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Systemic in vivo delivery of siRNA to tumours using combination polyethyleneimine and transferrin-polyethyleneimine conjugates

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Complete List of Authors:	Grabowska, Anna; University of Nottingham, Pre-Clinical Oncology, School of Medical and Surgical Sciences, Queens Medical Centre Kircheis, Ralf; Virologik GmbH, Sasso, Luana; University of Nottingham, School of Pharmacy Desai, Arpan; University of Nottingham, School of Pharmacy Kumari, Rajendra; Precos Ltd, McKenzie, Andrew; Precos Ltd, Clarke, Philip; The University of Nottingham, Alexander, Cameron; University of Nottingham, The School of Pharmacy Watson, Sue; University of Nottingham, Pre-Clinical Oncology, School of Medical and Surgical Sciences, Queens Medical Centre Mayne, Cerys; The University of Nottingham, Hughes, Jaime; University of Nottingham, Faculty of Medicine & Health Sciences

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Anna M Grabowska[1*], Ralf Kircheis[2], Rajendra Kumari[3], Philip Clarke[1], Andrew McKenzie[3], Jaime Hughes[1], Cerys Mayne[1], Arpan Desai[4], Luana Sasso[4], Susan A Watson[1†], Cameron Alexander[4*]

Materials for delivery of oligonucleotides need to be simple to produce yet effective *in vivo* to be considered for clinical application. Formulations of biomaterials based on combinations of existing demonstrated polymeric gene carriers with targeted derivatives are potential candidates for rapid translation but have not been fully explored for siRNA applications. Here we investigated formulations based on derivatised PEI for delivery of siRNA to gastrointestinal cancer cells. siRNA was complexed with linear PEI alone or with a mixture of linear PEI and transferrin-conjugated branched PEI (TfPEI), and knockdown of reporter genes was investigated. Overall, in vitro use of complexes containing TfPEI resulted in up to 93% knockdown at 72hrs post-transfection. Sustained knockdown was also achieved in a bioluminescent xenograft model. When complexes were delivered intra-tumorally, a 43% reduction in luminescence was achieved in the treated group compared with the control group 48 h after treatment. For systemic administration, only the intra-peritoneal route, and not the intra-venous route was effective, with 49% knockdown achieved at 72hrs and sustained up to 144hrs (44%) after a single administration of TfPEI-complexed siRNA. No toxicity or induction of the interferon response was observed. These findings demonstrate that simple formulations of transferrin-conjugated PEI with a 'parent' polymer such as linear PEI have potential as a method for therapeutic delivery of siRNA when administered either intra-tumorally or systemically.

Introduction

Small interfering RNAs (siRNAs) are a powerful tool for down-regulation of gene expression in mammalian cells[1]. Oligonucleotides of this type can be designed for selective targeting of specific mRNAs, leading to their cleavage and degradation, by the RNA-induced silencing complex (RISC). Cellular recycling of the RISC enables targeting of further mRNAs, making the overall gene silencing process highly efficient. These factors, together with the high sequence specificity of siRNAs, means they have potential as therapeutics,[2] and in particular, as anti-cancer agents for downregulation of oncogenes.[3]

Whilst *in vitro* delivery of siRNA can be readily achieved, *in vivo* delivery has proved more difficult.[4] For treatment of cancer, systemic delivery of siRNA is required to target distant metastases as well as primary tumour but potential losses via the kidney and liver or through degradation must be avoided. Delivery of uncomplexed siRNA has been achieved through the use of hydrodynamic injection or attachment of cell-targeting ligands[5, 6] but neither provides a complete solution[7]. Alternatively, siRNAs can be complexed with macromolecules to protect them from degradative enzymes and increase cellular uptake,[8] but liposomes, for example, activate the innate immune response and resulting in toxicity when used systemically[9].

The polycation polyethyleneimine (PEI) has been successfully used for DNA delivery[10, 11] and protects siRNA from serum-associated enzymes. Multiple protonatable amine groups make PEI efficient at condensing DNA by electrostatic interactions and separation of nitrogen atoms by a 2 carbon spacer along the polymer backbone modulates the overall basicity. The resulting strong pH buffering capacity of linear and branched PEI has been suggested to enhance endosomal escape leading to efficient release of DNA complexes into the cytoplasm[12]. Linear and branched PEI can be synthesised and different molar mass forms are readily available; linear and low molecular weight PEI have both been associated with lower toxicity[13, 14] and higher efficiency of DNA delivery[15, 16]. In addition, PEI can be readily functionalised,[17, 18] as the presence of primary amines enables standard bioconjugation chemistries to be used to introduce targeting ligands, self-assembly inducers and steric shielding groups. These functionalities can enhance circulation

times and specific tissue accumulation *in vivo,* although there is an inevitable trade-off between introduction of targeting functionality at PEI-amine groups and loss of binding affinity and buffering capacity. In addition, for a practical pharmaceutical application, there is a need to keep the use of expensive or delicate functional affinity ligands to the minimum level associated with effective targeting. Therefore, despite the many potential advantages of PEI as an oligonucleotide delivery system, the inherent lack of targeting of the unsubstituted parent polycation and the balances of introduced functionality, oligonucleotide binding ability, buffering capacity and toxicity have resulted in rather limited *in vivo* studies of this polymer as a carrier of siRNA.[19-21]

