Advances in the development of lanthanide macrocyclic complexes as luminescent bio-markers

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ABSTRACT

The development of peripherally substituted europium(III)-macrocycles suitable as luminescent bio-markers was continued in three related areas. (1) Protocols were established for the coupling of NCS-substituted Eu-macrocycles to proteins and for the mounting on microscope slides of particles labeled with luminescent Eu-macrocycles. The emission/excitation spectra of the dried, slide-mounted particles were investigated. (2) A procedure was developed for the synthesis of lanthanide-macrocycles having a single peripheral functionality. The structure and properties of the mono-functionalized macrocyclic complexes were established. (3) A study was undertaken to explore whether the emission intensity of the Eu-macrocycles can be increased by energy transfer from yttrium(III) complexes. Preliminary results have shown that a considerable luminescence enhancement can be achieved by this method.

The results obtained in these three areas are evaluated in the light of the research reported by other investigators.

Keywords: lanthanide, luminescence, chelate, imaging, time-gated, europium, fluorescence, macrocyle, stain, rare-earth

1. INTRODUCTION

Two limiting factors for immunodiagnostic nucleic acid hybridization cell imaging and assays are sensitivity and speed; these two factors are often related, as greater sensitivity can allow shorter incubation times. When the analysis of biological samples depends on fluorescence measurements, the sensitivity is often limited by the background signal due to autofluorescence or Raman scattering. As an example, a multilaboratory survey found the average autofluorescence of human lymphocytes to equal that of 657 fluorescein molecules[1]. These considerations have suggested[2] the use of assays based on the luminescence of certain complexes of the lanthanide ions, especially those of europium(III) and terbium(III). The luminescence of these ions is characterized by narrow band emissions, very large Stokes shifts, and long lifetimes --features that allow the lanthanide luminescence to be very effectively separated from that of the background. When performed in a direct time-gated mode, lanthanide-based luminescence assays maximize signal to noise by essentially eliminating the background[3]. In addition, luminescent lanthanide complexes undergo only minor concentration quenching. These luminescent compounds are suitable for clinical diagnostic tests and microscopic imaging[2].

The sensitivity of these labels should minimize the need for in situ PCR by permitting exquisitely sensitive, simple nucleic acid hybridization and FISH assays[4]. The long lifetimes and narrow bandwidths of the lanthanide macrocyles permit their detection and quantitation in the presence of other strong fluorochromes, such as DAPI. Automation of the Pap test and other exfoliated cytology by the combination of DAPI for DNA and Eu Macrocycle tagging for HPV and/or other antigen or FISH measurements are very exciting scientific and commercial possibilities.

2. Studies on Luminescent Lanthanide Bio-Markers

2.1. Luminescent Lanthanide Labels Reported by Other Investigators

The majority of the studies performed by other investigators on the development of lanthanide(III) complexes as luminescent bio-markers have dealt with thermodynamically stabilized complexes of anionic polychelating ligands.

Soini and Hemmila have very successfully utilized a multi-step complexation of Eu(III) to amino-polycarboxylate and β-diketonate ligands in the DELFIA (Dissociation-Enhanced Lanthanide Fluoroimmunoassay) technique[5,6]. This consists of first, reacting an antibody or other macromolecule which has been coupled with a non-luminescent Eu-amino-polycarboxylate with an antigen or other combining species. After the specific reaction has taken place, the Eu(III) ion is freed by acid and subsequently, coordinated to a chelating b-diketonate or other ligand, to form a complex capable of luminescence in aqueous micellar solution. This technique, however, is not applicable to microscopic imaging, for which localization is a major requirement.

Mukkala et al.[7] described the lanthanide complexes of ligands consisting of two N-donor heterocyclic moieties joined at the 2-carbon positions by a bridging group capable of forming a five-or six-membered ring chelate ring with the metal ion. Each of the N-donor heterocycles also carried at the 2'-carbon position a substituent terminating in a carboxylate or phosphonate group. In addition, a group carrying a coupling functionality was substituted for any one of the hydrogen atoms of the parent chelating structure. The luminescence properties of the Eu(III) and Tb(III) complexes of various ring-substituted 6,6'-bis(N,N)-bis(carboxymethyl)-aminomethyl)-2,2'-bipyridines were investigated. The excitation wavelengths were found to fall in the 272-344 nm range for the Eu(III) chelates and in the 272-325 nm range for the Tb(III) chelates, in borate buffer at pH 8.5 as well as in ethanol solution. Monoclonal antibodies to human laminine and keratin were labeled with a 100-fold molar excess of the isothiocyanate derivatives of two Eu(III) chelates. The labeled antibodies were applied to fixed cryostat sections which were subsequently dehydrated with ethanol and viewed with a fluorescence microscope; emission was observed at 613 nm under excitation in the 340-380 nm range.

