Time-Resolved Fluorescence-Based Europium-Derived Probes for Peroxidase Bioassays, Citrate Cycle Imaging and Chirality Sensing

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谨以此篇献给我的父亲母亲和儿子

This dissertation is dedicated to my parents and my son
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<td>$[\alpha]$</td>
<td>Optical activity</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline-phosphatase</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>Cit</td>
<td>Citrate</td>
</tr>
<tr>
<td>CL</td>
<td>Citrate lyase</td>
</tr>
<tr>
<td>CLIA</td>
<td>Chemiluminescent immunoassay</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CPL</td>
<td>Circular polarized luminescence</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DELFIA</td>
<td>Dissociation enhanced lanthanide fluoroimmunoassay</td>
</tr>
<tr>
<td>DIFP</td>
<td>Phosphate ester of diflunisal</td>
</tr>
<tr>
<td>DLCLLA</td>
<td>Direct lanthanide chelate label-based luminescence assay</td>
</tr>
<tr>
<td>Dy</td>
<td>Dysprosium</td>
</tr>
<tr>
<td>EALL</td>
<td>Enzyme-amplified lanthanide luminescence</td>
</tr>
<tr>
<td>ECIA</td>
<td>Electrochemical immunoassay</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ee</td>
<td>Enantiomeric excess</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic E. coli</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Eu</td>
<td>Europium</td>
</tr>
<tr>
<td>EuTc</td>
<td>Europium tetracycline complex</td>
</tr>
<tr>
<td>EuTc-Cit</td>
<td>Europium-tetracycline-citrate</td>
</tr>
<tr>
<td></td>
<td>(molar ratio of Eu$^{3+}$:Tc is 1 : 1)</td>
</tr>
<tr>
<td>EuTc-HP</td>
<td>Europium tetracycline hydrogen peroxide complex</td>
</tr>
<tr>
<td></td>
<td>(molar ratio of Eu$^{3+}$:Tc is 3 : 1)</td>
</tr>
<tr>
<td>F</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FIA</td>
<td>Fluorescent immunoassay</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging microscopy</td>
</tr>
<tr>
<td>FM</td>
<td>Fumarase</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FSAP</td>
<td>5-fluoresalicyl phosphate</td>
</tr>
<tr>
<td>Fum</td>
<td>Fumarate</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HP</td>
<td>Hydrogen peroxide, $H_2O_2$</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HST</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>iCit</td>
<td>Isocitrate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>----------</td>
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<tr>
<td>IDL</td>
<td>Interactive data language</td>
</tr>
<tr>
<td>KG</td>
<td>α-ketoglutarate</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>Mal</td>
<td>Malate</td>
</tr>
<tr>
<td>MDH</td>
<td>Malic dehydrogenase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulfonate</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>β-Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>β-Nicotinamide adenine dinucleotide reduction</td>
</tr>
<tr>
<td>NTA</td>
<td>β-naphthoyl trifluoroacetone</td>
</tr>
<tr>
<td>Oxa</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>pHPA</td>
<td>p-Hydroxyphenylacetate</td>
</tr>
<tr>
<td>pHPPA</td>
<td>4-hydroxyphenylpropionic acid</td>
</tr>
<tr>
<td>POx</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>QY</td>
<td>Quantum yield</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RLD</td>
<td>Rapid lifetime determination</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylaldehyde</td>
</tr>
<tr>
<td>SLT1</td>
<td>Shiga-like toxins</td>
</tr>
<tr>
<td>Sm</td>
<td>Samarium</td>
</tr>
<tr>
<td>Suc</td>
<td>Succinate</td>
</tr>
<tr>
<td>Tb</td>
<td>Terbium</td>
</tr>
<tr>
<td>TBDRH</td>
<td>Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate</td>
</tr>
<tr>
<td>Tc</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TCSPC</td>
<td>Time-correlated single photon counting</td>
</tr>
<tr>
<td>TOPO</td>
<td>Trioctylphosphine oxide</td>
</tr>
<tr>
<td>TRFI-ELISA</td>
<td>Time-resolved fluorescence imaging ELISA</td>
</tr>
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Chapter 1. Introduction

1.1. Characteristics of Fluorescence Spectra of Lanthanide

Fluorometry is a very useful tool in a variety of analytical regions since it can provide high sensitivity, good selectivity and multiparameter information, such as fluorescence intensity, lifetime, anisotropy and others. It has been widely applied in biomedical research and clinical diagnosis [1, 2], such as in astrospace living and environmental monitoring, interaction mechanisms of molecules, immunoassay, DNA sequencing, fluorescence in situ hybridization, and in cellular imaging. Developing new fluorescent reagents and analytical methods is one of main research directions for improving sensitivity and selectivity of bioanalysis. Lanthanide complexes have become a particularly attractive focus because of the specific features of their fluorescence.

1.1.1. Fluorescence Emission Mechanism of Lanthanide Complexes

Fluorescence [3, 4] of conventional fluorophores is the result of several processes as shown in the left of Fig. 1.1 (Jablonski diagram). In the excitation stage, the fluorophore absorbs energy supplied by an external radiation and is raised to any one of the vibrational multiplets of the first excited singlet state (S₁) from ground state (S₀). Most of the fluorophores in the S₁ state rapidly relax, through internal conversion (non-radiative decay), to the lowest vibrational level S₁, in which fluorescence emission originates. A photon is emitted on returning to the ground state (S₀). Other processes such as collisional quenching, energy transfer and solvent interactions may also occur. They are non-radiative decay. Conversion from S₁ to the first triplet state T₁ is intersystem crossing and transition from T₁ to ground state is forbidden, so its decay rate is slow, yields phosphorescence.
There are 15 elements in the lanthanide series, but only Sm$^{3+}$, Eu$^{3+}$, Tb$^{3+}$ and Dy$^{3+}$ enabling fluorescence. However, the absorption and fluorescence of these ions are very low and difficult to be used in analysis. They usually need to ligate an organic compound as antenna for energy transfer to metal ions.

![Diagram of fluorescence emission mechanism of Eu$^{3+}$ complex](image)

*Figure 1.1. Fluorescence emission mechanism of Eu$^{3+}$ complex. S$_0$, S$_1$, and T$_1$ are singlet ground state, singlet excited state, and triplet state, respectively.*

The fluorescence emission process of lanthanide complex [5, 6, 7] (in Fig. 1.1) is of some particularities that is absent in conventional fluorophores: (a) The ligand, not the lanthanide ion itself, absorbs energy from external source into S$_1$ from its S$_0$, then proceeds on the internal conversion. b) That intersystem crossing to T$_1$ from the lowest vibrational level S$_1$ can further proceed to intramolecular energy transfer from T$_1$ of the ligand to the localized appropriate 4f energy level of the central lanthanide ion, which in turn can moves up to its
own excited singlet state. It requires that deactivating ligand transition ($S_1 \rightarrow S_0$ and $T_1 \rightarrow S_0$) must be minimal and the energy level of ligand $T_1$ should be matched, namely close to, but just higher than the resonance level of lanthanide ion. c) The multiple emissions. For example in the europium complex, multiple emissions \cite{8} are observed as several electronic transitions $^5D_0 \rightarrow 7F_J$ ($J = 0, 1, 2, 3, 4$) and $^5D_1 \rightarrow 7F_J$ ($J = 1, 2, 3, 5, 6$), the most intense transitions are $^5D_0 \rightarrow 7F_2$ and $^5D_0 \rightarrow 7F_1$ with emissions around 610-660 nm and 585-600 nm.

The above mechanism results in the three main advantages of lanthanide complexes in fluorometry. First is the large Stokes’ shift (150 – 300 nm). Owing to energy dissipation during internal conversion, intersystem crossing, and intramolecular energy transfer, energy of photon emitted from lanthanide complexes is significantly different from that of the excitation radiation, and therefore the Stokes’ shift of lanthanide complexes is usually large. This feature can be applied to avoid the overlap between excitation and emission spectra of fluorophore itself (inner filter effect) or emission from biological matrix.

Second is the narrow emission bands (1 – 20 nm), namely line-like bands. It is because of the shielding of the f orbitals by the higher s and p orbitals of lanthanide. It is noted that there are three and five components of the emission splitting patterns \cite{9, 10} from $^5D_0 \rightarrow 7F_1$ and $^7F_2$, for example in Eu$^{3+}$ complexes. But the failure to observe such splitting may be due to spectral resolution limitations of instrument rather than to inherent structural properties of the system. In addition, the fluorescence intensity of main band of lanthanide complex is very strong although its quantum yield is usually lower than that of conventional fluorophores. The reason is that the transferred energy is largely emitted by the line-like main band. The narrow emission bands also offer the possibility for the multiplex assays without overlapping spectra.

Third is the long fluorescence decay time (10 – 2000 µs). The f-f electronic transitions of lanthanide are forbidden, leading to long excited state decay time. Decay times of
Lanthanide complexes are quite sensitive to the detailed nature of the ligand environment, and especially to the number of water molecules occupying inner coordination sites. The forbidden f-f transitions is also reflected in low extinction coefficients, making direct photoexcitation of lanthanide ions rather difficult, and requiring organic ligands for energy absorption. The relatively long decay times of lanthanide complexes have greatly facilitated the time-resolved fluorometry.

1.1.2. Time-Resolved Fluorescence Assays

The fluorescence decay time \([5, 8, 11]\) is one of the most important characteristics of a fluorophore. There are mainly two kinds of method used for the measurement of the fluorescence decay times, namely time-domain or pulse fluorometry and frequency domain or phase-modulation fluorometry. In this dissertation, only time-domain methods will be discussed.

Compared with the lanthanide complexes, the conventional fluorophores have relative short decay times between 5-100 ns. The decay times of most light source background, such as Tyndall, Raman scatter, Rayleigh scatter, and sample background, such as cuvette, plate, and sample matrix (protein, NADH, etc), are around 0.1-10 ns. So the gated fluorometry based on the lanthanide complexes can be used as shown in Fig. 1.2.

The gated fluorescence experiment starts with the excitation pulse, but does not measure the fluorescence emission until the background has decayed to zero or minimum by a temporal lag. As lanthanide complexes have longer lifetimes, the background should be possible to be eliminated entirely. The sensitivity and selectivity of determination can be therefore improved.
The µs scale decay time of lanthanide complexes have also greatly facilitated their decay time determination. The decay time based fluorometry has the following advantages: (1) decay time is the inherent characteristics of fluorophores, not affected by the concentration of fluorophores and photobleaching. (2) decay time is independence of the light source fluctuation. (3) decay time of lanthanide complex is only sensitive to its microenvironment, such as water in inner coordination field. With the development of fluorescence theories and instruments, there have been a lot of applications of lanthanide fluorescence as in different time–resolved determination [5-8] and imaging [12, 13]. There have been several techniques developed, such as lifetime based time-correlated single photon counting (TCSPC) and rapid lifetime determination (RLD) in time-domain fluorometry. Time-resolved fluorometry can also been applied in anisotropy (polarization) detection as time-resolved anisotropy [14].
1.2. Time-Resolved Detection of Lanthanide Fluorescence for Bioassays

Fluorescence lanthanide chelates have been successfully developed as labels and probes for the highly sensitive and selective bioassays in the past two decades. Time-resolved fluorescence detection \cite{15, 16} has been widely applied in fluoroimmunoassay, DNA hybridization assay, enzyme assay, cell activity assay, and fluorescence imaging microscopy. According to the necessity of analyte immobilization, these bioassays can be broadly classified into heterogeneous and homogeneous fluorescence determination. Although the latter, especially utilizing the principle of fluorescence resonance energy transfer (FRET) \cite{17, 18}, has shown powerful potentials, it does not yet surpass the solid phase fluorescence bioassay in sensitivities, selectivities and virtual applications now. In the following, the three main approaches for time-resolved lanthanide fluorescence in heterogeneous phase, direct lanthanide chelate label-based luminescence assay (DLCLLA), dissociation enhanced lanthanide fluoroimmunoassay (DELFIA) and enzyme-amplified lanthanide luminescence (EALL), will be discussed in some details.

1.2.1. Direct Lanthanide Chelate Label-based Luminescence Assay (DLCLLA)

In contrast to other analytical methodologies (DELFIA and EALL), the experimental protocol of DLCLLA is simple. Its measurement principle (in Fig.1.3.) is that recognition molecules are labeled with fluorescent lanthanide chelate, being used to capture an analyte. The fluorescence intensity from label reports the quantity of analyte captured after excess labeled recognition molecules are washed off.
But an ideal fluorescence lanthanide chelate for DLCLLA is not easily achieved because the lanthanide chelate as label must undergoes the whole experiment processes including labeling reaction, recognition reaction and several rinses. Thus, there are some strict requirements for lanthanide chelate [15]: (a) high luminescence in water, (b) chemically and photochemically stable, (c) high metal-chelate binding constant and the presence of contain reactive groups appropriate for effective labeling reactions.

Many synthetic organic chelators (see Fig. 1.4) have emerged. 4,7-Bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) [19, 20] as ligands binding Eu$^{3+}$ was the first commercial reagent for direct time-resolved fluorescence bioassay. It has been other applied in immunoassays [21], Western blots [22] and membrane based nucleic acid hybridization assay [23]. Subsequently, polyamino-polycarboxylate compounds and cage-type ligands have been synthesized, such as trisbipyridine cryptate (TBP), 4'-(3-isothiocyanato-4-methoxyphenyl)-6,6'-bis[N,N-bis(carboxymethyl)aminomethyl]-2,2';6',2''-terpyridine (TMT), and others. [Eu$^{3+}$-TBP] can be used the detection of specific amplified...
target DNA [24, 25], but it is more often used in homogeneous detection for biomolecular interaction via FRET [16]. [Eu\(^{3+}\)-TMT] was employed for the determination of protein and DNA samples [26] and its limit of detection is approx. \(10^{-16}\) mol. Many lanthanide \(\beta\)-diketonate chelates also display intensive fluorescence, but a few are suitable for the requirement of DLCLLA, for example, BHHCT [27] and BTOT [28] (Fig. 1.4.). They have been used in albumin and IgE assays [29]. Other \(\beta\)-diketonate compounds, e.g. 1,10-bis(thiophene-2'-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BTOT) [30] and 4,4'-bis(1',1',1'-trifluoro-2',4'-butanedione-6'-yl)-chlorosulfo-o-terphenyl) (BTBCT) [31] have also been reported.
1.2.2. Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA)

In lanthanide chelate based fluorescence detection systems, DELFIA [32, 33] is the most widely utilized approach. Its principle (Fig. 1.5) is that recognition molecule is labeled by the lanthanide chelate, which has a strong binding ability, but no or much weak fluorescence itself in the assay medium. After specific binding reaction for the analyte has been performed and the non-bound fraction of the label molecule has been efficiently washed away, lanthanide ion must be dissociated and released from the lanthanide chelate label, then enter an enhancement solution and micelle environment in which it coordinates new ligand and is measured via fluorescence.

\[ \text{analyte} \rightarrow \text{labeled recognition molecule} \rightarrow \text{bound capture molecule or group} \]

**Figure 1.5. Scheme of DELFIA**

*Recognitions include immunoaffinity or hybridization.*

In DELFIA [32-36] diethylene-triaminetetraacetic acid or the derivative of ethylenediaminetetraacetic acid (EDTA) as chelating agent coordinates with europium ion to form lanthanide chelate label, such as, isothiocyanatophenyl–EDTA-Eu$^{3+}$. Dissociative enhancement solution usually includes β-naphthoyltrifluoroacetone (NTA) as the energy transfer chelator, trioctylphosphine oxide (TOPO) as synergistic ligand, and Triton X-100 as detergent. In phthalate buffer at low pH 3.4, the stability of lanthanide chelate label is strongly
decreased in comparison to NTA present in the solution in large excess. Under these conditions the ligand exchange reaction is completed very fast. A new chelate is formed with NTA and the remaining empty coordination sites are occupied with TOPO, which prevents aqueous quenching. The hydrophobic chelate formed is dissolved in a micellar detergent solution (Triton X-100), in which the high fluorescence intensity can be determined.

This method has been employed in many areas, such as PCR [37], nucleic acid hybridization [38, 39], immunoassays [40] for hormones [41], steroid [42] and inhibitor [43], proteins in cells [44-45], and drug discovery [46].

In addition, multiplexing DELFIA has been also developed. Its protocol is (a) the same chelators are used to ligate several of lanthanide ions and to form differently colored fluorescent lanthanide chelates; (b) these lanthanide chelates are labeled on different recognition molecules (or groups) in same system; (c) “co-fluorescence enhancement” solution is employed for fluorescence measurement [47, 48]. Due to the narrow emission peaks of lanthanides at different wavelengths and their different fluorescence lifetime, the combination of spectral windows and temporal windows can be employed for the optimization of the measurement parameters. Thus, maximal sensitivity and minimal signal loss are obtained. All labels can be determined simultaneously, even if one is present in a significant excess, which is based on co-fluorescence enhancement. Multiplexing DELFIA assays, such as Eu$^{3+}$ and Sm$^{3+}$ [49, 50], Eu$^{3+}$ and Y$^{3+}$ [51], or Eu$^{3+}$, Sm$^{3+}$, Tb$^{3+}$ and Dy$^{3+}$ [52, 53], have been reported.

1.2.3. Enzyme Amplified Lanthanide Luminescence (EALL)

The concept and experiment of EALL [54] were first reported by Evangelista and co-workers in 1991. It is a bridge to connect the two sectors of enzyme assay and lanthanide
fluorescence assay. The basic principles of EALL is shown in Fig. 1.6. The label of recognition molecule is enzyme, not lanthanide chelate. After the analyte is recognized, substrate for enzyme is added. Its reaction product can associate with lanthanide ion to form fluorescence chelate to be detected.

As many enzymes can catalyze different substrates to form a lot of different products, the wide application area of EALL can be predicted. Up to now, Eu$^{3+}$ and Tb$^{3+}$ as main lanthanide element are employed in EALL. Some enzymes, such as alkaline-phosphatase (AP), glucose oxidase (GOx), peroxidase (POx), galactosidase, esterase and catalase, have been employed in EALL as summarized in Table 1.1. As these enzymes are of high stability and sensitivity, they have been usually used as label in many biological recognition molecules and have been commercialized.
On the other hand, since the property of substrate as media will influence the fluorescence of the final lanthanide chelate, there are some stipulations [54]: a) the substrate does not absorb the excitation light efficiently in the absorption spectrum of lanthanide chelate; b) the substrate does not efficiently transfer the excitation energy to the chelated lanthanide; c) the substrate does not efficiently chelate the lanthanide. Now some substrates have been used in different enzymes, for example, salicylaldehyde (SA), 5-fluorosalicyl phosphate (FSAP), phosphate ester of diflunisal (DIFP) and other salicyl phosphate-derived compounds for AP; 4-hydroxyphenylpropionic acid (pHPPA) for POx.

