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Research Article

Fluorine-18 labelling of small interfering RNAs (siRNAs) for PET imaging†

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Abstract: Small interfering RNAs (siRNAs), a class of macromolecules constituted by the association of two single-stranded ribonucleic acids of short sequences, have been labelled with the positron-emitter fluorine-18 ($T_{1/2}$: 109.8 min). The strategy involves (1) prosthetic conjugation of a single-stranded oligonucleotide with [^{18}F]FPyBrA (*N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide) followed by (2) formation of the target duplex by annealing with the complementary sequence, therefore, permitting parallel and combinatorial preparation of [^{18}F]siRNAs. Pure fluorine-18-labelled siRNAs (0.55–1.11 GBq, specific activity: 74–148 GBq/μmol at EOB) could be obtained within 165 min starting from 37.0 GBq of starting [^{18}F]fluoride (1.5–3.0%, non-decay-corrected isolated yields). Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: fluorine-18; siRNA; oligonucleotide; FPyBrA

Introduction

RNA interference (RNAi) is an evolutionary conserved, post-transcriptional gene-silencing pathway first observed in plants in 1990 but also recently in mammals.[1–3] The RNAi process, which is related to a natural defense against viruses and the mobilization of transposable genetic elements or transposons,[4] can also be a powerful tool in the hands of the biologist to efficiently and specifically block the expression of a gene at the RNA level. The general mechanism involves the cleavage of double-stranded RNA (dsRNA) by Dicer, a dsRNA-specific endonuclease, into short interfering RNA of 21 base-pairs (siRNA) with a two-nucleotide overhang on the 3′-ends. These siRNAs are then recognized by the RNA-induced silencing complex (RISC) that promotes their unwinding, guides the RNA–protein complex to the complementary mRNA and cleaves the mRNA at a defined site.[5–7] The siRNA approach is, however, more difficult to achieve in whole organisms than in cells because of the poor pharmacological properties of RNAs. When administered directly to a living mammal, RNA does not permeate well the cell membranes and is very rapidly metabolized by plasmatic RNAse, resulting in a very short half-life in the vascular system. Therefore, vectorization or stabilizing chemical modifications appear necessary for the systemic delivery of siRNAs. In particular, some chemical modifications on the RNA backbone or on the nucleobases enhance their *in vivo* stability and are also useful in reducing off-target effects[8–10] that lead to non-specific interferon response.[11–13] *In vivo* evaluations of the global biodistribution, pharmacokinetic parameters and activity of chemically modified siRNAs are necessary to verify that chemical modifications do not affect gene silencing[14,15] or induce non-specific effects such as accumulation in sink organs[16] and to define the best molecules and protocols for efficient *in vivo* RNAi.

The work presented herein represents to our knowledge the first labelling of this class of macromolecules,

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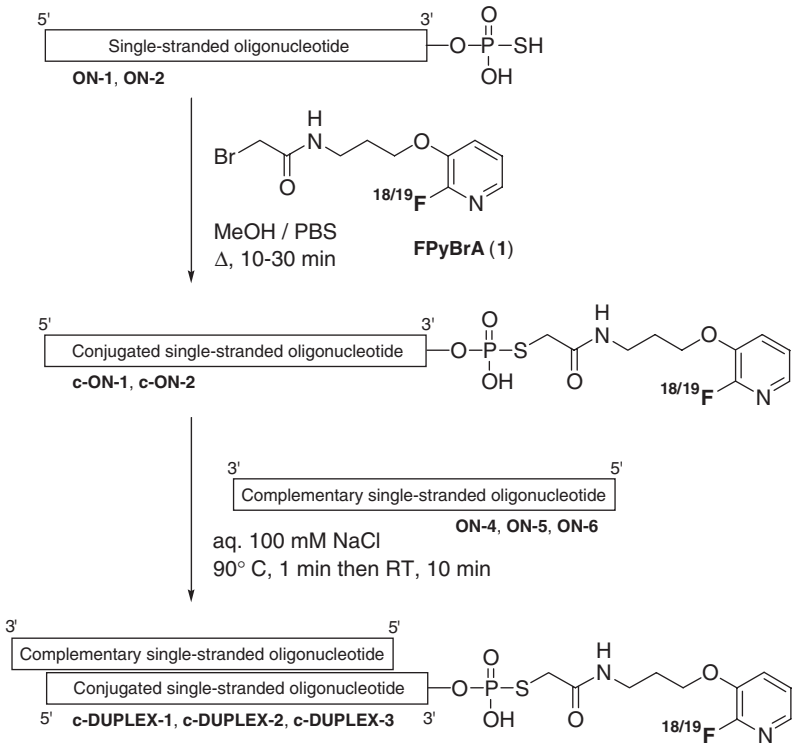
the siRNAs, with the short-lived positron-emitter fluorine-18 (half-life: 109.8 min). Three selected duplexes showing high *in vitro* cellular capacity to interfere with the target mRNA coding for Luciferase were labelled with this radioisotope based on a prosthetic conjugation approach using *N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide ([^{18}F]FPyBrA) as a reagent, now permitting *in vivo* dynamic and quantitative molecular imaging of siRNAs with positron emission tomography (PET).

