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TITLE:

Preparation of neutrally-charged, pH-responsive polymeric nanoparticles for cytosolic siRNA delivery

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SUMMARY:

Methods to prepare and characterize the physicochemical properties and bioactivity of neutrally-charged, pH-responsive siRNA nanoparticles are presented. Criteria for successful siRNA nanomedicines such as size, morphology, surface charge, siRNA loading, and gene silencing are discussed.

ABSTRACT:

The success of siRNA as a targeted molecular medicine is dependent upon its efficient cytosolic delivery to cells within the tissue of pathology. Clinical success for treating previously 'undruggable' hepatic disease targets with siRNA has been achieved. However, efficient tumor siRNA delivery necessitates additional pharmacokinetic design considerations, including long circulation time, evasion of clearance organs (e.g., liver and kidneys), and tumor penetration and retention. Here, we describe the preparation and in vitro physicochemical/biological characterization of polymeric nanoparticles designed for efficient siRNA delivery, particularly to non-hepatic tissues such as tumors. The siRNA nanoparticles are prepared by electrostatic complexation of siRNA and the diblock copolymer poly(ethylene glycol-*b*-[2-(dimethylamino)ethyl methacrylate-*co*-butyl methacrylate]) (PEG-DB) to form polyion complexes (polyplexes) where siRNA is sequestered within the polyplex core and PEG forms a hydrophilic, neutrally-charged corona. Moreover, the DB block becomes membrane-lytic as vesicles of the endolysosomal pathway acidify (< pH 6.8), triggering endosomal escape and cytosolic delivery of siRNA. Methods to characterize the physicochemical characteristics of siRNA nanoparticles such

as size, surface charge, particle morphology, and siRNA loading are described. Bioactivity of siRNA nanoparticles is measured using luciferase as a model gene in a rapid and high-throughput gene silencing assay. Designs which pass these initial tests (such as PEG-DB-based polyplexes) are considered appropriate for translation to preclinical animal studies assessing the delivery of siRNA to tumors or other sites of pathology.

INTRODUCTION:

Because siRNAs inhibit the translation of proteins from mRNA sequences, they can theoretically be used to drug all known pathologies[1-5]. However, the use of siRNA in medicine is limited by the comprehensively poor pharmacokinetic profile of siRNA molecules[6,7]. When injected intravenously, siRNAs are rapidly cleared through the kidneys and/or degraded by nucleases[8,9]. Due to its large size and negative charge, siRNA cannot enter cells or escape the endolysosomal pathway to access the RNA-Induced Silencing Complex (RISC) that resides in the cytosol[10-13]. Thus, extensive effort has focused on the design and implementation of siRNA delivery strategies[14]. This effort has largely focused on the development of lipid- and polymer-based nanoparticles which package siRNA, protect it from clearance and degradation in vivo, and initiate cellular uptake and endosomal escape through ionizable, cationic amine groups. Many pre-clinical successes have been reported and most recently, the first clinical success has been reported for nanoparticle-based hepatic siRNA delivery to treat hereditary transthyretin-mediated (hATTR) amyloidosis[15].

There are many cancer-causing genes that are currently "undruggable" by conventional pharmacology (i.e., small molecule drugs), motivating the design of polymeric siRNA nanoparticles (si-NPs) to treat cancer[16]. However, there are a separate set of design parameters that must be considered for non-hepatic siRNA delivery. The delivery system must shield the cationic charge of the polyplex which causes agglutination within the systemic circulation[17-19]. For tumor delivery, specifically, si-NP stability is essential to endow long circulation and thus increased accumulation within tumors via the enhanced permeability and retention (EPR) effect[20,21]. Moreover, control over si-NP size is essential since only nanoparticles approximately 20 – 200 nm diameter in size leverage EPR[22], and smaller si-NPs (~20 – 50 nm diameter) exhibit improved tumor penetration over larger sized nanoparticles and microparticles[23].

To address these additional design constraints for systemic tumor delivery of siRNA following intravenous administration, neutrally-charged, pH-responsive si-NPs have been developed (**Figure 1**)[24]. These si-NPs are PEGylated, or most recently, Zwitterionated[25], for neutral surface charge and resistance to protein adsorption and opsonization in circulation. Since they cannot rely solely on cationic character to drive intracellular delivery, extremely efficient endosomal escape is imperative for achieving potent gene silencing. Accordingly, the core of these si-NPs is composed of a highly endosomolytic core which is inert at extracellular pH (7.4), but which is triggered in a switch-like manner in the acidified conditions of the endolysosomal pathway [pH 6.8 (early endosomes) – 5.0 (lysosomes)]. Lastly, a mixture of cationic and hydrophobic content within the core of si-NPs provide both electrostatic and van der Waals stabilization forces, improving stability of the si-NPs in blood compared to merely cationic systems.

The integration of many functions into a relatively simple design is possible using Reversible Addition-Fragmentation chain Transfer (RAFT) controlled polymerization to produce polymers with complex architecture and precise composition. To produce si-NPs with neutral surface charge, pH-responsiveness, and NP stability, RAFT is used to synthesize poly(ethylene glycol-*b*-[2-(dimethylamino)ethyl methacrylate-*co*-butyl methacrylate]) (PEG-DB; **Figure 1A**). PEG-DB is electrostatically complexed with siRNA, forming si-NPs with a PEG corona and DB/siRNA core (**Figure 1B**). PEG forms an inert, neutrally-charged hydrophilic layer on the si-NP corona. The DB block consists of a 50:50 molar ratio of 2-(dimethylamino)ethyl methacrylate (DMAEMA) and butyl methacrylate (BMA). Cationic DMAEMA electrostatically complexes negatively-charged siRNA. BMA self-associates within the NP core by van der Waals interactions, increasing NP stability. Together, DMAEMA and BMA impart pH-dependent lipid bilayer-lytic behavior to the DB polymer block. At extracellular pH, the DB block is sequestered to the si-NP core and is inert to lipid bilayers. Under acidic conditions, such as those within the endolysosomal pathway, ionizable DMAEMA within the DB block facilitates the proton sponge effect, where endosomal buffering leads to osmotic swelling and rupture[26]. Additionally, hydrophobic BMA moieties within the DB block actively integrate into and lyse lipid bilayers, resulting in potent endosomolysis. Thus, siRNA is complexed with PEG-DB to form si-NPs that are neutrally-charged and highly stable at extracellular pH but which disrupt lipid bilayers at acidic pH, ensuring cytosolic delivery of the siRNA payload.

Herein are described the experimental procedures to produce si-NPs from PEG-DB. Methods to characterize the physicochemical parameters and bioactivity of si-NPs are presented and discussed. In order to rapidly assess si-NP bioactivity, luciferase is used as a model gene for knockdown studies. Firefly Luciferase is the protein responsible for the 'glow' of fireflies[27]. Accordingly, mammalian cells transfected with the firefly luciferase gene produce a bioluminescent 'glow' that can be captured using a luminometer to quantify levels of Luciferase expression. Here, we use Luciferase to assess bioactivity of si-NPs by delivering siRNA against Luciferase and quantifying the corresponding reduction in bioluminescence in Luciferase-expressing cells compared to cells that receive a scrambled siRNA.

PROTOCOL:

1. Preparation and characterization of si-NPs

1.1. si-NP preparation

1.1.1. Dissolve polymer in 10 mM citric acid buffer (pH 4.0) at 3.33 mg/mL. Polymer can first be dissolved at 10x concentration in ethanol to ensure dissolution.

NOTE: Polymer can be dissolved at lower concentrations, but use at concentrations above 3.33 mg/mL can prevent homogenous NP formation.

1.1.2. Add siRNA (50 μM in diH_2O) to result in N^+:P^- ratio of 10. Mix polymer and siRNA solutions thoroughly by pipetting and let incubate for 30 min. The N^+:P^- ratio represents the number of

positively-charged amine groups on the polymer to the number of negatively-charged phosphate groups on the siRNA and is calculated by the formula below:

$$N^+ : P^- = \frac{(mol\ Pol)(RU\ amine)}{(mol\ siRNA)(bp\ siRNA)(2)}$$

where, mol Pol is the molar amount of polymer, RU amine is the number of repeating units of positively-charged amines per polymer, mol siRNA is the molar amount of siRNA, and bp siRNA is the number of base pairs per siRNA molecule.

1.1.3. Add a 5-fold excess of 10 mM phosphate buffer (pH 8.0) and mix gently either by pipetting or inverting the tube. To confirm that the final pH is neutral (~7.2-7.5), pipette 10 μL of si-NP solution onto pH test strips.

