Photoacoustic Detection of Superoxide Using Oxoporphyrinogen and Porphyrin

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ABSTRACT: The superoxide ($O_2^{\bullet-}$) ion is a highly reactive oxygen species involved in many diseases, hence its non-invasive detection is desirable to identify the onset of pathological processes. Here, we employed photoacoustic (PA) spectroscopy, which enables imaging at ultrasound resolution with sensitivity of optical modality, for the first time to detect $O_2^{\bullet-}$, using stimuli-responsive contrast agents. *Meso* (3, 5-di-*tert*-butyl 4-hydroxyphenyl) porphyrins and oxoporphyrinogens were used as PA contrast agents, which trap the $O_2^{\bullet-}$ and enable its detection. The trapped $O_2^{\bullet-}$ increased the PA signal amplitude of chromophores up to 9.6-fold, and induced a redshift in the PA signal maxima of up to 225 nm. Therefore, these trigger-responsive probes may be highly valuable as smart diagnostic PA probes to investigate pathological events stimulated by $O_2^{\bullet-}$ species.

Reactive oxygen species (ROS) are highly aggressive, biochemically produced molecules that play important roles in physiological and pathological processes. ROS are a set of short-lived species comprised of $O_2^{\bullet-}$, H_2O_2 , $HO^{\bullet}$ and $HO_2^{\bullet}$, of which the primary superoxide ion ($O_2^{\bullet-}$) is produced from oxygen via cellular electron transfer processes. It is well-established that uncontrolled $O_2^{\bullet-}$ production is involved in diverse pathological processes, especially in inflammatory disorders like atherosclerosis, inflammatory bowel diseases, and rheumatoid arthritis as well as in cancer.¹ The generated $O_2^{\bullet-}$ undergoes further reactions to the secondary ROS species, hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$).² To image the onset of diseases and elevated production of ROS, previous studies employed ROS-reactive (chemically irreversible) probes, which give a readable signal upon reaction that is correlated to the amount of produced ROS.^{1,3} Among ROS-imaging, fluorescence based methods are highly prevalent in literature (up to 75% of previous reports), followed by electron paramagnetic resonance (EPR) spectroscopy and colorimetric methods.^{3,4} However, optical methods suffer from photon scattering and diffusion which limits the detection depth to ca. 0.1 mm; EPR as well as colorimetric methods are suitable only for *in vitro* applications.

Photoacoustic (PA) imaging circumvents these limitations by combining optical imaging with ultrasound (US), and is highly suitable for *in vivo* imaging.^{5,6} As ultrasound waves show less attenuation in tissues in comparison to light, PA imaging can be possible at higher depths (up to 7 cm) with US spatial resolution (up to 50 μm).^{7,8} Moreover, the generated PA signal is directly proportional to the amount of light absorbing material present, thus increasing PA sensitivity to the levels of optical modalities (up to sub-nano molar).⁸ The light absorbing agents can be of either endogenous or exogenous origin, which are irradiated with a non-ionizing, low energy laser light to generate PA signals via thermo-elastic expansion.^{9,10} PA imaging has already found potential clinical applications and several translational studies are ongoing,¹¹ e.g. for the early detection of malignant tumors,¹²⁻¹⁴ the characterization of inflammatory bowel diseases,¹⁵ the localization of sentinel lymph nodes,¹⁶ as well as the assessment of tissue hypoxia,¹⁷ tumor vasculature,¹⁸ and fat metabolism.¹⁹ Therefore, PA imaging of short-lived ROS is an exciting next step to advance this modality.

For the PA detection of ROS, it is necessary to develop probes selectively reactive to $O_2^{\bullet-}$, $HO^{\bullet}$ and/or H_2O_2 .²⁰ The latter species was imaged with the aid of phthalocyanine, that after reaction cleaved attached soluble groups and increased the nanoparticle size and thereby the PA signal intensity.²¹ Alternatively, horseradish peroxidase (HRP) and its substrate were entrapped in a liposome, which upon reaction with H_2O_2 gave a green dye with increased PA-signal.²² To make previously investigated exogenous PA probes ROS-sensitive, extensive optimization is necessary as they are ‘always PA active’.²³⁻²⁸ Hence, it is advantageous to explore new strategies to prepare ROS-responsive PA chromophores.^{29,30} Ideally, the desired trigger-responsive probes convert from a PA-off to a PA-on state and/or exhibit a shift in the absorption maxima (preferably into the NIR-range) after the reaction of a particular ROS species.^{31,32}

Figure 1. Conceptual illustration of reversible ROS-trapping by probe for PA generation (depicted absorption and PA spectra changes are only for hypothetical purpose).

