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Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro

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Abstract

The impetus to develop non-viral gene delivery vectors has led to examination of synthetic polycationic polymers as plasmid DNA (pDNA) condensing agents. Previous reports have highlighted superiority (up to $\times$10-fold) in the in-vitro transfection of pDNA complexes formed by poly-(L)-ornithine (PLO) compared to those formed with poly-(L)-lysine (PLL). The apparent basis for this consistent superiority of PLO complexes remains to be established. This comparative study investigates whether physico–chemical differences in the supramolecular properties of polycation:pDNA complexes provide a basis for their observed differential gene transfection. Specifically, particle size distribution and zeta potential of the above complexes formulated over a wide range of polycation:pDNA ratios were found to be consistent with a condensed (150–200 nm) cationic ($+30$–40 mV) system but not influenced by the type of cationic polymer used. A spectrofluorimetric EtBr exclusion assay showed that polycation:pDNA complexes display different pDNA condensation behaviour, with PLO able to condense pDNA at a lower polycation mass compared to both polylysine isomers, and form complexes that were more resistant to disruption following challenge with anionic counter species, i.e. poly-(L)-aspartic acid and the glycosaminoglycan molecule, heparin. We conclude that particle size and surface potential as gross supramolecular properties of these complexes do not represent, at least in a non-biological system, the basis for the differential transfection behaviour observed between these condensing polymers. However, differences in the ability of the polylysine and polyornithine polymers to interact with pDNA and to stabilise the polymer-pDNA assembly could have profound effects upon the cellular and sub-cellular biological processing of pDNA molecules and contribute to the disparity in cell transfection efficiency observed between these complexes. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The condensation of DNA involves a dramatic decrease in the volume occupied by a DNA molecule and is of immense biological impor-

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tance, as such the structural, physico–chemical and energetic aspects of the condensation process continue to receive much attention (Marquet and Houssier, 1991; Bloomfield, 1996). Recently, the impetus to develop non-viral gene delivery vectors has led to the examination of a range of synthetic polycationic polymers as plasmid DNA condensing agents. Such polymers have encompassed a wide range of characteristics, varying in mass, shape and nature of the cationic species, and include synthetic copolymers (Wolfert et al., 1996; Dash et al., 1997; Kabanov and Kabanov, 1998), branched or linear homopolymers (Zhou et al., 1991; Tang and Szoka, 1997; Adami et al., 1998) and polymers coupled to receptor targeting ligands (Wagner et al., 1990; Edwards et al., 1996; Lee and Huang, 1996). However, all are recognised to self-assemble with anionic DNA through electrostatic interactions leading to the formation of colloidal complexes; the condensed particles produced providing protection for the therapeutic gene against nuclease degradation and serving as a platform for enhancing the cellular delivery of DNA.

Evidence accumulated to date would indicate that in general the polycation polymer — DNA vectors provide for a lower degree of transfection compared with optimised cationic liposome formulations although some notable examples, such as the polymer polyethylenimine (Boussif et al., 1995; Remy et al., 1998), may be recognised as exceptions. Nevertheless, polymers are perceived to offer some advantages, including the homogeneity and smaller size of the resulting DNA complexes, and the expedience with which targeting 'signals' may be covalently attached to the polymer backbone, including ligands for cellular or subcellular targeting, e.g. endosome disrupting or for nuclear-localising functionality (Thomas et al., 1997; Zauner et al., 1998). This has led to the use of polycationic pDNA condensing agents in multicomponent lipid:polycation:DNA delivery vectors (Gao and Huang, 1996; Li and Huang, 1997; Sorgi et al., 1997).

The linear polymer poly-(L)-lysine (PLL), although not clinically exploitable, has to date been the most commonly utilised model cationic polymer in laboratory gene delivery investigations. Intriguingly, previous reports in a range of cell types have highlighted superiority (up to $\times 10$-fold) in the in-vitro transfection of complexes formed by poly-(L)-ornithine (PLO) compared to those formed with PLL, for example, in B16 murine melanoma cells (Pouton et al., 1998), HelaS3 — human cervix carcinoma cell (Hazemoto et al., 1995), and Chinese hamster ovary (CHO) cells (Farber et al., 1975). This is of note given that the monomers lysine and ornithine differ only by an additional $-CH_2-$ in the sidechain of lysine, and that both their oligomeric structures give rise to linear polymers with the protonated species constituted by a primary amine moiety. The apparent basis for the consistent superiority of PLO complexes remains to be established.

