Research Article

Fluorine-18 labeling of peptide nucleic acids

Bertrand Kuhnast[1,2], Frédéric Dolle[1,*] and Bertrand Tavitian[1,2]

[1] *Service Hospitalier Frédéric Joliot, Département de Recherche Médicale, CEA/DSV, 4 place du Général Leclerc, F-91401 Orsay, France*
[2] *INSERM ERM 0103, CEA/DSV4 place du Général Leclerc, F-91401 Orsay, France*

Summary

Peptide nucleic acids (PNAs) are a unique class of synthetic macromolecules, originally designed as ligands for the recognition of double stranded DNA. From a chemical point of view, the deoxyribose phosphate backbone of DNA is replaced by a pseudo-peptide *N*-(2-aminoethyl)glycyl backbone, while the nucleobases of DNA (adenine, guanine, cytosine and thymine) are retained. Due to the increasing interest in the labeling of peptide nucleic acids (PNAs) as potent diagnostic agents in nuclear medicine, we have used and adapted the reliable methodology developed for the fluorine-18 labeling of oligonucleotides and have now demonstrated that it is possible to label PNAs in sufficient quantity and with high specific radioactivity for PET studies in a time compatible with the half life of fluorine-18. Copyright © 2002 John Wiley & Sons, Ltd.

Introduction

Peptide nucleic acids (PNAs) are a unique class of synthetic macro-molecules, originally designed as ligands for the recognition of double stranded DNA.[1] In order to mimic an oligonucleotide binding to the double stranded DNA via Hoogsteen base pairing, PNAs were designed from a chemical point of view by replacing the deoxyribose phosphate

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*Correspondence to: F. Dolle, Service Hospitalier Frédéric Joliot, Département de Recherche Médicale, CEA/DSV, 4 place du Général Leclerc, F-91401 Orsay, France.

backbone of DNA by a pseudo-peptide *N*-(2-aminoethyl)glycyl back-
bone, while retaining the nucleobases of DNA (adenine, guanine,
cytosine and thymine).[2]
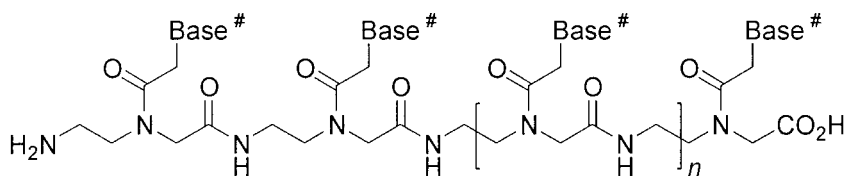
Base :[#] A (Adenine), C (Cytosine), G (Guanine), T (Thymine)

Combining within the same molecule the capacity of nucleic acids to
hybridize to their complementary sequence and the biostability of
pseudopeptides gives PNAs the properties of a powerful molecular tool.
They can be designed with a variety of chemical modifications[3] or
conjugated with carriers[4] and targeting agents,[5] and hold great promise
in an array of applications (reviewed in ref. 2), including several with
therapeutic potential such as antisense-,[6] antigene-,[7] antitumoral-[8] and
antibacterial[5] agents.

The therapeutic potential of PNAs deserves to be evaluated on the
basis of their *in vivo* behavior with adapted techniques. Positron
emission tomography (PET), a high-resolution, sensitive and non-
invasive imaging technique that can be used in humans, is the most
advanced technology currently available for studying *in vivo* molecular
interactions and represents a method of choice to asses the pharmaco-
kinetics of new therapeutic agents such as oligonucleotides or PNAs.[9] We
have recently described a general method for the labeling of oligonucleo-
tides with γ or β^+ emitters for *in vivo* imaging and also demonstrated its
potential for *in vivo* imaging in a primate PET study.[10–12]

We report here that this method can be applied to the labeling of
PNAs with the position emitter fluorine-18 (half-life: 109.8 min).

Results and discussion

Chemistry

The synthetic procedure that we have designed for the labeling of
oligonucleotides is based on the efficient conjugation of oligonucleotides
containing a single phosphorothioate monoester with an electrophilic
moiety such as 2-bromo-*N*-substituted acetamides.[13,14]

