Preparation of Curcumin-Containing α-, β-, and γ-Cyclodextrin/ Polyethyleneglycol-Conjugated Gold Multifunctional Nanoparticles and Their in Vitro Cytotoxic Effects on A549 Cells

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Curcumin is a compound in turmeric that has attracted significant attention as a promising treatment for many diseases, including cancer, due to its potent antioxidant effect, anti-inflammatory effect, and anti-cancer effect. Curcumin has antioxidant effects but its therapeutic application is limited due to poor water solubility. Three types of CDs (α-, β-, and γ-CDs) were conjugated with PEGylated GNPs and the curcumin-containing CD/PEG-conjugated GNPs (cur-CD-GNPs) were characterized. Transmission electron microscopy and dynamic light scattering results showed that these cur-CD-GNPs have a small gold nanocore (approximately 5 nm) and the average size of the three cur-CD-GNPs was approximately 25–35 nm. Curcumin was efficiently incorporated into the β-CD solution and the loading efficiency of curcumin in β-CD-GNPs was the highest of the three types of CD-GNPs prepared. The cytotoxic effect of cur-CD-GNPs was investigated using a human lung cancer cell line. All cur-CD-GNPs exhibited cytotoxic effects comparable to that of curcumin solution and CD-GNPs without curcumin were not cytotoxic. These results suggest that cur-CD-GNPs may be a useful multifunctional nanomedicine, although in vivo investigations are required.

Key words curcumin; cyclodextrin; gold nanoparticle; nanomedicine; PEGylation; poorly-water-soluble drug

Curcumin is a compound in turmeric that has attracted significant attention as a promising treatment for many diseases, including cancer, due to its potent antioxidant effect, anti-inflammatory effect, and anti-cancer effect. Curcumin has been considered for the treatment of various kinds of cancers and its combination with anticancer drugs has been studied. Curcumin affects various signaling pathways (nuclear factor κB, activator protein 1, tumor suppressor genes (e.g., phosphatase and tensin homolog deleted from chromosome 10 (PTEN)), apoptotic genes (e.g., p53, p21), histone deacetylases, and microRNAs). However, the therapeutic effects of curcumin are currently insufficient due to its low oral bioavailability (rapid metabolism) and poor water-solubility. Curcumin nanoparticles may address these inadequacies. The use of nanocarriers such as liposomes, polymer micelles, emulsions, and cyclodextrins, techniques such as nano-pulverization, and the preparation of nanocomposite particles may improve the dissolution of curcumin and its therapeutic effects. Additionally, nanoparticle curcumin formulations can be orally administered, as shown in a dose-escalation clinical trial. Nano formulations of curcumin hold promise as a drug delivery system.

Gold nanoparticles (GNPs) have been extensively studied as novel metal-based platforms for drug delivery, imaging, sensing, and photodynamic therapy. GNPs exhibit unique optical properties derived from surface plasmon phenomena (the absorption and interaction with light of various wavelengths). Surface modification of GNPs (e.g., size, morphology, and adjustment of the core shell type) can change the wavelength of light absorption. GNPs hold promise as carriers for photothermal therapeutics to kill cancer cells with heat, which requires efficient light absorption. The combination of therapy and diagnostic, called “theranostics,” is a novel therapeutic modality and the applications of GNPs as drug nanocarriers are expanding.

The large surface area and high surface energy of GNPs is a significant advantage in drug delivery. GNP chemical modification and surface conjugation with therapeutic molecules (e.g., small molecule drugs, nucleic acids, and proteins), homing compounds for pathological sites (e.g., antibodies and ligands), and compounds for imaging have all been investigated for future use as nanomedicines. In particular, surface modification with polyethyleneglycol (i.e., PEGylation) allows the nanoparticles to escape recognition by the immune system (e.g., the mononuclear phagocyte system) and prolong the circulation time in blood. Enhanced residence time in the blood circulation allows the passive accumulation of nanoparticles in cancer tissue, called the enhanced permeability and retention effect (EPR effect). The pharmacokinetics behavior of GNPs (5–30 nm) in the blood circulation differs from conventional larger drug nanocarriers such as liposomes and micelles. Although further investigation is necessary, smaller nanoparticles (30 nm) can penetrate deeper into cancer tissue following their accumulation via the EPR effect, while their renal clearance via the glomerulus can be controlled.

