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# A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids

**Martin L. Read*, Surjeet Singh[1], Zubair Ahmed, Mark Stevenson[2], Simon S. Briggs[2], David Oupicky[3], Lee B. Barrett[4], Rachel Spice, Mark Kendall[5], Martin Berry, Jon A. Preece[1], Ann Logan and Leonard W. Seymour[2]**

Molecular Neuroscience Group, Department of Medicine, University of Birmingham, Birmingham, B15 2TT, UK, [1]School of Chemistry, University of Birmingham, B15 2TT, UK, [2]Department of Clinical Pharmacology, University of Oxford, Oxford, OX2 6HE, UK, [3]Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI 48202, USA, [4]Department of Anesthesia and Critical Care, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA and [5]Department of Engineering Science, University of Oxford, Oxford, OX1 3PJ, UK

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## ABSTRACT

**Synthetic vectors based on reducible polycations consisting of histidine and polylysine residues (HIS RPCs) were evaluated for their ability to deliver nucleic acids. Initial experiments showed that RPC-based vectors with at least 70% histidine content mediated efficient levels of gene transfer without requirement for the endosomolytic agent chloroquine. Significant gene transfer was observed in a range of cell types achieving up to a 5-fold increase in the percentage of transfected cells compared to 25 kDa PEI, a gold standard synthetic vector. In contrast to 25 kDa PEI, HIS RPCs also mediated efficient transfer of other nucleic acids, including mRNA encoding green fluorescent protein in PC-3 cells and siRNA directed against the neurotrophin receptor $p75^{NTR}$ in post-mitotic cultures of rat dorsal root ganglion cell neurons. Experiments to elevate intracellular glutathione and linear profiling of cell images captured by multi-photon fluorescent microscopy highlighted that parameters such as the molecular weight and rate of cleavage of HIS RPCs were important factors in determining transfection activity. Altogether, these results demonstrate that HIS RPCs represent a novel and versatile type of vector that can be used for efficient cytoplasmic delivery of a broad range of nucleic acids. This should enable different or a combination of therapeutic strategies to be evaluated using a single type of polycation-based vector.**

## INTRODUCTION

There has been rapid progress in the development of different types of nucleic acids including protein-encoding genes, oligonucleotides and mRNA with the potential to form the basis of new treatments for genetic and acquired disorders in humans. In particular, the recent emergence of small interfering RNA (siRNA) to trigger RNA interference has initiated efforts to identify siRNA sequences capable of modulating the function of a vast number of genes (1). However, a major hurdle to the continued development and therapeutic application of nucleic acids is the lack of suitable vectors for their delivery. There are several issues that need to be addressed before efficient delivery vectors can be developed; including the toxicity of vectors observed in clinical studies (2,3) and the lack of targeting of vectors to specific cell types. In addition, it would be advantageous to design vectors that are capable of delivering a broad range of nucleic acids to evaluate different or a combination of therapeutic strategies.

Synthetic vectors based on polycations such as polyethylenimine (PEI) (4) or poly-L-lysine (PLL) (5,6) have been widely used to transfer nucleic acids to cells. The benefits of using synthetic vectors are their ability to complex and deliver large quantities of nucleic acids of virtually any size and they are also generally regarded as being safer than viral vectors. However, the transfection efficiency of synthetic vectors is typically poor compared to viral vectors and significant improvements are required to enable further clinical development. Several barriers that limit nucleic acid transfer both *in vitro* and *in vivo* have been identified, and include poor intracellular delivery (7), toxicity (8) and instability of vectors in physiological conditions (9). PLL, for example, binds electrostatically to the phosphate groups of DNA forming discrete

*To whom correspondence should be addressed. Tel: +44 121 627 2331; Fax: +44 121 472 0499; Email: mread@globalnet.co.uk

polyplexes with diameters in the range of 50–100 nm. However, PLL is incapable of mediating efficient escape from endocytic vesicles into the cytoplasm, and poor gene expression is observed *in vitro* without the use of endosomolytic agents such as chloroquine (10).

The transfection properties of polyplexes can be influenced by the type and molecular weight (mwt) of polycation used. We have previously demonstrated, for example, that plasmid DNA complexed with 211 kDa PLL was more stable to salt-induced aggregation than 20 kDa PLL and gave a 20-fold greater level of DNA in the plasma of mice 30 min after intravenous administration (11). However, there are several disadvantages in using higher mwt polycations for gene transfer including their reported ability to hinder unpacking of DNA from polyplexes (7) and toxicity. Intravenous injection of 211 kDa PLL/DNA polyplexes to Wistar rats, for example, resulted in hematuria that may have been mediated by direct erythrocyte lysis (11).

A strategy to reduce the toxicity of polyplexes but retain their stability in physiological conditions is to cross-link low mwt polycations bound to DNA such that cleavage by the intracellular environment facilitates the release of DNA (12). Recently, we developed a synthetic vector based on a linear reducible polycation (RPC) prepared by oxidative polycondensation of the peptide Cys-$Lys_{10}$-Cys (13). Cleavage of RPC by the intracellular reducing environment decreased toxicity of the polycation to levels comparable with low mwt peptides. In addition, efficient intracellular release of DNA and gene transfer was shown in a range of cell types using vectors based on RPC. A related approach has also been used by McKenzie *et al*. where low mwt peptides containing two to five cysteine groups, such as Cys-Trp-$Lys_8$-Cys-$Lys_8$-Cys, were mixed with DNA prior to spontaneous oxidation to form interpeptide disulfide bonds (14). However, a major drawback in both of these studies was that chloroquine (13,14) or cationic lipids (13) were required to facilitate endocytic escape of polyplexes and mediate efficient levels of gene transfer.

In the present study, we investigated whether efficient gene therapy vectors based on RPCs could be developed by the inclusion of histidine residues that have a known endosomal buffering capacity. We show that histidine-rich RPCs can be cleaved by the intracellular reducing environment enabling efficient cytoplasmic delivery of a broad range of nucleic acids, including plasmid DNA, mRNA and siRNA molecules without the requirement for the endosomolytic agent chloroquine. Furthermore, we demonstrate efficient delivery of nucleic acids in both rapidly dividing cells and in primary cultures of post-mitotic cells. These histidine-rich RPCs can be readily modified by the incorporation of targeting ligands and hydrophilic polymers to improve their utility and biocompatibility. Hence, these findings should help to facilitate the design and construction of efficient and versatile synthetic delivery vectors capable of evaluating therapeutic strategies with different or combinations of nucleic acids.

## MATERIALS AND METHODS

### Cell lines and primary cells

The human prostatic cell lines PC-3 and DU145, ovarian carcinoma cell line SKOV-3, liver carcinoma cell lines HepG2 and Alexander, breast carcinoma cell line MCF7, lung carcinoma cell line A549 and green monkey transformed cell line COS-7 were grown in DMEM containing 1 mM Glutamax, glucose (1g/l, Invitrogen) and 10% foetal calf serum (FCS). The human prostate cell lines LNCaP, PNT1a (15) and PNT2 (16,17) were grown in RPMI 1640 medium with 2 mM glutamine and 10% FCS. All cell lines were incubated at 37°C in a 5% $CO_2$ humidified environment. Dorsal root ganglion (DRG) cultures were prepared from adult Sprague–Dawley rats. Briefly, spinal ganglia were removed aseptically, washed twice in Neurobasal-A media (Invitrogen) and digested in 0.125% collagenase/supplemented Neurobasal-A (2% B27 supplement, 0.5 mM L-glutamine and 0.5% gentimicin) for 2 h at 37°C. Spinal ganglia were removed from the collagenase, washed in Neurobasal-A and purified by centrifugation for 10 min at 120 *g* on a 15% bovine serum albumin column.

### Sources of nucleic acids and polycations

The reporter gene expression plasmids pEGFPN1 (Clontech, Oxford, UK) and pCMVLuc1 (a gift from Dr Manfred Ogris, Munich, Germany) were used throughout these studies. Plasmid DNA was grown in *Escherichia coli* and purified using Qiagen Gigaprep kits (Crawley, West Sussex, UK). The concentration and purity of DNA was checked on a spectrophotometer at $A_{260}$ and $A_{280}$ absorbance wavelengths. mRNA encoding green fluorescent protein (GFP) was prepared by *in vitro* transcription of the plasmid pGEM4Z/GFP/A64 (a gift from Dr D. Boczkowski, Duke University Medical Center, NC) using the mMessagemMachine kit and capped with the anti-reverse cap analog (ARCA) from Ambion (Huntingdon, Cambridgeshire, UK). Construction of this plasmid is described elsewhere (18). Products were checked by gel electrophoresis and were ~900 bases in length. To design target-specific siRNA duplexes, we selected siRNA sequences directed against $p75^{NTR}$ using criteria set out by Elbashir *et al*. (19) from the open reading frame of rat $p75^{NTR}$ mRNA, NCBI accession number NM012610. The siRNA sequences used were Seq2, 5′-AACGCTTGATGCCCTTTTAGC-3′; and Scr2, 5′-AATCGCATGCGTTCCATTTCG-3′. Oligonucleotide templates and control scrambled sequences were chemically synthesized (Alta Biosciences, University of Birmingham, UK) and siRNA sequences constructed using the Silencer siRNA Construction kit (Ambion, TX, USA). GFP-22 siRNA was purchased from Qiagen, Crawley, UK. The cationic polymers used were branched polyethylenimine (PEI, 25 kDa; Aldrich), linear PEI (4xJetPEI; Qbiogene, Wiesbaden, Germany) and poly(L-lysine). HBr (PLL, 54 and 84 kDa; Sigma).

### Peptide synthesis and purification

The $CH_3K_3H_3C$ (HIS3 monomer), $CH_6K_3H_6C$ (HIS6 monomer) and $CK_{10}C$ peptides were synthesized using a Biosystems automated peptide synthesizer and standard Fmoc protocols. Cleavage from the polymeric support and deprotection of amino acid side chain residues were performed in 10 ml of trifluoroacetic acid/water/phenol/triisopropylsilane (88:5:5:2 v/v) for 2–4 h at room temperature. The soluble product was then concentrated *in vacuo* and precipitated by washing with cold diethyl ether. The peptides were then dissolved in distilled water and lyophilized to yield a white solid.

The peptides were redisolved in distilled water and purified by reversed-phase HPLC on a $C_{18}$ column. Once the peptides had been eluted, elution solvent was removed *in vacuo* followed by lyophilization. The peptides were then characterized by MALDI-TOF mass spectrometry and NMR. The purity of all peptides was shown to be >95% by analytical reversed-phase HPLC.

### Preparation and characterization of RPCs

Polycondensation reactions were performed with 10 mg of each peptide in phosphate-buffered saline (PBS) containing 30 vol% DMSO and allowed to react at room temperature for 4 days as previously described (20). At certain time intervals during the step growth polymerization, an aliquot of the reaction mixture was removed and terminated with 8 mol% excess of 2-aminoethanethiol. The mwts (weight averages) of resulting RPCs at various time points were measured by gel permeation chromatography (GPC) using calibration with commercially available PLL with mwts in the range 13.4–84 kDa. The standards and RPCs (10 µl) were injected on a CATSEC 300 GPC column and eluted in 300 mM NaCl + 0.1% TFA. RPCs were purified from DMSO and cyclic by-products using centrifugal ultra filters with mwt cut-off 10 000. The concentrations of RPCs were determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) using PLL calibration. The $^1$H NMR titration for the determination of p$K_a$ values of histidine moieties was performed at 300K using 10 mg of each peptide in deuterated water in a Brucker AC 300 MHz spectrometer. The chemical shifts of the H5 and H6 protons of the imidazole ring of the histidine residues were measured as a function of pH. The p$K_a$ values were determined by plotting the chemical shift against pH. The pH of the solution was adjusted by addition of 100 mM deuterated sodium hydroxide.