Results and discussion

The method of choice for a highly efficient fluorine-18-labelling of oligonucleotide-type macromolecules is today the conjugation of a prosthetic group, carrying the radioisotope, with a reactive function of the macromolecule.[17–21] This strategy allows for a wide choice of chemical routes, including drastic chemical conditions for the preparation of the labelled prosthetic group reagent, followed by the conjugation of the latter with a macromolecule using mild conditions needed to preserve its integrity. Recently, a new 2-bromoacetamide [^{18}F]reagent, coded [^{18}F]FPyBrA (*N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide, [^{18}F]-**1**, Scheme 1, was developed for the fluorine-18-labelling of oligonucleotides and derivatives.[21] In this reagent, the pyridinyl moiety carries the radioactive fluorine which can be efficiently incorporated via a nucleophilic *hetero*aromatic substitution,[22–25] and the 2-bromoacetamide function assures the efficient alkylation of a phosphorothioate monoester group borne at the 3′- or 5′-end of single-stranded oligonucleotides.[21]

Labelling of the target mRNA–Luciferase duplexes described herein is based on the following sequence: (1) labelling of a single-stranded, 3′-phosphorothiolated oligonucleotide using the [^{18}F]FPyBrA reagent; and (2) duplex formation (annealing) using the complementary sequence. This approach, besides being compatible with the half-life of fluorine-18, avoids the expected denaturation of the duplex under the conditions of a direct conjugation with [^{18}F]FPyBrA. This approach also permits parallel and combinatorial preparation of the duplexes and minimizes the number of radiochemical syntheses required. In the present work, for example, the selected duplexes **DUPLEX-1** and **DUPLEX-3** (see below) use the same antisense fluorine-18-labelled oligonucleotide **ON-1**. Finally, this approach also allows the parallel *in vivo* PET imaging of both single-stranded oligonucleotides and sense–antisense duplexes with one batch of labelled oligonucleotide.



Scheme 1

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Description and selection of the sense–antisense duplexes

All sense and antisense sequences used in the present work belong to a mRNA-Luciferase-targeted siRNA (sense: $^{5'}$ CGU ACG CGG AAU ACU UCG AUU $^{3'}$/ antisense: $^{5'}$ UCG AAG UAU UCC GCG UAC GUU $^{3'}$).[3]

As shown in Table 1, all single-stranded oligonucleotides (**ON-1** to **ON-6**) are 21-mer with the 3′-end uridine being 2′-*O*-methyl-modified. **ON-1** and **ON-4** are full RNA-based sequences. **ON-2** and **ON-5** are mixed RNA/2′-fluoropyrimidines-RNA, whereas **ON-3** and **ON-6** are mixed RNA/2′-*O*-methyl-RNA. **ON-1**, **ON-2** and **ON-3** are provided with a phosphorothioate function (–OP(OH)SH) at their 3′-end, allowing their conjugation with [$^{19/18}$F]FPyBrA.[21] Note that the presence of this phosphorothioate function imposes the concomitant use of a 2′-*O*-methylated base (2′methoxyuridine in this case) to circumvent the known and described cyclization between the unprotected 2′-OH function and the phosphorothioate group during the conjugation reaction.[26]

DUPLEX-1 is the combination of **ON-1** and **ON-4**. This full RNA duplex, which is not expected to show high plasmatic stability once injected *in vivo*, is the duplex of reference. Sequences bearing 2′-*O*-methyl-modified bases as well as 2′-fluoropyrimidines are described to be more resistant to nuclease degradation and an increase in the *in vitro* half-life of the corresponding duplexes have already been shown.[15,27] However, particular care should be taken when designing an alternative sequence bearing *O*-methyl-modified bases or 2′-fluoropyrimidines, since modifications along the sequence of duplexes might hamper the interference effect of these potential siRNAs with the target mRNA.[28,29] Besides **DUPLEX-1**, a number of other sense–antisense duplexes (combination of the single-stranded oligonucleotides (**ON-1** to **ON-6**)) can be designed based on the sequences described above (Table 1). Selection of the duplexes was based on their plasmatic stability toward nucleases and on their *in vitro* cellular capacity to still interfere with the target mRNA coding for Luciferase. This work, which will be reported elsewhere,[30] resulted in the selection of the following two siRNAs: **DUPLEX-2** (a combination of **ON-2** and **ON-5**) and **DUPLEX-3** (a combination of **ON-1** and **ON-6**). Noteworthy, all duplexes using **ON-3** as the antisense sequence were shown to have severe reduction of interference effect with the target mRNA and were therefore not further studied (for example, combination of **ON-3** and **ON-6** completely abolishes the interference effect described above). Table 2 shows sequences, chemistry and analytical data obtained for the three duplexes mentioned above (**DUPLEX-1**, **DUPLEX-2** and **DUPLEX-3**).

The nomenclature used throughout the text is the following: **c-ON** corresponds to the 3′-conjugated single-stranded oligonucleotide resulting from the coupling of [$^{19/18}$F]FPyBrA with the original 3′-phosphorothiolated sequence. **c-DUPLEX** corresponds to the combination of a 3′-conjugated single-stranded oligonucleotide (**c-ON**) with a single-stranded complementary sequence (**ON**).

Chemistry

N-[3-(2-Fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide (FPyBrA, **1**, Scheme 1) as reference compound and [3-(3-*tert*-butoxycarbonylamino-propoxy)-pyridin-2-yl]-trimethyl-ammonium trifluoromethane-sulfonate (**2**) as the precursor for labelling with fluorine-18 were prepared as previously described [21] from commercially available (3-hydroxypropyl)carbamic acid *tert*-butyl ester and 2-fluoro-3-hydroxypyridine or 2-dimethyla-

Table 1 Sequence, chemistry and analytical data of the starting and conjugated oligonucleotides