Here, we synthesized novel functional GNPs whose surface was chemically conjugated with PEG and cyclodextrin (CD). CD is a cyclic oligosaccharide that can encapsulate hydrophobic drugs. The conjugation of CD–drug moieties on inorganic nanoparticles has attracted attention as a promising drug delivery technique because conjugation endows the nanoparticles...
with the functionality of drug loading.\textsuperscript{21} We incorporated the therapeutic compound curcumin in the CD-conjugated GNPs both to provide drug-loaded nanoparticles and to improve solubility. Several articles have described the preparation of curcumin-loaded CD-conjugated metals and GNPs\textsuperscript{22,23} but more information is required. The functional drug-loaded GNPs were characterized and their \textit{in vitro} anticancer effect was investigated using a human cancer cell line.

**MATERIALS AND METHODS**

**Materials** Curcumin, coumarin-6, O-(2-mercaptoethyl)-O'-methyl-polyethylene glycol (molecular weight (MW): 10 kDa; PEG-SH) and 11-mercapto undecanoic acid (MUA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Hydrogen tetrachloroaurate(III) tetrahydrate (HAuCl\(_4\).4H\(_2\)O), sodium tetrahydroborate (NaBH\(_4\)) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3A-Amino-3A-deoxy-(2A\(\text{S}\),3A\(\text{S}\))-n-cyclodextrin hydrate (n=\(\alpha\), \(\beta\), or \(\gamma\); amino-CD) and N-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (WSC) was purchased from Dojindo (Kumamoto, Japan).

**Synthesis of CD/PEG-Conjugated GNPs (CD-GNPs)**
The synthesis scheme for cur-CD-GNPs is shown in Fig. 1. PEGylated GNPs (PEG-GNPs) were prepared as described previously.\textsuperscript{24} As a typical procedure, NaBH\(_4\) solution (630 mM, 1 mL) was added to 30 mL of HAuCl\(_4\) (264 \(\mu\)M) in a glass vial with stirring and the solution was incubated at room temperature (r.t.) for 24 h. Next, 21.1 \(\mu\)L of 10 mM PEG-SH solution was added to 6 mL of the resulting GNP solution with stirring and the sample was incubated for 1 h to conjugate the PEG chains with the GNPs. Free PEG-SH was removed by filter centrifugation (3000 \(\times\)g, 4°C, 10 min) through an Amicon Ultra filter. The total volume of PEG-GNP solution was then adjusted to 4 mL by adding pure water.

**Preparation of Curcumin-Containing CD/PEG-Conjugated GNPs (Cur-CD-GNPs)** Cur-CD-GNPs were prepared based on Heo’s method.\textsuperscript{22} Briefly, 50 \(\mu\)L of 2.5 mM curcumin dissolved in DMSO, was added to 1 mL of CD-GNP solution as prepared in the “Synthesis of Curcumin-Containing CD/PEG-Conjugated GNPs (Cur-CD-GNPs),” and the resulting solution was sonicated for 10 min. The cur-CD-GNPs solution was then filtered through a 0.2 \(\mu\)m membrane filter to remove crystallized curcumin nanoparticles. The loading efficiency of curcumin against the nanocarrier was calculated by measuring the absorbance of at 425 nm using a UV-Vis spectrometer (UV1800; Shimadzu, Kyoto, Japan).

**Particle Size and Zeta Potential** The mean diameters of the resulting nanoparticles were measured using a Malvern ZetaSizer Nano-S instrument (Malvern Instruments Ltd.,...
Malvern, U.K.) with temperature control. The measurements are based on dynamic light scattering (DLS). The mean zeta-potentials of the resulting nanoparticles were measured using a ZetaSizer (Malvern Instruments Ltd.).

