Study on technetium-99m labeling of graphene oxide nanosheets through click chemistry-99mTc labeling of graphene oxide nanosheets postprint

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Abstract

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 99mTc, 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 99mTc-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 99mTc 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 99mTc for SPECT imaging. High radiolabeling efficiency makes GO nanosheets suitable platforms for future molecular imaging research.

Full Text

Study on Technetium-99m Labeling of Graphene Oxide Nanosheets Through Click Chemistry

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Graphene oxide (GO) nanosheets possess several advantages, such as a large surface area, outstanding biocompatibility, and straightforward chemical modification capability, making them promising candidates for drug delivery applications. In this study, we radiolabeled GO nanosheets with 99mTc to satisfy the potential needs of micro-SPECT imaging probes in preclinical and clinical research. GO nanosheets were synthesized through the modified Hummers' method, then functionalized with azide groups in two steps and conjugated to DOTA (1,4,7,10-tetraazacyclododecane-N,N ,N ,N -tetraacetic acid) bearing an alkynyl group via click chemistry. Subsequent addition and reduction of technetium-99m yielded the 99mTc-DOTA-GO conjugate. DOTA-conjugated GO nanosheets with lateral dimensions of 500–600 nm were successfully synthesized. Both atomic force microscopy (AFM) and FT-IR were performed to characterize the GO-DOTA conjugates. The labeling efficiency of GO-DOTA with 99mTc exceeded 90%, and radiochemical purities were greater than 96% after purification. We successfully synthesized graphene oxide derivatives, DOTA-conjugated GOs, via click chemistry, and labeled them with 99mTc for SPECT imaging. The high radiolabeling efficiency makes GO nanosheets suitable platforms for future molecular imaging research.

Keywords: Graphene oxide nanosheets, 99mTc labeling, Click chemistry
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INTRODUCTION

Carbon-based nanomaterials, including graphene, single-walled carbon nanotubes (SWCNs), and multi-walled carbon nanoparticles (MWCNs), have demonstrated great potential in electronics, nanocomposites, nanocarriers, and energy applications due to their excellent physical properties [1–3]. Among these materials, graphene oxide (GO), an oxygen-rich, two-dimensional sp^2-bonded carbon sheet [4–7] with a large surface area, outstanding biocompatibility, and straightforward chemical modification capability [8–10], has attracted significant research interest.

Several groups have reported that GO nanosheets can serve as nanocarriers to deliver drugs [11] or biomolecules [12] into cells for imaging, biosensing, and therapeutic purposes [13,14]. PEGylated nano-graphene oxide is not only soluble in buffers and serum without agglomeration, but also exhibits photoluminescence in the near-infrared region with minimal background. The π-stacking capability can be exploited to load doxorubicin for selectively killing cancer cells in vitro [15]. A multi-functional graphene oxide-iron oxide hybrid nanocomposite

(GO-IONP) was developed with PEG and loaded with doxorubicin, enabling magnetically targeted drug delivery and localized photothermal annihilation of cancer cells under magnetic field guidance [16].

Given these advantages of GO nanosheets, we hypothesized that labeling them with radioactive isotopes could provide valuable in vivo information regarding biodistribution and imaging in animal models. Currently, GO nanomaterials have been radiolabeled with ^{64}Cu [14], ^{66}Ga [17], and ^{198},^{199}Au [18]. In these studies, the authors suggested that radiolabeled GO nanostructures could be among the most promising nanomaterials for future nanotechnology-based cancer diagnosis and therapy. However, GO nanosheets have not yet been utilized in SPECT imaging. Therefore, we aimed to label GO nanosheets with the single-photon emitting radioisotope technetium-99m (99mTc) to create a SPECT radiotracer. We believe that radiolabeling GO nanosheets with 99mTc would satisfy the potential need for micro-SPECT imaging probes in preclinical and clinical research [19–21].

To obtain a radiotracer based on GO nanosheets using a straightforward and efficient method, we designed and functionalized organic polydentate ligands based on 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA). DOTA [22], as a common chelator, can form stable complexes with radioactive metals such as 64Cu and 99mTc. The conjugation of the DOTA chelator to GO nanosheets was achieved through the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, known as "click chemistry," which offers high efficiency and specificity. After addition and reduction of technetium-99m, the 99mTc-DOTA-GO conjugate could be readily obtained with high efficiency.

In this study, we prepared GO nanosheets, conjugated them with DOTA chelators via click chemistry, and subsequently labeled the DOTA-GO nanosheets with 99mTc. We successfully obtained 99mTc-DOTA-GO with high labeling efficiency, demonstrating that this method could be readily applied to label other GO nanomaterials in the future.

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MATERIALS AND METHODS

A. General

Graphite (powder, < 20 μm) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Sodium azide was obtained 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 acquired 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 used as received. Unless otherwise specified, all chemicals were of analytical 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 stirring at 80 °C for 6 h, the resultant dark blue mixture was slowly cooled to room temperature over approximately 6 h. The cooled mixture was diluted with 500 mL of water and filtered through 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 an ice bath [24]. After stirring for 20 min at 5 °C, the mixture was further stirred at 35 °C for 8 h. Water (750 mL) and H_2O_2 (30 wt.%, 30 mL) were then slowly added to terminate the reaction. After standing overnight, the obtained precipitate was washed with diluted hydrochloric acid (1:10, v/v) and water three 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 for 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, and the mixture was stirred under nitrogen for 24 h. The reaction was terminated by adding methylene chloride (50 mL) to coagulate the product, which was subsequently washed with methylene chloride three 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) dissolved in 10 mL of DMSO. The mixture was stirred and refluxed for 48 h at 50 °C, followed by extraction with ethyl acetate to eliminate any residual DMSO. The black product was filtered and dried under 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 [Figure 1: see original paper] [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 thin layer chromatography (TLC). Upon completion, the reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated under reduced pressure to obtain the crude product, which was purified by column chromatography through silica gel (eluent: dichloromethane/methanol: 9/1, v/v) to afford the desired product as a brown oil (80% yield).

