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Short communication

Phototherapeutic functionality of biocompatible graphene oxide/dendrimer hybrids

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ABSTRACT

Hydroxyl-terminated fourth generation poly(amido amine) dendrimer and folic acid were chemically bound on graphene oxide. The resultant hybrids exhibited one-photon and two-photon fluorescence emission, since the excitation irradiation at 390 and 780 nm on the hybrids brought a fluorescence emission in the visible region around 450 nm. In addition, the photocytotoxicity study revealed that under the two-photon excitation at 780 nm, the hybrids can absorb near-infrared light and generate reactive oxygen species which can oxidize the HeLa cells and cause their death, suggesting the phototherapeutic behavior. Cytotoxicity measurement revealed the high biocompatibility of the hybrids toward HeLa cells. Thus, the present biocompatible hybrids consisting of only dendrimer, folic acid and graphene oxide have potentials as photodynamic therapeutic agents for medical treatment.

1. Introduction

Graphene oxide, which is an oxidized graphene composed of a graphene-like sheet, is chemically functionalized with oxygen-including groups such as hydroxyl, carboxylic acid and epoxide [1]. Hybrid materials of graphene oxide (GO) have been investigated as new promising materials for biomedical applications including cellular imaging [2], drug delivery [2,3], and photodynamic therapy [4]. Poly(amido amine) (PAMAM) dendrimers, highly-branched polymers with a multi-functionalized peripheral surface, have high degree of molecular uniformity, monomolecular weight, and specified size and shape [5–8]. PAMAM dendrimers also possess a strong fluorescence emission [5–8]. It has been confirmed by the visual observation of fluorescent dendrimers that fluorescent dendrimers-bound avidins interact selectively with biotins immobilized on the patterned substrates [9]. The fluorescent PAMAM dendrimers have revealed lower in vitro cytotoxicity than the non-fluorescent ones toward rat C6 glioma cells [10].

In the present work, hydroxyl-terminated PAMAM dendrimer (DEN-OH) (see Fig. S1) was chemically bound on GO as a vehicle, and folic acid as a target reagent to specific cells with folate receptors [3] was further attached on GO. Since it has been reported that neutral PAMAM dendrimers are less toxic than ionic dendrimers [11], DEN-OH should be preferable to utilize for preparing biocompatible novel nanohybrid materials. Biomedical characteristics of the prepared hybrids such as fluorescent property and cell cytotoxicity were examined. Moreover, the ability to generate reactive oxygen species (ROS) in the hybrids under laser irradiation was investigated for interpreting the obtained characteristics of photocytotoxicity toward HeLa cells under two-photon excitation at 780 nm of these hybrids. Altogether, the present work provides new hybrid materials consisting of GO, DEN-OH and folic acid (FA), GO/DEN-OH/FA, that should have a potential to use as a drug in the photodynamic therapy and also a drug carrier for the efficient delivery in target cancer cells, since GO is a new type of drug carrier, dendrimer is necessary as a stabilizing and photosensitizing drug and FA works as a cancer-targeting molecule.

2. Experimental

2.1. Binding of DEN-OH and FA on GO

Esterification reagents (*N*,*N*′-dicyclohexylcarbodiimide and 4-dimethylaminopyridine) were added in a dimethylformamide

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solution of dendrimer and GO under vigorous stirring, and the mixture was further vigorously stirred at room temperature for 3 days. The dispersion was centrifuged, and the centrifugate (GO/DEN-OH) was rinsed with dimethylformamide. *N*-hydroxysuccinimide and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride were added into an aqueous suspension of GO/DEN-OH, and the mixture was ultrasonicated for 2 h. Then, an aqueous $NaHCO_3$ solution (pH 8) of FA was added and the mixture was stirred overnight at room temperature. The purification of the product (GO/DEN-OH/FA) was carried out by the dialysis against a $NaHCO_3$ solution (pH 8.0) and water. The details are given in Supporting information.

