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Research Article

Photoconjugation of 3-azido-5-nitrobenzyl-[^{18}F]fluoride to an oligonucleotide aptamer

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Summary

Photoconjugation techniques are well established in biochemistry and molecular biology applications. We report here the first application of photoconjugation to label an oligonucleotide with positron emitting fluorine-18. ANBF, 3-azido-5-nitrobenzyl fluoride, was tagged with fluorine-18 in 30–50% yield from the corresponding tosylate. The HPLC purified [^{18}F]ANBF was photochemically conjugated to a hexylamine modified 15-base, single stranded, DNA aptamer with up to 20% radiolabeling efficiency, based on starting [^{18}F]ANBF. Separation by gel exclusion chromatography and HPLC analysis of the DNA fractions indicated that the benzyl fluoride was covalently bound to the aptamer. The total reaction time from the start of radiosynthesis was 135 min. Based on these results, the feasibility to photolabel oligonucleotide molecules with fluorine-18 labeled arylazides for PET applications has been established. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: fluorine-18; PET; photoconjugation; phenylazide; aptamer

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Introduction

The application of DNA and RNA oligonucleotides as molecular therapeutic agents is maturing rapidly. Likewise, the utilization of radiolabeled DNA, RNA or oligonucleotide probes for diagnostic imaging or evaluation of new therapeutic agents is an area of current intense interest. Oligonucleotides labeled with beta-emitting radioisotopes such as tritium, phosphorous-32 and sulfur-35 are well-established tools in cell and molecular biology. Methods for single photon (technetium-99m, indium-111, iodine-125) labeling of oligonucleotides were developed in the 1990s.[1–4] These methods are largely based on the labeling strategies designed for proteins and peptides.[5] More recently, fluorine-18 labeled DNA/RNA based molecules for quantitative imaging using positron emission tomography (PET) have been reported.[6–10] Most attractive for labeling with short-lived positron emitters are the small (15–50 mer) single-stranded DNA/RNA oligonucleotides, called aptamers, that are engineered with high target specificity and to possess favorable pharmacokinetic profiles.[11–13]

One reported strategy for fluorine-18 labeling of oligonucleotides is based on the two-step synthesis of N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide followed by S-conjugation to a 3′-phosphorotioate oligonucleotide.[9] This procedure, like many of the fluorine-18 labeling schemes for tagging proteins or peptides, is labor-intensive and time-consuming. We report here on our progress towards a two-step oligonucleotide labeling method based on photochemical conjugation of a fluorine-18 labeled molecule to a 5′-amino modified 15-mer. This approach is modeled after photoaffinity labeling reagents[14] that have found great utility in biochemistry and molecular biology applications such as tagging proteins, enzymes and receptors with fluorescent dyes or radioiodine. Initial studies towards photoiodination of monoclonal antibodies were described by Pandey *et al.*[15] Similarly, two photo-conjugation reactions for fluorine-18 tagging of proteins using 4-azidophenacyl-[^{18}F]fluoride[16] and [^{18}F]-3,5-difluorophenyl azide[17] have been reported. These bioconjugates are compared to the new azido compound described herein.

Results and discussion

Preparation of 3-azido-5-nitro-benzylfluoride (ANBF) and [18F]ANBF

The synthetic scheme for the production of the fluorine containing arylazide photolabeling reagent is shown in Figure 1. The nitro analog was chosen to facilitate photoconjugation at higher wavelengths to reduce potential UV damage to the oligonucleotide. Partial reduction of the dinitrobenzyl alcohol (**1**) was achieved with ammonium sulfide following a literature procedure[18] yielding the aniline (**2**). Diazotization of (**2**) followed by displacement with azide gave (**3**) in modest yield. Direct fluorination with (diethylamino)sulfur trifluoride gave the non-radioactive 3-azido-5-nitro-benzylfluoride (**4**). The precursor for radio-fluorination (**5**) was prepared by tosylation of the benzyl alcohol (**3**).

