

Reviews

Molecular Fluorescence, Phosphorescence, and Chemiluminescence Spectrometry

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This review covers the approximately two-year period of January 1, 2000, to December 31, 2001. Consequently, there is a gap between this review and our last review (A1), which covered roughly from November, 1997 through October, 1999. This gap is due to a change in computer search; STN was used primarily for this search. We have tried to minimize the gap by doing individual searches for each topic by use of SciFinder Scholar. If we have missed some important references, please let us know and we will consider them for our next review.

A computer search of *Chemical Abstracts* provided most of the references for this review. Coverage is limited to articles that describe new developments in the theory and practice of molecular luminescence for chemical analysis in the ultraviolet–visible and near-infrared region. Citations may be duplicated between sections

because of articles with contents that span several topics. However, in an effort to reduce the length of this review, we have tried to limit this duplication in citation. In general, citations are limited to journal articles and usually do not include patents, proceedings, reports, and dissertations.

To maintain this review at a reasonable size, we have tried to focus on important advances rather than extensions of previous advances. This reduction was done at the request of the journal in an attempt to significantly reduce the length of the biannual reviews. This reduction was also attempted with more of a focus on articles of general interest and relevance to the field of analytical chemistry. Although we are not able to provide extensive coverage of developments of relevance to broad areas, such as chromatography and biological sciences, we have tried to include major review articles and chapters relevant to these topics. However, if you feel that we have omitted an important article published during the above referenced time period, please forward the reference to one of us and we will be certain to consider it for the next review.

BOOKS, REVIEWS, AND CHAPTERS OF GENERAL INTEREST

Several books (B1–B4) and many review articles covering different theoretical and practical aspects of fluorescence spectroscopy and its applications in different fields appeared in the past two years. Of particular interest are *New Trends in Fluorescence Spectroscopy: Applications to Chemical and Life Sciences* (B1) and *Fluorescence Correlation Spectroscopy: Theory and Applications* (B2). A chapter on analytical fluorescence spectroscopy appeared in the *Encyclopedia of Analytical Chemistry* (B5). The review articles covered a wider range of topics, many of which are stimulating and visionary. Dual lifetime referencing was introduced as a new method that is capable of converting fluorescence intensity into frequency-domain or time-domain information (B6). Lakowicz used the term radiative decay engineering to describe the endless possibilities for controlling the radiative decay rates and even predicted high emission from “nonfluorescent” molecules under certain conditions (B7). Other methods and applications, such as fluorescence correlation spectroscopy (B9), optical glucose sensing (B8), applications of fluorescence resonance energy transfer to analyze carbohydrates (B10), fluorescence

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polarization for analyzing carbohydrate–protein interaction (*B11*), and nanoscale fluorescence sensors for intracellular analysis (*B12*), were also reviewed.

One of the limiting factors in the miniaturization of microfabricated analytical devices is the detection method. Recent developments of such fluorescence and nonfluorescence detection methods were discussed (*B13*, *B14*). The focus on chemiluminescence together with other optical spectroscopies in general and its promise as a detection method in new applications appeared in several reviews (*B13*, *B15*, *B16*). Patonay and Soper reviewed separately the analytical applications of near-infrared fluorescence in immunoassays (*B17*) and electrophoresis (*B14*). Of environmental interest is a review article on optical and photophysical characteristics of polychlorinated dibenzo-*p*-dioxin derivatives (*B18*).

GENERAL INSTRUMENTATION

Several advances were reported in the area of instrumentation. Of general interest is a review published by Schwarz and Hauser detailing developments in detection methods in microfabricated devices (*C1*). Bowser and Kennedy reported the development a system for in vivo monitoring of neuroactive amino acids and amines by a flow-gated interface that coupled a microdialysis sampling system with capillary electrophoresis using laser-induced fluorescence detection with precolumn derivatization (*C2*). In this instrument, sensitivity was increased significantly (15-fold) by using a sheath-flow cuvette. The authors report the capability of separating and detecting several neurotransmitters collected in vivo at 20-s intervals. Two publications (*C3*, *C4*) by Hanning et al. dealt with the development and application of a liquid core waveguide for fluorescence detection in DNA sequencing by capillary electrophoresis. The introduction of the excitation beam into the capillary is transverse to the capillary, and the fluorescence emission is collected and propagated by total internal reflection within the waveguide and detected at the end of the capillary. Very good sensitivity is reported, and four-color DNA sequencing is demonstrated. In a second publication, the authors report the use of the liquid core waveguide detector in a 91-capillary array (*C4*). Optical crosstalk between adjacent capillaries is reported to be minimal, and the system efficiently rejects stray light. Prince et al. report an advance in optical-fiber-based fluorescence sensors with multiple sensors on the same fiber (*C5*). By coupling an optical delay with a second fiber, the individual sensors can be placed closely together and still allow discrimination based on the time delay between the excitation pulse and the returning fluorescence, surpassing the typical spatial restraint imposed by the fluorescence lifetime of the sensor.

