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Synthesis of ^{13}N-labelled radiotracers by using microfluidic technology

Vijay Gaja,[a] Vanessa Gómez-Vallejo,[a] Mar Cuadrado-Tejedor,[b] José I. Borrell,[c] and Jordi Llop[a]*

Microfluidics has recently emerged as a useful alternative to traditional methods for the preparation of radiotracers labelled with positron emitters. Up to date, microfluidics technology has been applied to the radiosynthesis of ^{18}F-labelled and ^{11}C-labelled compounds; however, application to other shorter-lived positron emitters has not been investigated. The radiosynthesis of *S*-[^{13}N]nitrosothiols and *N*-[^{13}N]nitrosamines was approached in microfluidic system by reaction of the corresponding thiol or secondary amine, respectively, with [^{13}N]NO_2^- in the presence of mineral acid. The radiosynthesis of azo compounds was carried out by reaction of the same labelling agent with primary aromatic amines in acidic media to yield the corresponding diazonium salts, which were further reacted with aromatic amines and alcohols to yield the corresponding ^{13}N-labelled azo compounds. Radiochemical conversion values for *S*-[^{13}N]nitrosothiols and ^{13}N-labelled azo compounds calculated from chromatographic profiles improved our previous results by using conventional methods. The formation of *N*-[^{13}N]nitrosamines could not be detected, independently of experimental conditions. In conclusion, the preparation of *S*-[^{13}N]nitrosothiols and ^{13}N-labelled azo compounds was successfully achieved by using microfluidics technology. Higher radiochemical conversions than those previously reported using conventional synthetic strategies have been obtained.

Keywords: nitrogen-13; microfluidics; nitrosothiols; nitrosamines; azo; PET

Introduction

Positron emission tomography (PET) is a non-invasive *in vivo* imaging technique that produces a three-dimensional image of functional processes in a living organism, allowing the study of *in vivo* biochemistry and biology underlying disease and therapeutic intervention.[1,2] Despite the increasing number of biomedical cyclotrons installed all over the world[3] (estimated over 650 in 2010 according to the International Atomic Energy Agency), the possibility to perform studies with PET is limited by the production capabilities of radiochemistry laboratories, where the complexity of the processes associated to the preparation of the radiotracers (including radionuclide production, labelling, purification and formulation) traditionally collides with the real-time flexibility required to perform clinical or preclinical PET studies. Because of this fact, faster, more efficient and reliable radiosynthesis procedures are continuously developed, and in this scenario, the microfluidic technology offers a very promising approach for fast labelling optimization and dose-on-demand implementation.[4,5]

Among all microfluidic alternatives, the continuous flow concept was introduced in the market in a commercially available system by Advion (Figure 1 for schematic flowchart), being the main advantages of this system the elimination of temperature gradients within the reaction mixture and the possibility to perform reactions at high pressure, which lead to faster incorporation rates of the labelling agent into the final radiotracer while the amount of precursor can be decreased. Up to date, such system has been used for the preparation of ^{18}F-labelled radiotracers, including (among others) [^{18}F]fallypride,[6] [^{18}F]FIAU[7] and [^{18}F]EtDT, [^{18}F]PrDT and [^{18}F]CB-102.[5] In all cases, the cyclotron-produced $^{18}F^-$ was trapped in an anionic exchange resin cartridge and eluted with Kryptofix K2.2.2/K_2CO_3 into a conical shape vial where azeotropic evaporation of the solvent and reconstitution with acetonitrile left the ^{18}F-fluoride solution ready for multiple batch production by using the microfluidics system. More recently, the same system has been used to perform [^{11}C]carbonylation reactions by using solutions containing [^{11}C]CO in the form of the copper(I)tris(3,5-dimethylpyrazolyl)borate-[^{11}C]carbonyl complex (Cu(Tp*)[^{11}C]CO).[8]

Carbon-11 and fluorine-18 have been historically the most widely used positron emitters, and because of this fact, first applications of microfluidics technology have been focused in the preparation of ^{18}F-labelled and ^{11}C-labelled radiotracers. Although the use of other radioisotopes such as ^{13}N (half-life of 9.97 min, maximum positron energy of 1.19 MeV) might be very advantageous for the development of alternative synthetic strategies to label molecules in different positions, the short

[a]*Radiochemistry Department, CIC biomaGUNE, Paseo Miramón 182, San Sebastián 20009, Spain*

[b]*Division of Neuroscience, CIMA, University of Navarra, Pamplona, Spain*

[c]*Grup d'Enginyeria Molecular, Institut Químic de Sarrià, Universitat Ramon Llull, Via Augusta 390, 08017 Barcelona, Spain*

