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Bioreducible polymeric nanoparticles containing multiplexed cancer stem cell-regulating miRNAs inhibit glioblastoma growth and prolong survival

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Supporting Information

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Abstract

Despite our growing molecular understanding of glioblastoma (GBM), treatment modalities remain limited. Recent developments in mechanisms of cell fate regulation and nanomedicine provide new avenues to treat and manage brain tumors via delivery of molecular therapeutics. Here we have developed bio-reducible poly(beta-amino ester) nanoparticles that demonstrate high intracellular delivery efficacy, low cytotoxicity, escape from endosomes, and promotion of cytosol-targeted environmentally-triggered cargo release for miRNA delivery to tumor-propagating human cancer stem cells. In this report, we combined this nanobiotechnology with newly discovered cancer stem cell inhibiting miRNAs to develop self-assembled miRNA-containing polymeric nanoparticles (nano-miRs) to treat gliomas. We show that these nano-miRs effectively intracellularly deliver single and combination miRNA mimics that inhibit the stem cell phenotype of human GBM cells *in vitro*. Following direct intratumoral infusion, these nano-miRs were found to distribute through the tumors, inhibit the growth of established orthotopic human GBM xenografts, and cooperatively enhance response to standard-of-care γ -radiation. Co-delivery of two miRNAs, miR-148a and miR-296-5p, within the bio-reducible nano-miR particles enabled long-term survival from GBM in mice.

Graphical Abstract

Keywords

miRNA; polymer; bio-reducible; brain cancer; cancer stem cell; nanomedicine

More than 50,000 new cases of malignant brain cancer are diagnosed in the U.S. each year with glioblastoma (GBM) being the most common and deadly form.¹ Despite aggressive treatment consisting of surgical resection and radiotherapy/chemotherapy, the median life expectancy for GBM patients is only 14–20 months, highlighting the need for new therapeutic approaches.² Treatment options for GBM remain limited in part due to tumor cell resistance to chemotherapy/radiation and the difficulty in delivering newer targeting therapeutics to the brain.^{3–4} GBMs are highly heterogeneous at the cellular level and contain cells that vary in their capacity to propagate tumor growth as revealed through single cell sequencing, RNA-profiling⁵ and studies of intra-tumoral evolution.⁶ Among these different cell subpopulations are multi-potent stem-like cells (also referred to as cancer stem cells or CSCs) that are critical determinants of tumor propagation, therapeutic resistance, and recurrence following treatment.⁷ Epigenetic mechanisms that support this stem-like tumor-propagating phenotype represent a vulnerability amenable to therapeutic targeting.⁸ Non-coding RNAs, in particular miRNAs, are emerging as critical epigenetic regulators of cell fate and oncogenesis.⁹ miRNAs selectively inhibit gene expression primarily by targeting

mRNA for degradation usually via complementary 3'-UTR seed sequences. Numerous miRNAs have been found to regulate tumorigenesis and cancer cell stemness by targeting tumor-suppressing or tumor promoting transcripts.¹⁰ We recently showed that the coordinated actions of Oct4 and Sox2 induce a CSC state in GBM cells through a mechanism that involves the down-regulation of a network of miRNAs through promoter DNA methylation.^{11–12} We further showed that the repression of two of these miRNAs, miR-148a and miR-296-5p, is required for the induction of GBM tumor propagating capacity by Oct4/Sox2 and that their reconstitution using viral expression vectors efficiently inhibits the GBM stem-like phenotype.^{11–12}

Therapeutically translating these advances in the molecular drivers of GBM stem cells remains a challenge.¹³ Viral gene delivery is promising but there remain potential limitations to clinical translation due to factors such as scalability, limited cargo size, and potential tumorigenic and immunogenic effects.^{14–15} Non-viral vectors such as polymeric nanoparticles can be designed to circumvent many of these problems, but traditional cationic polymers such as poly(L-lysine) (PLL) and polyethylenimine (PEI) that encapsulate nucleic acid cargoes into nanoparticles by electrostatically-driven self-assembly are generally ineffective for utilization *in vivo* and have been shown to be minimally effective for delivery of relatively small RNA molecules.¹⁶ Poly(beta-amino ester)s (PBAEs) are newer synthetic cationic polymers that promote superior gene delivery versus PEI, in part, as they contain hydrolytically-cleavable ester bonds, which reduces cytotoxicity as well as enhances cargo release.¹⁷ Like many gene delivery vehicles, PBAEs were first optimized for DNA delivery. Because RNA oligos (e.g. miRNA) are shorter and stiffer than plasmid DNA, they are often harder to complex into nanoparticles,¹⁸ and the materials that are effective for DNA delivery are often ineffective for RNA delivery.¹⁹

