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Short Communication

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Hybrid lipid–polymer nanoparticles for sustained siRNA delivery and gene silencing

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Abstract

The development of controlled-release nanoparticle (NP) technologies has great potential to further improve the therapeutic efficacy of RNA interference (RNAi), by prolonging the release of small interfering RNA (siRNA) for sustained, long-term gene silencing. Herein, we present an NP platform with sustained siRNA-release properties, which can be self-assembled using biodegradable and biocompatible polymers and lipids. The hybrid lipid–polymer NPs showed excellent silencing efficacy, and the temporal release of siRNA from the NPs continued for over one month. When tested on luciferase-expressed HeLa cells and A549 lung carcinoma cells after short-term transfection, the siRNA NPs showed greater sustained silencing activity than lipofectamine 2000-siRNA complexes. More importantly, the NP-mediated sustained silencing of prohibitin 1 (PHB1) generates more effective tumor cell growth inhibition *in vitro* and *in vivo* than the lipofectamine complexes. We expect that this sustained-release siRNA NP platform could be of interest in both fundamental biological studies and clinical applications.

From the Clinical Editor: Emerging gene silencing applications could be greatly enhanced by prolonging the release of siRNA for sustained gene silencing. This team of scientists presents a hybrid lipid-polymer nanoparticle platform that successfully accomplishes this goal, paving the way to future research studies and potential clinical applications.

Key words: Lipid–polymer nanoparticle; siRNA; Sustained release; Gene silencing; Cancer

RNA interference (RNAi) has shown great potential to treat various diseases including cancer, through selectively silencing target genes.[1,2] To facilitate safe and effective delivery of RNAi therapeutics (e.g., siRNA) to tumor cells — a major hurdle for the clinical applications of RNAi, a few cationic lipid-/polymer-based nanoparticle (NP) platforms have been successfully developed and moved into clinical studies in cancer patients.[3-6] These NP systems, however, lack sustained siRNA release properties, and their therapeutic efficacy may be limited by transient gene silencing. We hypothesize that sustained siRNA delivery could lead to long-term, effective knockdown of target genes, thus avoiding frequent administration of therapeutic siRNAs to maintain the silencing action and minimizing systemic side effects.

Macroscopic biomaterial scaffolds and microspheres have been demonstrated with sustained siRNA release and prolonged gene silencing, but these strategies are mainly limited to local delivery applications due to their large size.[7,8] Therefore, the development of controlled-release NP platforms would be necessary to achieve sustained siRNA delivery for systemic

Conflicts of interest: O.C.F. has interest in BIND Therapeutics, Selecta Biosciences, and Blend Therapeutics, three biotechnology companies developing nanoparticle technologies for medical applications.

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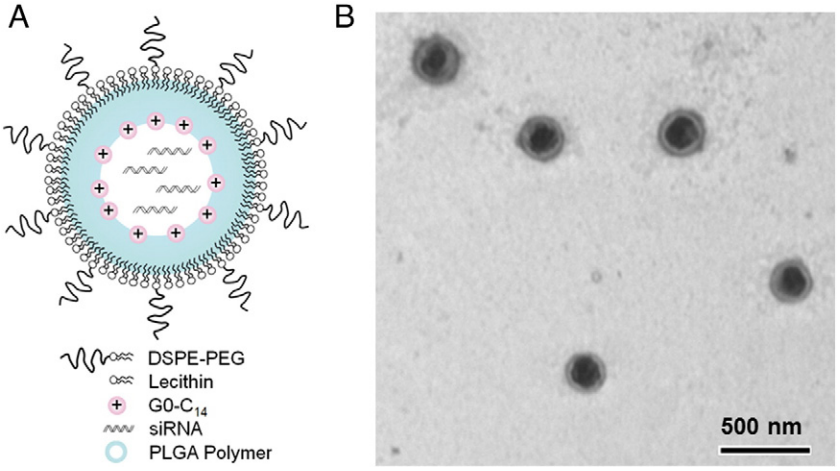

Figure 1. Hybrid lipid–polymer NPs for siRNA delivery. **(A)** Schematic and **(B)** TEM image of the NPs.

