Development Of A Modular Ratiometric Fluorescent Probe For The Detection Of Extracellular Superoxide

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**DEVELOPMENT OF A MODULAR RATIOMETRIC FLUORESCENT
PROBE FOR THE DETECTION OF EXTRACELLULAR SUPEROXIDE**

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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Summary

Reactive oxygen species (ROS) have undergone an exceptional change in the way they are perceived over the last two decades. Their role as simple antimicrobial agents during host defense has been broadened by a vast repertoire of signaling functions in health, disease and aging. Unravelling these short-lived species' diverse actions has concurrently increased the interest in them, emphasizing the need for reliable, specific and sensitive detection methods. Ratiometric fluorescent probes offer an elegant option to detect the analyte of interest and to normalize the obtained ROS-dependent signal to a second fluorescence emission. The ratio of the two signals is thus independent of the sensor concentration, and minimizes artefacts and intensity fluctuations. This Ph.D. thesis focuses on the design of a ratiometric probe for the detection of ROS in the extracellular space by linking a ROS-sensor (namely HCy5) and a reporter dye (Chromis500) with a well-defined, highly negatively charged macromolecule. As a further step, the linker was improved with a double-stranded DNA-analogue to produce a modular platform for ratiometric ROS detection.

In Chapter 1 ROS production is discussed in general and the action of neutrophils during intestinal inflammation is outlined.

Chapter 2 focuses on the advances in the field of ratiometric fluorescent probes for the detection of ROS and reactive nitrogen species. A summary of the probes published to date is provided, together with a critical discussion of their possible application in tissue culture and *in vivo*.

Before designing the ratiometric probe, the already existing ROS-sensor HCy5 was validated in cell-free and cell culture-based experiments relevant for the scope of this thesis, which are reported in Chapter 3. The detection of superoxide and hydroxyl radicals was successfully achieved in cell-free experiments and ROS-sensing in cell-culture based assays was exemplified. The sensing capacity of HCy5 was compared to other frequently used fluorescent ROS-sensors and it was found to be similar. Subsequently, to obtain an extracellular ROS-sensor, this intracellular indicator was modified with hydrophilic macromolecules.

A ratiometric fluorescent sensor was created by coupling Chromis500 to the hydrophilic linker as outlined in Chapter 4. For this purpose, the extracellular indicator that consisted of ten poly(glutamic acid) residues, was modified with a lysine residue to enable coupling of the reporter dye. By measuring the Förster resonance energy transfer efficiency, the optimal distance between the two fluorophores was determined, and six discrete PEG₁₀ units were found

to reduce the effect sufficiently. Uptake studies in a cell line mimicking the intestinal epithelial barrier proved the predominantly extracellular nature of the ratiometric probe. Finally, specific superoxide sensing was exemplified in a cell-free assay and an approximately 2-fold increase in fluorescence emission ratio was detected.

In Chapter 5, a double stranded peptide nucleic acid was introduced in the linker design. This allowed us to increase the modularity of the probe and also to manipulate the two fluorophores separately. After obtaining the fully assembled ratiometric probe, its cellular uptake was assessed and its internalization was found to be greatly reduced compared to the uncoupled Cy5 and Chromis500. As for the previous approach, the superoxide sensing was successfully investigated in cell-free assays and a 2-fold increase in fluorescence emission ratio was obtained. The analysis was completed by ROS sensing in cell culture, which also resulted in a doubling of the emission ratio.

The main findings of this Ph.D. work are outlined in Chapter 6, where the conclusions drawn from the main results are presented, together with a discussion of the system's limitations and possible improvements.

