PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 21/64 — A1

(11) International Publication Number: **WO 98/38496**

(43) International Publication Date: 3 September 1998 (03.09.98)

(21) International Application Number: PCT/US98/00148

(22) International Filing Date: 13 January 1998 (13.01.98)

(30) Priority Data:
08/808,563 28 February 1997 (28.02.97) US

(71)(72) Applicant and Inventor: LAKOWICZ, Joseph, R. [US/US]; 10037 Fox Den Road, Ellicott City, MD 21042 (US).

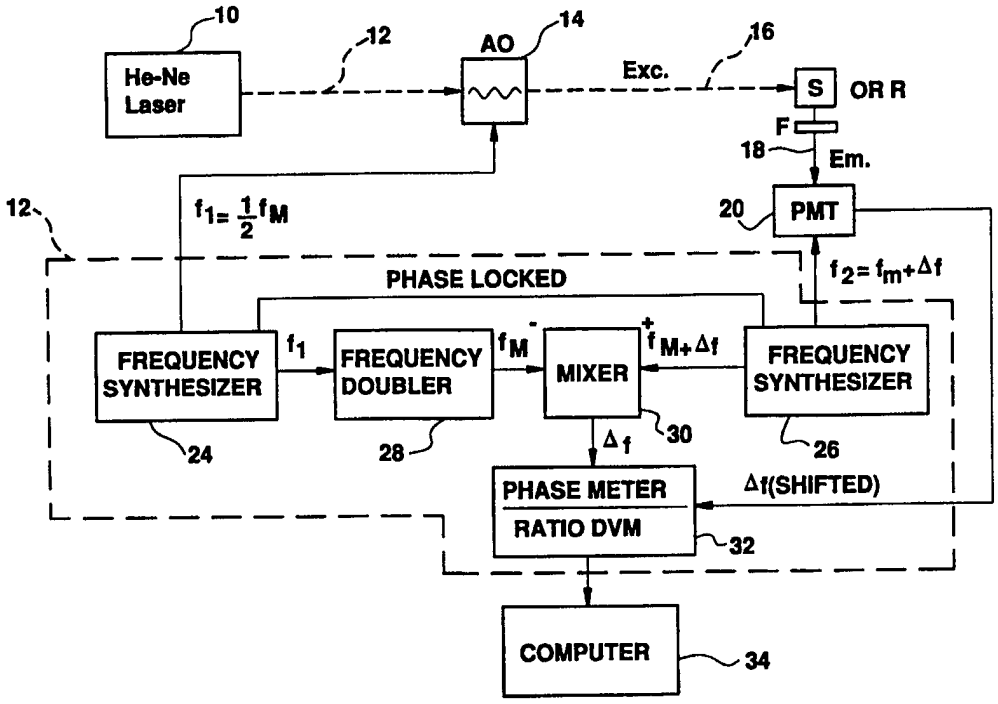
(72) Inventors: SZMACINSKI, Henryk; 8401 Glen Road, Baltimore, MD 21234 (US). TERPETSCHNIG, Ewald; University of Regensburg, Institute for Analytical Chemistry Bio Chemosensors, D-93040 Regensburg (DE). MURTAZA, Zakir; 900 S. Paca Street, Baltimore, MD 21230 (US).

(74) Agent: REPPER, George, R.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street N.W., Columbia Square, Washington, DC 20004 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MEASURING ANALYTES WITH METAL-LIGAND COMPLEX PROBES



(57) Abstract

A metal-ligand complex probe is contacted with an analyte in a sample to form an analyte-bound probe species in equilibrium with free probe species, at least one of which is fluorescent. The sample is excited with radiation (16) to produce an emission signal (18) which is detected and correlated to analyte concentration.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

MEASURING ANALYTES WITH METAL-LIGAND COMPLEX PROBES

BACKGROUND OF THE INVENTION

This work was supported by the National Institutes of Health and the National Science Foundation.

FIELD OF THE INVENTION

The present invention relates to the field of measuring analytes in a sample.

DESCRIPTION OF THE BACKGROUND ART

Measurement of certain analytes in blood, such as pH and carbon dioxide, is an important aspect of the clinical care of patients. Previously, such measurements have been made using gas chromatography and other chemical methods. These methods are disadvantageous in that it is necessary to ship the blood sample to a clinical laboratory for analysis, which often results in a delay of an hour or more. Moreover, since the blood gases change rapidly, the shipping time may cause the results to be invalid. Furthermore, these known methods cannot be used for continuous in vivo monitoring of blood.

Optical methods for measurement of blood chemistry, such as fluorescence-based methods, are of great interest because they offer the possibility of decreased cost and less handling of possibly contaminated blood.

During the past several years there has been increasing interest in fluorescence lifetime-based sensing. In this method the analyte concentration is determined from the decay time of the fluorophore and

its dependence on the analyte of interest. Lifetime-based sensing can be preferred over intensity-based methods because the lifetime is mostly independent of the probe concentration and can be unaffected by photo bleaching or washout of the probe. Lifetimes have been measured through skin and in turbid media, opening the possibility of transdermal sensing with long wavelength light sources. Lifetime sensors have now been identified from a large number of analytes, including pH, NH_3, CO_2, Ca^{2+}, Mg^{2+}, immunoassay and glucose. Lifetime sensing applications for pO_2 and pCO_2 in bioprocess control have also been described.

At present most lifetime-based fluorophores display lifetimes of from 1 to 10 ns, which requires relatively fast electronics for time-domain lifetime measurements, or modulation frequencies of from 10 to 100 MHZ for frequency-domain measurements. Additionally the auto-fluorescence from most biological specimens displays decay times near 1-10 ns, making it difficult to separate the desired signal from the interfering auto-fluorescence. Such short-lived probes present obstacles to providing simple instruments for point-of-care assays and off-gating the short-lived autofluroescence in circumstances which requires high sensitivity detection.

There remains a need in the art for new and improved methods for measuring analytes.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method of measuring an analyte in a sample comprises the following steps. A metal-ligand complex probe is

contacted with a sample containing analyte. The probe is bound to analyte in the sample to form an analyte-bound probe species. Both bound and unbound species of said probe exist in the sample. At least one of the bound and unbound species is fluorescent, with each of the bound and unbound species being optically distinguishable. The sample containing the bound and unbound species is excited with radiation, so as to produce a resulting emission from at least one of the bound and unbound species. The resulting emission is detected, so as to provide an optical measurement of the emission. Concentration of analyte in the sample is determined utilizing the optical measurement of the emission.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 graphically shows pH-dependent absorption spectra of $[Ru(deabpy)(bpy)_2](PF_6)_2$.

Figure 2 graphically shows pH-dependent emission spectra of $[Ru(deabpy)(bpy)_2](PF_6)_2$. Excitation at 414 nm.

Figure 3 graphically shows pH-dependent fluorescence intensities (557-750 nm) of $[Ru(deabpy)(bpy)_2](PF_6)_2$ in different buffer concentrations.

Figure 4 graphically shows pH-dependent absorption of $[Ru(deabpy)(bpy)_2]^{2+}$ at 450 nm.

Figure 5 graphically shows wavelength-ratiometric measurements of pH using the emission intensities at 620 and 650 nm.

Figure 6 graphically shows pH-dependent frequency-domain intensity decays of $[Ru(deabpy)(bpy)_2]^{2+}$ at pH 2.40 (●), 7.45 (■) and 13.53 (▲).

Figure 7 graphically shows pH-dependent amplitude of the two decay time global analysis (Table II).

Figure 8 graphically shows pH-dependent phase angles of $[Ru(deabpy)(bpy)_2]^{2+}$ with a modulation frequency of 700 kHz.

Figure 9 graphically shows pH-dependent modulation of $[Ru(deabpy)(bpy)_2]^{2+}$ with a modulated frequency of 700 kHz.

Figure 10 shows the structure of $[Ru(deabpy)(bpy)_2]^{2+}$.

Figure 11 shows structures of cation-sensitive metal-ligand probes.

Figure 12 schematically shows point-of-care assays based on metal-ligand probes with a LED light source.

Figure 13 is a schematic diagram showing instrumentation for use in accordance with one embodiment of the present invention.

Figure 14A shows structure of an alternative metal-ligand complex which displays a different pK_a value as well as absorption and emission wavelengths.

Figure 14B shows another potential metal-ligand pH sensor in accordance with the present invention.

Figure 15 graphically depicts data in connection with the metal-ligand complex $[Ru(bpy)_2(dcbpy)]Cl_2$ showing pH-dependent intensity, phase and modulation data.

Figure 16 graphically depicts data in connection with a metal-ligand complex $[Ru(deabpy)(bpy)_2](PF_6)_2$ obtained with a LED light source.

Figure 17 graphically depicts data in connection with a metal-ligand complex $[Ru(deabpy)(bpy)_2](PF_6)_2$ obtained with a LED light source.

Figure 18 graphically depicts data in connection with a metal-ligand complex $[Ru(deabpy)(bpy)_2](PF_6)_2$ obtained with a LED light source.

Figure 19 graphically depicts pH-dependent phase angles for $[Ru(deabpy)(bpy)_2](PF_6)_2$ obtained with a LED light source.

Figure 20 graphically depicts pH-dependent modulation data for $[Ru(deabpy)(bpy)_2](PF_6)_2$ obtained with a LED light source.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

According to the present invention, analytes can be sensed and measured using metal-ligand complexes utilizing lifetime measurements, intensity measurements, phase modulation fluorometry, time-domain fluorescence methods or ratiometric wavelength shifts. The invention is particularly applicable to transition metal-ligand complexes containing, for example, ruthenium, osmium, rhenium, rhodium, and the like.

In accordance with one embodiment, the present invention provides a method in which a luminescent ligand is added to the sample to be analyzed in the form of a photoluminescent metal-ligand complex probe having intrinsic analyte-induced lifetime changes. The lifetime measurements can be performed in optically dense samples or turbid media and are independent of and/or insensitive to photo bleaching, probe wash-out or optical loss. The lifetime changes can be measured using known time-resolved or phase-modulation fluorometry methods.

In accordance with one embodiment of the method of the invention, the probe can be either fluorescent or phosphorescent.

The step of adding a luminescent metal-ligand complex probe sample to be analyzed requires matching a

particular probe to a particular analyte, so that at least a portion of the sample will be bound (e.g., non-covalently bound) to the probe so that both bound and unbound species of the probe will exist. Thus, the invention differs from prior lifetime measurement methods which rely on a collisional quenching mechanism for measuring analytes. See, for example, U.S. Patent No. 4,810,655 to Khalil et al.; and Great Britain Patent No. 2,132,348 to Demas et al.

By definition, in collisional quenching, the probe does not bind to the analyte as required by the present invention. Instead, collisional quenching requires collisional contact between the fluorophore (probe) and the quencher (analyte). For collisional quenching to occur, the quencher must diffuse to interact with the fluorophore while the latter is in the excited state. Thus, the excited fluorophore returns to the ground state without emission of a photon.

In contrast, the present invention may have an "enhancement" of the luminescence. When the fluorescent ligand binds to the analyte, there may be an increase or decrease in intensity. It is also to be emphasized that the method of the present invention is not a Foerster energy transfer mechanism, and thus is different from the method disclosed in European Patent Application 397,641 to Wolfbeis.

The present invention thus differs from oxygen sensing with metal-ligand complexes, in that in the latter case, the quenching is due to diffusion controlled collisional encounters between the oxygen and the fluorophore. In the present invention, the change in intensity or lifetime is caused by

interaction of the analyte with the fluorophore resulting in a different decay time. In the case of intensity-based quenching, one of the forms can be non-fluorescent. In the case of lifetime-based sensing, both forms, with and without bound analyte, must be fluorescent so that a lifetime change can be detected upon complexation.

The method of the present invention may be useful for sensing a wide range of organic solutes such as pH, carbon dioxide, sodium ion, potassium ion, calcium ion, or magnesium ion concentrations and the like, in blood and other bodily fluids. Such measurements can be of intracellular analytes, or of extracellular analytes, depending on the location of the fluorophore.

The method of the invention is useful in either in vitro or in vivo applications, including, for example, blood gas catheters, including optical fibers, and other bedside monitors, and non-invasive blood gas measurements. Also, the invention may be used for sensors in fermentors and incubators.

As noted above, the method in accordance with certain embodiments of the invention determines and quantifies chemical analytes by changes in photoluminescence lifetimes. Embodiments of the invention can include adding a luminescent metal-ligand complex to the sample containing the analyte to be analyzed in the form of a photoluminescent probe. In accordance with certain embodiments, the probe can be either fluorescent or phosphorescent.

The invention generally requires matching a particular probe to a particular analyte, so that at

least a portion of the analyte will become bound (e.g., non-covalently bound) to the probe, so that both bound and unbound (i.e., free) species of the probe will then exist within the sample. The probe can be chosen to have intrinsic analyte-induced lifetime changes, i.e., when the probe is bound to an analyte, the naturally occurring fluorescent or phosphorescent lifetime changes. It is to be understood that throughout this application the term "lifetime" refers to the photoluminescent lifetime defined as the inverse of the decay rate of the probe. In the case where two lifetimes are displayed by the probe, the term "lifetimes" refers to the measured mean or apparent lifetimes. These changes in lifetime can be measured to determine the concentration of the analyte, as will become more apparent from the discussion below.

In the context of the present invention, the term "sample" refers to compounds, surfaces, solutions, emulsions, suspensions, mixtures, cell cultures, fermentation cultures, cells, tissues, secretions and/or derivatives or extracts thereof, as well as supercritical fluids. Samples, as defined above, which can be used in the embodiments of the present invention for sensing analytes based on fluorescence lifetimes also include samples that can be clear or turbid. Such samples to be measured according to these embodiments of the present invention require only that the fluorophore used be contacted with the sample such that the analyte to be sensed influences the lifetime of the fluorophore such that the lifetime varies with the presence or amount of the analyte.

Such samples can also include, e.g., animal tissues, such as blood, lymph, cerebrospinal fluid, bone marrow, gastrointestinal contents, and portions, cells or internal and external secretions of skin, heart, lung and respiratory system, liver, spleen, kidney, pancreas, gall bladder, gastrointestinal tract, smooth, skeletal or cardiac muscle, circulatory system, reproductive organs, auditory system, the autonomic and central nervous system, and extracts or cell cultures thereof. Such samples can be measured using methods of the present invention in vitro, in vivo and in situ.

Such samples can also include environmental samples such as earth, air or water samples, as well as industrial or commercial samples as compounds, surfaces, aqueous chemical solutions, emulsions, suspensions or mixtures.

Additionally, samples that can be used in the method of the present invention include cell culture and fermentation media used for growth of prokaryotic or eukaryotic cells and/or tissues, such as bacteria, yeast, mammalian cells, plant cells and insect cells.

The term "analyte" in the context of the present invention refers to elements, ions, compounds, or salts, dissociation products, polymers, aggregates or derivatives thereof. Examples of analytes that can be measured in the method of the present invention include, e.g., H^+, Ca^{2+}, Mg^{2+}, Na^+, K^+, NH_3^+, PO_4^{2-} and the like, or other compounds containing these ionic solutes, including salts, derivatives, polymers, dissociation products, or aggregates thereof.

