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Highly Photoresistant Chemosensors Using Acridone as Fluorescent Label

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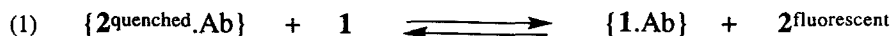
Abstract: *Highly photoresistant and selective chemosensors have been prepared using acridone as fluorescent label in combination with monoclonal antibodies.* © 1997 Elsevier Science Ltd. All rights reserved.

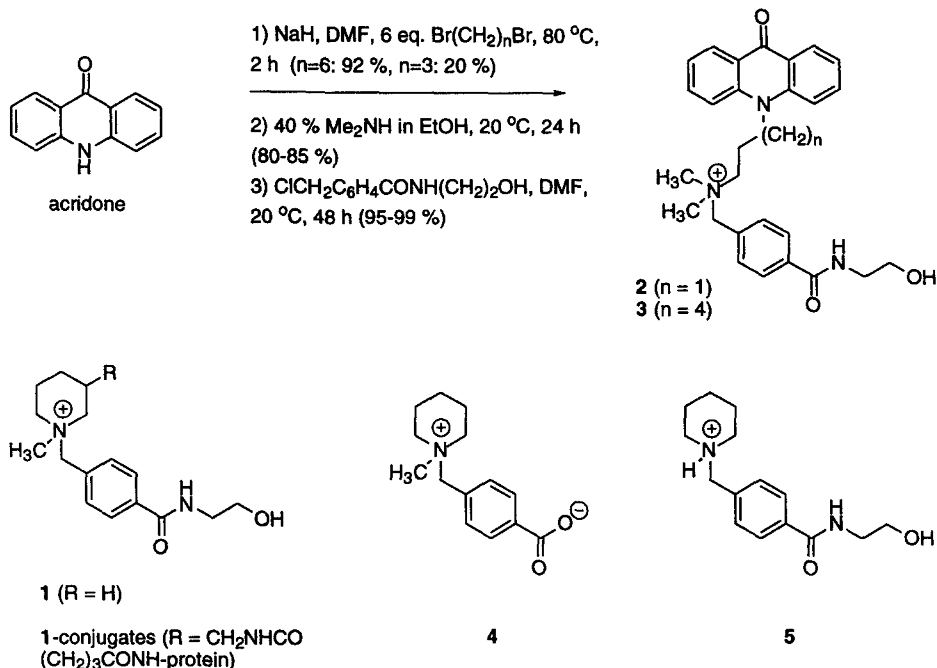
Fluorescent chemosensors modulate a fluorescent signal in response to the local concentration of an analyte, for example a drug,[1] metabolite,[2] or an inorganic ion.[3] Existing chemosensors generally suffer from photobleaching under irradiation at the excitation wavelength of the fluorophore, which severely limits their useful lifetime. While the use of short laser pulses somewhat limits the damage of photobleaching,[4] the problem can in principle be solved more directly by incorporating a photoresistant yet modulable fluorophore as light emitting component in the chemosensor. Herein we report upon the preparation of chemosensors using the fluorophore acridone in combination with antibodies. These chemosensors exhibit very high resistance to photobleaching and high sensitivity and selectivity for the analyte.

We recently reported upon an assay for screening catalysis in high-throughput format using substrates labeled with the fluorescent tag acridone.[5] Acridone was found to be highly resistant to bleaching, with no observable decrease in fluorescence upon continuous UV irradiation at 254 nm or 356 nm adsorbed on TLC plates for several weeks. By contrast fluorescein, dansyl, anthracene and rhodamine, which are most often used as fluorescent components in chemosensors, underwent complete bleaching within minutes under these conditions. We reasoned that acridone should allow to prepare photoresistant sensors if its fluorescence could be modulated by an analyte recognition process.

To test this hypothesis, we investigated if acridone might be used in a chemosensor for analyte **1** using antibodies raised against a protein conjugate of **1** as sensing component.[6] Acridone-labeled achiral hapten analogs **2** and **3** were prepared by alkylation of acridone and purified by preparative reverse-phase HPLC.[7] A series of forty-one anti-**1** monoclonal antibodies was then assayed for fluorescence quenching of **2** or **3**. Ten antibodies quenched the fluorescence of **2** or **3** by more than 80 % upon binding (eight for **2** and five for **3**). In all cases addition of analyte **1** restored fluorescence to the level corresponding to free **2** or **3** in solution. This showed that the quenching interactions were taking place in the combining sites of the antibodies, and established that the complexes of the quenching antibodies with **2** or **3** functioned as selective chemosensors for analyte **1** according to a reversible competitive displacement equilibrium (eq. 1, Ab = anti-**1** antibody).[8]



$$(1) \quad \{2^{quenched}.Ab\} + 1 \rightleftharpoons \{1.Ab\} + 2^{fluorescent}$$



