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REVIEW

Tanja Steinkamp · Uwe Karst

Detection strategies for bioassays based on luminescent lanthanide complexes and signal amplification

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Abstract Two attractive detection strategies for bioassays are reviewed in this article. Both approaches use the highly sensitive time-resolved luminescence detection of lanthanide complexes in combination with a signal amplification scheme. While enzyme-amplified lanthanide luminescence (EALL) has been an established technique for more than a decade, nanoparticles doped with luminescent lanthanide complexes have been introduced very recently. In this paper, the basic properties and major applications of both techniques are presented, and their future perspectives are discussed critically.

Keywords Lanthanides · Luminescence · Bioassays · Amplification

Introduction

Enzyme assays and immunoassays are commonly used tools in clinical chemistry and are applied to the analysis of a large number of important analytes [1, 2]. They are characterized by high selectivity and sensitivity. Improvements in the field of bioassays can be achieved either by optimizing the biochemical processes involved or by developing or improving detection schemes. Time-resolved lanthanide luminescence detection has been established as a powerful approach in recent years, and has been summarized in several reviews [3, 4]. This review focuses on a particular aspect in the field of time-resolved lanthanide luminescence detection: the combination with an amplification step yielding a gain in selectivity and sensitivity.

T. Steinkamp · U. Karst (✉)
Department of Chemical Analysis and MESA Institute for Nanotechnology, University of Twente,
P.O. Box 217, 7500 AE Enschede, The Netherlands
E-mail: u.karst@utwente.nl
Fax: +31-53-4894645

The four lanthanide ions Tb(III), Eu(III), Sm(III) and Dy(III) exhibit characteristic emission spectra with narrow line-shaped emission bands in the visible range of the electromagnetic spectrum. However, it is hard to evoke this emission because of the low molar absorptivity of the naked metal ions [5]. To circumvent this problem, the lanthanide ions are complexed to organic ligands possessing energy levels close to those of the metal ions. The principle of the energy transfer is depicted in Fig. 1. In these complexes, it is possible to excite the ligand by irradiation of light at its characteristic excitation wavelength. The absorbed energy is then transferred from the ligand to the central lanthanide ion, which subsequently emits light at its characteristic emission wavelengths [5]. Figure 2 shows the emission spectra of Tb(III) and Eu(III) complexes, respectively. The energy transfer, which has a low probability by the laws of quantum mechanics, accounts for the two great advantages of lanthanide luminescence: first, the extremely large Stokes' shift (often exceeding 200 nm) and second, the long-lived luminescence. Typical decay times of the lanthanide complexes are in the high microsecond or the low millisecond range, allowing time-resolved measurements with comparably simple and inexpensive instrumentation. The principle of time-resolved measurements is explained in Fig. 3. After excitation with a light pulse, mostly from a xenon lamp or a laser, a delay time is used to allow the (almost) complete decay of scattered excitation light and of background fluorescence of organic molecules, typically exhibiting lifetimes in the nanosecond range. Afterwards, the long-lived fluorescence of the lanthanide complexes is recorded selectively. For these reasons, time-resolved fluorescence measurements of lanthanide chelates are frequently more sensitive and more selective than conventional fluorescence detection methods.

Based on these properties, lanthanide luminescence has evolved to become a highly sensitive and selective detection method. Lanthanide chelates have become popular labels for immunoassays [6, 7], and attractive detection schemes have been developed and commer-

