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Analytica Chimica Acta 500 (2003) 21–69
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Review

Current status of modern analytical luminescence methods

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Received 24 March 2003; received in revised form 2 September 2003; accepted 2 September 2003

Abstract

Modern analytical luminescence methods and their recent applications are reviewed with emphasis on the most sensitive methods that can be expected to be useful in future microanalytical systems such as μ-TAS, lab-on-chip, point-of-care (POC) and high throughput screening (HTS) applications. Photoluminescence (PL) is presently the most important group of analytical techniques utilising luminescence. Because of the rapidly increasing popularity of electrochemiluminescence (ECL) and its applications, we have given particular attention to ECL mechanisms and techniques. Due to the present and future importance of capillary electrophoresis (CE) as a separation method, the CE detection methods based on luminescence are also considered in a relatively detailed way. For those researchers, designing novel experiments and assays, experimental set-ups, and apparatus we include web links to the manufacturers of some fairly rare reagents, as well as modern instrument components.

Keywords: Point-of-care; High throughput screening; Electrochemiluminescence

1. Introduction

A wide variety of luminescent phenomena lend themselves as tools in analytical chemistry. Table 1 lists the different types of luminescence. Of these, RCL [1], LL [2–5] and SL [6,7] have been studied to a clearly lesser extent than photoluminescence (PL) and CL. These three can be regarded as sub-classes of CL, and their common feature is that the excitation reactions are primarily induced by radical species in aqueous media.

Traditional CL does not necessarily involve radical species in the luminescence generation pathways, but this may occur. General reviews on CL are published very frequently and detailed information on the mechanisms and applicability of different CL systems is easily found from the literature [8–19]. Thus, the traditional CL methods are largely left outside the scope of this paper.

Regardless of the need for more or less expensive optics and light sources, PL will almost certainly always remain the most significant analytical luminescence method, and the different forms of CL will be able to fully compete with it only in certain special applications. PL can be divided into two main subclasses: fluorescence and phosphorescence, and these analytical methods are regularly reviewed in analytical chemistry (also including CL) [20,21] and frequently also in various other sources [22–28].

Probably, the most versatile type of CL in the future will be electrogenerated chemiluminescence, i.e. electrochemiluminescence (ECL) [29–35]. The advantage of ECL lies mainly in that it typically allows the excitation of the same luminophore molecule several

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0003-2670/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.aca.2003.09.004

Table 1
Different types of luminescence

Luminescence type	Caused by
Photoluminescence (PL)	Photo-excitation of compounds
Chemiluminescence (CL)	Chemical excitation of compounds
Electrochemiluminescence (ECL)	Electrogenerated chemical excitation
Radiochemiluminescence (RCL)	Radiation-induced chemical excitation
Lyoluminescence (LL)	Excitation induced by dissolution of an irradiated or other energy-donating solid
Sonoluminescence (SL)	Excitation of compounds by ultrasonication, either by energy transfer from the intrinsic SL centres of water or by chemical excitation by hydroxyl radicals and atomic hydrogen

times and easily offers possibilities for time-resolved measurements, whereas in the other CL methodologies the excitation event of a molecule is normally a single shot event and then the molecule is destroyed. Time-resolved measurements are often either totally useless or very difficult to achieve in CL regimes other than ECL. However, it can be assumed that time-resolved CL could be carried out, e.g. by fast and reproducible reagent microinjection in microanalytical systems.

High sensitivity is common to practically all of the luminescence methods and it is not extraordinary to have detection limits (limit of detection, LOD) even in sub-picomolar levels. In addition, the log–log intensity versus concentration calibration plots can be linear over several orders of magnitude of concentration.

The main aim of this review is to provide a brief description accompanied by only very brief discussions of usable or potentially usable basic principles of currently important luminescence methods and their instrumentation in miniaturised and other modern analytical systems. Readers are guided to the very latest reviews on each specific topic when such papers are available. These reviews then provide the exact historical background of the phenomena and pioneers in the field, plenty of clarifying figures, and leads to older review papers not mentioned here. However, some novel applications and issues felt generally important are given more detailed consideration. The focus is on bioanalytical applications.

2. Generation of luminescence

Molecules and metal chelates produce luminescence after photo-excitation relatively rarely, and usually the excitation energy of the molecule is just lost by non-radiative pathways. This is why the naturally photoluminescent molecules can quite often be directly determined selectively and sensitively also from relatively complicated solution matrices.

Fluorescence is a process in which a luminophore absorbs a suitable-energy light quantum (a photon) to raise an electron from an occupied orbital to a higher energy vacant orbital, followed by the electron returning back to the original ground state energy level, and emitting a quantum of light with an energy corresponding to the energy difference between the excited state and the ground state level, in such a way that the electron spin remains unchanged throughout the entire process. Thus, the molecule is all the time either in its ground or excited singlet states (the magnetic field does not split the energy levels of these electronic configurations). This process is very fast, and the luminescence lifetime is of the order of nanoseconds.

Only in very rare cases, a much slower radiative relaxation of the molecule can occur in a solution at room temperature. This requires rather special molecule properties, which allow the excited electron to reverse its spin while it is transitioned to a lower-energy state, called a triplet state because the singly occupied electrons in different orbitals are now in a parallel spin (the energy level is split into three levels in a magnetic field). When the electron transitions to a ground state level its spin must be reversed again, and therefore the time scale of the process is much slower than in the fluorescence process and is called phosphorescence. Typical luminescence lifetime of phosphorescence is from some tens of microseconds up to some milliseconds. Phosphorescence is a much more common phenomenon in a solid state than in a liquid solution phase.

In conclusion, spin-allowed transitions ($\Delta S = 0$) are called fluorescence and spin-forbidden transitions ($\Delta S = 1$) are called phosphorescence. Some metal chelates, especially lanthanide(III) chelates, produce

photoluminescence which cannot clearly be given the definition of fluorescence nor phosphorescence [36]. The spin multiplicity of the central ion during an emissive transition step may change, e.g. from 5 to 7, i.e. it neither remains unchanged as in fluorescence nor changes from 3 to 1 as in normal phosphorescence. Thus, some researchers [37] strongly emphasise that the measurement of the emission of long-lived lanthanide(III) photoluminescence should be called time-resolved photoluminescence (TR-PL) instead of time-resolved fluorescence. In some very rare cases, also other types of delayed or long-lived luminescence processes than phosphorescence can occur (see, e.g. ref. [38]).

Relatively recently, the advent of multiphoton excitation of luminophores has opened up a lot of new possibilities [39], which will be discussed below in Section 4.

When light is generated by chemical reactions there are two main pathway types giving the necessary excitation energy to the finally emitting species. One type is based on the disintegration of the luminophore itself when the energy is mainly available from the breakage of its bonds, and the other type is based on a sequence of electron transfer reactions.

One-electron redox reactions can lead to similar excited states as the photo-excitation of a molecule (or a metal ion or metal chelate, as well), which can be described by the schematic molecular orbital energy diagram presented in Fig. 1. There are three possible excitation pathways, namely (i) oxidation-initiated and (ii) reduction-initiated pathways and (iii) comproportionation pathway. Pathways (i) and (ii) can be shortened to *ox–red* and *red–ox* excitation routes. If the luminophore radical intermediates are produced in aqueous solution, there can be considerable differences in their stability, which alone may make either *ox–red* or *red–ox* excitation routes very unfavourable in which case the other one becomes predominant.

In the first pathway, an electron is first removed from the highest occupied molecule orbital of the compound (or from an occupied level of a metal ion). Then, a reducing agent may donate an electron to fill the hole in the HOMO levels of the compound (or low energy metal ion levels) and a ground state product is formed. However, if the energy levels of the lowest unoccupied orbitals of the oxidised form of the molecule (or high energy levels of an ion) and the reductant are

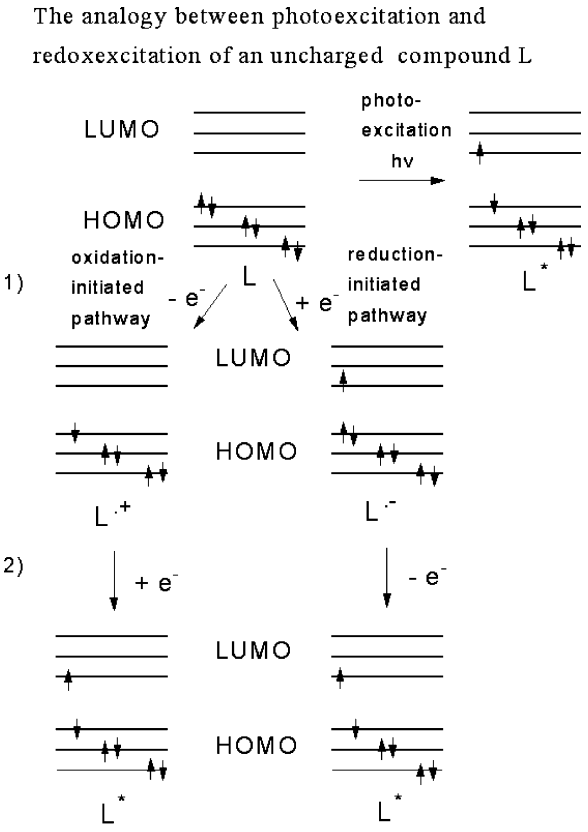

Fig. 1. Schematic energy level diagram of excitation processes of a luminophore L in photo- and redox luminescence processes. In the photo-excitation pathway, an electron is raised to a higher energy orbital by the absorption of a photon, thus forming the excited state. In the redox excitation pathway with one-electron steps, two excitation routes are possible. If the reducing/oxidising agent at the final step is sufficiently strong, both excitation routes result in similar excited final products.

suitably matched, an electron can be donated preferably to the lowest unoccupied level (LUMO), which results in the formation of a product in its excited state. Analogously, in the reduction-initiated pathway, the properties of the oxidant of the second step determine whether the end product is formed in its ground or excited state. The prerequisite for comproportionation pathway is that the oxidised and reduced forms of the molecule are produced simultaneously or successively within the lifetime of the radical first formed. Comproportionation pathway results in the formation

of one excited and one ground state molecule from the reacting radicals.

For simplicity, this figure was drawn in a way as if only singlet state excitation would be possible (electrons remain all the time "net-spin paired"), but actually electron transfer can result in either excited triplet or singlet state end products. In organic, non-aqueous, solvents some molecules produce ECL involving singlet state emission both by so-called energy-insufficient T-route via triplet–triplet annihilation, and by the energy-sufficient S-route [29–35].

In aqueous media, the necessary highly cathodic and/or highly anodic working electrode potentials can be reached only in very special cases, and in addition, if the luminophore anion and/or cation radicals can be formed at all, they are typically so reactive with water that most of them are consumed by unwanted side reactions. Those ECL methods requiring strictly non-aqueous conditions can have much value only in very limited areas of analytical chemistry.

In addition, some special molecules can be triggered to be disintegrated by pathways which produce light. The most commonly used molecules of this type are luminol, its relatives and derivates of acridine. The triggering step in the light emitting pathway can be based on chemical reaction, electrochemical reaction, light pulse, ultrasonic pulse, thermal pulse, etc., depending on the properties of the molecule in question.

3. Bioaffinity assays

More than 40 years ago, the technique of radioimmunoassays was introduced for the quantitative analysis of molecules of biological interest [40]. The impact of this technique on clinical endocrinology has been vast: for the first time, it was possible to quantify hormones with specificity and sensitivity. Later, with improvements and newer assay configurations, the technique was used in other medical areas, including hematology, oncology, microbiology and pharmacology. The power of this technique arises from: (i) the use of antibodies (Ab), which confer specificity and sensitivity because of their high binding affinity for the analyte, and (ii) the use of a label, which can be accurately determined quantitatively at very low concentrations. Antibodies are specific binding proteins functioning in the natural defence mechanism of animals against foreign intruders. Immunoassays are based on the unique recognition reaction between antibodies and the antigens (Ag), which elicit their production [41].

Recombination nucleic acid techniques have emerged relatively recently and strongly contribute to our understanding of the pathogenesis and diagnosis of genetic, neoplastic and viral diseases. The principles of these techniques rest upon the selective cleavage of nucleic acids by restriction endonucleases and by localisation of specific sequences of nucleotides after hybridisation with known DNA or RNA fragments (probes) labelled with a radionuclide or an alternative label [41].

The use of photoluminescent labels in both of these closely related techniques has many advantages in the use of radionuclide labels and is presently quite well developed. Especially the time-resolved detection of these labels gives extremely high sensitivity for the assays [41,42].

Immunoassays can be conducted on both heterogeneous and homogeneous basis. If the separation of an unbound label from a solid-surface-bound immunocomplex is carried out, the immunoassay is heterogeneous. If the separation is not necessary, the immunoassay is called homogeneous. Even though the sensitivity of heterogeneous assays is often higher, and the dynamic range of homogeneous assays is generally modest [43], homogeneous assays are usually preferred unless sensitivity is an issue.

General methods in immunoassays and DNA-probing assays are treated in the literature every now and then [44–51].

Quite often bioaffinity assays are carried out on the surface of small micro- or nanoparticles, which are easy to coat in large batches with desired biomaterial such as monoclonal antibodies. When, e.g. latex particles are used only as carriers they often contain paramagnetic material so that they can be easily separated with a magnetic field. A recent report of the state of the art of the different uses of particles in diagnostics is available from our National Technology Agency: http://www.tekes.fi/julkaisut/Particle.pdf (in English) [52]. If the nanoparticles are made from luminescent material they can be utilised as labels as well, or in encoding the different particle classes in multiplexed assays.

4. Photoluminescence, luminescent molecules and metal chelates, and their use as probes and labels

Photoluminescence has been used for a long time in the investigation of the properties of the excited states of atoms and molecules. Even though any field of material physics can take advantage of PL measurements, probably the most important area of PL is analytical chemistry.

Most analytes have no natural luminescence. In this situation, a derivatisation or labelling can be a very nice tool for sensitive analysis. Generally, the area of luminescent probes, indicators and labels (tags, markers) is continuously growing. Designing, synthesis and characterisation of luminescent molecules is in rapid progress due to the urgent needs for a wide variety of analytical applications, e.g. in the fields of biological, inorganic and organic analysis.

In biology and medicine, photoluminescent probes can be used in fluorescence or time-resolved imaging to study cell metabolism or detection of specific severe physiological conditions, etc. Thus, in principle, the same probes or markers can be used both in quantitative analytical chemistry and immuno-fluorescence microscopy [53]. Photoluminescence microscopy (mainly fluorescence microscopy) can give a lot of a very detailed information of species or microscopic specimens studied if (i) confocal detection (ii) two-photon excitation (TPE) or (iii) time-resolved detection is utilised [54]. In confocal microscopy, emission pinholes and descanning optics are necessary to achieve axial depth discrimination. Two-photon excitation (and multiphoton excitation in general) is naturally localised in the focal volume, and there is no significant off-local fluorescence to reject. In this case, an addition of a pinhole can enhance the resolution but at the expense of signal loss. The combination of these techniques creates an extremely selective detection method, time-resolved multi-photon excited photoluminescence, that can easily reveal a suitable single molecule from a solution.

Fluorescent probes are available from many manufacturers (see, e.g. http://www.probes.com/handbook/ and http://www.microscopy.bio-rad.com/fluorescence/fluorophora.htm and Table 3) and some of these luminophores are also electrochemiluminescent in aqueous solutions if excited by hot electron electrochemistry, although the manufacturers probably are not aware about this.

Table 2
Common fluorescent luminophores used for labelling

Luminophore	λ_{ex} (nm)	Luminophore	λ_{ex} (nm)
Fluorescein isothiocyanate (FITC)	494, 518	Allophycocyanin (APC)	650, 651
Phycoerythrin (PE)	565, 575	Cy3	548, 562
Rhodamine B	540, 625	Cy5	650, 670
Texas Red (TR)	596, 615	Cascade Blue	400, 420
Coumarin	384, 469		

Table 2 lists common luminophore labels. In PL and ECL methods stable metal chelates are also used; ruthenium labels being considered as the most important. Table 3 lists the manufacturers of luminophores.

Sometimes luminescent solid nanoparticles are also used as labels [55,56]. The luminescent dyes can also be bound on the surface of solid microparticles or—beads, and the resulting particles are used as labels. When the microbeads or—particles are fabricated to contain different luminophores so that each particle type has a different emission spectrum, the beads can be individually recognised if they can be detected in the volume one particle at a time. In this manner, assay multiplexing becomes possible and several analytes can be determined on the basis of a single one-step incubation [57].

The excitation mechanisms and the use of luminescent labels are discussed in the later sections of this paper. If we have missed an important manufacturer, please add it to the label-producers list located on our new web board at Graduate School of Chemical Sensors and Microanalytical Systems site: http://www.chemsem.hut.fi/.

4.1. Excitation step and side reactions of luminescence

Photoluminescence is typically regarded as a very soft way of excitation of molecules, although some photo bleaching of the irradiated molecules is normally recognised and taken into consideration. Especially biochemists often ignore that molecules and metal chelates in their excited states have properties totally different to those they have in their ground

Table 3
Distributors of labels

Distributor	Label	Website
Amersham	Cy3 and Cy5 NHS esters	http://www.mdyn.com/aptrix/upp00919.nsf/Content/DrugScr+CyDye+Fluors+introduction
Arctic Diagnostics	ArcDia BF NHS esters, metalloporphyrins	http://www.arcticdiagnostics.fi
Pierce Biotechnology		http://www.piercenet.com
ATTO-TEC GmbH		http://www.atto-tec.com
Chromagen		http://www.chromagen.com
Research Organics	Cy3	http://www.resorg.com/cy3.htm
Prozyme		http://www.prozyme.com
MoBiTec		http://www.mobitec-germany.com/mobitec_us/
Igen Inc.	Ruthenium chelates	http://www.igen.com/home.htm
Perkin-Elmer Life Sciences	Lanthanide chelates	http://www.lifesciences.perkinelmer.com/wallac.asp
CIS bio International	Lanthanide chelates	http://www.htrf-assays.com/reagents/index.htm
Research Organics	Lanthanide chelates	http://www.resorg.com/quantum.htm
Sigma-Aldrich	Lanthanide chelates	http://www.sigmaaldrich.com/Brands/Fluka_Riedel_Home/Analytical
BioPAL	Lanthanide chelates	http://www.biopal.com/Fluorescent.htm
Lumiphore	Lanthanide chelates	http://www.lumiphore.com
Newport Instruments	Lanthanide chelates	http://www.newportinstruments.com/quantum/quantum.html
Porphyrin Products	Phosphorescent metalloporphyrins	http://www.porphyrin.com/ppiweb.pdf
Luxcel Biosciences	Phosphorescent metalloporphyrins	http://www.luxcel.com
Porphyrin Systems	Phosphorescent metalloporphyrins	http://www.porphyrin-systems.de
Molecular Probes	Microspheres: Pt porphyrin- and Eu complex-labelled	http://www.molecularprobes.com
Seradyn	Lanthanide nanoparticles	http://www.seradyn.com

states. For instance, acid constants can be considerably changed and, most importantly, the redox properties are vastly changed. Orange light at the wavelength of 590 nm is composed of photons having energy of 2.0 eV. A luminophore capable in one-electron electron transfers in an excited state 2.0 eV above its ground state has a 2.0 V-shifted one-electron reduction and oxidation potential when compared to its ground state redox potentials. This is a huge difference and it is no wonder why excited state molecules often react with the other species existing in the medium, or most unfortunately, the label reacts with a molecule marked by the label in question. In this way, the excitation event may be totally wasted. The higher the energy of an incident photon is, the more different and possibly harmful photophysical and photochemical processes can occur in the excited luminophore.

