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## Research Article

# Enhancement of cancer therapy efficacy by trastuzumab-conjugated and pH-sensitive nanocapsules with the simultaneous encapsulation of hydrophilic and hydrophobic compounds

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## Abstract

Trastuzumab-conjugated pH-sensitive double emulsion nanocapsules (DENCs) stabilized by a single-component Poly (vinyl alcohol) (PVA) with magnetic nanoparticles can be fabricated through a two-step double emulsion process; these nanocapsules can be used to encapsulate hydrophilic doxorubicin (Dox) and hydrophobic paclitaxel (PTX) simultaneously. When PMA<sub>SH</sub> was attached to the shell of the DENCs, enhanced dual drug release of PTX/Dox was detected, specifically in intracellular acidic pH environments. The targeting ability of these Trastuzumab-conjugated DENCs was demonstrated with confocal images, which revealed a significantly elevated cellular uptake in HER-2 overexpressing SkBr3 cells. More importantly, an intravenous injection of this co-delivery system followed by magnetic targeting (MT) chemotherapy suppressed cancer growth *in vivo* more efficiently than the delivery of either PTX or Dox alone. The integration of the functionalities makes this combination therapy system a powerfully new tool for *in vitro/in vivo* cancer therapy, especially for in HER-2 positive cancers.

**From the Clinical Editor:** Trastuzumab-conjugated pH-sensitive nanocapsules were used in this study for simultaneous targeted delivery of hydrophobic (PTX) and hydrophilic (Dox) anti-cancer agents to HER-2 positive cancer cells. Additional use of magnetic targeting demonstrated superior efficacy of this delivery system compared to PTX or Dox alone.

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**Key words:** Emulsion; pH sensitive; HER-2 positive breast cancer; Trastuzumab; Co-delivery

The most common carcinoma in women worldwide is breast cancer, and it is expected to account for 29% (226,870) of all new malignancies in 2012.<sup>1</sup> Among the four classifications of breast cancer, the type with the over-expression of the human epidermal growth factor receptor-2 (HER-2) oncogene is more invasive and causes higher mortality<sup>2</sup>; thus, HER-2 positive breast cancer has become a major challenge for cancer therapy. Recent progress in combination chemotherapy for breast cancer has successfully improved the response rate (RR), overall survival (OS) rate, and relapse-free survival (RFS) rate of patients by incorporating two or more anti-cancer drugs into a single treatment.<sup>3</sup> For example, Wang et al. reported that the co-delivery of drugs and DNA from cationic core-shell nanoparticles can

suppress breast cancer tumor growth in a mouse model more efficiently than the delivery of either compound alone.<sup>4</sup> Nevertheless, the side effects caused by the toxic chemotherapeutics were still a concern,<sup>5</sup> and discovering treatments that cause fewer less severe side effects remained a crucial challenge.<sup>6</sup> Therefore, many drug delivery systems have emerged to improve the therapeutic efficacy and reduce the side effects by enhancing the accumulation of active molecules in tumor sites. Moreover, nanoparticles are capable of passive drug targeting because leaky tumor tissues display enhanced permeability and retention (EPR) effects.<sup>7–9</sup> However, the complex behavior of nanoparticles within the circulatory system still remains unknown, and the EPR effect is not a silver bullet. Therefore, designing nanoparticles equipped with efficient targeting ligands and multiple encapsulated anti-cancer drugs for use in tandem with the EPR effect will generate combination therapies that greatly enhance drug efficacy in tumors.<sup>10</sup>

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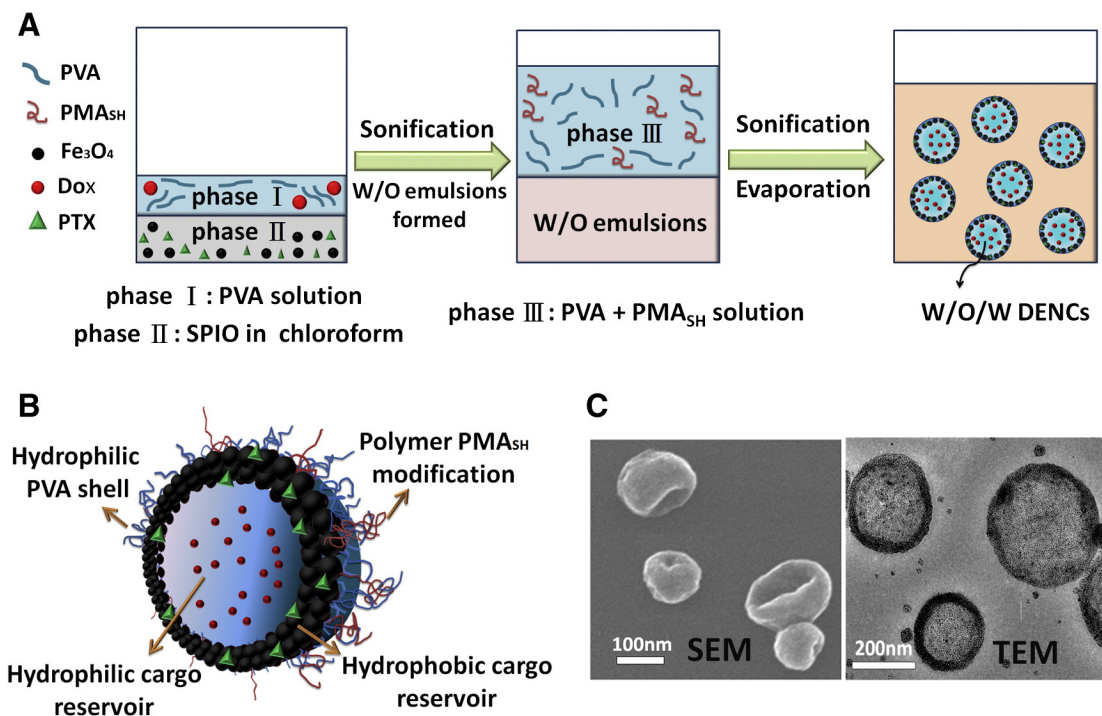


Figure 1. (A) Schematic of the synthesis of DENCs using the two-step emulsion process. The hydrophobic phase and the hydrophilic phase were stabilized using PVA polymer with a molecular weight of 16000 g/mol. (B) DENCs carried hydrophilic cargo in the inner core and hydrophobic cargo in the outer shell; PMA<sub>SH</sub> was modified out on its shell site, which entangled with the hydrophilic polymer PVA. (C) SEM and TEM images of dried DENCs.

