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Nonviral vectors are safer than viral vectors, easy to prepare and purify, capable of carrying large cargos, and highly flexible for structural and functional modifications. Polyplexes, complexed nucleic acids with cationic polymers via attractive electrostatic interactions, are the most widely investigated form of nonviral vectors, due to easy and efficient preparation. However, nucleic acid complexation by cationic polymers has not been studied as intensively as design and synthesis of cationic polymers, and a considerable level of inconsistency in preparing this simple form of nonviral vectors exists. During the seemingly simple mixing of nucleic acids with cationic polymers, a number of factors (e.g., molecular weight and structure of cationic polymer, size of nucleic acids, ionic strength in mixture, mixing kinetics, concentration of nucleic acids and cationic polymers, and incubation time) play crucial roles. In this study we report that a sequence of mixing nucleic acids and cationic polymers greatly affect complexation kinetics and determine the key characteristics of the resulting polyplexes, hence, transfection efficiency. Both plasmid DNA (5 kbp; double stranded; circular) and siRNA (23 bps; single stranded; linear) were mixed with polyethylenimine (PEI), the most commonly used cationic polymer to complex nucleic acids, in two opposite sequences: gradually adding PEI to nucleic acids vs. gradually adding nucleic acids to PEI. Resulting polyplexes were closely characterized by dynamic light scattering (DLS), ethidium bromide exclusion assay, gel electrophoresis, atomic force microscopy (AFM), and transmission electron microscopy (TEM). In addition, cell viability, transfection effi ciency, and gene silencing efficiency were also correlated with the characteristics of the polyplexes. It was found that gradual addition of PEI to nucleic acid-containing solution resulted in fewer but larger polyplexes, compared with those prepared in the reverse sequence. This finding suggests that at initial mixing period where nucleic acids were in excess, PEI condensed multiple copies of nucleic acids into a single polyplex. Further addition of PEI resulted in stabilization of polyplexes. The polyplexes prepared by gradually adding PEI to a nucleic acid-containing solution transfected cells more effi ciently than those prepared in a reverse manner. This study underscores that not only lumped processes but also initial complexation kinetics needs to be carefully considered for optimal preparation of polyplexes.