Previous trigger-responsive concepts are, typically, based on a cleavable group attached to the reporter probe (Figure 1, top, e.g. as in fluorophore-quencher conjugates). After the reaction of reactive species, the cleaved reporter molecule generates a stronger signal.³³ In this work, we describe a novel approach without any cleavable group, but trapping the trigger by the reporter molecule. In comparison to the cleavable probes it is of advantage that the consumption of the substrates does not continue and thus is not so much time dependent. Therefore, non-covalent trapping methods may generate a lower background signal. Our probe changes optical and photoacoustic properties by non-covalent interactions with the super oxide ion ($O_2^{\bullet-}$). To the best of our knowledge, this is the first time such a concept is described using a small molecule dye, and is applied to image $O_2^{\bullet-}$ by PA imaging. We report that oxoporphyrinogen **2** is suitable to trap the $O_2^{\bullet-}$, and gave a 9.6-fold increase in the PA signal accompanied by an 89 nm redshift, and is detectable in up to 3 μ M.

Experimental section

a) Free base porphyrin (1-2H): A dry round bottomed flask equipped with a magnetic stirring bar was charged with 725 mg (3.1 mmol, 1.02 eq.) of 3,5-di tert-butyl-4-hydroxy benzaldehyde, and 210 mg (3.02 mmol, 1 eq.) of hydroxylamine-hydrochloride ($NH_2OH \cdot HCl$) and dried on H.V. To this mixture 15 mL abs chlorobenzene was added, and the suspension was degassed for 10 mins by argon bubbling, 210 μ L (203.1 mg, 3.03 mmol, 1 eq.) of pyrrole was added. The mixture was stirred for 24h at r.t., followed by addition of 10 mL nitrobenzene and the mixture was placed in a pre-heated oil bath at 130 $^{\circ}C$ for 2h. After cooling the reaction, solvents were removed by vacuum distillation, and the residual solid was washed with $NaHCO_3$ (2 x 30 mL), extracted with (4 x 50 mL) CH_2Cl_2 , dried over Na_2SO_4 and concentrated on rotary evaporator. The dry mixture was dissolved in CH_2Cl_2 , precipitated by the addition of methanol (ca. 1:10 ratio) and centrifuged (10 min, 6000 rpm). The supernatants were removed and the obtained solids were dried in H.V. to obtain 218 mg (0.77 mmol, 25.5%) of microcrystalline product **1-2H**. 1H NMR (in CD_2Cl_2 , 400MHz) δ in ppm: 8.93 (s, 8H), 8.05 (s, 8H), 5.6 (s, 4H), 1.64 (s, 72H), -2.74 (br, 2H); ^{13}C NMR (in $CDCl_3$, 151 MHz) δ in ppm: 153.4, 133.9, 133.4, 131.8, 121, 34.6, 34.4, 30.7, 29.7.; HRMS (ESI, +Ve, $C_{76}H_{94}N_4O_4$, $M_{Calc.}$:1126.72751): found = 1127.73499 ($[M+H]^+$)

b) Synthesis of Oxoporphyrinogen (2) using TBAF:

A dry flask was charged with 19 mg (16.8 μ mol) of **1-2H** and 10 mL of abs. CH_2Cl_2 , followed by 70 mg of TBAF (221 μ mol, 13 eq.). The mixture was stirred at r.t. for 2 h, and the obtained dark blue mixture was poured in water, extracted with CH_2Cl_2 and dried over Na_2SO_4 . The solvent was removed on rotary evaporator and the reaction mixture was eluted through a short silicagel column using CH_2Cl_2 /hexane. The collected fractions were dried on rotary evaporator. The product was precipitated in CH_2Cl_2 /MeOH and dried in H.V. to obtain 13.2 mg (69.5 %) of blue microcrystalline product **2**.