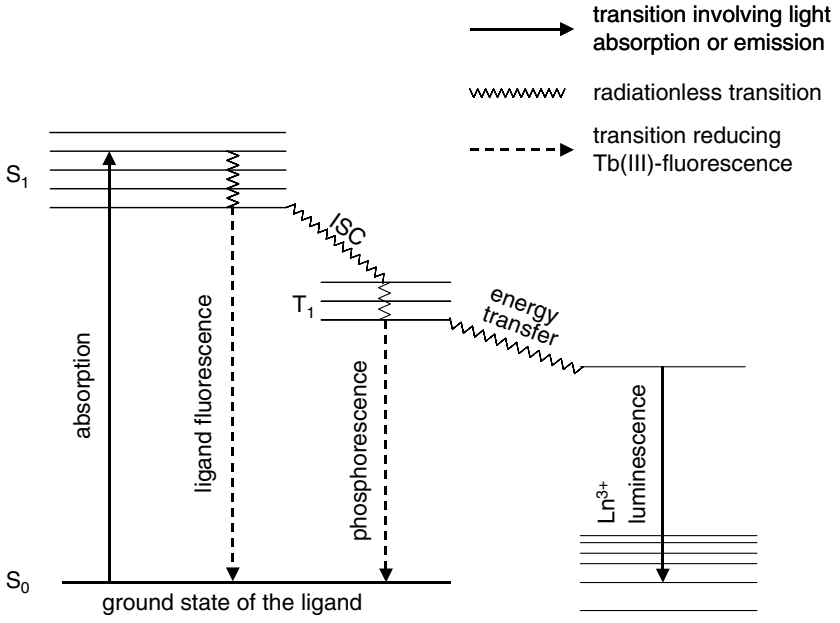


Fig. 1 Mechanism of the energy transfer from the ligand to the lanthanide(III) ion

cialized, including the DELFIA [8] and the FIAgen [9] techniques. However, a further improvement in the sensitivities of these methods would allow the detection of even lower analyte concentrations. Therefore, the combination of amplification strategies with lanthanide luminescence is a highly promising approach.

In recent years, two attractive techniques for the combination of lanthanide luminescence measurements with a coupled amplification step have become available. The first comprises the enzyme-catalyzed formation of a ligand, which is, in contrast to the substrate, able to transfer energy to a lanthanide cation. This strategy combines the enzymatic amplification of established enzyme-linked immunosorbent assays (ELISA) with the sensitivity of lanthanide luminescence techniques, and is known as enzyme-amplified lanthanide luminescence (EALL). The second uses luminescent lanthanide complexes that are entrapped in nanoparticles. If the nanoparticles are used as labels in immunoassays, every single particle comprises the luminescent properties of a large number of individual lanthanide complexes. Although both strategies were developed to fulfill similar requirements, they differ significantly with respect to possible applications. The EALL technique is not only capable of detecting affinity reactions with bound enzyme labels, such as antibody–antigen or biotin–streptavidin binding, but also enzymatic conversions themselves. Although one reaction step more (addition of the lanthanide solution) is required compared with assays based on conventional fluorophores, the total effort is only

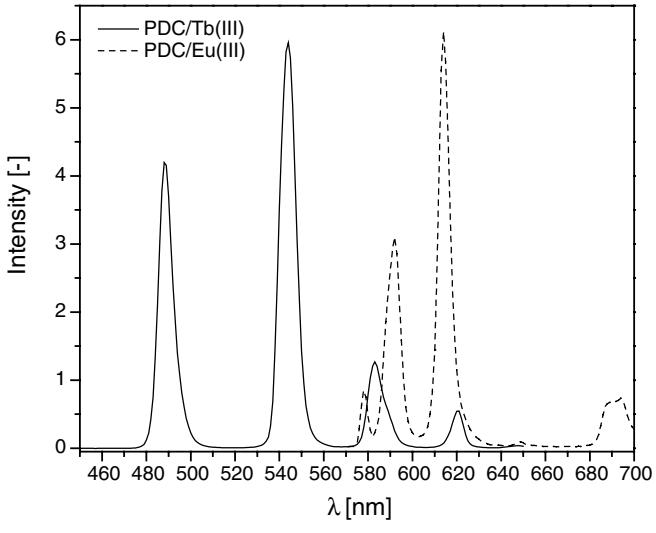


Fig. 2 Emission spectra of Tb(III) and Eu(III), complexed to 2,6-pyridinedicarboxylic acid (*PDC*)