Several significant advances in fluorescence methodology were reported in the literature. Cai et al. reported the development of a method based on fluorescence polarization to monitor the binding of an anti-thrombin aptamer with thrombin (*C6*). The change in polarization was reported to be linear in the 0.1–100 ng/mL range with a detection limit of 0.1 ng/mL. In an environmental application, an online preconcentration and flow-through fluorescence detection method for cadmium in marine waters was reported by Charles et al. (*C7*). The use of ion-exchange resin reduced interferences, and a detection limit of 35 pM is reported. Nakanishi et al. report a new method for imaging conformational

changes of proteins in live cells (*C8*). The method is based on the specific binding of a new fluorescent probe that is environment-sensitive, enabling the monitoring of protein conformational changes. Nivikov et al. published a comprehensive report detailing theoretical and experimental aspects of a new single molecule fluorescence analysis method based on photon arrival times (*C9*). The theory of single molecule impulse–response functions (SMIRFs) is developed, and the practical application is demonstrated by estimating the triplet lifetimes and intersystem crossing yields for the immobilized fluorescent dyes DiI and rhodamine 6G.

LASER-BASED TECHNIQUES

Laser based techniques continue to further use nonlinear optical spectroscopies. Some of these applications employ absorption and scattering phenomena in combination with fluorescence spectroscopy. For example, pump–probe spectroscopy, second- and third-harmonic generation, and hyper-Rayleigh scattering have been reported in the study of excited-state kinetics and ground-state aggregations and for the study of nonlinear optical materials, as well (*D1*, *D2*). Many of these applications were used for the study of nonlinear effects in glasses (*D3*), films (*D4*), liquid crystals (*D5*), and DNA (*D6*), and at interfaces, such as the water/air interface (*D7*). Two-photon excitation is leading the applications of multiphoton excitation for fluorescence imaging and microscopy, time-resolved fluorescence, and steady-state fluorescence. One of the limiting factors in using fluorescence spectroscopy is the availability of fluorescent probes. A noticeable research activity focuses on synthesizing and studying the two-photon characteristics as well as the lasing properties of new fluorophores (*D8*–*D10*). An interesting array of optical methods, including polarized pump–probe fluorescence and fluorescence anisotropy, were applied to study the conjugation and polyoligomerization of a class of bis(porphyrin) compounds (*D11*). Although many of these methods are utilized for other applications beyond analytical purposes, their potential use in analytical chemistry is apparent.

Other fluorescence applications continue to expand their horizons. Dual-laser fluorescence correlation spectroscopy was evaluated as a biophysical probe of binding (*D12*). Nanosecond and picosecond laser flash photolysis were used to study excited triplet states and determine photoacid generation quantum yields (*D13*). By using rhodamine B base as an acid sensor and a triazine derivative as the actinometer for 308-nm laser excitation, the authors suggested that photoacid generation originates in the singlet state.

Landgraf reviewed the application of semiconductor light sources in conjunction with phase fluoremetric methods to measure fluorescence lifetimes down to 100 ps and even lower (*D14*), whereas Baxter et al. surveyed instrumental strategies of optical parametric oscillator and amplifier devices for chemical processes in industrial, environmental, and other settings (*D15*).

SENSORS

The sensitivity of fluorescence continues to drive the development of fluorescence-based sensors. The array of such sensors spans over a wide range of applications from remote sensing for environmental purposes to miniaturized clinical sensors. Old sensing challenges continue to attract the development of more sensitive and selective techniques that employ newer technologies,

and new challenges are recognized and addressed (*E1–E3*). Of particular environmental interest is a remote detection of nitroaromatic explosives in soil. A group from Sandia National Laboratories designed fluorescence sensor particles, the fluorescence of which is strongly influenced by the absorption of nitroaromatic molecules into the particles (*E4*). The particles were sprayed onto contaminated soil and were capable of detecting TNT concentrations in the ppm range from a standoff distance of 0.5 km. Such sensing particles can as well be incorporated into fiber optics and other settings. The group of Wolfbeis, on the other hand, reported the synthesis of near-infrared fluorescent-labeled nucleic acid conjugates for the detection of nucleic acid (*E5*). Similar to immunoassays, such conjugates are known to combine the selectivity and high recognition of nucleotides with the sensitivity of fluorescence.

The Birch research group reported a study on glucose sensing for glucose concentrations in the range of 2.5–3.0 mM by using fluorescence resonance energy transfer and discussed the possibility of using this approach in an implanted glucose sensor in the body (*E1*). The continued interest in sensing saccharides yielded considerable research activity, especially with boronic acid fluorophores (*E2, E6*). Cyclodextrin-based sensors combined with boronic acid fluorophore were reported for selective sugar recognition in water (*E2*). Other cyclodextrin-based sensors were used for the detection and determination of biorelated analytes, such as bile acids and endocrine disruptors in solutions, and as immobilized chromophore-modified cyclodextrins on a cellulose membrane (*E7–E9*).

Optical sensors for oxygen and pH have a fair representation in the literature. One method used delayed fluorescence for probing singlet molecular oxygen, and another utilized phosphorescence quenching for an optical oxygen sensor (*E10, E11*). The first method made use of laser-induced generation of singlet molecular oxygen to study the strong singlet O-sensitized delayed fluorescence of a porphyrazine-based fluorophore in air saturated solutions (*E10*). The second used self-assembled film of a Pd porphyrin derivative on an alumina plate (*E11*). The phosphorescence intensity from the film was inversely proportional to the increased oxygen pressure. Such a device has response times of 36 s for oxygenation of the film and 148 s for deoxygenating it. Layer-by-layer self-assembly was also used for fabricating an optical pH sensor (*E12*). Polyanion layers mixed with 1-hydroxypyrene-3,6,8-trisulfonate were deposited on polycation layers, and the layered films were cross-linked to ensure the stability of the sensor. The sensor was applied to a wide range of pH, from 1 to 13, by following the fluorescence of the protonated and deprotonated forms.

