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Study on technetium-99m labeling of graphene oxide nanosheets through click chemistry–^{99m}Tc labeling of graphene oxide nanosheets*

JIANG Da-wei (江大卫),[1] PENG Cheng (彭程),[1] SUN Yan-Hong (孙艳红),[1]
JIA Li-Na (贾丽娜),[1] LI Jian-Bo (李剑波),[2] and ZHANG Lan (张岚)[1,†]

[1] *Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China*
[2] *Department of Nuclear Medicine, Inner Mongolia Medical University Affiliated Hospital, Hohhot, Inner Mongolia 010050, China*
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Graphene oxide (GO) nanosheets possess several advantages, such as a large surface, outstanding bio-compatibility, and straightforward chemical modification capability. They also have great potential as a drug-carrier. In this article, we radiolabeled GO nanosheets with ^{99m}Tc, which satisfies the potential needs of micro-SPECT imaging probes in pre-clinical and clinical research. GO nanosheets were synthesized through the modified Hummers' method, then GO nanosheets with azide group covalently functionalized in two steps were conjugated to DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid) and functionalized with an alkynyl group by means of click chemistry. Then through the addition and reduction of technetium-99m, the ^{99m}Tc-DOTA-GO were attained. DOTA-conjugated GOs with lateral dimensions of 500–600 nm were synthesized. Both atomic force microscopy (AFM) and FT-IR were performed to characterize the GO-DOTA. Labeling efficiency of GO-DOTA with ^{99m}Tc was $> 90\%$ and radiochemical purities were $> 96\%$ with purification. We successfully synthesized graphene oxide derivatives, DOTA-conjugated GOs, via Click Chemistry, and it was labeled with ^{99m}Tc for SPECT imaging. High radiolabeling efficiency makes GO nanosheets suitable platforms for future molecular imaging research.

Keywords: Graphene oxide nanosheets, ^{99m}Tc labeling, Click chemistry

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I. INTRODUCTION

Carbon-based nanomaterials, such as graphene, single-walled carbon nanotubes (SWCNs), multi-walled carbon nanoparticles (MWCNs), and so on, have shown great potential in the field of electronics, nanocomposites, nanocarriers, and energy sources for their excellent physical properties [1–3]. Among them, graphene oxide (GO), which is an oxygen-rich, two-dimensional sp^2-bonded carbon sheet [4–7] with a large surface, outstanding bio-compatibility, and straightforward chemical modification capability [8–10], has attracted more interest in research fields.

Several groups have reported that GO nanosheets could serve as nanocarriers to deliver drugs [11] or biomolecules [12] into cells for imaging, bio-sensing, and therapeutic purposes [13, 14]. Pegylated nano-graphene oxide is not only soluble in buffers and serum without agglomeration, but also found to be photoluminescent in the near-infrared with little background. The π-stacking could be used to load doxorubicin for selectively killing cancer cells *in vitro* [15]. A multi-functional graphene oxide-iron oxide hybrid nanocomposite (GO-IONP) was derived with PEG and loaded with doxorubicin, which could enable magnetically targeted drug delivery and could be utilized for localized photothermal annihilation of cancer cells guided by the magnetic field [16].

Due to the advantages of the GO nanosheets mentioned above, we hypothesized that labeling of GO nanosheets with radioactive isotopes could provide more *in vivo* information concerning biodistribution and imaging results in animal models. Currently, GO nanomaterials have been radiolabeled with ^{64}Cu [14], ^{66}Ga [17], and ^{198,199}Au [18]. In these articles [18], the authors believed that the radiolabeled GO nanostructure could be nominated as one of the most promising nanomaterials in upcoming nanotechnology-based cancer diagnosis and therapy. However, GO nanosheets have not been utilized in SPECT imaging yet. As a result, we would like to label GO nanosheets with single-photon emitting radioisotopes–technetium-99m (^{99m}Tc) to provide a SPECT radiotracer. We believe that the radiolabeling of GO nanosheets with ^{99m}Tc would satisfy the potential need of micro-SPECT imaging probes in preclinical and clinical research [19–21].

