Calcium Phosphate-Polymer Hybrid Nanoparticles for Enhanced Triple Negative Breast Cancer Treatment via Co-Delivery of Paclitaxel and miR-221/222 Inhibitors

Zilan Zhou^a, Carly Kennell^a, Joo-Youp Lee^{a,*}, Yuet-Kin Leung^b, and Pheruza Tarapore^b

^a Chemical Engineering Program, Department of Biomedical, Environmental, and Chemical Engineering, University of Cincinnati, Cincinnati, Ohio 45221-0012, U.S.A.

^b Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45221-0012, U.S.A.

* Corresponding author: Joo-Youp Lee

Tel.: +1 513-566-0018; Fax: +1 413-556-0018

E-mail: joo.lee@uc.edu

Financial Support: This study was supported by the Marlene Harris-Ride Cincinnati Breast Cancer Foundation, the Cincinnati Cancer Center, the Department of Defense (W81XWH-15-1-0353 (PT)) and the National Institutes of Health (P30ES006096). The authors appreciate their support.

Word count for abstract: 134

Word count for manuscript: 2,778

Number of references: 42

Number of figures: 5

Number of tables: 0

Number of Supplementary online-only files: 1

ABSTRACT

In this study, a development of a novel calcium phosphate-polymer hybrid nanoparticle system is reported. The nanoparticle system can co-encapsulate and co-deliver a combination of therapeutic agents with different physicochemical properties (i.e., inhibitors for microRNA-221 and microRNA-222 (miRi-221/222) and paclitaxel (pac)). miRi-221/222 are hydrophilic and were encapsulated with calcium phosphate by co-precipitation in a water-in-oil emulsion. The precipitates were then coated with an anionic lipid, dioleoylphosphatidic acid (DOPA), to co-encapsulate hydrophobic paclitaxel outside the hydrophilic precipitates and inside the same nanoparticle. The nanoparticles formed by following this approach had a size of about ≤ 100 nm and contained both lipid-coated calcium phosphate/miRi and paclitaxel. This nanoparticle system was found to simultaneously deliver paclitaxel and miRi-221/222 to their intracellular targets, leading to inhibit proliferative mechanisms of miR-221/222 and thus significantly enhancing the therapeutic efficacy of paclitaxel.

Keywords

Polymeric nanoparticle; RNA interference; co-delivery; synergistic effect; cancer therapy.

Abbreviations

miR, micro RNA; miRi, micro RNA inhibitor; pac, paclitaxel; ER, estrogen receptor; PR, progesterone receptor; HER2/neu, epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; PLGA, poly(D,L-lactide-co-glycolide); PEG, poly(ethylene-glycol); CaP, Calcium phosphate; TEM, transmission electron microscope; qRT-PCR, quantitative real-time polymerase chain reaction; DIEA, N,N-Diisopropylethylamine; NHS, N-hydroxysuccinimide; THF, tetrahydrofuran; DCM, Dichloromethane; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)-

carbodiimide hydrochloride; DOPA, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphate; FAM, Fluorescein amidite; NPs, Nanoparticles; EMEM, Eagle's Minimum Essential Medium; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4', 6-diamidino-2-phenylindole.

BACKGROUND

Breast cancer is one of the leading causes of cancer patient deaths in the U.S.¹ In particular, ~10-20% of breast cancer cells do not express Estrogen Receptor, Progesterone Receptor, and epidermal growth factor receptor 2 (HER2/neu), and are referred to as Triple-Negative Breast Cancer (TNBC). TNBC is very difficult to detect and target, and has a high potential for metastases.² Many conventional treatments are reported to have become ineffective, showing worse prognosis than other types of breast cancers in clinic.^{3, 4} Rational drug combinations exploiting the blockage of multiple survival pathways in tumor cells hold great promise to enhance the efficacy of the treatment. MicroRNA (miR) is a short non-coding RNA that can effectively regulate protein translation. One unique feature of miR is that one miR can target and regulate expressions of multiple genes at the transcriptional and translation level.⁵ A key miR can act as a master switch for multiple pathways. In breast cancer, miR-221/222 have been recently determined as crucial regulators in cancer initiation, progression, epithelial-to-mesenchymal transition, and tamoxifen-related drug resistance.⁶⁻¹¹ miR-221/222 were found to be overexpressed in TNBC, which makes them a potential therapeutic target for TNBC.⁶ In addition, miR-221/222 were found to be up-regulated in paclitaxel-resistant cancer cells, indicating their involvement in paclitaxel resistance.¹² miR inhibitor (miRi) is a synthetic RNA molecule with anti-sense sequence to mature miR, and is able to specifically bind to and inhibit the functions of miR. Therefore, it is anticipated that a combination of miR-221/222 inhibitors (miRi-221/222) and paclitaxel (pac) would be a promising strategy for TNBC treatment.