**Correspondence to: Jordi Llop, Radiochemistry Department, CIC biomaGUNE, Paseo Miramón 182, San Sebastián, 20009, Spain.*
E-mail: jllop@cicbiomagune.es

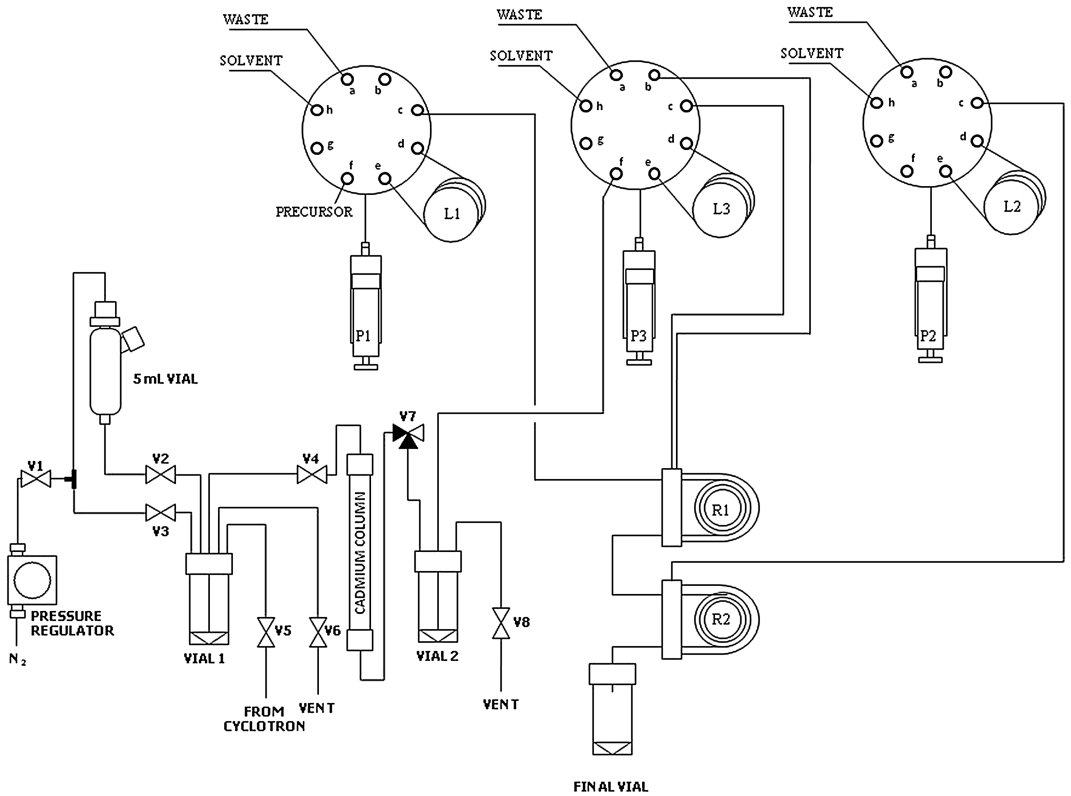


Figure 1. Flowchart of the automated system for the synthesis of ^{13}N-labelled radiotracers. V1–V8, two-way to three-way electrovalves; P1–P3, syringe pumps; L1–L3, loops; R1 and R2, reactors.

half-life of this isotope requires preparation of single dose batches, as well as the development of efficient synthetic strategies. In this context, the use of microfluidics could provide a very interesting alternative to facilitate the use of ^{13}N-labelled radiotracers in preclinical and clinical practice.

Recently, we have developed strategies for the generation of the labelling agent [^{13}N]NO_2^- starting from cyclotron-produced [^{13}N]NO_3^- and its incorporation into bioactive molecules such as *N*-[^{13}N]nitrosamines,[9] *S*-[^{13}N]nitrosothiols[10,11] and azo compounds.[12] Despite the short half-life of nitrogen-13, these ^{13}N-labelled compounds have been prepared with good radiochemical yields in short times. In the present paper, we describe for the first time the radiosynthesis of ^{13}N-labelled *S*-[^{13}N]nitrosothiols and azo compounds by using microfluidics technology, with improved radiochemical conversion (RCC) results compared with previously published data. Attempts to synthesize ^{13}N-labelled nitrosamines are also reported and briefly discussed. The potential application of microfluidics to the preparation of ^{13}N-labelled radiotracers and improvements to be implemented are also mentioned.

