

Research Article

The Synergistic Anticancer Effect of Dual Drug- (Cisplatin/Epigallocatechin Gallate) Loaded Gelatin Nanoparticles for Lung Cancer Treatment

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Received 2 April 2020; Revised 16 June 2020; Accepted 29 June 2020; Published 5 August 2020

Academic Editor: Ana Espinosa

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Lung cancer has the highest mortality of any cancer worldwide, and cisplatin is a first-line chemotherapeutic agent for lung cancer treatment. Unfortunately, cisplatin resistance is a common cause of therapeutic failure. The ability to overcome chemoresistance is crucial to the effective treatment of lung cancer. Recently, epigallocatechin gallate (EGCG), a type of polyphenol extracted from tea, has been shown to suppress the rapid proliferation of cancer cells, including lung cancer. We tested whether nanoparticles (NPs) carrying a dual drug load, cisplatin and EGCG, could overcome chemoresistance to cisplatin, by working together to kill lung cancer cells. Self-assembling gelatin/EGCG nanoparticles (GE) were synthesized, and cisplatin was then incorporated, to construct a dual drug nanomedicine (EGCG/cisplatin-loaded gelatin nanoparticle, named as GE-Pt NP). The particle size and zeta potential were examined by dynamic light scattering (DLS). The morphological structure of GE-Pt NPs was observed by transmission electron microscopy (TEM). *In vitro* testing was performed using a human lung adenocarcinoma cell line (A549). A cytotoxicity examination was performed, using a WST-8 cell proliferation assay. Intracellular cisplatin content was quantified by inductively coupled plasma mass spectrometry (ICP-MS). In conclusion, we successfully prepared GE-Pt NPs, as spherical structures, approximately 75 nm in diameter, with a positive charge ($+19.83 \pm 0.25$ mV). The encapsulation rate of cisplatin in GE-Pt was about 63.7%, and the EGCG loading rate was around 89%. A relatively low concentration of GE-Pt NPs (EGCG 5 $\mu\text{g/mL}$: cisplatin 2 $\mu\text{g/mL}$) exhibited significant cytotoxicity, compared to cisplatin alone. The GE-Pt NPs are freely taken up by cells via endocytosis, raising the intracellular cisplatin concentration to a therapeutic level. We consider that combination therapy of cisplatin and EGCG in nanoparticles (GE-Pt NPs) may help overcome cisplatin resistance and could effectively be used in the treatment of lung cancer.

1. Introduction

Lung cancer is one of the most common cancers. It has high mortality, worldwide, with estimated numbers of over two million new cases being diagnosed and more than 1.7 million deaths annually [1]. Currently, platinum-based chemotherapy regimens are used for broad-based treatment of lung cancer patients. The four platinum-containing compounds, cisplatin, carboplatin, oxaliplatin, and nedaplatin, have become common anticancer drugs [2]. Compared to other anticancer drugs, cisplatin can be used for many types of cancer, such as cancers of the lung, head, neck, and cervix, and can prolong the survival time of patients [2]. Cisplatin (*cis*-diammine-dichloro-platinum or cDDP) has a heavy metal complex, with a central platinum atom, surrounded by two chlorine atoms and two molecules of ammonia. The cytotoxic effect of cisplatin involves its reaction with DNA to form interstrand and intrastrand cross-linking, which inhibits DNA replication and RNA transcription, leading to DNA breakage and ultimately triggering apoptosis [3, 4]. However, cisplatin resistance is the major cause of therapeutic failure, so five-year survival rates of lung cancer are still too low to satisfy [5, 6]. Common side effects of cisplatin include tiredness, malaise, vomiting, nephrotoxicity, and hearing loss, which can also lead to patient weakness and treatment failure [7]. Therefore, ideally, a therapeutic agent is needed that can overcome chemoresistance and avoid the side effects of cisplatin, for maximum damage to cancer cells and a lasting therapeutic effect.

Green tea is one of the most popular beverages in the world. It contains various polyphenols, mainly catechins, which include epicatechin (EC) and epigallocatechin (EGC) as hydroxy derivatives, epicatechin gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG) [8, 9]. EGCG is one of the most abundant polyphenols in green tea. It contains three hydroxyl substituents on the B ring and a gallate moiety esterified on the third carbon (C3) on the C ring. These structures contribute to its activity as an antioxidant and iron chelation [10]. EGCG has several pharmacological and biological properties, including antioxidant, antibacterial, cardioprotection, antiatherosclerosis, anti-inflammation, neuroprotection, and anticarcinogenesis [11, 12]. Accumulating evidence has demonstrated that EGCG inhibits tumor growth, invasion, angiogenesis, cancer stemness, metastasis and affects multiple signaling pathways by binding to various molecular targets, such as mitogen-activated protein kinase (MAPK), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), and epithelial-mesenchymal transition (EMT) [13]. EGCG was also reported to enhance the effects of chemotherapy and target therapy in lung cancer cells [14]. Therefore, we hypothesize that therapeutic agents, in combination with EGCG, may provide an effective way to overcome drug resistance, exemplified by the resistance of tumor cells to cisplatin.

Nanomedicine is a field that combines nanotechnology, pharmaceutical, and biomedical sciences to develop drugs loaded in nanocarriers, with higher therapeutic effects and minor side effects, for the treatment of diseases, especially

cancer [15]. Nanocarriers have unique properties, such as nanoscale size and a high ratio of surface area to volume, and have the potential to modulate the pharmacokinetics and pharmacodynamics of drugs, thereby enhancing their therapeutic effectiveness. Loading the drug into the nanocarrier can enhance its stability in the body, prolong the circulation time of the compound in the blood, and control the rate of drug release. Due to specific physiological conditions in the cancerous tissue, there can be enhanced permeability and an enhanced retention effect (EPR) [15, 16]. In addition to having characteristics such as high permeability and a long lasting effect for cancer treatment, nanoparticle-based drug delivery systems also have many unique properties that can improve the antitumor response. For example, the encapsulation of a hydrophobic drug in hydrophilic nanocarriers can overcome the problem of solubility and chemical stability of anticancer drugs [17]. Active targeting or cell membrane targeting chimeric peptides of nanoparticles which can be achieved by surface modification of specific antibodies or peptides on nanoparticles can recognize and target cancer cells enhancing tumor eradication capacity [18–20]. Furthermore, for modulation of drug release, metal-based nanocarriers that can be pH-triggered, near-infrared (NIR) light-responsive, or photosensitive have been designed for drug release in high local concentrations at a suitable site, in tumor cells [21, 22]. Multifunctional nanocarriers that combine different functional antitumor drugs and are used for codelivery of multiple medications by nanoparticles to exhibit synergistic antitumor efficiency were also investigated [17, 19, 21]. Drug-loaded NPs can change the biodistribution of the drug, causing accumulation in the tumor area. The effectiveness of cisplatin-loaded gelatin nanoparticles for lung cancer treatment has been demonstrated in previous studies, both *in vitro* and *in vivo* [23, 24]. To reduce the resistance to cisplatin, we developed a dual drug delivery system based on cisplatin/EGCG-loaded gelatin nanoparticles (GE-Pt NPs), to exert synergistic antitumor activity. As mentioned above, multifunctional nanocarriers are an approach which involves the loading of two or more drugs into a single well-designed nanocarrier, which uses different mechanisms or targets multiple pathways to improve the therapeutic outcome [25]. Unlike in the case of a single regimen, multiple drugs can produce broad and sustained cellular toxicity that can provide the opportunity for overcoming intrinsic or acquired drug resistance [25–27]. Cisplatin and EGCG can work together to kill cancer cells by different mechanisms enhancing their toxicity to cells for treating tumorous cells/tissue. In this study, the optimal parameters for the preparation of GE-Pt NPs, to achieve high drug loading rates and slow drug release profiles, were investigated. The anticancer effect and the cellular uptake of the GE-Pt NPs were investigated in A549 cancer cells and compared to cisplatin solution, to evaluate GE-Pt NP's toxicity.

