

Optimizing the Luminescence of Lanthanide(III) Macrocyclic Complexes for the Detection of Anti5BrdU

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ABSTRACT

A Eu(III)-macrocyclic-mono-isothiocyanate, Quantum Dye[®], has been coupled to a monoclonal antibody against 5BrdU. Since Quantum Dyes do not undergo concentration quenching, the coupling conditions were optimized to achieve the maximum number of Eu(III) macrocycles bound to the antiBrdU, without decrease in solubility or loss of antigen-binding ability. In order to optimize the coupling conditions, a colorimetric method for the quantitation of the Eu(III)-macrocyclic-mono-isothiocyanate has been developed.

A simple mixture composed of an ethanolic solution and a Gd(III)-containing aqueous solution is now used to provide lanthanide enhanced luminescence, LEL. Under LEL conditions, the specific binding of Eu(III) macrocycles to apoptotic cells has been observed in both aqueous and mounted slide preparations. A comparison between measurements of the same LEL model system, obtained in both time-gated luminescence and standard fluorescence modes, has demonstrated that time-gating significantly improves the signal to noise ratio.

Keywords: Luminescence, macrocycle, lanthanide, europium, cofluorescence, peptide, digital microscopy, fluorescence lifetime, LEL.

1. INTRODUCTION

The art-science of cytopathology has benefited and will continue to benefit from quantitative measurements of the molecular species present in cells. The combination of morphology and/or relevant molecular biological parameters should be synergistic. This has certainly been the case in hematology¹. Three related parameters of interest are: apoptosis, DNA synthesis, and total DNA content. The precise measurement of these parameters in a single cell leads to a new simple paradigm. If a cell is undergoing apoptosis, it cannot cause cancer. If an abnormally high number of cells are synthesizing DNA or if there is a population of cells with an abnormal DNA index², then these cells are suspicious as either pre-malignant or malignant. A population with an abnormal DNA Index is one in which a significant number of cells contain the same abnormal amount of DNA and these cells are neither undergoing apoptosis nor synthesizing DNA. These parameters can be combined with monoclonal antibodies and nucleic acid probes to provide a very specific description of a cell, as a complement to the traditional morphological information.

Vallarino and Leif^{3,4} have developed a class of functionalized macrocycles, Quantum Dyes[®], that include a central luminescent lanthanide(III) ion. These ions offer the unique advantage of producing narrow-band, long-lived, luminescent emissions both in solutions and in the dry state. Quantum Dye[®] luminescent labels are uniquely sensitive because the emitting species remains localized on the analyte, and their emission bands are extremely narrow, less than 10 nm at half peak-height, and are well removed from the excitation bands. In addition, the narrow band-width and fixed location of their emissions minimizes noise and cross-talk with other dyes. The millisecond lifetimes of the Quantum Dyes' luminescence⁵ is also an advantage, as it provides absolutely minimal noise and spectral cross-contamination when the emissions are observed with time-gated instrumentation. Quantum Dyes with different central lanthanide ions (europium(III), samarium(III), and terbium(III) and dysprosium(III)) have identical chemical behaviors, and each has its own characteristic narrow band emissions, distinct from those of the others. Thus, the Quantum Dyes together and admixed with conventional fluorochromes offer the opportunity for the simultaneous detection of different targets within the same sample.

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Unlike conventional fluorescent dyes, the individual units of a Quantum Dye[®] do not transfer energy to each other and thus do not self-quench⁵. This absence of concentration quenching allows multiple units of a Quantum Dye[®] to be bound to a polymer for increased signal intensity. In addition, signal intensity can be greatly enhanced through the phenomenon of lanthanide enhanced luminescence, LEL, that permits a high concentration of light absorbing species (antenna molecules) to funnel energy into a very low concentration of Quantum Dye[®]-tagged molecules^{5,6,7,8}. The LEL micellar solution consists of: a surfactant, one or more luminescence enhancers, and a suitable trivalent ion--such as gadolinium(III), lanthanum(III) or yttrium(III)--as energy-transfer donor. To this LEL matrix, one or more Eu(III), Sm(III), Tb(III) or Dy(III) macrocycle(s) are added as emitter(s).

Simple, flash-lamp based instrumentation can be used to detect Quantum Dyes and selectively eliminate the background noise from autofluorescence and conventional organic fluorochromes. These characteristics of Quantum Dyes permit localized staining on slides with excellent signal-to-noise ratio. In turn, this simplifies the automated processing of samples and allows multiple dyes to be combined in one sample preparation. Taken together, these characteristics make Quantum Dye[®] technology ideal for the detection and examination of rare events.

1.1. Quantum Dye Nomenclature and Abbreviations

The following abbreviations will be used to describe the Quantum Dyes and related compounds. Specific metal ions will be given by their standard chemical symbols. The unfunctionalized, mono-functionalized and di-functionalized macrocyclic complexes will be abbreviated respectively as "Mac", "Mac-mono" and "Mac-di". The term "Macs" without the mono or di prefix will include the unfunctionalized, the mono-functionalized and di-functionalized macrocyclic complexes (Mac, Mac-mono and Mac-di). When a specific peripheral pendant substituent having at least one reactive site (reactive functionality) is specified, its abbreviation will be given as a suffix. The registered trademark, Quantum Dye, will refer to any and all of the macrocyclic species covered by US patents 5,373,093 and 5,696,240.

1.2. LEL Emulsion

The actual use of the europium(III) Quantum Dye monoisothiocyanate, EuMac-mono-NCS, with the LEL emulsion has required both optimization of the coupling conditions and improvements in the production of the LEL emulsion. The previously described⁶ LEL emulsion served to prove scientific feasibility; but was not suitable for commercial use. Detailed studies of the LEL emulsion established that it had an irreversible thermal transition at about 50 °C and that the addition of conventional antioxidants had the unexpected effect of decreasing the luminescence. Under the conditions described here, the LEL effect can be advantageously applied to detect very low concentrations of luminescent lanthanide(III) ions bound into the cavity of macrocyclic ligands⁸. It should be taken into account that LEL has the sensitivity restriction that even the purest commercially available gadolinium oxide contains approximately 0.3 ppm of europium oxide as a contaminant.