Materials and methods

General

Aniline (ACS reagent), 4-aminobenzenesulfonic acid (ACS reagent), phenol (ReagentPlus®, ≥99.5%), 4-phenylazophenol (98%), 4-phenylazoaniline, 4-(4-hydroxy-phenylazo)-benzenesulfonic acid sodium salt, ammonium formate (AMF) (reagent grade, 97%), formic acid (ACS reagent, >96%), acetic acid (purum, >99.0%), sodium acetate (ACS reagent, ≥99.0%), cyclohexanethiol (97%), 1-propanethiol (99%), glutathione (>98%), 1-adamantanethiol (>99%, GC quality), diisopropylamine (puriss. p. a., >99%, GC quality), trifluoroacetic acid (TFA) (puriss. p. a., for HPLC, >99%) and piperidine (puriss. p. a., >99%, GC quality) were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) and used without further purification. Ethanol, acetonitrile and methanol were of HPLC grade and purchased from Scharlab (Sentmenat, Barcelona, Spain). Water (ultrapure, Type I water, ISO 3696) was obtained from a Milli-Q® Integral system (Millipore Iberica S.A.U., Madrid, Spain).

Production of nitrogen-13

Nitrogen-13 was produced in an IBA Cyclone 18/9 cyclotron via the ^{16}O(p,α)^{13}N nuclear reaction. The target system consisting of an aluminium body with aluminium vacuum foil (thickness 25 μm, ∅ 23 mm) and havar target foil (thickness 25 μm, ∅ 29 mm) containing 1.75 mL of water was irradiated with 18 MeV protons at a beam current of 20 μA for 20 s (integrated current of 0.1 μA h). The irradiated solution was collected in a vial and passed through a glass column filled with cadmium to quantitatively reduce [^{13}N]NO_3^- into [^{13}N]NO_2^-. The cadmium column was further rinsed with 1 mL of water, and the final solution was transferred to a conical shape vial to be used in the microfluidics system. A small fraction of this solution (20 μL) was submitted to HPLC (see succeeding discussions for conditions) to determine the percentage of radioactivity as [^{13}N]NO_2^-.

A set of 10 preliminary experiments was carried out in order to determine the amount of radioactivity generated at the end of the irradiation process (381 ± 15 MBq) and the percentage of [^{13}N]NO_3^-, [^{13}N]NO_2^- and [^{13}N]NH_4^+ before the reduction step (86%, 5% and 9%, respectively).

The microfluidic platform

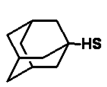
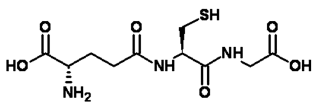
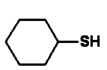
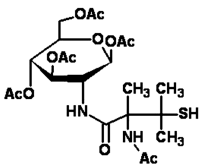
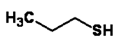
The radiosyntheses under microfluidic conditions were conducted using a NanoTek Microfluidic Synthesis System (Advion, USA), which consisted of two blocks: a reactor module and a pump module (Figure 1). The pump module block consisted of two high-pressure syringe pumps (P1 and P2), both connected to an eight-way bridged valve. The reactor module comprises a high-pressure syringe pump (P3) connected to an eight-way bridged valve and four thermostated slots where microreactors can be hosted. P1 and P2 were used to dispense the precursors, and P3 was used to dispense $[^{13}N]NO_2^-$. Reagents were loaded in loops 1–3 (L1, L2 and L3 in Figure 1) before being pushed to the reactor. For one-step reactions (preparation of *S*-[^{13}N]nitrosothiols and *N*-[^{13}N]nitrosamines), only P1 and P3 were used, whereas P1, P2 and P3 were used for conducting two-step reactions (preparation of ^{13}N-labelled azo compounds). Microreactors were made of fused silica tubing (100 μm ID; length = 2 m) rolled and held by a brass ring casted with a thermoresistant silicone polymer.

Synthesis of *S*-[^{13}N]nitrosothiols

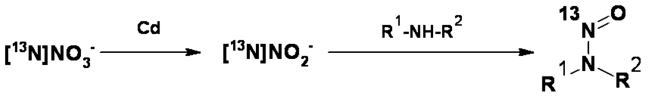
The synthesis of *S*-[^{13}N]nitrosothiols was approached by direct nitrosation of the corresponding thiols with $[^{13}N]NO_2^-$ in the presence of mineral acid (Scheme 1). The labelling agent ($[^{13}N]NO_2^-$) was loaded in storage loop L3 (Figure 1) whereas the precursor was dissolved in acidic acetonitrile solution and loaded in L1. The reactions were conducted using discrete bolus of reagents driven by the pertinent pumps (P3 for labelling agent, P1 for the precursor, Figure 1) in microreactor 1 (R1). Different relative ratios between bolus volumes and flow rates were assayed. Pure solvent was used to push the reaction crude to the final vial after reaction was finished and to clean the reaction pathway between consecutive runs.