In order to obtain the radiotracer based on GO nanosheets using a straightforward and easy method, we have designed and functionalized organic polydentate ligands on the basis that 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA). DOTA [22], as a common chelator, could provide stable and facile complexes with radioactive metals (such as ^{64}Cu and ^{99m}Tc). The conjugation of the DOTA chelator and GO nanosheets was obtained through the copper(I)-catalyzed azide-alkyne cyclo-addition (CuAAC) reaction, known as "click chemistry" with high efficiency and specificity. After the addition and reduction of Technetium-99m, ^{99m}Tc-DOTA-GO could be readily attained with high efficiency.

In this article, we have prepared GO nanosheets and conjugated GO nanosheets with DOTA chelators by click chemistry, then labeled DOTA-GO nanosheets with ^{99m}Tc. Finally,

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† Corresponding author, zhanglan@sinap.ac.cn

we obtained ^{99m}Tc-DOTA-GO with high labeling efficiency. We believe that this method could be readily used in the labeling of other GO nanomaterials in the future.

II. MATERIALS AND METHODS

A. General

Graphite (powder, $< 20\,\mu m$) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Sodium azide was purchased from Jingyan Chemicals Co., Ltd. (Shanghai, CHINA). Sodium pertechnetate was purchased from Shanghai Atom Kexing Pharmaceuticals Co., Ltd. (Shanghai, CHINA). DO3A, trifluoroacetic acid, and propargyl bromide were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, CHINA). Other chemical reagents ($K_2S_2O_8$, P_2O_5, $CuSO_4$, sodium ascorbate, H_2O_2, H_2SO_4, HCl, CH_2Cl_2, DMF, DMSO, ethyl acetate) were obtained from Sinopharm Chemical Reagent Co., Ltd., and were used as received. Unless otherwise specified, all chemicals were of analytic grade and commercially available. All aqueous solutions were prepared with water from a Millipore Milli-Q system (Millipore Corporation, Billerica, MA, USA).

B. Preparation of Pre-GO

In a typical experiment [23], graphite powder (4 g) was added to a mixture of concentrated H_2SO_4 (12 mL), $K_2S_2O_8$ (2.5 g), and P_2O_5 (2.5 g). After being stirred at 80 °C for 6 h, the resultant dark blue mixture was slowly cooled to room temperature over about 6 h. The cooled mixture was diluted with 500 mL of water and then filtered with a 0.22 μm filter membrane (Generay Biotech Co., Ltd., Shanghai, China). The filtered pre-oxidized graphene oxide (pre-GO) was dried overnight in a vacuum at 50 °C.

C. Preparation of GO

The pre-GO (2 g) was added to 92 mL of cold H_2SO_4 (0 °C), and 12 g of $KMnO_4$ was gradually added with mild stirring in the ice bath [24]. After stirring for 20 min at 5 °C, the mixture was further stirred at 35 °C for 8 h, then water (750 mL) and H_2O_2 (30 wt.%, 30 mL) were slowly added to terminate the reaction. After standing overnight, the obtained precipitation was washed by diluted hydrochloride acid (1:10, v/v) and water 3 times. For purification, the GO product was resuspended in water to form a brown dispersion, which was subjected to dialysis to remove residual metal ions and acids. The purified product was dried in a vacuum at 50 °C overnight and was then ready to be used in further experiments.

D. Preparation of 2-chloroethyl isocyanate-conjugated graphite oxides (Cl-GO)

GO (100 mg) and anhydrous DMF (10 mL) were added to a round-bottom flask to create an inhomogeneous suspension [25]. Then, 2-chloroethyl isocyanate (4 mmol) was added. The mixture was allowed to stir under nitrogen for 24 h. The reaction was terminated by the adding methylene chloride (50 mL) to coagulate the product. The product was washed with methylene chloride 3 times.

