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# Antibiofouling Polymer-Coated Gold Nanoparticles as a Contrast Agent for in Vivo X-ray Computed Tomography Imaging

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***Abstract:*** Current computed tomography (CT) contrast agents such as iodine-based compounds have several limitations, including short imaging times due to rapid renal clearance, renal toxicity, and vascular permeation. Here, we describe a new CT contrast agent based on gold nanoparticles (GNPs) that overcomes these limitations. Because gold has a higher atomic number and X-ray absorption coefficient than iodine, we expected that GNPs can be used as CT contrast agents. We prepared uniform GNPs (~30 nm in diameter) by general reduction of $HAuCl_4$ by boiling with sodium citrate. The resulting GNPs were coated with polyethylene glycol (PEG) to impart antibiofouling properties, which extends their lifetime in the bloodstream. Measurement of the X-ray absorption coefficient in vitro revealed that the attenuation of PEG-coated GNPs is 5.7 times higher than that of the current iodine-based CT contrast agent, Ultravist. Furthermore, when injected intravenously into rats, the PEG-coated GNPs had a much longer blood circulation time (>4 h) than Ultravist (<10 min). Consequently, CT images of rats using PEG-coated GNPs showed a clear delineation of cardiac ventricles and great vessels. On the other hand, relatively high levels of GNPs accumulated in the spleen and liver, which contain phagocytic cells. Intravenous injection of PEG-coated GNPs into hepatoma-bearing rats resulted in a high contrast (~2-fold) between hepatoma and normal liver tissue on CT images. These results suggest that PEG-coated GNPs can be useful as a CT contrast agent for a blood pool and hepatoma imaging.

## Introduction

With advances in the syntheses of a variety of nanomaterials, there has been a surge of interest in the use of nanoparticles as imaging probes for in vivo biomedical applications.[1−8] Typical examples include magnetic nanoparticles as magnetic resonance contrast agents,[9−11] quantum dots,[12−14] and gold nanoparticles (GNPs)[15−18] as optical imaging probes. The novel physical properties of such nanomaterials, for example, superparamagnetism, highly intense fluorescence, and surface plasmon resonance, give them advantages over conventional molecular imaging probes.

X-ray computed tomography (CT) is one of the most useful diagnostic tools in hospitals in terms of frequency of use and cost. Current contrast agents for CT are based on iodinated small molecules because, among nonmetal atoms, iodine has a high X-ray absorption coefficient.[19,20] Iodinated compounds, however,

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allow only very short imaging times due to rapid clearance by the kidney, which can also cause them to have renal toxicity.[21,22]

Very recently, novel nanoparticle-based CT contrast agents have emerged to overcome the shortcomings of iodine agents.[23−25] Polymer-coated bismuth sulfide ($Bi_2S_3$) nanoparticles may be useful as a CT contrast agent and have an efficacy/safety profile in vivo that is comparable to or better than those of iodinated imaging agents.[23] Despite several favorable properties, it is difficult to control the size and shape of $Bi_2S_3$ nanoparticles, and there is a lack of chemical methods to modify their surface, which may hinder their further clinical application. Therefore, we have attempted to develop other nanomaterial-based CT contrast agents.

Here we report on the feasibility of biocompatible polymer-coated GNPs as a potential CT contrast agent.[24,25] Unlike $Bi_2S_3$ nanoparticles, the synthesis, physical and biological properties, and surface chemistry of GNPs have been intensively studied. The size and shape of GNPs can be easily controlled, and they can be modified with various functional groups.[26−31] Furthermore, GNPs are biocompatible and nontoxic in vivo.[32−34] Most importantly, the fact that gold has a higher X-ray absorption coefficient than iodine (5.16 and 1.94 $cm^2/g$, respectively, at 100 keV) encouraged us to study the feasibility of GNPs as a potential CT contrast agent in vivo.[24,35]

Because plasma proteins and salts in blood nonspecifically adsorb onto the surface of bare nanoparticles, which causes the production of large aggregates, the direct use of GNPs in vivo leads to rapid clearance from the bloodstream due to uptake by the reticular endothelial system, including macrophages in the liver and spleen.[36−38] Therefore, for GNPs to be used in vivo, their surfaces should be modified with antibiofouling agents, such as polyethylene glycol (PEG).[39−45] In this report, we describe the synthesis of antibiofouling polymer-coated GNPs and their application as a CT contrast agent for angiography and hepatoma detection in vivo.

