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Luminescent probes for the bioimaging of small anionic species in vitro and in vivo
Luminescent probes for the bioimaging of small anionic species \textit{in vitro} and \textit{in vivo}

Trent D. Ashton,$^a$ Katrina A. Jolliffe$^b$ and Frederick M. Pfeffer$^{*a}$

The ability to spatiotemporally identify the formation of specific anionic species, or track changes in their concentration inside living systems, is of critical importance in deciphering their exact biological roles and effects. The development of probes (also called bioimaging agents and intracellular sensors) to achieve this goal has become a rapidly growing branch of supramolecular chemistry. In this critical review the challenges specific to the task are identified and for a select range of small anions of environmental and biological relevance (fluoride, chloride, iodide, cyanide, pyrophosphate, bicarbonate, hydrosulphide, peroxynitrite, hypochlorite and hypobromite) a comprehensive overview of the currently available \textit{in vitro} and \textit{in vivo} probes is provided.

1. Introduction

1.1 Overview

The study of anion recognition is now a relatively mature science in line with the closely related field of cation recognition.$^{1-5}$ Over the last 10–15 years sustained effort from the supramolecular chemistry community has refined the fundamental principles relating to how a host interacts with a negatively charged guest.$^{1-17}$

Similarly anion sensing has matured and an array of effective molecular detectors, operating by means of well understood principles, are now available.$^{11,18-32}$ An excellent recent tutorial review by Gale (see also other articles in this special issue) neatly highlights the strategies that are now widely employed in the detection and/or quantification of anionic species.$^{33}$ While the field has matured, challenges still exist for the detection of anions in water; the heavily hydrated nature of these species in aqueous environments makes strong binding difficult and also hinders their reactivity.$^{7,12,26,34}$

As the study of anion recognition and sensing has advanced supramolecular chemists have applied their fundamental knowledge to the detection of anions of biological significance.$^{14,21,35-46}$ Indeed the rise of anion recognition as a field of study was in no
small part due to the fact that the majority of intracellular operations involve anionic species. A natural extension of such efforts is the detection or sensing of biologically relevant anions in a biologically relevant setting such as inside living cells or in living organisms.52,53,55,56 Thus the field of anion imaging has emerged and supramolecular chemists now find themselves planning and executing the synthesis of reporters to selectively detect and indicate the presence of anions inside living cells and organisms. Probes capable of achieving this feat are amongst the most powerful resources available for elucidating the exact biological role of the target anion. While the number of probes capable of efficiently communicating an anion recognition event from such a venue is growing, it is still small when compared to the large number of intracellular sensors/probes for cationic species61 (see also other articles in this special issue). As such the biological role of the target anion. While the number of probes capable of efficiently communicating an anion recognition event from such a venue is growing, it is still small when compared to the large number of intracellular sensors/probes for cationic species61 (see also other articles in this special issue). As such the field provides fertile ground for both emerging and established researchers alike.

1.2 Challenges

The ideal anion sensor functioning in vitro or in vivo must satisfy a demanding set of criteria (outlined in brief in Table 1) and it is clear from this list that an imaging agent must ‘do more’ than a sensor. Key challenges include (i) selecting a suitable fluorophore, (ii) choosing an effective switching mechanism and (iii) catering for the biological environment in which the probe must function (see additional discussion for these three points below). Few, if any, of the currently available probes satisfy all of these criteria and given that sensors that are truly selective for specific anions in water have only emerged in the last 10–15 years it is no surprise that the development of selective anion sensors for bioimaging applications is currently at the forefront of applied supramolecular chemistry.

1.2.1 The fluorophore. An extensive range of fluorophores are now available, however, for in vitro work those that are not just bright (ε > Φ) but have NIR emission are of considerable benefit.54,65–67 A “window” of increased optical transparency exists in the range 650 to 950 nm and at these wavelengths tissue autofluorescence is minimised and the major interferents (haemoglobin, lipids and water) absorb to a less significant extent.66 The use of NIR emission also minimises light scattering and is far less likely to damage the living system. The use of multiphoton excitation (or inverse Stokes) techniques is also of particular relevance for in vivo studies68,69 and several multiphoton probes for anions are described herein. For in vitro studies which require little photon penetration the requirements are slightly more forgiving and probes with emission wavelengths ranging to 350 nm have been successfully used. In all cases a large Stokes shift is desirable to minimise light scattering. Emission wavelengths (and in turn Stokes shift) are known to be influenced by a number of factors including the structure of the excited state and solvent reorganisation upon excitation.70–72 Recently, quantum chemical calculations have been employed in the design of fluorophores with ca. 200 nm Stokes shift.73

While well-known fluorophores (such as rhodamine, fluorescein, BODIPY and cyanine) are common in anion imaging studies, several unconventional fluorophores (e.g. Si, Se, Ge and Te rhodamines and squaraine-rotaxanes) have successfully been used in recent years. An increasing number of effective probes also employ lanthanide based luminescence for signal transduction.72-75,80-83

1.2.2 The switch. The ideal probe must either “switch on” in the presence of the target analyte or if quantitation is required the probe should arrive with an “internal standard” present i.e. the active signalling luminophore should be coupled to a fluorophore that has a constant unwavering response in the biological environment. Such ratiometric sensing allows exact quantification of

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**Table 1** Design criteria for anion sensors versus anion probes in vitro and in vivo

| For recognition/sensing in water | For recognition/sensing in vitro and in vivo |
|----------------------------------|---------------------------------------------|
| Selective for the target in water | Selective for target in cells/small organisms |
| Strong binding/signalling and low detection limit | Sensitive at relevant biological concentrations (e.g. Cl⁻ vs.ONO²⁻) |
| Water soluble | Water soluble yet amphiphilic for cell permeability |
| “Switch on” or ratiometric | Localise in relevant compartment |
| Large extinction coefficient, quantum yield and Stokes shift. | Non-toxic |
| | “Switch on” or ratiometric |
| | Large extinction coefficient, quantum yield and Stokes shift. |
| | Red or NIR emissive |
| | Photostable and metabolically stable |

Fred Pfeffer received his BSc (Hons 1) in 1996 and PhD in 2001 from Deakin University. He then worked for three years at Trinity College Dublin, first as a lecturer then as postdoctoral fellow in the group of Prof Thorfinnur Gunnlaugsson. In 2004 he returned to Australia as lecturer and in 2010 was appointed Senior lecturer. Research interests include many aspects of supramolecular chemistry including anion recognition and sensing. A research focus is the synthesis and use of fused [n]polynorbornane scaffolds as preorganising elements for further applications including host:guest chemistry and the construction of metallo-supramolecular cages.

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the target as the response from the active signalling moiety can be measured against that of the “constant” fluorophore. Such requirements can also be met if both the unbound and bound probe are fluorescent at measurably distinct wavelengths. A recent article by Sessler highlights the use of ratiometric probes for bioimaging applications.43 Unfortunately, for many anions (such as Cl\(^-\), I\(^-\), HCO\(_3\)\(^-\) and BrO\(_3\)\(^-\)) the current list of selective “switch on” and ratiometric probes is limited.

A probe can also be classified according to the electronic event by which fluorescence is “switched” or modulated (for example ICT/PET modulation, FRET, heavy ion effect, and excimer formation). Many sensors—known as chemodosimeters—have been designed such that a chemical reaction controls this modulation\(^{30,33,35,49,86}\) and it is logical that these strategies have been adopted by those pursuing the goal of in vitro and in vivo imaging. While the chemodosimeter approach is by far the most popular (and is excellent for the selective detection of a specific species) the chemodosimeter is, in most instances, irreversibly transformed to the signalling moiety and as such true spatiotemporal information cannot be gleaned. Continued effort from the research community is required to achieve the goal of tracking rather than trapping the anionic species of interest. Another very popular approach to modulating fluorescence is the displacement approach.\(^{27,46,87}\) The luminophore is quenched by a species that is non-covalently attached and the luminophore–quencher combination is chosen such that the target anion interacts with the quencher more strongly than it does with the luminophore. Hence the quencher is displaced, the luminophore is released and fluorescence is “switched on”.

1.2.3 **The biological environment.** The solvent for life is water, hence the probe must possess a degree of water solubility and many hydrophobic probes have been made more biocompatible by attaching either a PEG\(^{88}\) or a sugar.\(^{89}\) Nevertheless, a hydrophilic/hydrophobic (amphipathic) balance must be struck such that the sensor can passively diffuse through cell membranes. In the wider field of cellular imaging a commonly employed manoeuvre to ensure uptake is to mask polar hydroxy groups and carboxylates as esters (especially acetoxymethyl (AM) esters)\(^{90}\) that are subsequently hydrolysed by one of the myriad of intracellular esterases to release the desired probe. This approach has been adopted in the field of anion imaging agents and three peroxynitrite probes are shown in Fig. 1 as examples. The early (1997) dichlorodihydrofluorescein was used as the diacetate diester DA-DCHF\(^{91}\) and the more recent (2010) HKGreen3 employed a single acetate.\(^{92}\) The tetra-acetoxymethyl ester AM-DTTA passively diffused into cells whereupon the tetracarboxylate ligand (DTTA) was liberated and in the presence of terbium and europium the desired lanthanide probes assembled in cellulo.\(^{93}\) An added benefit of this approach is that the unmasked probe is typically retained inside the cell, nevertheless, even probes containing carboxylates can be expelled from the cell by active anion transport mechanisms.\(^{94}\)

Ideally once the probe is inside the living entity it should localise in the most relevant sub-cellular compartment. Guidelines to predict the likely compartmentalisation of new probes are not unequivocally established\(^{83,95}\) and colocalisation studies with well-established dyes are generally required. Nevertheless some general trends exist: (i) cationic probes gravitate to the mitochondria\(^{96,97}\) as the mitochondrial membrane is negatively polarised and (ii) weakly basic probes accumulate within the more acidic lysosomes.\(^{98}\) These general guidelines have also been adopted in the field of anion bioimaging, for example, the recently described probes for hypochlorite Rh-TPP and Rh-Py (Fig. 2)\(^{99}\) employ a triphenylphosphonium and pyridinium appendage respectively for mitochondrial localisation. The intracellular sensor for hydrosulfide Lyso-NHS used a morpholine substituent for lysosomal localisation.\(^{100}\)

**Fig. 1** Examples of peroxynitrite probes DA-DCHF, HKGreen3 and AM-DTTA in which intracellular uptake and subsequent trapping was performed using a lipophilic ester (highlighted in red) that was cleaved in vivo by intracellular esterases.

**Fig. 2** Examples of probes that localise in the mitochondria (Rh-TPP, Rh-Py) and lysosome (Lyso-NHS).

1.3 **Structure of this review**

In conjunction with a comprehensive listing of recent examples (the majority of examples are from the last 5 years) the broad concept of this review is to provide both a “why” and “how to” target the specific anion of interest. For each of the anions covered herein a justification of the cellular relevance is first provided—even anions of obvious environmental importance (such as cyanide and fluoride) have considerable relevance and interest for intracellular studies (see Section 2). Also covered are...
a number of anions that are of relevance primarily at a cellular level (see Section 3), for example bicarbonate plays a critical role in living systems as a measure of CO₂ uptake/respiration (hypercapnia/hypercapnia = CO₂/HCO₃⁻ poisoning respiratory acidosis). Similarly, reactive oxygen and nitrogen species (such as hypochlorite ClO⁻ and peroxynitrite ONOO⁻) have critical in vivo roles and elevated levels of these species are associated with many disease states (see Section 4). Where possible, examples have been grouped by the means (mechanism) by which sensing is achieved and also whether the probes are: intensity modulated (“switch off” or “switch on”) or ratiometric (wavelength modulation). The terms fluorescent probe, anion imaging agent and intracellular sensor are all used interchangeably.

2. Anions of environmental and biological relevance

There now exists a number of excellent sensors for anions such as fluoride and cyanide, widely recognised as anions of environmental concern. While not commonly appreciated, these anions also have significant relevance in a biological setting and a number of intracellular probes have been developed for their detection. Chloride has a more passive, nonetheless important, role in the environment and, like iodide, plays an important physiological role. Anions covered in this section are fluoride, chloride, iodide and cyanide.

2.1 Fluoride

Fluoride is a very well-known anion due to its use in drinking water and toothpaste to prevent dental caries and osteoporosis. Nevertheless excess fluoride is responsible for a number of deleterious conditions including dental and skeletal fluorosis and is now linked to cancer and neurotoxicity. Probes capable of selectively indicating fluoride in vitro and in vivo may assist in clarifying the exact biological roles of this anion.

Given its “Janus” behaviour the recognition and sensing of fluoride has been a focus of supramolecular chemists. Two approaches that have been widely used in the design of both sensors and bioimaging agents are (i) deprotonation (Section 2.1.1) and (ii) desilylation (Section 2.1.2). Deprotonation, mediated by the strongly basic fluoride anion, leading to enhanced ICT of a luminophore, was one of the first means by which this anion was detected, and while the approach has been used for imaging, this design is prone to interference from other basic anions (such as acetates). By far the most common approach employs the fluoride mediated desilylation reaction of chemodosimeters that have been designed with a silyl ether.

2.1.1 Fluoride mediated deprotonation. The phenolic naphthalimide probe NIM (Fig. 3) was reported in 2014. The probe was both colourimetric (strong absorption band emerging at 641 nm in the presence of F⁻) and fluorescence “switch off” (λ_ex = 490 nm, λ_em = 582 nm decreases). In solution, a similar, yet less pronounced, change was also recorded with acetate—a common interferent for probes operating by means of deprotonation. Of interest, and hinting at an additional role for this probe, the fluorescence of the unreacted probe was considerably enhanced in the lysosomes of cancer cells as opposed to healthy cells. Preliminary experiments indicate that both a protein-rich and an acidic environment (such as in cell lysosomes) were required for the enhancement.

The ratiometric hydroxynaphthalene probe 2 (Fig. 4) was reported by Liu and Ke in 2014. A PEG cyanoacrylate was included to enhance ICT and also balance solubility. The probe was selective to fluoride (no significant fluorescent changes were elicited by AcO⁻) and the emission intensity ratio I₄₉₀/I₂₄₀ nm could be used to quantify fluoride up to 10 equivalents with a limit of detection (LOD) of 8.5 μM. The probe was cell permeable, non-toxic to prostate cancer (PC3) and epithelial cervical cancer (HeLa) cells and located in the cytoplasm of these cells (confirmed using the red nuclear stain propidium iodide—PI). In PC3 cells a clear change in emission colour was observed when cells pre-treated with 2 were exposed to fluoride.
of fluoride (and to a similar extent acetate) as weak emission at 425 nm increased and the ratio \( F_{425}/F_{512} \) was used to determine \( F/CO \) concentration (<200 equivalents). The N–H of indole has been used previously for the recognition and sensing of fluoride anions116,117 and for probe deprotonation significantly enhanced ICT and a clear change in fluorescence emission was recorded in murine macrophages (RAW264.7) upon addition of fluoride.

In 2013 Chellappa reported the rhodamine based probe RDF-1 (Fig. 6) that operates by means of deprotonation leading to spirocycle ring opening.118 A strong “switch on” fluorescence response at 557 nm was observed in the presence of \( F^- \). In HeLa cells RDF-1 was non-toxic and within 10 minutes of NaF addition significant fluorescence enhancement was observed.

2.1.2 Fluoride mediated desilylation. Many chemodosimeter have been designed to use the selective reaction of fluoride with silicon (incorporated as a Si–O–C bond) to form the exceptionally strong Si–F bond (> 800 kJ mol\(^{-1}\)). The approach is essentially identical to the fluoride mediated cleavage of silyl containing protecting groups.119 For the probes shown herein the hydroxy fragment is released as a phenoxide anion which is a component of an ICT fluorophore; hence the desilylation reaction leads to a dramatic fluorescence “switch on”.

One of the earliest “switch on” probes functioning by means of desilylation, a TBDPSO-coumarin (TBPCA, Fig. 7), was published by Park and Hong in 2009.120 The probe readily entered cells and was retained with no toxicity. Images in human epithelial lung carcinoma A549 cells show clear blue fluorescence upon exposure to NaF.

The “switch on”, red emitting probe 8 (Fig. 8) was recently published by Zhu (2014).121 Synthesis of the masked ICT fluorophore involved aldol type reaction of the potential electron donor 4-OTBPS-benzaldehyde with the electron withdrawn 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF). For probe 8 a linear emission “switch on” at 612 nm was observed upon reaction with fluoride and a LOD = 0.07 mM was determined. The absorption spectra could be used to quantify...
the amount of fluoride present in solution due to the linear relationship between the increase at 596 nm and the decrease at 438 nm. Imaging was performed in live HeLa cells and 10 μM NaF was readily visualised using fluorescence microscopy.

The highly selective, ratiometric, benzothiazolium hemicyanine 10 (Fig. 9) developed by Ma, Du and Zhang (2011)\textsuperscript{122} could monitor fluoride concentration using the ratio $F_{500}/F_{558}$. A limit of detection (0.08 nM) was identified and in live RAW264.7 macrophages a distinct ratiometric fluorescence response was observed upon addition of buffered NaF. The probe was also shown to penetrate rapidly (<5 minutes) and was non-toxic.

