Research Article

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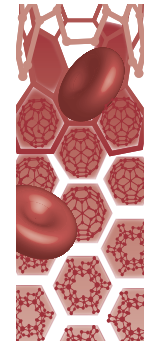

Nanoparticle–dendrimer hybrid nanocapsules for therapeutic delivery

Background: Nanocapsules can efficiently encapsulate therapeutic cargo for anticancer drug delivery. However, the controlled release of the payload remains a challenge for effective drug delivery. **Materials & methods:** We used dithiocarbamate-functionalized PAMAM dendrimer to cross-link the shell of arginine gold nanoparticles stabilized nanocapsule, and controlled the drug release from the nanocapsule. The ability of cross-linked nanocapsule to deliver hydrophobic paclitaxel to B16F10 cells was demonstrated both *in vitro* and *in vivo*. **Results:** Cross-linked nanocapsule possesses tunable stability and modular permeability, and can deliver paclitaxel with improved anticancer efficiency compared with free drug both *in vitro* and *in vivo*. **Conclusion:** Dithiocarbamate chemistry provides a new tool to harness multifactorial colloidal self-assembly for controlled drug delivery for cancer therapy.

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Keywords: cancer therapy • drug delivery • nanocapsule • paclitaxel • sustained drug release

Youngdo Jeong[‡,1], Sung Tae Kim[‡,1], Ying Jiang[1], Bradley Duncan[1], Chang Soo Kim[1], Krishnendu Saha[1], Yi-Cheun Yeh[1], Bo Yan[1], Rui Tang[1], Singyuk Hou[1], Chaekyu Kim[1], Myoung-Hwan Park[1,2] & Vincent M Rotello*[,1]

[1]Department of Chemistry, University of Massachusetts-Amherst, 710 North Pleasant Street, Amherst, MA 01003, USA

[2]Department of Chemistry, Sahmyook University, Seoul 139-742, South Korea

*Author for correspondence: rotello@chem.umass.edu

[‡]Authors contributed equally

Nanomaterials [1], featuring high internal volume, surface area and colloidal stability, are able to efficiently encapsulate therapeutic payloads for anticancer drug delivery [2,3]. Designing nanoparticles with controllable drug release properties has potential to improve the therapeutic index of various chemotherapeutics [4,5]. For example, Zink and Stoddart have designed mesoporous silica nanoparticles with tunable permeability, engineering the supramolecular interaction of the payload on the nanoparticle surface to release the drug in a controllable manner [6–8].

Nanoparticle-stabilized capsule (NPSC) is a new delivery platform that enables the direct cytosolic delivery of a large variety of therapeutics, including proteins, small-interfering RNAs and chemotherapy drugs [9]. Gold nanoparticles (AuNPs) were used in NPSCs as modular building blocks to incorporate particle properties to expand overall functionality. NPSCs were initially stabilized through supramolecular interactions between the outer nanoparticle shell and the inner complementary hydrophobic 'oil' components. The NPSCs were further stabilized on the outer surface through lateral interactions between nanoparticles and complementarily charged biomolecules [10,11]. Using this strategy, we have been able to produce nanocapsules capable of effective delivery of paclitaxel (PTX), a small molecule chemotherapy drug with limited clinical applications due to its poor aqueous solubility [12]. Additionally, we have previously demonstrated the controlled release of therapeutics from a nanoparticle carrier by cross-linking gold nanoparticles and poly(amido amine) (PAMAM) dendrimers using dithiocarbamate (DTC) chemistry [13,14]. We envisioned that cross-linking NPSCs with PAMAM dendrimers using similar DTC chemistry would generate new nanoparticle–dendrimer hybrid nanocapsules (NDHCs) for controlled drug delivery. The NDHC nano-

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capsules were assembled by adding 2 nm gold core arginine AuNPs to the surface of oil droplets, followed by cross-linking the AuNPs using DTC-functionalized dendrimer linker (Figure 1). We found that the stability and release of PTX from NDHC could be conveniently controlled by adjusting the molar ratio between dendrimer and nanoparticles. We further demonstrated the therapeutic applications of PTX-encapsulated NDHC for cancer therapy by delivering PTX to B16F10 melanoma cells *in vitro* and *in vivo* (Figure 1) [15,16].