NOTE: Citric acid and phosphate buffers are prepared according to the Millipore Sigma Buffer Reference Center Charts.

1.2. Physicochemical characterization of si-NPs

1.2.1. Record the size and surface charge of resulting si-NPs using dynamic light scattering (DLS). Prepare a DLS sample by filtering 1 mL of si-NPs (0.1 – 1.0 mg/mL) through 0.45 μm pore-size syringe filters into a square quartz or polystyrene cuvette. Record size and surface charge measurements using a DLS instrument according to the manufacturer's specifications.

1.2.2. Confirm the size and morphology of si-NPs by imaging analysis using transmission electron microscopy (TEM).

1.2.2.1 Add 5 μL of si-NP solution at 1 mg/mL to TEM grids and incubate for 60 s. Blot dry for 3 s.

1.2.2.2 Add 5 μL of 3% uranyl acetate solution and incubate for 20 s. Blot dry for 3 s. Dry grids overnight under desiccation.

1.2.2.3 Image grids according to the protocol established for the specific microscope to be used.

1.2.3. Characterize the loading of siRNA in si-NPs at various N^+:P^- ratios using agarose gel retardation.

1.2.3.1 To produce 2% agarose gel, add 2 g of electrophoresis grade agarose powder to 100 mL of 1x TAE (Tris-acetate-EDTA) buffer at pH 8.0. Stir to suspend agarose. Heat uncovered in microwave until all agarose is dissolved (1-3 min).

1.2.3.2 Once cooled, add 5 μL of ethidium bromide (10 mg/mL in H_2O), and mix well. Pour agarose into a gel tray and place comb to produce wells, letting dry for 30 min. Carefully remove comb to

leave behind loading wells, and fill the gel tray to the max fill line with 1x TAE buffer.

1.2.3.3 Generate si-NPs (according to the procedure above) at 0, 1, 2, 5, 7, 10, 20, and 40 N^+:P^- ratios. Place 2 μL aliquots of loading dye (no SDS and reducing agents) on paraffin film for each si-NP formulation. Mix 10 μL of si-NP solution with loading dye on paraffin film by pipette.

1.2.3.4 Add si-NP/loading dye solutions to agarose gel wells. Run voltage source at 100 V for 35 min (or until samples have traversed 80% of gel length).

1.2.3.5 Visualize siRNA bands on a UV transilluminator according to the manufacturer's specifications.

2. Determining in vitro bioactivity of si-NPs

2.1. Knockdown of the model gene luciferase

2.1.1. Generate luciferase si-NPs (according to the procedure above) using luciferase siRNA and scrambled si-NPs using a scrambled siRNA sequence as a control. Formualte both si-NPs at the same final N^+:P^- ratio and at the optimum ratio identified by agarose gel retardation studies. Example siRNA sequences are included in the **Table of Materials**.

2.1.2. Seed luciferase-expressing cells [MDA-MB-231/Luciferase (Bsd) stable cells] in 96-well black-walled plates at a density of 2,000 cells per well. Allow to adhere overnight in full media (DMEM, 10% FBS) in an incubator (37 °C, 5% CO_2, 95% humidity).

2.1.3. Dilute si-NPs into full serum media for a final volume of 100 μL per well and siRNA concentration of 100 nM. Treat cells for 24 h with si-NPs.

2.1.4. After 24 h, remove treatments and replace media with full serum media containing 150 μg/mL D-luciferin. Incubate cells for 5 min before measuring luminescence on a plate reader or in vivo optical imaging system according to the manufacturer's specifications.

2.1.5. Replace luciferin-containing media with fresh, full serum media, and incubate 24 h more. Repeat the step above, removing media and replacing with full serum media containing 150 μg/mL D-luciferin, followed by a 5 min incubation prior to measuring luminescence at the 48 h timepoint.

2.1.6. For longitudinal studies, maintain cells under sterile conditions while measuring luminescence and can continue to be cultured after replacing luciferin-containing media with fresh, full media.

NOTE: The appropriate siRNA concentration will vary with different si-NPs and siRNA molecules. When using neutrally-charged polyplexes with an endosomolytic core (e.g., PEG-DB), 100 nM is

typically well-tolerated by the cells and produces >75% luciferase knockdown. The mass ratio of PEG-DB to siRNA at 10 N^+:P^- ratio and 100 nM siRNA treatments (assuming 26 bp siRNA) is 23.3, i.e., add 23.3 ng of PEG-DB for every 1.0 ng of siRNA. For example, add 1.16 μL of 3.33 mg/mL polymer for 166.5 ng of siRNA to treat one well at 100 nM in a 96-well plate (100 μL media volume per well).

REPRESENTATIVE RESULTS:

Some essential characteristics of effective si-NPs for in vivo siRNA delivery are the proper size (~20 – 200 nm diameter), siRNA packaging, and gene silencing bioactivity. While this is not an exhaustive list (as addressed in the Discussion), these basic characteristics should be confirmed before considering further testing of a formulation.

Figure 2 illustrates the characterization of si-NP size and surface charge upon formulation. DLS and TEM are used as complementary methods to observe si-NP size (both), polydispersity (DLS), and morphology (TEM). DLS measurements show that si-NP 1 has an average diameter of 35 nm, unimodal distribution indicated by the presence of a single peak, and low polydispersity indicated by a relatively narrow peak width (**Figure 2A**). TEM measurements confirm the size measurement from DLS, suggest the presence of a uniform population of si-NPs, and reveal the spherical morphology of the si-NPs (**Figure 2C**). DLS and TEM measurements of si-NP 2 reveal that it has undesirable size (>200 nm diameter) of average diameter 1,500 nm, a multimodal and polydisperse population, and aggregates in solution, forming no distinct particle morphology (**Figure 2B,D**). Both si-NPs 1 and 2 display neutral surface charge, indicated by near-zero mean zeta potential values (**Figure 2E**).

Loading efficiency of siRNA into si-NPs is characterized using an agarose gel retardation assay. Un-complexed siRNA migrates through the agarose gel and is visualized (due to binding of ethidium bromide) at the gel bottom. When siRNA is complexed with the polymer to form si-NPs, migration through the agarose gel is obstructed and siRNA is visualized at the gel top (or where it was loaded into wells). For PEG-DB-based si-NPs, complexation increases with increasing N^+:P^- ratios until full complexation is achieved at ~10-20 N^+:P^- ratio (**Figure 3**).

Luciferase is used as a model gene for the rapid assessment of si-NP gene silencing bioactivity. Bioluminescence measurements via a plate reader or in vivo optical imaging system allow for the rapid, high-throughput quantification of luciferase protein expression in well-plate format. This technique is considerably faster, cheaper, and less burdensome than analyzing gene silencing through traditional molecular analyses such as PCR (gene expression) and western blot (protein expression). By this method, luciferase-expressing cells are treated with luciferase si-NPs and scrambled si-NPs (as a control), and %Luciferase Activity is calculated by comparing luminescence signal to untreated cells. Bioactive luciferase si-NPs will exhibit significantly diminished %Luciferase Activity when compared directly to scrambled si-NP control, as can be seen for si-NP 1 in **Figure 4**. In contrast, luciferase si-NPs that do not reduce %Luciferase Activity compared to scrambled si-NP control (such as si-NP 2) are not considered bioactive (**Figure 4**). Often 48 h is the time of maximum gene silencing (**Figure 4**), but we have observed significant gene silencing at time points ranging from 24 h to 240 h post-treatment.

FIGURE AND TABLE LEGENDS:

Figure 1. Polymer chemistry and si-NP schematic. (**A**) Chemical composition of PEG-DB diblock copolymer that composes si-NPs and endows neutral surface charge (PEG block) and pH-responsive behavior (DB block). (**B**) Self-assembly of si-NPs. At pH 4.0, DB block is water-soluble because DMAEMA tertiary amines are highly protonated. The positively-charged DB block electrostatically complexes negatively-charged siRNA molecules. The pH is then adjusted to ~7.4 by the addition of 5x pH 8.0 phosphate buffer, resulting in the "hydrophobization" of DB block and sequestration of siRNA and DB within the core of si-NPs.

Figure 2. DLS and TEM characterization of si-NP size, surface charge, and morphology. (**A, C**) si-NP 1 represents a uniform sample with appropriate size (~50-100 nm diameter), whereas (**B, D**) si-NP 2 has formed undesirable, large and polydisperse aggregates. (**E**) Both si-NPs display near-neutral surface charge (zeta potential). Error bars represent standard error.