A variation on the desilylation probe was reported by Zhang in 2013.\textsuperscript{123} In the presence of fluoride, desilylation of the functionalised naphthalimide chemodosimeter 12 (Fig. 10) was immediately followed by fragmentation to give the conjugate base of 4-hydroxynaphthalimide 13. In solution studies a linear fluorescence "switch on" ($\lambda_{em} = 560$ nm) was realised (20-fold increase in one hour with only 1.0 equivalent of F$^-$/C$_0$; LOD = 0.35 μg L$^{-1}$). After incubation of probe 12 with human epithelial carcinoma cells (A549) addition of a solution of NaF elicited a distinct green fluorescent response.

With an eye to enhanced solubility the PEG-thiourea-fluorescein probe PEG-FITC-Si (Fig. 11) was synthesised by Zeng and Wu (2013)\textsuperscript{88} in two steps from the corresponding fluorescein isocyanate. Again in the presence of fluoride a dramatic increase in fluorescence ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 526$ nm) was observed (LOD = 19 ppb). Imaging was successfully achieved in HeLa and murine fibroblasts (L929) only 15 minutes following addition of fluoride (100 μM) with perinuclear probe localisation.

The TBSO-benzothiazole BBTGA (Fig. 12), also deliberately designed for biocompatibility by conjugating glucosamine to improve solubility, was reported by Wang in 2013.\textsuperscript{89} A linear

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Fig. 9 Top: structure and reaction of benzothiazolium hemicyanine 10 with F$^-$. Bottom: images of RAW264.7 macrophages incubated with 10 and no added F$^-$. (a) bright-field, (b) blue channel at 490 ± 20 nm, (c) orange channel at 560 ± 20 nm, and (d) ratio image from (c) and (b). Images of RAW264.7 macrophages incubated with 6 after addition of NaF (e) bright-field, (f) blue channel at 490 ± 20 nm, (g) orange channel at 560 ± 20 nm, and (h) ratio image from (g) and (f). Scale = 20 μm. Image reproduced with permission.\textsuperscript{122}

Fig. 10 Top: functionalised naphthalimide probe 12 and its reaction with F$^-$. inset shows fragmentation mechanism. Bottom: (a) bright-field image of A549 cells incubated with 12 (20 μM) for 24 h (a) bright field (b) without NaF (c) with NaF (150 mM). Image reproduced with permission.\textsuperscript{123}

Fig. 11 Top: structure of fluoride chemodosimeter PEG-FITC-Si. Bottom: fluorescence imaging of L929 (top) and HeLa (bottom) cells incubated with the sensor before (A and C) and after (B and D) treatment with NaF. Image reproduced with permission.\textsuperscript{88}

Fig. 12 Structure of BBTGA and 15, showing reaction of BBTGA with F$^-$. 
30-fold fluorescence enhancement ($\lambda_{em} = 508$ nm) was noted 5 minutes after the addition of NaF in PBS buffer. The probe was water soluble, non-toxic and when a buffered solution of NaF (0.1 mM) was added to human nasopharyngeal epidermal carcinoma (KB) cells that had been pre-treated with a dilute solution of BBTGA strong fluorescence was observed.

Again with solubility and biocompatibility in mind the carbohydrate conjugate probe 15 (Fig. 12) was synthesised by Du (2011) using the well-known copper assisted azide alkyne cycloaddition (CuAAC). $^{124}$ A very strong (160 fold), linear, fluorescence “switch on” response ($\lambda_{em} = 520$ nm) was observed with increasing NaF (from 0 to 1.4 mM, probe concentration 50 $\mu$M) and a limit of detection of 10.5 $\mu$M was identified. A strong fluorescence response was observed upon addition of NaF solution to Hep2G cells that had been incubated with 15 (Fig. 13).

The pyrene dimer 16 (Fig. 14) containing a disilane (Si–Si) bond was designed and constructed by Li and Shen (2012). $^{125}$ Ratiometric measurement in solution (THF: H$_2$O) was possible as the well-known pyrene excimer fluorescence at 470 nm ceased upon reaction of the probe with fluoride and only monomer emission at 378 nm was present. Up to 6 equivalents of F$^-$ could be measured using $F_{378}/F_{470}$. Loading of 16 into HeLa cells was performed using polyactic acid nanoparticles and upon exposure to fluoride a clear change in emission was detected.

In 2012 Lee, Kim and Ahn published an interesting variant on the desilylation probe. $^{126}$ Upon desilylation the carefully functionalised aminonaphthalene P1 (Fig. 15) reacts in an additional intramolecular process to give the extended aminocoumarin 20. A fluorescence “switch on” ($\lambda_{em} = 595$ nm) was observed in both murine metastatic melanoma (B16F10) cells and also in live zebrafish. Imaging was accomplished using two photon microscopy (TPM) and the lower excitation energy associated with this technique is perfect for in vivo research to understand how fluoride is distributed in a whole body context. In the zebrafish, increased concentrations of F$^-$ in the tail and abdomen were observed at $t = 2$ h versus $t = 30$ min.

A recent report (2014) by Song also outlined a dicyanoacrylate “switch on” probe (21, Fig. 15) in which a desilylation cascade approach was used to create a red fluorescent iminocoumarin. $^{127}$ The probe itself was non-fluorescent and for the product a Stokes shift of more than 140 nm was recorded. Using fluorescence microscopy the probe was shown to be readily internalised and was capable of indicating the presence of fluoride in living human keratinocyte (HaCaT) cells (not shown).

The related probe FP (Fig. 15) from the group of Peng (2014), $^{128}$ also relies on an additional reaction occurring post Si cleaving. The probe was synthesised from the corresponding quinolinecarbaldehyde and the final product of the reaction sequence is a highly fluorescent ($\phi_F = 0.84, \lambda_{ex} = 441$ nm, $\lambda_{em} = 485$ nm) aminobenzopyranimine. The probe was shown to be relatively non-toxic, localised in the mitochondria of both breast cancer (MCF-7) and fibroblast-like (COS-7) cells and fluorescence was dramatically “switched on” when the cells were treated with dilute solutions of NaF (Fig. 16).

### 2.1.3 Miscellaneous
A Se–B bond can be selectively cleaved by fluoride and the unusual red emitting BODIPY probe 22 (Fig. 17) reported by Tang (2011) was designed on this principle. $^{129}$ The presence of Se quenched fluorescence but upon reaction with fluoride the “usual” F–B bond was formed (23) and red fluorescence ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 690$ nm) was “switched on” (ca. three-fold enhancement). The chemodosimeter was selective for fluoride and the increased fluorescent response was readily observed in human hepatoma cells (HepG2) pre-treated with F$^-$. 

### 2.2 Chloride
Chloride is the most abundant anion in living organisms and its transport across cellular membranes is essential for a number of physiological processes including the maintenance
of cell volume, acidification of internal compartments and even electrical excitability. Impaired transport of this anion due to a genetic mutation in a cAMP-regulated Cl⁻/C₀ channel defines the condition known as cystic fibrosis (CF). Indeed, the pursuit of biologically active chloride transporters to remedy this condition is an important current goal for supramolecular chemists. Other than some recent developments (Section 2.2.3) the strategy employed in the design of chloride imaging agents relies on halide mediated collisional quenching (Section 2.2.1) and as such the majority of Cl⁻ probes are “switch off”. Nevertheless by attaching such probes to “constant” fluorophores several ratiometric probes have been successfully designed and used (Section 2.2.2).

2.2.1 Chloride mediated collisional quenching. Due to its recognised physiological importance the development of functionalised quinolones (such as SPQ and MEQ, Fig. 18) as chloride imaging agents was accomplished long before other anions were targeted and several are commercially available. Nevertheless, these quinolone based probes are “switch off”; they are not selective for chloride amongst other halides and they also suffer from photobleaching. Furthermore, unless invasive techniques are used to deliver the probe into cells, probes such as MEQ must be reduced (using NaBH₄ to the charge neutral, cell permeable dihydro form (dHMEQ) first which following uptake is oxidised back to the Cl⁻ responsive form. Despite their limitations, functionalised quinolone...
Fig. 18  Top: structure of SPQ, MEQ and the cell permeable diHMEQ probes for Cl\textsuperscript{−}. Bottom: images of MEQ-loaded neurons (left) before and (right) 20 min after GABA application. Scale = 15 μm. Image reproduced with permission.\textsuperscript{146}

![SPQ, MEQ, diHMEQ](image1)

Green emission from the bisacridinium (λ\textsubscript{em} = 505 nm) was quenched on addition of Cl\textsuperscript{−} while red rhodamine emission remained constant. The ratiometric probe was successfully used for intracellular imaging to link chloride concentration to endosomal acidification.

More recently, the ratiometric probe MQAF (Fig. 21) was reported by Tang (2012).\textsuperscript{153} The structure consisted of the “switch off” methoxyquinolium combined with aminofluorescein and monitoring at two channels [chloride sensitive emission λ\textsubscript{ex} = 318 nm, λ\textsubscript{em} = 436 nm and insensitive λ\textsubscript{ex} = 494 nm, λ\textsubscript{em} = 519 nm] gave accurate measurements of Cl\textsuperscript{−} concentration. This probe was successfully used in ventricular myocytes to illustrate that induced ischemia results in increased Cl\textsuperscript{−} concentration. In 2014 the same group published the ratiometric methoxyquinolium dansyl combination (MQDS, Fig. 19) and the ratio λ\textsubscript{em} = 440 nm against λ\textsubscript{em} = 560 nm was used to monitor chloride concentration.\textsuperscript{154} Imaging of liver cancer cells (HepG2) was performed and intracellular chloride concentration was successfully monitored as the extracellular levels in the surrounding media were deliberately increased (Fig. 21).

2.2.3 Recent developments. A “switch on” probe selective for \textit{in vitro} or \textit{in vivo} chloride has yet to be reported however the groups of Fusi and also Smith are getting close. Fusi reported that both the Cd(II) complex with nitrobenzooxadiazole-tetraazacyclododecane \textsuperscript{25} (λ\textsubscript{ex} = 410 nm, λ\textsubscript{em} = 520 nm) and also the bis Zn(II) complex of 25 (λ\textsubscript{ex} = 325 nm, λ\textsubscript{em} = 543 nm) act as “switch on” sensors for halides (Cl\textsuperscript{−} and F\textsuperscript{−}) (Fig. 22).\textsuperscript{155,156} It was postulated that for both complexes the metal to nitrogen (nitrobenzooxadiazole) distance and M to N (cyclam) distances change upon binding of chloride and it is the balance between fluorescence enhancement (ICT to nitrobenzooxadiazole) from freeing the N-nitrobenzooxadiazole nitrogen and quenching (PET) of nitrobenzooxadiazole fluorescence by the cyclam that leads to the fluorescence modulation. The ligands have been internalised in human neuroblastoma (HeLa) cells to give fluorescence signals, however, images of the halogen sensitive metal complexes functioning in cells have not yet been published.

Another new, and very interesting, class of chloride probes are the squarine-rotaxanes developed by Smith (Fig. 23).\textsuperscript{157,158}

Fig. 19  Acridine and quinolinium “switch off” probes MACA and lucigenin.

![MACA, lucigenin](image2)

probes have proven very useful. Clear images were obtained by Inglefield who used MEQ (λ\textsubscript{em} = 320 nm) to confirm Cl\textsuperscript{−} transport (mediated by manipulating the GABA\textsubscript{A} ion channel, Fig. 18) into neuronal cells obtained from a rat brain slice,\textsuperscript{146} and Durack employed SPQ to measure intracellular chloride in porcine lymphocytes using flow cytometry.\textsuperscript{147} Recently this style of probe has also been shown to be compatible with two-photon excitation.\textsuperscript{148}

The identification of N-methylacridinium-9-carboxamide MACA (λ\textsubscript{em} = 500 nm, Fig. 19) and also the bisacridinium lucigenin (λ\textsubscript{em} = 506 nm, Fig. 19) as longer wavelength variants was a welcome development.\textsuperscript{149} However, while these compounds have been used successfully in vesicle/liposome based studies\textsuperscript{150} they were shown to be unstable in cell based studies.\textsuperscript{149}

2.2.2 “Switch on” and ratiometric probes. Ratiometric measurement of intracellular chloride has been achieved by conjugating the chloride sensitive methoxyquinoline to the “constant” dimethylaminoquinoline fluorophore to give bis-DMXPQ (λ\textsubscript{em} = 450 for MQ vs. λ\textsubscript{em} = 565 nm for DMO, Fig. 20).\textsuperscript{151} This dimer distributed uniformly in the cytoplasm and was reported to be stable and non-toxic. Related compounds could be reduced (in a similar process to that for MEQ) for non-invasive cell loading.\textsuperscript{152} While not classed as a small molecule the bisacridinium tetramethylrhodamine conjugate BAC-TMR-dextran (Fig. 20) was constructed from the carbohydrate aminodextran.\textsuperscript{152}

![bis-DMXPQ](image3)

![BAC-TMR-dextran](image4)

![Ratiometric probes bis-DMXPQ and BAC-TMR-dextran used for chloride imaging.](image5)
The squaraines have been somewhat overlooked as a biologically compatible fluorophore due to their susceptibility to hydrolysis. In contrast, hydroxysquaraines have been found to be much more stable and for rotaxane (Fig. 23) interaction with chloride shifts the surrounding macrocycle slightly along the squaraine “axle” which in turn leads to fluorescence modulation. While not yet demonstrated in an intracellular setting squaraine-rotaxane probes are red emissive, ratiometric (in acetone $\lambda_{em} = 698$ nm decreases and $\lambda_{em} = 665$ nm increases) and, unlike the first generation of probes they are selective for chloride ($I^-$ is not bound; $Br^-$ binding is 10 fold weaker than that of $Cl^-$) and thus have tremendous potential for further development in an intracellular setting.

2.3 Iodide

In the environment iodide occurs naturally in minerals (alutarite and iodargyrite) and it is interesting that AgI is used by humans as a as a nucleation agent in “cloud seeding” programs due a similarity in crystal structure to that of water ice. Iodide is also a common additive to table salt as deficiencies can lead to the condition known as goitre. Indeed the consumption of trace amounts of iodide is essential for human health—the anion is transported to, and accumulated in, the thyroid gland for incorporation into the iodine containing hormones.

Selective probes for iodide bioimaging are rare. It is interesting to note that the early probes for chloride such as SPQ and MEQ (see Fig. 18) were actually more sensitive to iodide than chloride, nevertheless the far greater concentration of chloride resulted in minimal interference from iodide.

2.3.1 Recent developments.

A recent publication from Mahapatra (2012) outlines a successful displacement approach for the detection of iodide in Candida albicans (IMTECH3018) cells. The complexation of Hg(II) by the thiosemicarbazole ligand 27 (Fig. 24) quenches the inherent fluorescence of the carbazole fluorophore (heavy metal effect). Iodide is capable of displacing Hg(II), liberating the highly fluorescent carbazole ($\lambda_{em} = 425$ nm) and a four-fold fluorescence “switch on” ($\lambda_{em} = 425$ nm)
was observed. Importantly, selectivity for iodide over other halides was excellent.

### 2.4 Cyanide

Cyanide has a long history of use in industry and is also well known as an environmental poison but for cystic fibrosis (CF) sufferers CN has a particularly sinister role. Infection with *Pseudomonas aeruginosa* (PA) is common amongst patients with CF and PA is a cyanogenic bacteria (synthesises CN) and in *in vivo* cyanide functions as a potent inhibitor of cellular respiration.\(^{164}\) Indeed PA-mediated cyanogenesis has an acknowledged role in the pathogenesis of CF lung disease.\(^{165}\) Less common, but more problematic is infection with *Burkholderia cepacia* complex (Bcc) which is typically multidrug resistant and is also cyanogenic.\(^{165,166}\) Hence diagnostics for CN in *in vivo* would be welcome for rapid identification of these problematic lung infections. Other sources of *in vivo* cyanide come from cyanogenic glycosides produced by some plants as part of their innate defence system (for example in almond seeds) and these glycosides can be enzymatically hydrolysed to produce free CN in living tissues.\(^{167}\)

A number of strategies exist for the detection of cyanide but the most common approaches for imaging CN rely on its (i) nucleophilicity or (ii) affinity for copper ions.\(^{160,168}\) The nucleophilicity of cyanide has been exploited in the design of chemodosimeters (see Section 2.4.1). Typically, nucleophilic attack of CN at a chemodosimeter incorporating a C—O (aldehyde), C—NR, C—C-CN or related functionality results in a product in which conjugation at some point of the probe is broken and in turn the fluorescence response is modulated. The other successfully used approach involves the displacement of copper (Section 2.4.2). These probes are functionalised with copper chelating groups such that when Cu(II) is introduced the resultant assembly exists in quenched form (heavy/transition metal ion induced quenching). Cyanide has a very high affinity for copper and is capable of selectively displacing the fluorophore. A very stable Cu(CN)\(_2\) species is formed and the fluorophore is liberated—"switched on".

