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Lanthanide Complex-Based Fluorescence Label for Time-Resolved Fluorescence Bioassay

Jingli Yuan[1,2] and Guilan Wang[1]

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Different from organic fluorescence dyes, fluorescent lanthanide complexes have the fluorescence properties of long fluorescence lifetime, large Stokes shift and sharp emission profile, which makes them favorable be used as the fluorescent labeling reagents for microsecond time-resolved fluorescence bioassay. Lanthanide complex-based fluorescence labels have been successfully used for highly sensitive time-resolved fluorescence immunoassay, DNA hybridization assay, cell activity assay, and bioimaging microscopy assay. Since the technique allows easy distinction of the specific fluorescence signal of the long-lived label from short-lived background noises associated with biological samples, scattering lights (Tyndall, Rayleigh and Raman scatterings) and the optical components (cuvettes, filters and lenses), the sensitivity of fluorescence bioassay has been remarkably improved. This paper summarized the recent developments of lanthanide complex-based fluorescence labels and their applications in time-resolved fluorescence bioassays mainly based on the authors' researches and relative publications.

KEY WORDS: lanthanide complex; fluorescence label; time-resolved fluorescence bioassay.

INTRODUCTION

In the areas of life science, biotechnology and clinical diagnostics, various bio-labeling reagents, including radio-isotopes [1], enzymes [2,3], fluorescence dyes [4,5], and chemiluminescent compounds [6,7], have been developed and widely used for the detections of biological molecules. Among these reagents, due to the high sensitivity and easy operation of fluorescence detection, fluorescence dyes have occupied the most important position in today's biotechnology, such as in human genome project and biochip technology [5,8–10]. However, the conventional fluorescence bioassay has a problem that the fluorescence detection is easily affected by the background noises caused by biological sample and analysis instrument [11], which has limited the effective application of fluorescence dyes for highly sensitive bioassay.

The fluorescence of some lanthanide ions (Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+}) can be dramatically enhanced when they are coordinated with appropriate organic ligands [12–15]. The fluorescence properties, including quantum yield, molar extinction coefficient, excitation wavelength and emission lifetime, are strongly dependent on the structure of the ligand. Compared with organic fluorescence dyes, fluorescent lanthanide complexes have several characteristic fluorescence properties including very long fluorescence lifetime (usually over several hundreds microseconds or more than 1 ms for Eu^{3+} and Tb^{3+} chelates, and 10–100 μs for Sm^{3+} and Dy^{3+} chelates), large Stokes shifts (fluorescent lanthanide complexes have absorption maximum generally below 400 nm, and emit fluorescence maximally around 615, 545, 643, and 574 nm for Eu^{3+}, Tb^{3+}, Sm^{3+}, and Dy^{3+} chelates, respectively), very sharp emission profile with the full width at half maximum of $\sim$10 nm. The excitation and emission spectra of an Eu^{3+} complex of N,N,N^1,N^1-[(4′-phenyl-2,2′:6′,

[1] Department of Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Zhongshan Road, Dalian, P. R. China.
[2] To whom correspondence should be addressed. E-mail: jingliyuan@yahoo.com.cn

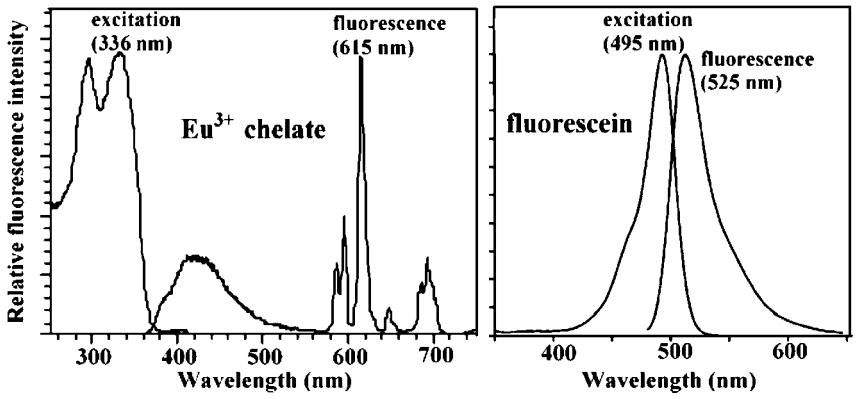

Fig. 1. Excitation and emission spectra of an Eu^{3+} complex with N,N,N′,N′-[(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo)]tetrakis(acetate) and fluorescein.