The one-step reaction to produce the fluorine-18 labeled ANBF is also shown in Figure 1. Tetrabutylammonium [18F]fluoride was prepared from the cyclotron-produced aqueous [18F]fluoride and was resolubilized in anhydrous acetonitrile. The acetonitrile solution was added to the tosylate (**5**) and the mixture was heated at 80°C for 5–10 min. Following reversed-phase semi-preparative HPLC purification, [18F]ANBF (**6**) was obtained in 30–50% decay corrected yield 40–50 min after the start of the synthesis. HPLC analysis confirmed that [18F]ANBF co-eluted with unlabeled ANBF (**4**).

The [18F]ANBF yields were superior to the 0–12% production of fluoroarylazides by ^{18}F-for-^{19}F exchange described by Hashizume *et al.*[17] and modest compared to the production of 4-azidophenacyl-[18F]fluoride by Wester *et al.*[16] in 71% radiochemical yield 15 min into

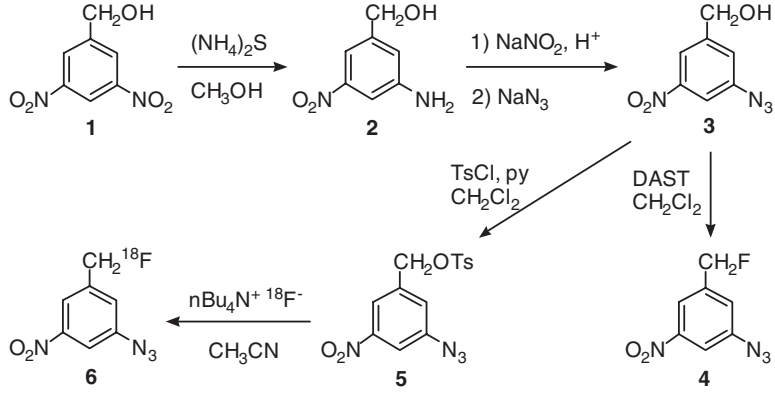

Figure 1. Synthesis of [19F/18F] 3-amino-5-nitro-benzylfluoride (ANBF)

the synthesis. The choice of either the tosylate leaving group or the position of the label may have contributed to our modest radiofluorination yields.

Photoconjugation of 3-azido-5-nitro-benzyl[^{18}F]fluoride ([^{18}F]ANBF) with an amino modified oligonucleotide

Commercially synthesized thrombin aptamer modified at the 5′-terminus with a hexylamine provided a primary amine target for interaction with the photolabeling reagent as shown in Figure 2. Based on the previous accounts, heptoazocyclotetraene.[16,19,20] of arylnitrene chemistry the proposed structures for the labeled oligonucleotide (**1**) is shown in Figure 2. Photolysis of the arylnitrene promotes ring expansion to the heptoazocyclotetraene. The hexylamine acts as a nucleophile and reacts with the intermediate to form a covalent bond. This is analogous to the case in protein labeling where the terminal amine of lysine forms a covalent bond with the heptoazocyclotetraene

