

# Increasing the Luminescence of Lanthanide(III) Macrocyclic Complexes by the Use of Polymers and Lanthanide Enhanced Luminescence

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## ABSTRACT

A Eu(III)-macrocyclic-isothiocyanate, Quantum Dye<sup>TM</sup>, has been reacted with lysine homo- and hetero-peptides to give polymers with multiple luminescent side chains. Contrary to the concentration quenching that occurs with conventional organic fluorophores, the attachment of multiple Quantum Dyes to a polymer results in a concomitant increase in luminescence. The emission intensity of the peptide-bound Quantum Dye units is approximately linearly related to their number. The attachment of peptides containing multiple lanthanide(III)-macrocycles to analyte-binding species is facilitated by employing solid-phase technology. Bead-bound peptides are first labeled with multiple Quantum Dye units, then conjugated to an antibody, and finally released from the bead by specific cleavage with Proteinase K under physiological conditions. Since the luminescence of lanthanide(III) macrocycles is enhanced by the presence of Gd(III) or Y(III) ions in a micellar system, a significant increase in signal can be achieved by attaching a polymer labeled with multiple Quantum Dye units to an analyte-binding species, such as a monoclonal antibody, or by taking advantage of the luminescence enhancing effects of Gd(III) or Y(III), or by both approaches concomitantly. A comparison between the integrated intensity and lifetime measurements of the Eu(III)-macrocyclic under a variety of conditions show that the signal increase caused by Gd(III) can not be explained solely by the increase in lifetime, and must result in significant part from an energy transfer process involving donors not directly bound to the Eu(III).

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

## 1. INTRODUCTION

We have previously reported<sup>1</sup> that the luminescence of lanthanide(III)-macrocycles can be increased by the presence of a second lanthanide(III) ion which, in conjunction with a suitable light-absorbing anion, transfers energy to the lanthanide(III) emitter. This phenomenon was originally described by Xu<sup>2</sup> for free lanthanide(III) ions and was named “cofluorescence”. However, since the ligand-mediated luminescence of the lanthanide(III) ions is considered to involve an initial singlet-to-triplet transition of the excited ligand(s), and the luminescence lifetime ranges from 100s of microseconds to milliseconds, the use of the term “fluorescence” in reference to this phenomenon is incorrect. Therefore the term “lanthanide enhanced luminescence”, LEL, will be employed in this paper. When luminescent lanthanide(III)-macrocycles are to be used as markers for bio-systems, another approach that can be successfully employed to increase signal intensity is the use of polymers carrying multiple lanthanide labels<sup>3,4,5</sup>. This is possible because lanthanide luminescence, in contrast to organic dye fluorescence<sup>6</sup>, does not undergo concentration quenching<sup>3,4,5</sup>. This freedom from concentration quenching has been attributed by Lakowicz<sup>7</sup> to the lack of overlap between the absorption and excitation spectra of the light-absorbing/light-emitting pair.

Inorganic phosphor particles<sup>8</sup> are another type of light emitting labels that do not suffer from concentration quenching. However, the absorption spectrum of these particles is narrow, resulting in the preferred method of illumination being two photon absorption of infrared laser light. The use of these particles is limited by nonspecific binding; furthermore, the total binding of rigid particles to solid substrates and cells is limited to a small contact zone. Semiconductor nanocrystals<sup>9,10</sup>, quantum dots<sup>10</sup>, have also been used as light emitting labels. These nanocrystals have broad absorptions that tail off into the ultraviolet. Although individual nanocrystals have been reported<sup>10</sup> to have very narrow emission spectra, for example 12 nm width at half maximum for ZnS-capped CdSe, the collective emissions from a population of these and other nanocrystals are significantly broader. As stated by Chan and Nie<sup>10</sup>, “The properties of Quantum Dots result from quantum-size confinement, which occurs when metal and semiconductor particles are smaller than their excitation Bohr radii (about 1 to 5 nm).” Since the wavelength of the emission maximum is proportional to a physical property, namely the size of the nanocrystal<sup>9</sup>, the size heterogeneity of Quantum Dots results<sup>9</sup> in a broadening of the emission band to 32 nm width at half maximum. By comparison, the emission of

the europium macrocycle in the lanthanide enhanced luminescence solution<sup>1</sup>, when observed at high resolution, had a 7 nm width at half maximum. In addition, intermittent dark states of individual nanocrystals can be a significant problem<sup>11</sup>. According to Lacoste et al.<sup>11</sup>, “They exhibit dark states that can span any duration from microseconds to seconds, sometimes accompanied by intermittent spectral jumps. This photophysical behavior can result in a strong nonlinear relationship between the laser excitation power and the fluorescence emission.” Although the previous quotation concerns single particles, it indicates that the precision of measurements could be compromised by one or more particles entering a dark state when only a small number of particles were present. Finally, both inorganic phosphor particles and nanocrystals have multiple antibody binding sites on their surfaces. Thus the conjugates of these particles could cross-link their antigens and, unless special care is taken, could form aggregates by two particles being bridged by a protein molecule.

Recently, Peterson and Meares<sup>12</sup> have reported on the Merrifield<sup>13</sup> synthesis of support-bound peptides that are substrates for cathepsin B and cathepsin D. These authors stated: “The solubility properties of the PEGA support allow enzymatic permeability in an aqueous environment”. Enzymatic cleavage liberates the peptide that is N-terminal to the cleavage site. The cathepsins were chosen because they are lysosomal endoproteases. In a subsequent paper<sup>14</sup>, these authors reported on the synthesis of peptides that were substrates for cathepsins B and D and contained a DOTA chelating group, capable of binding radioactive <sup>90</sup>Y(III), attached by a peptide bond to the N terminal amino acid. These peptides also contained a p-isothiocyanato-phenylalanine moiety attached by a peptide bond to the C terminal amino acid. The p-isothiocyanato functional group could serve to link the peptide to proteins, including antibodies, by reaction with to the side-chain amino groups of lysine moieties.

The work described in this paper differs from that of Petersen and Meares in that: 1) The peptides contain multiple luminescent labels. 2) The peptides are still bound to a bead when they are conjugated with a protein. 3) The multi-label peptides are subsequently cleaved from the support by an enzyme. Enzymatic cleavage is mild and is to be preferred to disulfide reduction<sup>15,16</sup> because enzymatic cleavage will leave intact disulfide groups that are essential for the structural integrity of many proteins and that serve as linkages to conjugates. An example would be the linkage between a bead bound peptide and a protein<sup>16</sup>, such as a monoclonal antibody.

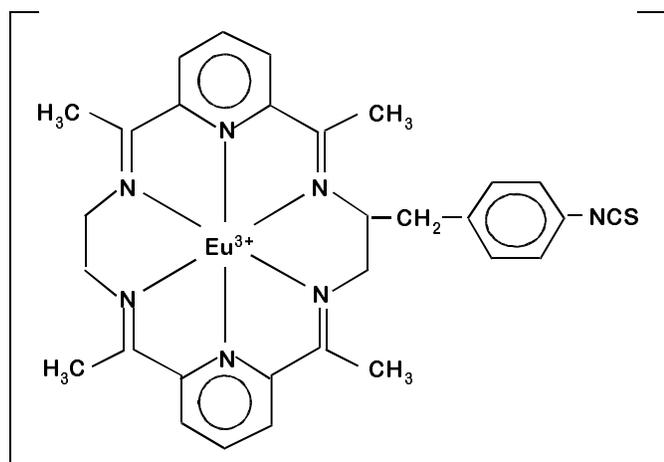


Figure 1. Structure of the cationic europium macrocyclic moiety containing a 4-isothiocyanate-benzyl-substituent on one of the aliphatic side-chains. The empirical formula of the moiety is  $C_{30}H_{31}N_7SEu$ . The trichloride of the mono-functionalized Eu(III) macrocyclic complex shown in Figure 1 was used in the solid phase coupling reactions described below.

