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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Glutathione-Triggered "Off–On" Release of Anticancer Drugs from Dendrimer-Encapsulated Gold Nanoparticles

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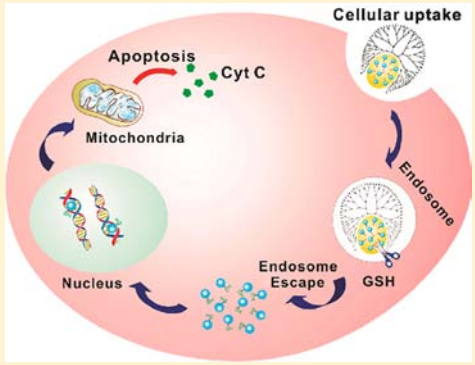
Supporting Information

ABSTRACT: Polymeric nanoparticles that can stably load anticancer drugs and release them in response to a specific trigger such as glutathione are of great interest in cancer therapy. In the present study, dendrimer-encapsulated gold nanoparticles (DEGNPs) were synthesized and used as carriers of thiolated anticancer drugs. Thiol-containing drugs such as captopril and 6-mercaptopurine loaded within DEGNPs showed an "Off–On" release behavior in the presence of thiol-reducing agents such as glutathione and dithiothreitol. Thiolated doxorubicin and cisplatin, loaded within the nanoparticle, showed much reduced cytotoxicity as compared to the free anticancer compounds. The toxicity of drug-loaded DEGNPs can be enhanced by improving the intracellular glutathione. Glutathione-triggered release of thiolated doxorubicin within cancer cells is further confirmed by flow cytometry and confocal laser scan microscopy studies. In addition, DEGNPs showed excellent biocompatibility on several cell lines. This study provides a new insight into biomedical applications of dendrimers and dendrimer-encapsulated nanoparticles.



INTRODUCTION

Dendrimers are one of the most promising and versatile drug carrier candidates which meet the need of ideal nanovehicles.[1] Drugs delivered by dendrimers showed several advantages as compared to free drugs, such as improved solubility, stability, therapeutic efficacy, and biocompatibility.[2] Despite these promising benefits, the release of drugs from the dendrimer matrix in a controlled fashion still present a challenge. Stimuli-responsive dendrimer-drug formulations have great potential to solve this problem due to their unique response to specific triggers.[3] Examples of the most widely used intracellular or extracellular stimuli in the design of stimuli-responsive dendrimers include pH, redox potential, light, temperature, and enzyme.[2a,3b,4] These smart drug delivery systems are designed to release their payloads at targeted organelles or tissues for in vitro and in vivo applications, respectively.[3a]

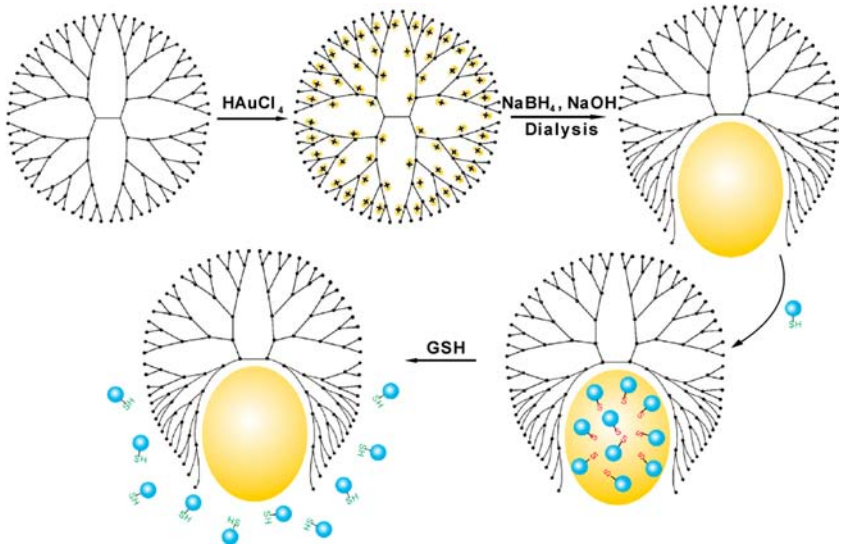
Gold nanoparticles have gained increasing interests in medicine and biology due to their unique optical and electronic properties and good biocompatibility.[5] They can act as vehicles of anticancer compounds and improve the therapeutic efficacy of the payload.[6] Doxorubicin (DOX)-loaded gold nanoparticles exhibited enhanced cytotoxicity of the anticancer drug on DOX-resistant cancer cells.[7] Gold nanoparticles can be easily functionalized with drugs and imaging agents through the Au–S bond.[5e,8] The formed Au–S bond is stable in physiological conditions, and the bound thiol-containing compound on gold nanoparticle surface can be replaced by other thiols.[9] Glutathione (GSH) is an abundant nonprotein thiol in the cytoplasm of live cells, often found in millimolar range. It is responsive for releasing thiol-containing payloads bound on gold nanoparticles.[10] However, the in vivo instability and short blood circulation time of monolayer protected gold nanoparticles,[11] and the difficulty in multifunctional modification on the nanoparticle surface still limits their applications in clinical cancer therapy.

