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Chemiluminescence quenching of pteroic acid–*N*-sulfonyl-acridinium-9-carboxamide conjugates by folate binding protein

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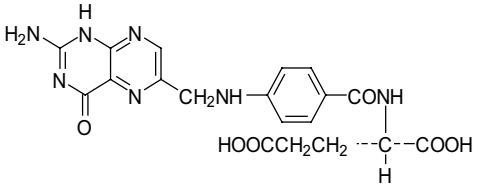
Abstract—N^{10}-Trifluoroacetylpteroic acid was conjugated to chemiluminescent *N*-sulfonylacridinium-9-carboxamide labels at the N^{10} or 9-position carboxamide. Upon binding to folate binding protein the light output of the N^{10} derivative (**9**) was quenched up to 62% upon triggering with basic peroxide, while the 9-position carboxamide conjugate (**7**) was quenched only 12%. The utility of this effect was demonstrated in a model homogeneous chemiluminescent assay for folic acid.

Chemiluminescent *N*-sulfonyl-acridinium-9-carboxamides are among the most efficacious labeling reagents used worldwide in modern medical diagnostics. They have achieved that status by virtue of the detection sensitivity (equal or exceeding radioisotopes), specificity (low sample matrix interference), dynamic range (10^6), short read time (flash kinetics), and ease of use (variety of reactive functional groups for conjugation, hydrophilicity, simple equipment requirements).[1]

Recently, we communicated for the first time that the chemiluminescence of acridinium salts can be quenched by specific binding to a protein.[2] In that example the signal from an N^{10}-biotinylated acridinium-9-carboxamide conjugate was quenched 92% in the presence of avidin, laying the foundation for a homogeneous chemiluminescent assay for biotin. In many respects avidin–biotin receptor–ligand pairing is unique. Avidin is a tetrameric glycoprotein that binds four biotin ligands with extraordinarily high affinity (10^{-15} M). The ligand binding pocket is very deep, estimated to be 15 Å.[3]

In this report, we investigated whether a lower affinity binding interaction could result in efficient chemiluminescent quenching. The folate/folate binding protein system was chosen as the test case. Folic acid (pteroylglutamic acid, PGA) (**1**) is the parent structure of a class of enzyme cofactors important in one-carbon metabolism and linked to serious disorders including congenital malformations, cardiovascular disease, and carcinogenesis. A number of endogenous folate binding proteins have been identified in a variety of tissues.[4] The commercially available folate binding protein (FBP) isolated from cow's milk is a 222 amino acid glycoprotein[5,6] with a reported association constant[7] of 2×10^7–9×10^9 M^{-1}. Even though, the architecture of the binding site has not been defined, FBP has been used successfully in many assay formats for the detection of folic acid.[7–10]



1, Folic acid

Two labeled ligands for FBP were prepared as shown in Scheme 1. Pteroic acid (**2**) was first acylated with trifluoroacetic anhydride to give N^{10}-trifluoroacetyl pteroic acid **3**.[11] Compound **3** was activated with *N*-hydroxysuccinimide (NHS) and 1-[3-(dimethyl-aminopropyl)]-3-ethylcarbodiimide hydrochloride (EDAC) and then

