JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2006; **49**: 1213–1221.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.1136

Research Article

Labeling G-rich oligonucleotides (GROs) with N-succinimidyl 4-[^{18}F]fluorobenzoate (S^{18}FB)

Junling Li[1,*], John O. Trent[2,3], Paula J. Bates[2,3] and Chin K. Ng[1,3]
[1] *Department of Diagnostic Radiology, University of Louisville School of Medicine, Louisville, KY 40202, USA*
[2] *Department of Medicine, University of Louisville School of Medicine, Louisville, KY 40202, USA*
[3] *James Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY 40202, USA*

Summary

Three G-rich oligonucleotides (GROs) were conjugated with N-succinimidyl 4-[^{18}F]fluorobenzoate (S^{18}FB). The yields for GRO5, the shortest sequence among those three GROs being tested, were 42, 70, 84, and 99% at GRO5 concentration of 59, 118, 236, and 472 nmol/100 µl, respectively. Reaction temperature (22 and 40°C), reaction time (15 and 30 min), and borate buffer concentration (25 and 50 mM) showed no noticeable effects on the labeling results. Two longer sequences of GROs, GRO15A and GRO26A, were also labeled with the same procedures and the corresponding yields were $80 \pm 2\%$ at 93 nmol/100 µl ($n = 3$) and $47 \pm 3\%$ at 98 nmol/100 µl ($n = 3$), respectively. Finally, CROa was conjugated with S^{18}FB to further confirm our results. The radiochemical yields were 13.7 ± 2.5, 40.9 ± 3.5, 70.1 ± 2.0, $93.4 \pm 1.4\%$ at 8.7, 17.5, 35 and 52.5 OD of CROa (non-isolated, decay-corrected and based on S^{18}FB, $n = 3$) in comparison to 7, 17, 22 and 28% at 5, 10, 20 and 40 OD of CROa, respectively, as reported by Hedberg *et al.* (*Acta Chem Scand* 1998; **52**: 1034–1039). Copyright © 2006 John Wiley & Sons, Ltd.

Received 30 March 2006; Revised 23 August 2006; Accepted 30 August 2006

Key Words: oligonucleotides; radiopharmaceutical; PET imaging; molecular imaging

*Correspondence to: Junling Li, Department of Diagnostic Radiology, Department of Medicine and James Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY 40202, USA. E-mail: junling.li@louisville.edu

Introduction

Guanosine-rich oligonucleotides (GROs) are emerging as a new class of non-antisense oligonucleotides (ONs) to exhibit anti-cancer effect. The formation of a secondary structure called G-quartets is thought to play a critical role in preventing GROs from degradation by nucleases, thus GROs in G-quartet configurations can be very stable in serum-containing conditions.[1,2] Moreover, many recently published results on the inhibitory effect of GROs on cancer growth indicate that GROs are a promising class of agents for the treatment of cancers.[3–6]

It has been shown that nucleolin is the primary target of GROs[1] and other molecules that bind to nucleolin are specifically taken up by tumors,[7] thus F-18 labeled GROs are expected to be trapped inside the cancer cells by binding to nucleolin. Because of the increasing interest in labeled oligonucleotides and derivatives as radiopharmaceuticals for PET, we have chosen the $S^{18}FB$ method to label this class of special molecules, GROs, with ^{18}F. ^{18}F-labeled GROs might serve as a novel class of radiopharmaceuticals for cancer diagnosis and could play an important role in monitoring the treatment efficacy of GROs.

There are two main methods for labeling ONs with F-18: (1) N-succinimidyl 4-[^{18}F]fluorobenzoate was used as the ligand to conjugate with alkylamine modified oligonucleotides at 5′- or 3′-end. Hedberg *et al.*[8] first used this method to label four types of ONs, but the conjugation yields were only 7–28% even at high concentration of ONs (40 OD). The main advantage of this method is that the conjugation reaction can be carried out at low temperature ($\leqslant 40^{\circ}C$) in a short time. (2) N-(4-halobenzyl)-2-bromoacetamide was used to conjugate with an ON having a phosphorothioate monoester group at its 3′- or 5′- end.[9–11] The advantage for this strategy is the usual high yield of 70–90%, so it was widely used to conjugate many ONs with ^{18}F.[9–13] But this method requires high reaction temperature (80–120°C), thus it cannot be used to label some temperature-sensitive molecules such as proteins or peptides. Some G-quartets were also reported to be not stable at high temperature,[1] therefore the $S^{18}FB$ method is more suitable for labeling various G-quartet oligonucleotides with ^{18}F.

