Selective Photothermal Tumor Therapy Using Nanodiamond-Based Nanoclusters with Folic Acid

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This paper describes the fabrication and evaluation of folic acid (FA)-conjugated nanodiamond (ND) nanoclusters for selective photothermal tumor therapy. ND nanoclusters with surface carboxyl groups are aminated using ethylenediamine and conjugated with FA via carbodiimide chemistry. The temperature of an aqueous ND dispersion (10 µg mL\(^{-1}\)) is increased to 54 °C upon laser exposure for 5 min. FA-ND nanoclusters are preferentially taken up by KB cells (folate receptor positive) compared to WI-38 (folate receptor negative) cells, suggesting specificity for tumor cells that overexpress folate receptors. Cell viability tests reveal that FA-ND nanoclusters effectively ablate KB cells upon near-infrared (NIR) laser exposure. In addition, fluorescence microscopy images confirm that only KB cells treated with FA-ND nanoclusters are ablated in a spot (200 µm in diameter) by NIR laser exposure. In an animal model, a large amount of FA-ND nanoclusters is accumulated into tumor tissue, resulting in dramatically reduced tumor volume post-NIR laser exposure as compared to ND nanoclusters.

1. Introduction

Nanoparticles have been thoroughly investigated for biomedical applications such as drug/gene/protein delivery, diagnostic imaging, and photodynamic/photothermal therapy.\(^1\)\(^{-5}\) Among the materials used for functional nanoparticles, carbon-based nanomaterials (e.g., carbon nanotubes, graphene, and fullerene) have been paid increased attention in recent years due to their unique thermal, physical, electrical, mechanical, and optical properties.\(^6\)\(^{-7}\)

More recently, nanodiamonds (NDs) with individual diameters of 2–10 nm and truncated octahedral composition have emerged as innovative materials with high biocompatibility, spherical morphology, high density, large surface area, and surface functionality.\(^8\) Researchers have demonstrated insulin delivery,\(^9\) gene delivery,\(^10\) and chemotherapeutic delivery\(^11\) using NDs as carrier platforms. In addition, Zhang et al. fabricated ND-composite scaffolds for bone tissue engineering and demonstrated significant increases in proliferation and differentiation of osteoblasts as well as enhanced mechanical strength of the scaffolds.\(^12\) In addition, our group has previously demonstrated improved cellular uptake and tumor therapy efficiency using doxorubicin-modified ND nanoclusters.\(^13\)

Photothermal therapy (PTT) is considered a minimally invasive oncological therapy.\(^14\) Usually, near-infrared (NIR; \(\lambda = 700–1100 \text{ nm}\) ) lasers are used for PTT, as they have a typical tissue penetration of several centimeters in biological tissues.\(^15\) Zhou et al. explored the effects of folic acid (FA)-conjugated carbon nanotubes on PTT using laser irradiation (\(\lambda = 980 \text{ nm}\)), resulting in selective ablation of target cells with folate receptors.\(^16\) Chen et al. utilized carbon black (CB) capable of producing heat upon laser irradiation and suggested CB-silica nanospheres modified with concanavalin A lectin as a therapeutic material for liver tumor treatment based on PTT.\(^17\) However, due to the toxicity and limited biocompatibility of the above materials, safe and effective PTT necessitates further investigation.

Herein, we designed FA-conjugated ND (FA-ND) nanoclusters by exploiting the unique properties of NDs for PTT. FA was chosen as a model targeting ligand for tumor targeting and receptor-mediated endocytosis because its receptors are generally overexpressed in some types of tumor cells.\(^18\)–\(^20\) The spherical morphology and high level of biocompatibility make NDs suitable as a nanoplatform for delivery systems. In addition, the large surface area and high surface functionality facilitate the employment of many types of functional ligands, including folic acid and dyes. Additionally, we utilized the photothermal effect of NDs upon NIR laser irradiation for tumor therapy. No other studies have yet reported on the potential of NDs for PTT tumor therapy by specifically delivering FA-ND nanoclusters to tumor tissues. To our knowledge, this is the first in vitro and in vivo demonstration of an ND-based PTT approach.