In order to achieve synergistic therapeutic effects, both anti-cancer drug and gene regulator should be co-delivered to the same cancer cells.^{13, 14} The development of a single carrier that can co-encapsulate and co-deliver both drug and miRi to the same cancer cells

emerges as a promising approach for enhanced cancer therapy.¹⁴⁻¹⁸ However, the synthesis of such a co-delivery system comprising miRi and chemotherapeutic drug is quite challenging in many cases, due to the hydrophilicity of miRi and the hydrophobicity of the drug.

Nanoparticles made of biocompatible and bio-degradable polymer, such as FDA-approved poly(D,L-lactide-co-glycolide) (PLGA) and poly(ethylene-glycol) (PEG), have been of particular interest.^{14, 19} Calcium phosphate (CaP) is a well-established reagent for *in vitro* cell transfection due to its high biocompatibility and ease of preparation. One advantage of employing CaP is that it dissolves at acidic pH, such as endosomal and lysosomal pHs. After dissolution, the osmotic pressure inside these compartments increases and thus induces endosomal escape. However, during synthesis, the precipitation of CaP is difficult to control and often causes aggregates. It was reported that by preparing CaP in a water-in-oil emulsion, the growth of CaP was well controlled.²⁰ In addition, a lipid coating could further stabilize the CaP precipitates in organic solvent.²¹ It is assumed that a combination of water-in-oil emulsion and lipid coating could generate organic solvent soluble CaP/miRi precipitates with controllable sizes so they can be co-encapsulated with pac inside the polymeric nanoparticles.

In this study, we report a novel calcium phosphate polymer hybrid nano-scaled particle system to co-deliver two model drugs, miRi-221/222 and pac, for enhanced TNBC treatment. The hybrid NPs can co-encapsulate miRi-221/222 and pac in the same entity. The NPs containing miRi-221/222 are able to reduce the level of miR-221/222, thus inhibiting the proliferative mechanism of miR-221/222. This inhibition is done by up-regulating the target of miR-221/222, p27^{Kip1} and tissue inhibitor of metalloproteinase 3 (TIMP3). The cell viability of MDA-MB-231 cells treated with the co-delivery NPs was drastically reduced compared to treatment of NPs containing negative control miRi or NPs containing pac only.

METHODS

A lipid/CaP/miRi complex was synthesized according to previous reports.^{21, 22} This lipid-coated complex is stable and soluble in non-polar or moderately polar solvents. Then the complex was co-encapsulated with pac by PLGA-*b*-PEG to form calcium phosphate polymer hybrid NP through nanoprecipitation.²³⁻²⁶ The morphology of the NPs was characterized by transmission electron microscope (TEM). The NPs were washed and un-encapsulated drugs (pac and miRi) were separated using Amicon® Ultra Centrifugal Filters filter (100 kDa MWCO). Then the amount of pac and miRi encapsulated in the NPs were measured by HPLC and fluorescence spectroscopy, respectively. The encapsulation efficiency was determined by dividing the amount of drugs encapsulated by the amount of drugs initially added when NPs were formed. The *in vitro* drug release kinetics was assessed by a dialysis method. The delivery of miRi into the TNBC cell line was verified by fluorescent microscopy. The gene regulation efficiencies and cytotoxicity of the co-delivery NPs were evaluated via quantitative real-time polymerase chain reaction (qRT-PCR) and MTS assay, respectively. A detailed description of the methods is included in the Supplementary Materials.