### Formation and characterization of polyplexes

Plasmid DNA, mRNA or siRNA were added to a polypropylene microcentrifuge tube at a final concentration of 50 µg/ml in ultra-pure water or 20 mM HEPES–NaOH, pH 7.4 as indicated. Polycation was added to the nucleic acid at the desired (w/w) ratio or N:P ratio and mixed by gently pipetting the solution up and down several times. Polyplexes were then allowed to form for at least 15 min at room temperature prior to use. In some cases, HIS RPC/DNA polyplexes were incubated in 150 mM NaCl for 30 min prior to addition to cells. In co-transfection experiments, pEGFPN1 and GFP-22 siRNA were condensed either by HIS6 RPC in water at (w/w) ratio of 24 or 4xJetPEI in 150 mM NaCl at N:P ratio of 5–10 and incubated for 30 min at room temperature prior to use.

The weight per charge of the $CH_3K_3H_3C$ and $CH_6K_3H_6C$ peptides were 257.2 and 254.9, respectively. Formation of HIS RPC/DNA polyplexes at (w/w) ratio of 40, for example, was equivalent to an N:P ratio of ~32. Condensation of plasmid DNA by polycations was determined using an ethidium bromide (EtBr) exclusion assay measured with an LS-50B fluorimeter (Perkin-Elmer, Bucks, UK) at 510 nm excitation and 590 nm emission wavelengths and by the agarose gel retardation assay as previously described (21). The hydrodynamic diameters of polyplexes were measured by dynamic light scattering using a Zetasizer 3000 (Malvern Instruments, Malvern, UK) equipped with a 50 mW internal laser. The measurements were performed in triplicate at 25°C in 7.5 mM HEPES–NaOH, pH 7.5 at the scattering angle 90 degrees and analysed assuming a monomodal distribution model.

The ability of reducing conditions to destabilize RPC/DNA polyplexes was examined by incubating polyplexes with 25 mM DTT at 37°C for 1 h and NaCl added at varying concentrations (0–1 M). The samples were then loaded on to a 1% agarose gel containing 0.5 µg/ml EtBr and run for 1 h at 120 V in 0.5× TBE buffer. A Typhoon gel scanner set at 533/610 nm wavelengths and 550 V was used to scan the gel and the quantity of DNA in particular bands analysed using ImageQuant™ software.

### Western blot analysis

To determine levels of $p75^{NTR}$ in siRNA treated DRG cultures, cells were washed twice with PBS and incubated for 15 min at 37°C with 0.25% trypsin/EDTA (Invitrogen), followed by trituration and centrifugation at 1300 r.p.m. for 5 min. The DRG cell pellet was re-suspended in ice-cold lysis buffer containing 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% NP-40 (Sigma) and protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. After a 30 min centrifugation at 13 400 *g* at 4°C, the lysates were normalized for protein concentration using a colorimetric DC protein assay (Bio-Rad, Hercules, CA, USA). Homogenates and cell lysates were stored at −70°C until used for western blot analysis. Each sample (40 µg total protein) was incubated with Laemmeli loading buffer at 90°C for 4 min and separated on a 12% SDS–polyacrylamide gel (Invitrogen). Proteins were transferred to PVDF membranes (Millipore UK, Gloucestershire, UK), blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk. Membranes were blotted overnight for the relevant antibody. For detection, an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) and HRP-conjugated secondary antibody (1:1000; Amersham) was used. Each blot was stripped and re-probed with relevant antibodies thereafter.

### Transfection studies

Polyplexes were added directly to a 48-well plate (13.5 µl per well) containing 2–5 × $10^4$ cells per well in 125 µl of DMEM or RPMI without FCS or containing 2–10% FCS as indicated (cells were plated at least 24 h before transfection). Transfection studies with HIS RPCs were performed both in the presence and absence of 100 µM chloroquine. After 4 h, the mixture containing polyplexes was discarded and replaced with 200 µl per well of fresh media containing 10% FCS. Cells were cultured for 24 h prior to analysis of reporter gene expression. In some experiments, free polycations were added to cells prior to addition of the polyplexes. To determine the effect of altering intracellular reducing environment on transfection properties of HIS RPC/DNA polyplexes, cells were incubated either in the presence of 5–10 mM glutathione–monoethyl ester (GSH–MEE) for 3 h or 20–500 µM DL-buthionine sulfoxamine (BSO) for 24 h prior to transfection. Intracellular levels of glutathione were determined using the method of Sebastia *et al*. (22). Briefly,

cells were incubated in 40 μM monochlorobimane (mBCl) in a total volume of 100 μl of culture media and returned to the incubator for 30 min. Fluorescence was measured on a Victor$^2$ plate reader (Wallac) at 360 nm excitation and 460 nm emission wavelengths and background values subtracted.

In some experiments, DRG cultures were transfected with siRNA using Oligofectamine reagent (Invitrogen) in 4-well tissue culture plates (Nunc). Briefly, 0.84 μg siRNA was mixed in 50 μl Opti-MEM (Invitrogen), and 3 μl Oligofectamine added. Lipoplexes were allowed to form at room temperature for 30 min and then added to cells. After 5 h of transfection, supplemented Neurobasal-A was added and incubated for a further 72 h prior to cell lysis for analysis by western blotting. Cell survival was determined using the MTS assay (Promega) after exposure to free polycations or polyplexes and normalized to values obtained in their absence as previously described (13).

### Assay of reporter genes

Luciferase expression following transfection was measured by a luminescence assay using cell lysates. The culture media was discarded and cell lysates harvested after incubation of cells for 30 min at 4°C in 100 μl of 1× lysis reagent buffer (Promega). The lysate was gently vortexed and 20 μl was diluted into 100 μl of luciferase reaction buffer (20 mM glycylglycine, 1 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 0.5 mM ATP and 0.27 mM coenzyme A lithium salt). The luminescence was integrated over 10 s on a Lumat LB9507 (Berthold Instruments, UK) and the results expressed as relative light units (RLU) per mg of cell protein, determined using the Advanced Protein Assay (Totam Biologicals, Northampton, UK). Analysis of GFP expression was carried out on a Coulter Epics XL flow cytometer. Cells were trypsinized at appropriate times after transfection, washed with PBS and then fixed in 2% paraformaldehyde. GFP was excited using the 488 nm line of an Argon laser and emitted light collected at 520 nm (green fluorescence) and 575 nm (red fluorescence) to enable correction for autofluorescence by diagonal gating. Background fluorescence and autofluorescence were determined using mock-treated cells. Cellular debris showing reduced side- and forward-scatter was excluded from analysis. The software programme WinMDI was used to analyse data and expressed as the percentage of GFP-positive cells or mean fluorescence per cell (MnX). Analysis of cells expressing GFP was performed on an inverted Zeiss axiovert 100 microscope (Zeiss, Germany) and images recorded on a SPOT camera using SPOT imaging software v2.0 (Diagnostic Instruments Inc).

### Analysis of GSH levels by multiphoton fluorescence microscopy

Cells were plated into 8 well Permanox chamber slides at a density of $2.5 \times 10^4$ cells per well and maintained in normal growth media. After 24 h, cells were washed with PBS and 200 μl of phenol-red free media added containing 40 μM mBCl prior to incubation of cells for 60 min at 37°C. Cells were then washed three times with phenol-red free media prior to analysis. Images of cells were acquired using a Bio-Rad multiphoton fluorescence microscope (MPM) based at the Department of Engineering Science, University of Oxford, UK. Relative GSH levels of individual cells were obtained by linear profiling of mBCl fluorescence from images captured by the MPM using the software programme Image J (National Institutes of Health). Background fluorescence was subtracted from the mean value.

## RESULTS

### Preparation of histidine-rich RPCs

Two histidine-rich RPCs (HIS3 RPC and HIS6 RPC) were prepared by oxidative polycondensation of the peptides Cys-$His_3$-$Lys_3$-$His_3$-Cys and Cys-$His_6$-$Lys_3$-$His_6$-Cys using the method previously described by Oupicky *et al*. (20). GPC analysis showed that a 48 h incubation period was sufficient to produce high mwt RPCs with mwts of 113 kDa for HIS6 RPC (Figure 1A) and 59 kDa for HIS3 RPC (data not shown). The peptide Cys-$Lys_{10}$-Cys was also used to prepare a lysine-based RPC of mwt 65 kDa ($RPC_{65}$) as a control polycation in this study. Details of RPC sequences used in this study are given in Table 1. Preliminary characterization by NMR to monitor changes in the chemical shift of $H_5$ and $H_6$ protons demonstrated that both the HIS6 RPC (Figure 1B) and HIS3 RPC (data not shown) had a buffering capacity with a p$K_a$ in the region of 5–6 corresponding to the p$K_a$ of the imidazole group.

### Triggered activation of histidine-rich RPC/DNA polyplexes

We next investigated the ability of histidine-rich RPCs to form polyplexes with DNA and their stability in reducing conditions. In the presence of the intercalating dye, ethidium bromide fluorescence was strongly quenched by addition of HIS6 RPC or HIS3 RPC to DNA at (w/w) ratios 4 and 7, respectively (Figure 1C) indicating efficient polyplex formation. Similar trends of complexation were observed using the agarose gel retardation assay, for instance, DNA was partially retained in the wells by HIS6 RPC at (w/w) ratio of 2 and completely retained at (w/w) ratios of 4 and above (Figure 1D). To determine the ability of polyplexes to be activated by reduction HIS6 RPC/DNA at (w/w) ratio of 4 was reduced with DTT and the amount of free DNA assessed by gel electrophoresis in the presence of salt. Figure 2 shows that a relatively low percentage of DNA (<13.4%) was retained in the wells by HIS6 RPC in non-reducing conditions even in the presence of 1 M salt. Reduction of HIS6 RPC by DTT, however, led to a significant increase in the amount of DNA released. Quantification of DNA showed that 71.9% of DNA was released in the absence of salt and that >95% of the DNA was released when the salt concentration was increased to 0.5 M or greater (Figure 2).