Dendrimer-encapsulated gold nanoparticles (DEGNPs) developed by Crooks et al. showed excellent biocompatibility and were used as contrast agents for computed tomography imaging.[12] DEGNPs are much more stable than gold nanoparticles due to the protection of the gold nanoparticle surface by the dendrimer.[13] Also, the dendrimers can be easily modified with targeting, imaging, and solubilizing ligands before the synthesis of gold nanoparticle.[2b,c] Since there are plenty of pockets remaining in DEGNPs, these nanoparticles can be loaded with thiolated compounds via the Au–S bond. Such a GSH-responsive drug delivery system can be realized without involving sophisticated synthesis. Though only a few drugs contain a thiol group, the functional groups on anticancer drugs such as amine, hydroxyl, and carboxyl can easily be converted

Received: March 22, 2013
Published: June 21, 2013

   dx.doi.org/10.1021/ja402903h | J. Am. Chem. Soc. 2013, 135, 9805–9810

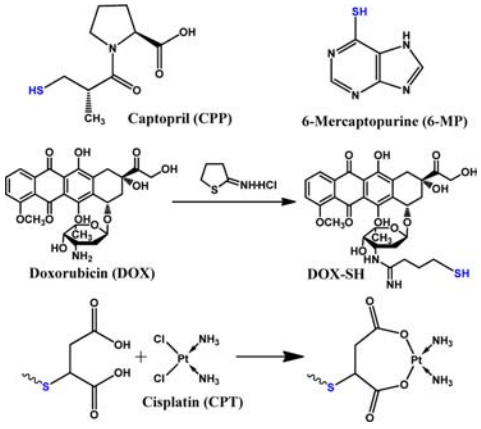
Scheme 1. Synthesis Route of DEGNPS and Their Applications As Scaffolds for the Design of GSH-Responsive Drug Delivery Systems



to the thiol group. Therefore, DEGNPs can be used as scaffold for a variety of anticancer drugs.

In the present study, we developed a novel GSH-triggered drug delivery system based on DEGNPs (Scheme 1). Thiol-containing drugs such as captopril (CPP), 6-mercaptopurine (6-MP), and thiolated anticancer drugs such as DOX and cisplatin (CPT) were used as model drugs (Chart 1). The GSH-triggered release behavior of DEGNPs was evaluated both in vitro and in live cells. To the best of our knowledge, this is the first report of using DEGNPs as a promising scaffold to develop stimuli-responsive drug delivery systems. The study also provides a new strategy to covalently bind bioactive molecules to dendrimer interiors through the Au−S linkage.