Code	Sequence	HPLC R_t[a] (min)	MS found (calculated)[b,c]
ON1/c-ON1	$^{5'}$ U C G A A G U A U U C C G C G U A C G U U $^{3'ps}$	8.50[d]/9.50[c]	6973.6 (6970.3)
ON2/c-ON2	$^{5'}$ Uf C G A A G Uf A Uf Uf Cf Cf G Cf G Uf A Cf G Uf Uf $^{3'ps}$	9.70[d]/10.50[c]	6984.5 (6992.2)
ON3	$^{5'}$ U C G A A G U A U U C C G C G U A C G U U $^{3'ps}$	—	—
ON4	$^{5'}$ C G U A C G C G G A A U A C U U C G A U U $^{3'}$	8.50[d]	—
ON5	$^{5'}$ Cf G Uf A Cf G Cf G G A A Uf A Cf Uf Uf Cf G A Uf Uf $^{3'}$	9.50[d]	—
ON6	$^{5'}$ C G U A C G C G G A A U A C U U C G A U U $^{3'}$	9.10[d]	—

C: cytidine; C: 2′methoxycytidne; Cf: 2′fluorocytidine; U: uridine; U: 2′methoxyuridine; Uf: 2′fluorouridine; A: adenosine; A: 2′methoxyadenosine; G: guanosine; G: 2′methoxyguanosine; ps: phosphorothioate.

[a] HPLC column and conditions: C18 μBondapak® Waters (300 × 7.8 mm, porosity 10 μm); triethylammonium acetate, 50 mM, pH 7.4 (TEAA) and acetonitrile; gradient elution: linear 5 min from 95/5 to 90/10 (TEAA/acetonitrile) then linear 15 min from 90/10 to 75/25 and washout 5 min at 50/50, flow rate: 6.0 mL/min.

[b] GSG-MALDI-TOF Spektrometer (Germany).

[c] Analytical data (HPLC, MS) concerning the conjugated oligonucleotides. c-ON refers to a conjugated ON.

[d] Analytical data (HPLC) concerning the starting oligonucleotides.

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Table 2 Sequence, chemistry and HPLC data of the selected duplexes

Code	Combination of	Sequence	HPLC R_t[a] (min)
DUPLEX-1	**ON-1**	$^{5'}$ U C G A A G U A U U C C G C G U A C G U U $^{3'ps}$	9.60
	ON-4	$^{3'}$ U U A G C U U C A U A A G G C G C A U G C $^{5'}$	
DUPLEX-2	**ON-2**	$^{5'}$ U C G A A G U A U U C C G C G U A C G U U $^{3'ps}$	10.70
	ON-5	$^{3'}$ U U A G C U U C A U A A G G C G C A U G C $^{5'}$	
DUPLEX-3	**ON-1**	$^{5'}$ U C G A A G U A U U C C G C G U A C G U U $^{3'ps}$	9.80
	ON-6	$^{3'}$ U U A G C U U C A U A A G G C G C A U G C $^{5'}$	

C: cytidine; C: 2′methoxycytidne; C: 2′fluorocytidine; U: uridine; U: 2′methoxyuridine; U: 2′fluorouridine; A: adenosine; A: 2′methoxyadenosine; G: guanosine; G: 2′methoxyguanosine; ps: phosphorothioate.
[a]HPLC column and conditions: C18 μBondapak® Waters (300 × 7.8 mm, porosity 10 μm); triethylammonium acetate, 50 mM, pH 7.4 (TEAA) and acetonitrile; gradient elution: linear 5 min from 95/5 to 90/10 (TEAA/acetonitrile) then linear 15 min from 90/10 to 75/25 and washout 5 min at 50/50, flow rate: 6.0 mL/min.

mino-3-hydroxypyridine, respectively (11 and 38% non-optimized yields).

Sequence and analytical data (high-performance liquid chromatography, HPLC, MS) belonging to the two conjugated single-stranded oligonucleotide (**c-ON-1** and **c-ON-2**) are described in Table 1. These two conjugated oligonucleotides were prepared as follows: reaction of 3′-phosphorothiolated oligonucleotide **ON-1** and **ON-2** (*circa* 90 nmol) was carried out using 60 eq. of FPyBrA (**1**) in a mixture of methanol and 0.1 M phosphate buffer saline (PBS, pH 7.4) at 80°C for 30 min (Scheme 1). Yields of conjugation were determined by HPLC (ratio between conjugated and non-conjugated oligonucleotides) and were reasonably high, 50–60%, especially when considering the size (21-mer) of the starting oligonucleotides (longer oligonucleotides generally are less reactive[31]). Potentially, the yields could even be pushed further, but in that case the level of by-products would complicate the final HPLC purification. The conjugated oligonucleotides (**c-ON-1** and **c-ON-2**) were then purified by semi-preparative reversed-phase HPLC (from both the excess of reagent (FPyBrA, **1**) and non-reacted starting material (**ON-1**, **ON-2**)) and finally desalted using a Sephadex® NAP-10 column before characterization. Final isolated yields for **c-ON-1** and **c-ON-2** were about 15–20%, since just the central section of the HPLC peak was collected.

Chemical purity of the conjugated oligonucleotides (**c-ON-1** and **c-ON-2**) was estimated by HPLC (Table 1) and was found to be greater than 95%. Mass spectrometry was in accordance with the expected calculated mass for both conjugated oligonucleotides (see Table 1) and clearly confirmed mono-conjugation with the 2-bromoacetamide reagent. Finally, both conjugated and starting oligonucleotides were 5′-[^{32}P]phosphorylated and the addition of the *N*-[3-(2-fluoropyridin-3-yloxy)-propyl]acetamide moiety was confirmed by non-denaturing 20% polyacrylamide gel electrophoresis (Figure 1).

