Modality switching between therapy and imaging based on the excitation wavelength dependence of dual-function agents in folic acid-conjugated graphene oxides

SEUNG WON JUN,1,4 JUNYOUNG KWON,1,4 SOO KYUNG CHUN,1 HYUN AH LEE,2 JAEBOM LEE,1 DAE YOUN HWANG,2 CHEN-YUAN DONG,3 AND CHANG-SEOK KIM1,*

1Department of Cogno-Mechatronics Engineering, Pusan National University, Busan 46241, South Korea
2Department of Biomaterials Science, Pusan National University, Miryang 50463, South Korea
3Department of Physics, National Taiwan University, Taipei 10617, Taiwan
4The first two authors contributed equally to this work
*ckim@pusan.ac.kr

Abstract: Owing to its near infrared (NIR) absorption, graphene oxide (GO) is promising for both photothermal (PT) therapy and multiphoton (MP) imaging. Novel therapy/imaging modality switching is proposed here based on the selected excitation wavelength of femtosecond (FS) laser. GO-based destruction of cancer cells is demonstrated when the laser power of 800-nm-wavelength FS laser is increased above 7 mW. However, GO-based imaging is mainly monitored without damaging the sample when using 1200-nm wavelength FS laser in the same laser power range. Folic acid (FA) conjugated graphene oxide (FA-GO) was synthesized for selective cancer cell targeting. Dual-function FA-GO-based cancer cell targeting agents were experimentally optimized to enable therapy/imaging modality switching.

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Traditionally, cancer has been treated by surgery, chemotherapy, radiation therapy, and hormonal therapy, alone and in combination, although these treatments often give rise to side effects. In the past few decades, research efforts on new cancer therapies have aimed at minimizing these side effects, reducing risk, and improving prognosis. Recently, a targeted therapy based on the photothermal (PT) effect has received significant attention as a new cancer treatment method, because it can yield highly selective and minimally invasive treatment of targeted cancer cells [1–3]. In the PT approach, a laser beam is irradiated onto nanoparticles to generate heat for targeted destruction of cancer cells. Because they offer deeper penetration into tissues and incur less photodamage on cells, excitation lasers with wavelengths in the near infrared (NIR) have been mostly utilized in the PT therapy. Therefore, several types of nanoparticles with strong NIR absorbance have been developed as PT agents for killing cancer cells, including carbon-based materials (carbon nanotubes and graphenes), gold-based structures, Pd sheets, CuS particles, and even organic particles [4–6].

1. Introduction
Various graphene-based materials, such as graphene oxide (GO), reduced GO (rGO), and GO nanocomposites, have been used in the PT therapy of cancer owing to their strong absorbance in the NIR range. GO has many advantages than some other NIR absorbing PT agents, such as gold nanoparticles. When the size of GO is reduced to nanometer and the band gap is formed by a quantum confinement effect, strong photoluminescence can be produced from nanosized GO. It has a low cost and relatively high biocompatibility since GO is mainly composed of carbon material [7,8]. To be used in the targeted PT therapy for destruction of cancer cells, nanoparticles need to be able to selectively attach to cancer cell membranes [9]. Since GO is a single monomolecular layer of graphite with a large specific surface area and many functional groups, it is suitable for specific/targeted PT therapy. In this work, folic acid (FA), a molecule that targets cancer cells, was conjugated to GO via a covalent amide bond, because FA is a promising candidate for specific targeting of folate receptors (FRs), with high specificity and high affinity. It has been known that FRs are overexpressed in the membranes of cancer cells in several human cancers, such as breast, ovary, lung, kidney, and endometrium cancers. It was also reported that FA-conjugated GO (FA-GO) can selectively target cancer cells by binding to FRs [10–12]. Previously, FA-GO was designed for a drug delivery system owing to its good biocompatibility, remarkable solubility, and stability in physiological media [13].

