RNA Interference using c-Myc-Conjugated Nanoparticles Suppresses Breast and Colorectal Cancer Models

Naveen K. Tangudu1†, Vinod K. Verma1†, Tristan D. Clemons2#, Syed S. Beevi1#, Trevor Hay3, Ganesh Mahidhara1, Meera Raja3, Rekha A. Nair4, Liza E. Alexander4, Anant B. Patel1, Jedy Jose1, Nicole M. Smith5, Bogdan Zdyrko6, Anne Bourdoncle5, Igor Luzinov6, K. Swaminathan Iyer2*, Alan R. Clarke3*, Lekha Dinesh Kumar1*

Affiliations:
1Cancer Biology, Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Uppal Road, Hyderabad-500 007, India;
2School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Crawley WA 6009, Australia;
3European Cancer Stem Cell Research Institute, Cardiff University, Hadyn Ellis Building, Maindy Road, Cathays, Cardiff, CF24 4HQ, UK;
4Dept of Pathology, Regional Cancer Centre, Medical College campus, Trivandrum 695011, India;
5Univ de Bordeaux, INSERM U869, IECB, ARNA laboratory. 2 Rue Robert Escarpit, 33607 Pessac, France;
6School of Materials Science and Engineering, Clemson University, Clemson SC 29634, USA;
† & # contributed equally to the work.
Funding: This work was funded by Department of Science and Technology (SR/SO/HS-51-2007), Department of Biotechnology (BT/PR10024/AGR/36/28/2007,) Ministry of Science and Technology, Government of India, CSIR 12th FYP- BSC 103 to L. Dinesh Kumar & group, the Australian Research Council (ARC), the National Health & Medical Research Council (NHMRC) of Australia and the National Science Foundation (CBET-0756457) as well as ANR P-NANO and F-DNA, the Conseil Régional d'Aquitaine and Association pour la recherche sur le Cancer (ARC) to S. Iyer & group. A.R. Clarke & group supported by CR-UK, the Welsh Government and Tenovus.

Corresponding Authors:

Dr. Lekha Dinesh Kumar  
Principal Scientist, E112  
Project Leader, Cancer Biology  
Centre for Cellular & Molecular Biology  
Hyderabad, A.P 500007  
Ph: +91-40-27192933,2576, Fax: +91-40-27160591, email: lekha@ccmb.res.in

Prof. Swaminathan Iyer  
ARC Future Fellow, Group Leader, BioNano UWA  
School of Chemistry & Biochemistry, Mailbag M310, Faculty of Sciences, The University of Western Australia, 35 Stirling Highway Crawley WESTERN AUSTRALIA 6009, T (+61 8) 6488 4470, F (+61 8) 6488 7330, E swaminatha.iyer@uwa.edu.au

Professor Alan R Clarke,  
Director, European Cancer Stem Cell Research Institute, (ECSCRI)  
Director, Cardiff CR-UK Centre,  
Head of Research, Cardiff School of Biosciences,  
Cardiff University, Hadyn Ellis Building  
Maindy Road, Cathays, Cardiff, UK, CF24 4HQ  
Ph: +44 (0)29 2087 4609, Fax: +44 (0)29 2087 4116, email: ClarkeAR@cf.ac.uk

Running Title: Suppression of breast and colon cancer models using RNAi
Keywords: Multimodal nanoparticles, RNAi, shRNA, colorectal cancer, breast cancer

Competing interests: The authors declare no competing financial interest.

No. of words
Abstract: 214
Main text: 5087
No. of main figures: 5
No. of main tables: Nil
Supplementary Figures: 6
Supplementary Tables: 2

List of abbreviations

Abbreviations
NPs - Nano Particles
SQUID - superconducting quantum interference device
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RES - Reticuloendothelial system
FTIR - Fourier Transform Infrared spectroscopy
FACS - Fluorescence-activated cell sorting
MRI - Magnetic resonance imaging
qPCR - quantitative Polymerase chain reaction
RhB-PGMA - Rhodamine B linked polyglycidal methacrylate
MGB - Minor groove binder
NFQ - non-fluorescent quencher
IHC - Immunohistochemistry
TEM - Transmission Electron Microscopy
ANOVA - Analysis of Variance
DNA - Deoxyribo nucleic acid
RNA - Ribo nucleic acid
PBS - Phosphate Buffer Saline
DMSO - Dimethyl sulfoxide
Fe$_3$O$_4$ - Iron(III) oxide or ferric oxide
ICC - Immunocytochemistry
Abstract: In this article we report the development and pre-clinical validation of combinatorial therapy for treatment of cancers using RNA interference (RNAi). RNAi technology is an attractive approach to silence genes responsible for disease onset and progression. Currently the critical challenge facing the clinical success of RNAi technology is in the difficulty of delivery of RNAi inducers, due to low transfection efficiency, difficulties of integration into host DNA and unstable expression. Using the macromolecule polyglycidal methacrylate (PGMA) as a platform to graft multiple polyethylenimine (PEI) chains, we demonstrate effective delivery of small oligos (antimiRs & mimics) and larger DNAs (encoding shRNAs) in a wide variety of cancer cell lines by successful silencing/activation of their respective target genes. Furthermore, the effectiveness of this therapy was validated for in vivo tumour suppression using 2 transgenic mouse models; firstly, tumour growth arrest and increased animal survival was seen in mice bearing Brca2/p53-mutant mammary tumours following daily intra-tumoural treatment with nanoparticles conjugated to c-Myc shRNA. Secondly, oral delivery of the conjugate to an Apc-deficient crypt progenitor colon cancer model increased animal survival and returned intestinal tissue to a non-wnt-deregulated state. This study demonstrates through careful design of non-viral NPs and appropriate selection of therapeutic gene targets, that RNAi technology can be made an affordable and amenable therapy for cancer.
Introduction

