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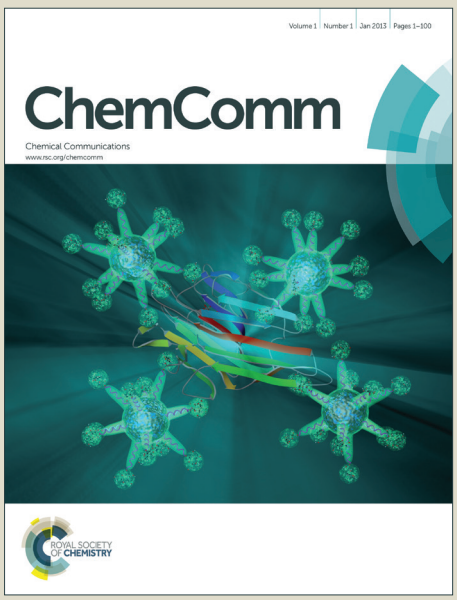

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Multi-layered polymeric nanoparticles for pH-responsive and sequenced release of theranostic agents

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In this study, multi-layered pH-responsive polymeric NPs are prepared by multiple (up to 4) emulsifications to encapsulate multiple hydrophilic and hydrophobic theranostic agents for controlled and sequenced release. It is found that the sequence of release of multiple chemotherapeutic agents from the NPs significantly affects their efficacy against cancer cells.

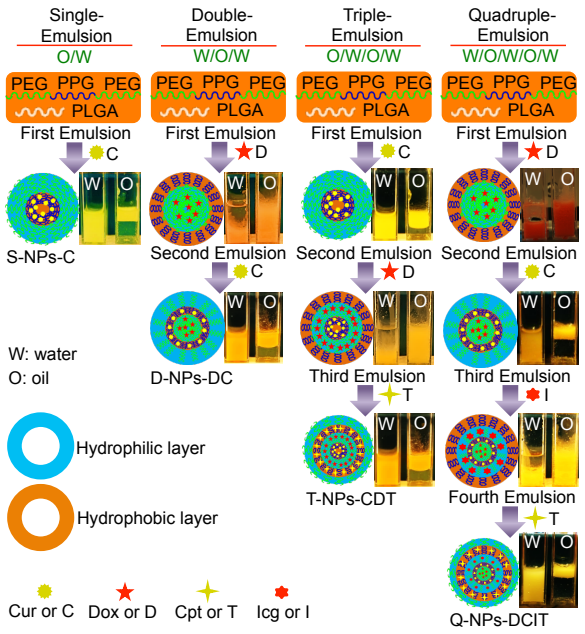
Combination chemotherapy, which is the use of two or more chemotherapeutic agents with different anticancer mechanisms or multiple treatment modalities (e.g., chemo and photothermal therapies), plays an important role in clinical cancer treatment.[1] However, due to the vastly differing physiochemical and pharmacokinetic properties of different agents including solubility, biodistribution, circulation time in blood, and membrane transport properties, the current practice of simply taking multiple free agents with no control of their delivery and release is far from optimal in making use of the therapeutic capacity of the agents for cancer treatment.[2] This deficiency also makes dosing and scheduling an optimal regimen of administering the multiple agents *in vivo* extremely difficult.[3] Therefore, it is of significance to co-deliver all the agents within the same carrier to synchronize the actions of the agents in a controlled fashion.

Over the past decade, nanoparticles (NPs) made of amphiphilic block copolymer have attracted much attention for the delivery of theranostic agents including chemotherapeutic drugs.[4] Among the different methods that have been reported so far for preparing polymeric NPs, two of them are widely used: the emulsion-solvent evaporation[5] and double-emulsion methods.[6] The former can be used to fabricate an "oil-in-water" structure for encapsulation of hydrophobic agents,[7] whereas the latter results in a "water-in-oil-in-water" configuration for encapsulating hydrophilic agents.[8] These methods have been extensively used to encapsulate and deliver chemotherapeutic agents for cancer treatment.[9] However, for most studies using the two methods, either hydrophobic or hydrophilic agents (but not both) were encapsulated in the NPs for delivery. Two recent studies reported that a hydrophilic and hydrophobic drug could be encapsulated into the hydrophilic core and the hydrophobic layer in the shell of single-layered core-shell polymeric NPs made using the double-emulsion method, respectively.[10,11] However, the single-layered NPs could not be used to achieve sequenced release of two (or more) agents that are all hydrophilic or hydrophobic. The capability of sequenced release is important because it has been shown that the therapeutic outcome of combination chemotherapy using multiple drugs is dependent on the sequence of drug administration.[12]