2. Materials and Methods

2.1. Materials and Reagents. Gelatin type A (porcine skin with bloom 110), EGCG ($\geq 95\%$), *cis*-diammineplatinum(II) dichloride (cisplatin), and Cell Counting Kit-8 (CCK-8) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). The o-phenylenediamine (oPDA) was acquired from Alfa Aesar Co. Ltd. (Lancashire, UK). Dimethylformamide (DMF) was purchased from J.T. Baker (Pittsburgh, PA, USA). The Spectra/Por® membrane dialysis products (MW 3500) were acquired from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). The Nutrient Mixture F-12 Ham and penicillin-streptomycin-neomycin (PNS) were obtained from Life Technologies (Eugene, OR, USA). Phosphate-buffered saline (PBS), insulin, trypsin-EDTA, and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, USA). The rest of the chemicals were purchased from Sigma-Aldrich.

2.2. Nanoparticle Synthesis and Characterization

2.2.1. Preparation of Cisplatin-Loaded EGCG-Gelatin Nanoparticles. An equal volume of gelatin (type A, 0.44% w/v) and EGCG solution (0.44% w/v) was mixed gently to form the self-assembly NPs, by stirring, as previously described [28, 29], to make GE NPs. Then, cisplatin solution (1.5 mg/mL in double-distilled water) was added to the GE NP mixture, stirring for another 6 hours. The cisplatin-loaded GE NPs were prepared, which are referred to as GE-Pt NPs. A diagram of the process of preparation and the ion exchange between gelatin and cisplatin.

2.2.2. Characterization of GE-Pt NPs. The size and zeta potential of these NPs were characterized by using a dynamic light scattering analyzer (DLS) (Zetasizer-ZS90 Plus; Malvern, UK) at 25°C with light scattering at an angle of 90°. The morphology of NPs was examined with a transmission electron microscope (TEM, Hitachi HT-7700, Tokyo, Japan). NPs for TEM examination were pretreated with 0.5% uranium acetate for good image contrast. The chemical components for each material were detected by Fourier transform infrared spectroscopy (FTIR, JASCO 4200, Tokyo, Japan). A thermogravimetric analyzer (TGA, Pyris 1, PerkinElmer, CT, USA) was used to examine the thermal behavior change of each component. Raw materials and the colloidal suspension of NPs, for FTIR and TGA examination, were lyophilized to obtain powders for the test. For the FTIR assay, each sample was mixed with KBr. For the TGA assay, 10.0 mg powder sample was used and heated in a TGA at a rate of 20°C/min, from room temperature to 800°C, in an O₂ environment.

2.2.3. Encapsulation Efficiency (EE) and Release Profiles of GE-Pt NPs. The GE-Pt NPs suspension was centrifuged at 12,500 rpm for 30 min in a centrifuge tube (MWCO 10 kD). The filtered suspension of GE-Pt NPs (100 µL) was used for quantifying the unloaded EGCG and cisplatin. The EGCG solution was examined with a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific) at 274 nm. The cisplatin concentration of GE-Pt NPs was quantified by the o-phenylenediamine (oPDA) method, by measuring the unencapsulated cisplatin in suspension [30]. The suspension was added to the oPDA solution (1.2 mg/mL in DMF) in the ratio of 1:1, then heated to 100°C in the dry bath for 10 minutes. The reacting solution was transferred into

a 96-well plate at 100 µL/well. The absorbance of each sample was measured at 704 nm, using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Equation (1) was used to calculate the encapsulation efficiency (EE) of cisplatin.

$$EE (\%) = \frac{\text{total cisplatin} - \text{unloaded cisplatin}}{\text{total cisplatin}} \times 100\%. \quad (1)$$

The same equation was also used for calculating the EE (%) of EGCG. The released profiles of EGCG and cisplatin from the GE and GE-Pt NPs were suspended in phosphate-buffered saline (PBS) at 37°C and were evaluated by a dialysis method. The colloidal suspension of GE NPs and GE-Pt NPs, of known EGCG and cisplatin concentration, was placed inside a dialysis bag and dialyzed against PBS at 37°C. The EGCG and cisplatin released outside the dialysis bag were sampled at indicated time intervals (0, 5, 10, 15, 30, ..., 1200, and 1400 minutes). The EGCG and cisplatin concentrations outside the dialysis bag were measured by the same methods as used in the EE examination.

2.3. In Vitro Examination

2.3.1. Cell Viability Test. To study the anticancer effect of the GE and GE-Pt NPs, for lung cancer treatment, human lung adenocarcinoma cells (A549) were used. The A549 cells were grown in Nutrient Mixture F-12 Ham medium with 10% fetal bovine serum and 1% PSN supplement. Cells were grown in an incubator at 37°C in a 5% CO₂ environment. The cell viability was determined by the CCK-8 assay. A549 cells were seeded in a density of 5 × 10³ cells/well in 96-well plates and incubated overnight. Then, various concentrations of EGCG, cisplatin, GE, and GE-Pt NPs colloidal suspensions were prepared by diluting stock formulations with culture media for EGCG (5, 20, and 80 µg/mL) and cisplatin (2, 8, and 32 µg/mL). At the indicated date (days 1, 3, and 7), the cell viability was detected by using the CCK-8 kit with a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) reduction assay. The working WST-8 agent was added into each well at 110 µL per well and then incubated for 3 h at 37°C. A microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA) was used to read the absorbance optical density (OD) at 450 nm. The percentage of viable cells was calculated in comparison to control cells, cultured with medium only. For the group treated for 7 days, the culture medium was replaced on day 3 by the addition of fresh medium alone. All the experiments were performed in six repeats (*n* = 6) and carried out in three batches. The statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA) using Excel (Microsoft). A probability (*p*) value ≤ 0.05 is statistically significant.

2.3.2. Intracellular Drug Accumulation. For the quantification of intracellular cisplatin accumulation, A549 cells were grown in 24-well culture plates at a concentration of 1.5 × 10⁵ cells per well. After incubation overnight, the cells were

cocultured with cisplatin solution or GE-Pt NPs at the same cisplatin concentration (15 $\mu\text{g}/\text{mL}$) and then incubated at 37°C for 6 and 24 hours. In the control group, the cells were cultured in the normal medium. At the indicated time, cells were washed 3 times with PBS and then detached by trypsinization. The cell pellets were collected and digested by 50% HCl solution at 80°C for 60 min, to release platinum (Pt) ions. The samples were diluted to the proper concentration for analysis. The intracellular Pt concentration in each tested condition was quantified ($n = 3$) using an inductively coupled plasma mass spectrometer, ICP-MS Thermo X-SERIES 2 ICP-MS (Thermo Scientific, Waltham, MA).

3. Results

3.1. Characterization of Dual Drug-Loaded Nanoparticles (GE-Pt NPs)

3.1.1. Size and Zeta Potential Results. The schematic drawing of the formation of gelatin/EGCG self-assembling nanoparticles (GE NPs) is shown in Figure 1(a). Due to the electrical attraction, these two components are forced together into aggregating particles; then, cisplatin is conjugated with the carboxyl group of gelatin, via ion exchange with the $-\text{NH}_2$ on cisplatin [24]. The colloidal solution of GE and GE-Pt is revealed in Figure 1(b), showing a good condition of semi-transparent suspension without precipitation (Figure 1).

In Table 1, the GE NPs were approximately 78.5 nm in diameter, with zeta potential at $+13.23 \pm 0.72$ mV. The EGCG encapsulation efficiency (EE) is good, at around 90%. After cisplatin loading (1.5 mg/mL), there was no significant difference in particle size (GE-Pt, 74.4 ± 9.7 nm), but the zeta potential increased to $+19.38 \pm 0.25$ mV. The addition of cisplatin left the EE unchanged, at around 90%, for GE/GE-Pt NPs. The EE of cisplatin is $63.67\% \pm 2.98\%$ in GE-Pt NPs, which is acceptable. Both NPs, suspended in water, had a low polydispersity index (PDI, ~ 0.15), indicating monodispersion.

