# A nanoparticle formula for delivering siRNA or miRNAs to tumor cells in cell culture and *in vivo*

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**To improve RNA delivery, we present a protocol to produce an RNA carrier based on a Zn(II)-dipicolylamine (Zn-DPA) analog, which is an artificial receptor for phosphate anion derivatives. We further functionalized this Zn-DPA analog to hyaluronic acid (HA)-based self-assembled nanoparticles (HA-NPs) with a hydrodynamic diameter of 100 nm by conjugating amine-functionalized Zn-DPA molecules onto the HA-NPs through amide formation, resulting in efficient tumor-targeted delivery of RNAs (siRNAs, miRNA or other short oligoribonucleotides) and small-molecule drugs. The functional group of Zn-DPA can be converted into other groups such as a carboxylic or thiol group, and the DPA analog can be covalently attached to a variety of existing and novel platforms or formulations for the development of multifunctional materials via standard bioconjugation techniques. Protocols for RNA formulation and delivery into tumor tissues and tumor cells are also described. Our design strategy offers a versatile and practical method for delivering both RNA and chemotherapeutics to tumor cells and expands existing nanomaterial capabilities to further the field of drug and gene delivery.**

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## INTRODUCTION

RNAi technology has emerged as a new gene therapy approach and revolutionary tool for experimental biology[1–3]. However, the delivery of RNA molecules (e.g., siRNA, miRNA or oligonucleotide) to a target still remains one of the biggest challenges in clinical translation and practical applications, even though it has been over a decade since Dr. Inder Verma proclaimed "There are only three problems in gene therapy: delivery, delivery and delivery"[4]. Although many carrier systems, such as cationic polymers, lipids or amino acids, have been developed to deliver RNAs into cells with promising results, the development of safe and versatile platforms for efficient RNA delivery is still a daunting task[5,6].

The most commonly used synthetic RNA carriers are based on polycations, such as polyethylene imine, poly-L-lysine and lipid-like particles with positively charged head groups (e.g., Lipofectamine 2000 or Lipofectamine RNAiMAX)[7,8]. Polycation systems can induce nano-sized polyelectrolyte complexes with negatively charged RNAs by electrostatic interactions. Unlike plasmid DNA, which forms stable and condensed complexes with cationic agents, RNA loosely binds to cationic molecules owing to its low charge density and molecular weight[9]. Strong cationic polymers or high doses of lipids can be used to increase RNA stability; however, undesirable side effects such as increased cellular toxicity and nonspecific accumulation induced by positive charges limit their therapeutic applications.

Here we describe a highly flexible protocol to design a sophisticated RNA carrier that we used for tumor-targeted RNA delivery in our previous studies, which is distinct from the reported gene delivery systems that are based on cationic derivatives or chemically modified RNAs[10,11]. This is an alternative RNA delivery system based on the use of Zn-DPA analog, an artificial receptor for phosphate anion derivatives. We and others have reported a series of fluorescent chemosensors for the detection of phosphate-containing molecules based on Zn-DPA analogs owing to the selective Zn-DPA-coordinated interaction associated with phosphate groups[12–15]. Similar Zn-DPA analogs were also used as probes for necrotic and apoptotic cells by targeting anionic phosphatidylserine on cell surfaces[16,17]. Recently, we applied Zn-DPA analogs as carriers for the delivery of therapeutic molecules, such as siRNA, using the selective and strong binding of Zn-DPA with the phosphodiester groups on the siRNA backbone[10,11]. In particular, this Zn-DPA platform for RNA delivery is highly flexible and can be used in conjunction with existing delivery systems such as NPs, polymers, proteins and antibodies by simply labeling the small-molecule Zn-DPA by established bioconjugation chemistry.

Sophisticated NPs developed for therapeutic or diagnostic applications[18–20] act as highly efficient drug delivery carriers and/or imaging probes; however, many of these systems lack RNA-binding capability and cannot be readily applied for RNA delivery. To overcome the difficulty of incorporating RNA into a sophisticated NP platform, we examined our recently developed, nontoxic, tumor cell–specific, drug-loadable and biodegradable HA-based self-assembled NPs for tumor therapy and imaging[21–23]. These are ideal nanoformulations for anticancer small-molecule delivery, as the NPs can target tumor cells by active targeting of CD44, which is overexpressed on the surface of a variety of tumor cells[24,25]. As a proof of principle, we demonstrated that a combination of the Zn-DPA analog and HA-based polymeric NPs allows efficient RNA delivery through high RNA binding affinity while maintaining the unique properties of NPs, such as high cellular delivery, drug-loadability and tumor targetability, as well as tumor-cell permeability[11]. The versatile NP system can deliver RNAi therapeutics along with chemotherapeutics to target sites such as tumor cells. In addition, the nanocarrier can release both payloads in the tumor cells in a controlled manner. These nanoformulations carrying the RNA-drug combinations hold

great potential given that they can provide a highly potent, yet less toxic, means of cancer treatment by compensating for each other. This would consequently result in synergistic therapeutic efficacy with relatively low side effects. For instance, RNA can sensitize specific cancer cells (e.g., drug-resistant cancer cells) to chemotherapeutics, so that the same amount of drug molecules can kill more cancer cells than conventional chemotherapeutics[26,27]. The nanocarrier system also has the potential to regulate cancer metastasis when specific RNAi therapeutics related to cancer metastasis are co-administrated with chemotherapeutics by RNA-drug co-delivery nanocarrier systems[28].

Furthermore, synthesis of the DPA-functionalized polymeric NPs is easy to scale up with cost-effective and readily available chemicals, which is a key issue in the development of practical applications. Because of its flexibility and ease of preparation, sophisticated and tailored carrier systems can be designed not only for siRNA but also for other phosphate anion–containing derivatives such as miRNA or oligonucleotide analogs.

In this protocol, we describe the detailed procedures for (i) synthesizing DPA-polymeric NP conjugate, (ii) formulating RNA complex, and (iii) delivering RNA into tumor tissues and tumor cells. Although we focus on the applications of DPA-functionalized HA-NPs for siRNA or miRNA delivery, which were developed in our previous study, potential designs for other DPA-functionalized NPs will be discussed in the Experimental design. Overall, this protocol will provide scientists with a practical tool to incorporate RNA delivery function to existing carrier systems for the development of multifunctional RNA carriers and to overcome technical obstacles of typical genetic delivery agents.

## Experimental design

The design concept of this protocol is to add an RNA delivery function to NPs that can deliver small-molecule drugs by simple labeling of an artificial phosphate receptor, a Zn-DPA analog (**Fig. 1**). The NP formula does not use highly cationic polymers, and it does not require expensive chemical modifications of RNA molecules. Because NPs and macromolecules labeled with small DPA moieties will gain the ability to bind RNAs, one can easily transform novel NPs to RNA carriers while maintaining the unique physicochemical properties of the original NP system, e.g., cell-penetrating, tumor-targeting, drug-loading and optical or magnetic characteristics[23,29,30]. Zn-DPA analogs can be modified with an appropriate terminal functional group such as a primary amine, carboxylic or thiol group, depending on the material to be labeled. This bioconjugation technique is similar to labeling fluorescent dyes to a protein.

Although Zn-DPA analogs are small molecules compared with the relatively large-sized NPs, the addition of Zn-DPA may still influence the solubility or stability of NPs. Therefore, formula optimization may still be required to achieve the best possible results. In this section, we discuss the design of DPA analogs and the coupling of these molecules with NPs. Once Zn-DPA-decorated NPs are prepared, RNAs can be loaded and routinely tested similarly to typical RNA delivery agents. Therefore, we will not discuss the general design strategies for RNA formulation or delivery in cells, but we will provide detailed instructions in the PROCEDURE section. It is important to recognize that the protocol introduced here uses a nanomaterial and details many steps outside the synthesis of the DPA compound.

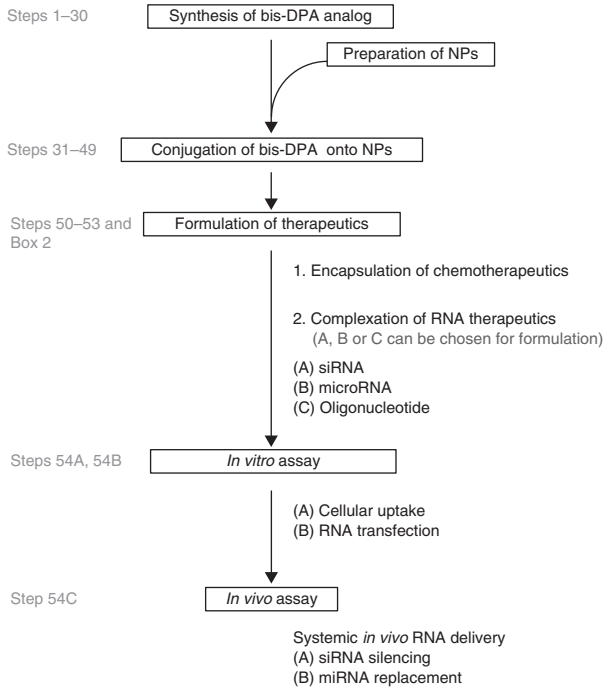


**Figure 1** | Flow diagram of the general procedure. Main steps are in solid boxes.

**DPA analogs.** Preparation of appropriate DPA analogs is the key to successful application of this protocol. It is well reported that rationally designed Zn-DPA complexes are highly selective for phosphate-containing biomolecules in aqueous solution through specific bindings between the coordinated Zn-DPA complexes and the anionic phosphate groups.

In this protocol, we used bis-DPA, **3**, as a model-binding ligand for RNA (**Fig. 2**). **3** contains a primary amine group that can be easily conjugated to materials containing carboxylic groups via conventional carbodiimide chemistry, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) or N,N′-dicyclohexylcarbodiimide (DCC) in the presence of N-hydroxysuccinimide (NHS). Here we introduce a bis-DPA with a primary amine group. This functional group can be converted into other groups such as a carboxylic or thiol group by established methods depending on the materials to be labeled by DPA molecules. This compound is currently not commercially available and therefore requires synthesis in the laboratory. We provide a detailed synthetic protocol for the DPA analog, and if synthesis of this compound is not directly accessible in biological laboratories, an organic chemistry or polymer science group can assist in reproducing zinc-DPA and nanocarriers.

