COMMUNICATION

Enhanced Intracellular siRNA Delivery using Bioreducible Lipid-Like Nanoparticles

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The discovery of RNA interference (RNAi) has had a transformative impact on the study of gene regulation for disease treatment. RNAi can knock down genes by targeting and cleaving complementary mRNA with a high efficiency using sequence-specific siRNA. It has the potential to silence any target gene, and to treat a variety of diseases, including cancers, viral infections, and hereditary disorders.[1] The therapeutic application of siRNA, however, is hindered by the lack of safe and efficient tools capable of delivering siRNA into the target cells. The past decade has witnessed substantive efforts in developing cationic lipids,[2] polymers,[3] and multifunctional inorganic nanoparticles[4,5] for siRNA delivery. A majority of these siRNA carriers encapsulate and escort siRNA into the cytosol in the form of nanoparticles and through an endocytotic pathway. These siRNA nanocomplexes, however, usually encounter a variety of biological barriers on their journey to their sites of action after entering cells, which could further affect their delivery efficiency. Notably, weak intracellular release of the siRNA from the nanocarriers limits the available siRNA that can initiate RNAi, and thus compromises gene silencing efficiency.[6] In general, siRNA is encapsulated into delivery vehicles in the form of nanoparticles, which can penetrate the cell membrane. However, binding between the siRNA and carrier that is too strong can retard the release of the siRNA, once the complex enters the cell. Therefore, designing smart siRNA delivery vehicles that can readily release siRNA in response to distinct intracellular environments has emerged as an effective approach to advance siRNA delivery.[7] Pioneering examples integrate components such as acid responsive units,[4,8] or reducible disulfide bonds[9] into cationic polymers. These complexes act as biodegradable siRNA nanocarriers by taking advantage of the acidic or strongly reductive environment that can be found within cells.

In this study, we report the design of bioreducible lipid-like materials (termed "lipidoid") integrating disulfide bonds, which are degradable in the presence of thiol-containing biomolecules, and can be used as a highly efficient siRNA delivery platform. It is generally known that glutathione (GSH) and/or other strongly reductive species are up-regulated inside cells, compared with levels found in blood plasma.[10] We believe that a reductive intracellular environment such as this could trigger the degradation of bioreducible siRNA nanocomplexes via a route of thiol exchange reactions with the lipidoid, releasing the siRNA inside the cells efficiently, and boosting the gene knock down efficiency. Recently, Anderson and co-workers[11] have developed a combinatorial strategy that has been used to synthesize a library of lipidoids for siRNA delivery, which has also aided in the study of the structure–function relationship in lipidoid-facilitated siRNA delivery. We have further expanded this class of materials for use as delivery agents of DNA, mRNA, and proteins.[12,13]

Although there are a few reports that indicate that biodegradable lipids can improve DNA plasmid delivery,[14] a thorough investigation on the use of bioreducible lipid, or lipid-like materials for siRNA delivery has not been reported yet. This is mostly due to the laborious synthesis normally required, and inefficiencies associated with integrating biodegradable units into conventional lipids. In this paper, we synthesized six bioreducible lipidoids via Michael addition of aliphatic amines and acrylate, incorporating disulfide bonds (**Scheme 1**). To evaluate the effect of disulfide bond integration on the capability of lipidoid-facilitated delivery, six nonbioreducible lipidoids were synthesized using a similar strategy by replacing the disulfide bond in the acrylates with carbon–carbon bonds. All of the lipidoids were able to encapsulate siRNA via electrostatic interaction, forming nanoparticles, and efficiently entering cells. However, the lipoplexes composed of the bioreducible lipidoids released siRNA more efficiently than nonbioreducible lipidoid under a reductive environment, as revealed by gel electrophoresis assay and intracellular siRNA trafficking studies. Using siRNA targeting green fluorescence protein (GFP) as a model, it was observed that the bioreducible lipidoids/siRNA complexes suppressed the GFP expression of GFP-expressing MDA-MB-231 cells, with a significantly enhanced efficiency when compared with nonbioreducible lipidoids. A representative bioreducible lipidoid, **1**-O16B, was able to deliver siRNA-targeting polo-like kinase 1 (Plk-1) into cancer cells, depleting Plk-1 and inhibiting tumor cell proliferation, demonstrating the lipidoid's potential for cancer therapy.

