel confirmed mixing sequence-independent, complete shielding of plasmid DNA and charge-compensation by PEI at an N/P ratio of 5 or higher (Fig. 1B, C, and D), consistent with other prior studies.^{26,27} The results shown in Fig. 1 imply lower molecular ratios of PEI to plasmid DNA incorporated in a polyplex prepared with DtoP, compared with the one prepared with PtoD, resulting in (1) a smaller polyplex size (Fig. 1A) and (2) a lower surface charge (Fig. 1A) at complete molecular shielding (EtBr exclusion; Fig. 1B) and charge-compensation (retarded electrophoretic migration; Fig. 1C and D). Since siRNA (21 bps) is about 250 times smaller than plasmid DNA (~ 5 kbps), siRNA complexation by PEI is expected to be different from DNA

complexation and may be significantly more affected by the mixing sequence. Similar to plasmid DNA/PEI polyplexes (Fig. 1), addition of siRNA to PEI (RtoP) resulted in smaller polyplexes (~60 nm) and lower zeta-potential (~+34 mV) than those prepared by adding PEI to siRNA (PtoR) (~180 nm and ~+45 mV) (Fig. 2A). Interestingly, PtoR was not able to condense siRNA in size at all N/P ratios, while RtoP condensed siRNA at N/P ratios of 7.5 or higher (Fig. 2A). This observation indicates that adding cationic PEI to siRNA at an N/P ratio of 1 or higher loosely aggregated siRNA/PEI complexes without size condensation. In contrast, adding siRNA to PEI initially generated loose siRNA/PEI complexes at an N/P ratio up to 2.5 but further addition of negatively charged siRNA condensed the polyplexes by attractive electrostatic interactions with PEI. Similar to plasmid DNA complexation (Fig. 1B, C, and D), the mixing sequence did not greatly affect the siRNA shielding by PEI and the electrophoretic mobility of siRNA/PEI complexes (Fig. 2B, C, and D), confirming mixing sequence-independent, complete compensation of siRNA's negative charge by cationic PEI. Results shown in Fig. 2 imply lower molecular ratios of PEI to siRNA incorporated in a polyplex prepared with RtoP than the one prepared with PtoR, resulting in a smaller polyplex size (Fig. 2A) and a lower surface charge (Fig. 2A) at similar siRNA shielding (Fig. 2B) and charge-compensation (Fig. 2C and D).

Among many factors determining polyplexes' transfection efficiency, including polyplex characteristics (size, number, and surface charge), enhanced cellular uptake, and improved intracellular trafficking, a significant polyplex size difference resulting from different mixing sequences presents a notable design consideration. It was observed that larger polyplexes exhibited a higher transfection efficiency than smaller ones, hypothetically due to efficient endosomal escape *via* a strong proton sponge effect and fast sedimentation onto the cells.^{21,22,28} As implicated by the results shown in Fig. 1 and 2, the mixing sequence may also affect the copy numbers of nucleic acids per polyplex, and hence polyplex densities at the same amount of nucleic acids. This necessitates the investigation of the morphology and concentration of nucleic acid/PEI polyplexes prepared in different mixing sequences (see the next section).

EtBr fluorescence quenching in plasmid DNA/PEI polyplexes (a relative fluorescence value of ~0.1 at an N/P ratio of 5 or higher) was higher than that of siRNA/PEI polyplexes (a relative fluorescence value of ~0.2 at an N/P ratio of 2.5 or higher), which implies a higher level of intermolecular intervention of cationic polymers (*e.g.*, PEI) with longer nucleic acids (*e.g.*, plasmid DNA) than shorter ones (*e.g.*, siRNA). However, at an N/P ratio of 1, molecular dynamics simulations and isothermal titration calorimetry showed stronger binding of siRNA to PEI than plasmid DNA (also evidenced by a relative fluorescence value of ~0.7 for plasmid DNA/PEI polyplexes and ~0.35 for siRNA/PEI polyplexes in Fig. 1B and 2B).²⁹ This can be explained by the fact that a significant conformational change is required for plasmid DNA to bind to cationic polymers, while small siRNA's binding to cationic polymers do not

require such a change. However, a sum of electrostatically attractive forces exerted on one plasmid DNA molecule is greater than those on some siRNA molecules, generating higher molecular shielding upon complete complexation (Fig. 1B and 2B). It is a widely accepted consensus that nucleic acid/PEI polyplexes prepared at an N/P ratio of 1 are often inefficient in complexing nucleic acids, highly unstable, and poor in transfection.^{30–32} At an N/P ratio of 10 and up to 6 hours of incubation in water, no changes in size, zeta-potential, and transfection efficacy were observed. Although thermodynamic kinetics play an important role in self-assembly/disassembly (complexation/decomplexation), particularly for reversible processes, electrostatic interactions between counter-charged polymers (*i.e.*, PEI and nucleic acids) could be strong enough to make the complexation considered relatively irreversible, unless there is significant counter-driving forces such as elevated temperatures and/or salt/electrolyte concentrations.