Since enzyme amplification cycling reactions, EALL has a very low limit of detection (Table 5.1). It is conceivable that, in the scheme of EALL, substrate may be also a strong fluorescent lanthanide chelate to yield no or weak fluorescence product by enzyme catalytically. So the determinations of GOx [67] and of catalase [68] can be considered as this scheme.

In addition, some improved schemes from enzyme and lanthanide chelates have been presented. For example, Ioannou and co-workers [55] reported the two-round enzymatic amplification, first combined with tyramide signal amplification [56], then with EALL, so that the selectivity and sensitivity have been increased.
<table>
<thead>
<tr>
<th>Target</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Detection Chelates</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Alkaline-phosphatase (AP)</td>
<td>AP</td>
<td>5-fluorosalicyl phosphate (FSAP)</td>
<td>FSA-Tb$^{3+}$-EDTA</td>
<td>0.2 amol</td>
<td>54</td>
</tr>
<tr>
<td>2 IgG</td>
<td>AP</td>
<td>5-fluorosalicyl phosphate (FSAP)</td>
<td>FSA-Tb$^{3+}$-EDTA</td>
<td>5 pg</td>
<td>54</td>
</tr>
<tr>
<td>3 Xanthine oxidase(XOD)</td>
<td>XOD</td>
<td>salicylaldehyde</td>
<td>SA-Tb$^{3+}$-EDTA</td>
<td>1 µUnits</td>
<td>54</td>
</tr>
<tr>
<td>4 β-Galactosidase (Gas)</td>
<td>GAs</td>
<td>salicyl- β-D-galactoside</td>
<td>SA-Tb$^{3+}$-EDTA</td>
<td>90 amol</td>
<td>54</td>
</tr>
<tr>
<td>5 Glucose oxidase (GOx)</td>
<td>GOx</td>
<td>1,10-phenanthroline-2,9-dicarboxylic acid dihydrazide (PDAdh)</td>
<td>PDA-Tb$^{3+}$-EDTA</td>
<td>2 fmol</td>
<td>54</td>
</tr>
<tr>
<td>6 DNA fragment</td>
<td>AP</td>
<td>salicylaldehyde</td>
<td>SA-Tb$^{3+}$-EDTA</td>
<td>4 pg</td>
<td>57</td>
</tr>
<tr>
<td>7 α-Fetoprotein (AFP)</td>
<td>AP</td>
<td>5-fluoresalicyl phosphate (FSAP)</td>
<td>FSA-Tb$^{3+}$-EDTA</td>
<td>0.15 pg/mL</td>
<td>58</td>
</tr>
<tr>
<td>8 α-Fetoprotein (AFP)</td>
<td>AP</td>
<td>phosphoester of diflunisal (DIFP)</td>
<td>DIF-Tb$^{3+}$-EDTA</td>
<td>5 pg/mL</td>
<td>59</td>
</tr>
<tr>
<td>9 Horseradish peroxidase (HRP)</td>
<td>HRP</td>
<td>4-hydroxyphenylpropionic acid (pHPPA)</td>
<td>pHPPA (dimer)-Tb$^{3+}$-EDTA +</td>
<td>2 $\times 10^{-12}$ mol / L</td>
<td>60</td>
</tr>
<tr>
<td>10 IgG</td>
<td>POx</td>
<td>4-hydroxyphenylpropionic acid (pHPPA)</td>
<td>pHPPA (dimer)-Tb$^{3+}$-EDTA +</td>
<td>3 µg/L</td>
<td>61</td>
</tr>
<tr>
<td>11 Porcine liver esterase</td>
<td>esterase</td>
<td>acetic acid eater of bis(2-pyrididmethyl)-(2-hydroxybenzyl)amine</td>
<td>bis(2-pyrididmethyl)-(2-hydroxybenzyl)amine-Tb$^{3+}$</td>
<td>$3 \times 10^{-9}$ M</td>
<td>62</td>
</tr>
<tr>
<td>12 DNA fragment (PCR)</td>
<td>AP</td>
<td>FSAP</td>
<td>FSA-Tb$^{3+}$-EDTA</td>
<td>$1 \times 10^{3}$-2$\times 10^{5}$ molecules</td>
<td>63</td>
</tr>
<tr>
<td>13 pBR322 plasmid DNA (dot-blot DNA hybridization)</td>
<td>AP</td>
<td>alkyl and aryl-substituted salicyl phosphates</td>
<td>xSA-Tb$^{3+}$-EDTA</td>
<td>125 pg</td>
<td>64</td>
</tr>
<tr>
<td>14 Interleukin 6</td>
<td>AP</td>
<td>DIFP</td>
<td>DIF-Tb$^{3+}$-EDTA</td>
<td>0.15 ng/L</td>
<td>65</td>
</tr>
<tr>
<td>15 Tumor necrosis factor- α (TNF- α)</td>
<td>AP</td>
<td>DIFP</td>
<td>DIF-Tb$^{3+}$-EDTA</td>
<td>0.2 ng/L</td>
<td>66</td>
</tr>
<tr>
<td>16 Glucose oxidase (GOx)</td>
<td>GOx</td>
<td>glucose</td>
<td>Tc-Eu$^{3+}$-HP</td>
<td>0.32 mUnits/mL</td>
<td>67</td>
</tr>
<tr>
<td>17 Catalase</td>
<td>catalase</td>
<td>tetracycline-Eu(III)-hydrogen peroxide</td>
<td>Tc-Eu$^{3+}$-HP → Tc-Eu(III)</td>
<td>0.046 Units /mL</td>
<td>68</td>
</tr>
</tbody>
</table>
From these discussions, some characterizations of these approaches can be concluded: 
(a) lanthanide chelate as labels are used in DLCLLA and in DELFIA, but requirements are different. Label in DLCLLA must have intense fluorescence, while that in DELFIA must have no (or weak) fluorescence. However, the lanthanide chelate in EALL is a probe, in which the label is an enzyme. (b) DLCLLA in principle and protocol is simple, some cases have even higher sensitivity than DELFIA. But its labeling chelates need much stronger fluorescence intensity and stability, thereby its applications are restricted. (c) DELFIA is a highly sensitive scheme as its processes of molecule recognition and enhance fluorescence are separated. However, this feature also prevents its application in cytofluorometry, in-situ immunostaining, etc although it has enough widely application area. (d) The good perspective of EALL may be conceivable as enzymes correspond to numerous substrates and products, and it can be connected to other technologies to improve its applications, but now the available substrates and lanthanide chelates are rather limited and need to further explored.

1.3. Aim of Research

The goal of this dissertation is to develop ternary europium-derived (different stoichiometry) fluorescent probe for bioassays in aqueous solution. There are three main aspects: the first is based on the europium-tetracycline-hydrogen peroxide (EuTc-HP) probe, which can be converted into europium-tetracycline (EuTc) by peroxidase (POx), so that the activity of POx and POx labeled biomolecules will be possible determined; The second is based on the changes of fluorescence of EuTc probe when ligated to hydroxy acid (or oxyanions). Citrate and the main intermediates in the Krebs cycle can be determined and imaged without enzymes or multi-enzyme systems. The third relates to fluorescent discrimination of enantiomeric malates in aqueous solution.
Different fluorescence technologies, namely conventional steady-state and time-resolved (gated) fluorescence detection, conventional and time-resolved (gated and rapid lifetime detection) imaging, are being applied.

1.4. References


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Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe

2.1. Introduction

Peroxidases are widely studied [1-3] across a range of scientific disciplines, and applied [4-6] in bioanalytical protocols, in chemical and biomedical research, and in the food and pharmaceutical industries in general. They are known for being sensitive to ultra-low concentrations of substrates and tolerant to relatively harsh conditions, and therefore probably among the most preferred enzyme labels in enzyme-linked immunosorbent assay (ELISA) [7], nucleic acid assay [8], high-throughput screening (HTS) [9], and histochemical staining imaging [10].

Quite a variety of methods is known for the determination of the activity of peroxidase (POx). Practically all are based on the fact that POx reacts with hydrogen peroxide and a second substrate. Numerous such second substrates [11, 12] (often referred to as hydrogen donors) are suitable for chromogenic, fluorogenic or other signal-generating purposes. As a result, kinetic assays have been developed based on spectrophotometry / reflectometry [13, 14], fluorometry [15, 16], chemiluminescence [17-19], electrochemiluminescence [20] and electroanalysis [21-23]. Among the fluorometric methods, those based on time-resolution [24] are the most sensitive tools in biological assays. Surprisingly, there is only one report [25] on the application of time-resolved lanthanide luminescence (using Tb$^{3+}$ ion) now, although it is a particularly attractive scheme for the determination of the activity of POx.

A new and easily accessible fluorescent probe, the europium-tetracycline-hydrogen peroxide (EuTc-HP) complex, is presented here for the time-resolved detection of the activity of peroxidase. It is based on the findings that (a) H$_2$O$_2$ forms a strongly fluorescent system
with the europium-tetracycline complex (EuTc) [26, 27], (b) this system (EuTc-HP) is
decomposed by POx and reversed to the weakly fluorescent EuTc, and (c) the average
lifetime of EuTc-HP (~60 µs) is about two times that of EuTc (~30 µs), which offers the
possibility for a time-resolved assay of POx. The results presented here demonstrate that
EuTc-HP can be used for a new type of fluorometric assay for the activity of POx.

2.2. Results and Discussion

2.2.1. Principle of POx Assay

2.2.1.1. Structure and Reaction

Mechanism of POx

Among of POx, horseradish
peroxidase (HRP) [1, 2] is the most
comprehensively studied. Its
structure [1, 2, 28] is shown in Fig.
2.1. HRP is specifically discussed in
this dissertation.

The processes [1, 29, 30]
through which hydrogen peroxide
oxidizes the second substrate
catalytically by HPR, compose of multi-step reactions in Fig. 2.2. In the figure, compound-I
and compound-II are enzyme intermediates, and AH\textsubscript{2} is the second substrate (hydrogen
donor). •AH is a radical product which has several possible fates, depending upon its

![Fig.2.1. HRP structure. The main components are the heme (blue), the essential calcium ions (red), and the three key aminoacid residues: the proximal (below the heme) and distal (above the heme) histidines (yellow) and the distal pocket arginine (cyan). (From: Radu L. Silaghi-Dumitrescu, ref.[12])]
chemistry and environment of the reaction, to become a dimer, to react with another substrate molecule, or to attack another species causing cooxidation.

Besides the Fe$^{3+}$ in the centre of heme, the functional role of the key residues in the active site of HRP are very important in the processes of catalysis [1, 2, 31, 32]. The three amino acids, histidine 170 (H170), histidine 42 (H42) and arginine 38 (R38), synergistic act for the decomposition of H$_2$O$_2$.

The kinetic mechanism scheme in Fig. 2.2 is generally considered as the following: (1) From HRP to compound-I is a complicated process [33, 34]. H$_2$O$_2$ forms the iron-peroxide bond via H170, then H42, R38 and H170 together promote heterolytic cleavage of the O-O bond leading to formation of the ferryl group, Fe=O, and formation of water which is a leaving group. (2) Second substrate joins the process of transferring compound-I to
compound-II. Electron transfer occurs to the porphyrin ring and the π-cation radical disappears [35, 36]. Proton transfer occurs to H42. (3) In the process of compound-II reaction to native HRP [37, 38], both proton and electron transfer occur to the ferryl group with second substrate, simultaneously reducing Fe(IV) to Fe(III) and forming water.

The step of reduction of compound-II to native HRP is often rate-limiting in the POx catalytic cycle [39]. Sometimes the compound-I formation can possibly become the rate-controlling step by limiting concentration of H₂O₂ and a large excess of second substrate, but the production of compound-II is never the rate-limiting step. Obviously, the different concentration and kind of substrates can be reflected in the change of velocity of catalytic reaction, which usually governs the activity of enzyme.

2.2.1.2. Detection Scheme for POx

EuTc is a fluorescent probe for hydrogen peroxide (H₂O₂) [26]. It is based on the finding that the complex formed between Eu³⁺ and tetracycline undergoes a large increase in fluorescent intensity on exposure to H₂O₂. The fluorescent complex formed between EuTc and H₂O₂ (referred to as EuTc-HP) can be reversibly decomposed by peroxidases to EuTc.

![Figure 2.3. Schematic diagram of the principle underlying the POx activity assay. EuTc and hydrogen peroxide form a strongly fluorescent complex that is decomposed by POx on addition of the second substrate phenol.](image)

23
Hence, the EuTc-HP reagent, which is easy to prepare, is highly promising for the kinetic assay of peroxidases, their substrates, activators, and inhibitors. The principle of the assay is shown in Fig. 2.3.

Phenol was used as the second substrate in this assay of POx because it is easily available, stable, does not absorb at the excitation wavelength for EuTc-HP (~400 nm), and does not give fluorescent products. In a system composed of EuTc-HP, POx, and phenol, the activity of peroxidase is proportional to the reaction rate, i.e. \( \Delta F/\text{min} \), where \( \Delta F \) is the difference between the initial fluorescence intensity (\( F_0 \)) and the final fluorescence intensity (\( F \)), ideally both corrected for a (conceivable) blank. Unless excited with light (of \( \lambda \) 350 – 440 nm), no light is emitted, thus excluding the possibility of chemiluminescence.

### 2.2.2. Spectral Characterizations

The excitation and emission spectra of EuTc-HP and EuTc are given in Fig. 2.4. Like in other lanthanide complexes, the photonic energy absorbed by the ligand (Tc) in the EuTc complex is transferred to the central Eu\(^{3+}\) ion with its typical emission [40-42] in the form of main line (\( ^5D_0 \rightarrow ^7F_2 \)) peaking at 613 and 618 nm (two peaks) and several side bands (Fig. 2.4). The appearance of two main peaks is a clear indication of the change of the crystal field [41] around Eu\(^{3+}\). From the findings presented so far the proposal is that \( \text{H}_2\text{O}_2 \), on addition to EuTc, replaces at least one water molecule ligated to Eu\(^{3+}\). However, no significant redox reactions are involved. Rather, water ligand (acting as a strong quencher) is replaced by \( \text{H}_2\text{O}_2 \) ligands. On addition of \( \text{H}_2\text{O}_2 \) and subsequent formation of the EuTc-HP complex, the intensity of the emission increases by a factor of about 15. On the other hand, the spectra of EuTc-HP do not significantly change on addition of phenol alone. In order to obtain the optimum fluorescence intensity of \( \text{H}_2\text{O}_2 \) in EuTc, the molar ratio of Eu\(^{3+}:\text{Tc} \) is kept at 3:1.
Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe

Figure 2.4. Fluorescence excitation (left) and emission spectra (right) of EuTc and the EuTc-HP complex in MOPS buffer of pH 6.9. 400 µL of EuTc stock solution, 160 µL of 5 mM H₂O₂ and 136 µL of 49 mM phenol, with MOPS to total volume 2 mL. (A), EuTc plus excesss HP; (B), EuTc-HP plus phenol; (C), EuTc.

Figure 2.5. Effect of the concentration of H₂O₂ on the fluorescence decay profile of EuTc-HP. From (A) to (E), the concentrations of H₂O₂ decrease from 300 to 60, 30, 12 and 0 µM, respectively. All samples contain 500 µL of EuTc stock solution in a total volume of 2 mL.
Fig. 2.5. shows the fluorescence decay profiles of EuTc on addition of increasing concentrations of H₂O₂ from curve (E) to curve (A). EuTc and EuTc-HP have rather different decay patterns and decay times. An analysis of the data in Fig. 2.5 has indicated [26] that the decay profile of EuTc-HP can be fitted to a three-component model. The respective decay times are 10 µs (relative amplitude 17%), 34 µs (18%) and 61 µs (65%). The average decay time is ~60 µs. EuTc also has three components as 7 µs (40%), 24 µs (54%) and 53 µs (6%), with its average decay time ~30 µs only. From these results it is obvious that time-resolved measurements are best performed with a lag time of ~ 60 µs in order to selectively detect the EuTc-HP complex and minimize interference from EuTc, proteins and plates.

2.2.3. Kinetic Studies

The activity of POx is directly related to the change in the fluorescence intensity of the EuTc-HP system as shown in Fig. 2.6. In the absence of POx (curve A) only small changes
are observed; these are ascribed to effects of temperature. As the activities of POx increase from (B) to (F), the slope increases, and this can be used to determine its activity. Curve (G) is a time trace of the system to which no H\textsubscript{2}O\textsubscript{2} and no POx have been added (i. e. that of plain EuTc). It is worth noting that in the assay described here, fluorescence does not drop to zero but only from the level of the fluorescence of EuTc-HP to that of EuTc. Once formed, EuTc is not affected by POx. Incubation at elevated temperature accelerates the reaction, so that fluorescence intensity changes more rapidly and strongly. Therefore, lower activities of POx can be detected. The dynamic range of the determination can also be adjusted by the incubation time.

![Figure 2.7. The influence of substrate-phenol to the catalytic cycle of POx. 50 µL of EuTc stock solution in each well (total volum 250 µL). Concentrations of H\textsubscript{2}O\textsubscript{2} and phenol are 0.5 and 4 mM, the activity of POx is 0.012 U/mL.](image)

It needs to be emphasized that POx as a protein itself does not induce the decrease of fluorescence intensity, and that there is no change of the fluorescence of EuTc-HP if only POx is added, without phenol as indicated in Fig. 2.7. Additionally, if POx is denatured by
heating, none of the kinetic effects were observed that are associated with the presence of active POx.

2.2.4. Effect of Substrates

\( \text{H}_2\text{O}_2 \) is not only a substrate of peroxidase, but also an enhancer of the fluorescence of EuTc. Fluorescence reaches a maximum on increasing the concentration of \( \text{H}_2\text{O}_2 \) from zero to 1.5 mM. The increase in fluorescence also depends on the concentration of EuTc. The optimal condition is obtained when a solution containing 20 µL of 5 mM \( \text{H}_2\text{O}_2 \) and 50 µL of the EuTc stock solution in a total of 250 µL is applied for POx activity assay.

As a result of the reaction mechanism of POx discussed in section 2.2.1.1, \( \text{H}_2\text{O}_2 \) is catalytically decomposed by POx, and the two phenoxy radicals formed undergo dimerization (and possibly other reactions). From a mechanistic point of view it is important to keep in mind that such assays work best if the concentration of the substrate (phenol) is much higher than that of hydrogen peroxide, since only in this case the activity of POx can be determined via the consumption of \( \text{H}_2\text{O}_2 \). No significant change in fluorescence was observed provided the concentration of phenol was \( \leq 3.5 \text{ mM} \) for the EuTc-HP system (0.4 mM \( \text{H}_2\text{O}_2 \)).