**Zn-DPA–functionalized NPs.** Once the DPA analog is synthesized, it can be conjugated to nanomaterials through relatively easy bioconjugation methods[31]. These techniques, which are used to form linkages between NPs and functional moieties such as bis-DPA, have been well developed by using commercially available chemical mediators or cross-linkers in buffers and/or organic solvents followed by purification to remove unreacted DPA analogs and reactants. As DPA is a small molecule, the unreacted amount can be removed by conventional methods using dialysis tubes, centrifugal filtration, ultrafiltration, size-exclusion

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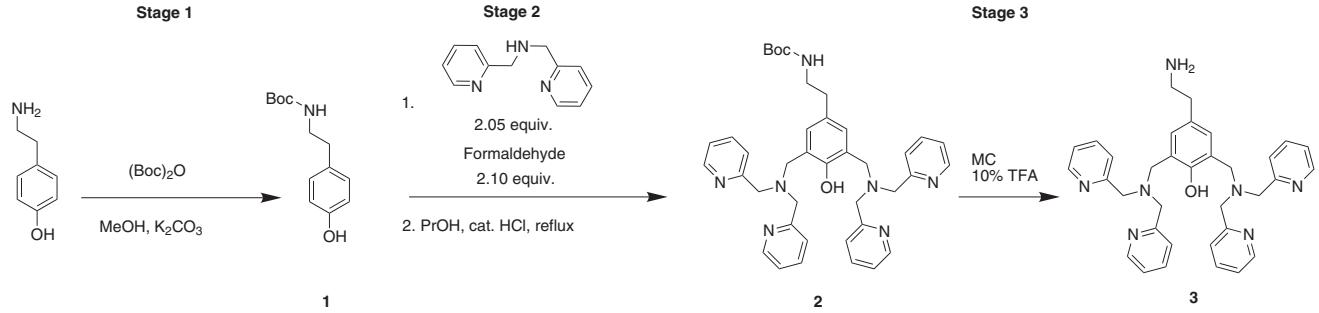


**Figure 2** | Synthesis of bis-DPA. Stage 1: Boc protection of amine. Stage 2: attachment of two 2,2′-dipicolylamines to tyramine via Mannich reaction. Stage 3: elimination of Boc protection with trifluoroacetic acid (TFA) in methylene chloride (MC).

columns or disposable desalting columns depending on the type of nanomaterials. Chelated Zn ions in the DPA analog have a crucial role in coordination between DPA and RNA. In this protocol, Zn ions are incorporated after preparation of the DPA-polymeric NP conjugate. It is also important to ensure that Zn ions are well incorporated into the DPA analog, as the release of free Zn ions can induce cellular toxicity.

In addition to applying DPA molecules into polymeric NPs or biomolecules, other inorganic NPs or materials can be functionalized with DPA molecules to create multifunctional siRNA carriers. As mentioned above, the end-functional group of DPA can be modified to a thiol group. The thiolated DPA allows for the ease of labeling the surface of gold NPs or quantum dots. Alternatively, DPA analogs can be labeled onto the surface of NPs through bifunctional linkers with PEG groups to improve the stability of the particles. For further potential applications, DPA functionality can be conjugated to polymer-coated, water-soluble magnetic NPs for magnetofection applications[32]. DPA molecules can also be functionalized onto materials such as carbon nanotubes, graphene sheets or gold nanocages for multifunctional roles, such as photodynamic therapy[33], while also delivering therapeutic RNAs. As discussed, various novel agents and/or carriers can be designed by adding an RNA delivery function to existing organic or inorganic materials without complex modification processes. In this protocol, we demonstrate how to add siRNA delivery function to our recently developed HA-NPs, overcoming the initial limitation relating to the highly anionic nature of the HA polymer.

**Zn-DPA–functionalized HA-NPs.** We exploit HA-NPs, as they are nontoxic, highly tumor cell–permeable, drug-loadable, biodegradable, easy to formulate and cost effective to manufacture. An amphiphilic HA conjugate can be prepared by coupling a hydrophilic biopolymer HA with a hydrophobic bile acid analog: 5β-cholanic acid (5β-CA; **Fig. 3**). In aqueous conditions, the amphiphilic HA conjugate readily forms self-assembled NPs by a sonication method. HA-NPs can efficiently accommodate hydrophobic small-molecule drugs in the hydrophobic inner core. Moreover, the nanocarrier system shows excellent tumor cell targetability and permeability. HA-NPs can recognize and actively endocytose tumor cells, given that a variety of

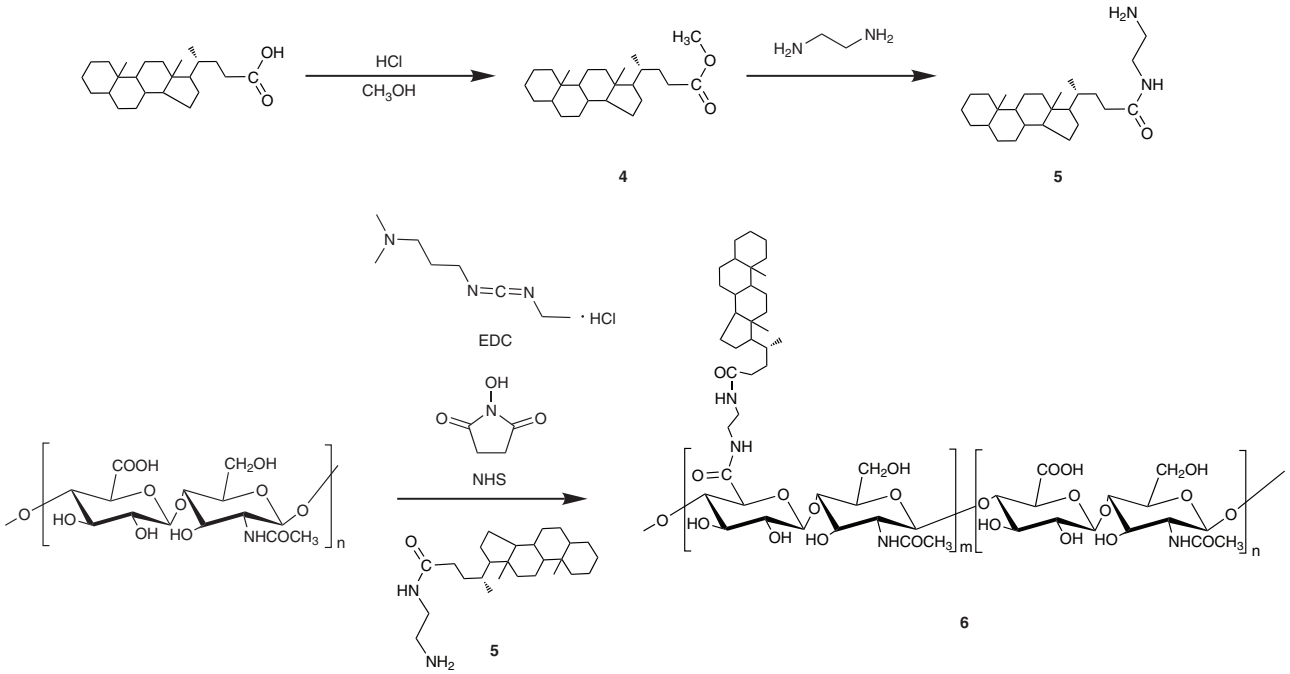


**Figure 3** | Synthesis scheme of aminoethyl 5β-cholanoamide 5 (EtCA) and HA-aminoethyl 5β-cholanoamide (HA-CA) conjugate **6**.

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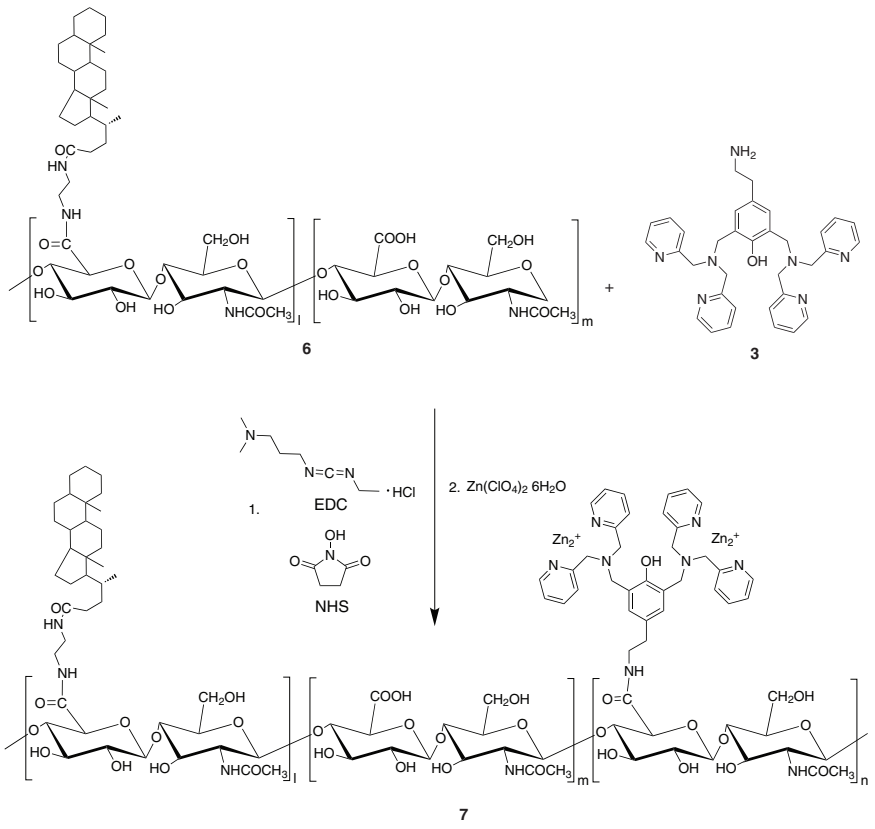


**Figure 4** | Synthesis scheme of bis-DPA-functionalized HA nanoparticles (HDz-NPs).

tumor cells are known to overexpress HA receptors, e.g., CD44 (refs. 24,25). Once HA-NPs penetrate tumor cells, the HA backbone is rapidly degraded by the HA-degrading intracellular enzyme hyaluronidase-1 (refs. 23,34), which substantially accelerates drug release in tumor cells[23]. We integrate the synthetic receptor, Zn-DPA, with the tumor-homing NPs into a nanocarrier system. Because HA has multiple functional groups including carboxylic and hydroxyl groups on its backbone, HA-NPs can be easily modified with multiple DPA molecules containing amine groups through amide formation. By adding $Zn^{2+}$ salt molecules into HA-DPA-NPs (HD-NPs), HA-DPA/Zn-NP complexes (HDz-NPs) can be prepared (**Fig. 4**; thin-layer chromatography (TLC) for compound **2** is shown in **Fig. 5**). The loading of DPA molecules onto the HA backbone can be calculated by $^1$H NMR. The UV-visible absorption spectra show a very weak bis-DPA absorbance and a high background absorbance in the presence of HA-DPA conjugates. RNA-binding capability can be tested in a retardation assay by agarose gel electrophoresis. Complexation of RNA molecules onto NPs should induce marked retardation of RNAs compared with that of free RNAs. The zeta-potential of NPs also reflects RNA binding. After RNA molecules are incorporated onto NPs, zeta potential of nanoformulations should decrease in comparison with the bare NPs without RNAs. In addition to loading siRNA molecules, DPA-functionalized NPs can bind to other molecules such as miRNAs and oligonucleotides (**Fig. 6**).

After RNA loading is optimized, complementary calcium and phosphate ions can be added onto the NPs (CaP-HDz/RNA-NPs), which facilitates intracellular RNA release and endosomal escape[35]. We confirmed cellular delivery using Cy3-labeled siRNA and analyzed transfection efficiency of siRNAs with model systems. Firefly luciferase (fLuc) gene-targeting siRNA (siLuc) and EGFP gene-targeting siRNA (siGFP) were transfected in stabilized cancer cell lines that express fLuc and GFP, 143B-fLuc and DU145-GFP. In addition, miRNA can also be effectively delivered with our system. We demonstrated transfection of miR-34a in HCT116 cells with a fLuc reporter vector containing the complementary target sequences of miR-34a. Cytotoxicity of NPs was evaluated by the alamarBlue cell proliferation assay.