Prior to any duplex formation, concentrations of the conjugated single-stranded oligonucleotides were measured (UV absorption at 260 nm). Duplex formation was performed as follows: the conjugated oligonucleotide (**c-ON-1** or **c-ON-2**, *circa* 500 pmol) was incubated at 90°C for 1 min with a stoechiometric amount of its complementary strand (**ON-4**, **ON-5** or **ON-6**) in aqueous 100 mM NaCl (pH 7.4); then the reaction mixture was left at room temperature for another 10 min. Chemical purity of the duplexes (**c-DUPLEX-1**, **c-DUPLEX-2** and **c-DUPLEX-3**) was verified by HPLC and was found to be greater than 90%. Retention times recorded for these duplexes were similar to those recorded for **DUPLEX-1, DUPLEX-2 and DUPLEX-3**, respectively (Table 2). Duplex formation was also performed using 5′-[^{32}P]phosphorylated conjugated oligonucleotides ([^{32}P]**c-ON-1** and [^{32}P]**c-ON-2**), permitting analyses using non-denaturing 20% polyacrylamide gel electrophoresis, which confirmed the formation of the duplexes with yields greater than 95% (Figure 1).

Radiochemistry

N-[3-(2-[^{18}F]Fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide ([^{18}F]FPyBrA, [^{18}F]-**1**, HPLC purified) was prepared according to slightly modified published procedure[21] in 18–20% non-decay-corrected yield (based on starting [^{18}F]fluoride) using a three-step radiochemical pathway in 80–85 min (Scheme 2). This [^{18}F]reagent replaces nowadays our previous work-horse *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]FBnBrA)[26,31–38] for the design and development of oligonucleotide-based radiopharmaceuticals [16,39–45] for PET).

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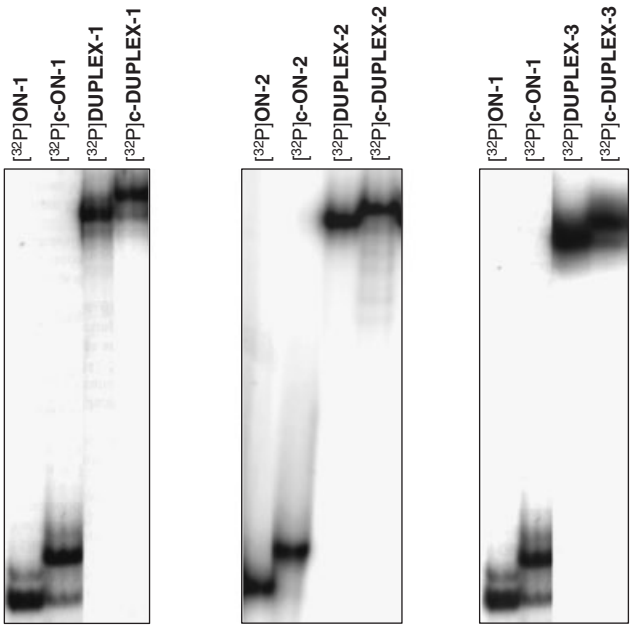
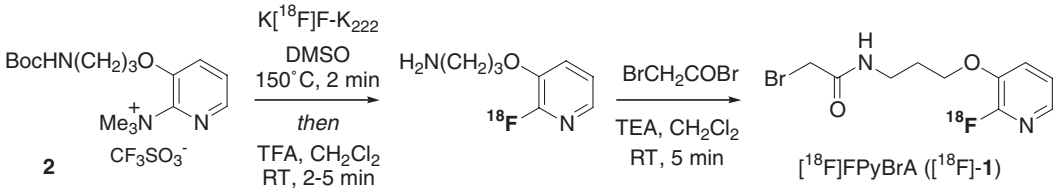


Figure 1 Analyses of [^{32}P]**ON**, [^{32}P]**c-ON**, [^{32}P]**DUPLEX** and [^{32}P]**c-DUPLEX** using non-denaturing 20% polyacrylamide gel electrophoresis.



Scheme 2

Briefly, the developed procedure involves (1) a high-yield nucleophilic *hetero*aromatic *ortho*-radiofluorination as the fluorine-18 incorporation step (70–85% radiochemical yield) and uses [3-(3-*tert*-butoxycarbonylamino-propoxy)-pyridin-2-yl]-trimethyl-ammonium trifluoromethanesulfonate (**2**) as a precursor for labelling and no-carrier-added [^{18}F]fluoride ion as its activated K[^{18}F]F–Kryptofix®222 complex,[45,46] followed by (2) rapid and quantitative TFA removal of the *N*-Boc-protective group and (3) condensation with 2-bromoacetyl bromide (45–65% radiochemical yield). Typically, 3.3–3.7 GBq (90–100 mCi) of HPLC-purified [^{18}F]FPyBrA could be obtained in *circa* 80–85 min, starting from 18.5 GBq (500 mCi) of a cyclotron production batch of [^{18}F]fluoride.

Conjugation of the 3′-phosphorothiolated oligonucleotides (**ON-1** and **ON-2**) with the HPLC-purified [^{18}F]FPyBrA ([^{18}F]-**1**) (Scheme 1) was carried out in a mixture of methanol and 0.1 M phosphate buffer saline (PBS, pH 7.4) at 120°C for 10 min. The labelled conjugated oligonucleotides ([^{18}F]**c-ON-1** and [^{18}F]**c-ON-2**) were then purified by semi-preparative RP-HPLC (from both the excess of reagent ([^{18}F]FPyBrA, [^{18}F]-**1**) and non-reacted starting material (**ON-1**, **ON-2**)) and finally desalted using a Sephadex® NAP-10 column. [^{18}F]**c-ON-1** and [^{18}F]**c-ON-2** were obtained within 60 min in 20–25% isolated and decay-corrected yields based on starting [^{18}F]FPyBrA ([^{18}F]-**1**). Radiochemical purity of the labelled conjugated oligonucleotides ([^{18}F]**c-ON-1** and [^{18}F]**c-ON-2**) was verified by HPLC and was found to be greater than 95%. Aliquots of the labelled conjugated oligonucleotide batches were also analyzed by non-denaturing 20% polyacrylamide gel electrophoresis (Figure 2), both confirming a high radiochemical purity and the addition of the *N*-[3-(2-fluoropyridin-3-yloxy)-propyl]acetamide moiety. Co-elution/co-migration with non-labelled conjugated oligonucleotides (**c-ON-1** and **c-ON-2**) was also demonstrated using HPLC and gel electrophoresis.

