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Research Article

# Factors affecting the labeling yield of F-18-labeled AS1411

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**Abstract:** The labeling of AS1411, a guanine (G)-rich oligonucleotide (ON), conjugated with [$^{18}$F]SFB was evaluated at different experimental conditions: ON concentration, base composition (G-rich [AS1411] vs cytosine-rich [CRO]), ON purity (desalted vs high-pressure liquid chromatography (HPLC)-purified) and buffers (tris-HCl, sodium borate, and potassium phosphate (PBK)). The labeling yield, defined as % F-18 incorporated into the ON, of HPLC-purified AS1411 was significantly improved with increasing concentration – $7 \pm 3$, $12 \pm 2$, and $25 \pm 5\%$ ($n = 5$) at 56, 93, and 160 nmol/100 μL, respectively. The labeling yields, however, were much higher if guanines of AS1411 were completely replaced by cytosines – $22 \pm 1$, $41 \pm 2$, and $59 \pm 3\%$ ($n = 3$), respectively, at the same corresponding concentration ($p < 0.05$). The specific activity of $^{18}$F-AS1411 and $^{18}$F-CRO was 4.2–12.6 and 4.2–25 mCi/μmol, respectively, after alcohol precipitation. If desalted grade of AS1411 and CRO was used instead for the conjugation, the yields were dropped to only 0–5 and 0–1 ($n = 5$), respectively, regardless of the concentration used. The labeling yields were $5 \pm 2$, $12 \pm 3$, and $31 \pm 4$ ($n = 3$) at 200 nmol/100 μL of AS1411; and $30 \pm 2$, $45 \pm 3$, and $68 \pm 4$ ($n = 3$) at 200 nmol/100 μL of CRO for tris-buffer, sodium borate, and PBK, respectively, at pH of 8.5 ($p < 0.05$). Copyright © 2007 John Wiley & Sons, Ltd.

**Keywords:** AS1411; G-quadruplex; radiopharmaceutical; PET imaging

## Introduction

Molecular targeting is of great interest for diagnosis and therapy, particularly in oncology.[1] Owing to their excellent targeting capacities and ease of synthesis in high diversity, oligonucleotides (ONs) are extensively used *in vitro* as ligands for various cellular targets and hold great potential for *in vivo* applications.[2] ON ligands with high affinities for a variety of molecular targets have been identified from combinatorial libraries.[3] They are also called the aptamers, which are comparable to antibodies in specificity and affinity for their target molecule.[1]

G-rich oligonucleotides (GROs) can form a higher order structure called G-quadruplexes.[4–7] This structural motif was found to substantially increase the *in vivo* stability of ONs because it is not rapidly degraded in serum.[8,9] Moreover, such higher order structures can potentially play a critical role in several biological processes including remarkable antiproliferative activity to many tumors, modulation of telomere activity, and *in vitro* DNA replication.[7,10–14] AS1411, formerly called AGRO100, is a GRO aptamer in G-quadruplex structure that has been shown to exhibit antiproliferative effect in cancer cells by binding specifically to a protein called nucleolin.[7,15] Results from the recent Phase I clinical trials demonstrated AS1411 to be a promising anti-cancer drug.[16,17] Although there is currently a general lack of success of *in vivo* aptamer imaging, it is our hope that labeling AS1411 in G-quadruplex structure with $^{18}$F would be useful for pharmacokinetic and pharmacodynamic studies of this drug in patients as well as to serve as a diagnostic imaging agent for cancer detection with positron emission tomography (PET).

Previously we labeled three single-stranded GROs (GRO5, GRO15A, and GRO26A) with N-succinimidyl 4-[$^{18}$F]fluorobenzoate ([$^{18}$F]SFB).[18] Depending on the sequence length, labeling yield of 100% could be achieved within 10 min at high ON concentration and room temperature for short GROs that did not form the G-quadruplex structure. Since AS1411 in the

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G-quadruplex structure has been shown to be a promising anti-cancer drug, we extended our previous work by labeling this GRO with the same technique. In order to assess various factors that can potentially affect the labeling yield of AS1411, we evaluated the labeling yield of the compound by changing ON concentration, base composition, ON purity, and buffer.

