RARE-EARTH CHELATES AS "FLUORESCENT" MARKERS IN CELL SEPARATION AND ANALYSIS

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The synthesis of a novel series of functionalized macrocyclic complexes of the lanthanide(III) ions is reported. The Eu(III) complexes possess a set of properties (water solubility, inertness to metal release, ligand-sensitized luminescence, reactive peripheral functionalities) that make them suitable as luminescent markers for bio-substrates. Currently employed organic fluorophores give efficient signal production, but this is accompanied by interference from background fluorescence and, if multiple fluorophores are used, from spectral overlap. The long lifetimes and narrow-band emissions of the luminescent lanthanide complexes will minimize background interference; however, long lifetimes will also result in a significantly reduced signal for flow cytometry or cell sorting.

Introduction

Cell separation and cell analysis are mutually dependent branches of cytophysics. The quality of a cell separation can be ascertained by analyzing the individual fractions to determine their cellular composition. In turn, the accuracy of a cell analysis procedure can be documented most easily for a cell population that has been enriched in a specific fraction by an effective separation procedure. In the past, it was sufficient to report the results of a cell separation in terms of morphology (*1-3*). Recently, however, the development of monoclonal antibodies and of recombinant DNA techniques has greatly increased the capability to analyze cells for specific antigens and even to observe single genes (*4*) thus permitting the use of these advanced detection procedures.

The Advantages of Narrow-Line Emitting Fluorophores

Current fluorescent histochemical techniques, whether based on flow analysis or on image cytometry, are limited by the chemical nature of the fluorescent markers. Traditional fluorophores are large, rigid organic molecules in which both the ground and

the excited electronic states are comprised of a multitude of closely spaced vibrational levels. In these molecules the electrons involved in light absorption are outer shell (valence) electrons; their energies are affected by atomic vibrations. The photons absorbed therefore have an energy distribution which corresponds to the many possible values of the energy difference between the lowest vibrational level(s) of the ground electronic state and various vibrational levels of the excited electronic state. Almost immediately after light excitation, these molecules relax to the lowest vibrational level of the first excited state. When fluorescence emission occurs, the energy distribution of the emitted photons corresponds to the many possible values of the energy difference between this lowest vibrational level of the first excited state and various vibrational levels of the ground state. At room temperature, therefore, both the excitation and the emission spectra are broad (*5*). With traditional organic fluorophores, overlap between the fluorescence of the marker and the broad autofluorescence of the cellular substrate represents a major limitation in the sensitivity of the optical measurements (*6-7*). The broadness of the emission-excitation spectra also severely reduces the number of fluorophores that may be measured simultaneously (*8*). The emissions of fluorescein and rhodamine, for example, overlap to such an extent that in flow microfluorometers and other analytical instruments special electronic circuitry must be employed to separate the individual signals (*9*). Although this type of dual measurement has been considerably facilitated by the substitution of rhodamine with phycoerythrin (*10*), which absorbs at lower wavelengths, the limitation remains.

The preceding considerations led Leif et al. to propose the use of rare-earth chelates as luminescent reporter molecules (*11*). In these compounds, the luminescence results from transitions within the 4f electron manifold of the metal ion. Since the inner shell 4f electrons are non-bonding and are effectively shielded from the environment by the outer electron shells, these emissions are free from vibrational broadening (*12*).

Figure 1 shows the most intense emissions of Eu(III) and Tb(III) superimposed for comparison on the emissions of fluorescein and rhodamine; clearly, these lanthanide emissions do not overlap.

The Question of Long Lifetimes. Another distinctive feature of the luminescent lanthanide chelates is their long excited-state lifetime, usually more than 300 μs (*13*). Since the lifetimes of natural as well as conventional synthetic fluorophores are usually of the order of 10 ns (*5*), the lanthanide luminescence may be completely freed from background interference by short-pulse excitation of the sample, followed by slightly delayed signal detection (delay, ca. 1 μs). These time-gated measurements based on the long-lived luminescence of the lanthanide marker are advantageous in automated immunoassays (*14*) and would also be of value in the microscopic examination of cells as well as in flow cytometry or flow cell sorting. In the latter case, however, previous analysis (*15*) has shown that the signal intensity will also be dimin-

ished. Even in the favorable situation of a 10 µs excitation pulse and a lanthanide chelate with a relatively short lifetime of 38 µs, the percentage of light emitted within the orifice will be only 34%. This includes the light signal collected while the cell is still being illuminated. True dark field observation with a conventional continuous wave laser would decrease this to less than 25%. With long-lived lanthanide fluorophores as markers, slowing the flow rate of the sample would significantly increase the efficiency of signal collection.