Zusammenfassung

Das Verständnis der Bedeutung reaktiver Sauerstoffspezies (RSS) hat sich in den letzten zwei Jahrzehnten stark verändert. Während diese kurzlebigen Moleküle früher vornehmlich im Zusammenhang mit Entzündungen und für ihre antimikrobielle Aktivität bekannt waren, umfassen neuerer Kenntnisse auch deren Rolle in der Signalübertragung in verschiedenen Geweben und bei Alterungsprozessen. Somit besteht heute ein grosses Interesse, diese Moleküle noch eingehender zu untersuchen. Der Nachweis von RSS stellt jedoch immer noch eine Herausforderung dar, was die Suche nach zuverlässigen, spezifischen und sensitiven Nachweismethoden intensiviert hat. Ratiometrisch auswertbare Fluoreszenzfarbstoffe scheinen für den Nachweis von RSS besonders vorteilhaft, da sie nicht nur den Analyten (RSS) direkt sichtbar machen können, sondern das erhaltene Signal mit einer zweiten Fluoreszenzemission zu normalisieren vermögen. Da das Verhältnis der beiden Fluoreszenzsignale unabhängig von der Konzentration des Fluoreszenzfarbstoffes ist, werden fluktuierenden Fluoreszenzemissionen und andere Artefakte vermieden. Die vorliegende Doktorarbeit befasst sich hauptsächlich mit der Entwicklung eines ratiometrischen Fluoreszenzfarbstoffs für den Nachweis von extrazellulären. Dazu wurde der bereits bekannte RSS-affine, intrazellulär aktive Farbstoff (HCy5) mit einem RSS-blinden Fluorophor (Chromis500) durch ein hydrophiles Makromolekül verbunden. Das makromolekulare Bindungsmolekül wurde in einem zweiten Schritt mit einem Doppelstrang eines DNS-Analogons modifiziert, womit ein flexibel kombinierbarer und ratiometrisch auswertbarer Farbstoff zur Verfügung stand.

In Kapitel 1 wird die physiologische RSS-Produktion grundsätzlich erklärt und für den Fall einer Entzündung im Darm spezifiziert.

Kapitel 2 fokussiert auf ratiometrisch auswertbare Fluoreszenzfarbstoffe, die RSS oder reaktive Stickstoffspezies sichtbar machen können. Bisher beschriebene Farbstoffe werden tabelliert und an einigen Beispielen wird deren mögliche Anwendung in Zellkulturen und *in vivo* kritisch diskutiert.

Experimentell wird der bekannte, intrazellulär aktive RSS-Farbstoff HCy5 zunächst in, im Zusammenhang mit der Doktorarbeit relevanten, zellfreien Medien und in Zellkulturen validiert (Kapitel 3). Der Nachweis von Superoxid- und Hydroxylradikalen in zellfreien Untersuchungen gelang und in einem Vergleich des Nachweisvermögens von HCy5 mit jenem anderer etablierter RSS-Sensoren wurden in verschiedenen Zellexperimenten ähnliche Ergebnisse

erzielt. Danach wurde der intrazellulär aktive Farbstoff HCy5 mit hydrophilen Makromolekülen modifiziert, um einen extrazellulären RSS-Sensor zu erhalten.

Kapitel 4 beschreibt die Synthese eines ratiometrisch auswertbaren Fluoreszenzfarbstoffes zum Nachweis von RSS. Dazu wurde HCy5 zunächst mit einer Oligomerkette von zehn Glutaminsäuren (Bindungsmolekül) und einem Lysin modifiziert; an letztere Aminosäure konnte danach das RSS-blinde Chromis500 gebunden werden. Weiter wurde die Distanz zwischen den Fluorophoren optimiert, um die Förster-Resonanzenergietransfer (FRET) Effizienz zu minimieren, was durch Einfügen von sechs einzelnen PEG₁₀-Oligomeren beim Bindungsmolekül gelang. Die extrazelluläre Aktivität des Farbstoffes wurde durch Zellaufnahmeexperimente mit einer von Darmepithelzellen abgeleiteten Zelllinie bestätigt. Schliesslich wurde die spezifische Superoxid Detektionskapazität in zellfreien Experimenten untersucht und eine 2-fache Zunahme des ratiometrischen Verhältnisses der Fluoreszenz Emissionen beobachtet.

Kapitel 5 beschreibt die Optimierung des aminosäure-basierten Bindungsmoleküls, indem es mit einem Doppelstrang einer Peptidnukleinsäure modifiziert wurde. Diese Änderung im Bindungsmolekül erhöhte die Flexibilität des Sensors und ermöglichte die voneinander unabhängige Synthese und Handhabung der zwei Fluorophore. Wie für den vorhergehenden Ansatz wurde zuerst die Aufnahme in Zellen untersucht; im Vergleich zu den nativen Farbstoffen Cy5 und Chromis500 wurde das Farbstoffkonjugat in deutlich geringerem Ausmass internalisiert. In zellfreien Experimenten mit Superoxid konnte wiederum, wie in Kapitel 4 beschrieben, eine 2-fache Zunahme des Verhältnisses der Fluoreszenzemissionen gemessen werden. Die Untersuchungen wurden mit RSS-Messungen an Zellen ergänzt, wo ebenfalls eine Verdoppelung des Fluoreszenz-Emissionsverhältnisses beobachtet wurde.