Figure 3. Agarose gel retardation assay to assess si-NP siRNA loading efficiency. The disappearance of siRNA bands at the gel bottom indicate complexation of siRNA to polymer. Polymer-complexed siRNA is unable to migrate through the gel and is thus visualized at the top of the gel nearby the loading wells. As the N^+:P^- ratio is increased, siRNA complexation increases, as indicated by decreased intensity of the siRNA band at the gel bottom.

Figure 4. Si-NP-mediated knockdown of the model gene Luciferase in Luciferase MDA-MB-231 cells. Luciferase activity at (**A**) 24 h and (**B**) 48 h post-treatment with either scrambled si-NPs or luciferase si-NPs at 10 N^+:P^- ratio and 100 nM siRNA dose. %Luciferase Activity is calculated by dividing the luminescence signal of treatment samples (scrambled and luciferase) by the luminescence signal of untreated cells. NOTE: si-NP 1 represents an effective formulation with gene silencing bioactivity, whereas si-NP 2 represents a formulation without gene silencing bioactivity. (*) indicates a statistically significant difference ($p < 0.05$) compared to the Scrambled group for a formulation at a given timepoint. Error bars represent standard error.

DISCUSSION:

The si-NPs described here are formed by electrostatic association of anionic siRNA and cationic polymers into polyion complexes (polyplexes). Electrostatic complexing of siRNA and the cationic DB block of PEG-DB polymers is facilitated by mixing at low pH (4.0). At pH 4.0, DMAEMA is highly protonated, and consequently the DB block is highly charged. This ensures that the polymers dissolve as unimers in solution as opposed to forming micelles and that DB complexes efficiently with siRNA. Subsequently, the pH of solution is adjusted to neutral (pH 7.4), causing 'hydrophobization' of the DB block, micelle formation, and entrapment of the complexed siRNA within the core of the resulting polyplexes. These polyplexes are designed such that PEG forms a hydrophilic, inert shell around the DB/siRNA core, resulting in neutral surface charge that is necessary for systemic administration. The polymer chemistry and process of polyplex formation are outlined in **Figure 1**.

Rigorous physicochemical characterization of si-NPs is essential for determining whether

formulations are appropriate for moving into biological testing. Size, surface charge, and particle shape/morphology are all important parameters that can impact biological performance. For systemic delivery to tumors, si-NP size should fall between 20 – 200 nm diameter[22], and most recent studies suggest 20 – 50 nm diameter particles are ideal[23]. Surface charge should be neutral or slightly negative to minimize protein adsorption and opsonization[28]. Studies have investigated the connection between particle shape and pharmacokinetics/particle clearance, suggesting particles with high aspect ratio are desirable over spherical particles[29-31]. However, to date spherical particles are still most commonly employed, and to our knowledge, are the only particle shape to have been translated to human studies in oncology.

It is important to note that the uniform and consistent formation of polyplexes, such as the si-NPs presented here, is dependent upon a range of physicochemical parameters. We have found empirically that polyplex concentration, pH of the solution, and the ratio of polymer to siRNA (N^+:P^- ratio) all drastically impact polyplex formation. In our hands, siRNA concentration does not have a large impact on polyplex formation, but using polymer concentrations in excess of 3.33 mg/mL results in formation of large aggregates and inconsistent polyplex size. As these si-NPs are pH-responsive, a variety of problems can arise when the final pH of si-NP solutions is too acidic (< pH 7.2). Two ways to prevent these complications are to lyophilize polymers in pure diH_2O so that there are no salts to change buffer composition upon dissolution and to re-make buffers frequently to prevent the "drifting" of buffer pH over time. To be cautious, the pH of buffers should be confirmed each time before making si-NPs, and the final pH of si-NP solutions should always be verified. Finally, the N^+:P^- ratio of si-NPs impacts siRNA loading efficiency and polyplex physicochemical parameters. There typically exists an ideal N^+:P^- ratio at which all siRNA is loaded but there is not an overabundance of un-complexed polymer which forms separate populations of micelles and increases cytotoxicity. For the si-NPs presented here, N^+:P^- 10-20 is the range in which optimum consistency and performance have been observed.

Luciferase reporter cell lines are used here for the rapid and high-throughput analysis of si-NP bioactivity. Luciferase reporter cell lines must be generated or purchased before starting bioactivity analysis, thus requiring an initial investment in time and expense. However, use of the luciferase reporter cells to assess gene silencing is faster, more amenable to high-throughput analysis, and cheaper over time than performing RT-PCR (to measure mRNA expression) or western blots (to measure protein expression). For instance, measuring luminescence in this assay typically takes just a few minutes as opposed to all-day or multi-day processes necessary to perform RT-PCR or western blots. Moreover, luminescence measurements can be conducted on well-plates, allowing for the simultaneous quantification of many samples (up to 96-well plates have been used by the authors). Lastly, D-luciferin is the only reagent needed above and beyond typical cell culture reagents, making the method much more affordable than RT-PCR or western blot. It should be noted however, that the luciferase reporter cell assay is limited to only assessing gene silencing of the model gene luciferase and cannot be used to measure gene silencing of other "therapeutic genes" of interest. Thus, the authors refer readers to several studies where RT-PCR and/or western blot has been used to assess gene silencing of therapeutic genes of interest[24,32-34].

In addition to bioactivity, researchers should ideally consider a comprehensive gamut of in vitro biological tests to characterize si-NP performance. The luciferase assay described above can also be used to assess cell viability by comparing the luminescence signal of scrambled si-NP treated cells to untreated cells. Since the siRNA is a non-targeting sequence, any change in luminescence can be attributed to non-specific impact of the si-NPs on cell viability, and this effect is typically dependent on the polymer chemistry and molar amount. Techniques in flow cytometry and fluorescent microscopy can be used to assess cell uptake of si-NPs using fluorescently-labeled siRNAs, polymers, or both. Additionally, molecular techniques such as stem-loop PCR and Argonaut 2 immunoprecipitation can be used to precisely measure intracellular siRNA levels. Since siRNA is only bioactive if delivered to the cytosol, where it is loaded into RISC, si-NPs must trigger endosomal escape of siRNA once internalized by the cell. Assays used to experimentally measure endosomal escape include the red blood cell hemolysis assay (described in detail by Evans et al.[35]), fluorescent imaging of the colocalization of fluorescently labeled si-NP cargo and LysoTracker[36], and most recently, fluorescent imaging of the recruitment of Galectin 8 to punctured endosomal vesicles[37,38]. Gathering data and synthesizing the results from in vitro experiments for cell viability, cell uptake, endosomal escape, and bioactivity provide the researcher complementary pieces of information from which to draw interpretations and garner mechanistic insight about si-NP (in)effectiveness.

In sum, nanoparticles capable of efficiently delivering siRNA have tremendous potential to treat disease. For example, in oncology, many genes that cause cancer progression, resistance to therapy, and metastasis are 'undruggable' by conventional pharmacology but could be treated using siRNA. However, prerequisite to their use in animal models of disease, siRNA nanomedicines (referred to here as si-NPs) require extensive physicochemical and biological characterization to ensure both their safety and effectiveness. To this end, methods have been described herein to produce and characterize si-NPs in vitro, by which the si-NPs can be assessed for suitability to carry forward into animal studies.

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DISCLOSURES:

The authors disclose no potential conflicts of interest.

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35 Evans, B. C. et al. Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. Journal of Visualized Experiments. (73), e50166 (2013).
36 Werfel, T. et al. Combinatorial Optimization of PEG Architecture and Hydrophobic Content Improves siRNA Polyplex Stability, Pharmacokinetics, and Potency In vivo. *Journal of Controlled Release.* (2017).
37 Kilchrist, K. V., Evans, B. C., Brophy, C. M., Duvall, C. L. Mechanism of Enhanced Cellular Uptake and Cytosolic Retention of MK2 Inhibitory Peptide Nano-polyplexes. *Cellular and Molecular Bioengineering.* **9** (3), 368-381 (2016).
38 Kilchrist, K. V. et al. Gal8 Visualization of Endosome Disruption Predicts Carrier-Mediated Biologic Drug Intracellular Bioavailability. *ACS Nano* (2019).

