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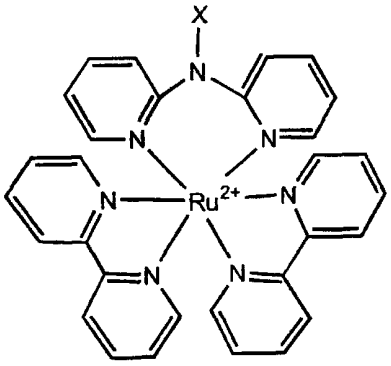
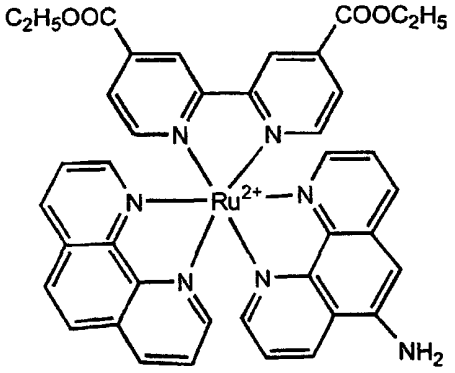
(54) Title: NEW RUTHENIUM METAL LIGAND COMPLEXES



Complex A when X = H,
Where X can be CH_2COOH or $CH_2CH_2NH_2$



Complex B

(57) Abstract: A fluorescent metal ligand complex includes a transition metal associated with a complement of diamine ligands, which may be the same or different, one of which is amino reactive for attachment to biological molecules. The surprisingly long lifetime and high anisotropy ratio of the complexes make them especially useful in fluorescence polarization assays and in fluorescence resonance energy transfer assays.

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Title: New Ruthenium Metal Ligand Complexes

Field Of The Invention

This invention relates to metal ligand complexes useful as fluorescent probes in assays for biological molecules and more specifically in immunoassays making use of fluorescence resonance energy transfer (FRET) and fluorescence polarization (FP) techniques.

Background Of The Invention

Optical assays based on detection of changes in fluorescence are not new. Such assays that use measurements of fluorescence lifetime are known to have advantages over other optical analytical methods because the fluorescence lifetime of excited probes is independent of the probe concentration. Thus, useful measurements can be made relatively independently of total intensity. Spectroscopic methods for FRET and FP based immunoassays using fluorescence lifetimes of probes are described by A. J. Ozinskas in Principles of Fluorescence Immunoassays, Topic in Fluorescence Spectroscopy, Vol. 4, 1994, page 449-490.

Optical assays based on fluorescence changes in certain metal ligand complexes are not new. Such assays are described by J. R. Lakowicz, *et. al.* in Recent Development in Fluorescence Spectroscopy, Near Infrared Dyes for High Technology Application, edited by Daehne *et. al.* (eds.) and published in 1998 by Kluwer Academic Publishers, page 3-19. The lifetimes of certain metal ligand complexes are relatively long compared with previously known fluorescent materials. In most cases the fluorescent lifetime of such complexes is more than 30 ns and in some cases up to micro-seconds. However, the longer lifetime probes present possibilities for new, improved and lower cost methods for optical sensing using phase modulation and frequency modulation analysis as described by J.R. Lakowicz in US Patent 5,660,991. There continues to be a

need for complexes that give better results in assays of the type described by Lakowicz.

Several examples of ruthenium metal complexes are disclosed by A. Juris *et .al.* in the article "Ru(II) Polypyridine Complexes: photophysics, photochemistry, electrochemistry and chemiluminescence" published in Coord. Chem. Rev. in 1988 at pages 84, 85-277. Some such complexes are emissive in response to radiation and show changes in emission when in the proximity of certain analytes, making them useful for sensing in the clinical chemistry as described by Z. Murtaza, *et. al.*. "Long lifetime Metal-ligand pH Probes", Anal. Biochem. 1997, 247, 216; by Lakowicz, J. R *et. al.* in "Development of Long lifetime Metal Ligand Probes for Biophyics and Cellular Imaging" J. Fluoresc,1997, 7, 17; and in PCT WO09838496A1, (09/03/1998) to J.R. Lakowicz, *et. al.*

