Europium and Terbium Chelators as Candidate Substrates for Enzyme-labelled Time-resolved Fluorimetric Immunoassays

Eleftherios P. Diamandis
Department of Clinical Biochemistry, Toronto Western Hospital, 399 Bathurst Street, Toronto, Ontario, Canada M5T 2S8, and Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario, Canada M5G 1L5

Thirty-three candidate fluorogenic chelators for Eu^{3+} and Tb^{3+} were examined. Ten were found to form highly fluorescent complexes with Eu^{3+} and five with Tb^{3+}. In all cases, the fluorescence observed was lanthanide-specific, long-lived and was monitored by microsecond time-resolved fluorimetry. The fluorogenic chelators could be quantified, in the presence of an excess of lanthanide, at levels of $< 1 \times 10^{-8}$ mol l^{-1} in all but one case. Two new fluorogenic chelators could form ternary complexes with Eu^{3+} and Tb^{3+}, in the presence of ethylenediaminetetraacetic acid. The structures of the chelators identified are such that enzyme substrates could be developed and used for enzyme-labelled time-resolved fluorimetric immunoassays. It was found that one of the new chelators identified, 4-methylumbelliferyl phosphate, which forms fluorescent, long-lived complexes with Eu^{3+}, could be split by alkaline phosphatase to phosphate and 4-methylumbelliferone, which does not form fluorescent complexes with Eu^{3+}. Based on this, highly sensitive immunoassays for thyroid stimulating hormone and thyroxine in human serum were developed. It is anticipated that some of the identified chelators could be transformed into enzyme substrates suitable for highly sensitive, enzyme-labelled time-resolved fluorimetric immunoassays.

Keywords: *Time-resolved fluorimetric immunoassay; enzyme label; europium and terbium; fluorogenic chelator; thyroxine and thyroid stimulating hormone*

The fluorescent europium and terbium chelates are interesting mainly because of their potential application as labels for highly sensitive time-resolved fluorimetric immunoassays.[1–4] The first commercially available time-resolved fluorescence immunoassay system, Delfia (Pharmacia–LKB, Turku, Finland), uses Eu^{3+} as the immunological label.[5,6] A second-generation time-resolved fluorimetric immunoassay system, FIAgen (CyberFluor, Toronto, Canada), uses the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid as the immunological label.[7,8] These two systems, along with the principles of time-resolved fluorimetry and its application to immunoassay and other bioanalytical techniques, have already been reviewed.[1–4]

Recently, many groups have also used Tb^{3+} and its chelates as immunological and nucleic acid labels.[9,10] In general, Tb^{3+} is inferior to Eu^{3+} in terms of detectability. However, Eu^{3+} and Tb^{3+} can be used simultaneously for dual analyte assays.[11]

The mechanism of fluorescence of the Eu^{3+} and Tb^{3+} chelates has been reviewed previously.[1–4] These two ions, when excited by radiation, emit very weak metal ion fluorescence, which is not analytically useful. The fluorescence is dramatically enhanced when the metal ion forms a chelate with appropriate organic ligands. An important property of these chelates is that the radiation is absorbed at a wavelength characteristic of the ligand and is emitted as a line spectrum characteristic of the metal ion. This is as a result of intramolecular energy transfer from the ligand to the central metal ion.[12] In general, it is difficult to predict theoretically which organic molecule ligands can form highly fluorescent complexes with Eu^{3+} and Tb^{3+}. Some classes of compounds, *i.e.*, the diketones, tetracyclines, phenanthrolines, acetylene derivatives, five-membered heterocyclic ring derivatives, benzoic acid derivatives, biphenyl derivatives, pyridine derivatives, pyrimidine and pyrazine derivatives, di- and tripyridyl derivatives, quinoline derivatives, azauracil and purine derivatives and phosphorimido derivatives have been identified as fluorogenic ligands of Eu^{3+} and/or Tb^{3+}.[13]

In time-resolved fluorimetric immunoassays, it is desirable to use a chelate label that can be detected down to the sub-picomolar level.[5] Alternatively, multiple labelling strategies can be used in order to achieve sub-picomolar analyte sensitivity.[14–16] More recently, there have been efforts to combine time-resolved fluorimetric immunoassay with enzymic catalysis. In one version of this approach, the primary immunological label is alkaline phosphatase (ALP); its substrate is the phosphate ester of 5-fluorosalicylic acid (FSAP). The non-hydrolysed ester (FSAP) and the hydrolysed ester (FSA) have different behaviour in Tb^{3+}–ethylenediaminetetraacetic acid (EDTA) solutions. When FSA is added to an aqueous alkaline solution containing Tb^{3+}–EDTA, a mixed complex is formed that emits long-lived fluorescence, which is characteristic of Tb^{3+}. The FSA is an appropriate ligand for energy transfer to the metal ion. An intact hydroxyl group on the FSA molecule is essential for these highly fluorescent mixed complexes to be formed. The FSAP does not form any fluorescent complexes with Tb^{3+}–EDTA. In a heterogeneous immunoassay design, ALP activity can be monitored using FSAP as substrate, by measuring the FSA released after adding an alkaline solution of Tb^{3+}–EDTA.[17] This system has been used for the highly sensitive and rapid quantification of α-fœtoprotein and thyroid stimulating hormone (TSH) in serum.[18,19]