2″-terpyridine-6,6″-diyl)bis(methylenenitrilo)]tetrakis (acetate) and a typical organic fluorescence compound, fluorescein are shown in Fig. 1.

To fluorescence bioassay, how to eliminate background noises caused by the autofluorescence from biological samples, the scattering light associated with Tyndall, Rayleigh and Raman scatterings, and the luminescence from the optical components such as the cuvettes, filters and lenses is a main problem for the improvement of detection sensitivity. For this purpose, the microsecond time-resolved fluorometric technique using lanthanide complexes as the fluorescence labels is a very effective method, since the background noises are short-lived with the luminescence lifetime of nanosecond to a few microseconds [16]. The principle of microsecond time-resolved fluorometric measurement is shown in Fig. 2. After the sample is excited by a flash lamp, the luminescence from all molecules and instrument begins to decay exponentially. Since the short-lived background is rapidly decreased, it can be effectively eliminated during the delay time. This permits the signal from long-lived lanthanide label to be only measured during the counting time. Generally, the measurement is cycled many times for one cuvette (usually one second per cuvette), which further improves the signal to noise ratio.

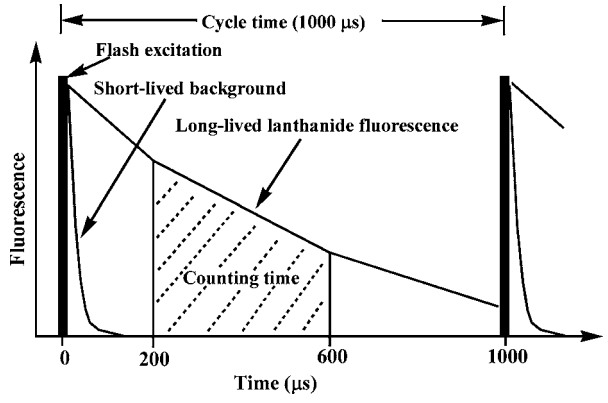

Fig. 2. Principle of the time-resolved fluorometric measurement with a delay time of 200 μs, a counting time of 400 μs, and a cycle time of 1000 μs.

It is a well-known fact that many lanthanide complexes, especially the complexes of Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+} with some β-diketones and aromatic amine derivatives, are strongly fluorescent. However, for biological molecule labeling, a labeling reagent should have good solubility and high stability in aqueous buffer, furthermore an appropriate coupling group is also necessary. Therefore, up to now only a few of fluorescent lanthanide complexes suitable to be used as bio-labels were developed successfully and used for highly sensitive time-resolved fluorescence bioassays. The principle, development and application of lanthanide fluorescence labels for time-resolved fluorescence immunoassay (TR-FIA), DNA hybridization assay, cell activity assay, and microscopy bioimaging have been summarized in several reviews [17–25]. In this paper, the developments of lanthanide complex-based fluorescence labels and their applications in bioassays are summarized mainly based on the authors' researches and relative publications.

LANTHANIDE COMPLEX-BASED FLUORESCENCE LABELS

Some bidentate β-diketones, such as 2-naphthoyltrifluoroacetone, 2-thenoyltrifluoroacetone, and pivaloyltrifluoroacetone, can form strongly fluorescent complexes with lanthanide ions. Since these complexes are not very stable (the formation constant only in the order of 10^3–10^6) [26,27], and will be dissociated at lower