In this study, we systemically explored the assembly of multi-layered core-shell polymeric NPs with multiple (up to 4) emulsifications (i.e., emulsions) for encapsulating different (two or more) hydrophilic and hydrophobic agents to achieve both pH-controlled and sequenced release. The strategy is outlined in Scheme 1. We have designed four types of polymeric NPs with one (single), two (double), three (triple), and four (quadruple) emulsifications during preparation. The last emulsion is designed to be always in water to obtain NPs with high water solubility or miscibility, for which the first emulsion has to be either in water (i.e., oil-in water for single and triple-emulsion) or in oil (i.e., water-in-oil for double and quadruple-emulsion). In addition, poly (vinyl alcohol) was used as the stabilizer to prevent potential aggregation during the procedure. Curcumin (Cur or C, hydrophobic), doxorubicin hydrochloride (Dox or D, hydrophilic), irinotecan (Cpt or T, hydrophobic), and indocyanine green (Icg or I, hydrophilic) were used as the model agents in this study to obtain agent-laden S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT for the single, double, triple, and quadruple-emulsion methods, respectively. Cur, Dox, and Cpt are chemotherapeutic agents and both Dox and Cpt have been clinically used. Icg is a clinically used agent for *in vivo* imaging and has been explored for photothermal therapy.

As illustrated in Scheme 1, for single-emulsion, an "oil-in-water" structure was fabricated and hydrophobic agents can be encapsulated into the hydrophobic core of the resultant single-emulsion NPs (S-NPs). For double-emulsion, a hydrophilic core was formed for encapsulating hydrophilic agents during the first emulsion of "water-in-oil" while hydrophobic agents could be loaded into the hydrophobic layer in the shell of the resultant double-emulsion NPs (D-NPs) during the second emulsion of "water-in-oil-in-water". For the triple-emulsion method, the first emulsion of "oil-in-water" was used to encapsulate hydrophobic agents in the hydrophobic core. An

"oil-in-water-in-oil" structure was formed during the second emulsion when hydrophilic agents could be encapsulated into the newly formed hydrophilic layer. Finally, an "oil-in-water-in-oil-in-water" structure was formed during the third emulsion when hydrophobic agents could be encapsulated into the newly formed hydrophobic layer. At least one hydrophilic and two hydrophobic agents could be encapsulated into the hydrophobic core and the two (one hydrophilic and one hydrophobic) layers in the shell of the resultant triple-emulsion NPs (T-NPs). For the quadruple-emulsion, a "water-in-oil" structure was formed first during the first emulsion to encapsulate hydrophilic agents. After three further alternate emulsions, a "water-in-oil-in-water-in-oil-in-water" configuration was formed to encapsulate at least two hydrophilic and two hydrophobic agents in the hydrophilic core and the three (one hydrophilic and two hydrophobic) layers in the shell of the resultant quadruple-emulsion NPs (Q-NPs).



Scheme 1 A schematic illustration of the procedures for preparing multi-layered polymeric nanoparticles (NPs) with up to four emulsions together with pictures of the NP samples in cuvettes after each emulsion showing their solubility in water (W) versus oil (O, i.e., dichloromethane or DCM in this study). A continuous homogeneous appearance of the sample in the cuvettes indicates miscible or soluble while phase separation with a two-layered appearance in the cuvettes indicates immiscible or insoluble. Successful assembly of a layer-by-layered configuration can be examined by the solubility of the sample in water or oil dependent on the hydrophilicity of the surface layer of the nano-assembly. During each emulsion, one hydrophobic or hydrophilic agent was added into the system for encapsulation to obtained S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT for the single, double, triple, and quadruple-emulsion methods, respectively. Cur or C: curcumin (hydrophobic); Dox or D: doxorubicin hydrochloride (hydrophilic); Cpt or T: irinotecan (hydrophobic); Icg or I: indocyanine green (hydrophilic); PEG: polyethylene glycol; PPG: polypropylene glycol; and PLGA: poly (D,L-lactide-co-glycolide).