Although it should be recognised that PLL or PLO will serve no more than model DNA condensing agents in the laboratory, the mechanisms of their differential in vitro gene transfection behaviour remains to be explained. This comparative study has, therefore, sought to elucidate if physico–chemical differences in the supramolecular structures of PLL- and PLO-pDNA complexes provide a basis for their observed differential gene transfection. Specifically, using the polymer PLL, it's isomer poly-(D)-lysine (PDL), and PLO we have shown that supramolecular properties, i.e. particle size and zeta potential, of complexes formed between pDNA and these polymers were comparable. Using an EtBr exclusion assay we demonstrate distinct interactions of the polymers with pDNA, with PLO able to condense pDNA at a lower polycation mass and form complexes more resistant to disruption following challenge with anionic counter species. Such differences may well influence the biological processing of pDNA molecules and hence contribute to the disparity in cell transfection efficiency observed between polyornithine and polylysine vectors.

2. Materials and methods

Unless otherwise stated, materials were obtained from Fisher Scientific UK, (Loughborough, UK).

2.1. Plasmid DNA

The 7.2 kb plasmid pCMVβ (code # 6177-1; Clontech, Palo Alto, USA) encoding β-galactosidase under the control of the human cytomegalovirus immediate early promoter was amplified using a transformed DH5α strain of *Escherichia coli*. The pDNA was harvested and purified using the Qiagen Plasmid Mega Kit (Qiagen Ltd., Crawley, UK). The concentration and purity of the pDNA was determined by the 260/280 UV absorbance ratio (GeneQuant II RNA/DNA calculator; Amersham Pharmacia Biotech, St. Albans, UK) and plasmid integrity by agarose gel electrophoresis and restriction enzyme digest. The pDNA was aliquoted in sterile nuclease free water and stored at −20°C prior to use.

2.2. Polycations and preparation of complexes with pDNA

Polycations were obtained from Sigma Aldrich Company Ltd. (Poole, UK) with polymer mass quoted based upon Low Angle Laser Light Scattering (LALLS) with average molecular weights (M_w) of: 50 200 for PLL (degree of polymerisation 240); 50 200 for PDL (degree of polymerisation 240); 50 700 for PLO (degree of polymerisation 260). The polydispersity (M_w/M_n) profiles were for: PLL, 1.15; PDL, 1.28; PLO, 1.24. The polycations were aliquoted in sterile nuclease free water and stored at −20°C until use. Polycation/pDNA complexes were formed by addition of the appropriate mass of polycation to pDNA, with mixing (vortex half-speed setting for 3 s) and incubation at room temperature for 20 min prior to experimentation.

2.3. Cell culture and transfection

The alveolar adenocarcinoma cell line, A549 (Smith, 1977) (European Collection of Animal Cell Cultures, Salisbury, UK) was cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and the antibiotics streptomycin (100 μg/ml) and penicillin G (100 IU/ml). The cells were grown at 37°C in a 5% CO_2/air humidified atmosphere. For transfection studies the cells (passage 94–97) were cultured to 80% confluency (70 h post-seeding) in 24-well tissue plates. Media was removed and the cells were bathed X2 with pre-warmed DMEM. Polycation/pDNA complexes were formed at mass ratios of 2:1, 2.5:1 and 3:1 and added in serum-free DMEM to the appropriate wells to achieve a final concentration of 5 μg pDNA per well. Transfection was allowed to proceed for 4 h after which pDNA complexes were removed and the respective cells bathed X2 with pre-warmed DMEM. Thereafter cells were incubated in DMEM supplemented with 10% FBS. At 48 h after the start of transfection the cells were analysed for β-galactosidase expression using a standard spectroscopic assay as described previously (MacGregor et al., 1991; Birchall et al., 1999) All the cell culture plastics were obtained from Corning Costar (High Wycombe, UK) with DMEM and supplements from Life Technologies Gibco BRL (Paisley, UK).

2.4. Zeta potential and particle sizing of polycation/pDNA complexes

The zeta potentials of the polycation/pDNA complexes formed at various mass ratios were determined by microelectrophoresis (Malvern ZetaSizer 3, Malvern Instruments Ltd., Malvern, UK). Polycation-pDNA complexes were prepared using the same concentration of pDNA with the addition of varying mass ratios of PLL, PDL and PLO. All the complexes were prepared in distilled, degassed water and consecutively measured ten times with instrument calibration (Malvern AZ55 Electrophoretic Standard) prior to each series of measurements. The same sample was then subjected to size analysis by photon correlation spectroscopy (PCS: Malvern ZetaSizer 3) using a 5 mW laser at an angle of incidence of 90°. This measurement was repeated three times in multimodal analysis. Both zeta potential and size determinations were performed at 25°C. Prior to use all glass and plasticware was prewashed with filtered water to minimise particulate contamination. Additional size analysis was performed using a Coulter N4 Plus (Coulter Electronics Ltd., Luton, UK) in Size Distribution Processor (SDP) mode.

