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Review Paper

The lanthanides as luminescent probes in investigations of biochemical systems

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Abstract

A review of the luminescence properties of lanthanides, their role as structural and analytical probes and the application of lanthanide complexes in fluoroimmunological and nucleic acid hybridization assays is presented.

Keywords: Biochemical systems; Lanthanides; Luminescent probes

1. Introduction

In natural biological systems, lanthanides are found only in trace amounts and, so far, no indication has been given of any significant role for these species. Nevertheless, for many years, studies have been conducted of reactions between different biological systems, such as amino acids, proteins, nucleotides, nucleic acids or plant and animal tissues, and lanthanides introduced into them [1–3]. There is increasing interest in the application of lanthanides in the investigation of the properties and function of biochemical systems and in the determination of biologically active substances.

The elements of this group have been employed in many methods and techniques of investigation, such as luminescence spectroscopy (including circularly polarized luminescence), nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, Mössbauer spectroscopy, magnetic circular dichroism spectroscopy, neutron activation analysis and electron microscopy. Luminescence methods are particularly important for the study of biochemical systems, because of the variety of possible applications and the relatively simple instrumentation required [4–11].

Lanthanides can form complexes with many biological substances, including donor groups with negatively charged oxygen atoms, e.g. carboxylic groups or phosphate groups as ligands (amino acids, nucleotides). Binding also occurs as a consequence of metal coordination to the oxygen of carbonyl or hydroxyl groups, e.g. in sugars or nucleosides.

Lanthanides enter into reaction with biologically active compounds, replacing the ions of calcium (they can also replace Zn(II), Mg(II), Mn(II), Fe(II) and Fe(III)), and due to their specific spectroscopic properties can serve as probes providing information on these materials and the biochemical processes occurring in them.

The ions of Ca(II) and Ln(III) are particularly alike in terms of their size, the nature of bond formation in biological systems, their coordination geometry and the favouring of donor atoms. The two ions have coordination numbers higher than six and similar values of the rate constants of ligand exchange (about $10^8\ s^{-1}$). Their ionic radii are very similar, e.g. 0.100–0.118 nm for Ca(II) and 0.086–0.122 nm for Ln(III) (for the coordination numbers 6–9). Similar to calcium, lanthanides are hard acids and show a particular affinity to oxygen as donor atom (O>N>S). Both Ca(II) and Ln(III) are characterized by a very elastic coordination geometry, and their bonds are mainly electrostatic in nature. Ln(III) ions bear greater charge than Ca(II) ions, which is responsible for the greater stability of lanthanide complexes compared with calcium complexes as a consequence of the greater Ln(III) charge density (the ratio of the atomic number to the ion radius). The above-mentioned similarities permit isomorphous replacement of Ca(II) ions by Ln(III) ions since successful substitution of metal ions in biologically active materials depends to a greater degree on their size than on the charge of the ions involved.

Significant differences between calcium and the lanthanides are revealed in their spectroscopic properties. Ca(II) ions are colourless, diamagnetic and do not show luminescence, whereas certain elements from the lanthanide series

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are coloured, paramagnetic and emit characteristic radiation. Owing to these particular features, lanthanides have been widely applied in biochemical investigations.

The specific physical and chemical properties of lanthanides which make them so useful in studies of biochemical systems are a consequence of their electronic structure. The electrons of the 4f shell of the lanthanide atoms and their trivalent ions are shielded by electrons from higher shells (5s, 5p) and thus protected from the influence of the environment. Owing to this, lanthanides in different chemical combinations show the same spectroscopic properties (related to the 4f electronic transitions) as their free ions in the gas state. The electronic transitions in the 4f shell are responsible for the characteristic absorption and luminescence spectra of very narrow bands (small half-intensity width) and the long lifetimes of the excited states (the order of milliseconds).

The most important role in biochemical studies falls to Eu(III) and Tb(III) ions, because of the suitable energy gap between the lowest emission level and the ground state. The Gd(III) ion is characterized by a highest excited state ($^6P_{7/2}$) which lies above the excited states of the majority of ligands.