Polarization immunoassays using measurements of the fluorescent lifetime of metal ligand complexes is described in US Patent 5.660,991, dated Aug 26, 1997, issued to J.R. Lakowicz, *et. al.* Successful use of this method is known to be dependent on selection of the complex. Proper selection can give better sensitivity. Selection of the complex is dependant, among other things, upon the size of the analyte. In cases where the analyte is a protein, such as in immunoassays, a complex having a longer fluorescent lifetime will give better results. However, it is known that in every case a high anisotropy value will give better results. As is well known, the anisotropy value is determined by the ratio of $I_{\equiv} - I_{\perp} / I_{\equiv} + 2I_{\perp}$ where $I_{\equiv}$ is the value when polarizers are parallel and $I_{\perp}$.is the value when polarizers are perpendicular.

A number of already known such metal ligand complexes contain functional groups enabling their conjugation with biological molecules ("biomolecules") such as antibodies and other proteins thus enabling their use in FRET and FP immunoassays. The fluorescence properties of these metal ligand complexes differ from each other based on the ligand used and on the group attached to the complex by the ligand. Known metal ligand complexes that can be linked with biomolecules usually produce anisotropy values of less

than 0.3. Fluorescent metal ligand complexes that would produce anisotropy values of greater than 0.3 would expand the usefulness of such complexes especially in FP immunoassays. It has been a problem that such complexes have not been known in the past.

It is an object of the present invention to overcome this problem.

It is also an object of the present invention to provide a fluorescent metal ligand complex having a lifetime sufficiently long to be useful in fluorescence assays, especially FRET and FP assays using inexpensive monitoring devices.

It is another object of this invention to provide such a fluorescent metal ligand complex that is also being capable of attachment to biological molecules.

It is still another object of this invention to present such a fluorescent metal ligand complex that also provides improved polarization values.

It is still another object of this invention to provide fluorescent metal ligand complexes having a fluorescent lifetime greater than about 10 ns and having an anisotropy value of greater than about 0.3.

Summary of the Invention

These and other objects are accomplished by the present invention which, in one aspect, comprises metal ligand complexes that are fluorescent in response to electromagnetic radiation and that include groups for attachment to biological molecules.

In another aspect the invention comprises FRET or FP assays for biological molecules that make use of such complexes.

The metal ligand complex of the present invention comprises a transition metal associated with a complement of diamine ligands, one of which is amino reactive for attachment to a biomolecule.

In one embodiment the complex of the present invention is $Ru(bpy)_2(dpa)](PF_6)_2$ ((A) in **FIG. 3.**)

In another embodiment the complex of the present invention is $Ru(phen)(aphen)(deebpy)](PF_6)_2$ ((B) in **FIG. 3.**)

Brief Description Of The Drawings

The invention is described below in more detail with reference to the drawings in which:

FIG. 1 shows the absorption and emission spectra of Ru(phn)(aphn)(decbpy), one complex according to the present invention, compared with that of $Ru(bpy)_2(dcbpy)$, a prior art material.

FIG. 2 shows the absorption and emission spectra of $Ru(bpy)_2(dpa)$, another complex according to the present invention, compared with that of $Ru(bpy)_2(dcpy)$, a prior art material.

FIG. 3 shows the structural formula for two complexes according to the present invention: $[Ru(phen)(aphen)(deebpy)](PF_6)_2$ and $[Ru(bpy)_2(dpa)(PF_6)_2$.