If a luminophore in its excited state becomes reactive with the sample matrix, there is no difference whether the excited state has been produced by absorption of a single high-energy photon or two or three lower-energy photons. This is sometimes forgotten in the studies.

The scheme in Fig. 2 reminds us of some of the processes possible for a solvated molecule after an absorption of UV (or visible) photon [58]. Luminophore AB might just relax radiatively producing a fluorescence emission, or after intersystem crossing a phosphorescence emission might be possible. Typically, luminophores that yield both fluorescence and phosphorescence produce the most phosphorescence the lower the temperature is. In addition, a photo ionisation of the molecule AB can occur, if the energy of the absorbed photon is sufficiently high. Photochemical reactions might also occur as well as radiative or non-radiative energy transfers to other species present in the solution. It has been shown that energy transfer processes can be very nicely utilised in analytical chemistry. All in all, a variety of competing processes can exist, while the only desired one would normally, for our purposes, be either fluorescence or phosphorescence or some other form of long-lived luminescence.

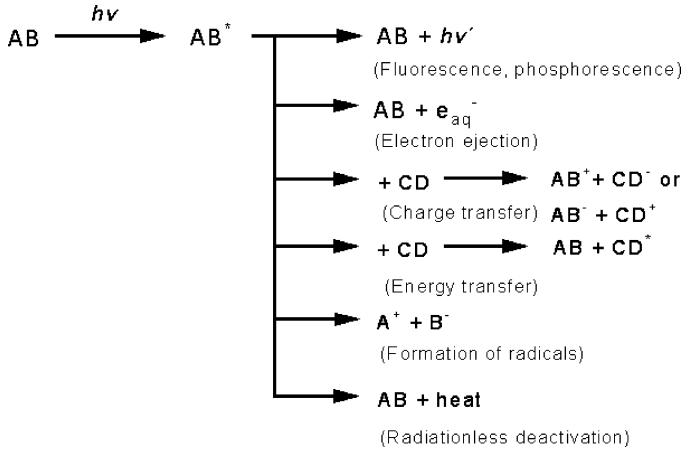

Fig. 2. Possible de-excitation processes of electronically excited molecules (AB*) in polar media [58]. Reprinted from [58], Copyright 1989, with permission from Elsevier.

Thus, the luminophore and the media have to be carefully chosen to avoid unwanted side reactions.

However, it is worthwhile to emphasise that all of the CL pathways for excitation of molecules normally have as much unwanted side reactions as PL processes. One of the strongest benefits of the PL really is that under appropriate conditions a luminophore can be continuously cycled between the ground state and an excited state emitting a photon on most cycles. Nevertheless, even typically very stable molecules seem to finally photo decompose after absorbing around 10^5–10^6 photons [59].

Fluorescence and phosphorescence of organic luminophores are described in a sufficiently detailed way already in the elementary analytical chemistry text books so these luminescence excitation and emission processes are not discussed further here.

4.2. Excited states and photoluminescence of lanthanide(III) chelates and metalloporphyrins

Lanthanide(III) chelates and metalloporphyrins are at the moment the most important labelling compounds showing long-lived luminescence necessary for the time-resolved detection of the luminescence, which greatly increases the signal-to-background ration.

Direct bonding typically observed in the transition metal series is not seen in the lanthanide series mainly due to the inaccessibility of the 4f electrons. The lanthanides behave as hard-acid cations in solution preferring interaction with hard-base donors like oxygen and fluoride to that with sulphur or heavy halide donors. Significant interaction with nitrogen donors is observed only when steric factors force the interaction as in the chelates of aminopolycarboxylates [60].

The lanthanide(III) ions, particularly those in the middle of the series, i.e. Sm(III), Eu(III), Tb(III) and Dy(III), form complexes that often emit visible radiation corresponding to the 4f $\rightarrow$ 4f intrashell transitions of the central metal ion when excited by the near-UV radiation. Generally the emission may occur (i) from the excited ligand perturbated by the cation, (ii) from the excited cation perturbated by the ligand, and (iii) as a consequence of a non-radiative energy transfer from the excited ligand to cation, followed by emission from the latter. These various types of emission are the consequence of differences in relative energy levels of the lowest excited states of the central ion and the ligand. Schematic energy level diagrams for three typical types of lanthanide(III) complexes are illustrated in Fig. 3 [61].

When the resonance level of the central metal ion is energetically higher than the lowest triplet state (T_1) of the ligand (case c) or there are no excited f levels as in the case of La(III) or Lu(III) (case b), the ligand-localised emission can be observed. However, in the case of low-lying radiative 4f* levels (case a), an intramolecular energy transfer from the ligand excited T_1 state to the 4f* level of the lanthanide(III)

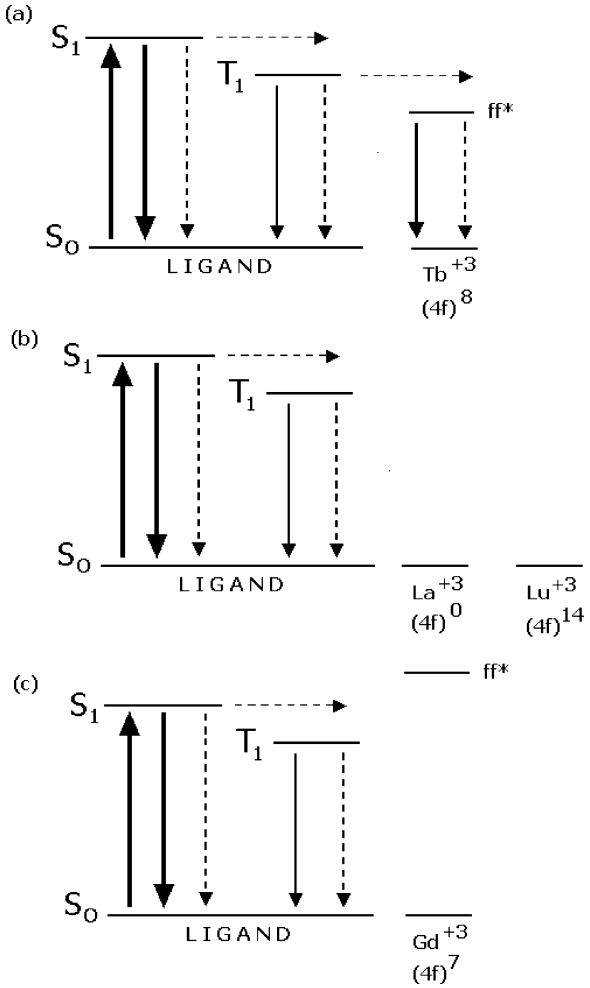

Fig. 3. Schematic energy level diagrams for three typical types of lanthanide complexes. The lowest excited 4f levels (ff*) of the central metal ion are located at energetically lower (a) or higher (c) states than the lowest triplet level (T_1) of a ligand. There are no excited 4f levels in La(III) or Lu(III) complexes (b). Radiative, very low efficient radiative and non-radiative processes are represented by (- - -▶), (→) and dashed arrow, respectively [61]. Reprinted from [61], Copyright 1990, with permission from Elsevier.

ion may occur. In this case lanthanide(III) ion specific luminescence is observed.

From the point of view of metal ion luminescence, the chelated lanthanide(III) ions can be classified into three groups [61]:

(i) Those ions that show no luminescence such as La(III) ($4f^0$) and Lu(III) ($4f^{14}$), and those for which luminescence is seldom observed such as Gd(III) ($4f^7$). Intra 4f → 4f transitions are, naturally, impossible for La(III) and Lu(III), whereas Gd(III) is luminescent, but its lowest lying excited level is energetically too high to accept energy of a typical ligand T_1 state.

(ii) Those ions that exhibit strong luminescence due to the fact that in each instance an excited energy state lies just below the ligand triplet state, and relatively large gaps exist between resonance levels and ground state, i.e. Sm(III), Eu(III), Tb(III) and Dy(III).

(iii) Those ions that show only weak luminescence because of the small energy differences between resonance levels and ground states, which increase the probability of non-radiative transitions, i.e. Pr(III), Nd(III), Ho(III), Er(III), Tm(III) and Yb(III).

The emissions of Sm(III), Tb(III) and Dy(III) almost exclusively originate from the resonance levels, which usually are the lowest levels of the lowest excited multiplet, to the vibrational levels of the ground state multiplet. Eu(III), however, quite often emits from both of the $^5D_1$ and $^5D_0$ levels, but the emissions can be resolved by time-resolved techniques due to the short lifetime of $^5D_1 \rightarrow {}^7F_J$ transitions [61].

Three mechanisms by which the excitation energy can be transferred from ligands to central ions have been proposed [61]:

Mechanism I: After an efficient intersystem crossing between the lowest singlet and triplet excited states of the ligand, an energy transfer occurs from T_1 level to a lower-energy state of Ln(III) (emitting level, denoted by $E_{Ln,e}$) which is finally radiatively relaxed

$$S_1 \rightarrow T_1 \rightarrow E_{Ln,e} \rightarrow \text{ground state multiplet} + h\nu_J \quad (1)$$

Mechanism II: There is a direct energy transfer from the S_1 level of the ligand to the emitting level of the Ln(III) ion

$$S_1 \rightarrow E_{Ln,e} \rightarrow \text{ground state multiplet} + h\nu_J \quad (2)$$

Mechanism III: There is an energy transfer from the S_1 of the ligand to an upper intermediate level of the lanthanide(III) ion ($E_{Ln,i}$), then back to the T_1 of the ligand to return finally to a lower, emitting level of the

lanthanide(III) ion:

$$S_1E_{Ln,i} \rightarrow T_1 \rightarrow E_{Ln,e} \rightarrow \text{ground state multiplet} + h\nu_J \quad (3)$$

Aromatic Tb(III) and Eu(III) chelates usually follow mechanism I, where the emitting level $E_{Ln,e}$ is normally the resonance level of Ln(III). Mechanism II is usually not efficient due to the short lifetime of singlet states, and an efficient energy transfer generally precludes the presence of an intramolecular triplet state sensitiser, which leads to mechanism I. In addition to this intramolecular energy transfer, aromatic triplet sensitisers can also promote intermolecular energy transfer to the lanthanide ions and chelates. Mechanism III is possible especially for Eu(III), which usually has several low-lying levels between the S_1 and T_1 levels of ligands.

Mechanism I is normally valid for the Ln(III) chelates used as labels in bioaffinity assays [42,62]. This mechanism is often called ligand-sensitised excitation mechanism because the ligand is first excited either by photo-excitation or by a chemical reaction and then it transfers the energy intramolecularly to the central ion finally emitting its specific emission lines.

Porphyrins are a large group of compounds having a tetrapyrrolic structure with side substituents. These compounds have been known for a long time since they widely occur in nature (e.g. chlorophylls, heme, their precursors and some vitamins). A large variety of porphyrins and their derivatives have also been synthesised in the laboratories. Porphyrins form stable complexes with metal ions to form metalloporphyrins. Metalloporphyrins display high quantum yields, intense absorption bands in the 360–400 nm region (Soret band) and in the 500–550 nm region (Q-band) accompanied by red phosphorescence emission (600–750 nm) with Stokes shifts greater than 100 nm, and long luminescence lifetimes (10–1000 μs) [63–65]. These properties allow effective spectral and/or decay resolution of luminescence from background light. The sensitivity in detection is higher if these dyes are excited at Soret band due to the higher molar absorption coefficient in comparison to that of Q-band. In the metalloporphyrin group, Pt(II)- and Pd(II)-porphyrins are of prime interest since they have the highest values of phosphorescence quantum yields.

4.3. Time-resolved photoluminescence ("time-resolved fluorescence")

Time-resolved measurements can be made mainly by two approaches. The first concept is to use sinusoidically modulated excitation with different frequencies (phase-resolved fluorometry) and following either demodulation or phase shift of excitation [66]. The second, perhaps a more common method, especially with slower decaying luminophores, is the use of fast pulsed UV-light source and a gated detection of light after each excitation pulse. The delay time after the nominal excitation UV-pulse end must be sufficiently long to allow the lamp to decay to zero intensity. These principles are discussed in detail with clarifying figures already in modern basic analytical chemistry textbooks, e.g. in Harris textbook *Chemical Quantitative Analysis* [67]. In this text, time-resolved photoluminescence is used instead of TRF, i.e. TR-PL covers all the photoluminescence phenomena that allow the use of time-resolved measurements, irrespectively to the actual luminescence mechanism.

The power of this methodology lies mainly in two principles: (i) The scattering phenomena do not disturb the measurements because the excitation light is off during the emission measurement and (ii) the fluorophores existing in the serum matrix or originating from the buffers usually do not show any phosphorescence. Thus, the use of long-lived PL yielding labels generally induce a vast increase in the S/N ratio in comparison to those obtained with fluorescent labels, whose emission must be measured already during the excitation UV-pulse.

Most important labels for time-resolved luminescence measurements are certain lanthanide chelates that typically have luminescence lifetimes in the range from a few hundred microseconds to around 2 milliseconds [68]. Nicely chelated lanthanides typically show a single-exponential decay that is easy to measure and utilise. They have a large Stokes shift, which makes them also useful with instruments not capable of time-resolved PL measurements. An advantage of these chelates is that they are normally insensitive to the presence of triplet oxygen, which is generally not the case for true phosphorescent probes.

Another group of labels suitable to analogous use is metalloporphyrin labels [69–72], which show true

phosphorescence. Typically, these labels need careful scavenging of the oxygen from the solution prior to as well as during the luminescence measurements. Oxygen concentration in an air-saturated buffer solution is around 0.2 mmol/l at room temperature [73]. The benefit of these probes is that the emission occurs in the red end of visible or NIR range, which is well suited to low-cost semiconductor-based detectors and diode lasers (DL).

The quenching effect of oxygen on metalloporphyrin phosphorescence can also be useful in analytical chemistry. This allows to construct oxygen sensors based on the oxygen-induced change of luminescence lifetime of immobilised metalloporphyrin dyes [74–79]. Also biosensors can be developed on this basis [80,81]. These types of oxygen sensors are developed by a small spin-off company of the University College Cork (http://www.luxcel.com/).

4.3.1. *Delfia*

The oldest TR-PL technology was called Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFIA® [42,68]). In this technology, non-luminescent lanthanide chelates (derivatives of EDTA and DTPA) were used as labels, and after the immunoassay lanthanide(III) ions were dissociated from the original ligands and new luminescent chelates were formed by a development solution prior to TR-PL measurement. Mainly, Eu(III) and Tb(III) ions were used as the central ions of the labels. Most of the important patents in this area are probably no longer valid and this technology is beginning to be available to others rather than Perkin-Elmer Life Sciences alone.

Wallac Oy (Turku, Finland) developed a fully automated version of DELFIA® called AutoDELFIA®, and totally different system called Wallac Aio! (all-in-one) which is based on the use of strongly photoluminescent lanthanide(III) labels and measuring the TR-PL from the surface of single polystyrene sample vials. This technology is presently being commercialised by a small company called Innotrac Anal. Chem., Turku, Finland (http://www.innotrac.fi/). The automated random-access instrument Innotrac AIO Satelite carries out an immunoassay in 18–20 min [82,83]. The instrument seems to be quite expensive (US$ 36,000) to naturally fit in many of the fields of point-of-care (POC).

4.3.2. *Photoluminescence resonance energy transfer*

Energy transfer phenomena can be nicely utilised in the development of fast and sensitive homogeneous binding assays [84]. True fluorescence resonance energy transfer (FRET) using organic luminophores as donors and acceptors is being studied and carried out [85]. However, with FRET only wavelength discrimination is normally available. More powerful methodology can be achieved, if long-lived luminescence displaying luminophores are used as energy donors [86].

Canberra-Packard (Meriden, CT, USA) together with CIS Bio International (Bagnolis, France) have developed homogeneous time-resolved fluorescence (HTRF®) technology, as they call it [87]. A logical name for this technology would have been time-resolved photoluminescence resonance energy transfer (TR-PRET) or less preferably time-resolved fluorescence resonance energy transfer. This technology uses Eu(III) cryptate (chelate) in which the ligand contains 2,2′-bibyridines as light absorbers. This Eu-cryptate is used as a label of one party of a binding assay. One other party of the assay is labelled by an allopchycocyanin related dye (XL665) which emits fluorescence at 665 nm. After excitation by pulsed nitrogen laser, Eu-cryptate shows long-lived PL by its characteristic sharp emission lines $^5D_0 \rightarrow {}^7F_J$, one of which is at 620 nm. When the labelled parties are brought in close proximity by a bioaffinity assay, the excited Eu(III) can transfer energy non-radiatively to the XL665, which can then emit long-lived luminescence at 665 nm by following “by transmission” the relatively long luminescence lifetime of Eu(III) in its excited state $^5D_0$. Fig. 4 displays the emission spectra of these luminophores and the FRET process during the detection step of a binding assay. In the figure, Eu(III) emission is not measured with high spectral resolution, thus the peaks are much sharper in reality.

During the time-resolved measurement of the signal at 665 nm, the time-resolved emission intensity at 620 nm is also measured. The former signal gives information about the concentration of an end product of a binding assay such as an immunocomplex, and the latter signal gives information about the concentration of the unbound label. Thus, instead of absolute signal values the ratio of intensities at 665 and 620 nm can also be utilised, which increases the precision and accuracy of the assays.

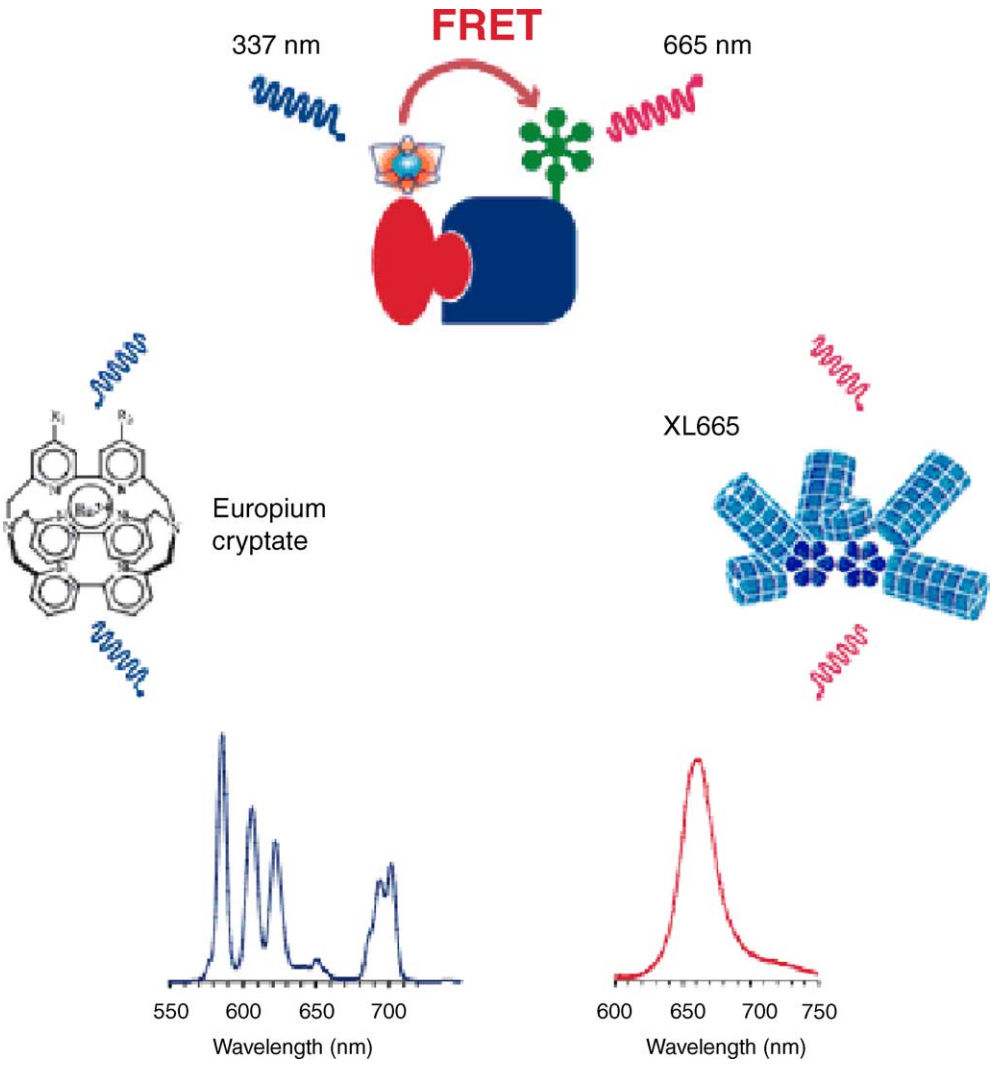

Fig. 4. Eu(III) cryptate used by CIS Bio International as a long-lived PL displaying label, and the resonance energy transfer to the acceptor molecule XL665 in the detection step of a binding assay (http://www.htrf-assays.com/techno/index.htm/). Reproduced with permission from CIS BIO International.

