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Optochemical Sensors Based on Luminescence

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CONTENTS

1. INTRODUCTION

In the past two decades researchers have shown a great interest in the area of optical chemical sensors resulting in rapid development of such sensors with applications in fields such as industrial, environmental and medical. In general, the transducer in optochemical sensors is converted into a optical signal for real-time and on-line information about the presence of specific compounds or ions in complex samples. Depending on the origin of the optical signal, the devices are classified as absorbance, reflectance, luminescence, Raman, light scattering, or chemoluminescence sensors [1, 2]. Whereas at the beginning of the era of optochemical sensors the detection of absorption or fluorescence with conventional spectrometer was the center of interest, the developments in telecommunication have paved the way for cheaper and miniaturized sensor readout equipment. These developments have also been over the past two decades the driving force for the success of luminescence based sensors, to which this chapter is dedicated to.

Optical-sensing techniques have been widely used for quantitative measurements of various analytes such as H^+ [3–5], carbon dioxide [6, 7], oxygen [8–11], metal ions [12, 13], ammonia [14, 15], glucose [16, 17] and humidity [18, 19] in environmental, industrial, clinical, medical and biological applications. Optochemical sensors in general offer many advantages over other sensing techniques [20, 21] in the way that optical chemical sensing does not consume analytes, no reference is required and the signal is insensitive to sample flow rate, stirring speed and exterior interferences. Additionally, optical sensors have the potential for miniaturization, remote sensing and easy installation when optical fibers are used [12, 22].

Among the various optical methods, fluorescence detection seem to offer the advantages of high sensitivity and ion-selective fluorescence probes. During the past several years there has been increased interest in lifetime-based sensing [23], which is preferred over intensity-based methods because the lifetime is mostly independent of the probe concentration and can be unaffected by photobleaching or washing out of the probe. As an analytical tool for life-time based sensors there is an increasing interest in the use of time-resolved fluorescence [23]. The basic idea is to identify fluorophores or sensing schemes in which the decay time of the sample changes in response to the analyte concentration. Such lifetime-based sensing is most often performed using the phase-modulation method or frequency domain. The use of phase angles or decay times are mostly independent of the signal level and can be measured in turbid media.

The aim of this contribution to the Encyclopedia of sensors is to provide the reader the basic principles of lifetime-based optochemical sensors focusing on phase-modulation fluorometry as the measurement principle. Section 2 deals with detection principles for luminescence based sensors, where the differences between intensity and luminescence lifetime (time-domain and frequency-domain) measurements are described. Section 3 discusses instrumentation for the phase measurement technique focused on the principle set-up of phase measurements, different phase detection principle and limiting factors for performance of phase measurements as well as optical components in instrumentation set-up. Last Chapter 4 describes sensor schemes for lifetime-based optochemical sensors using the phasemodulation method. This chapter reviews chemical detection principles used in lifetime-based sensors for monitoring pH, oxygen and carbon dioxide.

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2. DETECTION PRINCIPLES FOR LUMINESCENCE BASED SENSORS

2.1. Luminescence Lifetime versus Intensity Measurements

Luminescence is observed when the energy of an electronically excited state species (luminophore or dye) is released in form of the light. Luminescence is divided into two subcategories, fluorescence and phosphorescence. They differ from the photophysical processes which occurs in the molecule during excitation and emission [1]. Depending on the whether the excited state is singlet or triplet, the emission is called fluorescence (in this case the molecule returns directly to the ground state) or phosphorescence [24, 25]. This results in completely different lifetimes of these two categories. Fluorescence lifetime or decay time typically occurs over tens of nanoseconds (10^{-9} to 10^{-7} s), phosphorescence occurs over much longer time periods (milliseconds; 10^{-5} to 10 s). One has to differentiate between the cases in which the luminophore is the analyte itself (emitting the luminescence signal for detect) from the cases in which the analyte quenches the luminescence signal of the luminophore (in this case the luminophore acts as an indicator for the analyte).

In opto-chemical luminescence based sensors a reduction of luminescence caused by the analyte is detected. Two characteristics of the luminophores can be applied for measuring this reduction of luminescence. On the one hand there is the possibility to measure luminescence intensity, which can be generally applied in sensors with all available luminophores. In principle the measurement of the luminescence intensity offers an easy and cost effective sensor. However, the measurement of luminescence intensity, suffers from optic interferences caused by changes of turbidity, refractive index or color of the sample, fiber bending and microbending of the fiber tip. Intensity signals generally are strongly disturbed by fluctuations in the opto-electronic system (drifts of the light source or photodetector).

Furthermore, degradation of the indicator/dye in the sensitive membrane caused by photobleaching and leaching is very critical. Consequently for practical applications some important properties of such instrumentations have to be considered to get a stable system. Gruber et al. [26] introduced an instrumentation based on luminescence intensity measurement which tries to compensate opto-electronic interferences with a second photodiode. Consequently deviations of the excitation light, caused by temperature effects or ageing of the light source, can be compensated. Optical interferences and interferences resulting from the sensitive membrane, however, cannot be compensated by this technique.

On the other hand there is the possibility to overcome most of these effects by measuring the luminescence lifetime[1], as a parameter which is almost independent of the absolute signal height. Intensity changes in the excitation light as well as deviations in the optical path do not cause changes in the measurement signal and furthermore the influence of the photobleaching behavior is reduced dramatically. Unfortunately, luminescence lifetime based sensors are restricted only to a few dyes with certain characteristics. However, sensing based on luminescence lifetime provides certain benefits [27]. This approach is able to overcome the problems which affect intensity-based sensors such as indicator concentration, photodegradation or leaching of the dye, ageing of the light source or any changes in the optical path which also influences the intensity of the detected luminescence. In particular, photobleaching of the dye is one of the main factors which limits the stability of optochemical sensors in practical applications. With the assumption that the resulting photoproducts do not gain luminescence and if the changes in microenvironment of the dye are not relevant, the photobleaching causes only a decrease of luminescence intensity but does not show an effect on the lifetime behavior of the sensor. Developments in the last years have enabled some of these restrictions to be overcome and consequently luminescence lifetime based sensing schemes are available for a significant amount of analytes today.

In Table 1 the major distinctions between intensity and lifetime measurements from an instrumentation point of view are summarized. It can be concluded that lifetime measurement is the more advantageous detection principle because of its insensitivity to fluctuations of instrumental

Table 1. Major distinctions between intensity and lifetime measurements with particular consideration to properties which are relevant for practical applications.

Property	Intensity measurement	Lifetime (phase-) measurement
Excitation intensity	Proportional to excitation intensity	Theoretically independent
	Dependent on deviations of the excitation light source (temperature, ageing)	(τ ... intrinsic dye property)
Optical arrangement	Deviations in the optical path (spatial distribution, soiling) results directly into an intensity change	Theoretically independent
Sensor production	Luminescence is proportional to the amount of dye and the thickness of the sensing layer	oxygen characteristic shows little dependence on dye concentration in the polymer
Calibration	2 point calibration necessary	In-production and 1 point re-calibration
Background sensitivity	Direct relationship	Vector like addition
Photobleaching	Direct proportional	Theoretically independent, practically lower than in intensity measurements (problems with background signal)
Signal/noise ratio		Worse (higher measurement frequencies)
Resolution		Worse at the same irradiation intensity

[1] Lifetime is defined as the average time a molecule remains in the excited state.

artifacts and the lower photobleaching effects together with the benefits concerning sensor production and calibration. In contrast to absorption methods, no reference measurement is necessary, and, in contrast to fluorescence-intensity measurements, no compensation for variation of the mentioned instrumental parameters is necessary.

2.2. Luminescence Lifetime Measurement Methods

The luminescence lifetime of a sample is the mean duration of time the luminophore remains in the excited state after excitation of the luminophore with a short light pulse (see Fig. 1). Following pulsed excitation, the intensity decays of many luminophores are single exponential [28]:

$$I(t) = I_0 e^{-(t/\tau)} \tag{1}$$

where I_0 is the intensity at $t = 0$ and τ is the lifetime.

For the detection of the luminescence lifetime there are in principle two available techniques. Firstly, the direct (*time domain*) method and secondly, the indirect (*frequency domain* also referred as *phase modulation*) method. The time domain method monitors the emission signal in time domain and evaluates the decay profile of the signal. On the other hand the frequency domain method uses sinusoidally modulated excitation signals at certain frequency and detects the time delay (phase shift) of the emission signal of the sensor dye to calculate the lifetime. Both approaches provide the same information for an assumed single-exponential decay characteristic, because of their Fourier-transform relationship.

2.2.1. Time Domain Measurements (Direct Method)

Measurement of the time dependent emission which follows excitation with a brief pulse of light as shown in Fig. 1 is the so called time domain or direct method. For these short excitation pulses flash-lamps, lasers or synchron radiation sources are commonly used as light sources. Furthermore, detectors (photomultiplier tubes PMTS) with a wide bandwidth (high speed) are necessary in combination with fast signal sampling circuits (oscilloscope). Another variation of the time domain measurement is the so called step-measurement. In this case instead of the short-pulse excitation the sensor is illuminated with a rectangular signal and the responding signal from the sensor follows the step with an exponential rise. Instead of the fact that in this case can be used slower components for the excitation, the detection electronics has to remain the same and consequently the high cost instrumentation is inevitable. In addition to this large and costly instrumentation it is often necessary to use deconvolution treatment to extract the decay curve if the excitation pulse duration and the signal transition times of the detectors are not negligible [29]. All these facts point to an instrumentation which uses frequency domain measurements which can be realized with less cost and printed circuit board (PCB) size, which is quite useful for application of optochemical sensors in industrial and biochemical environments.

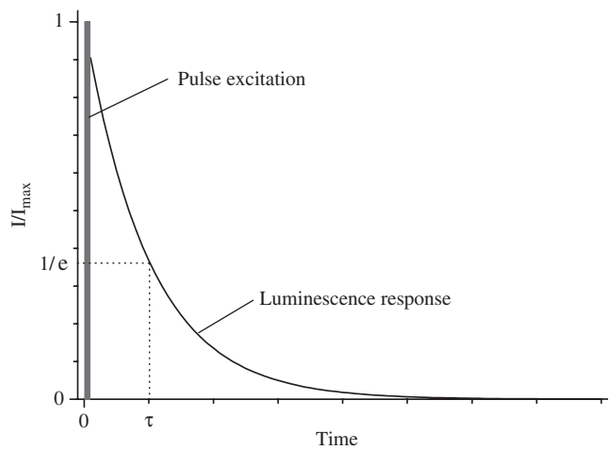

Figure 1. Characteristic luminescence decay curve after pulse excitation.

2.2.2. Frequency Domain Measurements or Phase-Modulation Fluorometry (Indirect Method)

In contrast to time domain measurements in phase-modulation fluorometry an intensity modulated (sinusoidally) light source at certain frequency can be used instead of pulse sources. Because of the time lag between absorption and the emission of the dye, the emission is delayed in time relatively to the excitation signal. This delay is described by the phase shift between these two signals (see Fig. 2).

With the following equations the relationship between excitation signal and emission signal of the system is described.

The excitation function $E(t)$ can be expressed as the sum of a constant intensity term and a sinusoidally varying intensity term [30].

$$E(t) = E_0(1 + M_E \sin(\omega t)) \tag{2}$$

where the steady state DC-fraction with an angular frequency of c0 is represented by E_0 and M_E is the modulation

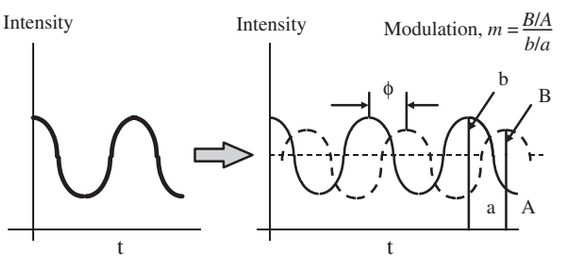

Figure 2. Scheme of the phase-modulation fluorimetry for lifetime-based sensing: the fluorophore is continuously excited by an intensity-modulated light beam. The emission is consequently modulated at the same frequency. Due to the finite fluorescence lifetime of the fluorophore, the emission is phase shifted (ϕ) and demodulated ($m = bA/aB$) with respect to the excitation. The fluorescence lifetime is calculated from either the phase shift or the demodulation values.

index of the excitation signal. It is defined as ratio of the AC-amplitude to the DC-intensity and can be set $M_E \leq 1$.

The emitted time dependent luminescence response coming from the sensor remains unchanged in frequency and is sinusoidal as well. However, the luminescence signal is time delayed (phase shifted) relative to the excitation signal. This can be expressed as follows:

$$L(t) = L_0(1 + M_L \sin(\omega t + \phi)) \tag{3}$$

Modulation index of the luminescence signal M_i, will be less than that of the excitation signal. On the subject of the intensity of the signal L_0 it must be said that it is smaller but proportional to E_0 and is determined by the absorption coefficient, concentration and quantum efficiency of the luminescent dye, the used matrix and the thickness of the layer and as well by an instrumental factor representing the optical arrangement.

2.2.3. Phase-Lifetime Relationship

The relationship between the luminescence lifetime and the measurement of the phase shift is described by the following equations.