2.2.5. Optimization of the POx Assay

Any changes in pH will produce two effects on the system. The first is on tetracycline which has several dissociable groups that also may bind europium ion [44]. Both the absorption and emission spectra of tetracycline are highly sensitive to pH. The maximum enhancement in the fluorescence intensity on addition of \( \text{H}_2\text{O}_2 \) occurs at pH 6.9 (6.7 – 7.2 are acceptable). The second effect is that on the enzyme, even though POx is rather robust and maintains its activity over the pH 5 – 10 range, albeit with varying activity [45]. A pH of 6.9
was chosen for further experiments since it results in a fairly strong fluorescence of EuTc-HP at acceptable enzyme activity.

The buffers MOPS, HEPES, Tris, and phosphate were tested in the assay. It shows that MOPS buffer is the best, while HEPES has a slight quenching effect. Phosphate interferes most strongly since 12 µM of phosphate cause a quenching by 21% of the fluorescence intensity of EuTc-HP. Tris buffer does not significantly affect. However, its best buffer capacity is between pH 7.5 and 9.0, which is outside our preferred pH range. Therefore, a 10 mM MOPS buffer of pH 6.9 was used throughout the experiments.

Even though the temperature optimum for POx is reported [45] to lie between 40 and 50 °C, the experiment was performed at 30 °C since this already gave adequate kinetics. Conceivably, higher temperatures may be applied in certain cases or if shorter reaction times are desired.

The interferences by common cations and anions were studied and no significant effects were found [26] except for phosphate and citrate. Known interferants [42, 45] for the EuTc-HP system, such as ascorbic acid, uric acid, and bilirubin if present in concentrations of > 6, 40, 7, and 16 µM, respectively.

2.2.6. Steady-state Fluorescence Intensity Assay

For the conventional fluorescent intensity assay, 50 µL of EuTc stock solution in each well (total volume 250 µL), the dynamic range is between $8.5 \times 10^{-5}$ and $4.5 \times 10^{-2}$ Units/mL, with the limit of detection $7.0 \times 10^{-5}$ Units/mL after incubating for 10 min. Such a low LOD suggests that this probe be used for enzyme-linked immunosorbent assays using POx as a label.
2.2.7. Time-resolved Fluorescence Assay

The principle of time-resolved fluorescence has been demonstrated in Fig. 1.2. Due to the long decay times of europium complexes, they are often used in time-resolved fluorescent assays which can effectively eliminate background fluorescence, such as the intrinsic short-lived fluorescence of proteins and microtiter plates. In the assay presented here, it also can largely reduce the fluorescence of EuTc. From the decay profile and the experimental optimization, a lag time of 60 µs and an integration time of 40 µs were found to be the most appropriate for the POx assay. No significant improvement is found when the integration time is increased from 40 to 100 µs. For the determination of high activities of POx, a short incubation time is adequate as can be seen from Fig. 2.8. For the gated assay, the dynamic range is from $4.0 \times 10^{-5}$ to $5.9 \times 10^{-3}$ Units/mL, with a limit of detection (S/N = 3) of $1.0 \times 10^{-5}$ Units/mL.

![Figure 2.8. Calibration curve of the activity of POx by time-resolved fluorescence determination. 50 µL of EuTc stock solution in each well (total volum 250 µL)](image)

Figure 2.8. Calibration curve of the activity of POx by time-resolved fluorescence determination. 50 µL of EuTc stock solution in each well (total volum 250 µL)
2.2.8. Inhibitors of POx

Cyanide is a strong but reversible inhibitor of POx [1]. It binds to the sixth coordination site of the ferric ion heme complex of POx which also binds H₂O₂. Thus, it retards or prevents the catalytic cycle [46, 47]. In order to demonstrate inhibition, the kinetics due to inhibition of POx as monitored by the EuTc-HP system is shown in Fig. 2.9. Curve (A) shows a time trace where cyanide was added to the EuTc-HP/phenol system, but in absence of POx. Fluorescence remains unaffected, and this is also true for cyanide concentrations of less than 0.44 mM. Curve (E) shows an uninhibited kinetics, while curves (B), (C), and (D) demonstrate that POx is inhibited if cyanide is present in concentrations of 2.2 µM or higher. This indicates that (a) the rate of the decomposition of H₂O₂ by POx decreases with increasing concentrations of cyanide; and (b) the probe may also be used to determination the concentration of inhibitors cyanide and to monitor the change of activity of POx.

Figure 2.9. Effect of cyanide on the activity of POx. Each well (250 µL total volum) includes 22 µl of EuTc, 20 µl of 5 mM HP and 17 µl of 49 mM phenol. (A), cyanide added to the system, but no POx; (B) – (D), in presence of 0.2 Units/mL of POx plus cyanide in final concentrations of 440, 67, or 2.2 µM, respectively; (E), plus 0.2 Units/mL of POx added, but no cyanide.
Other known inhibitors of POx include sodium azide, whose effect on the EuTc-HP system (both in presence and absence of POx) is the same as that of cyanide. Hydroxylamine, in contrast, exerts a quenching effect on fluorescence of the EuTc-HP even in the absence of POx. Consequently, azide (but not hydroxylamine) can be also assayed via its inhibitory effect on POx.

**2.2.9. Comparison with Known Fluorescent Methods for POx**

The main fluorescence detections for the activity of POx are summarized in Table 2.1. Since H₂O₂ is not directly detectable by optical signals (which is contrast to electrochemical detection), substantial efforts have been made to identify chromogenic, fluorogenic and chemiluminogenic second substrates. They express the relationship between the second substrate and the activity of POx. Among the fluorogenic substrates in Table 2.1, homovanillic acid [11], p-hydroxyphenylacetic acid [15], p-hydroxyphenyl-propionic acid [16], Amplex Red [48] are widely used. Recently, new substrates such as 4-(N-methylhydrazino)-7-nitro-1,2,3-benzooxadiazole [49] and 10-methylacridan-9-carboxyhydrazide [50] have also been developed. None of them have been explored for the time-resolved determination of POx. However, Meyer and Karst [25] have presented an enzyme amplified lanthanide luminescence (EALL) method. It utilizes the ternary complex formed between Tb³⁺, EDTA and the oxidation product of ρ-hydroxyphenylpropionic acid which has a decay time of about 100 µs, thus enabling the gated determination and working best in presence of an enhancer such as CsCl.

On the other hand, the direct determination of H₂O₂, rather than the optimization of the second substrate for POx, has been not fully explored yet. Theoretically, direct H₂O₂ detection, which is independent of possible effects of the sometimes complicated second
Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe

substrate on POx, offers more advantages than the above-mentioned methods if sensitivity is comparable. Certain metal ion complexes also have been used for the determination of H₂O₂ [51, 52] for example titanium complexes. While these methods enable the assay of H₂O₂ by photometry, they do not enable fluorescent or gated assays since the probes are not fluorescent. Conceivably, they also may be used for POx assay, but this has not been demonstrated yet.

Compared to the above methods, the POx assay presented here is first direct H₂O₂ – based time-resolved fluorometric assay with comparable sensitivity. It does not require the addition of other enhancers, works best at neutral pH and can be applied to both intensity-based and time-resolved determination of the enzyme.

2.3. Conclusion

The EuTc-HP probe presented here represents the first lanthanide probe suitable for POx determination via H₂O₂ at neutral pH. It enables a convenient and sensitive assay for POx, is easily accessible, and has the typical virtues of a europium probe including large Stokes shifts (thereby reducing background luminescence) and µs decay times. It demonstrates, as well as in follow works, the suitability of EuTc-HP as a probe for POx-associated ELISAs, nucleic acid hybridization assays, and in other bioassays.
<table>
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<tr>
<th>Name</th>
<th>Structure</th>
<th>ex/em (nm)</th>
<th>LOD (reported)</th>
<th>range (reported)</th>
<th>ref.</th>
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<td>1</td>
<td>Homovanillic acid (HVA)</td>
<td><img src="image" alt="HVA structure" /></td>
<td>315/425</td>
<td>1 mU</td>
<td>1-10 mU</td>
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<tr>
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<td>326/410</td>
<td>500 µU</td>
<td>500 µU-10 mU</td>
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<tr>
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<td>3-((p-hydroxyphenyl) propionic acid (HPPA)</td>
<td><img src="image" alt="HPPA structure" /></td>
<td>320/404</td>
<td>7.8 µU</td>
<td>7.8 µU-1 mU</td>
</tr>
<tr>
<td>4</td>
<td>p-hydroxyphenethyl alcohol (p-tyrosol)</td>
<td><img src="image" alt="p-Tyrosol structure" /></td>
<td>320/404</td>
<td>15.6 µU</td>
<td>15.6 µU-1 mU</td>
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<td>N,N'-Dicyamomethyl-o-phenylenediamine (DCM-OPA)</td>
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<tr>
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<td>10-methylacridan-9-carboxylatehydrazide</td>
<td><img src="image" alt="10-Methylacridan-9-carboxylatehydrazide structure" /></td>
<td>357/510</td>
<td></td>
<td>4.6 x 10^{-14} M</td>
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<tr>
<td>7</td>
<td>o-phenylenediamine</td>
<td><img src="image" alt="o-Phenylenediamine structure" /></td>
<td>428/560</td>
<td></td>
<td>0.56 µU/mL (2mL)</td>
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<tr>
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<td>10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red)</td>
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<td>0.0-2 mU/mL</td>
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<td>9</td>
<td>4-(N-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH)</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>470/547</td>
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<td>determination glucose with POx</td>
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<td>tetra-substituted amino aluminium phthalocyanine (TAAIPc)</td>
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<td>5.9 x 10^{-13} mol·L^{-1}</td>
<td>0.0-3.94 x 10^{-11} mol·L^{-1}</td>
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<tr>
<td>11</td>
<td>oxidation of pHPPA-Tb(III)-EDTA, CsCl</td>
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<td>2 x 10^{-12} M</td>
<td>2·10^{-12} – 1.0 x 10^{-8} M</td>
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<tr>
<td>12</td>
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<td></td>
<td>390-410/618</td>
<td>10 µU/mL</td>
<td>14µU/mL - 2 mU/mL</td>
</tr>
</tbody>
</table>
2.4. Experimental Section

2.4.1. Reagents

Peroxidase (EC 1.11.1.7., type I, from horseradish, 148 U/mg solid) was purchased from Sigma (Deisenhofen, Germany; www.sigmaaldrich.com). The activity unit used in this article is based on the one defined by Sigma: one unit will form 1.0 mg purpurrogallin from pyrogallol in 20 s at pH 6.0 at 20 °C. All inorganic salts were obtained in analytical purity from Merck (Darmstadt, Germany; www.vwr.com) unless otherwise stated. All solutions were prepared in 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer of pH 6.9 (Roth, Karlsruhe, Germany, www.carl-roth.de) unless otherwise specified. High-purity hydrogen peroxide (H₂O₂) as a 30% solution was from Merck. Europium(III) trichloride hexahydrate was from Alfa Products (Danvers, USA; www.alfa.com), tetracycline hydrochloride from Serva (Heidelberg, Germany; www.serva.de). Tris- (hydroxymethyl) aminomethane (TRIS) and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were from Sigma-Aldrich.

The stock EuTc solution was prepared by mixing of 10 mL of 6.3 mM Eu³⁺ solution with 10 mL of a 2.1 mM tetracycline solution, then diluting to 100 mL with MOPS buffer. This reagent is also available from Chromeon GmbH (Regensburg, Germany; www.chromeon.com) and may be diluted to the concentration required. A 5 mM solution of hydrogen peroxide was prepared fresh daily. The 0.49 M phenol solution was stored at 4 °C and diluted as required.
2.4.2. Apparatus

Absorption spectra were acquired on a Cary WinUV photometer (Varian, Australia, www.varian.com). Fluorescence studies of the effect of $\text{H}_2\text{O}_2$ and phenol on spectra the EuTc and EuTc-HP were performed on an SLM AB2 luminescence spectrometer (Spectronic Unicam; Rochester, New York, USA; www.thermospectronic.com). Fluorescence intensity and kinetics were acquired on either a Fluoroskan Ascent micro titer plate reader (from Thermo Labsystems, Helsinki, Finland; www.labsystems.com) or on a Tecan GENios+ micro plate reader (Tecan, Groedig, Austria, www.tecan.com). The excitation/emission filters were set to 405/620 nm, or 405/612 nm respectively. The 96-well micro titer plates were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany; www.greiner-lab.com).

The luminescence lifetimes of different concentration of $\text{H}_2\text{O}_2$ in EuTc were detected with a pulsed 392-nm laser (LDH-C-400, PicoQuant GmbH, Berlin, Germany; www.picoquant.de), and an H5783-P04 PMT detector (Hamamatsu) with multiphoton-counting board in a multipass cuvette. Data were processed wither by FluoFit (PicoQuant GmbH).

2.4.3. Recommended POx Assay Protocol

Place, in each well of a thermostatted (30 °C) 96-well micro titer plate, 50 µL of EuTc stock solution, 20 µL of 5 mM $\text{H}_2\text{O}_2$ solution, 17 µL of 49 mM phenol solution, and with MOPS buffer to make up to a final volume of 250 µL. After 10 min, POx solutions of activities ranging from $4.0 \times 10^{-5}$ to $4.5 \times 10^{-2}$ Units/mL are added, and the decrease in the time-resolved fluorescence intensity is recorded on the Tecan GENios + reader over typically 5 to 60 min, depending on activity of POx, with a lag time of 60 µs and the integration time of 40 µs. Conventional steady-state fluorescence was detected with the same samples and micro plates as described above.
Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe

2.5. References


Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe


[41] Richardson, F. S. Terbium(III) and europium(III) ions as luminescent probes and stains for biomolecular systems. Chemistry Review 1982, 82, 541-552.


Chapter 2. Determination of the Activity of Peroxidase via the EuTc-HP Probe


3.1. Introduction

Immunoassays and DNA hybridization assays as two main members of broad category of bioassays have been widely utilized in biochemical research [1-5], clinical diagnosis [6-8] and pharmaceutical industries [9,10]. With the development of the electronic and analytical technologies, immunoassays and DNA hybridization assays have been combined to improve \textit{de novo} chip technologies [11-13], including the protein chip [14, 15] arrays.

There are different analytical schemes in immunoassays, such as radioimmunoassay (RIA) [16], spectrophotometric immunoassay, fluorescence immunoassay (FIA) [17], chemiluminescence immunoassay (CLIA) [18] and electrochemical immunoassay (ECIA) [19]. RIA is the most sensitive method in all biological and medical assays, but its isotopic hazard potential have recently limited its wide application. The photoluminescence approaches are main stream for bioassays because they are more sensitive than ECIA. These technologies are also used in DNA hybridization assays [20-22].

Fluorescence imaging is a powerful means for presenting the distribution of species, but is possible only (a) if the species of interest has a fluorescence by its own (such as NADH, many flavins and porphyrins) [23, 24], (b) if the species of interest can be rendered fluorescent by attaching a label (for instance, in immunofluorescence and DNA fluorescence studies) [25, 26] or (c) if appropriate fluorescent probes are available for the species of interest (e.g. probes for pH, oxygen, ions, as in this case hydrogen peroxide) [27-29].
In this chapter, POx as a label has been further explored in IgG and oligonucleotide detection, based on the measurement of the activity of POx studied in chapter 2. The fluorescence of EuTc-HP decreases if POx catalyzes the consumption of H$_2$O$_2$. The time-resolved fluorescent assay for biological specimen has more advantages than the conventional steady-state fluorescence assay because of its ability to eliminate the background fluorescence of proteins and biological matrix. On the other hand, this probe can be used as a reversible molecular sensor for the imaging of POx-ELISA.

3.2. Results and Discussion

3.2.1. Principle of Fluorescence Detection of POx-ELISA

IgG, as a model, is employed for POx-ELISA with EuTc-HP. Scheme of principle of POx as a label for sandwich ELISA has been shown in Fig. 3.1. Anti-IgG is first coated onto...
Chapter 3. Peroxidase as a Label for ELISA and Oligonucleotide Hybridization Assay

the polystyrene surface of microtiter plate, different concentrations of analyte IgG react with it after BSA has blocked the extraneous binding sites. EuTc-HP is added when the secondary POx labeled anti-IgG has recognized IgG, then after incubating, IgG can be determined via the fluorescence decrease of EuTc-HP because H₂O₂ is catalytically decomposed by POx.

POx as a label for direct ELISA is shown in Fig. 3.2. Instead of anti-IgG, analyte IgG is directly coated on the microtiter plate. Other processes are the same as Fig. 3.1.

Detail protocols of sandwich POx-ELISA and direct POx-ELISA are in experimental Section 3.4.3.

3.2.2. Kinetic Studies of Sandwich POx-ELISA

Fig. 3.3. presents the fluorescence change of the decomposition of EuTc-HP probe by IgG versus POx labeled anti-IgG. Curve(A) is a time trace for blank, which coated anti-IgG same as other wells without target molecular IgG. (B) to (D) are curves of kinetic process of the concentrations of IgG versus the activity of POx-labeled anti-IgG. With the increasing amount of POx-anti-IgG, from (B) to (D), the slope of curve is increasing, which can be used to indicate the activity of POx. Therefore, the concentration of IgG can be determined by this method. The kinetic curve of the direct POx-ELISA is similar as this case.
3.2.3. POx – ELISA for IgG via the EuTc-HP Probe

3.2.3.1. Steady-state fluorescence POx – ELISA

The activity of peroxidase labeled on anti-IgG can be measured with the EuTc-HP fluorescence probe. The calibration curves (after incubating for 20 min, expressed as ΔF = (F₀ - F) of fluorescence) are shown in Fig. 3.4. It indicates that (a) for the sandwich POx-ELISA, its limit of detection is 0.1 ng/mL, and the linear range is between 0.3 - 6 ng/ml, r = 0.98; (b) for the direct POx-ELISA, its limit of detection is 2 ng/mL, and the linear range is between 10 - 2000 ng/ml, r = 0.99; (c) the sandwich POx-ELISA with EuTc-HP fluorescent probe is more sensitive than the direct one.
3.2.3.2. Time-resolved fluorescence detection of POx-ELISA

One of the advantages of time-resolved fluorescence of ELISA is suppression of the background fluorescence, especially that from biological samples. This experiment is performed and recorded with a time lag of 60 µs after the pulsed excitation light source is switched off. The best integration time was found to be 40 µs. Fig. 3.5. shows the resulting normalized calibration plot for this linear range, which was obtained by plotting the normalized fluorescence \([F_0 - F] / F_0\) versus the concentration of IgG. The limit of detection of this time-resolved fluorometric sandwich POx-ELISA is calculated to be 0.1 ng/ml of IgG. The linear range is from 0.1 to 8.0 ng/ml. For direct POx-ELISA, the linear range is from 1.0 to 88.0 ng/mL, with 1.0 ng/mL of limit of detection. Obviously, the sandwich POx-ELISA is more sensitive than direct POx ELISA whether in conventional steady-state or in time-
resolved fluorescence detection. Both methods have enough good sensitivity and is suitable for the requirements of regular clinical assay.