In order to explore the possibility of labeling GROs with ^{18}F, we have chosen to label three single-stranded GROs with N-succinimidyl 4-[^{18}F] fluorobenzoate ($S^{18}FB$). CROa, the same oligonucleotide used by Hedberg *et al.* [8] was also conjugated with $S^{18}FB$ to confirm our findings.

Results and discussion

Chemistry

In order to further characterize our conjugation methods with ESI-MS, GRO5 and GRO15A were selected to conjugate with ^{19}F SFB at room temperature.

DOI: 10.1002/jlcr

^{19}F-conjugated GRO5 and ^{19}F-conjugated GRO15A were analyzed by ESI-MS. Prior to the MS-ESI experiment, pH of the conjugated mixtures were adjusted to 7–7.5 with sodium acetate (3 M, pH 5.5), then precipitated with ethanol. The pellets were resolubilized with water prior to the ESI-MS analysis. The measured MS values of 1808.2 Da for ^{19}F SFB-GRO5 and 5003 Da for ^{19}F SFB-GRO15A were in good agreement with the expected MS values of 1810.2 and 5002 Da, respectively.

Similar work as described above was performed with CROa, a longer sequence of oligonucleotide used by Hedeberg *et al.* ^{19}F-conjugated CROa was further analyzed by ESI-MS. The measured MS value for ^{19}F SFB-CROa was 6393.0 Da, corresponding well to the expected MS value of 6393.2 Da.

Radiochemistry

Synthesis of S^{18}FB. S^{18}FB was synthesized mainly according to Wester[14] in three radiochemical steps within 40–45 min. The overall yield was 55–65% (decay corrected and based on [^{18}F]F$^-$) and the radiochemical purity was determined by HPLC to be $>99\%$.

^{18}F Labeling of oligonucleotides

Labeling of GRO5. Experiments were performed with GRO5 first in order to optimize the conjugation process with S^{18}FB. Figure 1 showed the comparison among the various HPLC chromatograms of S^{18}FB-DMF, S^{18}FB-GRO5 conjugation reaction, and S^{18}FB reference experiment (same experimental conditions as S^{18}FB-GRO5 conjugation reaction, except that no GRO5 was added) (panels A, B & C, respectively). All S^{18}FB ($t_R = 28.4$ min, Figure 1(A)) disappeared and two new peaks that may be related to the hydrolysis of S^{18}FB were found in Figure 1(C), demonstrating that S^{18}FB was very instable in the presence of the borate buffer in less than 15 min. A new detected peak with $t_R = 18.2$ min in Figure 1(B) was later confirmed to be ^{18}F-conjugated GRO5 by co-injection with ^{19}F-conjugated GRO5 reference compound and comparing it with all the other peaks shown in Figures 1(A)–(C).

Experiments using various GRO5 concentrations, borate buffer concentrations, reaction temperature and time were performed in order to optimize the labeling process. GRO5 concentration was found to affect the labeling yields significantly. As the GRO5 concentration increased, the yields were improved significantly (42–70%), reaching a plateau at 120 nmol/100 µl. The specific activity ranged from 366 to 699 MBq/µmol. According to the results of Figure 1, the hydrolysis process probably competed with the conjugation reaction. Moreover, it was found that the ratio of ^{18}F-conjugated yield/S^{18}FB hydrolysis was 4/6 at 59 nmol/100 µl and 9/1 at 474 nmol/100 µl, indicating that S^{18}FB hydrolysis was highly suppressed and labeling yields were enhanced with higher GRO5 concentration.