A pulsed light source would also provide a significant increase in collected signal (*16*). Pulsed excitation would reduce the time, during the observation of the cell in the orifice, when the detection system must be either electronically gated off or optically obscured in order to achieve dark field illumination. Two suitable pulsed light sources are short arc xenon strobe lamps and noble gas lasers. Both argon and neon lase with markedly greater efficiency when pulsed and produce ultraviolet emissions suitable for the excitation of lanthanide complexes.

The luminescence lifetime of europium chelates is longer than the transit time through a transducer (10-30 µs). Therefore, the question may be raised of whether such chelates, when used as markers in a flow system, would produce a significantly lower signal than conventional short-lived fluorophores. Mathies and Stryer (*17*) have stated that, under saturating laser excitation, a fluorophore is in principle capable of being excited once for each lifetime; for fluorescein, this would mean approximately 200 times per µs. The excitation conditions assumed by Mathies and Stryer in their calculations were different from those employed in a modern flow cytometer such as the EPICS Elite (EPICS is a trademark of the Coulter Corp.). These authors assumed a beam with a circular 4 µm cross-section at the $1/e^2$ points and 65 mW of 488 nm radiation energy. With these parameters, Mathies and Stryer calculated that one fluorescein molecule could be excited approximately 97 times per µs.

The situation is quite different in a flow cytometer. At the flow rates required to obtain statistically significant data, the thickness of the sample stream is sufficient to permit some lateral position dispersion of the cells in the flow chamber. To provide consistent laser excitation of cells with imperfect trajectory, the gaussian laser beam is broadened in the direction perpendicular to the flow. The size of the laser beam at the $1/e^2$ points in an EPICS Elite is approximately 35X75 μm^2, an area 164 times greater than that assumed in the calculation by Mathies and Stryer. The air-cooled argon ion laser in the EPICS Elite is usually set to produce 15 mW of 488 nm radiation. Correction for both the increased area of the beam cross-section and the decreased radiation intensity results in a 711 fold decrease in excitation energy to the sample. Under the best conditions, this is equivalent to 0.129 exciting photons per molecule per µs. With the same geometric parameters, a 50 mW water-cooled UV Argon laser would excite 0.32 molecules per µs and a 9 mW UV He-Cd laser would excite 0.05 molecules per µs. If a combined DNA and Eu-chelate analysis were to be performed, the beam would be broadened. Multimode He-Cd lasers produce larger beams than gaus-

sian lasers. In a ten µs excitation interval with the 50 mw UV Argon laser, approximately (10x0.32)=3.2 photons would be available to excite each chelate. This number could be increased by employing a high powered continuous or pulsed laser; however, such sources are not as yet available in clinical flow cytometers.

The above calculations, based on the cited work of Mathies and Stryer, indicate that the inability of the Eu-macrocycle to undergo multiple cycles of excitation and emission during the time of flow detection may result in an approximate 3-fold decrease in useful signal. However, preliminary studies indicate that luminescent Eu-macrocycles undergo less concentration quenching than traditional organic fluorophores. Thus, this limitation may in principle be overcome by greater loading of the luminescent marker on the substrate.

Work by Other Investigators

The usefulness of lanthanide complexes as biological markers has been recognized by other workers. Soini and Hemmila (*18*) and later Soini and Lovgren (*14*) have described an analytical procedure involving the Eu(III) complex of the polyaminocarboxylate ligand DTPA (*19*) (Fig. 2(a)), which is non-luminescent in aqueous solution. In this procedure, the Eu-DTPA chelate was decomposed with acid and the solubilized Eu(III) was complexed with a ß-diketonate in a micellar phase (*20*). These dissociation-combination steps complicated the analytical procedure and increased the volume of solution to be monitored for Eu(III) emission, with consequent decrease in optical sensitivity. Since this protocol involved separation of the fluorophore from the specific bio-substrate, the technique was unsuitable for immuno-luminescence and other measurements on single cells or particles by either flow cytometry or microscopy.

