*Journal of Fluorescence, Vol. 15, No. 4, July 2005 (© 2005)*
DOI: 10.1007/s10895-005-2830-x

# Emerging Applications of Phosphorescent Metalloporphyrins

**Dmitri B. Papkovsky[1,2,3] and Tomás C. O'Riordan[1]**

*Received November 4, 2004; accepted March 7, 2005*

The subject of phosphorescent metalloporphyrins is reviewed, focusing mainly on the development and application of Pt- and Pd-porphyrins. A summary of their general chemical and photophysical properties, and guidelines for rational design of the phosphorescent labels, bioconjugates and probes is given. Examples of different detection formats and particular bioanalytical applications developed in recent years are presented. The potential of phosphorescent porphyrin label methodology is discussed and compared to that of the long-decay fluorescent lanthanide chelates and other common fluorophores.

**KEY WORDS:** Metalloporphyrins; labels; probes; phosphorescence; time-resolved fluorescence; bioanalytical applications.

## INTRODUCTION

Metalloporphyrins relate to a large family of compounds termed tetrapyrrols, which include porphyrins, chlorins, phthalocyanines, some other structures, and their metallocomplexes [1]. The parent compound, termed porphine, contains four pyrroles linked by methine bridges, making up an aromatic 16 membered ring with 18 $\pi$-electrons. Porphyrin molecules may be obtained by substitution of the porphine at any of the eight pyrrole exo hydrogens (numbered 1–8) or the methine hydrogens ($\alpha$, $\beta$, $\gamma$, $\delta$). Synthesis of porphyrins is usually achieved by condensation of substituted pyrrols, which produces symmetrical polyfunctional structures, and subsequent chemical modification and/or metallation [1]. Other members of the family may be synthesized from corresponding pyrrol analogs (e.g. benzoporphines, phthallocyanines), or by selective reduction or oxidation of one or two pyrrol rings of the macrocycle, while preserving its aromatic structure (chlorins, bacteriochlorins, porphyrin-ketones). Some porphyrin dyes can also be obtained from natural sources. Tetrapyrrols can coordinate transition metals producing metalloporphyrins, in which the macrocycle usually occupies four coordination sites of the central atom, while the remaining sites, if any, are available for axial ligands. The structures of representative porphyrin compounds are shown in Fig. 1. Early work (1930s–1960s) led to the synthesis and photophysical study of a large variety of porphyrin dyes and their metallocomplexes [1]. This was generally completed by the early 1970s, when a complete 'periodic table' of metalloporphyrins was described [2–9].

Early interest in porphyrin dyes was due to their involvement in functioning of living systems. Thus, naturally occurring chlorophyll (Mg(II)-chlorin), heme (Fe(II)-protoporphyrin IX), vitamin B-12 (Co(II)-corrin), are the key components in processes such as photosynthesis, electron transport, red-ox catalysis, oxygen transport in blood, etc. Therefore, research with porphyrin dyes

[1] Biochemistry Department/ABCRF, University College Cork, College Road, Cork, Ireland.
[2] Luxcel Biosciences Ltd., G17, Lee Maltings, Prospect Row, Cork, Ireland; www.luxcel.com.
[3] To whom correspondence should be addressed. E-mail: d.papkovsky@ucc.ie

ABBREVIATIONS: CP, coproporphyrin; EtioP, etioporphyrin; OEP, octaethylporphyrin; TPP, tetraphenylporphyrin; TFPP, tetra (pentafluorophenylporphyrin); OEPK, octaethylporphyrin ketone; OEPDK, octaethylporphyrin diketone; CPK, coproporphyrin ketone; TBP, tetrabenzoporphyrin; TCPP, tetra(carboxyphenyl)porphyrin; TRF, time-resolved fluorescence; MeCP, metallocoproporphyrin; CTAB, cetyl trimethyl ammonium bromide; Pt/Pd-NCS, Pt/Pd coproporphyrin isothiocyanate; PS, polystyrene; CYP450, cytochrome $P_{450}$.



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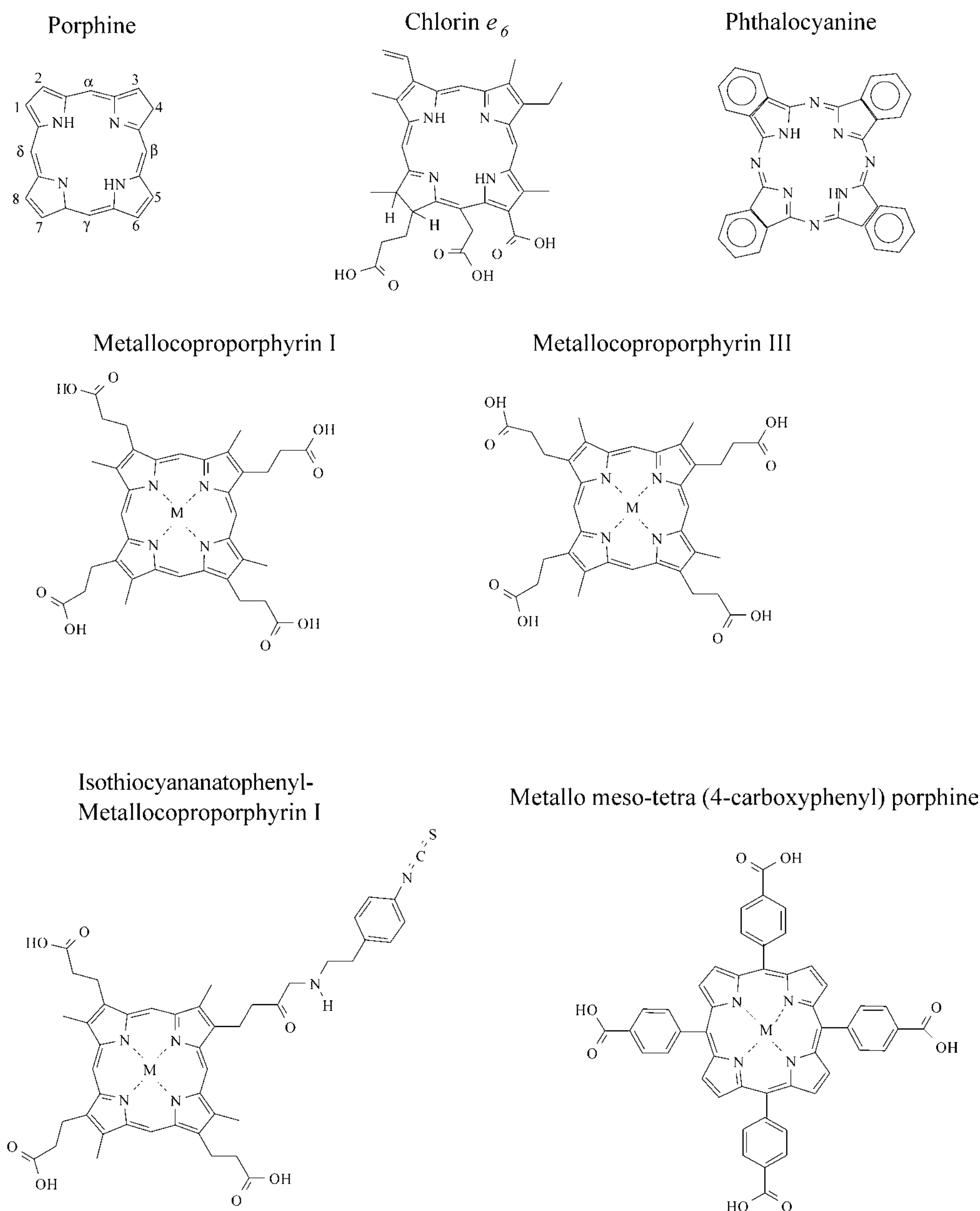


**Fig. 1.** Structures of some porphyrins and metalloporphyrins.

has been largely focused on the studies and mimicking of biological molecules and processes occurring in living organisms, with the view of their general understanding and reconstruction *in vitro*.