If a single-exponential decay is assumed for the general case, the impulse response of the luminescence signal $a(t)$ is given as follows:

$$a(t) = \alpha \cdot e^{-(t/\tau)} \tag{4}$$

where α is the intensity at time $t = 0$. To describe this time-domain behavior in the frequency domain, the Fourier-transformation can be used and following equation is obtained:

$$A(j\omega) = \frac{\alpha \cdot \tau}{1 - j\omega \cdot \tau} = \frac{\alpha \cdot \tau}{1 + \omega^2 \cdot \tau^2} - j \cdot \frac{\alpha \cdot \omega \cdot \tau^2}{1 + \omega^2 \cdot \tau^2} \tag{5}$$

where $\omega = 2\pi f$ is the circular frequency. If this expression's relationship with signal processing theory is considered, this function describes a first order low-pass filter.

Consequently the phase shift can be derived as follows:

$$\phi = \arctan\frac{\mathrm{Im}(j\omega)}{\mathrm{Re}(j\omega)} = \arctan(\omega \cdot \tau) \tag{6}$$

This equation represents the basic relationship of phase shift Φ and lifetime τ of the system.

The magnitude of the signal can be written as:

$$|A(j\omega)| = \frac{\alpha \cdot \tau}{\sqrt{1 + \omega^2 \cdot \tau^2}} \tag{7}$$

The magnitude of the signal also depend on the frequency. This behavior can be expressed by the degree of modulation M:

$$M = \frac{1}{\sqrt{1 + \omega^2 \cdot \tau^2}} \tag{8}$$

After rearrangement of Eq. (6) and incorporation of this in Eq. (8), the following relationship between the modulation and phase shift Φ is obtained:

$$M = \frac{1}{\sqrt{1 + \omega^2 \cdot \tau^2}} = \frac{1}{\sqrt{1 + \tan^2 \Phi}} = \cos \Phi \tag{9}$$

These are the relationships of lifetime, phase and modulation data for ideal single-exponential decay behavior. In this case the determination of lifetime with phase or modulation measurement must be equal and independent of the modulation frequency [31]. Unfortunately, for practical sensor measurements an ideal single-exponential model can rarely be assumed. If the emission is characterized by multi exponential decays, the above calculations have to be modified. Therefore, the lifetimes cannot be determined using a single modulation frequency. In order to analyze the lifetime fractions, multi-frequency phase and modulation measurements are required. For a general case the multi-exponential system can be represented by a sum of N exponential decays:

$$\alpha(t) = \sum_{i=1}^{N} \alpha_i \cdot e^{-(t/\tau_i)} \tag{10}$$

where α_i is the contribution to the amplitude of the i-th component and τ_i is the lifetime of the i-th component.

This is a shortly description of the theoretical background for implementation of a phase measurement system. For real world applications, the instrumentation for the phase measurement technique in detail is described and presented with a schematic block diagram in the next section. Afterwards, the major components and the key factors for a successful realization of such an instrument are presented.

3. INSTRUMENTATION FOR THE PHASE MEASUREMENT TECHNIQUE

The main objective of the phase-sensitive detection for phase fluorometric applications is the determination of the phase shift of the luminescence signal relative to the modulation signal. Practically that means, that the measurement is performed at known frequencies, which are generated by circuits in the same instrument. In consequence all systems consist of a signal generation unit, a signal detection unit and one unit to compare these two signals to obtain the phase shift. A principle set-up of such a phase fluorometric instrument is shown and described in Section 3.1, with remarks concerning some practical aspects like temperature dependence and component tolerances which can be eliminated by a referencing system.

Since the heart of a phase fluorometric instrument is the phase detector, several principle setups with particular blocks are described in Section 3.1.

3.1. Principle Setup of Phase Measurement Systems

A short diagram overview of the setup is given in Fig. 3 to identify all the relevant components of a phase fluorometric instrument. Based on this scheme the characteristics and relevant specifications of the used functional blocks are explained.

A frequency generator provides a signal of a certain frequency to modulate the intensity of a light source using an optimized driver circuit. The light illuminates the sensitive dye via an optical excitation filter. A photodetector in combination with another optical filter collects the luminescence

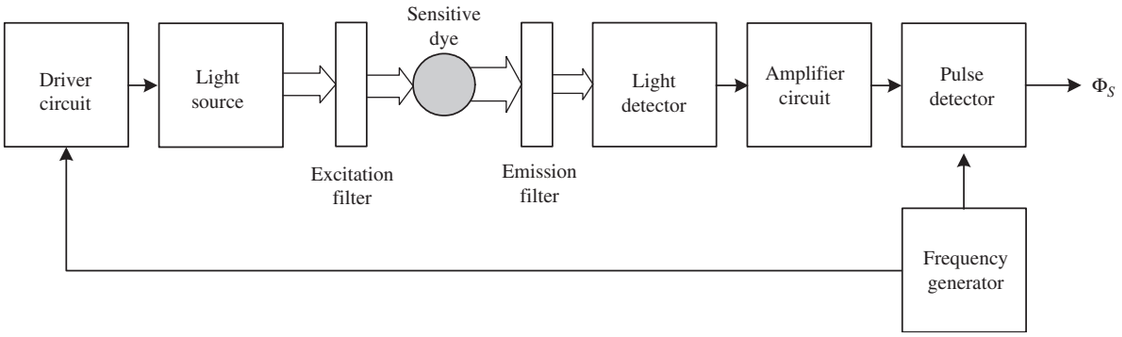

Figure 3. Principle set-up of a phase fluorometric instrument.

coming from the sensitive dye. These obtained signals are then amplified with appropriate amplifier circuits and are fed afterwards to the phase detection unit, which determines the phase shift of the incoming signal relative to the modulation signal produced by the frequency generator.

Considering the signal path of Fig. 3, the measured phase shift of the system, which occurs in the set-up between signal generation and multiplication with the reference signal, consists of the combination of:

- the phase shift due to the modulation of the light source. It depends on the time response characteristic of the used light source (e.g., LED) in combination with a suitable driver circuit;
- actual phase shift due to change at the sensitive dye;
- phase shift of the detection circuitry including the photodetector (e.g., photodiode), photodetector amplifier, additional amplifier and signal conditioning circuits;
- phase shift of the multiplier stage in the phase detection unit (in particular the phase shifts resulting from generation of the in-phase and quadrature reference signals which can be neglected, when using a digital implementation).

Furthermore the phase shifts caused by electronic and optoelectronic components are changing with temperature, ageing and also the component tolerances cause errors which cannot be neglected. So there is the need for a referencing system to eliminate all these undesired phase shifts of the system. In principle therefore several implementation strategies can be used to overcome this problem:

- The use of a second photodetector and amplifier circuit, which exclusively detects the excitation light. The phase shift of the sensitive dye can be calculated by subtracting this second phase shift from the phase shift of the luminescence detector. Unfortunately this method only works under the assumption that the detection circuits behave exactly equally. In reality this is difficult to implement, because of component tolerances and temperature behavior of the sensitive components.
- The measurement of the excitation light by removing the optical emission filter in front of the photodetector and preventing that the light can excite luminescence of the sensor. This is a laboratory method, and can hardly be applied in practical setups.
- Usage of a luminescence standard (sensor foil with a specified fluorescence time independent of the analyte concentration) instead of the indicator to calculate the instrumentation phase shift. For this method a similar restriction for practical setup occurs as mentioned above.
- The use of a second light source (e.g., a reference LED) with an emission spectrum in the range of the luminescence emission spectrum. The light of this second light source does not excite any luminescence, but can easily pass the emission filter in front of the photodetector. This method allows the exclusive determination of the detector circuitry alone. It keeps the phase shift of the light source-driver circuit as well as with the light source itself, which characteristic is easier to handle and therefore this referencing method, which is illustrated in Fig. 4 as a good choice for a real setup.

With an alternatively phase measurement of the reference (Φ_S) as well as the signal (Φ_R), the phase shifts caused by the system can be eliminated by subtraction of the signal and reference signals:

$$\Phi = \Phi_S - \Phi_R \tag{11}$$

In case of using this referencing method the developer has to keep in mind that the signal and the reference light sources have to be very carefully selected, because different characteristics of the switching times (also over temperature) of these two components may drastically affect the phase measurement.

3.2. Phase Detection Principles

Since the beginning of phase measurement in the late 19th century many systems based on different technologies and principles have been developed. A detailed historical perspective is given by C. Kolle in Ref. [32]. In present times analogue instruments have been almost replaced by digital based measuring devices. However for every application one should be mindful to maintain the complexity and therefore the costs as low as possible for the requirements of the system. Recent developments in logic-cells, μ-Controller and digital signal processor (DSP) technologies caused a shift of the detection techniques to digital techniques with a significant improvement on resolution and detection limits. Nevertheless on this point a short overview of the most

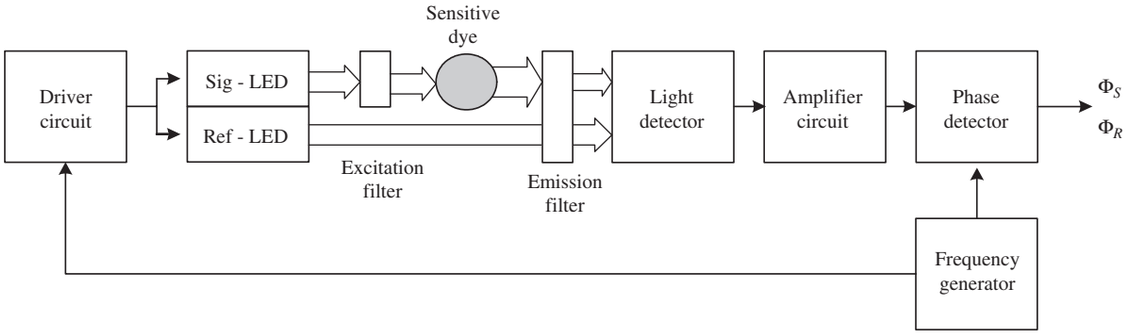

Figure 4. Set-up of a phase fluorometric instrument with a second LED for a system reference measurement.

common phase detection principles will be given in following sections.

3.2.1. Zero-Crossing Detection

The principle of the zero-crossing detection is based on the measurement of time shift of the zero-crossings of DC-less modulation and luminescence signals. Therefore, comparators can be employed as zero-crossing detectors to produce rectangular digital signals and out of them the phase shift measurement can be reduced to a precision time measurement.

In the majority of cases for time measurements counting devices with a precision time reference (quartz oscillators) are used. Another approach is the simple technique of conversion of the time shift into a duty-cycle using one "exclusive-or" gate. If the phase shift is zero, the output signal is zero as well, otherwise if the phase shift is 180° then the output signal is constant one. All phase shifts in between these extremes are representing a pulse-width modulated signal, which is proportional to the phase shift of the luminescence signal in comparison to the modulation (reference) signal. The principle is also shown in Fig. 5.

The signal coming from the optical detector is amplified and band-pass filtered (A) to eliminate the DC fraction and the higher harmonics of the signal. With the comparator (C) a phase shifted transistor-transistor-logic signal (TTL) is produced, which is fed into an "exclusive-or" gate (EX-OR) as well as the excitation square wave. This acts as an phase discriminator whose output gives a duty-cycle signal. This out-coming pulse train can be averaged by a low-pass filter (LP) whose output represents a voltage signal corresponding to the measured phase-shift. In general this principle is called as "time-to-amplitude converter" and represents a simple and powerful method for a phase sensitive detection system in several applications [33, 34].

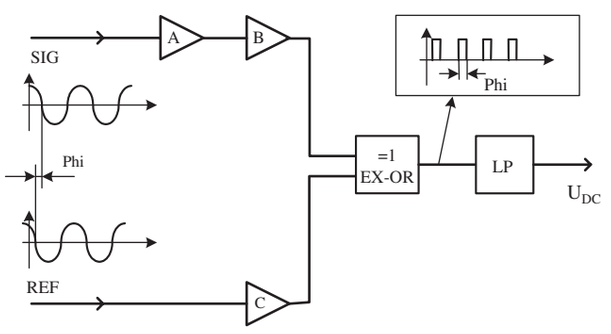

Figure 5. Basic principle of phase shift measurement using an "exclusive-or" gate.

3.2.2. Synchronous Demodulation

Another promising technique for the fulfilling the demands of phase sensitive detection is the synchronous demodulation. The principle of this method is based on a high correlation of the signal of interest and a reference signal, which is locked to the signal of interest and therefore the term "lock-in system" is used for such systems. This detection is often applied to recover signals from noise, because the method captures only a very narrow band signal energy contained in the fundamental harmonic of the signal. A comprehensive and practical insight into this technique was given by Meade [35] and Mandelis as well [36]. All lock-in systems can be decomposed into the basic structure which is shown in Fig. 6.

