Colloidal gold nanoparticle conjugates of gefitinib

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ABSTRACT

Gefitinib (GF) is a US Food and Drug Administration-approved epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor for treating the lung cancers. We fabricated colloidal gold nanoparticle (AuNP) conjugates of the GF anticancer drug by self-assembly to test their potency against A549, NCI-H460, and NCI-H1975 lung cancer cells. GF adsorption on AuNP surfaces was examined by UV–vis absorption spectra and surface-enhanced Raman scattering. Density functional theory calculations were performed to estimate the energetic stabilities of the drug-AuNP composites. The N1 nitrogen atom of the quinazoline ring of GF was calculated to be more stable than the N3 in binding Au cluster atoms. The internalizations of GF-coated AuNPs were examined by transmission electron and dark-field microscopy. A cell viability test of AuNP–GF conjugates with the EGFR antibody exhibited much higher reductions than free GF for A549, NCI-H460, and NCI-H1975 lung cancer cells after treatment for 48 h.

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1. Introduction

Recently, understanding and controlling interfacial phenomena in biotechnology has become increasingly important in both fundamental research and industry [1]. Noble metal nanoparticles (NPs) have been introduced as novel drug delivery systems for anticancer therapy [2–5]. Gold nanoparticles (AuNPs) after binding with the drugs may overcome antibiotic drug resistance [6]. AuNPs are known to be less toxic and can possibly be used in a hospital environment for immunotherapy [7]. Surface-enhanced Raman scattering (SERS) has been used to examine the adsorption structures in bioconjugate chemistry [8,9]. Quantum mechanical calculations can be helpful to better understand the adsorption structures of self-assembled monolayers on metal cluster atoms [10].

Lung cancer is one of the leading causes of cancer-related deaths worldwide [11]. Expressed epidermal growth factor receptor (EGFR) can be utilized in lung cancer as a prime candidate for the development of targeted therapeutics. Targeting EGFR is thus one of the most promising strategies for lung cancer therapy. In association with EGFR, tyrosine kinase inhibitor (TKI) has emerged as an effective cure for some patients [12–16]. An EGFR-targeting small-molecule inhibitor, gefitinib (GF), received approval from the US Food and Drug Administration as treatment for lung cancer patients, because it specifically targets the tyrosine kinase activity of EGFR, is one of the most popular and commercially available drugs to treat lung cancers [17–19].

Despite previous studies [2–7] of nanoparticle conjugates with anticancer drugs, there have been no reports on the interfacial behavior of GF on AuNPs. GF may coordinate with AuNP surfaces via the quinazoline ring. In this work, we fabricated an AuNP conjugate of GF by self-assembly. Density functional theory (DFT) calculations were introduced to predict the plausible geometries of GF on an Au atom cluster. The adsorption behavior of GF on AuNP surfaces was studied mainly by means of SERS. A cell viability test was performed to examine their potency against A549, NCI-H460, and NCI-H1975 lung cancer cells. Our work may be potentially useful in the application of AuNP conjugates in lung cancer therapy.
2. Experimental

2.1. Chemicals

HAuCl₄ and citrate were purchased from Sigma–Aldrich (St. Louis, USA). GF and mouse monoclonal anti-EGFR antibody (catalog number: Ab62, concentration 1.0 mg/mL) were purchased from Selleck Chemicals (Houston, USA) and Abcam (Cambridge, UK), respectively. Dimethyl sulfoxide (DMSO) was purchased from Daejung (Sihheung, Korea). The (ortho-pyridyl) disulfide-poly(ethylene glycol)–N-hydroxysuccinimidy ester (OPSS-PEG-NHS) linker was purchased from CreativePEGWorks (Winston Salem, USA).