The emission spectra of Eu(III) complexes reveal bands related to the $^5D_0$–^7F_J ($J=0$–4) transitions. In water solutions, the most intense are $^5D_0$–$^7F_1$ (585–600 nm) and $^5D_0$–$^7F_2$ (610–630 nm) transitions. The $^5D_0$–$^7F_2$ transition is hypersensitive ($\Delta J=\pm 2$). The physical and chemical properties of Eu(III) complexes are characterized by the parameter $\eta = I(^5D_0$–$^7F_2)/I(^5D_0$–$^7F_1)$ which provides information on the symmetry of the complexes [12,13]. The $^5D_0$–$^7F_0$ transition (577–581 nm) between the non-degenerate states is characteristic of a given chemical environment of the Eu(III) ion. Since it is forbidden, the intensity of the corresponding band is very low and its observation requires laser excitation. The emission lifetime of the $^5D_0$ level in water solutions is in the range 0.1–1.0 ms.

Tb(III) ions in solutions of its complexes emit from the $^5D_4$ level. The highest intensity bands correspond to the transitions $^5D_4$–$^7F_5$ (540–555 nm) and $^5D_4$–$^7F_6$ (485–500 nm). The band attributed to the transition $^5D_4$–$^7F_5$ is sensitive to the environment of the metal ion in the complex (the inner and outer spheres). In water solutions, the emission lifetime of the $^5D_4$ level is in the range 0.4–5 ms.

The emission spectra of Sm(III) complexes reveal bands corresponding to the transitions $^4G_{5/2}$–$^6H_{7/2}$ (590–600 nm) and $^4G_{5/2}$–$^6H_{9/2}$ (640–650 nm), with emission lifetimes of the order of about 50 μs; in contrast, the spectra of Dy(III) complexes reveal bands corresponding to transitions $^4F_{9/2}$–$^6H_{15/2}$ (470–480 nm) and $^4F_{9/2}$–$^6H_{13/2}$ (570–580 nm), with emission lifetimes of about 10 μs. A relatively small energy gap between the emission and ground levels of Sm(III) and Dy(III) ions favours radiationless deactivation of their excited states, so that the intensity of the observed emission is lower than that of Eu(III) and Tb(III) ions.

The 4f electrons of the lanthanides can be excited directly by irradiation, e.g. with a laser beam at a wavelength corresponding to the maximum absorption of a given lanthanide, or indirectly through the process of intermolecular (sensitization) or intramolecular energy transfer. When employing intramolecular energy transfer from the ligand to the lanthanide ion, the complex is irradiated with the wavelength corresponding to the maximum absorption of the ligand. When a sensitizer is used, a wavelength specific for this sensitizer is employed.

2. Lanthanides as structural and analytical luminescent probes

Luminescence revealed by a sample (biological system) can provide important structural and analytical information. If a studied system does not emit radiation, luminescent molecules or ions, e.g. Eu(III) or Tb(III), can be introduced into it and employed as a luminescent probe sensitive to the influence of the environment. In certain cases, the emission from the sample and the probe and the energy transfer between them can be studied simultaneously.

Information on the composition and structure of the coordination sphere can be obtained from the emission and excitation spectra of Eu(III) or Tb(III) complexes. The excitation spectra permit the identification of donor groups in the ligand and metal binding sites. Analysis of changes in the intensity of lanthanide emission bands or ligand luminescence quenching provides information on the distance between the ligand donor group and the lanthanide ion as well as the complex stoichiometry. Investigation of the quenching of europium (or terbium) luminescence by other metal ions permits the determination of the distance between the two metals. Measurement of the emission lifetimes of Tb(III) or Eu(III) in H_2O and D_2O provides information on the number of water molecules in the inner coordination sphere of the metal (the hydration number). From the ratio of the intensities (η parameter) and the structure of the lanthanide emission bands, conclusions can be drawn about the symmetry of bonds and the conformational properties of the ligand at the metal binding sites. Additional information on the same subject can be obtained for optically active (chiral) systems characterized by circularly polarized luminescence [14]. The above information can be supplemented by data from studies of the chemiluminescence of biological systems containing europium ions as a chemiluminescent probe [15–18].

Measurements of the luminescence of lanthanide ions originating from intramolecular energy transfer from the ligand to the Ln(III) ion have been employed as a fundamental technique for the determination of various substances of biological importance.

