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# Nanographene oxide labeling with $^{188}$Re

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**Abstract** Nanographene oxide (NGO) is currently being explored for various biomedical applications. However, little information is known about its biological behaviors *in vitro* and *in vivo*. For further studying its pharmacokinetics and related biological behaviors in living systems, an effective and convenient tracing method is particularly demanded. In this work, NGO was labeled with radionuclide $^{188}$Re ($^{188}$Re-NGO). To obtain high labeling yield and purity, a number of labeling conditions, including concentration of $SnCl_2$ and ascorbic acid, reaction time and temperature, and pH were optimized, and stability of the $^{188}$Re-NGO *in vitro* and *in vivo* was evaluated. The results showed that NGO could be effectively labeled with high yield. The purified $^{188}$Re-NGO showed high stability *in vitro* and *in vivo*. A pretest of NGO biodistribution with single photon emission computed tomography showed that the $^{188}$Re-NGO was rapidly taken by organs such as lungs, liver, and spleen. The biodistribution of $^{188}$Re-NGO differs significantly from the free radionuclide, indicating that the labeling procedure is highly suitable for investigating its biological behavior in living systems.

**Key words** Graphene oxide, Radio-labeling, $^{188}$Re, SPECT images, Biodistribution

## 1 Introduction

Nanographene oxide (NGO), comprised of $sp^2$-bonded carbon atoms, is of highly regular and robust structures with defined periodicity. Due to its unique electronic, thermal, mechanical and optical properties, potential applications of NGO may include nanoelectronic devices, transparent conductors, composite materials, and biomedine[1–4]. In particular, recent studies have shown that NGO can be a better candidate for targeted drug/gene delivery, biosensing and bioimaging[4–6]. For studying pharmacology and toxicology of this novel nanomaterial, it is essential to know its biological behavior *in vitro* and *in vivo*. However, due to the special characteristics of NGO, limited techniques are available to trace NGO in living systems. Therefore, the development of reliable analytical methods is needed to overcome the experimental difficulties of e.g. the detection and quantitative measurement of NGO in biological medium, cells and animals.

Techniques of radio-tracer technique, mass spectrometry, fluorescence imaging, nuclear magnetic resonance, and ultrasonic imaging, have been used to determine the biological behavior of nanomaterials *in vitro* and *in vivo*[7]. Radio-tracer technique is especially good in terms of the high sensitivity and accuracy, fast analysis and interference-free. Since labeling the fullerenol with $^{99m}$Tc by Li *et al* in 2002[8], numerous studies have been reported on radio-labeling of various carbon nanomaterials, including fullerene derivatives[8], single walled carbon nanotubes (SWCNT)[9], and multi-walled carbon nanotubes (MWCNT)[10]. To authors' knowledge, however, no reports are available so far on radio-labeling of NGO.

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In this study, we established an effective and convenient method to trace NGO *in vitro* and *in vivo,* The radionuclide $^{188}$Re[11] was used to label NGO. The labeling conditions of $SnCl_2$ and ascorbic acid concentrations, the reaction time and temperature, and pH value of the solution, were optimized. Stability *in vitro* and *in vivo* of the labelled $^{188}$Re-NGO was determined by paper chromatograph. Based on the radio-labeling technique, NGO biodistribution in mice was investigated by single photon emission computed tomography (SPECT). The results showed high labeling yield, radiochemical purity and stability of the $^{188}$Re-NGO. These suggest that $^{188}$Re-NGO is suitable for studying cellular uptake of NGO *in vitro* and biodistribution *in vivo*. We believe that the radio-labeling procedure is of importance for investigating its toxicology profiles and biomedical applications.

## 2 Materials and Methods

### 2.1 Reagents and animals

The $^{188}$Re was obtained from an alumina-based $^{188}$W /$^{188}$Re generator (Shanghai Ke-Xing Pharmaceutical Co.), with the $^{188}$W solution supplied by the Oak Ridge National Laboratory (Oak Ridge, TN). The NGO was prepared by a modified Hummers method[12]. Characterized by atomic force microscopy (AFM), it was of mainly single layered NGO (>70%), about 1.0 nm in topographic height and 10–800 nm in lateral width. No significant amounts of particles were found on the substrate, indicating a good purity of the NGO aqueous solution, which was stable for over a month without agglomeration. Raman spectroscopy of the samples showed that the ratio of ID/IG increased significantly after treating them with $H_2SO_4$ and $KMnO_4$, suggesting that much disorder had been introduced onto the planar graphite. Details of the NGO characterization can be found in our previous paper[13].

