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Systemic delivery of therapeutic small interference RNA using a pH-triggered amphiphilic poly-L-lysine nanocarrier to suppress prostate cancer growth in mice

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Abstract:

Prostate cancer is associated with high mortality and new therapeutic strategies are necessary for improved patient outcome. The utilisation of potent, sequence-specific small interfering RNA (siRNA) to facilitate down-regulation of complementary mRNA sequences *in vitro* and *in vivo* has stimulated the development of siRNA-based cancer therapies. However, the lack of an effective siRNA delivery system significantly retards clinical application. Amphiphilic polycations with 'stealth' capacity have previously been synthesised by PEGylation of poly-L-lysine-cholic acid (PLL-CA). The benzoic imine linker between PEG and PLL-CA was designed to be stable at physiological pH but cleavable at lower pHs, consistent with the extracellular environment of tumours and the interior of endosomes/lysosomes. The selective hydrolysis of the PEG linker at these targeted sites should provide enhanced cellular uptake and endosomal escape while simultaneously ensuring prolonged blood circulation times. In this study, physicochemical profiling demonstrated nano-complex formation between the PLL derivatives and siRNA (200 to 280 nm in diameter). At physiological pH only a slight cationic surface charge (<20 mV) was detected, due to the masking effect of the PEG. In contrast, significantly higher positive charges (~20 to 30 mV and > 40 mV) were detected upon hydrolysis of the PEG linker at acidic pHs (pH = 6.8 and 5.5, respectively). The PEGylated complexes were stable in serum without significant aggregation or decomplexation of siRNA for up to 48hours. At the cellular level, PEG-PLLs were comparable with the commercial carrier INTERFRin™, in terms of cellular uptake, endosomal escape and *in vitro* reporter gene knockdown. In vivo, utilising a mouse model grafted with prostate carcinoma, significant tumour suppression was achieved using PEGylated complexes without marked toxicity or undesirable immunological response, this was accompanied by a simultaneous reduction in target mRNA levels. In summary, the advantages of these vectors include: the *in vitro* and *in vivo* silencing efficiency, and the low toxicity and immunogenicity.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in the male population of the United States, with over 33,720 fatalities annually (Siegel et al., 2011). Recently, highly sequence-specific gene-silencing via the RNA interference (RNAi) mechanism has become a powerful method for down-regulating the expression of disease-related genes ([Elbashir et al., 2001], [Bumcrot et al., 2006] and [De Fougerolles et al., 2007]). RNAi-induced silencing of cancerous genes related to tumour transformation, development, and metastasis has been considered as a promising strategy for cancer gene therapy ([Dassie et al., 2009], [Kortylewski et al., 2009] and [Santel et al., 2011]). Synthetic siRNA is one of the approaches used to therapeutically affect this post-transcriptional mechanism ([Elbashir et al., 2001], [Bumcrot et al., 2006] and [De Fougerolles et al., 2007]). However, the key bottleneck to the development of such a therapy for cancer remains the lack of safe, efficient and controllable siRNA delivery systems ([Whitehead et al., 2009] and [Guo et al., 2011]). Although viral vectors have shown potential, well documented safety issues limit their clinical application (Thomas et al., 2003). This is evident from the number of viral vector trials that has steadily dropped over the years (Edelstein et al., 2007). In contrast, advances in nanotechnology have facilitated the development of non-viral delivery constructs to achieve clinically appropriate, safe, and efficient delivery of siRNA ([Whitehead et al., 2009], [Oh and Park, 2009], [Howard 2009] and [Guo et al., 2010]).

Among the variety of polymers designed for gene delivery, polycationic monodispersed poly(L-lysine) (PLL) emerged as a promising carrier in early studies ([Walsh et al., 2006], [Yusuke et al., 2007], [Meyer et al., 2009] and [Watanabe et al., 2009]) due to controllable molecular size, shape, and the potential for flexible chemical modification. However, because of relatively low transfection efficiency, PLL-based polyplexes are unlikely to be used in clinical applications (Guo et al., 2011). This is mainly due to poor endosomal escape (pH 5-6.5), and subsequent degradation of the nucleic acid cargo in the late lysosomes (pH ~4.5) by a variety of degradative enzymes (Dominska and Dykxhoorn 2010). It has been recently reported that fusogenic/synthetic peptides and pH-sensitive moieties can be attached to various polymers to assist endosomal escape ([Meyer et al., 2009], [Hatakeyama et al., 2009],

[Carmona et al., 2009] and [Benoit et al., 2010]). These compounds are normally pH-triggered amphiphiles that undergo a surface change in the acidic environment, leading to the release of nanoparticles into the cytoplasm. Previously our group has published the synthesis and characterisation of a new class of 'stealth' amphiphilic PLLs (Gu et al., 2008). These PLLs were chemically modified with cholic acid (CA, a hydrophobic moiety) on one side and subsequently grafted with poly(ethylene glycol) (PEG) on the other side, via a novel acid sensitive linker (benzoic imine) (see Figure 1). This novel structure has the potential to form cationic micelles that comprise of a) the hydrophobic core in which water-insoluble molecules can be encapsulated and b) the hydrophilic surface where nucleic acids can be complexed via the electrostatistic interaction (see discussions in Gu et al., 2008). The chemistry used for this vector facilitated the formation of a benzoic imine linkage which is stable at physiology pH (7.4) but is responsive to small pH fluctuations under physiological conditions. For example, at acidic conditions such as the extracellular environment of tumours and endosolysosomes, the linkage cleaves detaching the PEG; in addition, it has been shown that this PEG-deshielding process is reversible if the pH changes back to 7.4 (Gu et al., 2008). It is postulated therefore that when circulating in blood vessels these amphiphilic derivatives will act as 'stealth' delivery systems where the PEG shields the charge at physiological pH, while PEG deshielding under acidic pH will facilitate cellular internalisation at tumour tissues and membrane disruption in the endosome/lysosome.

In this work two of these amphiphilic PLLs, namely PEG17-PLL-CA14 and PEG17-PLL-CA32, were investigated as vectors for siRNA delivery. The physicochemical properties of the complexes were monitored. Cellular uptake and intracellular trafficking mechanisms, including the role of endolysosomal release, and gene silencing efficiency were assessed. In addition, the *in vivo* anti-tumour efficacy was confirmed by systemic administration of siRNAs targeting the vascular endothelial growth factor (VEGF) gene in a prostate carcinoma mouse model.

Experimental Section

Materials. PC-3 cell line (human prostate cancer cell line) was purchased from the

European Collection of Cell Cultures (ECACC), UK. TRAMP C1 cell line (transgenic adenocarcinoma of the mouse prostate cell line) was kindly donated from Cork Cancer Research Centre (CCRC), Ireland. Luciferase GL3 siRNA (sense sequence 5' – CUU ACG CUG AGU ACU UCG A – 3'), fluorescein-siRNA (sense sequence 5' – UUC UCC GAA CGU GUC ACG U – 3', modified by 3'-fluorescein on sense sequence) and Negative control siRNA (sense sequence 5' – UUC UCC GAA CGU GUC ACG U – 3', no modification) was obtained from QIAGEN, USA. Mouse VEGF siRNA (sense sequence 5'-AGG UUU CAA UAU ACA UUU ATT-3') was purchased from Sigma. BCA® Protein Assay Reagent was purchased from Pierce, Thermo Scientific, USA. Luciferin and Reporter Lysis Buffer was obtained from Promega, WI, USA. LysoTracker Red was purchased from Molecular Probes, Invitrogen, USA. All other materials were purchased from Sigma-Aldrich. All reagents were used as advised by suppliers.

Polymer self-assembly and formation of complexes. PLL-CA and PEG-PLL-CA polymers were synthesised as previously described (Gu et al., 2008). Briefly, PLL (Molecular weight, MW=15-30 kDa) was modified with CA at substitution levels of either 14 or 32 mol %, to produce PLL-CA14 and PLL-CA32 respectively, in addition, these derivatives were further modified by a monomethoxy-PEG (mPEG, MW=2 and 5 kDa) with grafting level of 17 mol %, to produce PEG17-PLL-CA14 and PEG17-PLL-CA32 respectively. The benzoic imine, a pH triggerable linker, was located between the PLL and the PEG (Figure 1).

PLL particles were formed by dissolving the PLL derivatives in aqueous solutions (i.e., phosphate buffer saline (PBS) for particle size and zeta potential, and deionised (DI) water for all other experiments), followed by sonication in a bath sonicator at room temperature (RT) for 1 hour. PLL.siRNA complexes were formed by aliquoting the PLL solution into the siRNA solution (siRNA solution was prepared in RNase-free water following the QIAGEN and Sigma recommendation) at different mass ratios (MRs) of PLLs and siRNA. The mixture was incubated for 30 minutes at room temperature (RT) to achieve complexation.

Determination of siRNA binding by gel retardation assay. The ability of PLLs to complex siRNA was analysed by gel retardation (Xiong et al., 2009). Briefly, complexes of PLL and siRNA (containing 0.5 μg siRNA) at different mass ratios

were prepared as described above and loaded onto 1% (w/v) agarose gels in Tris/Borate/EDTA (TBE) buffer (Sigma) containing ethidium bromide. Electrophoresis was performed at 120 V for 30 minutes and the resulting gels were photographed under UV.

Particle size and zeta potential of PLL.siRNA complexes. Particle size and zeta potential measurements were carried out using a Malvern Nano-ZS (Malvern Instruments, U.K.) (Gu et al., 2008). Briefly, PBS solutions (pH = 7.2, 6.8 and 5.5, were filtered through a 0.2 μm membrane) were added to PLL.siRNA complexes to make 1 ml of final volume, and incubated for 1 hour prior to particle size and zeta potential measurements. The concentration of PLLs was fixed at 0.2 mg/ml.