In recent years, porphyrins have been used in the development of new supramolecular structures, drugs, medical procedures (e.g. photodynamic therapy), and bioanalytical applications. The scope of this review is to detail the properties of *phosphorescent* metalloporphyrins and some related structures, and discuss the emerging applications in which they are used as highly sensitive, selective, and versatile labels or probes.

## PROPERTIES OF PHOSPHORESCENT METALLOPORPHYRINS

### Absorption Characteristics

Electronic spectra of metalloporphyrins originate from the aromatic tetrapyrrolic macrocycle. But the metal

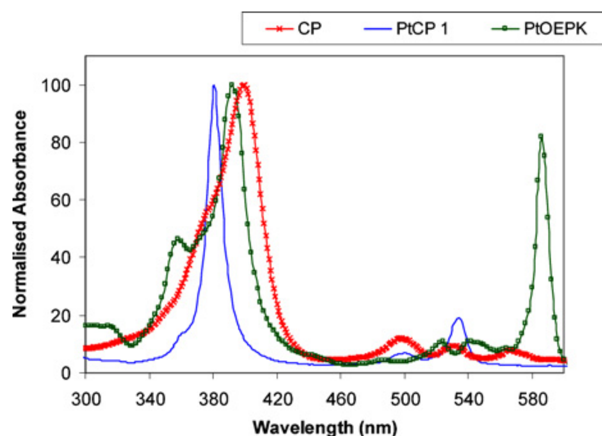


**Fig. 2.** Absorption spectra of CP-I free base (×), PtCP-I (–) measured in DMSO, and PtOEPK (□) measured in $CHCl_3$.

ion also plays a central role, due to 'heavy atom effect,' i.e. spin–orbit coupling between the $\pi$-electrons of the macrocycle and the electrons of the central atom [10,11]. UV-visible absorption of metalloporphyrins (Fig. 2) shows an intense band at approximately 400 nm, which is termed the Soret (also called B) band and which is a result of electronic excitation from the ground state to a vibrational level of the second excited singlet state ($S_0 \rightarrow S_2$). Two further absorbance bands (termed Q bands) can be observed between 500 and 600 nm. The lower energy Q (0,0) band results from promotion of ground state electrons to the lowest vibrational level of the first excited singlet state ($S_1$). The slightly higher energy Q(1,0) band possesses one moment of vibrational energy at the $S_1$ level. Further weak absorption bands, termed L, M and N, may be observed between 210 nm and the Soret band. These compounds display high absorptivity, with molar extinction coefficients ranging 1–5 × $10^5$ $M^{-1}$ $cm^{-1}$ at the Soret band [5].

Metalloporphyrins may be classified into *regular* and *irregular* on the basis of their electronic spectra. For *regular* metalloporphyrins (groups 1A–5A of the periodic table) absorption and emission spectra suffer only minor interference of the central atom on the $\pi$-electron system of the porphyrin macrocycle. As a result, their spectra are termed 'normal' due to close resemblance to the spectra of the metal-free porphyrin dication. In contrast, *irregular* metalloporphyrins are those whose absorption characteristics are considerably altered by the central atom, resulting in either *hypsochromic* (i.e. blue-shifted) or *hyperchromic* (red-shifted) absorption peaks or the appearance of prominent L and M absorbance bands at approximately 320 nm [5].

## Emission Characteristics

In terms of luminescent emission, metalloporphyrins may be categorized into those which display fluorescence, delayed fluorescence or phosphorescence [5]. Emission of prompt fluorescence from the second singlet state has been reported for a number of metallobenzporphyrins and metal phthalocyanines [12–14]; however, in the majority of cases, the molecule will undergo internal conversion to the first singlet state over a picosecond timescale. From here the molecule may emit light as prompt fluorescence or undergo intersystem crossing to the triplet state ($T_1$), from which it may emit light as phosphorescence or undergo re-excitation to the $S_1$ state in which case delayed fluorescence will be observed. The latter may occur from thermal repopulation, giving E-type delayed fluorescence, or from triplet–triplet collisions, giving P-type delayed fluorescence [15].

The efficiency of triplet state formation in porphyrins is generally high [5]. This is increased with the presence of a metal of higher molecular weight, which increases the probability of the molecule decaying to $S_0$ via the triplet state, i.e. emitting phosphorescence. Furthermore, this results in a decreased phosphorescence lifetime. *Regular* metalloporphyrins display normal absorption spectra indicating a negligible effect of the central metal substituent. In these cases, fluorescence is the dominant type of emission, like in metal-free porphyrins. Low levels of phosphorescence emission may be observed, but usually only at $-196$°C, i.e. when thermal desactivation pathways and various quenching processes are greatly reduced.

*Irregular* metalloporphyrins display a marked effect by the central atom on the electronic spectra, resulting in *hypsochromic* or *hyperchromic* shifts in absorption spectrum [5]. Many *hyperchromic* complexes, including Fe(II), Co(II), and Ni(II) undergo radiationless transition to the ground state producing non-luminescent compounds. However, a number of complexes displaying *hypsochromic spectra* including Pt(II) and Pd(II), and to a lesser extent, Rh(III), Ru(II), and Os(II) display phosphorescence at $-196$°C. For Pt(II)- and Pd(II)-porphyrins, the efficiency of triplet states formation and the balance between pathways for their deactivation are such that these dyes display strong phosphorescence [7] which is preserved at room temperatures and even above. The heavy atom effect can be illustrated by examining differences in photophysical characteristics between Pt(II) and Pd(II) complexes (see Fig. 3a and b). Complexes of normal substituted porphyrins with Pt(II) (MW = 195.23) have emission maxima at ∼650 nm, lifetime of ∼100 $\mu$s and a quantum yield of ∼20%, while for Pd(II) (MW = 106.7)