#### 2.4.1 Cyanide as a nucleophile

A relatively early example (2007) was the coumarin aldehyde 28 (Fig. 25) by Kim and Hong.\(^{169}\) This probe incorporated a structure known to function by means of the excited state intramolecular proton transfer (ESIPT) principle.\(^{170}\) Attack at the aldehyde occurs in the vicinity of an acidic OH (phenol) which ultimately leads to formation of a cyanohydrin and a phenoxy anion. As the phenoxy is part of an ICT fluorophore, its formation leads to a dramatic enhancement of fluorescence (\(\lambda_{em} = 450\ nm\)). Proton transfer to form 30 was confirmed by the significant upfield shift in the \(^1\)H NMR spectrum as the phenolic proton (\(\delta_H = 10.1\ ppm\)) was "transferred" to the cyanohydrin (\(\delta_H = 6.1\ ppm\)). While microscopy was not performed a fluorescent plate reader was used to confirm selective "switch-on" (\(\lambda_{ex} = 350\ nm\), \(\lambda_{em} = 450\ nm\)) cyanide sensing in murine embryonal carcinoma (P19) cells.

Yoon, designed the ESIPT hydroxylfluorescein aldehyde probes (both mono and di were synthesised; dialdehyde 31 shown in Fig. 26) for microfluidic sensing of cyanide as well as both *in vitro* and *in vivo* probes for cyanide.\(^{171,172}\) Formation of the phenoxy in this instance leads to spirocyclic ring opening and strong fluorescence "switch on" (\(\lambda_{em} = 520\ nm\)). Using dialdehyde 31 the imaging of cyanide in BALB/c nude mouse model was accomplished (Fig. 26) *in vivo*.\(^{172}\) This probe was

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**Fig. 25** Coumarin based ESIPT sensor 28 for the detection of cyanide.

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**Fig. 26** Top: fluorescein dialdehyde chemodosimeter 31 for CN. Bottom: *in vivo* images of CN in the lungs after various incubation times after infection with PA and Bcc. Colour images were reconstructed from inverted fluorescence images. Image reproduced with permission.\(^{172}\)
able to detect, in the lungs of the mice, increased levels of cyanide due to infection caused by PA and Bcc. The probe itself did not cause adverse effects when injected as a DMSO solution directly into the lungs of the mice.

The salicylaldehyde functionalised fluorene ESIPT probe FSal (Fig. 27) was reported by Malik in 2014.\textsuperscript{173} The sensor could detect cyanide in solution at very low concentration (<0.1 ppb) by reaction to form the corresponding cyanohydrin leading to a strong fluorescence ‘switch-on’ ($\lambda_{\text{ex}} = 329$ nm, $\lambda_{\text{em}} = 520$ nm). The probe was capable of imaging cyanide (as tetra-butylammonium cyanide, TBACN) in human neuroblasts (SH-SY5Y) and the probe was both highly selective and non-toxic.

The ‘switch off’ BODIPY dialdehyde 34 (Fig. 28) was reported by Ravikanth in 2013.\textsuperscript{174} Using NMR spectroscopy the probe was clearly shown to react with two equivalents of $\ce{CN}$ and fluorescence ($\lambda_{\text{em}} = 554$ nm) was quenched with the addition of 2.2 equivalents of the anion. In human breast adenocarcinoma cells (MDA-MB-231) the probe was non-toxic and the intense green fluorescence of the probe was quenched upon treatment of the cells with $\ce{CN}$.

The ratiometric aminocoumarin probe Coum-1 (Fig. 29) reported by Li (2012)\textsuperscript{175} possesses a reactive dicyanoacrylate appendage (readily installed using the reaction of malononitrile with the corresponding coumarin aldehyde). A distinct response (both colourimetric and fluorescent) was observed following the conjugate addition reaction of cyanide to the alkene. The diminished length of the ICT system leads to a shift in both absorption and emission maxima and using excitation at $\lambda = 447$ nm ratiometric measurement of cyanide could be performed [initial coumarin ($\phi_F = 0.45$, $\lambda_{\text{em}} = 585$ nm), product Coum-CN ($\phi_F = 0.33$, $\lambda_{\text{em}} = 495$ nm)]. An impressive 470 fold increase in $F_{495}/F_{585}$ was realised with the addition of only 1.0 equivalent of $\ce{CN}$. The probe was successfully used for the detection of cyanide in HeLa cells by comparing emission from the red and green channels.

Also relying on the conjugate addition of $\ce{CN}$ the BODIPY chemodosimeter 36 (Fig. 30) was developed by Jang (2012).\textsuperscript{176} The reaction of cyanide with the dicyanoethylene appendage interrupts the ICT and as a consequence both visible and fluorescent properties were modulated. Interference from fluoride was noted in CH$_2$Cl$_2$ but in water the strong solvation of fluoride rendered it less competitive. A clear “switch on” response ($\lambda_{\text{ex}} = 480$ nm, $\lambda_{\text{em}} = 520$ nm) was observed in the cytoplasm of HeLa cells that had been incubated with the probe for 20 min then treated with NaCN.

A “switch-on” phenothiazine–hemicyanine probe Phc (Fig. 31) was reported by Yang and Li (2014).\textsuperscript{177} The cyanide anion readily
attacked the C\textequal N bond of the indolium and a 20 fold enhancement in fluorescence ($\lambda_{em} = 488 \text{ nm}$) was observed when solutions of the probe were exposed to only 3.0 equivalents of $\text{CN}$. The probe was selective amongst other anions tested and was used in both human breast cancer (GES) and HeLa cells to demonstrate a quick (15 min) “switch on” effect in the presence of in vitro cyanide. Furthermore in adult zebra fish exposed to $\text{Phc}$ and cyanide (30 $\mu$M) a strong fluorescent response was observed, particularly in the gills and abdomen.

The related red-emitting phenazine(di)cyanine-based chemodosimeters $\text{PMI}$ and $\text{PDMI}$ (Fig. 32) were reported by Hua (2014)\textsuperscript{178} and again these probes rely on nucleophilic attack of cyanide on a indolium cation. As $\text{PDMI}$ has two indolium appendages an excess of $\text{CN}$ was required before the fluorescence “switch on” ($\lambda_{ex} = 425 \text{ nm}$, $\lambda_{em} = 580 \text{ nm}$) occurred.

In contrast, for $\text{PMI}$ an instantaneous response was observed ($\lambda_{ex} = 530 \text{ nm}$, $\lambda_{em} = 620 \text{ nm}$). Of interest, given that aldehydes are also used as a reactive group for $\text{CN}$, $^1$H NMR spectroscopy was used to monitor the intact CHO even as an excess of cyanide was added. The probe located in the cytoplasm In HeLa cells (confirmed using co staining) and a clear “switch on” response to cyanide was noted within 30 minutes of exposure.

2.4.2 Cyanide mediated displacement. Several papers from the group of Yoon describe probes for cyanide bioimaging based on Cu(II) displacement (shown schematically in Fig. 33—the probe is introduced to the cells in the Cu(II) complexed quenched state (heavy metal effect) and upon cyanide mediated displacement of the Cu(II) from the complex fluorescence is restored). The fluorescein tetracarboxylate “switch-on” probe $38$-Cu(II) was published in 2009\textsuperscript{179} and was one of the first to prove that the copper displacement method could be used both in vitro and in vivo. When $38$-Cu(II) was trialled in solution studies, the addition of 100 equivalents of $\text{CN}$ increased the quantum yield from 0.057 to 0.53 ($\lambda_{ex} = 505 \text{ nm}$, $\lambda_{em} = 522 \text{ nm}$). When Caenorhabditis elegans nematodes (a model host for studying microbial pathogenesis and innate immunity)\textsuperscript{180} that were initially incubated with $38$ and Cu(II) were subsequently incubated with $\text{CN}$ a strong fluorescence “switch on” was observed. Of interest when these fluorescent nematodes were again incubated with Cu(II), fluorescence was quenched.

In additional work from the group of Yoon, the NIR emissive cyanine fluorophore ($\lambda_{ex} = 680 \text{ nm}$, $\lambda_{em} = 748 \text{ nm}$) functionalised with picolylamino groups $39$ (Fig. 34) was synthesised in a short overall sequence from commercially available cyanine IR-780.\textsuperscript{181} In the presence of Cu(II) no significant fluorescence was observed ($\phi_F < 0.01$) and initial solution based studies confirmed selective, “switch on” sensing ($\phi_F = 0.65$, $\lambda_{em} = 748 \text{ nm}$)
of cyanide. Again using the *C. elegans* nematode as a model organism, aqueous NaCN was readily detected in vivo. Of significant interest when the nematodes were infected with *P. aeruginosa* (PA14) labeled with green fluorescent protein, the cyanide that the bacteria are known to produce was also detected in vivo. Such a result neatly conveys the significance of such small fluorescent probes for medically relevant assays.

The Cu(II) displacement approach has also been used by Ghosh and Das to develop a “switch on” probe for cyanide, however, in this instance the emitting species (λ<sub>ex</sub> = 380 nm, λ<sub>em</sub> = 583 nm) was a phosphorescent DPA-functionalised iridium complex 40 (Fig. 35).<sup>182</sup> Detection of cyanide was achieved inside live HeLa cells within 2 minutes using cells pre-incubated with 40-Cu(II) then exposed to a 0.2 ppm aqueous solution of NaCN. A Cu(II) displacement probe operating by both a colour change and also fluorescent enhancement was reported by Kim.<sup>183</sup> Coumarin imine 41 (Fig. 36) was synthesised in four steps from *m*-anisidine with the last step involving condensation with 2-aminophenol. A crystal structure of the stable non-fluorescent 41-Cu(II) (F<sub>f</sub> = 0.02) confirmed that the metal was complexed by both oxygen atoms and the imine nitrogen atom as shown in Fig. 36. Similar to the previous examples the probe operates by means of Cu(II) displacement, however, unlike the Cu(II) complex, the free coumarin imine 41 is prone to hydrolysis (cyanide actually enhances the rate of hydrolysis) and ultimately it is the coumarin aldehyde 42 (λ<sub>ex</sub> = 479 nm, λ<sub>em</sub> = 514 nm, F<sub>f</sub> = 0.65) that functions as the reporting species. Again, excellent selectivity for cyanide amongst a selection of anions was reported (CN detected at 10<sup>-8</sup> M). No adverse effects were noted when human hepatoma cell line HepG2 cells were treated with the complex and a strong intracellular fluorescence response was detected when cells were treated with solutions of KCN.

An interesting copper displacement probe for cyanide was devised by Zheng (2014).<sup>184</sup> The non-fluorescent Cu(n) schiff base complex of benzimidazole hydroxynaphthalene 43 (Fig. 37) was itself formed by displacing Zn(n) from the corresponding, highly fluorescent Zn complex (both the Cu and the Zn complexes were characterised by means of X-ray diffraction). Displacement of copper from the 43-Cu(n) complex was effected by cyanide to give the fluorescent free 43 (λ<sub>ex</sub> = 366 nm, λ<sub>em</sub> = 425 nm).
Displacement of Cu(II) was also effected by $S_2^-$ and it should be noted that this anion (known to have an affinity for Cu) is often omitted from the standard suite of anions used to evaluate the selectivity of many Cu based probes. In HeLa cells incubated with the Zn(II) complex addition of Cu(II) quenched the fluorescence, however, the corresponding addition of $CN^-$ to restore fluorescence was not performed.

The Hg(II) complex of benzimidazole 44 (Fig. 37) was described by Zhang and Liu (2013) for the intracellular sensing of $CN^-$. The complex is non-fluorescent (heavy metal effect) and in the presence of cyanide a strong fluorescence "switch on" ($\lambda_{ex} = 345$ nm, $\lambda_{em} = 467$ nm) was observed due to displacement of Hg(n). Unfortunately both sulphide and also iodide displaced the cation to give an equivalent response. While these competitors might ultimately limit in vivo applications (toxicity was also not evaluated), successful in vitro sensing of cyanide was demonstrated when HeLa cells that had been incubated with 44-Hg(n) were treated with cyanide.

2.4.3 Miscellaneous. Very few in vitro or in vivo probes that function by means of H-bonding exist—a testament to the lack of truly selective anion sensors that function by H-bonding in highly competitive media. The bispyrenecarbohydrazide 45 (Fig. 38) reported by Yoon (2014) bound cyanide such that excimer emission ($\lambda_{ex} = 500$ nm, $\lambda_{em} = 550$ nm) was "switched on". A proposed "clamshell" H:G complex of 45 with cyanide was proposed leading to close proximity of both pyrene moieties. In aprotic solvents fluoride also elicited a "turn on" response, however, when small amounts of protic solvents were present $CN^-$ was targeted more selectively. Again HeLa cells were used for imaging and, in addition to rapid uptake, a distinct fluorescence "switch on" was observed when $CN^-$ was added.

3. Anions of biological relevance

Many crucial intracellular processes involve anionic species and the dysregulation of these species is known to accompany a number disease states. Indeed, the dysregulation of intracellular pyrophosphate (PPI) levels is associated with many conditions including cancer (see Section 3.1). Imaging agents for specific anionic targets in vitro and in vivo can be used to confirm the exact biological role of these anions and importantly they can also function as diagnostics for specific medical conditions. Anions covered in this section are: pyrophosphate, bicarbonate and hydrosulfide.

3.1 Pyrophosphate

Pyrophosphate (PPI) is produced or used in many cellular metabolic processes, such as ATP hydrolysis and DNA/RNA polymerisation reactions. Intracellular PPI concentrations can provide information on important cellular processes and have recently been suggested as a means of cancer diagnosis. The concentration of PPI in other physiological fluids, such as...
synovial fluid and urine, can also be used to identify diseases such as chondrocalcinosis or calcium pyrophosphate dihydrate (CPPD) crystal deposition disease.188

This knowledge has led to the recent development of numerous colourimetric and fluorescent sensors for PPI (see also the review by Yoon in this special issue).14,36,189 However, significant challenges remain in the development of such probes, due to the difficulties associated with binding PPI in water and distinguishing it from related polyphosphates such as ATP. As many of the fluorescent sensors developed for PPI to date are “switch-off” or only exhibit weak fluorescence they are of only limited use in bioimaging applications. Nevertheless, there are several examples where such compounds have been successfully used to image the presence of cellular PPI and there are a handful of recent examples where “switch on” sensors have been developed and effectively used in imaging applications.

3.1.1 PPI binding to metal complexes. One of the earliest examples of the use of a PPI sensor in cellular imaging was reported by Kim.190 The 1,8-naphthalimide–DPA–Zn(II) complex 46-2Zn(n) (Fig. 39) was prepared in good overall yield over five steps and in CH₂CN:HEPES buffer, ligand 46 exhibited weak emission at 476 nm (λex = 360 nm), characteristic of the 4-amino-1,8-naphthalimide fluorophore. Formation of the 46-2Zn(n) complex resulted in a 59-fold fluorescence enhancement together with a 29 nm bathochromic shift to 505 nm, attributed to suppression of PET from the DPA amine. The addition of PPI to 46-2Zn(n) resulted in a hypsochromic shift (23 nm) and ca. 50% quenching of fluorescence (10 equivalents of PPI). Calculations indicated that PPI bound to only one Zn(n)DPA centre and PET quenching was mediated by PPI. Imaging was performed using mouse myoblasts (C2C12) and cells were first incubated with 5 μM Zn(OAc)₂, then with 1 μM 46. Subsequent addition of PPI led to a dose-dependent decrease in cellular fluorescence (Fig. 39).

Hong and co-workers reported the three step synthesis of the NIR emissive benzothiazolium hemicyanine ligand 47 (Fig. 40), which readily forms a bis Zn(n) complex as a PPI binding site.191 In aqueous buffer (pH 7.4) Probe 47-2Zn(n) showed weak emission at 548 nm (λex = 500 nm, ΦF = 0.08) and addition of PPI (1.0 equivalent) “switched on” emission (ΦF = 0.10) with a bathochromic shift to 558 nm. While ATP also gave a measurable response, 47-2Zn(n) was used to image PPI uptake in a C2C12 myoblast cell line with a clear increase in intracellular fluorescence observed 30 minutes following addition of 2.5 equivalents of PPI. Importantly, the cells remained viable as determined using Hoechst nuclear stain and the probe had good cell permeability.

3.1.2 PPI mediated displacement. The [DCCA]₂Cu complex, comprising both a dicyanomethylene-4H-chromene fluorophore and a copper(n) complex of iminodiacetic acid group for PPI binding, has been reported as a NIR emissive “switch on” sensor for PPI.192 In aqueous buffer DCCA (Fig. 41) is highly fluorescent (λex = 500 nm; λem = 675 nm; ΦF = 0.79), but upon addition of Cu(n) the fluorescence intensity decreased and complete quenching was observed with five equivalents of Cu(n). On addition of
PPi to a 1:5 mixture of DCCA and Cu(ClO₄)₂ fluorescence “switch on” was observed, and peaked after 15 equivalents PPI (Φ_F = 0.48). The enhancement was attributed to the displacement of one of the ligands from the Cu(II) complex. This probe was evaluated in KB cells and almost no intracellular fluorescence was observed for the [DCCA]₂Cu complex alone. After incubation with PPI, a significant increase in cellular fluorescence was observed within 30 minutes with signals localized in the perinuclear area of the cytosol, indicating a subcellular localisation of PPi and good cell membrane permeability of the [DCCA]₂Cu complex.