The energy transfer efficiency at a distance of 7.5 nm is said to be approximately 75% and this diminishes extremely rapidly as the distance is increased since the rate of energy transfer depends on the inverse sixth power of the distance between an excited donor and an acceptor molecule. Thus, only a bound XL665-label has a contribution to the time-resolved signal at 665 nm, because the direct fluorescence induced by the excitation light pulse has already decayed before the time-resolved emission measurement is started [86]. Therefore, extremely rapid binding assays in a homogeneous solution phase without any washing steps can be carried out. Time-resolved measurements allow discarding the Raman and Raleigh scattering and also background fluorescence originating from sample matrix. This system is usable not only in vitro diagnostics but also in many types of binding assays used in high throughput screening (HTS) in the development of new drugs. Later, trade marks TRACE® (Time-Resolved Amplified Cryptate Emission) and

HTRF® have been used to denote this type of technology developed for immunoassays, protein–protein interactions, cell surface receptor studies, nucleic acid hybridisation and enzyme assays [86]. Bazin et al. have illustrated their different developments in some quite clarifying figures. The availability of the reagents and technology can be checked on the web: http://www.cisbiointernational.com/Ang/index.htm.

Wallac (Perkin-Elmer Life Sciences) has developed closely analogous assays with other lanthanide chelates and luminophores and their own instrumentation [43,88]. Largely, the same types of applications are possible and already in commercial use. Either Eu(III) or Tb(III) ions are used as the central ions of the long-lived luminescence-generating primary label chelates, and the acceptors are Cy5 and rhodamine, respectively [43,88]. This company has used the name LANCE® (Lanthanide Chelate Exitation Technology) as an umbrella of different developments within their company. The availability of the reagents and instruments and the concepts of LANCE® variants are displayed: http://www.lifesciences.perkinelmer.com/wallac.asp.

4.4. Fluorescence polarisation

Homogeneous bioaffinity assays can be developed also on the basis of fluorescence polarisation (FP) [89–91]. In FP excitation, light is plane-polarised, and large and small molecule interactions enhance or diminish the polarised fluorescence signal. In FP, the polarization of the emitted light depends on how far the luminophore rotates during the lifetime of its excited state. The smaller the molecule, the faster it rotates, and the smaller the FP signal will be. Binding of a fluorophore-labelled ligand to its receptor in a solution or on the surface of the living cell will result in slower rotation and, thus, an increase in FP signal. FP is independent of fluorescence intensity and is more tolerant of fluorescence quenching and light scattering than the traditional methods of fluorescence measurements. FP is a sensitive technology for the determination of molecular size and microviscosity and for monitoring pharmacokinetics in body fluids. At least the following companies utilise this technology: Abbott Laboratories (http://www.abbottdiagnostics.com), Molecular Devices (http://www.moleculardevices.com), Perkin-Elmer, PanVera (http://www.panvera.com) and Jolley Consulting and Research (http://www.jolley.com).

4.5. Fluorescence correlation spectrometry

Fluorescence correlation spectrometry (FCS) is a very sensitive technique for the measurement of fluorescent ligand concentration, and for quantification of ligand–receptor binding in homogeneous assays. For instance, Evotec OAI (Hamburg, Germany; http://www.evotecoai.com) is developing this technology to achieve miniaturised ultra-HTS systems based on confocal nano-fluorescence read-out techniques. In FCS single molecules are measured as they diffuse through the extremely small volume of around 1 fl. Free ligands diffuse through this measurement volume more rapidly than ligand–receptor complexes due to the latter's greater molecular mass. The whole measurement process takes only a few seconds. FCS has recently been thoroughly reviewed [92–94].

4.6. Multi-photon excitation

The term multi-photon excitation means two or more photon excitation processes [95]. In analytical use, typically only two-photon excitation is applied. TPE of luminophores is a non-linear process involving the absorption of two photons, whose combined energy is sufficient to induce a transition to an excited electronic state. Fig. 5 displays a comparison between one- and two-photon absorption [96]. A key feature of two-photon microscopic systems is the limitation of PL excitation to within a femtolitre size focal volume.

To achieve a TPE a high photon flux needs to be delivered to the sample. This is typically achieved with ultrashort pulsed laser excitation. Femtosecond and picosecond laser sources have been used but also continuous wave (cw) lasers. One of the most commonly used laser sources for multi-photon excitation is femtosecond titanium-sapphire laser systems. These are capable of generating a 100 fs pulse train at repetition rates of around 80 MHz. The tuning range of Ti-sapphire laser extends from 7000 to 1000 nm. Other commonly used femtosecond laser sources are CrLiSAF and pulse-compressed Nd:YLF lasers, and in the picosecond range, mode-locked Nd:YAG and pulsed-dye lasers [96].

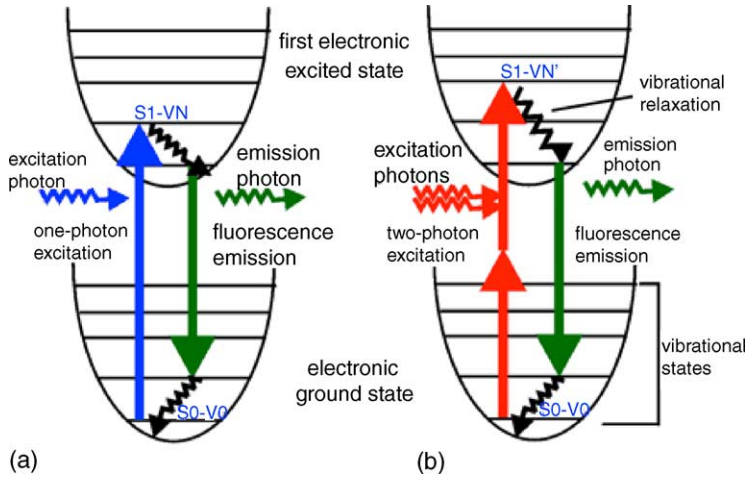

Fig. 5. Jablonski diagram for one-photon (a) and two-photon (b) excitation. Excitations occur between the ground state and the vibrational levels of the first electronic excited state. One-photon excitation occurs through the absorption of a single photon. Two-photon excitation occurs through the absorption of two lower-energy photons via short-lived intermediate states. After either excitation process, the fluorophore relaxes to the lowest energy level of the first excited electronic states via vibrational processes. The subsequent fluorescence emission processes for both relaxation modes are the same [96]. Reproduced with permission from Annual Reviews.

In general, most luminophores can be excited in two-photon mode at twice their one-photon absorption maximum. However, one- and two-photon absorption processes have different quantum mechanical selection rules. Thus, a luminophore's two-photon excitation spectrum shape is not necessarily equivalent to its one-photon excitation spectrum.

A sensitivity necessary for detection of single molecules by PL has become more or less routinely achieved in recent years [97]. Although most of the single molecule work has used one-photon excitation, TPE can offer improvement in the signal-to-background ratio, owing to the excitation volume localisation and the wide spectral separation of the emission, excitation and Raman bands. The first demonstrated single-molecule detection in an aqueous solution was carried out using Rodamine B in 1995 [98]. Single molecules have been detected by TPE both in free solution [99,100] and in flow cells [101].

Soini and co-workers have developed methods for detecting biomolecules by TPE [102–104]. In their method, microparticles are used as solid phase in binding the target molecules. The degree of binding is then quantified from individual microparticles by use of two-photon excitation of PL. They have demonstrated the effectiveness of the TPE method (called TPX by them) using the human α-fetoprotein (AFP) immunoassay. The sensitivity and dynamic range obtained with this assay suggests that this method can provide a cost-effective and simple way to measure various biomolecules in solution [102,103]. Typically, they use Q-switched Nd:YAG laser emitting 1 ns pulses at 1064 nm, and the detection is normally based on CPMs. When a microparticle appears in the focal volume, the confocally arranged scattering detector monitors the arrival of the particle. Then the observed scattering signal triggers the stopping of the flow and switches the PL or TR-PL measurement on. At least 10–100 particles have to be measured to obtain statistically sufficient values for calculation of the analyte concentration. Fig. 6 shows the detection principle on the moment of a particle arriving to the focal volume around 1 fl.

The microparticles may be encoded with different photoluminescent dyes as explained above, and thus separation-free multiparametric assays are possible on the basis of single assay incubation step.

Another homogeneous method applicable with the same instrumentation is being developed by the same group. This coincidence particle assay is based on a formation of particle-particle pairs (or particle–luminophore pairs as well) [52]. The analyte

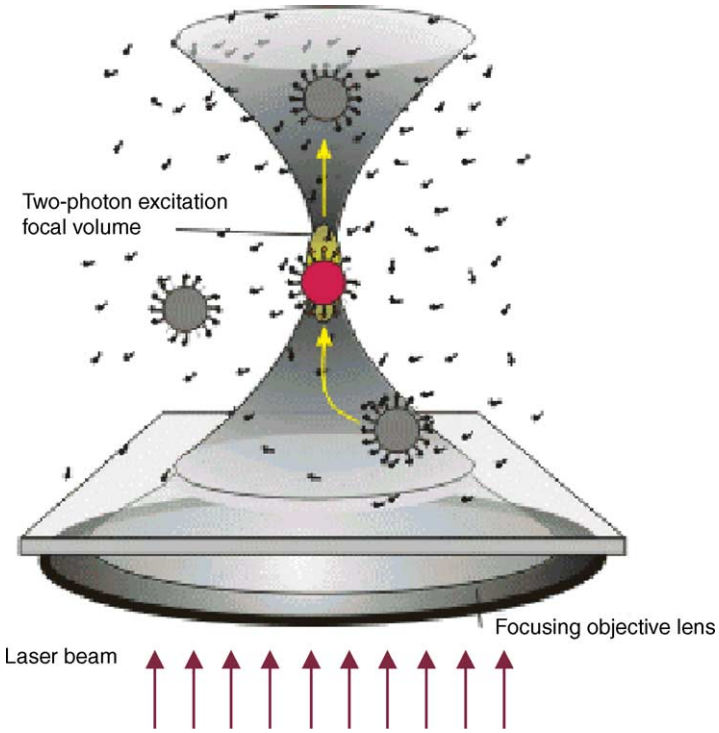

Fig. 6. A latex particle moving through the detection volume during a two-photon excitation.

concentration is measured by detecting the presence of particle pairs, each having a different photoluminescent encoding. For example, one particle is coated with one antibody and the other particle is coated with a different monoclonal antibody having an affinity to the same biomolecule but to a different epitope. When the analyte is trapped between the two particles, the particles are bound together to form a pair. If only one of the particles is observed (only one type of emission) no analyte was present in the focal volume, but when both of the emissions are observed simultaneously in the focal volume an analyte molecule is detected. These methods are being commercialised by Arctic Diagnostics Oy, Turku, Finland (http://www.arcticdiagnostics.fi).

4.7. Solid labels

If solid particles are sufficiently small and their other chemical properties do not interfere, they can be used as labels instead of single molecules. The solid state quite easily provides bases for phosphorescence due to the principles of photophysics, and thus wider variety for labelling in time-resolved PL format can be naturally expected. Of course fluorescence is useful as well if the background signal is not problematic for the sensitivity range required.

Chan and Nie [105] have reported the use of CdSe quantum dots in biochemical analysis. They state that the emission brightness of their single nanoparticles corresponds to that of approximately 20 rhodamine molecules. Quantum Dot Corporation, Hayward, USA, has commercialised this technology (http://www.qdots.com). Quantum dot-encoded larger carrier particles are available from the former company and Crystaplex Corporation, Pittsburgh, USA (http://www.crystalplex.com).

Beverloo et al. [56] introduced the use of Eu(III)-doped yttrium oxysulfide phosphorus particles as labels, and on the same basis, plenty of inorganic lanthanide(III) phosphorus particles can be selected for potentially good label materials for TR-PL

detection. Latex particles containing lanthanide(III) chelates can also be efficiently used on the same basis [106,107]. Also, metalloporphyrins can be incorporated inside the particles offering an alternative label particle branch for similar time-resolved usage. Commercial porphyrin-doped particles have been available at least from Molecular Probes, Leiden, The Netherlands.

Lanthanide(III)-containing inorganic or organic nanoparticles probably allow the analogous use already explained in connection to TR-PRET. In this case, the nanoparticle is a long-lived PL displaying label and the acceptor is a suitable dye molecule as in the cases of HTRF® and LANCE® discussed above. This technology is going to be commercialised by Innotrac Oy, Turku, Finland (http://www.innotrac.fi) [52].

An interesting method based on photochemical excitation has been created with the aid of microparticles [108]. This method involves singlet oxygen generation from a photosensitiser and time-resolved detection of CL at a shorter wavelength than is used to photogenerate the primary reactants and is called AlphaScreen™ (Amplified Luminescence Proximity Homogeneous Assay). This assay format was commercialised by the former Packard Instruments, presently Perkin-Elmer Life Sciences (http://www.packardinst.com/packardbioscience.asp).

5. Electrochemiluminescence

Electrochemiluminescence is treated here as chemiluminescence produced directly or indirectly as a result of electron transfer between an electrode and some solution species or species bound to the electrode surface. Thus, electrochemiluminescence as a term is considered to have no difference with the term electrogenerated chemiluminescence. As a final luminophore-exciting step, ECL can have (i) a heterogeneous electron transfer between the luminescent species and an electrode or (ii) a homogeneous electron transfer between a solvated species and the luminescent species. In analytical chemistry, ECL can be used for determination of electrochemiluminescent compounds. Also, ECL can be used for detection of other compounds of high practical interest. This technique employs electrochemiluminescent labels in analogous protocols developed earlier for bioaffinity assays utilising radioactive, photoluminescent or chemiluminescent labels.

For bioaffinity assays, it is essential that an aqueous medium can be used, and the methods that cannot tolerate the presence of water traces are practically of no value. Therefore, the classical ECL of organic compounds in aprotic solvents is not generally applicable in bioaffinity assays. On the other hand, employment of an aqueous solution severely restricts the potential window to quite a narrow region if the traditional electrochemistry and conventional metal electrodes are used. However, for some luminophores the conventional electrode materials are also applicable, especially if appropriate radical-forming coreactants are used. At present, the use of semiconductor electrodes already widens the available potential window or at least offers more attractive potential window edges. However, the widest potential window known so far can be obtained if thin insulating film-coated cathodes are used together with traditional metal or oxide anodes. The wider the obtainable potential window is, the more energetic emissions are possible.

ECL, in common with PL, has many advantages in bioaffinity assays over the other existing bioaffinity assay detection techniques. For both ECL and PL, the labels are very stable and have long shelf lives, and their handling and disposal are easy in comparison to radioactive tracers. The dynamic range of determination of the labels typically spans over several orders of magnitude in concentration, and the determination limits are extremely low. Normally, e.g. the immunoreactivity of antibodies remains intact if five to six or fewer label molecules are conjugated with each labelled antibody.

The commercially utilised anodically produced ECL methods based on $Ru(bpy)_3^{2+}$ labels (discussed in Section 5.3) do not easily allow the use of multiple labels or internal standards. On the contrary, the newest available ECL technology, hot electron-induced ECL, is expected to provide a better basis for internal standardisation and for multilabel assays than the other presently known ECL methods. A special advantage of the hot electron injection into aqueous solution from tunnel emission electrodes is that this method enables wavelength and time discrimination or, even their combination, in efficient separation of the signals emerging from different

labels. The time-resolved electrogenerated chemiluminescence (TR-ECL) of aromatic Tb(III) chelates at thin insulating film-coated electrodes allows a sensitive detection of Tb(III), along with biological compounds using aromatic Tb(III) chelates as labels in bioaffinity assays. The obtained low LODs are mainly based on the long luminescence lifetime of chelated Tb(III) and the use of time-resolved measuring techniques in connection with pulsed electrical excitation of the label chelates.

5.1. Electrochemiluminescence mechanisms

General ECL reviews have been published quite frequently [29–34], and therefore only the essentials of the ECL principles and theory are dealt with here. Here the emphasis is put on the commonly used luminescent labels and their ECL. Quite often, ECL excitation pathways are very closely related to the basic PL excitation mechanisms.

In principle, e.g. for aromatic compounds (Ar) the above-mentioned two excitation pathways, *ox–red* and *red–ox* excitation routes, equations can be suggested:

$$\mathrm{Ar} \rightarrow \mathrm{Ar}^{+} + \mathrm{e}^{-} \quad (4a)$$

$$\mathrm{Ar}^{+} + \mathrm{e}^{-} \rightarrow \mathrm{Ar}^{(1\,\mathrm{or}\,3)*} \quad (4b)$$

$$\mathrm{Ar} + \mathrm{e}^{-} \rightarrow \mathrm{Ar}^{-} \quad (5a)$$

$$\mathrm{Ar}^{-} \rightarrow \mathrm{Ar}^{(1\,\mathrm{or}\,3)*} + \mathrm{e}^{-} \quad (5b)$$

It is assumed that the working electrode is rapidly pulsed between anodic and cathodic potential by a potentiostat following a pulse generator. It is noteworthy that a direct triplet state excitation can be obtained via redox reaction at room temperature [109–112].

However, in practice the ECL mechanisms are generally not as simple as described above, due to the usage of aqueous solutions. In aqueous solution, the usable potential window for conventional metal electrodes is restricted by cathodic hydrogen evolution and anodic oxygen evolution reactions and their overvoltages [113]. Hence, the usable potential window normally would allow one to obtain excitation with emission only in the IR range. To avoid this, coreactants that produce highly reducing radicals upon oxidation or highly oxidising radicals upon reduction can be used in some cases. Another problem is that one-electron oxidised or reduced luminophores are normally extremely unstable having very short lifetimes in aqueous solutions.