On the other hand, GO has received attention as a cell imaging probe for single-photon microscopy (SPM) or multiphoton microscopy (MPM) applications, owing to the strong photoluminescence of GO induced by continuous wave (CW) lasers or ultrafast pulsed lasers [14–16]. When the energy of the laser beam is absorbed by nanoparticles, the electrons in the particles are excited and then return back to the ground state by releasing the absorbed energy, which can be manifested as radiation, vibration, or heat, depending on the properties of the nanoparticles. Those nanoparticles that support two modes of energy release can be used as dual-function agents. Some types of nanoparticles, such as gold nanoparticles and carbon-based nanomaterials, have been considered for use in both cancer cell imaging and therapeutic applications, owing to their photoluminescence and photothermal effects [17,18]. However, possible dual-function nature of GO been properly addressed yet, with only a few works reporting on this issue [19,20]. For example, an 800-nm-wavelength centered femtosecond (FS) laser was used as a common excitation source of dual-function agents of GO, for both therapy and imaging. A lower laser power was applied for two-photon fluorescence imaging of cancer cells, while for the PT therapy of cancer cells the laser power was increased. However, this method required a delicate power control for separating the imaging and therapeutic modes. When a higher laser power is required to obtain deeper in vivo tissue imaging, it becomes difficult to determine the threshold on the laser power that would distinguish two-photon fluorescence imaging from PT therapy, which limits practical applications.

![Fig. 1. Schematic representation of the excitation-wavelength dependent modality switching processes activated when a FS laser beam interacts with dual-function FA-GO-based cancer cell targeting agents. An 800-nm-wavelength centered laser used for PT therapy and A 1200-nm-wavelength centered laser was adapted for MP imaging.](image-url)
In this study, we suggest an approach that utilizes the excitation wavelength dependence to ensure definite therapy/imaging modality switching of dual-function GO agents. The approach is based on the fact that FA-GO exhibits a relatively low absorption at the wavelength of 1200 nm compared with 800 nm. This allows to dramatically suppress the PT effect by using 1200 nm centered FS lasers, which are available for three-photon fluorescence imaging. In other words, non-tissue-damaging in vivo imaging of deep tissue can be easily performed by adapting three-photon fluorescence imaging owing to the strong MP fluorescence property of FA-GO with respect to the laser irradiation at 1200 nm. Yet, the PT effect can be obtained by centering the wavelength of the FS laser at 800 nm. We experimentally demonstrate the novel modality switching between the PT therapy and MP imaging by switching the excitation wavelength of the FS laser from 800 nm to 1200 nm, while using the same power.

2. Materials and methods

2.1 Material

The major agents used in this study were the following: graphite flakes, chloroacetic acid (CICH₃COOH), dimethyl sulfoxide (DMSO), N-hydroxysuccinimide (NHS), and FA, which were purchased from Sigma–Aldrich Co.; DMEM cell culture medium, penicillin, phosphate buffered saline (PBS) and streptomycin, which were purchased from Gibco Invitrogen. The dialysis bags (MWCO = 10,000) were purchased from G&P Life Science.

2.2 Preparation and synthesis of FA-GO

GO samples were synthesized using the modified Hummer method [21,22] which starts by adding graphite and sodium nitrate to sulfuric acid, after which potassium permanganate and water are added and the resulting mix is stirred. Finally, impurities are cleaned using phosphorus pentoxide.

We used a previously reported method [23–25] for conjugation of FA with GO. To obtain nanoscale GO samples, GO was cracked by ultra-sonication in a water solution for 2 h. Then, 5 g of NaOH and 5 g of CICH₃COONa were added to 100 mL of the GO solution (1 mg/mL). After sonication for 2 h, the resulting product (GO-COOH) was neutralized with diluted HCl and washed five times with DD water by centrifugation. The GO-COOH suspension was dialyzed against DD water for over 48 h to remove any unreacted ions. 200 mg of sulfanilic acid and 80 mg of sodium nitrite were dissolved in 20 mL of 0.25% NaOH. The resulting solution was added dropwise to 26 mL of 0.1 M HCl solution in an ice bath. The diazonium salt solution was added to the dispersion of GO-COOH in an ice bath with stirring for 2 h. After dialyzing against DD water for over 48 h, the sulfonated GO-COOH (GO-SO₃H) was stored at 4 °C. GO-SO₃H was then conjugated with FA using a modification of the standard EDC-NHS. 100 mg of GO-SO₃H dispersion was activated by the EDC/NHS solution and treated by ultrasonication for 2 h. 20 mL of 0.5% FA was added to form a mixed solution and allowed to react at room temperature for 12 h. The unreacted materials were separated out by dialysis against the sodium bicarbonate solution (pH 8.0) for 48 h, which was followed by dialysis against DD water for 24 h.

