



Role of DNA topology in uptake of polyplex molecules by dendritic cells

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ABSTRACT

Dendritic cells (DCs) are an attractive target for DNA vaccines as they are potent antigen presenting cells. This study demonstrated how non-viral gene delivery to DCs involving complexes of poly-L-lysine (PLL) and plasmid DNA (pDNA) (polyplexes) showed dependence on DNA vector topology. DNA topology is of importance from both production and regulatory viewpoints. In our previous study with CHO cells we demonstrated that polyplex uptake was dependent on DNA topology whereby complexes containing supercoiled (SC) pDNA were smaller, more resistant to nucleases and more effectively condensed by PLL than open circular (OC) and linear-pDNA complexes.

In this study polyplex uptake in DCs was measured qualitatively and quantitatively by confocal microscopy along with gene expression studies and measurement of DC phenotype. PLL is known for its ability to condense DNA and serve as an effective gene delivery vehicle. Quantification studies revealed that by 1 h following uptake 15% ($\pm 2.59\%$ relative standard error [RSE]) of SC-pDNA polyplexes were identified to be associated (fluorescent co-localisation) with the nucleus, in comparison to no nuclear association identified for OC- and linear-pDNA complexes. By 48 h following uptake, 30% ($\pm 1.82\%$ RSE) of SC-pDNA complexes associated with the nucleus in comparison to 16% ($\pm 4.40\%$ RSE) and 12% ($\pm 6.97\%$ RSE) of OC- and linear-pDNA polyplexes respectively. Confocal microscopy images showed how DNA and PLL remained associated following uptake by dual labelling. Polyplex (containing 20 μg pDNA) gene expression (plasmid encoded *lacZ* [β -galactosidase] reporter gene) in DCs was greatest for SC-pDNA polyplexes at 14.12% unlike that of OC- (9.59%) and linear-pDNA (7.43%). DCs express cell surface markers which contribute towards antigen presentation. Polyplex gene expression did not alter DC phenotype through surface marker expression. This may be due to the pDNA dose employed (20 μg) as other studies have used doses as high as 200 μg pDNA to induce DC phenotypic changes. Although no change in DC phenotype occurred, this could be advantageous in terms of biocompatibility. Collectively these results indicate that DNA topology is an important parameter for DC vector design, particularly pDNA in the SC conformation in regards to DNA vaccination studies.

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1. Introduction

Dendritic cells (DCs) are key components of the immune system which function by binding and collecting antigens. Following recognition, DCs present the antigen of interest through selective surface markers to T-cells in order to activate a specified immune response [1]. Antigen presentation also stimulates the differentiation of T-cells to B cells which release antibodies specific for the antigen of interest. It is these functions that researchers aim to exploit in the production of vaccines.

Non-viral gene delivery to DCs is an attractive approach for DNA vaccination to elicit immune responses towards encoded antigenic sequences [2]. Non-viral techniques often entail delivery of nucleic acids that are bound to a cationic polymer (polycations) resulting in plasmid DNA (pDNA) – polymer products, known as polyplexes [3]. Polycations operate by binding and condensing pDNA into smaller structures thereby facilitating uptake. Poly-L-lysine (PLL) is a widely used polycation DNA condensing agent which has been shown to be effective in inducing confined pDNA structures. Benefits offered by PLL over other polycations include the ease and rapid ability by which it binds with DNA, and versatility to undergo chemical modification allowing successful delivery of genes [4,5].

Key factors that affect polyplex uptake in DCs should be considered in regards to vaccine design. One parameter is the influence of pDNA topology. Plasmids naturally confer to a dense compact form referred to as supercoiled (SC), whereas a single strand nick can generate an open circular (OC) conformation. Restriction digestion of the double stranded pDNA results in a linearised form [6].

Abbreviations: DCs, dendritic cells; DNA, deoxyribonucleic acid; 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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Few studies have analysed the effect of pDNA topology on polyplex gene expression, with some identifying superior reporter gene expression for SC-pDNA [6–8]. We have previously reported DNA topology dependent uptake of polyplexes within Chinese hamster ovary (CHO) cells [9]. However polyplex uptake and the influence of DNA topology in DCs have not been studied in great depth. This study addresses polyplex uptake within DCs to deduce whether parameters such as pDNA topology affect uptake, gene expression and DC phenotype, which are important considerations for vaccine design.

2. Materials and methods

2.1. Plasmid DNA preparation and purification

The plasmid; pSV β – 6.8 kb (Promega, Southampton, UK) was propagated within *Escherichia coli* (*E. coli*) DH5 α cells. Plasmids were purified and quantified as previously reported by Dhanoya et al. [9].

