Analysis of PEI transfection by subcellular fractionation and radiolabeling reveals surprising differences between the intracellular trafficking of DNA and PEI

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ABSTRACT SUMMARY
Differential and density-gradient centrifugation techniques were used to determine the intracellular distribution of radiolabeled 25 kD branched polyethylenimine (bPEI)/plasmid DNA complexes (“polyplexes”) in HeLa cells over time. By differential centrifugation, [14C]bPEI was found mostly in the lighter fractions whereas [3H]DNA was found mostly in the heavier fractions. A majority of the intracellular polymer (~60%) and DNA (~90%) was found in the nuclear fraction. Polymer and DNA also differed in their distribution to heavier and denser organelles (lysosomes, mitochondria) in density-gradient centrifugation studies indicating that these components traffic separately after cell internalization. An unexpected finding from this study was that while DNA accumulated in cells over time to 18-50% of input amount, polymer concentrations remained relatively constant at only 1-6% of input amount. These results suggest that a significant amount of cationic polymer is dissociated from the DNA cargo early on in the transfection process.

INTRODUCTION
Non-viral vectors, such as cationic polymers and lipids, have been extensively investigated for gene delivery. Although non-viral vectors are generally considered safer than viral vectors, they are much less efficient at delivering nucleic acids to cells than viruses. Non-viral vectors encounter several barriers to gene delivery that viruses readily overcome, such as cellular uptake, endosomal escape, cytoplasmic translocation, and gene expression. In order to enhance the design of non-viral vectors, studies to investigate the uptake and intracellular mechanism of these vectors are needed.

Subcellular fractionation methods can provide quantitative data on intracellular distribution of materials and has been used to determine amount of delivered DNA in various intracellular compartments with liposomal formulations and PEI, and to provide rate constants for quantitative models. The goal of this work is to quantify the intracellular distribution of cationic polymer and plasmid DNA (pDNA) complexes, or polyplexes, in native cell environments. We used differential and density-gradient subcellular fractionation methods combined with radiolabeling to track both branched polyethylenimine (bPEI) and pDNA in HeLa cells. We described here a detailed approach to intracellular polyplex quantification, in which, for the first time to our knowledge, both polymer carrier and cargo DNA are followed in major organelles involved in polyplex trafficking.

EXPERIMENTAL METHODS
PEI (branched, 25 kD) and luciferase-expressing plasmid DNA were radiolabeled using acetylation with [3H]deoxyctydine triphosphate, respectively. Unreacted reagents were removed using spin columns. The resultant eluent was characterized using a Cu(II) acetate assay and scintillation counting.

Radiolabeled materials were used for uptake and fractionation studies. HeLa cells were used for all studies. For uptake studies, polyplexes were formulated at N/P 5 by mixing 10 µL [3H]DNA/unlabeled DNA mixture (0.1 g/L) with 10 µL bPEI (65.3 µg/mL) for 10 min at room temperature prior to a 10-fold dilution in OptiMEM. Polyplexes were then added onto seeded cells in 24-well plates, and incubated for various amounts of time (up to 4 h). At 4 h, the supernatant was removed and replaced with complete media for up to an additional 20 h. For fractionation studies, 5-20 × 10⁶ cells were treated with [3H]DNA/[14C]bPEI polyplexes (N/P 5) similarly to uptake studies and homogenized to generate a nuclear fraction, post-nuclear supernatant (PNS). The PNS was further fractionated via differential and/or density-gradient centrifugation to obtain organelle populations. In differential centrifugation experiments, the PNS was subjected to increasing g-forces (3000g, 15,000g, 100,000g) to obtain heavy mitochondrial (HM), light mitochondrial (LM), microsomal (MF), and cytosolic (C) fractions. In density-gradient centrifugation, the vesicular fraction was applied to a 5-20% iodixanol density gradient and centrifuged for 90,000g overnight. Fractions were collected off the gradient. All washes and fractions were analyzed by marker enzyme activity and immunoblotting to determine organelle populations. Washes and fractions were also analyzed for radioactivity.