The lipidoids, featuring bioreducible components were synthesized by reacting aliphatic amines with appropriate acrylates in a similar strategy to previous reports (Scheme 1).[13] Briefly, amines and acrylates were heated at molar ratios of 1:2.4 without any solvent, at 80 °C for 48 h. They were then cooled, and the crude products used directly for preliminary siRNA delivery studies. *N,N*-Dimethyl-1,3-propanediamine (**1**) and 2,2′-(3-aminopropyl-azanediyl) diethanol (**2**) were selected as representative amines based on our knowledge that they have the potential to form lipidoids with efficient gene delivery

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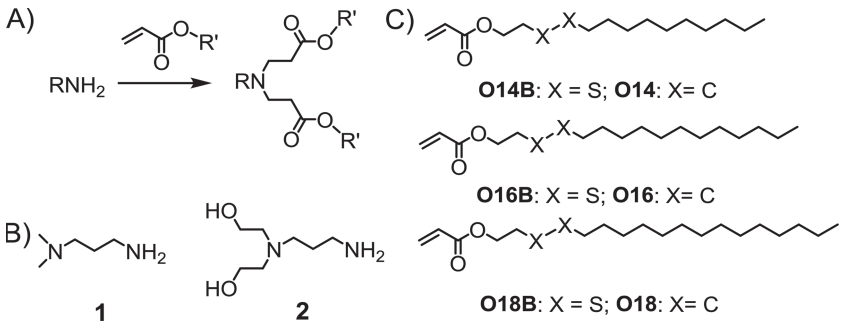

Scheme 1. A) Synthesis of lipidoids via Michael addition conjugation of an amine and an acrylate, B) Chemical structures of amines, and C) acrylates used for lipidoid synthesis. The lipidoids are named by the amine number, followed by the acrylate number.

ability.[13] Meanwhile, the incorporation of disulfide or carbon–carbon bonds into the acrylates allows us to evaluate the effect of bioreducible bond incorporation on the efficacy of lipidoid-facilitated siRNA delivery (Scheme 1). For simplicity, lipidoids were named using the following method: the first number indicates the amine number, followed by "O" indicating "acrylate," and the number of carbon atoms that comprises each hydrophobic tail of the acrylate. A "B" was added to the end of the name when referring to a bioreducible lipidoid. For example, **1**-O16 represents the lipidoid synthesized from amine **1** and O16, while **1**-O16B indicates the lipidoid prepared by reacting amine **1** and acrylate O16B, which features two disulfide bonds in its hydrophobic tail.

We first evaluated the capability of lipidoids to deliver siRNA by transfecting a breast cancer cell line that stably expresses GFP (GFP-MDA-MB-231) with siRNA targeting the GFP gene (siGFP). Cells were exposed to siGFP complexes (24×10^{-9} M of siRNA) formulated with one of the 12 lipidoids in the library (Scheme 1), and the cellular GFP expression profile following each lipidoid/siGFP treatment was measured 48 h post-transfection using flow cytometry analysis. The GFP expression was calculated by dividing the mean fluorescence intensity of treated cells to that of untreated controls. As shown in **Figure 1**, naked siGFP treatment has no effect on suppressing GFP expression, suggesting that naked siRNA has inefficient cellular uptake or instability in serum. The lipidoid/siGFP-treated cells, however, display varying levels of GFP expression depending on the specific lipidoid used in the lipidoid/siRNA lipoplexes. All non-bioreducible lipoplex treatments suppressed GFP expression slightly; the most efficient nonbioreducible lipidoid, **2**-O14, reduced GFP expression to 80% of controls. In contrast, the bioreducible lipidoid/siRNA complexes silenced GFP expression with much higher efficiency than the nonbioreducible lipidoids that contained the same amine head and hydrophobic tail length. For example, **1**-O16B/siGFP suppressed GFP expression down to 28% compared with 90% expression by **1**-O16/siGFP. Similarly, the exposure of cells to the other five bioreducible lipidoids/siGFP complexes suppressed GFP expression by greater percentages than their nonbioreducible counterparts. More noteworthy, four of the six bioreducible lipidoids, **2**-O14B, **1**-O16B, **2**-O16B, and **1**-O18B, suppressed GFP expression with an efficiency outperforming the commercial gene transfection reagent, Lipofectamine 2000 (Figure 1). The preliminary siRNA delivery screening results indicate that the integration of bioreducible disulfide bonds into lipidoids enhanced their capability as siRNA delivery vehicles.