Materials & methods

General

Paclitaxel was purchased from LC Laboratory. All the other chemicals were purchased from Sigma-Aldrich or Fischer Scientific and used as received, unless otherwise specified. Dichloromethane (DCM) used for chemical synthesis was dried according to standard procedures. Transmission electron microscopy (TEM) images were acquired on a JEOL CX-100 operating at 80 keV. Dynamic light scattering data were measured with a Malvern Zetasizer Nano ZS. Fluorescent images were taken on Olympus IX51 microscope. Cell images were obtained on Zeiss LSM510 Meta confocal microscope. The fluorescence from the Alamar blue assay was measured in a SpectraMax M2 microplate spectrophotometer and analyzed by Origin 8 to determine the cell viability. Flow cytometry analysis was performed in a BD LSR-II flow cytometer equipped with FACSDiva (BD Sciences, USA) by counting 10,000 events.

Synthesis of arginine functionalized gold nanoparticles

Arginine functionalized gold nanoparticles were synthesized as previously reported [10]. In a typical reaction, 10 mg of 1-pentanethiol protected gold nanoparticles was dissolved in 10 ml distilled DCM and purged with argon for 10 min. Subsequently, 30 mg of arginine ligand in 5 ml of methanol was added to the nanoparticle solution. The reaction mixture was allowed to stirrer for 2 days followed by removal of solvents. The resulting black colored residue was washed with a mixture of hexanes (90%) and DCM (10%) for five-times to remove 1-pentanethiol and excess arginine ligand. This nanoparticle residue was dissolved in distilled water and purified by dialysis with skin membrane (10,000 MWCO) in distilled water for 12 h. Finally, molecular cut off filtration (10,000 MWCO) for three-times were used to ensure the purity of arginine functionalized gold nanoparticles (see Figure S1–S3 in Supporting Information for synthesis and characterization).

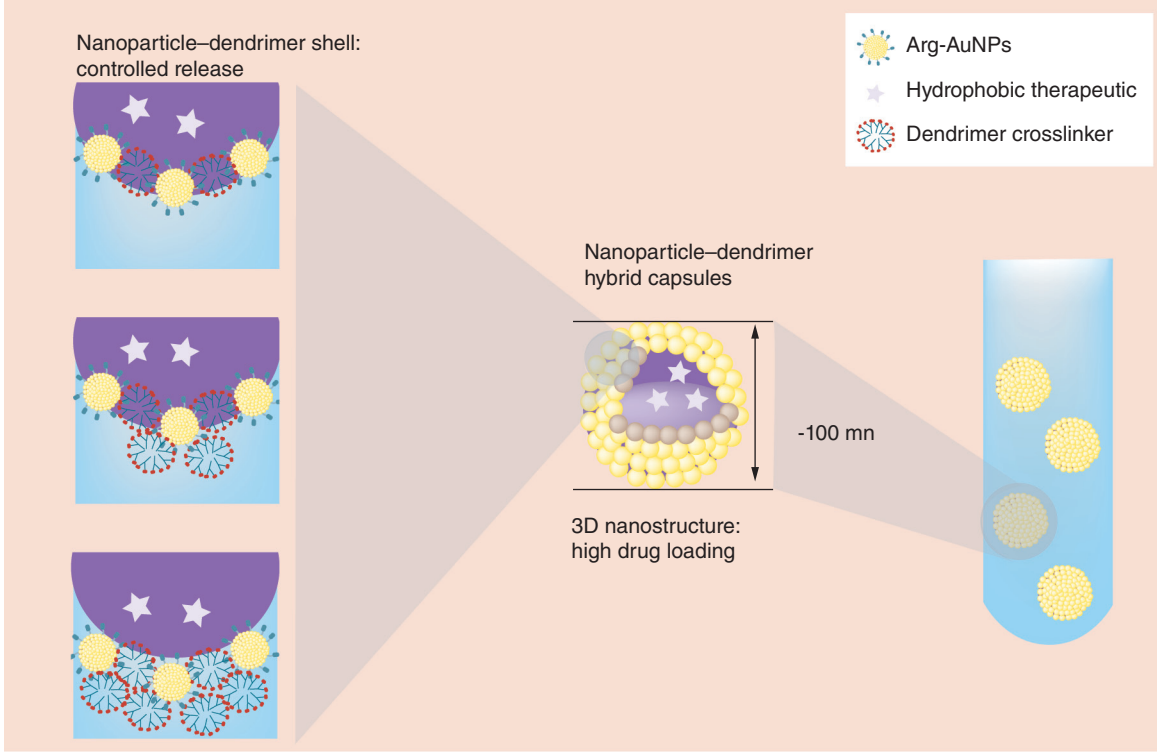

Figure 1. Schematic illustration of the strategy used to treat melanoma using the nanoparticle–dendrimer hybrid nanocapsules.