Kankare et al.[8] reported a number of polychelating ligands capable of forming luminescent complexes with a variety of metal ions, including the lanthanides. These polychelate ligands consisted of a 2,2':6',2" terpyridine as the parent structure, with substituents terminating in carboxylate groups at each of the 6 and 6'' positions. In addition, a group containing a coupling functionality was substituted for any one of the hydrogen atoms of the parent chelating structure. The europium(III) and terbium(III) complexes of 6,6"-Bis[N,N-bis(carboxymethyl)aminomethyl]-4,4"-diphenyl-2,2,40;6'2"-terpyridine were excited at 340 nm and emitted respectively at 614 nm and 544 nm.

Savitsky et al.[9] reported a Eu(III) complex formed from the products of the reaction of the amino group of aminobenzoyltrifluoracetone (ABTFA) with either the cyclic dianhydride of diethylenetriaminepentaacetic acid (DA DPTPA) or pentasucciniimidyl ester (PSE DPTA). The reaction of equimolar concentrations of ABTFA and DA DPTPA in anhydrous DMSO produced an intermediate with one remaining cyclic anhydride group of the DA DPTPA available for reacting with the protein. A conjugation ratio of 5 to 1 was achieved with bovine serum albumin by starting with a 100 to 1 molar ratio of chelate to protein. This chelate was excited in the 330-340 nm range. The reaction of 4 moles of ABTFA with PSE DTPA in anhydrous DMSO and subsequent conjugation with BSA produced BSA-DTPA-$(ABTFA)_4$, which was the brightest conjugate.

A 10-fold molar excess of "free" Eu(III) was required in solution to prevent dissociation of the complex; a detergent was added to form a micellar phase for protection of the excited Eu(III) from vibrational deactivation by water molecules. The intensity of the luminescence was found to be independent of the nature of detergent; the optimum detergent concentration was reported to be 0.02%, which was said to be the "critical concentration of micellation of detergent". The solution also contained trioctylphosphinoxide, at an optimum concentration of 5. 5 x 10^{-5}M --a value similar to that employed in the previously mentioned DELFIA technique. Under these conditions, as low as 0.1 ng/ml of human IgG could be detected, in spite of loss due to binding at the microtiter tray cell wall. A limitation of this assay was the requirement for the presence of "free" Eu(III) In the solution; although Eu(III) is only minimally luminescent when complexed to proteins, DNA or other plasma and cellular constituents, it still provides a significant background.

Sabbatini et al.[10] have recently reviewed a series of lanthanide complexes of encapsulating cage-like organic ligands, synthesized and investigated by her group. The spectroscopic study of these complexes was of significant value in explaining and predicting lanthanide luminescence; however, the complexes themselves appeared to have limited potential for utilization as bio-markers, for they either did not bind the lanthanide ions sufficiently

strongly or had excitation maxima below 320 nm, a region that requires the use of expensive and not commonly available fused silica optics.

Shakhverdov et al.[11] obtained near-infrared luminescence from neodymium(III) and ytterbium(III) in aqueous media at room temperature, employing eosin as energy transfer dye. In a model study, the concentration of eosin was maintained at 10^{-3} M for single photon excitation and at 10^{-1} M for two-photon excitation with a 1064 nm Nd(III) laser. The luminescence was found to be strikingly solvent-dependent. In water, the excited-state lifetime was estimated to be 0.17 microseconds on the basis of the low quantum yield. In deuterium oxide, the lifetime was 5 microseconds and increased to 270 microseconds in the presence of the deuterated phosphate triester, $(C_4D_9O)_3PO$. Other dyes, such as rubrene, acriflavine and related molecules, were also found to transfer energy to Nd(III); for these systems the Authors stated, “energy transfer to Nd(III) takes place not only from triplet, but from excited singlet state of ligand too”.