2.5. Ethidium bromide spectrofluorimetric exclusion assay

Quantitation of ethidium bromide (EtBr) intercalation between DNA base pairs was determined through fluorescence spectroscopy (Aminco-Bowman Series 2 Luminescence Spectrometer, SLM-Aminco Spectroscopic Instruments, Rochester, NY). The plasmid pCMVβ (15 μg) in double distilled nuclease free water was mixed in a 3 ml cuvette with 3 μg of EtBr (Pharmacia Biotech.) and the resultant fluorescence emission (at excitation and emission wavelengths of 516 and 598 nm, respectively) measured and taken to represent 100%. Polycation was then added incrementally to a final volume of 3 ml. After each addition, the sample was mixed and the decreased fluorescent signal, resulting from EtBr intercalation between pDNA base pairs, was allowed to stabilise prior to reading. The fluorescent signals were corrected at each dilution and expressed as a percentage of the signal attributed to that for pDNA alone (Sorgi et al., 1997).

Adaptations to the above assay were made to examine the differential effects of anionic polymers upon the displacement of polycation from pDNA binding. Specifically, polycation:pDNA complexes equivalent to a mass:mass ratio of 2.5:1 were prepared in double distilled nuclease free water and the fluorescent signal arising from co-incubation with EtBr was allowed to stabilise. This complex was then challenged by incremental additions of a linear anionic polymer, either heparin (four ionisable groups per repeating unit) or poly-(L)-aspartic acid (PAA; single negative charge per monomer), or the monomeric anionic surfactant sodium dodecyl sulphate (SDS). Heparin and PAA were obtained from Sigma Aldrich company Ltd. SDS was obtained from Pharmacia Biotech.

2.6. Gel electrophoresis pDNA migration and nuclease digestion assay

Diiferential displacement of polycation from pDNA binding following anion challenge was also examined by gel electrophoresis. A solution of pCMVβ (4 μg) in dH_2O and an equal volume of dH_2O containing 10 μg of polycation were mixed to form pDNA:polycation complexes as described in Section 2.2. The complexes were challenged with either heparin or PAA (at mass and charge ratio conditions documented in the respective figure legends) and aliquotes run on a 0.8% agarose (Promega Ltd., Southampton, UK) gel with electrophoresis conditions of 100 V for 2 h in 0.5X TBE running buffer (0.045M Tris–borate, 0.001M EDTA). The pDNA signal was visualised under UV light by staining with ethidium bromide (2 μg/μl) and imaged using a Gel Doc 1000 with Molecular Analyst software (Biorad Laboratories, Hercules, CA, USA).

The amount of protection afforded to the pDNA following anionic disruption of polycation-pDNA complexes was determined by nuclease digestion. Briefly, heparin or PAA was added to pre-formed polycation:pDNA (2.5:1 w/w; 4 μg pDNA) complexes at an anion:cation ratio of 5, prior to incubation with 1 U of DNase I (Fluka, Gillingham, UK) for 5 and 15 min periods at 37°C. After incubation the reaction was stopped by addition of an equal volume of Tris-EDTA buffer pH 8.0 and the pDNA liberated by incubation with assay buffer (10 mM Tris Cl pH 8.0; 5 mM EDTA pH 8.0; 1% SDS; 100 mM NaCl).

3. Results and discussion

Although PLL has been widely exploited in in-vitro gene transfection studies the application of PLO as an experimental vector has in comparison received only limited attention (Hazemoto et al., 1995; Pouton et al., 1998). In the present study we show significantly ($P > 0.05$) enhanced reporter gene expression (approximately × 5-fold) with PLO–pDNA complexes compared to those formed with either of the polylysine isomers (Fig. 1). The mass ratios of polcation:pDNA in complexes shown in Fig. 1 (between 2:1 and 3:1 for each polycation polymer) represent the optimum constituent ratios of polycation:pDNA for the transfection of A549 cells, with complexes formulated within this range consistently leading to the highest level of gene transfection independent of polymer molecular weight tested (between 10 000

and 50 000 MW). We also observed that at mass ratios above (up to 5:1 w/w) and below (down to 1:1 w/w) this 'optimal range', transfection mediated by PLO still remained significantly greater than that mediated by PLL or PDL complexes ($P < 0.05$), although the difference was less pronounced and paralleled the overall decline in the reporter gene expression. We have noted similar observations using COS-7 cells, although for the same treatments reporter gene expression observed in COS-7 cells was generally inferior to that in A549. A standard MTT assay, following the method described elsewhere (Plumb et al., 1989), was used to assess the viability of A549 cells after exposure to polycations alone or when complexed with pDNA. Under the transfection conditions of our experiment we found no significant ($P > 0.05$) loss in cell viability with any treatment (viability $\geq 85\%$ of control for each treatment).

