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The *in Vitro – in Vivo* Translation of Lipid Nanoparticles for Hepatocellular siRNA Delivery

Kathryn A. Whitehead[1], **Jonathan Matthews**[1], **Philip H. Chang**[1], **Farnaz Niroui**[1], **J. Robert Dorkin**[2], **Mariano Severgnini**[3], and **Daniel G. Anderson**[1,4,5,*]

[1]David H. Koch Institute for Integrative Cancer Research, Cambridge, MA

[2]Department of Biology, Cambridge, MA

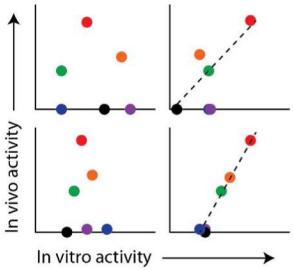
[3]Alnylam Pharmaceuticals, Cambridge, MA

[4]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139 USA

[5]Harvard-MIT Division of Health Science Technology Massachusetts Institute of Technology, Cambridge, MA 02139 USA

Abstract

A significant challenge in the development of clinically-viable siRNA delivery systems is a lack of *in vitro – in vivo* translatability: many delivery vehicles that are initially promising in cell culture do not retain efficacy in animals. Despite its importance, little information exists on the predictive nature of *in vitro* methodologies, most likely due to the cost and time associated with generating *in vitro – in vivo* data sets. Recently, high-throughput techniques have been developed that have allowed the examination of hundreds of lipid nanoparticle formulations for transfection efficiency in multiple experimental systems. The large resulting data set has allowed the development of correlations between *in vitro* and characterization data and *in vivo* efficacy for hepatocellular delivery vehicles. Consistency of formulation technique and the type of cell used for *in vitro* experiments was found to significantly affect correlations, with primary hepatocytes and HeLa cells yielding the most predictive data. Interestingly, *in vitro* data acquired using HeLa cells was more predictive of *in vivo* performance than mouse hepatoma Hepa1-6 cells. Of the characterization parameters, only siRNA entrapment efficiency was partially predictive of *in vivo* silencing potential, while zeta potential and, surprisingly, nanoparticle size (when < 300 nm) as measured by dynamic light scattering were not. These data provide guiding principles in the development of clinically-viable siRNA delivery materials and have the potential to reduce experimental costs while improving the translation of materials into animals.



*Correspondence should be addressed to D.G.A. (dgander@mit.edu) 500 Main St., 76-653, Cambridge, MA 02139, 617-258-6843 (phone) and 617-258-8827 (fax).

Keywords

siRNA delivery; liver delivery; nanoparticles; liposomes; lipidoids

The development of biomaterials for applications in drug delivery, stem cell therapy, and tissue engineering can be a laborious, resource-intensive process. In an attempt to maximize research productivity while reducing laboratory expenses, many biomaterials development efforts test materials *in vivo* only if favorable effects are first achieved *in vitro*.[1-3] In principle, this allows a lab to test and optimize a larger number of compounds in cell culture while minimizing costs associated with animal testing. Unfortunately, *in vitro* experiments often fall short in the prediction of *in vivo* activity, and the translation of initially promising cell culture results into animals remains a critical bottleneck in the development of clinically viable biomaterials.

A significant but understudied factor in the translation process is the potential for correlation between *in vitro* and *in vivo* experimental systems. It has been well-documented that many *in vitro* models do not recapitulate important features of native tissue, which may contribute to an inability to translate promising materials or drugs into higher-order animals.[4-6] In the area of materials development for applications in siRNA delivery, this translational challenge persists.[7,8] Unfortunately, little information is available on how *in vitro* and characterization data affect ultimate material utility in animals. Should sufficient data be available, it may be possible to make improvements to the experimental design of traditional *in vitro* models by analyzing empirical data sets.

Over the course of the last several years, efforts have been dedicated towards the high-throughput synthesis, testing, and translation of a class of lipid-like siRNA delivery materials, termed 'lipidoids'.[9-12] As a result of these studies, a large amount of data on the transfection performance of these materials both *in vitro* and *in vivo* has been collected. Herein, we describe the correlations, and lack thereof, that have been observed between the transfection ability of lipidoids in a number of *in vitro* systems, their characterization parameters and their *in vivo* efficacy. It is our hope that the conclusions drawn from this data set will better inform the choice of *in vitro* model systems and the selection of candidate materials for *in vivo* study, thereby improving our ability to identify efficacious materials with long-term potential for applications in siRNA delivery.