![Graph](image)

*Figure 3.5. Calibration plot of time-resolved detection for IgG by POx-Anti-IgG versus the decrease in fluorescence intensity (F-F₀) over 20 min, with 60 µs lag time and 40 µs integration time. F are fluorescence intensities of blank and sample.*

### 3.2.3.3. Time-resolved fluorescence imaging ELISA (TRFI-ELISA)

The pathway of light of imaging [29] is indicated in Fig. 3.6. As in conventional fluorescence reader, all spots of the microtiter plate can be excited simultaneously with a pulsed 96-LED array (λ<sub>max</sub> = 405 nm). The light from the pulsable LED array passes an excitation filter and hits the wells of the microtiter plate. The emission from the fluorescent sample is filtered by an excitation filter and is detected.

![Diagram](image)

*Figure 3.6 Scheme of imaging*
by the CCD camera.

The camera is gated during the total exposure by an external trigger signal. The lag time and integration time are 60 and 40 µs after the LEDs were switched off (Fig. 3.7). The detailed protocol is described as in section 3.4.7.

![Scheme of time-resolved imaging](image)

**Figure 3.7. Scheme of time-resolved imaging**

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>$c_{IgG}$ [ng ml$^{-1}$]</td>
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<td>0.5</td>
<td>1</td>
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<td>10</td>
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</table>

**Figure 3.8. TRFI-ELISA for IgG.**

$F$ is the intensity value calculated from an area in the centers of the spots and averaged over three spots of equal concentrations. SD is the standard deviation in the spot regions.
Time-resolve fluorescence imaging-ELISA (TRFI-ELISA) for IgG was performed on a microtiter plate (protocol of sandwich POx-ELISA) after 45 min incubation of EuTc-HP probe at room temperature. The result is shown in Fig. 3.8 (false-color, value as arbitrary intensity). The dynamic range of this TRFI-ELISA is from 5 to 100 ng/mL for IgG. At high concentration of IgG, the complete decomposition of the EuTc-HP complex is readily observable. The time-gated fluorescence intensities from imaging decreased nearly to the values of the EuTc reference samples in spot column 1. The fluorescence intensities from imaging were calculated and averaged from the regions in the centers of the spots. The standard deviations between three different spots with the same IgG concentration are in the same magnitude (between 5-10 %).

The advantage of imaging ELISA is the fast data acquisition and the clear and concise data visualization, which makes this method suitable for high-throughput screening applications.

**3.2.4. Principle of Competitive POx-Oligonucleotide Hybridization Assay**

Competitive oligonucleotide hybridization is a very popular method in nucleic acid assays [30-31], which can improve the selectivity of detection. The scheme of competitive oligonucleotide hybridization is shown in Fig. 3.9. Anti-biotin is coated on the surface of microtiter plate, then affinitively reacts with oligonucleotide conjugated biotin after BSA has blocked the extraneous binding sites. Complementary oligonucleotides with or without POx are added to undergo competitive reaction, after adding EuTc-HP, incubating, the fluorescence changes can be recorded for calculating the concentration of oligonucleotide.
3.2.5. Fluorescence Detection of POx-Oligonucleotide Hybridization

In this experiment, the 20 base sequence (TA) from a fragment of SLT1 (Shiga-like toxins), as a model, is employed. The sequence of TA is 5'-AAG TAG TCA ACG AAT GGC GA-3'. Horseradish peroxidase labeled TA is denoted as POx-TA. The complementary oligonucleotide for TA is cTA, which is biotinylated to form BcTA. (see section 3.4.1.)

TA and POx-TA compete to hybridize with immobilized BcTA. The fluorescence change of the decomposition of EuTc-HP probe by POx-TA is used to evaluate the concentration of TA. In the low concentration of TA from 0-2.8 nM, a linear relationship with fluorescence change is obtained, but in high concentration of TA, the results are not so good. The possible reason is that TA is only a 20 base pairs nucleotide, while POx-TA has a
macromolecular protein label, thus competition of POx-TA at low concentration is weaker than that of TA.

![Graph showing calibration curve of competitive hybridization of TA and PTA.]

*Figure 3.10. Calibration curve of competitive hybridization of TA and PTA, total concentration*[TA] + [PTA] = 5 nM*

### 3.3. Conclusion

EuTc-HP as a typical europium probe for bioassay, it need not label or conjugate on protein or DNA. So it is easily prepare and procedure besides it has the merits of lanthanide complex fluorescence. The rate of consumption of the EuTc-HP system as monitored via the decrease in fluorescence intensity is a direct parameter for (a) the activity of the POx, (b) IgG versus POx-labeled-anti-IgG, and (c) oligonucleotide versus POx-oligonucleotide (competitive reaction). From above experiments, it is a evidence that the EuTc-HP can be utilized in bioassays via fluorescence intensity and imaging technologies.
3.4. Experimental Section

3.4.1. Reagents

Rabbit anti-bovine IgG, bovine IgG, rabbit anti-bovine IgG / peroxidase conjugate, BSA were purchased from Sigma (Deisenhofen, Germany; www.sigmaaldrich.com) and used without further purification. Sequence of TA from a fragment of SLT1 (Shiga-like toxins) in EHEC (Enterohemorrhagic E. coli) is 5’-AAG TAG TCA ACG AAT GGC GA-3’. There are no self-complementarity and no hairpin-loop in TA. POx labeled TA (POx-TA) is HRP-5’-AAG TAG TCA ACG AAT GGC GA-3’ and biotinylated complementary oligonucleotide for TA (BcTA) is Bio-5’-TCG CCA TTC GTT GA C TAC TT-3’. They were purchased from Thermo Hybaid (www.thermohybaid.com)

All inorganic salts were obtained in analytical purity from Merck unless otherwise stated. Europium(III) trichloride hexahydrate was from Alfa Products, tetracycline hydrochloride from Serva.

The stock EuTc solution was prepared by mixing of 10 mL of a 6.3 mM Eu³⁺ solution with 10 mL of a 2.1 mM tetracycline solution, then diluting to 100 mL with MOPS buffer. High-purity hydrogen peroxide (H₂O₂) as a 30% solution was from Merck. A 5 mM solution of hydrogen peroxide was prepared fresh daily. The 0.49 M phenol solution was stored at 4 °C and diluted as required.

PBS buffer (0.26 g KH₂PO₄, 2.17 g Na₂HPO₄·7H₂O and 8.71 g of NaCl in 800 mL distilled water, adjusted to pH 7.4 with 1.0 M HCl or NaOH) to the corresponding working concentrations, then dilute to 1000 mL. 20 x SSC buffer: dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL, adjusted to pH 7.0 with HCl or NaOH, then dilute to 1000 mL.
According to requirements, diluted to 1 x SSC (saline sodium citrate), 1 x SSC + 1% SDS (sodium dodecyl sulfate), 1 x SSC+1% triton-X-100.

3.4.2. Apparatus

Fluorescence intensity and kinetics were acquired on either a Fluoroskan Ascent microtiter plate reader (from Thermo Labsystems, Helsinki, Finland; www.labsystems.com) or on a Tecan GENios+ micro plate reader. The excitation/emission filters were set to 405/620 nm, or 405/612 nm respectively.

3.4.3. Protocol of POx-ELISA

The scheme of sandwich POx-ELISA is shown in Fig. 3.1.

1) Coating antibody on the microtiter plate: Use 200 µl of Rabbit anti-Bovine-IgG at a concentration of 5 µg/mL in 10mM of PBS at pH 7.4, add to each well of a polystyrene microtiter plate, incubate at 37 °C for 1 hour. Alternatively, incubate at 4 °C overnight.

2) Blocking extraneous binding sites: Block remaining binding sites in each well by incubating with 200 µl of 1% BSA in 10mM of PBS at pH 7.4, incubate at 37 °C for 30 min.

3) Reacting with analyte: Add 200 µl of different concentrations Bovine IgG to each well, incubate at 37 °C for 1 hour.

4) Reacting with POx labeled secondary antibody: Add 200µl of rabbit anti-bovine IgG peroxidase conjugate at 1:1500 in each well, incubated at 37°C for 1 hour.

Between above two times coating, rinse 3 times by 10 mM PBS at pH 7.4.
Last rinse before fluorescence assay, MOPS buffer was used to avoid residual PBS contaminating the EuTe-HP, and ready for detection (section 3.4.5)

For direct POx-ELISA, analyte IgG was coated onto the microtiter plate, other processes are same as sandwich POx-ELISA.

3.4.4. POx-Oligonucleotide Hybridization Assay

The scheme of POx-oligonucleotide hybridization assay is shown in Fig.3.9.

1). Coating antibody on the microtiter plate: Use 200 µl of Rabbit anti-biotin-IgG at a concentration of 5 µg/mL in 10 mM pH 7.4 of PBS, add to each well of a polystyrene microtiter plate, incubate at 37 °C for 1 hour. Alternatively, incubate at 4°C overnight.

2). Blocking extraneous binding sites: Block remaining binding sites in each well by incubating with 200 µl of 1% BSA in 10mM pH 7.4 of PBS, incubate at 37 °C for 30 min.

3). Affinity reaction with biotin-oligonucleotide: Add 200 µl of 5 nM BcTA in 10 mM pH 7.4 of PBS in each well, incubate at 37 °C for 1 hour.

Between above two times coating, need rinse 3 times by 10 mM pH 7.4 PBS.

4). Competitive hybridization: Mix different concentration of oligonucleotides of TA and POx-TA in hybridization solution (5 x SSC and 1% SDS ) in each well. Incubate for 10 min at 50 °C or 30 min at 45 °C with agitation.

5). Post-hybridization washes

(a) add 200 µl of preheated wash solution 1 (1 x SSC, 1% SDS) in each well, at 40 °C for 10 min, with shaking
(b) add 200 µl of wash solution 2 (1 x SSC, 1 % Triton X-100) in each well, at 40 ºC for 10 min, with shaking

(c) add 200 µl of preheated wash solution 3 (1 x SSC) in each well, at 40 ºC for 10 min, with shaking.

Last rinse before fluorescence assay, MOPS buffer was used to avoid residual SSC contaminating the EuTc-HP, and ready for detection (section 3.4.5)

### 3.4.5. Fluorescent Intensity Detection

Time-resolved fluorescence detection: 250 µL of EuTc-HP- phenol analytical solution, which including 50 µL of EuTc standard solution, 20 µL of 5 mM H2O2, 17 µL of 49 mM phenol and other 10 mM of MOPS in 6.9 pH, was added to each well of samples (immunoassay and oligonucleotide hybridization assay). The kinetic curve and the end – point of fluorescence intensity of EuTc-HP system after incubating 20-60 min was record, depending on POx-anti-IgG or POx-TA. Time-gated fluorescence was with a lag time of 60 µs and the integration time of 40 µs. Steady-state fluorescence was detected with the same samples and micro plates as described above without lag time.

### 3.4.6. Imaging Set-up

The set-up of the self-developed imaging system was used for time-resolved imaging of the emission intensity as described by Liebsch et al [29] and Schaeferling et al [32] in Fig. 3.11. Imaging detection system consists of a fast gatable CCD-camera, a pulsable LED array with 96 UV light emitting diodes (λ_max = 405 nm), a 96 fiber light-guiding adapter, a pulse generator, optical excitation and emission filters, and a personal computer for controlling and visualization of the experiments. The pulsed data were acquired process with the
corresponding software modules. The images were processed, visualized and evaluated with the IDL software module (Research Systems, Inc., Boulder, CO).

![Figure 3.11. Scheme of the imaging setup](image)

Optical system in imaging set-up:

1) CCD camera: The camera had a black/white CCD chip with 640x480 pixels (307200 pixels, VGA resolution) and a 12-bit resolution, equivalent to 4096 gray-scale values. The CCD chip can be gated directly with a minimal trigger time of 100 ns, additional image intensification is not required. and camera is triggered by a pulse generator.

2) LED array: The LEDs ($\lambda_{\text{max}} = 405 \text{ nm}$) were arrayed fitting exactly to wells of a 96-well microtiter plate for single illumination of each well by one LED, and LED array was pined to the electronic board, which enables a quick exchange by LED emitting of different wavelengths.
3) Light-guiding adapter: There are 96 optical fibers in light-guiding adapter for reduces the imaged area to fit the standard optics of the imaging set-up. This enables the simultaneous evaluation of 96-well at the same time.

3.4.7. Imaging

The camera is gated during the total exposure by an external trigger signal. The excitation pulse had a width of 90 µs, the lag time was fixed to 60 µs, with a time window from 150-190 µs after the LEDs were switched off. The resulting image is an added superimposition of this single pictures. The corresponding background images were recorded in a second acquisition cycle within the same time gates without prior excitation and finally subtracted from the emission signals. The whole imaging process for the read-out of a microtiter plate is accomplished, using three consecutive acquisition cycles for data evaluation and quantification.

3.5. References


Chapter 3. Peroxidase as a Label for ELISA and Oligonucleotide Hybridization Assay


Chapter 4. Fluorescence Determination and Imaging of Citrate

4.1. Introduction

Citrate (Fig. 4.1) is an ubiquitous natural compound that occurs in all living cells since it is an important intermediate in the Krebs cycle (also referred to as the citrate cycle or the tricarboxylic acid cycle) [1, 2], which is the central metabolic hub of the cell. Besides its function in cell metabolism, citrate is also widely studied for other fields. In environmental sciences, as a popular chelating agent that assists in the elimination of heavy metal ions through being uptaken and biotransformed by bacteria [3-6]. In medicine, citrate is used as an anticoagulant [7-9] to prevent blood clotting, and for the evaluation of urinary citrate excretion which in turn is related to kidney stones, renal tubular acidosis and certain bone diseases [10-12]. It is also widely utilized as an additive in the food and pharmaceutical industry [13, 14].

Citrate does not have significant physical and chemical properties suitable for direct determination in complex systems, and therefore is difficult to detect and visualize. There are many indirect methods have been exploited, such as by separation technique (such as high performance liquid chromatography [15-18] and capillary electrophoresis [19-21]), or by enzymes for transformation and synthetic receptors for recognition (Table 4.4).
In most of enzyme-based assays [22], two enzymes, citrate lyase (CL) and malic dehydrogenase (MDH), are usually employed and the decomposition of NADH is detected by absorbance at 355 nm or fluorescence at 445 nm. However, this method would have serious limitations including (a) the need for UV excitation at 355 nm (where almost all materials display fluorescence and background therefore would be strong); (b) the short decay time of NADH (3-5 ns) which hinders the application of time-resolved fluorescence assay and imaging with its unique advantages in terms of background suppression; and (c) the method needs reactions by enzyme catalytically and has rather complicated protocols. So far, however, no direct, intensity based or time-resolved fluorescence assays and imaging for citrate has been reported.

The fluorescence enhancement of citrate on EuTc has been unexpected observed during the studies of interference on EuTc-HP system. This offers a direct method for the fluorescent time-resolved detection of citrate without the involvement of enzymes. So here a europium derived fluorescent probe for the detection and visualization of citrate will be presented. The method is based on the finding that the weak fluorescent europium-tetracycline (EuTc), associates with citrate to form a strongly fluorescent europium-tetracycline-citrate (EuTc-Cit) complex in neutral pH. The features of lanthanide fluorescence of EuTc-Cit are suitable for the time-resolved determination and rapid life detection imaging.

4.2. Results and Discussion

4.2.1. Characterization of EuTc-Cit

4.2.1.1. Spectra of EuTc-Cit
The fluorescence properties of EuTc have been presented before [23, 24] including its application for detection and visualization of hydrogen peroxide [25, 26]. The absorption and fluorescence spectra of the EuTc-Cit system are shown in Fig. 4.2 and their characteristics in Table 4.1.

![Figure 4.2. Spectra of citrate (150 µM) in EuTc(50 µM of Eu³⁺ and Tc)](image)

**Figure 4.2. Spectra of citrate (150 µM) in EuTc(50 µM of Eu³⁺ and Tc)**

**Table 4.1. The characteristic parameters of in EuTc-Cit**

<table>
<thead>
<tr>
<th></th>
<th>EuTc</th>
<th>EuTc-Cit</th>
</tr>
</thead>
<tbody>
<tr>
<td>absorption peak (nm)</td>
<td>390</td>
<td>389 and 401</td>
</tr>
<tr>
<td>molar absorptivity ε (L mol⁻¹ cm⁻¹)</td>
<td>1.76 x 10⁴</td>
<td>2.05 x 10⁴</td>
</tr>
<tr>
<td>emission (nm)</td>
<td>616</td>
<td>615</td>
</tr>
<tr>
<td>average lifetime (µs)</td>
<td>44</td>
<td>83</td>
</tr>
<tr>
<td>QY (%)</td>
<td>0.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The maximal absorbance of EuTc-Cit are from 381 to 408 nm, similar to that of EuTc. In contrast, the intensity of the emission of EuTc-Cit at 615 nm is much stronger than that of
EuTc. This line-like emission is due to the $^5D_0\rightarrow^7F_2$ electronic transition which is typical for Eu$^{3+}$ fluorescence, with other side bands at 580, 590, 651, and 697 nm, respectively. The quantum yield (QY) of EuTc-Cit increases to 3.2 % from 0.4 % in case of EuTc, (as referring to tris (2,2'-bipyridyl) dichlororuthenium(II) hexahydrate) [27]. In EuTc-Cit complex system, citrate, in being a polydentate ligand, can chelate with Eu$^{3+}$ to form poly-ring via the oxygen atoms of carboxyl and hydroxyl groups [28-30]. It is assumed that citrate displaces water molecules, which ligate to the inner coordination field of Eu$^{3+}$ and quenching its fluorescence. Obviously, the energy transfer in EuTc-Cit is much more effective than that in EuTc.

4.2.1.2. Decay time of EuTc-Cit

The decay profile of EuTc-Cit can be fitted to a three-component model, with decay time components of 15 µs (relative amplitude 12 %), 58 µs (33 %) and 95 µs (55 %), respectively, with an average lifetime (important with respect to imaging) being 83 µs. For EuTc, the three-component model is composed of 8 µs (41 %), 24 µs (55 %) and 123 µs (4 %), respectively, with a average lifetime of 44 µs. The time-resolved fluorescence detection of citrate will benefit from these features of the decay times.