G. Click Chemistry Between N_3-GO and Alkynyl-DO3A

The N_3-GO (1 mg/mL) and alkynyl-DO3A (2 µmol/L) solutions were prepared in a mixed solvent (water/tert-butyl alcohol: 5/3, v/v). Copper sulfate (2 µmol/L) and sodium ascorbate (10 µmol/L) were then added to the solution, which was 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, as shown in Fig. 2 [Figure 2: see original paper]. 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, which was then washed thoroughly with chloroform (50 mL × 3) and quantitatively adjusted to a concentration of 1 mg/mL.

I. Radiolabeling of DOTA-GO

Sodium pertechnetate (50 µL, 37 MBq) and stannous chloride solution (1–2 µL, 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 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 99mTc-DOTA-GO Conjugate

After completing the radiolabeling procedure, the 99mTc-DOTA-GO was washed with 0.9% saline three times to remove free 99mTc and colloids. The radiolabeling efficiency was determined by TLC on ITLC-SG using 100% acetone as the mobile phase. Each TLC plate was cut into 1 cm fragments,

and the radioactivity of each segment was measured. The percentage of 99mTc-DOTA-GO could thus be calculated.

RESULTS

Pre-GO and GO nanosheets were prepared by the modified Hummers' method with a yield of approximately 80%. The lateral morphology of GO nanosheets was investigated by atomic force microscopy (AFM), as shown in Fig. 3 [Figure 3: see original paper]. The lateral sizes of GO nanosheets primarily ranged from 500 to 600 nm, and the thickness of the GO sheets ranged from 0.8 to 1 nm.

Cl-GO was prepared as described, and the products were analyzed by Fourier Transform Infrared Spectroscopy (FT-IR). As shown in Fig. 4 [Figure 4: see original paper], the most characteristic features of Cl-GO in the FT-IR spectrum include the C=O stretching vibration at 1703 cm^{-1}, which can be assigned to the carbamate esters of the surface hydroxyls, the stretch at 1646 cm^{-1} corresponding to amide carbonyl stretching, and the absorption at 1111 cm^{-1} attributable to Cl-C stretching. Upon treatment with sodium azide, the most obvious feature of N_3-GO in the FT-IR spectrum was a new band at 2040 cm^{-1}, which can be attributed to the azide functional groups.

Alkyne-DO3A was obtained according to Fig. 1 and characterized by ^1H NMR and MS. ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 1.40(s, 27H), 2.70–2.82(m, 16H), 2.15(s, 1H), 3.31(s, 6H), 3.46(s, 2H); MS(ESI^+): m/z = 553$[M+H]^+$, as shown in Fig. 5 [Figure 5: see original paper].

DOTA-GO was obtained according to Fig. 2. The radiolabeling efficiency of DOTA-GO was investigated by 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 99mTc-DOTA-GO exceeded 90%, as shown in Fig. 6 [Figure 6: see original paper].

DISCUSSION

Graphene oxide nanosheets contain hydroxyl, epoxyl, and carboxyl functional groups that serve as sites for chemical modification. The GO nanosheets were first dispersed in DMF and functionalized with 2-chloroethyl isocyanate, which derivatized the edge carboxyl functional groups via formation of amides or carbamate esters. Cl-GO nanosheets dispersed well in DMSO or DMF but not in water or other conventional polar protic solvents due to the carbon-chloride bonds. Sodium azide was then added to substitute the chloride atoms in Cl-GO, forming azide-functionalized graphene oxide nanosheets of various sizes. 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 include absorptions at 1733 cm^{-1} and 1039 cm^{-1} (C=O carbonyl stretching and C-OH stretching in COOH groups), while the absorption at 1620 cm^{-1} can be assigned to skeletal

vibrations of unoxidized graphitic domains.

The prepared N_3-GO (Fig. 2) could be well dispersed in water due to the azide functional groups; however, alkyne-DO3A needed to be dissolved in tert-butyl alcohol to ensure the click reaction proceeded. After mixing N_3-GO nanosheets with alkyne-DO3A, copper sulfate solution and excess sodium ascorbate solution were added in proper sequence. Following the reaction, the resultant DO3A-GO nanosheets were deprotected by adding trifluoroacetic acid. The obtained DOTA-GO was analyzed by FT-IR spectroscopy, which confirmed the disappearance of azide functional groups through the absence of the peak at 2040 cm^{-1}.

The radiolabeling process for GO nanosheets was convenient and time-efficient, with the entire procedure taking approximately 30 min. Radiolabeled products could be used for in vitro analysis or in vivo evaluation without requiring a purification step after the labeling reaction. However, recent reports have raised safety concerns regarding PEGylated GO for cellular investigations, suggesting that further safety examination would be necessary when applying these materials to SPECT/CT imaging.

CONCLUSION

GO nanosheets hold great potential in molecular imaging and drug delivery. When functionalized appropriately, they enable diverse in vitro and in vivo applications. In this work, we successfully prepared GO nanosheets and conjugated them with DOTA chelators through click chemistry. We then successfully radiolabeled the GO nanosheets with 99mTc. Overall, we have prepared DOTA-chelator conjugated GO nanosheets and believe that these as-prepared DOTA-GO nanosheets could be labeled not only with 99mTc but also with other radioactive metals (such as 64Cu or 68Ga) for use in molecular imaging.

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Note: Figure translations are in progress. See original paper for figures.