Figure 1

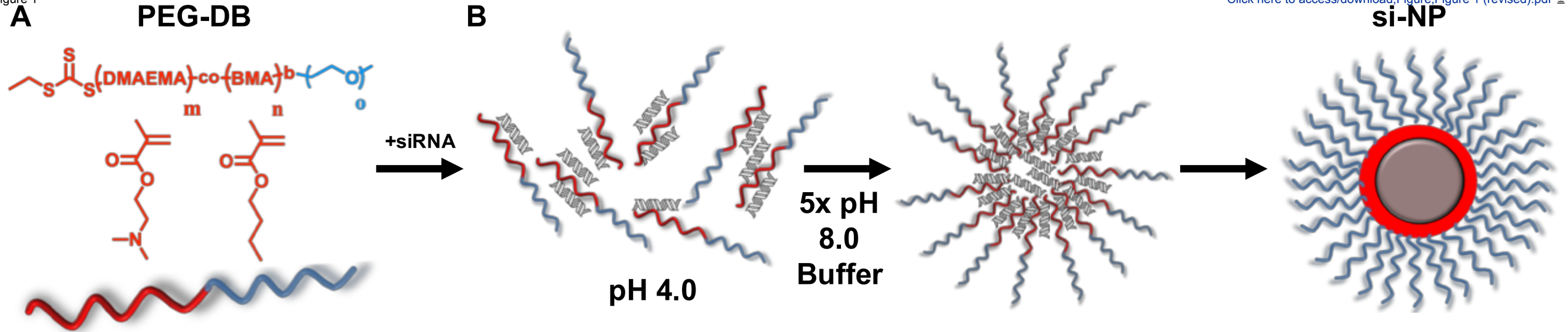
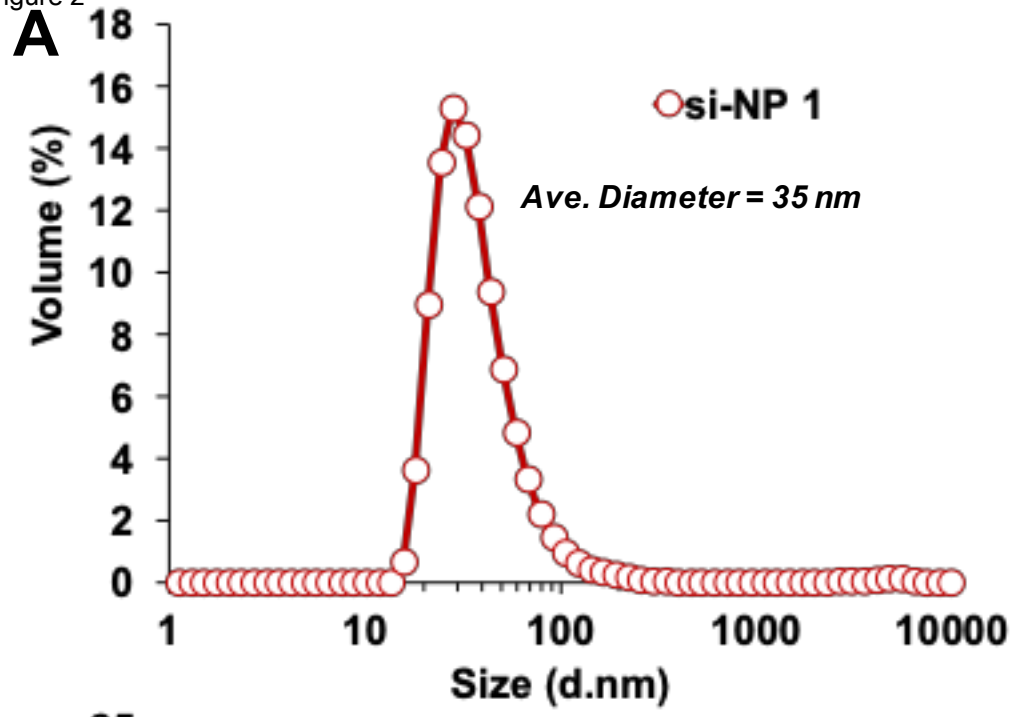
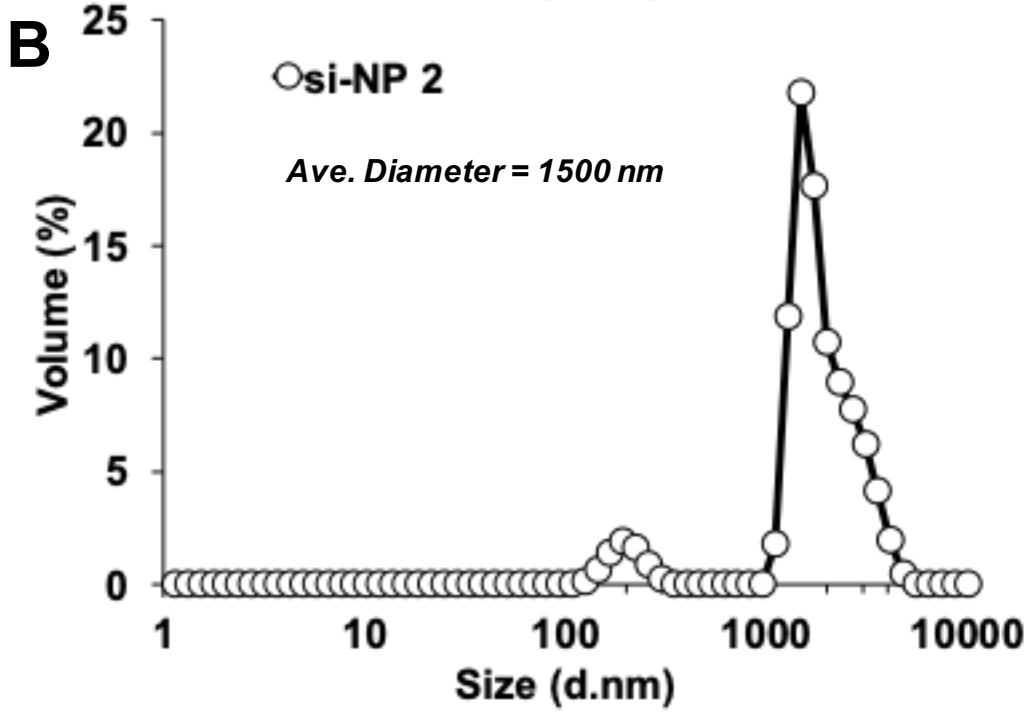
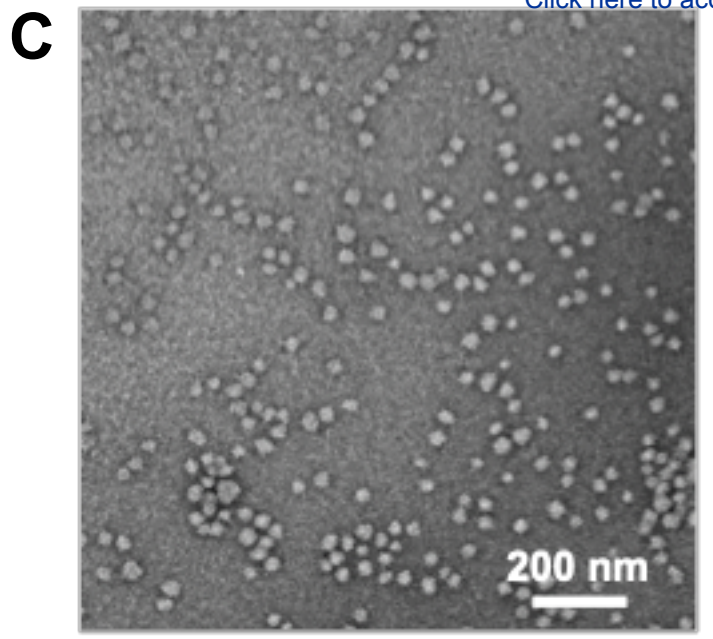
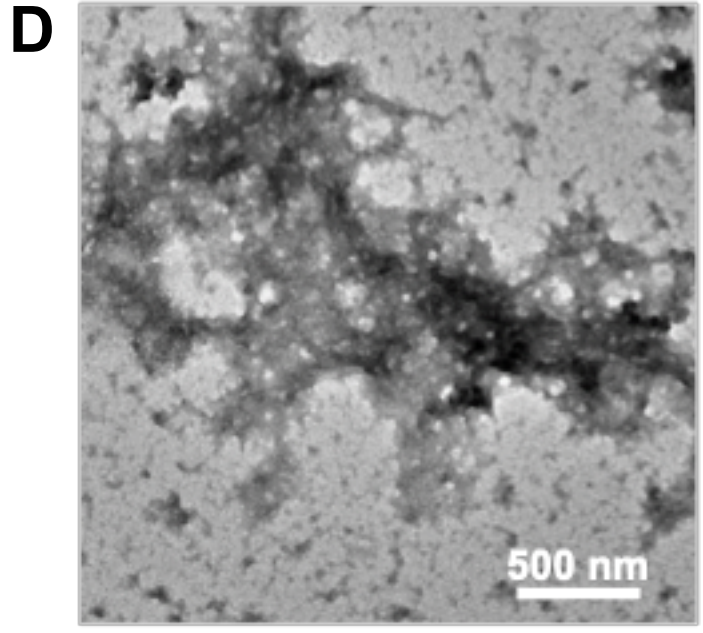