Detailed Description

The invention will be described in detail in connection with embodiments (A) and (B) shown in **FIG. 3.**

The complex of embodiment (A), $[Ru(bpy)_2(dpa)](PF_6)_2$, was synthesized by refluxing, $Ru(bpy)_2Cl_2$ with dpa (where dpa is 2,2'-dipyridylamine) (both chemicals purchased from Aldrich Chemical Co. Inc.) ligand in the 1:1 ethanol water mixture for 24 hours. The resulting reaction mixture was filtered while hot and reduced to half using a vacuum evaporator. At this point saturated aqueous solution of ammonium hexafluorophosphate was added which resulted the brown precipitates, which were filtered and washed with water and than with ether and dried in air. The compound, $[Ru(bpy)_2(dpa)](PF_6)_2$,

was further purified by column chromatography on alumina using acetonitrile toluene solvent mixture. The compound was characterized by elemental analysis which were agreed with theoretical values.

Complex B was synthesized in three steps. The first intermediate is $Ru(phen)Cl_4$ it was synthesized by using following method:

Synthesis of $Ru(phen)Cl_4$ (I). Tetrachloro-mono-1,10-phenanthroline-ruthenium(II) was obtained on mixing 1:1 molar ratio of $RuCl_3$ and 1,10-phenathroline (purchased from Aldrich Chemical Co. Inc.) in 0.01N HCl. a dark brown precipitates of $Ru(phen)Cl_4$ was obtained in few hours, this dark compound was filtered and washed with water and dried in air. It was the starting material for the second intermediate which was synthesized by using following method.

Synthesis of $Ru(aphen)(phen)Cl_2$ (II). Compound II was prepared by refluxing 1:1 ratio of (I) and ligand 5,amino-1,10-phenanthroline (aphen) (purchased from GFS Inc.) respectively in tetrahydrofuran for 5 hours. On cooling and reducing the volume to one third, which gives dark brown crystals, crystals were filtered and washed with cold water and dried in air. This product was the starting material for the third and final compound B by using following procedure.

Synthesis of $[Ru(phen)(aphen)(deebpy)](PF_6)_2$ (B). The target complex was obtained by reaction of II with 4,4'-diethylacetate-2,2'-bipyridine, deebpy, ligand in 1:1 ratio respectively in ethanol water (1:1) mixture at reflux for 24 hours. The reaction mixture was filtered while hot and reduced to half in volume by vacuum evaporator than aqueous solution of saturated ammonium hexafluorophosphate was added which gives brown precipitates. The dark brown color precipitates was filtered and washed with water followed by ether and dried in air. The brown compound, $[Ru(phen)(aphen)(deebpy)](PF_6)_2$ was further purified by column chromatography using alumina and acetonitrile/toluene solvent mixture.

The absorption and emission spectra, quantum yield and lifetime of synthesized [Ru(phen)(aphen)(deebpy)]$(PF_6)_2$ and [Ru(bpy)$_2$(dpa)]$(PF_6)_2$ were measured by standard techniques using a spectrophotometer from ISS, Champaign-Urbana, IL, and the results are shown in FIGS. 1 and 2. The spectral properties of these compounds were also compared with the spectral properties of the preferred metal ligand complex of the prior art, Ru(bpy)$_2$(dcbpy)]$(PF_6)_2$. The resulting spectral data suggests that the compounds of the present invention have better properties than reported compounds and are better candidates for FP probes. For example, compound B in **FIG. 3** has about twice the lifetime and quantum yield values as does the prior art material, making it better for FP based immunoassays.

Table 1 shows the photophysical parameters, measured by well known techniques on the spectrophotometer, for the embodiments of the present invention (as shown in **FIG. 3**) and those for Ru(bpy)$_2$(dcbpy)]$(PF_6)_2$. The complexes (A) and (B) in **FIG. 3** according to the present invention have anisotropy values of 0.32 and 0.36, respectively, (maximum possible value is 0.4) compared with 0.24 for the prior art material. Fluorescent lifetimes for the complexes of the present invention are 155 ns and 557 ns compared with 373 ns for the prior art material, confirming that complex (B) of **FIG. 3** is more suitable for FP immunoassays than the prior art material for large molecules. While material (A) will to be useful in FP assays for small molecules.

Table 1. Photophysical parameters of the probes at room temperature.

