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ABSTRACT

THE PHOTOPHYSICAL PROPERTIES OF LANTHANIDE LUMINESCENT PROBES

by
Ke Li

A detailed study of the luminescence quantum yield, lifetimes and the number of water molecules coordinated to lanthanide luminescent probes based on biphenylyl-7-amino-4-methyl-2(1H)-quinolinon (Bi-cs124) derivatives have been carried out using a time-resolved fluorescence assay (TRFA). Bi-cs124 was conbined with different chelating agents, specifically diethylene triamine pentaacetic acid (DTPA), ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and 3,6,9,12-Tetrakis carboxymethyl)-3,6,9,12-tetraazatetradecane-1,14-dioic acid (TTHA). The results indicate that Bi-cs124-TTHA has a longest lifetime and smallest number of coordinated water molecules because TTHA has larger number of chelating groups. The dependence of the luminescence lifetimes on the probes concentrations was investigated. The luminescence lifetime is shorter at higher probes concentrations due to self-quenching.

Images of E-coli cells labeled with one of the lanthanide luminescent probes have been obtained by TRFA excited by excimer laser radiation (351 nm) using the total internal reflection fluorescence microscopy (TIRFM). The results indicate feasibility of using lanthanide luminescent probes for time-gated luminescence microscopy.

THE PHOTOPHYSICS PROPERTIES OF LANTHANIDE LUMINESCENT PROBES

by
Ke Li

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Otto H. York Department of
Chemical, Biological and Pharmaceutical Engineering

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CHAPTER 1

INTRODUCTION

1.1 Rare Earth Elements

The elements from the 57th element lanthanum to the 71th element lutetium and IIIB family elements scandium and yttrium are defined as rare earth elements by International Union of Pure and Applied Chemistry (IUPAC).

Except scandium and yttrium, other rare earth elements have the same outer shell electronic structure like $4f^{0\text{-}14}5d^{0\text{-}10}6s^{1\text{-}2}$.The new electrons fill into 4*f* subshell by the increase of nuclear charge number. Consequently, they are also called "*f*-block" elements. In these elements, however, the outermost *d* and *f* subshells lie close together in energy, leading to some irregularities in electronic structure. The lanthanides are chemically similar. Except for cerium and europium, the lanthanides occur naturally in ionic compounds as ions with a +3 charge (lost two electrons in 6*s* and one in 5*d* subshells). Going down the series, the radii of the Ln^{3+} decrease—a phenomenon known as the "lanthanides contraction." An energy level diagram of some rare earth elements is shown in Figure 1.1.[1]

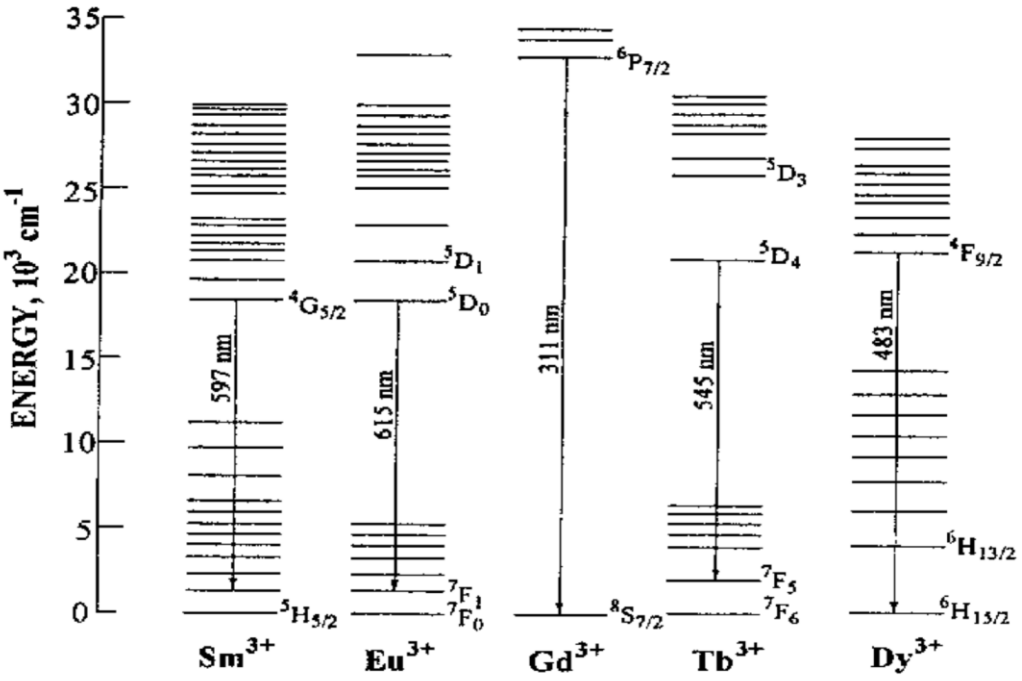


Figure 1.1 Electronic energy levels of some rare earth elements ions.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.2 Lanthanides

The majority of the rare earth element ions do not exhibit luminescence. However, four lanthanide hydrated ions Sm^{3+}, Eu^{3+}, Tb^{3+}, Dy^{3+}, fluoresce weakly when excited by ultraviolet light (UV-light) or visible light. Their fluorescence is significantly enhanced when lanthanide ions are combined with organic ligands. This is due to the energy transfer between the ligands and lanthanide ions.

1.2.1 Mechanism of Lanthanide Probes Luminescence

When the electrons of the ligand compounds absorb excitation light, they are promoted from the ground state S_0 to excited state S_1. After that, excitation is transferred from S_1 to triplet state T_1 due to the intersystem crossing. Finally, excitation from the low-lying

triplet state is transferred to the excited state of chelated lanthanide ion, which emits light. The luminescence mechanism is shown in Figure 1.2.[3]

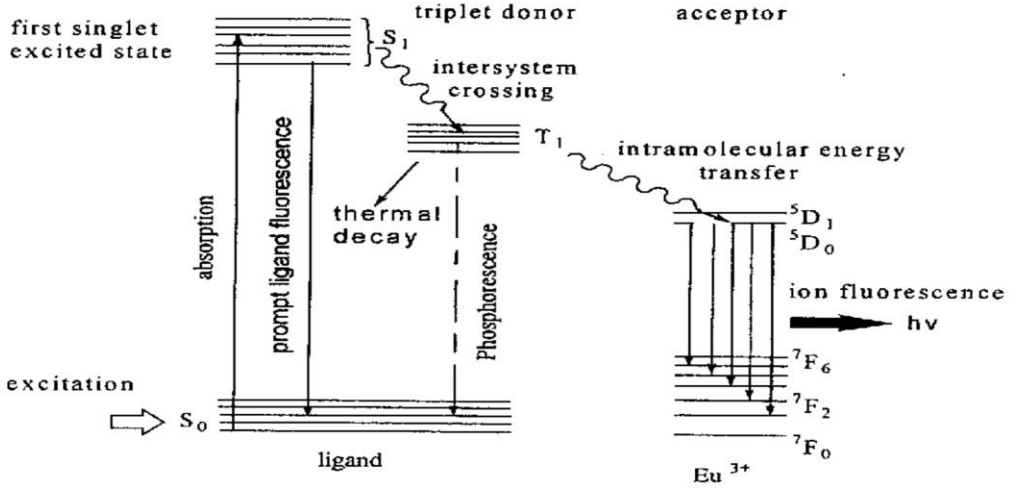


Figure 1.2 The mechanism of luminescence of chelated Eu^{3+}.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

The photophysical properties of lanthanide luminescent probes depend on the match of the energy levels of the excited state of the lanthanide ion and the triplet state T_1 of the organic ligand. The luminescence intensity, the wavelength range and the spectra vary for different lanthanide ions. Sm^{3+}, Eu^{3+}, Tb^{3+} and Dy^{3+} have the strongest luminescence among all lanthanide ions. The transition in the lanthanide ions is forbidden; therefore the luminescence lifetimes are long. In addition, the excited electron is shielded from the solvent which reduces quenching and results in high quantum yield and "atomic-like" spectra of the luminescence. The photophysical properties of a lanthanide probe ($[EU(\beta\text{-}NTA)_3]$) and several luminescent organic compounds are compared in Table 1.1.[4-6]

Table 1.1 Photophysics Properties of Some Organic Fluorescent Compounds in Comparison with a Lanthanide Luminescent Compound ([EU(β-NTA)$_3$])

Compound	Luminescence lifetime (ns)	λex,max (nm)	λem,max (nm)	Stokes shift (nm)	Quantum yields(Q)	ε (mol^{-1} L cm^{-1})
FITC	4.5	492	518	26	0.85	7.0×10^4
RBITC	3	550	585	35	0.70	1.2×10^4
Cy5	NA	650	667	17	0.27	2.5×10^5
[EU(β-NTA)$_3$]	7×10^5	340	613	273	0.21	3.6×10^4

FITC: Flourescein isothuocyanate; RBITC: Rhodamine-B200-isothiocyanate; Cy5: cyanine dye; β-NTA: 2-naphthoyltrifluorobutanedione. λex,max: wavelength of the maximum absorption; λem,max: wavelength in the maximum of emission; ε: coefficient of molar absorption (extinction coefficient), base 10.

Source: Tan, M., Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.2.2 Photophysical Characteristics of Lanthanide Probes

1.2.2.1 The Luminescence Emission Spectrum is Lanthanide Ion Dependent. The luminescence emission spectra of luminescent lanthanide compounds depend upon the lanthanide ion but not on the organic ligands. The function of the organic ligands is to absorb excitation light and subsequently to transfer excitation energy to the lanthanide ion. The luminescence light is emitted by lanthanide ions. Different ligands chelated with the same lanthanide ion exhibit similar emission spectra.

1.2.2.2 Long Luminescence Emission Lifetime. Lanthanide probes with Tb^{3+} ion have the luminescence lifetime ca. 1 ms in regular (light) water used as a solvent and ca. 2 ms in heavy water (D_2O). Eu^{3+} based probes have luminescence lifetimes about 0.5 ms in regular water and about 2 ms in heavy water. The luminescence from lanthanide probes is a delayed luminescence because energy transfer between the ligand and the lanthanide

ion requires time. Because of this the lifetime is even longer than a phosphorescence lifetime of the organic ligand. In Table 1.1, the luminescence lifetime of Tb^{3+} probe is ca. 10^5 times longer than the fluorescence lifetimes of organic fluorescent compounds. Long luminescence lifetimes together with high quantum yield make lanthanide luminescent probes ideal for time-gated detection.

1.2.2.3 Large Stokes Shift. The majority of lanthanide probes have Stokes shifts larger than 200 nm. This provides an additional way to suppress the interference caused by the excitation light and induced luminescence of the medium via spectral filtering.

1.2.2.4 Characteristics of the UV Absorption and Luminescence Emission Spectra. Lanthanide probes have relatively wide absorption bands which allows flexibility in the choice of the source of excitation light. However, the luminescence spectra are structured with sharp peaks (the full width at half maxima are usually about 10 - 15 nm). The characteristic line-like spectra can serve as "fingerprints" and could be useful in application which require identification and discrimination of the emitting species.

1.3 Lanthanide Luminescent Probes

The first studies of lanthanide luminescent probes began in 1942. Weissman discovered Eu^{3+} characteristic spectra when he measured β-diketone-Eu^{3+} under UV-light. After that a significant volume of research has been done on the properties of lanthanide luminescent. In 1966, Crosby [7] and Sinha summarized the photophysical properties of lanthanide chelates. In 1984, Horrock [6] and Albin introduced lanthanide probes as a tracer agents into biochemistry. Presently, different kinds of lanthanide luminescent probes have been developed by many research teams, including β-diketone, carboxylic

acid derivatives, macrocyclic ligands, heterobiaryl ligands, cryptands, podands, calix-arenes, terphenyl ligands and proteins. Most of them are based on Eu^{3+} (red luminescence) and Tb^{3+} (green luminescence). These compounds were used as luminescent probes to detect biomolecules or live cells. They also can be used in time-resolved fluorescence assays.

1.3.1 β-diketone Derivatives Luminescent Probes

In the early stage study, 2-naphthoyltrifluoroacetone (β-NTA) [8], 2-thenoyltrifluoroacetone (TTA) and pivaloyltrifluoroacetone (PTA) ligands could form strong fluorescent complexes with lanthanide ions. In these earlier studies these β-diketone derivatives did not have any groups that can be used to attach them to proteins or other biomolecules. Introduction of groups to provide possibility of attachment of these probes to biomolecules lead to significant deterioration of the luminescence intensity.

In further development of the β-diketone based probes, chlorosulfonylation tetradentate β-diketone derivatives have been introduced (Figure 1.3) [9, 10]. These compounds have chlorosulfonylation group which can bound with proteins.

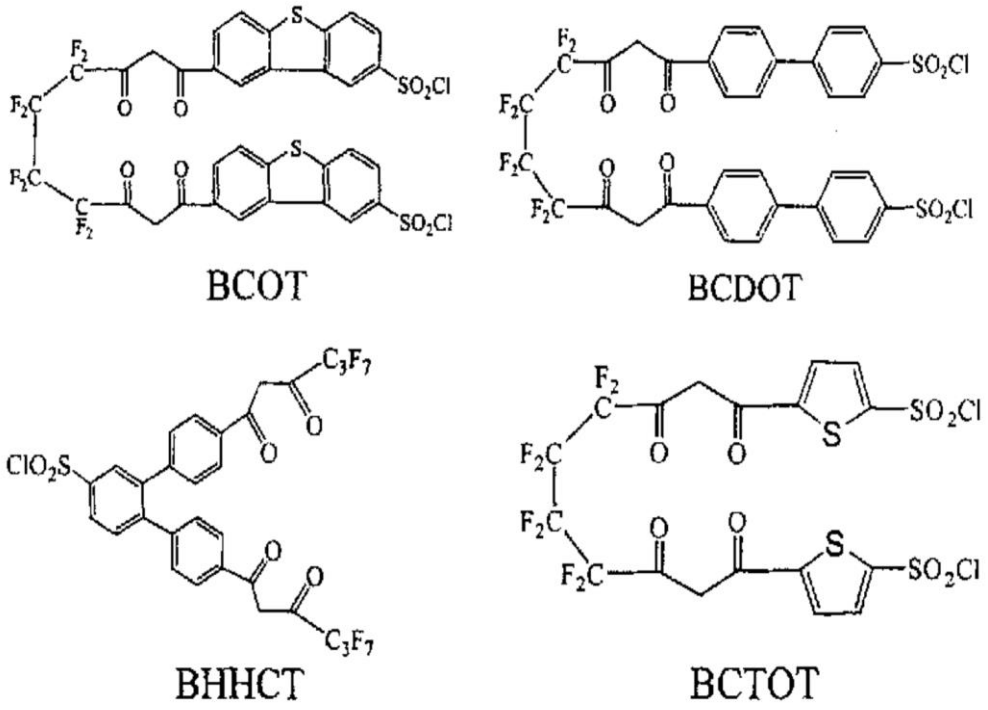


Figure 1.3 The structure of some chlorosulfonylation tetradentate β-diketone derivatives.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.3.2 Macrocyclic Ligands Luminescent Probes

In 1988, Evangelista et al. [11] reported a macrocyclic ligand 4,7-di(chlorine sulfonylbenzene)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA, Figure 1.4A) and its application in time-resolved fluorescent assay. BCPDA chelated with Eu^{3+} has luminescence lifetime ca. 0.7 ms. The detection limit of BCPDA-Eu^{3+} was around 10 pmol L^{-1} [12].