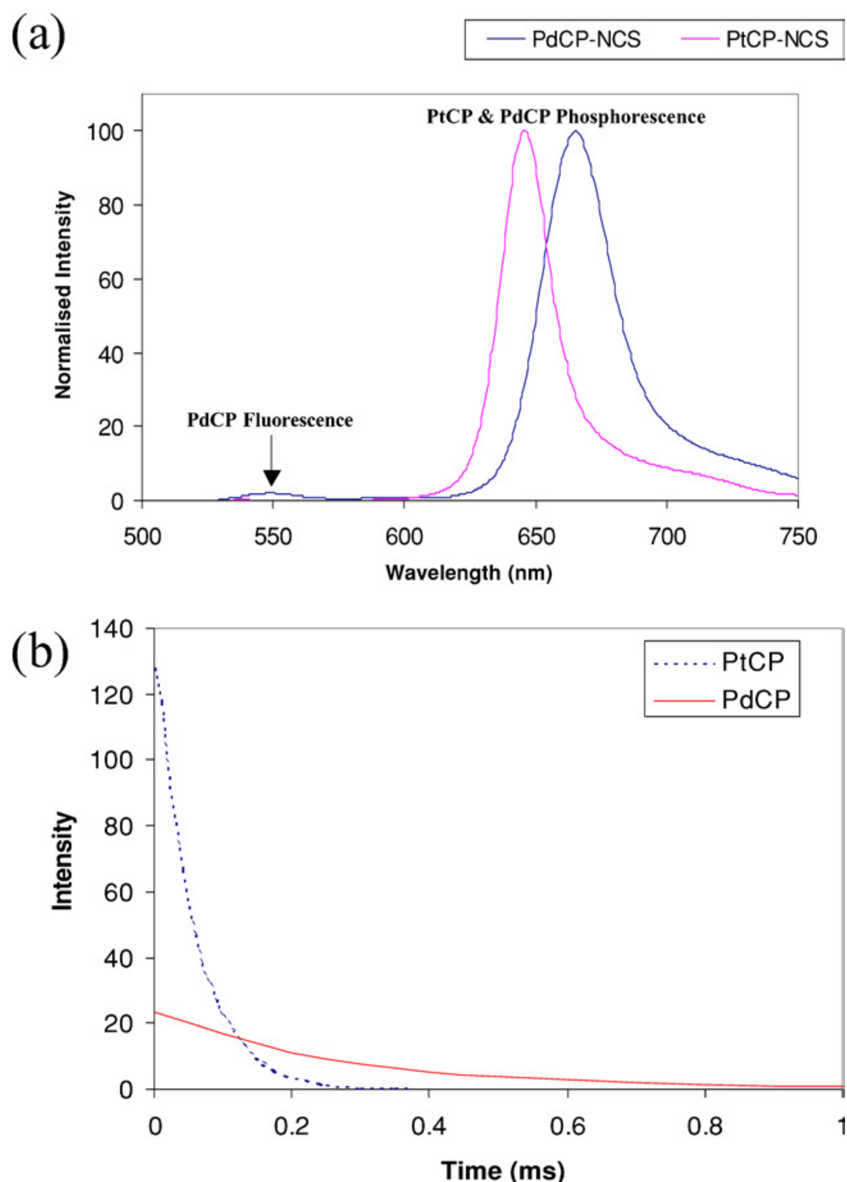


**Fig. 3.** (a) Normalized emission spectra of the PtCP and PdCP labels measured in deoxygenized solution of CTAB, and (b) phosphorescence decay curves of PtCP- and PdCP-labeled oligonucleotide probes (10 nM) measured at 381/648 and 396/668 nm (excitation/emission) respectively.

emission is at ~670 nm, lifetime ~900 $\mu$s and yield ~10% (see Fig. 3). The emission spectrum of PdCP also shows a small band at 548 nm which has been attributed to E-type delayed fluorescence, i.e. thermal repopulation of the $S_1$ state from the triplet state (see Fig. 3a) [9]. This effect is rare for Pt(II) complexes but has been shown in the Pt(II) complex of etioporphyrin I [9]. Finally, a number of Cu(II) porphyrins also display high levels of phosphorescence at low temperatures but completely lose this ability at increasing temperatures.

The reduction or oxidation of one or two pyrrols of the macrocycle (e.g. in chlorines, bacteriochlorins, or porphyrin-ketones) causes changes in electronic spectra, both in the metal-free forms and in metallocomplexes [16,17]. A gradual red-shifting and considerable enhancement of the absorption Q-band are observed, often with some reduction in intensity of the B-band. Such changes in absorption spectra of metalloporphyrin analogs are accompanied with greater red-shifting of their phosphorescence spectra and reduction of emission yields and

**Table I.** Phosphorescence Characteristics of Different Metalloporphyrins (at RT)

| Dyes | $\lambda_{abs}$ (nm) | $\lambda_{Phos}$ (nm) | $\phi_{phos}$ | Lifetime (ms) | References |
|---|---|---|---|---|---|
| Porphine[a] | 610 | 785 | 5.5 | 20.0 | [5] |
| Pt-EtioP[a] | 379 | 641 | 90 | 0.121 | [7] |
| Pt-OEP | 383 | 647 | 40 | 0.095 | [82] |
| Pt-CP | 380 | 646 | 54 | 0.090 | [79] |
| Pt-OEPK | 396 | 760 | 12 | 0.060 | [17] |
| Pt-CPK | 394 | 763 | 8 | 0.031 | [83] |
| Pt-OEPDK | 402 | 870 | 1.6 | | [16] |
| Pd-EtioP[a] | 389 | 660 | 50 | 1.93 | [7] |
| Pd-OEP | 395 | 670 | 20 | 990 | [83] |
| Pd-CP | 395 | 667 | 17 | 1.10 | [21,79] |
| Pd-OEPK | 408 | 789 | 1 | 0.455 | [17] |
| Pd-CPK | 406 | 793 | 0.8 | 0.237 | [83] |
| Pd-Chlorin[a] | 590 | 770 | 0.12 | 5,00 | [5] |
| Pd-OEPDK | 414 | 930 | 0.3 | | [16] |
| Pt-TPP | 400 | 671 | 19 | 0.055 | [84] |
| Pt-TFPP | 390 | 653 | 1.9 | 0.027 | [22,84] |
| Pd-TBP | 407 | 767 | 7.9 | 0.26 | [31] |
| Pd-TCPP | 416 | 687 | 6 | 0.69 | [30] |
| Zn-EtioP[a] | 388 | | 0.07 | 57 | [5] |

[a] Phosphorescence only observed at −196°C.

lifetimes [5,17]. Examples of these spectral effects are shown in Fig. 2. Similar changes are seen in conjugated tetrapyrrols such as tetrabenzoporphines, phthallocyanines, and in meso-substituted tetraphenylporphines, in which electronic interactions between the macrocycle and aromatic substituents result in more longwave absorption and emission bands, compared to normal metalloporphyrins.

A cross-section of different phosphorescent metalloporphyrins and their properties is given in Table I. The following sections of this review will be focused mainly on Pt- and Pd-porphyrins.

Pt- and Pd-porphyrins represent stable chemical compounds. Both Pt(II) and Pd(II) ions require planar coordination geometry, which is matched by the porphyrin macrocycle. These ions are coordinated very tightly making it is practically impossible under normal conditions to dissociate them from the macrocycle. These complexes withstand various solvents, buffers with extreme pH and ionic strength, the presence of mild reducing and oxidizing agents (strong reductants and oxidants can modify the macrocycle), and they are stable at high temperatures—up to 200°C and even higher. Furthermore, Pt(II) and Pd(II) do not tend to change their valency and coordination state in such complexes, and their tendency to bind axial ligands is very weak. Due to this, the photophysical and phosphorescent properties of Pt- and Pd-porphyrins are stable and predictable, as opposed to many other metalloporphyrins, for which dissociation of metal ion and exchange of axial ligands can often take place.

For Pd-porphyrins, excitation and emission spectra are slightly red-shifted (10–20 nm) with respect to corresponding Pt-porphyrins, and their phosphorescence lifetimes are approximately 10 times longer. Examples of the phosphorescence spectra and decay curves are shown in Fig. 3. Together with the other porphyrin analogs (meso-substituted porphyrins, porphyrin-ketones and benzoporphyrines), this pair gives a diverse selection of dyes with different spectral and kinetic properties of phosphorescence. The relatively sharp excitation and emission bands and absence of other types of emission (fluorescence is usually negligible) facilitate their spectral identification and sensitive phosphorescent detection using time and wavelength discrimination.