The imino-thiophenyl calix[4]arene derivative 48 (Fig. 42) has been used to image both Zn(II) and PPi in HeLa cells. Ligand 48 was prepared in three steps from p-tert-butylcalix[4]arene and upon addition of Zn(II) in a fluorescence turn-on response (λ_ex = 390 nm, λ_em ~ 450 nm) was observed. Subsequent addition of PPI resulted in complete quenching of the emission, attributed to the displacement of Zn(II) from the 48-Zn(II) complex as a result of the higher binding affinity of PPI for Zn(II). In HeLa cells incubated with 10 μM 48, very low fluorescence intensity was observed. After subsequent incubation with ZnSO₄/pyrithione for 20 minutes, the cells exhibited highly intense blue fluorescence (4 times higher than with 48 alone). Further treatment with PPI resulted in a decrease in fluorescence intensity (1.5 times higher than 48 alone).

The bis Zn(II) complex of the pyridine-naphthalene based SPHN (Fig. 43) has been reported as a PPi selective fluorescent chemosensor. Compound SPHN was readily prepared by condensation of the bis-glycine adduct of 2,6-diaminopyridine and 2-hydroxy-1-naphthaldehyde. In 7:3 CH₂CN:aqueous HEPES buffer the addition of ZnCl₂ to SPHN resulted in a “switch-on” of fluorescence (λ_ex = 400 nm; λ_em = 450 nm, Φ_F = 0.940) which was suggested to be a result of the formation of a 1:2 L₂:Zn(n) complex. The addition of PPI to SPHN-2Zn(n) led to SPHN-2Zn(n)+PPI which showed intense blue fluorescence in the presence of both SPHN and Zn²⁺ (d) and did not show any fluorescence in the absence of Zn²⁺ (a) and in the presence of PPI (g). Corresponding differential interference contrast (DIC) images (e, b and h) and merge images (f, c and i) of the cells are shown. Image reproduced with permission.
quenching of this fluorescence as a result of displacement of Zn(II) from SPHN and importantly, a selective response for PPI was observed in the presence of ATP. In HeLa cells preincubated with exogenous Zn(II) the addition of SPHN elicited a fluorescence response, however, when PPI was added at the same time as SPHN, significantly lower levels of fluorescence were observed.

More recently, Rissanen and co-workers have reported a terpyridine–Zn(II) complex 49-ZnCl₂ (Fig. 44) capable of the detection of nanomolar PPI concentrations in water, together with the first example of a small molecule probe to image native PPI concentrations in cells (i.e. without the addition of exogenous PPI). The complex was prepared by mixing 4,4′-(4-N,N′-dimethylaminophenyl)-2,2′:6′,2″-terpyridine in a 1:1 ratio with ZnCl₂ and an X-ray crystallographic structure, confirmed the formation of a 1:1 complex. While 49-ZnCl₂ is fluorescent in the solid state, in water the fluorescence is quenched. The addition of PPI to a solution of 49-ZnCl₂ in 0.01 M HEPES buffer resulted in an approximately 500 fold increase in fluorescence (λ<sub>ex</sub> = 440 nm; λ<sub>em</sub> = 591 nm), attributed to the formation of a 1:3 complex between PPI and 49. Cellular imaging was performed in HeLa cells. Cells were treated with 10 μM 49-ZnCl₂ for 30 min and bright orange-yellow emission was observed that allowed mapping of PPI concentration in different parts of the cells with the maximum emission observed in the nuclei as well as the cytoplasmic membranes.

3.2 Bicarbonate

The bicarbonate anion is the primary species responsible for maintaining cellular acid–base homeostasis. The enzyme carbonic anhydrase (CA) produces HCO₃⁻ inside cells from dissolved CO₂ and dysregulation of CA is associated with a number of tumour types. The bicarbonate anion also plays a role in physiological processes such as cyclic AMP regulation, osteoporosis and kidney disease. Unfortunately only indirect methods have been available for measuring bicarbonate in cells including total H⁺CO₃⁻ concentration or estimates based on pH; each of these are prone to significant error and do not provide spatiotemporal information. As such a more direct means for the bioimaging of this anion using fluorescent probes would be a welcome advance.

3.2.1 Recent developments. Very few small molecule probes exist for the cellular imaging of bicarbonate and these can be divided into two classes: dipyridylalkylbenzenes and luminescent lanthanide complexes.

The dialkynylbenzene probe 50 for the imaging of bicarbonate in vitro was reported by Murphy, Wong and Lee in 2011 (Fig. 45). A NIR multiphoton approach was used for excitation and probe emission also tailed into the NIR region. Solution studies identified strong binding of bicarbonate (log<sub>K</sub> = 7.13) and a four-fold enhancement in fluorescence intensity (λ<sub>em</sub> = 450 nm) was observed as well as a redshift of 30 nm. Binding was tentatively assigned to the δ of the amide N leading to enhanced electron transfer (1:2 H:G binding stoichiometry supported this theory). In solution, binding of citrate was also observed (log<sub>K</sub> = 7.83), nevertheless, in vitro imaging of HCO₃⁻ was performed in both HeLa and A549 cells (λ<sub>ex</sub> = 900 nm and λ<sub>em</sub> = 400–650 nm) and after 3 h the probes had localised in the cytoplasm and the emission profiled matched the 30 nm red shift observed in solution indicating binding of the target anion.

With the aim of sensing bicarbonate and other oxyanions (such as malate and citrate) “in cellulo” a large body of research...
effort has been undertaken by the group of Parker using luminescent lanthanide probes.\textsuperscript{83,199} The cellular uptake, localisation, stability and toxicity of these probes have all been studied in detail.\textsuperscript{45,81,200–202} These sensors have sharp emission bands ($\Delta J = 1, 2, 3$ and $4$) and as they rely on energy transfer from a chromophore/sensitiser (normally incorporated as part of the ligand) to the metal the emission can be modulated by the target anion disturbing either the metal excited state or the ligand singlet or triplet states (for excellent overviews of the photophysical properties of lanthanides and how they can be manipulated for sensing and imaging see the excellent recent reviews by Meade,\textsuperscript{82} Parker,\textsuperscript{83} and Pierre\textsuperscript{203}). Lanthanide complexes are capable of selectively binding anions if both the ligand and metal are judiciously selected. Typically, but not always, the mode of action involves displacement of a bound water ($q$) by the target anion leading to a modulated emission profile in which the $\Delta J = 2$ band (e.g. Eu at ca. 620 nm) is considerably altered whereas other bands such as $\Delta J = 1$ (e.g. Eu at ca. 590 nm) are not. Changes in these two distinct outputs allows the probe to function in a very useful ratiometric fashion.\textsuperscript{82,83,203} Combinations of individual Tb and Eu complexes (one complex is responsive, the other is not) can also function in a ratiometric manner.\textsuperscript{204}

The detection of bicarbonate by the lanthanide complexes 51-Eu(m) and 52-Eu(m) containing the 1-azaxanthone-4-carboxyl sensitiser was reported by Parker in 2011 and in a follow up study in 2012 (Fig. 46).\textsuperscript{205,206} The probes were non-toxic and indicated the presence of bicarbonate ($\text{HCO}_3^-$) formed by exposing the cells to a CO$_2$ atmosphere) in the mitochondria of a number of cell lines including A549, MCF-7 and HeLa cells. While solution based binding studies indicated little selectivity of the probes over the common carboxylate interferents citrate and lactate, in cells the concentration of bicarbonate is typically 10 fold greater than lactate and 100 times greater than citrate. Of additional interest the same ligands with terbium were 10 fold greater than lactate and 100 times greater than citrate. Nevertheless the challenge of targeting HS$^-$/H$S$ in living systems is a rapidly developing field.\textsuperscript{52,213–216} Given the large number of examples this review will highlight bioimaging agents that either (i) present significant advances in sensitivity, (ii) target particular cellular locations, (iii) emit in the NIR range

### 3.3 Hydroxysulphide

Hydrogen sulphide (H$_2$S), along with NO and CO, is considered to be the third gaseous signalling agent, or gasotransmitter.\textsuperscript{267} Endogenous hydrogen sulphide is produced from homeocysteine (Hcy) by the cytosolic enzymes; cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CGL).\textsuperscript{208} In addition hydrogen sulphide is also produced from cysteine (Cys) by 3-mercaptoppyrurate sulfurtransferase (3MST) mediated metabolism; 3MST is present in both the cytosol and mitochondria.\textsuperscript{208} Considering the first and second pK$_{a}$ values for H$_2$S are 7.65 and 15 respectively (at 25 °C and pH 7.4 the ratio of H$_2$S/HS$^-$/S$^2-$ can be calculated as $30:70:0.00002$ respectively).\textsuperscript{207} As such, for the purpose of this review we can assume that appreciable quantities of HS$^-$ are present. Nevertheless the challenge of targeting HS$^-$ is complicated as pH varies between subcellular compartments and as such the H$_2$S/HS$^-$ ratio will also vary accordingly.

Hydrogen sulphide has been linked to a number of physiological processes such as inflammation, angiogenesis, respiration, ischaemic reperfusion injury as well as oxidative stress.\textsuperscript{208,209} As such the development of HS$^-$/H$S$ releasing drugs is an active area of research.\textsuperscript{210}

Due to the rapid catabolism of HS$^-$ by sulphide quinone oxidoreductase (SQR), persulfide dioxygenase (SDO), thiosulfate reductase (TR) and sulphite oxidase (SO$_4^{2-}$) any probe must react quickly and emit brightly (high quantum yield ($\phi$)) and molar absorptivity ($\varepsilon$)). This formidable challenge is made more difficult by the fact that the typical concentration of HS$^-$ in blood are in the order of $10^{-6}$ M to $10^{-9}$ M and 30–300 μM in tissues have been reported.\textsuperscript{207,212} There is still debate over the physiologically active form of hydrogen sulphide,\textsuperscript{212} therefore a selective means to detect low concentrations of the anionic form could unlock some of the secrets of its remarkable biology.

The development of fluorescent indicators to sense HS$^-$ in living systems is a rapidly developing field.\textsuperscript{52,213–216} Given the large number of examples this review will highlight bioimaging agents that either (i) present significant advances in sensitivity, (ii) target particular cellular locations, (iii) emit in the NIR range

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**Fig. 46** Top: structure of HCO$_3^-$ sensitive probes 51-Eu(m), 52-Eu(m) and the bound 51-Eu(m)-CO$_3^-$ Bottom: images of HeLa cells incubated with 52-Eu(m), localised in the mitochondrial region under 3, 4 and 5% CO$_2$ atmosphere. Image reproduced with permission.\textsuperscript{211}
and (iv) have been demonstrated to be applicable to in vivo studies.

For probes that target hydrosulphide, three strategies are commonly employed to modulate fluorescence. The first two are chemodosimetric approaches that rely on HS\(^-\) as a nucleophile or HS\(^-\) mediated reduction. The third strategy is sulphide mediated metal displacement. Nucleophilic reactions (typically S\(_n\)Ar or nucleophilic addition) are used to restore ICT or remove a group involved in a PET process, displace a trigger or interrupt a conjugated system. Anionic HS\(^-\) is a superior nucleophile compared to thiols (pK\(_a\) > 8.5) at physiological pH. When two proximal electrophiles are present interference from endogenous sulphur species such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) is further circumvented. Reduction of an azide is an often utilised approach as the azide can be introduced directly to a fluorophore bearing an arylamine as a component of an ICT system, or as a sulfonylazide. Reduction of nitro groups, hydroxyl amines, and N-oxides are related examples of this strategy, however the reduction of the nitro-group invariably suffers from poor reaction kinetics. The displacement of a metal, typically Cu(II) or Zn(II), from a chelating ligand has also been widely used for bioimaging HS\(^-\). There are obvious parallels between this approach and the probe design for CN selective probes (see Section 2.4).

3.3.1 Hydrosulphide as a nucleophile. Lin et al. made use of both the donor photoelectron transfer (d-PET) properties and HS\(^-\) reactivity of the 2,5-dinitrophenylether in their hybrid cyanine–BODIPY probe, NIR-H\(_2\)S (Fig. 47).\(^{217}\) NIR-H\(_2\)S is essentially non-fluorescent due to the d-PET process, however, upon treatment with HS\(^-\) the fluorescent phenol 53 (\(\lambda_{\text{ex}} = 650\) nm, \(\lambda_{\text{em}} = 708\) nm) is revealed. NIR-H\(_2\)S has a detection limit of 30 nM although this value was determined at pH 7.0. Using probe NIR-H\(_2\)S visualisation of varying concentrations of exogenous HS\(^-\) in MCF-7 cells was achieved. Notably higher fluorescent intensity at the mitochondria was observed which was confirmed by colocalisation studies using Hoechst 33342.

Fig. 47 Structure and reaction of cyanine–BODIPY hybrid probe NIR-H\(_2\)S with HS\(^-\); INSET shows fragmentation mechanism.

Zheng and Cui (2014) functionalised nile red with this trigger to give a NIR-emissive (\(\lambda_{\text{ex}} = 488\) nm, \(\lambda_{\text{em}} = 655\) nm) HS\(^-\) responsive probe NR-HS\(_2\) (Fig. 48).\(^{218}\) Upon reaction with HS\(^-\) the quantum yield of NR-HS\(_2\) (\(\Phi_\text{F} = 0.05\) in simulated physiological conditions) increased to 0.32 as nile red was regenerated. Maximum fluorescence was obtained after 20 minutes with a limit of detection of 270 nM. Fluorescence microscopy experiments were carried out using MCF-7 cells and NR-HS\(_2\) responded to exogenous HS\(^-\).

The 2,5-dinitrophenylether trigger was incorporated onto a 4-hydroxynaphthalimide fluorophore by Liu et al. to give Lyso-NHS (Fig. 48). The probe also contained a basic morpholine substituent (Lyso-NHS pK\(_a\) = 3.12) which was responsible for compartmentalisation of the sensor into the lysosome (Fig. 49).\(^{100}\) The reaction of HS\(^-\) with Lyso-NHS led to a significant increase in fluorescent intensity (\(\lambda_{\text{ex}} = 450\) nm, \(\lambda_{\text{em}} = 555\) nm) with a maximal response after 20 minutes. The probe had a nanomolar (480 nM) detection limit and was used to visualise exogenous HS\(^-\) in MCF-7 cell lysosomes (confirmed by co-staining with neutral red (NR) a known lysosomal stain) and was not toxic (MTT assay) at the concentrations used.

Also using the 2,5-dinitrophenylether trigger, the group of Govindaraju synthesised the HS\(^-\) probe DNOPCy (Fig. 50).\(^{219}\) Dislodging the 2,5-dinitrophenyl group of DNOCy (\(\lambda_{\text{em}} = 555\) nm) gave Cy-quinone which has a red-shifted emission maximum

![Fig. 48](image1.png) Structures of NR-HS\(_2\) and Lyso-NHS.

![Fig. 49](image2.png) Colocalisation images of Lyso-NHS in MCF-7 cells. (a) Lyso-NHS with HS\(^-\) (green channel. (b) Neutral red (red channel. (c) Merged images of (a) and (b). Image reproduced with permission.\(^{100}\)

![Fig. 50](image3.png) NIR emissive HS\(^-\) probe DNOPCy.
at \( \lambda = 695 \) nm. This change in fluorescence emission was ideal for ratiometric detection and visualisation of exogenous NaSH in human embryonic kidney cells (HEK293T) was successfully accomplished.

Through the judicious placement of a proximal aldehyde, Feng (2014) developed the dinitrophenyl ether probe 54 (Fig. 51) that reacted fully within two minutes of exposure to HS\(^-\) to give maximum fluorescence.\(^{220}\) The reaction generates the modified HBMT releasing the fluorescent probe \( \text{HS-Cy} \) at the aldehyde of 54 (Fig. 51), which incorporates a proximal electrophile.\(^{221}\) Nucleophilic addition of HS\(^-\) at concentrations as low as 48 nM were detected in human embryonic kidney cells (HEK293T) was successfully accomplished.

In 2012 Qian and co-workers reported a rapidly reacting HBMT based HS\(^-\) activated fluorescent probe (Scheme 1).\(^{221}\) Probe 1 can undergo thiol exchange with both HS\(^-\) and thiols, however only the persulfide generated from HS\(^-\) can cyclise to release the fluorescent HBMT \((\lambda_{\text{ex}} = 295 \text{ nm}, \lambda_{\text{em}} = 487 \text{ nm})\). Probe 1 was weakly fluorescent due to PET from the pendant dithiol and reacted rapidly (2 \text{ min}) with HS\(^-\) with an detection limit of ca. 120 nM. Once again this probe readily detected exogenous HS\(^-\) in HeLa cells.