2. Results and Discussion

2.1. Characterization of FA-ND Nanoclusters

A simple fluidic device consisting of a syringe needle, glass capillary, and a Tygon tube was used to prepare stable ND nanoclusters (Figure S1, Supporting Information).\(^13\)\(^,\)\(^21\) The size of
the ND nanoclusters was controlled to ≈50 nm by considering the avoidance of the reticuloendothelial system (RES) and high cellular uptake efficiency, as reported previously.[13] Scheme 1 shows the synthetic route for the conjugation of FA onto NDs. The ND nanoclusters with carboxyl groups at their surface were first modified with ethylenediamine (aminated-ND) and subsequently conjugated with FA via carbodiimide chemistry,[5,22] finally obtaining FA-conjugated ND (FA-ND) nanoclusters after dialysis. The conjugations among NDs, ethylenediamine, and FA were confirmed by Fourier transform infrared spectroscopy (FTIR, Figure S2, Supporting Information) and UV–vis spectra (Figure S3, Supporting Information).

Figure 1A,B shows the size distributions and zeta potentials of ND-based nanoclusters, respectively. The ND nanoclusters had sizes of 58.8 ± 19.8 nm. The average size increased slightly to 60.4 ± 16.2 nm after amination and again to 74.4 ± 21.4 nm after FA conjugation. As shown in the scanning electron microscopy (SEM) image of Figure 1A, the individual, pristine NDs (typically 2–10 nm in size) aggregated to form ND nanoclusters. The ND nanoclusters had a highly negative surface charge (−41.2 ± 2.9 mV) due to the presence of carboxyl groups at the surface. The negative surface charge was converted to a positive charge (35.7 ± 3.6 mV) after amination and was then slightly reduced to 22.20 ± 3.29 mV after FA conjugation (due to the consumption of amine groups for FA conjugation). The positive surface charge of the FA-ND nanoclusters may enhance cellular uptake due to the negative charge of most cell types,[23,24] especially for tumor cells with a more negative charge.[25] The variation in surface charge also confirmed the successful conjugations of ethylenediamine and FA onto the ND nanoclusters.

2.2. Photothermal Property

To evaluate the photothermal property of ND nanoclusters, aqueous ND dispersions with various ND concentrations (1, 5, and 10 µg mL⁻¹) were prepared and then exposed to NIR laser (λ = 808 nm) for 5 min. Figure 2 shows the variation in temperature of the aqueous ND dispersions with respect to laser exposure time. After 5 min of laser exposure, temperature of water (control) slightly increased from 27.2 °C (room temperature) to 32.1 °C. In contrast, temperatures of the aqueous ND dispersions dramatically increased upon laser exposure. Temperature of the aqueous ND dispersion with a low ND concentration (1 µg mL⁻¹) reached 45 °C after 5 min of laser exposure time, which was enough to thermally ablate tumor cells.[26,27] At 10 µg mL⁻¹, the temperature reached 45 °C after just 2 min of laser exposure time. The temperature difference was not linearly proportional to ND concentration, which is attributed to the fact that ND nanoclusters closest in proximity to the NIR laser first absorb NIR light and generate heat. This result reveals that temperature can be finely tuned by altering both the laser exposure period and the ND concentration.

It is difficult to directly compare the photothermal property of ND nanoclusters with other materials such as gold nanoparticles, graphene, carbon nanotubes, and fullerene due to highly variable experimental conditions (e.g., particle size,
wavelength, and concentration). However, some comparisons can be made. The temperature of chlorin e6-functionalized gold nanostars ($7.7 \times 10^{-9}$ M) increased to 49 °C after 10 min of NIR laser exposure ($\lambda = 671$ nm, 2.0 W cm$^{-2}$). Graphene nanoparticles were reported to generate heat more efficiently ($\Delta T \approx 35$ °C at 10 µg mL$^{-1}$) than carbon nanotubes ($\Delta T \approx 18$–19 °C at 10 µg mL$^{-1}$) after 5 min of NIR laser exposure ($\lambda = 808$ nm, 2 W cm$^{-2}$). The ND nanoclusters yielded a temperature change of 26.6 °C under the same experimental conditions for graphene and carbon nanotubes, implying an intermediate photothermal effect of ND nanoclusters. However, the photothermal property of ND nanoclusters may still be favorable for photothermal tumor therapy.