Accordingly, for this study we aimed to evaluate if a 'minimal-functionalized' PEI formulation could be made to enable cancer cell targeting without systemic toxicity, while retaining strong RNA binding during transit *in vivo* to maintain therapeutic efficacy. Here we describe the use of a simple linear PEI co-formulated with ligand-conjugated branched PEI for delivery of siRNA to gastrointestinal (GI) cancer cells *in vitro* and *in vivo.* For these studies we utilised an siRNA sequence for knockdown of luciferase in bioluminescent xenograft models, and transferrin as the ligand for cancer cell targeting. The receptor for transferrin is expressed on proliferating cells[22] and has been used for delivery of a range of therapeutic agents[23] to cancer cells, including siRNA[24]. Incorporation of transferrin-conjugated PEI into complexes has also been shown to improve DNA delivery to tumour cells[25, 26]; the mechanism may involve shielding the positive charge on PEI-DNA complexes *in vivo*, reducing accumulation in 'first pass' organs and enabling them to reach target tissues including tumours[27]. However, while there have been several *in vitro* and *in vivo* studies of Tf-conjugated PEI alone with siRNA,[20, 21] the combination of the linear 22k PEI with branched PEI conjugated to Tf had not been evaluated prior to this study. Therefore, as a first step towards a low cost targeted siRNA formulation, a mixture of free, and transferrin-conjugated PEI, was assessed for systemic delivery of siRNA to cancer cells *in vivo*.

Methods

Cells

Bioluminescent HCT116 (colorectal, ECACC Ref No. 91091005) and MGLVA1 (gastric, ascitic variant of MKN 45G[28]) cells, stably transfected with firefly luciferase (pORF-LucSh-CpG, InvivoGen, Toulouse, France) were routinely cultured in RPMI1640 culture medium (Gibco, Paisley, UK) containing 10% (v/v) heat inactivated foetal bovine serum (FBS, Sigma, Poole, UK) at 37°C in 5% CO_2 and humidified conditions.

siRNA and polycations

For *in vitro* studies, siRNAs were purchased from Eurogentec, and for *in vivo* studies siRNAs synthesised by Dharmacon (Lafayette, CO, USA). A TAMRA-labelled control siRNA[29] was used to investigate uptake. To investigate siRNA activity, a luciferase siRNA (target sequence: UCAGAGUGGUGCUGAUGUA) that targets the pORF-LucSh-CpG-encoded low CpG luciferase was used; a non-targeting siRNA (SR-CL000-005, sequence not given, Eurogentec, Southampton, UK) or one targeting wild-type luciferase, not effective against the low CpG luciferase (sequence: CGAGUCGUCUUAAUGUAUA) were used. Branched PEI (25kD, Aldrich, Milwaukee, WI, USA) was conjugated to transferrin and purified as described previously[27]. Quantities of protein to PEI were chosen in accord with these protocols to generate Tf-PEI25 at a 1:1 molar ratio[25]. Linear PEI (22kD), obtained from MBI-Fermentas (St. Leon-Rot, Germany); this form of PEI was chosen based on the better *in vivo* properties observed when PEI mixed with TfPEI was used for DNA delivery previously[27].

Transfection

Transfections were carried out as previously described[27]. $5x10^4$ cells were transferred to each well of a 24-well plate the day before transfection in 1ml of complete medium (RPMI + 10% fetal calf serum). On the day of transfection, medium was replaced with 0.3ml of fresh complete medium 2 h prior to transfection. A range of N/P ratios (the ratio between the number of nitrogen groups, N, in the polymer and the number of phosphate groups, P in the nucleic acid) was used. Details of the amounts of siRNA and polymer and resulting N:P ratio are given in each figure for each of the individual conditions tested. Amounts used for the *in vivo* work are in the Materials and Methods section '*In vivo siRNA delivery*'. The appropriate quantity of siRNA was diluted to 50µl in Opti-MEM1 (Gibco, Paisley, UK). The required amount of linear PEI and transferrin-conjugated PEI (TfPEI), when used, was thawed, vortexed, mixed in the required ratio, diluted to 50µl in Opti-MEM1, added to the nucleic acid and immediately mixed by repeated pipetting. Following incubation at room temperature for 20 mins, the complexes were added to cells and cells were returned to the incubator. Each experiment was repeated on at least two occasions and representative data are shown in the figures. For competition experiments, free transferrin (Sigma) was added to the cells immediately prior to addition of the transfection mix at a range of concentrations between 500 and 10µg/ml.

Flow Cytometry

Cells were removed from the plate with trypsin/EDTA, washed in fresh medium, resuspended in 4% formalin and analysed on a Beckman-Coulter XL-MCL flow cytometer; data were analysed using WinMdi (http://facs.scripps.edu/).

Luciferase Reporter Assays

Cells were analysed using the Luciferase assay system (Promega, UK). After washing in PBS, cells were lysed in Passive Lysis Buffer (Promega), 5µl was added to 25µl of Luciferase Assay Reagent and luminescence measured using a MicroLumi XS luminometer (Hartalabs, USA).

Dynamic Light Scattering (DLS)

Hydrodynamic radii of polymer-siRNA complexes were determined via scattered light recorded at a 90° angle to the incident radiation using a Viscotec Model 802 Dynamic Light Scattering (DLS) instrument equipped with an internal laser

(825–832 nm) with a maximum radiation power of 60mW. Samples were diluted with filtered, deionized water and at least five measurements of each sample taken. Mean and standard deviation were calculated. Data processing was performed using OmniSize2.

Zeta potential measurement

Zeta (ζ-) potentials measurements were derived from electrophoretic mobilities determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Complexes were prepared in 10mM HEPES buffer adjusted to pH 7,4 and diluted to a concentration of 4μg/ml. Three measurements of each sample with at least 10 repeat data capture points each were carried out. Mean and standard deviations of ζ-potentials were calculated.

