Self-assembled complex of probe peptide – E. Coli RNA I conjugate and nano graphene oxide for apoptosis diagnosis

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Abstract

Caspase-3 plays an important role in the initiation and propagation of apoptosis which is involved in various kinds of diseases including neurodegenerative diseases and inflammatory diseases. The caspase-3 cleavable site of Asp-Glu-Val-Asp (DEVD) connected to a partial sequence of Escherichia coli RNAI was labeled with tetramethyl-6-carboxyrhodamine (TAMRA) as an optical probe. Graphene oxide (GO) was synthesized by a modified Hummer’s method and exploited for the preparation of nano-sized GO (NGO) conjugated with polyethylene glycol (PEG). After binding the NGO-PEG by π-π stacking, the quenched fluorescence of TAMRA-DEVD-single stranded DNA (ssDNA) conjugate was recovered via the enzymatic cleavage by caspase-3 in live A549 cells. The comparative study with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay clearly confirmed the specific detection of apoptosis by the non-covalent TAMRA-DEVD-ssDNA/NGO-PEG complex. Furthermore, the self-assembled NGO complex was successfully exploited for in vivo diagnosis of apoptosis-related diseases like hypoxic-ischemic encephalopathy (HIE) and liver cirrhosis.

1. Introduction

The progress of diagnosis is highly dependent on the development of nanomaterials that offer a simple, rapid, cost-effective, and in situ monitoring platform for biosensing and bioimaging applications [1–5]. Graphene oxide (GO), a special member of carbon nanomaterials family [2,6,7], has unique physical property to bind biomolecules, such as nucleic acids [8], peptides [9], and aromatic chemical compounds [10,11], by π-π stacking. The complex formation can cause a long range fluorescence resonance energy transfer (FRET) from an attached dye on the biomolecule to graphene resulting in the fluorescence quenching [12]. Accordingly, nano-sized GO (NGO) derivatives have been widely exploited for a variety of biomedical applications. NGO has been investigated for controlled delivery of gene and cancer therapeutics with a high drug loading efficiency on both sides of the graphitic domain [10,11]. NGO can work as a photosensitizer for a photothermal ablation therapy [13]. In addition, NGO based hybrid nanomaterials have shown a great potential as attractive and powerful tools for biosensing and bioimaging to detect nucleic acids [14], peptides [9], proteins [15], viruses [16], and small molecules [17].

Apoptosis is an important physiological event of cell loss which depends on pre-existing and de novo proteins for the highly regulated pathway [18]. Among them, caspase-3 is particularly known to be directly involved in the initiation and propagation of apoptosis [18,19]. Apoptosis is highly related with various kinds of diseases such as neurodegenerative diseases, inflammatory diseases, cancer, autoimmune diseases, and hematologic diseases [18,20–22]. However, the conventional assays for the detection of apoptosis including terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Annexin V assays are inefficient and time-consuming due to the nonspecificity and the complicated multiple processes [23]. Despite wide investigations [19,23–26], there are few reports on in vivo monitoring of caspase for diagnostic applications.

In this work, taking advantages of π-π stacking between graphene and single stranded DNA (ssDNA), and the fluorescence quenching by FRET, we developed a facile platform of the near-infrared fluorescent (NIRF) probe peptide − ssDNA/polyethylene glycol (PEG) conjugated NGO (NGO-PEG) complex for the diagnosis of apoptosis related diseases. Tetramethyl-6-carboxyrhodamine
2. Materials and methods

2.1. Materials

Natural graphene powder, sulfuric acid (H2SO4), sodium nitrate (NaNO3), potassium permanganate (KMnO4), chloroacetic acid (CICH2COOH), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), staurosporine (STS), 4,6-diamidino-2-phenylindole (DAPI), bovine serum albumin (BSA), caspase-3 and its inhibitor, Z-DEVD-FMK, were purchased from Sigma–Aldrich (St. Louis, MO). 5′-Thiol-modified partial sequence of E. coli RNA I (5′-ATC TGG CTT GCT CTA GCG-3′) was purchased from Bioneer (Daejon, Korea). C-terminal amine functionalized fluorescent caspase-3 specific probe peptide (TAMRA-GDEVDAP) was purchased from Peptone (Daejon, Korea). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and N-(E-nalimidocaproyloxy)sulfosuccinimide (Sulfo-EMCS) were purchased from Pierce (Rockford, IL). Hydrogen peroxide (H2O2) was purchased from Junsei Chemical (Tokyo, Japan) and polyethylene glycol (PEG) was purchased from Sunbio (Seoul, Korea). All reagents were used without further purification.

