Platinum(II) Ring-fused Chlorins as Near-infrared Emitting Oxygen Sensors and Photodynamic Agents

Nelson A. M. Pereira,[a] Mafalda Laranjo,[c,d,e] João Casalta-Lopes,[c,d,e] Arménio C. Serra,[b] Marta Piñeiro,[a] João Pina,[a] J. Sérgio Seixas de Melo,[a] Mathias O. Senge,[f] M. Filomena Botelho,[c] Liliana Martelo,[a,g] Hugh D. Burrows[a] and Teresa M. V. D. Pinho e Melo[a]*

[a]CQC, Department of Chemistry, University of Coimbra, 3004-535 Coimbra, Portugal.

[b]Department of Chemical Engineering, CEMUC, University of Coimbra, Rua Silvio Lima Polo 2, P-3030 290 Coimbra, Portugal.

[c]Biophysics Unit, Faculty of Medicine of University of Coimbra, Azinhaga de Santa Comba, Celas, 3004-548 Coimbra, Portugal

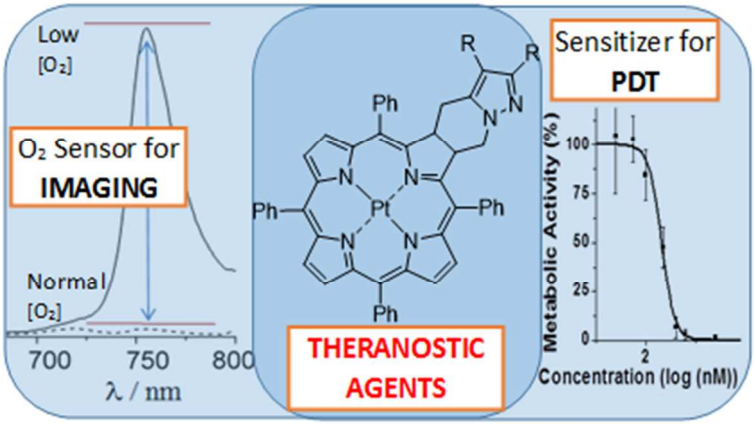
[d]CIMAGO - Center of Investigation in Environment, Genetics and Oncobiology, Faculty of Medicine of University of Coimbra, Azinhaga de Santa Comba, Celas, 3004-548 Coimbra, Portugal

[e]IBILI – Institute for Biomedical Imaging and Life Science, Faculty of Medicine of University of Coimbra, Azinhaga de Santa Comba, Celas, 3004-548 Coimbra, Portugal

[f]School of Chemistry, SFI Tetrapyrrole Laboratory, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College Dublin, The University of Dublin, Dublin 2, Ireland

[g]Centro de Química-Física Molecular (CFQM), and the Institute of Nanoscience and Nanotechnology (IN), Instituto Superior Técnico, University of Lisbon, 1049-001 Lisbon, Portugal.

Supporting Information Placeholder



KEYWORDS: PDT, theranostics, Pt(II)chlorins, photosensitizers, oxygen sensing

ABSTRACT: Novel near-infrared luminescent compounds based on platinum(II) 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine–fused chlorins are described. These compounds, have high photostability, and display light emission, in particular simultaneous fluorescence and phosphorescence emission in solution at room temperature, in the biologically relevant 700-850 nm red and near-infrared (NIR) spectral region, making them excellent materials for biological imaging. The simultaneous presence of fluorescence and phosphorescence emission at room temperature, with the phosphorescence strongly quenched by oxygen whereas fluorescence remains unaffected, allows these compounds to be used as ratiometric oxygen sensors in chemical and biological media. Both steady-state (fluorescence vs phosphorescence intensities) and dynamic (dependence of phosphorescence lifetimes upon oxygen concentration) luminescence approaches can be used. Photocytotoxicity studies against human melanocytic melanoma cells (A375) indicate that these compounds display potential as photosensitizers in photodynamic therapy.

Luminescence sensing and imaging provides a sensitive and low-cost approach for the *in vivo* study of biological systems.[1] Near-infrared (NIR) emitters in the therapeutically relevant 700-850 nm region are particularly important, as their spectral features span a region where tissues are nearly transparent.[2,3] Valuable applications include NIR luminescence imaging of cells for diagnosis of cancer and other abnormal conditions.[4-7] Examples of the most important classes of NIR emitters are inorganic fluorophores, such as quantum dots; up-converting fluorophores containing lanthanide ions; organic dyes, such as cyanines, squaraines, phthalocyanines, porphyrins, boron dipyrromethanes (BODIPYs), perylene dyes; and carbon nanotubes.[2,6] However, although a number of materials have been reported which luminesce in this region, the only long wavelength emission compound approved by the US Food and Drug Agency (FDA) for direct usage in medical diagnostics is the cyanine dye indocyanine green.[2] There is, therefore, the urgent need to develop new and efficient NIR emitting materials.