DOI: 10.1002/jlcr

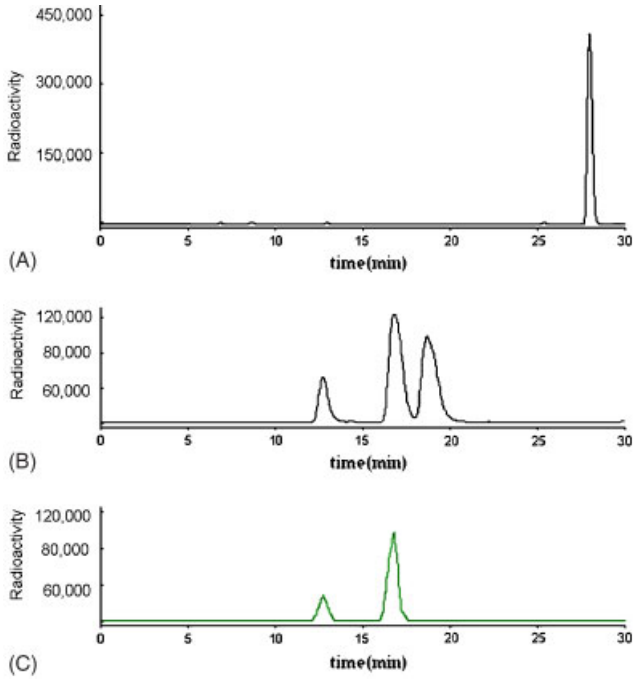

Figure 1. HPLC chromatograms for S^{18}FB-DMF, GR05 labeled with S^{18}FB and S^{18}FB reference experiment (S^{18}FB-DMF-borate buffer, same conditions as the labeling except no GR05 was added) (panels A, B and C, respectively)

The stability of S^{18}FB was studied in the borate concentration of 25 and 50 mM. The results indicated that lower borate buffer concentration improved the stability of S^{18}FB, but the labeling yields were almost the same, 41% vs 42% at 59 nmol/100 µl. Since the main focus of the work was on percent yield and higher concentration has better buffering capacity, therefore 50 mM was used to assess the conjugation step.

Two different reaction temperatures, 22 and 40°C, were also investigated, but no noticeable effect was detected, 40% vs 42% at 59 nmol/100 µl. These results are important for labeling temperature sensitive biomolecules with F-18.

Finally, reaction time of GRO5 with S^{18}FB, 15 and 30 min, was investigated as well, and the results showed no significant difference.

Labeling of GRO15A and GRO26A with S^{18}FB. Two longer GROs, GRO15A and GRO26A, were also labeled with S^{18}FB by the same method described above. The yields were $80 \pm 2\%$ at 93 nmol/100 µl ($n = 3$) and $47 \pm 3\%$ at 98 nmol/100 µl ($n = 3$), respectively. These results indicated that longer sequence tended to have lower yields. The HPLC peaks for labeled GROs

DOI: 10.1002/jlcr

and non-labeled GROs on the UV chromatographs were difficult to be resolved due to a possible formation of secondary structures in longer sequences of GROs,[1] therefore, the specific activity for GRO15A and GRO26A was estimated to be between 453 and 764 MBq/μmol.

Labeling of CROa with $S^{18}FB$. Hedberg *et al.*[8] first reported the labeling results for CROa with $S^{18}FB$ but the conjugation yields were in the range of 7–28%. The specific activity ranged from 133–581 MBq/μmol. We, however, got very high labeling yields for three different sequences of GROs using the same conjugation method. To further verify our results, CROa, the same oligonucleotide used by Hedberg *et al.*, was also labeled with $S^{18}FB$ in our experiments. The effect of CROa concentrations on the labeling reaction was studied in details. Figure 2 showed a direct comparison of our results to those obtained by Hedberg *et al.*[8] Our yields were clearly much higher, especially at higher concentrations.