Vallarino and Leif have reported<sup>17</sup> on asymmetrically mono-functionalized, water soluble macrocyclic complexes of rare-earth, actinide and yttrium ions. These complexes, an example of which is illustrated in Figure 1, are particularly promising as labels for proteins and other bio-molecules because they include a single coupling functionality, thus avoiding the risk of crosslinking to the substrate.

The following abbreviations will be used to describe molecular structures related to those shown in Figure 1. Specific metal ions will be given by their standard chemical symbols. The mono-functionalized and di-functionalized macrocyclic complexes will be abbreviated respectively as “Mac-mono” and “Mac-di”. The term Mac without the mono or di prefix will include both the mono-functionalized and di-functionalized macrocyclic complexes (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. Thus, the compound shown in Figure 1 is abbreviated as EuMac-mono-NCS. The generic term, M-Mac, will refer to any and all of the macrocyclic species covered by US patents 5,373,093 and 5,696,240.

The present work had two primary goals. The first was to establish that multiple EuMac-di-NCS could be attached to lysine homo- and hetero-polymers, that the red luminescence of the resulting peptide would be proportional to the number of bound EuMacs, and that the luminescence of the peptide bound EuMacs could still be enhanced by the LEL effect. As part of this study, luminescent lifetime measurements which are relevant to the mechanism of the LEL were also carried out. The sec-

ond goal was to establish that the EuMac-mono-NCS could be coupled to a peptide bound to a solid support, e.g. a bead, and that the EuMac-labeled part of the peptide could then be released from the bead by enzymatic hydrolysis under conditions that would not significantly degrade either the luminescence of the EuMacs or the activity of a monoclonal antibody.

## 2. EXPERIMENTAL

### 2.1. Materials

The following were purchased from Aldrich Chemical Co.: Hexamethylenetetramine (HMTA), ACS Reagent, Catalog No. 39,861-0 (1999); Tris(hydroxymethyl)aminomethane (TRIS), ACS Reagent, Catalog No. 25,285-9 (1996-97); Dimethylsulfoxide (DMSO), ACS Reagent, spectrophotometric grade, Catalog No. 15,493-9 (1996-97), used for the lysine homo- and hetero-polymer experiments; 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione (thenoyltrifluoroacetone, HTTFA), Catalog No. T2,700-6 (1996-97). For the lysine homo- and hetero-polymer experiments, commercial HTTFA was purified by recrystallization from ethanol(charcoal)/hexane and stored at 4°C in a dark glass container. For the peptide bound to bead experiments, 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); Trioctylphosphineoxide (TOPO), Catalog No.22,330-1 (1999).

The following were purchased from SIGMA: Aspartic acid, > 99%. Catalog No. A8949 (1998); L-Lysine homo-polymer Catalog No. P-1274, m.wt. 93,000; L-Lysine-L-phenylalanine (4:1) random copolymer (m.wt 47,200), Catalog No. P-3150 (Lysine-phenylalanine); L-Lysine-L-tryptophan (4:1) random copolymer (m.wt. 38,000), Catalog No. P-9285, (Lysine-tryptophan); Proteinase K Molecular Biology, 23 mg/mL protein, 1,100 units, solution in 40% glycerol (v/v) containing 10 mM Tris-HCl, pH 7.5, and 1 mM calcium acetate, Catalog. No. P-4850 (2000); Dimethylsulfoxide (DMSO) ACS Reagent, Sigma Catalog No. D-8779, used for the peptide bound to bead experiments.

TRIS, Amersco Ultra Pure Grade, Catalog No. 0497-1Kg was used for the bead-bound peptide experiments. Sephadex G-25 Superfine, Amersham Pharmacia, Code No. 17-0031-01 (1998-99). High purity Gd(III) trichloride hydrate,  $GdCl_3 \cdot n(H_2O)$ , was used for the lysine homo- and hetero-polymer experiments; it was prepared from the oxide,  $Gd_2O_3$  99.999% REO, Alfa Aesar, Catalog No. 11289 (1999-2000), by dissolving it in 15% aqueous HCl, followed by evaporation to dryness with mild heating under reduced pressure. For the bead-bound peptide experiments,  $GdCl_3 \cdot 6H_2O$  99.99%, Alfa Aesar, Catalog No. 11287 (1999) was used as received.

The TRIS and HMTA buffers were adjusted to the desired pH by the appropriate additions of aqueous HCl and NaOH. The lanthanide enhanced luminescence (LEL) solution was prepared according to J.R. Quagliano et al.<sup>1</sup> (LEL solution).

The hydrophilic support for the synthesis and manipulation of the peptides was PL-PEGA Resin (Polymer Laboratories), described by the vendor as Acryloylated bis(2-aminopropyl)polyethylene glycol/dimethyl acrylamide copolymer, nominal particle size 300-500 um, nominal loading 0.2 g, abbreviated as PEGA. The H-Cys(NpyS)Trp(Lys)<sub>3</sub>ProAlaProPhe(Ala)<sub>3</sub>-LC-PEGA resin and H-Cys(Npys)Trp(LysAla)<sub>3</sub>ProAlaProPhe(Ala)<sub>3</sub>-LC-PEGA resin were custom synthesis products from AnaSpec. The peptide attached to the LC-PEGA resin will be abbreviated respectively as (Lys)<sub>3</sub>Peptide-PEGA-Bead and (LysAla)<sub>3</sub>Peptide-PEGA-Bead. NpyS is the abbreviation for 3-nitro-2-pyridinesulfonyl.

The EuMac-di-NCS and EuMac-mono-NCS<sup>17</sup> were prepared according to US Patent 5,696,240 and are commercially available from Research Organics, Cleveland, Ohio. Common inorganic acids, bases, and salts were obtained from ordinary commercial sources.

Eppendorf Safe-Lock 1.5 mL microcentrifuge tubes, Catalog Number 22 36 320-4 (Eppendorf tubes) were used in all operations with the PEGA beads with bound peptide.

PRB-1, an antibody specific for the 5BrdU marker for DNA (Anti5BrdU) and labeled with a fluorescein analog, available from Phoenix Flow Systems, Catalog No. ABFM18, San Diego, California and the reagents in the Phoenix Flow APO-BRDU kit, Catalog No: Au1001, were used for the measurement of the resistance of the antibody to Proteinase K.

In reporting quantities and concentrations, the term "micro" will be conventionally abbreviated as u; for example, microgram will be abbreviated as ug.

### 2.2. Instrumentation

Atomic absorption analyses of europium were performed on a Varian SpectraAA instrument, using as reference the elemental standards from Aldrich Chemical Co. (Eu, Catalog No. 20,712-8, 1996-97); selected samples were analyzed by ICP-AES (Schneider Laboratories, Richmond, VA). Fluorescence spectra of solutions were obtained with an SLM Model 8000 photon-

counting spectrofluorometer. Fluorescence decay curves of solutions were obtained with a Varian Cary Eclipse spectrofluorometer equipped with a Hamamatsu R3896 (red sensitive) PMT. Both the excitation and emission slits of the Cary were held constant at 5 nm. The lifetime measurements were performed 30 minutes after mixing the specified components. The 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. Visible/ultraviolet absorption spectra of solutions in homo and hetero peptide experiments were obtained with a Shimadzu UV-265 ultraviolet-visible recording spectrophotometer, using stoppered quartz cuvettes. In the experiments with the PEGA-Bead bound peptides, spectra were obtained with a Shimadzu UV 2401 PC model # 206-82301-92 spectrophotometer. 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 electronically-photograph the PEGA-Bead bound peptides. 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 450 and above 600 nm emission filter (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 an uncooled EDC-1000N CCD camera (652 x 494). The gray levels of the images were inverted for display. Darkness indicates strong luminescence.