The zeta potential associated with polycation-pDNA complexes was determined by microelectrophoresis. As both the zeta potential and particle diameter of polycation-pDNA complexes are dependant on pDNA concentration, all the complexes analysed in this study were prepared using the same concentration of pDNA. The zeta potential of the polycation/pDNA complexes passed through zero close to theoretical neutrality, i.e. $1{:}1 +/-$ calculated as equivalent molecules of monocationic monomer, i.e. lysine or ornithine, and monoanionic nucleotide and equating to a mass ratio (polymer:pDNA) of approximately 0.6:1 w/w. Above theoretical neutrality the complexes proved to be strongly electropositive with, at the maximum mass ratio examined of 5:1 w/w (corresponding to $7.9{:}1 +/-$ for PL complexes, and $8.45{:}1 +/-$ for PLO), zeta potentials approaching $+30$ mV for PLL and PLO complexes and approximately $+37$ mV for PDL complexes (Fig. 2a). Statistical analysis (ANOVA) revealed that the zeta potentials for PDL, PLL and PLO complexes were not significantly ($P > 0.05$) different when analysed at equivalent theoretical charge ratios. Fig. 2b shows photon correlation spectroscopy (PCS) of polycation-pDNA complexes and reveals a particle size distribution that provided for mean diameters of 150–200 nm. Statistical investigations (ANOVA) indicated that there was no significant difference ($P > 0.05$) in the mean size of particles as a function of either the nature of polycation in the pDNA complex or the polycation:pDNA mass ratio. More detailed analysis of the sizing data (Coulter N4 Plus: Size Distribution Processor function) revealed that the particle size distribution of both the polylysine-pDNA and PLO-pDNA complexes was bimodal with two distinct particle populations of approximately 60 and 400 nm mean diameter (data not shown). This was consistent with the observed polydispersity indices of between 0.3 and 0.4 for all samples tested, indicating disparity from a normally distributed particle size population. This bi-modal profile of polycation:pDNA size is in agreement with the previous reports for complexes comprising PLL and pDNA over a range of polymer molecular weights and assessed by PCS and atomic force microscopy (Lee and Huang, 1996; Wolfert and Seymour, 1996; Pouton et al., 1998). The data in Fig. 2a and b, therefore, would indicate that, at least prior to interaction with cell membranes, the surface potential and particle size distribution of pDNA complexes formulated with the poly-lysine or PLO polycation polymers does not provide a

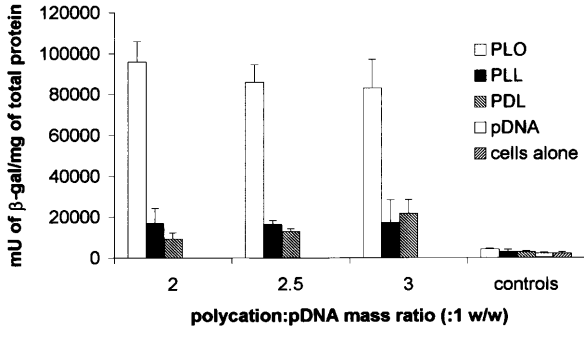

Fig. 1. Transfection of A549 cells with polycation-pDNA complexes. Freshly prepared complexes of poly-(L)-lysine (PLL), poly-(D)-lysine (PDL) and poly-(L)-ornithine (PLO) with pCMVβ (5 μg) were incubated at 37°C with 80% confluent cells in the absence of serum for 4 h. β-galactosidase activity was determined 48 h post-transfection (MacGregor et al., 1991) and standardised against cellular protein content (Kruger, 1991). Data represented as mean ± S.D., $n = 9$. Statistical analysis by one-way analysis of variance (ANOVA) and Duncan's multiple range test. Significance level $P < 0.05$.