SAMPLE PREPARATION, QUENCHING, AND RELATED PHENOMENA

There were several papers that reported the use of fluorescence quenching to detect explosives. Goodpaster and McGuffin reported the development of a novel method for the detection of nitrated explosives based on the fluorescence quenching of pyrene (*F1*). The samples were first separated chromatographically and were detected using indirect laser-induced fluorescence, resulting in enhanced sensitivity and selectivity over methods based on UV absorbance. La Grone et al. (*F2*) reported the development of a chemical sniffer based on fluorescence quenching of fluorescent thin films in the presence of ultratrace quantities of TNT vapors.

The authors report that the device is portable and has near-real-time response to femtogram quantities of nitroaromatic explosives.

There were reports of advances in the theoretical treatment of fluorescence quenching. A theoretical treatment of reversible fluorescence quenching was presented by Naumann (*F3*). A modified Smoluchowski formulation was developed on the basis of well-defined non-Markovian rate equations. This treatment results in positive curvature, nonlinear plots that accurately represent quenching at high quencher concentrations. A comprehensive theoretical analysis was reported by Bandyopadhyay, Seki, and Tachiya that examined the effect stochastic gating on fluorescence quenching on the basis of diffusion-influenced electron transfer (*F4*). A model that takes various transient effects into account was developed, and numerical results from this model were in excellent agreement with those predicted from molecular dynamics simulations on the same system. Of related interest was a study that provided the first experimental evidence of nonexponential kinetic data for an acid–base reaction, which was shown to conform to Smoluchowski theory (*F5*).

There have been several advances in the area of biological applications of fluorescence quenching. Hossain et al. have developed and characterized a cyclodextrin–peptide conjugate that acts as a chemosensor (*F6*). Pagliari et al. reported on the development of a new chiral sensor based on a modified cyclodextrin (*F7*). The modified cyclodextrin is composed of a dansyl moiety and a metal binding site. In the presence of Cu(II), enantioselective fluorescence quenching allowed for the determination of the optical purity of praline. A significant advance in the application of fluorescence quenching was reported by Zhuang et al. in a study that observed single-molecule protein-folding (*F8*). In this study, titin was labeled with several fluorophores that undergo self-quenching in the folded state and yet are fluorescent in the unfolded state. This allowed measurement of the folding dynamics of a single molecule in real time.

There were two reports of developments in the detection of DNA and RNA. Kurata et al. have developed a new application based on a BODIPY FL-tagged oligonucleotide that can quantitatively detect specific DNA or RNA molecules on the basis of fluorescence quenching by guanine when the probe is hybridized to the target oligonucleotide (*F9*). In a more general detection scheme, Yang et al. reported a method for the rapid determination of DNA and RNA based on the fluorescence quenching of magdala red (*F10*). In the cases examined, the detection limits ranged from 6 to 15.0 ng/mL.

DATA REDUCTION

There were several papers involving the use of neural networks in fluorescence analysis. Capitan-Vallvey et al. described a method for the quantitative detection of three drugs that have significant spectral overlap (*G1*). Partial-least-squares multivariate calibration and Kohonen artificial neural networks were used to resolve the mixture, and a calibration matrix was constructed for the analysis. Lerner et al. report the development of a neural network classification scheme for signal counting in fluorescence in situ hybridization images (*G2*). The system allows rapid rejection of images that cannot be analyzed. Wolf et al. reported on a method for the on-line monitoring of biological processes using two-dimensional fluorimetry interpreted by artificial neural networks

(G3). As a demonstration of the technique, the degradation of chlorinated organic compounds was monitored using an extractive membrane bioreactor and the fluorescence of the sample.

Amador-Hernandez et al. proposed a flow injection-based method for the screening of polycyclic aromatic hydrocarbons in water (G4). The method uses a preconcentration step and fluorescence detection with a Nd:YAG laser for excitation. The data are categorized by soft independent modeling of class analogy and partial-least-squares regression. An interesting application was reported by Herbert et al. that detailed the identification of soft cheeses by front face fluorescence spectroscopy (G5). Multivariate statistics and principal component analysis was used to characterize protein structure in relation to the cheese texture. Tellinghuisen reported a study that used Monte Carlo calculations to evaluate the effect of data bias and inconsistency in linear regression analysis (G6). This is most relevant in cases in which data are transformed prior to analysis, which can result in normally distributed data becoming nonnormally distributed. Calculations were performed for several cases of equilibrium and kinetics data, and the bias was found to be a significant factor.

There were two papers dealing with the characterization of dissolved organic matter by fluorescence excitation–emission matrixes. Persson and Wedborg evaluated the use of 3-dimensional fluorescence as a characterization tool for dissolved organic matter using principle component analysis and partial-least-squares regression (G7). Marhaba et al. report the characterization of dissolved organic matter by principal component analysis of 3-dimensional fluorescence spectra (G8). In this study, fractions obtained by solid-phase adsorption were used to create a 3-dimensional fluorescence spectral library.