Kun Ming mice, from Shanghai SLAC Laboratory Animal Corp. Ltd., China, were checked for absence of infection for one week prior to experiment. They were housed in plastic cages, fed a commercial diet, and given water *ad libitum*. Permission of the local ethics committee was obtained, and all animal experiments were performed according to Chinese law and accepted international standards in biomedical research.

### 2.2 Labeling of NGO with $^{188}$Re

The NGO was radiolabeled with $^{188}$Re based on reduction of Re (VII) into Re (V) by stannous chloride[14]. First, aliquots of ascorbic acid (40 $mg{\cdot}mL^{-1}$) and stannous chloride (60 $mg{\cdot}mL^{-1}$ in 1 M HCl) were added to a 100 μL NGO aqueous solution (1 $mg{\cdot}mL^{-1}$). After adjusting pH with NaOH/HCl, radioactive $Na^{188}ReO_4$ solution (about 30 μCi) was added, and the total volume of reaction system was maintained to approximately 0.5 mL. Then the mixture was gently mixed and incubated in water bath.

After the reaction, the mixture (10 μL) was used to determine the labeling yields by paper chromatograph (PC) with Whatman NO.1 (1×13 $cm^2$). The $^{188}$Re distribution on PC was measured with a $\gamma$-ray counter. The radioactivity of $^{188}$Re-NGO and $ReO_2$ colloid (if any) were at the originals on the chromatography paper developed by saline solution (retardation faction, $R_f$=0), with $R_f$ value for free $ReO_4^-$ ions being at about 0.9–1. The labeling yield of $^{188}$Re-NGO was calculated as Labeling yield=[$Y_{segment\ 0}/Y_{segment(0–10)}$]×100%, where $Y_{segment\ 0}$ is counts per minute (cpm) of segment 0, and $Y_{segments\ (0–10)}$ is total cpm of all segments.

To optimize the labeling procedures, the effects of labeling conditions were examined, including final concentrations of ascorbic acid and stannous chloride, the reaction time, incubation temperature and the pH value.

### 2.3 Purification of $^{188}$Re-NGO and examination of its stability

The $^{188}$Re-NGO with high labeling yield was prepared by utilizing the optimized labeling procedure. The $^{188}$Re-NGO was washed by saline for three times to remove free ascorbic acid and stannous chloride, and was dispersed in Millipore water, RPMI-1640 or RPMI-1640 culture medium (with 10% fetal bovine serum, FBS) at room temperature. The radiochemical purity of each suspension (10 μL portion) were determined as described in §2.2, thus obtaining the labeled $^{188}$Re-NGO at various time intervals to examine its stability *in vitro*.

To assess the stability of $^{188}$Re-NGO *in vivo*, 20 Kun Ming mice (male 20±2 g, 6–8 w) were intravenously injected with the $^{188}$Re-NGO suspension (200 μL) containing 1-mCi radioactivity, and were anesthetized with pentobarbital sodium at 1, 3, 6, 12 and 24 h post injection. The collected anticoagulant blood was used to measure its radioactivity with the $\gamma$-ray counter. The blood was centrifuged at $10^4$ rpm for 5 min, the supernatant was discarded with the remaining solid being washed with deionized water, and the measured radioactivity was compared with that of the blood.

### 2.4 Biodistribution of $^{188}$Re-NGO in mice

SPECT was used to assess the tissue biodistribution of $^{188}$Re-NGO in mice dynamically. The animals were anaesthetized by pentobarbital sodium, and the $^{188}$Re -NGO of about 1 mCi was intravenously administrated into mice via tail vein. Immediately after injection, projection images were recorded by placing a mouse directly on the HEGP collimator surface of a gamma camera (Picker CX 250) at 5, 15, 30 and 60 min. Each image took 5 min with energy window of 155 keV and matrix of 64×64.

## 3 Results and Discussion

### 3.1 The NGO labeling with $^{188}$Re and its optimization conditions

The NGO labeling with $^{188}$Re was conducted by the reduction Re (VII) into Re (V) using stannous chloride. The optimized labeling conditions on the $^{188}$Re-NGO yield and radiochemical purity were pH=4, 3 mg·mL$^{-1}$ $SnCl_2{\cdot}2H_2O$, 1.6 mg·mL$^{-1}$ ascorbic acid, incubation time of 25 min, and reaction temperature of 80°C. The results of experiments on the reaction parameters are shown in Fig.1.

#### 3.1.1 The $SnCl_2$ concentration (Fig. 1A)

The NGO labeling yield increased with the $SnCl_2$ concentration, reaching about 92.5% at 3 mg·mL$^{-1}$ $SnCl_2$. Compared with MWCNTs or $C_{60}$ derivatives labeling with $^{99m}$Tc, the NGO labeling with $^{188}$Re requires more $SnCl_2$, indicating that the $^{188}$Re and $^{99m}$Tc have different labeling feasibility.