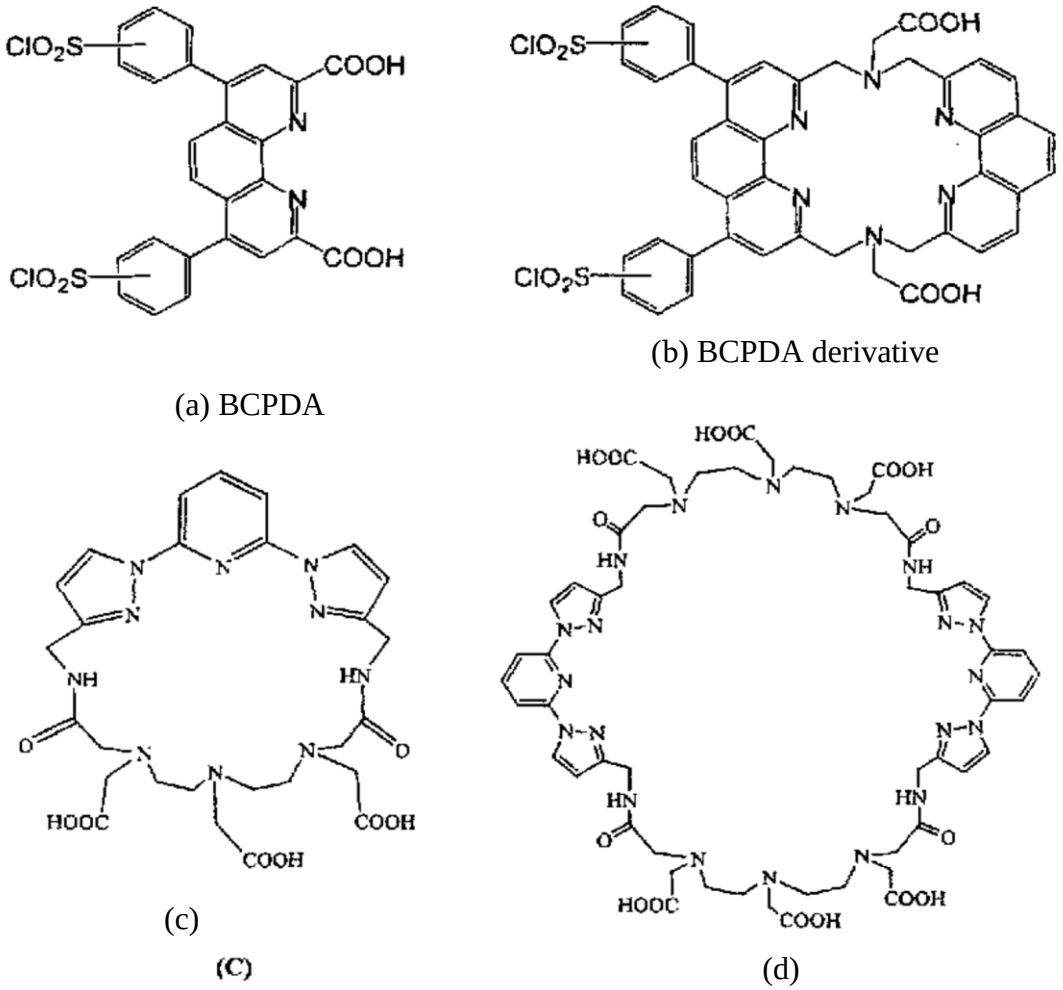


Figure 1.4 Structures of macrocyclic ligands.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

In 1992, Horiguch et al. modified BCPDA structure and obtained a new BCPDA derivative containing di-phenanthroline and di-sulfonylchloride (Figure 1.4B). This new derivative has similar luminescence emission intensity as the original BCPDA. In 1994, the same research team use modified BCPDA[13] to detect a human chorionic gonadotropin (hCG) with a detection limit of around 0.01 I. U. hCG ml^{-1}.

In 2002, J.C. Rodriguez-Ubis team [14] synthesized macrocyclic derivatives containing di-pyrazole-pyridine or DPTA (Figure 1.4 C,D). They all have excellent photophysics properties and solubility in water.

1.3.3 Heterobiaryl Ligands Lanthanide Luminescent Probes

The heterobiaryls (such as pyridine, dipyridyl and pyrazol) have stable structures, and the heteroatom coordinates lanthanides ions. They could serve as prototype structures of lanthanide luminescent probes. Probes based on polycarboylic acid combined with lanthanide ions have are stable in aqueous solutions. The sulfonylchloride, isothiocyano group or active ester could attach to heterobiaryl, therefore heterobiaryl derivatives can bound with protein directly. Structures of heteroboaryl ligand lanthanide luminescent probes are shown in Figure 1.5[15].

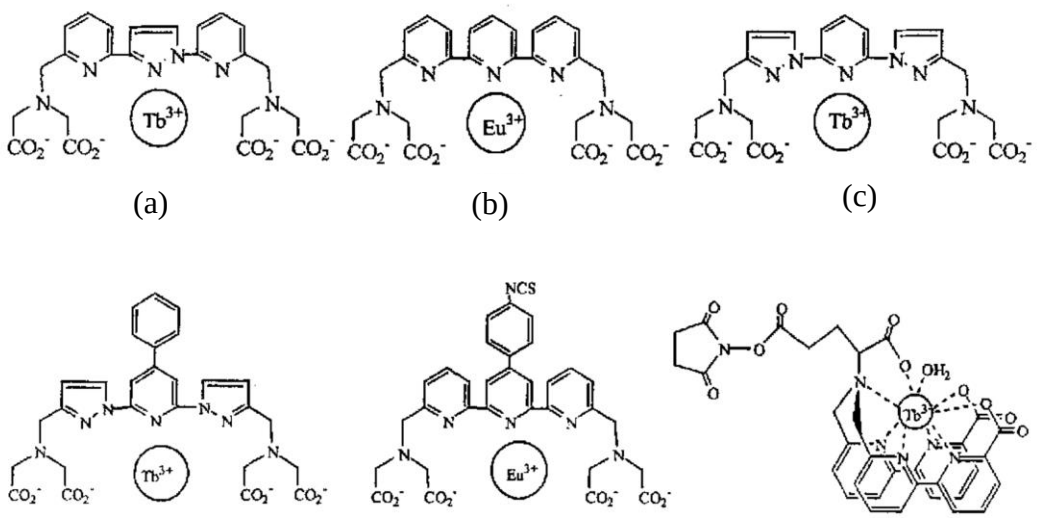


Figure 1.5 Structures of heteroboaryl ligands lanthanide luminescent probes.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.3.4 Polycarboxylic Acid Derivatives Lanthanide Probes

This type of ligands first made by Selvin [16] in 1995. The original structure is DTPA-cs124 (Figure 1.6). Recently, DPTA-cs124 is modified with CF_3 and isothiocyanate, in order to label biomolecules or improve photophysical properties. The luminescence emission intensity of DTPA-cs124-CF_3-Eu^{3+} is 3 times higher than that of DTPA-cs124 - Eu^{3+}.

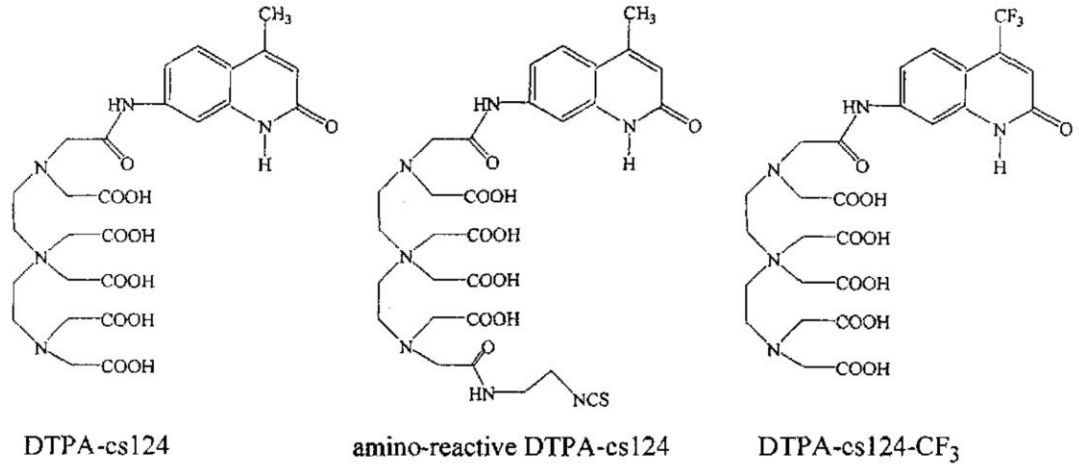


Figure 1.6 Structures of DTPA-cs124 derivatives.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.4 Time-Resolved Fluorescence Assay

One of the key applications of the lanthanide luminescent probes is bioanalysis. The main advantage is the long luminescence lifetime (ms to sub-ms range). For comparison, fluorescence lifetimes of organic compounds as well as bio-tissue interference luminescence occur in the nanosecond time domain. The lanthanide luminescence can be selectively measured even in the presence of other luminescent substances by time-gating of the signal with the delay sufficient for virtually complete decay of interfering

fluorescence. The background luminescence interference arises from various sources such as luminescence of the medium in biological assays. Biological samples contain a multitude of luminescent substances, e.g., proteins and NAD+/H [17, 18]. However the majority of interfering luminescence has short lifetimes and can be discriminated using time-gated detection. Generally, a time-resolved fluorescence assay requests an excitation sources, filters and/or monochromators, a time-gating device, and light collecting optics. The excitation can be performed by conventional pulse light sources or lasers. Time gating could be performed using modern detection devices (such as gated ICCD cameras, etc.) or even using mechanical shutters. The principle of the time-resolved fluorescence assay is shown in Figure 1.7.

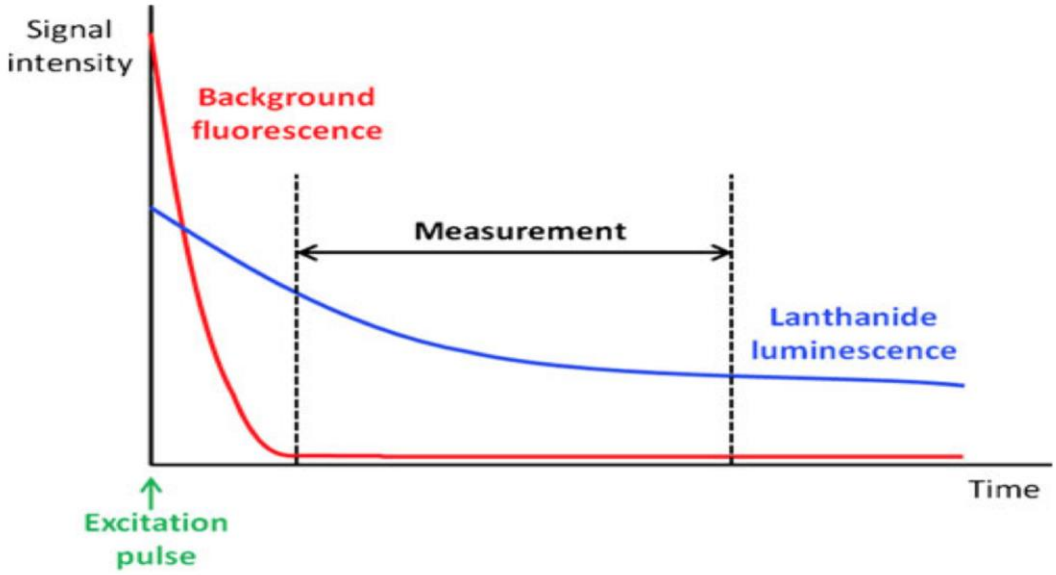


Figure 1.7 Principle of time-resolved fluorescence assay.

Source: Hagan, A.K. and T. Zuchner, *Lanthanide-based time-resolved luminescence immunoassays.* Anal Bioanal Chem, 2011. **400**(9): p. 2847-64.

1.4.1 Dissociation Enhanced Lanthanide Fluorimmunoassay System (DELFIA)

The DELFIA system developed by Wallac LLC[19]. This is a kind of high sensitivity lanthanide fluoroimmunoassay. SCN-Ph-DTTA-Eu^{3+} and SCN-Ph-EDTA-Eu^{3+} are used

as labels. The Compound SCN is the so-called cross-linking group, which can combine with biomolecules in an immunoreaction. After an immunoreaction, β- NTA or TOPO or Triton X-100 (pH=3.2) is added to the solution to enhance fluorescence. These compounds take the lanthanides ion away from the immune complexes and form fluorescence complexes. Subsequently, the fluorescence is measured by using time-resolved fluorescence.

In DELFIA system, the detection limit is about 10^{-13} - 10^{-14} mol L^{-1}. Due to the large quantity of fluorescence enhancing additives in the solution, this system can be strongly influenced by Eu^{3+} contaminations. The principle of the DELFIA system is shown in Figure 1.8.