Size, morphology, and density of polyplexes

Since the mixing sequence greatly affects the size of nucleic acid/PEI polyplexes (Fig. 1 and 2) and the same amounts of PEI and nucleic acids were mixed, it was hypothesized that the mixing sequence determines the nucleic acid copy numbers per polyplex and affects the density/concentration of polyplexes. The size and morphology of nucleic acid/PEI polyplexes prepared by different mixing sequences were observed by using AFM and TEM. AFM and TEM also allow us to estimate the density of polyplexes when all samples were prepared at the same concentrations and observed under the same conditions. AFM images obtained under a wet condition showed that plasmid DNA/PEI polyplexes prepared by adding plasmid DNA to PEI (DtoP) were ~100 nm in size while adding PEI to plasmid DNA resulted in polyplexes of ~250 nm (Fig. 3A), which are larger (~40 and 25%, respectively) than those measured by DLS. This might be due to partially flattened polyplex attachment on the mica surface as well as size exaggeration generated by the AFM tip convolution effect. TEM images showed plasmid DNA/PEI polyplexes in agreement with AFM, except that the polyplexes in TEM images looked smaller than those observed by AFM (Fig. 3C and D), because TEM samples need to be dried under vacuum prior to imaging. AFM images also showed a significantly wide and flat periphery, in contrast to their highly dense core, of the polyplexes prepared by adding PEI to plasmid DNA (PtoD) (Fig. 3B). This may also explain why the polyplexes under the TEM looked smaller than those by AFM because of the insufficient contrast provided by the amorphous polymer in the periphery. This can be explained that a PEI-rich outer layer was formed when additional PEI (150 times excess PEI to plasmid DNA at an N/P ratio of 10) was added to loosely complexed plasmid DNA/PEI polyplexes. Addition of plasmid DNA to excess PEI (DtoP), contrarily, formed dense polyplexes without a flat outer layer (Fig. 3A). This means that gradually added plasmid DNA was completely complexed by excess PEI. The most striking difference observed by AFM between plasmid

Fig. 3 AFM images of the morphology of plasmid DNA/PEI polyplexes prepared at an N/P ratio of 10 (A: plasmid DNA/PEI polyplexes prepared by DtoP mixing; B: plasmid DNA/PEI polyplexes prepared by PtoD mixing) and TEM images (C: plasmid DNA/PEI polyplexes prepared by DtoP mixing; D: plasmid DNA/PEI polyplexes prepared by PtoD mixing).

DNA/PEI polyplexes prepared by different mixing sequences is density. While ~ 12 polyplexes prepared by adding plasmid DNA to PEI (DtoP) were counted in $1 \mu\text{m}^2$ area, only 2 polyplexes prepared by adding PEI to plasmid DNA (PtoD) were found in the same size area (Fig. 3A and B, respectively). TEM images showed ~ 42 and 5 polyplexes prepared by DtoP and PtoD, respectively, per unit area (Fig. 3C and D, respectively). Assuming that all plasmid DNA was complexed (indicated by no migration in agarose gel; Fig. 1C and D), this result clearly shows a 6–8 fold difference in plasmid DNA copy numbers per polyplex prepared by different mixing sequences. It is infeasible to accurately measure the exact nucleic acid copy numbers per polyplex but the results shown in Fig. 3 clearly indicate that adding PEI to plasmid DNA forms polyplexes containing 6–8 times more plasmid DNA copies, hence a significantly larger size than the polyplexes prepared by adding plasmid DNA to PEI.