In this report we create new nanoparticles consisting of state-of-the-art polymeric nanobiotechnology with newly discovered cancer stem cell inhibiting miRNAs to develop miRNA delivering nanoparticles (nano-miRs) to treat gliomas. We develop and characterize nanoparticles utilizing bioreducible and cationic PBAE polymers capable of safely and efficiently shuttling miRNA into GBM cells, enabling escape out of the endosomes into the cytosol, and exhibiting an environmentally-triggered release of miRNA upon entering the cytosolic compartment. For the first time, we demonstrate the use of modified PBAE-based polymers for the effective delivery of miRNA mimics and observed that they inhibit the stem cell phenotype of human GBM cells. Further, we show that this new bioreducible PBAE-based nanomedicine spreads through established tumors *in vivo* and can be effective for therapeutic *in vivo* delivery of miRNA, and consequently, oligonucleotides in general. Critically, the delivery of these tumor-suppressing miRNAs using these biomaterials inhibited the growth of established GBM xenografts and led to significant long-term survival in mouse models. Our findings demonstrate that identifying and validating stem cell-regulating miRNAs in combination with advances in nanomedicine can impact the development of therapies for targeting the human CSC population and treating GBM.

Bioreducible PBAE nano-miRs encapsulate miRNAs into nanoparticles, effectively release them in a reducing cytosolic environment and deliver miRNAs to human GBM *in vitro*

To investigate the miRNA delivery capabilities of bioreducible PBAE nano-miRs and compare their performance with previous iterations of non-reducible PBAEs, we synthesized bioreducible polymer R646, its non-reducible analog 646, and C32 – a non-reducible PBAE that has been shown to be very effective at delivering DNA²⁰ (Figure 1). The bioreducible monomer 2,2'-disulfanediyldis(ethane-2,1-diyl)diacrylate (BR6) was copolymerized with 4-amino-1-butanol (S4) via a Michael Addition reaction at a 1.01:1 BR6:S4 ratio, and the resulting acrylate-terminated polymer was endcapped with 2-(3-aminopropylamino)ethanol (E6) to synthesize the polymer BR6-S4-E6 (R646). Polymer 646 was synthesized using the same procedure, but using the non-reducible hexane-1,6-diyl diacrylate (B6) instead of BR6. Finally, polymer C32, which has been shown to successfully deliver plasmid DNA to human prostate cancer xenografts in mouse models, was synthesized by copolymerizing 1,4-butanediol diacrylate (B4) and 5-amino-1-pentanol (S5) at a 1:1.2 B4:S5 ratio following the method reported by Anderson et al.²⁰ Polymers were characterized with gel permeation chromatography for molecular weight and polydispersity (Table S1 and Figure S14) and NMR for polymer structure (Figures S15–S17). YO-PRO®-1 Iodide competition binding assay, in which YO-PRO®-1 Iodide fluoresces upon binding miRNA and is quenched as it is displaced by polymer, shows that these PBAEs bound miRNA with equivalent binding affinity. pH titration curves were determined for the polymers using acid-base titration and showed they have equivalent buffering capacity in the physiologically-relevant range of pH 6–7.4, as indicated by a gradual slope at this pH range (Figure 1). As all three polymers have a similar structure (linear PBAE polymers that contain a similar tertiary amine repeat unit in the backbone, Figure 1D), their buffering capacity is similar. To determine the optimal nano-miR formulations required to deliver miRNA to GBM cells, we prepared nano-miRs at increasing polymer: miRNA weight-weight ratios (w/w). We optimized nano-miR w/w ratios *in vitro* in human GBM1A CSCs at a miRNA dose of 90 nM with a minimum acceptable cell viability of 75%. At this dosage, R646 nano-miRs maintained cell viability of greater than 75% at 150 w/w while 646 and C32 achieved the same at 37 w/w (Figure 2A). Additionally, incubating GBM1A or GBM1B neurospheres with R646 nano-miRs using the conditions described above for 3 hrs or 24 hrs did not have adverse effects on cell viability (Figure S1). At these optimal w/w ratios, cellular uptake of fluorescently labeled miRNAs was assessed via flow cytometry. We found that R646 nano-miRs achieved nearly 60-fold higher cellular uptake of miRNA compared to 646 and C32 nano-miRs encapsulating the same amount of miRNA (Figure 2B). This is most likely due to the fact that R646 attenuated cytotoxicity, allowing us to formulate R646 nano-miRs at a much higher w/w ratio and resulting in smaller and more stable nanoparticles compared to C32 and 646. Lastly, we assessed functional delivery of bio-active miRNAs using the different nano-miR formulations. Polymers were complexed with either a non-targeting control miRNA (Ctrl) or a combination of miRNA mimics miR-148a and miR-296-5p (Comb). Three days after transfection, functional delivery was assessed through qRT-PCR analysis of the expression of Dnmt1 and Hmga1, which are known targets of these two miRNAs.^{11–12}