In 2013 the Tang group reported the HS\(^-\) responsive ratiometric probe HS-Cy (Fig. 52) which incorporates a proximal electrophile.\(^{222}\) Nucleophilic addition of HS\(^-\) at the aldehyde of HS-Cy leads to a rapid (within 3 \text{ min}) loss of fluorescence emission \((\lambda_{\text{em}} = 780 \text{ nm}, \Phi_F = 0.24)\). This decrease in fluorescence was speculated to be caused by a PET process from the free hydroxyl or sulfhydryl groups of the intermediate qHS-Cy. The intramolecular cyclisation between the free sulfhydryl and the ester the releases the ketone cyanine (ketone-Cy) resulting in a 155 nm blue-shifted emission \((\lambda_{\text{em}} = 625 \text{ nm})\). The cyclisation step is a much slower process, with the emission at 625 nm increasing over 35 min. In the mitochondria of HepG2 cells (pH 8.0) the nucleophilic addition and substitution occurred within 30 \text{ s} and 5 \text{ min} respectively. The fluorescence intensity ratio \(F_{625}/F_{780}\) rises from 0.01 to 24.8 following the addition HS\(^-\). Probe HS-Cy was used to image exogenous HS\(^-\) in HepG2 cells and could detect endogenous HS\(^-\) in A549 cells stimulated with sodium nitroprusside (SNP). A decrease in the \(F_{625}/F_{780}\) ratio was observed when an inhibitor (DL-propargylglycine, PPG) of the HS\(^-\) producing enzymes (CBS and CES) was added to the cells, confirming HS\(^-\) as the analyte responsible for the response.

Guo and co-workers reported a fast reacting flavylum derived ratiometric HS\(^-\) probe 59 (Fig. 53).\(^{223}\) Nucleophilic attack of HS\(^-\) on this NIR emissive probe \((\lambda_{\text{ex}} = 450 \text{ nm}, \lambda_{\text{em}} = 690 \text{ nm})\) disrupts conjugation and the fluorescent product is essentially a substituted aminocoumarin \((\lambda_{\text{ex}} = 485 \text{ nm})\). In pure PBS buffer, the reaction was complete in 20 \text{ s}, making probe 59 one of the fastest probes yet reported. Upon treatment with HS\(^-\) a 694-fold increase in the ratio \(F_{694}/F_{690}\) \((0.07-83.90)\) was noted allowing a detection limit of 140 nM. While this probe did react with mercaptooethanol, selectivity for HS\(^-\) over cysteine and GSH was apparent. It was suggested that electrostatic repulsion between the benzopyrylium ion and the protonated amines of Cys and GSH prevents addition. Probe 59 was found to be non-toxic (MTT assay) and was subsequently used for the ratiometric imaging of exogenous HS\(^-\) in HeLa cells.
The mitochondria selective HS\(^-\) probe **CouMC** (Fig. 54) was reported by the groups of He and Guo in 2013.\(^{224}\) The coumarin-hemicyanine probe **CouMC** has two fluorescent emissions (\(\lambda_{\text{em}} = 510\) and 652 nm) with the red emission, which corresponds to the full conjugated system, being the more intense. Nucleophilic attack of HS\(^-\) at the indolium C=N interrupts the conjugated system of **CouMC** and the resultant truncated \(\pi\)-system of **CouMC-SH** exhibits coumarin-like fluorescence (\(\lambda_{\text{em}} = 510\) nm). Maximum fluorescence emission was achieved in 30 s in simulated physiological conditions. The red fluorescence could be reinstated when the media containing **CouMC-SH** was acidified to pH 2.5; **CouMC** itself, was stable over the pH range of 2.5 to 8.0. In *vitro* ratiometric imaging studies with **CouMC** in MCF-7 cells revealed high localisation in the mitochondria (colocalisation with Deep Red 633). The intracellular reaction of the probe with HS\(^-\) occurred rapidly (<80 s) and as such this probe may find utility in the spatiotemporal tracking of this anion.

### 3.3.2 Hydrosulfide mediated reduction.

In 2012 Cho reported **FS-1** (Fig. 55), the earliest example of a two-photon probe for HS\(^-\).\(^{225}\) The sensor employed the azide “trigger”, which was pioneered by the Chang group for bioimaging.\(^{226}\) When treated with HS\(^-\), a 21-fold increase in fluorescent enhancement (\(\lambda_{\text{em}} = 548\) nm, \(\Phi_F = 0.46\)) was observed.\(^{225}\) The probe also displayed an increase in two-photon cross section under the same conditions (HEPES buffer, \(\Phi = 15\)–302 GM). Endogenous HS\(^-\) was visualised in live HeLa cells using **FS-1**, pre-treatment with cysteine and GSH both led to enhanced two-photon excited fluorescence (TPEF) (\(\lambda_{\text{ex}} = 750\) nm, \(\lambda_{\text{em}} = 520\) nm). Phorbol 12-myristate 13-acetate (PMA) induced oxidative stress led to a decrease in observed emission intensity.

In 2012 Han and co-workers reported the NIR-emitting ratiometric cyanine-azole probe **Cy-N\(_3\)** (Fig. 56).\(^{227}\) Upon treatment with NaSH the azide probe (\(\lambda_{\text{ex}} = 625\) nm, \(\lambda_{\text{em}} = 710\) nm \(\Phi_F = 0.11\)) is reduced to give **Cy-NH\(_2\)** (\(\lambda_{\text{em}} = 750\) nm, \(\Phi_F = 0.12\)) with a concomitant increase in molar absorbptivity (\(\varepsilon_{660} = 130 000 \text{ M}^{-1} \text{ cm}^{-1}\)). The maximum fluorescence response was achieved in 20 min which was superior to related examples at that time. The ratio of emission intensities (\(F_{750}/F_{710}\)) increased from 0.6–2.0 with the addition of 10 equivalents of NaSH, and a detection limit of 80 nM was established. The probe was successfully used to detect endogenous HS\(^-\) in live RAW264.7 macrophages (stimulated using PMA). The fluorescence emission intensity also increased in cells treated with NaSH and **Cy-N\(_3\)** was also used to study the time dependent decomposition of the HS\(^-\) releasing agent 5-(4-hydroxyphenyl)-1,2-dithiole-3-thione (ADT-OH) in fetal bovine serum.

In 2013 the groups of Xu and Peng independently reported the red emitting dicyanomethylenobenzopyran probe **DCMC-N\(_3\)** (Fig. 57).\(^{218,219}\) Probe **DCMC-N\(_3\)** is essentially non-fluorescent, however, upon reaction with HS\(^-\) fluorescence was “switched on” (\(\lambda_{\text{em}} = 670\) nm in 1 : 1 PBS buffer : DMSO or \(\lambda_{\text{em}} = 655\) nm in 1 : 1 phosphate buffer : MeCN). The Xu group showed that
DBMC-N$_3$ could be used to indicate the presence of exogenously administered HS$^-$ in human umbilical vein endothelial cells (HUVEC). Similarly, preliminary experiments by the Peng group used DBMC-N$_3$ to image exogenous HS$^-$ in HeLa cells. The Peng group also utilised the favourable properties of DCMC-N$_3$ (NIR, large Stokes shift and good $\Phi_{\text{Fmax}} = 50\%$ at 820 nm in DMSO) to visualise the presence of HS$^-$ in MCF-7 cells using two photon microscopy. Furthermore, a skin-pop injection of probe DCMC-N$_3$ and NaSH (25 equiv.) into ICR mice revealed that the probe could be used in vivo as an enhanced fluorescent response ($\lambda_{\text{ex}} = 530$ nm, $\lambda_{\text{em}} = 655 \pm 20$ nm) was observed. The development of the fluorescent response over 4 h is shown in Fig. 57.

Through a simple two-step azidation procedure Ma (2012) converted cresyl violet to the ratiometric azide probe 61 (Fig. 58).$^{230}$ Both 61 ($\lambda_{\text{em}} = 566$ nm, $\Phi_F = 0.54$) and cresyl violet ($\lambda_{\text{em}} = 620$ nm, $\Phi_F = 0.44$) were strongly fluorescent ($\lambda_{\text{em}} = 620$ nm). The emission ratio ($F_{620}/F_{566}$) ranged from 0.34–10.5 upon addition of HS$^-$ and a detection limit of 100 nM was determined. Probe 61 was used to visualise exogenous HS$^-$ added to MCF-7 cells, and the emission could be quenched with addition of ZnCl$_2$ to the cells, confirming that switching is a result of azide reduction. The utility of 61 to visualise HS$^-$ in vivo was demonstrated in live zebrafish. As the concentration of HS$^-$ was increased (10–500 μM) fluorescence emission ratio ($F_{620}/F_{566}$) of the probe (10 μM) ranged from 0.73–1.64 with no visible fluorescence decrease on standing for elongated times, indicating that probe 61 was stable in vivo.

Chang in 2013 reported probe SF7-AM; which incorporated AM-esters to facilitate passive diffusion into cells. Intracellular ester hydrolysis gave the free carboxylates and the probe was subsequently retained within the cells (Fig. 59).$^{231}$ The inclusion of two azide triggers to the rhodamine-based fluorophore gave enhanced sensitivity and SF7-AM was used as a tool to study vascular endothelial growth factor (VEGF) stimulated HUVEC HS$^-$ production as a model for angiogenesis.
As an advancement on their Lyso-NHS probe (Fig. 48) Cui and Xu developed the lysosome targeting probe Lyso-AFP (Fig. 60).232 When HS\(^-\) mediated reduction of the essentially non-fluorescent azide (\(\Phi_F = 0.012\)) occurred an increase in fluorescence response (\(\Phi_F = 0.263\), \(\lambda_{ex} = 426\ \text{nm}, \lambda_{em} = 535\ \text{nm}\)) was observed over 20 min. As with Lyso-NHS the pendant morpholine of Lyso-AFP lead to lysosomal localisation.

Another lysosome specific probe; rhodamine-based SulpHensor (Fig. 61) was described by Yang (2014).233 No fluorescent response at physiological pH was observed for this probe upon treatment with HS\(^-\). The acidic lysosomal environment is required to open the spirocycle which results in a weakly fluorescent species (\(\lambda_{ex} = 540\ \text{nm}, \lambda_{em} = 550\ \text{nm}, \Phi_F = 0.05\)). Subsequent azide reduction by HS\(^-\) greatly enhances the ICT fluorescent emission at 550 nm (\(\Phi_F = 0.05\)) and a limit of detection of 300 nM for HS\(^-\) was determined. The HS\(^-\)/H\(^+\) induced fluorescent response of SulpHensor was demonstrated in vitro using HeLa cells. Lysosomal accumulation was confirmed by co-staining with Lyso-Tracker green and comparing the intensity profiles (Fig. 61) from the red (SulpHensor) and green channels (LysoTracker) over a selected region.

Bae et al. employed the 4-azidobenzylcarbamate trigger in their two photon fluorescent probe SHS-M2 (Fig. 62). Hydro-sulphide mediated reduction initially yields the aminobenzyl-carbamate which subsequently fragments to give the observed products. In addition, the attachment of a pendant triphenyl-phosphonium moiety lead to mitochondrial accumulation in astrocytes.234 Upon reduction with HS\(^-\) the emission wavelength of SHS-M2 (\(\lambda_{em} = 464, \Phi_F = 0.24\)) was red-shifted by 81 nm (\(\lambda_{em} = 545\ \text{nm}, \Phi_F = 0.12\)) and despite a decrease in quantum yield the two photon cross section was improved (\(\Phi_{0\text{max}} = 17–55\ \text{GM}\)). The probe was successfully used to detect mitochondrial HS\(^-\) in HeLa cells; metabolic precursors (GSH or Cys) of HS\(^-\) were added prior to the SHS-M2 and an increase in the \(F_{545}/F_{464}\) ratio was recorded. The probe was also used to demonstrate the relationship between the DJ-1 gene and CBS mediated HS\(^-\) production in astrocytes as a model of Parkinson’s disease.

The 4-aminonaphthalimide-based HS\(^-\) probes NAP-1 and AcSH-2 also bearing the 4-azidobenzylcarbamate trigger were independently reported by Zhao and Song in 2014.235,236 Reaction of NAP-1 (AcSH-1) with HS\(^-\) gives the fluorescent aminonaphthalimide and a corresponding red-shift in emission (from \(\lambda_{em} = 474\ \text{nm to } \lambda_{em} = 540\ \text{nm}\)).235 A low limit of detection for HS\(^-\) was determined (50 nM at pH 7.0 and 110 nM at pH 7.4) and the probe was used by Song for monitoring endogenous HS\(^-\) production in sodium nitroprusside stimulated MCF-7 cells (Fig. 63) as well as quantifying HS\(^-\) levels in murine hippocampus. Zhao used AcSH-2 to visualise exogenous HS\(^-\) in MCF-7 cells using TPM (\(\lambda_{ex} = 800\ \text{nm}, F_{530}/F_{468} = 0.38–2.90\)). It was also evident that the N-(2-hydroxyethyl)-imide of AcSH-2 promoted mitochondrial localisation.236

Chen and co-workers built the \(m\)-nitrophenyloxyycyanine Cy-NO\(_2\) (Fig. 64) as a d-PET based probe for HS\(^-\).237 The 3-nitrophenylether attached to the cyanine framework could accept an

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**Fig. 59** Structure, intracellular esterase and sulphide reactions of SF7-AM.

**Fig. 60** Structure and reaction of Lyso-AFP.

**Fig. 61** Top: structure and reaction of SulpHensor. Bottom: HeLa cells stained with (a) LysoTracker Green (Ch1, green) and (b) SulpHensor (Ch2, red) with NaSH solution (c) overlay of (a) and (b). Scale = 10 \(\mu\)m. Image reproduced with permission.233

**Fig. 62** Top: two-photon fluorescent probe SHS-M2 developed by Kim. Bottom: (A–D) Pseudocoloured ratiometric TPM images \(F_{545}/F_{464}\) of (A, D) HeLa cells with (A) SHS-M2 or (D) SHS-M2 and (B, C) HeLa cells pretreated with (B) GSH or (C) cysteine prior to SHS-M2. Image reproduced with permission.234
electron from the excited state of the cyanine fluorophore. Reduction of the nitro group with HS\(^{-}\) disrupts the d-PET process resulting in significantly enhanced emission (from \(\Phi_F = 0.05\) to \(\Phi_F = 0.11\), \(\lambda_{ex} = 755\) nm, \(\lambda_{em} = 789\) nm). The probe localised in the cytosol of RAW264.7 macrophages and was used to detect exogenous HS\(^{-}\). A significant drawback associated with unassisted nitro group reduction is that it is a kinetically slow process, and in this instance fluorescence intensity peaked only after 60 min, even at 37 °C.

### 3.3.3 Hydrosulfide mediated metal displacement

In 2014 Huang and Deng reported a NIR emitting probe based on Cu(II) displacement from the Cu(II)-cyclen complex \(68\)-Cu(II) (Fig. 65).\(^{238}\) The probe was originally designed to function by means of HS\(^{-}\) mediated NO\(_2\) reduction, but no reaction occurred at ambient temperatures. The probe was redesigned and the nitro group was reduced to an amine and functionalised with the cyclen-macrocycle. Complexation of \(68\) with Cu(II) gave the non-fluorescent probe which, when treated with HS\(^{-}\) in buffered solution studies gave a significant fluorescence “switch on” response (\(\lambda_{ex} = 716\) nm, \(\lambda_{em} = 765\) nm). Visualisation of exogenous HS\(^{-}\) in RAW264.7 macrophages was successfully demonstrated as was the detection of endogenous HS\(^{-}\) which was produced by the overexpression of wild-type (WT) cystathionine-\(\gamma\) lyase (CSE) in human embryonic kidney (HEK293). In vivo studies were then performed by injecting imprinting control region (ICR) mice with \(68\)-Cu(II) by skin-pop injection. One \(68\)-Cu(II) treated mouse was then injected with Na\(_2\)S (as source of HS\(^{-}\)) and the fluorescence images were recorded (\(\lambda_{ex} = 670\) nm, \(\lambda_{em} = 790\) nm). The fluorescence signal increased seven-fold 1 h after treatment with HS\(^{-}\) (compared to the control) and continued to rise gradually over 5 h.