Three possibilities were explored in order to better understand the differences: esterifcation reagents used in $S^{18}FB$ synthesis (disuccinimido carbonate by Hedeberg vs O-(N-succinimidyl) N, N, N′, N′-tetramethyluronium tetrafluoroborate (TSTU) in the present study), different elution steps in $S^{18}FB$ purification (8 ml 25% CH_3CN by Hedeberg *et al.* vs 2 ml 35% CH_3CN in the present study) and two reaction durations (30 min by Hedeberg *et al.* vs 15 min in the present study). All those factors were found to have no obvious effects on the yields.

During the conjugation reaction of GRO5 with F-18, labeling yields were found to be suboptimal when the GRO5 samples were kept in solution for a

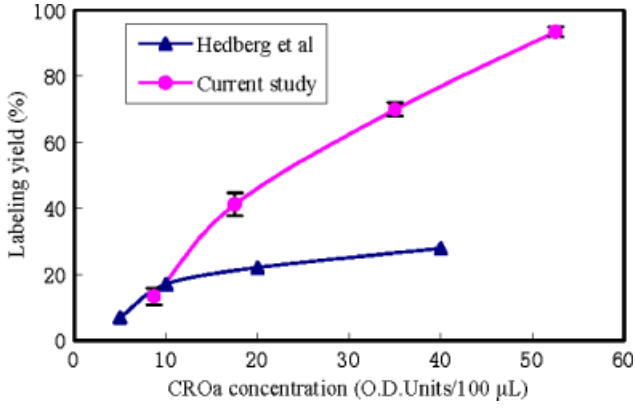

Figure 2. Comparison of results obtained from the current study ($n = 3$ for each concentration, reaction time: 15 min) with those reported by Hedberg *et al.* [8] (reaction time: 30 min) on the relationship between CROa concentration and labeling yield. Reaction temperature 40°C

DOI: 10.1002/jlcr

certain period. When GRO5-borate buffer samples were kept at 4°C for over 2 months, the yields were only 10–20% and a new HPLC peak was also detected ($n > 5$). In addition, the labeling yield decreased if the sample was kept at 4°C for even 1 week compared with the freshly dissolved GRO5 sample (15% vs 42% at 59 nmol/100 µl), but no same side-product peak was detected. Similar results were also reported by Hedberg *et al.*: three radiochemical peaks were detected after the reaction of CROa with $S^{18}FB$, with the first two being attributed to $S^{18}FB$ hydrolysis, the third one to ^{18}F–CROa but the labeling yield was low (7–28%). Comparing to the use of freshly made sample for the conjugation reaction, the yield was almost unchanged at ~100% for a higher concentration of GRO5 (474 nmol/100 µl) which was kept for 1 week at 4°C. The reason might be partially related to GRO5 being denatured when kept in solution for some time. If GRO5 concentration is low, the remaining amount may be not enough for achieving a reasonable yield. Thus it is plausible that the quality of the ONs may play a very important role in the entire labeling procedure. ONs are usually recommended to store as dried or in solution in −20°C. Amino modification makes the ONs less stable. These types of ONs are generally stable for only about 6 months at room temperature and cannot endure multiple freeze thaw cycles. Some data showed that those modified ONs are stable in solution only for 2–3 days.[15]

Conclusion

This preliminary study demonstrates that GROs can be labeled with $S^{18}FB$ with reasonable yields at low temperatures. The labeling yields appeared to be lower with the longer sequence of GROs. This method overcomes the disadvantage of N-(4-halobenzyl)-2-bromoacetamide method and provides a useful method for labeling GROs with F-18.

Experimental

General

Chemicals. All chemicals used were commercially available and without further purification. SFB was prepared by a similar esterification procedure as reported by Li *et al.*[16] Solid phase extraction (SPE) columns were C18 Sep-Pak cartridges (Waters, USA) and LiChrolut EN (Merck, Germany). The oligonucleotides: 5′-TGTTG-3′-NH_2 (GRO5), 5′-GTT-GTT-TGG-GGT-GG T-3′-NH_2 (GRO15A), 5′-GGT-TGG-GGT-GGG-TGG-GGT-GGG-TGG-GG-3′-NH_2 (GRO26A), NH_2-5′-GAA-CCT-CTG-AGA-GTT-CAT-CT-3′ (CROa), which had a phosphodiester DNA backbone and 3′- or 5′-$(CH_2)_6$-NH_2 modification, were purchased from Intergrated DNA Technologies (IDT), Inc. (Coralville, IA, USA).