To understand the mechanism of enhanced siRNA delivery using bioreducible lipidoids, **1**-O16B and **1**-O16 were selected and purified as representative bioreducible and nonbioreducilbe lipidoids, respectively. The chemical structures of **1**-O16B and **1**-O16 were confirmed by ^{1}H NMR (Figure S1, Supporting Information) and electrospray ionization-mass spectrometry (ESI-MS). Both **1**-O16B and **1**-O16 can effectively bind siRNA, as revealed by an agarose gel retardation assay (**Figure 2**a). The complexation of siRNA with **1**-O16B or **1**-O16 at an N/P ratio of 24 completely retarded the migration of siRNA (10 pmol), though the nonbioreducible lipidoid (**1**-O16) has a slightly higher binding affinity toward siRNA. The encapsulaion of siRNA by **1**-O16B and **1**-O16 efficiently protects siRNA from nuclease degradation. As shown in Figure 2b, naked siRNA (10 pmol) was completely degraded within 2 h upon exposure to RNase A (4 μg mL^{-1}). However, when the same amount of siRNA was complexed with **1**-O16B or **1**-O16 (at an N/P ratio of 24), the siRNA remained intact for more than 2 h of RNase A treatment showing that both bioreducible and nonbioreducible lipidoid can protect siRNA from enzymatic degradation at comparable efficiencies.

To understand whether the bioreducible lipidoid/siRNA complexes can release siRNA more efficiently than nonbioreducible lipidoids under a reductive intracellular environment, a gel retardation assay (Figure 2c) was used. It is generally known that glutathione (GSH) is up-regulated over 1000-fold in the cytoplasm compared with blood plasma.[10] This high concentration of intracellular GSH can degrade the bioreducible lipidoids

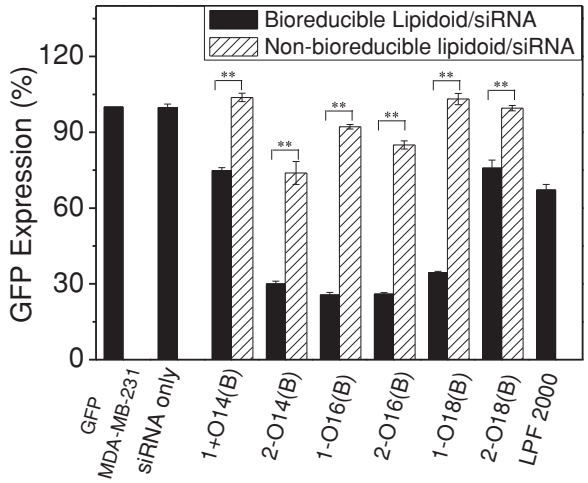

Figure 1. GFP expression of GFP-MDA-MB-231 cells treated with naked siGFP, lipidoid/siGFP nanocomplexes, and Lipofectamine 2000 (LPF 2000)/siGFP complexes. The siRNA complexes were prepared by mixing siGFP (24×10^{-9} M) with lipidoids at N/P ratios of 5:1 or LPF2000 at weight/weight ratio of 6:1. Data are presented as mean ± SD ($n = 3$, the two asterisks refer to statistical significance between bioreducible and nonbioreducible lipidoid facilitated siRNA delivery, $P < 0.05$, Student's *t*-test).