Successful formation of the hydrophilic and hydrophobic layers during each emulsion can be checked first by the solubility of the newly formed NPs in water or an organic solvent (i.e., oil that was dichloromethane or DCM in this study). As demonstrated by the pictures of the samples in cuvettes in Scheme 1, if the newly formed layer in the NPs consisted of the hydrophilic blocks of amphiphilic polymers, the NPs could dissolve in water but not in oil. On the contrary, the NPs could dissolve in oil but not water when the newly formed layer consisted of hydrophobic blocks. We performed all single and multiple-emulsion studies and synthesized the NPs using poly (D,L-lactide-co-glycolide) (PLGA) and Pluronic F127 (PF127) because the combination could be used to obtain NPs with much better stability in aqueous solutions. As shown in Fig. S1 (see ESI†), NPs prepared by the double-emulsion method using PLGA alone tend to form aggregates with two major peaks of size distribution for the single and aggregated NPs, which is probably due to the hydrophobic nature of the PLGA NP surface.

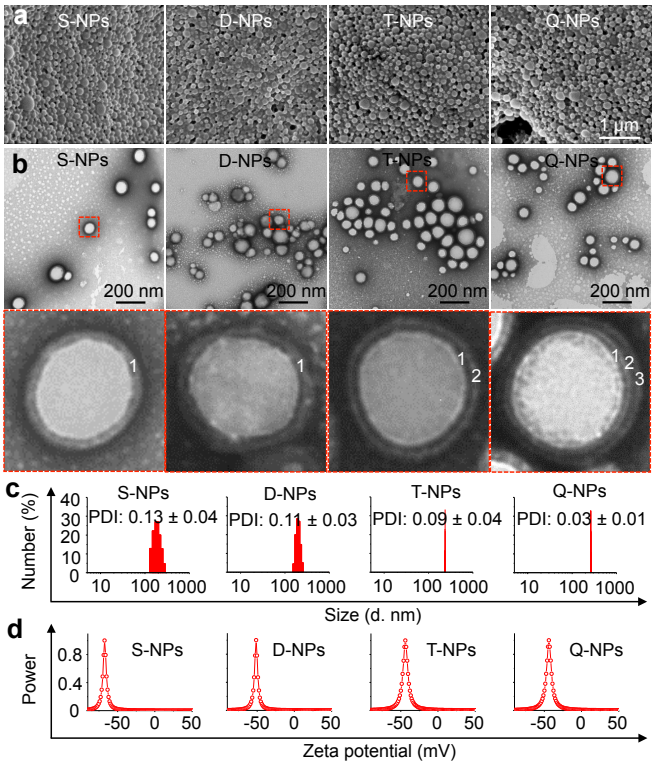


Fig. 1 Characterization of NPs prepared by single, double, triple, and quadruple-emulsion methods (S-NPs, D-NPs, T-NPs, and Q-NPs, respectively). (a) SEM and (b) TEM images of the four types of NPs. The insets in (b) are zoom-in images showing the single (for single and double-emulsion) and multiple (for triple and quadruple-emulsion) layers (labeled with numbers 1, 2, and 3) in the shell of the NPs. (c) Size distribution and (d) zeta potential of the four types of NPs determined by dynamic light scattering (DLS) at 22 °C.

The morphology and size of the NPs made with PLGA and PF127 using the single, double, triple, and quadruple-emulsion methods (S-NPs, D-NPs, T-NPs, and Q-NPs for short) were visualized by transmission (TEM) and scanning (SEM) electron microscopy. As shown in Fig. 1a (SEM) and b (TEM), the NPs are well dispersed with a spherical and core-shell morphology and are ~100 nm in diameter. The single (for single and double-emulsion) and multiple (for triple and quadruple-emulsion) layered structures in the shell of the NPs are visible in the insets of Fig. 1b. We further checked the size of the S-, D-, T- and Q-NPs in DI water using dynamic light scattering (DLS) to be 186.2 ± 3.9 nm, 203.7 ± 3.2 nm, 238.9 ± 1.5 nm, and 262.7 ± 0.4 nm in diameter, respectively (Fig. 1c). The average size of NPs slightly increases as more polymeric layers form