The monodispersion of GE and GE-Pt NPs can also be confirmed from the DLS results by its narrow peak distribution, as shown in Figure 2(a). The morphologies of GE and GE-Pt NPs were revealed by TEM, shown in Figure 2(b), round and spherical structures of the particles were observed, with no aggregation, and the particle size of these two NPs was around 50–100 nm. Some smaller particles were found, but this may due to dehydration during the preparation of TEM samples.

3.1.2. FTIR and TGA Results. Figure 3(a) shows the FTIR pattern of the raw materials (gelatin, EGCG, and cisplatin) and their nanoparticles (GE, GE-Pt). The spectra of the gelatin reflecting the C=O bending band at 1640 cm^{-1} and the amide band (N-H) at 1550 cm^{-1} were observed [31]. In the gelatin, a weak carboxyl group stretching vibration was noted at 1160 cm^{-1} [31, 32]. Characteristic peaks of EGCG were found at wavelengths of 1700 ($-\text{COO}$), 1620 (C=C aromatic ring), and 1234 ($-\text{OH}$ aromatic ring) [28, 33, 34]. The peak of cisplatin (NH) was found at 1299 cm^{-1} . The amine peaks of cisplatin were seen at 1625 cm^{-1} (NHN) for asymmetric

bending, 1316 cm^{-1} (NHN) for symmetric bending, and 1299 cm^{-1} (NHN) for symmetric bending [35]. Although GE and GE-Pt NPs had EGCG and cisplatin incorporated, the EGCG and cisplatin amount in total gelatin-based NPs was too low to be detected and distinguished by FTIR. Their FTIR patterns were similar to that of gelatin. Thermal analysis was used to identify the metal (Pt) component of the cisplatin.

The result of the TGA assay is provided in Figure 3(b). The weight loss between 50 and 200°C in the GE is due to the adsorbed water lost during heating. Weight lost from 200 to 450°C is due to the decomposition of proline in gelatin. Glycinin was lost from gelatin between 450 and 620°C. All of the organic components were lost around 800°C, and the total weight loss reached 100% with no residues above 700°C. A similar weight loss pattern was found in GE-Pt from 50 to 200°C. There is a dramatic weight loss seen in GE-Pt above 200°C, from the $\text{Cl}-\text{NH}_2$ decomposition of cisplatin, since cisplatin decomposes from 225 to 249°C [36]. The fraction remaining above 700°C ($\sim 26\%$) was the platinum from the cisplatin, loaded into GE-Pt, since it has been previously reported that the residual weight in CDDP, above 1000°C, is Pt [37]. From this result, we confirmed the cisplatin loading in GE-Pt NPs, by TGA, calculated as 0.57 mg/mL cisplatin.

3.1.3. Dual Drug Release from Variant Nanoformulations. The *in vitro* release of EGCG and cisplatin from the two NPs (GE or GE-Pt) was determined by placing them in a dialysis bag, followed by immersion in phosphate buffer (PBS, pH 7.4) at 37°C for 1400 min. Figure 4(a) shows the accumulated EGCG release rate from EGCG solution, GE, and GE-Pt NPs as $78.2 \pm 0.1\%$, $45.0 \pm 0.2\%$, and $33.6 \pm 0.2\%$, respectively, at 120 min ($*p < 0.05$ compared with EGCG solution). The release rate slowed after 240 min and later reached a sustainable and stable rate of EGCG release for more than 1400 min. The EGCG had been completely released by the EGCG solution group at 960 min. Meanwhile, the EGCG release rate in these two nanoformulations was $< 60\%$ with a slow EGCG release pattern. At 1440 min, the release of cisplatin from GE-Pt NPs was $30.1 \pm 0.3\%$ compared with that from cisplatin solution ($*p < 0.05$). For the same amount of time, the release of cisplatin solution was higher ($37.8 \pm 0.1\%$). The rapid initial cisplatin release, from both formulations, was followed by a slower cisplatin release, which continued for up to 24 hours.

3.2. Dual Drug-Loaded NPs for Treating Lung Cancer Cells

3.2.1. Anticancer Effect of Gelatin/EGCG Self-Assembled Nanoparticles (GE NPs). The anticancer activity of EGCG has been widely reported, both *in vitro* and *in vivo*. In this test, A549 cells were treated with EGCG solution and gelatin/EGCG nanoparticles (GE NPs) to examine the cellular effect in different EGCG conditions. Low (5 $\mu\text{g}/\text{mL}$), medium (20 $\mu\text{g}/\text{mL}$), and high (80 $\mu\text{g}/\text{mL}$) concentrations of EGCG and GE NPs were tested to determine cell viability, to evaluate their potential anticancer effect. An EGCG concentration of 80 $\mu\text{g}/\text{mL}$ showed high toxicity to A549 cells, both in solution and in nanoform (GE NPs). It caused low cell viability

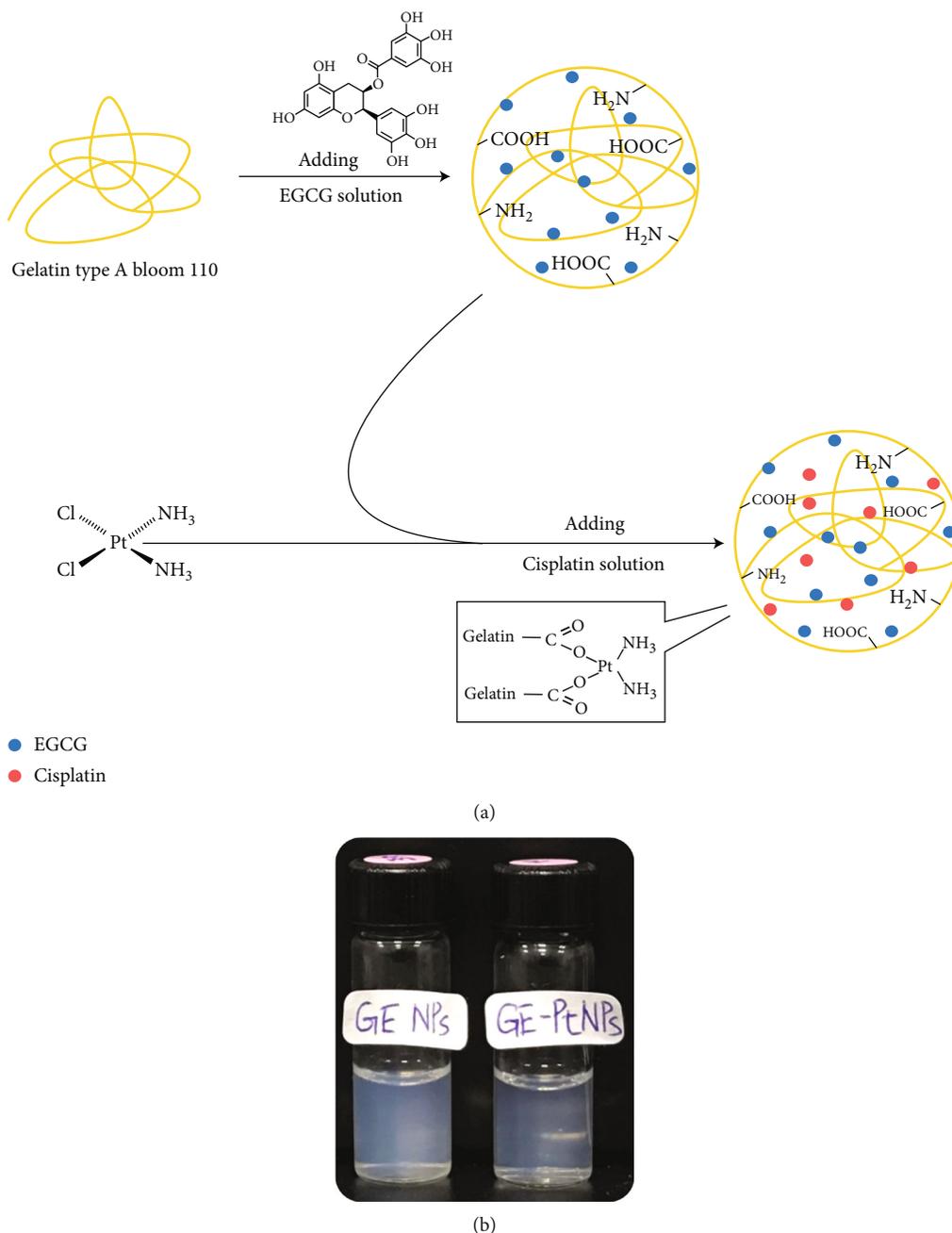


FIGURE 1: (a) Diagram of the GE-Pt nanoparticle synthesis process. (b) Photograph of colloidal suspensions.