1.3. Stability of Isothiocyanates

Isothiocyanates have been used as functional groups in coupling protocols since the work of Riggs' group⁹; however, the stability of these species in the basic solutions employed for coupling has never been completely established, especially in the presence of heavy metal ions, such as the Eu(III) of the EuMac. Riggs et al. stated that fluorescein isothiocyanate, FITC, "was stable in solid form." The effects of pH, temperature, and concentration on the conjugation of FITC to polyclonal horse anti-rabbit-IgG was studied by The and Feltkamp¹⁰, who reported that, "Maximal labeling was obtained in 30-60 minutes at room temperature, pH 9.5 and an initial protein concentration of 25 mg/ml." Gradient elution of DEAE Sephadex A 50 medium columns with increasing concentration of 1 M NaCl and 0.01 M phosphate (pH 7.2) admixed with phosphate buffered saline was used to separate the immunoglobulin conjugates according to their degree of concentration. In a second paper, The and Feltkamp¹¹ reported that the more heavily conjugated species eluted at the higher salt concentration. These authors stated¹⁰ that under the basic conjugation conditions, "To avoid considerable hydrolysis the (fluorescein-NCS) solution was used within 3 hours." Although the absolute amount of bound FITC increased with FITC concentration, the percentage of bound FITC decreased with concentration. "More heavily coupled IgG molecules apparently had a lower affinity for FITC than still unlabeled and the more lightly coupled IgG molecules." A simple explanation for this effect is that, as the number of bound negatively charged FITC molecules to the negatively charged protein increased, the repulsive force between the two negatively charge species also increased. This effect would not be expected for the EuMac-mono-NCS, which has a charge of +3. These authors also reported¹¹ that the non-specific binding of the FITC-IgG conjugates significantly increased at a ratio of 3 to 4 fluoresceins per IgG and above. They commented, "Eosi-

nophilic tissues or cells especially were stained non-specifically.” This observation is easily explained by the fact that eosin is a brominated derivative of fluorescein.

Devani et al.¹² described a method to detect and quantitate thioureas, thiosemicarbazides and mono-thiosemicarbazones by a colorimetric method in an ethanolic solution of ammonia. Under the conditions of this protocol, both alkyl and aryl isothiocyanates react with ammonia to produce a thiourea-type linkage, which in turn reacts with the naphthoquinone to produce a chromophore with an absorption maximum in the range of 500 to 570 nm. Although the product of this reaction, because of its spectral overlap with fluorescein isothiocyanate, FITC, is presently unsuited for measurements of FITC, the method can be applied to the Quantum Dye isothiocyanates, which do not absorb in this region. Accordingly, phenyl isothiocyanate was used as a model to develop a variation of the analytical procedure of Devani et al.¹², adapting it to the different solvent system used for the coupling of the EuMac-mono-NCS. The new analytical procedure was found to be effective for the quantitation of the NCS functionality present in the EuMac-mono-NCS.

2. EXPERIMENTAL

2.1. Materials

The following were purchased from Sigma-Aldrich Chemical Co.: Hexamethylenetetramine (HMTA), ACS Reagent, Catalog No. 39,861-0 (1999); 4,4,4-Trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA), Catalog No. T2,700-6 (1996-97); 2,3-Dichloro-1,4-naphthoquinone (98%), Catalog No. D67200; N,N-Dimethylformamide (DMF), 99.8%, A.C.S. spectrophotometric grade, Catalog No. 15,481-4; Ammonium hydroxide, A.C.S. reagent, Catalog No. 221228; Hydroxylamine HCL, Catalog No. H-9876; Fluorescein, Catalog No. F245-6; and Drierite® (calcium sulfate, anhydrous) 4 mesh with indicator, catalog No. 238961, and without indicator, Catalog No. 238910. For the LEL studies, commercial HTTFA was purified by recrystallization from ethanol(charcoal)/hexane and stored at 4°C in a dark glass container. For the experiments with cells, the HTTFA was used as received. 1,10-Phenanthroline (Phen), Catalog No.13,137-7 (1999); Cetyltrimethylammonium bromide (CTAB), Catalog No.85,582-0 (1999). Tris(hydroxy-methyl)-amino-methane (TRIS) was purchased from Amresco, Catalog No. 0497. Positive and negative control apoptotic cells were from the Phoenix Flow Systems APO-BRDU™ Kit.

The HMTA buffers were adjusted to the desired pH by the addition of aqueous HCl or NaOH.

The prototype europium(III) complex with no peripheral functionalities, EuMac, was prepared according to De Cola et al.¹³ EuMac-di-NCS and EuMac-mono-NCS were prepared according to US Patent 5,696,240⁴ and are commercially available from Research Organics, Cleveland, Ohio¹⁴. The monoclonal anti5BrdU was obtained from Phoenix Flow Systems. Common inorganic acids, bases, and salts were obtained from ordinary commercial sources.

In reporting quantities and concentrations, the term “micro” will be conventionally abbreviated as μ ; for example, microgram will be abbreviated as μg .

2.1.1. LEL Emulsion

The LEL emulsion is made by a proprietary process, which is presently being optimized by Newport Instruments. The concentrations in moles/L of the components in the LEL emulsion used for the studies described in this paper were: 1,10-phenanthroline, 9.46×10^{-5} ; cetyltrimethylammonium bromide, 7.57×10^{-6} ; hexamethylenetetramine buffer, 0.108; 1,1,1-trifluoro-4(2-thienyl)-2,4-butanedione (HTTFA), 5.68×10^{-4} ; Gd(III) chloride. 1.1×10^{-4} ; and 8.35% ethanol. The concentrations of the EuMac are stated in the figures and texts below. Briefly, the emulsion was prepared by mixing 1.9 mL of a pre-made de-aerated buffered, aqueous GdCl_3 solution, 0.03 mL of Eu Mac stock solution diluted in ethanol, and 0.07 mL pre-made ethanolic concentrate of the organics. The EuMac stock solutions were diluted so that an identical aliquot of each could be used in the final working solution. This maintained the ethanol concentration at 8.35% within a constant solution matrix. Upon mixing, each concentration of Eu Mac was decanted into a triangular quartz cuvette and its luminescence measured.