Ligand-conjugating nanoparticles can distinguish specific tumor cells from normal tissues and therefore accumulate drugs at the proper sites with their active targeting mechanisms.<sup>11</sup> Monoclonal antibodies (mAbs) have served as one of the most promising receptor-sensitive agents in clinical applications. In particular, Trastuzumab, which is a monoclonal antibody that was approved in 1998, exhibits an outstanding ability to target HER-2 positive breast cancer cells.<sup>12</sup> Studies utilizing Trastuzumab for combination therapy with other compounds,<sup>13,14</sup> as well as in conjugation with micelles,<sup>15</sup> nanoscale gold particles,<sup>16</sup> and liposomes<sup>17,18</sup> that incorporate anti-cancer drugs have been performed. Recently, Lee et al. reported the co-delivery of paclitaxel and Trastuzumab with cationic micelles to achieve the targeted delivery of paclitaxel to HER-2 overexpressing human breast cancer cells, but the delivery still resulted in incomplete tumor death because the delivery system lacked a proper drug-triggered release capability.<sup>15</sup> Therefore, utilizing a combination therapy that integrates the drug cocktail with the targeting ligand may be the solution to these problems.<sup>19</sup> Furthermore, the concept of magnetic targeting with external magnetic field applied locally on the nidus was introduced to strengthen the accumulation in tumor, where the delivery of only relying on pure molecular targeting may not have been completely achieved.<sup>20,21</sup> Therefore, in this study, we propose a novel magnetic targeting and pH-sensitive nanocapsule that simultaneously encapsulates hydrophobic paclitaxel (PTX) and hydrophilic doxorubicin (Dox) in preparation for triggered release within an acidic environment; moreover, the nanoparticles were equipped with dual targeting system

combining HER-2-specific trastuzumab and magnetic targeting with an applied external magnetic field. A combination therapy exhibiting a controlled dose release from DENCs was tested both *in vitro* and *in vivo*.

## Methods

### Materials and reagents

The materials and reagents utilized in the study are provided in the supplementary document.

### Synthesis of double emulsion nanocapsules (DENCs)

As illustrated in Figure 1, A, DENCs were fabricated through a two-step, double-emulsion processes utilizing three different phases. First, hydrophilic phase I (0.2 mL of 2 wt% PVA-16 k solution) and hydrophobic phase II (0.5 mL of chloroform containing 5 mg SPIO) were mixed and emulsified via ultrasonic treatment at a frequency of 20 kHz at 60 W for 30 s, forming water-in-oil (W/O) emulsions. Hydrophilic phase III (1.5 mL of mixed 2 wt% PVA solution and various 0.2, 0.5 or 1 wt% PMA<sub>SH</sub>) was then added and subjected to further ultrasonic emulsification at a frequency of 20 kHz at 160 W to form a W/O/W emulsion. After being vigorously stirred for 4 h to remove the organic solvent, the blend was purified and collected by centrifugation at 9000 rpm and re-dispersed in DI-water.

### Conjugation of targeting antibody Trastuzumab to DENCs

Trastuzumab was conjugated with thiol groups via the SMCC method (Figure S1). The Bradford method for identifying and quantifying Trastuzumab is described in the supporting information (Figure S2).

### Characterization

Transmission electron microscopy (JEOL, Japan, JEM-2100) and field-emission scanning electron microscopy (FE-SEM, JEOL-6500, Japan) were utilized to analyze the morphology of the DENCs. Before microstructure analysis, the DENCs were dried on a silicon wafer with a thin platinum coating for SEM and were fixed and dried in Cu mesh for TEM. The particles sizes of the DENCs were determined using dynamic light scattering (Brookhaven Inc., Holtsville, NY, BI-200SM Goniometer), while the zeta potential was measured via DLS (BECKMAN COULTER Delsa™ Nano C particle analyzer). Flow cytometry (Becton Dickinson, San Jose, CA, USA) was used to analyze the binding ability.

### Drug loading and nature release measurement of PTX and Dox

The detail methodology of encapsulating PTX and Dox were provided in the supplementary. In brief, these two drugs were encapsulated in DENCs through emulsion process described above, and the amount of PTX and Dox in the DENCs were 3 mg and 1 mg, approximating 2:1 in molar ratio, which was demonstrated to be the most effective combination to SkBr3 cells (the detail was discussed in Figure S10).

### Cytotoxicity of DENCs

SkBr3 (Human breast adenocarcinoma) cells were seeded at a density of  $10^5$  cells/well on a 96-well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and were incubated at 37 °C in a 5% CO<sub>2</sub>-humidified atmosphere. A previously generated complex solution containing the free drugs, pure DENCs, or drug-loaded DENCs at different concentrations was added to the cells and incubated. After 24 and 48 hours, the medium was removed, and 20 μL of a 10% MTT solution with 100 μL of fresh medium was added to the wells and incubated for 4 hours. The absorbance was read with an ELISA reader (Tecan, Sunrise™, Australia) at 590 nm. In addition, to observe the relationship between the intracellular drug release behavior and cytotoxicity, confocal microscopy was used with FITC incorporated into the DENCs to serve as a model drug (green signal). The FITC was encapsulated in the DENCs similar to method that encapsulated PTX, that the PTX were replaced by 2 mg of FITC. The nuclei were dyed using DAPI (blue signal), and the lysosome, which is known to be an acidic organelle in cells that can be used to observe the pH-triggered release behavior, was labeled with LysoTracker® (red signal).