## 2.3. Methods

### 2.3.1. Coupling of the EuMac-di-NCS to a lysine homo-polymer

EuMac-di-NCS (3.78 mg,  $4.0 \times 10^{-3}$  mol) was dissolved in 0.900 mL of DMSO (EuMac-di-NCS DMSO solution). A 0.267 M HMTA aqueous solution was adjusted to pH 9.4 with NaOH (0.267 M HMTA pH 9.4 buffer). Lysine homo-polymer (5.1 mg,  $5.5 \times 10^{-5}$  mol) was dissolved in a mixture consisting of 0.400 mL DMSO and 1.00 mL 0.267 M HMTA pH 9.4. buffer (polylysine HMTA solution). The EuMac-di-NCS DMSO solution (0.150 mL, 0.62 mg EuMac-di-NCS) was added with gentle shaking to the polylysine HMTA solution. The mixture was allowed to stand at room temperature for 45 min, after which time 0.100 mL of  $2.0 \times 10^{-2}$  M aspartic acid was added with gentle shaking and the mixture was allowed to stand at room temperature for an additional 15 min. It was then chromatographed through a column (17 cm height, 7 mm id) of Sephadex G-25 in 0.267M HMTA pH 7.5 buffer. Elution with the same HMTA buffer gave the coupled peptide as a colorless solution. The eluate was divided into several portions. One portion was quantitatively analyzed for Eu by flame atomic absorption. Another portion was analyzed for polylysine by absorbance, using the Biuret technique. (Dr. V. Katiyar/ [yishwa@alacran.metro.inter.edu](mailto:yishwa@alacran.metro.inter.edu)) The third portion was analyzed for Eu-luminescence as follows: 0.100 mL of eluate, 0.400 mL of  $5 \times 10^{-2}$  M HHTFA and 1.00 mL of a 0.71 M HMTA pH 6.0 were diluted with ethanol to 25.0 mL and the emission spectrum was obtained with excitation at 350 nm. This procedure was repeated twice. first using 5.3 mg of polylysine and 0.300 mL of the EuMac-di-NCS DMSO solution (1.23 mg EuMac-di-NCS) and then using 4.8 mg of polylysine and 0.470 mL of the EuMac-di-NCS DMSO solution (1.93 mg EuMac-di-NCS).

### 2.3.2. Coupling of the EuMac-di-NCS to a lysine-phenylalanine copolymer

The coupling and chromatography experiments described for the lysine homo-polymer were repeated using a lysine-phenylalanine (4:1) random copolymer, with the flow detector set for absorbance at 250 nm (phenylalanine absorption). The following quantities were used for the coupling reactions: 1) Lysine-phenylalanine, 4.8 mg; EuMac-di-NCS, 0.566 mg; Eu/peptide reagents mole ratio = 5.32. 2) Lysine-phenylalanine, 5.3 mg; EuMac-di-NCS, 1.13 mg; Eu/peptide reagents mole ratio = 9.67. 3) Lysine-phenylalanine, 5.3 mg; EuMac-di-NCS, 1.81 mg; Eu/peptide reagents mole ratio = 15.5. The average yield of coupling-elution was ca. 18% relative to initial peptide. The eluates were analyzed for peptide using the BioRad technique (BioRad Laboratories, Inc., US/EG Bulletin 1069), and for Eu-luminescence as described for the polylysine analog.

### 2.3.3. Coupling of the EuMac-di-NCS to a lysine-tryptophan copolymer

The coupling and chromatography experiments described for the lysine homo-polymer were repeated using a lysine-tryptophan copolymer, with the flow detector set for absorbance at 280 nm (tryptophan and EuMac absorptions). The following quantities were used for the coupling reactions: 1) Lysine-tryptophan, 4.9 mg; EuMac-di-NCS, 0.4 mg; Eu/peptide reagents mole ratio = 3.26. 2) Lysine-tryptophan, 4.9 mg; EuMac-di-NCS, 0.8 mg; Eu/peptide reagents mole ratio = 6.51. A precipitate formed during the coupling reactions and the solutions were filtered prior to chromatography. The average yield of Eu-coupled

peptide was less than 10% relative to the initial peptide. The eluates were analyzed for peptide by absorbance at 282 nm and for Eu-luminescence as described above.

### **2.3.4. Removal of the Supernatant from Peptide-Bound PEGA Beads**

In experiments with peptide-bound PEGA beads, removal of the supernatant was performed as follows: the PEGA beads with bound peptide were allowed to settle by gravity for approximately one minute prior to removing the supernatant fluid with a Gilson Pipetman P200 and Fisher Brand 200uL pipetting tips (Fisher Scientific Catalog No. 21-197-2K). The fine bore of the pipetting tips prevented any uptake of the beads.

### **2.3.5 Coupling of the EuMac-mono-NCS to the bead-bound peptide**

The (Lys)<sub>3</sub>Peptide-PEGA-Beads (2.3 mg) were weighed in a 1.5 mL Eppendorf tube. A mixture consisting of 0.20 mL of DMSO and 0.50 mL of the 0.267 M HMTA pH 9.45 buffer was added to the (Lys)<sub>3</sub>Peptide-PEGA-Beads, which were then dispersed by Vortex-mixing for approximately 2 minutes. EuMac-mono-NCS was added to DMSO to produce a  $5.4 \times 10^{-3}$  M, 4.6 mg/mL solution. An aliquot of this solution (0.150 mL, 0.69 mg of EuMac-mono-NCS) was slowly added with gentle tapping to suspend the Peptide-PEGA-Beads. The total volume was 850 uL. The Peptide-PEGA-Beads were allowed to stand at room temperature for 45 min and allowed to settle by gravity. Subsequently the buffer was removed with a 200uL tip Pipetman. The Peptide-PEGA-Beads were then exposed to the EuMac-mono-NCS solution two more times and the beads were allowed to settle by gravity at room temperature for respectively 45 and 53 min. Subsequently the buffer was removed with a 200uL tip Pipetman. The EuMac labelled (Lys)<sub>3</sub>Peptide-PEGA-Beads were then washed four times with 150 uL of HMTA pH 7.55 buffer. This washing restored the EuMac to neutrality and removed contaminants, such as any unbound EuMac-mono-NCS. The EuMac-Peptide-PEGA-Bead samples were stored at 8°C.

### **2.3.6. Release of the EuMac-labeled peptide from the PEGA beads by enzymatic hydrolysis**

The EuMac labelled (Lys)<sub>3</sub>Peptide-PEGA-Beads were washed twice with 150 uL of Tris-Ca pH 8.0 buffer and then suspended in 426 uL of Tris-Ca pH 8.0 buffer. Proteinase K was diluted with a pH 8.0 Tris-Ca buffer to 0.46 ug/uL in Tris-Ca pH 8.06 buffer (Proteinase K solution). The Proteinase K solution (25 uL, 11.5 ug) was added to the EuMac-Peptide-PEGA-Beads resulting in a total volume of 451 uL and a Proteinase K concentration of 27.0 ug/mL. The EuMac-Peptide-PEGA-Beads were allowed to settle for approximately one minute, 70 ul aliquots of the supernatant were removed at 40, 80, 115, and 124 minutes using a 200 uL tip Pipetman, and the absorbance spectrum of each aliquot was obtained with a spectrophotometer employing stoppered 40 uL fused silica cuvettes (Starna, 16.40-Q-10). The EuMac-Peptide-PEGA-Bead samples were stored at 8°C.