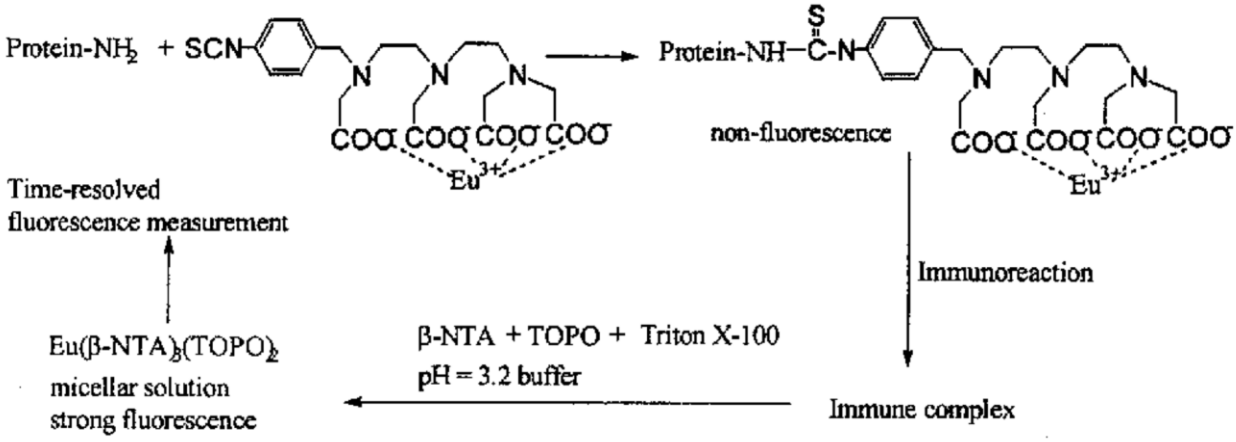


Figure 1.8 Principle of dissociation enhanced lanthanide fluorimmunoassay system.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.4.2 Enzyme Reaction Time-Resolved Fluorescence Assay

In 1991, Evangelista et al. [20] reported an assay to measure concentrations of enzymes or substrates. Before the reaction, a substrate cannot form a fluorescent complex with lanthanide ions. After the enzymatic reaction, the substrate is converted and forms a

stable fluorescent complex with a lanthanide ion. The complex is detected by time-resolved fluorescence. For example, the salicylaldehyde catalyzed by xanthine oxidase is converted to salicylic acid, and then chelated with EDTA-Tb^{3+}, as shown in Figure 1.9.

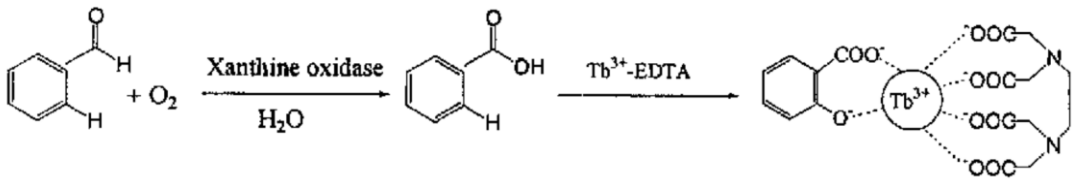


Figure 1.9 Synthesis of salicylate-EDTA-Tb^{3+} catalyzed by xanthine oxidase.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

1.4.3 Homogeneous Time-Resolved Fluorescence Assay (HTRFIA)

The TRACE (time-resolved amplified cryptate emission) system is based on energy transfer in homogeneous phase [21]. The resonance energy transfers between a long luminescence lifetime lanthanide luminescent probe and normal organic luminescence compounds. In this system, the TBP-Eu^{3+} as a fluorescence energy donator and allophycocyanin as an energy acceptor, this has a maximum fluorescence wavelength at 665nm, quantum yield about 0.7. When those two luminescence compounds marked a monoclonal antibody and an anthelion, the complexes form sandwich immune complexes. Two luminescence compounds get closer; the energy could transfer from lanthanide probes to normal organic compounds. Finally, the allophycocyanin can fluorescence at 665nm. Depending on this system, the detection limit of PSA is around 30$pgml^{-1}$, also be used for a HIV protease detection. The principle of HTRFIA is shown in Figure 1.10.[22]

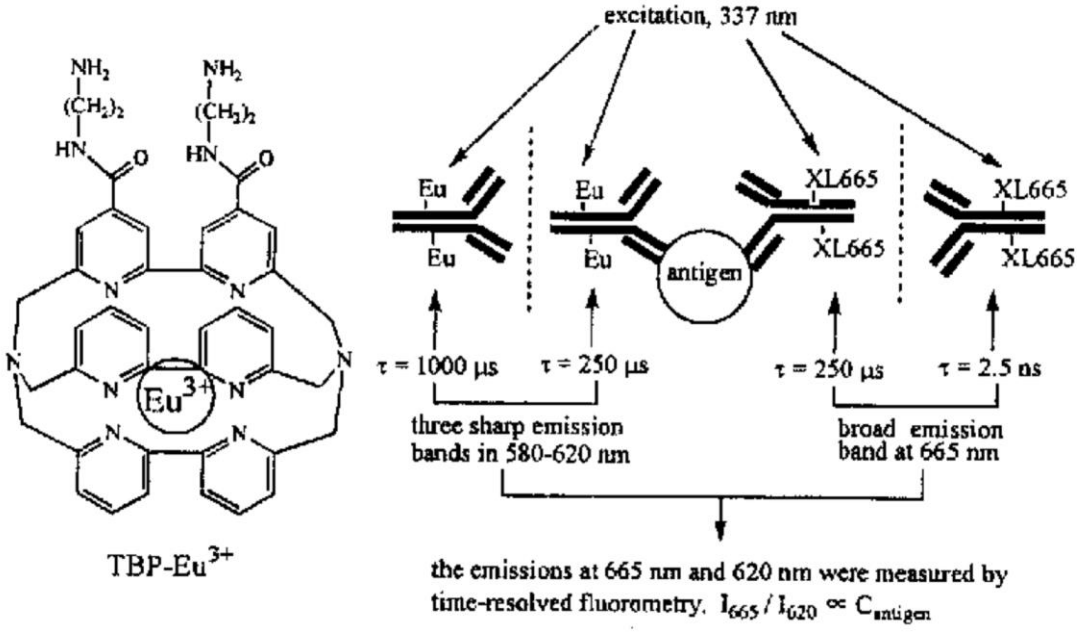


Figure 1.10 Principle of homogeneous time-resolved fluorescence assay.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

CHAPTER 2

RELATIONSHIP BETWEEN LUMINESCENCE LIFETIME AND CONCENTRATION OF LANTHANIDE LUMINESCENT PROBES

2.1 Luminescence Lifetime

The lifetime is a basic photophysical property of a lanthanide luminescent probe. The luminescence lifetime of the probes based on Eu^{3+} is around 0.5 ms and that of Tb^{3+} is around 1 ms.

The luminescence lifetime can be derived from the following expression.

$$I_t = I_0 \exp(-t/\tau) \tag{2.1}$$

I_t is the luminescence emission intensity after time t of excitation and I_0 is the luminescence emission intensity at excitation. The luminescence lifetime is τ [23].

2.2 DTPA-cs124 Derivatives

In this thesis, the lanthanide luminescent probe is a DTPA-cs124 (Figure 2.1a) derivatives whose structure is DPTA-cs124-CF_3-$CH_2C(O)$-$NH(CH_2)_6NHC(O)CH_2Br$-Eu^{3+} (Figure2.1b). This derivative has been synthesized in a previous study [24] and is named Probe 11 (Probe 11 as the same below).

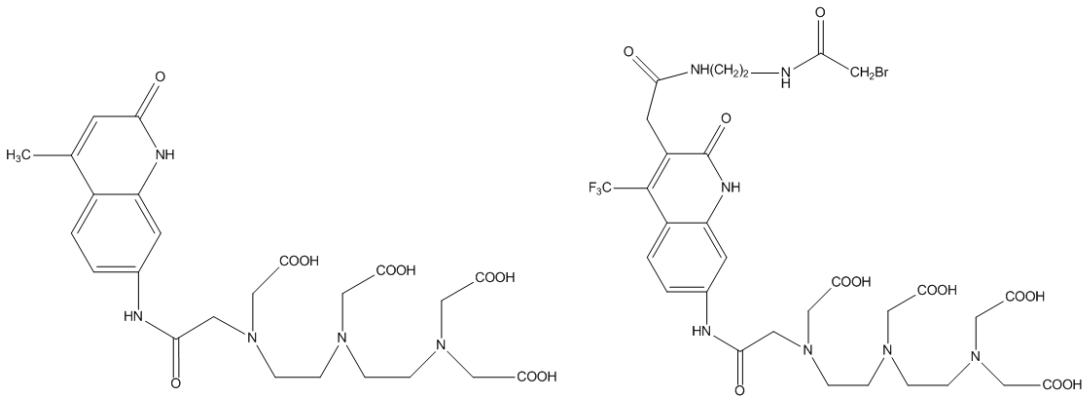


Figure 2.1 Structure of DPTA-cs124(a); Probe 11(b).

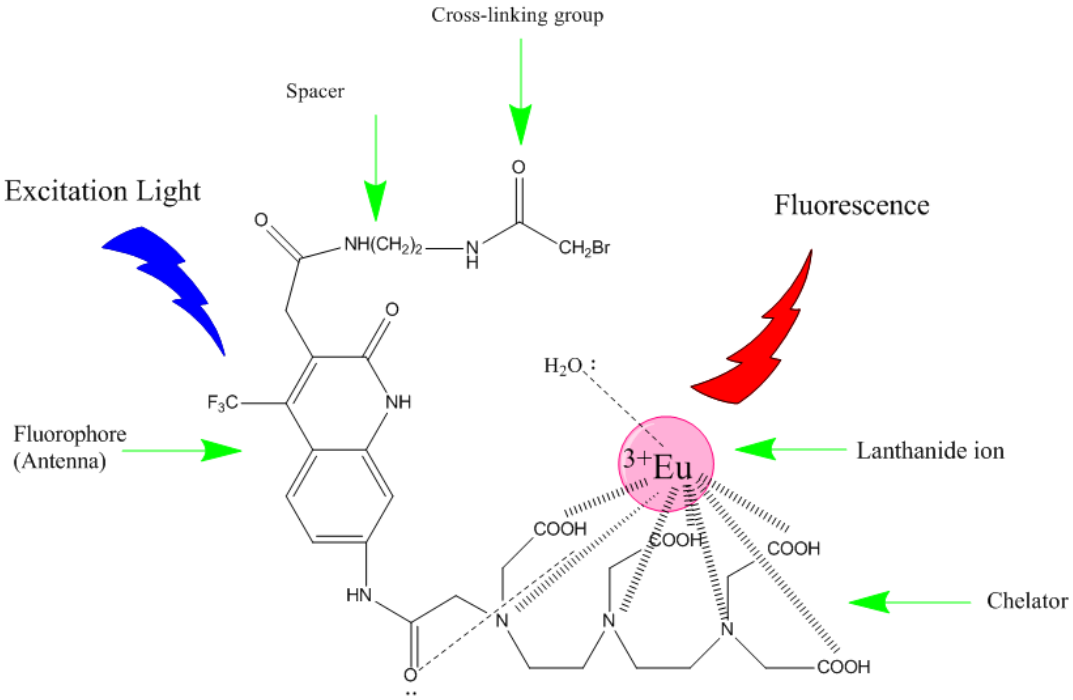


Figure 2.2 Function groups of lanthanide ion probe (DTPA-cs124-CF3-Eu^{3+} with cross-lingking group).

2.2.1 Function of Different Groups in Lanthanide Luminescent Probes

2.2.1.1 Chelating Group. The main purpose of using chelating group is attaching lanthanides ion to luminescent compounds. For lanthanides, the trivalent captions Ln^{3+} are hard Lewis acids with an optimum coordination number of nine [23, 25]. In order to fit cations, the chelating group requires donors. For example, phosphonates, β-diketonates and carboxylates (were measured in this thesis) which contain nitrogen and oxygen donor atoms as donors. For another requirement, the most applications of luminescent probes rely on the ability to carry the lanthanide ion on the biomolecule. This demands that the chelating groups have a high stability under various experimental conditions, and necessitates the use of multidentate chelating groups.

The high stability of carboxylates-lanthanide complexes relay on the ability of multiple amine and carboxylate coordinating centers, which wrap around the lanthanide ion. An additional advantage of a high denticity carboxylates is that minimizing water molecules on the lanthanide coordination sphere.

The diethylenetriaminepentaacetic acid (DTPA), which used in Probe 11 has eight coordinators. The typical stability constant log*K* for DTPA-lanthanide complexes is 10^{19} - 10^{23} [26].

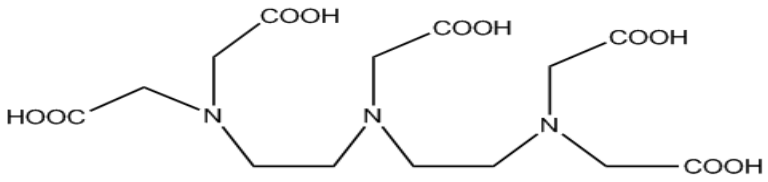


Figure 2.3 Structure of DTPA.

2.2.1.2 Antennas. The antennas are energy receivers. The antenna groups consist a highly π-conjugated aromatic or a heteroaromatic system which efficiently absorbs light and transfers its energy to the lanthanide ion, increasing the molar extinction coefficient from ca. 1-10mol^{-1} L cm^{-1} for the incomplexed trivalent cation to ca $10^4 – 10^5$ mol^{-1} L cm^{-1} for a typical lanthanide–antenna complex[25].

After study 41 lanthanide luminescence complexes, the crucial factor of sensitization efficiency of an antenna is the energy gap between the ligand triplet excited state and the lowest excited state of the lanthanide ions which should be at least 1850 cm^{-1}. A small energy gap leads to decreased quantum yield because of back energy transfer and quenching of the antenna triplet level. Large energy gap is desirable for the reason that the energy transferred from the antenna transitions through the non-radiative excited states until it reaches the emitted level. In order to match this energy gap strategy, the choice of antenna depends on what lanthanide ion is used. And the optimum antenna for a specific lanthanide ion is rarely is the optimum choice for a different lanthanide ion.

In Probe 11, carbostyril, cs124-CF3 (figure 2.4) [27, 28] acts as the antennas group. The cs124 derivatives represent attractive choice as ligands for lanthanide probes for detection of biomolecules due to high quantum yield and high solubility in water.