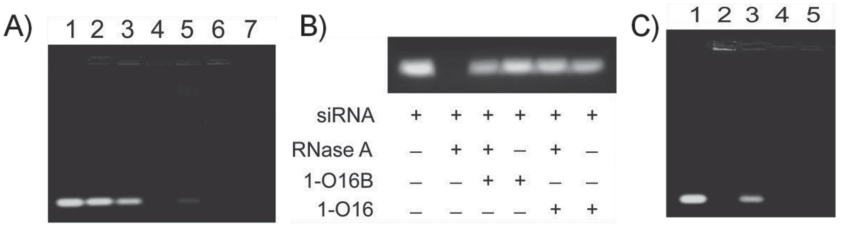

Figure 2. A) Gel electrophoresis assay of siRNA/lipidoid complexes at varying N/P ratios. 1) siRNA only; 2–4: **1**-O16B/siRNA complexes mixed at N/P ratios of 6 (lane 2); 12 (lane 3), 24 (lane 4); 5–7: **1**-O16/siRNA complexes mixed at N/P ratios of 6 (lane 5); 12 (lane 6), 24 (lane 7); B) Lipidoid **1**-O16B and **1**-O16 protected siRNA from nuclease degradation; C) GSH-triggered siRNA release from lipidoid/siRNA complexes. Lane 1: siRNA; 2–3: siRNA/**1**-O16B complex in the absence (lane 2) and presence of 5×10^{-3} M GSH (lane 3); 4–5: siRNA/**1**-O16 complex in the absence (lane 4) and presence of 5×10^{-3} M GSH (lane 5).

through a route of disulfide bond exchange with GSH, and such a degradation process can facilitate the release of siRNA from the lipoplexes, potentially improving the siRNA delivery. As shown in Figure 2c, the treatment of **1**-O16B/siRNA or **1**-O16/siRNA complexes with GSH (5×10^{-3} M) results in differences in siRNA release. The **1**-O16B/siRNA lipoplex releases a significant amount of siRNA (lane 3) upon GSH treatment, while in contrast, this siRNA release is not observed from **1**-O16/siRNA complexes in the presence of the same concentration of GSH (lane 5).

The nanoparticle structures of the lipidoid/siRNA complexes as well as the GSH-treated bioreducible lipoplexes after degradation were further characterized by dynamic light scattering (DLS) analysis and transmission electron microscopy (TEM) studies. The complexation of **1**-O16B and **1**-O16 with siRNA formed nanoparticles with mean sizes of 160 nm and 106 nm, respectively. The treatment of **1**-O16B/siRNA nanoparticles with 5×10^{-3} M GSH degraded the lipoplexes and broadened the particle size distribution, as measured by DLS analysis (**Table 1**). However, no significant nanoparticle size variation was observed for the **1**-O16/siRNA complexes in the absence or presence of GSH. The degradation of the lipidoid/siRNA complexes in the presence of GSH was further confirmed by TEM imaging. **1**-O16B/siRNA and **1**-O16/siRNA complexes form nanoparticles with diameters of ≈100 nm (**Figure 3** and Figure S2, Supporting Information). However, following the addition of GSH to the above lipoplexes, no discrete nanoparticles were observed for **1**-O16B/siRNA complexes (Figure 3), while nanoparticles that retained their original diameter were observed for the **1**-O16/siRNA complexes (Figure S2, Supporting Information). Taken together, the gel electrophoresis assay and nanoparticle characterization reveal that the intracellular reductive environment can trigger bioreducible lipidoid degradation and facilitate intracellular siRNA release. We hypothesize that this accounts for the enhanced gene silencing efficiency observed in the bioreducible lipidoid-facilitated siRNA delivery.