### Chloroquine-independent transfection using histidine-rich RPCs

Having established the ability of histidine-rich RPC/DNA polyplexes to be destabilized by reduction, we next evaluated their transfection activity compared to PEI/DNA polyplexes, a gold standard non-viral vector, using luciferase as a reporter gene. For these experiments PEI/DNA polyplexes were formed at N:P ratio of 10, which we (data not shown) and others (23,24) have shown mediates efficient gene transfer. Initial transfections showed that HIS3 RPC/DNA polyplexes

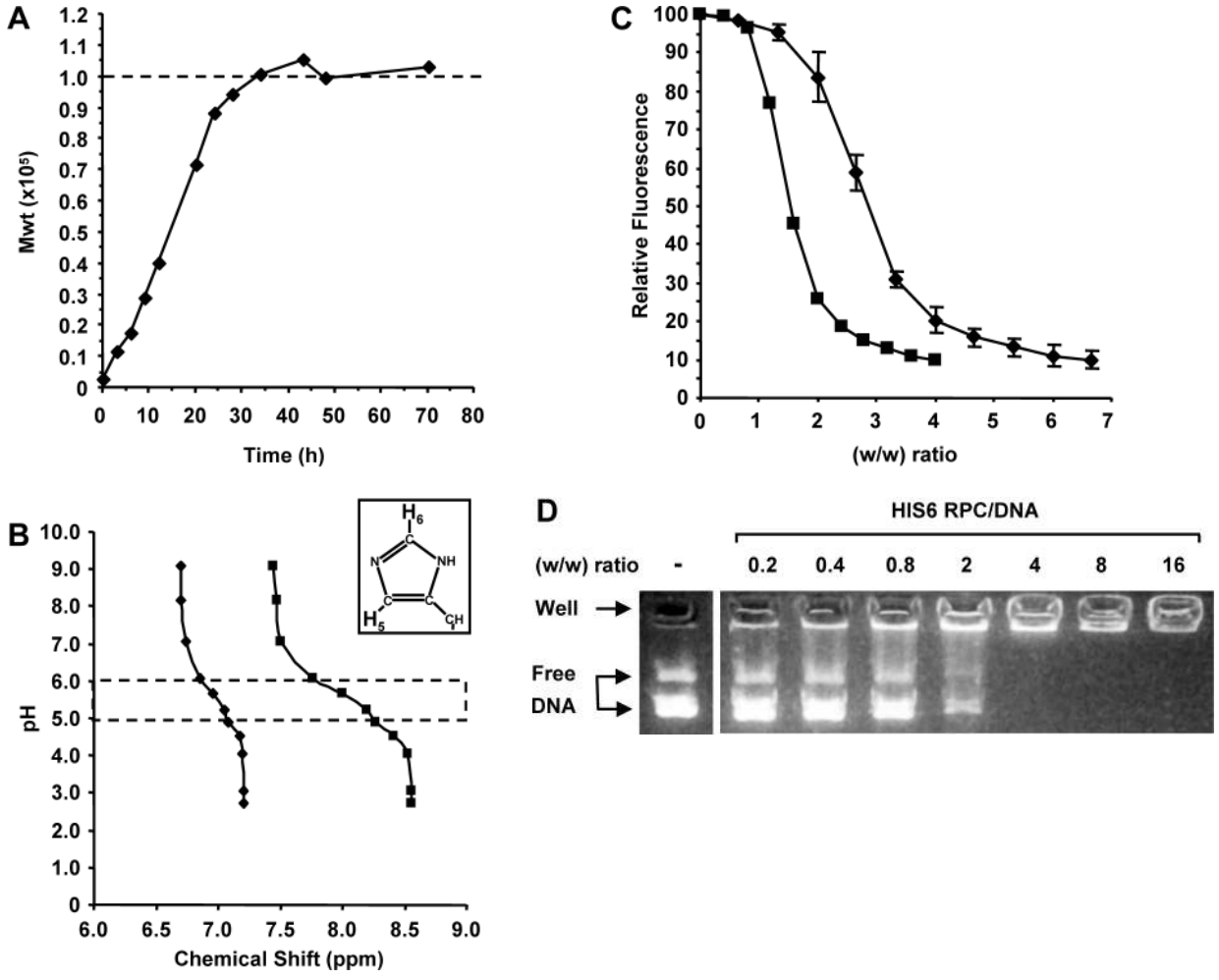



**Figure 1.** Characterization of HIS RPCs and polyplex formation. (**A**) Mwt growth of oxidative polymerization of HIS6 RPC in DMSO over time. Dialysis of HIS RPCs was performed in Centricon filters (10 000 mwt cut off) with a final volume of 400–500 µl. (**B**) pH titration curves of histidine residues of HIS6 RPC measured by NMR spectroscopy. The pH titration was achieved using 10 µM HIS6 RPC dissolved in deuterated water and basified using deuterated sodium hydroxide. The chemical shift for the H5 and H6 protons in the imidazole ring were plotted (relative positions of H5 and H6 protons are shown in inset). (**C**) EtBr exclusion assay was performed as previously described (21) with HIS6 RPC (filled squares) and HIS3 RPC (filled diamonds). Results are shown as mean and SD values from at least three samples. (**D**) Agarose gel electrophoresis of pCMVLuc1 complexed with HIS6 RPC at the indicated (w/w) ratio. The control lane (-) contains DNA alone. The relative locations of wells and free DNA are indicated.

**Table 1.** Reducible polycations used in study

| Polycation | Sequence | Mwt (kDa)[a] | No. of monomers per RPC[b] |
|---|---|---|---|
| HIS3 RPC | $[Cys\text{-}His_3\text{-}Lys_3\text{-}His_3\text{-}Cys]_n$ | 59 | 41 |
| HIS6 RPC | $[Cys\text{-}His_6\text{-}Lys_3\text{-}His_6\text{-}Cys]_n$ | 113 | 50 |
| $RPC_{65}$ | $[Cys\text{-}Lys_{10}\text{-}Cys]_n$ | 65 | 43 |
| HIS3 monomer | $Cys\text{-}His_3\text{-}Lys_3\text{-}His_3\text{-}Cys$ | 1.4 | —[c] |
| HIS6 monomer | $Cys\text{-}His_6\text{-}Lys_3\text{-}His_6\text{-}Cys$ | 2.2 | —[c] |

[a]The polydispersity index of RPCs was ~2.
[b]The approximate number of monomers per RPC is shown. The mean number of monomers incorporated for all RPCs used in study was 44.7 ± 4.7.
[c]Not applicable.

gave modest levels of gene expression in PC-3 cells at levels 15- to 440-fold lower than those achieved with PEI/DNA (Figure 3A). Chloroquine enhanced the transfection activity of HIS3 RPC/DNA polyplexes producing a 3.2- to 12.7-fold increase in reporter gene expression. By comparison, higher luciferase reporter gene activity was observed using HIS6 RPC/DNA polyplexes at (w/w) ratio ⩾24 at levels equivalent to PEI/DNA (Figure 3B). Furthermore, the addition of chloroquine prior to transfection with HIS6 RPC/DNA polyplexes at (w/w) ratios ⩾24 did not enhance reporter gene expression. Fluorescence-activated cell sorting (FACS) analysis was used to investigate the transfection properties of HIS RPC in more detail and showed that a significantly higher percentage of PC-3 cells were transfected by HIS6 RPC/DNA polyplexes (57.3 ± 3.6%) at (w/w) ratio of 40 than PEI/DNA (46.6 ± 1.0%) (Figure 4A). By comparison, a lower percentage of PC-3 cells were transfected using HIS3 RPC/DNA (18.9 ± 1.4%), HIS6 monomer/DNA (11.0 ± 0.9%) or $RPC_{65}$/DNA (<2%). Fluorescence microscopy also confirmed that a higher proportion of PC-3 cells were transfected by the GFP expression plasmid using HIS6 RPC at (w/w) ratio of 40 compared to PEI (Figure 4B). We next compared the transfection activity of HIS6 RPC, HIS3 RPC and PEI in a range of different cell types. Figure 4C shows that in 7 out of 10 cell

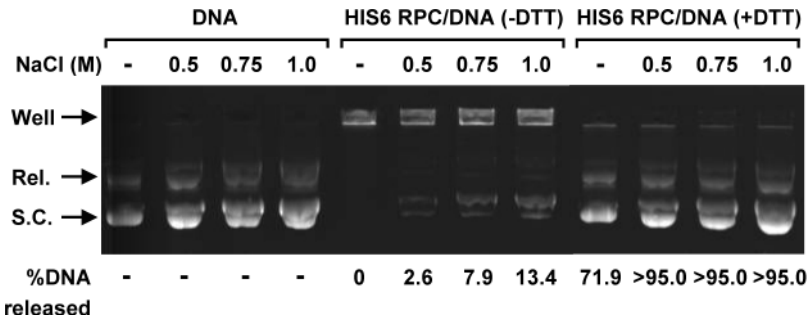
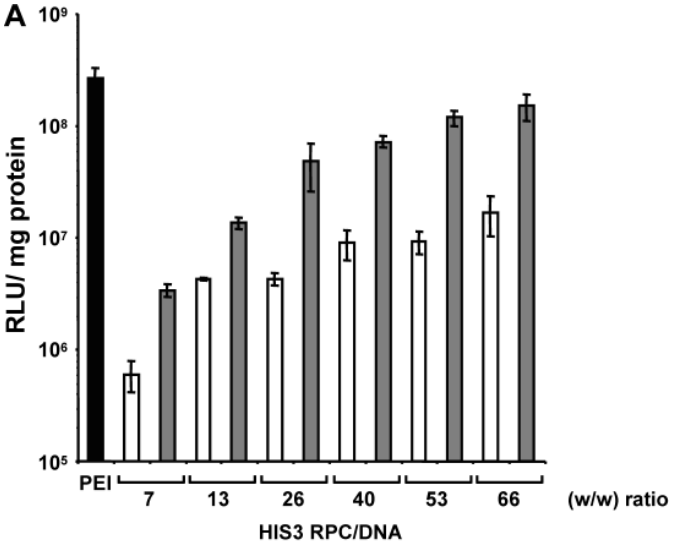



**Figure 2.** Reduction destabilizes HIS6 RPC/DNA polyplexes. To determine the ability of polyplexes to be activated by reduction HIS6 RPC/DNA polyplexes formed at (w/w) ratio of 4 were incubated with 25 mM DTT for 1 h. NaCl was added at a range of concentrations (0–1.0 M) and the amount of free DNA assessed by gel electrophoresis. ImageQuant™ software was used to quantify the amount of DNA released (Rel. = relaxed DNA; S.C. = supercoiled DNA).



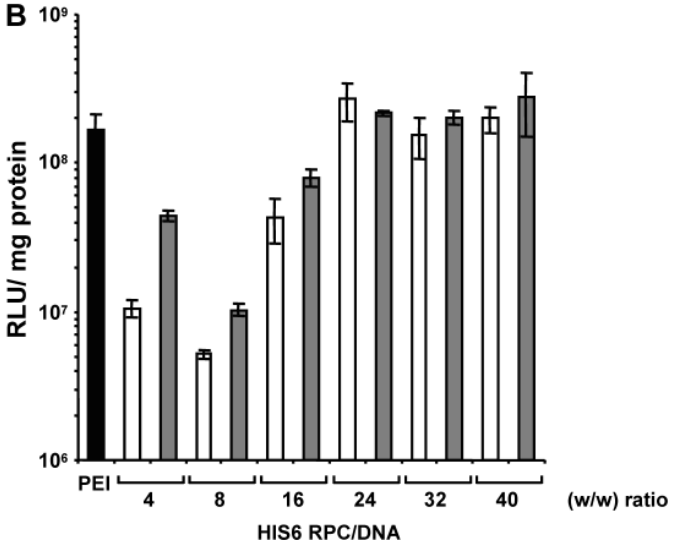




**Figure 3.** Histidine-rich RPCs facilitate chloroquine-independent gene transfer. PC-3 cells were transfected with 0.5 μg pCMVLuc condensed by either (**A**) HIS 3 RPC or (**B**) HIS6 RPC at the indicated (w/w) ratio. For control transfections 25 kDa PEI/DNA polyplexes were formed at N:P 10. Cells were incubated with polyplexes for 4 h in the absence (white bars) or presence (dark gray bars) of 100 μM chloroquine and the media was discarded and replaced with fresh media containing 10% FCS. Luciferase activity was measured after 24 h. Results are shown as mean and SD values from at least three samples.

lines used HIS6 RPC mediated significantly higher frequency of gene transfer compared to PEI achieving up to a 5-fold greater number of GFP-positive cells. Similar levels of gene transfer were observed using HIS6 RPC and PEI in A549 and MC7 cells, and PEI mediated higher levels of GFP-positive cells than HIS6 RPC only in liver carcinoma Alexander cells. These results demonstrate that in many cell types HIS6 RPC possesses superior transfection capabilities than HIS3 RPC and PEI.