Figure 2









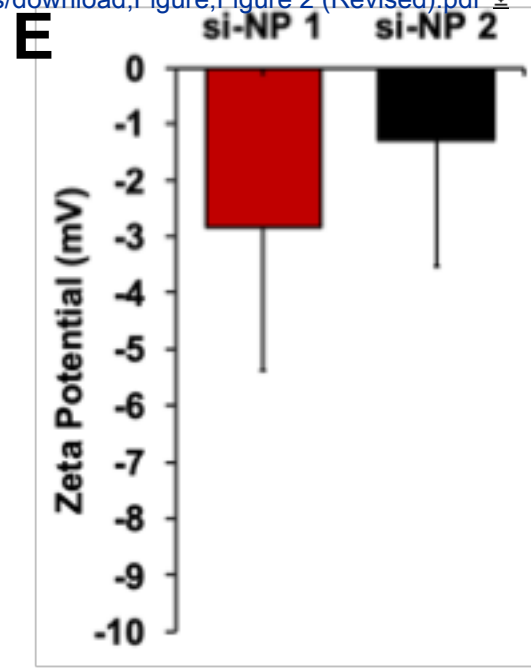


Figure 3

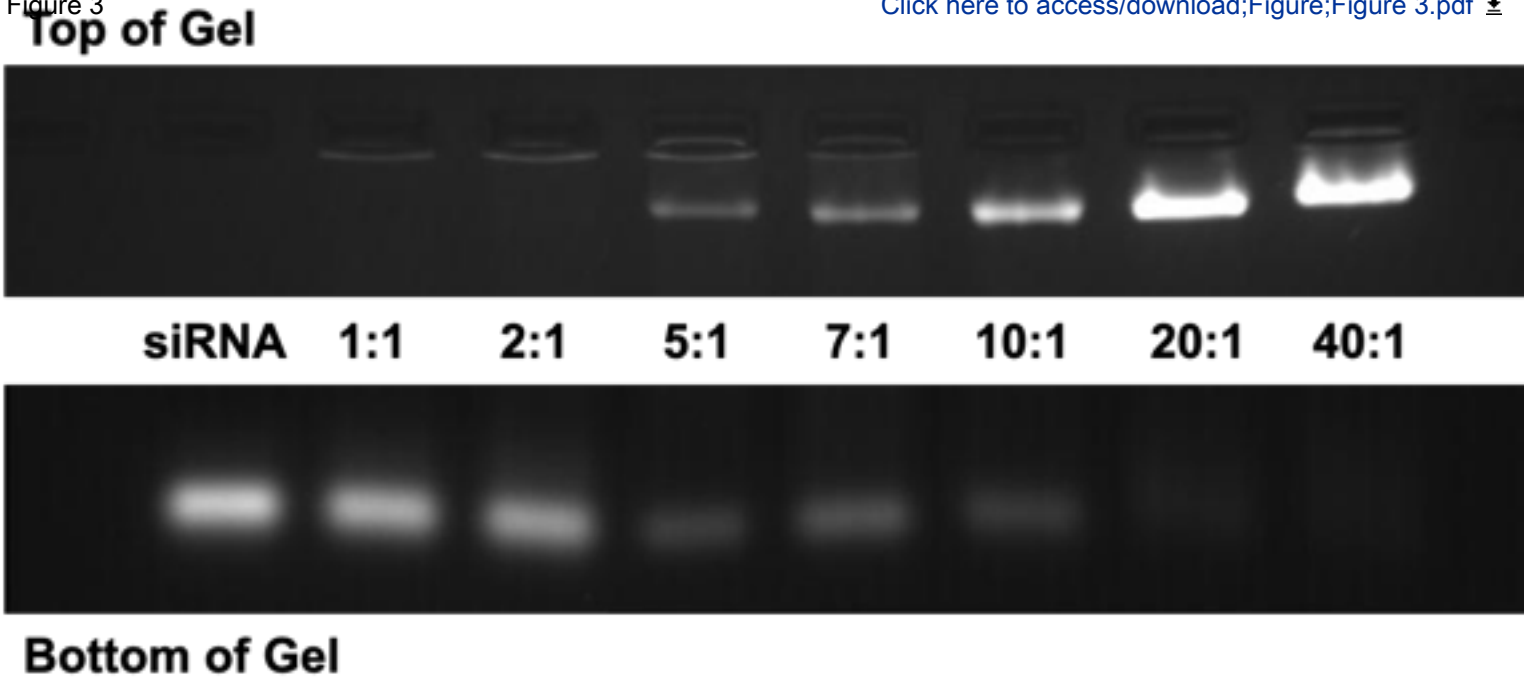


Figure 4

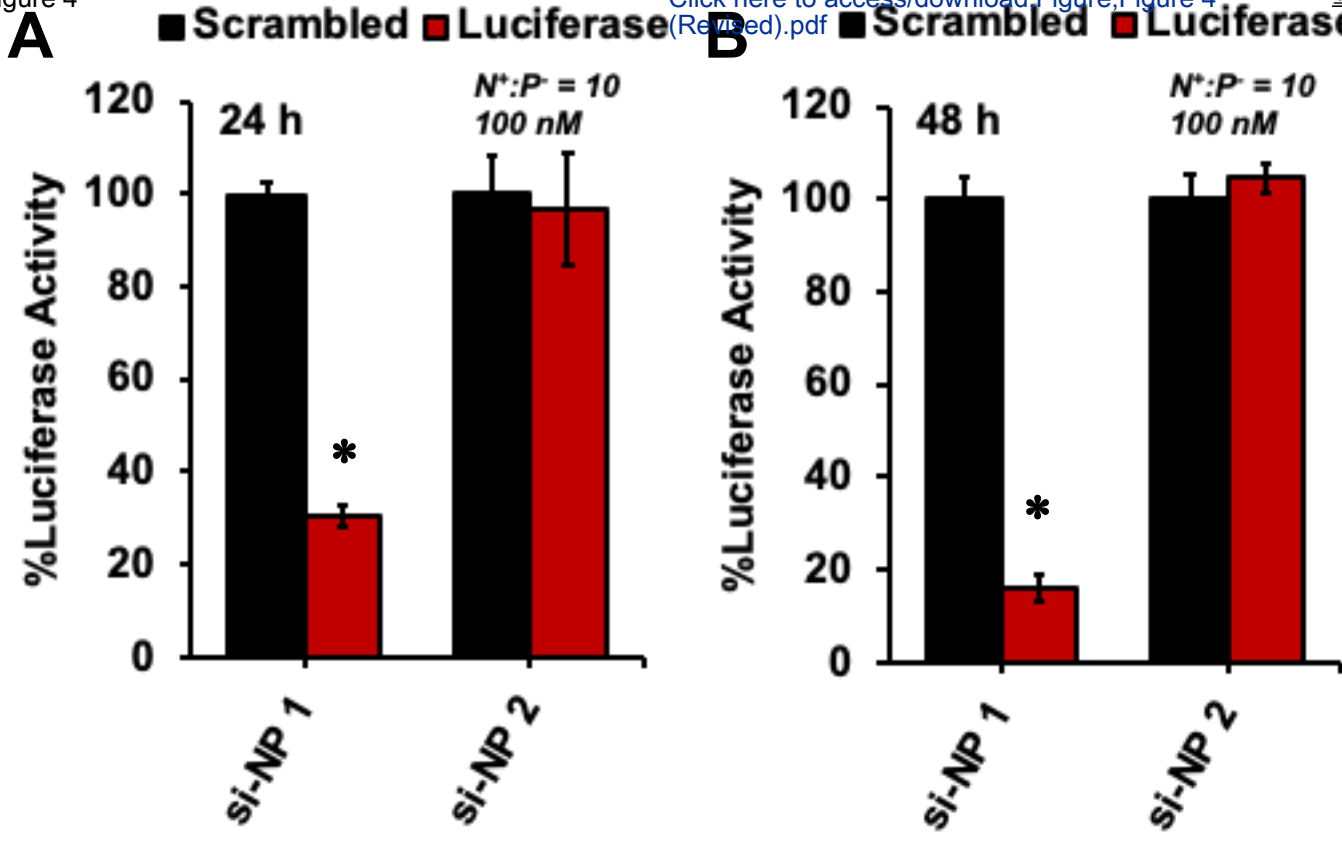


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.45 μm pore-size syringe filte	Thermo Fisher Scientific	F25133	17 mm diameter, PTFE membrane
0-14 pH test strips	Millipore Sigma	P4786	
10x TAE buffer	Thermo Fisher Scientific/Invitrogen	AM9869	
6-7.7 pH test strips	Millipore Sigma	P3536	
96-well black walled plates	Corning	3603	Tissue-culture treated
Agarose Powder	Thermo Fisher Scientific/Invitrogen	16500	
Citric acid monohydrate	Millipore Sigma	C1909	
dibasic sodium phosphate dihydrate	Millipore Sigma	71643	
D-luciferin	Thermo Fisher Scientific	88294	Monopotassium Salt
DMEM	Gibco	11995065	High glucose and pyruvate
Ethanol	Millipore Sigma	459836	
ethidium bromide	Thermo Fisher Scientific/Invitrogen	15585011	
FBS	Gibco	26140079	
loading dye	Thermo Fisher Scientific/Invitrogen	R0611	