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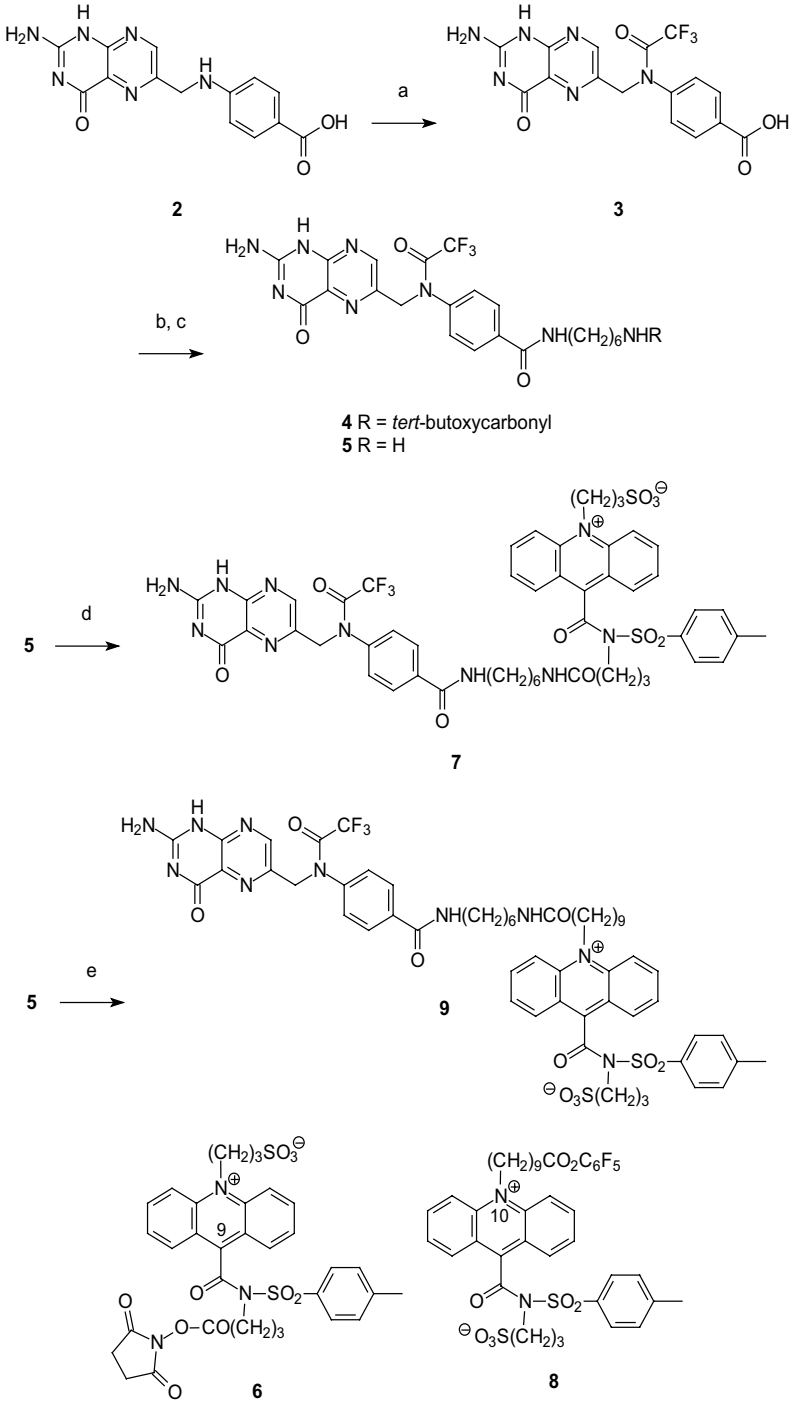

Scheme 1. Preparation of FBP ligands. (a) i. Trifluoroacetic anhydride, 60–65 °C, sealed tube, 72 h; ii. aq ethanol, reflux, 1 h, 89%; (b) i. diisopropylethylamine, NHS, EDAC, DMF, 20 h; ii. $BocNH(CH_2)_6NH_2$, 24 h, 94%; (c) trifluoroacetic acid, dichloromethane, 2 h, 76%; (d) **6**, 50 mM phosphate buffer (pH 8)/DMF, 16 h, 46%; (e) **8**, DMF, triethylamine, 3 h.

condensed with 6-(*N*-*tert*-butoxycarbonylamino)hexylamine to yield the N-protected pteroic acid derivative **4**. Acidolysis of the Boc group gave the amino functionalized intermediate **5** as a trifluoroacetic acid salt.

The acridinium-9-carboxamide active esters **6**[12] and **8**[2] bearing linkers extending from the 9- and 10-positions, respectively, were conjugated to pteroylamine **5** to give the chemiluminescent folate tracers **7** and **9**.