The phosphorescence of Pt- and Pd-porphyrins is conserved only when the dye molecules are in a monomeric form. Even metalloporphyrins with highly hydrophilic substituents tend to form dimers and aggregates in aqueous solution, a process which is dependant on concentration, temperature, pH, and ionic strength [18]. Aggregation can be seen as a ’blue shifting’ and a decrease in intensity of absorption bands [19,20], and isobestic points are often not seen due to process complexity. Aggregation can be reversed by the addition of surfactants such as Triton X-100 and cetyl trimethyl ammonium bromide (CTAB), which solubilise porphyrin molecules and thus restore true absorbance and emission [19]. Quenching of Pd- and Pt-porphyrins in aqueous solutions, including complex biological samples, is insignificant. Water, common salts, chemical and biological species including

proteins and nucleic acids were shown not to cause any major quenching, and the phosphorescence usually remains strong. Self-quenching in solutions can be seen for Pd- and Pt-porphyrins at high ($\geq\mu$M) concentrations, which is thought to be partially due to dynamic (collisional) quenching [21]. Temperature has relatively small impact on the phosphorescence, causing its gradual reduction.

Photochemical stability of metalloporphyrins is moderate and comparable to many other organic fluorophores. These dyes are generally stable under typical conditions of phosphorescence measurements. Some structures (e.g. chlorins—reduced porphyrins) are more unstable and susceptible to photodegradation. A number of porphyrin compounds have been produced which display enhanced photostability, so they can be used in conditions requiring high-intensity irradiation or prolonged exposure to light. These include metallocomplexes of TFPP, in which the pentafluorophenyl substituents were shown to increase the oxidation potential and reduce the electron density [22]. Also porphyrin-ketones (partly oxidized porphyrins) were shown to have higher photostability than corresponding porphyrins, and they are far more stable than chlorines, with whom they have close spectral similarity [17]. On the other hand, many porphyrins including Pd- and Pt complexes are known to be efficient sensitizers of singlet oxygen photogeneration [23]. This property is actively exploited in photodynamic therapy [24], but it can also result in photodamage of the porphyrin dyes causing their photo-oxidation and bleaching.

## PHOSPHORESCENT MATERIALS

### Oxygen-Sensitive Materials

Pt- and Pd-porphyrins have been actively exploited in quenched-phosphorescence sensing of oxygen. Their excited triplet states are effectively quenched by molecular (triplet) oxygen, both in solutions and in solid-state oxygen-permeable materials. Pt(II) porphyrins, with lifetimes in the order of 50–100 $\mu$s, are only moderately quenched by ambient oxygen, which makes them very suitable for oxygen sensing in physiological range [17]. Pd(II) porphyrins with approximately 10 times longer lifetimes are more suited for measuring low oxygen levels [25].

Dynamic quenching by oxygen is a non-chemical collisional process, which is described by the Stern–Volmer equation [26]:

$$I_0/I = \tau_0/\tau = 1 + k_q\tau_0[Q] \quad (1)$$

where $I_0$, $I$, and $\tau_0$, $\tau$ are the intensities and lifetimes in the absence and presence of quencher [Q], and $k_q$ is the bimolecular quenching rate constant. By optimizing combination of the phosphorescent dye and quenching medium (i.e. parameters $\tau_0$ and $k_q$), one can develop efficient oxygen-sensitive probes, and tune their sensitivity to the desired oxygen range.

Solid-state polymeric materials used in conjunction with hydrophobic Pt-porphyrins have been particularly successful in optical oxygen sensing, due to their long-wave spectral characteristics, optimal sensitivity at ambient oxygen range, compatibility with common polymers and with LED/laser excitation. Different types of oxygen sensors, e.g. in the form of thin film coatings, membranes, fiber-optic probes and inserts have been described. Pt(II)- and Pd(II) complexes of OEPK, TFPP, as well as OEP, TPP, TBP embedded in polystyrene, polysulfone, PVC, some other polymers have been used [27], which have appropriate permeability for molecular oxygen and which act as good quenching media. PtOEPK-PS and PtTFPP-PS composites are among the most commonly used solid-state oxygen-sensitive luminescent materials, as they display convenient spectral characteristics and good photostability. Using polymeric materials loaded with metalloporphyrins (up to 5% w/w), one can eliminate self-quenching effects and achieve high phosphorescent signals, and reduce possible cross-sensitivity effects. Such materials are currently well established and compete successfully with the other oxygen-sensitive materials based on fluorescent complexes of ruthenium(II) and osmium(II), and polycyclic aromatics [27,28].

Hydrophilic Pt- and Pd-porphyrins are moderately quenched by oxygen in aqueous solutions, a fact which can be exploited for oxygen sensing in biological samples [21]. However, the use of free metalloporphyrins as oxygen sensors and probes is somewhat difficult, due to their considerable hydrophobicity and tendency to aggregate in aqueous solutions (described earlier). These undesirable effects can be partly overcome by complexing the dyes with hydrophilic macromolecules, such as polypeptides, polysaccharides or synthetic polymers. Thus, albumin, the key carrier protein in serum, and other proteins, are known to bind porphyrins with relatively high affinities ($\mu$M–nM range), mainly due to hydrophobic interactions [21]. Such binding can help solubilise even hydrophobic metalloporphyrins and keep them in monomeric form in aqueous media. In complexes with macromolecular carriers, metalloporphyrins retain strong phosphorescence and sensitivity to dissolved oxygen, while self-quenching is considerably reduced. The dyes used in such systems include Pd-meso-tetra-(4-sulfonatophenyl)-porphine [21], Pd-coproporphyrin

[21,29], Pd-meso-tetra-(4-carboxyphenyl)porphine [30], and sulfonated Pd-tetrabenzoporphine [31]. However, the limitations of such oxygen probes include variable and unstable chemical composition and the possibility of migration of the dye to other sample components (e.g. cells, surfaces, other proteins). Considerable heterogeneity in photophysical properties and sensitivity to oxygen has been reported for these oxygen-sensitive materials [21].

Many of these issues can be addressed by using conjugated water-soluble oxygen probes, in which the oxygen-sensitive metalloporphyrin molecules are covalently bound to a macromolecular carrier. Optimization of the dye, carrier, conjugation chemistry, composition, and choice of the dye attachment site for the resulting conjugate allows one to achieve fine tuning of the sensing characteristics and phosphorescent properties of such probes [32].

## Phosphorescent Labels and Bioconjugates

Luminescent dyes are widely used either as labels or in the form of bioconjugates. For use on a large scale, corresponding labeling reagents and procedures should produce conjugates with a well-defined chemical composition, optimal phosphorescent characteristics and preserved biological functionality and stability. The subsequent application should be a simple, reproducible, cost-efficient procedure, which is compatible with widely accepted assay formats, detection, and liquid handling instrumentation.

The use of phosphorescent metalloporphyrins in bioassays requires their conjugation to biomolecules such as proteins, peptides, and oligonucleotides. The conjugation of Pt- and Pd-porphyrins to other molecules, and particularly to hydrophilic biopolymers such as proteins and nucleic acids has proven somewhat difficult. This was due to the initial lack of suitable hydrophilic derivatives for labeling in aqueous solution (preferred medium for biomolecules). Many synthetic porphyrins are too hydrophobic, while those which are suitable as labels, such as Pd- and Pt complexes of polycarboxylic porphyrins or sulfonated TPPs, have suffered from the lack of convenient and stable labeling chemistries, and from their polyfunctionality.

