

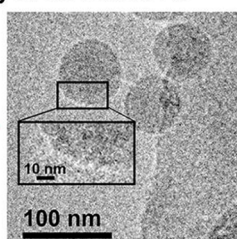
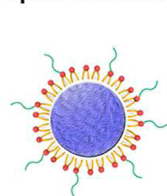
Microfluidic Synthesis of Hybrid Nanoparticles with Controlled Lipid Layers: Understanding Flexibility-Regulated Cell–Nanoparticle Interaction

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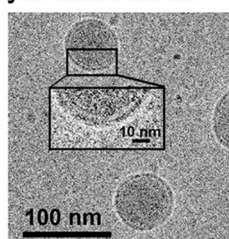
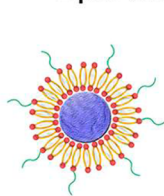
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ABSTRACT

Lipid-monolayer covered NP



Lipid-bilayer covered NP



The functionalized lipid shell of hybrid nanoparticles plays an important role for improving their biocompatibility and *in vivo* stability. Yet few efforts have been made to critically examine the shell structure of nanoparticles and its effect on cell–particle interaction. Here we develop a microfluidic chip allowing for the synthesis of structurally well-defined lipid-polymer nanoparticles of the same sizes, but covered with either lipid-monolayer-shell (MPs, monolayer nanoparticles) or lipid-bilayer-shell (BPs, bilayer nanoparticles). Atomic force microscope and atomistic simulations reveal that MPs have a lower flexibility than BPs, resulting in a more efficient cellular uptake and thus anticancer effect than BPs do. This flexibility-regulated cell–particle interaction may have important implications for designing drug nanocarriers.

KEYWORDS: nanostructures · lipids · microfluidics · interfaces · drug deliver

Lipid-polymer hybrid nanoparticles (LNPs) loaded with drugs are emerging as sophisticated therapeutic agents.^{1–4} These NPs present high drug encapsulation efficiency, prolonged circulation half-life, high stability, and efficient cellular uptake.^{5–7} To fabricate these LNPs, two categories of approaches have been developed: (1) The two-step method by separately preparing the polymeric nanoparticles and aqueous lipid films/liposomes, followed by constructing core–shell nanoparticles through extrusion, sonication, or electrostatic interaction;^{8,9}

(2) the one-step method combining nanoprecipitation of polymeric nanoparticles and simultaneous self-assembly of the lipid shell.^{10,11} It is worth noting that the different fabrication strategies may result in the formation of multilamellar or unilamellar lipid shell covering the polymeric core.^{12,13} The different lipid structures may affect the physicochemical properties of LNPs. Yet few efforts have been made to critically examine the shell structure of nanoparticles and its effect on cell–particle interface or the efficacy in disease treatment. This might

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be due, to a large extent, to the lack of adequate techniques to precisely control the lipid shell structures of LNPs.

In our previous study,⁵ we synthesized two kinds of core-shell hybrid NPs with different rigidity by modulating the amount of interfacial water between polymeric core and lipid shell. The correlation between the rigidity of NPs and their cellular uptake efficiency has been investigated, with rigid NPs being easier to be internalized by cells than soft ones. In addition to the interfacial water inside NPs that regulates the cell-particle interface, we postulate that the distinct lipid shell structure of hybrid NPs may also mediate the particle flexibility and its efficiency in cancer therapy. To investigate the role of lipid shell, the accurate assembly of varying lipid layers onto the polymeric cores is a prerequisite, which might be difficult to achieve by traditional approaches.

In this work, *via* a specifically designed microfluidic chip, we realize the synthesis of controllable core-shell nanoparticles with polymer cores and lipid-monolayer-shells (MPs) or lipid-bilayer-shells (BPs). The difference in MPs and BPs is characterized by cryo-transmission electron microscope (Cryo-TEM),

fluorescence spectrum, and other methods. The distinct lipid shell structures may lead to a tunable flexibility of LNPs, which has been experimental and theoretically confirmed by atomic force microscope (AFM) experiments and molecular dynamics (MD) simulations. The flexibility-dependent *in vitro* and *in vivo* uptakes of MPs and BPs are investigated using a cell coculture model and *in vivo* imaging. The anti-cancer efficacy of dual drug-loaded MPs and BPs is evaluated for a comprehensive understanding of flexibility-regulated cell-nanoparticle interaction.

RESULTS AND DISCUSSION

Fabrication of MPs and BPs. To fabricate LNPs with controlled lipid shell structures, we adopt a two-stage microfluidic chip.^{5,14,15} The first stage of the chip consists of three inlets and one straight synthesis channel, while the second stage has one middle inlet and a spiral synthesis channel (Figure 1, details for design and fabrication of microfluidic chip in Materials and Methods). In mode A, we introduce poly(lactide-co-glycolide) (PLGA) solution into the first stage of chip to precipitate intermediate PLGA NP and inject lipid solution into the second stage to assemble lipid monolayer-