Chemiluminescence of **7** and **9** were first evaluated in the absence of FBP. As shown in Figure 1a, the time/intensity profiles of the two compounds dissolved in buffer (10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.3) were similar upon reaction with basic hydrogen peroxide, regardless of the regiochemistry of attachment to the pteroic acid nucleus. Both tracers exhibited flash-type luminescent profiles with all light being generated within 5 s after addition of the triggering reagent. The

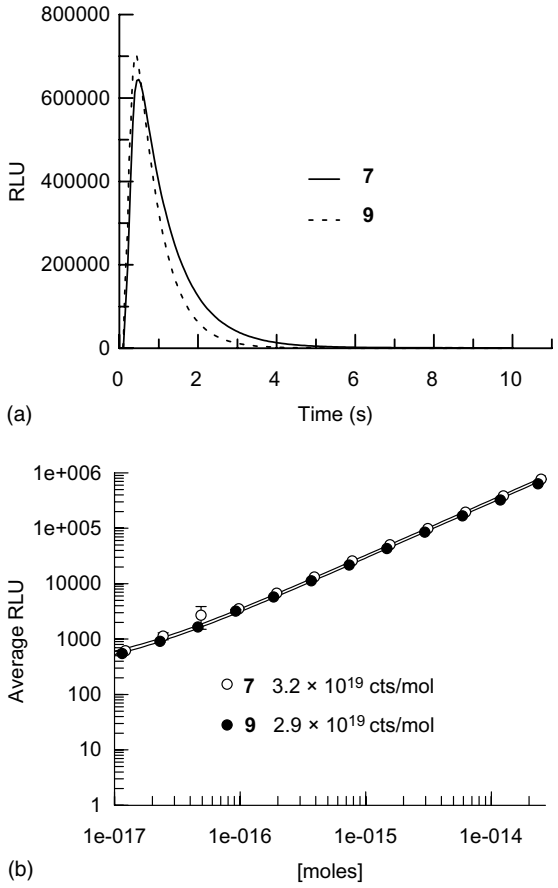

Figure 1. Chemiluminescence of ligands **7** and **9**. (a) Time/relative light units profile. The labeled ligands were triggered with 200 μL 0.18 N NaOH, 0.7% H_2O_2, 1% Triton X-100, 0.05% diethylenetriaminepentacetic acid. (b) Dilution linearity. Ligands were diluted in 10 mM borate, 2 mM EDTA, 0.1% Tween-20, pH 7.3, and triggered as above.

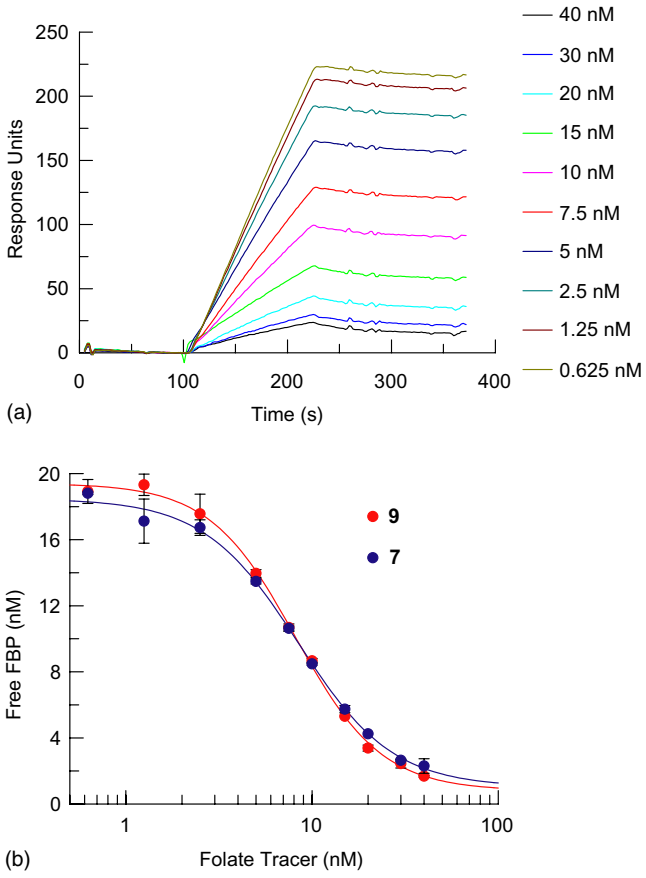

Figure 2. Binding of FBP to ligands **7** and **9**. (a) BIACore sensorgram: FBP (20 nm), ligand **9** (0.625–40 nM). (b) IC_{50} plot. (Data points represent the average of duplicate values. The curve represents the best nonlinear fit of the data using a four-parameter logistic.)

integrated signal for compound **7** was 3.2×10^{19} counts/mol while compound **9** was essentially the same, 2.9×10^{19} counts/mol (Fig. 1b).