## Results and discussion

### Chemistry

AS1411 in G-quadruplex structure was confirmed by UV thermal denaturation studies.[6,7,9,19] This method is based on the fact that dissociation of an antiparallel G-quadruplex structure leads to a decrease in absorbance at 295 nm.[19]

To verify $^{19}$F-AS1411 as the cold reference compound, one sample was divided into three parts for the following experiments: (1) ESI-MS. 8573.2 Da for a $^{19}$F-conjugated single-stranded AS1411 was found; (2) Analytical high-pressure liquid chromatography (HPLC). A new peak with $t_R$ of 20–20.5 min was found compared with 18.5 min for the AS1411 peak. No AS1411 peak was detected after conjugation; (3) UV melting. Figure 1 showed the UV melting results of AS1411 in potassium phosphate (PBK) (a), $^{19}$F-AS1411 in PBK (b) and $^{19}$F-AS1411 redissolved in deionized water after the removal of PBK with alcohol precipitation (c).[20] Compared with curve a, the conjugation did not affect the G-quadruplex structure as shown in curve b. As shown in curve c, the removal of potassium ions appeared to alter the structural characteristics of AS1411. Since the G-quadruplex structure of AS1411 has been extensively studied in potassium,[6,7,9,19] our objective is to optimize the labeling of AS1411 with [$^{18}$F]SFB in PBK. Based on the results of ESI-MS, HPLC analysis, and UV melting, we therefore concluded that $^{19}$F-AS1411 in PBK could be used as the cold reference for the following radiochemical experiments.

### Radiochemistry

The reaction mechanism for [$^{18}$F]SFB and AS1411 is shown in Scheme 1. The specific activity of [$^{18}$F]SFB was 0.1–1 mCi/nmol and the radiochemical purity determined by analytical HPLC was >99.9%. The peak for $^{18}$F-labeled AS1411 was identified by the cold reference with HPLC. The specific activity was 4.2–12.6 mCi/μmol. The radiochemical purity was >99% based on analytical HPLC.

**Effect of ON concentration and base composition.** Figure 2 showed that the ON concentration and base composition greatly affected the labeling yield. As AS1411 concentration increased, the labeling yields were correspondingly improved at ambient reaction temperature: $7 \pm 3$, $13 \pm 2$, and $25 \pm 5\%$ ($n = 5$) at 56, 93, and 160 nmol/100 μL, respectively. When all the guanines of AS1411 were replaced by cytosines (C-rich oligonucleotide, CRO), the labeling yields became $22 \pm 1$, $41 \pm 2$, and $59 \pm 3\%$ ($n = 3$), respectively, at the same corresponding concentration ($p < 0.05$). The labeling yield difference between AS1411 and CRO may be due to the tendency for AS1411 in PBK to form a G-quadruplex structure blocking the 3′ end for full access to conjugation.

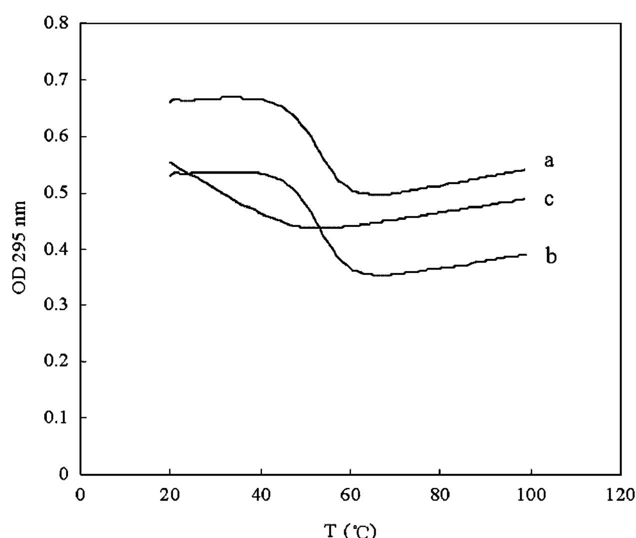
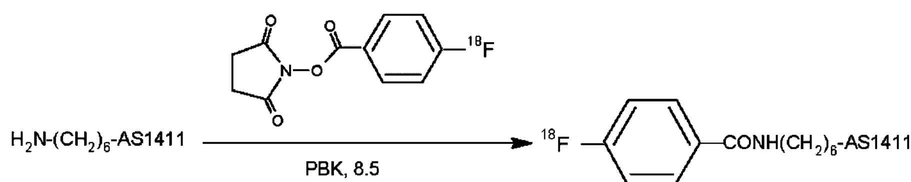



**Figure 1** Comparison of UV melting studies for AS1411 in PBK, S$^{19}$FB-conjugated AS1411 in PBK, and S$^{19}$FB-conjugated AS1411 redissolved in deionized water after the removal of PBK. a, SFB-AS1411 in PBK; b, AS1411 in PBK; c, SFB-AS1411 redissolved in water.