## Experimental Section

**Materials.** Sodium citrate tribasic dihydrate, hydrogen tetrachloroaurate ($HAuCl_4$), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution were purchased from Sigma (St. Louis, MO). PEG−SH (molecular weight, 5000) was purchased from Nektar (Huntsville, AL).

**Measurements.** The hydrodynamic particle size of the PEG-coated GNPs in distilled water was measured using an ELS 8000 (Otsuka Electronics Korea, Seoul, South Korea). The size of the PEG-coated GNPs at dried state was measured by scanning electron microscopy (SEM) using an S4700 electron microscope (Hitachi Ltd., Tokyo, Japan). The surface atom composition of GNPs was assessed by X-ray photoelectron spectroscopy (XPS) (VG Multilab ESCA 2000 system, Waltham, U.K.). Thermal gravimetric analysis (TGA) was carried out using TGA-50H (Shimadzu Co., Kyoto, Japan). The temperature of the sample gradually increased from 20 to 800 °C at a rate of 10 °C/min.

**Synthesis of PEG-Coated GNPs.**[46,47] In a 250 mL round-bottomed flask equipped with a condenser, 100 mL of 0.01 wt % $HAuCl_4$ was heated to a boil with vigorous stirring. Next, 2 mL of 1 wt % sodium citrate was added quickly, which resulted in a color change from blue to burgundy. After further stirring at the same temperature for 10 min, the resulting solution was cooled to room temperature and then mixed with PEG−SH (1 mg) and stirred for 1 h to covalently modify the surface of the GNPs with PEG. The resulting PEG-coated GNPs were collected by centrifugation at 16,800*g* for 30 min and washed twice with distilled water.

**Cell Culture.** The rat hepatoma cell line N1S1 and the hepatocyte cell line HepG2 were originally obtained from American Type Culture Collection (Manassas, VA). N1S1 cells were maintained as a suspension culture in Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY), and HepG2 cells were maintained as adherent culture in Dulbecco's modified eagle medium containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air. The viable cell concentration was determined by counting the cell number trypan blue-excluding cells.

**Preparation of Rat Hepatoma Models.**[48] Sprague−Dawley rats (male, average body weight: 300 g) were anesthetized with 0.5 mL of ketamine/0.05 mL of rompun and implanted intrahepatically with $10^6$ N1S1 cells in 0.1 mL. This procedure did not result in surgical or postoperative mortality. Animals were cared for according to the guidelines of the Institutional Animal Care and Use Committee at GIST.

**In Vitro Cytotoxicity Assay (MTT Assay).** Cytotoxicity of PEG-coated GNPs was determined using an MTT colorimetric assay. HepG2 cells were seeded in 96-well plates at a density of $8 \times 10^4$ cells/well in 100 $\mu$L of medium and incubated overnight at 37 °C in an atmosphere of 5% $CO_2$/95% air. The medium of each well was then replaced with 100 $\mu$L of fresh medium containing various concentrations of the nanoparticles. All concentrations were tested in replicates of six. After 24 h, the medium was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS) to eliminate remaining particles. Next, 100 $\mu$L of fresh culture medium was added to each well, followed by 20 $\mu$L of MTT solution (2.5 mg/mL in PBS). The cells were then incubated for a further 4 h at 37 °C, and then the medium was carefully