Chart 1. Chemical Structures of Thiol-Containing and Thiolated Drugs Used As Model Drugs in This Study



RESULTS AND DISCUSSION

Characterization of the DEGNPs and Drug-Loaded DEGNPs. DEGNPs were synthesized according to the well-established method reported by Crooks et al.[12a,c,14] Hydroxyl-terminated generation 3, 4, and 5 (G3-OH, G4-OH, and G5-OH) polyamidoamine (PAMAM) dendrimers were used as templates in the synthesis of gold nanoparticles. As shown in Figure 1, gold nanoparticles synthesized within G3-OH, G4-OH, and G5-OH dendrimers (G3-OH/Au NPs, G4-OH/Au NPs, G5-OH/Au NPs) are globular-shaped and have an average size around 3 nm. This is in accord with previous results wherein gold nanoparticles synthesized using low-generation dendrimers as templates have a larger size than the theoretical size.[15] The UV−vis spectra of the synthesized DEGNPs in Figure 1d further confirmed the formation of DEGNPs.[16]

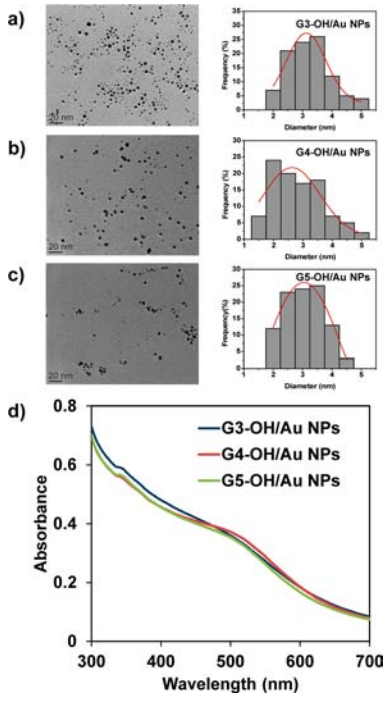


Figure 1. TEM images and size distributions of the synthesized G3-OH/Au NPs (a), G4-OH/Au NPs (b), and G5-OH/Au NPs (c). (d) UV−vis spectra of the synthesized DEGNPs.

The synthesized DEGNPs were further characterized by ^{1}H NMR. As shown in Figure 2, the resonance signals of G5-OH

dx.doi.org/10.1021/ja402903h | J. Am. Chem. Soc. 2013, 135, 9805−9810

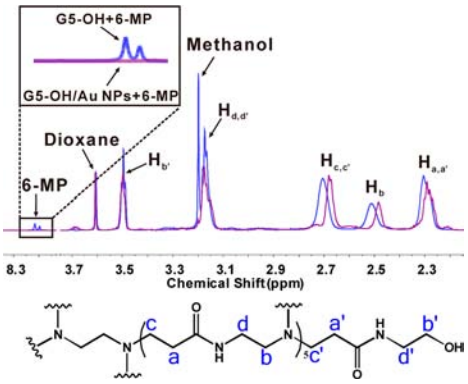


Figure 2. ^{1}H NMR spectra of G5-OH (blue line) and G5-OH/Au NPs (purple line) loaded with 6-MP. The concentrations of 6-MP are equal to each other, and the molar ratio of 6-MP to G5-OH is 10 in the samples.

dendrimer are slightly lower than those of G5-OH/Au NPs. This is due to the close proximity of G5-OH protons to gold nanoparticles in the synthesized G5-OH/Au NPs. The decrease in resonance signals of $H_{d,d'}$ and $H_{a,a'}$ is more significant than those of $H_{b'}$ located on the G5-OH dendrimer surface. This phenomenon can be recognized as evidence of metal nanoparticle encapsulation within the dendrimer.[17] Interestingly, the proton signals of 6-MP disappear in the presence of G5-OH/Au NPs, indicating that most of the 6-MP molecules are bound to the gold nanoparticle surface. These results confirm that the synthesized DEGNPs are capable of loading thiol-contaning drugs (Scheme 1).