**Solubility Phase Diagram** Excess curcumin was added to the amino-CD solutions (0–20mm) and each mixture was shaken at 20°C for 2h. The solution was filtered and the absorbance (at 425nm) was measured using the UV-Vis spectrometer (Shimadzu).

**Optical Properties** The absorbance of the samples in the range of 300 to 600 nm was scanned using the UV-Vis spectrometer (Shimadzu).

**Transmission Electron Microscopy (TEM)** The cur-CD-GNPs were observed using a JEOL JEM-1400 plus microscope (JEOL Ltd., Tokyo, Japan). All cur-CD-GNP samples were negativity stained with 2% uranium acetate solution. An appropriate amount of nanoparticle dispersion was added onto a carbon grid and the nanoparticles were observed at 100kV.

**Cell Culture** The A549 human lung carcinoma cell line was obtained from ATCC (Manassas, VA, U.S.A.). Cells were cultured in Dulbecco’s modified Eagle medium (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum with antibiotics (penicillin and streptomycin). The cells were cultured in Dulbecco’s modified Eagle medium (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum maintained in a CO₂ incubator (SCA-80D, ASTEC Co., Ltd., Fukuoka, Japan) at 37°C and in 5% CO₂.

**In Vitro Cytotoxic Effects** The cytotoxic effects of the nanoparticles were evaluated using the WST assay as described previously, with modifications. In brief, a cell suspension was added to the wells of a 96-well plate (5000 cells/well) and the cells were pre-incubated at 37°C for 24h, then drug formulations (free curcumin solution, CD-GNPs, or cur-CD-GNPs) were added. Free curcumin solution was prepared by dissolving in DMSO and medium. The cells were exposed to the drug formulations at 37°C for 24h. Cell viability was evaluated using a cell counting kit (CCK-8; Dojindo, Kumamoto, Japan). The absorbance of the solution in each well at 450nm was measured using a plate reader (Wallac ARVO multi-label counter; PerkinElmer, Inc., Waltham, MA, U.S.A.).

**Observation of Cellular Uptake of Fluorescence Marker-Containing CD-GNPs** A cell suspension was added to 35 mm glass-base dishes (100000 cells/dish) and pre-incubated at 37°C for 24h, then drug formulations consisting of fluorescence marker (coumarin-6; free coumarin-6 solution or coumarin-6-containing CD-GNPs) were added and the cells were incubated for 4h. Coumarin-6-containing CD-GNPs were prepared as described in the “Preparation of Curcumin-Containing CD/PEG-Conjugated GNP.”

After incubation, each dish was washed with PBS, then 4% paraformaldehyde was added and the cells were incubated at r.t. for 30min. Intracellular fluorescence was observed using a confocal laser microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

**RESULTS AND DISCUSSION**

Curcumin-containing CD/PEG-conjugated gold nanoparticles (cur-CD-GNPs) were synthesized as shown in Fig. 1. First, GNPs were formed under reducing conditions, then PEGylated using PEG-SH by producing a coordination bond between the GNPs and thiol groups. Next, MUA was conjugated with the PEG-GNPs to produce the scaffold for further conjugation with amino-CD through a coupling reaction in the presence of WSC/NHS. MUA has a role of spacer between GNP and CD. It is expected that the room to conjugate GNP with CD is produced by using MUA and that the further amount of conjugation between them is formed compared with other methods (e.g., the use of thiolated CD) to conjugate GNP with CD directly.

The characteristics of the GNP-based functional conjugates are shown in Table 1. The mean diameter of the GNPs was approximately 5 nm while that of the PEG-GNPs was approximately 30 nm, consistent with our previous study on PEG/biosimilar-conjugated GNPs. In that previous study, we used two lengths of PEG chain (5, 10k) to conjugate GNPs. The mean diameters of the PEGylated GNPs differed from the length of the PEG chain used: PEGylated GNPs with the 10k PEG chains were approximately 25–33.6 nm while those with 5k PEG chains were approximately 15.7–21.9 nm. In another study, the thickness of the PEG layer using 2k PEG on the surface of PEGylated liposomes. Modifications on PEG can