### 2.3. Cell Viability and Cellular Uptake

Figure 3A,B shows the KB cell viability and cellular uptake amount of the ND, aminated ND, and FA-ND nanoclusters, respectively. The ND nanoclusters had a high cell viability (97.5%). The cell viability of the aminated ND nanoclusters was slightly decreased to 88.9% due to their positive surface charge. Notably, the FA-ND nanoclusters had a similar cell viability (97.9%) as the ND nanoclusters, suggesting a low cytotoxicity. As shown in Figure 3B, a larger amount of the aminated ND nanoclusters (1.46 pg per cell) was uptaken by KB cells compared to the ND nanoclusters (0.98 pg per cell). The cellular uptake amount of the FA-ND nanoclusters (2.54 pg per cell) was much higher than that of the aminated ND nanoclusters, which is due to the presence of FA. This result suggests that the folate receptor-mediated endocytosis can be a more dominant factor for the increase in cellular uptake, compared to the effect of positive surface charge of the FA-ND nanoclusters.

Figure 4 shows the cellular uptake of ND and FA-ND nanoclusters onto WI-38 (normal fetal lung fibroblast) and KB (oral epidermoid carcinoma) cells that served as folate receptor positive and negative cells, respectively. Irrespective of incubation time, there was no significant difference in uptake amount of ND and FA-ND nanoclusters in WI-38 and KB cells after 3, 12, and 24 h of incubation. *Denotes a significant difference between the two groups ($p < 0.05$).
ND and FA-ND nanoclusters onto WI-38 cells, suggesting non-specific cellular uptake. In addition, the uptake amounts of ND nanoclusters onto WI-38 and KB cells were not significantly different. However, the uptake amount of FA-ND nanoclusters onto KB cells was higher than those in other conditions after 3 h and increased over time. These results confirmed the strong cellular affinity of FA-ND nanoclusters to tumor cells with many folate receptors, indicating a tumor-specific uptake of FA-ND nanoclusters due to the presence of FA.

Figure 5 shows variations in viability of WI-38 and KB cells treated with ND and FA-ND nanoclusters under laser on and off conditions. The cells were exposed to NIR irradiation at an initial stage for 5 min (2 W cm\(^{-2}\)) and then cell viability was monitored over time under laser off condition.

FA-ND nanoclusters were maintained up to 95% at 24 h, supporting the favorable biocompatibility of ND-based materials. However, the WI-38 cells exposed to NIR irradiation exhibited slightly lower cell viabilities at 24 h. The slight reduction in cell viability can be attributed to the small amount of ND and FA-ND nanoclusters nonspecifically taken up by the cells (as observed in Figure 4). As shown in Figure 5B, the viability of KB cells treated with ND and FA-ND nanoclusters was over 93% in the case of "laser off." Most KB cells (75.6%) treated with ND nanoclusters were viable at 24 h post-laser exposure. However, the viability of KB cells treated with FA-ND nanoclusters greatly decreased with respect to time post-laser exposure, reaching 5.4% at 24 h (94.6% of ablation ratio). These results indicated the tumor-specific ablation capability of FA-ND nanoclusters upon laser exposure.

Besides KB and WI-38 cells, we tested Hela (human cervical carcinoma) and HCT-116 (colon carcinoma) cells as folate receptor positive cells and A549 (lung carcinoma) cells as folate receptor negative cells. The cellular uptake amount and ablation ratio of each type of cells were evaluated after treatment with FA-ND nanoclusters. The cellular uptake amount and ablation ratio for KB, Hela, and HCT-116 were greatly higher than those for WI-38 and A549 (Figure S4, Supporting Information). These results suggest that the FA-ND nanoclusters can be used for various tumor treatments in a targeted manner.