To further study whether the increased siRNA delivery efficiency of the bioreducible lipidoids was a result of the triggered release of siRNA intracellularly, the cellular uptake and intracellular siRNA trafficking of **1**-O16B/siRNA and **1**-O16/siRNA nanoparticles were monitored by delivering fluorescently labeled siRNA (FAM-siRNA) into MDA-MB-231 cells, coupled with confocal laser scanning microscopy (CLSM) imaging. As shown in **Figure 4**, MDA-MB-231 cells treated with fluorescent lipoplexes have a significant amount of green fluorescence, arising from FAM-siRNA, indicating the efficient cellular uptake of **1**-O16B/siRNA or **1**-O16/siRNA complexes. The endosome/lysosome were then stained using LysoTracker@Red, which showed that **1**-O16B/siRNA lipoplexs escaped from the endosome/lysosome after 6 h of incubation (Figure 4A), as the green fluorescence (FAM-siRNA) was no longer co-localized with the red endosome/lysosome fluorescence. However, when the cells were treated with **1**-O16/siRNA complexes, the green fluorescence remained co-localized with the endosome/lysosome (yellow spots in Figure 4b), suggesting the entrapment of siRNA within

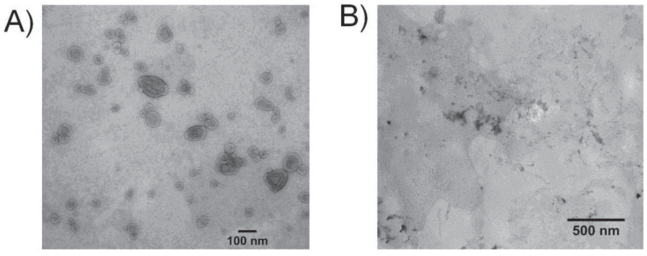

Figure 3. TEM images of siRNA and **1**-O16B complexes at N/P = 5 A) before and B) after GSH treatment.

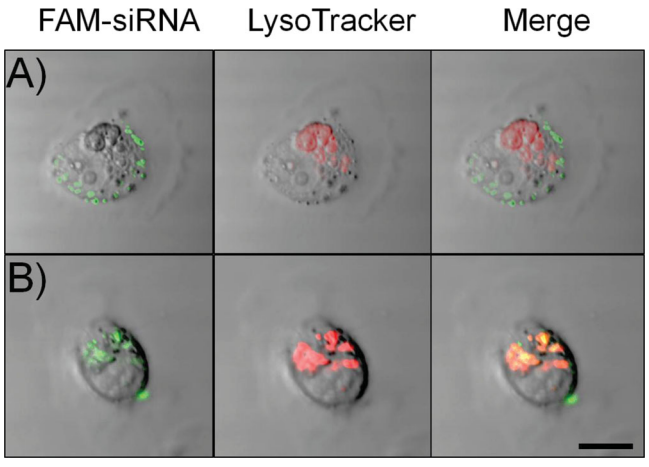

Figure 4. CLSM studies of MDA-MB-231 cells treated with A) **1**-O16B/FAM-siRNA complexes and B) **1**-O16/FAM-siRNA complexes for 6 h. The endosome/lysosome of cells were counter-stained using LysoTracker@Red. Scale bar: 12 μm.

Table 1. Hydrodynamic mean diameters of lipidoid-siGFP nanoparticles in the absence and presence of glutathione.

	1-O16B	1-O16
Lipidoid	74.7 ± 7.4 nm	117.2 ± 3.1 nm
Lipidoid+siGFP	160.8 ± 1.4 nm	106.7 ± 3.7 nm
Lipidoid+siGFP+GSH	Broad distribution	114.6 ± 16.4 nm