2.2. Preparation of nanoparticles

Citrate-modified AuNPs were prepared according to the previous procedure [20]. The AuNP–GF sample was prepared by adding 10 μL of GF to 1 mL of AuNPs via a self-assembly process. The mixture was centrifuged at 10,000 rpm for 5 min. To estimate the loading efficiency of GF onto AuNPs, the UV band of 1 mL of supernatant solution was compared with that of 1 mL of the precipitated solution redispersed in DMSO. The loading efficiency of AuNPs–GF (10⁻⁵ M) in DMSO was estimated to be 26–46%, depending on the solvents and concentrations. To attach the EGFR targeting moiety to AuNPs, we suspended 10 μL of antibody (1 mg/mL) in 90 μL of NaHCO₃ (pH 8.5) according to the previous report [21]. The OPSS-PEG-NHS linker was prepared with a concentration of 1 mg/mL (1 mg of OPSS in 1 mL of NaHCO₃ of pH 8.5). Then, 100 μL of antibody and 100 μL of OPSS-PEG-NHS in a ratio of 1:1 was mixed and maintained at 4 °C for approximately 3 h. Subsequently, 1 mL of AuNP–GF was added to 200 μL of antibody-OPSS-PEG-NHS and maintained at 4 °C overnight. The mixture solution was then centrifuged at 10,000 rpm for 5 min. After collecting the precipitate, it was dissolved again in 1 mL of DMSO solution. AuNPs–GF-antibody was prepared with a GF concentration of 10⁻⁴ M.

2.3. Equipment and characterization methods

This amount of AuNP conjugates was diluted to 10⁻⁵ M by DMSO solvent and used for cell counting kit (CCK) experiment. The UV–vis absorbance spectrum of the surface plasmon band from the AuNP solution was obtained using a Scino Mecasy 3220 spectrophotometer. The size distributions of AuNPs were checked using either a JEOL JEM-3010 or JEM-1010 transmission electron microscope (TEM). Raman spectra equipped with dark-field microscopy (DFM) were obtained using a Renishaw RM 1000 microRaman spectrometer as in the previous report [22]. The excitation wavelength was 632.8 nm. The spontaneous scattering from the sample was dispersed through a holographic notch filter and collected via a CCD camera.

2.4. DFT calculations

DFT calculations on Raman frequencies and assignments for the six Au atom cluster models were performed with B3LYP/LanL2DZ functional/basis set using Gaussian 09 software [23]. The solvent effect caused by water molecules was considered by using the polarized continuum model (PCM) implemented in Gaussian 09 software. Our PCM model recognized the aqueous environment, where all the experiments were performed. Regarding the possibility of a parallel orientation, the geometry of 16 Au atom cluster was optimized with B3LYP/LanL2DZ functional/basis set. The binding structures of GF to the gold cluster were obtained while the geometry of gold cluster was fixed.

2.5. Cell culture methods and cytotoxicity

A549 (American type cell culture (ATCC), chronic lymphocytic chronic (CCL)-185), NCI-H460 (ATCC human tumor bank (HTB)-177, National Cancer Institute), and NCI-H1975 (ATCC th bronchogenic cancer (TCP)-1016, National Cancer Institute) were kindly provided by the National University. These are assumed to be GF-resistant-cancer cell lines [12]. They were grown on Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS and antibiotics at 37 °C in a 5% CO₂ atmosphere incubator. RPMI and fetal bovine serum were obtained from WelGene (Daegu, Korea). The lung cancer cells were plated at a concentration of 1 × 10⁶ cells per dish on a SPL 100 mm diameter cell culture dish (Seoul, Korea) containing growth medium. AuNPs were added and the cells were incubated at 37 °C in 5% CO₂. After 48 h, the cells were harvested and washed with Dulbecco’s phosphate buffered saline solution. A cell viability test was performed using a Donjindo CCK-8 kit. A Tecan Model F300 microplate reader was used to measure the absorbance change.

3. Results and discussion

3.1. Molecular structure and DFT calculations

Fig. 1 illustrates the molecular structure of GF, which mainly consists of aromatic quinazoline and phenyl rings. We expect that GF may adsorb on the AuNPs via the nitrogen atoms of the quinazoline ring [22]. Considering that most strong SERS bands can be ascribed to the quinazoline ring, we may assume that GF may bind on Au via its nitrogen atoms. It is recognized that a cluster of just six atoms may be insufficient to explain the realistic adsorption behaviors of GF on AuNPs. Unlike erlotinib with a strong anchoring unit of the acetylenic group [22], GF may weakly bind on AuNPs with its quinazoline ring and lead to a rather flat bound geometry. Our DFT calculations of GF on 16 Au atom predicted that the binding energy would be 11.69 kcal/mol, which would be quite comparable to those of the standing geometries despite the difference of the Au substrate sizes.