### **2.3.7. Kinetics of the cleavage of the Peptide-Bound PEGA Beads by Proteinase K**

The Proteinase K solution was diluted 100-fold with distilled water to reach a concentration of 230 ug/mL. One tenth mL of 0.1M (LysAla)<sub>3</sub>Peptide-PEGA beads were transferred into a 1.5 mL Eppendorf tube, washed 3 times with 0.5 mL of an aqueous solution containing TRIS (0.01M) and CaCl<sub>2</sub> (0.001M) adjusted to pH 7.07 (Tris-Ca Buffer), and resuspended in 0.30 mL of Tris-Ca buffer. Preliminary experiments with the (Lys)<sub>3</sub>Peptide-PEGA-Beads indicated that the washes resulted in less than 7.3% of the peptide being released prior to the addition of Proteinase K. The suspension of beads was transferred to a Starna Stirring Cell, Semi-Micro Rectangular cuvette (9-Q-10-MS) equipped with a small magnetic stirring bar and 0.70mL of Tris-Ca buffer was added. The Starna Spinette Cell Stirrer was turned on at approximately 70% of maximum speed for the rest of the experiment. The spectrum was obtained in duplicate. The Proteinase K solution (0.05 mL) was added resulting in an enzyme concentration of 11 ug/mL. All subsequent spectra were obtained in sequential groups of 5.

In order to reduce the noise due to the random distribution of the PEGA beads in the beam, each set of multiple readings was averaged. The absorbance of the two chromophores tryptophan and NpyS is low while the peptide is bound to the bead. Release of the peptide from the bead increases the absorbance of the two chromophores.

### **2.3.8. Digestion of a monoclonal antibody with Proteinase K**

The containers used for the experiment were Fisher 5 mL polystyrene round bottom tubes, 12 x 75 mm style, Fisher Scientific Catalog No. 2008. Proteinase K was diluted with a pH 8.0 Tris-Ca buffer to concentrations of 24 and 240 ug/mL. The Anti-5BrUdR was diluted to 0.1 ug/uL with the pH 8.0 Tris-Ca Buffer, according to the published instructions. Proteinase K was added and the samples were incubated at room temperature for 58 min. Within less than 5 minutes after the end of the incubation, one mL,  $1.0 \times 10^6$  cells, of previously prepared BrdUrD labeled control cells were added to a mixture of 90 uL of Rinse Buffer of the Phoenix Flow kit and 10 uL of the Anti5BrdU solution. The cells with the labeled Anti5BrdU solution were incu-

bated in the dark for 30 minutes at room temperature. Propidium Iodide/RNase A Solution (0.5 mL) was added to stain the DNA. The 5mL tubes were wrapped with aluminum foil and the cells were incubated in the dark for 30 minutes at room temperature. After incubation, a FACScan (Becton Dickinson) flow cytometer equipped with a 488 nm laser and logarithmic amplifiers was used to measure the cells fluorescence arising from both the fluorescein analog labeled Anti5BrdU and the Propidium Iodide.

#### 2.4 Lifetime Measurements

The excitation and emission wavelengths were respectively 370 and 620 nm. The start of data acquisition was 90 usec after triggering the flash-lamp and the instrument was set to make a measurement every 50 usec. The data from 100 flashes was averaged by the instrument. The total decay time, measurement period, was set to 2,000 usec. The emission filter was set to open and the excitation filter was set to auto. The PMT potential was set at 1000 volts.

### 3. RESULTS AND DISCUSSION

#### 3.1. Labeling of lysine homo- and hetero-polymers with multiple europium(III)-macrocycles

Under the conditions described in the Methods, Sections 2.3.1-2.3.3, the EuMac-di-NCS reacted with lysine homo- and hetero-polymers to form multi-label peptides with an average yield of 15-20% relative to the starting peptide. This series of experiments gave the following results, illustrated in Figures 2-4: A) The average EuMac-to-polylysine mole ratio in the coupled peptide, referred to as Eu-polylysine loading in the following, increased proportionally to the Eu/polylysine mole ratio used in the coupling reaction, Figure 2. B) The emitted photon count, when normalized to account for different peptide concentrations, increased proportionally to the average Eu-peptide loading, Figure 3. C) The emission spectra of EuMac-polylysine samples with different Eu-polylysine percentage loadings had identical patterns, Figure 4, confirming that the emitting species are the same in each case. These results, summarized in Table 1 and Figure 5, showed that the normalized Eu-luminescence increased proportionally to the Eu-peptide loading, which in turn increased proportionally to the Eu-Mac-di-NCS/peptide reagent mole ratio.

The combined results of both the homo- and hetero-polylysine experiments clearly demonstrate that the polymer bound EuMacs does not concentration quench and therefore the use of EuMac and other lanthanide optical-labels attached to a polymer is both scientifically and commercially feasible.

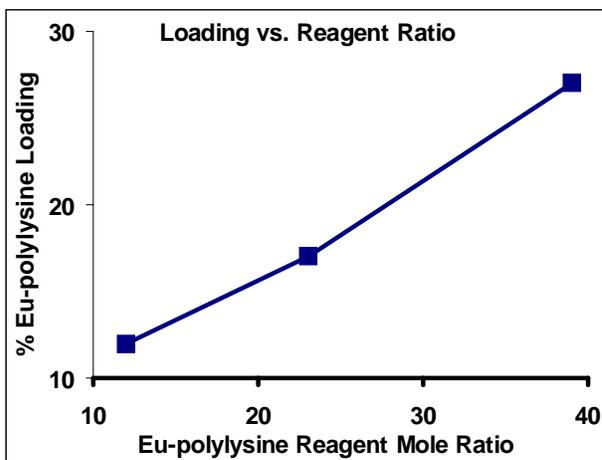


Figure 2. Plot of the average EuMac-to-polylysine loading in coupled polylysine, as a function of the EuMac-di-NCS-to-polylysine mole ratio used in the coupling reaction. The loading is expressed as percentage of EuMac-coupled lysine residues.

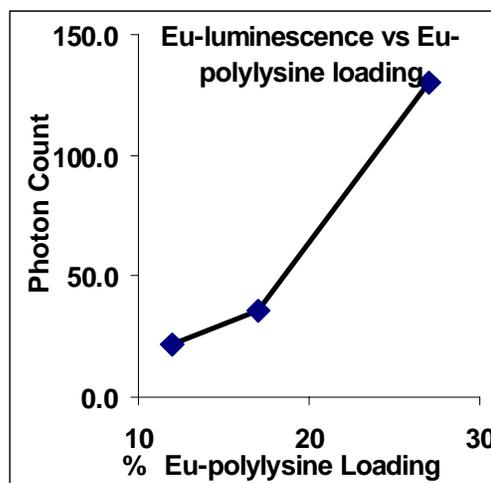


Figure 3. Eu(III) emission at 618 nm, normalized to  $1 \times 10^{-6}$  mmol polylysine /mL, as a function of the Eu-polylysine percentage loading.