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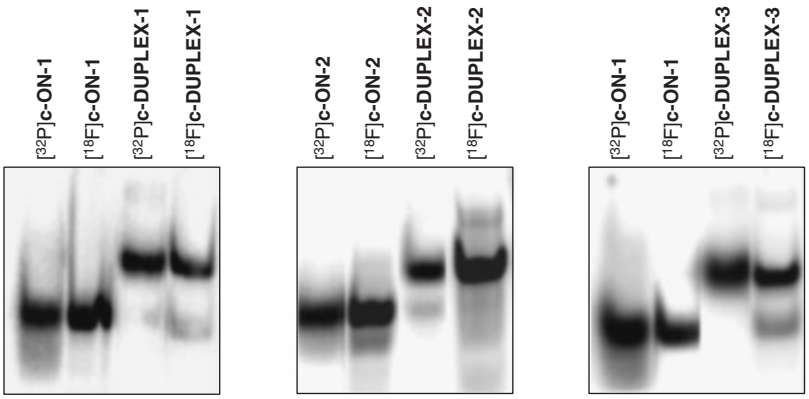

Figure 2 Analyses of [^{32}P/^{18}F]**c-ON** and [^{32}P/^{18}F]**c-DUPLEX** using non-denaturing 20% polyacrylamide gel electrophoresis.

Concentrations of the labelled conjugated single-stranded oligonucleotides were measured (UV absorption at 260 nm) and duplex formation was performed as mentioned above. Briefly, the labelled conjugated oligonucleotides ([^{18}F]**c-ON-1** or [^{18}F]**c-ON-2**, *circa* 5 nmol) were incubated at 90°C for 1 min with a stoechiometric amount of its complementary strand (**ON-4**, **ON-5** or **ON-6**) in aqueous 100 mM NaCl (pH 7.4), then the reaction mixture was left at room temperature for another 10 min. [^{18}F]**c-DUPLEX-1**, [^{18}F]**c-DUPLEX-2** and [^{18}F]**c-DUPLEX-3** were obtained within 20 min in nearly quantitative isolated and decay-corrected yields based on starting [^{18}F]**c-ON-1** or [^{18}F]**c-ON-2**. Radiochemical purity of the labelled duplexes ([^{18}F]**c-DUPLEX-1**, [^{18}F]**c-DUPLEX-2** and [^{18}F]**c-DUPLEX-3**) was verified by HPLC (Table 2) and was found to be greater than 90%. Non-denaturing 20% polyacrylamide gel electrophoresis was also performed, confirming the formation of the duplexes with yields greater than 95% and a radiochemical purity greater than 90% (Figure 2). Co-elution/co-migration with the corresponding non-labelled duplexes (**c-DUPLEX-1**, **c-DUPLEX-2** and **c-DUPLEX-3**) was also demonstrated using HPLC and gel electrophoresis.

Conclusion

A quantity of 0.55–1.11 GBq (15–30 mCi) of pure fluorine-18-labelled duplexes (**c-DUPLEX**, specific activity : 74–148 GBq/μmol at EOB) could be obtained within 165 min starting from 37.0 GBq (1 Ci) of starting [^{18}F]fluoride (1.5–3.0%, non-decay-corrected isolated yields). The work presented herein represents the first fluorine-18-labelling of siRNAs, now permitting *in vivo* dynamic and quantitative molecular imaging of this class of macromolecules with PET. The strategy involved, based on a prosthetic conjugation of one single-stranded oligonucleotide with [^{18}F]FPyBrA followed by formation of the target duplexes, proved to be compatible with the half-life of the positron-emitter used and permits parallel and combinatorial preparation of [^{18}F]siRNAs.

Experimental

General

Chemicals, flash chromatography and TLC analysis. Chemicals were purchased from standard commercial sources (Aldrich- , Fluka- or Sigma France) and were used without further purification, unless stated otherwise. Single-stranded 21-mer RNAs (chemically synthesized and gel purified) were purchased from Eurogentec (Belgium).

Oligonucleotide solution concentration determination. The concentration of all conjugated oligonucleotide solutions was measured by UV absorption at 260 nm on a UV/Vis spectrophotometer (UVIKON XL, Bio-Tek Instruments, France). The molar absorptivities (ε_0) were 217 100 L/mol/cm for **ON-1**, **ON-2** and **ON-3** and 208 200 L/mol/cm for **ON-4**, **ON-5** and **ON-6** (Eurogentec, Belgium).