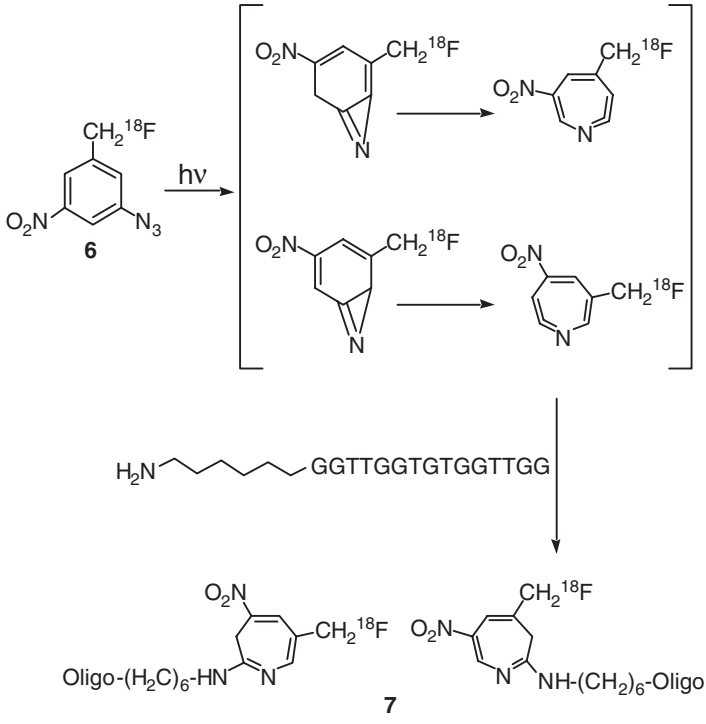

Figure 2. Synthesis of the radiofluorinated aptamer

intermediate. Given the substitution pattern on the phenyl ring it is possible to form the two species as shown in Figure 2. No attempt was made to determine if one or both of the positional isomers constitute the final product.

Purified [18F]ANBF was extracted from the HPLC solvent using a C18 SepPak® and eluted in methanol. The methanol was evaporated and the aptamer, in a buffer solution, was added to the [18F]ANBF residue. The photolysis was carried out at 0°C for 10 min using a 275 W mercury lamp. Purification and analysis of the labeled aptamer was performed using gel exclusion chromatography and reversed-phase HPLC. The specific activities of the intermediate [18F]ANBF and the labeled aptamer were not measured.

The labeled aptamer and free [18F]ANBF were easily separated by gel exclusion chromatography. An eluent profile is shown in Figure 3. The labeled aptamer eluted shortly after the blue dextran void volume marker band at about 40% of the column volume with the radioactivity and DNA absorbance peaks coinciding. Free ANBF was retained by the column and eluted at 80–90% of the column volume. The photolabeling efficiency ranged from 7 to 20% (based on starting ANBF), increasing linearly ($R^2 = 0.949$) with increasing DNA concentration (see Figure 4). These yields compare favorably with the 15–30% protein photolabeling yields reported by Wester *et al.*[16] and exceeds the 7% yield achieved by Hashizume *et al.*[17] Wester found that the labeling efficiency increased as a function of the number of lysines and terminal amino groups in the protein being labeled. While the radiochemical

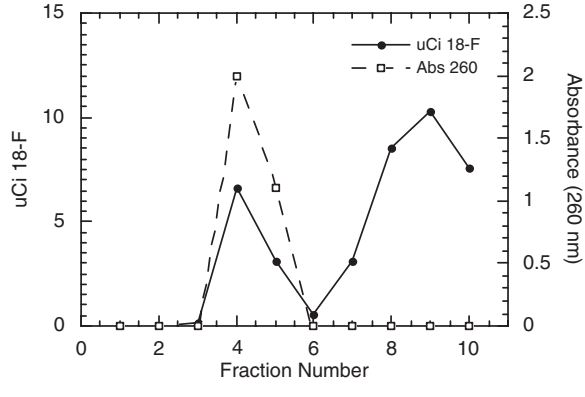

Figure 3. Gel exclusion chromatogram demonstrating separation of the labeled aptamer and free [18F]ANBF

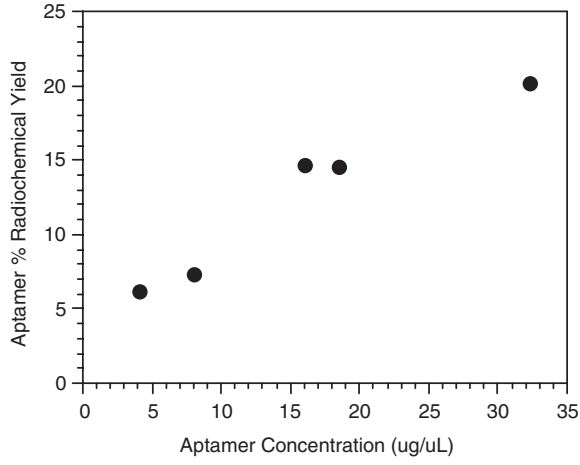

Figure 4. Radiochemical yield, based on the starting [^{18}F]ANBF, of the labeled aptamer as a function of aptamer concentration

yield plateau was never reached in our study (Figure 4), we achieved a comparable yield with far less amino density. Further enhancement of the photolabeling efficiency might be possible at higher oligonucleotide concentrations.