All polychelating ligands mentioned above have one feature in common. They are organic compounds capable of independent existence as metal-free species and their lanthanide(III) complexes are formed by reaction of a metal salt with the pre-synthesized ligands. Such complexes remain somewhat labile even when their formation constants are very high, and therefore readily undergo metal-exchange reactions when placed in solutions containing other metal ions --such as calcium ions --with high affinity for the same ligands. Such metal exchange would result not only in partial loss of the lanthanide marker but also in the release of “free” lanthanide(III) ions that could then bind to functional groups of the substrate producing spurious luminescence data. Most complexes of these polychelating ligands have another limitation. The absorption of light by the ligands, which is the first step in the energy pathway leading to lanthanide emission, occurs at low wavelengths (272-342 nm). In this spectral region, conventional fluorescence microscope objectives absorb the exciting radiation, requiring, as stated above, the use of expensive fused silica optics.

A different approach was used by Beverloo et al.[12], who reported labeling cells with microcrystals of the phosphor yttrium(III) oxysulfide doped with europium(III). Cells 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 was their tendency to agglutinate. Coating the particles with a polycarboxylic acid, which could then be displaced by negatively charged ions such as phosphate, citrate, or ethylenediamine-tetracarboxylate, served to prevent aggluntination; however, the negative charge of the particles then caused nonspecific binding to common slide-coating agents such as poly-S-lysine or bovine serum albumin. In a subsequent paper[13], another difficulty was reported: because of the short excitation wavelengths of these phosphors (below 300 nm), optics completely made of quartz were required. The authors concluded, “Nevertheless, there is a need to develop soluble time-resolved dyes with long luminescence life times and high quantum efficiency which do not significantly fade upon excitation”.

2.2. Lanthanide Complexes of Six-Nitrogen-Donor Macrocyclic Ligands

2.2.1. Complexes of Symmetrically Bi-Functionalized Macrocycles.

These complexes, synthesized[14,15] in a one-pot reaction by the Eu(III)-templated 2:2 cyclic Schiff-base condensation of a carbon-substituted 1,2-diaminoethane and 2,6-diacetylpyridine are air- and light-stable microcrystalline solids soluble in a variety of polar solvents. In these complexes, the cationic Eu(III)-macrocycle moieties retain their identity in dilute aqueous solution and over a wide pH range; furthermore, they do not release the Eu(III) ion in the presence of competing ligands nor exchange it in the presence of competing metal ions. These Eu(III)-macrocycles, therefore, do not dissociate under the experimental conditions employed for the formation of antigen-antibody complexes or for the hybridization of oligonucleotides to DNA or RNA.

In contrast to the Eu(III)-macrocycle moieties, the exocyclic counterion-ligands present in these complexes are labile and easily exchanged. Thus it is possible to “switch on” the typical Eu(III) luminescence simply by replacing the counterion-ligands (usually acetates) originally present in the complexes with luminescence-inducing ligands, such as chelating β-diketonates[14,15]. To optimize the luminescence of the europium(III) macrocyclic complexes, a variety of substituted β-diketonates with absorption maxima in the 340-380 nm region was

screened[16], using as model the X-ray analyzed {Eu($C_{22}H_{26}N_6$} triacetate complex. The anion of 1,1,1-trifluoro-4-(2thiophene)-2,4-butanedione was found to be the most effective luminescence enhancer, followed closely by the anion of 1,1,1-trifluoro-4-(2naphthyl)-2,4-butanedione, which is the chelating enhancer reported by Hemmila et al.[6] and currently in use in the DELFIA immunological assays.

As often observed for compounds of this type, the emission intensity of the Eu(III)-macrocycle-(β-diketonate) complexes was found to be somewhat solvent dependent. Thus, although significant emission was observed in 5% polyethyleneglycol (MW1,500), 95% water, an approximate 5-fold enhancement was observed[16] in 80% ethyleneglycol, 20% water and also in 90% ethanol, 10% water. These changes in emission intensity were not accompanied by any appreciable change in either the patterns or the energies of the emission and excitation spectra, which remained the same in different solvents as well as in solid-mounted samples.