**Multicargo nanocarriers loaded with RNAs and small-molecule drugs.** To show that HDz-NPs can act as a multifunctional carrier for both hydrophobic drug molecules and for RNAi therapeutics, the hydrophobic anticancer drug paclitaxel (PTX) was loaded in the interior of HDz-NPs as a model compound, and siRNA molecules were complexed on the surface of NPs[11]. For preclinical applications, PTX can be substituted with various other anticancer drugs such

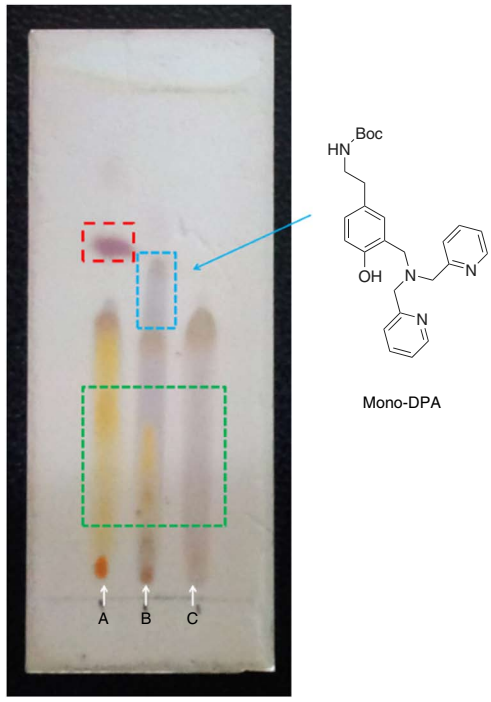


**Figure 5** | TLC analysis of compound **2**. Photograph of a silica gel TLC plate. TLC plates were eluted with methylene chloride:methanol = 10:1, stained with ninhydrin and heated. Pillar A shows a mixture of all reactants. Pillar B shows the completed reaction mixture. Pillar C shows the isolated compound **2**. The pink spot in the dashed red box is compound **1**. The yellow streak in the dashed green box is remaining 2,2′-picolylamine. Pillar C does not show a yellow streak. A gray band in the dashed blue box includes mono-DPA and impurities.

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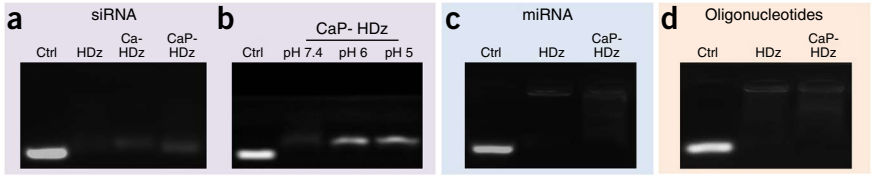


**Figure 6** | Formulation of RNAs with CaP-HDz-NPs. (**a**–**d**) Electrophoretic retardation analysis of nucleotides. Analysis of siRNA (**a**,**b**), miRNA (**c**) and oligonucleotides (**d**) (all at 10 pmol) binding with HDz and CaP-HDz-NPs (all at 2 μg of HDz-NPs). siRNA, miRNA and oligonucleotides are securely complexed with HDz and CaP-HDz at neutral pH (pH 7.4). However, rapid siRNA release from CaP-HDz-NPs was observed in endosomal/lysosomal pH conditions (pH 6 and 5) (**b**). This figure is adapted with permission from ref. 11, Copyright (2014) American Chemical Society. Ctrl, control.

as doxorubicin or camptothecin depending on established treatment regimens. To monitor simultaneous siRNA/drug delivery in cells, Cy3-labeled siRNA and Oregon green (OG)-labeled PTX were loaded onto the HDz-NPs, and the NPs were then applied to CD44-positive HCT116 cells. The cells were monitored by multichannel fluorescence microscopy at 30 min post treatment. In addition, therapeutic activity of RNA/drug-incorporated NPs on HCT116 cells was evaluated using a standard alamarBlue assay. Drug-loaded NPs can then be associated with therapeutic RNAs, and they may show synergistic therapeutic effects induced by both small molecules and RNAi. In our initial proof-of-principle studies[11], we tested siRNA silencing and miR-34a delivery effects of CaP-HDz/siLuc and CaP-HDz/miR34a in HCT116-miR34a tumor xenograft mice after i.v. injection of the formulas. Silencing of the fLuc-encoding gene and intracellular delivery of miR-34a were monitored by *in vivo* bioluminescence imaging (BLI). We advise that you vary the doses of RNA-containing NPs and image at different time points when optimizing this protocol in your setting. When anticancer drugs or therapeutic siRNAs are used instead of dye and siLuc, therapeutic effects can be investigated by monitoring tumor volume.

Overall, modification of HA-NPs with Zn-DPA endows HA-NPs with a unique function to bind RNAs. Furthermore, multiple DPA molecules on HA-NPs may result in HDz-NPs with strong RNA interaction. Unlike many other conventional RNA delivery carriers involving chemical conjugation or physical complexation of RNA with polycations, HA-NPs are highly biocompatible because they are based on a naturally occurring anionic biopolymer HA. Together with the excellent tumor-targetability of HA-NPs and the distinctive loadability of RNA therapeutics onto the surface and hydrophobic drug molecules in the core of HDz-NPs by a simple mixing method, this RNA and small-molecule drug delivery system is a promising, safe and efficient avenue for targeted-gene anticancer drug therapy.

## MATERIALS

### REAGENTS

**! CAUTION** Most of the reagents and solvents used in the protocol require the use of protective goggles, gloves and lab coats.

**▲ CRITICAL** It is strongly recommended to perform all the reactions in a fume hood. Solid and liquid waste generated during all the procedures should be disposed of properly, according to institutional guidelines.

**▲ CRITICAL** All animal experiments should be conducted in accordance with a US National Institutes of Health (NIH)-approved protocol.

- Athymic nude mice (female, 5–6 weeks old; Harlan Laboratories)
- Agarose (Bio-Rad, cat. no. 161-3102EDU)
- Calcium chloride dihydrate (Sigma-Aldrich, cat. no. 223506)
- 143B Human osteosarcoma cells (ATCC, cat. no. CRL-8303)
- 5β-Cholanic acid (5β-CA; Sigma-Aldrich, cat. no. C7628)
- Cy3-labeled siRNA (Invitrogen, cat. no. AM4621)
- Deionized water
- *N*,*N*-Dimethylformamide (DMF; Sigma-Aldrich, cat. no. 270547) **! CAUTION** It is flammable and harmful. It may cause skin and serious eye irritation.
- DMSO (Sigma-Aldrich, cat. no. D8418)
- 3,3′-Dioctadecyloxacarbo-cyanine perchlorate (Sigma-Aldrich, D4292) **! CAUTION** It may cause skin, eye and respiratory irritation.
- 2,2′-Dipicolylamine (TCI, cat. no. D2228)
- Di-*tert*-butyl dicarbonate (TCI, cat. no. D1547) **! CAUTION** It is an irritant.
- D-Luciferin (Gold Biotechnology, cat. no. LUCNA-1)
- DU 145 human prostate cancer cells (ATCC, cat. no. HTB-81)
- Ethidium bromide (Bio-Rad, 161-0433) **! CAUTION** It is a toxic chemical and a mutagen.
- Ethyl acetate (Sigma-Aldrich, cat. no. 34858) **! CAUTION** It is volatile and flammable.
- 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich, cat. no. E6383) **! CAUTION** It may cause skin irritation, serious eye damage and respiratory irritation.
- Ethylenediamine (EDA; Sigma-Aldrich, cat. no. 03550) **! CAUTION** It is flammable and harmful. It may cause severe skin burns and eye damage.
- EDTA (Sigma-Aldrich, cat. no. E9884) **! CAUTION** It is an irritant and can cause serious eye irritation. It is harmful to aquatic life.
- Formaldehyde, 37 wt% in water solution (Sigma-Aldrich, cat. no. 252549) **! CAUTION** It is a toxic chemical.
- Geneticin selective antibiotic (G418 sulfate; Invitrogen, cat. no. 10131035)
- Hexane (Sigma-Aldrich, cat. no. 270504) **! CAUTION** It is a volatile and flammable chemical.
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. 320331) **! CAUTION** It is toxic and corrosive. It causes severe skin burns and eye damage when in contact with skin and eyes. It may cause respiratory irritation.
- N-hydroxysuccinimide (NHS; Sigma-Aldrich, cat. no. 56480)
- Isoflurane (RxElite, cat. no. NDC60307-120-25) **! CAUTION** It is a profound respiratory depressant. Wear goggles, a lab coat and a face mask during experiments. Handle isoflurane inside a hood when appropriate. Closely seal the bottle after use.
- Lipofectamine 2000 (Lipo2K; Invitrogen, cat. no. 11668-019)
- Lipofectamine RNAiMAX (LipoMax; Invitrogen, cat. no. 13778030)
- Luciferase siRNA (siLuc; Invitrogen, cat. no. 12935-146)
- Magnesium chloride (Sigma-Aldrich, cat. no. M8266)
- McCoy's 5A (Cellgro, cat. no. 10-050-CV)
- Methanol (Sigma-Aldrich, cat. no. 32213) **! CAUTION** It is highly flammable and toxic.
- Methylene chloride (Sigma-Aldrich, cat. no. 66740) **! CAUTION** It is volatile, flammable and a potential carcinogen.
- Minimum essential medium (MEM; Cellgro, cat. no. 10-010-CV)
- miR-34a Mimic (Invitrogen, cat. no. 4464067; see **Table 1** for RNA sequences)
- miR-34a negative control (miR-NC; Invitrogen, custom synthesis; see **Table 1** for RNA sequences)
- Negative control siRNA (siNC; Invitrogen, custom synthesis; see **Table 1** for RNA sequences)
- Ninhydrin (TCI, cat. no. I0015) **! CAUTION** It may cause skin, eye and respiratory irritation.