LUMINESCENCE IN ORGANIZED MEDIA

The term organized media or constrained environment has not been widely used in the published literature. However, systems that can be classified in this category, such as micelles, zeolites, calixarenes, and cyclodextrins, continue to attract the attention of many researchers. Choi et al. compared the solubilization power of gemini surfactant micelles and concluded that the longer aliphatic chain induces greater solubility of analytes than the shorter chain (H1), yet the solubilization power of these micelles was proven to strongly depend on the structure of the analyte and not only on the nature of the surfactant micelle. In addition, the authors reported that the dye molecules, solubilized by these micelles, were not located in the inner core, but rather, close to the surface of the micelle. Another study of fluorescence energy transfer from the phenyl groups of Triton X-100 to pyrene butyric acid concluded that the pyrene molecules are uniformly distributed in the core of the micelle (H2). Other studies aimed at utilizing fluorescent probes as well as fluorescence quenching for studying the structure and microenvironment of micelles (H3–H5). The group of Dogra used dual fluorescence for the study of Triton X-100 reversed micelles in cyclohexane and the dependence of these micelles on the water content, surfactant, pH, and on trifluoroacetic acid (H4). As a conclusion, the authors suggest that protons do not seem to penetrate the reversed micelles. An interesting study by Morrissey et al. addressed the question of whether the structure of surfactant complexes on the DNA surface depends on the nucleotide sequence and suggested two stages

of forming oligonucleotide–surfactant complexes, the first of which is independent of the nucleotide sequence, and the second of which is sequence-dependent (H5). Singh et al. synthesized several diphenylpolyenes and used them in different micellar systems to study the fluorescence of the excited-state twisted intramolecular charge transfer of these compounds (H6). The Warner group continues to be active in synthesizing, characterizing, and utilizing amino acid-based surfactants in analytical applications, and comparing between the monomeric and polymeric micelles of these surfactants (H7, H8). An interesting study from the same group reported the correlation between fluorescence anisotropy measurements and separation results from capillary electrophoresis. The authors introduced the fluorescence anisotropy method as a tool for studying enantioselective reactions and chiral molecular recognition in polymerized surfactant micelles (H9).

The interest in studying inclusion complexes of calixarenes and cyclodextrins is apparent in the wide range of publications of these host molecules (H10–H12). Naphthoquinone derivatives were used in a fluorescence study of calixarene inclusion complexes, and the possibility of forming hydrogen bonds between the hydroxyl groups of the host and the carbonyl groups of the guest was postulated (H10). Cyclodextrins, native and derivatized, continue to play a very active role in analytical applications, for example, microplate-based detection of amino acids (H13) and the determination of biological toxins (H14). Zeolites have also been used in many applications; they were used as probes, and their microenvironment was studied by luminescent probes (H15, H16). For optical sensing, solvatochromic dyes were introduced into the zeolite pores by ion exchange, inclusion synthesis, and direct adsorption for size-selective vapor sensing (H15). Various organic analytes were introduced to such zeolites, and the fast response of the optical changes was demonstrated. The group of Ramamurthy demonstrated the use of luminescence in the study of the unique “superpolar” characteristics of zeolite supercages and suggested a potential use of such zeolites for excited-state switching of carbonyl compounds (H16).

LOW-TEMPERATURE LUMINESCENCE

A modest level of activity was evident in the area of low-temperature luminescence. Two reviews recently appeared in the literature. Hofstraat and Wild reviewed the use of low-temperature to achieve high spectral resolution with excitation–emission matrixes (I1). Jankowiak reviewed the fundamentals of fluorescence line-narrowing experiments (I2).

Further development of Shpol'skii techniques has resulted from the collaborative efforts of Bystol, Campiglia, and Gillispie at North Dakota State University. Improved methods for laser-excited, time-resolved, Shpol'skii fluorescence is reported for several polycyclic aromatic hydrocarbons at 77 K (I3). This was accomplished using a narrow band, frequency-doubled, and tunable dye laser along with a gated intensified charge-coupled device (ICCD) camera. In this case, the distal end of the bifurcated fiber optic probe was frozen directly into the sample matrix. A similar approach for the rapid acquisition of relatively long (>200 ms) phosphorescence lifetimes has been reported (I4). Using this methodology, 20 decays could be recorded from a frozen sample in <5 min. The same groups also demonstrated the use of liquid

helium (4.2 K) for the rapid freezing of Shpol'skii matrixes within a few seconds (15).

Leviniskii et al. report a unified procedure for the simultaneous detection of polycyclic aromatic hydrocarbons and their nitro derivatives at low temperature (16). Sensitivities comparable to that of high performance liquid chromatography with fluorescence detection were achieved without the need for derivatization of the nitro analogues. Low-temperature (77 K) luminescence detection has been combined with capillary electrophoresis for the on-line identification of some structural and stereoisomers (17).

TOTAL LUMINESCENCE AND SYNCHRONOUS EXCITATION SPECTROSCOPIES AND RELATED TECHNIQUES

Total luminescence, synchronous excitation, or other similar multiwavelength methods have appeared in a number of unique analytical applications. Synchronous scanning fluorescence has been combined with principal components analysis (PCA) to evaluate the condition and identity of transformer oils from various manufacturers (11). It was shown that regulatory conformance could be determined along with identification of the manufacturer and the history of use for a wide range of transformer oils, both new and used. Synchronous scanning has also been applied for the direct characterization of dissolved organic matter in natural water samples without the need for sample preparation (12). The content of aromatic structures and the extent of humification determined using synchronous scanning fluorescence was comparable and more rapid than the standard characterization of isolated humic substances. Schimmel et al. reported the use of synchronous scanning fluorescence with 13 unique fluorescence microspheres to determine blood flow (13). Kim and Ivanov investigated the use of synchronous fluorescence with a whole cell, fluorescence in situ hybridization (FISH) assay (14). Although nonspecific binding of the hybridization probes presents practical limitations with the approach, it was shown to be an effective method for monitoring nitrifying bacteria in microbial communities. Also of note is development of a simultaneous, dual analyte immunoassay using synchronous scanning fluorescence (15). Simultaneous determination of serum albumin and transferrin is reported with good precision (5–6% RSD) and high sample throughput (20 h⁻¹).

