Video Article

Synthesis of ^{68}Ga Core-doped Iron Oxide Nanoparticles for Dual Positron Emission Tomography /(T$_1$)Magnetic Resonance Imaging

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Abstract

Here, we describe a microwave synthesis to obtain iron oxide nanoparticles core-doped with ^{68}Ga. Microwave technology enables fast and reproducible synthetic procedures. In this case, starting from FeCl$_3$ and citrate trisodium salt, iron oxide nanoparticles coated with citric acid are obtained in 10 min in the microwave. These nanoparticles present a small core size of 4.2 ± 1.1 nm and a hydrodynamic size of 7.5 ± 2.1 nm. Moreover, they have a high longitudinal relaxivity (r_1) value of 11.9 mM^{-1}·s^{-1} and a modest transversal relaxivity value (r_2) of 22.9 mM^{-1}·s^{-1}, which results in a low r_2/r_1 ratio of 1.9. These values enable positive contrast generation in magnetic resonance imaging (MRI) instead of negative contrast, commonly used with iron oxide nanoparticles. In addition, if a ^{68}GaCl$_3$ elution from a ^{68}Ge/^{68}Ga generator is added to the starting materials, a nano-radiotracer doped with ^{68}Ga is obtained. The product is obtained with a high radiolabeling yield (> 90%), regardless of the initial activity used. Furthermore, a single purification step renders the nano-radiomaterial ready to be used *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/58269/

Introduction

The combination of imaging techniques for medical purposes has triggered the quest for different methods to synthesize multimodal probes[1,2,3]. Due to the sensitivity of positron emission tomography (PET) scanners and the spatial resolution of MRI, PET/MRI combinations seem to be one of the most attractive possibilities, providing anatomical and functional information at the same time[4]. In MRI, T$_2$-weighted sequences can be used, darkening the tissues in which they accumulate. T$_1$-weighted sequences may also be used, producing the brightening of the specific accumulation location[5]. Among them, positive contrast is often the most adequate option, as negative contrast makes it much harder to differentiate signal from endogenous hypointense areas, including those often presented by organs such as the lungs[6]. Traditionally, Gd-based molecular probes have been employed to obtain positive contrast. However, Gd-based contrast agents present a major drawback, namely their toxicity, which is critical in patients with renal problems[7,8,9]. This has motivated research in the synthesis of biocompatible materials for their use as T$_1$ contrast agents. An interesting approach is the use of iron oxide nanoparticles (IONPs), with an extremely small core size, that provide positive contrast[10]. Due to this extremely small core (~2 nm), most of the Fe^{3+} ions are on the surface, with 5 unpaired electrons each. This increases longitudinal relaxation time (r_1) values and yields much lower transversal/longitudinal (r_2/r_1) ratios compared to traditional IONPs, producing the desired positive contrast[11].

To combine IONPs with a positron emitter for PET, there are two key issues to take into account: radioisotope election and nanoparticle radiolabeling. Regarding the first issue, ^{68}Ga is an alluring choice. It has a relatively short half-life (67.8 min). Its half-life is suitable for peptide labeling since it matches common peptide biodistribution times. Moreover, ^{68}Ga is produced in a generator, enabling the synthesis in bench modules and avoiding the need for a cyclotron nearby[12,13,14]. In order to radiolabel the nanoparticle, surface-labeling radioisotope incorporation is the prevalent strategy. This can be done using a ligand that chelates ^{68}Ga or taking advantage of the affinity of the radiometal toward the surface of the nanoparticle. Most examples in the literature concerning IONPs use a chelator. There are examples of the use of heterocyclic ligands such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)[15], 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA)[16,17], and 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NODAGA)[18], and the use of 2,3-dicarboxypropane-1,1-diphosphonic acid (DPD), a tetradentate ligand [19]. Madru *et al.*[20] developed a chelator-free strategy in 2014 to label IONPs using a chelator-free method used by another group posteriorly[21].