Synthesis of *N*-[^{13}N]nitrosamines

The synthesis of *N*-[^{13}N]nitrosamines was approached by direct nitrosation of the corresponding secondary amines with $[^{13}N]NO_2^-$ in the presence of mineral acid (Scheme 2). The labelling agent ($[^{13}N]NO_2^-$) was loaded in storage loop L3 (Figure 1) whereas the precursor was dissolved in acidic solution and loaded in L1. The reactions were conducted using discrete bolus of reagents driven by the pertinent pumps (P3 for labelling agent, P1 for precursor, Figure 1) in microreactor 1 (R1). A second

R	Compound	R	Compound
	1		4
	2		5
	3		

Scheme 1. Radiosynthesis of *S*-[^{13}N]-nitrosothiols.



R_1	R_2	Compound
	$-CH_2-(CH_2)_3-CH_2-$	6
$(CH_3)_2-CH_2-$	$(CH_3)_2-CH_2-$	7

Scheme 2. Radiosynthesis of *N*-[^{13}N]-nitrosamines.

approach, in which the precursor was loaded in L1 and the acid was added to the labelling agent solution and loaded in L3, was also assayed.

^{13}N-Labelled azo compounds

For the synthesis of ^{13}N-labelled azo compounds, a two-step reaction was used (Scheme 3). The labelling agent ($[^{13}N]NO_2^-$) was loaded in storage loop L3 (Figure 1), the aromatic amine (precursor A, Scheme 3) was loaded in L1, and the phenol or aromatic amine (precursor B, Scheme 3) was loaded in L2. The reactions were conducted using discrete bolus of reagents driven by the pertinent pumps (P3 for labelling agent, P1 and P2 for precursors for steps 1 and 2, respectively) in microreactors R1 (step 1) and R2 (step 2). Different relative ratios between bolus volumes, flow rates and reactor temperatures were assayed. Pure water was used to push the reaction crude to the final vial after reaction was finished and to clean the reaction pathway between consecutive runs.

Determination of radiochemical conversion

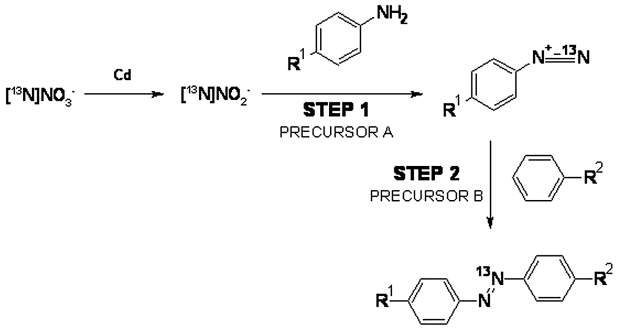
The determination of the RCC was carried out by HPLC. An Agilent 1200 series HPLC equipped with a quaternary pump, a variable wavelength detector (λ = 220 nm) and a radiometric detector (Gabi, Raytest) was used in all cases.

Chromatographic conditions for $[^{13}N]NO_2^-$ determination: stationary phase: HP Asahipak ODP-50 column (4.0 × 125 mm, 5 μm); mobile phase: solution containing additive for ionic chromatography (P/N 5062–2480, Agilent Technologies, 15 mL) in a mixture water/acetonitrile (86/14), adjusted to pH = 8.6 with 1 M NaOH solution at a flow rate of 1 mL/min.

Chromatographic conditions for *S*-[^{13}N]nitrosothiols: stationary phase: Mediterranea Sea_{18} column (4.6 × 150 mm, 5 μm); mobile phase: water/methanol/acetonitrile (10/15/75) at a flow rate of 1 mL/min for compounds **1–3** and **5**; aqueous 0.1% TFA solution/acetonitrile (95/5) at a flow rate of 1 mL/min for compound **4**.

Chromatographic conditions for *S*-[^{13}N]nitrosamines: stationary phase: YMC J'sphere ODS-H80 column (4.6 × 120 mm, 4 μm); mobile phase: water/acetonitrile/methanol (28/18.5/3.5) at a flow rate of 1 mL/min.

Chromatographic conditions for ^{13}N-labelled azo compounds: stationary phase: Zorbax Eclipse XDB C18 column (4.6 × 150 mm, 5 μm); mobile phase: AMF 0.1 M aqueous solution (pH = 3.9)/methanol/acetonitrile (40/15/45) at a flow rate of 1 mL/min for compound **8**; AMF 0.1 M aqueous solution (pH = 3.9)/methanol/acetonitrile (40/15/45) at a flow rate of 1 mL/min for compound **9**; AMF 0.1 M aqueous solution (pH = 3.9)/acetonitrile (83/17) at a flow rate of 1 mL/min for compound **10**.



