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The radiolabeling of proteins by the [^{18}F]AIF method

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Abstract

A new (^{18}F)AIF²⁺-binding ligand that contains 1,4,7-triazacyclononane-1,4-diacetate (NODA) attached to a methyl phenylacetic acid group (MPA) was conjugated to *N*-(2-aminoethyl)maleimide (EM) to form NODA-MPAEM. The NODA-MPAEM was labeled with (^{18}F)AIF²⁺ at 105 °C in 49–82% yield and conjugated at room temperature to an antibody Fab' fragment in 69–80% yield (total time ~ 50 min) and with retention of immunoreactivity. These data indicate that the rapid and simple [^{18}F]AIF-labeling method can be easily adapted for preparing heat-sensitive compounds with ^{18}F quickly and in high yields.

Keywords

aluminum fluoride; antibody; CEA; ^{18}F fluorine; radiolabeling; PET

1. Introduction

^{18}F -labeling of proteins has been performed usually in a multistep process by first labeling a prosthetic group and conjugating that to the protein, which can take several hours (Miller et al., 2008; Schirmacher et al., 2007; Wester et al., 2007; Day et al., 2010; McCabe et al., 2010). Recent improvements have led to simpler and automated methods that can be performed more rapidly. Most often, $^{18}\text{F}^-$ is bound to the para-position of benzaldehyde or benzoic acid, and the prosthetic group is then activated to attach the label to the side-chain of lysine, an activated C-terminus, or a thiol on the protein. Other ^{18}F -labeled groups, such as fluorodeoxyglucose (^{18}F)FDG can be attached in a similar manner (Cai et al., 2006; Cai et al., 2007; Flavell et al., 2008; Wuest et al., 2008; Wuest et al., 2009; Bejot et al., 2011). Another promising approach is the radiolabeling of a silicon-containing prosthetic group, which can be achieved in good yield under mild conditions and then conjugated to a peptide or protein (Schirmacher et al., 2007; Mu et al., 2008; Iokova et al., 2009; Rosa-Neto et al., 2009; Wangler et al., 2008).

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We have pursued a method that captures a $([^{18}\text{F}]\text{AlF})^{2+}$ complex, using a NOTA-derived ligand bound on a peptide, and showed these labeled peptides were stable *in vivo* and retained their binding abilities (McBride et al., 2009; McBride et al., 2010; Laverman et al., 2010; D'Souza et al., 2011a; McBride et al. 2011). Although this procedure allows peptides to be fluorinated in one simple step within 30 min, it requires agents to be heated to $\sim 100^\circ\text{C}$, which is unsuitable for most proteins and some peptides. We and others have found that an aromatic group attached to one of the nitrogen atoms of the cyclononane ring of the NOTA can enhance the yield for the $([^{18}\text{F}]\text{AlF})^{2+}$ complexation compared to some alkyl and carboxyl substituents (D'Souza et al., 2011b; McBride et al., 2010; Shetty et al. 2011). In this report, we explore the potential for labeling heat-labile compounds with $([^{18}\text{F}]\text{AlF})^{2+}$, using a new $([^{18}\text{F}]\text{AlF})^{2+}$ -binding ligand that contains 1,4,7-triazacyclononane-1,4-diacetate (NOTA) attached to a methyl phenylacetic acid group (MPA). This was conjugated to *N*-(2-aminoethyl)maleimide (EM) to form NODA-MPAEM. The NODA-MPAEM derivative is labeled in a 2-step process with reasonably high yields.

2. Materials and methods

2.1. Materials

All commercially obtained chemicals were of analytical grade and used without further purification. $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, *N*-(2-aminoethyl)maleimide and sodium hydroxide (99.99%) were purchased from Sigma-Aldrich (Milwaukee, WI). α, α -Trehalose and acetic acid were acquired from J. T. Baker (Phillipsburg, NJ). Trifluoroacetic acid was obtained from Creosalus (Louisville, KY). The analytical reverse-phase HPLC (RP-HPLC) and size-exclusion HPLC (SE-HPLC) columns were purchased from Phenomenex (Torrance, CA). The USP $^{18}\text{F}^-$ in saline was obtained from PETNET Solutions (Hackensack, NJ). The Oasis hydrophilic-lipophilic balanced (HLB) solid-phase extraction (SPE) cartridges were acquired from Waters (Milford, MA). CEA was obtained from Scripps (San Diego, CA).