Total luminescence spectroscopy was used to monitor seasonal variations of natural organic matter (NOM) in six Georgia rivers over a period of two years (16). These data were normalized to carbon content and compared with the dissolved organic carbon (DOC) determined by fractionation with ultrafiltration. In a separate study, total luminescence was used in concert with infrared spectroscopy (IR), atomic absorption (AA), and inductively coupled plasma-mass spectrometry (ICPMS) to compare the physical and chemical properties of humic acids found in the drinking water of the blackfoot disease areas of Taiwan with that from the endemic arsenicosis areas of inner Mongolia (17). Excitation–emission spectra have also been applied for analysis of human dental tartars (18). The parallel factor analysis model (PARAFAC) was used to achieve spectral resolution and reveal porphyrin-like fluorescence spectra. A novel use of total luminescence spectroscopy for the detection and possible identification of biohazard materials has been reported (19). Anderson et al. recorded excitation–emission spectra of homogenized wasps

following feeding on honey containing various concentrations of *Bacillus megaterium* endospores. Some potential as a rapid diagnostic tool for biohazards was indicated. Total luminescence was used to optimize the linear trajectories for cross-section fluorimetry in the simultaneous determination of a three-component aqueous mixture of pesticides (110). Carbendazim, fuberidazole, and thiabendazole were simultaneously detected at trace levels with better than 5% precision in water samples using cross sections through the total luminescence spectrum.

SOLID SURFACE LUMINESCENCE

Eroglu et al. reported the use of filter paper pretreated with CdCl₂ as a solid substrate for detection of H₂S using a fiber-optic sensor (K1). A number of alternate substrates were also investigated. The observation of fluorescence enhancement at a silver colloid surface is the basis for a homogeneous immunoassay format using standard microplates that have been coated with silver colloid via silanization of the plastic surface (K2). The enhanced fluorescence from surface-bound probes relative to those in free solution provides the necessary discrimination for directly monitoring biorecognition events. The fact that nothing more sophisticated than standard microplates and microplate readers are required provides a competitive advantage over the more technically complex surface plasmon resonance (SPR) methods.

The solid-substrate room temperature phosphorescence (RTP) of two commercially available metalloporphyrins was investigated for use as DNA probes (K3). Stable RTP signals were observed on filter paper without the need for external heavy-atom quenchers. Yeung's group at Iowa State University utilized total internal reflection fluorescence (TIRF) microscopy to monitor the adsorption/desorption behavior of individual DNA molecules at a liquid–solid interface in real time (K4). At a C₁₈ surface, adsorption was driven primarily by hydrophobic interactions rather than electrostatic interactions. The dynamic behavior of individual molecules was correlated to elution behavior in capillary liquid chromatography and capillary electrophoresis. A possible alternative to colorimetric detection with Western blot assays has been described using chemiluminescence on a membrane substrate (K5). The method utilized a camera luminometer and instant photographic film to detect the visible chemiluminescence from the luminol–H₂O₂–horseradish peroxidase (HRP) reaction. HRP (0.2 ng) was detected with a 10-min exposure time.

The use of solid substrates for luminescence detection of immunoassays continues to be of interest. Trevanich et al. reported the use of a membrane filter to collect bacteria and serve as a substrate for chemiluminescent detection (K6). Monoclonal antibodies for *Escherichia coli* O6:H16 were used to successfully screen against 15 different *E. coli* strains and 19 non-*E. coli* bacteria. A photon-counting television camera was used to detect the chemiluminescent spots, which produced a linear dynamic range of 1–300 CFU. A RTP immunoassay was described using dye-embedded polyglutaraldehyde nanoparticles (K7). A polyamide membrane was used as a solid substrate for the sandwich-type immunoassay. Surugiu et al. reported the use of disposable thin-glass capillaries for a sensitive chemiluminescent ELISA for 2,4-dichlorophenoxyacetic acid (K8). The glass capillaries were first derivatized with an aminosilane to which carboxymethyl dextran

was covalently attached. Monoclonal antibodies were coupled to the dextran.

The Hurtubise group continues to be active in the general area of solid matrix luminescence. The quenching effects of oxygen on the RTP of perdeuterated phenanthrene using two different filter paper substrates were reported (*K9*). Similarly, dynamic quenching of RTP contained within glucose glasses was investigated (*K10*). The same group also reported the use of both room temperature fluorescence and phosphorescence on filter paper for the human biomonitoring of three aromatic metabolites in urine (*K11*). In general, SMP proved to be more sensitive than SMF.

LUMINESCENCE IN CHROMATOGRAPHY, ELECTROPHORESIS, AND FLOW SYSTEMS

A review with 36 references covered applications of laser-induced fluorescence (LIF) detection in capillary electrophoresis (CE) to analytes exhibiting native fluorescence upon irradiation with UV light or using multiphoton excitation (*L1*). Pulsed and cw laser sources were compared, and environmental applications were discussed, along with multiphoton excitation for biological fluorophores. Another review with 54 references covered the use of noncovalent labels for LIF detection of proteins in CE (*L2*). Noncovalent protein labels for use in the UV, visible, and near-IR (NIR) spectral regions were enumerated. A review with 83 references focused on NIR detection in CE, including instrumentation, NIR labels, and applications for DNA sequencing and fragment analysis (*L3*).

