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Advances in Reactive Oxygen Species Imaging

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ABSTRACT

Reactive oxygen species (ROS) play a pivotal role in many cellular processes and can either be beneficial or harmful. Design of ROS sensitive fluorophores has allowed for imaging of specific activity and has helped elucidate mechanisms of action for ROS. Understanding the oxidative role of ROS in the many roles it plays allows us to understand the human body. This review provides a concise overview of modern advances in the field of ROS imaging. Indeed, much has been learned about the role of ROS throughout the years, however it has recently been shown that using nanoparticles, rather than individual small organic fluorophores, for ROS imaging can further our understanding of ROS.

INTRODUCTION

In nature, reactive oxygen species (ROS) are unavoidable byproducts of aerobic metabolism. These oxygen-containing chemical species include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), the hydroxyl radical ($^{\bullet}OH$), peroxides (O_2^{2-}), and superoxides ($O_2^{\bullet-}$). Additionally, reactive nitrogen species (RNS), such as nitric oxide (NO), nitric dioxide (NO_2), and peroxynitrite ($OONO^-$), also fall under the category of reactive species. The presence of ROS in the body is vital to functions ranging from killing foreign microbes, to playing pivotal roles in cell signaling pathways(*1*), it has also become a biomarker for oxidative stress caused by diseases such as Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, depression, etc.(*2*)

Due to their involvement in a myriad of diseases, ROS probes for both *in vivo* and *in vitro* detection are vital tools for clinical diagnostics. Although many probes have been developed, several problems need to be addressed to successfully yield a multipotent ROS probe. Some of the considerations in designing *in situ* ROS fluorescent probes include water solubility, aggregation, quantum yields, singlet excited state lifetime, and excitation wavelength, which determines tissue penetration. Additionally, biological considerations such as cell permeability, ROS selectivity, and reaction rates are important for meaningful detection. Due to the complex cellular environments in which ROS are monitored, numerous diverse probes have been developed. In this review, we will focus on recent developments in the field of ROS imaging.

REDUCED DYES FOR ROS IMAGING

A commonly used strategy to trigger a fluorescent readout in response to ROS, is by using a non-fluorescent reduced dye, which when oxidized by ROS yields a fluorescent product. This strategy has been widely used for decades, with compounds such as Scopoletin being used to detect

H_2O_2 in the presence of peroxidase in the 1950s(*3*). Reduced dyes are particularly attractive for ROS detection since they often can be generated from commercially available fluorescent dyes. This allows for trivial synthesis of a wide range of reduced dyes that can be used to detect and image ROS. This section will cover the most common reduced dyes that are used for ROS imaging.

Hydrocyanines

Since their discovery in 2009(*4*), hydrocyanine probes have been used for detection of superoxide and hydroxyl ROS both *in vitro* and *in vivo*(*4*,*5*), with detection limits down to nanomolar concentrations. A wide range of hydrocyanines with desired properties can be prepared in a single synthetic step, making them accessible even to labs with limited synthetic infrastructures.

Hydrocyanines were first discovered by Kundu et. al.(*4*) in 2009 by reducing commercially available cyanine dyes with $NaBH_4$. The hydrocyanine hydro-Cy3 successfully imaged ROS in rat aortic smooth muscle cells after treatment with the angiotensin II peptide, a system which mimics the development of atherosclerosis and hypertension. In addition, hydro-Cy7, a near-infrared (NIR) dye, was used to image ROS in mice during a lipopolysaccharide-mediated inflammatory response. In the years following, hydrocyanines were extensively investigated for ROS imaging, most of which were covered in a previous review.(*5*) We will therefore focus on recent developments in the field of hydrocyanines in the past three years.

Although hydrocyanines have many uses, they do have some limiting factors. These limitations include high auto-oxidation, low Stokes shifts, and low solubility, as well as their product cyanine dyes' lability to ROS(*6*). A strategy for reducing some of these shortcomings was published by Maity et. al.(*6*), where a new class of thiophene-bridged hydrocyanines (TBHC) was introduced

(Fig 1A). TBHC showed superior stability to auto-oxidation, with 89% of probe remaining after a 48hr incubation in phosphate-buffered saline (PBS), compared to 42% for hydro-Cy5 (Fig 1B). After oxidation, THBC generated a fluorescent product with significant stability towards ROS-mediated degradation (Fig 1C). However, despite their promise, THBC-derivatives have not been thoroughly studied, due in part to their relative complex synthesis compared to regular hydrocyanines. Regardless, the increase in chemical stability as well as improved photophysical properties yields a promising scaffold that should be further investigated.