### Cell uptake

To measure the uptake into the SkBr3 cells, the cells were seeded onto glass coverslips (Nunc, USA) at a density of  $2 \times 10^5$  cells per well and cultured in 2 mL of growth medium overnight.

The next day, the growth medium was removed, and the cells were treated with 2 mL of serum-free medium containing doxorubicin, IgG-conjugated-Dox-DENCs (IgG-Dox-DENCs) or Trastuzumab-conjugated-Dox-DENCs (T-Dox-DENCs) to achieve a final doxorubicin concentration of 0.8 μg/mL. After incubation at 37 °C for 0.5 h, the medium was removed from the cells, which were washed with PBS, fixed with 4 w/w% of paraformaldehyde in PBS before being imaged at a magnification of 400× with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY, USA).

### HER-2/neu receptor expression in SkBr3 cells

Using Trastuzumab (1: 5000) for 1 h,  $3 \times 10^5$  SkBr3 cells with HER-2/neu receptors were detected and subsequently incubated for 30 min with fluorescein-conjugated rabbit anti-human IgG (1:10,000). The fluorescence was assayed with flow cytometry (Becton Dickinson, San Jose, CA, USA).

### Cell binding ability

SkBr3 cells containing HER-2/neu receptors at a density of  $3 \times 10^5$  per tube or MDA-MB-231 cells without HER2/neu receptors were treated with Dox-loaded T-DENCs (T-Dox-DENCs) or IgG-Dox-DENCs at 4 °C for 0.5 hour in the dark. Following a wash and resuspension in 1 mL of DI water, the cells were analyzed to reveal the signal of fluorescent Dox by flow cytometry.

### In vivo experiments

All animals were obtained from BioLASCO Taiwan Co. (Taiwan) and were handled in accordance with the guidelines of the Animal Care and Use Committee of the Institute of Life Sciences of National Chiao Tung University. Human breast adenocarcinoma SkBr3 cells ( $1 \times 10^7$  cells in 100 mL PBS) were mixed with matrigel and injected subcutaneously into female nude mice (CAnN.Cg-Foxn) at ages of 6 weeks. After the tumors developed up to a size of approximately 100 mm<sup>3</sup>, a two-part experiment was conducted. First, to observe the biodispersity, the accumulation sites, and the effect of MT in mice, the DENCs were modified with the Cy5.5 labeling reagent (Figure S5) and injected via the tail vein. Next, a Caliper IVIS system (IVIS Imaging System 200 Series, Caliper LifeScience, USA) was operated based on the fluorescence (emission: 740 nm, excitation: 675 nm) of Cy5.5 to analyze the amounts of DENCs in the nude mice. Then, pure DENCs, PTX-DENCs, Dox-DENCs, T-DENCs, T-PTX-DENCs, and T-PTX-Dox-DENCs were dispersed in separate saline solutions and injected via the tail vein every 4 days for 4 cycles (n = 5) with magnets at the tumor sites to direct the particles. Additionally, a group of nude mice were treated without magnets to check effectiveness of the magnetic targeting (MT).

## Results

### Characterization of DENCs

As illustrated in Figure 1, A, double emulsion nanocapsules (DENCs) were fabricated using a two-step double emulsion



process with amphiphilic poly(vinyl alcohol) (PVA) and hydrophobic superparamagnetic iron oxide (SPIO).<sup>22</sup> In the water phase, PVA with a molecular weight of 16,000 g/mol (PVA-16 k) served as the surfactant used to stabilize both the O/W and W/O interfaces. In the organic phase, 5 nm iron oxide nanoparticles coated with oleic acid were dissolved in chloroform. Then, these two phases were emulsified by pulsed ultrasound sonication to form the water-in-oil structure. Subsequently, the pH-responsive polymer PMA<sub>SH</sub> (poly(methacrylic acid) (PMAA) functionalized with thiol group<sup>23–25</sup>) was added to modify the shell of the DENCs to enable a pH-triggered drug release in the second step of emulsification. Moreover, the thiol group on the PMA<sub>SH</sub> side chain and the primary amines in Trastuzumab can be conjugated efficiently with SMCC (FT-IR analysis in Figure S3). By conjugating Trastuzumab with the DENCs, HER-2 positive carcinomatous cells can be specifically targeted. To investigate applications in combination therapy, Dox and PTX were selected as two model drugs and encapsulated stably inside the DENCs at a neutral pH; release could be triggered in acidic environments. The schematic drawing in Figure 1, B illustrates the purpose of each component in the nanocapsule. The double emulsion nanocapsules (bowl-like) are composed of PVA-16k, have a mean diameter of approximately  $174 \pm 13$  nm (verified by dynamic light scattering measurements, Figure S6), and disperse well in water solutions without any additional surfactant. The SEM image on left side of Figure 1, C depicts the nanostructure as morphologically similar to a collapsed balloon, suggesting that the double emulsion nanocapsule is a hollow structure in solution. The formation of the collapsed hollow structure is attributed to the drying process necessary for sample preparation under vacuum and water evaporation of the inner core. The corresponding TEM image of the DENCs shown on the right side of Figure 1, C clearly reveals that the iron oxide nanoparticles appeared to assemble and pack uniformly in the shell of the double emulsion capsules.