RESULTS

Development and Characterization of Co-delivery Nanoparticles

The purpose of the hybrid NPs developed in this study is to co-encapsulate anti-cancer drug and miRi in the same NP. Due to the two different properties of hydrophobic chemo drug and hydrophilic miR or miRi, a challenge arose when encapsulating both entities in a single carrier. The calcium phosphate-polymer hybrid NPs are developed in order to co-encapsulate

and co-deliver the entities to the same site. As illustrated in Fig. 1A, miR-221/222 inhibitors (miRi-221/222) are first mixed with calcium chloride and sodium phosphate and then added to cyclohexane/IGEPAL® CO-520 (70/30 (v/v)). With the help of strong surfactant IGEPAL®CO-520, a water-in-oil emulsion is formed, and miRi-221/222, calcium, and phosphate are well dispersed in the water droplets, which control the growth of CaP/miRi. The excess positive charges of calcium on the surface of precipitates are neutralized by negatively charged lipid DOPA, forming a lipid coating. The lipid/CaP/miRi complex shows a narrow size distribution (Fig. 1B(a)) and could be co-encapsulated with pac in PLGA-*b*-PEG NPs through nanoprecipitation. The lipid-coated CaP/miRi and pac were encapsulated inside the PLGA block copolymer due to hydrophobic interactions, and the PLGA core was stabilized by the hydrophilic PEG (Fig. 1A).

The TEM images of the lipid/CaP/miRi complex and co-delivery NPs (NP (miRi/pac)) are shown in Fig. 1B (a) and (b), respectively. The particle size distributions for the lipid/CaP/miRi complex and NP (miRi/pac) were also obtained by analyzing the TEM images shown in Fig. 1B. As shown in Fig. 1C, the diameter of lipid/CaP/miRi is in a range of about 10-25 nm. Because lipid and miRi are electron transparent, only the calcium phosphate precipitates can be visualized under TEM without staining.^{27, 28} As shown in Fig. 1B (a), the lipid/CaP/miRi complex has formed a hollow core, and it is speculated that miRi is encapsulated in the hollow core and protected by the calcium shell.²¹ The structure of lipid/CaP/miRi is still preserved even after co-encapsulation with paclitaxel by PLGA-*b*-PEG, shown in Fig. 1B (b). The final nanoparticles were in a range of about 30-80 nm in size. a single PLGA-*b*-PEG NP was found to encapsulate up to about 16 lipid/CaP/miRi complexes as shown in Fig. 1B (b).

Encapsulation Efficiency and Release Kinetics of Co-Delivery Nanoparticles

The encapsulation efficiencies of a single agent (pac or miRi)-encapsulated NP and co-delivery NPs were examined (Fig. 2A). All NPs encapsulating miRi or miRiNC were prepared with lipid-coated CaP. The NP sample encapsulating only pac (NP(pac)) was prepared with the PLGA-*b*-PEG polymer and does not have lipid-coated CaP. The loading efficiency was about 70% regardless of the number or type of the agent encapsulated in the NPs. The sequence of miRi (i.e. miRi-221/222 and scrambled negative control miRi) has no effect on the encapsulation efficiency. Pac and miRi loadings result in ~15 µg/mg of nanoparticles and 300 pmoles/mg of nanoparticles, respectively.

The *in vitro* release kinetics of miRi and pac from co-delivery NPs were investigated by dialysis against PBS at 37 °C (Fig. 2B). To simulate the extracellular and acidic endolysosomal environment, two pH conditions were employed. The quantity of pac encapsulated inside the NPs was determined from HPLC by dissolving an aliquot of NPs in acetonitrile. FAM-labeled miRi was measured in a fluorescence plate reader. About 80% of pac was released within 20 hours regardless of the pH condition. This is attributed to the fact that the dissolution of the PLGA-*b*-PEG block copolymer is not dependent on pH.²⁶ However, the miRis were released at slower and different rates with respect to pH: 40% released at pH 5 and 20% at pH 7 after 20 hours. The release rate was almost doubled at acidic pH 5 compared to physiological pH 7. This behavior is attributed to the high dissolution rates of calcium phosphate at low pH conditions.^{29,}
³⁰ Lipid coating was reported to be able to delay the release of hydrophilic agents and avoid the burst release.³¹