Compounds	λ_{ab} (nm)	λ_{em} (nm)	τ^a (ns)	τ^b (ns)	ϕ (air)	r_0^c
[Ru(bpy)$_2$(dpa)]$(PF_6)_2$						
In water	455	630	155	--	(0.01)	0.32

In acetonitrile	450	650			
[Ru(aphen)(phen)(deebpy)]$(PF_6)_2$					
In water	455	630	557	630	0.078(0.068) 0.36
In acetonitrile	450	650		1554	
[Ru$((bpy)_2$(dcbpy)]$(PF_6)_2$[d]					
In water	460	650	472	373	0.042(0.039) 0.24
In acetonitrile	455	680	--	--	--

a) Measured in aerated sample b) measured in nitrogen flushed sample c) measured in PVA

d) Already reported dye. τ = lifetime in nano seconds, ϕ = quantum yield, r_0 = fundamental anisotropy in rigid media and λ = wave length in nano meters

In polarization immunoassay the complexes of the present invention are conjugated to an antibody that is specific to an antigen (the target analyte). After labeling to the antibody the complex gives a value of anisotropy, due to the rotational motion of complex and the antibody with which it is conjugated. On adding this labeled antibody to a target antigen, its anisotropy changes because the rotation is further slowed by the increased size of the resulting system (antibody and antigen). The change in the anisotropy value can be use to study information about the target analyte. The molecular weight of the target antigen, based on the complex (A) of **FIG. 3** should be in the range of 10^4- 10^5 (dalton) and for the dye B it should be in the range of 10^5- 10^6 (dalton). The anisotropy of labeled analyte can be determined by the Perrin equation, r = r_0-/1+(/), where r is anisotropy of the analyte, r_0 = anisotropy of dye (in the absence of rotational diffusion), τ is lifetime of the probe and θ is rotational

correlation time, as described in Lakowicz, J. R., Principles of Fluorescence Spectroscopy Second edition, Kluwer Academic Press, 1999, page 583.

In FRET based assays these complexes can be used as the donor, and an appropriate acceptor can be selected based on the emission maximum of the chosen complex. In a sandwich assay, for example, these complexes may be attached to the one of antibodies and will act as donors. An acceptor dye can be attached to the other antibody. Upon adding antigen, (an analyte) these two antibodies bind to the antigen and show energy transfer. The energy transfer is sensed and used to determine the presence or amount of analyte. As described by J.R. Lakowicz in Principles of Fluorescence Spectroscopy Second Edition, Kluwer Academic Press, 1999, page 368-391.

Other embodiments of the metal ligand complex of the present invention may use other transition metals, such as, for example, Os and Re, and these embodiments are intended to be within the scope of the claims, below.

Claims:

1. A fluorescent metal ligand complex comprising:

a transition metal; and

a complement of diamine ligands wherein one of such ligands is amino reactive.

2. The metal ligand complex of claim 1 wherein the transition metal is Ru.

3. The metal ligand complex of claim 2 having the formula $Ru(bpy)_2(dpa)](PF_6)_2$.

4. The metal ligand complex of claim 2 having the formula $Ru(phen)(aphen)(deebpy)](PF_6)_2$.

5. A fluorescence polarization assay wherein a fluorescent metal ligand complex according to claims 1-4 is used as an indicator.

6 A fluorescence energy resonance transfer assay wherein a fluorescent metal ligand complex according to claims 1-4 is used as a donor.