2.1.2. Spectral Comparison of EuMac and Fluorescein

Two solutions were compared: (1) Fluorescein (1.00×10^{-5} M in HMTA, buffered at pH 8.03 with 1.0×10^{-3} M HCl) and (2) EuMac 1.00×10^{-5} M in LEL emulsion. Excitation and emission spectra, in fluorescence mode, were obtained with the SLM instrument.

2.1.3. Isothiocyanate Color Test Studies

The color test solutions for the dilution studies of EuMac-Mono-NCS and for the mock coupling studies with the phenyl-NCS were prepared by a modification of the procedure by Devani et al.¹² For the EuMac-mono-NCS dilution studies, the stock solution was prepared by dissolving 77 mg of the EuMac-mono-NCS in 10 mL of spectrophotometric grade DMF (9.05 mM). The solution for each color test measurement was prepared by adding from 50 to 1,000 μ L of the stock EuMac-mono-NCS solution to 3.00 mL of 1.145 mM (0.26 mg/mL) 2,3-dichloro-1,4-naphthoquinone in DMF. Next, sufficient DMF was added to bring the total volume of DMF to 4.40 mL. Finally, 0.600 mL 28-30% aqueous ammonia was added and the solution was left to stand at room temperature for 15-30min. The final concentrations of the EuMac-Mono-NCS ranged from 40 to 200 μ M. All samples were analyzed using an Agilent 8453 UV-Vis Spectrophotometer.

For the phenyl-NCS stability studies, a stock solution of phenyl-NCS was prepared on the day of use to prevent decomposition of the -NCS functionalities in DMF. The solution was prepared by diluting 71.8 μ L of phenyl-NCS to a total volume of 10 mL with DMF. To avoid decomposition by atmospheric conditions, this dilution was performed under a stream of nitrogen and over a bed of Drierite. Two experimental solutions were prepared: (1) A "phenyl-NCS coupling solution" containing 2.414 mL of a 10:1 mixture of NaHCO₃ (20 mM) and NaN₃ (2 mM), pH ca. 8.5; 0.240 mL of a 80:20 mixture of NaHCO₃ (1.00 M) and Na₂CO₃ (1.00 M), pH ca. 9.0; 0.250 mL of 60 mM (8.11 mg/mL) of stock phenyl-NCS solution, and 0.410 mL of DMF. (2) The "control coupling solution" had the same composition, except that 0.250 mL of DMF was substituted for the phenyl-NCS solution. Each color test solution was prepared at time intervals by mixing the following: 3.00 mL of 2,3-dichloro-1,4-naphthoquinone (1.145 mM, 0.26 mg/mL) in DMF, 1.2 mL of DMF, 0.200 mL of either the "phenyl-NCS coupling solution" or the "control coupling solution", and 0.600 mL 28-30% aqueous ammonia (added last). The spectrum was recorded on the Agilent 8453 UV-VIS Spectrophotometer, immediately after each solution was prepared (temperature, 23.5 °C), and the spectrum of the control solution was subtracted from that of the coupling solution.

2.1.4. Coupling of the EuMac-mono-NCS to the Anti5BrdU.

The anti5BrdU (140 μ L of a 12 mg/mL solution) and 14 μ L of 1 M NaHCO₃ pH 9.0 buffer were added to a 2 mL plastic tube (Fisher Scientific Cat no. 02-681-343) with cap (Fisher Scientific Cat no. 05-664-59), equipped with a micro stirring bar. Stirring was started and was continued during the entire experiment. A first aliquot of EuMac-NCS (4.76 μ L of a 10 mg/mL DMSO solution) was slowly and continuously added. Three minutes later a second aliquot of 4.76 μ L of the same EuMac-NCS solution was similarly added. (The total EuMac-mono-NCS to anti5BrdU ratio to was 10:1.) The solution was incubated for 60 minutes at room temperature, ca. 27°C. After 60 minutes, any remaining isothiocyanate was destroyed by the addition of 1 μ L of a 1.5 M hydroxylamine HCl solution (pH 8.5). After an additional 30 minutes to complete the quenching reaction, the solution was transferred to a 1.5 mL centrifuge tube. The reaction vial was rinsed with 10 μ L of TBS-Azide, and the rinse was added to the centrifuge tube. This was followed by centrifugation for 2 min at 17,000 x g (Hermle Z 180M microcentrifuge) to remove any precipitate that may have formed during conjugation. The clear solution was transferred into another vial and purified by size-exclusion chromatography on a Sephadex G-25 in an XK16 column (Amersham Biosciences), using TBS-Azide as the eluent and a UV detector. The solvent front (10.6 mL) containing the first peak was collected in TBS-Azide and was then concentrated using a 10,000 molecular weight cut off filter (Millipore, Catalog No. PBGC02510) with a 3 mL stir cell (Millipore model 8003, Catalog No. 5125) under inert gas pressure. Helium was used in this experiment. The UV spectrum of the conjugate had a strong absorption at 270 nm, which showed the presence of coupled Quantum Dyes. However, since the Quantum Dyes absorb much more strongly than the antibody at 270 nm, these spectral measurements could not be used to determine the ratio of Quantum Dye to antibody. The conjugate was stored in the refrigerator at ca. 11°C. Later experiments have indicated that this is not a good method for prolonged storage.

2.1.5. Apoptosis Staining:

A pair of Leif Centrifugal Cytology Buckets¹⁵ for a Beckman Coulter model GPR centrifuge, each of which holds 2 inserts, were assembled with aminosilane treated slides (Silane-Prep Slides, Sigma Catalog No. S4651). Four chamber inserts were used. Separate positive and negative control apoptotic cells were resuspended by swirling the cells in the vendors' vials. A 0.5 mL aliquot of each cell suspension (approximately 0.75×10^6 cells) in 70% (v/v) ethanol was pipetted into each bucket chamber and centrifuged at 300 x g for 5 minutes at room temperature. The supernatant was removed