However, major drawbacks of this approach include a high risk of *in vivo* transmetalation, low radiolabeling yields, and lengthy protocols unsuitable for short-lived isotopes[22,23,24]. For this reason, Wong *et al.*[25] developed the first example of core-doped nanoparticles, managing to incorporate ^{64}Cu in the core of the IONPs in a 5-min synthesis using microwave technology.

Here, we describe a rapid and efficient procedure to incorporate the radionuclide into the core of the nanoparticle, eluding many of the drawbacks presented by traditional methods. For this purpose, we propose the use of a microwave-driven synthesis (MWS), which reduces reaction times considerably, increases yields, and enhances reproducibility, critically important parameters in IONP synthesis. The refined performance of MWS is due to dielectric heating: rapid sample heating as molecular dipoles try to align with the alternating electric field, being polar solvents and reagents more efficient for this type of synthesis. In addition, the use of citric acid as a surfactant, together with microwave technology, results in extremely small nanoparticles, producing a dual T_1-weighted MRI/PET[26] signal, herein denoted as ^{68}Ga Core-doped iron oxide nanoparticles (^{68}Ga-C-IONP).

The protocol combines the use of microwave technology, ^{68}GaCl$_3$ as positron emitter, iron chloride, sodium citrate, and hydrazine hydrate, resulting in dual T_1-weighted MRI/PET nanoparticulate material in hardly 20 min. Moreover, it yields consistent results over a range of ^{68}Ga activities (37 MBq, 111 MBq, 370 MBq, and 1110 MBq) with no significant effects on the main physicochemical properties of the nanoparticles. The reproducibility of the method using high ^{68}Ga activities extends the field of possible applications, including large animal models or human studies. In addition, there is a single purification step included in the method. In the process, any excess of free gallium, iron chloride, sodium citrate, and hydrazine hydrate are removed by gel filtration. Total free isotope elimination and the purity of the sample ensure no toxicity and enhance imaging resolution. In the past, we have already demonstrated the usefulness of this approach in targeted molecular imaging[27,28].

Protocol

1. Reagent Preparation

1. **0.05 M HCl**
   1. Prepare 0.05 M HCl by adding 208 µL of 37% HCl to 50 mL of distilled water.

2. **High-performance liquid chromatography eluent**
   1. Prepare high-performance liquid chromatography (HPLC) eluent by dissolving 6.9 g of sodium dihydrogen phosphate monohydrate, 7.1 g of disodium hydrogen phosphate, 8.7 g of sodium chloride, and 0.7 g of sodium azide in 1 L of water. Mix well and check the pH. Pass the eluent through a 0.1-µm cutoff sterile filter and degas before use. Acceptance range: pH 6.2 - 7.0 (if not, adjust with NaOH [1 M] or HCl [5 M]).

2. Synthesis of Citrate-coated Iron Oxide Nanoparticles

1. Dissolve 75 mg of FeCl$_3$·6H$_2$O and 80 mg of citric acid trisodium salt dihydrate in 9 mL of water.
   NOTE: These quantities provide 12 mL of final purified nanoparticles ([Fe] ~1.4 mg·mL^{-1}). Quantities can be scaled down to obtain a final volume of 2.5 mL.
2. Put the mixture in the microwave-adapted flask.
3. Load a dynamic protocol in the microwave. Set the temperature to 120 °C, the time to 10 min, the pressure to 250 psi, and the power to 240 W.
4. Add 1 mL of hydrazine hydrate to the reaction.
   NOTE: Hydrazine hydrate starts iron reduction. Therefore, a change in the appearance of the solution, from light yellow to brown, is observed.
5. Start the microwave protocol.
6. Meanwhile, rinse a gel filtration desalting column with 20 mL of distilled water.
7. Once the protocol has finished, allow the flask to cool at room temperature.
8. Pipette 2.5 mL of the final mixture onto the column and discard the flow-through.
   NOTE: The microwave stops the protocol at 60 °C; the nanoparticles can be added directly to the gel filtration column at 60 °C.
9. Add 3 mL of distilled water to the column and collect the nanoparticles in a glass vial.
   NOTE: Nanoparticles can be stored at room temperature for 1 week. After this time, nanoparticle aggregation appears, increasing their hydrodynamic size.