In Vitro GSH-Triggered Release of Drugs from DEGNPs. After demonstrating the drug-loading capacity of DEGNPs, the profiles of the in vitro release of the loaded drugs from the nanoparticles were investigated. CPP is an antihypertensive drug with a thiol group (Chart 1). As shown in Figure 3a, 97.0% of the CPP was released from G5-OH in PBS buffer (pH = 7.4) after 3 h, while only 18.4% of the drug was detected at 12 h in the presence of G5-OH/Au NPs. Although PAMAM dendrimer/drug inclusion complexes were able to prolong the release of bound drugs in aqueous solutions, burst release still occurs in physiological conditions if the drugs are loaded via noncovalent interactions. The presence of Au NPs significantly retarded the release of CPP from G5-OH dendrimer. This result suggests that the formed Au−S bond between the Au NPs and the thiol group of CPP is stable in physiological conditions. Similarly, CPP loaded within G3-OH/Au NPs and G4-OH/Au NPs showed a much slower release rate than that within G3-OH and G4-OH (74.8% vs 86.0% for G3-OH, 49.7% vs 90.5% for G4-OH at 12 h) (Figure S1 and Figure S2 in Supporting Information [SI]). A lower amount of CPP loaded within G3-OH-encapsulated Au NPs than that within G4-OH- and G5-OH-encapsulated Au NPs is observed, which is attributed to the smaller pocket size of G3-OH and the lower ratio of Au/dendrimer added for G3-OH (25:1 for G3-OH vs 50:1 for G4-OH and 100:1 for G5-OH).

6-MP, an antileukemic drug with a thiol group (Chart 1), was also employed as a model drug.[10b] As shown in Figure 3b, only 16.6% of the 6-MP loaded within G5-OH/Au NPs was released at 24 h in PBS buffer, while most of the drugs were released from G5-OH and G5-NH_2 dendrimers within 2 h (Figure S3 in SI). Although the 6-MP release from G5-OH/Au NPs stopped in 4−24 h, the addition of a thiol-reducing agent (dithiothreitol, DTT) to the complex solution at 24 h can rapidly release 88.5%

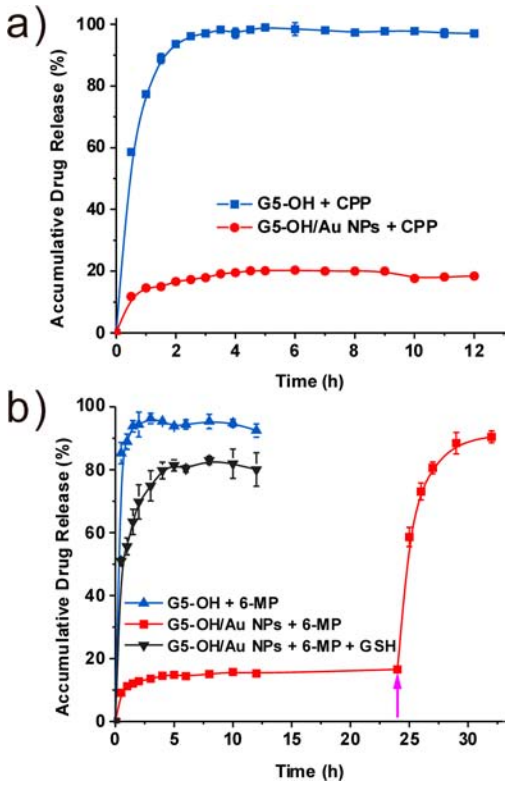


Figure 3. In vitro release profiles of CPP (a) and 6-MP (b) from G5-OH and G5-OH/Au NPs. The purple arrow (b) indicates the addition of DTT into the complex solution at 24 h.

of the loaded 6-MP within 5 h. Also, if the 6-MP-loaded G5-OH/Au NPs were first incubated with 1 mM GSH, 79.8% of the loaded 6-MP was released within 4 h. It is worth noting that this GSH concentration (~1 mM) is within the intracellular GSH concentration (1−10 mM) in human cell lines. The release kinetics of the drugs from DEGNPs can be easily tailored by changing the GSH concentration and the compositions of DEGNPs (Figure S1 in SI). These results suggest that the drugs loaded within DEGNPs exhibit an "Off−On" release behavior upon exposure to thiol-reducing agents, such as GSH (Figure S4 in SI).