The main advantage of the lanthanide luminescent probes is their high sensitivity; this refers to both structural and analytical probes. Their application permits the detection of very small changes in the structure of the studied compounds, and allows the detection limit of the determined substances

to be lowered. The above problems can be illustrated by a few examples.

2.1. Structural probes

Measurements of the efficiency of energy transfer from tryptophan to Tb(III) or Eu(III) ions substituted in place of calcium in parvalbumin (protein found in skeletal muscles in most vertebrates) permits the determination of the distance between the donor (tryptophan) and the acceptor (Tb(III) or Eu(III) ions) in molecules of this protein [19].

Analysis of the excitation spectra and emission lifetimes of Eu(III) and Tb(III) ions in complexes with calmodulin (multifunctional protein with four Ca(II) binding sites), as well as energy transfer from Eu(III) and Tb(III) (as donors) to ions of other lanthanides (acceptors), enables the distances between the calcium binding sites in a molecule of this compound to be established [20,21].

Laser excited Eu(III) ions were used for the identification of metal binding sites in bovine α-lactalbumin. The results of luminescence titration demonstrated the presence of at least two different metal binding sites in this protein. Analysis of the $^5D_0$–$^7F_0$ band in the excitation spectrum of Eu(III) in a complex with α-lactalbumin revealed the presence of three kinds of chemical surroundings of the complexed Eu(III) ion. Two involved four negatively charged groups and were related to the Ca binding sites, while the third was ascribed to a non-specific metal binding site taken by Eu(III) after the first type of site had been saturated. The possibilities and limitations of this method when applied to the investigation of biological materials are discussed in Ref. [22].

Studies were performed of the emission intensity and lifetime of the excited state of Tb(III) in a complex with a synthetic heptapeptide corresponding to bovine prothrombin residues 17–23 containing γ-carboxyglutamic acid. Prothrombin is a good model for the investigation of the interactions of metal ions with many proteins involved in the process of blood coagulation. The composition and dissociation constants of the above complexes were determined. A comparison of the results with the properties of metal ion binding of natural prothrombin led to the conclusion that the structural fragment 17–23 is essential for the functional properties (i.e. bonding of metal ions) of this protein [23].

Information about the dynamics of molecules of bovine brain calmodulin (the calcium activated regulatory protein) and human serum transferrin was obtained from measurements of the efficiency of fluorescence energy transfer vs. temperature. These energy transfer processes were from tyrosine (donor) to Tb(III) (acceptor) in calmodulin and from Tb(III) donor to Fe(III) (acceptor) in transferrin [24].

The luminescence properties of terbium ions were used in an investigation of the interaction of adriamycin (doxorubicin hydrochloride) and cisplatin (*cis*-dichlorodiammineplatinum(II)) with GH3/B6 pituitary tumour cells. The two above-mentioned compounds are inhibitors of tumour cell development. Analysis of the emission spectrum of the system Tb(III)–GH3/B6 and the absorption spectrum of adriamycin proved that the quenching of Tb(III) luminescence by adriamycin was caused by resonance dipole–dipole energy transfer, and permitted the location of the adriamycin receptor to be determined at a distance of about 40 Å from the attached Tb(III) probe at the lipid–protein interface. The quenching of Tb(III) luminescence by cisplatin was demonstrated to occur as a result of static energy transfer, and the cisplatin acceptor was located near the Tb(III) binding site at the outer surface of the cell membrane. These data indicate that the receptors of adriamycin and cisplatin in the plasma membrane of tumorigenic cells are bonded to the same calcium binding protein [25,26].

The DNA structure was investigated by Eu(III) ion luminescence. In complexes of Eu(III) with oligodeoxynucleotides and nucleotides, two kinds of binding site were identified. At one site, the coordination sphere of Eu(III) contained six to seven water molecules and one to two oligomer atoms, whereas at the other the coordination sphere contained two water molecules and six to seven oligomer atoms. In the latter case, the Eu(III) ion was bound with the dimer or polymer structure of the oligonucleotide [27,28].