Examination of this and other enzyme immunoassay systems reveals that immunological assays with sub-picomolar analyte sensitivity and speed can be developed if the substrate (*i.e.*, FSA) can be quantified at a concentration level of $\approx 1 \times 10^{-8}$ mol l^{-1}. The analyte detectability at levels $< 1 \times 10^{-12}$ mol l^{-1} is due to the enzymic amplification introduced by ALP or other enzymes.

In this paper, 33 compounds were examined as candidate Eu^{3+} and/or Tb^{3+} fluorogenic chelators. These molecules were selected with two criteria in mind: (*i*) that their structure contained aromatic or heteroaromatic rings capable of absorbing electromagnetic radiation, which could then be used to excite the lanthanide internally; and (*ii*) that the structure also contains a lanthanide binding site, which preferably, but not necessarily, consisted of at least one carboxyl group and a hydroxyl group, the hydroxyl group being available for conversion to either a phosphate ester or a galactoside. These derivatives could act as substrates for ALP or β-galactosidase, respectively. The search identified five molecules that are good fluorogenic chelators for both Eu^{3+} and Tb^{3+} and five

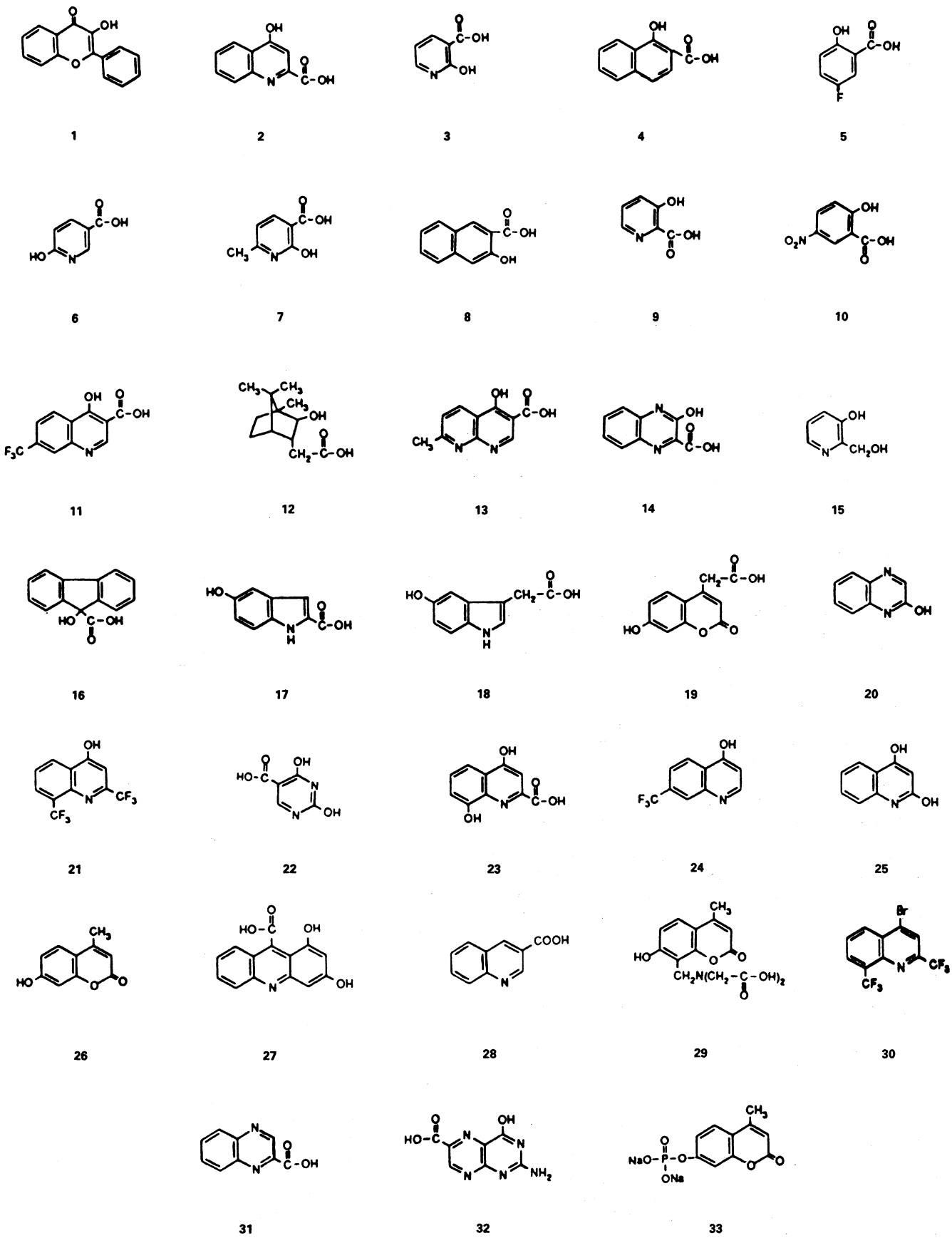

Fig. 1 Chemical structures of the fluorogenic lanthanide chelators tested. (The names of all compounds are listed under Appendix)

molecules that are good fluorogenic chelators for Eu^{3+} only. Under optimized measuring conditions of pH and presence or absence of EDTA, these chelators could be detected at levels of $< 1 \times 10^{-8}$ mol l^{-1}. Thus, they are candidate substrates for highly sensitive enzymically amplified time-resolved fluorimetric immunoassays. One chelator identified, 4-methylumbelliferyl phosphate, which is already a substrate for ALP, was used as a model to develop new heterogeneous time=resolved fluorescence immunoassays for TSH and thyroxine (T4) in serum.

Experimental

Instrumentation

All time-resolved fluorimetric measurements were carried out in white polystyrene microtitre wells obtained from Dynatech Laboratories, (Alexandria, VA, USA) using the 615 Immunoanalyzer. The instrument was a microsecond time-resolved fluorimeter commercially available from CyberFluor. The emission filter used, at 615 nm, was found to be suitable for both Eu^{3+} and Tb^{3+}, as previously described.[18] The excitation source was a nitrogen laser providing excitation radiation at 337.1 nm. No effort was made to optimize the excitation wavelength for each chelator tested.

