Room temperature phosphorescence in the liquid state as a tool in analytical chemistry

Jacobus Kuijt, Freek Ariese, Udo A.Th. Brinkman, Cees Gooijer*

Department of Analytical Chemistry and Applied Spectroscopy, Vrije Universiteit Amsterdam, de Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Received 28 April 2003; accepted 27 May 2003

Abstract

A wide-ranging overview of room temperature phosphorescence in the liquid state (RTPL1) is presented, with a focus on recent developments. RTPL techniques like micelle-stabilized (MS)-RTP, cyclodextrin-induced (CD)-RTP, and heavy atom-induced (HAI)-RTP are discussed. These techniques are mainly applied in the stand-alone format, but coupling with some separation techniques appears to be feasible. Applications of direct, sensitized and quenched phosphorescence are also discussed. As regards sensitized and quenched RTP, emphasis is on the coupling with liquid chromatography (LC) and capillary electrophoresis (CE), but stand-alone applications are also reported. Further, the application of RTP in immunoassays and in RTP optosensing—the optical sensing of analytes based on RTP—is reviewed. Next to the application of RTPL in quantitative analysis, its use for the structural probing of protein conformations and for time-resolved microscopy of labelled biomolecules is discussed. Finally, an overview is presented of the various analytical techniques which are based on

Abbreviations: 1-BrN, 1-bromonaphthalene; 2-BrN, 2-bromonaphthalene; 4-MSA, 4-maleimidylsalicylic acid; 6Br2N, 6-bromo-2-naphthol; α-CDE, 6-ido-6-deoxy α-cyclodextrin; β-CDE, 6-ido-6-deoxy β-cyclodextrin; A, adenine; acac, acetylacetone; AOT, di-2-ethylhexylsulfosuccinate sodium salt; AP, alkaline phosphatase; β-CD, heptakis (6-bromo-6-deoxy β-cyclodextrin); BSA, bovine serum albumin; C, cytochrome; CCD, charge-coupled device; CD, cyclodextrin; CD-RTP, cyclodextrin-induced room temperature phosphorescence; CE, capillary electrophoresis; CSO, cholesterol oxide; CPB, cetylpyridinium bromide; CPM, coproporphyrin I ketone; CPK, coproporphyrin I ketone tetraethyl ester; CTAB, cetyltrimethylammonium bromide; CTAC, cetyltrimethylammonium chloride; cyclic AMP, cyclic 3′,5′-adenosine monophosphate; CZE, capillary zone electrophoresis; dansyl chloride, 5-dimethylaminonaphthalene sulfonyl chloride; DMF, dimethylformamide; DNA, deoxyribonucleic acid; DOM, dodecyl-β-d-maltoside; EA, electron affinity; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ET, energy transfer; ferron, 8-hydroxy-7-iodo-5-quinolinesulfonic acid; FIA, flow injection analysis; G, guanine; HAI-RTP, heavy atom-induced room temperature phosphorescence; HAP, heavy atom perturber; HRP, horseradish peroxidase; IC, inner conversion; IP, ionization potential; ISC, intersystem crossing; LC, liquid chromatography; LED, light-emitting diode; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MIP, molecularly imprinted polymer; MS-RTP, micelle-stabilized room temperature phosphorescence; NS, naphthalene sulfonate; OEPK, octaethylporphine ketone; PAR, perylene-3,4-carboxylic acid; Ph4TBP, meso-tetraphenyltetrabenzoporphyrin; PMP, pinacolyl methylphosphonate; RNA, ribonucleic acid; R.S.D., relative standard deviation; RTPL, room temperature phosphorescence in the liquid state; SDBS, sodium dodecylbenzene sulfonate; SDS, sodium dodecyl sulfate; S/N, signal-to-noise ratio; SS-RTP, solid surface room temperature phosphorescence; T, thymine; TBP, tetrabenzoporphyrin; UV, ultraviolet; VASS, variable-angle synchronous scanning

* Corresponding author. Tel.: +31-204447540; fax: +31-204474743.
E-mail address: gooijer@chem.vu.nl (C. Gooijer).

0003-2670/03/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
the closely related phenomenon of long-lived lanthanide luminescence. The paper closes with a short evaluation of the state-of-the-art in RTP and a discussion on future perspectives.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Room temperature phosphorescence; Luminescence; Lanthanides; Liquid state; Analytical applications

1. Basic aspects of RTPL

1.1. Introduction

In the past decades, room temperature phosphorescence in the liquid state (RTPL) has evolved into a sensitive and versatile tool in analytical chemistry, based on many different detection principles and solvent systems suitable for the generation of RTPL [1]. In this review, developments from the past decade will be highlighted. Topics of interest include micelle-stabilized room temperature phosphorescence (MS-RTP), cyclodextrin-induced RTP (CD-RTP) and the recently developed heavy atom-induced RTP (HAI-RTP) technique. In addition, attention will be devoted to the interesting subject of RTP optosensing, and to the versatile coupling of indirect RTP modes (sensitized and quenched phosphorescence) to various separation techniques. Tryptophan RTP will be shown to be a valuable and sensitive tool for the structural analysis of proteins. Finally, the subject of long-lived lanthanide luminescence will be discussed. This technique is applied in optosensing and immunoassays, for the detection of biomolecules, and as a detection method in liquid chromatography (LC) and capillary electrophoresis (CE). Low-temperature phosphorescence (Shpol’skii phosphorescence spectroscopy) and solid surface (SS)-RTP—neither of which are RTPL techniques—will not be discussed. For a review on Shpol’skii phosphorescence spectroscopy, the reader is referred to [1], while SS-RTP is dealt with in [2].

In this review, emphasis will be on fundamental aspects of RTPL that are important for an assessment of its present and future potential, and on novel applications and detection principles. The sensitivity of the electronically excited triplet state to external factors generally requires efficient protection from dissolved molecular oxygen as well as blocking of non-radiative decay. Hence, much research is still devoted to finding ways to induce or enhance RTP. As will be seen in this review, the range of conditions compatible with RTPL keeps expanding, thereby broadening the application range of RTPL.

It should be noted that the sensitivity of the (excited) triplet state to external factors also creates possibilities for detection, especially in the field of RTP optosensing, quenched phosphorescence detection and the structural probing of proteins. Consequently, the application range of RTPL is larger than the group of compounds that displays native phosphorescence: any compound or solvent parameter that is able to influence the phosphorescence signal, either positively or negatively, can be detected.

1.2. Theory

Phosphorescence of organic molecules can be defined as the radiative transition originating from the lowest excited triplet state, T1, to the (singlet) ground state, S0. In contrast to fluorescence (singlet-to-singlet transition), phosphorescence is a spin-forbidden process. Nevertheless, it can be observed under specific conditions, due to internal or external spin–orbit coupling which mixes pure singlet and triplet states to produce states with a mixed character in spin multiplicity [3,4].

The photophysical processes relevant to directly excited RTP are presented in Fig. 1. In order to obtain direct phosphorescence, the phosphorophore is excited by light of the appropriate wavelength. After excitation, rapid vibrational deactivation and internal conversion (IC)2 occur. Fluorescence will therefore generally be emitted from the lowest vibrational level of the lowest electronically excited singlet state, S1. Many organic molecules also show IC/vibrational relaxation to the S0 ground state. In addition, a significant fraction of the excited molecules will undergo intersystem crossing (ISC) to T1. The intersystem crossing quantum efficiency, ϑISC, can be enhanced by internal or external spin–orbit coupling—the
so-called heavy atom effect. However, population of
the triplet state is not the only requirement for obtain-
ing RTP: its subsequent deactivation by phosphores-
cence should also be efficient, i.e. it should be able to
compete with the other deactivation pathways open to
the triplet state—deactivation by non-radiative decay
and bimolecular quenching from dissolved molec-
ular oxygen or other quenchers. In this context, it
is important to note that strong spin−orbit coupling
usually also enhances the rate constant of phosphores-
cence, $k_p$, resulting in reduced competition from the
other deactivation pathways. Alternatively, the rela-
tive importance of RTP can be increased by reducing the
rates of the non-radiative deactivation pathways.

This can be achieved by enhancing the rigidity of the
environment—for example, by inclusion of the phos-
phorophore into micelles or CDs—and by efficient
deoxygenation of the sample. The phosphorescence
quantum yield, $\phi_p$, or $\phi_{ISC} \times \phi_p$, is given by

$$
\phi_p = \phi_{ISC} \times \phi_p = \frac{k_{ISC}}{k_p + k_{ad} + \sum k_{q,p}}
$$

where $k_{ISC}$ is the intersystem crossing rate constant,
$k_f$ and $k_p$ are the rate constants of fluorescence and
phosphorescence, respectively, $k_{ad}$ and $k_{q,p}$ the rate
constants of non-radiative decay, and $\sum k_{q,p}$ the sums of all effective (unimolecular)
quenching rate constants of fluorescence and phos-
phorescence, respectively. Due to the slow kinetics of phosphorescence compared to fluorescence, bi-
molecular quenching of the triplet state by molecular
oxygen ($k_q$ ca. $10^9 \text{M}^{-1} \text{s}^{-1}$) is highly efficient, in
contrast to collisional quenching of the excited singlet
state. Therefore, dissolved molecular oxygen should
be removed efficiently in order to achieve a large
value for $\phi_p$ and, thus, for $\phi_p$. Model calculations
based on Eq. (1) readily show that direct RTP will
be shown only by a limited number of compounds.

For a molecule displaying phosphorescence, $k_p$ is
typically $1 \text{s}^{-1}$ or less. Therefore, for such a molecule
a phosphorescence quantum yield of ca. 0.5 can be
achieved only if the oxygen concentration is below
$10^{-9} \text{M}$—even if it is assumed that $\phi_{ISC}$ equals unity
and that the other triplet state deactivation pathways
can be neglected. However, such a low oxygen con-
centration is not easily maintained in practice. For
exceptional phosphorophores, $k_p$ may be as large as
$10^2 \text{s}^{-1}$. Using the same assumptions as above, this
means that $\phi_p$ will be about 0.5 even when the oxygen
concentration is $10^{-7} \text{M}$—a concentration level that
is easily achieved when using standard equipment.

As noted above, there are two important modes of
indirect phosphorescence that can be used for detec-
tion purposes, sensitized RTP and quenched RTP. An
eample of sensitized RTP is given in Fig. 2. This

![Fig. 1. Schematic representation of photophysical processes rele-
vant to directly excited RTP. $S_0$, singlet ground state; $S_1$, lowest
excited singlet state; $T_1$, lowest excited triplet state; exc, excita-
tion; flu, fluorescence; v, vibrational relaxation; ISC, intersystem
crossing; q, bimolecular quenching; phos, phosphorescence.

![Fig. 2. Spectra of $5 \times 10^{-6} \text{M}$ 2,6-naphthalenedisulfonic acid in
demineralized and distilled water: (a) fluorescence (1) and sen-
sitized phosphorescence (2) excitation spectrum, with emission
wavelengths of 346 and 513 nm, respectively; (b) fluorescence
emission spectrum; (c) sensitized phosphorescence emission spec-
trum ($10^{-4} \text{M}$ biacetyl). The emission spectra were obtained with
excitation at 230 nm. Figure taken from [5].](image-url)
figure clearly illustrates the large wavelength difference, generally observed in sensitized RTP, between the excitation spectrum (a) and the emission spectrum (c). Obviously, the fluorescence emission band (b) is at a much shorter wavelength, which results in a much smaller wavelength difference for fluorescence. Sensitized RTP can be obtained if a sensitizer (analyte) molecule is excited that is able to provide triplet–triplet energy transfer (ET) to the phosphorophore. Obviously, for efficient ET to take place the rate constant, \( k_{\text{ET}} \), should be large. This implies that the triplet level of the donor molecule should be higher than the triplet state of the acceptor \([3,4,6]\). In addition, there should be significant spectral overlap between the phosphorescence emission spectrum of the donor and the \( S_0 \rightarrow T_1 \) absorption spectrum of the acceptor. Even if these conditions are fulfilled, efficient ET occurs only in case of sufficiently long donor triplet lifetimes and high acceptor concentrations—as can be concluded from Eq. (2)

\[
\phi_{\text{ET}} = \frac{k_{\text{ET}}[A]}{(\tau_0^D)^{-1} + k_{\text{q}}[A]} \tag{2}
\]

where \( \tau_0^D \) is the triplet lifetime of the donor and \([A]\) the acceptor concentration. Assuming that \( k_{\text{ET}} = 10^{10} \text{M}^{-1} \text{s}^{-1} \), \([A] = 10^{-2} \text{M} \), and \( \tau_0^D = 0.1 \mu\text{s} \)—such values are frequently encountered in sensitized RTP experiments—\( \phi_{\text{ET}} \) is already close to its maximum value of unity \([5]\). The intensity of sensitized phosphorescence is given by

\[
I_p(\text{sens}) = 2.303I_{\text{exc},\lambda}(D)\sigma_{\text{exc},\lambda}^D\phi_{\text{ET}}\phi_{\text{ISC}}^D
\]

where \( I_{\text{exc},\lambda}(D) \) is the intensity of the excitation light, while the next three symbols represent the extinction coefficient of the donor, its concentration, and the optical path length, respectively, and the final three the intersystem crossing efficiency of the donor, the efficiency of energy transfer, and the phosphorescence efficiency of the acceptor, respectively \([5]\). In many cases biacetyl has been used as the phosphorophore: strong enhancement compared to direct RTP can thus be achieved since biacetyl has a very low absorption over its entire spectrum \([7]\), whereas in favorable cases the donor (analyte) may have a high extinction coefficient.

Bimolecular quenching is not only a problem that has to be solved in RTP-based methods. It can also be used for detection; in this case, the directly excited or sensitized RTP from exceptionally strong phosphorophores like biacetyl or bromonaphthalenes is decreased due to the collisional deactivation provided by the analytes (quenchers). Quenching is based either on triplet–triplet energy transfer (requirements are identical to those for sensitized RTP) or on electron transfer. Triplet–triplet energy transfer is generally considered to proceed via the exchange (or collisional) mechanism proposed by Dexter \([3,4]\), a mechanism that essentially requires molecular contact (typical critical interaction radius, \( R_{\text{c}} \), ca. 10 Å) \([4]\).

In most cases, the long-range (coulombic) mechanism according to Förster should be ruled out, because it is spin-forbidden (only interactions in which both spins are preserved are allowed, e.g. singlet–singlet energy transfer). However, as will be discussed, under certain conditions the Förster mechanisms can also be relevant for quenching interactions involving a change in spin, even though this is forbidden \([3,4]\).