The method of the invention further includes exciting the tagged sample with radiation from any suitable radiation source, such as a laser, an light emitting diode or the like. Light sources suitable for use in the methods of the present invention, also include noble gas light sources such as helium, neon, argon, krypton, xenon, and radon, and combinations, thereof. Light sources can include gas lamps or lasers which provide uniform light that has been filtered, polarized, or provided as a laser source, such as a coherent wave (CW) laser or a pulsed laser. Specified impurities can be added to the above described noble gas light sources to provide suitable light sources for use in the present invention with varying wavelengths such as emission and excitation wavelengths. Such impurities include Group II metals, such as zinc, cadmium, mercury, strontium, selenium and ruthenium. A green helium-neon laser can be used in accordance with one embodiment of the present invention, and is inexpensive and reliable.

In one embodiment, the intensity of the excitation radiation is modulated at a particular modulation frequency and the lifetime determined using known phase-modulation, i.e., frequency domain, techniques. Alternatively, a pulsed radiation source may be used and the lifetime of the sample determined using known time resolved methods. Both phase-modulation and time-resolved fluorometry methods are well known in the prior art, see Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, 1983, Chapter 3. However, current instrumentation renders the phase modulation method more expedient. The phase-modulation method is further discussed below, but it is to be understood

that these same principles generally apply to time-resolved measurements.

When the sample is excited with radiation whose intensity is modulated, for example, in a sinusoidal manner, the time lag between absorption and emission causes the emission to be delayed in phase and demodulated relative to the excitation radiation. As discussed above, when a luminescent ligand is added to the sample, at least a portion of the analyte will bind with the ligand, i.e., probe, so that both bound and unbound species of the probe will exist within the sample. The probe is preferably chosen so that there will be a significant difference in the luminescent lifetime between the bound and unbound species. The phase shift and the corresponding demodulation factor m can be measured and used to calculate the photoluminescent lifetime based on well known formulae. See, Lakowicz, supra. It is desirable to select the modulation frequency in a range that coincides with the frequency at which the differences between the measured phase angles and the demodulations of the bound and unbound ligand are maximal.

Thus, according to a method according to the invention, the emission radiation is detected, the phase shift (in degrees) and the demodulation factor m (as a percentage change) are measured, and the apparent photoluminescent lifetime may be calculated therefrom. An absolute value of difference in phase angle between the bound and free unbound forms of the ligand of at least about 10°, e.g., on the order of 10-60° at a preselected frequency, and a difference in modulation factor of at least about 10%, e.g., on the order of about 10-87%, can be utilized. These ranges of phase

angles and modulation factors offer a combination of precision and dynamic range.

The absolute values of the frequency-dependent phase differences and demodulations can be determined by the photoluminescent lifetimes of the free and bound ligand. In addition, if the excitation and emission spectra are not congruent, effects can occur whereby at particular wavelengths of excitation or emission one form or the other of the probe is preferentially excited or its emission preferentially observed. In such cases, the apparent analyte sensitive concentration range (for pH, the apparent pKa) varies with excitation or emission wavelength. This can be advantageous where the method of the present invention allows a range of concentrations that can be accurately measured with a single probe to be easily varied by selection of the appropriate excitation and/or emission wavelengths.

The probes described in the present application display absorption in the blue region of the spectrum and long decay times over 100 ns. As a result it is possible and preferable to use amplitude modulated light emitting diodes (LEDs) as the excitation source. It is known that LEDs are inexpensive and reliable, and easily modulated at the frequencies needed for use with long decay time probes.

EXAMPLE 1

ABBREVIATIONS:

bpy	2,2'-bipyridine
deabpy	4,4'-diethylaminomethyl-2,2'-bipyridine
LED	light emitting diode

MLC metal-ligand complex

SUMMARY

Herein is described the synthesis and fluorescence spectral characterization of a pH-sensitive metal-ligand complex, $[Ru(deabpy)(bpy)_2]^{2+}$, where deabpy is 4,4'-diethylaminomethyl-2,2'-bipyridine. This metal-ligand complex (MLC) was found to display pH dependent intensities, emission spectra, and decay times, with the changes centered near the physiological useful pH value of 7.5. The apparent pK_a values were not found to be dependent on ionic strength. The compound was found to be useful for lifetime-based sensing by phase-modulation fluorometry. Global analysis of the intensity decays over a range of pH values revealed two decay times of 235 and 380 ns, associated with the protonated and unprotonated forms, respectively. Because of its long decay time pH sensing was accomplished by phase-modulation fluorometry with a conveniently low modulation frequency of 700 kHz. The lifetime data were obtained with either an amplitude-modulated laser and with an amplitude-modulated blue light emitting diode. This pH sensitive complex also displays a modest spectral shift with change in pH allowing its use as a wavelength-ratiometric MLC probe. One can imagine lifetime sensors for a variety of blood cations and point-of-care assays based on long lifetime metal-ligand complexes with simple solid state light sources and detectors.

Among the various optical methods, fluorescence detection offers the advantages of high sensitivity and ion-selective fluorescence probes. The current status of fluorescence sensing has described in recent

literature. See, e.g., Proceedings of the 2nd European Conference on Optical Chemical Sensors and Biosensors, EUROPT(R)ODE II (F. Baldini, Ed.). Florence, Italy, April 1994, *Sensors and Actuators B.*, pp. 439. Proceedings of the 1st European Conference on Optical Chemical Sensors and Biosensors, EUROPT(R)ODE I (O.S. Wolfbeis, Ed.). Graz, Austria, April 1992, *Sensors and Actuators B.*, pp. 565. Topics in Fluorescence Spectroscopy, Vol. 4: Probe Design and Chemical Sensing (1994). (J.R. Lakowicz, Ed.), Plenum Press, New York. pp. 501. Fluorescent Probes in Cellular and Molecular Biology (1994). Slavik, J., CRC Press, Boca Raton, pp. 295. Advances in Fluorescence Sensing Technology II (1995). (J.R. Lakowicz, Ed.) San Jose, CA., *SPIE Proc.*, Vol. 2388. pp. 598. Potential Applications of Lifetime-Based Phase-Modulation Fluorometry for Bioprocess and Clinical Sensing (1995). Bambot, S.B., J.R. Lakowicz and G. Rao. *Trends in Biotechnology*, 13:106-116.

During the past several years there has been increasing interest in lifetime-based sensing. See, e.g., Lifetime-Based Sensing. Szmacinski, H. and Lakowicz, J. R. (1994) In: Topics in Fluorescence Spectroscopy, Vol. 4: Probe Design and Chemical Sensing (J. R. Lakowicz, Ed.), Plenum Press, 295-334. Luminescence Decay Time-Based Optical Sensors: Principles and Problems (1993). Lippitsch, M.E and S. Draxler. *Sensors and Actuators B*, 11:97-101. Time-resolved Fluorescence Spectroscopy for Chemical Sensors (1996). Draxler, S. and M.E. Lippitsch. *Appl. Optics*, 35(21):4117-4123.

In this method the analyte concentration is determined from the decay time of the fluorophore and

its dependence on the analyte of interest. Lifetime-based sensing can be preferred over intensity-based methods because the lifetime is mostly independent of the probe concentration and can be unaffected by photobleaching or washout of the probe. As noted above, the possible mechanisms of lifetime-based sensing have been reviewed. Lifetimes have been measured through skin and in turbid media, suggesting the possibility of trans-dermal sensing with long wavelength light sources. Lifetime sensors have now been identified from a large number of analytes, including pH, NH_3, CO_2, Ca^{2+}, Mg^{2+}, Cu^{2+}, immunoassays and glucose. Practical sensing applications for pO_2 and pCO_2 in bioprocess control have also been described. See, e.g., Sensing Oxygen Through Skin Using a Red Diode Laser and Fluorescence Lifetimes (1995). Bambot, S.B., G. Rao, Romauld. M., G.M. Carter, J. Sipior, E. Terpetschnig and J.R. Lakowicz. *Biosensors & Bioelectronics*, 10(6/7):643-652. Frequency-domain Lifetime Measurements and Sensing in Highly Scattering Media (1995). Szmacinski, H. and J.R. Lakowicz. *Sensors and Actuators B.*, 30:207-215. Optical Measurements of pH Using Fluorescence Lifetimes and Phase-Modulation Fluorometry (1993). Szmacinski, H.and J.R. Lakowicz. *Anal. Chem.*, 65:1668-1674. Lifetime-Based Optical Sensing of pH Using Resonance Energy Transfer in Sol-Gel Films (1994). Bambot, S., J. Sipior, J.R. Lakowicz and G. Rao. *Sensors and Actuators B: Chemical*, 22:181-188. A Lifetime-Based Fluorescence Resonance Energy Transfer Sensor for Ammonia (1995). Chang, Q., J. Sipior, J.R. Lakowicz and G. Rao. *Anal. Biochem.*, 232:92-97. A Lifetime-Based Optical CO_2 Gas Sensor with Blue or Red Excitation and Stokes or Anti-Stokes Detection (1995). Sipior, J., S. Bambot, Romauld M., G.M. Carter, J.R. Lakowicz and G. Rao. *Anal. Biochem.*,

227:309-318. Possibility of Simultaneously Measuring Low and High Calcium Concentrations Using Fura-2 and Lifetime-Based Sensing (1995). Szmacinski, H. and J.R. Lakowicz. *Cell Calcium*, 18:64-75. Fluorescence Lifetime Characterization of Magnesium Probes. Improvement of Mg^{2+} Dynamic Range and Sensitivity Using Phase-Modulation Fluorometry (1996). Szmacinski, H. and J.R. Lakowicz. *J. Fluoresc.*, 6 (2): 83-95. Fluorescence Lifetime Sensor of Copper Ions in Water (1996). Birch, D.J.S., O.J. Rolinski and D. Hatrick. *Rev. Sci. Instrum.*, 67(8):2732-2737. Homogeneous Model Immunoassay of Thyroxine by Phase-Modulation Fluorescence Spectroscopy (1992). Ozinskas, A.J., H. Malak, J. Joshi, H. Szmacinski, J. Britz, R.B. Thompson, P.A. Koen and J.R. Lakowicz. *Anal. Biochem.*, 213:264-270. Optical Sensing of Glucose Using Phase-Modulation Fluorometry (1993). Lakowicz, J.R. and B.P. Maliwal. *Anal. Chim. Acta.*, 271:155-164. Phase Fluorometric Sterilizable Optical Oxygen Sensor (1994). Bambot, S., R. Holavanahali, J.R. Lakowicz, G. Carter and G. Rao. *Biotechnol. Bioeng.*, 43:1139-1145. A Phase Fluorometric Optical CO_2 Gas Sensor for Fermentation Off-Gas Monitoring (1996). Sipior, C.J., L. Randers-Eichhorn, J.R. Lakowicz, G. Carter and G. Rao. *Biotech. Prog.*, 12:266-271. Non-Invasive Oxygen Measurements and Mass Transfer Limitations in Tissue Culture Flasks (1996). Randers-Eichhorn, D.L., R. Bartlett, D. Frey and G. Rao. *Biotechnol. Bioeng.*, 51:466-478.

As further noted above, at present most lifetime-based fluorophores display lifetimes from 1 to 10 ns, which requires relatively fast electronics for time-domain lifetime measurements or modulation frequencies from 10 to 100 MHZ for frequency-domain measurements. Additionally, the auto-fluorescence from most

biological specimens displays decay times near 1-10 ns, making it difficult to separate the desired signal from the interfering auto-fluorescence. The availability of longer lived probes permits design of simple instruments for point-of-care assays and off-gating the short lived autofluorescence in circumstances which requires high sensitivity detection. In fact, a solid state phase-modulation fluorometer has already been reported. See, Detection of Fluorescence Lifetime Based on Solid State Technology and Its Application to Optical Oxygen Sensing (1995). Gruber, W.R., O'Leary, P. And Wolfbeis, O.S., *SPIE Proc.*, 2388:148-158.

Use of long-lifetime metal-ligand probes may be useful in fluorescence microscopy and for chemical imaging by lifetime imaging. See, e.g., Fluorescence Lifetime-Imaging of Intracellular Calcium in COS Cells Using Quin-2 (1994). Lakowicz, J.R., H. Szmacinski, K. Nowaczyk, W.J. Lederer, M.S. Kirby and M.L. Johnson. *Cell Calcium*, 15:7-27. Fluorescence Lifetime Imaging of Free and Protein-Bound NADH (1992). Lakowicz, J.R., H. Szmacinski, K. Nowaczyk and M.L. Johnson. *Proc. Natl. Acad. Sci. U.S.A.*, 89:1271-1275. High-Speed Fluorescence Microscopy: Lifetime Imaging in the Biomedical Sciences (1995). Periasamy, A., X.F. Wang, P. Wodnick, G.W. Gordon, S. Kown, P.A. Diliberto and B. Herman. *J.S.M.A.*, 1(1):13-23.

Herein is described the synthesis and spectral properties of a long lived metal-ligand complex (MLC) which displays pH sensitive emission in the physiological range from 6 to 8. This compound $[Ru(deabpy)(bpy)_2]^{2+}$ (Figure 10) was found to display pH sensitive intensities, phase angles and modulations with an apparent pK_a near 7.5. This pH-dependent MLC can

be regarded of the first of a series of cation-sensitive probes which display decay times in excess of 200 ns.

MATERIALS AND METHODS

Synthesis of $[Ru(deabpy)(bpy)_2]$ $(PF_6)_2$

The synthetic procedure for preparation of $[Ru(deabpy)(bpy)_2](PF_6)_2$ followed published procedures [see, Luminescent pH Sensors Based on Di(2,2'bipyridyl) (5,5' diaminomethyl-2,2' bipyridyl)-ruthenium (II) Complexes (1992). Grigg, R. and W.D.J. Amilaprasadh Norbert. *J. Chem. Soc. Chem. Commun.*, 1300-1302; Formation of Thin Polymeric Films by Electropolymerization. Reduction of Metal Complexes Containing Bromomethyl-Substituted Derivatives of 2,2' Bipyridine. Gould, S., G.F. Strouse and B.P. Sullivan. *Inorg. Chem.*, 30:2942-9.] with slight modification. The $(CH_2Br)_2$bpy was prepared by following reported methods [see, Formation of Thin Polymeric Films by Electropolymerization. Reduction of Metal Complexes Containing Bromomethyl-Substituted Derivatives of 2,2' Bipyridine. Gould, S., G.F. Strouse and B.P. Sullivan. *Inorg. Chem.*, 30:2942-2949; Synthesis and Coordination Chemistry of 1-(2',2"-bipyridyl-5-yl-methyl)-1,4,8,11-tetrazacyclotetradecane of Ni^{II} or Cu^{II} in Cyclam Cavity (bpy = 2,2' bipyridine; cyclam = 1,4,8,11-tetra-aza cyclotetradecane (1992). Rawle, S.C., P. Moore and N.W. Alcock. *J. Chem. Soc. Chem. Commun.*, 684-] and resulting compound was purified by column chromatography [30]. The ligand 4,4'-diethylaminomethyl-2,2'-bipyridine (deabpy) was prepared by refluxing $(CH_3CH_2)_2$ NH with $(CH_2Br)_2$bpy in CCl_4 and purified by using a silica column using acetone/dichloromethane solvent mixture. $Ru(bpy)_2Cl_2$ was stirred in acetone for about two hours with silver

triflate, the white precipitate of silver chloride was removed, and the resulting red color solution was stirred with deabpy ligand for about six hours. The acetone was removed and the residue redissolved in water, precipitated with ammonium hexafluorophosphate and filtered. The brick red color solid was redissolved in acetonitrile and chromatograph with an acetonitrile/toluene mixture over alumina. The $[Ru(deabpy)(bpy)_2](PF_6)_2$ was characterized by proton NMR.