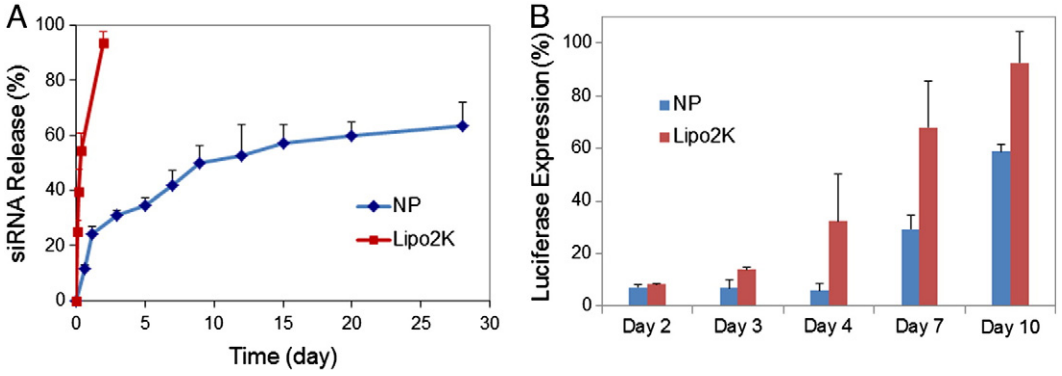

Figure 2. Sustained siRNA release and luciferase silencing. **(A)** *In vitro* siRNA release profiles of the NPs *vs.* Lipo2K complexes. **(B)** Luciferase expression *vs.* time after 6-h transfection with NP(siLuc) and Lipo2K(siLuc).

cancer treatment.[9,10] We have recently developed an innovative lipid–polymer hybrid NP platform for siRNA delivery,[11] and herein demonstrate that our ameliorated hybrid NPs can release siRNA continuously for periods longer than one month and achieve sustained gene silencing for at least two weeks. The growth of tumor cells treated with our NPs containing siRNA specifically targeting prohibitin 1 (siPHB1) was drastically inhibited both *in vitro* and *in vivo*.

Methods

Hybrid lipid–polymer NPs were prepared by a modified double-emulsion solvent evaporation technique and self-assembly method.[11] In this work, a new cationic lipid-like compound ($G0\text{-}C_{14}$) was synthesized by reacting 1,2-epoxytetradecane with PAMAM dendrimer (generation 0), and used for siRNA NP formulation. The siRNA release profile was obtained by measuring the dye-labeled siRNA remaining in the NPs or lipofectamine 2000 (Lipo2K) at different time points. Luciferase expression change was measured in luciferase-expressing HeLa (Luc-HeLa) cells transfected with NP(siLuc) or Lipo2K(siLuc) for 6 h. Similarly, the expression level of PHB1 in A549 lung cancer cells was tested by immunoblotting over a period of 14-24 days, after treatment with NP(siPHB1). *In vitro* A549 cell proliferation was monitored by AlamarBlue assay for 12 days. The A549 xenograft tumor growth was studied using 6-week-old BALB/C nude mice. A detailed description of the methods and experiments is included in the Supplementary Materials.

Results

The hybrid lipid–polymer NPs (Figure 1) are composed of an aqueous siRNA core stabilized by the positively charged lipid-like compound $G0\text{-}C_{14}$, a middle hydrophobic PLGA polymer shell, and a relatively neutral-charge lipid-PEG surface layer. With the use of $G0\text{-}C_{14}$ and selected formulation parameters, these NPs can have a sustained siRNA release for more than one month (Figure 2, *A*). The siRNA half-release time can be extended to ~9 days, as compared to ~8 h for Lipo2K. To evaluate the silencing efficacy of these sustained-release NPs, we introduced NP(siLuc) or Lipo2K(siLuc) into Luc-HeLa cells and measured luciferase expression at different time points. As

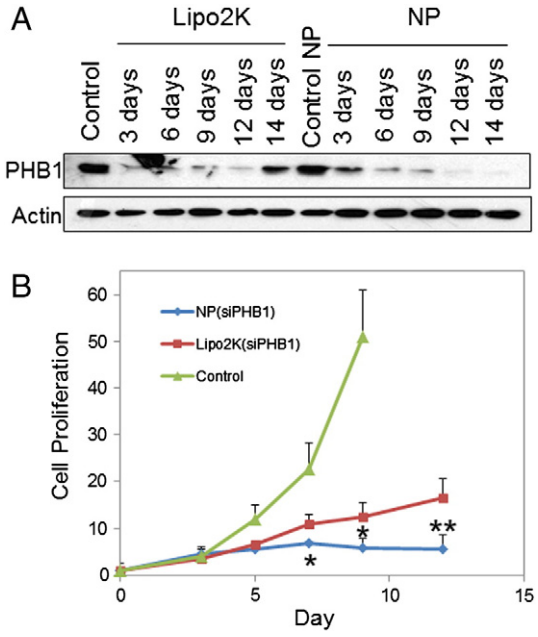

Figure 3. Sustained PHB1 silencing and its effect on A549 cell proliferation. **(A)** The PHB1 expression change in A549 cells was measured by Western blot analysis after 6-h transfection. Actin was used as an internal standard. **(B)** A549 cell proliferation *vs.* time after transfection. *$P < 0.05$, **$P < 0.01$, NP *vs.* Lipo2K.