We found that R646 nano-miRs significantly reduced the expression of both targets while 646 and C32 nano-miRs did not (Figure 2C–D). Unlike R646 nano-miRs, canonical PBAEs did not show meaningful, statistically significant target gene knockdown, demonstrating the need for improved materials for polymeric nanoparticle-mediated miRNA delivery (Figure S2). In order to rule out the possibility that differences in polymer molecular weight contributed to the observed difference in transfection efficacy, we synthesized R646 and 646 of similar molecular weight and used these polymers to deliver control or combination miRNA mimics. Our results showed that R646 nano-miRs again achieved significantly higher target gene knockdown and lower cytotoxicity compared to 646 nano-miRs formulated with matching molecular weight polymers, indicating that polymer characteristics beyond molecular weight are responsible for the superior performance of bio-reducible R646 nano-miRs (Figure S3). Furthermore, target gene knock-down by R646 nano-miRs was found to be somewhat more efficient than that achieved by commercially available RNAiMax and substantially more efficient than that achieved by commercially available PEI and Lipofectamine 3000 (Figure S4).

We characterized the physical properties of the three nano-miR formulations by measuring nanoparticle hydrodynamic diameter via dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA), zeta-potential by electrophoretic light scattering, and nanoparticle morphology by transmission electron microscopy (TEM). Diluted with PBS to a buffer condition of 150 mM and pH 7.4 to mimic physiological conditions, nano-miR size measured via NTA showed number-averaged hydrodynamic diameters of approximately 100 nm for all three formulations while DLS showed that R646 nano-miRs have an intensity-weighted z-average hydrodynamic diameter of approximately 200 nm, while 646 and C32 nano-miRs had z-average hydrodynamic diameters of greater than one micron, indicating the presence of aggregates in their particle distributions (Figure 3A). Interestingly, we also performed DLS size measurements before PBS dilution (measurement in 25 mM sodium acetate buffer, pH 5) and found that all nano-miRs were below 200 nm in diameter (Figure S5). Thus, the canonical PBAE nano-miRs were prone to aggregation after the initial self-assembly and following dilution into neutral physiological buffer. This suggests that the high w/w ratio that we were able to use with bio-reducible polymer R646 (as this polymer was engineered to be less cytotoxic) resulted in nano-miRs that had complexed miRNA more strongly, which reduced nano-miR aggregation in higher salt and pH environments. Zeta-potential was slightly positive for R646 nano-miRs and essentially neutral for 646 and C32 nano-miRs (Figure 3B). TEM images show that all formulations formed spherical nanoparticles with R646 nano-miRs appearing slightly smaller than the 646 nano-miRs and C32 nano-miRs (Figure 3C and Figure S6). As R646 nano-miRs degrade in water due to the hydrolytic ester linkages within the R646 polymer, we wanted to evaluate whether the R646 nano-miRs could be formulated dry in a manner suitable for storage that would also facilitate *in vivo* use. We utilized a lyophilization procedure with sugar as a cryoprotectant and found that R646 nano-miRs maintained their physical properties following lyophilization with no significant change in nanoparticle diameter assessed by DLS or TEM (Figure S7).²¹

To determine miRNA release kinetics, we performed a gel retention assay, in which nano-miRs were loaded into an agarose gel, and tightly bound RNA would be unable to

electrophoresis under an applied voltage. We incubated the nano-miRs in 5 mM glutathione (GSH) to mimic the reducing cytosolic space in the intracellular environment.²² In the presence of GSH, miRNA from bio-reducible R646 nano-miRs began to release almost instantaneously, and was completely released within 5 minutes; non-reducible 646 and C32 nano-miRs, in contrast, did not release miRNA even after 2 hr incubation (Figure 3D). We incubated the nanoparticles in artificial cerebrospinal fluid (aCSF) to mimic the redox and ionic environment in the brain extracellular space²³ for longer times and found that for all nano-miR formulations, miRNA began to release after 5 hr and was completely released by 10 hr. (Figure 3E) These results indicate that R646 nano-miRs are able to release miRNA cargo rapidly in a stimuli-responsive manner upon entry into the reducing intracellular space due to the reduction of disulfide bonds in the polymer backbone, while non-reducible 646 and C32 nano-miRs hold on to their cargo for much longer and eventually release miRNA after the slower hydrolysis of ester bonds.