N-(4-halobenzyl)-2-bromoacetamide was designed as a universal and radiochemically feasible reagent, the benzyl function offering the opportunity to act as the carrier of various radioactive halogens such as bromine-76 (a positron emitter with a relatively long half life ($T_{1/2}$: 16.1 h)) or iodine-125 (a low energy Auger electron emitter used for high resolution autoradiography, ($T_{1/2}$: 59.9 days)), but also and especially fluorine-18, the most widely used positron emitter ($T_{1/2}$: 109.8 min).[15] Using this labeled reagent, the procedure designed for the labeling of oligonucleotides can be reliably and routinely applied[10,11] to all the popular chemical modifications of oligonucleotides. Besides natural phosphodiester DNA oligodeoxyribonucleotides, full length phosphorothioate diester internucleosidic bond deoxyribonucleotides (the modification most favoured by industry for human antisense therapy[16]) were successfully labeled as well as hybrid methylphosphonate/phosphodiester internucleosidic bond deoxyribonucleotides (a mixed backbone oligonucleotide with interesting potential imaging properties[17,18]) or 2′O-methyl modified oligoribonucleotides (confering resistance to nucleases and high efficiency of duplex formation with the complementary RNA).[19,20]

In order to adapt the methodology used for the preparation of labeled oligonucleotides, a cysteine was added to the CO_2H terminus of the original PNAs to benefit the high and selective reactivity of the nucleophilic thiol function towards 2-bromo-*N*-substituted acetamides. A selected 18mer PNA (**1**, sequence CCCTAACCCTAACCCTAA, bearing a L-cysteine at its CO_2H terminus) was conjugated with *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) using the thiol function.
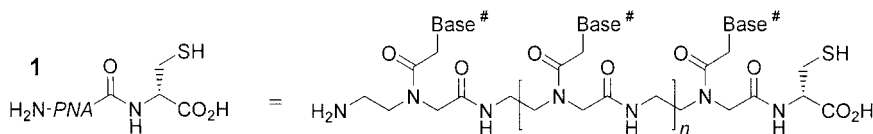
N-(4-Fluorobenzyl)-2-bromoacetamide (**2**) was prepared in 60–65% non-optimized yields from commercially available 4-fluorobenzylamine (**3**) and 2-bromoacetylbromide in the presence of *N*-methyl-morpholine.

The 18mer PNA **1** was then reacted with *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) in a mixture of MeOH and phosphate buffer saline (0.1 M aq., pH 8) for 20 min at 80°C. The modified PNA was purified by semi-preparative reverse-phase HPLC using a dedicated Poros® column and was characterized by mass spectrometry analysis. Analytical data
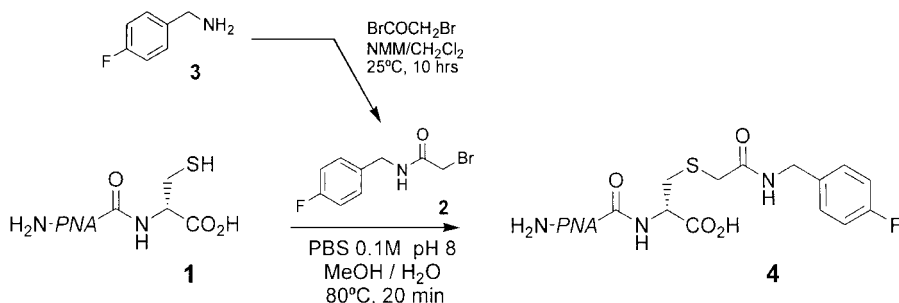
were in accordance with the expected structure (MS (electrospray): 4998.9 (theoretical), 4999.2 (experimental)).

Radiochemistry

N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(**2**), was synthesized in 3 steps from 4 cyano-N,N,N-trimethylanilinium trifluoromethane-sulfonate (**5**, prepared from commercial 4-dimethylaminobenzonitrile). The first radiochemical step, the introduction of fluorine-18 into the benzonitrile ring, was performed in hot DMSO, using the K[^{18}F]F-K$_{222}$ complex by microwaves activation at 100 W for 1 min. The desired [^{18}F]fluorobenzonitrile (**6**) was obtained in 80–85% yields.

   The second step, the reduction of the cyano function, was performed with LiAlH$_4$ in refluxing THF (140°C) for 2 min, giving the desired labeled 4-fluorobenzylamine ([^{18}F]-**3**). The final step, the condensation with bromoacetyl bromide, occurred cleanly in 2 min at room temperature in a 10/1 (v/v) mixture of CH$_2$Cl$_2$/H$_2$O. Semi-preparative HPLC gave pure N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide [^{18}F]-(**2**). (Co-elution with authentic synthesized unlabeled reference compound) Typically, 60–90 mCi (2.2–3.3 GBq) of pure N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) could be obtained in 85–95 min starting from a 550–650 mCi (20.3–24.0 GBq) aliquot of a cyclotron [^{18}F]F$^-$ produc-tion batch (9–16% non decay-corrected yield based on the starting [^{18}F]fluoride).

