Multiple fluorescent chemical sensing and imaging

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Optical sensors, unlike most others, enable multiple sensing of (bio)chemical species by making use of probes whose signals can be differentiated by spectral and/or temporal resolution. Multiple sensors are of substantial interest for continuous monitoring of chemical parameters in complex samples such as blood, bioreactor fluids, in the chemical industry, aerodynamic research, and when monitoring food quality control, to mention typical examples. Moreover, such sensors enable non-invasive, non-toxic and online detection. We discuss in this *critical review* the state of the art in terms of spectroscopic principles, materials (mainly indicator probes and polymers), and give selected examples for dual and triple sensors along with a look into the future (109 references).

1. Introduction

Optical chemical sensors (OCS) have experienced increasing success during the last decade.[1,2] They have distinctive features and merits compared to other sensor systems as they enable for non-invasive measurements, remote and online sensing, exhibit often unmatched sensitivity, are non-toxic in use, easy to handle, and can be applied in hazardous environments and in point-of-care diagnostics. Most sensors are based on the measurement of one of the various parameters known in fluorescence (*e.g.*: intensity, lifetime, quenching, anisotropy, FRET).

Analytes of wider interest include O_2, CO_2, pH, Na^+, K^+, Ca^{2+},Cl^-, or glucose, just to mention a few of the clinically relevant species for which there is a particularly large market.[3–7]

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In environmental and water analysis, ions like Hg^{2+}, Pb^{2+}, Zn^{2+}, or UO_2^{2+} can be detected and quantified.[8–11] Organic molecules like alcohols,[12,13] amines,[14,15] polycyclic aromatic hydrocarbons (PAHs),[16,17] warfare agents[18,19] and drugs/toxins,[20,21] in turn, can be sensed in even complex samples such as food. OCS also can serve as transducers in biosensors, often by making use of immobilized enzymes, antibodies, or cells, for example in the case of the determination of biochemical oxygen demand or in glucose sensors, where the consumption of oxygen is measured using oxygen sensors as a transducer.

Such sensors respond in a reversible way and thus can be used to continuously monitor the respective species, for example in the blood stream, a bioreactor, or the environment. Other "sensors" (mainly those based on high-affinity interactions such as between strands of complementary oligomers, between antigen and antibody, or between metal ions and ligands) respond irreversibly and thus can be used as single-shot probes only (which are appropriate in many situations).

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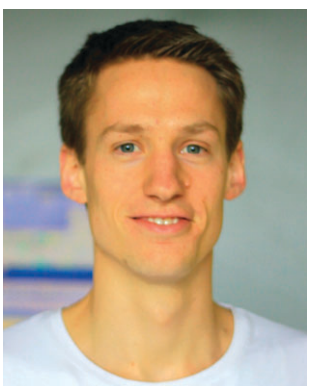

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OCS often have been combined with fiber optics.[2,22] It is reminded at this point that this review covers OCS in its strict ("Cambridge") definition as outlined by McDonagh *et al.*:[1] *Chemical sensors are miniaturised devices that can deliver real time and on-line information on the presence of specific compounds or ions in even complex samples*. It is mainly organic chemists that often use the term sensor for a probe (an indicator) by ignoring the long way that one has to go until a probe has become a sensor. In fact, numerous probes never have become sensors.

As the number of available OCS increased, new spectroscopic methods of interrogation were invented to meet the requirements of the respective application. Common optical techniques range from measurement of absorption (from the UV to the IR)[23] and steady-state fluorescence[24] to the determination of luminescence lifetimes in the frequency or time domain.[25–28] More recently, sensor materials have been interrogated by methods of fluorescence imaging to gain a maximum of temporal and spatial resolution. In aerodynamic research, for example, whole aircraft models are coated with optical oxygen probes in order to image pressure (actually oxygen partial pressure) and its variation on the surface within milliseconds.[29,30]

OCS technology is materials science to a large extent. Setting aside purely spectroscopic methods (that are exploiting the intrinsic optical properties of an analyte), the materials (sometimes referred to as "stimuli-responsive materials") respond to an analyte by a change in their optical properties. They often are based on smart combinations of (permeation-selective) polymers and optical probes. However, materials such as quantum dots and other nanoparticles, semiconductor materials, metal films, fullerenes, (carbon) nanotubes and the like are increasingly employed. The material—or their solutions in a solvent ("cocktails")—are deposited on an optically transparent support (including waveguides) and then interrogated by spectroscopic means. Luminescent reference dyes may be added to the cocktail[31–33] in order to obtain referenced signals.

One advantage of OCS over electro-analytical devices relies on the possibility of multi-analyte monitoring. Respective multiplexed sensor systems can come in various modifications and are referred to as multisensors. There are also multiplexing approaches with electrochemical techniques, but most of them result in electrode arrays or in complex surface modification of the electrodes.[34] These systems also suffer from the spatial distance between the electrodes. This renders such arrays less suitable for clinical investigations, not least due to the limited sample volume available. Furthermore, the parameters of interest are not detected and determined at exactly the same site, which also limits the feasibility in micro fluidic applications, significantly in samples such as blood vessels with their concentration gradients of O_2 and pCO_2. Optical multisensors enable simultaneous determination of analytes at exactly the same point. By sensing n parameters with one sensor spot, the number of sensors required will be reduced by a factor of $1/n$.

There are several formats of multisensors. The simplest one is based on multispot sensing which is however not a multiplexed technique in its strict sense. Rather, it is an array of different OCS in close proximity (*e.g.* fiber optics or wells of microplates). In sensor arrays, individual sensors are aligned such that the sample volume is contacted simultaneously. Like in the case of electrode multisensors, the resolution of these arrays is limited due to the spatial distance between the individual sensors.

OCS offer the unique possibility of determining a variety of species simultaneously at exactly the same spot. Two principle formats of respective multiple sensors exist: the multi-layer sensors and the single layer sensor (see Fig. 1). In the multi-layer (mostly dual layer) approach, several indicators (one for each analyte) are embedded in appropriate polymers. The two discrete indicator-polymer blends are then superimposed on a solid support, *e.g.* glass or metal substrates, polymer foils, or fiber tips. Single layer OCS, in contrast, consist of homogeneously distributed indicator molecules, often embedded in beads, but incorporated in one single polymer.[1,2] The third format has several advantages in that inner filter effects (in either absorption or emission) are minimized, response times are faster and not highly different for the various analytes, and fabrication is more reproducible.