Another example of luminescence analysis based on a Eu(III) complex has been reported by Evangelista et al. (*21*). In this case the ligand was the dinegative ion of 4,7-bis(chlorosulfenyl)-1,10-phenanthroline-2,9-dicarboxylic acid, BCPDA (Fig. 2(b)), which formed a luminescent chelate with Eu(III). The luminescence, however, was not detectable in aqueous solution at the concentrations of the analytes of interest and the sample had to be dried prior to measurement. Furthermore, the intrinsic luminescence of the Eu-BCPDA complex was rather low; in order to measure analytes present in minor quantities, it was necessary to increase sensitivity by binding the chelate to thyroglobulin (*22*) in a multilayer system. Soini et al. (*23*) have observed luminescence from a chelate attached by antibody to a cell, but do not report the chemical structure of the chelate. Beverloo et al. (*24*) have reported the use of microcrystals of the phosphor yttrium(III) oxysulfide doped with europium(III). Cells specifically labeled with this phosphor could be detected in a time- gated mode even in the presence of the very bright, red emitting DNA stain, ethidium bromide. A disadvantage of these 50-500 nm phosphor particles is that they must be coated to prevent agglutination with a polycarboxylic acid, which may then be displaced by negatively

charged ions such as phosphate, citrate, or ionized ethylenediamine tetracarboxylic acid (EDTA). The negative charge of these coated phosphors also causes nonspecific binding to slide coating agents such as poly-l-lysine or bovine serum albumin.

Results and Discussion

This section describes our systematic efforts to design and synthesize water-soluble, chemically stable, luminescent complexes of the Eu(III) and Tb(III) ions, equipped with peripheral functional groups for coupling to a bio-substrate. Details of the experimental methods and results are to be found in the references cited.

Our search for lanthanide derivatives suitable as luminescent markers initially focused (*11,15*) on the tris-chelate complexes of ß-diketonates (Fig. 2(c)). These compounds possessed three important favorable features. First, the tris-ß-diketonates of both Eu(III) and Tb(III) were known to exhibit ligand-sensitized luminescence, with emission intensity depending largely on the efficiency of the energy transfer from an excited triplet state of the ß-diketonate ligand to the emission level(s) of the metal ion (*25*). Second, the lanthanide tris-ß-diketonates were thermodynamically stable, with cumulative stability constants, $ß_3$, ranging from 18 to 21 log units (*26-27*). Third, the tris-ß-diketonates contained several coordinated water molecules that could be replaced by uncharged N-donor heterocyclic ligands (*28*) to give complexes having decreased vibrational quenching and hence increased luminescence. It was reasonable to conclude that, if the N-donor heterocycle were a chelating ligand previously coupled to a bio-substrate, this substitution reaction could provide a straightforward way of attaching the luminescent complex to the intended target.

To explore this possibility, we developed the synthesis of the previously unknown bifunctional chelate, 5-isothiocyanato-1,10-phenanthroline (Fig. 2(d)) (*29*). This was coupled to a model protein (BSA and γ-globulin) via a thiourea linkage, following the procedure established for fluorescein isothiocyanate. We then proceeded to attach a preformed Eu(III) or Tb(III) tris-ß-diketonate to the protein-coupled phenanthroline. However, this last step met with failure, in that the highly luminescent protein-lanthanide conjugate initially formed dissociated upon washing or dialysis (*29*). The phenanthroline moiety remained covalently attached to the protein, while the lanthanide complex broke down and luminescence was lost. Obviously, the thermodynamic stability of these complexes, however high, was not sufficient to prevent their dissociation in a very dilute, buffered aqueous solution.

These first unsuccessful attempts emphasized an important generalization, namely, that the time frame of ligand exchange and metal exchange kinetics becomes a major consideration for complexes to be used as probes in biological systems. In the very dilute aqueous or aqueous-organic media required for such systems, often involving contact with potentially competing ligands, even ordinarily "stable" metal complexes, if labile, may dissociate. When such dissociation occurs, the value of the

probe is diminished or lost. The task, therefore, was to design and synthesize a new class of Eu(III) and Tb(III) complexes that would retain their identity in dilute aqueous solution through kinetic inertness rather than through thermodynamic stability alone.

To achieve this objective, we decided to take advantage of the so-called macrocyclic effect. Examples of metal-macrocyclic complexes that remain undissociated in solution, even though containing inherently labile metal ions, abound both in nature and in the synthetic chemical literature (*30*). The vast majority of these macrocyclic complexes contain a "small" metal ion bound inside the four-donor-atom cavity of an organic ligand, a striking example being the Mg(II) of chlorophyll. A limited number of inert complexes of larger metal ions with five-and six-donor-atom macrocyclic ligands have also been reported. Among these, the most relevant were the complexes of La(III) and Ce(III) with the six-nitrogen ligand L^1 (Fig. 2(e)), obtained by Backer-Dirks et al. (*31*) from the metal-templated cyclic Schiff-base condensation of 1,2-diaminoethane and 2,6-diacetylpyridine. Although these authors had been unable to similarly synthesize the corresponding complexes of other lanthanides, an appropriate change in experimental procedure, together with use of the lanthanide acetates instead of the nitrates as templates, allowed us to obtain the Eu(III) and Tb(III) complexes in high yields and excellent purity (*32*).