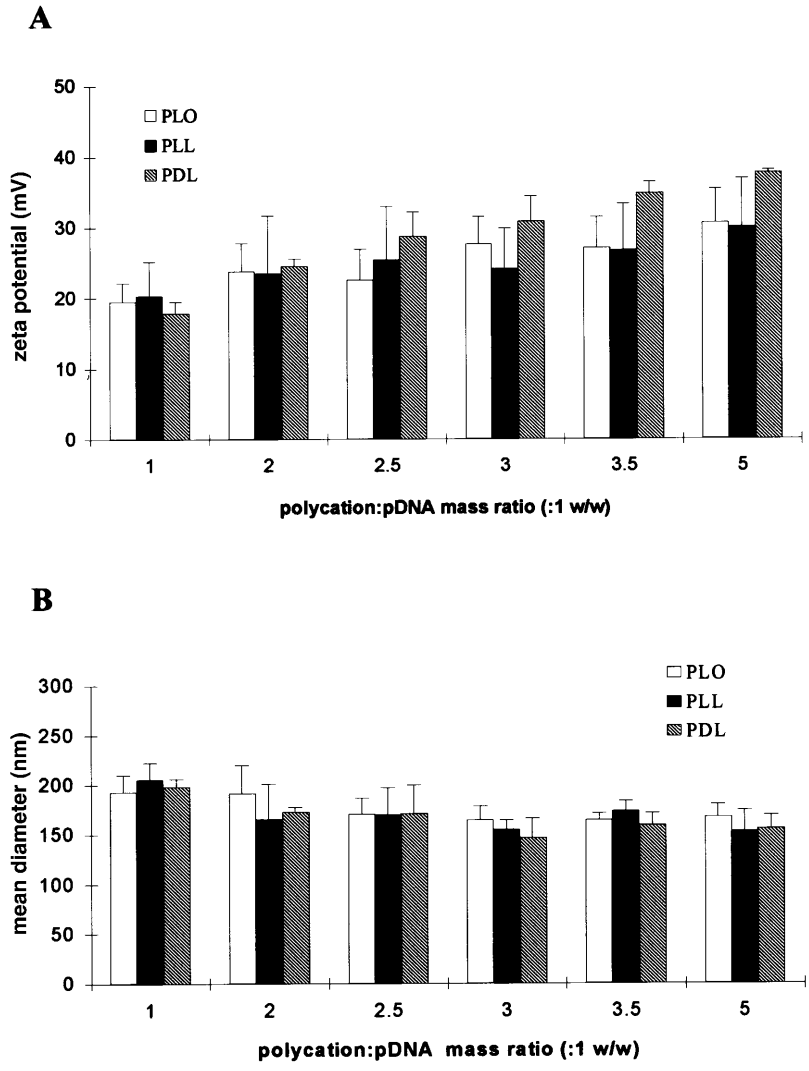

Fig. 2. Zeta potential and size analysis of polycation–pDNA complexes. Complexes were formed between PLL, PDL, PLO and pCMVβ in degassed double-distilled water at polycation:pDNA mass (w/w) ratios of 1:1, 2:1, 2.5:1, 3:1, 3.5:1 and 5:1. The hydrodynamic diameter and zeta potential of each polycation/pDNA complex was determined by microelectrophoresis (Malvern ZetaSizer 3). Data represented as mean ± S.D., $n = 10$ (zeta), $n = 3$ (size). Statistical analysis by one-way ANOVA and Duncan's multiple range test. Significance level $P < 0.05$.

basis for the differential in-vitro transfection efficiency observed between these non-viral vectors.

As a measure of the efficiency of the respective polycations in condensing pDNA we used a fluorimetric EtBr exclusion assay as described previously (Gershon et al., 1993). Fig. 3 shows the relative fluorescence of polycation:pDNA complexes as a function of their calculated theoretical (+/−) charge ratios with each fluorescent profile representative of at least three study replicates (the S.E.M. data being excluded to avoid the figure being overly complicated). Maximal pDNA condensation, represented as a plateauing of signal between 5 and 10% of relative fluorescence, was achieved at (+/−) charge ratios of 0.8:1, 1.2:1 and 1.5:1 for PLO, PLL and PDL, respec-

tively, with corresponding IC50 values of 0.63, 1.03 and 1.23 where IC_{50} is defined as the polycation:pDNA charge ratio at 50% relative fluorescence. For each polycation:pDNA complex the fluorescence remained constant at charge ratios above that required for maximal condensation, indicating that polycation:pDNA complexes formulated at mass ratios of between 2:1 and 3:1 (equivalent to +/− ratios between 3.2:1 and 5.1:1) as used in the transfection studies (Fig. 1) reflect conditions of equivalent and maximal pDNA condensation by all three polymers. However, it is clear from the IC_{50} values and mass of polycation required to achieve maximal condensation that polycation:pDNA complexes display disparate pDNA condensation behaviour, with PLO appearing to induce pDNA condensation more readily than both polylysine isomers. Polyamino acids can exist in various conformations from random coil to an α-helix and both these conformations are known to exist for PLL in an aqueous solution (Shibata et al., 1992). It has been suggested (Blauer and Alfassi, 1967) that the additional $-CH_2-$ group contained within the lysine may make the α-helix conformation of polylysine more stable than its polyornithine counterpart. Given the comparable p*K*a values of the primary amine groups for lysine and ornithine (i.e. p*K*a 10.5–10.7; Morgan et al., 1989), it is probable that conformational differences rather than protonation *per se* provides a basis for the differential behaviour of PLO-mediated pDNA condensation. The least effective condensing polycation was PDL. The L-isomer is the natural orientation of nuclear enzymes and proteins and, therefore, it is possible that DNA interactions with other macromolecules are biased towards L-isomer conformations (Reich et al., 1990).