### Delivery of mRNA and siRNA using HIS6 RPC

Efficient non-viral vectors are also needed to deliver RNA molecules for a wide range of therapeutic applications (25). mRNA and siRNA are active within the cytoplasm and therefore bypass the requirement for nuclear delivery. However, studies so far have demonstrated that unmodified PLL and PEI-based vectors are generally too stable to release mRNA molecules to enable efficient translation to occur (26). We therefore investigated whether histidine-rich RPCs can be used to deliver RNA molecules such as mRNA and siRNA. Figure 5A shows that HIS6 RPC mediated high levels of transfection with mRNA encoding GFP in PC-3 cells. Increasing the amount of HIS6 RPC used gave a greater percentage of GFP-positive cells with highest levels of transfection achieved using a (w/w) ratio of 24 (91.6 ± 0.76%) or 40 (96.7 ± 0.4%). In contrast, GFP mRNA delivered by PEI at N:P 10 produced only 32.9 ± 2.4% GFP-positive PC-3 cells. Fluorescence microscopy also confirmed high levels of GFP mRNA transfection using HIS6 RPC compared to PEI (Figure 5B).

Recent results from our group and others have implicated the low-affinity neurotrophin receptor $p75^{NTR}$ as a key regulator of CNS axonal growth (27,28) involved in growth cone collapse following CNS injury (29). A potential therapeutic strategy to promote axon regeneration is therefore to down-regulate levels of $p75^{NTR}$ (30,31). We therefore investigated whether histidine-rich RPCs can be used to deliver siRNA targeted against $p75^{NTR}$ in primary cultures of adult rat DRG neurons. Results from western blotting indicated virtually complete knockdown in the level of $p75^{NTR}$ following delivery of siRNA (Seq2) targeted to the mRNA of this receptor using HIS6 RPC (Figure 5C), whereas there was no decrease in $p75^{NTR}$ using a scrambled version of the siRNA (Scr2). This level of knockdown in the level of $p75^{NTR}$ was equivalent to using Oligofectamine. By comparison, PEI and HIS3 RPC were less effective at delivering the Seq2 siRNA

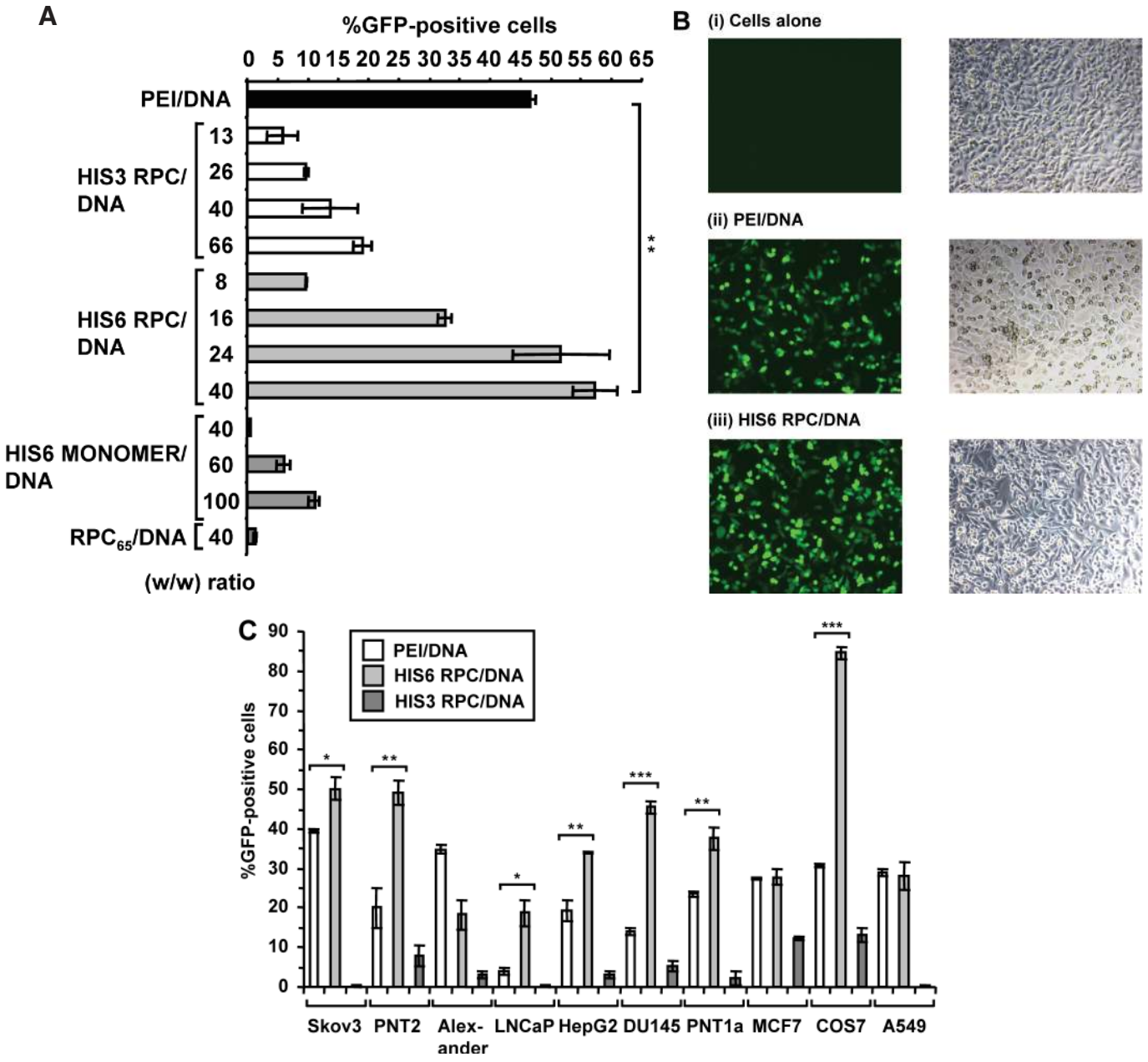



**Figure 4.** Enhanced frequency of gene transfer with histidine-rich RPCs. (**A**) PC-3 cells were transfected with 0.5 μg pEGFPN1 condensed by either HIS3 RPC, HIS6 RPC, HIS6 monomer or $RPC_{65}$ at the indicated (w/w) ratio, and by 25 kDa PEI at N:P 10 ($P = 0.0005$). (**B**) Fluorescent images of PC-3 cells expressing GFP following transfection with (i) nothing or 0.5 μg pEGFPN1 condensed with (ii) 25 kDa PEI at N:P 10 or (iii) HIS6 RPC at (w/w) ratio of 40. Phase-contrast images of transfected PC-3 cells are shown in the corresponding right-hand column. (**C**) A range of cell types were transfected as indicated with 0.5 μg pEGFPN1 condensed by 25 kDa PEI at N:P 10 (white bars), HIS6 RPC at (w/w) ratio of 40 (light grey bars) or HIS3 RPC at (w/w) ratio of 66 (dark grey bars). GFP expression was measured after 24 h by flow cytometry analysis. Results are shown as mean and SD values from at least three samples. Significant differences between HIS6 RPC/DNA and 25 kDa PEI/DNA transfected cells are indicated (***, $P \leqslant 0.0001$; **, $P \leqslant 0.001$; *, $P \leqslant 0.01$).

with knockdowns of 56 and 25%, respectively, in the levels of $p75^{NTR}$.

A recent study demonstrated efficient siRNA delivery using linear PEI, also known as JetPEI, in a subcutaneous mouse tumour model (32). We therefore directly compared the ability of HIS6 RPC and JetPEI to mediate down-regulation of GFP expression following co-transfection of PC-3 cells with the GFP reporter plasmid, pEGFPN1 and GFP-22 siRNA. Figure 5D shows that at a concentration of 5 nM GFP-22 siRNA, there was >80% reduction in the mean fluorescence per cell (MnX) mediated by HIS6 RPC polyplexes formed at a (w/w) ratio of 24. By comparison, higher concentrations of GFP-22 siRNA (⩾50 nM) were required to mediate similar levels of knockdown with JetPEI at either N:P 5 or 10 indicating that silencing of GFP fluorescence was less efficient using this cationic polymer as the delivery vector (Figure 5D). At a concentration of 50 nM GFP siRNA, for example, there was a 90% reduction in the MnX using HIS6 RPC compared to