In the figure shown above the signal of interest $s(t)$ is fed into two multiplier stages which are the key elements of this systems. The goal is to determine the phase shift of signal $s(t)$ to an reference signal $r(t)$ which has to be precisely synchronized with the signal of interest. The signal $s(t)$ is on the one hand multiplied by an in-phase reference signal $r_i(t)$ and

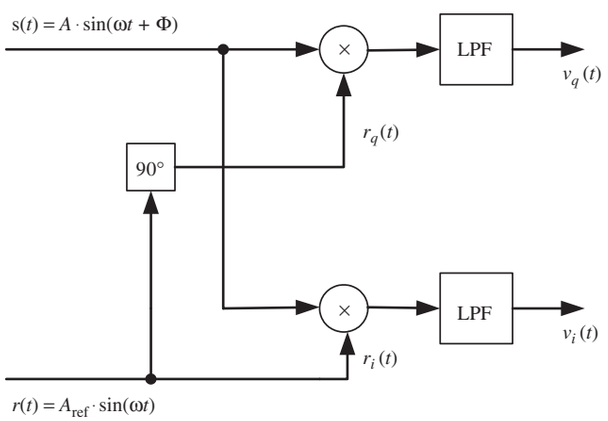

Figure 6. Structure of the synchronous demodulation principle.

on the other hand by an quadrature reference signal $r_q(t)$, which is shifted by 90° with respect to $r_i(t)$. The outputs of the two multipliers are then introduced into low-pass filters to firstly eliminate the second harmonic signal of the multiplier and secondly to operate as integrator which allows very narrow bandwidth detection. The two output signals v_i and v_q constitute the in-phase and the quadrature components of a vector-representation which can be described by a magnitude and a phase. Mathematically this circuit is described with following equations:

$$v_i(t) = s(t) \cdot r(t) = A \cdot \sin(\omega t + \Phi) \cdot A_{\text{ref}} \cdot \sin(\omega t) \quad (12)$$

With assumption of $A_{\text{ref}} = 1$, one obtains

$$v_i(t) = \frac{A}{2} \cdot (\cos(\phi) - \cos(2\omega t + \Phi)) \quad (13)$$

The signal averaging, which is performed by the low-pass filter, eliminates in the case of ideal lock-pass filter the second harmonic signal at the frequency 2ω. Hence the output signal is expressed as:

$$v_i(t) = 2 \cdot \cos \Phi \quad (14)$$

The similar treatment of the quadrature reference signal $r_q(t)$ yields to

$$v_q(t) = s(t) \cdot r(t) = A \cdot \sin(\omega t + (\Phi)) \cdot A_{\text{ref}} \cdot \cos(\omega t) \quad (15)$$

and eventually

$$v_q(t) = \frac{A}{2} \cdot \sin(\Phi) \quad (16)$$

From these results the magnitude R and the phase Φ can be calculated using the following equations:

$$R = \sqrt{v_i^2 + v_q^2} \quad (17)$$

$$\Phi = \arctan \frac{v_q}{v_i} \quad (18)$$

In opposite to other methods the synchronous detection has the essential advantage in providing the determination of a phase shift as well as a value for the signal magnitude, which is able to deliver important information on the system. During many stages of the development of the optoelectronic, optic and chemical parts of the system and also during operation of the optochemical measurement system, the magnitude can provide information on quality and stability of the optic/optoelectronic parts, ageing of the optical components (LEDs), quantum yield and photobleaching of the sensitive dye.

3.2.3. Cross Correlation Technique

A common approach, which is employed for phase measurements with high frequencies, is so called the "cross-correlation" technique. This method is characterized by multiplication of the high frequency signal with a signal, whose frequency is only slightly different. The different frequency, which is called the cross-correlation frequency maintains the phase shift information. The actual phase shift detection can then be performed at a low frequency with improved sensitivity and precision.

Such a system, which operates at two frequencies, was first implemented by Spencer and Weber [37]. Another continuous multi-frequency cross-correlation phase and modulation fluorometer was reported by Gratten and Limkemann [38], where a detailed mathematical treatment of this cross correlation technique was given. This cross-correlation method is now state of the art in all frequency-domain fluorometers and modulation frequencies of several Gigahertz can be achieved using this technique [39].

However this heterodyning technique is dominant at high frequency applications with high-speed optical detectors like PMTS. In practical sensor applications the frequencies are lower and low-cost solid state photodetectors can be used. In 1995 Gruber et al. [40] described a heterodyning demodulator in which the polarity of the photodiode is switched, which corresponds to a multiplication with $+1$ and -1.

3.2.4. Phase Locked Detection

The phase locked detection of fluorescence lifetimes differs in several aspects from common synchronous detection schemes. It is based on phased locked loops and the lifetime is converted to a repetitive output signal, which serves as modulation signal as well. In this approach the frequency of the signal is directly proportional to the luminescence lifetime. The frequency or the period of the resulting signal is a parameter which can be easily determined with high precision. However, this technique is only suitable for lifetimes greater than 1 μs because of the restriction by the electronics available.

3.3. Performance Limiting Factors for Phase Measurement Systems

As already mentioned at the beginning of this document the using of a phase measurement instrumentation instead of an intensity measurement has several advantages. Nevertheless for such instrumentations some special properties caused by the sensitive dye and by the measurement at a certain frequency have to be considered to get an optimized instrument. Therefore in this section some essential properties will be pointed out.

3.3.1. Optimum Modulation Frequency

The determination of the optimum modulation frequency is often carried out in an easy experimental way with regard to the maximum phase shift at the output. However this optimization procedure does not automatically imply that the resolution of the system is maximized, because of different signal to noise ratios (SNR) at different measurement frequencies. The SNR depends to a large value on the excitation light intensity and unfortunately a high illumination intensity causes fast photobleaching of the dye. So the

major goal is to optimize the modulation frequency to maximize the SNR and keep the excitation light intensity as low as possible.

For the theoretical approach an ideal single-exponential decay of the sensor luminescence is assumed. After rearranging the Stern-Volmer equation

$$C_x = \frac{\tau_0/\tau - 1}{k_q/k} \tag{19}$$

the luminescence lifetime as a function of the quencher concentration can be expressed as:

$$\tau = \frac{\tau_0}{1 + K_{SV} \cdot C_x} \tag{20}$$

where τ_0 and τ denotes the lifetimes in the absence and the presence of quencher (e.g., oxygen), respectively, k_q the transition due to radiationless deactivation by a quencher, k decay rate, C_x the concentration of the quencher (e.g., oxygen) and K_{SV} is termed as Stern-Volmer quenching constant.

The lifetime sensitivity (i.e., phase change due to lifetime change) can be derived as:

$$s^{\tau}_{O_2} = \frac{d\tau}{dO_2} = -\frac{\tau_0 \cdot K_{SV}}{(1 + K_{SV} \cdot O_2)^2} = -\frac{\tau^2 \cdot K_{SV}}{\tau_0} \tag{21}$$

For the special case at 0% of quencher the sensitivity simplifies to:

$$s^{\tau}{}_{O_2}{}^{*} = -\tau_0 \cdot K_{SV} \tag{22}$$

These are the basic relationships calculating the lifetime sensitivity when the Stern-Volmer constant and the quencher free lifetime are known. In a phase-fluorometric measurement the phase sensitive information is required. Using the Eq. (6) the phase sensitivity s^{ϕ}_{τ} (i.e., phase change due to lifetime change) can be expressed as follows:

$$s^{\phi}_{O_2} = \frac{d\phi}{d\tau} = \frac{\omega}{1 + \omega^2 \cdot \tau^2} \tag{23}$$

From this equation it can be seen that the sensitivity not only depends on the lifetime but also on the modulation frequency. In order to find the optimum frequency for maximum sensitivity at a specific lifetime one can differentiate Eq. (23) with respect to co and set the result equal to zero, i.e.,

$$\frac{d}{d\omega}\left(\frac{d\phi}{d\tau}\right) = \frac{1 - \omega^2 \cdot \tau^2}{(1 + \omega^2 \cdot \tau^2)^2} = 0 \tag{24}$$

As a result one obtains $\omega_{opt}\tau = 1$. From this follows a phase shift of 45° (= arctan1). Hence the optimum frequency f_{opt1} is given by

$$f_{opt1} = \frac{1}{2 \cdot \pi \cdot \tau} \tag{25}$$

This relationship is optimized to obtain the maximum phase shift for a given luminescence lifetime. Practically τ varies with analyte concentration and therefore an optimum frequency across a lifetime range must be chosen. In order to select a frequency for a given measuring range limited by the lifetimes τ_1 and τ_2, the optimum frequency for a maximum phase shift difference $\Delta\phi$ can be derived as follows:

$$\Delta\phi = \arctan(\omega \cdot \tau_1) - \arctan(\omega \cdot \tau_2) \tag{26}$$

$$\frac{d(d\phi)}{d\omega} = \frac{\tau_1 - \tau_2 + \omega^2 \cdot \tau_2^2 \cdot \tau_1 - \omega^2 \cdot \tau_1^2 \cdot \tau_2}{(1 + \omega^2 \cdot \tau_1^2) \cdot (1 + \omega^2 \cdot \tau_2^2)} = 0 \tag{27}$$

Therefore, the optimum modulation frequency f_{opt1} to maximize the phase difference across the measuring range is given by:

$$f_{opt1} = \frac{1}{2 \cdot \pi} \cdot \sqrt{\frac{1}{\tau_1 \cdot \tau_2}} \tag{28}$$

3.3.2. Phase Noise Using the Synchronous Demodulation

Actually the resolution of the system is limited by the noise of the detected luminescence signal and the sensitivity, whereby in case of phase-fluorometry can be defined as:

$$s^{\phi}C_x = \frac{d\phi}{dC_x} \tag{29}$$

That means the phase shift change per quencher change and this property is given by the used quencher dye. In practice the noise is described by its root mean square value or the standard deviation, respectively. However the noise distribution is problematic and therefore the maximum peak-to-peak value, which determines the resolution, cannot exactly be given. Therefore it can be described by a probability that the actual signal will exceed nominal peak-to-peak values. A very common specification is a confidence interval of $\pm 3\sigma$ whereby 0.27% of all data points will exceed the limit, whereby 99.73% remain within the bounds. While in amplitude measurements the root mean square value of phase noise is defined by the system alone, in phase measurements it is a function of the actual amplitude of the signal.

In order to find the relationships of amplitude and phase noise a phasor representation is displayed in Fig. 7 for the modulated luminescence signal A.

As it can be seen from Fig. 7 the noise phasor $n(t)$ can be represented by an in-phase $n_i(t)$ and an quadrature $n_q(t)$

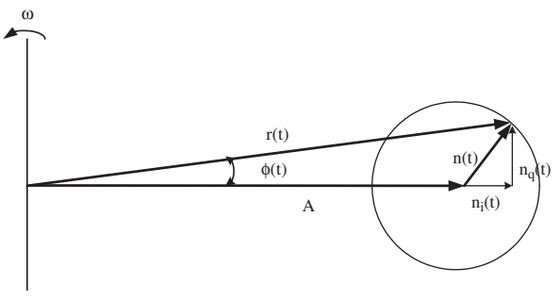

Figure 7. Phasor diagram of the sum of signal and narrowband noise.

part. The signal phasor A and the noise phasor $n(t)$ together yield in the measured signal $r(t)$, which can be described as:

$$r(t) = [A + n_i] \cdot \sin \omega t - n_q \cdot \cos \omega t = R(t) \cdot \sin(\omega t + \phi(t)) \tag{30}$$

This is allowed because the synchronous demodulation captures only the very narrowband signal energy contained in the fundamental harmonic of the signal.

The result of the vector like addition of the signal and the noise phasor is the introduction of both, amplitude noise [in $R(t)$] and phase noise [in $\phi(t)$]. Thus the Eq. (30) can also be written in form:

$$r(t) = \sqrt{[A + n_i(t)]^2 + n_q^2(t)} \cdot \sin\left(\omega t + \arctan\left(\frac{n_q(t)}{A + n_i(t)}\right)\right) \tag{31}$$

Assuming that the noise is small $n_i(t)$, $n_q(t) \ll A$, the amplitude can be approximated by

$$r(t) \approx A + n_i(t) \tag{32}$$

and the value of the phase noise angle is

$$\phi(t) \approx \arctan\frac{n_q(t)}{A} \approx \frac{n_q(t)}{A} \tag{33}$$

The phasor diagram in Fig. 7 is a "snapshot." Considering that the noise parts $n_i(t)$ and $n_q(t)$ are gaussian random processes, their mean values are zero and that the noise phasor spends an equal amount of time in any place within the envelope of the circle, it is clear that the mean values of them are equal. Furthermore with assumption of uncorrelated noise the following relationship can be established:

$$\overline{n^2(t)} = \overline{n_i^2(t)} = \overline{n_q^2(t)} \tag{34}$$

or

$$\sigma_n^2 = \sigma_{n_i}^2 = \sigma_{n_q}^2 \tag{35}$$

what means that the variances (mean square values or power) of $n(t)$, $n_i(t)$ and $n_q(t)$ are equal.

The SNR of the amplitude, which is represented by the ratio of the root mean square value of the signal to the root mean square value of the noise, can be defined as:

$$\mathrm{SNR}_V = \frac{A}{\sigma_n} \tag{36}$$

Considering the variance (mean square value) of the phase noise (Eq. (33)) one can express it as:

$$\sigma_\phi^2 = \frac{\sigma_n^2}{A^2} \tag{37}$$

Thus the root mean square value (standard deviation) of phase noise yields to:

$$\sigma_\phi = \frac{\sigma_n}{A} = \frac{1}{\mathrm{SNR_V}} \tag{38}$$

From this relationship it can be easily seen that the phase noise is directly linked to the amplitude of the signal.