Chemicals and Solutions

The candidate organic chelators tested are shown in Fig. 1. All of them were obtained from Aldrich, Milwaukee, WI, USA. Calf intestinal ALP was obtained from Boehringer Mannheim Canada, Montreal, Quebec, Canada. Alkaline phosphatase-labelled streptavidin (SA–ALP) was obtained from Zymed Laboratories, San Francisco, CA, USA, as a 0.75 mg ml^{-1} solution. White, opaque 12 well polystyrene microtitre strips coated with monoclonal anti-TSH antibody (for the TSH assay) or a thyroxine–globulin conjugate (for the T4 assay) were obtained from CyberFluor. The biotinylated detection antibodies, standards and buffers needed for the immunoassays were also from CyberFluor. Europium(III) chloride hexahydrate and terbium(III) chloride hexahydrate were from Aldrich. All other chemicals were from Sigma, St. Louis, MO, USA.

All chelators were dissolved in a 0.1 mol l^{-1} tris(hydroxymethyl)methylamine (Tris) buffer, pH 9.0, to prepare stock 1×10^{-3} mol l^{-1} solutions. More dilute solutions were prepared in the same buffer as needed. The alkaline phosphatase substrate buffer was 0.1 mol l^{-1} Tris, pH 9.0, containing 0.1 mol l^{-1} NaCl and 1 mmol l^{-1} $MgCl_2$. The diluent for the biotinylated detection TSH antibody and the SA–ALP conjugate was a 6% m/v solution of bovine serum albumin in a 50 mmol l^{-1} Tris buffer, pH 7.40, containing sodium azide at 0.5 g l^{-1}. The wash solution was prepared by dissolving 9 g of NaCl and 0.5 ml of polyoxyethylenesorbitan monolaurate (Tween 20) in 1 l of distilled water.

Procedures

All chelators were screened by mixing 100 μl of a chelator solution and 100 μl of an Eu^{3+} or Tb^{3+} solution (4×10^{-3} mol l^{-1} in water) and measuring the delayed fluorescence on the 615 Immunoanalyzer. The most promising chelators were further tested with Eu^{3+} or Tb^{3+} solutions of various pH, in the absence or presence of EDTA. The optimization experiments conducted are described under Results and Discussion. Calibration graphs and detection limits were constructed or calculated by adding 100 μl of an aqueous Eu^{3+} or Tb^{3+} solution to 100 μl of a chelator solution in a 0.1 mol l^{-1} Tris buffer of pH 9.0. For chelator **5**, we added 100 μl of a Tb^{3+}–EDTA solution, 1×10^{-3} mol l^{-1} in a 0.5 mol l^{-1} Tris buffer[18] of pH 12.5. For chelators **11** and **13**, we added 100 μl of a 4×10^{-3} mol l^{-1} solution of Eu^{3+} or Tb^{3+} in a 0.1 mol l^{-1} Tris buffer, pH 11, containing 5×10^{-3} mol l^{-1} EDTA.