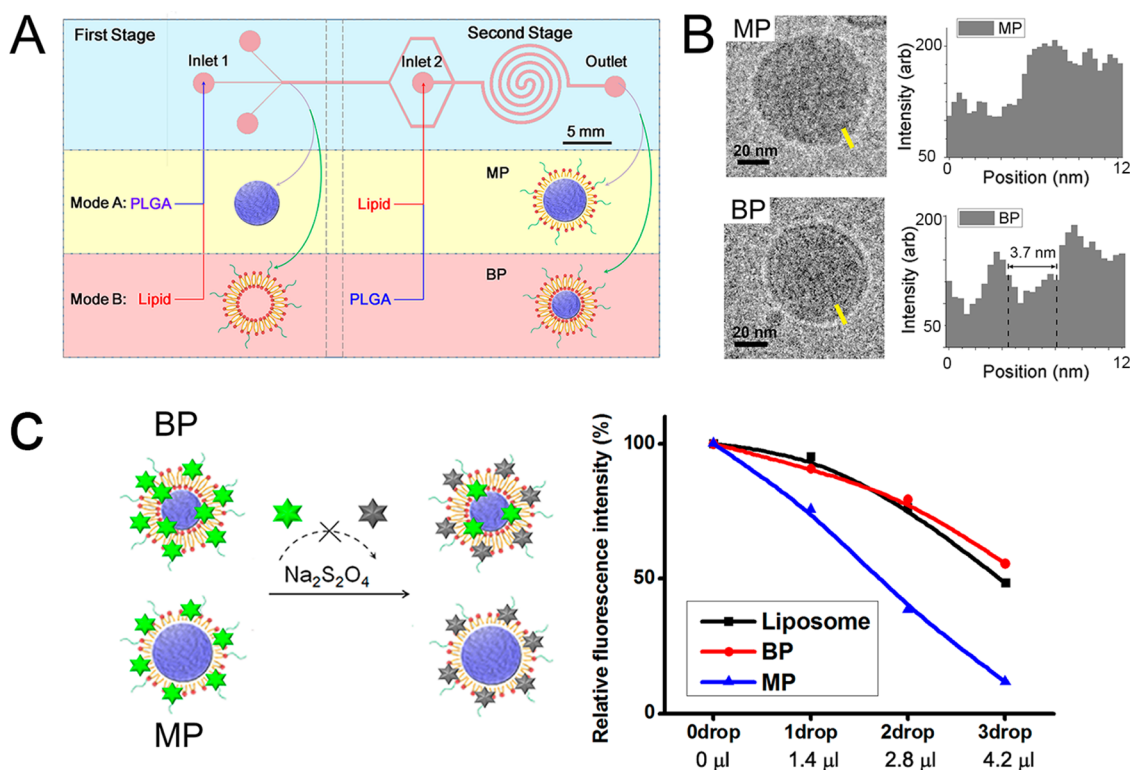


Figure 1. Schematic of the two-stage microfluidic chip and characterization of hybrid nanoparticles with varying lipid shells. (A) In mode A, we introduce PLGA solution into the first stage and inject lipid solution into the second stage to assemble lipid monolayer-covered PLGA nanoparticles (MPs). In mode B, we inject lipid solution into the first stage, and PLGA solution into the second stage to prepare lipid bilayer-covered PLGA nanoparticles (BPs). (B) Cryo-TEM images of MPs and BPs. The electron density across the lipid layer structure (indicated by the short yellow line) shows the lipid bilayer structure of BPs, which is 3.7 nm thick. (C) The reduction of fluorescence of NBD-DSPE labeled MPs, BPs, and liposomes caused by adding sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) dropwise to quench the NBD in the outer lipid layer (excitation wavelength 460 nm, emission wavelength 530 nm). Each drop corresponds to 1.4 μL of $\text{Na}_2\text{S}_2\text{O}_4$. Left: illustration to show the quenching mechanism; right: experimental measurements.

shell onto the surface of PLGA NP by hydrophobic attraction between lipid tail and PLGA (Figure 1 and Materials and Methods). In mode B, we generate an intermediate liposome by injecting lipid solution into the first stage, which can reassemble onto the PLGA NP when PLGA solution is injected into the second stage (mode B, Figure 1). We note that such assembly is assisted with the spiral structure at the second stage of microfluidics.¹⁶ From the synthesis process we may deduce that in mode A the LNPs are covered by lipid-monolayer-shell (named as MPs, monolayer nanoparticles), while in mode B, LNPs are coated by lipid-bilayer-shell (named as BPs, bilayer nanoparticles, Figure 1). To explore the practicality of process, we characterize the LNPs with Cryo-TEM and observe a structural difference of outer lipid shells between MPs and BPs (Figures 1 and S1). The electron density across the lipid layer structure shows that BP has a bilayer shell structure with the thickness of ~ 4 nm (Figures 1 and S1). We should note that due to the existence of interfacial water between PLGA core and lipid bilayer shell in BPs,⁵ the interaction between PLGA and lipid bilayer is relatively weak, so that we can observe a corona of lipid bilayer with a characteristic thickness of ~ 4 nm by Cryo-TEM.¹⁷ For MPs covered with lipid monolayer, the hydrophobic tails of lipids are embedded into the PLGA surface. In this case, the lipid monolayer cannot be distinguished from TEM due to the tight contact between the PLGA core and the hydrophobic tail of lipid (Figure 1).

Characterization of MPs and BPs by DLS. We next evaluate the amount of lipids for complete coverage on the PLGA core of MPs and BPs by dynamic light scattering (DLS). Previous studies and our experiments indicate that only LNPs completely covered by lipids are stable in both water and PBS, while PLGA nanoparticles or liposomes may experience aggregation in PBS, resulting in an increase in size (Figure S2, Materials and Methods).¹⁸ By varying the PLGA/lipid (w/w) ratio from 8 to 1, we observe a similar size distribution of MPs (mode A) in both water and PBS at a ratio of 4. Interestingly, to generate stable lipid-covered BPs in mold B, the PLGA/lipid ratio should be decreased to 2 (Figure S3). This result suggests that BPs require twice the amount of lipids compared to MPs for completely covering the PLGA cores, to form the lipid bilayer shell. We should note that to prepare pure PLGA nanoparticles *via* a microfluidics-based nanoprecipitation, we introduce the PLGA solution and two water sheaths into the first stage, without the injection of stabilizer into the second stage, resulting in a large size distribution of PLGA nanoparticles (Figure S2). To prepare lipid-coated core-shell PLGA nanoparticles, we use lipids as a stabilizer during the precipitation, leading to a smaller size distribution of lipid-covered PLGA nanoparticles (Figure S3), when compared with that of pure PLGA nanoparticles (Figure S2). The DLS results in

Figure S3 also indicate both MPs and BPs have similar sizes: the average diameter of MPs is 103.67 ± 8.51 nm and that of BPs is 106.43 ± 6.75 nm.

Characterization of MPs and BPs by Fluorescence Quenching.

We further adopt the fluorescence quenching method to characterize the lipid monolayer or bilayer shell.¹⁹ We synthesize the NBD-DSPE labeled BPs and MPs by adding NBD-DSPE labeled lipids (NBD-lipids) into the lipid solution. The final concentration of NBD-lipids in both MPs and BPs is $1.25 \mu\text{g/mL}$. Due to the lipid bilayer structure in BPs and liposomes, the amount of NBD-lipid at the outer lipid layer is $\sim 0.62 \mu\text{g/mL}$. This NBD quenching method is based on the fact that sodium dithionite reacts more rapidly with NBD in the outer lipid layer than that in the inner lipid layer. By controlling the reaction time and the amount of sodium dithionite that is added dropwise into the MPs, BPs, or liposomes, the fluorescence quenching reaction could be limited to the NBD-lipids in the outer lipid layer (details in Materials and Methods).²⁰ If an adequate amount of sodium dithionite is added, all NBD, both in the inner and outer lipid layers, could be quenched, and the fluorescence reduces to background. In our experiments, we add the sodium dithionite dropwise, and the fluorescence in MPs is quenched to a constant value after 3 drops due to the complete reaction of NBD at the outer lipid layer, while for the liposomes and BPs, the constant fluorescence value is obtained after 6 drops. Thus, we define the fluorescence after 6 drops as the background and compare the fluorescence values of MPs, BPs, and liposomes from 1 to 3 drops (Figure 1C). As expected, the fluorescence signal of MPs decreases to almost zero after 3 drops of sodium dithionite because all NBD-lipids from the exposed hydrophilic end are quenched, while the fluorescence of BPs and liposomes is quenched to around 50% after 3 drops of sodium dithionite as a result of the remaining NBD fluorescence from the inner lipid layer (Figures 1C and S4, Materials and Methods). The variation of fluorescence quenching of MPs and BPs proves the lipid monolayer shell of MPs and the lipid bilayer shell of BPs.