4.2.1.3. Composition of EuTc-Cit

The continuous-variations (or Job’s) method (Fig. 4.3) has been employed for the detection of molar ratio of Eu : Tc : Cit. In Fig. 4.3(a), while the concentration of citrate is excess, the mole fraction of Eu$^{3+}$ : Tc are continuously changed while keeping the total concentration at 25 µM. The peak of curve is at 0.5 of mole fraction of $[Eu^{3+}] / ([Eu^{3+}] + [Tc]$, namely molar ratio of Eu:Tc is 1:1. Changing total concentration to 37.5 and 50 µM, the same result is obtained. Similar case is shown in Fig. 4.3(b) with mole fraction of $[Tc] / ([Tc] + [Cit])$ being 0.33. Other total concentration of Tc and citrate, 37.5 and 50 µM, are also
employed in this experiment. The result indicates that the molar ratio of Tc: Citrate is 1:2. In combination of Fig. 4.3(a) and (b), the mole ratio of Eu:Tc:Cit is 1:1:2, namely EuTc(Cit)$_2$.

Figure 4.3. Continuous-variations method (Job’s plot)

It is noted that the molar ratio of Eu$^{3+}$ : Tc in EuTc-HP system (chapter 2 and 3) is 3:1. The 1:1 of molar ration of Eu$^{3+}$ : Tc will be used in EuTc - hydroxy acid system (chapter 4, 5, and 6).
Since the molar ratio of (EuTc):citrate is 1:2, there are two dissociation constants $K_{d1}$ and $K_{d2}$ for EuTc-Cit. The disassociation constants are roughly estimated by the Benesi-Hildebrand type equation for a two binding site saturation [31-33]: the $pK_{d1}$ and $pK_{d2}$ are in the range of 4.0 – 5.0.

4.2.1.4. Spectra Circular Dichroism

The circular dichroism (CD) spectra are mainly used for the characterization of chiral compounds and secondary structure of proteins or other biologically important molecules. Tetracycline [34] as chiral molecule, $[\alpha]_{D}^{25}$ - 257.9° (0.1 N HCl), $[\alpha]_{D}^{25}$ - 239° (methanol), has five chiral carbons. The spectral change in CD from tetracycline binding Eu$^{3+}$ is shown as in Fig. 4.4. The CD spectrum of tetracycline consisted of two intense exciton couplets
(maximum at 296 nm, minimum at 271 nm and at 324 nm) with two crossover at 282 nm and at 311 nm. The CD spectra of Tc in ligand of Eu$^{3+}$ or in free state have significant differences. The spectral changes from EuTc are expressed that three bands at 242, 267, and 290 nm instead of one crossover at 282 nm from Tc; a new crossover at 385 nm is occurred (maximum at 408 nm and minimum at 373 nm) which is assumed from the absorption of EuTc around 400 nm (Fig. 4.2). But spectra of EuTc in absence and in present citrate is similar, with only have a little changes. In this case, EuTc is an asymmetric probe, citrate ligating EuTc form ternary complex which is also asymmetric. It indicates that citrate does not significant influence the structure of EuTc at least at ground state.

### 4.2.1.5. Solid form of EuTc-Cit

The EuTc-Cit shows strong fluorescence not only in solution, but also in solid. Fig. 4.5 (left) is a microscopic photo for the solid EuTc-Cit under UV light. Significant red emission from Eu$^{3+}$ is evident. Under white light, the common solid form of EuTc-Cit can be observed in Fig. 4.5 (right).

*Figure 4.5. Solid form of EuTc-Cit in UV light (left) with 470 nm cut-off objective lens; same EuTc-Cit in white light (right) with common objective lens. Two photos were obtained on Leica DMR fluorescence microscope.*
4.2.2. Optimal Experimental Conditions

In EuTc-Cit, tetracycline and citrate as ligands have been deprotonated and are sensitive to pH. The acid dissociation constants of tetracycline [35-38] are pKa1 ~ 3-4, pKa2 ~ 7.3-8.1, and pKa3 ~ 8.8-9.8, that of citric acid [39] are pKa1 = 3.1, pKa2 = 4.7, and pKa3 = 6.4. But once the EuTc-Cit system is formed, it is stable in wide pH range from 7.4 to 9.2 in Fig. 4.6. In this pH range, Tc and citrate, can lose two and three protons, respectively, to become anions with multi-negative charges, and this facilitates binding to europium ion.

Three different buffers (HEPES, MOPS and TRIS) were exploited (Fig. 4.6). All of them are suitable for the system. Since the optimal buffer range of MOPS is not coincidental with EuTc-Cit, and TRIS buffer is largely temperature dependent, a 10 mM of HEPES buffer solution of pH 8.0 is used in these experiment.

Figure 4.6. The influence of pH for EuTc-Cit. Concentration of Eu³⁺, Tc and citrate are 50, 50 and 150 µM, respectively.
The time trace (Fig. 4.7) of EuTc binding citrate shows that the increasing of fluorescent intensity depends on time and the concentration of citrate. The higher concentration of citrate, the longer the time to form stable fluorescence.

The fluorescence of EuTc-Cit is inversely proportional to the temperature, as most of the fluorophores. The temperature curve can be describe as \( y = 14.4 - 0.3 \times x \), (\( x \) and \( y \) are temperature (°C) and fluorescence intensity).

4.2.3. Interferences

The interferences for EuTc-Cit systems, about 40 common cations in Table 4.2, anions, gases and small biomolecules, were studied. Oxygen has a small effect as its fluorescence in saturated air is 90% of the maximum intensity of that in nitrogen. Alkali, halide, sulfate, nitrate, ammonium, small organic molecules in serum, and human serum albumin (HAS) do not interfere. For heavy metals ions, at pH 8.0, since some of them are precipitated by \( \text{OH}^- \), only \( \text{Ni}^{2+} \), \( \text{Co}^{2+} \) and \( \text{Cu}^{2+} \), which can complex citrate and tetracycline [40-44], have a constant
Chapter 4. Fluorescence Determination and Imaging of Citrate

affect if their concentration are larger than 16, 16 and 2 \( \mu M \), respectively, at 40 \( \mu M \) of citrate in EuTc-Cit. Moreover, for phosphate compounds and ions, just 280 \( \mu M \) of phosphate and 8 \( \mu M \) of ATP interfere. It is noted that hydrogen peroxide (which can be probed by EuTc) does not affect the fluorescence of EuTc-Cit, probably because of the much weaker coordinating ability of \( \text{H}_2\text{O}_2 \) to \( \text{Eu}^{3+} \), and of different stoichiometry (molar ratio of \( \text{Eu:} \text{Tc} \) is 1:1 here, but the optimal molar ratio of EuTc is 3:1 when probing \( \text{H}_2\text{O}_2 \)).

**TABLE 4.2. INTERFERENCES OF COMMONS SUBSTANCES FOR EU\text{Tc}-\text{Cit}**

(interference is maximally -10 % of initial fluorescence intensity)

<table>
<thead>
<tr>
<th>EuTc-Cit</th>
<th>Maximum tolerable concentration ratio (compare with 40 ( \mu M ) of citrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NO}_3^- ), ( \text{F}^- ), ( \text{Br}^- ), ( \Gamma ), ( \text{SO}_4^{2-} ), ( \text{HCO}_3^- ), ( \text{NH}_4^+ ), ( \text{Na}^+ ), ( \text{K}^+ )</td>
<td>1000</td>
</tr>
<tr>
<td>L-glutamic acid, glucose, glycerol, succinate, acetate</td>
<td>500</td>
</tr>
<tr>
<td>( \text{Cd}^{2+} ), L-malate, lactate</td>
<td>200</td>
</tr>
<tr>
<td>ascorbate</td>
<td>120</td>
</tr>
<tr>
<td>tartrate, fumarate, ketoglutarate, pyruvate</td>
<td>50</td>
</tr>
<tr>
<td>AMP</td>
<td>40</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} )</td>
<td>32</td>
</tr>
<tr>
<td>( \text{Ca}^{2+} ), uric acid, malonate</td>
<td>20</td>
</tr>
<tr>
<td>D-malate,</td>
<td>16</td>
</tr>
<tr>
<td>NADH</td>
<td>10</td>
</tr>
<tr>
<td>phosphate</td>
<td>7</td>
</tr>
<tr>
<td>isocitrate</td>
<td>4</td>
</tr>
<tr>
<td>( \text{Fe}^{3+} ), ( \text{Zn}^{2+} ), oxaloacetate</td>
<td>3</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
</tr>
<tr>
<td>( \text{Co}^{2+} ), ( \text{Ni}^{2+} )</td>
<td>0,4</td>
</tr>
<tr>
<td>ATP</td>
<td>0,2</td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>0,05</td>
</tr>
<tr>
<td>HSA</td>
<td>(1.56 mg/mL)</td>
</tr>
</tbody>
</table>
4.2.4. Quantitative Assay of Citrate

4.2.4.1. Lifetime based assay

Decay time can be used as a parameter for citrate assay, e.g. by time-correlated single photon counting (TCSPC) as shown in Fig. 4.8.

![Figure 4.8. Lifetime assay of citrate.](image)

The decay time is increasing with the increase of citrate concentration in EuTc. The decay times of three components from different concentrations of citrate in EuTc are compiled in Table 4.3. The relative amplitudes of third component from each samples at ~100 µs increase from 4 % on going to ~ 50 % with concentration of citrate increasing, at last it becomes stable in 100 µM of citrate in EuTc. In contrast to $\tau_3$, the relative amplitudes of first component is decreasing with the increase of citrate concentration.
Chapter 4. Fluorescence Determination and Imaging of Citrate

Table 4.3. The decay time assay of EuTc-Cit
(Fitting function: 3-exponential)

<table>
<thead>
<tr>
<th>Samples</th>
<th>No.</th>
<th>Decay (µs)</th>
<th>Rel. Amplitude (%)</th>
<th>Av./µs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EuTc(50 µM:50 µM)</td>
<td>τ1</td>
<td>8.3</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ2</td>
<td>24.6</td>
<td>55.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ3</td>
<td>123.4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>EuTc-Cit (4 µM)</td>
<td>τ1</td>
<td>13.8</td>
<td>59.1</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>τ2</td>
<td>29.9</td>
<td>34.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ3</td>
<td>115.9</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>EuTc-Cit (16 µM)</td>
<td>τ1</td>
<td>17.0</td>
<td>38.0</td>
<td>78.2</td>
</tr>
<tr>
<td></td>
<td>τ2</td>
<td>58.7</td>
<td>35.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ3</td>
<td>106.3</td>
<td>26.8</td>
<td></td>
</tr>
<tr>
<td>EuTc-Cit (100 µM)</td>
<td>τ1</td>
<td>15.9</td>
<td>13.4</td>
<td>83.6</td>
</tr>
<tr>
<td></td>
<td>τ2</td>
<td>61.2</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ3</td>
<td>96.3</td>
<td>51.2</td>
<td></td>
</tr>
<tr>
<td>EuTc-Cit (150 µM)</td>
<td>τ1</td>
<td>15.1</td>
<td>12.5</td>
<td>83.4</td>
</tr>
<tr>
<td></td>
<td>τ2</td>
<td>58.5</td>
<td>32.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>τ3</td>
<td>95.0</td>
<td>54.8</td>
<td></td>
</tr>
</tbody>
</table>

4.2.4.2. Conventional steady-state fluorescence assay

Figure 4.9. Calibration curve citrate via steady-state fluorescence intensity. Concentration of EuTc is 50 µM. F and F₀ are fluorescence intensities of EuTc in presence and absence citrate.
In contrast to TCSPC, which is usually tedious and requires expensive instrumentation, conventional steady-state fluorescent spectroscopy is widely used. For citrate assay, the dynamic range is from $8 \times 10^{-7}$ to $4 \times 10^{-5}$ M, with $4 \times 10^{-7}$ M of the limit of detection (defined as $3\sigma$/slope) and the relatively small error bar (for $n=3$) in Fig. 4.9.

### 4.2.4.3. Time-resolved fluorescence assay

The time-resolved fluorometry has also been studied for citrate assay. This method records the fluorescence intensity after a lag time, so it can eliminate the background fluorescence. The effect of different lag times on $F/F_0$ of the EuTc system in absence and in presence of citrate is shown in Fig 4.10(a). $F/F_0$ increases on going from 0 µs to a 100 µs lag time. The $F/F_0$ value reaches a maximum at a lag time from 100 to 150 µs and decreases after 150 µs lag time. This is in accordance with the decay profile of EuTc-Cit because the main component in the decay profile of EuTc-Cit has a 95 µs decay time. Therefore, most of the increase in fluorescence is detected if the integration time window is opened after a lag time of 100 µs.

![Figure 4.10](image)

**Figure 4.10.** (a) Fluorescence of $F/F_0$ in EuTc-Cit at different lag time, with a 40 µs integration time (b) Fluorescence ($F/F_0$) in EuTc-Cit at different integration time, with 100 µs. $F$ and $F_0$ are fluorescence intensity of EuTc(50 µM) in presence and absence citrate (150 µM).
The integration time is defined as the length of the time period, the detector is exposed to the emission light. Fig. 4.10(b) shows the effect of the integration time on $F / F_0$, while $100 \mu s$ lag time was employed. If the integration time is increased from $20 \mu s$ to $200 \mu s$, $F / F_0$ decreases slowly. Considering that the major component of the luminescence decay of EuTc-Cit, a $40 \mu s$ integration time was regarded to be appropriate. Obviously, the integration time is not really critical.

The time-resolved assay of citrate with a lag time of $100 \mu s$ and integration time of $40 \mu s$ has a dynamic response between $1.6 \times 10^{-7}$ and $5.6 \times 10^{-5}$ M, with a detection limit (defined as $3\sigma / \text{slope}$) of $6.0 \times 10^{-8}$ M of citrate.

4.2.4.4. Imaging

Two imaging schemes were employed for the determination of citrate, the first is conventional steady-state imaging in Fig. 4.11. It shows significant fluorescence changes while EuTc probe was added in the different concentrations of citrate. But the images are greatly affected by fluctuations of the light source and light scatter and leaded to the pictures of substantial heterogeneity.

![Image](image.png)

*Figure 4.11. Imaging of citrate in EuTc (50 µM) probe. Steady-state imaging, one window for 0-50 µs. Citrate concentrations (from left to right) are 0, 0.16, 0.4, 1.0, 1.6, 4.0, 10.0, 16.0, 20, 40.0, 60.0 and 80.0 µM, respectively.*

The second is rapid lifetime determination (RLD) imaging [45-47] as shown in Fig. 4.12. The principle of RLD is shown in Fig. 4.12(a), two imaging windows are used in the
decay period of the fluorophore. The LED pulses lasted from 0-50 µs. Image 1 was recorded after a time lag of 50 µs (gating time $t_1$: 100-180 µs) and image 2 after a time lag of 150 µs (gating time $t_2$: 200-240 µs). The ratio is calculated to:

$$R_{RLI} = \frac{(RLD_1)}{(RLD_2)}$$  \hspace{1cm} \text{(Equation 4-1)}

The RLD images require that the fluorophore has a microsecond scale lifetime, EuTc-Cit is suitable for this approach. In contrast to steady-state imaging, RLD (Fig. 12 b) offers better homogeneity since the lifetime is relatively independent of the scattering and fluctuations in the intensity of the light source and advantages in terms of signal generation and of excluding artifacts including local inhomogeneities of the concentration of fluorescent probes.

Figure 4.11. (a) Scheme of rapid lifetime detection (RLD) imaging, (b) RLD for citrate in EuTc (50 µM) two windows from 100-180 µs and from 200-240 µs. citrate concentrations (from left to right) are 0, 0.16, 0.4, 1.0, 1.6, 4.0, 10.0, 16.0, 20., 40.0, 60.0 and 80.0 µM, respectively.
4.2.4.5. Comparison with other chemical methods for citrate assay

The main methods for citrate assay are summarized in Table 4.4. These approaches focus on amperometry, colorimetry, fluorescence and chemiluminescence: (a) most of them usually need two or three reaction steps by enzymes or catalysts to yield products which have significant properties suitable for determination. These experimental processes are tedious and some side reactions occur. (b) enzyme for citrate assay via NADH as monitor, which has been commercialized, can be used in colorimetry or fluorometry. But its sensitivity is still lower than that of the EuTc-Cit method. (c) synthetic receptor is also used to recognize citrate, but its selectivity and sensitivity is not good enough. (d) EuTc-Cit method has simple reaction step and facilitation manipulation. It can be used in time-resolved fluorescence detection, which can suppress the background fluorescence, for improve selectivity and sensitivity to obtain a low the limitation of detection.
<table>
<thead>
<tr>
<th>methods</th>
<th>principle</th>
<th>linear range</th>
<th>LOD</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 enzyme (spectrophotometry)</td>
<td>citrate $\xrightarrow{citrate lyase}$ oxaloacetate + acetate oxaloacetate + NADH + $H^+$ $\xrightarrow{malic dehydrogenase}$ malate + NAD$^+$</td>
<td></td>
<td>0.02 µmol</td>
<td>48</td>
</tr>
<tr>
<td>2 enzyme-solid</td>
<td>same method 1. (spectrophotometry)</td>
<td>1-20 mg L$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 amperometry</td>
<td>Citrate $\xrightarrow{citrate lyase, Mg^{2+}, Zn^{2+}}$ acetate + oxaloacetate oxaloacetate $\xrightarrow{oxaloacetate decarboxylase, Mg^{2+}, Mn^{2+}}$ pyruvate + CO$_2$ pyruvate + H$_3$PO$_4$ + O$_2$ $\xrightarrow{pyruvate oxidase, Mg^{2+}, Mn^{2+}, FAD, TPP}$ acetylphosphate + CO$_2$ + H$_2$O$_2$</td>
<td>0.25-5.00 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 amperometry (flow injection)</td>
<td>same method 3</td>
<td>0.015-0.5 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 potentiometry</td>
<td>polymeric membranes + quaternary ammonium ion exchanger</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 chemiluminescence</td>
<td>Fe(III)-citrate $\xrightarrow{hv}$ Fe$^{2+}$ + CO$_2$ luminol + O$_2$ + 2HO$^{-}$ $\xrightarrow{Fe^{2+}}$ 3-aminophthalate + N$_2$ + 2H$_2$O + hv</td>
<td>2.0x10$^{-7}$-1.0x10$^{-4}$ M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 chemiluminescence</td>
<td>Ru(bpy)$_3$$^{2+}$ +citrate + Ce$^{4+}$ $\xrightarrow{Fe(III)^{2+}}$ [Ru(bpy)$_3$$^{2+}$] $\xrightarrow{hv}$ Ru(bpy)$_3$$^{2+}$ + hv</td>
<td>0.38-38 µg mL$^{-1}$</td>
<td>0.1 µg mL$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>8 spectrophotometry</td>
<td>2,4-diaminophenol + H$_2$O$_2$ $\xrightarrow{Fe(III)}$ 2,4-diaminophenol (dimer) + O$_2$ + H$_2$O citrate as inhibitor, citrate + Fe(III)$\rightarrow$Fe(III)-Citrate complex</td>
<td>0-1000 mg L$^{-1}$</td>
<td>0.96 mg L$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>9 colorimetry</td>
<td>Fe(III)-citrate $\xrightarrow{hv}$ Fe(II) + CO$<em>2$ Fe(II) + 1,10-phenanthroline $\xrightarrow{[Fe(phen)</em>{3}]^{2+}}$</td>
<td>1-120 µg L$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 colorimetry</td>
<td>competitive indicator for host – guest complexes</td>
<td></td>
<td>55 µM</td>
<td></td>
</tr>
<tr>
<td>11 ion chromatography</td>
<td>polymeric anion exchange column, trimesic acid mobile phase</td>
<td>1-12 µg</td>
<td>0.26 µg</td>
<td></td>
</tr>
<tr>
<td>12 fluorometry, imaging</td>
<td>europium-tetracycline-citrate (this method)</td>
<td>1.6x10$^{-7}$-5.6x10$^{-5}$ M</td>
<td>6.0 x10$^{-8}$M</td>
<td></td>
</tr>
</tbody>
</table>
4.2.5. Different Kinds of Tetracyclines in Eu-xTc-Cit

Further studies were performed for different tetracycline derivatives as shown in Table 4.5.