Multiplexed sensing in its strict sense is making use of one single sensor material only but resulting in a multitude of optical information. This review will focus on luminescent two-layer and single-layer dual sensors. These are the most feasible ones in terms of medical and industrial applications. One important feature of dual sensors, no matter which format, is based on the fact that the signals of even single OCS need to be corrected for other effects such as temperature or external quenchers. In such multiple OCS, a probe for the analyte is combined with a probe for the potentially interfering species. For example, oxygen probes are always interfered with by temperature. Thus, a temperature probe is incorporated into the oxygen sensor system to compensate for effects of temperature.[35]

Otto S. Wolfbeis

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Fig. 1 Formats for multiple optical chemical sensing. (1) Multispot sensor or sensor array, with two different distinct sensing areas aligned in close proximity. (2) Two-layer (or sandwich) sensor, with two sensor layers superimposed on a solid support. (3) Single layer sensor, with different probes (often contained in particles) incorporated into a single (polymer) binder.

2. Sensor formats

2.1 Multispot sensors and arrays

Such OCS are composed of single sensor systems for given analytes, merged and miniaturized to a compact optical sensing device. If a large sample volume is available, microplates are adequate for multi analyte sensing, and the bottom of the wells can be coated with sensor spots in a typical thickness of 2–10 μm. Aliquots of the sample volume are then added to the wells and the plate is read out (using commercially available readers) or imaged. The sensor layer may be mechanically fixed in the well, or deposited in the form of a cocktail after which the solvent is evaporated so to obtain a thin sensor film. This is a cost-effective and easy method to prepare parallel sensors for multi analyte detection, *e.g.* in water analysis.[36] One drawback of this approach is the fact that all the desired parameters or analytes are not determined in the same compartment. Deviations between the single wells of the plate may cause erroneous data.

Other approaches of multiple OCS include chemically modified optical fibres or fibre bundles.[37,38] Optical fibres can be made chemically sensitive by depositing a "sensor chemistry" on their tip. Thus, they enable remote monitoring and online sensing. The indicator–binder combination at the distal end of the light guides is brought into contact with the sample volume. Fiber optic microsensors for oxygen have been most successful. With a diameter in the lower μm range, fibres for several analytes can be combined to a miniaturized sensor array or bundle. It also is possible to deposit several sensor spots on the tip of one fiber.

One common problem when dealing with optical sensors is the cross-sensitivity of almost any indicator towards interferents. If two or more indicators in a sensor array respond to several quenchers but to a different extent, this can be utilized to quantify several quenchers. If *n* analytes have to be sensed, *n* probes with different quenching constants have to be applied.[39] The concentration of the species can then be calculated using modified Stern–Volmer equations. This may, in fact, be used to generate multiple analyte arrays by placing different probes on a solid support. Each probe is sensitive to the analytes to a different extent. Exposure of the array to the sample results in a typical intensity pattern of the sensor spots on the array. Chemical noses and arrays for related groups of compounds (*e.g.* nitroaromatics, explosives,[40] organic vapours,[41] heavy metals[36]) can be fabricated that way. However, all the OCS systems described above are not dual sensors in a strict sense. Rather, they are a combination of single sensors combined to a miniaturized device that do not provide the opportunity for multiple sensing at one given point. Furthermore, they do not enable the spatial distribution of analytes to be imaged over certain areas.

2.2 Multisensor systems

On a first glance, it would appear that a chemical multisensor can be assembled simply by mixing the respective sensor chemistries ("stimuli-responsive polymers") for the analytes of interest. However, this turned out not to work for several reasons: (a) Many indicators display rather broad absorption and emission bands that often overlap so that unambiguous assignment is difficult. (b) If two luminescent probes are located in close (<10 nm) proximity, fluorescence resonance energy transfer (FRET) can occur which is highly undesirable. In order to overcome these limitations, indicators have been incorporated in micro- or nanoparticles. These prevent FRET from occurring and can be chosen such that the indicator probes are well soluble and that a maximum of permeation selectivity is achieved so to improve selectivity. For example, temperature indicators with cross-sensitivity towards oxygen can be shielded from oxygen by making use of oxygen-impermeable polymer beads. Additionally, the dynamic range can be fine-tuned by the permeability and kind of the polymers used.[42] Finally, the signal of such materials can be easily adjusted by proper variation of the sensor components. Fig. 2 gives a cross-section of a typical dual sensor based on the use of sensor particles.

Interestingly, most dual OCS described so far contain one oxygen sensor combined with a probe for another analyte, examples O_2/CO_2, O_2/pH, and O_2/T. Determination of oxygen partial pressure (pO_2) is of special interest because oxygen is virtually omnipresent and often acts as an interferent in sensors for other species. Since oxygen is consumed or produced during chemical or enzymatic reactions (mainly oxidases and peroxidases), they often are employed in (enzymatic) biosensors.[43]

2.2.1 Dual sensing of temperature and oxygen. Dual sensors for simultaneous determination of temperature and oxygen are needed for chemical process control, bioreactor monitoring, sensing of blood oxygen and glucose, in the gas industry in general, but also in microbiology and medical research. Another application is in aerodynamic research.[44] Here, sensor paints ("stimuli-responsive polymers") for determination of air pressure (= oxygen partial pressure) are sprayed onto aircraft models for investigation in wind tunnels. Respective sensor layers are referred to as pressure-sensitive paints (PSPs).

Practically all optical sensors for oxygen rely upon collisional quenching of luminescence by oxygen. The sensor response is directly related to oxygen partial pressure (pO_2) in a gas or of a liquid. Higher oxygen levels result in lower intensity (and hence luminescence lifetime). Typical probes for oxygen are listed in Table 1.

The majority of the indicators applied in oxygen sensing schemes exhibit a rather strong cross-sensitivity towards

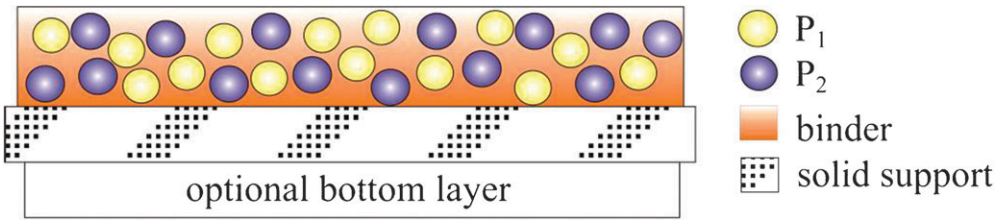
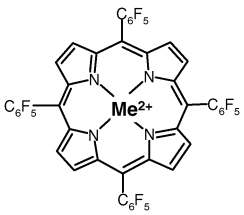
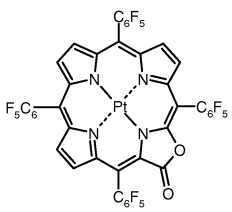
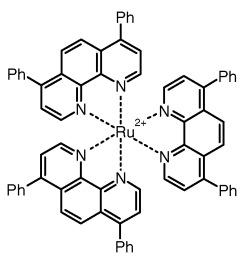
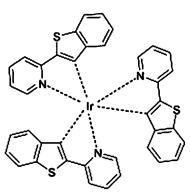