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

Purified supercoiled (SC)-pDNA samples were both nicked and digested to generate open circular (OC) and linear topologies respectively. This method was carried out according to our protocol, previously reported in Dhanoya et al. [9].

2.3. Production of PLL/DNA polyplexes

Plasmids were bound with poly-L-lysine hydrobromide (PLL) (Sigma) of molecular weight, 9600 according to Dhanoya et al. [9]. A total volume of 100 μ l was used for polyplexes prior to the addition of cells for transfection.

2.4. Fluorescent tagging of PLL

PLL was labelled with Oregon Green 488, succinimidyl ester (Invitrogen) according to a previous study [10]. Unbound dye was removed by spin column purification in accordance to the manufacturer's protocol (Invitrogen).

2.5. Labelling of naked pDNA

Naked pDNA was labelled via the nucleic acid fluorescent stain; TOTO-3 (Dimeric Cyanine Nucleic Acid Stains–Invitrogen) at a final concentration of 4 μ M as carried out by Dhanoya et al. [9]. The fluorescent stain exhibits excitation and emission spectra of 642 and 660 nm, respectively for analysis via confocal microscopy.

2.6. Generation of human monocyte-derived dendritic cells

This study was approved by the joint University College London/University College London Hospitals National Health Service Trust Human Research Ethics Committee and written informed consent was obtained from all participants.

Venous blood was sampled in heparinized tubes. Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation using Lymphoprep (Axis-Shield). Monocytes were isolated through magnetic positive selection using CD14 MACS MicroBeads (Miltenyi Biotech) according to manufacturer's instructions. After purification the isolated cells were \geq 95% CD14⁺ cells. Cultures were established in RPMI-1640 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (PAA laboratories), 100 U/ml penicillin/streptomycin (Gibco), 100 ng/ml recombinant human GM-CSF and 50 ng/ml rhIL-4 (both gifts from Schering-Plough Research Institute, Kenilworth, NJ).

Dendritic cells were harvested after 4–7 days culture and were greater than 90%CD1a positive.

3. Reverse transfection of DCs

Polyplexes were spotted (each spot contained either 2 μ g pDNA for confocal microscopy analysis or 20 μ g pDNA for gene expression studies [total DNA mass as deduced from nanodrop spectrophotometer analysis]) on PLL (50 μ g/ml) coated 22 \times 22 mm coverslips (VWR International) for 1 h at room temperature in the dark. Approximately 1×10^6 DCs were seeded in DC differentiation media on the PLL coated coverslips and incubated at 37°C for the desired time within 6-well plates (Helena Biosciences). Subsequently media was aspirated and replaced with fresh media lacking serum and incubated at 37°C. Following the desired duration of transfection, samples were extracted and media aspirated. Cells were washed once with HBSS. Subsequently cells were treated with 1 ml 3.8% paraformaldehyde and incubated for 15 min. This was followed by washing with PBS. In regards to confocal microscope analysis, coverslips were removed and mounted onto a microscope slide with DAPI mounting medium (Vectashield). In the case of transfected samples which were to be analysed by flow cytometry, samples were processed in BD FACS Calibur tubes (BD FACSCalibur) whereby washing steps entailed centrifugation at 1400 rcf for 5 min.

3.1. Labelling of DCs

DCs were stained following transfection with HCS CellMask™ Stains (Invitrogen) for a period of 30 min according to the manufacturer's protocol. The stain displays excitation and emission spectra of 556 and 572 nm respectively.

3.2. Gene expression analysis

DCs seeded in 6-well plates (Helena Biosciences) were reverse transfected with polyplexes containing 20 μ g DNA for 48 h. Subsequently cells were analysed for β -galactosidase expression. Expression was detected using a colorimetric β -Gal Assay Kit (Invitrogen). The number of blue cells detected under a light microscope in 5 fields of view was expressed as a percentage of total cells.

3.3. Confocal microscopy

A Leica SP2 confocal microscope was used to view cells that were mounted on the appropriate slides. Fluorescence images were collected using a scan speed of 400 Hz and 8 frame averaging. Nuclei were detected using 4,6-diamidino-2-phenylindole (DAPI) (Vectashield) (excitation: 405 nm, emission: 400–450 nm). DNA was detected via TOTO-3 (Dimeric Cyanine Nucleic Acid Stains–Invitrogen) (excitation: 642 nm, emission and emission: 660 nm). PLL was detected via Oregon Green 488 (Invitrogen) (excitation: 488 nm, emission 524 nm) and cell labelling was detected by HCS CellMask™ (Invitrogen) (excitation: 556 nm, emission: 572 nm).