Several specific applications of luminescence detection in CE were described. A noncompetitive immunoassay for insulin antibodies employed insulin labeled with a NIR dye, which formed a labeled immunocomplex with the antibodies (*L4*). CE with LIF detection was used to separate and detect the free labeled insulin and the labeled immunocomplex. A scheme was described for on-line chemiluminescence detection in microchip CE in which dansylated amino acids (dansyl-lysine and dansyl-glycine) were separated and reacted with a reagent to generate chemiluminescence (*L5*, *L6*).

DYNAMIC MEASUREMENTS AND FLUORESCENCE POLARIZATION

The interest in studying the dynamics of a system stems from the need to characterize various phenomena like aggregation, adsorption, association. Among these systems, one can count organized media and constrained environment, biorelated systems, and macromolecules ranging from peptides and proteins to membranes and intact cells, as well as polymers, thin or thick films, micelles, and host-guest complexes, to name a few. Fluorescence spectroscopy is one of the most versatile methods and has been widely used in the study of analytical, bioanalytical, and physical chemistry of such systems. Many of the concerned applications have been reviewed in other sections, and the literature here is complementary to those sections. Among the methods that are frequently used, one can find fluorescence quenching, time-resolved fluorescence and fluorescence anisotropy, fluorescence resonance energy transfer, and fluorescence recovery after photobleaching.

To study the dynamic environment of fluorescent-labeled DNA oligomers upon their adsorption onto cationic layers on a glass

surface, two-photon time-resolved fluorescence anisotropy (TRFA) was used to detect the slow rotational motion of the DNA on the surface (*M1*). Furthermore, the addition of up to 0.1 M salt concentrations was proven to further slow the rotational motion. A similar setup was used to study the local dynamics of the backbone of anthracene-labeled polystyrene adsorbed onto a quartz surface (*M2*). The fluorescence anisotropy did not reach zero, even after a long time, because of the hindered rotational motion of the fluorophores. A more complex behavior of TRFA was reported for pyrene dissolved in a solution of a thermoresponsive polymer (*M3*). The anisotropy initially dropped to a finite value after 40 ns, and it was followed by an increase over a time scale of hundreds of nanoseconds. Other liquid-liquid interfaces were studied in a similar manner, and the effect of surfactants on the in-plane and out-of-plane rotational dynamics of octadecylrhodamine B was studied at the toluene-water interface (*M4*). The group of Birch used TRFA as a new approach for particle sizing of nanometer resolution of silica hydrogel (*M5*). On the theoretical front, Edman et al. reported a new approach to quantitative analyses of TRFA based on the extended Forster theory of donor-donor energy migration in bifluorophoric macromolecules (*M6*). Another approach used Monte Carlo simulations for the dynamics of homo- and heterotransfer of excitation energy in inclusion complexes of multichromophoric cyclodextrins with fluorescent dyes (*M7*). The latter showed that the efficiency of transfer from the antenna chromophores to the fluorescent dye was close to 100%.

Fluorescence recovery after photobleaching (FRAP) is widely used in real biological (*M8*) and biomimetic (*M9*) systems. However, the use of FRAP in other systems is very apparent. A review article appeared on confocal FRAP and discussed its use for studying molecular interactions at high concentrations and the formation of molecular networks and their permeability to other probe molecules (*M10*). Weiss et al. used confocal microscopy and FRAP for the characterization of sol-gel glasses (*M11*). The authors introduced the fluorescent dyes into the glass by diffusion within wet samples, demonstrating by that the potential use of a noninvasive optical characterization method of sol-gel glasses. An interesting hybrid of FRAP, fluorescence detected linear dichroism and fluorescence polarization setup was used to simultaneously measure the electrophoretic mobility, band broadening, and the molecular orientation of DNA during steady-field gel electrophoresis (*M12*). Lateral diffusion of phospholipid bilayers supported on a polyion/alkylthiol layer pair was estimated by the use of FRAP to be on the order of 10^{-9} cm²/s at room temperature (*M13*). The group of Turro used FRAP for determining the segmental scale mobility in a confined polymer melt and concluded that changes in the local packing near the free surface has an enormous effect on the mobility (*M14*). Another hybrid of total internal reflection with FRAP was capable of measuring the velocity of a liquid with a resolution of ~ 100 nm (*M15*).

CHEMILUMINESCENCE

An initial attempt to apply molecular connectivity calculations for prediction of chemiluminescent behavior has been reported (*N1*). Successful prediction of new chemiluminescent methods could significantly reduce development time and provide enhanced analytical utility. Multivariate calibration using nonlinear principal

component analysis and nonlinear partial least squares was applied to nonlinear chemiluminescence data (N2). Rose and Waite investigated the uniqueness of the relation between the chemiluminescence intensity of luminol and the total concentration of Fe(II) in aqueous samples (N3). The authors have developed a kinetic model that describes the two-step oxidation of luminol and applied it to investigate the effects of naturally occurring fulvic acids on the chemiluminescence reaction. Jonsson and Irgum described a series of new nucleophilic catalysts for fast chemiluminescence reaction kinetics (N4). This is of particular interest for application of chemiluminescence detection miniaturized devices in which detection volumes, residence times, or both are limited. Both single- and dual-component catalysts were investigated. Maximum emission was typically observed in <10 ms. The use of a CCD camera with a continuous flow manifold has also been described for rapid acquisition of chemiluminescence spectral profiles (N5). The authors propose that the system could be employed to simultaneously monitor multiple detection channels using separate detection zones on the CCD. Bobbitt's group has described the use of a novel labeling strategy for detection of proteins using chemiluminescence (N6). They labeled the protein avidin with a tertiary amine attached to biotin. The tertiary amine sensitized the protein to produce chemiluminescence using Ru(bpy)³²⁺. Chemiluminescence intensity increased with increasing number of tertiary amine labels per mole of protein.