c) Oxoporphyrinogen (2) using KO_2 : To 9.6 mg (8.5 μmol) of **1-2H** in CD_2Cl_2 (0.65 mL) and 18-crown-6 (47 μmol), was added KO_2 (total 8 mg, 110 μmol , in 8 steps over 3 h) in a NMR tube for titration. Finally, the reaction mixture was poured into water and extracted with CH_2Cl_2 . The reaction mixture was eluted through a short silicagel column and the product was precipitated in excess $\text{CH}_2\text{Cl}_2/\text{MeOH}$ to obtain 5 mg (52%) of **2**. ^1H NMR (in CD_2Cl_2 , 400MHz): 10.11 (br s, 4H), 7.53 (s, 8H), 6.81 (s, 8H), 1.33 (s, 72H); ^{13}C NMR (in CDCl_3 , 151 MHz) δ in ppm: 147.7, 134.4, 131.4, 119.7, 35.5, 30.7, 29.7, 29.4; MS:(ESI, +Ve, $\text{C}_{76}\text{H}_{92}\text{N}_4\text{O}_4$, Calc. 1124.71186): 1125.72034 for $[\text{M}+\text{H}]^+$)

d) Photoacoustic Imaging: The preclinical PA device VEVO LAZR 2100 (from Fujifilm Visual Sonics Inc., Amsterdam, NL) was equipped with a LZ 250 transducer (from Visual Sonics Inc.), having a centre frequency of 21 MHz was used for all measurements (26 db gain). The VEVO LAZR built-in pulse laser (wavelengths between 680-970 nm) was used with a step size of 5 nm in spectroscopy mode. The device was warmed up for 30 min and its energy was calibrated using an internally programmed and built-in sensor by placing it *ca* 10-11 mm above the sensor before the measurements. Tube phantoms were measured at 10-11 mm depth, i.e. the distance between the transducer and tube, in a water chamber and a total of 59 frames (5 nm steps) were recorded. The Vevo 2100 software was used for data processing. During the experiments, all variable parameters were kept constant i.e. photoacoustic gain, laser power, focus depth, frame averaging, and frame rate.

Results and discussion

Previously, the oxoporphyrinogen (OxP, **2**) was synthesized via aerial oxidation of *meso* tetrakis (3,5-di *tert*-butyl-4-hydroxyphenyl) porphyrin (**1-2H**).³⁴ **2** was also obtained by treating **1-2H** with fluoride ions (tetrabutylammonium fluoride, TBAF), followed by removing F^- from the complex.³⁵ Both, porphyrinoids **2** and **1-2H**, showed a weak absorption band in the range > 700 nm, but the absorption maxima upon complexation with F^- was shifted to above 800 nm (**2:F**). As basicity of F^- is similar to that of $\text{O}_2^{\bullet-}$,³⁶ we believed that $\text{O}_2^{\bullet-}$ could perform a similar complexation with **2** (and with **1-2H**), and thus motivated us to carry out $\text{O}_2^{\bullet-}$ -responsive PA imaging studies.

Chart 1. Structures of the *meso*(3,5-di *tert*-butyl-4-hydroxyphenyl) porphyrin **1**, and from it derived oxidized forms **2** and **3**.

Synthesis of tetrapyrrole probes

The synthesis of **1-2H** was reported by Milgrom in a one-pot condensation of 3,5-di *tert*-butyl-4-hydroxy benzaldehyde and pyrrole in hot propanoic acid (classical Adler-Longo conditions), which gave 13% yield after chromatography and recrystallization.³⁴ Lindsey *et al* reported generally applicable modified conditions for the synthesis of porphyrins using $\text{BF}_3 \cdot \text{OEt}_2$,³⁷ but those did in our case not result in **1-2H**. However, **1-2H** was successfully synthesized by us using $\text{NH}_2\text{OH} \cdot \text{HCl}$ catalyzed condensation (see SI).³⁸ The product **1-2H** was obtained in 25% yield without chromatography but by precipitation in methanol, as purple micro crystals, and identified with ^1H -NMR, UV-vis absorption and MS spectra. Metal ions ($\text{Ni}^{(\text{II})}$, $\text{Zn}^{(\text{II})}$) were incorporated by refluxing **1-2H** with $\text{Ni}(\text{OAc})_2$ and $\text{Zn}(\text{OAc})_2$ (see SI), and **1-Ni**, **1-Zn** were obtained as red and dark green micro crystals, respectively.

