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In vivo targeting and positron emission tomography imaging of tumor vasculature with ^{66}Ga-labeled nano-graphene

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ABSTRACT

The goal of this study was to employ nano-graphene for tumor targeting in an animal tumor model, and quantitatively evaluate the pharmacokinetics and tumor targeting efficacy through positron emission tomography (PET) imaging using ^{66}Ga as the radiolabel. Nano-graphene oxide (GO) sheets with covalently linked, amino group-terminated six-arm branched polyethylene glycol (PEG; 10 kDa) chains were conjugated to NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid, for ^{66}Ga-labeling) and TRC105 (an antibody that binds to CD105). Flow cytometry analyses, size measurements, and serum stability studies were performed to characterize the GO conjugates before in vivo investigations in 4T1 murine breast tumor-bearing mice, which were further validated by histology. TRC105-conjugated GO was specific for CD105 in cell culture. ^{66}Ga-NOTA-GO-TRC105 and ^{66}Ga-NOTA-GO exhibited excellent stability in complete mouse serum. In 4T1 tumor-bearing mice, these GO conjugates were primarily cleared through the hepatobiliary pathway. ^{66}Ga-NOTA-GO-TRC105 accumulated quickly in the 4T1 tumors and tumor uptake remained stable over time (3.8 ± 0.4, 4.5 ± 0.4, 5.8 ± 0.3, and 4.5 ± 0.4 %ID/g at 0.5, 3, 7, and 24 h post-injection respectively; $n = 4$). Blocking studies with unconjugated TRC105 confirmed CD105 specificity of ^{66}Ga-NOTA-GO-TRC105, which was corroborated by biodistribution and histology studies. Furthermore, histological examination revealed that targeting of NOTA-GO-TRC105 is tumor vasculature CD105 specific with little extravasation. Successful demonstration of in vivo tumor targeting with GO, along with the versatile chemistry of graphene-based nanomaterials, makes them suitable nanoplatforms for future biomedical research such as cancer theranostics.

1. Introduction

Graphene, a 2-D sp^2-bonded carbon sheet with desirable electrical/mechanical/chemical properties, has attracted enormous interest in biomedicine [1–4]. Recently, functionalized nano-graphene with ultra-high surface area has been used as a nanocarrier for loading and delivery of various drugs and genes [2,5–7]. In vivo applications of nano-graphene for cancer therapy have been further explored, with encouraging therapeutic effects in animal models [8,9]. The potential toxicity of graphene has also been investigated [2,10–13], and it is generally agreed that the toxicity of graphene is closely associated with its surface chemistry. For example, a recent study suggested that polyethylene glycol (PEG) functionalized nano-graphene could be gradually excreted from mice after intravenous injection, without rendering noticeable toxicity to the treated animals [10].

In this study, we explored the use of nano-graphene for in vivo tumor targeting and quantitatively evaluated the pharmacokinetics and tumor targeting efficacy through serial non-invasive positron emission tomography (PET) imaging. To ensure in vivo stability of the nano-graphene conjugates, we used 10–50 nm graphene oxide (GO) sheets with six-arm branched PEG (10 kDa) chains covalently attached to the surfaces [5,8], which have ample amino groups for covalent conjugation of various functional entities (imaging labels, antibodies, etc.).

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Recently, we have produced high specific activity ^{66}Ga ($t_{1/2}$ = 9.3 h, 56.5% β^+, 43.5% EC) from ^{nat}Zn and ^{66}Zn targets with a cyclotron, using proton irradiations between 7 and 16 MeV. The reactivities of ^{66}Ga for common bifunctional chelators exceeded 70 GBq/μmol [14], which were significantly higher than the previously reported values (<4.6 GBq/μmol) [15]. The relatively long half-life of ^{66}Ga makes it a suitable radiolabel for nanomaterials such as GO, whose in vivo kinetics is poorly matched by the much shorter half-life of ^{68}Ga ($t_{1/2}$ = 68.3 min). Labeling chemistry with radiogallium has been well studied because of the popularity of ^{68}Ga from ^{68}Ge/^{68}Ga generators, and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) is generally agreed to be one of the most suitable chelators [16].