RESULTS AND DISCUSSION
As a first approach to determine the time dependency of cellular uptake and internalization of polyplexes, a pulse-chase experiment was performed using [3H]DNA/bPEI polyplexes. During the 4 h pulse period, the rate of surface-association and cellular internalization of polyplexes, as determine by scintillation counting for the radiolabeled plasmid, appeared linear with time, with ~50% of polyplexes associating with cells after 4 h (Figure 1). During the chase period, the percentage of cell surface-associated polyplexes decreased over time as the percentage of internalized polyplexes increased over time. The percentage of polyplexes also increased slightly in the chase media over time, indicating possible gradual dissociation of polyplexes from the cell surface or polyplex exocytosis. These results suggest that the intracellular distribution of polyplexes is shifting from association with the plasma membrane to trafficking through intracellular organelles over the duration of 24 h.
Cellular fractionation studies have been used to quantitatively assess the intracellular distribution of a number of polymer/liposome conjugates. In particular, differential centrifugation, in which organelles are separated on the basis of mass, is commonly used for cellular fractionation. Cells were treated with \[^{3}H\]DNA/\[^{14}C\]bPEI polyplexes for 1 h at 4 °C for binding, then 30 min and 4 h at 37 °C for internalization. Cells were then fractionated using differential centrifugation. After 30 min, the total percentage of \[^{3}H\]DNA and \[^{14}C\]bPEI associated with the cells was 15.5% and 1.2%, respectively. After 4 h, the percentage increased to 48.0% and 5.6%, respectively. The overall effective intracellular N/P is ~0.3-0.6, indicating either rapid exocytosis of displaced polymer after cellular uptake, or partial polyplex unpackaging before cellular uptake. After both 30 min and 4 h, a majority of \[^{3}H\]DNA and \[^{14}C\] polymer was found in the nuclear fraction (Figure 2a-b). Of the post-nuclear fractions, \[^{3}H\]DNA was found mostly in the HM, and then distributed slightly throughout the lighter fractions by 4 h (Figure 2c). In contrast, \[^{14}C\]bPEI was found distributed throughout all fractions, with more polymer found in the HM and LM at 30 min, and then shifts to the LM and MF fractions by 4 h (Figure 2d). These results indicate polymer, but not DNA, accumulates in vesicular organelles over time.

![Figure 2](image2.png)

**Figure 2.** Intracellular distribution of \[^{3}H\]DNA/\[^{14}C\]bPEI polyplexes in treated cells fractionated using differential centrifugation. (a,c) \[^{3}H\]DNA; (b,d) \[^{14}C\]bPEI.

As an alternative fractionation method, density-gradient centrifugation, in which organelles are separated by buoyant density, was evaluated for improved organelle separation. Prior to density-gradient centrifugation, the cytosol was initially separated from the vesicular compartments. To further investigate the polyplex distribution in the vesicular fraction, an iodixanol gradient was used to separate plasma membrane, endosomes, and lysosomes after cells were treated for 4 h with radiolabeled polyplexes. Similar trends to those observed in differential centrifugation experiments were seen in uptake of \[^{3}H\]DNA and \[^{14}C\]bPEI in density-gradient centrifugation. Overall, a bimodal distribution of both DNA and polymer was detected in the vesicular fraction as a function of density (Figure 3). The first peak (less dense) corresponded to fractions enriched in plasma membrane and endosomes. The second peak (more dense) corresponded to fractions enriched in lysosomes was offset between DNA and polymer, confirming that DNA and polymer traffic differentially in the cell.

![Figure 3](image3.png)

**Figure 3.** Distribution of radiolabeled polyplexes after 4 h pulse in cells fractionated using a 5-20% continuous iodixanol gradient.

**CONCLUSION**

We demonstrate the use of subcellular fractionation methods to quantitatively assess both polymer and DNA in intracellular compartments. By radiolabeling both the synthetic carrier and the cargo DNA, we were able to quantify the amount of each component in the media, cell-associated fractions, as well as various intracellular organelles, such as the plasma membrane, nuclei, cytosol, endosomes, lysosomes, and mitochondria. These studies described general method development for the quantitative analysis of polyplex intracellular distribution, and will be applied for studying the effect of various chemical moieties on polymeric gene carriers.

**REFERENCES**


**ACKNOWLEDGMENTS**

This work is supported by the Intracellular Delivery of Biologics through the Washington State Life Sciences Discovery Fund. JS is supported by NSFGRFP and the HHMI/UW Molecular Medicine Scholarship. JLC and BC are supported by the Mary Gates Undergraduate Research Fellowship.