Then, oxoporphyrinogen chromophore **2** was prepared by treating **1-2H** with excess TBAF at r.t. for 2h and subsequently filtering it through a short silicagel plug followed by precipitation in MeOH.³⁵ Similarly, the product **2** can be obtained by reaction of **1-2H** with excess $\text{KO}_2/18\text{-crown-6}$ (18-C-6), subsequent aqueous washing and silica gel filtration. 18-C-6 was required to complex

K⁺ ion and to generate 'free' O₂^{•−} in organic solution. The obtained product **2** from TBAF or KO₂ reaction with **1-2H** has identical characteristics (Figure. S37). The ¹H-NMR spectrum of **2** in the presence of F[−] showed no loss of any protons, but only upfield shift of core NH protons (Figure. S38), confirming the non-covalent complexation of F[−]. The changes in ¹H-NMR chemical shifts of **2** to the added KO₂/18-C-6 (Figure S39) were similar to that of TBAF –upfield shift of internal NH protons, indicating a similar mode of binding. Furthermore, changes in ¹H-NMR chemical shifts during the titrations of **1-2H** with KO₂/18-C-6 and with TBAF confirmed that both ionic species oxidize **1-2H** to **2** and formed a complex with the core NH protons (see Figure S41, Figure S42). The absorption spectra of **2** in the presence of O₂^{•−} (and F[−]) showed a stronger redshifted band than that of pristine **2**, thus also confirming the complexation (Figure. 2b). Therefore, it can be concluded that **2** traps the O₂^{•−} (and F[−]) non-covalently and is suitable for PA imaging of O₂^{•−}.

In order to evaluate whether the internal pyrrolic NHs are necessary for O₂^{•−}-binding, and whether metallated porphodimethenes (**3-Ni** and **3-Zn**) exhibit superior PA properties than **2**, the porphyrins **1-Ni** and **1-Zn** were oxidized via treatment with excess TBAF at r.t. for 2h, and purified by a short-plug chromatography. ¹H-NMR analysis of **1-Ni**, **1-Zn** titration with TBAF (Figure S43, Figure S44) showed similar spectral changes as in **1-2H**, and yielded **3-M** as the final products. This confirms that metallo-porphyrins can be activated by F[−]. However, when the oxidation of **1-Zn** was carried out with KO₂/18-C-6, **3-Zn** could not be isolated but a decomposition process was observed. Moreover, the stability of **3-M** was found to be lower than that of **2**, possibly due to disproportionation to the respective porphyrins in solution (as observed in ¹H-NMR, >5% of **1-Ni** in **3-Ni**, Figure S45). This lability of **3-M** was also perceived by visible colour changes to the eye (from bright green to a reddish shade) and UV-vis (appearance of Soret band). This implies that metal-free **2** is better suited as O₂^{•−}-responsive PA probe than the metallated ones.

Photoacoustic and absorption spectra measurements

Photoacoustic spectroscopy experiments were carried out using DMF solutions of oxoporphyrinogen **2**, **3-Ni**, **3-Zn**, and porphyrin **1-M** (M = 2H, Zn, Ni), and ICG as standard. The Vevo LAZR 2100 (Visual Sonics Inc.) photoacoustic scanner was used to measure PA signals in polythene tubes (i.d = 0.38 mm) filled with different porphyrinoid solutions in a water chamber phantom at excitations between 680-970 nm.

Figure 2. a) The change in absorbance at 750 nm in response to given reactive trigger species (25 μM in DMF) is shown. b) UV-vis (full lines, left axis) and PA spectra (dotted lines, right axis) of ICG (7.8 μM), **2** (100 μM), **2**+KO₂ (25 μM) and **2**+TBAF (25 μM). To induce

trigger response, to 25 μM solution of **2**, excess $\text{KO}_2/18\text{-C-6}$ and TBAF were added, and the other triggers were added to 125 μM solutions of **2**, c) PA images of tube phantoms containing ICG, **2**, **2**+ KO_2 and **2**+TBAF, for excitation at their PA_{Max} , and DMF as control.