TABLE 1: Characterization of GE and GE-Pt NPs.

Group	Size (nm)	Zeta (mV)	PDI	EGCG EE (%)	Cisplatin EE (%)
GE	78.47 ± 0.65	13.23 ± 0.72	0.16 ± 0.01	88.77 ± 2.52	/
GE-Pt	74.43 ± 9.71	19.83 ± 0.25	0.15 ± 0.01	89.16 ± 0.39	63.67 ± 2.98

$n = 3$. EE: encapsulation efficiency.

(between 13 and 41%, see Figures 5(a)–5(c)) depending on the period of treatment (1–7 days). If $20 \mu\text{g}/\text{mL}$ was used, the EGCG solution was more effective for inhibiting cancer proliferation than GE NPs ($*p < 0.05$), in all culture

periods, due to quick access in the medium. However, in the lowest EGCG-treated group ($5 \mu\text{g}/\text{mL}$), GE NPs were more effective than free EGCG in reducing cell viability on day 3 ($98.6\% \pm 2.3\%$, $*p < 0.05$) and day 7 ($93.7 \pm 10.9\%$).

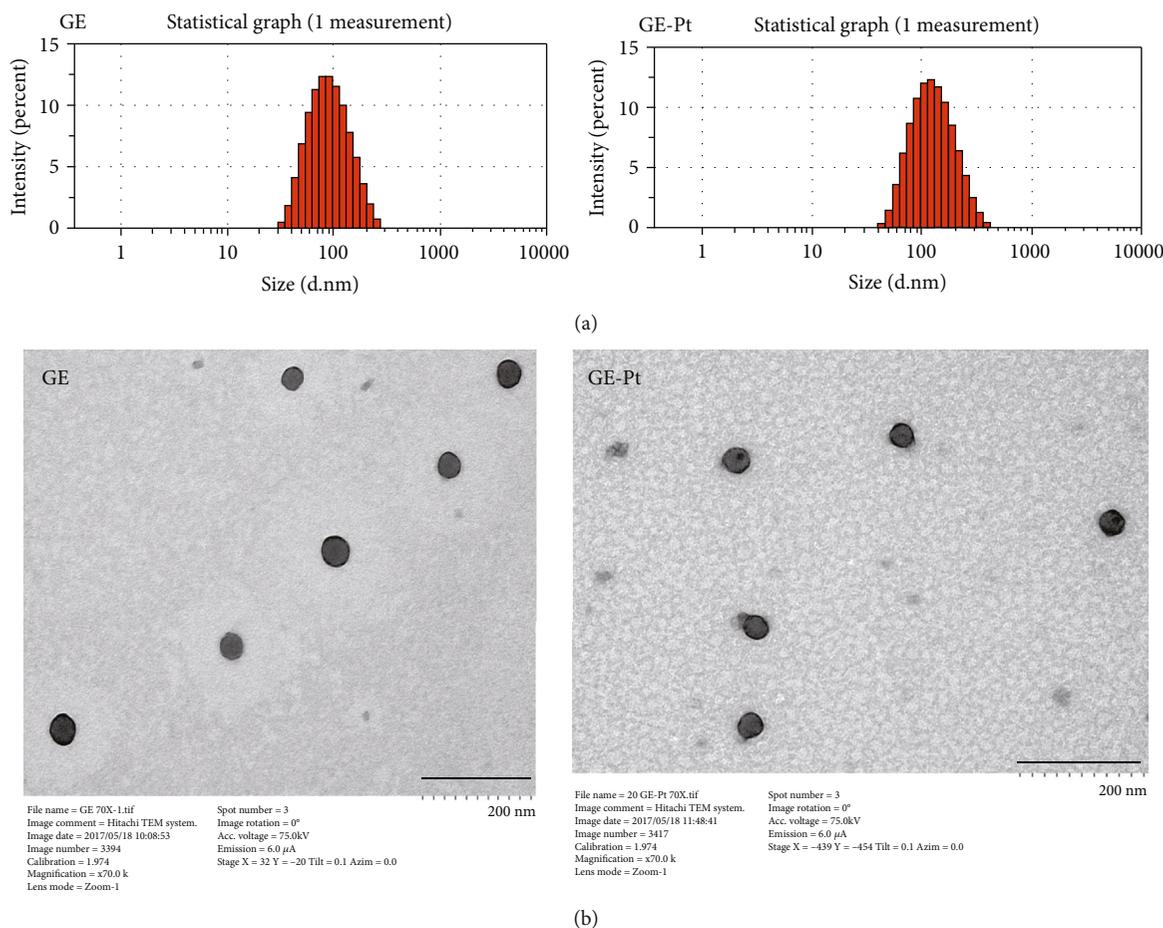


FIGURE 2: (a) Size distribution and (b) morphology of GE and GE-Pt NPs acquired from DLS and TEM examination. TEM scale bar: 200 nm.

However, at lower EGCG concentrations ($<20 \mu\text{g/mL}$), EGCG is not effective for inhibiting A549 cell viability, either in solution or in nanoform.

3.2.2. Anticancer Effect of the Dual Drug- (EGCG/Cisplatin) Loaded Gelatin Nanoparticles (GE-Pt NPs). To examine the synthetic effect of dual drug- (cisplatin/EGCG) loaded gelatin nanoparticles (GE-Pt NPs), A549 cells were treated with cisplatin solution, cisplatin+EGCG solution, and GE-Pt NPs at high (cisplatin: $32 \mu\text{g/mL}$, EGCG: $80 \mu\text{g/mL}$), medium (cisplatin: $8 \mu\text{g/mL}$, EGCG: $20 \mu\text{g/mL}$), and low (cisplatin: $2 \mu\text{g/mL}$, EGCG: $5 \mu\text{g/mL}$) concentrations. In high drug concentrations, all groups were effective in reducing cell viability (9.1~28.8%, Figures 6(a)–6(c)) due to the toxicity of cisplatin ($32 \mu\text{g/mL}$). On day 1 (Figure 6(a)), GE-Pt significantly reduced the A549 cancer cell viability, at all tested concentrations, compared with cisplatin alone or cisplatin+EGCG ($*p < 0.05$). When treated with the medium cisplatin solution ($8 \mu\text{g/mL}$) for 3 days (Figure 6(b)), the cell viability was higher ($41.8 \pm 7.4\%$) than that of the dual drug solution (cisplatin ($8 \mu\text{g/mL}$)+EGCG ($20 \mu\text{g/mL}$) at a viability of $15.3 \pm 0.1\%$). The synergistic effect of the dual drug was confirmed from this data. We changed the culture medium on day 3, without adding any drug, for culture from day 3 to day 7. Overall, the anticancer effect of the dual drug nanoformulation (GE-Pt NPs) was higher than those of the other formu-

lations, in any concentration, for treating A549 cells over a longer time (day 7) with the lowest cell viability of 12~24% (Figure 6(c), $*p < 0.05$). The higher anticancer effect may be caused, in part, by the slow release of cisplatin from GE-Pt NPs uptaken into cells at day zero when added in the medium.