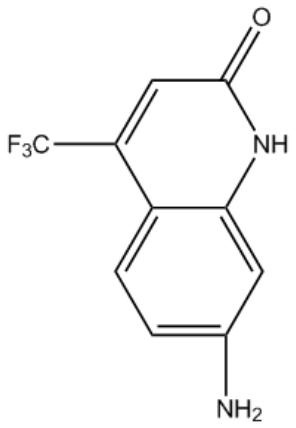


Figure 2.4 Structure of cs124-CF_3.

2.2.1.3 Cross-linking Group. The main purpose of lanthanide probes is labeling biomolecules via activated derivatives which are stable under anhydrous conditions and can be easily coupled to biomolecules. Such like Lys amino groups (e.g., active esters, isothiocyanates) or Cys thiols (e.g., maleimides, iodoacetamide).

2.3 Time-resolved Fluorescence Assay Devices

The home built system includes an excimer laser (OPTex, Lambda Physik), a pulse generator, a PMT preamplifier, an oscilloscope (9304A, LeCroy), and a spectrograph (Acton Spectra Pro, Princeton Instruments).

2.3.1 Excimer Laser

The excimer laser takes their name from an excited state dimers from which lasing occur. The most important excimers are rare gas halides such as Argon Fluoride (ArF), Krypton fluoride (KeF), Xenon Chloride (XeCl), and Xenon Fluoride (XeF). These produce intense UV light on distinct spectral lines between 193 nm and 351 nm.

Xenon Fluoride (XeF) gas type is used in this thesis, which give out a laser light at 351 – 353 nm. The active medium in this excimer laser is a mixture of the rare gas, a halogen gas and a buffer gas. The gas mixture supplied from a premix gas cylinder. For XeF, the gas mixture is Xe, He, F_2 and buffer (Ne). In addition a cylinder of an inert gas (Helium) is required for flushing.

2.3.2 Monochromator, Photomultiplier (PMT) and Amplifier

The monochromator and PMT deal with fluorescence from the lanthanide probes. And by the function of amplifier, selecting wavelength and amplify weak signal.

2.3.2.1 Monochromator. A monochromator is an optical device that transmits a mechanically selectable narrow band of wavelengths of light or other radiation chosen from a wider range of wavelengths available at the input. A device that can produce monochromatic light has many uses in science and in optics because many optical characteristics of a material are dependent on wavelength. Although there are a number of useful ways to select a narrow band of wavelengths (which, in the visible range, is perceived as a pure color), there are not as many other ways to easily select any wavelength band from a wide range.

The lanthanide luminescent ligands measured in this thesis, they all base on Eu^{3+} and Tb^{3+}. The europium ion has four characteristic peaks at 595 nm, 615 nm, 655 nm, 688 nm (Figure 2.5) and for the terbium ion also has four characteristic peaks at 489 nm, 545 nm, 583 nm, 621 nm (Figure 2.6). But to measure the luminescence lifetime, only the peak has the highest emission intensity, which is 615 nm for the europium ion and 545 nm for the terbium ion were concerned about. Peak width for the europium ion (615 nm) is around 13nm, for the terbium ion (545 nm) is around 15 nm.

Diffraction grating used was 50 groove/mm with blazed at 600 nm. The dispersion is 62.3 nm/mm based on the manual. The slits were set at 0.25 mm.

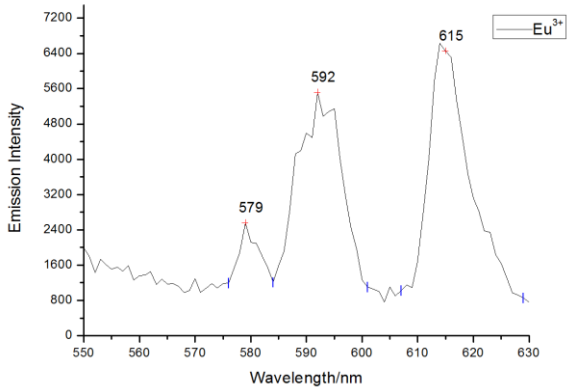


Figure 2.5 Luminescence emission spectrum of Eu^{3+}.

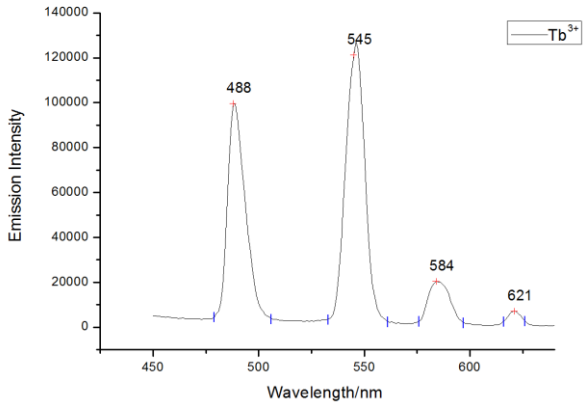


Figure 2.6 Luminescence emission spectrum of Tb^{3+}.

2.3.2.2 Photomultiplier (PMT). Photomultipliers are constructed from a glass envelope with a high vacuum inside, which houses a photocathode, several dynodes, and an anode. Incident luminescent photons strike the photocathode material, which is present as a thin deposit on the entry window of the device, with electrons being produced as a consequence of the photoelectric effect. These electrons are directed by the focusing electrode toward the electron multiplier, where electrons are multiplied by the process of secondary emission.

2.3.2.3 Amplifier. The amplifier is an electronic device that increases the power of signal. This is a high voltage device and the amplify voltage no more than 1600 V. Without the amplifier, oscilloscope cannot detect signal from PMT.

2.3.2.4 Oscilloscope. The oscilloscope is a type of electronic test instrument that allows the observation of constantly varying signal voltages, usually the two-dimensional plot of one or more signals as a function of time. The non-electrical signals can be converted to voltages and a displayed. In our case, a lanthanide probe is excited by laser beam and the luminescence is selected by monochromator, then photons have been collected by the PMT, amplified by an amplifier and finally display on the Oscilloscope.

2.4 Experiment

The lifetime of lanthanide luminescent probes is based on the matching degree between the lanthanide ion and the ligand. The Probe 11, which is based on the europium ions, has a luminescence lifetime around 0.5 ms. But the luminescence lifetime is getting shorter in a high concentration due to concentration quenching.

The concentration quenching is not an important influence factor to lanthanide luminescent probes, and this phenomenon is already accepted as common sense.

2.4.1 Set-up Devices

Connecting devices by data cables, every device should use an ext-trigger as the pulse generator; the frequency is set at 500 ms per period. Turn on the laser and wait for it to warm up. Turning on the PMT and set the amplifier to 1600 V. Turning on the

monochromator and adjusting input and output slit to 0.25 mm. Turning on the oscilloscope at channel 2 and math setup using the trace B as an average type.

2.4.2 Time-resolved Fluorescence Assay Measure Luminescence Lifetime

The Probe 11 has been diluted by a concentration gradient. Then these solutions have been measured in a cuvette one by one. Switch on the laser, let the laser beam hit the solution then emit red fluorescence. Switch on the monochromator control software, set grating at 50 g/mm with 600 nm blaze and set the wavelength at 615 nm for Eu^{3+}.

The oscilloscope was put in the auto setup, screen shows a fast trigger on channel 2. Press trigger setup and select ext-trigger, cancel channel 2 and bring trace B up. Put time-delay at 2us due to wiping out an undesired signal. Then an exponent decay showed on screen. Set trace B to summary type. Wait for an exponent curve until it gets into a steady state. Then press stop to lock on the exponent curve. Using software and layout trace B into the ASCII-word model and generate a '.txt' file. Import this txt file into Origin. Draw a line base on these data, Origin will give out a solid line similar to the curve on the oscilloscope.

To find the luminescence lifetime by this curve, at first determine the maximum emission intensity (y0) by linear fit with zero slopes. Then use non-linear fit, exponent decay function (Equ 2.2). Set y0 equal to what software get in linear fit, and it will not change. Getting 100-lifter fit until no change on t1, the t1 is the lifetime. An example of lifetime calculation is shown in Figure 2.7.

$$y=A1\bullet\exp(-x/t1)+y0 \qquad (2.2)$$

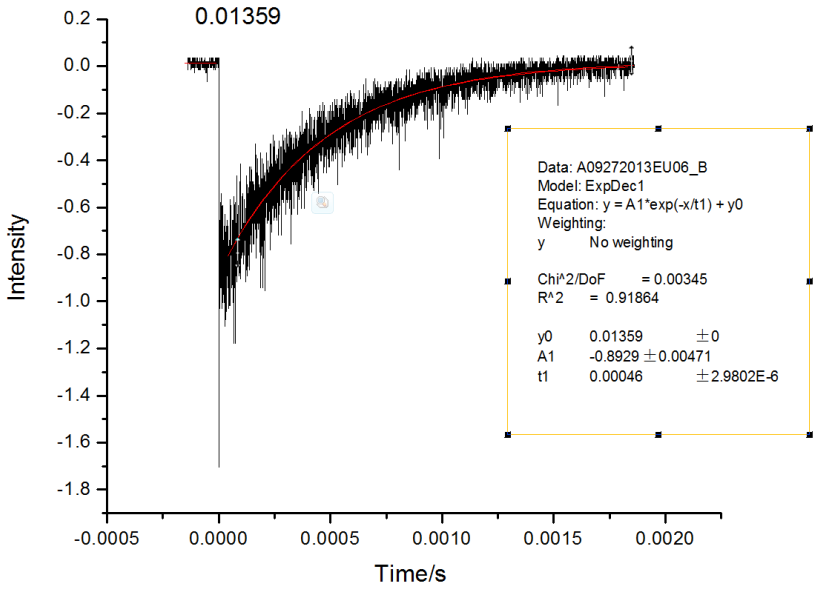


Figure 2.7 Example of luminescence lifetime calculation.

2.5 Results

Table 2.1 Eu^{3+}Probe Luminescence Lifetime vs. Concentrations

Concentration/M	6.80E-06	5.70E-06	4.50E-06	3.40E-06	2.20E-06	1.10E-06	2.20E-07
Lifetime/ms	0.45	0.48	0.48	0.5	0.52	0.5	0.52
	0.45	0.48	0.47	0.51	0.53	0.51	0.52
	0.46	0.46	0.47	0.5	0.52	0.51	0.52
	0.46	0.46	0.46	0.52	0.51	0.52	0.52
	0.48	0.47	0.47	0.54	0.5	0.5	0.53
	0.46	0.45	0.48	0.55	0.51	0.56	0.53
	0.46	0.48	0.48	0.51	0.51	0.53	0.53
	0.47	0.46	0.46	0.54	0.53	0.53	0.53
	0.47	0.48	0.49	0.52	0.53	0.53	0.53
	0.47	0.49	0.47	0.5	0.53	0.54	0.5
Average Lifetime/ms	0.46 ±0.0090	0.47 ±0.012	0.47 ±0.0090	0.52 ±0.018	0.52 ±0.10	0.52 ±0.018	0.52 ±0.0090

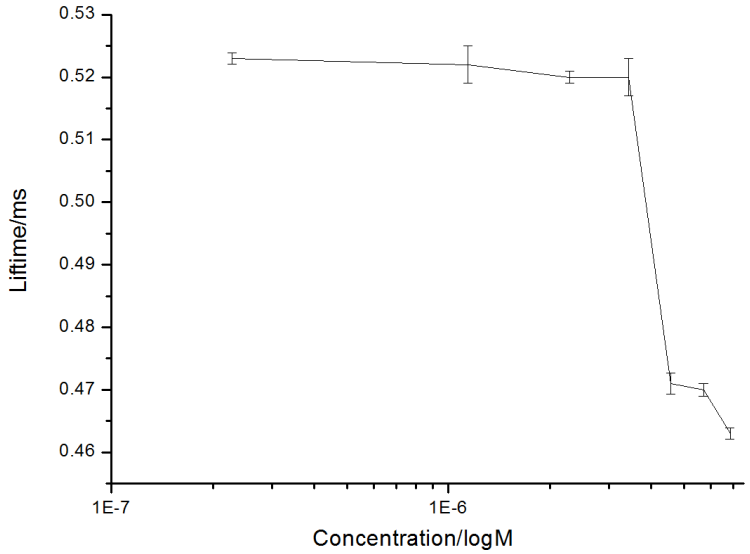


Figure 2.8 Luminescence lifetime of Probe 11 at different concentrations.

In this measurement system, there are some instability factors that influence the results. In this view, it is good to do this measurement after sunset. In addition, in order to avoid these factors, every sample has been measured ten times and then the average lifetime will be the lifetime of the sample. This particular Probe 11-Eu^{3+} has a lifetime around 0.55 ms. But at a higher concentration, the lifetime sharply decreased to 0.46 ms. It can be seen from Figure 2.8, the quenching concentration is around $3\text{-}4 \times 10^{-6}$ M.

2.6 Discussion

The results indicate concentration self-quenching. Excited state reactions, energy transfer, complex-formation and collisional quenching, all these processes can result in luminescence quenching. Quenching depends on temperature and concentration. Temperature remained the constant in the measurement. The only variable was concentration.

2.6.1 Static Quenching

The static quenching is about remaining energy transferring mechanism. Static quenching occurs when the molecules form a complex (reporter-quencher dimer) in the ground state, i.e., before excitation occurs. The complex has its own unique properties, such as being nonfluorescent and having a unique absorption spectrum. Dye aggregation is often due to hydrophobic effects—the dye molecules stack together to minimize contact with water. Planar aromatic dyes that are matched for association through hydrophobic forces can enhance static quenching. Pure static quenching reduces the intensity of fluorescence but does not necessarily decrease the lifetime of luminescence emission.

2.6.2 Dynamic Quenching

Dynamic quenching decreases the luminescence lifetime and the intensity of the emission but the shapes of the absorption and the luminescence spectra of the dyes are unchanged.

2.6.2.1 Förster Resonance Energy Transfer. The Energy can be transferred non-radioactively (without absorption or photons emission) between a donor and an acceptor. The energy transition of the donor and the acceptor is extremely depending on the donor-acceptor distance, R. This mechanism also depends on the donor–acceptor spectral overlap (Figure 2.9) and the relative orientation of the donor and the acceptor transition dipole moments. Förster resonance energy transfer can typically occur over distances up to 100 Å [29]. FRET quenching can be reduced by dilution.