Immunoassay of TSH

Pipette 100 μl of the TSH standards (0, 0.25, 0.50, 1, 2, 4, 8 and 16 mU l^{-1}) into monoclonal antibody coated wells and then add 50 μl of biotinylated monoclonal detection antibody diluted 50-fold in the 6% BSA diluent. Incubate with mechanical shaking for 1 h at room temperature and wash ×4. Add 100 μl per well of the SA–ALP conjugate diluted 3000-fold in the 6% BSA diluent, incubate for 15 min as above and wash ×4. Add 100 μl per well of the 4-methylumbelliferyl phosphate substrate diluted to a concentration between 5×10^{-6} and 5×10^{-5} mol l^{-1} (see under Results and Discussion) in the alkaline phosphatase substrate diluent and incubate as above for 30 min. Then, add 100 μl of a Eu^{3+} solution, 4×10^{-3} mol l^{-1} in water, mix and measure the fluorescence within 5 min with the 615 Immunoanalyzer. The calibration graph is constructed and data reduction carried out automatically by the Immunoanalyzer.

Immunoassay of T4

Pipette 10 μl of thyroxine standards or serum samples into thyroxine-coated wells and then add 100 μl of biotinylated T4 antibody diluted 20-fold in the T4-antibody diluent. Incubate with mechanical shaking for 1 h at room temperature and wash ×4. Add 100 μl per well of the SA–ALP conjugate and complete the assay as described for TSH but incubate the substrate for only 15 min.

Results and Discussion

The candidate Eu^{3+} and Tb^{3+} chelators tested are shown in Fig. 1. Many chelators possess a hydroxyl group that can be converted to a phosphate ester or galactoside for the purpose of using them as substrates of ALP or β-galactosidase as has already been reported for chelator **5**, which is included for comparison.[17–19] In some cases, sets of candidate chelators

Table 1 Optimum assay conditions of chelators in the presence of Eu^{3+} or Tb^{3+}

Europium chelator	Works in presence of EDTA?	Detection limit/ mol l^{-1}	Stability of fluorescence	pH optimum*
2	No	5×10^{-9}	Poor	11
9	No	5×10^{-9}	Good	9–11
11	Yes	2×10^{-9}†	Good†	11†
13	Yes	2×10^{-9}†	Good†	11†
14	No	2.5×10^{-9}	Good	11
19	No	2.5×10^{-9}	Poor	9–11
29	No	4×10^{-9}	Good	9–11
31	No	7×10^{-9}	Poor	11
32	No	2.5×10^{-9}	Good	9–11
33	No	2.5×10^{-9}	Poor	9–11
Terbium chelator				
5	Yes	5×10^{-9}†	Poor†	12.5†
9	No	5×10^{-9}	Good	11
11	Yes	3×10^{-9}†	Good†	11†
13	Yes	5×10^{-9}†	Good†	11†
29	No	2×10^{-9}	Good	11
33	No	3.9×10^{-8}	Poor	11

* Solutions of Eu^{3+} and Tb^{3+} are stable at alkaline pH only in the presence of an excess of EDTA. Detection limits were thus established at optimum pH only for chelators **5**, **11** and **13**. In all other cases, aqueous solutions of Eu^{3+} and Tb^{3+} of pH 6.5 were used as described under Procedures.

† Studies carried out in the presence of Eu^{3+} or Tb^{3+} and EDTA.

