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The impact of DNA topology on polyplex uptake and transfection efficiency in mammalian cells

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ABSTRACT

The effect of DNA vector topology when complexed to poly-L-lysine (PLL) and its quantification in transfection efficiency has not been fully addressed even though it is thought to be of importance from both production and regulatory viewpoints. This study investigates and quantifies cell uptake followed by transfection efficiency of PLL:DNA complexes (polyplexes) in Chinese hamster ovary (CHO) cells and their dependence on DNA topology. PLL is known for its ability to condense DNA and serve as an effective gene delivery vehicle. Characterization of PLL conjugated to a 6.9 kb plasmid was carried out. Dual labeling of both the plasmid DNA (pDNA) and PLL enabled quantitative tracking of the complexed as well as dissociated elements, within the cell, and their dependence on DNA topology. Polyplex uptake was quantified by confocal microscopy and image analysis. Supercoiled (SC) pDNA when complexed with PLL, forms a polyplex with a mean diameter of 139.06 nm (±0.84% relative standard error [RSE]), whereas open circular (OC) and linear-pDNA counterparts displayed mean diameters of 305.54 (±3.2% RSE) and 841.5 nm (±7.2% RSE) respectively. Complexes containing SC-pDNA were also more resistant to nuclease attack than its topological counterparts. Confocal microscope images reveal how the PLL and DNA remain bound post transfection. Quantification studies revealed that by 1 h post transfection 61% of SC-pDNA polyplexes were identified to be associated with the nucleus, in comparison to OC- (24.3%) and linear-pDNA polyplexes (3.5%) respectively. SC-pDNA polyplexes displayed the greatest transfection efficiency of 41% which dwarfed that of linear-pDNA polyplexes of 18.6%. Collectively these findings emphasize the importance of pDNA topology when complexed with PLL for gene delivery with the SC-form being a key pre-requisite.

1. Introduction

Non-viral gene delivery into mammalian cells is widely used in the biotechnology industry for the production of recombinant proteins requiring post translational modifications as well as considered for clinical trials in gene therapy vaccination. Gene transfer through such means often entails delivery of nucleic acids that are bound to a cationic polymer (polycations) resulting in plasmid DNA (pDNA) – polymer products, often referred to as polyplexes (Elouahabi and Ruysschaert, 2005). There are numerous polycations which have been found to operate by binding and condensing pDNA which facilitates uptake either as polyplexes alone or by encapsulation within liposome structures (Tsai et al., 1999; Lee and Huang, 1996). Such polycations have been shown to condense pDNA into confined structures of approximately 100–200 nm. These include that of polyethylenimine (PEI), poly-L-lysine (PLL), poly(amidoamine) (PAAs) and dendrimers amongst others (Ko et al., 2009; Zhang et al., 2009; Hartmann et al., 2008; Tsai et al., 1999; Dutta et al., 2008). Non-viral methods have been tested within mammalian cells such as Chinese hamster ovary (CHO) cells, as these cells allow high protein expression and can be successfully scaled up in culture (Liu et al., 2008; Derouazi et al., 2004). Short term transient transfection of CHO cells using polycation polyplexes has been reported to result in high protein yields (Reisinger et al., 2009; Schlaeger and Christensen, 1999) and successfully scaled up.

There are various factors which may affect the efficiency of polyplex uptake, and hence transfection efficiency, but remarkably the detailed mechanisms remain poorly understood. One area which has been neglected is the measure of the influence of pDNA topology. Previous reports focusing on DNA topology identified greater

Abbreviations: CHO cells, Chinese hamster ovary cells; DNA, deoxyribonucleic acid; EtBr, ethidium bromide; linear-pDNA, linear plasmid DNA; OC, open circular; OC-pDNA, open circular plasmid DNA; pDNA, plasmid DNA; PLL, poly-L-lysine; SC, supercoiled; SC-pDNA, supercoiled plasmid DNA.

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gene expression for supercoiled (SC) – pDNA through end point experiments (Akbuga et al., 2003; Remaut et al., 2006). However questions do remain concerning factors such as polyplex stability, biophysical characteristics and monitoring complexes post transfection, and whether such parameters are affected by DNA topology. Conventionally transfection studies usually employ SC-pDNA which exhibits a condensed shape which has been assumed to facilitate cellular uptake (Hsu and Uludag, 2008). However alternative plasmid topologies may be of interest. Plasmids can be linearized by single site digestion, which may increase DNA accessibility and thus favor gene expression, but may also be prone to aggregation and exposure to nuclease attack (Anada et al., 2005). Nicked plasmids (open circular – OC) containing a strand break adopt conformations intermediate between those of supercoiled and linear. Interestingly OC-pDNA have been reported to exhibit similar sizes to those of SC-pDNA when bound to the polymer; poly((2-dimethylamino)ethyl methacrylate) (Cherng et al., 1999).