In vivo siRNA delivery

The study was carried out according to the UK Coordinating Committee of Cancer (UKCCCR) guidelines[30] under a UK Home Office project licence. MGLVA1-DLuX tumours were established subcutaneous grafting into the flank of female MF1-nude mice under anaesthesia (Hypnorm, Roche/Hypnovel Jannsen). Tumour size was monitored by calliper measurements and imaged under anaesthesia using the IVIS®100 imaging system (Caliper Life Sciences) 15 minutes after administration of luciferase substrate, D-Luciferin (intraperitoneal, 60 mg/kg in sterile PBS, Xenogen, New Jersey, USA). Areas of luminescence were identified as Regions of Interest (ROIs) and quantified as photons emitted using Living Image/Igor Pro Software (Caliper Life Sciences). Mice with tumours of luminescence of greater than 10^6 were used; bioluminescence is linear over the range used in this study (Figure S1).

Mice were divided into groups, to be injected with luciferase or control siRNA. For the intra-tumoral route of injection, 20μg of the appropriate siRNA was complexed with a 1:4 mixture of TfPEI and PEI as described above but glucose was added to a final concentration of 3%, in a total volume of 50μl and the complexed siRNA was injected into two sites within each tumour. For systemic delivery, single intra-peritoneal injections of 50μg of siRNA in a final volume of 200μl were used. The mice were imaged again for bioluminescence at timepoints between 24 and 144hrs after administration as indicated; bioluminescence post-treatment was expressed as a percentage of bioluminescence prior to siRNA injection.

Real-time PCR

RNA extraction, cDNA synthesis and real-time RT-PCR performed as described previously,[31] using Sybr Green for detection (Eurogentec). Sequences of the primers are shown in Table S1. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

Tumour tissues were formalin-fixed and paraffin-embedded, sectioned, mounted onto Polysine coated microscope slides (Thermoscientific, Loughborough UK), dewaxed in xylene, rinsed in sequential alcohols and rehydrated prior to staining with mouse monoclonal anti human transferrin receptor (AbCam, UK) using the animal research kit (Dakocytomation, UK) according to manufacturer's protocol, or rat anti-mouse CD34 antibody (AbCam, UK) as follows: endogenous peroxidise was blocked with 3% hydrogen peroxide, antigen retrieval carried out in 10mM citric acid at 98ºC for 20 mins, blocked using the Avidin:Biotin blocking kit (Vector Labs, UK) using concentration matched rat IgG2aκ (BD pharmingen, UK) as a control. Primary labelling was visualised with biotinylated goat anti-rat secondary (BD pharmingen, UK), followed by streptavidin binding complex (Vector Labs Ltd, UK) and Diaminobenzidine tetrahydrochloride chromogen (Dakocytomation, UK). Following counterstaining with Mayer's Haemalum, sections were coverslipped using DPX. Microvessel density was analysed by assessment of vessel hotspots across the surface area of the section at 10x magnification.

Results

Characterisation of complexes

The biological activity of polymer/oligonucleotide polyelectrolyte complexes is strongly dependent on particle size charge and method of preparation.[32] Previous studies on Tf-PEI complexes with DNA prepared at a Tf:PEI25 molar ratio of 1:1 had shown greater transfection efficiency for particles of diameters of < 200 nm and positive zeta potentials,[27] thus we utilised Tf:PEI at a 1:1 molar ratio for the targeted siRNA delivery systems. Characterisation of siRNA complexes prepared using mixtures of TfPEI:PEI(1:4) and TfPEI:PEI(1:15) at N/P ratios of 8 and 12, was thus carried out using dynamic light scattering in comparison with PEI complexes (Table 1a). The particle size distributions of PEI complexes were well-defined with hydrodynamic diameters of 20 or 45nm diameter dependent on N:P ratio, based on the number distributions of particles sizes calculated from scattered light intensities. Inspection of correlation functions and calculated particle sizes from intensity distribution data revealed the presence of weakly-scattering species in the PEI complexes with hydrodynamic diameters of <10 nm. Features in this size range were observed in the absence of siRNA, suggesting that these signals most likely corresponded to free PEI. Use of TfPEI:PEI(1:4) also resulted in particles in the 20-40nm diameter range, with essentially all of the particulate content being of these sizes by number distribution, although the presence of larger aggregates was apparent in the scattered light intensity distributions. These variations in apparent size were expected for DLS intensity distributions of heterogenous particle populations, owing to the sixth power dependency of scattered light with particle diameter. There was no detectable free polymer-protein conjugate (i.e. TfPEI in the presence of the polyplexes) and nor were signals corresponding to free PEI observed. For non-PEI complexes, increased N/P ratios decreased the observed hydrodynamic diameters, implying better condensation of the oligonucleotide with increased polycation. By contrast, for TfPEI-siRNA complexes at TfPEI:PEI of 1:4, increased N/P ratios resulted in an apparent increase in the sizes of the most abundant particles, which may have reflected the increased content of Tf (which has an isolectric point of 5.2-5.5 dependent on pH) and the resulting

potential for interparticle association between polycation and negative domains on the Tf surface. However, the differences in the sizes of PEI and TfPEI:PEI(1:4) complexes over this N/P range (i.e. ~10 nm for both N/P 8 and 12) were considered unlikely to result in any major changes in biological behaviour, for example in cell uptake pathways, based on these size variations alone. The TFPEI:PEI-siRNA complexes at N:P 8 and N:P 12 were of similar size distributions to the PEI:siRNA complexes, indicating the likely predominance of the unconjugated PEI in the physicochemical behaviour of these species.