Serum stability of PLL.siRNA complexes. Complexes containing 0.5 μg siRNA at MR20 were incubated for different time intervals in 50% (v/v) fetal bovine serum (FBS) at 37ºC (Kim et al., 2006). Following incubation, samples were treated for 1 hour with excess heparin (1,000 I.U./ml) to release the siRNA from the PLLs at RT and then loaded onto 1.5% (w/v) agarose gels in TBE buffer containing ethidium bromide. Electrophoresis was performed at 120 V for 30 minutes and the resulting gels were photographed under UV.

In addition, complexes (MR20) were incubated in 90% (v/v) FBS at 37ºC for 48hr and the particle sizes were measured using Malvern Nano-ZS (Malvern Instruments, U.K.). FBS on its own and complexes (MR20) incubated in DI water (DI water was filtered by 0.2 μm membrane) at 37ºC for 48 hour were used as controls. The concentration of PLLs was fixed at 0.2 mg/ml.

Cell culture and molecular biology. PC-3 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS. TRAMP C1 cells were maintained in Dulbecco's modified Eagle's medium high glucose with L-glutamine and without sodium pyruvate (Gibco, UK) supplemented with 5% FBS, 5% Nu Serum IV (BD Biosciences, UK), insulin from bovine pancreas, 10^{-8} mol/l 5a-Androstan-17b-ol-3-one (Ahmad et al., 2009). All cells were grown in an incubator (ThermoForma) at 37ºC with 5% CO_2 and 95% relative humidity.

The pGL3-luc plasmid contains the firefly luciferase gene under the control of the simian vacuolating virus 40 (SV40) promoter/enhancer. The pGL3-luc plasmid was

maintained and propagated in competent *E. coli*. It was isolated by alkaline lysis, purified by anion exchange chromatography using the Plasmid Mega kit (QIAGEN) in accordance with the manufacturer's instructions and stored in DI water at -20°C. Plasmid concentration was determined from the absorbance at 260 nm using a GeneRay UV-photometer Biometra®. Plasmid with A260/A280=1.8-2.0 was used for experiments.

MTT assay. 5000 PC-3 cells/well and 2500 TRAMP C1 cells/well were seeded in 96-well plates one day before transfection. Subsequently, complexes (MR20) at different concentrations were incubated with cells for 48 hours. At the end of this period, complexes were replaced with 200 µl fresh growth medium, and 20 µl MTT stock (5 mg/ml in PBS) was added and incubated with cells for 4 hours at 37ºC. The contents were removed and 100 µl DMSO was added to dissolve the purple formazan products. The results were measured at 570 nm using a microplate reader. The concentration of siRNA complexed with PLLs leading to 50% cell growth inhibition (IC50) was estimated from the plot of the percentage of viable cells versus the concentration of siRNA for each treatment.

Fluorescence activated cell sorting (FACS). 100,000 PC-3 cells/well were seeded in 12-well plates and incubated for 24 hours under normal growth conditions. Cells were then transfected by fluorescein-siRNA complexed with PEG-PLL-CAs (MR20) at different concentration and incubated for 24 hour in 10% FBS containing growth medium. Naked fluorescein-siRNA was used as the negative control, and siRNA complexed with INTERFRin™ (Polyplus) (prepared following the manufacturer's recommendation) was used as the positive control. On the following day, cells were first treated with CellScrub™ (Genlatins) to remove complexes associated with cell surfaces (uninternalised complexes) according to manufacturer's instructions. The medium was then removed by aspiration, and cells were washed twice with PBS and trypsinised. Cells were subsequently centrifuged (1000 rpm for 5 minutes), the supernatant was carefully discarded and the pellets were re-suspended in 1000 µl cold PBS in Polystyrene Round-Bottom Tubes (Becton Dickinson). 10,000 cells were measured for each sample following the application of Becton Dickinson FACScalibur manual. Fluorescein-positive cells (%) were displayed by Dot Plot and the data were also analysed in Histogram Plot (data not shown).

Confocal laser scanning microscopy (CLSM). 50,000 TRAMP C1 cells/well were seeded in 12-well plates with glass bottoms (MatTek™). Various formulations containing fluorescein-siRNA were applied to cells with a final siRNA concentration of 50 nM and incubated at 37°C for 4 hours. In order to label late endosomes and lysosomes, 75 nM LysoTracker Red was added to cells for 30 minutes at 37°C. The media was then replaced with fresh growth medium and observed using an Olympus FV 1000 microscope. Fluorescein-labeled siRNA was detected using excitation at 488 nm and emission of 505-530 nm (green). LysoTracker Red was detected using excitation at 543 nm and an emission band pass filter 560 nm (red) (Xiong et al., 2009).

***In vitro* reporter gene knockdown.** PC-3 cells were seeded at a density of 50,000 cells/well in 24-well plates. Following 24-hour incubation, the media was replaced with 10% FBS containing growth medium and transfected with 0.4 μg of pGL3-luc plasmid DNA complexed with FuGENE6® DNA transfection reagent (Roche). Following 8-hour incubation, cells were replaced with 10% FBS containing growth medium, and luciferase GL3 siRNA formulated with PEG-PLLs-CAs (MR20) was applied to the cells following a further 24 hour transfection at 37°C. Naked Luciferase GL3 siRNA (50nM), negative control siRNA formulated with PEG-PLL-CAs and Luciferase GL3 siRNA complexed with INTERFERin™ were utilised as negative, negative and positive controls respectively. At the endpoint of transfection, cells were washed with pre-warmed PBS, treated with 250 μl of cell lysis buffer (Promega) and a freeze-thaw cycle (Wang et al., 2009). Cellular debris was removed by centrifugation at 13,000 rpm for 5 minutes. The luciferase activity in cell lysate (20 μl) was measured with 100 μl of luciferin substrate in a luminometer (BERTHOLD Technologies) after 10 seconds. The protein content was determined with Pierce BCA® Assay according to manufacturer's instructions (Thermo Scientific). Luciferase gene expression was expressed as relative light unit (RLU) per microgram protein.

Experimental animals, tumour induction, treatment schedule, quantification of VEGF mRNA and determination of serum IFN-α and IL-12 levels in mice. Male C57 BL/6 mice (Harlan Laboratories, UK) were used in all experiments. The animal ethics committee of University College Cork approved all experiments. The mice

were kept at 22ºC with a natural day/night light cycle in a conventional animal colony. All mice were maintained in a pathogen free animal facility for at least 2 weeks before the experiments. Male C57 BL/6 mice in good condition, 6-8 weeks of age, were used in experiments. The tumour-bearing animal model was established by subcutaneous injection of 5 x 10^6 TRAMP C1 cells into the right flank of a mouse (Ahmad et al., 2009). Tumour growth and body weight was recorded regularly, and tumour volume was calculated using a formula $a^2b(\pi/6)$, where a is the minor diameter of the tumour and b is the major diameter perpendicular to diameter a.

When the tumour volume reached ~50 mm^3 (day 0), mouse VEGF siRNAs at a dose of ~1 mg/kg formulated with PEG-PLL-CA (MR20), or with in-vivo-JetPEI™ (positive control, prepared following the manufacturer's recommendation) was administer to tumour-bearing mice (5 mice/group) by intravenous (i.v.) injection on day 1, 2, 6, 10, 14 and 18. PBS, VEGF siRNA on its own and PEG-PLL-CA on its own were used as negative controls. On day 19, the mice were sacrificed and the tumours were collected. The tumours were homogenised using a MagNA Lyser Instrument (Roche). The tissue homogenates were centrifuged at 15,000 rpm for 2 minutes at 4°C to remove the insoluble tissue debris and the supernatant was collected for reverse transcription polymerase chain reaction (RT-PCR) to determine the expression of VEGF mRNA in each solid tumour. Briefly, total RNA was isolated from the above supernatant using an Absolutely RNA Miniprep Kit (Stratagene). First-strand cDNA was generated from total RNA samples using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative real-time RT-PCR was performed using a Light Cycler System (Roche). RT-PCR was carried out at the following thermal conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, annealing for 5 seconds at 55°C, and 15 seconds at 72°C. The primers used were: mouse 18S ribosomal RNA (rRNA) (forward 5'-gcaattattccccatgaacg-3' and reverse 5'-gggacttaatcaacgcaagc-3', Eurofins), and mouse VEGF-a (forward 5'-gcagcttgagttaaacgaacg-3' and reverse 5'-ggttcccgaaaccctgag-3', Eurofins). Quantitative level of each VEGF mRNA was measured as a fluorescent signal corrected according to the signal for 18S rRNA.

For the *in vivo* study of immune response to siRNAs, serum Interferon-α (IFN-α) and Interleukin-12 (IL-12) levels were determined in male C57 BL/6 mice (6-8 weeks of

age). Groups of five mice were intravenously injected with the desired VEGF siRNA formulations (50 μg per mouse) in a 0.1 ml injection volume. Negative control mice were treated with 0.1ml PBS, and 100 μg of polyinosinic:polycytidylic acid (poly (I:C)) alone as a positive control ([Kim et al., 2008] and [Choi et al., 2010]). Blood samples were collected at 4 hours post treatment. Serum IFN-α was measured by Verikine™ Mouse Interferon Alpha ELISA Kit (PBL, InterferonSource) and serum IL-12 were measured by Mouse IL-12 Platinum ELISA (eBioscience).

Statistical analysis. An unpaired Student's t-test (two-tailed) was used to test the significance of differences between two mean values. One-way ANOVA was used to test the significance of differences in three or more groups. Two-way ANOVA with repeated measures was used to test the significance of differences in measurements of tumour growth and body weight. In all cases, $p<0.05$ was considered statistically significant.