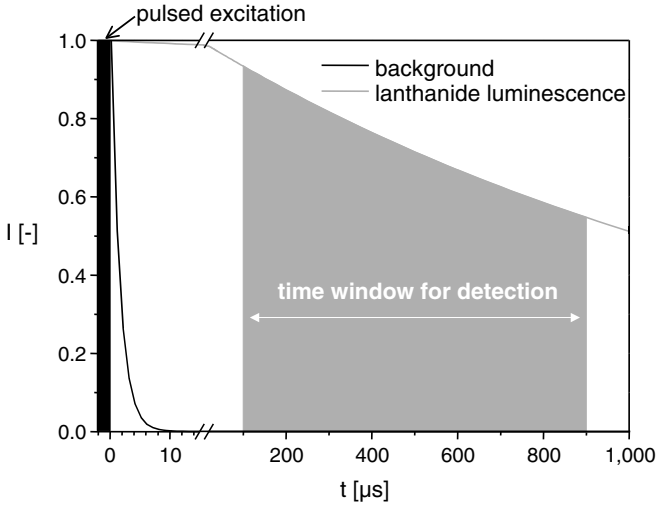


Fig. 3 Principle of time-resolved luminescence measurements

slightly higher for an EALL system. It should also be noted that this additional step is not significant in an automated analyzer system. Luminescent nanoparticles, however, cannot change their fluorescent properties upon an enzymatic reaction, and are therefore limited to the detection of affinity assays. On the other hand, the detection scheme for affinity assays using nanoparticles is simpler and can be easily transferred to related applications.

Within this review, the most prominent features and applications of both techniques are introduced and critically discussed with particular emphasis on possible future trends. Related techniques utilizing other types of nanoparticles, including those based on semiconductor materials ("quantum dots") [10, 11, 12], lanthanide-doped solid-state materials [13, 14], or polymers containing ruthenium complexes with long-lived fluorescence [15] are characterized by attractive properties as well, but are not the subject of this review.

Enzyme-amplified lanthanide luminescence

The term EALL was introduced by Evangelista et al [16, 17] in 1991. In a pioneering article, they introduced a new detection scheme for highly sensitive bioanalytical assays. Here, enzymes are either used as a label or as the analyte of interest. A method for the determination of alkaline phosphatase (aP) with the use of 5-fluorosalicylic phosphate (FSAP) and Tb(III) has been developed (see Fig. 4) and a model immunoassay for Rat IgG has been performed with alkaline phosphatase as marker. The effectiveness of the EALL principle was further demonstrated by applying the detection scheme to the determination of other hydrolytic and redox enzymes, namely xanthine oxidase, β-galactosidase and glucose oxidase. For xanthine oxidase and β-galactosidase, two other salicylic acid (SA) derivatives, salicylaldehyde and salicyl-β-D-galactoside, were employed as substrates for the enzymatic reaction. Both were, after conversion to SA, complexed to Tb(III). In contrast, for the determination of glucose oxidase, 1,10-phenanthroline-2,9-dicarboxylic acid dihydrazide has been used as substrate. In this case, the resulting 1,10-phenanthroline-2,9-dicarboxylic acid forms a luminescent complex with Eu(III). This article indicated that EALL had the potential to be used as an alternative to ELISA and radioimmunoassays (RIAs).

The same authors used the EALL principle for detection in nucleic acid hybridization assays in microwell, dot-blot and Southern blot formats [18]. For the assay, which was performed in microwells, the denatured target DNA sequence (pBR322 linearized with Hind III enzyme) was hybridized with a biotinylated probe (pBR322 BioProbe), and after washing and blocking the unspecific binding sites, aP-labeled avidin was added. For the detection of the aP label, FSAP solution in buffer was given into the well. After incubation, in which the FSAP was cleaved in an aP-catalyzed reaction to FSA (see Fig. 4), a solution containing Tb(III) and EDTA was added and the resulting lanthanide luminescence was recorded. In the dot-blot and Southern blot assays, which were performed on nylon membranes, 5-*tert*-octylsalicyl phosphate was used as reagent instead of the FSAP. The authors report a higher sensitivity of the non-isotopic EALL method in comparison to radioisotopic detection. Various bio- and immunoassays were developed, which routinely use the EALL principle as the detection method. Those assays employ aP as marker and use the cleavage of a salicylic acid phosphate derivative to the respective SA derivative with subsequent formation of a luminescent complex with Tb(III) and EDTA. Figure 5 illustrates the basic principle of most of those assays.