3. Synthesis of ^{68}Ga Core-doped Iron Oxide Nanoparticles (^{68}Ga-C-IONP)

1. Put 75 mg of FeCl$_3$·6H$_2$O and 80 mg of citric acid trisodium salt dihydrate into the microwave-adapted flask.
2. Elute the ^{68}Ge/^{68}Ga generator using the recommended volume and concentration of HCl, according to the vendor (in our case, 4 mL of 0.05 M HCl). After the injection of that volume in the self-shielded generator, (4 mL of) ^{68}GaCl$_3$ is obtained, ready to use without further processing.
   NOTE: Follow the corresponding radioactivity safety measures for steps 3.2 - 3.12. ^{68}Ga is a positron and gamma emitter isotope. The use of the appropriate safety measures to avoid exposure to radiation by the operator is crucial. Researchers must follow an ALARA (as low as reasonably achievable) protocol using typical shielding and radionuclide-handling procedures. Moreover, the use of a ring, body badges, and a contamination detector is mandatory.
3. Add 4 mL of ^{68}GaCl$_3$ to the microwave-adapted flask. This volume can be smaller, depending on the generator activity and desired activity of final nanoparticles.
4. Pipette 5 mL of distilled water into the flask and mix well.
5. Load a dynamic protocol in the microwave. Set the temperature to 120 °C, the time to 10 min, the pressure to 250 psi, and the power to 240 W.

6. Add 1 mL of hydrazine hydrate to the reaction.
   NOTE: Hydrazine hydrate starts iron reduction. Therefore, a change in the appearance of the solution, from light yellow to brown, is observed.
7. Start the microwave protocol.
8. Meanwhile, rinse a gel filtration desalting column with 20 mL of distilled water.
9. Once the protocol has finished, allow the flask to cool at room temperature.
10. Pipette 2.5 mL of the final mixture onto the column and discard the flow-through.
    NOTE: The microwave stops the protocol at 60 °C; the nanoparticles can be directly added to the gel filtration column at 60 °C.
11. Add 3 mL of distilled water to the column and collect the nanoparticles in a glass vial.
12. Calculate radiolabeling efficiency using a NaI well-type detector. This parameter typically measures the activity of the ^{68}Ga incorporated in the reaction. After synthetic and purification processes, the activity of the purified sample is measured. Because of the short half-life of ^{68}Ga, the initial activity has to be corrected at time (t). Normalization with time follows the standard equation:
    $N_T = N_0 \cdot e^{-\lambda t}$
    Here,
    N_T: Counts at time (t)
    N_0: Counts at time (t) = 0
    λ: Decay constant
    t: Elapsed time

$$\text{Radiolabeling efficiency} = \frac{\text{MBq purified product (at time = t)}}{\text{MBq initial radioactivity (corrected at time = t)}} \times 100$$

NOTE: Radiolabeling efficiency should be between 90% - 95%.

4. Analysis of [68]Ga Core-doped Iron Oxide Nanoparticles (^{68}Ga-C-IONP)

1. **Dynamic light scattering**
   1. Use dynamic light scattering (DLS) to measure the hydrodynamic size of ^{68}Ga-C-IONP. Pipette 60 µL of the sample into a cuvette and perform three size measurements per sample. To ensure reproducibility, this should be repeated with several nanoparticle batches.