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**TABLE 1** | RNA sequences.

| RNAi | Sense sequence 5′–3′ | Antisense sequence 5′–3′ |
|---|---|---|
| siRNA Firefly luciferase (siLuc) | GCACUCUGAUUGACAAAUACGAUUU | AAAUCGUAUUUGUCAAUCAGAGUGC |
| siRNA GFP (siGFP) | GGGCACAAGCUGGAGUACAACUACA | UGUAGUUGUACUCCAGCUUGUGCCC |
| siRNA-negative control (siNC) | AAUUCUCCGAACGUGUCACGU | ACGUGACACGUUCGGAGAAUU |
| miR34a | UGGCAGUGUCUUAGCUGGUUGUU | AACAACCAGCUAAGACACUGCCA |
| miRNA-negative control (miR-NC) | UUGUACUACACAAAAGUACUG | CAGUACUUUUGUGUAGUACAA |
| Oligonucleotide (Oligo-623) | GTTATTCTTTAGAATGGTGC | |

- Oligonucleotide (Oligo-623; Invitrogen, custom synthesis; see **Table 1** for RNA sequences)
- Paclitaxel, Oregon Green 488 conjugate (OG-PTX; Invitrogen, cat. no. P22310)
- pcDNA3.1 (Invitrogen, cat. no. V790-20)
- Potassium carbonate ($K_2CO_3$; Sigma-Aldrich, cat. no. 209619)
- 1-Propanol (Sigma-Aldrich, cat. no. 402893) ! **CAUTION** It is volatile and flammable.
- RNA annealing/dilution buffer (Invitrogen, cat. no. P22310)
- RPMI 1640 medium (Cellgro, cat. no. 10-041-CV)
- Silica gel 60, 0.040–0.063 mm (Merck, cat. no. 1093859025) ! **CAUTION** It is a hazardous dust.
- siRNA GFP reporter control (siGFP; Invitrogen, cat. no. 12935-145; see **Table 1** for RNA sequences)
- siRNA luciferase reporter control (siLuc; Invitrogen, cat. no. 12935-146; see **Table 1** for RNA sequences)
- siRNA negative control (siNC; Invitrogen; see **Table 1** for RNA sequences)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Sodium hyaluronate (MW = 234.4 kDa; Lifecore Biomedical, cat. no. HA200K-1)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045) ! **CAUTION** It may cause severe skin burns and eye damage.
- Sodium phosphate (Sigma-Aldrich, cat. no. 342483) ! **CAUTION** It may cause irritation to the eyes and skin.
- Sodium phosphate dibasic ($Na_2HPO_4$; Sigma-Aldrich, cat. no. S7907)
- Sodium phosphate monobasic ($NaH_2PO_4$; Sigma-Aldrich, cat. no. S8282)
- Sodium sulfate ($Na_2SO_4$; Sigma-Aldrich, cat. no. 239313)
- Trifluoroacetic acid (Sigma-Aldrich, cat. no. T6508) ! **CAUTION** Exposure to the liquid or spray-mist may cause severe skin and eye damage and/or respiratory irritation.
- Tris-acetate-EDTA, 10× (Advanced Biotech, cat. no. 08-514-001)
- Trizma base (Sigma-Aldrich, cat. no. T1503) ! **CAUTION** It may cause skin, eye and respiratory irritation.
- Tyramine (Sigma-Aldrich, cat. no. T90344) ! **CAUTION** It is an irritant and mutagen.
- UltraPure DNase/RNase-free distilled water (UPD; Invitrogen, cat. no. 10977-015)
- Vectashield mounting medium with DAPI (Vector Laboratories, cat. no. H-1200) ! **CAUTION** It is harmful if inhaled, swallowed or on contact with skin.
- Z-fix solution (Anatech, cat. no. 170) ! **CAUTION** It contains formaldehyde, which can be toxic.
- Zinc nitrate hexahydrate (ZNH; Sigma-Aldrich, cat. no. 228737) ! **CAUTION** It is corrosive and harmful.

**EQUIPMENT**

- Access to a NMR spectrometer such as a Fourier 300 (Bruker)
- Centrifugal filter unit, 100 kDa MWCO (Millipore, Amicon Ultra-15, cat. no. UFC910008)
- Bath sonicator (Branson)
- Beakers (200, 500 and 1,000 ml)
- Conical tubes (15 and 50 ml)
- Dialysis tubing, 12–14 kDa MWCO (Spectrum Laboratories, cat. no. 132678)
- Dynamic light-scattering (DLS) spectrometer such as a nanoparticle analyzer (Horiba, cat. no. SZ-100)
- Eight-well multichamber Permanox slides (Nalgene Nunc International, cat. no. 177445)
- Enduro Gel XL complete electrophoresis system (Labnet International, cat. no. E0160)
- Flow cytometer (Becton Dickinson, Accuri C6)
- Freezer (−20 °C) and 4 °C refrigerator
- Glass capillary
- Glass chromatography columns (cylindrical pillar shape, diameter: 5 cm)
- Glass reflux condenser
- Hand lamp (254 and 365 nm)
- Gel documentation system (Biospectrum 810, UVP)
- Confocal laser scanning microscope (FluoView FV10i, Olympus)
- Magnetic stirrer bars
- Magnetic stirrer hot plate
- Membrane filter, 0.45-µm pore size (Millipore, cat. no. HAWP04700F1)
- Oil bath
- Probe-type sonifier (Sonics & Materials Vibracell VCX130)
- Rotary evaporator
- Separatory funnels (250 ml)
- Septum
- Single-neck round-bottom flasks (50, 100 and 250 ml)
- Squeeze bottle
- Syringe filter, 0.45-µm pore size (Whatman, cat. no. 6971-2504)
- Thick-walled balloon
- TLC plates (Merck, cat. no. 1055540001)
- Vacuum pump
- Vortex mixer
- Xenogen IVIS-Lumina system (Caliper Life Sciences)

**REAGENT SETUP**

▲ **CRITICAL** Unless otherwise indicated, the buffers listed in this section can be prepared in advance and stored at 4 °C for several months.

**HEPES-phosphate buffer** Dissolve 11.9 g of HEPES, 212.9 mg of $Na_2HPO_4$ and 8.2 g of NaCl in ultrapure water (1 liter). Adjust the pH to 7.5 with HCl.

**Phosphate buffer (10 mM)** Dissolve 2.8 g of $Na_2HPO_4$ in deionized water (1 liter) and 2.4 g of $NaH_2PO_4$ in deionized water (1 liter). Mix 68.5 ml of $Na_2HPO_4$ solution and 31.5 ml of $NaH_2PO_4$ solution to prepare 10 mM phosphate buffer (PB). Adjust the pH to 6.5 with HCl.

**PBS (10× final concentration)** Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4$ and 2.4 g of $KH_2PO_4$ in 800 ml of deionized water. Adjust the pH of PBS buffer solution to 7.4 with HCl.

**PBS (1× final concentration)** Dilute 10× PBS tenfold with deionized water (vol/vol).

**Tris-calcium buffer** Dissolve 12.2 g of Trizma base and 147.01 g of calcium nitrate tetrahydrate in 1 liter of deionized water. Adjust the pH to 7.6 with HCl.

**Tris-acetate-EDTA buffer (50× final concentration)** Mix 242 g of Trizma base and 750 ml of deionized water. Carefully add 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 liter. This stock solution can be stored at room temperature (20–23 °C). The pH of this buffer is not adjusted and should be ~8.5.

**Tris-acetate-EDTA buffer (1× final concentration)** Dilute the 50× Tris-acetate-EDTA buffer 50-fold with deionized water (vol/vol).

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## Box 1 | 143B-fLuc–, DU145-GFP– and HCT116-fLuc-miR34a–stabilized cell lines ● TIMING 3–4 weeks

1. Modify pcDNA3.1 expression vector with fLuc or GFP, and include one miR34a perfect binding site (5′-acaaccagctaagacactgcca-3′) in the 3′ untranslated region of the fLuc sequence.
2. Transfect each cell line using lipo2K in six-well plates with 10% (vol/vol) FBS cell culture medium (without antibiotics), according to manufacturer's protocol. Briefly, mix 2.5 µg of the plasmid with 100 µl of Opti-MEM and 10 µl of Lipo2K with 100 µl of Opti-MEM in separate tubes, and incubate for 5 min. Combine both tubes, mix it gently, further incubate the mixture for 20 min at room temperature and add the 200 µl of Lipo2K/plasmid complexes to the well cells. After 4–6 h of transfection, replace the cell medium.
**! CAUTION** Antibiotics can be toxic to the cells with Lipo2K.
■ **PAUSE POINT** Transfection time may vary; resistant cells may be transfected for 24 h.
3. After 24–48 h, transfer the cells to 10-cm tissue culture dishes with 1:10; 1:100 and 1:1,000 dilutions to obtain individual clones.
4. Culture the cells in selection medium, supplemented with 500 µg/ml geneticin (G418).
5. Incubate the cells for 2–3 weeks; once single colonies become visible, mark fLuc or GFP-positive isolated colonies.
**! CAUTION** It is important to select isolated colonies to avoid contamination from neighboring negative colonies when collecting the clones.
6. To detach the colonies, wash the dish with PBS, add 20–50 µl of trypsin to the selected colonies and incubate for 2–5 min. With 20–50 µl of medium, gently aspirate the cells and transfer them to 12-well culture plates.
**! CAUTION** At least 8–10 clones should be selected, and fLuc or GFP signals from the selected clones need to be tested before and after transfection of sifLuc, siGFP or miR34a to determine if the clones can be used for *in vitro* or *in vivo* transfection assays (Steps 54B, 54C).
7. Culture the selected clones on T-75 flasks, and prepare stocks for *in vitro* or *in vivo* transfection assays (Steps 54B, 54C).

**143B-fLuc–, DU145-GFP– and HCT116-fLuc-miR34a–stabilized cell lines** Follow the steps in **Box 1**.

**HCT116-fLuc-miR34a tumor xenograft models** Subcutaneously inject $5 \times 10^6$ HCT116-fLuc-miR34a cells in the right flank of 24 female athymic nude mice (18–20 g). The 24 mice are divided into five groups, with four mice per group (CaP-HDz/siNC, CaP-HDz/miNC, CaP-HDz/siNC and CaP-HDz/miNC, PBS group) and four extra mice. **! CAUTION** Obtain appropriate training regarding animal handling. Animal protocols must be in place before performing animal studies.

**EQUIPMENT SETUP**

**Preparation of glassware** Dry all glassware in an oven overnight at >100 °C and allow it to cool down to room temperature before use.

## PROCEDURE

### Part 1, synthesis of bis-DPA (3): Synthesis of compound 1 ● TIMING 4–6 h

**1|** Add a Teflon-coated magnetic stir bar to a single-neck round-bottom flask (250 ml) and add 40 ml of methanol.

**2|** Add 2.74 g of tyramine (20.0 mmol). Dissolve tyramine with stirring.

**3|** Immerse a bottle of di-tert-butyl dicarbonate (melting point 22–24 °C) in a water bath at ~40 °C.

**4|** Take out 4.84 ml (4.6 g; density 0.95 g/ml) of di-tert-butyl dicarbonate from the bottle in a water bath at ~40 °C using a 5-ml micropipette, and transfer it to a 20-ml vial. Next, dissolve di-tert-butyl dicarbonate in the vial with 5 ml of methanol.

**5|** Slowly add the dissolved di-tert-butyl dicarbonate methanol solution prepared in Step 4 into the tyramine solution prepared in Step 2.
**! CAUTION** Gas evolution will take place.

**6|** Slowly add 8.3 g of $K_2CO_3$ (60.0 mmol) into the solution prepared in Step 5. Next, add 10–15 ml of distilled water with a squeeze bottle carefully to dissolve $K_2CO_3$. Stir the reaction mixture for 4 h at room temperature.

**7|** Check whether tyramine is completely consumed by TLC analysis. In a 10:1 (vol/vol) mixture of methylene chloride and methanol, the $R_f$ value of the product is ~0.8 and that of tyramine is negligible. Remove volatiles from the reaction mixture under low pressure using a rotary evaporator at 60 °C for 30 min.