These include the determination of thyrotropin (thyroid-stimulating hormone, TSH) in serum [19]. The samples are pipetted into the wells of a microtiter strip, which was coated previously with monoclonal anti-TSH antibody. Biotinylated detection antibody is added, which subsequently reacts with the aP-labeled streptavidin. Detection is performed as described above with FSAP as substrate.

The same assay, and a related one for thyroxine, was used to prove the applicability of 4-methylumbelliferyl phosphate, which was selected from 33 candidate fluorogenic chelators for Eu(III) and Tb(III) as substrate for EALL with Eu(III) [20]. In this case, the fluorescence is quenched with the ongoing enzymatic reaction because only the phosphate forms luminescent complexes with Eu(III), whereas the generated 4-methylumbelliferone is not a sensitizer for lanthanide luminescence.

Later, it was found that phosphotyrosine but not tyrosine itself forms luminescent complexes with Tb(III). Based on this, fluorometric and time-resolved immunofluorometric assays for acidic phosphatase and protein-tyrosine phosphatase activity have been developed [21].

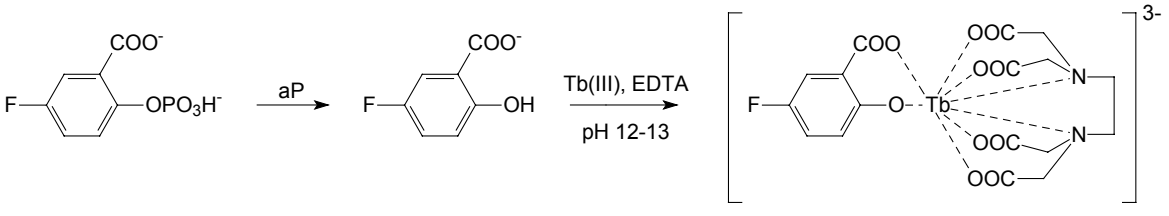


Fig. 4 Reaction sequence of the aP-catalyzed cleavage of FSAP to FSA with subsequent complexation to Tb(III)/EDTA

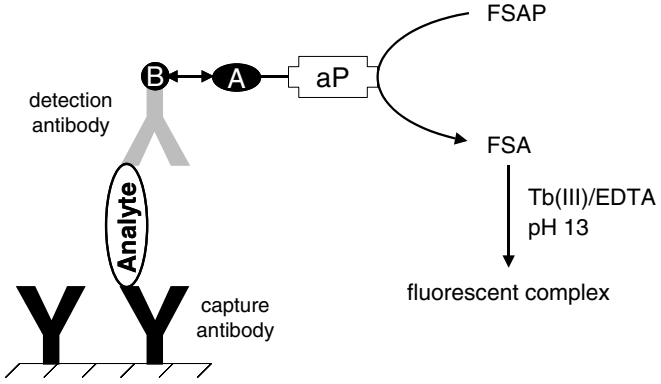


Fig. 5A, B Principle of an immunoassay with detection by means of EALL. The detection antibody is marked with biotin (**B**), which interacts with aP labeled avidin (**A**)

Some assays for the determination of α-fetoprotein have also been performed using the EALL technique [22, 23, 24]. These assays are based, as in the case of the thyrotropin assays, on the biotin-streptavidin interactions, and aP was again used as label. However, the measurement principles and the employed enzyme substrates were evolved in order to enhance the sensitivity. Lianidou and coworkers for example, used second derivative synchronous scanning fluorescence spectrometry for the screening of 14 different bidentate ligands, some of which are compounds of pharmaceutical interest. The most promising, diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid), has been converted to its phosphate ester, which has been found useful in the EALL-type immunoassay for α-fetoprotein. The same group introduced a sandwich-type immunoassay for interleukin 6, a cytokine that has been implicated in the pathology of several diseases, using diflunisal phosphate as enzyme substrate for aP [25]. In another paper, they described the determination of tumor necrosis factor-α [26], a potent pre-inflammatory and immunoregulatory cytokine, which is produced by a large number of different cell types in response to bacterial toxins and inflammatory and other invasive stimuli. Therefore, it is a critical mediator for the diagnosis of a wide variety of diseases.