Die wichtigsten Erkenntnisse dieser Doktorarbeit sind im Kapitel 6 zusammengefasst. Mögliche Einschränkungen des Systems werden diskutiert und Verbesserungsvorschläge gemacht.

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Chapter 1

Background and Purpose

Traditionally, reactive oxygen species (ROS) were viewed as antimicrobial agents that were mainly produced during host defense mechanisms, such as the oxidative burst of neutrophils.^[1–3] The activity of ROS was long believed to be unspecific and their oxidative action towards lipids, amino acids and other biomolecules was accepted as a necessary evil.^[4] This perception has changed during the last two decades due to the discovery of various signaling functions of ROS.^[5] The ever widening spectrum now includes, but is not limited to, mediation of information during inflammatory responses, stem cell differentiation, cancer and ageing.^[6–10]

In this work the term reactive oxygen species is used to describe oxygen containing reactive molecules.^[11] The primary species that is generated from molecular oxygen is its one-electron reduction product superoxide ($\text{O}_2^{\bullet-}$), and is mainly produced by NADPH oxidases^[12] or through leakage in the electron transport chain in the mitochondria.^[5] Nitric oxide ($\text{NO}^{\bullet}$) is the primary nitrogen containing ROS and is produced by NO synthases.^[13] Further downstream ROS are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical ($\text{HO}^{\bullet}$), singlet oxygen ($^1\text{O}_2$), ozone (O_3), various carbon peroxides and the nitrogen containing peroxynitrite (ONOO^-), nitroxyl (HNO) and nitrogen dioxide (NO_2).^[10,14] $\text{O}_2^{\bullet-}$ can either be converted into H_2O_2 by superoxide dismutase (SOD) or can react with nitric oxide ($\text{NO}^{\bullet}$) to produce ONOO^- . H_2O_2 is the precursor of HOCl , which is generated by myeloperoxidase (MPO) and the $\text{HO}^{\bullet}$ that is formed upon catalytic cleavage of the dioxygen bond by Fe^{2+} or Cu^{2+} (**Figure 1-1**).^[15] $\text{NO}^{\bullet}$ is a gaseous free radical that can recombine in a diffusion-controlled reaction with $\text{O}_2^{\bullet-}$, giving rise to ONOO^- or can be reduced and protonated leading to HNO (**Figure 1-1**).^[16,17] Distinguishing between the different ROS is a challenging task from a chemical point of view. Powerful analytical methods are therefore needed to elucidate the signal/stress dichotomy of these species.

Figure 1-1: ROS generation in cells.

Endogenous ROS can originate from different sites such as leakage from mitochondrial respiratory chain, xanthine oxidase, lipoxygenase, cytochrome P450 or by release of iron and copper ions from iron-sulphur clusters. However, NADPH oxidases (NOXs) are the only source that generates $O_2^{\bullet-}$ not through leakage but on purpose.^[18] NOX is a multicomponent transmembrane protein of which seven mammalian isoforms have been described, all sharing the feature of transferring electrons from NADPH across a membrane to reduce molecular oxygen to $O_2^{\bullet-}$ (**Figure 1-2 a**).^[19,20] NOX2 is the best studied homolog of this family and because it was first discovered in neutrophils and macrophages it is also called phagocyte NADPH oxidase. The required subunits to obtain a functional enzyme are a Rho guanosine triphosphatase (Rac1 or Rac2), the transmembrane proteins gp91^{phox} and p22^{phox}, and the cytosolic components p47^{phox}, p67^{phox} and p40^{phox}.^[21] Electron transfer is ensured by a seven-step mechanism starting from cytosolic NADPH that donates two electrons to FAD, which is reduced to FADH₂. By forming a semi-quinone, one electron is transferred to the inner heme, then to the outer heme and finally to molecular oxygen. The same cascade is repeated with the second electron from the FAD semi-quinone, reducing a second molecule of oxygen (**Figure 1-2 a**). Overall, this process is energetically favorable ($\Delta E_m = 160$ mV), except for the step from the inner to the outer heme, which can only occur when oxygen is bound to the outer heme to accept the electron.^[19] The assembly of NADPH oxidase on the phagosomal and plasma membrane is triggered by the activation of neutrophils, which represents an important mechanism in host defense (**Figure 1-2 c**).