Table 1 – Dynamic Light Scattering by polymer/siRNA complexes

Polycation	N/P	D_H (number distribution± SD)/nm	D_H (intensity distribution± SD)/nm
PEI	8	45 ± 2 (100)	48 ± 2 (6), 204 ± 23 (89), >1µm (5)
PEI	12	24 ± 1 (100)	22 ± 1 (1), 270 ± 27 (87), >1µm (12)
TfPEI:PEI (1:4)	8	20 ± 1 (100)	20 ± 1 (2), 300 ± 31 (43), 462 ± 88 (27), >1µm (28)
TfPEI:PEI (1:4)	12	38 ± 2 (100)	44 ± 3 (6), 276 ± 40 (88), >1µm (6)
TfPEI:PEI (1:15)	8	23 ± 9 (100)	30 ± 14 (29), 136 ± 60 (68), > 1µm (2)
TfPEI:PEI (1:15)	12	21 ± 12	36 ± 9 (63), 274 ± 92 (35), aggregates (2)

(Figure in parentheses is the percentage of the sample population)

Complexes obtained with an N/P ratio of 8 were also characterised in terms of their surface charge (Table 2). PEI complexes exhibited significantly higher mean zeta potentials (24 mV) compared to TfPEI:PEI(1:4) complexes at the same N/P ratio (zeta potential of 17 mV, $p<0.01$). These data were in accord with those reported previously for Tf-PEI / DNA complexes,[33] and suggested a partial shielding of the high surface charge of PEI in the polyelectrolyte complexes, even at high N:P ratios, by the attached transferrin (molar mass 80 kDa, pI = 5.5-5.8, dependent on Fe content).

Table 2 – Zeta potentials of polymer/siRNA complexes

Polycation	N/P	Zeta potential (mV)[a]	Conductivity (mS/cm)
PEI	8	+24 ± 8	0.26
TfPEI:PEI(1:4)	8	+17 ± 11	0.33
TfPEI:PEI(1:15)	8	+22 ± 6	0.18

Recorded in 10mM HEPES buffer adjusted to pH 7.4.

Uptake of TfPEI:PEI-conjugated siRNA

Initial *in vitro* experiments investigated the effects of different amounts of transferrin within the PEI-siRNA complexes on the uptake of fluorescently-labelled siRNA by HCT116 cells, chosen as a model cell line well-characterised for expression of the transferrin receptor, TfR2[34, 35]. Cells were transfected with complexes containing 1:15 mixtures of TfPEI:PEI, at a range of N/P ratios, and analysed by flow cytometry at 24 hrs. Transfection efficiencies, assessed as mean fluorescence, for complexes containing transferrin were similar to those obtained using PEI alone. There was an increase in fluorescence at N/P ratios of 8 and 16 compared with cells treated with no siRNA which was significant when 2µg siRNA was used (Figure 1a, $p<0.05$ and $p<0.01$ for PEI alone and TfPEI:PEI ratio of 1:15) and a decrease when the N/P ratio was further increased. Cells transfected with fluorescent siRNA were also examined microscopically 1, 4 and 24 h after transfection. In cells transfected using PEI only, at the earlier time-point, a positive signal was observed only in a proportion of the cells, and in positive cells, the fluorescence was localised to vesicles which were widely distributed in the cytoplasm. When TfPEI was included in the complex, almost all cells were fluorescent at 1 h after transfection. By 4 h, in cells treated with PEI alone, fluorescence was clustered into a single area of the cell outside the nucleus, similar to the pattern in cells treated with complexes containing TfPEI. By 24 h, the fluorescence signals were reduced with all treatments, with the strongest reduction found when PEI alone was used. Representative images are shown in Figure 1b. These data together show that despite the reduced protonatable nitrogen content of PEI following conjugation of transferrin the overall uptake of PEI-siRNA complexes into this cell line was not diminished. In turn, this implied that the loss of non-specific charge-mediated uptake through Tf conjugation was at least partially offset by receptor-mediated endocytosis, even when the TfPEI:PEI ratio was as low as 1:15.

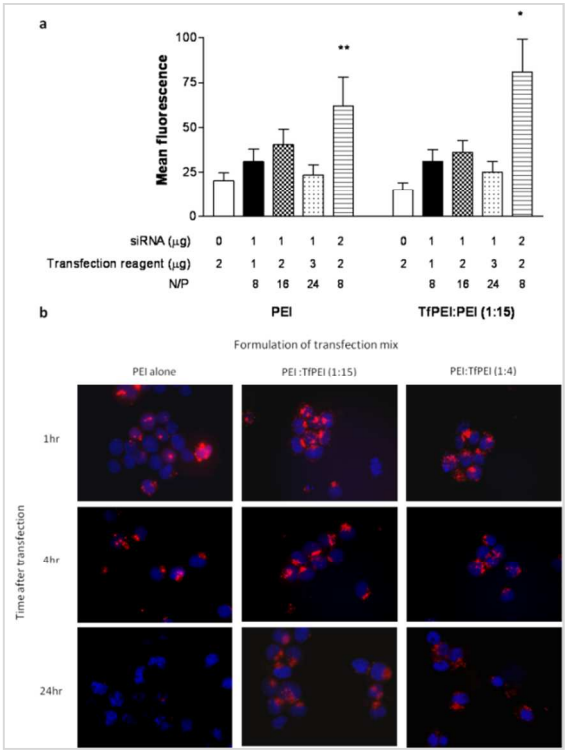

Figure 1: Uptake of PEI-conjugated siRNA by gastrointestinal cancer cells. In (a) mean fluorescence is shown 24 hrs after transfection of HCT116 cells with 1 or 2 µg of TAMRA-labelled siRNA using PEI alone or transferrin-conjugated PEI (TfPEI) mixed with PEI at a ratio of 1: 15 TfPEI:PEI. * and ** indicate a significant increase ($p<0.05$ and $p<0.001$ respectively) in fluorescence compared with cells treated with transfection reagent alone (One way ANOVA with Bonferroni multiple comparison post-test). In (b) fluorescence microscopy images are shown of cells at 1, 4 or 24 h after transfection with 2µg TAMRA-labelled siRNA complexed with PEI alone, or TfPEI:PEI (1:4 or 1:15 mixture, at N/P of 8).