With the goal of simultaneously delivering multiple miRNA constructs, we investigated whether R646 nano-miRs were able to co-deliver two different miRNA mimics (miR-148a and miR-296-5p) to the same cell. To this end, we made nano-miRs containing either Cy3-labeled miRNA, Cy5-labeled miRNA, or both miRNAs (comb.). For nano-miRs containing both miRNAs, we mixed the two anionic RNAs together before adding the cationic polymer for nanoparticle self-assembly to enable miRNA multiplexing. Flow cytometry experiments investigating cellular uptake showed that cells treated with nano-miRs carrying both miRNAs increased proportionally in fluorescence (Figure 4A, B). Confocal imaging showed that endosomes in these cells contained both Cy3 and Cy5 fluorescence, confirming that the nano-miRs not only delivered both miRNAs into the same cell but also into the same endosomes, which indicates that two different miRNAs can be complexed into the same nano-miR delivery system (Figure S8A). We also saw detectable levels of diffuse Cy5 fluorescence distributed throughout the cytosol and nucleus at 2h, suggesting that the R646 nano-miRs effectively escaped the endosome (Figure S8B). In contrast, cytosolic RNA molecules have previously been difficult to image with scanning laser confocal microscopy following leading non-viral gene delivery methods such as lipid nanoparticle mediated delivery.^{24–25} We also stained cells treated with Cy5-labeled miRNA nano-miRs with a lysosomal dye (pKa 4.5) and found that endosomes with Cy5 signal did not colocalize with lysosomes at 2h, consistent with efficient endosomal escape prior to detectable lysosomal targeting. Our results show that a significant number of R646 nano-miRs effectively avoided lysosomal degradation and released their miRNA cargo in the cytosol. This is a significant improvement over traditional cationic polymers and nanomedicine delivery systems, for which lysosomal degradation has been a limitation.^{26–27} Overall, these results indicate that bio-reducible R646 nano-miRs outperformed nano-miRs constructed from non-reducible polymers 646 and C32 by attenuating cytotoxicity, increasing cellular uptake of miRNA cargo, and effectively delivering bio-active miRNAs to the cytoplasm of GBM cells. We therefore chose to use R646 nano-miRs to assess *in vitro* and *in vivo* delivery of our GBM-regulating miRNA mimics.

Mature miRNA mimics labeled with Dy547 were complexed with bio-reducible R646 to formulate PBAE nano-miRs and used to transfect multicellular GBM neurospheres. Fluorescence from the Dy547-labeled miRNA was readily detectable in the multicellular

spheres starting 3 hrs after the transfection and persisted for at least 9 days (Figure S9A and Figure S19). We recently showed that miR-148a and miR-296-5p are repressed as part of an epigenetic program by which GBM cells become stem-like and tumor propagating.^{11–12} We also found that reconstituting these miRNAs individually using viral vectors inhibits the stem cell phenotype and tumor-propagating potential of GBM cells.^{11–12} To assess the bioactivity of R646-delivered miRNAs, nano-miRs carrying control miRNA (miR-Ctrl), miR-148a mimic, miR-296-5p mimic, or miR-148a+miR-296-5p (comb.) were used to transfect GBM neurospheres. Total RNA concentrations were held fixed at 120 nM, and miR-148a or miR-296-5p were either blended with miR-Ctrl or one another, so that the total amount of each functional miRNA remained at 60 nM in all conditions.

Expression of mature miRNAs was measured using qRT-PCR 3 days after transfection. Transfecting GBM neurospheres with miR-148a or with miR-296-5p nano-miRs increased intracellular miRNA levels by 24-fold and 27-fold, respectively (Figure 4C), and these miRNAs remained substantially elevated (15–20 fold) for up to 12 days (the last time point examined) (Figure S19C). The combination nano-miRs simultaneously increased miR-148a and miR-296-5p levels by 16-fold and 30-fold respectively (Figure 4C). To directly evaluate the effects of these nano-miRs on the GBM stem cell phenotype, two patient-derived neurosphere lines were transfected using nano-miRs and sphere forming capacity, a quantitative marker of cell stemness and self-renewal, was measured. miRNA delivery by this approach significantly inhibited sphere-forming capacity (Figure 4E and 4F and Figure S9C and S9D) concurrent with the decreased expression of stem cell markers Sox2, Nanog, Bmi1, and Olig2 (Figure 4D and Figure S9B) and also inhibited previously described miR-148a and miR-296-5p targets, Dnmt1 and Hmga1, respectively (Figure 2C and 2D). These results supported the use of our novel bio-reducible PBAE polymeric nano-miRs to deliver bio-active miRNAs to GBM xenografts *in vivo*.