Instrumentation and Procedures

Chemicals and solvents were purchased from Aldrich and used without further purification. Absorption spectra were measured using a Perkin Elmer lambda 6 UV/Vis spectrophotometer. Observed were two isosbestic points at 414 nm and 475 nm. The absorbance at 450 nm of different samples were measured to obtain the ground state pK_a.

$Ru(bpy)_2(deabpy)(PF_6)_2$ solutions at different pH value were prepared by dissolving equal amount of aqueous $Ru(bpy)_2(deabpy)(PF_6)_2$ in different buffer solutions. From pH 2 to pH 4.7, we used citrate buffer; from pH 4.8 to pH 6.3 we used acetate buffer; from pH 6.4 to pH 7.8 we used phosphate buffer; from pH 7.8 to pH 9.0 we used tris buffer, from pH 9.2 to pH 11 we used carbonate/bicarbonate buffer, from pH 11 to pH 12 we used dibasic sodium phosphate/sodium hydroxide buffer. Unless stated otherwise the buffer concentrations were 20 mM and contained 0.1 M potassium chloride to maintain ionic strength. In order to determine if the pK_a was dependent on the buffer concentration, we also prepared sample solutions in 50 mM and 100 mM phosphate with and buffer with pH values

between 6 and 8 and compared their total fluorescence intensity from 550 nm and 750 nm.

The fluorescence emission spectra were measured using Aminco Bowman Series AB2 Luminescence Spectrometer with the excitation wavelength of 414 nm. The acidic sample (at low pH) had an emission maximum at 650 nm, and the basic sample had an emission maximum at 620 nm. The emission intensity ratios (620 nm/650 nm) of the different sample were also recorded.

For the phase/modulation measurements (Figures 6-9), an air-cooled Argon ion laser (output at 488 nm) was used as the excitation light source, and the modulation frequency was set at 700 kHz. On the emission side, a long-wave pass filter was used to collect the fluorescence with wavelength longer than 600 nm. The reference solution used here was an aqueous solution of Texas Red with a reference lifetime of 4 ns. For the lifetime measurements, we used the same instrumentation and reference solution, and 23 different modulation frequencies ranging from 11 kHz to 2 MHZ.

Phase modulation measurements (Figures 16-20) were also performed with a Nichia blue LED (NLPB500, Nichia America Co., Lancaster, Pa. with maximum output at 450 nm) as the excitation light source. In this case the measurements were performed on an ISS K2 Multifrequency Phase and Modulation Fluorometer (Champaign, IL). A set of Andover 500FL07, 600FL07 and 700FL07 short-wave pass filters (Salem, NH) was added in the excitation path to ensure the cut-off of light from the LED with wavelengths longer than 500 nm. On the emission side, an Andover 600FH90 long-wave-pass filter was used to

collect the fluorescence with wavelengths longer than 600 nm. An 650FH90 or 700FH90 filter was used to replace the 600FH90, where necessary. The reference solution used for the lifetime measurement was a 0.5% solution of Du Pont Ludox HS-30 colloidal silica in water, with the intensity matched to that of the sample by using neutral-density filter(s) in its emission path. All experiments were performed at an ambient temperature. In this case single frequency measurements were performed at 823 kHz.

Single and multi-exponential intensity decays were recovered from the multi-frequency data as described previously [see, Construction and Performance of a Variable-Frequency Phase-Modulation Fluorometer (1985). Lakowicz, J.R. and B.P. Maliwal. *Biophys. Chem.*, 21:61-78; Frequency-Domain Fluorescence Spectroscopy (1991) in Topics in Fluorescence Spectroscopy, Vol. 1: Techniques, (Lakowicz, J.R., Ed.). Plenum Press, New York, pp. 293-355]. The data were analyzed globally in terms of the multi-exponential model

$$I_k(t) = \sum_{i=1,\,k}^{2} \alpha_{ik}\, e^{-t/\tau_i} \qquad (1)$$

where $I_k(t)$ are the intensity decays at each pH (k) value, τ_i are the decay times and α_{ik} are the amplitudes. For the global analysis we assumed that the α_{ik} values would depend on pH, but that the two lifetimes would be independent of pH. The goodness-of-fit was judged by the usual χ^2_R criterion with assumed uncertainties in phase $\delta p = 0.2°$ and modulation $\delta m = 0.005$.

RESULTS

Absorption and emission spectra of $[Ru(deabpy)(bpy)_2]^{2+}$ at pH values from 2 to 12 are shown in Figures 1 and 2, respectively. Only modest changes are seen in the absorption spectrum, but the emission spectrum increases about 3-fold as the pH increases from 2.52 to 11.8. The pH-dependent intensity changes are shown in Figure 3, and reveal a pKa value near 7.5. This pKa value is ideally suited for measurements of blood pH, where the clinically relevant range is from 7.35 to 7.46, with a central value near 7.40. In addition, much cell culture work is performed near pH 7.0-7.2. We attribute the changes in absorption and emission to deprontonation of the amino groups on $[Ru(deabpy)(bpy)_2]^{2+}$ (Figure 10).

We were pleasantly surprised by the pKa value near 7.5, as we expected a higher pKa near 9 based on the structures shown in Figure 10. In fact, we initially attempted to synthesize a different structure which contained hydroxyl groups on the terminal methyl groups. These hydroxyl groups were thought to be needed to obtain a pKa near 7.5, based on their presence in the widely used buffer tris, tris(hydroxymethyl)aminomethane. However, we had difficulties synthesizing or isolating the hydroxyl containing compound, and decided to synthesize and test $[Ru(deabpy)(bpy)_2]^{2+}$ as an initial step.

We also considered whether the pK_a observed by fluorescence is in fact the ground state pK_a. The ground state pK_a was determined from the changes in absorption at 450 nm (Figure 4). In this case the pK_a was found to be 7.14, somewhat lower than the value of 7.5 observed by fluorescence. This small difference in pK_a values is

not surprising, as metal ligand complexes with ionizable diimine ligands often display different pK_as in the ground and excited states. Importantly, the difference is not large, and our probe does not seem to be sensitive to ionic strength. The same apparent pK_a values were observed in 20, 50 and 100 mM phosphate buffer (Figure 3). We will describe fluorescence changes as due to ionization events at pK_a values, but we recognize that the ground state and excited state pK_a values may be slightly different.

Figure 2 shows that the emission spectrum shifts to longer wavelengths as the amino groups are protonated at low pH. This suggests the use of $[Ru(deabpy)(bpy)_2]^{2+}$ as a wavelength-ratiometric probe. Such ratiometric probes are already in widespread use for measurement of Ca^{2+} [34-35] and pH [36-37], but these are not MLC probes and they display ns decay times. We used the emission spectra at various pH values to obtain a wavelength-ratiometric calibration curve (Figure 5). To the best of our knowledge $[Ru(deabpy)(bpy)_2]^{2+}$ is the first MLC probe which can be used as a ratiometric probe. See, e.g., Fluorescent Indicators of Ion Concentrations (1989). Tsien, R.Y. *Meth. Cell Biol.*, 30:127-156; Fluorescence Ratio Imaging: A New Window into Intracellular Ionic Signalling (1986). Tsien, R.Y. and M. Poenie. *Trends Biochem. Sci.*, 11:450-455; Optical Measurements of pH Using Fluorescence Lifetimes and Phase-Modulation Fluorometry (1993). Szmacinski, H. and J.R. Lakowicz. *Anal. Chem.*, 65:1668-1674; Spectral and Photophysical Studies of Benzo[c]xanthene Dyes: Dual Emission pH Sensors (1991). Whitaker, J.E., R.P. Haughland, F.G. Prendergast. *Anal. Biochem.*, 194:330-344.

The emission shift to longer wavelengths at low pH (Figure 2) seems to be generally understandable in terms of the electronic properties of the excited MLCs. The long wavelength emission is thought to result from a metal-to-ligand charge transfer (MLCT) state in which an electron is donated from Ru to the ligand. The protonated form of deabpy is probably a better electron acceptor, lowering the energy of the MLCT state, shifting the emission to longer wavelengths and thereby decreasing the lifetime. We observed that the emission spectrum of $[Ru(dcbpy)(bpy)_2]^{2+}$ where dcbpy is 4,4'-dicarboxy-2,2'-bipyridine, displays a red shift relative to $Ru(bpy)_3$ [see, Metal-Ligand Complexes as a New Class of Long-Lifetime Fluorophores for Protein Hydrodynamics (1995). Terpetschnig, E., H. Szmacinski, H. Malak and J.R. Lakowicz. *Biophys. J.*, 68:342-350; Fluorescence Polarization Immunoassay of a High Molecular Weight Antigen Based on a Long-Lifetime Ru-Ligand Complex (1995). Terpetschnig, E., H. Szmacinski and J.R. Lakowicz. *Anal. Biochem.*, 227:140-147].

The dcbpy ligand is probably a better electron acceptor than bpy. These results suggest a general approach to designing wavelength-ratiometric MLC probes based on cation-dependent changes in the electron affinity of the ligand. In fact, $[Ru(deabpy)(bpy)_2]$ displays pH-dependent lifetimes (Figure 15), but the pk_a may be too low for medical applications. It could be used at a pH in the range of pH 2 to 5.

Examination of the emission spectra (Figure 2) reveals that the probe is fluorescent in both forms, protonated and unprotonated. It meets the requirements as a lifetime probe because each form is fluorescent and may display distinct decay times. Frequency-domain

intensity decays of $[Ru(deabpy)(bpy)_2]^{2+}$ are shown in Figure 6. At low pH with protonated amino groups the mean decay time is near 240 ns, and at high pH the mean decay time increases to near 390 ns. The intensity decay was found to be reasonably fit to a single exponential at each pH value (Table I). The decay times were difficult to resolve at a single pH value, so that only a modest decrease in χ_R^2 was found for the double exponential fit (Table I). However the apparent decay time found from the single exponential fit increases with increasing pH. Similar intensity decays were found whether the entire emission above 600 nm was observed, or if one just observed the red side of the emission above 700 nm.

The intensity decay may be a multi-exponential at intermediate pH values, where both species are present. Hence we examined the intensity decays at several pH values between 2 and 12. Because of the closely spaced decay times it was difficult to recover the two decay times at each pH. Hence we performed a global analysis in which the pre-exponential factors (α_{ik} values) were assumed to be pH dependent, and the same two decay times would be present at all pH values. For a global analysis the frequency-domain data were only poorly fit by the single lifetime model, as seen from the elevated of $\chi_R^2 = 124$ value (Table II). Analysis in terms of the two lifetime models resulted in a reduced value of $\chi_R^2 = 4.66$. The results of this global analysis are shown graphically in Figure 7. These data show that the amplitude associated with the 235 ns decay time decreases with increased pH near pH 7.5, and that the amplitude associated with the 380 ns component increases at this same pH value. Importantly, the two recovered decay times were comparable to those observed

at pH 2 and pH 12 (Table I), supporting the assignment of the 235 ns decay time to the protonated form and the 380 ns decay time to the unprotonated form of $[Ru(deabpy)(bpy)_2]^{2+}$.

To use $[Ru(deabpy)(bpy)_2]^{2+}$ as a pH sensor we measured its pH-dependent phase and modulation, with a light modulation frequency of 700 kHz, Figures 8 and 9, respectively. As the pH is increased, the phase angles increased from 45 to 57 degrees, and the modulation decreases from 0.69 to 0.52. Similar data were obtained using the blue LED excitation source at 823 kHz (Figures 19 and 20). Conveniently, these changes occur over the pH range from 6 to 8. With present instrumentation one can expect the phase angles and modulation to be accurate to 0.1 degrees and 0.005 in modulation. Hence, the pH can be measured to about one part in 50, or ± 0.04 from pH 6 to 8. Such resolution is acceptable by medical standards [see, Continuous Intravascular and On-Demand Extravascular Arterial Blood Gas Monitoring. Mahutte, C.K.], however one can expect improved phase and modulation occurring with dedicated instrumentation and/or the use of multiple light modulation frequencies. This capability should find immediate applications for non-invasive pH sensing in tissue culture vessels, analogous to that recently reported for oxygen sensing [see, Non-Invasive Oxygen Measurements and Mass Transfer Limitations in Tissue Culture Flasks (1996). Randers-Eichhorn, D.L., R. Bartlett, D. Frey and G. Rao. *Biotechnol. Bioeng.*, 51:466-478].

DISCUSSION

The MLC probe described is sensitive to pH, but a variety of other ions are of medical interest.

However, the invention is applicable to metal-ligand probes for a wide variety of analytes. For instance, the lifetimes of ns probes with ion-chelating groups display changes in lifetime upon chelation. Such ns probes include Ca^{2+}, Mg^{2+}, Na^{2+} and K^{+}. Coupling of the appropriate chelating groups, such as BAPTA or an aza-crown ether, to a metal-ligand complex should result in metal-ligand probes which display ion-sensitive lifetimes. The structures of such potential probes are shown in Figure 11. It is understood that this invention includes other luminescent metal-ligand complexes which include metals such as Re, Os, or Rh. Metal-ligand complexes can be used in immunoassays based on polarization or lifetimes modified by resonance energy transfer. See, e.g., Calcium Concentration Imaging Using Fluorescence Lifetimes and Long-wavelength Probes (1992). Lakowicz, J.R., H. Szmacinski and M.L. Johnson. *J. Fluoresc.* 2(1):47-62; Lifetime-based sensing of sodium (1996). Szmacinski, H. and J.R. Lakowicz. submitted for publication; Fluorescence Lifetime-Based Sensing and Imaging (1995). Szmacinski, H. and J.R. Lakowicz. *Sensors and Actuators B*, 29:16-24; Fluorescence Energy Transfer Immunoassay Based on a Long-Lifetime Luminescent Metal-Ligand Complex (1995). Youn, H.J., E. Terpetschnig, H. Szmacinski and J.R. Lakowicz. *Analy. Biochemistry*, 232:24-30; Fibre-Optic Oxygen Sensor with the Fluorescence Decay Time as the Information Carrier (1988). M.E. Lippitsch and O.S. Wolfbeis.

Long-lifetime cation probes open-up many applications with simple instrumentation. Because of the long decay time, the light modulation frequencies can be near 1 MHZ or lower. Hence, the light source can be a amplitude-modulated light emitting diode (LED). If necessary, signal detection can be performed

simultaneously with electronic off-gating of the detector to suppress the ns components due to autofluorescence from the samples. Such probes and simple instrumentation may allow sensing in blood serum or whole blood, as shown in Figure 12. Hence, the development of metal-ligand complexes can enable simple instrumentation for point-of-care clinical chemistry.

TABLE I. Single Decay Time Analysis of $Ru(bpy)_2(deabpy)$

pH	τ (ns)	χ^2_R
2.40	236	5.22 (4.74)[a]
5.89	252	3.51 (3.19)
6.91	262	2.91 (1.82)
7.45	284	6.24 (3.69)
8.02	327	3.13 (3.18)
13.5	392	2.77 --

[a] The values in parenthesis are for the double exponential fit.