At present, the most important ECL label molecules are the derivatives of ruthenium(II) tris-(2,2′-bipyridine) chelate ($\mathrm{Ru(bpy)_3}^{2+}$). Its PL properties, photophysics and photochemistry have been discussed several times in great detail [114,115]. It is of course also usable as a photoluminescent label, and its ^{3}MLCT excited states decay sufficiently slowly to allow time-resolved phosphorescence (TR-P) measurements with suitable instruments. Single molecules have been detected by using a derivative of this chelate as a label by TR-P [116].

This chelate and its derivatives are normally excited using an *ox–red* pathway in which tripropyl amine or some other aliphatic amine is used as a coreactant. At an appropriate pH, this results in the production of a highly reducing radical by a deprotonation of the one-electron oxidation product of this coreactant [117]. The *ox–red* excitation scheme is as follows:

$$\mathrm{R{-}H_2C{-}NR'R''} \rightarrow \mathrm{R{-}H_2CN^{\bullet}} + \mathrm{R'R''} + \mathrm{e}^{-}\ (\text{to anode}) \quad (6a)$$

$$\mathrm{R{-}H_2CN^{\bullet}} + \mathrm{R'}R'' \rightarrow \mathrm{R{-}HC^{\bullet}N : R'R''} + \mathrm{H}^{+} \quad (6b)$$

$$\mathrm{Ru(bpy)_3}^{2+} \rightarrow \mathrm{Ru(bpy)_3}^{3+} + \mathrm{e}^{-}\ (\text{to anode}) \quad (6c)$$

$$\mathrm{Ru(bpy)_3}^{3+} + \mathrm{R{-}HC^{\bullet}N : R'R''} \rightarrow \mathrm{Ru(bpy)_3}^{2+*} + \mathrm{R{-}CH{=}NR'}R'' \quad (7)$$

$$\mathrm{Ru(bpy)_3}^{2+*} \rightarrow \mathrm{Ru(bpy)_3}^{2+} + h\nu \quad (8)$$

At the first step, amine is one-electron oxidised and deprotonated simultaneously with oxidation of $\mathrm{Ru(bpy)_3}^{2+}$ (reactions (6a)–(6c)). Second, the resulting radical reduces $\mathrm{Ru(bpy)_3}^{3+}$ to $\mathrm{Ru(bpy)_3}^{2+}$ in its ^{3}MLCT excited state, which finally emits light at around 620 nm (reactions (7)–(8)) [118,119]. However, in parallel, a *red–ox* excitation pathway may occur, i.e. Eqs. (9) and (10) followed by (8):

$$\mathrm{Ru(bpy)_3}^{2+} + \mathrm{R{-}HC^{\bullet}N : R'R''} \rightarrow \mathrm{Ru(bpy)_3}^{+} + \mathrm{R{-}CH{=}NR'R''} \quad (9)$$

$$\mathrm{Ru(bpy)_3}^{+} \rightarrow \mathrm{Ru(bpy)_3}^{2+*} + \mathrm{e}^{-}(\text{to anode}) \quad (10)$$

An excited reaction product is only formed if the anode potential is sufficiently positive.

This type of ECL is normally generated with potentiostatic control using a conventional three-electrode cell [117–121]. When amines are used as coreactants the working electrode material is usually gold or platinum. The electrodes are normally reusable (non-disposable). However, our preliminary results have shown that either disposable carbon or transparent indium tin oxide-coated (ITO-coated) glass or plastic electrodes are also applicable with this chemistry for designing low-cost, single-use cells, e.g. for point-of-care or physician's office analysis.

The *red–ox* excitation pathway using peroxodisulfate as coreactant with noble metal electrodes is applicable in a mixture of acetonitrile and water [120,121], but not in a fully aqueous solution. Apparently, this is due to the practically concerted two-electron reduction of peroxodisulfate at these electrodes and very short lifetime of the one-electron reduced chelate [120,121]. However, the *red–ox* excitation pathway is applicable in cases of a hot electron injection into an aqueous solution (see below). The *red–ox* excitation pathway includes generation of a sulphate radical that is one of the strongest ($E^\circ = 3.4$ V versus SHE) known one-electron oxidants [122]:

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{-} + SO_4^{2-} \quad (11)$$

$$Ru(bpy)_3^{2+} + e^- \rightarrow Ru(bpy)_3^{+} \quad (12)$$

$$Ru(bpy)_3^{+} + SO_4^{-} \rightarrow Ru(bpy)_3^{2+*} \quad (13)$$

Also in this case, a parallel *ox–red* excitation pathway of the chelate may occur. It is described with Eqs. (14)–(16) followed by (8):

$$Ru(bpy)_3^{+} + S_2O_8^{2-} \rightarrow Ru(bpy)_3^{2+} + SO_4^{-} + SO_4^{2-} \quad (14)$$

$$Ru(bpy)_3^{2+} + SO_4^{-} \rightarrow Ru(bpy)_3^{3+} \quad (15)$$

$$Ru(bpy)_3^{3+} + e^- \rightarrow Ru(bpy)_3^{2+*} \quad (16)$$

An additional comproportionation excitation pathway is in principle possible, but in bioaffinity assays it is typically insignificant:

$$Ru(bpy)_3^{+} + Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+} \quad (17)$$

It has been recently demonstrated that the lifetime of $TPA^{\bullet+}$ cation radicals is much longer than previously assumed [123], which at least partially explains the anomalities of Igen's ORIGEN® technology, based on this chemistry, and the use of quite large (either 2800 or 4500 nm) optically non-transparent paramagnetic beads as a site of their assays [117]. The applications of this technology are discussed below in Section 5.3. In this technology, an immunometric immunoassay is carried out on the surface of the above-mentioned beads, and then the beads are collected for ECL detection on a gold disc electrode by a magnetic field (which also keeps the beads in a rigid position) to be followed by an ECL measurement from above.

Bard and his students explain that $TPA^{\bullet+}$ under the excitation conditions has a half life of 0.2 s [123]. Thus, $TPA^{\bullet+}$ would have sufficient lifetime to diffuse around the beads, and these species being sufficiently strong oxidants, they can one-electron oxidise the label chelates all around the beads. Now, the relatively stable Ru(III) species [115] just sits and waits until a deprotonoted $TPA^{\cdot}$ radical encounters it within a tunnelling distance and excites it by reaction (7).

In a particular system, the energetics of the luminophore and the other intermediates along with the obtainable electrode potentials determine the possibility of the occurrence of the emission in accordance with the basic thermodynamics. The enthalpies of the excitation steps of each pathway can be evaluated using the equation:

$$-\Delta H^\circ = -\Delta G^\circ - T\,\Delta S^\circ = E^\circ(Ox^{\bullet+}/Ox) - E^\circ(Red/Red^-) - T\Delta S^\circ \quad (18)$$

where $E^\circ(Ox^{+\bullet}/Ox)$ and $E^\circ(Red/Red^-)$ denote the standard potentials of the oxidant and the reductant of the excitation step (or electrode potential, if the heterogeneous electron transfer occurs), respectively, and the other symbols have their usual meanings. The entropic term of Eq. (18) is about 0.16 eV [124] (9). If the calculations show that the $-\Delta H^\circ$ value in the excitation step is higher than the energy difference between the luminophore ground and excited states, the excitation is possible on the basis of energetics. Juris et al. [115] summed up the luminescence and redox properties of $Ru(bpy)_3^{2+}$ (Fig. 7).

Anodically induced ECL of $Ru(bpy)_3^{2+}$ is analytically quite versatile, and its use is typically based either on the use of its suitable derivative as

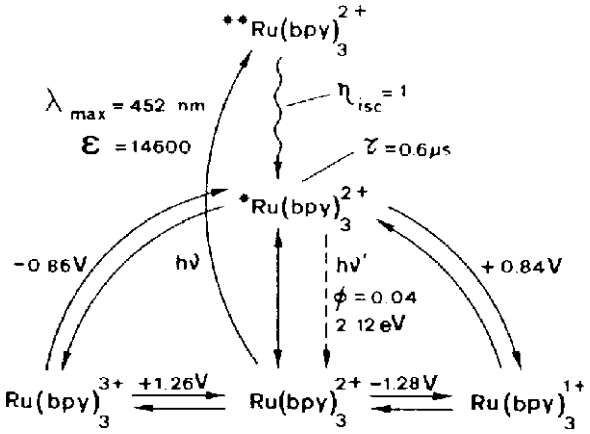

Fig. 7. The properties of $Ru(bpy)_3^{2+}$ in a nut shell [115]. Juris et al. [115] have denoted the metal-to-ligand charge transfer excited triplet state (^{3}MLCT) with one asterisk and the excited singlet state (^{1}MLCT) with two asterisks. Reprinted from [115], Copyright 1988, with permission from Elsevier.

electrochemiluminescent label, or the analyte replacing the TPA's role in the above excitation pathways (mainly different types of amines or amino groups-containing molecules can be determined [34,35]). In addtion, $Ru(bpy)_3^{3+}$ has such a long lifetime in aqueous solutions that in flow analysis systems it can be generated anodically elsewhere and just joined and mixed to the analyte stream in the detection zone. In this contribution we are, however, only dealing with labelling applications of this chelate, except in the section considering novel applications of capillary electrophoresis (CE).

An alternative system that often has a higher technical simplicity for certain applications is based on the use of cathodically pulse-polarised disposable thin insulating film-coated electrodes such as oxide-covered aluminium or silicon electrodes combined with almost any counter electrode. According to previous studies, strongly oxidising species such as sulphate and hydroxyl radicals can be cathodically generated in fully aqueous solutions on pulse-polarized oxide-covered aluminium electrodes [125–127]. As a primary step, the cathodic pulse polarization of thin oxide film-covered aluminium electrodes induces a tunnel emission of hot electrons (e_{hot}^-) into an aqueous electrolyte solution. This apparently results in a subsequent generation of hydrated electrons (e_{aq}^-) and oxidising radicals (such as sulphate radicals $SO_4^{\bullet -}$) from added coreactants [128,129]. Hot and hydrated electrons can react with compounds very hard to reduce. Therefore, cathodic reductions can occur that are usually impossible in aqueous solutions [130]. Similar phenomena have also been observed with oxide-covered silicon electrodes [131]. During the high amplitude cathodic pulse polarization, apparently not every emitted hot electron reacts at the aluminium oxide/solution interface with solute species. If tunnel-emitted electrons have enough energy, they may enter the conduction band of water and turn into hydrated electrons as a result of the thermalisation and solvation processes [132,133]. This precludes that the concentration of the coreactant added to produce oxidising radicals is not too high and electron species are not too efficiently scavenged by oxidising radical precursors, such as peroxodisulphate ions, hydrogen peroxide or molecular oxygen. These react with hydrated or presolvated hot electrons, at or near diffusion controlled rate, and produce highly oxidising sulphate or hydroxyl radicals upon one-electron reduction [134]. Hence, highly reducing and oxidising conditions are simultaneously achieved in the vicinity of the electrode surface by appropriate selection of the cathodic coreactant concentration [125–130]. $Ru(bpy)_3^{2+}$ based labels can be efficiently excited in fully aqueous solutions by using hot electron chemistry in the presence of peroxodisulphate ions [135]. The special feature of hot electron injection into aqueous solution is that luminophores having very different optical and redox properties can be simultaneously excited (Fig. 8). The mechanism of tunnel emission of hot electrons into aqueous solution is described in detail elsewhere [128–131].

The scheme in Fig. 1 is also applicable for metal chelates and some metal ions, providing that HOMO levels are taken as occupied low-energy orbitals and LUMO levels as unoccupied high-energy orbitals in ground state species. In the case of $Ru(bpy)_3^{2+}$, its one-electron reduced form, $Ru(bpy)_3^{+}$, has an additional electron in the ligand centred orbital. This makes the ligand reactive with water and determines a very short lifetime of this species in comparison with the one-electron oxidised form of $Ru(bpy)_3^{2+}$. $Ru(bpy)_3^{3+}$ lacks an electron at a metal centred orbital due to a true oxidation state change of the central ion. This species is quite stable in an aqueous environment [115]. Therefore, for some applications, $Ru(bpy)_3^{3+}$

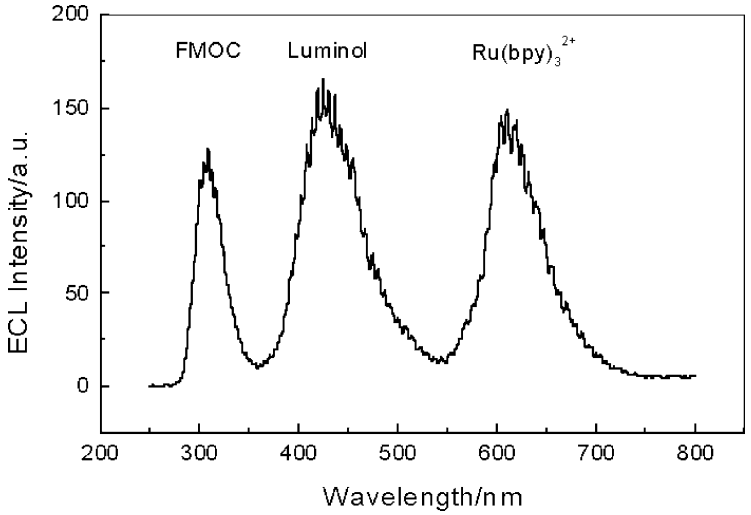

Fig. 8. ECL spectrum of a solution containing 1 μM 9-methyl chloroformate (FMOC, emits at about 309 nm), 10 μM luminol (emits at about 425 nm) and 0.1 μM $Ru(bpy)_3^{2+}$ (emits at about 620 nm). An oxide-covered Al-plate working electrode and Pt-wire counter electrode were used in a 1 cm quartz cuvette equipped with electrode supports made from PTFE. Conditions: 0.2 M borate buffer, pH 9.2, 0.5 mM $K_2S_2O_8$, pulse charge 120 μC, pulse voltage 40 V, pulse frequency 40 Hz. All measurements were carried out with a Perkin-Elmer LS 5 luminometer.

can be electrochemically generated outside the luminescence measuring cell and then transported through tubing toward desired area [136].

An injection of hot electrons into an aqueous solution provides a possibility to excite aromatic lanthanide chelates electrochemically by a ligand-sensitised mechanism [125–127,137]. The ligand is excited by redox reactions, and it transfers energy to the central ion that finally emits due to its typical f–f transitions. However, this is directly applicable only for Tb(III) chelates.

Of aromatic lanthanide chelates, those of Tb(III), Eu(III), Sm(III), Dy(III) and Yb(III) are applicable as labels. Currently, Tb(III) phenolic chelates are the best labels known [137–140]. Tb(III) is very redox inert [141] and has its resonance level at an appropriate energy for the energy transfer to occur from the triplet states of various aromatic compounds [141,142]. In addition, the luminescence lifetime of the Tb(III) ion that is well-screened from water molecules by a multidentate ligand is of the order of 1.7–2.4 ms, thus allowing the efficient use of time-resolved measuring techniques [125–127]. Time-resolved ECL measurements provide excellent signal-to-noise ratio and about the same sensitivity of detection as the time-resolved PL of lanthanide(III) chelates. The benefits of ECL appear mainly in development of small-sized low-cost devices for POC or doctor's office use. An ECL apparatus presently requires simple electronics costing less than US$ 100 instead of the UV-flash lamp system necessary in TR-PL that costs around US$ 2000. However, the competition situation will be quite different when pulsed diode lasers emitting sufficiently short wavelengths become commercially available at sufficiently low price.

The ECL excitation mechanisms for Tb(III) chelates are as follows [125–127,137]:

$$\mathrm{Tb(III)} - L + \mathrm{Ox}^{\bullet} \rightarrow \mathrm{Tb(III)} - L_{\mathrm{ox}} + \mathrm{Ox}^{-} \quad (19)$$

$$\mathrm{Tb(III)} - L_{\mathrm{ox}} + \mathrm{e}_{\mathrm{quasi\ free}}{}^{-}\ (\mathrm{or}\ \mathrm{e}_{\mathrm{aq}}{}^{-}) \rightarrow \mathrm{Tb(III)} - L^{1*} \quad (20)$$

$$\mathrm{Tb(III)} - {}^{1}L^{*} \rightarrow \mathrm{Tb(III)} - {}^{3}L^{*} \quad (21)$$

$$\mathrm{Tb(III)} - {}^{3}L^{*} \rightarrow^{*} \mathrm{Tb(III)} - L \quad (22)$$

$$^{*}\mathrm{Tb(III)} - L \rightarrow \mathrm{Tb(III)} - L + h\nu_J \quad (23)$$

where L is an aromatic multidentate ligand, and $\mathrm{Ox}^{\bullet}$ is a either hydroxyl or sulphate radical.

Other luminescent lanthanide ions cannot be considered as redox inert [141]. In particular, a relatively easy reducibility of Eu(III), Sm(III) and Yb(III) causes

problems since the emission intensity and luminescence lifetime of their chelates are poor in comparison to those of Tb(III).

Luminol is well known to be excited anodically at platinum and carbon electrodes [143,144], and also cathodically at either ITO electrodes [145] or thin insulating film-coated electrodes [146]. The ECL of luminol is apparently among the first observations of an ECL phenomenon. Harvey [147] was the first to study the ECL of luminol. Epstein and Kuwana published a series of important papers in the 1960s [148]. Later, the anodic luminol ECL was studied at the rotating ring-disc electrode by Haapakka and Kankare [149]. A few years later, Sakura and Imai [143,144] found that the ECL mechanism is different at around 0.7 V versus SCE, and at potentials above 1 V versus SCE at the glassy carbon working electrode.

Luminol chemiluminescence (CL) mechanisms are relatively complex. Luminol normally exhibits a strong CL only in alkaline solutions. The pK_{a1} and pK_{a2} of luminol are 6.2–6.7 and 15.1, respectively [150–152]. Hence, the luminol CL is efficient only if the luminol's singly deprotonated form, LH^- predominates. The chemiluminescence pathway is usually commenced by a hydrogen atom abstraction (24), or by a one-electron oxidation followed by a rapid deprotonation of the formed $LH^\bullet$ radical (25) (pK_a of $LH^\bullet$ is 7.7) [153–156]:

$$LH^- + Ox^\bullet \rightarrow L^{\bullet-} + Hox \quad (24)$$

$$LH^- + Ox^\bullet \rightarrow L^{\bullet-} + H^+ + Ox^- \quad (25)$$

where $Ox^\bullet$ is a one-electron oxidant or an oxidising electrode. $L^{\bullet-}$ radicals can either disproportionate to yield LH^- and diazaquinone (L) (26), or they can be oxidised for the second time by a one-electron oxidation to yield L (27).

$$L^{\bullet-} + L^{\bullet-} + H^+ \rightarrow LH^- + L \quad (26)$$

$$L^{\bullet-} + Ox^\bullet \rightarrow L + Ox^- \quad (27)$$

In the presence of oxyradicals or hydrogenperoxide, an endoperoxide species, $LO_2{}^{2-}$ or LO_2H_2, respectively, is produced as a result of the reaction of $L^{\bullet-}$ with a superoxide radical (28) or the reaction of L with a hydrogen peroxide (29)

$$L^{\bullet-} + O_2{}^{\bullet-} \rightarrow LO_2{}^{2-} \quad (28)$$

$$L + H_2O_2 \rightarrow LO_2H_2 \quad (29)$$

After this, $LO_2{}^{2-}$ or LO_2H_2 eliminates molecular nitrogen (or LO_2H^-, pK_{a1} and pK_{a2} of LO_2H_2 being 10.4 and 16, respectively) [157], generating excited 3-aminophtalate dianion (AP^{2-*}) which finally emits light (30, 31).

$$LO_2{}^{2-} \rightarrow N_2 + AP^{2-*} \quad (30)$$

$$AP^{2-*} \rightarrow AP^{2-} + h\nu \quad (425\,nm) \quad (31)$$

In the cathodically generated ECL, hydrogen peroxide is usually added as a coreactant that produces the hydroxyl radical needed for the first common step of CL routes (24, 25) and for the reaction (27) to produce diazaquinone, L. In the presence of oxygen, its one-electron reduction supplies a superoxide radical for the reaction (27). The reaction (29) can occur due to the presence of hydrogen peroxide. A superoxide radical is too weak as an oxidant (formal reduction potential 0.75 V versus SHE at pH 9) [113] to initiate the CL pathway by reactions (24, 25) [E° ($LH^\bullet/LH^-$) = 0.87 V versus SHE, E° ($L/L^{\bullet-}$) = 0.24 V] [158]. However, a hydroxyl radical having a reduction potential range from 2.2 to 1.8 V versus SHE at pH from 7 to 14 [113] is a sufficiently strong one-electron oxidant. Therefore, the luminol ECL can be formed by at least two parallel CL pathways, both requiring the presence of hydroxyl radical as a trigger. On the other hand, for the anodically generated ECL, an electrode itself can serve as oxidant in steps (25) and (27). Alternatively, water and hydroxide ions can serve as sources of hydroxyl radical, hydrogen peroxide and superoxide radical, if sufficiently high anodic potentials are used at platinum or gold electrodes, which are oxide-covered under these conditions.