RESULTS

The *in vitro* – *in vivo* translation of siRNA delivery data presents a considerable challenge in the development of siRNA-based therapeutics. Ideally, transfection results from cell culture experiments would correlate directly with siRNA-mediated gene silencing in animal models. To understand the potential for the establishment of *in vitro* – *in vivo* transfection trends, let us consider Figure 1a, which displays relative *in vitro* gene expression on the x-axis and relative *in vivo* gene expression on the y-axis for a gene being targeted by an siRNA formulation. Perfectly correlated data would fall along a straight line at a 45° angle. Since the goal of this exercise is the identification of functional, *in vivo* siRNA delivery formulations, those that result in expression patterns where points fall above the 45° line would be classified as false positives, meaning that they display higher transfection potential in cell culture than they do in animals. Because siRNA delivery materials must negotiate far more obstacles *in vivo* (*e.g.* extravasation, avoidance of phagocytosis) than they do in cell culture, false positives can be expected and would be difficult to eliminate completely during experimental design. On the other hand, points that fall below the 45° line are false negatives, meaning that better gene silencing is mediated *in vivo* than *in vitro*. These points

are problematic, as many studies rely on cell culture data to identify the most promising candidates for animal studies. Accordingly, these 'false negative' delivery materials would be inadvertently eliminated from consideration, and their *in vivo* transfection potential would remain untapped.

One experimental design parameter that can influence the *in vitro* – *in vivo* trend is the choice of formulation technique of the delivery material. Most active delivery materials, including the lipidoids described in this study, are formulated into nanoparticles containing polyethylene glycol and other "helper" materials for *in vivo* use.[13-16] These additional formulation components have been shown to improve *in vivo* efficacy while reducing clearance and immune cell uptake. Because the preparation of nanoparticles, which traditionally involves time-consuming formulation steps such as extrusion, in-line mixing, and dialysis,[9] *in vitro* experiments sometimes make use of complexes formed through the simple mixing of negatively-charged siRNA and cationic delivery material.[9,17,18] These complexes are often larger, more heterogeneous in size, and may precipitate over time.[19,20]

We first asked whether or not the type of formulation procedure used *in vitro* (nanoparticle *versus* complex) affected the correlation of *in vitro* to *in vivo* silencing efficacy. Figures 1b and c show the correlation between *in vivo* and *in vitro* efficacy for complex-mediated and nanoparticle luciferase silencing, respectively, for over 100 lipidoids. These lipidoids were synthesized from the conjugate addition of alkyl-amines to either alkyl-acrylates, alkyl-acrylamides or alkyl-epoxides *via* a Michael addition reaction.[9,10] Lipidoids employed for this study possessed diverse chemical structure as is described in Figure 2. Alkyl-amines included primary, secondary and tertiary amines with linear and/or branched alkyl chains, benzene and/or piperazine rings, alcohols and/or ethers. All doses in Figure 1 were chosen to maximize the data spread. *In vivo* efficacy was determined by the ability of lipidoid nanoparticles (LNPs) to silence Factor VII (FVII), a model protein produced in hepatocytes in mice. Relative FVII activity values of 1 and 0 correspond to 0% and 100% silencing, respectively. *In vitro* efficacy was determined by quantifying luciferase knockdown in HeLa cells. Figure 1b reveals a lack of materials efficacy correlation when lipoplexes are used for transfection experiments in cell culture. Of most significant concern is the large population of false negatives in Figure 1b, particularly those falling along the x-axis.

Fortunately, the use of identical nanoparticle formulations both *in vitro* and *in vivo* resulted in a significant reduction in the number and severity of false negative data points (Figure 1c). Based on this data, animal testing could be performed on any LNPs producing >50% silencing in HeLa cells without losing any top materials. While the trend observed between *in vitro* LNP and *in vivo* LNP data (Figure 1c) leaves room for improvement, it is a better predictor of *in vivo* efficacy than when employing lipoplexes for cell culture experiments. Furthermore, the differences between Figures 1b and 1c were not simply due to a shifting of data points in the lower right quadrant. *In vitro* transfection results were globally altered when moving from lipidoid complex to LNP formulations (Figure 1d).

