Acta Biomaterialia 37 (2016) 120–130

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Quantification of cellular and nuclear uptake rates of polymeric gene delivery nanoparticles and DNA plasmids via flow cytometry

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form 17 March 2016
Accepted 24 March 2016
Available online 24 March 2016

Keywords:
Gene delivery
Nanoparticle
Polymer
Computational modeling
Brain cancer

ABSTRACT

Non-viral, biomaterial-mediated gene delivery has the potential to treat many diseases, but is limited by low efficacy. Elucidating the bottlenecks of plasmid mass transfer can enable an improved understanding of biomaterial structure–function relationships, leading to next-generation rationally designed non-viral gene delivery vectors. As proof of principle, we transfected human primary glioblastoma cells using a poly(beta-amino ester) complexed with eGFP plasmid DNA. The polyplexes transfected $70.6 \pm 0.6\%$ of the cells with $101 \pm 3\%$ viability. The amount of DNA within the cytoplasm, nuclear envelope, and nuclei was assessed at multiple time points using fluorescent dye conjugated plasmid up to 24 h post-transfection using a quantitative multi-well plate-based flow cytometry assay. Conversion to plasmid counts and degradation kinetics were accounted for via quantitative PCR (plasmid degradation rate constants were determined to be $0.62\ h^{-1}$ and $0.084\ h^{-1}$ for fast and slow phases respectively). Quantitative cellular uptake, nuclear association, and nuclear uptake rate constants were determined by using a four-compartment first order mass-action model. The rate limiting step for these poly(beta-amino ester)/DNA polyplex nanoparticles was determined to be cellular uptake ($7.5 \times 10^{-4}\ h^{-1}$) and only 0.1% of the added dose was taken up by the human brain cancer cells, whereas 12% of internalized DNA successfully entered the nucleus (the rate of nuclear internalization of nuclear associated plasmid was $1.1\ h^{-1}$). We describe an efficient new method for assessing cellular and nuclear uptake rates of non-viral gene delivery nanoparticles using flow cytometry to improve understanding and design of polymeric gene delivery nanoparticles.

Abbreviations: 447, B4-S4-E7; bp, base pair (nucleotides); CT, cycle threshold; DAPI, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine; DMSO, dimethyl sulfoxide; DTS, DNA-targeted sequence; EDTA, ethylenediaminetetraacetic acid; eGFP, enhanced green fluorescent protein; EtOH, ethanol; ER, efficiency of replication; FBS, fetal bovine serum; $Fluor_{PR}$, Fluorescence according to the plate reader; FRET, Förster resonance energy transfer; gDNA, genomic DNA; GPC, gel permeation chromatography; k_{bd}, rate constant of plasmids being recycled from either the nuclear envelope or being internal to the nucleus to the cytoplasm; k_{cell}, rate constant of plasmids into the cell; k_{deg1}, fast degradation constant of plasmid DNA; k_{deg2}, slow degradation constant of plasmid; k_{ne}, rate constant of plasmid onto the nuclear envelope; k_{ni}, rate constant of plasmids entering nucleus; M_n, number-average molecular weight; M_w, weight-average molecular weight; N:D, nucleotide to dye ratio; NaAc, sodium acetate; NGM, normalized geometric mean; NLS, nuclear localization signal; PBAE, poly(beta-amino ester); NMR, nuclear magnetic resonance; P_{cyto}, plasmid number within the cytoplasm; PDI, polydispersity index; pDNA, plasmid DNA; $Plasmid_{corr}$, corrected plasmid number; $Plasmid_{PR}$, plasmid number according to the plate reader calibration; $Plasmid_{qPCR}$, plasmid number according to qPCR; P_{ne}, plasmid number on the nuclear envelope; P_{ni}, plasmid number within the nucleus; SDS, sodium dodecyl sulfate; SRS, sum of the residuals squared; SV40, simian virus-40; THF, tetrahydrofuran; $\mathfrak{A}$, Heaviside function.

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http://dx.doi.org/10.1016/j.actbio.2016.03.036
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Statement of Significance

In this work, a quantitative high throughput flow cytometry-based assay and computational modeling approach was developed for assessing cellular and nuclear uptake rates of non-viral gene delivery nanoparticles. This method is significant as it can be used to elucidate structure–function relationships of gene delivery nanoparticles and improve their efficiency. This method was applied to a particular type of biodegradable polymer, a poly(beta-amino ester), that transfected human brain cancer cells with high efficacy and without cytotoxicity. A four-compartment first order mass-action kinetics model was found to model the experimental transport data well without requiring external fitting parameters. Quantitative rate constants were identified for the intracellular transport, including DNA degradation rate from polyplexes, cellular uptake rate, and nuclear uptake rate, with cellular uptake identified as the rate-limiting step.