Electron transfer reactions are based on the oxidizing and reducing properties of the quencher and the phosphorophore. In a simplified approach for ground-state molecules in the gas phase, the ability to release an electron depends on the (first) ionization potential (IP), while the ability to accept an electron is given by the electron affinity (EA). For efficient electron transfer to occur, the energy released should be larger than the energy required for ionization of the donor (IP), i.e. the reaction should be exothermic. Donor molecules like amines can be oxidized quite easily, since they have non-bonding nitrogen orbitals at a relatively high energy level. In addition, photo-excited molecules are usually much more susceptible to redox reactions than their ground-state counterparts. This implies that excited donor molecules are oxidized more easily (IP* < IP), while the propensity for excited acceptor molecules to accept electrons is also enhanced (EA* = EA) \([8]\).

Regardless of the specific mechanism involved, the quenching interaction will be governed by the well-known Stern–Volmer equation for bimolecular quenching

\[
\frac{I_p}{I_p} = 1 + \frac{k_{\text{q}}[A]}{k_{\text{ET}}[A]} \tag{3}
\]

where \( k_{\text{q}} \) is the quenching rate constant, \([A]\) the quencher concentration, and \( I_p \) the intensity of the phosphorescence.

3 For electron transfer reactions in solution, redox potentials have to be used, while also solvent energy terms have to be taken into account. Nonetheless, the variations in the redox potentials often parallel those in the values of IP and EA, i.e. a plot of redox potential versus IP or EA will generally be linear.
quenching of luminescence

\[ \frac{I}{I_0} = 1 + k_q [Q] \tau_0 \]  

where \( k_q \) is the rate constant for bimolecular quenching, \([Q]\) the quencher (analyte) concentration, and \( \tau_0 \) the triplet lifetime in the absence of quenchers. According to Eq. (4), even low concentrations of quenchers will exhibit efficient quenching if the triplet lifetime, \( \tau_0 \), and the associated quenching rate constant, \( k_q \), are sufficiently high; for collisional quenching (triplet–triplet energy transfer, electron transfer), \( k_q \) is at its maximum if the quenching interaction is diffusion-controlled, i.e. if there is no energy barrier involved. In such a case, \( k_q \) equals \( k_{\text{diff}} \), which is approximated by the Debye equation [3,4]

\[ k_{\text{diff}} \approx \frac{8 \pi R T^3}{3000 \eta} \]  

where \( R \) is the gas constant, \( T \) the temperature, and \( \eta \) the viscosity of the solvent. In aqueous solutions at room temperature, \( k_{\text{diff}} \) is about \( 10^{10} \text{ M}^{-1} \text{ s}^{-1} \), which enables sensitive detection of many compounds by quenched RTP. This is readily illustrated by a model calculation based on Eq. (4). If the lifetime of the phosphorophore in the absence of analytes (quenchers) is \( 10^{-4} \text{ s} \), an analyte concentration as low as \( 10^{-7} \text{ M} \) will cause a 10% reduction of the phosphorescence intensity.

2. Micelle-stabilized RTP

2.1. Introduction

As early as 1980, Cline Love et al. [10] explored the use of sodium dodecyl sulfate (SDS) micelles for the inclusion of phosphorophores (the test analytes, naphthalene, pyrene and biphenyl), in order to reduce the rate of non-radiative decay and, thus, extend the application range of direct RTP to analytes with \( k_q \lesssim 1 \text{ s}^{-1} \). The heavy atom perturbers (HAP), Ag(I) and Tl(I), were added to the micellar solutions to increase the ISC efficiency, \( \varphi_{\text{ISC}} \), and—more importantly—the phosphorescence efficiency, \( \varphi_p \), thereby enhancing the RTP quantum yield and intensity. In all instances, no RTP was obtained in the absence of HAP ions. Due to the ability of the metal ions to replace sodium ions from the Stern layer of the micelles, a close proximity of HAP and phosphorophore was obtained, which is important for a strong enhancement of ISC. For completeness it is noted here that in some unfavorable cases \( k_{np} \) will be enhanced more strongly than \( k_p \); obviously, \( \varphi_p \) will not be enhanced in such cases by the addition of HAP ions.

It should also be stressed that micelles do not prevent quenching by molecular oxygen, apart from the reduction in \( k_q \) due to the increased viscosity (cf. Eq. (5)). Therefore, high RTP quantum yields and intensities require efficient deoxygenation of the sample. The use of nitrogen purging for deoxygenation in micellar systems is complicated by the continuous formation of foam during purging. Therefore, alternative deoxygenation methods have been developed. In 1986, the use of sodium sulfite as an oxygen scavenger in micellar solutions (of naphthalene) was proposed [11]. The low oxygen concentrations required to obtain MS-RTP were achieved by the consumption of oxygen according to

\[ 2\text{SO}_3^{2-} + \text{O}_2 \rightarrow 2\text{SO}_4^{2-} \]  

Sodium sulfite is not only applied for deoxygenation purposes in virtually all MS-RTP studies, but also in techniques such as CD-RTP and HAI-RTP (Sections 3 and 4, respectively).

Other deoxygenation methods have also been reported for RTP in the liquid state: Zn(s)/HCl and Na_2 CO_3/HCl systems were used to remove oxygen from cyclodextrin (CD)-containing solutions, based on the purging action of the evolved gases (H_2 and CO_2, respectively) [12]. Deoxygenation was reported to be rapid, but required strongly acidic conditions (pH < 1). Moreover, the RTP signal was stable only over a limited period of time (at least 10 min), since the purging action ceases when the reaction stops.
Table 1

Detection of naproxen and propanolol in pharmaceutical preparations by MS-RTP and LC

<table>
<thead>
<tr>
<th>Pharmaceutical preparation</th>
<th>Declared content (mg)</th>
<th>Found (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS-RTP</td>
<td>LC</td>
</tr>
<tr>
<td><strong>Naproxen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antalgin (tablets, Syntex Latino)</td>
<td>275</td>
<td>290</td>
</tr>
<tr>
<td>Propanol 250 (capsules, Valio Mestre/Farma 86)</td>
<td>250</td>
<td>217</td>
</tr>
<tr>
<td>Proxen (capsules, Berenguer-Inale)</td>
<td>250</td>
<td>263</td>
</tr>
<tr>
<td>Proxen (vials, Berenguer-Inale)</td>
<td>617</td>
<td>909</td>
</tr>
<tr>
<td><strong>Propanolol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betadipresan (tablets, Fides)</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Betadipresan-DIU (tablets, Fides)</td>
<td>100</td>
<td>73</td>
</tr>
<tr>
<td>Sumial 10 (tablets, ICI-Farma)</td>
<td>10</td>
<td>10.3</td>
</tr>
<tr>
<td>Sumial Retard (capsules, IC1-Farma)</td>
<td>160</td>
<td>155</td>
</tr>
</tbody>
</table>

Adapted from [14].

Therefore, these methods will probably not find widespread application in RTP-based techniques.

2.2. Stand-alone measurements

There are many examples of MS-RTP detection based on measurement with a conventional luminescence spectrometer. Due to the inherent selectivity of MS-RTP, detection of target analytes in pharmaceutical and agricultural samples is frequently possible without (extensive) sample preparation. SDS has been used most often (concentration, 10–100 mM). In most cases, ca. 20 mM thallium nitrate was used as HAP and 10–20 mM sodium sulfite for deoxygenation. Some examples are given below.

Naproxynl was determined in Praxilene tablets with a nominal content of 100 mg; analysis yielded 104.2 mg with an R.S.D. of 2.4% [13]. Naproxen and propanolol [14] were detected in pharmaceutical products with satisfactory accuracy (Table 1), although the presence of hydralazine in Betadipresan resulted in quenching of phosphorescence and, thus, rather low values for the propanolol concentration. For comparison, the samples were also analyzed using LC with UV absorption detection. In general, the LC data were somewhat closer to the declared concentrations. Naproxen was also determined in human serum and urine (recovery, 97–101%), spiked with the analyte at concentrations (20–100 mg/l) typically found within 12 h after application of the drug [15].

The pesticide napropamide was determined in commercial pesticide formulations and in spiked tomato and pepper samples [16]. The determination of 1-naphthaleneacetic acid in spiked canned pineapple was also reported [17]. In both cases, satisfactory recoveries were achieved.

Some metal ions, in particular Al(III), Ga(III) and In(III), can be detected by RTP after complexation with 8-hydroxy-7-iodo-5-quinolinesulfonic acid (ferron) due to the ensuing enhancement of $\theta_{ISC}$ and $\theta_p$ [18]. Detection of the metal–ferron (1:3) complexes was performed using several surfactants. For the detection of Ga(III), neutral Brij-35 and the cationic surfactants cetylpyridinium bromide (CPR) and cetyltrimethylammonium bromide (CTAB) provided the best detectability. The latter surfactant provided a limit of detection (LOD) of 5 ng ml$^{-1}$, an R.S.D. of 4% (at 50 ng ml$^{-1}$) and linearity over two orders of magnitude. Detection is selective since only a few metals give stable complexes. Nevertheless, metal ions like Cu(II), Ni(II), Co(II) and Fe(II) can interfere because they are able to quench RTP. Fortunately, these ions can be masked with 9,10-phenanthroline or EDTA, while the signal from Al–ferron can be suppressed by adding fluoride ions. Alternatively, mixtures of Ga(III) and Al(III) can be analyzed by using Kalman filtering [18]. The remaining problem—the presence of In(III) in Ga(III)-containing samples—was addressed in a subsequent study by using time-resolved detection to discriminate between the two RTP lifetime components (126 and 158 ms for In(III) and Ga(III)).

6 Although this method is an example of MS-RTP, it can also be classified as an RTP optosensing technique.
respectively) [19]. Thus, binary mixtures could generally be analyzed with 10% accuracy or better.

Detection of Pt(II) after complexation with ferron or ferron-like compounds such as 8-quinolinol and 5-sulfo-8-quinolinol, was performed in the presence of several cationic (CTAC, CTAB) or neutral (Brij-35, Triton X-100, DOM) surfactants for micelle formation [20]. The best result (LOD, 6 ng ml\(^{-1}\)) was obtained with n-dodecyl-β-D-maltoside (DOM). In another study, Pd(II) ions were detected using the RTP of its coproporphyrin III complex after cloud-point precipitation [21]. After heating of the sample to 95 °C, the Triton X-100 phase containing the analyte separated from the aqueous phase, which resulted in a 10-fold enhancement of the detectability (LOD, 2 nM).

2.3. Stopped-flow mixing

A convenient and easily automatable procedure to obtain MS-RTP was reported by Panadero et al. [22]: by using a stopped-flow mixing technique, they obtained RTP from the pesticide carbaryl within 2–3 s after mixing of the solutions (Fig. 3). In the set-up used (Fig. 4), a syringe solution containing thallium nitrate, sodium sulfite and buffer, and a syringe solution containing SDS, sodium sulfite, buffer and carbaryl were mixed. Although the RTP signals are obtained much faster than in conventional MS-RTP batch measurements, it should be noted that both solutions contain sodium sulfite. That is, the time needed to obtain RTP is not the deoxygenation time but, rather, the time the thallium ions need to replace the sodium ions from the micellar Stern layer [22]. Both
the slope (before the curve starts levelling off) and the amplitude (signal after levelling off) were used for analysis. With the same set-up, RTP from dipyridamole [23] and naproxen [24] was obtained within 1–10 s. In all cases, LODs were in the 10–20 ng ml\(^{-1}\) range [22–24]. Naproxen was successfully determined (recovery, 91–106%) in serum spiked within the therapeutic doses (25–70 mg/l) [24], while the analysis of Asasantin 75 and Persantin 100 tablets required no sample pretreatment—apart from simple dissolution of the tablets and dilution in 0.1 M SDS [23].

A different syringe filling scheme than the one considered above was reported in [25]: one syringe solution contained SDS and the analyte (nafronyl), and the other one, a buffer with sodium sulfite and thallium nitrate. Since the deoxygenant and HAP are separated from the analyte and SDS before mixing, the time to obtain phosphorescence (5 s for full signal) includes not only the time for the thallium ions to replace the sodium ions from the micelles, but also the time required for adequate deoxygenation. That is, the stopped-flow mixing technique is by far the fastest way to obtain RTP to date.

2.4. Sensitized RTP

As noted before, bimolecular interactions are not seriously hindered by the inclusion of the analytes in micelles. Therefore, sensitized RTP—a technique requiring frequent and efficient collision of donor and acceptor molecules—can also be applied in micellar systems. The acridine dyes trypaflavine, acridine yellow and acridine orange were used to generate sensitized phosphorescence from several polycyclic aromatic hydrocarbons (PAHs) included in SDS micelles [26], with sodium sulfite as deoxygenant and Ti(I) as HAP. Selective detection of some target PAHs in the presence of other PAHs was possible due to the differences in the \(\Phi_\text{1}\) energy levels. For instance, pyrene (\(\Phi_\text{1}, 16,900 \text{ cm}^{-1}\)) could be selectively determined—with selectivity factors ranging from 350 to 5000—in the presence of fluorene, naphthalene and phenanthrene (\(\Phi_\text{1}, 21,000–22,000 \text{ cm}^{-1}\)) upon sensitization by trypaflavine (\(\Phi_\text{1}, 17,800 \text{ cm}^{-1}\)). For these PAHs, the selectivity was improved about 2, 7, and 35-fold, respectively, compared to fluorescence and direct MS-RTP.

Sensitized RTP from biacetyl included in di-2-ethyl hexylsulfosuccinate sodium salt (AOT) reversed micelles was also studied [27]. In these micelles, the hydrophobic tails are directed towards the apolar (or low-polarity) bulk solution, while the ionic groups surround the water pool present in the micellar center. Both the sensitizer, \(\alpha\)-naphthylacetic acid, and biacetyl are present in the micellar water pools. The energy transfer efficiency, \(\Phi_\text{ET}\) (cf. Eq. (2)) was found to decrease with an increasing relative size of the water pool, \(W = [\text{H}_2\text{O}]/[\text{AOT}]\), at higher values of \(W\), due to the increased average distance between donor and acceptor molecules; at low values of \(W\), the decrease in \(\Phi_\text{ET}\) was absent, most probably because of the concomitant decrease in microviscosity. Compared to (normal) SDS micelles, a 13-fold higher RTP signal was obtained. Another advantage of using AOT reversed micelles is that no foam is formed during purging with nitrogen [27]. Sensitized RTP from biacetyl included in AOT reversed micelles was also obtained using either sodium naphthoate as the sensitizer or cetyltrimethylammonium ions (CTA\(^+\)) as co-surfactant and ‘anchored’ naphthoate counterions as the sensitizer [28]. Comparable results were obtained with both sensitizers.

2.5. Novel micellar systems

In the last decade, studies on micro-emulsion-RTP and block-copolymer-based micelles were published. The use of micellar agents other than SDS should be explored since a higher sensitivity and analyte solubility may thus be achieved. In addition, foaming problems associated with nitrogen purging may be reduced.