Fig. 2 Cross section of a dual optical chemical sensor (OCS). The particles (P_1, P_2) are responsive to the respective analytes and yield the optical information. The particles are incorporated into a matrix polymer (the binder) whose choice is quite critical. The sensor matrix usually is deposited on an optically transparent support so that the optical signal also may be gathered from the bottom. If gathered from the top, the sensor layer also may contain a reflective bottom layer that prevents sample fluorescence (which is strong in the case of most biomatter) from interfering with the optical readout.

temperature.[48] In order to compensate for this undesired effect, temperature probes are added to the sensor system. These resulting materials may be employed in various forms including planar sensors, fiber optic systems, or as pressure sensitive paints (PSPs). In each case they enable measurements of oxygen partial pressure (including air pressure and its gradients) to be corrected for effects of temperature. It also enables the study of heat transfer characteristics on surfaces.[44] This is illustrated in Fig. 3 where a hollow glass sphere (diameter = 5 cm) was first spray-painted with a dual PSP/TSP and then tested for its response to temperature and air pressure (actually partial pressure of oxygen). In order to generate a temperature gradient on the surface, the sphere was placed on a heating plate. An area of lowered pO_2 was created by directing a jet stream of nitrogen gas onto the top of the same sphere.

Table 1 Representative luminescent (and quenchable) probes for sensing oxygen

Compound	Acronym	Chemical structure	Reference
Palladium (or platinum)-5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)porphyrin	PdTFPP or PtTFPP		35
Platinum(II)-5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)porpholactone	PtTFPL		45
Ruthenium(II)-tris(4,7-diphenyl-1,10-phenanthroline)	$Ru(dpp)_3$		46
Iridium(III) [tris[2-(benzo[b]thiophen-2-yl)pyridinato-C^3,N]]	$Ir(btpy)_3$		47

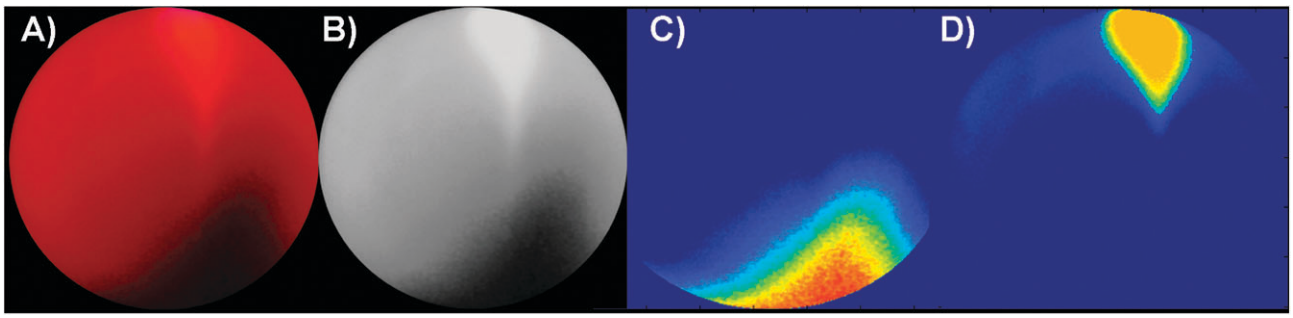


Fig. 3 Visualization of the temperature and pO_2 distribution on a glass sphere (diameter = 5 cm) coated with a dual sensor for oxygen and temperature. The coating consists of the oxygen probe PtTFPL and polyacrylonitrile (PAN) microparticles doped with temperature indicator $Ru(phen)_3$ in a film of a fluoropolymer (FIB; see text). The sphere was placed on a heating plate with a temperature of ~60 °C. In a parallel experiment, a stream of nitrogen was directed onto the sphere from above, thereby inducing an area of lower pO_2. (A) Luminescence image taken with a commercially available digital camera under UV-excitation of the dual sensor. (B) Picture taken with a cooled b/w camera used for imaging applications. (C) Pseudo-colour image of the temperature gradient. (D) Pseudo-colour image of the area where nitrogen gas is blown onto the sphere.

The first approaches towards dual sensing utilized inorganic compounds, the so-called thermographic phosphors, as probes for temperature, for example $La_2O_2S:Eu^{3+}$ with its green and strongly temperature dependent emission. It was combined with the red luminescent oxygen probe PtTFPP.[35] Both were incorporated into the highly fluorinated poly(heptafluoro-*n*-butyl methacrylate-*co*-hexafluoroisopropyl methacrylate) (FIB) polymer. Fluorinated polymers (but also silicones, polystyrene and ethyl cellulose) are often applied in sensors for oxygen because of their outstanding permeability.[49,50] Both probes can be photoexcited at the same wavelength. Their emissions can be separated by using optical filters. However, the sensors suffer from the rather poor brightness (absorbance multiplied with quantum yield) of the inorganic compound.

Metal ligand complexes (MLCs) with their much larger brightness therefore have been used more recently for sensing temperature (see Table 2). They cover the relevant temperature range from 0 to ~80 °C, some even up to 200 °C. Other MLCs also can be used for cryogenic applications (*i.e.* in the range from −175 to −85 °C).[51] A dual sensor for oxygen (in fact air pressure) and temperature exclusively based on metal ligand complexes has been reported by the groups of Gouterman and Khalil.[52] Oxygen was sensed *via* PtTFPP, and temperature *via* the silicon octaethylporphyrin complex. Both were contained in FIB polymer that acts as a binder. In a similar approach,[53] two porphyrin derivatives, metalated with magnesium and platinum, respectively, were employed. The chemistry of the metal ion strongly governs the quenching by oxygen of the MLC. Platinum(II) *meso*(pentafluorophenyl)porpholactone is highly sensitive to oxygen, but also to temperature. Magnesium *meso*-tetrakis(pentafluorophenyl)porphyrin (MgTFPP), in contrast, remains virtually unaffected by molecular oxygen, but is a good probe for temperature.