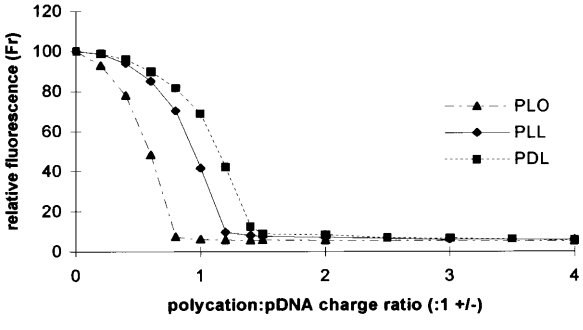

Fig. 3. Ethidium bromide exclusion assay. Relative fluorescence (Fr) of polycation-pDNA complexes in double distilled water is expressed as the percentage, following incremental addition of polycation, of the initial fluorescence of ethidium bromide (3 μg) in the presence of pCMVβ (15 μg). The polycation-pDNA mass ratios were converted to equivalent charge ratios assuming 1 μg pDNA is equivalent to 3.03 nmol of negative charge and 1 μg of polylysine and polyornithine provide 4.78 and 5.13 nmol of positive charge, respectively. Data represented as mean ± S.D., $n = 4$.

To further probe for differential properties of the polycation-pDNA complexes, we adapted the above spectrofluorimetric EtBr exclusion assay to assess the stability of the respective complexes to challenge with anionic counter species. Specifically, pre-formed polycation:pDNA (2.5:1 w/w) complexes were challenged with (i) the low charge density linear anionic polymer, poly-aspartic acid (PAA); (ii) the high charge density linear anionic polymer, heparin, or (iii) the anionic surfactant SDS. Fig. 4a illustrates the change in relative fluorescence as a function of the incremental addition of PAA, where the additions were expressed as the molar ratio of negative charge derived from anion counter species (i.e. PAA) to the positive charge contributed by the polycation condensing agent, i.e. either PLL, PLO or PDL. The resistance to PAA challenge at anion:cation molar ratios $\leq 1{:}1$ was comparable for each of the polycation–pDNA complexes with recovery of relative fluorescence (Fr) from a value of less than 10% to a value of 60%. The further addition of PAA resulted in a continued gradual increase in fluorescence until the signal plateaued at an anion:cation molar ratio between 5:1 and 6:1. Slightly greater recoveries in Fr (implying greater dissociation of complex under PAA challenge) were observed with the PLL and PDL complexes (80–85% Fr) compared to those comprised of PLO (75% Fr), with the fluorescence recovery of PLO complexes nevertheless significantly ($P < 0.05$) lower than that observed for polylysine-based complexes at anion:cation ratios of 2:1 through to 9:1. Fig. 4b illustrates the change in Fr as a function of heparin challenge, where for PLL and PDL complexes the Fr recovered to approximately 60% at an anion: cation ratio of 1:1,