One of the more dynamic growth areas of analytical development in recent years has been associated with the efforts to understand and apply electrogenerated chemiluminescence (ECL). A survey of the recent literature indicates that work in this area continues to be quite active. Much of the fundamental understanding of ECL continues to be produced from Bard's group at the University of Texas at Austin. Zu and Bard described the effects of electrode surface hydrophobicity and surfactant on ECL intensity (N7). They have also assessed the role of direct coreactant oxidation and the effect of halide ions on ECL intensity (N8). Factor et al. investigated the effects of nonionic surfactant chain length on ECL intensity (N9). An ~5-fold increase in ECL efficiency is reported with surfactant. A slight decrease in ECL intensity was observed with increasing surfactant chain length. Khramov and Collinson investigated the use of Nafion and Nafion-SiO₂ composite films for the fabrication of ECL-based sensors (N10). ECL of immobilized Ru(bpy)³²⁺ was observed to be strongly dependent on the Nafion content. Collinson et al. also studied the role of the reductant on Ru(bpy)³²⁺ ECL signal magnitude and stability while encapsulated in a silica sol-gel monolith (N11). Immobilized ultramicroelectrodes were utilized to generate the ECL-potential curves. The tertiary amines showed a significant dependence on scan rate, whereas sodium oxalate did not, as a result of the differences in mobility within the host matrix. McCall and Richter reported the efficient quenching of Ru(bpy)³²⁺ ECL by phenol and substituted phenols (N12). The efficiency of quenching was dependent on the position of substitution on the aromatic ring with meta derivatives providing the greatest effect.

Recent developments in the microfabrication of miniature analytical devices has also stimulated interest in chemiluminescence as a compatible technology for on-chip detection. Tsukagoshi et al. demonstrated the use of chemiluminescence with

capillary electrophoresis microchips constructed of two quartz plates with a Si membrane (N13). The same group also demonstrated a microchip electrophoresis separation of two dansylated amino acids with chemiluminescence detection (N14). Although the detection sensitivity observed was not as good as that with conventional capillary electrophoresis with chemiluminescent detection, the improvement in analysis speed (<40 s) was significant. Hybridization assays have also been demonstrated on a three-dimensional microchip format using chemiluminescence with the CCD imaging detector (N15). Three orders in linear dynamic range was obtained with a detection limit of 250 amol.

NEAR-INFRARED FLUORESCENCE

Near-infrared (NIR) fluorescence continues to increase in use. Application of this technique to immunoassays was reviewed in an article with 60 references (O1).

The photophysical properties of a NIR molecule that could be used as a sensor for pH or solvent acidity were studied using steady-state and time-resolved techniques (O2). Another NIR fluorescent dye, allophycocyanin 1, was studied as a tracer in a fluorescence resonance energy transfer system (O3). The dye forms a trimeric structure that is stabilized by the presence of a peptide linker in its core. Cross-linked dye was conjugated to antibodies or streptavidin and associated with a europium complex donor for homogeneous, time-resolved techniques based on fluorescence resonance energy transfer. In another study, a difluoroboron dipyrromethene dye with an analyte-responsive donor group conjugated to the core was synthesized and investigated for molecular switching in the NIR (O4).

A cationic cyanine molecule was studied as a NIR probe for nucleic acid detection (O5). The fluorescence of the probe decreases in the presence of DNA. A NIR-labeled ssDNA probe that exhibits enhanced fluorescence upon binding to a strand containing its complementary sequence was described for use in DNA hybridization detection (O6). Both single- and dual-labeled probes were studied; it was found that self-quenching of the dye labels in the dual-labeled probe is reduced upon hybridization, leading to greatly increased fluorescence.

The noncovalent association of a NIR squarylium dye with bovine serum albumin was studied using fluorescence-detected circular dichroism spectroscopy (O7). It was found that the FDCD signal of the dye/protein complex, but not of the dye itself, was diminished upon addition of urea, which denatured the protein. It was concluded that the FDCD spectral features arise from noncovalent interactions between the dye and the secondary structure of the protein. Average association constants of two differently substituted squarylium dyes were compared.

LUMINESCENCE TECHNIQUES IN BIOLOGICAL AND CLINICAL ANALYSIS

A review with 51 references discussed the UV-visible absorption and fluorescence spectral properties of polypeptides and proteins (P1). The characteristics of light sources for photoactivation of sensitizers for photodynamic therapy and fluorescence diagnosis in dermatology were discussed (P2). Fluorescence diagnosis of tumor cells in dermatology in which a heme metabolite is applied to the tissue was described (P3). The metabolite is taken up selectively in neoplastic tissue and metabolized, leading to the accumulation of fluorescent porphy-

rins, primarily in the tumor cells.

There were several developments in the area of UV-visible fluorescent dyes (near-IR dyes are discussed in the section on near-IR fluorescence). The synthesis and photophysical characterization of derivatives of acridine dyes were described (*P4*). The dyes had high fluorescence quantum yields. The use of analogues that bear a free carboxyl group to probe the conformation of peptides was suggested. The binding interactions of a tricationic cyanine dye with duplex and triplex DNA as a function of DNA sequence was studied using optical spectroscopy and viscometry (*P5*). Multiple DNA binding modes, including minor groove binding of dye monomers and at higher dye concentrations, dimers, were observed.