The labeled aptamer was analyzed by radio-HPLC. A gradient solvent system was used to elute the aptamer from the C18 column. The radioactive peak co-eluted with the UV active oligonucleotide peak. The retention time for free [^{18}F]ANBF was nearly 30 min longer than the labeled aptamer. The results from the size exclusion chromatography and the analytical HPLC indicate that the benzyl fluoride was covalently bound to the aptamer. Additionally, there was no evidence on the HPLC of aptamer breakdown as a result of the photochemical reaction.

The specificity of the ANBF for the amine on the 5′-terminus was indicated by the inability of the ANBF to bind to a oligonucleotide with the same sequence but constructed without the 5′-hexyl amine modification. The covalent nature of the bond was tested with [^{18}F]ANBF added in excess to the reaction, and then treated with high levels of lysine (3 M) after completion of the photoconjugation reaction in an attempt to displace any charged reaction products off the poly-anionic backbone of the oligonucleotide molecule. An HPLC comparison of the ANBF–aptamer complex in the presence and absence of lysine showed that there was no change in either the retention or

spectral characteristics of the ANBF–aptamer product, strongly suggesting that the association between the aptamer and labeling agent was not an ionic interaction.

Experimental

Materials and methods

All chemicals and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI), with the exception of HPLC grade CH_3CN and MeOH from J.T. Baker (Phillipsburg, NJ) and were used without further purification. Flash chromatography was performed using silica gel (60 Å, 230–400 mesh) from Aldrich. Melting points were taken on a Mel-Temp^{TM} apparatus and are reported uncorrected. Proton magnetic resonance spectra were obtained on a Bruker AMX 300 instrument at 300 MHz; chemical shifts are referenced to tetramethylsilane. Purification of the radiolabeled compounds was carried out by HPLC (column and conditions noted below) with a Waters 560 pump and an in-line Linear^{TM} UV-106 spectrophotometer (254 nm) to detect mass and a NaI detector and Ortec NIM components to measure radioactivity, or on a Thermo Separation Products 4000 HPLC system equipped with a photo diode array (PDA) detector and a column oven.

3-Amino-5-nitrobenzyl alcohol **2** was prepared by reduction of 3,5-dinitrobenzyl alcohol with ammonium sulfide according to the procedure of Meindl *et al.*[18]

3-Azido-5-nitrobenzyl alcohol (3)

The following reaction was carried out with stirring under reduced light. In a 100 ml flask, 3-amino-5-nitrobenzyl alcohol (0.97 g, 5.8 mmol) was added to 12 ml 12 N hydrochloric acid to form a slurry. The mixture was chilled in an ethylene glycol/dry ice bath (−15°C). Acetic acid (10 ml) was added, followed by careful addition of sodium nitrite (0.64 g, 9.3 mmol) dissolved in 3 ml of water. The temperature was not allowed to rise above 10°C. The mixture was stirred for 30 min. Sodium azide (0.65 g, 10 mmol) was dissolved in 3 ml water and added dropwise to the reaction mixture. The mixture was gradually allowed to warm to room temperature following the addition of 15 ml of water. A light tan precipitate was collected by filtration and washed with water. After

drying *in vacuo*, 0.71 g (64%) of tan powder was recovered: mp 104°C. ^{1}H NMR (CD_3OD): δ 4.70 (s, 2H, CH_2OH), 7.44, 7.74, 8.00 (m, 3H, *aromatic*) HRMS(EI): $C_7H_6N_4O_3$ calcd: 194.043990 found: 194.044419.