Water-soluble copolymers of 1-vinylnaphthalene and methacrylic acid were used for the first time in 1991 to generate RTP (from pyrene and benzophenone) [29]. With this type of micelles, foam formation during nitrogen purging was reported to be much less problematic. Poly(ethylene oxide)—poly(propylene oxide)—poly(ethylene oxide) block-copolymer micelles and mixed-block-copolymer/SDS micelles were also used [30]. In the block-copolymer micelles, RTP was slightly enhanced compared to free-solution RTP. With the mixed aggregates, RTP was significantly enhanced even below the critical micellar concentrations of both species.
Lin et al. [31] studied the RTP obtained from 2-bromonaphthalene (2-BrN) in SDS rod-like micelles. At high electrolyte concentrations, a change from spherical to rod-shaped micelles was reported to take place. The generation of RTP was considered to result from a dramatic increase of the microviscosity inside the micelles during the sphere-to-rod transition. It was reported that no RTP was obtained from 2-BrN in spherical micelles, but a convincing explanation for this observation was not presented.

Corroborating earlier work by Ramos et al. [32], various analytes such as PAHs [33–36], carbaryl [37] and the plant growth regulators 2-naphthoxyacetic acid and 1-naphthaleneacetamide [38] were determined by micro-emulsion RTP. In all instances, thallium nitrate was used as HAP. The micro-emulsions consist of SDS micelles (size, ca. 10–20 nm) in water, with higher linear alcohols (typically 0.01–0.02% 1-butanol or 1-pentanol) as co-surfactants [32–38] and linear alkanes (0.01–0.02% n-heptane) [32–36] or 0.02% dichloromethane [37,38] incorporated in the micellar core. Because the particles are quite small, no scattering of visible light occurs and the solutions are clear to the eye. It was stated that an advantage of using micro-emulsions instead of normal micelles is the faster and better solubilization of non-polar compounds. Furthermore, an increased protection against oxygen quenching was reported. However, deoxygenation still appeared to be necessary in all cases and the phosphorescence lifetimes of, typically, 1–3 ms were not significantly longer than those reported for conventional micellar systems [32,35].

Carbaryl was successfully determined in spiked soil samples [37]; the LOD in standard solutions was 24 ng ml$^{-1}$. Naphthalene and phenanthrene were detected with LODs of 17 and 12 ng ml$^{-1}$, respectively [36]. For acenaphthene, an LOD of 5 ng ml$^{-1}$ was reported and the analyte was successfully determined in spiked (75 ng ml$^{-1}$) seawater [33]. Variable-angle synchronous scanning (VASS) phosphorometry was applied in combination with time-resolved detection in order to detect PAHs in mixtures containing three to five PAHs [34,35]; the PAHs were successfully determined in spiked coffee [34] and spiked road-dust samples [35]. Derivative VASS micro-emulsion phosphorometry was applied to the determination of 2-naphthoxyacetic acid and 1-naphthaleneacetamide in spiked soil [38]. In most of the above cases, the spiking levels were not indicated, but probably they were quite high (mg/l level). Therefore, far-reaching conclusions about the selectivity achieved in real-life analysis should not be drawn from these data.

2.6. Concluding remarks

Most recent MS-RTP studies either merely confirm the findings from earlier reports or exemplify the application of MS-RTP in real-life analysis. However, there are also some new developments: stopped-flow mixing was shown to be a convenient and rapid way to obtain RTP, which should find a more widespread use. Sensitized RTP in micelles provides improved selectivity compared to direct MS-RTP and fluorescence, but the enhancement is too small to enable direct analysis of most samples encountered in practice. In addition, novel MS-RTP techniques based on rod-like micelles, micro-emulsions, reversed micelles, and micelles from co-block polymers were developed. An advantage of the latter two micellar systems is that there is no foaming during nitrogen purging. However, it should be noted here that the use of sodium sulfite for deoxygenation completely eliminates foaming problems. The reported enhancements of detectability for the novel micellar systems are rather limited, except for the AOT reversed micelles used with sensitized RTP. Such micelles may also be useful for coupling with LC, since they are compatible with high concentrations of organic modifier. Despite the progress that has been made, the overall impression is that the advantages offered by most of the new MS-RTP techniques discussed above are rather limited.

3. Cyclodextrin-induced RTP

3.1. Introduction

Another approach to obtain direct RTP uses cyclodextrins (CD) for inclusion of the phosphorophores. It was found in the 1980s that inclusion of a phosphorophore in the hydrophobic cavity of a CD induces RTP under deoxygenated solvent conditions, or at least enhances RTP compared to homogeneous solutions. By using the differences in phosphorescence lifetime and intensity between free and complexed phosphorophores, Turro et al. [39] studied the kinetics of
inclusion, using bromo- and chloronaphthalenes as the phosphorescent probes. Addition of acetonitrile resulted in the formation of ternary complexes, and in an enhanced RTP intensity and lifetime for 1-bromonaphthalene and 1-chloronaphthalene. It was also found that quenching by the aqueous-phase quencher nitrite was reduced five-fold upon addition of β-CD. Another early study [40] was dedicated to the RTP from n-(4-bromo-1-naphthoyl)alkyl trimethylammonium bromide surfactants included in β- and γ-CDs. Here, the surfactant part of the phosphorophore is partly included in the cavity with the positively charged end coiling at the mouth of the CD, thus providing additional protection against molecular oxygen and Co(NH₃)₆³⁺—a purely aqueous-phase quencher.

The inclusion of guest molecules in the cavity of host CDs and the requirements for obtaining direct RTP have been studied extensively [41]. It is well known that a neat fit of the guest molecule and host cavity is important for obtaining strong (van der Waals) binding of apolar guest molecules. For polar compounds, electrostatic interactions such as hydrogen bonding are important as well [41]. RTP from included phosphorophores is obtained—or enhanced compared to homogeneous solutions—due to the effective shielding from dissolved molecular oxygen and the more rigid environment (high microviscosity) provided by the cavity of the CD. The phosphorescence can also be enhanced by co-inclusion of a HAP; due to its close proximity to the analyte after binding, both the ISC and the phosphorescence rate constants will be increased. Co-inclusion of a sensitizer molecule instead of HAP will provide sensitized RTP; this technique will also be discussed here.

3.2. Ternary complexes of halogenated phosphorophores

In the early studies on CD-RTP [39,40], phosphorescence was obtained from binary complexes of halogen-containing phosphorophores and CD molecules. As discussed above, the addition of acetonitrile resulted in stronger RTP due to the formation of ternary complexes. Many other compounds able to form ternary complexes with CDs and (halogen-containing) phosphorophores have been used since then, including various linear, branched and cyclic alcohols [42–44] and cationic, anionic or neutral surfactants [44–46]. Strong RTP was obtained even under aerated conditions.

Among the alcohols studied, the branched and cyclic alcohols (tert-butanol and cyclohexanol, respectively) provided the strongest RTP, which suggests that steric factors dominate the protection against quenching by dissolved oxygen [43]. Probably, linear alcohols have the apolar tail in the cavity and the hydroxy group outside, while branched alcohols favor binding at the outside of the CD. In the latter case, the phosphorophores are effectively protected from dissolved oxygen due to capping of the CD mouth [47]. In general, the RTP signal from the binary complexes was strongly enhanced with increasing concentrations of alcohol, except for the less soluble alcohols, which displace the analyte from the CD cavities at high alcohol concentrations [43].

The enhancement was used to develop an RTP-based optosensing scheme for the detection of alcohols in the low mM range [42]. Here, the RTP of a binary glucosyl-β-CD:1-bromonaphthalene (1-BrN) complex dramatically increased (up to 10⁴-fold) in the presence of various branched and non-branched alcohols. The highest sensitivity again was obtained for tert-butanol and cyclohexanol; the primary and secondary alcohols showed poorer detectability.

Among the surfactants used to enhance the RTP of 1-BrN-β-CD complexes, CTAB, sodium dodecyl-benzene sulfonate (SDBS) and polyethylene tert-octyl phenyl ether were observed to provide much higher RTP signals than Tween-20, SDS and CPB [46]. This can be partly explained by the bulky head groups of the former surfactants: their long hydrophobic chain is partly included in the cavity, while the part with the polar head group is coiling at the mouth of the cavity [46]. A bulky group in this position provides better protection against molecular oxygen.

Escandar and Muñoz de la Peña [44] optimized the concentrations of β-CD and surfactants (SDS, Triton X-100) or alcohols (cyclopentanol, cyclohexanol and 1-pentanol) in order to obtain maximum RTP from ternary 1-BrN complexes in aerated solutions. The optimization was straightforward in case of the alcohols, but more complicated for the surfactants due to the existence of multiple species and the formation of micelles above the critical micellar concentration. It was observed in several studies [44–46] that the
RTP initially increases with increasing surfactant concentration, but then rapidly decreases to zero again. This is probably due to the formation of micelles that start to compete with the CDs for $1$-BrN; the formation of micelles was indicated by a decrease of the surface tension of the solution, which reached a minimum above a certain concentration of surfactant [45, 46].

3.3. Ternary complexes of non-halogenated phosphorophores

The group of halogen-containing phosphorophores is rather small and a more versatile technique should also enable the detection of non-halogenated analytes. Such a technique, based on the formation of ternary complexes, is discussed here.

With non-halogenated phosphorophores deactivation due to phosphorescence is slow. As noted before, both $\vartheta_{ISC}$ and $\vartheta_p$ are enhanced in most cases if a heavy atom-containing molecule is brought into close proximity to the phosphorophore, which results in stronger RTP. It was found that ternary complex formation of CD, phosphorophore, and HAP is a convenient way to achieve this goal: due to the small volume in which phosphorophore and HAP are organized, strong RTP was obtained from the ternary complexes of several PAHs and related compounds [48–52]. The HAP used in [48]—$1,2$-dibromoethylene—not only enhanced $\vartheta_{ISC}$ and $k_p$, but also caused increased protection against quenching by molecular oxygen, most probably due to the formation of suspended microcrystals (also see Section 3.4). Thus, RTP was obtained even in aerated solutions, although higher signals were obtained after purging with nitrogen. The residual phosphorescence obtained under aerated solvent conditions was lower for polar compounds such as nitrogen heterocyclics than for less polar compounds such as PAHs, because of the lower complexation constants and higher exit rates [49]. Interestingly, selectivity was derived from the relative sizes of the phosphorophore and CD cavity, in presence of the HAP as a second guest [48].

The intrinsic selectivity of CD-RTP was also addressed in a study on the ternary complex of fluorene by DeLucia and Cline Love [53] who explored the sensitized phosphorescence emitted by biacetyl included, together with a sensitizer, in $\alpha$-, $\beta$-, or $\gamma$-CD. Due to the confinement of sensitizer and phosphorophore to the cavity of the CD, the effective concentrations of both were increased and RTP was strongly enhanced. The actual enhancement was found to be dependent on the molecular dimensions of the analytes: biphenyl, naphthalene, and phenanthrene were detected in $\alpha$- and $\gamma$-CD with increasing sensitivity, while chrysene and triphenylene were detected only when complexed with $\gamma$-CD.

3.4. Analyte:CD:precipitant complexes

Several studies deal with ternary complexes consisting of analyte, $\beta$-CD and precipitating agent [54–57]. Due to the addition of the precipitating agent—generally an apolar compound without a heavy atom—

<table>
<thead>
<tr>
<th>Species added</th>
<th>Toleration ratio (species to fluorene (m/m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorescence</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>200</td>
</tr>
<tr>
<td>9-Bromo fluorene</td>
<td>100</td>
</tr>
<tr>
<td>Naphthalene, acenaphthene</td>
<td>40</td>
</tr>
<tr>
<td>Anthracene, benz[a]pyrene, benz[e]pyrene</td>
<td>20</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4</td>
</tr>
<tr>
<td>Fluoranthen, 1-naphthol</td>
<td>2</td>
</tr>
<tr>
<td>Benz[a]fluoranthen</td>
<td>2</td>
</tr>
<tr>
<td>1,2,5,6-Benzanthracene, 1,2-benzanthracene</td>
<td>2</td>
</tr>
<tr>
<td>Acridine, dibenzoferan, dibenzothiophene</td>
<td>1</td>
</tr>
<tr>
<td>Carbazole, phenazine, triphenylene</td>
<td>1</td>
</tr>
<tr>
<td>2-Naphthol, 2-bromo fluorene</td>
<td>1</td>
</tr>
<tr>
<td>Phenanthrene, chrylene</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Adapted from [50].
a microcrystalline suspension with microcrystals of ca. 1 μm diameter is formed in the aqueous solution [55]. Remarkably strong and long-lived phosphorescence can be observed from analytes incorporated in such microcrystalline suspensions even under aerated conditions. In this respect, the method may be compared with the technique introduced by Weinberger and Cline Love [58], in which RTP was obtained from aqueous suspensions of PAHs that were previously dissolved in methanol, THF or dimethylformamide (DMF). It should be noted that no HAP was involved in most cases; in combination with the aggregation induced by the precipitating agent this results in ultra-long lifetimes under deoxygenated as well as aerated conditions [54,55].

Jin et al. [56] studied the RTP of some PAHs and nitrogen heterocyclics, induced in ternary analyte-β-CD-precipitant complexes. The precipitants tested included n-alkanes, bromobenzene and cyclohexane. Likewise, Nazarov et al. [54,55] studied the RTP from the test phosphorophores naphthalene, deuterated naphthalene and phenanthrene. Decalin, n-hexane, chloroform, cyclohexane and isoctane were used as precipitating agents. Under deoxygenated conditions at room temperature, the RTP lifetimes were about 2–15 s, while even in aerated solutions (at 274 K) the lifetimes exceeded 100 ms [54]. Further measurements indicated that, due to the formation of microcrystals, the value of the bimolecular quenching rate constant, \( k_q \) for molecular oxygen was reduced 10^6-fold, i.e. \( k_q \) was about 10^3 M^{-1} s^{-1} [55].

A study on ternary complexes of 6-bromo-2-naphthol (6Br2N), β-CD and the precipitants cyclohexane, n-hexane, chloroform and benzene was also performed [57]. To investigate if the formation of microcrystals is a prerequisite for obtaining RTP, experiments were performed with cyclohexanol and 2-bromoethanol as the third component of the ternary complex. With these compounds, no precipitation occurred and no RTP was obtained, indicating that precipitation is indeed required.