![Figure 4.12. Comparison with different tetracyclines and ratio in Eu-xTc-Cit](image)

*xTc: tetracycline hydrochloride (Tc), chlortetracycline hydrochloride (cT), rolitetracycline hydrochloride (rT), oxytetracycline hydrochloride (oT), demeclocycline hydrochloride (deT), doxycycline hydrochloride (doT), minocycline hydrochloride (miT), meclocycline sulfosalicylate salt (meT). Molar ratio is Eu/xTc, concentration of citrate and xTc is 100 µM and 48 µM, respectively, concentration of Eu³⁺ is changed according to ratio.*

According to Fig. 4.12, it concludes that a) among tetracycline analogs, the fluorescence intensity of oxytetracycline, tetracycline and rolitetracycline chelating europium ion with citrate are strong, that of demeclocycline, doxycycline, chlortetracycline and meclocycline sulfosalicylate are medium, and that of minocycline is very weak. These refer to their structures of molecule. Obviously, it is favorite that OH groups in R3 of tetracyclines.
TABLE 4.5. THE STRUCTURES OF FAMILY OF TETRACYCLINE

<table>
<thead>
<tr>
<th>No.</th>
<th>Chemicals</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tetracycline</td>
<td>H</td>
<td>CH₃</td>
<td>OH</td>
<td>H</td>
<td>(CH₃)₂N-</td>
<td>H</td>
</tr>
<tr>
<td>2</td>
<td>chlortetracycline</td>
<td>Cl</td>
<td>CH₃</td>
<td>OH</td>
<td>H</td>
<td>(CH₃)₂N-</td>
<td>H</td>
</tr>
<tr>
<td>3</td>
<td>rolitetracycline</td>
<td>H</td>
<td>CH₃</td>
<td>OH</td>
<td>H</td>
<td>(CH₃)₂N-</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
<td>oxytetracycline</td>
<td>H</td>
<td>CH₃</td>
<td>OH</td>
<td>OH</td>
<td>(CH₃)₂N-</td>
<td>H</td>
</tr>
<tr>
<td>5</td>
<td>demeclocycline</td>
<td>Cl</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>(CH₃)₂N-</td>
<td>H</td>
</tr>
<tr>
<td>6</td>
<td>doxycycline</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>OH</td>
<td>(CH₃)₂N-</td>
<td>H</td>
</tr>
<tr>
<td>7</td>
<td>minocycline</td>
<td>(CH₃)N⁻</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>(CH₃)₂N⁻</td>
<td>H</td>
</tr>
<tr>
<td>8</td>
<td>meclocycline sulfosalicylate salt</td>
<td>Cl</td>
<td>=CH₂</td>
<td>\</td>
<td>OH</td>
<td>(CH₃)₂N⁺SO₃⁻</td>
<td>H</td>
</tr>
</tbody>
</table>

tetracycline hydrochloride (Tc), chlortetracycline hydrochloride (cT), rolitetracycline hydrochloride (rT), oxytetracycline hydrochloride (oT), demeclocycline hydrochloride (deT), doxycycline hydrochloride (doT), minocycline hydrochloride (miT), meclocycline sulfosalicylate salt (meT)
However, R₁ group except H is hindrance, for example, R₁ = Cl⁻ in demeclocycline, and (CH₃)₂N⁻ in minocycline. But R₆ = group was no influence on the coordination of Tc and Eu³⁺, such as R₆ = N in oxytetracycline. b) the optimal molar ratio of Eu:Tc is at 1:1 regardless of any tetracycline. c) The orders of fluorescence intensity of coordination of Tc and Eu³⁺ with citrate are different. For example, at ½ (or 0.5/1) of mole ratio of Eu³⁺:Tc, the order is oxytetracycline > rolitetracycline > tetracycline > doxycycline > demeclocycline > mecloxycline sulfosalicylate > chlortetracycline > minocycline. But at 2:1 molar ratio, the order is change, tetracycline > oxytetracycline > rolitetracycline > demeclocycline > doxycycline > chlortetracycline > mecloxycline > minocycline. These knowledge on structures will greatly benefit further studies, especially the optimization of Eu-xTc fluorescence probes for citrate.

4.3. Conclusion

A europium derived fluorescent probe for the measurement and visualization of citrate is first time presented. Besides a straightforward direct detection using a simple reagent and without the need for multi-enzyme assays, many lifetime and imaging techniques are utilized in this system. This probe is simple to prepare, stable both in solution and in solid, and compatible with the blue laser diodes. It will be most useful for monitoring citrate-related bioprocesses.
4.4. Experimental Section

4.4.1 Reagents

Tri-sodium citrate dihydrate and other inorganic salts were obtained in analytical purity from Merck unless otherwise stated. All solutions were prepared in 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer of pH 8.0 (Sigma-Aldrich, Deisenhofen, Germany) unless otherwise specified. Europium(III) trichloride hexahydrate was from Alfa Products, tetracycline hydrochloride from Sigma.

The EuTc standard solution was obtained by dissolving Eu$^{3+}$ chloride and tetracycline (each in 0.5 mM concentration) in 10 mM HEPES buffer of pH 8.0. This reagent is stable for at least 2 months if stored at 4 °C in the dark.

4.4.2. Apparatus

Absorption spectra were acquired on a Cary WinUV photometer. Fluorescence studies of the effect of citrate on spectra the EuTc were performed on an SLM AB2 luminescence spectrometer. Fluorescence intensity was acquired on either a Fluoroskan Ascent micro titer plate reader or on a Tecan GENios+ micro plate reader. The excitation/emission filters were set to 405/620 nm, or 405/612 nm respectively. The 96-well black, transparency, flat bottom microtiter plates for imaging were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany; www.greiner-lab.com). The luminescence lifetimes of different concentration of citrate in EuTc were determined with a pulsed 392-nm laser and an H5783-P04 PMT detector with multiphoton-counting board in a multipass cuvette. Circular dichroism (CD) spectra were performed on JASCO model J – 710 spectropolarimeter (www.jasco.de). DMR fluorescence microscopy is from Leica (www.light-microscopy.com)
4.4.3. Fluorescence Microscopic Observation of Solid form EuTc-Cit

Efforts have been made to crystalize EuTc-Cit. 20 µL of EuTc (50 µM) in citrate (150 µM) forms a drop on the cover slide, which is hung over the reservoir in which includes 40% (v/v) polyethylene glycol 400. After one week, a thin solid member (instead of crystal) is observed under fluorescence microscopy. Two photos of solid EuTc-Cit were obtained on a Leica DMR fluorescence microscopy. One is in UV light with objective lens of PL FLUORTAR (cut off 470 nm) at 4.0 s exposure time and 4.1 gain; the other in white light with objective lens of N PLAN at 45.8 ms exposure time and 4.1 gain.

4.4.4. RLD Imaging

Imaging set-up was as described in section 3.4.6. Scheme of RLD with two windows of fluorescence detection at 100-180 µs and 200-240 µs are recorded. The manipulation and calculation of images, such as the rotation and crop of the images, the subtraction of the dark image (blank, without illumination) from the fluorescent image respectively, the ratio of the images and filtration of the background noise, were done by a self-developed program based on Matlab (6.1, Mathwork, Natick, MA, USA).

4.5. References


Chapter 4. Fluorescence Determination and Imaging of Citrate


[22] UV-method for the determination of citric acid in foodstuffs, Boehringer Mannheim, Cat.no. 139076, 1998.


[27] Van Houten, J.; Watts, R. J. **Effect of ligand and solvent deuteration on the excited state properties of the tris(2,2'-bipyridyl)ruthenium(II) ion in aqueous solution. Evidence for electron transfer to solvent.** Journal of the American Chemical Society 1975, 97(13), 3843-4.


[34] The Meck Index


Chapter 5. Fluorescence Imaging and Detection of Main Intermediates in the Krebs Cycle

5.1. Introduction

The Krebs cycle [1, 2] (Fig. 5.1) is a key series of metabolic reactions in aerobic cellular respiration, occurring in the mitochondria of animals and plants. It is the central metabolic hub of the cell not only for harvesting chemical energy [3, 4], but also for building the basic blocks of amino acids, nucleotide bases, porphyrins and others [5-7].

Figure 5.1. The Krebs cycle (adapted from Berg, J. M ref.[2])
There are many intermediates in the Krebs cycle had been studied. By exploring the interactions between the Krebs cycle and other metabolism systems [8-10], intermediates are still medium. The most of studies of the Krebs cycle have been developed basically through isotropic labels [11-13] especially for the mechanism research. The main intermediates in the Krebs cycle, such as citrate, isocitrate, α-ketoglutarate (KG), succinate, fumarate, L-malate and oxaloacetate (their structures are in Fig. 5.1), are usually absent of significant physical and chemical properties suitable for direct non-radioactive determination, and therefore the Krebs cycle is difficult to visualize and to detect directly. Besides separative chromatography [14-16] and electrophoresis [17-19], enzyme or multi-enzymatic methods [20-23] are mostly employed by coupling with reactions involving NADH. These methods always depend upon the change of NADH [24-26] as a monitor to detect reactants or products. But the disadvantages of detection through NADH in biosamples are obvious: short decay times, UV excitation at 355 nm and complicated reaction processes.

Here, a europium derived fluorescent probe has been used for the detection and visualization of main intermediates. The method is based on the finding that the weakly fluorescent europium-tetracycline (EuTc) [27-29] can reversibly associate with intermediates to form differently fluorescent europium-tetracycline-ligands (EuTc-L) at neutral pH. As these complexes have the merits of lanthanide fluorescence, time-resolved fluorescence detection can be used. Fluorescence imaging is applied for the visualization of intermediates. In addition, the stepwise determination of the formation and decomposition of intermediates can be performed via the kinetic fluorescence changes.
Chapter 5. Fluorescence Imaging and Detection of Main Intermediates in the Krebs Cycle

5.2. Results and Discussion

5.2.1. Characterization of EuTc Complexes with Main Intermediates

5.2.1.1. Absorbance and fluorescence spectra

The absorption and fluorescence spectra of the EuTc-L systems are shown in Fig. 5.2. The maximal absorbances of EuTc-L are from 381 to 408 nm, similar to that of EuTc. In contrast, the fluorescence intensity of the emissions of EuTc-L are stronger than that of EuTc from 615 to 619 nm. These intermediates can act as polydentate ligands and thus may be expected to form ternary complexes with EuTc. The coordination with Eu$^{3+}$ involve oxygen atoms from carboxyl and hydroxy group, probably resulting in formation of 5-, 6-, or 7-member ring with Eu$^{3+}$ [30-32]. Different molecule structures lead to different fluorescence intensities (Fig. 5.2), as well as in decay times and quantum yields (Table 5.1)

![Figure 5.2. Spectra of EuTc-L complex. Eu$^{3+}$: 50 µM, Tc: 50 µM L:150 µM](image-url)
### TABLE 5.1. THE DECAY PROFILES AND QUANTUM YIELD OF EuTc-L

<table>
<thead>
<tr>
<th>Samples</th>
<th>Components</th>
<th>Decay (µs)</th>
<th>Rel. Amplitude (%)</th>
<th>Av.(µs)</th>
<th>QY(%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EuTc</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8</td>
<td>40.7</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>24</td>
<td>55.4</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>123</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-Cit</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>15</td>
<td>12.5</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>58</td>
<td>32.7</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>95</td>
<td>54.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-iCit</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>16</td>
<td>57.4</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>40</td>
<td>27.6</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>111</td>
<td>15.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-KG</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8</td>
<td>40.5</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>21</td>
<td>56.1</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>112</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-Suc</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>7</td>
<td>36.3</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>21</td>
<td>57.4</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>88</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-Fum</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8</td>
<td>28.0</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>22</td>
<td>49.3</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>90</td>
<td>22.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-Mal</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>17</td>
<td>53.3</td>
<td></td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>61</td>
<td>24.2</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>109</td>
<td>22.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EuTc-Oxa</td>
<td>τ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>13</td>
<td>14.7</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>49</td>
<td>60.1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>τ&lt;sub&gt;3&lt;/sub&gt;</td>
<td>71</td>
<td>25.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Eu<sup>3+</sup>: 50 µM, Tc: 50 µM, for the purpose of the Krebs cycle study, only 150 µM of L (ligand) is used here.

<sup>b</sup> Fitting function: 3-exponential decay

<sup>c</sup> tris (2,2’-bipyridyl) dichlororuthenium(II) hexahydrate as the reference, ref [33]

<sup>d</sup> In this case, the concentration of L-malate for EuTc is not saturated.

### 5.2.1.2. Fluorescence Decay times and Quantum Yields

The decay profiles of EuTc-L complexes, as obtained through TCSPC, can be fitted into a three-component model as summarized in Table 5.1. The quantum yields of EuTc-Cit and EuTc-Oxa show higher than that of other intermediates. For decay times, the average lifetime...
of EuTc-Cit and EuTc-Mal are longer than others. EuTc-iCit, EuTc-Fum and EuTc-Oxa have a lifetimes longer than that of EuTc, while EuTc-Suc and EuTc-KG are almost same as EuTc. As KG and succinate cannot effectively form complexes with Eu\(^{3+}\), no significant fluorescent intensity and lifetime enhancement are expected and indeed observed. Decay times of different components for EuTc-L are important in the time-resolved and RLD imaging. Furthermore, by choosing different lag times for “gated” detections, different intermediates can be determined in different time windows.

### 5.2.2. Imaging for the Krebs Cycle

Imaging [34-36] as a potential “mapping” technique has been used to show the Krebs cycle as in Fig. 5.3. From Fig. 5.3(a), the different Krebs cycle intermediates in steady-state imaging have been observed at time window from 0 – 50 µs according to their fluorescence intensity. The fluorescence of EuTc-Cit is significantly higher than others, while those of succinate and KG are almost the same as the background. The order of the rest is oxaloacetate > malate > isocitrate.

RLD imaging, which has been discussed in section 4.2.4.4, was also employed to depict the Krebs cycle. Two windows from 100 to 150 µs and from 160 to 210 µs were imaged in Fig. 5.3(b), the dramatic fluorescence changes of EuTc-L complexes were observed. Due to different decay times of EuTc-L in Table 5.1, the relative ratios (\(R_{RLD}\)) of the integrations between two windows for EuTc-L are also different. The relative ratios of L-malate and fumarate in EuTc obviously increase, however, that of oxaloacetate is significantly decreasing to a level almost as KG. RLD imaging can further explain the possibility of distinguishing intermediates by the lifetime-based fluorescence.
Chapter 5. Fluorescence Imaging and Detection of Main Intermediates in the Krebs Cycle

Figure 5.3. Fluorescence imaging of main intermediates in the Krebs cycle. (a) steady-state imaging, one window from 0-50 µs; (b) rapid lifetime determination (RLD) imaging, two time windows from 100-150 µs and from 160-210 µs. Intermediates (150 µM) were added in EuTc (50 µM).
5.2.3. Conversions Between Intermediates in the Krebs Cycle

5.2.3.1. Stepwise visualization of decomposition of citrate

The characteristics of the EuTc complexes with main intermediates of the Krebs cycle have made it possible for the stepwise visualization of decomposition of citrate. Oxaloacetate, L-malate and fumarate can be produced according to the following enzymatic reactions (Eq. 5-1, 5-2, 5-3),

\[
\text{citrate} \xrightarrow{\text{CL}} \text{oxaloacetate} + \text{acetate} \quad \text{(Equation 5-1)}
\]

\[
\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+ \quad \text{(Equation 5-2)}
\]

\[
\text{L-malate} \xrightarrow{\text{FM}} \text{fumarate} + \text{H}_2\text{O} \quad \text{(Equation 5-3)}
\]

Figure 5.4. Kinetic curve of the formation and stepwise decomposition of EuTc-Cit complex. Blank solution including 200 µL of 0.5 mM EuTc and 60 µL of 4.8 mM of NADH in 1.70 mL of HEPES buffer, then 40 µL of 2 mM citrate, 70 µL of 3.4 U/mL citrate lyase (CL), 50 µL of 1588 U/mL malic dehydrogenase (MDH) and 80 µL of 618 U/mL fumarase (FM) were added, respectively.
Citrate lyase (CL), malic dehydrogenase (MDH) and fumarase (FM), respectively, were employed to catalyze these reactions. Fig. 5.4 indicates the stepwise processes via fluorescence: the fluorescence of a blank solution, composed of EuTc and NADH only, is stable over time. (In this experiment, NADH does not interfere the fluorescence of EuTc and EuTc-Cit because they have different emissions and excitations.) While adding citrate, the fluorescence increases due to the formation of the EuTc-Cit complex. After fluorescence of EuTc-Cit reached a plateau, CL, MDH and FM were added, respectively. Fluorescence decreases step by step (in three stages), thereby indicating the complete consumption of citrate and formation of EuTc-oxaloacetate, EuTc-malate and EuTc-fumarate, respectively. This experiment manifests that (a) EuTc-L acts as a reversible fluorescent probe, and the change of concentrations of the main intermediates of the Krebs cycle can be directly monitored via fluorescence, (b) Despite the fact that the reaction of Eq 5-2 can also be determined by the NADH, the dual simultaneously fluorescent measurements by EuTc-oxaloacetate and NADH have indicated the former is much more sensitive (see section 5.2.4.2). (c) The stepwise decompositions of citrate, oxaloacetate, and L-malate can be clearly visualized through their complexes with EuTc. NADH can only indicate one of the decomposition steps or the overall changes without the capability of stepwise indication of the reactions.