DOI: 10.1002/jlcr

Analytical methods

Electrospray ionization mass spectroscopy (ESI-MS) was performed using a Q-TOF mass spectrometer (Waters, Milford, MA). Data were acquired in the negative ionization mode by scanning from m/z 100 to m/z 2000. Samples were dissolved in 2.5 mM imidazole and 2.5 mM piperidine in aqueous 50% isopropyl alcohol.[8,17]

For on-line radioactivity and UV measurements, the HPLC column was connected to a UV-visible detector (Varian Inc., Walnut Creek, CA, USA) and then to a NaI(Tl) scintillation flow-through detector (Bioscan, Washington DC, USA), and the data were recorded and processed by Galaxie software system (Varian Inc., Walnut Creek, CA, USA) for determination of radiochemical yields. The HPLC column used for the identification of standards and separation of radioactive products was Jupiter 5 μm C18 250 × 4.6 mm (Phenomenex, Torrance, CA, USA). The gradient protocol used was: 0–5 min: 5–10% CH_3CN, 95–90% 100 mM triethylammonium acetate (TEAA, pH 7.0); 5–10 min: 10–25% CH_3CN, 90–75% 100 mM TEAA; 10–20 min: 25–40% CH_3CN, 75–60% 100 mM TEAA; 20–30 min:40–100% CH_3CN, 60–0% 100 mM TEAA. The flow rate was fixed at 1 ml/min.

Chemistry

Syntheses of chromatographic standard: oligonucleotide conjugation with N-succinimidyl 4-fluorobenzoate (SFB). 20 μl SFB-DMF (700 nmol) was mixed with 80 μl oligonucleotides in 50 mM borate buffer (pH 8.5, 200 nmol), vortexed for a few seconds, then put at room temperature (22°C) for 20 min.

Radiochemistry

Production of [^{18}F]Fluoride. [^{18}F]Fluoride was produced via the ^{18}O-(p, n) ^{18}F nuclear reaction by bombardment of [^{18}O]H_2O by 11 MeV protons in a high-pressure silver target using a RDS-111 Cyclotron (Siemens, Knoxville, TN, USA). The crude [^{18}F]F^- provided together with [^{18}O]H_2O was loaded onto a Sep-Pak® light QMA cartridge (Waters Corporation, Milford, MA, USA) (pretreated with 5 ml $NaHCO_3$ and 10 ml water). The cartridge was then eluted with 500 μl of the aqueous mixture consisting of Kryptofix® 2.2.2. (10 mg, 26.5 μmol) and K_2CO_3 (3.45 mg, 25.2 μmol). The water was finally removed by repeated azeotropic evaporation with acetonitrile at 110°C.

Preparation of N-succinimidyl 4-[^{18}F]fluorobenzoate ($S^{18}FB$). The procedure used in the current study was modified from that reported by Wester *et al.*[14] In brief, the above dry residue was redissolved with 10 mg of the triflate salt of ethyl trimethylammonium benzoate in 300 μl DMSO and heated at 100°C for 7 min. 100 μl of 2 M NaOH was then added and kept at 100°C for 7 min before 650 μl of 1 M HCl was added. The mixture was then diluted to 3 ml with water

DOI: 10.1002/jlcr

before loading onto a Plus C18 Sep-Pak cartridge pretreated with 10 ml of ethanol and 10 ml of water. 3 ml of CH_3CN was used to elute the sample into a vial with 10 µl of 45% tetramethylammonium hydroxide after the first 10 ml wash with water. The solvent was evaporated with N_2 at 110°C and repeated twice by an addition of 0.5 ml CH_3CN. 10 mg of TSTU in 500 µl of anhydrous CH_3CN was added to the dry radioactive residue, heated at 100°C for 2 min. 8 ml of 5% HOAc was put to quench the reaction. The solution was passed through another pretreated C18 plus Sep-pak cartridge (Waters Corporation, Milford, MA, USA) and then the final product was eluted out with 3 ml of CH_3CN after 10 ml of water and 2 ml of 35% CH_3CN/H_2O. The radiochemical purity was detected by the HPLC system.

