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# Dual Analyte Detection Using Tandem Flash Luminescence

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**Abstract**—A heterogeneous, dual analyte-binding assay which makes use of the flash luminescence from both aequorin and an acridinium-9-carboxamide label is presented. The signal generating species were triggered both differentially and sequentially using $Ca^{2+}$ followed by basic peroxide. Both signals were resolved readily using a single photomultiplier tube without the need for multi-wavelength detection. To demonstrate the tandem luminescence concept in a model assay system, dose–response curves for two analytes, biotinylated BSA and myoglobin, were generated using a competitive binding format. Because of the relatively short assay time and the well-resolved signals, this format will be useful in the development of dual analyte high-throughput assays. © 2002 Elsevier Science Ltd. All rights reserved.

A variety of assays used for biological research and clinical diagnostics employ detection reagents labeled with species capable of generating a luminescent signal.[1] Luminescent labels are desirable as they provide high sensitivities, in part due to their low naturally occurring backgrounds in biological systems and their relatively high luminescent yields. Not surprisingly, methods for generation of luminescent signals have been recently combined for use in dual analyte detection formats.[2–13] Several methods have been described in which multiple luminescent signals are generated simultaneously and differentiated by differences in the kinetic profile of the respective luminescent reactions (kinetic resolution).[2–5] Differentiation of multiple luminescent signals has also been achieved through monitoring differences in the wavelength of light produced from a specific luminescent species.[5,6] Alternatively, methods of differential induction have been described which generate well-resolved signals sequentially that are easily distinguishable by the time frame in which they are generated.[7–13] This later approach has been used in conjunction with commercially available luminometers without the need for kinetic resolution or multi-wavelength detection.

While methods of differential induction have been shown to be effective, there are limitations to their use in the area of high-throughput detection. All of the systems described employ an enzymatically-derived response for at least one of the luminescent signals being generated. All the enzymatically-derived signals exhibit glow kinetics. For high throughput applications such long-lived kinetics can be disadvantageous because the wait time to collect enough light to achieve a needed sensitivity can be of the order of tens of minutes. While the cutoff can vary from application to application, a desirable time frame for high-throughput applications is typically of the order of seconds.

We describe here a dual analyte assay employing tandem *flash* luminescence detection derived from the photoprotein aequorin and an acridinium-9-carboxamide label. Specifically, a model combination assay for biotinylated BSA and myoglobin was developed. The two analytes were measured in a competitive heterogeneous assay format with biotinylated aequorin and acridinium-labeled myoglobin binding to immobilized avidin and anti-myoglobin pAbs, respectively.

Thus, biotinylated aequorin was prepared in a manner analogous to a previously described procedure.[14] Briefly, recombinant apoaequorin was purified by a combination of ion-exchange and size exclusion chromatographies from cultures of *Escherichia coli* BL21(DE3)pLysS transformed with a pET-derived apoaequorin construct. The active photoprotein was generated by incubation of the purified apoaequorin with a 10-fold molar excess of coelenterazine overnight at 4 °C in 30 mM Tris, 2 mM EDTA, 5 mM DTT, pH 7.6 (storage buffer). The resulting aequorin was dialyzed into 100 mM $NaHCO_3$, 200 mM NaCl, 0.5 mM EDTA, pH 8.6 and biotinylated

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using biotin active ester **1** (Pierce; Fig. 1). Excess labeling reagent was subsequently removed by exhaustive dialysis against storage buffer. Using an electrospray ionization mass spectrometry (ESI-MS) technique the ratio of biotin-to-aequorin was determined to be 0.8.[15]

Acridinium-labeled myoglobin was prepared by the addition of the acridinium-9-carboxamide active ester **2** dissolved in DMF (0.2 mL, 4 mg/mL)[16,17] to a solution of horse skeletal muscle myoglobin (2.5 mL, 2.5 mg/mL) in 100 mM sodium borate, pH 9.0. The resulting reaction was allowed to stand at ambient temperature in the dark for 2 h, after which unconjugated label was removed using a desalting column (MWCO = 6 kDa) equilibrated with 100 mM sodium borate, pH 9.0. The ratio of acridinium-to-myoglobin was determined to be 3.6 using ESI-MS.[15]

Microtiter plates containing immobilized avidin and anti-myoglobin pAbs were prepared as a capture surface for the biotinylated aequorin and acridinium-labeled myoglobin, respectively. Avidin (Pierce, ImmunoPure, 10 μg/mL) and affinity purified goat anti-horse myoglobin (Bethyl Laboratories, 20 μg/mL) were mixed 1:1 in 100 mM sodium phosphate, 150 mM NaCl, pH 8.5 (PBS) and applied to 96-well microtiter plates containing an oxosuccinimide activated surface (DNA-Bind, Costar). The immobilization reactions were incubated for 2 h at ambient temperature and unbound proteins were subsequently removed by three successive washes with PBS containing 0.2% Tween-20. The remaining unreacted sites on the microtiter plates were blocked by incubation with PBS containing 2% BSA for 1 h. Control surfaces in which the avidin or anti-myoglobin antibodies were individually replaced with BSA were also prepared in an identical manner.