#### *pH-triggered drug release behavior*

PMAA is a pH-sensitive polymer that is hydrophilic and “randomly coiled” in neutral solution but easily transforms and shrinks to a “globule-like structure” for several reasons, including ionization, hydrophobic interaction, and hydrogen bonding as the pH in the environment decreases.<sup>26</sup> The PMA<sub>SH</sub> generated by modifying PMAA<sup>24</sup> was not only functionalized with thiol groups but also retained the pH-sensitivity of PMAA (Figure S7). As schematically illustrated in Figure 2, A, PMA<sub>SH</sub> is entangled in the shell under acidic conditions, which can lead to the deformation of the DENCs and trigger the drug release. The pH-sensitive drug release profiles of PTX and Dox from PTX-DENCs and Dox-DENCs, respectively, were dependent on the varied amounts of PMA<sub>SH</sub>, which were investigated; the results are presented in Figure 2, B and C, respectively. These two drug release behaviors displayed distinct release models caused by their different degrees of pH-sensitivity. PTX located in the shell of the DENCs was directly affected by the shrinkage of PMA<sub>SH</sub> such that a dramatic increase in cumulative release was detected with increasing amounts of PMA<sub>SH</sub> under acidic conditions. In contrast, the Dox

housed in the core was influenced to a lesser extent by the shell deformation, but the crevices or cracks caused by the shrinkage of PMA<sub>SH</sub> still enhanced the amount of Dox released relative to the capsules without PMA<sub>SH</sub> modification. PTX-Dox-DENCs with 1 wt% of PMA<sub>SH</sub> incorporated during the emulsion process were used to study the drug release profiles at pH 7 and pH 4. At pH = 7, the greater release of Dox from the core relative to the release of PTX from the shell is caused by (a) the positive charge of Dox in the core and negative PMA<sub>SH</sub> from the outer shell (attractive), and (b) the affinity relationship between these drugs and the environmental solution (PTX is hydrophobic and is less thermodynamically unfavorable to the solution even with the tween-80 added). Figure 2, D shows that the amount and rate of released PTX and Dox are higher at pH 4. However, the release behavior profiles of dual-drug-containing nanocapsules were similar to those of the nanocapsules containing only a single drug, indicating that the drugs do not interfere with each other's release mechanism. Figure 2, E reveals that the DENCs prevented the drugs from releasing in a neutral environment, demonstrating that less than 10% of the PTX and less than 15% of Dox were lost to leakage. However, as the pH value changed from 7 to 4, the acid-induced release behavior was clearly observed, although the enhanced PTX release was more dramatic than the Dox release.

#### *In vitro cytotoxicity*

The cytotoxicity of the PTX-DENCs, Dox-DENCs, T-DENCs, T-PTX-DENCs, and T-PTX-Dox-DENCs containing a total of 0.1, 0.5, 1, 2, 4, 8, 10, 20  $\mu\text{M}$  of drugs were cultured with SkBr3 cells to evaluate their therapeutic effect (Figure S8). The IC<sub>50</sub> assay is provided in Table S2. As schematically depicted in Figure 3, A, the cytotoxicity of the various DENCs containing 2  $\mu\text{M}$  of different drugs were tested. No cytotoxicity was observed in the SkBr3 cells treated with DENCs without a drug payload, indicating that the DENCs were biocompatible. However, the cell viability decreased to  $56.4 \pm 4.4\%$  and  $34.8 \pm 4.3\%$  after incubation with PTX-DENCs and Dox-DENCs for 24 h, respectively. The viability of the Trastuzumab-conjugated PTX-DENCs (T-PTX-DENCs) notably decreased to  $26.9 \pm 3.5\%$ , suggesting that Trastuzumab and PTX acted synergistically to kill the cancer cells. Furthermore, when the dual drug payload was targeted with Trastuzumab, a successful combination *in vitro* therapy was generated because T-PTX-Dox-DENCs had killed almost all of the SkBr3 cells within 24 h ( $12.2 \pm 2.89$ ). Additionally, we used FITC-loaded (green fluorescence) T-DENCs to verify the endocytosis behavior in the SkBr3 cells as presented in Figure 3, B. After incubation for 4 h, the DENCs had detectably entered the lysosomes, where the regions were dyed red by LysoTracker®. The lysosome is an acidic compartment in a cell, and a speedy drug release was assumed to have occurred. After incubation for 8 h, spot-like green fluorescence was found to have transformed into a mist-like texture and spread throughout the cytoplasm, suggesting that the fluorescence was released from the DENCs.

#### *Targeting ability*

The biospecificity of the Trastuzumab toward HER-2 over-expressed breast cancer cells might result in rapid binding caused