3.3.3. Influence of Background Light

Although an optical filter is introduced directly in front of the photodetector to prevent the detection of the excitation light (set as primary light), still some influences of the primary light cannot be avoided. In particular, the residual primary light, which is not blocked by the optical filter, and the fluorescence light of the optical filter, which is also excitated by the light source, give rise to the so-called "falselight." Because the fluorescence light of the optical filter typically shows short lifetimes, both contributions give rise to a background signal which is in-phase with the excitation and contribute to the phase shift. The falsification of the measured values is demonstrated in Fig. 8.

The background signal (BG) is drawn slightly exaggerated to highlight the effects. It can be easily seen that due to the background signal the amplitude is increased from A to A_{BG} and the phase shift (Φ) changes correspondingly to a lower value (Φ_{BG}). Another effect, which is related to the background signal, is that the error on the phase shift increases when the signal amplitude decreases. This effect is also shown in Fig. 8: the decrease of the original amplitude (A) to a new amplitude (N) causes that the measured phase shift decreases from Φ to $\Phi_{\mathrm{BG}+N}$, so that the error ($\Phi - \Phi_{\mathrm{BG}+N}$) increases. This is observed for example in the case of photobleaching of the dye.

In this case the task of the developer is to optimize the system in a way to reduce the influence of this effect to a minimum.

3.4. Practical Aspects for Implementation (Aspects for Designing Phase Measurement Periphery)

As mentioned in the previous sections the various aspects have to be considered for an optimized instrumentation of an optochemical sensor. Considering Fig. 4, it can be seen that an accurate phase measurement block is just one important part of such a complex instrumentation. To optimize the resolution of the system the developer also has to pay attention to the analogue parts of the system, because every noise source which is introduced in this sensitive parts limits the SNR and therewith the resolution of the system. The goal of this section is to show some important aspects in selection of the optoelectronic components

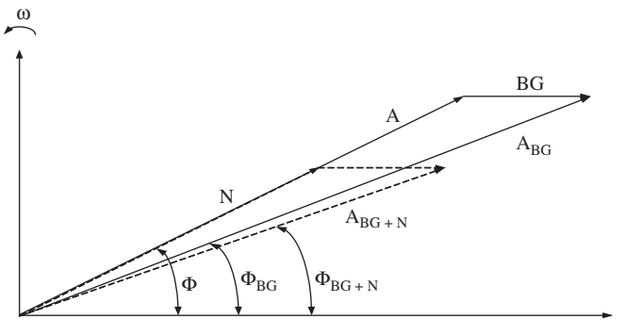

Figure 8. Influence of background signal on the phase shift performance.

and designing the analogue parts of the phase measurement system.

Basically the requirements of the optical set-up do not concern only the resolution and accuracy of the measurement, but also the temperature behavior of the opto-electronic components and the properties of long time stability of the sensitive element containing the luminophore [32]. The time stability of the sensitive element depends on its photo-stability and this is affected by the dose of the exciting light which is received by the luminescent dye (photo-bleaching). This means that the photo-stability of the sensitive element increases with decreasing excitation intensity [24, 32, 41]. However it is not possible arbitrarily to decrease the intensity of the excitation light source without decreasing the signal-to-noise ratio and worsening the resolution of the measurement. As a consequence, an optical set-up is demanded, which is able to efficiently collect the luminescence signal with an excitation intensity as minimum as possible.

The optical set-up of an opto-chemical sensor typically consists of the following parts:

- an excitation module, consisting of a suitable light source for the excitation of the luminescent signal (excitation source) and one or more optical filters set in front of the excitation source to separate the excitation radiation emitted by the excitation source from the luminescent signal (excitation filter);
- a reference module, which may consists of a second light source (reference source) or of a second photodetector, to account for measurements errors due to changes with temperature of the operational parameters of the electronic components and eventually one optical filter set in front of the reference source to select the wavelength emission in the same range as the luminescence signal (reference filter);
- a detection module, consisting of one photodetector to detect the luminescent signal emitted by the sensitive element and of one optical filter set in front of the photodetector to separate the luminescent signal emitted by the luminophore from the excitation light and other optical background contributions (emission filter);
- a collecting optics, which may consist of refractive optical components (prisms or lenses) and/or light-guides (glass rods or optic fibers) to carry the excitation light to the luminophore and the luminescent signal to the photodetector.

Additionally, together with the selection and optical characterization of the light sources and of the photodetector, the following tasks must be also fulfilled for the optimization of the measurement system:

- selection and characterization of the driver circuits for the chosen light sources and
- selection and characterization of amplifier circuits for the chosen photo-detector in order to maximize the SNR of the setup.

All these components have to be fitted together in a appropriate housing with respect to interferences and cross-talk to get an user friendly instrument.

3.4.1. Selection of Light Sources

a. Excitation Sources Many kinds of different optical sources are nowadays available for applications in the sensor field, like for instance wideband continuous spectrum sources (e.g., incandescent lamps), light emitting diodes (LED), laser diodes or lasers. The choice of the proper source depends on several factors: the spectral characteristics of the luminescent dye (luminophore) which is used for sensing purposes, the luminescence parameter which is going to be measured (e.g., intensity, lifetime, polarization), the measuring technique that is going to be adopted, like for instance intensity measurement, direct recording of luminescent decay times by pulsed excitation technique or indirect determination of luminescence lifetime by phase technique. Other properties which have to be taken into consideration for choosing the light sources are the luminescence lifetime, the quantum yield and photo-stability of the luminophore. The latter parameters are also important in order to determine the amount of exciting radiation which the luminophore can tolerate without degradation [24, 25, 32].

Lasers due to their extremely high optical output power and beam collimation are used for excitation of molecular systems which show narrow absorption bands or peaks with low quantum yield and high photo-stability [25, 42]. They are suitable light sources in all those applications in which fast switching time or high modulation frequency of the exciting light are necessary. Typically such features are highly valued in direct intensity measurement of luminescence or in direct decay time measurement by pulsed excitation of luminescence from luminophores with short lifetimes (order of magnitude of a few nanoseconds). However lasers can be quite voluminous and expensive, therefore, for some applications laser diodes offer an interesting alternative and are preferred, showing a good monochromaticity and beam collimation, fast switching time and possibility of high modulation frequency like lasers, but being less voluminous and expensive. Besides they are typically tunable light sources.

Contrarily to lasers and laser diodes, LEDs exhibit a non-monochromatic incoherent emission (characterized by a much lower optical output power and poor beam collimation) and they do not allow fast switching time and modulation frequency as lasers and diode lasers. Nevertheless LEDs are suitable light sources for excitation of luminophores with broader absorption bands and with typical luminescent lifetime in the order of milliseconds up to microseconds. In comparison to lamps (Tungsten, Tungsten-halogen, Xenon and Quartz-halogen lamps) LEDs are advantageous due to their lower power consumption, high stability and reliability, small size, robustness and lower costs. Moreover incandescent lamps are typically modulated by mechanical chopper, while semiconductor sources can be easily modulated up to several hundred kHz by simple modulation of the applied voltage or current. LEDs have already proved to be particularly suitable for instrumentation based on the indirect measurement of the luminescence lifetime of a large variety of opto-chemical sensors by phase technique [32]. An example of such opto-chemical sensor is given by a Palladium (Pd)-porphyrin complex immobilized in a polystyrene matrix, whose absorption and luminescence spectra are shown in Fig. 9.

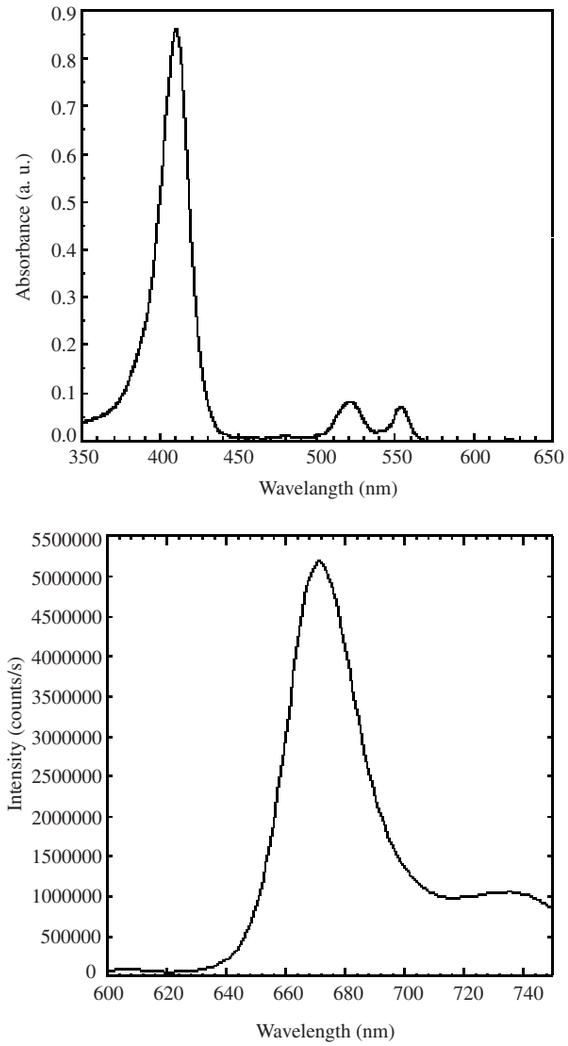

Figure 9. Absorption (top) and luminescence (bottom) spectra of a Pd-porphyrin complex immobilized in a solid matrix of polystyrene. The luminescence spectrum has been recorded under excitation at 522 nm by a Shimadsu spectrometer.

The absorption spectrum of this dye (top picture) shows three main absorption bands: a principal one with maximum around 400 nm and two minor bands centered at about 520 nm and 560 nm, respectively. The emission is shifted with respect to the absorption to longer wavelengths (Stokes shift). The luminescence emission of this luminophore for an excitation at 517 nm shows a broad band with maximum at about 670 nm (see bottom picture). The so-called Stokes shift of this dye is therefore of the order of 150 nm. For excitation of an optochemical sensor based on a Pd-porphyrin complex dye like in this example, LEDs with emission bands matching the absorption bands of the dye should be used. The excitation at 400 nm is particularly efficient due to the high absorbance shown by the dye. As a consequence the dose of excitation light to produce a measurable luminescence signal can be kept lower for this band than for the other two. However it must be also considered that the excitation efficiency of the luminescence is a wavelength dependent function which increases with decreasing wavelength. Therefore, at excitation wavelengths round 400 nm, also other luminescence emissions from materials close to the dye can be efficiently excited. Materials which form together with the luminophore the sensitive element or which are just close to it, as well as materials of the optical set-up (optical filters for instance) can emit a photoluminescence which increases the optical background. For this reason a blue-green LED, which matches the second absorption band of the dye at about 520 nm, is often preferred as excitation source for this luminophore. A typical emission spectrum of one blue green LED from Nichia is shown in the Fig. 10. The emission band is centered at about 517 nm and has a width of about 30 nm at half peak maximum.

The choice of the excitation source requires a detailed investigation not only of its spectroscopic properties, but also of its electronic characteristics. This is generally important for all kinds of light sources but in particular for LEDs, since these devices are developed for applications in displays, lighting, indicator lights, traffic lights, etc. and they are not optimized with particular regard to emission and wavelength stability and temperature stability. Unfortunately this kind of information is not given by the producer in the LED data sheets. That is why dedicated investigations are normally carried out to characterize the spectral and electric behavior of the source (e.g., peak wavelength, current–voltage characteristics, junction capacitance) at various operating conditions (temperature and current).

b. Reference Source To compensate the measured luminescence for the effect produced on the excitation source by changes of the operating conditions, it is necessary to use a reference signal to monitor the behavior of the light source [32].

In many opto-chemical sensor instrumentation, a reference concept is adopted, which is based on the use of a second light source. It is important to select the reference

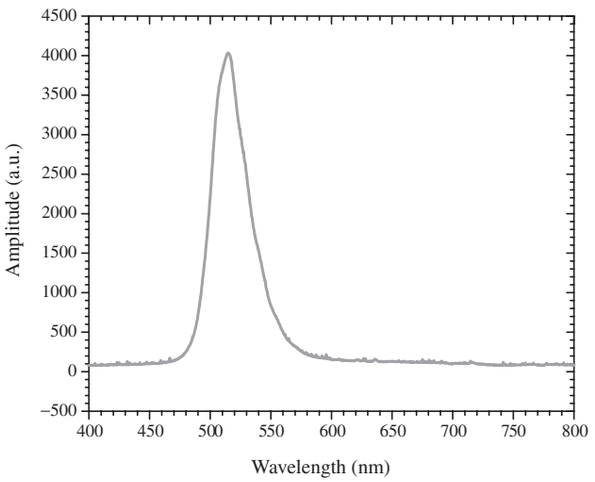

Figure 10. Emission spectrum of a LED used as excitation source for the sensitive element based on Pd-porphyrin luminophore. The measurement was performed at a forward current of 20 mA and at constant ambient temperature of 25°C.

source with an emission spectrum in the same spectral range as the luminescence signal, because in such instrumentation the photo-detector is typically equipped with one set of long-pass optical filters to prevent the detection of the exciting light and consequently to reduce the optical background. Moreover, this second light source must not excite any luminescence from the sensitive element. The selection of a proper reference light source must keep into consideration also its electric properties. In particular, the switching time characteristics and the temperature behavior of the switching time of this light source must match those of the excitation source as best as possible. By the assumption that the two driver circuits of the light sources behave equally, the contribution to the luminescence phase shift due to the detection circuit can be determined.