Cancer is a major global health problem and is a leading cause of death both in developed and developing countries. Most commonly practiced therapies in patients target cancer cells by the use of non-specific and non-selective treatments that cause significant off target effects. In particular chemotherapy is the preferred clinical method for cancer treatment since the 1940s, despite its many side effects. Biological therapies are in limited use in augmenting standard therapies, but this approach has thus far not been effective enough to replace the traditional methods of chemotherapy in the majority of cancers. The shortcomings in efficacy and safety associated with the current treatment regimens emphasize the need for highly specific and targeted alternative therapies which could maximize patient survival and minimize the limitations of the existing strategies.

The discovery of the c-Myc gene has changed our fundamental understanding of malignancy and it is now well established that c-Myc is deregulated in the majority of cancers (1). Mechanistically, the basis of this association appears to stem from the plethora of roles played by c-Myc in regulating cell death, growth and proliferation, as well as in regulating important biochemical functions such as the uptake and metabolism of cellular glucose (2). c-Myc therefore appears to act as a cellular master switch, controlling a network of oncogenes and tumour suppressor genes through a variety of signal transduction pathway molecules. Mutations in c-Myc or gross changes in its expression levels, clearly have the potential to derail the otherwise precise checkpoints that maintain cells in their normal quiescent state and lead to the development of cancer. Therapeutically, approaches that would allow the reprogramming and retuning of c-Myc activity within cancer cells are attractive strategies for disease control and prevention (3). One such approach is the use of RNA interference (RNAi) technology. Recent
breakthroughs in RNAi technology have vastly improved our understanding of how gene expression can be specifically modulated and can be employed as a potential therapeutic tool (4-7). Harnessing small RNA molecules to silence genes involved in the development and growth of cancer cells is an important step forward in developing a new and target-focused cancer therapy (8). The biggest challenge to the use of RNAi technology lies in achieving effective intracellular delivery of therapeutic molecules, in the form of either short interfering RNA (siRNA) or short hairpin RNA (shRNA) (9). Recently, spherical nucleic acid-gold nanoparticle conjugates were shown to cross the blood brain barrier and induce apoptosis in glioma cells, thereby reducing tumour burden (10). While the pre-formed 21 base siRNA duplexes are highly unstable, with a transient period of expression, shRNAs are more robust and better suited for long term effectiveness, due to their ability to produce siRNAs continuously within the cell and hence bring about prolonged knock-down of the target genes. Although a shRNA based approach would be ideal for cancer-related therapeutic development, they have thus far only been delivered effectively in vivo using viral vectors. However, with viral-based delivery it is important to bear in mind their potential immunogenicity and the risk of becoming pathogenic due to mutations. The idea of using a non-viral agent stems from its ability to mimic a viral function to infect cells, while avoiding the dangers of virus-associated pathogenesis. The use of non-viral transfecting agents has been proposed for several years because they are theoretically safer and easier to produce (11). However, their clinical success has been limited in the delivery of shRNA due to low transfection efficiency, difficulties of integration and unstable expression.

Currently, polyethyleneimine (PEI) is the most widely used non-viral agent and is considered the gold standard in vitro (12). The major drawback of PEI as a transfecting agent is its cytotoxicity, and the observed correlation between increased transfection efficacy with increased molecular
weight and concentration of PEI (13-17) makes it a difficult proposition to improve efficiency without its deleterious effect on cells. We have previously demonstrated that anchoring multiple PEI chains to PGMA nanoparticles can dramatically decrease their toxicity even at higher concentrations, whilst achieving efficient nanoparticle endocytosis (16). In the current study, various types of nucleic acids, designed to target cancer-related genes, were tested in different cancer cell lines after conjugation to these nanoparticles. shRNAs of c-Myc oncogene were selected to test the in vivo efficacy of this biological drug complex in targeting knock down of the oncogene and related signaling cascades, thus triggering suppression of growth in established mouse models of colorectal and breast cancer.

**Materials and Methods**

**Preparation and characterization of the PEI-PGMA NPs**

The PEI-PGMA NPs were prepared following the procedure outlined by Evans *et al* (16) with the magnetic cores synthesized as per the procedure outlined by sun *et. al* (18). The NPs were characterized using TEM (JOEL 2100) at 120kV, Dynamic light scattering for size and surface charge, and fluorescence spectrometry. Nitrogen concentration was obtained using elemental analysis of the NPs before and after PEI attachment. Nitrogen concentration was determined to be 765μM at a NP concentration of 1mg/ml. The NPs were superparamagnetic as determined by SQUID magnetometry, as there was no hysteresis at 300K (with specific saturation magnetization of 6 emu g⁻¹), and the relaxivity r² of the NP was determined, based on the iron content inside the polymer, to be 340 s⁻¹ mM⁻¹ Fe. The NPs were centrifuged (16000g, 30 min), the supernatant was removed and the NP pellet was resuspended in 20mM HEPES buffer containing 150mM NaCl. They were then sterilized under UV for 20 min and mixed with DNA in different ratios and incubated at room temperature for 45min. To assess optimal binding, a gel
retardation assay was performed for the different ratios (W/V) of PEI-PGMA (NPs) with short oligos and plasmid DNA (D) (N: D ratio). It was determined that the ratio of (N:D) 4:1 and 25:1 were optimal for binding oligos and plasmid DNA, respectively. The appropriate ratios (N:D) were determined as 4:1 for oligos and 25:1 for larger DNA molecules. This ratio was maintained throughout the study.