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**Fig. 63** Confocal fluorescence images of endogenous HS\(^{-}\) in living MCF-7 cells with NAP-1. Cells were pre-stimulated with SNP, then with NAP-1 (1A, 1B, 1C). Cells were pre-stimulated with SNP, and then with NAP-1 (2A, 2B, 2C). Cells were pre-treated with \(L\)-propargylglycine (PPG), and then with NAP-1 (3A, 3B, 3C). Cells in (3A, 3B, 3C) were thereafter treated with SNP (4A, 4B, 4C). Cells were incubated with NAP-1 alone (D). The average fluorescence intensity from the regions of interest a, b and c in (D) was recorded with 60 s intervals (E) \(\ln = 3\). Ratiometric images \((F_{530}/F_{468})\) generated by the Olympus software (1C, 2C, 3C, 4C). Scale bars = 10 \(\mu\)m. Image reproduced with permission.\(^{235}\)

**Fig. 64** Structure and reaction of hydrosulphide probe Cy-NO\(_2\).

**Fig. 65** Top: NIR emissive cyclen dosimeter \(68\) by Deng and Huang. Bottom: representative fluorescence images of mice (pseudocolour) that were injected with \(68\)-Cu(II) (40 mm), followed by Na\(_2\)S. The images were recorded after the injection of Na\(_2\)S within 5 h. Image reproduced with permission.\(^{238}\)
4. Reactive anions

In addition to the well-known oxidative stress caused by reactive oxygen and nitrogen species, it is becoming increasingly apparent that these moieties are involved in a wide range of physiologically essential processes (such as cellular migration and circadian rhythm). Reactive oxygen/nitrogen species can be neutral, anionic, radicals or even radical anions (Table 2) and as many of these species exist only fleetingly they are exceptionally challenging targets for small molecular probes.

At physiological pH (ca. 7.4) several of these species exist to a considerable extent as an anion (peroxynitrite, hypochlorite and hypobromite) and can therefore be considered biologically relevant anionic targets. Given their diverse roles in detecting these species in vitro and in vivo has been strong and several reviews on the topic are available.

4.1 Peroxynitrite

Peroxynitrite is formed from the rapid reaction of two other RO/N species: superoxide $O_2^-$ and nitric oxide $NO^+$ (Fig. 66) and its detection in living systems is hampered by a number of factors including (i) its reactivity (it is a potent nucleophile and is a more powerful oxidant than superoxide) with a number of in vitro targets such as proteins, nucleic acids and lipids (especially those containing thiols) and (ii) degradation to other highly reactive species (including both the hydroxyl radical and the nitrogen dioxide radical). The pKₐ of ONOO⁻ is 6.8 and as a result ~80% of the species is found in the anionic form at pH 7.4. Typically the anion adopts a cisoid structure that is responsible for its ‘relative’ stability compared to the parent acid.

Elevated levels of this species are associated with cardiovascular and neurodegenerative disorders, metabolic diseases, inflammation, pain, and cancer. Hence the development of probes for its detection in vivo is an important task en route to fully understanding its diverse physiological roles.

Table 2 Reactive oxygen/nitrogen species

| Neutral | Anionic | Radical anion | Radical |
|---------|---------|---------------|---------|
| Hydrogen peroxide HOOH | Peroxynitrite ONOO⁻ | Superoxide $O_2^-$ | Nitric oxide $NO^+$ |
| Singlet oxygen $O_2$ | Hypochlorite $ClO^-$ | Hydroxyl $HO^+$ | Hydroxyl $HO^+$ |

A percentage of this species can also be found in neutral form (HOBr pKₐ = 8.7).

Peroxynitrite has long been known as a cellular oxidant and hence sensors to monitor this anion emerged last century. Probes for peroxynitrite rely on the same reactivity that the anion exhibits in vivo; ONOO⁻ is both nucleophilic (reacts with $CO_2$ in vivo to form carbonate) and highly oxidative (by one and two electron processes) and is a known nitrating agent.

All three of these reaction types has been employed in the design of imaging agents for the species however probes that employ the oxidation power of ONOO⁻ are by far the most prominent (Section 4.1.1). Amongst these, probes based on the oxidation of B, Se, and Te form an interesting subset (Section 4.2.2). Common interferents with the detection of ONOO⁻ are typically other ROS in particular hypochlorite ($ClO^-$) and nitroxide radical ($^*NO$).

Early anion sensors for peroxynitrite inside cells include the ‘switch on’ probes dichlorodihydrofluorescein DCHF and dihydrorhodamine DHR-123 (Fig. 67). While many imaging studies, and even flow cytometry assays, were performed using these, (and similar) probes they are somewhat non-selective and in some instances light sensitive.

4.1.1 Peroxynitrite mediated oxidation/fragmentation. A recent report by Shivanna (2013) detailed the use of rhodamine B phenyl hydrazide RBPH (Fig. 68). The probe was readily oxidised by ONOO⁻ to give the highly fluorescent ($\lambda_{em} = 580$ nm) ring open rhodamine-B; the product confirmed by $^1$H NMR spectroscopy ESMS. Interferents such as $H_2O_2$ did not elicit the same reaction ($ClO^-$ was not tested) and as such some selectivity was noted. Live MCF-7 cells incubated with the probe gave a clear “switch on” response within 30 min when exogenous ONOO⁻ was added.

A strategy developed by Yang involves ONOO⁻ nucleophilic attack on a reactive trifluoromethyl ketone group followed by...
formation of a spirohemiacetal via a dioxirane intermediate. Three separate probes have been developed: employing dichloro-fluorescein HKGreen1, BODIPY HKGreen2 and rhodol HKGreen3 fluorophores (Fig. 69). For these chemodosimeters fluorescence is quenched in the ketone form but following spirohemiacetal formation (and for HK1 and HK3 fragmentation) fluorescence is “switched on”. For HKGreen2 a 21-fold enhancement of fluorescence (λem = 539 nm) was observed upon reaction with just one equivalent of ONOO⁻/C0⁺. The probes react to a small extent with HO⁻/C1⁵ but not the common ClO⁻/C0⁺ interferent. Evaluation in cells was performed for all probes (Results for HKGreen3 in RAW264.7 macrophages shown in Fig. 69). This cell line is known to produce ROS/RNS, including ONOO⁻, in response to immunological and inflammatory stress and after stimulation of endogenous ONOO⁻ production using bacterial endotoxin lipopolysaccharide (LPS), interferon-γ (IFN-γ) and phorbol 12-myristate 13-acetate (PMA) strong fluorescence was noted. No fluorescence enhancement was noted when ONOO⁻ production was inhibited by (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO) a superoxide scavenger or aminoguanidine (AG) an inhibitor of NO synthase confirming that endogenous production of ONOO⁻ was being detected.

In a more recent article from the Yang group a functionalised rhodol chemodosimeter HKGreen3 (Fig. 70) was described in which the oxidation of electron rich aromatics was exploited. The “switch on” probe (290-fold increase in λem = 535 nm) was capable of discriminating between peroxynitrite and other reactive oxygen species including hydroxyl radical and hypochlorite. HKGreen4 also performed in the presence of CO₂, a molecule known to react quickly with ONOO⁻. The probe was water soluble and was successfully used with either conventional or two photon microscopy to image endogenous ONOO⁻ production in E. coli challenged RAW264.7 cells. In conjunction with a number of enzymatic inhibitors the probe was used to further confirm the generation of endogenous ONOO⁻ production in response to the presence of E. coli. Generation of ONOO⁻ is thought to be an immune effector for bacterial clearance, and the results of this study suggest that the ONOO⁻ formation in E. coli challenged macrophages is enzymatically regulated.

The “switch on” coumarin pyridinium probe C-Py-1 was reported by Yu in 2014 (Fig. 71). The probe was selective for peroxynitrite amongst other reactive oxygen/nitrogen species with a 25-fold enhancement of emission at λem = 493 nm. The emitting species was identified (¹H NMR spectroscopy and ESI-MS) to be the coumarin aldehyde formed from oxidative cleavage of the alkene in the presence of ONOO⁻. Excellent cell
membrane permeation and low cytotoxicity were noted and the probe was successfully applied to the imaging of endogenous ONOO⁻/C⁰⁻ in RAW264.7 cells. In vitro fluorescence was "switched on" within 30 min of the addition of lipopolysaccharide (LPS) to the cells to stimulate endogenous production of ONOO⁻/C⁰⁻. The related hemicyanine probe CHCN (Fig. 72) has recently been published by Yoon (2015).²⁸⁹ Oxidative cleavage of the alkene gives coumarin aldehyde 73 and 1,3,3-trimethyloxindole; both products confirmed using ESMS and ¹HN M NMR spectroscopy. This probe indicated ONOO⁻/C⁰⁻ in a linear ratiometric fashion (F₅₁₅/F₆₃₅ increase), had a low LOD (49.7 nM) and displayed excellent selectivity—only a very slight response to a large excess of hypochlorite was noted. The probe was shown to operate successfully in RAW264.7 cells when the cells were stimulated to produce ONOO⁻ using a number of agents (LPS, IFN-γ and PMA). No response was observed when inhibitors of endogenous ONOO⁻ production were introduced (TEMPO and aminoguanidine).

A multichannel probe PN600 (Fig. 73) that can clearly distinguish ONOO⁻ from ClO⁻ has been developed by Yang (2012).²⁹⁰ When the cells were stimulated to produce ONOO⁻, a clear fluorescent response was observed. PN600 is powerful enough to oxidise 74 to the red emitting iminoquinone 75. Due to differing excitation yet similar emission wavelengths either of OCl⁻ or the ONOO⁻ could be inferred by monitoring either at 420 nm and selectively exciting at 355, 465 or 575 to identify which fluorophore had formed and hence which oxidant was present. In human glioma cell line U87 the probe was shown to quickly penetrate the cell membrane and was non-toxic (MTT assay). Using 3-morpholinosydnonimine (SIN-1), a compound known to decompose to NO⁻/C⁰⁻ which in turn forms ONOO⁻, a clear fluorescent response was observed. The nuclease sensitivity of ONOO⁻ leads to initial formation of a C-O-B bond in place of the original C-B bond of PN600. The nucleophilicity of ONOO⁻ leads to initial formation of a C-O-B bond in place of the original C-B bond of PN600.
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Bond (Scheme 2). This insertion is followed by hydrolysis to afford C–O–H. The process is similar to the established hydroboration/oxidation using alkaline H2O2 however the reaction of boronates with ONOO• is nearly 1 x 107 times faster than the reaction with H2O2 and hence discrimination between these species is possible.293

The naphthalimide-based “switch off” probe 80 was reported by James in 2014 (Fig. 74). The fluorescent α-fructose complex (λem = 525 nm) of 79 in which PET from the spacer N is prevented was “switched off” by ONOO•-mediated oxidation to the corresponding phenol 81. The product again has a free amine and PET quenches fluorescence. All other ROS except ClO• did not elicit a significant response. In RAW264.7 macrophages ONOO• production was stimulated by a number of immunological factors (again including LPS) and the “switch off” was easily visualised. When TEMPO or aminoguanidine was added to the stressed cells prior to addition of the probe fluorescence was maintained due to a lack of endogenous ONOO•.

In 2012 Han reported the “switch on” dioxaborolane pyrene probe PyBor (Fig. 75) which was synthesised in three steps from pyrene.295 Reaction with ONOO• was complete in seconds (PyBor is converted to PyOH) and was accompanied by a significant increase in quantum yield from ϕF = 0.08 to 0.60 (PyOH, λex = 347, λem = 410 nm). While slightly sensitive to H2O2, ClO• and BrO• co-staining confirmed PyBor localised in the cytoplasm of RAW264.7 cells and intense intracellular fluorescence was observed when cells that had been incubated with PyBor were treated with LPS, IFN-γ and PMA. When the cells were pre-treated with aminoguanidine, only weak fluorescence was detected.

4.1.2 ONOO• mediated chalcogen oxidation. Fully reversible, NIR emissive, phenylselenylaniline probes BzSe-Cy, Cy-PSe and a related Te containing cyanine probe Cy-NTe have been developed by Tang and Han (Fig. 76). The first, benzylselenylecyanine BzSe-Cy,296 is a “switch off” probe that can be recycled with ascorbic acid. In BzSe-Cy the selenium is an electron donating
component of the cyanine system, however, in its oxidised Se=O form (readily accomplished by ONOO\(^-\)) electron donation is "switched off" and fluorescence is quenched. The second probe, \textit{Cy-PSe}\(^{297}\) is a "switch on" probe that uses PET from the dibenzyldiselenoether to quench BODIPY fluorescence. When oxidised to Se=O, electron transfer is inhibited and fluorescence is "switched on". The third, \textit{Cy-NTe}\(^{298}\) another "switch on" probe also operates using this principle. The Te component required six steps to construct prior to coupling with commercially available cyanine chloride. Both \textit{Cy-PSe} and \textit{Cy-NTe} are easily oxidised by ONOO\(^-\) and also easily reduced by glutathione (GSH) which makes them mimics of the Se containing glutathione peroxidase enzymes (GPx). In the non-oxidised state the metals quench fluorescence by PET, however PET is not possible in the oxidised forms and strong fluorescence enhancement is observed (23-fold for \textit{Cy-PSe} and 13-fold for \textit{Cy-NTe}). The successful "switch on" oxidation, "switch off" reduction cycles for both \textit{Cy-PSe} and \textit{Cy-NTe} were demonstrated in RAW264.7 cells by successive treatment with LPS then an ROS scavenger glutathione S-transferase (GST) to "switch off" fluorescence. Mitochondrial localisation of \textit{Cy-NTe} was reported as was a lack of toxicity (MTT assay) and the switching behaviour of the probe was sensitive enough to be visualised \textit{in vivo} using BALB/c mice.

The selenium containing "switch off" BODIPY probe \textit{BOD-Se} (Fig. 77) was reported by Han in 2012.\(^{299}\) The authors proposed that, in light of the long time required to eliciting a response, oxidation of the Se itself by ONOO\(^-\) did not modulate fluorescence but that the product containing Se=O was hydrolysed (Fig. 78) to liberate a new selenium free fluorescent BODIPY 82. The \textit{BOD-Se} probe itself was highly fluorescent (\(\lambda_{\text{em}} = 572\) nm, \(\phi_F = 0.96\)) and a colour change from red to blue was noted upon exposure to ONOO\(^-\) with an isosbestic point at 567 nm. Fluorescence intensity decreased more than 200-fold along with a shift of the emission maximum (\(\lambda_{\text{em}} = 680\) nm, \(\phi_F = 0.05\)). Again murine macrophage RAW264.7 cells were stressed using LPS/IFN-\(\gamma\) and PMA to induce endogenous production of ONOO\(^-\) prior to imaging.

4.1.3 Peroxynitrite mediated nitration/nitrosylation. The \textit{Ds-DAB} probe (Fig. 78) was described by Wang in 2013 and its synthesis was remarkably easy—one step from commercially available materials.\(^{300}\) Fluorescence was initially quenched
A probe for nitrative stress (NiSPY-3, Fig. 79) was rationally designed by Nagano (2006) and is an interesting example in which nitration leads to fluorescence “switch on” ($\lambda_{\text{ex}} = 505 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) based on an a-PET process. The probe was selective for peroxynitrite and was successfully used to visualise exogenous ONOO$^-$ in live HeLa cells.

### 4.1.4 Miscellaneous

An interesting lanthanide-based probe for ONOO$^-$ bioimaging was reported by Guan in 2010 (see Section 3.2 for the general principles by which Ln emission is modulated). The DTTA ligand (a combination of dimethoxyphenyl and terpyridinetetraacid, Fig. 80) was designed to be both a Ln chelator and also sensitive to a d-PET process. In practice the terbium complex $\left([\text{DTTA}]\text{Tb}\right)$ ($\lambda_{\text{ex}} = 335 \text{ nm}$, $\lambda_{\text{em}} = 612 \text{ nm}$) was sensitive to ONOO$^-$ whereas the corresponding europium complex ($\lambda_{\text{ex}} = 335 \text{ nm}$, $\lambda_{\text{em}} = 541 \text{ nm}$) was not. The authors postulated that quenching by charge transfer, not PET, was responsible for the quenching, however, the electron rich dimethoxyphenyl substituent of DTTA is similar to that of NiSPY-3 (shown in Fig. 79). Thus, with a “cocktail” of both lanthanides $\left([\text{DTTA}]\text{Eu(III)}\right)$ and $\left([\text{DTTA}]\text{Tb(III)}\right)$ ratiometric measuring was possible with only very slight interference noted from nitrate. The tetraacetoxymethyl ester form AM-DTTA readily entered HeLa cells esters along with solutions of both lanthanides. Ester hydrolysis inside cells was followed by assembly of the lanthanide complexes and their formation could be visualised using microscopy. Again using SIN-1 as a source of NO$^+$ (reacts immediately with superoxide to form ONOO$^-$) a clear decrease in Tb emission in proportion to increasing SIN-1 concentration was observed.

An interesting example of a probe for the sensing of ONOO$^-$ by means of luciferin bioluminescence was reported in 2013 by...
Bonini using the peroxy-caged luciferin PCL-1 (Fig. 81). Unfortunately, cell images were not shown in this instance. The boronic acid based chemodosimeter PCL-1 (originally designed and used by Chang for the in vivo bioimaging of \( \text{H}_2\text{O}_2 \) in mice) reacts far more rapidly with \( \text{ONOO}^- \) than it does with \( \text{H}_2\text{O}_2 \) to form luciferin which in turn forms oxyluciferin with concomitant emission of light \( (\lambda_{\text{em}} = 560\,\text{nm}) \).