The measured PA spectrum of **2** (100 μM) was in agreement with the absorption spectrum, featuring a sharply decreasing band from 680 nm to 750 nm (see Figure 2b). We then tested the PA properties of **2** in the presence of $\text{O}_2^{\bullet-}$ and F^- , as well as with other reactive oxygen and nitrogen species (Figure 2a). An excess of TBAF, $\text{KO}_2/18\text{-C-6}$ was added to a solution of **2** (25 μM) and the PA signal was measured. To our satisfaction, $\text{O}_2^{\bullet-}$ addition (**2**+ KO_2) resulted in a 9.6-fold stronger PA-signal (PA signal of **2** at 25 μM was only at background level, see Figure S19, hence 100 μM solution was taken and the concentration normalized for comparison), with a redshift of 89 nm (observed PA maximum, PA_{Max} : 745 nm) (Figure 2b). When PA spectroscopy of increasingly diluted solutions of $\text{O}_2^{\bullet-}$ -activated **2** was performed, we found that PA signals were detectable down to 3 μM (Figure S20). Furthermore, we investigated the detection limit of $\text{O}_2^{\bullet-}$ by **2** using UV-vis and found that 550 nmol/mL of $\text{O}_2^{\bullet-}$ can be detected by 100 nmol/mL of **2** in solution. The $\text{O}_2^{\bullet-}$ concentration in healthy mitochondria is 0.1 pmol/mL, but increases in varicosity as high as >69 nmol/mL.³⁹ As the PA detection of **2** (with $\text{O}_2^{\bullet-}$) is possible in 3 nmol/mL, **2** is suitable to image 16 nmol/mL concentration $\text{O}_2^{\bullet-}$. The effect of other ROS triggers (H_2O_2 , $^t\text{BuOOH}$, OCl^- , $^{\bullet}\text{OH}$, and $^t\text{BuO}^{\bullet}$; radicals were generated by $\text{Fe}^{(II)}$ addition) and RNS (NO_2^- , NO_3^- , ONOO^-) species were studied in DMF solution by UV-vis and PA spectroscopy (Figure 2a, Figure S5). Insignificant changes in absorbance and PA gain to these reactive species lead to the conclusion that only $\text{O}_2^{\bullet-}$ induce this trigger effect. It was also found that the $\text{O}_2^{\bullet-}$ -containing solution of **2** was stable for > 7 days (Figure S15a) and **2** can be regenerated by treating the solution with citric acid (Figure S15b).

Then, we performed PA spectroscopy with **2** and F^- using TBAF, which showed a 16-fold intense PA-signal and a redshift of over 160 nm (observed PA_{Max} : 815 nm) (Figure 2b) compared to pure **2**. The dilution series PA spectra confirmed F^- -activated **2** solutions could be detected down to 3 μM (Figure S21). Furthermore, the detection limit of F^- by **2** was also investigated by UV-vis, and found that 400 nmol/mL of F^- could be detected using 100 nmol/mL of **2**. Complete activation of UV-vis solution and thus saturation was achieved by around 2 $\mu\text{mol/mL}$.

In addition to ROS, we investigated the effect of halides (Cl^- , Br^- and I^- as $\text{Bu}_4\text{N}^+\text{X}^-$ salts) since **2** resembling calix[4]pyrroles are known to bind anions.⁴⁰ Furthermore the effects of potassium and tetrabutyl ammonium cations (as KBr, and as hexafluoro phosphate salt $\text{Bu}_4\text{N}^+\text{PF}_6^-$) on **2** were investigated (Figure S5). None of the above ions changed the absorption spectra further indicating that the trigger effect was selectively caused by $\text{O}_2^{\bullet-}$, and its mimic fluoride ion.

Figure3. PA spectra of ICG (12.5 μM), **3-M** (**3-Ni**: starred, **3-Zn**: dotted lines) and **3-M**+ KO_2 , **3-M**+TBAF (all in 25 μM solutions). To induce trigger response, excess $\text{KO}_2/18\text{-C-6}$ and TBAF were added to 25 μM solutions of **3-M** and recorded PA immediately.

Table 1. Change in optical and photoacoustic properties of **1-M**, **2**, **3-Ni** and **3-Zn**, in response to added triggers

No.	UV-vis λ_{Max} ^[a] (PA Avr at 685nm ^[b])	+ $\text{O}_2^{\bullet-}$ ^[c] PA_{Max} nm (PA Avr)	$\text{O}_2^{\bullet-}$ in- duced red shift in nm ^[d] (PA signal gain, fold)	+ F^- ^[c] PA_{Max} in nm (PA Avr)	F^- induced red shift in nm ^[d] (PA signal gain in- fold) ^[e]
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			in fold) ^[e]		
2	654 (0.23)	745 (0.95)	89 (9.6)	815 (1.27)	161 (16)
3-Ni	661 (0.40)	760 (0.95)	99 (4.9)	680 (0.85)	19 (2.1)
3-Zn	690 (0.44)	740 (1.43)	50 (6.4)	875 (1.63)	185 (6.5)
1-2H	655 (0.25)	755 (0.69)	100 (4.1)	790 (0.87)	135 (5.8)
1-Ni	535 (0.22)	760 (0.95)	225 (5)	680 (0.76) ^[f]	110 ^[f] (3.5)
1-Zn	606 (0.18)	740 (0.84)	134 (5)	875 (1.41)	269 (10.5)