5.2.3.2. Formation of citrate in the Krebs cycle

The application and specificity of EuTc-L system for the Krebs cycle is further explored by the kinetic change in the intermediate steps in Eq.5-4 and Eq.5-5, which are usually considered as the last step and the first step in the Krebs cycle.
Chapter 5. Fluorescence Imaging and Detection of Main Intermediates in the Krebs Cycle

\[ \text{L-malate} + \text{NAD}^+ \xrightarrow{MDH} \text{oxaloacetate} + \text{NADH} + H^+ \quad (\text{equation 5-4}) \]

\[ \Delta G^\circ = +29.7 \text{ kJ mol}^{-1} \]

\[ \text{Acetyl CoA} + \text{oxaloacetate} + H_2O \xrightarrow{CS} \text{citrate} + \text{CoA} + H^+ \quad (\text{equation 5-5}) \]

\[ \Delta G^\circ = -31.4 \text{ kJ mol}^{-1} \]

Unlike the other steps in the Krebs cycle, the standard free energy of Eq. 5-4 is significantly positive, the formation of citrate from malate is possible when coupled with Eq. 5-5, driven by the utilization of the products oxaloacetate by citrate synthase (CS) and NADH by the electron-transport chain. As shown in Fig. 5.5, the fluorescence signal of blank with L-malate, MDH, acetyl CoA, CS and EuTc is stable and low (although a litter bit higher than EuTc) over time, no reaction happened. The products in Eq. 5-4, and 5-5, NADH and CoA do

---

*Figure 5.5. Kinetic curve of the formation and decomposition of citrate.*

Blank including 0.2 mL of 0.5 mM EuTc, 0.4 mL of 0.5 mM L-malate, 0.16 mL of 9.9 U/mL MDH, 0.16 mL of 1 U/mL CS, 0.16 mL of 0.23 mM acetyl CoA and 0.8 mL of 10 mM HEPES buffer at 8.0 pH in cuvette, plus 54 µL of 14 mM NAD\(^+\), then add 55 µL of 4.3 U/mL CL when the maximum fluorescence was reached.
not introduce the fluorescence change. After the addition of NAD$^+$, the whole reaction from Eq.5-4 to Eq. 5-5 is initiated and resulted in the production of citrate, consequently the increase of the fluorescence due to the formation of the EuTc-Cit system. To further indicate the specificity of the EuTc-L system, CL is used to testify the existence of citrate in Eq. 5-6.

\[
\text{citrate} \xrightarrow{\text{CL}} \text{oxaloacetate + acetate} \quad \text{(equation 5-6)}
\]

The addition of CL causes the decrease of citrate and a drop of the fluorescence. This experiment has realized (a) citrate was product by the pathway in the Krebs cycle, this process can be kinetically monitored via fluorescence. (b) Dual fluorescence determinations for EuTc-Cit and NADH can also be simultaneously monitored, they depict the reaction processes of Eq. 5-4 and Eq. 5-5, respectively.

### 5.2.4. Fluorescence Detection of Main Intermediates in the Krebs Cycle

**5.2.4.1. Time-resolved fluorescence assays**

As the different decay times of EuTc-L, the discrimination of intermediates can be performed in different time windows. In steady-state fluorescence measurement (with 0 µs lag time in Fig. 5.6), the order of the normalized intensity [defined as $(F - F_0) / F_0$] is EuTc-Cit > EuTc-Oxa > EuTc-Ma > EuTc-iCit > EuTc-Fum > EuTc-Suc $\approx$ EuTc-KG $\approx$ EuTc. Gating obviously can be used to fine tune between selectivity and sensitivity. On increasing the lag time from 0 to 100 µs, the normalized intensity of all species is increased (Fig. 5.6.) except for KG. On increasing the lag time to 250 µs, oxaloacetate is widely suppressed and citrate is reduced by 40 %, while isocitrate, fumarate and malate, remain much less affected. Obviously, L-malate and oxaloacetate can be nicely discerned. By comparing citrate with
isocitrate, it is noted that decay times of the main components of EuTc-Cit (95 µs) and EuTc-iCit (16 µs) are quite different (Table 5.1).

It should be emphasized that the main intermediates can be sensed and imaged directly through their complexes with EuTc, and that no enzymes or multi-enzyme systems are needed. However, assays for other intermediates have to exclude any interference by citrate.

5.2.4.2. Dual fluorescence detection the decomposition process of oxaloacetate

Dual fluorescence measurement here refers to detection of two fluorophores which have different excitation wavelengths and emissions. This method not only offers multi-parameters for analytes, but also benefits for monitor of the kinetic processes of reaction. In the decomposition of oxaloacetate (Eq. 5-2), the fluorescence intensities of EuTc-Oxa and NADH were detected, the excitation and emission of EuTc-Oxa are at 405 and 620 nm, the respective
data for NADH are at 355 and 460 nm. The fluorescence kinetic changes of EuTc-Oxa and NADH express the oxaloacetate consumed by MHD catalytically. The time trace of EuTc-Oxa is $Y = 7.73 + 0.38X \ (R = 0.97)$, that of NADH is $Y = 162 + 0.14X \ (R = 0.96)$, $Y$ and $X$ denote corrected fluorescence intensity and time. But the former method is more sensitive because of steeper slope.

![Figure 5.7. Dual fluorescence determination of the decomposition of oxaloacetate via the fluorescence changes of EuTc-Oxa (exc: 400 nm, em: 620 nm) and NADH (exc: 355 nm, em: 460 nm). Concentration of EuTc, oxaloacetate and NADH are 48, 96 and 185 µM, respectively. The activity of MDH is 7 U/mL. $F$ and $F_0$ are the fluorescence intensity of EuTc-Oxa and NADH at $t$ and 0 min.](image)

5.3. Conclusion

The time-resolved fluorescence detection and imaging of main intermediates of the Krebs cycle has been made possible for the first time. Some lifetime and imaging techniques are utilized in this system as the discrimination of their characteristics of fluorescence. The process of the stepwise decompositions of intermediates, such as citrate, isocitrate, oxaloacetate and L-malate can be also directly visualized. This probe will be widely used in other bioassays.
5.4. Experimental Section

5.4.1. Reagents

Citrate lyase (EC: 4.1.3.6, from Enterobacter aerogenes), citrate synthase (EC: 4.1.3.7, from porcine heart), mitochondrial malic dehydrogenase (EC: 1.1.1.37, from porcine heart), and fumarase (EC: 4.2.1.2 from porcine heart) were from Sigma and used without further purification.

Tri-sodium citrate dihydrate, α-ketoglutaric acid (α-oxoglutaric acid), fumaric acid, di-sodium succinate and other inorganic salts were obtained in analytical purity from Merck unless otherwise stated. Ds-isocitric acid, oxaloactic acid, L-sodium malate were in analytical purity from Sigma-Aldrich. All solutions were prepared in 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer of pH 8.0 unless otherwise specified. Europium(III) trichloride hexahydrate was from Alfa Products, and tetracycline hydrochloride from Sigma.

The EuTc standard solution was obtained by dissolving Eu$^{3+}$ chloride and tetracycline (each in 0.5 mM concentration) in 10 mM HEPES buffer of pH 8.0. This reagent is stable for at least 2 months if stored at 4 °C in the dark.

5.4.2. Apparatus

Absorption spectra were acquired on a Cary WinUV photometer. Fluorescence studies of the effect of intermediates (L) in the Krebs cycle on the EuTc spectra were performed on an SLM AB2 luminescence spectrometer. Fluorescence intensity (steady-state and time-gated) were acquired on a Tecan GENios+ micro plate reader. The excitation/emission filters were set to 405/612 nm respectively. The 96-well black, transparence, flat bottom microtiter plates
for imaging were obtained from Greiner Bio-One GmbH. The decay times of EuTc-L were
detected with a pulsed 392-nm laser and an H5783-P04 PMT detector with multiphoton-
counting board in a multipass cuvette. Data were processed with the FluoFit. Dual
fluorescence detection, EuTc-Oxa (excitation is at 405 nm, emission is at 620 nm) and NADH
(excitation is at 355 nm, emission is at 460 nm) were simultaneously were performed in
FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany, www.bmglabtech.com).
Imaging data were evaluated by a self-developed program based on Matlab (6.1, Mathwork,
Natick, MA, USA)

5.5. References

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Chapter 6. Chiral Fluorescence Discrimination of L-/D-Malate

6.1. Introduction

Chiral discrimination is of central importance in the field of molecular chirality. It is a key technology in the molecular elucidation of the processes of chemical biology [1], in the characterization and optimization of new therapeutic drugs [2, 3], as well as in the development of sensory agents for chiral biological molecules.

Fluorescence for chiral recognition [4-8] is known for over 20 years, especially as an interesting topic in supramolecular chemistry. However, most of them so far has been confined to synthetic receptors in organic solvents or water-organic solvent mixtures. Lanthanide complexes [9, 10] have been widely utilized in chiral discrimination based on chiroptical scheme (such as circular dichroism [11-13] and circular polarized luminescence [14-16]), nuclear magnetic resonance [17-19] and in mass spectroscopy [20-22]. But none research based on lanthanide fluorescence intensity and lifetime is reported now.

Malates are optically active α–hydroxy acids that exist abundantly in nature [23] and serve as convenient starting materials for the synthesis of homochiral compounds [24]. Chiral

![Sterochemistry of L- and D-malate](image)

*Figure 6.1. Stereochimetry of L-/ D-malate. H atom (cyan), carbon atom (grey), and oxygen atom (red). The specific optical rotation of malate: $\alpha_D -2.3^\circ$*
discrimination and resolution of malates and other hydroxy acids are mostly achieved through HPLC [25-28] or electrophoresis [29-31]. Only a few fluorescent methods have been reported based on the use of synthetic receptors in organic solvents, such as the bisbinaphthyls for differentiating enantiomeric mandelic acids [32].

Here a novel fluorescent molecular chirality sensor based on the finding that both the fluorescence intensity and the fluorescence decay time are quite different for ternary complexes formed between the europium-tetracycline complex (EuTc) [33-35] and the enantiomeric malates in aqueous solution at neutral pH. As a result, one can discriminate chiral malates by either intensity-based or decay-based fluorescence.

6.2. Results and Discussion

6.2.1. Fluorescent Spectra of Enantiomeric Malate in EuTc

The different spectral characteristics of EuTc complexes with enantiomeric malates are shown in Fig. 6.2. The absorbances of the ternary EuTc-L-malate and EuTc-D-malate complexes peak both at 381 nm, with shoulders at around 405 nm. The maximal emissions of EuTc-L-malate and EuTc-D-malate are also similar at 619 nm and at 618 nm as the europium emission. Side bands are formed at 580, 591, 651, and 697 nm. The different mainband splittings of the two complexes result from different effects of the enantiomers on the crystal field [36]. The maximal capability of discrimination \( [(F_L - F_0)/(F_D - F_0)] \) for chiral malates is found to be 5.9 at 619 nm, where \( F_0, F_L, \) and \( F_D \) denote the fluorescence intensities of EuTc, EuTc-L-malate and EuTc-D-malate, respectively. The quantum yields (QY) [37] of energy transfer from Tc to Eu\(^{3+}\) increase from 0.4% in case of EuTc to 1.7 % of EuTc-L-malate and 0.7 % EuTc-D-malate.
6.2.2. Optimal Experimental Conditions

The fluorescence of the EuTc-malate system is stable between pH 7.7 and 8.7 for EuTc-L-malate and from 7.2 to 8.7 for EuTc-D-malate in Fig. 6.3. The acid dissociation constants [38] of malic acid are $\text{pK}_a = 3.4$ and $\text{pK}_a = 5.1$. In above-mentioned pH range, both protons of malic acid are dissociated, and malate dianion can readily chelate with Eu$^{3+}$. On the other hand, tetracycline [39] (section 4.2.2) may also lose two protons in this case. A 10 mM HEPES buffer solution at 8.0 pH was used throughout this work. It is important to note that phosphate buffer may not be used since phosphate forms a complex with EuTc.
Figure 6.3. The effect of pH on fluorescence intensities of EuTc (50 µM) in D-malate (160 µM) or L-Malate (160 µM).

The time traces of chelation of enantiomeric malates with EuTc are shown in Fig. 6.4. Their fluorescent intensities reach a plateau, usually forming the stable fluorescence in 10 min when malate (either L-malate or D-malate).

Figure 6.4. Time trace of the chelation of enantiomeric malate in EuTc (50 µm). E: Blank, EuTc, A, B, C, and D with malate are 1.6, 0.4, 0.16 and 0.04 mM, respectively.
6.2.3. Fluorescence Decay Times of EuTc-L-malate and EuTc-D-malate

The decay profiles of the emissions of EuTc-L-malate and EuTc-D-malate are also different as detected by TCSPC (Fig. 6.5 a). It reveals a complex decay pattern from which three components can be extracted for either complex. EuTc-L-malate has components of 16 µs (with a relative amplitude of 28 %), 78 µs (48 %) and 104 µs (24 %). The respective data for EuTc-D-malate are 16 µs (62%), 49 µs (35 %) and 123 µs (3 %). Obviously, the average lifetimes of EuTc-L-malate and EuTc-D-malate are largely different (84 and 48 µs, respectively). The distributions of their decay times (Fig. 6.5b) are the basis for their

![Graph (a) showing decay profiles of EuTc-L-malate and EuTc-D-malate](image)

![Graph (b) showing distribution of decay time](image)

*Figure 6.5. The decay profiles of EuTc-L-malate and EuTc-D-malate (a) decay time; (b) distribution of decay time. Eu²⁺: 50 µM; Tc: 50 µM; L-malate: 2 mM; D-malate: 2 mM*
discrimination by time-resolved fluorescence. Consequently, chiral discrimination may also be achieved by TCSPC, despite the presentation of six decay times in a mixed solution and the complication of instrumentation.

6.2.4. Optimal Lag Time for Discrimination of Chiral Malates

As TCSPC is complex, alternatively, time-resolved (“gated”) fluorescence, which is experimentally easier, can be applied to chiral differentiation by measuring fluorescence intensity after a certain delay time, since the effect is much more pronounced for the longer decaying EuTc-L-malate. It is based on the selection of time-delay (lag time) before detection of the selected window (gate) after excitation. The difference of lifetimes of EuTc-L-malate and Eu-Tc-D-malate result in the different intensities in the detection windows. The effect of different lag times on F / F₀ of the EuTc with enantiomeric malates are displayed in Fig. 6.6. For L-malate, the value of F/F₀ increases on going from 0 µs to a 100 µs lag time with a 80 µs integration time, and decreases after 150 µs; for D-malate, the value of F / F₀ has only a minimal increase up to 300 µs lag time. The changes of fluorescence intensity in different lag

![Figure 6.6. The change of F / F₀ versus lag time. F₀ is the fluorescence of EuTc. F is the fluorescence of enantiomer malate (150 µM) in EuTc (50 µM)](image-url)
time can be explained by the distribution of decay time in Fig. 6.5 b. The most efficient fluorescent discrimination of enantiomeric malates is at 120 µs decay time.

6.2.5. Fluorometric Determination of Enantiomeric Excess of Chiral Malate

The applicability of EuTc as a fluorescent probe for chiral discrimination of malate is best presented in the quantitative determination of their optical purity. Steady-state fluorescence can be used to determine the enantiomeric excess ($ee$, defined as in Eq. 6-1) of a system in Fig. 6.7. The $ee$ of malates is indicated by normalized fluorescence intensity according to $(F-F_0)/F_0$ when EuTc was added to a solution of a mixture of L- and D-malate. A linear relationship exists between normalized fluorescence intensity and $ee$.

$$ee\% = \left(\frac{\text{mole of one enantiomer} - \text{mole of other enantiomer}}{\text{total moles of both enantiomers}}\right) \times 100\%$$  \hspace{1cm} \text{(equation 6-1)}

![Figure 6.7](image_url)  

Figure 6.7. Relationships between ee % of malate and fluorescence. $F$ and $F_0$ are the fluorescence intensities of EuTc (50 µM) in presence and absence of a mixture of ([L-malate]+[D-malate]=500 µM).
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The time-resolved fluorescence is also applied to chiral differentiation. Therefore, a 120 µs delay time and a 80 µs integration time were chosen in order to suppress the contribution of D-malate to a substantial extent. Fig. 6.7 shows the resulting plot of gated fluorescence intensity versus $ee$. It is obvious that gated discrimination is more sensitive than the steady-state fluorescence determination for $ee$. The linear correlation coefficients are 0.99 for both graphs when the total concentration is kept constant. Thus, this relationship can be employed and detect the optical purity of malate.

6.2.6. Fluorescence Imaging of Enantiomeric Malates

Fluorescent imaging is a viable tool for two-dimensional presentation (“mapping”) of analytes [40-43]. Here for the first time, chiral discrimination can also be accomplished through time-resolved fluorescence imaging. As shown in Fig. 6.8, both the steady-state and the time-resolved fluorescence imaging can be utilized to visualize the $ee$ of malates. Fig. 6.8

Figure 6.8. Imaging (2D) of enantiomer malates([L-malate]+[D-malate] = 500 µM) in EuTc (50 µM). (a) steady-state fluorescence imaging, window from 0-50 µs; (b) time-gated fluorescence imaging, window from 140-220 µs.
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Figure 6.9. Imaging (3D) of enantiomer malates ([L-malate] + [D-malate] = 500 µM) in EuTc (50 µM). (a) time-resolved imaging, one time window from 160-240 µs; (b) steady-state imaging, one time window from 0-50 µs; Composition of ee (%), in (b) is the same as in (a).

(top) gives the data as a conventional fluorescence two-dimensional graph (false color), and Fig. 6.8 (bottom) as a time-resolved (gated) fluorescence imaging. The latter has large discrimination capability for enantiomeric malates. These data can also be shown as three-dimensional graphs as in Fig. 6.9. Contrast to Fig. 6.8, the conventional and time-gated fluorescence imaging in Fig. 6.9 have more direct and clear expression of the relationship of ee % and imaging.

This probe shows the potential to map the chiral-specific spatial arrangement of ligands on the surface. In addition to, the several promising features of the time-resolved fluorescence imaging of chiral lanthanide complex have been expressed, such as large chiral selectivity (which facilitates quantitative analysis) and the capability of chiral recognition independent of the relative concentrations of analytes and the chirality probe.

6.2.7. Calibration Curves for L-/D-malates

Both steady-state (Fig. 6.10 a) and time-resolved (Fig. 6.10 b) fluorometries were used in the determination of L-/D-malate. In the steady-state method, linear ranges of L-malate and
D-malate are 4.6x10^-6 - 1.8x10^-4 M and 4.6x10^-6 – 7.3x10^-5 M, with the limits of detection (S/N = 3) of 1.8x10^-6 M and 4.6x10^-6 M, respectively. In the time-gated mode, linear ranges are from 7.3 x 10^-7 to 7.3 x 10^-5 M and from 4.6 x 10^-6 to 7.2 x10^-5 M, with the limits of detection of 4.4 x 10^-7 M and 4.6 x 10^-6 M for L-malate and D-malate, respectively. Obviously, measurement of L-malate with EuTc probe by time-resolved fluorescence, contrast to steady-state fluorescence, can improve sensitivity and limit of detection.