**In vitro transfection studies**

Adherent HEK293 (Human Embryonic Kidney), MCF7 and MDA-MB 231 (Breast cancer), semi-adherent COLO205 (colon cancer) and non-adherent Jurkat (Leukemia) cell lines were obtained from ATCC (American Type Culture Collections). The cells were 4 to 6 months old at the time of experimentation and mycoplasma testing (Look out mycoplasma PCR detection kit, Sigma) was performed regularly and assured that these cell lines are free of mycoplasma. DNA constructs used were GIPZ c-Myc shRNA Transfection Starter Kit (RHS4287-EG17869) and GIPZ non-silencing shRNA (RHS4346), which was used as negative control to rule out off-target effects (Open Biosystems, USA). In the case of the shRNAs, the best out of the four which showed maximum knock down was selected for *in vivo* experiments. Mimics and antimiRs were purchased from Exiqon, Denmark. Cells were seeded in complete media (RPMI for Jurkat and DMEM for all other cell lines, with 10% FBS) in 24 well plates in triplicates at a density of 2 × 10^5 cells/ml for all transfection studies. After 12-24 h, 50-60% confluent cells were transfected with DNA/RNA NP complex in the above stated ratios, depending on the types of DNA/RNA used. The complexes were incubated at 37°C for 4h (small oligos) and 6 h (for larger plasmids), replaced with fresh complete media and incubated in a CO2-regulated incubator for another 48 or 72 h for q-PCR or immunoblot analysis respectively. The transfection with lipofectamine 2000
(Invitrogen, USA) was performed in an identical manner to that of the NPs. All the experiments were repeated a minimum of 3 times for statistical analysis.

For immunocytochemistry, cells were seeded on a cover slip in triplicates at the rate of $2 \times 10^5$ cells/ml and the transfected cells were fixed with 4% formaldehyde diluted in phosphate-buffered saline (PBS). Fixed cells were permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min. After blocking with 10% normal goat serum in PBS for 1 h, cells were incubated overnight at 4°C with either the rabbit monoclonal anti-p53, anti-c-Myc (Millipore, USA) or anti-GAPDH antibodies (Calbiochem, USA) at 1:200 dilution. These were then washed four times with PBS for 10 min each. The cells were subsequently incubated with appropriate secondary antibody conjugated with alexa 633,488 (Invitrogen, USA) at 1:400 dilutions for 1 h at room temperature. Following a further set of four 10 min washes with PBS, the cells were mounted on DAPI mounting media containing antifade (Vectashield, Vector lab, USA). Confocal images were obtained on Leica laser scanning microscope at 63x. Transfection efficiency was performed in triplicates and calculated. One hundred cells were counted per field of view for a total of 4 fields per experiment, per treatment. All experiments were repeated 3-5 times and data transformed using arcsine transformation method before statistical analysis.

RNA isolation and Quantitative Real Time PCR analysis

At the end of each time point, media was removed and cells were scraped off in TRIzol reagent (Invitrogen, USA). RNA from homogenized tissue samples as well as cell lysates were isolated using RNAeasy kit (Qiagen, USA) and RNA concentrations were measured using nanodrop spectrometer (Thermo-scientific). A 260nm/280nm absorption ratio of 2.0 confirmed the RNA to be pure and protein free. The quality of RNA was also checked using 1% agarose gel electrophoresis. One microgram of RNA was transcribed to cDNA using random primers and
further used for both SYBR green assay and Taqman gene expression assay of all the genes. Total RNA was DNase-treated and one microgram RNA was transcribed to cDNA using RT-PCR Reagents kit (Applied Biosystems). Quantitative PCR analysis was performed using SYBR® Green PCR master mix following standard MIQE protocols as well as TaqMan® Gene Expression Assays (Applied Biosystems), following the manufacturer's instructions, and the reactions were carried out in a 7900HT Thermo cycler (Applied Biosystems). TaqMan® Gene Expression Assays included a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ dye label on the 5’ end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3’ end. 18S rRNA, β-actin and nono genes were used as endogenous controls to normalize the data (Supplementary Table S1). The fold expressions of particular genes are represented as $\log_{10} 2^{\Delta\Delta CT}$. Real Time StatMiner® Software was used for data analysis. This software performs parametric, non-parametric and paired tests for relative quantification of gene expression, as well as a 2-way ANOVA for two-factor differential expression analysis.

**Immunoblot analysis**

Total cellular protein from all cell lines transfected with respective miRNA mimics/knock down and shRNAs was extracted after 72hrs using lysis buffer containing 1x protease inhibitor cocktail (Calbiochem USA). Protein concentrations were measured by the Bradford (Sigma) method and equal amounts (~25-30 μg) of cellular extract/lysate were separated on 10-12% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk for 1h at room temperature, before incubation with appropriate primary antibodies for c-Myc, β-catenin, GAPDH, p53 or β-actin (1:3000) overnight at 4°C. Membranes were then incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody for 1 h, washed and signal visualized with ECL plus reagent.
following exposure onto X-ray film. All human and mouse primary antibodies used were raised in rabbit with a dilution of 1:4000 for all the westerns and 1:50 for ICC. The secondary antibodies were raised in mouse/donkey with a dilution factor of 1:10,000 for western and 1:200 for ICC.