Intracellular GSH-Triggered Release of Thiolated Anticancer Drugs Loaded within DEGNPs. To confirm if the GSH-responsive drug delivery system based on DEGNPs also works in cancer cells, G5-OH/Au NPs were loaded with thiolated DOX (DOX-SH). The amine group on DOX was reacted with 2-iminothiolane to give a thiol group (Chart 1).[7b,18] As shown in Figure 4a, DOX-SH-loaded G5-OH/Au NPs showed decreased cytotoxicity in HeLa cells (a cervical cancer cell line) as compared to free DOX-SH, which is attributed to the sustained release of DOX-SH from the nanoparticles due to the stable Au−S linkage. However, if the DOX-SH-loaded G5-OH/Au NPs were first incubated with 5 mM GSH before cellular uptake, the cytotoxicity of the nanoparticles increases to the levels of free DOX-SH at different concentrations, suggesting that the released DOX-SH triggered by GSH retains its anticancer activity. We further investigated whether the activity of DOX-SH-loaded G5-OH/Au NPs is enhanced by increasing intracellular GSH concentration. As shown in Figure 4b, the drug-loaded nanoparticles showed much improved cytotoxicity in HeLa cells when the cells were preincubated with 20 mM monoethyl

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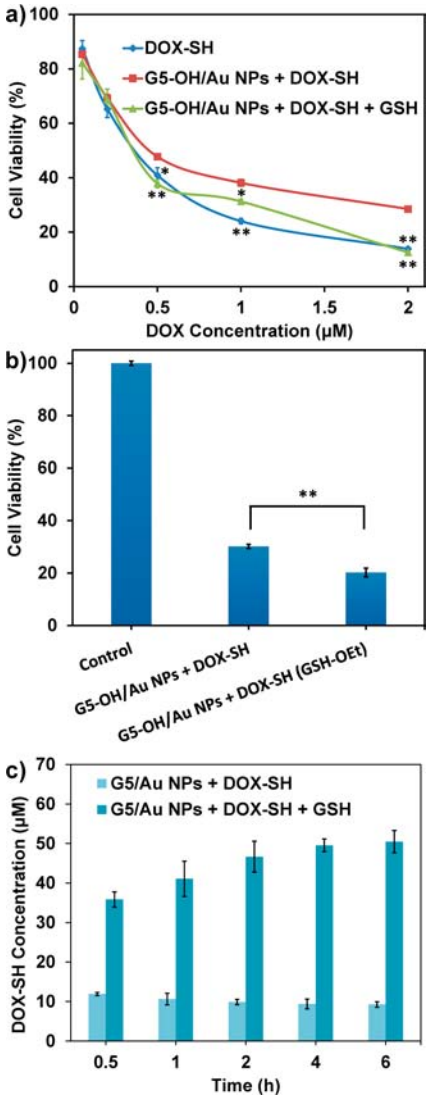
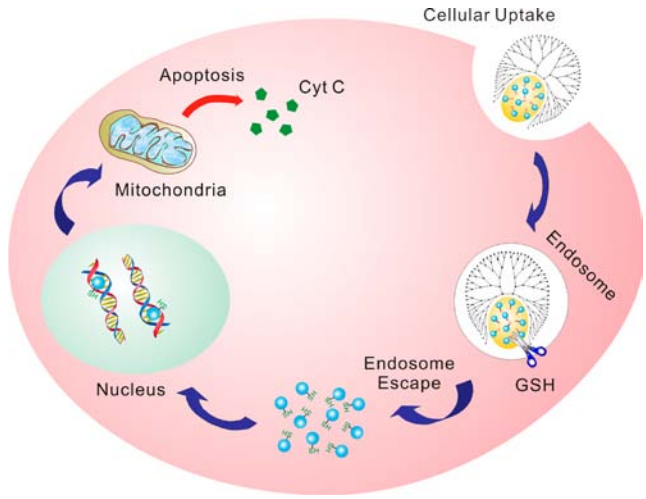


Figure 4. (a) Cytotoxicity of DOX-SH-loaded G5-OH/Au NPs vs free DOX-SH on HeLa cells. GSH was added at a concentration of 5 mM. (b) Cytotoxicity of DOX-SH-loaded G5-OH/Au NPs on HeLa cells tested in the absence and presence of 20 mM GSH-OEt, which was added to enhance the intracellular GSH concentration. The results indicate significant differences in the cytotoxicities of DOX-loaded nanoparticles in the presence and absence of GSH or GSH-OEt, (*) $p < 0.05$, (**) $p < 0.01$, respectively. (c) In vitro release of DOX-SH from drug-loaded G5-OH/Au NPs in the absence and presence of GSH.