HPLC analysis. [*HPLC A*]: Equipment: a Waters 600 Controller Gradient system, a Hewlett Packard 1100 series UV-multi-wavelength detector and a Packard Flow One Scintillation Analyzer equipped with a positron-dedicated cell for radioactivity monitoring; column: semi-preparative C18 μBondapak® Waters (300 × 7.8 mm, porosity: 10 μm); solvents and conditions: aq. 50 mM triethylammonium acetate, pH 7.4 (TEAA buffer) and acetonitrile; gradient elution: linear 5 min from 95/5 to 90/10 (TEAA buffer/acetonitrile, v:v) then linear 15 min from 90/10 to 75/25 (v:v) and washout 5 min at 50/50 (v:v); flow rate: 6.0 mL/min;

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temperature: RT; absorbance detection at $\lambda = 254$ nm. [*HPLC B*]: Equipment: a Waters 510 pump, a Shimadzu SPD10-AVP UV-multi-wavelength detector and a Geiger–Müller detector; column: semi-preparative SiO_2 Zorbax® Rx-SIL, Hewlett Packard (250 × 9.4 mm; porosity: 5 μm); solvents and conditions: isocratic elution with CH_2Cl_2/EtOAc: 50/50 (v:v); flow rate: 5 mL/min; temperature: RT; absorbance detection at $\lambda = 254$ nm. [*HPLC C*]: Equipment: a Waters 600 Controller Gradient system, a Shimadzu SPD10-AVP UV-multi-wavelength detector and a Geiger–Müller detector; column: semi-preparative C18 μBondapak®, Waters (300 × 7.8 mm; porosity: 10 μm); solvents and conditions: aq. 50 mM triethylammonium acetate, pH 7.4 (TEAA buffer) and acetonitrile; gradient elution: linear 5 min from 95/5 to 90/10 (TEAA buffer/acetonitrile, v:v) then linear 10 min from 90/10 to 70/30 (v:v) and washout 5 min at 50/50 (v:v); flow rate: 6 mL/min; temperature: RT; absorbance detection at $\lambda = 254$ nm.

Spectroscopies. NMR spectra were recorded on a Bruker AMX (300 MHz) apparatus using the hydrogenated residue of the deuterated solvents (DMSO-d_6, $\delta = 2.50$ ppm; CD_2Cl_2, $\delta = 5.32$ ppm) and/or TMS as internal standards for ^{1}H NMR as well as the deuterated solvents (DMSO-d_6, $\delta = 39.5$ ppm; CD_2Cl_2, $\delta = 53.8$ ppm) and/or TMS as internal standards for ^{13}C NMR. The chemical shifts are reported in ppm, downfield from TMS (s, d, t, m for singlet, doublet, triplet and multiplet, respectively). The mass spectra were measured on a Nermag R10-10 apparatus or a GSG-MALDI-TOF spectrometer.

Electrophoresis gel analyses. Non-denaturing 20% polyacrylamide gels were prepared and run according to our in-house procedures (20 W, 1–4 h in tris borate EDTA buffer, Invitrogen, France). Gels were analyzed using a Phosphorimager Storm 860 (Microsystem Electronics, now GE Healthcare, France).

Radioisotope availability/production. No-carrier-added aqueous [^{18}F]fluoride ion was produced via the [^{18}O(p,n)^{18}F] nuclear reaction by irradiation of a 2 mL [^{18}O]water (>97%-enriched, CortecNet, Paris, France) target on an IBA Cyclone-18/9 cyclotron (18 MeV proton beam) and was transferred to the appropriate hot cell. *Target hardware*: commercial, 2-mL, two-port, stainless steel target holder equipped with a domed-end niobium cylinder insert. *Target to hot cell liquid-transfer system*: 60 m PTFE line (0.8 mm internal diameter; 1/16 in external diameter), 2.0 bar helium drive pressure, transfer time 3–6 min. Typical production of [^{18}F]fluoride ion at the end of bombardment for a 20 μA, 30 min (10 μA h) irradiation: 750–800 mCi (27.7–29.6 GBq). [γ-^{32}P]ATP (triethylammonium salt of aqueous solution, 370 MBq/mL, 222 TBq/mmol) was purchased from Amersham (now GE Healthcare, France).

Miscellaneous. Radiosyntheses using fluorine-18, including the HPLC purifications, were performed in a 7.5-cm-lead shielded cell using a computer-assisted Zymate robot system (Zymark corporation, USA).

Chemistry

***N*-[3-(2-Fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide (FPyBrA, 1)**. Synthesized in three steps from (3-hydroxypropyl)carbamic acid *tert*-butyl ester (commercially available) and 2-fluoro-3-hydroxypyridine[47,48] as previously described.[21] ^{1}H NMR (CD_2Cl_2, 298 K): δ: 7.73 (dd, $J = 3.3$ and 1.8 Hz, 1H); 7.32 (td, $J = 7.8$ Hz, $J < 1.5$ Hz, 1H); 7.14 (dd, $J = 7.8$ and 4.8 Hz, 1H); 4.12 (t, $J = 6.0$ Hz, 2H); 3.85 (s, 2 H); 3.49 (q, $J = 6.3$ Hz, 2H); 2.05 (q^5, $J = 6.3$ Hz, 2H). ^{13}C NMR (CD_2Cl_2, 298 K): δ: 166.2 [C]; 153.8 [d, $J^1_{F-C} = 235$ Hz, C]; 142.3 [d, $J^2_{F-C} = 25$ Hz, C]; 137.5 [d, $J^3_{F-C} = 13$ Hz, CH]; 123.0 [CH]; 122.3 [CH]; 67.7 [CH_2]; 37.8 [CH_2]; 29.5 [CH_2]; 28.9 [CH_2]. MS (DCI/NH_4^+) $C_{10}H_{12}Br_1F_1N_2O_2$: 292 [M + H$^+$]; 290 [M + H$^+$].