DNA recognition by a single zinc finger tethered to thiazole orange dye was studied (*P6*), including characterization of the photophysical and photochemical properties of the conjugate. Dye fluorescence was observed upon binding of the zinc finger, which was derived from the DNA binding domain of the glucocorticoid receptor, to the native glucocorticoid receptor element in DNA. Little fluorescence was observed from the dye-zinc finger conjugate alone or in the presence of DNA lacking the receptor element sequence.

The application of fluorescence to nucleic acid hybridization detection continues to be an active field. A dual-labeled DNA probe (see *P6*) was used to detect ssRNA of plant viral genome through fluorescence resonance energy transfer (*P7*). Another paper described the combination of two amplification techniques, tyramide signal amplification followed by enzyme-labeled fluorescent substrate amplification, for hybridization detection of leptin receptor mRNA (*P8*). The combined techniques allowed for fluorescence in situ hybridization (FISH) detection of scarce mRNAs that could not be detected using either amplification technique alone. FISH was also employed for detection, identification, and enumeration of *E. coli* bacteria (*P9*). The technique employed cyanine 3-labeled peptide nucleic acid probes and an array scanner to detect bacteria collected on membrane filter surfaces.

A two-wavelength fluorescence method was described for measuring cellular DNA repair capacity (*P10*). In a host cell, the expression of a UV-irradiated plasmid carrying the marker gene for enhanced green-fluorescent protein is compared to the expression of an undamaged plasmid containing the gene for a red-shifted-fluorescent protein. The proteins are excited at 488 nm but have different emission maximums, allowing simultaneous determination of the fluorescent proteins.

Charge-coupled device (CCD) cameras were employed for detection of proteins and DNA. A CCD-based image-analyzing system was used to detect proteins dotted on nitrocellulose membranes (*P11*). The immobilized proteins were biotinylated and then visualized via chemiluminescence or fluorescence using streptavidin-peroxidase or streptavidin-fluorescein isothiocyanate, respectively. A mutation DNA microarray was marked with cyanine 3 and read by means of an intensified CCD camera that acquired time-delayed fluorescence images (*P12*). Time-resolution provided a means by which interference from background could be minimized.

REAGENTS AND PROBES

The area of fluorescent probes or indicators continues to grow exponentially. There is a continued interest in designing, synthesizing, and characterizing such fluorescent indicators for a wide variety of analytical applications, for example, inorganic, organic, and biological (*Q1*, *Q2*). Noticeably, many such probes are used in sensing (*Q3*) and for developing nonlinear probes (*Q4*). Fluorescent Ca^{2+} indicators were used to focus on detecting low levels of intracellular Ca^{2+} concentrations; the dissociation constant of such indicators is on the order of 10^7 M. Liepouri et al. reported the synthesis and characterization of low-affinity ($K_d < 30$ M) iminocoumarin-based fluorescent Ca^{2+} indicators applicable to high Ca^{2+} concentrations (*Q1*). In comparison, only little research activity focuses on fluorescent probes that can specifically probe zinc. Hirano et al. reported the synthesis of fluorescent zinc probes for measuring the cellular regulation of zinc (*Q5*). Fang et al. covered an interesting evolving area of novel fluorescent probes described as molecular beacons (*Q6*). Such probes are necessary for ultrasensitive protein detection in the proliferating area of proteomics and genomics. In their native form, the beacons are nonfluorescent as a result of the close proximity of the attached fluorophore and quencher. Upon binding, the beacon is unfolded, and the fluorophore is distant from the quencher, yielding enhanced fluorescence. A similar approach can be adopted by replacing the quencher with a donor or an acceptor and following the fluorescence resonance energy transfer of the donor-acceptor pair.

OTHER TECHNIQUES AND APPLICATIONS

There were several notable advances that are related to theoretical aspects of fluorescence. Cohen et al. reported experimental observation of nonexponential kinetic data for a direct acid-base reaction by measuring the excited-state proton transfer from a naphthol sulfonate to an acetate anion (*R1*). It was shown that this reaction quantitatively conforms to predictions by Smoluchowski theory. Edman presented a theoretical framework for the treatment of classical fluorescence correlation spectroscopy of multiple molecules and that of single molecules (*R2*). Molski described a 5-state model of the kinetics of bleaching in single-molecule fluorescence spectroscopy (*R3*). Exact formulations were developed that describe the rate constants for the bleaching number and the bleaching time.

A comprehensive report detailing theoretical and experimental aspects of a new single molecule fluorescence analysis method based on photon arrival times appeared in the literature (*R4*). Novikov et al. derived a theory for single molecule impulse response functions based on photon arrival times under both pulsed and steady-state excitation conditions. Practical application is demonstrated by estimating the triplet lifetimes and intersystem crossing yields for the immobilized fluorescent dyes DiI and rhodamine 6G.

Several reports appeared in the literature detailing schemes for the detection of explosives. Goodpaster and McGuffin described a method based on fluorescence quenching that is capable of analysis of nitrated explosives (*R5*). Stern-Volmer plots were constructed for the quenching of pyrene fluorescence, and the Stern-Volmer constants were found to increase with the degree of nitration, which allowed for the differentiation of various explosives. Another sensing application based on quenching was reported by la Grone et al. (*R6*). The fluorescence quenching of

a polymer film in the presence of vapors from explosives was used to detect TNT and other nitroaromatic compounds from landmines. The prototype sensor was able to detect femtogram levels of these explosives. In a related report, Rose et al. detailed results from and investigation of the design and synthesis of sensing materials for the detection of explosives by fluorescence (R7).

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