2.2.2. Spectral Studies and Microscopic Imaging of Microsphere Labeled with Eu(III)-macrocycles.

As a model for cytological imaging, a protocol was developed for the coupling of the Eu(III)-macrocycles to avidin, for the subsequent affinity-binding of the Eu(III)-coupled avidin to biotinylated agarose microspheres, and for the observation of the latter by reflectance luminescence on slide-mounted samples or luminescence microscopy.

Coupling[4] between avidin and a Eu(III)-macrocycle with two peripheral isothiocyanato functionalities was carried out at room temperature in a 85-to-15 mixture of carbonate/bicarbonate buffer (pH 9.6) and dimethylsulfoxide. After the avidin and Eu-macrocycle had been allowed to incubate for approximately 20 min., a solution of aspartic acid (5-fold molar ratio relative to the Eu-macrocycle) in the same carbonate/bicarbonate buffer was added. The mixture was allowed to incubate for an additional 10 min.; it was then absorbed on a column filled with Sephadex G25 (Pharmacia LKB, Uppsala, Sweden) in tricine buffer (pH 7.6), and eluted with the same buffer using a slight nitrogen gas pressure. The first eluate fraction, identified by flow-absorption at 280 nm, was collected and checked for Eu(III) content by spot-testing on (non-fluorescent) filter paper impregnated with 1,1,1-trifluoro-4-(2thiophene)-2,4-butanedione. Independent analyses of avidin (by absorbance at 280 nm and Eu(III) (by atomic absorption with nitrogen oxide flame) showed the avidin-to-Eu(III) to vary between 1,7 and 3.5, depending on the coupling conditions. Samples of {Eu(III)-macrocycle}-avidin in tricine buffer showed no decomposition over a period of several weeks when stored at ca. 4 ^{o}C.

Biotinylated agarose beads (SIGMA, No. B-6885) were incubated in fresh skim milk at 4 ^{o}C (twice for 12 hr) to block non-specific binding of proteins and washed thoroughly. The beads were then incubated with {Eu(III)-macrocycle}-avidin in tricine buffer (pH 7.6) for 30 min. After thorough washing and addition of the enhancer 1,1,1-trifluoro-4-(2thiophene)-2,4-butanedione (also in tricine buffer at ph 7.6), the beads showed the intense red luminescence typical of Eu(III). When an excess of beads was used, no Eu-macrocycle-avidin remained in the supernatant, showing that even at the highest macrocycle loading the biotin-binding sites of avidin had not been blocked[4].

For reflectance luminescence measurements, a sample of agarose beads labeled with {Eu(III)-macrocycle}-avidin, as described above, was dehydrated by repeated extraction with an ethanol/enhancer solution and air dried at 60 ^{o}C. The dry beads were embedded in Accumount 60 (Stephens Scientific, Riverdale, NJ) on a microscope slide with coverslip; air dried again at 60 ^{o}C, and observed by reflectance in a fluorometer (SLM 8000) with excitation at 360 nm and an incidence angle of 55 degrees. The pattern and energies of the Eu(III) emissions observed in this manner (Figure 1) for the slide-mounted beads were identical, within experimental error, to the emission spectrum of a solution of {Eu(III)-macrocycle}-avidin in tricine buffer with added enhancer.