TABLE II. Global pH-Dependent Intensity Decay Analysis of $[Ru(deabpy)(bpy)_2]^{2+}$

pH	τ_1 (ns)	τ_2 (ns)	α_1	α_2	χ^2_R
2.4	235	380	0.964	0.036	
5.8	235	380	0.893	0.107	
6.91	235	380	0.828	0.172	4.66[a]
7.45	235	380	0.687	0.313	
8.02	235	380	0.380	0.620	
13.5	235	380	0	1.0	

[a] For the global single decay time analysis the χ^2_R value was 124, with a mean decay of 294 ns.

One embodiment of instrumentation for use with the method of the invention is schematically shown in Figure 13. However, any suitable instrumentation can be used, reference being made to instrumentation disclosed in U.S. Patent No. 4,937,457 to Mitchell, and those disclosed in Lakowicz, "A Review of Photon-Counting and Phase-Modulation Measurements of

Fluorescence Decay Kinetics", Applications of Fluorescence in the Biomedical Sciences, pp. 29-67 (1986), the contents of which are incorporated herein by reference.

As shown in Figure 13, radiation source 10, emits excitation beam 12 which is modulated by acoustooptic modulator 14 at a frequency f1 to create sinusoidally-modulated excitation beam 16. It is to be understood that modulator 14 need not be an acoustooptic modulator, but that any suitable modulator may be used, such as an electrooptic modulator. Moreover, the modulation need not be sinusoidal, but of any desired shape. Also, the modulator need not be external, but instead the light source may be intrinsically modulated.

Sinusoidally-modulated excitation beam 16 irradiates sample S, which contains the analyte to be measured and the appropriate probe, with both bound and unbound species of the probe being contained within the samples. The irradiated sample emits emitted beam 18 which is detected at photo multiplier tube 20. Emitted beam 18 is amplitude modulated at the same frequency as the excitation but it is phase shifted and demodulated with respect to the excitation. It may be desirable to filter emitted beam 20 with optical filter F in order to change the effective sensitivity range of the detector, as explained above.

Cross-correlation circuit 22 includes first frequency synthesizer 24 which generates frequency f1, equal to one-half of a modulation frequency fM to drive acoustooptic modulator 14, and the PMT dynode chain. Cross-correlation circuit 22 also includes second

frequency synthesizer 26 which generates a frequency f2 equal to the modulation frequency fM plus a cross-correlation frequency Δf to drive photo multiplier tube 20. First frequency synthesizer 24 is coupled to frequency doubler 28, which directs a signal having a frequency equal to the modulation frequency fM to mixer 30. Second frequency synthesizer 26 also directs a signal having frequency f2 equal to the modulation frequency fM plus the cross-correlation frequency Δf to mixer 30. Mixer 30 produces an output signal having a frequency equal to Δf, the difference between fM and f2.

Mixer 30 and photo multiplier tube 20 are each connected to phase meter/digital voltmeter 32. Phase meter/digital voltmeter 32 compares the output signal having a frequency Δf received from mixer 30 and the signal having a frequency Δf(shifted) received from photo multiplier tube 20 to calculate the phase shift θ, and the demodulation factor m which is stored in computer 34.

Since many modifications, variations and changes in detail may be made to the described embodiments, it is intended that all matter in the foregoing description and shown in the accompanying drawings be interpreted as illustrative and not in a limiting sense.

CLAIMS

1. A method of measuring an analyte in a sample, comprising the steps of:

contacting a metal-ligand complex probe with sample containing analyte, wherein the probe is bound to analyte in the sample to form an analyte-bound probe species, wherein bound and unbound species of said probe exist in said sample, at least one of said bound and unbound species are fluorescent, each of said bound and unbound species being optically distinguishable;

exciting the sample containing the bound and unbound species with radiation, so as to produce a resulting emission from at least one of the bound and unbound species;

detecting the resulting emission so as to provide an optical measurement of the emission; and

determining concentration of analyte in the sample utilizing the optical measurement of the emission.

2. The method of claim 1 wherein said analyte is pH, CO_2, sodium ion, potassium ion, calcium ion or magnesium ion.

3. The method of claim 1 wherein said analyte is pH or CO_2.

4. The method of claim 1 wherein the concentration of analyte in the sample is determined by calculating apparent luminescence lifetime of said emission.

5. The method of claim 4 wherein said lifetime is calculated using phase-modulation fluorometry or time-resolved fluorometry.

6. The method of claim 1 wherein said probe comprises $[Ru(deabpy)(bpy)_2]^{2+}$.

7. The method of claim 1 wherein said probe comprises $[Ru(deabpy)(bpy)_2](PF_6)_2$.

8. The method of claim 1 wherein said probe is a transition metal-ligand complex probe.

9. The method of claim 8 wherein said probe includes ruthenium, osmium, rhenium, or rhodium.

10. A method of measuring an analyte in a sample, said analyte being selected from the group consisting of pH, CO_2, sodium ion, potassium ion, calcium ion and magnesium ion, the method comprising the steps of:

contacting a metal-ligand complex probe with said sample containing said analyte, wherein photoluminescence of the probe is affected by said analyte;

exciting the sample with radiation so as to produce a resulting emission;

detecting the resulting emission so as to provide an optical measurement of the emission; and

determining concentration of analyte in the sample utilizing the optical measurement of the emission.

11. The method of claim 10 wherein the concentration of analyte in the sample is determined by calculating apparent luminescence lifetime of said emission.

12. The method of claim 11 wherein said lifetime is calculated using phase-modulation fluorometry or time-resolved fluorometry.

13. The method of claim 10 wherein said probe comprises $[Ru(deabpy)(bpy)_2]^{2+}$.

14. The method of claim 10 wherein said probe comprises $[Ru(deabpy)(bpy)_2](PF_6)_2$.

15. The method of claim 10 wherein said probe is a transition metal-ligand complex probe.

16. The method of claim 15 wherein said probe includes ruthenium, osmium, rhenium, or rhodium.

17. The method claim 1, wherein the bound and unbound species have a difference in phase angle of at least about 10°, and a difference in modulation factor of at least about 10%.

18. The method claim 10, wherein the bound and unbound species have a difference in phase angle of at least about 10°, and a difference in modulation factor of at least about 10%.

19. The method of claim 1, wherein the bound and unbound species have a difference in phase angle of about 10-60°, and a difference in modulation factor of about 10-87%.

20. The method of claim 10, wherein the bound and unbound species have a difference in phase angle of

about 10-60°, and a difference in modulation factor of about 10-87%.

FIG. 1

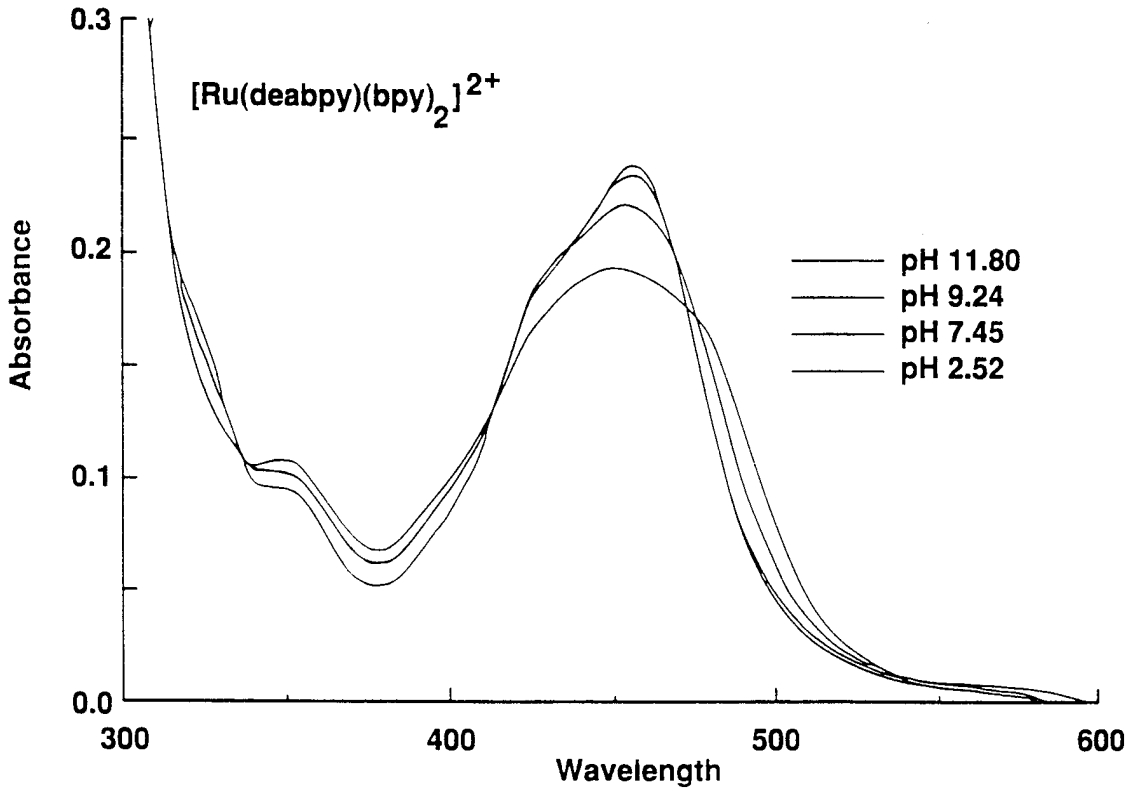

[Ru(deabpy)(bpy)2]2+
Absorbance
0.3
0.2
0.1
0.0
300
400
500
600
Wavelength
pH 11.80
pH 9.24
pH 7.45
pH 2.52

FIG. 2

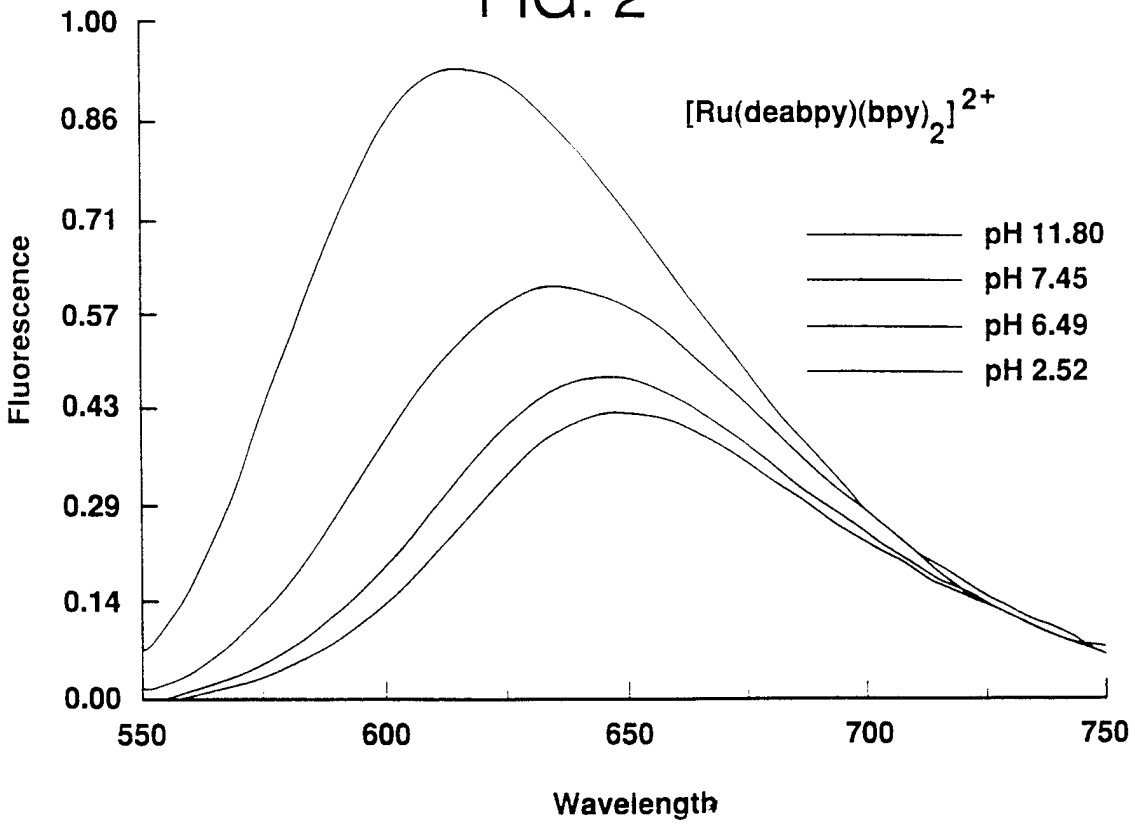

[Ru(deabpy)(bpy)2]2+
Fluorescence
1.00
0.86
0.71
0.57
0.43
0.29
0.14
0.00
550
600
650
700
750
Wavelength
pH 11.80
pH 7.45
pH 6.49
pH 2.52

FIG. 3

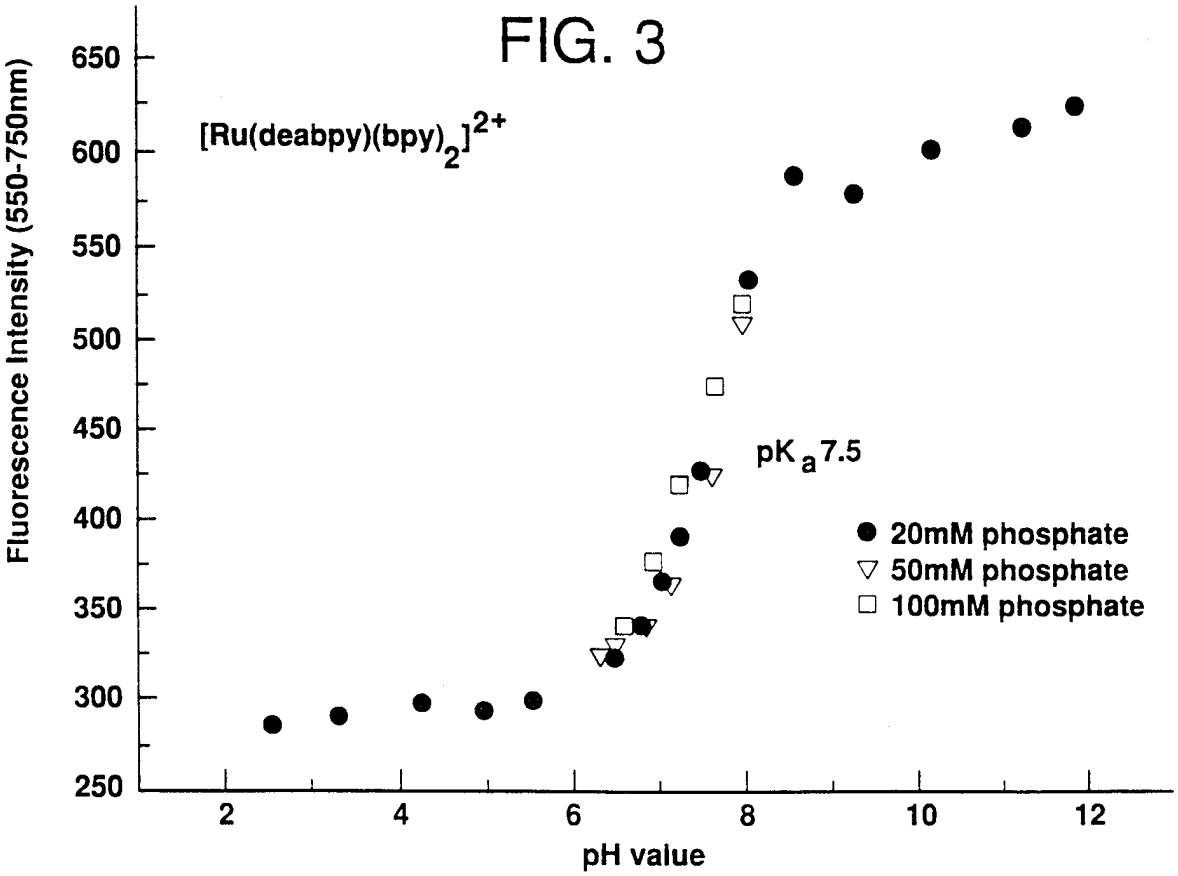

[Ru(deabpy)(bpy)2]2+
Fluorescence Intensity (550-750nm)
650
600
550
500
450
400
350
300
250
2
4
6
8
10
12
pH value
pKa 7.5
20mM phosphate
50mM phosphate
100mM phosphate