Pastore et al. [159] have recently studied the ECL behaviour of luminol/H_2O_2 and luminol/O_2 systems at Pt electrode by using different electroanalytical techniques such as chronoamperometry, cyclic and rotating disk electrode voltammetry in addition to ECL measurements, and Itagagi et al. [160] by electrochemical impedance spectroscopy.

During the last few years, the ECL of luminol has been used quite frequently in flow injection analysis (FIA) for determination of various compounds and metal ions which can be coupled one way or another with the luminol ECL mechanisms [161–167]. ECL of luminol has also been utilised to reveal defects in non-conductive metal coatings [168]. Also, biosensors

for determination of cholesterol, glucose and related substrates producing H_2O_2 based on the ECL FIA have been constructed and studied [169–171].

Low-cost screen-printed electrodes that can be used in disposable biosensors have been fabricated and studied [172–175]. In addition, some other inexpensive methods for making new type of electrodes for generation of ECL from luminol have been tested and investigated [175–177]. The use of luminol and its derivatives as labels is considered in the next chapter.

5.2. Organic compounds and metalloporphyrins as labels

Hot electron electrochemistry allows excitation of a variety of organic luminophores such as derivatives of fluorescein and coumarines, methyl chloroformate or water soluble naphthalene derivatives that emit in the range from UV to NIR and have very different redox properties [178,179]. In principle, many of the common labels in fluoroimmunoassay methodology are anticipated to be applicable as ECL labels excitable by the hot electron electrochemistry. However, examples of actual immunoassays utilising the organic labels in such way have not yet been described in the literature.

Derivatives of isoluminol and luminol are the most popular pure organic labels that are currently applied in ECL, but acridine derivatives lucigenin and acridinium esters have recently also been used in ECL methods.

Relatively recently, a synthesis and an ECL study of a new derivative, *N*-(β-carboxylpropionyl)luminol (CPL), has been published [180]. CPL can be coupled to a hydroxyl or amino group due to the presence of a carboxyl group. The linear response range of CPL is shown to be 10 pM to 400 nM, with R.S.D. of 5.8% for 5 nM of CPL [180].

Campbell and Patel have described the synthesis of ABEI and its isothiocyanate derivative in detail, as well as the conjugation procedure of SCN-ABEI with proteins [181,182]. In contrast to luminol, ABEI labels do not markedly lose their chemiluminescence efficiency if conjugated with proteins. Arai et al. [183] have carried out electrochemiluminoimmuno assays with a flow injection analysis system using ABEI-isothiocyanate as a label. The LOD of ABEI alone was 6 fmol (S/N = 2), and R.S.D. was 1.7% at 1.5 pmol (n = 10). The authors used a labelled anti-hIgG–hIgG system as a model and demonstrated that the formation of immunocomplexes had an ECL enhancing effect. The new homogeneous immunoassay method has a better performance than either single-radial immunodiffusion or nephelometric immunoassay methods [183].

Yang et al. [184] have shown recently that ABEI could be detected in the range of 6.5 pM to 1.3 μM, and a LOD of 2.2 pM (S/N = 3) for ABEI was obtained when oxidised at a +1.0 V (versus Ag/AgCl) potential in alkaline solution. ABEI was also used as an oligonucleotide marker to label a DNA probe. The intensity of the ECL was linearly related to the concentration of the complementary sequence in the range 96 pM to 96 nM, and the LOD was 30 pM.

Yoshimi et al. [145] used luminol that was coupled to antibodies using glutaraldehyde, and showed an enhancement of the cathodically generated ECL at ITO electrodes caused by immunocomplex formation. At certain cathodic conditions, luminol was observed to be electrochemically inert, and the ECL generation was based on the generation of hydroxyl radicals from hydrogen peroxide [145]. Luminol induces strong chemiluminescence in the presence of either hydroxyl or sulphate radicals [185,186] and luminol, ABEI and AHEI can be sensitively detected by hot electron chemistry [146].

Although luminol CL and ECL has been investigated for a long time, we believe that luminol still has an unknown cathodic excitation pathway which needs to be studied, because it has been observed to show CL in highly reducing conditions in the absence of any apparent oxidants [187,188].

Littig and Nieman have shown that acridinium esters can be excited by electrochemically producing hydrogen peroxide from dissolved oxygen, the optimal pH being just below 12. The working curve dynamic range covered four decades in concentration and the LOD for acridinium ester-labelled lysine was 10 fmol [189]. Later, Lin and Yamada [190] showed that the ECL of methyl-9-(*p*-formylphenyl) acridinium carboxylate fluorosulfonate (MFPA) at platinum electrode (2.5 V versus SCE) was affected by the presence of minute amounts of chloride and hydrogen peroxide in the solution. A positive square wave pulse was exerted on the reaction cell, which allowed MFPA to be determined in the range 1.0–400 nM and the detection limit was reported to be 0.21 nM.

The application of this method to immunoassay was also demonstrated by the determination of human gonadotropin using MFPA as label. A reaction mechanism study suggested that MFPA decomposed in alkaline aqueous solution containing hydrogen peroxide to 4-hydrobenzaldehyde and *N*-methylacridone. The latter could be oxidised to form an excited state molecule at the surface of the electrode. Other acridinium ester studies have been carried out by Wilson et al. [191] and Yang et al. [192]. Wilson proposes that in their case electrochemical oxidation of the acridan 2′,6′-difluorophenyl 10-methylacridan-9-carboxylate produces the corresponding acridinium ester, which reacts with hydrogen peroxide forming a dioxetanone intermediate. Decomposition of this dioxetanone generates light at 430 nm. At pH 8.0 and a hydrogen peroxide concentration of 10 mM, light emission from the ECL reaction was used to determine the acridan concentration with a LOD of 54 pM. Thus, acridan esters were considered to be useful labels in ECL immunoassays and nucleic acid assays.

Lucigenin (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate) has been found out to be cathodically excitable at about −0.5 V versus Ag/AgCl at glassy carbon electrodes [193] and at around −0.65 V at platinum electrodes [194]. This cathodic ECL has been also studied at modified gold electrodes [195]. In this study, the ECL intensity increased in the micellar solutions considerably. The light emitters are *N*-methylacridone and the excited lucigenin [193].

Wilson et al. [196] have made comparisons between an acridan ester (2′,3′,6′-trifluorophenyl 10-methylacridan-9-carboxylate), luminol and $Ru(bpy)_3^{2+}$ ECL at optically transparent ITO electrodes. At ITO electrodes, $Ru(bpy)_3^{2+}$ had the strongest emission at 1.5 V versus Ag/AgCl. They also discuss the advantages and disadvantages of ITO as an electrode material.

ECL of hemin at a platinum electrode in the alkaline solutions has been observed [197]. Under the optimum conditions the linear response range of hemin was 1.0×10^{-5} to 1.0×10^{-8} g ml^{-1}, the LOD was around 1.0×10^{-8} g ml^{-1}, and the R.S.D. for 1×10^{-7} g ml^{-1} hemin was 2.8%. It was also found out that hemin could catalyse the ECL of lucigenin at a platinum electrode in a neutral solution in the presence of hydrogen peroxide, the catalytic ECL intensity was linear with the concentration of hemin in the range of 1.0×10^{-14} to 1.0×10^{-10} g ml^{-1}. IgG labelled with hemin was used to determine that hemin retained ECL catalytic activity when conjugated to protein.

Some porphyrins have recently been shown to produce ECL by the means of traditional electrochemistry [198,199]. In addition, porphyrins and metalloporphyrins and their protein conjugates seem to be electrochemiluminescent in aqueous solution during cathodic hot electron injection [178,200], which suggests that metalloporphyrins can be used as ECL labels in bioaffinity assays, some of which even allow the use of time-resolved detection [200].

5.3. $Ru(bpy)_3^{3+}$ and other transition metal chelates as labels

Although the use of $Ru(bpy)_3^{2+}$-based labels was proposed a long time ago [120,121], practical applications in immunoassays and closely related DNA-probing assays have only appeared during the last decade. This area has presently a tight patent coverage by Igen Inc. (Gaithersburg, USA) and their applications based on these patents are marketed under the ORIGEN® technology umbrella. In 1991, Blackburn et al. reported first immunoassays based on the use of tris(2,2′-bipyridyl)ruthenium NHS ester, [4-(*N*-succimidyloxycarbonylpropyl)-4′-methyl-2,2′-bipyridine]bis(2,2′bipyridine)-ruthenium(II) dihexafluorophosphate [117]. The structure of $Ru(bpy)_3^{2+}$ was chemically modified with reactive groups on one of the bipyridyl ligands to form activated species able to react with proteins, haptens, nucleic acids, etc. Utilising the $Ru(bpy)_3^{2+}$ NHS ester, Blackburn et al. carried out heterogeneous ECLIAs for carcinoembryonic antigen and α-fetoprotein as well as competitive electrochemiluminoimmuno assays for digoxin and thyrotropin [117]. They also demonstrated that their methods were applicable for the determination of the HIV-1 gag gene by detection of a polymerase chain reaction (PCR) product [117].

Roche Diagnostics (formerly Boehringer-Mannheim) offer ECL instruments and commercial ECL kits based on Igen's ORIGEN® technology. In both commercially available models, Elecsys 1010 and 2010, the whole ECLIA procedure is fully automated. For example, the Elecsys hTSH ECLIA is based on the immunometric sandwich principle where the overall assay time is only 18 min. The test reproducibility

is shown to be improved by daily BlankCell procedures [201,202]. According to our observations, the nice performance of these commercial instruments is difficult to reproduce with our laboratory instruments. This is mainly caused by the high blank emission induced by inappropriately preconditioned gold or platinum electrodes, which become oxide-coated in the ECL generation conditions. In the evaluation test of the Boehringer-Mannheim (presently Roche Diagnostics) Elecsys 2010 immunoanalyser, the system showed an acceptable analytical performance [203–206].

Various immunoassays have been developed for Elecsys 2010 since the year 1999, and lot of comparisons between different assay formats have been made [207–219]. Generally, ECLIA has always had at least an equal analytical performance in these comparisons to other present competing technologies.

Kenten et al. used the $Ru(bpy)_3^{2+}$ NHS ester as a label in the quantification of PCR-amplified products from viruses, cloned genes and oncogenes [220]. Other PCR applications based on the use of the same label have been developed by Gudibande et al. [221] for specific amplified DNA sequences. Similar techniques were also used by other groups for determination of biotoxoids, viruses, bacterial spores [222,223], and both HIV-1 DNA and RNA [224,225]. An alternative $Ru(bpy)_3^{2+}$-based label was developed by DiCesare et al. (Perkin-Elmer label) [226]. It is applicable mainly for DNA and RNA probing assays [222,227–240].

An alternative method for PCR is nucleic acid sequence-based amplification (NASBA) which was commercialised by Organon Teknika BV, Boxtel, The Netherlands, using Igen's ORIGEN® technology. This method utilises simultaneously three different enzymes in isothermal conditions and it allows to selectively amplify either RNA or DNA. Quantitative NASBA (QNASBA) allows to determine HIV-1 virus in approximately in 1.5 h and the measuring range covers several orders of magnitude which nicely allows to monitor the effectiveness of antiviral medication [224]. New NASBA papers are continuously being published [241–250]. ORIGEN® technology has been applied also in drug discovery [251].

In all $Ru(bpy)_3^{2+}$ systems mentioned above, the excitation mechanisms are mainly based on the anodic *ox–red* excitation pathway where the chelate is partly oxidised by electron transfer from the electrode, and partly via $TPA^{\bullet+}$ cation radical-mediated pathway. However, the hot electron electrochemistry is also applicable, but it has less sensitivity due to the solid-state blank emission produced by the insulating films under high-field current injection conditions. The cathodic ECL at oxide-covered aluminium electrodes permits the detection of $Ru(bpy)_3^{2+}$ and its derivatives below nanomolar concentration levels yielding linear log–log calibration plots that span over several orders of magnitude of concentration. The applicability of this method has been demonstrated by a heterogeneous immunometric assay of hTSH as a model analyte [135].

In addition to ruthenium chelates, some other transition metal chelates are also applicable as labels for ECLIA. Thus far, the ECL of rhenium, osmium and iridium complexes has been studied, but no actual immunoassay utilising these labels has been published yet [198,252–256].

5.4. Lanthanide chelates as labels

Some lanthanide(III) chelates can be detected by the hot electron chemistry at the pM level with the linear calibration range spanning over five to six orders of magnitude of concentration [125–127]. Within this method the working electrode is composed of a conducting base material (C) and a thin good-quality insulating film (I) on its surface. The insulating film can consist of one or several layers of the same or different insulating material. The use of super-lattice type multi-potential barriers is also possible. The working electrode can be either optically transparent or non-transparent. In the case of transparent working electrodes, the light measurement can be performed through the working electrode. A two-electrode cell is usually sufficient, though a conventional three-electrode cell, composed of a working, an auxiliary and a reference (such as Ag/AgCl electrode) electrode, can also be used. With optically non-transparent working electrodes, the geometry of a working electrode or an auxiliary electrode has to be designed to ensure that the light detection can be performed from the counter electrode direction. If both working and counter electrodes are sufficiently optically transparent, the dual labelling and the light detection at two different wavelengths (filters F_1 and

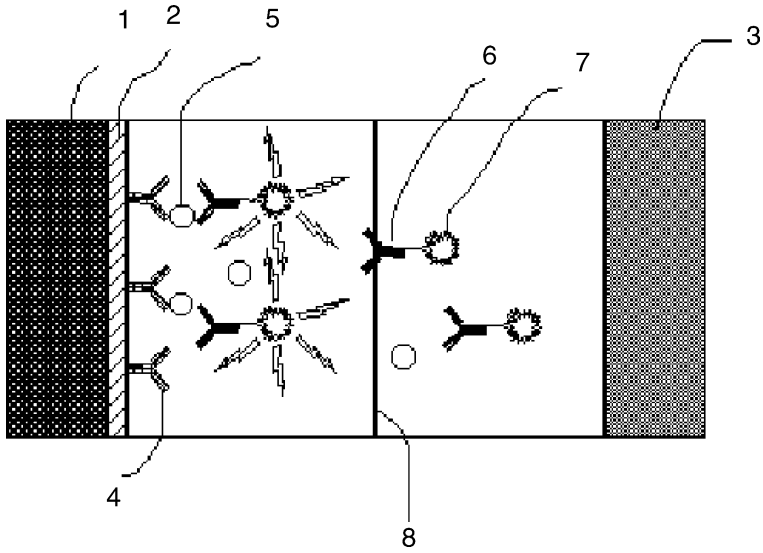

Fig. 9. Excitation of luminophores at thin insulating film-coated tunnel emission electrodes. Luminophores can be excited only at a distance up to ca. 50 nm from the tunnel emission electrode surface. This allows carrying out homogeneous assays. However, heterogeneous assays always provide a higher sensitivity. The best detection limits are obtained if the luminophores display sufficiently long-lasting ECL (e.g. the decay time solid-state electroluminescence of thin insulating films is typically of the order of 6–10 μs), thus easily permitting the time-resolved measurements with lanthanide(III) labels.

F_2) can be easily performed without expensive optics or a beam splitter.

The measuring principles of an immunochemical reaction employing the insulator electrodes are presented in Fig. 9. The working electrode consists of a base material C (1) and an insulating film I (2). Most commonly, the immunoassay is performed using an immunometric principle; thus, the insulating film I (2) is coated with antibodies (4) that are specific to an analyte (5). To perform the immunoassay, the mixture of a sample and a label (6)–(7), a labelled second antibody specific to the analyte (5), is incubated in the buffer solution in contact with the working electrode surface. This results in the formation of immunocomplexes I–(4)–(5)–(6)–(7) on the surface of the insulating film. If the sensitivity requirement of the analyte determination is not that high, the analyte level can be quantified after this reaction step by electric pulse excitation of the label molecules (7) involved in the complex I–(4)–(5)–(6)–(7) formation. This homogeneous assay principle is applicable because the excitation of the label molecules only occurs at a certain distance (8) from the surface of the insulating film, whereas more distant label molecules are not excited. In the case of heterogeneous assays, the labelled antibodies (the entities (6)–(7)) not bound to complexes I–(4)–(5)–(6)–(7) are washed away, providing a better sensitivity than in the case of homogeneous assays. The chemical reactions occurring on the counter electrode (3) do not usually produce luminescence with the luminophores used in these applications nor the dissolution of anode material has sufficient time to induce problems at the cathode during the short ECL measurement.

If the working electrode is fabricated from ITO-glass electrode by coating ITO with an insulating film, e.g. by atomic layer deposition (ALD), an optically transparent working electrode is created and light can be detected both through an optically transparent cathode and anode. ITO serves nicely as an anode material, but in many cell designs stainless steel wires, grids or tubes can serve as much cheaper counter electrodes. However, there is no need to have more than one detector and one optical path for detection if only a single label is used in the assay, or if the detector is able to record both the emission spectra and the time dependence of the ECL of several

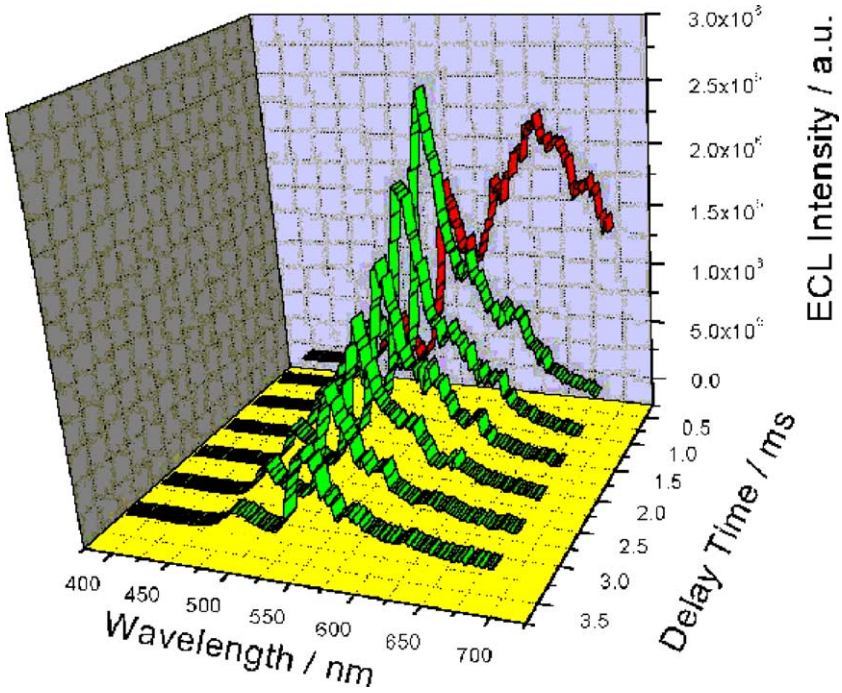

Fig. 10. Simultaneous ECL excitation of $Ru(bpy)_3^{2+}$ and a Tb(III) chelate. Reprinted with permission from [135], Copyright 1999, American Chemical Society.

labels (Fig. 10). Plenty of different cell designs have been tested by us, but these solutions must still remain unpublished.