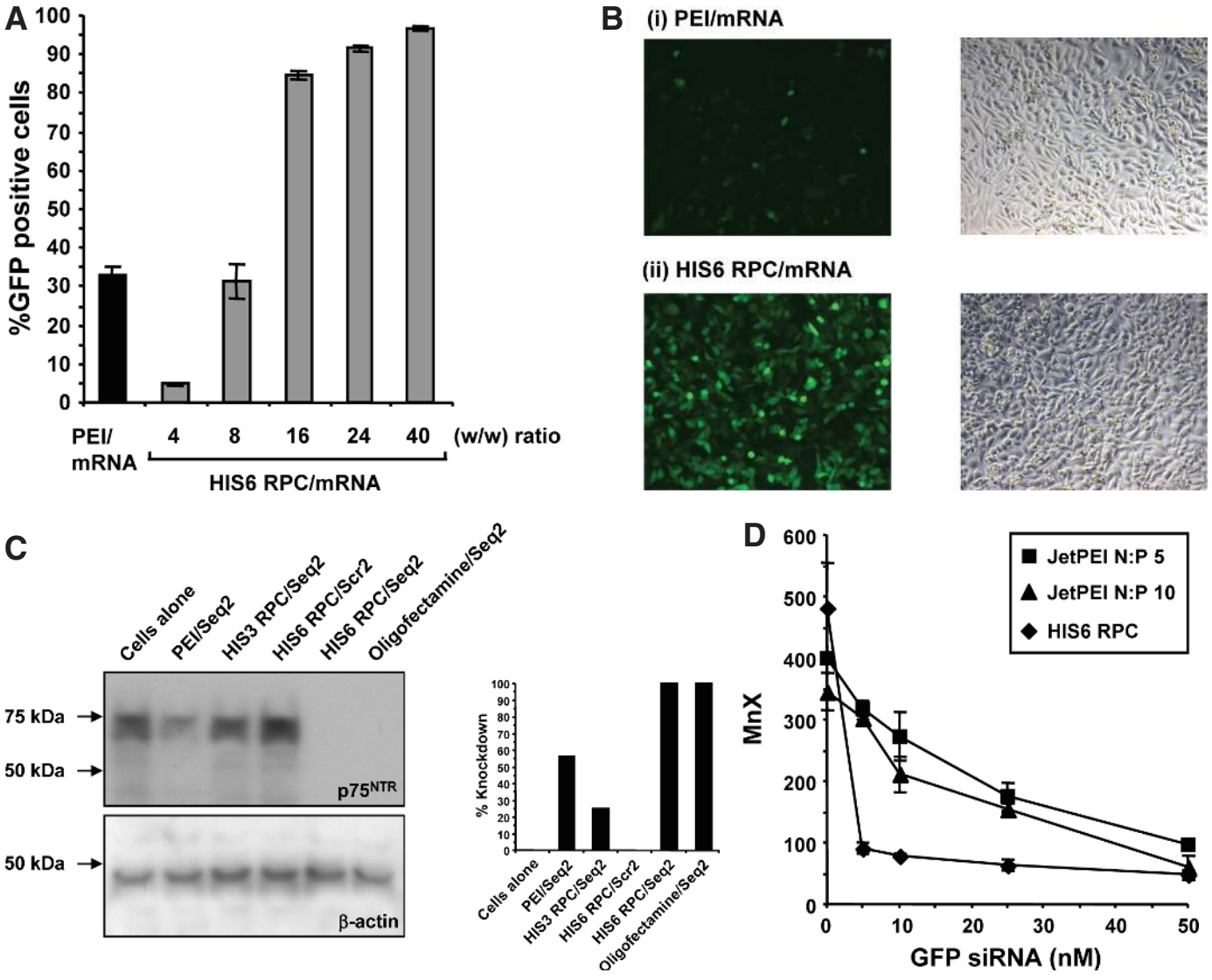



**Figure 5.** Histidine-rich RPCs mediate efficient delivery of mRNA and siRNA. (**A**) PC-3 cells were transfected with 0.5 μg cap-*GFP*-$A_{64}$ mRNA condensed either by PEI at N:P 10 (black bar) or HIS6 RPC at the indicated (w/w) ratio (dark grey bars). (**B**) Fluorescent images of PC-3 cells expressing GFP following transfection with 0.5 μg cap-*GFP*-$A_{64}$ mRNA condensed with (i) 25 kDa PEI at N:P 10 or (ii) HIS6 RPC at (w/w) ratio of 40. Phase-contrast images of transfected PC-3 cells are shown in the corresponding right-hand column. (**C**) Western blot analysis of $p75^{NTR}$ (upper panel) and β-actin (lower panel) using DRG culture lysates following transfection with siRNA directed against $p75^{NTR}$ (Seq2) or a scrambled sequence (Scr2) and condensed either by 25 kDa PEI at N:P 10, HIS3 RPC at (w/w) ratio of 66, HIS6 RPC at (w/w) ratio of 40 or Oligofectamine at (w/w) ratio of 3.5 as indicated. The relative% knockdowns in $p75^{NTR}$ levels are shown in the bar graph on the right-hand side. (**D**) PC-3 cells were co-transfected with 0.5 μg pEGFPN1 and GFP-22 siRNA at the indicated dose condensed either by JetPEI at N:P 5 (squares), JetPEI at N:P 10 (triangles) or HIS6 RPC at (w/w) ratio of 24 (diamonds). In panels (A) and (D), flow cytometry analysis was used to determine %GFP positive cells and mean fluorescence per cell (MnX) after 24 h. Results are shown as mean and SD values from at least three samples.

83% with JetPEI at N:P 10. Altogether, these results demonstrate that histidine-rich RPCs can efficiently deliver siRNA molecules to mediate gene silencing at levels similar or greater than using commercially available transfection reagents.

### Effect of salt-induced aggregation of particles on transfection activity

The inability of chloroquine to enhance reporter gene expression using HIS6 RPC indicated that intracellular delivery of DNA to the cytoplasm had been significantly improved compared to PLL/DNA. However, previous studies have shown that particle size (33), cellular toxicity (8), the intracellular reducing environment (13) or the presence of free polycation (34) can all influence the transfection properties of polycations. We therefore investigated whether any of these factors might contribute to the high transfection efficiency of histidine-rich RPCs. First, photon correlation spectroscopy showed that discrete particles with diameters in the range of 36–78 nm were formed between HIS3 RPC, HIS6 RPC, HIS3 monomer, HIS6 monomer or $RPC_{65}$ with DNA (Figure 6A and Supplementary Figure 1). The addition of salt to histidine-rich RPC/DNA polyplexes resulted in rapid aggregation of particles with diameters of 1273.9 nm

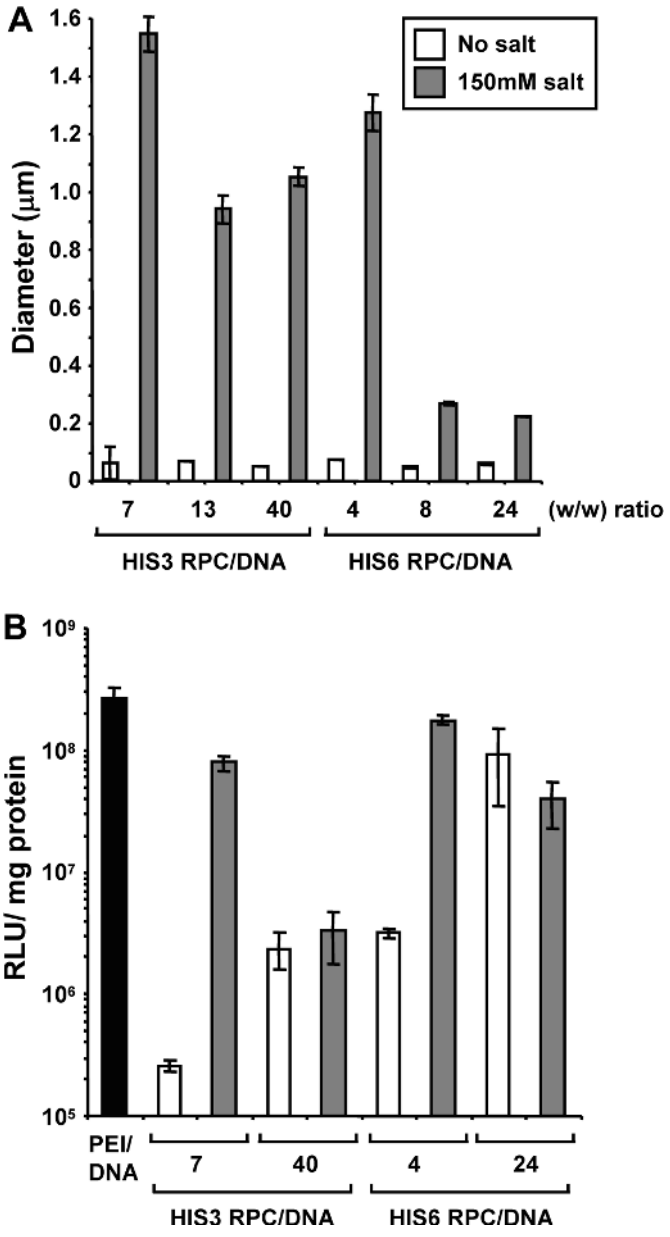



**Figure 6.** Effect of salt-induced aggregation of particles on transfection activity. (**A**) Photon correlation spectroscopy was used to assess changes in the diameter of polyplexes formed between DNA condensed with either HIS3 RPC or HIS6 RPC at the indicated (w/w) ratio in the absence (white bars) or presence (dark grey bars) of 150 mM NaCl for 2.5 h. (**B**) PC-3 cells were transfected with 0.5 μg pCMVLuc1 condensed by either PEI at N:P 10 (black bar), HIS3 RPC or HIS6 RPC at the indicated (w/w) ratio. Polyplexes were incubated in the absence (white bars) or presence of 150 mM NaCl for 30 min (dark grey bars) prior to addition to cells. Luciferase activity was measured after 24 h. Results are shown as mean and SD values from at least three measurements.

(HIS6 RPC) and 1545.8 nm (HIS3 RPC) after 2.5 h at (w/w) ratios of 4 and 7, respectively (Figure 6A). However, HIS6 RPC/DNA polyplexes at higher (w/w) ratios ⩾8 were generally stable in salt with average diameters between 223.0 ± 1.2 and 267.3 ± 4.7 nm (Figure 6A). Similarly, polyplexes formed with the lysine-based $RPC_{65}$ were also relatively stable to salt-induced aggregation, even at low (w/w) ratios, with diameters of ~200 nm (Supplementary Figure 1). In contrast, polyplexes formed with low mwt HIS6 monomer or HIS3 monomer aggregated rapidly in salt with diameters >1600 nm when formed at (w/w) ratios of 10–60 (Supplementary Figure 1).

Histidine-rich RPC/DNA polyplexes were then incubated in salt for 30 min prior to transfection to determine whether salt-induced aggregation directly influenced their transfection capability. A 54-fold (HIS6 RPC) and 300-fold (HIS3 RPC) increase in gene expression was observed using salt-aggregated polyplexes at (w/w) ratios of 4 and 7, respectively. In contrast, the addition of salt to histidine-rich RPC/DNA polyplexes at higher (w/w) ratios of 24 and 40 did not significantly alter the level of reporter gene expression (Figure 6B). These results indicate that preincubation of histidine-rich RPC/DNA polyplexes at low (w/w) ratios in salt is a useful strategy to enhance gene transfer. However, the inability of salt to enhance transfection of HIS6 RPC/DNA polyplexes at higher (w/w) ratios indicated that salt-induced aggregation was not the mechanism responsible for mediating efficient gene transfer with this vector.

### Cellular toxicity of PEI and histidine-rich RPCs

Cellular toxicity is often associated with efficient transfection of cells using non-viral vectors such as 25 kDa PEI (35), which is likely to be due to the membrane damaging effects of polycations (36). We therefore evaluated the toxicity of histidine-rich RPC/DNA polyplexes at high (w/w) ratios where there is likely to be a large excess of free polycation. Results using the MTS viability assay demonstrated negligible cellular toxicity with either HIS3 RPC/DNA or HIS6 RPC/DNA at (w/w) ratio of 40 (Figure 7A). No significant toxicity was also detected with PEI/DNA at N:P 10 using the MTS assay, although a significant proportion of cells had a rounded morphology indicating a loss of adherence (Figure 4B). Significant toxicity was however observed using polyplexes based on $PLL_{54}$ or $PLL_{85}$ at the same (w/w) ratio with an 88% reduction in cell viability. We next evaluated the toxicity of free polycations using PC-3 cells and showed that increasing the dose of PEI from 0.2 to 3 μg per $5 \times 10^4$ cells resulted in a 92.7% reduction in cell viability. In contrast, there was no significant decrease in viability of PC-3 cells even when a 4-fold higher dose of 12 μg per $5 \times 10^4$ cells of HIS6 RPC was used (Figure 7B). These results demonstrate that histidine-rich RPCs have negligible toxicity and are significantly less toxic than free PEI.