2.2. Synthesis of fluorescently labeled caspase-3 specific probe

To prepare fluorescently labeled caspase-3 specific probe, 50 nmol of NIRF TAMRA-labeled DEVD peptide (f-DEVD) with a free amino group at C-terminal was reacted with 50 nmol of sulfo-EMCS in PBS (pH 7.2). After vigorous stirring at room temperature for 1 h, the resulting maleimide-functionalized f-DEVD peptide was reacted with 0.9 equimolar amount of 5-sulfhydryl-modified ssDNA for 6 h with mild stirring. The remaining cross-linker and unreacted peptide fragment were eliminated using a dextran desalting column (MWCO = 5 K) and further purified by dialysis (MWCO = 10 K) against deionized water. The resulting products were collected, concentrated using a SpeedVac evaporator (Thermo, Madison, WI), and analyzed by 25 agarose gel electrophoresis in 0.5 TBE buffer and gel permeation chromatography (GPC).

2.3. Synthesis of GO and NGO-PEG

GO was synthesized with natural graphite flake using a modified Hummer’s method as reported elsewhere [2,19]. To prepare NGO, exfoliation was carried out by sonication 1.0 mg/ml of GO suspension for 4 h with a small tip of Branson sonicator (Fisher, Pittsburgh, PA) in a refrigerated circulator (Jeoil Tech, Korea). After that, 10 mL of 2.0 mg/mL NGO suspension was mixed with chloroacetic acid (1.0 g) and NaOH (1.2 g) which resulted in carboxylic acid functionalized NGO. The resulting NGO–COOH was neutralized, and purified by repeated rinsing and filtration. PEG-amino (2.0 kDa) was added to the NGO–COOH suspension both at a final concentration of 1.0 mg/mL and sonicated in a bath sonicator for 5 min. NGO-PEG was prepared by the addition of EDC (5.0 mm), which was sonicated for 30 min and finally stirred at room temperature for 12 h. The reaction was quenched with mercaptoethanol and the final product was dialyzed against deionized water for 24 h. The prepared samples were characterized by Fourier transform – infrared spectroscopy (FT-IR, Thermo Nicolet, Madison, WI), UV–visible spectroscopy (Thermo Evolution Array, Madison, WI), transmission electron microscopy (TEM, Philips CM200, Madison, WI), and atomic force microscopy (AFM, Veeco Multimode Scanning Probe Microscope with the NanoScope IV Controller, Santa Barbara, CA).

2.4. Assessment of fluorescence quenching and recovery

The concentration of f-DEVD was estimated by measuring the concentration of ssDNA in the f-DEVD-ssDNA conjugate using NanoDrop N-1000 (Thermo, Madison, WI). One unit of the absorbance at 260 nm corresponded to ca. 33 μg of f-DEVD. To the 10 μL of aliquot of the probe solution containing 10 ng of the probe, 50 μL of pre-determined amount of NGO-PEG conjugate in PBS was added with mild stirring. After incubation at room temperature for 6 h, the residual fluorescence signal intensity in the region of interest (ROI) was recorded as photons per sec per square centimeter per steradian (p/sec/cm²sr) and analyzed with a fluorescence imaging system (Xenogen, Alameda, CA). The f-DEVD-ssDNA/NGO-PEG complex was also characterized with 310 nm excitation wavelengths and UV–visible spectroscopy. Then, the specific fluorescence recovery by capase-3 was assessed with the f-DEVD-ssDNA/NGO-PEG complex formulated at a weight ratio of 79/21 as described above. An aliquot of the resulting complex (20 μL, 2 ng probe) was slowly added to 80 μL of the PBS containing various amounts of recombinant human caspase-3. After incubation for 30 min, fluorophotometric analysis was carried out with a Fluoroskan Ascent FL microplate fluorometer (Thermo, Madison, WI) at the excitation/emission wavelength of 540/575 nm.