Chemosensors {**2**.34F7} and {**2**.83B8} were characterized in detail. In both cases two equivalents of acridone derivative **2** were bound per antibody molecule. Fluorescence of **2** was quenched by 90% with antibody 34F7 and by 98 % with antibody 83B8 compared to the intensity of free **2** in solution. The dissociation constants were K_D({**2**.34F7}) ~ 5 nM and K_D({**2**.83B8}) ~ 50 nM as estimated by serial dilution of the 2:1 complexes. The fluorescence increase upon addition of analyte followed a sigmoidal curve as a function of log([**1**]) (fig. 1 and 2). While signal stabilization required approximately 2 minutes with {**2**.34F7}, chemosensor {**2**.83B8} responded within mixing time upon addition of analyte. Sensor {**2**.34F7} displayed a 250-fold binding selectivity for analyte **1** (EC_{50} = 2 μM)[9] against its carboxyl analog **4**[6] (EC_{50} = 530 μM), and a 20-fold selectivity against the piperidine analog **5**[6] (EC_{50} = 40 μM). The selectivity of sensor {**2**.83B8} was complementary, with only a marginal discrimination between **1** (EC_{50}= 8 μM) and **4** (EC_{50} = 18 μM) but a 120-fold selectivity against the piperidine analog **5** (EC_{50} = 980 μM). These selectivities were combined by using the ternary combination **2**+34F7+83B8, which discriminated analyte **1** (EC_{50} = 9.5 μM) 105-fold against **4** (EC_{50} = 1000 μM) and 80-fold against **5** (EC_{50} = 750 μM). Thus these acridone-based chemosensors performed satisfactorily in terms of response times, structural selectivity, and sensitivity for analyte **1**.

We then investigated the behavior of the chemosensors under sustained light irradiation to probe their resistance to photobleaching. Samples of free **2**, {**2**.34F7}, {**2**.83B8} and **2**+34F7+83B8 (each 10 μM in aqueous buffer pH 7.4) were exposed to continuous UV irradiation from a TLC lamp (1350 μw/cm^2, 254 nm or 356 nm) or daylight at 20 °C.. Treatment at 254 nm for 12 hours did not affect the acridone nucleus itself but

resulted in destruction of the antibodies and the haptenic part of **2**, as evidenced by the fact that the solutions showed a fluorescence spectrum expected for free acridone (10 μM), but which could not be quenched by addition of fresh antibody 34F7 or 83B8. By contrast *the samples were totally unaffected after exposure to either 356 nm UV light or daylight for four days.* The fluorescence of the free labeled analyte **2** was unchanged, and underwent complete quenching upon addition of antibody 83B8, proving that the molecule was intact. The resulting sensor and the treated antibody-sensors, which were still fully quenched after the prolonged irradiation, returned to full fluorescence in presence of analyte **1** with apparent EC_{50} identical to that of unexposed control samples of the sensors.

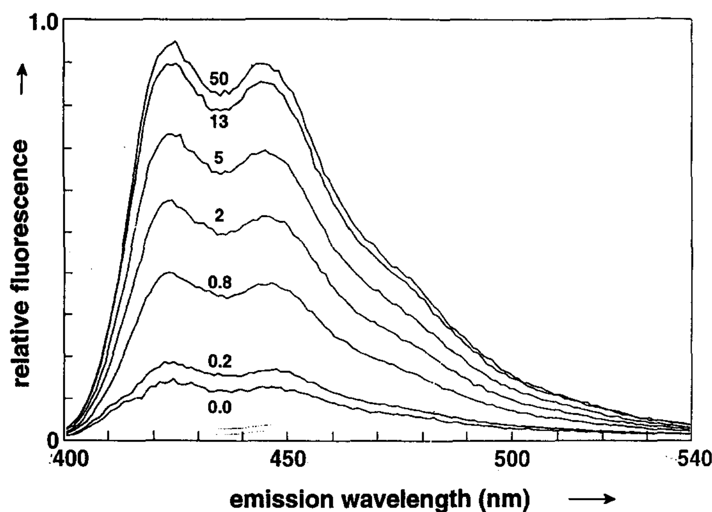


Figure 1.[10] Fluorescence emission spectrum of chemosensor {**2**.34F7} upon addition of analyte **1**. λ_{exc} = 356 nm, 0.5 μM 34F7 + 1 μM **2** in aq. 160 mM NaCl, 10 mM phosphate, pH 7.4.