Radiolabeling of oligonucleotides with $S^{18}FB$. The procedure is similar to the cold conjugation described above, except that different amount of oligonucleotides (50–500 nmol/100 µl) were added. The optimal experiments including parameters such as different oligonucleotide concentrations, reaction temperature, and time were also investigated to assess the possible requirements for optimal conjugation yields. To further confirm our results, we performed the experiments with another oligonucleotide, CROa used by Hedberg *et al.*[8] in their experiments. Radiochemical yields were non-isolated, decay-corrected and determined by HPLC chromatograms since over 99% of radioactivity was eluted out within the analysis time of 30 min based on $S^{18}FB$.

Acknowledgements

The authors wish to thank PETNET Pharmaceuticals, Inc, Louisville, KY for providing F-18 fluoride ions for our experiments.

References

1. Bates PJ, Kahlon JB, Miller DM. *J Biol Chem* 1999; **274**(37): 26369–26377.
2. Dapic V, Bate PJ, Miller DM. *Biochemistry* 2002; **41**: 3676–3685.
3. Gomez D, Aouali N, Lacroix L, Trentesaux C, Morjani H, Mergny JL, Riou JF. *J Biol Chem* 2003; **278**: 50554–50562.
4. Jing NJ, Xiong WJ, Sha W, Jing L, Tweardy DJ. *Cancer Res* 2004; **64**: 6603–6609.
5. Xu H, Hamhouyia F, Thomas SD, Burke TJ, Girvan AC, McGregor WG, Trent JO, Miller DM, Bates PJ. *J Biol Chem* 2001; **276**(16): 43221–43230.
6. McMicken HW, Bates PJ, Chen Y. *Cancer Gene Ther* 2003; **10**: 867–869.
7. Christian S, Pilch J, Porkka K, Laakkonen P, Ruosalahti E. *J Cell Biol* 2003; **163**: 871–878.
8. Hedberg E, Langstrom B. *Acta Chem Scand* 1998; **52**: 1034–1039.
9. Kuhnast B, Klussmann S, Hinnen F, Boisgard R, Rousseau B, Furste JP, Tavitian B, Dolle F. *J Label Compd Radiopharm* 2003; **46**: 1205–1219.
10. Kuhnast B, Hinnen F, Boisgard R, Tavitian B, Dolle F. *J Label Compd Radiopharm* 2003; **46**: 1093–1103.

DOI: 10.1002/jlcr

11. Erik FJ, De Vries, Vroegh J, Dijkstra G, Moshage H, Vaalburg W. *Nucl Med Biol* 2004; **31**: 605–612.
12. Erik FJ, Vroegh J, Elsinga PH, Vaalburg W. *Appl Radiat Isot* 2003; **58**: 469–476.
13. Boisgard R, Kuhnast B, Vonhoff S, Younes C, Hinnen F, Verbavatz JM, Rousseau B, Furste JP, Wlotza B, Dolle F, Klussmann S, Tavitian B. *Euro J Nucl Med Mol Imaging* 2005; **32**: 470–477.
14. Wester HJ, Hamacher K, Stocklin G. *Nucl Med Biol* 1996; **23**: 365–372.
15. Alan S. Gerstein (ed.). *Molecular Biology Problem Solver: A Laboratory Guide*. Wiley-Liss: New York, 2001.
16. Li JL, Wang YX, Tian HB, Zhou W, Zhang XL, Lin YW, Yin DZ. *J Radioanaly Nucl Chem* 2002; **254**: 415–419.
17. Greig M, Griffey RH. *Rapid Commun Mass Spectrom* 1995; **9**: 97–102.

DOI: 10.1002/jlcr