#### 3.1.2 The reaction time and temperature (Figs. 1B and 1C)

The curves of the reaction time and temperature effects on labeling yield of the $^{188}$Re-NGO show that at 25 min and 80°C the reaction yield was over 90%, without further increases at longer time and higher temperature.

#### 3.1.3 The pH value (Fig.1D)

The labeling yield of $^{188}$Re-NGO was almost the same at pH=2–8, but high pH solution obviously decreased the labeling yield. This may be related with the stannous chloride hydrolyzing in alkaline labeling system.

#### 3.1.4 The ascorbic acid concentration

The effect of ascorbic acid on NGO labeling with $^{188}$Re is complex. The labeling yield decreased from 99% to 73.5% when the ascorbic acid concentration increased from 0 to 2.4 mg·mL$^{-1}$ (Fig.1E). The $^{188}$Re(V) could be bound to NGO by their chemical coordination or the $^{188}$Re(V) physical deposition, but the extra ascorbic acid remarkably inhibits the labeling yield.

Also, the added ascorbic acid can stabilize the $SnCl_2$ in the solution. On reducing the Re (VII) into Re (V) in NGO labeling with $^{188}$Re, the $SnCl_2$ is easily over-oxidized by Re (VII), forming the radioactive colloids of Sn-Re(IV), which cannot be separated by paper chromatograph or centrifugation, and this interferes greatly with the tracing of $^{188}$Re-NGO nanoparticles. However, the radioactive colloids can be avoided from the ascorbic acid (Fig.1F). The radioactive level was up to be 3.6±0.7% of total activity at 0.8 mg·mL$^{-1}$ ascorbic acid in the labeling system, being high enough for experimental study on cell uptake and biodistribution.

### 3.2 Purification of the labeled $^{188}$Re-NGO

Prior to biodistribution, the $^{188}$Re-NGO was purified by triple cycles of centrifugation and saline wash to removal the free $SnCl_2$, ascorbic acid, and $NaReO_4$, and the PC analysis showed that the radiochemical purity increased from 92.2 to 98.5% (Fig.2).