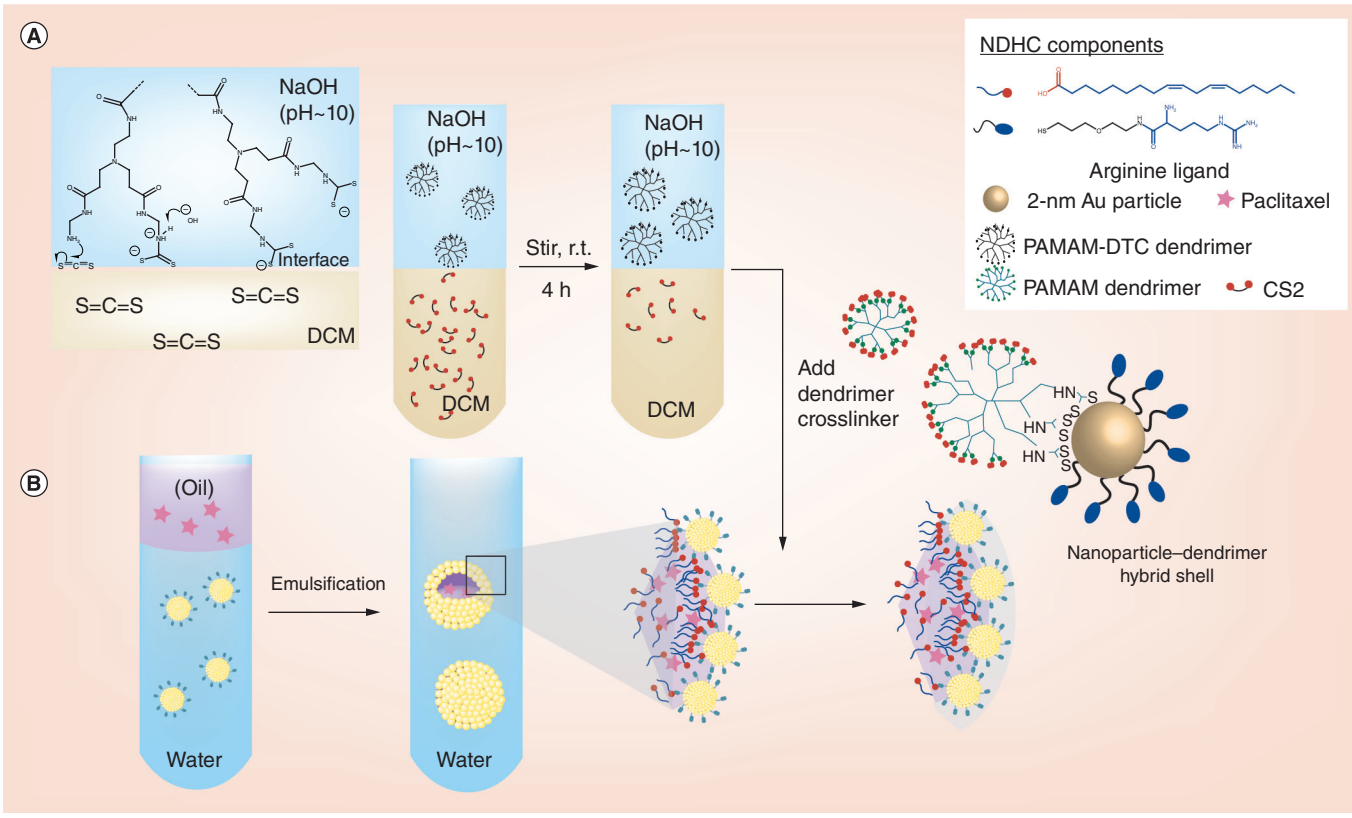

Figure 2. Schematic illustration of the formation of (A) dithiocarbamate functionalized dendrimers, (B) NDHCs. The dithiocarbamate functionalized dendrimer cross-linkers were fabricated by the interfacial reaction between PAMAM dendrimer and CS_2, with the resulting cross-linkers covalently assembling with AuNPs at the interface of therapeutic oil droplets.NDHC: Nanoparticle–dendrimer hybrid nanocapsule.