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1. Introduction

Gene therapy has the potential to treat inheritable diseases such as cystic fibrosis [1], Duchenne muscular dystrophy [2,3], and hemophilia [4] as well as acquired diseases such as cancer [5,6]. In general, viruses are highly efficient in delivering nucleic acid with a multiplicity of infection as low as one viral particle per cell [7], but are immunogenic and can cause insertional mutagenesis [8]. Non-viral methods, although generally less efficient at delivering nucleic acids than viral methods, are easier to scale-up from a manufacturing standpoint, are easily chemically modified for optimization of function, and can have low toxicity and host immune response [9,10].

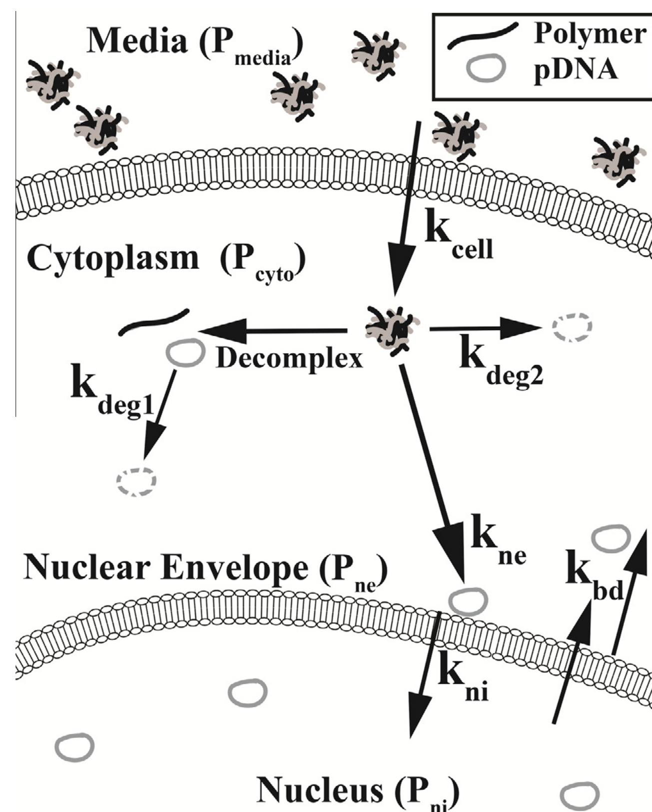
Although high-throughput synthesis and screening methods can enable the evaluation of a large library of biomaterials for non-viral gene delivery [11], an improved understanding of polymeric structure–function relationships could enable improved rational design of next-generation non-viral gene delivery vectors. Quantifying plasmid cellular and nuclear uptake rates and differences can elucidate salient functional differences between biomaterial structures and can reveal bottlenecks associated with specific vectors. PCR is a well-established method to quantify the number of plasmids within the cell or nucleus [12,13]. However, lysing cells and nuclei, isolating and purifying DNA, and then subsequently running PCR on the samples is a time-consuming process that is less amenable to scale up for semi-high throughput analysis of multiple time points and multiple structures.

This work describes a new approach to quantify the number of plasmids within the cell's cytoplasm (P_{cyto}), associated with nuclei (P_{ne}), and internalized by nuclei (P_{ni}) based on flow cytometry. Through the use of mathematical fitting to a system of differential equations and a four-compartment model, this method also allows the quantitative determination of cellular and nuclear uptake rate constants. We synthesized a newly discovered and leading non-viral polymeric vector, poly(beta-amino ester) (PBAE) B4-S4-E7 or 447 [14,15] to transfect human primary glioblastoma cells and subsequently evaluated its intracellular delivery properties using this flow cytometry-based method. This technique can be utilized to quantify the rate constants associated with cellular entry (k_{cell}), nuclear envelope association (k_{ne}), and nuclear internalization (k_{ni}) for a given non-viral vector (Scheme 1). This tool could be used to further elucidate biomaterial structure–function relationships of non-viral gene delivery vectors [16,17] and quantitative characterization could enable improved design of next generation polymer vectors [18].

2. Materials and methods

2.1. Polymer synthesis

Polymer B4-S4-E7 (447) was synthesized via a Michael addition reaction (Scheme 2) by adding 4-amino-1-butanol (S4) neat to 1,4-butanediol diacrylate (B4) in a 1.2:1 B4:S4 monomeric ratio, immediately vortexed, and placed in a 90 °C oven for 24 h in the dark using a stir bar greater than 100 RPM, resulting in a B4-S4



Scheme 1. Depiction of plasmid transfer rates between compartments of interest. The four compartments of interest are: the media (P_{media}), the cytoplasm (P_{cyto}), nuclear envelope-associated (P_{ne}), and nuclei-internalized (P_{ni}). The associated transfer rate constants between these four compartments are k_{cell}, k_{ne}, and k_{ni} respectively. k_{bd} is the rate to the cytoplasm from the nuclear envelope and from within the nucleus. The fast and slow degradation rate constants are k_{deg1} and k_{deg2} ,which are hypothesized to be associated with decomplexed and complexed plasmids, respectively.

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