J|A|C|S COMMUNICATIONS

Published on Web 12/10/2008

Targeted Paclitaxel by Conjugation to Iron Oxide and Gold Nanoparticles

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Selective targeting of cancer cells has limited success by application of modern chemotherapeutic methods. Paclitaxel **1** (i.e., Taxol) is one of the most popular chemotherapeutic agents used nowadays for treatment of breast, ovarian, and lung cancers.[1,2] Being able to promote tubulin assembly into microtubules,[2,3] paclitaxel brings significant impact mainly because of its mechanism of action.[4] On the other hand, its drawbacks come from the lack of tumor specificity and low solubility in water. To overcome these barriers, we planned to attach paclitaxel onto the biocompatible nanoparticles. Herein we report our success in the development of paclitaxel-conjugated Fe_3O_4 nanoparticles, which possessed phosphate moieties for selectively targeting cancer cells. They also functioned as prodrugs with magnetism and good hydrophilicity.

Among the broad diversity of nanoparticles, iron oxide nanoparticles (Fe-NPs) and gold nanoparticles (Au-NPs) are the most intensively studied.[5,6] Inherent merits of Fe-NP are strong magnetization[7] and that it exhibits little to no toxicity in vivo.[8] In the clinical area of human medicine, these particles are used as delivery vehicles for drugs, genes, and radionuclides.[7] Being ferrofluids, they could function as contrast agents in magnetic resonance imaging. These superparamagnetic Fe-NPs in combination with an external magnetic field allow particles to be delivered to the desired target area and be fixed at a specific site while the medication is released and acts locally.[7,9] Recent examples include immobilization of the enzyme glucose oxidase on Fe-NPs as reported by Rosenzweig et al.[10] A novel method is also developed by Kondo et al.[11] for separation of neutrophils from acute inflammatory neutrophils and macrophages by thermoresponsive magnetic nanoparticles conjugated with a macrophage-specific anti-F4/80 antibody. Moreover, several examples related to the use of magnetic nanoparticles to solve problems during their clinical applications are also reviewed by Berry et al.[12]

Functionalized Au-NPs are promising candidates for drug delivery because of their unique dimensions, tunable functionalities on the surface, and controllable drug release.[13] Recently, Wang et al.[14] have revealed the application of 3-mercaptopropionic acid capped Au-NPs in drug delivery and as biomarkers of drug-resistant cancer cells. Our longstanding thrust in the development of new chemotherapeutic agents[15,16] and syntheses of functional nanobiomaterials[17] prompted us to conjugate paclitaxel onto superparamagnetic iron oxide and gold nanoparticles. We planned to apply the nanotechnology for solving three problems associated with paclitaxel as an effective anticancer drug: (1) low solubility in water, (2) low bioavailability for selectively targeting cancer cells, and (3) lack of a reliable method for its detection and tracking.

Our syntheses of conjugated paclitaxel-containing nanoparticles are shown in Scheme 1. First, we protected the thiol terminal of tetraethylene glycol monothiol (**3**)[18] with a stoichiometric amount of (mono-4-methoxy)trityl chloride (MMTrCl) in the presence of triethyl amine to give **2** in 65% yield. Then paclitaxel (**1**) was treated with $(MeO)PCl_2$ (1.54 equiv) and collidine in THF, (monomethoxy)tritylated thiol **2** (1.0 equiv), I_2 (2.0 equiv), and water in sequence to provide the desired pro-paclitaxel **4** as the major product in 72% yield. The "one-flask method"[15] in the conversion of **1** + **2** → **4** allowed three steps accomplished in situ: coupling of the paclitaxel with the PEG-SH spacer, oxidation of the phosphite center, and deprotection of the (monomethoxy)trityl group.

Second, we modified the ammonium groups in Fe_3O_4-nanoparticles [Fe-NP-$(NH_3)^+_n$][19] by using *N*-succinimidyl 3-(*N*-maleimido)propionate[20] (1.2 equiv) in DMSO to produce the functionalized Fe-NP **5**. The water-soluble and dispersed Fe_3O_4-nanoparticles were prepared from two solutions containing Fe^{II} and Fe^{III} as well as an organic acid containing an amino group. Then the pH of the solution was adjusted, and the proper amount of adherent was added to achieve complete coating of the particle surface with $-NH_3^+$ groups.