FIG. 4

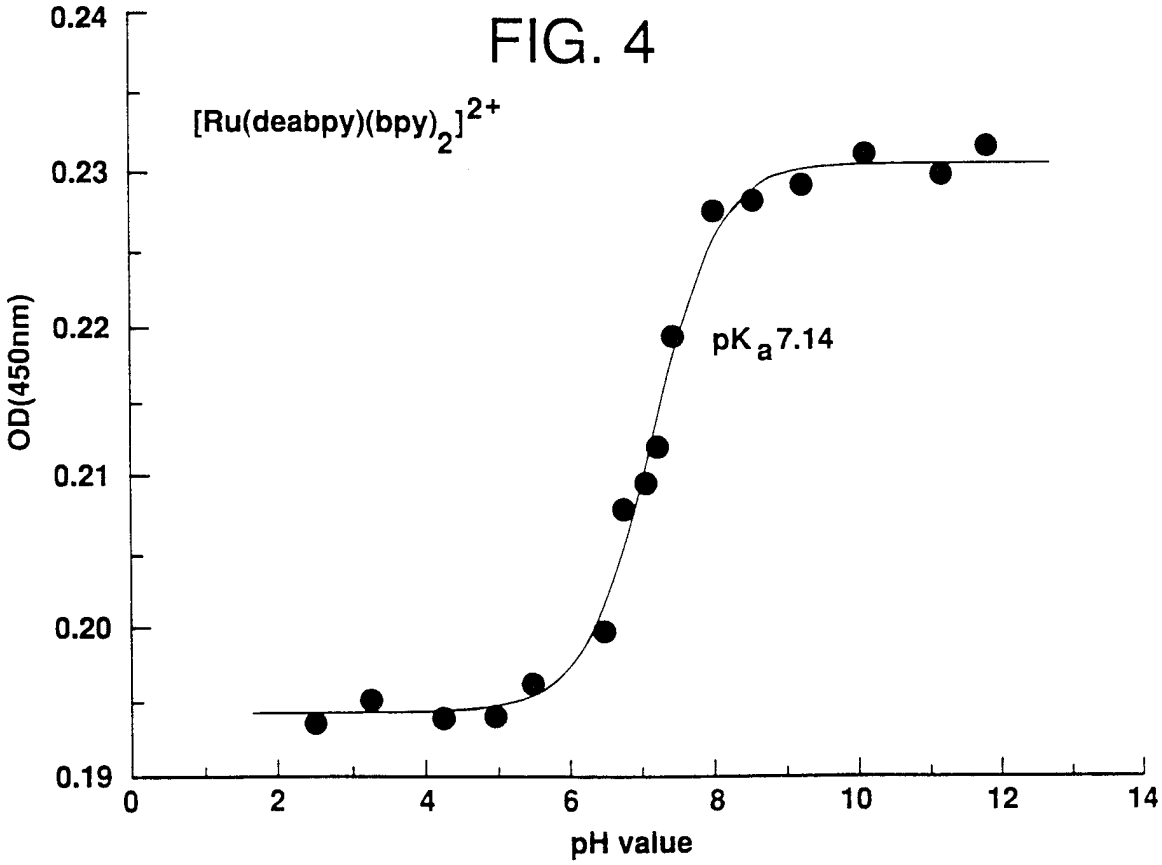

[Ru(deabpy)(bpy)2]2+
OD(450nm)
0.24
0.23
0.22
0.21
0.20
0.19
0
2
4
6
8
10
12
14
pH value
pKa 7.14

FIG. 5

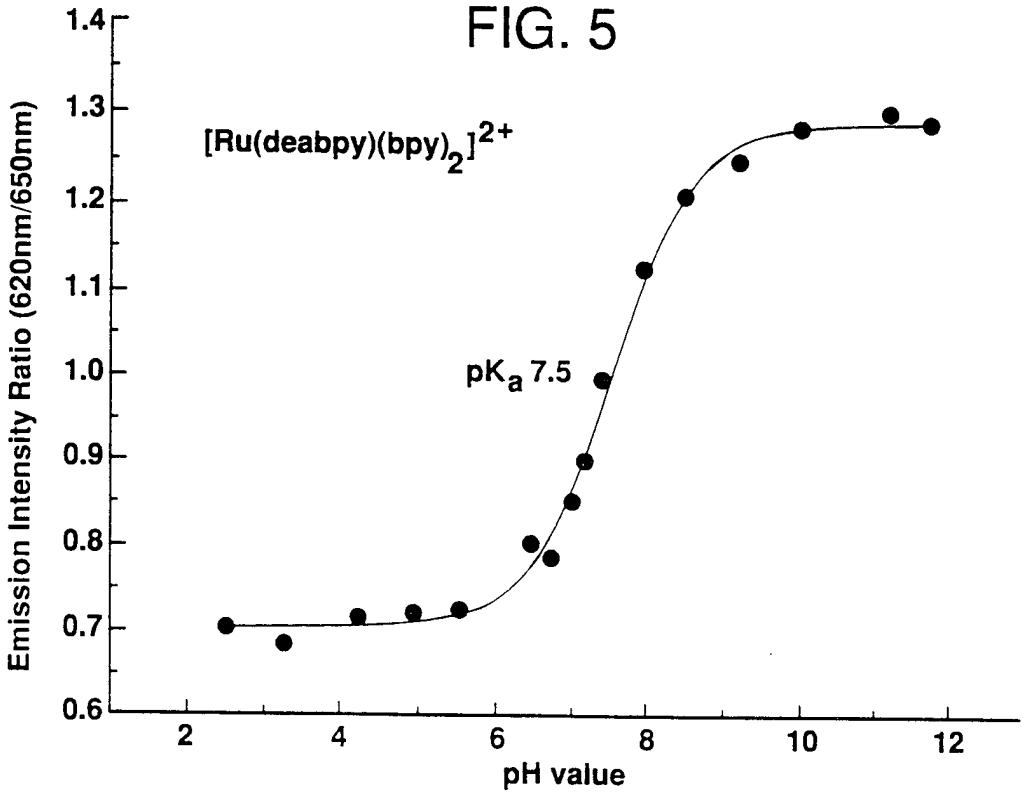

[Ru(deabpy)(bpy)2]2+
pKa 7.5
Emission Intensity Ratio (620nm/650nm)
pH value
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
2
4
6
8
10
12

FIG. 6

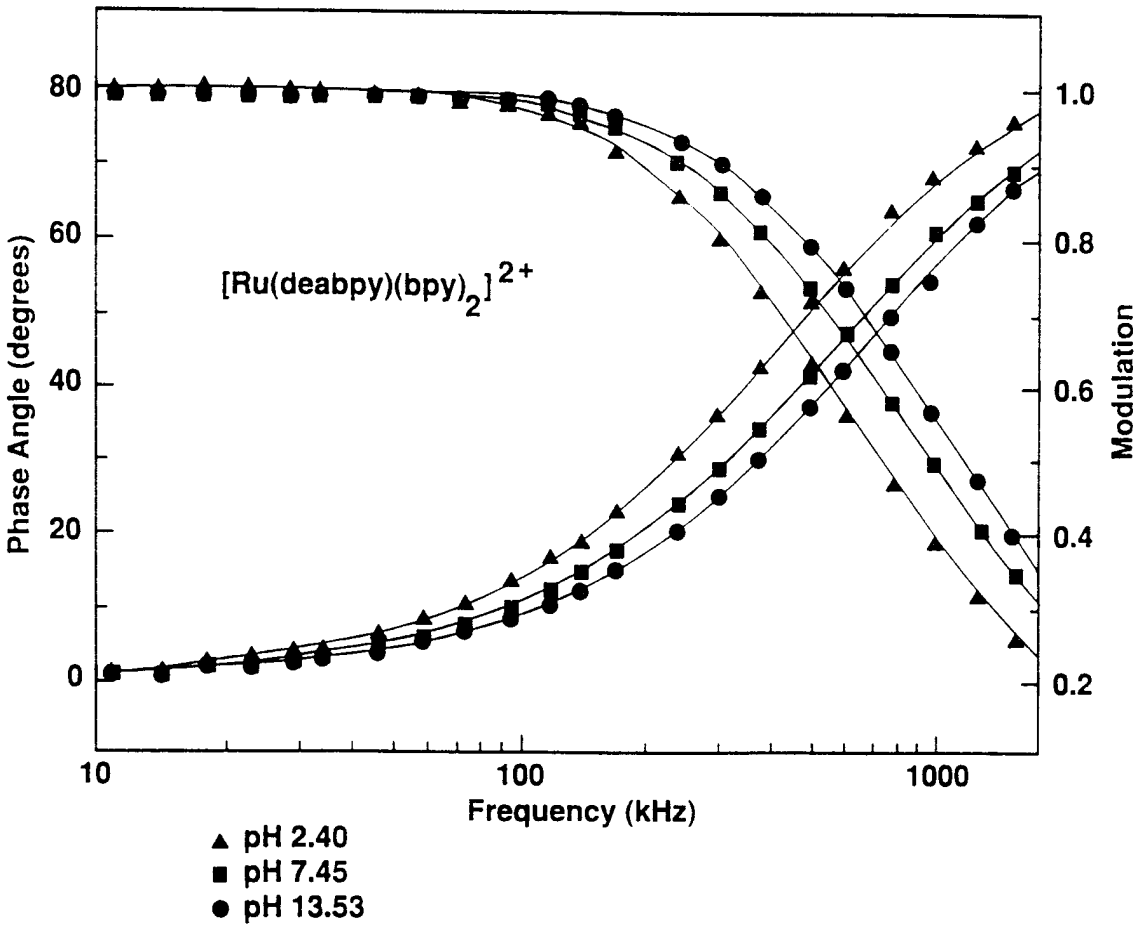

[Ru(deabpy)(bpy)2]2+
Phase Angle (degrees)
Modulation
Frequency (kHz)
80
60
40
20
0
1.0
0.8
0.6
0.4
0.2
10
100
1000
▲ pH 2.40
■ pH 7.45
● pH 13.53

FIG. 7

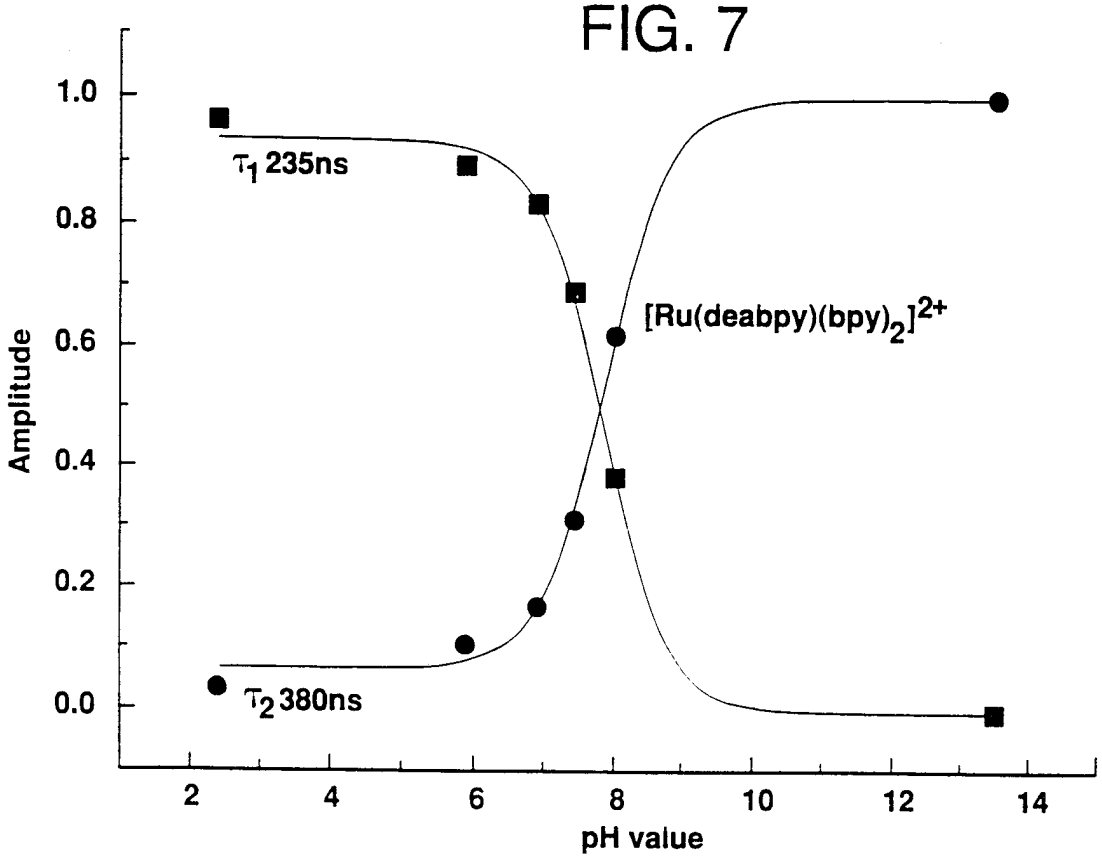

1.0
0.8
0.6
0.4
0.2
0.0
Amplitude
τ_1 235ns
τ_2 380ns
[Ru(deabpy)(bpy)$_2$]$^{2+}$
2
4
6
8
10
12
14
pH value