Synthesis of dithiocarbamate-functionalized dendrimer linker

To synthesize the dendrimer linker, we followed the previous report [13]. In brief, 8.8 mM of PAMAM dendrimer in an NaOH solution (pH ~10, 3 ml, 0.4 M) were mixed with 500 µl CS_2 in DCM (3 ml). The mixture was stirred for 4 h at room temperature. At the end of the reaction, the linker was isolated from the aqueous layer.

Fabrication of the NDHCs

We followed similar procedures to fabricate the NPSCs [10]. Briefly, 2.0 µl of linoleic acid was emulsified in 1 ml of arginine-functionalized nanoparticles (1.0 µM) in a phosphate buffer solution (5.0 mM, pH 7.4) using an amalgamator (speed 5000 rpm for 200 s). This process results in oil droplets having a 12.8 nM concentration. Next, 200 µl of the 6.4 nM droplets were incubated with arginine nanoparticles (5 µM) in 800 µl phosphate buffer (5.0 mM, pH 7.4) for 10 min. To prepare the NDHCs, the above made NPSCs were cross-linked through the addition of the dithiocarbamate functionalized dendrimers (12.8 nM for 1:1 ratio, 51.2 nM for 1:4 ratio and 204.8 nM for 1:16 ratio) for 12 h. To fabricate the PTX-loaded NDHCs, PTX was initially dissolved in linoleic acid, then PTX-containing linoleic acid was emulsified and stabilized in arginine-functionalized nanoparticles solution at the same condition for making NPSCs as above mentioned. 10 mg/ml PTX in linoleic acid for *in vitro* or 50 mg/ml PTX in linoleic acid for *in vivo* drug release was used.

In vitro release study

After fabricating the NDHCs with different ratios of NP to dendrimer, PTX-loaded NDHC was added to a dialysis cassette (Side-A-Lyzer® 3.5 K, Thermoscientific, IL, USA) and dialyzed against phosphate buffered saline (PBS, pH 7.4) at 37°C with constant stirring (100 rpm, Lab-Line® Orbit Environ-Shaker, Lab-Line Instruments, Inc.) for 3 days in the absence of any solubility enhancers [17]. At given time intervals, each supernatant (0.3 ml) of three different NDHC formulations was taken and then resuspended in 25% acetonitrile after a drying process (Vacufuge™, Eppendorf, Germany). Each supernatant was quantitatively analyzed by a high-performance liquid chromatography system (DGU-20AS, Shimadzu, Japan). Separation