Almost exclusively expressed on proliferating tumor endothelial cells, CD105 (endoglin) is an ideal marker for tumor angiogenesis (i.e. new blood vessel formation) [17–19]. It holds tremendous clinical potential as a prognostic, diagnostic, and therapeutic vascular target in cancer, since high expression level of CD105 correlates with poor prognosis in more than 10 solid tumor types [20]. In addition, the fact that CD105 is not readily detectable in resting endothelial cells or most normal organs makes it a universally applicable target for molecular imaging and therapy applications targeting the tumor vasculature. For most nanomaterial-based tumor targeting and imaging, efficient extravasation is the key hurdle [21,22]. In this regard, CD105 is highly desirable for nanomaterial-based tumor targeting, such as the functionalized GO used in this study, where extravasation is not required to observe the tumor signal.

TRC105, a human/murine chimeric IgG1 monoclonal antibody (mAb) which binds to CD105 with high avidity, is used here as the targeting ligand for CD105 [17]. A multicenter Phase 1 first-in-human dose-escalation trial of TRC105 was recently completed in the United States and multiple Phase 2 cancer therapy trials are underway [23]. These promising clinical data warranted the development of TRC105-based imaging/therapeutic agents, which can play important roles in multiple facets of future cancer patient management. Therefore, the goal of this study is to investigate whether TRC105 can be used as the ligand for CD105 targeting of covalently functionalized GO in an animal tumor model, which can open up new possibilities for future image-guided drug delivery, cancer therapy, as well as establishing GO as a promising nanoplatform for cancer theranostics. To validate the in vivo data, various in vitro/in vivo/ex vivo studies and control experiments were carried out to confirm CD105 specificity of the GO conjugates.

2. Materials and methods

2.1. Reagents

TRC105 was provided by TRACON pharmaceuticals Inc. (San Diego, CA). S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) and fluorescein isothiocyanate (FITC) were purchased from Macrocyclics, Inc. (Dallas, TX) and Sigma–Aldrich (St. Louis, MO) respectively. Chelex 100 resin (50–100 mesh; Sigma–Aldrich, St. Louis, MO), succinimidyl carboxymethyl PEG maleimide (SCM-PEG-Mal, molecular weight: 5 kDa; Creative PEGworks, Winston Salem, NC), rat anti-mouse CD31 primary antibody (BD Biosciences, San Diego, CA), AlexaFluor488- and Cy3-labeled secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, CA), and PD-10 desalting columns (GE Healthcare, Piscataway, NJ) were all acquired from commercial sources. Water and all buffers were of Millipore grade and pre-treated with Chelex 100 resin to ensure that the aqueous solution was heavy metal-free. All other reaction buffers and chemicals were obtained from Thermo Fisher Scientific (Fair Lawn, NJ).

2.2. Cell lines and animal model

4T1 murine breast cancer, MCF-7 human breast cancer, and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). 4T1 and MCF-7 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and incubated at 37 °C with 5% CO_2. HUVECs were cultured in M-200 medium (Invitrogen, Carlsbad, CA) with 1 × low serum growth supplement (Cascade Biologics, Portland, OR) and incubated at 37 °C with 5% CO_2. Cells were used for in vitro and in vivo experiments when they reached ~75% confluence.

All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. Four- to five-week-old female BALB/c mice were purchased from Harlan (Indianapolis, IN) and 4T1 tumors were established by subcutaneously injecting 2 × 10^6 cells, suspended in 100 μL of 1:1 mixture of RPMI 1640 medium and Matrigel (BD Biosciences, Franklin lakes, NJ), into the front flank of mice. Tumor sizes were monitored every other day and mice were used for in vivo experiments when the diameter of tumors reached 5–8 mm.

2.3. Syntheses and characterization of GO conjugates

The synthesis of PEGylated GO (termed "GO-PEG-NH_2"), starting from graphite oxide, has been reported previously [5,8]. Four conjugates of GO-PEG-NH_2 were prepared and investigated in this study: NOTA-GO, NOTA-GO-TRC105, FITC-GO, and FITC-GO-TRC105 (Fig. 1A). Since all conjugates contain the same six-arm branched PEG chains covalently linked to GO, "PEG" was omitted from the acronyms for clarity considerations. NOTA-GO and NOTA-GO-TRC105 were subsequently labeled with ^{66}Ga for in vivo PET imaging and biodistribution studies, while FITC-GO and FITC-GO-TRC105 were employed for in vitro evaluation of CD105 binding affinity and specificity through fluorescence techniques.