2.3 Cells culture and treatment

Human breast cancer cell (MCF7) lines and non-tumorigenic epithelial breast cell lines (MCF10A) were obtained from the Korean Cell Line Bank (Seoul, Korea). MCF-7 cells were grown with Dulbecco's Modified Eagle's Medium (DMEM, Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, S001-01, Welgene, Gyeongsan-si, Korea), L-glutamine, penicillin, and streptomycin (Thermo Scientific) in a humidified incubator at 37°C under 5% CO₂ and 95% air. Also, MCF10A cells were cultured in DMEM/F12 Ham’s Mixture supplemented with 5% Equine Serum (Gemini Bio), EGF 20 ng/mL.
(Sigma–Aldrich), insulin 10 µg/mL (Sigma–Aldrich), hydrocortisone 0.5 mg/mL (Sigma–Aldrich), cholera toxin 100 ng/mL (Sigma–Aldrich), 100 units/mL penicillin and 100 µg/mL streptomycin (Thermo Scientific) in a humidified incubator at 37°C under 5% CO₂ and 95% air.

2.4 Chemical characterization

The absorbance characteristics of GO and FA-GO were measured using an ultraviolet-visual (UV/Vis) spectroscope (S310, Scinco, Korea). The surface zeta potentials of GO, GO-COOH, GO-SO₃H, and FA-GO were monitored using a zeta-sizer (Nano ZS, Malvern Instruments, UK). In addition, a Fourier transform infrared (FTIR) spectroscope (FT/IR-6300, JASCO, Tokyo, Japan) was used for validation of successful functionalization of GO samples with different functional groups in the 1500–3500 cm⁻¹ range. The morphologies and sizes of GO samples were studied using an atomic force microscope (AFM; XE-7, Park System, Korea).

2.5 Multi-photon microscopy

A custom-built MP microscope (MPM) was used to acquire two-photon and three-photon induced fluorescence images. Compared with conventional microscopes with limited imaging depth owing to the fact that light is strongly scattered by biological tissue, the MPM ensures high-resolution imaging of deep tissue layers based on the nonlinear excitation characteristics. Three-photon microscopy is significantly advantageous compared with two-photon microscopy, because longer wavelengths afford relatively weak absorption and scattering in biological samples. The three-photon induced auto-fluorescence of cells and tissues can be significantly attenuated, which cannot be achieved using either confocal or two-photon microscopy. This makes the MPM useful for weak-background and high-contrast imaging of
deep tissue layers [26–28]. Figure 2 schematically shows our custom-built MPM, where a Ti-Sapphire FS pulse laser (680–1080 nm, 140 fs, 80 MHz) and an optical parametric oscillation FS pulse laser (1050–1600 nm, 200 fs, 80 MHz) are used as laser sources for nonlinear absorption. The average power of the two laser sources can be adjusted by using a half-wave plate and a linear polarizer, because these two laser sources yield a horizontally polarized beam. For the validation of power effect on the sample target, the average power of each laser beam was measured at the location of specimen below objective lens using photodiode power sensor (S132C, Thorlabs, USA). Subsequently, the laser beam was circularly polarized using a quarter-wave plate. Two-dimensional (2D) images were obtained using galvanometer-driven x-y scanners for raster scanning of the beam, and three-dimensional (3D) images were acquired using a motorized stage for z-stacking of the focal plane. The laser beam was expanded using a beam expander and was reflected to the back aperture of the objective lens by a short-pass dichroic mirror. The expanded laser beam was focused onto the sample by a high NA (1.0, water) objective lens. Two- or three-photon induced fluorescence signals were produced by the focused beam, and the signals from the sample were recollected in the backward direction by the focusing objective lens and further guided by long-pass dichroic mirrors set for four channels: red, green, blue, and second harmonic generation (SHG). For all of the three colors, the fluorescence signals were isolated using band-pass filters and detected using photomultiplier tubes (PMTs). The PMT detectors were utilized for photon counting of fluorescence signals. All device operations as well as the readout of the fluorescence signals were controlled using a custom-written LabVIEW code running on a personal computer (PC) DAQ.