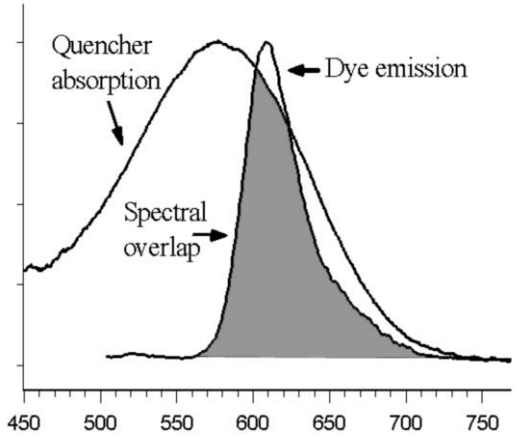


Figure 2.9 The principal of spectral overlap.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

2.6.2.2 Dexter Electron Transfer. Dexter electron transfer is another dynamic quenching mechanism, also called collision mechanism. Dexter energy transfer is a short-range phenomenon that falls off exponentially with distance (proportional to e^{-R}) and depends on the spatial overlap of the donor and the quencher molecular orbitals. In the most donor-fluorophore–quencher-acceptor situations, the FRET mechanism is more important than the Dexter mechanism.

2.6.3 Quenching mechanism in Probe 11

The absorption and the luminescence spectra of the Probe 11 at different concentrations are not measured in this thesis, which indicates that whether new luminescence complexes had been formed is not clear.

The wavelength of luminescence emission peak of Eu^{3+} is 615 nm and has narrow band; the absorption spectrum band is at 380 nm. These indicate that the quenching not cause by the spectral overlap.

A special case of the energy transfer is cross-relaxation, where the original system loses the energy (E3 − E2) by obtaining the lower state E2 (which may also be the ground state E1) and another system acquires the energy by going to a higher state E2′. Cross-relaxation may take place between the same lanthanide ions (being a major mechanism for quenching at higher concentration) or between two differing elements, which happen to have two pairs of energy levels separated by the same amount. The cross-relaxation between a pair of ions is graphically presented in Figure 2.10. The two energy gaps may be equal or can be matched by one or two phonons. Cross-relaxation has been measured in a variety of ions and it is a dominating factor in nonradioactive relaxations at high concentration.

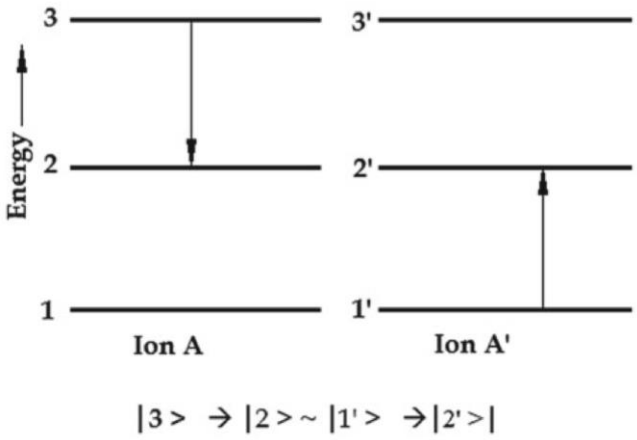


Figure 2.10 The principal of Cross-relaxation mechanism.

2.7 Conclusions

2.7.1 The Luminescence Lifetime

The luminescence lifetime of Probe 11 were measured in the regular water. At low probe concentrations, the luminescence lifetimes are around 0.55 ms and around 0.46 ms at high concentrations.

2.7.2 Self- quenching

A concentration quenching was discovered. The critical concentration of Probe 11 is 6.85×10^{-6} M. There is no spectral overlap after quenching occurring. Even if there is no evidence indicates that the shape of absorption and the luminescence spectra of the Probe 11 at different concentrations changed, the pure static quenching theory may not give a suitable explanation due to the formation of new complexes do not reduce luminescence lifetime. The Cross-relaxation quenching mechanism could give an explanation of this concentration quenching.

CHAPTER 3

MICROSCOPY OF LANTHANIDE LUMINESCENT PROBES LABELED E-COLI CELLS

3.1 Immunoassays

3.1.1 Fluoroimmunoassay (FIA)

Fluoroimmunoassay is a kind of immunoassay, the strong luminescence compounds have been used as tracer agent. In the early 1940s [30], the fluorescein already been used to label the antigen or antibody. For example, the antibody labeled with a carbimide fluorescein to detect polysaccharide antigen of pneumococcus. But this assay is not widely used due to the short luminescence lifetime and the low quantun yield [31]. Fluorescein isothiocyano (FITC) came out ten years after carbimide, and the method of labeling has been improved, then FIA has real practical value. Higher sensitivity and better selectivity are the two advantages of FIA compared to spectrophotometry. The detection limit of FIA is lower than spectrophotometry by about 2~4 order of magnitude. Fluorescein, rhodamine and its derivatives, and cyanine dyes are widely used in organic luminescence dyes (Figure 3.1) [32].

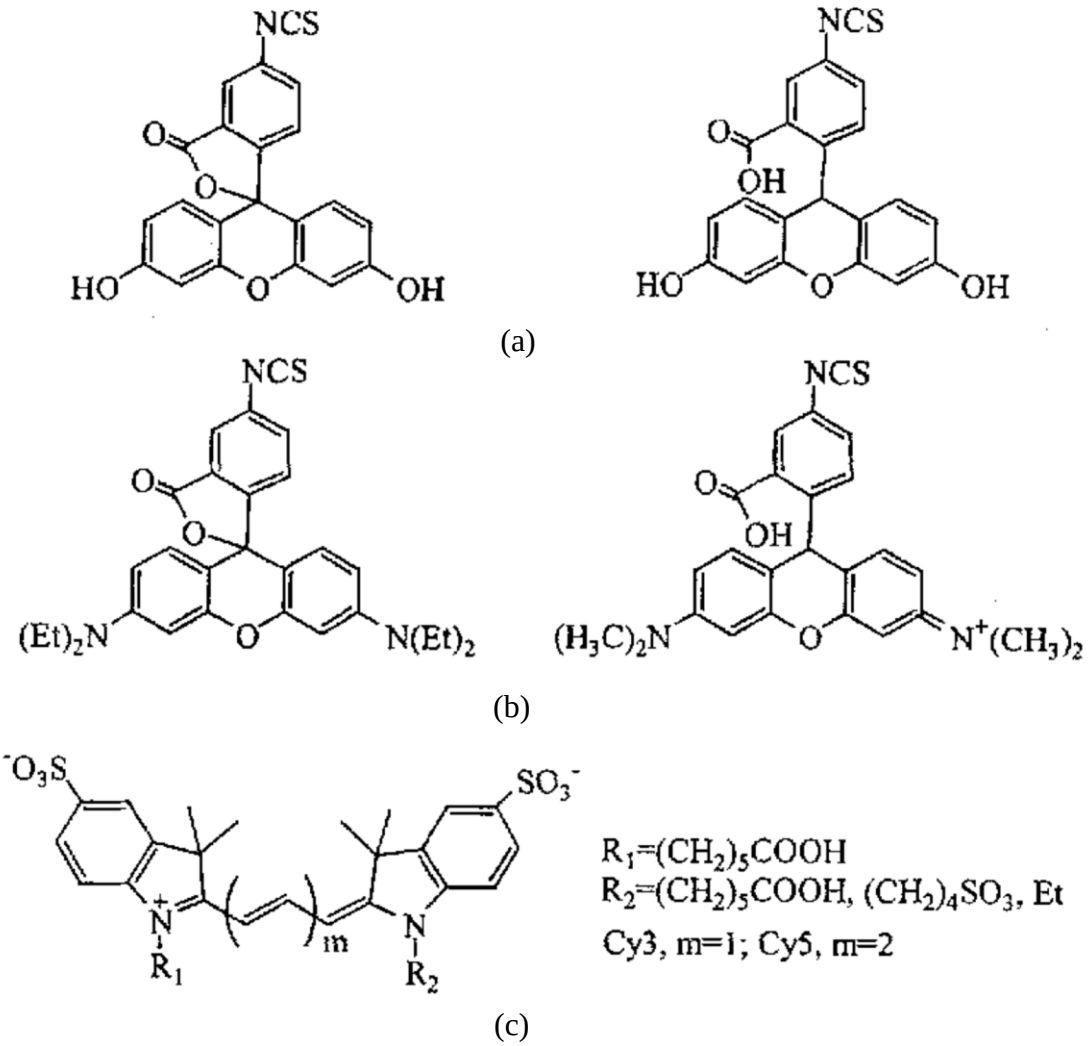


Figure 3.1 Structures of fluorescein (a), rhodamine (b) and cyanine dyes derivatives(c).

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

These compounds have high quantum yield and stable structures. But their negative aspects are also apparent, the detection limit is around 10-10mol L^{-1}, and Stokes shift between the excitation and the luminescence emission spectrum is small (less than 50 nm), the background luminescence and stray light (Tyndall, Rayleigh, Raman) have strongly disturb the signal of desire luminescence. Besides, the influences from surroundings like pH, polarity, oxygen content, under the excitation light for a long time

and getting closer to other absorption group will lead to luminescence quenching or bleaching.

3.1.2 Radioimmunoassay (RIA)

The radioactive material is served as tracer agent, which has high detectability and combines with the immunoassay, gives out the radioimmunoassay. The applications of RIA are disease diagnosis, observation of the curative effect and medical research due to the outstanding microanalysis ability [33]. The detection limit is ca. 10^{-12} - 10^{-15}mol L^{-1} it is so-called ultratrace analysis. For example, tyrosine labeled by NaI^{125}, which is replaced by hydrogen on the aromatic. RIA has advantages: the spatial size of radioactive complexes is small (less steric hindrance), the strong signal, the low detection limit and the high sensitivity. But the limitations of RIA are obvious. The transport and storage are subject to legal restriction due to health and safety considerations. And the radioisotope is not stable, the half-life period of I^{125} is 59.4 days [34]. The luminescence lifetime of labeled protein is just a few weeks. The working curve should be re-measured before assay every time [35].

3.1.3 Enzyme Immunoassays (EIA)

The process of EIA, at first biomolecule labeled with enzyme, then substrate and enzyme have a chromogenic reaction, and product concentration is in direct proportion to enzyme concentration. That indicates labeled protein concentration. Enzyme is the first successful non-radio tracer agent instead of the radioactive material.

For now, the enzymes used in EIA are horseradish peroxidase (HRP, widely distributed in plants), alkaline phosphatase (AP, hard to purify), galactosidase (GLA).

Based on these three enzymes, the immunoassay for them is colorimetric method (Figure 3.2), fluorescence method (Figure 3.3), chemiluminescence (Figure 3.4).

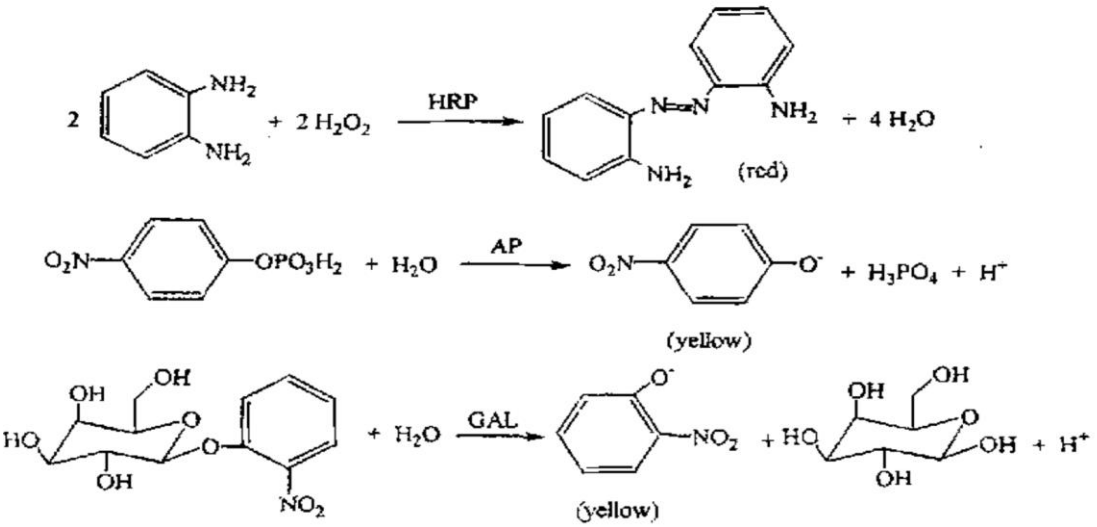


Figure 3.2 Enzyme- catalytic reactions for colorimetric method.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

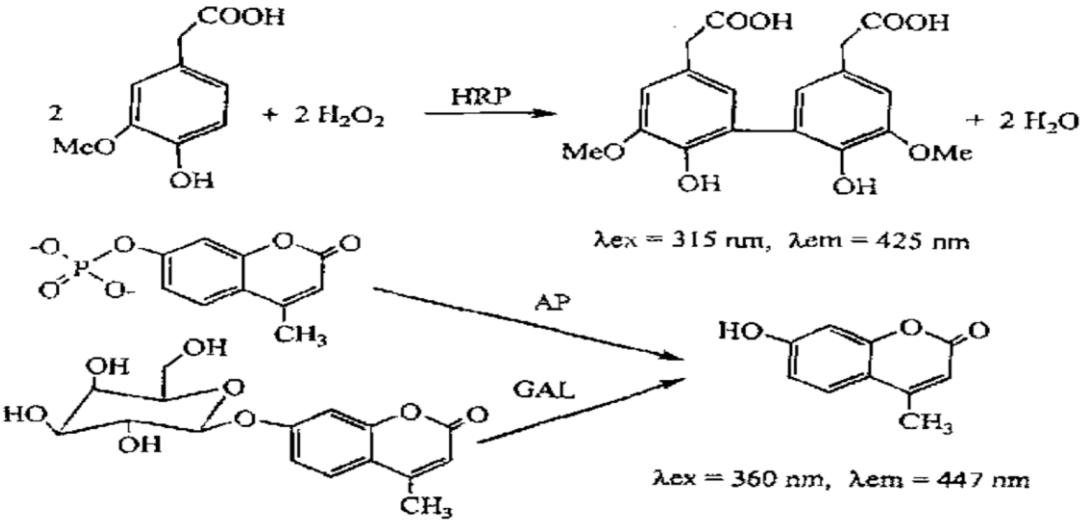


Figure 3.3 Enzyme-catalytic reactions for fluorescence method.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