Bioreducible PBAE nano-miRs spread through brain tumor xenografts to deliver miRNAs

To circumvent the delivery obstacle posed by the blood brain barrier, we used direct intra-tumoral delivery to test the biological effects of our nano-miRs *in vivo*.²⁸ Trans-cranial cannulas were placed with their tips within the right caudate/putamen of mice. One week after cannula placement, animals were implanted with Oct4/Sox2 induced cancer stem cells (iCSCs)¹¹ via the cannula. iCSCs generate aggressive rapidly growing xenografts and represent a demanding model to assess miRNA biodistribution and *in vivo* bioactivities. R646 nano-miR delivery was started 3 weeks after cell implantation. Twice per week for 3 weeks animals received slow infusions of nano-miRs containing either Dy547-labeled control miRNA or miR-148a via the cannula. Brains were collected and histopathologic sections were visualized using fluorescence microscopy and compared to the adjacent H&E stained counterparts. Dy547-labeled miRNA was found to be distributed through approximately 60% of these large rapidly growing tumors when evaluated 25 days after the first nano-miR infusion and 3 days after the last infusion (Figure 5A). To determine if the miRNAs delivered by this protocol retained their biological function, we measured the expression of Dnmt1 and Dnmt3b, two well described miR-148a targets.¹¹ Tumors treated

with miR-148a nano-miRs had significantly lower expression levels of both Dnmt1 and Dnmt3b compared to control treated animals (Figure S10). To investigate the therapeutic potential of miRNA delivery using R646 biodegradable PBAE nanoparticles in comparison to ionizing radiation (I.R.), a standard of care treatment modality for GBM, tumors were established as described above and treated with 148a nano-miR +/- I.R. (Figure S11). Intratumoral delivery of miR-148a nano-miRs to a pre-established tumor more effectively decreased tumor size and tumor vascularity and increased tumor cell apoptosis as measured by caspase 3 activation than I.R. treatment alone. Combining 148a nano-miRs with I.R. generated cooperative and potentially synergistic anti-tumor responses.

miRNA co-delivery via bio-reducible PBAE nano-miRs inhibits tumor growth and prolongs animal survival in an orthotopic model of human GBM

We have recently reported that viral-based transgenic expression of either miR-148a or miR-296-5p differentiated GBM stem cells and inhibited their self-renewal as spheres. We also found that these miRNAs independently inhibit the capacity of GBM stem cells to propagate glioma xenografts *in vivo*.¹¹⁻¹² We asked if reconstituting both of these stem cell inhibiting miRNAs using non-viral R646 nano-miRs would have cooperative effects on pre-established GBM xenografts. Human GBM derived neurospheres (GBM1A), which generate tumors that closely recapitulate the growth pattern and pathology of clinical GBM,²⁹ were implanted in animals using an experimental paradigm similar to the one described above (Figure 5B). Tumors were then treated with R646 nano-miRs containing control miRNA, miR-148a, miR-296-5p, or a combination of miR-148a+miR-296-5p. Tumor burden in brains collected after 28 days of treatment quantified by computer-assisted morphometry was significantly decreased in all three groups treated with active miRNA, with the most profound effect seen in animals treated with the miRNA combination (Figure 5C and Figure S12A). We also saw an increase in tumor tissue necrosis (Figure 5D) and apoptosis (Figure S12B), as measured by histopathology and cleaved caspase 3 immunohistochemistry, respectively. We find that while miR cooperativity was limited during *in vitro* evaluation, miR cooperativity is potent *in vivo* and consistent with our understanding of these two miRs targeting complementary tumor-promoting mechanisms. The substantial decrease in tumor burden observed in mice treated with the multiplex nano-miRs and the more modest effects observed when delivering miR-148a alone in both this patient-derived GBM model and the more aggressive engineered iCSC model predicted the therapeutic survival advantage of miR-148a + miR-296-5p multiplex nano-miRs.