It was anticipated that the choice of cell line used for *in vitro* experimentation may also have a significant effect on translation. Because the *in vivo* system used in our lab involves hepatocellular liver delivery in the mouse, we asked whether or not the HeLa cells used to generate Figures 1b-d were the most appropriate choice of cell culture model. To test the potential value of using a cell line more closely related to our *in vivo* target, we examined *in vitro* transfection in the mouse hepatoma cell line Hepa1-6. All lipidoids for these experiments were formulated as nanoparticles containing siRNA specific against luciferase, which was stably expressed in the Hepa1-6 cells. Figure 3 demonstrates the correlation between relative Factor VII activity *in vivo* and relative luciferase activity in immortalized mouse hepatocytes *in vitro*. Surprisingly, the trend observed between *in vitro* and *in vivo*

data worsened when using this cell model. While there was a small area in the lower right quadrant of the graph where false negatives had been eliminated, this area was not as large as the one found in Figure 1c for HeLa cells.

In order to perform *in vitro* experiments using cells that were as morphologically similar to mouse liver cells as possible, we chose to work with freshly-isolated mouse hepatocytes. For these experiments, we were able to use Factor VII as a target (the same target used *in vivo*) since isolated hepatocytes retain some native gene function for 1-2 days post-isolation.[21,22] Dose-response data was collected for six LNPs in primary hepatocytes in order to identify the most appropriate siRNA dose for comparison with *in vivo* data (Figure 4a). A dose of 10 nM was selected, as it provided the maximal range of transfection results. Figure 4b shows an excellent correlation between *in vivo* and primary hepatocyte *in vitro* data ($R^2 = 0.99$) compared to other cell types for a set of six lipidoids (each represented by a different color). HeLa cell data for these six lipidoids also demonstrated fair correlation ($R^2 = 0.50$).

The effect of various nanoparticle characterization parameters on *in vivo* efficacy was also evaluated. The LNPs examined in this study possessed surface charges of −5.5 to 20 mV, as determined by zeta potential measurements under neutral pH conditions. These charges did not correlate to *in vivo* hepatocellular delivery in our system (Figure 5a). Additionally, we investigated the effect of nanoparticle diameter on *in vivo* efficacy, as size is often regarded as an important factor in delivery ability to various physiological targets.[23,24] All LNPs formulated in this study had diameters less than 300 nm. For these diameters, no correlation between size and *in vivo* efficacy was observed at an siRNA dose of 5 mg/kg (Figure 5b) or at lower siRNA doses (< 0.5 mg/kg, data not shown). It may be possible that particles larger than 200 nm experience reduced efficacy, although there are not enough data points in Figure 5b to state this conclusively. Most LNP populations were relatively monodisperse in diameter, with 90% of all formulations having a polydispersity index of < 0.2. To further explore the effect of size on efficacy, we formulated the lipidoid C12-200[10] into nanoparticles of varying size ranging from 65 to 135 nm in diameter. Each nanoparticle formulation was compositionally identical. All C12-200 particles achieved the same level of Factor VII silencing *in vitro*, independent of size (Figure 5c).

Entrapment efficiency as determined by a ribogreen assay, which quantifies the percentage of siRNA that is protected from dye-binding, and thus presumably encapsulated into an LNP upon particle formation, is often used to characterize drug-loaded nanoparticles.[25,26] The relationship between entrapment and *in vivo* hepatocellular delivery efficacy is shown in Figure 6. Although no clear trends exist for high siRNA dose (5 mg/kg) *in vivo* data (Figure 6a), the lower siRNA dose data (≤ 0.5 mg/kg) in Figure 6b demonstrates that the most efficacious LNPs have an siRNA entrapment of approximately 75%. While high entrapment values will provide more substantial delivery payloads, this may be potentially offset by hindered siRNA release once inside the target cell due to strong electrostatic interactions between lipidoid and siRNA molecules.

DISCUSSION

An ideal *in vitro* experimental system is low-cost, high-throughput, and accurate in its identification of efficacious materials for application in animals. While the former two characteristics are more readily optimized during method development, the latter poses a significant challenge. This challenge in the identification of efficacious materials *in vivo* is often ascribed to the additional barriers present in the physiological environment preventing the translation of materials that had possessed delivery ability *in vitro*. Failure in translation, however, may also be due to flaws in the *in vitro* models themselves. Such flaws are difficult to identify because there is limited data describing the successful translation of

siRNA delivery materials from *in vitro* to *in vivo* systems. The availability of more abundant efficacy data, both in cell culture and in animals, would assist in determining if translation difficulty is due to biological challenges or to issues in method development.