**8|** Dissolve the remaining residue in 50 ml of ethyl acetate. Transfer it to a 250-ml separatory funnel containing 100 ml of deionized water. Shake the mixture vigorously to mix well and allow it to rest for separation of the organic layer. Carefully decant the separated organic layer into a flask.

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**9|** Add 20–30 g of $Na_2SO_4$ and manually stir the flask at 100 r.p.m. for 1–2 min to remove residual water from the decanted organic layer.

**10|** Filter and remove volatiles using a rotary evaporator at 60 °C for 30 min to obtain a viscous brown liquid. The expected yield is 4.0–4.5 g (85–95%).
■ **PAUSE POINT** Compound **1** can be stored at 4 °C for several months.

**Synthesis of compound 2** ● **TIMING 2–3 d**
**11|** Add a Teflon-coated stir bar to a single-neck round-bottom flask (100 ml) and add 3.7 ml of 2,2′-dipicolylamine (20.56 mmol, density: 1.107 g/ml).

**12|** Add 1.57 ml of formaldehyde solution (21.08 mmol, density: 1.09 g/ml, 37 wt% water solution). Add 1 ml of 1 N HCl. Stir the mixed solution until the mixture becomes clear.

**13|** Dissolve 2.37 g of compound **1** (10.0 mmol) in ~5 ml of 1-propanol. Next, add it to the reaction mixture. Add 30 ml of 1-propanol.

**14|** Attach a reflux condenser to the flask containing the reaction mixture, seal it with a septum and insert a needle with a balloon filled with $N_2$ gas.

**15|** Stir and reflux the reaction mixture for 2–3 d. Check the products by TLC; care must be taken because of the difficulty of confirming the completion of the reaction and purifying the product (**Fig. 3**). The completed reaction mixture is concentrated under low pressure using a rotary evaporator with a water bath at 60 °C to provide a dark brown mixture.
▲ **CRITICAL STEP** Check TLC to see whether Boc-protected tyramine is completely consumed. If the remaining 2,2′-dipicolylamine is negligible, terminate the reaction.

**16|** Dissolve the dark brown mixture in 50 ml of methylene chloride, and transfer it to a 250-ml separatory funnel containing 100 ml of deionized water. Neutralize it with saturated aqueous $K_2CO_3$ solution. Shake the mixture well.

**17|** Carefully decant the organic layer into a flask. Add 20–30 g of $Na_2SO_4$ into the flask and shake the flask manually for 1–2 min (~100 r.p.m.) to remove residual water.

**18|** Filter and evaporate the organic solvent under low pressure using a rotary evaporator at 40 °C to obtain a sticky dark brown liquid. Dissolve the residue in a small amount of methylene chloride (2–3 ml). Load the dissolved residue onto a silica gel column (300–400 ml) filled with methylene chloride.

**19|** Elute the silica gel column with 300 ml of methylene chloride. Next, elute it with 300 ml of hexane, and then elute it with 500 ml of ethyl acetate. A light yellow solution is eluted immediately. Continue until clear eluent emerges. Then elute with hexane (300 ml) again to clear off ethyl acetate from the silica gel column.

**20|** Elute with a mixture of methylene chloride and methanol. Gradually increase the ratio of methylene chloride to methanol from 100:0, 100:1.0, 100:1.5, 100:2.0, 100:2.5, 100:3.0, 100:3.5, 100:4.0, 100:4.5 to 100:5. Elute with 300 ml of methylene chloride. At each ratio containing methanol, elute with a mixture of 300 ml of methylene chloride and methanol. Note that silica gel column is eluted under air pressure.

**21|** Check the eluent coming out of the column by TCL periodically (~5 times per min), and when a dark spot appears on TLC under 254-nm UV light start to collect the eluent. When the dark spot on TLC seems blurred, then stop collecting the eluents.

**22|** Remove volatiles from the collected eluents under low pressure using a rotary evaporator at 40 °C to provide a sticky brown liquid compound **2** (Boc-bis-DPA). The estimated yield is up to 80% (5.3 g). TLC (silica, methylene chloride:methanol = 10:1), $R_f$ = 0.5.
■ **PAUSE POINT** Compound **2** can be stored at 4 °C for several months.

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**Synthesis of compound 3** ● **TIMING 6 h**

**23|** Add a Teflon-coated stir bar to a single-neck round-bottom flask (250 ml).

**24|** Dissolve 1 g (1.5 mmol) of compound **2** in 10 ml of methylene chloride.

**25|** Add dissolved compound **2** into the single-neck round-bottom flask (250 ml).

**26|** While stirring, slowly add a mixture of 26 ml of methylene chloride and 4 ml of trifluoroacetic acid.

**27|** After 4 h, confirm the products with TLC ($R_f$ compound **2** = 0.5, $R_f$ compound **3** = 0.05, silica, methylene chloride:methanol = 10:1) and then completely remove volatiles under low pressure using a rotary evaporator at 40 °C.

**28|** While stirring, dissolve the remaining residue in 20 ml of methylene chloride and add 10 ml of saturated aqueous $K_2CO_3$ solution slowly to basify the mixture.
**! CAUTION** Gas evolution will take place.

**29|** Transfer the mixture to a 50-ml separatory funnel, shake it well and carefully decant the organic layer into a flask. Pour 20 ml of methylene chloride to the remaining aqueous layer in a separatory funnel. Shake it well and decant the organic layer into a flask.

**30|** Dry the combined organic layer with $Na_2SO_4$. Add 10–15 g of $Na_2SO_4$ into the flask and shake the flask manually for 1–2 min (~100 r.p.m.) to remove residual water. Filter and remove volatiles under low pressure using a rotary evaporator at 40 °C to obtain a gummy brown solid compound **3** (bis-DPA). Expected yield is over 95%.
**? TROUBLESHOOTING**
■ **PAUSE POINT** Compound **3** can be stored at 4 °C for several months.

**Part 2, synthesis of aminoethyl 5β-cholanoamide (5): synthesis of compound 4** ● **TIMING 7 h plus drying time**

**31|** Add CA (1 g, 2.8 mmol) into a round-bottom flask (500 ml) filled with 50 ml of methanol. Add concentrated HCl (180 µl, 2.16 mmol). Allow the reaction to proceed with stirring under reflux at 60 °C for 6 h.
**! CAUTION** Methanol is highly flammable and toxic. Use protective goggles, gloves, a mask and a lab coat. Perform the procedure inside a fume hood.

**32|** Dry the solution prepared in Step 31 using a rotary evaporator under aspirator vacuum to obtain compound **4** (5β-CA methyl ester, MeCA). Characterize its chemical structure using $^1$H NMR in $CDCl_3$ at a concentration of 10 mg/ml.
■ **PAUSE POINT** MeCA can be stored at –20 °C for several months.

**Synthesis of compound 5** ● **TIMING 7 h plus drying time**

**33|** Add compound **4** (0.9 g, 2.4 mmol) into a round-bottom flask (50 ml) containing EDA (6 ml, 89.7 mmol). Let the reaction occur with stirring under reflux at 130 °C for 6 h. Use a glass pipette to add the solution dropwise into a 200-ml beaker containing 100 ml of cold deionized water.
**! CAUTION** EDA is flammable and harmful if swallowed or if it comes in contact with skin. Handle it inside a fume hood and avoid contact with skin and eyes. Use protective goggles, gloves, a mask and a lab coat.

**34|** Centrifuge the solution obtained in Step 33 at 25,000*g*, 4 °C for 30 min and remove the supernatants. Wash the precipitates thoroughly with deionized water. Repeat the centrifuging and washing steps five times to remove residual EDA.

**35|** Dry the product (compound **5**, EtCA) under vacuum. Characterize its chemical structure using $^1$H NMR in $CDCl_3$ at a concentration of 10 mg/ml.
■ **PAUSE POINT** EtCA can be stored at –20 °C for several months.

**Synthesis of HA-CA conjugate (6)** ● **TIMING 1 d plus dialysis and lyophilization time**

**36|** Dissolve HA (120 mg, 0.5 µmol) in formamide (24 ml); EDC (72.8 mg, 379.8 µmol) in formamide (500 µl); NHS (43.7 mg, 379.7 µmol) in DMF (500 µl); and compound **5** (39.8 mg, 98.8 µmol) in DMF (48 ml).

**37|** Add EDC solution into the HA solution prepared in Step 36. Allow the reaction to proceed with stirring at room temperature for 30 min.

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**38|** Add NHS and compound **5** solution slowly into the solution prepared in Step 37. Allow the reaction to proceed with stirring at room temperature overnight.

**39|** Dialyze the resulting solution prepared in Step 38 using dialysis tubing (12–14 kDa MWCO) against water/methanol (2 volumes/1 volume) for 1 d and distilled water for an additional 1 d. Lyophilize the solution to obtain compound **6** (HA-EtCA (HA-CA) conjugate).
■ **PAUSE POINT** The HA-CA sample may be stored at –20 °C for several months.

**40|** Characterize compound **6** using $^1$H NMR after dissolving it in $D_2O/CD_3OD$ (1 volume/1 volume) at a concentration of 5 mg/ml. To prepare NMR samples, weigh 5 mg of HA-CA conjugate in a 1.5-ml tube. Add 500 µl of $CD_3OD$ and vigorously vortex it for 120 min. Add 500 µl of $D_2O$ and vortex until the solution is completely clear. Transfer the solution (700 µl) into an NMR tube.

**Part 3, preparation of bis-DPA-functionalized HA NPs (HDz-NPs; 7)** ● **TIMING 1 d plus dialysis and lyophilization time**
**41|** Weigh 60 mg of compound **6** (HA-CA; $M_w$ = 258,000 Da) prepared in Step 39 in a 15-ml conical tube. Add 12 ml of PB (pH 6.5). Sonicate the HA-CA solution for 20 min to prepare HA-NPs using a probe-type sonifier.

**42|** Dissolve EDC (10.5 mg, 54 µmol) in PB (pH 6.5, 500 µl); sulfo-NHS (17.6 mg, 81 µmol) in DMSO (500 µl); and compound **3** (bis-DPA; 15 mg, 27 µmol) in DMSO (1 ml) by agitation.

**43|** Add EDC solution into the HA-NPs solution prepared in Step 41. Allow the reaction to proceed with stirring at room temperature for 30 min.

**44|** Add NHS and compound **3** solution into the solution prepared in Step 43. Allow the reaction to proceed overnight at room temperature.

**45|** Dialyze the resulting solution prepared in Step 44 using dialysis tubing (12–14 K MWCO) against water/methanol (1 volume/1 volume) for 1 d and distilled water for an additional 1 d to completely remove unreacted EDC, NHS and DPA residues.

**46|** After the dialysis step, lyophilize the solution prepared in Step 45.
■ **PAUSE POINT** The HA-CA-DPA (HD) conjugate sample prepared in Step 46 may be stored at -20 °C for several months.