Similar to plasmid DNA/PEI polyplexes, the size and morphology of siRNA/PEI polyplexes were greatly affected by the mixing sequence. Adding siRNA to PEI (RtoP) formed much smaller polyplexes (~ 120 nm) than those prepared by adding PEI to siRNA (PtoR) (~ 400 nm) (Fig. 4A and B). Almost doubled sizes of siRNA/PEI polyplexes measured by AFM than DLS (Fig. 2A) can be explained by more significantly flattened siRNA/PEI polyplexes on the mica surface than plasmid DNA/PEI polyplexes. This indicates weaker intermolecular intervention of siRNA with PEI than plasmid DNA, which was also implied by lower EtBr fluorescence exclusion in siRNA/PEI polyplexes (Fig. 2B) than plasmid DNA/PEI polyplexes (Fig. 1B). The relatively inefficient siRNA complexation by PEI was also indicated by the absence of a core in the siRNA/PEI polyplexes (no white [high] central area) prepared by adding

Fig. 4 AFM images of the morphology of siRNA/PEI polyplexes prepared at an N/P ratio of 10 (A: siRNA/PEI polyplexes prepared by RtoP mixing; B: siRNA/PEI polyplexes prepared by PtoR mixing) and TEM images (C: siRNA/PEI polyplexes prepared by RtoP mixing; D: siRNA/PEI polyplexes prepared by PtoR mixing).

siRNA to PEI (RtoP) (Fig. 4A) and a substantially wider peripheral flat outer layer around the siRNA/PEI polyplexes prepared by adding PEI to siRNA (PtoR) (Fig. 4B), in comparison with plasmid DNA/PEI polyplexes (Fig. 3A and B). siRNA/PEI polyplexes in TEM images (Fig. 4C and D) were smaller than those in AFM images (Fig. 4A and B) but consistent with mixing sequences: smaller and more siRNA/PEI polyplexes generated by RtoP mixing than those formed by PtoR mixing. AFM image analyses counted an average of 7 and 2 siRNA/PEI polyplexes prepared by RtoP and PtoR mixing, respectively, per μm^2 area (Fig. 4A and B). In TEM images, an average of 20 siRNA/PEI polyplexes prepared by RtoP mixing were found, while only 3 of those prepared by PtoR mixing were observed (Fig. 4C and D, respectively). The difference in siRNA/PEI polyplex numbers in AFM and TEM images indicates that about 4–7 fold more siRNA copies per polyplex were complexed by PEI when PEI was added to siRNA (PtoR) than adding siRNA to PEI (RtoP), resulting in larger polyplexes (Fig. 4B).

Despite high interest, employing siRNA in basic research and clinical therapy has been hampered by its inefficient delivery^{33–35} that requires preparing stable siRNA-containing carriers such as polyplexes. It is known that cationic polymers with a high molecular weight are more efficient in complexing nucleic acids, particularly oligonucleotides (*e.g.*, siRNA), compared to small molecular weight polymers.^{36,37} Therefore, not only electrostatic interactions, which depend on the charge density, but also nucleic acid molecular weights play a crucial role in forming polyplexes in a collaborative manner. The results shown in Fig. 3 and 4 demonstrate that molecular ratios of nucleic acids to polymers in the initial mixing stage (*i.e.*, nucleic acid-dominant [PtoD and PtoR] *vs.* cationic polymer-dominant [DtoP and RtoP]) greatly affect the physical

characteristics of the resulting polyplexes, such as size, structure, degree of complexation, nucleic acid copy numbers per polyplex, and polyplex density, which affect biological properties (gene transfer efficiency).

Transfection, silencing, and cytotoxicity

Biological outcomes of nucleic acid delivery can be determined by many factors, including cellular uptake, intracellular trafficking, nucleic acid stability, and gene expression/silencing efficiency. A fundamental question raised in this study was whether internalization of many polyplexes that contain few nucleic acid copies (polyplexes prepared by DtoP and RtoP) would generate higher transfection/silencing than taking up a few polyplexes that contain many nucleic acid copies (polyplexes prepared by PtoD and PtoR). Answering this question could depend on the choice of measurement: the number of transfected/silenced cells (transfection rate) *vs.* the level of gene expression/silencing per cell. In Boussif *et al.*'s study, the authors used luciferase, a transgene model which allowed the measurement of total luciferase expression but prevented them from assessing the transfection rate.¹⁴ Therefore, they were not able to conclude whether the increased transfection by polyplexes prepared by gradually adding PEI to DNA was due to the higher number of cells expressing luciferase at similar levels or similar number of cells expressing luciferase at higher levels. In this study, GFP-encoding plasmid DNA and anti-GFP siRNA were used for conclusive findings. Whether polyplexes prepared with different mixing sequences would affect the cell viability, which may also be relevant to transfection/silencing levels, was tested as well.