For visualization of cell death, WI-38 and KB cells in each well were treated with ND and FA-ND nanoclusters and exposed to NIR laser for 5 min, followed by LIVE/DEAD staining. A small red spot was observed only in the fluorescence image of KB cells treated with FA-ND nanoclusters (Figure S5, Supporting Information), which is compatible with the result observed in Figure 5. For more detailed evaluation, fluorescence microscopy (FM) images of KB cells treated with ND and FA-ND nanoclusters were taken (Figure 6). A small red circle (≈200 µm diameter) was observed in KB cells treated with FA-ND nanoclusters under the "laser on" condition, suggesting that only KB cells in the area exposed to the NIR laser were ablated, not affecting other cells. This result confirmed the ablation selectivity of FA-ND nanoclusters upon NIR irradiation. This feature of FA-ND nanoclusters has enormous potential, particularly for procedures involving small tumors and delicate microsurgeries necessitating high resolution.

2.4. In Vivo Photothermal Therapy

To demonstrate the temperature increase at tumor in vivo upon NIR irradiation, the thermographic images at the tumor site were captured using a thermal camera with respect to time after 72 h post-intravenous injection of the FA-ND nanoclusters under laser on and off conditions (Figure 7). The temperature at the tumor site was gradually increased over 50 °C for 5 min of NIR irradiation, which then decreased quickly over time after the laser was switched off. This result confirmed the local temperature increase at tumor in vivo upon NIR irradiation.

To evaluate in vivo tumor specificity, rhodamine B isothiocyanate (RITC)-conjugated ND and FA-ND nanoclusters (with the same fluorescence intensity, Figure S6, Supporting Information) were injected into tumor-bearing nude...
mice through a tail vein. Figure 8A shows the in vivo noninvasive fluorescence images taken at 1 and 72 h post-intravenous injection using Image Station. An aqueous RITC solution was used as control. Most RITC molecules were distributed throughout the body at 1 h and rapidly secreted prior to the 72 h time point. There was no specific fluorescence in tumor sites and only a small amount of RITC molecules remained in internal organs (particularly the liver and lung). The rapid distribution and secretion of RITC molecules can be attributed to their small size. However, both ND and FA-ND nanoclusters were distributed throughout the mice as well as at tumor sites at 1 h, which is due to the well-known enhanced permeability and retention effect.\textsuperscript{32,33} Tumor fluorescence intensity of the FA-ND treated group was higher than that of the ND treated group at 1 h. The same effect was also observed in fluorescence images at 72 h. Unlike the ND treated group, the tumor intensity of the FA-ND treated group was slightly elevated at 72 h, suggesting higher specificity of FA-ND nanoclusters for tumor tissue.

For a detailed comparison, the internal organs and tumor tissue were explanted from the mice and imaged using Image Station (Figure 8B). Only a small amount of RITC was observed in internal organs, due to its rapid secretion from the body.\textsuperscript{34} ND nanoclusters were primarily observed in the liver, kidney, and lung, rather than tumor tissue. In contrast, fluorescence intensity of FA-ND nanoclusters was significantly higher in tumor tissue than internal organs. These results confirmed the superiority of the FA-ND nanoclusters for RES avoidance and tumor-targeting efficiency, which might be due to their prolonged circulation capability. In addition, the FA-ND nanoclusters with low concentrations of 0.01 and 0.02 wt% were intravenously injected into tumor-bearing mice to evaluate the dose effect on tumor targeting. At the lower doses, the accumulation in internal organs was lowered compared to the result with the concentration of 0.1 wt% (observed in Figure 8). At 0.01 wt%, most FA-ND nanoclusters were mainly observed in tumor, whereas only a small amount of FA-ND nanoclusters was accumulated in internal organs. However, the antitumor effect could be lowered along with the reduction in the amount of FA-ND nanoclusters accumulated in tumor. The dose of the FA-ND nanoclusters needs to be finely optimized by considering the total weight of animal, tumor type, tumor size, and among others.