As hydrocyanines are well established in their use for ROS detection, recent advances in the field have focused on building multi-functional systems to increase selectivity and introduce additional functions. For example, Al-Karmi et. al.(*7*) introduced a ^{18}F-label on IR780, allowing for positron emission tomography (PET)-based biodistribution studies of hydrocyanine probes. Andina et. al.(*8*) utilized a modular design of a reported dye connected to hydro-Cy5 via two complimentary peptide nucleic acid moieties for measuring extracellular ROS. The use of a reporter dye allowed them to account for distribution differences by utilizing a ratio-based readout. In a study by Zhang et. al.(*9*), glioblastomas were selectively imaged by conjugating hydro-Cy5 to the integrin αvβ3-targeting peptide Arg-Trp-(D-Arg)-Asn-Arg. Using this strategy, tumors could be imaged selectively in the presence of other inflammatory tissues.

Xanthene Probes

Xanthene dyes are some of the most frequently used probes for ROS detection, with reduced derivatives of fluorescein and rhodamine being the most abundant. They can be easily prepared through direct reduction of their oxidized derivatives. Xanthene dyes were initially used as H_2O_2-selective probes, but have been shown to react readily with hydroxyl and peroxyl radicals

as well as several RNS(*10*). This has reduced their practicality in favor of more selective probes, although they are still frequently used.

Although fluorescein and rhodamine-based dyes are heavily used for ROS detection, they do not possess any specificity towards the ROS source. One way to achieve organelle specificity was explored by Zhang et. al(*11*) where Si-rhodamine-based NIR fluorescent probes were used to target lysosomal ROS. These probes showed high specificity towards the highly reactive ROS, HClO, HO•, and ONOO•. This allowed for imaging of ROS in the lysosomes of cancer cells. This strategy was further explored by Wang et. al, when they created a NIR probe for *in vivo* imaging of HClO/ONOO• in an idiopathic pulmonary fibrosis mouse model(*12*).

ROS-SENSITIVE FUNCTIONAL GROUPS

Several ROS probes possess a protecting/quenching group that is cleaved upon ROS exposure. These probes depend largely on the nucleophilic properties of ROS such as H_2O_2 and superoxides. This mechanism of action allows for ROS specificities that are complementary to reduced dyes. The main classes that will be covered in this section are sulfonyls, arylboronates, and phosphinates.

Sulfonyls

Sulfonyl-protected dyes were developed to battle shortcomings associated with other protecting groups such as acetyls, which show significant deprotection from hydrolysis in addition to perhydrolysis(*13*). The superoxide-selective sulfonyl probes were introduced when Maeda et. al(*14*) developed a di-protected fluorescein analogue, although the observed selectivity depended on the sulfonyl substituent. Further studies were done to eliminate the impact of side reactions by

modifying the leaving group. In particular, the triflate-protected probe HKSOX-1 showed excellent resistance to thiol-mediated cleavage, which allowed for selective detection of superoxide in zebrafish embryos(*15*). Sulfonyls are still being developed for use in selective superoxide detection, with recent applications including superoxide detection in mitochondria(*16*), and the use of two-photon excitation microscopy (TPEF) probes(*17*).

Arylboronates

Arylboronic acids have long been known for their reactivity towards H_2O_2, in which addition of peroxide to the boron leads to an aryl group migration and subsequent phenol formation(*18*). Boronic acids mask the fluorescence of fluorophores, yielding H_2O_2-responsive probes. This method was pioneered by the Chang lab(*19*) when they synthesized a di-boronic acid-modified fluorescein that selectively reacted with H_2O_2 in the presence of other ROS. Following this seminal study, aryl boronic acid-capped fluorophores remains a prevalent strategy in the selective detection of H_2O_2. Recent advances include detection of H_2O_2 in ischemia-reperfusion injury(*20*), chemiluminescent detection through a capped luciferin substrate(*21*), and the use of boronate-capped probes that trigger 1,6-elimination reactions upon phenol generation(*20*).