NIH 3T3 cells were incubated with GFP plasmid DNA/PEI polyplexes prepared by adding plasmid DNA to PEI (DtoP) or adding PEI to plasmid DNA (PtoD) at a concentration of 0.6 μg plasmid DNA mL^{-1} at an N/P ratio of 10. As shown in Fig. 5A, a comparable number of cells were transfected by GFP plasmid DNA/PEI polyplexes, regardless of the mixing sequence. This is possibly due to the fact that both polyplexes greatly outnumbered the cells (Fig. 3 and 4). Noticeably, the cells transfected by GFP plasmid DNA/PEI polyplexes prepared

by PtoD mixing showed significantly higher GFP expression, possibly attributed to the more efficient intracellular trafficking due to faster sedimentation,^{21,28} the higher proton sponge effect,²² and the more plasmid DNA copies per polyplex than those transfected by the polyplexes prepared by DtoP mixing (Fig. 5B). However, the GFP plasmid DNA/PEI polyplexes prepared by PtoD mixing turned out to be more cytotoxic than those prepared by DtoP mixing (Fig. 5C). Since there were less viable cells upon incubation with the polyplexes prepared by PtoD mixing than those incubated with the polyplexes prepared by DtoP, lumped transfection by both the number of transfected cells (Fig. 5A) and the expression level of transgene per transfected cell (Fig. 5B) would be similar, independent of the mixing sequence. As demonstrated in Fig. 5, depending on the method of transfection efficiency quantification, it is possible for one to not find a difference in transfection by the polyplexes prepared by different mixing sequences.²² The results shown in Fig. 5 indicate that less cells were transfected with more copies of plasmid DNA when incubated with the polyplexes prepared by PtoD mixing, which resulted in higher gene expression and cytotoxicity than those prepared by DtoP. Therefore, adding PEI to plasmid DNA (PtoD) incorporated more copies of plasmid DNA in the resulting polyplexes in comparison with those prepared by adding plasmid DNA to PEI (DtoP), but their larger size and higher surface charge (Fig. 1A) generated a significantly higher cytotoxicity.^{38,39}

NIH 3T3 cells expressing GFP (NIH 3T3/GFP cells) were incubated with siRNA/PEI polyplexes at 0.6 μg siRNA mL^{-1} at an N/P ratio of 10 (Fig. 6). Similar to GFP plasmid DNA/PEI polyplexes, GFP siRNA/PEI polyplexes prepared by adding PEI to siRNA (PtoR) were more efficient in silencing GFP expression than those prepared by adding siRNA to PEI (RtoP) (Fig. 6A). However, unlike GFP plasmid DNA/PEI polyplexes, the viability of the cells incubated with GFP siRNA/DNA polyplexes was not affected by the mixing sequence (Fig. 6B). The finding is interesting because GFP siRNA/PEI polyplexes prepared by RtoP mixing were smaller (~ 60 nm; better complexes) (Fig. 2A) in a significantly larger number (Fig. 4A and C) than GFP siRNA/PEI polyplexes prepared by PtoR mixing (~ 180 nm) (Fig. 2B, 4B, and D). It is a general consensus that smaller particles are more efficiently endocytosed than larger ones

Fig. 5 Transfection of NIH 3T3 cells by GFP plasmid DNA/PEI polyplexes prepared by adding plasmid DNA to PEI (DtoP) or adding PEI to plasmid DNA (PtoD) at an N/P ratio of 10, represented by (A) the ratio of transfected cells to total cells (transfection rate), (B) the mean GFP expression level of transfected cells, and (C) the relative viability of the cells. The cell viability and the GFP expression were measured after 1 and 3 days, respectively, after incubation with the polyplexes.

Fig. 6 *In vitro* studies of siRNA/PEI polyplexes prepared at an N/P ratio of 10 incubated with NIH 3T3 cells stably expressing GFP. (A) GFP silencing measured after three days of incubation. (B) Relative viability of cells measured after one day of incubation.

(a rough cut-off to be ~ 150 nm).^{40,41} The fact that larger polyplexes prepared by PtoR mixing exhibited higher gene silencing can be explained by the faster sedimentation of larger particles on cells *in vitro* and fast siRNA release into the cytoplasm from the loosely formed polyplexes. Also, a high load of siRNA per polyplex provides abrupt release of siRNA into the cytosol for complete and faster mRNA silencing. It is known that cytosolic release of siRNA is a key efficiency-determining step in gene silencing.⁴² It should be noted that GFP silencing is a lumped quantification based on the extent of the decrease in overall fluorescence signals because the unequal GFP expression levels in individual cells prevents from quantitatively estimating how many individual cells were undergoing GFP silencing. In other words, significant silencing in a few cells may significantly contribute to overall gene silencing. Therefore, it can be inferred that PtoR polyplexes in a larger size and a smaller number than RtoP polyplexes encapsulated more copies of siRNA for more efficient gene silencing.