**Scheme 1**

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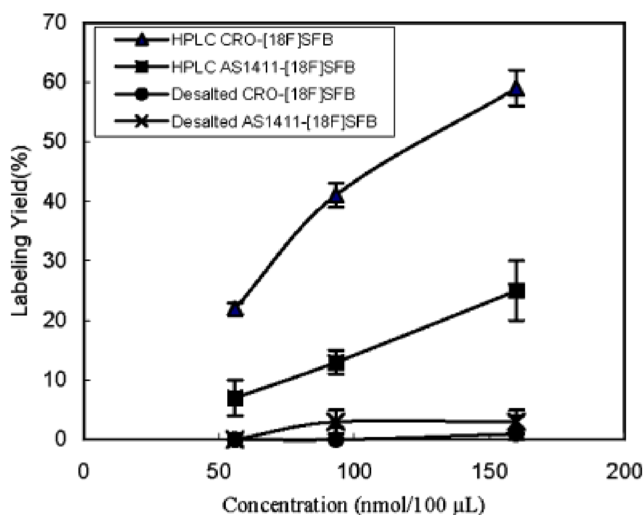


**Figure 2** Comparison of the labeling yields of HPLC-purified AS1411, desalted AS1411, HPLC-purified CRO and desalted CRO ($n \geqslant 3$). Reaction temperature: 22 °C. Reaction time 10 min.

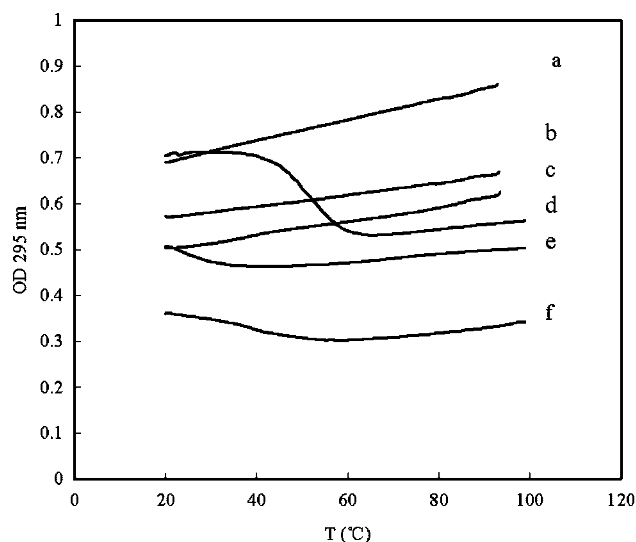


**Figure 3** UV melting results of AS1411 and CRO in three different buffers (pH = 8.5): a, CRO in PBK; b, CRO in sodium borate; c, CRO in tris-HCl; d, AS1411 in PBK; e, AS1411 in sodium borate; f, AS1411 in tris-HCl.

**Effect of ON purity.** Desalted and HPLC-purified AS1411 and CRO were used to conjugate with [$^{18}$F]SFB, respectively, and the corresponding results are shown in Figure 2. The yields were only between 0 and 5% ($n = 5$) for desalted AS1411, regardless of the concentration used. The yields for the HPLC-purified grade, however, were improved significantly as the AS1411 concentration increased. Results were reproducible with the samples stored in PBK at −20°C for over 3 months. Results were similar for CRO. Only 0– 1% ($n = 5$) was obtained with the desalted grade, compared with much higher yields for the HPLC-purified grade.

In our previous study,[18] labeling yields (>90%) for single-stranded desalted ONs were reported to be higher than those obtained by Hedberg *et al.* (<30%).[21] We later discovered that the desalted grade tended to give inconsistent labeling yields of AS1411 or CRO in all concentrations; therefore, HPLC-purified ONs are preferred in order to achieve consistent labeling yields. The effect of ON purity on the labeling yield, however, was present regardless of whether the ON was in a single-stranded form or in a G-quadruplex form.

**Effect of buffer.** The labeling steps for AS1411 and CRO in concentration of 200 nmol/100 μL of ON were tested in three different buffers: tris-HCl, sodium borate, and PBK (50 mM, pH 8.5). The labeling yields of $5 \pm 2$, $12 \pm 3$, and $31 \pm 4$ ($n = 3$), were obtained, respectively, for AS1411; however, the yields were $30 \pm 2$, $45 \pm 3$, and $68 \pm 4$% ($n = 3$), respectively, for CRO under the same experimental conditions ($p < 0.05$). Figure 3 shows the UV melting results of AS1411 and CRO in three different buffers using the denaturation method. AS1411 could readily form a G-quadruplex structure in the presence of potassium ions (d) and perhaps form different or less stable G-quadruplex structures in the presence of other ions (e and f). On the other hand, CRO (a–c) clearly exhibited a different structural conformation as compared with AS1411 in all buffers. The choice of buffer appeared to affect the labeling yields of both AS1411 and CRO in the same order, with the best yield obtained with potassium ions. However, the tendency to form G-quadruplex structures may be responsible for the lower labeling yields obtained in AS1411 compared with CRO under the same experimental conditions. The reason as to why potassium ions are more favorable for higher labeling yields will require further investigation.