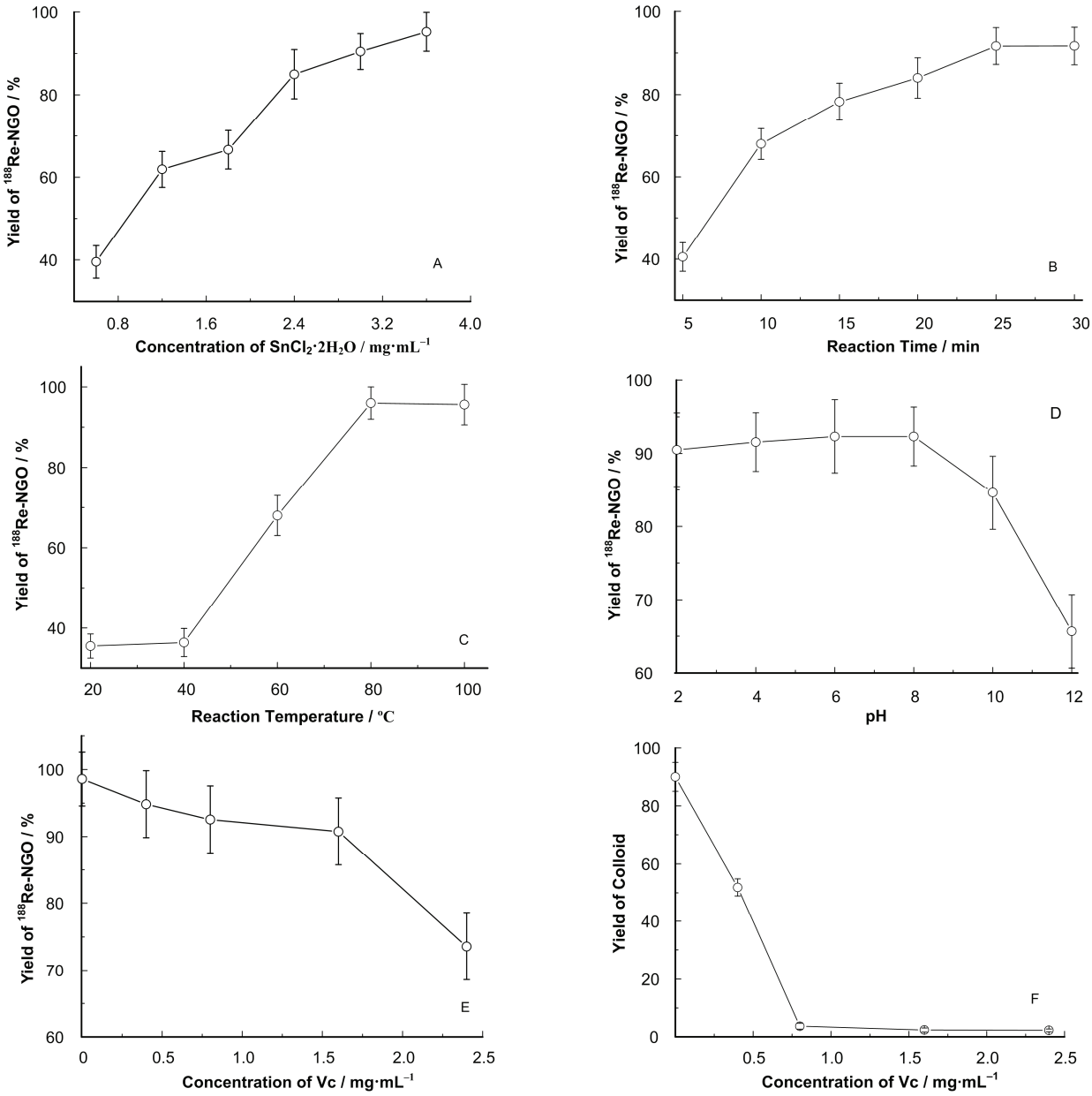


**Fig.1** Effects of the reaction conditions on labeling yield of the $^{188}$Re-NGO. (A) $SnCl_2$ concentration. (B) Incubation time. (C) Incubation temperature. (D) pH value of the solution. (E) Ascorbic acid concentration. (F) Yield of colloid. The data in mean ±SD were averaged from three independent experiments ($n$=3).

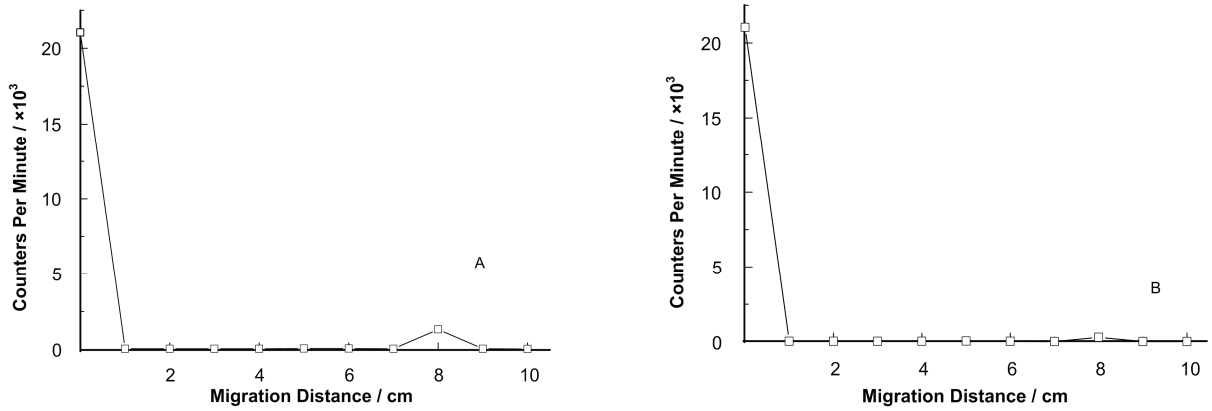


**Fig.2** The radiochemical purity of $^{188}$Re-NGO determined by PC developed by saline, the distribution of radioactivity before (A) and after (B) purification.

### 3.3 Stability of the $^{188}$Re-NGO

*In vitro* stability of $^{188}$Re-NGO was checked by their radiochemical purities at various elapsed times in the PC analysis. The results showed that the $^{188}$Re-NGO was stable in pure water, RPMI-1640, and RPMI-1640 with 10% FBS cell culture medium, and the radiochemical purity was still over 90% after 48 h (Fig.3A). The $^{188}$Re-NGO had excellent stability in mice, too. Most $^{188}$Re-NGO was intact in blood at 24 h post injection (Fig.3B), indicating that the radioactivity counts were from the $^{188}$Re-NGO distribution in mice, rather than that of radionuclide $^{188}$Re which might fall from the $^{188}$Re-NGO.