To quantify uptake approximately 30 cells from each slide were randomly selected under the DAPI filter and the number of cell associated polyplexes were counted and classified on the basis of their intracellular location (cell periphery, cytosol or nuclei of the respective cell) using ImageJ software. The number of polyplexes within each cellular compartment was expressed as a percentage of the total number of polyplexes counted within the group of 30 cells. The number of cells (30) was selected as this was found to be statistically sufficient for quantification as recommended by previous studies [11,12]. Each experiment was repeated in triplicate as previously reported by Dhanoya et al. [9]. Slides were blinded

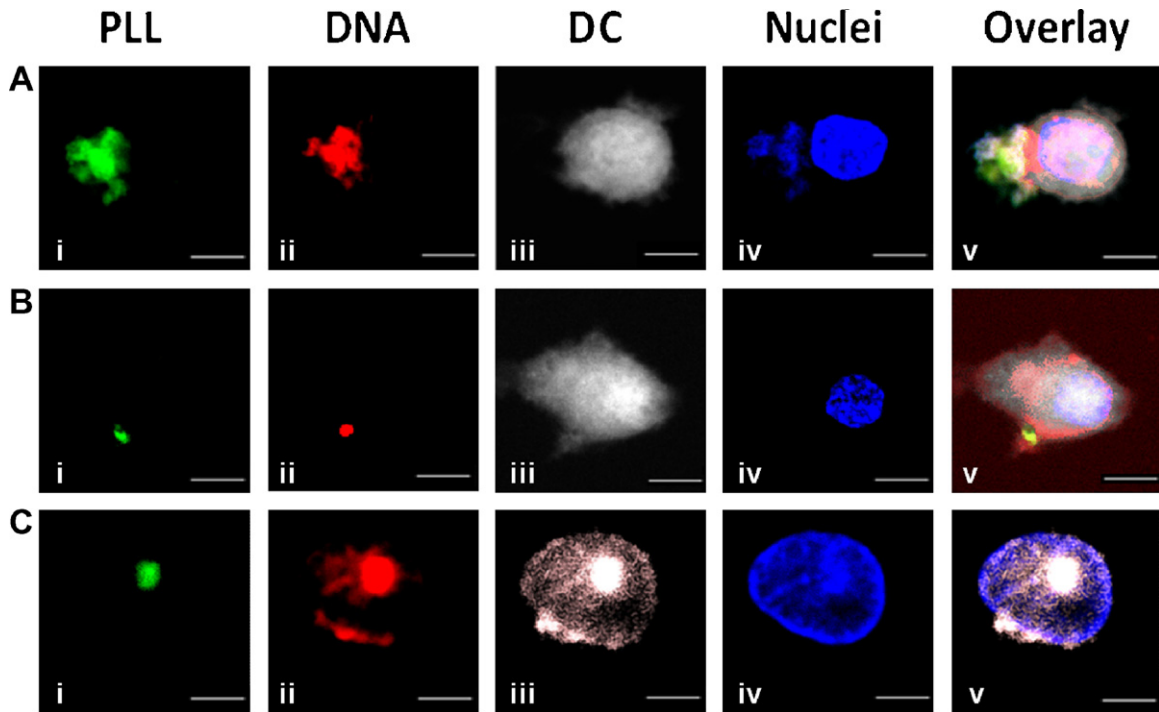


Fig. 1. Classification of polyplex cellular location in DCs. A: Linear-pDNA polyplexes. B: OC-pDNA polyplexes. C: SC-pDNA polyplexes. Individual components of polyplexes were stained; (i) PLL, (ii) DNA, (iii) DC, (iv) nuclei and (v) overlay of fluorescent images to show polyplex cellular location. A(v): periphery. B(v): cytosol. C(v): nuclear association. Polyplexes (containing 2 μg DNA) were prepared at charge ratios (ratio of PLL to DNA) of; +1.6 for SC- and OC-pDNA, and +5 for linear-pDNA. Scale bar represents 5 μm . DCs were fixed with paraformaldehyde, stained and coverslips were mounted on slides.