Another approach has been chosen for the determination of chronic myelogenous leukemia-specific mRNA sequences, also named the Philadelphia translocation. This DNA abnormality can be directly associated with malignancy. Here, the analyte is additionally amplified by a polymerase chain reaction (PCR), after labeling of the PCR primers with biotin or a hapten [27, 28, 29, 30], The immunoassay technique is analogous to the previously mentioned assays, and detection is performed with the FSA/Tb(III)/EDTA-system too. Similar attempts have been made to determine prostate-specific antigen mRNA [31, 32]. Improved quantification of the PCR products was achieved using an internal standard procedure [33]. As template, a 308 bp DNA fragment was used, and an internal standard of identical size and identical primers, but with a different centrally located sequence of 26 bp, was synthesized. As the target analyte is co-amplified with a known amount of the internal standard, the latter compensates for the varying efficiency of the amplification. Again, the FSA/Tb(III)/EDTA system is used for the final detection after hybridization with digoxigenin-modified probes and attachment of an anti-digoxigenin-aP conjugate. The same group later published a similar approach for sandwich-type DNA hybridization assays [34]. A related two-cycle amplification scheme led to a 30-fold improvement of the signal and a tenfold improvement of the signal-to-noise ratio compared to a single cycle of amplification [35]. However, the repeatability for the two-round amplification scheme is only moderate, with relative standard deviations of over 10%.

Barrios and Craik [36] recently used the FSA/Tb(III)/EDTA system to assay the substrate specifity of proteases. In a library of tetrapeptides, FSA was bound to the *N*-terminus of each peptide. Upon cleavage of the respective amide bond by a protease (compare to Fig. 6), FSA is released and detected as described above. In principle, this approach should also allow a rapid quantitative analysis of other proteases based on dedicated FSA-modified substrates.

Other groups made attempts to further increase the number of applications for EALL by using different enzymes and substrates. Xie et al [37] described an EALL method that uses *p*-hydroxybenzoic acid as

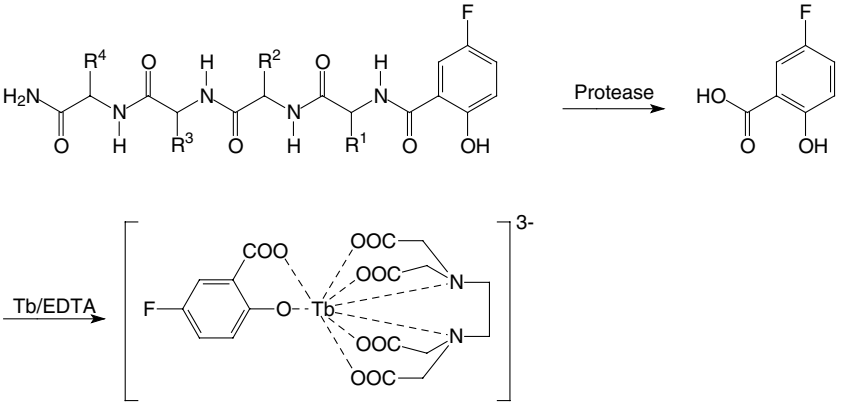


Fig. 6 Reaction sequence employing the FSA/Tb(III)/EDTA system to evaluate the substrate specifity of proteases (R^1–R^4 are the rest of the respective amino acids)