The signals derived from both aequorin and acridinium were well resolved within a relatively short time frame. Biotinylated aequorin and acridinium-labeled myoglobin in 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.2% Tween-20, pH 7.6 (TBS/Tween-20) were simultaneously captured on an immobilized avidin/anti-myoglobin surface. Subsequent removal of unbound proteins by three successive washes with TBS buffer and triggering of luminescence with $Ca^{2+}$ ($t = 0$) followed by basic peroxide ($t = 10$ s) provided two cleanly resolved signals (Fig. 2). Under these optimized conditions the flash kinetics permitted the detection of a majority of the light from both species well within a total read time of 20 s.

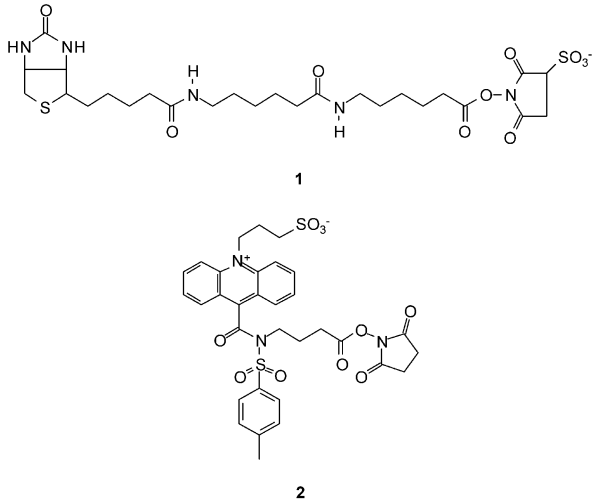


**Figure 1.** Structures of the biotin active ester (**1**) and acridinium-9-carboxamide active ester (**2**) utilized in the preparation of aequorin and myoglobin conjugates.

Several control experiments were conducted to ensure the observed signals were the result of specific binding events. When BSA was immobilized on the plate in place of anti-myoglobin pAbs, no significant acridinium derived signal was generated upon incubation with biotinylated aequorin and acridinium-labeled myoglobin (Fig. 3, column A). Likewise, when BSA was immobilized on the plate in place of avidin, no significant aequorin derived signal was generated (Fig. 3, column B). Importantly, data from the two control surfaces also indicated that there is no acridinium response upon addition of $Ca^{2+}$ or any luminescent response generated from the products of aequorin bioluminescence upon addition of basic peroxide. The luminescent response obtained when all components of the system were present and were triggered in tandem is depicted in Figure 3, column C.

In order to maximize the dynamic range of detection in the model assay, the dependence of the luminescent response on labeled conjugate concentration was investigated. Specifically, a solution of equal concentrations of biotinylated aequorin and acridinium-labeled myoglobin (360 nM each) in TBS/Tween-20 buffer was serially diluted two-fold across an avidin/anti-myoglobin pAb immobilized microtiter plate. The mixtures were incubated for 2 h and non-specifically bound materials were removed by three successive washes with TBS buffer. Triggering using the standard conditions outlined in Figure 2 and integration of the aequorin and acridinium signals provided the curves shown in Figure

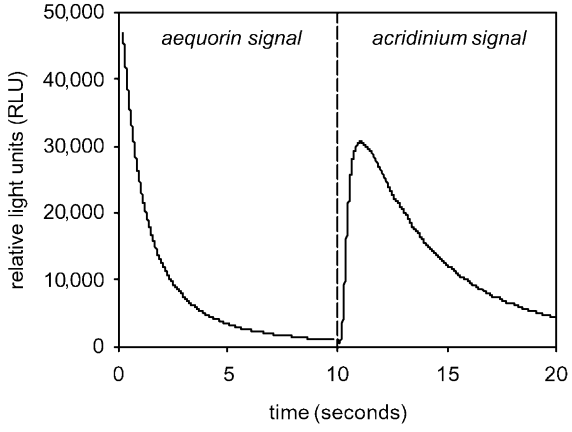


**Figure 2.** Representative luminescence kinetic profile obtained upon sequential triggering of surface-bound biotinylated aequorin followed by surface-bound acridinium-labeled myoglobin. All luminescence readings were taken using a microplate luminometer (MicroLumat Plus, Perkin-Elmer). Aequorin trigger solution: 100 μL 5 mM Tris, 1 mM $CaCl_2$, pH 7.6. Acridinium trigger solution: 125 μL 0.18 N NaOH, 0.7% $H_2O_2$, 1% Triton X-100, 0.05% diethylenetriaminepentacetic acid.