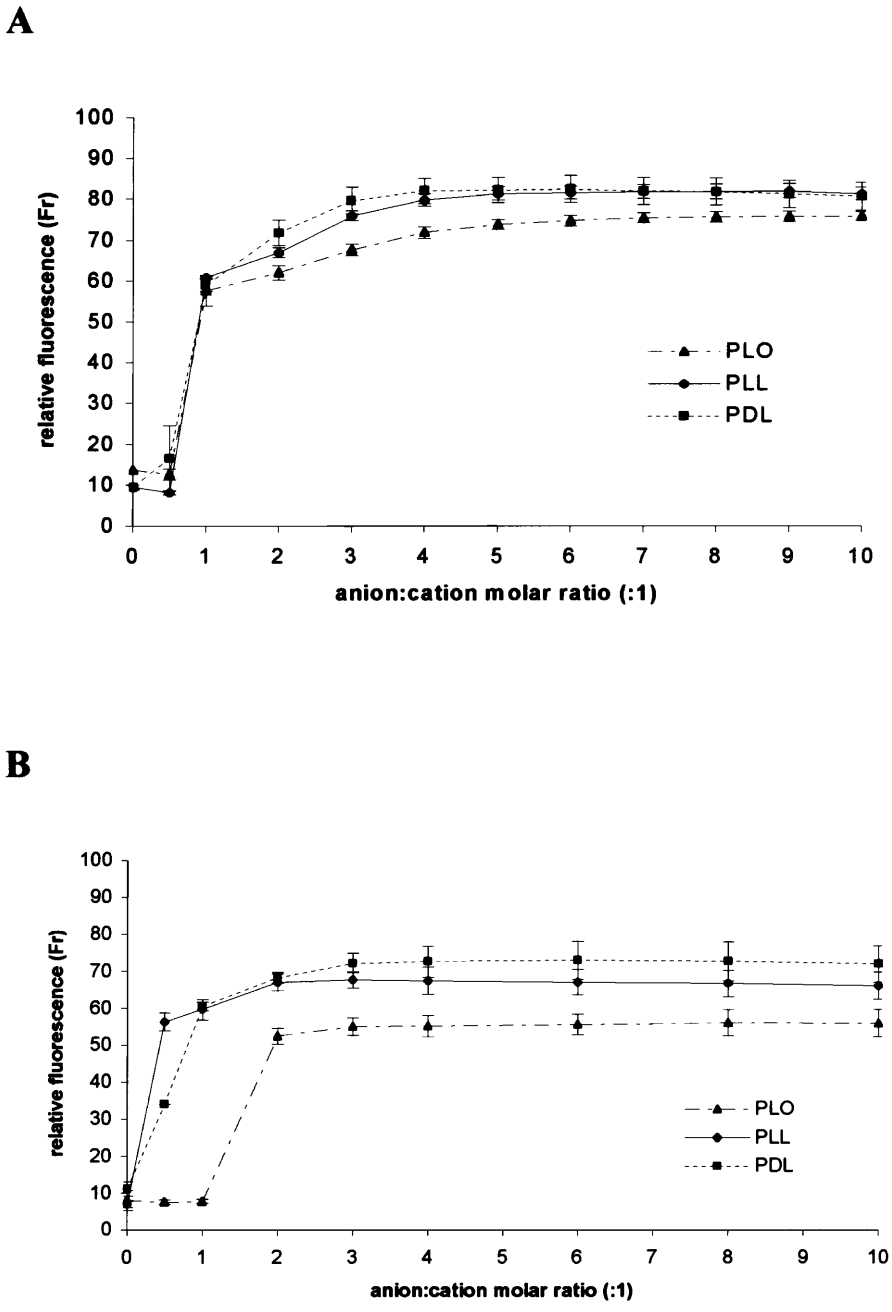

Fig. 4. Anionic displacement of PLL, PDL and PLO from pDNA. Polycations were complexed with pCMVβ at a peptide:pDNA mass ratio of 2.5:1. Complexes were challenged by incremental addition of the linear anionic polymers poly-(L)-aspartic acid (PAA; low charge density) (A) or heparin (high charge density) (B). Data represented as mean ± S.D., $n = 8$.

before plateauing at approximately 70%. However, heparin challenge at an anion:cation ratio of 1:1 had no measured effect on the Fr associated with the PLO complex. Indeed, for the PLO complex the concentration of heparin had to be increased to an anion:cation ratio of 2:1 before

any recovery in Fr was observed, with the Fr eventually plateauing at much lower values for PLO complexes (55–60%). At all the points from 0.5:1 through to 10:1 anion:cation ratio the fluorescence recovery of PLO complexes was significantly ($P < 0.05$) lower than that observed for the polylysine-based complexes. While challenge with both PAA and heparin showed PLO complexes to display greater resistance to polyanion-induced disruption, the differential effect between PLO and the polylysines was more pronounced for heparin. This may be of relevance given the potential for interactions between polycation–pDNA complexes and other glycosaminoglycan molecules at the plasma membrane cell surface. Challenge with the monomeric anionic surfactant SDS showed little consistent difference between complexes comprising PLO or polylysine (data not shown). In each case the recovery of EtBr fluorescence was more gradual with SDS challenge than that seen with PAA or heparin and clearly may reflect different mechanisms of SDS-induced destabilisation of polycation-pDNA complexes to that mediated by challenge with the anionic polymers, i.e. competing interactions other than those based upon electrostatic forces may impact upon the stability of polycation: pDNA complexes (Kabanov and Kabanov, 1995; Tang and Szoka, 1997). It should be mentioned that complexes formed with PLO, PDL or PLL at an equivalent mass ratio, for example 2.5:1 (w/w) ratio as used in the gene transfection and spectrofluorimetric challenge studies, gives rise to complexes with slightly different charge equivalents, i.e. respectively, 4.23:1 $+/-$ for PLO and 3.95:1 $+/-$ for PDL or PLL. Whilst it is important that this be noted, it is also clear that this difference in charge equivalents between PLO and the polylysines at a fixed mass ratio is not a basis for the differing responses of PLO and PLL or PDL complexes. This is exemplified by the fact that across a broad range of charge ratios a consistent differential response (i.e. transfection as in Fig. 1 or anion challenge as in Fig. 4a and b) of PLO compared with the poly-lysines is maintained. Taken in its entirety the spectrofluorimetric EtBr exclusion data would support the hypothesis that PLO interactions with pDNA, and the stability of the resulting complexes are distinct from those of PLL and PDL complexes.