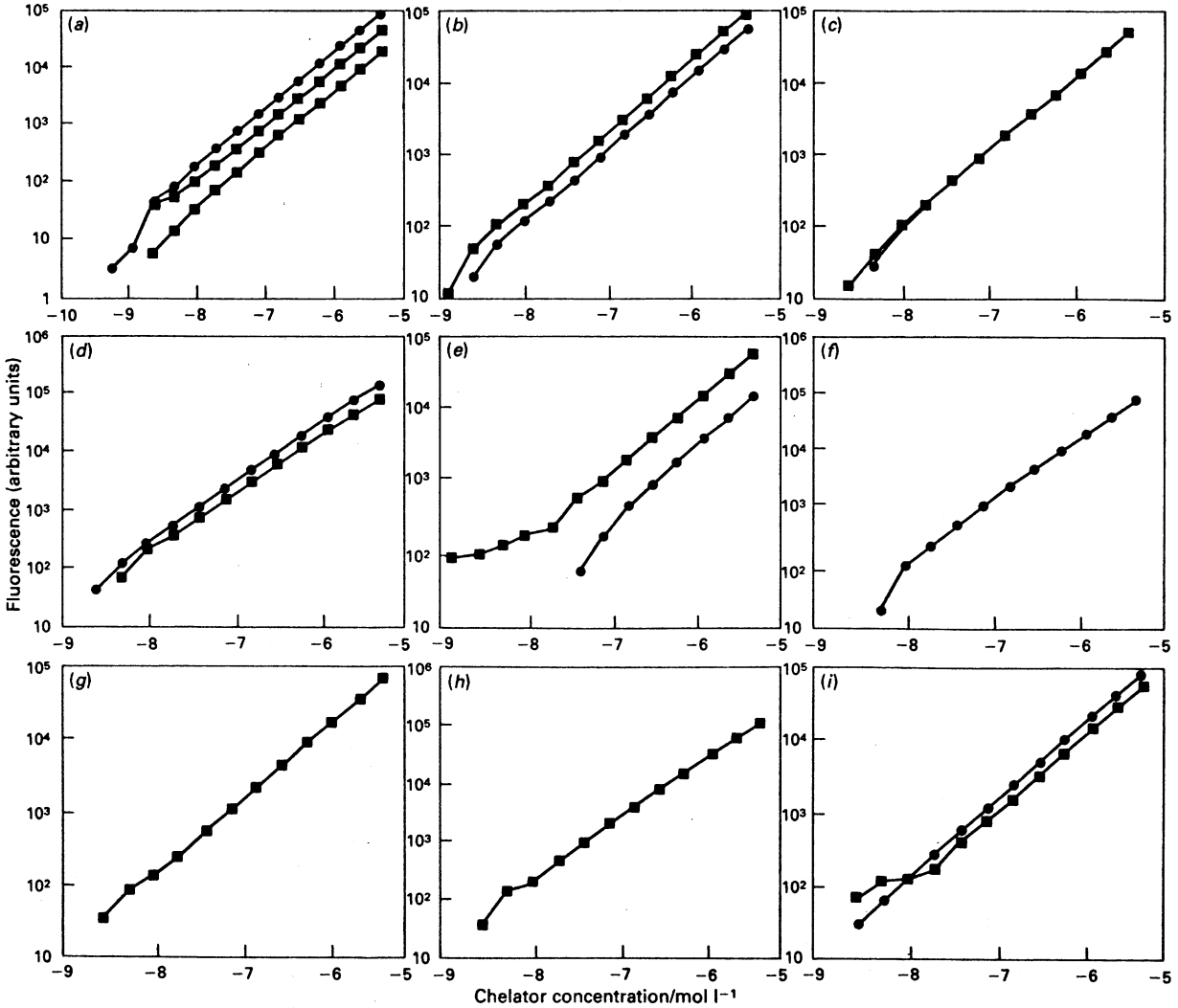

Fig. 2 Logarithmic plots of fluorescence *versus* concentration of fluorogenic lanthanide chelator. In all cases (*a*)–(*e*) except (*d*), the fluorescence of each Eu^{3+} chelate was higher or equal [(*c*)] to the corresponding fluorescence of the Tb^{3+} chelate; the Eu^{3+} chelate fluorescence curve in plots (*a*)–(*e*) is thus on top of the Tb^{3+} chelate fluorescence curve, except in case (*d*). The chelators represented are **11** [(*a*) the third curve representing the lowest fluorescence is due to the Tb^{3+}–EDTA–fluorosalicylate complex as described in ref. 17]; **13** (*b*); **9** (*c*); **29** (*d*); **33** (*e*); **2** [(*f*) with Eu^{3+}]; **14** [(*g*) with Eu^{3+}]; **19** [(*h*) with Eu^{3+})]: **31** [(*i*) lower curve with Eu^{3+}]; **32** [(*i*) upper curve with Eu^{3+}]

were included with only one group difference, *i.e.*, **14**, **20** and **31**. On other occasions, derivatives of a particular structure, *i.e.*, 4-methylumbelliferone (**26**, **19**, **29** and **33**), were tested. Quinoline derivatives (**2**, **11**, **21**, **23**, **24**, **25**, **28** and **30**) were also thoroughly tested. From the structures in Fig. 1, ten compounds were identified that could form highly fluorescent complexes with Eu^{3+}. Five of these chelators could also form fluorescent complexes with Tb^{3+}, as shown in Table 1. Only chelator **5** was found to be unique for Tb^{3+}. Chelator **29** was superior in terms of fluorescence intensity under the conditions of measurement, with Tb^{3+} in comparison with Eu^{3+}. Two chelators, **11** and **13**, exhibited the property of working in the presence of an excess of EDTA, *i.e.*, they form fluorescent complexes with Eu^{3+} and Tb^{3+} chelated to EDTA. This property is also seen with chelator **5** (previously reported[17]). In all other cases, fluorescence of the complexes decreases dramatically in the presence of EDTA. In all cases, the fluorescence measured is long-lived and lanthanide-specific. Native chelator fluorescence might also exist but is not detected by the time-resolved fluorimeter.