In 1992, the results of studies of the satellite tobacco necrosis virus, with a complex structure and three calcium binding sites, performed using laser excited luminescence of Eu(III) ions, were reported. Analysis of the excitation spectrum and measurements of the lifetimes of the excited states of Eu(III) in a complex with the virus permitted the determination of the Eu(III) binding site (involving five carbonyl ligands), its structure, symmetry and dissociation constant. Moreover, changes in the affinity of lanthanide ions when complexed with the studied virus, as a function of the ionic radii, were found. The greatest affinity was established for Sm(III) and Eu(III) ions, whose ionic radii are close to that of the Ca(II) ion occurring in natural systems [29].

The Tb(III) binding to human erythrocyte spectrin was studied by Tb(III) fluorescence titration, circular dichroism spectroscopy and fluorescence scattering measurements. The terbium binding sites (low and high affinity) and the influence of terbium binding on the conformational changes and aggregation of spectrin are described in Ref. [30].

2.2. Analytical probes

For the determination of tetracycline and its derivatives, the luminescence of Eu(III) complexed with the relevant antibiotic was used [31]. This method was later combined with chromatographic separation of tetracyclines and improved by introducing a surfactant (Triton X-100) and a synergistic agent (tri-*n*-octyl phosphine oxide (TOPO)) into the system [32,33]. A method for the determination of tetracyclines in serum samples in the system Eu(III)–tetracycline–thenoyltrifluoroacetone, in the presence of Triton X-100, was also proposed [34].

The emission of Tb(III) complexed with ciprofloxacin was used for the determination of this antibiotic (fluoroquin-

olone group) in biological fluids, in combination with liquid chromatography [35]. Intramolecular energy transfer from bleomycins and nalidixic acid to Tb(III) allowed the fluorometric determination of these antibiotics on separation by liquid chromatography with reversed phases [36].

The fluorescence of cerium(III) ions (λ_{em} = 350 nm) in complexes with polysaccharide sulphates, such as heparin or carrageenans, was used for their determination on separation [37].

Another interesting application of the specific lanthanide properties involves the determination of steroids in biological fluids, based on sensitized Tb(III) emission in the Tb(III)–steroids–sodium dodecylsulphate system. In combination with high performance liquid chromatography, this method permits the determination of steroids containing α,β-unsaturated carbonyl groups in the A-ring (e.g. testosterone, progesterone, cortisone) [38]. This method of steroid determination was also used in cooperation with capillary electrophoresis/micellar electrokinetic chromatography [39].

Novel heptadentate chelating agents, 2,6-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4-benzoylphenol and 4-(phenylethynyl)-2,6-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]pyridine, capable of forming stable Eu(III) chelates have been described. These chelates can be used as luminescent labels in capillary electrophoresis separation of a variety of samples of biological interest (e.g. amino acids, peptides and proteins) [40].

Sensitized emission of Tb(III) ions complexed with orotic acid (2,6-dihydroxypyrimidine-4-carboxylic acid, an intermediate in the biosynthesis of pyrimidine) and reversed-phase ion-pair chromatography were used for the determination of orotates in urine [41]. Introduction of surfactants, in particular cetyltrimethylammonium bromide (CTAB), into the system was found to lower the detection limit [42].

For the determination of plasma salicylate [43] and diflunisal (a derivative of salicylic acid with analgesic and anti-inflammatory properties similar to aspirin) in serum and urine [44], a method based on the fluorescence of terbium in ternary complexes with ethylenediaminetetraacetic acid (EDTA) and the above compounds was applied.

Fast fluorometric kinetic methods were developed for the determination of diphacinone (pesticide, indane derivative) in serum (based on the sensitized emission of europium in the complex Eu(III)–diphacinone–ammonia in the presence of Triton X-100) [45], and for the determination of propyl gallate (antioxidant) in edible and cosmetic oils (based on the sensitized emission of terbium in the system Tb(III)–propyl gallate–sodium dodecylsulphate) [46].

A number of specific applications of the sensitized luminescence of lanthanides for the determination of various organic compounds are given in Ref. [47].

One more important specific application of ytterbium complexes with porphyrins is worth mentioning. These complexes can be used as fluorescent labels of malignant tumours in mice (in vivo) [48]. The use of ytterbium complexes (emitting in the range 900–1050 nm), instead of free porphyrins, eliminates the background luminescence from biological tissue and enhances the luminescence contrast value of the tumours. Moreover, porphyrins, when complexed with heavy metals, are not phototoxic (unlike free porphyrins), which is important from the viewpoint of diagnostic applications.