Several libraries of lipid-like materials, termed 'lipidoids', have been developed for applications in siRNA delivery, with a key goal of silencing hepatocellular gene targets.[9-12] During this time, we have experimented with various *in vitro* models in the hopes of improving our ability to successfully translate effective delivery vehicles into mice. Because hundreds of formulations have ultimately been tested in a Factor VII hepatocellular gene silencing mouse model, we have been able to draw numerous conclusions regarding how to best identify materials candidates for *in vivo* testing.

It was anticipated that maximizing the similarities between *in vitro* and *in vivo* systems should result in the best correlations. This study, in general, confirms such thinking: using nanoparticles for both animal and cell culture work provided significant improvements in translation, as did the use of primary hepatocytes *in vitro*. The addition of PEG to nanoparticle formulations has been previously demonstrated to alter cell uptake patterns and transfection efficiencies;[27,28] therefore, its inclusion *in vitro* would likely result in better translation. Interestingly, however, the immortalized mouse hepatocyte Hepa1-6 cell line was less predictive of *in vivo* delivery to hepatocytes than HeLa cells, which have been derived from cervical carcinoma cells. One possible explanation is that immortalized hepatocytes are almost as dissimilar to native hepatocytes as are HeLa cells. Immortalized hepatocytes are highly dysfunctional and capable of recapitulating < 1% of normal liver cell function. Freshly-isolated primary hepatocytes, on the other hand, retain many of the characteristics of liver cells *in vivo*, including secretory functionality, polarization, quiescence, and active phase I/II metabolism.[29]

Such results imply that the use of primary hepatocytes *in vitro* will result in the best correlations with animal experiments. However, researchers must weigh the cost, time, and expertise required to isolate and quantify gene silencing in viable primary hepatocyte populations against the quality of data obtained. For small-scale experiments (say, less than 5 compounds), it is likely more economical to test each material *in vivo* than to work with primary cells. It is also possible that HeLa cells may provide enough selection pressure to warrant their use for *in vitro* screening. In our *in vitro* studies of thousands of lipidoid compounds, for example, only 5% of materials mediate gene silencing of greater than 50% in HeLa cells,[12] which is the criteria for *in vivo* testing, as identified in Figure 1c.

The influence of nanoparticle size on the liver delivery process has been explored for decades, with some studies reporting the superior efficacy of smaller nanoparticles, often with diameters less than the size of liver fenestrae (~100 nm).[30-33] Yet, many other reports describe the efficacious hepatocellular delivery of larger particles (100 – 200 nm) while observing little to no influence of nanoparticle size on the delivery process.[12,34-36] In this study, which examined particles less than 300 nm in diameter, no correlation was observed between *in vivo* FVII silencing and nanoparticle diameter at any siRNA dose for the 100+ lipidoids tested (Figure 6b). Furthermore, when we examined the delivery of the lipidoid C12-200 formulated into nanoparticles between 65 and 135 nm in diameter, we found efficacy to be independent of LNP size. For cationic nanoparticles, there may be a tradeoff between the enhanced liver uptake and increased nanoparticle disassembly experienced by smaller nanoparticles upon intravenous administration. Zuckerman and colleagues recently reported that cationic siRNA-loaded nanoparticles less than ≤ 100 nm in size are rapidly cleared from circulation and disassembled *via* the glomerular basement membrane within the kidney.[37] Therefore, nanoparticles of larger size may be able to avoid disassembly in the kidneys while experiencing sufficient uptake into hepatocytes to achieve therapeutic effect.

The results of this study offer valuable insight into what can often be a frustrating materials translation process. Much of the inherent value in these data comes from their quantity; by studying over 100 nanoparticles in multiple *in vitro* systems and in mice, we have been able to more accurately correlate experimental parameters and characterization data with efficacy. Most importantly, we have shown that consistency of formulation and the use of primary hepatocytes generate the most predictive *in vitro* data, while LNPs with entrapments of about 75% have an increased potential for siRNA delivery ability *in vivo*. Most surprisingly, it was found that when the diameter of LNPs are less than 200 nm, size does not correlate with *in vivo* efficacy in hepatocellular targets. Although caution should be used when extrapolating these data to alternative delivery material and experimental systems, these findings may offer guiding principles in the design of siRNA delivery experiments and the choices made when attempting materials translation into animals.