Nonviral gene delivery in cell culture is often poorly correlated in animal models and clinical settings. Although the polyplexes prepared by PtoD and PtoR mixing showed significantly higher gene expression and silencing, respectively, than those prepared by DtoP and RtoP *in vitro* (Fig. 5 and 6), those preparations may not guarantee direct translation *in vivo*. For example, the large polyplexes prepared by PtoD and PtoR could be inefficient for tissue penetration or sequestered fast due to their higher cationic charge (Fig. 1A, 2A, 3B and 4B).^{43–47} In addition, they might be relatively unstable under physiological conditions due to loose complexation. Serum triggers polyplex aggregation and may diminish the difference of performance of the two sets of polyplexes under physiologically relevant conditions. Therefore, the optimized mixing sequence verified in cell culture in this study needs to be further evaluated for additional criteria applicable to *in vivo* gene delivery. There have been approaches to address this potential concern such as PEGylation. Although the primary scope of the study was to investigate the mixing sequence-dependent nucleic acid complexation by cationic polymers, it would still be interesting to investigate whether the mixing sequence of the nucleic acid complexation by PEG-conjugated cationic polymers would result in similar observations.

A model for nucleic acid complexation by cationic polymers

As shown in Fig. 1 and 3, adding plasmid DNA to PEI (DtoP) formed polyplexes that are smaller with a lower zeta-potential in a significantly larger number than those prepared by adding PEI to plasmid DNA (PtoD). As shown in Fig. 3B, adding PEI to plasmid DNA formed large polyplexes with a dense core and flat outer layer. Based on these observations, we developed a model explaining how the mixing sequence affects the physico-chemical properties of plasmid DNA/PEI polyplexes. At an N/P ratio of 10, 6.3×10^{14} PEI molecules (25 kDa) were mixed with 4.0×10^{12} plasmid DNA molecules (~ 3000 kDa) (~ 150 times more PEI molecules than plasmid DNA molecules). At the very moment when the first drop of plasmid DNA-containing solution is added to a PEI-containing

solution, PEI that is significantly smaller than plasmid DNA but excess in number immediately surrounds plasmid DNA and compact it. As more plasmid DNA is added, abundant PEI condenses them into small particulates. Cationic PEI in excess stabilizes the particles and prevent from further aggregation.⁴⁸ As a result, only a small number of plasmid DNA is surrounded by PEI per polyplex. Since PEI remains in excess relative to plasmid DNA, further addition of plasmid DNA continues forming small polyplexes containing a few copies of plasmid DNA as illustrated in Fig. 7 and experimentally observed in Fig. 3A and C. In the case of adding PEI to plasmid DNA, PEI plays a role as an electrostatic glue and aggregates abundant plasmid DNA. As more PEI is added, reaching charge neutrality, secondary rearrangement of particles through bridging PEI occurs, leading to forming a PEI-rich outer layer around the large plasmid DNA/PEI aggregates with higher copies of plasmid DNA per particle as illustrated in Fig. 7 and also experimentally implied in Fig. 3B and D. Schatz *et al.* observed similar results with polyelectrolyte complexes of chitosan and dextran sulfate.^{48,49} However, it was found that when oppositely charged components were added in one-shot, rather than the dropwise approach as in this study, the process was independent of mixing sequences.⁵⁰ The interaction between siRNA and PEI molecules is anticipated to be different from that of plasmid DNA and PEI. Since the persistence length of double-stranded RNA is ~ 260 bps, siRNA (21 bps) behaves as a rigid rod.^{51–53} Therefore, whereas

Fig. 7 A proposed model for mixing sequence-dependent formation of plasmid DNA/PEI polyplexes. At very initial stages of mixing ($t = \Delta t$, $\Delta t \rightarrow 0$), plasmid DNA is surrounded by a cationic polymer (PEI) in excess (above), while addition of PEI to plasmid DNA results in aggregation (below). Further addition of plasmid DNA to PEI or PEI to plasmid DNA ($t \gg 0$) generates small polyplexes containing fewer copies of condensed plasmid DNA in a large number (above) or large polyplexes containing more copies of condensed plasmid DNA in a small number, respectively (below).