**47|** Disperse the HD conjugate in ultrapure water (2 mg/ml) by sonication, as described in Step 41, for 20 min to prepare HD-NPs. Dissolve ZNH in ultrapure water (3 mg/ml, 10.1 mM). Add Zn stock solution (5 ml) into the HD-NPs solution (5 ml). Incubate the resulting mixture at 40 °C under agitation for 20 min to prepare HA-CA-DPA/Zn NPs (HDz-NPs).

**48|** Freeze-dry the solution to obtain compound **7**.
■ **PAUSE POINT** Compound **7** may be stored at –20 °C for several months.

**49|** Characterize compound **7** using $^1$H NMR after dissolving it in $D_2O/CD_3OD$ (1 volume/1 volume) at a concentration of 5 mg/ml.

**Part 4, formulation of RNA therapeutics** ● **TIMING 1.5 h**
▲ **CRITICAL** For co-delivery of both RNA and hydrophobic small-molecule drugs, the encapsulation step (**Box 2**) should be followed by the siRNA/miRNA/oligonucleotide formulation step (Step 51).

**50|** Dissolve compound **7** (HDz-NPs) prepared in Step 48 or the drug-loaded HDz-NPs prepared in **Box 2** in ultrapure water at 1 mg/ml by sonication, as described in Step 41, for 20 min.

**51|** Mix 2 µl of the solution prepared in Step 50 with 1 µl of 10 µM siRNA (luciferase siRNA: siLuc), miRNA (miR-34a) or oligonucleotide (Oligo-623; 10 pmol) by gentle vortexing. Incubate the resulting HDz/RNA-NPs at room temperature for 30 min.
■ **PAUSE POINT** The HDz/RNA-NPs can be stored at 4 °C until it is required, but for no longer than 30 d.

**52|** Mix 3 µl of the solution prepared in Step 51 with 1 µl of Tris-calcium buffer; add 4 µl of HEPES-PB and mix the solution by vigorously pipetting up and down (CaP-HDz/RNA-NPs).
■ **PAUSE POINT** The CaP-HDz/RNA-NPs solution can be stored at 4 °C until it is required, but for no longer than 30 d.

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## Box 2 | Steps for encapsulation of lipophilic small molecules ● TIMING 1.5 h plus dialysis and lyophilization time

1. Disperse HDz-NPs in ultrapure water (5 mg/ml) by sonication, as described in Step 41, for 20 min.
2. Dissolve lipophilic small molecules (e.g., PTX, doxorubicin or camptothecin) in DMF (4 mg/ml).
3. Add the solution (1 ml) dropwise into the HDz-NPs solution (2 ml) with sonication for 20 min.
4. Agitate the solution prepared in step 3 for 30 min with stirring.
5. Dialyze against distilled water for 12 h using dialysis tubing (12–14 kDa MWCO) to remove DMF and unloaded residues. After the dialysis step, lyophilize the solution.

**53|** Prepare a 3% (wt/vol) gel in Tris-acetate-EDTA buffer with agarose powder. Run the CaP-HDz/RNA-NPs complex and the corresponding free RNAs (1 µl of 10 µM siLuc, miR-34a or Oligo-623 (10 pmol)) as controls in a retardation assay by agarose gel electrophoresis (6 × 6 cm gel, 100 V, 15 min). Analyze the result using a gel documentation system.
**? TROUBLESHOOTING**

**Part 5, experiments in cells and animals**

**54|** The RNA therapeutics prepared as described above (Step 51) can be used for experiments in cells and animals. To determine cellular uptake, perform the steps in option A. The fluorescence signal of the samples will depend on various factors, such as the exposure time, imaging contrast, dye quenching and staining effect. Therefore, it may be difficult to quantify the cellular uptake of different samples. The cellular uptake can also be determined using a flow cytometer, which may give accurate information regarding the amount of cellular uptake. To show that the NPs can be used for transfection, perform the steps in option B. For *in vivo* experiments, follow the guidelines in option C, which is a procedure developed for mice.

**(A) Cellular uptake ● TIMING 2 d**

(i) Preseed 2 × $10^5$ cells (original HCT116 cell line without transfection; McCoy's 5A, 10% (vol/vol) FBS) in each well of an eight-well multichamber Permanox slide and incubate the cells overnight.

(ii) Prepare CaP-HDz/Cy3-labeled siRNA solution as described in Step 51. Wash the cells twice with 400 µl of PBS buffer (1×). Dilute the formulation (8 µl; siRNA: 10 pmol) with 200 µl of medium and add the CaP-HDz/Cy3-labeled siRNA solution into each well and incubate at 37 °C in a humidified 5% $CO_2$ atmosphere for predetermined time points (e.g., 1, 3 or 6 h). Perform parallel experiments using Lipofectamine 2000 (Lipo2K, Invitrogen)-complexed Cy3-labeled siRNA, and free Cy3-labeled siRNA only as a control group.

(iii) To confirm that the HA receptor CD44 mediates endocytosis of the nanoformulations, CD44 receptors are blocked with 200 µl of free HA molecules (10 mg/ml) dissolved in the culture medium (McCoy's 5A, 10% (vol/vol) FBS); next, the CaP-HDz/Cy3-siRNA solution (8 µl) prepared in Step 54A(ii) is added into the well.
▲ **CRITICAL STEP** Use the formulations immediately after synthesis. This will reduce the chance of siRNA degradation and bacterial contamination.

(iv) Wash the cells three times with 400 µl of PBS buffer (1×) per well.

(v) Add 200 µl of Z-fix solution to each well and incubate at room temperature for 20 min.

(vi) Wash the cells three times with 300 µl of PBS buffer (1×) per well.

(vii) Add one drop of Vectashield mounting medium with DAPI to stain the nucleus, cover it with a glass coverslip and secure it with nail polish. Wait for 30 min at room temperature and examine the cells with a confocal microscope.
■ **PAUSE POINT** The cells may be stored at 4 °C protected from direct light for several days before fluorescence imaging without altering the imaging results.

**(B) *In vitro* transfection assay of RNAs ● TIMING 2–3 d**

(i) Seed 143B-fLuc, DU145-GFP or HCT116-miR34a cells in a 96-well plate at 100 µl per well. The cell density should be 5 × $10^4$ cells per ml.

(ii) Prepare CaP-HDz/siLuc solution (8 µl) containing 1 µl of 5 µM siLuc, siGFP or miR-34a (5 pmol) and 2 µl of HDz-NPs (2 µg; Step 51). Predetermined concentrations of siRNA or miRNA can be used to evaluate the concentration-dependent silencing effect of CaP-HDz/RNA nanoformulations.

(iii) Dilute the formulation solution (8 µl) with 92 µl of the cell culture medium (MEM, 10% FBS).
▲ **CRITICAL STEP** Use the formulations immediately after synthesis. This will reduce the chance of siRNA degradation and bacterial contamination.

(iv) Change the cell culture medium with 100 µl of the transfection complexes in the wells. Incubate the cells at 37 °C for at least 24 h. Parallel experiments for positive control should also be carried out by Lipo2K according to the recommended protocol from the manufacturer (Invitrogen).
**? TROUBLESHOOTING**

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(v) Evaluate GFP expression levels in the DU145-GFP cells after the treatment using the flow cytometer.
(vi) Set up the Xenogen IVIS-Lumina imaging system for 143B-fLuc or HCT116-miR34a cells.
(vii) Add 20 µl of 3 mg/ml D-luciferin substrate per well and wait for 5 min.
(viii) Image the luciferase expression of the cells in the plate using a Xenogen IVIS-Lumina system.
(ix) The bioluminescence intensity of untreated 143B-fLuc cells is set as the 100% signal intensity, and the relative signal intensity is calculated to estimate the fLuc-encoding gene expression level. The relative signal intensity is further normalized with live cell numbers.

**(C) *In vivo* delivery of siRNA or miRNA ● TIMING 1–3 d**

(i) Prepare CaP-HDz/RNA solution (280 µl) containing 35 µl of 20 µM siLuc, siNC, miR-34a or miR-NC (RNA: 700 pmol) and 70 µl of HDz-NPs (70 µg; Step 51).
(ii) Check and make sure that the diameter of the tumor is ~8 mm
**! CAUTION** Obtain appropriate training regarding animal handling. Animal protocols must be in place before performing animal studies.
(iii) Set up the IVIS imaging system.
(iv) Anesthetize the animals using the rodent anesthesia system with isoflurane (2% (vol/vol) in 0.2 liter/min $O_2$ flow).
(v) Intraperitoneally inject D-luciferin solution dissolved in 1× PBS into HCT116-fLuc-miR34a tumor-bearing mice at a dose of 150 mg/kg. Image and quantify the luciferase expression by the IVIS system 15 min after administration. BLI is quantified as total photon counts from the region of interest after subtraction of background luminescence.
**? TROUBLESHOOTING**
(vi) Intravenously inject siLuc or miR-34a complexed with CaP-HDz (280 µl; RNA: 700 pmol). In parallel, CaP-HDz/siNC, CaP-HDz/miNC (280 µl; RNA: 700 pmol) or 280 µl of PBS should also be injected as control groups. The BLI should be visualized by Xenogen IVIS-Lumina100, and the relative fLuc expression level should be calculated.
**▲ CRITICAL STEP** Use the formulations immediately after preparation. Long sample storage times will increase the chance of siRNA degradation and bacterial contamination.
(vii) Intraperitoneally inject D-luciferin solution again, and scan the mouse with the IVIS imaging system at serial time points after injection. We typically choose 1, 2 and 3 d after injection. Image acquisition time ranges from a few seconds to a few minutes per scan.

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

**TABLE 2** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 30 | Improper separation of compound **2** from unreacted residues | Product is viscous oil; it drags along the silica gel | Mono-DPA is separated easily using ethyl acetate. The remaining 2,2′-dipicolylamine is not eluted with methylene chloride:methanol (20:1). Never use a mobile phase that is more polar than methylene chloride:methanol (15:1 (vol/vol)) |
| 53 | RNA degradation | RNA is highly susceptible to degradation by exogenous RNase introduced during handling | RNase-free reagents, barrier pipette tips and tubes should be used. RNA oligonucleotides should not be handled with ungloved hands |
| | Rapid RNA release from HDz/RNA-NF | Complexation between RNA and HDz-NPs can be disturbed by EDTA (1 mM) in TAE buffer, which can remove zinc ions from Zn-DPA moieties | For the gel retardation assay of HDz/RNA-NF, excess ZNH (0.3 µmol) can be added into the NF solution before electrophoresis |
| 54B(iv) | Cell contamination after adding the particles | CaP-HDz/siRNA-NPs solution is not well sterilized before being added into the medium | This problem can be resolved by using sterilized containers and solutions during experiments |
| 54C(v) | No detectable signal after injection of luciferin | Biodistribution of luciferin is rapid but the kinetics may be tissue dependent | The optimal time to image the model should be determined after luciferin injection. Continue to take images every 5–10 min for up to 40 min to generate a kinetic curve for luciferin expression in the model |

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● TIMING

Steps 1–30, synthesis of bis-DPA: 3–4 d
Steps 31–40, preparation of HA-NPs: ~2 d plus dialysis and lyophilization time
Steps 41–49, preparation of DPA-functionalized NPs: ~1 d plus lyophilization time
Steps 50–53, formulation of gene therapeutics: ~1.5 h for each formulation
Step 54A, cellular uptake: ~2 d
Step 54B, *in vitro* transfection assays: 2–3 d
Step 54C, *in vivo* delivery of siRNA or miRNA: 1–3 d
**Box 1**, preparation of stabilized cell lines: 3–4 weeks
**Box 2**, encapsulation of lipophilic small molecules: 1.5 h plus dialysis and lyophilization time

## ANTICIPATED RESULTS

### Synthesis of DPA analogs (3)

After Step 30, a gummy brown compound **3** of bis-DPA should be obtained. The anticipated analytical results are as follows:

Compound **1**: $^1$H NMR spectrum of **1** (300 MHz, $CDCl_3$): $\delta$ = 7.00 (d, J (H,H)= 8.4 Hz, 2H), 6.80 (d, J (H,H)= 8.6 Hz, 2H), 3.33 (t, J (H,H)= 6.7 Hz, 2H), 2.70 (t, J (H,H)= 6.7 Hz, 2H), 1.46 p.p.m. (s, 9H).