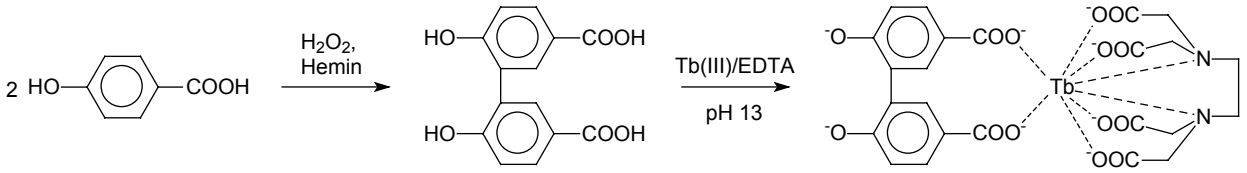


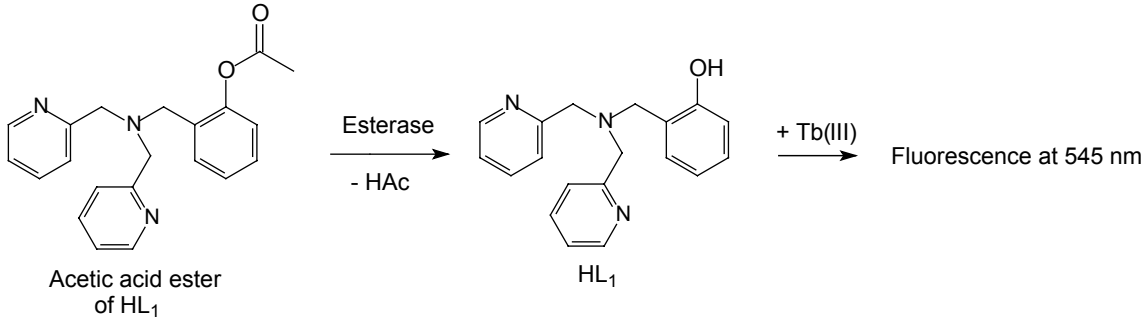
Fig. 7 Hemin-catalyzed dimerization of *p*-hydroxybenzoic acid in the presence of H_2O_2; the dimer is subsequently complexed to Tb(III)/EDTA

substrate for horseradish peroxidase (HRP) with subsequent detection by means of Tb(III) luminescence. The method has been used for the determination of tuberculosis antibodies. The same group used *p*-hydroxybenzoic acid to study hemin catalysis [38]. Hemin is often used as substitute for HRP and in this case, it catalyzes the dimerization of *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid and *p*-hydroxyphenylpropionic acid in the presence of H_2O_2. The reaction scheme is depicted in Fig. 7. It was found out that only the dimer of *p*-hydroxybenzoic acid, di-*p*,*p*'-hydroxybenzoic acid, forms a luminescent complex with Tb(III), whereas all educts and dimers of the two other substrates do not sensitize Tb(III). This implies that – in respect to Tb(III) – the complexing site was the two carboxylic acid groups of the dimer of *p*-hydroxybenzoic acid. Additionally, a simple label assay for a hemin-BSA-conjugate was performed which exhibited a detection limit for the conjugate of 2×10^{-10} mol/l. In contrast to these results, it was later found that the dimers of a variety of substrates for the above described reaction, in this case catalyzed by HRP, do form luminescent complexes with Tb(III) in the presence of CsCl [39, 40]. Hence, the authors of this article suggest that the complexation sites for the lanthanide ion are not the carboxylic acid groups but the two hydroxyl groups of the dimer. The limit of detection for HRP with *p*-hydroxyphenylpropionic acid as enzyme substrate was in this case 2×10^{-12} mol/l. This reaction was further applied to the detection of two different ELISAs, one for the determination of goat anti-rabbit IgG, the other for human anti-gliadin IgG in serum. It turned out that detection by means of EALL was applicable to both assays with good results.

Until now, most EALL schemes have been performed at alkaline pH, where the luminescence intensities yield the highest values. However, addition of EDTA at high pH is required in order to avoid precipitation of lanthanide hydroxide. This is very unfavorable in terms of the limits of detection of the EALL methods, because EDTA itself is able to sensitize the lanthanide ions to a minor extent. One of the first EALL methods that can be performed at neutral pH was developed by Steinkamp et al [41] after screening a small library of tripod ligands. The ligand bis(2-pyridylmethyl)-(2-hydroxybenzyl)-amine (HL_1) has been converted to its acetic acid ester, which was subsequently cleaved in an esterase-catalyzed reaction to the acid. Detection was performed by means of Tb(III)-sensitized luminescence. Figure 8 shows the ester hydrolysis with subsequent complexation. Owing to the fact that the tetradentate tripod ligand forms highly stable complexes with Tb(III) and that the luminescence measurements are performed at neutral pH, the addition of co-complexing agents like EDTA can be avoided and background fluorescence is further decreased. With this detection principle, a limit of detection of 10^{-9} mol/l for esterase from porcine liver could be achieved [41]. Based on this reaction, a method for the direct monitoring of an enzymatic conversion has been developed, which was compared to reaction monitoring by means of electrospray ionization mass spectrometry (ESI-MS) [42].