large and flexible plasmid DNA is condensed in size by electrostatic attraction with PEI, short and rigid siRNA is not likely to be further condensed due to its stiffness. At an N/P ratio of 10, there are ~ 1.5 times more siRNA molecules (13 kDa ; 9.3×10^{14}) than PEI molecules (25 kDa ; 6.3×10^{14}). At an initial stage of adding siRNA to PEI (RtoP), incoming siRNA is immediately complexed by flexible PEI abundant in solution. Steric crowding of the polymer on the finite surface area of siRNA impedes further binding of PEI resulting in a small polyplex with a few siRNA copies. Continued addition of siRNA to PEI ends up forming many small polyplexes that contain a few copies of siRNA. In contrast, when PEI is added to a siRNA solution (PtoR), several siRNA molecules that are abundant in solution immediately interact with incoming PEI, which is larger than siRNA, resulting in many copies of siRNA being incorporated per polyplex. As more PEI comes in, pre-existing polyplexes rearrange and aggregate due to PEI working as intermolecular glue resulting in larger polyplexes with more siRNA per polyplex as well as loosely bound PEI molecules on the periphery. If a multimeric siRNA possessing a higher charge density and relative flexibility is used, higher gene silencing *via* significantly improved complexation by PEI, similar to the illustration in Fig. 7, can be obtained.⁵⁴ How multi-linked siRNA is complexed with PEI by different mixing sequences in comparison with plasmid DNA and monomeric siRNA would be an interesting subsequent study.

According to the proposed model, adding PEI to nucleic acids (PtoD and PtoR) leaves free PEI molecules not engaged

in polyplex formation (Fig. 7 and 8). This is particularly more significant in PtoD mixing of ~ 150 times excess PEI to plasmid DNA than PtoR mixing of siRNA and PEI in roughly comparable numbers, at an N/P ratio of 10. As reported previously, the cytotoxicity of nucleic acid/polymer polyplexes is attributed to free cationic polymers.^{55–58} Therefore, the significantly higher cytotoxicity of the GFP plasmid DNA/PEI polyplexes prepared by adding PEI to plasmid DNA (Fig. 5C) can also be explained by free PEI in excess upon complete complexation (Fig. 7). Our model illustrating the effect of the mixing sequence on the polyplex preparation stresses the importance of controlling molecular interactions between nucleic acids and cationic polymers in determining physico-biological properties of the resulting polyplexes. Employing a novel method that allows precise, differential control on nucleic acids and cationic polymers would further validate the proposed model. For example, microfluidic devices would be able to provide in depth description of how polyplex formation is affected by the mixing sequences since they offer many advantages, including small scale preparation, easy manipulation of the concentration and environment, fast response to external stimulations, and continuous monitoring and analysis.

Conclusions

Mixing sequence-dependent complexation of nucleic acids (plasmid DNA and siRNA) by a cationic polymer (PEI) and its effect on the gene transfer efficiency were investigated. Gradually adding a plasmid DNA-containing solution to a PEI-containing solution resulted in the formation of many small polyplexes, while fewer and larger polyplexes were formed in the reverse method (*i.e.*, adding PEI to plasmid DNA), indicating a significant difference in plasmid DNA copy numbers per polyplex. The cells transfected by the plasmid DNA/PEI polyplexes prepared by adding PEI to plasmid DNA showed higher transgene expression, but with higher cytotoxicity, than those prepared by the other. Similar phenomena, such as higher gene silencing by siRNA/PEI polyplexes prepared by adding PEI to siRNA, which are larger in size but smaller in number, to those prepared by adding siRNA to PEI were also observed, however with comparable cytotoxicity. Experimental findings led to the development of a model explaining the crucial roles of molecular interactions between nucleic acids and cationic polymers in determining physical and biological properties of polyplexes. This study can further be applied to other popularly used nucleic acids/cationic polymer polyplexes and may provide insightful information about designing and evaluating novel nonviral gene delivery carriers.

Acknowledgements

This work was financially supported by the National Science Foundation (CHE-0748912), Gabrielle's Angel Foundation for

Fig. 8 A proposed model for mixing sequence-dependent formation of siRNA/PEI polyplexes. At very initial stages of mixing ($t = \Delta t$, $\Delta t \rightarrow 0$), siRNA is paired by a cationic polymer (PEI) in excess (above), while addition of PEI to siRNA results in aggregation (below). Further addition of siRNA to PEI or PEI to siRNA ($t \gg 0$) generates small polyplexes containing a few copies of siRNA in a large number (above) or large polyplexes containing many copies of siRNA in a small number, respectively, without condensation (below).