Table 1. Particle Size, Polydispersity Index (PDI), and Zeta Potential of GNPs, PEG-GNPs, MUA/PEG-GNPs, α-CD-GNPs, β-CD-GNPs and γ-CD-GNPs

| Formulation          | Particle size (nm) | PDI   | Zeta potentials (mV) |
|----------------------|-------------------|-------|----------------------|
| GNPs                 | 5.3±0.9           | 0.39±0.20 | N.D.                |
| PEG-GNPs             | 34.5±0.4          | 0.24±0.02 | −9.9±2.0            |
| MUA/PEG-GNPs         | 31.3±1.1          | 0.42±0.07 | −14.5±0.3           |
| α-CD-GNPs            | 26.5±4.0          | 0.36±0.17 | −9.7±0.5            |
| β-CD-GNPs            | 26.3±0.3          | 0.45±0.10 | −9.1±1.5            |
| γ-CD-GNPs            | 29.2±2.2          | 0.34±0.10 | −9.5±1.3            |

Data are shown as the mean±standard deviation (S.D.) (n=3).

**Fig. 2. TEM Images of the Cur-α-CD-GNPs (α=α, β, γ)**

Scale bars indicate 20 nm.
affect the thickness of the layer and the thickness of the PEG layer on the liposome was estimated to be 3.5–4.5 nm. Thus, the PEGylation of nanoparticles can affect dynamic light scattering results. Further conjugation of CD with the GNPs resulted in particle diameters similar to that of simple PEG-GNPs (Table 1). The zeta potentials of PEGylated nanoparticles and cur-CD-GNPs were nearly neutral (<10 mV) whereas MUA/PEG-GNPs had a slight negative charge (approximately −15 mV). These results suggested that PEGylation greatly affects the surface change of nanoparticles. Setua et al. reported that surface modification with MUA prior to conjugation with cisplatin and GNPs made the GNPs negatively charged.26)

TEM images of cur-CD-GNPs are shown in Fig. 2. The cores of the GNPs, with a mean particle size of approximately 5 nm, were evident and the dispersion appeared uniform. The white contrast around the GNPs is likely the PEG-layer. The total diameter of the cur-CD-GNPs was less than 10 nm, which is inconsistent with the results obtained using dynamic light scattering (Table 1, around 30 nm) and may be due to drying process of the GNPs prior to TEM imaging.

Then we assessed the amount of curcumin incorporated into the GNPs. Solubility phase diagrams of curcumin with different amino-CD solutions are shown in Fig. 3 and indicate that the order of solubility was $\beta$ amino-CD > $\alpha$ amino-CD > $\gamma$ amino-CD. Thus, curcumin is efficiently incorporated into $\beta$ amino-CD. Most reports to date describe the use of $\beta$-CD for incorporating drugs, including curcumin, and there is little information regarding the use of other CDs.21) Curcumin is incorporated into $\beta$-CD at a molar ratio of 1/2.27,28)

Next, we measured the UV-Vis spectra of free curcumin, CD-GNPs, and cur-CD-GNPs to investigate curcumin incorporation into the CD-GNPs (Figs. 4A–G). Optical peaks

Fig. 3. Phase-Solubility Diagrams of the Curcumin Amino-CD Complexes
Data are shown as the mean±S.D. (n=3).

Fig. 4. UV-Vis Spectra of Free Curcumin, CD-GNPs, and Cur-CD-GNPs
A) Curcumin, B) $\alpha$-CD-GNPs, C) $\beta$-CD-GNPs, D) $\gamma$-CD-GNPs, E) cur-\(\alpha\)-CD-GNPs, F) cur-\(\beta\)-CD-GNPs, G) cur-\(\gamma\)-CD-GNPs.
corresponding to GNPs and curcumin were observed in the spectra of cur-CD-GNPs. The loading efficiency of curcumin into CD-GNPs was measured by comparison of the CUR UV-Vis absorptions in CD-GNP solution and cur-CD-GNP solution and the approximate loading efficiency of CUR in α-CD, β-CD and γ-CD was 7.4±0.9, 13.1±3.1, and 5.2±1.1%, respectively, consistent with the phase diagram (Fig. 3).