Fluorescence intensity of the complexes varies with pH as shown in Table 1. However, solutions of Eu^{3+} and Tb^{3+} in buffers of alkaline pH rapidly deteriorate due to hydroxide precipitation. For this purpose, complexes are preferably formed with the addition of an aqueous solution of Eu^{3+} or Tb^{3+}(pH ≈6.5), which is stable for at least 2 weeks at room temperature. For chelators **5**, **11** and **13**, the Eu^{3+} and Tb^{3+} solutions were prepared in the presence of an excess of EDTA at pH 12.5 (**5**) or 11, respectively. These solutions were also very stable when stored at room temperature. In all cases studied, the fluorescence intensity of the complexes was highest when the Eu^{3+} and Tb^{3+} concentration in the final solution was around 2×10^{-3} mol l^{-1}. Lower concentrations resulted in lower fluorescence signals.

The stability of fluorescence intensity with time was studied in all cases. Fluorescence intensity remained stable (±10%) for at least 3 h for chelators **9**, **11**, **13**, **14**, **29** and **32** and Eu^{3+} and/or Tb^{3+}. Fluorescence intensity decreased with time (change ≥ 10% h^{-1}) for chelators **2**, **19**, **31** and **33**. This decrease was also observed for chelator **5** and previously reported.[18] The reasons for this decrease were not studied further.

Calibration graphs with all chelators that form highly fluorescent complexes with Eu^{3+} and/or Tb^{3+} were constructed and the detection limits were calculated as the concentration that could be distinguished from zero with 95% confidence. The detection limits are reported in Table 1. Representative calibration graphs are shown in Fig. 2. In all

cases but one, the detection limits were $< 1 \times 10^{-8}$ mol l^{-1}. Some chelators were clearly superior to **5** in terms of detectability; this chelator has already been used as a phosphate ester to devise highly sensitive immunoassays.[18,19]

It is anticipated that modification of the structures of these chelators, *i.e.*, transformation into phosphate esters or galactosides, will result in spectroscopic changes and/or chelation changes and/or energy transfer changes, the net result being loss of fluorescence in the presence of the lanthanides. This principle has been shown to work with chelator **5** and Tb^{3+}. Work is now in progress that addresses this possibility with the other compounds listed in Table 1.

Another interesting possibility is to have a pair of compounds A and B one of which, A, is an enzyme substrate that forms fluorescent complexes with Eu^{3+} or Tb^{3+}. Upon enzymic cleavage of a group from A, which is then transformed to B, the ability of A to form fluorescent complexes is lost. One such pair reported here is **33** and **26**. Only **33** forms long-lived, highly fluorescent complexes with Eu^{3+}.

When ALP cleaves off phosphate to produce **26**, the latter does not exhibit long-lived fluorescence in the presence of Eu^{3+}. The compound **33** is a widely used non-fluorescent ALP substrate; when ALP cleaves off phosphate, the product, **26**, exhibits blue, short-lived fluorescence. The pair **33**–**26** and Eu^{3+} were used to develop two model time-resolved fluorimetric immunoassays for TSH and T4 in serum. The design of the TSH assay is of the non-competitive heterogeneous immunoassay format where an immunocomplex of the type solid phase–coating antibody–TSH detection antibody–biotin –streptavidin–alkaline phosphatase is formed in polystyrene microtitre wells.

The activity of ALP can then be measured. In this work, ALP has been detected by adding the substrate **33** for 30 min, followed by addition of a 4×10^{-3} mol l^{-1} Eu^{3+} solution to form the fluorescent complex with the unreacted substrate. The calibration graphs obtained are shown in Fig. 3. The sensitivity and range of assay can be adjusted by adjusting the concentration of the substrate added. In the system proposed here, there is an inverse relationship between TSH concentration and signal because the greater the concentration of ALP present in the immunocomplex, the smaller is the signal observed with the proposed substrate. The detection limit of the TSH assay is about 0.1 mU l^{-1}. Precision was excellent at 2–3% within the measuring range.