2.2. In vitro study of the photocytotoxicity of GO/DEN-OH/FA

An aqueous suspension (100 μl) of GO/DEN-OH/FA (or GO/FA) was incubated with HeLa cells (1×10^6 cells), which were seeded onto coverslips in a 6-wells plate for 2 h. Microscopic cell viability was investigated by using a multiphoton and high-velocity spectral confocal microscope under the laser irradiation at 780 nm (1.07 W) for 15 min at five different positions.

3. Results and discussion

3.1. Hybridization of DEN-OH and FA on GO

DEN-OH and FA were attached to GO using condensing agents for esterification and amidation, respectively (Fig. 1). TEM and AFM images revealed that after DEN-OH and FA were conjugated on GO, the resultant hybrids exhibited an aggregates flat sheet (see Fig. 1 and Fig. S2). The average zeta potential and particle size of GO, and the resultant hybrids were then measured by DLS and zeta potential as shown in Table S1. The increased in the average particle size of GO/DEN-OH/FA might be due to the aggregation of GO hybrid materials. Zeta potential value of the aggregated GO/DEN-OH was 23.7 mV when compared with the well-dispersed GO (−78 mV); while after FA was conjugated on GO/DEN-OH, zeta potential value was −59.7 mV, indicated GO/DEN-OH/FA was formed a stable dispersion in water.

IR spectrum of GO/DEN-OH exhibited the absorbance of the C=O band at 1735 cm^{-1} of GO [12] which was significantly decreased after the immobilization of DEN-OH (Fig. S3 and Table S2). Meanwhile, GO/DEN-OH showed absorption bands of amide I and amide II vibration modes at 1629 and 1555 cm^{-1}, respectively, of DEN-OH [13]. Moreover, the bands at 1694 and 1284 cm^{-1}, respectively, can be attributed to C=O and C—O stretching modes of ester bond.

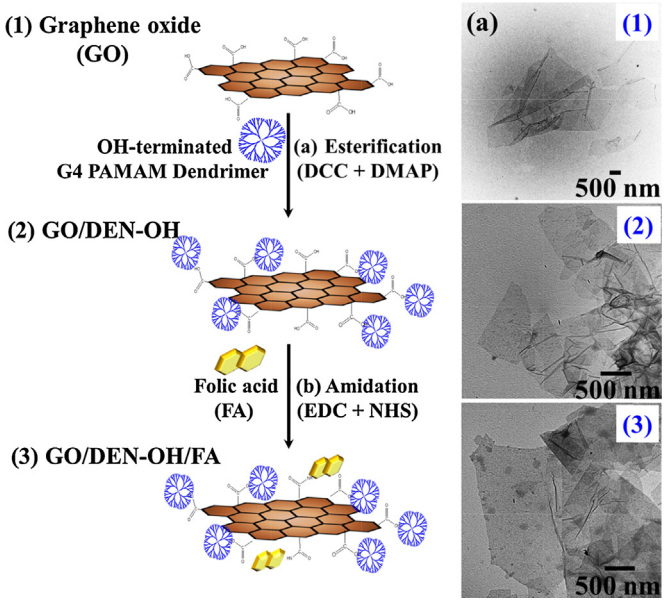

Fig. 1. Schematic illustration of immobilization of OH-terminated PAMAM dendrimer (DEN-OH) and folic acid (FA) on graphene oxide (GO) and TEM images of (1) GO, (2) GO/DEN-OH and (3) GO/DEN-OH/FA.

Thus the covalent immobilization of DEN-OH on GO through the ester linkage was proved. When FA was bound on GO/DEN-OH, GO/DEN-OH/FA showed a band shift of amide I from 1629 cm^{-1} of GO/DEN-OH to 1643 cm^{-1}. Contrary, the NH_2 bending band of FA at 1605 cm^{-1} disappeared. These results mean the successful formation of amide linkage between GO/DEN-OH and FA.

Fig. S4 shows Raman spectra of GO and the resultant hybrids at an excitation wavelength of 633 nm. It was observed that both GO/DEN-OH and GO/DEN-OH/FA exhibited Raman D and G bands in common at 1334 and 1590 cm^{-1}, respectively, similar to GO. The I_D/I_G values of GO/DEN-OH (1.03) is almost comparable to I_D/I_G values of GO (1.06); however, I_D/I_G values of GO/DEN-OH/FA was increased up to 1.29 (Table S3). Since I_D/I_G values provide information of structural defect of carbon materials, it can be indicated the covalent functionalization of DEN-OH and FA on GO might take place at the carboxylic group at the edge of GO sheet.