In case of a sensor based on Ruthenium-diphenyl phenanthroline (Ru-dpp) dye, for example, the most suitable excitation source is a blue LED emitting at 460 nm. Possible reference sources are LEDs with an emission peak at about 620 nm (the luminescence peak of this dye). Consequently, LEDs emitting at 590, 594, 645 and 650 nm were selected and investigated. Unfortunately, a very good matching of the switching times of signal and reference LED is hardly obtainable. However, the changes of the switching time over the temperature of both LEDs is more important than the absolute values. Table 2 shows the rate of change of the switching time of the excitation LED with temperature and rate of change of four candidates as reference LED. It can be easily seen that the temperature behavior of the LED emitting at 594 nm better matches that of the excitation LED. The worst candidates are the red LEDs emitting at 645 and 650 nm, since their switching time decrease with increased temperature, whereas the excitation LED has an increasing switching time with increasing of temperature.

If one LED has more than one emission band, the temperature behavior of all emission bands, and in particular of their switching time, must be investigated, because it may occur that they behave differently from each other. This is for example the case of the LED emitting at 594 nm, which according to Table 2 represents the best candidate as reference LED for Ru(dpp) based sensor instrumentation. This LED shows a second emission band at about 850 nm with a much slower switching time. Therefore, if this LED has to be used as reference, it is necessary to prevent that the light of this second emission band reaches the photo-detector, for example by filtering the light by means of a proper optical filter.

c. LED Modulation Circuits The major property of the LED driver circuits is to provide the LEDs with an adequate prepared square wave or sinusoidal signal from the frequency generation unit of the phase detection unit. For a optimum performance (accuracy and stability) the absolute switching times are not that important. The key of success in this case is a reproducible switching of both circuits (Signal and Reference).

In an ideal case the LED emits light directly after asserting the signal. In practical there is a delay which is dependent on:

- temporal activity of LED itself is limited by the carrier lifetime in pn junction,
- junction capacitance of the LED has to be loaded before emitting light. This behavior can be affected with the LED modulation circuit,
- reproducibility of switching time.

Table 2. Rate of change of LED phase with changing the temperature in the range 10–70 °C.

Reference LED	Emission peak	Rate of change of the phase with temperature
Excitation	460 nm	$+6.7\times10^{-4\circ}/^{\circ}C$
LED 1	590 nm	$+1.1\times10^{-4\circ}/^{\circ}C$
LED 2	594 nm	$+5.0\times10^{-4\circ}/^{\circ}C$
LED 3	645 nm	$-1.0\times10^{-2\circ}/^{\circ}C$
LED 4	650 nm	$-3.5\times10^{-3\circ}/^{\circ}C$

3.4.2. Optical Filters

One important task that any optical set-up in sensor instrumentation has to perform, is the separation between the luminescence signal, which has to be collected and transmitted to the photo-detector and all other optical contributions, such as for instance the exciting radiation, other luminescent emissions due to materials close to the luminophore or composing the optical set-up, ambient light.

Usually when a luminophore is characterized by a broad luminescence band and by a Stoke shift large enough, optical set-ups based on optical filters are used. It is possible also to use dispersive instrumentation [34, 43], often in combination with an array of photodiodes as a photo-detector. This instrumentation comprises prism-based or holographic grating-based spectrometer. Sometimes it is also possible to use spectrometers with more than one dispersive element, such as a holographic grating sandwiched between two prisms, in order to increase the spectral resolving power (the minimum separation between two spectral lines that can be resolved). However the use of optical filters is often necessary also in case of a sensor instrumentation based on dispersive optical components.

For all those applications (and among these optochemical sensor instrumentation) where the stokes shift is large, the optical filter based solution is preferred to the use of dispersive optical components due to its higher robustness, stability, smaller size and lower costs.

The separation of luminescence signal from the excitation can appear at the first sight a rather easy task, since the luminescence emission is shifted toward longer wavelengths with respect to the exciting radiation. However, light sources typically show a broad emission. Even in the case of LEDs, the emission at longer wavelengths beside the nominal peak are significantly high. For this reason one or more short-pass optical filters (excitation filter) must be used in front of the excitation source to cut the emission at wavelengths close to the luminescence band of the luminophore. In addition a long-pass optical filter or a set of long-pass optical filters (emission filter) should be set in front of the photodetector to avoid that the radiation of shorter wavelengths than the luminescence band is also detected.

For example, in case of a Ru-dpp based sensor with absorption spectrum at around 460 nm and emission around 600 nm, a blue LED emitting at 460 nm is used as excitation

source. So an optical high-pass filter with an edge wavelength of 550 nm is used to separate the excitation from the emission light. Consequently, the filter at the exciting LED has to suppress the light of the LED at wavelengths higher than 550 nm, the so called "red-tail" of the LED.

Even more complicated is the reduction of the contribution of the optical background coming from luminescence emission of other material than the luminophore. Since luminescence emissions typically occur in the red spectral region, it is not really possible to distinguish these contributions from the luminescence signal. So the only possible strategy to cope with the luminescence contribution from other materials is to avoid their excitation.

The choice of the excitation and emission filters is of fundamental importance for the good functioning of the optical set-up and it must be performed taking into account not only the spectral characteristics of the excitation source and luminophore, but also other properties of the filters, such as the self-fluorescence (in particular for the emission filter), the thermal and mechanical stability and the chemical inertness against the organic solvents typically used for cleaning of optic components (e.g., acetone, ethanol, isopropanol).

A wide range of different commercially available optical filters exist. They can be classified according to their function in:

1. band-pass filters, which allow required wavelength ranges to pass through selectively;
2. long-pass filters, which block light of short wavelengths and transmit light of long wavelengths;
3. short-pass filters, which block the light of longer wavelengths and transmit light of short wavelengths;
4. neutral density filters, which attenuate the visible radiation in the same way for all the wavelengths of the spectrum.

Filters can be classified according their composition in absorption based (glass and gelatine filter) or interference based filter.

a. Glass Filters Colored glass filters are characterized by selective absorption in a defined range of the visible spectrum. They are obtained by adding impurities to glass material, which are otherwise transparent. This can be achieved in different ways, by adding heavy metal ions or ions of rare earths to obtain ionically colored glass, or by temperature treatment of particular color carriers (impurities) present in the colorless glass to obtain colloidal colored glasses. In both cases the color of the filter depends on the type and concentration of the added species and of the color carriers. In the case of colloidally colored glasses the basic glass used and the course of the temperature during the activation process play also a significant role in determining the final properties of the filter.

Glass filter can be produced in different size and thickness, typically of one or more millimeters. They show a very good properties in terms of mechanical and temperature stability and a high grade of chemical resistance against many solvent, but due to the presence of colorants they can exhibit a fluorescence emission under UV or visible (blue) excitation.

The measurement of the filter transmission and its eventual self luminescence can be performed by measuring first the transmission with the filter far away from the detector (in this case the contribution of the eventual luminescence of the filter is negligible) and second by repeating the measurement with the filter placed in front of the detector. The apparent transmission, which is measured in this second case, is the sum of the real transmission and of the luminescence contribution emitted by the filter. Figure 11 shows one example of the apparent transmission of three long-pass glass filters used in opto-chemical sensor instrumentation under direct green excitation from a typical LED used as excitation source.

b. Gelatine Filters Gelatine filters are manufactured by dissolving suitable organic dyes in liquid gelatine and by coating the proper amount of solution onto a glass surface. After the drying of the coating, the gelatine film is stripped from the carrier material and coated with a lacquer.

This kind of filters have a typical thickness of about 0.1 mm and a maximum working temperature of 180 °C. Because of their uniform thickness, gelatine filters have an excellent optical quality and are suitable for precise works in which an increment in length of the optical path cannot be tolerated. However, like glass filters they exhibit fluorescence emission under exposition to visible radiation of short wavelengths.

Moreover the dyes used in gelatine filters may alter their spectral properties with time and may be degraded by heat, humidity and light (for example UV radiation). Gelatine filters are attacked by most solvents and are dissolved by acetone.

c. Interference and Dichroic Filters Interference filters are obtained by depositing a multi-layer structure of thin films of dielectric material, called spacer, between two reflecting surfaces. The light entering the filter undergoes multiple reflections and the reflected beams interferes with each other, experiencing constructive or destructive interference depending on their wavelengths. The condition for constructive interference is determined by the thickness of the spacer. Therefore, by selecting the proper thickness of the spacer it is possible to decide which wavelengths can pass through the filter and which on the contrary are blocked by destructive interference.

If the back layer is totally reflective, then the arrangement is called dichroic filter. These devices let the light of a selected spectral region pass through and reflect the light belonging to the complementary range. They combine therefore the behavior of the filter with that of a wavelength selective beam-splitter.

In comparison with colored glass and gelatine filters, interference filters exhibit both narrow transmission bands and they show very sharply defined transmission bands like long-pass and short-pass filters. The blocking capability of interference filters (the measure of the transmission outside the band-pass region) is therefore much better than in case of glass or gelatine filters. Since no light absorption takes place in the material of filter, they are also characterized by no luminescence emission. Beside they exhibit very stable spectral characteristics with respect to temperature and humidity changes, a shift of the nominal transmission band occurs when the filter is tilted with respect to the direction of incidence of the light. In particular it is found that as

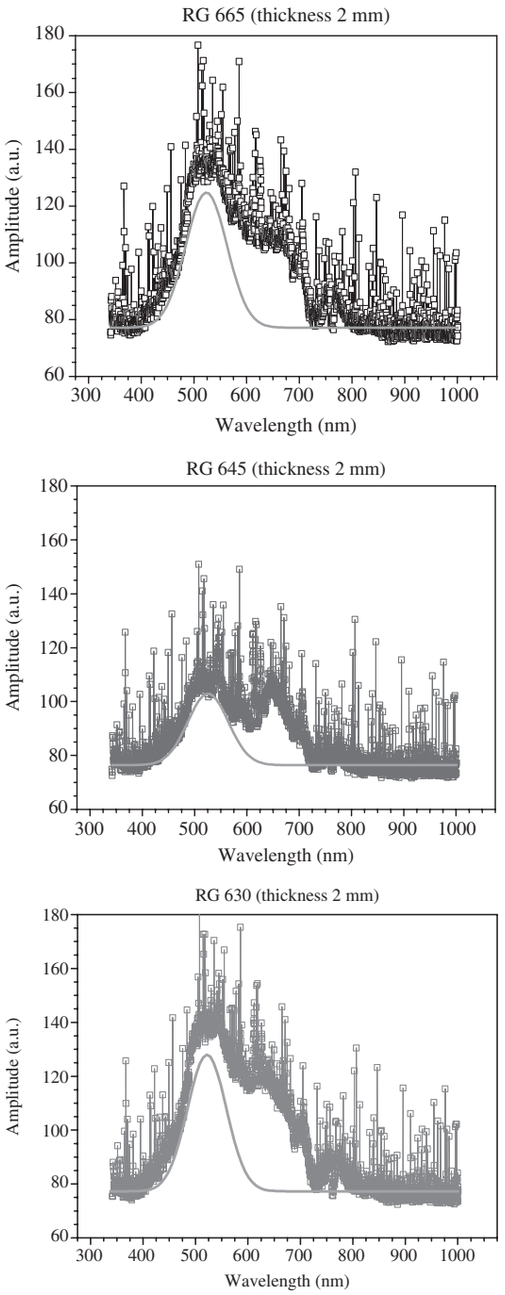

Figure 11. Apparent transmission of three long-pass glass filters with edge wavelengths at 665, 645 and 630 nm, respectively. The apparent transmission is measured by setting the filter directly in front of the photo-detector and by detecting the transmitted light of a proper source. In the present case a LED emitting at 522 nm was used as a light source. The recorded spectra show the emission peak of the source, which is transmitted by the filter, and a second broad emission, which is given by the photoluminescence of the filter itself.

the tilt angle increases, the center of the transmitted band moves toward shorter wavelength.

The selection of the most suitable filters for the sensor instrumentation is principally subjected to optical and economical criteria: on the one hand the blocking capability (the ability of the filter to block the radiation of wavelengths outside the nominal transmission band) and the reduction of self-luminescence (especially if filters are set directly in front of the photo-detector) are highly valued, and on the other hand, the cost of the sensor instrumentation must be kept as low as possible. The best optical properties are shown by interference filters, which are the most expensive components, while the most affordable price is that of gelatine filters, which on the contrary are the most critical components in term of mechanical, chemical and temperature stability of their optical properties. For many applications glass filters represent therefore a good compromise between optical and economical aspects.