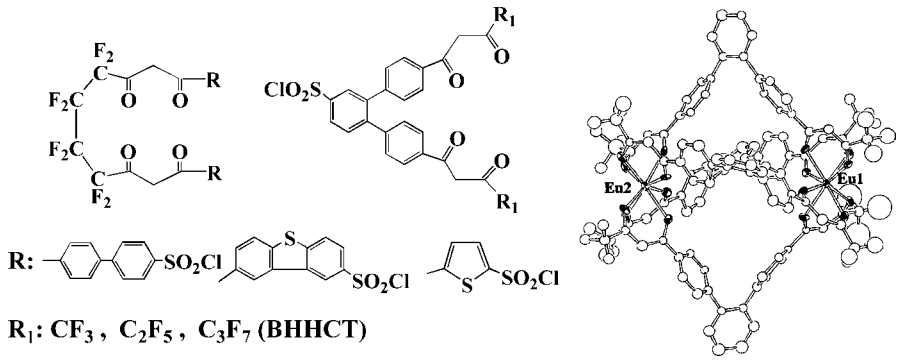

Fig. 3. Structures of some chlorosulfonylated tetradentate β-diketones and an Eu^{3+} complex with 4,4″-bis(4,4,4-trifluoro-1,3-dioxobutyl)-*o*-terphenyl.

concentration in aqueous solution to cause the fluorescence to be extinguished, the lanthanide complexes of conventional bidentate β-diketones are not suitable to be used as the bio-labels. To circumvent this problem, we synthesized several chlorosulfonylated tetradentate β-diketones, and developed their applications for protein labeling and time-resolved fluorescence bioassays [28–30], their analogues were also synthesized and applied in bioassays recently [31–33]. The structures of these β-diketones are shown in Fig. 3. Different from bidentate β-diketones, the tetradentate structure in these β-diketones can increase the stabilities as well as the fluorescence intensities of their Eu^{3+} complexes, since the coordinated water molecules in the first coordination sphere of Eu^{3+} can be effectively decreased. The crystal structure determination [34] of an Eu^{3+} complex with 4,4″-bis(4,4,4-trifluoro-1,3-dioxobutyl)-*o*-terphenyl has shown that the bis(β-diketonyl)-*o*-terphenyl-Eu^{3+} complex is dinuclear having a cage structure, and no water molecule was coordinated to Eu^{3+} ion in crystal state (Fig. 3).

The chlorosulfonylated β-diketone-Eu^{3+} complexes are easy to be conjugated to proteins through the formation of sulfonamide (protein-NH-SO_2-label). The main drawback of these complexes as bio-labels is their low solubility in aqueous buffer, and in some case the solubility of a labeled protein is lower in aqueous buffer if the labeling ratio is heavy. Because the β-diketone-Eu^{3+} complex-labeled bovine serum albumin (BSA) is highly soluble in aqueous buffer, BSA can be employed as a carrier protein for increasing the labeling ratio. In this way, the BSA conjugate of antibody, hapten or streptavidin (or avidin) can be prepared and used for coupling to chlorosulfonylated β-diketone-Eu^{3+} complex.

Some aromatic amine derivatives can also form strongly fluorescent complexes with lanthanide ions. These ligands mainly include the derivatives of pyridine, 2,2′-bipyridine, 2,2′,2″-terpyridine, and 1,10-phenanthroline [35–45]. Fig. 4 shows the structures of some fluorescent lanthanide complexes with aromatic amine derivatives that can be used as bio-labels, in which the energy collected by the 'antenna' effect of aromatic amine skeleton can be transferred to the central lanthanide ion, and the structure of polyacid makes a highly stable lanthanide complex be formed in aqueous solution. The labels shown in Fig. 4 can be coupled to proteins or other amino-compounds through their isothiocyanato, chlorosulfonyl, N-hydroxysuccinimide ester or (4,6-dichloro-1,3,5-triazin-2-yl)amino group in an aqueous buffer. Generally, the reaction of chlorosulfonyl, N-hydroxysuccinimide ester or (4,6-dichloro-1,3,5-triazin-2-yl)amino group with a amino-compound is fast (1–3 h), and that of isothiocyanato group is slow (24–48 h).

The conjugate of DTPA (diethylenetriamine pentaacetic acid) with 7-amino-4-methyl-2(1H)-quinolinone (DTPA-cs124) was also developed as a bio-label for time-resolved fluorescence bioassays [46–48]. It can be conjugated to a protein by using its anhydride (DTPA anhydride-cs124), the amino- and thiol-reactive forms. Its Eu^{3+} and Tb^{3+} complexes are fluorescent having the fluorescence lifetimes of over 500 μs (Eu^{3+} complex) and 1000 μs (Tb^{3+} complex). The structures of DTPA-cs124, its amino- and thiol-reactive forms are shown in Fig. 5.