### 4.2 Hypochlorite

Hypochlorite plays an important physiological role defending against invading pathogens and endogenous hypochlorite is produced from the reaction of \( \text{H}_2\text{O}_2 \) with \( \text{Cl}^- \) and is catalysed by the heme enzyme myeloperoxidase (MPO). Over stimulation of the MPO immune response, and increased hypochlorite levels can lead to host tissue damage and inflammation that is associated with a number of serious disorders such as cancer, neurodegeneration, arthritis and cardiovascular disease.

\[
\text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{H}_2\text{O} + \text{ClO}^-
\]

Bioimaging agents must be selective for ClO\(^-\) over \( \text{H}_2\text{O}_2 \) if the MPO–\( \text{H}_2\text{O}_2–\text{Cl}^- \) enzymatic system is used to generate endogenous ClO\(^-\). Otherwise the fluorescent signal may be due to exogenous \( \text{H}_2\text{O}_2 \). Another consideration is \( p\text{H} \); the parent acid HOCl has a \( pK_a \) of 7.463 at \( 35 \, ^\circ\text{C} \), therefore at physiological \( p\text{H} \) approximately 50% of the acid is dissociated; so it possible that either the hypochlorite anion or the protonated form could be the detected species. Most researchers evaluate their probe over a \( p\text{H} \) range to identify whether hypochlorite or hypochlorous acid is being detected.
The majority of chemodosimeter for hypochlorite rely on oxidation; indeed ClO⁻ is well known, even at a household level, as a strong oxidising agent. The most common triggers include the oxidation of p-hydroxy and p-aminophenyl ethers to give quinones or iminoquinones (Section 4.2.1) although the p-aminophenyl ethers typically elicit a larger response to ClO⁻ and are more selective over ONOO⁻ (versus p-hydroxy). Oximes provide a pathway for non-radiative decay and are also readily react with hypochlorite (Section 4.2.2). As for probes that target ONOO⁻, the oxidation of S, Se, and Te has been employed in spirocyclic ring opening triggers for fluorescein and rhodamine dyes. This class of probe (notably the Se and Te based examples) are conveniently reversible as they can be reduced back to the parent structure by thiols (e.g. Cys, GSH) or hydrosulfide.

### 4.2.1 Hypochlorite mediated oxidation/fragmentation.

An early example of a “switch on” probe for ClO⁻ was reported by Nagano in 2003. Nago and colleagues.

Nagano's fluorescein system used hypochlorite to oxidise either a pendant p-hydroxyphenyl ether (HPF) or p-aminophenyl ether (APF) attached to the fluorescein fluorophore. Removal of the phenyl ethers (as the corresponding quinone or aminoquinone) restored ICT and gave rise to high fluorescence emission ($\lambda_{\text{em}} = 514$ nm, $\Phi_F = 0.81$). Unfortunately, and confirming the challenging nature of selective ClO⁻ detection, HPF was more reactive towards HO⁻ and to a lesser extent ONOO⁻. The second probe APF also gave a fluorescence response to HO⁻ and ONOO⁻ which was greater than HPF but unlike HPF, APF gave a large response when treated with hypochlorite. While HPF and APF were not completely selective, the difference in reactivity was demonstrated in neutrophils which contain azurophilic granules that are abundant in the ClO⁻ generating MPO enzyme. Both APF and HPF loaded neutrophils were stimulated with PMA to stimulate endogenous ClO⁻ production and fluorescence images were collected after 10 minutes. Only the cells containing APF exhibited a significant increase in fluorescent emission.

An extension on the Nagano fluorescein system was reported in 2007 by Libby, whereby the p-aminophenol ether moiety was attached to a water soluble sulphonaphtho-fluorescein (SNAPF, Fig. 82). Oxidation with hypochlorite resulted in the expulsion of the aminoquinone moiety as a hypochlorite responsive PET trigger to modulate the fluorescence of a wavelength-matched control dye, SNAPF, when injected into mice with peritonitis compared to saline-injected animals ($n = 4$ per group). Image reproduced with permission.

In conjunction with a rhodamine fluorophore the p-aminophenol ether trigger (MitoAR, Fig. 82) was also exploited by Nagano (2007). It was envisaged that the inherent positive charge of the ring open rhodamine would lead to accumulation of the probe within the mitochondria of living cells. In this instance the phenyl ether was located in the 2-position of the phenyl substituent in an effort to facilitate PET to the xanthene. Hypochlorite mediated oxidation of the p-aminophenol ether correlated with a large increase in fluorescence response ($\lambda_{\text{em}} = 574$ nm) but only moderate selectivity over HO⁻ was noted. Nevertheless, MitoAR was used to monitor the MPO-catalysed production of mitochondrial ROS in HL-60 cells using H₂O₂ stimulation (Fig. 84).

Yuan et al. successfully employed the 4-amino-3-nitrophenyl moiety as a hypochlorite responsive PET trigger to modulate the luminescence of terpyridine polycacid lanthanide complexes, ANMTTA-Tb(III) and ANMTTA-Eu(III) (Fig. 85). Oxidation of the 4-amino-3-nitrophenol substituent gave the benzo[5,1-b:3,4-b']dibenzo[1,4]oxazine (BFO), which reacted with a further equivalent of ClO⁻ to restore the luminescent terbium and europium complexes HTTA-Tb(III) and HTTA-Eu(III). Probe ANMTTA-Eu(III) was used for the time-gated spatiotemporal luminescence visualisation.
of exogenous hypochlorite in HeLa and in LPS/IFN-γ/PMA-stimulated RAW264.7 macrophages. Addition of the MPO inhibitor, ABAH, to cells resulted in no fluorescence emission.

An elegantly designed system HKOCI-1 (Fig. 86), related to the aforementioned probes, was reported by Yang in 2008.309 This example used a BODIPY fluorophore which was suitably quenched ($\Phi_F < 0.01$) by a $p$-methoxyphenol substituent by means of PET. Oxidative demethylation of HKOCI-1 to the quinone (HKOCl) was effected by hypochlorite; the greater oxidation potential of the quinone makes PET unfavourable and fluorescence is “switched on” ($\lambda_{em} = 541$ nm). This process was selective for hypochlorite, with ONOO$^-$ eliciting a response only at a 10-fold higher concentration. The probe was used for the visualisation of live MPO producing RAW264.7 murine macrophages stimulated using lipopolysaccharide (LPS), interferon-γ (IFN-γ) and PMA. Stimulated cells showed an increase in the fluorescent output compared to the control. Additionally, when cells were stimulated in the presence of 2,2,6,6-tetramethylpiperidinoxy (TEMPO), much weaker fluorescent was observed. TEMPO is a known superoxide (O$_2^-$) scavenger which is an intermediate in hypochlorite synthesis from the MPO system.

In 2012 Yao reported the dihydrofluorescein based probe FCN2 (Fig. 87) which, unlike preceding examples, is triggered by oxidation dealkylation rather than spirocycle ring opening.310 Ether cleavage from FCN2 restores the ICT of the dihydrofluorescein system (86) and results in a 1643-fold increase in fluorescent intensity ($\lambda_{em} = 485$ nm, $\Phi_F = 0.71$). FCN2 was completely soluble in aqueous solution and, although some autooxidation was noted (over 24 h), reacted rapidly enough (30 min) to be applicable for in vitro (NIH3T3 cells) and in vivo applications (larval and adult zebrafish). The fluorescent response generated from the treatment of 3 month old zebrafish with exogenous hypochlorite and FCN2, indicated the accumulation of hypochlorite in gall bladder, intestine, eye, liver and eggs.
Peng and co-workers reported the oxidation-activated “enhanced PET” BODIPY-based probe BClO (Fig. 88) which was synthesised in an exquisite two step procedure from 2,4-dimethylpyrrole.\textsuperscript{311} The PET quenching was mediated by a pendant pyrrole unit which was envisaged to provide “enhanced PET” compared to single electron donors (such as heavy metal containing probes). The resultant probe, BClO was essentially non-fluorescent at $\lambda_{\text{em}} = 505$ nm ($\Phi_F = 0.006$), however, oxidation with hypochlorite afforded the pyrrol-3-one BOCIO in which PET was restricted and a 56-fold “switch on” fluorescence response was observed ($\Phi_F = 0.347$). In solution studies BClO was highly sensitive to hypochlorite with a limit of detection of 0.56 nM. Facilitated by the remarkable sensitivity of BClO the authors determined the basal hypochlorite levels in MCF-7 and HeLa cancer cell lines. When incubated with BClO (1 $\mu$M) for 20 min at 37 °C, both MCF-7 and HeLa displayed an increased fluorescence response (in relation to healthy COS-7 and RAW264.7 cells) corresponding to intracellular ClO$^\cdot$ concentrations of 9.45 nM and 8.23 nM respectively. In both of the cancer cell lines visualised, pre-treatment with the antioxidant GSH or 4-aminobenzoic acid hydrazide (ABAH, an MPO inhibitor) resulted in a significant drop in fluorescence response. The authors suggest that it may be possible to use BClO in a diagnostic capacity; differentiating healthy and cancer cells based on endogenous hypochlorite concentration. The utility of BClO was further demonstrated by the detection of hypochlorite produced in MCF-7 cells stimulated with elesclomol (an ROS generating anticancer agent).

4.2.2 Hypochlorite mediated oxime/imine oxidation. An early example of a probe that used the oxime oxidation approach (Flu-1, Fig. 89) was published by the Li group in 2011.\textsuperscript{312} The emissive species ($\lambda_{\text{em}} = 530$ nm, $\Phi_f = 0.65$) was an aldehyde containing fluorescein derivative (Flu-0) and when the aldehyde was protected as the oxime (Flu-1), fluorescence was effectively quenched ($\Phi_f = 0.01$). Probe Flu-1 displayed good selectivity against a suite of anionic species, however screening against ROS such as ONOO$^-$, HO$^-$, O$_2^\cdot$ etc. was not performed. Importantly, Flu-1 failed to illicit a fluorescent response with H$_2$O$_2$ and bioimaging of exogenous hypochlorite was successfully demonstrated using HeLa cells incubated with Flu-1.

Wu, Zeng and Wu (2013) employed the same oxime/aldehyde strategy using a BODIPY fluorophore.\textsuperscript{313} The water soluble BOD-OXIME (Fig. 89) was poorly fluorescent ($\Phi_f = 0.04$) but reacted in a dose dependant “switch on” manner (LOD = 17.7 nm) with hypochlorite to give BOD-CHO which exhibited extraordinary fluorescence in aqueous solutions ($\Phi_f = 0.96$, $\lambda_{\text{em}} = 525$ nm). The visualisation of both exogenous (ClO$^-$) and endogenous hypochlorite (PMA stimulation) in RAW264.7 macrophages was successfully demonstrated. Selectivity for ClO$^-$ in vitro was excellent as no emission was observed when ABAH or taurine (a ClO$^-$ scavenger) were added to the PMA stimulated cells.

A recent report from the Kumar group in 2014 identified 4-dimethylaminocinnaldehyde oxime 87 ($\Phi_f = 0.008$, Fig. 89)
as a selective “switch on” probe for hypochlorite. The formation of the nitrile oxide was confirmed by spectroscopic means (1H and 13C NMR) and by in situ trapping using 2-butene to give the 4,5-dihydroisoxazole. The ability of probe 87 to visualise endogenous and exogenous hypochlorite was demonstrated using the brain resident murine macrophages (BV2 microglial) and C6 glial cell lines (Fig. 90). Addition of exogenous NaOCl and also LPS stimulated endogenous hypochlorite production led to increased fluorescence.

The first ratiometric fluorometric probe for hypochlorite was developed by Yuan using an analogous approach to those above. The probe consists of a diamino maleonitrile derived imine 88 of aminocoumarin aldehyde 89 (Fig. 91). The weakly fluorescent imine ($\lambda_{\text{em}} = 585$ nm, $\Phi_F = 0.02$) reacted with hypochlorite to give the parent aldehyde and a bathochromic shift to an emission maximum centred at $\lambda_{\text{em}} = 505$ nm ($\Phi_F = 0.59$) was observed. At physiological pH a marked increase in emission ratio ($F_{505}/F_{585}$) from 0.12 to 28.2 was observed after the probe was treated with hypochlorite. Probe 88 was successfully used to visualise exogenous hypochlorite in MCF-7 cells.

**4.2.3 Hypochlorite mediated ring opening.** A number of rhodamine based systems which react specifically with hypochlorite have been reported in recent years. Work in this area was pioneered by Nagano in 2007 with the rhodamine thioether spirocycle HySOx. The thioether was critical for the stability of the closed form of the HySOx probe, particularly at higher pH. Hypochlorite oxidation of the thioether triggers ring opening and fluorescence “switch on” ($\lambda_{\text{em}} = 575$ nm, $\Phi_F = 0.34$). Utilising this sensitivity to hypochlorite the authors were able to image phagocytosis in porcine neutrophils.

In an extension of this work, Nagano (2011) reported the silyrhodamine analogue MMSiR (Fig. 92). The isosteric replacement of oxygen with silicon resulted in a significant red-shift in the emission wavelength to the near-infrared (NIR) region ($\lambda_{\text{em}} = 670$ nm, $\Phi_F = 0.31$). Similar to HySOx, the MMSiR probe was also used in the imaging of phagocytosis. In addition the hydrophilic probe wsMMSiR was synthesised and applied to the in vivo imagining of hypochlorite in a mouse peritonitis model (Fig. 93). In this study C57BL/6 mice were treated with an intraperitoneal injection of zymosan to stimulate neutrophil invasion of the peritoneal cavity. Injection of wsMMSiR and PMA resulted in enhanced fluorescence emission in the abdomen.

Using a more traditional rhodamine fluorophore, Yoon (2007) developed both a thio- and selenoester trigger for selective ClO− sensing. Upon reaction with hypochlorite, the non-fluorescent R19-S, R19-Se and R101-S undergo ring opening and fluorescence is “switched on” at $\lambda_{\text{em}} = 550$ nm, 545 nm and 585 nm respectively (Fig. 94). Probe R19-S was used to visualise hypochlorite production in phagocytes and microbial hypochlorite generation in intestinal epithelia of Drosophila melanogaster.
In the last few years a number of diacylhydrazine–rhodamine probes have been reported. These probes rely on selective oxidation by hypochlorite to give a diacyl diimide, then subsequent hydrolysis reveals the fluorescent rhodamine. An early example of this approach was reported by Ma and co-workers although high pH was required (pH 12). Modifications to the benzoyl substituent can be used to tailor probes which are more suited for imaging purposes. For example, by using a pendant alkoxyquinoline moiety (RHQ, Fig. 95) Goswami and co-workers were able to image endogenous hypochlorite in human peripheral blood mononuclear cells (PBMCs). A similar fluorescein based system (90 R = O and 91 R = S, Fig. 95) from the Li group was used to monitor hypochlorite in *Rhodobacter ferrooxidans* prokaryotes as a potential model for hypochlorite induced stress.

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In 2014 Hou *et al.* employed this trigger in the mitochondria targeting probes Rh-TPP and Rh-Py. The incorporation of a phosphonium (Rh-TPP) or pyridinium ion (Rh-Py) onto the probe facilitated the imaging of exogenous hypochlorite in the mitochondria of HeLa cells. The ability of Rh-TPP and Rh-Py to visualise hypochlorite in vivo was also demonstrated (Fig. 97). Nude mice were injected with either probe followed by an injection with a ClO⁻.
solution. In each case a persistent fluorescent response was elicited from hypochlorite.

A hydroxamic acid variant of this approach was outlined by Shin and Tae in 2013. In this case it was proposed that the hydroxamic acid was oxidised to the corresponding ring opened acyl nitroso group and it was anticipated that this product was rapidly hydrolysed to rhodamine 19 ($\lambda_{em} = 547$ nm). Probe 92 (Fig. 98) could detect hypochlorite concentration at ca. 25 nM. Of interest, when the exocyclic amines had an additional ethyl substituent, or were replaced by hydroxyl groups (fluorescein) the probes did not respond to hypochlorite, even when a 20-fold excess was administered.

Probe 92 could be used to detect exogenous hypochlorite in A549 lung cancer cells with a clear dose responsive fluorescence "switch on" with increasing ClO$^-$ concentration (Fig. 99). Furthermore 92 was successfully used for the in vivo detection of exogenous hypochlorite added to live zebrafish.