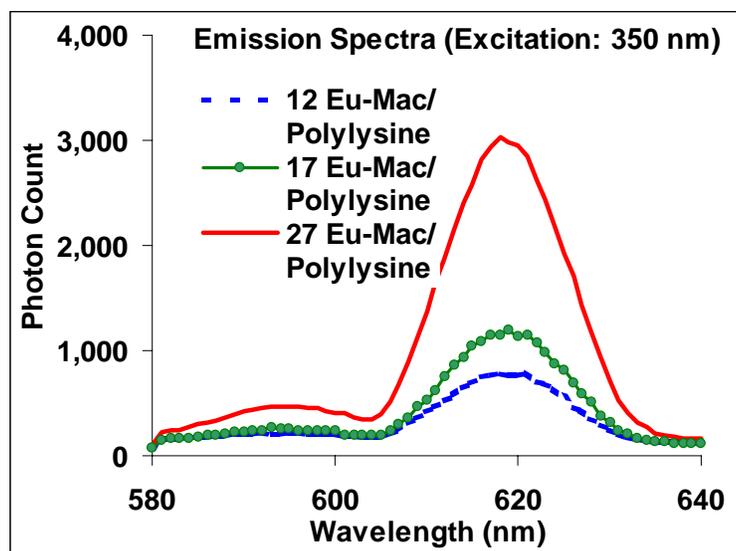


Figure 4. Eu-Emission spectra of EuMac-polylysine conjugates with different Eu-polylysine percentage loadings.

Table 1: Comparison of the emission intensities (as normalized photon counts) of EuMac-polylysine-phenylalanine copolymers obtained with different Eu-to-peptide reagent ratios.

Eu-Peptide Reagent Ratio	Normalized Photon Count
5.32	$4.69 \times 10^8$
9.67	$6.03 \times 10^8$
15.5	$7.9 \times 10^8$

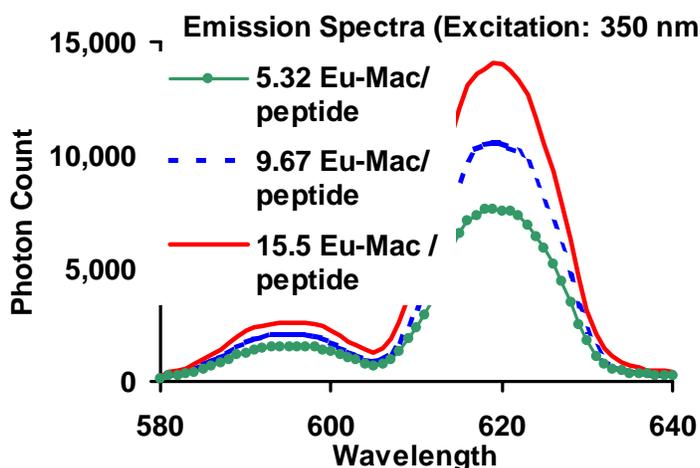


Figure 5. Eu-Emission spectra of EuMac-Polylysine-phenylalanine conjugates as a function of different Eu MacNCS/poly-lysine-phenylalanine ratios used in the coupling reactions.

Table 2: Comparison of the emission intensities (as normalized photon counts) of EuMac-lysine-tryptophan copolymers obtained from different Eu-to-peptide reagent ratios.

Eu-Peptide Reagent Ratio	Normalized Photon Count
3.26	$8.5 \times 10^7$
6.51	$34. \times 10^7$

### 3.2 Studies on peptides bound to solid supports

#### 3.2.1 Enzymatic release of a peptide carrier from PEGA Beads

The direct coupling of a lanthanide(III)-labeled peptide to an analyte binding species requires both a simple coupling technique and a means to minimize any possible interference of the labeled peptide with the final binding to the analyte. One approach to achieving both goals is to employ a solid phase system, such as the peptide-bound PEGA beads described in Methods, Section 2.3. In addition, the chemical reactions employed, including those required to cleave the peptides from the beads, should be sufficiently mild to maintain the integrity of the lanthanide(III) label and of the analyte binding species. Since the lanthanide(III) macrocycles are Schiff bases and the analyte binding species are often proteins, cleavage by use of strong acids or bases is precluded and enzymatic cleavage was the method of choice. Proteinase K was selected as the enzyme because it has been used in histochemistry and its reactivity with specific peptide sequences had been described<sup>18</sup>.

The peptide used in this work (Figure 6) contained a ProAlaProPhe(Ala)<sub>3</sub> segment, which is peptide VII of Table III of Bromme et al.<sup>18</sup> Peptide VII was chosen as the enzyme substrate segment of the peptide because it has the highest ratio, 133,000 sec.<sup>-1</sup>mole<sup>-1</sup>, between the rate of catalysis and the Michaelis constant; Bromme et al.<sup>18</sup> describe this ratio as a measurement of protease activity.

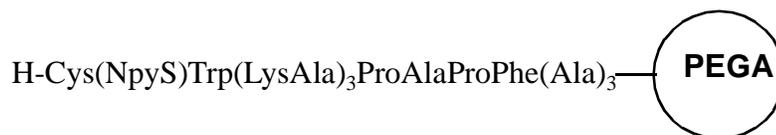


Figure 6. Proteinase K cleavable peptide bound to a PL-PEGA Resin bead. The presumed cleavage site for Proteinase K is between the phenylalanine and the first of the 3 alanines.

The peptide of Figure 6 also included three lysines, selected because they can react with an isothiocyanato group or other reactive functionality, such as those present on functionalized luminescent EuMacs. The 3-nitro-2-pyridinesulfonyl (NpyS) group was bound to the cysteine by a disulfide link; this, according to reports of Menzo et al.<sup>19</sup>, should subsequently undergo a facile disulfide exchange with an available cysteine or other sulfhydryl of an analyte-binding species, such as an antibody, with the formation of the peptide-antibody disulfides being favored.

It was noted that the absorbance of the two chromophores present in the peptide, tryptophan and NpyS, was low as long as the peptide was bound to the bead and increased as soon as the peptide was cleaved from the bead. The decreased absorbance of the peptide while attached to the bead is an example of the distribution error<sup>20</sup>. Since absorbance is a logarithmic property, the negative log of the intensity of each light wave is proportional to the number of chromophores traversed. Therefore, if one percent of the light rays have their intensity reduced a thousandfold by interacting with a bead and the other 99% pass through unaffected, the resulting absorbance will be  $\text{Log}(1.00/99)$  or 0.004. After hydrolysis, all of the light rays pass through and are attenuated by a homogenous solution of peptides containing both tryptophan and NpyS.

As shown in Figure 7, the absorbance of the bead-containing solution increased with time after the addition of Proteinase K; The 276 nm and 350 nm peaks are characteristic of the tryptophan and NpyS chromophores. The time dependence of the release of the peptide from the beads is shown in Figure 8. The plateau reached after approximately 200 minutes indicates that the enzymatic hydrolysis has effectively ceased. Similar results (data not shown) were obtained in two other experiments carried out at pH 7 and pH 8, respectively. In these experiments, aliquots were removed from two samples, one with and one without Proteinase K. In all experiments, there was a small continuous release of the peptide without the enzyme; however, the release of the free peptide was much greater in the Proteinase K samples.