**In vivo studies**

All animal models were used strictly in accordance with the animal ethical committees of the participating institutes, CCMB and Cardiff University, and were housed in the transgenic facilities of the respective institutes. Two types of animal models were used in the study. The breast cancer model (conditional *Brca2*/p53 knockout under control of *Blg-cre* transgene) develops autochthonous tumours on any of the 5 pairs of mammary glands in a 6-15 month time window. PCR conditions for genotyping of the *Blg-cre* transgene and the conditional alleles for *Brca2* and *p53* have already been described (19). Mammary tumours were measured at 0.5cm$^3$ before starting treatment and all tumours in each cohort were treated identically by giving intratumoral injections of 10µg of c-Myc DNA (GIPZ c-Myc shRNA-vector vehicle, Open Biosystems, USA) complexed with 250µg of NPs (PEI-PGMA) (in a volume of 100 µl) along with the control cohorts (c-Myc shRNA alone, scrambled + NPs, NPs alone, untreated). All tumours of a single mouse were given identical treatment and tumours of similar size were taken into consideration while transforming the data. The *Apc* knockout model (*AhCre-ErT Apc$^{fl/fl}$*) is an inducible colorectal cancer model which develops a c-Myc-dependent “crypt progenitor” phenotype in the intestine upon injection with β-naphthoflavone and tamoxifen, causing death within a few days of induction (20). The *cre* and *Apc* alleles were brought together by extensive breeding and genotyping performed using the primers stated in Supplementary Table S2. 6-8 week old mice were IP-injected with 80mg/kg β-naphthoflavone and tamoxifen (dissolved
together in corn oil at 10mg/ml each) once daily for 5 days to cause recombination of the targeted alleles. Apc knockout mice were treated with a daily oral dose of 50μg c-Myc shRNA (in pGIPZ vector)-encoding plasmid DNA complexed to 1.25mg NPs (in a total volume of 250 μl ) from day 1 of induction until the end time point as described above. WT refers to healthy wild type mice (C57/BL6) where control tissues were harvested for comparison with treated mice in all experiments.

**Fourier Transform Infrared Spectroscopy (FTIR)**

To analyze the toxicity of the nanoparticles **in vivo**, mice were divided into 4 groups consisting of 5 mice each. The different cohorts received 0, 250, 500 or 1000μg daily dose of NPs by oral gavage for a period of 21 days in this sub-chronic toxicity study. The control cohort (no NPs) was treated with just buffer. Following treatment, animals were sacrificed as per the ethics guidelines and tissues (liver, kidney, lung, spleen) were removed and immediately frozen in liquid nitrogen. They were freeze-dried using a Labconco system (Missouri, USA) and crushed in aseptic conditions. The freeze dried pellets were analyzed for the characteristic peaks that represent intactness of macromolecular organization in the organs. Scanning was performed using a Bruker Vertex-70 FTIR spectrometer, with the ATR mode in the range of 400-4000 cm$^{-1}$ to obtain the characteristic peaks.

**In vitro Cytotoxicity assay of PEI-PGMA-NPs**

Adherent (HEK293, MCF7 and MDA-MB 231), semi-adherent (COLO205) and non-adherent (Jurkat) cells were obtained from ATCC (American Type Culture Collections) and maintained in the recommended growth media. **In vitro** cytotoxicity of PEI-PGMA-NPs was analyzed by MTT assay. Briefly, cells were seeded in 96-well plate at a concentration of $2 \times 10^4$ cells/well in 100μl volume, and incubated O/N at 37°C in a humidified atmosphere containing 5% CO$_2$. Cells were
treated with predetermined concentrations (0–300 µg/ml) PEI-PGMA-NP diluted appropriately with culture media in triplicates and grown as above for 48h. After the treatment, media containing PEI-PGMA-NP were carefully removed by aspiration. 100 µl of 0.4mg/ml MTT in PBS was added to each well and incubated in the dark for 4 h. After the incubation period, formazan crystals were solubilized by the addition of 100µl of DMSO to each well and kept in an incubator for 45 min. Amounts of formazan were determined by measuring the absorbance at 540 nm. The data were presented as percentage post treatment recovery (% live cells), whereas the absorbance from non-treated control cells was defined as 100% live cells. The percentage recovery (% live cells) was plotted against the concentration of PEI-NP. Cells grown in normal growth medium without treatment served as control. Measurements were made in triplicate, the experiments were repeated thrice with 3 biological replicates. The signal to background ratio was calculated using the formula S/B = mean signal/mean background. Student’s one tailed test was used to determine the statistical significance of the treated vs untreated cells and the error bars represent standard deviation.

Statistical analysis

The data of percentage cells transfected was subjected to arsine transformation, as the values ranged from 4-98% and the transformed data was subjected to statistical analysis as per the factorial completely randomized design, with cell type taken as one factor and transfection agent type taken as the other. In vivo data obtained are shown as mean values ± s.e.m. Significant difference between means were performed using two tailed students t-test. N is the number of independent animals used in all cases with p<0.05.