The reduced, humanized, monoclonal anti-CEACAM5 antibody Fab' fragment, hMN-14 Fab' and the CEA-Scan® kit (arcitumomab) were provided by Immunomedics, Inc. (Morris Plains, NJ). Female nude mice (NCr nu-m), 23.1 ± 2.3 g, were procured from Taconic Farms (Germantown, NY).

2.2 HPLC methods

Analytical HPLC was performed using a Waters 2695 system equipped with a Phenomenex Gemini C_{18} reverse-phase column (250×4.6 mm, $5 \mu\text{m}$, $110 \text{ \AA}$) or a Phenomenex Jupiter C_4 reverse-phase column (250×4.6 mm, $5 \mu\text{m}$) (reverse-phase HPLC, RP-HPLC). A Phenomenex Biosep-SEC-3000 column (300×7.8) was used for the size-exclusion HPLC (SE-HPLC) stability studies.

Method 1: For routine HPLC analysis of the radiolabeled NODA-MPAEM, a Phenomenex Gemini column was used with a flow of 1 mL/min and a gradient of 100% A (0.1% TFA) to 100% B (0.1% TFA 90% CH_3CN , 10% H_2O) over 30 min. Absorbance was detected at 220 and 254 nm using Waters 2996 photodiode array (PDA) detector and radioactivity detected with a Perkin Elmer 610TR Radiomatic Flow scintillation analyzer.

Method 2: The protein conjugation reaction was followed by RP-HPLC using the same buffers and gradient as method 1, but with a Phenomenex C-4 column in place of the C-18 column.

Method 3: For serum stability studies, a Phenomenex Biosep-SEC-3000 column (300×7.8) was eluted at a flow rate of 1 mL/min with 0.2 M phosphate buffer at pH 6.8. Absorbance was detected at 220 and 254 nm, and radiation with an in-line Packard A-100 detector.

Method 4: The NODA-MPAEM was purified using Waters PrepLC 4000 system with Sunfire Prep C₁₈ optimal bed density (OBD) reverse-phase column (150 × 30 mm, 5 μm), using a linear gradient of 100% A (0.1% TFA) to 15% B (90% acetonitrile, 10% water, 0.1% TFA) over 80 min at a flow rate of 45 mL/min (absorbance 220 nm).

2.3 Methods

2.3.1 Synthesis of Bis-*t*-butyl-NODA-MPAA NHS ester: (tBu)₂NODA-MPAA NHS ester

The bis-*t*-butyl-NODA-MPAA was prepared as described previously (D'Souza et al. accepted). To a solution of (tBu)₂NODA-MPAA (175.7 mg, 0.347 mmol) in CH₂Cl₂ (5 mL) was added 347 μL (0.347 mmol) DCC (1 M in CH₂Cl₂), 42.5 mg (0.392 mmol) *N*-hydroxysuccinimide (NHS), and 20 μL *N,N*-diisopropylethylamine (DIEA). After 3 h, dicyclohexylurea (DCU) was filtered off and solvent evaporated. The crude product was purified by flash chromatography on (230–400 mesh) silica gel (CH₂Cl₂:MeOH, 100:0 to 80:20) to yield 128.3 mg (61.3%) of the NHS ester [HRMS (ESI) calculated for C₃₁H₄₆N₄O₈ (M+H)⁺ 603.3388, found 603.3395].