Although not shown here, the υ(CH) band at ~3050 cm⁻¹, an indicator of the upright geometry, was barely identified, suggesting a rather flat geometry [24]. Considering that the aliphatic linker is distant from the quinazoline ring, the conformational variance would not affect the coordination at the surface. Based on DFT calculations of energetic stabilities using the PCM model, the N1 nitrogen atom position in the quinazoline ring of GF is predicted to be more stable in binding with the Au cluster atoms than those of the N3 position by 4.75 kcal/mol (summarized in Table 1). According to the Boltzmann distribution formula, the population of the adsorbed states with the energetic difference of 4.75 kcal/mol, may be calculated to be as low as ~0.03%. The bond length of the N1 atom was calculated as 2.20 Å, which was shorter than 2.25 Å in the case of the N3 atom.

<table>
<thead>
<tr>
<th>Configurations</th>
<th>B.E. (kcal/mol)</th>
<th>Distance (Å)</th>
</tr>
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<tr>
<td>GF + Au6 - N3</td>
<td>13.11</td>
<td>Au - N3 2.25</td>
</tr>
<tr>
<td>GF + Au6 - N1</td>
<td>17.86</td>
<td>Au - N1 2.20</td>
</tr>
<tr>
<td>GF + Au16 parallel</td>
<td>11.69</td>
<td>Au - N3 4.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Au - N1 4.07</td>
</tr>
</tbody>
</table>
Fig. 1. (a) Molecular structure of GF. Optimized structures of GF with (b) N3 and (c) N1 binding onto the six Au atoms. (d) A parallel geometry of GF on 16 atom Au surfaces.

Fig. 2. (a) UV spectra of free and supernatant GF. UV–vis spectra after conjugating GF with the antibody and applying 2 mM GSH on AuNPs. (b) TEM images of GF-coated AuNPs. The scale bars in the left and right images are 200 nm and 5 nm, respectively.
3.2. **UV–vis absorption spectra of GF on AuNPs and loading efficiency**

The UV absorption band at 330 nm may be ascribed to the aromatic ring mode of GF. The loading efficiency of GF at the initial concentration of $10^{-5}$ M was estimated as 26–46% by subtracting the unbound GF in the supernatant solution from the absorbance measurements. Depending on the initial concentrations of GF and solvents, the loading efficiencies appeared to be calculated slightly differently. The surface plasmon band could be ascribed to a collective motion of electrons on the surface of AuNPs in aqueous colloidal solutions. The surface plasmon band redshifted after the adsorption of GF on AuNPs. After the assembly of antibodies, the UV–vis spectra appeared to return almost to the original positions of the pristine AuNPs owing to the extended interparticle separation via the PEG linker. The TEM images in Fig. 2(b) suggest a slightly aggregated structure of AuNP–GF conjugates. The images on the right show an aggregated structure. In order to better understand the interfacial phenomena of GF on AuNPs, we performed Raman spectroscopy.

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**Fig. 3.** (i): Ordinary Raman (OR) in the solid state and (b) SERS spectra after GF adsorption on AuNPs. (ii) Concentration-dependent SERS spectra of GF on AuNPs and (iii) intensity plots of the bands at ~690 cm$^{-1}$. SERS spectra of pristine AuNPs and 2 mM GSH are also listed for comparison. The error bars show the standard deviations of the three independent measurements.

**Fig. 4.** (a) SERS spectra of GF on AuNPs in cell culture media of 0–100% according to the procedures described in Ref. [22]. (b) Time-dependent SERS spectra after treating 2 mM GSH for 0, 5, 15, 30, and 60 min.
3.3. Raman spectra