Phosphinates

The nucleophilic property of superoxide can also be exploited to cleave phosphinate groups. This strategy was spearheaded by Xu et. al(*22*) when they synthesized diphenylphosphinate-capped fluorescein and naphtofluorescein analogues, which were able to detect superoxide down to 0.1nM. These probes are also useful for *in vitro* imaging, which was illustrated by imaging superoxide formation in macrophages after stimulation with phorbol 12-

myristate 13-acetate, an oxidative burst stimulant. This strategy was further expanded on by Zhang et. al.(*23*) who created a NIR-dye which allowed for *in vivo* imaging of superoxide. Recently, this strategy was also expanded to include bioluminescence by Liu et. al.(*24*), who utilized a phosphinate-capped luciferin to detect polystyrene-induced superoxide formation in cells. Furthermore, a mitochondria-targeting probe (NA-T) that saw a shift in fluorescence after phosphinate-deprotection and subsequent 1,6-elimination to release an unmodified diketopyrrolopyrrole dye has been developed(*25*). NA-T showed high specificity towards superoxide-mediated cleavage in the presence of a wide range of ROS, and was used for imaging of PMA/LPS-triggered superoxide formation in *Daphnia magna*.

TWO-PHOTON MISCROSCOPY

TPEF is a type of fluorescence microscopy that uses two NIR photons to electronically excite a fluorophore from the ground to the singlet excited state, as opposed to directly exciting the fluorophore with a high energy light source. Because long wavelengths penetrate deeper into tissue, using TPEF, rather than confocal microscopy, offers many benefits. Aside from deeper tissue penetration, TPEF also offers reduced photobleaching and photodamage, suppressed background signal, and minimized scattering.

Common organic fluorophores, such as fluorescein derivatives, have been modified with ROS-sensitive moieties that quench fluorescence, however, upon ROS-mediated cleavage they possess fluorescence suitable for TPEF(*26*). Furthermore, Lewis and team recently developed an azulene fluorophore for TPEF bioimaging of RNS and ROS(*27*). The boronic ester is cleaved in the presence of H_2O_2 and $ONOO^-$, and due to strong electron donating groups the azulene derivative undergoes an intramolecular charge transfer which shifts the absorption and emission

of the polyaromatic hydrocarbon to the visible range. Due to the immense list of chromophores that have been extensively studied, which absorb in the visible spectra, modifying current chromophores for TPEF has been a popular choice.

ULTRASOUND IMAGING

Ultrasound imaging, or sonography, is a technique that uses high frequency sound waves for real time *in vivo* imaging. Currently, microbubbles are one of the most used ultrasound contrast agents (UCAs) for clinical ultrasound imaging. However, for imaging ROS, there are limitations for using microbubbles as UCAs.

Microbubbles

Microbubbles vary in size from 1-10μm in diameter. Conventional microbubbles are composed of lipids, polymers, and surfactants, etc., which encapsulates a gas. However, by chemically modifying hydrazine, N_2 encapsulation as an organic compound is possible. The Murthy lab has previously demonstrated the use of chemically generated microbubbles to image *in vivo* oxidative stress via ultrasound.(*28*) Allylhydrazine encapsulated within a liposome proved very efficient in detecting 10 mM of ROS *in vitro* and *in vivo*.(*28*) In the presence of ROS, the amine from allylhydrazine oxidizes into 2-propenyl-diazene and thus allows for a retro-ene reaction that results in the release of nitrogen and propylene gas. This increase in gas concentration creates acoustic impedance which can be detected acoustically.

Photoacoustic Imaging Using Nanoparticles

When tissue absorbs light and converts it to heat, via vibrational relaxation, this may lead to thermoelastic expansion that then generates ultrasound waves. This is known as the photoacoustic effect. Previously researchers have used polymers and nanoparticles as a nanoplatform for developing photoacoustic probes(*29*). *In vivo* ratiometric photoacoustic imaging is a relatively new method that has been used to monitor ROS and RNS(*29*,*30*). When using nanoparticles, coupled with ROS sensitive dyes, the nanoparticle emits an optoacoustic signal. In the presence of ROS, absorption/fluorescence of the chromophore is blue shifted due to degradation while absorption/fluorescence of the nanoparticle remains unaffected. Overlaying the images when monitoring at NIR versus visible wavelength generates a representative image.

Using nanoparticles to image and generate ROS in cancer cells is of great importance for theranostics. By incorporating multifunctional groups on semiconducting perylene diimide (PDI), Chen and team developed a theranostic nanoparticle, PDI-IR790s-Fe/cisplatin(*31*), for ratiometric photoacoustic imaging. IR790s, a cyanine derivative, absorbs light at 790 nm but decomposes in the presence of ROS, losing its absorptivity. The change in absorption ratio between PDI and IR790s allows for ratiometric imaging. The cisplatin prodrug activates NOX thus forming superoxides followed by H_2O_2 generation by superoxide dismutase. Fe^{3+} then converts H_2O_2 into hydroxyl radicals via the Fenton reaction which reacts with IR790s to quench absorption. This technique to induce ROS for cancer therapy also allows for real-time monitoring during treatment.