ester of GSH (GSH-OEt) for 2 h. GSH-triggered release of DOX-SH loaded within G5-OH/Au NPs is further confirmed by in vitro release studies as shown in Figure 4c. These results clearly demonstrate that DEGNPs are potentially useful for the delivery of thiolated anticancer drugs into cancer cells (Scheme 2).

The prolonged delivery of anticancer drugs bound by DEGNPs is further demonstrated using CPT as a model drug. It is reported that dicarboxylic acids are able to covalently bind CPT, which is widely used in the preparation of CPT prodrugs.[19] Here, mercaptosuccinic acid (MSA) with a thiol group and two carboxylic acid groups was first conjugated to DEGNPs via the Au−S bond. The resulting nanoparticles with the dicarboxylic acid structure were incubated with CPT to form CPT-loaded DEGNPs (Chart 1). As shown in Figure 5,

Scheme 2. Intracellular GSH-Triggered Release of Anticancer Drugs Loaded within DEGNPs



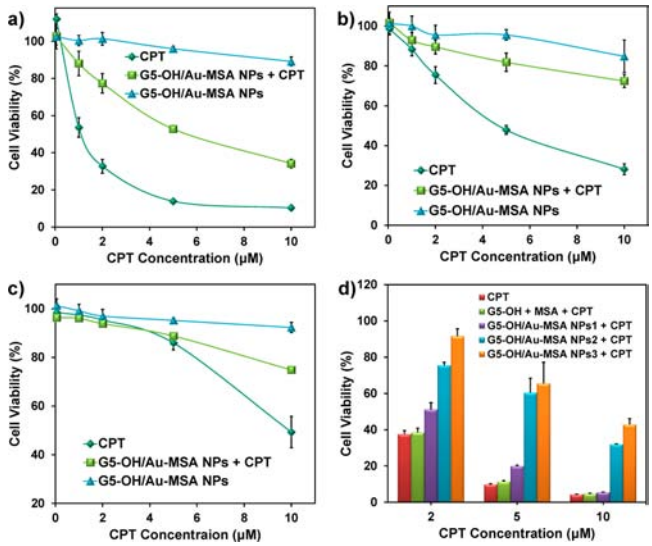


Figure 5. Cytotoxicities of CPT and CPT-loaded G5-OH/Au-MSA NPs on HeLa (a), U2OS (b), and MCF-7 (c) cells. G5-OH/Au-MSA NPs were used as controls. (d) Cytotoxicities of CPT loaded within different G5-OH/Au-MSA NPs on HeLa cells. The ratios of Au and G5-OH are 10:1, 50:1, and 100:1 for G5-OH/Au NPs1, G5-OH/Au NPs2, and G5-OH/Au NPs3, respectively.

G5-OH/Au-MSA NPs are almost nontoxic to the three cancer cell lines including HeLa, U2OS (an osteosarcoma cell line), and MCF-7 (a breast cancer cell line), and CPT-loaded G5-OH/Au-MSA NPs showed much reduced cytotoxicity on these cancer cells as compared to free CPT molecules. This phenomenon is in accord with that observed for DOX-SH-loaded DEGNPs in Figure 4. The absence of MSA did not influence the cytotoxicity of CPT (Figure S5 in SI), suggesting that MSA plays an important role in the attachment of CPT molecules to the nanoparticle. To prove the role of Au NPs on the reduced cytotoxicity of CPT in the formulation, we reduced the Au NP contents in the DEGNPs and tested the activity of CPT loaded within different DEGNPs. As shown in Figure 5d, the decrease of Au/G5-OH molar ratio in the DEGNPs can improve the toxicity of loaded CPT, and the CPT loaded within G5-OH/MSA showed similar toxicity with free CPT, which is due to the increased amount of free CPT molecules in

dx.doi.org/10.1021/ja402903h | J. Am. Chem. Soc. 2013, 135, 9805−9810

the formulations. We also investigated whether CPT-loaded DEGNPs are able to kill cancer cells by GSH-triggered release of CPT. Surprisingly, further addition of GSH decreased the cytotoxicity of the CPT-loaded nanoparticle in the cancer cells (Figure S5 in SI), which can be explained by the inactivation of CPT by GSH molecules.[20]