The comparison of two nanomedicines (GE and GE-Pt) is shown in Figure 7. With dual drug loading in one of the nanoparticles, GE-Pt shows a significant increase in anticancer effect by reducing cell viability for 7 days of treatment ($24.0 \pm 2.9\%$, $*p < 0.05$, Figure 7). For EGCG, even in nanoform (GE), the cell viability was decreased to $62.0 \pm 11.0\%$ on day one, but no further effect was seen after a longer culture period (days 3 and 7) during which cell viability rose to around 94%.

3.3. Intracellular Platinum Content Increased by Treating with Nanoformulation. In order to examine cisplatin accumulation in the cell, the concentration was measured by ICP-MS (Figure 8). The platinum concentration in the cells was 6.4 ppb for the cells treated with cisplatin solution and 14.2 ppb for the cells cultured with GE-Pt after 1 hour of treatment ($*p < 0.05$). After 3 hours, the platinum concentration was slightly increased (~ 11.0 ppb) in the group treated with the cisplatin solution. However, the platinum concentration in cells treated with GE-Pt NPs for 3 hours reached

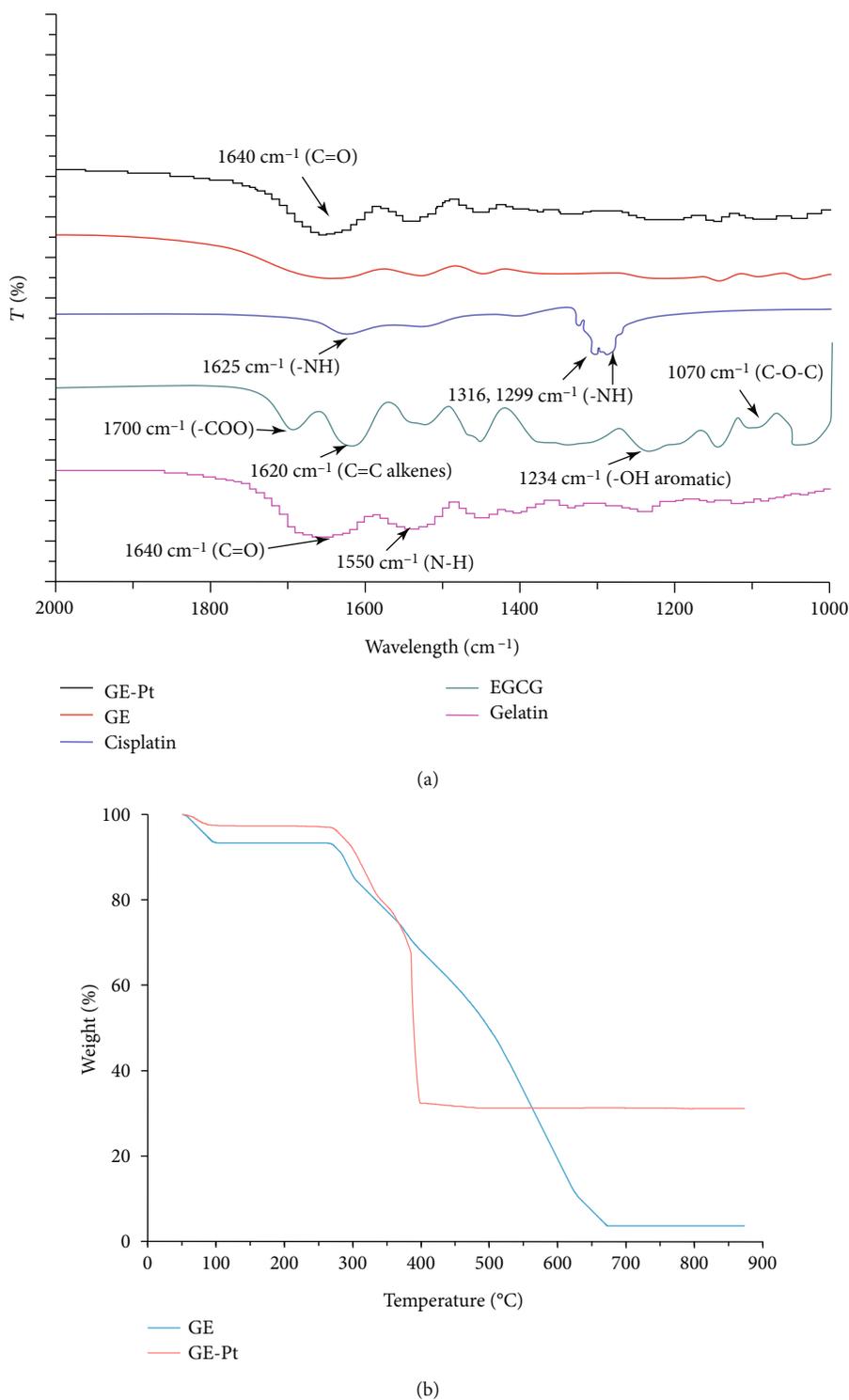


FIGURE 3: (a) FTIR spectra and (b) TGA pattern of two NP formulations.

40.1 ppb (2.8-fold compared with the GE-Pt at 1 h). The platinum concentration of cells in GE-Pt NPs was 3.6-fold higher than that in the cisplatin solution, after 3 h of treatment, which is a significant difference ($*p < 0.05$). The difference is evidence that the nanoparticles (GE-Pt NPs) are transporting drugs (cisplatin) more efficiently into the cells, compared with free cisplatin.

4. Discussion

Cisplatin resistance and the severe side effects of treatment are major causes of treatment failure in lung cancer patients. Here, we provide a novel strategy for antitumor treatment by developing dual drug-containing nanoparticles for treating lung cancer, to overcome the chemoresistance of cisplatin.

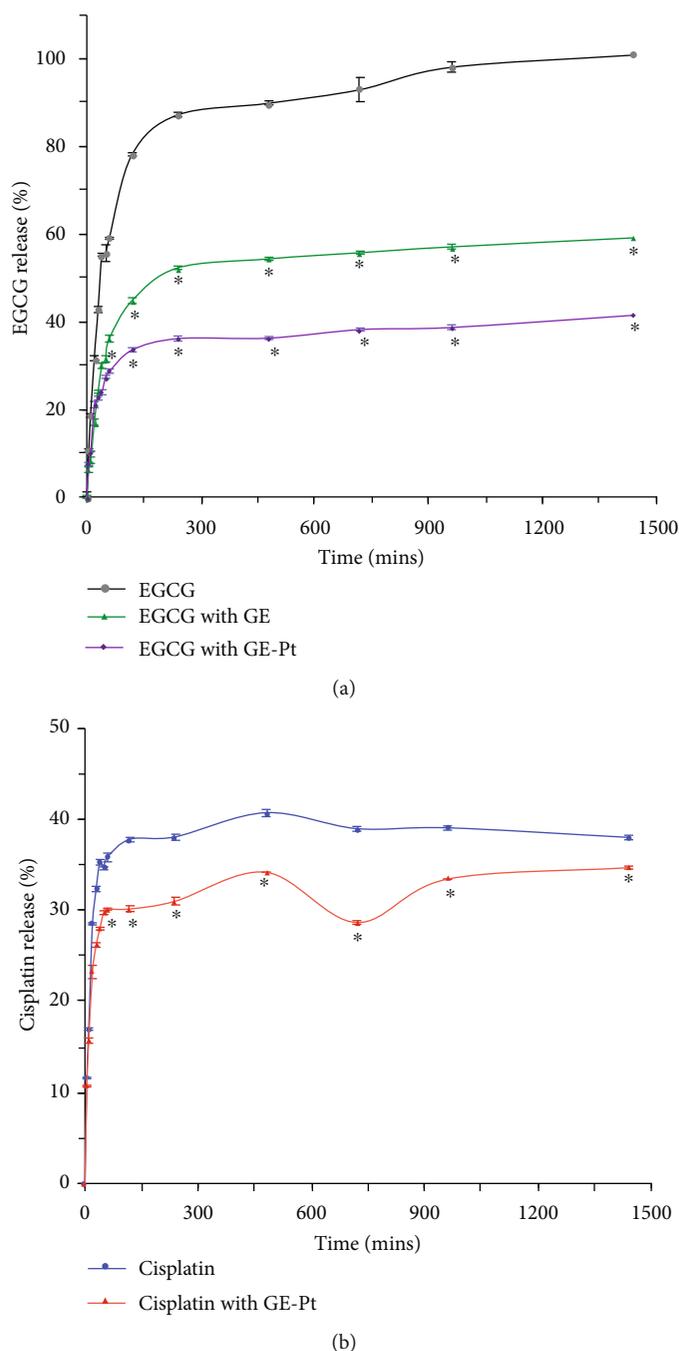


FIGURE 4: (a) EGCG release pattern from different EGCG formulations (EGCG solution, GE, and GE-Pt NPs) and (b) cisplatin release from different formulations (cisplatin solution, GE-Pt NPs).