These complexes were white crystalline solids, thermally very stable and soluble in water as well as common organic solvents. They were unique among the derivatives of the lanthanide(III) ions in that the metal-macrocycle entities remained undissociated in dilute aqueous solution, even under conditions that would result in rapid decomposition of most other lanthanide complexes. In contrast, the exocyclic ligands, whether anions or solvent molecules, were labile and readily exchangeable. For example, prolonged contact of the Eu-L^1 acetate complex with a ten-fold excess of hydrochloric acid in methanol resulted in replacement of the acetates by chlorides without degradation of the Eu-L^1 entity. The behavior of the M-L^1 complexes in solution is consistent with their structure as established by single-crystal X-ray analysis (Benetollo, F.; Bombieri, G.; Fonda, K.K.; Polo, A.; Vallarino, L.M. *Polyhedron*, in press).

In $EuL^1(CH_3COO)_2Cl{\cdot}4H_2O$ (Fig. 3), the Eu ion is bound to the six N atoms of the L^1 macrocycle in a nearly planar arrangement, with Eu-N internuclear distances corresponding to the sum of the individual radii. The two bidentate chelating acetate ligands occupy axial positions on opposite sides of the Eu-macrocyle unit, the organic portion of which is slightly folded in a "butterfly configuration" that minimizes steric strain.

The ease of exchange of the exocyclic anions proved to be a major asset in view of the potential use of these compounds as luminescent markers. The Eu-L^1 and Tb-L^1 acetate-chloride salts, initially obtained from the metal-templated synthesis,

exhibited only a weak luminescence (*33-34*); however, substitution of the acetates by a variety of chelating carboxylates or ß-diketonates resulted in highly luminescent species. The most effective luminescence enhancers were mononegative ligands with rigid, _-bonded structures and hard donor atoms (O, aromatic N) (*35*). Figure 4 illustrates the increase in emission intensity observed upon addition of 2-furoic acid to the Eu-L^1 diacetate-chloride. The stoichiometric character of this effect is evident from the luminescence titration graph. Substituted macrocycle-enhancer complexes were also isolated and characterized as pure crystalline solids (*34-35*).

Having succeeded in synthesizing water-soluble complexes of Eu(III) and Tb(III) that could retain their identity in dilute solution and exhibit ligand-sensitized luminescence, our next goal was to introduce into the backbone of the macrocycle a functional group suitable for coupling to a bio-substrate. We chose the synthetic scheme shown in Fig. 5, which utilized a carbon-substituted 1,2-diaminoethane precursor. This synthesis had the advantage of introducing the coupling groups into the flexible -CH_2-CH_2- side-chains, at a position and at a distance where it would be less likely to disturb either the conformation of the macrocyclic ligand or the electronic character of the ligand-metal bonding. There was no precedent in the literature for this kind of synthetic procedure. However, we anticipated that under appropriate conditions the template action of the metal ion, favoring the formation of the macrocyclic complex, would prevail over the competitive side-reactions arising from the functional group of the diamine precursor. This expectation proved to be true and lanthanide complexes were obtained in good yields for the three macrocyclic ligands L^2 (with functional group X = -CH_2-OH), L^3 (with X = -CH_2-C_6H_4-OH), and L^4 (with X = -CH_2-C_6H_4-NH_2).

These functionalized metal-macrocycles closely resembled their non-functionalized analogs. They were microcrystalline solids with excellent thermal stability. They were soluble in water as well as in common organic solvents, and they exhibited the same inertness to metal release in solution as well as the same lability of the exocyclic anions. Also, similar to their L^1 analogs, the lanthanide complexes of the L^2, L^3, and L^4 macrocyclic ligands were only modestly luminescent as the acetate salts but became intense emitters in the presence of a suitable enhancer (*36-37.*). Figure 6 illustrates the marked increase in emission intensity that occurs when the Eu-L^3 triacetate is treated with gradually increasing amounts of 4,4,4-trifluoro-1(2-thienyl)butane-1,3-dione. The shift in the excitation maximum of the macrocycle-enhancer system, relative to that of the original macrocycle acetate alone, clearly shows that the chelating enhancer not only provides better protection from vibrational quenching by the solvent but also acts as a radiation absorber and promotes effective energy transfer to the metal ion.