There is a major advantage if the same luminescent material which is used for imaging can also be used as fluorescent probe and sensor for both qualitative and quantitative analysis of a chemical species.[8-10] One important target in cellular studies is molecular oxygen, whose concentration can provide information on the cell state. Quenching of the excited states by molecular oxygen can lead to reactive oxygen species, such as singlet oxygen or superoxide radical anions, which can be harmful for malignant cells. Combining this ability with diagnostic properties endows these systems with potential as theranostic agents.

The incorporation of high atomic number metal ions, *e.g.*, platinum, into porphyrins, phthalocyanines, chlorins and bacteriochlorins can enhance triplet state formation, and, in many cases, lead to room temperature phosphorescence within the biological spectral window.[11-17]

Chlorins (dihydroporphyrins) show suitable spectral properties in the NIR region. However, when prepared by the classical route of porphyrin diimide reduction, they have limited chemical stability, and reoxidation to the porphyrin occurs easily. We have recently shown that their stability can be dramatically enhanced by the introduction of a fused ring. Indeed, stable 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-fused chlorins were obtained *via* an unprecedented [8π+2π] cycloaddition of diazafulvenium methides with porphyrins.[18,19] Furthermore, preliminary studies on the photodynamic activity of 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-fused chlorins against melanoma cells showed that they are very active photodynamic agents.[20] Interestingly, a dihydroxymethylchlorin derivative was particularly active against human melanocytic melanoma A375 cells. This is of particularly importance considering the known resistance of melanoma to conventional chemotherapy and radiotherapy, and the fact that PDT of melanoma can be compromised due to the natural resistance mechanism of some melanotic melanomas.[21,22] In fact, high melanin levels in such tumors can lead to optical interference *via* competition with the photosensitizer for light absorption.

To enhance the potential using the results already achieved with above chlorins, their Pt(II) complexes were prepared with the aim of developing NIR luminescence probes as well as new photosensitizers for photodynamic therapy (PDT) of cancer.

The platinum complex of 5,10,15,20-tetraphenylporphyrin was synthesized following a known general procedure, replacing $Pt(acac)_2$ as the metal source with $PtCl_2$.[23] 5,10,15,20-Tetraphenylporphyrin (**1**, TPP) reacted with $PtCl_2$ in benzonitrile under microwave irradiation at 250 ºC for 20 min, giving the Pt-complex **2** in 90 % yield. The metallated porphyrin **2** (2 equiv.) reacted with 2,2-dioxo-1*H*,3*H*-pyrazolo[1,5-c]thiazole **3** under microwave irradiation at 250 ºC for 20 min giving the novel Pt complex of 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine–fused chlorin **5** in 20 % yield (50 % of porphyrin recovered). Thus, Pt-complex **2** participated in a [8π+2π] cycloaddition with diazafulvenium methide **4**, generated *in situ* from sulfone **3** through thermal extrusion of sulfur dioxide. To improve the hydrophilicity of the complex for application in biological systems, Pt(II)-chlorin **5** was converted into the corresponding dihydroxymethyl derivative **6** using $LiAlH_4$ as reducing agent (Scheme 1).

The absorption spectra of the Pt complexes **5** and **6** (in toluene solution) display wavelength maxima at ~590 nm, together with additional absorption bands at shorter wavelengths (Figure 1 and Table 1). Note, for the compounds investigated the absorbance of the low energy band is much greater than those found for TPP or its Pt(II) complex (see Figure 1 and Figure S3).