from the Centrifugal Cytology Bucket sample block by aspiration with narrow stem transfer pipette; and the bucket was disassembled. The cells were washed with the Phoenix Flow Systems ApoBrdU wash buffer Catalog No. ABWB13 for 5 minutes by dipping the slides in a coplin jar. The wash buffer was removed by blotting the edge of the slide with Kimwipes[®] and drying the regions surrounding the cell monolayers. The DNA Labeling solution was prepared according to the APO-BrdU Protocol¹⁶. The apoptotic breaks were tailed with 5BrdU by layering each monolayer area with 25 μ L of DNA Labeling solution for 60 minutes at 37 °C in a humid chamber (a pipette tip box containing moist Kimwipes[®]). It is essential to make sure the solution covers the entire cell area. The cells were then washed for 10 minutes with a TRIS version of the Phoenix Flow Systems rinse buffer¹⁶ and the rinse buffer was removed, as above. The entire area of each individual monolayer was covered with 50 μ L of the Quantum Dye labeled anti5BrdU in TRIS rinse buffer. The slides were then incubated for 30 minutes at room temperature in a humidity-saturated container. The cells were washed for 10 minutes with rinse buffer and the rinse buffer was removed, as above. The cells were treated with LEL for 5 minutes then washed with a 1:1 mixture of Ethanol and LEL. The cells were finally washed and dehydrated with 100% Ethanol and mounted with Clearium Mounting Medium[®] (Surgipath[®]) and coverslipped.

2.2. Instrumentation

Absorption spectra were obtained with an Agilent 8453E UV-Visible Spectroscopy System. High resolution emission spectra of solutions were obtained in the fluorescence mode with an SLM Model 8000 photon-counting spectrofluorometer. Emission spectra of both solutions and dried material were obtained in both the fluorescence and the phosphorescence mode with a Varian Cary Eclipse spectrofluorometer. Both the SLM and Cary instruments were equipped with a Hamamatsu R3896 (red sensitive) PMT. For time-gated luminescence studies with the Cary instrument, the emission was obtained from a single flash. No data were collected during the first 100 microseconds after the flash, after which the emission signal was integrated for a gate time of 2 milliseconds. The averaging time was set to 100 milliseconds, and the number of averaged readings (averaging time divided by gate time) was 50. The data interval was 1 nm and the scan rate was 600 nm/min.

The Cary spectra reported here are uncorrected because, at the time of the experiments, the software for spectra correction had not been implemented in the wavelength range studied. However, the lack of correction should not affect the results because of the narrow (30 nm) spectral range investigated.

Appropriate blank samples were used to zero the instrument (i.e. blank subtraction) prior to each scan. Samples for both fluorometers were examined in stoppered triangular quartz cuvettes, so oriented that the excitation beam entered the diagonal face at a 45 degree angle and the emitted light was collected through the bulk of the sample at 90 degrees relative to excitation. For the dry samples, the Cary Eclipse Microplate reader accessory was used. All experiments and measurements were performed at ambient temperature unless stated otherwise. Spectra were transferred to Microsoft[®] Excel and graphed.

A fluorescence microscope equipped with a 10X objective 0.25 N.A was employed to observe and to electronically-photograph the cells. UV illumination was provided by a 100 watt Mercury-Xenon short arc. The fluorescence was excited at 365 nm and the emitted light was observed through an Omega Optical Ploemopak cube, UV DAPI, equipped with the following: a 365 nm narrow-band-width excitation filter (Omega 365HT25), a 400 nm Beamsplitter (Omega 400DCLP02), and a two-band emission filter (450 and above 600 nm, Omega 450DF65). The CCD optical path was equipped with a 619 nm narrow-band, 5.6 nm half-width, emission filter (Omega 618.6NB5.6). The images were obtained with a peltier cooled Quantitative Imaging Corp. MicroImager II, Monochrome, 12 bit ADC, CCD camera (1280 x 1024). The gray levels of the images were inverted for display. Darkness indicates strong luminescence.

3. RESULTS AND DISCUSSION

3.1. Enhancement of the EuMac Emission Intensity by LEL:

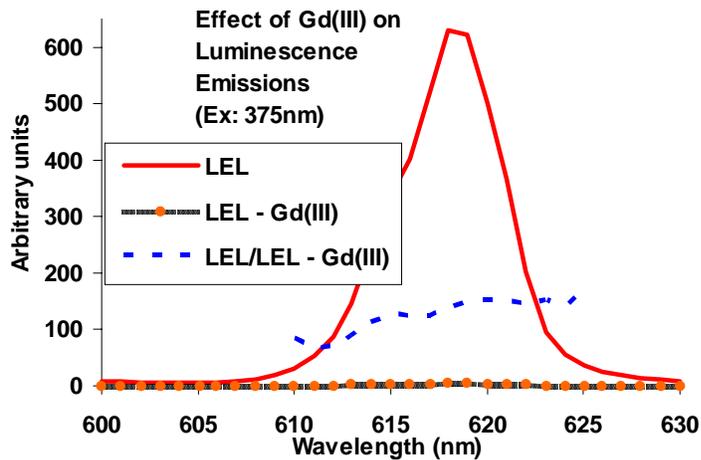


Figure 1. The luminescence intensity of 1.5×10^{-7} M EuMac in a gadolinium-containing enhancer solution, LEL, is over 100 times greater than when the gadolinium is omitted, LEL - Gd(III). The PMT voltage was 750 volts. The excitation and emission slit widths were respectively 20 and 2.5 nm. The dashed line, LEL/LEL - Gd(III), shows the ratio of the luminescence intensity between solutions with and without gadolinium. The ratio is 137 at the emission maximum, 618 nm.

The luminescence enhancement produced by the LEL emulsion vs. the control solution without Gd(III), when measured with the Varian Cary instrument in the time-gated phosphorescence mode (Figure 1), is over one-hundred fold. This result is in agreement with the previous measurements obtained on the SLM instrument⁶ (fluorescence mode).

3.2 Linearity Studies:

A series of experiments was performed to demonstrate the linearity of time-gated luminescence measurements of the EuMac in gadolinium-containing LEL emulsions. In order to maintain a linear response from the instrument for these concentration studies, the photomultiplier voltage was kept constant at 580 volts.

Figure 2 shows the emission spectra at the two concentration extremes of this series, 1.36×10^{-5} M and 1.36×10^{-9} M. The figure illustrates that well resolved spectra could be routinely obtained even at 1.36×10^{-9} M EuMac. At this low concentration, however, the presence of the Eu(III) contaminant in the Gd(III) becomes evident.