It should be noted that a disubstituted macrocycle such as L^2, L^3, or L^4 can exist as two regioisomers, as illustrated in Fig. 7. One isomer is the <u>cis</u> form, in which

the two functional groups X occupy positions adjacent to the same pyridine ring and thus are "on the same side" relative to the metal center. The other isomer is the trans form, in which the two X groups occupy positions adjacent to different pyridine rings and thus are "on opposite sides" relative to the metal center. Furthermore, the carbon atoms of the diimine side-chains which carry the X groups are chiral; thus, additional isomers become possible if the diamine precursor is racemic. The presence of isomers most likely is responsible for the failure we have so far encountered in obtaining single crystals of these functionalized macrocycles, suitable for X-ray analysis.

The peripheral -OH and -NH_2 functional groups of the macrocycles were found to exhibit their normal reactivity. For example, reaction of the metal macrocycle acetates with acetic anhydride, in the presence of 4-dimethylaminopyridine as catalyst, gave the corresponding ester or amide in good yields (*38*). No degradation of the metal-macrocycle entity occurred even under the drastic conditions required for this reaction and the resulting derivatives were soluble and inert to metal release. Figure 8 shows the infrared spectrum of the acetate ester of the M-L^3 triacetate complex and illustrates the characteristic features of this type of compound.

Conclusions

The series of functionalized macrocyclic complexes of Eu(III) and Tb(III) reported in this paper fulfill the fundamental requirements of a luminescent marker for cytology and immunology. These complexes are soluble in water and water-compatible solvents; they do not release the metal ion even in the presence of acids, bases, or competing ligands; they can be made instantaneously luminescent in aqueous solution by the simple addition of an enhancer; finally, they contain primary hydroxy or amino functionalities that can be used for coupling to a desired bio-substrate. These functionalized macrocycles exhibit the narrow-emission, long-lived luminescence that is typical of traditional Eu(III) and Tb(III) chelates, and thus offer the same advantages in regard to sensitivity and lack of mutual or background interference. Future work will focus on the attachment of the functionalized macrocycles to bio-substrates and on the evaluation of the stability and luminescence of the resulting conjugates.

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FIGURES

Figure 1. Emission profiles of fluorescein, rhodamine, and of Eu(III) and Tb(III) nitrates in aqueous solution. (Emission intensities on arbitrary scales.)

Figure 2. Schematic formulas of: (a) Diethylenetriamine-pentacetate, DTPA; (b) 4,7-bis(chlorosulfenyl)-1,10-phenanthroline-2,9-dicarboxylate, BCPA; (c) A generic ß-diketonate (R and R' may be equal or different); (d) 5-isothiocyanato-1,10-phenanthroline; (e) Eu-L^1 complex, where L^1 represent the macrocyclic ligand $C_{22}2H_{26}N_6$.

Figure 3. Structure of the $[EuL^1(CH_3COO)_2]Cl{\cdot}4H_2O$ complex in the crystalline state, showing the atom labeling scheme. The oxygens of the clathrated water molecules are omitted for clarity.

Figure 4. Luminescence "titration" of $[EuL^1(CH_3COO)_2]Cl{\cdot}4H_2O$ with 2-furoic acid in methanol. Inset shows the excitation spectra of: (a) original complex and (b) complex with added enhancer (1:1 mole ratio).

Figure 5. Synthetic scheme for functionalized lanthanide macrocyclic complexes. The functional group X is $-CH_2-OH$ for ligand L^2, $-CH_2-C_6H_4-OH$ for ligand L^3, and $-CH_2-C_6H_4-NH_2$ for ligand L^4.

Figure 6. Luminescence "titration" of $EuL^3(CH_3COO)_3{\cdot}nH_2O$ with 4,4,4-trifluoro-1(2-thienyl)butane-1,3-dione in methanol: (a) emission spectra of solutions containing increasing Eu to enhancer mole ratios, (b) excitation spectrum of 1:1 complex-enhancer solution.

Figure 7. Computer-generated schematic formulas of the two [S,S]-regioisomers of the Eu-L^2 macrocycle, in which X = $-CH_2-OH$. Structures (a) and (b) are front and side views, respectively, of the cis isomer; (c) and (d) are the corresponding views of the trans isomer. For these molecules, the functional groups of the cis isomer occupy opposite "hemispheres" relative to the plane of the macrocycle, whereas those of the trans isomer occupy the same "hemisphere". The carbon-attached hydrogen atoms of the macrocycle and the exocyclic ligands are omitted for clarity.

Figure 8. Infrared spectrum of the diacetylester of $EuL^3(CH_3COO)_3 \cdot nH_2O$. The peak at 1750 cm^{-1} represents the stretching absorption of the carbonyl ester group. The two peaks at 1634 and 1589 cm^{-1} represent the C=N stretching absorptions of the pyridine and imine groups, respectively, and are a diagnostic feature of this kind of macrocycle.