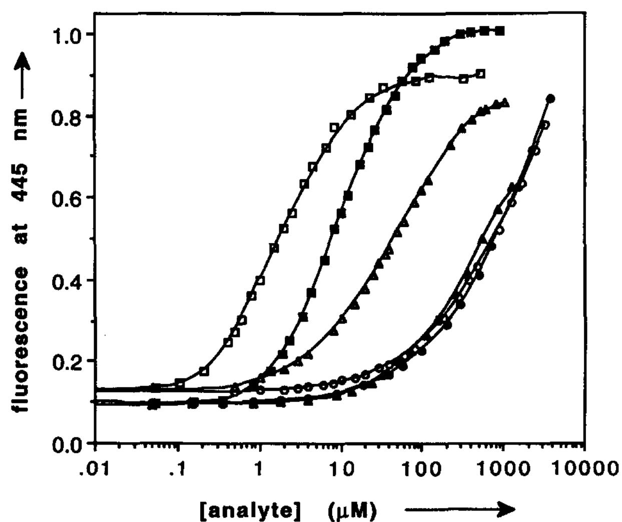


Figure 2. Fluorescence readings of chemosensors at 445 nm as a function of analyte concentration, as logarithmic plot. Analyte **1** using {**2**.34F7} (□) and **2**+34F7+83B8 (■); analog **5** using {**2**.34F7} (Δ) and **2**+34F7+83B8 (▲); analog **4** using {**2**.34F7} (o) and **2**+34F7+83B8 (•). The lines were obtained by interpolation from the experimental points.

In summary we have shown that acridone is a useful building block for the preparation of fluorescent chemosensors. Acridone is readily derivatized by simple alkylation chemistry, and in combination with antibodies leads to chemosensors which are highly selective and have short switching times. Acridone fluorescence is almost quantitatively modulable by the analyte recognition process, and provides chemosensors with very high resistance to photobleaching.

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[7] **2** (trifluoroacetate salt): pale yellow crystalline solid, m.p. 206-208 °C (dec.), HRMS (FAB+): $C_{28}H_{32}N_3O_3^+$, calc.458.2444, obs. 458.2461. ^{1}H-NMR (300 MHz, D_2O): 8.13 (dd, ^3J = 8.5, 2 Hz, 2H); 7.72 (ddd, ^3J = 9, 7, 2 Hz, 2H); 7.47 (d, ^3J = 9 Hz, 2H); 7.33 (d, ^3J = 8.5 Hz, 2H); 7.26 (t, ^3J = 7 Hz, 2H); 7.01 (d, ^3J = 8.5 Hz, 2H); 4.40 (t, ^3J = 7 Hz, 2H); 4.31 (s, 2H); 3.80, 3.53 (2 t, ^3J = 6 Hz, 2 x 2H); 2.96 (s, 8H); 2.28 (m, 2H).^{13}C-NMR (100 MHz, D_2O + 30 % CD_3OD): 179.3, 170.1, 141.7, 136.5, 136.1, 133.4, 131.0, 128.5, 127.5, 123.2, 121.6, 116.0, 68.5, 61.0, 51.3, 43.1, 42.1, 21.9. **3** (trifluoroacetate salt): yellow oil, HRMS (FAB+): $C_{31}H_{38}N_3O_3^+$, calc.500.2913, obs. 500.2894. ^{1}H-NMR (300 MHz, D_2O): 8.12 (dd, ^3J = 8.5, 2 Hz, 2H); 7.72 (d, ^3J = 8.5 Hz, 2H); 7.65 (ddd, ^3J = 9, 7, 2 Hz, 2H); 7.49 (d, ^3J = 9 Hz, 2H); 7.47 (d, ^3J = 8.5 Hz, 2H); 7.20 (dd, ^3J = 8.5, 7 Hz, 2H); 4.37 (s, 2H); 4.14 (t, ^3J = 7.5 Hz, 2H); 3.73, 3.49 (2t, ^3J = 6 Hz, 2 x 2H); 3.05 (m, 2H); 2.94 (s, 6H); 1.78-1.57 (m, 4H); 1.40-1.20 (m, 4H).^{13}C-NMR (100 MHz, D_2O + 10% CD_3OD): 178.9, 170.0, 141.6, 136.3, 135.4, 133.7, 131.3, 128.6, 127.1, 122.5, 121.4, 116.0, 67.3, 64.7, 60.9, 50.9, 46.3, 43.05, 27.3, 26.3, 26.2, 23.0.

[8] For a general introduction on antibodies as fluorescent chemosensors, see: Smith, D.S.; Al-Hakiem, M.H.H.; Landon, J. *Ann. Clin. Biochem.* **1981**, *18*, 253.

[9] EC_{50} is defined here as the concentration inducing 50% of the maximum fluorescence increase.

[10] Fluorescence measurements were carried out at 20 °C in a 1x1 cm cuvette on a SPF-500C spectrofluorometer from SLM Instruments, Inc. with l_{exc} = 356 nm, set to give maximum fluorescence reading (2.0) at 445 nm for 2 µM free **2**. Aliquots of 4 µL of properly prediluted solutions of analyte **1**, **4** or **5** were added to 2 mL of sensor (1 µM **2** + 0.5 µM antibody 34F7 and/or 0.5 µM antibody 83B8) in aqueous 160 mM NaCl, 10 mM phosphate, pH 7.4. The solution was then mixed using a 1 mL pipetter and allowed to stand for 2 minutes before recording fluorescence.

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