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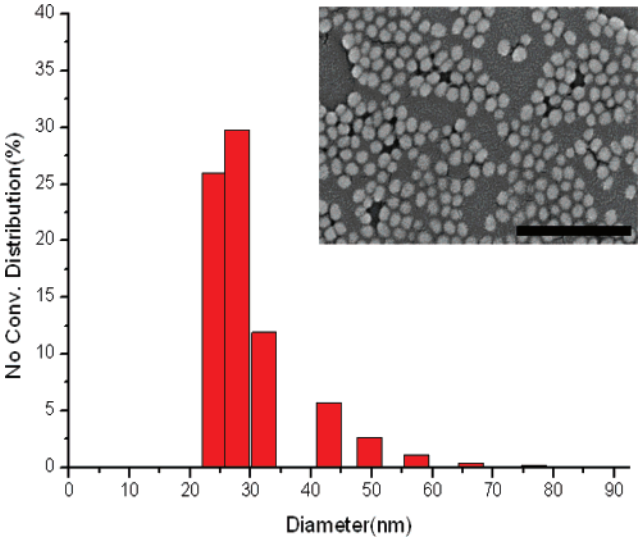


***Figure 1.*** Hydrodynamic size distribution and SEM image of the PEG-coated GNPs. The scale bar in the SEM image indicates 300 nm.

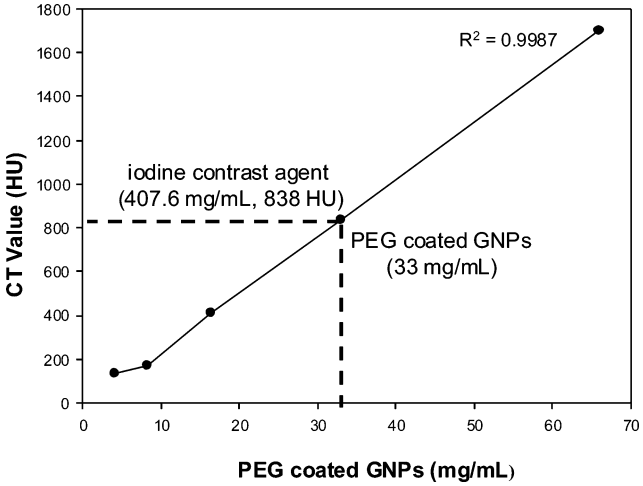


***Figure 2.*** HU measurements of the PEG-coated GNPs in vitro. The measurements show that 33 mg/mL of the PEG-coated GNPs gives an equivalent X-ray absorption as 407.6 mg/mL (189 mg iodine/mL) of the conventional iodine contrast agent, Ultravist.

aspirated. The cells were solubilized in 200 $\mu$L of DMSO, and the absorbance in each well at 570 nm was measured using a FL600 microplate reader (Bio-Tek Inc., Winooski, VT). The data are expressed as the percent of viable cells compared to the control group.

**CT Imaging.** CT images were taken prior to injection of the PEG-coated GNPs and at appropriate time points after tail vein injection. Rats were anesthetized using general inhalation anesthesia (1.5% isoflurane in 1:2 $O_2/N_2$). For cancer imaging, 400 $\mu$L of PEG-coated GNPs (100 mg/mL) was injected through the tail vein into rats bearing hepatomas. For blood pool imaging, 500 $\mu$L of PEG-coated GNPs (140 mg/mL) was injected into normal rats through the tail vein. CT data were acquired using a GE Light Speed VCT 64-detector CT (GE Amersham Healthcare System, Milwaukee, WI). Imaging parameters were as follows: slice thickness, 0.625 mm; pitch, 0.984:1; 120 kVp, 500 mA; field of view, 512 × 512, gantry rotation time, 0.4 s; table speed, 40 mm/rotation. All animals were scanned in the cranial to caudal direction from the lower chest to the pelvis. CT data were analyzed using the Hounsfield units (HU) for regions of interest, including liver, hepatoma, and spleen.