Recently, some fluorescent lanthanide complex-conjugated macromolecules were developed as bio-labeling reagents for time-resolved fluorescence bioassays. These kinds of the labels include $(biotin)_x$-polyvinylamine-$(BCPDA\text{-}Eu^{3+})_y$ [BCPDA is 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; $x = 5$–10; $y = 50$–100] [49–51], {4-[2-(4-isothiocyanatophenyl)ethynyl]-2,6-bis{[N,N-bis(carboxymethyl)amino]methyl}pyridine}-Eu^{3+} complex conjugated poly(Glu:Lys)-streptavidin or poly(Glu:Lys)-BSA-streptavidin [52], fluorescent lanthanide complex-doped

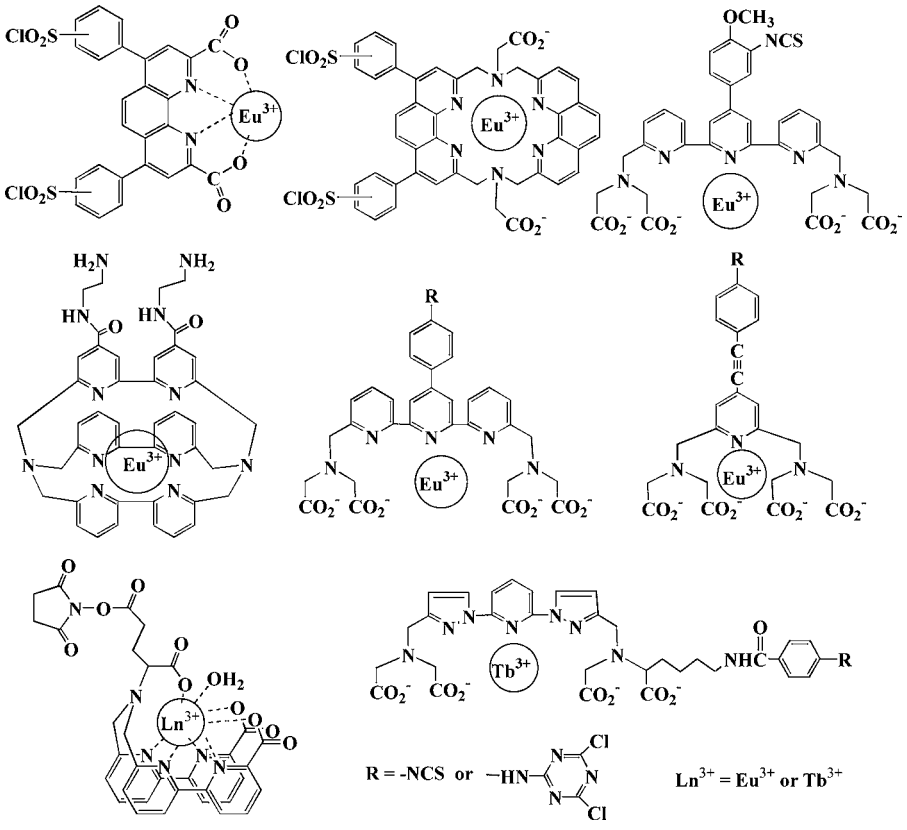

Fig. 4. Structures of some fluorescent lanthanide complexes with aromatic amine derivatives that can be used as bio-labels.

polystyrene latex particles [53–57] and silica nanoparticles [58–61]. Since many fluorescent lanthanide complex molecules were attached to these labels, which effectively enhanced the fluorescence of the labels, and thus the sensitivities of time-resolved fluorescence bioassays using these labels have been remarkably improved.