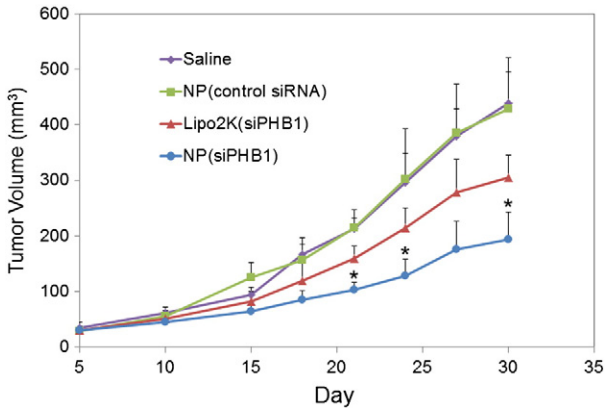

Figure 4. Xenograft tumor growth of A549 cells pre-treated with NP(siPHB1) *vs.* Lipo2K(siPHB1), saline, and control NP (n = 4-5). *$P < 0.05$, NP *vs.* Lipo2K.

shown in Figures 2, *B* and S1, the luciferase signal was significantly decreased to less than 10% at day 2 by both NP (siLuc) and Lipo2K(siLuc). Notably, by day 4, the luciferase signal remained less than 10% in NP(siLuc)-treated cells, while it recovered to over 30% in Lipo2K(siLuc)-transfected cells. This result suggested that the sustained siRNA release from NPs could contribute to the prolonged silencing activity. As the Luc-HeLa cells proliferate rapidly (Figure S2), the internalized NPs can be highly diluted with time. This resulted in the recovery of luciferase expression back to ~30% and 60% at day 7 and 10, respectively, although still much lower than those (~68% and 92%) in Lipo2K(siLuc)-transfected cells.

In addition to luciferase silencing, we also evaluated the sustained knockdown of PHB1, a protein involved in cell proliferation, apoptosis, chemoresistance, and other biological process.[12-14] Figure 3, *A* shows that after single transfection with Lipo2K(siPHB1) for 6 h, the PHB1 expression in A549 cells can be effectively silenced after 3 days. However, it recovered after 14 days. In contrast, the impressive PHB1 silencing was maintained by NP(siPHB1) over 2 weeks, and PHB1 expression was recovered at 24 days (Figure S3). No significant difference was observed in the cellular uptake of siRNA with either the NP or Lipo2K formulation after 6-h incubation (Figure S4). This result further suggested that the prolonged silencing effect of NPs is more likely due to the sustained siRNA releasing.

To determine whether the sustained silencing could lead to better efficacy in inhibiting cancer cell growth, we first studied the *in vitro* proliferation of A549 cells after transfection with NP(siPHB1) or Lipo2K(siPHB1). Cell number was measured using AlamarBlue assay, which allows continuously monitoring of cell number in real time. Figure 3, *B* demonstrates that the A549 cell growth in the control group is very rapid with a ~50-fold increase of cell number on day 9 relative to day 0, much faster than that in the Lipo2K(siPHB1) and NP(siPHB1) groups. More impressively, the cell proliferation was significantly inhibited after a week in the NP(siPHB1) group, whereas the Lipo2K(siPHB1) group showed continuous cell growth. This means that sustained silencing of PHB1 inhibits A549 cell growth *in vitro* more efficiently than short-term silencing. Furthermore, we studied *in vivo* growth of A549 cells using a xenograft mouse model. Consistent with our *in vitro* results, the mean tumor volumes of NP(siPHB1) group were significantly smaller compared to Lipo2K(siPHB1) or to the two control groups (saline and control NP), as shown in Figure 4.

Discussion

Controlled-release polymer technologies have benefited many branches of medicine over the past four decades, as they can enhance the *in vivo* therapeutic efficacy, reduce the administration frequency, and maximize the patient compliance.[15] Recently, we have developed self-assembled polymeric NP technologies with sustained-release and targeting properties, which were brought from conception to clinical studies for cancer treatment and smoking cessation.[16-18] The hybrid lipid–polymer NPs are designed based upon these polymeric nanotechnologies, and combine the unique properties of lipoplexes. It is noteworthy that majority of reported siRNA NP platforms, including those in clinical studies, do not have the sustained siRNA release property. While more tests (e.g., release kinetics effect and systemic delivery) will be needed to clarify the potential of this NP system for further improvement of RNAi therapy, the present results are promising and have demonstrated its capability for sustained, long-term gene silencing and effective inhibition of tumor cell growth. Furthermore, we postulate that the anti-tumor efficacy of our RNAi NPs could be greatly enhanced by incorporating tumor-specific targeting

ligands, and/or by simultaneously delivering synergistic siRNA combinations against multiple pathways or drug-siRNA combinations.[3,6,19,20]

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.03.006.

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