MATERIALS AND METHODS

Lipidoid Synthesis

Lipidoids were synthesized as described previously.[9,10] Briefly, alkyl-acrylates or alkyl-epoxides (Sigma Aldrich, St. Louis, MO) were added stoichiometrically to alkyl-amines, which were purchased from either Scientific Polymer Products (Ontario, NY) or Hampford Research, Inc. (Stratford, CT). Each mixture was stirred without solvent in a glass scintillation vial at 90°C for three days.

In Vitro Transfection with Lipidoid Complexes

Testing was performed on HeLa cells that had been stably modified to express both firefly and Renilla luciferase. Cells were maintained at 37°C in a 5% carbon dioxide atmosphere in high glucose Dulbecco's Modified Eagles Medium without phenol red (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Prior to transfection, cells were seeded in white 96-well plates (Corning Life Sciences, Corning, NY) at a density of 15,000 cells per well and were allowed to attach overnight. Each lipidoid was mixed with 50 ng of anti-firefly luciferase siRNA (Dharmacon, Lafayette, CO) at a weight ratio of 5:1 in 25mM sodium acetate buffer. The siRNA-lipidoid complexes were then applied to the seeded HeLa cells at an siRNA concentration of 20 nM. 10% FBS was maintained in the culture medium during transfection. Relative firefly luciferase silencing was assessed 24 hours post-transfection using a Dual-Glo® Luciferase Assay kit (Promega, Madison, WI). Normalization to Renilla luciferase values served as a control for potential toxicity and/or off-target effects. Transfections were performed in quadruplicate. Lipofectamine RNAiMax™ (Invitrogen) was used according to manufacturer's instructions as a positive control.

Formulation of LNPs

Lipidoid nanoparticles were formed by mixing lipidoids, cholesterol (Sigma Aldrich), DSPC (Avanti Polar Lipids, Alabaster, AL) and mPEG2000-DMG (MW 2660, gift from Alnylam Pharamceuticals, Cambridge, MA) at a molar ratio of 38.5: 50: 10: 1.5 in a solution of 90% ethanol and 10% 10 mM sodium citrate (by volume). An siRNA solution was prepared by diluting siRNA in 10 mM sodium citrate such that the final weight ratio of total lipid (lipidoid + cholesterol + DSPC + PEG): siRNA was 10: 1. Equal volumes of lipid solution and siRNA solution were rapidly mixed together using either a microfluidic device or by pipet to form nanoparticles. Particles were diluted in phosphate buffered saline (PBS, Invitrogen) and then dialyzed against PBS for 90 minutes in 3,500 MWCO cassettes (Pierce/Thermo Scientific, Rockford, IL).

In vitro Transfection of Cell Lines with LNPs

For experiments using Dual HeLa cells, the cells were maintained and seeded as described above. For experiments with immortalized hepatocytes, mouse Hepa1-6 cells were stably transfected using a mouse retrovirus with firefly luciferase (pGL3).[38] Hepa1-6 cells were maintained identically to the Dual HeLa cells, and were seeded in white 96-well plates at a density of 10,000 cells/well and allowed to attach overnight. LNPs were prepared as described in the preceding paragraph with siRNA specific against firefly luciferase (Dharmacon, Lafayette, CO) and cells were transfected at an siRNA concentration of 40 nM. For HeLa cells, firefly luciferase silencing was assessed with a Dual-Glo® Luciferase Assay kit with Renilla silencing serving as a control. For Hepa1-6 cells, firefly luciferase silencing was assessed with a Bright-Glo® Luciferase Assay kit (Promega), with nanoparticles containing non-targeting siRNA serving as a control.

In vitro Transfection of Primary Hepatocytes with LNPs

Mouse hepatocytes were isolated from C57BL/6 mice as described previously[39] with viability >90%. LNPs were prepared as described above with siRNA specific against Factor VII. A reverse transfection was performed in which 80 ul of cell suspension (12,000 cells) was pipetted onto 20 ul of LNP solution in clear flat-bottom 96-well plates that had been treated with 0.1% rat collagen (Sigma). Cells were examined 24 hours post-transfection to ensure viability. 48 hours post-transfection, cells were lysed and Factor VII gene expression was quantified using a branched DNA kit (Affymetrix, Santa Clara, CA). Factor VII expression was normalized to GAPDH expression to control for potential toxicity and/or off-target phenomena.