### Effect of intracellular GSH levels on transfection activity

We next investigated whether the transfection ability of HIS6 RPC/DNA polyplexes was influenced by the intracellular reducing environment. A fluorescence assay based on the dye mBCl was used to determine intracellular glutathione content (22). Using this assay, we established that cells preincubated for 3 h in the presence of GSH–MEE, a cell permeable form of GSH, contained a 2- to 3-fold higher amount of GSH. Also, preincubation of cells for 24 h in the presence of DL-BSO, an agent which depletes glutathione by inhibiting its synthesis, resulted in a 50–80% reduction in GSH depending on the dose used (Figure 8A).

Up to a 210-fold decrease in gene expression was observed when PC-3 cells were preincubated in 10 mM GSH–MEE and

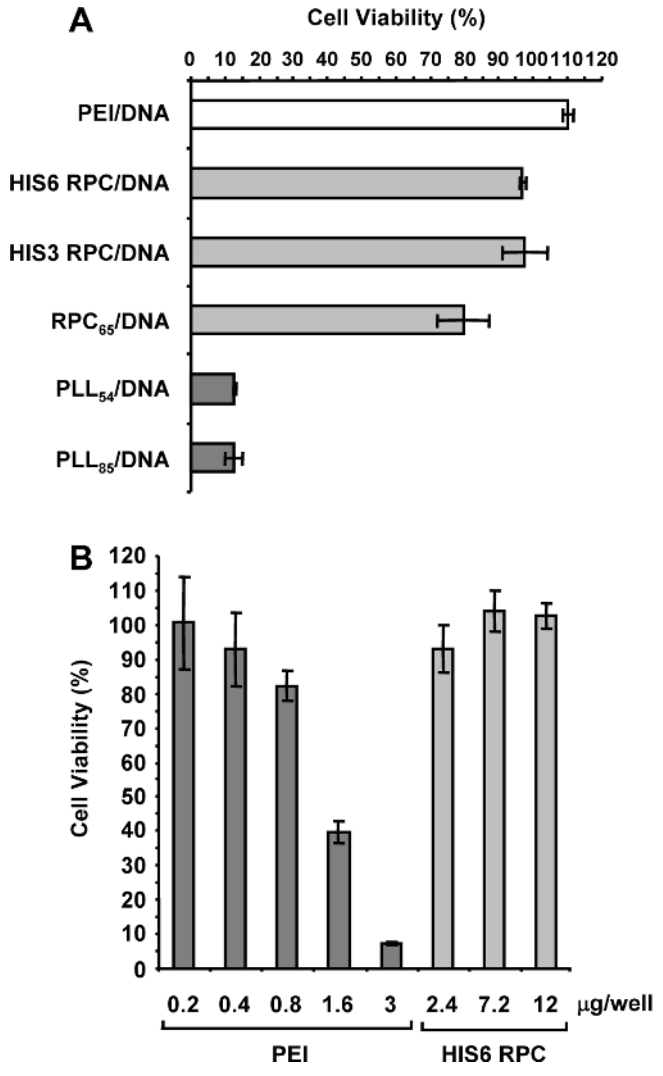



**Figure 7.** Cellular toxicity of PEI and histidine-rich RPCs. (**A**) PC-3 cells were transfected with 0.5 µg of pCMVLuc1 condensed either by 25 kDa PEI at N:P 10 or HIS6 RPC, HIS3 RPC, $RPC_{65}$, $PLL_{54}$ and $PLL_{85}$ at (w/w) ratio of 40. The cells were incubated with polyplexes for 4 h in serum-free media and the media discarded and replaced with fresh media containing 10% FCS. The MTS assay was used to assess the viability of transfected PC-3 cells after 24 h. (**B**) PC-3 cells were incubated in the presence of 25 kDa PEI (dark grey bars) or HIS6 RPC (light grey bars) at the indicated dose for 4 h in serum-free media. The media was discarded and replaced with fresh media containing 10% FCS and the MTS assay used to assess cellular viability after 24 h. Results are shown as mean and SD values from at least three samples.

transfected with HIS6 RPC/DNA polyplexes (Figure 8B). In contrast, there was only a 3.1-fold decrease in gene expression following transfection of cells with PEI/DNA after exposure to 10 mM GSH–MEE. The most significant change in gene expression in BSO-treated PC-3 cells was a modest 3.9-fold decrease in luciferase reporter gene expression following transfection with HIS6 RPC/DNA polyplexes. No significant changes in gene expression were observed by transfection with PEI/DNA or HIS6 monomer/DNA polyplexes following treatment of PC-3 cells with BSO (Supplementary Figure 2).

The relative intracellular levels of GSH in a range of cell types following treatment with mBCl were then studied by multiphoton fluorescence microscopic analysis (Figure 8C and Supplementary Figures 3 and 4). In most cell types, such as PNT2, PNT1a, COS7, HepG2, PC-3 and Skov3, small punctate spherical bodies were observed that were present throughout the cell. However in others, such as Alexander cells, these punctate bodies were not as apparent (Figure 8C and Supplementary Figure 3). Previous work has indicated that these punctate spherical bodies may correspond to mitochondria, which contain high intracellular stores of cellular sulfhydryls (37). Linear profiling of mBCl fluorescence in several cell types demonstrated heterogeneity in GSH levels both in individual cells and between different cell types. Alexander cells, for example, had one of the highest GSH contents with a low degree of heterogeneity, whereas PNT2 cells had the lowest GSH content (Figure 8D).

Altogether, these results indicated that there was sufficient reducing capacity within cells to activate HIS RPC-based vectors as BSO-treatment resulted in only a modest fall in transfection activity. Furthermore, high levels of gene transfer were still observed in cells, such as PNT2, that had one of the lowest GSH contents (Figure 8D). Unexpectedly, increasing the intracellular GSH with GSH–MEE significantly diminished transfection with HIS6 RPC/DNA polyplexes, which is in contrast to a previous study using the RPC-based vector $RPC_{187}$/DOTAP (13). The transfection activity of histidine-rich RPCs may therefore depend on the rate of cleavage of the polycation as this will most likely be accelerated in cells with elevated intracellular GSH.

### Free HIS6 RPC enhances PLL-mediated gene transfer

In the present study, highest levels of gene expression were observed using polyplexes at higher (w/w) ratios where there is likely to be free polycation in solution. To determine whether free HIS6 RPC can directly influence gene transfer PC-3 cells were transfected in the absence or presence of histidine-rich polycations with non-viral vectors such as PLL/DNA and $RPC_{65}$/DNA that are known to mediate only low levels of gene transfer. FACS analysis showed that addition of free HIS6 RPC to PC-3 cells prior to transfection with $RPC_{65}$/DNA polyplexes resulted in a 22.4-fold increase in GFP-positive cells from $2.24 \pm 0.3\%$ to $50.2 \pm 1.3\%$ (Figure 9A). Similarly, a 2.5-fold increase in GFP-positive cells was observed from $9.7 \pm 0.2\%$ to $30.5 \pm 2.3\%$ when free HIS6 RPC was added prior to transfection with PLL/DNA polyplexes. In contrast, there was no significant increase in %GFP-positive cells by addition of the HIS6 monomer to cells prior to transfection with either $RPC_{65}$/DNA or PLL/DNA.

We next incubated PC-3 cells in the presence of HIS6 RPC for varying durations prior to addition of PLL/DNA polyplexes. The simultaneous addition of free HIS6 RPC with PLL/DNA resulted in a 21.5-fold increase in gene expression using the LUC reporter gene (Figure 9B). After a 1 h preincubation with the HIS6 RPC, there was still an improvement in gene expression but this was decreased to 5.9-fold. However, after a 2 or 4 h preincubation with HIS6 RPC, there was no significant enhancement in reporter gene expression. These results demonstrate that the presence of free HIS6 RPC can influence the transfection activity of gene therapy vectors, especially polyplexes based on PLL residues that are known to be relatively inefficient at mediating escape from endocytic vesicles.

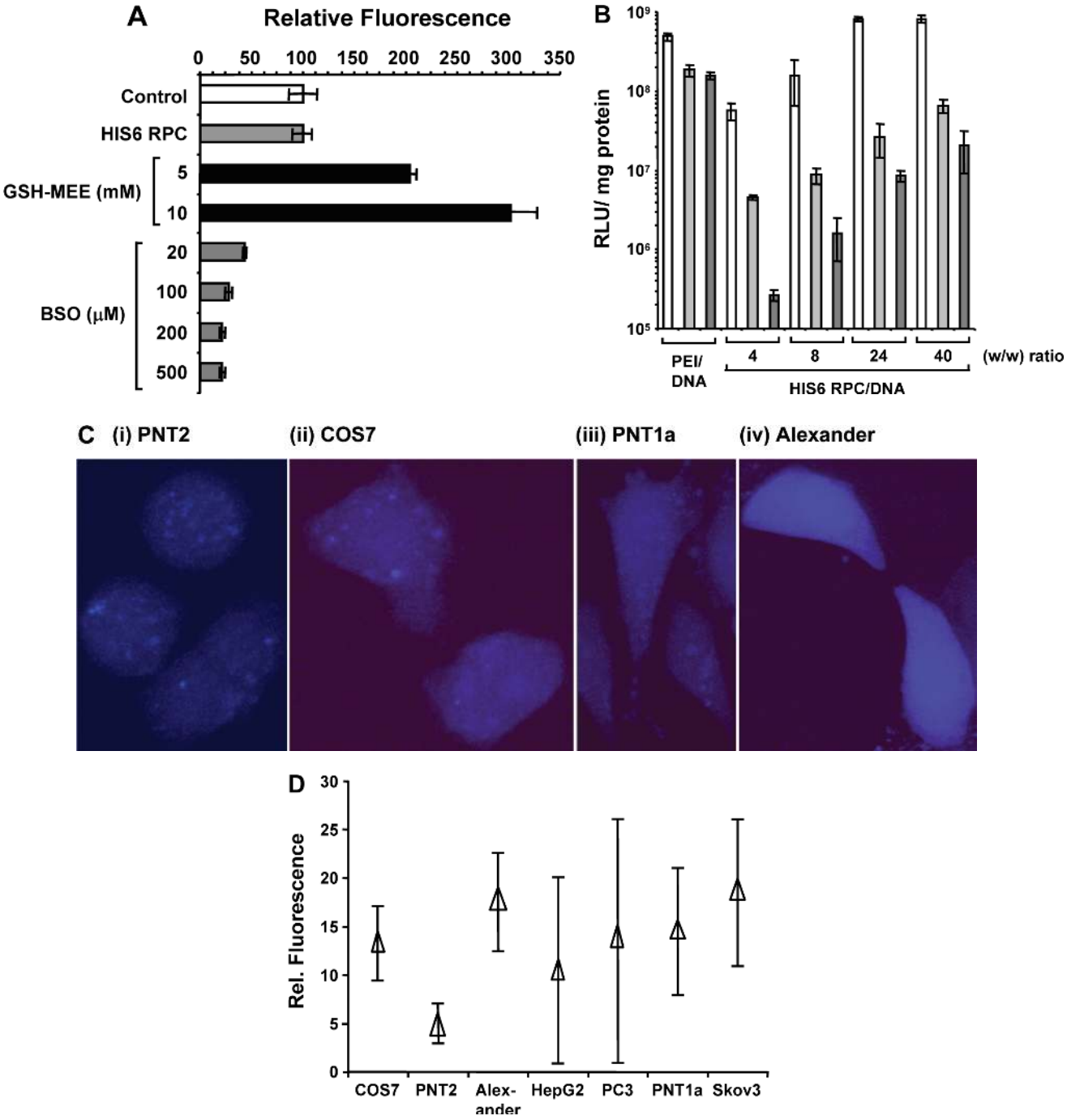



**Figure 8.** Effect of intracellular GSH levels on transfection activity. (**A**) PC-3 cells were incubated in the presence of GSH–MEE for 3 h (black bars) or BSO for 24 h (dark grey bars) at the indicated concentrations and GSH levels determined as described by Sebastia *et al*. (22). For control experiments, cells were incubated on their own (white bars) or in the presence of HIS6 RPC (light grey bar). (**B**) PC-3 cells were incubated for 3 h in serum-free media containing 0 mM (white bars), 5 mM (light grey bars) or 10 mM GSH–MEE (dark grey bars) prior to transfection with 0.5 μg pCMV-Luc1 condensed with either 25 kDa PEI at N:P 10 or HIS6 RPC at the indicated (w/w) ratio. Luciferase activity was measured after 24 h. Results are shown as mean and SD values from at least three samples. (**C**) Microscopic comparison of GSH distribution in isolated (i) PNT2, (ii) COS7, (iii) PNT1a and (iv) Alexander cells based on mBCl fluorescence. (**D**) Relative fluorescence of mBCl-treated cells obtained by linear profiling from at least ten stacks in the indicated cell type. The empty triangle represents the mean fluorescence, whereas vertical bars represent the maximum and minimum levels of fluorescence. Background fluorescence was subtracted from the mean value.