Luciferase knockdown by TfPEI:PEI-conjugated siRNA in gastrointestinal cancer cells

We next investigated siRNA-mediated knockdown *in vitro* using TfPEI:PEI complexed siRNA. Luciferase siRNA, in complexes containing TfPEI (at ratios of 1:15 or 1:4) at a range of N/P ratios, was transfected into HCT116 and MGLVA1 cells (chosen as an established gastric cancer cell line *in vitro* and *in vivo*)[36] stably expressing luciferase and luciferase activity measured at d3. Initial experiments demonstrated that TfPEI:PEI complexes with siRNA were well-tolerated by both cell lines, with no significant loss in metabolic activity as measured by MTT assays in HCT-116 cells, and less than 20 % loss in activity in MGLVA-1 cells after 24 hr (Figure S1, Supporting Information). Knockdown of approximately 60% was achieved in HCT116 cells at either TfPEI:PEI ratio, and knockdown was generally maintained at d3 (Figure 2c). In MGLVA1 cells, there was delayed knockdown, especially at TfPEI:PEI(1:15) but knockdown of nearly 90% could be achieved by d3. Overall, maximal, sustained knockdown was achieved at TfPEI:PEI(1:4) using N/P 8-12.

Under these conditions, we observed a small but significant inhibition of knockdown when free transferrin was added to the cells immediately before transfection at concentrations of 500 or 100µg/ml suggesting at least part of the uptake is mediated via transferrin receptors (Figure 2e).

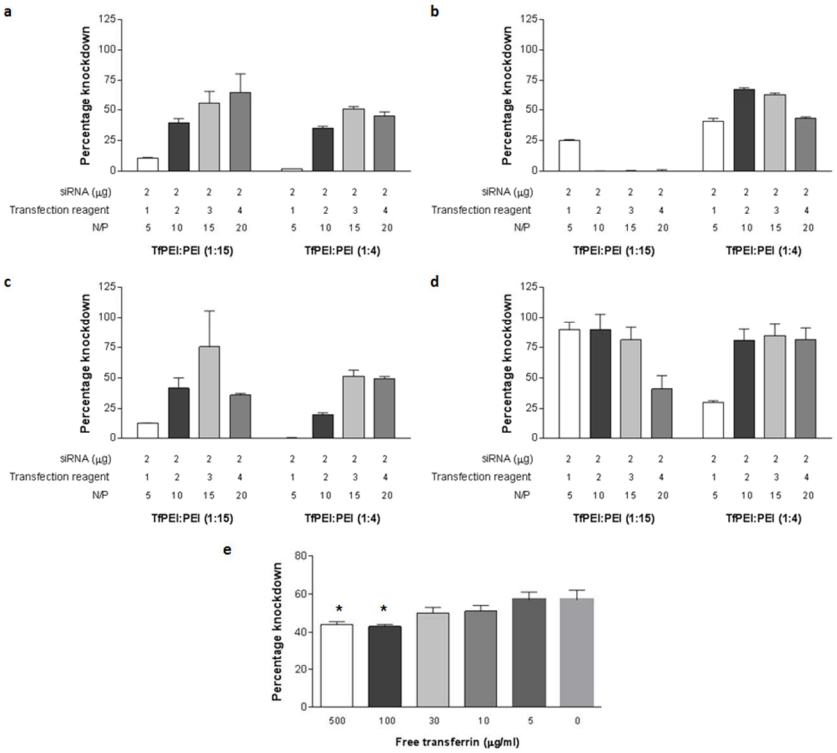

Figure 2 Knockdown of luciferase activity using TfPEI:PEI siRNA complexes. Percentage knockdown of luciferase activity achieved 24 h (a,b) and 72 h (c,d) after transfection of HCT116 (a,c) or MGLVA1 cells (b,d) using a luciferase-specific siRNA complexed with transferrin-conjugated PEI (TfPEI) mixed with PEI at a ratio of 1:4 or 1: 15 TfPEI:PEI relative to a control siRNA. Inhibition of knockdown by free transferrin in MGLVA1 cells transfected with 2ug 1:4 TfPEI:PEI and 2µg of siRNA and transfection reagent (e). * indicates concentrations of transferrin that gave significant knockdown relative to 'no transferrin' control ($p<0.05$)

In vivo delivery of siRNA using TfPEI-PEI complexes

Intratumoral delivery: Having established that incorporation of transferrin into the complexes and the concomitant change in physical properties did not interfere with siRNA uptake and knockdown *in vitro,* the activity of TfPEI:PEI complexed luciferase siRNA was investigated in an *in vivo* bioluminescent MGLVA1 subcutaneous tumour. These tumours showed good vascularisation and strong staining for transferrin receptor (Figure S2). A dose of 20µg of luciferase or control siRNA complexed TfPEI:PEI(1:4) at an N/P ratio of 8, which had given sustained knockdown in MGLVA1 cells *in vitro*, was initially administered directly into each tumour and bioluminescence monitored over 48 h. Representative images of tumours from both groups are shown in Figure 3a. Bioluminescence at each time-point was expressed as a percentage of the initial bioluminescence (24hrs prior to treatment, Figure 3b). Over the whole experiment, taking all timepoints into consideration, there was a significant difference between the bioluminescence in the two groups ($p=0.005$, Two-way ANOVA). In the control siRNA group, luminescence increased over this period (+ 29%), reflecting continued tumour growth, while in the luciferase siRNA group, luminescence levelled out at 24 h post-injection, then decreased by 48 h (32%

decrease), equating to 47.3% lower luminescence in the treated compared with the control group at 48 h ($p<0.05$, One-way ANOVA with Bonferroni correction).