[3-(3-*tert*-Butoxycarbonylamino-propoxy)-pyridin-2-yl]-trimethyl-ammonium trifluoromethanesulfonate (2). Synthesized in two steps from commercially available (3-hydroxypropyl)carbamic acid *tert*-butyl ester and 2-dimethylamino-3-hydroxypyridine[47,48] as previously described.[21] ^{1}H NMR (CD_2Cl_2, 298 K): δ: 8.10 (bd, $J = 3.3$ Hz, 1H); 7.66 (d, $J = 8.1$ Hz, 1H); 7.60 (dd, $J =$ 6.1 and 4.2 Hz, 1H); 4.31 (t, $J = 6.3$ Hz, 2H); 3.71 (s, 9H); 3.31 (q, $J = 6.3$ Hz, 2H); 2.12 (q^5, $J = 6.3$ Hz, 2H); 1.38 (s, 9H).^{13}C NMR (CD_2Cl_2, 298 K): δ:156.6 [C]; 147.7 [C]; 142.6 [C]; 139.0 [CH]; 129.0 [CH]; 124.6 [CH]; 121.2 [q, $J = 319$ Hz, CF_3]; 79.3 [C]; 68.1 [CH_2]; 54.8 [3 × CH_3]; 37.5 [CH_2]; 30.0 [CH_2]; 28.4 [3 × CH_3].

Conjugation of oligonucleotides with *N*-[3-(2-fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide (FPyBrA, 1). Preparation of c-ON-1 and c-ON-2. *Circa* 90 nmol of oligonucleotide (**ON-1** or **ON-2**, 15 OD (1 OD is the UV absorbance of 38 μg of an oligonucleotide regardless of the sequence of bases)) dissolved in water (100 μL) was mixed with an excess (60 eq.) of *N*-[3-(2-fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide (FPyBrA, **1**) dissolved in 150 μL of methanol and 750 μL of PBS (0.1 M, pH 7.4). The reaction mixture was heated at 80°C for 30 min, concentrated and purified by semi-preparative RP-HPLC (HPLC A). The fraction containing pure conjugated oligonucleotides was collected and concentrated to dryness. The residue was taken up in

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1 mL of water and transferred onto a NAP10® G25 Sephadex column (Amersham Pharmacia Biotech). The conjugated oligonucleotides (**c-ON-1** or **c-ON-2**) were eluted in 1.5 mL of water according to manufacturer's instructions.

c-ON-1: R_t (HPLC A): 9.50 min. MS (MALDI-TOF, positive mode): theor.: 6970.3; exp.: 6973.6. Gel electrophoresis: see Figure 1.
c-ON-2: R_t (HPLC A): 10.50 min. MS (MALDI-TOF, positive mode): theor.: 6992.2; exp.: 6984.5. Gel electrophoresis: see Figure 1.

Duplex formation. Annealing of c-ON-1 with ON-4 or ON-6 and c-ON-2 with ON-5. Preparation of c-DUPLEX-1, c-DUPLEX-2 and c-DUPLEX-3. *Circa* 500 pmol of the conjugated oligonucleotide **c-ON-1** (see above for oligonucleotide solution concentration determination) dissolved in water (< 10 μL) was mixed with a stoechiometric amount of its complementary strand (**ON-4** or **ON-6**, 500 pmol, dissolved in water (10 μL)) and 100 μL of 100 mM aq. NaCl (pH 7.4). The reaction mixture was heated at 90°C for 1 min, and then left at room temperature for another 10 min. The procedure described above was also used with the conjugated oligonucleotide **c-ON-2** and the complementary strand **ON-5**.

c-DUPLEX-1 (**c-ON-1/ON-4**): R_t (HPLC A): 9.60 min. Gel electrophoresis: see Figure 1.
c-DUPLEX-2 (**c-ON-2/ON-5**): R_t (HPLC A): 10.70 min. Gel electrophoresis: see Figure 1.
c-DUPLEX-3 (**c-ON-1/ON-6**): R_t (HPLC A): 9.80 min. Gel electrophoresis: see Figure 1.

Radiochemistry

Phosphorus-32 radiochemistry. *Enzymatic phosphorus-32 5′-labelling of oligonucleotides*: *Circa* 100 pmol of oligonucleotide (**ON-1**, **ON-2**, **c-ON-1** or **c-ON-2**) dissolved in water (10 μL) were mixed with 10 units of T4 PNK enzyme (1 μL, 10 units/μL in the following storage buffer: 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM DTT, 0.1 mM ATP, 0.2 mg/mL BSA, 50% (v/v) glycerol, Invitrogen, France), [γ-^{32}P]ATP (1 μL, 10 μCi see Radioisotope availability/production section), T4 PNK forward buffer 5X (5 μL, composition: 350 mM Tris–HCl (pH 7.6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol, Invitrogen, France) and water (9 μL). The solution was incubated for 10 min at 37°C and then quenched by heating another 10 min at 65°C. Water (25 μL) was added and the mixture was transferred onto a Biospin-30® steric exclusion column (Bio-Rad, France). The [^{32}P]-labelled oligonucleotides were eluted in 50 μL of water according to manufacturer's instructions.

Fluorine-18 radiochemistry. *Preparation of the K[^{18}F]F–K_{222}-complex*: In order to recover and recycle the [^{18}O]water target, 2 mL of aqueous [^{18}F]fluoride ion from the target holder was passed through an anion exchange resin (Sep-pak® Light Waters Accell™ Plus QMA cartridge (chloride form, beforehand washed with aq. 1 M $NaHCO_3$ (2 mL) and rinsed with water (20 mL) and CH_3CN (10 mL)) by helium pressure (1.5–2.0 bar). Helium was blown through the column to maximally extract [^{18}O]water. The [^{18}F]fluoride ion was then eluted from the resin, using an aq. K_2CO_3 solution (1.0 mL of a 4.5 mg/mL solution), into a Vacutainer® tube containing Kryptofix®222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane, 12.0–15.0 mg). The resulting solution was then gently concentrated to dryness at 145–150°C under a nitrogen stream for 10 min to give no-carrier-added K[^{18}F]F–K_{222} complex as a white semi-solid residue.