The ability of the acridinylated ligands to bind to FBP was then tested independently of the chemiluminescent signal from the acridinium moiety in a solution competition surface plasmon resonance format on the BIAcore instrument (Fig. 2).[13] Thus, a high-density biosensor was prepared by conjugating pteroylamine **5** to a CM-5 carboxylated chip according to the manufacturer's protocol. A solution of FBP (20 nM) was incubated with varying concentrations of **7** or **9**, and then injected onto the biosensor. The resulting sensorgrams (Fig. 2a) were used to construct a plot of the concentration of free FBP versus ligand (Fig. 2b). The IC_{50} for each ligand was determined from the plot. The values for **7** (8.43 ± 0.46 nM) and **9** (8.14 ± 0.29 nM) were essentially the same.

The effect of binding of the ligands to FBP on the chemiluminescent signal from the acridinium-9-carboxamide label was next investigated. Specifically, the acridinium-derivatized FBP ligands **7** and **9** (1 nM) were individually mixed with serially diluted folate binding protein (0.08–40 nM) in assay buffer. After the solutions were incubated for 45 min, the chemiluminescence response from each mixture (25 μL) was recorded. Plots of the normalized relative light units (RLU) obtained versus folate binding protein concentration are shown in Figure 3.

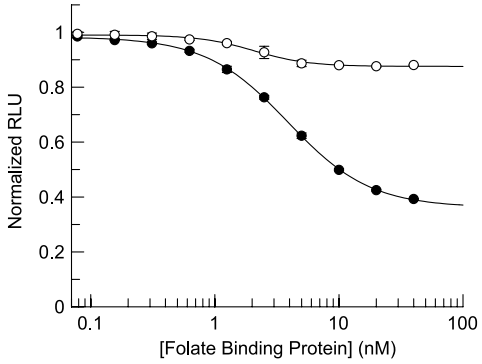

Figure 3. Plot of normalized chemiluminescent response for (**9**) (•) and (**7**) FBP ligands (○) versus folate binding protein concentration. (Data points represent the average of duplicate values. The curve represents the best nonlinear fit of the data using a four-parameter logistic.)

The data show that chemiluminescence from an acridinium–FBP ligand can be quenched by folate binding protein in a concentration-dependent manner. Significantly, the chemiluminescent output derived from ligand **9** is quenched up to 62% upon titration with folate binding protein. In contrast, ligand **7** was quenched only 12% upon titration with folate binding protein. Identical studies utilizing the ligands **7** and **9** and bovine serum albumin (BSA) were also conducted as controls. Chemiluminescent signal produced from these controls over the entire BSA concentration range tested (0.08–40 nM) did not vary significantly. The results demonstrate that chemiluminescence quenching is due to a specific binding interaction.

The FBP specific modulation of acridinium chemiluminescence was further demonstrated in a model homogeneous assay for folic acid. Thus, a fixed concentration of ligand **9** (1 nM) was added to several concentrations of folic acid (0.75–10 nM) in assay buffer. The folate containing mixtures were subsequently added to a fixed concentration of folate binding protein (20 nM) also in assay buffer. The solutions were allowed to incubate for 45 min, and 25 μL of each sample was triggered as above. The dose–response curve obtained is depicted in Figure 4. The average % quenching value reported in Figure 4 is relative to the chemiluminescent response obtained for ligand **9** in buffer alone. As expected, acridinium chemiluminescence quenching due to association of folate binding protein with the folate tracer decreases with increasing concentrations of folic acid.