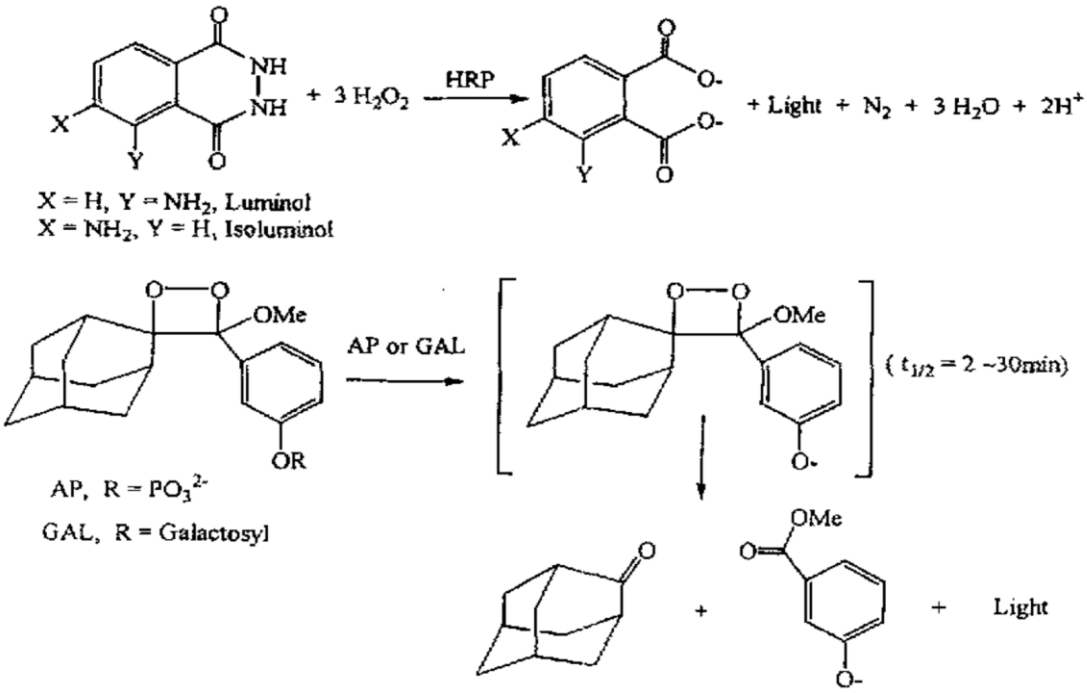


Figure 3.4 Enzyme-catalytic reactions for chemiluminescence.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

The activity of protein-enzyme complexes and a protein is strongly influenced by enzyme-catalytic reactions. In addition, temperature, pH and concentration of ion (Zn^{2+}, Mg^{2+}, N^{3-}) also have influence on activity.

3.1.4 Chemical Immunofluorescent Assay (CLIA)

Some small size chemical compounds can absorb energy from the chemical reaction, and then fluoresce. Chemical immunofluorescent assay combines this phenomenon and immunofluorescent assay together[36].

Acridinium ester and luminol derivatives are tracer agents in CLIA. And luminol derivatives weed out due to less sensitivity than acridinium ester. If acridinium expose in NaOH-H_2O_2 solution, the solution will luminescence immediately. This intensity

luminescence occurs in one second. The principle of acridinium ester chemical reaction luminescence is shown in Figure 3.5. The chemical reaction in CLIA is simple and fast, it is a beneficial to measure in a high flux automatic device. The detection limit of this kind of tracer agent could reach 10^{-18} M (attomolar). Soon afterwards, acridinium sulfonamide ester is invented then used in the automatic immunodetection system which analysis speed can reach 200 tests/h [37].

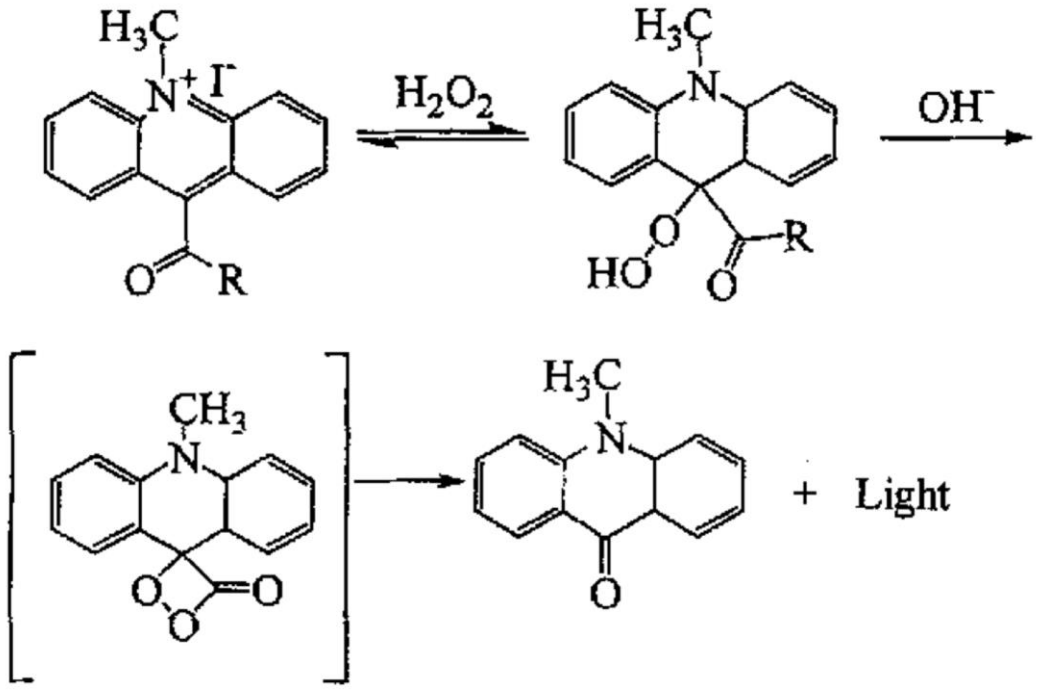


Figure 3.5 Chemical immunofluorescent assay reactions of acridinium derivatives.

Source: Tan, M., *Novel Lanthanide Fluorescence Probes and Their Application to Time-Resolved Fluorescence Bioassay*, J. Prof. Dr. Yuan, Editor. 2005, Dalian Institute of Chemical Physics, Chinese Academy of Sciences: Dalian P.R. China.

3.2 Avidin-Based Lanthanide Luminescent Probes Label Escherichia Coli Cells

There are two parts of samples of lanthanide probe labeled Escherichia coli cells (E-coli cells). One is preparing E-coli combine with biotin and another is avidin-based lanthanide

probes. The samples are the combination of these two parts. Avidin-Probe 4-Tb^{3+} (from PHRI) has been synthesized before, properties can be found in the reference [38] .

3.2.1 Preparation of E-coli Cells Labeled with Modified Avidin

Ten uL suspension of E-coli (RL721 strain) inoculate in 100mL LB broth and incubated in a 500 mL Erlenmeyer flask overnight at 37 ℃. The cells were harvested by a centrifugation (9000 rpm, 3 min), washed with PBS (Phosphate Buffered Saline) and re-suspended in the same buffer. Sixty microliters of this suspension containing ca. 1 mg of cells was washed 3 times with 1 mL of 0.1 mol L^{-1} sodium borate buffer, pH 8.5, and each time collected by the centrifugation. After the last wash, the cells were suspended in 50uL of the same buffer and 8 uL of 100 mmol L^{-1} DMSO solution of NHS-dPEG12-biotin was added. After incubation at room temperature for 30 min the cells were washed 4 times with 500 uL of PBS. After the final wash, cells were sus- pended in 30 uL of PBS buffer and supplemented with 30 uL of 5 umol L^{-1} avidin modified with one of the lanthanide labels [AV – Probe 4 -Tb^{3+} (n = 15) from PHRI]. After 30 min of the incubation at room temperature, cells were washed by PBS 4 times with 500 uL PBS and suspended in 100 uL of the same buffer.

3.2.1.1 Streptavidin. Streptavidin (SA) is a kind of protein, which has the similar biological characteristics with avidin (AV). SA is the excretion of Streptomyces avidinii, and SA has the similar molecular weight and combining capacity with biotin compare with avidin in the egg white, isoelectric point is 6.0. But nonspecific binding is less than avidin.

SA purified by iminobiotin chromatography. One liter of Streptomyces avidinii nutrient solution has 10 - 60mg of SA. SA is a tetramer protein, the molecule weight is 66 kDa. One molecule of SA has high specific binding with biotin due to extremely strong affinity between them.One peptide chain of SA has 159 amino acid residues, molecule weight 16450. SA has 4 same peptide binding and glycine and alanine are the main parts of amino acid. Tryptophan residues are active groups, which combine with biotin[39].

3.2.1.2 Biotin. Biotin also called vitamin H, which comes from many food such as strawberry, beer and food with plenty of protein. Fat and protein cannot homergy without biotin. The main function of biotin in medical is anti-alopecia. And in biochemistry, biotin is a bridge between the tracer agent and the protein of bacteria[40].

3.3 Experiment

3.3.1 Set up Devices

Laser, ICCD camera (Princeton Instruments) and microscope are used in this assay. Change laser light route using two mirrors and a gate slit, the laser light could reach microscope. Adjust angle and height of mirrors that laser beam can hit the center of the prism.

Connecting PC, laser and ICCD camera with Ext-trigger with data cable. Make sure laser energy above 10 mJ.

The 1024 × 1024 camera pixels are 2 × 2 binned resulting in 512 × 512 pixel^2 images. The microscope used an objective with the magnification of ×56 and the numerical aperture of 0.90. Combined with the intermediate ‘‘ocular’’ lens with the magnification of ×10 it provides the field of view of 14 × 14 um^2.

3.3.2 Observe E-coli on Object Slide

To ensure measure microscope in a right way, first observe E-coli on object slide. In this preliminary experiment, on the object slide, stick-like bacteria crowds should be found which are tiny and bright.

Dispersing 10 uL of E-coli on the object slide with a cover glass (3.8 cm^2). Dry in the air 15 min. Turn on Xe arc lamp. Move cover glass, put slide on the objective stage. Switch light from top, coarse focusing and fine focusing to get image of E-coli.

3.3.3 Microscopic Detection of E-coli Utilizing Avidin Labeled with Multiple Lanthanide Luminescent Probes using Total Internal Reflection Fluorescence Microscopy (TIRFM)

One uL of glycerol disperse on the hypotenuse side of the prism with a cover slide. Remove cover slide, the 10 uL of E-coli suspension place on glycerol, disperse by the cover slide. Dry it in the air for 15 min. Remove cover slide. Put the prism on the microscope base and move to the objective stage. Ensure laser beam hit the center of prism, and laser beam at a 90 ° with objective lens, ICCD camera is switch off. Adjust position of the prism; switch light into ICCD camera, at last minute switch on ICCD camera and set time-gate at 10 ms leads focused the image of E-coli presence on the PC

screen, the light source from Xe arc lamp. Set time-gate at 2 ms and time-delay at 100 us (for Tb^{3+}, lifetime1.5 ms for Probe 4). Switch off Xe arc lamp and turn on laser light. Then the lanthanide luminescent probe excited by laser light totally internally reflected from the hypotenuse side inside the prism. The fluorescence collected by photon count mode, threshold at 70. One thousand pulses were accumulated (at trigger of 500 ms per period).

3.4 Results

After combine the avidin-base Probe 4 and the biotin affinity E-coli together, sample has been judged by visual inspection under UV light. Biotin and E-coli, avidin and Probe 4, avidin and biotin are connected to each other. A normal microscopy and luminescence accumulate image acquired by ICCD camera. Time-gate 2ms and time-delay 100 us are justified for Probe 4 (Tb^{3+}). The maximum of photon per pixel is around 60 by 1000 pulses accumulation. As expected, the images were highly contrasted (Figure 3.6).

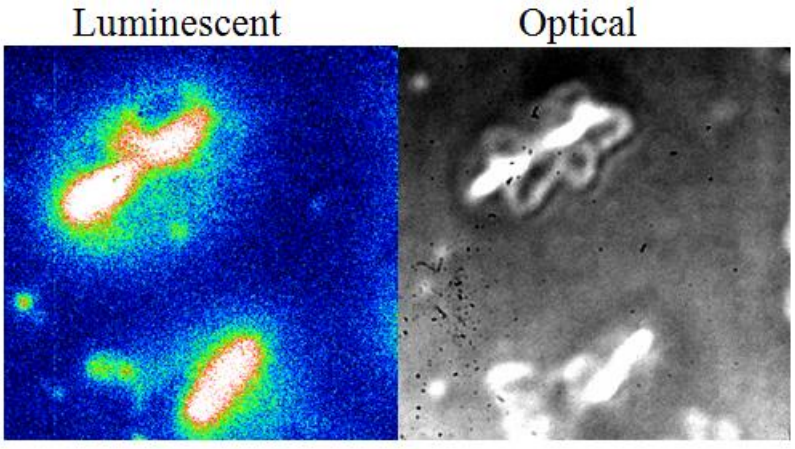


Figure 3.6 Imaging of the labeled Escherichia coli cells using total internal reflection fluorescence microscopy.

3.5 Discussion

There are several critical applications of this assay, threshold, glycerol, time-gate and time-delay, TIRFM.

3.5.1 Total Internal Reflection Fluorescence Microscopy (TIRFM) and Application of Glycerol

Total internal reflection is an optical phenomenon (Figure 3.7 (a)). When a beam of light propagates through the interface of a dense medium and a less dense medium, a little of the light is refracted if light meet the interface at a small angle, and the rest of the light is reflected back into the dense medium. At a certain angle, all the light is reflected. This critical angle is known as angle of total reflection which depends on the refractive indices of two mediums, the refraction law (n1, n2): $\boldsymbol{\Theta}c = \sin^{-1}(n1/n2)$. But a bit of energy of the beam goes through interface and propagates a short distance (a few hundred nanometers) into less dense medium, generating an evanescent wave. This evanescent wave can excite the fluorophore molecule, if evanesent wave is not absorbed; it goes back into the dense medium. In this way, it is possible to get luminescence with a very low background of excitation light. Based on this principle, an experimental set-up for imaging of small luminescent objects has been built [41, 42].

The probes labeled E-coli were excited only by the evanescent wave shows that the photon per pixel is less than 30. It is hard to distinguish from background noise. To improve the quality of the image, glycerol is introduced.