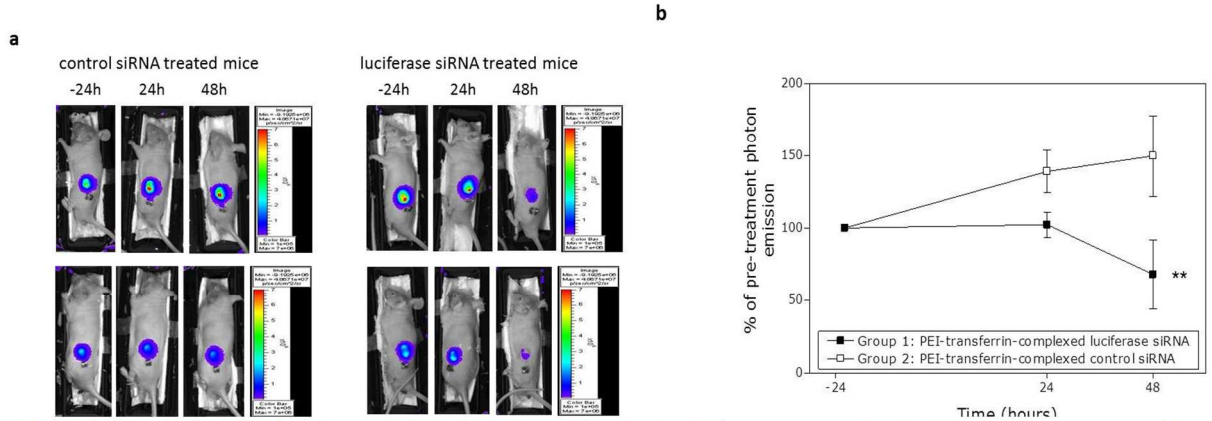

Figure 3 siRNA knock-down *in vivo* using the intra-tumoral route. MGLVA1 cells expressing luciferase were used to establish sub-cutaneous xenografts in nude mice and imaged 24 h before, and 24 and 48 h after intra-tumoral injection of a luciferase or control siRNA complexed with TfPEI:PEI at a ratio of 1:4 and an N/P ratio of 8. (a) Representative images of 2 mice treated with luciferase or control siRNA taken 24 h before, 24 h after or 48 h after treatment. (b) Percentage change in bioluminescence at 24 and 48 h post-injection. A significant reduction in bioluminescence was observed in the luciferase siRNA-treated group (** indicates p=0.005, 2-way ANOVA, n=6) relative to the control siRNA-treated group (n=7) over the whole course of the experiment, and a significant reduction in the luciferase siRNA compared with the control siRNA-treated group at 48hrs ($p<0.05$, one-way ANOVA with Bonferroni correction) but not at 24hrs.

Systemic delivery: In a pilot study to investigate the most appropriate route of delivery for systemic delivery, 4 mice were injected intra-tumorally, intra-venously, or intra-peritoneally with complexes containing the luciferase siRNA or intra-venously with the control siRNA, and luminescence examined at 42 and 68 h after administration of siRNA. Bioluminescence in each group compared to the control group is illustrated in Fig. 4a. There was no effect at 42 h post-injection but at 68 h, there was a reduction in bioluminescence in the groups in which siRNA was administered intra-tumorally or intra-peritoneally, but not in the group in which the siRNA was administered intra-venously.

Next, a larger study was set up in which complexed luciferase or control siRNA was administered intra-peritoneally and bioluminescence monitored at 24, 72 and 144hrs after administration. As in the intra-tumoral study, whilst luminescence in both groups increased due to tumour growth, there was significant lower bioluminescence in the luciferase siRNA-treated group compared with the control group, taking into account all time-points ($p=0.008$, Two-way ANOVA). At 24hrs, the reduction in bioluminescence (30%) was not significant, but by 72hrs the reduction was significant (49%, $p=0.045$, one-way ANOVA). Knockdown was sustained through to 144hrs after siRNA administration (44% decrease, not sig.) and the lower bioluminescence in the luciferase siRNA group at end-point compared with the control group was also reflected in a 39% reduction in luciferase mRNA level (data not shown). General animal condition was monitored daily and there was no apparent toxicity associated with delivery of siRNA to the animals with body weights maintained in both groups. There was also no significant difference in the growth of the tumours in the two groups (Fig. S3a) and no significant induction of the interferon response at end-point (Fig. S3b,c).

Discussion

The data together show that a relatively simple co-formulation of linear PEI with transferrin-conjugated PEI can be used for delivery of siRNA to colonic (HCT116) and gastric (MGLVA1) gastrointestinal cancer cells leading to specific knockdown of the target gene. The siRNA formulation which gave sustained knockdown *in vitro* was also effective *in vivo* at knocking down luciferase activity in MGLVA1 tumours growing subcutaneously in MF1 nude mice when delivered either intra-tumorally or systemically via the intra-peritoneal route.