The observation that linkage of pteroic acid through the N^{10}-position of the acridinium-9-carboxamide nucleus

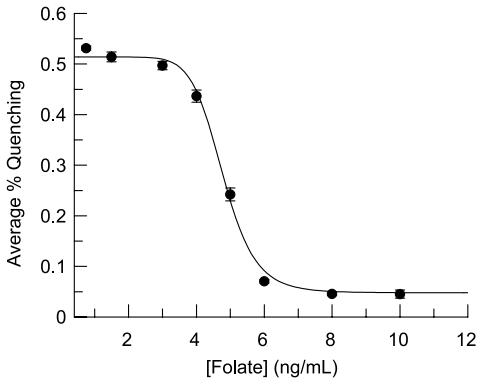

Figure 4. Dose–response curve generated for folic acid. (Data points represent the average of triplicate values. The curve represents the best nonlinear fit of the data using a four-parameter logistic.)

(**9**) results in more efficient chemiluminescence quenching than that of the 9-position analog (**7**) is consistent with the results previously reported in the biotin-acridinium-9-carboxamide–avidin system[2] albeit falling short in magnitude. Based on the accepted mechanism of acridinium salt chemiluminescence, we rationalize that in both systems, the acridinium-ligand–protein complex, when reacted with basic peroxide, gives rise to an excited state acridone chemiluminophore, which is either free in solution or left bound to the protein. This is illustrated for compounds **7** and **9** in Figure 5.

In the bound state, the excited acridone from **9** must be held in the proximity of a quenching element while the acridone from **7** is free to dissociate from the FBP-ligand complex.

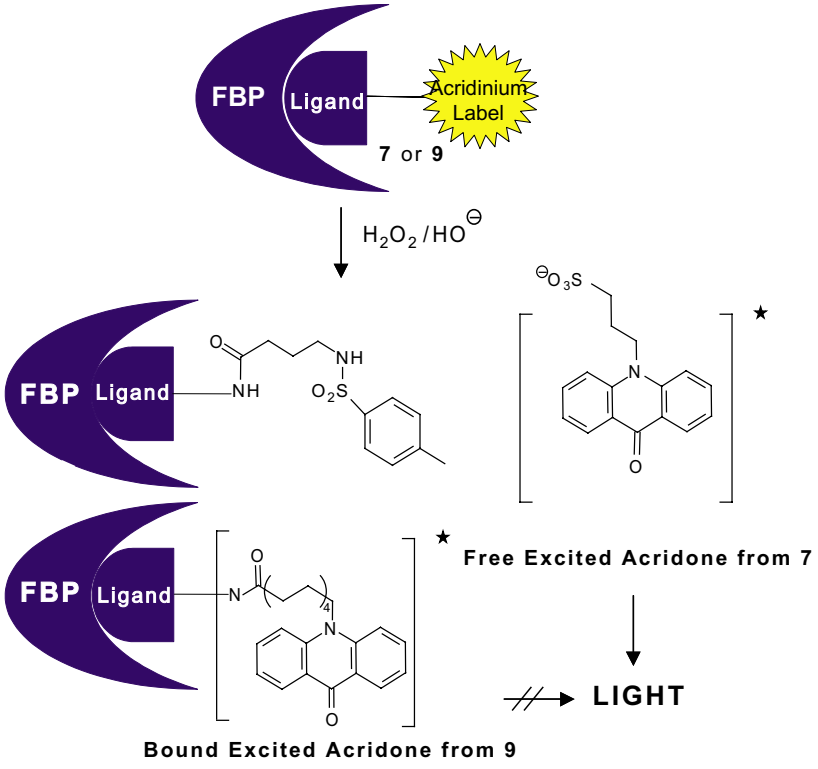

Figure 5. FBP-mediated quenching of ligands **7** and **9**.

In summary, the 62% chemiluminescence quenching efficiency observed in the N^{10}-acridinium-9-carboxamide-pteroic acid–FBP binding system was sufficient to demonstrate a homogeneous chemiluminescent assay format for the detection of folic acid.

We continue to explore other binding protein/ligand-acridinium-9-carboxamide pairs to further define the general scope of this technology.

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