3-Azido-5-nitro-benzylfluoride (4)

Alcohol **3** (0.26 g, 1.3 mmol) was dissolved in 12 ml dichloromethane. An excess of (diethylamino)sulfur trifluoride (DAST) (0.72 g, 4.5 mmol) was dissolved in 4 ml dichloromethane and cooled in a dry ice/ethylene glycol bath (−15°C). The alcohol solution was added to the cooled DAST solution over the course of 10 min. The mixture was allowed to warm to room temperature and stirred for an additional 30 min. The reaction mixture was poured over 3 g of ice. The organic layer was separated. The aqueous layer was extracted with 2×5 ml portions of dichloromethane. The combined organic layers were washed with 1 ml water and dried over magnesium sulfate. After filtration the solvent was removed *in vacuo*. The crystalline residue, recrystallized from dichloromethane/hexane, gave 110 mg (52%) of light brown crystals: mp 66–67° C. ^{1}H NMR ($CDCl_3$): δ 5.41 (d, 2H, CH_2F, $J = 47$ Hz), 7.27, 7.80, 7.91 (m, 3H, *aromatic*) HRMS(EI): $C_7H_5FN_4O_2$ calcd: 196.039654 found: 196.040194.

3-Azido-5-nitrobenzyl tosylate (5)

Alcohol **3** (0.25 g, 1.3 mmol) and pyridine (0.60 ml, 7.4 mmol) were mixed in 10 ml dichloromethane and chilled in an ice bath. *p*-Toluenesulfonyl chloride (1.11 g, 5.8 mmol) was dissolved in 10 ml dichloromethane and added to the stirring alcohol solution via a syringe. The reaction mixture was placed in the freezer and allowed to stand for 2 days. The mixture was quenched by adding to 100 ml of water and extracted with dichloromethane. After removal of solvent by rotary evaporation, the oily residue was purified by flash chromatography (20% ethyl acetate/hexane). Removal of solvent gave 0.26 g (58%) of a viscous oil which crystallized on standing. A portion of this material was additionally purified by recrystallization (ethyl acetate/hexane) to give a light yellow crystalline solid: mp 73–74°C. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, Ar-CH_3), 5.11 (s, 2H, CH_2OTs), 7.22 (m, 1H, *aromatic*), 7.35–7.38, (m, 2H, *aromatic*), 7.79–7.82 (m, 4H, *aromatic*). HRMS(FAB/EI): $C_{14}H_{12}N_4O_5S$ calcd: 348.052842 found: 348.052949.

Production of [^{18}F]-fluoride

[^{18}F]fluoride ion was produced via the $^{18}O(p,n)^{18}F$ reaction by irradiation of ^{18}O-enriched water with a 11 MeV proton beam using the Center for Functional Imaging CTI RDS 111 cyclotron.

3-Azido-5-nitrobenzyl-[^{18}F]-fluoride (6)

The aqueous [^{18}F]-fluoride was added to a vacutainer containing 2 µl of tetrabutylammonium hydroxide (1 M in water). The water was azeotropically removed with acetonitrile under a gentle stream of nitrogen. The residue was dissolved in acetonitrile (2×150 µl) and transferred to a vial containing the tosylate precursor (**5**) (1 mg, 2.9 mmol). The mixture was heated to 80°C for 5–10 min. Water (300 µl) was added and the entire solution was injected onto a reversed-phase HPLC column (Phenomenex Bondclone-10 C18 column, 3.9×300 mm, mobile phase: 50% CH_3CN/H_2O, flow rate: 1 ml min^{-1}) for purification. [^{18}F]ANBF (**6**) was separated ($R_t = 15$ min) from unreacted tosylate (**5**) ($R_t = 32$ min). The [^{18}F]ANBF fraction was passed through an activated C18 SepPak® and the [^{18}F]ANBF was eluted with methanol (1 ml). The methanol was then evaporated leaving the [^{18}F]ANBF residue. Purified [^{18}F]ANBF was obtained in 40–50 min with decay corrected yields ranging from 30 to 50% from the starting [^{18}F]fluoride ion.