2.3.2 Synthesis of NODA-MPAEM: (MPAEM = methyl phenyl acetamido ethyl maleimide)

To a solution of (tBu)₂NODA-MPAA, NHS ester (128.3 mg, 0.213 mmol) in CH₂Cl₂ (5 mL) was added a solution of 52.6 mg (0.207 mmol) *N*-(2-aminoethyl) maleimide trifluoroacetate salt in 250 μL DMF and 20 μL DIEA. After 3 h, the solvent was evaporated and the concentrate treated with 2 mL TFA. The crude product was diluted with water and purified by preparative RP-HPLC to yield (49.4 mg, 45%) of the desired product. HRMS (ESI) calculated for C₂₅H₃₃N₅O₇ (M+H)⁺ 516.2453, found 516.2452 ¹H NMR (500 MHz, DMSO-*d*₆, 25 °C) δ 2.6–3.25 (m, 15H), 3.30–3.40 (m, 4H), 3.42–3.60 (m, 5H), 4.32 (s, 2H), 6.97 (s, 2H), 7.27 (d, 2H), 7.43 (d, 2H), 8.19 (t, 1H); ¹³C (125.7 MHz, DMSO-*d*₆) 37.4, 37.6, 42.3, 47.2, 49.7, 50.7, 55.2, 58.0, 115.2, 117.5, 130.0, 130.5, 134.9, 137.7, 158.6, 158.9, 170.6, 171.5, 172.9.

2.3.3 ¹⁸F-radiolabeling of NODA-MPAEM—The NODA-MPAEM ligand (20 nmol; 10 μL), dissolved in 2 mM sodium acetate (pH 4), was mixed with AlCl₃ (5 μL of 2 mM solution in 2 mM acetate buffer, 200 μL of ¹⁸F[−] (0.73 and 1.56 GBq) in saline, and 200 μL of acetonitrile. After heating at 105–109°C for 15–20 min, 800 μL of deionized (DI) water was added to the reaction solution, and the entire contents removed to a vial (dilution vial) containing 1 mL of deionized (DI) water. The reaction vial was washed with 2 × 1 mL DI water and added to the dilution vial. The crude product was then passed through a 1-mL HLB column, which was washed with 2 × 1 mL fractions of DI water. The labeled product was eluted from the column using 3 × 200 μL of 1:1 EtOH/water.

2.3.4 Conjugation of [¹⁸F]AIF-NODA-MPAEM to hMN-14 Fab'—Fab' fragments of the humanized MN-14 anti-CEACAM5 IgG (labetuzumab) were prepared by pepsin digestion, followed by TCEP (tris(2-carboxyethyl)phosphine) reduction, and then formulated into a lyophilized kit containing 1 mg (20 nmol) of the Fab' (2.4 thiols/Fab') in 5% trehalose and 0.025 M sodium acetate, pH 6.72. The kit was reconstituted with 0.1 mL PBS, pH 7.01, and mixed with the [¹⁸F]AIF-NODA-MPAEM (600 μL 1:1 EtOH/H₂O). After incubating for 10 min at room temperature, the product was purified on a 3-mL Sephadex G50-80 spin column in a 0.1 M, pH 6.5 sodium acetate buffer (5 min). The isolated yield was calculated by dividing the amount of activity in the eluent by the total activity in the eluent and the activity on the column.

Immunoreactivity of the purified product was analyzed by adding an excess of CEA and separating on SE-HPLC, comparing to a profile of the product alone. The product was also analyzed by RP-HPLC to assess percent-unbound [¹⁸F]AIF-NODA-MPAEM (Phenomenex

Jupiter C-4 column 250 × 4.6 mm; 0.1% TFA buffers 100% H₂O to 90% CH₃CN over 30 min, 1 mL/min).

2.3.5 ^{99m}Tc-CEA-Scan®—A CEA-Scan® kit containing 1.2 mg of IMMU-4, a murine anti-CEACAM5 Fab' (anti-CEA, 2.4 × 10⁻² μmol), was labeled with 453 MBq ^{99m}TcO₄⁻Na⁺ in 1 mL saline according to manufacturer's instructions and used without further purification.