3. Application of lanthanide complexes in fluoroimmunoassays and nucleic acid hybridization assays

An interesting and increasingly developing area in which the luminescence properties of lanthanides have been successfully used is for the analysis of biological substances by immunological methods whose sensitivity and specificity ensure their advantage over other techniques. These methods have been applied, first of all, in clinical studies of substances occurring in very low concentrations for whose detection no specific chemical methods have been developed. Such substances are enzymes, hormones or drugs.

The technique of immunological assay is based on the process of binding of an analyte (antigen) with an appropriate antibody. As a result of this specific reaction an immunological complex is formed. Usually the immunological reaction is carried out on the plastic surface to which the antibody is attached, e.g. microtitration strip wells, microbeads or electrophoretic gel. The binding with the solid phase can be realized via a protein which is well adsorbed on this phase and with which the antibody forms a covalent bond.

To determine the amount of analyte, the label is introduced to one of the substrates of the immunological reaction. This label must be easily detectable and identifiable. Such labels can be radioactive isotopes in radioimmunoassays or fluorescence emitting compounds in fluoroimmunoassays. The advantage of fluoroimmunoassays is that they are safer, frequently more sensitive and the method of measurement is relatively fast and simple [49,50]. The chelates of europium, terbium, dysprosium and samarium, characterized by intense emission, have been used as labels of antigens or antibodies. The advantages of using lanthanide labels in fluoroimmunoassays over fluorescence emitting organic compounds include the significant Stokes shift and the long lifetimes of lanthanides in their excited states. These characteristics permit the elimination of disturbances due to the background radiation originating from the emission of biological compounds and scattered excitation light. Measurements of the laser excited luminescence of an appropriately complexed lanthanide are performed using the time-resolved method, i.e. with a delay of the order of 200–400 ms with respect to the moment of excitation. In this way, it is ensured that only the

lanthanide emission is measured, as the emission lifetimes of the lanthanide ions are substantially longer (milliseconds) than the lifetimes of excited organic molecules (nanoseconds).

A lanthanide chelate suitable for fluoroimmunoassays should be water soluble and should contain functional groups capable of entering into a covalent bond with an immunologically active compound (antigen or antibody). Moreover, it should be characterized by a high stability constant and a suitable excitation spectrum (high absorbance of a ligand), as well as a high yield of energy transfer from the ligand to the lanthanide ion.

3.1. Immunological assays

Lanthanide chelates are employed as labels in two different configurations of commercially available fluoroimmunoassay. Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) [51,52] covers two stages with the participation of two different lanthanide complexes. In the first stage, the lanthanide ions (mainly europium), in the form of complexes with the isothiocyanate derivative of EDTA (or diethylenetriaminetetraacetic acid), form a stable bond with the immunoreactive agent supported on the surface of the solid phase, via the formation of a covalent bond between the thiocyanate group and the free amine group of the protein. After the immunological reaction, the antigen–antibody conjugate labelled with the lanthanide is treated with enhancement solution containing 2-naphthoyltrifluoroacetone, detergent and a synergistic agent (TOPO). At the low pH of this solution, the lanthanide dissociates off from the primary complex, forming a chelate with 2-naphthoyltrifluoroacetone, which emits intense luminescence. In this way, by measuring this luminescence intensity, the content of Eu(III) in the solution can be determined in the range 5×10^{-14} to 1×10^{-8} mol l^{-1}. This method has been used in many diagnostic procedures, e.g. for the determination of hormones (prolactin, thyrotropin, cortisol, testosterone), in serological analyses for the determination of rubella or tetanus antibodies and for the determination of virus antigens. Apart from the above applications, the method has been employed for the determination of triazine herbicides in water [53,54], steroids in animal preparations [55] and benzodiazepine receptor binding drugs [56].