3. Result and discussion

3.1 Chemical characterization

![Fig. 3. Chemical characterization of FA-GO. (a) AFM image of FA-GO, (b) UV-Vis spectra of GO and FA-GO, (c) FTIR spectra of GO, GO-COOH, GO-SO3H, FA-GO and FA, (d) Zeta potential of GO, GO-COOH, GO-SO3H and FA-GO.](image)

Figure 3 shows the chemical characterization results for FA-GO. Ultrasound was used to crack the synthesized FA-GO. As shown in the AFM image in Fig. 3(a), the resulting FA-GO nanoparticles were 100 nm in diameter, and their height was in the 1–2 nm range. This height
is characteristic of a single layer or two layered sheets. The UV-Vis FTIR spectra were measured to determine the conjugation of FA. As shown in Fig. 3(b), the UV-Vis spectra of GO and FA-GO reveal a new peak at 190 nm owing to the conjugation of FA with GO. This peak reflects the \( n \rightarrow \sigma^* \) transition in R-NH\(_2\) molecules. The FTIR spectra of the FA-GO samples were also measured, to validate the successful conjugation between FA and GO (Fig. 3(c)). The black color in the spectrum corresponds to the ester, hydroxyl, and epoxide groups of GO sheets. It is necessary to convert these groups into COOH groups to improve the aqueous solubility of the graphene derivatives and to facilitate chemical binding of biomolecules to GO via EDC chemistry. In our strategy, the hydroxyl, epoxide, and ester groups of GO were converted into the presence of SO\(_3\)H groups in GO, which was also confirmed by FTIR measurements. Conjugation of FA with GO-SO\(_3\)H through formation of an amide bond by the reaction between the NH\(_2\) groups of FA and the COOH groups of GO-SO\(_3\)H is reflected in the FTIR spectra in Fig. 3(c). On the green curve, a new peak appeared in the 1640–1560 cm\(^{-1}\) region, which was observed at the same location as the FA peak. The new peak in FA-GO can be assigned to the vibration mode of CO-NH. The FTIR spectra also clearly reveal the successful conjugation of FA with GO. In Fig. 3(d), the zeta potential was measured to understand the difference in the stability of graphene derivatives. The zeta potentials of GO, GO-COOH, GO-SO\(_3\)H, and FA-GO were determined to be \(-40.9\), \(-46.5\), \(-46.4\), and \(-48.6\) mV, respectively. The lower zeta potentials for FA-GO compared with GO indicate a significant difference in the surface charge, and thus in the colloidal stability, of these graphene derivatives.

3.2 MP induced fluorescence properties of FA-GO

Two- and three-photon induced fluorescence spectra of FA-GO were also measured and are shown in Fig. 4. The 800-nm center wavelength of the Ti-Sapphire ultrafast FS laser was used for the two-photon excitation (2PE), while the 1200 nm center wavelength of the OPO laser was used for the three-photon excitation (3PE). The fluorescence spectra are similar in the 400–650 nm range, and their peak intensities are at \(~590\) nm, for both 2PE and 3PE cases.
To determine whether the three-photon induced fluorescent FA-GO can enhance the imaging depth, we investigated different thicknesses of epoxy-based optical tissues. Biomimetic phantoms were fabricated using epoxy-based composites. Epoxy was used as a base material to fabricate the tissue phantoms owing to its good formability for making various shapes. Tissue phantom formation started by mixing epoxy and viscosity depressant. The viscosity depressant was used to reduce the high viscosity of epoxy. Next, TiO$_2$ was added to the mixture to provide the scattering property, which is a compatible tissue characteristic. The mixture was stirred and sonicated. Finally, a hardener was added to solidify the tissue phantom. The optical tissue phantom consisted of epoxy that contained TiO$_2$ as scatters to adjust the reduced scattering coefficient to $1 \text{ mm}^{-1}$, which is similar to the reduced scattering coefficient of actual human tissue. A tissue phantom with adjustable depth was fabricated using a different height mold. Two- and three-photon induced fluorescence images of FA-GO in the epoxy-based biomimetic phantom were acquired, to demonstrate the advantage of three-photon deep tissue imaging. The same incident laser power was used for all depths, to facilitate the comparison between the two- and three-photon cases. Figure 5(a) shows strong three-photon induced fluorescence signals that were detected at the depth of $1350 \mu$m. In contrast, it was difficult to detect two-photon induced fluorescence signals at the depth of $750 \mu$m. Therefore, using three-photon microscopy and FA-GO, information about deeper tissue layers can be obtained. Our results suggest that three-photon induced fluorescence of FA-GO has potential applications and likely represents a novel approach to bioimaging.
3.3 PT properties of FA-GO