2.5. Monitoring of intracellular caspase-3 by confocal microscopy

As schematically shown in Fig. 1a, PECylated NGO makes a stable complex with 18-nucleotide partial sequences of E. coli RNA
I which is conjugated to TAMRA labeled caspase-3 specific peptide substrate of f–NH₂–DEVD. The complex formation by π-π stacking results in the fluorescence quenching by FRET. E. coli RNA I is involved in the replication of ColE1-type plasmid in E. coli. Because mammalian cells do not have a complementary nucleic acid to the sequence of RNA I oligo-DNA, the ssDNA does not hybridize with complementary sequences nor displace it from the NGO surface during its diagnostic applications [27]. At the same time, ssDNA anchor might be protected from enzymatic attack by the complex formation with NGO [28]. The probe peptide f-ssDNA conjugate was prepared by the coupling reaction of f-DEVD and sulfhydryl group at 5'-end of ssDNA using a bifunctional linker of sulfo-EMCS (Fig. 1b). The resulting f-DEVD-ssDNA conjugate was characterized by agarose gel electrophoresis and GPC. After staining with ethidium bromide, the f-DEVD peptide exhibited a mobility toward (+) direction and formed a clear band confirming the successful conjugation of the peptide to negatively charged ssDNA (Fig. S1). With the band migration, color change from red to orange color was observed, which might be attributed to the co-localization of ethidium bromide and TAMRA. The retention time of the major peak on the GPC chromatogram corresponding to the DNA was shifted revealing the successful conjugation of ssDNA with f-DEVD peptide (Fig. S2).

In order to prepare NGO-PEG, GO was first synthesized with graphite powder by using a modified Hummer’s method [2,19]. TEM of GO samples showed the characteristic translucent sheets of GO with wrinkles and folds in a lateral dimension of micrometer scale (Fig. S3). NGO was prepared with the GO flakes by intensive sonication in a bath circulator equipped with a refrigerator. FT-IR of NGO samples showed the characteristic aromatic C=C bonds and C=O stretch at the wavelength of 1700 cm⁻¹ and 1580 cm⁻¹, respectively (Fig. S4). AFM images showed the well dispersed morphology of NGO flakes with a lateral width of less than 150 nm and a topological height of less than 1 nm (Fig. 2). Then, the NGO was activated with chloroacetic acid in a strong basic condition to introduce carboxyl groups on NGO (NGO–COOH), which was further modified with PEG-amine by amide bond formation using EDC. FT-IR showed the characteristic peak of carboxyl group at 1630 cm⁻¹, and those of –CH₂- and –CO─NH- at 2860 cm⁻¹ and 1650 cm⁻¹, respectively (Fig. S4). In addition, the morphology of NGO–COOH and NGO-PEG was assessed by AFM. The topological height of NGO–COOH and NGO-PEG slightly increased from ca. 1.0 nm–1.2 nm and 2.3 nm (Fig. 2), which might be attributed to the carboxyl modification and the PEGylation. From the results, we could confirm the successful preparation of NGO-PEG.

3.2. Preparation and characterization of self-assembled f-DEVD-ssDNA/NGO-PEG complex

We prepared and characterized the noncovalent complex of the fluorescent probe and NGO-PEG by π-π stacking. When the predetermined amount of f-DEVD-ssDNA (100 ng/mL) was mixed with NGO-PEG at various concentrations up to 79.2 µg/mL in PBS (pH = 7.4), the fluorescence of f-DEVD-ssDNA gradually decreased in an NGO concentration-dependent manner due to the fluorescence quenching by FRET with increasing formation of f-DEVD-ssDNA/NGO-PEG complex (Fig. 3a). The quenching did not evolve significantly at an NGO-PEG concentration higher than 79.2 µg/mL. The f-DEVD-ssDNA/NGO-PEG complex exhibited a big increase in the topological height from ca. 2.3 nm–4.3 nm, when compared with bare NGO-PEG (Fig. 2). The hydrodynamic diameter of the complexes also slightly increased from ca. 78 nm–92 nm in comparison with NGO-PEG (Fig. S5a). In addition, the zeta potential of TAMRA-DEVD-ssDNA/NGO-PEG complex slightly decreased in
Fig. 2. AFM height profiles of (a) NGO, (b) NGO-COOH, (c) NGO-PEG, and (d) TAMRA-DEVD-ssDNA/NGO-PEG complex.
comparison with that of NGO-PEG reflecting the binding of negatively charged ssDNA to the surface of NGO-PEG (Fig. S5b). As a representative protein in the serum, BSA did not cause any significant increase of fluorescence intensity after incubation up to a concentration of 300 \( \mu g/mL \) for 1 h (Fig. 3b) confirming the serum stability of the complex for diagnostic applications. To verify the specific cleavage and the following fluorescence recovery of f-DEVD-ssDNA/NGO-PEG complex by active caspase-3 during apoptosis, the complex at an f-DEVD-ssDNA concentration of 100 ng/mL was incubated with increasing concentrations of recombinant human caspase-3 for 1 h. The f-DEVD-ssDNA/NGO-PEG complex showed a gradual and significant increase in fluorescence intensity with increasing concentration of caspase-3 up to 350 ng/mL (Fig. 3c).