E. Preparation of azide-functionalized GO (N_3-GO)

To prepare the azide-functionalized GO [26], 6 mmol of sodium azide was mixed with Cl-GO (50 mg), which was dissolved in 10 mL of DMSO. The mixture was stirred and refluxed for 48 h at 50 °C, and followed by extraction with ethyl acetate to eliminate any residual DMSO. The black product was filtered and dried with a vacuum.

F. Synthesis of (4, 7-bis-tert-butoxycarbonylmethyl-10-prop-2-ynyl-1, 4, 7, 10 tetraaza-cyclododec-1-yl)-acetic acid t-butyl ester (Alkynyl-DO3A)

Alkyne-DO3A was prepared according to Fig. 1 [27]. Potassium carbonate (0.134 g) was added under nitrogen to a solution of 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-tris (t-butyl acetate) (DO3A, 50 mg) in acetonitrile (5 mL). The reaction temperature was raised to 70 °C following the addition of propargyl bromide (23.1 mg). The reaction was stirred at 70 °C and monitored by a thin layer of chromatography (TLC). When completed, the reaction mixture was cooled to room temperature and filtered. The filter liquor was evaporated under reduced pressure to obtain the crude product. It was purified by column chromatography through a silica gel (eluent: dichloromethane/methanol: 9/1, v/v) to afford the desired product as a brown oil (80%).

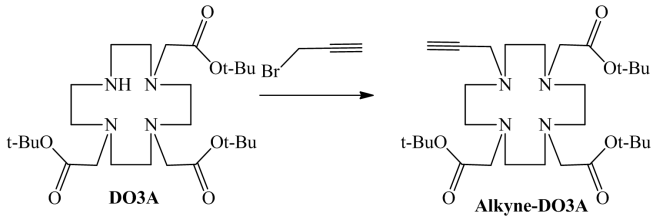

Fig. 1. Schematic display of synthesis of Alkyne-DO3A.

G. Click chemistry between N_3-GO and alkynyl-DO3A

The N_3-GO (1 mg/mL) and alkynyl-DO3A (2 μmol/L) solution were prepared in a mixed solvent (water/tert-butyl alcohol: 5/3, v/v), then copper sulfate (2 μmol/L) and

sodium ascorbate (10 μmol/L) were added to the solution and stirred for 24 h at room temperature to obtain the DO3A-functionalized GO (DO3A-GO).

H. Preparation of DOTA-GO

Synthesis of DOTA-GO was conducted via Cu(I) catalyzed click chemistry, according to Fig. 2 Trifluoroacetic acid (5 mL) was added to a solution of DO3A-GO in water and the reaction was stirred for 24 h at room temperature. The solvent was evaporated to dryness under reduced pressure to obtain the crude product, then the product was washed well with chloroform (50 mL × 3 times) and quantitatively set to the concentration of 1 mg/mL.