The synthesis of 2,6-bis[*N*,*N*-bis(carboxymethyl)-aminomethyl]-4-benzoyl phenol-chelated Tb(III) and its isothiocyanate derivative has been described [257,258]. Suitable electrochemiluminescent Tb(III) chelates for labelling are commercially available from Perkin-Elmer Life Sciences as isothiocyanate, iodoacetetamido-, or amino derivatives. Their Labelling Services may be able to offer also other Tb(III) chelates carrying these moieties for efficient coupling with various biomolecules. Tb(III) chelated by 2,6-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-4-(phenylethyl)phenol as an isothiocyanate derivative is the best electrochemiluminescent label so far synthesised for TR-ECL.

Actual immunoassays utilising hot electron electrochemistry in the detection step of the bioaffinity assay have been demonstrated so far only a few times [127,257,259,260], and no commercial reagent kits or instruments are available yet.

5.5. Novel solid labels?

Nanocrystals (quantum dots) have very recently been shown to be electrically excitable in non-aqueous media [261,262], but as far as we know, no ECL studies using aqueous nanocrystal suspensions have been carried out. Already now, fullerens are known to be excitable in aqueous solution with hot electron chemistry [263] thus, we cannot see any reasons why nanocrystals having a suitable band gap would not be electrochemiluminescent during cathodic hot electron injection in the presence of suitable coreactants.

6. Developments in the instrumentation

General reviews on fluorescence mentioned in the introduction and in the Sections 4 and 5 typically contain chapters on instrumentation, but some other specific and relatively recent reviews also exist [264–266].

6.1. Light detectors

An efficient and accurate light detection is a common need to all analytical luminescence methods. The size of the required photosensitive area of the detector varies depending on the application. Usually a high detection sensitivity is needed, but the detector must also be able to handle many orders of magnitude higher light fluxes than those corresponding to the LOD of the wavelengths in question.

In photo-cathode devices, the photons are incident on the active region of the material forming the detector. The surface material of the photo-cathode plays a key role in the functioning of a photo-emissive light detector and the wavelength range of sensitivity is dictated primarily by the absorption band of the cathode material and to some extent the work function of the surface [267]. One of the strengths of the devices based on the photo-cathodes is their very low noise level.

In all semiconductor photo-detectors, one or more semiconductor layers are typically grown on a suitable substrate. The simplest of semiconductor photo-detectors, the photo-conductive types, are not very usable in analytical applications. The other type requires a junction, either of the p–n junction variety or of the Schottky barrier variety. In both cases, the light must be allowed to penetrate to the region designed for absorption. When the energy of the absorbed photon is large enough to raise an electron from the valence band to the conduction band, they create holes in the valence band. The existing electric field separates then the electrons and holes and causes the terminal current to flow in proportion to the photon flux.

When low light intensities need to be detected and measured, single-photon counting using photo-cathode devices, photomultiplier tubes (PMT), has for long been the first choice. However, the traditional way of measuring just the photocurrent of the PMT is often perfectly satisfactory. If high magnetic fields are problematic for vacuum PMTs the gas-filled PMTs can be a solution [268]. When the specific needs of an application are not very wide, the price of the detectors can be decreased by an appropriate choice of usable wavelength range and properties of the PMT (window material, side-on or end-on PMT, photo-cathode material, size of the photo-cathode, cooling, etc.). The novel commercial solutions can be looked at, e.g. Hamamatsu Ltd, http://www.hpk.co.jp/Eng/products/etdpro.htm and Electron Tubes limited, Ruislip, England, http://www.electron-tubes.co.uk/menu.html. Presently, also multi-anode photomultipliers are available. For instance, Hamamatsu has produced a compact 16-channel multi-anode linear array PMT.

Channel photomultipliers (CPM, or microchannel plate photomultipliers, MCP) are performing better than ordinary PMTs. In these devices, electrons from the photo-cathode pass through a narrow semi-conductive channel. Multiple secondary electrons are emitted each time the electrons on their way to the anode hit the inner wall of the curved channel. This effect occurs multiple times along the path, leading to an avalanche effect with a gain exceeding 10^8. These detectors also have extremely low dark current (much lower than the traditional PMTs), but they require special high voltage sources due to the needed very high operating voltage of 2400–3000 V. For instance, Perkin-Elmer produces both CPMs and also single photon counting modules based on CPMs (http://optoelectronics.perkinelmer.com). CPMs presently cost between US$ 800–2300 depending on their cathode and window materials and other properties. Rockwell International Corporation has produced solid-state photomultipliers based on the impurity band to conduction band ionisation [269].

We all know how great the impact on the diode array detectors has been on the UV-Vis spectrophotometry and UV-detectors in HPLC during the last decades. However, in applications of spectrophotometry it is sufficient to measure changes in a rather high intensity light beam passing through the cuvette. Luminescence methods typically need measuring ability of many orders of magnitude lower light intensities with sufficient precision and accuracy. The different applications need very different methods of light collection from the sample area to the detector, and the cost and the needed versatility of the instruments varies vastly from one application to another.

Scientific instruments typically need a full selectability of wavelength and bandwidth of the light beam, and typically they allow the use of large size components, cooling of the detector, several alternative expensive light sources, etc. In this area, most problems can be solved with traditional instrumentation. Sometimes, traditional arc and lamp sources

cannot give sufficient excitation intensity but lasers or dye-lasers are required. Often, discrete wavelength laser light sources are sufficient but sometimes cw-light sources are necessary. In this area, the low cost is typically not very important.

The economically most promising area of the rapidly developing analytical chemistry lies in the area of analysis made outside of big central laboratories, in the actual place needing the analysis: in the points of environmental problems, at home, at the site of a chemical production processes, etc. Most often these applications would require a very inexpensive apparatus, and often the methods utilised should preferably allow for reliable use by untrained personnel or final end users. The apparatus should normally also be small in size and optionally battery operated. Fortunately, the new emerging technologies seem to realise these needs better and better.

Silicon photodiodes with effective areas of a few square millimetres have proved to be inexpensive and useful in some applications of steady state fluorometry. These are based on a reverse biased p–n junction. The reverse bias voltage has the effect of increasing the voltage across the depletion layer compared with a forward bias voltage so that any photo-induced electron–hole pairs are swept rapidly across the junction to create a current pulse. However, these diodes are not suitable for single-photon counting purposes.

Avalanche photodiodes (APD) have been developed for photon counting purposes and are then called single-photon avalanche diodes (SPAD) [270,271]. These devices consist of a reverse biased p–i–n junction and operate in a non-proportional multiplication mode analogous to a Geiger Müller tube. The reverse bias is held slightly above rather than below the breakdown voltage for the junction. The electric field is sufficiently high to sustain an avalanche of carrier multiplication via secondary ionisation once a primary electron–hole pair has been photo-induced by absorption in the depletion layer. The diode current is either turned off passively by limiting the current flowing with a suitable resistor, or actively by lowering the bias voltage after the onset of the avalanche [272,273].

In the PMT or MCP the primary photoelectron is emitted from the photo-cathode into vacuum and then multiplied by secondary electron emission. The shower of secondary electrons is collected by the anode and produces a current impulse at the output. To operate a PMT or MCP as a single photon detector, the gain (controlled by the operating voltage) must be set to a level to produce output pulses (from single primary electrons) in excess of the threshold of the timing device. In the SPAD a conduction electron is excited internally, which triggers an avalanche breakdown.

A characteristic feature of a SPAD is the extremely small variance of the amplitude of the output pulses even if more than one primary photo-electron was to be excited by the light pulse. The PMT produces, on the other hand, output pulses with high variance (depending on the type) and the mean amplitude is proportional to the number of primary photo-electrons. An important parameter of all detectors is the percentage of photo-electrons produced per photon, the quantum efficiency. It ranges from a few percent (MCP) to more than 50% (SPAD).

The spectral response of a SPAD is determined on the long wavelength side by the band gap of the semiconductor employed. Single-photon counting modules (SPCMs) [273] are commercially available, see, e.g. Perkin-Elmer Optoelectronics SPCM-AQR-13 or equivalents (http://optoelectronics.perkinelmer.com/Downloads/spcmaqr.pdf) which produce ca. 25 ns TTL pulses induced by single photons. SPCMs are presently priced between US$ 4500–12,000 depending on the specifications of the modules.

In addition to silicon, semiconductor nitrides such as those of aluminium, gallium and indium are promising materials for both light detectors and emitters. These materials and their ternary and quaternary alloys cover the band gap range of 1.6–6.2 eV, suitable for band-to-band light detection and generation from red to ultraviolet wavelengths [267].

For those making their microanalytical chips from silicon, a promising alternative could be a photodetector based on porous silicon [274]. A relatively new detector type in the NIR range is superlattice avalanche photodiodes, which have quite recently been reviewed by Taguchi et al. [275]. Also superconducting NbN detectors for visible and NIR range have been fabricated as well [276], but cooling the detector down to 4.2 K is not attractive for most of the applications. Quite interestingly, also single-photon field effect transistors have been recently designed for light detecting purposes [277,278].

Sometimes an array of tiny detectors is not sufficient but a two-dimensional matrix of detectors is needed.

Charge-coupled device (CCD) sensors and cameras are available for both of these purposes. CCD cameras are useful, e.g. in the areas of HTS and astronomy. CCD-detection has also been taken in use in atomic emission spectrometry [279], and in fact it has also been predicted that in atomic absorption spectrometry the Charge-coupled array detector together with a high intensity continuum light source would be in the future the choice number one [280]. A CCD is best described as a semiconductor chip, one face of which is sensitive to light. Typically, it is fabricated from p-doped silicon layer on an n-doped substrate. This structure is then capped with an insulating layer of SiO_2 (easily obtainable by thermal oxidation), on top of which is placed a pattern of highly doped and strongly conducting silicon electrodes. The light sensitive face is rectangular in shape and subdivided into a grid of discrete rectangular areas, pixels. The arrival of a photon on a pixel generates a small electrical charge, which is stored for later read-out. The size of the charge increases cumulatively as more photons strike the surface. By using a few clocking circuits, an amplifier and a fast analogue-to-digital converter (ADC), it is possible to evaluate the amount of light that has fallen onto each pixel by examining the amount of charge it has stored. The chip will usually be cooled to reduce the noise level. The whole instrument is often referred to as a CCD camera. The electronics controlling the CCD chip are interfaced to a computer, which in turn controls them. Thus, the images observed by the CCD are transferred directly to computer memory without an analogue stage, hence they can be plotted on an image display device or written to magnetic disk.

The principal advantages of CCDs are their high sensitivity, dynamic range and linearity. It is common for CCDs to achieve a quantum efficiency of about 80%. CCDs are also sensitive to a broad range of wavelengths and are more sensitive to red light than typical PMTs. The typical dynamic range of CCDs is about 10^5, and the response in this range is essentially linear.

For instance, Johansson and Pettersson [281] have constructed a spectrometer for the range 250–975 nm, which is based on the use of CCD camera and holographic gratings. The instrument provides spectral information from each selected 5 nm wavelength band depicted with its corresponding geometric location, i.e. a single pixel on the CCD chip. Hence, objects can be visualised in different wavelength bands and a spectrum can be produced for each location of the object. The imaging spectrometer system provides a powerful combination of spectroscopy and image processing. Messler et al. [282] have constructed an instrument for multi-wavelength excitation imaging composed of fluorescence ratio-imaging system which is based on a 12 bit, 2 MHz slow scan CCD camera and a fast wavelength selector based on the use of a diffraction grating mounted onto a galvanometric scanner.

Charge transfer detectors (CTDs) is a larger class that includes CCDs and charge injection devices (CIDs). In atomic spectrometry, CIDs allow a better separation than CCDs of strong and weak emission lines situated closely to each other in the emission spectrum [279].

Presently, photon counting using PMTs still seems to be the most cost-effective way to detect very low light levels and they have quite large photo-cathode areas, if such are needed by the application. Somewhat more expensive and better quality choice is the use of CPMs for the same purposes. SPAD-detectors will surely become more and more attractive as time goes by and their development proceeds further and the prices decrease. Also, CCD detectors surely will find their way to the relatively low-cost instruments later on.

6.2. Light sources

Traditional light sources for PL instruments are not expected to develop much because their technology has taken such a long time to reach present quality standards. The greatest impact for low-cost instrumentation has lately been created by the development of light emitting diodes (LEDs), diode lasers (DL) and Diode pumped solid-state lasers (DPSSL) [283]. The use of NIR DLs in analytical chemistry has recently been discussed by Imasaka [284]. Blue [285] and violet [286] DLs have been in use for a while now. Very recently, also UV-LEDs and UV-LED pointers have appeared on the markets. Table 4 sums up some of the currently available light sources, their distributors and prices.

Hirayama and Aoyagi have reported the fabrication of 330 nm UV-LEDs [287] and 300 nm UV-LEDs [288] based on the InAlGaN. They state that also LDs can be fabricated from this alloy. Thus, it is possible

Table 4
Light sources and commercial manufacturers

Light source	λ (nm)	*P* (mW)	Price (US$)	Manufacturer	Other
UV-LED pointer	370	0.75	120	Roithner, http://www.roithner-laser.com	
UV-LED "Money checker"	370	1	50	Roithner	
RLT350-30	350	30 μW	32	Roithner	Based on AlGaN
RLT370-10	370	0.75	45	Roithner	
LED370-66-60-10 illuminator	370	60	990 per piece		60 InGaN diode chips, 110° output angle
PDL 800-B pulsed diode laser	375	1 W peak power	7000/driving unit, 375–8900 per head LDH	PicoQuant GmbH, http://www.psplc.com	70 ps pulses, repetition rates 1–40 MHz
PLS 370 pulsed UV LED	370	2.5	2000 per head		
UV LED	365	100	Ready to be distributed by end of 2003	Nischia, http://www.nischia.co.jp/info/news/new20020926.htm	
UV DL	375	10			
NCCU001E	380	100	85	Nischia	
NDHU2000APAE2	375	2	2700		
NDHV310APB	405	30	1900		
QUV349-100	349	100	27000	CrystaLaser, http://www.crystalaser.com	Diode pumped Q-switched lasers
QUV349-50	349	50	20000		
QUV349-25	349	25	17000		
QUV266-10	266	10	28000		
QUV266-5	266	5	23000		
QUV266-2	266	2	20000		
Spectra-Physics Millennia solid-state laser	266	200	175000		

UV LED and UV DL are both manufactured by Nischia.

that also 300 nm UV LEDs and DLs might be commercially available within a couple of years. Earlier, Nishida et al. have reported fabrication of 352 nm UV LEDs from the same material [289]. In this paper, they forecast that this material allows to fabricate efficient UV LEDs covering the range between 200 and 360 nm, which is interesting to those who need reasonably hard UV light for their applications. This group has later manufactured UV LEDs emitting at around 342 nm [290]. Other researchers have anticipated that AlInGaN LEDs emission peak can be tuned between 305 and 340 nm by varying the alloy constituents [291].

Very interestingly, Kipshide et al. [292] have grown AlGaN/GaN and AlN/AlGaInN superlattices on Si(1 1 1). These LEDs emit in the range from 290 to 334 nm, which is quite attractive especially for the users of many lanthanide chelates. Thus, the same chip could now be integrated to contain highly efficient SPAD for single photon counting together with a hard UV emitting pulsed light source. Fabrication of UV LEDs has been carried out from several other materials, and possibly we will hear more about them in the future.

We are convinced that LEDs and DLs or arrays formed from these devices will be in a paramount role in the inexpensive PL instruments of the future. Today, a nice site to search suitable companies and manufacturers in the different areas of optical instrumentation is The OpticPages Buyers Guide (www.opticspages.com/bg/pd228.htm).

6.3. Optical fibres, waveguides, filters and wavelength dispersing elements

Optical fibres and waveguides are often much more convenient to use than traditional lense systems

in optical paths. Optical fibres are composed of a high-refractive index core and transparent cladding which is finally enclosed typically in a black plastic jacket. In analytical chemistry, many types of probes or optodes ("optical electrodes") [293] have been constructed for getting light in or out to and from various devices, reactors and sample cells. Also, living cells can be studied from inside with miniaturised optodes by PL [294]. Novel microstructured optical fibres, holey optical fibres, have been recently discussed by Monro et al. [295].

Potyrailo et al. have extensively reviewed the area of optical waveguide sensors in analytical chemistry a few years ago [264]. At about the same time, advances in miniature optical waveguide sensors were discussed by Klainer et al. [296]. In addition to PL-based probes, also optodes and sensors based on ECL have recently appeared [297,298].

In miniaturised systems, optical fibres are typically replaced by flat waveguides, which also work on the same total reflectance principle as optical fibres. When light is totally reflected, the electric field of the light penetrates the interface to some extent. When the oscillating electric field encounters the reflective interface, the field decays exponentially inside the cladding. The part of the light that penetrates the wall of an optical fibre or waveguide is called an evanescent wave. This evanescent wave can be used to PL excitation of luminophores situated on the surface or in the close proximity of the surface. Evanescent field can be also utilised in light absorption measurements by attenuated total reflectance.

One of the application areas has been capillary waveguide sensors [299]. These are multifunctional devices: optical waveguides, sample cavities, sampling devices, flow-through cells, mechanical support for sensor coatings and wavelength discriminators. The optical properties of the inner coating vary with the analyte to be measured and can be detected by methods including measurement of refractive index, absorption, reflection, luminescence and luminescence lifetime. In many cases, this is accomplished by evanescent wave spectroscopy. Bioaffinity sensors for trace analysis based on luminescence excitation by utilising planar waveguides have been developed to provide a novel generation of optical bioaffinity sensors for ultra trace analysis [300]. These sensors are based on luminescence generation in the evanescent field of high-refractive index single-mode planar waveguides. With the waveguiding layers and the grating parameters chosen, a very sharp discrimination of bulk against surface-confined excitation in combination with high excitation intensities in the evanescent field can be achieved, leading to extremely high sensitivity. Incoupling of excitation light is performed using diffractive gratings.

An important issue is the outcoupling and incoupling of the light in different parts of light transportation chain, which is usually carried out by gratings. Barnes has treated the problems in fluorescence near surfaces and the roles of surface and waveguide modes [301], and e.g. Duveneck et al. discuss the available different methods for the detection of both transmitted and luminescence light [300,302]. The transmitted excitation light can be detected either at the distal waveguide chip end or using a second outcoupling grating. Both isotropically emitted luminescence, collected by a lens located below the transducer substrate ('volume detection'), and emission coupled back into the waveguiding layer can be monitored, the latter via a second outcoupling grating. With these devices 100 amol of fluorophore-labelled DNA can be detected [300]. Later, planar waveguides have been developed for even more sensitive analysis of nucleic acids [302].