Photoconjugation of [^{18}F]ANBF to the aptamer (7)

All aqueous reaction solutions and buffers used with the aptamer were prepared with DNAse-free water (Sigma). The thrombin aptamer was commercially prepared (Genset Corp) as the single-stranded native deoxyribonucleotide oligomer of the sequence GGTTGGTGT-GGTTGG.[13] The 5′-terminus was modified by the attachment of a hexylamine linker. Immediately prior to use the aptamer was diluted to the desired final concentration using water and the appropriate amount of potassium phosphate buffer to obtain a final buffer concentration of 10 mM (pH 7.4). The aptamer in 0.11 ml of buffer was added to the vial containing the dried [^{18}F]ANBF (**6**) and mixed thoroughly. The photolysis was conducted at 0°C with a 275 W mercury lamp (General Electric Model RSM 275; emission max = 297 nm) for 10 min. The reaction vial was kept 10 cm from the lamp surface, and the reaction was

mixed with a magnetic stirrer. The aptamer was purified by gel exclusion chromatography. The reaction mixture was loaded directly onto a Exocellulose GF-5 column (Pierce; MW exclusion limit = 5000) and was eluted with water. A couple of drops of a blue dextran solution (Pharmacia) was added to visualize the void volume. Fractions (0.5 ml) were collected and the absorbance was measured at 260 nm on a Perkin Elmer Lambda 3 UV/VIS spectrophotometer and ^{18}F activity was determined using a Capintec CRC30 dose calibrator. Purified labeled aptamer was obtained in 1–7% decay corrected yield (based on starting [^{18}F]fluoride ion), 135–150 min from the start of synthesis.

The specific binding of the ANBF to the hexylamine bridging group was examined by repeating the above reactions with an aptamer that without the amine group attached to the terminus.

The specificity of ANBF for the amine moiety on the 5′-terminus was further examined using non-radioactive ANBF. The reaction was carried out under the same conditions as with the radiolabeled ANBF, except a 10-fold excess of the ANBF was added to the reaction. Following photoconjugation, the reaction was split into two aliquots, and lysine was added to one of the aliquots to a final concentration of 3 M. HPLC was then carried out to determine if the conjugated ANBF remained with the aptamer or was displaced by the lysine.

HPLC Conditions

HPLC analysis of the labeled aptamer was carried out using two different methods. In the first, a Nova-Pak C-18 3.9×300 mm column (Waters) was used. A linear gradient of 10% CH_3CN/0.1 M TEA to 50% CH_3CN/0.1 M TEA was used at a rate of 1 ml min^{-1}. The eluent was analyzed by UV absorbance and radioactivity detection. The retention time of the labeled aptamer was 10–11 min, while ANBF was retained for 43 min by this system. The second system utilized a 4×250 mm PA100 DNAPac column (Dionex). A linear gradient of 25 mM Tris (pH 8.0)/200 mM NH_4Cl/1% acetonitrile to 25 mM Tris (pH 8.0)/800 mM NH_4Cl/1% acetonitrile was used at 1.5 ml min^{-1}. The column was maintained at 36°C. Eluent was monitored with a PDA detector over a 210–600 nm wavelength range. The aptamer was retained for 10 min, the ANBF–aptamer conjugate for 7.5 min, and free ANBF (both prior and after exposure to UV light) eluted with the solvent front.

Conclusion

We have prepared a positron labeled arylazide [18F]ANBF and demonstrated the ability to couple the labeled intermediate with an amine modified DNA aptamer. The best overall non-decay corrected yield for the entire reaction sequence was ~3% at 135 min. While improvement in either the fluorination and/or photolabeling yields are necessary for this labeling strategy to find widespread applicability, the feasibility of photolabeling oligonucleotide molecules with a fluorine-18 labeled arylazide has been established.

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