2.3.6 Animal Study—Nude mice were inoculated subcutaneously with CaPan-1 human pancreatic adenocarcinoma (ATCC HTB-79TM, Manassas, VA). When tumors were visible, the animals were injected intravenously with 100 μL of the radiolabeled Fab'. The [¹⁸F]AIF-NODA-MPAEM-hMN-14 Fab' was diluted in saline to 3.7 MBq/100 μL containing ~2.8 μg of Fab'. A ^{99m}Tc-IMMU-4 Fab' aliquot (16.9 MBq) was removed and diluted with saline (0.85 MBq/100 μL containing ~2.8 μg of Fab'). The animals were necropsied at 3 h post injection, tissues and tumors removed, weighed, and counted by gamma scintillation, together with standards prepared from the injected products. The data are expressed as percent injected dose per gram.

3. Results

3.1. Synthesis and reagent preparation

The NODA-MPAEM was produced as shown in Figure 1, where the (tBu)₂NODA-MPAA (D'Souza et al. 2011b) was coupled to 2-aminoethyl-maleimide and then deprotected to form the desired product. The crude product was diluted with water and purified by preparative RP-HPLC to yield (49.4 mg, 45%) of the desired product [HRMS (ESI) calculated for C₂₅H₃₃N₅O₇ (M+H)⁺ 516.2453, found 516.2452].

3.2. Radiolabeling

The NODA-MPAEM (20 nmol) was mixed with 10 nmol of Al³⁺ and labeled with 0.73 GBq and 1.56 GBq of ¹⁸F⁻ in saline. After SPE purification, the isolated yields of [¹⁸F]AIF-NODA-MPAEM were 82% and 49%, respectively, with a synthesis time of about 30 min. The [¹⁸F]AIF-NODA-MPAEM-hMN-14 Fab' conjugate was isolated in 74 % and 80% yields after spin-column purification for the low and high dose protein labelings, respectively. The total process was completed within 50 min. The specific activity for the purified [¹⁸F]AIF-NODA-MPAEM-hMN-14 Fab' was 19.5 GBq/μmol for the high-dose label and 10.9 GBq/μmol for the low dose label.

SE-HPLC analysis of the labeled protein for the 0.74-GBq run showed the ¹⁸F-labeled Fab' as a single peak and all of the activity shifted when excess CEA was added (Figure 2). RP-HPLC analysis on a C4 column showed the labeled maleimide standard eluting at 7.5 min, while the purified ¹⁸F-protein eluted at 16.6 min (not shown). There was no unbound [¹⁸F]AIF-NODA-MPAEM in the spin-column purified product.

3.3. Serum stability

The [¹⁸F]AIF-NODA-MPAEM-hMN-14 Fab' was mixed with fresh human serum and incubated at 37 °C. SE-HPLC analysis over a 3-h period, with and without CEA showed that the product was stable and retained binding to CEA (Figure 2).

3.4. Biodistribution

The biodistribution of the [¹⁸F]AIF-NODA-MPAEM-hMN-14 Fab' and the ^{99m}Tc-IMMU-4 murine Fab' was assessed in nude mice bearing Capan-1 pancreatic cancer xenografts. At 3

h post-injection, both agents showed an expected elevated uptake in the kidneys, since Fab' is renally filtered from the blood (Table 1). The [^{18}F]AIF-Fab' concentration in the blood was significantly ($P < 0.0001$) lower than the $^{99\text{m}}\text{Tc}$ -Fab', with a correspondingly elevated uptake in the liver and spleen. The faster blood clearance of the [^{18}F]AIF-Fab' likely contributed to the lower tumor uptake as compared to the $^{99\text{m}}\text{Tc}$ -Fab' (2.8 ± 0.3 vs. 6.8 ± 0.7 , respectively), but it also resulted in a more favorable tumor/blood ratio for the fluorinated Fab' (5.9 ± 1.3 vs. 0.9 ± 0.1 , respectively). Bone uptake for both products was similar, suggesting the Al ^{18}F -NODA was tightly held by the Fab'.