In this paper, the dual drug- (cisplatin/EGCG) loaded gelatin nanoparticles (GE-Pt NPs) were successfully synthesized and examined. The *in vitro* anticancer effect was confirmed in human adenocarcinoma cells (A549), with inhibition of cell viability, via effective transport and subsequent high concentrations of intracellular cisplatin.

Nanomedicine is an emerging field that combines nanotechnology, pharmaceutical, and biomedical sciences and has the capacity to improve therapeutic efficacy and reduce side effects [15, 16]. In this study, gelatin was used as the carrier component because of its nontoxicity, low cost, ready

availability, biodegradability, and biocompatibility [38–40]. Various models of chemoresistance to cisplatin have been reported, concerning decreased DNA-adduct formation, enhanced DNA repair, decreased susceptibility to induction of apoptosis, inhibition of efflux transporters, generation of reactive oxygen species (ROS), increased detoxification of the drug, and increased drug inactivation by sulfur-containing molecules [26]. Therefore, coadministration of two therapeutic agents, involved in different mechanisms, may offer a synergistic therapeutic effect, leading to the eradication of resistant cancer cells.

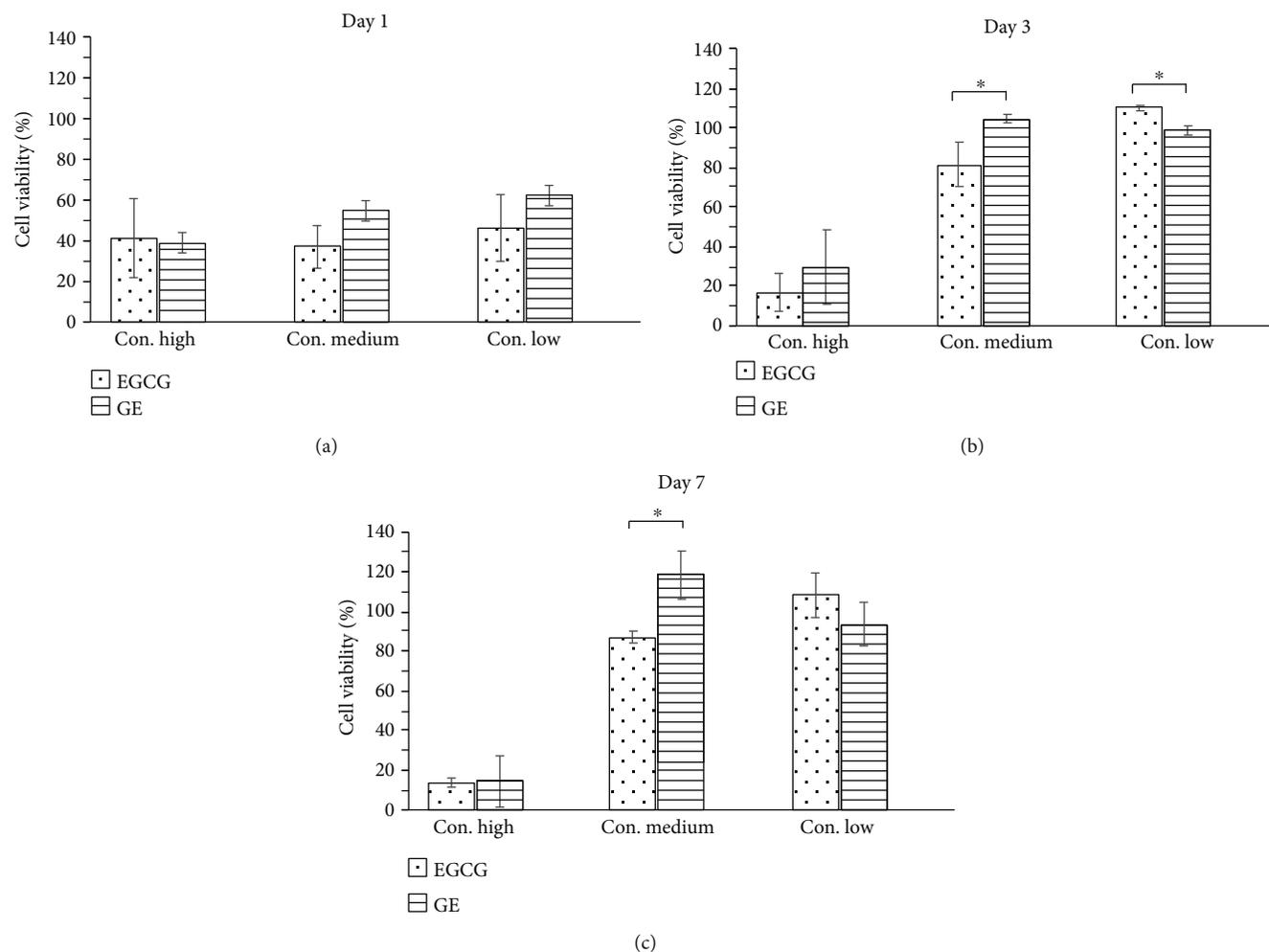
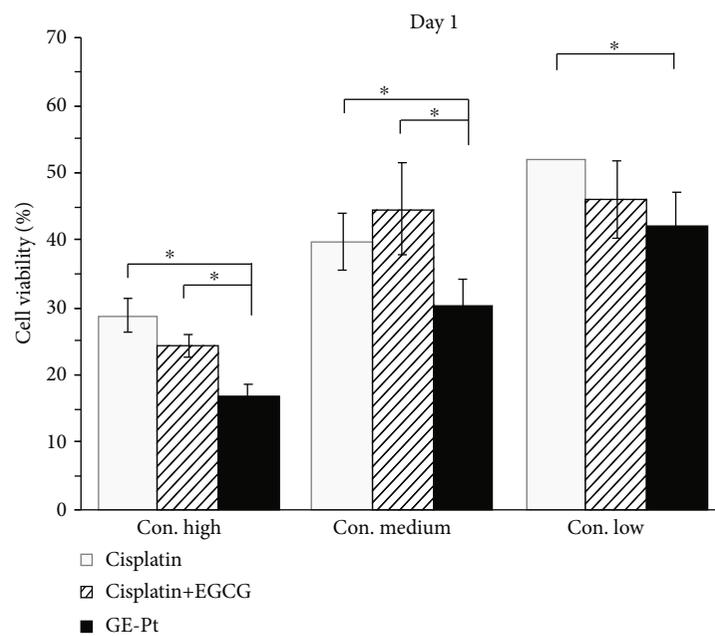


FIGURE 5: Cell viability of A549 cells treated with variant EGCG formulations, on (a) day 1, (b) day 3, and (c) day 7. Group explanation: EGCG: EGCG added to the medium; GE: gelatin/EGCG self-assembled (GE) NPs. High concentration means that cells were cultured with EGCG at $80 \mu\text{g}/\text{mL}$. Medium concentration means that cells were cultured with EGCG at $20 \mu\text{g}/\text{mL}$. Low concentration signifies that cells were cultured with EGCG at $5 \mu\text{g}/\text{mL}$ ($*p < 0.05$).