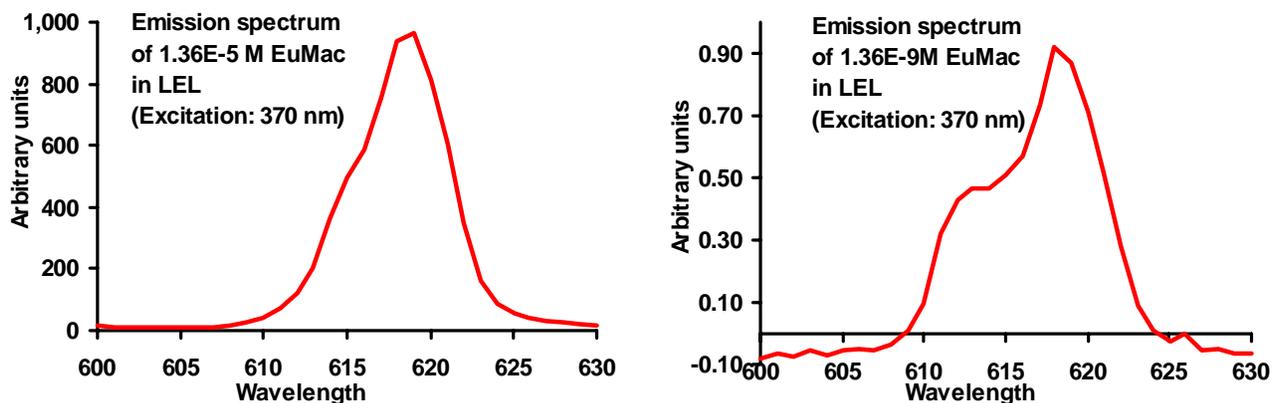


Figure 2. Luminescence emission spectra of EuMac in LEL. Left: At 1.36×10^{-5} M EuMac, a small shoulder can be detected at 612nm. Right: At 1.36×10^{-9} M, the shoulder at 612nm is more pronounced. This shoulder is primarily the result of the Eu(III) contaminant in the Gd(III) component of the LEL emulsion. The excitation and emission slit widths were respectively 10 and 2.5 nm.

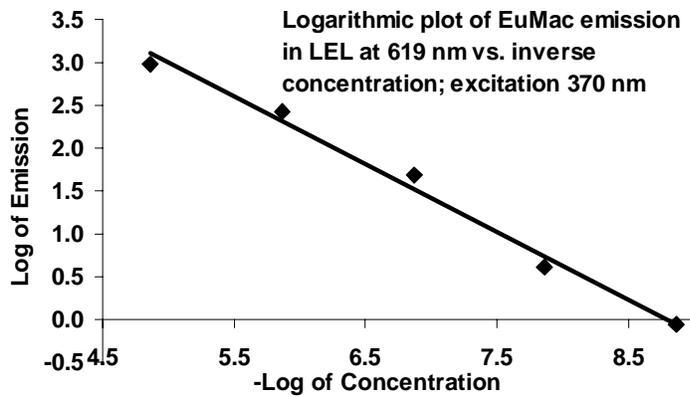


Figure 3. Plot of the log of the emission intensity at the maximum, 619 nm, versus the negative log of the concentration of EuMac in LEL. In order to maintain a linear response from the instrument for concentration studies, the photomultiplier voltage was kept constant at 580 volts. The equation of the least squares straight line fit was:

$$y = -0.7914x + 6.9633. \text{ The } R^2 \text{ of the fit was } 0.9892.$$

the luminescence.

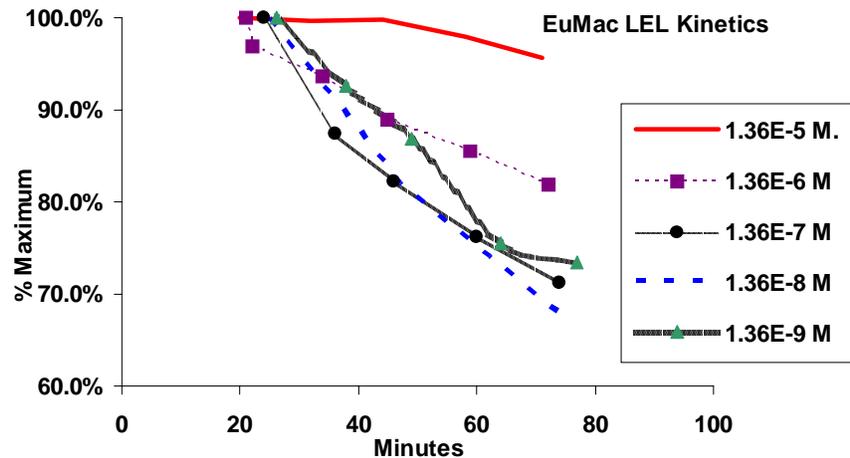


Figure 4. Kinetics of emission intensity loss of EuMac in LEL emulsions with Gd(III). The two higher concentration solutions 1.36×10^{-5} and 1.36×10^{-6} appear to be more stable than the three solutions of lower concentration.

In another set of experiments, the luminescence intensity of four EuMac samples (3×10^{-8} to 1.2×10^{-7} M) was measured over a period of 80 minutes in the usual gadolinium-containing LEL micellar system. As illustrated in Figure 5, the linearity of the average emission intensity versus concentration is excellent even in this low concentration range. Both the fit of the 5 averaged data points for each concentration, and the results of the fourth data point for each concentration, taken about 80 minutes after mixing, had $R^2 = 0.9997$. The results for only the first data points, taken about 30 minutes after mixing, had $R^2 = 0.9924$.