## Experimental

### General

**Chemicals.** All chemicals used were commercially available and were used without further purification. SFB was prepared by a similar esterification procedure as reported by Cai *et al.*[22] and Li *et al.*[23] Solid phase extraction (SPE) columns were C18 Sep-Pak cartridges (Waters, USA) and LiChrolut EN (Merck, Germany). AS1411 with the sequence of 5′-GGT GGT GGT GGT TGT GGT GGT GGT GG-3′ and CRO with the sequence of 5′-CCT CCT CCT CCT TCT CCT CCT CCT CC-3′,

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which have phosphodiester DNA backbones and 3′-$(CH_2)_6$-$NH_2$ modification, were purchased from Intergrated DNA Technologies (IDT), Inc. (Coralville, IA, USA).

**Analytical methods**. Thermal denaturation experiments were carried out using an Ultrospec 2000 instrument equipped with a Peltier effect heated cuvette holder and temperature controller (Amersham Pharmacia Biotech). Absorbance at 295 nm was monitored over a temperature range of 20–99°C at a heating/cooling rate of 1°C/min. 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. ESI was analyzed by IDT Inc. (Coralville, IA).

## Chemistry

**G-quadruplex preparation**. AS1411 in G-quadruplex structure was prepared according to the previously published method[8] prior to the conjugation. Typically, 100 nmol of HPLC-purified AS1411 was dissolved in 80 μL, 50 mM of PBK with pH 8.5. The sample was annealed by boiling for 5–10 min and cooled down to room temperature before overnight incubation at 4°C. The formation of the G-quadruplex structure was monitored by UV melting.

**Synthesis of chromatographic standard: AS1411 conjugation with N-succinimidyl 4-fluorobenzoate (SFB)**. Twenty μL of SFB-DMF (1 μmol) was mixed with 80 μL of 100 nmol HPLC-purified AS1411 in 50 mM of PBK (pH 8.5), vortexed and then reacted at 22°C for 20 min. The mixture was precipitated by dropping 1 mL of *n*-butanol into it, vortexed and centrifuged. The pellet was washed with 95% of ethanol and then re-constituted with different solutions according to different purposes. The conjugated product was identified by the results obtained from analytical HPLC, UV melting, and ESI-MS.

**Labeling AS1411 with N-succinimidyl 4-[$^{18}$F]fluorobenzoate ([$^{18}$F]SFB)**. Typically, 1 mCi of [$^{18}$F]SFB, which was synthesized based on a published method,[24] was resolubilized in 20 μL of DMF before adding 100 nmol/80 μL AS1411 in PBK. The mixture was vortexed and reacted at room temperature for 10 min and then precipitated with alcohol as described above. The radioactivity pellet was re-constituted with PBK for further use. Different factors including AS1411 concentration, base composition (AS1411 vs CRO), ON purity (desalted vs HPLC-purified) and different buffers (tris-HCl, sodium borate, and PBK) were investigated to optimize the labeling yields. The labeling yield was decay corrected and calculated as follows: % (radioactivity in pellet)/(radioactivity in the supernatant + radioactivity in pellet). Non-significant amount of radioactivity was found to be bound to the centrifugation tube in control experiments without any ON ($n = 3$). The radiochemical purity was monitored by analytical HPLC.

**Statistical analysis**. Student's *t*-test with Bonferronic correction was used for comparison of any two groups in experiments with multiple groups. One-way ANOVA was used to determine whether there was a trend in the data. A *P*-value of $< 0.05$ was considered significant.

## Conclusion

ON concentration, ON purity, and buffer choice greatly affected the labeling yields of AS1411 with [$^{18}$F]SFB. HPLC-purified CRO, which is an ON with a base composition different from AS1411, showed higher labeling yields than HPLC-purified AS1411 under the same experimental conditions. The labeling yields for desalted AS1411 and CRO were much lower than those for HPLC purified. The presence of cations and higher order structure such as G-quadruplex can significantly influence the labeling yield of ONs with [$^{18}$F]SFB. The highest yield for labeling AS1411 with [$^{18}$F]SFB was 35% when 200 nmol/100 μL of HPLC-purified AS1411 in PBK was used.

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