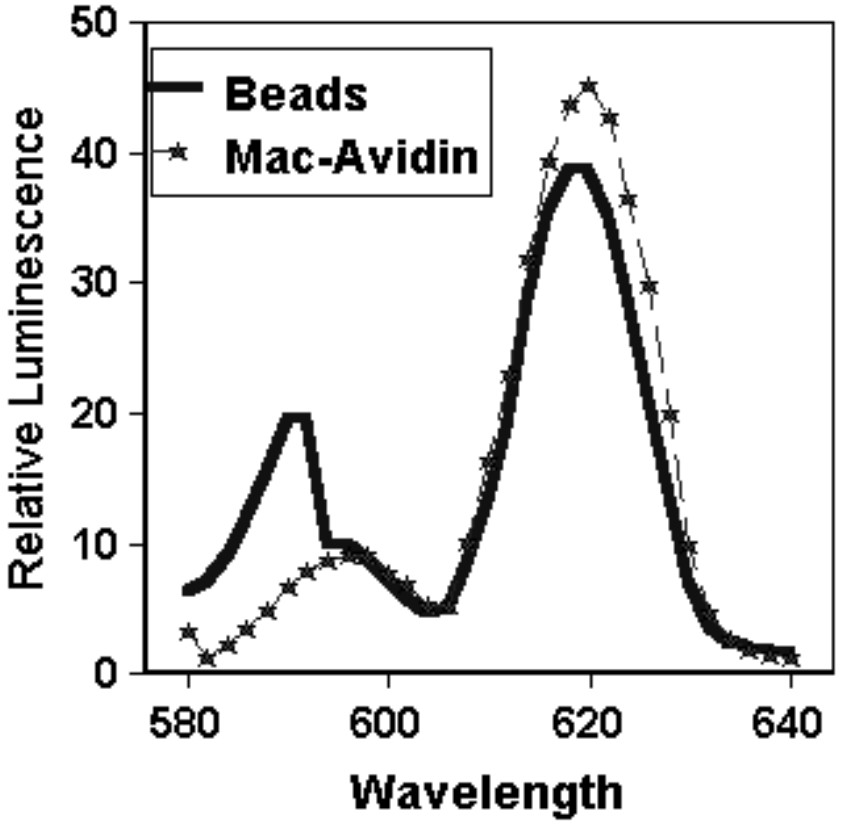

Figure 1, Comparison of the emission spectra of Eu(III)-macrocycle}-avidin bound to biotinylated agarose beads mounted in Accumount 60 with Eu(III)-macrocycle}-avidin in tricine buffered aqueous solution. The area under the individual spectra were scaled to be approximately equal. The heterogeneity of the agarose beads on the slides precludes any quantitative measurements.

2.3. Enhancement of the Luminescence of Eu(III)-Macrocycles by Energy Transfer from Yttrium(III).

Enhancement of the Luminescence of Eu(III)-Macrocycles by Energy Transfer from Yttrium(III). The interaction between B-diketonate complexes of luminescent lanthanide(III) ions and yttrium(III), in the presence of a synergistic compounds, has been employed by Xu[17] to provide a "cofluorescence" effect which significantly increases the emission intensity. Xu[17] stated, "The strong fluorescence of the lanthanide chelates is based on the fact that the ligand absorbs the excitation energy, whereafter the energy is transferred from the triplet level of the ligand to the resonance level of the lanthanide." Xu further stated that, "The cofluorescence is based on an intermolecular energy transfer that occurs from the chelate of the ion increasing fluorescence, the energy donor, to the chelate of the fluorescent ion, the energy acceptor, provided that the cofluorescence complex is in solution as a suspension or in solid form as aggregated particles and that the solution contains a large excess of the chelate containing ion increasing the fluorescence."

The systems described by Xu require that the original chelate containing the luminescent lanthanide(III) ion be dissociated from the biomolecule prior to the addition of the Y(III) "cofluorescence species", and therefore would not be applicable to cell imaging. However, the method is, at least in principle, susceptible of extension to other lanthanide complexes, including the Eu(III)-macrocycles. A preliminary, unoptimized study with the parent unfunctionalized macrocyle {Eu($C_{22}H_{26}N_6$} triacetate complex as prototype, has shown an approximate three fold increase in luminescence.

2.4. Synthesis and Characterization of Lanthanide Complexes of Mono-Functionalized Macrocyclic Ligands.

The coupling of the bi-functional Eu(III)-macrocycles to avidin, when carried out as described in Section 2.2.1., has not resulted in any appreciable cross-linking. However, the possibility of either intra- or inter-molecular cross-linking is inherent in the use of any bi-functional reagent. Accordingly, it was of interest to develop a synthesis for a novel class of lanthanide(III) complexes containing mono-functionalized six-nitrogen-donor macrocyclic ligands.

Several members of this of this class have now been obtained[18] in good yields by the 1:1 Schiff-base con-

densation of a carbon-substituted 1,2-diaminoethane with a pre-formed non-fuctionalized diketone-diimine, in the presence of lanthanum(III) acetate as templating agent (Figure 2). Lanthanum(III) was used in the development of the synthesis, because it is diamagnetic and the proton nuclear magnetic resonance spectra of its complexes are structurally diagnostic. Because of the extreme chemical similarity among the larger lanthanides, any results obtained with lanthanum can then be confidently extended to europium or other neighboring