Results

Formation of PLL.siRNA complexes. Novel amphiphilic PLLs have been previously described where chemical substitution of PLL backbones with cholic acid (hydrophobic moiety) produced PLL-CA14 and PLL-CA32 (see Materials and Methods), and subsequent grafting with PEG produced PEG17-PLL-CA14 and PEG17-PLL-CA32, via an acid sensitive linker (benzoic imine) (Figure 1) (Gu et al., 2008). These constructs can self-assemble into nanoparticles in physiological environments and complex siRNAs via electrostatic interactions. The interactions between siRNA and PLLs (non-PEGylated and PEGylated) were analysed by a gel retardation assay (Figure 2a). Complete complexation of siRNA with PLL-CA14/CA32 took place from MR5 onwards; in contrast, in the case of the PEGylated derivatives complete complexation of siRNA occurred from the higher MR20. The results imply that the complexation between siRNA and the PLL derivatives was influenced by PEG grafting.

The size and charge of complexes formed, at different pHs, between siRNA and PLLs were measured by dynamic light scattering (DLS). The particle size (200-280 nm) did

not significantly change with pH (Table 1). However, particularly in the case of PEG-PLLs, the polydispersity index (PDI) showed a slight increase (not statistically different) at pH 6.8 and 5.5 compared to pH 7.2 (Table 1), this may be due to cleavage of the benzoic imine linker at the acidic pHs thus affecting the size distribution. The zeta potential of the non-PEGylated complexes did not change with mass ratio (5, 20 and 50) and no change was detected over the pH range studied (7.2 6.8 and 5.5). In contrast, the zeta potential of PEGylated complexes at individual mass ratios (5, 20, and 50) varied significantly with pH. The surface charge of complexes at low pH was significantly ($p<0.05$) higher (>40 mV) compared to pH 6.8 (~20 to 30 mV) and 7.2 (<20 mV) (Table 1). A similar trend was reported by Gu et al., (2008) for the non-complexed polymers. This increase in charge maybe explained by the gradual unmasking of the PEG group as the pH decreases, thus exposing the cationic surface of the PLLs. The stability of the benzoic imine at pH 7.4 and the subsequent hydrolysis to release the free PEG chains when the pH decreased to 6.5 were previously shown by FT-IR and H^1 NMR (Gu et al., 2008). These combined results support the hypothesis of a pH-triggered delivery system. As the particle size and zeta potential of PLL.siRNA complexes did not change remarkably with increasing mass ratios (Table 1) a mass ratio of 20 was used for all further experiments.

Stability study of PLL.siRNA complexes. It has been reported that naked siRNA is degraded in human plasma with a half-life of minutes (Bumcrot et al., 2006). However, formulations with various carrier systems can protect siRNA against nuclease degradation (Guo et al., 2010). To verify whether PLL derivatives can protect siRNA from serum nucleases, naked siRNA and PLL.siRNA complexes were incubated in 50% FBS at 37ºC (Kim et al., 2006). Naked siRNA was not stable and was completely degraded after 4hour incubation (Figure 2b). In the case of PLL.siRNA complexes, siRNA appeared to be stable for up to 24 hours (Figure 2b). PEGylation did not appear to significantly increase the protective effect on siRNA, as slight nuclease-mediated degradation of siRNA formulated with either PLL-CAs or PEG-PLL-CAs was evident after 48 hours (Figure 2b).

Systemically administered highly cationic particles are eliminated rapidly from the circulation by either the lung endothelial capillary bed or the reticuloendothelial

system (RES, also known as mononuclear phagocytic system (MPS)), as the particles can non-specifically bind serum proteins to form aggregates (Li and Szoka, 2007). When chemically grafted onto the cationic outer shell layer as a 'brush', PEG chains can stabilise particles against nonspecific binding of proteins as well as particle self-aggregation ([Li and Szoka, 2007] and [Li and Huang]). Particle size distribution data generated from DLS measurements demonstrated that aggregation (>1 μm) occurred when non-PEGylated complexes were incubated in 90% FBS at 37ºC. In contrast, PEG17-PLL-CA32 was resistant to aggregation up to 48 hour under the same conditions (Figure 2c); the same trend was observed with PEG17-PLL-CA14 (data not shown).

Cytotoxicity of PLL.siRNA complexes. It has been reported that a significant increase in cytotoxicity is caused by highly cationic complexes compared to their PEGylated derivatives and this cytotoxicity, which generally results from aggregation and precipitation on the cell surface, can induce necrosis. In this study, the cytotoxicity of PLL.siRNA complexes, at a set mass ratio of 20, was studied in PC-3 and TRAMP C1 cell lines using an MTT assay. In the PC-3 cell line, PLL, PLL-CA14, PEG17-PLL-CA14, PLL-CA32, and PEG17-PLL-CA32 complexes displayed IC_{50} values equal to 150, 100, 300, 120 and 340 nM (siRNA) (Figure 3a). In the TRAMP C1 cell line, alternatively, PLL, PLL-CA14, PEG17-PLL-CA14, PLL-CA32, and PEG17-PLL-CA32 complexes displayed IC50 values equal to 165, 114, 320, 135 and 350 nM siRNA respectively (data not shown). The data indicates that PEGylated complexes alleviated the cytotoxicity relative to their unmodified counterparts. Due to higher cytotoxicity, non-PEGylated PLLs were not used in the following *in vitro* and *in vivo* investigations (unless otherwise mentioned). In all further *in vitro* experiments ≤50 nM siRNA was used with PEGylated PLLs (MR20) thus ensuring >90% cell viability.

Internalisation and intracellular trafficking of PLL.siRNA complexes. Fluorescein-siRNA was used to study the internalisation of PLL.siRNA complexes in PC-3 cells. Fluorescein-siRNA on its own and complexed with INTERFERin™ were used as negative and positive controls respectively. The siRNA formulated with PEG-PLL-CAs at all concentrations tested significantly increased cellular uptake compared to naked siRNA (Figure 3b). At 10 and 25 nM, only PEG17-PLL-CA14 achieved

similar cellular uptake compared to INTERFERin™. In contrast, at 50 nM, approximately 60%~65% fluorescein positive cells were detected with complexes of both PEG17-PLL-CA14 and PEG17-PLL-CA32; these results were, comparable to the uptake levels seen with INTERFERin™ (Figure 3b). In addition, the PEGylated PLL complexes demonstrated similar intracellular uptake in TRAMP C1 cells (~50% fluorescein-positive cells were achieved at 50 nM of siRNA, data not shown).

The intracellular trafficking of these complexes in TRAMP C1 cells was investigated using a marker for late endosomes and lysosomes, LysoTracker Red (red) (Figure 3c). Four hours after treatment, there was no evidence of internalisation by non-complexed siRNA (Figure 3c), and this is in agreement with data from Figure 3b. In contrast, following transfection with PEG-PLL-CA a definite fraction of siRNA (green) was detected in the cytoplasm which was not co-localised with LysoTracker (red). The green fluorescence was relatively stronger than the red fluorescence, implying that this fraction was released from endolysosomes (Figure 3c) (Xiong et al., 2009), a similar pattern was observed for INTERFERin™, a polyethylenimine (PEI)-based reagent (Figure 3c). It was reported by Gu et al. that PEG-PLL-CA displays a pH-dependent membrane destabilising effect, where the degree of haemolytic activity increased as the pH decreased from 7.4 to 6.0; this result also indicated the potential for endosomal escape (Gu et al., 2008). In contrast, in the case of non-PEGylated PLL-CA32, only yellow staining was detected in the cytoplasm of the cells. This yellow colour is consistent with co-localision of LysoTracker Red with fluorescein siRNA.

The intracellular trafficking of the PEGylated PLL complexes in PC-3 cells was similar to that observed in TRAMP C1 cells (data not shown).

***In vitro* reporter gene knockdown with PEGylated complexes.** The ability of PEGylated PLL-CAs to deliver functional siRNA for RNAi was confirmed using Luciferase GL3 siRNA to silence pGL3 plasmid expression in PC-3 cells (Figure 3d). Compared with negative control siRNA, PEG17-PLL-CA32 using 50 nM targeted siRNA produced a nine-fold reduction in gene expression (Figure 3d); the high cell viability following exposure to this vector (Figure 3a) implies that inhibition of gene expression was a result of RNAi rather than cytotoxicity. This level of gene silencing was similar to that recorded with INTERFERin™ (Figure 3d). The *in vitro* luciferase

gene silencing of the PEGylated PLL complexes in TRAMP C1 cells was similar to that recorded in PC-3 cells (data not shown).

***In vivo* anti-tumour effect of PEGylated complexes.** Since PEG17-PLL-CA14/CA32 presented similar physicochemical properties and *in vitro* gene silencing characteristics, and PEG17-PLL-CA32 is slightly less toxic consequently it was chosen for the *in vivo* therapeutic study. The effect of systemic administration of anti-VEGF siRNA formulated with PEGylated nanocarriers was assessed after intravenous injections through the tail vein of TRAMP C1 tumour-bearing mice (n=5mice/group). Monitoring of tumour growth showed that naked siRNA and PEG-PLL-CA alone did not have a significant effect on tumour suppression, compared to the negative control (PBS) (Figure 4a). In contrast, VEGF siRNA/PEG-PLL-CA achieved a significant inhibitory effect (~2-fold reduction in tumour volume relative to PBS control), which was comparable to the therapeutic effect achieved with In-vivo JetPEI™. In addition, monitoring of mouse body weight demonstrated no significant loss compared to PBS controls over the treatment period (Figure 4b), indicating the absence of PEGylated PLL.siRNA induced toxicity. In addition to tumour volume, tumour weights recorded at the end of the study (data not shown), showed that treatment with the PEGylated complexes resulted in 40.98±11.5% tumour reduction compared to the PBS control.