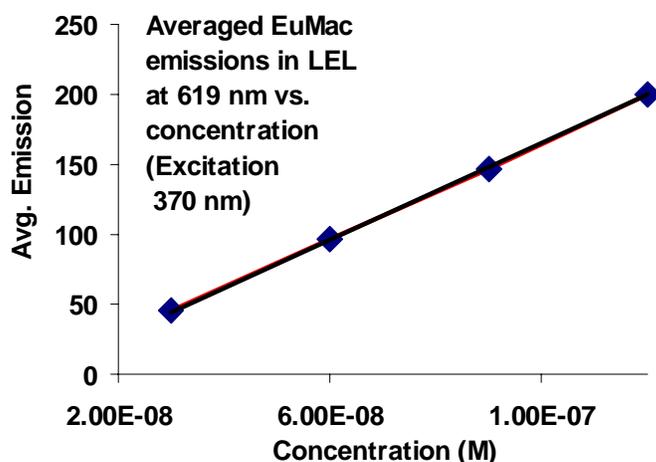


Figure 5. The emission intensity readings (at the 619 nm maximum) obtained over a period time from four different concentrations of EuMacI in LEL emulsions with Gd(III) were averaged and are shown plotted versus concentration. The excitation and emission slit widths were respectively 10 and 2.5 nm. The PMT voltage was 800. The equation of the least squares straight line fit was: $y = 2 \times 10^9 x - 6.7$. The R^2 of the fit was 0.9997.

3.3 Comparison of the Spectral Patterns of EuMac and Fluorescein:

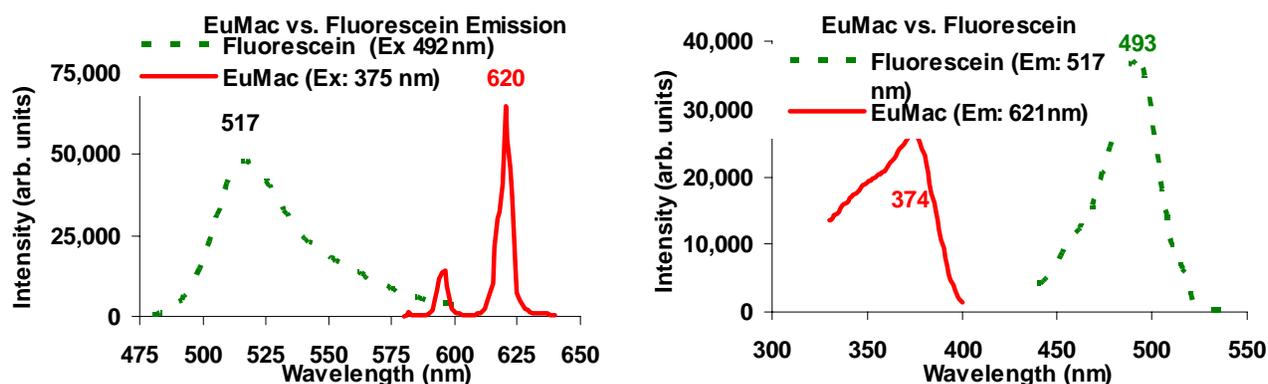


Figure 6. Left, Emission spectra obtained with the SLM spectrofluorometer. Fluorescein (1.0×10^{-5} M) in HMTA buffer (1.0 M, pH 8.03). EuMac (1.0×10^{-5} M) in LEL emulsion. The excitation and emission slits were respectively 16 and 1 nm.

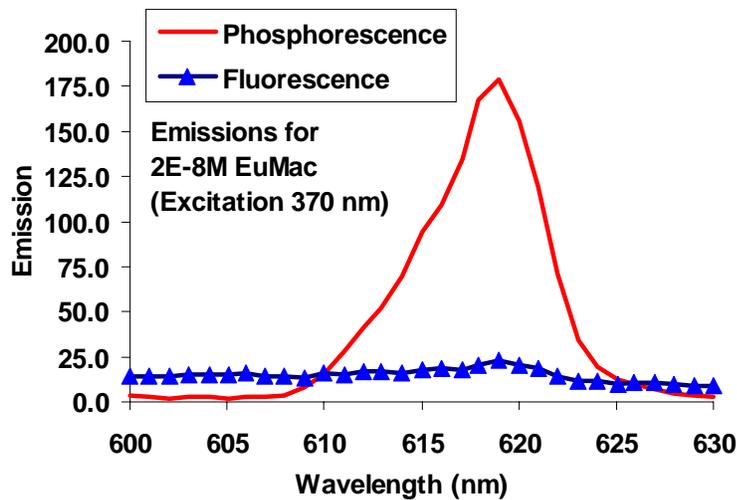
Right, Excitation spectra obtained with the SLM spectrofluorometer. Fluorescein (1.0×10^{-5} M) in HMTA buffer (1.0 M, pH 8.03). EuMac (1.0×10^{-5} M) in LEL emulsion. The excitation and emission slits were respectively 4 and 8 nm.

It is instructive to compare the emission and excitation spectra of the EuMac with those of a common organic fluorophore such as fluorescein, when both compounds are at the same concentration and the spectra are obtained under the same instrumental conditions. Figure 6 (left) shows that the emission band of EuMac in the LEL emulsion containing gadolinium(III) is very narrow, with a width at half-maximum of 5.2 (nm) compared to 37.5 nm for fluorescein. As is shown in Table 1, the width at half maximum of the EuMac is only 14% of that of fluorescein; and the summed emission under the peak between the half maxima is 19%. The excitation spectrum of the EuMac (Figure 6, right) is similar in shape and width to that of fluorescein because the light is first absorbed by the enhancer HTTFA, which is an organic molecule, and the energy is then transferred to the europium(III) emitter. The excitation-emission separation (Stoke's shift, Table 1) is ten times greater for the EuMac than for fluorescein.

Table 1: Emission spectra of Fluorescein vs. EuMac in LEL emulsion with Gd(III)

Emitting Compound	Half-Max Width (nm)	Relative Areas at Half-Max	Max Emission (nm)	Excitation-Emission Separation (nm)
Fluorescein	37.5	1.00	517	24
EuMac	5.2	0.19	620	246

3.4. The Advantage of Time-gated Luminescence:



The emission spectra of EuMac in the LEL emulsion matrix, obtained with the Cary instrument (Figure 7) demonstrated that the sensitivity of time gated luminescence (phosphorescence mode) was superior to a continuous measurement (fluorescence mode). However, the spectral resolution obtained with the SLM spectrofluorometer, as evident in Figure 6, was superior to that obtained with the Varian Cary instrument in either fluorescence or luminescence mode.

Figure 7. Comparison of the sensitivity of measurement obtained with the Varian Cary fluorometer in the fluorescence and phosphorescence modes. The excitation and emission slit widths were respectively 10 and 2.5 nm. The PMT voltage was 900 volts. The EuMac was in the LEL emulsion.