To understand the potential therapeutic effects of cur-CD-GNPs, we assessed the cytotoxicities of cur-CD-GNP formulations incorporating different CDs on a lung cancer cell line. As shown in Fig. 5, CD-GNPs which did not contain curcumin did not exhibit cytotoxicity. These results mean that gold nanoparticle is a safe nanocarrier. In our previous study, PEGylated gold nanoparticle with or without therapeutic antibody-fragment did not exhibit marked cytotoxicity. These results support that gold nanoparticle is useful nanocarrier. In contrast, all three cur-CD-GNPs exhibited a significant cytotoxic effect compared with control. The cytotoxic effect of cur-CD-GNPs was similar to that of curcumin solution. The intracellular behavior of CD-GNPs was investigated using coumarin-6, a lipophilic fluorescent dye (Fig. 6). The coumarin-6 solution rapidly permeated throughout the A549 cells whereas coumarin-6 incorporated into CD-GNPs localized in the cells, suggesting that coumarin-6 localizes into the endosome after being taken up into the cells. A large amount of curcumin solution rapidly permeated into the cells and curcumin crystallized during incubation in medium due to the poor solubility of the drug. We previously showed that the cytotoxic effect of curcumin solution is limited, and that nanopulverized curcumin and the incorporation of curcumin into CD using ultra-short laser pulses improved cytotoxicity.

In contrast, cur-CD-GNPs are taken up into cells via endocytosis. We estimated that PEGylation on the surface of on Cur-CD-GNPs could prevent the intracellular uptake like other nanoparticles such as liposomes and polymer micelles, but the high specific gravity of the GNPs (specific gravity of Au is 19.32 g/cm³ at r.t.) increased the interaction with cell surface, resulting in cellular uptake in in vitro conditions. After the addition of coumarin-6-CD-GNPs into A549 cells, several fluorescent dots were found on the images (e.g., α-CD-GNPs, especially), which means that the nanocarrier is localized in endosome, and the particles were taken up via endocytosis. The cur-CD-GNPs seemed to be released curcumin gradually in the cells. This controlled release of curcumin may result in a cytotoxic effect similar to that of curcumin solution (i.e., rapid cytotoxicity). Although we did not conduct the experiment of drug release, the release of curcumin from CD-GNP is expected to follow the mechanism of equilibrium reaction between host molecule (CD) and guest molecule (curcumin). For example, Heo et al., developed paclitaxel-included CD-GNP and the drug was released over 24h. We think that similar tendency could be obtained. The therapeutic effect of curcumin is time-dependent and concentration-dependent. The results of intracellular behavior partially mention the mechanism cytotoxic effect of cur-CD-GNPs.

CONCLUSION

In conclusion, a multifunctional gold nanoparticle-based nano-formulation was developed and its properties were characterized. The relation between curcumin and three types of amino-CDs (α, β, γ) was clarified. While there was difference in encapsulation efficiency among these CDs, there was similar cytotoxicity observed between three CDs in the current study. These results suggested that intracellular behavior of curcumin release from CDs also affected the therapeutic effect. The use of amino-CDs is still rare information for the conjugation with gold nanoparticle carrier. So, the information obtained in the present study is useful for the readers in this region. The conjugation of PEG and CDs with gold nanoparticles has potential as an effective strategy for drug delivery and imaging. The poor water solubility of curcumin was improved by loading it into CD-conjugated gold nanoparticles. The methodology described in the present study is ap-

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Fig. 5. Cytotoxic Effects of the Cur-CD-GNPs
The curcumin concentration was 50µg/mL. Data are shown as the mean±S.D. (n=3). *p<0.05, statistically significant.
Applicable to other poorly-water-soluble drugs and compounds. PEGylation of the nano formulation should enhance the in vivo therapeutic effect via the EPR effect, although further in vivo investigation is necessary.

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Conflict of Interest The authors declare no conflict of interest.

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