Figure 2 presents the emission spectra for chlorins **5** and **6**. The fluorescence emission (in the 644-655 nm range) is structured, with a maxima at 598 nm (Table 1). However, the most interesting feature of these compounds is the additional occurrence of room temperature phosphorescence, with maxima at 755 nm (see Figure 2). The assignment of this long wavelength band to phosphorescence was based on its similarity with the RT phosphorescence emission spectra collected with 5 μs delay after flash and the time resolved phosphorescence spectra obtained in a laser flash photolysis setup (see Figures 2 and S4-S6 in SI). For the Pt(II) derivatives (chlorins **5** and **6**) at 293 K, the RT phosphorescence spectra display an unstructured long wavelength emission band centered at ~755 nm (Figures S4 and S7). This provides the possibilities for using these compounds for NIR imaging. There is a strong overlap between the phosphorescence and the fluorescence emission bands. However, the phosphorescence is highly sensitive to oxygen, and increases when the concentration of O_2 in solution is reduced (see Figure S4 in SI). This allows determination of the fluorescence quantum yields for chlorins **5** and **6** using oxygen saturated solutions, where the phosphorescence is effectively quenched, see Figure 2, while the fluoresecence is little affected. The fluorescence quantum yields (ϕ_F) for chlorin **5** and **6** were obtained using TPP (ϕ_F = 0.11)[24] in toluene as standard (see experimental section), showing a lower ϕ_F = 0.0001 value, see Table 1. This is particular relevant when compared to the free base chlorins of **5** and **6** where the fluorescence quantum yields are more than 3-orders of magnitude higher. In addition, a blue-shift of the fluorescence was also observed upon going from the free base chlorins to the corresponding Pt(II)-chlorin derivatives (see Table 1).

Degassed solutions of the Pt(II) chlorins in toluene show a much stronger phosphorescence at 756 nm compared with aerated ones, while the fluorescence emission intensity at 600 nm remains unaltered, as can be seen in Figure S4. The

phosphorescence quantum yields (ϕ_{Ph}) for chlorins **5** and **6** were obtained by using tris(2,2'-bipyridyl)ruthenium (II) in water (ϕ_F = 0.042)[25] as standard. For further details please see the experimental Section in SI. The values of the phosphorescence quantum yields (Table 1) depend on the dissolved oxygen concentration in the solution, varying from 0.0002 in oxygen saturated toluene (3 hours of bubbling with oxygen); through 0.0028 with an air equilibrated solution ($[O_2]= 1.8\times10^{-3}$ M), finally to 0.088 with a nitrogen saturated solution. The strong quenching of the phosphorescence band of chlorins **6** and **5** by molecular oxygen, and the increase in its intensity by over two orders of magnitude on going from oxygen saturated to degassed solutions (see Table 1 Figure S4) opens the possibility of sensing oxygen in chemical and biological media by studying the ratio of the intensity of phosphorescence to fluorescence bands (at ~600 nm)[26]. One particularly important application of such ratiometric sensing is in cancer diagnosis, since it could, in principle, enable the determination of intracellular oxygen concentration, thereby allowing distinction between normoxic and hypoxic cells. This also opens the way for *in vivo* oxygen tumor imaging by phosphorescence quenching.[15]

To mimic biological conditions, a micellar system of the surfactant polysorbate 80 (Tween 80) in aqueous DMSO solution (Tween80/DMSO/water (2/2/96, v/v/v)) was used. In this medium similar spectroscopic properties (absorption and emission wavelength maxima) were observed as those in toluene solution spectra (Figure 3).

The potential for oxygen sensing of chlorin **6** was analyzed quantitatively using phosphorescence intensities at different oxygen concentrations and the Stern-Volmer equation (eqn. 1),[24]

$$\frac{I^{\circ}}{I} = 1 + K_{SV} pO_2 \quad \text{(eqn. 1)}$$

where K_{SV} is the Stern-Volmer constant, pO_2 is the oxygen partial pressure, I^0 = (luminescence intensity at 755 nm / luminescence intensity at 600 nm) and I = (luminescence intensity at 755 nm / luminescence intensity at 600 nm) at several pO_2 from 0 to 21%. A good linear fit was observed up to 21 % oxygen, with a K_{SV} value of 0.18 (Figure 4). The fluorescence intensity monitored at 600 nm was practically unchanged, showing that chlorin **6** can be used as a ratiometric phosphorescence probe for measuring oxygen pressures and, consequently, solution concentrations.