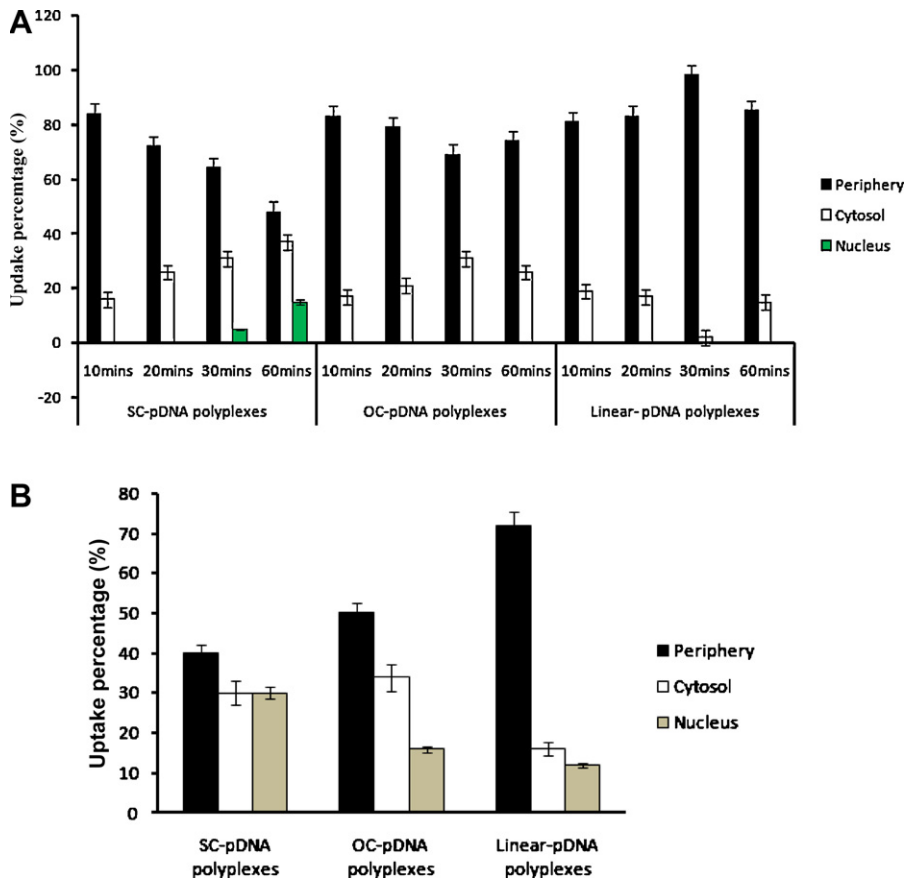


Fig. 2. Quantification of polyplex uptake in DCs in various cellular compartments. A: Confocal image quantification at 1 h. B: Confocal image quantification at 48 h. Polyplexes (containing 2 μg pDNA) were prepared at charge ratios (ratio of PLL to DNA) of; +1.6 for SC- and OC-pDNA, and +5 for linear-pDNA. The figure shows the mean and standard error (SE) of 3 individual experiments ($p < 0.05$).

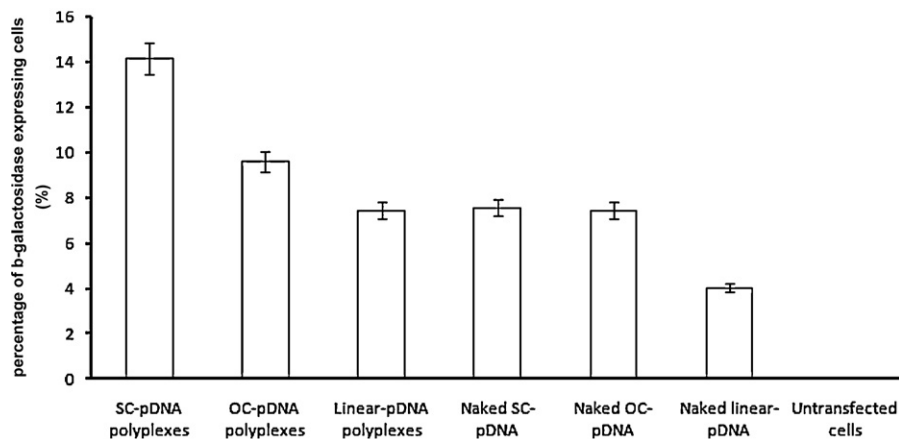


Fig. 3. Polyplex gene expression within DCs. Polyplexes (containing 20 μ g pDNA) of similar surface charge were transfected, i.e. charge ratios of +1.6 employed for SC- and OC-pDNA polyplexes and +5 for linear-pDNA polyplexes. The figure shows the mean and SE of 3 individual experiments ($p < 0.05$).

with regard to experimental condition before counting to reduce possible bias.