Cancer Research (Award 56), and the National Science Foundation (DMR-0956091).

Notes and references

- 1 E. R. Burnight, L. A. Wiley, A. V. Drack, T. A. Braun, K. R. Anfinson, E. E. Kaalberg, J. A. Halder, L. M. Affatigato, R. F. Mullins, E. M. Stone and B. A. Tucker, *Gene Ther.*, 2014, **21**, 662.
- 2 M. W. Huston, A. R. A. Riegman, R. Yadak, Y. van Helsdingen, H. de Boer, N. P. van Til and G. Wagemaker, *Hum. Gene Ther.*, 2014, **25**, 905.
- 3 J. Guo, M. R. Cahill, S. L. McKenna and C. M. O'Driscoll, *Biotechnol. Adv.*, 2014, **32**, 1396.
- 4 C. W. Olanow, *Nat. Rev. Neurol.*, 2014, **10**, 186.
- 5 R. C. Mulligan, *Science*, 1993, **260**, 926.
- 6 S. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494.
- 7 C. A. Stein and Y. C. Cheng, *Science*, 1993, **261**, 1004.
- 8 M. A. Kotterman and D. V. Schaffer, *Nat. Rev. Genet.*, 2014, **15**, 445.
- 9 R. Keswani, K. Su and D. W. Pack, *J. Controlled Release*, 2014, **192**, 40.
- 10 S. Li and L. Huang, *Gene Ther.*, 2000, **7**, 31.
- 11 H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, *Nat. Rev. Genet.*, 2014, **15**, 541.
- 12 S. C. De Smedt, J. Demeester and W. E. Hennink, *Pharm. Res.*, 2000, **17**, 113.
- 13 E. Wagner, M. Zenke, M. Cotton, H. Beug and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 3410.
- 14 O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297.
- 15 W. T. Godbey, K. K. Wu and A. G. Mikos, *J. Controlled Release*, 1999, **60**, 149.
- 16 K. Suh, H.-J. Paik and B. K. Hwang, *Bioorg. Chem.*, 1994, **22**, 318.
- 17 B. Abdallah, A. Hassan, C. Benoist, D. Goula, J. P. Behr and B. A. Demeneix, *Hum. Gene Ther.*, 1996, **7**, 1947.
- 18 D. Goula, C. Benoist, S. Mantero, G. Merlo, G. Levi and B. A. Demeneix, *Gene Ther.*, 1998, **5**, 1291.
- 19 W. T. Godbey, K. K. Wu and A. G. Mikos, *J. Biomed. Mater. Res.*, 1999, **45**, 268.
- 20 M. X. Tang and F. C. Szoka, *Gene Ther.*, 1997, **4**, 823.
- 21 M. Ogris, P. Steinlein, M. Kurs, K. Mechtler, R. Kircheis and E. Wagner, *Gene Ther.*, 1998, **5**, 1425.
- 22 R. Kircheis, L. Wightman and E. Wagner, *Adv. Drug Delivery Rev.*, 2001, **53**, 341.
- 23 M. S. Shim and Y. J. Kwon, *Bioconjugate Chem.*, 2009, **20**, 488.
- 24 A. Boletta, A. Benigni, J. Lutz, G. Remuzzi, M. R. Soria and L. Monaco, *Hum. Gene Ther.*, 1997, **8**, 1243.
- 25 D. Finsinger, J. S. Remy, P. Erbacher, C. Koch and C. Plank, *Gene Ther.*, 2000, **7**, 1183.
- 26 M. Ogris, S. Brunner, S. Schuller, R. Kircheis and E. Wagner, *Gene Ther.*, 1999, **6**, 595.
- 27 P. Erbacher, T. Bettinger, P. Belguise-Valladier, S. Zou, J. Coll, J. P. Behr and J. Remy, *J. Gene Med.*, 1999, **1**, 210.
- 28 O. Boussif, M. A. Zanta and J. P. Behr, *Gene Ther.*, 1996, **3**, 1074.
- 29 M. Zheng, G. M. Pavan, M. Neeb, A. K. Schaper, A. Danani, G. Klebe, O. M. Merkel and T. Kissel, *ACS Nano*, 2012, **6**, 9447.