Delivery of miRi into TNBC Cell Line

To verify the delivery of miRi into TNBC cells, a fluorescence imaging study was carried out using MDA-MB-231 cells incubated with NP(miRi) with a miRi concentration of 20 pmole/ml for 5 min, 30 min, 2 hrs and 4 hrs at 37 °C. DAPI was used to stain the nuclei and phase contrast images were taken to show the cell morphology. In addition, endolysosomes were labeled with LysoTracker® to examine whether NP(miRi) could escape from endolysosomes. As shown in Fig. 3, from 5 min to 2 hrs, the amount of miRi delivered inside the cells was found to increase based on a color change from red to orange. The orange color was derived from an overlap between green miRi and red endolysosomes. It indicates that those NPs(miRi) were also trapped inside the endolysosomal compartments after ~0.5-2 hrs. In contrast, the white arrows show the poor co-localization of miRi and endolysosomes, suggesting that these miRi escaped from the endolysosomes after 4 hrs. This can be attributed to the calcium phosphate precipitate used to load miRi. It is well known that calcium phosphate dissolves in acidic conditions.³⁰ As NPs(miRi) enter the cells through endocytosis and localize in acidic endolysosomes, the dissolution rate of calcium phosphate binding miRi increases. Then the dissolved calcium and phosphate ions increase osmotic pressure and cause a swelling of the compartments. This swelling eventually ruptures the endolysosomal membranes and releases miRi into cytoplasm.^{30,}

32

Nanoparticle-Mediated miRNA and mRNA Regulation

After validating the delivery of miRi into cells by NPs, a qRT-PCR study was performed to evaluate the capability of miRNA and messenger RNA (mRNA) regulation of the miRi-encapsulated NPs. The PLGA-PEG NPs encapsulated only the miRi-221/222 without pac. The miRi-221/222 are short single stranded RNA molecules with the anti-sense sequences of miR-

221/222. The binding of miRi to miR would decrease the activities of miR and alter the translation. The level of miR-221/222 was assessed by qRT-PCR after treating MDA-MB-231 cell line for three days (Fig. 4 (A) and (B)). Cells without treatment were used as a control. The results show that the NPs encapsulating miRi-221/222 successfully inhibited the miR-221/222 in two of the miRi dosages of 5 and 12.5 pmoles/ml. In addition, the scrambled sequence did not cause any significant unspecific alteration of the miR expression. Next, the mRNA levels of the two targets, p27^{Kip1} and TIMP3, were examined. Cell cycle inhibitor p27^{Kip1} and TIMP3 are direct targets of miR-221/222.^{9, 10, 33, 34} Both p27^{Kip1} and TIMP3 are considered tumor suppressors.^{7, 10, 11, 34, 35} These previous studies have shown that the overexpression of TIMP3 in vascular smooth muscle cells and melanoma cell lines inhibits the invasion and promotes an apoptotic cell death. The down-regulation of p27^{Kip1} was found to be responsible for the oncogenesis and progression of prostate carcinoma. The knockdown of miR-221/222 in MDA-MB-231 cell line with miRi-221/222 could significantly increase the expression of mRNAs of p27^{Kip1} and TIMP3, suggesting a successful NP-mediated miRNA regulation (Fig. 4 (C)).

Cytotoxicity of Co-Delivery Nanoparticles

The cytotoxic efficacy of the NPs was examined using the MDA-MB-231 cell line as an *in vitro* TNBC model. The cells were treated with pac-encapsulated NPs (NP(pac)), co-delivery NPs (NP(pac/miRi) or NP(pac/miRi NC)) and free pac. The cells without treatment served as a control. After 72 hours of incubation, the viability of the cells was assessed by MTS assay (Fig. 5). The difference between the cell viability of the encapsulated form (NP(pac)) and free form of pac was minimal. Pac decreased cell viability by ~40% at a high dosage of 0.67 µg/ml. However, with the co-delivery strategy (NP(pac/miRi)), the cell viability went down by ~80% at

the same dosage of 0.67 $\mu\text{g/ml}$. In addition, the paclitaxel cytotoxicity was examined in a broader range of the concentrations (Fig. S1). The result shows that the NP(pac/miRi) requires only ~1% of pac used in a free form to achieve the same ~80% cytotoxicity. In order to make sure the enhanced efficacy comes from the synergistic effect of miRi-221/222 and pac, a scrambled miRi was used as a negative control and encapsulated in the co-delivery NPs (NP(pac/miRiNC)) using the same method as miRi-221/222. As expected, the NP(pac/miRi) showed a much higher cytotoxic efficacy than NP(pac/miRiNC). The NP encapsulating miRi alone, NP(miRi), was also tested in a wide range of miRi concentrations in order to verify that the enhanced efficacy was derived from the co-delivery of miRi and pac. Indeed, the NP(miRi) showed only a moderate inhibition effect on the cell viability (Fig. S2). These results using the negative control and NP(miRi) confirm the synergistic effect of miRi-221/222 and pac. It is worth noting here that the co-delivery of miRi-221/222 and pac also works for estrogen positive (MCF-7) and HER2/neu positive (SKBr-3) breast cancer cell lines (Fig. S3).