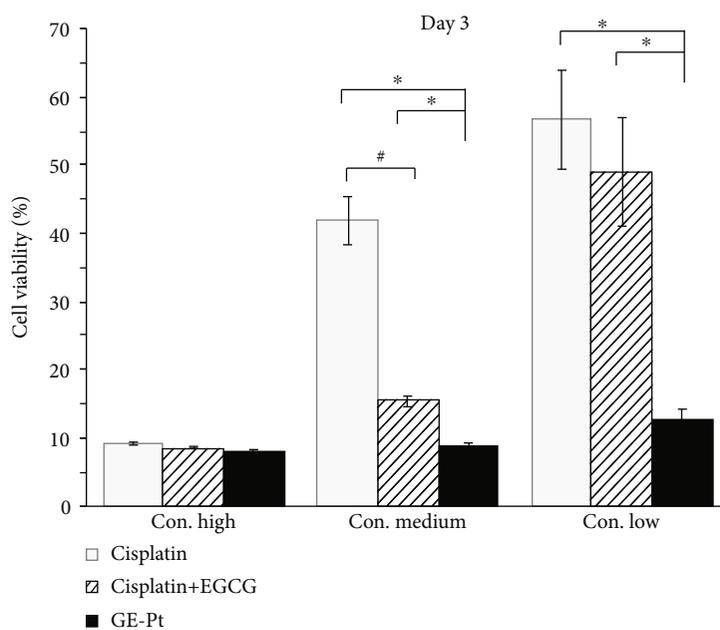
The EGCG exhibits its antitumor effect in lung cells, including the inhibition of cell proliferation, migration, and angiogenesis [13, 41]. However, EGCG has some pharmacological limitations, such as poor drug stability and membrane permeability [42], resulting in low bioavailability and effectiveness for treating cancer cells. Oxidation of the polyphenol structure leads to EGCG degradation [43, 44]. Chemoprevention is a promising approach for cancer management, especially through the use of naturally occurring phytochemicals capable of impeding the process of one or more steps in carcinogenesis [45]. Siddiqui et al. encapsulated the green tea polyphenol epigallocatechin-3-gallate (EGCG) in polylactic acid- (PLA-) polyethylene glycol (PEG) nanoparticles. They observed that encapsulated EGCG retains its biological effectiveness with over 10-fold dose advantage for reducing the viability of human prostate cancer (PCa) cells. This could serve as a basis for the use of nanoparticle-mediated delivery, to enhance bioavailability and limit any unwanted toxicity of chemopreventive agents, EGCG [45]. In our study, the EGCG in nanoform (GE

NPs) was more effective in reducing the viability of A549 cells than the EGCG solution on days 3 and 7 of treatment (Figure 5) at low EGCG concentration.

Cisplatin is one of the first-line chemoagents for various kinds of cancer treatment, including lung cancer [24, 46]. Cisplatin is a conventional treatment for most advanced non-small-cell lung cancer (NSCLC) patients, but it has no significant therapeutic effect due to chemoresistance. Its clinical use is also limited due to severe adverse effects, including nephrotoxicity. Different types of nanoparticles with cisplatin encapsulation have been developed to increase the therapeutic effect of cisplatin, with fewer side effects. Gelatin nanoparticles (GP), loaded with cisplatin and decorated with biotinylated epidermal growth factor (bEGF), have previously been developed (GP-Pt-bEGF). These have a lower half-maximal inhibitory concentration ($IC_{50} = 1.2 \mu\text{g}/\text{mL}$) for the inhibition of A549 cell growth, and *in vivo* testing shows that GP-Pt-bEGF has higher antitumor activity and lower toxicity than cisplatin solution, in a subcutaneous model [24]. The investigation of cisplatin-loaded poly



(a)



(b)

FIGURE 6: Continued.

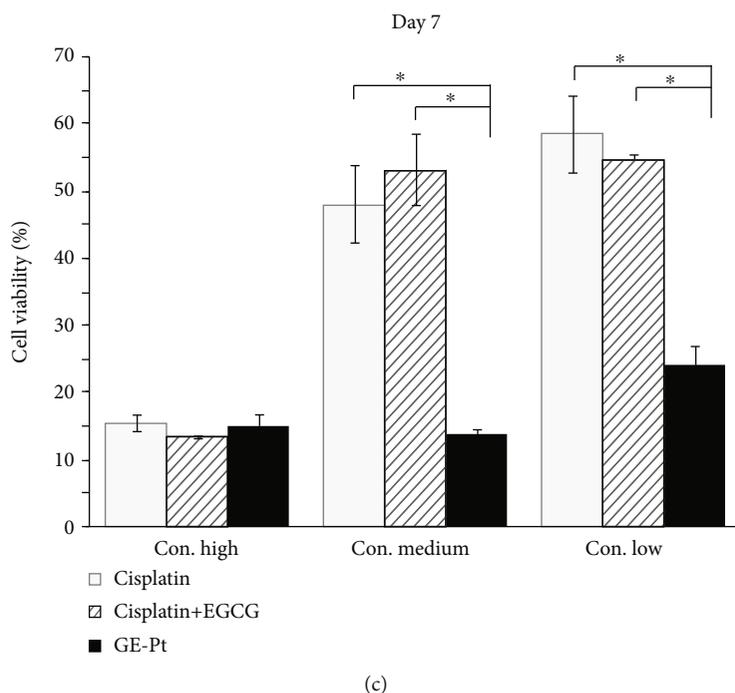


FIGURE 6: Viability of A549 cells treated with variant cisplatin formulations on (a) day 1, (b) day 3, and (c) day 7. Group explanation: cisplatin: only cisplatin added to the medium; cisplatin+EGCG: both cisplatin and EGCG added in the medium as dual drugs; and GE-Pt: GE NPs with cisplatin loading (* $p < 0.05$ compared with GE-Pt, # $p < 0.05$ compared with cisplatin).

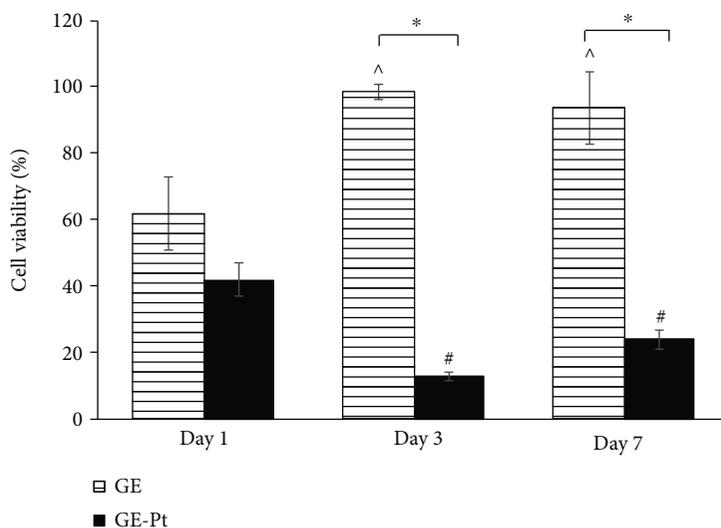


FIGURE 7: Cell viability of A549 cells treated with single (GE) or dual drug- (GE-Pt) loaded NPs on day 1, day 3, and day 7 at low concentration (GE with EGCG at $5 \mu\text{g/mL}$ or GE-Pt with EGCG at $5 \mu\text{g/mL}$ combined with cisplatin at $2 \mu\text{g/mL}$). ^ $p < 0.05$, GE-treated cells compared with GE day 1, # $p < 0.05$, GE-Pt-treated cells compared with GE-Pt day 1, and * $p < 0.05$, GE-Pt compared with GE.

(lactic-co-glycolic acid) (PLGA) nanoparticles demonstrated an average particle size and zeta potential of 284.8 nm and -15.8 mV, respectively. The release profile of cisplatin from PLGA NPs showed a biphasic characteristic: an initial burst release on day 1 followed by a controlled release phase [47]. A similar cisplatin release pattern was seen in our GE-Pt NPs (Figure 4(b)).