in the NPs. The size determined by DLS is larger than that from TEM and SEM, probably because the hydrophilic blocks of the polymers on the surface of the NPs are swollen in water and contribute more to the hydrodynamic diameter determined by DLS than the size of the dry NPs used for TEM and SEM studies. Interestingly, the NPs have much better polydispersity index (PDI) or their sizes are more homogeneous when more emulsions are conducted. This might be because more polymers could be added in the new layers of smaller than larger nanoparticles during the intermediate steps of multiple emulsions to make the larger multistep nanoparticles more homogeneous. All four types of NPs have similar zeta potentials (Fig. 1d), which is probably due to the fact that the outer surface of the NPs is composed of the hydrophilic blocks of the two amphiphilic polymers used and PLGA contributes to the negative zeta potential of the resultant nanoparticles. The negative zeta potential (less than -25 mV) suggests that all the four types of NPs should have high stability in aqueous solution. These results indicate that we have successfully prepared core-shell NPs with one or multiple layers in the shell using the single, double, triple, and quadruple-emulsion methods.

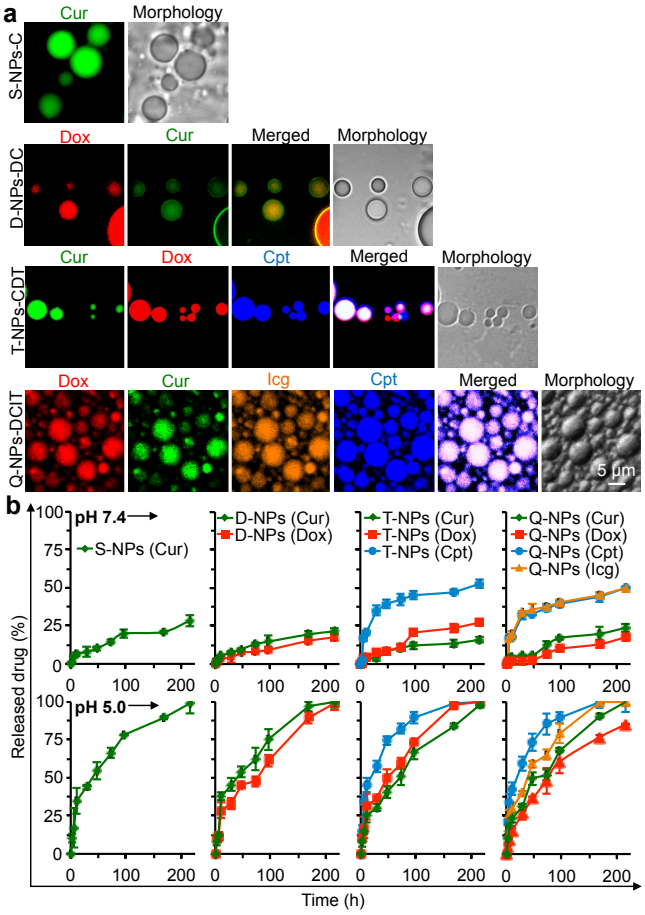


Fig. 2 (a) Fluorescence and gray scale micrographs showing Cur in S-NPs-C, Dox and Cur in D-NPs-DC, Cur, Dox, and Cpt in T-NPs-CDT, and Dox, Cur, Icg, and Cpt in Q-NPs-DCIT. (b) *In vitro* release of encapsulated agents from S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT at pH 5.0 and 7.4 showing not only pH-responsive but also sequenced release profile.

In order to confirm that both hydrophilic and hydrophobic agents could be encapsulated into the NPs, we performed fluorescence microscopy studies first. As shown in Fig. 2a, the green fluorescence of curcumin (Cur) could be seen in the nanoparticles from single-emulsion (S-NPs-C) before rotary evaporation to remove organic solvent. Both red (Dox) and green (Cur) fluorescence were detectable in all nanoparticles made with the double-emulsion method (D-NPs-DC). For the triple or quadruple-emulsion methods, fluorescence of all the three (Cur, Dox, and Cpt) or four (Dox, Cur, Icg, and Cpt) different agents shows up in each nanoparticle. Successful encapsulation of the various agents using the single and multiple-emulsion methods is further evidenced by fluorescence spectra and UV-Vis absorption of the agent-laden NPs (Figs. S2-S5, see ESI†). When co-encapsulating multiple agents, the total amount (in weight) of each agent was equally distributed among the multiple agents. The encapsulation efficacy and loading content of the different agents using the four different methods are high (Table S1, see ESI†). Interestingly, the encapsulation efficiency of the agents in the core or inner layers increases with the number of emulsions, probably because more of the agents were encapsulated into the nanoparticles during the steps of forming outer layers.