3.4. DC phenotype measurements

Polyplexes containing 20 μ g of pDNA were reverse transfected into DCs (approximately 1.9×10^6 cells per well). Cells were cultured for a period of 48 h, and then detached from the PLL coated coverslips by gentle pipetting. Cells were washed and assayed for β -galactosidase activity via an ImaGene Green™ C₁₂FDG *lacZ* Gene Expression Kit (Invitrogen) according to the manufacturer's protocol. Cells were then centrifuged and resuspended in PBS, to which 100 μ l was aliquoted to FACS tubes each containing 2 μ l antibodies for the following DC surface markers; IgG1, IgG2b, CD1a, DC-SIGN, CD11c, MHC-I, MHC-II, CD40, CD80, CD83 and CD86 (BD Pharmingen). Antibodies were fluorescently labelled with phycoerythrin (PE) or allophycocyanin (APC). After 20 min incubation period at room temperature in the dark the cells were washed, resuspended in 300 μ l PBS and analysed by flow cytometry.

3.5. Statistical analysis

One-way ANOVA was employed to deduce levels of statistical significance. Level of significance selected was $p = 0.05$.

4. Results and discussion

4.1. Qualitative and quantitative analysis of DC polyplex uptake

Polyplexes (containing 2 μ g pDNA) were transfected into DCs and uptake was monitored qualitatively by confocal microscopy over an initial 60 min period (Fig. 1). Polyplexes were dual labelled with DNA stained red, while PLL was tagged green. DC cytosol was stained light grey to highlight passage of polyplexes (using CellMask™). Polyplexes were transfected at differing charge ratios (ratio of PLL to DNA) of +1.6 for SC- and OC-pDNA, and +5 for linear-pDNA, as in Dhanoya et al. [9]. Dual staining was maintained indicating both DNA and PLL remained associated following uptake. SC-pDNA polyplexes were often observed to be associated with the nuclei whereas OC- and linear-pDNA complexes were usually located at the cell periphery indicating DNA topology may influence uptake (Fig. 1).

Polyplexes in each cell were classified as being at the cell periphery (Fig. 1a(v)), cytosol (Fig. 1b(v)) or nucleus (Fig. 1c(v)). If no

fluorescent overlap between the polyplex and the CellMask™ occurs, complexes are defined as being at the cell periphery. If some overlap between the polyplex and the CellMask™ occurs, complexes are classified as being in the cytosol. Complete overlap between polyplex and nuclear stain is classified as nuclear association. Using these criteria the number and localisation of cell associated polyplexes was counted and classified. Initially (10–20 min following uptake) the majority of polyplexes, regardless of DNA topology, were observed to be within the periphery of DCs (Fig. 2a). However by 1 h uptake of SC-pDNA complexes was

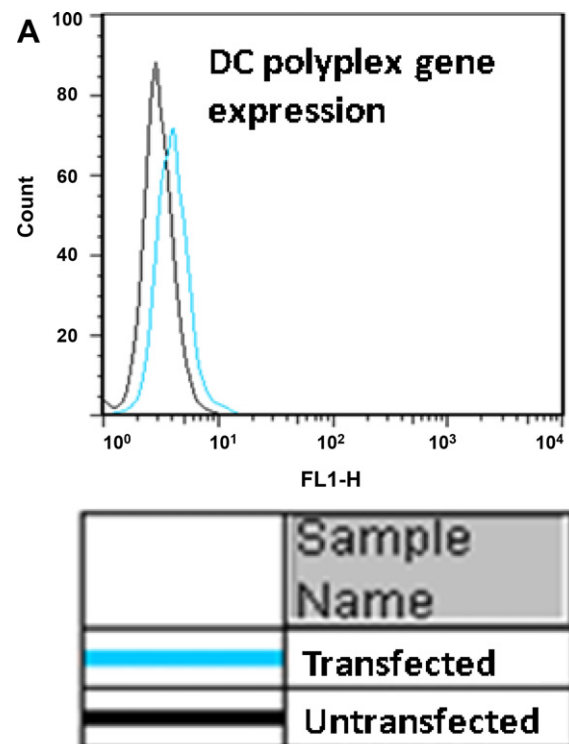


Fig. 4. The effect of polyplex gene expression on DC phenotype. A: β -galactosidase expression (FL1) in control (untransfected) and transfected (20 μ g DNA). B: Scatter plots displaying the effect of polyplex gene expression on DC surface marker expression in transfected and nontransfected cells. β -galactosidase expression (measured in FL1, y-axis) is plotted against cell surface marker expression (x-axis). C: β -galactosidase positive and negative cells from transfected cultures were gated and the expression of cell surface markers analysed.