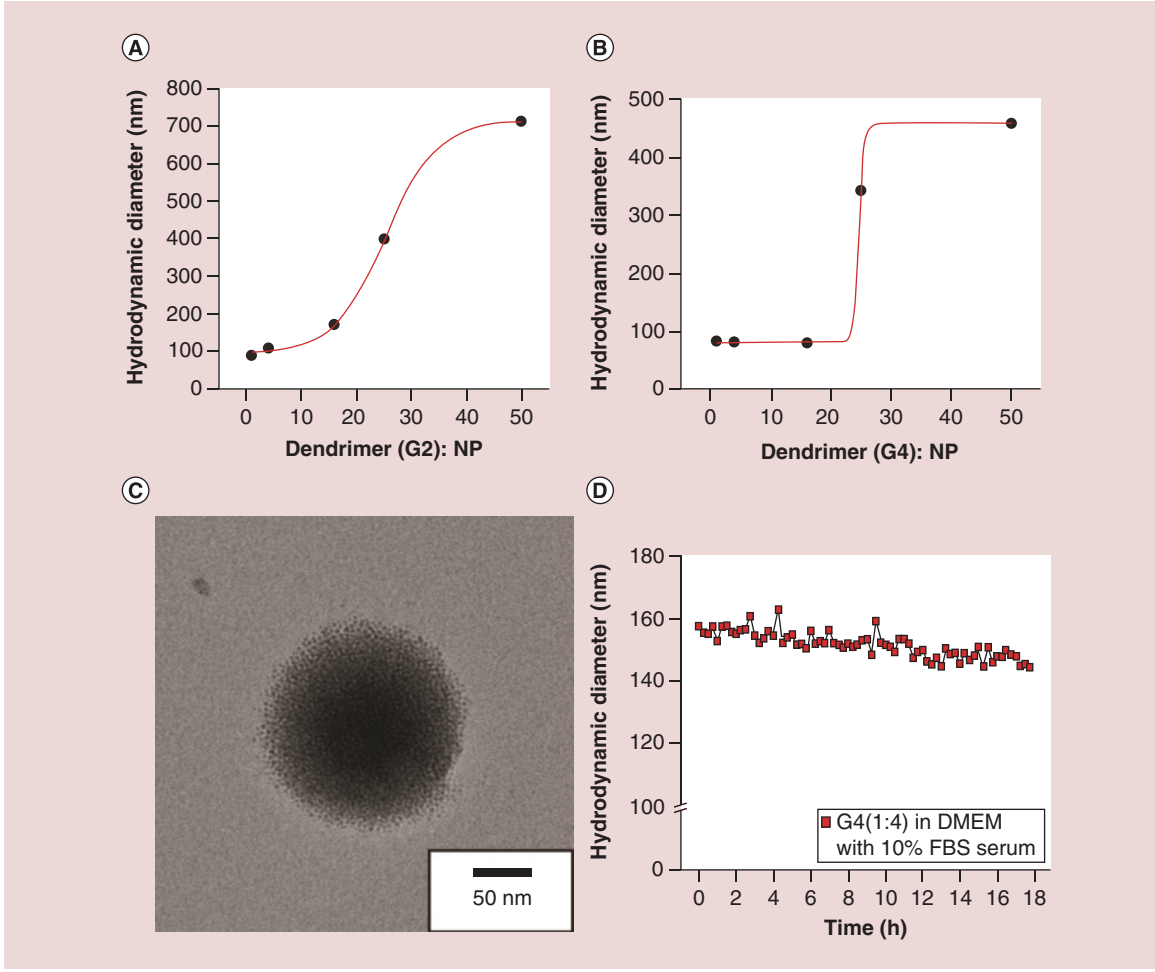

Figure 3. The hydrodynamic diameters of the NDHCs fabricated by various molar ratios when (A) generation 2 dendrimers, and (B) generation 4 dendrimers were used. Each point is the mean value obtained by DLS measurement, **(C)** representative TEM image of NDHC and **(D)** average diameter of NDHCs fabricated using a 1:4 AuNP:dendrimer (G4) ratio incubated in DMEM media with 10% serum for 18 h.
DLS: Dynamic light scattering; DMEM: Dulbecco's Modified Eagle's Medium; NDHC: Nanoparticle–dendrimer hybrid nanocapsule; TEM: Transmission electron microscopy.

was achieved using C18 reverse phase column (ODS, 4.6 mm × 250 mm) under a mobile phase consisting of two eluents, 0.1% trifluoroacetic acid (TFA) in H_2O and 0.1% TFA in ACN (68:32, v/v). The flow rate was 1 ml/min and the injection volume was 20 µl. Released paclitaxel was detected at 273 nm using an ultraviolet detector (SPD-20AV).

Cytotoxicity of capsules

The cell viability was measured using AlamarBlue assay (Invitrogen, CA, USA). Briefly, B16F10 were seeded in a 48-well plate (5×10^4 cells/well) 24 h prior to the experiment. At the day of experiment, cells were washed with cold PBS and treated with varied concentration of either NDHC, or NDHC-PTX, or PTX for 24 h. Following incubation, the cells were washed with PBS and treated with 10% Alamar blue dye in a low glucose Dulbecco's Modified Eagle's Medium (DMEM) medium with 10% fetal bovine serum. After incubation, fluorescence intensity was measured (Ex: 560 nm, Em: 590 nm) using a SpectroMax M2 microplate reader (Molecular devices, CA, USA) to determine cell viability.

Cellular uptake of nanocapsule

B16F10 were cultured using DMEM, 1 ml and plated in 12-well microplates with a density of 40,000 cells/well and the cells were grown for 24 h. The Nile red encapsulated NSPEs or NDHCs in DMEM media were then added and incubated for 0.5, 1 and 6 h, respectively. The media of the cells was removed and the cells washed with PBS (3×) to eliminate any extracellular capsules. After completing incubation, the cells were washed twice with PBS, harvested with tryp-

sin/EDTA and resuspended in 2% FCS in PBS buffer. These samples were analyzed by BD™ LSR II.