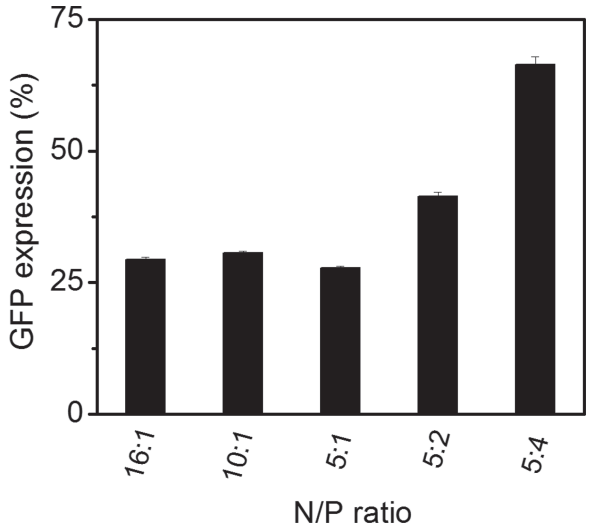

Figure 5. The GFP expression of MDA-MB-231-GFP cells treated with 1-O16B/siGFP complexes with different N/P ratios. The siRNA complexes were prepared by mixing siGFP (15 pmol) and varying amounts of 1-O16B. Data are presented as mean ± SD ($n = 3$).

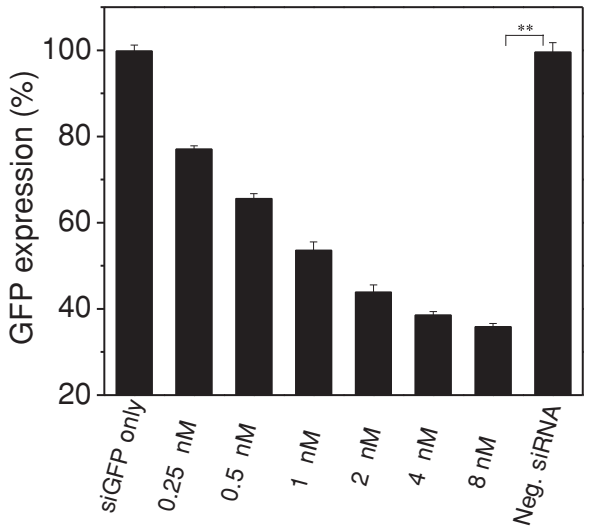

Figure 6. siGFP dose-dependent gene silencing of MDA-MB-231-GFP cells after transfection with 1-O16B/siGFP complexes. **1**-O16B and siRNA were mixed at N/P ratios of 5:1. For control experiments, 8×10^{-9} M of siGFP or scrambled siRNA was used. Data are presented as mean ± SD ($n = 3$). The asterisks refer to statistical significance between **1**-O16B-facilitated siGFP and scrambled siRNA delivery ($P < 0.05$).

the subcellular compartments, which leads to less efficient gene silencing by the nonbioreducible lipidoid.

Next, the conditions for siRNA delivery were optimized using the bioreducible lipidoid **1**-O16B. GFP-MDA-MB-231 cells were treated with **1**-O16B/siGFP lipoplexes with N/P ratios in the range of 1.25 to 16. As shown in **Figure 5**, an increase in the N/P ratios of **1**-O16B/siRNA complexes from 1.25 to 5 enhances the efficiency of GFP gene silencing, from 60% to 25%, while a further increase of the N/P ratio above 5 does not improve siRNA delivery efficacy. Therefore, the N/P ratio of **1**-O16B and siRNA complexes was fixed at 5 for the remainder of the study. Similarly, the GFP expression of GFP-MDA-MB-231 cells following **1**-O16B/siGFP treatment was found to be siRNA-dose dependent. As the cells were exposed to increased concentrations of siGFP from 0.25×10^{-9} to 8×10^{-9} M, cellular GFP expression decreased from 78% down to 28% (**Figure 6**). However, the treatment of cells with **1**-O16B/scrambled siRNA complexes only had a minor effect on suppressing GFP expression, suggesting that siRNA sequence-specific gene knock down is involved in the RNAi.