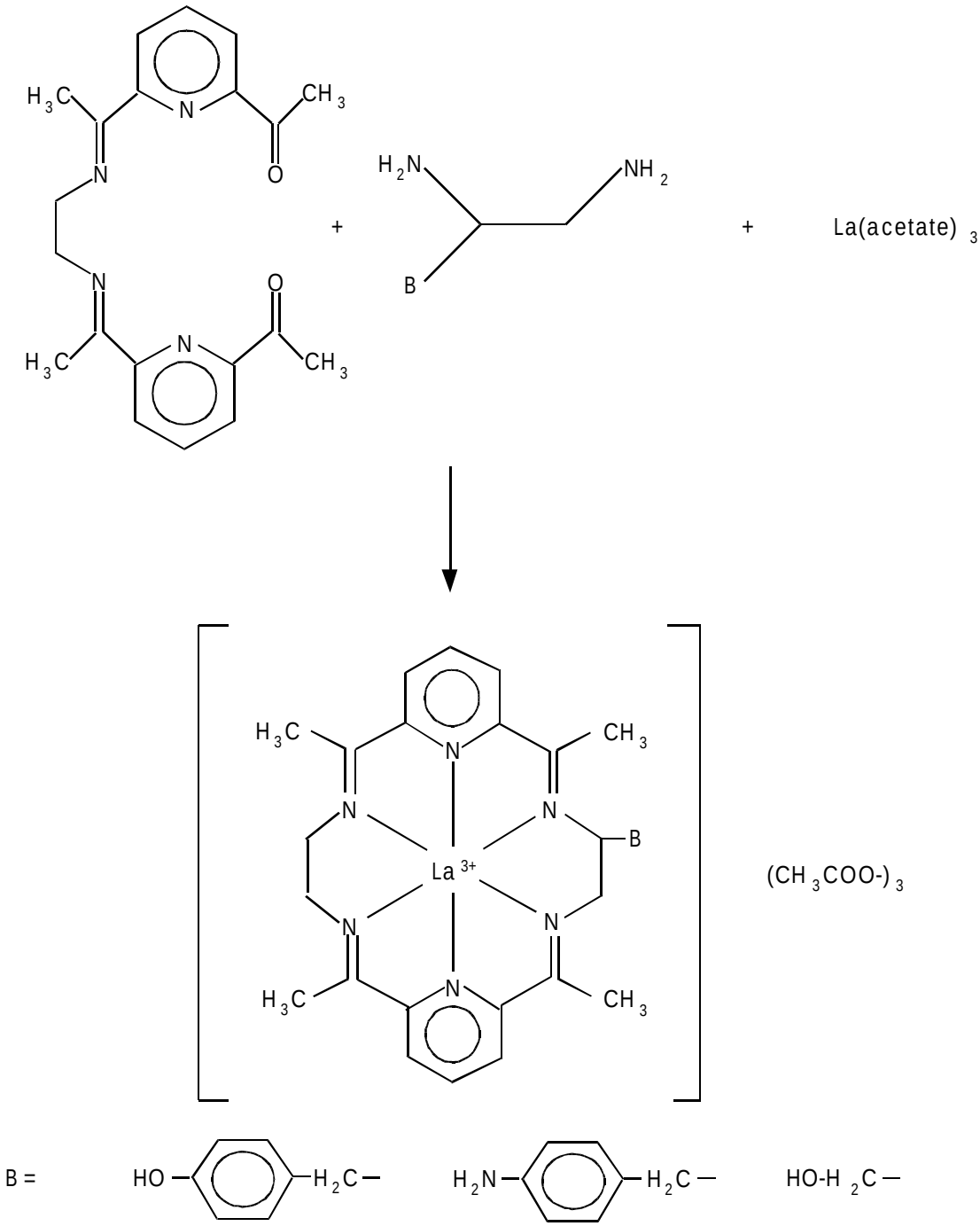

Figure 2. Lanthanide ion templated synthesis of mono-functionalized macrocyclic ligand. the B group attached to the diimine side-chain provides the functionality. Macrocycles with hydroxy-benzyl, amino-benzyl and hydroxy-methyl groups have been synthesized.

elements.

The La(III) complexes of mono-functionalized macrocycles are essentially identical in their general physical as well as chemical characteristics to their bi-functional analogs. They are air-stable, light-stable microcrystalline solids, soluble in a variety of polar solvents and resistant to ligand hydrolysis as well as to metal release or exchange even in the presence of competing reagents. Extension of the synthesis to the Eu(III) species is in

progress.