3.3. Monitoring of intracellular caspase-3 by confocal microscopy and fluorophotometry

The f-DEVD-ssDNA/NGO-PEG complex at a weight ratio of 79/21 was exploited for real-time monitoring of caspase-3 protease activity in live A549 cells. The prepared complexes were taken up to A549 cells within 1 h and then apoptosis was induced by the treatment with STS (1.8 \( \mu g/mL \)) or TRAIL (300 ng/mL). During incubation with the apoptosis inducer of STS for 3 h, the morphology of the cells was changed and the formation of apoptotic-bodies was clearly observed by optical microscopy (Fig. S6). While the control cells without apoptosis inducer treatment did not show any detectable NIRF signals, the cells treated with STS or TRAIL exhibited fluorescent signals on the confocal microscopic images (Fig. S6c and 4). The signals were thought to result from the cleavage of fluorescent substrate peptide immobilized on the surface of NGO-PEG by caspase-3. The DNA fragmentation also represents a characteristic hallmark of apoptosis. It is well known that activated caspase-3 translocates from cytoplasm into nucleus and initiates apoptotic DNA fragmentation. As shown in Fig. 4, TUNEL staining revealed a good co-localization together with the NIRF signals in the nucleus.

As a colorimetric assay system, the dose dependent response of TRAIL was monitored in a multiwell plate with the f-DEVD-ssDNA/NGO-PEG complex. With increasing concentration of TRAIL from 50 to 300 ng/mL, the fluorescence intensity was enhanced in the cells treated with f-DEVD-ssDNA/NGO-PEG complex (Fig. 5a). Furthermore, the effect of caspase-3 inhibitor of Z-DEVD-FMK on intracellular fluorescence activation was also investigated with the same procedures (Fig. 5b). NIRF signals were significantly reduced upon exposure to the caspase-3 inhibitor before the treatment with TRAIL, which was in good agreement with the previous report [19]. The f-DEVD-ssDNA/NGO-PEG complex was cytocompatible in A549 cells with increasing concentration of NGO-PEG up to 100 \( \mu g/mL \) (Fig. S7). From the results, we could confirm that the non-covalent f-DEVD-ssDNA/NGO-PEG complex effectively detected the caspase-3 activation during the progress of apoptosis in live cells.

3.4. Bioimaging of cerebral hypoxic-ischemia and liver cirrhosis

The f-DEVD-ssDNA/NGO-PEG complex was applied to in vivo diagnosis of hypoxic-ischemic encephalopathy (HIE) as a model
system of apoptosis-related diseases using 7 days old SD rat pups. The HIE model was prepared by carotid-artery ligation, which exacerbates neuronal apoptosis and gradually enhances temporal distribution of caspase-3 in the brain [29]. The ischemic time dependent brain injury could be correlated with the fluorescence signal intensity by NIRF imaging after injection of the f−DEVD−ssDNA/NGO-PEG complex (Fig. 6a). Then, the brain tissues were cryo-sectioned to analyze the apoptosis in a cellular level. Fig. 6b shows representative confocal microscopic images of the non-infarcted and infarcted hemispheres 30 min after injection of the f−DEVD−ssDNA/NGO-PEG complex. In case of the non-infarcted hemisphere, cells were arranged concretely in extracellular matrix (ECM) and did not show the fluorescence of TAMRA. In contrast, the cells in the infarcted hemisphere were arranged roughly in destroyed ECM showing the fluorescence of TAMRA (Fig. 6b). TTC and H&E staining confirmed that the fluorescent site of f−DEVD−ssDNA/NGO-PEG complex was identical to the site of HIE (Fig. 7a). After TUNEL and TB staining, and TEM imaging, photomicrographs of the brain treated by the carotid-artery ligation also confirmed the occurrence of hypoxic-ischemic damage and the consequent apoptosis in the brain (Fig. 7b). Moreover, the systemically delivered f−DEVD−ssDNA/NGO-PEG complex could