3.4.3. Photodetectors

The selection of the proper detector must take into account the following properties [25, 42]:

- the spectral relative response, which determines the spectral range where the detector can be used;
- the absolute spectral sensitivity, defined as the ratio of the output signal (a voltage or a current according to configuration in which the detector is used) to the incident radiation power at the different wavelengths;
- the signal to noise ratio which is also a function of the wavelength;
- the maximum intensity range where the detector has a linear response (where the output signal of the detector is linearly dependent on the incident radiation);
- the time response of the detector, which is a property particularly important for applications where the incident radiation is modulated (the output signal of the detector typically fall with increasing modulation frequency of the incident light).

Existing different optical detectors can be classified into two classes: (a) the thermal detectors, such as bolometers, pyroelectric or thermoelectric detectors, and (b) the photodetectors, like for instance photo-multiplier tubes, photoconductors, junction photodiodes or avalanche photodiode [25]. In thermal detectors the energy absorbed from the incident radiation raises the temperature and causes changes in the temperature dependent properties of the detector. Thermal detectors are characterized by a wavelength independent sensitivity and therefore are very useful for calibration purposes, like for example for absolute measurements of the radiation power of lasers.

Photo-detectors are based either on the emission of photo-electrons from photo-cathodes (photo-multiplier tubes) or on changes of the conductivity of semiconductor due to the incident radiation, or either on photovoltaic devices where a voltage is generated by internal photo-effect. Generally they show a spectral response which depends on the work function of the emitting surface or on the band gap in semiconductors. Photo-multiplier tubes are often used in laser spectroscopy and are suitable for all the applications in which very low radiation powers must be detected. A possible alternative for photo-multiplier tubes is represented by avalanche photodiodes. They are reversed biased semiconductor diodes in which the free carriers acquire sufficient energy in the accelerating field produced

by the applied bias voltage to produce additional carriers on collisions with the semiconductor lattice. The advantage of the avalanche photodiodes is their fast response time which decreases with increasing bias voltage.

However, photomultiplier tubes and avalanche photodiode are often excluded for application in sensor instrumentation due to economic considerations, especially when the applications do not required extreme performance of the photo-detector in terms of sensitivity and response time. Silicium (Si)-photodiodes have proved to be particularly suitable for instrumentation based on the indirect measurement of the luminescence lifetime of opto-chemical sensors by phase-shift technique [32]. They actually show a good sensitivity at the operating wavelength (the luminescence maximum of the luminophore emission is typically in the range 600–800 nm depending on the luminophore) and a response time fast enough for such applications. More stringent for Si-photodiode in opto-chemical sensor instrumentation are other properties, such as detector capacitance (especially for measurement by phase shift technique), noise, homogeneity of the active area, temperature behavior and stability of the photodiode [32]. In particular, the active surface of the photodiode has to be as large as possible to get as much light as possible, but the capacitance of the photodiode must be as low as possible to reduce the noise. Unfortunately, these two requirements are in contrast, since the capacitance is directly proportional with the area of the active surface and a compromise has to be found. For example, for a homogeneous distribution of the luminescence light on the photodiode surface, a large active surfaces give a better signal-to-noise ratio than smaller ones with lower capacitance. That is why in this case it is important to use a photodiode with a high spatial homogeneity of the active area. It is known that, specially at the margins of the photodiode surface, deviations in the spectral sensitivity and response time from their values in the center are possible. Spatial homogeneity is a very important parameter, since it is impossible to have a change of spatial distribution of the luminescence on the photo-detector with time and temperature, as a consequences of changes in the light source emissions or in the optical path: lens, filters, light guide, optic fibers, and in general any optical component of the measuring set-up are affected by temperature variations and may change with time. If the spectral sensitivity and the response time of the photo-detector change as a consequence of the change of the luminescence distribution, the measure of the luminescence intensity or lifetime (phase shift) also changes.

a. Photodiode Amplifier Circuits One key component of the instrumentation for optical chemical sensors is the conversion of the optical signal coming from the fluorescent dye back into electrical domain. Therefore several photon devices exist to perform this conversion. Photodiodes are the most common devices for this purpose and have a good price/performance characteristics for the application in optochemical sensors.

The goal of the photodiode amplifier circuits is to convert the signal from the photodiode into an adequate voltage signal which can be further processed at the analog-digital converter (ADC) stage. In fact, this is the key to success for an optochemical sensor based on phase measurement technique, because every noise which is introduced in this part of instrumentation is amplified through all stages and the SNR can not be risen later without losing bandwidth. On the other hand this part should be as sensitive as possible to be able to reduce the intensity of the illumination light as much as possible and consequently to reduce the photobleaching of the dye.

Concerning these assignments the photodiode amplifier circuit has to feature the following requirements:

- high signal to noise ratio of the photodiode amplifier circuit,
- phase shift independent of the signal magnitude,
- no or low gain peaking to avoid undesired oscillations,
- no or low influence of power supply noise,
- no or low influence of electrostatic, magnetic and radio Frequency Interference (RFI) coupling.

To achieve all these goals a consideration of all the possible noise sources would help to find the selection criteria for the components and circuits.

In case of using the photodiode in photoconductive mode the noise sources of amplifier can be classified as:

- Thermal noise, intrinsic behavior of feedback resistors which can only be reduced by cooling the device.
- Voltage noise of the operational amplifier inputs.
- Current noise of the operational amplifier inputs and the photodiode itself.
- Power supply noise which can be suppressed by an high power-supply-rejection-ratio (PSRR) of the operational amplifiers.
- Electrostatic, magnetic and RFI coupling noise, which can be reduced by a careful layout and adequate shielding of the circuits.
- In the case of using the synchronous demodulation, cross-talk of the demodulation signal is normally produced on the same PCB and adds to the signal from the dye in a vector like addition and falsifies the output in a similar way as the background signal (see Section 3.3.3 Influence of Background Light).

Furthermore, in order to optimize the performance of the system several methods can be used to stabilize the photodiode parameters and to avoid undesired changes of the phase measurement. In this context the photodiode capacitance change as a function of temperature has to be especially observed.

4. SENSOR SCHEMES FOR LIFETIME-BASED OPTOCHEMICAL SENSORS USING PHASE-MODULATION METHOD

Optical sensors based on the measurement of the luminescence intensity suffer from interferences by changes of turbidity, refractive index or color of the sample. Changes in the optoelectronic system such as drifts of the light source and the photodetector, bending of the optical fibers and displacement or delamination of the sensing layer may also cause signal changes to occur. Furthermore, degradation of the indicator caused by photobleaching and leaching are critical. Decay-time sensing is advantageous over the intensity-based sensing because such measurements are independent

of probe concentration, photobleaching and drifts in lamp intensity, of inner filter effects, all of which are major limitations of current fluorescence-intensity-based optrodes. The measurement of the luminescence decay time as a parameter which is almost independent of the absolute signal height can solve such problems and therefore has substantial advantages in practice. The advantages of lifetime-based sensing (time-domain and frequency-domain sensing) are illustrated in Fig. 12 [44]. In contrast to intensity measurements, lifetime measurements depend on the signal during a short period of time, 1–20 ns, depending on the probe's lifetime. The decay time is obtained from the slope of the intensity decay following pulsed excitation (Fig. 12, middle). The intensity-based sensing depends on reliable measurements of probe's intensity. However, lifetime measurements based on pulse method are presently too costly and complex. Fluorescence lifetimes can be conveniently measured by the phase-modulation technique, where the sample is excited by light which is intensity-modulated at frequencies (f) ranging from 1 to 200 MHz. The possible mechanisms of lifetime-based sensing have been reviewed [23] and have been described for a large number of analytes, including pH [45], oxygen [46, 47, 48], carbon dioxide [49], NH_3 [50] and glucose [51].

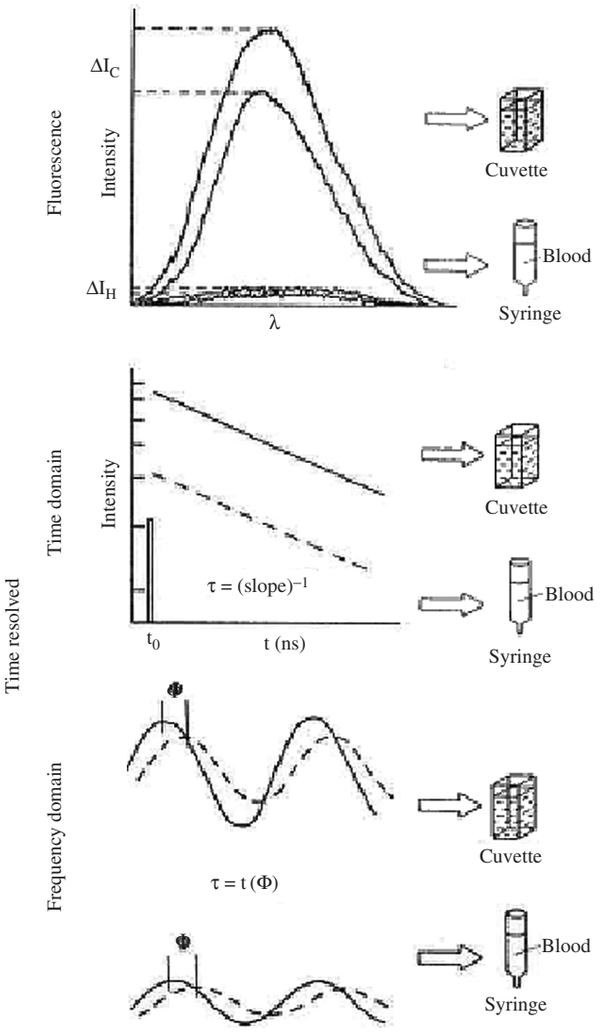

Figure 12. Intensity, time-domain and frequency-domain sensing, as applied in the laboratory, a cuvette and blood sample in a clinical setting. Reprinted with permission from [44], J. R. Lakowicz, "Probe Design and Chemical Sensing in Topics" in "Fluorescence Sensing," Vol. 4, p. 6. Plenum Press, New York, 1994. © 1994, Plenum.

4.1. Optical Sensing of pO_2 Using a Phase-Modulation Fluorimetry

The simplicity and robustness of phase fluorimetry is best exemplified by the optical oxygen sensor. The availability of long-lifetime synthetic probes has been particularly instrumental in reducing costs in this case, permitting low-frequency modulation using simple electronics. As of now, lifetime determination (both time or frequency-domain) of dyes with long-lived excited state is the method of choice for high-accuracy oxygen measurements [47, 48].

Oxygen optosensing is in general based on the efficient quenching of a photoexcited luminophore immobilized in a polymer matrix used as a solid support. If quenching occurs only by a dynamic (collisional) mechanism, then the ratio τ_0/τ is equal to I_0/I and is described by the classic Stern-Volmer equation (see Eq. (20)) [31], which predicts a first-order type response to oxygen tension resulting in higher oxygen sensitivities at low oxygen concentrations. This equation is directly related to the intensity and can be expressed as:

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV} \cdot pO_2 \tag{39}$$

where, I_0 and I are the luminescence intensities in the absence and the presence of quencher (oxygen).

The overall dynamic quenching constant K_{SV} (Stern-Volmer constant) can be described by:

$$K_{SV} = k_q \cdot \tau_0 \tag{40}$$

where k_q is the bimolecular quenching rate constant which, for a diffusion-limited reaction can be described by the Smoluchowski equation:

$$k_q = 4\pi N_p (D_A + D_B) \tag{41}$$

where N is the number molecules per millimole, p is a factor related to the probability of each collision causing quenching and to the radius of interaction between the donor and the quencher. D_A and D_B are the diffusion coefficients for the donor and acceptor, respectively.

Using Eq. (33) it is possible to relate intensity or lifetime of phosphorescence (or fluorescence) to the oxygen partial pressure. The oxygen sensing properties of phosphorescence intensity based sensors are characterized by the ratio I_0/I_{100}, where I_0 and I_{100} represent the detected phosphorescence intensities from the film exposed to argon and oxygen saturated conditions, respectively (Fig. 13) [52]. The phosphorescence intensity changes under various oxygen pressures and Stern-Volmer plots are shown in Fig. 14 [52]. The K_{SV} value is obtained from the slope of I_0/I versus pO_2. The highly sensitive oxygen sensor has a larger I_0/I_{100} and K_{SV}, respectively.

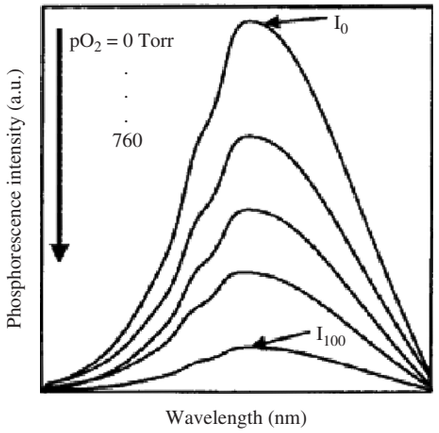

Figure 13. Typical phosphorescence spectrum changes of probe molecule immobilized in polymer film under various oxygen partial pressure conditions. Reprinted with permission from [52], Y. Amao, *Microchim. Acta* 143, 1 (2003). © 2003, Springer.