## Results and Discussion

Antibiofouling polymer-coated GNPs were prepared through two steps. First, GNPs were synthesized using a well-known citrate reduction method.[46] Second, the GNPs were covalently conjugated to PEG by reaction of the thiol group of PEG−SH and the gold surface in an aqueous solution. PEG−SH was chosen as a coating material because it is biocompatible and spontaneously forms a chemisorbed surface layer on gold surfaces.[49,50] In addition, we predicted that the high resistance of PEG to protein adsorption would provide a long plasma circulation time.

Figure 1 shows the hydrodynamic size of the PEG-coated GNPs as measured by dynamic light scattering as well as SEM image of the same nanoparticles. As seen in the SEM images, the size and shape of GNPs without PEG layers are homogeneous, with a diameter of approximately 30 nm and a narrow size distribution. In general, hydrodynamic size of polymer-coated nanoparticles is greater than the size measured in the dried state by SEM. Contrary to our expectations, we observed only a slight difference between the size measured by dynamic light scattering (31 ± 7.5 nm) and SEM for the PEG-coated GNPs; however, we confirmed the presence of a PEG layer on the GNPs by both XPS and TGA analysis (Figures S1 and S2, Supporting Information). TGA measurement revealed that the polymer layer constitutes approximately 4.5% of the weight of a PEG-coated GNP. High-resolution C(1s) XPS analysis showed that the intensity of the characteristic peak that corresponds to the C−O ether bond of PEG increased significantly in the PEG-coated GNPs compared to that of the citrate-stabilized GNPs (34.9% and 5.4%, respectively).[51,52]

We next compared the X-ray absorption of the PEG-coated GNPs and Ultravist, a popular iodine-based CT contrast agent currently used in the clinic (Figure 2). HU values were obtained for increasing amounts of GNPs. Figure 2 shows that 33 mg/mL of the PEG-coated GNPs gave an equivalent X-ray absorption as 407.6 mg/mL of Ultravist (corresponding to 189 mg I/mL). In other words, at the same concentration, the attenuation coefficient of the PEG-coated GNPs is 5.7 times higher than that of the current iodine-based CT contrast agent and approximately 2 times higher than that of the $Bi_2S_3$ nanoparticle-based CT contrast agent.[23] This result clearly indicates that the PEG-coated GNPs have a high potential for use in in vivo CT imaging.

To examine the feasibility of the PEG-coated GNPs as a CT contrast agent in vivo, we performed blood pool imaging of rats following intravenous injection of the nanoparticles (280 mg/kg). Figure 3 shows the three-dimensional CT image of the heart and great vessels including the aortic arch, aorta, inferior vena cava, and hepatic veins. The HU values of several regions of interest were recorded at different time points. As seen in Figure 3a, the heart and great vessels can be distinguished on the GNP-enhanced CT image with good contrast. Quantitative analysis of the CT values for the heart and great vessels (Figure 3b) reveals that the PEG-coated GNPs circulate in the blood-

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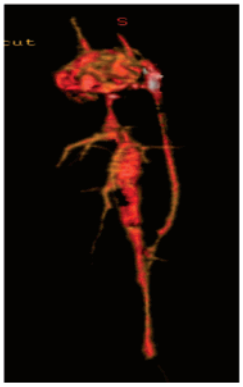
(a)



(b)

| | LV | Aortic arch | IVC | Liver | Spleen |
|---|---|---|---|---|---|
| Pre | 59 | 32 | 44 | 68 | 62 |
| 10min | 160 | 161 | 156 | 91 | 98 |
| 15min | 144 | 158 | 165 | 91 | 94 |
| 30min | 164 | 172 | 143 | 84 | 99 |
| 1h | 151 | 146 | 159 | 95 | 96 |
| 2h | 155 | 147 | 146 | 102 | 104 |
| 4h | 150 | 145 | 140 | 98 | 110 |
| 12h | 131 | 104 | 111 | 103 | 138 |
| 24h | 67 | 120 | 82 | 108 | 152 |