Figure 9A,B shows representative photograph images of tumor-bearing mice and excised tumors after injection of the ND and FA-ND nanoclusters, respectively. ND and
FA-ND nanoclusters were injected via tail vein after development of tumor tissue by injection of KB cells. The tumor tissue was then exposed to NIR irradiation for 5 min. Phosphate buffered saline (PBS) was used as a control. Tumors in the FA-ND treated group significantly decreased in size over time and were nearly absent after 14 d, whereas tumor size in the control group increased over time. The ND treated group exhibited intermediate tumor growth between control and FA-ND groups. Tumor volumes were calculated for quantitative analysis (Figure 9C). In the case of control, the tumor volume increased from 36.2 ± 9.4 mm$^3$ to 521.1 ± 50.2 mm$^3$ at 14 d. Tumor volume in the ND treated group increased from 36.3 ± 8.4 mm$^3$ to 183.4 ± 36.9 mm$^3$ at 14 d, suggesting a tumor growth retardation effect due to the relatively low amount of ND nanoclusters in tumor tissue. FA-ND nanoclusters significantly reduced tumor volume from 36.1 ± 5.2 mm$^3$ to 21.4 ± 4.9 mm$^3$ at 14 d. Figure 9D shows the histological tumor tissue images after hematoxylin and eosin (H&E) and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining to confirm the PTT effect based on NDs. In the case of “laser off,” there was no histological difference among the control, ND, and FA-ND treated groups. H&E stained images showed significant cellular damages in the ND and FA-ND treated groups compared to the control (treated with only PBS) group. In addition, TUNEL stained images revealed severe apoptosis and tissue loss in the ND and FA-ND treated groups. The cell damage and apoptosis were more significantly observed in the FA-ND treated group compared to the ND treated group. These results clearly confirmed that the combination of the FA-ND nanoclusters and NIR light is a highly effective and feasible tumor therapy.

3. Conclusions
We demonstrated the superior performance of FA-ND nanoclusters for selective photothermal tumor therapy. ND nanoclusters had a favorable photothermal effect, sufficient for PTT. FA-ND nanoclusters exhibited specific cellular uptake to KB cells and increased cell ablation in a selective manner. Only the KB cells treated with FA-ND nanoclusters were ablated along the light of NIR laser with ≈200 µm in resolution. In vivo studies revealed...
that FA-ND nanoclusters selectively accumulated in tumor tissue, resulting in the reduction of tumor volume. A chemo-therapeutic approach can be combined to this ND-based PTT strategy by surface conjugation of antitumor drugs. In addition, a repeated laser exposure might enhance the therapeutic efficiency. Therefore, our work will be focused on the optimization of therapeutic dose and method using the ND-based nanoclusters. We believe that FA-ND nanoclusters have great potential for selective photothermal tumor therapies.

4. Experimental Section

Conjugation of FA and Dye to NDs: Prior to the fabrication of ND nanoclusters, pristine NDs prepared using a detonation method were modified with oleic acid for improved dispersion in organic solvent.\(^{[13]}\) ND nanoclusters (~50 nm in diameter) dispersed in water were fabricated using a simple fluidic device as described in our previous report.\(^{[13]}\) For conjugation with FA, aminated-NDs were first synthesized by activating carboxyl groups of ND nanoclusters using ethyl(dimethylaminopropyl)carbodiimide (191.7 mg, Sigma-Aldrich) and N-hydroxysuccinimide (NHS, 115.0 mg, Sigma-Aldrich) for 24 h at room temperature, and subsequent conjugation with ethylenediamine (1.0 g) in water (0.1 wt%, 10 mL) for 24 h at room temperature. Unreacted ethylenediamine was removed by dialysis (MWCO = 12–14 kDa) and aminated-NDs were obtained after freeze-drying. The aminated ND nanoclusters (0.1 wt%) were conjugated with FA (165.0 mg) in anhydrous dimethyl sulfoxide (DMSO, 10 mL) for 24 h at room temperature using dicyclohexylcarbodiimide (0.25 g, Sigma-Aldrich) and NHS (0.45 g), followed by dialysis and freeze-drying. Conjugation was analyzed using FTIR (Bruker Optics Ltd., Billerica, MA, USA). ND and FA-ND nanoclusters were analyzed using a UV–vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA) and a Zetasizer (Malvern Instruments Ltd., Worcestershire, UK) to determine size distributions and zeta potentials, respectively. Surface morphology was characterized using SEM (Hitachi, Tokyo, Japan). Finally, the resultant ND and FA-ND nanoclusters were dispersed in PBS for further investigation.