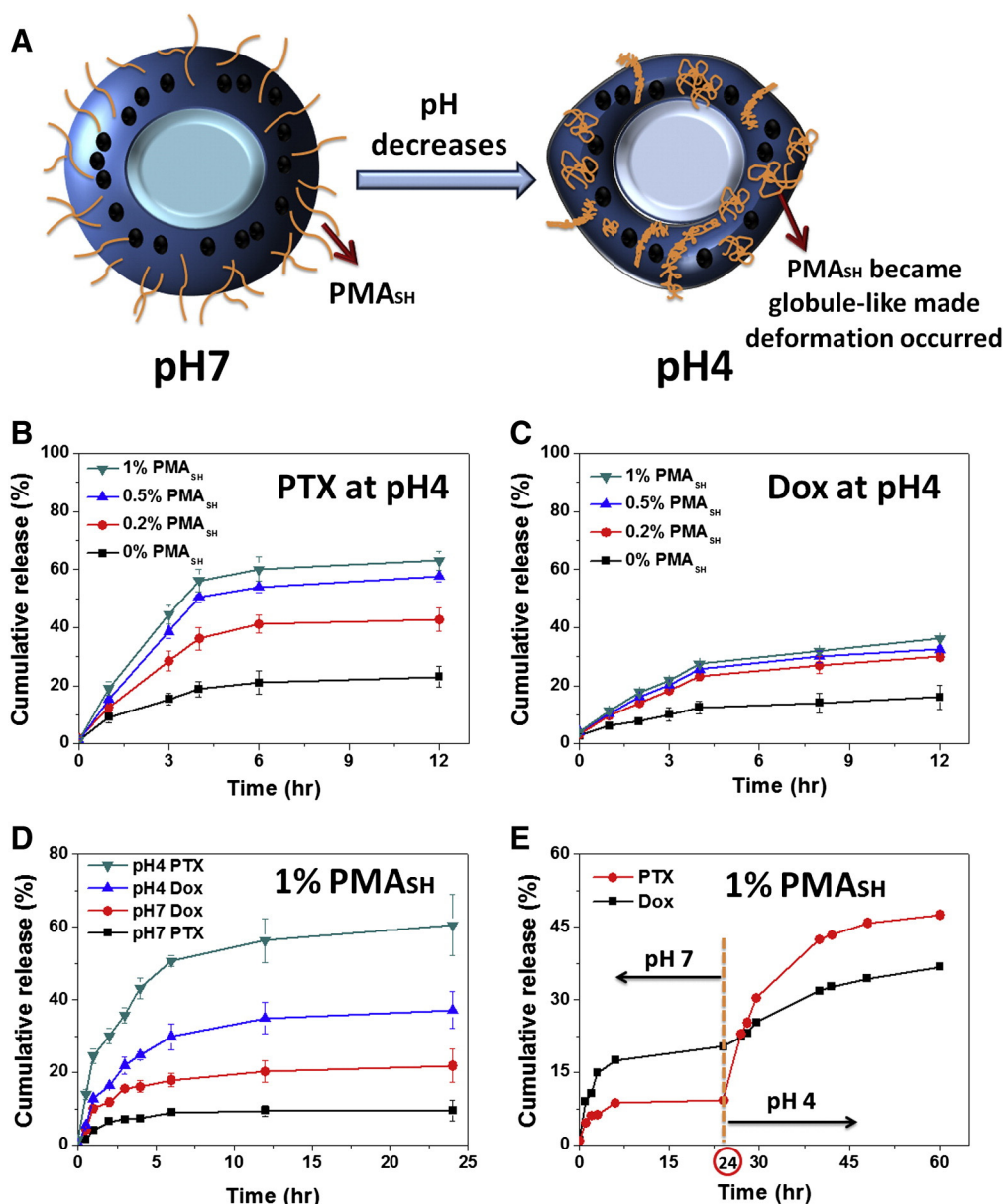


Figure 2. Illustration of the morphological transformation and drug release behavior of DENCs caused by pH-sensitive PMA<sub>SH</sub>. (A) PMA<sub>SH</sub> tended to stretch at neutral pHs and shrink in an acidic environment; therefore, when the pH decreased, the shell experienced a global pulling force due to the shrinkage of PMA<sub>SH</sub>, and deformation of DENCs took place, inducing drug release. The cumulative drug release of (B) PTX-DENCs and (C) Dox-DENCs from separate DENCs composed of uniform amounts of PVA but various concentrations of PMA<sub>SH</sub> at pH 4. (D) Cumulative release of PTX-Dox-DENCs composed of 1 wt% PMA<sub>SH</sub>, while the pH induced release behavior is illustrated in (E), which depicts the buffer exchange experiment from pH 7 to pH 4.

by the antigen-antibody reaction, which was supported by the flow cytometry data in Figure 4, A; Figure 4, A illustrates the enhanced fluorescence intensity of the concentrated T-Dox-DENCs and the rapid binding generated between SkBr3 cells and T-Dox-DENCs. In contrast, IgG was not specific to HER-2 positive cells, and the intensity of fluorescence was not concentration dependent relative to IgG-Dox-DNEC, as observed in Figure 4, B. Because the targeting is receptor mediated, SkBr3 and the triple-negative (ER-, PR-, HER-2-) MDA-MB-231 cell line were compared to establish the targeting ability of trastuzumab. In Figure 4, C and D, the SkBr3 with saturated

HER-2 antigens demonstrated excellent binding ability toward the T-Dox-DENCs via the obvious shift of fluorescent intensity; however, the HER-2-negative MDA-MB-231 did not display any intensity shift during analysis, indicating that no binding had occurred between the cells and the T-Dox-DENCs. For further validation, the targeting ability of T-DENCs was examined via confocal microscopy using SkBr3 cells incubated alone, with free Dox, with IgG-Dox-DENCs, and with T-Dox-DENCs for 30 min. In Figure 4, E, the first row represents the nucleus, the second row represents the fluorescence of Dox, and the third row represents the merging of the first two rows. The four columns