***Figure 3.*** (a) Three-dimensional in vivo CT angiogram image of the heart and great vessels obtained 10 min after injection of 500 $\mu$L of PEG-coated GNPs (140 mg/mL) into the tail vein of a Sprague−Dawley rat. (b) HU values of the left ventricle (LV), aortic arch, inferior vena cava (IVC), liver, and spleen before injection (pre) and at the indicated times after injection.

stream for at least 4 h without an appreciable loss of contrast. The maximum HU values for the heart and aortic arch were observed approximately 30 min after injection with GNPs and indicate a 3- to 4-fold enhancement compared to the preinjection CT image. The much longer half-life in blood vessels of GNPs compared to the iodine-based contrast agent Ultravist (<10 min) may be due to the PEG coating and should help improve the diagnosis of diseases.[45,49]

In addition, we observed the gradual accumulation of the nanoparticles by the liver for up to 2 h, whereas the spleen continued to accumulate the PEG-coated GNPs even after 2 h. This accumulation in the spleen may be due to the uptake of GNPs by macrophages.[38] We did not, however, observe any apparent toxicity associated with this accumulation, even after up to a month.

Because the PEG-coated GNPs can be accumulated by the liver, more specifically the Kupffer cells and hepatocytes, we next examined the use of the nanoparticles for the detection of hepatoma in vivo. A hepatoma rat model was prepared as described previously.[48] Figure 4 shows serial CT images of a rat liver bearing a hepatoma. Arrows and arrowheads indicate the hepatoma region and aorta, respectively. Although it was difficult to identify the hepatoma in the pre-enhanced CT image (Figure 4a), we obtained good contrast enhancement (~2-fold) between the hepatoma and the surrounding normal liver 5 min after intravenous injection of GNPs. Furthermore, the relative contrast difference remained unchanged for up to 24 h (data not shown). These results suggest that the GNPs developed here can be used as a CT contrast agent for the detection of hepatoma. In addition, the GNPs clearly enhanced the CT signal for the aorta (arrowhead) for at least 4 h (Figure 4e), which confirms their long circulation time. This agrees well with the results obtained from blood pool imaging in normal rats (Figure 3b).

Although GNPs are known to be biocompatible and nontoxic, we performed an MTT assay to determine the toxicity of the