### *Conjugation to Proteins*

Early attempts to obtain phosphorescent conjugates with proteins (antibodies) were made with water-soluble metallocomplexes of tetracarboxylic coproporphyrins. Pd-coproporphyrin-I was used in a multi-step method (carbodiimide-mediated condensation), to achieve linkage of the carboxylic groups of porphyrin to amino groups on protein molecules [33]. Improvements to this labeling scheme were later made using carbodiimide to activate a tetracarboxyl porphyrin (either coproporphyrin or tetra(4-carboxyphenyl)porphyrin in the presence of an active ester forming alcohol such as *N*-hydroxysuccinimide or *N*-hydroxybenzotriazole to produce activated porphyrin molecules [34–36]. The drawback of these systems was poor reproducibility of the conjugation process (due to the instability of reactive intermediates and limited storage stability), partial crosslinking of protein (due to the use of polyfunctional porphyrin reagent), and production of bioconjugates with compromised activity (due to protein inactivation and aggregation during labeling) [35]. Despite these drawbacks, these phosphorescent protein conjugates have been used with certain success in several assay formats, where they have demonstrated excellent sensitivity, potential for new applications but also the need for further improvement. It was shown that metallocomplexes of coproporphyrins are more optimal labels than those of tetra(4-carboxyphenyl)porphine, as the former are less damaging to the activity and stability properties of labeled proteins [34]. This can be attributed to the higher hydrophilicity and smaller size of coproporphyrins, making them preferred structures for further label design.

The synthesis of monofunctional derivatives of Pt(II)- and Pd(II)-coproporphyrins with a single isothiocyanatophenyl moiety has been described recently [37–39], with claims to overcome the problems associated with the conjugation schemes described previously. Reactive water-soluble derivatives of MeCP bearing single *p*-isothiocyanatophenyl moiety (PtCP-NCS and PdCP-NCS, see Fig. 1) enabled facile site-specific labeling of biomolecules via their primary amino groups in one step under mild conditions in aqueous solutions. These structures were optimized with respect to linker arm to conjugates displaying optimal activity and photophysical characteristics [40].

These reagents enabled synthesis of phosphorescent bioconjugates with different biomolecular structures. Their initial application was in labeling of polypeptides, and conjugates with antibodies, avidin [41], serum albumin [32] were produced. All these conjugates displayed excellent performance including high emission efficiency, uncompromised sensitivity of detection by time-resolved fluorescence (TRF) (down to ∼0.1 pM), consistent yields, chemical composition, good functional activity and storage stability [42].

However, in protein conjugates of PdCP and PtCP synthesized by the aforementioned methods, a

considerable degree of internal quenching of the label by polypeptide carrier was observed, causing a ∼3–4-fold reduction of yield and ∼20% reduction in phosphorescent lifetime compared to the free label in solution [42]. The exact origin of this quenching is still unclear, but it can be eliminated by using surfactants solubilising MeCP label, such as CTAB or Triton X-100 [42], or potentially by using other conjugation chemistries.

### *Conjugation to Oligo- and Mononucleotides*

Metalloporphyrin conjugates of nucleic acids (including mono-, oligo-nucleotides and large fragments of DNA/RNA) display high potential for detection schemes in molecular biology. As with protein labeling, carbodiimide conjugation chemistry was initially employed for labeling of oligonucleotide sequences for imaging applications [34,43,44]. The drawbacks of these conjugation schemes have been overcome by the development of the stable monofunctional reagents described previously. Optimized procedures for labeling of synthetic oligonucleotides with MeCP-NCS have recently been developed [45], which allowed preparation of phosphorescent oligonucleotides with high yields, purity, and homogeneity. Various oligonucleotides, bearing 5′-, 3′- or an internal amino-modification, were single and dual-labeled with MeCP-NCS, and underwent a detailed spectroscopic investigation. In such conjugates, the MeCP label was shown to be practically unquenched, retaining high emission efficiency.

More recently, labeling of nucleotide bases (aminoallyl-dUTP) with MeCP-NCS and procedures for incorporation of MeCP labels into large DNA fragments and in a polymerase chain reaction (PCR) have been developed [46], while several other conjugates, including biotin-PtCP [41] have been described. Furthermore, monofunctionalised maleimide derivatives (reactive with thio groups) and *b*-cyanoethylphosphoramidates of MeCP (for direct labeling of oligonucleotides in solid-phase DNA synthesis) have been synthesized, which are currently under evaluation in our laboratory. Development of the phosphorescent oligopeptide probes for use in enzymatic assays is also underway.

It is clear that many of the problems associated with conjugation of the porphyrin dyes to various biomolecules have been successfully solved or addressed in recent years. A number of generic phosphorescent labeling reagents, bioconjugates, procedures for their preparation, purification and subsequent use have been developed and optimized, in accordance with basic specifications and requirements of particular assay formats and applications for which they were designed.

## DETECTION OF METALLOPORPHYRIN PHOSPHORESCENCE

Strong longwave long-decay emission of Pt- and Pd-porphyrins and corresponding materials and bioconjugates, facilitate their sensitive and selective detection by fluorescent spectroscopy. Different detection formats can be used, and the choice of optimal detection system is usually driven by the application, type of the phosphorescent material, bioconjugate or probe used, and practical requirements.

Steady-state phosphorescence intensity measurements of MeCP labels in solution under continuous excitation usually provide decent (sub-nM) sensitivity, which is quite comparable to the other conventional fluorophors such as FITC and Cy5. The sensitivity is limited by optical interferences (light scattering, autofluorescence, sample effects, etc.), causing relatively high and variable background signals. Standard monochromator or filter-based fluorescent spectrometers, microplate readers and imagers equipped with a flash-lamp or laser as excitation source and an appropriate red-sensitive photodetector, can be used. For the porphyrins emitting at longer wavelengths (see Table I), the use of PMT-based spectrometers can be problematic, as the latter have considerably reduced sensitivity at above 700 nm.

These limitations of steady-state phosphorescence detection of Pt- and Pd-porphyrin labels can be eliminated by using TRF detection with pulsed excitation and microsecond time resolution. Time-resolved fluorescence has been shown to provide radical improvement in sensitivity and S:N ratio for the long-decay luminescent labels such as fluorescent lanthanide chelate and cryptate [47], and for the phosphorescent MeCP labels [42]. A number of commercial TRF instruments have become available, which can be used without any major modifications for sensitive detection of MeCP labels. Standard TRF plate readers, such as the Victor$^2$ (PerkinElmer Life Sciences), equipped with xenon flash-lamp and a set of samarium filters (340/642 nm) allows measurement of PtCP label within a broad dynamic range, with sensitivity of 0.1–1 pM for small samples (Fig. 5). A number of other fluorescent readers optimized for TRF detection of lanthanide labels are also quite appropriate for use with MeCP labels (e.g. selected models from BMG, Tecan).