6.2.8. Origin of the Enantioselectivity

6.2.8.1. Characteristics of chirality of EuTc-L-malate and EuTc-D-malate

Unlike some coordinatively saturated binary europium complex [44-45], such as that of cryptands, calixarene ligands, EuTc itself is not coordinatively saturated and therefore is prone to form fairly stable ternary complexes to replace the water molecules in the inner coordination field, which is a quencher of the lanthanide fluorescence. As in the case of EuTc-malates, the chirality of EuTc creates a chiral environment for the binding of enantiomeric malates, which would result in different fluorescence. For the characterization of the
asymmetric coordination, the circular dichroism can offer more information about ligands or the relationship between lanthanide and ligands.

![Graphs](a) L-malate and D-malate; (b) Eu-L-malate and Eu-D-malate; (c) Tc and EuTc; (d) EuTc, EuTc-L-malate and EuTc-D-malate. Concentrations of Eu$^{3+}$, Tc, L-malate and D-malate are 50 µM, 50 µM, 2 mM and 2 mM, respectively. In (a) and (b), concentration of malate was used to calculate the value of Mol. CD; in (c) and (d), concentration of Tc was used to calculate the value of Mol. CD.

In Fig. 6.11 a, each enantiomeric malate has a corresponding CD peak which have opposite signs. Enantiomeric malates binding Eu$^{3+}$ still keep the character of chiral in Fig. 6.11b. As the chirality of Tc, this leads to the asymmetric EuTc in Fig. 6.11 c (see Section 4.2.1.4). In Fig. 6.11 d, the values of molar CD in the positive Cotton effects of EuTc, EuTc-L-malate and EuTc-D-malate at 242, 266 and 408 nm have a little different, other Cotton effects (positive and negative) are almost same except that at 222 nm, which is the opposite signals of CD peak from enantiomeric malates (compare with Fig. 6.11b). They can be
discriminated if the value of molar CD is calculated by the concentration of Tc. Nevertheless, if calculated by the concentration of malate, the CD spectra of enantiomeric malates will be not quite different since that can be overshadowed by the strong spectra of EuTc or Tc. It means that the CD spectra of EuTc or Tc can overlap that of enantiomeric malate and make them difficult to distinguish between L-malate and D-malate. On the other hand, it shows that the influences of L-/D-malate in EuTc in the ground state (S₁) are not significant, the different fluorescence intensity may be due to their excited state, because they coordinate differently and the spatial orientation with the tetracycline ligand is different.

6.2.8.2. Composition of EuTc-malate

Job’s method has been employed for determination of the molar ratio of Eu⁺⁺⁺:Tc :malate. In Fig. 6.12 a, the maximum fluorescence intensity is reached at 1:1 molar ratio of Eu⁺⁺⁺: Tc in excess malate, regardless of L-malate or D-malate. In Fig. 6.12b, the concentration of Eu⁺⁺⁺ is excess, the molar ratio of Tc:malate is approximately at 1:2. In combination of Fig. 12 a and b, the molar ratio of Eu:Tc:malate is 1:1:2, or (EuTc):Tc is 1:2.

![Figure 6.12. Job’s plot for molar ration of EuTc-malate.](a) ratio of Eu⁺⁺⁺ to Tc, concentrations of malate and (Eu⁺⁺⁺+Tc) are 333 and 83 µM; (b) ratio of Tc to malate, concentrations of Eu⁺⁺⁺ and ([Tc]+[malate]) are 130 and 50 µM)
Rough estimations of the disassociation constants of EuTc-malates (EuTc:malate = 1:2) are calculated by the following Benesi-Hidebrand type equation [32, 46, 47] for a two binding site saturation.

\[
\frac{F - F_0}{F} = \frac{B_{\text{max}1}[M]}{K_{d1} + [M]} + \frac{B_{\text{max}2}[M]}{K_{d2} + [M]}
\]  

(equation 6-2)

where \(F_0\) and \(F\) are the fluorescence intensities of EuTc in the absence and present of malate, \([M]\) is the concentration of malate, \(B_{\text{max}1}\) and \(B_{\text{max}2}\) are constants. The dissociation constants are \(K_{d1}\) of \(4 \times 10^{-4}\) M, \(K_{d2}\) of \(3 \times 10^{-5}\) M for EuTc-L-malate and \(K_{d1}\) of \(6 \times 10^{-4}\) M, \(K_{d2}\) of \(7\times 10^{-5}\) M for EuTc-D-malate.

6.2.9. Other \(\alpha\)-Hydroxy Acids and Amino Acids

The \(\alpha\)-hydroxy acids lactate and tartrate were also studied. Lactate does not undergo significant changes in fluorescence on addition of EuTc. The enantiomeric tartrates, in contrast, cause an increase in fluorescence intensity of EuTc. The fluorescence intensities of the EuTc complex with enantiomeric tartrates at different lag time are shown in Fig. 6.13a, EuTc-R-tartrate and EuTc-S-tartrate have a maximum discrimination at 60 \(\mu\)s lag time.

(a)  

(b)  

*Figure 6.13. (a) The fluorescence intensities at different lag time for enantiomeric tartrates(5mM) in EuTc; (b) the calibration curves for enantiomeric tartrates in EuTc, with 60 \(\mu\)s lag time. Concentration of Eu\(^{3+}\) and Tc are 50 and 50 \(\mu\)M.*
The calibration curves by time-resolved fluorescence determination are depicted in Fig. 6.14b, but their discriminative ability \([ (F_R - F_0)/(F_S - F_0) ] \) is only 30% of that of enantiomeric malate in EuTc.

Amino acids, such as L-histidine, phenylalanine, arginine, lysine, glutamine, cysteine, asparagine, aspartic acid, threonine, proline, isoleucine, glutamine acid, tryptophan, serine, methionine, valine, leucine and tyrosine, were investigated in EuTc, but no (or only insignificant) fluorescence enhancements were observed.

6.3. Conclusion

The probe EuTc represents a novel lanthanide-based sensing probe for chiral discrimination using time-resolved fluorescence, and for direct imaging of enantiomeric hydroxy acids in aqueous solution of near-neutral pH. Chiroselective imaging is of great potential with respect to mapping the spatial arrangement of (chiral) ligands on solid surfaces, i.e. in biosensor arrays and in high-throughput screening.

6.4. Experimental Section

6.4.1. Reagents

L-sodium malate, D-malic acid were obtained in analytical purity from Sigma-Aldrich. (2R, 3R)+) tartaric acid, (2S, 3S)-(−) tartaric acid, and other inorganic salts were obtained in analytical purity from Merck unless otherwise stated. All solutions were prepared in 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer of pH 8.0 unless otherwise specified. Europium(III) trichloride hexahydrate was from Alfa Products, and tetracycline hydrochloride from Sigma.
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The EuTc standard solution was obtained by dissolving Eu\(^{3+}\) chloride and tetracycline (each in 0.5 mM concentration) in 10 mM HEPES buffer of pH 8.0. This reagent is stable for at least 2 month if stored at 4 °C in the dark.

6.4.2. Apparatus

Absorption spectra were acquired on a Cary WinUV photometer. Fluorescence studies were performed on an SLM AB2 luminescence spectrometer. Fluorescence intensity (steady-state and time-resolved) were acquired on a Tecan GENios+ micro plate reader. The excitation/emission filters were set to 405/612 nm respectively. The decay times of EuTc-malate were detected with a pulsed 392-nm laser and an H5783-P04 PMT detector with multiphoton-counting board in a multipass cuvette. Data were processed with by FluoFit (PicoQuant). Circular dichroism (CD) spectra were acquired on JASCO model J – 710 spectropolarimeter (www.jasco.de). The 96-well black, transparence, flat bottom microtiter plates for imaging were obtained from Greiner Bio-One GmbH. Imaging data were evaluated by the IDL software module.

6.5. References


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7. Summary

The dissertation describes the development of europium-derived fluorescence probes for biological substances in aqueous solution. A scheme is shown in Fig. 7.1. Europium-tetracycline-hydrogen peroxide and europium-tetracycline-hydroxy acid probes have the merits of large Stokes’ shift, line-like emission and long lifetime.

Figure 7.1. Scheme of research.

HP: hydrogen peroxide; EuTc: europium-tetracycline complex; HA: hydroxy acid.

In chapter 1 the mechanism of lanthanide complex luminescence is introduced. The three main approaches for time-resolved fluorescence in heterogeneous phase, namely direct lanthanide chelate label-based luminescence assay (DLCLLA), dissociation enhanced lanthanide fluoroimmunoassay (DELFIA) and enzyme-amplified lanthanide luminescence (EALL) are reviewed.

The time-resolved assay of the activity of peroxidase (POx) by the fluorescent probe europium-tetracycline-hydrogen peroxide (EuTc-HP) is presented in chapter 2. At first, the catalytic mechanism of POx, as a widely studied enzyme across a range of scientific disciplines, is discussed. Quantification is based on the finding that the strongly fluorescent complex EuTc-HP (which is in equilibrium with unbound H$_2$O$_2$) is indirectly decomposed by
POx to give the weakly fluorescent EuTc following the consumption of H$_2$O$_2$ in MOPS buffer at neutral pH. The rate of consumption of the EuTc-HP as monitored via the decrease in fluorescence intensity (or change in decay time) is a direct parameter for the activity of the POx. The time-resolved assay can detect as little as 1.0 × 10$^{-5}$ Units/mL of POx, with a dynamic range from 4.0 × 10$^{-5}$ to 5.9 × 10$^{-3}$ Units/mL. The effects of cyanide, hydroxylamine, and azide (all known to inhibit POx) are also studied.

When POx is exploited as a label and EuTc-HP as a probe, ELISA and oligonucleotide hybridization assays can be developed as shown in chapter 3. The time-resolved fluorescent assays for biological specimens have advantages over the conventional steady-state assays. Two schemes for POx-ELISA, sandwich and direct, have been investigated. The linear range is from 0.1 to 8.0 ng/ml for IgG in POx-sandwich ELISA, with 0.1 ng/ml of the limit of detection in time-gated method. EuTc-HP also can be used as a reversible molecular sensor for the imaging of POx-ELISA. The competitive oligonucleotide hybridization assay by the POx label is discussed as well.

The direct fluorescence detection and imaging of citrate are demonstrated in chapter 4. The method is based on the fact that the weakly fluorescent europium-tetracycline (EuTc) complex reversibly associates with citrate to form the strongly fluorescent europium-tetracycline-citrate (EuTc-Cit) complex in HEPES buffer at pH 8.0. Average fluorescence lifetime is also increased from 44 µs (for EuTc) to 88 µs (for EuTc-Cit). Steady-state fluorometry, TCSPC, time-resolved fluorometry and RLD imaging have been used in citrate determination. The time-resolved assay has a dynamic response between 1.6 x 10$^{-7}$ and 5.6 x 10$^{-5}$ M, with a detection limit of 6.0 x 10$^{-8}$ M for citrate. Compared with other main analytical methods for citrate, it is the most sensitive and simplest scheme available up to now. Different
tetracycline derivatives are also studied. In addition, this probe is simple to prepare, stable both in solution and in solid, and compatible with the blue laser diodes.

The Krebs cycle, a key series of metabolic reactions in aerobic cellular respiration, is occurring in the mitochondria of animals and plants. Fluorescence imaging of main intermediates in the Krebs cycle and the some bioprocesses of the intermediates are depicted for the first time in chapter 5. Intermediates, i.e. oxaloacetate, citrate, isocitrate, α-ketoglutarate (KG), succinate, fumarate and L-malate, can reversibly associate with EuTc to form EuTc-L complexes having different fluorescent intensities and lifetimes in neutral pH. The steady-state fluorescence intensity and rapid lifetime determination imaging have been employed in the visualization of the Krebs cycle. The stepwise detection of the formation and decomposition of intermediates can be performed via kinetic fluorescence changes. In addition, dual fluorescence method, monitoring both NADH and EuTc-L, is exploited.

Fluorescence chirality sensing is presented in chapter 6. EuTc as a lanthanide chelate is employed for the fluorescence discrimination of enantiomeric malates in aqueous solution. The fluorescence discriminating ability \((F_L - F_0) / (F_D - F_0)\) is 5.9 at 619 nm, which is more sensitive than any other chiral fluorometry for α-hydroxy acids reported so far. The average lifetimes and quantum yields of EuTc-L-malate and EuTc-D-malate are 84 and 48 µs, 1.7 % and 0.7 %, respectively. It is important that this probe can be also applied for the time-resolved fluorescent determination of the optical purity (ee %) of malate with 120 µs lag time. Chiral malates can also be achieved by time-resolved imaging. Other chiral α-hydroxy acids, lactate and tartrate are also discussed. It is the first report on using lanthanide ternary complexes as a “turn-on” fluorescence chiral molecular sensor.
8. Zusammenfassung


Abbildung 7.1. Schematische Zusammenfassung der Forschungsarbeit.
HP: Wasserstoffperoxid; EuTc: Europium(III)-tetracyclin; HA: Hydroxysäure.

Im ersten Teil der Arbeit werden die grundlegenden Eigenschaften und Mechanismen der Lumineszenz von Lanthanid-Komplexen erläutert. Im Mittelpunkt stehen dabei die drei wichtigsten Methoden zeitaufgelöster Fluoreszenzmessungen in heterogenen Systemen, zum einen der „direct lanthanide chelate label-based luminescence assay“ (DLCLLA), zum anderen der „dissociation enhanced lanthanide fluorimmunoassay“ (DELFIA), und schließlich die „enzyme-amplified lanthanide luminescence“ (EALL).

In Kapitel 2 wird ein neuer zeitaufgelöster Fluoreszenztest zur Bestimmung der Aktivität von Peroxidase (POx) eingeführt, der auf dem System Europium-Tetracyclin-
Wasserstoffperoxid (EuTc-HP) als molekulare Sonde basiert. Zunächst wird der Katalyse-
mechanismus von POx beschrieben, einem Enzym, das in der wissenschaftlichen Forschung 
bereits eingehend untersucht wurde. Die Quantifizierung der Enzymaktivität erfolgt über den 
direkten Abbau des stark fluoreszierenden EuTc-HP-Komplexes. Dieser steht im Gleichge-
wicht mit ungebundenen H₂O₂ in der gepufferten Lösung, das durch den enzymatischen Ein-
fluss von POx umgewandelt wird. Die Umsatzrate von EuTc-HP kann als direkter Parameter
für die Enzymaktivität durch die Abnahme der Fluoreszenzintensität oder Veränderungen in
der Fluoreszenzabklingzeit detektiert werden. Der zeitlich aufgelöste Fluoreszenztest hat eine 
Nachweissgrenze von 1 x 10⁻⁵ U/mL POx mit einem dynamischen Messbereich von 4.0 x 10⁻⁵ 
bis 5.9 x 10⁻³ U/mL. Der Einfluss von Inhibitoren wie Cyanid, Hydroxylamin und Azid auf 
die Aktivität von POx wurde ebenfalls untersucht.

Wird POx als Biomarker und EuTc-HP als molekulare Sonde eingesetzt, können 
ELISAs und DNA-Hybridisierungsassays detektiert werden. Kapitel 3 verdeutlicht, dass 
zeitaufgelöste Fluoreszenztests für Biomoleküle den gebräuchlichen Intensitätsmessungen 
überlegen sind. Ein Sandwich- und ein direkter POx-ELISA wurden im Rahmen dieses 
Projekts untersucht. Dabei wurde ein linearer Messbereich für IgG von 0,1 bis 8.0 ng/ml mit 
einer Nachweissgrenze von 0,1 ng/ml erhalten. EuTc-HP kann auch als reversibler molekula-
rer Sensor für das Imaging von POx-ELISAs verwendet werden. Ein kompetitiver Hybridisie-
 rungstest für Oligonukleotide mit Hilfe von POx-Markern wird ebenfalls diskutiert.

Ein direkter Fluoreszenznachweis auf Citrat, einem in der Natur allgegenwärtigen Stoff, 
mittels bildgebender Verfahren wird in Kapitel 4 entwickelt. Die Methode basiert auf der 
Fähigkeit des schwach fluoreszierenden Europium-Tetracyclins reversibel Citrat zu binden 
und dabei einen stark fluoreszierenden Europium-Tetracyclin-Citrat-Komplex (EuTc-Cit) zu 
bilden. Die mittlere Fluoreszenzlebensdauer wird in Folge ebenfalls von 44 µs (EuTc) auf 88
Zusammenfassung

µs (EuTc-Cit) erhöht. Stationäre Fluorometrie, TCSPC, zeitaufgelöste Fluorometrie und RLD Imaging können so zur Bestimmung von Citrat herangezogen werden. Der zeitaufgelöste Fluoreszenztest ergibt einen dynamischen Messbereich von $1.6 \times 10^{-7}$ bis $5.6 \times 10^{-5}$ M bei einer Nachweigrenze von $6.0 \times 10^{-8}$ M Citrat. Verglichen mit anderen Bestimmungsmethoden für Citrat ist dies momentan das empfindlichste und einfachste verfügbare analytische Verfahren. Darüber hinaus ist dieser molekulare Sensor einfach zu synthetisieren, stabil sowohl in gelöster als auch in fester Form und kompatibel zur Anregungswellenlänge eines blauen Diodenlasers. Die Eigenschaften anderer Tetracyclin-Derivate wurden ebenfalls untersucht.


Schließlich werden in Kapitel 6 chirale Fluoreszenzsensor beschrieben. EuTc kann zur Unterscheidung der beiden enantiomeren Formen von Malat in wässriger Lösung benutzt werden. Der Unterschied in der Fluoreszenzintensität nach der Koordination beider Isomerer
(F_L-F_0) / (F_D-F_0) beträgt 5,9 bei einer Emissionswellenlänge von 619 nm. Das ist ein höherer Faktor als bei allen anderen bekannten chiralen fluorimetrischen Verfahren für α-Hydroxy-säuren. Die mittleren Fluoreszenzabklingzeiten von EuTc-L-Malat und EuTc-D-Malat betragen 84 bzw. 48 µs, die Quantenausbeuten 1,7 % bzw. 0,7 %. Die enantiomeren Formen von Malat können auch mittels zeitaufgelöstem Fluoreszenzimaging angezeigt werden. Weitere chirale α-Hydroxysäuren, sowie Laktat und Tartrat werden ebenso diskutiert. Somit ist diese die erste Arbeit, bei der ternäre Lanthanid-Komplexe als „einschaltbare“ molekulare chirale Fluoreszenzsensoren eingesetzt werden.

* Please notice that the German summary is just for your information. For the details, please check the English version.
9 Recent Publications and Patent

9.1. Publications


9.2. Patent


US patent in application
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