DELFIA can be used for simultaneous multianalyte determinations taking into account the β-diketone (aromatic or aliphatic) chelates of Eu(III), Tb(III), Sm(III) and Dy(III) (at different maximum emission wavelengths and emission lifetimes) as well as the so-called co-fluorescence effect [57–60]. The co-fluorescence effect is based on intermolecular energy transfer from the chelates of the enhancing ion (e.g. Y(III)) to the chelates of the emitting ion when the chelates are present in the form of a suspension in an aqueous solution or in a micellar environment. The enhancing ion must not contain excited 4f or 4d levels situated below the excited triplet level of the β-diketone used. The co-fluorescence enhancement solution also contains synergistic ligands (e.g. 1,10-phenanthroline and its derivatives, pyridine derivatives, TOPO), which exchange water molecules in the lanthanide coordination sphere, and detergent (e.g. Triton X-100). The micellar environment protects the chelates against non-radiative processes and can solubilize the particles and stabilize the solutions by preventing the sedimentation of the particles. The use of the co-fluorescence effect increases lanthanide chelate detectability and also allows Sm(III) and Dy(III) emission measurements. In the system containing pivaloyltrifluoroacetone, 2,2′-bipyridine, Y(III) and Triton X-100, the detection limits are 0.019 pmol l^{-1} for Eu(III), 0.27 pmol l^{-1} for Tb(III), 3.8 pmol l^{-1} for Sm(III) and 20 pmol l^{-1} for Dy(III) [59].

The double-label fluoroimmunoassays with Eu(III) and Sm(III) or Eu(III) and Tb(III), as well as the quadruple-label assay with chelates of Eu(III), Sm(III), Tb(III) and Dy(III), have been presented [57–61]. Owing to the differences in the emission lifetimes and maximum emission wavelengths, all labels can be determined simultaneously, even when one is present in a significant excess.

Recently, the luminescence enhancement of Eu(III) by Y(III) ions has been described in aqueous solutions of complexes with new binucleating complexing agents, i.e. bis(4-pyridine-2,6-dicarboxylic acids), without other additives and micelle formation. In this solution, a polymeric structure, where Eu(III) and Y(III) ions are linked together by ligands, has been generated. Luminescence enhancement of Eu(III) ions is a result of the transfer of absorbed energy along the polymer chain through several ligands [62].

In the second configuration of fluoroimmunoassays with the participation of lanthanides, called FIAgen [63–66], the europium chelate with 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) acts as a label. BCPDA carries two sulphonyl chloride groups capable of reaction with the amino groups of protein molecules. The detection limit for Eu(III) complexed with BCPDA is 3×10^{-13} mol l^{-1}. This label is more effective when bonded to streptavidin and used against the antibody bonded with biotin. An excess of Eu(III) ions is added after the completion of the immunoreaction, and the amount of immunocomplex formed is determined in the solid phase. This method has been applied for the determination of α-fetoprotein in serum, prolactin, thyrotropin and growth hormone.

Moreover, very sensitive fluoroimmunoassays based on the participation of europium and terbium chelates and alkaline phosphatase (ALP enzyme) have been devised. In one of such assays [67], ALP bonded to streptavidin cleaves phosphate from 5-fluorosalicyl phosphate, yielding 5-fluorosalicylic acid (FSA), which forms a ternary complex FSA–Tb(III)–EDTA characterized by intense fluorescence. This method was applied for the determination of α-fetoprotein in serum. In another assay [68], developed for the determination of thyroid stimulating hormone and thyroxine in human serum, ALP is employed to cleave a phosphate group from 4-methylumbelliferyl phosphate. As a result, 4-methylum-

belliferone is formed, whose complex with Eu(III) does not show fluorescence. Added Eu(III) forms a complex with unreacted phosphate which is characterized by intense fluorescence. Measurement of this fluorescence enables the determination of the amount of ALP bonded to the appropriate antibody.

Other lanthanide complexes have also been applied in fluoroimmunological assays. For example, a complex of Tb(III) with transferrin was used for the determination of the antibiotic gentamycin [69]; for the determination of human serum albumin, a complex of Tb(III) with the reagent formed in the reaction of diethylenetriaminepentaacetic acid anhydride with *p*-aminosalicylic acid was used [70,71]. A method based on the synergistic luminescence of Eu(III) (in the presence of Tb(III) ions) was used for the determination of human immunoglobulin G. Initially, an Eu(III) complex with diethylenetriaminepentaacetic acid is bound to immunoglobulin G, but after immunological reaction, Eu(III) dissociates from the complex under the influence of a solution containing thenoyltrifluoroacetone, 1,10-phenanthroline and Triton X-100. In this system, the emission of Eu(III) is measured. The intensity of this emission shows a fivefold increase in the presence of Tb(III) ions in the system [72].