A dedicated ArcDia® reader has recently been developed by Luxcel Biosciences Ltd. (Cork, Ireland) for sensitive TRF detection of MeCP labels [41]. It uses a simple low-cost optical setup with a solid-state green laser (532 nm) operating in a pulsed mode (pulses ∼1 $\mu$s and above), a photon-counting detector, fast signal processing optoelectronics allowing measurement of steady-state

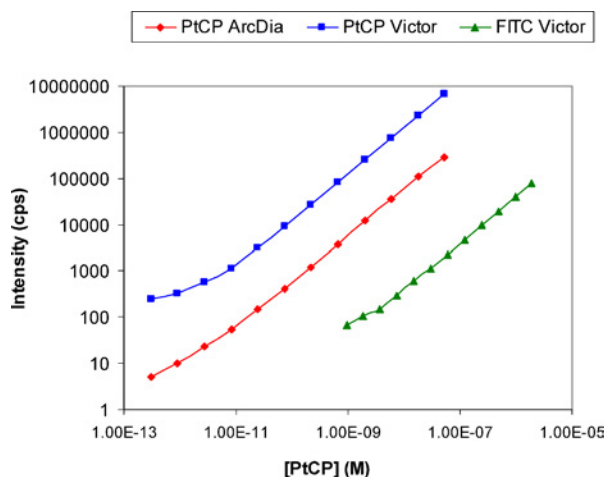


**Fig. 4.** Measurement of PtCP label on two time-resolved fluorescence plate readers, Victor$^2$ (340/642 nm) and ArcDia (532/650 nm), using delay time 50 $\mu$s, gate time 100 $\mu$s, 100 $\mu$l samples in a black 96-well plate, and comparison with FITC label measured on Victor$^2$ in steady-state mode using FITC filter set (488/530 nm).

and time-resolved phosphorescence (minimal delay time of 1 $\mu$s), and also emission decay (multichannel scaler with 0.05 $\mu$s bins). Its mechanical block with a *X–Y* stage allows rapid measurement of multiple samples in microplates, slides, gels, other substrates, and has capabilities for two-dimensional scanning. This versatile instrument provides superior performance, particularly for PtCP for which the laser matches the longwave absorption band. It achieves background-free TRF detection with sensitivity of ~0.1 pM for PtCP labels, and slightly lower but still comparable sensitivity for PdCP label, in various formats and applications. A comparison of different labels and instruments is shown in Fig. 4.

To attain maximal sensitivity of MeCP labels, it is important to eliminate possible quenching interferences, particularly quenching by oxygen. The presence of oxygen (~200 $\mu$M in air-saturated solutions or 21 kPa in air) will considerably reduce the phosphorescence (2–10-fold for PtCP and much greater for PdCP), thus compromising sensitivity. This can be avoided (if required) by rather simple means. The most common method is to use deoxygenators such as sodium sulfite or glucose/glucose oxidase as additives in common biological buffers [48], which provide rapid, effective, and continuous removal of dissolved oxygen from aqueous samples over long periods (hours and even days). These reagents are mild, convenient to use and biocompatible, they do not interfere with many biological samples (e.g. antigen-antibody and ligand–receptors binding, DNA hybridization, even whole cells). Another possibility is to degas samples or apply phosphorescent detection from dry surfaces (surface-enhanced phosphorescence).

For the samples producing relatively high levels of phosphorescence, removal of oxygen is not necessary and also less sophisticated and more robust detectors can be used. For solid-state materials used in oxygen sensing, simple LED photodiode based detectors have been developed, including those which operate with a flexible optic–optic probe.

A number of phosphorescent materials and probes have the ability to respond to changes in micro- and macro-environment by altering their phosphorescence lifetime. When lifetime changes are considerable and analyte specific, lifetime-based detection provides an attractive alternative to the intensity measurements. Emission lifetime is an intrinsic parameter of the fluorophore. It is generally independent of the concentration and measurement setup, thus allowing simple internal referencing, and effective elimination of optical interferences such as instability of the light source and photodetector, label photobleaching, dispensing errors, inner filter and meniscus effects, etc. It is widely recognized that lifetime-based detection is a preferred method for such systems and applications.

The two main formats of luminescence lifetime measurements include direct lifetime measurements and phase fluorometry. The first method employs a short-pulse excitation of the sample, and subsequent measurement with a high-speed detector and multi-channel scaler of the complete decay curve, which is then reconstructed and analyzed to determine emission lifetime(s). Such measurement of microsecond lifetimes for the porphyrin dyes is technically simpler than for conventional fluorescent dyes (lifetimes 1–10 ns) and ruthenium labels (lifetimes 0.2–2 $\mu$s). A number of commercial spectrometers (e.g. LS-50B, PerkinElmer Life Sciences; Cary Eclipse, Varian) are suitable for measuring decay curves of metalloporphyrin dyes. However, relatively long pulses of xenon flash-lamp (10–20 $\mu$s) makes such instruments less suitable for measuring lifetimes shorter than 20 $\mu$s. The laser-based ArcDia® reader described previously is free from these limitations.

Phase fluorometry employs excitation with modulated light (sine or square wave of constant frequency) and measurement of delay in the emission signal (also modulated in this case). In this case, measurements are carried out in a quasi-continuous mode, providing a more simple real-time readout. The measured parameter phosphorescence phase shift (degrees angle) is related to emission lifetime according to a simple equation [49].

Simple LED photodiode based phase-fluorometric detectors are popular in quenched-phosphorescence

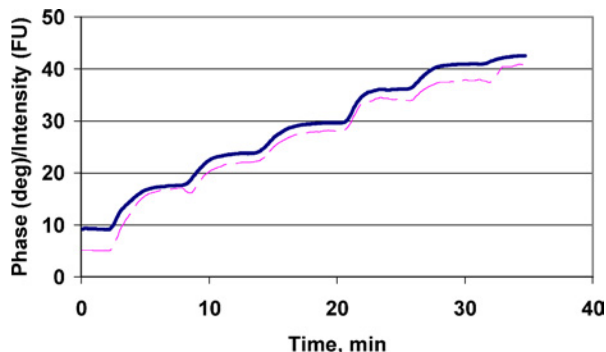


**Fig. 5.** Phosphorescence phase (solid) and intensity measurements (dashed), with the PtOEPK-PS based oxygen sensor and instrument described in [17]. Curves show sensor response to different oxygen standards (from left to right): 100.0, 20.5, 10.0, 5.0, 2.0, 0.5, 0.00 kPa. Plateau regions correspond to sample equilibration.

sensing, of oxygen, as they provide stable, reliable, calibration-free measurements with solid-state phosphorescent materials. Measurements can be carried out under ambient light, on a small scale and in a contact-less fashion in a sealed or closed vessel through a semi-transparent wall or surface. Phase-fluorometry at a single excitation frequency provides less information than direct lifetime measurements, and it can also suffer from interferences by short-lived light components. These drawbacks can be overcome by efficient optical and frequency filtering and by performing measurements at several different frequencies. An example of the phase-fluorometric sensor response to oxygen is shown in Fig. 5.

## APPLICATIONS

The characteristic luminescent properties of Pt- and Pd-porphyrins have attracted high interest in using them as labels (tags) for biomolecules. It is considered that these labels are in many respects advantageous over the conventional short-decay fluorescent labels (such as FITC, rhodamines, cyanine dyes), and they are also seen as alternatives to the lanthanide chelate and cryptate labels [33,50,51]. They provide luminophores with new spectral and temporal combinations, thus enabling expanded multi-parametric and multiplexed luminescence detection systems.

Thus far, a considerable number of applications employing phosphorescent Pt- and Pd-porphyrins have been described (see Table II). Most of them relate to quenched-phosphorescence oxygen sensing and high-sensitivity bioaffinity assays.