Results

Cytotoxicity and Bio-distribution studies did not show NP toxicity effects in vitro or in vivo
The ability of a non-viral transfection agent (Fig. 1A and Supplementary Fig. S1) to bind plasmid DNA, and in turn induce RNAi efficiently, is primarily governed by its ability to successfully bind nucleic acids (RNAs, small oligos and plasmid DNA encoding shRNAs) at high loading concentrations. Using gel retardation assays, the ratios \([\text{PEI-PGMA(N): DNA(D)}]\) of 4:1 and 25:1 (weight/volume (W/V)) were found to be optimum for binding oligos and plasmid DNAs respectively. These ratios were ideal for achieving high transfection efficiency in all types of cell lines (Fig. 1B). Once the binding ratios were optimized, cytotoxicity of the nanoparticles (NPs) was evaluated in 4 different cancer cell lines; MCF-7, MDA-MB-231 (both adherent), COLO-205 (semi-adherent) and Jurkat (non-adherent) along with the human cell line HEK-293 and mouse cell line NIH-3T3 as controls (Supplementary Fig. S2). This study ascertained that NP concentrations up to 300\(\mu\)g/ml did not induce any significant cytotoxicity on the cell lines investigated. A major concern of all nanoparticulate agents in translational research is their potential toxicity \(\textit{in vivo}\) and as a result it was important to carefully assess the bio-distribution and toxicity profile of our NPs \(\textit{in vivo}\) before validating therapeutic efficacy. The reticuloendothelial system (RES) is primarily involved in the identification, biodegradation and removal of foreign particulate matter in the body. A thorough investigation of the fate of the key organs involved in RES after treatment with the NPs was performed using standard techniques such as Fourier Transform Infrared spectroscopy (FTIR) and Liver function tests (21). Histological data obtained by H&E staining of control tissue (no NPs) compared with tissue from mice acutely treated for 6 weeks on weekdays with a dose of 1250 \(\mu\)g (or 50 mg/kg) NPs showed integrity in liver, spleen and kidney (Supplementary Fig. S3A). This was further supported by FTIR analysis of the respective organs following treatment with the same dose of the NPs (Supplementary Fig. S3B). Finally, liver function tests between
control and NP-treated mice showed no significant difference with respect to the enzymes alanine transaminase or alkaline phosphatase following prolonged treatment with 1250 μg NPs (Supplementary Fig. S3C).

**Efficient induction of RNA interference in vitro**

Achieving high levels of transfection using non-viral agents remains a challenge in most semi-adherent and non-adherent cell lines, due to the lack of syndecans, unlike in adherent cells (22). The majority of transfection reagents currently available for RNAi delivery show maximum efficiencies of 60-70% in normal adherent cell lines such as HEK293, and 20-30% in refractory cell lines such as NIH3T3 and cancer cell lines, including MDA-MB231 and MCF7. Semi-adherent cell lines (e.g. COLO 205) and non-adherent cell lines (e.g. Jurkat) continue to be difficult to successfully transfecy with commercial reagents (23). Electroporation is considered the only alternative to transfet semi-adherent and non-adherent cells, although with limited success and little potential for clinical translation. The ability of our NPs to transfet different types of cancer cell lines well below the toxicity level was assessed. To test the functional and practical utility of the NPs, small RNA and DNA molecules, along with larger plasmid shRNAs, were employed for silencing different target genes by RNAi. The recovery/knockdown of different target proteins was analyzed *in vitro* using different types of nucleic acids in combination with the NPs. miRNAs are 22-23nt RNA molecules which predominantly bind to the 3'-UTR of the target mRNA, resulting in knock down of target gene expression (24). To test the ability of the NPs to efficiently deliver miRNAs, transfetion studies were done using various mimics and knockdown probes of human microRNAs (miR-200c, miR-105, miR-432*, miR-659, miR-662, miR-921 and antimiR-21) in different cell lines. Efficient modulation of their corresponding target genes (p53, β-catenin and caspase3) was observed using
immuno-blotting (Fig. 2A and Supplementary Fig. S4). The ability of our NPs to transfect a large DNA construct was tested by using a pGIPZ vector encoding shRNA against the ubiquitous GAPDH gene as a positive control and scrambled shRNA as negative control. Successful transfection of the cells was indicated by the knock down of the target gene in all three cell lines investigated (Fig. 2B). Similarly, knockdown of the target oncogene c-Myc was observed when pGIPZ vector coding for c-Myc shRNA was used, demonstrating the effective knockdown of the target c-Myc oncogene as verified by q-PCR, immuno-blotting and immunocytochemistry (Fig. 2C).

**Suppression of autochthonous mammary tumours**

In the *Blg-cre Brca2/p53* conditional knockout breast cancer model, mice develop spontaneous mammary tumours between 6 and 15 months of age (Fig. 3A). Injections to the periphery of tumours were carried out using c-Myc shRNA (pGIPZ vector) complexed with PEI-PGMA (25:1, N:D) in a cohort of 6 mice, along with appropriate control cohorts (n=6), to test the penetrability and distribution of the NPs in the tumour and the efficacy of treatment (Fig. 3B). Tumours treated with shRNA bound to the NPs showed a markedly reduced tumour growth rate compared to controls (Fig. 3C). The median survival of the cohort treated with c-Myc shRNA-NP complex was over 30 days, compared to control cohorts that had median survivals of between 10 and 14 days (Fig. 3D). A concomitant 30-fold reduction in c-Myc levels was confirmed by q-PCR, immuno-blotting analysis and immunohistochemistry for the c-Myc shRNA bound to the NP treatment group in comparison to both wild type and untreated cohorts (Fig. 3E-G). Importantly, although injection was done at the tumour periphery, equal distribution of NPs was observed throughout the tumour core and extended periphery by magnetic resonance imaging (MRI) and fluorescent confocal analysis, confirming effective...
penetration of these complexes throughout the tumour 24 hours post injection (Supplementary Fig. S5A & B). In addition, no trace of the NPs was observed in off-target organs even at the end of treatment, as assessed by fluorescence (Supplementary Fig. S5C and D).