Whereas, the requirement for performing simultaneously multi-analyte detection is solved by the approach of nucleic acid microarrays, requirements on sensitivity may often remain unsatisfied by classical detection technologies. Inherent limitations of conventional fluorescence excitation and detection schemes can be often overcome by the implementation of planar waveguides as analytical platforms for nucleic acid microarrays, with fluorescence excitation in the evanescent field with the guided excitation light. Duveneck et al. [302] discuss the relevant parameters for an optimisation of sensitivity and the specific formats of their planar waveguide platforms, which are compatible with established industrial standard formats allowing the integration into industrial high throughput environments. They also explain their optical system for fluorescence excitation and detection. In a direct comparison with a state-of-the-art scanner, they demonstrate that the implementation of genomic microarrays on planar waveguide platforms allows for unprecedented, direct detection of low-abundant genes in very limited amounts of sample.

Placing multiple sensing regions (sensing pads) on a single chip is a powerful means for referencing and multi-component analysis by miniature integrated optical sensors. Duebendorfer and Kunz [303] describe on-chip referencing experiments performed with sensitive grating coupler sensor chips consisting of compact TiO_2 and Ta_2O_5 waveguiding films on fused silica and with TiO_2 films on replicated polycarbonate substrates. They see that their results are not only relevant for grating coupler sensor chips but also for many other evanescent wave sensors.

Recently, Knight and Russell have reviewed the novel possibilities to guide light [304] and Charlton et al. have reviewed the recent developments in the design and fabrication of visible photonic band gap waveguide devices [305]. The design and fabrication considerations of silicon integrated optical waveguides has been reviewed by Benaissa and Nathan [306].

Organic polymers as waveguide and optical fibre materials and their processing and use have been recently reviewed several times [307–309]. Discussion includes optical interconnects, directional couplers, array waveguide grating (AWG) multi/demultiplexers, switches, tunable filters, variable optical attenuators (VOAs), and amplifiers. Several integrated planar lightwave circuits, such as tunable optical add/drop multiplexers (OADMs), photonic-crystal superprism waveguides, digital optical switches (DOSs) integrated with VOAs, travelling wave heterojunction phototransistors and three-dimensionally (3D) integrated optical devices are also highlighted. The devices described include a variety of passive and thermo-optically active elements that can be used in various coupling, routing, and filtering applications. These devices can be either individually pigtailed and packaged components, or they can be part of a massively parallel photonic integrated circuit on the multichip module (MCM), board, or backplane level [309].

Inorganic glasses for optical waveguides has been reviewed by MacFarlane [310] and fluoride glasses by Rault et al. [311]. Agnihotri et al. [312] have considered the advances in low temperature processing of silicon nitride based dielectrics and their applications in surface passivation of silicon and integrated optical devices. The possibilities that oxidised porous silicon offers for integral optical waveguides are reviewed by Yakovtseva et al. [313] and Canham et al. [314]. Sol–gel materials have been reviewed by Mac Craith et al. [315], Righini and Pelli [316] and Forastiere et al. [317]. The last of these reviews is concentrated on techniques for producing SiO_2 on Si in integrated optical devices.

IR transmitting waveguides and infrared optical sensors have been discussed by Harrington [318] and Saito and Kikuchi [319].

Photonic crystals consist of dielectric materials that serve as electrical insulators in which an electromagnetic field could be propagated with low loss [320]. Holes are arranged in a lattice-like structure in the dielectric and repeated identically and at regular intervals, a property known as periodicity. If built accurately enough, the resulting crystal would have a photonic band gap, a range of frequencies within which a specific wavelength of light is blocked. Future applications of photonic crystals are believed to include photonic-crystal lasers, light emitting diodes and photonic-crystal thin films. Advances in photonic crystals are also discussed by Weisbuch et al. [321,322]. These authors believe that 2D and 3D photonic crystals provide a viable route to high-performance photonic integrated circuits (PICs).

Incoupling and outcoupling of light in the devices has been widely discussed in the literature [323–325]. Parriaux et al. [323] have reviewed the use of gratings as coupling elements with planar waveguides. Wiki et al. [324] have developed an optical biosensor based on a grating coupler triplet and Maims et al. [325] have fabricated injection moulded polymer waveguides that are useful in on-chip fluorescence and absorption measurements to be undertaken online in μ-TAS. Their devices involved embossed diffractive elements incorporated in such a way that effective in- and out-coupling of light from flow channels was achieved without the need for prism coupling and index matching solutions. The system was demonstrated using laser and LED sources to obtain fluorescence and absorption spectra in the visible region. A relatively novel type of grating is chirped grating couplers [326]. These can be used as a powerful basic building block for constructing miniature sensor modules. Kunz et al. [326] discuss the feasibility of fabricating these chips by means of very low-cost processes such as replication and thin film deposition. The most important theory fundamentals for calculating the performance of these chips are given by them, and they

report experimental results obtained with replicated polycarbonate chips used in a flow cell.

Wavelength discrimination and dispersion is often needed in analytical instruments. Many of the methods and solutions developed for optical communication devices surely can be adopted and developed further in microanalytical devices. Many applications of integrated optics, related especially to multi-wavelength telecommunications, require the use of dichroic reflectors to achieve narrow or broad-band wavelength-selective filters. Bragg mirrors in particular are very good candidates for that purpose. Ferriere and Benkelfat [327] describe the design of a holographic set-up used to record a photolithographic mask directly on the substrate, enabling the inscription of periodic and aperiodic parameters waveguides for these purposes. Wavelength-division multiplexing waveguide hologram devices and photonic-crystal superprism waveguides have been discussed by Chen et al. [328] in combination with the fabrication of polymer waveguides and their application in optoelectronic systems. Bragg grating technology in silica-based optical fibres as well as other fibre materials has been reviewed by Reekie and Dong [329]. Chen et al. [330] have discussed spectrometers on a chip.

Chekhlova and Tcheremiskin [331] have recently reviewed waveguide spectral multiplexers/demultiplexers. They discuss the practical implementation of wavelength-division multiplexers in specific devices based on SiO_2 and InP. SiO_2-based multiplexers exhibit lower losses, whereas devices based on InP make it possible to use the technologies of microelectronics and integrating optics and to fabricate radiation sources and passive components on the same substrate for optical communication purposes. Their multiplexers/ demultiplexers are fabricated by conventional lithography. Devices such as the planar arrayed-waveguide grating or the distributed Bragg reflector (AWG and DBR, respectively) are assumed to have increasing importance in the areas of fibre point-to-point communication and networking. In the particular context of dense wavelength-division multiplexing (DWDM), these devices play a well-established role as wavelength-selective elements. Parker and Walker [332] have reviewed this area. Cremer et al. [333] have developed a Grating spectrograph in indium gallium arsenide/indium phosphide for dense wavelength-division multiplexing purposes in IR range. This spectrograph is suited for monolithic integration with photodiodes, laser diodes, or optical amplifiers on a single chip.

Micromachined free-space integrated micro-optics have been discussed by Wu and co-workers [334–336]. The optical axes of these optical elements are parallel to the substrate, which enables the entire free-space optical system to be integrated on a single substrate. Microscale Fresnel lenses, mirrors, beam-splitters, gratings and precision optical mounts have been successfully fabricated and characterized. In addition, micropositioners such as rotary stages and linear translational stages are monolithically integrated with the components using the same surface-micromachining process to provide on-chip optical alignment or optomechanical switching. The groups propose that their free-space micro-optical bench (FSMOB) technology could significantly reduce the size and cost of most optical systems, and could have a significant impact on optical switching, sensing and data storage systems.

In-line fibre evanescent field electro-optic modulators have been reviewed by Afrt et al. [337]. They consider electro-optic modulators that consist of an optical fibre waveguide coupled to an electro-optic waveguide. Desirable attributes of these devices are that the optical fibre is uninterrupted and the interaction with the electro-optic region occurs only where the optical properties are modulated. Dalton et al. [338] have discussed the polymeric electro-optic modulators quite recently. Reactive ion etching and/or multicolor photolithogaphy are used to fabricate buried channel waveguide structures out of the resulting polymeric electro-optic materials and to integrate polymeric waveguides with SiO_2 optical fibres. Dalton et al. have demonstrated both vertical and horizontal integration of polymeric electro-optic modulator circuitry with semiconductor very large scale integration (VLSI) circuitry.

We are sure that plenty of research carried out in the field of optical communication can be quite directly adopted in the field of microanalytical optical systems [339–347]. There must be loads of additional papers considering methods applied in wavelength dispersion, multiplexing, routing, mixing, filtering, etc. that real experts in this field could serve on a silver platter to analytical chemists developing microsystems.

7. Luminescence-based detection methods in regular and on-chip capillary electrophoresis

Commercially available CE instruments are generally equipped with an UV-Vis absorbance detector, which is relatively inexpensive. However, while the mass LOD in UV detection is impressive, the concentration LOD is limited by the light path length generally determined by the capillary's inner diameter. Luminescence can offer lower LODs than absorbance-based detection.

Several luminescence-based methods have been developed for CE, and a number of reviews have been published since early 1990's on their application in the detection system of CE. Review articles on the different techniques are referred to where appropriate.

This section describes the PL, CL and ECL detection in both regular and microchip CE (MCE or μ-TAS). Table 5 compares the different luminescence-based detection techniques, concentrating on the most recent research.

7.1. Photoluminescence detection methods in CE

A number of methods employing laser-induced fluorescence (LIF) have been developed for CE and studied in several reviews (e.g. refs. [348–352]). Since this technique can be expected to be familiar to many readers and background material is easily found, in this article CE–LIF is covered superficially.

The advantages of LIF are the low background noise and the intensity of radiation produced by the lasers at narrow bandwidths, high spatial coherence and insignificant chromatic aberration [353]. In addition, the output of the laser is easily focused on to the capillary. As a result, the LODs are typically very low. Already in 1994, Chen and Dovichi [354] reported yoctomole LODs for CE–LIF, in other words, detection of just a few molecules of the analyte. Since then, detection of single molecules with CE–LIF has been reported [355].

With single-wavelength LIF detection little structural information of the analyte is obtained, but wavelength-resolved fluorescence detection, described in a review by Zhang et al. [353], is able to give spectroscopic information about fluorescent species while maintaining low LODs. Multidimensional LIF such as wavelength-resolved, time-resolved or polarization fluorescence detection also reduces matrix interference [353]. However, the main disadvantages of LIF as well as wavelength-resolved fluorescence detection have been the high cost of UV lasers and the need for pre- or post-column derivatisation for most molecules.

Indirect LIF or fluorescence detection of non-fluorescent molecules in a fluorescent electrolyte solution is also possible [356], but as with indirect detection in general, the sensitivities are mostly lower than with direct detection. Indirect fluorescence has also been made on a chip [357] but it is not recommended for charged analytes in MEKC [358]; MEKC–LIF of neutral analytes, on the other hand, can give higher fluorescence yields than the analyte would exhibit without the presence of the micelles [358].

Kuijt et al. [359] have introduced a method for indirect phosphorescence detection after CE separation. The system is based on the quenching of biacetyl phosphorescence in the background electrolyte (BGE) by the analytes via various mechanisms. The system was fit for use in a wide pH range, and for phenols, benzoic acid derivatives and naphtalenedisulfonic acids the LODs were between 10 and 80 nM.

Fluorescence detection in CE has produced a few noteworthy reviews. Jankowiak et al. [360] have reviewed fluorescence line narrowing detection in CE and chromatography, and Matysik [361] has reviewed fluorescence detection under non-aqueous conditions.

7.2. Chemiluminescence detection methods in CE

Generally, CL is very sensitive technique, and if coupled to an efficient separation technique such as CE [362], it offers outstanding selectivity and sensitivity. The LODs can be as low as with LIF, but with a simpler and much less costly instrumentation. The sensitivity of CL mainly stems from the absence of a light source, which significantly lowers the noise levels from those attained with, e.g. UV detection as well as eliminates Rayleigh and Raman scattering [363]. The linear range is also wide [364] and the reactions typically have fast kinetics, making them well suited to low-volume on-line detection [365].

CL is accomplished by reaction of added compounds with the analytes. The CL reagents can be classified into two groups: chemiluminescence labels and chemiluminogenic compounds. As their names imply, the first mentioned are used to label the analytes

Table 5
Comparison of luminescence detection techniques in CE

Technique	Compounds	LOD	Other information	Ref.
LIF	Anilines	0.3–0.9 nM	MEKC, pre-column derivative	[392]
LIF	Anilines	57–490 nM	MEKC, pre-column derivative, fast and quantitative reaction	[393]
LIF	Proteins, indocyanine green label	0.3 μM		[394]
LIF	Amino acids and peptides, fluorescamine labels	2.1–8.0 nM	Two solid-state UV lasers	[395]
LIF (optical fibre)	(±)-FLEC	40 nM	Enantiomeric impurity determination	[396]
LIF	Thiols and phytochelatins	250 nM		[397]
LIF polarisation	Immunoassays	1 nM		[398]
LIF	Oligosaccharides	0.85 nM (0.895 fmol)	NACE with N-methylformamide	[399]
LIF (red diode laser)	Cyanine-5 dye	9 pM	900 molecules detected of 4560 injected	[400]
LIF	Rhodamine 6G	50 nmol		[354]
LIF (laser-excited confocal FL scanner)	DNA genotyping		12 parallel samples in 160 s	[401]
LIF scanned	Immunoassays	30 pM fluorescein, 4.3 nM anti-estradial assay	Multi-channel μfluidic system, 30 s analysis	[402]
I-LIF	Valproic acid	0.9 mg/l (6 μM)		[356]
I-LIF	Se and Sb compounds	2.6–7.5 μM		[403]
I-LIF	Aliphatic carboxylic acids C2 – C18	0.56–1.55 μM	NACE (ACN, EtOH, water), merocyanine 540	[404]
FL	*Cis* and *trans* resveratrol	Not given	NACE (MeOH-ACN and SC micelles), enantiomeric sepn. at 77 K	[405]
FL line narrowing spectroscopy	Depurinating DNA adducts	0.2 nM	CE at 4.2 K	[406]
FL	Biogenic amines, FITC-labelled	2.95–6.57 μM	MCE, 80 s sepn.	[384]
FL (UV-light emitting diode induced FL)	Fluorescamine-derivatives of bradykinin and lysine	3 fmol (bradykinin), 18 fmol (lysine)		[407]
FL	Immunoglobulins	17 nM of sheep IgM	ELISA, PDMS chip	[408]
FL (variable wavelength)	FITC-labelled amines, rhodamines	1 nM (amines), 500 pM (rhodamines)	MCE, 50 s sepn. for amines, 6 s sepn. For rhodamines	[385]
FL	Immunoassays	50 nM lowest mentioned concentration	μfluidic chips	[409]
FL	Antibodies and theophylline	30 pM fluorescein	MCE	[410]
I-FL	Film developing agents	0.2–0.4 mM	MCE, 30 s separation	[411]
I-FL	Biogenic amines	6.5 μM	MCE, 90 s sepn.	[357]
Quenched phosphorescence	Naphtalenesulfonic acids, amino acids and others	10–1000 nM, mainly below 100 nM	Biacetyl in BGE, wide pH range	[359]
CL/L-HP	Nb(V), Ta(V)	0.41 nM (Nb), 32 nM (Ta)	Stacking	[374]
CL/L-HP	Fluorescein conjugate of HRP	9–35 nM	MCE, different channel depths	[387]
CLND	Aminoglycosides	1.4 μM of nitrogen	Stacking; nebuliser and CL reaction chamber optimised	[412]
CL	Cu(II)	4 pM	Stacking, water plug before sample	[366]

CL	Co(II), dansyl-glycine	0.49 μM, 0.39 μM	PDMS-chip with 3 patterns, L-HP and dansyl-conjugated peroxalate-HP reactions	[389]
CL	Co(II), Cu(II), Ni(II), Fe(III), Mn(II)	0.5 pM, 0.1 nM, 5 nM, 50 nM, 8 μM	Stacking	[375]
CL	Atropine and pethidine	3.8 nM, 77 nM	μTAS with chemical $Ru(bpy)_3^{2+}$ oxidation	[413]
CL	Glucose	100 μM	Microfluidic system, immobilised reagents	[414]
CL	Dansyl amino acids	10 μM	MCE–CL, 40 s sepn.	[388]
CL	Dansyl-tryptophan	0.43 fmol (lowest linearity point 30 nM)	Batch-type cell; in flow-type cell the LOD of same was 1.3 fmol	[372]
CL	Luminol	0.25 nM without stacking, 15 pM stacked	ACN stacking, *p*-iodophenol enhancer, batch-type cell	[373]
I-CL	Monoamines and catechol	0.5–3.1 μM	L-HP with Cu(II)	[377]
I-CL	Amino acids, catecholamines	0.77–43.7 μM (AA:s), 1.9–7.5 μM (CA:s)	L-HP system with Co(II) as enhancer, analytes suppress the enhancer	[378]
ECL	β-Blockers	2 μM (0.6 mg/l)	$Ru(bpy)_3^{2+}$ in BGE, oxidised post-column	[369]
ECL	Diphenhydramine	20 nM at S/N = 10	$Ru(bpy)_3^{2+}$ as ECL reagent, wide linear range	[367]
ECL	Ru(bpy) and Ru(phen), indirect detn. of amino acids	0.2–0.8 μM, AA:s ca. 300 μM	MCE (MEKC), floating Pt electrode	[368]
ECL	Proline, valine, phenylalanine	1.2–25 μM	Flow injection, falling drop interface, reagent $Ru(bpy)_3^{2+}$	[379]
ECL	Tripropylamine, lidocaine	50 pM, 20 nM	CE current did not affect ECL of $Ru(bpy)_3^{2+}$, wide linear range	[380]
ECL	Tripropylamine, proline	2 nM, 2 μM	ECL reagent immobilised	[381]

with intense chemiluminophore groups. The last mentioned are themselves only weakly chemiluminescent but their reaction with analytes generates intense CL. Indirect detection, in which the analytes reduce the CL efficiency, is also possible but generally less sensitive than direct detection.

A challenge in CE–CL is the sensitivity of CL emission intensity to environmental factors such as pH, ionic strength, solvent, separation temperature and other species present in the system. This requires optimisation of the CE conditions to achieve separation under the optimal CL emission conditions, or alternatively, choosing the reagents and reactions such that the CL efficiency is highest under the separation conditions. In practice the modification of the CE conditions is usually the more easy approach. The equipment also needs to be set to measure the signal during the short time when the emission intensity of the CL reaction is at its maximum; the emission versus time profile is different for different compounds.

CL reagents such as luminol and related compounds, peroxyoxalate and its esters, acridinium esters, lophine derivatives, adamantyldioxetane compounds, ruthenium complexes, permanganate and firefly luciferase have been successfully used in CE–CL with excellent sensitivity [364–367]. Several reviews have been written on the applicability of these reagents. In their extensive review, Yamaguchi et al. concentrated on describing the reactions of various luminol-type reagents with different types of analytes [364]. In ECL detection of CE, mainly $Ru(bpy)_3^{2+}$ [367–369] has been used as a reagent.