Luciferase siRNA	IDT	N/A	Antisense Strand Sequense: GAGGAGUUCAUUAUCAGUGCAAUUGUU Sense Strand Sequense: CAAUUGCACUGAUAAUGAACUCCT*C* *DNA bases
MDA-MB-231 / Luciferase (Bsd) stable cells	GenTarget Inc	SC059-Bsd	Luciferase-expressing cells sued to assess si-NP bioactivity
monobasic sodium phosphate monohydrate	Millipore Sigma	S9638	
Scarmbled siRNA	IDT	N/A	Antisense Strand Sequense: AUACGCGUAUUAUACGCGAUUAACGAC Sense Strand Sequense: CGUUAAUCGCGUAUAAUACGCGUA*T* *DNA bases
square polystyrene cuvettes	Fisher Scientific	14-955-129	4.5 mL capacity
TEM grids	Ted Pella, Inc.	1GC50	PELCO Center-Marked Grids, 50 mesh, 3.0mm O.D., Copper
Trisodium citrate dihydrate	Millipore Sigma	S1804	
uranyl acetate	Polysciences, Inc.	21447-25	

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Title of Article: Preparation of neutrally-charged, pH-responsive polymeric nanoparticles for cytosolic siRNA delivery

Author(s): John Hendershot, Adam E Smith, Thomas A Werfel

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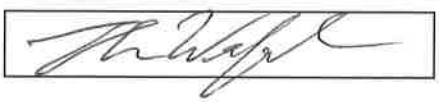
Signature:	
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Response to Review of JoVE59549 - Preparation of neutrally-charged, pH-responsive polymeric nanoparticles for cytosolic siRNA delivery

We thank the reviewers for their insightful reviews of our work. We believe that we have adequately addressed all reviewer concerns and accordingly, this revision is greatly improved. Particularly, both reviewers requested that we include more information of the luciferase reporter cell line used for assessing siRNA gene silencing in Protocol Section 2. Thus, we have included the information and source of the luciferase cell line in the protocol and have added this information to the table of materials as well. Moreover, we have taken care to update the narrative of the manuscript such that it highlights in vitro si-NP characterization. The goal of this manuscript is to highlight the imperative experiments for in vitro si-NP characterization prior to performing in vivo studies. We believe that the use of methods presented herein will help researchers ensure that novel si-NP formulations are appropriate for carrying forward into in vivo studies.

In addition to the reviewers' concerns, we have taken care to address all formatting issues brought to light by the JoVE editorial staff. We thank you for the opportunity to contribute a timely and important topic to the journal.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
We have thoroughly proofread this revised version of the manuscript.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in imperative voice: 1.2.1.
2) Please split up steps so that each step contains no more 3-4 actions and 4 sentences. For example, 1.2.3.1 is too long and needs to be split up.
We have, to our knowledge, changed all text to the imperative tense and broken up steps that were longer than 3-4 actions or 4 sentences.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 2.1.2: which cell line was used? Mention culture conditions such as temperature and humidity.
We have added information on the cell line to both the manuscript and table of materials.

2) Please describe the imaging step in detail. Mention acquisition conditions etc.
For details about specific instrument use, we have referred readers to the manufacturer's specifications for the particular instrument that researcher is using. We do this because there are so many different companies and models which produce various instruments that to go into detail about our specific process is 1) very difficult due to the restriction of not using "commercial sounding language", and 2) irrelevant to any researcher using a different model instrument.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion

should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
We believe that the discussion section of the revised manuscript addresses each of these categories.

• **Figure/Table Legends:**
1) Fig 4: define error bars.
We have defined the error bars in the figure legend. The error bars represent standard error of the means.

• **References:** Spell out journal names.
We have fully spelled out all journal names in the references section.

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We have removed both instances of commercial sounding language mentioned above.

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All figures are original to this publication.

Reviewer #1:

Manuscript Summary:

Hendershot et al. describe the synthesis of pH-responsive diblock copolymers for delivery of siRNA and methods to characterize polymer/siRNA nanoplexes. They further offer a method for screening knockdown activity in cell culture.

Major Concerns:

The use of Luciferase reporter cells is provided as a mechanism for assessing knockdown. However, no details are provided as to how to generate these cells; at minimum, the plasmid used for the luciferase gene should be provided as this will be important for matching to this siRNA sequence. Ideally, a commercially available Luc expressing cell line would be used to screen knockdown, but if unavailable some additional details and/or relevant references where adequate

details are provided should be included for generation of Luc expressing cells. Also, assessment of KD via PCR or western analysis is more common; while its not necessary to include methods on these, it would be helpful to the reader to mention these and direct them to good protocols (for example, other Jove articles or Methods-type papers) in case they wanted to evaluate KD via another method.

Thank you for bringing this omission to our attention. We agree that it is essential to provide information about the source of the luciferase reporter cells and have thus added that information to the protocol and table of materials. Moreover, we agree that the use of a commercially available cell line is ideal to expand access of this assay and to minimize lab-to-lab variability. Accordingly, we use MDA-MB-231 / Luciferase (Bsd) stable cells (GenTarget Inc., San Diego, Ca) to conduct screens of luciferase knockdown by siRNA. These cells are generated by GenTarget Inc. by transduction with lentivirus expressing the firefly luciferase gene. Luciferase is expressed under a constitutive CMV promoter, and Blasticidin resistance is expressed under Rsv promoter to maintain stable luciferase expression.

Updated Section of Protocol:

> *"Seed luciferase-expressing cells [MDA-MB-231 / Luciferase (Bsd) stable cells (GenTarget Inc., San Diego, Ca)] in 96-well black-walled plates at a density of 2,000 cells per well. Allow to adhere overnight in full media (DMEM, 10% FBS) in an incubator (37 ℃, 5% CO_2, 95% humidity).*
>
> *Dilute si-NPs into full serum media for a final volume of 100 μL per well and siRNA concentration of 100 nM. Treat cells for 24 h with si-NPs.*
>
> *After 24 h, remove treatments and replace media with full serum media containing 150 μg/mL D-luciferin. Incubate cells for 5 min before measuring luminescence on a plate reader or in vivo optical imaging system according to the manufacturer's specifications.*
>
> *Replace luciferin-containing media with fresh, full serum media, and incubate 24 h more. Repeat the step above, removing media and replacing with full serum media containing 150 μg/mL D-luciferin, followed by a 5 min incubation prior to measuring luminescence at the 48 h timepoint."*

Additionally, we have added to the discussion to briefly examine the use of western blotting (to assess protein expression) and RT-PCR (to assess mRNA levels) to screen siRNA knockdown, endeavoring to highlight how these methods compare and contrast with the use of luciferase reporter cells. Specifically, we have described the advantages and disadvantages of using the luciferase reporter cells for knockdown assays as compared to using western blotting or PCR.