GO revealed a main absorption band at 232 nm and a shoulder around 300 nm, which correspond to $\pi \rightarrow \pi^*$ transition of aromatic CC bonds and $n \rightarrow \pi^*$ transition of C=O bonds, respectively,

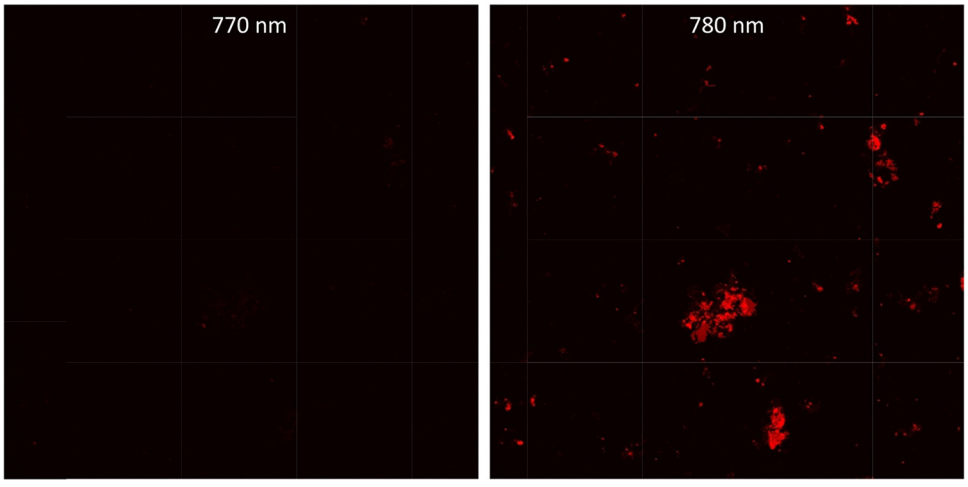

Fig. 2. Two-photon fluorescence images of GO/DEN-OH/FA (DEN-OH = 54 μM) at excitation wavelengths of 770 and 780 nm.

in GO as shown in Fig. S5(a) and Table S4 [12,2]. After DEN-OH was attached on GO, a spectrum changed to a main band below 200 nm and a shoulder at 288 nm. These bands attributed to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively, of amide group in DEN-OH. In addition, these bands were intensified with increasing the concentration of DEN-OH on GO. After FA was loaded onto GO/DEN-OH, an absorption band at 283 nm was sharpened, although its absorbance scarcely changed from that of GO/DEN-OH, and an intrinsic absorption band at 359 nm of FA was not apparent (Fig. S5(b) and Table S4). Although these results seem to suggest no contribution of FA on UV–vis spectra of GO/DEN-OH, a band below 200 nm of DEN-OH was weakened, indicating the hypochromic effect of FA on it.

GO exhibited an emission band at 371 nm and a weak band at 570 nm, respectively, when excited at 282 and 390 nm (Fig. S6), similar to the previous report [2]. Since GO is a 2D network consisting of sp^2 and sp^3 carbon atoms, the recombination of electron–hole pairs localized within finitely-sized sp^2 carbon clusters embedded within a sp^3 carbon matrix can behave as the luminescence center and chromophore [14] and give rise to fluorescence [15,16]. Meanwhile, DEN-OH revealed an emission band around 450 nm at the excitation wavelength of 390 nm (Fig. S6) [7]. This band comes from the photoluminescence of oxygen-doped DEN-OH. The photoinduction of oxygen exciplex or peroxy radical in $1 \rightarrow 2$ N-branched dendrimers is assumed [8,17,18].