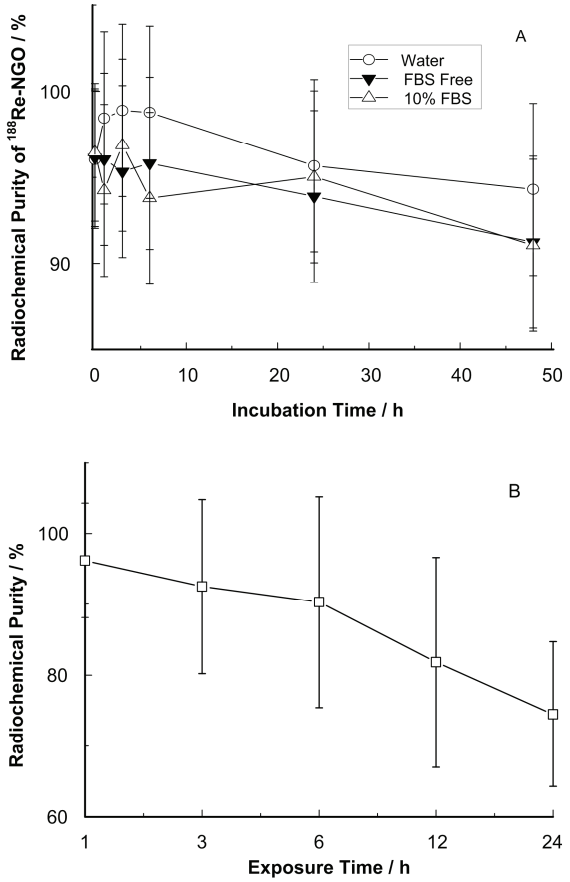


**Fig.3** (A) The radiochemical purity of $^{188}$Re-NGO in pure water, RPMI-1640 and RPMI-1640 with 10% FBS cell culture medium at the time points of 1, 3, 6, 24, and 48 h, (B) *in vivo* stability of $^{188}$Re-NGO by determining the blood radiochemical purity at different time points post-injection within 24 h.

### 3.4 SPECT imagings of $^{188}$Re-NGO in mice

To study the biological behavior of NGO in living systems, a pretest of $^{188}$Re-NGO biodistribution was conducted by using SPECT. The NGO biodistribution in Kun Ming mice were obtained after intravenous injection. The outline of the mouse and the images of other organs were undefined because of intensive background signals of high energy $\beta$-particles (2.1 MeV) originated from the $^{188}$Re[15]. The most distinguished feature in the projection images of SPECT showed high lung uptake of $^{188}$Re-NGO(Fig. 4) which is consistent with the biodistribution of major carbon nanomaterials, such as $C_{60}$[8], SWCNT[16], MWCNT[10], and ND[13], indicating that the $^{188}$Re-NGO is to suitable for investigating the NGO biological behavior in living systems.

The NGO distribution with well water-dispersion is different from the free Na$^{188}$ReO$_4$, it can be quickly accumulate in lung after injection. And the bright spots on the projection images in 5 min were of almost the same level in 60 min due to its slow clearance. Contrarily, the free $^{188}$Re can be rapidly cleared from blood, and does not accumulate in any examined organs after intravenous administration, further evidencing that the $^{188}$Re-NGO is stable *in vivo*.

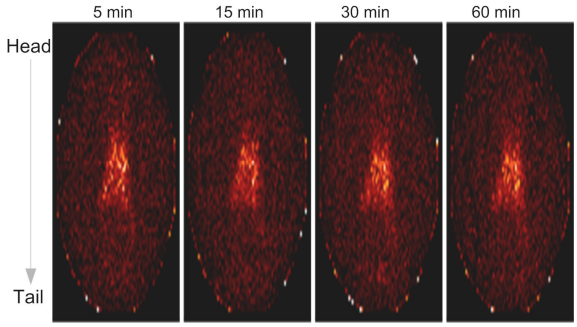


**Fig.4** Projection images of Kun Ming mouse after intravenous injection of $^{188}$Re-NGO via tail vein at the time points of 5, 15, 30, and 60 min, the bright dots represent the mouse lung.

## 4 Conclusions

The NGO labeling with $^{188}$Re is convenient and effective. The high labeling yield and purity of $^{188}$Re-NGO are obtained by optimizing the $SnCl_2$ and ascorbic acid concentration, reaction duration and temperature, and pH. The $^{188}$Re-NGO is stable *in vitro* and *in vivo*, and rapidly uptake in lungs, liver, and spleen, indicating that the labeling procedure is useful for studying the NGO biodistribution in living systems. Further efforts are underway to study the NGO adsorption, distribution, metabolism and excretion in mice.

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