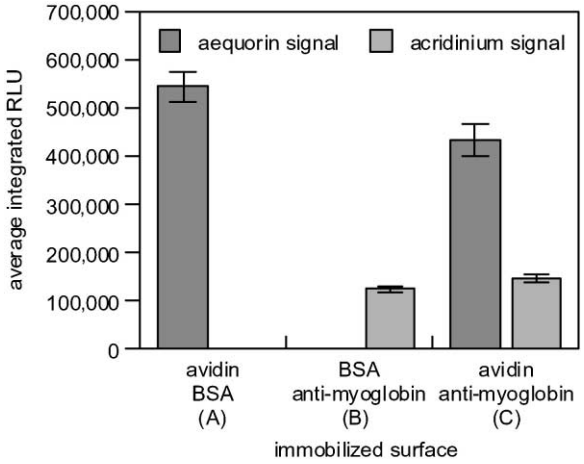


**Figure 3.** Luminescent response versus immobilized protein surface. Data represent the average of quadruplicate values generated using 50 nM biotinylated aequorin/20 nM acridinium-labeled myoglobin and the triggering conditions outlined in Fig. 2.

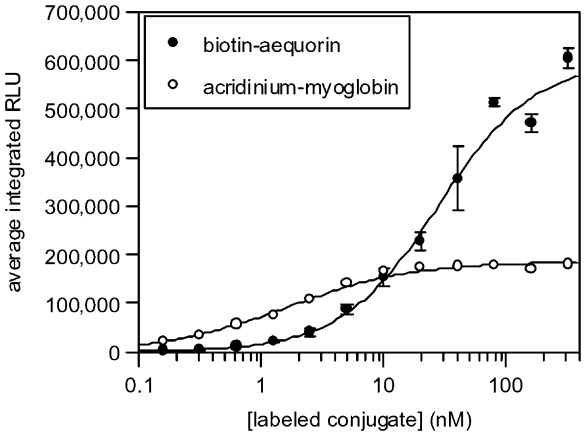


**Figure 4.** Average integrated luminescent response of aequorin and acridinium signals versus the concentration of biotinylated aequorin and acridinium-labeled myoglobin, respectively, using the triggering conditions outlined in Figure 2. Data points represent the average of triplicate values and the curves represent the best fit of the data using a four-parameter logistic.

4. Fixed concentrations of biotinylated aequorin (50 nM) and acridinium-labeled myoglobin (20 nM) were subsequently chosen for the development of dose–response curves.

To demonstrate the application of tandem flash luminescence to a model combination assay, dose–response curves for two model analytes, biotinylated-BSA and myoglobin, were generated (Fig. 5). Mixtures of biotinylated-BSA (0.78–800 nM) and myoglobin (640–0.62 nM) were prepared such that their concentration gradients were oriented in opposite directions. The final mixtures were applied to immobilized avidin/anti-myoglobin pAb surfaces and incubated for 2 h. Thus, the first well contained the highest concentration of myoglobin and the lowest concentration of biotinylated BSA; the last well contained the highest concentration of biotinylated BSA and the lowest concentration of myoglobin. Non-specifically bound materials were subsequently removed by three successive washes with TBS buffer as above and signals were generated using the

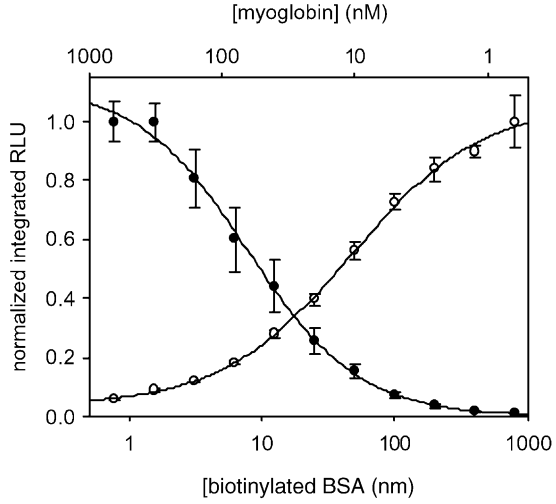


**Figure 5.** Simultaneous dose–response curves generated for biotinylated-BSA (●) and myoglobin (○) generated using the tandem luminescence combination assay format. Data points represent the average of triplicate values and the curves represent the best fit of the data utilizing a four-parameter logistic.

standard triggering conditions described in Figure 2. As expected, an inverse relationship between concentration of the analyte and signal was observed for both analytes. Higher concentrations of myoglobin result in the greater displacement of surface bound acridinium-labeled myoglobin. Likewise, higher concentrations of biotinylated BSA result in a reduced aequorin signal.

The simultaneously generated dose–response curves shown in Figure 5 demonstrate the utility of the aequorin/acridinium label combination for tandem flash luminescent assays. The format retains all advantages associated with the use of chemiluminescent/bioluminescent labels while providing conditions suitable for sequential, rapid signal generation. The system is suitable for a wide variety of analyte combinations and signals can be readily detected using commercially available luminometers without the need for multi-wavelength detection. Efforts to apply this format to high-throughput screening are in progress.

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