### Quenched Phosphorescence Oxygen Sensing and Oxygen Respirometry

Solid-state oxygen-sensitive materials were actively researched since mid-1980s for applications such as pressure-sensitive paints and pressure imaging in aerospace industry [52], optic–optic microsensors for measurement oxygen in biological samples such as suspension of cells, tissue, blood [53], non-destructive measurement of residual oxygen in packaged products, breadth gas analysis, process control and bioreactor applications [54–56], biological screening [57], environmental, and general oxygen sensing in gas phase and in solution [27]. A number of simple instruments (these materials are compatible with green and yellow LEDs) have been developed, which usually employ phase measurements such, but also intensity or direct lifetime measurements [58].

Quantifying of molecular oxygen in living systems was first demonstrated in the mid-1980s by Wilson and co-workers, who employed water-soluble Pd-porphyrins as oxygen sensors and phosphorescence lifetime measurements as the method of detection [21,29,59,60]. Later this group developed a number of near-infrared soluble phosphorescent oxygen probes based on Pd-benzoporphyrins and PdTCPP conjugated to hydrophilic carriers (dendrimeric structures). These probes have been applied to imaging of oxygen distribution in tissue and tumors [53,61–64].

In recent years, a strong interest is emerging in the use of quenched-phosphorescence oxygen sensing in screening applications, particularly in the areas of drug discovery, general cell biology, medicine and biotechnology. Molecular oxygen and rates of oxygen uptake can serve as valuable biomarker, which reflects metabolic status, viability of aerobic cells and living systems, and their alterations as a result of disease, effects of endogenous and exogenous stimuli and response to drugs. Such applications require high sample throughput, low cost, together with flexibility and the possibility to integrate such assays in existing screening platforms and processes.

Notwithstanding their operational performance, solid-state oxygen-sensitive materials have certain limitations with respect to biological screening. They suffer from the lack of biocompatibility and flexibility, relatively high costs, which limit their use, particularly in cell-based assays with adherent cells. The soluble near-infrared oxygen probes are also not very suitable for these applications, as they are not compatible with many instruments (PMTs are not sensitive in this region). Additional factors for biological applications include intrinsic cyto- and phototoxicity of the oxygen-sensitive material, cell permeability, friendliness and compatibility, migration of its

**Table II.** Outline of Applications Based on Metalloporphyrin Labels and Probes

| Application | Phosphorescent label/probe | Detection system | References |
|---|---|---|---|
| *Oxygen sensing* | | | |
| Pressure sensing, imaging | PtTFPP/PdTFPP-PS (solid state), PtTFPP–fluoroacrylic polymer | Lifetime-based sensing | [52] |
| Biological oxygen sensing | PdCP, PdTCPP–BSA complexes (soluble) | Lifetime-based imaging | [29,30,85,86] |
| Oxygen distribution in tissue | PdTBP-based (soluble) | Lifetime-based | [31,62,87] |
| General $O_2$ sensing | PtOEP-PS (solid state) | Lifetime based sensing | [82] |
| General $O_2$ sensing | PtOEPK-PS (solid state) | Phase fluorometry | [17] |
| Packaging, food | PtOEPK-PS (solid state) | Phase fluorometry | [55] |
| Breadth gas analysis | PtTFPP-PS (solid state | Intensity-based | [56] |
| Trace oxygen analysis | PdOEPK-PS, PdTFPP-PS | Phase fluorometry | [25] |
| Enzymatic glucose sensing | PdCP + glucose oxidase enzyme (soluble) | Intensity measurements | [68] |
| Immunosensor | PtOEPK-PS + Ab-glucose oxidase (heterogeneous) | Phase fluorometry | [69] |
| Cell viability assays | PtOEPK-PS (solid state) | Phase fluorometry | [57] |
| Cell viability assays | PtCP-based (soluble) | Intensity, TRF | [32] |
| Cell-based, animal-based toxicity screening | PtCP-based (soluble) | Intensity, TRF | [88] |
| Enzymatic assays, CYP450, metabolites, ADME-tox | PtCP-based (soluble) | Intensity, TRF | [67] |
| Relative air humidity (RH) | PtCP-LB films (solid state coatings) | Lifetime-based sensing | |
| *Phosphorescent labels* | | | |
| Solid-phase immunoassays | PdCP-Ab | TRF | [33,73] |
| Solid-phase immunoassay | PtCP-Ab, PtCP-avidin, PtCP-biotin | TRF | [41] |
| FISH | PtCP-oligonucleotide, PtCP-Uracil | TRF imaging microscopy | [43] |
| Immunostaining | PtCP-avidin, PtTCPP-avidin | TRF imaging microscopy | [34,79] |
| Multi-color imaging | PtCP, PdCP-Ab, lanthanides, fluorescent stains | TRF and prompt fluorescence microscopy | [89,90] |
| Immunoassay with label-specific antibodies | PdCP | TRF | [74] |
| Homogeneous immunoassay | PtCP-dioxin | Fluorescence quenching, TRF | [77] |
| Post-PCR detection of DNA in gels | PtCP-oligo, PtCP-Uracil | TR-F scanning | [46] |
| Homogeneous hybridization assays, real-time PCR | PtCP-oligonucleotide, PdCP-oligonucleotide | TR-F, prompt fluorescence | [45,80] |
| Whole cell based immunoassays | PtCP-Ab | TRF | [75] |
| Homogeneous immunoassays | PtCP-peptide | TRF (RET) | [76] |
| Homogeneous enzymatic assays (proteases) | PtCP-peptide, PtCP-Ab | TR-F (proximity quenching) | [76] |
| Electrochemiluminscent label | PtCP-Ab | Photon counting (ECL) | [91] |

components, high sensitivity to detect small oxygen gradients associated with cellular respiration, etc.

An integrated approach to using optical oxygen sensing and respirometry in biological screening which addresses all these issues was developed by our group. Respirometric Screening Technology (RST) employs specially designed and optimized phosphorescent probes and formats of oxygen respirometry [32]. The PtCP-based probes are water soluble, cell friendly (non-toxic, impermeable), have convenient spectral characteristics and provide optimal response to oxygen in the physiological range. The probes can be used in a broad range of working concentrations ($\mu$M–nM) using standard fluorescence detectors (prompt and TRF spectrometers, readers, imagers). They provide monitoring of oxygen consumption in multiple samples containing cells using standard microtiter plates and equipment routinely used in biochemical and screening laboratories. Special low-volume sealable microplates [65] provided further enhancement of sensitivity, miniaturization, and improved performance of such RST assays. The possibility of time-resolved detection allows elimination of interferences by scattering, fluorescent compounds, cells in such assays, and reliable probe detection at low concentrations and on a micro scale [66].

Using the RST probes and assay formats, a number of customized cell-based, enzyme-based, and animal-based screening assays have been developed and validated with practical targets. These assays include basic cell viability and cell proliferation assays [57], monitoring of toxic effects of chemicals on cells [57]; *in vitro* assessment of

hepato- and nephrotoxicity of drug candidates [67]; measurement of activity and inhibition of oxygen-dependent enzymes (e.g. drug metabolism by $CYP_{450}$ enzymes [66]); measurement of oxygen uptake by small aquatic animals and mouse embryos and animal-based acute toxicity studies [66], studies of cellular metabolic responses, and mechanisms of cell death (apoptosis) [67].

Other applications of quenched phosphorescence oxygen sensing using metalloporphyrin dyes include flow-injection systems for measurement of activity of oxygen-dependent enzymes and their metabolites, using water-soluble oxygen probe (PdCP coupled with glucose oxidase to detect glucose), and quenched phosphorescence detection [68]; immuno-sensor application [69].