4. Discussion

We reported a facile method for ^{18}F -radiolabeling of peptides previously that involves the binding of a highly stable [^{18}F](AIF) $^{2+}$ complex to a metal-binding ligand bound to a peptide (McBride et al., 2009; McBride et al., 2010; Laverman et al., 2010; D'Souza et al., 2011a, 2011b; McBride et al., 2011), and two other groups have recently reported their results using this labeling approach (Liu et al. 2011; Shetty et al. 2011). This radiolabeling approach has the advantage of rapid binding to the ligand, does not require a dry-down step, and high specific activities can be obtained after a simple SPE purification step to remove unbound ^{18}F . The procedure does not require expensive automated equipment, but could be easily adapted for use with such equipment.

All of these products have been highly stable in human serum *in vitro* and *in vivo* in mice. However, the procedure requires the ligand-peptide to be heated to $\sim 100^\circ\text{C}$ to facilitate the formation of the [^{18}F]AIF-ligand bond, limiting the method to peptides that can tolerate high temperatures. The purpose of this investigation was to determine the potential for developing an [^{18}F]AIF-based labeling procedure that could be used with heat-labile peptides and proteins.

Others have reported methods for attaching ^{18}F to a small prosthetic group, which is then conjugated to a protein. The prosthetic group is commonly attached to the protein through a thiol or a lysine side-chain on the protein, although several other attachment methods have been used (Schirmacher et al., 2007; Wester et al., 2007; Flavell et al., 2008; Wuest et al., 2008). In many cases, this has involved multistep syntheses that have taken hours to perform, but with new automated methods, such as microfluidics (Bejot et al., 2011), the preparation times have been shortened considerably.

In order to provide proof-of-principle that the [^{18}F]AIF technique could be adapted in a similar manner, we prepared a simple NODAMPAEM ligand for attachment to thiols on small proteins. To avoid exposing the heat-labile compound to high temperatures, the NODA-MPAEM was first mixed with Al^{3+} and $^{18}\text{F}^-$ in saline and heated at $100\text{--}115^\circ\text{C}$ for 15 min to form the [^{18}F]AIF-NODA-MPAEM intermediate. This intermediate was rapidly purified by SPE in 49–82% isolated yield ($67.7 \pm 13.0\%$, $n=5$), depending on the amount of activity added to a fixed amount (20 nmol) of the NODA-MPAEM. The [^{18}F]AIF-NODA-MPAEM was then efficiently (69–80% isolated yield, 74.3 ± 5.5 , $n=3$) coupled to a reduced Fab' in 10–15 min, using a spin column gel filtration procedure to isolate the radiolabeled protein, in this case an antibody Fab' fragment. The entire two-step process was completed in ~ 50 min, and the labeled product retained its molecular integrity and immunoreactivity. Thus, the feasibility of extending the simplicity of the [^{18}F]AIF-labeling procedure to heat-sensitive compounds was established.

The [^{18}F]AIF-ligand complex has been shown to be very stable in serum *in vitro*, and in animal testing, minimal bone uptake is seen (McBride et al., 2009; D'Souza et al., 2011a). In this series of studies, ^{18}F associated with the NODA-MPAEM compound conjugated to a

Fab' was stable in serum *in vitro*, and the conjugate retained binding to CEA. When injected into nude mice, there was selective localization in the tumor, providing a ~6:1 tumor/blood ratio. Bone uptake was similar for the [^{18}F]AIF-hMN-14 Fab' and the $^{99\text{m}}\text{Tc}$ -IMMU-4 murine Fab', again reflecting *in vivo* stability of the ^{18}F or Al^{18}F complex. However, [^{18}F]AIF-Fab' hepatic and splenic uptake was higher as compared to the $^{99\text{m}}\text{Tc}$ -IMMU-4. Splenic uptake might be related to difference between a humanized and murine Fab', but hepatic uptake suggests other differences exist. Thus, the derivative and labeling procedure will require further examination and depending on the agent of interest, may require optimization to achieve favorable biodistribution (Tolmachev et al., 2011). Indeed, the specific NODA derivative can be modified in different ways to accommodate conjugation to other reactive sites on peptides or proteins. However, this particular derivative provided proof-of-principle required to show that the Al^{18}F -labeling procedure can be adapted for use with heat-labile compounds.