GO-PEG-NH_2 was mixed with p-SCN-Bn-NOTA or FITC, which has the same chemical reaction between the SCN group and the $-NH_2$ group, at a molar ratio of 1:10 at pH 9.0 for 2 h. The resulting NOTA-GO (or FITC-GO) was purified by centrifugation filtration using 100 kDa cutoff Amicon filters (Millipore, Billerica, MA). NOTA-GO (or FITC-GO) was subsequently reacted with SCM-PEG-Mal at pH 8.5 at a molar ratio of 1:30 for 2 h. After removing the unreacted SCM-PEG-Mal and other reagents by centrifugation filtration, the resulting reaction intermediates were named as NOTA-GO-Mal or FITC-GO-Mal.

In parallel, TRC105 was mixed with Traut's reagent at a molar ratio of 1:25 at pH 8.0 for 2 h. The thiolated TRC105 (i.e. TRC105-SH) was purified by size exclusion chromatography using phosphate-buffered saline (PBS, pre-treated with Chelex 100 resin to prevent oxidation of the thiol) as the mobile phase. Subsequently, NOTA-GO-Mal or FITC-GO-Mal was mixed with TRC105-SH at a molar ratio of 1:5 at pH 7.5 in the presence of tris(2-carboxyethyl)phosphine (TCEP; to avoid disulfide formation between TRC105-SH). The final products were purified by centrifugation filtration, which were named NOTA-GO-TRC105 and FITC-GO-TRC105. To characterize the GO conjugates, atom force microscopy (AFM), dynamic light scattering (DLS), and zeta-potential measurements were carried out.

2.4. Production of ^{66}Ga

Targets of ^{nat}Zn or ^{66}Zn were electrodeposited from 0.05 N hydrochloric acid solution onto gold or silver target backings with dimensions approximately matched to the cyclotron beam of a General Electric (Waukesha, WI) PETtrace cyclotron [14]. Targets were irradiated with 20–30 μA of 13 MeV protons for between 1 and 3 h, dissolved in concentrated HCl, and purified by cation exchange chromatography with successive additions of 10 N and 7 N HCl to recover the zinc target material and elute trace contaminant metals. The product was collected in 4 N HCl and evaporated to dryness before being redissolved in 0.1 N HCl prior to buffering. If ^{nat}Zn was used as the target, ^{68}Ga was allowed to decay overnight before target processing commenced. In this case, the only radioisotopic contaminant was ^{67}Ga ($t_{1/2}$ = 78.3 h), present as <5% of the radioactivity at 16 h after the end of bombardment (EoB). On the other hand, radioisotopic purity of ^{66}Ga produced from ^{66}Zn target exceeded 99.9%.

2.5. Radiolabeling of GO conjugates

74–111 MBq of ^{66}Ga-acetate (pH 5.5) was prepared from 0.1 N HCl solution by addition of 0.25 M NH_4OAc solution (pH 7.2), and added to a solution of NOTA-GO-TRC105 or NOTA-GO, at a ratio of 30 μg of each GO conjugate per 37 MBq of ^{66}Ga. The reaction mixture was incubated for 30 min at 37 °C with constant shaking. ^{66}Ga-NOTA-GO-TRC105 and ^{66}Ga-NOTA-GO were purified by size exclusion chromatography using normal saline buffered with 0.25 M NH_4OAc (pH 7.2) as the mobile phase. The radioactive fractions containing ^{66}Ga-NOTA-GO-TRC105 or ^{66}Ga-NOTA-GO were collected and passed through a 0.2 μm syringe filter prior to injection into 4T1 tumor-bearing mice.

2.6. In vitro studies of GO conjugates

To evaluate the CD105 targeting characteristics of the GO conjugates, HUVECs (that express high levels of CD105 [24–26]) and MCF-7 cells (that do not express CD105) were used for flow cytometry analysis of FITC-GO and FITC-GO-TRC105. Cells were harvested and suspended in cold PBS with 2% bovine serum albumin at a concentration of 5 × 10^6 cells/mL, incubated with FITC-GO-TRC105 or FITC-GO (at a concentration of 50 μg/mL based on GO) for 30 min at room temperature, washed three times with cold PBS, and centrifuged at 1000 rpm for 5 min. Afterwards, the