FIG. 8

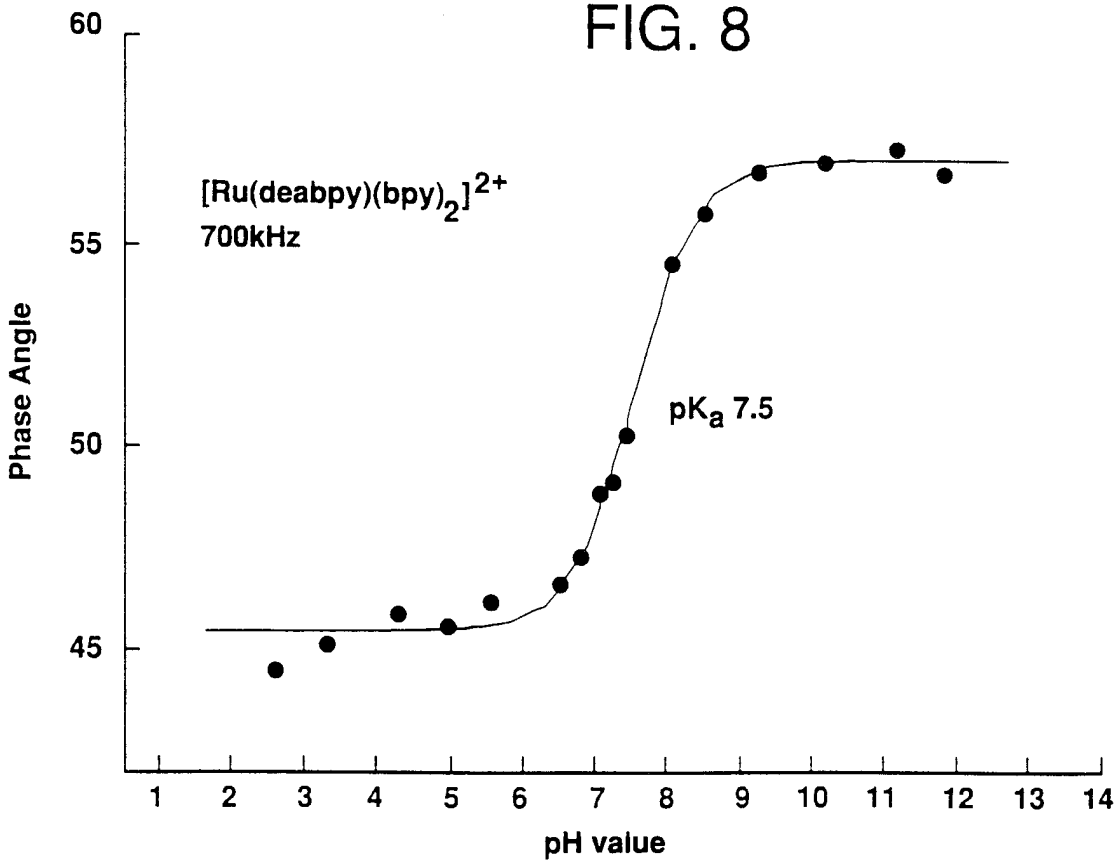

60
55
50
45
Phase Angle
[Ru(deabpy)(bpy)$_2$]$^{2+}$
700kHz
pK$_a$ 7.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH value

FIG. 9

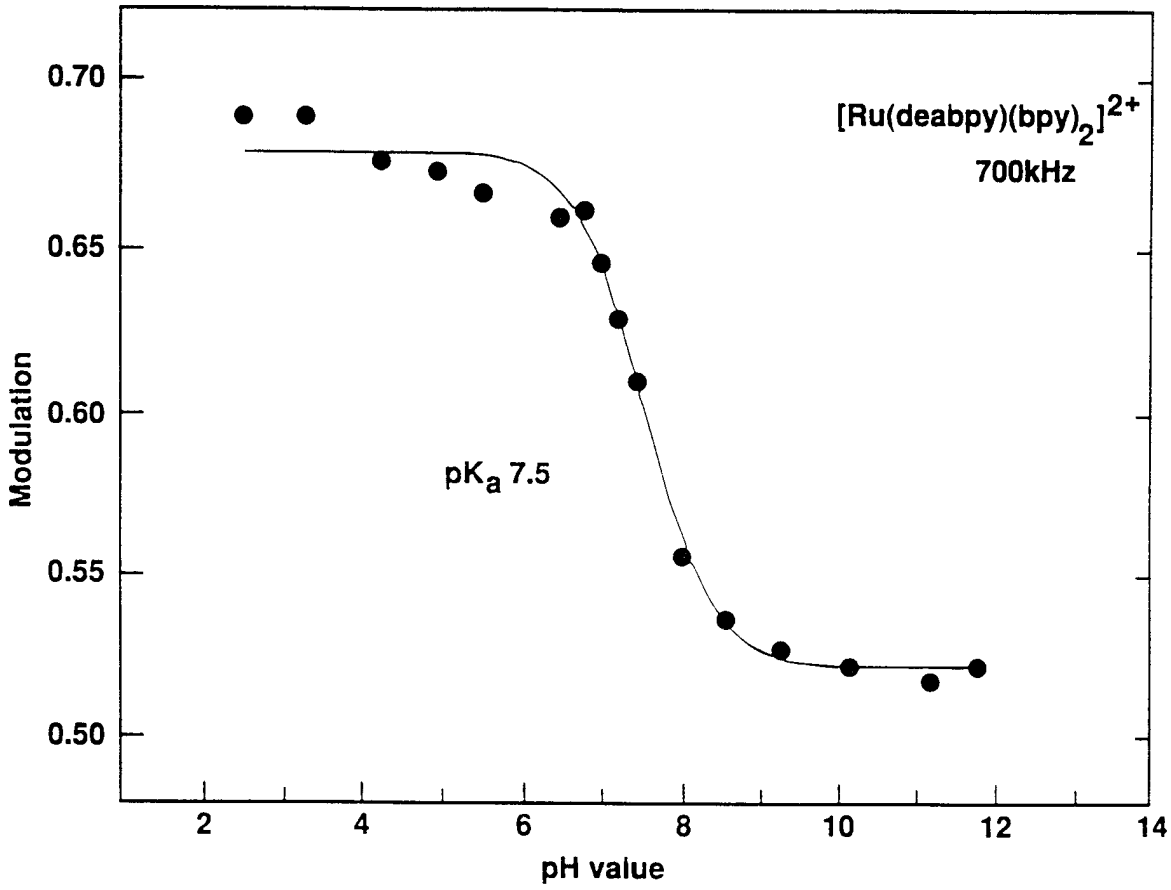
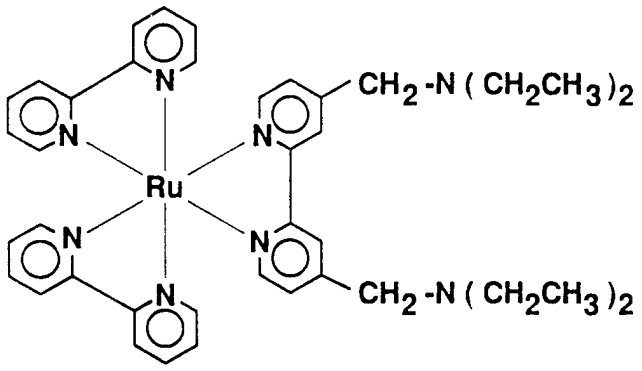


FIG. 10

MLC pH SENSOR



$[Ru(bpy)_2(deabpy)](PF_6)_2$

FIG. 11

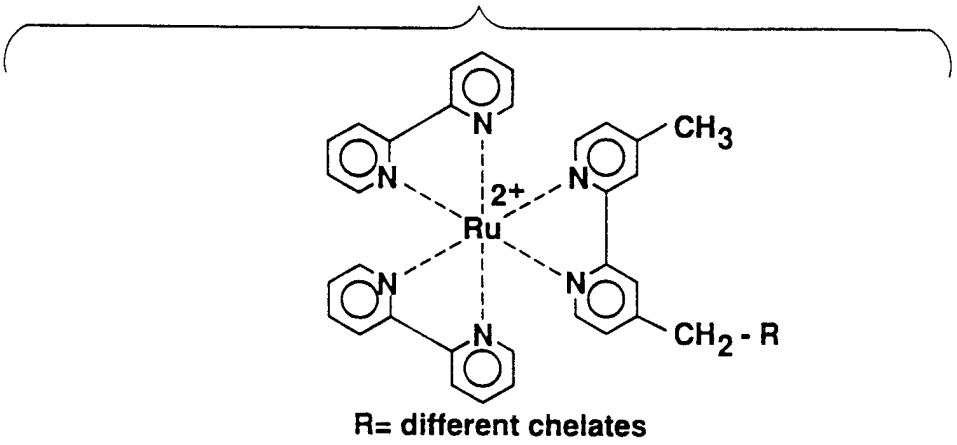

N
CH3
N
2+
N
Ru
N
N
N
CH2 - R
R= different chelates
CH3
HOOC
HOOC
HOOC
HOOC
O
O
O
NH2
H
N
O
O
O
N
H
H
N
O
O
O
O
N
H
For Ca probe
For Na probe
For K probe

FIG. 12

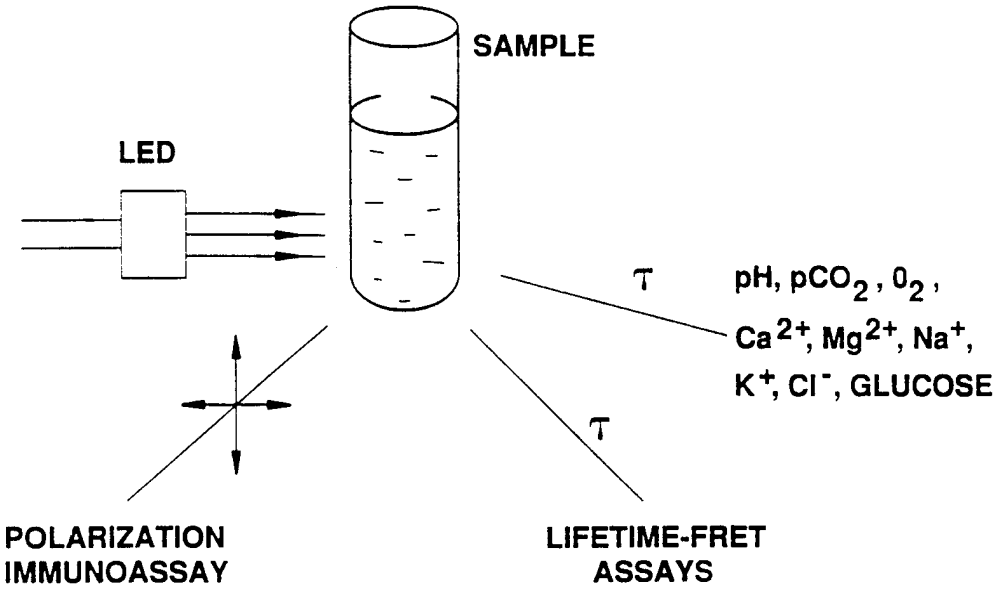

SAMPLE
LED
τ
pH, pCO2 , 02 ,
Ca2+, Mg2+, Na+,
K+, Cl-, GLUCOSE
τ
POLARIZATION
IMMUNOASSAY
LIFETIME-FRET
ASSAYS

FIG. 13

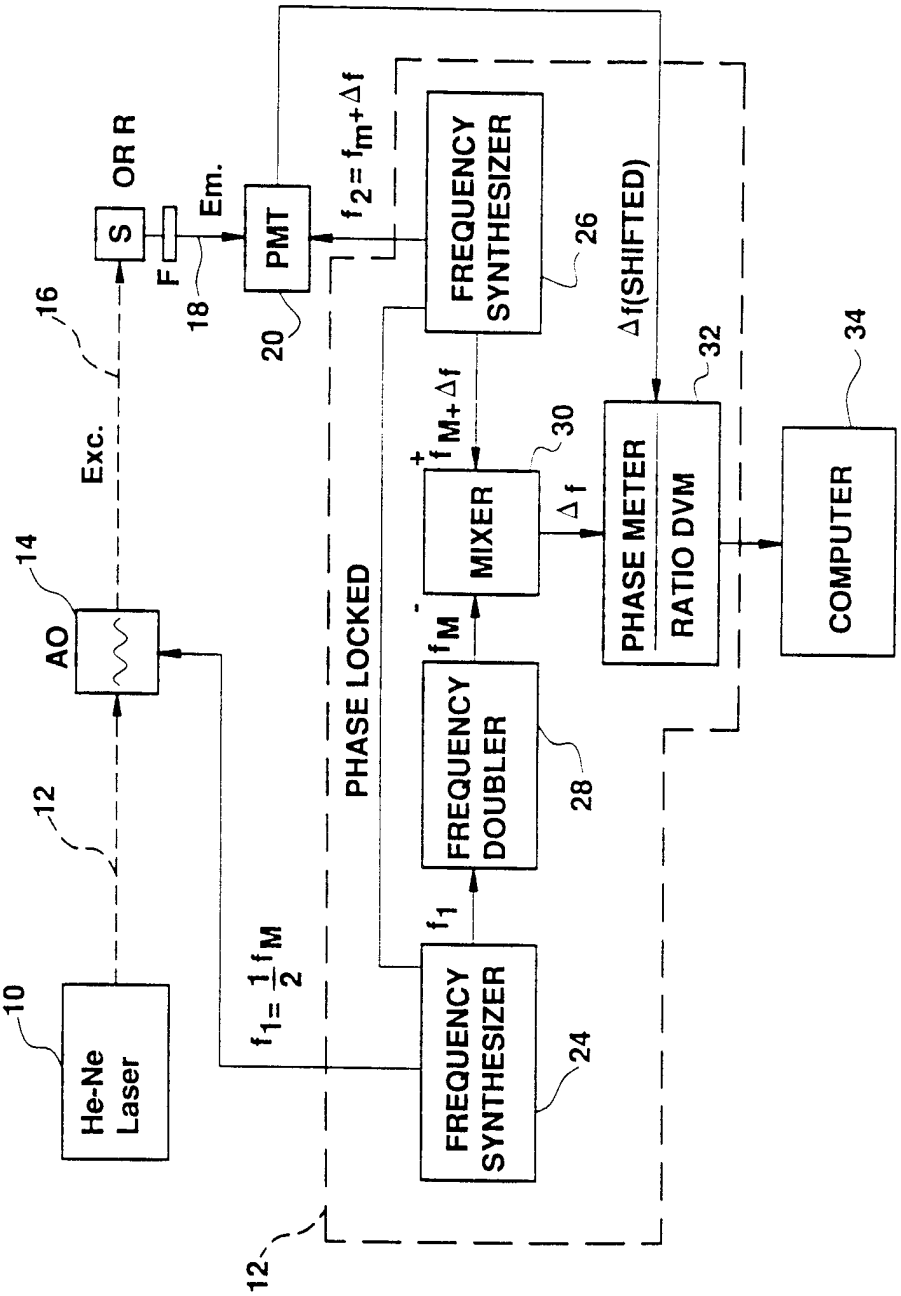

He-Ne Laser
10
12
AO
14
Exc.
16
S
OR R
F
Em.
18
PMT
20
$f_2 = f_m + \Delta f$
FREQUENCY SYNTHESIZER
26
Δf(SHIFTED)
$f_{M+\Delta f}$
MIXER
30
Δf
PHASE METER
RATIO DVM
32
COMPUTER
34
f_M
FREQUENCY DOUBLER
28
f_1
FREQUENCY SYNTHESIZER
24
$f_1 = \frac{1}{2} f_M$
PHASE LOCKED
12