To rigorously test the efficacy of our nano-miR therapy in a survival study, animals bearing pre-established intracranial GBM1A glioma xenografts received either control or miR-148a + miR-296-5p multiplex nano-miRs beginning on post-implantation day 45 and continued twice per week for 6 weeks. Surviving animals began to show signs of weight loss so treatment was stopped after 6 weeks (12 injections) on post-implantation day 87. All treated animals regained weight one week after ending treatment. All 9 animals treated with control nanomiRs were either dead or pre-morbid requiring euthanasia by post-implantation day 90. In contrast, 6 of 9 nano-miR-treated animals remained alive and healthy by post-implantation day 133, at which time the experiment was terminated (Figure 5E).

Histological analysis of the surviving animals euthanized at post-implantation day 133 revealed that 4 out of the 6 had no detectable tumor (Figure S13).

There is a need to develop and translate new treatment strategies for glioblastoma that are designed to target the subpopulation of CSCs that drive tumor propagation, therapeutic resistance, and tumor recurrence following conventional treatments.³⁰ These CSCs are highly plastic and exist in a state of dynamic flux between CSC and non-CSC states.³¹ The epigenetic mechanisms driving these phenotypic transitions represent unexploited vulnerabilities amenable to therapeutic biological targeting.^{4, 8} Approaches based on the premise that reconstituting CSC-inhibiting miRNA toward the goal of normalizing dysregulated networks in cancer hold great promise.³² As is becoming evident, miRNAs regulate cell phenotypes by modulating multiple gene targets simultaneously. The broad targetome of naturally occurring miRNAs has numerous advantages over siRNAs that are engineered to target individual genes within multi-genic processes such as CSC regulation.³² We recently identified two candidate therapeutic miRNAs, miR-148a and miR-296-5p, based on their repression during the induction of GBM stemness and tumor-propagating capacity by Oct4/Sox2.^{11–12} We now show that reconstituting these miRNAs using non-viral bio-reducible PBAE nano-miRs offers a therapeutic approach distinct from conventional cytotoxic therapy (e.g ionizing radiation or temozolomide) that has been shown to be insufficiently effective against CSC pools and clinical GBM.^{30, 33}

A central challenge to developing nucleic acid-based modalities for targeting tumor-propagating CSCs is identifying and developing a suitable delivery vehicle. An optimal miRNA carrier for intra-tumoral delivery should be able to stably encapsulate miRNA, including combinations of miRNA, and protect it while in the extracellular environment and then quickly release it in the cytosolic environment. Our bio-reducible R646 nano-miRs were found to fulfill these requirements. Using a gel retention assay, we were able to show that the R646 nano-miRs fully encapsulated the miRNA in non-bio-reducible conditions and then released the miRNA in a triggered manner within 5 minutes when in a cytosol-like reducing environment. The quick intracellular release of miRNA is dually important, as it significantly reduces cytotoxicity compared to non-reducible PBAEs and cationic polymer such as PEI (Figure 2A and S4A), allowing higher polymer-to-miRNA weight/weight ratios to be used and smaller, more stable nanoparticles to be formed (Figure 3A and S5). Furthermore, it allows the miRNA to enter into cellular pathways necessary for miRNA function.^{34–35}

Particle sizing analysis revealed that the R646 nano-miRs are approximately 100 nm in diameter (Figure 3A). *In vivo* tumor distribution studies revealed that these nanoparticles can spread through brain tumor tissue following direct intra-tumor infusion, distributing particles through established tumors with at least 60% coverage (Figure 5A). This represents a length scale of ~2 mm from the cannula infusion site following a 5 μ L infusion to reach the tumor margins. The finding that the nano-miRs could spread through the GBM tumors is consistent with the finding that this treatment led to long-term survivors in the majority of the combination miRNA treated animals (Figure 5E). In comparison, non-bio-reducible PBAE/DNA nanoparticles delivered via an intratumoral infusion were recently observed to spread through a similar length-scale of approximately 2 mm in 9L rat glioma tumors

following a larger volume 25 μ L infusion³⁶ and PEGylated non-bioreducible PBAE/DNA nanoparticles spread through a length-scale of $\sim$ 2 mm in the brains of Fischer 344 rats following a 20 μ L infusion.³⁷ Thus, for non-viral nucleic acid delivery to brain tumors, the R646 polymeric nanoparticles, small and with relatively neutral surface charge, appear to be potent vehicles that are able to spread through brain tumors sufficiently to have therapeutic effect. In our current study, the functional efficacy of bioreducible nano-miRs *in vivo* was validated and is especially significant as oligonucleotides are known to be more difficult to deliver by electrostatic polyplex nanoparticles than plasmid DNA molecules are, as oligonucleotides are typically 100-fold smaller and consequently orders of magnitude less multivalent than plasmids.