An optimal NP delivery system is desired to be capable of controlling the drug release at the desired location such as tumor with an acidic pH (~5-6) to reduce their side effects.[13] In addition, polymeric NPs are usually taken up by cells via endocytosis first in endosomes and then in lysosome with a low pH of ~4-5.[14] Therefore, we investigated the *in vitro* drug release at 37 °C from the four types of NPs at pH 7.4 (pH of blood) and 5.0. At the neutral pH, the release of Cur and DOX is sustained in both S-NPs-C and D-NPs-DC (Fig. 2b). The Cur and Dox in T-NPs-CDT and Q-NP-DCIT have similar release profiles to that in S-NPs-C and D-NPs-DC. In contrast, the release of Cpt in T-NPs-CDT or Icg and Cpt in Q-NP-DCIT is faster than Cur and Dox, probably because Cpt and/or Icg were encapsulated in the outer layers of the NPs (Fig. 2b). Moreover, the release of all the agents from all the NPs was faster at pH 5.0 (which is probably due to the faster degradation or hydrolysis of PLGA under acidic pH) and the release of agents encapsulated in the outer layer was faster than that in the inner layer or core. These observations demonstrated that multiple agents could be encapsulated into one NP using our multi-emulsion approach to achieve not only pH-responsive but also sequenced release.

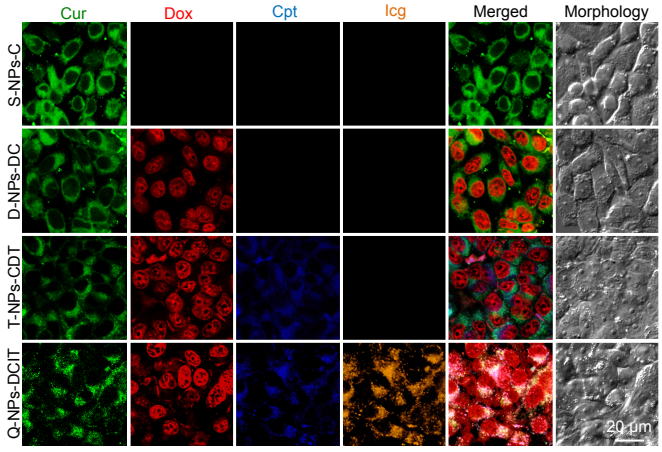


Fig. 3 Confocal micrographs of MDA-MB-231 cancer cells after incubated with S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-CDIT for 5 h at 37 °C, showing successful delivery of the encapsulated agents in the NPs into the cancer cells.

To examine the capability of delivering multiple agents using the four types of NPs into cancer cells, we incubated MDA-MB-231 human breast and PC-3 human prostate cancer cells with the S-NPs-C, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT for 5 h. As shown in

Fig. 3 (for MDA-MB-231 cells) and Fig. S6 (for PC-3 cells, see ESI†), the four types of NPs could be used to successfully deliver the four different agents into both MDA-MB-231 and PC-3 cells. To study intracellular trafficking of the NPs, all the NPs were encapsulated with Cur only. As shown in Fig. S7 (see ESI†), there was significant overlap between the fluorescence of Cur and the red stain of late endosomes/lysosomes in PC-3 cancer cells after 5 h incubation at 37 °C. These observations together with the minimal (< 15%) release of the encapsulated agents during the first 5 h incubation at neutral pH (Fig. 2b) suggest that the cancer cells actively take up all the four types of NPs via endocytosis.