Updated Section of Discussion:

> *"Luciferase reporter cell lines are used here for the rapid and high-throughput analysis of si-NP bioactivity. Luciferase reporter cell lines must be generated or purchased before starting bioactivity analysis, thus requiring an initial investment in time, expense, or both. However, use of the luciferase reporter cells to assess gene silencing is faster, more amenable to high-throughput analysis, and cheaper over time than performing RT-PCR (to measure mRNA expression) or western blots (to measure protein expression). For instance, measuring luminescence in this assay typically takes just a few minutes as opposed to all-day or multi-day processes necessary to perform RT-PCR or western blots. Moreover, luminescence measurements can be conducted on well-plates, allowing for the*

simultaneous quanitification of many samples (up to 96-well plates have been used by the authors). Lastly, D-luciferin is the only reagent needed above and beyond typical cell culture reagents, making the method much more affordable than RT-PCR or western blot. It should be noted however, that the luciferase reporter cell assay is limited to only assessing gene silencing of the model gene luciferase and cannot be used to measure gene silencing of other "therapeutic genes" of interest. Thus, the authors refer readers to several studies where RT-PCR and/or western blot has been used to assess gene silencing of therapeutic genes of interest[24,31-33]."

Minor Concerns:

1. The choice of PEG-PDB as an abbreviation is a bit confusing considering the second block contains only 2 monomers - D and B.
We agree that the 'P' is misleading as used. We have changed all instances of 'PEG-PDB' to 'PEG-DB'.

2. "The siRNA nanoparticles are prepared by electrostatic complexation of siRNA and the diblock copolymer poly([(ethylene glycol)-b-[(2-(dimethylamino)ethyl methacrylate)-co- (butyl methacrylate)]) (PEG-b-PDB) to form polyion complexes (polyplexes) where siRNA is sequestered within the polyplex core and PEG forms a hydrophilic, neutrallycharged corona." -open square bracket, before ethylene glycol, either remove or place before poly and before b to define the block, or remove
Thank you for the suggestions to edit the full chemical name for clarity and accuracy. We have updated the diblock polymer nomenclature to:
poly(ethylene glycol-b-[2-(dimethylamino)ethyl methacrylate-co-butyl methacrylate])

3. "Cationic DMAEMA condenses negatively-charged siRNA."
97-98 It's more accurate to say that cationic DMAEMA electrostatically complexes negatively charged siRNA, which facilitates siRNA condensation. At least use the term on line 239, electrostatically condenses.
Yes, it is more accurate to use the term electrostatically complexes to describe this phenomenon. We have changed the term 'condenses' to 'electrostatically complexes.'

4. "Under acidic conditions, such as those within the endolysosomal pathway, the PDB block both facilitates the proton-sponge effect through ionizable amines of DMAEMA and actively integrates into and lyses lipid bilayers through hydrophobic BMA moieties, resulting in potent endosomolysis."
101-104 Proton sponge effect has no dash, and this is the first mention of proton sponge in the methods and therefore should have a source and/or further explanation of proton sponge.
We have removed the dash from proton sponge, referenced prior literature that explains the mechanism in detail, and added a sentence here for clarity and readability. Thank you for pointing this out.

Updated Text Reads:
"Under acidic conditions, such as those within the endolysosomal pathway, ionizable DMAEMA within the DB block facilitates the proton sponge effect, where endosomal buffering leads to osmotic swelling and rupture[26]. Additionally, hydrophobic BMA moieties within the DB block actively integrate into and lyse lipid bilayers, resulting in potent endosomolysis."

5. 123 Almost every transfection agent provides a mass ratio for their products in a table (i.e. add X uL of 3.33 mg/mL polymer for 25ug RNA injection). This protocol could be enhanced with an example table for a 25 BP siRNA and a carrier.
We agree that clarifying the mass ratio will help with ease of formulation and provide a good reference to other commonly employed transfection reagents. We have added this information in the following text (lns. 200-202):

> *"The mass ratio of PEG-DB to siRNA at 10 N^+:P^- ratio and 100 nM siRNA treatments (assuming 26 bp siRNA) is 23.3, i.e. add 23.3 ng PEG-DB for every 1.0 ng siRNA. For example, add 1.16 μL of 3.33 mg/mL polymer for 166.5 ng siRNA to treat one well at 100 nM in a 96-well plate (100 μL media volume)."*

6. 1.2 (141) You should probably test at an NP ratio according to your gel results from 1.2.3. It should be around 10 N:P, but depending on the polymer properties it could be different. If the number is 10, you probably don't need to include all of the NPs for the gel, just test N:P of 10 and state that if the band runs, you need to run multiple NPs on a gel to determine the NP for in vitro bio-activity. You should run the gel first in case the NP of 10 is not appropriate for the synthesized polymer, and you can do sizing, surface charge, and morphology determination at the appropriate NP.
We agree with the approach laid out here by the reviewer. This is the approach we typically take when developing new si-NPs and which we have highlighted within this manuscript. With regards to the N^+:P^- ratio, we do start by running the gels first to confirm the optimal N^+:P^- ratio (or range of N^+:P^- ratios) for efficiently complexing siRNA. Accordingly, we tested for the knockdown of si-NPs at 10 N^+:P^- ratio, which shows siRNA complexation and as we have discovered empirically elicits an optimal combination of gene silencing and biocompatibility. We have updated Figure 4 and accompanying text to clarify that these results were obtained with si-NPs formulated at 10 N^+:P^- ratio.

7. 163,159 TAE is Tris-acetate-EDTA buffer, and I believe it should be about pH 8 to run a gel. Please confirm these details and include them in the protocol
Yes, the reviewer is correct. We have updated the manuscript to define TAE and indicate that it is used at pH 8 for running agarose gel electrophoresis.

8. 178 - whichever N:P ratio is appropriate. You even write in the conclusion that 10-20 is usually optimal, so it would make more sense to explore which NP to use for a new polymer with similar properties.
The reviewer brings up another good point about the N^+:P^- ratio here. We should change the wording to indicate that ideal N^+:P^- ratios should be determined by the gel electrophoresis assay and used accordingly. For the si-NPs used here, we have empirically found that 10 N^+:P^- ratio displays an ideal balance of target gene silencing and biocompatibility. However, as the reviewer points out, this could change for different polymers and/or si-NP formulations. We have updated the text in line 178 accordingly.

9. 203/Figure 2 should include surface charge characterization, as it was mentioned in the abstract, intro, and throughout the method.
We apologize for this omission. We have added surface charge characterization data to Figure 2, as shown below.

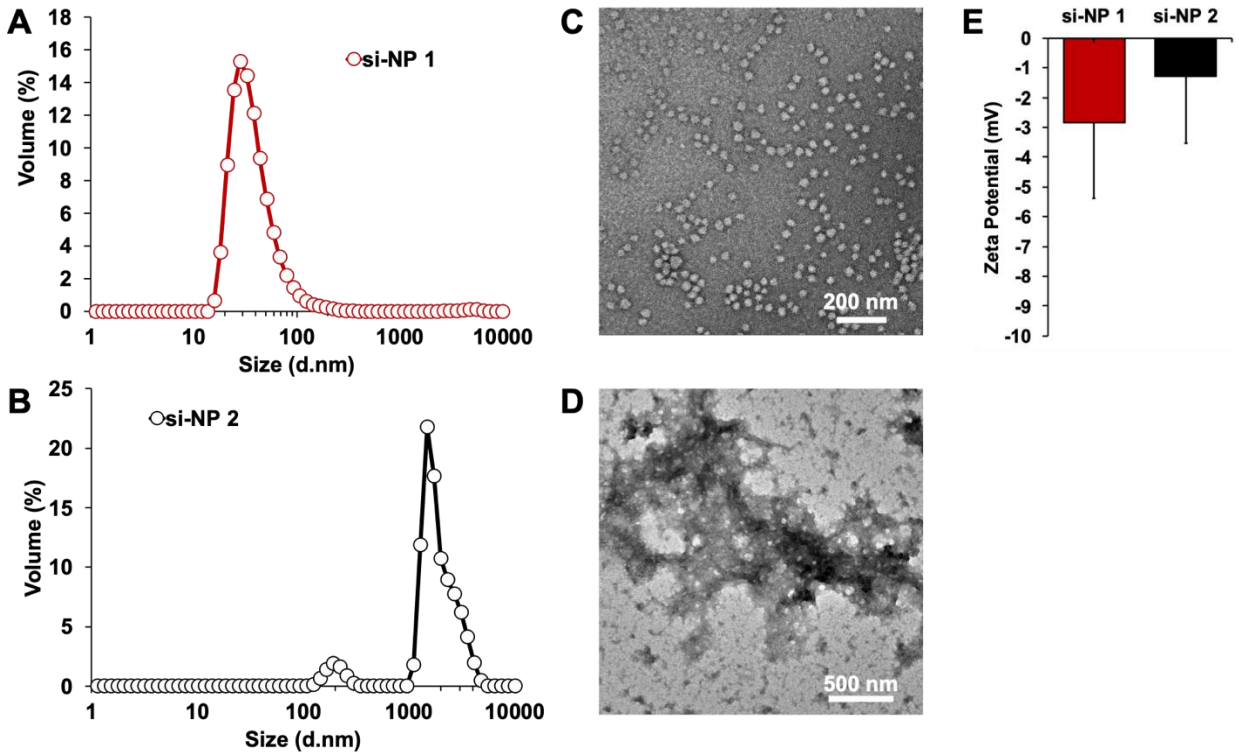

Figure 2. DLS and TEM characterization of si-NP size, morphology, and surface charge. *(A, C) si-NP 1 represents a uniform sample with appropriate size (~50-100 nm diameter), whereas (B, D) si-NP 2 has formed undesirable, large and polydisperse aggregates. (E) Both si-NPs display neutral surface charge (zeta potential).*

10. Figure 1 - The final si-NP in 1B looks to be part of 1A. Consider reformatting the figure for clarity.
We have reformatted Figure 1 to make it less confusing. Thank you for this suggestion.