Inductively coupled plasma mass spectrometry analysis

After NDHCs treatment, mice organs were harvest and initially digested overnight using a mixture of HNO_3 and H_2O_2 (3:1, v/v) under constant shaking/sonication. Then the digested solutions were incubated with additionally 0.5 ml of fresh aqua regia for an additional 1 h prior to the inductively coupled plasma mass spectrometry (ICP-MS) measurement [18]. A series of gold standard solutions ranging from 20 to 0 ppb was prepared prior to each experiment. ICP-MS analyses were performed on a Perkin-Elmer NexION 300X ICP mass spectrometer as follows: nebulizer flow rate: 0.95–1 l/min; rf power: 1600 W; dwell time: 50 ms.

Animal care

All animal experiments were conducted in accordance with the guidelines of Institutional Animal Care and Use Committee at University of Massachusetts-Amherst. Female C57BL6 mice (8–10 weeks, 20–25 g) were purchased from Jackson Laboratory (ME, USA). Food and water intake were assessed.

In vivo delivery of NDHCs

After 1 week of acclimatization, female C57BL/6 mice were anesthetized intraperitoneally by Avertin (tribromoethanol, Acros, 200 mg/kg). Then, B16F10 cells (50 µl, 2 × 10^5 cells) were inoculated subcutaneously in the right flank after removing hair with a trimmer (Braintree Scientific, MA, USA). After the tumors were allowed to grow to over 100 mm^3 (10 days), mice were randomly rearranged and divided into the following groups: control group (saline), PTX, 1 mg/kg dose)-treated group, the NDHC-treated group and PTX-loaded NDHC-treated group. Each agent sample was intravenously administered on day 1 and day 3. Changes in mouse weight were monitored and tumor volumes were measured by an electric digital caliper. In order to measure each tumor volume by the external caliper, both the greatest longitudinal diameter (= length) and the greatest transverse diameter (= width) were determined. Based on these measurements, each tumor volume was calculated by the following formula: Tumor volume = ½ (length × $width^2$).

Results & discussion

The starting template for NDHCs was prepared through the guanidinium-carboxylate self-assembly between arginine-functionalized gold nanoparticles (Arg-AuNP) and linoleic acid nanodroplets (Figure 2) [10]. Arg-AuNP was prepared and thoroughly characterized using previously reported method (Supplementary Figures 1–3 & Supplementary Table 1). The precursor capsules were further cross-linked using dithiocarbamate (DTC)-functionalized dendrimers. These cross-linkers were prepared through the reaction of PAMAM dendrimers and CS2 in a biphasic solution (water: DCM, pH = 10, Figure 2A). [13]. TEM and scanning electron microscopy indicated that the NDHCs were structurally stable, retaining their 3D structures and size after drying (Supplementary Figure 4).

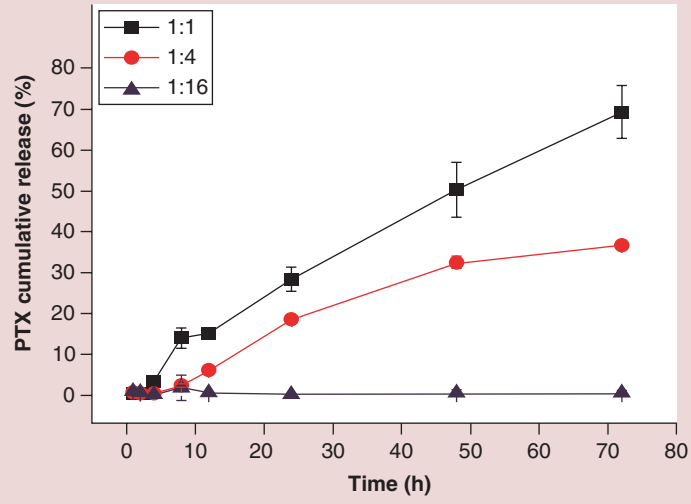

Figure 4. Drug release profiles of nanoparticle–dendrimer hybrid nanocapsules fabricated by using different dendrimer molar ratios in terms of time. NDHCs were suspended in dialysis cassettes and released PTX was measured using HPLC. Higher molar ratios of dendrimer to AuNPs reduced the rate of drug release.
HPLC: High-performance liquid chromatography; NDHC: Nanoparticle–dendrimer hybrid nanocapsule; PTX: Paclitaxel.