FIG. 14A

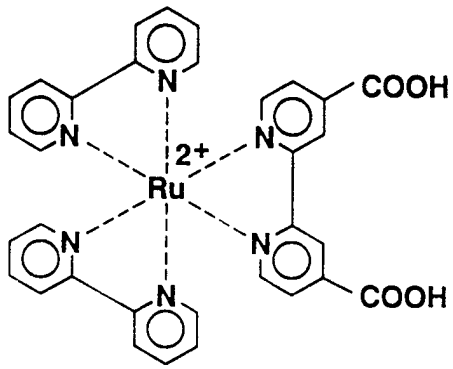


FIG. 14B

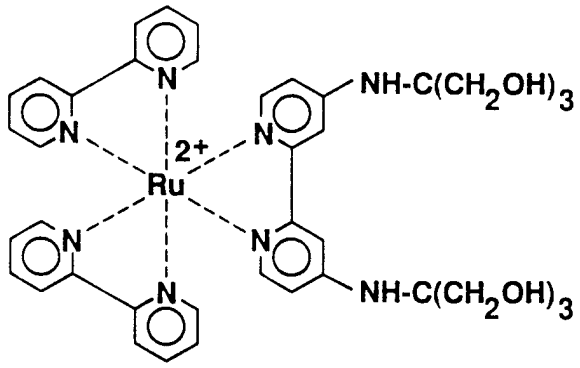


FIG. 15A

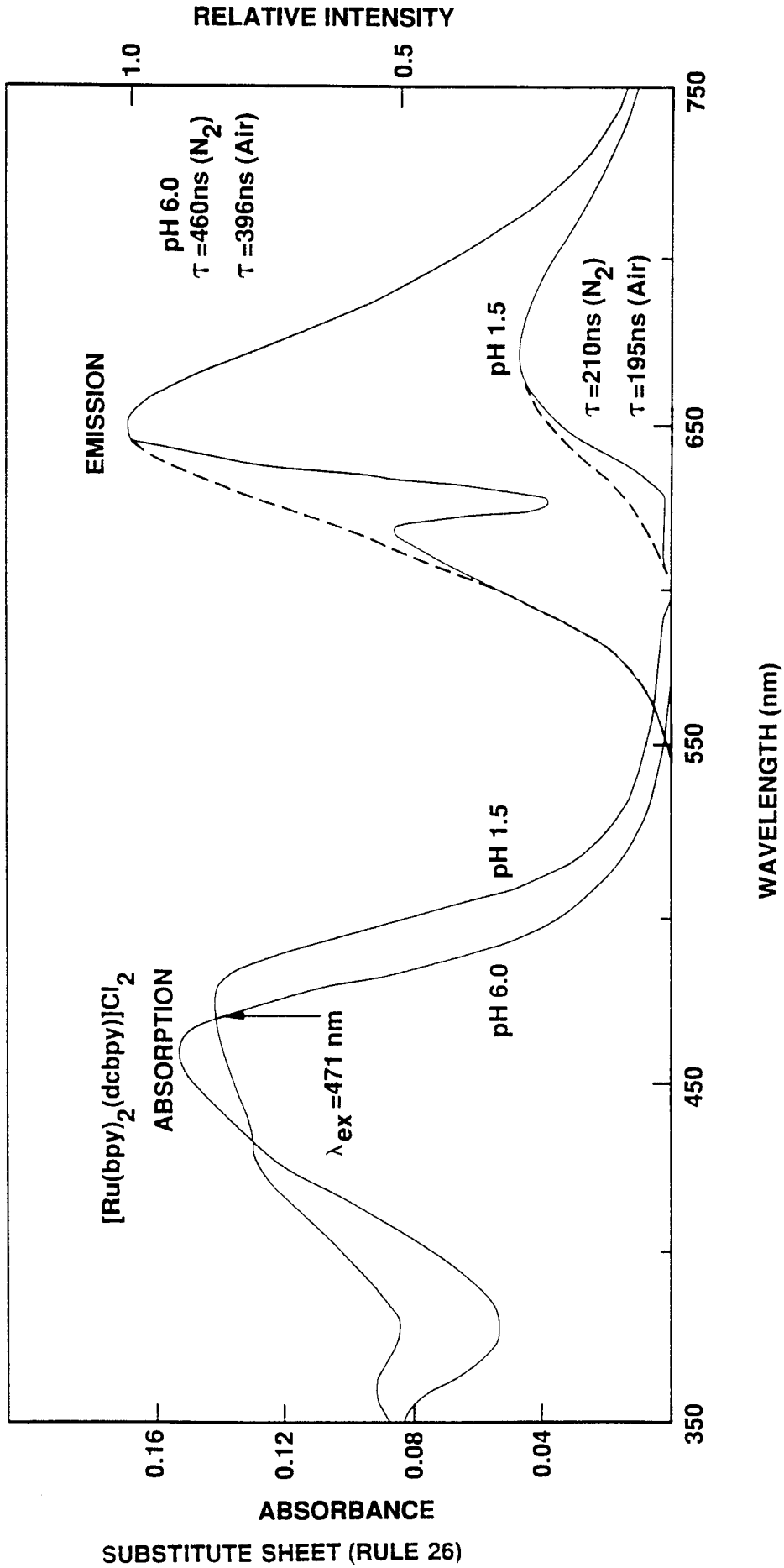

RELATIVE INTENSITY
1.0
0.5
EMISSION
pH 6.0
τ =460ns (N2)
τ =396ns (Air)
pH 1.5
τ =210ns (N2)
τ =195ns (Air)
[Ru(bpy)2(dcbpy)]Cl2
ABSORPTION
λex =471 nm
pH 1.5
pH 6.0
0.16
0.12
0.08
0.04
ABSORBANCE
350
450
550
650
750
WAVELENGTH (nm)

FIG. 15B

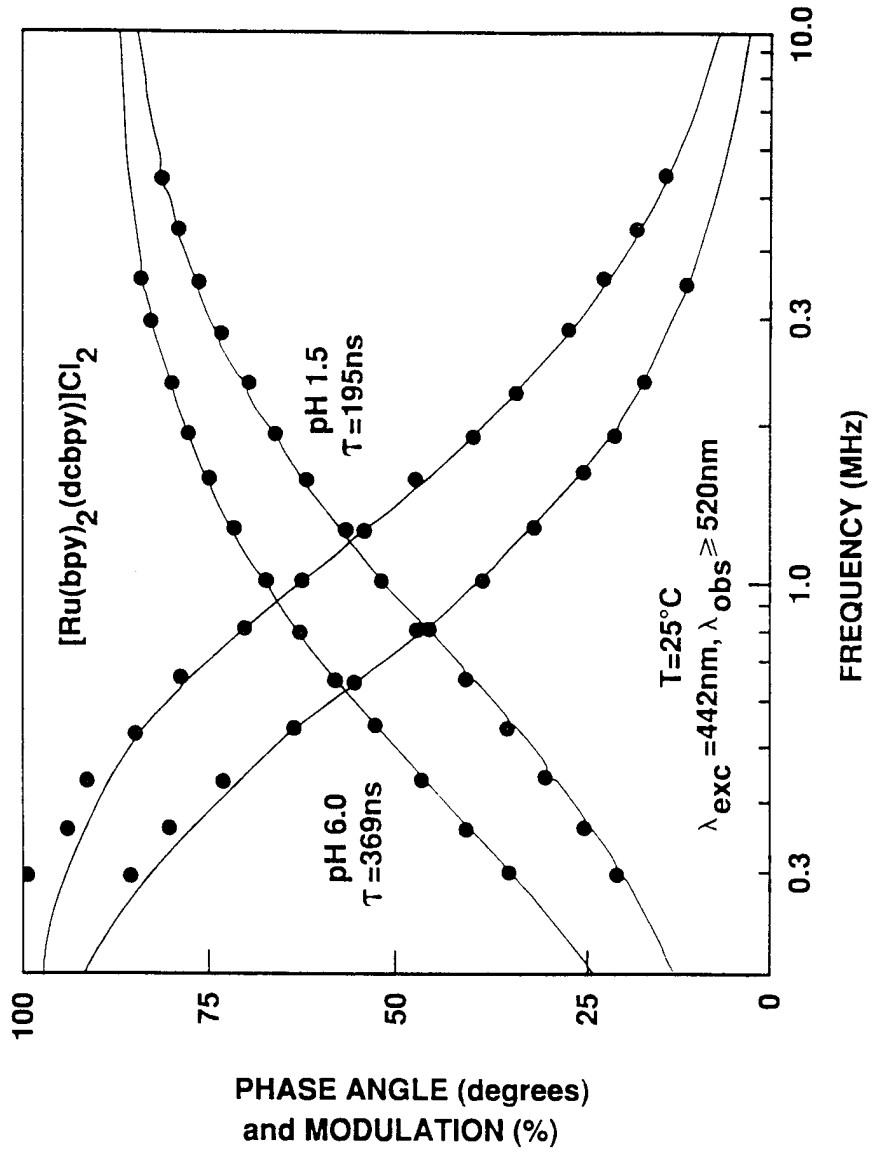

[Ru(bpy)2(dcbpy)]Cl2
pH 1.5
τ=195ns
pH 6.0
τ=369ns
T=25°C
λexc =442nm, λobs ≥ 520nm
100
75
50
25
0
0.3
1.0
0.3
10.0
FREQUENCY (MHz)
PHASE ANGLE (degrees)
and MODULATION (%)

FIG.16

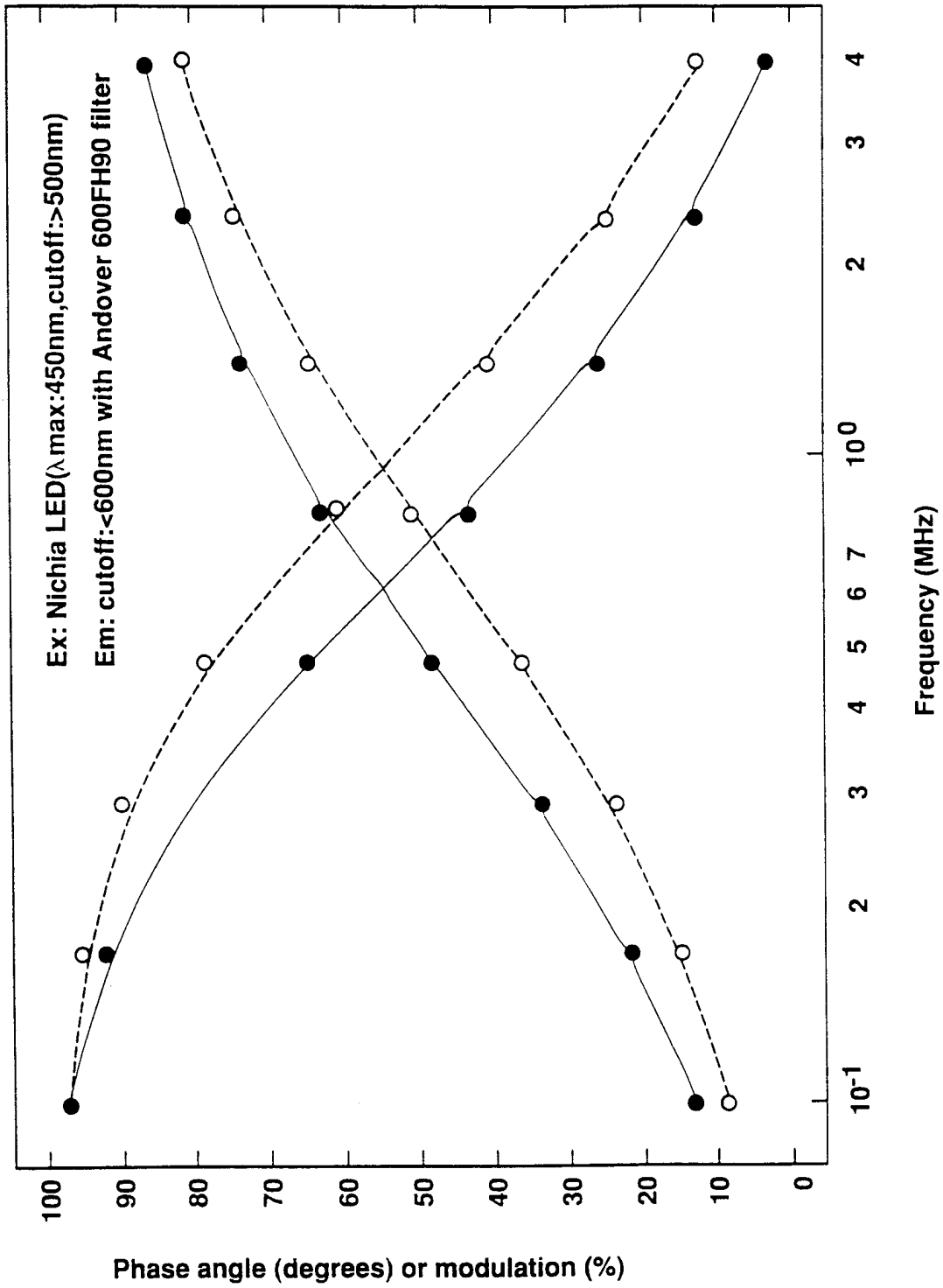

Ex: Nichia LED(λmax:450nm,cutoff:>500nm)
Em: cutoff:<600nm with Andover 600FH90 filter
Phase angle (degrees) or modulation (%)
100
90
80
70
60
50
40
30
20
10
0
Frequency (MHz)
10-1
2
3
4
5
6
7
100
2
3
4