Fig. 3(i) shows Raman spectra before and after the adsorption of GF on AuNPs. The spectral changes were not found to be dramatically different after adsorption of the solid GF on the solution phase AuNPs. Despite the drug adsorption on the AuNPs, the intrinsic SERS intensity of GF appeared to be rather weak in comparison to other simpler aromatic adsorbates such as thiophenol. The vibrational bands between 1300 and 1425 cm$^{-1}$ can be ascribed to the quinazoline ring modes. These bands appeared to change significantly upon adsorption on AuNPs, suggesting interaction with the surfaces. One of the strongest bands at 1337 cm$^{-1}$ appeared to red shift to 1301 cm$^{-1}$ upon adsorption on Au. AuNPs were covered and stabilized with the organic citrate anions. Tripeptides with aliphatic chains do not have any strong Raman bands even at their concentrations higher than the aromatic drug compound of GF. The comparative Raman spectra of 2 mM GSH on AuNPs and pristine AuNPs showing no strong SERS bands were also compared in Fig. 3(ii). Spectral data with the appropriate vibrational assignments for GF based on the DFT calculations are summarized in Table 2. Concentration-dependent spectra indicated that the SERS intensities would show their maximum values at $\sim 10^{-5}$ M of GF as shown in Fig. 3(iii) and (iv). This concentration of $\sim 10^{-5}$ M can correspond to full-monolayer coverage of aromatic adsorbates on AuNPs. Fig. 4(a) and (b) shows that GF may detach easily in 2 mM glutathione (GSH) solution but not in cell culture medium solution. The concentration of the cell culture media solution was adjusted by the previously reported method [25]. This result suggests that GF may be released inside cells but not by the cell culture media solution after the endocytosis of the AuNP conjugates.

3.4. Cellular uptake

Fig. 5(a) shows TEM images of GF-assembled AuNPs in A549 cells after the uptake. The arrows indicate the locations of internalized AuNPs. We found that GF-assembled AuNPs were internalized in either endosomal or lysosomal compartments indicated by the arrow. Fig. 5(b) of the DFM image also exhibited the internalized AuNPs inside cells. Although not shown here, we could not observe any discernible GF Raman peaks inside cells, presumably owing to the intracellular release of GF via glutathione as in the previous report [25]. Considering that GF would release in cytosols via intracellular GSH, the endosomal encapsulation may not affect its efficacies significantly. In order to check the potential application of AuNP–GF conjugates in drug delivery, we performed a cell viability test.

3.5. Cytotoxicity of GF-assembled AuNPs in A549, NCI-H460, and NCI-H1975 cells

A549, NCI-H460, and NCI-H1975 cells are known to have resistance to GF owing to the mutation in EGFR [11,12]. Fig. 6 shows

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Spectral data and vibrational assignments for GF.</th>
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<tr>
<td>Cal.</td>
<td>OR</td>
</tr>
<tr>
<td>693</td>
<td>690</td>
</tr>
<tr>
<td>770</td>
<td>774</td>
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Unit in cm$^{-1}$. Abbreviations: Cal., calculated values; ν, stretching; β, in-plane bending; γ, out-of-plane bending. The assignment is based on the present DFT calculation of Raman frequencies for the GF N1-coordinated six Au atom cluster models with B3LYP/LanL2DZ functional/basis set. No scale factor was applied in the calculated vibrational positions. OR positions were listed after the baseline correction.
the viability of A549, NCi-H460, and NCi-H1975 cells in 5–100 nM GF. We have chosen a GF-resistant cell to investigate the efficacies of the AuNPs–GF-antibody. The used AuNPs did not affect the cell viabilities significantly as in the previous report [26]. We have to mention that only GF–AuNP conjugates without any targeting moiety showed similar cell viability reduction to free GF. Introducing the antibody was thus beneficial to reduce cell viability. It was found that cell viability decreased by 30–80% after assembling the GF–AuNP–antibody than those that used only free drugs at the concentrations of 0.05 and 0.1 μM for the three lung cancer cell lines. We plan to utilize our method as a potentially useful drug delivery system to enhance the efficacy of treating lung cancer cells.

4. Conclusions

Interfacial structures of GF on AuNPs were examined mainly by Raman spectroscopy. DFT calculations predicted plausible adsorption structures on Au cluster atoms. A cytotoxicity test indicated that a complex with the targeting antibody could damage lung cancer cell lines more effectively. Cell viability appeared to decrease by 30–80% after treating antibody-conjugated AuNPs with GF concentrations of 0.05 and 0.1 μM, if compared to using only free GF. Our results suggest that AuNPs can be successfully employed for conjugating the TKI inhibitor of GF.

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References


Fig. 6. Cell viability tests of free GF and AuNP–GF conjugates attached with the antibody for (a) A549, (b) NCi-H460, and (c) NCi-H1975 lung cancer cells after 48 h. The error bars show the standard deviations of three independent measurements.