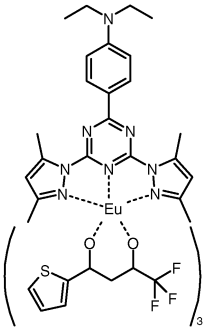
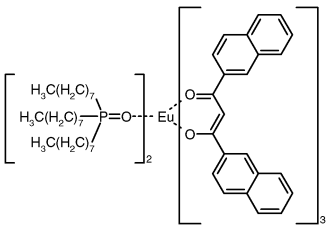
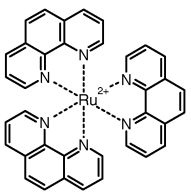
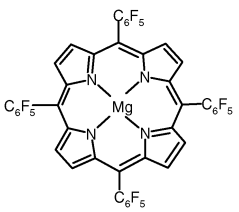
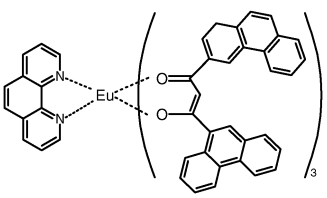
MLCs of the Eu(III) diketonate type have narrow emission bands, with a strong peak at ~615 nm.[54,55,57] This makes them ideal probes for combination with others, for example PtTFPL.[45] However, they also display weak side bands in emission which can interfere with signals from other probes in the case of multiple sensors. One further drawback of many Eu(III) MLCs (and also of Ru(II) MLCs) is their cross-sensitivity towards oxygen. This can be overcome by incorporating the probes into gas-impermeable polymer beads. The system ruthenium(II)-tris(1,10-phenanthroline) [Ru(phen)] in poly(acrylonitrile) particles, for example, turned out to be a sensitive probe for temperature with little cross-sensitivity to oxygen because poly(acrylonitrile) is virtually impermeable to this gas.[58] Ru(phen) displays a broad absorption band that ranges from 350 to 550 nm, so that it can be combined[56,59] with red to near IR emitting platinum or palladium (benzo-) porphyrins, all of which have overlapping absorption (and excitation) bands (between 400 nm and 460 nm).

Luminescent metal–ligand complexes of the Ir(III) ion also are viable probes for sensing both oxygen and temperature. They display bright tunable emissions, luminescence lifetimes ranging from 1 to 10 μs, fairly good molar absorbances, and high photostability in most cases. One good example is represented by the $Ir(btpy)_3$ complex (see Table 1) which was found[47,60] to be a very useful probe for oxygen that can be applied in dual sensors (T/O_2).

Besides their narrow emission band, the Eu(III)-diketonates exhibit luminescence decay times in the order of several hundreds of microseconds. This makes them amenable to both spectral and temporal signal separation (see section 3.2.3.). In contrast to the ruthenium polypyridyl complexes, they usually exhibit both higher molar absorbance and luminescence quantum yield, provided that a proper antenna ligand is attached.[61] The probe $Eu(tta)_3(dpbt)$ (see Table 2) exhibits good temperature sensitivity in the range from 0 and 70 °C. Unfortunately, its luminescence is also efficiently quenched by oxygen. Shielding of the indicator from oxygen by using appropriate polymers is therefore mandatory. Different systems are described in literature, specifically a two-layer system with the complex in poly(vinyl methyl ketone) (PVMK) and a particle system approach using poly(acrylonitrile) (PAN) as a gas-blocking polymer. This temperature probe was combined[62–64] with PtTFPP or PdTFPP as the oxygen indicator to give excellently performing dually sensing paints.

Cocktails for use in dually sensitive sensor films recently[65,66] have been manufactured without the use of organic solvents.

Table 2 Representative luminescent probes for sensing temperature

Compound	Acronym	Chemical structure	Reference
$La_2O_2S:Eu^{3+}$	—	Solid state	35
Europium(III)-tris(thenoyltrifluoroacetylacetonato)-(2-(4-diethylaminophenyl)-4,6-bis(3,5-dimethylpyrazol-1-yl)-1,3,5-triazine)	$Eu(tta)_3(dpbt)$		54
Europium(III)-tris(dinaphthoylmethane)-bis(trioctylphosphine oxide)	Eu-DT		55
Ruthenium(II)-tris(1,10-phenanthroline)	$Ru(phen)_3$		56
Magnesium *meso*-tetra(pentafluorophenyl)porphyrin	MgTFPP		53
Europium(III)-1,10-phenanthroline-tris(3-(3-phenanthryl)-1-(9-phenanthryl)propane-1,3-dione)	EuD_2		45

This was accomplished by dispersing, in water, core–shell nanoparticles composed of a polystyrene (PS) core and a poly(vinyl pyrrolidone) (PVP) shell. They were dyed with a luminescent probe for oxygen and suspended in plain water. The cocktail also contained PAN microparticles that were dyed with a luminescent temperature-sensitive probe. The particles can be easily dispersed in water and then sprayed onto a support using a spray gun. The material is highly adhesive to various surfaces, especially aluminium which is often used to manufacture models of aircrafts and cars. Depending on the application, either the PVP shell or the PS core can be chosen as the host for the luminescent probe. The PS-PVP particles are forming films of high mechanical stability.

2.2.2 Dual sensing of carbon dioxide and oxygen. Most sensors for carbon dioxide are making use of pH indicators

dyes. They are exploiting the equilibrium that is established between carbon dioxide, water, and carbonic acid. In other words, the more carbon dioxide that is present, the more carbonic acid is formed, this results in lower pH values. A patent was found to be relevant in this context.[67] The process is fully reversible. Examples of pH-sensitive probes for use in sensors for CO_2 are given in Table 3. In fact, the first dual sensor system ever reported in the literature was a two-layer sensor for determination of carbon dioxide and oxygen.[68] It was composed of 8-hydroxypyrenetrisulfonate (HPTS)[69,70] covalently bound to cellulose particles in an hydrogel matrix as probe for carbon dioxide. This layer was covered with a layer of silicone containing the oxygen probe ruthenium(II) tris(2,2′-bipyridyl) dichloride (see Table 1) adsorbed on silica gel microparticles. A polyester foil served as a solid support. An optical isolation layer was superimposed on this two-layer system to prevent interferences by ambient light and sample fluorescence. Both probes can be excited at 460 nm. Signal separation was performed by means of spectral separation by using optical filters.