Compound **2**: $^1$H NMR spectrum of **2** (300 MHz, $CDCl_3$): (300 MHz, $CDCl_3$): $\delta$ = 8.52 (d, J (H,H)= 4.5 Hz, 4H), 7.60 (t, J (H,H)= 7.5 Hz, 4H), 7.50 ( d, J (H,H)= 7.7 Hz, 4H), 7.12 (t, J (H,H)= 6.0 Hz, 4H), 7.03 (s, 2H), 3.87 (s, 8H), 3.79 (s, 4H), 3.33 (t, J (H,H)= 6.7 Hz, 2H), 2.70 (t, J (H,H)= 6.7 Hz, 2H), 1.46 p.p.m. (s, 9H), $^{13}$C NMR spectrum of **2** (75 MHz, $CDCl_3$): $\delta$ = 159.2, 155.9, 154.3, 148.9, 136.5, 129.5, 128.6, 124.0, 122.9, 122.0, 78.3, 59.8, 54.8, 43.6, 39.0, 28.2 p.p.m. MALDI-TOF: m/z calculated for $C_{39}H_{46}N_7O_3$ $[M+H]^+$: 660.366; found: 660.588.

Compound **3**: $^1$H NMR spectrum of **3** (300 MHz, $CDCl_3$): δ = 8.52 (d, J (H,H)= 4.5 Hz, 4H), 7.60 (t, J (H,H)= 7.5 Hz, 4H), 7.50 ( d, J (H,H)= 7.7 Hz, 4H), 7.12 (t, J (H,H)= 6.0 Hz, 4H), 7.03 (s, 2H), 3.87 (s, 8H), 3.79 (s, 4H), 2.89 (t, J (H,H)= 6.7 Hz, 2H), 2.63 p.p.m. (t, J (H,H)= 6.7 Hz, 2H), $^{13}$C NMR spectrum of **3** (75 MHz, $CDCl_3$): $\delta$ = 159.2, 154.3, 148.9, 136.5, 129.5, 124.0, 122.9, 122.3, 122.0, 59.8, 54.8, 43.6, 39.0 p.p.m. MALDI-TOF: m/z calculated for $C_{34}H_{38}N_7O$ $[M+H]^+$: 560.314; found: 560.500.

### Preparation of DPA-functionalized NPs (7)

After Step 48, a white, cotton-like compound 7 (HDz-NPs) is obtained. Typical $^1$H NMR spectrum (300 MHz, $D_2O/CD_3OD$ (1 vol/1 vol)) of compounds **5** and **7** is shown in **Supplementary Figures 1** and **2**, respectively. The characteristic peaks of HA should be observed at 2.0 p.p.m. (the methyl group at the C2 position of N-acetyl glucosamine) and 3.3–4.8 p.p.m. (methylene and hydroxyl groups at the sugar unit). Those of CA are found in the range of 0.6–1.8 p.p.m. (methyl and methylene groups of the ring structure). The peaks of bis-DPA (**3**) are confirmed at 6.8–8.3 p.p.m. (methylene groups of the ring structure). The degree of conjugation of compound **3** on the HA polymer backbone can be quantitatively characterized by the integration ratio between the characteristic peaks of HA at 2.0 p.p.m. and compound **3** at 6.8–8.3 p.p.m. Approximately 30 molecules of **3** can be conjugated to 100 repeating sugar units of HA. In addition, NP formation, size distributions and zeta potential of HDz-NPs **7** can be analyzed by the DLS method (**Supplementary Fig. 3a,b**). An average hydrodynamic diameter of HDz-NPs is ~100 nm.

### Formulation of RNAs with CaP-HDz-NPs

In Steps 50–53, HDz-NPs readily form nanocomplexes with three kinds of RNAs, including siRNA, microRNA and oligonucleotides. The complexation of HDz-NPs with siRNA, microRNA or oligonucleotides can be confirmed by a retardation assay using agarose gel electrophoresis. The anticipated results are shown in **Figure 6**. HDz-NPs are able to bind siRNA, microRNA and oligonucleotides, whereas RNA molecules can easily be released from HDz-NPs after addition of phosphate ions to the complexes. This indicates that the complexation is based on coordination between the phosphate groups of RNAs and Zn-DPA. In contrast, siLuc can be secured, even after the addition of phophate ions, on HDz/siLuc capped with complementary calcium ions or calcium phosphate ions, i.e., Ca-HDz/siLuc or CaP-HDz/siLuc, respectively; the addition of phosphate ions does not trigger siLuc release from Ca-HDz/siLuc or CaP-HDz/siLuc. It is important to note that RNA release can be markedly accelerated in acidic conditions, i.e., endosomal/lysosomal pH (pH 6 or 5). As an example, we present a typical result in **Figure 6**.

### Cellular uptake of CaP-HDz-NPs complexed with Cy3-labeled siRNA

We present, as examples, the cellular uptake of CaP-HDz nanoformulations complexed with Cy3-labeled siRNA in CD44-positive cancer cells (HCT116 cells) and compare cellular uptake with Lipofectamine 2000 (Lipo2K) complexed with Cy3-labeled siRNA as a positive control and Cy3-labeled siRNA only as a negative control (Step 54A). **Supplementary Figure 4** shows typical cellular uptake in confocal micrographs of CaP-HDz/siRNA, Lipo2K/siRNA and siRNA in HCT116 cells. Strong and prominent

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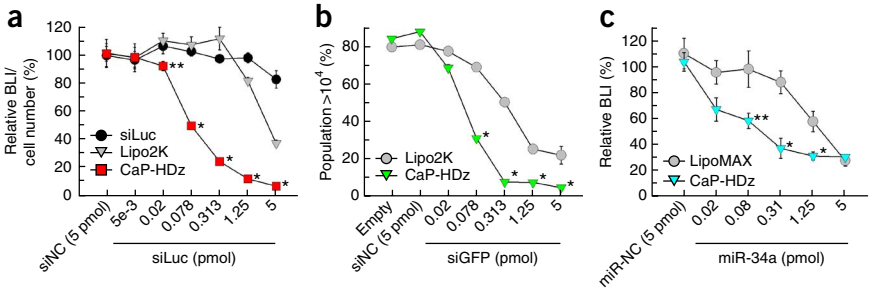


**Figure 7** | *In vitro* RNA transfection effect of CaP-HDz/RNA-NFs. (**a**,**b**) Suppression of fLuc on 143B-fLuc cells (**a**); or GFP on DU145-GFP cells (**b**) after treatment with siLuc only, Lipo2K or CaP-HDz-NPs complexed with varying concentrations of siLuc or siGFP, siNC and empty carriers. (**c**) Suppression of fLuc signals in HCT116-fLuc-miR34a cells by LipoMAX or CaP-HDz-NPs complexed with miR-34a, miR-NC. $*P < 0.005$, $**P < 0.05$ versus control, Lipo2K/siRNA or LipoMAX/miRNA. The CaP-HDz/RNA group suppresses fLuc and GFP gene expression more efficiently than the free siRNA, Lipo2K/siRNA or LipoMAX/miRNA groups. This figure is adapted with permission from ref. 11, Copyright (2014) American Chemical Society.

red fluorescence signals from Cy3-labeled siRNA can be observed in HCT116 cells incubated with CaP-HDz/siRNA. In contrast, far less fluorescence signal should be detected from the control groups than those for the CaP-HDz/siRNA group. To confirm that the HA receptor CD44 mediates endocytosis of the nanoformulations, cellular uptake of CaP-HDz/siRNA can be monitored in HCT116 cells, in which CD44 receptors are blocked with an excess amount of free HA molecules in a competitive binding study. After treatment of excess HA, cellular uptake of CaP-HDz/siRNA considerably decreases. Cellular uptake of CaP-HDz/Cy3-siRNA should also be confirmed in another CD44-positive cancer cell line (DU145 cells). Transfection efficiency of the CaP-HDz/siRNA group can be quantitatively evaluated by flow cytometry.

### Formulation and intracellular delivery of RNAs and drugs with CaP-HDz-NPs

CaP-HDz-NPs should be able to accommodate both biological therapeutics (RNAs) and chemotherapeutics (small-molecule hydrophobic drugs) onto a single platform (**Box 2**). Small-molecule drugs can be encapsulated within the hydrophobic interior of HDz-NPs owing to interaction between hydrophobic CA molecules of NPs and hydrophobic drug molecules using a sonication method in aqueous solution. Drug loading efficiency and drug contents can be quantified using an HPLC technique after the dissolution of the nanostructure in a co-solvent of distilled water and methanol (1:1 (vol/vol)). The loading efficiency should be >85%, and loading contents can vary in the range of 5–50% depending on the feeding amount of drug molecules. After hydrophobic small-molecule drugs are loaded, RNAs can be complexed as described in Steps 50–53. Cellular uptake and also colocalization of NPs, siRNA and drugs can be monitored by multichannel confocal fluorescence microscopy after treatment of HCT116 cells with the Cy5.5-labeled CaP-HDz-NPs that are loaded with both Cy3-labeled siRNA and OG-PTX (CaP-HDz/RNA+PTX). The anticipated results are shown in **Supplementary Figure 5**. The nanoformulations containing RNA and drug molecules can be rapidly internalized; colocalization of the three components of the nanoformulations (NPs, RNAs and drugs) should be observed in the HCT116 cells at 30 min after treatment.