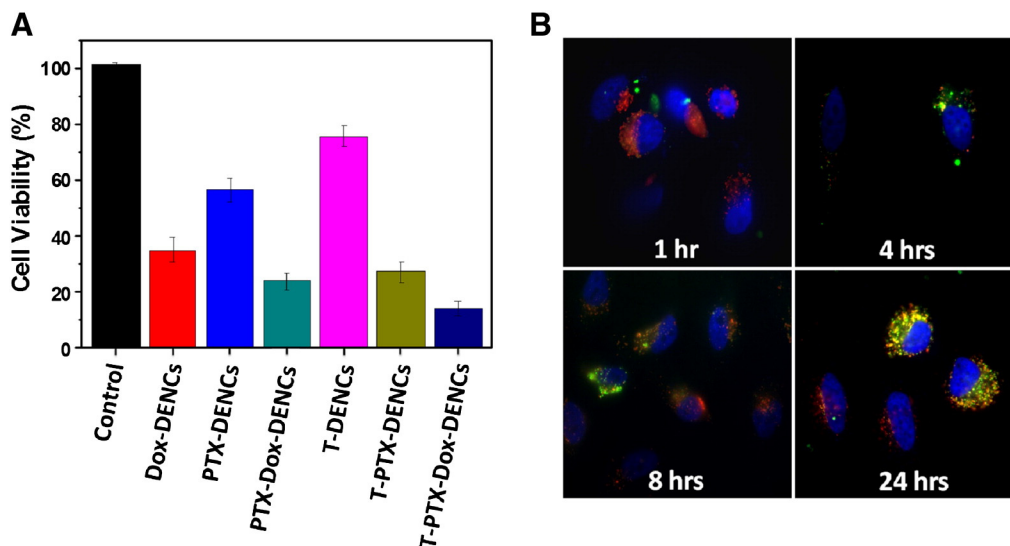


Figure 3. (A) Cell viability after incubating SkBr3 cells with pure DENCs, Dox-DENCs, PTX-DENCs, T-DENCs, T-PTX-DENCs, and T-PTX-Dox-DENCs for 24 hours at 37 °C (n = 6), (B) FITC (labeled in green) was used to detect the release from DENCs in SkBr3 cells after 24 h incubation (red is LysoTracker<sup>®</sup>, which is a label specific for lysosomes, and blue is DAPI for labeling the nucleus).

represent SkBr3 alone, free Dox, IgG-Dox-DENCs, and T-Dox-DENCs. It was discovered that only the T-Dox-DENCs clearly displayed the visible fluorescence of Dox in the SkBr3 cells, which responded positively to the flow cytometry test, demonstrating that the T-Dox-DENCs were rapidly taken up by the HER-2 positive SkBr3 cells.

#### *In vivo animal experiment*

To investigate the magnetic targeting (MT) effect further in an *in vivo* animal model with a solid tumor, we injected 100  $\mu$ L of Cy5.5-labeled DENCs into the tail veins of nude mice bearing SkBr3 tumors to assess the nanocapsule accumulation in the tumor 24 h post-injection, as shown in Figure 5. A magnet with strength of 2000 G was wrapped with a bandage on the left tumor for 6 hours immediately after injection. As illustrated in Figure 5, B, after injecting DENCs into the solid tumor, clear fluorescence can be observed at both locations of the tumor under the IVIS imaging system after one day, but an obvious contrast between the left and right tumor appeared after 3-day post-injection; the intensity of the left tumor, which was treated with the magnet, was 2.4-fold higher than the other. Given their local targeting and high accumulation of drug concentrations observed in real time, the DENCs provide a very promising MT chemotherapy method to treat solid tumors. To use the treatment most efficiently, a magnet was wrapped with a bandage at the tumor site to enhance therapy efficacy.

To analyze the therapeutic effect, we compared the tumor growth for up to 30 days in mice receiving the following treatments: 100  $\mu$ L of 2 wt% (i) saline (control), (ii) DENCs, (iii) Dox-DENCs, (iv) PTX-DENCs, (v) T-Dox-DENCs, (vi) T-PTX-DENCs, and (vii) T-PTX-Dox-DENCs injected via the tail vein into nude mice with MT performed with a 2000 G magnet. Additionally, a control group of nude mice without magnetic targeting were injected with T-PTX-Dox-DENCs to compare with those applied with magnets. As shown in Figure 6, all drug-

containing DENCs demonstrated an inhibition effect by suppressing the tumor size, while pure DENCs displayed an effect similar to that of saline, meaning that no obvious harm occurred after 4 doses were administered. The combination therapy formed by integrating the active targeting of the Trastuzumab and a single drug system generated a more obvious effect in the tumor than in the non-targeted therapy. Moreover, the therapeutic efficacy of the system containing both drugs and Trastuzumab was even more dramatic than the Trastuzumab/single drug complex. T-PTX-DENCs have a better therapeutic effect than PTX-DENCs because Trastuzumab enhances intracellular cell uptake via the antigen-antibody reaction with the HER2 receptor. T-PTX-Dox-DENCs achieved the best therapeutic efficacy due to the targeting ligand and the co-delivery system, which inhibited the growth of SkBr3 tumor the most. The effect of MT is also revealed by comparing the disparity of T-PTX-Dox-DENCs + MT with T-PTX-Dox-DENCs. In Figure S11, both of the excised tumors showed significant fluorescence of accumulated Dox; however, in the ones applied with MT, the accumulation of Dox in lung, kidney, and heart reduced. Simultaneously, the result of the treatment also tells the fact of enhancing therapy efficacy by applying an external magnetic field.

#### **Discussion**

Co-delivery of therapeutics via the incorporation of anti-tumor drugs with either protein,<sup>27</sup> siRNA<sup>28</sup> or using multiple drugs<sup>29</sup> with nanotechnology for breast cancer therapy to enhance the anti-tumor activity has been suggested as a potential method.<sup>30,31</sup> Among these strategies, the treatment of multi-chemotherapeutic drugs was proven to reduce drug resistance and improve the response rate due to synergistic effects. However, great cytotoxicity toward normal cells and tissue was a serious side effect. Consequently, attempts have been made to carry multiple