Suppression of colorectal cancer model

In our colorectal cancer model, induction of \( Apc \) loss by tamoxifen and \( \beta \)-naphthoflavone injection results in the development of a widespread, aggressive c-Myc-dependent ‘crypt progenitor’ phenotype. This mimics early tumour development along the entire length of the small intestine and results in the death of the animals within 7 to 10 days. We hypothesized that the high buffering capacity of the multiple PEI chains on the NPs would render significant protection to the shRNA from the harsh environment of the gastrointestinal tract. Additionally, the adhesive property of the cationic polymer with the proteoglycan-coated proteins, coupled with the ability of PEI to effect endocytosis, would result in significant transfection in the distal large intestine. Therapy was orally administered to a cohort of 6 mice, and the response was again compared with appropriate control cohorts (n=6). Confocal imaging tracked the presence of the NPs all along the intestinal tract, with higher doses localized within the distal large intestine and comparatively only trace amounts observed in the small intestine and proximal large intestine (Fig. 4A). Despite the disproportionate presence of the NPs in these tissues, effective c-Myc transcript and protein level reduction was observed by both q-PCR and western blotting across these major components of the gastrointestinal tract (Fig. 4C). The high levels of NPs present in the large intestine is most likely a result of the timing of tissue collection and represents a snapshot of their presence in the latter stages of the normal digestive process. The cohort of animals which received oral treatment with the shRNA conjugated to the NPs showed a remarkable increase in survival (median survival greater than
40 days) when compared to control cohorts, where median survival was less than 15 days in all cases (Fig. 4B). Immunohistochemical analysis at various time points throughout the treatment reflected a progressive reduction in the expression levels of the c-Myc protein (Fig. 4D). Additionally, re-localization of \( \beta \)-catenin from nucleus to cytoplasm was observed during treatment, indicating a return to a non-wnt-deregulated state (Fig. 5A). Site triggering of the NPs in the distal large intestine was shown by significant expression of turbo-FP co-localized with the NPs as assessed by confocal analysis (Fig. 5B), as well as \textit{ex vivo} multispectral imaging of the entire gut of the mouse following oral delivery (Fig. 5C). Protein ratios were calculated based on densitometric quantification and were carried out using the GeneTools program (SynGene), with \( \beta \)-actin used for normalization (Supplementary Fig. S6).

**Discussion**

Cancer therapy has been moving through a slow process of development, hitherto hindered by limitations in drug targeting technologies. The focus on cancer drug development has shifted from toxic and non-specific chemotherapeutic drugs to non-toxic and target-specific biological drugs with reduced side effects. The discovery of RNAi technology has significantly enhanced our understanding of how gene expression can be modulated as a potential therapeutic tool and high throughput screening method for targets against many cancers, although delivery of such therapy to autochthonous tumors still remains largely elusive (25). The efficiency of RNAi depends on the mode of delivery to the target, especially for diseases such as cancer. Solid tumors, such as those of the intestine, pancreas and liver, are difficult to treat at their natural place of origin. The extravascular tumour tissue often has a limited blood supply that may render the core necrotic tissue inaccessible for drug penetration following intravenous administration,
thus leading to sub-optimal treatment and potential relapse (26). In this report, through both \textit{in vitro} and \textit{in vivo} studies, we have demonstrated the effective use of a simple non-viral nanoparticle, at a non-cytotoxic level, for the targeted delivery of nucleic acid molecules as biological drug cargos into different cancer tissues.

The successful transfection of the nanoparticles presented in this study is the result of PEI’s ability to associate with membrane proteins, which in turn results in endocytosis of the PEI-conjugated nanoparticles. The propensity of PEI to act as a “proton sponge” allows for endosomal escape of its incorporated polyplexes. Following endocytosis, natural acidification within the endosome protonates PEI, inducing chloride ion influx, osmotic swelling and destabilization of the vesicle, leading to the release of the polyplex into the cytoplasm. Grafting multiple PEI chains on to a macromolecular core significantly enhanced the NPs capability to transfect, protect and deliver DNA/RNA molecules. The glycidyl methacrylate units, located in the “loops” of the PGMA core with multiple free epoxy groups, serve as reactive sites for the subsequent attachment of the PEI sub-units. The major difference between this method and the traditional method of anchoring PEI to the surface of a nanoparticle lies in the mobility of the epoxy functional groups located on the PGMA core. This increased mobility ensures better access to the epoxide functional groups, resulting in a 2-3 fold greater grafting density when compared to a monolayer of epoxy groups on a nanoparticle surface of similar dimension (27). Furthermore, the emulsification method used for the synthesis of the nanoparticle core allows for the encapsulation of magnetite (Fe$_3$O$_4$) nanoparticles within the core. The efficacy of our nanoparticle formulation was demonstrated as a non-viral agent through the high density covalent binding of PEI onto a rhodamine B linked polyglycidal methacrylate (RhB-PGMA) reactive nanoparticle core. A polymer with epoxy functionality was chosen as the core, since the
reactions of epoxy groups are quite universal, affording ease of attachment of the amino functionalized PEI and carboxylic functionalized RhB. In addition, the epoxy groups of the polymer can cross-link to provide structural integrity to the core. This renders the NPs as multimodal, with rhodamine B allowing for fluorescence imaging and magnetite for MRI contrast, thus providing suitability for use in both \textit{in vitro} and \textit{in vivo} studies.