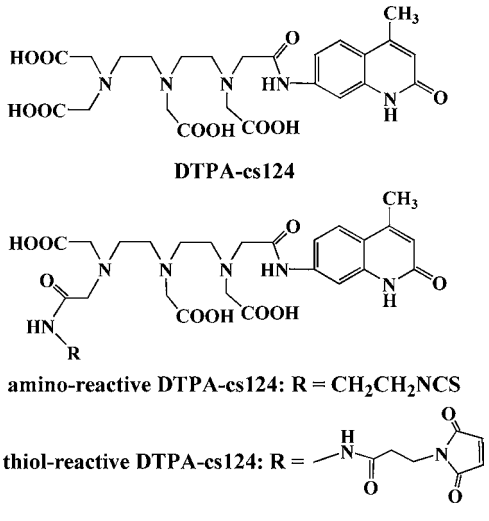

Fig. 5. Structures of DTPA-cs124 and its amino- and thiol-reactive derivatives.

We recently established a covalent binding-copolymerization method to prepare silica-based fluorescent europium nanoparticles that can be used for biolabeling and highly sensitive time-resolved fluorescence bioassay [62]. This kind of fluorescent nanoparticles prepared in a water-in-oil microemulsion consisting of a conjugate of (3-aminopropyl)triethoxylsilane (APS) bound to BHHCT-Eu^{3+}, free APS, tetraethyl orthosilicate (TEOS), Triton X-100, *n*-octanol, water, cyclohexane and ammonia water are uniform and small size (<40 nm in diameter), highly photostable, strongly fluorescent having a fluorescence quantum yield of over 50% and a long fluorescence lifetime of 384 μs. Different from the luminophore-doped nanoparticle labels, since the Eu^{3+} complex molecules were covalently bound to silicon atoms in the nanoparticles, the dye-leaking problem in bioassay processes using the luminophore-doped nanoparticle labels can be effectively resolved. Furthermore, since free amino groups have been directly introduced to the surface of the nanoparticle label by using

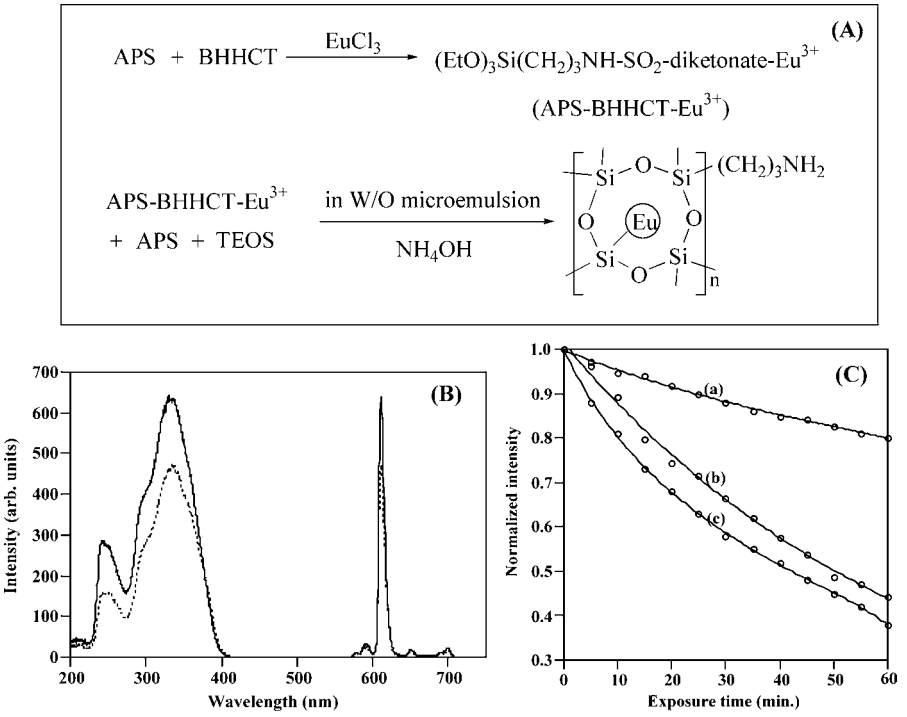

Fig. 6. Preparation principle of silica-based fluorescent europium nanoparticle label (A), its time-resolved excitation and emission spectra (B, *solid line*: the spectra of the nanoparticles; *dashed line*: the spectra of free BHHCT-Eu^{3+} complex), and photobleaching experiment results (C, a: the nanoparticles; b: free BHHCT-Eu^{3+} complex; c: rhodamine 6G) (modified from Ref. [62]).

free APS in the preparation, its bio-labeling procedure is very simple and reproducible. Preparation principle of the nanoparticle label, its excitation and emission spectra and photobleaching experiment result are shown in Fig. 6.