Due to the formation of microcrystals, the protection against dissolved molecular oxygen is better than with conventional, non-precipitating complexes. Therefore, rather high residual RTP signals were obtained in aerated solutions, i.e. compared with deoxygenated solutions about 20% of the RTP was still present for nitrogen heterocyclics [56]. All quoted studies reported that by far the largest RTP enhancements were achieved with cyclohexane as the precipitant. This may be a space-matching effect: due to the neat fit of both guests, maximal displacement of water occurs and the rigidity is increased, which will cause a decrease of non-radiative decay [56].

### 3.5. α-CD complexes

Hamai [59,60] studied the RTP of solutions containing 6Br2N and α-CD. In such solutions, binary complexes as well as ternary 2:1 α-CD:6Br2N complexes are present, depending on the concentration of α-CD. From a plot of RTP versus the calculated concentration of the ternary 2:1 α-CD:6Br2N complex it was concluded that RTP is obtained from the ternary complexes only; in other words, efficient protection of the triplet state is provided only after complexation with two α-CD molecules [60]. RTP from the 2:1 α-CD:6Br2N complex was obtained even in aerated solutions, but the triplet lifetime substantially increased, from 160 to 290 μs, after purging with nitrogen [59]. In another study on the same complexes [61], a 13-fold enhancement was reported after purging. The addition of sodium sulfite (for deoxygenation; cf. Eq. (6)) resulted in the quenching of phosphorescence. It is doubtful if this effect can be attributed to bimolecular quenching, since the sulfite concentration used was less than 1 mM and effective protection by the CDs should be expected.

In contrast to experiments involving binary β- and γ-CD complexes, co-complexation with alcohols such as 2-bromoethanol, 2-bromopropanol, 2,3-dibromo-propanol, cyclopentanol and benzylic alcohol did not enhance RTP. On the contrary, quenching occurred due to displacement of the phosphorophore from the rather small α-CD cavity.

The (water-soluble) 2:1 α-CD:6Br2N complex was used for RTP optosensing of the temperature by Brewster et al. [62]. With increasing temperature, the ternary complex became less stable, which resulted in a decrease of the RTP lifetime since only the ternary complex yields phosphorescence; over the temperature range from 1.6 to 60 °C, the lifetime was reduced from 1.25 to 7.5 μs. Due to the high sensitivity of the sensing system differences as small as 0.1 °C could be measured.
3.6. Heavy atom-derivatized CD complexes

As noted above, the addition of a HAP is necessary to obtain RTP from non-halogen analyte:CD complexes, except when precipitant ternary complexes are involved. Interestingly, halogen-derivatized CDs can be used as an alternative to these halogenated second-guest molecules. This was first demonstrated as early as 1985 by Femia and Cline Love[63]. The authors obtained RTP from PAHs such as naphthalene, fluorene, phenanthrene and pyrene, included in heptakis (6-bromo-6-deoxy-β-cyclodextrin (Br-β-CD))—synthesized by replacing the primary hydroxyls of the sugar groups with bromine. In order to solubilize Br-β-CD, a mixture of water and a low-polarity solvent, dimethylformamide, had to be used. Maximum RTP was obtained when using 80 vol.% DMF. In the absence of water, no RTP was obtained because in that case the hydrophobic driving force for inclusion into the Br-β-CD cavity is too low.

The photophysical properties of analytes included in iodine-substituted CDs were also studied [64,65]. Compared to the corresponding parent CD complex, a 20% enhancement of RTP was obtained for the 6-iodo-6-deoxy-β-cyclodextrin (β-CDI):2-chloronaphthalene complex [64]. For the inclusion complexes of 6Br2N and 3-bromoquinoline with 6-iodo-6-deoxy-α-CD (α-CDI), a slight (10–18%) reduction of RTP was observed compared to the corresponding 2:1 α-CD:analyte complexes [65]. Probably, the low degree of substitution (one iodine atom per CD) in the above cases [64,65] seriously compromises the ability of α- and β-CDI to induce RTP. On the other hand, it effects a much better water solubility than with Br-β-CD (degree of substitution, 7). It should also be noted that a better indication of the potential of the iodine-derivatized CDs to induce RTP would probably be obtained if experiments with non-halogenated phosphorophores were performed. The use of CDs derivatized with several heavy atoms as well as ionic groups to provide adequate water solubility should give better results. Interestingly, such CDs could simultaneously be used for separation and detection purposes, for instance in electrophoretically driven separations. By contrast, due to the slow dissociation kinetics associated with ternary complexes—consisting of CD, analyte and HAP—such complexes are probably not suitable for application in separation techniques because of severe peak broadening [66]. In conclusion, more research will be required for a full assessment of the analytical potential of halogen-derivatized CDs.

3.7. Concluding remarks

CD-RTP has been obtained from several types of binary and ternary inclusion complexes. Based on the RTP enhancement observed after co-inclusion of several alcohols, an optosensing scheme for alcohols was proposed. Ternary precipitant complexes of PAHs have ultralong phosphorescence lifetimes due to the efficient protection from dissolved molecular oxygen. Interestingly, as will be seen in Section 5, such complexes can be used for RTP-based optosensing. Halogen-derivatized CDs were used to enhance the RTP from included phosphorophores. However, the potential of such derivatized CDs for the detection of non-halogenated analytes has not been fully explored yet and deserves further study, particularly concerning the eventual coupling of CD-RTP with separation techniques. Finally, it should be noted that—in contrast to MS-RTP—almost none of the many CD-RTP studies was dealing with the application of CD-RTP in real-life analysis. Such studies, however, will be required to establish the practicability of CD-RTP; at present, no evaluation can be given.

4. Heavy atom-induced RTP

4.1. Introduction

For a long time it was generally accepted that RTP in homogeneous liquid solutions is restricted to a few exceptional compounds such as biacetyl and brominated naphthalenes. For all other compounds, the presence of some kind of protective medium such as micelles or CDs was believed to be required. However, recently RTP was also obtained (under deoxygenated conditions) from several phosphorophores (analytes) in homogeneous solutions, by applying HAI-RTP[67]. It has been suggested in [67] that the designation ‘non-protected (fluid)-RTP’ more adequately describes the specific nature of this technique. We fully agree with this argument, but prefer to use the acronym ‘HAI-RTP’ since it is commonly used in the literature.
requires the presence of a HAP at rather high concentrations. 5-Dimethylaminonaphthalene sulfonyl chloride (dansyl chloride) and 16 dansylated amino acids were studied as target analytes; for dansyl chloride an LOD of 5 ng ml\(^{-1}\) was obtained. In this study, TINO\(_2\) (50 mM) was used as HAP; other salts such as KI, KBr, and TlHCOO were reported not to induce RTP. A neutral pH was suitable for deoxygenation by sodium sulfite (10 mM) and for obtaining RTP.

### Table 3

Survey of LODs, triplet lifetimes, and type of HAP used for the detection of various compounds by HAI-RTP

<table>
<thead>
<tr>
<th>Compound</th>
<th>HAP</th>
<th>LOD (ng ml(^{-1}))</th>
<th>Triplet lifetime ((\mu s))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substituted naphthalenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dansyl chloride</td>
<td>TINO(_2)</td>
<td>5</td>
<td>–</td>
<td>[67]</td>
</tr>
<tr>
<td>Dansylamide</td>
<td>TINO(_2)/KI</td>
<td>86</td>
<td>222</td>
<td>[68]</td>
</tr>
<tr>
<td>Dansylated amino acids</td>
<td>TINO(_2)</td>
<td>–</td>
<td>263–640</td>
<td>[67]</td>
</tr>
<tr>
<td>1-Naphthyleaetic acid</td>
<td>TINO(_2)/KI</td>
<td>15</td>
<td>236</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>TINO(_2)</td>
<td>110</td>
<td>418</td>
<td>[69]</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>TINO(_2)/KI</td>
<td>11</td>
<td>180</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>TINO(_2)/KI</td>
<td>3</td>
<td>180</td>
<td>[70]</td>
</tr>
<tr>
<td>a-Naphthalene acetamide</td>
<td>TINO(_2)/KI</td>
<td>8</td>
<td>251</td>
<td>[68,71]</td>
</tr>
<tr>
<td>Naproxen</td>
<td>TINO(_2)/KI</td>
<td>16</td>
<td>1028</td>
<td>[68,72]</td>
</tr>
<tr>
<td>Naphazoline</td>
<td>TINO(_2)/KI</td>
<td>12</td>
<td>156</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>TINO(_2)/KI</td>
<td>3</td>
<td>300</td>
<td>[73]</td>
</tr>
<tr>
<td>a-Naphthoxyacetic acid</td>
<td>TINO(_2)</td>
<td>2</td>
<td>340</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>2</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>β-Naphthoxyacetic acid</td>
<td>TINO(_2)/KI</td>
<td>31</td>
<td>–</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>TINO(_2)</td>
<td>2</td>
<td>1230</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>2</td>
<td>630</td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>KI</td>
<td>44</td>
<td>632</td>
<td>[76,77]</td>
</tr>
<tr>
<td>1-Naphthyleaetic acid</td>
<td>TINO(_2)/KI</td>
<td>10</td>
<td>–</td>
<td>[78]</td>
</tr>
<tr>
<td>1-Hydroxy-2-naphtholic acid</td>
<td>TINO(_2)</td>
<td>28</td>
<td>240</td>
<td>[69]</td>
</tr>
<tr>
<td>1-Amino-5-naphthalene-sulfonic acid</td>
<td>TINO(_2)</td>
<td>7</td>
<td>464</td>
<td>[69]</td>
</tr>
<tr>
<td><strong>PAHs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>TINO(_2)/KI</td>
<td>65</td>
<td>417</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>TINO(_2)/KI</td>
<td>15</td>
<td>130</td>
<td>[68]</td>
</tr>
<tr>
<td>Fluorene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nitrogen heterocyclics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-4-Thiazolyl-benzimidazole</td>
<td>TINO(_2)/KI</td>
<td>15</td>
<td>89</td>
<td>[68]</td>
</tr>
<tr>
<td>Carbazole</td>
<td>TINO(_2)/KI</td>
<td>4</td>
<td>255</td>
<td>[68]</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>TINO(_2)/KI</td>
<td>20</td>
<td>90</td>
<td>[68]</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>TINO(_2)/KI</td>
<td>11</td>
<td>166</td>
<td>[68]</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid</td>
<td>TINO(_2)/KI</td>
<td>25</td>
<td>145</td>
<td>[68]</td>
</tr>
<tr>
<td>8-Hydroxy-4-methoxyacridine</td>
<td>TINO(_2)/KI</td>
<td>21</td>
<td>91</td>
<td>[68]</td>
</tr>
</tbody>
</table>

* HAP used to obtain the given LODs and triplet lifetimes, i.e. optimal HAP.
* Optimal HAP not specified.

4.2. Application range and analytical merits

Soon after its introduction, HAI-RTP from other substituted naphthalenes, as well as from nitrogen heterocyclics and some PAHs, was also reported; an overview is given in Table 3. For all substituted naphthalenes, the RTP emission spectra were very similar since the RTP originates from the naphthalene part of the molecule. The LODs in Table 3 range from a few to about 100 ng ml\(^{-1}\). Therefore, trace analysis of...
real-life samples will often require preconcentration. PAHs such as naphthalene, anthracene, phenanthrene and pyrene\cite{69} give only weak RTP signals (no LODs were presented), which was attributed to low \( k_p \) values (a \( \pi-\pi^* \) transition instead of an \( n-\pi^* \) transition is involved in this case). However, for some PAHs (fluorene and acenaphthene) rather low LODs were achieved (Table 3).

The linear detection range is generally limited to about one order of magnitude. This may be acceptable in routine analyses of pharmaceutical products, but it is a clear disadvantage in, for instance, environmental analysis where analytes can be present at widely different concentrations.

\( \text{TINO}_3 \) (25–250 mM) and KI (typical concentration, 1 M) were used in all cases to induce intersystem crossing (Table 3). KI was most suitable for generating RTP with nitrogen heterocyclics; for the substituted naphthalenes, the optimal HAP is dependent on the individual compound. Sodium sulfite was used for deoxygenation in all studies, at concentrations of 1–15 mM\cite{67–79}. At lower sulfite concentrations longer stabilization times were needed\cite{70–78}, while at higher concentration levels the RTP intensity was lower\cite{72–78}, possibly due to sulfite-induced quenching. Stable RTP signals were generally obtained within a few minutes and the triplet lifetimes were in the range of ca. 0.1–1 ms (Table 3), which is quite close to the lifetimes generally encountered in MS-RTP and CD-RTP.

Due to the inherent selectivity of RTPL, in several cases analysis could be performed without (extensive) sample pretreatment. For instance, the determination of 1-naphthoxylic acid in spiked serum and urine (200 ng ml\(^{-1}\)) was performed successfully (recovery, 97%) after mere dilution of the sample\cite{78}. Likewise, naproxen\cite{72} and nafronyl\cite{73} were determined in pharmaceutical formulations. It should be noted that the composition of the latter samples is not very complex and analyte concentrations were quite high; sample pretreatment will remain necessary in many other cases.

An advantage of HAI-RTP is the intrinsic simplicity of the method: no ‘pseudophase’ is involved and the solutions are stable and clear. Moreover, the method is directly applicable to biological samples containing high sodium ion concentrations, in contrast to MS-RTP which suffers from displacement of the thallium ions from the micelles by the sodium ions present in the sample\cite{78}.

Some authors mentioned that the technique, in contrast to MS-RTP and CD-RTP, is well suited for combination with flow injection analysis (FIA), LC and CE\cite{67,69}. Indeed, as noted before, CD-RTP cannot be combined with separation methods unless highly dynamic CD complexes are involved. However, MS-RTP detection has been applied in LC in the past\cite{80}, despite the foaming problems associated with nitrogen purging. Moreover, the use of sodium sulfite for deoxygenation, as introduced by Díaz-García and Sanz-Medel\cite{11}, eliminates this problem. Finally, it should be noted that the combination of separation methods and HAI-RTP itself will be seriously hindered by organic solvent effects: several commonly used solvents such as acetone and methanol strongly reduce the RTP intensity even at the 1 vol.% level or—as is the case with the addition of 1 vol.% acetonitrile—extend the required stabilization time to more than 30 min\cite{74}. Therefore, although 20% acetone\cite{67} or 10% methanol\cite{73} can be tolerated in specific cases—possibly depending on the analyte—the coupling of HAI-RTP and separation techniques seems most promising for separation procedures aimed at polar or ionic compounds, which do not require high concentrations of organic solvent.