DISCUSSION

The miR regulates multiple molecular pathways of cancer cells and is considered a promising therapeutic target. The synergistic effects of combining traditional chemo drug and RNAi that inhibits proliferative miR or restores cancer suppressive miR have attracted numerous attentions.³⁶ In this study, miR-221/222 as oncomiRs are chosen as targets for treating TNBC. The miRi-221/222 and pac are co-encapsulated in the CaP polymer hybrid NPs.

Unlike the widely used cationic polymer or lipid for gene delivery which are inherently cytotoxic and unable to release incorporated RNA or DNA molecules upon arrival at the cancer cell (i.e. unpacking issue), CaP has many advantages.³⁷ First, CaP is present in body and

considered highly biocompatible.^{38,39} In addition, CaP exhibits pH dependent solubility and thus is designed to release the co-precipitated miRi upon a pH change. As shown in Fig. 2B, miRi are released much faster at the pH 5 condition compared to pH 7 due to the fast dissolution of CaP in acidic conditions. Furthermore, CaP dissolves in acidic endosomes, increasing the osmotic pressure inside and finally causing endosomal membrane rupture to release the miRi into cytoplasm.^{30,40} The endosomal escape is virtualized by fluorescence microscope, as shown in Fig. 3. Despite the advantages of CaP, it often suffers huge variations in transfection due to the uncontrollable growth of CaP precipitates. By performing the condensation of miRi with CaP in the water-in-oil emulsion, the precipitation of CaP and miRi is well controlled.^{20,41,42} The lipid coating onto CaP/miRi precipitates further stabilizes the whole CaP/miRi complex in the oil. In addition, the C18 chain of lipid provides enough hydrophobicity to the CaP/miRi so it can be encapsulated by polymeric nanoparticles via nano-precipitation.

Once the co-delivery of NPs were successfully formulated, *in vitro* tests on the MDA-MB-231 cell line were performed to determine if the NP(miRi/pac) was able to achieve its designed objectives, RNAi and synergistic effects. The levels of miR-221/222 were reduced and the functions of miR-221/222 were inhibited by examining the expression of p27^{Kip1} and TIMP3 in the cells treated with NP(miRi) (Fig. 4). The p27^{Kip1} was down-regulated by miR-221/222. With the miR-221/222 inhibited by NP(miRi), MDA-MB-231 restores p27^{Kip1} and TIMP3 which are considered tumor suppressors. Finally, the strategy of co-delivery miRi-221/222 and pac shows superior outcomes when compared to other delivery methods (i.e. free pac, NP(pac), NP(miRi), and NP(miRiNC/pac)) (Figs. 5, S1, S2).

In summary, a novel CaP-polymer hybrid nanoparticle system was developed in order to achieve enhanced therapeutic effects of the co-delivery of pac and miRi-221/222 for TNBC. The

nanoparticle system forms a PLGA-*b*-PEG polymer matrix comprising pac and lipid-coated CaP precipitates binding miRi-221/222 for the co-delivery. The NP system was proven to be effective in up-regulating the gene expressions of p27^{Kip1} and TIMP3 responsible for tumor suppression in the MDA-MB-231 cell line. It was demonstrated that the co-delivery NP system had a superior tumor cell suppression capability when compared to free pac or NPs delivering a single drug (i.e. miRi only or pac only) that we tested. This suggests the synergistic effects of combining RNA interference (miRi-221/222) and traditional therapeutic drug (paclitaxel). It is anticipated that a co-delivery strategy of this kind can help reduce the dosage of potent and cytotoxic drugs while achieving a desired cytotoxic efficacy.

ACKNOWLEDGMENTS

We also gratefully acknowledge Dr. Shuk-Mei Ho's generous support allowing for accessing various instruments in her lab facilities.