2. **Colloidal stability**
   1. Assess the colloidal stability of ^{68}Ga-C-IONP by measuring the hydrodynamic size of the sample after incubation in different buffers (PBS, saline, and mouse serum) for different times, ranging from 0 to 24 h. Incubate 500 µL of the sample in each buffer at 37 °C. At the selected times, take 60-µL aliquots and pipette them into DLS cuvettes to measure their hydrodynamic size.

3. **Electron microscopy**
   1. Analyze the core size of ^{68}Ga-C-IONP using transmission electron microscopy (TEM) and annular dark-field imaging (STEM-HAADF) (ref TEM protocol: NIST - NCL Joint Assay Protocol, PCC-X, Measuring the Size of Nanoparticles Using Transmission Electron Microscopy).

4. **Gel filtration radio-chromatogram**
   1. Fractionate the elution into 500-µL aliquots during the gel-filtration purification step and measure the radioactivity present in each one using an activimeter; thus, rendering a gel-filtration chromatogram.

5. **Radiochemical stability of ^{68}Ga-C-IONP**
   1. Incubate ^{68}Ga-C-IONP in mouse serum for 30 min at 37 °C (repeated 3x). After that time, purify the nanoparticles by ultrafiltration and measure the radioactivity present in the nanoparticles and filtrate. No activity should be detected in the different filtrates.

6. **Relaxometry**
   1. Measure longitudinal (T_1) and transverse (T_2) relaxation times in a relaxometer at 1.5 T and 37 °C. Four different concentrations of ^{68}Ga-C-IONP (2 mM, 1 mM, 0.5 mM, and 0.25 mM) should be measured. Plot relaxation rates (r_1=1/T_1, r_2=1/T_2) against iron concentration. The slope of the curve obtained renders r_1 and r_2 values.

7. **MR and PET phantom images**
   1. Acquire *in situ* MR (T_1-weighted sequence) and PET phantom images for a series of dilutions of ^{68}Ga-C-IONP (0 mM, 1 mM, 6.5 mM, and 9.0 mM) to observe the increasing signal in correlation with the PET activity and MRI.

Representative Results

[68]Ga-C-IONP were synthesized by combining $FeCl_3$, [68]$GaCl_3$, citric acid, water, and hydrazine hydrate. This mixture was introduced into the microwave for 10 min at 120 °C and 240 W under controlled pressure. Once the sample had cooled down to room temperature, the nanoparticles were purified by gel filtration to eliminate unreacted species ($FeCl_3$, citrate, hydrazine hydrate) and free [68]Ga (**Figure 1**).