The coupling of CL to CE is challenging mainly as a result of the very small flow volumes of CE and difficulties in addition of CL reagents into the separation capillaries, but a number of approaches to efficient CE–CL detector configurations have been developed. The most popular configurations are sheath flow, coaxial sheath flow, on-column and post-column CL. These have been described in detail in Kuyper and Milofsky's [363] and Huang and Fang's [365] review articles. Staller and Sepaniak [362] grouped the instrumental configurations into three groups, one of which comprised both sheath flow and coaxial sheath flow and the second group being end-column detection. The third configuration was a moulded reactor system, in which a low dead volume junction was moulded between a single reaction capillary and multiple inlet capillaries. Separation was made in one of the inlet capillaries, while the others carried reagents.

Tsukagoshi et al. [370–373] have developed several detection cells for post-column CE–CL. The cells can be divided into two distinct groups: batch- and flow-type cells. In the batch-type cells the glass cuvette detection cell also works as the outlet CE reservoir. The group has widely compared the properties of the cell types. Generally, their experience has been that batch-type cells offer more sensitive detection and require less complicated instrumentation than the other cell types. With this cell type the exact positioning of the capillary and the electrode are not critical for the peak height and the plate number. The flow-type cell, on the other hand, requires optimisation of the reaction cell dimensions, capillary tip position and reagent flow rate.

The batch-type cell gives LODs of about 1/3 of the values obtained with the flow-type cell (0.43 fmol versus 1.3 fmol in a TDPO acetonitrile–hydrogen peroxide CL reagent [372], probably as a result of dilution of the sample with the CL reagent in the flow-type cells. However, the flow-type cell was the better suited of these two to continuous repeated injection [372]. Tsukagoshi et al. studied mainly dansyl amino acids and phenolic compounds, and they reported detectable ranges for the compounds in the 0.01–10 μM range [370]. The LODs of the phenolic compounds were in the 0.1 μM range with use of batch-type cells [371]. For luminol in batch-type cells, the group obtained LOD of 0.25 nM (7 amol), which could yet be lowered to 15 pM with acetonitrile stacking and further enhanced by *p*-iodophenol under weakly alkaline conditions [373]. The authors reported that with stacking and enhancement the sensitivity of the signal could be increased more than 400 times.

In the development of the detector configurations, it is necessary to remember that all reagents need not be mixed with the background electrolyte only near the detection point. For example, in luminol–hydrogen peroxide systems the luminol can be a part of the separation solution [374,375]. In designing the BGE one must remember, however, to choose the BGE compounds so that they do not affect the luminescence intensity. A few of the most commonly used surfactants, such as SDS, have been found to diminish ECL intensity [369] and so MEKC–ECL requires optimisation and careful thought. However, cyclodextrins and

Triton X-100 do not normally affect the intensity [369] and can be used in the BGE.

Sample pre-concentration on-line by stacking, sweeping or other similar techniques, although common in, e.g. CE–UV systems, is still rarely seen in CE–post-column-CL. Apparently the LODs obtained with the system have been so good that sample pre-concentration has not been found necessary for practical analyses. Some studies have contained also a pre-concentration step [373–376], but further research in this area would yet be needed since the LODs can improve quite dramatically with stacking. As an example, Liu et al. [376] have reported ultrasensitive CE–CL of cobalt ions with luminol–hydrogen peroxide reaction, detecting even sub-femtomolar concentrations. At so low concentration levels, the contamination control plays a considerable role in the experiment design. The group improved the performance of the detection first by transferring the grounding electrode upstream of the detection point, then by employing a new mixing mode for the analyte and the reagent, and finally by field-amplified sample stacking. The signal was increased by several orders of magnitude until, theoretically, single Co(II) molecules could be detected. In practice, the injection volume probably contained more than just one molecule of Co(II) as a result of the stacking.

Indirect chemiluminescence detection has also been reported. Tsai and Whang [377] have employed indirect luminol–hydrogen peroxide based CL detection for CE analyses. In the indirect CL detection the analytes suppress the signal by complexation with the CL enhancer, usually a metal ion such as Co(II). Tsai and Whang's LODs for monoamines and catechol were in the range 0.5–3.1 μM and thus significantly lower than usually obtained by indirect detection. Also Zhang et al. [378] used the indirect luminol CL detection with CE. In these reports the sensitivity of the method seems to be good despite the indirect detection.

While chemiluminescence is beginning to show some signs of popularity as CE detector, electrochemiluminescence detection in CE is still a rare combination. Forbes et al. [369] were the first to use ECL of $Ru(bpy)_3^{2+}$ for detection of β-blockers separated with CE. $Ru(bpy)_3^{2+}$ was a part of the BGE. It was oxidised post-column and in the oxidised form reacted with the analytes producing light. The CE separation voltage had to be isolated from the vial where the ECL was made to avoid decomposition of water. Huang et al. [379] have combined flow injection sample introduction to CE–ECL. The flow injection functioned with a falling-drop interface, which isolated the flow injection system from the CE operating voltage. The LODs in these two works were roughly the same as in most CE–CL systems (see Table 5). In two recent articles, Cao et al. [380,381] have presented two new CE–ECL couplings. In their end-column ECL system [380] they showed that the electrophoretic current did not affect the ECL reaction of $Ru(bpy)_3^{2+}$ and that the high voltage field only resulted in a shift of the detection potential. They also introduced a solid-state ECL detector [381], in which the reagent was immobilised in a film. The sensitivity of both systems was very good.

These reports show that CE–ECL has potential to become a sensitive and easily applicable analysis system. A great advantage of $Ru(bpy)_3^{2+}$ *red–ox* reactions is that derivatisation is not required for many classes of compounds, since e.g. many amines participate in the reaction.

7.3. Luminescence detection methods in microchip-based CE

Microchip CE, here covering all capillary electromigration techniques applicable on a chip, is growing to be as versatile and easily applicable as ordinary CE. So far most of the reports on this area of research have used CZE in their MCE–CL applications. In future, we will probably see an increasing number of applications in which the separation involves modified surfaces of the microchannels.

The benefits of miniaturisation are extremely fast analysis times, low sample and reagent consumption and the possibility of having a portable instrument, literally a lab-on-a-chip. On the other hand, the low sample consumption requires very sensitive detection systems to be used. In her recent review, Verpoorte [382] has listed the microchip applications involving clinical or forensic samples. Luminescence-based detection techniques are well represented especially in the immunoassays.

For literal lab-on-a-chip approach a LIF detector is too large, but the technique is feasible for use in MCE detection because of its sensitivity and ease of focusing. LIF is becoming one of the most common

detectors for MCE especially with use of glass chips. With use of plastic chips, the LIF detection often has some background noise, which, however, decreases under near-IR wavelengths. For more information on MCE–NIR–LIF the reader is encouraged to turn to McWhorter and Soper's [383] review.

Other photoluminescence–MCE approaches have also been published. For the analysis of amines, Rodriguez et al. [384] have reported 75 s separation times for a MEKC-on-a-chip analysis with fluorescence detection, and Belder et al. [385] have achieved 6 s separation times for other amines with MCE–FL. On-chip enzymatic assays, reviewed by Wang [386], mostly use fluorescence detection.

With CL detection, microchip systems avoid the need for complicated post-separation reagent mixing interfaces and consequent dead volumes. In Mangru and Harrison's development [387], a sample injector, CE separation channel and post-separation CL reactor were fitted on a 7.5 cm × 3 in. × 1.95 mm chip and used for the analysis of mouse IgG. They obtained LODs in the range 9–35 nM using horseradish peroxidase catalysed reaction of luminol in systems with different channel depths. Deeper channels (40 μM versus 10 μM) allowed lower LODs. Hashimoto et al. [388] have also presented a MCE–CL system, in which they analysed dansyl amino acids. All these groups used glass as the chip material.

Liu et al. [389] have studied CL detection for a microchip CE (MCE) system fabricated in poly(dimethylsiloxane). They used Co(II)-catalysed luminol-peroxide reaction and dansyl-conjugated peroxalate-peroxide reaction to compare three chip patterns: cross, cross combining with Y and cross combining with V. The cross-Y combination was promising for the luminol-peroxide CL system and the cross-V combination for the other system. LODs for a dansyl amino acid were 0.39 μM (59 amol) to 8.56 μM (1310 amol) depending on the used MCE strategy. Chiral forms of compounds (dansyl-phenylalanine) were also separated and detected at 5 μM concentration with their MCE–CL system.

Arora et al. [368] have studied the use of a floating platinum electrode in MCE–ECL. The floating working electrode has an electrolytic contact only, not an electronic contact, i.e. the working electrode is in an electric field produced by two auxiliary electrodes and the other end of the floating working electrode is serving as an anode and the other as a cathode [390,391]. The separation [368] involved MEKC on a glass chip. The sensitivity of direct detection of ruthenium complexes was good, that of indirect detection of amino acids was not mentioned but the lowest sample concentrations were 300 μM.

8. Other on-chip applications

Most of the present on-chip applications are based on CE, but we are sure that many other techniques will be emerging in the very near future. Present microchip applications generally utilise optical, electrochemical or mass spectrometry detection. Optical methods involve absorption, refractive index, and turbidance measurements in addition to luminescence methods. Andreas Manz et al. has nicely shown what miniaturisation actually means for LODs when the detection volume drastically decreases (Fig. 11) [415]. The figure displays how the sensitive fluorescence will finally be beaten by potentiometry and refractive index measurements when detection volumes are decreased sufficiently small. In addition, the figure shows an area where less than one molecules exist. Thus, there is nothing to detect unless you have sufficiently time to wait that your analyte will diffuse into your detection volume, or you are able to force the whole sample through your miniature detection volume.

In principle, it is relatively easy to design different functions of a very complicated μ-TAS on a chip, but much more difficult to get it actually work in real world (see, e.g. Burns et al. [416]). However, Webster et al. [417] have fabricated a really working monolithic CE device with an integrated fluorescence detector for separation of DNA fragments. Also single molecules have been detected on a chip [418]. In microscale, there are various different studied ways of mixing solutions by passive and active mixers [419–422], and transport or filtration of liquids or nanoparticles. There are solutions for pre- and post-column derivatisation, cell lysis, extraction, pre-concentration and dialysis [423,424]. Also active and passive microvalves, pumps and microreactors have been developed. You just name it, and maybe can already now find the solution for your problem from the literature.

For instance, PCR has been carried out on chip by Northrup et al. [425], and PCR products have been

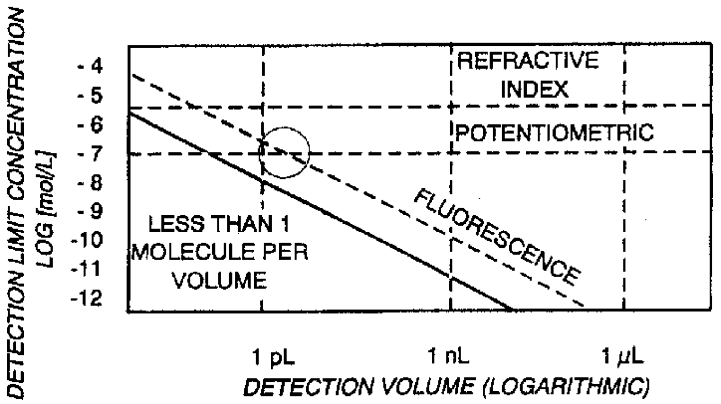

Fig. 11. Detection limits as a function of the detection volume, for refractive index, potentiometric and fluorescence detectors [415].

analysed by Woolley et al. [426]. Also monolithic integrated PCR/CE system has been fabricated [427] and continuous flow PCR on a chip has been carried out [428]. Automated multi-step genetic assays have been described by Andersson et al. [429]. PL-based nucleic acid detection and microarrays has been recently reviewed by Epstain et al. [430].

Different applications need their own solutions for chip-based sample preparation and no universal answer can be given. Sometimes monolithic integration is worthwhile and sometimes discrete components are more economical and reasonable [382,431,432].

Poly(dimethysiloxane) (PDMS) is a nice optically transparent and versatile material for many purposes in micro systems. For instance, Chabinyc et al. have fabricated a PDMS-based chip with integrated optical fibres and an avalanche photodiode [433]; Yang et al. [434] have fabricated microchannels from PDMS for rapid on-chip immunoassay with fluorescence detection. Linder et al. have carried out electrokinetically driven heterogeneous immunoassays in a PDMS/glass microfluidic chip with Cy5 as a tracer [435]. Camou et al. [436] have fabricated 2D optical lenses from this material, and Jensen-McMullin et al. [437] have used PDMS in their chip in which microbeads were trapped for fluorescence detection.

SU-8 is another interesting material, which is originally a resist developed for photolithography purposes but which allows to fabricate relatively thick layers upon smooth substrates, such as silicon and glass wafers, which layers can be channelled as desired. L'Hostis et al. [438] have constructed an ECL detector and a microenzymic reactor combining Si and SU-8 technologies. Both devices were fabricated using standard processing techniques to produce on-wafer sensor elements, which were composed of a platinum or, alternatively, carbon interdigitated electrode array. The platinum array was resting on top of a Si p–n photodiode. After these elements were completed, the entire wafer was modified with SU-8, which was structured to form a series of shaped spacers surrounding each device. For the ECL detector a simple flow-channel was defined, whereas for the microreactor a more complex layout defining two chambers separated by a series of SU-8 columns was employed. The upstream chamber of the microreactor was packed with porous glass beads modified with immobilised enzyme glucose oxidase, whereas the downstream chamber contained the detector. The performance of the ECL detector was assessed by the detection of codeine using $Ru(bpy)_3^{2+}$. A LOD of 100 μM was obtained. Glucose was determined with detection a limit 50 μM by the ECL response.

Also, Fiaccabrino et al. [439] have used $Ru(bpy)_3^{2+}$ in demonstration of their chip. They fabricated a miniaturised and fully integrated probe for assays based on ECL. It combined both the electrode transducer and the photodetector in a single 5 mm × 6 mm Si chip. The device accommodated two identical cells, each consisting of a Au interdigitated microelectrode array (IDA) resting on top of a p–n photodiode, and of which one was used as an active cell and the other as a dummy. Light-intensity measurements were taken in a differential mode to increase the overall

system performances. The device was characterised using Ru(II) tris(2,2′-bipyridyl) and tripropylamine as model system. The highest signal intensities were detected in a phosphate buffer of pH 7.5 and an IDA gap of 0.8 μm. The observed excellent correlation between the peak light intensity and the concentration of the Ru complex over a 0.5–50 μM range.

Using comparable microelectrode array Horiuchi et al. [440] carried out a real-time visualisation of ECL in a microscale region using IDA in a thin layer flow cell. The authors studied the ECL reaction, which was based on the annihilation of cations and anions of 9,10-diphenylanthracene at the IDA, from the spatial and temporal emission patterns in the thin layer (not possible with macroelectrodes). Michel et al. [441] have shown practically same phenomena by demonstrating that $Ru(bpy)_3^{2+}$ label can be excited by redox-cycling of a ruthenium chelate covalently linked to a protein and placed in an aqueous medium, which is not possible at macroelectrodes. ECL measurements were performed in phosphate buffer at carbon interdigitated microelectrode arrays. The light intensity represented 31% of that obtained using free $Ru(bpy)_3^{2+}$ in phosphate buffer containing proteins. No actual immunoassay was carried out probably because the sensitivity would have been insufficient.

Greenwood et al. [442] have constructed a portable μ-TAS drug screening device utilising again the tris (2,2′-bipyridine)ruthenium(II) reaction using both chemiluminescence and electrogenerated chemiluminescence. Zhan et al. have made novel use of $Ru(bpy)_3^{2+}$ ECL [443]. They describe a microfluidics-based sensing system that relies on electrochemical detection and ECL reporting. The important result is that the ECL reporting reaction is chemically decoupled from the electrochemical sensing reaction. That is, the electrochemical sensing reaction does not participate directly in the ECL process, but because electrochemical cells require charge balance, the sensing and ECL reactions are coupled. This provides a means for direct photonic readout of electrochemical reactions that do not directly participate in an ECL reaction and thus broadens the spectrum of redox compounds that can be detected by ECL. The system was used to electrochemically detect benzyl viologen present in solution and report its presence via $Ru(bpy)_3^{2+}$ luminescence.

In addition to the luminescence systems, even a plasma emission has been successfully miniaturised to be carried out on a chip [444].

9. Future developments and prospects

Presently, analytical chemistry has reached a stage where individual molecules can be detected, identified, counted and even their physical and chemical properties can often be measured. The first single molecule that could be detected was Rhodamine 6G [445]. This was obtained by fluorescence, and a derivative of this molecule, tetramethyl rhodamine isothiocyanate (TRITC) is thus useful for extremely sensitive labelling of biomolecules. The most sensitive PL methods are based either on: (i) confocal excitation, (ii) pulsed excitation with time-resolved or time-correlated detection, (iii) two-photon excitation, (iv) sophisticated energy-transfer scheme, or their combinations.

In addition, Bard and Fan [446] has seen two alternative ways to achieve a single-molecule detection: (i) either trapping an electroactive molecule in solution between a small ultramicroelectrode tip and a conductive substrate in a scanning electrochemical microscope, or (ii) the use of ECL, in which the product of the electron transfer of the electroactive molecule reacts to form an excited state that emits a photon.

One field where the ability to detect single molecules is required is HTS, in which extremely fast and low-volume assays are presently being developed. In this field, detection based on luminescence will surely be dominating and at least PL (PL intensity screens, PL polarization screens, PL resonance energy transfer assays, homogeneous time-resolved PL, PL correlation spectroscopy, photoluminescent reporter assays, PL imaging), CL and ECL methods will have an interesting future, but with a hard competition [447].

The strength of CL and ECL methods is a low-cost excitation step because there is no need for expensive lasers or accurate excitation optics. However, the solid-state lasers are becoming all the time better and cheaper, and the price of the instrument in HTS is typically not the most important issue. In direct search, only a couple of references can presently be found with combination of HTS and ECL [448,449]. However,

plenty of patent applications have been filed which consider different types of electrode arrays useful in the HTS applications, and in addition, in other miniaturised analysis methods.

We believe that miniaturisation will induce a revolution in analytical chemistry analogously to that which occurred in electronics earlier but with a less dramatic impact. The benefits of miniaturisation are easy to see: (i) decreased sample volume, (ii) decreased reagent cost, (iii) faster analysis, (iv) parallelisation, integration and automation becomes possible and (v) portable instruments can be constructed. Lab-on-chip (μ-TAS, Micro Total Analysis System) applications will surely have uttermost importance in many areas of analytical chemistry. Very recent excellent reviews can be found virtually in any areas of miniaturisation: State of the art in miniaturised separation techniques [450], microfluidics in general [451,452], bioanalysis in microfluidic devices [453], life science in general [454,455], chemistry in general [456], sample manipulation [431], etc.

Nano- and microfabrication plays an ever increasing role in science and technology and will soon allow the scientists to build systems of the same complexity as found in nature. Conventional methods that emerged from microelectronics are now used for the fabrication of structures for microelectromechanic systems, micro-optics and microanalytical devices [457–459].

The areas of environmental analysis and POC desperately need portable low-cost analysers and the new emerging technologies seem to make the construction of these devices both technically and economically easier and easier [460,461]. Luminescence methods together with electroanalytical methods will be in the most important role also in these areas.

We believe that electrochemistry with ultramicroelectrodes using voltammetric or amperometric instrumentation will very often lose the race with luminescence-based methods, but potentiometry will be strong in the areas and concentration levels in which it can be applied. The ECL and other CL methods will dominate in certain applications, but PL will be the most popular tool for analysis, at least for the present decade, and possibly for ever. All in all, luminescence methods seem to have very bright lights ahead, although very low light levels are being detected.

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