This two-layer system approach was substantially improved by placing HPTS in ethyl cellulose particles that were incorporated in a poly(dimethyl siloxane) matrix as the upper sensor layer. The bottom layer consisted of the highly sensitive oxygen probe PtTFPP (see Table 1) dissolved in polystyrene.[46,73] The dual lifetime referencing method (DLR, see section 3.2.1.) was applied in order to achieve precision sensing. This dual sensor was applied to monitor carbon dioxide and oxygen over growing bacterial cultures of *Pseudomonas putida*.[74]

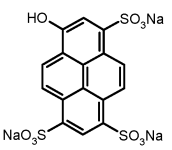
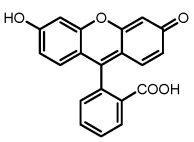
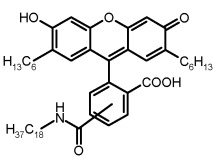
One negative effect that may occur when dealing with two-layer sensors is the phenomenon of cross-leaching of the components. This is even more pronounced if plasticizers are being applied. Furthermore, diffusion of the analytes through the different polymers may be retarded, resulting in unacceptably long response times, especially for the bottom layer. Single layer sensor systems are therefore preferred. However, such sensors occasionally display increased photo-decomposition, and thus signal drifts, especially if oxygen is to be determined. This is due to the formation of (highly reactive) singlet oxygen as a result of the quenching process.[75] This may be reduced, to a certain extent, by addition of compounds like diazabicyclo-octane or carotene, however.

Spatial mapping of the partial pressure of carbon dioxide and oxygen was performed with two-dimensional sensor layers, whose luminescence was imaged with CCD cameras. One system[76] for the determination of the distribution and gradients of oxygen and carbon dioxide in seawater and marine mats makes use of HPTS as a probe for carbon dioxide and of Ru(dpp) (see Table 1) as a probe for oxygen in a single layer system with ethyl cellulose as a matrix polymer.

One crucial aspect in sensing carbon dioxide results from the need of such sensors to work in the presence of water in the interior of the sensor matrix. This is accomplished, in most cases, by inclusion of water into polymer beads.[77] Alternatively, minute droplets of buffer solution may be entrapped.[68] This technique often causes, however, aging of the system due to loss of water, with negative effects on response times and reproducibility. Recent approaches[78,79] make use of room temperature ionic liquids (IL) rather than aqueous buffer. ILs display very low vapour pressure and are more stable than water-based sensors for carbon dioxide. It is expected that IL-based carbon dioxide sensors combined with other probes (such as for oxygen) will result in new multiple OCS.

2.2.3 Dual sensing of pH and oxygen. Dual sensing of pH and oxygen is more difficult from a materials point of view. Oxygen sensing requires materials that are highly permeable for oxygen but—ideally—impermeable to protons and ions. In the case of sensing pH, the contrary is the case. The problem was solved by analogy to the method for mapping gradients of carbon dioxide and oxygen in marine sediments.[72] The pH

Table 3 Representative fluorescent probes for sensing carbon dioxide and pH

pH probe	Acronym	Chemical structure	Reference
8-Hydroxypyrene-1,3,6-trisulfonate	HPTS		69,70
Fluorescein	—		71
2′,7′-Dihexyl-5(6)-*N*-octadecyl-carboxamidofluorescein	DHFA		72

indicator DHFA (see Table 3) is lipophilic enough not to be washed out from the polymer. The use of polymer beads is not necessary in this case. The highly luminescent platinum complex PtTFPP was used as a probe for oxygen. Both indicators were incorporated into a hydrogel matrix polymer, which is permeable to both protons and oxygen. The same matrix was used in a dual sensor for pH and oxygen with optical fibers.[71] Here, carboxyfluorescein was covalently immobilized on poly(hydroxyethyl methacrylate) particles. Oxygen was probed with Ru(dpp) (Table 1) dissolved in particles made from organically modified silica. The sensor cocktails were then deposited at the tip of an optical fiber.

The signals of the probes for pH and oxygen in most cases are interrogated *via* measurements of fluorescence intensity (rather than lifetime).[80,81] This is rather easy to perform but prone to errors due to photobleaching, leaching, and instrumental drifts. As most of the common pH indicators are organic fluorophores, determination of their fluorescence lifetime (which yields a self-referenced signal by ratioing the weighed contributions of the acid form and the conjugated base form) is an alternative, but the determination of lifetimes in the ns time regime is cumbersome and involves more sophisticated instrumentation than in the case of probes that have decay times in the order of micro- to milli-seconds. In the case of sensing pH (where pH indicators do exist in the acidic and conjugate base forms), ratiometric (*i.e.* 2-wavelength) measurement of absorbance (or fluorescence intensity) is the method of choice and gives a well-referenced signal.[82] One alternative consists in the use of pH-sensitive luminescent metal–ligand complexes that exhibit luminescence decay times in the microsecond time regime.[83,84]

2.2.4 Dual sensing of pH and temperature. Surprisingly, there are hardly any optical dual sensor systems for sensing pH values and temperature simultaneously. Probes for sensing pH usually have bands for the acid form and the conjugate base form and thus cover a good fraction of the visible spectrum, this making signal separation in the presence of other signals (in dual sensors) rather difficult. However, the Eu(III) diketonates with their narrow bands were found[85] to give temperature dependent signals that can be well separated from the signals of a pH probe, and thus could be used in a luminescent pH/T sensor. In another approach,[86] a polymer was used that changes colour with changing temperature and/or changing pH. The polymer is based on a combination of a pH-sensitive and solvatochromic azo dye with a thermo-responsive polymer. On exceeding a certain temperature (the so-called lower critical solution temperature), the polymer begins to precipitate. As a result, the absorbance of the dye changes due to the different polarity of the environment. Thus, pH and temperature can be sensed by means of colour changes of the system.

2.2.5 Triple sensors for pH, oxygen and temperature. Optical triple sensors are rather scarce. The major challenge when designing such systems is to find probes for three different analytes that can be excited at the same wavelength, but whose signals can be entirely separated. Two approaches have been described[87] for simultaneous determination of pH, temperature, and oxygen partial pressure. In the first, a material is used that is composed of poly(hydroxyethyl methacrylate) particles dyed with 8-hydroxypyrenetrisulfonate (for pH), poly(vinyl chloride) particles dyed with a Eu(III) probe (for temperature), and poly(styrene-*co*-acrylonitrile) particles dyed with PtTFPL (for oxygen). The three kinds of fluorescent particles (all photo-excitable at 405 nm) are contained in a hydrogel binder that is spread as a thin (10–20 μm) sensor film. The signals can be completely separated by optical emission filters.

In the second system,[87] the porpholactone oxygen probe was replaced by PtTFPP. Its emission overlaps the emission of the temperature probe, but both signals can be distinguished by making fluorescence measurements in the time domain because the two MLCs have highly different luminescence decay times (also see section 3.2.3.).