It is also possible to use the same detection principle for the 'competitive-type' heterogeneous assay formats. In the proposed thyroxine assay, the immobilized antigen approach as described elsewhere was used.[20] When the bound labelled reagent is quantified with this type of assay, there is an inverse relationship between analyte concentration and signal.[20] In the proposed ALP detection system, the greater the concentration of ALP present in the bound form, the smaller is the

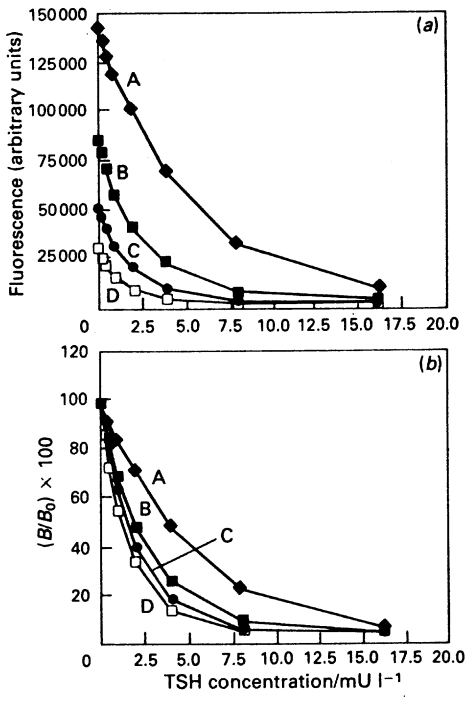

Fig. 3 Calibration graphs for the proposed TSH assay. (*a*) Fluorescence *versus* TSH concentration on linear axes. (*b*) A percentage plot, where B_0 is the fluorescence of the zero standards and B the fluorescence of all other standards. The concentration of the substrate used was: A, 5×10^{-6}; B, 1×10^{-5}; C, 2×10^{-5}; and D, 5×10^{-5} mol l^{-1}

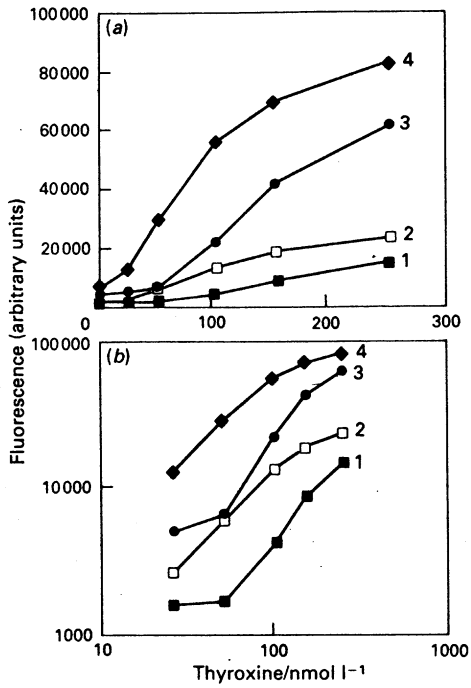

Fig. 4 Calibration graphs for the proposed thyroxine assay. (*a*) Double linear plots. (*b*) Double logarithmic plots. The substrate concentration and antibody dilutions used were: 1, 5×10^{-6} mol l^{-1} and 10-fold; 2, 5×10^{-6} mol l^{-1} and 20-fold; 3, 2×10^{-5} mol l^{-1} and 10-fold; 4, 2×10^{-5} mol l^{-1} and 20-fold

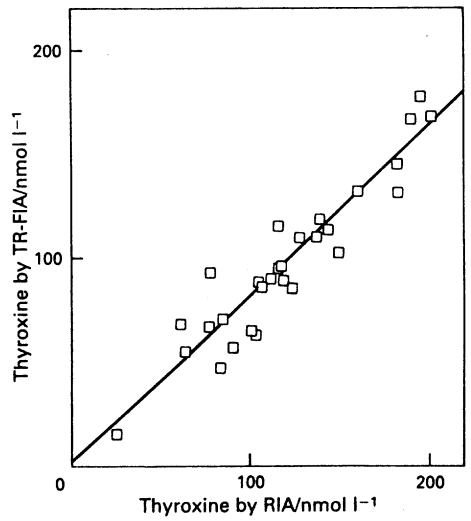

Fig. 5 Correlation between serum thyroxine results obtained with a radioimmunoassay (RIA) and the proposed time-resolved fluorescence immunoassay method (TR-FIA). $y = 0.5262 + 0.8088x$; $r = 0.94$

signal generated. Subsequently, calibration graphs will show proportionality between analyte concentration and signal. To demonstrate this, the thyroxine assay was designed as described in ref. 20 and at the end of all washing steps the following immunocomplex was formed on the solid phase: polystyrene–T4 conjugate–antibody–biotin–SA–ALP. The activity of ALP can then be quantified with use of the substrate, **32**. With a conventional detection system, the magnitude of the signal generated by ALP is inversely related to the analyte concentration and the calibration graphs have a shape similar to those of Fig. 3