3.5. Isothiocyanate Color Test Studies

As described in the Experimental section, a colorimetric method was developed for the quantitative measurement of the -NCS functionality of the EuMac-Mono-NCS, and of the phenyl-NCS under coupling conditions. Figure 8 shows the absorption spectrum of the EuMac-Mono-NCS at various concentrations in the NCS-color test matrix. It should be noted that this spectrum is unequivocally diagnostic of the -NCS functionality, because no absorption between 450 and 750 nm occurs in the spectra of the non-functionalized EuMac prototype (Figure 6), of the EuMac-Mono-NCS alone, and of the NCS-color test control solution (data not shown). For each color test solution containing the EuMac-Mono-NCS, the absorption intensity between 555 and 565 nm increased linearly with increasing concentration (Figure 9). Similar results (data not shown) were obtained with the phenyl-NCS.

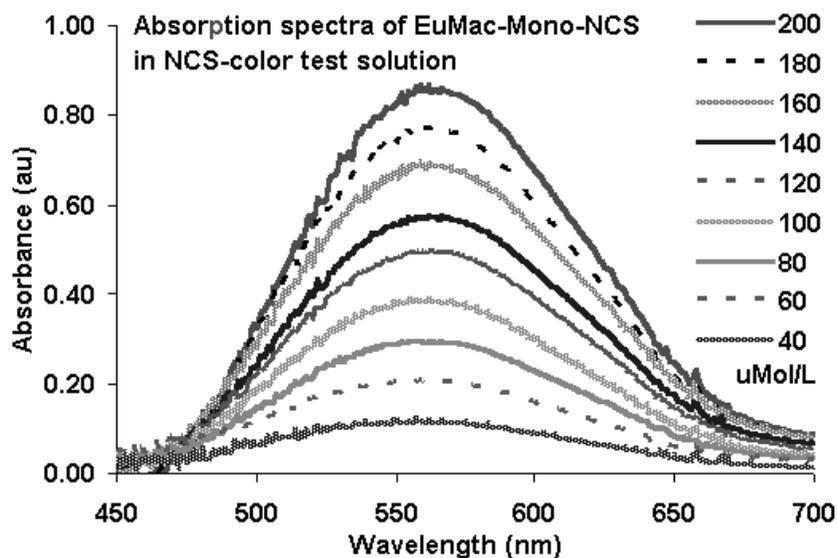


Figure 8. Absorption spectra of different concentrations of EuMac-mono-NCS in the NCS-color test solution. The absorbance increases with concentration. The key, at the right, shows the EuMac-mono-NCS concentration in descending order, which is the same as the order of the spectra.

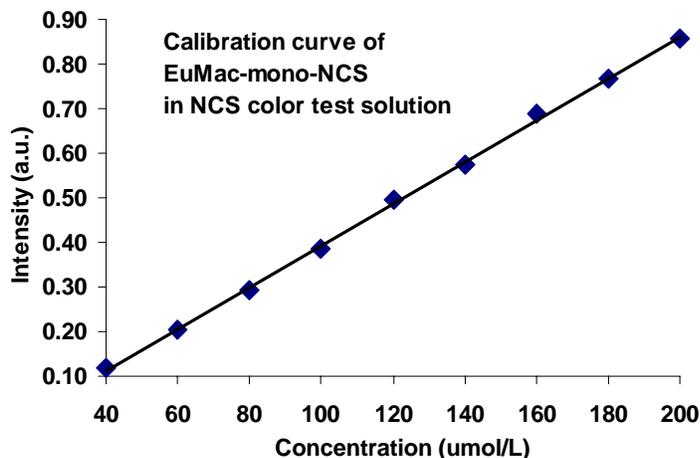


Figure 9. Plot of the average absorbance (555-565 nm) of the EuMac-mono-NCS in the color test solution, vs. the concentration expressed in micromoles. The equation of the best straight-line fit is $y = 0.0047x - 0.076$.

As shown by the value of R^2 , 0.9992, the data agree well with the straight line fit.

Figure 10 shows the results of a study intended to assess the stability of the model compound phenyl-NCS in a buffer solution of the type used in coupling reactions of the EuMac-Mono-NCS. The concentration of the -NCS functional group was measured over a period of time using the NCS-color test method described in the Experimental section. The absorbance in the range of 450 to 700 nm was found to decrease with time (Figure 11), and the time dependence of the average absorbance (558-568 nm range) is complex. There appears to be an initial 60 min incubation period during which there is only a very slow decrease in -NCS concentration, followed by a much steeper decline. These results are in general agreement with the reaction kinetics reported by The and Feltkamp¹⁰ at 25 °C, which show that most of the reaction of FITC with horse IgG occurred during the first 30 minutes and that the reaction had stopped after 90 minutes.

Similar preliminary studies with the EuMac-mono-NCS provided the unexpected results (data not shown) of an increase in absorbance with time. Further studies will be required to provide a rationalization for these results.