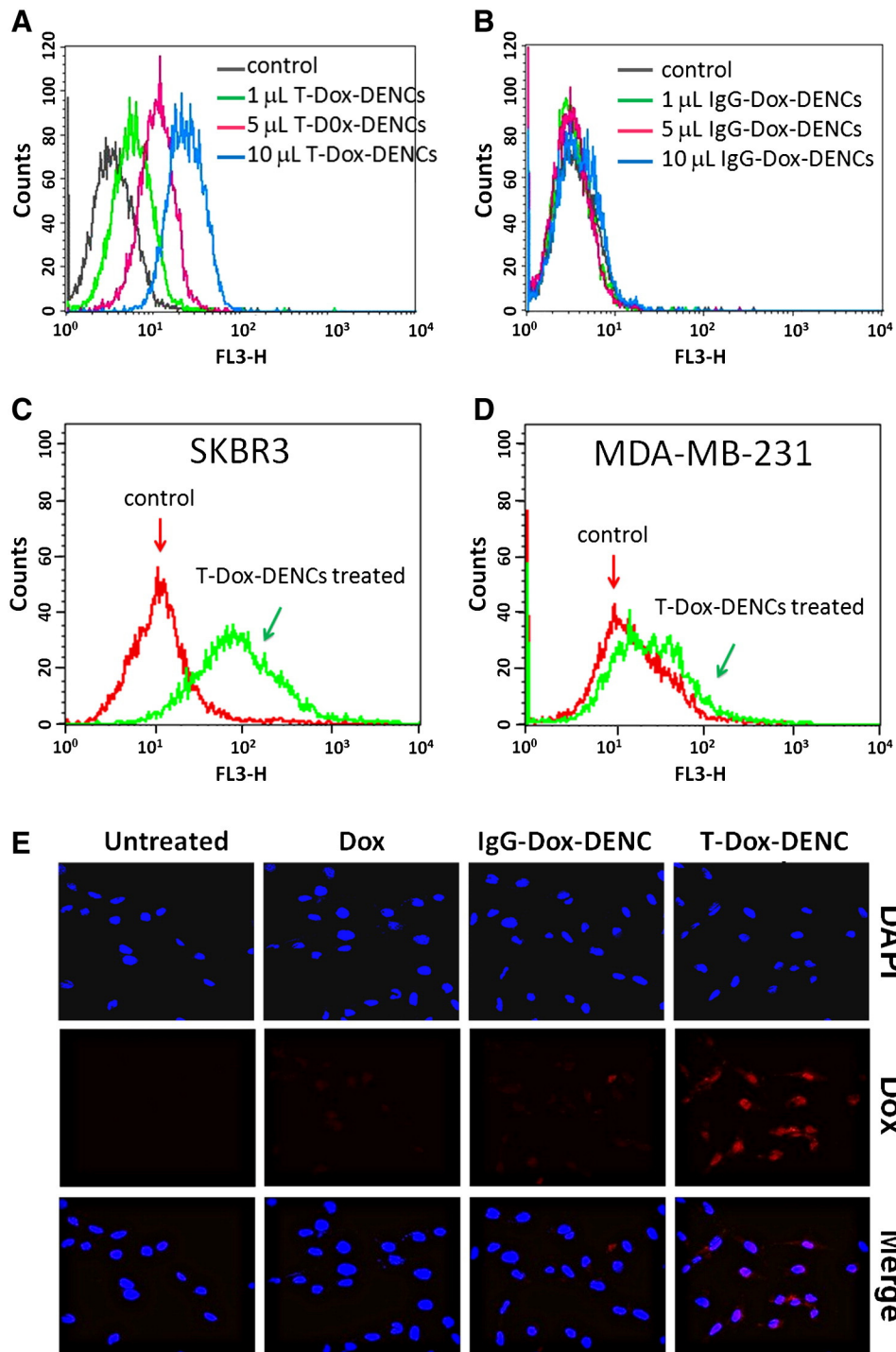


Figure 4. Flow cytometry analysis and confocal microscopy for studying the targeting ability of Trastuzumab toward SkBr3 cells over 30 min at 37 °C. DENCs are conjugated to IgG and Trastuzumab separately. After different concentrations of (A) Trastuzumab-conjugated DENCs (T-DENCs) and (B) IgG-conjugated DENCs (IgG-DENCs) were incubated with SkBr3 cells, they were detected by flow cytometry. SkBr3 cells (C) and MDA-MB-231 cells (D) were treated with Q-T-DENCs to verify the receptor mediated targeting. (E) Confocal microscopy for the detection of endocytosis in untreated, free Dox treated, IgG-Dox-DENCs treated, and T-Dox-DENCs incubated SkBr3 breast cancer cell contained obvious fluorescence levels for Dox.

drugs in single nano-scale drug delivery systems to achieve therapeutic effects and reduce toxicity.<sup>3,29</sup> Furthermore, obtaining high selectivity toward tumor tissues has become desirable when designing a drug delivery system, while ligand-targeted

chemotherapy is gaining attention due to the cooperative anticancer effect. Previous reports have demonstrated that a synergistic interaction occurs between the targeting agent Trastuzumab and therapeutic drugs, including PTX and Dox.<sup>32</sup>



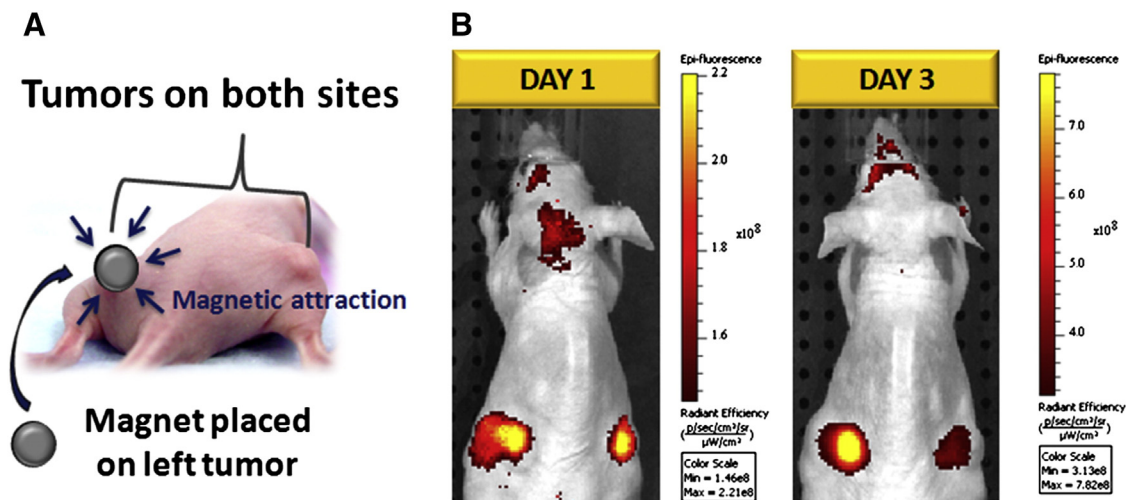


Figure 5. (A) A magnet was attached to the left tumor to evaluate the MT effect. (B) The variation in fluorescence intensity between the left side and right side tumors was observed under IVIS. The left side displayed a higher accumulation than the other, which was due to the magnetic treatment.