Our \textit{in vitro} studies showed that nucleic acids conjugated to NPs enhanced transfection efficiency and knockdown or recovery of various oncogenes and tumour suppressor genes in a number of cell lines. Using our NPs, microRNAs, anti-miRs (in the form of short oligos) and larger plasmid DNAs encoding shRNAs against targeted oncogenes/tumour suppressor genes were transfected into different cancer lines at high levels of efficiency, similar to those observed with viral agents. RNAi effects of shRNAs, antimiRs and mimics were evident with the efficient knock down and modulation of their respective targets. PEI-PGMA can act as a proton sponge which delays acidification and fusion with lysosome osmotic swelling and finally the rupture of some of the endosomes will in turn allow the escape of these NP-DNA complexes into the cytosol (28).

Following the demonstration of effective transfection \textit{in vitro}, we next tested the ability of c-Myc shRNA (pGIPZ) bound with NPs to suppress tumours \textit{in vivo} using an established murine transgenic \textit{Brca2/p53-mутант} breast cancer model (19). Unlike xenografted tumour models, solid autochthonous tumours are relatively inaccessible for most treatment regimes and frequently pose challenges due to their inherent site of origin and difficulties with delivery to tumour cores. In addition, transgenic models mirror the actual mechanism of tumour progression in humans, enabling insight into the loss or gain of function of genes at specific stages of tumour growth. \textit{In vivo} in site delivery of shRNAs directly within mammary
tumours enabled targeting of the extravascular tumour necrotic core, facilitating efficient knockdown of c-Myc. This genetic knockdown of a specific oncogene suppressed tumour growth effectively, thereby increasing survival, and also prevented off target silencing in other organs. Oral delivery to mice deficient for the Apc gene within the intestine, allowed the encapsulated bio-therapeutic to be specifically delivered to tumour-like cells therein, thus protecting it from degradation under the harsh conditions of the stomach. Again, this allowed the drug to accumulate at the right therapeutic concentration, thereby triggering the inhibition of neoplastic spread. The persistent and efficient knockdown brought about by c-Myc shRNA returned the small intestine to a near normal state, the effect of which was translated as markedly increased survival in the treatment cohort. This anti-cancer drug cargo thus promises an efficient bio-therapeutic regime for currently undruggable targets at problematic sites without obvious cytotoxicity. This observation has great implications for the treatment of solid tumours at their natural site of origin. A key factor that will contribute towards the successful translation of this platform will be the development of a robust, scalable production of the nanoparticle formulations in a good manufacturing practice facility, with further control over particle size distribution as described in previous polymer formulation reports (29). In the present case, we believe this is achievable by fine-tuning the emulsion polymerization process. Although we demonstrate a proof of principle, our approach would be further enhanced by the development of moieties which would enable targeted, site specific delivery so as to avoid off-target effects.

In conclusion, we have demonstrated that we can use macromolecular grafting approaches to design efficient non-viral formulations that have in vivo capacity to deliver long term effective RNAi therapy against cancer. Furthermore, this work also demonstrates in-site delivery of a
biological drug, for effective accumulation to therapeutic levels, the most desirable and preferred method for translation from bench to the bedside in cancer therapy.

**Acknowledgments:** The authors acknowledge the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterization & Analysis, and The University of Western Australia, funded by the University, State and Commonwealth Governments. We acknowledge Dr. Dinesh Kumar for help with statistical analysis and critical evaluation of the manuscript, Abdul Rawoof for helping with formatting text and figures, Prof. J.L. Mergny and Velumani Selvaraj for editing the manuscript, N.Mahesh Babu and Avinash Raj for their help in tissue processing, IHC and animal handling during toxicity studies, G.Srinivas for flow cytometry analysis, Dr. E.R. Prasad and Ch. Kiran for their assistance in western blotting analysis.

**Data and materials availability:** All reasonable requests for collaboration involving materials used in the research will be fulfilled provided that a written agreement is executed in advance between the requester (and his or her affiliated institution) and the Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, India; The University of Western Australia, Australia and Cardiff University, UK.
References


11. Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene


Legends for Figures

Figure 1. Comparison of transfection efficiencies of lipofectamine and PEI-PGMA NPs in adherent, semi-adherent and non-adherent cell lines. (A) Schematic representation of the multimodal NPs formed by covalently binding multiple PEI chains on a macromolecular PGMA-RhB modified core. (B) Representative confocal merged images of various cell lines transfected with the plasmid DNA vector encoding GFP reporter gene using lipofectamine or NP as transfection agent. GFP expression is shown in green in the lipofectamine panel and rhodamine fluorescence from the vector in red in the NP panel. Note: Rhodamine from NPs and not GFP is chosen to demonstrate that every cell in the field of view is transfected with the macromolecular vector. Cell nuclei are visualized with DAPI (blue) in all images.