The efficacy of the formulation derives from incorporation of transferrin into PEI/siRNA complexes. Although some inhibition of knockdown by free transferrin was observed, suggesting that at least some of the uptake is also via specific receptor binding, most probably the uptake is also due to a reduction in polyelectrolyte complex surface charge by the large (80kDa) and negatively charged conjugated transferrin, as shown by the reduced zeta potentials of the Tf-PEI siRNA complexes compared to those with PEI-siRNA only. Shielding of the positive charge of PEI in polyelectrolyte complexes has been hypothesised for PEGylated polymer/DNA complexes *in vivo,* through a reduction in plasma protein and red blood cell binding, leading to prolonged blood circulation and prevention of erythrocyte aggregation.[37] However, there are significant differences in physical properties, particularly persistence length and compaction, of shorter oligonucleotides such as siRNA compared to DNA plasmids.[24] These in turn mean that it is not always possible to use polymers optimised for plasmid DNA therapeutics directly for oligonucleotide delivery. However, by simple mixing of linear PEI and branched PEI-Tf conjugates we were able to generate complexes of appropriate

size and charge for siRNA delivery, and demonstrated particle uptake *in vitro* in cancer cell lines. These data indicated that incorporation of transferrin and the resultant reduction in positive charge was not detrimental to cellular uptake, and were able to identify TfPEI:PEI and N/P ratios that were effective in promoting luciferase knockdown. The particles incorporating TfPEI were similar in size to those using PEI alone and while zeta potential measurements confirmed that incorporation of transferrin into the complexes reduced the positive charge of the particles for complexes prepared at N/P = 8, addition of transferrin did not generate polyplexes with an overall negative charge.

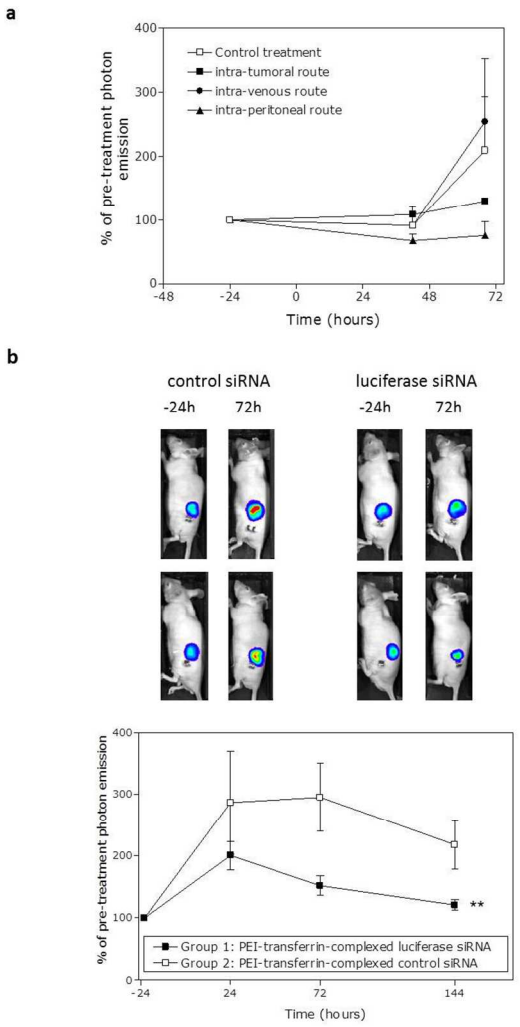

Figure 4: Systemic delivery of TPEI-complexed siRNA *in vivo*. Bioluminescent sub-cutaneous MGLVA1 xenografts were established in nude mice. (a) TfPEI-complexed luciferase siRNA (TfPEI:PEI ratio of 1:4 and an N/P ratio of 8) was administered via intra-tumoral, intra-venous or intra-peritoneal routes (n=4 mice per group) as a pilot study; a control siRNA was administered intra-venously as a control treatment. Bioluminescence at 42 and 68 h post-administration is shown relative to bioluminescence at 24hrs pre-administration. Knockdown at 68 h was greatest when the intra-tumoral or intra-peritoneal routes of administration were used. (b) TfPEI-complexed luciferase or control siRNA (TfPEI:PEI ratio of 1:4 and an N/P ratio of 8) was administered via the intra-peritoneal routes (n=7 and 8 respectively) and imaged at 24hrs before and 24, 72 and 144 hrs after injection. Representative images of 2 mice treated with control or luciferase siRNA taken 24 h before and 72 h after treatment are shown and the data for all mice are illustrated graphically. A significant reduction in bioluminescence was observed in the luciferase siRNA-treated group (** indicates p=0.008, 2-way ANOVA) relative to the control siRNA-treated group.

In this study we used 22kD linear PEI and 25kDa branched PEI as similar molecular weight PEIs (<25kD) have previously been shown to be more effective than higher molecular weight forms (~800kD) for delivery of DNA[15, 38] and were associated with lower toxicity.[13, 39] Low molecular weight linear PEI has been successfully used for intraperitoneal delivery of siRNA in mice, and reduced the size of subcutaneously-grown tumours as well as achieving a ~ 50% knockdown in target gene expression. However, in these studies, gene silencing effects were apparent after 11 days and multiple injections were required.[40] The majority of studies investigating the use of PEI for siRNA delivery have used the branched form, either alone[38] or conjugated to a peptide e.g. one targeted to integrin AvB3.[41] In a study comparing branched and linear PEI for siRNA delivery, whilst binding of linear PEI to siRNA was similar to binding of branched PEI and uptake by cells was also observed, the authors did not achieve knockdown of the target gene.[13] This contrasts with our findings in which we achieved 80 - 90% knockdown in both the colonic and gastric cell-lines by day 1 and this was maintained up to day 3 of the assays. Differences in the efficacy of knockdown is likely due to differences in the characteristics of the particles used, including size, charge and shielding which are dependent on particle formulation, or characteristics of the target cells. The formation of smaller complexes may lead to more efficient uptake due to increased mobility of the complexes and increased interaction with negatively charged cell-surface proteoglycans, but at very high ratios, release of the nucleic acid into the cytoplasm may be impaired (i) by a very tight/strong (in extreme cases even non-reversible) condensation of nucleic acid and/or (ii) due to reduced ability of small-sized particles to act as a proton sponge.[42]

The effect of using complexes containing TfPEI has previously been shown to vary with different cell-types.[15, 25] In this study, in MGLVA1s there was a greater delay in knockdown which increased markedly between day 1 and day 3, using a number of the formulations, but in particular the TfPEI:PEI ratio of 1:15. This may mean that the route of uptake is different in the 2 cell-lines used or that release of the siRNA from the endosomes may be slower in the MGLVA1s.