Figure 6(a) shows the quadratic relationship between the two-photon induced fluorescence intensity of FA-GO and the excited laser power, as low as 7 mW, at the location of specimen for the wavelength of 800 nm. An abnormal relationship was observed for the laser power as high as 7 mW. For the laser power values is increased above 7 mW, two-photon induced fluorescence intensity did not increase proportionally to the input power squared, and thermal energy release was dominant. On the other hand, Fig. 6(b) shows the cubic relationship between the three-photon intensity of FA-GO and the excitation laser’s power, for the wavelength of 1200 nm. In this case, the signal increased proportionally to the input power cubed, suggesting that the PT effect is suppressed in 3PM.

To confirm the PT effect in the wavelengths of 800 nm and 1200 nm, we performed the micro-bubbling test. The micro-bubbling of water, in the presence of FA-GO when excited by a FS laser beam, was examined for samples on glass cover slips. This experiment and the experiments for cell therapy were performed on the custom-built MPM. For 2PM, extensive micro-bubbling occurred in case of the 800 nm laser power of 9 mW. This was not observed in the case of 3PM with the same laser power of 9 mW with 1200 nm wavelength, owing to the lower absorbance of FA-GO at 1200 nm. Figure 7 shows the 2PM and 3PM images (left) and the transmission images (right) for FA-GO, after raster-scanning at 9 mW. The scanning area was fixed at 150 µm × 150 µm and the pixel dwell time was fixed at 45 kHz. This means that the reduction of FA-GO occurs quickly, within a fraction of one microsecond. Such a fast reduction is attributable to the high peak power of laser pulses. For a pulsed laser with a repetition rate of 80 MHz and a pulse width of 140 fs, the pulse peak power was 0.8 kW for the average laser power of 9 mW. In addition, the instantaneous and strong increase in the temperature also contributed to the ultrafast thermal reduction of FA-GO. Localized micro-bubbling and boiling can yield high-pressure shock waves, which in turn can kill cells.
The PT heat generation for the laser wavelengths of 800 nm and 1200 nm was compared using a FS laser with the average power of 40 mW. As shown in Fig. 8, in both cases FA-GO dependent temperature increases were observed in response to laser irradiation. To reveal the PT property of FA-GO, we collected temperature increase data for different solutions of FA-GO in DI water. Here, we show the results for only a low-concentration (1 mg/mL) FA-GO solution, to demonstrate the extraordinary PT energy conversion efficiency for irradiation with 800 nm. The temperature rapidly increased within 550 s of irradiation. Owing to the water absorption at 1200 nm, the temperature increase of the DI water sample was higher for irradiation with this wavelength than with 800 nm. Figure 8(c) shows wavelength dependence of PT heat generation for FA-GO, suggesting much more efficient PT heat generation for irradiation with 800 nm.
3.4 Uptake and internalization of FA-GO by cellular targets