The application of glycerol.

The reason for low intensity of the image is that the evanescent decays too fast to provide efficient excitation of the large cell. The evanescent wave has characteristic

thickness of only ca. 100 nm from the interface and cannot excite efficiently much larger E-coli cell (the size of E-coli cells is ca. 0.5 um $\times$ 3 um.

To improve the coupling still preventing laser light reaching the objective lens coupling medium (glycerol) was used. In this case, the cell is submerged completely or partially in glycerol, and complete internal reflection does not occur at the cell location. Electromagnetic wave excites the whole volume of the cell, which leads to a drastic enhancement of the image brightness.

Based on the refraction law above, refraction depends on the refractive indices of the two mediums, if n1 and n2 are not appeared much different, the laser light pass through the interface with a small refraction angle, which can hit the E-coli directly. The refractive index of the quartz prism under 351 nm laser is around 1.473 and the refractive index of the glycerol is around 1.472.

Originally, without the glycerol (Figure 3.7 (b)), the laser beam hits the hypotenuse of the prism, then total reflect back to the prism. The evanescent wave run over and absorbed by the probes labeled E-coli. The luminescence from the E-coli is collected by the objective lens. The laser light is stopped by the prism and does not influence the luminescence signal.

With dispersed the glycerol on the hypotenuse of the prism (Figure 3.8 (c)), the interface of the prism and glycerol instead of the interface of the prism and air. The laser beam could go through the layer of the glycerol. The total reflection occurs at the interface of glycerol and air. In this view, the laser light is stopped by the interface of glycerol and air; do not influence the luminescence signal either. In this way laser light could hit the probes labeled E-coli [43].

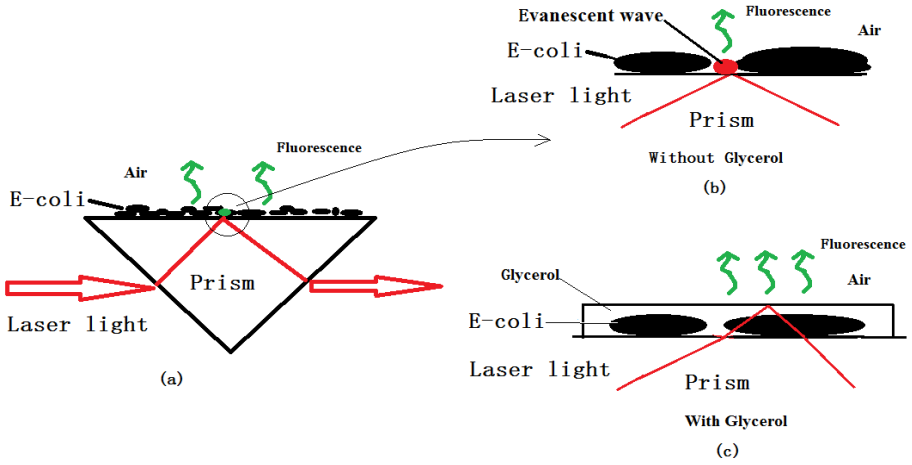


Figure 3.7 The principles of TIRFM(a), without glycerol(b), with glycerol(c).

3.5.2 Value of Threshold

At photon count mode, the strongest interference is the photon from environment. Threshold applies to select the photon into counting which energy is higher than some value. Input different values of threshold under photon count mode until finding a critical value, which can filtrate most of noises but not that high to exclude desire fluorescence.

3.5.3 Time-gate and Time-delay

Time-gate and time-delay are the fundamental control values in the time-resolved fluorescence assay. Recall Figure 1.6 (Principle of time-resolved fluorescence assay). The luminescence lifetime of Probe 4-Tb^{3+} is around 1.5 ms. The time-gate (2 ms in this assay) needs to cover whole lifetime. Time-delay (50 us in this assay) applies to exclude the short-live background fluorescence (less than 2 us) [38].

3.6 Conclusions

Time-resolved fluorescence assay combine with the total internal reflection fluorescence microscopy give us a highly sensitive assays to image single molecules, biological micro objects, and body tissues in which the signal cannot be amplified. The application of glycerol gives a way to improve the quality of image.

CHAPTER 4

PHOTOPHYSICS PROSPERITIES OF Bi-cs124 DERIVATIVES LANTHANIDE LUMINESCENT PROBES

4.1 Introduction

Every lanthanide luminescent probe contains several function groups, chelates, antennas, spacer, cross-linking groups and lanthanide ion. The photophysics prosperities of these function groups are relatively independent from each other, which they could be chosen individually. Aiming at obtain good photophysics prosperities, cross-linking groups were excluded. Bi-cs124 derivatives synthesized in previously studies. Luminescence lifetime, quantum yield and number of water molecules in the complexes of these derivatives have been measured in the assay. Base on those data, the best one has been chosen to combine with a cross-linking group then apply to label biomolecules.

Quantum yield should be measured at the same concentration of the samples. Ultraviolet-visible absorption spectrum gives out a way to measure the concentration of samples based on the Beer-lambert law.

The area under the luminescence spectrum is the so-called quantum yield. There is a way to calculate unknown relative quantum yield by compare the quantum yield between the samples and the reference compound. The relative quantum yield of reference compound is known.

4.1.1 Ultraviolet-visible (UV-V) Absorption Spectrum

Ultraviolet and visible absorption spectrums are molecular spectrum. They are all caused by jump of valence electrons. Based on UV-V absorption spectrum, composition, concentration and structure of analyses can be determined or inferred. The application of UV-spectrum in this assay is to determine concentration of lanthanide probes in solution. This principle is based on Beer-Lambert's law. The concentrations of probes and absorbance are proportional, the unknown concentration are calculated from known concentration and absorbance. The molar absorptivity of cs124 (antenna group which absorb energy) is $19000 m^{-1} cm^{-1}$.

Beer-lambert law[23]:

$$A=\varepsilon Lc \tag{4.1}$$

A is absorbance, and ε is molar absorptivity. L is optical distance.

4.1.2 Luminescence Emission Spectrum

The luminescence emission spectrum is also a molecular spectrum. Some specific molecules can emit photons after being excited by a specific wavelength photon. Electrons promote to different vibrational levels of excited state from a ground state. Electron at the excited state collide with other electron, which consume energy, then the electrons fall back to different vibrational levels of ground state and release energy as different wavelength luminescence.

4.2 Experiment

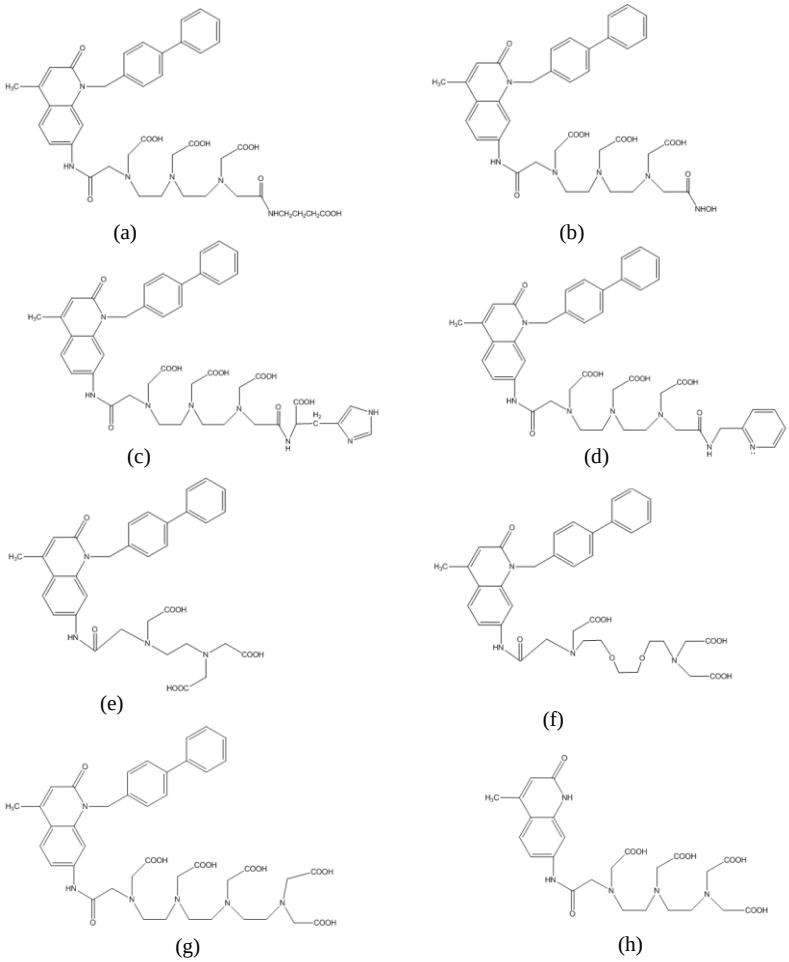
4.2.1 Synthesis of Reference Compounds

The reference compounds were made in this assay, which are DTPA-cs124-Eu^{3+} for Eu^{3+} probes and DTPA-cs124-Tb^{3+} for Tb^{3+} probes.

Eight point seven milligrams (0.05 mmol) [27] of cs124 were added to a solution of 40 mg (0.15 mmol) of DTPA dianhydride in 0.4 ml of DMSO. After incubation (25 min at 25 ℃), the mixture was supplemented with five ml of ether, and the resulting precipitate was spun down, washed with ether, air dried, dissolved in 0.5 ml of DMF, and mixed with 0.15 ml of water. After incubation for 10 min at 45 ℃, the mixture was diluted with 2.5 ml of water and extracted with 20 ml of butanol. The organic phase was separated and divided into two equal parts. Each portion was mixed with 0.3 ml of a 0.1 M solution of a lanthanide trichloride ($TbCl_3$ and $EuCl_3$). After vigorous agitation, aqueous phase was collected. Analytical thin layer chromatography, using an acetonitrile: water system (3:1) as the developing solvent, show that only one main Ln^{3+} product. The products were purified using preparative thin layer chromatography under the same condition. The luminescent material was eluted with 50% aqueous ethanol. After agitation, liquid phase was collected as reference RTb and REu in this essay.

The concentration of RTb and REu were measured under ultraviolet-visible absorption spectrum. Absorbance is 0.51 for RTb and 0.88 for REu. Comparing with absorbance at 1 M, the concentration of RTb is 26.8 uM and REu is 46.3 uM. The reference compounds were made this section. Photophysics prosperities of these samples can be measured comparably.

4.2.1.1 Relative Brightness and Quantum Yield. There are 16 samples (Figure4.1) of Bi-cs124 derivatives and two reference compounds, dilute them at the same concentration 10 uM. The concentration of dilute solution was determined by the UV-V spectrum. The luminescence emission spectra were measured in fluorospectro photometer. Origin supplies a way to calculate the area under the spectrum as the total luminescence emission intensity (Figure 4.2). Relative brightness defined as ratio between total luminescence emission intensity of samples and total luminescence emission intensity of reference compound. The quantum yield is defined as relative brightness multiply quantum yield of reference compounds. Results are showed in Table 4.1.



(a) (b) (c) (d) (e) (f) (g) (h)

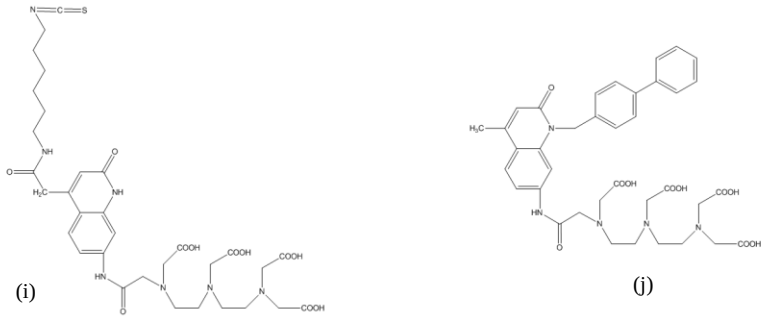


Figure 4.1 Structure of samples Bi-cs124-DTPA-Ala(a) sample 1, 7; Bi-Cs124-DTPA-Ha(b) sample 4; Bi-cs124-DTPA-His(c) sample 2, 3; Bi-cs124-DTPA-Py(d) sample 5,6; Bi-cs124-EDTA(e) sample 10, 16; Bi-cs124-EGTA(f) sample 11, 12; Bi-cs124-TTHA(g) sample 13, 14, 15; cs124-DTPA(h) sample RTb,REu; cs124-DTPA-NCS(i) sample 9; Bi-cs124-DTPA(j) sample 8.

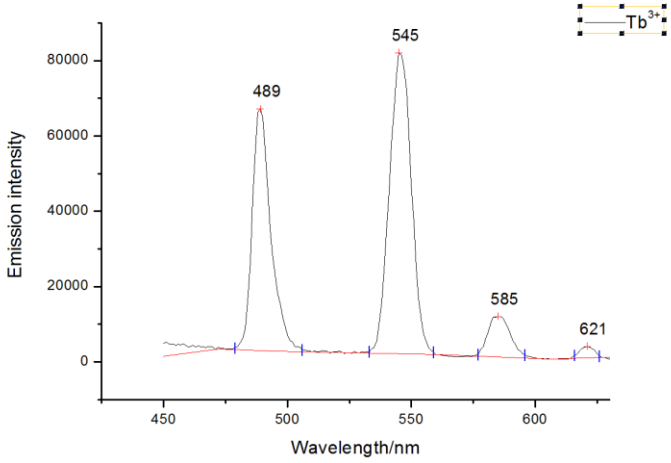


Figure 4.2 Example for total luminescence emission intensity calculation (sample 13 in heavy water c=10 nM).