We further investigated the anti-cancer capability of S-NPs-T, D-NPs-DC, T-NPs-CDT and Q-NPs-DCIT using PC-3 and MDA-MB-231 cancer cells. The viability data after one-day treatment are shown in Fig. 4a. All the drug formulations showed toxicity to the two cancer cells in a concentration-dependent manner. Because Icg is an *in vivo* imaging agent with no cytotoxicity, the anticancer capability of Q-NPs-DCIT is not better than T-NPs-CDT or D-NPs-DC under the same total dose of all agents. However, it is still significantly better than S-NPs-T at a total dose of 8.6 and 17.2 μM. Although Icg is not a chemotherapeutic drug, it can be used for photothermal therapy. Indeed, when near infrared (800 nm) laser was applied for 3 minutes at 1.5 W/cm^2, the Q-NPs-DCIT could induce significantly higher toxicity to both types of cancer cells (Fig. 4a). Furthermore, the Q-NPs-DCIT with laser irradiation exhibits significantly better anticancer capability than free Dox (Fig. S8, see ESI†).

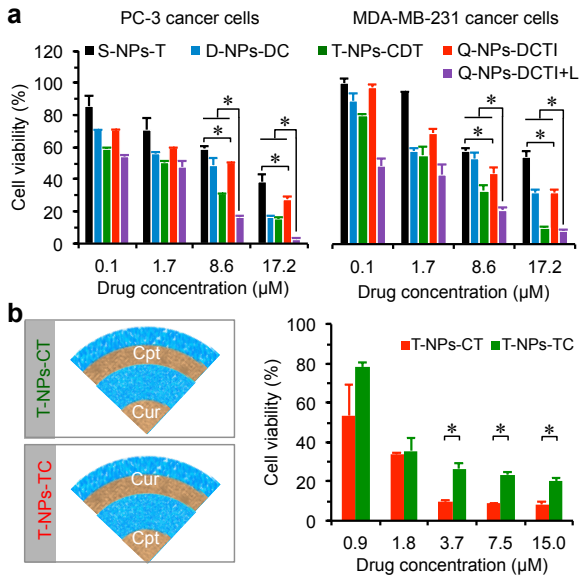


Fig. 4 (a) Cell viability of PC-3 and MDA-MB-231 cancer cells after treated with S-NPs-T, D-NPs-DC, T-NPs-CDT, and Q-NPs-DCIT with different concentrations for 24 h. The treatment with Q-NPs-DCIT was further combined with photothermal therapy by utilizing the photohermal effect of Icg (Q-NPs-DCIT+L) upon irradiation with near infrared laser (L, 800 nm) for 3 minutes at 1.5 W/cm^2. (b) Cell viability of MDA-MB-231 cells after exposure to T-NPs-TC and T-NPs-CT at different concentrations at 37 °C for 48 h. The asterisk indicates $p < 0.05$ between the indicated groups.

To study the effect of release sequence on the cytotoxicity of multiple drugs, we used the triple-emulsion method to encapsulate two hydrophobic drugs (Cur and Cpt) to obtain T-NPs-CT and T-NPs-TC. As shown in Fig. 4b, for T-NPs-CT, Cur and Cpt were encapsulated in the core and hydrophobic layer, respectively. For T-NPs-TC, Cpt was encapsulated in the hydrophobic core while Cur was in the hydrophobic layer. The T-NPs-CT showed significantly higher cytotoxicity than T-NPs-TC to MDA-MB-231 cells at concentrations higher than 3.7 μM. This observation is consistent with previous studies suggesting that the treatment sequence of multiple drugs for combination chemotherapy affects its therapeutic outcome.[12] The multi-emulsion approach developed in this study makes it possible to deliver multiple drugs in one nanoparticle to achieve the desired sequenced release inside tumor and cancer cells.

In conclusion, we systemically studied and developed the emulsion-based multi-layered core-shell NPs for encapsulation and delivery of multiple hydrophilic and/or hydrophobic agents for both therapy (e.g., Dox) and *in vivo* imaging (e.g., Icg). The agents could be encapsulated into the core and the different layers in the shell to achieve not only pH-responsive but also sequenced release. Cell studies confirmed that these NPs could help to deliver multiple agents into PC-3 and MDA-MB-231 cancer cells for combination (chemo and/or photothermal) therapy. Moreover, the chemotherapeutic outcome of multiple drugs was found to be dependent on their release sequence. Since combination chemotherapy is very important and increasingly used in the clinic for treating cancer and possibly many other diseases, this study will have a significant impact on the broad field of drug delivery by providing a universal approach for controlling the release of multiple theranostic agents to achieve the optimal outcome of combination therapy using either multiple drugs or multiple treatment modalities.

Notes and references

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