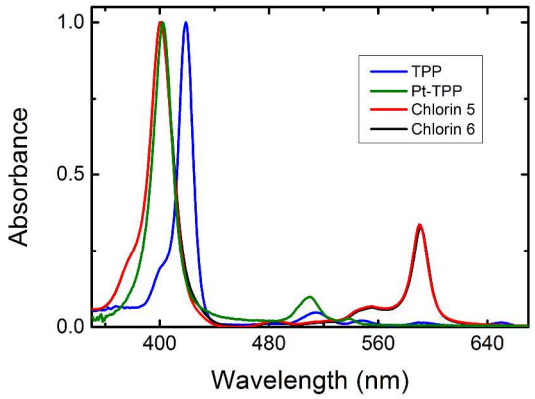
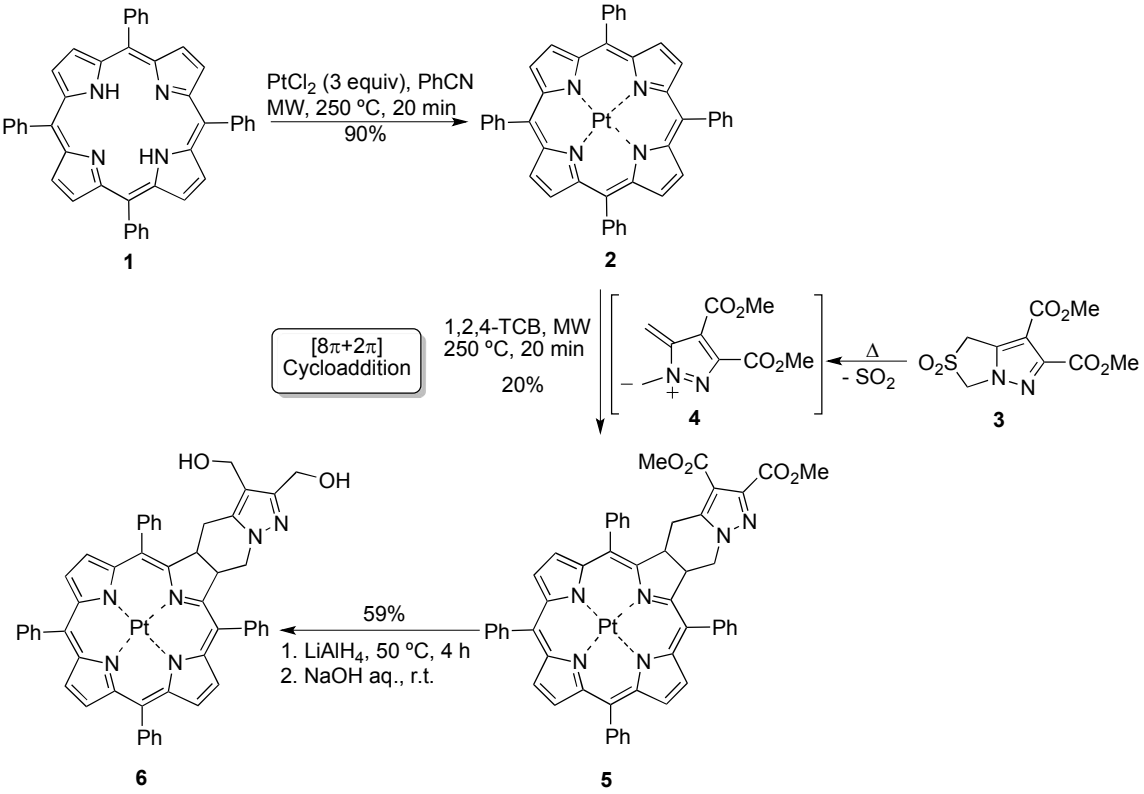


Figure 1. Normalized absorption spectra of Pt(II) chlorins **5** and **6**, presented with TPP and Pt(II)-TPP (considered as references compounds) in toluene solution at T = 293K..



Scheme 1. Synthesis of Pt(II) 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine–fused chlorins 5 and 6.

Table 1. Absorption and photophysical data (absorption, fluorescence and phosphorescence maxima, fluorescence ϕ_F and phosphorescence quantum yields, ϕ_{Ph} and phosphorescence lifetimes, τ_{Ph}) for the Pt(II) chlorins and the corresponding free base chlorins in solution at room temperature.