R_1	R_2	Compound
H	OH	8
H	NH_2	9
SO_3H	OH	10

Scheme 3. Radiosynthesis of ^{13}N-labelled azo compounds.

Radiochemical conversion values were calculated in all cases from chromatographic profiles, referred to the percentage of $[^{13}N]NO_2^-$ in the solution resulting from the reduction step.

Results and discussion

Synthesis of *S*-[^{13}N]nitrosothiols

The synthesis of *S*-[^{13}N]nitrosothiols was first reported by our research group in 2009. The first attempts consisted of synthesizing [^{13}N]GSNO by dissolving glutathione in aqueous hydrochloric acid, which was reacted with $[^{13}N]NO_2^-$ in a reaction vial.[10] In a more recent work,[11] the labelling strategy was based on trapping $[^{13}N]NO_2^-$ in an anion exchange resin, which was further reacted (in the solid support) with a group of thiols to yield the desired *S*-[^{13}N]nitrosothiols in short reaction times (60 s) with good RCCs (48.7–74.5% with respect to $[^{13}N]NO_2^-$, depending on the starting thiol). All reactions were assayed at room temperature and with the same acid concentration. Because the formation of *S*-nitrosothiols is a very straightforward reaction that works very well both in non-radioactive and in radioactive conditions, our first attempts to implement microfluidics technology to the preparation of ^{13}N-labelled radiotracers were performed for the synthesis of *S*-[^{13}N]nitrosothiols. In order to obtain comparative results to previously published data, the same concentration of precursors and reaction temperatures were used. However, only HCl (which could not be employed in solid phase support synthesis because of displacement of the labelling agent) was used as acid.

For optimization of the experimental conditions, several acid concentrations and flow rates were assayed. A significant dependence of RCC with acid concentration was found, as expected, in all cases. Almost 100% RCC values (Table 1) were obtained at room temperature for compounds **2**, **3** and **4** at HCl concentrations of 0.05 M (compounds **2** and **3**) and 0.1 M (compound **4**). These are milder conditions than the ones used in solid phase support synthesis (acid concentration = 1 M). Flow rates of 20, 40 and 60 μL/min (P1 + P3) were assayed, but effects on RCC could not be observed, probably because of the fast reaction rate. When the acid concentration was decreased by a factor of 10, significantly lower RCCs, which were dependent on flow rate (the lower the flow rate, the higher the RCC) were obtained (results not shown). Optimal conditions offered better RCC values (99.5 ± 0.2, 98.4 ± 1.1 and 99.6 ± 0.3 for **2–4**) than those obtained using solid phase support synthesis (60.3 ± 5.5, 74.5 ± 2.1 and 48.7 ± 6.3, respectively), thus confirming the faster incorporation rates of the labelling agent into the final radiotracer by using the microfluidics approach (Table 1).

Lower RCC values were obtained in the case of compounds **1** and **5**. In both cases, no conversion was observed when HCl was not added to the precursor solution (Figure 2); an increase in acid

Table 1. Optimized experimental conditions and radiochemical conversion for the preparation of ^{13}N-labelled nitrosothiols by microfluidics and by solid phase support synthesis

	Solid phase support		Microfluidics	
Compound	Acid	RCC (%)[f]	Acid	RCC (%)[f]
1	TFA[a]	72.5 ± 3.9	HCl[b]	53.6 ± 8.8
2	TFA[a]	60.3 ± 5.5	HCl[c]	99.5 ± 0.2
3	TFA[a]	74.5 ± 2.1	HCl[c]	98.4 ± 1.1
4	HCl[a]	48.7 ± 6.3	HCl[d]	99.6 ± 0.3
5	TFA[a]	66.2 ± 5.8	HCl[e]	57.3 ± 6.5

Solid phase support results have been obtained from Gómez-Vallejo *et al.*[11] RCC, radiochemical conversion.
[a]Concentration: 1.0 M.
[b]Concentration: 0.01 M.
[c]Concentration: 0.05 M.
[d]Concentration: 0.1 M.
[e]Concentration: 0.5 M.
[f]Calculated as the ratio between the amount of radiotracer before purification and the amount of $[^{13}N]NO_2^-$ obtained after the reduction step.