Once at the surface of individual cancer cells, the nano-miRs are able to be internalized effectively as demonstrated in our *in vitro* cellular uptake studies (Figure 2 and Figure S9). Once internalized into a cell, the tertiary amines of the PBAE polymers can facilitate avoidance of lysosomes and endosomal escape by a sufficiently large fraction of nano-miRs that the released miRNA can be detected in the cytosol (Figure 1E and Figure S8B). Through these mechanisms, the nano-miRs are able to effectively increase the intracellular levels of multiplexed cargo miRNA 15–30 fold (Figure 4C) with retention of anti-CSC bioactivity (Figure 4D–F).

Combinations of miRNAs that inhibit multiple pathways required for tumorigenesis should more efficiently impede tumor growth and propagation while at the same time reducing the emergence of resistance.^{30, 32} To reduce the possibility of cytostatic effects or tumors developing resistance, we explored two treatment modalities by either combining I.R. treatment with nano-miR-148a delivery or targeting two parallel pathways that contribute to GBM cell stemness and tumor propagation using R646 biodegradable PBAE nanoparticles to co-deliver miR-148a and miR-296-5p. miR-148a nano-miRs and ionizing radiation cooperatively inhibited tumor xenograft growth. This result is consistent with the relative resistance of GSCs to cytotoxic therapeutics and our current and previous findings that miR-148a inhibits GBM cell stemness.^{11, 33} Co-delivering both miR-148a and miR-296-5p as multiplexed nano-miRs *in vivo* increased tumor cell death and reduced tumor burden more significantly than either miRNA delivered alone (Figure 5 and Figure S13). This cooperative therapeutic effect *in vivo* is consistent with the concept that cancers will be more responsive to strategies designed to target multiple complementary tumor-promoting pathways by normalizing miRNA networks and their multiple targetomes than to single miRNA or highly specific siRNA therapeutics.

To our knowledge, our current report is the first time that a bioreducible PBAE-based system has been evaluated for oligonucleotide delivery *in vivo*. Moreover, it is the first time that PBAE-based nanomaterials have been formulated for miRNA delivery *in vitro* or *in vivo*. The results presented in this study demonstrate the promise of using R646 nano-miR systems in combination with cancer stem cell-inhibitory miRNAs as nanomedicine to impact the development of biological therapies for treating GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PBAE synthesis, miRNA complexation and buffering capacity

(A) PBAE monomer structures are shown. (B) Polymer R646 was synthesized using a Michael addition reaction between monomers BR6 and S4 at a 1.01:1 BR6:S4 ratio. The resulting acrylate-terminated polymer was endcapped with monomer E6 to yield BR6-S4-E6 (R646). Polymer 646 was synthesized via a similar method using monomer B6 instead of BR6. (C) Polymer C32 was synthesized by reacting B4 with S5 at a 1:1.2 B4:S5 ratio, resulting in an amino alcohol terminated polymer. (D) Chemical structures of R646, 646, and C32. (E) Polymer-miRNA competitive binding assay; polymer to miRNA binding strength is assessed by quenching of YO-PRO®-1 Iodide fluorescence over increasing polymer concentrations. (F) Acid-base titration curves for PBAE polymers with 150 mM aqueous NaCl for comparison. pH was adjusted to pH 3 with HCl and titrated with NaOH.

Figure 2. Polymer R646 attenuates cytotoxicity compared to non-reducible PBAE and effectively delivers miRNAs to GBM cells

(A) Nano-miR formulations were screened in GBM1A cells to identify optimal nano-miR formulation with >75% relative viability. Numbers on the x-axis indicate polymer-miRNA w/w ratios. (B) Nano-miR uptake was measured using flow cytometry after treating cells with nano-miRs loaded with Cy5-labeled miRNA. R646 nano-miRs had significantly higher cell uptake (**** $P < 0.0001$) than all other conditions assessed by One-way ANOVA with Tukey post hoc tests. (C) qRT-PCR analysis of expression of *Dnmt1* and *Hmga1* in GBM1A 3 days after treatment with nano-miRs delivering a non-targeting control miRNA (Ctrl) or a combination of miRNA mimics miR-148a and miR-296-5p (Comb). Fold expression was normalized to cells treated with Ctrl miRNA only. R646 showed statistically significant knockdown in expression of (C) *Dnmt1* and (D) *Hmga1* assessed by Holm-Sidak corrected multiple t-tests between matched Ctrl and Comb (** $P < 0.01$; *** $P < 0.001$). Bars show mean + SEM of three (qRT-PCR) or four wells (viability and uptake). For each target, R646 nano-miRs delivering the combination of miRNA mimics also showed significantly higher knockdown than all other conditions as assessed by One-way ANOVA with Tukey post hoc tests (* $P < 0.05$).