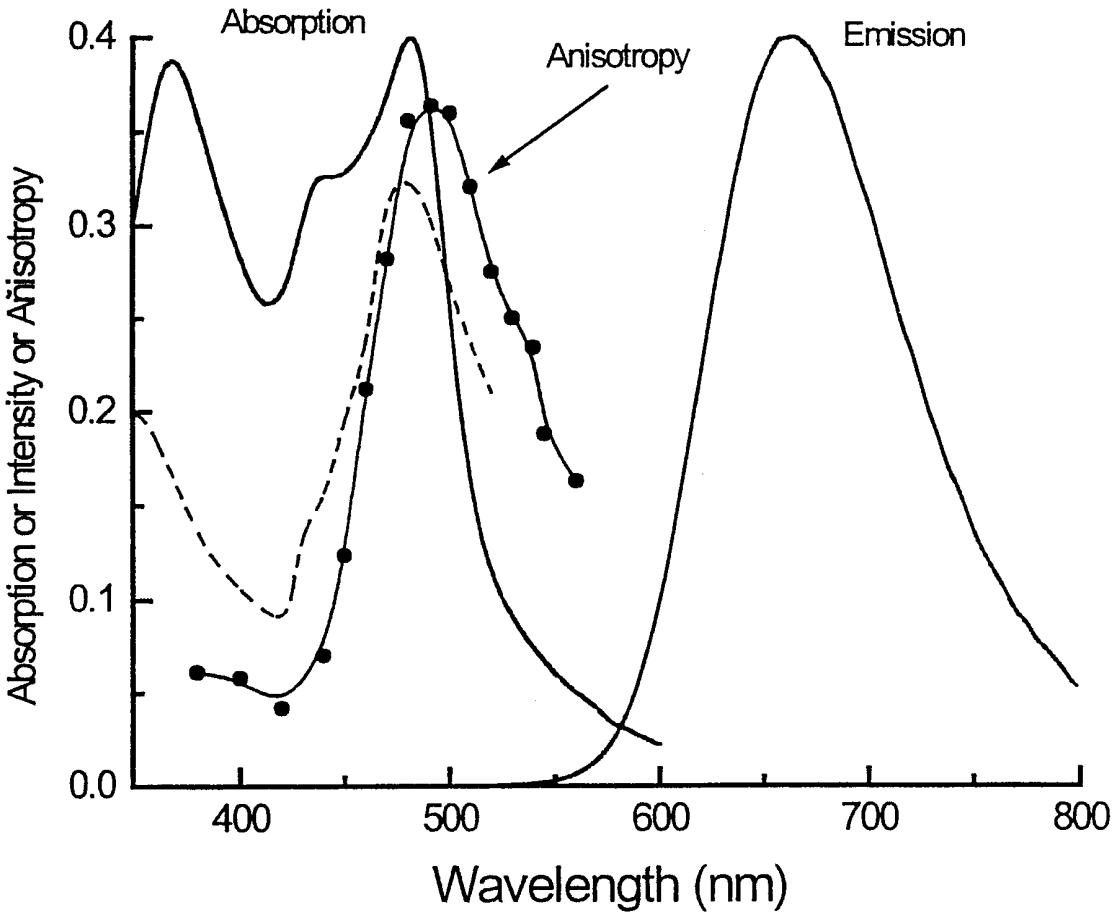


Figure 1. Absorption and emission spectra of Ru(phn)(aphn)(decbpy) in aqueous solution. Excitation anisotropy spectrum (—●—) is compared with that of $Ru(bpy)_2(dcbpy)$ (- - - -. Both anisotropy spectra were measured in PVA films.