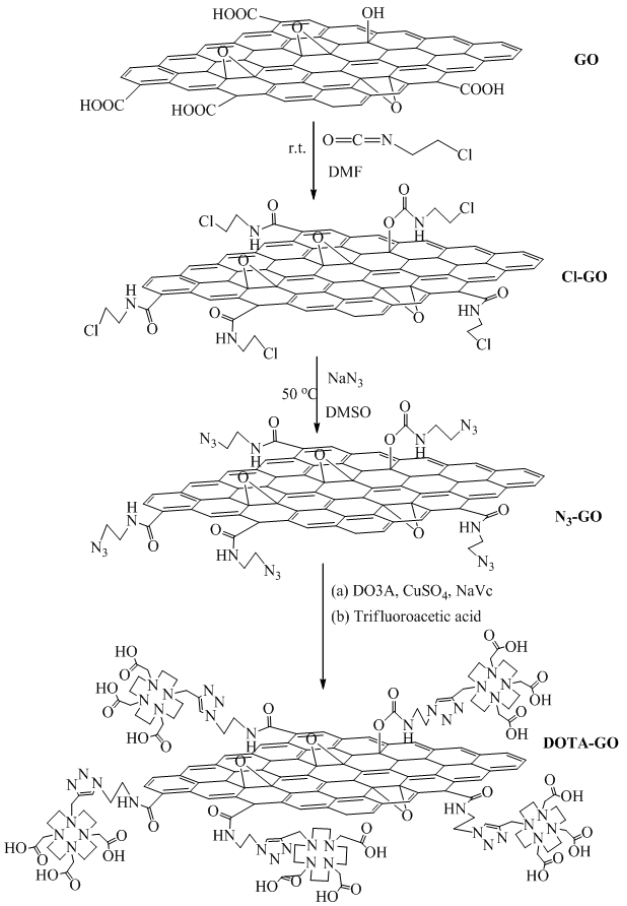

Fig. 2. Schematic display of preparation of DOTA-GO via Cu(I) catalyzed click chemistry.

I. Radiolabeling of DOTA-GO

50 μL of sodium pertechnetate (∼ 37 MBq) and 1–2 μL of stannous chloride solution (1 mg/mL) in HCl were added to a 200 μL DOTA-GO solution (1 mg/mL). The pH of the solution was adjusted to 7–7.5 using 0.1 M of sodium bicarbonate solution. The mixture was allowed to stand for 30 min at room temperature. Standard safety procedures were employed during the radiolabeling process.

J. Radiochemical purity of the ^{99m}Tc-DOTA-GO conjugate

When the radiolabeling procedure was finished, the ^{99m}Tc-DOTA-GO was washed with 0.9% saline 3 times to remove the free ^{99m}Tc and colloid. The radiolabeling efficiency was determined by TLC on ITLC-SG using 100% acetone as the mobile phase. Each TLC was cut into 1 cm fragments and counts of each segment were taken. As a results, the percentage of ^{99m}Tc-DOTA-GO could be calculated.

III. RESULTS

Pre-GO and GO nanosheets were prepared by the Modified Hummers' method with a yield of about 80%. The lateral morphology of GO nanosheets was investigated by atom force microscopy (AFM). AFM images of GO nanosheets were shown in Fig. 3. The lateral sizes of GO nanosheets mainly ranged from 500 to 600 nm, and the thickness of the GO sheets ranged from 0.8 to 1 nm.

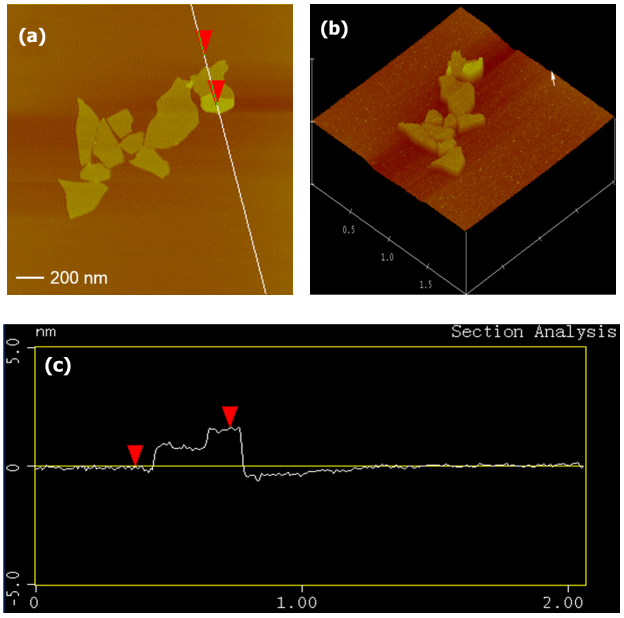

Fig. 3. (Color online) AFM images of GO (0.1 mg/mL) in water. (a) Taping-mode AFM image of GO; (b) 3D AFM image of GO; (c) Height changes between the two red arrows show that as-prepared GO nanosheets were under a single-layer state.