Since we had identified conditions that allowed efficient and prolonged siRNA delivery *in vitro* using complexes incorporating TfPEI, we investigated their function in an *in vivo* model which enabled real-time monitoring of the effect of siRNA administration. Both intra-tumoral delivery and systemic delivery via the peritoneum were effective at reducing luciferase activity in the xenografts whilst, in a pilot study, intra-venous delivery was not effective. Biodistribution studies in which the intravenous delivery route have been used previously showed that siRNA complexed with RGD-PEG-PEI complexes accumulates in the liver, and to a lesser extent in the lung, spleen, heart and kidney,[43] while other studies with

modified PEI complexes have shown accumulation in the lung predominantly,[44] thus reducing the effectiveness of delivery. The intra-peritoneal route has previously been successfully used for delivery of nucleic acids.[40, 45] In a study using PEI-complexed siRNA administered intra-peritoneally and a subcutaneous ovarian cancer xenograft model, a HER-2 specific siRNA significantly reduced tumour growth over 14 days compared with a control siRNA.[40] Further studies would be needed to verify the low efficiency of delivery by the intra-venous route observed in our small pilot study, to determine the underlying mechanisms and to investigate whether intravenous delivery of TfPEI-siRNA complexes can be achieved; however, the current study provides proof-of-principle that systemic delivery using TfPEI-siRNA complexes is feasible.

The downregulation of a luciferase gene as a result of siRNA administration in this study appears to be specific. The effects were observed only with an siRNA targeting the luciferase gene expressed by the cells and not when an siRNA, complexed in an identical manner and targeting a closely related luciferase gene, was used. The reduction in the bioluminescent signal observed in the luciferase siRNA group compared with the control group at end-point following systemic administration (44%) was paralleled by a reduction in the luciferase mRNA of the same order of magnitude (39%). Interestingly, as in the *in vitro* studies, little knockdown was achieved at 24hrs but there was an increase in knockdown at 72hrs, which, in the systemic study, was maintained through to 144hrs after administration of the siRNA. This may be a result of delayed release of the siRNA from the particles and subsequent gene knockdown following uptake of the siRNA/polycation particles.

In addition, there were no apparent adverse effects in the animals as a result of administration of the siRNA complexes; in the study where siRNA was administered systemically, no toxicity was observed in the animals over 6 days following injection and there was no effect on tumour growth, as anticipated since the siRNA used targets the luciferase gene and thus should not affect tumour cell proliferation. The absence of free PEI in the TfPEI-containing complexes in contrast with those containing PEI only, as observed from the light scattering studies, may have contributed to this low toxicity in addition to the reduced overall charge of the Tf-PEI complexes compared to PEI/siRNA complexes alone. Lastly, although we did not use siRNAs modified to prevent induction of the innate immune response,[46, 47] we saw only a small non-significant increase in expression of OAS1 and downregulation of the STAT1 gene. These genes are both involved in activation of the innate immune response,[48] suggesting that the siRNAs used do not contain "trigger" sequences[49] or that the formulation used protected them from uptake by immune cells. However, if delivery were shown to be mediated through the transferrin receptor rather than through non-specific uptake as a result of transferrin-mediated shielding, further toxicity studies would be required to demonstrate that the absence of toxicity in this model was not a result of poor binding of transferrin to mouse transferrin receptor.

This study provides proof-of-principle that linear PEI (22kDa) co-formulated with transferrin-conjugated branched PEI (25kDa) can be used systemically for knockdown of tumour-expressed genes and therefore has potential as a delivery agent for treatment of GI cancer. Future studies will investigate the route of uptake, *in vivo* targeting[50] and the relative efficacy of complexes containing a broader range of polymers. These include other derivatives of PEI, such as temperature and/or acid-responsive PEI conjugates,[51] alternative cell-surface ligands[41] and new polymer backbones and reporter groups[52] which might further improve the efficacy and control of siRNA delivery and release *in vivo*.

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†This paper is dedicated to the memory of Professor Susan Watson.

Notes and references

[1] Cancer Biology, Division of Cancer and Stem Cells, University of Nottingham, NG7 2UH, UK.
[2] Virologik GmbH, Erlangen, Germany.
[3] PRECOS Ltd, Loughborough, UK
[4] School of Pharmacy, University of Nottingham, NG7 2RD, UK

email: anna.grabowska@nottingham.ac.uk:
cameron.alexander@nottingham.ac.uk
Tel: +44 (0)115 8231135
Fax: +44 (0)115 8231137

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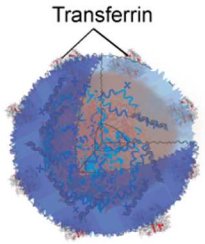
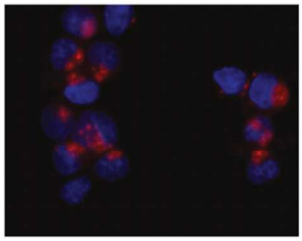
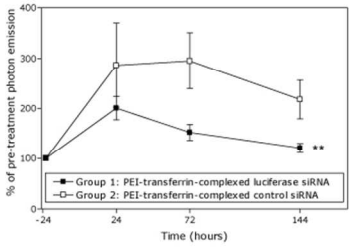


PEI-siRNA complexes



Enhanced tumour cell uptake due to targeted formulation



Reduced tumour bioluminescence due to luciferase knockdown