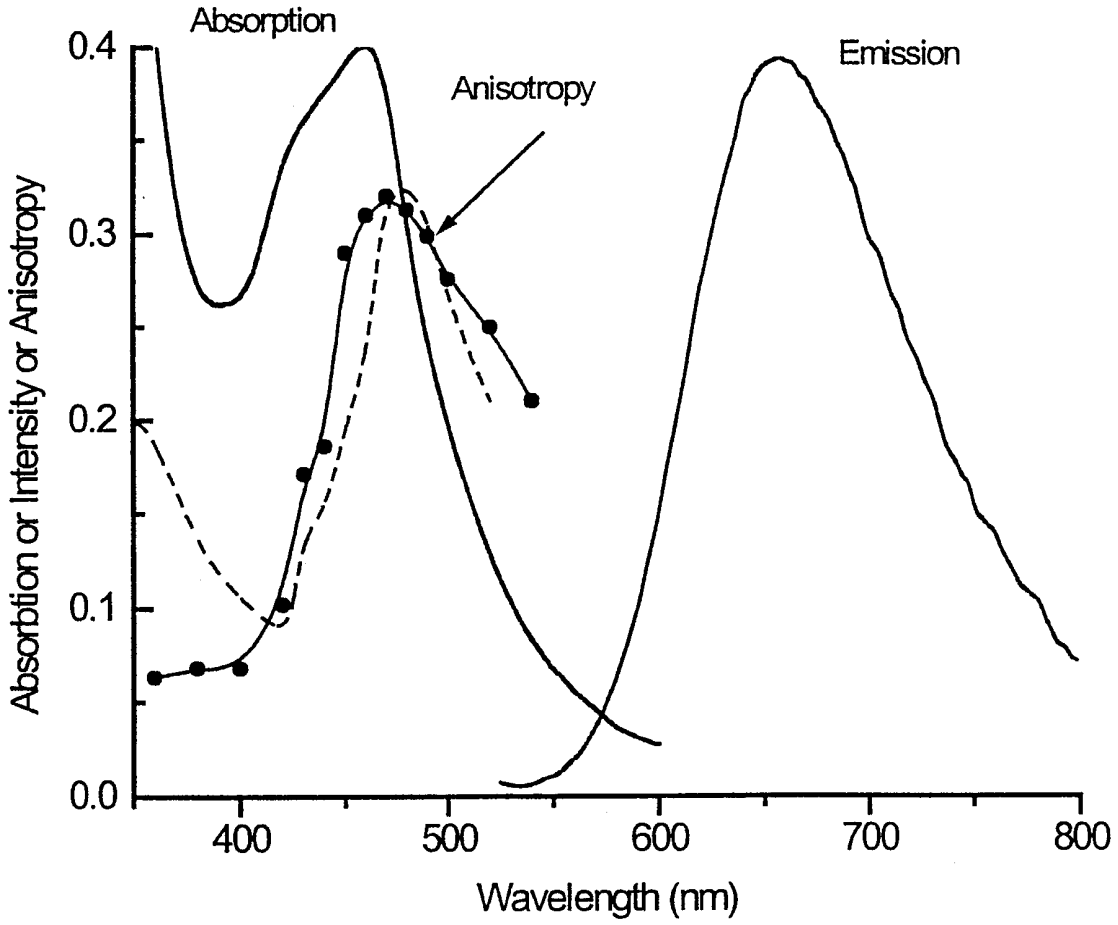
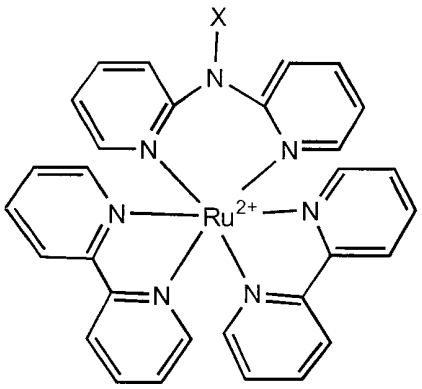


Figure 2. Absorption and emsission spectra od $Ru(bpy)_2(dpa)$ in aqueous solution. Anisotropy excitation spectrum (—●—) is compared with that for $Ru(bpy)_2(dcbpy)$- - - -). Both anisotropy spectra were measured in PVA films.



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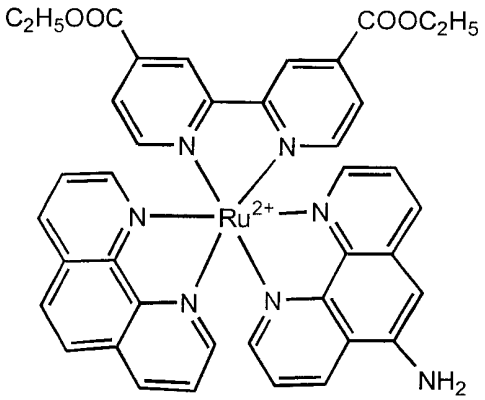

Complex B