The size of the NPs also plays a key role in efficient drug delivery into cells. The adsorption of NPs onto the cell membrane leads to an increase in drug concentration near the cell surface, offering a gradient that would favor drug influx into the cells [48]. Cisplatin-PLGA NPs exhibited *in vitro* anticancer activity against A549 cells, comparable to that of cisplatin solution [47]. Alavi et al. revealed that cisplatin-loaded poly

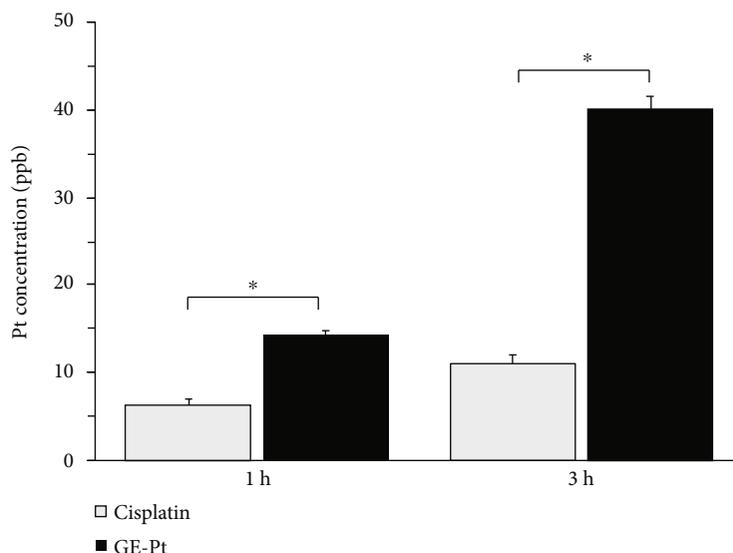


FIGURE 8: Intracellular Pt concentration quantified by ICP-MS examination (* $p < 0.05$).

(butylcyanoacrylate) (PBCA) NPs significantly increased its cytotoxicity. The IC_{50} of this cisplatin-PBCE NPs was around $10\sim 15\ \mu\text{M}$ in the lung cancer cell line (LCC1) [49], and the cisplatin-PBCE NPs significantly enhanced the therapeutic effects of the drug *in vivo*, evidenced by the survival time of lung cancer-bearing mice compared to the cisplatin solution receiver group [49]. The cytotoxicity of the cisplatin NPs may partly be caused by their efficient uptake by target cells. This study has shown that a high platinum concentration was acquired in A549 cells cocultured with the GE-Pt NPs (Figure 8), suggesting that cisplatin loaded into NPs is more effective in delivery into cancer cells than free cisplatin for the reduction of cancer cell viability, with fewer side effects. The GE-Pt is much more effective than cisplatin or cisplatin+EGCG solution (Figure 6), which could be due to the addition of EGCG.

Cisplatin exerts its anticancer effect through multiple steps. Therefore, resistance to cisplatin has been attributed to multiple mechanisms, such as decreased cisplatin accumulation and increased nucleotide excision repair (NER) [26, 50]. Thus, EGCG might overcome cisplatin resistance via the regulation of downstream molecules, in each of these distinct resistance mechanisms. For example, a mechanism that increases cisplatin uptake and accumulation in cancer cells is the involvement of copper transporter 1 (CTR1), a membrane protein participating in copper homeostasis, in cisplatin uptake [26]. EGCG could induce the expression of CTR1, leading to the accumulation of cellular cisplatin and cisplatin-DNA adducts and enhanced sensitivity of ovarian cancer cells to cisplatin. These findings provide a new strategy for green tea polyphenol, EGCG, as an adjuvant for the treatment of ovarian cancer [51]. Excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC-1) is a single-strand DNA endonuclease, which participates in the NER system that repairs cisplatin-induced DNA lesions [26]. High expression of ERCC-1 in clinical samples correlates with cisplatin responsiveness and

patient survival [52, 53]. Heyza et al. found that EGCG is a potent inhibitor of ERCC-1 activity, which inhibits DNA repair ability and enhances the therapeutic efficacy of cisplatin [54]. These findings support EGCG as an ideal candidate to modulate various molecules in cisplatin resistance mechanisms. The result in Figure 7 reveals that the GE-Pt NPs highly reducing the A549 cell viability may be explained by these mechanisms.

It is reported that cisplatin, combined with EGCG, shows a synergistic inhibitory effect in an *in vitro* lung cancer model, and the effective doses of cisplatin and EGCG were $12\ \mu\text{M}$ and $100\ \mu\text{M}$, respectively [14]. *In vivo* xenografts also gave a similar result; with the injection of dual drugs several times during the treating period, tumor size was dramatically reduced [5, 14, 55]. In our study, cisplatin and EGCG were co-loaded into one nanoparticle, to form GE-Pt NPs. Even at low drug concentration (cisplatin/EGCG at $2\ \mu\text{g}/\text{mL}$ + $5\ \mu\text{g}/\text{mL}$), GE-Pt NPs possessed high cytotoxicity compared to the cisplatin solution and cisplatin+EGCG solution (Figures 6 and 7). Also compared with a previous study, gelatin nanoparticles with cisplatin (cDDP) loading (GPs-CDDP) have no effect for reducing cell viability ($\sim 100\%$) of A549 at $5\ \mu\text{g}/\text{mL}$ cisplatin after one-day treatment [24]. In this study, when A549 cells treated with low concentration of GE-Pt (EGCG $5\ \mu\text{g}/\text{mL}$ +cisplatin $2\ \mu\text{g}/\text{mL}$) for the same time period, their cell viability downed to 40%. The cisplatin concentration in GE-Pt NPs ($2\ \mu\text{g}/\text{mL}$) here was lower than GPs-CDDP ($5\ \mu\text{g}/\text{mL}$), but with higher toxicity to A549 cells. These results indicate that dual drug-loaded NPs (GE-Pt NPs) provide a stable, sustainable release of cisplatin/EGCG (Figure 4) and an improved uptake for a better accumulation of cisplatin in cells (Figure 8). And the synergistic effect of cisplatin/EGCG was shown, in effective tumor cell eradication, even for a very low dose. The combination therapies of cisplatin with EGCG together can be considered to overcome drug resistance and reduce the toxicity of cisplatin for lung cancer treatment.

5. Conclusions

The gelatin nanoparticles with cisplatin and EGCG loading (GE-Pt) were designed and prepared to overcome the chemoresistance of cisplatin by synergistic action for the effective killing of lung cancer cells. In this study, we successfully synthesized spherical GE-Pt NPs in nanosize (~75 nm), possessing a positive charge (+20 mV). The encapsulation rate of cisplatin and EGCG in GE-Pt was about 64% and 89%, respectively. Even in a low concentration of GE-Pt NPs (EGCG at 5 $\mu\text{g}/\text{mL}$ and cisplatin at 2 $\mu\text{g}/\text{mL}$), these GE-Pt NPs still exhibited significant inhibition of cancer cell viability. These effects were caused, in part, by the ease of uptake into cells, facilitated by endocytosis. The synergistic effect of the combination with cisplatin, EGCG, and nanomedicine (GE-Pt NPs) was shown to reduce A549 cell viability *in vitro* to 24% after 7 days. This provides an effective anticancer formulation for further evaluation in an animal model.

Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

The authors acknowledge the technical support provided by TMU Core Facility for the TEM and ICP-MS Thermo X-SERIES 2 ICP-MS (Thermo Scientific, Waltham, MA). This work was supported in part by a grant from the University System of Taipei Joint Research Program (USTP-NTUT-TMU-107-04) and also partially supported by the Ministry of Science, Taiwan (MOST 106-2628-E-038-001-MY3), and the Higher Education Sprout Project by the Ministry of Education (MOE) (grant no. DP2-108-21121-01-0-01-01/02/03).

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