2.2.6 Triple sensors for glucose, oxygen and temperature. A dual fibre optic sensor for glucose and oxygen was described by Li and Walt.[80] The addition of temperature as a third parameter is easily conceivable. Following the design[62] of a dual sensor for oxygen and temperature that enabled the monitoring of an enzymatic reaction even at varying temperature it became obvious that the triple sensor described in 2.2.5. can be converted into a triple sensor for glucose, temperature and pH, respectively, by incorporating the enzyme glucose oxidase into the hydrogel binder. The enzyme is well retained by this matrix after treatment with the cross-linker glutaraldehyde. The lifetime-based triple sensor ($pH/O_2/T$) described above was used to report the consumption of oxygen that is caused by the presence of glucose according to

$$\text{glucose} + O_2 + H_2O \rightarrow \text{gluconolactone} + H_2O_2$$

A cross section of the triple sensor is shown in Fig. 4. It is primarily intended for sensing glucose and to indicate any deviations of pH (which adversely affect response and the calibration graph) and changes in temperature (which have the same effect). The hydrogen peroxide formed is harmful to the probes used but can be efficiently destroyed by adding traces of catalase to the polymer matrix. The dual and triple sensor systems for oxygen, temperature, pH and carbon dioxide as described in the literature so far are summarized in Table 4.

3. Spectroscopic methods of interrogation

Multiple and dual optical chemical sensors/sensing (OCS) require/s sophisticated methods of interrogation which are derived from interrogation of single OCS. There are various methods and techniques to monitor and evaluate the response of a fluorescent sensor. The easiest and seemingly least complicated technique is to measure the steady-state luminescence intensity of the respective probes. However, this method is prone to errors in that absolute values are hardly reproducible, and data obtained with two different instruments or setups may not be consistent. This can be fatal in case of industrial or medical applications. These drawbacks can be overcome by determination of luminescence lifetimes or other self-referenced parameters.[89] Several approaches and techniques are established. They have their merits and drawbacks which are listed

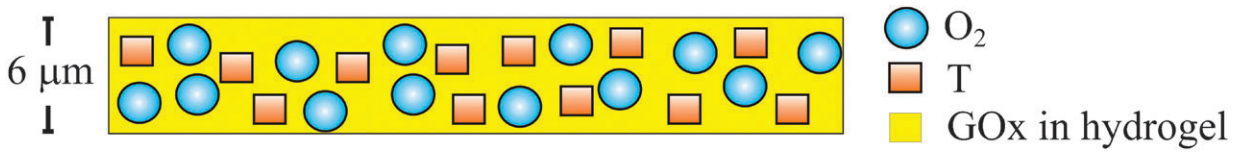


Fig. 4 Cross-section of a triple sensor for glucose, temperature and pH. The sensor layer consists of a polyurethane hydrogel into which luminescent sensor beads ("stimuli-responsive beads"; typically 3 µm in diameter) for temperature (T) and oxygen (O_2) were incorporated along with cross-linked glucose oxidase (GOx). The size of the beads is 3 ± 1 µm. The yellow coloration of the hydrogel matrix symbolizes the presence of GOx.

Table 4 A selection of materials as used in dual sensor or triple sensor systems. For chemical structures and acronyms of the probes and polymers see Tables 1–3

Parameter and probe/polymer combination					
O_2	T	pH	CO_2	Matrix/binder	Ref.
PtTFPP/PSAN[a]	Eu(tta)$_3$(dpbt)/PVC[a]	—	—	Hydrogel	88
PtTFPP in PS	Eu(tta)$_3$(dpbt) in PVMK	—	—	Two-layer	64
PdTFPP/PSAN[a] in hydrogel	Eu(tta)$_3$(dpbt) in PVMK	—	—	Two-layer	62
PdTFPP/PSAN[a]	Ru(phen)/PAN[a]	—	—	Hydrogel	63
PtTFPP	Ru(phen)/PAN[a]	—	—	Poly(^{t}BS-*co*-TFEM)	88
PtTFPL	MgTFPP	—	—	FIB	53
PtTFPL	EuD_2	—	—	FIB	45
PtTFPP	SiOEP	—	—	FIB	79
PtTFPP	$La_2O_2S:Eu^{3+}$	—	—	FIB	35
PtTFPL/PSAN[a]	Eu(tta)$_3$(dpbt)/PVC[a]	HPTS/pHEMA[a]	—	Hydrogel	87
PtTFPP/PSAN[a]	Eu(tta)$_3$(dpbt)/PVC[a]	HPTS/pHEMA[a]	—	Hydrogel	87
PtTFPP	—	DHFA	—	Hydrogel	72
Ru(dpp)/ormosil[a]	—	Carboxyfluorescein/ pHEMA[a]	—	Hydrogel	71
Ru(bpy)/silicagel beads in silicone[a]	—	—	HPTS/cellulose[a] in hydrogel	Two-layer	68
PtTFPP in PS	—	—	HPTS/cellulose[a] in PDMS	Two-layer	74
Ru(dpp)	—	—	HPTS	Ethyl cellulose	77

[a] Particle system.

and discussed in the following sections. The main parameters (intensity and lifetime) are compared in more detail in Table 5.

3.1 Measurement of fluorescence intensity

In the simplest case, the luminescence of a probe or label is photo-excited continuously and the resulting intensity is measured.[90,91] In the case of multiple OCS, the intensity method requires the use of optical probes whose signals can be differentiated by their emission wavelength. Unfortunately, luminescence intensity is not only dependent on the concentration of the analyte of interest, but also is affected by other parameters. This becomes apparent when looking at Parker's law:

$$I_L = I_0 \cdot \phi \cdot k \cdot \varepsilon \cdot l \cdot c \quad (1)$$

where I_L is the intensity of luminescence, I_0 the intensity of the exciting light, ϕ the quantum yield of the fluorophore, k a geometrical parameter characteristic of the experimental setup, ε the absorption coefficient of the fluorophore, l the length (or thickness) of the (evenly) penetrated layer, and c the concentration of the fluorophore in the medium, all at a given temperature.

As can be seen from this law, luminescence intensity is directly proportional to the intensity of the excitation light. On the one hand, this is advantageous because very high luminescence intensities can be achieved by making use of strong light sources like lasers. On the other, small inhomogeneities and deviations in the excitation light field will cause local fluctuations, particularly in the case of imaging non-planar surfaces such as those of organs, skin, or aircraft. Moreover, light intensity is a function of distance. Intensity drops by a factor of (distance)$^{-2}$. This effect holds for both the exciting light and emitted light, and is particularly serious in imaging again. Intensity data also will be falsified by local variations in the concentration of a fluorophore.