APPLICATIONS OF LANTHANIDE FLUORESCENCE LABELS TO TR-FIA

The BHHCT-Eu^{3+} complex has been successfully used as a label for both noncompetitive and competitive TR-FIA. In these assays, BHHCT-Eu^{3+}-labeled streptavidin (SA), SA-BSA conjugate, and hapten-BSA conjugate were used for the assays. A comparison of human immunoglobulin E (IgE) TR-FIA using BHHCT-Eu^{3+}-labeled SA and SA-BSA showed that the use of the labeled SA-BSA can increase the detection sensitivity and improve the detection precision [63]. Furthermore, the labeled SA-BSA is more suitable for long preservation. It has been confirmed that BHHCT-Eu^{3+} labeled SA-BSA can be stored at -20°C for over two years without losing the biological activity and the fluorescence intensity. For noncompetitive immunoassay, BHHCT-Eu^{3+} was used for the TR-FIAs of α-fetoprotein (AFP) [30], IgE [63], thyroid-stimulating hormone (TSH) [64], stromal cell-derived factor 1 protein (SDF-1) [65], cytokines in human serum (or plasma) [66], and type IV collagen in mouse urine [67]. In which, the method of SDF-1 detection was used for the detection of SDF-1 level in normal and HIV-infected human plasma (Fig. 7). The results firstly reveal that the SDF-1 level in human plasma can be significantly increased by HIV infection. For competitive immunoassay, BHHCT-Eu^{3+} was used for the TR-FIAs of methamphetamine (MA) in human urine and hair [68], p21 protein in human serum [69], bensulfuron-methyl [70], 17β-estradiol and estriol in water [71].

Since BHHCT-Sm^{3+} complex is also fluorescent, the dual-label TR-FIA using BHHCT-Eu^{3+} and BHHCT-Sm^{3+} was developed and used for the simultaneous measurement of AFP and carcinoembryonic antigen (CEA) [72], and the psychopharmaceuticals and stimulants [73] in serum samples. However, since BHHCT-Sm^{3+} is weak fluorescent and its fluorescence lifetime is relatively shorter (30–70 μs), the above Eu^{3+}-Sm^{3+} dual-label TR-FIA needed a complicated procedure and gave a lower sensitivity for BHHCT-Sm^{3+} measurement. To overcome this drawback, a new fluorescent Tb^{3+} complex, N, N, N^1, N^1-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine]tetrakis (acetate)-Tb^{3+} (BPTA-Tb^{3+}) was

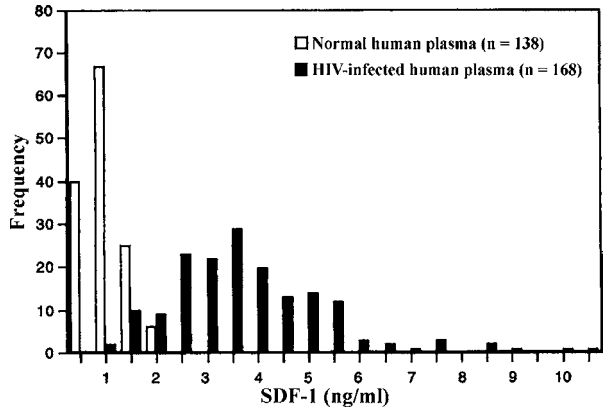

Fig. 7. Plasma SDF-1 level in normal and HIV-infected human plasma (modified from Ref. [65]).

synthesized and used for the simultaneous measurement of AFP and CEA in human serum by using an Eu^{3+}-Tb^{3+} dual-label TR-FIA method [74]. The structure of BPTA (BPTA can be bound to SA by using its succinimidyl monoester form) and the assay format for AFP and CEA simultaneous measurement are shown in Fig. 8. Since BHHCT-Eu^{3+} and BPTA-Tb^{3+} are all strongly fluorescent, simultaneous measurement method of AFP and CEA was improved with a simpler procedure and higher sensitivity.