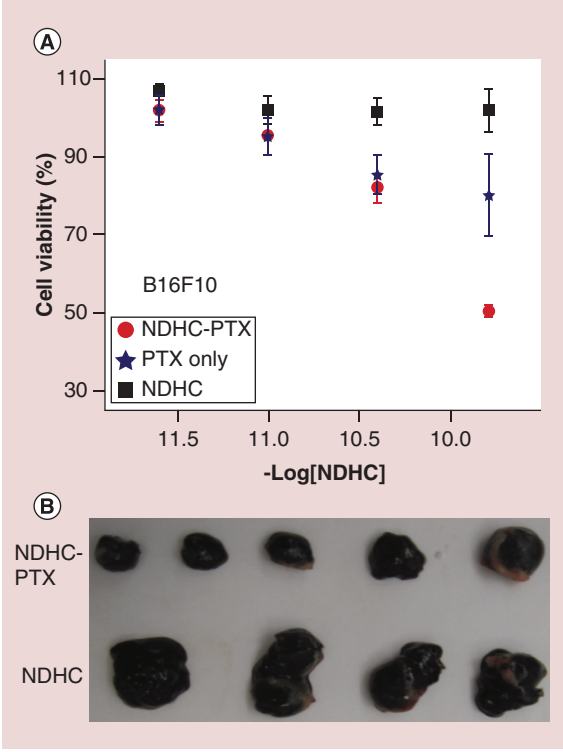

Figure 5. (A) *In vitro* cytotoxicity of NDHCs, PTX-loaded NDHCs and free PTX after 24 h incubation with B16F10 cells measured by Alamar blue assay. Cell viability experiments were performed in triplicate and the error bars represent the standard deviations of these measurements. **(B)** Images of tumors harvested from mice after two-times intravenous administration of PTX-loaded NDHCs (five mice, top photo) and NDHCs (four mice, bottom photo), respectively.
NDHC: Nanoparticle–dendrimer hybrid nanocapsule; PTX: Paclitaxel.

We next studied the effect of dendrimer generation on NDHC stability. Using capsule diameter as a means to evaluate NDHC stability, we varied the ratios of Arg-AuNP: dendrimer using both G2 and G4 PAMAM dendrimers as cross-linkers (Figure 3A & B). At AuNP:dendrimer molar ratios below 1:16 for G2 and G4, the diameter of NDHCs remained around 100 nm, with higher ratios presumably causing inter-capsule cross-linking (Supplementary Figures 5 & 6). We then investigated the colloidal stability of NDHC following 18 h of incubation with 5 mM phosphate buffer. The diameter of NDHCs fabricated using G2 dendrimer cross-linkers increased dramatically (Supplementary Figure 7), while the G4 analogs were stable with no significant size change, presumably due to the higher cross-linking density using G4 dendrimer (Figure 3D). NDHCs cross-linked using G4 dendrimer were then selected for the following studies.

We next determined the role of NP: dendrimer ratio on capsule permeability and drug release. To this end, precursor capsules encapsulating PTX were cross-linked with varying amounts of G4 dendrimer, followed by drug release study. NDHCs were placed in a dialysis cassette and dialyzed against PBS (pH 7.4) at 37°C with constant stirring for 3 days in the absence of any solubility enhancers, and PTX release kinetic was monitored by high-performance liquid chromatography. As shown in Figure 4, increasing the NP-dendrimer ratio decreased the rate of drug release, with higher ratios (1:16) resulting in essentially no release, which is consistent to our previous report [14]. The slow drug release from NDHCs with higher dendrimer-NP ratio was probably due to the thicker and denser nanocapsule shell that caused by the high Arg-AuNP: dendrimer linker ratio. Importantly, the PTX release kinetic study showed no evidence of burst release of drug, making NDHCs excellent candidates for controlled drug delivery [16].