REFERENCES

1. Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015; 65: 5-29.
2. Badve S, Dabbs DJ, Schnitt SJ, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol.* 2011; 24: 157-67.
3. Lee MJ, Ye AS, Gardino AK, et al. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell.* 2012; 149: 780-94.
4. Dent R, Trudeau M, Pritchard KI, et al. Triple-Negative Breast Cancer: Clinical Features and Patterns of Recurrence. *Clinical Cancer Research.* 2007; 13: 4429-34.
5. Ghildiyal M and Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet.* 2009; 10: 94-108.
6. Stinson S, Lackner MR, Adai AT, et al. TRPS1 Targeting by miR-221/222 Promotes the Epithelial-to-Mesenchymal Transition in Breast Cancer. *Sci Signal.* 2011; 4: ra41.
7. Shah MY and Calin GA. MicroRNAs miR-221 and miR-222: a new level of regulation in aggressive breast cancer. *Genome Med.* 2011; 3: 56.
8. Garofalo M, Di Leva G, Romano G, et al. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell.* 2009; 16: 498-509.
9. Miller TE, Ghoshal K, Ramaswamy B, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. *J Biol Chem.* 2008; 283: 29897-903.
10. le Sage C, Nagel R, Egan DA, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J.* 2007; 26: 3699-708.
11. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J Biol Chem.* 2007; 282: 23716-24.
12. Zhou M, Liu Z, Zhao Y, et al. MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. *J Biol Chem.* 2010; 285: 21496-507.
13. Xiong XB and Lavasanifar A. Traceable multifunctional micellar nanocarriers for cancer-targeted co-delivery of MDR-1 siRNA and doxorubicin. *ACS Nano.* 2011; 5: 5202-13.
14. Xu X, Xie K, Zhang XQ, et al. Enhancing tumor cell response to chemotherapy through nanoparticle-mediated codelivery of siRNA and cisplatin prodrug. *Proc Natl Acad Sci U S A.* 2013; 110: 18638-43.
15. Chen Y, Wu JJ and Huang L. Nanoparticles Targeted With NGR Motif Deliver c-myc siRNA and Doxorubicin for Anticancer Therapy. *Mol Ther.* 2010; 18: 828-34.
16. Qian X, Long L, Shi Z, et al. Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma. *Biomaterials.* 2014; 35: 2322-35.
17. Shi S, Han L, Deng L, et al. Dual drugs (microRNA-34a and paclitaxel)-loaded functional solid lipid nanoparticles for synergistic cancer cell suppression. *J Control Release.* 2014; 194: 228-37.
18. Jia H-Z, Zhang W, Zhu J-Y, et al. Hyperbranched-hyperbranched polymeric nanoassembly to mediate controllable co-delivery of siRNA and drug for synergistic tumor therapy. *Journal of Controlled Release.* 2015; 216: 9-17.