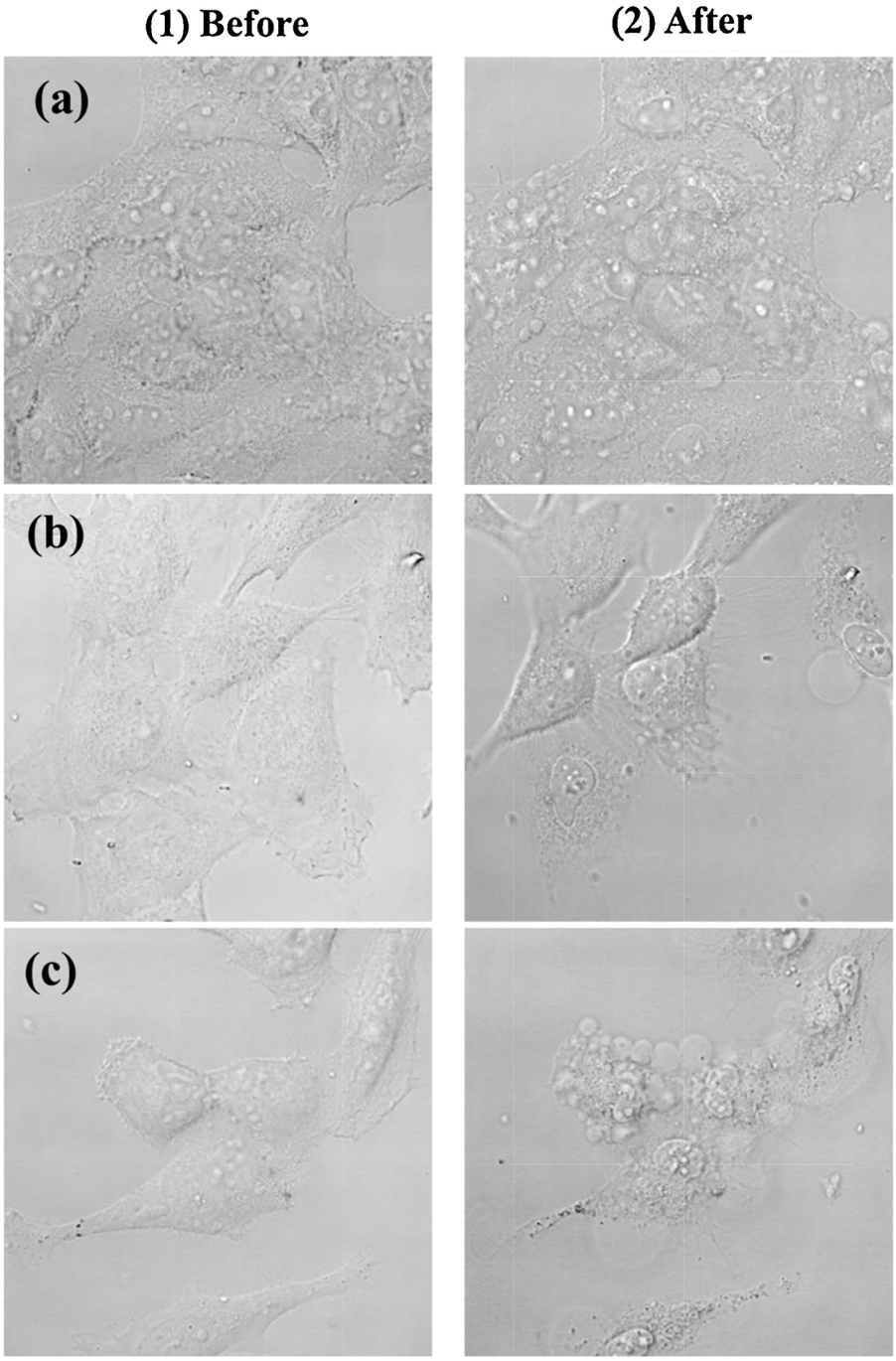

Fig. 3. Two-photon confocal microscopic images of HeLa cells (a) without (control) and (b) and (c) with GO/DEN-OH/FA before and after two-photon irradiation at 780 nm for 15 min. Concentration (μM) of DEN-OH: (b) 18, (c) 90.