APPLICATIONS OF LANTHANIDE FLUORESCENCE LABELS TO DNA HYBRIDIZATION ASSAY

DNA hybridization assay is one of the most widely used research tools for gene expression analysis and diagnosis of infectious, genetic, and neoplasmic diseases and microbial taxonomy [75–77]. However, the conventional DNA hybridization assay method is tedious and time-consuming since the method requires many steps including immobilization of the DNA on a solid matrix, prehybridization, hybridization, washings, and detection. To circumvent this problem, a homogeneous assay method would be highly desirable since the steps of immobilization, prehybridization, and washing are not necessary. A homogeneous DNA hybridization assay method based on fluorescence resonance energy transfer (FRET) has been developed [78,79], in which two DNA probes modified with a donor fluorescence dye and an acceptor fluorescence dye, each being complementary to the two different sequences of a target DNA, were used. After the hybridization of two DNA probes with target DNA in a homogeneous solution, the donor and the acceptor come close to each other, thus the target DNA can be detected by measuring the sensitized emission of the acceptor. However in such system using organic fluorescence dyes as donor and acceptor pairs, sensitivity is low due to the strong effect of background emission caused by donor emission and the directly excited acceptor emission at measurement wavelength.

A homogeneous time-resolved fluorescence DNA hybridization assay format based on FRET from lanthanide complex (BHHCT-Eu^{3+} or BPTA-Tb^{3+}) to Cy dye (Cy5 or Cy3) is shown in Fig. 9 [80,81]. In the assay, two DNA probes are labeled with biotin on the 3′-terminus and Cy dye on the 5′-terminus, respectively. After the hybridization of two DNA probes with the target sequence in a homogeneous solution, biotin and Cy dye are brought into close proximity. In the presence of the lanthanide complex-labeled SA, the biotin-SA binding makes the lanthanide complex and Cy dye come close to each other, which results in the FRET from the lanthanide chelate to Cy dye, and thus the sensitized emission of Cy dye can be detected by time-resolved fluorescence measurement. The detection limits using FRET format for the target DNA are 200 (BHHCT-Eu^{3+}-Cy5) [80] and 30 pM (BPTA-Tb^{3+}-Cy3) [81], respectively.

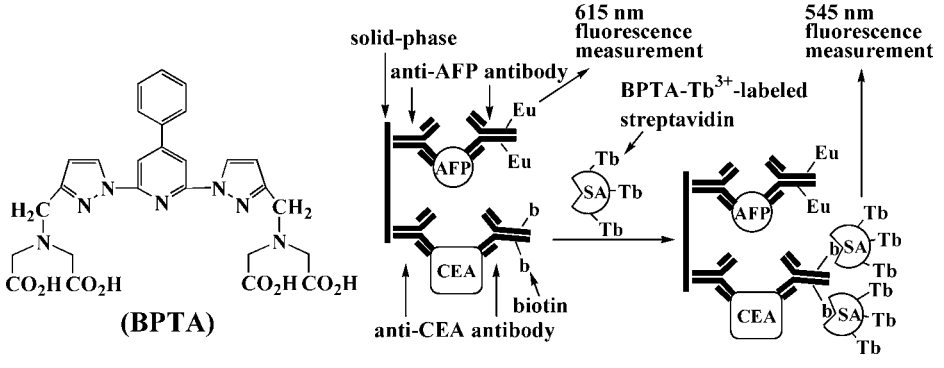

Fig. 8. Structure of BPTA and principle of Eu^{3+}-Tb^{3+} dual-label TR-FIA for the simultaneous measurement of AFP and CEA in human sera (modified from Ref. [74]).

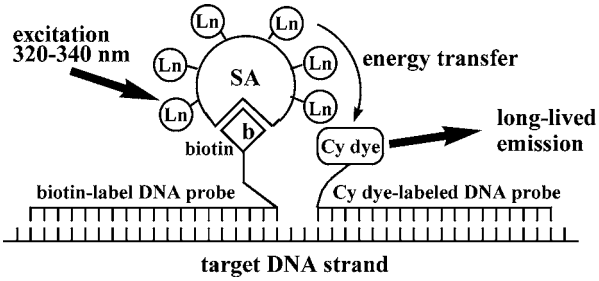

Fig. 9. Principle of the homogeneous time-resolved fluorescence DNA hybridization assay based on fluorescence resonance energy transfer from lanthanide complex to Cy dye. Ln is BHHCT-Eu^{3+} or BPTA-Tb^{3+}; Cy dye is Cy5 or Cy3.