The biocompatibility and cytotoxicity of delivery vehicles has also posed a challenge for the safe delivery of siRNA, along with the success of RNAi therapeutics. In order to evaluate the cytotoxicity of the bioreducible lipidoids as a siRNA delivery platform, MDA-MB-231 cells were incubated with varying concentrations of **1**-O16B/siGFP nanocomplexes. Cell viability measurements via AlamarBlue assay indicated that viability remained at 90% or above following treatment with **1**-O16B/siGFP nanocomplexes (Figure S3, Supporting Information). This result was observed for siGFP as concentrations increased from 1 to 32×10^{-9} M (lipidoid concentration increased from 0.16 to 5.3 μg mL^{-1}), suggesting that bioreducible lipidoids are highly biocompatible for siRNA delivery.

Having identified the bioreducible lipidoid **1**-O16B as a safe and highly efficient siRNA delivery nanocarrier, the ability of **1**-O16B to deliver therapeutic siRNA into cancer cells, and its ability to interfere with cell-cycle progression as a potential cancer therapy strategy was investigated. To this end, polo-like kinase 1 (Plk1), a protein kinase that plays a crucial role in cell proliferation and cancer progression, was selected as a target. Plk1 is overexpressed in a broad spectrum of cancer cells, and previous investigations have suggested Plk1 as a highly potent target for cancer therapy.[15] Additionally, RNAi[16] and chemical inhibitors[17] interfering with Plk1 function have been shown to induce cell apoptosis in vitro, and suppress tumor growth in vivo. In this study, MDA-MB-231 cells were treated with a **1**-O16B/siPlk-1 complex formulated at the optimized siRNA delivery conditions (N/P ratio of 5). The viability of the cells was determined 48 h post-transfection and compared with that of the cells treated with **1**-O16B only, **1**-O16B/scrambled siRNA complex, or Lipofectamine 2000/siPlk-1. As shown in **Figure 7**, treatment of MDA-MB-231 cells with **1**-O16B/siPlk-1 complexes (siRNA concentration: 50×10^{-9} M) reduced cell viability down to 25%, outperforming Lipofectamine 2000 facilitated siPlk-1 delivery under the same conditions (50% cell viability). Control experiments conducted by exposing cells to **1**-O16B or **1**-O16B/scrambled siRNA complex had a negligible effect on cell viability. Moreover, the proliferation of cells treated with the **1**-O16B/siPlk-1 complexes was dependent on the dose of siRNA that the cells were exposed to. Cells treated with 32×10^{-9} M had a reduced viability of 45%, while 16×10^{-9} M siPlk-1-treated cells had a viability of 75% (Figure 7A). To examine whether Plk-1 depletion accompanied **1**-O16B/siPlk-1 treatment and subsequent cell proliferation inhibition, Plk-1 protein expression of MDA-MB-231 cells was detected by Western blot analysis. As shown in Figure 7B, **1**-O16B/siPlk-1 treatment significantly reduced Plk-1 expression in MDA-MB-231 cells, while no similar reduction in Plk-1 expression was observed for cells treated with **1**-O16B or **1**-O16B/scrambled siRNA complexes.