## DISCUSSION

Progress in the field of gene therapy is hindered by the lack of suitable delivery vectors. Synthetic vectors based on polycations are promising vectors for gene delivery as they are relatively safe and can be readily modified by the incorporation of ligands for targeting to specific cell types (38,39). However, the levels of gene expression mediated by synthetic vectors are typically low compared to viral

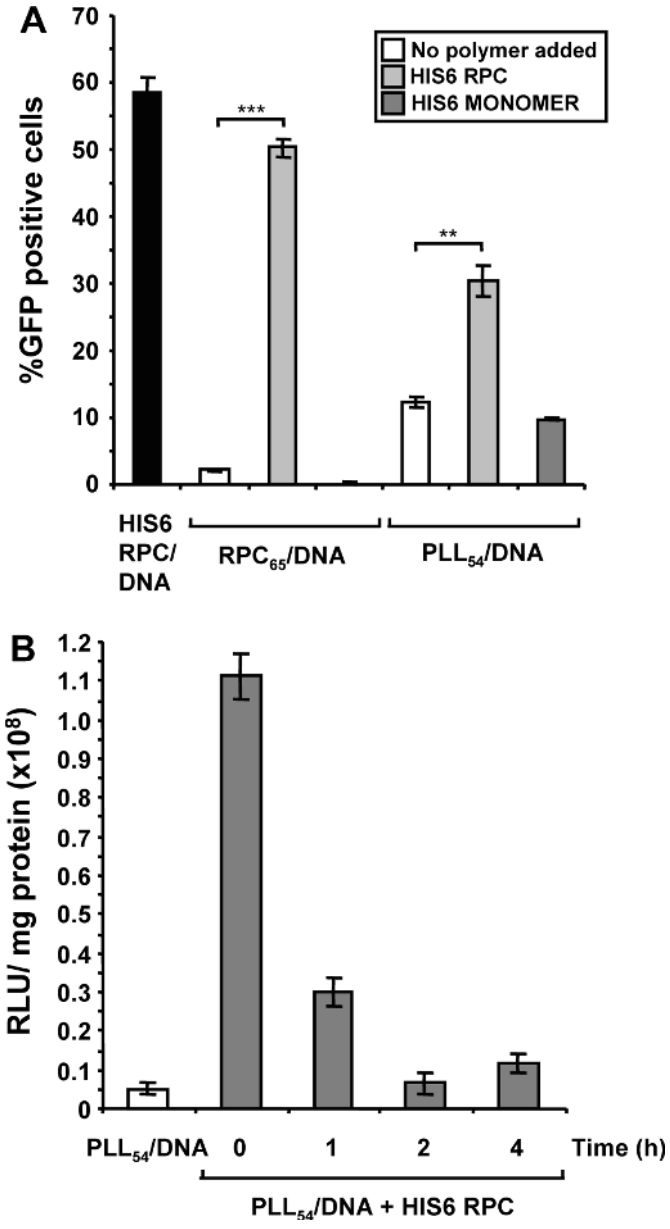



**Figure 9.** Free HIS6 RPC enhances $PLL_{54}$- and $RPC_{65}$-mediated gene transfer. (**A**) PC-3 cells were transfected with 0.5 μg pEFGPN1 condensed with HIS6 RPC at (w/w) ratio of 40, $RPC_{65}$ at (w/w) ratio of 2 or $PLL_{54}$ at (w/w) ratio of 2. In these experiments, polyplexes were added to cells on their own (white bars) or by prior addition of free HIS6 RPC (light grey bars) or HIS6 monomer (dark grey bars). (**B**) Same as in (A) except that free HIS6 RPC was added to PC-3 cells at the indicated number of hours prior to transfection with 0.5 μg pCMV-Luc1 condensed with $PLL_{54}$ at (w/w) ratio of 2. Luciferase activity was measured after 24 h. Results are shown as mean and SD values from at least three samples. Significant differences between RPC/DNA and PLL/DNA transfected cells in the absence or presence of HIS6 RPC are indicated (***, $P \leq 0.0001$; **, $P = 0.0002$).

vectors. In addition, the type and molecular weight of polycation used to deliver a particular type of nucleic acid has to be carefully selected to give optimal transfection levels. For instance, we recently showed that polyplexes based on low mwt peptides, such as 2 kDa PEI, were significantly more efficient at delivering mRNA than 25 kDa PEI, which was presumably due to fewer electrostatic interactions enabling greater access of mRNA to the translational machinery (26). In this study, we attempted to produce a more versatile polycation-based vector by evaluating the properties of reducible polycations consisting of histidine and lysine residues. Transfection experiments showed efficient gene transfer in a range of cell types achieving up to nearly a 5-fold increase in the percentage of transfected cells compared to PEI. In contrast to PEI, histidine-rich RPCs also mediated efficient transfer of a broad range of nucleic acid molecules including mRNA encoding for GFP in PC-3 cells and a 21mer siRNA directed against the low affinity neurotrophin receptor $p75^{NTR}$ in primary cultures of post-mitotic DRG neurons.

Uptake of polyplexes into cells typically occurs by endocytosis where polyplexes are internalized into endocytic vesicles and follow the lysosomal trafficking pathway eventually leading to degradation of the polymer or DNA, or both, by the acidic pH and by various lysosomal enzymes. The inability of polyplexes to avoid this fate is a major barrier to efficient transfection following internalization by endocytosis (10). Chloroquine is widely used to enhance polyplex-mediated gene transfer *in vitro* by favouring the survival of polyplexes in endocytic vesicles and promoting their release into the cytoplasm (10,40). The precise role of chloroquine on gene transfer is still not fully understood, although recent data from flow cytometric analysis of pH-sensitive fluorophores conjugated to PLL confirmed that chloroquine most likely acts by enhancing escape from endocytic vesicles, but this mechanism does not appear to involve pH buffering (40). Previous results from our group indicated that lysine-based RPCs were inefficient at mediating endocytic escape since chloroquine produced a 10- to 130-fold increase in reporter gene expression (13). In this study, we demonstrated that histidine-rich RPCs produced levels of gene expression $>10^8$ RLU/mg per protein in the absence of chloroquine, which is at least 10-fold higher than levels observed previously using lysine-based RPCs in PC-3 cells (13). Furthermore, the inability of chloroquine to enhance transfection with HIS6 RPC/DNA polyplexes at (w/w) ratios $\geqslant$24 indicated that transfer of DNA from endocytic vesicles to the cytoplasm had been significantly improved.

Histidine residues were incorporated into RPCs as the imidazole group has a $pK_a$ ~6.0 which becomes cationic in a slightly acidic medium and may facilitate escape from endocytic vesicles. Indeed, data from several groups have shown that poly-L-histidine mediates an acid-dependent fusion and leakage of negatively charged liposomes (41,42). In addition, Goncalves *et al*. recently showed that histidylated polylysines complexed with DNA had prolonged stability inside early endocytic vesicles that was likely to favour DNA escape into the cytosol (43). In this study, the content of histidine residues appeared to be an important factor in determining the transfection activity of RPCs. Highest transfection activity was mediated by HIS6 RPC which contained ~600 histidine residues per polymer (70% histidine content) compared to 246 in the HIS3 RPC (55% histidine content). These results are in contrast with those of McKenzie *et al*., where optimal gene transfer was observed with disulfide cross-linked peptides containing 10–30% histidine content. Increasing the histidine content to 40–50% gave progressively lower levels of gene transfer in HepG2 and CHO cells (44). A possible explanation

given by McKenzie *et al*. was that peptides with a histidine content of 40–50% produced larger particles of 205–320 nm in diameter. In contrast, in this study the advantage of using RPCs with 55–70% histidine content was that polyplexes formed with DNA remained small with diameters still in the range of 50–60 nm.

A requirement for effective gene delivery is that vectors are relatively stable in physiological conditions. It has been shown, for instance, that polyplexes formed with unmodified low mwt polycations, such as 20 kDa PLL, are unsuitable for *in vivo* applications as they tend to aggregate rapidly in the presence of 150 mM salt (11). Similarly, in this study polyplexes formed with either HIS3 or HIS6 monomers with mwts of 1.4–2.2 kDa formed large aggregates >1.66 μM in diameter in salt (data not shown). An important finding in this study was that polyplexes formed with HIS6 RPC were stabilized to salt-induced aggregation at (w/w) ratios ⩾8. Indeed average diameters of HIS6 RPC-based polyplexes were ∼200 nm in 150 mM salt. Similarly, polyplexes formed with lysine-based RPCs were also relatively stable in physiological salt conditions with average diameters of 210 nM. The transfection activity of lysine-based RPCs was, however, significantly different to histidine-rich RPCs producing <2% GFP-positive cells. These results therefore indicate that the enhanced transfection efficiency mediated by histidine-rich RPCs compared to lysine-based RPCs was likely to be due to the direct influence of histidine residues and not to differences in the size or stability of polyplexes.

Although polyplexes formed with higher mwt polymers have improved stability in physiological conditions, data from studies by Schaffer *et al*. (7) and ourselves (13) have indicated that high mwt polymers, such as 211 kDa PLL, can hinder vector unpacking. An important consideration in developing vectors based on high mwt histidine-rich polycations was therefore to include cysteine residues to enable them to be triggered by reduction to facilitate release of nucleic acids. This was confirmed by initial experiments using gel electrophoresis which showed that incubation of HIS6 RPC with the reducing agent DTT facilitated release of DNA even in low salt conditions. We studied the effect of reduction on gene transfer in more detail by altering intracellular levels of GSH with the reagents GSH–MEE and BSO. The most significant finding was a decrease of up to 210-fold in gene expression following gene transfer with HIS6 RPC/DNA polyplexes in GSH–MEE treated PC-3 cells. The rate of degradation of reducible polycations will most likely be greater in cells with elevated GSH levels, which suggests that HIS6 RPC needs to be relatively intact to mediate efficient gene transfer and that cleavage of the polymer by reduction diminishes transfection activity. This finding was further supported by results from transfection of PC-3 cells using the low mwt HIS6 RPC monomer where only low levels of gene expression were observed. Hence, it may be possible to improve the transfection properties of HIS RPCs by increasing their mwts.