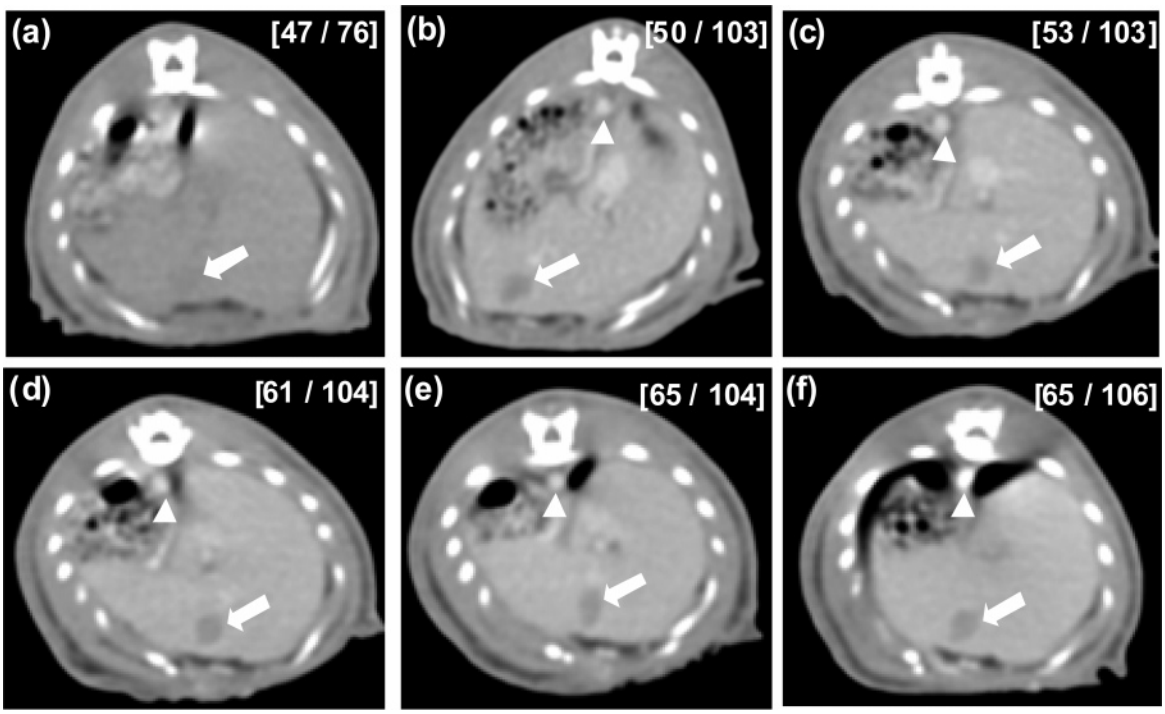



***Figure 4.*** Serial CT images in a rat hepatoma model following injection of 400 $\mu$L of PEG-coated GNPs (100 mg/mL) into the tail vein. Images were obtained at (a) 0 h (before injection) and (b) 5 min, (c) 1 h, (d) 2 h, (e) 4 h, and (f) 12 h after injection. Arrows indicate the hepatoma regions, and the arrowheads indicate the aorta. Numbers in brackets are the HU values of the hepatoma regions (left) and the surrounding normal liver parenchyma (right).

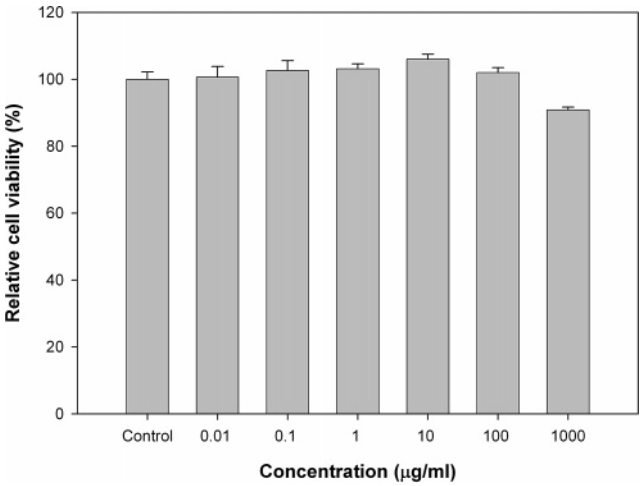



***Figure 5.*** Cell viability of HepG2 cells after 24 h of incubation with increasing amounts of the PEG-coated GNPs. Cell viability was measured using an MTT assay.

PEG-coated GNPs in vitro. We used the HepG2 hepatocyte cell line because an appreciable amount of the nanoparticles accumulate in the liver. Figure 5 shows cell viability data for HepG2 cells after 24 h of incubation with increasing amounts of PEG-coated GNPs. As expected, the nanoparticles did not show any appreciable toxicity, even at 1 mg/mL, which is probably a much higher concentration than encountered in vivo. More detailed toxicity studies, such as measurement of the cytotoxicity to other cell lines in vitro and analysis of long-term organ toxicity, are needed before the present nanoparticles can be used in the clinic.

## Conclusion

In conclusion, we examined the feasibility of PEG-coated GNPs as a CT contrast agent in vivo. The simple combination of the antibiofouling property provided by PEG and the high X-ray absorption property of gold results in an efficient CT contrast agent with a long circulation time that may avoid the shortcomings of current iodine-based CT contrast agents such as short imaging times and renal toxicity. Furthermore, we showed that the PEG-coated GNPs can be used not only as a blood pool imaging agent but also as a detection agent for hepatoma. We anticipate that the biocompatible, antibiofouling PEG-coated GNPs developed here will be a clinically useful imaging agent.

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**Supporting Information Available:** XPS data and TGA graph of the PEG-coated GNPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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