Figure 2. Silencing of target genes using RNAi. (A) Up-regulation of p53 using anti-miR-21 oligos linked to the rhodamine labelled macromolecular vector. RNAi effect of miR-21 is demonstrated by up-regulation of p53 mRNA by q-PCR and protein by immunofluorescence and immunoblotting respectively in HEK293 and MDA-MB-231 cell lines, as compared to untreated control cell lines or scrambled shRNA treated controls. Similarly, knockdown of β-catenin by mimics of miR-200c and miR-105 is demonstrated by immunoblotting in Jurkat cell lines. (B) Down-regulation of GAPDH using GAPDH shRNA linked to the NPs. RNAi effect of delivered shRNA on GAPDH expression is demonstrated by q-PCR, immunofluorescence and immunoblotting in HEK293, MDA-MB-231 and Jurkat cell lines as compared to controls. (C) Knock down of the functional oncogene c-Myc using c-Myc shRNA linked to the macromolecular vector. RNAi effect of delivered shRNA on c-Myc is demonstrated by q-PCR, immunofluorescence and immunoblotting in HEK293, MDA-MB-231 and Jurkat cell lines.
231 and Jurkat cell lines as compared to controls (untreated or scrambled shRNA treated). β-actin was used as a loading control in all experiments. All q-PCR experiments were conducted in triplicates, each experiment having 3 biological replicates and 2 technical replicates, as assessed by fluorescence. The relative quantitation (RQ) of expression of the respective genes in the treated cells in relation to their respective controls is presented here. All controls were without treatment of macromolecular vector or by treating with scrambled (scr) shRNA. Representative confocal images show the Rhodamine B labeled macromolecular vector (red), nuclei labeled with DAPI (blue), p-53, c-Myc and GAPDH labeled with anti-p53, anti-c-Myc (green, Alexaflour 488) and anti GAPDH (yellow) respectively. All images were captured at 10μm scale.

Figure 3. Suppression of autochthonous mammary tumours by in site delivery of nanoparticles to a mouse model of breast cancer (Brca2/p53 knockout). (A) Example of mammary tumour regression in conditional Brca2/p53 knockout mouse model upon treatment with c-Myc shRNA complexed with NPs (B) For the purpose of live multispectral imaging, 4 similarly-sized mammary tumours which appeared within a span of 10 days on the same mouse, were treated individually for a period of 25 days, resulting in suppression of tumours treated with c-Myc shRNA linked-nanoparticles (3rd and 5th mammary) compared to continued growth of untreated tumour (1st mammary) and tumour treated with scrambled shRNA-linked NPs (4th mammary). The red/yellow colour in the tumour core vs periphery shows the intensity of the rhodamine expression as shown in the intensity scale. Suppression of tumour growth (C) and increased survival (D) in mice treated with c-Myc shRNA-NPs, as compared to control cohorts. (E-G) Reduction in c-Myc transcript and protein expression
levels in tumours from the treated cohorts compared to wild type tissue and tumours from untreated mice as shown by RT-PCR (RQ) using TaqMan gene expression assays, western blotting and immunohistochemistry respectively.

**Figure 4. Non-viral nanoparticle is suitable for oral delivery of shRNA to a mouse model of colorectal cancer (Apc knockout).** (A) Confocal imaging of the fluorescent nanoparticles in Apc-deficient gut, following oral administration of 50µg c-Myc shRNA-encoding plasmid DNA complexed with 1250µg nanoparticles, showing maximum retention of complex in the distal end of the large intestine. (B) Prolonged survival (up to 47 days) of mice treated with c-Myc shRNA-NP complex as compared to other treated cohorts (scrambled shRNA + NPs, shRNA alone, NPs alone and induced but untreated mice (control)). Wild type was taken as control tissue for all analyses. (C) Decreased c-Myc transcript and protein levels on 12th, 32nd and 47th days post-treatment, as demonstrated by RT-PCR using TaqMan gene expression assays and western blotting respectively. Mice were healthy but culled at these time-points specifically to analyse expression. (D) Immunohistochemistry for c-Myc protein in treated cohort compared to other treated groups and control cohort (as described in panel B), again demonstrating gradual reduction in c-Myc protein levels during treatment.

**Figure 5. IHC analysis, reporter gene assay and bio-distribution studies on mice deleted for Apc in the intestine.** (A) β-catenin staining in samples from untreated wild type, induced and untreated, or induced and NP:c-Myc DNA complex-treated apc-deficient small intestines at various time-points. The gut rolls were processed as described in the methods section. Marked circles denote recombined (apc-deficient) areas showing dark nuclear staining (control) or fading
nuclear stain with higher cytoplasmic staining in treated mice culled at different time-points (up to 47 days). (B) Cryo-sectioning and confocal imaging of *apc*-deficient small intestine after oral delivery of the NP:c-Myc DNA complex. The gut roll was made as described in methods and immediately frozen using embedding media in dry ice for cryo-sectioning. These frozen gut rolls were sectioned (4-5μm) at constant temperature (-20°C) in a Freezing Microtome and visualized using confocal microscopy at 63x. The fluorescence of rhodamine B linked to the vector (red) and turbo-FP (reporter gene) expression (yellow) was localized within the cytoplasm, the nucleus was stained with DAPI (blue). (C) *In vivo* multispectral imaging was performed to analyse the bio-distribution of orally-delivered NPs in mice. After successive treatment for 15 days, mice were culled and the intact intestines were imaged under the multispectral imager using a rainbow filter. Comparison of the treated intestine (right panel) with untreated (left panel) shows a high level of distribution throughout the intestine.
Figure 2.
Figure 5.
Molecular Cancer Therapeutics

RNA Interference using c-Myc-Conjugated Nanoparticles Suppresses Breast and Colorectal Cancer Models


Mol Cancer Ther Published OnlineFirst February 18, 2015.