   The conjugation reaction of PNA **1** with N-(4-[^{18}F])fluorobenzyl)-2-bromoacetamide ([^{18}F]-(**2**)) occurred in 10 min at 120°C in a mixture of MeOH and phosphate buffer saline (0.1 M aq., pH 8). Starting from a 10 mCi (740 MBq) aliquot of radiosynthesized [^{18}F]-(**2**), 2 mCi (74 MBq) of labeled 18mer PNA [^{18}F]-(**4**) could be isolated after the purification by reverse-phase HPLC. Finally, pure labeled 18mer PNA was desalted
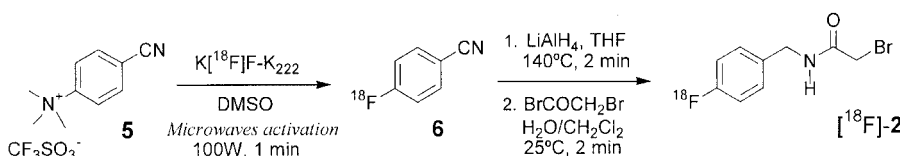
and formulated for *in vivo* injection on an NAP-10® G25 Sephadex Column (Amersham Pharmacia Biotech). The whole fluorine-18 labeling procedure lasted 180 min. Specific radioactivity for the labeled PNA [^{18}F]-(**4**) was about 1 Ci/µmol (37 GBq/µmol) at the end of the procedure.
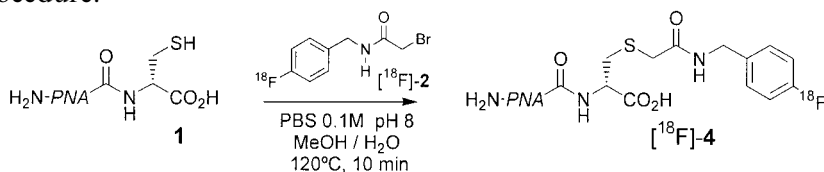
As a control, an aliquot of the radioactive probe was co-injected with unlabeled reference onto analytical reverse-phase-HPLC. Radioactivity and UV detection demonstrated that [^{18}F]-labeled PNA ([^{18}F]-**4**) eluted at the same retention time as authentic synthetized unlabeled reference compounds (**4**).

Experimental

General

Chemicals. Chemicals were purchased from Aldrich, Fluke, Sigma and ICN Biochemicals and were used without further purification. The CO_2H-terminus cysteine-derived 18mer PNA (^{NH_2}CCCTAACCCTAA CCCTAA-Cys^{CO_2H}) was purchased from Perseptive Biosystems (France).

Analytical methods. Thin layer chromatography (TLC) was run on precoated plates of silica gel 60F$_{254}$ (Merk). The compounds were localized at 254 nm using a UV lamp. Flash chromatography was conducted on silica gel 63–200 µm (Merck) at 0.3 bar (compressed air). HPLC systems: HPLC **A**: semi-preparative RP-HPLC: column Poros® R2/H (100 × 4.6 mm), 600 Controller Gradient system Waters, UV detector (254 nm) multiwavelength 490E Waters and Geiger–Müller detector; solvents: 1% aq. TFA and acetonitrile; HPLC **B**: semi-

preparative normal phase HPLC: column Prep Nova-Pak® HR Silica Waters (300 × 7.8 mm, 60 Å, 6 μm), UV detector Waters, a Geiger–Müller detector; solvents: CH_2Cl_2/EtOAc (95/5); HPLC **C**: analytical RP-HPLC: column Poros® R2/H (100 × 2.1 mm), a 600 Pump and a 600 Controller Waters, a UV detector Series 1100 (254 nm) Hewlett Packard and a Flow One Scintillation Analyzer Packard equipped with a positron-dedicated cell for radioactivity monitoring; solvents: 1% aq. TFA and acetonitrile. 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 were reported in ppm, downfield from TMS (^{1}H and ^{13}C) or TMP (^{31}P) (s, d, t, q, dd, m, b for singlet, doublet, triplet, quadruplet, doublet of doublet, multiplet (or multi sharp-peak system) and broad, respectively). The mass spectra were measured on a Nermag R10-10 apparatus and a Quattro VG (Fison, Manchester, UK) for electrospray ionization (negative mode).

Miscellaneous. Radiosyntheses using fluorine-18 were performed in a 7.5 cm-lead shielded cell using a computer assisted Zymate robot system (Zymark corporation, USA). Microwave activation was performed with a MicroWell 10 oven (2.45 GHz), Labwell AB, Sweden.