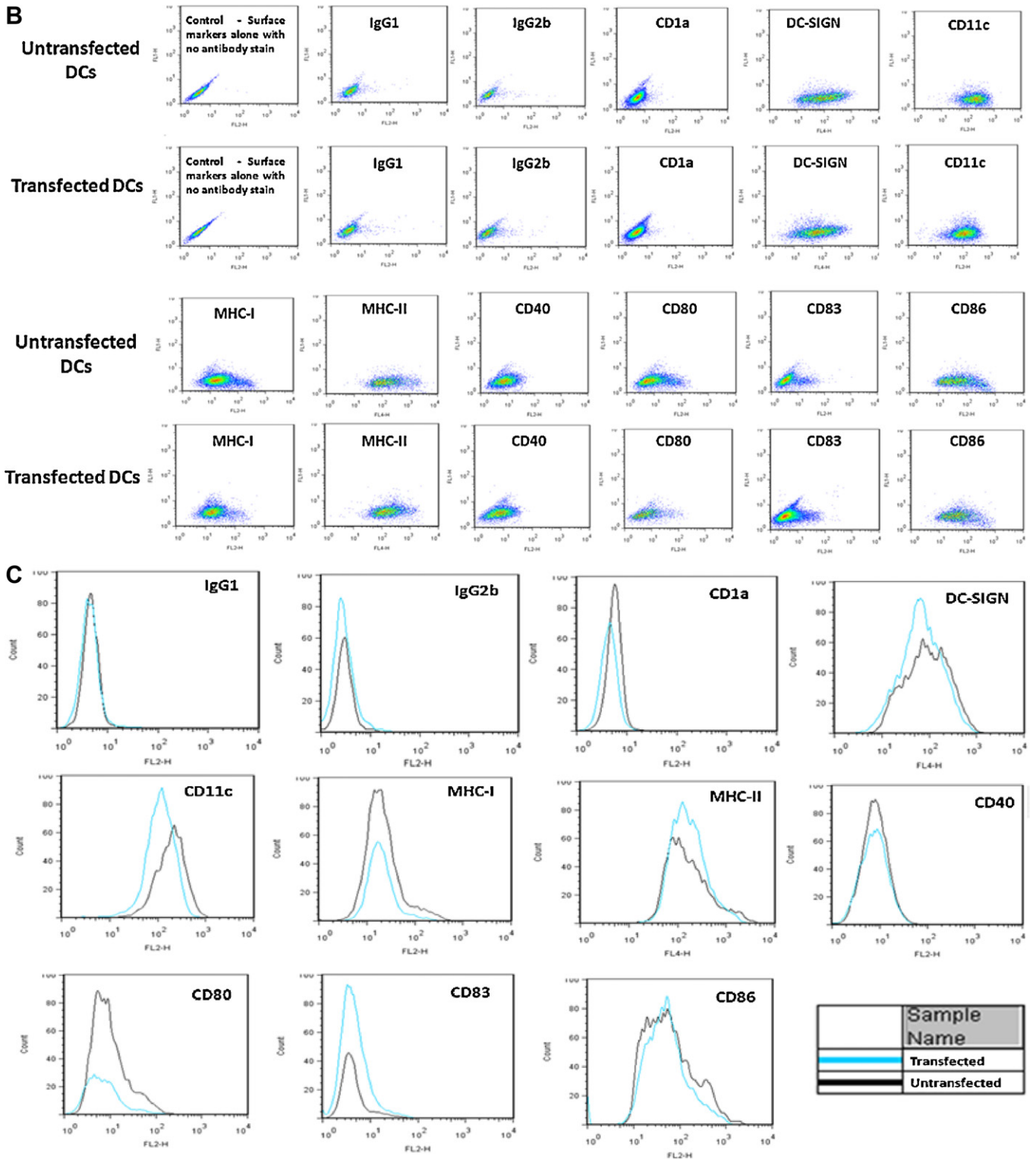


Fig. 4. (Continued).

much more efficient, with 15% ($\pm 2.5\%$ RSE) of complexes associated with the nuclei (polyplex fluorescence overlaid with nuclear stain). In contrast no nuclear association was observed for OC- and linear-pDNA polyplexes, indicating topology dependent uptake. Uptake also showed dependence on DNA topology at longer periods (Fig. 2b). The optimum percentages observed were still small compared to previous studies with CHO cells [9] ($61\% \pm 1.67\%$ RSE),

24.3 ($\pm 2.72\%$ RSE) and 3.5% ($\pm 7.12\%$ RSE) for SC-, OC-, and linear-pDNA polyplexes).