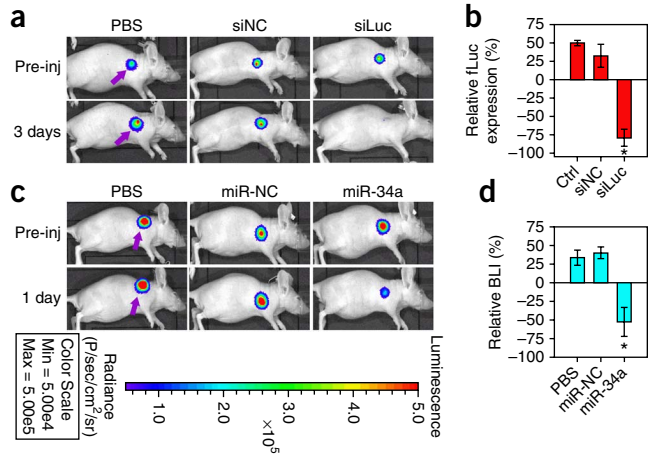


**Figure 8** | *In vivo* gene silencing effect of CaP-HDz/RNA-NFs. (**a**) *In vivo* bioluminescence imaging (BLI) of fLuc gene expression in HCT116-fLuc-miR34a tumor-bearing mice after i.v. injection of PBS, free siLuc or CaP-HDz/siLuc-NFs (280 µl; RNA: 700 pmol). (**b**) Quantitative analysis of fLuc expression at tumors after i.v. injection of CaP-HDz/siLuc-NFs into HCT116-fLuc-miR34a xenografts ($n = 4$). The BLI intensities from tumors of the CaP-HDz/siLuc-NF group significantly decreased, implying effective suppression of fLuc gene expression, but not from tumors of the other groups (PBS, CaP-HDz/siNC-NF). Purple arrows indicate tumor site. (**c**) *In vivo* bioluminescence imaging (BLI) of HCT116-fLuc-miR34a tumor-bearing mice i.v. injected with PBS, free miR-34a and CaP-HDz/miR34a-NFs (280 µl; RNA: 700 pmol). (**d**) Quantitative analysis of miR-34a delivery efficiency at tumors after i.v. injection of CaP-HDz/miR34a-NFs into HCT116-fLuc-miR34a xenografts ($n = 4$). The BLI signals from tumors of the CaP-HDz/siLuc-NF group significantly decreased *in vivo*, suggesting effective miR-34a delivery, but not from tumors of the other groups (PBS, CaP-HDz/siNC-NF). $*P < 0.005$, versus control. This figure is adapted with permission from ref. 11, Copyright (2014) American Chemical Society.

### *In vitro* RNA transfection assay

At Step 54B, the ability of CaP-HDz to deliver RNAs (siGFP, siLuc and miR-34a) can be confirmed by measuring the expression of GFP or fLuc in DU145-GFP cells, 143B-fLuc cells or HCT116-fLuc-miR34a treated with CaP-HDz/siGFP, CaP-HDz/siLuc or CaP-HDz/miR-34a nanoformulations (**Supplementary Fig. 6**). **Figure 7a**,**b** show typical results of the suppression effect of CaP-HDz/siLuc or CaP-HDz/siGFP on fLuc or GFP gene expression. **Figure 7c** shows the transfection effect of CaP-HDz/miR-34a on the suppression of

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fLuc signals in HCT116-fLuc-miR34a cells. The BLI signal intensity of untreated DU145-GFP cells, 143-fLuc cells or HCT116-fLuc-miR34a is set as the maximum GFP and fLuc expression, respectively. In the cells treated with CaP-HDz/RNAs, remarkably high gene silencing or transfection efficacy should be detected in a dose-dependent manner. The silencing efficacy of CaP-HDz/RNAs should also be superior to the Lipo2K or RNAiMAX complexes. Control Lipo2K or RNAiMAX complexed with nonspecific siNC, siRNA (siLuc and siGFP) or miR-34a should show significantly lower silencing/transfection effects.

### *In vivo* delivery of CaP-HDz complexed with siRNA or miRNA

At Step 54C, tumor-specific, *in vivo* gene silencing activity of CaP-HDz/RNA nanoformulations can be confirmed by *in vivo* BLI. The miR-34a reporter system is based on fLuc reporter genes, and thus the reporter system can be used for a dual imaging application to assess delivery efficiency of the CaP-HDz/RNA nanoformulations complexed with siLuc or miR-34a. fLuc gene silencing and intracellular miR-34a delivery efficacy of CaP-HDz/NRA nanoformulations can be evaluated by monitoring the BLI signals from tumors of HCT116-fLuc-miR34a xenograft mice after systemic administration of RNA nanoformulations. **Figure 8** shows typical *in vivo* BLI results to appraise the suppression effect of CaP-HDz/siLuc or CaP-HDz/miR34a. The BLI intensities from tumors of CaP-HDz/siLuc group should be decreased 24 h after injection and should be drastically dropped 72 h after injection. The tumors of the CaP-HDz/miR34a group should also show a substantial decrease in BLI signal from 24 h after injection. In contrast, The BLI intensities from tumors of control groups injected with PBS, CaP-HDz/siNC or CaP-HDz/miR-NC nanoformulations should be amplified owing to tumor growth.

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*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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1. Elbashir, S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
2. de Fougerolles, A., Vornlocher, H.P., Maraganore, J. & Lieberman, J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* **6**, 443–453 (2007).
3. Dykxhoorn, D.M., Palliser, D. & Lieberman, J. The silent treatment: siRNAs as small molecule drugs. *Gene Ther.* **13**, 541–552 (2006).
4. Jaroff, L. Fixing the genes. *TIME* (Jan. 11, 1999).
5. Whitehead, K.A., Langer, R. & Anderson, D.G. Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discov.* **8**, 129–138 (2009).
6. Liu, G., Swierczewska, M., Lee, S. & Chen, X. Functional nanoparticles for molecular imaging guided gene delivery. *Nano Today* **5**, 524–539 (2010).
7. Yoo, J.W., Irvine, D.J., Discher, D.E. & Mitragotri, S. Bio-inspired, bioengineered and biomimetic drug delivery carriers. *Nat. Rev. Drug Discov.* **10**, 521–535 (2011).
8. Tseng, Y.C., Mozumdar, S. & Huang, L. Lipid-based systemic delivery of siRNA. *Adv. Drug Deliv. Rev.* **61**, 721–731 (2009).
9. Bolcato-Bellemin, A.L., Bonnet, M.E., Creusat, G., Erbacher, P. & Behr, J.P. Sticky overhangs enhance siRNA-mediated gene silencing. *Proc. Natl. Acad. Sci. USA* **104**, 16050–16055 (2007).
10. Liu, G. *et al.* Sticky nanoparticles: a platform for siRNA delivery by a bis(zinc(II) dipicolylamine)-functionalized, self-assembled nanoconjugate. *Angew Chem. Int. Ed. Engl.* **51**, 445–449 (2012).
11. Choi, K.Y. *et al.* Versatile RNA interference nanoplatform for systemic delivery of RNAs. *ACS Nano* **8**, 4559–4570 (2014).
12. Lee, J.H., Jeong, A.R., Jung, J.H., Park, C.M. & Hong, J.I. A highly selective and sensitive fluorescence sensing system for distinction between diphosphate and nucleoside triphosphates. *J. Org. Chem.* **76**, 417–423 (2011).
13. Rhee, H.W. *et al.* A bifunctional molecule as an artificial flavin mononucleotide cyclase and a chemosensor for selective fluorescent detection of flavins. *J. Am. Chem. Soc.* **131**, 10107–10112 (2009).
14. Rhee, H.W. *et al.* Detection of kinase activity using versatile fluorescence quencher probes. *Angew Chem. Int. Ed. Engl.* **49**, 4919–4923 (2010).
15. Kwon, T.H., Kim, H.J. & Hong, J.I. Phosphorescent thymidine triphosphate sensor based on a donor-acceptor ensemble system using intermolecular energy transfer. *Chemistry* **14**, 9613–9619 (2008).
16. Smith, B.A. *et al.* Optical imaging of mammary and prostate tumors in living animals using a synthetic near infrared zinc(II)-dipicolylamine probe for anionic cell surfaces. *J. Am. Chem. Soc.* **132**, 67–69 (2010).
17. Bae, S.W. *et al.* Apoptotic cell imaging using phosphatidylserine-specific receptor-conjugated $Ru(bpy)_3^{2+}$-doped silica nanoparticles. *Small* **6**, 1499–1503 (2010).
18. Xie, J., Lee, S. & Chen, X. Nanoparticle-based theranostic agents. *Adv. Drug Deliv. Rev.* **62**, 1064–1079 (2010).
19. Choi, K.Y., Liu, G., Lee, S. & Chen, X. Theranostic nanoplatforms for simultaneous cancer imaging and therapy: current approaches and future perspectives. *Nanoscale* **4**, 330–342 (2012).
20. Yu, M.K., Park, J. & Jon, S. Targeting strategies for multifunctional nanoparticles in cancer imaging and therapy. *Theranostics* **2**, 3–44 (2012).
21. Choi, K.Y. *et al.* Self-assembled hyaluronic acid nanoparticles for active tumor targeting. *Biomaterials* **31**, 106–114 (2010).
22. Choi, K.Y. *et al.* PEGylation of hyaluronic acid nanoparticles improves tumor targetability *in vivo*. *Biomaterials* **32**, 1880–1889 (2011).
23. Choi, K.Y. *et al.* Smart nanocarrier based on PEGylated hyaluronic acid for cancer therapy. *ACS Nano* **5**, 8591–8599 (2011).
24. Platt, V.M. & Szoka, F.C. Jr. Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor. *Mol. Pharm.* **5**, 474–486 (2008).
25. Choi, K.Y., Saravanakumar, G., Park, J.H. & Park, K. Hyaluronic acid-based nanocarriers for intracellular targeting: interfacial interactions with proteins in cancer. *Colloids Surf. B Biointerfaces* **99**, 82–94 (2012).

26. Shapira, A., Livney, Y.D., Broxterman, H.J. & Assaraf, Y.G. Nanomedicine for targeted cancer therapy: towards the overcoming of drug resistance. *Drug Resist. Updat.* **14**, 150–163 (2011).
27. Palakurthi, S., Yellepeddi, V.K. & Vangara, K.K. Recent trends in cancer drug resistance reversal strategies using nanoparticles. *Exp. Opin. Drug Deliv.* **9**, 287–301 (2012).
28. Liu, C. *et al.* The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat. Med.* **17**, 211–215 (2011).
29. Choi, K.Y. *et al.* Theranostic nanoparticles based on PEGylated hyaluronic acid for the diagnosis, therapy and monitoring of colon cancer. *Biomaterials* **33**, 6186–6193 (2012).
30. Lee, D.E. *et al.* Amphiphilic hyaluronic acid-based nanoparticles for tumor-specific optical/MR dual imaging. *J. Mater. Chem.* **22**, 10444–10447 (2012).
31. Xie, J., Liu, G., Eden, H.S., Ai, H. & Chen, X. Surface-engineered magnetic nanoparticle platforms for cancer imaging and therapy. *Acc. Chem. Res.* **44**, 883–892 (2011).
32. Mykhaylyk, O., Antequera, Y.S., Vlaskou, D. & Plank, C. Generation of magnetic nonviral gene transfer agents and magnetofection *in vitro*. *Nat. Protoc.* **2**, 2391–2411 (2007).
33. Young, J.K., Figueroa, E.R. & Drezek, R.A. Tunable nanostructures as photothermal theranostic agents. *Ann. Biomed. Eng.* **40**, 438–459 (2012).
34. Stern, R. & Jedrzejas, M.J. Hyaluronidases: their genomics, structures, and mechanisms of action. *Chem. Rev.* **106**, 818–839 (2006).
35. Pittella, F. *et al.* Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity. *Biomaterials* **32**, 3106–3114 (2011).


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