3. Microscopic Imaging of Rare Earth Chelate Luminescent Labels

3.1. Gated CCD Studies

Seveus et al.[19] demonstrated that time-gated Eu(III) luminescence could be detected in strongly autofluorescent cells. Cultured human colon cancer cells were fixed with glutaraldehyde and permanently mounted. The Wallac Oy W-8044 chelate, for which no structure was reported, was conjugated to a monoclonal antibody which bound to the colon cancer cells. A fluorescein-conjugated antibody served as control. The combination of a mechanical chopper and a flash lamp provided the time gating. The signal to noise ratios for the fluorescein and chelate-labeled cells were, respectively, about 6 and 2,400 or better. The europium-labeled cells showed no decrease in luminescence intensity after a 77-day storage in daylight at room temperature. It should be noted that glutaraldehyde fixation, because it induces autofluorescence, is seldom if ever used for immunofluorescence studies. Although glyoxal is not as good a fixative as glutaraldehyde, glyoxal fixation does maintain acceptable morphology for light microscopy and minimal autofluorescence[16].

3.2. Gated Image Intensifier

Wang et al.[20] employed an image intensified CCD camera to obtain a time-gated signal from a Human Papillomavirus Type 16 DNA probe that had been coupled to avidin labeled with Soini's (Wallac Oy) Eu(III) chelate. The use of this probe in conjunction with a DNA stain is a rational approach to automating the Pap test. It could also serve as a model for detecting cellular HIV.

3.3. Film and CCD

Vallarino et al.[4] photographed microscopically imaged biotinylated-agarose beads labeled with a luminescent Eu-macrocyle, as discussed in Section 2.2.2. A 400 ASA film and a DAGE MTI CCD 725 camera operated at room temperature were employed in this study.

3.4. Future Instrumentation

3.4.1. Cameras

The present real limitation to the use of rare earth chelates is the lack of a commercially available, electronically gated CCD camera. The charge build up that occurs during the excitation light flash should be drained either via antiblooming diodes or other means. Rotating choppers loose the strongest part of exponentially decaying signal, which occurs right after the end of the excitation flash. Compared to image intensifiers, CCD cameras have the advantage of much higher quantum efficiency in the red, higher resolution, lower pixel response heterogeneity, and lower cost.

3.4.2. Light Sources

Although the performance of xenon flash lamps appears to be adequate, the potential increase in ultraviolet emission intensity provided by a flashed mercury arc should represent a significant motivation to develop this technology. Quantitative microscopy also requires that the present excitation optical train be improved to provide homogenous illumination of the specimen.

4. Conclusions

The NCS-functionalized macrocyclic complex of Eu(III) reported in this paper fulfills all fundamental requirements of a luminescent marker for cell imaging and solid-phase immunoassays: it does not release Eu(III) even in very dilute aqueous solution and the presence of competing ligands. Since the macrocycle is formed around the lanthanide ion, the species is kinetically stable and will not dissociate under the experimental conditions employed for the formation of antigen-antibody complexes or hybridizing an oligonucleotide to DNA or RNA. It can be made instantaneously luminescent in aqueous solution by the simple addition of an enhancer. It may be conveniently coupled to proteins, or any other bio-substrate containing primary amino groups, by the well-established protocol used for fluorescein isothiocyanate. The attachment of the Eu(III)-macrocycle is compatible with the function of immunological reagents. If any significant cross-linkage is observed with the bifunctional macro-

cycle, this should be eliminated by the use of the monofunctional macrocycle. The capacity to synthesize asymmetrically functionalized macrocycles will eventually permit the covalent linkage of enhancers and other species of interest to the macrocycle.

The luminescence of the protein-bound Eu(III)-macrocycle retains the narrow-band, long-lifetime character typical of other Eu(III) chelates in both aqueous and nonaqueous solutions, as well as in the solid state. The surrounding conditions have minimal effect on the shape of the emission spectrum. The capacity of the macrocycle to emit in mounting media permits the use of standard histological and cytological permanent mounting procedures.

Rare earth based time-gated luminescence will provide a means to both increase the sensitivity of one or more measurements and the total number of simultaneous luminescent and fluorescent species that can be measured.

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