Table 4.1 Photophysics Properties of Lanthanide Probes

No.	Total emission intensity[a] in regular water	Relative brightness in regular water	Quantum yield	Total emission intensity[a] in heavy water	Relative brightness in heavy water	Quantum yield	Lifetime inD2O/lifetime inH2O	Lifetime[b] in regular water (ms)	Lifetime[b] in heavy water (ms)	No.water molecules coordinated
Eu^{3+}										
1	4.55E+04	84.28%	0.08	1.94E+05	107.72%	0.46	3.93	0.56 ±0.0083	2.2 ±0.09	1.40 ±0.047
2	6.02E+04	111.40%	0.11	2.94E+05	163.35%	0.70	3.67	0.55 ±0.0026	2.02 ±0.03	1.39 ±0.017
5	3.20E+04	59.33%	0.06	9.68E+04	53.75%	0.23	4.98	0.54 ±0.0070	2.69 ±0.22	1.55 ±0.057
8	5.92E+04	109.58%	0.11	2.48E+05	137.76%	0.59	3.52	0.54 ±0.0053	1.9 ±0.04	1.39 ±0.031
10	2.29E+04	42.32%	0.04	1.44E+05	79.84%	0.34	3.48	0.29 ±0.02	1.01 ±0.05	2.58 ±0.302
12	3.02E+04	55.99%	0.06	7.31E+04	40.61%	0.18	2.70	0.57 ±0.0078	1.54 ±0.4	1.16 ±0.215
14	1.37E+05	253.11%	0.25	2.21E+05	122.78%	0.53	1.80	0.89 ±0.01	1.6 ±0.02	0.52 ±0.021
15	8.70E+04	161.03%	0.16	9.66E+04	53.65%	0.23	1.87	0.86 ±0.01	1.61 ±0.1	0.57 ±0.055
EuR	5.40E+04	100.00%	0.10	1.80E+05	100.00%	0.43	3.40	0.62 ±0.0051	2.11 ±0.02	1.20 ±0.019

[a]all samples measure at 10nM.
[b]all samples measure at 300nM

Table 4.1 Photophysics Properties of Lanthanide Probes (continued)

No.	Total emission intensity[a] in regular water	Relative brightness in regular water	Quantum yield	Total emission intensity[a] in heavy water	Relative brightness in heavy water	Quantum yield	Lifetime inD2O/lifetime inH2O	Lifetime[b] in regualr water (ms)	Lifetime[b] in heavy water (ms)	No.water molecules coordinated
Tb^{3+}										
3	1.76E+06	68.58%	0.22	3.08E+06	79.38%	0.40	1.51	1.08 ±0.01	1.63 ±0.03	1.31 ±0.083
4	4.26E+05	16.64%	0.05	8.18E+05	21.08%	0.10	1.43	1.08 ±0.02	1.54 ±0.03	1.16 ±0.13
6	1.02E+06	39.69%	0.13	1.42E+06	36.54%	0.18	1.64	0.99 ±0.01	1.62 ±0.09	1.65 ±0.19
7	1.59E+06	62.24%	0.20	2.51E+06	64.62%	0.32	1.53	1.16 ±0.0068	1.78 ±0.0088	1.26 ±0.033
9	2.08E+06	81.04%	0.26	3.30E+06	84.95%	0.42	1.49	1.03 ±0.0046	1.53 ±0.0090	1.33 ±0.034
11	6.96E+05	27.15%	0.09	1.12E+06	28.87%	0.14	1.48	1.14 ±0.0082	1.69 ±0.01	1.20 ±0.041
13	9.78E+05	38.16%	0.12	1.52E+06	39.23%	0.20	1.22	1.47 ±0.01	1.8 ±0.02	0.52 ±0.045
16	3.03E+05	11.82%	0.04	4.67E+05	12.02%	0.06	1.82	0.76 ±0.02	1.38 ±0.02	2.48 ±0.19
TbR	2.56E+06	100.00%	0.32	3.88E+06	100.00%	0.50	1.66	1.46 ±0.0089	2.43 ±0.03	1.15 ±0.039

[a]all samples measure at 10nM.
[b]all samples measure at 300nM.

4.2.1.2 Luminescence Lifetime and Number of Water Molecules Coordinated in the Ion's Sphere. The number of water molecules coordinated in the ion's sphere can be calculated by lifetime in the regular water and the heavy water[44].

$$\text{No. of water molecules} = q(\tau_{H2O}^{-1} - \tau_{D2O}^{-1}) \qquad q = 1.05 \text{ for } Eu^{3+},\ 4.2 \text{ for } Tb^{3+} \qquad (4.2)$$

Luminescence lifetime of 16 samples and reference compounds were measured by the same way as in Chapter 2. Samples and reference compounds dilute at the same concentration. Results are showed in Table 4.1.

4.2.1.3 Paper Chromatography. Paper chromatography is a kind of chromatography base on paper. Stationary phase is the water molecules absorb by paper, moving phase is organic solvent which incompatible with water. The sample drops on a side of paper that has been put in a closed container then developed by organic solvent. After organic solvent moves to the other side of paper, taking out paper and dry. Every different compound has a specific position on the paper called Rf. Chromatography bases on the principle of the dissolution in the similar material structure. The partition coefficient of different compounds is different, diffusion rate is also different. In this assay silicon gel-pad is used as chromatography paper. It has 10~100 times higher sensitivity than paper. When purify reference compounds with high concentration solution cause two compounds show on the silicon gel-pad, after dilute 100 times it comes to one compounds on the pad.

4.2.1.4 HEPES and Na-borate Buffer. HEPES has a high buffer ability of hydrogen ion. The aqueous phase collected after vigorous agitation, the solution is muddy at first, after putting several drops of 0.1M pH=8.0 HEPES, solution comes to limpidity. HEPES

keeps the hydrogen ion away from carboxylic acid, and improves the dissolving capacity of carboxylic acid in water.

Luminescence emission intensity measured in ultraviolet spectrophotometer, the Na-borate pH=9.0, 1 mM as solvent and buffer keep pH stable. If solution is acidic, hydrogen replace lanthanide ion on the carboxylate.

4.3 Results and Discussion

4.3.1 The Number of Water Molecules Combine with Probes

It is so-called coordinated water molecules on the ions' sphere. Ln^{3+}, they have nine coordination positions could coordinate nine water molecules. The vibration of O-H absorbs most excited energy of Ln^{3+}, that indicate Ln^{3+} have rare luminesce (water quenching) in regular water. Ln^{3+} is chelated by chelators (polycarboxylic acid in this thesis), the N and O as the donor of lone electron, which replace water molecules on the Ln^{3+} sphere. [23]

The energy absorbed by vibration of O-H is much greater than vibration of O-D. In heavy water, probes are excited under the same energy; more energy had to be used to excite Ln^{3+} luminescence. That gives an explanation for why luminescence lifetime in heavy water is longer than in regular water. For example, sample 16 and 13, which is Bi-cs124-EDTA-Tb^{3+} (lifetime 0.76 ms in regular water, number of water molecules is 2.48) and Bi-cs124-TTHA-Tb^{3+} (lifetime 1.47 ms in regular water, number of water molecules is 0.52).

The number of coordinated water molecules in the Eu^{3+} probes is greater than in Tb^{3+} probes. For example, samples 1 (Eu^{3+}) and 7 (Tb^{3+}), which all have a structure like Bi-cs124-Ala, but different lanthanide ions. The number of water molecules of sample 1 (Eu^{3+}) is 1.4 and for sample 7 (Tb^{3+}) is 1.26. The number of coordinated water molecules explains stability of products (lanthanide probes) in chaletor-water substitution reaction. More water molecules are coordinated less stability lanthanide probes. Stability depends on the diameter of the probes. The lower diameters are the more stability of probes. The diameter of Eu^{3+} is 95 pm and for Tb^{3+} is 92.3 pm. The diameter of Eu^{3+} is larger than Tb^{3+}, which causes number of coordinated water molecules of Eu^{3+} is greater than Tb^{3+}.

Bi-cs124-DTPA (1, 4, 5, 6, 7, and 8) derivatives and two reference samples (REu, RTb), these eight samples all have seven chelating groups (N and O). Compared with optimum nine position on the ions' sphere, there are two water molecules coordinate with lanthanide ions. But this number is between one and two. Chelate effect, carbonyl between cs-124 and DTPA transform to hydroxyl, which gives out another chelating group and easier to ring isomerism, which has a more stable resonance structure. Based on this principle, number of water molecules in probes may less than two. But for large diameter ions like lanthanide ions, tension of complexes will be enhanced when rings formation. While there are eight chelating groups (addition of ring isomerism one), assume that chelating groups chelate lanthanide ion one by one, for last one or two chelating groups is not stable with ion. The number of water molecules coordinated get higher base on tension theory. In other way, introduce dynamic equilibrium theory. There two complexes [Bi-cs124-DTPA (H_2O)] and [Bi-cs124-DTPA ($H_2O)_2$] in the

solution at a dynamic equilibrium. Due to hydrophobicity of cs-124, water molecules cannot go into inner coordination sphere.

Bi-cs124-DTPA-Ha (4 Tb^{3+}) and Bi-cs124-DTPA-Py (6 Tb^{3+}). DTPA residues modify by –Ha and –Py. The diameter of sample 6 is larger than sample 4 because of -Py occupying a larger space than -Ha do. In the substitution reaction, -Py and -Ha will compete with water molecules, -Ha occupy a smaller space and easier to replace water molecules than -Py does. This is steric hindrance theory.

Bi-cs124-DTPA-His (2, 3) samples have eight chelating groups, but the number of water molecules coordinated not much different with those Bi-cs124-DTPA derivatives simples, which have seven chelating groups. This phenomenon is explained by tension theory and steric hindrance theory.

Bi-cs124-EDTA (10 Eu^{3+}, 16 Tb^{3+}). Original EDTA has six chelating groups and form five-membered ring with Ln^{3+}. However, Ln^{3+} also can chelate with three water molecules. In the Bi-cs124-EDTA, only five chelating groups left and base on chelate effect carbonyl convert to carboxyl. At least, there are approximately six chelating groups, and hydrophobicity of cs-124, the number of water molecules coordinated is round 2.5.

Bi-cs124-EGTA (11 Tb^{3+}, 12 Eu^{3+}). The number of water molecules coordinated can be explained by the same theories as Bi- cs124-DTPA derivatives due to they all have seven chelating groups. Since no modify groups on EGTA residues, it better to compare with Bi-cs124-DTPA (8 Eu^{3+}), sample 8 and 12. The number of water molecules coordinated is 1.39 and 1.16 for sample 8 and 12. EGTA is better for

europium ions, which have larger diameter, and can be proved by compare number of water coordinated in samples 12 (Eu^{3+}, number of water molecules coordinated is 1.16), 11 (Tb^{3+}, number of water molecules coordinated is 1.2). Europium ions has larger diameter than terbium ions.

Bi-cs124-TTHA (13 Tb^{3+}, 14 Eu^{3+}, 15 Eu^{3+}). A TTHA residue has nine chelating groups, which match nine optimum coordinators. But it is also more than 0.5 water molecules in the ligand. This phenomenon is explained in tension theory due to nine chelating groups.

4.3.2 Quantum Yield

Quantum yield can be calculated by

$$\frac{\Phi_R}{\Phi} = \frac{A_R}{A} \tag{4.3}$$

Φ_R is quantum yield of reference compound and A_R is total luminescence emission intensity of reference compound. A is total luminescence emission intensity of samples, Φ is unknown quantum yield of samples [23]. The quantum yield for RTb is 0.32 in regular water, 0.5 in heavy water; quantum yield for REu is 0.1 in regular water and 0.43 in heavy water.

Quantum yield of probes compared base on different compounds with same lanthanide ion. Except sample 9 and two reference compounds, these samples all connected with biphenyl, which can enhance UV absorption.

For most samples had been measured in the assay except Bi-cs124-TTHA probes, same ligand but different lanthanide ion, the Tb^{3+} probe has greatly higher quantum yield than Eu^{3+} probes.

4.3.2.1 Quantum Yield of Terbium Ion Probes. Sample RTb (DTPA-cs124-Tb^{3+}) is the reference compound in this assay and also the highest quantum yield base on terbium ion. In Bi-cs124-DTPA derivatives the best on is sample 3 Bi-cs124-DTPA-His that has eight chelating groups. The energy transferred from triplet state of ligand to vibrational level, it can only happen if triplet state is higher than lowest vibrational level at least 1850 cm^{-1}. The lowest vibrational level of Tb^{3+} is 20400 cm^{-1} and for Eu^{3+} 17500 cm^{-1} For these probes which ligand triplet state are higher than both lowest vibrational level of Tb^{3+} and Eu^{3+}, energy easier transfer to higher vibrational level, that leads quantum yield of Tb^{3+} probes is higher than Eu^{3+} probes[33].

But for Bi-cs124-TTHA probes, the triplet state of ligand is not very high but can also transfer energy to both vibrational levels. The difference between triplet state of ligand and vibrational level of Tb^{3+} is close to 1850 cm^{-1}, the energy is hard to transfer to Tb^{3+} but easier to Eu^{3+} [25]. In view of this quantum yield of Bi-cs124-TTHA-Eu^{3+} is higher than Bi-cs124-TTHA-Tb^{3+}.

The difference between sample 9 and RTb is sample 9 has N-Chlorosuccinimide (NCS) group which has double bond could absorb the energy from fluorophore (cs-124). In this view, the quantum yield of sample 9 is less than RTb.

4.3.2.2 Quantum Yield of Europium Ion Probes. Bi-cs124-DTPA (8), Bi-cs124-EDTA (10), Bi-cs124-EGTA (12) and Bi-cs124-TTHA (14), these are Bi-cs124 compounds with different chelators. The number of chelating groups for them is seven, five, seven, and

nine. The quantum yield for them is 0.11, 0.04, 0.06 and 0.25. The more chelating groups the higher the quantum yield, and for same number of chelating groups the sample 8 (Bi-cs124-DTPA) is better than sample 12 (Bi-cs124-EGTA).

4.4 Conclusions

Based on the experimentally measured luminescence lifetimes, the numbers of water molecules coordinated with lanthanide ions were determined. The quantum yields were determined based on the total luminescence emission intensity in comparison with reference compounds. The number of chelating groups in the ligand has strong influence the photophysical properties of the lanthanide probes. Presence of double bonds and O-H bonds in the ligands lead to shorter luminescence lifetimes.

CHAPTER 5

CONCLUSIONS

The luminescence lifetime of Probe 11 was measured as a function of the probe concentration. The concentration quenching was observed. The characteristic concentration for the self-quenching offset of Probe 11 is about 3×10^{-6} M (lifetime drops of ca. 25%). The microscopic images of E-coli cells labeled by lanthanide probes were obtained in time-gated mode. Glycerol was used to improve coupling of the evanescent wave in the complete internal reflection technique. Luminescence quantum yields and the number of water molecules coordinated by lanthanide ions were determined. Strong impact of the number of coordinated water molecules on the lifetime as well as quantum yield was demonstrated. The results of this study could be used in the development of novel lanthanide ion based luminescent probes.

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