In the proposed system, there is a direct relationship between signal and analyte concentration. Calibration graphs obtained with the proposed system are shown in Fig. 4. Sensitivity can be adjusted by changing the antibody dilution, the substrate concentration and the substrate incubation time. Under optimized conditions, we have analysed 30 clinical samples by the proposed method and by a commercially available radioimmunoassay (RIA) procedure. The comparison is shown in Fig. 5. A good correlation exists between the two methods. Additionally, precision studies with three samples gave the following results (n = 12): T4 of 112 nmol l^{-1}, relative standard deviation (RSD) = 8.1%; 59 nmol l^{-1}, RSD = 6.2%; 25.9 nmol l^{-1}, 8.2%. Samples diluted 2- or 4-fold gave values between 93 and 105% of the expected results, confirming a good linearity of the proposed procedure.

Conclusions

Enzymically amplified time-resolved fluorescence immunoassays exhibit excellent sensitivity and can be used for the assay of many analytes in biological fluids. The first system reported is based on ALP and the substrate FSAP in combination with Tb^{3+}.[17–19] Some other potential new chelators are proposed. A system based on the pair 4-methylumbilliferyl phosphate (4-MUP) and 4-methylumbelliferone (4-MU) in combination with Eu^{3+} is examined in detail. This pair works in the opposite way in comparison with the reported FSAP–FSA–Tb^{3+} system.[17–19] Here, 4-MUP forms the fluorescent complex with Eu^{3+}; cleavage of phosphate by alkaline phosphatase releases 4-MU, which does not form fluorescent complexes with Eu^{3+}. This system works well in both 'non-competitive-type' and 'competitive-type' immunoassays.

Appendix

Chelators Chemical Names and Chemical Abstracts Service Registry Numbers

1, 3-hydroxyflavone, 577-85-5; **2**, 4-hydroxyquinoline-2-carboxylic acid, 492-27-3; **3**, 2-hydroxynicotinic acid, 609-71-2; **4**, 1-hydroxy-2-naphthoic acid, 86-48-6; **5**, 5-fluorosalicylic acid, 345-16-4; **6**, 6-hydroxynicotinic acid, 5006-66-6; **7**, 2-hydroxy-6-methylpyridine-3-carboxylic acid, 38116-61-9; **8**, 3-hydroxy-2-naphthoic acid, 92-70-6; **9**, 3-hydroxypicolinic acid, 874-24-8; **10**, 2-hydroxy-5-nitrobenzoic acid, 96-97-9; **11**, 4-hydroxy-7-trifluoromethyl-3-quinolinecarboxylic acid, 574-92-5; **12**, 3-hydroxy-4,7,7-trimethylbicyclo[2.2.1]heptane-2-acetic acid, 81925-04-4; **13**, 4-hydroxy-7-methyl-1,8-naphthyridine-3-carboxylic acid, 13250-97-0; **14**, 3-hydroxy-2-quinoxalinecarboxylic acid, 1204-75-7; **15**, 3-hydroxy-2-(hydroxymethyl)-pyridine, 14173-30-9; **16**, 9-hydroxy-9-fluorenecarboxylic acid, 467-69-6; **17**, 5-hydroxyindole-2-carboxylic acid, 21598-06-1; **18**, 5-hydroxyindole-3-acetic acid, 54-16-0; **19**, 7-hydroxycoumarin-4-acetic acid, 6950-82-9; **20**, 2-quinoxalinol, 1196-57-2; **21**, 2,8-bis(trifluoromethyl)quinolin-4-ol, 35853-41-9; **22**, 2,4-dihydroxypyrimidine-5-carboxylic acid, 23945-44-0; **23**, 4,8-dihydroxyquinoline-2-carboxylic acid, 59-00-7; **24**, 7-(trifluoromethyl)quinolin-4-ol, 322-97-4; **25**, quinoline-2,4-diol, 86-95-3; **26**, 7-hydroxy-4-methylcoumarin, 90-33-5; **27**, 1,3-dihydroxy-9-acridinecarboxylic acid, 28332-99-2; **28**, quinoline-3-carboxylic acid, 6480-68-8; **29**, calcein blue, 54375-47-2; **30**, 4-bromo-2,8-bis(trifluoromethyl)quinoline, 35853-45-3; **31**, quinoxaline-2-carboxylic acid, 879-65-2; **32**, pterin-6-carboxylic acid, 948-60-7; **33**, 4-methylumbelliferyl phosphate, sodium salt, 22919-26-2.

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