Intracellular Monitoring of the Release of Anticancer Drugs Triggered by GSH. The intracellular GSH-triggered release of anticancer drug such as DOX-SH in HeLa cells was evaluated by flow cytometry. As shown in Figure 6, cells treated

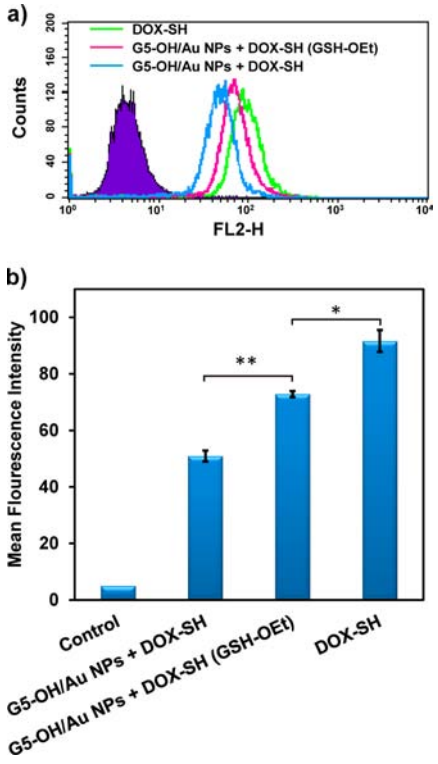


Figure 6. (a) Flow cytometry studies of HeLa cells incubated with DOX-SH and DOX-SH-loaded G5-OH/Au NPs. The cells were also preincubated with GSH-OEt for 2 h to determine the influence of intracellular GSH on the release of DOX-SH from the nanoparticle. (b) Intracellular mean fluorescence intensity of HeLa cells treated with DOX-SH and DOX-SH-loaded G5-OH/Au NPs. The results indicate significant differences on the fluorescence intensities of cells treated with or without GSH-OEt: (*) $p < 0.05$, (**) $p < 0.01$, respectively.

with DOX-SH-loaded G5-OH/Au NPs showed much lower fluorescence as compared to DOX-SH-treated cells. DOX emits red fluorescence and is able to penetrate into the nucleus of cells within several hours. In the case of DOX-SH-loaded DEGNPs, the fluorescence of DOX is quenched by the Au NPs due to the binding of DOX on the nanoparticle surface. Therefore, much weaker fluorescence is observed for the DOX-SH-loaded DEGNPs. When the cells were pretreated with GSH-OEt, the fluorescence of the cells treated with DOX-SH-loaded nanoparticles significantly increased. This result clearly proves the intracellular GSH-triggered release of DOX-SH loaded within DEGNPs. The intracellular DOX released in response to GSH is also monitored by confocal laser scanning microscopy (CLSM). As shown in Figure 7, the red fluorescence intensity of DOX-SH-loaded nanoparticle-treated cells is weaker than that of free drug-treated ones but is significantly increased if the cells were pretreated with GSH-

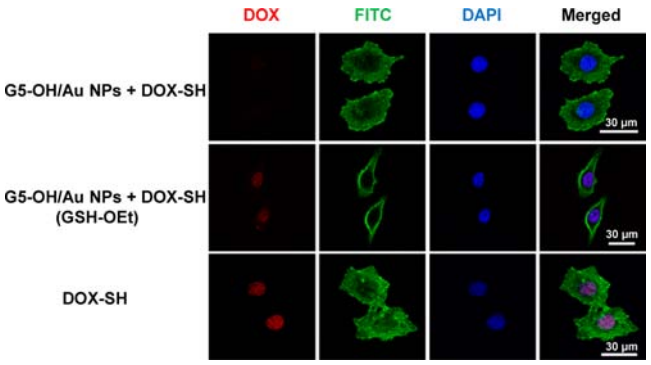


Figure 7. CLSM images of HeLa cells incubated with DOX-SH and DOX-SH-loaded G5-OH/Au NPs. The cells were preincubated with or without 20 mM GSH-OEt for 2 h. The nucleus and cytoskeleton of the cells were stained by DAPI (blue) and phalloidin-FITC (green), respectively.