We next investigated *in vitro* delivery using NDHCs. Based on above PTX release kinetic study, we focused our efforts on NDHCs fabricated with a 1:4 NP: G4 dendrimer molar ratio. Flow cytometry and ICP-MS analysis of B16F10 cells treated with nile red-encapsulated NDHCs both confirmed efficient delivery and cellular uptake of NDHCs by B16F10 cells (Supplementary Figure 8). Cross-sectional cell-TEM images of B16F10 cells incubated for 3 h with NDHCs were obtained to visualize the mechanism of delivery. NDHCs preserved their shape and size after entering cells (Supplementary Figure 9), indicating that the capsule structure is stable during the cellular uptake process.

We next studied the *in vitro* therapeutic delivery efficacy of NDHCs by measuring the cell growth rate of B16F10 cells treated with different NDHCs. cytotoxicity of NDHCs, PTX-loaded NDHCs and free PTX against B16F10 cells was evaluated by analar blue assay. As shown in Figure 5A, PTX-loaded (10 mg/ml in the linoleic acid core) NDHCs were more effective than the free PTX to prohibit the growth of B16F10 cells. Moreover, minimal cytotoxicity was observed when the cells were treated with NDHCs alone (Figure 5A).

The *in vivo* antitumor efficacy of PTX-loaded NDHC was preliminarily investigated with a B16F10 melanoma tumor model that has a high level of angiogenesis [19,20], providing a model for passive tumor targeting. We initially studied the biodistribution of NDHCs in mice organs, and found that a substantial amount of NDHC was accumulated in tumor, as well as other organs including liver, spleen and kidneys (Supplementary Figure 10). As the size of NDHCs was around 100 nm, it probably accumulated in the tumor through a well-studied enhanced permeation and retention effect [21,22]. The antitumor efficacy of PTX-loaded NDHC delivery was then performed by

intravenous injection of PTX-loaded NDHCs, into C57BL6 mice bearing melanoma tumors. Additionally, mice injected with NDHC only, PTX and PBS were used as controls. As shown in Figure 5B and Supplementary Figure 11, significant suppression of tumor growth was observed for PTX-loaded NDHCs treatment compared with NDHCs alone, PBS- and PTX-treated group, demonstrating the effectiveness of NDHCs for *in vivo* chemotherapeutic delivery The improved antitumor efficiency of PTX-loaded NDHCs than free PTX injection was consistent with the *in vitro* results, and probably resulted from the enhanced uptake of PTX at the tumor site. Significantly, the body weight of mice in all groups remained unchanged throughout the experiment (Supplementary Figure 12), indicating NDHCs have no substantial side effects. Taken together, these data demonstrate that NDHCs can successfully deliver hydrophobic PTX to tumor site and suppress tumor growth.

Conclusion

In summary, we have developed a strategy to assemble nanoparticle–dendrimer nanocapsules for controlled drug delivery for cancer therapy. These NDHCs feature a nanoparticle–dendrimer composite shell and an oil core that can encapsulate hydrophobic PTX. A key feature of this system is the use of DTC chemistry to cross-link the nanocapsule with dendrimer on NDHCs shell, enabling the stabilization of NDHCs as well as releasing PTX in a controllable manner. Additionally, the stability of NDHCs could be conveniently tailed via the structure of the dendrimer used to cross-link NDHCs. The PTX-encapsulated NDHCs have shown superior therapeutic effect than free drug to prohibit melanoma cell growth both *in vitro* and *in vivo*. Taken together, the strategy and methods reported herein provide new approaches to harness multifactorial colloidal self-assembly for controlled drug delivery in cancer therapy.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0034

Financial & competing interests disclosure

This research was supported by the NIH (EB014277 and GM077173). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Porous nanomaterials have abilities to effectively encapsulate therapeutic drugs make them particular attractive as a drug delivery systems, however, a controlled release of the payload remains a challenge.

Aim

- Controlling the dimensions and payload interactions at the surface of carriers provides a potent strategy for engineering controlled drug delivery platforms.

Results

- Dithiocarbamate chemistry was successfully applied to create porous nanoparticle–dendrimer nanocapsules.
- Dendrimer generation and NP: dendrimer ratio determine the stability of resulting nanocapsule.
- NP: dendrimer ratio controls the drug release profiles of nanoparticle–dendrimer hybrid nanocapsules.
- PTX-loaded nanoparticle–dendrimer hybrid nanocapsules show superior therapeutic effect to free PTX-treated cells both *in vitro* and *in vivo*.

Conclusion

- Dithiocarbamate chemistry provides a new tool to engineering porous nanoparticle–dendrimer nanocapsules for controlled release applications in cancer therapy.

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