[a] The farthest absorption band maxima in UV-vis; [b] Found PA Avr (25 μ M, in DMF) at 685 nm was taken when no λ_{Max} in the PA measured range was available; [c] The observed PA Avr (a.u) of excess trigger contained 25 μ M solutions injected into a tube phantom (0.38 mm i.d.); [d] The redshift was calculated as difference from absorption and PA maxima, when no PA band above 680 nm was available; [e] Gain ratios for triggers were determined by dividing found PA Avr (a.u) at PA_{Max} with that of neat solution at the same wavelength; [f] PA signal at 680 nm was taken as the observed λ_{Max} in UV-vis was at 645 nm.

and 15.7-fold for $\text{O}_2^{\bullet-}$ and F^- triggers, respectively. The metallated porphodimethenes (**3-M**) solutions gave over 5-fold gain for $\text{O}_2^{\bullet-}$. This indicates that **2** can be highly suitable for superoxide sensing PA imaging applications.

Subsequently, PA spectroscopy of metallated porphodimethenes (**3-M**) was performed along with the added $\text{O}_2^{\bullet-}$ and F^- triggers (Figure 3). The DMF solutions of neat **3-Zn**, **3-Ni** showed their PA_{Max} below 680 nm with sharply decreasing PA bands resembling their absorption spectral bands (Figure S29, S30). When $\text{O}_2^{\bullet-}$ was added to the **3-Zn** solution, an immediate 50 nm redshift (PA_{Max} : 740 nm) with a 6.4-fold gain in PA signal was observed. However, the stability of $\text{O}_2^{\bullet-}$ containing **3-Zn** solution was severely minimized, as the observed PA gain sharply decreased from a fresh (3 minutes) to 30 minutes old solution. This is due to decomposition of the **3-Zn** in the presence of $\text{O}_2^{\bullet-}$, as also observed in the synthetic process. The PA signal of **3-Zn** solutions after addition of F^- was remarkably redshifted by 170 nm (PA_{Max} : 875 nm) with a 6.5-fold gain (Figure S32), whereas in **3-Ni**, the F^- ion induced only a minimal red-shift (19 nm) and a minor gain in PA signal (2.1-fold). However in **3-Ni**, to our satisfaction, $\text{O}_2^{\bullet-}$ redshifted the PA maximum by 100 nm (PA_{Max} : 760 nm) with a 5-fold gain in PA signal (Figure S33) compared to **3-Ni** at the same wavelength. This unusual change in redshift in response to F^- could be explained by the strong electronegative nature of the Ni^{II} metal ion that could withdraw the porphyrin anion electron density better than Zn^{II} . For $\text{O}_2^{\bullet-}$, a radical formation is expected, which preferentially resides at meso-aryl, hence the effect of the metal ion is nominal, thus, **3-Ni**, **3-Zn** gave similar redshift in PA values.

The gain ratio in PA signals induced by triggers at 800 nm (PA_{800}) was calculated (Figure 4). The maximum at 800 nm (PA_{800}) was chosen for comparison as it is closest to the isosbestic point of oxy- and deoxy-hemoglobin and the PA_{Max} of ICG. We found that the gain in PA_{800} signal was highest for **2**, i.e. 8.8-fold

Figure 4. Gain in PA signals at 800 nm for compounds **2**, **3-Zn** and **3-Ni** (in 25 μ M solutions) after adding $\text{O}_2^{\bullet-}$ and F^- triggers. PA gain ratio was taken as 1, by default, for without trigger added solutions. Excess KO_2 /18-C-6 and TBAF were added to 25 μ M solutions of **2**, **3-M** to induce trigger response. Standard deviation from three measurements of all the solutions was below 5%.

Subsequently, we explored the response of porphyrins **1-M** (M= 2H, Ni, Zn) to both triggers. For this purpose, all **1-M** were first titrated with KO_2 in the presence of 18-crown-6 (18-C-6) (in DMF or CH_2Cl_2) and UV-vis were obtained and compared with those from TBAF titration (Figure S2, S3, S9-S12). In detail, the UV-vis absorption pattern of **1-M** changed similarly in response to $\text{O}_2^{\bullet-}$ and F^- ions: a loss of Soret and Q bands of porphyrin and appearance of a broad absorption band above 650 nm were observed. Moreover, there was a lower redshift for $\text{O}_2^{\bullet-}$ than for F^- in **1-2H**, **1-Zn**, whereas with **1-Ni**, a higher redshift for $\text{O}_2^{\bullet-}$ than for F^- was found.