19. Cheng J, Teply BA, Sherifi I, et al. Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. *Biomaterials*. 2007; 28: 869-76.
20. Roy I, Mitra S, Maitra A and Mozumdar S. Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. *International Journal of Pharmaceutics*. 2003; 250: 25-33.
21. Li J, Yang Y and Huang L. Calcium phosphate nanoparticles with an asymmetric lipid bilayer coating for siRNA delivery to the tumor. *J Control Release*. 2012; 158: 108-14.
22. Tang J, Li L, Howard CB, Mahler SM, Huang L and Xu ZP. Preparation of optimized lipid-coated calcium phosphate nanoparticles for enhanced in vitro gene delivery to breast cancer cells. *Journal of Materials Chemistry B*. 2015; 3: 6805-12.
23. Legrand P, Lesieur S, Bochot A, et al. Influence of polymer behaviour in organic solution on the production of polylactide nanoparticles by nanoprecipitation. *Int J Pharm*. 2007; 344: 33-43.
24. Fessi H, Puisieux F, Devissaguet JP, Ammourey N and Benita S. Nanocapsule Formation by Interfacial Polymer Deposition Following Solvent Displacement. *International Journal of Pharmaceutics*. 1989; 55: R1-R4.
25. Quintanar-Guerrero D, Allemann E, Fessi H and Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. *Drug Dev Ind Pharm*. 1998; 24: 1113-28.
26. Zhou Z, Badkas A, Stevenson M, Lee JY and Leung YK. Herceptin conjugated PLGA-PHis-PEG pH sensitive nanoparticles for targeted and controlled drug delivery. *Int J Pharm*. 2015; 487: 81-90.
27. Li WM, Chen SY and Liu DM. In situ doxorubicin-CaP shell formation on amphiphilic gelatin-iron oxide core as a multifunctional drug delivery system with improved cytocompatibility, pH-responsive drug release and MR imaging. *Acta Biomater*. 2013; 9: 5360-8.
28. Li WM, Su CW, Chen YW and Chen SY. In situ DOX-calcium phosphate mineralized CPT-amphiphilic gelatin nanoparticle for intracellular controlled sequential release of multiple drugs. *Acta Biomater*. 2015; 15: 191-9.
29. Bisht S, Bhakta G, Mitra S and Maitra A. pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery. *International Journal of Pharmaceutics*. 2005; 288: 157-68.
30. Li J, Chen YC, Tseng YC, Mozumdar S and Huang L. Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. *J Control Release*. 2010; 142: 416-21.
31. Miao L, Guo S, Zhang J, Kim WY and Huang L. Nanoparticles with Precise Ratiometric Co-Loading and Co-Delivery of Gemcitabine Monophosphate and Cisplatin for Treatment of Bladder Cancer. *Adv Funct Mater*. 2014; 24: 6601-11.
32. Behr J-P. The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit. *CHIMIA International Journal for Chemistry*. 1997; 51: 34-6.
33. Lu Y, Roy S, Nuovo G, et al. Anti-microRNA-222 (anti-miR-222) and -181B suppress growth of tamoxifen-resistant xenografts in mouse by targeting TIMP3 protein and modulating mitogenic signal. *J Biol Chem*. 2011; 286: 42292-302.
34. Gan R, Yang Y, Yang X, Zhao L, Lu J and Meng QH. Downregulation of miR-221/222 enhances sensitivity of breast cancer cells to tamoxifen through upregulation of TIMP3. *Cancer Gene Ther*. 2014; 21: 290-6.

35. Nassirpour R, Mehta PP, Baxi SM and Yin MJ. miR-221 promotes tumorigenesis in human triple negative breast cancer cells. *PLoS One*. 2013; 8: e62170.
36. Dai X and Tan C. Combination of microRNA therapeutics with small-molecule anticancer drugs: Mechanism of action and co-delivery nanocarriers. *Advanced Drug Delivery Reviews*. 2015; 81: 184-97.
37. Pack DW, Hoffman AS, Pun S and Stayton PS. Design and development of polymers for gene delivery. *Nat Rev Drug Discov*. 2005; 4: 581-93.
38. Kingston RE, Chen CA and Rose JK. Calcium Phosphate Transfection. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., 2001.
39. Chowdhury EH, Kunou M, Nagaoka M, Kundu AK, Hoshiba T and Akaike T. High-efficiency gene delivery for expression in mammalian cells by nanoprecipitates of Ca-Mg phosphate. *Gene*. 2004; 341: 77-82.
40. Giger EV, Puigmartí-Luis J, Schlatter R, Castagner B, Dittrich PS and Leroux J-C. Gene delivery with bisphosphonate-stabilized calcium phosphate nanoparticles. *Journal of Controlled Release*. 2011; 150: 87-93.
41. Kester M, Heakal Y, Fox T, et al. Calcium phosphate nanocomposite particles for in vitro imaging and encapsulated chemotherapeutic drug delivery to cancer cells. *Nano Lett*. 2008; 8: 4116-21.
42. Morgan TT, Muddana HS, Altinoglu EI, et al. Encapsulation of organic molecules in calcium phosphate nanocomposite particles for intracellular imaging and drug delivery. *Nano Lett*. 2008; 8: 4108-15.

Figure legend:

Figure 1. A: Synthesis route of co-delivery nanoparticles (NP(miRi/pac)); B: TEM images of (a) lipid-coated calcium phosphate miRi complex (lipid/CaP/miRi) and (b) co-delivery nanoparticle NP(miRi/pac); and C: particle size distributions of (a) lipid/CaP/miRi complex and (b) NP(miRi/pac). Arrows indicate the lipid/CaP/miRi complex inside the NP(pac/miRi).