DCs are key sentinels of the immune system which engulf foreign antigens [13]. Nanoparticle uptake by DCs has been reported previously which led researchers to focus on polyplexes due to similarity in size [14,15]. Our previous study regarding PLL/DNA polyplexes reported sizes of 139.06 nm ($\pm 0.84\%$ RSE), 305.54 nm

($\pm 3.2\%$ RSE) and 841.5 nm ($\pm 7.2\%$ RSE) for SC-, OC- and linear-pDNA polyplexes respectively [9], which are clearly within the size criterion to be taken up by DCs (up to 1 μm [14]). This may account for the uptake observed in Fig. 1.

4.2. Polyplex gene expression in DCs shows dependence on DNA topology

Uptake of DNA does not necessarily correlate to gene expression, so reporter gene β -galactosidase expression was measured directly. In this study complexes containing 20 μg pDNA were transfected into DCs for 48 h to induce gene expression. Although 2 μg pDNA was used for confocal image studies, there was no significant difference between uptake profiles of complexes containing 2 and 20 μg (data not shown). Gene expression (*lacZ* reporter gene encoding β -galactosidase) was highest for SC-pDNA polyplexes at 14% (Fig. 3). This was significantly greater than OC- (9.59%) and linear-pDNA polyplexes (7.43%) ($p < 0.05$).

The ability of SC-pDNA polyplexes to diffuse through cells more efficiently than the other pDNA forms may contribute towards higher gene expression. We previously reported how polyplexes containing SC-pDNA displayed smaller sizes and greater nuclease resistance than other DNA forms [9]. This is pivotal as DCs have been found to express various nucleases [16]. Gene expression was modest compared to a similar study with CHO cells [9], which may be due to premature phagocytic clearance thereby reducing nuclear uptake [15,17–19]. Other researchers have attempted to improve DC gene expression with immature DCs to increase cell viability [17]. A mannosylating complex has been found to enhance interaction with DC surface receptors [20]. Block copolymer systems which shield, internalise and release DNA cargo can also improve gene expression [21]. However these systems are poly-disperse (combination of polymers), are prone to aggregation and can be cytotoxic at high polymer concentrations [21]. Non-viral gene expression within DCs has been reported previously and summarised in Table 1. With the exception of Landi et al. [17] and Faham et al. [22], findings from Table 1 confirm that non-viral DC gene expression is dependent on DNA dosage and the size of polyplex used. Although one study [23] employed pDNA doses of up to 10 μg gene expression was only 0.005%. This may be due to the size of such complexes which ranged between 7 and 11.6 μm (Table 1). Another analysis [24] employed pDNA doses of >5 μg and reported $<0.05\%$ gene expression. In the present study a dose of 20 μg led to up to 14% gene expression. A smaller dose of 10 μg was also used; however this led to extremely low gene expression (data not shown). This may be due to the prevalence of nucleases within DCs [16] that degrade nucleic acids as previous gene expression studies using 10 μg in CHO cells reported higher gene expression profiles than complexes transfected into DCs [9]. This implies that at least three factors play a role in uptake and gene expression, these being; size, dosage and DNA topology. It is clear from this study that DNA topology is an important parameter to consider for non-viral gene delivery to DCs for vaccination strategies. For polyplex gene expression this study recommends the use of SC-pDNA when complexed with PLL.

4.3. The effect of polyplex gene expression on DC phenotype

DCs express various cell surface markers which contribute towards antigen presentation [2]. Fig. 4 shows flow cytometry scatter plots displaying the population of DCs and the level of expression of 9 surface markers following transfection of DNA polyplexes. SC-pDNA polyplexes were analysed, as these gave clear distinguishable population of cells positive for β -galactosidase that can be detected by flow cytometry (Fig. 4a). A comparison of the bulk transfected and nontransfected populations showed no

Table 1
Summary of studies focusing on the parameters effecting non-viral gene delivery into DCs. Previous findings were compared with that of the present study. Where fields are denoted 'N/A' the respective study did not report the value. Nuclear association was quantified via confocal microscopy image analysis. Nuclear association was defined by complete overlap between polyplex fluorescence and nuclear stain.