Preparation of N-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide ([^{18}F]FPyBrA, [^{18}F]-1): DMSO (600 μL) containing [3-(3-*tert*-butoxycarbonylamino-propoxy)-pyridin-2-yl]-trimethyl-ammonium trifluoromethanesulfonate (**2**, 4.0 mg) was directly added into the Vacutainer® tube containing the dried K[^{18}F]F–K_{222} complex. The tube (not sealed) was then thoroughly vortexed (15 s) and then placed in a heating block (at 145°C, for 2 min) without stirring the contents. The reaction vessel was then cooled using an ice-water bath, diluted with water (1 mL) and transferred on the top of a C18 cartridge (PrepSep™ R-C18, Fisher Scientific, beforehand activated with EtOH (2 mL) and then rinsed with water (10 mL)). The tube was rinsed twice with water (1 mL) which was also transferred and added to the diluted reaction mixture on top of the cartridge. After addition of another 2 mL of water, the whole was passed through the cartridge. The cartridge was washed with water (1 mL) and partially dried for 0.5 min by applying a nitrogen stream. The intermediate [3-(2-[^{18}F]fluoropyridin-3-yloxy)propyl]carbamic acid *tert*-butyl ester was eluted from the cartridge with CH_2Cl_2 (3 mL) into a 5 mL reaction vial containing TFA (0.1 mL). Twice 1 mL of CH_2Cl_2 was used to wash the cartridge and to completely transfer the fluorine-18-labelled ester. The resulting CH_2Cl_2/TFA solution (50/1, v/v) was concentrated to dryness (at 65–75°C under a gentle nitrogen stream for 4–6 min) giving the desired 3-(2-[^{18}F]fluoro-pyridin-3-yloxy)-propylamine. This residue was first redissolved in CH_2Cl_2 (2 mL) and concentrated again to dryness to minimize TFA presence (at 65–75°C under a gentle nitrogen stream for

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another 2–3 min), then redissolved in 800 μL of a solution of TEA in CH_2Cl_2 (40 μL/mL or 220 μmol of TEA). After addition of 400 μL of a solution of bromoacetyl bromide in CH_2Cl_2 (50 μL/mL or 220 μmol of $BrCOCH_2Br$), the reaction mixture was allowed to react for 5 min at room temperature with a smooth air-bubbling agitation. Finally, the clear solution was diluted with the HPLC solvent (500 μL) used for the purification and the crude was injected onto a semi-preparative HPLC. Isocratic elution (HPLC B) gave pure labelled *N*-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide ([^{18}F]FPyBrA, [^{18}F]-**1**), R_t: 7.0–8.0 min.

*Conjugation of oligonucleotides with N-[3-(2-[^{18}F]fluoropyridin-3-yloxy)-propyl]-2-bromoacetamide ([^{18}F]FPyBrA, [^{18}F]-1). Preparation of [^{18}F]**c-ON-1** and [^{18}F]**c-ON-2***: To 500 μL of MeOH containing HPLC-purified [^{18}F]FPyBrA ([^{18}F]-**1**, freed from HPLC solvents by concentration to dryness at 65–75°C under a gentle nitrogen stream) was added *circa* 90 nmol of oligonucleotide (**ON-1** or **ON-2**, 15 OD (1 OD is the UV absorbance of 38 μg of an oligonucleotide regardless of the sequence of bases)) dissolved in water (50 μL) and PBS (0.1 M, pH 7.4, 500 μL). The reaction mixture was heated for 10 min at 120°C, partially concentrated (at 65–75°C under a gentle nitrogen stream) and purified by semi-preparative RP-HPLC (HPLC C). The fraction containing the pure labelled conjugated oligonucleotide ([^{18}F]**c-ON-1** or [^{18}F]**c-ON-2**) was collected, concentrated under reduced pressure to a volume of 1 mL and transferred onto a NAP10® G25 Sephadex column (Amersham Pharmacia Biotech). The labelled conjugated oligonucleotide ([^{18}F]**c-ON-1** or [^{18}F]**c-ON-2**) was eluted in 1.5 mL of water according to manufacturer's instructions.

[^{18}F]**c-ON-1**: R_t (HPLC A): 9.50 min. Gel electrophoresis: see Figure 2.
[^{18}F]**c-ON-2**: R_t (HPLC A): 10.50 min. Gel electrophoresis: see Figure 2.

*[^{18}F]Duplex formation. Annealing of [^{18}F]**c-ON-1** with **ON-4** or **ON-6** and [^{18}F]**c-ON-2** with **ON-5**. Preparation of [^{18}F]**c-DUPLEX-1**, [^{18}F]**c-DUPLEX-2** and [^{18}F]**c-DUPLEX-3***: *Circa* 5 nmol of the labelled conjugated oligonucleotide [^{18}F]**c-ON-1** (see above for oligonucleotide solution concentration determination) dissolved in water (1.5 mL) was mixed with a stoechiometric amount of its complementary strand (**ON-4** or **ON-6**, 5 nmol dissolved in water (< 10 μL)) and 375 μL of 500 mM aq. NaCl (pH 7.4). The reaction mixture was heated at 90°C for 1 min, then left at room temperature for another 10 min. The procedure described above was also used with the conjugated oligonucleotide [^{18}F]**c-ON-2** and the complementary strand **ON-5**.

[^{18}F]**c-DUPLEX-1** ([^{18}F]**c-ON-1/ON-4**): R_t (HPLC A): 9.60 min. Gel electrophoresis: see Figure 2.
[^{18}F]**c-DUPLEX-2** ([^{18}F]**c-ON-2/ON-5**): R_t (HPLC A): 10.70 min. Gel electrophoresis: see Figure 2.
[^{18}F]**c-DUPLEX-3** ([^{18}F]**c-ON-1/ON-6**): R_t (HPLC A): 9.80 min. Gel electrophoresis: see Figure 2.

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