AFM Characterization of Flexibility Difference between MPs and BPs. One may speculate intuitively that BPs have high flexibility due to the existence of lipid bilayer shell, in contrast to MPs of lipid monolayer shell. To verify this hypothesis, the two kinds of nanoparticles are characterized by AFM-based nanomechanical mapping.^{21–23} Figure 2A,D is the typical topographies of MPs and BPs (more AFM images in Figure S5). Both MPs and BPs show the ball shape, the diameter of which is similar to Cryo-TEM observation. However, BPs have a higher deformation and energy dissipation than MPs (Figure 2B,C,E,F).²¹ The large particle deformation may be attributed to the high flexibility of BPs containing lipid bilayer shell, since previous study indicates that the self-assembled lipid bilayer structure could display fluidic and elastic

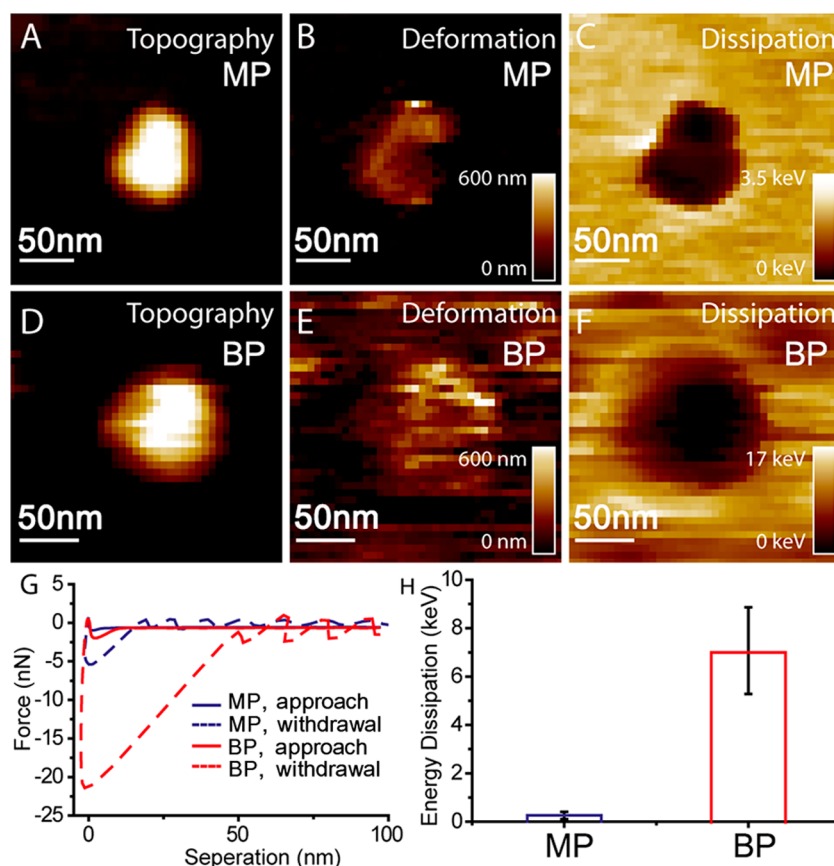


Figure 2. Quantitative nanoscale mechanical characterizations of MPs and BPs via AFM. (A) A typical topography of MPs. (B,C) The corresponding deformation and energy dissipation images of (A). (D) A typical topography of BPs. (E,F) The corresponding deformation and energy dissipation images of (D). (G) Typical approach and withdrawal force–distance curves recorded on the top of MPs (blue) and BPs (red), respectively. (H) The energy dissipated in MPs (blue) and BPs (red) during every tapping cycle by AFM tip ($P < 0.01$).

properties.²⁴ In addition to particle flexibility, we also discuss the mechanical variation of MPs and BPs from an energetic aspect. The energy dissipation refers to the energy which is dissipated from AFM tip into the NPs during every cycle as the AFM tip is approached and withdrawn from the NPs. The quantity of dissipated energy during one cycle is equal to the net area between the approach force–distance curve (FDC, solid line) and withdrawal FDC (dash line, Figure 2G). Measuring tens of NPs, the energy dissipation of MPs and BPs is summarized, showing that the energy dissipated in MPs is much smaller than the value recorded on BPs (Figure 2H). In this case, we propose that the huge difference of dissipated energy in MPs and BPs is related to the varying lipid structure and flexibility:²⁵ more flexible BPs result in a larger contact area at the AFM tip–particle interface than MPs, thus leading to a higher dissipation energy caused by the frictional force between the tip and the nanoparticle.

MD Simulations of Flexibility-Regulated Interaction. To verify the different energy dissipation of MPs and BPs in the AFM experiments, we also perform indentation by molecular dynamics (MD) simulations.^{26–28} The simulation system consists of NPs with a diameter of

40 nm, a rigid substrate, and a spherical indenter with a diameter of 20 nm. The simulation box size is $50 \times 75 \times 50$ nm with periodic boundary condition applied along x , y , and z directions. The PLGA core, the indenter, and the substrate are created by a fcc lattice with a Lennard-Jones reduced density of 1.414 (more details in Materials and Methods). Figure 3A,B shows the process when the indenter approaches and withdraws from the modeled MPs and BPs. Figure 3C,D records the forces as a function of indenter–NPs separation to simulate the interaction between NPs and AFM tips. When the loading force is 700 pN, the deformation of MPs is apparently smaller than BPs (see Movie S1 for MPs and Movie S2 for BPs), agreeing well with the AFM observation (Figure 2B,E). Moreover, the net area between the approach and withdrawal FDC is remarkably higher for BPs structure than that of MPs. We note that in the MD simulations we have selected a relatively small model system so that the dissipation energies do not agree with the experimental ones in their exact numbers. However, through the simulations, the mechanism of structure difference between MPs and NPs induced different dissipation energy, and the process of particle deformation during the interaction is clarified.