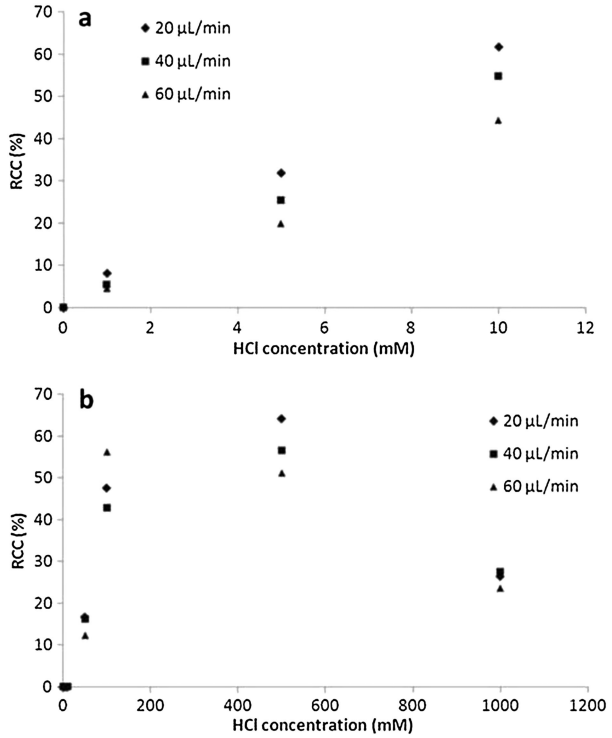


Figure 2. Radiochemical conversion values for compounds **1** (a) and **5** (b) as a function of HCl concentration and flow rate.

concentration yielded higher RCCs up to a certain level; in the case of compound **1** (Figure 2(a)), RCC increased up to 61.8 ± 2.3% for HCl concentration of 10 mM. However, at higher HCl concentrations, the RCC reached a plateau (65.3 ± 3.4% at HCl concentration of 1 M, result not shown in the figure). A clear effect of the flow rate on the RCC was observed, independently of the HCl concentration (lower flow rates yielding higher RCC values). Interestingly, measurable values of RCC were only observed for compound **5** when HCl concentration was ≥50 mM (Figure 2(b)). A maximum in RCC was obtained for HCl concentration of 0.5 M at a flow rate of 20 µL/min (64.1 ± 4.8%), which decreased at higher acid concentrations. This result suggests the decomposition of the *S*-[^{13}N]nitrosothiol in strong acidic conditions. For both compounds, the results obtained in our previous work (solid support synthesis, RCC values of 72.5 ± 3.9 and 66.2 ± 5.8% for **1** and **5**, respectively) could not be reached, although lower flow rates at the optimal HCl concentration (0.5 M) might lead to higher RCC values; however, lower flow rates require longer overall reaction times and could thus compromise decay-corrected radiochemical yield.

Synthesis of *N*-[^{13}N]nitrosamines

The synthesis of *N*-[^{13}N]nitrosamines has been also previously reported by our research group.[9] In our previous work, our first attempts consisted of the reaction of nitrous acid (generated from nitrite solution and mineral acid in water) with secondary amines. In this previously reported work, the presence of the corresponding *N*-[^{13}N]nitrosamine could not be detected irrespective of the starting secondary amine. Thus, a second combined approach (resin-supported NO_2^- + Ph_3P/Br_2/amine strategy) was assayed on a series of aliphatic secondary amines with good results (RCC values in the range 45.6 ± 7.4 to 53.4 ± 1.3). In the current work, the use of the latter strategy was anticipated to be not convenient; therefore, the approach based on direct reaction of the amines with [^{13}N]NO_2^- in acidic media was assayed using piperidine as secondary amine. In first instance, a solution of the amine in aqueous acidic solution was loaded in L1, and the labelling agent ([^{13}N]NO_2^-) was loaded in L3. Different acid concentrations (0.001–1 M) and reaction temperatures (60–120 °C) were assayed while the flow rate was maintained at 40 µL/min. Unfortunately, the presence of *N*-[^{13}N] nitrosopiperidine could not be detected in any case; thus, a second approach in which the acid was added to the labelling agent and the resulting solution was loaded in L3 while the solution of the amine was loaded in L1 was assayed with identical negative results, independently of the acid concentration (0.001–1 M) and reaction temperature (60–120 °C).

Despite that unsuccessful results could be expected, ^{13}N-nitrosation of diisopropylamine was also tested here by using microfluidics conditions. No *N*-[^{13}N]nitrosodiisopropylamine could be detected, irrespective of the experimental conditions used. These results suggest that the preparation of ^{13}N-labelled nitrosamines under microfluidic conditions might require activation of the secondary amine via similar strategies to those previously reported by our research group.