RT-PCR was used to measure the expression of VEGF mRNA in tumour samples. Compared to PBS control, a marked (~70%) downregulation of VEGF mRNA level was revealed by PEGylated PLL.siRNA complexes (Figure 4c). This anti-tumour efficacy was comparable with the commercial reagent, In-vivo JetPEI™ (Figure 4c). Furthermore, serum levels of two established cytokines IFN-α and IL-12 were investigated as a measure of unwanted immunotoxicity (2 mg siRNA/kg mouse body weight, 4-hour post-injection) (Figure 5a and 5b). Poly (I:C), a well-known synthetic inflammatory stimulant, was used as a positive control. The PEGylated siRNA complexes did not elicit increased IFN-α and IL-12 response relative to PBS and naked siRNA, whereas poly (I:C) induced a strong response (Figure 5a and 5b). These results confirmed that the suppression of tumour growth post systemic administration of the PEGylated PLL.siRNA vector was mediated mainly through inherent VEGF gene downregulation, and not some nonspecific immunosimulatory effects.

Discussion

The systemic application of therapeutic siRNAs has been proposed as a treatment for many serious diseases, such as genetic diseases, viral infections and cancer ([Whitehead et al., 2009], [Guo et al., 2010] and [Guo et al., 2011]). A key restriction to the development of human gene therapy remains the lack of safe, efficient and controllable methods for nucleic acid delivery ([Whitehead et al., 2009] and [Guo et al., 2010]). In past decades, numerous synthetic materials have been described which provided opportunities for enhanced safety, more efficiency, greater flexibility and simple fabrication ([Oh and Park, 2009] and [Guo et al., 2010]). Although significant progress has been made there is still need for improvement to ensure targeted delivery to diseased sites including tumours.

Systemic gene delivery vectors face an initial set of obstacles before reaching target cells. Highly cationic particles are eliminated rapidly from the circulation either by the lung endothelial capillary bed or by the RES (Li and Szoka, 2007). Modification of such polymers with PEG chains can stabilise particles against non-specific binding of serum proteins as well as particle self-aggregation, resulting in longer blood circulation times ([Li and Szoka, 2007] and [Li and Huang, 2010]). Previously our group reported the synthesis of a new class of 'stealth' amphiphilic PLLs grafted with PEG and CA at different molar ratios (Gu et al., 2008). In the current work, two of the poly(L-lysine) derivatives namely PEG17-PLL-CA14 and PEG-PLL-CA32, self-assembled into nanoparticles and demonstrated efficient complexation with siRNA through electrostatic interactions.

The average hydrodynamic diameter (200-280 nm) of PLL.siRNA complexes suggests that these nanoparticles can enter inflammation sites and solid tumours through the enhance permeation and retention (EPR) effect, and also attain reduced liver filtration ([Pirollo et al., 2006] and [Li and Szoka, 2007]). Pegylation of these amphiphilic PLL nanoparticles (NPs) improved the delivery potential of this material in a number of ways. For example, masking the cationic charge density in PLL derivatives with PEG significantly reduced non-specific cytotoxicity compared to the non-PEGylated derivatives. PEGylated complexes were not significantly affected by

decomplexation or aggregation implying that PEGylated PLL-CAs protected the siRNA against serum enzymatic degradation and prevented protein adsorption of complexes. The PEG induced protective effect may lead to long-term circulation in blood vessels and provide more opportunity to accumulate in tumour tissues following an EPR effect (Phillips et al., 2010).

One of the design features inbuilt into this vector is the pH sensitive linker used for the attachment of the PEG. This facilitates reversible masking of the positive charge by the PEG. The linker is bioresponsive and is cleaved at low pH such as in the extracellular environment of the tumour or in the endosome. Cleavage to release the PEG will facilitate cellular uptake and allow passive targeting to tumour cells by exploiting the physiological environment of the diseased site (Guo et al., 2011). The *in vitro* results show significant levels of cellular uptake with the PEGylated PLL NPs. The relatively high cellular uptake may be explained by the slightly positive surface charge and the amphiphilic nature of the vector. In the case of tumour cell culture models, it is likely that the extracellular pH will decrease thus promoting a degree of PEG cleavage resulting in an increase in charge. At pH 7.2 a slight positive charge was detected (< 20 mV); in contrast, as the pH decreased to 6.8 the charge increased (~20 to 30 mV). In vivo, at the lower pH, reported for the tumour site, this reversible masking effect will result in an increase in cell membrane association and enhanced cellular uptake ([Wang et al., 2009] and [Shi et al., 2010]).

Following cell internalisation, gene-delivery vectors encounter a new set of intracellular challenges. Non-specific electrostatic binding of complexes to the surface of cells results in internalisation via adsorptive pinocytosis (Dominska and Dykxhoorn 2010). Complexes subsequently become trapped inside endocytic vesicles. Only siRNA that escapes from the late endosome/lysosome can enter the cytoplasm and undergo the RNAi pathway. In this study, the pH-sensitive PLL NPs, appeared to achieve significant levels of endosomal escape, in a similar fashion to that observed with the PEI-based commercial vector. The increase in charge density detected at the low pH 5.5 (zeta potential > 40 mV) taken together with the confocal images suggest that the benzoic imine linker of the PEGylated PLL-CA NP was further cleaved in the acidic environment enabling endolysosomal disruption and release of siRNA into the cytoplasm. It has been reported by Gu et al. that PEG-PLL-CAs displayed pH

dependent haemolytic activity and exhibited a strong membrane-destablisation effect at a pH representative of the early endosome (pH = 6), thus indicating the potential to promote endosomal escape (Gu et al., 2008). The ability of such pH triggered vector systems to enhance endosomal/lysosomal disruption has previously been reported (Wang et al., 2009).

The confocal data suggests a different intracellular distribution pattern for siRNA complexed with the non-PEGylated PLL. While the precise reasons for this difference is unclear and would require further investigation the following is a possible explanation. A number of distinct endocytic pathways including clathrin mediated endocytosis, caveolae mediated endocytosis and macropinocytosis may be involved in the uptake of these nanoparticles/complexes (O'Neill et al., 2011). At physiological pH the non-PEGylated particles are highly charged relative to the PEGylated and it is known that the surface characteristics of nanoparticles may influence endocytosis behaviour. It is feasible that the endocytic route favoured by the PEGylated complexes may be more productive in terms of intracellular trafficking and endosomal release following PEG cleavage (Douglas et al., 2008).

The concept of successful siRNA endosomal release, to promote RNAi activity, by the PEG-PLL-CA NP was subsequently supported by high levels of reporter gene knockdown. No such knockdown was detected with the control containing scrambled siRNA, suggesting that the gene silencing effect was not related to cytotoxicity (Akhtar and Benter 2007).

Inhibition of tumour vasculature development (angiogenesis), by suppressing angiogenesis activators, is an effective strategy to treat cancer ([Kim et al., 2008] and [Choi et al., 2010]). The vascular endothelial growth factor (VEGF), one of the most important angiogenesis oncogenes, has been investigated as an anti-angiogenesis target ([Jiang et al., 2009] and [Mok et al., 2010]). In a TRAMP C1 prostate cancer mouse model intravenously injection of VEGF siRNA complexed with PEGylated PLL-CA at a relatively low dose (1 mg siRNA/kg mouse body weight) demonstrated significant tumour inactivation (~2-fold tumour size reduction) compared to naked VEGF siRNAs and empty PEG-PLL-CAs. The anti tumour response was similar to that observed following treatment with VEGF siRNA formulated with In Vivo-JetPEI. The therapeutic activity of the PEGylated siRNA formulation was accompanied by a

dramatic reduction in VEGF mRNA levels in the tumour tissue. While the tumour growth was significantly retarded by our siRNA nanotherapeutics as well as the commercial vector, neither of these treatments could completely inhibit the tumour growth. It has been reported that several oncogenes and oncosuppressors influence the development of cancer to varying degrees, and therefore it may be necessary to silence multiple genes simultaneously ([Chen et al., 2010] and [Guo et al., 2011]).

It has been recently reported that the immunostimulatory activity of siRNA is highly dependent on the nucleotide sequence and/or vector type ([Whitehead et al., 2009] and [Guo et al., 2011]). In this study, a partial analysis of immune stimulation (IFN-alpha and IL-12) by our pH-sensitive formulations demonstrated no evidence of immunotoxicity (Figure 5a and 5b), further confirming that gene silencing was a direct consequence of the RNAi pathway. The lack of an immunogenic response to this vector is a significant therapeutic advantage particularly if chronic administration is required.

PEGylation of the PLL masks the positive charge at physiological pH and prevented aggregation is serum, and relative to highly cationic vectors such as PEI it will be less toxic *in vivo* and therefore has the potential to achieve a longer circulation time in blood. Future studies will examine the pharmacokinetic and biodistribution profiles of these delivery vectors.

In conclusion, the pH-triggered PEG-PLL-CAs can form stable complexes with siRNA. These PEGylated complexes demonstrated physicochemical characteristics, in terms of size and charge, compliant with the requirements for prolonged circulation *in vivo*. Intracellular trafficking using living cells, together with membrane internalisation and reporter gene knockdown, supported the hypothesis of effective endolysosomal escape. Our preliminary *in vivo* results indicate the potential of siRNA-based therapeutics for prostate cancer therapy.

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Table 1. Particle size (nm) and zeta potential (mV) of PLL.siRNA complexes at MR5, 20, and 50 in PBS (pH 7.2, 6.8 and 5.5) using DLS (mean±SD, PDI±SD, n=3). The final PLL concentration was used at 0.2 mg/ml. – represented not measured.