Non-viral gene delivery system (plasmids employed 5–9 kb)	Size (μm)	Total dose (μg)	Zeta potential (mv)	Nuclear association at 48 h (%)	Optimal non-viral gene expression in DCs	pDNA topology (% content)	Change in DC phenotype? (Y/N)	References
Lipid/DNA	N/A	5	N/A	N/A	20% GFP expression at 48 h	SC (N/A)	N	[17]
PLL/SC-pDNA	<0.2	20	+13	30	14% β -galactosidase expression	SC-100%	N	This study
PLL/OC-pDNA	<0.4	20	+23	16	9% β -galactosidase expression	OC-100%	N	
PLL/Linear-pDNA	<1.0	20	+27	12	7% β -galactosidase expression at 48 h	Linear-100%	N	
Lipid/DNA	N/A	200	N/A	N/A	$>1.6\%$ EGFP expression at 24 h	SC (N/A)	Y	[22]
Lipid/DNA	N/A	4	N/A	N/A	$<1\%$ GFP expression at 24 h	SC (NA)	N	[19]
PEI/DNA encapsulated within PLGA particles	0.120	<5	+40	N/A	$<0.05\%$ GFP expression at 24 h	SC (N/A)	N/A	[24]
PEI/DNA encapsulated within PLGA particles	7.82–11.6	10	N/A	N/A	0.005% GFP expression at 48 h	SC (N/A)	N/A	[23]
Studies quantifying DC gene expression via luminescence: PEI/DNA	<0.3	10	N/A	N/A	$<1 \times 10^6$ RLU/mg protein (luciferase) at 24 h	SC (N/A)	N	[21]
PEI/DNA/PEG	0.125–0.132	6	+45.8	N/A	$<10^4$ RLU/mg protein (β -galactosidase) at 28 days	SC (N/A)	N	[15]
Lipid/DNA	0.128	5	+46.7	N/A	<1 ng/mg protein (luciferase) at 24 h	SC (N/A)	N	[20]

evidence of increased expression of any of the markers (Fig. 4b). β -galactosidase expressing cells were gated, and the expression of the cell surface marker on gated and non-gated cells was compared directly (Fig. 4c). Markers such as DC-SIGN, which mediates T-cell activation [25] did not change with polyplex gene expression (Fig. 4c). This could be due to the low DNA dosage employed whereby 20 μ g may not be enough to pass a certain threshold to elicit phenotypic changes. Table 1 summaries how previous studies employing similar DNA doses for non-viral DC gene delivery, failed to induce phenotypic changes, with the exception of one study which employed up to 0.2 mg DNA [22]. This suggests greater DNA dosage may be required for DC activation. PEI/DNA complexes were also reported to fail in inducing DC phenotypic changes [21]. Measuring such changes is important for clinical applications. Vaccines targeting DCs incorporate adjuvants that are designed to elicit phenotypic changes that activate DCs [21]. Therefore the findings from Fig. 4 reveal how PLL/DNA complexes could incorporate components (adjuvants) to induce DC activation. Alternatively by not activating DCs, PLL/DNA polyplexes could display biocompatibility allowing safe and effective nucleic acid delivery [21].

5. Conclusions

We have shown that both uptake and gene expression (transcription of reporter gene) of PLL/DNA polyplexes are dependent on DNA topology. Complexes containing SC-pDNA were most efficient in associating with the nucleus (polyplex fluorescence overlaid with nuclear stain) as observed by confocal microscopy studies (15% [2.59% RSE] associated with the nucleus in comparison to no nuclear association reported for OC- and linear-pDNA at 1 h). However confocal quantification via fluorescence overlay does not directly correspond to gene expression, as nuclear uptake of DNA can still be hindered by the presence of nucleases [9]. Complexes containing SC-pDNA displayed significantly higher gene expression (14%) than other topological forms (9.59% and 7.43% for OC- and linear-pDNA polyplexes) ($p < 0.05$), although expression was modest in comparison to that reported for CHO cells [9]. This may be due to DCs predominately expressing nucleases which restrict uptake and gene expression. Lack of DC surface marker expression may be explained by low dosage (20 μ g) used. This in itself may be considered advantageous in terms of biocompatibility and safe delivery of DNA in vivo [21].

In terms of bio-processing and vaccine production, the application of SC-pDNA is a key pre-requisite. The findings of this study show how pDNA in the SC conformation is more efficient in terms of both uptake and gene expression than OC- and linear-pDNA. Therefore DNA topology does impact on processing and vaccine manufacture. This is in agreement with current regulatory bodies such as the FDA which require 80% SC content (Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications – FDA, 2007) [26].

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