4.3. Concluding remarks

HAI-RTP is a recently introduced method for obtaining RTP in which no protective medium is needed. Many substituted naphthalenes and nitrogen heterocyclics show RTP in the presence of Th(I) or I\(^{-} \) ions (HAP) and sodium sulfite (deoxygenation). HAI-RTP can therefore be regarded as a useful method for expanding the application range of direct RTP. Also here, studies involving real-life samples are required to establish the practicability of HAI-RTP. The coupling with separation methods may be simplified since no micelles or CDs are involved. On the other hand, the coupling may be limited to aqueous-phase separations, since organic solvents were found to strongly reduce phosphorescence in several HAI-RTP experiments. More research on the nature and extent of the solvent effect will be required, however, before a full evaluation can be given.
5. Indirect phosphorescence detection

5.1. Introduction

All RTP methods that were discussed above, except for some examples of MS-RTP, are based on direct phosphorescence, i.e. phosphorescence obtained after direct excitation of the phosphorophore. In general, these methods require not only efficient sample de-oxygenation but also efficient protection of the excited triplet state by CDs, micelles, or another kind of organizing medium—with HAI-RTP as the exception.

Some special compounds, such as biacetyl and various bromonaphthalenes, can yield strong RTP in liquid solutions even in the absence of a protective medium. Based on these phosphorophores, indirect RTP modes like sensitized and quenched phosphorescence can also be used in free solution. This opens wide perspectives, since under these conditions the indirect RTP methods can easily be combined with a FIA set-up or with a liquid separation method. Of course, low oxygen concentrations in the LC eluents or CE buffers are required to obtain indirect RTP; in addition, for LC analysis the samples also have to be deoxygenated. Hence, adapted set-ups have to be used.

Already in the early 1980s it was shown that many compounds, including (poly)chlorinated biphenyls and naphthalenes, as well as biacetyl, can be detected by LC with sensitized phosphorescence detection. On the other hand, the coupling of RTP detection and CE is in principle possible. The recent development of such a technique will be highlighted in Section 5.2.

5.2. Sensitized and quenched RTP detection in CE

Due to the short optical pathlengths and small detection cell volumes available in CE, optical methods such as absorption generally offer limited detectability; therefore, alternative detection strategies are of prime interest. An intrinsic difficulty associated with the coupling of RTP detection and CE is the generation of molecular oxygen at the anode (injection side) during electrophoresis, due to the electrolysis of water. In the first study on sensitized and quenched phosphorescence detection in CE, the separation voltage polarity was reversed, i.e. a negative voltage was applied during electrophoresis. Accordingly, injection of samples and deoxygenation of the anodic buffer was performed outside the CE instrument (Fig. 5). To maintain low oxygen levels, an LC pump was used to continuously pump the previously deoxygenated buffer through the anodic (24 μl) interface, which alternatingly functioned as the buffer and the sample vial. In later studies, a simplified set-up was used which reduced the start-up time from 2 h to 20 min. However, the first set-up may well provide easier on-line coupling with LC for sample pretreatment or analyte enrichment. Interestingly, as can be seen in Fig. 6 (traces a and b, respectively), sensitized (biacetyl) phosphorescence was obtained for non-substituted naphthalene sulfonates (NS), whereas (near) diffusion-controlled quenching of biacetyl phosphorescence was observed for the hydroxy- and/or amino-substituted NS; LODs were in the $4 \times 10^{-7}$ to $5 \times 10^{-8}$ M range.

In quenched RTP, the signal is governed by the Stern–Volmer equation (cf. Eq. (4)). Hence, the degree of quenching induced by a certain analyte, present at a certain concentration, is independent of the height of the phosphorescence background. Therefore, LODs can be improved by enhancing the RTP background signal, which increases its signal-to-noise ratio (S/N). Unfortunately, the molar absorbance of biacetyl is very low over its entire absorption spectrum. While this is an advantage in sensitized RTP, where direct excitation has to be avoided, it is a clear disadvantage in quenched RTP (Fig. 6, trace b). For this reason, 1,5-dinaphthalenesulfonate was
Fig. 5. Instrumental set-up for CE with sensitized and quenched phosphorescence detection, consisting of an ultrasonic bath and a nitrogen gas supply for deoxygenation, an LC pump for pumping the CE buffer and the sample (1 ml/min), a luminescence detector, and a CE system. Sample introduction in the CE capillary is performed by hydrodynamic injection at a fixed time interval after switching the six-way valve. Inset: interface with the injection end of the capillary and the positive electrode inserted in it. Figure taken from [5].

used in a subsequent study to sensitize biacetyl prior to measuring its (quenched) phosphorescence [86]. Combined with the use of the total emission mirror of the luminescence spectrometer, this provided a 25-fold enhancement of S/N and, thus, of the LODs (Fig. 7). Several buffers commonly used in CE, i.e. borate, phosphate, malonate, acetate and succinate (pH 4.7–9.0), were found to be compatible with quenched RTP detection. However, the method could not be used at extreme pH values, because enolization of biacetyl occurs in both strongly acidic and basic solutions. Another water-soluble phosphorophore was therefore used in later studies (vide infra). The method was used to detect substituted NS, nitrophenols, hydroxybenzoic acids, thiocarbamates and sulfur-containing amino acids, with LODs down to 10 nM [86].

In both of the above studies, a pulsed xenon lamp was used to enable time-resolved detection (delay time, 50 μs; gating time, 0.5–1.0 ms). This enabled the easy removal of scatter and fluorescence and caused an increase of S/N of the long-lived RTP background signal (τ0, ca. 70 μs). CE-quenched phosphorescence was also applied to non-derivatized, low-molecular-weight amino acids, which are not readily detected with high sensitivity by most other detection techniques [87]. Since quenched phosphorescence detection of amino acids is based on electron transfer originating from the deprotonated amino groups, the pH of the separation buffer should be higher than 9. Since biacetyl cannot be used at pH > 9, a pH-insensitive and water-soluble compound, 1-bromo-4-naphthalenesulfonic acid, was used as phosphorophore instead. Also in this case, nearly diffusion-controlled bimolecular quenching was observed, with LODs close to 10 nM [87].

To expand the applicability of phosphorescence detection in CE to neutral analytes, the compatibility of quenched RTP detection and cyclodextrin-based electrokinetic chromatography (CD-EKC) was studied...
Fig. 7. Electropherogram of mixture of 22 NS (10^{-6} M each). The NS are indicated by the positions of their functional groups on the naphthalene skeleton, i.e. amino/hydroxy/sulfonic acid. Buffer: 25 mM boric acid, pH 9.0, 0.02 M biacetyl, 1×10^{-3} M 1,5-naphthalenedisulfonic acid. Injection volume equivalent to 288 mbar. Figure taken from [86].

<table>
<thead>
<tr>
<th>Peak assignment:</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4: 5/0/1; 5/0/2; 4/0/1; 8/0/2</td>
<td>15: 0/3/2,7</td>
</tr>
<tr>
<td>5-7: 6/0/1; 7/0/1; 6/4/2</td>
<td>16: 7/0/1,3</td>
</tr>
<tr>
<td>8/9: 2/0/1; 8/0/1</td>
<td>17/18: 2/0/1,5; 3/0/2,7</td>
</tr>
<tr>
<td>10: 0/0/2</td>
<td>19: 0/7/1,3</td>
</tr>
<tr>
<td>11: 0/1/2</td>
<td>20: 4/6/2,7</td>
</tr>
<tr>
<td>12: 0/4/1</td>
<td>21: 0/4/2,7</td>
</tr>
<tr>
<td>13: 0/6/7/2</td>
<td>22: 0/4/5/2,7</td>
</tr>
</tbody>
</table>

[84] Nitro-aromatics were chosen as test analytes, because the nitro group provides (near) diffusion-controlled quenching. A priori, it may be expected that inclusion of the analytes in the (charged) CDs will reduce the sensitivity of quenched RTP by reducing the bimolecular quenching rate constants. However, due to the low capacity factors generally encountered in CD-EKC, a large fraction of the analytes (quenchers) will be present in the aqueous phase. Consequently, the resulting bimolecular quenching rates were found to be high and LODs low (ca. 20 nM).

The experiments discussed above were all performed using conventional light sources. The most straightforward method to improve LODs in CE-quenched phosphorescence may seem to be enhancement of the continuous phosphorescence background by using a laser for excitation. Indeed, it was observed that the RTP background was significantly enhanced.
when using a small-size, 7.8 kHz-pulsed Nd–YAG laser with a highly stable output and a home-built detector instead of a conventional luminescence detector [85]. However, the LODs were not better than with the lamp-based system: under low irradiance conditions the triplet lifetime was long ($\tau$, ca. 300 ms), but quenching between two subsequent laser pulses was restricted to a short period of time only (ca. 130 ms), which reduced the observed quenching efficiency. At high irradiance, the efficiency of bimolecular quenching was strongly reduced due to the competition offered by triplet–triplet annihilation. When using a cylindrical lens for excitation (1 mm detection window), LODs similar to those obtained by pulsed lamp excitation (10 nM) were achieved [85]. Therefore, laser-induced RTP will be more appropriate than lamp-based RTP (4 mm detection window) if highly efficient separations are involved.

5.3. Concluding remarks

Quenched phosphorescence detection was shown to be an interesting detection mode for CE and CD-EKC. Its LODs are in the $10^{-7}$ to $10^{-8}$ M range, which is beyond the possibilities of many other techniques, especially for compounds with poor chromophores; absolute detection limits are about 1 fmol. Interestingly, such LODs were obtained by using conventional, lamp-based detection systems. The use of a pulsed, solid-state laser did not improve the detectability. However, more research will be required to fully assess the usefulness of laser-based detection. The relative simplicity and high sensitivity of the method may well render it suitable for detection in chip-based separation systems; in terms of detectability, favorable results should be expected compared to commonly used indirect detection methods such as indirect absorption and indirect fluorescence.

6. RTP optosensing

6.1. Introduction

Although some examples of RTP-based optosensing were already given in Sections 2.2, 3.2, and 3.5, the topic no doubt deserves a more comprehensive evaluation. RTP optosensing methods, based on various detection schemes, have been developed to determine analytes such as oxygen (transition) metals, lanthanides, organic compounds, and enzyme substrates like glucose. Most RTP optosensing detection schemes are based on FIA set-ups, but probe-type experiments are also used. According to Sanz-Medel [88], an important advantage of the FIA mode is the possibility of using irreversible chemistry to achieve selective binding prior to detection. Low-cost automation involving integrated sample pretreatment or enrichment can also be achieved quite easily [89]. Costa-Fernández and Sanz-Medel [89] suggested that the development of sensing phases based on molecularly imprinted polymers (MIP) can be useful for optosensing purposes, since MIP sensing phases are robust, possess high operational stability, and chemical (acid, organic solvents) and thermal resistance. Moreover, MIP sensing phases allow for selective interaction prior to detection and can, in principle, be designed for any target analyte while their production for low-molecular-weight compounds is almost routine [90,91]. Some examples [92–94] of using MIP phases for sensitized and quenched luminescence detection will be discussed below. Interestingly, these examples point to the feasibility of using MIP phases with sensitized and quenched RTP detection.

6.2. Oxygen sensing

The sensing of molecular oxygen based on luminescence quenching is regarded as one of the most typical and widespread optosensing applications. In all luminescence quenching detection schemes, a—preferably strong and low-noise—quencher is used as a background signal in the presence of molecular oxygen or other quenchers (cf. Eq. (4)). Although sensing is performed both in the gaseous and the liquid phase, the present discussion is restricted to detection in the liquid phase.

In most cases, Ru(II)–a-diimine complexes (lifetime, several hundreds of nanoseconds to tens of microseconds) or Pt(II)–or Pd(II)–porphyrin complexes (lifetime, hundreds of microseconds) are used as luminophores. Advantages are their long luminescence

---

*The term ‘luminescence’ is used in this case since not only RTP but also (long-lived) fluorescence is involved here.*
lifetimes, convenient excitation in the visible range, and large Stokes’ shifts (wavelength difference between excitation and emission maxima). Next to standard intensity measurements, lifetime measurements based on time-resolved detection and phase modulation are used [95]. The use of RTP intensity is straight-forward but is not as reliable as the other methods, which are independent of changes in illumination and collection efficiencies (light source intensity [96–98], local differences in transparency of the samples and changes in optical properties of the tissues studied [99,100], variations in the luminoaphore concentration (photobleaching [96,98,99], circulation concentration of the luminoaphore in tissues [99]), inhomogeneities of the sensor phases [97], and membrane positioning in probe-type experiments [98].

As regards the luminoaphore and the matrix used to immobilize it, the new Pt(II)− and Pd(II)− porphyrin ketone dyes provide a 10-fold increased photochemical stability compared to Pr(II)−octaethylporphine [101]. In addition, favorable overlap with yellow and orange light-emitting diodes (LEDs) was achieved and, thus, convenient excitation in the visible range for sensing purposes [101]. The position of the absorption bands can also be important for the detection of oxygen in tissue, since other chromophores present in tissue can limit the penetration depth of the excitation light to less than 1 mm [102].

As for the matrix, materials such as polymer films, sol−gel phases, zeolites and siloxanes have been tested [95]. A good matrix for inclusion of the sensing molecules is chemically inert and optically transparent, possesses photochemical and thermal stability, and shows negligible intrinsic fluorescence. Many polymer and sol−gel phases largely fulfill these requirements [89]. The porosity of the sensing phase is also important since the quencher should be able to interact with the immobilized luminoaphore [103,104].

Diaz-Garcia et al. [105] used FIA with four flow-through cells packed with an Al−ferron doped sol−gel phase to sense dissolved molecular oxygen in water. An intensified charge-coupled device (CCD) camera and fiber-optics were used for detection and excitation/collection, respectively. Other sensing phases were also applied in the FIA format: anion-exchange resin beads with immobilized Al−ferron, encapsulated in a highly oxygen-permeable silicone rubber film provided high operational stability (no substantial decrease in RTP intensity during 20 h of continuous operation) and response times of about 2.5 min for sensing in the liquid phase [103]. Sol−gel glasses doped with Al−ferron were also used; high operational and storage stability were reported and an LOD of 6 ng ml−1 in water was obtained [104].

Lähdesmäki et al. [106] applied bead injection spectroscopy for the determination of oxygen consumption by adherent cell cultures. For detection, microcarrier beads with immobilized Pr(II)−porphyrin were transiently retained at the bottom of a flow-through microscope chamber. The bead format was used to ensure fast consumption of oxygen. Three minutes before and during RTP intensity measurements the flow was stopped so that the oxygen in the buffer was depleted.

Probe-type sensing was also applied in many instances: Papkovsky et al. [98] used the well-plate reader accessory of a standard luminescence spectrometer for oxygen detection in aqueous samples. The Pr(II)−octaethylporphine complex used for optosensing was contained in a polystyrene film deposited at the bottom of the well-plates. With a comparable set-up, the metabolic activity (oxygen consumption) of living yeast cells on microtiter plates was monitored [107].