Figure 1. Illustrations of chemical structures of PEGylated PLL-cholic acid. (see reference of Gu et al., 2008)

Figure 2. Physicochemical evaluations of PLL.siRNA complexes. (**a**) Complexation of siRNA (0.5 μg) with PLL-CA14, PEG17-PLL-CA14, PLL-CA32 and PEG17-PLL-CA32 at different mass ratios using gel retardation (1% agarose gel at 120 mV for 30 minutes). Naked siRNA was used as control, and mass ratios between PLLs and siRNA of 2.5, 5, 7.5, 10, 20, 30, 40 and 50 were investigated. (**b**) Serum stability of nude siRNA (0.5 μg) and siRNA (0.5 μg) complexed with PLL-CA14, PEG17-PLL-CA14, PLL-CA32and PEG17-PLL-CA32 at MR20 following 50% FBS incubation for 1, 2, 4, 6, 8, 12, 24 and 48 hours at 37°C. Untreated siRNA (at 0 hour) was used as a control (**c**) Aggregation of PLL-CA32.siRNA and PEG17-PLL-CA32.siRNA complexes (MR20) incubated in 90% FBS for 48hr. The final PLL concentration was used at 0.2 mg/ml. Size distribution of serum on its own and complexes incubated in DI water were used as controls.

Figure 3. In vitro investigations of PLL.siRNA complexes in prostate cancer cell lines. (**a**) Viability of PC-3 cells treated with various formulations containing siRNA complexed with unmodified PLL, PLL-CA and PEG-PLL-CA (MR=20) (mean±SD, n=3). (**b**) Fluorescein-positive cells (%) with naked siRNA, INTERFERin® versus PEGylated PLL.siRNA complexes (MR20) at a series of siRNA concentrations, analysed by Dot Plots in PC-3 cells (* $P<0.05$, NS=no significance) (mean±SD, n=3). (**c**) Intracellular distribution of naked fluorescein siRNA (50 nM), siRNA.PLL-CA32 (MR20), siRNA.PEG17-PLL-CA32 (MR20) and siRNA.INTERFERin® in TRAMP C1 cells at 4hour post-transfection by confocal microscope. The images, upper left (UL), upper right (UR), lower left (LL) and lower right (LR) represented fluorescein, LysoTracker Red, merged images of fluorescein and LysoTracker Red, and transmission light. The areas shown by arrow (white colour) are expanded separately. (**d**) Luciferase reporter gene silencing in PC-3 cells by naked anti-luciferase siRNA (50 nM), anti-luciferase siRNA/scramble siRNA (50 nM) complexed with PEG17-PLL-CA14 (MR20), PEG17-PLL-CA32 (MR20) and INTEFERin® (* $P<0.05$, compared to formulated negative siRNAs) (mean±SD, n=3).

Figure 4. Effect of anti-tumour therapy by intravenous injection of VEGF siRNA.PEGylated PLL complexes in the TRAMP C1 mouse model. The mice were injected with formulations containing PEG17-PLL-CA32.VEGF siRNA (1 mg/kg) and In-vivo JetPEI.VEGF siRNA (1 mg/kg, positive control, not shown for clarity),

PBS, naked VEGF siRNA (1 mg/kg, negative control) and empty PEG17-PLL-CA32 (negative control) at different intervals through tails on day 1, 2, 6, 10, 14 and 18 when tumour volume reached ~50 mm^3 (day 0). (**a**) Tumour growth (mean±SD, n=5 mice/group) was evaluated by measuring tumour volume, (**b**) body weight (mean±SD, n=5 mice/group), and (**c**) tumour VEGF mRNA evaluated by semi-quantitative RT-PCR (mean±SD, n=5).

Figure 5. IFN-α (**a**) and IL-12 (**b**) responses to PEGylated siRNA complexes (mean±SD, n=5). Mice were injected by PBS, naked VEGF siRNA (2 mg/kg siRNA), VEGF siRNA (50 μg siRNA) mediated with PEG17-PLL-CA32, and poly (I:C) (100 μg) in a 0.1 ml volume through the tail vein. Serum was collected 4hour post injection, and IFN-α and IL-12 levels were measured by ELISA.

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Table 1.

Complexes	MR5 (PBS pH=7.2)	MR5 (PBS pH=6.8)	MR5 (PBS pH=5.5)	MR20 (PBS pH=7.2)	MR20 (PBS pH=6.8)	MR20 (PBS pH=5.5)	MR50 (PBS pH=7.2)	MR50 (PBS pH=6.8)	MR50 (PBS pH=5.5)
PLL-CA14.siRNA	272±8 nm (0.315±0.02) 47±2 mV	270±3 nm (0.314±0.02) 47±1 mV	269±4 nm (0.312±0.04) 48±1 mV	270±5 nm (0.305±0.01) 49±6 mV	268±2 nm (0.315±0.02) 50±2 mV	258±4 nm (0.325±0.02) 47±2 mV	268±6 nm (0.302±0.02) 53±2 mV	265±4 nm (0.311±0.01) 51±2 mV	274±3 nm (0.319±0.02) 50±5 mV
PEG17-PLL-CA14.siRNA	- - -	- - -	- - -	210±4 nm (0.311±0.01) 19±2 mV	213±1 nm (0.321±0.03) 26±1 mV	200±2 nm (0.410±0.05) 42±2 mV	212±2 nm (0.308±0.01) 21±1 mV	214±4 nm (0.328±0.02) 29±3 mV	198±5 nm (0.420±0.02) 45±3 mV
PLL-CA32.siRNA	272±5 nm (0.354±0.01) 56±1mV	268±3 nm (0.348±0.01) 53±2mV	270±7 nm (0.334±0.02) 54±2 mV	268±4 nm (0.333±0.02) 50±5 mV	258±3 nm (0.320±0.02) 49±3 mV	270±2 nm (0.355±0.03) 50±2 mV	260±2 nm (0.336±0.03) 54±1 mV	261±4 nm (0.336±0.02) 53±1 mV	265±5 nm (0.345±0.02) 52±2 mV
PEG17-PLL-CA32.siRNA	- - -	- - -	- - -	246±3 nm (0.328±0.02) 13±1 mV	252±4 nm (0.333±0.01) 21±3 mV	255±8 nm (0.395±0.01) 38±4 mV	245±9 nm (0.342±0.04) 11±4 mV	239±3 nm (0.361±0.02) 24±1 mV	232±6 nm (0.407±0.03) 42±3 mV

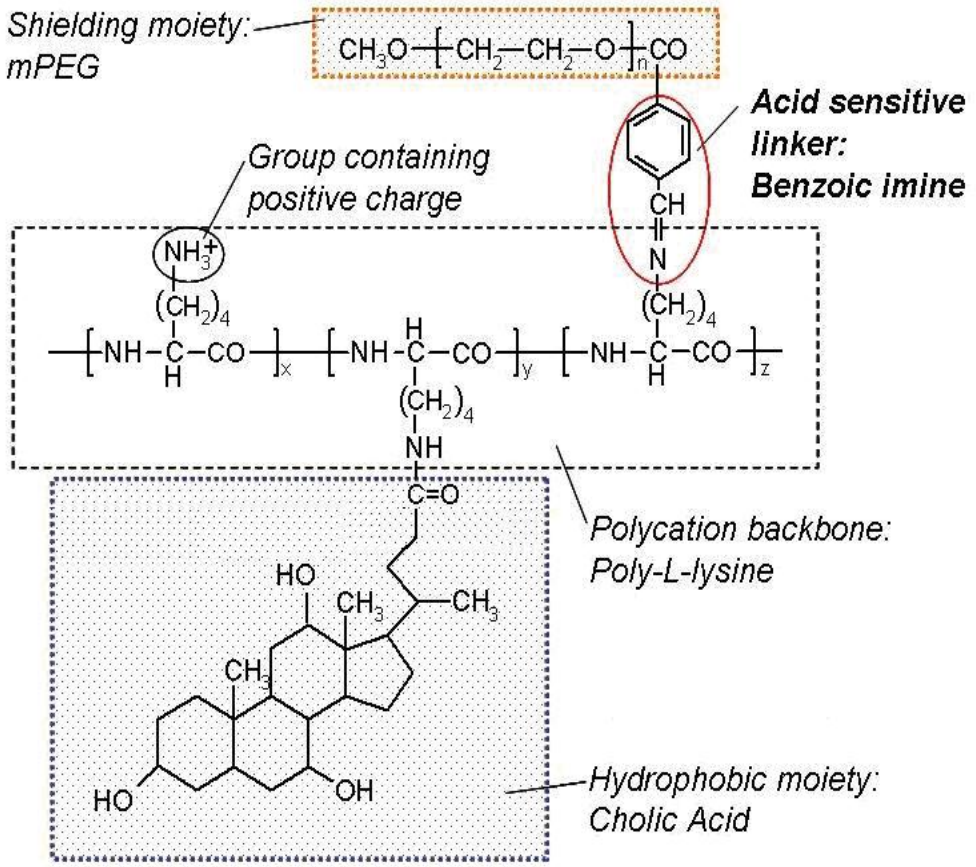

Shielding moiety:
mPEG
Acid sensitive
linker:
Benzoic imine
Group containing
positive charge
Polycation backbone:
Poly-L-lysine
Hydrophobic moiety:
Cholic Acid

Figure 1.