4.2.4 Hypochlorite mediated chalcogen oxidation. In 2013 Wu and Liu described the PET based BODIPY probe HCSe (Fig. 100) for hypochlorous acid. In this instance the BODIPY fluorophore was functionalised with a 2-(phenylselenyl)phenyl substituent and the presence of the selenide quenches fluorescence by PET ($\Phi_F = 0.005$). When oxidised by hypochlorite, the corresponding selenoxide (HCSeO) is formed and PET is unfavoured and BODIPY fluorescence is restored ($\lambda_{em} = 526$ nm, $\Phi_F = 0.690$). Using RAW264.7 cells the utility of this probe in vitro was explored; cells were pre-treated with HCSe and upon addition of ClO$^-$ enhanced fluorescence emission was detected. Subsequent treatment of these cells with glutathione (GSH) resulted in the loss of fluorescence intensity, indicating that the selenoxide (HCSeO) could be reduced back to its non-emissive precursor. Similarly, the endogenous

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**Fig. 97** Representative fluorescence images (pseudocolor) of nude mice given a skin-pop injection of (a) Rh-TPP and (b) Rh-Py (region A and C, respectively) and a subsequent skin-pop injection of ClO$^-$. Representative fluorescence images (pseudocolor) of nude mice given a skin-pop injection of (a) Rh-TPP and (b) Rh-Py region B and D, respectively. Images were taken after incubation for 20 and 40 min, respectively. Image reproduced with permission.

**Fig. 98** Structure and reaction of 92 with hypochlorite. Inset shows proposed acylnitroso intermediate 93.

**Fig. 99** Bright field image of A549 cells treated with (a) 92 in the absence of ClO$^-$. (b) With both ClO$^-$ and 92. Fluorescence image of A549 cells treated with (c) 92 in the absence of ClO$^-$. (d) Both ClO$^-$ and 83. Fluorescence images of zebrafish treated with (e) 92 in the absence of ClO$^-$. (f) Bright field image of zebrafish treated with 92 and ClO$^-$ (g) fluorescence images of zebrafish treated both 92 and ClO$^-$. Image reproduced with permission.

**Fig. 100** Structures of Se and Te containing probes for ClO$^-$. 
production of hypochlorite could be visualised in RAW264.7 cells that were stimulated using PMA.

Structurally similar annulated BODIPY chalcogenides (discovered somewhat serendipitously during the synthesis of dichalcogenides such as 93) were reported by Churchill in 2013. These unexpected annulated heterocycles were formed by base induced S_{2}Ar reaction of the dipyrrole dichalcogenide (93) and their structure confirmed by means of an X-ray structure of the selenide derivative (94). It is of note that the diselenide 93 has been utilised as a reversible superoxide probe. Despite not being applied to a living system, the annulated tellurium BODIPY (95) was shown to be at least 62-fold selective for ClO\textsuperscript{−} over other ROS (O_{2}\textsuperscript{−}*, H_{2}O_{2}, BuOOH, ClO\textsuperscript{−}, HO\textsuperscript{·}, and BuO\textsuperscript{·}) under physiological conditions (0.1 M PBS/EtOH (99:1 v/v), pH 7.5). Following oxidation with hypochlorite, PET from the tellurium was blocked and fluorescence was “switched on” (λ\textsubscript{em} = 597 nm, \(\Phi\textsubscript{F}\) increased from 0.06 to 0.23). Similar to HCSe\textsuperscript{−}, telluride oxidation could be reversed by treatment with GSH.

\(\text{MPhSe-BOD}\) is weakly fluorescent (\(\Phi\textsubscript{F} = 0.13, \lambda\textsubscript{em} = 510\text{ nm}\)) due to a Se modulated PET process. Hypochlorite oxidation interrupts the PET process and restores strong fluorescence (\(\text{MPhSeO-BOD}, \Phi\textsubscript{F} = 0.96\)). The reverse process (reduction) is selectively performed by HS\textsuperscript{−}. The probe was used to visualise endogenously produced hypochlorite in PMA stimulated RAW264.7 macrophages. The role of hypochlorite was confirmed by comparison to taurine and xanthine–xanthine oxidase controls. Imaging of the redox cycling between hypochlorite and HS\textsuperscript{−} was also demonstrated in RAW264.7 cells.

The tellurium containing rhodamine system (2-MeTeR, \(\Phi\textsubscript{F} < 0.001\)) presented by Nagano in 2012 has the advantage that its oxidised form (2-MeTeOR) is red emissive (\(\lambda\textsubscript{em} = 686\text{ nm}, \Phi\textsubscript{F} = 0.18\)). However, this probe suffers from interference from other ROS such as HO\textsuperscript{·} and ONOO\textsuperscript{−}. Nevertheless the probe was used to visualise endogenously produced ROS in human promyelocytic leukemia (HL-60) cells following stimulation with H_{2}O_{2}. As the added H_{2}O_{2} was consumed by MPO and/or the reaction was reversed by intracellular reductants, the increased fluorescent intensity was short lived. Fluorescence was restored following a second addition of H_{2}O_{2} (Fig. 101). Fluorescence intensity was also reduced when the cells were treated with aminobenzoic acid hydrazide (ABAH).

In 2013 the Han group reported the 4-aminonaphthalimide-based hypochlorite sensor \(\text{Ni-Se}\), shown in Fig. 102. While related to the aforementioned examples, the selenide was non-fluorescent (\(\Phi\textsubscript{F} = 0.04\)) and oxidation gave the fluorescent selenoxide \(\text{Ni-SeO}\) (\(\lambda\textsubscript{em} = 523\text{ nm}, \Phi\textsubscript{F} = 0.45\)). Unlike the previously described selenide–selenoxide systems fluorescence was not modulated by a PET mechanism. Instead, the 2-[phenylselenyl]benzyl moiety induced an excited state configurational twist which was not present in the oxidised \(\text{Ni-SeO}\). The reduction of the selenoxide could be reversed by the addition of HS\textsuperscript{−} to the system; this process was repeated up to six times with a decrease in fluorescence intensity of 50%. Cellular imaging of \(\text{Ni-Se}\) was performed using mouse macrophage RAW264.7 cells stimulated with LPS and PMA and enhanced fluorescence was observed. Weak fluorescence was also observed when cells were pre-treated with salicyldihydroxamic acid (SHA), a known inhibitor of MPO. A similar result was obtained with the use of a ROS scavenger glutathione S-transferase (GST, EC: 2.5.1.18). Co-staining with Hoechst 33342 revealed that probe was located mainly in the cytosol.

\(\text{Ni-Se}\) was also used in living mice for the \textit{in vivo} imaging of hypochlorite produced in a LPS model of acute inflammation (Fig. 103). Similar to the \textit{in vitro} study, when LPS was injected,
followed by Ni-Se, an enhanced signal was collected compared to the control (Ni-Se only). Injection of HS\(^-\) resulted in a decrease in fluorescence emission.

A new strategy for the detection of hypochlorite involving a selenide containing probe was presented by Li et al. in 2013.\(^{329}\) The partially reduced coumarin derivatives CM1 and CM2, (Fig. 104) each bearing a 3-phenylelenyl moiety were synthesised and both were non-fluorescent (\(\Phi_F < 0.001\)). In this instance the selenoxide produced from hypochlorite oxidation underwent a cope-type elimination to give the corresponding fluorescent reporters 96 (\(\lambda_{em} = 480 \text{ nm}, \Phi_F = 0.036\)) and 97 (\(\lambda_{em} = 468 \text{ nm}, \Phi_F = 0.047\)). The utility of probe CM1 was demonstrated in vitro, by the quantitative determination of exogenous hypochlorite in NIH3T3 cells. CM1 was also used to indicate the endogenous formation of hypochlorite in H\(_2\)O\(_2\) stimulated HL-60 human progranulocytic leukemia cell lines and RAW264.7 macrophages stimulated with lipopolysaccharide (LPS). In both of these instances there was a significant enhancement of the fluorescent response in relation to the controls.

A PET-based Ru(bpy)\(_3\)\(^{2+}\) based probe for the monitoring of ClO\(^-\)/HS\(^-\) redox cycle was developed by the Sun group in 2014 (Fig. 105)\(^{330}\). A pendent phenothiazine (PTZ) was attached to one of the 2,2'-bipyridine ligands, which suppressed fluorescence emission from the complex. Upon oxidation of the PTZ sulfur with ClO\(^-\) the fluorescence emission was restored (\(\lambda_{em} = 605 \text{ nm}, \lambda_{ex} = 450 \text{ nm}, \Phi_F = 0.39\)). This process could be reversed with HS\(^-\) in a recyclable fashion (12 cycles). The authors were able to visualise this ClO\(^-\)/HS\(^-\) redox cycle in live mice; weak fluorescence was observed after administration of probe 98 and this response was modulated during alternating additions of ClO\(^-\) and HS\(^-\).

The groups of Wang and Peng reported a cyanine-phenothiazine hybrid probe, PTZ-Cy2 which was sensitive to both hypochlorite and HO\(^*\).\(^{331}\) The sulphur atom of the non-fluorescent probe was initially oxidised by OCl\(^-\) and/or HO\(^*\) to give the fluorescent sulfoxide OPTZ-Cy2 which gave a pink emission (\(\lambda_{em} = 595 \text{ nm}\)). Continued addition of OCl\(^-\) to OPTZ-Cy2 led to a blue shifted emission (\(\lambda_{em} = 470 \text{ nm}\)) resulting from the degradation of the conjugated cyanine alkenne to the aldehyde OPTA. This blue shifted emission was not seen during HO\(^*\) addition, rather, excessive HO\(^*\) lead to a decrease in the total emission indicating decomposition. PTZ-Cy2 was used to visualise the ROS in PMA stimulated HeLa cells (Fig. 106). Co-staining with MitoTracker Deep Red FM indicates that fluorescence resulting from PTZ-Cy2 and OPTZ-Cy2 was localised in the mitochondria. Non-mitochondrial fluorescence was ascribed to the non-charged OPTA diffusing away from the mitochondria.

**4.2.5 Miscellaneous.** Lin and co-workers developed a series of rhodamine–merocyanine hybrid probes which absorb and...
emit in the NIR range. The non-fluorescent spirocyclic benzoyl thiosemicarbazide 100 (Fig. 107), in the presence of hypochlorite undergoes oxidative cyclisation to give the 2-amino-1,3,4-oxadiazole (101) which simultaneously opens the spirocycle and restores ICT (λ_{em} = 690 nm, λ_{ex} = 746 nm). Imaging of hypochlorite in LPS and PMA stimulated RAW264.7 macrophages established the viability of probe 100 in vitro and also revealed mitochondrial localisation. Probe 100 was applicable as a hypochlorite responsive probe in imprinting control region (ICR) mice treated with LPS; higher fluorescent emission was recorded when compared with the control or 100 alone.

Ma (2010) described the PET system 9-AEF consisting of an anthracene linked by an alkene to ferrocene (Fig. 108). The electron rich ferrocene instigates PET to the anthracene and fluorescence is quenched (Φ_F < 0.001). Hypochlorite could oxidise the alkene, and in the product (not characterised) PET is unfavoured and anthracene-like emission was observed (λ_{em} = 441 nm, Φ_F = 0.12). It should be noted that no single product was identified, nevertheless, the response to hypochlorite was dose dependant and the probe was successfully used to image hypochlorite in HeLa cells. No fluorescence was observed in cells containing 9-AEF unless they were pre- or post-treated with hypochlorite.

4.3 Hypobromite

In an analogous manner to hypochlorite, hypobromous acid/ hypobromite can be produced endogenously by macrophages and eosinophils from the reaction between H_{2}O_{2} and bromide which is catalysed by eosinophil peroxidase (EPO) in response to invading pathogens. Overproduction of HOBr can lead to diseases such as cancer, arthritis, cardiovascular disease and asthma. There is a demonstrated correlation between clinical severity in asthma patients and serum EPO levels. While the fraction of HOBr/BrO\(_{2}\) is likely to be high (the pK\(_{a}\) for HOBr is 8.7–8.8 at 25 °C) a small percentage of the anionic species is likely to exist at physiological pH and in certain compartments such as mitochondria a considerable percentage of BrO\(_{2}^{-}\) is likely. The design of fluorescent probes for in vitro detection of this species has recently received attention.

4.3.1 Recent developments. In 2012, the Han group reported two reversible fluorescent probes for the in vitro imagining of redox stress caused by HOBr. Both probes, mCyTem-OH and CyTem-OH (Fig. 109), respond through the HOBr mediated oxidation of a 2,2,6,6-tetramethylpiperidine-N-oxyl moiety to the corresponding oxaziridinium cation which leads to a donor-excited PET (d-PET) quenching mechanism (Φ_F decrease from 0.11 to 0.02 for mCy-TemOH). Oxidation of mCyTemOH (λ_{em} = 550 nm) results in a red-shifted emission centred at 632 nm. In vitro investigations used mCy-TeOH to monitor changes in endogenous HOBr concentration in
RAW264.7 macrophages; treatment with EPO, H$_2$O$_2$ and KBr resulted in an increase in the $F_{632}/F_{550}$ ratio from 2.8 to 12.9. Addition of ascorbic acid to the system resulted in reduction of the oxyammonium cation and a decrease in the $F_{632}/F_{550}$ ratio.

The full cyanine probe, Cy-TemOH, exhibited NIR absorption ($\lambda_{abs} = 702$ nm) and emission ($\lambda_{em} = 755$ nm). Cy-TemOH also responded to the HOBr/ascorbic acid redox cycle but suffered from severe bleaching after three cycles. Nevertheless, Cy-TemOH was successfully used to visualise the redox cycle in RAW264.7 cells. Both Cy-TemOH and mCy-TemOH localised in the cytoplasm and cells remained viable (MTT assay).

The same group then developed a NIR-reversible BODIPY-based probe which could monitor the redox cycle between HOBr and HS$^-$. The probe, diMPhSe-BOD (Fig. 110) absorbs strongly ($\epsilon_{672} = 22,770$ M$^{-1}$ cm$^{-1}$) but was weakly fluorescent ($\lambda_{em} = 711$ nm, $\Phi_F = 0.00083$) due to the electron transfer (PET) from the diarylselenides. Oxidation with HOBr leads to a blue-shifted emission ($\lambda_{em} = 635$ nm) and 118-fold increase in the $F_{635}/F_{711}$ ratio. The reverse reaction is accomplished by HS$^-$ (selectively over other RSS such as Cys, Hcys and GSH). It is also notable that the emission ratio generated from the reaction with HOBr was ca. 6-fold greater than with ClO$^-$. The HOBr/HS$^-$ redox cycle could be visualised in RAW264.7 cells and using diMPhSe-BOD HOBr and H$_2$S could be detected at concentrations as low as 50 and 100 nM respectively.

5. Conclusion and future work

One of the key purposes of this review was to highlight that the strategies employed for the design of small fluorescent anion sensors can be applied to in vitro and in vivo bioimaging. As such further developments in fluorophore design and strategies for signal modulation are likely to have immediate imaging applications. The emerging use of multiphoton excitation is already having an impact. Developments in the use of fluorescence lifetime will also broaden the range of approaches that can be used. Probes that are compatible with a super-resolution approach would also be beneficial for the ultimate aim of pinpointing the origin and/or fate of the species of interest.

Despite some impressive examples, many of the probes described herein do not meet the highly demanding criteria set out for an “ideal probe” in Section 1.2. Complete selectivity for the analyte of interest is not always realised and probes that are not dependent on a chemical transformation are currently the most prone to interference. Indeed it is a truly formidable
challenge to design a selective, reversible, recognition process that operates by means of non-covalent forces in an exceptionally competitive media! Further refinement of fundamental recognition principles and the ability to readily synthesise (or assemble) sophisticated structures is required to overcome this hurdle. While the selectivity of chemodosimeters is often excellent the reactions are typically irreversible and hence they are incapable of mapping in a truly spatiotemporal manner.

Furthermore, despite the obvious potential that these probes offer, many are destined to be research tools only; the widespread use of fluorescent probes in clinical settings is hampered by expensive operational set-up and sample by sample analysis. Nevertheless, several of the probes included herein have been successfully used in flow systems and with the current rapid progress in the field of micro-fluidics and micro-optics the goal of cost effective, “smartphone style”, point of care diagnostics using selective fluorescent probes is sure to be realised.

It is also interesting to note that for many small anions (\(\text{Cl}^{-}\), \(\text{I}^{-}\), \(\text{BrO}^{-}\)) the complete list of probes is very short and hence any progress would be welcome. Nevertheless, the field of anion sensing is rapidly advancing, and for the researchers involved in this field these challenges should be seen as nothing more than an excellent opportunity to further the fundamental understanding of our natural world.

**Glossary of cell lines**

- RAW264.7: Murine/mouse macrophages
- PC3: Prostate cancer
- HUVEC: Human umbilical vein endothelial cell
- NIH3T3: Murine/mouse fibroblast
- HeLa: Epithelial cervical cancer
- MCF-7: Breast cancer
- HEK-293: Human embryonic kidney cells
- L929: Murine/mouse fibroblast
- A549: Adenocarcinomic human alveolar basal epithelial cells
- COS-7: Fibroblast-like
- B16-F10: Murine/mouse skin melanoma
- U266: Human myeloma
- HepG2: Hepatocellular carcinoma
- PMN: Human polymorphonuclear neutrophils
- BV2 microglial: Brain resident murine macrophages
- MDA-MB-231: Human breast adenocarcinoma
- SH-SY5Y: Human neuronal neuroblasts
- HL-60: Human promyelocytic leukemia
- GES: Human breast cancer cells

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