![Fig. 4. Confocal microscopic images of A549 cells after incubation with TAMRA-DEVD-ssDNA/NGO-PEG complex for 1 h, followed by the treatment with (a) PBS and (b) TRAIL for 3 h (TAMRA: fluorescence image of TAMRA, DAPI: fluorescence image after DAPI staining, TUNEL: fluorescence image after TUNEL staining, TI: transmitted light image, and Merge: confocal image merged with transmitted light image).](image)

![Fig. 5. NIRF images and the fluorescence intensity values of the rectangular ROI in micro-plates after incubation of A549 cells with TAMRA-DEVD-ssDNA/NGO-PEG complex for 1 h, followed by the treatment with increasing concentration of TRAIL for 3 h (a) In the absence and (b) In the presence of a different concentration of caspase-3 inhibitor (Z-DEVD-FMK) (n = 8, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).](image)
visualize the degree of apoptotic liver damage in SD rats by the treatment with NDMA for 5 days (Fig. 8). The NIRF images of harvested liver samples clearly showed the NDMA concentration dependent gradual increase in the fluorescent signal intensity. The fluorescence intensity was also slightly increased in kidney samples, which might be attributed to the renal extravasation of enzymatically cleaved fragment of peptide substrate. From the results, the self-assembled NGO complex was thought to be successfully applied to the molecular diagnosis of HIE in neonatal rats and liver cirrhosis in SD rats for the first time reflecting the

Fig. 6. a) NIRF and the corresponding H&E staining images of apoptotically damaged brains with a different degree of hypoxic-ischemia after injection of TAMRA-DEVD-ssDNA/NGO-PEG complex. From right to the left, the hypoxic-ischemic brain images by carotid-artery ligation for 0, 24, 36, and 48 h. (b) Representative confocal microscopic images of the non-infarcted and infarcted hemispheres after treatment by the injection of TAMRA-DEVD-ssDNA/NGO-PEG complex for 30 min.

Fig. 7. a) Representative NIRF image, and photomicrographs of the hypoxic-ischemic brain after triphenyltetrazolium chloride (TTC) staining and H&E staining. The white arrow heads indicate the area of infarction. (b) Photomicrographs of hypoxic-ischemic brain after TUNEL staining and Toluidine Blue (TB) staining, and the corresponding TEM images. The black arrow head indicates brain cells undergoing cell death by necrosis and the white arrow heads indicate brain cells undergoing cell death by apoptosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web).
feasibility for the facile diagnosis of apoptosis-related diseases like neurodegenerative diseases, inflammatory diseases, cancer, hematologic diseases, and autoimmune diseases. The multiplex diagnostic systems by multiple stacking of cleavable fluorescent probe peptides-ssDNA conjugates on the NGO-PEG might simultaneously detect various kinds of biomarkers contributing to the more accurate diagnosis of apoptotic diseases. With efficient drug loading on the graphitic domain, the NGO complex can be also exploited as a theranostic system for a diagnostic therapy.

4. Conclusions

We successfully developed the non-covalent TAMRA-DEVD-ssDNA/NGO-PEG complex as a simple, rapid, and efficient platform for real-time imaging and monitoring of caspase-3 activation during the progress of apoptosis in vitro and in vivo. The NIRF TAMRA labeled capase-3 cleavable peptide substrate of TAMRA-DEVD was conjugated to a partial sequence of E. coli RNAI, which was self-assembled for the complex formation with NGO-PEG via π-π stacking. The self-assembled NGO complex made possible the monitoring the caspase-3 activity in live A549 cells, and the molecular diagnosis of hypoxic-ischemic encephalopathy in neonatal SD rats and liver damage in SD rats. From the results, we could confirm the feasibility of the NGO complex for caspase-specific bioimaging and the diagnosis of various apoptosis-related diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2012.06.086.

References