Several groups performed in vivo oxygen determinations based on RTP quenching: the partial oxygen pressure was determined in prepared male Wistar rats that were allowed to breathe ambient air, using in vivo RTP lifetime measurements [96]. RTP was obtained from a Pr(III)−porphyrine solution, which was injected intravenously. Non-invasive oxygen determination in arterioles and venules of unanesthetized hamsters was achieved by using an implanted transparent skin fold chamber [100]. The pO2 values found were similar to those measured using microelectrodes. Spatial in vivo measurements of oxygen levels in rat liver tissue during photodynamic therapy were also reported [99]; the oxygen levels near the excitation fiber were observed to decrease from 20 to 2 mmHg. Photosensitizer and phosphorophore were injected prior to measurements. The optical fibers used for excitation and detection were closely spaced (a few mm apart) and positioned just above the exposed tissue, i.e. the technique is non-invasive (Fig. 8). It should be emphasized that RTP intensity measurements cannot be used in this case because of problems related to photodegradation and variations in the circulation
concentration levels of the RTP probe, and changes in the optical properties of the tissue. In contrast, RTP lifetime can be used without such problems.

6.3. Heavy atoms

FIA was combined with RTP for the detection of several metal ions [108–110], the method is based on the RTP obtained from a complex of the metal with a ferron-like compound, due to the ensuing enhancement of $\vartheta_{ISC}$ and $\vartheta_{p}$ (also see Section 2.2). The phosphorescent complex was transiently retained on an anion-exchange resin, which is stable for 2–3 months, in the flow-cell of the luminometer. Regeneration of the resin was performed by injecting a small volume of a strongly acidic (HCl) solution. Sodium sulfite was used for deoxygenation.

Using 8-hydroxy-7-quinolinesulfonic acid as the ligand, Pb(II) was determined in seawater spiked at a concentration of 6–100 ng ml$^{-1}$ (recovery, 94–109%) [108]. An LOD of 0.1 ng ml$^{-1}$ and a linear range of three to four decades were achieved for standard solutions. Some selectivity against other metals was reported (Table 4), but the interference of these metal ions should be studied more thoroughly in order to fully understand its nature and extent. Likewise, Al(III) was detected (LOD, 2 ng ml$^{-1}$) by the RTP obtained from its complex (Al–ferron) with 8-hydroxy-7-iodo-5-quinolinesulfonic acid (ferron) [109]. The method showed selectivity against many ions at levels normally present in dialysis fluids and was successfully applied to the determination of Al(III) in such fluids; Fe(III) interferes but can be masked by adding 9,10-phenanthroline. RTP op- tosensing was also applied to the detection of Hf(IV) using ferron or 8-hydroxy-5-quinolinesulfonic acid as the ligand [110]. With the latter ligand moderate selectivity against Ga(III), In(III), and Zr(IV) was obtained: thus, reliable determination of Hf(IV) was possible in the presence of a 5–30-fold excess.

Iodide is also a well-known enhancer of ISC in organic compounds. Of course, simultaneously with the enhancement of $\vartheta_{ISC}$, the fluorescence quantum yield will be reduced upon complexation of iodide. Using the luminescence of immobilized 8-hydroxy-5-quinolinesulfonic acid, both the reduction of fluorescence and the enhancement of RTP were applied to monitor iodide (Fig. 9). LODs were 5 and 10 mg/l.
Table 4
Effect of metal ions on RTP optosensing of lead (100 ng ml$^{-1}$), with 8-hydroxy-7-quinolinesulfonic acid as complexing agent

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Concentration (mg/l)</th>
<th>Intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>0.2</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>Mg(II)</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>88</td>
</tr>
<tr>
<td>Al(III)</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>Hg(II)</td>
<td>0.2</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>94</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>0.4</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>92</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>0.2</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>77</td>
</tr>
<tr>
<td>Co(II)</td>
<td>0.2</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>86</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>–</td>
<td>–*</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>0.1</td>
<td>79</td>
</tr>
<tr>
<td>Ca(II)</td>
<td>5</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>173</td>
</tr>
</tbody>
</table>

Taken from [108].

* Addition of 50 mg/l 1,10-phenanthroline in the buffer eliminates interference of 5 mg/l Fe(II).

respectively [111]. The method was over 200-fold more sensitive for iodide than for other halides. The RTP signal was strongly reduced by organic solvents such as ethanol and acetone, but the fluorescence signal was not significantly influenced even at concentrations as high as 25 vol.%. Fe(III) and Cu(II) ions interfered in both detection modes and should be removed prior to analysis.

6.4. Lanthanide–ligand complexes

Some trivalent lanthanide ions show narrow luminescence emission bands with up to millisecond lifetimes. However, detection based on direct excitation is quite unfavorable since their absorptivities are very low. Because of this, the use of sensitized lanthanide luminescence from lanthanide–ligand complexes is a better option, which enables detection of the rare-earth metals as well as the ligands.

Eu(III) and Tb(III) are important rare-earth metal ions present in the earth’s crust, while gadolinium chelates are being evaluated for their use as contrast-enhancing agents in magnetic resonance imaging, which requires analytical methods for the determination of Gd(III) in biological fluids and tissues. Sensitized lanthanide luminescence was applied in a FIA set-up for the determination of Eu(III) [112], Gd(III) [113] and Tb(III) [114]. For detection, the luminescent lanthanide–ligand complex was transiently immobilized on a resin in a 25 μl flow-cell. Because the energy transfer process is intramolecular by nature, oxygen does not affect the emission. Immobilization of the complex on the resin resulted in increased luminescence intensities and lifetimes, which was attributed to the increased rigidity of the environment and removal of water from the first coordination sphere [113]. The best results were obtained for Eu(III) and Tb(III) with LODs of 1 and 3 nM, respectively.

Conversely, ligands such as tetracyclines [115] and anthracyclines [116] were detected using the luminescence of their complexes with Eu(III). Immobilization
of the tetracyclines resulted in four-fold lower LODs (0.25–0.4 ng ml\(^{-1}\)) than with liquid-phase detection; for the anthracyclines, LODs of 6–9 ng ml\(^{-1}\) were achieved. The method was successfully applied to the determination of these compounds in spiked urine (recovery, 96–101%) and pharmaceutical formulations (accuracy better than 0.3%) [115,116].

6.5. Biosensing

The experimental set-ups for the detection of molecular oxygen can also be used for the detection of enzyme substrates like glucose [98,107,117–119], phenolic compounds [120] and cholesterol [121]. In the case of glucose, RTP was obtained after the consumption of oxygen due to the enzymatic reaction

\[
glucose + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2 \quad (7)
\]

which implies that the obtained RTP is a measure of the glucose concentration. Also in this case, both FIA [117–121] and probe-type [98,107] analyses were developed. With FIA, air-equilibrated carrier streams were used to guarantee equal initial oxygen concentrations for all samples; the response times were generally 2–5 min. With the probe experiments for glucose detection, steady-state signals were obtained in less than 2 min [98] or even in 10–15 s [107]. The samples were exposed to the ambient air, so that the oxygen levels at the membrane result both from consumption at the membrane and diffusion from the bulk phase. Both with FIA and probe experiments, fiber-optics were used for excitation and collection of the emission. Papkovsky et al. [117] published a study on RTP optosensing of glucose, based on a FIA set-up consisting of a peristaltic pump, injection loop, glucose oxidase column and flow-through cell. Water-soluble Pd(II)– and Pt(II)–porphyrins were used to obtain RTP; with the Pt(II) complex detection was possible down to ca. 0.5 mM. Other workers used a FIA set-up with a glucose oxidase mini-column positioned before the flow-cell [118]. The cell was packed with an anion-exchange resin to immobilize the phosphophore (Al\(^{-}\)–ferron). An LOD of 80 \(\mu\)M was obtained for glucose and the method was successfully applied to serum, fruit juice, and lemonade samples which were injected after mere dilution. No interference from fructose, saccharose, or lactose was observed, while the interference from galactose (due to galactosidase impurities in glucose oxidase) was only minor. Ovchinnikov et al. [119] used two designs: a FIA set-up in which the glucose oxidase was immobilized directly on the oxygen membrane sensor and a set-up using a glucose oxidase microparticle column with a sensing membrane at the column outlet; the latter provided the best detectability (LOD, <0.1 mM); RTP lifetimes were measured using phase modulation. Probe-type experiments for glucose determination were performed using the same set-up as for oxygen detection [98,107] (cf. Section 6.2), but glucose oxidase was immobilized on the sensing membranes in addition to the phosphophore. With RTP intensity and lifetime measurements, LODs of 20 and 50 \(\mu\)M were obtained, respectively [98]. Phase modulation was also used to determine RTP lifetimes [107]; in this case, detection in serum was possible down to 0.3 mM glucose.

Polyphenolic compounds were detected in a FIA set-up including an immobilized laccase enzyme column and an oxygen-sensing membrane with Pt(II)–octaethylporphine [120]. The best results were obtained for di- and polyphenols (LODs down to 0.2 mM). The method was successfully applied to the determination of the total polyphenol content in tea (expressed in mM hydroquinone). Likewise, the determination of cholesterol was studied using a cholesterol oxidase (ChO) column in front of a flow-cell with Al–ferron on an anion-exchange resin [121]: the LOD was 50 \(\mu\)M. A system using a flow-through cell with mixed ChO and Al–ferron phases was also used but found to be less sensitive. To ensure proper solubilization of cholesterol, a hexane/chloroform (95:5 (v/v)) carrier was used. The method was applied to the determination of cholesterol in butter and eggs; the results were consistent with those of the Boehringer test (visible/UV enzymatic method). Unfortunately, the operational stability of the ChO-column was limited to about 60 assays.

6.6. Various sensing schemes

An interesting FIA-RTP optosensing method for the determination of anionic surfactants—in particular for SDBS—was presented by Badía and Díaz-García [122]. The active RTP sensing phase consisted of
Al−ferron and bovine serum albumin (BSA) immobilized on a strongly basic anion-exchange resin (Fig. 10). Under deoxygenated conditions, the presence of SDBS was observed to generate RTP due to surfactant-induced stretching of the BSA molecules. This unfolding was considered to result in RTP due to the increase in local microviscosity and protection against molecular oxygen. No RTP was obtained in the presence of cationic, non-ionic or amphoteric detergents. Consequently, selective detection of anionic detergents was achieved, although some (cationic and zwitterionic) detergents were observed to quench RTP by about 40% when present in three-fold excess.

Sensing of DNA was performed using the RTP enhancement obtained from a Pd(II)−porphine complex in the presence of double-stranded DNA—most probably due to increased rigidity [123]. Sodium sulfite was used for deoxygenation. A good selectivity against RNA and (surface-active) albumin was reported and the LODs were in the attomolar range. The results obtained in the DNA analysis of human colon tissue were consistent with the results from UV absorption measurements at 265 nm.

Optosensing schemes based on luminescence quenching have already been discussed in Sections 6.2 and 6.5. In some cases, the ability of a certain compound to quench RTP depends on solvent conditions, or it can be influenced by complexation with an analyte of interest. Jin et al. [124] reported an interesting pH optosensing scheme in which the quenching of RTP was enhanced when the pH increased, thus enabling monitoring of pH in the range 4–8. The enhancement of quenching due to ET was attributed to the increased overlap of the RTP emission spectrum of the ternary phosphorophore:β-CD-cyclohexane complex and the combined absorption spectrum of the pH-sensitive dyes (phenol red, bromocresol purple and bromophenol blue); no direct influence of the pH
A similar principle was reported for the detection of Hg(II) [125]: complexation of Hg(II) with dithizone was observed to effect a distinct shift in the absorption spectrum of dithizone, which resulted in increased spectral overlap with the emission spectrum of the 1-BnNβ-CD-cyclohexane complex and, thus, in increased quenching of RTP. An LOD of 14 ng ml$^{-1}$ was achieved, with moderate selectivity towards a number of metal ions. Cu(II) and Ag(I) strongly interfered since they can also form strong complexes with dithizone [125].

Interestingly, ET between the precipitant phosphorescent complex and the acceptor molecules appeared to be efficient enough for detection purposes in both studies, although dissolved molecular oxygen was not inhibiting (less than 5% difference between deoxygenated and aerated solutions). This suggests that a long-range, coulombic energy transfer process may be involved. Although such a process is spin-forbidden in this specific case, it can become important when other routes are blocked [3]. This may well be the case for the precipitant ternary complexes considered: due to the highly rigid environment of the phosphorophores in such complexes and adequate shielding from molecular oxygen, non-radiative deactivation will be largely inhibited so that extremely long triplet lifetimes of, typically, several seconds are observed [54,55].

Temperature, pH and pCO$_2$ were monitored on microtitre plates using sensor films with various Ru(II) complexes [97]. Due to the rather short luminescence lifetime of the Ru(II) complexes (0.33–4 μs), the presence of molecular oxygen in the sample did not inhibit detection. Also in this case, optosensing was based on modulation of ET. Variation of pH caused changes in the protonation state and absorption spectrum of the acceptor, bromothymol blue, and, thus, differences in spectral overlap with the excited state donor. Sensing of pCO$_2$ was performed in a similar way. Temperature-sensing was based on the increased thermal deactivation of the luminescent states at higher temperatures. The methodologies were not tested with real-life samples. Nevertheless, such tests will be required to evaluate their selectivity.

6.7. Molecularly imprinted polymers

A few examples of quenched fluorescence detection and sensitized lanthanide luminescence detection are presented here to illustrate the potential of molecularly imprinted polymer (MIP) phases for sensitized and quenched RTP detection. In one study, the fluorescence of the functional monomer, trans-4-[p-(N,N-dimethylamino)styryl]-N-vinylbenzylpyridinium chloride was quenched upon binding of cyclic 3', 5'-adenosine monophosphate (cyclic AMP) [92]. That is, the functional monomer was involved in recognition as well as detection. Problems were the long response time of about 30 min and the observed heterogeneity of the binding sites with regard to accessibility and binding affinity. Likewise, the fluorescence of a Zn(II)–porphyrin signalling functional monomer was quenched upon binding of 9-ethyladenine (Fig. 11) [93]; as regards structural analogues, no binding of adenine was observed, while 2- and 4-aminopyridine were bound 37- and 13-fold less strongly, respectively. As a final example, sensitized luminescence of Eu(III), that was partly complexed by a functional monomer, was used to detect pinacolyl methylphosphonate (PMP), a hydrolysis product of the nerve gas soman [94]. The MIP sensing phase was applied at the tip of an optical fiber. LODs as low as 1 and 7 ng/l were obtained for a bench-top and portable set-up, respectively. Although selectivity undoubtedly is an important characteristic of MIP phases, the observed selectivity against organophosphorus pesticides, which may interfere with the determination of PMP, is mainly based on the detection part (unique emission band at 610 nm) and only partly on selective binding.