Third, attachment of thiol **4** (4.3 equiv) to **5** in methanol at room temperature produced the desired Michael adduct paclitaxel-Fe-NP **6**, of which the mean diameter was 6.1 ± 0.8 nm as determined by TEM. Before and after conjugation, we measured the magnetization loops of Fe-NP-$(NH_3)^+_n$ and paclitaxel-Fe-NP **6** at room temperature; their curves are shown in Figure 1. The saturation magnetization for **6** was determined as 4.0 emu/mg, which indicates its magnetic detectability and the tracking feasibility. On the other hand, our results from thermogravimetric analysis (TGA) of hybrid nanoparticles **5** and **6** reveal that the estimated average numbers were 92 for the succinimido linkers and 83 for paclitaxel attached on the iron oxide cores.

Furthermore, we incorporated pro-paclitaxel **4** (500−1000 equiv) through its thiol terminal onto colloidal Au-NPs in water at room temperature, which was prepared by reduction of $HAuCl_4$ with sodium citrate.[21] The desired hydrophilic **7** was obtained as indicated by a UV/visible peak with a hyperchromic and bathochromic shift of 19 nm. The Au-NP **7** contained 201 functional paclitaxel sites on average as determined by the TGA method. The TEM micrographs in Figure 2b indicate that **7** with a diameter of 14.6 ± 0.7 nm was well dispersed. While the conjugated Au-NP **7** possesses good hydrophilicity, we attempted to obtain hydrophobic paclitaxel-conjugated Au-NPs.

Accordingly dodecanethiol ligands in the clusters **8**[22] were exchanged with paclitaxel-containing thiol **4** in toluene at room

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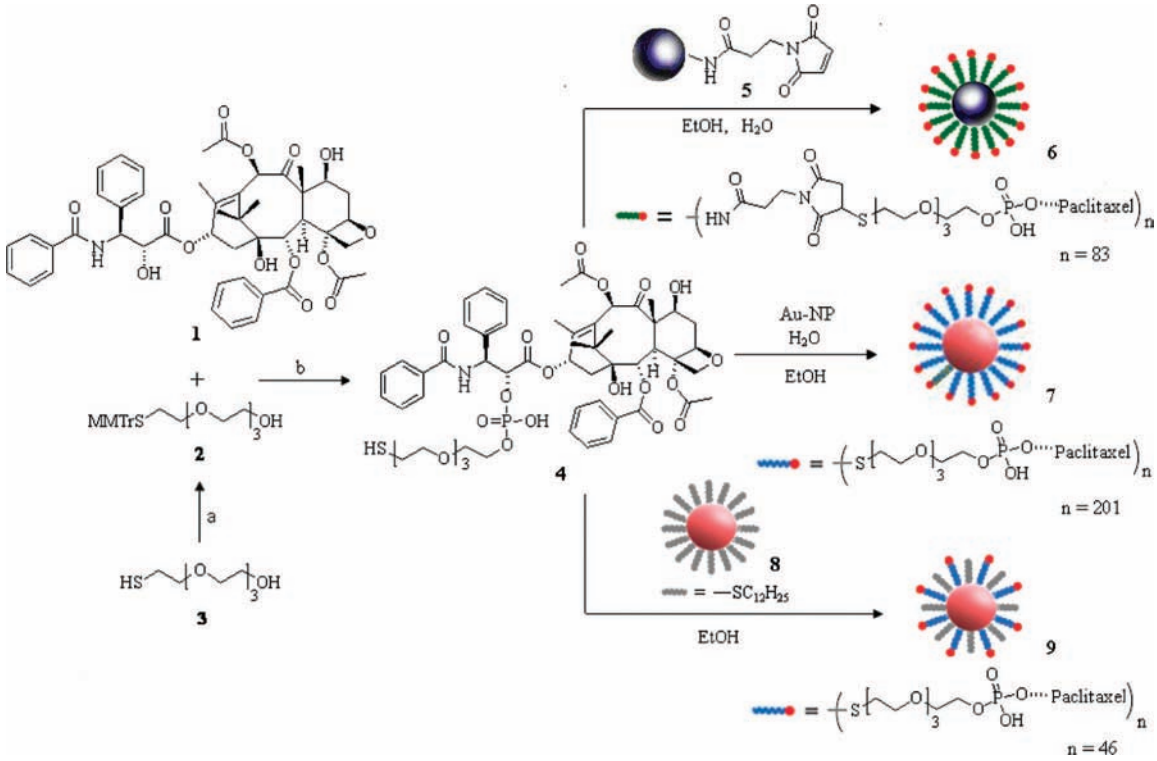
10.1021/ja804947u CCC: $40.75

Scheme 1[a]



[a] Reagents and conditions: (a) MMTrCl, Et_3N, THF, 25 °C. (b) $(MeO)PCl_2$, collidine, THF, 0 °C, I_2, H_2O.