Figure 3. Polymer R646 forms nanoparticles with miRNA and effectively releases miRNA in a reducing environment

(A) Nanoparticle hydrodynamic diameter as measured using intensity-weighted (DLS) or number-average (NTA) measurement showed that R646 nano-miRs had a statistically smaller hydrodynamic diameter via DLS as assessed by One-way ANOVA with Tukey post hoc tests (** $P < 0.001$) (B) Nanoparticle zeta-potential as measured via electrophoretic light scattering showed R646 nano-miRs had a statistically significantly higher zeta potential assessed by One-way ANOVA with Tukey post hoc tests (**** $P < 0.0001$). (C) TEM images of nano-miRs showed dried particle size and spherical morphology. (D) Gel retention assay performed in 5 mM glutathione mimicking the intracellular environment showed short-term miRNA release in reducing conditions. miRNA that was tightly bound within non-bioreducible PBAE nanoparticles was unable to run down the gel. (E) Gel retention assay performed in artificial CSF mimicking the extracellular environment in the brain showed long-term miRNA release in non-reducing conditions. Bars show mean + SEM of three independently prepared samples.

Figure 4. R646 nano-miRs can deliver multiple different miRNAs and inhibit the GBM stem cell phenotype

(A) Flow cytometry data of GBM1A neurospheres treated with R646 nano-miRs carrying either Cy3-labeled miRNA (Cy3-miR), Cy5-labeled miRNA (Cy5-miR), both RNAs (comb.) and completely untreated (Unt.). Fluorescence signal of cells treated with nano-miRs carrying both miRNAs were normalized against the single miRNA groups. Bars show mean + SEM of four wells. (B) Flow cytometry plot of cells treated with nano-miRs carrying both labeled miRNAs compared to the untreated population shows proportional uptake of both miRNAs. (C) Expression of mature miR-148a and miR-296-5p was measured by qRT-PCR 3 days after transfection. (D) qRT-PCR analysis to measure expression of stem cell markers in GBM1A neurospheres transfected with nano-miRs. (E) Equal numbers of GBM1A transfected with nano-miRs were cultured in neurosphere medium containing EGF/FGF for 12 days and neurosphere numbers (>100μm diameter) were quantified by computer-assisted image analysis. (F) Limiting dilution analyses of GBM1A transfected with control (Ctrl.) or miR-148a+miR-296-5p combination (Comb.) nano-miRs. Untransfected cells (Unt.) were used as negative control. Cells were plated in a limiting dilution manner, and the number of wells containing spheres was counted after 14 days to compare stem cell frequencies. One-way ANOVA with Tukey post hoc tests was used when performing multiple comparisons and $p < 0.05$ considered statistically significant. * $p < 0.05$

Figure 5. miR-148a and miR-296-5p co-delivery using R646 nano-miRs inhibits GBM tumor growth and extends survival *in vivo*

(A) miR-Ctrl labeled with Dy547 was visualized 3 days after the last infusion using fluorescence microscopy and compared to adjacent H&E stained sections. The intra-tumoral distribution of the nano-miRs was calculated as the ratio of fluorescence area divided by tumor area X 100 in brain sections with the highest cross-sectional area of tumor (N=3 mice per group; right panel). (B) Schematic summarizing treatment schedule for *in vivo* delivery of nano-miRs. Animals were sacrificed 42 days after cell implantation and maximum tumor cross-sectional areas following treatment with nano-miRs representing viable tumor tissue (C) and necrotic tumor tissue (D) were quantified from H&E stained sections using ImageJ software. For each cohort, R646 nano-miRs delivering the bioactive miRNA mimics showed significantly lower viable tumor area and higher necrotic area than the animals receiving control (Ctrl.) nano-miR as assessed by One-way ANOVA with Tukey post hoc tests (**p<0.01 and *p<0.05). (E) Kaplan-Meier survival curves comparing mice treated with control nano-miRs (miR-Ctrl) or miR-148a+miR296-5p nano-miRs (miR-Comb.). Therapy in the survival study was initiated 45 days after tumor cell implantation. Survival was compared across arms using the log-rank test (N=9). (**p<0.01, *p<0.05).