Then Cl-GO was prepared as seen in Fig. 2, and the products were analyzed by Fourier Transform Infrared Spectroscopy (FT-IR). As shown in Fig. 4, the most characteristic features of Cl-GO in the FT-IR spectrum are the C=O stretching vibration at 1703 cm^{-1} that could be assigned to the carbamate esters of the surface hydroxyls, the stretch at

1646 cm^{-1} should be the contribution of the amide carbonyl-stretching, and the absorption at 1111 cm^{-1} could be assigned to the Cl−C stretching. Upon treatment of sodium azide, the most obvious feature of N_3-GO in the FT-IR spectrum was the new band at 2040 cm^{-1}, which could be due to the contribution of the azide functional groups.

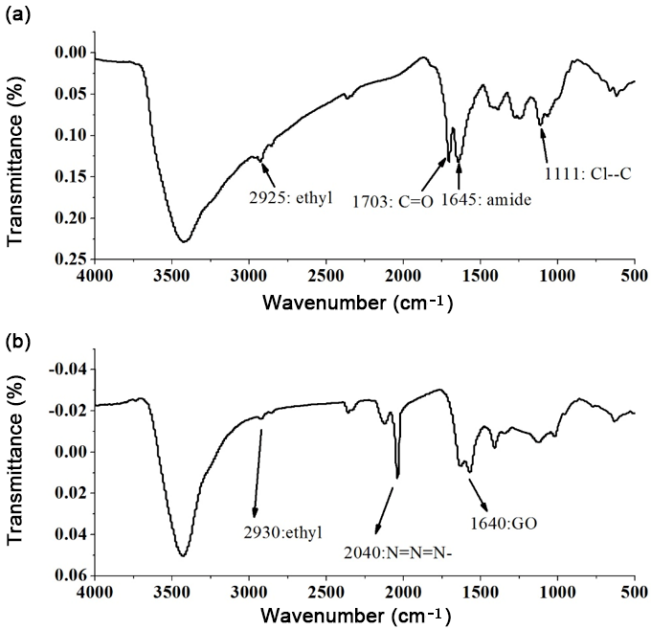

Fig. 4. Infrared spectra of Cl-GO (a) and N_3-GO (b).

The alkyne-DO3A was attained according to Fig. 1 and the final product was characterized by ^{1}H and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 1.40(s, 27H), 2.70–2.82(m, ^{16}H), 2.15(s, ^{1}H) 3.31(s, ^{6}H), 3.46(s, ^{2}H); MS(ESI+): m/z = 553[M+H]+, which is shown in Fig. 5.

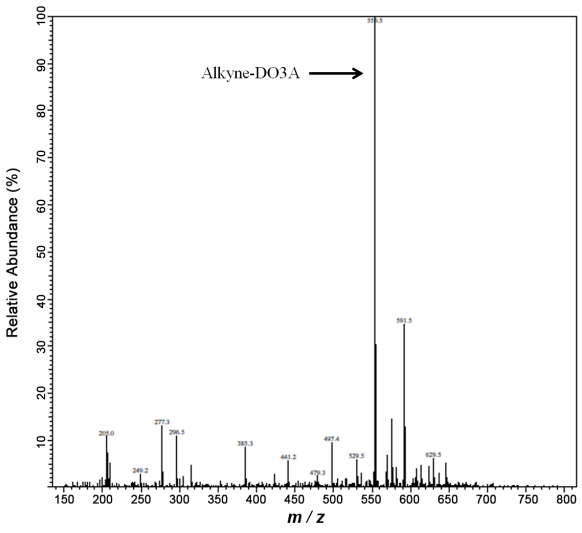

Fig. 5. The Mass spectrum characterization result of alkyne-DO3A.