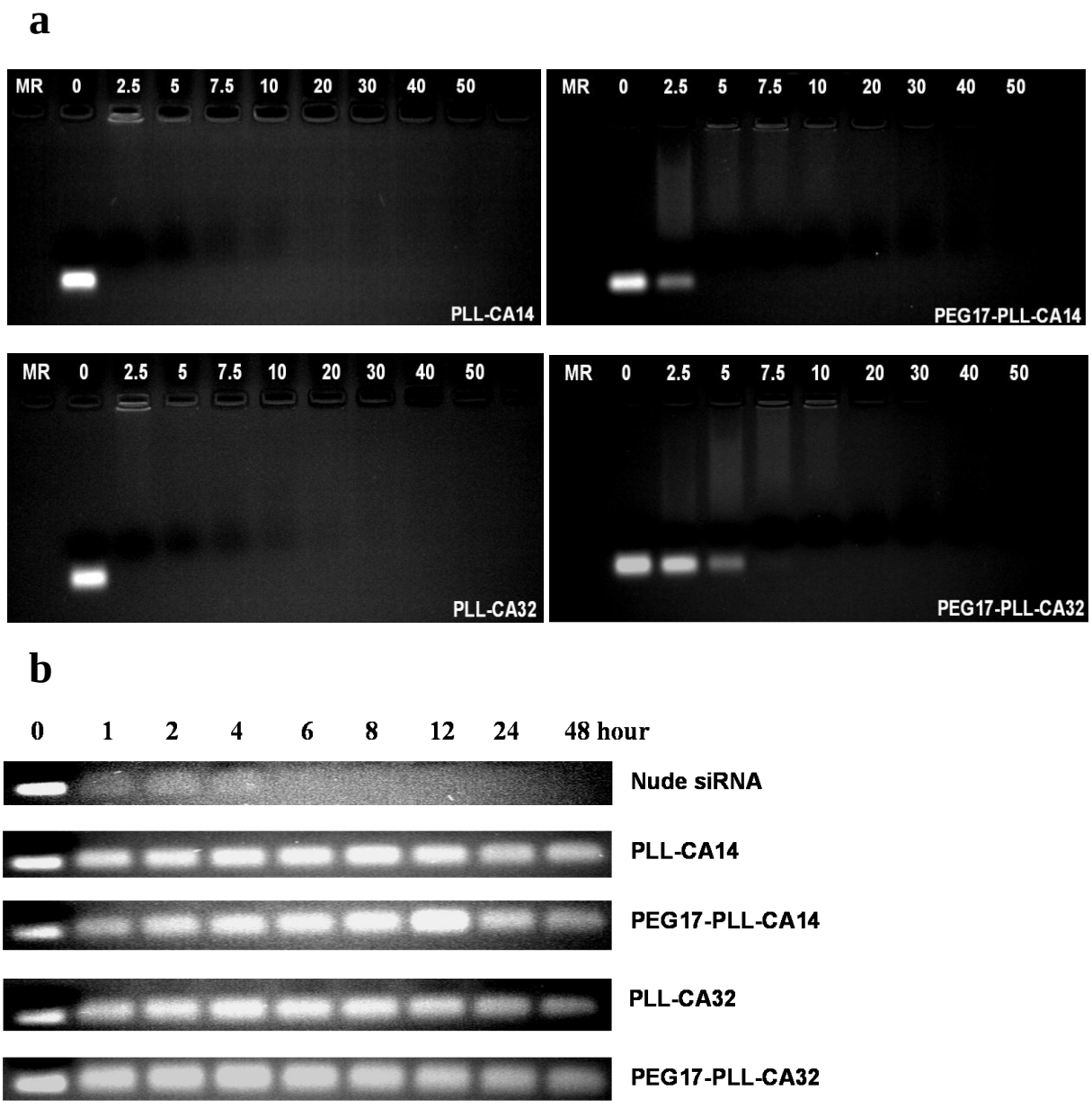

a
MR 0 2.5 5 7.5 10 20 30 40 50
PLL-CA14
MR 0 2.5 5 7.5 10 20 30 40 50
PEG17-PLL-CA14
MR 0 2.5 5 7.5 10 20 30 40 50
PLL-CA32
MR 0 2.5 5 7.5 10 20 30 40 50
PEG17-PLL-CA32
b
0 1 2 4 6 8 12 24 48 hour
Nude siRNA
PLL-CA14
PEG17-PLL-CA14
PLL-CA32
PEG17-PLL-CA32

Figure 2.

c

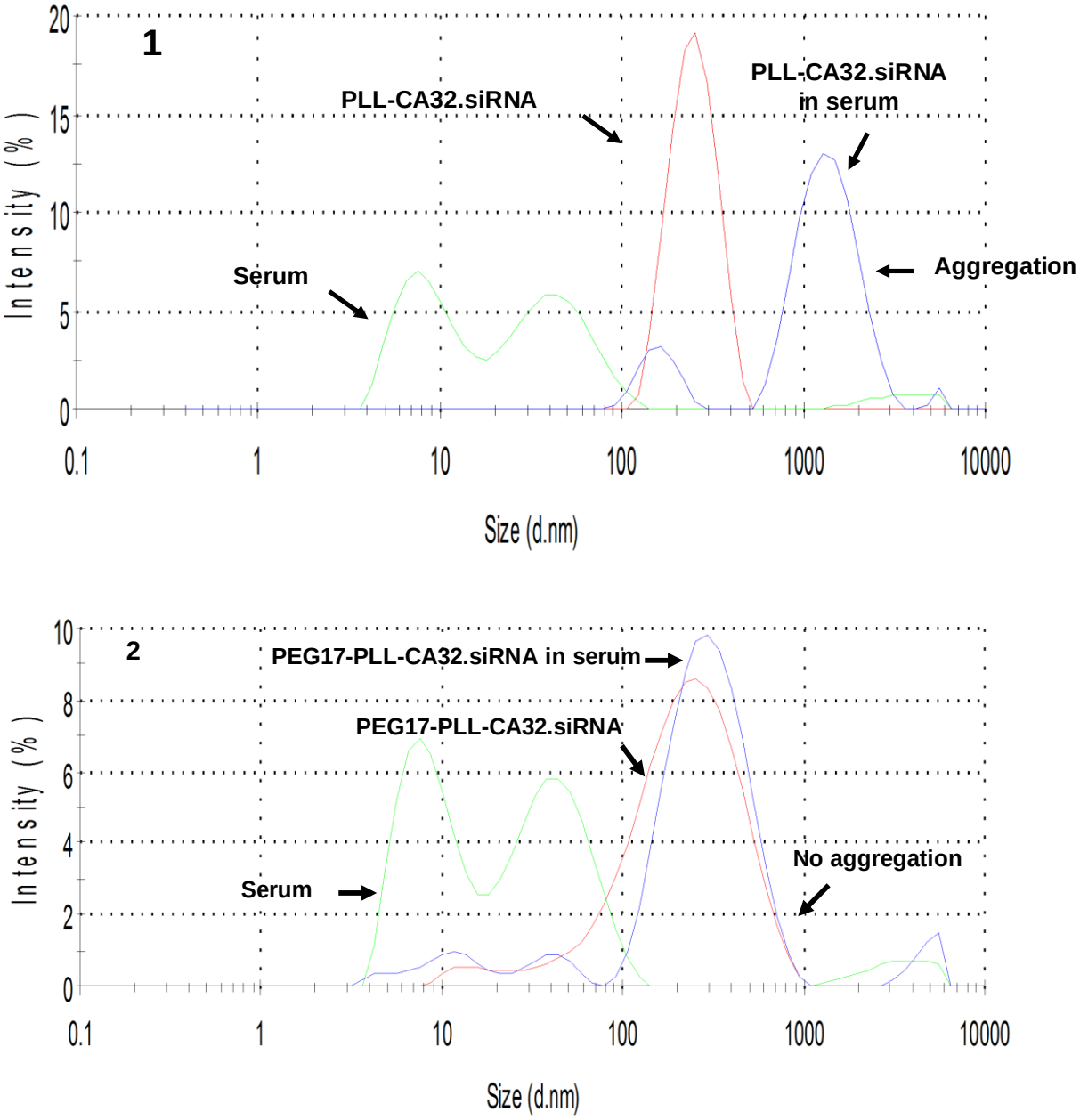

1
PLL-CA32.siRNA
PLL-CA32.siRNA
in serum
Serum
Aggregation
Intensity (%)
Size (d.nm)
2
PEG17-PLL-CA32.siRNA in serum
PEG17-PLL-CA32.siRNA
No aggregation
Serum
Intensity (%)
Size (d.nm)

Figure 2.