Synthesis of ^{13}N-labelled azo compounds

The synthesis of ^{13}N-labelled azo compounds, which could be potentially used as *in vivo* β-amyloid markers, was reported recently by our research group for the first time.[12] The synthetic approach consisted of a two-step process; the first step involved the reaction of anion exchange resin trapped [^{13}N]NO_2^- with primary aromatic amines in acidic conditions to yield the corresponding ^{13}N-labelled diazonium salt, which was then pushed to a reactor pre-charged with a solution of an aromatic amine or phenol to yield the ^{13}N-labelled azo compound. In the present work, a parallel strategy was used, although both steps were performed under microfluidic conditions; the diazonium salt was formed in reactor R1 (Figure 1) and was mixed with the corresponding aromatic amine or phenol (Scheme 3) in reactor R2 (Figure 1). The formation of the diazonium salt takes place under acidic conditions (pH < 2), whereas the formation of the azo compound gives better yields when carried out under moderate acidic or basic conditions (pH > 4, depending on the structure of the final tracer). In order to obtain comparative results to those previously reported, the same media and precursor concentrations were used; thus, a solution of the aromatic amine (precursor A) in 1 M (compounds **8** and **9**) or 0.1 M (compound **10**) aqueous HCl was loaded in L1, the labelling agent ([^{13}N]NO_2^-, aqueous solution) was loaded in L3 and a solution of the aromatic amine or phenol (precursor B, Scheme 3, see Table 2 for solvent) was loaded in L2.

First assays were performed on compound **8**. As expected, because of the aforementioned dependence of reaction rate with pH, the ratio between the volumes of precursors A and B had a big effect on RCC (Figure 3). Almost quantitative RCC values were obtained when ratios (precursor B)/(precursor A) = 2/1 were used, with minor effect of flow rate (RCC = 91.6 ± 1.7, 93.1 ± 2.3 and 93.9 ± 4.1% for flow rates of 40, 60 and 80 µL/min in step 1). When the (precursor B)/(precursor A) ratio was decreased to 1 and 0.5, lower RCC values (around 35% and 5%, respectively) were obtained, with again minor effect due to modifications in the flow rate. Interestingly, under the same experimental conditions as described in our previous work[12] (same precursor

Table 2. Optimized experimental conditions and radiochemical conversion for the preparation of ^{13}N-labelled azo compounds by microfluidics and by solid phase support synthesis

Compound	C_A (M)[a]	Solvent$_A$[b]	C_B (M)[c]	Solvent$_B$[d]	RCC (%)[e]	RCC (%)[f]
8	0.25	1.0 M aqueous HCl	0.45	1.0 M aqueous NaOH	58.3 ± 5.5[g]	93.9 ± 4.1[g]
9	0.25	1.0 M aqueous HCl	0.60	2.5 M AMF/HCOOH (pH = 4.0)	40.9 ± 11.0[h]	57.0 ± 2.3[i]
10	0.25	0.1 M aqueous HCl	0.45	1.0 M aqueous NaOH	40.3 ± 1.5[h]	49.3 ± 6.0[j]

Solid phase support results have been obtained from Gómez-Vallejo *et al.*[12]
[a]Concentration of precursor A.
[b]Solvent used for precursor A solution.
[c]Concentration of precursor B.
[d]Solvent used for precursor B solution.
[e]RCC with solid phase support approach.
[f]RCC with microfluidics approach.
[g]Steps 1 and 2 at room temperature.
[h]Step 1 at room temperature, step 2 at 90 °C.
[i]Step 1 at room temperature, step 2 at 80 °C.
[j]Steps 1 and 2 at 80 °C.

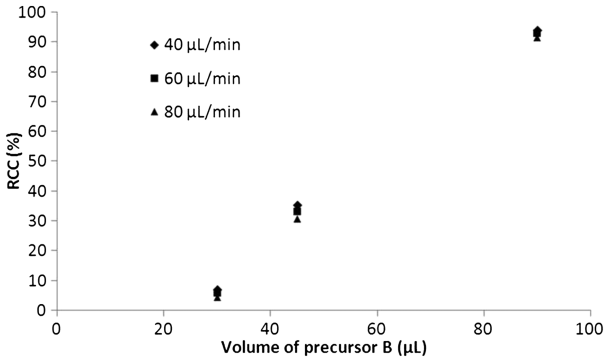


Figure 3. Radiochemical conversion values for compound **8** as a function of volume of precursor B (volume of precursor A = 45 µL/min in all cases) and flow rate in step 1.

concentrations, reaction temperature for steps 1 and 2), higher RCC values were obtained ($93.9 \pm 4.1\%$ vs. $58.3 \pm 5.5\%$), which is a clear indication of the advantages of using microfluidics in this particular reaction (Table 2).