GO/DEN-OH and GO/DEN-OH/FA exhibited emission bands at 355 and 450 nm with different intensities at 282 nm excitation, but only one 450 nm band was observed at 360 and 390 nm excitation (Fig. S6-1 and S6-2). It can be noticed that an emission band at 355–371 nm occurs only from the chemical structure of GO, although it is intensified strongly by the attachment of DEN-OH in comparison with DEN-OH/FA undergoing the quenching effect of FA. In addition, it was observed that the strong blue photoluminescence from GO/DEN-OH and GO/DEN-OH/FA can also visualize under fluorescence microscope as shown in Fig. S7, similar to the previous report [9,10]. On the other hand, although the photoluminescence in the visible region can take place from three components, the emission band around 450 nm can be contributed by DEN-OH, because the emission of GO is weak and the emission of FA is quenched by GO (Fig. S6-3). Thus, it can be confirmed that the fluorescence emission of GO/DEN-OH and GO/DEN-OH/FA originated from DEN-OH.

One-photon and two-photon excitation fluorescence spectra of GO/DEN-OH/FA are shown in Fig. S6-2 where a quite similar fluorescence emission band around 450 nm at 390 and 780 nm excitation was observed for all three hybrids of GO/DEN-OH, GO/DEN-OH/FA, and GO/FA, demonstrating that one-photon and two-photon excitation populate the same fluorescing excited state [19]. The remark is that the band intensity was one order stronger for GO/DEN-OH/FA than for GO/DEN-OH and GO/FA and one order weaker at 780 nm excitation than at 390 nm excitation. These observations indicate that GO/DEN-OH/FA exhibited one-photon and two-photon fluorescence emission. Moreover, bright red spots of visible fluorescent GO/DEN-OH/FA were visualized under multi-photon confocal microscopy at the excitation of 780 nm (Fig. 2). The number of visible fluorescent depended on the excitation wavelength, and GO/DEN-OH/FA revealed the fluorescence emission at 760–920 nm (Fig. S8). Strong two-photon fluorescence emission of GO/DEN-OH/FA suggests possible applications as a two-photon fluorescence marker in biomedical imaging [20] as well as for the two-photon photodynamic cancer therapy [21].

3.2. Photocytotoxicity of GO/DEN-OH/FA

The photocytotoxicity of GO/DEN-OH/FA hybrid materials was examined in vitro toward HeLa cells under two-photon excitation at 780 nm. FA is a targeting ligand to afford the specific delivery of GO/DEN-OH to bind with a folate receptor existing on the surface of HeLa cells. Two-photon confocal microscopic images before and after irradiation by a laser beam at 780 nm are shown in Fig. 3. While the control was not affected by the laser irradiation, the existence of GO/DEN-OH/FA hybrids could cause the death of HeLa cells, and the killing effect became strong with increasing the content of DEN-OH (partially for 18 μM and almost complete for 90 μM of DEN-OH) (Fig. S9). This tendency is consistent with the concentration-dependent increase of the fluorescence intensity (Fig. S5). These observations indicate that GO/DEN-OH/FA can be utilized in the two-photon photodynamic therapy, since GO/DEN-OH/FA itself is non-toxic, namely, it does not affect cells, if there is no photo irradiation.

The cytotoxicity of GO/DEN-OH/FA was evaluated by using MTT assay and compared with those of GO and DEN-OH as shown in Fig. 4. DEN-OH indicated high viability toward HeLa cells (>80% at concentrations up to 20 μg/ml). This result can be compared with the behavior of NH_2-terminated PAMAM dendrimer with the low viability toward C6 glioma cells and its improvement by fluorescent dendrimer [10]. Although the cell viability of GO was lower than that of DEN-OH, the loading of DEN-OH obviously improved the biocompatibility of GO, since the cell viability of GO/DEN-OH/FA was close to that of DEN-OH. These results can be compared with