PA spectroscopy was performed, analogously to **2**, with **1-2H** (in DMF, conc.: 67 μ M), **1-Ni**, **1-Zn** (in DMF, conc. : 100 μ M) and ICG (in DMF conc.: 7.8 μ M) to assess its potential as trigger responsive imaging agent. Pristine **1-2H**'s PA signal was almost at background level due to the lack of absorption bands beyond 680 nm (Figure 5a). Solutions of **1-2H** with F^- and $\text{O}_2^{\bullet-}$ showed

trigger-induced photoacoustic signals, the PA maximum at 755 nm and 790 nm, with an average 6- and 4-fold increase in PA signals, respectively (see Table 1). In order to evaluate the sensitivity of PA imaging for **1-2H**+KO₂ and for **1-2H**+TBAF, dilution series were generated,

Figure 5. O₂^{•-} and F⁻ trigger characteristic shifts in the UV-vis (full lines, left axis) and PA spectra (dotted lines, right axis) of the chromophores **1-M** (M = 2H, Zn, Ni) in DMF solution (excess TBAF, KO₂/18-C-6 were added to the **1-M** solutions to induce trigger response). a) **1-2H**, UV-vis spectra at conc. 10.5 μM, PA spectra at conc. 66 μM and ICG PA spectra at conc. 7.8 μM in DMF; b) **1-Zn**, UV-vis spectra at conc. 10 μM and PA spectra at conc. 25 μM; c) **1-Ni**, UV-vis spectra at conc. 10 μM and PA spectra at conc. 25 μM. d) PA images of tube phantoms filled with **1-M** solutions with and without activation by KO₂ or TBAF recorded at their respective maxima. ICG and DMF loaded phantoms were measured along as controls.

and PA signal could reliably be detected for concentrations of 8.3 μM.

Further, PA spectroscopy measurements were performed using the metallated porphyrins **1-Zn**, **1-Ni** in DMF solutions, analogously to **1-2H**. As expected, **1-Ni**, **1-Zn** generated no PA signals even in concentrations as high as 100 μM (Figure S22, S23). This is due to the absence of absorption bands in the PA imaging range (i.e. > 680 nm). In response to O₂^{•-} and F⁻, the absorption maximum of **1-Zn** was red shifted by 134 nm (PA_{Max} : 740 nm) and 269 nm (PA_{Max} : 875 nm), and exhibited a 5-fold and a 10.5-fold gain in PA signals, respectively (Figure 5b, and Figure S24, S26). Moreover, we also observed that the stability of O₂^{•-} containing **1-Zn** solution was lower and the PA signal intensity of approximately 30 min old solution was far below that of a freshly prepared one (see Figure S25, S26). This was due to oxidative decomposition of the formed **3-Zn** in the presence of O₂^{•-}, which was also observed in the synthetic process. In contrary, the solution of **2** in the presence of O₂^{•-} exhibited high stability, for more than 7 days at 4 °C (see Figure S6) without any decomposition.

In the case of **1-Ni**, the above triggers showed an opposite effect, i.e. a redshift of 225 nm for O₂^{•-} (PA_{Max} : 760 nm), and only 105 nm for F⁻ (λ_{Max} : 645 nm). The observed gain in PA signal for O₂^{•-} trigger was 5-fold (calculated at 760 nm) and 3.5-fold for F⁻ (calculated from 680 nm PA signal). Furthermore, to explore the detection limit of the trigger activated **1-Ni**, **1-Zn** solutions, dilution series were prepared and PA spectroscopy was performed. We found that the PA signal can be detected down to a 3.1 μM concentration (Figure S24-S28). Therefore, we conclude that porphyrins **1-M** (M = 2H, Zn, Ni) might also be suitable for O₂^{•-} trigger responsive PA imaging applications.

Biocompatibility and signal attenuation

The porphyrins and oxoporphyrinogens investigated above are hydrophobic and thus need to be formulated with excipients to obtain aqueous solubility. Cremophore EL (CrEL) formulation is a commonly used approach to enhance the water solubility of hydrophobic pharmaceutical compounds, and thus was applied here. As the stability and trigger response of the oxoporphyrinogen **2** was found to be higher than the others, we used **2** in CrEL formulation (**2:CrEL**) in further studies.