In vivo Factor VII silencing

All animal experiments were conducted using institutionally-approved protocols. Female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were warmed under a heat lamp and weighed before receiving tail vein injections of either PBS (negative control) or lipidoid nanoparticles containing anti-Factor VII siRNA diluted in PBS at a volume of 0.01 ml/g. For efficacious LNPs, an additional negative control was performed using noncoding siRNA to help to ensure that reductions in protein expression were due to sequence-specific gene downregulation and not due to the delivery vehicle. Animals were dosed at 5 mg/kg total siRNA with the exception of the C12-200 experiments shown in Figure 5c (0.03 mg/kg) and the low dose data shown in Figure 6b (0.5, 0.02 or 0.01 mg/kg). The sequence of the siFVII provided by Alnylam Pharmaceuticals was: sense: 5′-GGAucAucucAAGucuuAcT*T-3′ antisense: 5′-GuAAGAcuuGAGAuGAuccT*T-3′ where 2′-fluoro-modified nucleotides are in lower case and phosphorothioate linkages are represented by asterisks. Two days post-injection, mice were anesthetized *via* isoflurane inhalation, and 100 – 200 ul of blood was collected retroorbitally into microtainer tubes (BD Biosciences, Franklin Lakes, NJ). Blood was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was analyzed for Factor VII using a Biophen FVII assay kit (Aniara Corporation, Mason, OH).

Nanoparticle Characterization

LNPs were diluted to an siRNA concentration of ~ 5 ug/ml in 0.1x PBS, pH 7.3. siRNA entrapment efficiency was determined using the Quant-iT™ RiboGreen® RNA assay (Invitrogen). Particle sizes were measured with a ZETAPals analyzer (Brookhaven Instruments, Holtsville, NY). Sizes reported are the average effective diameter of each LNP. Zeta potential measurements were acquired on a Zetasizer Nano ZS (Malvern, Westborough, MA), and reported values were the average of 10 – 25 runs.

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ABBREVIATIONS

FVII	Factor VII
LNP	lipid nanoparticle
PBS	phosphate buffered saline
PEG	polyethylene glycol

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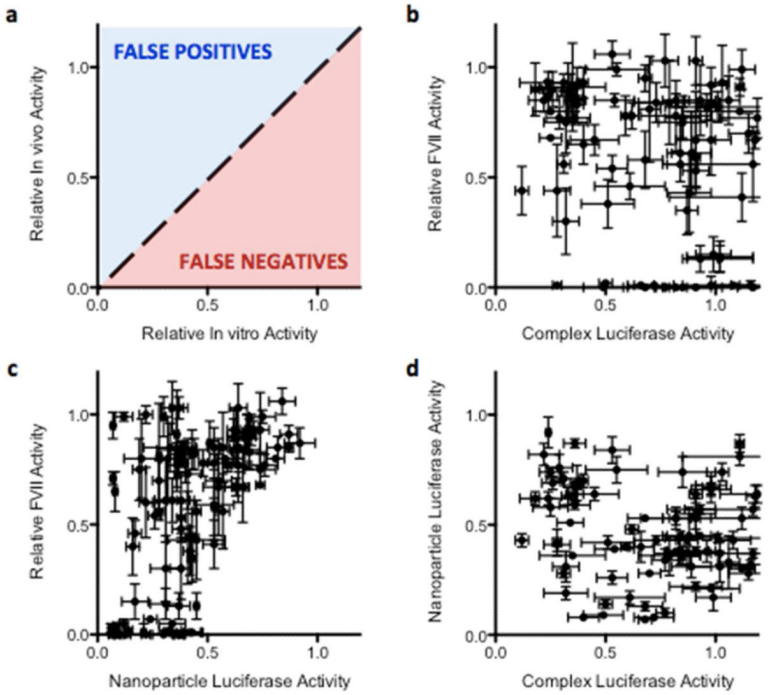

Figure 1.