Photoacoustic Imaging Using Upconversion Nanocrystals

Detecting multiple radical species independently is invaluable in ROS imaging. By incorporating two cyanine dyes, hydro-Cy5 and Cy7, that are sensitive to two specific reactive species onto the surface of an upconversion nanoprobe, Xing and team were able to screen the

presence of ROS and RNS via multispectral optoacoustic tomography by ratiometrically monitoring multiple radicals(*32*). Upconversion nanocrystals have been used due to their anti-stokes shift properties. Unlike TPEF, upconversion nanocrystals rely on sequential, rather than simultaneous, absorption of photons. Therefore, it is possible to excite the nanocrystal with NIR light and observe emission in the visible spectra(*33*). Due to the emission of the nanocrystal overlapping with the absorption of the cyanine dye, individual monitoring of the cyanine dye is possible. In the presence of ROS there is a fluorescence increase, thus an increase in optoacoustic (OA) signal. Conversely, in the presence of RNS there is a decrease in fluorescence and thus a decrease in the OA signal (Fig 2).

LUMINESCENCE

ROS imaging usually requires a light source to photoexcite a fluorophore, thus resulting in photoluminescence. However, there are processes that result in spontaneous light emission without photoexcitation, known as chemiluminescence and bioluminescence. While chemiluminescence involves a chemical process, bioluminescence involves luciferase enzymes to oxidize luciferin to oxyluciferin thus producing luminescence. Luminescence microscopy minimizes background emission and thus provides better signal-to-noise ratios and eliminates phototoxicity and photobleaching.

Chemiluminescence

Unlike fluorophores, chemiluminescent (CL) probes do not auto-fluoresce, thus making them desirable for bioimaging. However, some drawbacks are short emission time and wavelengths. To address the issue of short emission time, Ren and team synthesized a

chemiluminescent polymer dot probe (hemin-Pdots), that relies on chemiluminescence resonance energy transfer (CRET), to enhance the chemiluminescence lifetime to about 10 hours in the presence of a L-012, a luminol analogue, and H_2O_2(*34*). In the presence of H_2O_2, L-012 oxidizes, and chemiluminescence that is 100 times greater compared to luminol is observed(*34*). Slow diffusion of the oxidized L-012 through the hemin-Pdots nanoparticles increases the chemiluminescence lifetime via CRET, where the luminol is the energy donor and the hemin-Pdot is the acceptor. Indeed, slow diffusion dynamics of such chemiluminescent systems show a 700-fold enhanced CL over 10 hrs.

One of the problems in ROS imaging via luminescence, is correlating the readout to ROS concentration. Contag and team have previously reported a reporter probe, coelenterazine, to measure superoxide dynamics (*35*). Interestingly they were able to observe a change in chemiluminescence during cellular respiration as well as in response to variant glucose concentrations. By generating an *in vitro* baseline using coelenterazine, their system shows promise not only for *in vivo* imaging but also for quantification of superoxide concentrations.

CONCLUSION

ROS continues to play a central role in biology and medicine and there is great interest in imaging their concentration in cell culture and *in vivo*. Although significant progress has been made towards imaging ROS, several challenges still remain. In particular, although a wide variety of turn-on probes have been developed that can indicate the presence of ROS in tissues and in cells, their analysis is always made indirectly, via comparison against control cells. A central goal in the field of ROS imaging, which has still not been achieved, is the development of ROS probes that can quantitatively measure the concentration of ROS in cells, similar in function to calcium

chelating probes. Ratiometric ROS probes provide a significant step towards this goal, but are still unable to provide true quantitation. In addition, ROS probes that can measure tissue ROS concentrations via PET, MRI or ultrasound imaging are also greatly needed because these imaging modalities are widely used in the clinic. The vast majority of existing ROS probes are based on fluorescent imaging, and fundamentally new chemical strategies for imaging ROS has to be developed to detect ROS via PET, MRI, and ultrasound. The field of ROS imaging has made remarkable progress in the last 10 years, and we anticipate that there will be many exciting new developments in the years ahead.

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CONFLICTS OF INTEREST STATEMENT

No potential conflicts of interest relevant to this article exist.