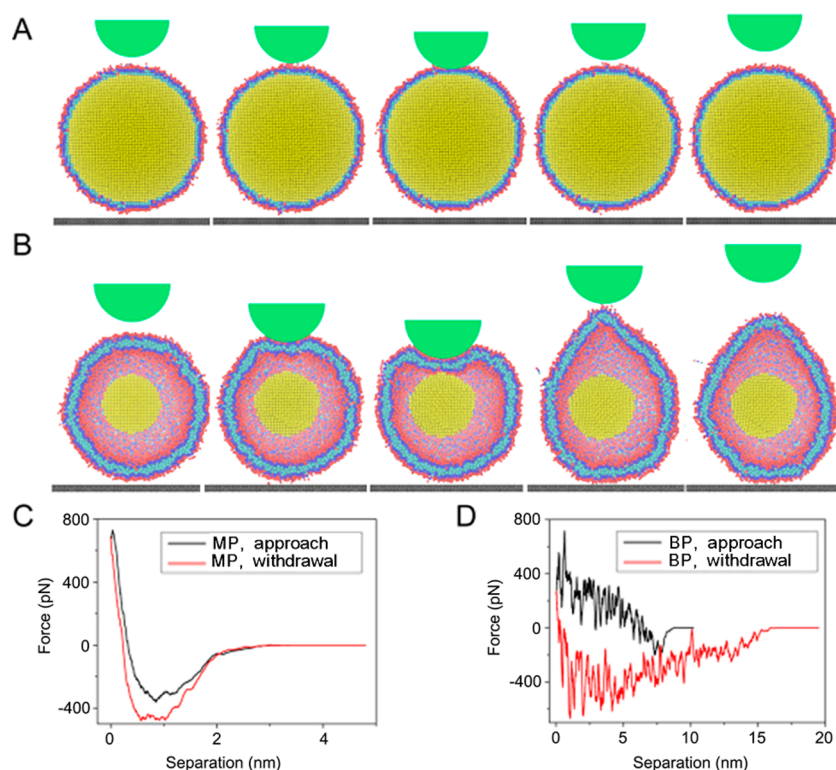


Figure 3. MD simulations to show indenter–nanoparticle interactions. (A) An indenter approaching and withdrawing from a MP. (B) The indenter approaching and withdrawing from a BP. (C,D) The force–displacement curves corresponding to (A) and (B), respectively.

Flexibility-Regulated *in Vitro* Uptake. To investigate how the flexibility of LNPs influences the capability in treating cancer, we fabricate hybrid dual drug-loaded MPs and BPs in which the PLGA core encapsulates doxorubicin (Dox, a drug used in cancer chemotherapy) and the lipid shell contains combretastatin A4 (CA4, a drug used to damage the vasculature of cancer tumors). In this work, we use hydrophobic Dox instead of the water-soluble doxorubicin hydrochlorate (Dox·HCl) for the following two considerations: (1) The high risk of cardiotoxicity by Dox·HCl treatment hinders its clinical application for cancer chemotherapy;²⁹ (2) the hydrophobic PLGA core exhibits a high encapsulation efficiency of hydrophobic Dox due to the hydrophobic interaction. The drug concentration and the entrapment efficiency are presented in Materials and Methods. We use a coculture chip to incubate HeLa (a type of cancer cell) and HUVEC (a type of blood capillary cell) with drug-loaded MPs or BPs,³⁰ allowing for a simultaneous observation of therapeutic effects. We choose this model because the inhibition of both cancer cells and blood capillary cells is important for the eradication of most types of solid tumors.³¹ This chip allows controlled adhesion of both HeLa and HUVEC cells to test their response to NPs (Materials and Methods). Confocal fluorescent observation of HeLa and HUVEC cells (stained with Hoechst33342) after treatment indicates that hybrid dual drug-loaded (Dox and CA4) MPs have a more significant cytotoxic

effect on HeLa and HUVEC cells than BPs and free drugs of the same concentrations (Figures 4A,B and S6, Table S2, and Materials and Methods). We speculate that BPs of high flexibility dissipate a larger energy at the LNPs–cell interface and are more difficult to be internalized by cancer cells than MPs. Our previous investigation also proves that MPs show an enhanced cellular uptake compared with BPs, albeit *via* the same internalization pathway.⁵ We should note that stiffer NPs have higher cellular uptake efficiency than softer ones; however, the similar structured core–shell microparticles with diameter of several micron show the opposite biological effect where softer microparticles are more efficient.³² This might be due to the distinct pathways of cellular uptake of particles with different sizes. In our case the NPs are internalized *via* endocytosis, despite the passive diffusion of microparticles. The detailed mechanisms for such contrasting results between particles at different size regimes warrant further investigation.

Flexibility-Regulated Anticancer Efficacy and *in Vivo* Imaging. We also perform *in vivo* investigation into the effects of drug-loaded MPs or BPs for tumor growth inhibition in a cervical carcinoma xenograft model. LNPs loaded with two drugs (Dox: 0.129 mg/kg, CA4:0.741 mg/kg) efficiently slow down the tumor growth. Meanwhile, mice treated with drug-loaded MPs have much smaller tumors than those with BPs (Figures 4C, S7, and S8). In comparison, LNPs without drugs (MPs Blank, and BPs

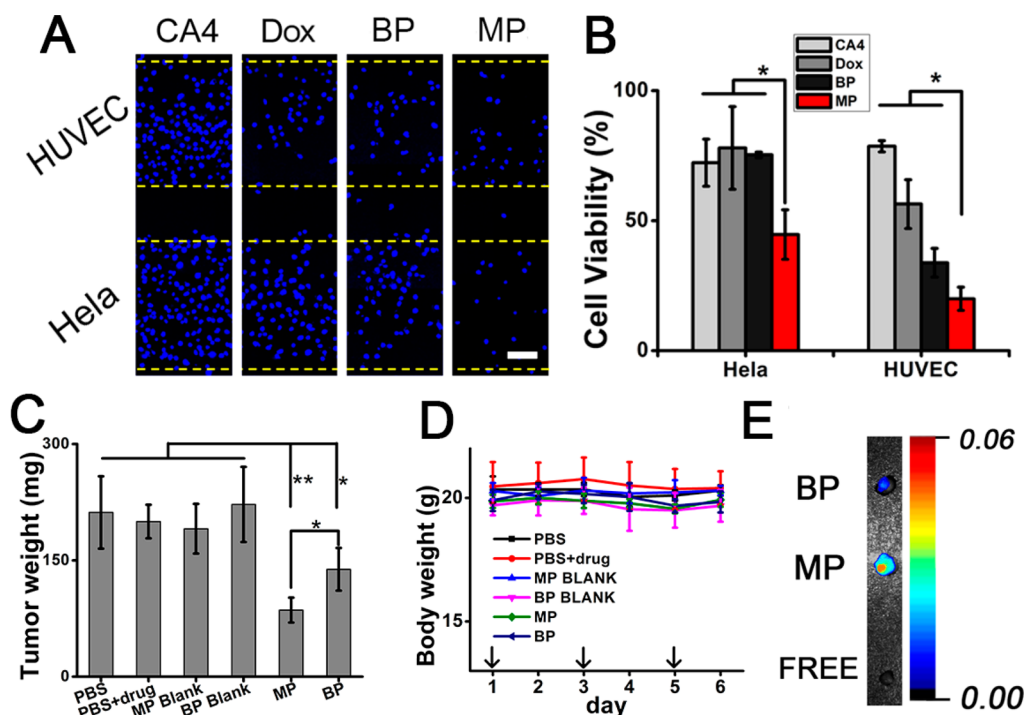


Figure 4. Treatment of cancer models by drug-loaded MPs and BPs. (A) Confocal fluorescent images of HeLa and HUVEC cells stained with Hoechst33342 and incubated with the CA4, Dox, dual drug-loaded (Dox and CA4) BPs and MPs, scale bar 100 μ m. (B) Statistical result of the cell viability after being treated by MPs, BPs, and free drugs. (C) Weight of the excised tumors in different treatment groups. (* $P < 0.05$; ** $P < 0.01$). (D) Body weight of the mice. There is no significant weight loss of mice in all groups. (E) Fluorescence imaging of *ex vivo* tumors. DiR (2.5 μ g/kg) labeled MPs and BPs are intravenously administered to mice *via* the tail vein, and the tumors are excised after 16 h treatment. FREE is the free DiR at 2.5 μ g/kg.