## Phosphorescent Bioaffinity Assays

The development of first TRF immunoassay system in early 1980s, by Wallac Oy (Turku, Finland, now part of PerkinElmer Life Sciences) [70], has demonstrated the potential of long-decay luminescent labels in diagnostic applications. DELFIA™ (dissociation enhanced lanthanide fluorescent immunoassay) still remains one of the most sensitive fluorescence-based platforms of bioaffinity assays [47], using europium chelates which display lifetimes approaching 1 ms. However, this heterogeneous system has its drawbacks, and a number of homogeneous TRF platforms, such as LANCE™ [71] and HTRF™ [72], all employing fluorescent lanthanide chelate or cryptate labels, have been developed. Although these systems have extended areas of applications of TRF, they are usually less sensitive than DELFIA™. Multiple patents on lanthanide labels and applications result in complex IP situations and very high costs associated with these assays, often making them unaffordable for users. The phosphorescent metalloporphyrin labels (emission characteristics—see section 'Emission Characteristics') have the potential to overcome these hurdles and compete with the lanthanides, both in terms of assay sensitivity, flexibility and costs [41]. They can also complement lanthanides, thus allowing multi-parametric detection, assay multiplexing, and new applications.

In the initial studies PdCP-labeled antibodies were evaluated in model heterogeneous immunoassays [50]. Later termed time-resolved phosphorescent immunoassay (TR-PIA) or PORphyrin PHosphorescent ImmunoAssay (PORPHIA) [33,50]. A TRF immunoassay for human chorionic gonadotropin (hCG) using a commercially available fluorometer was described [73]. Antibodies to the PdCP I label have been produced and used in what was termed a 'universal' phosphorescence immunoassay [74].

The introduction of PtCP labels, which are more sensitive than PdCP, and the development of monofunctional labeling reagents on the basis of MeCP have advanced this area. Antibodies, neutravidin and biotin labeled with PtCP-NCS have been evaluated comparatively in model immunoassays [42], and then a solid-phase sandwich immunoassay $\alpha$-fetoprotein (AFP) using PtCP-labeled monoclonal antibodies and ArcDia® TRF reader (see earlier paragraph) [41] was described. In these systems the PtCP label and antigen detection with sub-picomolar sensitivities was demonstrated, which is comparable, if not superior, with those achieved in lanthanide-based formats, including DELFIA™. A detailed optimization of the phosphorescent antibody conjugates for general immunoassay applications was carried out, using analogs of PdCP-NCS with different spacer arm as labeling reagents [40]. Furthermore, phosphorescently labeled antibodies were used in solid-phase assays with live cells, in which induction of expression of specific markers (cell adhesion molecules) on the surface of adherent cells by cytokines was monitored [75].

Development of homogeneous formats of TRF bioaffinity assays using MeCP labels and close proximity quenching and resonance energy transfer (RET) schemes is another challenge and application niche, particularly in life sciences and biomolecular screening. Our group has recently succeeded in the development of two of such systems [76]: a RET-based TRF immunoassay employing PtCP label as a donor, in which significant signal enhancement upon binding was achieved and the development of phosphorogenic oligopeptide substrates of protease enzymes (e.g. caspase-3), which provide marked signal enhancement upon enzymatic cleavage. Other homogeneous assays described include a TRF immunoassay of dioxins based on the quenching of PdCP-labeled antigen upon binding to antibodies [77]. Other systems are in development, including assays for phosphatases and kinases.

The nucleic acid conjugates (see earlier paragraph) have stimulated DNA detection systems based on TRF and MeCP labels. Similar to the immunoassays, DNA detection systems may be divided into those employing separation steps (heterogeneous) and separation-free (homogeneous) systems. The former involves label incorporation into target DNA strands by hybridization or in a polymerase chain reaction (PCR), capturing or separating the labeled target (on a gel, microplate or gene array), and subsequent TRF detection. The homogeneous formats, such as 'real-time PCR,' employ special probes (e.g. TaqMan™, Molecular Beacons™, Linear Probes™), which are incorporated in the enzymatic target amplification process (PCR) and which alter their emission in the presence of target DNA [78].

The probes based on RET or proximity quenching are sensitive to probe conformation (double-stranded/single-stranded) or chemical composition (cleaved/uncleaved), thus allowing monitoring DNA amplification process and quantifying the target in a closed tube format.

MeCP-labeled oligonucleotides have initially been applied to fluorescence *in situ* hybridization (FISH, heterogeneous format), for imaging of phosphorescently stained DNA and mRNA in fixed cells by TRF microscopy [79]. PtCP label was also applied to post-PCR DNA detection in electrophoretic gels [46], using several common methods of label incorporation into DNA (labeled primers, labeled base or modified base and post-PCR labeling). For these systems, sensitivity of ∼10 pg DNA was reported, which is far better than using FITC label or ethidium bromide staining.

The possibility of using the phosphorescent oligonucleotide probes in homogeneous hybridization assays has also been demonstrated. Using pairs of single-labeled oligos, strong and target-specific quenching of MeCP was observed upon hybridization of complementary sequences, i.e. when the quencher moiety was brought to close proximity of MeCP label [45,80]. Screening of quenchers, studies of distance effects were carried out, followed by the development of dual-labeled hybridization probes and their integration in a PCR with TRF detection on a plate reader.

Time-resolved fluorescence imaging/microscopy facilitates increased signal/noise when compared to steady-state fluorescence microscopy, especially when imaging biological samples. With respect to imaging of metalloporphyrins, the convenient visible absorption bands in the 500–650 nm range (see Table I) and long-decay red/very-near infrared emission of metalloporphyrins provide them certain advantages over lanthanides, which are only excitable with the UV light, and over conventional fluorescent stains. In addition to the oxygen imaging and FISH applications (see earlier discussion), multi-color imaging of cell surface, lysosomal or nuclear targets, using up to five stains (phosphorescent and fluorescent probes) [43]. Imaging was carried out using custom-built or modified microscopes allowing microsecond TRF mode and spectral compatibility with metalloporphyrin labels and with lanthanides [81].

Furthermore, we anticipate that multi-parametric assays and assay multiplexing will be one of the main avenues of further research with phosphorescent porphyrins and their *in vitro* diagnostic, screening, imaging, and sensing applications. It has already been demonstrated earlier that in such systems the porphyrin labels and probes can be coupled with each other (e.g. PtCP and PdCP, or MeCP and MeCP-ketone pairs, see Table I), and with lanthanides and conventional fluors.

## CONCLUSIONS

Overall, one can see that phosphorescent metalloporphyrins have reached rather high level of development. Although their practical use still remains not as widespread as for the other common labels, including lanthanides, we believe that this situation will change very soon. The availability of a spectrum of labeling reagents, phosphorescent materials, bioconjugates and probes, measurement instrumentation, demonstration assays and integrated systems are making this label and detection technology available to many researchers. A number of systems and applications have reached relatively wide practical use, and companies such as Luxcel Biosciences Ltd., Arctic Diagnostics Oy are actively involved in their commercialization. But many applications still have to be discovered, explored and developed to the levels acceptable for users in research laboratories and industry.

## ACKNOWLEDGMENTS

Support of this work by the Irish Higher Educational Authority and National Development Plan, 2001–2006 is gratefully acknowledged.

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