Very recently, another EALL system that operates at nearly neutral pH without the addition of EDTA has been developed for the determination of esterases or of xanthine oxidase (XOD) [43]. It employs PDC, which is able to sensitize Tb(III) as well as Eu(III) in a very efficient way. PDC has been converted to a variety of alkyl esters, which are then cleaved in an esterase-catalyzed reaction to the diacid again. The reaction scheme is shown in Fig. 9. 2,6-pyridinedicarboxaldehyde has been used for the determination of XOD, which also yields PDC when oxidized in the presence of XOD.

Fig. 8 Esterase-catalyzed ester cleavage of the acetic acid ester of HL_1; the resulting HL_1 is complexed to Tb(III) and the luminescence intensity is read out



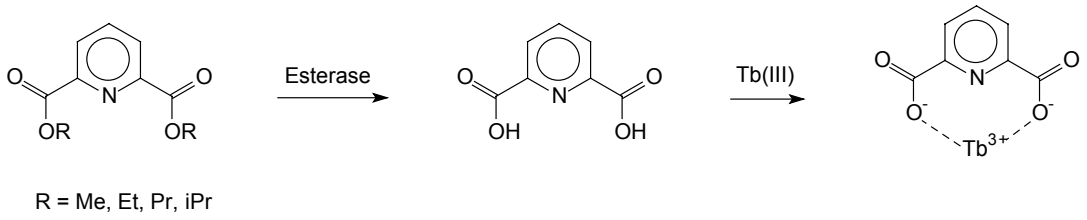


Fig. 9 Reaction sequence of the esterase-catalyzed hydrolysis of PDC esters with subsequent complexation to Tb(III)

Luminescent lanthanide complexes in nanoparticles

An attractive approach to enhancing the signal intensity of lanthanide luminescence is to enclose a large amount of luminescent lanthanide chelates in one nanoparticle, which is then used as a label in an affinity assay. The majority of the commercially available nanoparticles have until now contained Eu(III)/β-diketonate chelate complexes with a polystyrene cover, exhibiting similar spectroscopic properties to the free chelate complexes in solution.

Härma et al [44, 45, 46, 47, 48, 49, 50] applied these nanoparticles to different approaches to the determination of prostrate specific antigen (PSA). A commercially available nanoparticle with an average diameter of 107 nm and tris(naphthyltrifluorobutanedione) as ligand for Eu(III) was employed. The assay was performed in anti-PSA microplates. Biotinylated PSA was given onto the plate and the streptavidin-coated nanoparticle was added after incubation and washing. Evaluation was performed by means of TRF imaging after the plate was dried [44]. The principle of the described assay is explained in Fig. 10. The assay was further investigated with respect to solid-phase association and dissociation, non-specific binding, and affinity constants of the various binding site density nanoparticle-antibody bioconjugates [47]. Later, the assay was applied to the determination of real samples, namely PSA in serum [49]. Recently, a paper from these authors described the replacement of the microtiter plate by microparticles of size of 60–920 μm in diameter [50].

Eu(III) chelate-dyed nanoparticles with a size of 92 nm have recently been used as donors in a homogeneous proximity-based immunoassay for estradiol [51]. Regarding this competitive assay, the nanoparticles were initially coated with 17β-estradiol (E2) specific recombinant antibody Fab S16 fragments. The coated particles were placed into the BSA-blocked wells of a microtitration plate. 17β-estradiol was then added which binds to the nanoparticles, and after incubation of the plate an E2-Alexa680-conjugate was pipetted into the wells. AlexaFluor 680 is a near-infrared fluorescent label whose excitation spectrum overlaps with the emission spectrum of Eu(III). Hence, after the E2-Alexa680-conjugate is bound to the remaining free binding sites on the nanoparticle, it is possible to excite the Alexa label with the emission light of the nanoparticle due to the