The DOTA-GO was attained according to Fig. 2. Then, the radiolabeling efficiency of DOTA-GO was investigated by a radio thin layer chromatography (Radio-TLC) using 100% acetone as the mobile phase. The Rf value of free pertechnetate ions ranged from 0.8 to 0.9, while the Rf value of radiolabeled DOTA-GO was 0–0.1. The radiolabeling efficiency of ^{99m}Tc-DOTA-GO was higher than 90%, as shown in Fig. 6.

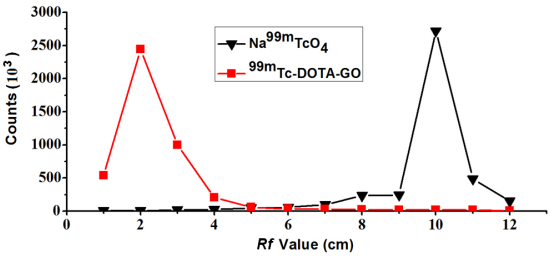

Fig. 6. (Color online) Radiolabeling efficiency of DOTA-GO. ■ represents the radio-TLC images of radiolabeled DOTA-GO, ▲ represents the un-reduced sodium pertechnetate.

IV. DISCUSSION

Graphene Oxide nanosheets contain hydroxyl, epoxyl, and carboxyl functional groups as the positions for chemical modification. The GO nanosheets were first dissolved in DMF and functionalized with 2-chloroethyl isocyanate, which could lead to the derivatization of the edge carboxyl functional groups via the formation of amides or carbamate esters. Cl-GO nanosheets dissolve well in DMSO or DMF, but not in water or other conventional polar protic solvents because of the carbon-chloride bonds. Then, sodium azide was added to substitute the chloride atoms in Cl-GO, and a different size of azide functionalized graphene oxide nanosheets was formed. The chemical nature of Cl-GO and N_3-GO was investigated by FT-IR spectroscopy. The characteristic features in the FT-IR spectrum of GO are the absorption at 1733 cm^{-1} and 1039 cm^{-1} (C=O carbonyl stretching and C−OH stretching in COOH groups). The absorption at 1620 cm^{-1} could be assigned to the skeletal vibrations of unoxidized graphitic domains.

N_3-GO prepared (Fig. 2) could be well dispersed in water as a result of azide functional groups, however, the alkyne-DO3A should be dissolved in tert-butyl alcohol to ensure the proceeding of the click reaction. After blending of N_3-GO nanosheets with alkyne-DO3A, the copper sulfate solution and excessive sodium ascorbate solution were added in proper order. After the reaction, the resultant DO3A-GO nanosheets were deprotectionized with the addition of trifluoroacetic acid. The attained DOTA-GO could be investigated by the FT-IR spectrums. From the FT-IR spectrums, we confirm the disappearance of azide functional groups through the missing peak at 2040 cm^{-1}.

The radiolabeling process of GO nanosheets were handy and time-saving, the whole procedure takes about 30 min. Radiolabeled products could be used for *in vitro* analysis or *in vivo* evaluation, and no purification process should be oper-

ated after the labeling reaction. However, recent reports have addressed the safety concerns of PEGlated GO when used for cellular investigation, thus when applied to SPECT/CT imaging, further safety examination would be necessary.

V. CONCLUSION

GO nanosheets have great potential in the field of molecular imaging and drug delivery. When applied with GO nanosheets, it would be possible to achieve diverse application *in vitro* and *in vivo*. In this paper, we successfully prepared GO nanosheets and conjugated these GO nanosheets with DOTA chelators through click chemistry. Finally, we successfully radiolabeled GO nanosheets with ^{99m}Tc. Overall, we have successfully prepared DOTA-chelator conjugated GO nanosheets and we believe that the as-prepared DOTA-GO nanosheets could not only be labeled with ^{99m}Tc, but with other radioactive metals (such as ^{64}Cu or ^{68}Ga) for use in molecular imaging.

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