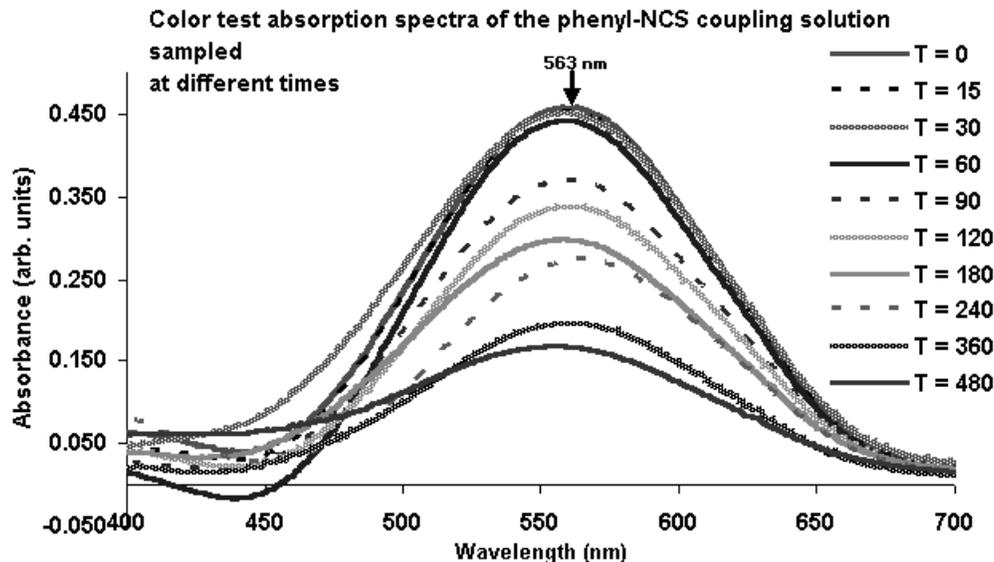


Figure 10. Study of phenyl-NCS stability in a coupling solution at pH 9. The times in minutes (shown in the key at the right of the figure) correspond to the time elapsed since preparation of both the “phenyl-NCS coupling solution” and the “control coupling solution”. The time is shown in ascending order, which is the same as the order of the spectra. In each color test measurement, the spectrum obtained from the “control coupling solution” was subtracted from that obtained from the “phenyl-NCS coupling solution”. The concentration of phenyl-NCS in the coupling solution was $1.8 \times 10^{-4} \text{M}$ (25ug/mL).

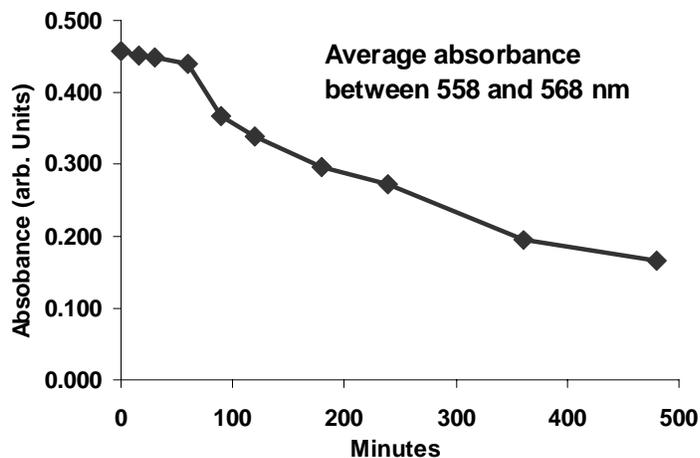


Figure 11. This plot shows the time dependence of the average absorbance (558-568 nm) for the individual spectra reported in Figure 10. The graph shows that the kinetics of decomposition of phenyl-NCS in a pH 9.0 coupling solution is complex, and cannot be represented as a simple linear function.

4. Apoptosis Study

The first expected use of Quantum Dye[®] kits is the measurement of apoptosis and the second is the measurement of S-phase. At present, a Quantum Dye labeled monoclonal antibody against 5BrdU can be used for both assays. The use of a europium(III) Quantum Dye labeled anti5BrdU should permit either apoptosis and DNA synthesis to be measured simultaneously with total DNA content. Figure 12 demonstrates the feasibility of detecting apoptosis. Luminescence apoptotic cells have been imaged with a conventional fluorescence microscope, which employed a standard 100 watt mercury arc. These were the positive control cells from a commercial kit¹⁶, which has only been modified by the substitution of europium Quantum Dye for the conventional fluorochrome label and TRIS for phosphate in the rinse buffer. As shown in Figure 7, the sensitivity of the microscope imaging system should be able to be improved over a hundred-fold by the use of an efficient time-gated imaging system.

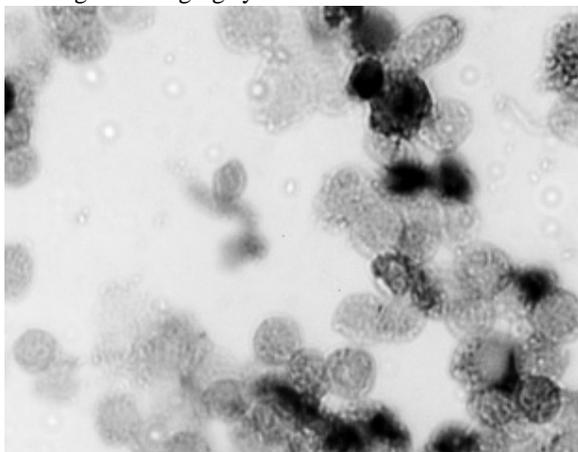


Figure 12. Apoptotic cells in LEL emission. The luminescence image was collected through a 619 nm filter, inverted, and merged with a brightfield image, which had been autoleveled. The dark areas are the Eu Quantum Dye tagged anti-5BrdU. The exposure time for the luminescence, using a cooled QIMAGING MicroImager II, was 5 sec.

5. Conclusions and Comments

Quantum Dyes are small molecules, and thus have several advantages over particulate inorganic tags, such as Quantum Dots. The Quantum Dyes do not interfere sterically with the binding of antibodies and other biomolecules. They do not interfere with intracellular staining. They have the narrowest emission known, 5.2 nm at half maximum. This emission is caused by transitions between quantum electronic states of the lanthanide(III) ion of the Quantum Dyes and thus does not change from batch to batch.

A new combination of three fluorescent tags has previously been described⁷. The DNA-specific stain DAPI can be combined with two functionalized, narrow-band emission lanthanide(III) macrocycles. All three fluorophores can be simultaneously excited using the same excitation filter and dichroic mirror, the only necessary difference being the emission

filter. In many instances, time-gated technology associated with a fluorescence microscope will produce the best signal to noise ratio for lanthanide luminescence. However, the major enhancement produced by Gd(III) on the emission intensity of europium Quantum Dye now makes it possible to obtain useful images with a conventional microscope employing a ca. 365 nm light that excites both the narrow bandwidth, red emitting europium Quantum Dye and a blue emitting DNA dye, DAPI.

Precise measurements of the ratio between the europium Quantum Dye and the samarium Quantum Dye, and of the ratio between each individual Quantum Dye and DAPI, should also be possible. These ratios, which are independent of both object (chromosome) size and of reasonable fluctuations in excitation intensity, should serve as excellent descriptors for nucleic acid measurements including: chromosome painting, comparative genomic hybridization, and FISH. Furthermore, the Eu and Sm Quantum Dyes can be used advantageously as tags for proteins.

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