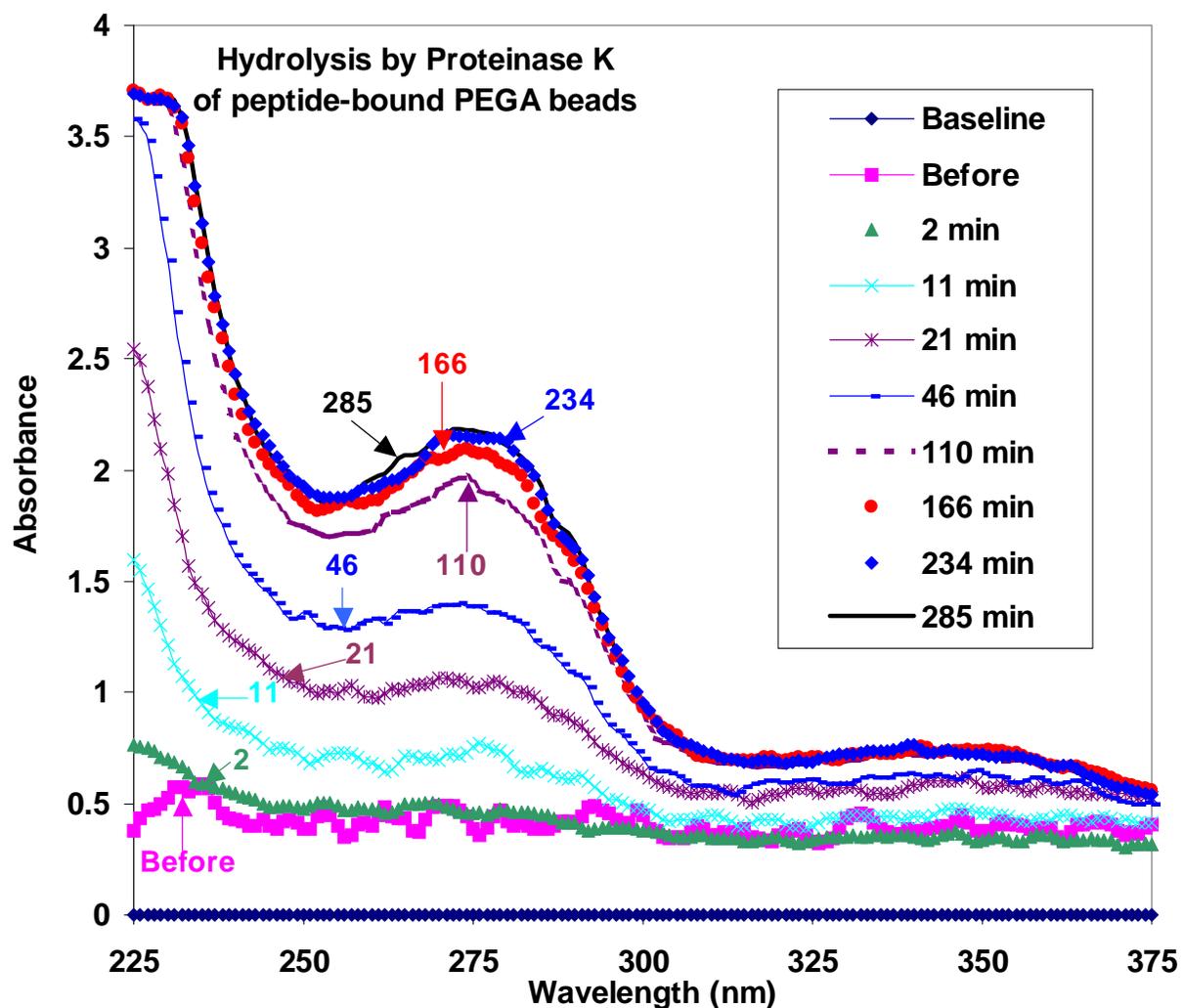


Figure 7. Absorbance spectra obtained during the hydrolysis of Peptide-PEGA beads. The TRIS-Ca buffer baseline is coincident with the abscissa. The curve identified as “Before” shows the absorbance prior to the addition of Proteinase K. In order to reduce the noise due to the random distribution of the PEGA beads in the beam, each set of multiple readings was averaged. The zero-time spectra were obtained in duplicate and 5 replicate spectra were obtained for data after the enzyme was added. Each time value represents the midpoints of the times range of the averaged spectra. Both the tryptophan peak at ca. 280 nm and the NpyS peak at ca. 350 nm increase with time as the hydrolysis proceeds.

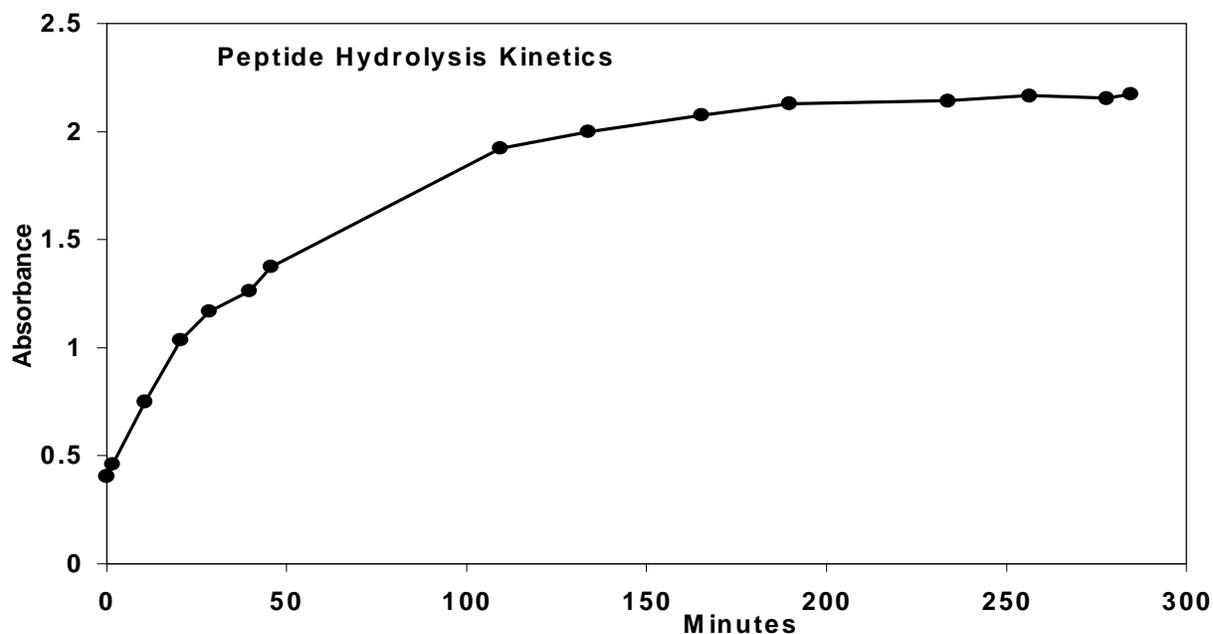


Figure 8. Time dependence of the release of the peptides from the beads. Absorbance measurements were made in nanometer increments. In order to reduce the noise due to the random distribution of the PEGA beads in the beam, each set of multiple readings was averaged as described in Figure 7. Each absorbance value is the average of the values obtained between 274 and 278 nm for the averaged replicate spectra. Each time value represents the midpoints of the time range of the averaged spectra.

### 3.2.2. Coupling of a Functionalized Europium Macrocycle to the Bead Bound Peptide

The peptide used in these experiments (Figure 9) was similar to the peptide shown in Figure 6; however, the 3 lysines were grouped together rather than being separated by an alanine. Figure 9 is a schematic representation of the Peptide-PEGA-Beads with EuMacs bound to the lysine residues; this structure shall be referred to as EuMac-Peptide-PEGA beads. The presence of functionalized europium macrocycles on the peptide did not significantly interfere with the capacity of Proteinase K to release the labeled peptide from the PEGA-bead.

The EuMac-Peptide-PEGA-Beads can be stored in either dimethylformamide or ethanol at  $-20^{\circ}\text{C}$  or below.