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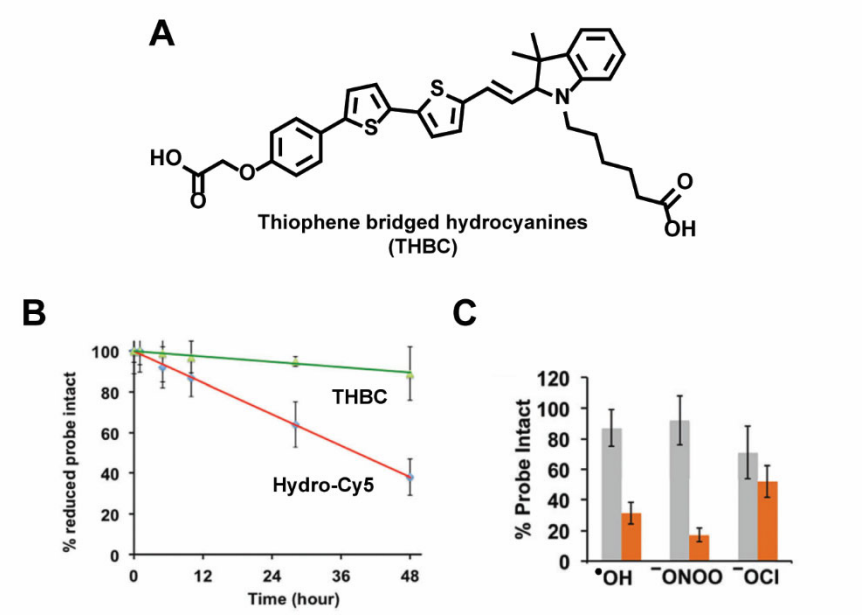

Fig 1: Hydrocyanines have been modified for increased stability and in vivo sensitivity. A) Structure of the recently reported thiophene bridged hydrocyanines (THBC). B) THBC has increased stability to auto-oxidation in PBS. C) Active thiophene bridged cyanines (TBC, grey) have a higher stability towards ROS-dependent degradation compared to Cy3 (orange). The graph represents % probe intact after incubation with 250μM of indicated ROS. Reproduced from Maity et. al.(6) with permission from The Royal Society of Chemistry.

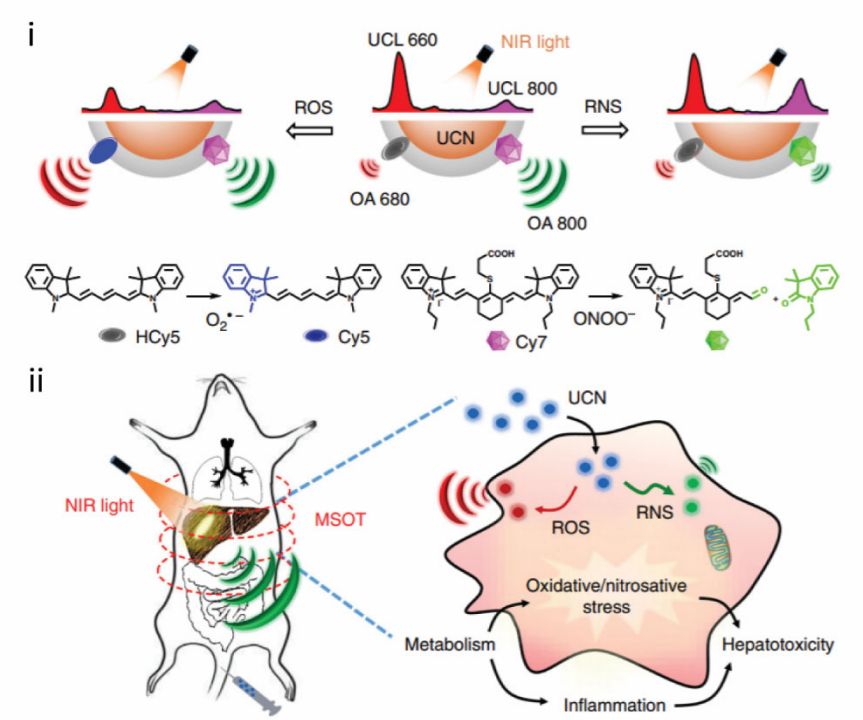

Fig 2: Illustration of i) HCy5,ROS, and Cy7, RNS on upconversion nanocrystal (UNC) surface. In the presence of ROS, non-fluorescent HCy5 gets converted into fluorescent Cy5 while in the presence of RNS, fluorescent Cy7 decomposes causing a signal reduction at 800 nm. ii) Schematic representation of UCN in live mice for bioimaging. Figure reprinted from Ai et al.(32) with permission from Springer Nature.