11. Figure 2 - Would it be possible to use the same scale for both TEM images?
These images are acquired at different magnification and thus, should not be manipulated to appear at the same scale. The images were acquired at different levels of magnification because of the extremely large size of the aggregates of "si-NP 2". It was difficult to appreciate the scale and morphology of the aggregates at the higher magnification that was used to image "si-NP 1".

12. Figure 4 - It would be very nice to show significance here, but probably not necessary.
Statistical significance is now indicated on Figure 4.

We thank the reviewer for their detailed and constructive review of the manuscript. We are confident that the revision is much improved due to their insightful comments.

Reviewer #2:

Manuscript Summary:

The article described the preparation of siRNA nanoparticles (siNP) by electrostatic complexation of siRNA and the diblock copolymer (PEG-b-PDB) to form polyion complexes where siRNA is

sequestered within the polyplex core and PEG forms a hydrophilic, neutrally charged corona. The complex with a relative small size should have better tumour tissue penetration and can exploit a charge reversal in tumour which allow endosomal escape, thus release siRNA effectively. This is excellent formulation design to the reviewer, and is a fine example of how drug delivery science helps deliver siRNA to the target, make the undruggable large molecules druggable. In general terms the manuscript is well-prepared and easy to follow, especially the Introduction. I believed the authors have followed the journal format, structure (but no video was found).

We thank the reviewer for these positive comments about our manuscript and are glad they found our work both interesting and useful.

Major Concerns:

*48 h results are presented but information in PROTOCOL is missing
Thank you for pointing out this unintentional omission. We have updated the protocol to include the 48 h timepoint in addition to the existing text (ln. 189) that informs the reader how to perform a longitudinal version of the assay.

*Long-circulation is curtail for site target delivery, it would be useful if a simple release study demonstrate this property is included using the biological test.
*We agree with the reviewer that long-circulation is an essential **in vivo** characteristic for si-NPs to achieve efficient delivery to tumors. However, it is not our goal in this manuscript to describe the in vivo properties of these si-NPs. We believe that doing so would cloud the focus of the current manuscript and a separate article is warranted to discuss in vivo experimental approaches. Our goal in the current manuscript is to highlight the imperative **in vitro** characterizations that are necessary to ensure newly-developed si-NP formulations are appropriate to carry forward for in vivo administration. This has always been our approach, and we believe that the experiments described in the current manuscript will assist researchers as they develop novel si-NP formulations in vitro with future ambitions of administering the si-NPs in vivo in pre-clinical animal models. We refer the reviewer elsewhere to several published studies within which we have described in vivo results using these si-NPs in detail[1-5].*

Minor Concerns:

Luciferase siRNA should be introduced in Introduction.
We agree that this would help the flow of the manuscript and reduce confusion when the luciferase gene silencing assay is presented later in the protocol. We have updated the Introduction with the following text:

> *"In order to rapidly assess si-NP bioactivity, Luciferase is used as a model gene for knockdown studies. Firefly Luciferase is the protein responsible for the 'glow' of fireflies[27]. Accordingly, mammalian cells transfected with the firefly luciferase gene produce a bioluminescent 'glow' that can be captured using a luminometer to quantify levels of Luciferase expression. Here, we use Luciferase to assess bioactivity of si-NPs by delivering siRNA against Luciferase and quantifying the corresponding reduction in bioluminescence in Luciferase-expressing cells compared to cells that receive a scrambled siRNA."*

SUMMARY is misleading as there was no in vivo study to prove this conclusion.
We agree that our development of the narrative for this manuscript could be misleading. We have revised the summary (as shown below) to de-emphasize the focus form in vivo studies and have

*done the same throughout various parts of the manuscript. Again, our goal is to highlight that the goal of our research is to use these si-NPs to treat disease in pre-clinical animal models aimed at future clinical translation, but also that the focus of this manuscript is to highlight the steps that must be taken to properly characterize si-NPs **in vitro** to ensure their suitability for administration to animals.*

Updated Summary:

> *"Methods to prepare and characterize the physicochemical properties and bioactivity of neutrally-charged, pH-responsive siRNA nanoparticles are presented. Criteria for successful siRNA nanomedicines such as size, morphology, surface charge, siRNA loading, and gene silencing are discussed."*

loading efficiency: Is N+:P ratio molar or weight ratio? And why name the ratio of polymer to siRNA 'N+:P ratio'?

The N^+:P^- ratio is a molar ratio. As described in Protocol section 1.1.2.:

> *"N^+:P^- ratio represents the number of positively-charged amine groups on the polymer to the number of negatively-charged phosphate groups on the siRNA and is calculated by the formula below:*
>
> $$N^+ : P^- = \frac{(mol\ Pol)(RU\ amine)}{(mol\ siRNA)(bp\ siRNA)(2)}$$
>
> *Where, mol Pol is the molar amount of polymer, RU amine is the number of repeating units of positively-charged amines per polymer, mol siRNA is the molar amount of siRNA, and bp siRNA is the number of base pairs per siRNA molecule."*

The authors stated the optimal size and gave a Figure, but is hard to read the real size range from a Figure.

We have updated Figure 2 to include the average size for each formulation and have added this information to the manuscript text as well.

Please provided cell type for the luciferase-expressing cells

We apologize for the omission of this information from the original manuscript. As described in detail in response to a major concern of Reviewer 1 above, we have revised the manuscript to include information on the cell line and its source.

The conclusions are not supported by adequate experimental data. Authors need to revised by focusing on the existing results.

As outlined above, we have updated the manuscript to de-emphasize in vivo aspects of si-NP development and focus on the initial in vitro characterization of si-NPs. We believe that adequate data is provided to support any claims made within the manuscript about in vitro si-NP characterization.

References are not all up to date. Many newer publications are easily found.

*We have endeavored to reference and include as many relevant and recent publications as possible. We are not aware of where we have omitted relevant references but are more than willing to add appropriate references if identified by the reviewer. Moreover, we have taken care to reference original articles where findings were **first** published. We do not believe the age of the reference is the primary concern, rather its content and relevance. However, many references to papers published within the last five years are included.*

We thank the reviewer for their constructive feedback and assistance in focusing the narrative of our manuscript on in vitro si-NP characterization.

1 Miteva, M. *et al.* Tuning PEGylation of mixed micelles to overcome intracellular and systemic siRNA delivery barriers. *Biomaterials.* **38** 97-107, doi:http://dx.doi.org/10.1016/j.biomaterials.2014.10.036, (2015).

2 Sarett, S. M. *et al.* Hydrophobic interactions between polymeric carrier and palmitic acid-conjugated siRNA improve PEGylated polyplex stability and enhance in vivo pharmacokinetics and tumor gene silencing. *Biomaterials.* **97** 122-132, doi:http://dx.doi.org/10.1016/j.biomaterials.2016.04.017, (2016).

3 Jackson, M. A. *et al.* Zwitterionic Nanocarrier Surface Chemistry Improves siRNA Tumor Delivery and Silencing Activity Relative to Polyethylene Glycol. doi:10.1021/acsnano.7b01110, (2017).

4 Werfel, T. *et al.* Combinatorial Optimization of PEG Architecture and Hydrophobic Content Improves siRNA Polyplex Stability, Pharmacokinetics, and Potency In Vivo. *Journal of Controlled Release.* (2017).

5 Werfel, T. A. *et al.* Selective mTORC2 Inhibitor Therapeutically Blocks Breast Cancer Cell Growth and Survival. *Cancer Res.* **78** (7), 1845-1858, doi:10.1158/0008-5472.CAN-17-2388, (2018).