The current study systematically compares the biophysical properties of SC, OC and linear forms of the same plasmid complexed with poly-L-lysine (PLL), focusing on the intracellular uptake of the three types of polyplexes by CHO cells. PLL was specifically selected as a gene carrier due to its effective ability to transfect cells and recommendation by key studies (Tsai et al., 1999). Advantages of using PLL over other polymers include its ease of DNA binding and condensation (Luo and Saltzman, 2000). Further benefits of PLL over its counterparts include its versatility whereby it can undergo chemical modification for tailor made transfection studies (Luo and Saltzman, 2000; Fu et al., 2011; von Erlach et al., 2011). By labeling both nucleic acid and polycation components of the polyplexes, the uptake of the vector could be followed by confocal microscopy. SC topology resulted in the formation of not only much tighter and more compact vectors, but also more nuclease resistant polyplexes. These highly condensed complexes resulted in much faster and more effective transport into the cell nucleus, and consequently significantly enhanced transgene expression.

2. Materials and methods

2.1. Plasmid DNA preparation

The plasmid; pSVβ – 6.9 kb (Promega, Southampton, UK) was propagated within *Escherichia coli* (*E. coli*) DH5α cells. Luria Bertani (LB) medium (Lennox, Fisher Bioreagent, Loughborough, Leicestershire) was used for cell culturing along with Nutrient Agar CM0003 (Oxoid, Basingstoke, Hampshire). LB medium was inoculated with plasmid-containing cells and grown for approximately 16 h in the presence of 100 μg/ml ampicillin at 37 °C within a rotary incubator at 250 rpm.

2.2. Plasmid DNA purification

Plasmids were routinely purified via Qiagen mini and maxi kits according to the manufacturer's protocol (Qiagen, Crawely, West Sussex). Purified plasmids were analyzed via agarose gel electrophoresis and quantified by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.3. Production of linear and nicked (open circular) plasmids

Purified SC-pDNA samples were both nicked and digested to generate open circular (OC) and linear topologies respectively. Plasmids were cut by restriction digestion via the Pst*I* enzyme (New England Biolabs). This enzyme was chosen as it cuts at a single site upon the respective plasmid (pSVβ). OC forms were obtained by treatment with the enzyme; Nt.B*st*NBI as this catalyses a single strand break (New England Biolabs). Generation of both linear- and OC-pDNA was carried out in accordance to the manufacturer's instructions. Following restriction digestion and nicking, enzymatic reactions were halted by heat treatment at 80 °C for 20 min followed by purification of the DNA via chloroform-phenol extraction (Sigma). DNA was further purified via ethanol precipitation which entailed treatment of DNA with 2.5 volumes of 100% ethanol followed by 0.1 volume of 3 M sodium acetate (Sigma) and incubation at −80 °C for 1 h. Samples were centrifuged on a bench top centrifuge for 20 min at maximum speed. DNA pellets were than washed with 70% ethanol and centrifuged for 10 min and DNA pellets were resuspended in endotoxin free TE buffer (10 mM Tris–Cl, pH 8.0, 1 Mm EDTA) (Qiagen).

2.4. 1% agarose gel electrophoresis

1% agarose gels were prepared with Tris–Borate EDTA (TBE) 10× concentrate (Sigma). Gels were stained with 500 μg/ml ethidium bromide (Sigma). Agarose gels were run at 100 V and 100 mA for 1 h.

2.5. Southern blot analysis

Biotinylated DNA samples (refer to *direct labeling of naked DNA*) were analyzed via electrophoresis. The gel was then treated with 0.2 M HCl (Sigma) at room temperature for 10 min. The gel was then rinsed in double distilled water (ddH_2O), followed by three 15 min period washes with 0.4 M NaOH (Sigma Aldrich). Transfer was carried out by the conventional wick procedure with 10× SSC buffer (saline sodium citrate stock [300 mM sodium citrate, pH 7.0, containing 1 M sodium chloride] (Sigma)) as the transfer agent with a 0.45 μm nitrocellulose membrane (Protran BA-85, Whatman) cut to the size of the gel to facilitate transfer overnight. The membrane was removed and rinsed briefly with ddH_2O and then cross linked within a CL-1000 ultraviolet cross linker (UVP). The membrane was then blocked for at least 1 h in 1% BSA/PBS followed by addition of streptavidin–horse radish peroxidase (Amersham Bioscience) for 15 min. Three 15 min washes with PBS (phosphate buffered saline [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4]) was then followed by treatment with ECL reagent (Amersham ECL Plus™, GE healthcare).

2.6. Production and characterization of PLL–pDNA polyplexes

Plasmids were bound with poly-L-lysine hydrobromide (PLL) (Sigma) of molecular weight, 9600 (Tsai et al., 1999). A constant amount of DNA (20 μg) was added to PLL to vary the charge ratio between the DNA and polycation in the presence of 1 mM HEPES buffer (pH 7.5) and Millipore filtered (0.2 μm filter) water. The charge ratio refers to the ratio of positively charged amine groups of PLL to that of the negatively charged phosphate groups of DNA. The amount of PLL which yields the desired charge ratio (2.67–66.67 μg) was obtained from Lollo et al. (2002). Individual aliquots of DNA and PLL of equal volume were prepared and then mixed and left at room temperature for 30 min. A total volume of 100 μl was used for polyplexes prior to the addition of cells for transfection.

2.7. Zeta potentials measurements

Zeta potentials were recorded via a Malvern Zetasizer Nanoseries instrument (Malvern Instruments, Malvern). A polystyrene standard (Malvern Instruments) was employed to calibrate the system. Following this 700 μl of the polyplex (20 μg/ml) was loaded within the system and zeta potentials were recorded. The temperature was set to 25 °C, along with the dispersant cell viscosity (to which values for water were used).