6.8. Concluding remarks

In the last decade, RTP optosensing methods based on principles such as bimolecular quenching, enhancement of ISC, sensitized lanthanide luminescence, and modulation of ET have been developed. In addition, some analytes of interest were detected by interactions enhancing the rigidity of their environment. The ET modulation technique is based on variations in the spectral overlap of the RTP emission spectrum

Fig. 11. Schematic representation of molecular imprinting of 9-ethyladenine (9EA) using a Zn(II)–porphyrin complex (1) and methacrylic acid (MAA) as a functional monomer. Figure taken from [93].

and the absorption spectrum of the quenchers (triplet-to-singlet energy transfer). It is not yet known to which extent the inclusion of the phosphorophores in such complexes reduces the bimolecular quenching rate constants. However, the quenching was shown to be adequate for analytical purposes and—of particular interest from a practical point of view—dissolved oxygen hardly plays any role.

The main attractiveness of optosensing schemes derives from their selectivity, for instance, in biosensing of enzyme substrates, detection of DNA, and luminescence detection after complexation. However, some methods will not be selective enough as such for all types of samples. For instance, although no serious interferences should be expected in the detection of oxygen in air, its detection in aqueous samples may well be hindered by organic or inorganic quenchers. Apart from selectivity, improved sensitivity and sensing phase stability are still required. That is, RTP optosensing is still in its infancy, despite the interesting detection principles that have been demonstrated. Finally, the examples of MIP sensing phases using sensitized and quenched RTP detection.

7. RTP measurement of biomolecules

7.1. Introduction

As was mentioned in Section 6.6, RTP can be used for the detection of (double-stranded) DNA in aqueous solutions. In this section, the application of phosphorescent labels for the detection of biomolecules such as proteins, antigens, RNA and DNA—particularly in (immuno)assays and time-resolved microscopy—will be discussed. The use of tryptophan RTP lifetime as a structural probe for proteins will also be considered.

7.2. RTP labels and assays

Fluorescent labels are by far the best choice for the sensitive detection of many classes of compounds, but under certain conditions, i.e. when strongly autofluorescent material is involved, the use of labels based on long-lived luminescence can be advantageous. In this case, improved sensitivity of assays may result, since time-resolved detection can be applied. In the past, almost exclusively lanthanide labels were used for derivatization [126]; more recently, various metalloporphyrins were also applied. These compounds not only exhibit long phosphorescence lifetimes, but also large Stokes’ shifts, high absorptivities and high phosphorescence quantum yields.

An overview of the photophysical properties of various metalloporphyrins is given in Table 5. For Pt(II)– and Pd(II)–porphyrin ketones, the extinction coefficients were ca. 45,000–170,000 M$^{-1}$ cm$^{-1}$ and the phosphorescence quantum yields ca. 0.01–0.12; the emission maxima were significantly red-shifted towards the range of ca. 760–800 nm. The photostability of these labels was reported to be significantly higher than for other porphyrin derivatives [127]. Pd(II)– and Lu(III)–tetrabenzoporphyrrin (TBP), and Pd(II)–tetrakis(phenyltetrabenzoporphyrrin
Table 5
Photophysical data for selected phosphorescent porphyrin ketone–metal complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption bands</th>
<th>RTP quantum yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>$\epsilon$ (M$^{-1}$ cm$^{-1}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt(II)–OEPK$^a$</td>
<td>398</td>
<td>6200</td>
</tr>
<tr>
<td>Pt(II)–CPKTEE$^a$</td>
<td>397</td>
<td>62700</td>
</tr>
<tr>
<td>Pt(II)–CPK$^b$</td>
<td>394</td>
<td>n.m.</td>
</tr>
<tr>
<td>Pd(II)–OEPK$^a$</td>
<td>410</td>
<td>82800</td>
</tr>
<tr>
<td>Pd(II)–CPKTEE</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>Pd(II)–CPK$^b$</td>
<td>406</td>
<td>n.m.</td>
</tr>
<tr>
<td>Zn(II)–OEPK$^a$</td>
<td>422</td>
<td>170000</td>
</tr>
<tr>
<td>Pd(II)–TBP$^c$</td>
<td>407</td>
<td>179000</td>
</tr>
<tr>
<td>Lu(III)–TBP$^c$</td>
<td>426</td>
<td>95500</td>
</tr>
<tr>
<td>Pd(II)–Ph4TBP$^c$</td>
<td>442</td>
<td>119000</td>
</tr>
</tbody>
</table>

OEPK, octaethylporphine ketone; CPK, coproporphyrin I ketone; CPKTEE, coproporphyrin I ketone tetraethyl ester; TBP, tetrabenzoporphyrin; Ph4 TBP, meso-tetraphenyltetrabenzoporphyrin; n.m., not measured.

$^a$ Solvent: chloroform, [127].
$^b$ Solvent: methanol, [127].
$^c$ Solvent: DMF, [102].

(Ph4TBP) were also studied [102]. The highest absorptivities and RTP quantum yields were obtained for Pd(II)–TBP (Table 5); emission was in the range of ca. 770–800 nm.

Pd(II)–coproporphyrins were used in a competitive, universal immunoassay for the detection of insulin (down to the sub-nM level) and other antigens [128]. With an extinction coefficient of 200,000 M$^{-1}$ cm$^{-1}$ and a phosphorescence quantum yield, $\Phi_p$, of 0.17 the Pd(II)–coproporphyrins are among the most powerful RTP labels available (cf. Table 5). Monofunctional $p$-isothiocyanatophenyl derivatives of Pt(II)– and Pd(II)–coproporphyrins were used for the covalent labelling of proteins [129]. The antibody–label conjugates essentially retained the activity and long-term storage stability of the unbound label; labels and antibody–label conjugates were evaluated in solid-phase immunoasays and found to be highly sensitive (detection down to 1–10 pM) compared to many other assays. Finally, dansyl chloride can be used as an RTP label for amino acids [130]. Using SDS micelles, MS-RTP from the dansylated amino acids was observed for pH 7–10. For deoxygenation 0.016–0.02 M sodium sulfite was used, while 0.03–0.05 M TINO3 was used as HAP. The authors suggested that the RTP detection of dansylated amino acids may be coupled with some separation technique involving SDS micelles. In a more recent study [67], the potential of dansyl chloride for RTP labelling in homogeneous solutions, in the presence of high concentrations of HAP, was addressed (cf. Section 4.1).

Phosphorescent metalloporphyrins can also be used for labelling of biomolecules in time-resolved microscopy; the application of time resolution is advantageous since many tissues are strongly autofluorescent [131,132]. N-Hydroxysuccinimide ester porphyrin derivatives were synthesized and bound to the antibodies avidin, streptavidin, and neutravidin. Glucose and glucose oxidase were added for deoxygenation [131]. The streptavidin–porphyrin derivative conjugates were used to study 28S rRNA in HeLa cells and CD4 membranes of lymphocytes [131]. In a further study [132], label–antibody conjugates were used to detect prostate-specific antigen, glucagon, human androgen receptor, p53 and glutathione transferase, again in autofluorescent tissues. In addition, metallocoproporphyrin-labelled dUTPs were synthesized and applied to the detection of DNA in metaphase spreads. In all these cases, the contrast was significantly improved compared to fluorescence microscopy.

Another interesting study reported on phosphorescence anisotropy measurements, which were performed to monitor the binding of antibodies to a BSA antigen labelled with eosin-5′-isothiocyanate.
Upon binding of the antibody—monoclonal anti-BSA (mouse IgG 2a)—the degree of phosphorescence anisotropy from the eosin-5′-isothiocyanate label was enhanced. In other words, after excitation with linearly polarized light some polarization was retained during RTP emission because the rotational movements were strongly reduced. Thus, a direct immunoassay format was achieved. A (pulsed) dual-channel phosphorimeter was used to monitor both polarizations simultaneously. Consequently, the measuring time was shortened and photodegradation problems were reduced compared to earlier, sequential studies [134]. An advantage of phosphorescence anisotropy over fluorescence anisotropy is the reduced interference from the excitation pulses. In addition, when using phosphorescence anisotropy rotational movements in the micro-to-millisecond timescale can be measured, while with fluorescence anisotropy one is restricted to the nanosecond timescale. Therefore, the phosphorescence anisotropy technique enables monitoring of much larger molecules [133].

7.3. RTP lifetime as a structural probe

The potential of tryptophan RTP lifetime measurements for probing conformational changes in proteins was first recognized in 1985 by Strambini and Gonnelli [135]. The authors suggested that the tryptophan phosphorescence lifetime is mainly influenced by differences in the rigidity (microviscosity) of the indole environment. Changes in the accessibility of proteins to molecular oxygen were supposed to play only a minor role, if any, since proteins in solution are quite permeable to molecular oxygen, independent of their actual conformation. More importantly, in all probing studies considered the dissolved oxygen was efficiently removed by purging with high-purity argon or nitrogen. Because of the strong dependence of the tryptophan RTP lifetime on the rigidity of the environment, RTP lifetime is a very sensitive probe that can detect subtle conformational changes [136].

As a first example, tryptophan phosphorescence lifetime (Trp 109) and activity measurements were used to monitor structural changes in *Escherichia coli* alkaline phosphatase (AP) induced by the addition of several denaturants (EDTA, acid, and guanidine·HCl) [137]. RTP lifetimes were reduced upon unfolding of the protein. An initial response was obtained within seconds after addition of the denaturant, but the total denaturation process took up to several hours (Fig. 12). In general, several binding interactions, including hydrophobic and electrostatic interactions, van der Waals forces, hydrogen bonds, and covalent bonds between cysteins, determine the structure of a protein. Experiments involving a variety of denaturants may therefore help to indicate which interactions are most important in specific cases. The experiments with AP indicated a complex behavior which involved several slowly interconverting conformations with different RTP lifetimes and activities. These two measured parameters did not correlate in all cases. The phosphorescence decay could best be described by a lifetime distribution even in the initial state [137]. However, upon unfolding the distribution was observed to broaden, which indicates increased heterogeneity, i.e. the presence of several conformations.

In another study, changes in pH were used to induce conformational changes in the protein azurin, which were studied by monitoring the RTP from the tryptophan residue [138]. Two different, slowly interconverting, conformations were shown to exist, as indicated by the two RTP lifetime components (417 and 592 ms) observed in the phosphorescence decay curves. The pre-exponential factors appeared to change with pH (one going up and one going down); the changes in conformation were ascribed to the protonation of the azurin His 35 residue at low pH. Interestingly, this is not a denaturation study since the conformational change at low pH is required for the functioning of the protein.
Finally, tryptophan phosphorescence was used to perform structural assessments on the bovine heart mitochondrial F$_1$-ATPase ε-subunit, which in vitro can still hydrolyse ATP [136]. Measurements of the band-width of the (vibrationless) 0–0 band at low temperature, as well as the heterogeneous RTP decay kinetics in fluid solution, indicated the existence of multiple, rather stable conformations of the ε-subunit. The exposure of the ε-subunit to 6 M urea, which is strongly dissociating, did not result in shorter decay times; this strongly suggests that the tryptophanyl side-chain was not solvent-exposed upon dissociation. By contrast, the conformational changes accompanying the binding of Mg–ATP were clearly indicated by changes in the phosphorescence lifetime distribution—most notably in a decrease of the observed lifetimes.

7.4. Concluding remarks

RTP labels are useful probes, especially for the application of time-resolved detection in immunoassays. In addition, time-resolved microscopy is a promising technique to monitor labelled biomolecules in strongly autofluorescent tissues. Phosphorescence anisotropy is an interesting technique, since it provides a direct assay format for monitoring antigen–antibody binding. Finally, tryptophan lifetime measurements can be considered a valuable tool in the structural analysis of proteins, enabling the detection of subtle conformational changes.

8. Lanthanide luminescence

8.1. Introduction

A discussion of lanthanide luminescence is relevant because long-lived lanthanide luminescence shows strong similarities with RTP, although the nature of the radiative transitions is fundamentally different for both luminescence modes. As regards the similarities, long luminescence lifetimes are involved in both types of luminescence, so that time-resolved detection can be applied simply by using pulsed xenon sources for excitation. Phosphorescence anisotropy has the inherent advantage of showing very large Stokes' shifts: the difference between the excitation wavelength of the organic sensitizers and the emission wavelength of lanthanide luminescence generally exceeds 250 nm, which is rather similar to the Stokes' shifts typically observed in RTP. Moreover, all modes present in RTP (direct, sensitized and quenched mode) are, in principle, also available in lanthanide luminescence.

In contrast with phosphorescence, which is based on emission from the T$_1$ to the S$_0$ state of an organic molecule, the luminescence from Eu(III) and Tb(III)—these lanthanides are used almost invariably—involve emission from the $^5$D$_0$ (and, to some extent, $^5$D$_1$) and $^5$D$_2$ levels to the $^7$F ground-state level. Moreover, these atomic emission bands are very narrow (a few nanometers) due to the protected nature of the lanthanide f shells. In addition, in a sensitizer–lanthanide complex intramolecular energy transfer is fast (deyr, ca. $10^{10}$ s$^{-1}$ [139]). Both factors strongly reduce the quenching efficiencies for dissolved molecular oxygen, so that in practice sample deoxygenation is not required at all. Instead, quenching by water due to energy transfer from the excited lanthanide to the surrounding water molecules—resulting in vibrational excitation of water—plays a prominent role [140]. Although many analytical applications of lanthanide luminescence involve the use of a separation technique, analytical methods based on immunoassays or involving batch measurements or FIA, have also been reported. Sensitized lanthanide luminescence obtained from Eu(III) or Tb(III) is used most often, with the sensitizer being coordinated by the lanthanide ion; occasionally, quenched lanthanide luminescence detection is applied. The present overview is far from exhaustive; it is intended to give an indication of the most important methods based on lanthanide luminescence, and of its potential in practical analysis.

8.2. Stand-alone measurements

Due to the selective nature of sensitized lanthanide luminescence detection (synthetic) mixtures and samples that are not too complex can be analyzed without any previous separation, especially when special scanning techniques are used. For example, second-derivative synchronous scanning fluorimetry was used for the simultaneous determination of salicylic acid and diflumisal in spiked urine and serum [141].
the analysis results were quite accurate (recovery, 87–111%). Benzoic acid and saccharin were simultaneously determined in soft drinks using a stopped-flow technique (cf. Fig. 4) [142]. One syringe contained Tb(III), Triton X-100, and trioctylphosphine oxine, while the other contained the sample. The Tb(III) luminescence was enhanced due to inclusion of the Tb(III)–analyte complex in the mixed aggregate. The extra dimension added (the luminescence curves have characteristic slopes and amplitudes) and the inherent selectivity of lanthanide luminescence enabled the direct analysis of soft drinks. Glucose, cyclamic acid, caffeine, citric acid and ascorbic acid could be tolerated in a 7–100-fold excess. As mentioned by the authors, easy automation of sensitized lanthanide luminescence detection can be achieved in this way.