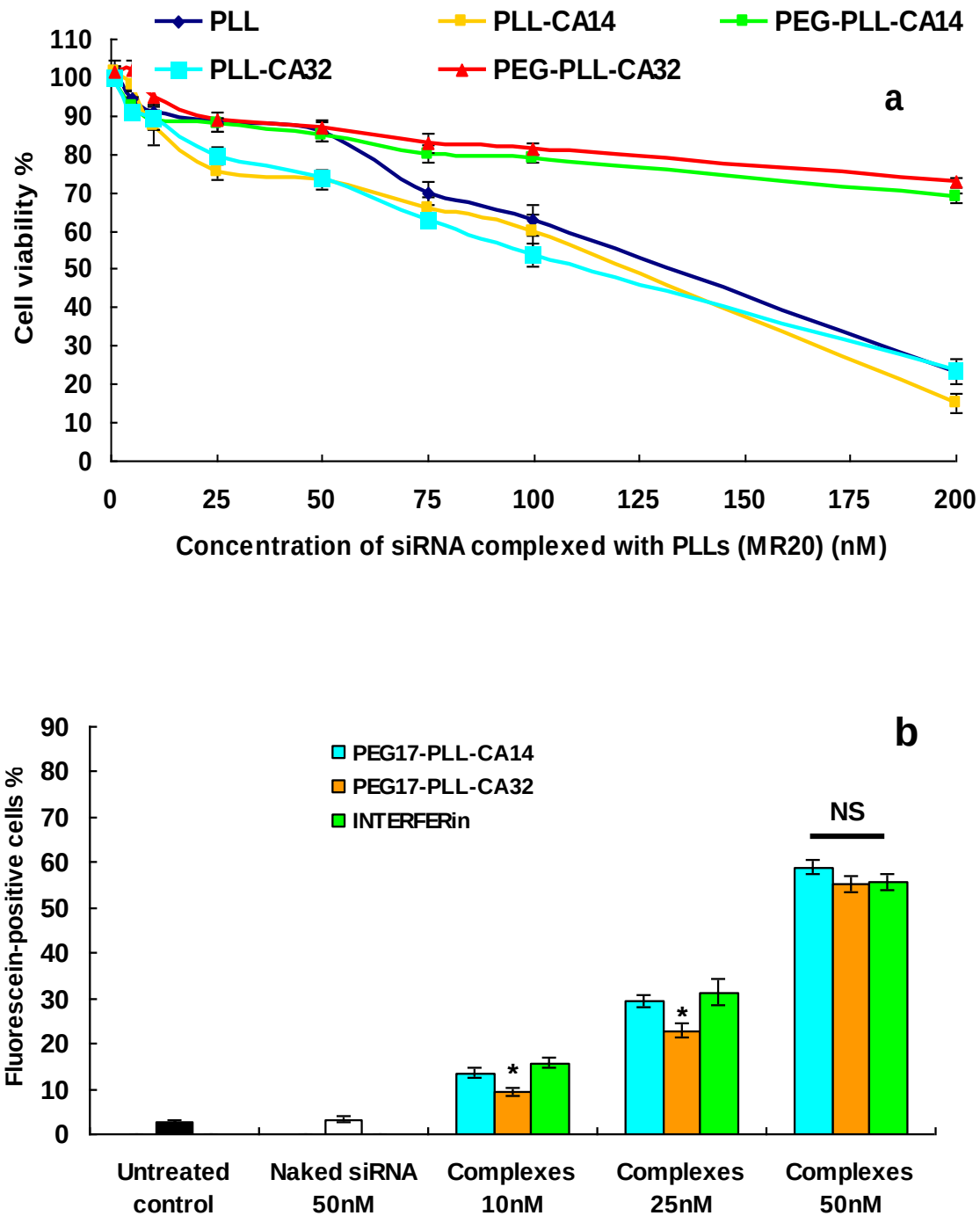
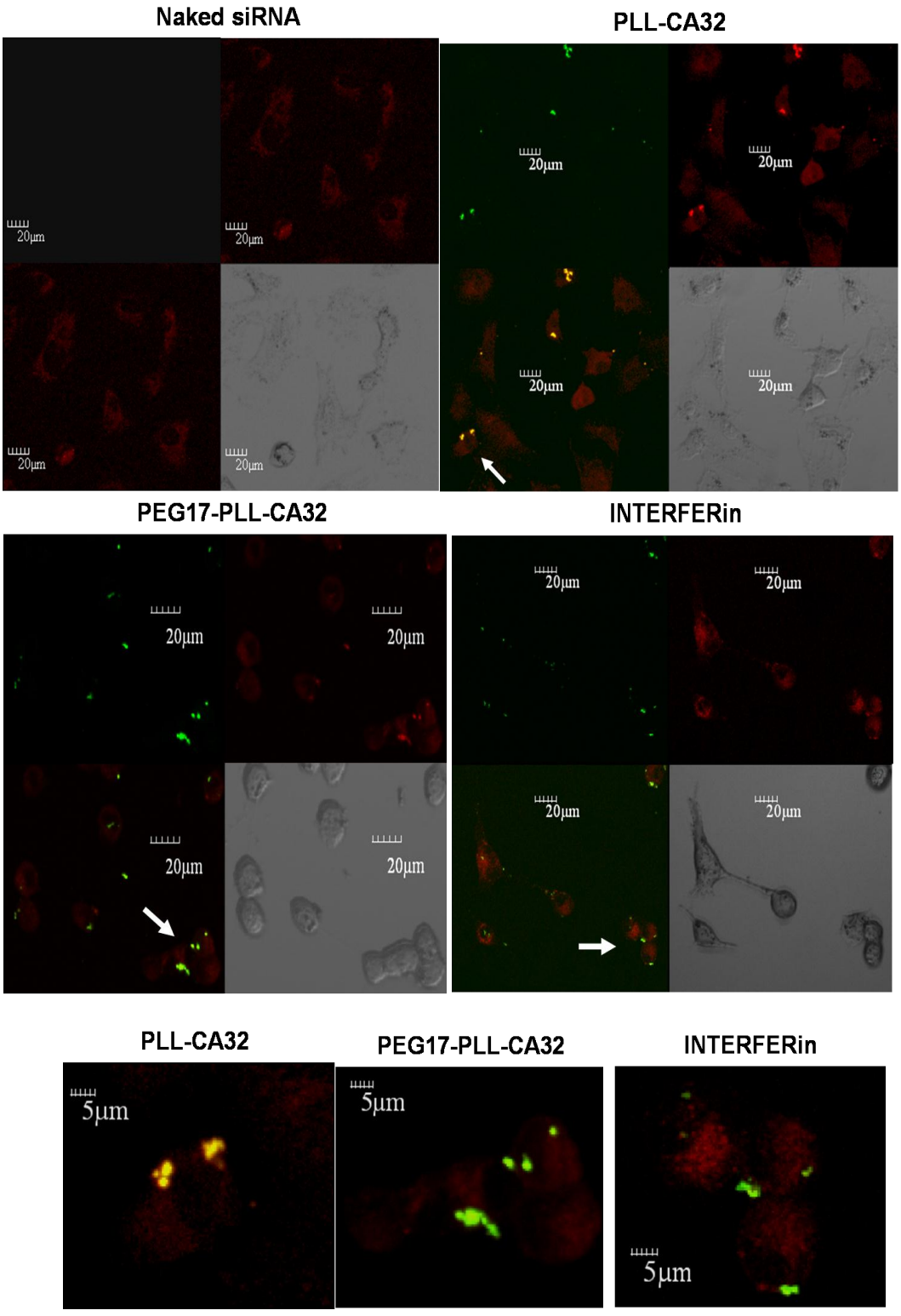

PLL
PLL-CA14
PEG-PLL-CA14
PLL-CA32
PEG-PLL-CA32
a
Cell viability %
Concentration of siRNA complexed with PLLs (MR20) (nM)
b
PEG17-PLL-CA14
PEG17-PLL-CA32
INTERFERin
NS
Fluorescein-positive cells %
Untreated control
Naked siRNA 50nM
Complexes 10nM
Complexes 25nM
Complexes 50nM

Figure 3.

C



(The highlighted areas as indicated by the white arrows)

Figure 3.

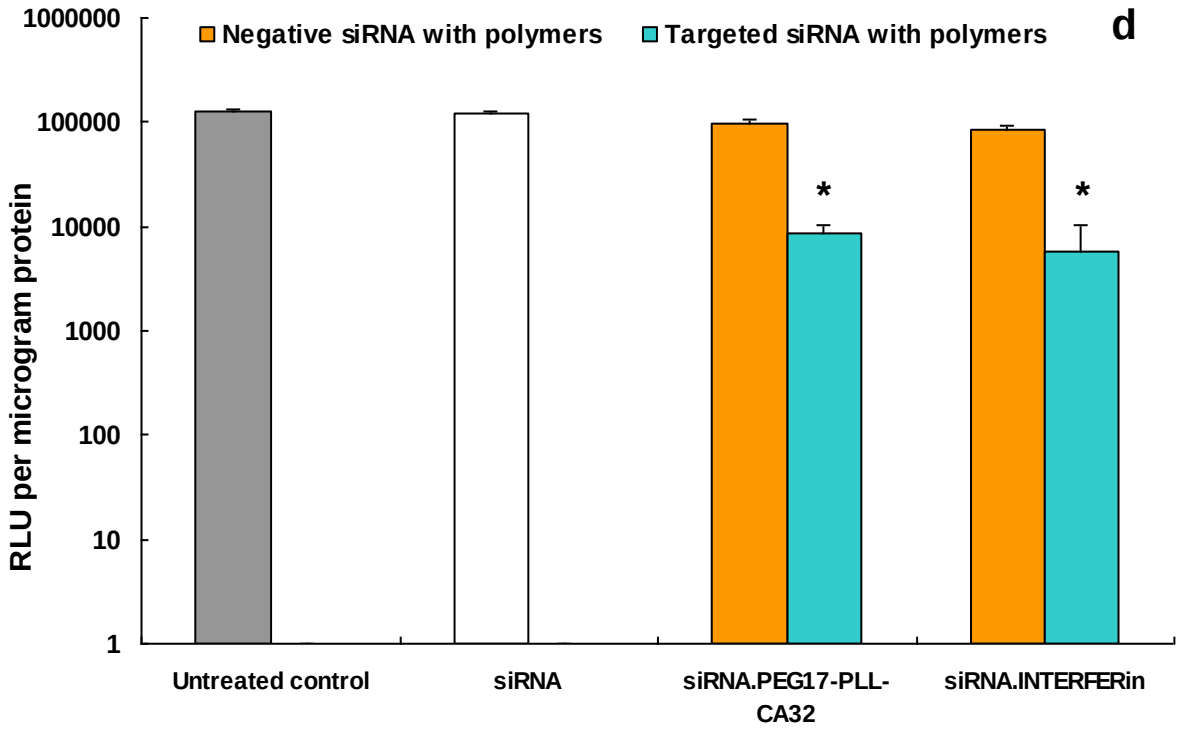

d
Negative siRNA with polymers
Targeted siRNA with polymers
RLU per microgram protein
1000000
100000
10000
1000
100
10
1
*
*
Untreated control
siRNA
siRNA.PEG17-PLL-CA32
siRNA.INTERFERin

Figure 3.