Time-resolved fluorometry also measures luminescence intensity, but only after a certain delay after having turned off the light source, typically several microseconds. This method (sometimes referred to as gated fluorometry) enables short-lived background luminescence to efficiently being eliminated, but of course requires probes with longer lifetimes, typically 5 to 10 times the gating time.

3.2 Measurement of luminescence lifetime

The measurement of fluorescence lifetime is superior to the intensity-based approach because it is not affected by scattering, reflection, the intensity of the excitation light field, or by inhomogeneous distribution of the indicator, at least in a first approximation. Lifetime can be measured in the time domain or the frequency domain.[92] In the frequency domain approach,

Table 5 Advantages and disadvantages of fluorometric sensing based on either intensity or lifetime

Fluorescence	Advantages	Disadvantages
Intensity	High intensity in the case of strong light sources; instrumentally simple; inexpensive; numerous indicators available	Dependent on the intensity of the light source; very homogeneous light field required in the case of imaging; not a self-referenced method
Lifetime	Independent of the intensity of the excitation light; self-referenced; widely independent of the optical setup;	More complex experimental setup; complex data acquisition; limited number of indicators available that are possessing long (> 1 μs) lifetime

the luminophore is excited by sinusoidally modulated light. The luminescence of the probe follows the excitation frequency with a lifetime-dependent delay. This phase shift $\Delta\phi$ is measured. It shall be recalled here that semiconductor components (such as LEDs, diode lasers, and photodiodes) are by far preferred in practical sensing for reasons of costs and size. Probes requiring UV excitation (such as the many pyrenes reported in the literature) are unlikely to be accepted in practice because (a) bright UV light sources are considerably more expensive, and (b) UV light often induces strong background luminescence.

Time domain fluorometry requires a square-shaped light pulse to excite the fluorophore. Luminescence rises and drops with a typical characteristic that is dependent on the luminescence decay time of the indicator. In principle, the two methods can be transferred into each other by Fourier transformation. The setup for detecting lifetimes in the time domain is less complex and less expensive compared to the frequency domain approach. Time-domain methods also may be coupled to gated measurements, with the additional benefit of eliminating short-lived background fluorescence. In the case of dual sensing, the method of dual lifetime determination (DLD) makes the need for spectral separation of signals superfluous.

3.2.1 Dual lifetime referencing (DLR). This is a relatively simple method to convert fluorescence intensity data into referenced data, for example a phase shift or a ratio of two intensities. This is particularly useful in the case of probes with decay times of <100 ns. In the DLR scheme, a long decaying reference luminophore is added to the probe. Both the probe and the indicator have to be excitable at the same wavelength and have to display overlapping emission spectra. In the time domain scheme (Fig. 5a), the first gate (A_1) is located in the excitation phase of the system, detecting the overall mixed intensity of both fluorophores. The second gate (A_2) is opened in the emission phase of the system with an appropriate delay after the excitation pulse, *i.e.* at the time when the luminescence of the indicator (Fig. 5a) has already decayed. The two signals are then used to generate a decay-dependent parameter R (in most cases the ratio between the intensities in the two gates) which serves as the analytical information.

The DLR scheme also works in the frequency domain (see Fig. 5b). However, the discrete signals of the single fluorophores cannot be distinguished. The total signal is composed of contributions by the reference luminophore (which is constant), and the variable contribution by the indicator. Depending on the intensity of the latter, the phase shift varies. The phase shift ($\Delta\phi$) therefore also decreases with the decrease in luminescence intensity of the indicator. One example for this approach is the referencing of the signal of HPTS as a carbon dioxide probe against an analyte-independent signal of a long-decaying iridium(III) metal ligand complex.[74]

The sensitivity of DLR to any kind of background luminescence, no matter whether conducted in the frequency domain or time domain makes this technique less applicable to samples like tissue or blood with their strong intrinsic fluorescence. DLR also suffers from effects of photobleaching: if one of the fluorophores (probe or reference dye) bleaches stronger than the other, the mixed intensity will falsify the parameters R and $\Delta\phi$, respectively.[93,94] Other than that, the DLR is a superb method to obtain referenced (*i.e.* more reliable) data.

One distinct (and highly welcome) feature results from the fact that DLR is applicable to all the numerous probes that have rather short (ns) fluorescence lifetimes because now they can be interrogated with pulses (or modulation frequencies) in the easily accessible kHz domain.

3.2.2 Rapid lifetime determination (RLD). The RLD method is a time-resolved technique where intensity data are used to calculate lifetimes. Luminescence is detected in two different gates, both located in the emission phase of the indicator (A_1 and A_2, see Fig. 6a) after a square-shaped excitation pulse has been applied. The lifetime-dependent ratio of the two intensity images represents the intrinsically referenced sensor response which is thus empirically calibrated against the analyte concentration. The lifetime τ of the luminescence can be calculated according to:

$$\tau = \frac{t_2 - t_1}{\ln \frac{A_1}{A_2}} \qquad (2)$$

where τ is the lifetime, t_1 and t_2 are the times when the different gates are opened (relative to the end of the excitation pulse), and A_1 and A_2 the intensities gathered in the two gates, respectively. In other words, the lifetime-dependent ratio of the two intensity images represents the intrinsically referenced response of the sensor system. It is often empirically determined.[93,95] For calculating the lifetime according to eqn (2), the two time windows A_1 and A_2 have to have the same length. True values for the decay time can only be obtained if the decay profiles are mono-exponential.[27,96] However, for the majority of applications it is often adequate to work with such data even if decays are multi-exponential.[97,98]

RLD is hardly prone to interferences because the ratio of the two areas A_1/A_2 (and luminescence decay time in general) is independent of the intensity of the exciting light and drifts in the sensitivity of the photodetectors. On account of this, effects of inhomogeneous indicator distribution within the sensor layer, coloration, turbidity, reflections, variations in the opto-electronic system, background fluorescence, and varying distance between surface and the detector also do not

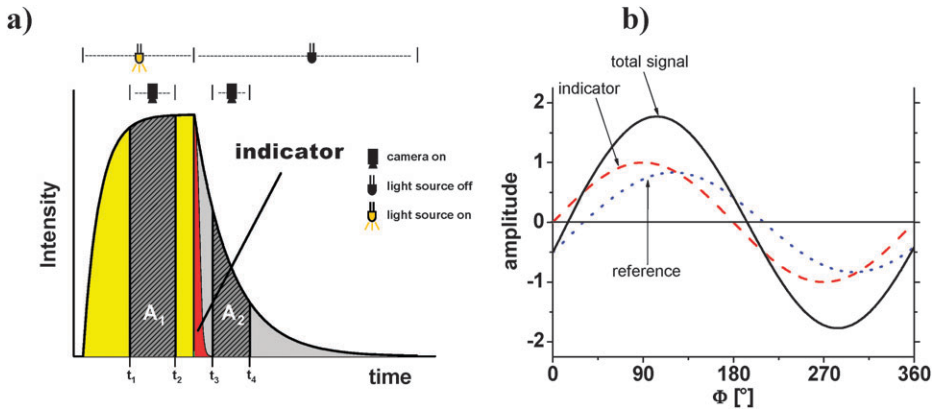


Fig. 5 Schematic drawing of the Dual Lifetime Referencing technique in (a) the time-domain, and (b) the frequency domain.