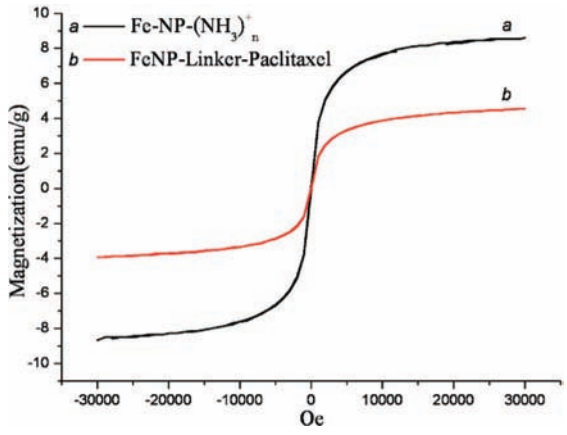


Figure 1. Magnetization curves for Fe-NP before and after conjugation with thiolated PEG-paclitaxel: (a) Fe-NP-$(NH_3)^+_n$ and (b) **6**.

temperature for 120 h. The dispersed hybrid **9**, as shown in Figure 2c, was generated with an average diameter of 2.1 ± 0.3 nm. We determined the average number of the paclitaxel molecules bound on each Au-NP **9** as 46 by the displacement method[23] involving the use of mercaptoethanol.

The paclitaxel-conjugated nanoparticles **6** and **7** exhibited good hydrophilicity, of which dispersion was 312 and 288 μg/mL, respectively. In comparison with the parent paclitaxel molecule (0.4 μg/mL),[24] their hydrophilicity was increased 780 and 720 times. In comparison with PEG-paclitaxel **4** (3.26 μg/mL), their hydrophilicity was increased 96 and 88 times. Because the PEG linker possesses good water solubility and Fe-NP-$(NH_3)^+_n$ is miscible with water, the improvement in hydrophilicity of **6** should be attributed to both the PEG spacers and the Fe-NP-$(NH_3)^+_n$ species. Our use of the flexible PEG spacer may also offer advantages to aid prodrugs **6** and **7** in penetrating cell membranes as well as to increase their biocompatibility.[25]

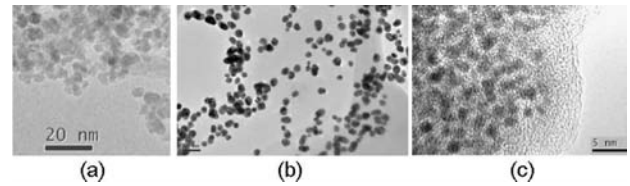


Figure 2. TEM images of paclitaxel-functionalized nanoparticles: (a) **6**, (b) **7**, and (c) **9**.

An advantage associated with our design by incorporation of the phosphodiester moiety in paclitaxel-containing nanoparticles is their capability of selective targeting. Chemotherapeutic agents possessing a phosphate unit would preferentially interact with the cancer cells.[26] Moreover, dephosphorylation often takes place more easily in cancer cells than in normal cells. Hydrolysis of the phosphodiester moieties with the aid of phosphodiesterase[27] could liberate free paclitaxel from nanoparticles. The feasibility of this hypothesis was confirmed by our experiments, in which up to 91% of paclitaxel-containing ligand in prodrug **6** were hydrolyzed by phosphodiesterase after 10 days to give free paclitaxel molecules as detected by HPLC (see Figure 3, curve *a*). Consequently, paclitaxel-Fe-NP **6** acted as "biofunctional material." Such a design is conceptually different from that reported by Zubarev et al.[28]