OEt. Most of the drugs were observed in the nucleus (stained by diamidino-2-phenylindole, DAPI, a blue fluorescent probe) of HeLa cells, suggesting that DEGNPs are able to deliver anticancer drugs such as DOX to its active sites in the nucleus. The results obtained from flow cytometry and CLSM are in accord with those obtained from the cytotoxicity studies.

Biocompatibility of the DEGNPs. It is reported that PAMAM dendrimers are cytotoxic on different cell lines, and the cytotoxicity of a dendrimer depends much on its surface functionality.[21] Hydroxyl-terminated PAMAM dendrimers with a neutral surface have the lowest toxicity among cationic, anionic, and neutral dendrimers.[21] In this study, the DEGNPs were synthesized using hydroxyl-terminated PAMAM dendrimers; thus, the cytotoxicity of the dendrimer is minimal. As shown in Figure 8 and Figure S6 in SI, G3-OH/Au NPs, G4-

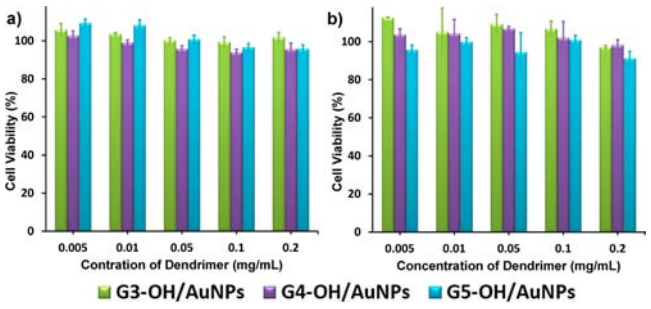


Figure 8. Biocompatibility of the synthesized DEGNPs in HeLa (a) and NIH3T3 (b) cells. The cells were incubated with the nanoparticles for 24 h.

OH/Au NPs, and G5-OH/Au NPs show extremely low cytotoxicity (>90% cell viability) even at a relatively high dendrimer concentration of 0.2 mg/mL in HeLa and NIH3T3 cell lines. This result indicates that DEGNPs can be used as biocompatible vehicles of anticancer drugs.

CONCLUSIONS

In summary, DEGNPs are able to load thiol-containing and thiolated drugs via the Au−S bond. The loaded drugs exhibit an "Off−On" release behavior in response to thiol-reducing agents such as GSH. Intracellular GSH can trigger the release of loaded anticancer drugs within DEGNPs and increase the activity of the loaded drugs. The GSH-triggered release of drugs loaded within DEGNPs is further confirmed by flow cytometry

dx.doi.org/10.1021/ja402903h | J. Am. Chem. Soc. 2013, 135, 9805−9810

and CLSM studies. The synthesized DEGNPs are biocompatible in several cell lines. This study provides a new strategy in the preparation of interior-functionalized dendrimer−drug conjugates. The DEGNP can be developed as a versatile nanocarrier of anticancer drugs with stimuli-responsive properties. We are now using PEGylated dendrimers as templates to synthesize Au NPs, and the resulting nanoparticles can further improve the drug-loading capacity and in vivo blood circulation time of the DEGNPs in the present study.

■ ASSOCIATED CONTENT

Supporting Information

Further data on the in vitro release of drugs from DEGNPs and the cytotoxicity of drug-loaded nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank financial supports from the Science and Technology of Shanghai Municipality (11DZ2260300), the National Natural Science Foundation of China (No. 21274044), the Program for New Century Excellent Talents in University of Ministry of Education of China (No. NCET-11-0138), and the Innovation Program of Shanghai Municipal Education Commission (No.12ZZ044) on this project.

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