	[a] Absorption λ_{max} (nm)					[a] Fluorescence λ_{max} (nm)		[a]Phosphorescence λ_{max} (nm)	ϕ_F *[a]	ϕ_P *[a]	Phosporescence lifetime (μs)
	Q_x (0-0)	Q_x (1-0)	Q_y (0-0)	Q_y (1-0)	B (0-0)	Q (0-0)	Q (0-1)	T (0-0)			
Chlorin **5**	-	590	554	484	400	598	655	756	0.0001[e]	0.0002[c] 0.0028[c] 0.088[d]	0.4[a,c] 26.1[a,d]
Chlorin **6**	-	591	555	486	400	598	644	756	0.0001[c]	0.0002[e] 0.0019[c] 0.068[d]	0.5[a,c] 25.7[a,d] 30.9[b,d] 11.3[b,c]
Free base chlorin of **5**[19]	649	595	545	518	416	654	698, 721	-	0.27	-	-
Free base chlorin of **6**	651	601	543	514	409	654	696, 718	-	0.23	-	-

*Fluorescence quantum yields (λ_{exc} = 413 nm) were determined by using TPP in toluene (Φ_F = 0.11)[24] and tris(2,2'-bipyridyl)ruthenium (II) in water (ΦF = 0.042)[25] as a standard,; [a]in toluene, [b]in aqueous micellar system (Tween80/DMSO/water (2/2/96, v/v/v)), [c]presence of O_2, [d]deaerated solution; [e] O2 saturated solution; values presented in parenthesis were obtained in the ns-TA setup.

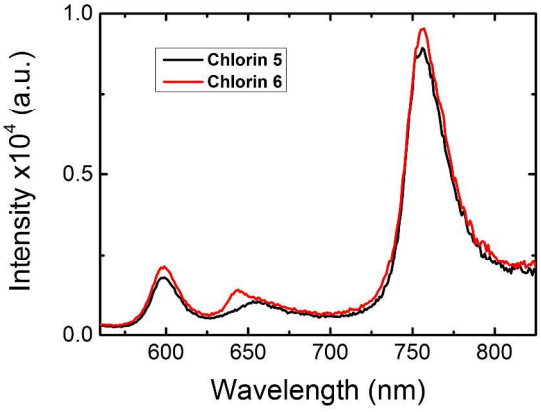


Figure 2. - Room temperature luminescence spectra (λ_{exc}= 400 nm) for chlorins **5** and **6** in oxygen saturated toluene solutions prepared by bubbling oxygen for 3 hours.

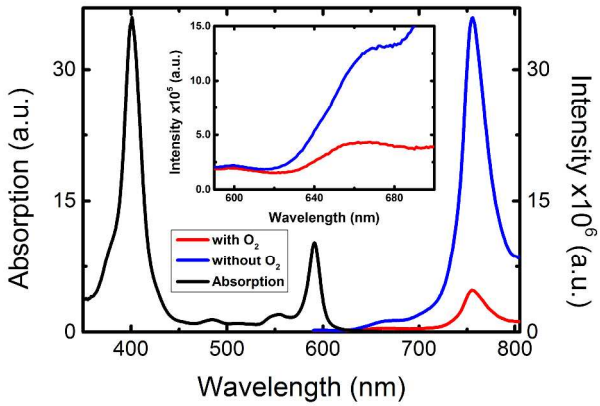


Figure 3. Room temperature luminescence spectra (λ_{exc} = 410 nm) together with the normalized absorption spectra for Pt(II) chlorin **6** in aqueous micellar system (Tween80/DMSO/water (2/2/96, v/v/v)), collected in the presence (air saturated solution) and absence of O_2 (after deoxygenation by bubbling N_2 for 20 min). Inset: Magnified view of the fluorescence emission bands present in the 590-700 nm range.

The phosphorescence lifetime of chlorin **6** was also determined in the presence and absence of oxygen in DMSO:Tween80:H_2O (2:2:96) (Table 1), and changed from 11.3 μs (aerated solution with O_2) to 30.9 μs (degassed solution), see Figure S6A and S6B in SI. The observed decrease in phosphorescence lifetime makes this class of compounds good candidates for applications in phosphorescence lifetime imaging microscopy, [27] in addition to their use as steady-state ratiometric O_2 sensor probes.

The photostability of chlorin **6** was determined by monitoring the phosphorescence emission of a deaerated solution in water at 756 nm. The phosphorescence intensity at 756 nm remained unchanged over ~4.5 h irradiation. Finally, the thermal stability of chlorins **5** and **6** was evaluated, using isothermal thermogravimetric analysis of the samples at 60 ºC during 3 h. Negligible mass loss (less than 1 %) was observed for the two compounds studied (SI, Figure S8). These results demonstrate that chlorins **5** and **6** have high thermal stability and photostability.

Quenching of the phosphorescence may be expected to produce singlet oxygen ($^1\Delta_g$). Its formation was confirmed by direct measurement of the characteristic singlet oxygen phosphorescence emission centered at 1270 nm following irradiation of aerated solutions of chlorin **6**. The singlet oxygen quantum yield (ϕ_Δ) was obtained by comparing the sensitized phosphorescence emission spectra from singlet oxygen (Figure S9 in SI), obtained with optically matched solutions of the samples and that of the reference phenazine[28] (for further details see the Experimental section).