5. Conclusions

The NODA-MPAEM is labeled rapidly with $^{18}\text{F}^-$ in saline and then conjugated to the immunoglobulin Fab' protein in high yield. The labeling method uses only inexpensive disposable purification columns, and while not requiring an automated device to perform the labeling and purification, it can likely be easily adapted to such systems. Thus, the NODA-MPAEM derivative provided proof-of-principle that this or another NODA-containing derivative can extend the capability of facile (^{18}F]AIF) $^{2+}$ fluorination to heat-labile compounds.

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Highlights

- The radiolabeling of the 1,4,7-triazacyclononane-1,4-diacetate (NODA)-methyl phenylacetic acid N-(2-aminoethyl)maleimide (NODA-MPAEM) with Al^{18}F is a high yield (49–82%), fast, one-step reaction with no dry-down step.
- The $[\text{F}^{18}]\text{AlF-NODA-MPAEM}$ was conjugated to an antibody Fab'-SH fragment in high yield (69–80%) at room temperature in 15 min.
- The $[\text{F}^{18}]\text{AlF-NODA-MPAEM-antibody Fab'}$ was stable *in vitro* and *in vivo*.

Figure 1.
Synthesis of NODA-MPAEM.

Figure 2. Stability and immunoreactivity of $[^{18}\text{F}]\text{AIF-NODA-MPAEM-hMN-14}$ anti-CEA Fab' as determined by SE-HPLC. (A) immediately after purification; (B) after 1 h in fresh human serum at 37 °C; (C) 3 h in serum; (D) 1.7 h in serum + excess CEA; (E) 3.4 h in serum + excess CEA.

Table 1

Biodistribution of [^{18}F]AIF-NODA-MPAEM-hMN-14 Fab' and $^{99\text{m}}\text{Tc}$ -IMMU-4 Fab' at 3 h after injection with 0.37 MBq ($\sim 3\ \mu\text{g}$) of each conjugate in nude mice bearing Capan-1 human pancreatic cancer xenografts (N = 6).

Tissue	[^{18}F]AIF- NODA-MPAEM-hMN-14 Fab'		$^{99\text{m}}\text{Tc}$ CEA Scan IMMU-4 Fab'	
	%ID/g	T/NT	%ID/g	T/NT
Capan-1 (weight $\pm$ SD)	2.8 ± 0.3 (0.22 ± 0.08 g)	----	6.8 ± 0.7 (0.16 ± 0.05 g)	----
Liver	17.5 ± 3.8	0.2 ± 0.04	4.6 ± 0.4	1.5 ± 0.1
Spleen	11.3 ± 1.6	0.3 ± 0.04	3.5 ± 0.5	2.0 ± 0.3
Kidney	216 ± 30.9	0.0 ± 0.0	183 ± 22.5	0.04 ± 0.01
Lung	4.2 ± 1.6	0.8 ± 0.5	4.4 ± 0.8	1.6 ± 0.3
Blood	0.5 ± 0.1	5.9 ± 1.3	7.6 ± 0.9	0.9 ± 0.1
Stomach	0.6 ± 0.1	4.7 ± 1.2	2.4 ± 0.3	2.9 ± 0.4
Sm. Int.	2.2 ± 0.2	1.3 ± 0.1	3.4 ± 0.4	2.0 ± 0.2
Lg. Int.	1.1 ± 0.4	2.8 ± 0.7	5.2 ± 1.0	1.3 ± 0.3
Muscle	0.4 ± 0.1	6.7 ± 1.3	1.1 ± 0.2	6.1 ± 1.0
Scapula	1.6 ± 0.4	1.8 ± 0.4	2.0 ± 0.2	3.4 ± 0.5