Lipidoid formulation procedure can significantly impact *in vitro – in vivo* correlations. **a)** *In vivo – in vitro* data points can be classified as either correlating (dashed line), false positives (blue area), or false negatives (red area). **b)** *In vitro* data generated *via* lipidoid complexes did not correlate with *in vivo* Factor VII silencing data. **c)** *In vitro* data generated *via* lipidoid nanoparticles (LNPs) was more predictive, with a reduction in the quantity and severity of false negatives. **d)** The transfection ability of LNPs when formulated as complexes or nanoparticles varied significantly. Error bars in each panel represent standard deviation (n = 3 and 4 for FVII and luciferase activity data, respectively).

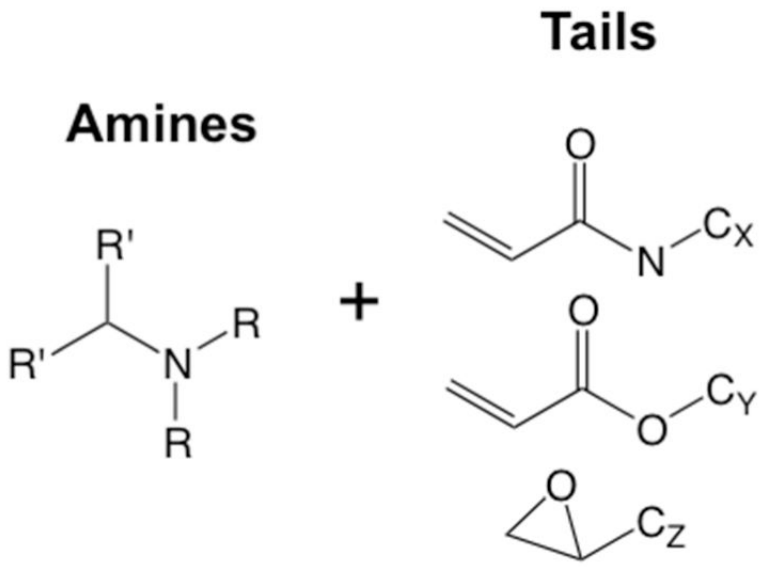

Figure 2.
The 100-lipidoids tested in this study were structurally diverse. Lipidoids were synthesized *via* the conjugate addition of alkyl-amines to alkyl-acrylamides, alkyl-acrylates, or alkyl-epoxides where X = 9 and 12, Y = 10-14 and Z = 12.

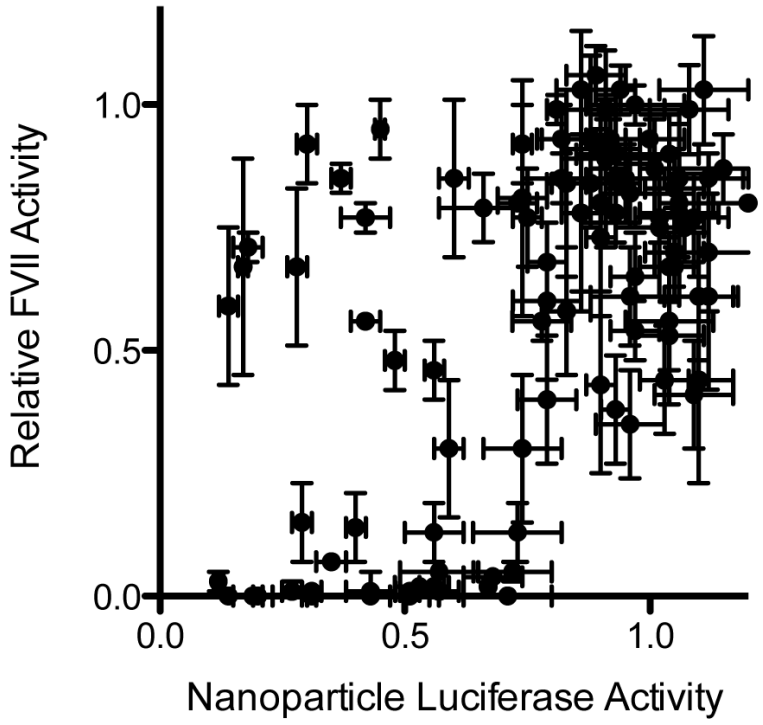

Figure 3.
The use of an immortalized hepatocellular line, Hepa1-6, for *in vitro* transfection experiments did not improve the trend observed between *in vivo* and *in vitro* data as compared to the data shown for HeLa cells in Figure 1c. Error bars represent standard deviation (n = 3 and 4 for Y and X error bars, respectively).