- 30 D. L. Gebhart and A. V. Kabanov, *J. Controlled Release*, 2001, **73**, 401.
- 31 H. Peterson, P. M. Fechner, A. L. Martin, K. Kunath, S. Stolnik, C. J. Roberts, D. Fischer, M. C. Davies and T. Kissel, *Bioconjugate Chem.*, 2002, **13**, 845.
- 32 Z. Dai and C. Wu, *Macromolecules*, 2012, **45**, 4346.
- 33 M. S. Shim and Y. J. Kwon, *FEBS J.*, 2010, **277**, 4814.
- 34 S. D. Li and L. Huang, *Gene Ther.*, 2006, **13**, 1313.
- 35 K. A. Whitehead, R. Langer and D. G. Anderson, *Nat. Rev. Drug Discovery*, 2009, **8**, 129.
- 36 A. Zintchenko, A. Philipp, A. Dehshahri and E. Wagner, *Bioconjugate Chem.*, 2008, **19**, 1448.
- 37 A. C. Richards Grayson, A. M. Doody and D. Putnam, *Pharm. Res.*, 2006, **23**, 1868.
- 38 S. Werth, B. Urban-Klein, L. Dai, S. Hobel, M. Grzelinski, U. Bakowsky, F. Czubayko and A. Aigner, *J. Controlled Release*, 2006, **112**, 257.
- 39 K. Kunath, A. Harpe, D. Fischer, H. Peterson, U. Bickel, K. Voigt and T. Kissel, *J. Controlled Release*, 2003, **89**, 113.
- 40 S. D. Conner and S. L. Schmid, *Nature*, 2003, **422**, 37.
- 41 C. He, Y. Hu, L. Yin, C. Tang and C. Yin, *Biomaterials*, 2010, **31**, 3657.
- 42 G. Sahay, W. Querbies, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer and D. G. Anderson, *Nat. Biotechnol.*, 2013, **31**, 653.
- 43 R. K. Jain and T. Stylianopoulos, *Nat. Rev. Clin. Oncol.*, 2010, **7**, 653.
- 44 R. L. Kanasty, K. A. Whitehead, A. J. Vegas and D. G. Anderson, *Mol. Ther.*, 2012, **20**, 513.
- 45 M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul and J. P. Benoit, *Biomaterials*, 2008, **29**, 3477.
- 46 F. Alexis, E. Pridgen, L. K. Molnar and O. C. Farokhzad, *Mol. Pharm.*, 2008, **5**, 505.
- 47 D. Bazile, C. Prud'homme, M. T. Bassoullet, M. Marlard, G. Spenlehauer and M. Veillard, *J. Pharm. Sci.*, 1995, **84**, 493.
- 48 C. Schatz, J. M. Lucas, C. Viton, A. Domard, C. Pichot and T. Delair, *Langmuir*, 2004, **20**, 7766.
- 49 A. Drogoz, L. David, C. Rochas, A. Domard and T. Delair, *Langmuir*, 2007, **23**, 10950.
- 50 C. Schatz, A. Domard, C. Viton, C. Pichot and T. Delair, *Biomacromolecules*, 2004, **5**, 1882.
- 51 P. Kebbekus, D. E. Draper and H. Hagerman, *Biochemistry*, 1995, **34**, 4354.
- 52 D. J. Gary, N. Puri and Y. Y. Won, *J. Controlled Release*, 2007, **121**, 64.

- 53 A. Noy, A. Perez, F. Lankas, F. J. Luque and M. Orozco, *J. Mol. Biol.*, 2004, **343**, 627.
- 54 H. Mok, S. H. Lee, J. W. Park and T. G. Park, *Nat. Mater.*, 2010, **9**, 272.
- 55 J. P. Clamme, J. Azoulay and Y. Mely, *Biophys. J.*, 2003, **84**, 1960.
- 56 Y. Yue, F. Jin, R. Deng, J. Cai, Y. Chen, M. C. M. Lin, H. F. Kung and C. Wu, *J. Controlled Release*, 2011, **155**, 67.
- 57 S. Boeckle, K. von Gersdorff, S. van der Piepen, C. Culmsee, E. Wagner and M. Ogris, *J. Gene Med.*, 2004, **6**, 1102.
- 58 J. Fahmeir, M. Gunther, N. Tietze, E. Wagner and M. Ogris, *J. Controlled Release*, 2007, **122**, 236.