For compound **9**, low RCC values were obtained when steps 1 and 2 were performed at room temperature, independent of the flow rate of steps 1 and 2 (range: $3.9 \pm 0.4\%$ to $20.0 \pm 3.6\%$). An increase in the temperature of step 1 to 80 °C had moderate negative effects on RCC (probably due to decomposition of the intermediate diazonium salt), whereas an increase in the temperature of step 2 offered optimal RCC values (52.0 ± 4.8 to $57.0 \pm 2.3\%$, depending on the flow rates of steps 1 and 2). Again, significantly higher RCC values than those obtained using the solid phase support synthesis were obtained (Table 2).

A different trend was observed for the preparation of compound **10**. Moderate RCC values were obtained when room temperature was used for steps 1 and 2 (29.7 ± 3.8 to $30.1 \pm 3.0\%$ depending on flow rates). These values were slightly improved when the temperature for step 2 was increased up to 80 °C (32.6 ± 2.0 to $34.2 \pm 3.3\%$) and reached optimal values (44.9 ± 5.8 to $49.3 \pm 6.0\%$) when the temperature of step 1 was also increased to 80 °C. This increase in the RCC when T_1 was increased suggests a slower reaction rate in the formation of the corresponding diazonium salt and higher stability of the latter. Interestingly, although RCC values obtained using microfluidics at 40 and 80 °C for steps 1 and 2, respectively, are lower than those obtained using solid phase support synthesis, the use of microfluidics allowed heating in step 1 (more difficult to implement in solid phase support synthesis) and thus RCC values could be improved under optimal conditions (Table 2).

In view of the results reported here, microfluidics is a promising tool for the synthesis of ^{13}N-labelled radiotracers. In all cases, equivalent or higher RCC values were obtained when the microfluidics technology was applied to the preparation of ^{13}N-labelled nitrosothiols and azo compounds in comparison with traditional (in vial or solid support) synthetic methods. However, further investigation has to be carried out in order to establish microfluidics as a routine tool for the preparation of ^{13}N-labelled radiotracers in the preclinical and/or clinical environments. As a matter of fact, two different issues still remain to be solved. First, a purification step should be included in the whole preparation flowchart. As small volumes are usually handled, the reaction mixture could be potentially directed into a loop, and HPLC might be performed under analytical conditions. Eventually, UPLC could be applied with the subsequent reduction in chromatographic retention times. Second and more important, a pre-concentration step for the labelling agent ($[^{13}N]NO_2^-$) should be developed. Currently, 1.75 mL of water is irradiated in the cyclotron, and after the reduction step, the cadmium column is further eluted with 1 mL of water (total starting volume = 2.75 mL); amounts of activity in the range 150–200 MBq of $[^{13}N]NO_2^-$ are typically obtained with 0.1 µA h integrated current, which means an activity concentration around 60 MBq/mL. Taking into account that 20–100 µL/min reaction flows are used in the microfluidics system, a maximum of 1.2–6.0 MBq of radiotracer can be produced per minute (assuming 100% RCC), which is clearly insufficient for further application to imaging studies. In order to obtain starting volumes more appropriate for microfluidics, the pre-concentration step should be implemented after reduction of $[^{13}N]NO_3^-$ to $[^{13}N]NO_2^-$; such procedures might be based on ion exchange resins or, as recently published for the pre-concentration of ^{18}F, on electrochemical methods[13]; in this scenario, the improved RCC obtained under microfluidics conditions would be translated into higher overall radiochemical yields. Implementation of these two key steps is currently under development in our laboratory.

Conclusions

In this study, the procedures for the preparation of *S*-[^{13}N]nitrosothiols and ^{13}N-labelled azo compounds have been implemented using a microfluidics system for the first time. In general terms, under similar or identical experimental conditions, higher RCC values were obtained when compared with our previously reported solid phase support synthesis procedure[11,12] in similar overall reaction times. The preparation of *N*-[^{13}N]nitrosamines was also approached under several experimental conditions, without positive results. However, activation of the secondary amine[9] could also be assayed in the microfluidics system as an alternative method to obtain significant RCC values.

The here reported method, coupled to an adequate purification system (currently under development in our laboratory), should allow the preparation of a wide range of *S*-[^{13}N] nitrosothiols and ^{13}N-labelled azo compounds on a dose-on-demand basis, although a pre-concentration step of the labelling agent might be required. This dose-on-demand concept becomes relevant in the case of nitrogen-13, in which because of its short half-life, multi-dose batch preparation is out of the scope of research and clinical PET centres.

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Conflict of Interest

The authors did not report any conflict of interest.

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