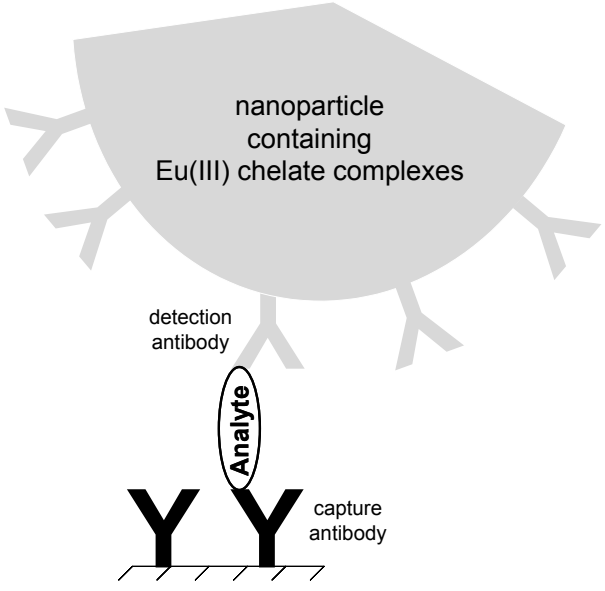


Fig. 10 Principle of an immunoassay employing nanoparticles containing Eu(III) chelate complexes

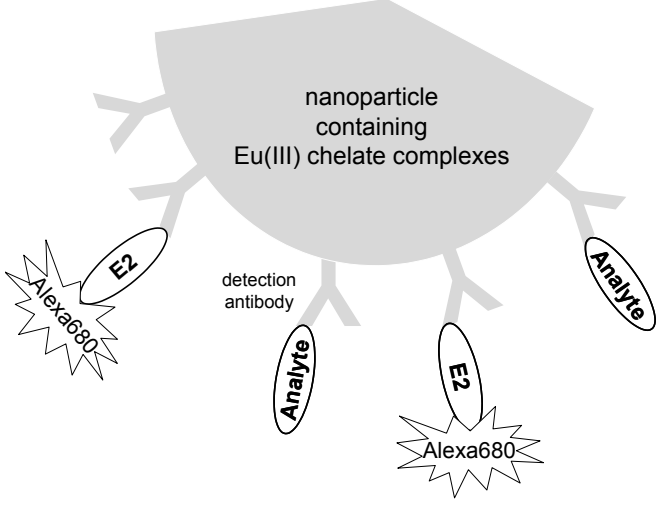


Fig. 11 Principle of the competitive proximity-based nanoparticle assay for the determination of 17β-estradiol

Förster energy transfer taking place between the two luminescent substances [52]. The principle of this assay is depicted in Fig. 11. Detection limits of 70 pmol/l for E2 could be achieved.

Conclusions and future perspectives

Despite the attractive properties of both approaches presented in this review, they are still only used by a limited number of scientists. With respect to the EALL technique, this is mainly due to the need for laborious development steps in order to apply the principle to other enzymes beyond those described in the literature. It is not trivial to develop a new EALL system, as the potential substrate must, aside from the required interaction with the enzyme, be characterized by a change from no observed energy transfer to a strongly sensitizing effect for one of the four lanthanide cations upon conversion. This certainly hampers the application of EALL to a broader range of enzymes. Furthermore, the EALL technique requires a little more experimental effort when compared to classical ELISA detection, and it provides significant advantages only in those cases where the sensitivity of the detection scheme is the bottleneck for the total sensitivity of a bioassay. On the other hand, many situations exist in which EALL could solve important analytical problems and would certainly be worth the experimental work – mainly the cases of enzyme determination where ultimate sensitivity is required.

A large expansion in the field of luminescent nanoparticles based on lanthanide chelates may be expected within the next few years. The reason for this is the possibility of applying this method in a more general way, making it easier for industry to provide commercial solutions. Furthermore, the complexity of the detection scheme is lower compared to ELISAs and it should facilitate an at least slight increase in the speed of analysis. Furthermore, multiplexing with at least four different colors should in principle be possible and will add new tools for multianalyte measurements in the future.

For both methods, however, their potential application to the field of bioassays is far from being fully exploited, and new highly selective and sensitive detection schemes based on these approaches can certainly be expected soon.

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