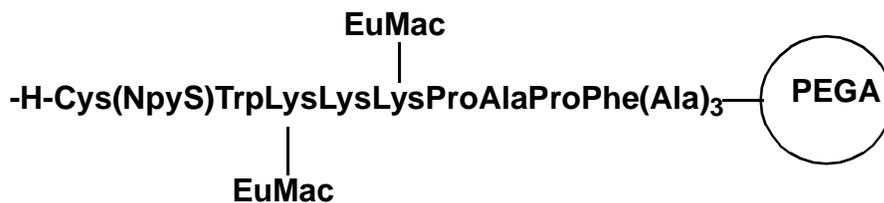


Figure 9. The position and number of the EuMacs in this figure is diagrammatic. The number of EuMacs bound on each peptide ranged from 0 to 3.

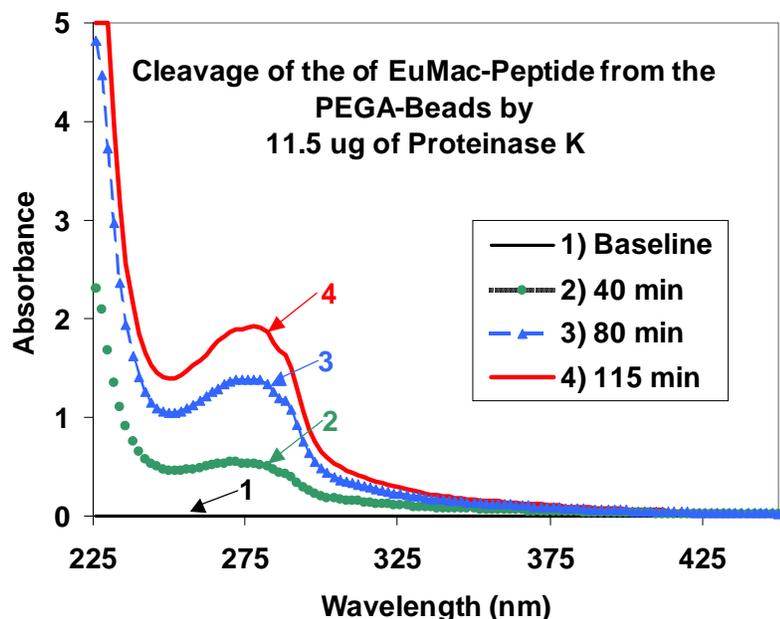


Figure 10. Absorbance spectra of supernatant samples from Proteinase K hydrolysis of EuMac-(mono)-NCS and NIRL-2 beads conjugate. The graph shows that the absorbance at about 280 nm increases with time for all samples. This demonstrates that Proteinase K cleaves a product (peptide) containing tryptophan from the PEGA-Beads.

### 3.2.3. Release of the Europium Macrocycle Labeled Peptide from the PEGA Bead by Enzymatic Hydrolysis

In these experiments, small samples of beads were mixed with the LEL solution for observation under an episcopic fluorescence microscope. Both the pre-hydrolysis sample of the EuMac-Peptide-PEGA-Beads and the sample hydrolyzed for 115 min showed the characteristic EuMac luminescence under UV excitation (Figure 11). However, as shown in Figure 11, the luminescence from the pre-hydrolysis sample was strong and the luminescence from the sample hydrolyzed for 115 min was weak. The strong luminescence of the pre-hydrolysis beads demonstrated that a significant amount of EuMac had coupled to the peptide. The drastic difference in luminescence before and after Proteinase K hydrolysis demonstrated that the EuMac-labeled part of the peptide was released from the bead.

The periphery of the pre-hydrolysis sample bead also showed luminescence, although not as bright as that of the bead itself (Figure 11 Left). This luminescence “halo” from the solution immediately surrounding the pre-hydrolysis bead can reasonably be ascribed to the EuMac-Peptide attached to the polyethylene glycol pendant polymer side chains that emanate from the PEGA-BEAD. The amount of EuMac-Peptide contained in this halo could have been considerable because the image observed through a microscope is a two-dimensional section of a three-dimensional object. To test for luminescence in the supernatant, a spot-test was performed by placing 2  $\mu$ L of the hydrolyzed supernatant sample (115 minutes into the hydrolysis) on a slide with 2  $\mu$ L of the LEL solution; the spot did luminesce when irradiated at approximately 365 nm.

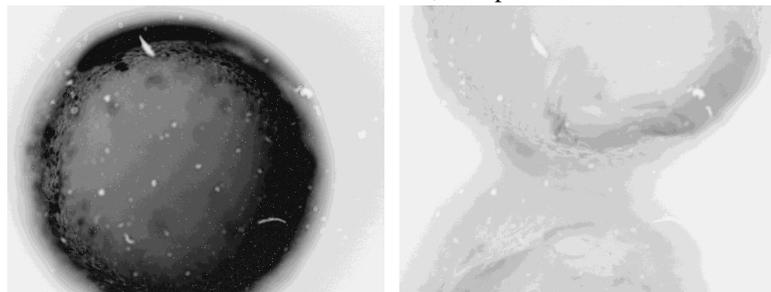


Figure 11. CCD Images (619 nm emission) of EuMac-Peptide-PEGA-Beads with added LEL solution, before hydrolysis with Proteinase K (Left) and after 115 minutes of hydrolysis (Right). The exposure was 500 ms with a 10X, N.A 0.25 objective. The bead on the left luminesces much more strongly than the two beads on the right. The white spots on the bead on the left are pixel artifacts.

### 3.2.4. Resistance of a monoclonal antibody to digestion with Proteinase K

An antibody specific for the 5BrdU marker for DNA (Anti5BrdU) and labeled with a fluorescent dye excited at 488 nm was used in these experiments; this antibody is part of a commercially available (Phoenix Flow, San Diego CA) flow cytometry kit. A comparison was made between the antibody before and after enzymatic digestion with two concentrations of Proteinase K. After digestion, the antibody was still able to stain BrdU labeled apoptotic cells.

The results shown in Table 3 demonstrate that incubation with 24 ug/mL of Proteinase K had negligible effect on the antibody labeling capacity and that even after exposure to a 10 times greater Proteinase K concentration, 65% of the positive cells could still be detected. For the 0 (control), 24, and 240 ug/mL Proteinase K treatments, the differences between the positive and negative channels were 440, 432, and 289 respectively. Note that a concentration of 24 ug/mL is approximately twice the 11.0 ug/mL concentration used to free the peptide from the bead and approximately equal to the 27.0 ug/mL concentration used to free the EuMac labeled peptide from the bead. Thus, virtually all of the biologically active antibody survived the conditions for enzymatic release of the peptide.

**Table 3: Effect of Proteinase K Treatment on Anti5BrdU**

Prot-K (ug/mL)	Anti-5BrdU ug/uL	% Fluor. Cells	Mean Channel of Pos. Cells	Mean Channel of Neg. Cells	Pos. - Neg. Mean Channel
0	0.1	36.7	675	235	440
24	0.1	35.5	681	249	432
240	0.1	24.0	524	235	289

### 3.3 EuMac Lifetimes

A study of the luminescence lifetime of the non-functionalized Eu(III)-macrocyclic under a variety of conditions was undertaken to provide additional information on the various parameters that determine the LEL effect for Eu(III) complexes of this class. The lifetimes of the non-functionalized EuMacs were measured in: 1) a nonaqueous environment (ethanol) with  $8.00 \times 10^{-4}$  mol/L HTTFA added as the diketone enhancer, 2) in a complete LEL aqueous micellar solution, and 3) in a LEL aqueous micellar solution without Gd(III). In each case the concentration of the Eu(III)-macrocyclic was the same, 28.1 umol/L. The lifetimes of the non-functionalized EuMac in water and deuterium oxide, without diketone enhancers, had been previously reported<sup>21</sup>. The data and the calculated monoexponential fit to the data are shown in Figure 12 The calculated results are shown in Table 4.