Blank), the PBS plus free drugs of the same amount, and PBS control cannot inhibit tumor growth (Figure 4C, Materials and Methods). The PBS plus free drugs are not effective in suppressing the tumor growth because we chose a relatively low concentration of drugs to reduce the toxicity of the drug and the potential side-effects of treatment. In comparison, the hybrid nanoparticles, especially MPs, exhibit an efficient anticancer therapy due to the enhanced cellular uptake. All treatments do not significantly affect the weight of the mice, indicating that all LNPs have similarly low levels of toxicity (Figure 4D). Drug-loaded MPs are thus the most effective in treating cancer in our mouse model. We also use the *in vivo* imaging technology to investigate the biodistribution of the DiR labeled MPs and BPs administered to mice *via* the tail vein (Materials and Methods).^{33–35} *In vivo* imaging shows that MPs tend to accumulate in tumor sites more rapidly than BPs (Figure S9). The comparison of *ex vivo* tumors after 16 h treatment indicates a higher uptake amount of MPs than BPs (Figure 4E).

Biocompatibility of MPs and BPs. To examine whether this mono/bilayer lipid coating will affect the cell membrane, we first use the membrane staining method to investigate the interaction between the lipid-coated NPs and the cell membrane. After co-incubation of HUVEC cells with MPs or BPs, HUVEC cells are stained with DiD (1 μ M, red fluorescence) to label the cell

membrane and Calcein-AM (2 μ M, green fluorescence) to label the cytoplasm. The confocal microscopic images show that HUVEC cells incubated with MPs or BPs maintain integrated morphology of cell membrane and cytoplasm, the result of which is similar to HUVEC cells under standard culture conditions (Figure 5A–C). We further perform the hemolysis assay to prove that the lipid coating may not rupture the cell membrane. After incubating drug-loaded (0.4 μ g/mL of Dox, and 1.1 μ g/mL of CA4) MPs or BPs or blank MPs and BPs in red blood cells (RBCs) solution, we measure the released hemoglobin from RBCs, which serves as the indicator of membrane disruption. The measurement of hemoglobin shows that neither MPs nor BPs can induce hemolysis (Figure 5D). Thus, we can conclude that the lipid coating of NPs does not affect the integrity of cell membrane.

In vivo biocompatibility of lipid-coated NPs is investigated by alanine transaminase (ALT) assay and histopathological staining. As lipid-coated NPs are prone to accumulate in the liver, we first measure the ALT level (a clinical biomarker for liver health) in the serum after Balb/c nude mice are administrated with PBS, free drug, drug-loaded MPs or BPs, blank MPs or BPs for 1 week. No significant change in ALT level is observed in all groups after treatment (Figure 5E), indicating that our lipid-coated core–shell NPs have negligible liver toxicity. We further evaluate the toxicity

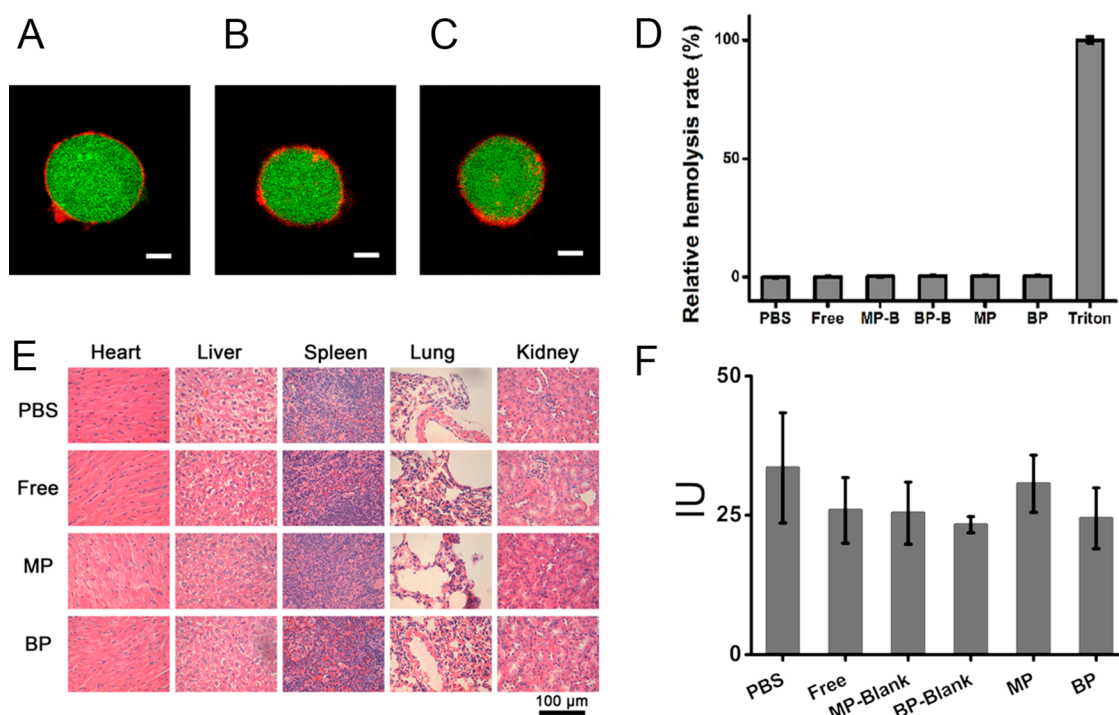


Figure 5. (A) Confocal fluorescence image of DiD-labeled cell membrane (red) and Calcein-AM-labeled cytoplasm (green) of HUVEC cells under standard culture conditions. (B) Confocal fluorescence images of stained HUVEC cells after co-incubation with MPs or (C) BPs. The scale bar is 5 μm . (D) Hemolysis assay showing that both MPs and BPs will not induce the rupture of cell membrane. (E) H&E staining of sections from major organs of mice after different treatments. (F) ALT assay of the serum samples from mice treated by PBS, free drug (Dox and CA4), blank MPs or BPs, and drug-loaded MPs or BPs. MP-B or BP-B represents blank MPs or BPs, and MP or BP represents drug-loaded MPs or BPs.

of drug-loaded MPs or BPs to major organs including heart, liver, spleen, lung, and kidney by hematoxylin and eosin (H&E) staining method. No abnormal pathology or noticeable organ damage caused by drug-loaded MPs or BPs is observed (Figure 5F). From the above two experiments, we can conclude that both MPs and BPs show good *in vivo* biocompatibility.