Linear profiling of images of mBCl-treated cells captured by multiphoton fluorescence microscopy showed heterogeneity in GSH levels in a range of different cell types. There was no direct correlation between GSH content and levels of gene transfer mediated by HIS RPC. In particular, efficient gene transfer occurred in PNT2 cells with a low GSH content suggesting that there was sufficient reducing capacity within cells used in this study to activate HIS RPC-based vectors. However, based on the observations with PC-3 cells treated with GSH–MEE, it is possible that cells with high GSH content may limit the ability of HIS RPC to mediate efficient gene transfer. Indeed, this may have contributed towards the inability of HIS RPC to mediate higher levels of gene transfer than PEI in Alexander cells that have a high GSH content with low heterogeneity. Although, other factors are also likely to influence gene transfer with RPC-based vectors such as the level of cytoplasmic nucleases and the rate of cell cycling of different cell-types (45).

The ability of GSH–MEE to diminish HIS RPC-mediated gene transfer was unexpected as previously we observed a 33-fold increase in gene expression using the lysine-based $RPC_{187}$ at a (w/w) ratio of 12.8 in combination with DOTAP in GSH–MEE treated HeLa cells (13). However, this is likely to reflect the different roles of RPCs in mediating gene transfer between these gene delivery vectors. HIS6 RPC is most likely facilitating endosomal escape of DNA, whereas the lysine-based $RPC_{187}$ condenses DNA but the DOTAP component is responsible for mediating the escape from endosomes. Hence, the improvement in transfection observed previously with $RPC_{187}$/DOTAP in cells with elevated GSH levels is likely to be due to improved DNA release from the polycation (13). A schematic detailing the proposed mechanism for HIS RPC-mediated delivery of nucleic acids is outlined in Figure 10.

Recently, the usefulness of delivering RNA has been gaining greater interest and many groups are now focused on improving the stability and delivery of RNA molecules (25). One reason for this has been the recent emergence of small interfering RNA (siRNA) to trigger RNA interference with many potential therapeutic applications (46). In addition, there is considerable interest in delivering mRNA to dendritic cells to stimulate potent CTL responses and induce antitumour immunity (47). In the present study, we showed that histidine-rich RPCs were efficient at delivering both mRNA and siRNA molecules. In comparison to 25 kDa PEI, HIS6 RPC gave a 3-fold higher percentage of GFP-positive cells with GFP mRNA and ∼2-fold greater level of gene silencing of $p75^{NTR}$ in DRG cultures. Indeed, western blot analysis showed virtually complete knockdown of $p75^{NTR}$ following delivery of siRNA by HIS6 RPC. Recent studies have also shown efficient siRNA delivery using peptide-based systems such as JetPEI (32), Penetratin 1 (48) and a fusion peptide domain of HIV-1 gp41 protein and the nuclear localization sequence of SV40 large T antigen (49). However, to the best of our knowledge this is the first demonstration of effective delivery of an siRNA molecule by a reducible polycation-based system in post-mitotic cells. Furthermore, we were able to demonstrate in co-transfection experiments the ability of HIS RPCs to deliver GFP-22 siRNA and silence GFP expression at siRNA concentrations significantly lower than those required using JetPEI.

Throughout this study, the ability of histidine-rich RPCs to deliver nucleic acids was compared with 25 kDa PEI (4), which is one of the most widely studied polycations and is generally regarded as a gold-standard synthetic vector. Our results demonstrated that HIS RPCs shared several important transfection properties similar to PEI. For instance, Ogris *et al*. showed that salt-induced aggregation of 800 kDa branched PEI/DNA polyplexes at low N:P ratios was associated with

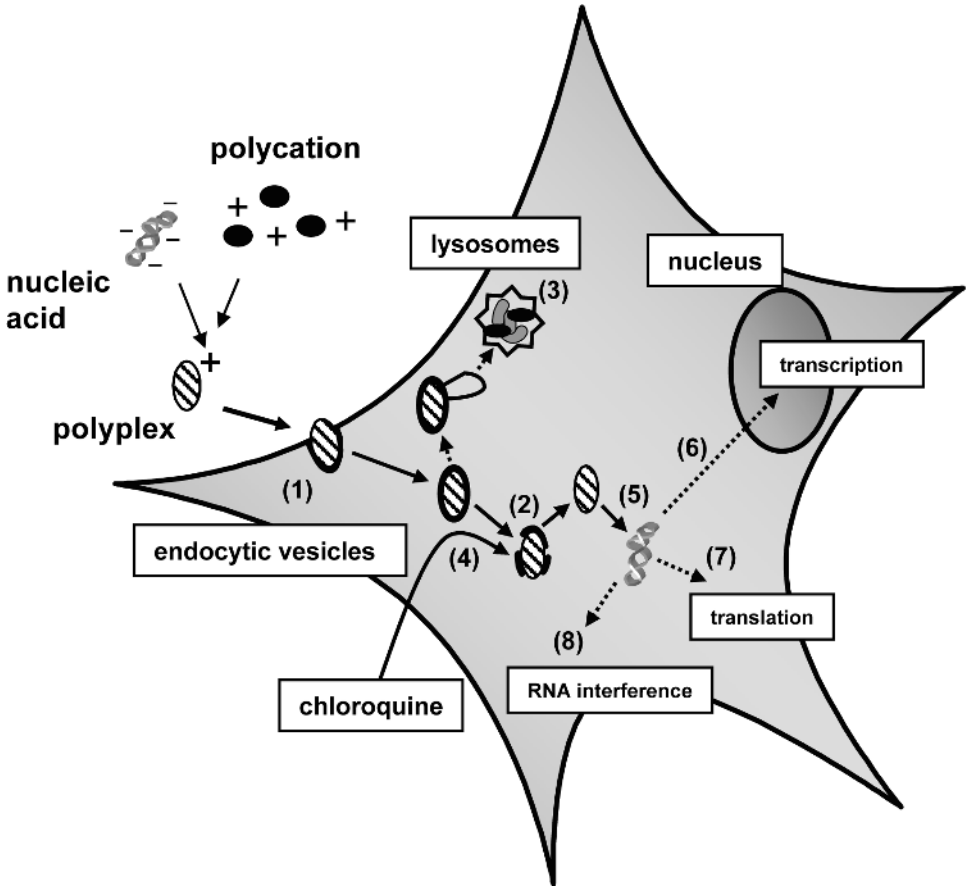



**Figure 10.** Proposed mechanism for HIS RPC-mediated delivery of nucleic acids. HIS RPC/DNA polyplexes are first internalized by endocytosis (1) and mediate escape from endocytic vesicles into the cytoplasm (2) thus avoiding degradation in lysosomal compartments (3). In the case of HIS3 RPC-based vectors, endosomal escape can be triggered by the buffering agent chloroquine (4). Intracellular reduction of HIS RPC-based polyplexes enables efficient cytoplasmic release (5) enabling transcription (6), translation (7) or RNA interference (8) to proceed depending on the nucleic acid payload.

a 10- to 100-fold increase in gene expression (33). In our study, salt-induced aggregation of HIS RPC/DNA polyplexes at low (w/w) ratios enhanced reporter gene expression by up to 300-fold. Polyplexes formed at higher (w/w) ratios were, however, relatively unaffected by the presence of salt with no significant changes in gene expression. Free PEI has also been shown to enhance the transfection activity of polyplexes (34,50), especially those formed with polycations that are known to have a poor ability to facilitate escape from endocytic vesicles. In this study, the addition of HIS6 RPC to cells prior to transfection mediated by $RPC_{65}$ resulted in a 22.4-fold increase in the percentage of GFP-positive cells. This level of enhancement was, however, diminished by increasing the duration of exposure of HIS6 RPC to cells prior to transfection, which is likely to be associated with the rate of turnover of the polycation in cells.

Despite the high levels of gene transfer that can be achieved with PEI both *in vitro* (4) and *in vivo* (51), there are concerns over its use due to toxicity. For instance, PEI has been shown to be toxic in many cell lines leading to reduced cell metabolic activity (52) and cell adherence (53). A goal of the present study was to develop a polycation capable of mediating nucleic acid transfer at levels similar or greater than PEI but without causing significant toxicity. We were able to demonstrate superior nucleic acid transfer capabilities of histidine-rich RPCs compared to PEI in the majority of cell types used and in primary cultures of post-mitotic cells. Furthermore, there was negligible toxicity following incubation of cells with HIS6 RPC compared to a 90% reduction in cell viability when a 4-fold lower dose of free PEI was used. Previous studies have shown that the cytotoxicity of cationic polymers such as polylysine and poly(amidoamines) increases with increasing mwt (54). Hence, the negligible toxicity observed with HIS6 RPC may well reflect its capacity to be cleaved by reduction into shorter peptides with a lower toxicity profile.

To date many studies have demonstrated that existing delivery vectors can be modified or novel types of vectors developed to overcome specific barriers to transfection. The challenges now lie in integrating many of these different functionalities into a single type of efficient delivery vector. We have shown that HIS RPCs can be developed that incorporate multiple features to overcome several barriers to transfection, including the ability to mediate escape from endocytic vesicles, efficient intracellular release of nucleic acids and a low toxicity profile. We also demonstrated that HIS RPCs can be used for efficient cytoplasmic delivery of a broad range of nucleic acids. This will enable different or a combination of therapeutic strategies to be evaluated with a single type of polycation-based vector.

Currently, we are assessing whether HIS RPC polyplexes can be used directly for *in vivo* gene transfer or whether further modifications are required. In this study, transfections were performed in the absence of serum to avoid masking the ability of histidine residues to enhance gene transfer that may have arisen from binding of serum proteins to polyplexes restricting cellular uptake (9). Preliminary experiments indicate that the transfection properties of HIS RPC polyplexes can be affected by the presence of serum proteins with a 50% decrease in GFP-positive cells observed in 10% FCS (Supplementary Figure 5). We also observed a similar decrease in the proportion of GFP-positive cells in serum using JetPEI as the transfection reagent, which has been used successfully *in vivo* (32). However, in contrast to HIS6 RPC, with JetPEI mediated gene transfer there was no significant reduction in the mean fluorescence levels per cell. Therefore, based on these observations it is envisaged that hydrophilic polymers and targeting ligands will need to be attached to HIS RPC-based polyplexes to further improve their stability and biocompatibility for *in vivo* applications. Towards this goal, we have previously shown that lysine-based RPCs can be readily modified by attachment of the hydrophilic polymer poly-[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) and basic fibroblast growth factor (bFGF) (20).

In conclusion, we have developed histidine-rich reducible polycations for safe and efficient intracellular delivery of nucleic acids, such as siRNA, with the potential to be readily modified for use in therapeutic applications. These properties should help to facilitate the design and construction of efficient and versatile non-viral vectors for gene transfer applications.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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