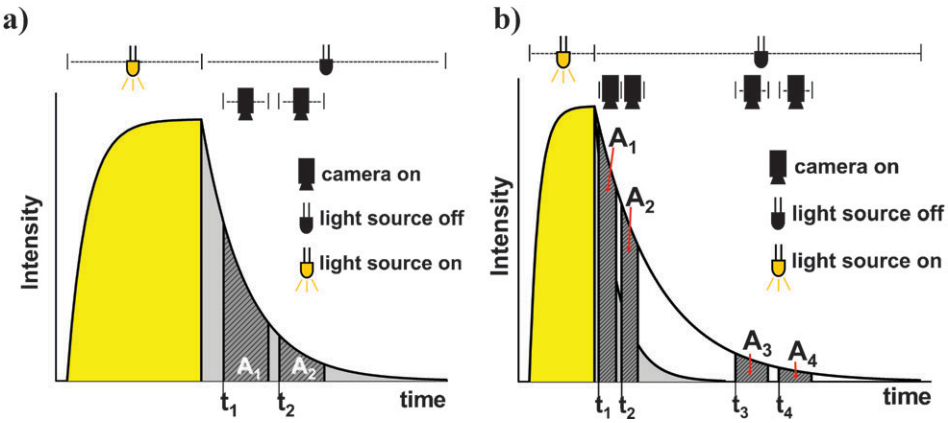


Fig. 6 Schematic presentation of (a) the Rapid Lifetime Determination method and (b) the Dual Lifetime Determination method.

adversely affect accuracy. The method even tolerates low levels of ambient light and displacements in the optical setup in the time period between calibration and actual measurements.[93,99] In contrast to the DLR method, the rapid lifetime determination is not affected by effects of photobleaching. Even if the fluorophore has lost *e.g.* 50% of its luminescence intensity due to bleaching, the relative intensities in the two gates A_1 and A_2 are reduced to the same extent. A final feature is its efficiency in gating out background luminescence.

3.2.3 Dual lifetime determination (DLD). In the majority of cases, the signals obtained with dually sensing paints are separated *via* spectral differences of the indicators, either by absorbance, excitation, or emission. However, the signal may be separated because of differences in the spectra and lifetimes, or of lifetimes only.[72,100] The DLD method is applicable if the decay time of one indicator is at least 10 times longer than the decay time of the other indicator. It has no analogy in the frequency domain.

In the DLD scheme, the intensities of four gates are taken in the emission phase of the luminophores, as shown in Fig. 6b. The first two gates (A_1 and A_2) have the same gate width and are opened at a time where both luminescences still are decaying, thus detecting a mixed intensity. The second two gates (A_3 and A_4) also have the same gate width (not necessarily the same as in the first two gates) and are positioned at a time when the luminescence of the first probe has decayed. They are exclusively acquiring the luminescence of the long-decaying indicator. The first two windows (acquiring the mixed emission) of course do not reflect the true decay time of the first indicator with its shorter lived emission. Rather, the results calculated from the first two gates according to eqn (2) are a quantity for relative changes in lifetime. The DLD method is a two-fold RLD method, capable of detecting the change in the lifetimes of two different indicators at the same time.[63,64]

4. Outlook

Multiple sensing based on the use of indicator probes is one of the highly attractive specific features of optical chemical sensing and imaging. It is not provided by any other method. While current research is mainly focused on dual sensors there is no reason not to assume that sensors for even more analytes can be envisioned. However, this will require substantial efforts in terms of new materials and spectroscopies. Probes with unusual spectral properties (such as narrow bandwidths or with decay times that can be easily differentiated from each other) are highly attractive. In that context, lanthanide-derived probes are particularly promising in view of their (a) almost line-like emissions (notwithstanding a number of weak side bands); (b) long (and highly different) decay times; and (c) highly variable colours.[101–103] Lanthanide ions, if incorporated in certain kinds of nanoparticles, also display the phenomenon of upconversion (the conversion of near infrared

light into visible luminescence).[104,105] Upconversion-based sensing additionally offers low autofluorescence, anti-Stokes signals, and high penetration depth and temporal resolution. This has led to exciting new aspects in terms of biosensing[106,107] and may lead to new routes in multiple sensing. For example, an optical pH sensor based on upconversion of 980-nm light was described quite recently.[108] It may be coupled to one of the known upconversion-based temperature sensors[109] to result in a fully temperature-compensated pH sensor. If combined with conventional (not upconvertible) probes, signals may be generated in two ways, *i.e.* by conventional photoexcitation and by upconversion so that the resulting signal can be spectrally separated by means of different excitation sources. One further option is to incorporate enzymes other than the ones described above to end up with a quite new generation of multiple biosensors.

5. Conclusion

Despite the large variety of established optical chemical sensors being available for single analytes, multiple sensing and imaging remains a challenging task. Seemingly trivial stipulations such as (a) the compatibility (and mutual solubility) of probes and polymers; (b) the need for unambiguously separating optical signals; (c) photostable, bright and specific probes; (d) compatibility with semiconductor opto-electronic components; (e) signal changes that are quite short and fully reversible; and (f) proper dynamic (analytical) ranges have not been fulfilled so far.

Other (but less "chemical") aspects include the miniaturization of the sensing system (for example if sample volumes are limited), and the adaption to either microfluidics or fiber optics technology.

Chemical sensing based on the determination of luminescence decay (in either the frequency or the time domain) outmatch conventional intensity measurements in many respects. High reproducibility, precision, and accuracy are typical features. Predictably, other spectroscopies known in fluorometry (including polarized emission, two-photon excitation, STED spectroscopy, fiber optic and distributed sensing, evanescent wave spectroscopy, and plasmonic excitation at metal interfaces) will be combined with existing schemes and further widen the technology of multiple sensing.

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