CONCLUSIONS

In conclusion, we have synthesized LNPs with either monolayer or bilayer of lipid shells (MPs and BPs) by a two-stage microfluidic chip. MPs and BPs show a clear variation in flexibility and energy dissipation, which is examined by AFM and also validated by atomistic

simulations. The low flexibility of MPs (lipid monolayer shell) might be responsible for efficient cellular uptake at the cell–particle interface, compared with BPs (lipid bilayer shell) of high flexibility. Both *in vitro* and *in vivo* experiments show that dual drug-loaded MPs exhibit a more significant anticancer effect than BPs, due to this flexibility-regulated cell–particle interaction. This work suggests that the difference in lipid shells of fine-structured LNPs might lead to a considerable variation of cell–nanoparticle interaction and the consequent therapeutic effects for cancer treatment. The underlying mechanism of flexibility-mediated interaction may open a new window in rational design of drug nanocarriers and other materials used to target the cell.

MATERIALS AND METHODS

Materials. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DSPE) were purchased from Avanti. Poly(*D,L*-lactide-co-glycolide) (PLGA, lactide:glycolide = 75:25) was purchased from SurModics. Doxorubicin (Dox), combretastatin A4 (CA4), sodium dithionite, phosphotungstic acid, ethanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and trifluoroethanol (TFE) were purchased from Sigma. Hoechst 33342 was purchased from Invitrogen. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and 10 $\times$ phosphate buffered saline (PBS) were purchased from Gibco.

Design and Fabrication of the Microfluidic Chip. The microfluidic chip consists of two stages: (1) the first stage has three inlets and a straight synthesis microchannel; (2) the second stage has one centered inlet and a spiral mixing channel (Figure S1). The three inlet channels at the first stage are 100 μm in width and 50 μm in depth. The main horizontal channel at the first stage is 300 μm in width and 50 μm in depth. The inlet channel at the second stage is 300 μm in width and 50 μm in depth. The double spiral channel at the second channel is 300 μm in width and 50 μm in depth, which first rotates in the counterclockwise direction for 3 loops, changes the direction through an S-shaped junction, and then rotates in the clockwise direction.

The microfluidic device was fabricated using standard soft lithography. SU-8 2050 photoresist (MicroChem Corp) was first spin-coated on a 4 in. silicon wafer at 500 rpm for 10 s followed

by 3000 rpm for 30 s to obtain the desired thickness (50 μm). The coated wafer was soft-baked for 1 h at 95 $^{\circ}\text{C}$ and then exposed to ultraviolet light with exposure energy of 150 mJ/cm^2 through a photomask containing the pattern of microfluidic channels. After exposure, the wafer was baked again at 95 $^{\circ}\text{C}$ for 25 min and soaked in SU-8 developer (MicroChem Corp) to dissolve the unexposed photoresist. The patterned SU-8 master mold was then hard-baked at 150 $^{\circ}\text{C}$ for 15 min to anneal microcracks in SU-8.

Degassed PDMS (mixed in a 10:1 ratio of PDMS base with curing agent, Sylgard 184, Dow Corning Inc.) was coated over the mold and baked at 80 $^{\circ}\text{C}$ for 1 h in an oven. The PDMS with embedded channels was bonded with glass substrate after oxygen plasma treatment. Five plastic tubes were inserted through the inlet and outlet ports and secured by a small amount of adhesive sealant (Dow Corning 3145 RTV). The assembled device was finally placed into an oven at 70 $^{\circ}\text{C}$ for 30 min.

Nanoprecipitation of Hybrid Nanoparticles. 100 mg PLGA was dissolved in 6.5 mL DMF and 3.5 mL TFE to prepare 1% PLGA solution (10 mg/mL). Lipid solution was composed of DPPC (4.55 mg/mL), DSPE-PEG (0.85 mg/mL), and cholesterol (0.48 mg/mL) with a molar ratio of 80:4:16. For generation of LNPs with lipid-monolayer-shell (MPs), the ratio of PLGA to lipid solution is 4, whereas the PLGA/lipid ratio is 2 for preparation of LNPs with lipid-bilayer-shell (BPs).

To prepare LNPs with lipid-monolayer-shell (MPs), we employed syringe pumps (PHD Ultra, Harvard Apparatus) to introduce the PLGA solution (0.5 mL/h) and two water sheaths (20 mL/h each) through the middle inlet and two side inlets of the first stage, while simultaneously injecting the lipid solution (0.5 mL/h) via the centered inlet of the second stage (mode A). To generate LNPs with lipid-bilayer-shell (BPs), we change the injection order by introducing the lipid solution (0.5 mL/h) at the first stage and the PLGA solution (0.5 mL/h) at the second stage, while keeping the water sheath at 20 mL/h each (mode B).

Transmission Electron Microscopy. Cryo-transmission electron microscope (Cryo-TEM, Tecnai 12 electron microscope) was used to differentiate the structure of lipid layers of MPs and BPs. The sample for Cryo-TEM was prepared by placing the LNPs onto a carbon-coated copper grid and freezing the sample in liquid ethane. The Cryo-TEM observation was carried out at ~ -170 $^{\circ}\text{C}$ with an acceleration voltage of 200 kV.

Dynamic Light Scattering. The size distribution of LNPs were studied using DLS (Zetasizer 3000HS, Malvern Instruments Ltd.). One mL LNPs suspension was put in a DLS cuvette and measured with detection optics arranged at 173 $^{\circ}$. Three measurements were performed on each sample. Only LNPs completely covered by lipids are stable in both water and PBS, while PLGA NPs or liposomes by themselves may experience aggregation in PBS, resulting in an increase in size shift (Figure S2). By varying the PLGA/lipid ratio from 8 to 1, we observe a homogeneous size distribution of MPs (mode A) in both water and PBS at a ratio of 4 (Figure S3). A higher or lower ratio leads to a small shift of peak position in PBS versus water due to the aggregation of excess PLGA NPs or liposomes in PBS. In comparison, we need to decrease the PLGA/lipid ratio to 2 to prevent aggregation of BPs (mode B) in PBS (Figure S3).