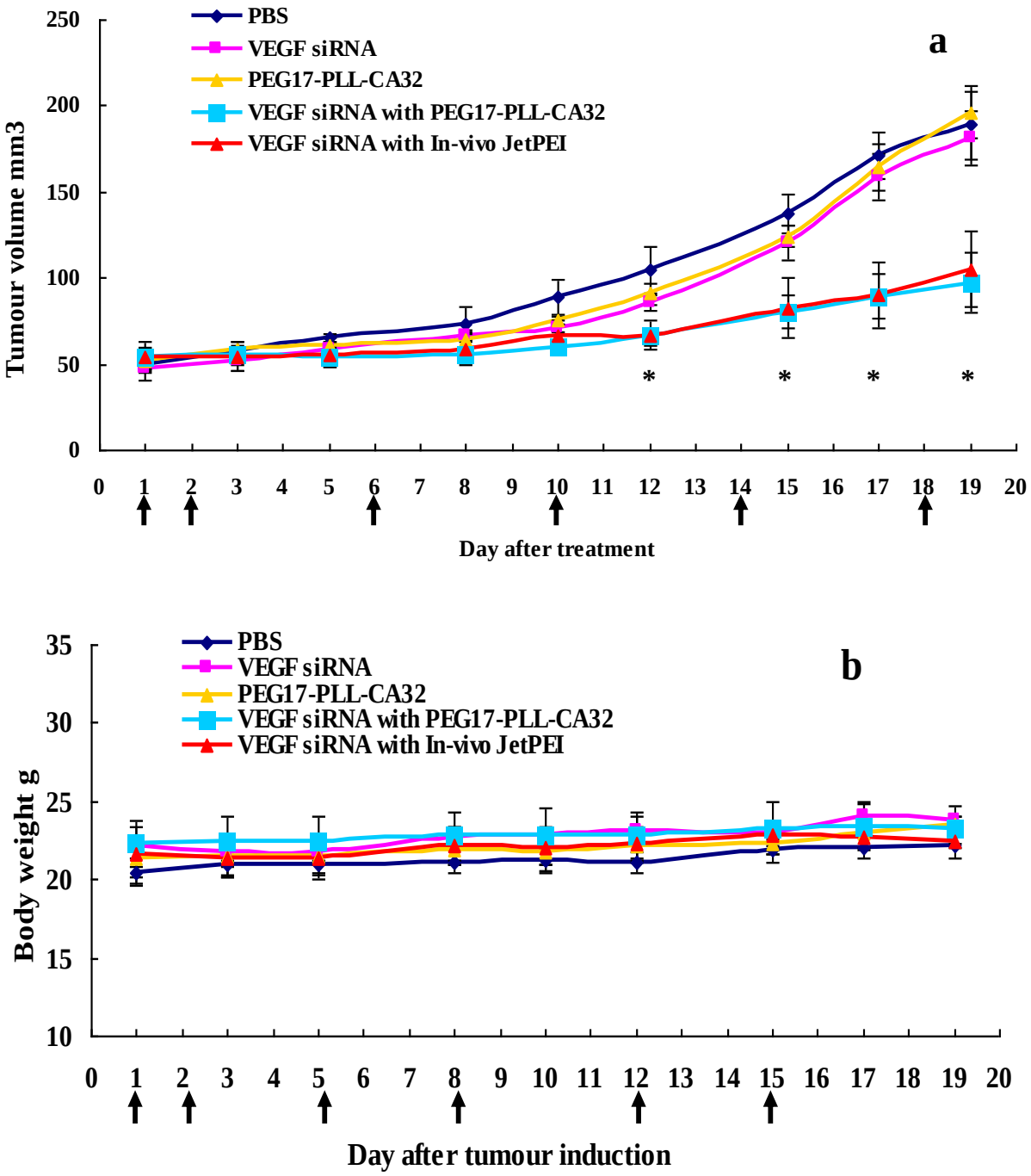

PBS
VEGF siRNA
PEG17-PLL-CA32
VEGF siRNA with PEG17-PLL-CA32
VEGF siRNA with In-vivo JetPEI
a
Tumour volume mm3
Day after treatment
b
Body weight g
Day after tumour induction

Figure 4.

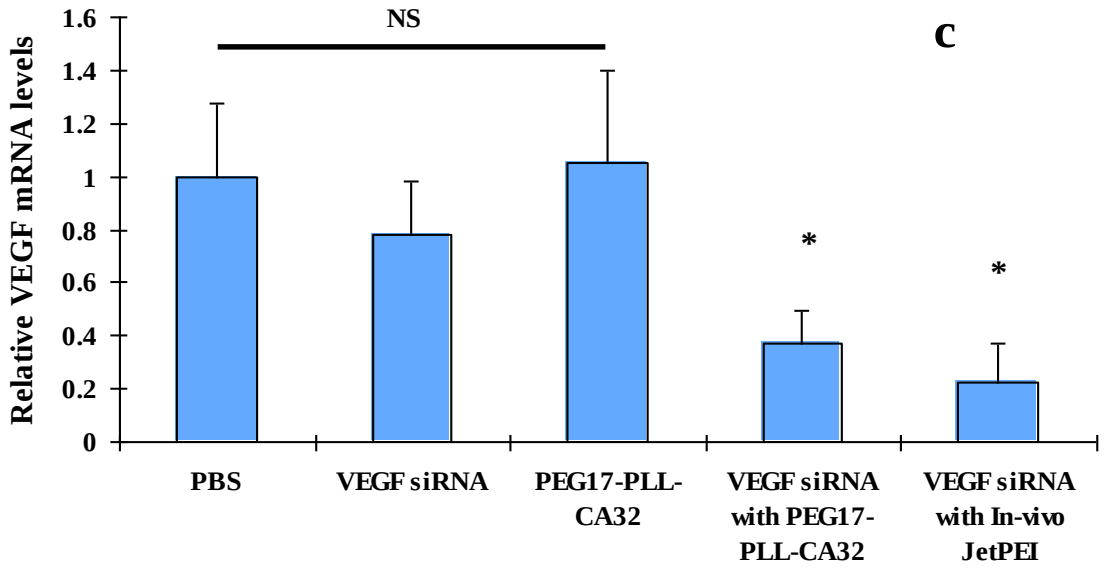

C
NS
Relative VEGF mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
PBS
VEGF siRNA
PEG17-PLL-CA32
VEGF siRNA with PEG17-PLL-CA32
VEGF siRNA with In-vivo JetPEI

Figure 4.

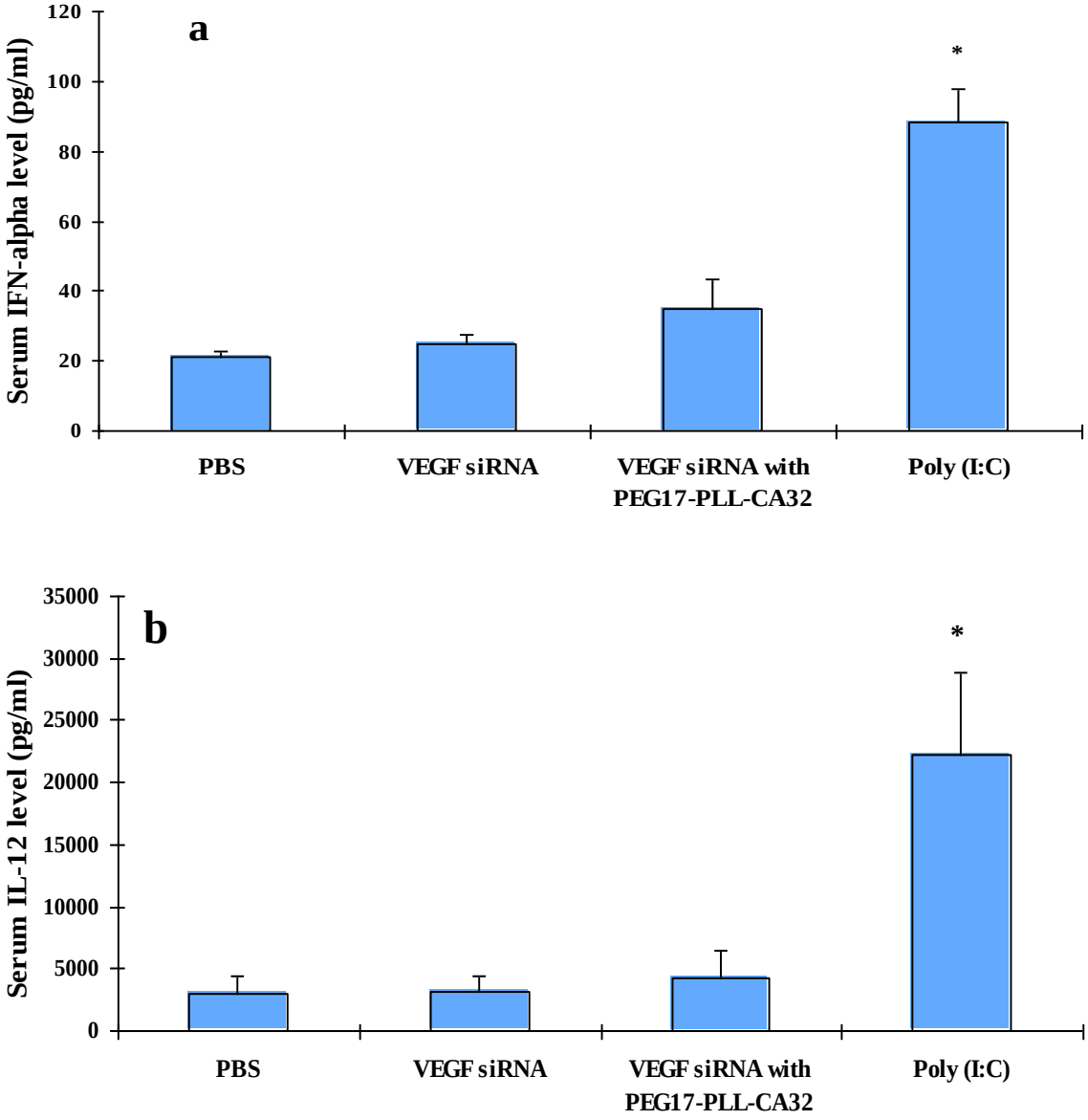

a
Serum IFN-alpha level (pg/ml)
120
100
80
60
40
20
0
*
PBS
VEGF siRNA
VEGF siRNA with PEG17-PLL-CA32
Poly (I:C)
b
Serum IL-12 level (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
*
PBS
VEGF siRNA
VEGF siRNA with PEG17-PLL-CA32
Poly (I:C)

Figure 5.