The first phase fluorimetric sensing of oxygen based on above described principle was reported by Woflbeis and co-workers [53]. This phase fluorimetric oxygen sensor measures the quenching by oxygen of the transition metal complex, tris[4,7-diphenyl-1,10-phenanthroline] ruthenium(II)$^{2+}$ (Ru-dpp)$^{2+}$. The fluorophore was immobilized in a silicone rubber membrane, which is an ideal matrix for oxygen determination because of its high oxygen solubility. The silicone matrix selects for gaseous analytes because of its hydrophobic nature, adding to sensor specificity. In addition, the hydrophobic matrix and the water insolubility of the ruthenium complex add to long-term sensor stability by preventing the leaching of the probe.

The sterilizable oxygen sensor with new polymer (polysulfone (PSU) or polyetherimide (PEI)/dye (Ru(II)-tris(4,7-diphenyl-1,10-phenanthroline) combinations, was developed at Joanneun Research [54] for use in bioreactors. The measurement system detected the luminescence lifetime of the dye in microsecond range by means of the phase-modulation technique. The light source was continuously modulated at a frequency of 20 kHz. Schematic optical set-up and components are illustrated in Fig. 15.

Beside Ru(II) complexes, a variety of luminescent species can be used as indicators, particularly those exhibiting long-lived singlet or triplet states for maximum sensitivity to the analyte [27]. Among the most frequently used luminophores for O_2 monitoring are the polycyclic aromatic hydrocarbons (PAHs) [55], because of their long excited-state lifetimes (40–300 ns) and high solubility in the extremely oxygen permeable silicone matrices. However, their UV excitation wavelengths and easy photodecomposition have prevented so far a wide usage. The strong room-temperature phosphorescence ($\phi_m > 0.1$) and long emission lifetimes (>10 μs) of Pd(II) and Pt(II) porphyrin complexes [56, 57] make them promising indicator dyes, but they often undergo facile oxidation when illuminated in the presence of O_2. Highly luminescent Ru(II) complexes [58–60] with polyaza heterocyclic chelating ligands are currently the most widely used oxygen-sensitive indicators. Their advantages

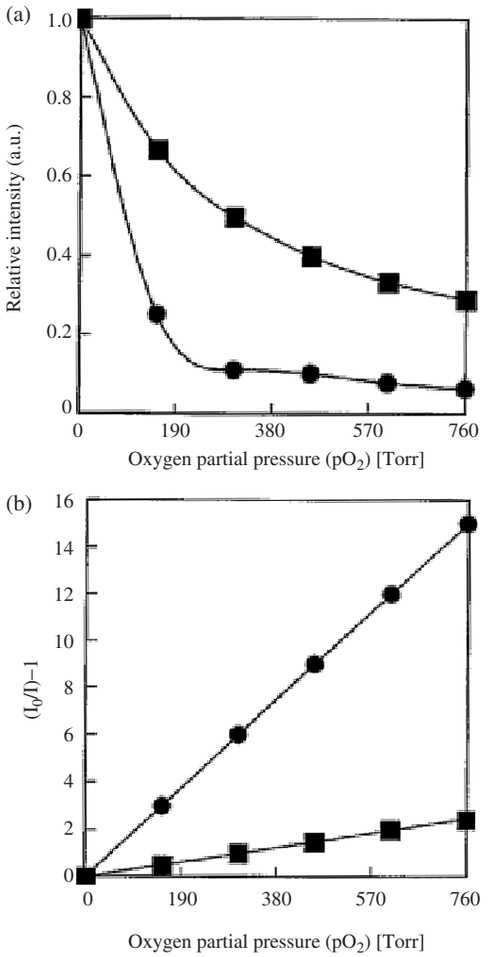

Figure 14. (a) Phosphorescence intensity changes and (b) Stern-Volmer plot of probe molecule immobilized in polymer film under various oxygen pressures. Reprinted with permission from [52], Y. Amao, *Microchim. Acta* 143, 1 (2003). © 2003, Springer.

include a strong absorption in the blue, intense luminescence quantum field ϕ_{em} up to 0.4 in the 550–800 nm region with a large Stokes shift (>150 nm) due to the long-lived (0.1–7 μs) metal-to-ligand charge transfer (MLCT) excited state, close to diffusion-limited O_2 quenching and the possibility of tuning their photophysical and immobilization properties by a judicious choice of the chelating ligands(typically 2,2′-bipyridine (bpy) or 1,10-phenanthroline (phen)).

In fluorescence sensing there is an entirely different class of methods for measurements, based on ratio determinations. Some of them do not require modulating the excitation light. The use of such methods requires oxygen-sensitive dye with specific characteristics. Two-dye molecules have been employed for similar purpose [61]; however a true dual-emitting dye is required to overcome the variations in bleaching [62]. The synthesis of a new oxygen sensitive dye [(dppe)Pt{$S_2C_2(CH_2CH_2$-N-2-pyridinium)}] [BPh_4] [63], that exhibits both fluorescence and phosphorescence emissions, made ratio oxygen

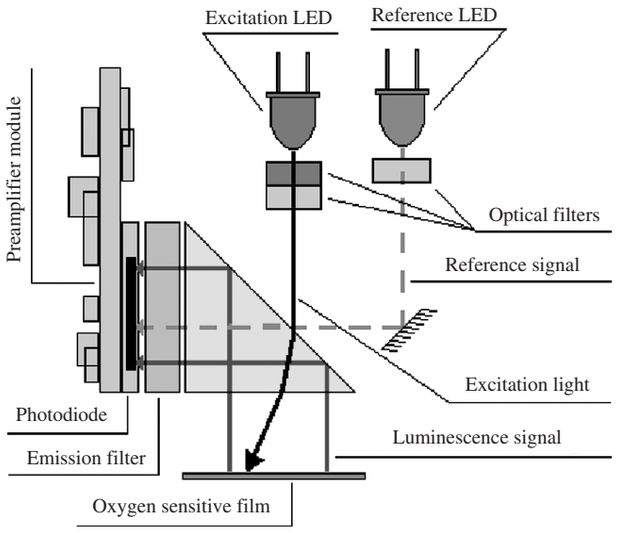

Figure 15. Optical set-up and components.

measurements more feasible. Recently, the same dual-emission dye immobilized in cellulose acetate, has been employed for development of ratio-based oxygen sensor [64]. The immobilized dye exhibits two emissions with greatly different lifetimes. The triplet intensity in film is significantly quenched by oxygen; this makes the dye suitable for for oxygen ratiometric measurements. It allows the peak ratio to be measured through any of the existing fluorescence methods (intensity, lifetime or polarization).

Room temperature phosphorescence (RTP) quenching-based sensors have become the focus of recent attention as they posses several advantages over those based on fluorescence. The longer excited-state lifetimes of phosphorescent indicators give rise to high quenching efficiency by oxygen. The long excitation and emission wavelengths are more compatible with available optical monitoring technology. Most recently, RTP quenching-based sensors have utilized metal chelates such as tetrakis (pyrophosphito)diplatinate(II) [65] and 8-hydroxy-7-iodo-5-quinoline sulfuric acid (ferron) chelates [66] and a range of modified Pd(II) and Pt(II) porphyrins [67]. When the porphyrin dye is used for oxygen measurement *in vivo* by intravenous infusion, a palladium series, especially Pd-meso-tetra-(4carboxyphenyl)-porphyrin (Pd-TCPP), is employed [68]. Its phosphorescence is sensitive to oxygen, it is chemically stable, its lifetime is relatively longer than that of other porphyrins and its phosphorescence emits in near infrared, where biological tissues absorb such wavelengths very little. This porphyrin dye was doped in silicone-based polymer and fixed at the edge of an optical fiber for development of an optical oxygen sensor of medical catheter-type for medicine or animal experiments [69]. The use of phosphorescence lifetime did not reduce the measurement accuracy and there was an excellent correlation between the pO_2 values measured through phosphorescence lifetime using oxygen sensors and those measured as calibration data using oxygen electrodes.

4.2. Optical Sensing of pH and pCO_2 Using a Phase-Modulation Fluorimetry

Lifetime-based sensing of parameters such as pH and pCO_2 is of great interest but it is difficult to realize in practice. Unlike in oxygen sensors (where the population of the excited state of a single indicator is reduced due to the collisional quenching by oxygen), decay-time pH sensors are based on the measurement of the relative contributions of the acid and base form of an indicator to the total decay time. Their decay times should differ so that the change in pH will change the relative contribution of each form to the overall decay [31]. The relative contribution of each form to the overall emission can be altered by selection of the excitation or emission wavelength, which will allow the pH-sensitive range to be altered by changing the wavelength [70]. Nevertheless, there are different schemes described in the literature to design such sensors. One approach for lifetime-based pH sensing uses the pH dependent photoinduced electron transfer (PET) of diethylaminomethyl pyrene [71]. This indicator shows a lifetime in the range of 100 ns but needs to be excited in the UV, where no inexpensive solid-state light sources are available. The use of the pH-dependent fluorescence decay time of seminaphtofluorescein (SNAFL) and seminaphtorhodafluors (SNARF) using phase-modulation fluorometry was suggested by Szmacinski and Lakowicz [72] and Thompson et al. [73]. In this case, phase and modulation values were found to be strongly pH-dependent in the physiological pH range, over easily accessible range of light modulation frequencies from 10 to 300 MHz. Both indicators are fluorescent in protonated and deprotonated forms, with lifetimes ranging from 0.5 to 5 ns; such short lifetimes require modulation frequencies of >100 MHz [74].

Another indirect approach to realize lifetime-based pH optodes is the use of fluorescence ("Forster") resonance energy transfer (FRET) from a pH-insensitive luminescent donor to a pH-sensitive colored acceptor [75, 76], whose absorbance overlaps significantly with the donor's emission. FRET is a non radiative, distance dependent dipole-dipole type interaction in which the acceptor quenches the donor fluorescence [77, 78]. In this technique the color change of a colorimetric pH indicator is converted into lifetime information. Optimal energy transfer is obtained when the donor-acceptor pair is within 40–70 Å of each other. The excited state of donor is deactivated by the acceptor, thereby causing a decrease in both the quantum yield and the decay time of donor.

FRET-based optodes have been realized by several groups by measuring either intensity or decay time as the analyte-dependent parameter but only in the range of a few nanoseconds [79]. The use of transition metal complexes (ruthenium complexes) as donor opens a promising way to obtain LED-compatible pH sensors with longer lifetimes in the microsecond range and was first suggested by Lakowicz [76] and Wolfbeis et al. [80]. They have presented a pH optode with decay times of a few microseconds by co-immobilization of luminescent ruthenium(II) complexes as a donor with pH indicators (bromothymol blue and reactive azo dye) as a acceptor, which paves the way to frequency domain pH sensing at low modulation frequencies (75–200 kHz). The phase

signal was not influenced by fluctuations of the LED or the sensitivity of the PMT. The effect of molecular oxygen was found to be crucial for practical applications. There is a great interest for other luminescent transition metal complexes which are less quenchable by oxygen to overcome major drawbacks of the described sensor and to make them more suitable for practical use. The same research group reported a year later about first decay time-based fiber optic pH microsensors which were without cross-sensitivity to molecular oxygen [81]. Energy transfer from a luminescent ruthenium(II)-tris(1,10-phenanthroline) (acting as donor) to the colored pH sensitive indicator (sulfonephthalein acceptor dye) converts the pH-dependent color change into a decay time information. The sensing scheme was insensitive to fluctuations of the optoelectronic setup, fiber bending, microbending and variations of the optical parameters of the sample.

The luminescence quenching by proton transfer is one of the two indirect approaches for sensing of CO_2 as a target analyte. This approach was described by Orellana and co-workers [82]. The proton transfer agent present in the indicator solution can be any Bronsted acid (HA), including dihydrogen phosphate or dihydrogenphtalate [82, 83]. The lumophore tris(2(2-pyrazinyl)thiazole)-ruthenium(II) cation, $Ru(pzth)_3^{2+}$ was immobilized onto CMSephadex, saturated with 0.1 M hydrogen phtalate buffer and covered with a silicone membrane. With an aqueous solution of $Ru(pzth)_3^{2+}$, which absorbs strongly at 331 nm, the observed emission at 652 nm decreases with increasing concentration of HA through an irreversible proton transfer quenching reaction. The sensor allows the measurement of pCO_2 through lifetime or intensity measurements, but it is temperature sensitive as most CO_2 optical sensors and exhibits slight sensitivity toward oxygen. Figure 16 illustrates the normalized luminescence intensity or emission lifetimes as a function of pCO_2 [83].

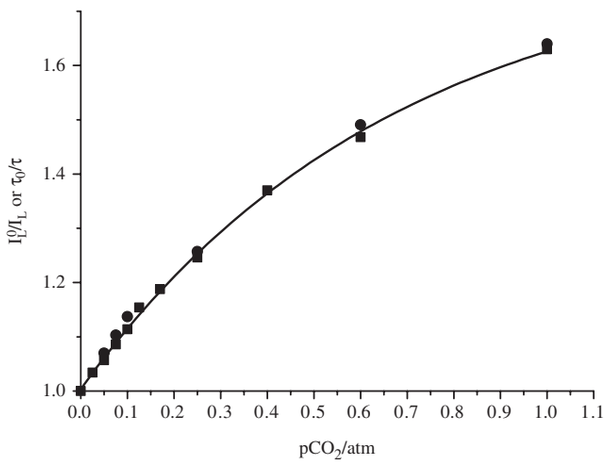

Figure 16. Stern-Volmer plot of the luminescence intensity (I_0/I) (■) or emission lifetime (τ_0/τ) (•) as a function of pCO_2 based on $Ru(pzth)_3^{2+}$ immobilized onto CM-Sephadex soaked in a pH 7.25 hydrogen phthalate solution and covered with silicone membrane. Reproduced with permission from [83], G. Orellana et al., *SPIE* 2508, 1 (1995). © 1995, SPIE.