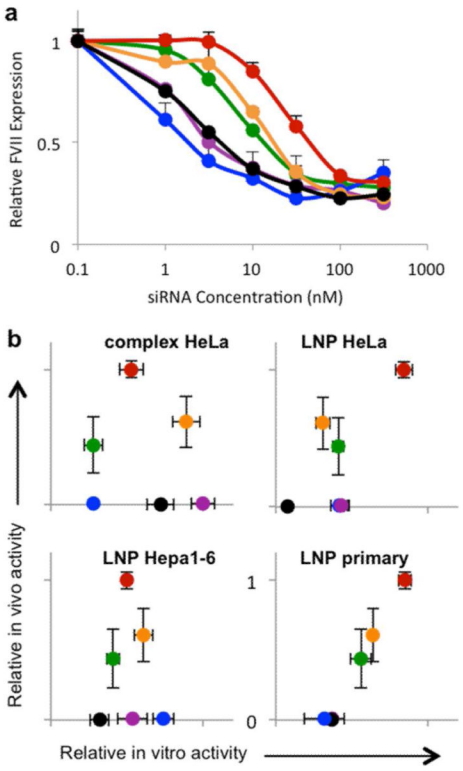

Figure 4.

Primary hepatocytes, which are morphologically most similar to mouse hepatocytes, offer the best correlation between *in vitro* and *in vivo* transfection data. **A)** Transfection results for freshly-isolated primary hepatocytes were dose-dependent for 6 LNPs. A dose of 10nM was determined to be most appropriate for comparison with *in vivo* data. **B)** For the 6 lipidoids studied in **A)**, primary cells transfected with LNPs offered the best correlation with *in vivo* activity, followed by HeLas transfected with LNPs. HeLa cells transfected with lipidoid complexes and Hepa1-6 cells transfected with LNPs did not correlate well with *in vivo* activity. Error bars represent standard deviation (n = 3).

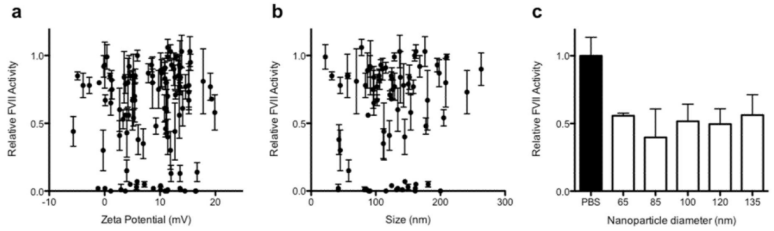

Figure 5.

The surface charge **(a)** and size (when less than 300 nm) **(b, c)** of lipidoid nanoparticles did not have any discernable effect on siRNA delivery to hepatocellular targets *in vivo*. Each data point in panels **(a)** and **(b)** represents a distinct lipidoid formulation. Panel **(c)** shows an unchanged silencing effect for the lipidoid C12-200 formulated into nanoparticles with identical chemical composition but varying diameter. Error bars in each panel represent standard deviation (n = 3).

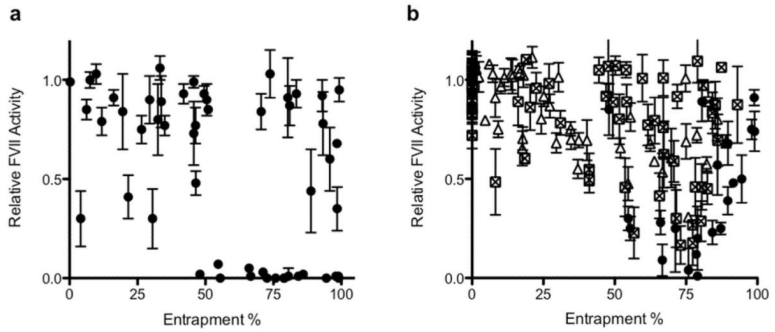

Figure 6.

While entrapment did not correlate with efficacy at high siRNA doses of 5 mg/kg **(a)**, low dose data revealed that LNPs possessing entrapment efficiencies of ~75% perform the best *in vivo* **(b)**. *In vivo* data collected at 0.5, 0.02, and 0.01 mg/kg siRNA doses are denoted in **(b)** by black circles, hatched squares, and white triangles, respectively. Error bars represent standard deviation (n = 3).