Owing to its high affinity for FRs, FA can be a promising candidate for cancer cell targeting. We demonstrated three-photon induced fluorescence of FA-GO using a custom-built three-photon microscope to obtain three-photon induced fluorescence images of FA-GO. To demonstrate cancer cell targeting imaging, we used human breast cancer cell (MCF7) lines and normal human mammary epithelial cell (MCF10A) lines. Cells in each of the considered cell lines were pretreated using a 10 µg/mL FA-GO solution to determine the role of FRs. Excess FA-GO was then removed by washing three times with PBS. To confirm the location of the analyzed cells, bright field images were acquired and multi-color images were acquired using DAPI as nuclear staining. Figures 9(a) and 9(c) show that cancer cells were successfully targeted using FA-GO. Figures 9(b) and 9(d) show that normal cells were not targeted by FA-GO. Though there are various confluence degrees at same culture plate in this duty, in most areas of the same culture plate for each cell line, most of the cells showed similar patterns for uptake and internalization of FA-GO. Our results suggest that highly localized targeting of cancer cells can be achieved using FA-GO.
3.5 NIR PT therapy using FA-GO

To demonstrate the excitation-wavelength dependent modality switching between therapy and imaging, we performed live cell imaging using MCF7 cancer cells and fluorescein diacetate staining. We acquired twenty raster scans at the laser wavelengths of 800 nm and 1200 nm by
2PM and 3PM, and acquired 2PL and 3PL in each scan. Figure 10 shows the images for in vitro cell therapy, acquired using the FS laser. Fluorescein diacetate (FDA) was used to examine the effects of laser irradiation on cell viability. Non-fluorescent FDA was taken up by cells and converted into the green fluorescent metabolite fluorescein. The measured signal served as an indicator of cell viability, as the conversion was esterase dependent [29,30]. Living cells emitted green fluorescent light, with the fluorescence intensity corresponding to the extent of cell viability (compromised cells had lower fluorescence intensity), which was not observed for cells that were not labeled with FA-GO (Fig. 10(a)). Interestingly, when cells were labeled with FA-GO, a few scans for the laser power above 9 mW were sufficient to induce therapeutic effects (Fig. 10(b)). The FDA fluorescence disappeared from some cells during the raster scanning, indicating that these cells were instantly damaged by the hosted nanoparticles. Significant reduction in cell viability was achieved when the cells were scanned over 15 times at the laser power of 9 mW. Unlike the case of 3PM, we did not observe cell death after 20 repeats of laser scanning (Fig. 10(c)). Furthermore, FDA signals did not disappear when the laser power was increased over 30 mW. Figure 10(d) shows the numerical variation of FDA fluorescence signal according to each laser scan. The normalized fluorescence was obtained from the regions of interest (ROIs) illustrated as three small squares in each fluorescence image. When cancer cells labeled with FA-GO were scanned with laser wavelengths of 800 nm, we clearly observed a sharp decrease of contrast.

4. Conclusion

We successfully synthesized FA-GO for selective targeting of cancer cells. The conjugation of FA was confirmed based on the UV-Vis and FTIR spectra. For the localized targeting of cancer cells with dual-function agents of FA-GO, the new modality switching between therapy and imaging was experimentally demonstrated using the excitation wavelength dependence. As the laser power of 800-nm-wavelength centered laser is increased above 7 mW, this laser was used for PT therapy to demonstrate that the ultrafast reduction of FA-GO can promote the micro-bubbling of water that helps to destruct cancer cells. The in vitro test in this study showed that the microbubbles were produced only in cells labeled with FA-GO and the micro-bubbles collapsed instantly. However, the 1200-nm-wavelength centered laser with the laser power range above 7 mW was used on the same sample target for three-photon fluorescence imaging without damaging the imaged samples. Since three-photon microscopy exhibits suppressed PT effect owing to low absorbance of FA-GO at 1200 nm, it can avoid occurrence of cell death with micro-bubbling till we increase the laser power values over 30 mW. Furthermore, since three-photon microscopy exhibits small absorption and low scattering owing to the longer wavelengths associated with biological tissue, it enables imaging of deep tissue layers with weaker background noise and higher contrast. The results described in this work indicate that the combination of PT therapy and MP imaging using FA-GO agents can provide a precise margin for minimally invasive treatment of malignant cancer cells. The suggested excitation-wavelength dependent modality switching between therapy and imaging is promising for applications of targeted cancer treatment and guided bioimaging. In addition, the proposed technique can be potentially used not only with FA-GO but also with other nanoparticles that satisfy dual-function switching.

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Disclosures

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