For the labelling of protein molecules, e.g. immunoglobulin G, the use of 4-iodoacetamidodipicolinic acid has been proposed, whose complexes with Tb(III) and Eu(III) are characterized by intense fluorescence [73]. For the determination of cortisol, a chelate of Eu(III) with ((chlorosulphonyl)thenoyl)trifluoroacetone (CTTA) is employed. The procedure is as follows. The CTTA labelled immunological complex in the solid phase is moved to a solution containing sodium dodecylsulphate, Eu(III) ions and TOPO; the emission of the formed Eu(III)–CTTA chelate is measured [74].

Some investigations of novel multidentate chelating agents, bipyridine and terpyridine derivatives, (arylethynyl)pyridines [75–78] and linear and macrocyclic polyaminocarboxylate coupled to a 7-amino-4-methyl-2(1*H*)-quinolinone chromophore [79], capable of forming lanthanide chelates which can be used as luminescent labels in fluoroimmunoassays, have been published.

More promising for the further development of fluoroimmunological assays with lanthanide detection is the use of lanthanide complexes with macrocyclic ligands [80,81]. In such complexes, the lanthanide ion is in a ligand cage and thus is protected from the effect of the solvent. Such complexes are stable, water soluble and emit intense luminescence; therefore their use is expected to simplify the procedure and increase the sensitivity of fluoroimmunological assays.

3.2. *Nucleic acid probes*

In a similar manner to fluoroimmunological assays, lanthanide complexes are used for the examination and determination of nucleic acids in nucleic acid hybridization assays [49,52,65,82]. In immunological assays, the detection reagent is a specific antibody, whereas in the nucleic acid hybridization assays, such a reagent is a specific sequence of DNA or RNA nucleotides labelled by a suitable lanthanide complex showing intense emission as a result of energy transfer from the ligand to the lanthanide ion. The DNA (RNA) whose sequence is to be determined is initially cut by restriction enzymes into defined fragments; then individual fragments of the double-stranded DNA are denatured by heating, i.e. split into single strands which, on cooling, can hybridize with the complementary labelled DNA sequence. This technique can be used in different areas of application, e.g. for the detection of the presence of genetic diseases and pathogenic organisms, such as viruses, bacteria and parasites, and for the identification of persons.

An example of the use of lanthanide complexes for the detection of nucleic acids is the technique involving a ternary complex of Tb(III) with diethylenetriaminepentaacetic and *p*-aminosalicylic acids bound with single-stranded DNA [83,84]. In such a system in the liquid phase, the intensity of Tb(III) emission increases with increasing concentration of DNA.

A method involving the use of a complex of Eu(III) with BCPDA has been proposed for the direct determination of nucleic acids in agarose gel [85], whereas for the determination of nucleic acids as well as antibodies, a complex of Eu(III) with terpyridine-bis(methyleneamine)tetraacetic acid has been used [86].

It should be mentioned that the sensitized luminescence of Eu(III) and Tb(III) has found application in liquid chromatography for the detection of single-stranded nucleic acids and polynucleotides containing guanine, thiouridine or xanthine [87]. These bases are characterized by suitable energy levels of excited states and act as donors in intramolecular energy transfer to the lanthanide ions.

A Tb(III) complex with phenanthroline was used for the determination of single-stranded nucleic acids [88] and nucleotides [89]. The use of an Eu(III) complex with tetracycline may be helpful in the selective determination of double-stranded and single-stranded DNA in the presence of RNA [90].

3.3. *Final note*

The information presented in this paper, based on reports published mainly in journals of chemical and physical–chemical profile, provides clear evidence of the profound importance of the lanthanide ions and complexes used as luminescent probes in the investigation of biological materials. Recently, a review of the problems related to the practical application of lanthanide complexes in bioanalytical assays has been published, in which the principles and mechanisms involved in particular kinds of assays have been presented [91]. That review has been prepared chiefly on the basis of papers and reports published in journals of medical profile.

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