Fluorescence Quenching. The fluorescence spectra of NBD-DSPE-labeled nanoparticles were detected with LS55 luminescence spectroscopy (PerkinElmer) from 500 to 700 nm with the excitation at 460 nm. We synthesized the NBD-labeled BPs and MPs by adding NBD-lipid to the lipid solution. The final concentration of NBD-lipid in both MPs and BPs is 1.25 $\mu\text{g}/\text{mL}$. Due to the lipid bilayer structure in BPs and liposomes, the amount of NBD-lipid at the outer lipid layer is ~ 0.62 $\mu\text{g}/\text{mL}$. In comparison, this value is 1.25 $\mu\text{g}/\text{mL}$ for MPs due to the lipid monolayer structure.

This NBD quenching method is based on the fact that sodium dithionite reacts more rapidly with NBD in the outer lipid layer than that in the inner lipid layer. By controlling the reaction time and amount of sodium dithionite, the reaction could be limited to NBD in the outer lipid layer. We added the sodium dithionite dropwise (1.4 μL per drop, 200 mM, pH 10) to

400 μL nanoparticle suspensions, and the fluorescence spectra were continuously detected until the reduction of fluorescence was stable (Figure S4). The fluorescence in MPs was quenched to a constant value after 3 drops due to the complete reaction of NBD at the outer lipid layer, while for the liposomes and BPs, the constant fluorescence value was obtained after 6 drops. The reduction of fluorescent intensity at 530 nm with increasing amount of sodium dithionite was compared to determine the structure of the lipid layer. Thus, we defined the fluorescence after 6 drops as the background and compared the fluorescence values of MPs, BPs, and liposomes from 1 to 3 drops (Figure 1C). The rate of chemical reaction was determined by the kinetics equation ($v = k \times c[\text{NBD}] \times c[\text{Sodium dithionite}]$), indicating that the reaction rate for MPs was faster than that of BPs and liposomes due to the higher NBD concentration at the outer lipid layer.

Atomic Force Microscope (AFM). The solution of MPs or BPs was dropped onto the surface of mica and air-dried at room temperature (25% humidity) for 1 h. The samples were characterized with Multimode VIII AFM (Bruker, CA) in PeakForce QNM mode. ScanAsyst-Air probe was utilized in the experiments, and the deflection sensitivity, tip radius, and spring constant of each probe were calibrated according to the released notes from Bruker. All images were processed and analyzed by the software Nanoscope Analysis (Bruker, CA).

MD Simulation. The simulation system consists of NPs with a diameter of 40 nm, a rigid substrate, and a spherical indenter with a diameter of 20 nm. The simulation box size is $50 \times 75 \times 50$ nm with periodic boundary condition applied along x , y , and z directions. Solvent-free coarse-grained (CG) lipids model developed by Deserno *et al.* is adopted.^{36,37} The PLGA core, the indenter, and the substrate are created by a fcc lattice with a Lennard-Jones reduced density of 1.414. The following potentials are used in the simulations:

$$U_{\text{LJ}}(r) = 4\alpha\epsilon \left[\left(\frac{b}{r} \right)^{12} - \left(\frac{b}{r} \right)^6 \right] \quad (0 < r < r_{\text{cut}} = 2.5b)$$

$$U_{\text{WCA}}(r) = 4\epsilon \left[\left(\frac{b}{r} \right)^{12} - \left(\frac{b}{r} \right)^6 + \frac{1}{4} \right] \quad (0 < r < r_{\text{cut}} = 2^{1/6}b)$$

$$U_{\text{cos}}(r) = \begin{cases} \beta[\epsilon + U_{\text{WCA}}(r)], & (r < r_{\text{cut}} = 2^{1/6}b) \\ -\beta\epsilon \cos \frac{\pi(r - r_{\text{cut}})}{2w}, & (r_{\text{cut}} < r < r_{\text{cut}} + w) \end{cases}$$

$$U_{\text{FENE}}(r) = -\frac{1}{2}k_{\text{FENE}}r_{\infty}^2 \ln \left(1 - \frac{r^2}{r_{\infty}^2} \right) \quad (0 < r < r_{\infty})$$

In the following, ϵ and σ are used as the units of energy and length. Compared to a typical membrane thickness of 5 nm, the bead diameter, σ , is set at 1 nm. The parameter ϵ can be deduced from the temperature, which is set as $k_{\text{B}}T = 1.1\epsilon$. There are one head bead and two tail beads in one lipid. The neighboring beads are connected by FENE bond with $k_{\text{FENE}} = 30\epsilon$ and $r_{\infty} = 1.5\sigma$, the head bead and the second tail bead are also connected by a harmonic spring with rest length $r_0 = 4\sigma$, and force constant $k_{\text{bend}} = 10\epsilon$. The nonbonded interactions are shown in Table S1.

We use a velocity-Verlet algorithm to perform time integration and Langevin thermostat to control the system temperature at $k_{\text{B}}T = 1.1\epsilon$, where k_{B} is the Boltzmann constant and T is the temperature. The integration time step is $\delta t = 0.006\tau$, where $\tau = (m\sigma^2/\epsilon)^{1/2}$ is the units of time and τ is about 10 ns. We set the velocity of the indenter as $0.05\sigma/\tau$ (5×10^{-3} m/s) to simulate the indentation of NPs. When the force on the indenter by NPs is about 700 pN, we set the indenter with an opposite velocity of $-0.05\sigma/\tau$ to retract the indenter. The simulations are implemented with LAMMPS package.³⁸

Encapsulation Efficiency of Dox and CA4 by NPs. To measure the encapsulation efficiency of Dox and CA4 by NPs, the drug-loaded MPs and BPs are filtered by Amicon Ultra-4 ultrafilter (MWCO = 15k, Millipore, USA) after synthesis by microfluidics. The concentration of CA4 in the diffusate is measured by HPLC

using a C18 column (5 mm 4.6×250 mm, Phenomenex, USA).⁵ The concentration of Dox in the diffusate is determined using an Infinite M200 microplate reader (Tecan) with the excitation wavelength at 490 nm and emission wavelength at 590 nm.⁵ Given the initial drug concentration of 1.22 $\mu\text{g/mL}$ for CA4 (in lipid solution) and 0.4 $\mu\text{g/mL}$ for Dox (in PLGA solution), we can determine that the amounts of CA4 loaded in the shell of MPs and BPs are 1.108 and 1.162 $\mu\text{g/mL}$, and the amounts of Dox loaded into the core of MPs and BPs are 0.392 and 0.389 $\mu\text{g/mL}$. The entrapment efficiencies of Dox and CA4 in MPs and BPs are higher than 90%.

Cell Culture in a Microfluidic Chip. A human cervical carcinoma cell line (HeLa) and a human umbilical vein endothelial cell line (HUVEC) were grown in DMEM with 10% FBS, and 1% penicillin/streptomycin at 37 °C, 5% CO₂.

A two-channel culture chip was reversibly bonded with a cell culture dish, and HeLa/HUVEC cells were seeded into the different channels. After a 12 h incubation, we removed the chip and treated both types of cells with drug-loaded MPs or BPs for 20 h. Cells were stained with Hoechst 33342 and then incubated with free Dox, free CA4, dual-drug MPs and BPs. The concentrations of free CA4 and Dox are 1.22 and 0.4 $\mu\text{g/mL}$.