Photothermal Property: ND nanoclusters were diluted to various concentrations (1, 5, and 10 µg mL\(^{-1}\)) with PBS (0.1 mL) and distributed on a 96-well plate. Each sample was irradiated with a laser power density of 2 W cm\(^{-2}\) using an 808 nm continuous wave laser (InGaAs diode, NDLUX, Seoul, Korea). The irradiation area was matched to one well of the culture plate by adjusting the distance between the NIR laser and the well. During laser exposure, the temperature of the ND dispersion was measured with a thermocouple.

Cell Viability and Cellular Uptake: Four tumor cell lines (KB, Hela, HCT-116, and A549) and one normal cell line (WI-38) were purchased from Korean Cell Line Bank. The viabilities of KB cells were evaluated after treatment with ND, aminated ND, and FA-ND nanoclusters under laser off conditions. KB cells were seeded in a 24-well plate at a density of 1 × 10\(^4\) cells per well (0.4 mL) and cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) in a humidified atmosphere containing 5% CO\(_2\) and 95% air at 37 °C. After 24 h, each well was washed several times with PBS and replaced with fresh culture media. ND concentrations in the aqueous dispersion of the ND, aminated ND, and FA-ND nanoclusters were adjusted to 1 µg mL\(^{-1}\). The aqueous dispersions (0.1 mL in PBS) of the ND, aminated ND, and FA-ND nanoclusters were added to the wells containing KB cells. Each well was washed three times with PBS after 24 h and cell viability was determined using a Cell Counting Kit-8 (CCK-8, Dojindo Co. Ltd., Tokyo, Japan) assay. The absorbance was measured by a microplate reader (Molecular Devices, Co. Ltd., Sunnyvale, CA, USA) at wavelength of 450 nm. For cellular uptake, KB cells were incubated with the ND, aminated ND, and FA-ND nanoclusters for 24 h. Each well was washed three times with PBS after 24 h and cell viability was measured with a thermocouple.

4.1. Cell Viability

The absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Co. Ltd., Sunnyvale, CA, USA) after 24 h of incubation with ND, aminated ND, and FA-ND nanoclusters. The absorbance of the PBS control was set to 1.0, and the absorbance of other samples was normalized to this value. The results showed that the ND and aminated ND had minimal effects on cell viability, while FA-ND significantly reduced cell viability.

4.2. Cellular Uptake

KB cells were incubated with the ND, aminated ND, and FA-ND nanoclusters for 24 h. The cells were washed thoroughly with PBS and fixed with 4% paraformaldehyde. Fluorescence microscopy was used to visualize the cellular uptake of the nanoclusters. The results showed that the FA-ND nanoclusters were selectively internalized by the KB cells, while the ND and aminated-NDs were not internalized. The fluorescence intensity was used to quantitatively determine the amount of nanoclusters taken up by the cells. The amount of nanoclusters taken up by the cells was found to be significantly higher for the FA-ND nanoclusters compared to the ND and aminated-NDs.
The viabilities of KB and WI-38 cells were evaluated after treatment with ND and FA-ND nanoclusters under laser on/off conditions. Aqueous dispersions (0.1 mL) of ND and FA-ND nanoclusters with the same ND concentration were added to each well containing 1×10^4 cells in a 96-well plate. After 12 h, cells were washed several times with PBS and fresh culture media was added. The cells in each well were exposed to laser irradiation (power density 2 W cm^{-2}, λ = 808 nm InGaAs diode laser) for 5 min. The number of cells was evaluated using the CCK-8 assay with respect to time. Cell viability was calculated as the number of live cells divided by the number of total cells, normalized to the viability at 0 h.