In parallel with the spectrofluorimetric EtBr exclusion assay we assessed the integrity and release of pDNA from the polycation:pDNA complexes by gel electrophoresis following challenge with either heparin or PAA at an anion:cation (i.e. heparin or PAA: polycation) ratio of 5:1 (data not shown). This allowed us to investigate if the nature of the disruption of polycation:pDNA complexes seen in spectrofluorimetric exclusion assay was different when challenged with either PAA or heparin. Significantly, only challenge with the high charge density polymer heparin was able to liberate free pDNA from the PLO–pDNA, PLL–pDNA and PDL–pDNA complexes allowing a subpopulation of pDNA to migrate through the agarose gel; no obvious difference was observed in the gel pattern between PLO or the polylysines when challenged with heparin. In contrast there was no evidence of migration of the liberated pDNA from complexes challenged with PAA, although PAA challenge did result in fluorescence in the gel loading well (as indeed was also the case for heparin) indicating pDNA still associated with polycation but where disruption or destabilisation by PAA challenge, although sufficient to allow EtBr access to pDNA base pairs, was insufficient to liberate free migrating pDNA. Further evidence for the disruption of the polycation–pDNA complexes in the presence of the anionic counter species was obtained using a DNase I digestion assay. As in the case of the gel migration study, heparin appeared to disrupt the polycation–pDNA complexes to a greater extent than PAA, i.e. complexes challenged with heparin were prone to endonuclease degradation of the pDNA while PAA challenged complexes provided a degree of protection of the pDNA following 5 min incubation with DNase I (data not shown). However, as in the migration assay, no obvious difference was observed in the gel pattern between PLO or the polylysines when exposed to a given anionic disrupting agent.

It is clear that the spectrofluorimetric assay and the gel-based migration assay have the potential to provide different facets of information on the same process, i.e. the spectrofluorimetric assay

provides a global assessment of EtBr-pDNA fluorescence not discriminating between the fluorescence associated with pDNA that has been liberated from the original polycation–pDNA complex, and that arising from EtBr access to pDNA that still remains associated with polycation but where some degree of destabilisation has occurred. Nevertheless, spectrofluorimetric assays provide a more reproducible and quantitative assessment of EtBr intercalation of pDNA than EtBr gel migration and DNase-based assays where quantitation of relative pDNA band intensity on an agarose gel is subject to inaccuracy.

In this study we confirm a significant increase in the in vitro transfection of reporter gene with PLO–pDNA complexes compared with PLL–pDNA and PDL–pDNA complexes and have aimed to identify a physico–chemical basis for this previously documented differential transfection behaviour. Specifically, particle size distribution and zeta potential of the above complexes formulated over a wide range of polycation:pDNA ratios were found to be consistent with a condensed polycationic system but not influenced by the type of cationic polymer used. Therefore, we conclude that particle size and surface potential as gross supramolecular properties of these complexes do not represent, at least in a non-biological system, the basis for the differential transfection behaviour observed between these condensing polymers. A spectrofluorimetric EtBr exclusion assay showed that polycation:pDNA complexes display different pDNA condensation behaviour, with PLO able to condense pDNA at a lower polycation mass compared to both polylysine isomers. Further, PLO–pDNA complexes were more resistant to disruption following challenge with anionic counter species, i.e. poly-(L)-aspartic acid and the glycosaminoglycan molecule, heparin. Differences in the ability of the polylysine and polyornithine polymers to interact with pDNA and to stabilise the polymer–pDNA assembly against anion challenge could have profound effects upon the cellular and sub-cellular biological processing of pDNA molecules, including cell uptake, sub-cellular trafficking and indeed nuclear localisation, and as such may well contribute to the disparity in cell transfection efficiency observed between these complexes. Further work will seek to address these cell biological issues.

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