The probe **2** was encapsulated in 0.15% CrEL (w/w) and the resulting **2:CrEL** formulation was prepared in phosphate buffer saline solution. The biocompatibility of **2:CrEL** was studied on human prostate cancer PC3 cells using the XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxyanilide salt) assay. We found that doses of up to 50 μM were compatible (Figure 6). For trigger activated PA imaging, as proven in dilution series experiments, concentrations of up to 3 μM are sufficient to generate a PA signal, hence, **2** is suitable for further studies.

Table 2. Compilation of observed properties with respective to optimal need

No.	Stability	Biocompatibility	Red shift in λ_{PAMax} (in nm) for $\text{O}_2^{\bullet-}$	Gain in PA signal (fold) for $\text{O}_2^{\bullet-}$
2	+++	+++	89	9.6
3-Ni	+	n.a.	99	4.9
3-Zn	+	n.a.	50	6.4
1-2H	+++	++	100	5.8
1-Ni	+++	++	225	5
1-Zn	+++	++	134	5

n.a.: not measured; +++: very good

++: good ; +: moderate

Figure 6. Viability of PC3 cells after incubation with **2**:CrEL in different concentrations (XTT-assay). CTRL: cells in culture medium with only 0.15% CrEL.

Subsequently, to mimic *in vivo* light absorption and ultrasound attenuation conditions, we performed PA imaging in a chicken muscle phantom. Polythene tubes containing solutions of **2** and **2**+KO₂ (conc.: 125 μM and 20 μM) we replaced within a chicken muscle and PA imaging was performed (Figure 7). The O₂^{•−}-activated solution was clearly visible in 11-16 mm depths (Figure S35). Higher depth is not possible with the commercial Vevo LAZR device, as it is engineered to produce a focussed light at 11 mm depth with diffused light a further few millimeters deeper. The neat **2** in chicken muscle showed no PA signal at 750 nm even at 125 μM, but gave a very weak signal at 700 nm. These confirm that probe **2** in combination with superoxide trigger may be well suited for *in vivo* PA imaging applications.

Figure 7. Ultrasound B-mode (top) and (bottom) PA images (at their PAMax) of neat **2**(left, conc. : 125 μM) and **2**+KO₂/18-C-6 (right, conc. : 20 μM) in chicken muscle.

Photostability

The oxoporphyrinogens were investigated for their photostability using red light irradiation (680-700 nm LED, with power output 100 mJcm^{−2}).⁴¹ After irradiation of the solution of **2** (in DMF, 125 μM) for 40 min (total power: 240 Jcm^{−2}) an 18 % lower absorbance than in the initial solution was found by UV-vis spectroscopy (Figure S7). On the contrary, irradiation of **3-Zn** and **3-Ni**, for 40 min showed only 1-3 % absorbance loss. For PA imaging, the applied power produces intensity in the range of 20-100 mJs^{−1} and imaging can be completed within 30 sec. Hence, the photobleaching of **2** is expected to be less 0.3 % in PA studies, which is minimal.

Conclusions

Here we showed for the first time superoxide sensing probes for photoacoustic imaging based on porphyrin, oxoporphyrinogen and porphodimethene dyes. These porphyrins are robust and biocompatible molecules, which can be used to visualize the presence of highly reactive species such as O₂^{•−} by photoacoustic imaging. We found that the oxoporphyrinogen **2** and its porphyrin precursor (**1-2H**) exhibit similar stimuli-responsive characteristics with a gain in PA signals and a red-shift in PA maxima. In detail, in response to O₂^{•−}, probe **2** exhibited the highest gain in the PA signal (9.6 fold at PAMax:745 nm), while **1-Ni** showed the highest red shift (225 nm) with a 5-fold gain in PA signal. The metal-free **1-2H** reacted as well with O₂^{•−} and showed an over 100 nm red shift and an up to 5-fold increase in PA signal. Of those, the metal-free oxoporphyrinogen **2** was convinced by its high biocompatibility and stability towards O₂^{•−} after activation. Thus, we are planning to explore its potential, with *in vivo* suitable compounds for superoxide imaging applications in future studies.

ASSOCIATED CONTENT

Supporting Information. UV-vis, fluorescence spectra, titrations, NMR and ESI-MS spectra and photoacoustic imaging data. "This material is available free of charge via the Internet at <http://pubs.acs.org>."

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ABBREVIATIONS

PA, photoacoustic; H.V., high vacuum; MS, mass spectrometry; DMF, N,N-dimethylformamide, μ M, micromolar.

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TOC Figure: