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Application Note

Using the FL600 Fluorescence Microplate Reader for Chemiluminescence Detection

What's a lumin anyway?

Chemiluminescence is a highly sensitive technique that has been employed in a wide variety of applications primarily in the biological sciences. Here we describe the use of the FL600 fluorescence microplate reader for "glow" chemiluminescent determinations.

Introduction

Molecules that release light do so as a result of being in a state of high energy, often referred to as an "excited state", and then returning to a lower energy or ground state with a subsequent release of energy (Figure 1). The production of light from excited molecules, as a means to release excess energy, is unusual. In the case of fluorescence the energy necessary to achieve the excited state is provided by illumination with a specific wavelength of light. Chemiluminescence, on the other hand, does not require the input of exogenous light, but rather utilizes the energy contained within specific chemical reactions to provide the necessary energy. With very few exceptions, the efficient chemiluminescence of organic compounds is the result of oxidation (1), but specialized "high energy" oxygenated-compounds have also been developed that will lumninesce when fragmented (2).

The enzyme alkaline phosphatase catalyses the hydrolysis of phosphate moieties from a variety of different molecules. Quantitation of alkaline phosphatase enzyme activity is generally required when alkaline phosphatase has been conjugated to an antibody as part of an immunodetection system. In regards to measurement of alkaline phosphatase several different substrates have been developed that allow direct measurement of the product. For example, the hydrolysis of p-nitrophenyl phosphate (pnpp) by alkaline phosphatase results in the formation of a colored compound that absorbs light maximally at 405 nm (3). Likewise, the action of alkaline phosphatase on methylumbelliferone phosphate (MUP) results in the formation of methylumbelliferone, which can be detected by its fluorescence (4). In order to achieve greater sensitivity substrates have been developed that emit light (luminesce) after being acted upon by alkaline phosphatase.

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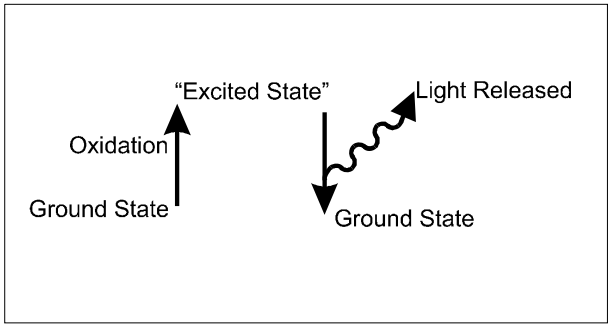

Figure 1. Schematic diagram depicting the generation of light from luminescent molecules in the excited state. Note that destabilization or oxidation of the ground state molecule can accompany the change from ground state to excited state.

One group of compounds that emit light are the 1,2-dioxetanes. As seen in Figure 2, when the 1,2-dioxetane compound CSPD is dephosphorylated by alkaline phosphatase an unstable intermediate anion is formed.

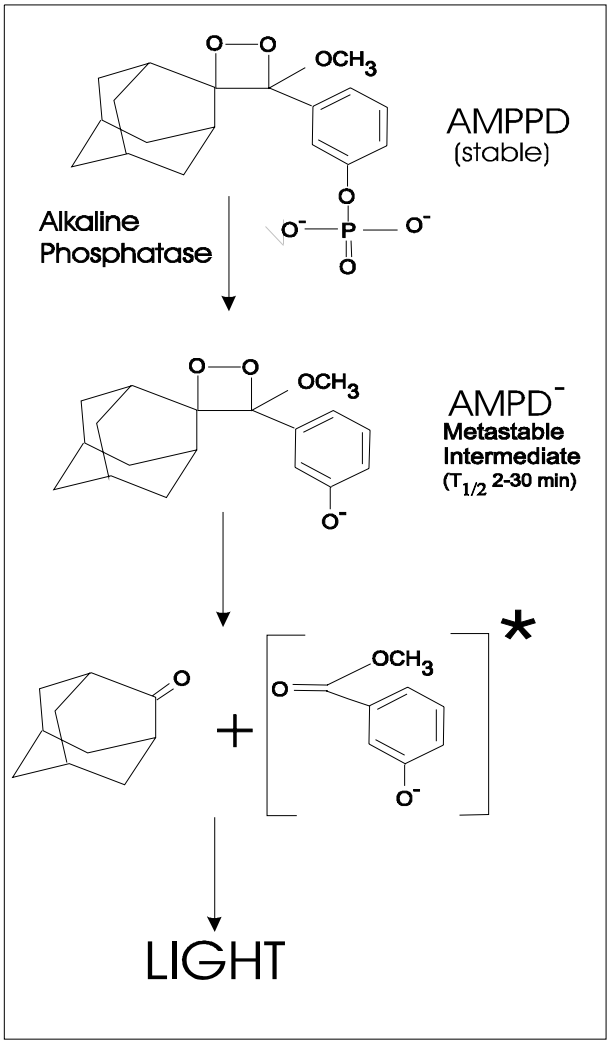

Figure 2. Chemical reaction of substrate (AMPPD) and alkaline phosphatase yielding products that break down and eventually emit light. 1,2 Dioxetane structure that can act as a substrate to several different enzymes depending on the side groups attached for specificity. Regardless of enzyme, after removal of stabilizing side group, compound breaks down into unstable intermediates and after a series of steps eventually emits light.

This intermediate subsequently decomposes, to a carbonyl compound that eventually releases its energy as 466 nm light. Because the intermediate anion decomposes slowly and at a fixed rate, the two step process allows for steady-state chemiluminescence that is dependent on the concentration of alkaline phosphatase.

Other enzymatic activities can be measured with the same basic 1,2 dioxetane structure. For example, a substrate used for b-galactosidase determinations has the same 1,2 dioxetane compound structure present, but has a galactose moiety substituted for the phosphate group seen in Figure 2. Hydrolysis of the galactose group from the molecule leads to the same decomposition, resulting in the emission of light.

The chemiluminescent signal can be further increased by the addition of macromolecular enhancers. Aqueous environments reduce the chemiluminescent signal by water-induced quenching. Macro-molecular enhancers exclude water from the site of chemiluminescence production, thus increasing emission efficiency. Interestingly, many enhancers will alter the emission characteristics of the generated light.

In this application note we describe the use of the FL600 fluorescence microplate reader for the determination of glow-type chemiluminescence with 1,2 dioxetane substrates in conjunction with enhancers.

Materials and Methods

Alkaline phosphatase (catalogue number P-5521) and b-galactosidase (catalogue number G-3153) were from Sigma Chemical Company (St. Louis MO). CSPDä, Galacton-Starä, and Sapphire IIä enhancer were purchased from Tropix (Bedford, Mass.). Opaque white microplates, catalogue number 3912, were obtained from Costar (Cambridge, Mass.).

Alkaline phosphatase assays were performed as follows. A series of dilutions ranging from 0.0 to 2000 ng/ml of calf intestinal alkaline phosphatase were made using alkaline phosphatase assay buffer as the diluent. Alkaline phosphatase assay buffer consisted of 1 mM $MgCl_2$, 100 mM DEA pH 9.0 in deionized water.

After dilution, 10 ml aliquots of samples and standards were pipetted into microplate wells in replicates of four. Substrate-enhancer mix was prepared fresh by diluting Concentrated Sapphire IIä enhancer 1:10 with assay buffer then adding 17 ml of concentrated CSPD substrate solution to each 1.0 ml of diluted enhancer mix resulting in a final substrate concentration of 0.4 mM. The reactions were then initiated by the addition of 200 ml of enhancer-substrate mixture. The reactions were then incubated at ambient temperature for various times dependent upon the initial enzyme concentration. Luminescence determinations were made using a FL600F fluorescence microplate reader. The sensitivity setting was at 150 and the data collected from the top with a 5 mm probe using static sampling with a 0.35 second delay, 50 reads per well. The lamp was turned off and the emission filter was removed.

b-Galactosidase assays were performed as follows. A series of dilutions ranging from 0.0 to 500 mg/ml of b-galactosidase enzyme were made using b-gal assay buffer as the diluent. b-Gal assay buffer consisted of 100 mM sodium phosphate pH 7.5 in deionized water. After dilution, 10 ml aliquots of samples and standards were pipetted into microplate wells in replicates of four. Enhancer-substrate mixture was made by diluting concentrated Sapphire IIä enhancer 1:10 with b-gal assay buffer. Concentrated Galacton-starä substrate solution was then added to a final substrate concentration of 0.1 mM. The reactions were then initiated by the addition of 200 ml of enhancer-substrate mixture. The reactions were then incubated at 37°C for various times dependent upon the initial enzyme concentration. Luminescence determinations were made using a FL600FA fluorescence microplate reader. The sensitivity setting was at 100 or 150 and

the data collected from the top with a 3 mm probe using static sampling with a 0.35 second delay, 50 reads per well. The lamp was turned off and the emission filter was removed.

Results

The importance of appropriate incubation intervals is demonstrated in Figure 3. When the luminescence of an alkaline phosphatase reaction was measured kinetically, an initial increase in luminescence, which peaks and eventually drops off. This increase is most evident and quite rapid with high enzyme concentrations suggesting that the drop in luminescence is the result of substrate consumption by the enzyme. Incubation times that allow determinations prior to the peak are necessary in order to achieve linearity.

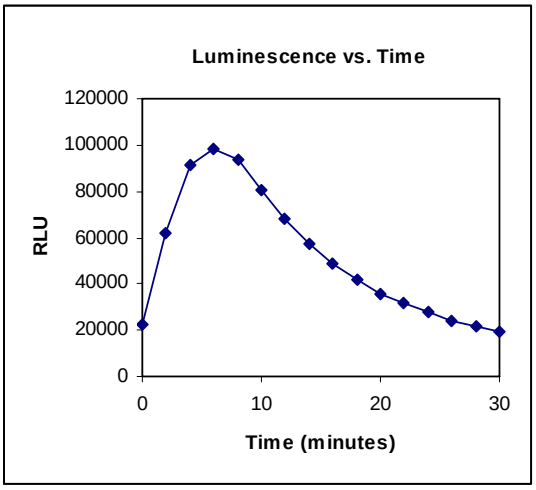

Figure 3. Change in luminescence over time. Luminescence determinations of alkaline phosphatase reactions (5 x10-2 DEA units/well) were made kinetically every 2 minutes for 30 minutes. Reactions were prepared as described in materials and methods.

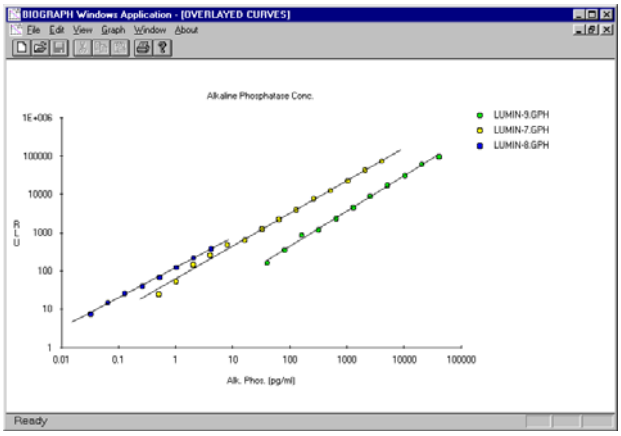

Figure 4. Linearity of alkaline phosphatase enzyme activity at various incubations times. Luminescence determinations of alkaline phosphatase reactions ranging from 0 to 2 X104 pg/well were made kinetically every 2 minutes for 30 minutes. At three different times (2 minutes; 10 minutes; and 30 minutes) linear portions of the concentration curve were plotted using KC4 data reduction software.

Figure 4 demonstrates the importance of incubation time in obtaining a useful calibration curve. When unknown concentrations are suspected to be quite low, then a longer incubation time of 30–40 minutes is suggested to allow for enzyme activity to generate an amount of luminescent product that can be measured, while very high enzyme concentrations require an incubation time of 2 minutes or less. In our hands an incubation time of 10-15 minutes provided the broadest range of linearity. With a 10 minute incubation,

enzyme dilutions over 5 orders of magnitude were found to be linear. As stated previously, increased sensitivity was obtained with longer incubations, but at the expense of being able to determine higher concentrations.
In terms of sensitivity, a 30 minute incubation time allowed for the detection of 25 pg/ml (p=0.005) of alkaline phosphatase. Taking into consideration the sample volume in the well (10 ml), the detection limit represents the determination of as little as 2.5 attomoles of alkaline phosphatase per well.

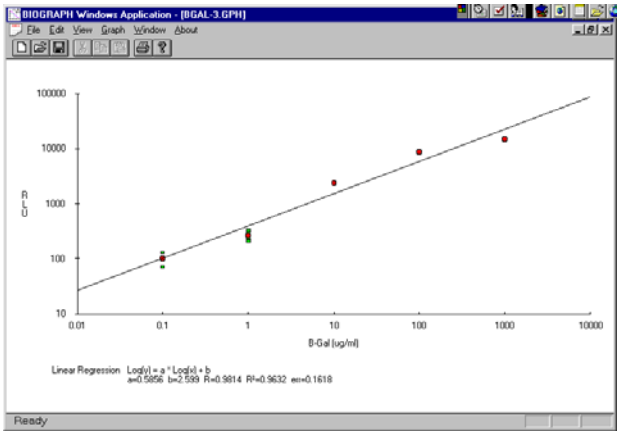

Figure 5 Linearity of b-galactosidase chemiluminescence over four orders of magnitude. The chemiluminescence of b-galactosidase reactions was determined for b-galactosidase enzyme concentrations ranging from 0 to 1000 mg/ml after a 5-minute incubation.

b-Galactosidase enzyme activity was determined using Galacton-StarÔ substrate. Similar to the CSPDÔ substrate used for alkaline phosphatase determinations, luminescence increases in a linear fashion with increasing amounts of enzyme (Figure 5). Using a 5-minute incubation, a linear range of four orders of magnitude was observed. When the reaction was incubated for 15 minutes a linear relationship is observed for b-galactosidase concentrations from 0 to 62.5 mg/ml (Figure 6). As with alkaline phosphatase determinations, high enzyme concentrations generally require shorter incubation times, while very low enzyme concentration naturally require longer incubation times (data not shown). In terms of sensitivity, b-galactosidase concentrations as low as 0.01 mg/ml (p=0.03) can be detected using a 30 minute incubation. Taking into account the volume placed in the well, this represents the ability to detect 100 pg of b-galactosidase enzyme.

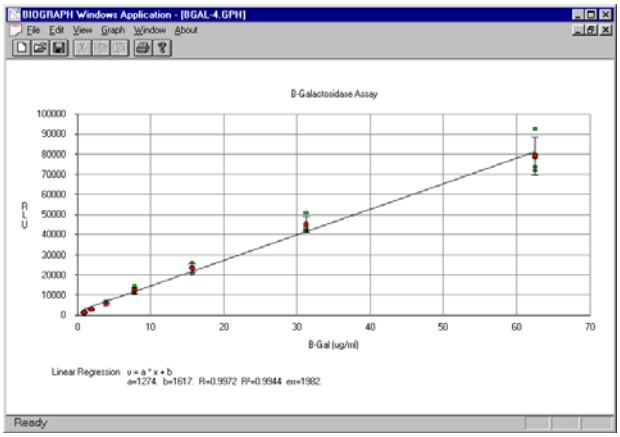

Figure 6. Concentration curve of low concentrations of b-Galactosidase activity. Concentrations of b-Galactosidase enzyme ranging from 0 to 62.5 mg/ml were assayed using the chemiluminescence of a 1, 2-dioxetane substrate following a 30 minute incubation.

Discussion

This application note demonstrates the capability of the FL600 fluorescence microplate reader to perform glow type chemiluminescent determinations. While not specifically designed for this application, the FL600 with the excitation lamp turned off and the emission filter removed provides the same instrument platform as a specific luminescence microplate reader.
As with any experiment, optimization of the reaction provides the best results. In the case of 1,2 dioxetane chemiluminescence, the correct incubation time is important for linear results. High enzyme concentrations, which tend to consume substrate quickly, require very short incubation times, while low enzyme concentrations have a better signal to blank ratio if a longer incubation time is chosen.

Unlike fluorescence, which requires the input of light at specific wavelengths for emission, chemiluminescence does not require a light source for the sample to emit light. Therefore, wells adjacent to the location of the detector are capable of emitting light and potentially influencing the determined luminescence of the well the detector is measuring. This phenomenon, often referred to as crosstalk, can be a problem with luminescence determinations if very intense chemiluminescence reactions are adjacent to reactions with considerably less chemiluminescence.

When using 1,2 dioxetanes substrates for chemiluminescence, the concurrent use of enhancers is important to provide adequate signal. Macromolecular enhancers provide a non-aqueous environment, which increases the emission efficiency of light production by preventing water-induced quenching. These enhancers also alter peak wavelength of emission. For example Sapphire-IIÔ enhancer will shift the emission maximum of 1,2-dioxetanes from 461nm to 475 nm, while the enhancer Emerald-IIÔ shifts the maximum to 542 nm. Depending on the application, different enhancers are more appropriate than others. Because Emerald-IIÔ provides a greater absolute intensity than Sapphire-IIÔ at its peak emission wavelength, it is the optimum choice if very low concentrations or maximal signal intensity is required. On the other hand, Sapphire-IIÔ provides a greater dynamic range since the photodetector is less likely to be saturated.

In this application note we have described the use of the FL600 for chemiluminescence determinations. Although the 3 mm detector probe provided very good results, in our hands the 5 mm detector probe was slightly superior in regards to detection limit performance. Although we only utilized white opaque microplates with reading from the top, clear bottom plates could also be utilized for bottom reading. The use of opaque sides is still recommended. Regardless of the read mode, only glow-type chemiluminescent reactions are appropriate for measurement with the FL600. Flash-type reactions occur within seconds of adding substrate; requiring a fluidics injector located within the instrument. Glow-type reactions, take place at much slower rates allowing the user to add reagents to the microplates and begin luminescence determinations before the reactions have gone to completion.

References

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