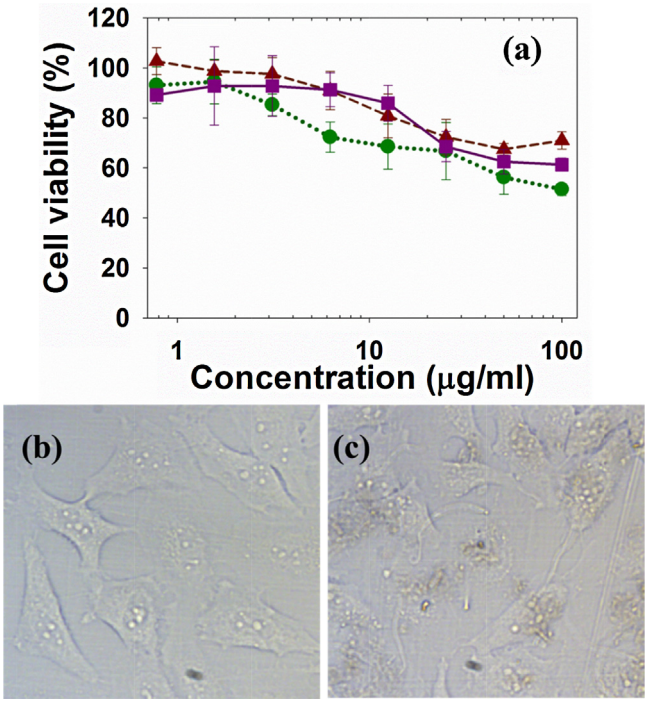

Fig. 4. (a) Cell viability of ● GO, ▲ DEN-OH and ■ GO/DEN-OH/FA and (b) and (c) microscopic images of HeLa cells (b) before and (c) after incubation with GO/DEN-OH/FA at the concentration of 6.25 μg/ml for 1 day.

those of GO loaded FA and anticancer drug, doxorubicin, which revealed the cell viability of >∼76% at concentrations up to 20 μg/ml [3]. Fig. 4(b) and (c) shows the microscopic images of HeLa cells before and after treatment with GO/DEN-OH/FA, respectively. No breakdown of cells can be seen.

In the present work, the generation of reactive oxygen species (ROS) by GO/DEN-OH/FA upon laser irradiation was examined by using *N*,*N*′-dimethyl-*p*-nitroso-aniline (RNO) as a probe to detect ROS, as described in SI. Since the oxidation by radical leads to the bleaching of an absorption band of RNO at 440 nm [22], the generation of ROS by GO/DEN-OH/FA upon laser irradiation were then monitored by observation of the bleaching of an absorption band of RNO at 440 nm. On the investigation of GO/DEN-OH/FA for generation of ROS upon laser irradiation, the UV–vis spectra of a dispersion of GO/DEN-OH/FA showed a hypochromism at 440 nm over laser irradiation of 2 h. Namely, it was observed that the bleaching of RNO increased almost linearly with irradiation time (Fig. S10), supporting the generation of ROS upon laser irradiation. The bleaching of RNO in a dispersion of GO/DEN-OH/FA at an absorption band of 440 nm was indicated that ROS was generated upon laser irradiation in consistence with previous reports [22]. This observation indicates that after GO/DEN-OH/FA absorbed NIR light upon laser irradiation, the excited GO/DEN-OH/FA transfers its energy to oxygen-doped DEN-OH and leads to the generation of ROS. Thus ROS kills the HeLa cells. These results indicate the suitability of GO/DEN-OH/FA for phototherapy.

4. Conclusions

Hybrid materials consisting of GO, DEN-OH and FA were successfully prepared. It was observed that GO/DEN-OH/FA exhibited a one-photon and two-photon fluorescence emission in the visible region at 450 nm but at different intensities, under one- and two-photon excitation of GO/DEN-OH/FA at 390 and 780 nm, respectively. The photocytotoxicity study revealed that when HeLa

cells were incubated with GO/DEN-OH/FA and irradiated by a 780 nm laser for 15 min, the death of the HeLa cells was observed. This occurred upon two-photon excitation at 780 nm: GO/DEN-OH/FA can absorb NIR light and generate ROS that can oxidize the HeLa cells and cause their death. Thus, GO/DEN-OH/FA could be utilized as photosensitizer in the two-photon photodynamic therapy for cancer treatment. In addition, GO/DEN-OH/FA hybrids are promising candidates to be used as carriers for drug delivery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.06.010.

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