The hydrodynamic size of ^{68}Ga-C-IONP was measured using dynamic light scattering (DLS). This revealed a narrow size distribution (PDI 0.2) and mean hydrodynamic size of 7.9 nm. Measurements of five different syntheses proved method reproducibility (**Figure 2a**). The zeta potential of several ^{68}Ga-C-IONP syntheses was measured to analyze nanoparticle surface charge; the mean value obtained was -36.5 mV. ^{68}Ga-C-IONP was incubated in different media at 37 °C during different times to ensure nanoparticle stability in biological solutions. The hydrodynamic size was measured at different times, revealing ^{68}Ga-C-IONP hydrodynamic size suffers no significant changes, meaning the sample is stable in different buffers and serums (**Figure 2b**). Because of the fast heating achieved using microwave technology, nanoparticles present ultra-small core sizes of about 4 nm. Electron microscopy images revealed homogeneous core sizes and the absence of aggregation (**Figure 2c**). A gel filtration chromatogram of ^{68}Ga-C-IONP shows a main radioactivity peak corresponding to the nanoparticles, followed by a reduced peak that corresponds to free ^{68}Ga (**Figure 2d**). The radiolabeling yield calculated after sample purification was 92%. This excellent radiolabeling yield was translated into a specific activity relative to an iron amount of 7.1 GBq/mmol Fe. The potential of ^{68}Ga-C-IONP as a contrast agent for MRI was checked by measuring longitudinal (r_1) and transversal (r_2) relaxation times. These were measured for five different ^{68}Ga-C-IONP syntheses at 37 °C and 1.5 T. An excellent mean r_1 value of 11.9 mM$^{-1}\cdot$s^{-1} and a modest r_2 value of 22.9 mM$^{-1}\cdot$s^{-1} were obtained, yielding an average r_2/r_1 ratio of 1.9, meaning ^{68}Ga-C-IONP is ideal for T$_1$-weighted MRI (**Figure 2e**). To confirm this hypothesis, the capability of ^{68}Ga-C-IONP to produce T$_1$ contrast in an MRI and PET signal was checked with the acquisition of PET and MR phantom images at different ^{68}Ga-C-IONP concentrations. As the iron concentration increases, so does the positive contrast in MR phantom. An increasing iron concentration implies an increasing ^{68}Ga concentration as well; hence, the PET signal is increasingly intense (**Figure 2f**).
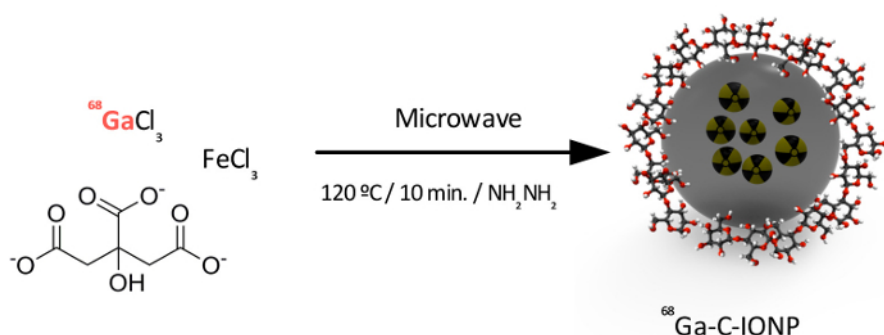

Figure 1: Synthetic steps followed in the protocol. Precursors are added in a microwave flask and introduced into the microwave upon hydrazine hydrate addition at 120 °C for 10 minutes, after which nanoparticles are obtained. Please click here to view a larger version of this figure.

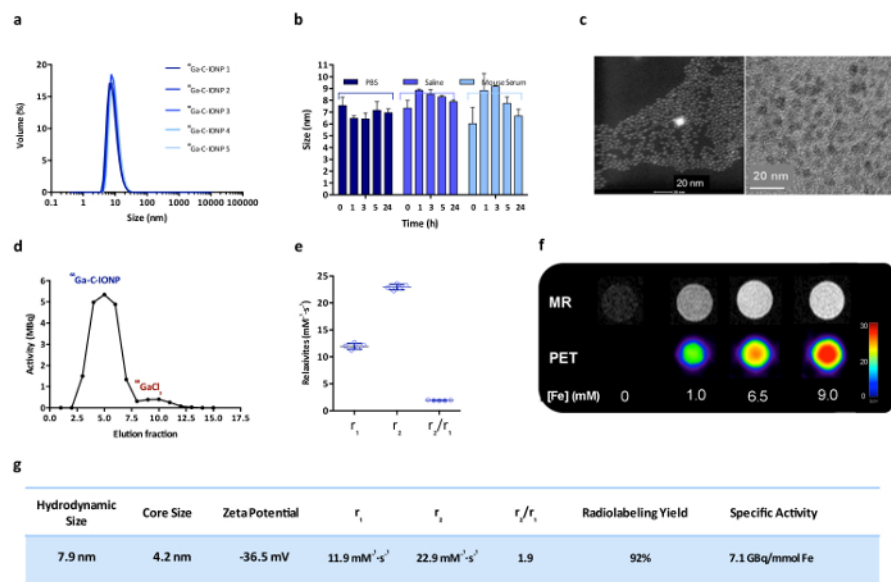| Hydrodynamic Size	Core Size	Zeta Potential	r_1	r_2	r_2/r_1	Radiolabeling Yield	Specific Activity
7.9 nm	4.2 nm	-36.5 mV	11.9 mM$^{-1}\cdot$s^{-1}	22.9 mM$^{-1}\cdot$s^{-1}	1.9	92%	7.1 GBq/mmol Fe