Isotope availability. No-carrier-added aqueous [^{18}F]fluoride ion was produced on a CGR-MeV 520 cyclotron by irradiation of a 2 ml water target using a 17 MeV proton beam on 95% enriched [^{18}O]water by the [^{18}O(p,n)^{18}F] nuclear reaction and was transferred to the appropriate hot cell. Typical production: 550–650 mCi (20.3–24.0 GBq) of [^{18}F]F$^-$ at the end of bombardment for a 20 μA, 30 min (36,000 μC) irradiation. A complete description of the target hardware and operation can be found in ref. 21.

Chemistry

N-(4-fluorobenzyl)-2-bromoacetamide (**2**). Synthesized from commercially available 4-fluorobenzylamine according to ref. 10. Rf (heptane/EtOAc: 50/50): 0.35. ^{1}H NMR (DMSO-d_6, 300.0 K): δ: 8.80 (bt, 1H); 7.32 (dd, *J*: 8.1 and 5.70 Hz, 2H); 7.15 (t, *J*: 8.1 Hz, 2H); 4.31 (d, *J*: 6 Hz 2H); 3.92 (s, 2H). ^{13}C NMR (DMSO-d_6, 300 K): δ: 166.1 [C]; 161.3 [C,

d, J: 249 Hz]; 135.0 [C, d, J: 2 Hz]; 129.2 [CH, d, J: 8 Hz]; 115.1 [CH, d, J: 23 Hz]; 41.9 [CH$_2$]; 29.4 [CH$_2$]. MS: 265 [M + NH$_4^+$]; 263[M + NH$_4^+$]; 248 [M + H$^+$]; 246 [M + H$^+$].

4 -Cyano-N,N,N-trimethylanilinium trifluoromethanesulfonate (**5**). Synthesized from commercially available 4-dimethylaminobenzonitrile according to ref. 10. Rf (MeOH/AcOH: 50/50): 0.4. ^{1}H NMR (DMSO-d$_6$, 338.0 K): δ: 8.30–8.00 (2b, 4H); 3.70 (s, 9H). ^{13}C NMR (DMSO-d$_6$, 338.0 K): δ: 150.0 [C]; 133.9 [CH]; 121.8 [CH]; 120.6 [CF$_3$, q, J: 325 Hz]; 116.9 [C]; 113.2 [C]; 56.4 [CH$_3$].

N-(4-fluorobenzyl)-2-(^{NH_2}CCCTAACCCTAACCCTAA-Cys^{CO_2H})-acetamide (**4**). 5 OD (1 OD is the UV absorbance of 33 μg of an oligonucleotide regardless of the sequence of bases) of the 18mer PNA (**1**) were reacted with an excess (3 eq) of *N*-(4-fluorobenzyl)-2-bromoacetamide (**2**) in 1.0 ml of a 1/1 mixture of methanol and phosphate buffer saline (0.1 M aq., pH 8) (v/v) for 20 min at 80°C. The solvents were evaporated and the conjugated PNA **4** was purified by RP-HPLC to give *N*-(4-fluorobenzyl)-2-(^{NH_2}CCCTAACCC-TAACCCTAA-Cys^{CO_2H})-acetamide (**4**) in a yield of 85% (determined by HPLC). HPLC A: gradient elution: linear in 30 min from 99/1 to 60/40 (1% aq. TFA/acetonitrile); flow rate: 6.0 ml/min; retention time 15.2–15.5 min. HPLC C: gradient elution: linear in 30 min from 99/1 to 60/40. (1% aq. TFA/acetonitrile); flow rate: 1.5 ml/min; retention time 15.0 min. MS (electrospray): 4998.9 (theoretical), 4999.2 (experimental).

Radiochemistry

K[^{18}F]F-K$_{222}$-complex. In order to recover and recycle the [^{18}O] water target, the 2 ml of aqueous [^{18}F]fluoride from the target was passed through an anion exchange resin (Sep-pak Plus "light" cartridge, Waters). See ref. 22 for more practical details. The [^{18}F]fluoride ion was then eluted from the resin using 1.0 ml of a 4.5 mg/ml aqueous K$_2$CO$_3$ solution. After an addition of 11.0–15.0 mg of Kryptofix$^®$ K$_{222}$ (4, 7, 13, 16, 21, 24-hexaoxa-1, 10-diazabicyclo[8.8.8]hexacosane), 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.