During our investigations on liberation efficacy of the pro-paclitaxel drugs, we found with surprise that Fe-NP **6** acted differently from Au-NPs **7** and **9**. Less than 5% of paclitaxel was hydrolyzed from Au-NPs **7** and **9** by phosphodiesterase within 10 days (Figure 3, curves *b* for **7** and *c* for **9**). Difficulty of liberation associated with **9** may be due to its hydrophobic property. However, it is unclear to us why **7** with its hydrophilicity failed to release paclitaxel. Furthermore, our results indicate that Fe-NP **6** was

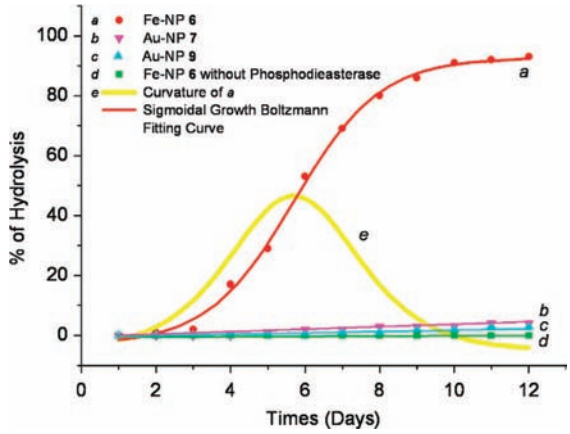


Figure 3. Hydrolysis of paclitaxel-conjugated NP **6**, **7**, and **9** (2.49×10^{-4} M in ammonium acetate buffer, pH 6.5, 20.0 μL) by phosphodiesterase[27] (20 units) in a sodium pyrophosphate butter (pH 6.5, 1.0 mL) at room temperature to give free paclitaxel, as detected by HPLC: Fitting curves (a) for **6**, (b) for **7**, (c) for **9**, and (d) for **6** in the absence of phosphodiesterase. Curve (e) is the curvature to the fitting curve (a).

sustained for three days and then liberated free paclitaxel (~70%) with high efficiency on days 4−8 (see the curvature curve *e*). We conceive that once phosphodiesterase started to hydrolyze some phosphodiester moieties in **6**, the congestion on the surface of nanoparticles was gradually relieved and thus more room was available to accommodate phosphodiesterase with ease.

Furthermore, we performed an efficacy evaluation of the pro-drug paclitaxel-Fe-NP **6** on human cancer cells (OECM1) and human normal cells (HUVEC) by the MTT assay. Our results showed significant (i.e., 10^4) enhancement of cytotoxicity resulting from the pro-drug to cancer cells in comparison with normal cells within 6 days. Their IC_{50} values were 5.03×10^{-7} and 3.58×10^{-3} μg/mL, respectively. Moreover, we did not detect any significant amount (i.e., <0.50%) of free paclitaxel (**1**) from paclitaxel-Fe-NP **6** in FCS (calf serum, 2.50×10^{-4} M) after 12 days. Thus a small amount of, if any, preleaching of paclitaxel will not cause a problem during the development of **6** as a pro-drug.

Among the newly synthesized paclitaxel-conjugated nanomaterials, the Fe-NP **6** was superior to Au-NP **7** during the antitumor studies in vitro. Aggregation often took place when an isotonic solution of hydrophilic Au-NP **7** was prepared for application to various lung cancer cell lines. We circumvented this problem by using paclitaxel-PEG-Fe-NP **6**. In addition, its isolation required a shorter period of time for centrifuge in comparison with the corresponding Au-NP **7**. During preparation of the TEM samples by sonication, hydrophobic paclitaxel-PEG-Au-NP **9** was also aggregated easily. The in vivo antitumor studies of these conjugates for various cancer lines will be carried out in due course.

In conclusion, three paclitaxel-conjugated nanoparticles were synthesized by use of Fe_3O_4 and Au as the cores. By possessing the PEG-SH spacer and the phosphate joining unit, these new paclitaxel-P(=O)(OH)-PEG-S-Fe-NP nanomaterials (i.e., **6**) functioned as a prodrug of paclitaxel, which was liberated in the presence of phosphodiesterase. It also possessed magnetic tracking capability and good hydrophilicity. Furthermore, the Au-NPs were synthesized through different methods for the production of both hydrophilic and hydrophobic paclitaxel conjugates. These conjugated nanomaterials constitute a new class of candidates as anticancer drugs.

Acknowledgment. We are grateful to the National Science Council of Republic of China for support of this research.

Supporting Information Available: Synthetic and experimental procedures, analytical data, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA804947U