The enhancement of lanthanide luminescence, due to ET after binding of Tb(III) ions to the nucleobases guanine (G), adenine (A), thymine (T) and cytosine (C), can be used for the detection of single-stranded oligonucleotides or single-stranded regions of DNA; double-stranded DNA effects no enhancement, which has been attributed to perturbation of the electronic structure upon base-pair formation [143]. In a recent report, an approx. 10-fold increase of the luminescence intensity was obtained for single-stranded oligonucleotides (10-mer) [144]. Interestingly, the presence of single mismatched base-pairs in 10-mer duplexes also leads to enhancement of Tb(III) luminescence [144]. As is the case with detection of single-stranded oligonucleotides, the enhancement here is due to ET from the nucleobases. The largest enhancement was provided by GG mismatches followed by GA and CA; low enhancements were observed for TT and GT mismatches (Table 6).

Detection of higher-ordered sequences in double-stranded DNA could be performed, by using a modified 14-mer single-stranded oligonucleotide with a Tb(III)-chelating group attached on a flexible hexamethylene chain, [143]. In the presence of double-stranded DNA with specific purine tracts, triple-stranded DNA sections were formed—so-called Hoogsteen-type base-pairing. As a result, the DNA base-sensitized lanthanide luminescence was quenched two to four-fold, which again was attributed to perturbation of the electronic levels of the DNA bases. Thus, double-stranded oligonucleotides with sections of 14 subsequent T–A base-pairs could be detected, using a single-stranded, all-T, 14-mer probe.

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
<th>Mismatch</th>
<th>Relative intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>T</td>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>GG</td>
<td>9.6</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
<td>GA</td>
<td>7.0</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>CA</td>
<td>6.7</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>CC</td>
<td>6.3</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>TC</td>
<td>4.7</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>AA</td>
<td>4.5</td>
</tr>
<tr>
<td>T</td>
<td>T</td>
<td>TT</td>
<td>3.6</td>
</tr>
<tr>
<td>G</td>
<td>T</td>
<td>GT</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Taken from [144].

Table 6: Influence of single base-pair mismatch on RTP emission intensity with 25 μM Tb(III) and 50 μM base.

8.3. Reversed micelles

As was described in Section 2.4, surfactants may form reversed micelles in apolar solutions. After addition of a small amount of water, water pools are formed inside the micellar core. The analytical potential of sensitized lanthanide luminescence detection in AOT reversed micelles, which contained Tb(III) or Eu(III) as counterions, was discussed in several papers [146–148]. A strong enhancement of the lanthanide luminescence was achieved, due to fixation of the lanthanide ions at the water/micelle interface, since this results in closer proximity of donor (analyte) and acceptor (lanthanide) and, thus, enhances energy...
transfer \[146\]. Since analytes with ionic groups and polar compounds can penetrate the micellar core more effectively than non-polar compounds, the former can be detected much more sensitively: LODs were $10^{-7}$ to $10^{-9}$ M for polar and $10^{-5}$ M for less polar compounds \[147\]. The technique was applied both in stand-alone measurements and in normal-phase LC for the determination of theophylline in Primatene tablets \[148\]; using the LC method, an LOD of 9 ng ml$^{-1}$ was achieved for standard solutions. Ephedrine, which is also present in Primatene tablets, did not interfere with the detection of theophylline. Therefore, the stand-alone measurements were sensitive enough to provide accurate results and yielded 94–100% of the nominal content—similar to the LC method. By contrast, the presence of theobromine interfered and LC should be used for the detection of theophylline in theobromine-containing samples.

8.4. Immunoassays and DNA hybridization assays

The long-lived luminescence of lanthanide chelates was also used in immunoassays and DNA hybridization assays. Also here, the application of time-resolved detection resulted in improved detectability. Meyer and Karst \[149,150\] proposed an enzyme-linked immunosorbent assay (ELISA) detection scheme, which utilizes the lanthanide luminescence from ternary lanthanide chelates formed after a reaction catalyzed by horseradish peroxidase (HRP). After enzymatic amplification, HRP catalyzes the dimerization of the substrate, 4-hydroxyphenylpropionic acid, after which the dimer forms a luminescent ternary complex with Tb(III)–EDTA chelate; for goat anti-rabbit IgG an LOD of 3 ng ml$^{-1}$ was achieved. The proposed methodology was tested in an ELISA for the determination of human anti-gliadin IgA in serum; the results agreed well with those from ELISAs using photometric detection \[150\]. For the determination of DNA in hybridization assays, the immobilized target DNA was hybridized with a derivatized probe oligonucleotide. After either a single \[151\] or a double \[152\] enzyme amplification step, conversion of a suitable substrate, 5′-fluorosalicyl phosphate, into the corresponding fluorosalicylate was catalyzed by bound AP. After complexation of 5′-fluorosalicylate with Tb(III)–EDTA, long-lived lanthanide luminescence was obtained. The assay with two amplification steps, showed a 10-fold improved S/N over the one using a single amplification step \[152\].

8.5. Liquid chromatography

The coupling of sensitized lanthanide luminescence detection and LC was first reported by DiBella et al. \[140\]. The lanthanides Tb(III) or Eu(III) were added in the post-column mode using a second LC pump and a mixing tee followed by a mixing coil. The quenching by water molecules could be reduced by the post-column addition of acetate ions, which remove water from the first coordination sphere of the lanthanides.

The analytes to be detected should be able both to form a complex with the lanthanide ion and to transfer their excitation energy to it via an intramolecular pathway \[153,154\]. This seriously limits the application range. Nevertheless, there are a number of useful applications, e.g. the analysis of orotate, theophylline, tetracyclines, steroids, nucleic acids and thiols \[153\]. A typical example, which serves to highlight the advantages of the technique, is the determination of orotate in urine \[155\]. For separation, ion-pairing reversed-phase LC was used; Tb(III) was added post-column. The method was sensitive (LOD, 10 nM) as well as selective: no sample pretreatment was necessary, except for a simple pH adjustment, filtration and dilution of the sample. A disadvantage of post-column addition is the inevitable dilution of the analyte \[153,154\], but in some cases the use of post-column addition is necessary in order to achieve optimum conditions for both separation and detection (complexation) \[153\]. As an alternative to the pre- or post-column addition of lanthanide ions, the detection of carboxylic acid-containing analytes was performed using a detection cell containing immobilized (partly complexed) Tb(III) \[154\]. The reported LODs (ca. 2 µM) were about 10-fold higher than with post-column addition of Tb(III), but it was noted by the authors that considerable enhancement of detectability may be anticipated with an improved set-up. Interestingly, a pre-column derivatization reaction with 4-maleimidylsalicylic acid (4-MSA), normally used to obtain fluorescent products from thiols, was extended to generate long-lived lanthanide
luminescence [156]. After reaction of the thiols with 4-MSA, the fluorescent product was complexed with Tb(III) and long-lived luminescence was obtained while the fluorescence was strongly suppressed. Compared with fluorescence detection, enhanced selectivity and a 5-fold improved sensitivity were obtained for glutathione and l-cysteine (LOD for both compounds, 1.5 × 10⁻⁷ M). The method was successfully applied to the determination of l-cysteine in urine spiked at a concentration of 10⁻⁶ M (Fig. 13); apart from 10-fold dilution and filtration, no sample pretreatment was used.

Dynamic quenching of Eu(III) and Tb(III) luminescence was also applied as a highly selective detection mode in FIA and LC [157], with different responses for Eu(III) and Tb(III). When using Eu(III), nitrite, sulfite, chromate, Fe(CN)₆³⁻ and Fe(CN)₆⁴⁻ could be detected with good sensitivity; no response was observed for halides, thiocyanate, sulfate and nitrate. When using Tb(III), thiocyanate and Pt(Cl)₄²⁻ could additionally be monitored, while sulfite could not be measured due to precipitation. For nitrite, an LOD of 50 nM was achieved in LC.

Finally, ligand exchange was also applied for detection in LC: organic phosphates, which are able to compete with the original ligand, acetylacetone (acac), for the Tb(III) ions, were detected with satisfactory LODs of 30-70 nM [158].

8.6. Capillary electrophoresis

The combination of sensitized lanthanide luminescence detection and micellar electrokinetic chromatography (MEKC) was first studied by Milefsky et al. [159], who used the steroids testosterone and progesterone as test analytes and a HeCd laser for excitation. Likewise, orotic acid was determined in urine, without any sample pretreatment (apart from dilution, filtration, and pH adjustment to 6.0) [160]. Compared to LC, the run time was reduced more
than 10-fold to as little as 1.5 min. At the same time, the LOD increased only five-fold (to 50 nM), which is quite acceptable in view of the optical pathlength available for detection (75 µm). In both MEKC studies, Tb(III) was added to the separation buffer; therefore, the capillary had to be conditioned for ca. 5 h every week since the lanthanide ions strongly interact with the negatively charged capillary wall [160].

Such problems can be avoided by applying post-column addition of the lanthanide ions. With this approach, capillary zone electrophoresis (CZE) was used to determine catecholamines and related compounds in urine [139]; for standard solutions, LODs of about 10^{-7} M were achieved. In addition to Tb(III), EDTA was added in order to induce the formation of a stable ternary complex at high pH. The post-column reactor was filled with a Tb(III)/EDTA solution and contained a porous tube surrounding the closely spaced ends of the separation and detection capillaries (Fig. 14). The solution was introduced into the detection capillary by applying a 30 mbar pressure on both the reactor and the inlet vial (the latter for compensation). A similar reactor, but without the porous tubing and with a space of about 30-50 µm between the capillaries, was used for the determination of diflunisal and salicylic acid [161]; the distance between the two capillaries should be carefully controlled in order to avoid peak broadening. The post-column reactor solution contained Tb(III) and CTAB; the latter compound was added to enhance the luminescence by compartmentalization and protection against water.

Dynamic quenching of (sensitized) lanthanide luminescence was also used as a detection mode in CZE [162]. The luminescence signal of the Tb(III)-acac complex was effectively quenched by nitrite, which resulted in LODs of 200 and 3 nM under normal and stacking injection conditions, respectively. Unfortunately, the peaks of other ions such as chromate were severely broadened. In addition, the surfactants nonyl- and dodecylsulfate were detected by the decrease in luminescence caused by the electrophoretic displacement of acac by the non-luminescent surfactants, while EDTA was detected by the ligand-exchange mode; unfortunately, the peaks were also broadened in the latter case.

8.7. Concluding remarks

Relevant applications of lanthanide luminescence detection have been discussed here; they range from coupling with separation techniques like LC and CE to application in immunoassays and stand-alone measurements; high selectivity and analyte detectability are the most notable advantages of lanthanide luminescence, as proven in several examples involving real-life samples. Interestingly, this luminescence mode can also be used for the detection of double-stranded DNA and higher-ordered DNA sections, as well as for single-stranded DNA and single DNA base-pair mismatches. Since RTP can only be used for the detection of double-stranded DNA (cf. Section 6.6), Therefore, lanthanide luminescence appears to be a more versatile method for the analysis of DNA than RTP, although the latter technique provides more selective detection of double-stranded DNA in the presence of RNA. Finally, long-lived lanthanide luminescence also has potential for application in MIP sensing phases, as was discussed in Section 6.7.
9. Evaluation and future perspectives

In this review, various RTPL techniques were discussed with emphasis on recent developments. Although some useful new methods were reported in MS-RTP and CD-RTP, progress in these fields is rather limited. In almost all cases, direct RTPL is still applied without coupling to a separation technique. Because of the rather high selectivity of RTPL, such an approach can be used successfully provided that the sample matrix is not too complex. Nevertheless, the practicability of stand-alone MS-RTP and CD-RTP measurements for real-life samples appears to be rather limited. This also applies for the newly developed HAI-RTP technique, which provides a useful addition to MS-RTP and CD-RTP.

The coupling of direct RTP techniques with a separation technique appears to be feasible. However, certain problems have to be solved: in MS-RTP, foaming problems are generated by purging with nitrogen unless alternative micellar systems are used; as an alternative, chemical deoxygenation can be applied. In CD-RTP, coupling with a separation method may be hindered by slow mass transfer, especially when stable ternary complexes are involved. Heavy atom-derivatized, water-soluble CDs may offer a solution here. In the case of HAI-RTP, such coupling seems to be simpler since no ‘pseudophase’ is involved. However, in this case coupling may be limited to separation techniques that use aqueous-phase buffers or eluents, because of the negative effects induced by several organic solvents. For a full evaluation of this effect further study will be required. In any case, the coupling of direct RTP with separation techniques will undoubtedly increase its value as a tool in analytical chemistry.

For the indirect RTP modes (sensitized and quenched RTP), successful coupling with LC and CE has been reported and was shown to provide high sensitivity. In this context, it should be noted that quenched phosphorescence detection provides low concentration detection limits in CE even for analytes with poor chromophoric properties. In addition, extremely low absolute detection limits (ca. 1 fmol) were obtained, due to the small capillary dimensions.

RTP optosensing is an interesting and rapidly expanding field of research, which—based on many different detection principles—allows for the determination of various analyte classes as well as for measurement of solvent parameters such as temperature and pH. Also in this case, selectivity (as well as sensitivity and sensing phase stability) remains a critical issue, since no separation is involved. Nevertheless, in several cases, depending on the optosensing principle and the sample matrix, adequate selectivity was achieved for reliable analysis. In this context, the use of MIPs for selective binding may become of interest.

The use of labels with long luminescence lifetimes—either providing RTP or lanthanide luminescence—provides clear advantages in immunoassays and microscopic monitoring of biomolecules, especially when a strongly fluorescent background is present. In addition, tryptophan RTP lifetime measurements have become a valuable tool in the structural analysis of proteins. A more widespread use of such labels and probes in the near future can therefore be anticipated.

Compared to RTP (sensitized) lanthanide luminescence has been applied more widely in LC and CE. It provides enhanced selectivity compared to absorption detection—as was proven in the analysis of several real-life samples. Its use in immunoassays will undoubtedly continue even though porphyrin RTP labels were recently introduced as an interesting alternative. The application of lanthanides for the detection of single-stranded DNA and DNA single mismatches appears promising and conveniently complements RTP, which is able to detect double-stranded DNA only.

It should be realized that most RTP studies discussed in this review do not involve real-life (complex) samples. Consequently, the potential of RTP for selective detection in complex samples (e.g. soil, biota) cannot be assessed yet. Nevertheless, the most promising RTP methods to handle real-life samples will undoubtedly involve coupling with a separation technique. The coupling of quenched RTP and CE has already been shown to provide useful separations as well as LODs in the low ng ml\(^{-1}\) range.

References