Figure 2. A: encapsulation efficiencies of miRi-221/222 (miRi), scrambled miRi (miRiNC) and paclitaxel (pac) by nanoparticles. Data represent mean $\pm$ standard deviation, n = 4; and B: *in vitro* release kinetics of miRi (black) and pac (red) from co-delivery nanoparticle NP (miRi/pac) at pH 7 (■) and pH5 (●), the amounts of miRi and paclitaxel remained inside NP(pac/miRi) were measured at 20 hrs, 42 hrs and 66 hrs using fluorescence plate reader and HPLC respectively. Data represent mean $\pm$ standard deviation, n = 3.

Figure 3. Intracellular distribution of FAM-labeled miRi (green) delivered by NP(miRi) over MDA-MB-231. Nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI) (blue); Endolysosomes were stained by Lysotracker (red). The cells were incubated with NP(miRi) at a miRi concentration of 20 pmole/ml for 5 min, 30 min, 2 hrs, and 4 hrs at 37 °C. Arrows indicate miRi in the cytoplasm.

Figure 4. microRNA (miR) expression levels of (A) miR-221 and (B) miR-222 after 3-day treatment with NP(miRi) and NP(miRiNC) and mRNA expression levels of (C) p27 and (D) TIMP3 after 3-day treatment with NP(miRi) and NP(miRiNC). miRiNC with scrambled

sequence serves as a negative control. Untreated (UT) cells were used as a control. Data represent mean $\pm$ standard deviation, n = 3. *p < 0.05 vs. UT.

Figure 5. Cell viability for MDA-MB-231 after 3-day treatment with free drug (pac), NP(pac), NP(pac/miRiNC) and NP(pac/miRi). miRiNC with scrambled sequence serves as a negative control. For NP(pac/miRi) and NP(pac/miRiNC), the pac to miRi ratio is 1 μ g pac to 20 pmole miRi. Data represent mean $\pm$ standard deviation, n = 3. *p < 0.05 vs. pac.

Down regulation of miR-221/222 through NP mediated delivery of miRi-221/222

Enhanced efficacy through co-delivery of miRi-221/222 and paclitaxel

Figure 1. A: Synthesis route of co-delivery nanoparticles (NP(miRi/pac)); **B:** TEM images of (a) lipid-coated calcium phosphate miRi complex (lipid/CaP/miRi) and (b) co-delivery nanoparticle NP(miRi/pac); and **C:** particle size distributions of (a) lipid/CaP/miRi complex and (b) NP(miRi/pac). Arrows indicate the lipid/CaP/miRi complex inside the NP(pac/miRi).

Figure 2. A: encapsulation efficiencies of miRi-221/222 (miRi), scrambled miRi (miRiNC) and paclitaxel (pac) by nanoparticles. Data represent mean $\pm$ standard deviation, $n = 4$; and **B:** *in vitro* release kinetics of miRi (black) and pac (red) from co-delivery nanoparticle NP (miRi/pac) at pH 7 (■) and pH5 (●), the amounts of miRi and paclitaxel remained inside NP(pac/miRi) were measured at 20 hrs, 42 hrs and 66 hrs using fluorescence plate reader and HPLC respectively. Data represent mean $\pm$ standard deviation, $n = 3$.

Figure 3. Intracellular distribution of FAM-labeled miRi (green) delivered by NP(miRi) over MDA-MB-231. Nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI) (blue); Endolysosomes were stained by Lysotracker (red). The cells were incubated with NP(miRi) at a miRi concentration of 20 pmole/ml for 5 min, 30 min, 2 hrs, and 4 hrs at 37 °C. Arrows indicate miRi in the cytoplasm.

Figure 4. microRNA (miR) expression levels of (A) miR-221 and (B) miR-222 after 3-day treatment with NP(miRi) and NP(miRiNC) and mRNA expression levels of (C) p27 and (D) TIMP3 after 3-day treatment with NP(miRi) and NP(miRiNC). miRiNC with scrambled sequence serves as a negative control. Untreated (UT) cells were used as a control. Data represent mean $\pm$ standard deviation, n = 3. *p < 0.05 vs. UT.

Figure 5. Cell viability for MDA-MB-231 after 3-day treatment with free drug (pac), NP(pac), NP(pac/miRiNC) and NP(pac/miRi). miRiNC with scrambled sequence serves as a negative control. For NP(pac/miRi) and NP(pac/miRiNC), the pac to miRi ratio is 1 µg pac to 20 pmole miRi. Data represent mean $\pm$ standard deviation, n = 3. *p < 0.05 vs. pac.