In addition, a more sensitive homogeneous time-resolved fluorescence DNA hybridization assay method based on the formation of an EDTA-Eu^{3+}-β-diketonate ternary complex in the DNA hybrid was developed [82]. The principle of the method is shown in Fig. 10, in which two DNA probes labeled with a bidentate β-diketone on the 3′-terminus and EDTA-Eu^{3+} complex on the 5′-terminus were used, respectively. The two DNA probes are complementary to the contiguous regions of a target DNA. After the hybridization of two DNA probes with the target sequence in a homogeneous solution, β-diketone and EDTA-Eu^{3+} come close to each other to form a strongly fluorescent EDTA-Eu^{3+}-β-diketonate ternary complex, thus the target DNA can be directly detected by time-resolved fluorescence measurement. This assay format gives the detection limit of 6 pM for a target DNA.

APPLICATIONS OF LANTHANIDE FLUORESCENCE LABELS TO TIME-RESOLVED FLUORESCENCE BIOIMAGING

Time-resolved fluorescence bioimaging microscopy technique using lanthanide complex-based fluorescence labels has been developed and used for immunohistochemistry, in situ DNA hybridization and detections of environmental pathogens. Since the fast decaying autofluorescence in the biological specimens can be easily suppressed, the biological image can be clearly observed without interference from autofluorescence. By using the Eu^{3+} complex-labeled antibodies or SA, the time-resolved fluorescence microscope has been employed for the detections of tumor associated antigen C242 in the malignant mucosa of human colon, type II collagen mRNA in developing human cartilaginary growth plates and the HPV type specific gene sequences in the squamous epithelium of human cervix [83], the biotinylated concanavalin A at the cell surface [84], and environmental pathogens [31,85–87]. The detections of environmental pathogens have shown that the signal-to-noise ratio of fluorescence microscopy detection can be increased several ten times by using a time-resolved fluorescence mode.

CONCLUSIONS

Through 20 years' development, lanthanide complex-based fluorescence labels and time-resolved fluorescence bioassay technique have become a powerful tool for diagnostics and biotechnology. The combination of a lanthanide fluorescence label and time-resolved fluorescence technique has been proved to give dramatic high sensitivity for bioassays.

Furthermore, the recent development of fluorescent lanthanide nanoparticle bio-labels has shown that this kind of bio-labels would be favorably useful for highly sensitive time-resolved fluorescence bioimaging and biochip technologies. The signal amplification and the photostability enhancement of fluorescent lanthanide nanoparticle labels make the labels suitable be used for the detection of long-time continuous excitation. In this field, the conventional organic fluorescence labels and other luminescent nanoparticle labels have the problems of photobleaching

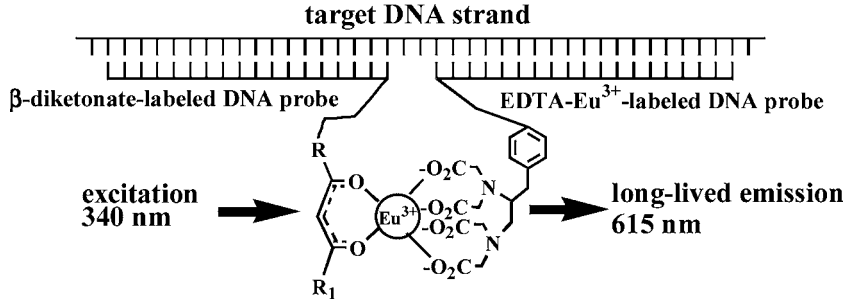

Fig. 10. Principle of the homogeneous time-resolved fluorescence DNA hybridization assay by DNA-mediated formation of an EDTA-Eu^{3+}-β-diketonate ternary complex (modified from Ref. [84]).

and interference of strong background noises [88,89], whereas which can be easily solved by using a lanthanide nanoparticle label combined with a time-resolved fluorescence detection technique.

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