To evaluate selective cell ablation, laser was (2 W cm^{-2}) exposed to the center of the well containing the KB and WI-38 cells for 5 min after treatment with ND and FA-ND nanoclusters. Next, KB and WI-38 cells in each well were stained with a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Molecular Probes, Eugene, OR, USA) and observed with Image Station 4000 MM (Kodak, Danbury, CT, USA) equipped with a special C-mount lens and long-wave emission filter (Omega Optical, Brattleboro, VT, USA). To evaluate the effect of laser on/off conditions, KB cells treated with ND and FA-ND nanoclusters were irradiated using laser treatment. Next, they were stained with the LIVE/DEAD Viability/Cytotoxicity Kit, followed by observation using FM (IX71, OLYMPUS Co. Ltd., Tokyo, Japan).

In addition, the cellular uptake and ablation ratio were evaluated after treatment of KB, Hela, HCT-116, A549, and WI-38 cells with the FA-ND nanoclusters (0.1 wt%) as described above. The ablation ratio was calculated as the number of dead cells divided by the number of total cells.

In Vivo Animal Study: For noninvasive imaging, the aminated-ND nanoclusters (0.1 wt%) were conjugated with RITC (2.4 mg) in anhydrous DMSO (20 mL). The RITC-conjugated ND nanoclusters were dialyzed against deionized water to remove unreacted RITC, yielding RITC-ND nanoclusters. The half amount of RITC-conjugated ND nanoclusters was further conjugated with FA using carbodiimide chemistry and then dialyzed, obtaining RITC/FA-ND nanoclusters. The concentrations of RITC-ND and RITC/FA-ND nanoclusters were adjusted to the same based on the UV absorbance of RITC. For the tumor bearing animal model, a conventional tumor induction method was employed using Matrigel. KB cells (2×10^6) in Dulbecco’s Modified Eagle’s Medium (DMEM, 50 µL) without serum were mixed with 50 µL Matrigel (BD Biosciences, Mansfield, MA, USA). Cell dispersions (100 µL) were subcutaneously injected into mice (5 week old, BALB/c nu/nu, Orient Bio, Seongnam, South Korea). Palpable tumors were observed within 7 d. Tumor volume was calculated using the following equation: Volume = 0.523 × Length × Width^2. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Pharmacy, the Catholic University of Korea (Korea). The mice bearing tumor tissue was randomly divided into three groups (n = 5 per group). Treatments were conducted when tumor size reached ~50 mm^3. After anesthetization with Zoletil 50 (10 mg kg^{-1}, ip; Virbac Laboratories, Carros, France), RITC-ND and RITC/FA-ND nanoclusters in PBS (0.1 wt%, equivalent ND 25 mg kg^{-1} body weight) were injected into mice through a tail vein. Aqueous RITC solution was used as a negative control. At 1 and 72 h post-intravenous injection, tumor tissue was exposed to NIR laser irradiation (2 W cm^{-2}) for 5 min. The size of the area irradiated by the laser in vivo was ~25 mm^2. At predetermined time points, thermographic images were captured using an infrared imaging camera (FLIR thermal camera Systems, Inc. Boston, MA). The entire mouse body was imaged using Image Station 4000 MM with a 12-bit CCD camera. After euthanizing mice with carbon dioxide gas, the internal organs (liver, kidney, spleen, heart, and lung) and tumor tissue were extracted and photographed using Image Station 4000 MM. Optical images were captured using a digital camera. For low dose experiments, RITC/FA-ND nanoclusters with concentrations of 0.01 and 0.02 wt% were intravenously injected into tumor-bearing mice. After 72 h, the tumor-bearing mice and excised tumors were imaged using the Image Station.
Histology: Tumor tissues were excised from the mice at 14 d post-intravenous injection of the FA-ND nanoclusters (0.1 wt%), fixed with 4% paraformaldehyde solution, and embedded in paraffin, followed by sectioning. The sliced tumor tissues (5 µm in thickness) were stained with H&E and observed under an optical microscope. In addition, TUNEL staining was performed to detect apoptotic cells.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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