N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-2). Freshly distilled DMSO (600 μl) containing 8 mg of the labeling precursor (the triflate

salt of 4-trimethylammoniumbenzonitrile (**5**)) was directly added into the tube containing the dried K[^{18}F]F-K$_{222}$ complex. The tube (not sealed) was placed in a microwave oven. Microwaves, 100 W, were applied to the system for 1 min. The resulting yellow-brown reaction mixture was diluted with 1 ml of water and transferred on a C18 Seppak cartridge (Waters). The tube was rinsed twice with 1 ml of water which was also transferred and added to the diluted reaction mixture on the cartridge. The whole mixture was then passed through the cartridge. The cartridge was washed with 1 ml of water and partially dried for 3 min by applying a nitrogen stream. 4-[^{18}F]Fluorobenzonitrile was eluted with 3 ml of THF onto a column containing 1.0 g of oven-dried 4 Å ground molecular sieve and the cartridge was washed twice with 1 ml of THF. The mentioned THF solution (5 ml) was left on the molecular sieve column for 3 min and then eluted into a reaction vial containing 20 mg of dry powdered LiA1H$_4$. 1 ml of THF was used to wash the column for another 3 times and to completely transfer the 4-[^{18}F]fluorobenzonitrile. The vessel was then tightly closed and heated for 2 min at 140°C (strong reflux). The resulting gray suspension was cooled for 2 min using an ice/water bath and concentrated to dryness under a nitrogen stream. The amine–aluminium complex as well as the excess of LiA1H$_4$ were destroyed by adding 300 μl of H$_2$O. The white aqueous residue was then diluted with 3 ml of CH$_2$Cl$_2$. After an addition of 1 ml of a solution of bromoacetyl bromide in CH$_2$Cl$_2$ (10 μl/ml or 114 μmoles of BrCOCH$_2$Br), the white milky suspension was allowed to react 1 min at room temperature with a smooth air-bubbling agitation. The reaction mixture was then filtered on cotton and the precipitate washed twice with 1 ml of CH$_2$Cl$_2$. The filtrate was concentrated to dryness at 80°C under a nitrogen stream, the residue dissolved in 1 ml of the HPLC solvent was used for purification and the crude was injected onto a SiO$_2$ semi-preparative HPLC. HPLC B: isocratic elution: flow rate: 5 ml/min, RT: 10.0–10.5 min.

Typically, 60–90 mCi (2.2–3.3 GBq) of pure *N*-4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F-**2**]) could be obtained in 85–95 min starting from a 550–650 mCi (20.3–24.0 GBq) aliquot of a cyclotron [^{18}F]F$^-$ production batch.

N-(4-[^{18}F]fluorobenzyl)-2-(^{NH_2} CCCTAACCCTAACCCTAA-Cys^{CO_2H})-acetamide ([^{18}F]-**4**). The HPLC-collected fraction containing *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) was concentrated to dryness at 80°C under a nitrogen stream and then diluted with 0.5 ml

of MeOH. 10 OD of the 18mer PNA (**1**) in 0.5 ml of water and 0.1 ml of phosphate buffer saline (0.1 M aq., pH 8) were successively added. The reaction vial was then tightly sealed with a Teflon cap and heated in a heating block without stirring at 120°C for 10 min. After cooling for 2 min using an ice/water bath, the reaction mixture was concentrated at 80°C under a nitrogen stream. Labeled PNA [^{18}F]-**4** was separated from unlabeled PNA (**1**) and unreacted *N*-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide ([^{18}F]-**2**) by RP-HPLC using the same conditions as for the purification of the cold reference 18mer PNA **4**. HPLC A: gradient elution: linear in 30 min from 99/1 to 60/40 (1% aq. TFA/acetonitrile see *analytical methods*); flow rate: 6.0 ml/min; retention time 15.2–15.5 min. Finally, the HPLC-fraction containing the labeled PNA was concentrated and transferred in a volume of 1 ml onto a NAP-10® G25 Sephadex Column (Amersham Pharmacia Biotech). Pure labeled and desalted PNA *N*-(4-[^{18}F]fluorobenzyl)-2-(^{NH_2}CCCTAACCCTAA-CCCTAA-Cys^{CO_2H})-acetamide ([^{18}F]-**4**) was eluted with 1.5 ml of water according to manufacturer's instructions.

Labeled synthesized PNA [^{18}F]-**4** co-elutes with authentic systhesized unlabeled reference compound **4**. HPLC C: gradient elution: linear in 30 min from 99/1 to 60/40 (1% aq. TFA/acetonitrile); flow rate: 1.5 ml/min; retention time 15.0 min.

Conclusion

Due to the increasing interest in the labeling of peptide nucleic acids (PNAs), for use as potent diagnostic agents in nuclear medicine, we have adapted and used the reliable methodology developed for the fluorine-18 labeling of oligonucleotides. We have now demonstrated that it is possible to label PNAs in sufficient quantity and with high specific radioactivity for PET studies in a time compatible with the half life of fluorine-18.

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