FIG. 17

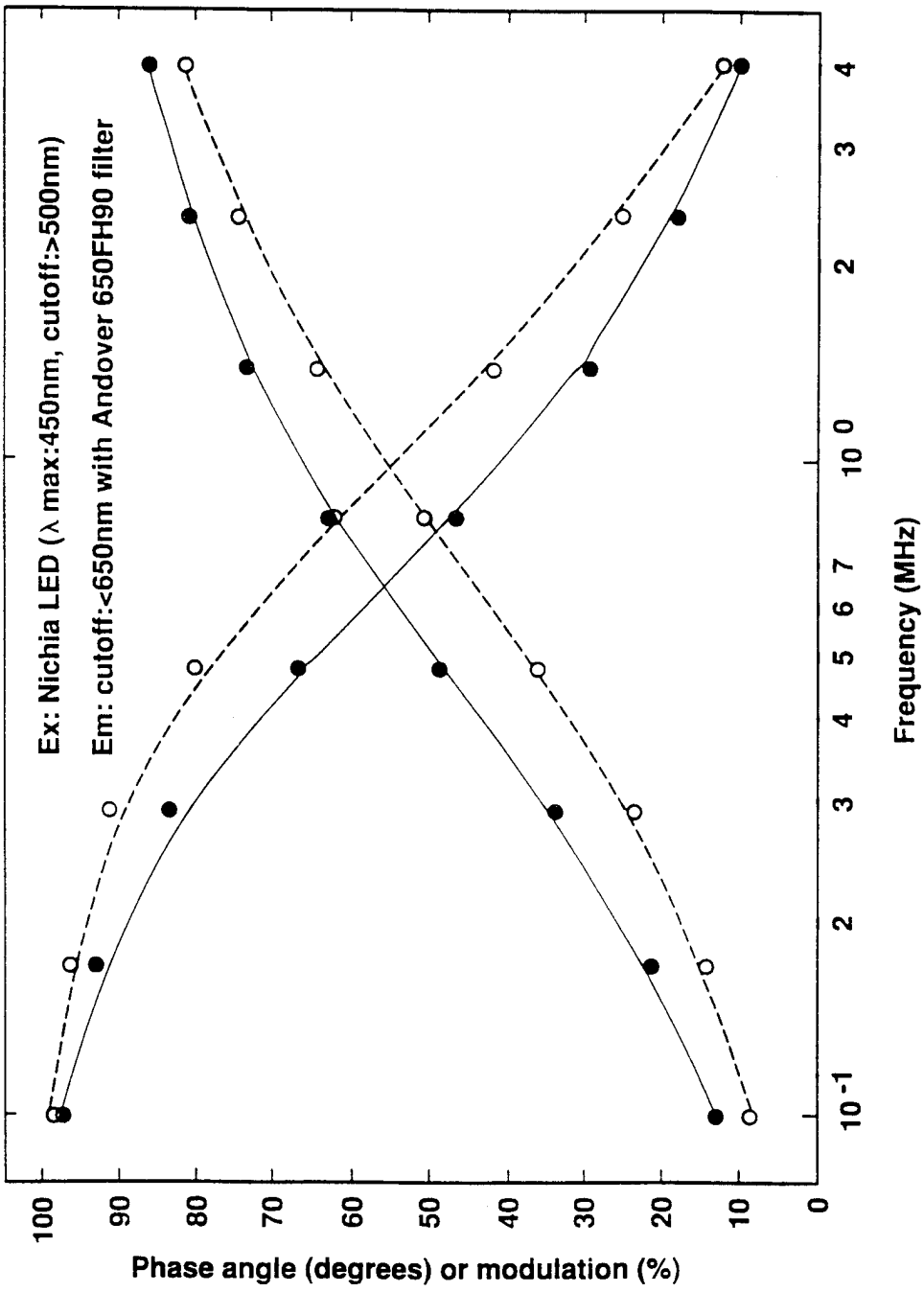

Ex: Nichia LED (λ max:450nm, cutoff:>500nm)
Em: cutoff:<650nm with Andover 650FH90 filter
Phase angle (degrees) or modulation (%)
Frequency (MHz)
100
90
80
70
60
50
40
30
20
10
0
10 -1
2
3
4
5
6
7
10 0
2
3
4

FIG. 18

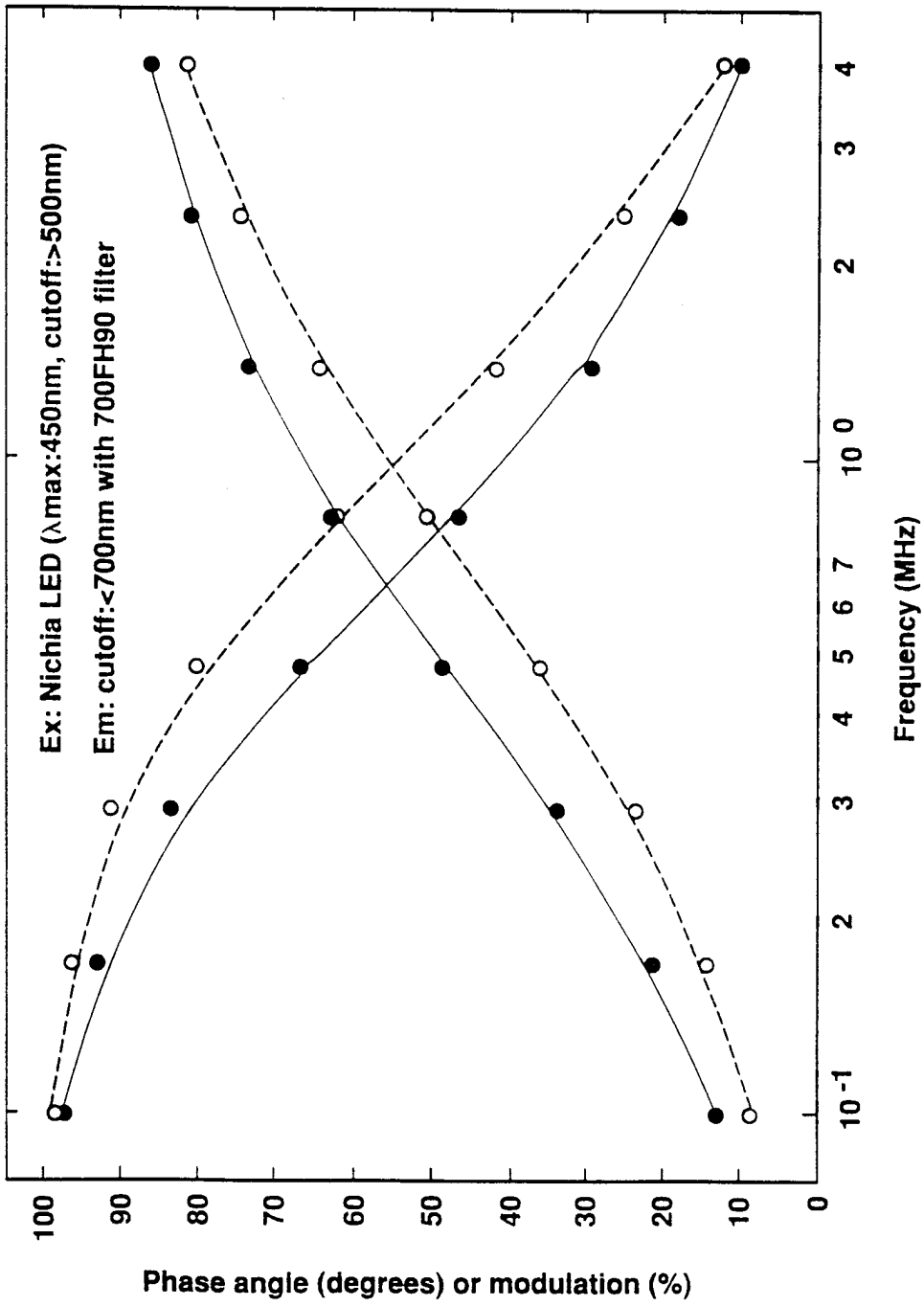

Ex: Nichia LED (λmax:450nm, cutoff:>500nm)
Em: cutoff:<700nm with 700FH90 filter
Phase angle (degrees) or modulation (%)
Frequency (MHz)
100
90
80
70
60
50
40
30
20
10
0
10 -1
2
3
4
5
6
7
10 0
2
3
4

FIG. 19

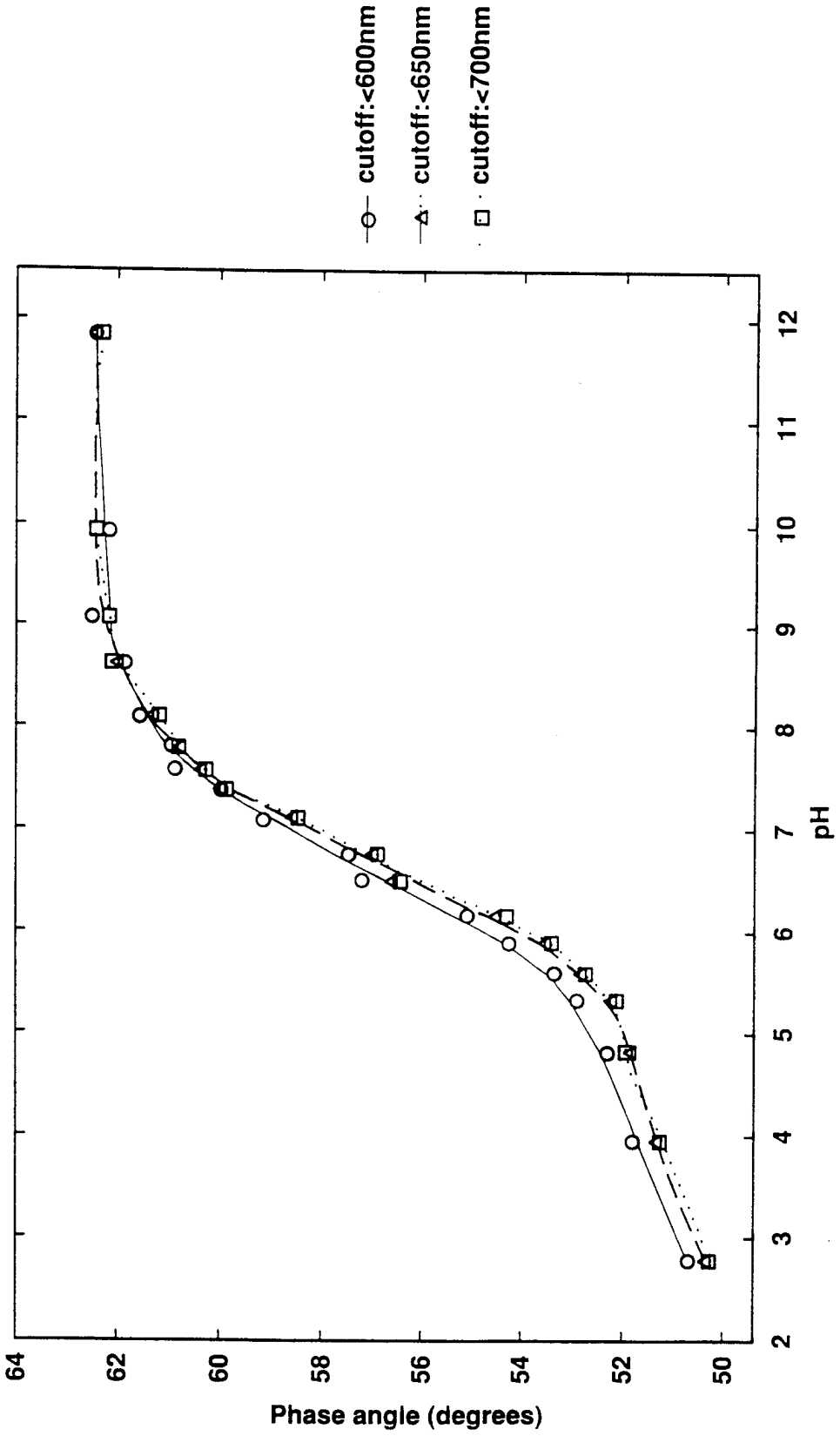
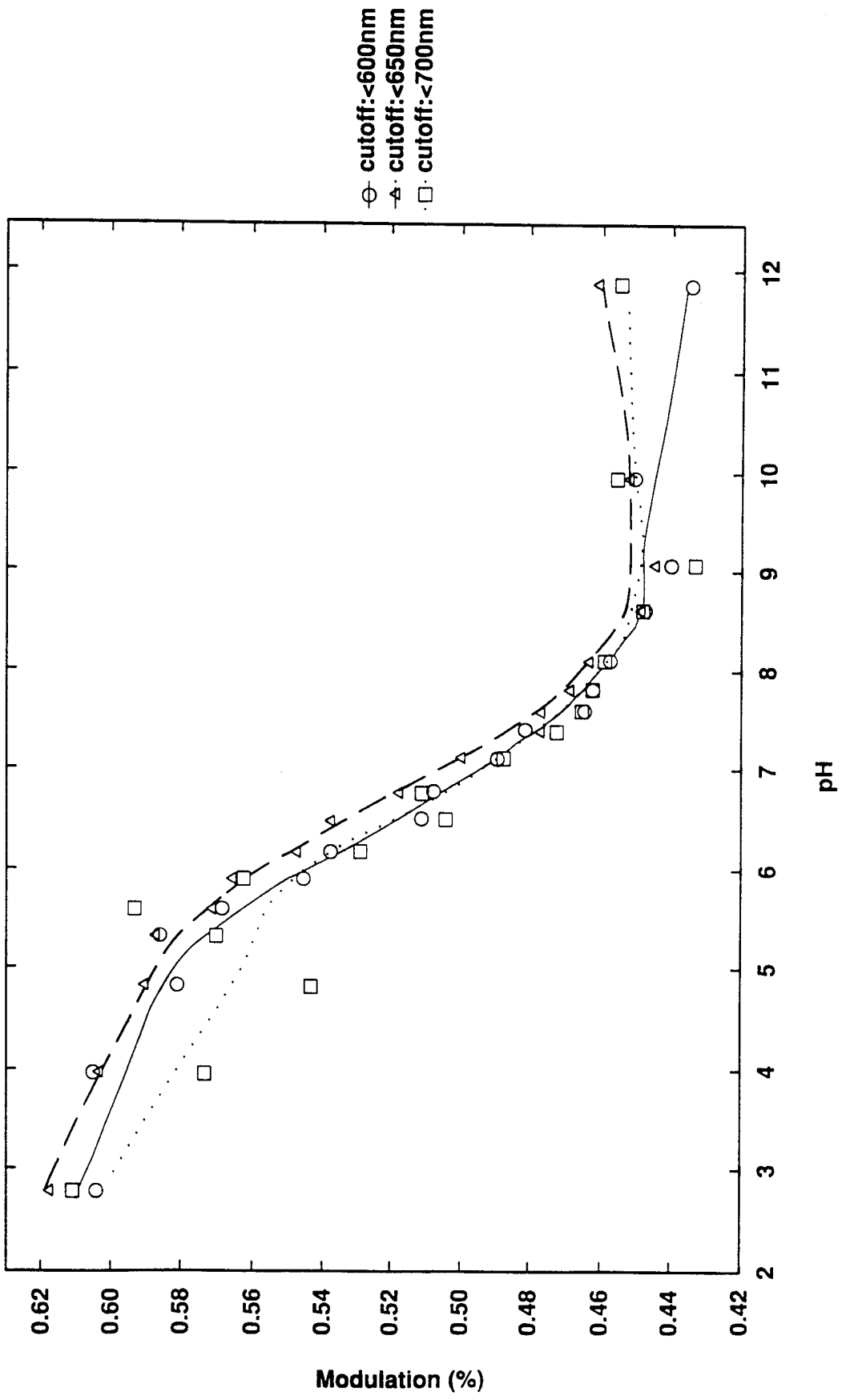

cutoff:<600nm
cutoff:<650nm
cutoff:<700nm
64
62
60
58
56
54
52
50
Phase angle (degrees)
2
3
4
5
6
7
8
9
10
11
12
pH

FIG. 20


cutoff:<600nm
cutoff:<650nm
cutoff:<700nm
0.62
0.60
0.58
0.56
0.54
0.52
0.50
0.48
0.46
0.44
0.42
Modulation (%)
2
3
4
5
6
7
8
9
10
11
12
pH

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00148

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 21/64
US CL :436/74, 76, 79, 133, 163, 164, 172
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/74, 76, 79, 133, 163, 164, 172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SZMACINSKI ET AL. Lifetime Based Sensing. Topics in Fluorescence Spectroscopy, Volume 4: Probe Design and Chemical Sensing. Edited by Joseph R. Lackowicz. New York: Plenum Press, 1994, pages 295-333, especially pages 307-313.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 MARCH 1998	Date of mailing of the international search report 21 APR 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAN M. LUDLOW Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00148

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE
search terms: metal ligand or diethylaminomethyl or deabpy or bpy or bipyridine; fluoresc?; transition or ruthenium or osmium or rhenium or rhodium