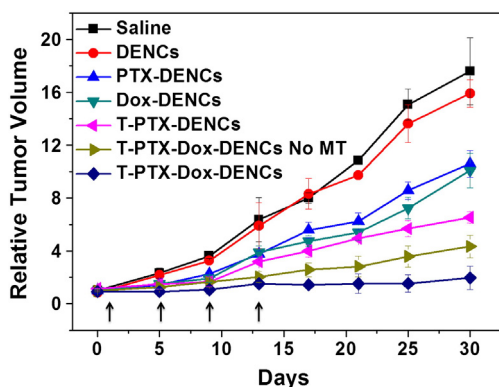


Figure 6. Tumor therapy of varies drug-loaded DENCs administration (4 times, where the arrows labeled) for treating nude mice with subcutaneous breast cancer ( $n = 5$ ). A control group without magnetic targeting (MT) and administrated T-PTX-Dox-DENCs is used to compare to the one with MT. Among all types of treatments, the T-PTX-Dox-DENCs with MT had the best therapeutic efficacy. The volume of tumors was expressed as relative tumor volume, which was compare to the volume of day 0 (analyzed tumor volume/day 0 tumor volume).

In this study, we encapsulated PTX and Dox simultaneously into pH-sensitive, Trastuzumab-modified double emulsion nanocapsules (DENCs) and applied MT to achieve efficient therapy while simultaneously reducing side effects. Trastuzumab, which is specifically targeted toward HER-2 positive breast tumor tissues, was conjugated to the DENCs, enhancing the cell uptake efficiency as evidenced by flow cytometry. The confocal microscopy image in Figure 4E also demonstrates that, after being exposed to the SkBr3 cells for 30 min, the T-DENCs were easily taken up by the cells because of the rapid targeting. A well-controlled pH-responsive drug carrier is one of the most effective means by which to trigger drug release *in vivo*.<sup>33</sup> Figure 3, B indicates that after incubation for 4 h, the DENCs entered lysosomes, which are acidic compartments in a cell, at detectable levels, and a speedy drug release was assumed to have occurred. By targeting with Trastuzumab and utilizing pH-triggered drug release, the cell

viability resulting from the co-delivery chemotherapy of T-PTX-DENCs decreased significantly, 25–30% at 2  $\mu\text{M}$  in Figure 3, A, therefore indicating greatly enhanced chemotherapeutic efficiency relative to the PTX-DENCs, Dox-DENCs and T-DENCs. Once the combination of PTX and Dox was loaded into the T-DENCs, the cell viability decreased to about 10%, demonstrating the strong synergistic effect of the combined anticancer drugs (See Figure S10). The possible mechanism for the synergistic therapeutic effect, which was reported by Wang and co-workers, suggests that the combination of Dox and PTX can significantly degrade the tubulin in tumor cells, leading to cell death; however, PTX alone can also inhibit the disassembly of microtubules. Moreover, nearly complete cytotoxicity was induced using the T-PTX-Dox-DENCs delivery system, which was attributed to two key factors. First, the rapid release of PTX and Dox can be achieved through the distortion and deformation of the DENCs due to the pH-sensitive effect of PMA<sub>SH</sub>, as was discussed in the previous section, to ensure that a high dose of anti-cancer drugs is delivered to specific sites instantaneously. Second, the biospecificity and targeting supplied by the Trastuzumab also contributed significantly to killing the cancer cells.

With the magnetic field applied on the tumor site, an obvious increase of amount of accumulation could be observed (Table S3); approximately 25.8% ( $77.49 \pm 12.4 \mu\text{g}$ ) of PTX and 20% ( $20.09 \pm 6.3 \mu\text{g}$ ) of Dox from the initial dose were accumulated in the tumor for T-PTX-Dox-DENCs with MT, which was 2.47-fold and 1.87-fold higher than that of PTX-Dox-DENCs and T-PTX-Dox-DENCs, respectively. Delivery using dual targeting (magnetic and molecular targeting) may provide a more specific accumulation of nanomedicine at the desired tumor site, which can contribute to lower drug doses and spontaneously reduce the side effects of chemotherapy. The combination chemotherapy using T-PTX-Dox-DENCs notably suppressed tumor growth relative to the bare nanocapsules; moreover, the tumors were significantly suppressed after treatment with Trastuzumab/Dox/PTX+MT therapy once every 4 days for 4 cycles, which demonstrated the efficacy of the therapy. In summary, with or without MT, T-PTX-Dox-DENCs can accumulate at the desired



place, but the combination uses of MT can achieve the best therapy efficacy as well as reduce the side effect caused by focusing the DENCs into the tumor site. These results indicate that a drug delivery system using pH-responsive DENCs for triggered co-delivery of PTX and Dox release may provide a platform to encapsulate other hydrophobic and hydrophilic cargo for treating various cancer cell lines.

In summary, the proper use of combination therapy is one of the most promising approaches to cancer treatment. We demonstrated cell death *in vitro* and tumor growth suppression *in vivo* with pH-responsive co-delivery multi-drug chemotherapy using drug-containing tumor-targeting nanocapsules that cause few side effects. The nano-platforms developed here can facilitate the development of various integrated theranostic protocols that combine multiple hydrophobic/hydrophilic drugs, molecular targeting, and MT to enhance the cumulative amount in the tumor; these systems will enhance the cumulative payload in the tumor, which may open new avenues for cancer treatment, leading to possible clinical applications.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2013.07.009>.

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