The numbers of adhered HeLa/HUVEC cells before and after incubation with different MPs, BPs, and free drugs were determined using a confocal microscope (LSM710, Zeiss) and Image-Pro Plus software (Media Cybernetics). The ratio of the number of cells after incubation to that before treatment was the cell viability (%). The viabilities (%) of HeLa and HUVEC cells incubated with free CA4, free Dox, dual-drug MPs and BPs for 3 and 20 h are listed in Table S2.

In Vivo Tumor Growth Inhibition Study. Female BALB/c nude mice (6–8 weeks old) were purchased from Vital River Laboratory Animal Center. A dose of 2×10^7 HeLa cells was inoculated subcutaneously in the right flank of BALB/c nude mouse to establish the xenografted cervical carcinoma model. When the tumor volume was above 50 mm³, mice were randomly divided into 6 groups (5 animals per group): (1) PBS, (2) PBS + drugs, (3) MPs without drugs (MPs Blank), (4) BPs without drugs (BPs Blank), (5) MPs NPs encapsulating drugs, and (6) BPs NPs encapsulating drugs. The dose of CA4 was 0.741 mg/kg and that of Dox was 0.129 mg/kg. Each dose was given every other day (total for 3 doses) by tail vein injection. The day before the first dose was specified as day 0. A vernier caliper was used to measure the longest (L) and shortest (S) diameters. Tumor size was calculated according to the formula of $V = 0.5LS^2$. Tumor size and body weight of each mouse were measured daily. One day after the last injection, the animals were sacrificed to harvest tumor tissues. All care and handling of animals was performed with the approval of Institutional Authority for Laboratory Animal Care of Institute of Process Engineering, Chinese Academy of Science.

In Figures 4, S7, and S8, the PBS + drug was not effective in suppressing the tumor growth because we chose a relatively low concentration of drugs (0.129 mg/kg for Dox and 0.741 mg/kg for CA4) to reduce the toxicity of the drug and the potential side-effects of treatment. We should note that the concentration of encapsulated drugs inside hybrid nanoparticles is the same as that of free drugs; however, the hybrid nanoparticles, especially monolayer-coated nanoparticles (MPs), exhibit an efficient anticancer therapy due to the enhanced cellular uptake.

For drug-loaded PLGA NPs, there are three key factors to ensure their therapeutic effects: (1) The size of PLGA NPs. NPs smaller than 200 nm in diameter display an improved enhanced permeability and retention (EPR) effect to make the NPs highly target to tumor tissues.³⁹ (2) The surface modification of PLGA NPs. The hydrophobic PLGA NPs are more likely to be aggregated in serum and exhibit a low cellular uptake efficacy. To lipid-PEG-coated PLGA NPs show much better long-term stability than the hydrophobic PLGA NPs.⁵ (3) The degradation of PLGA NPs. Both previous studies and our own investigations reveal that in an acidic environment (the intracellular pH of tumor cells, especially in lysosomes, is generally below 5), the PLGA NPs could degrade rapidly and release the encapsulated drug to achieve an enhanced antitumor efficiency.^{1,40} Since the NPs in our work are rationally designed to fulfill the above requirements, they could

show much better cell inhibition performance than free drug and lead to an enhanced cancer therapy.

In Vivo Imaging. *In vivo* imaging system was used to observe the real-time distribution and accumulation of nanoparticles in the female BALB/c mice (18–20 g) xenograft HeLa tumor model. A 2×10^7 HeLa cell was inoculated into the right flank of mice. When tumor size reached 50 mm³, the mice were administered with DiR-labeled nanoparticles (DiR = 2.5 $\mu\text{g/kg}$) via the tail vein, and the fluorescence distribution was monitored at determined time points (16 and 30 h) using a CRI Maestro 2 *in vivo* imaging system (CRI Maestro 2, USA) with appropriate wavelength (NIR mode). The tumors were excised and imaged after 16 h treatment. All images were normalized and analyzed using the imaging station CRI Maestro software.

MPs tend to accumulate in tumor tissue more rapidly than BPs, indicated by the higher fluorescence signal at the tumor site from MPs after 16 h treatment. If the treatment time is prolonged to 30 h, the accumulation of MPs and BPs at the tumor site is almost the same (Figure S9).

Cell Membrane Staining. The HUVEC cells were incubated with MPs or BPs at the same concentration (PLGA of 6.25 $\mu\text{g/mL}$, and lipid of 3.68 $\mu\text{g/mL}$) for 1 h. After incubation, HUVEC cells were washed twice with PBS and stained with DiD (1 μM , red fluorescence, to label the cell membrane), and Calcein-AM (2 μM , green fluorescence, to label the cytoplasm) for 30 min, and observed using a confocal microscope (LSM710, Zeiss). The exciting wavelengths are 488 nm (for Calcein) and 633 nm (for DiD).

Hemolysis Assay. RBCs were resuspended with prechilled PBS solution to obtain the 2% (v/v) RBC solution. 180 μL of the RBC solution was seeded into each well of the 96-well plate, and 20 μL of PBS, Triton X-100 (1%, v/v), drug-loaded (0.4 $\mu\text{g/mL}$ of Dox, and 1.1 $\mu\text{g/mL}$ of CA4) MPs and BPs, and blank MPs and BPs solutions was added. The 96-well plate was incubated for 1 h at 37 °C. After incubation, the intact RBCs were removed by centrifugation, and the released hemoglobin in the supernatant was measured by detecting the absorbance at 540 nm.

In Vivo Biocompatibility of NPs. Balb/c nude mice were administered with PBS, free drug (Dox: 0.129 mg/kg, CA4: 0.741 mg/kg), drug-loaded (Dox and CA4 with the same concentration as the free drug) MPs or BPs, blank MPs or BPs for three times every other day. One day after the last dose, the mice were sacrificed, and the serum samples were collected for the measurement of alanine transaminase (ALT) using an ALT Assay Kit (Nanjing Jiancheng Bioengineering Institute). To further investigate the toxicity of drug-loaded MPs or BPs, major organs from the same mice were harvested. We fixed the heart, liver, spleen, lung, and kidney by formalin and embedded the organs in paraffin before section. The sections were mounted on glass slides and stained using the H&E method for histological analysis. The stained sections were observed by light microscopy (Leica DM4000M).

Conflict of Interest: The authors declare no competing financial interest.

Supporting Information Available: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nano.5b05792.

Details about the characterizations of core-shell nanoparticles covered with lipid-monolayer-shell (MPs) or lipid-bilayer-shell of lipid (BPs), and their therapeutic effects (PDF)
Deformation of MPs (AVI)
Deformation of BPs (AVI)

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