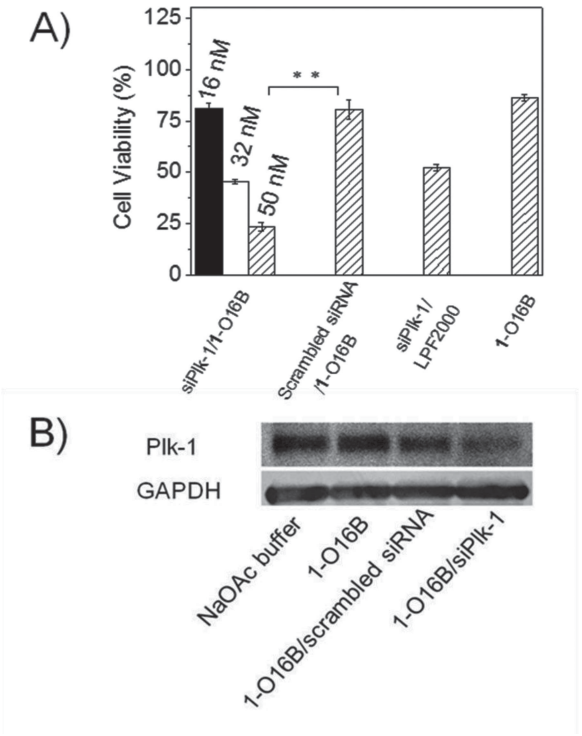

Figure 7. A) The viability assay of MDA-MB-231 cells after transfection with varying concentrations of siPlk-1/**1**-O16B complexes. For controls, the cells were dosed with scrambled siRNA/**1**-O16B, siPlk-1/LPF2000, or **1**-O16B. The cell viability was measured by AlamarBlue assay. Data are presented as mean ± SD ($n = 3$), the asterisks refer to statistical significance between **1**-O16B facilitated siPlk-1 and scrambled siRNA delivery ($P < 0.05$); B) Plk-1 protein expression in MDA-MB-231 cells after transfection with siPlk-1 or scrambled siRNA complexes of **1**-O16B. 50×10^{-9} M of siRNA was mixed with 1-O16B at N/P ratios of 5:1 prior to the cells being exposed.

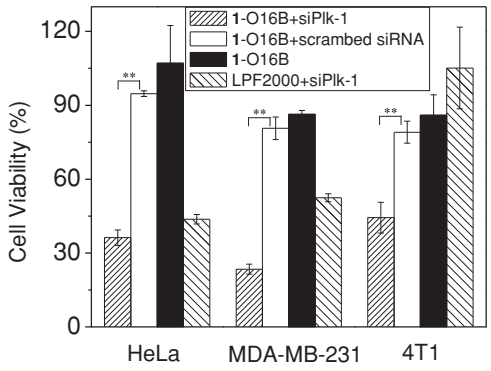

Figure 8. Bioreducible lipidoid **1**-O16B facilitated siPlk-1 delivery reduced the viability of different cancer cells. siRNA (50×10^{-9} M) was mixed with **1**-O16B at a N/P ratio of 5:1, or LPF2000 at a weight/weight ratio of 3:1. The cell viability was measured by AlamarBlue assay. Data are presented as mean ± SD ($n = 3$, $P < 0.05$).

An efficient siRNA delivery tool should be able to transfect different cell lines, as variations in cell surface and extracellular environments may affect the siRNA nanoparticle internalization and gene-silencing efficiency. To further demonstrate bioreducible lipidoids as an efficient siRNA delivery platform, siPlk-1 delivery facilitated by **1**-O16B was investigated on several different cell lines. Human cervical carcinoma cells (HeLa) and murine breast cancer cells (4T1) were selected as another two representative cancer cell lines. As shown in **Figure 8**, the treatment of HeLa and 4T1 cells with **1**-O16B/siPlk-1 complexes inhibited the proliferation of tumor cells, similar to MDA-MB-231 cells, while also being siRNA dose dependent. No obvious cell proliferation inhibition was observed for **1**-O16B or **1**-O16B/scrambled siRNA complex-treated cells. Moreover, **1**-O16B is more efficient at delivering siPlk-1 and inhibiting cell proliferation than Lipofectamine 2000 on all cell lines investigated.

In summary, we report the development of combinatorially designed bioreducible lipid-like nanoparticles as a novel siRNA delivery platform. The integration of bioreducible disulfide bonds into lipidoids, facilitated lipidoid degradation and siRNA release in response to intracellular GSH, which enhanced the siRNA delivery efficiency. A representative bioreducible lipidoid selected in this study, **1**-O16B can deliver siRNA-targeting GFP to suppress GFP expression of MDA-MB-231 cells, and siRNA targeting Plk-1 into cancer cells to interfere with cell progression and prohibit rapid tumor cell proliferation. These findings provide an efficient approach towards developing biodegradable nanocarriers for siRNA delivery for clinical translation as well as a novel approach in studying the structure–functionality relationships of lipid-based siRNA delivery system.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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