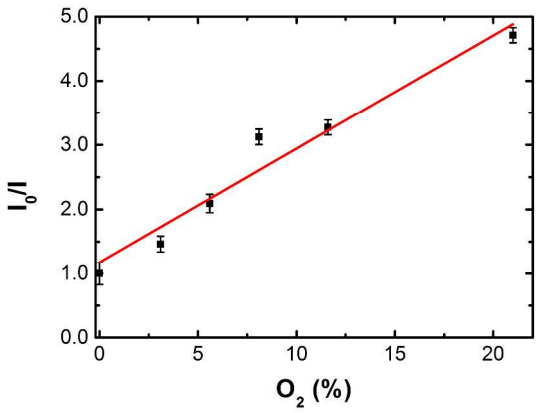


Figure 4. Plot of I^o(755 nm/600 nm)/I(755 nm/600 nm) as function of O2 concentration - from 0 to 21 % (gas phase) - for chlorin **6** in aqueous micellar system (Tween80/DMSO/water (2/2/96, v/v/v)).

A value of ϕ_Δ=0.58 was obtained for chlorin **6**. This strongly suggests that the platinum(II) ring-fused chlorins can also be potentially interesting photosensitizers for photodynamic therapy.

In this context, the photocytotoxicity of chlorins **5** and **6** against human melanocytic melanoma cells (A375) was evaluated. It was observed that the di(hydroxymethyl)-substituted chlorin **6** shows high photodynamic activity with an IC_{50} of 144 nM (confidence interval at 95 %: [121.8;171.0]) (Figure 5), comparable to the IC_{50} value of 156 nM obtained for Photofrin@ under the same conditions. Additionally, this chlorin **6** was significantly more active than the diester-substituted chlorin **5** (IC_{50} > 5 μM) as was previously also observed for the corresponding metal-free chlorins.[20] Experiments with A375 cells in the dark confirmed that the cytotoxicity is light-dependent. In the case of chlorin **6,** some cytotoxicity was observed in the absence of light, but with an IC_{50} value of 22.29 μM. Thus, the potential of

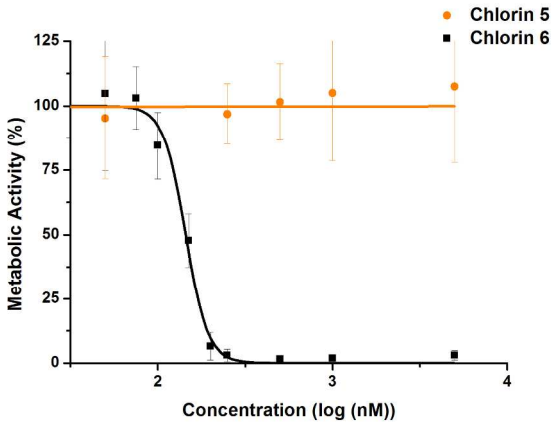


Figure 5. Metabolic activity of A375 human melanoma cells submitted to photodynamic treatment using the photosensitizer chlorins **5** and **6**. The values represent the average and standard deviation for each concentration.

platinum(II) 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine–fused chlorins as photosensitizers for PDT of melanocytic melanoma cells has been demonstrated.

In conclusion, new near infrared luminescent oxygen sensors based on platinum(II) 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine–fused chlorins are reported. Their high thermal and photochemical stability, attractive photophysical features, including oxygen dependent room temperature phosphorescence and potential as ratiometric emission measurements, make them excellent compounds to be used as probes for molecular oxygen, biological imaging and photodynamic therapy. This implies that the described compounds are true, stable Pt-chlorin-type theranostic agents.

ASSOCIATED CONTENT

Supporting Information

Experimental section, ^{1}H NMR, ^{13}C NMR, absorption and fluorescence emission spectra for 5,10,15,20-tetraphenylporphyrin (TPP) and Pt(II) complex, luminescence spectra of chlorins 5 and 6 (in the absence and presence of oxygen), thermal stability, testing of photodynamic activity of chlorins **5** and **6** and singlet oxygen formation on photolysis of chlorin 6.

AUTHOR INFORMATION

Corresponding Author

* E-mail address: tmelo@ci.uc.pt

Author Contributions

All authors have given approval to the final version of the manuscript.

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