FRET pioneered by Lakowicz and co-workers [84, 85] is mostly used technique to realize pCO_2 optical sensor, employing phase modulation technique, rather than a pulsed light source technique. An inert fluorescent dye (donor D), whose emission overlaps the absorption band of the pH indicator (acceptor A) is co-immobilized in the sensor film. The lifetime and emission spectrum of the lumophoric donor are usually pH independent quantities. The excited state of the lumophore is quenched via FRET by the deprotonated form of the pH-sensitive dye. An increase in the level of pCO_2 decreases the concentration of the deprotonated form of the acceptor A, leading to the an increase in the fluorescence intensity and lifetime of the donor lumophore D. Lakowicz and co-workers [84] used a pH-insensitive lumophore, sulforhodamine 101 (SR 101) as donor and m-cresol purple or thymol blue as pH-dependent acceptor, entrapped in an ethyl cellulose. Unfortunately, the lifetime of SR 101 is very short (in the range from 0.5 to 1.7 ns), therefore modulation frequencies higher than 100 MHz are required as well as expensive and complex detection instrumentation. Recently, Neurauter et al. [86] have overcome this problem by development FRET-pCO_2 sensor uses ruthenium(II) tris-(4,4′-diphenyl-2,2′-bipyridyl), $Ru(dpby)_3^{2+}$ as a donor, which shows lifetimes up to several hundred nanoseconds. The lipophilic pH sensitive acceptor was thymol blue, which was together with luminophore encapsulated in silicone rubber by an ion-pair technique [87] with 3-trimethylsilyl-1-propane sulfonate anions and tridodecylmethyl ammonium cations to create sensor for low-level CO_2 detection in sea water, that is compatible with low-cost phase modulation techniques known from lifetime-based oxygen sensors. TOA^+OH^- was added as a lipophilic counter ion stabilizing the anionic (deprotonated) form of pH indicator in the matrix. The absorbance maximum of the TOA^+TB^- ion pair overlaps with emission spectrum of Ru(II) complex at 620 nm, where the energy transfer occurs. Further development of this sensor led to time-resolved two-dimensional luminescence lifetime imaging of planar optical sensors using CCD cameras [88].

The major limiting factor for long-term use of above described sensor [86] is cross sensitivity toward oxygen, what is common problem in case of sensors for CO_2 detection, as well as temperature effect, causing the sensitivity decreases with increasing temperature. The high oxygen cross-sensitivity was also the case in FRET-based CO_2 sensor reported by research group of MacCraith [89]. In this case, Sudan III was employed as a pCO_2-sensitive acceptor dye, which was co-immobilized with a long-lifetime ruthenium polypyridyl complex in an ormosil/ethylcellulose hybrid material. The use of diazo dye Sudan III has broadened the dynamic range up to 100% carbon dioxide. The increased sensitivity at high humidity levels limit the applications of this sensor to environment of constant high humidity such as in food packages.

Although this kind of sensors can overcome the problems associated with intensity measurements, some drawback still remain unsolved. The main problem is oxygen cross sensitivity, which is introduced by the use of ruthenium dyes as a long-lifetime reference luminophore quenched by oxygen, and can be minimized by encapsulating the dye in oxygen impermeable polymer nano-beads [90]. Furthermore, a

novel highly attractive indirect sensing scheme introduced by Klimant [91, 92], called Dual Luminophore Referencing (DLR), converts the analyte-sensitive fluorescence intensity signal into the phase- domain or time-domain information. This internal ratiometric method make use of two fluorescent dyes, one being the short-lifetime indicator (for example pH indicator HPTS), the other being inert long-lifetime reference luminophore, e.g., a ruthenium-ligand complex) with similar spectral characteristics. DLR scheme is not based on the measurement of intensity but rather of the phase-shift that is modulated by the varying contributions of the amplitude of the indicator dye to the total signal amplitude. The spectra of the two dyes need to overlap, and the phase shift of the total signal is measured with respect to the phase of the modulation frequency of the light source. In this sensing scheme, the indicator and reference material are immobilized on common support. The layer facing the sample of the indicator and reference material is covered by a blackened layer, that allows contact between the indicator material and sample but is impermeable to the light used for exciting the indicator and reference material and improving the sensitivity of the sensor. The problem of oxygen cross-sensitivity, which is introduced by the use of Ruthenium dyes as a long-lifetime reference luminophore can be minimized by encapsulating the dye in oxygen-impermeable nanoparticles made from poly(acrylonitrile). An optical sensor for CO_2 using DLR approach was prepared by encapsulation of pH indicator HPTS and $Ru(dpp)_3^{2+}$ doped nano-beads in hydrophobic organically modified silica matrix [93]. Excitation and emission wavelengths of ruthenium complexes and HPTS dye sufficiently well match to make them appropriate candidates for a DLR-type carbon dioxide sensor that exhibits excellent compatibility with phase fluorometric oxygen sensor technology [94]. Quaternary ammonium base cethyltrimethylammonium hydroxide was used as ion-pairing agent for polar pH indicator as well as an internal buffer system and has increased the dynamic range up to 100% CO_2 with the resolution of $\pm 1\%$.

Considering the advantages and disadvantages of so far reported indirect opto-chemical sensing methods, the novel luminescent Eu(III) chelate (3-amino-6,7-dimethoxy-4-trifluoromethyl-2(1 H)-quinolinone as antenna molecule combined with diethylenetriaminepentaacetic acid (DTPA) as the complex forming site) developed by Uray et al. [95] offers the opportunity to overcome several problems of the previous sensing methods and enable the direct determination of CO_2. The dye comprises an excitation wavelength of 370 nm, large Stokes shift (>150 nm), spiked emission and luminescence decay time up to the millisecond range. The luminescent dye exhibits a pH dependence of both, the luminescence intensity and lifetime in the region suitable for carbon dioxide measurements. Additionally, the Eu(III)-chelate is completely insensitive to oxygen, what is in contrast to previously used ruthenium dyes. This dye dissolved in alcohol solution of polyurethane was employed in development of the sensing scheme for dissolved carbon dioxide in aqueous solutions in range from 8 hPa to 145 hPa pCO_2 based on luminescence lifetime measurement without the employment of an additional indicator dye [96]. The phase measurement technique developed at JOANNEUM RESEARCH was adopted for the requirements of the new dye, with respect to the excitation light source, modulation frequency, optical filters and a suitable reference system, is in detailed described in Ref. [96].

GLOSSARY

Band-pass optical filter A filter which allows required wavelength ranges to pass through selectively.

Cross-correlation technique An approach for phase measurements with high frequencies. This method is characterized by multiplication of the high frequency signal with a signal with a slightly different frequency. This different frequency, called as cross-correlation frequency, maintains the phase shift information.

Dichroic filter A filter obtained by depositing a multi-layer structure of thin films of dielectric material between two reflective surface. In particular, the black surface is a totally reflective one. Therefore, the light of a selected spectral region passes through the filter whereas the complementary spectral range is reflected. Dichroic filters combine the behavior of the filter with that of wavelength selective beamsplitter.

DLR (Dual Luminophore Referencing) Indirect sensing technique which converts the analyte-sensitive fluorescence intensity signal into the phase-domain or time-domain information. Internal ratiometric method uses two fluorescent dyes, short-lifetime indicator (e.g., pH indicator) and inert long-lifetime reference luminophore (e.g., ruthenium-ligand complex) with similar spectral properties.

Exclusive-Or gate The technique for time measurement which converts of the time shift into a duty-cycle. If the phase shift is zero, the output signal is also zero, otherwise if the phase shift is 180° then the output signal is constant one.

Falselight Instrumental intrinsic optical background which originates from the opto-chemical sensor instrumentation (e.g., optical filters, light sources and sensitive layer where the luminophore is immobilized).

Frequency domain or phase modulation method Second method for the detection of the luminescence lifetime; the sample is excited with sinusoidally modulated light at certain frequency and phase shift (time delay) between the sine function of luminescence to that of exciting light is determined.

Frequency generator The unit which provides a signal of a certain frequency to modulate the intensity of a light source using an optimized driver circuit.

FRET (Fluorescence resonance energy transfer) Non radiative distance dependent dipole dipole interaction in which the acceptor quenches the donor fluorescence. The rate depends on the fluorescence quantum yield of the donor and the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor.

Gelatine filter A filter which is manufactured by dissolving suitable organic dyes in liquid gelatine, by coating the proper amount of solution onto a glass surfaces and after drying by covering by a lacquer.

Glass filter Colored glass based filters. They are obtained by adding impurities (heavy metal or rare earth ions) to glass

material or by temperature activating impurities known as color carriers.

Interference filter A filter obtained by depositing a multilayer structure of thin films of dielectric materials between two reflecting surface. In contrast to dichroic filters none of the external surface is totally reflective. The light entering the filter undergoes constructive or destructive interference from the multi-layer structure and therefore only selected wavelengths can pass through the filter.

LED Acronym for light emitting diode. Solid state light source which exhibit a nonmonochromatic incoherent emission band, characterized by a typical broadening of some tenths of nanometer.

LED modulation circuit Its major task is to provide the LEDs with an adequate prepared square wave or sinusoidal signal from the frequency generation unit of the phase detection unit.

Long-pass optical filter A filter which blocks visible light of short wavelengths and let the long wavelengths range through.

LP Low-pass filter. Its output represents a voltage signal corresponding to the measured phase-shift.

Luminescence Released energy of an electronically excited state species in the form of light. Depending on whether the excited state is singlet or triplet, the emission is called fluorescence or phosphorescence.

Luminescence lifetime The average time a molecule remains in the excited state after excitation. It is an intrinsic property of an indicator and is virtually independent of fluctuations in light intensity, detector sensitivity and light path of the optical system

Neutral density filter A filter which attenuate the visible radiation in the same way for all wavelengths of the spectrum.

Phase fluorometric instrument An instrument consisting of a signal generation unit (light source, driver circuit), a signal detection unit (phase detector) and unit to compare these two signals to obtain the phase shift.

Phase locked detection of fluorescence lifetimes is based on phased locked loops, where the lifetime is converted to a repetitive output signal, which serves as modulation signal. The signal frequency is directly proportional to the luminescence lifetime.

Phase shift In case of two sinusoidal modulated signals of the same frequency, the difference between the angles at which the two signals reach the maximum. In case of indirect luminescence lifetime measurements a sinusoidal modulated radiation is used to excite the luminescence. As a consequence, the emission is also a sinusoidal modulated signal at the same frequency but with a phase shift with respect to the excitation. This phase shift relates to the luminescence lifetime of the luminophore.

Photobleaching of the dye Decrease in the luminescence intensity due to exposure to light.

Photodetector, Photo-detector Any device which converts the incoming light in an electrical signal. Photodetectors are based either on the emission of electrons from photocathodes due to light (photo-multiplier tubes) or on changes of the conductivity of a semi-conductive device or either on the generation of a voltage by internal photo effect.

Photo-detector A devices based on the emission of photoelectrons from photo-cathodes or on changes of the conductivity of semiconductor due to the incident radiation.

Photodiode A kind of photo based on a pn junction.

Photodiode amplifier circuits A devices to convert the signal from the photodiode into an adequate voltage signal which can be further processed at the analog-digital converter stage.

Quartz oscillator A counting device with a precision time reference used for time measurements.

RTP Room temperature phosphorescence is the emission of the light of an electronically excited triplet state. Phosphorescence can be observed at room temperature as well when the indicator is placed on a rigid support such as silica gel, sometimes even in the presence of oxygen.

Short-pass optical filter A filter which blocks visible light of long wavelengths and let the short wavelengths range through.

Silicium photodiode A photodetector based on a silicium based pn junction.

SNR Acronym for signal to noise ratio. It is a measure of the power of the signal (information) compared to the noise level given by an optical background.

Synchronous demodulation The technique for the phase shift measurement. It is based on a high correlation of the signal of interest and a reference signal, which is locked to the signal of interest and the "lock-in system" is used for such systems.

Thermal detectors A devices such as bolometers, pyroelectric detectors, in which the absorbed energy from the incident radiation raises the temperature and causes changes in temperature dependent properties of the detector.

Time domain or pulse method The method for the detection of the luminescence lifetime, where the sample is excited with brief pulse of light and the time-dependent decay of luminescence intensity is measured.

TTL Transistor-transistor-logic signal. A comparator produces a phase shifted signal TTL, which is fed into an "exclusive-or" gate as well as the excitation square wave.

Zero-crossing detection is based on the measurement of the time shift of the zero-crossing of DC-less modulation and luminescence signals.

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