**Table 4: Lifetimes and Calculated Values**

EuMac Solution	Lifetime (usec)	Summation of Experimental Points	Intercept at Zero Time (I)	Exponent (E)
HTTFA in EtOH	1,204	717	47.7	-0.0008
LEL with Gd(III)	955	5,336	402.2	-0.001
LEL without Gd(III)	344	325	52.1	-0.0024

The equation for the monoexponential fits was: Intensity =  $Ie^{ET}$ , where I is the intercept at time zero, E is a constant (see Table 4), and T is the time in microseconds. The range of the experimental points was 115 to 2,035 us. The relative intensity measured is the summation of these points. The intercept at zero time is equal to the relative intensity extrapolated to zero time.

The results summarized in Table 4 clearly show that the increase in integrated intensity (Summation of Experimental Points) for the EuMac in the LEL solution with Gd(III), relative to the LEL solution without Gd(III), cannot be related solely to the observed increase in lifetime. The ratio of the integrated intensities for the EuMac with and without Gd(III) is 16.4, while the ratio of the lifetimes is only 2.8. The ratio of the relative intensities for EuMac with and without Gd(III), extrapolated to zero time, is 7.72. Equally significant is the comparison with the ethanol/diketone solution, which has a longer lifetime than the LEL solution with Gd(III), 1,204 usec versus 955 usec for, but a much lower integrated intensity, 717 versus 5,336. Thus, it is evident that the LEL effect observed for the Eu(III)-macrocyclics results primarily from the role that the non-luminescent Gd(III)-diketonate species play in the absorption and transfer of energy to the Eu(III)-macrocyclics.

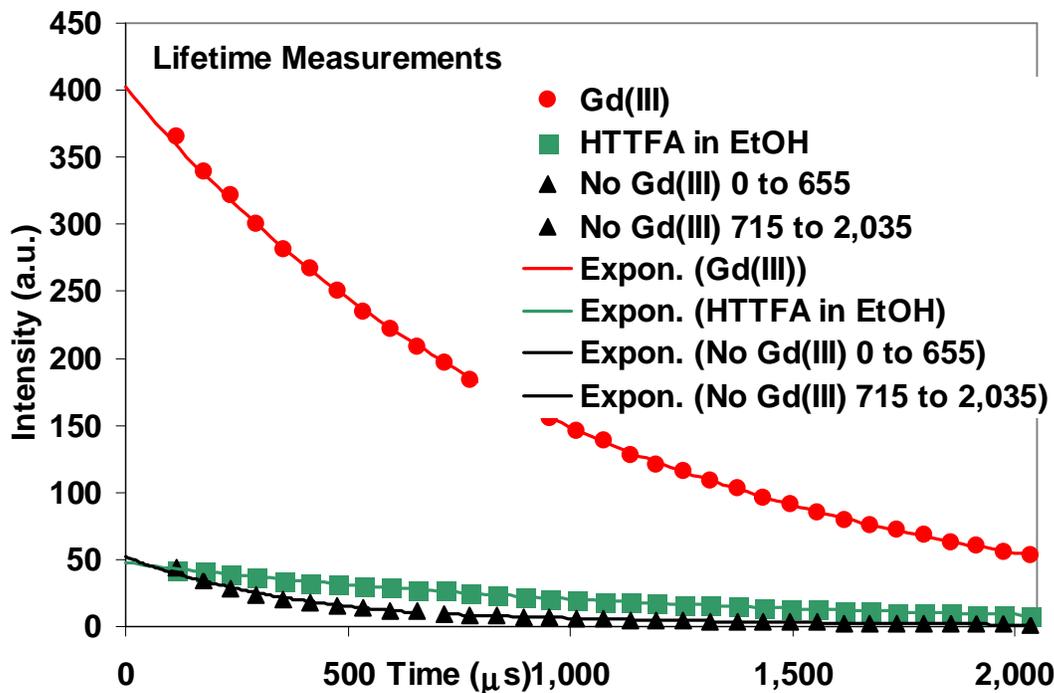


Figure 12. Graph of the data obtained with the Cary Eclipse spectrofluorometer. The individual data are shown by the symbols and the lines are the mono exponential fits calculated by Excel. The data from the experiment without Gd(III) required two separate monoexponential fits.

#### 4. CONCLUSIONS

A peptide bound to a solid-phase support was synthesized, conjugated with a lanthanide(III) macrocycle as luminescent label, and the labeled peptide was then released from the support by enzymatic cleavage. The very mild conditions of the enzymatic-cleavage did not significantly reduce the activity of an analyte-binding species, in this case an antibody, and did not affect the luminescence properties of the lanthanide-macrocycle. The peptide bound to the support included a cleavage site for an enzyme, one or more sites for covalently binding labels, and a site for selective coupling to a macromolecule--in this case a group capable of forming a disulfide link with the macromolecule. Under these conditions, the macromolecule to which the peptide is to be attached need not be subjected to the conditions of the peptide-labeling reactions and is unaffected by the labels themselves. The peptide described in this paper is the prototype of a new class of polymers that will permit multiple labels to be attached to a macromolecule with only one amino acid being modified. In contrast to inorganic phosphor particles or nanocrystals, these labeled peptides have only a single functionality capable of reacting with the macromolecule. Thus, peptides carrying multiple lanthanide macrocycles can be coupled to macromolecules with minimal possibility of structural modification and consequent functional change, as well as cross-linking. As a further advantage, the attachment of a flexible multi-label peptide to a single location in a macromolecule should not sterically hinder the binding of the macromolecule to a cell, whereas the attachment of rigid inorganic particles can limit the number of macromolecules that can bind to a cell or similar analyte.

It should be noted that, although a single inorganic nanoparticle may emit more intensely than a single lanthanide macrocycle under comparable excitation flux, the lanthanide macrocycle labels reported here can produce a more intense signal because: 1) Multiple lanthanide macrocycles attached to a polymer carrier can be contained in a volume equal to that of a single inorganic particle and their combined emission intensity is proportional to their number, since they do not suffer from concentration quenching. 2) The LEL effect permits various species, other than those directly bound to the lanthanide ion, to act as photon traps and energy-transfer donors. Thus, the luminescence intensity of the labels can be optimized in each case by selecting an amino acid sequence that permits the optimum positioning of energy transfer pairs, such as enhancers with lanthanide(III) macrocycles or energy transfer pairs of conventional organic fluorochromes.

In conclusion, the labeled peptides described in this paper present a number of useful and original features. In the case of luminescent labels that do not concentration quench, such as the lanthanide(III)-macrocycles, multiple labels can be bound to the peptide for increased signal intensity. Since the label(s) are coupled to the peptide prior to its attachment to the monoclonal antibody or other analyte binding species, the conditions for the labeling reaction need not be limited to those required to maintain biological activity of macromolecule. Consequently, the subsequent linkage of the already labeled peptide to a macromolecule will result in minimal, if any, reduction in biological activity. Finally, the use of polymers, such as peptides that have predictable, specific conformations permits the design and synthesis of labels for which the transfer of energy from photons can be controlled and optimized.

## 5. ACKNOWLEDGEMENTS

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