Figure 2: ^{68}Ga-C-IONP characterization. (**a**) This panel shows the hydrodynamic size distribution (volume weighted) of five different syntheses of ^{68}Ga-C-IONP. (**b**) This panel shows the hydrodynamic size (maximum peak in volume, mean ± SD) of ^{68}Ga-C-IONP in PBS, saline, and mouse serum (from t = 0 h to t = 24 h). (**c**) These are STEM-HAADF (left) and TEM (right) images of ^{68}Ga-C-IONP. The scale bars are 20 nm. (**d**) This panel shows a gel filtration radio-chromatogram. (**e**) This panel shows the longitudinal (r_1) and transversal (r_2) relaxivity values, and the r_2/r_1 ratio for five ^{68}Ga-C-IONP syntheses (mean ± SD). (**f**) These are MR and PET phantom images of different ^{68}Ga-C-IONP concentrations. (**g**) This is a table summarizing the main ^{68}Ga-C-IONP characteristics. Please click here to view a larger version of this figure.

Discussion

Iron oxide nanoparticles are a well-established contrast agent for T_2-weighted MRI. However, due to the drawbacks of this type of contrast for the diagnosis of certain pathologies, T_1-weighted or bright contrast is many times preferred. The nanoparticles presented here not only overcome these limitations by offering positive contrast in MRI but also offer a signal in a functional imaging technique, such as PET, *via* [68]Ga incorporation in their core. Microwave technology enhances this reproducible nanoparticle synthesis, considerably reducing the reaction time to a total of approximately 20 minutes (including a purification step). It also allows radioisotope incorporation at once in the core of the nanoparticle; suppressing an extra step required in a surface-labeling approach that would markedly extend the reaction time. This is a major advantage, especially when working with short half-live isotopes as [68]Ga ($t_{1/2}$ = 68.8 min). Moreover, the radiolabeling yield obtained (92%) is almost threefold the one obtained by the pioneering study using this nanoparticle-radiolabeling approach (Wong *et al.*[25]). This also represents a considerable improvement with respect to previous approaches, as in less than 20 minutes intrinsically radiolabeled nanoparticles with an excellent radiolabeling yield can be obtained; thus, eliminating *in vivo* radioisotope detachment or transmetalation risk and ensuring that the PET signal obtained comes from the nano-radiotracer and not from free [68]Ga. This will ease their potential use as contrast agents.

As ^{68}Ga-C-IONP are stable in different media at physiological temperature, no aggregation *in vivo* will take place; therefore presenting long blood circulating times. The gel filtration purification step eliminates the free ^{68}Ga fraction that has not been incorporated into nanoparticle cores, ensuring the PET signal is entirely provided by the ^{68}Ga-C-IONP. The outstanding r_1 value, together with the low r_2/r_1 ratio, the high radiolabeling yield, and specific activity, will allow the ^{68}Ga-C-IONP dose that is required to obtain an appropriate signal in PET and contrast in MRI to be diminished.

The nano-radiotracer presented here demonstrates that the combination of nanotechnology and radiochemistry can render a new tool that can be used for the *in vivo* detection of biological processes or diverse pathologies by means of PET and T_1-weighted MRI. It has already been used successfully in the detection by PET and MRI of angiogenesis in a murine model using RGD peptide as targeting moiety[27]. ^{68}Ga-C-IONP has also been employed, combined with a formyl peptide receptor 1 (FPR-1) antagonist, to target neutrophils in the detection of lung inflammation by PET in a non-invasive manner[28].

Disclosures

The authors have nothing to disclose.

Acknowledgements

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