Phagocytic cells are a major endogenous source of ROS in the body, such as the neutrophils accounting for 70% of all white blood cells in circulation in humans.^[1,18] The migration of these polymorphonuclear leukocytes from the blood stream to the site of the microbial invasion is one of the first defense mechanisms during innate immune response.^[22] Upon reaching the infected site, neutrophils can harness a vast repertoire of antimicrobial weapons with the main objective of killing the intruders. Their diverse intra- and extracellular mechanisms include phagocytosis of the microorganism followed by antibacterial protein- and ROS-dependent killing (**Figure 1-2 b (1)**), degranulation of vesicles filled with antimicrobial agents (**Figure 1-2 b (2)**), and release of neutrophil extracellular traps (NETs) to immobilize and kill pathogens (**Figure 1-2 b (3)**).^[23] The generation of ROS, *i.e.* $O_2^{\bullet-}$, by neutrophils is called respiratory or oxidative burst and is accompanied by a suddenly increased O_2 consumption due to NADPH oxidase activity. The generated $O_2^{\bullet-}$ leads to downstream ROS as described in **Figure 1-1**, which may also have a higher

Figure 1-2: a) Active phagocytic NADPH oxidase, b) Neutrophil (1) phagocytosis and ROS-dependent killing of microbes, (2) degranulation, (3) NETosis, c) neutrophil migration from blood to infected site in healthy and inflamed intestine.

antimicrobial activity than $O_2^{\bullet -}$. Upon engulfment of the pathogens, ROS are generated in the phagosome and granules containing a plethora of peptides and proteins with detrimental effects on microbes fuse with the phagosomal membrane. ROS also functions as a second messenger during a process called lethal NETosis. During these events, neutrophils die upon releasing their complete DNA structures into the extracellular space, triggered by chromatin decondensation and spreading. Many bactericidal components of neutrophil granules adhere to these fibrous structures and make

this active form of cell death a potent weapon to immobilize and kill especially large pathogens that are difficult to phagocytose. Additionally, two non-suicidal forms of NETosis have been discovered up to date and since in both cases the neutrophils remain alive, the processes have been referred to as vital NETosis (in contrast to lethal NETosis). The ROS-independent form of vital NETosis involves the release of DNA through nuclear decondensation but without excretion of the nuclear or plasma membrane. After these events, the neutrophils are still capable of engulfing pathogens and their lifespan is not affected by the loss of DNA. During the other variation of vital NETosis, only mitochondrial DNA is released and neutrophil nuclei are not involved in the process.^[24,25]

The importance of NADPH oxidase-derived ROS becomes apparent in patients diagnosed with chronic granulomatous disease (CGD), a rare inherited disorder characterized by a (partly) dysfunctional NADPH oxidase.^[26] Young patients usually suffer from recurrent life-threatening bacterial and fungal infections due to defective neutrophils that cannot generate $O_2^{\cdot-}$ nor downstream ROS (*e.g.* H_2O_2). As a result, formation of granulomata and dysregulated inflammation in the genitourinary and gastrointestinal (GI) tracts are frequently observed.^[27] The manifestation of the disease in the GI tract is characterized by ulcers, abscesses, fistulae and obstructive symptoms. A similar pathological appearance is observed in certain cases of inflammatory bowel disease (IBD), which is a multifactorial inflammatory chronic disorder of the GI tract. IBD patients suffer from relapsing and chronic bowel inflammation and have a ten times greater risk of developing colorectal cancer. In IBD patients, neutrophils accumulate aberrantly within intestinal crypts during bowel inflammation and cause a prolonged inflammatory response.^[28] The very effective means of neutrophils to fight microbes backfire in these situations, and lead to an excessive recruitment of inflammatory cells and tissue damage (**Figure 1-2 c**). The abnormal accumulation of neutrophils observed in IBD leads to a tremendously elevated ROS generation and results in the oxidation of DNA, proteins, lipids and other biomolecules.

The role of ROS production and its influence on the course of disease is still not unequivocally established. Hence, tools with high spatio-temporal resolution to specifically detect ROS at the site of inflammation would provide more evidence to this debate and would be of great value. Fluorescent probes represent a unique mean to detect changes in analyte concentration in real-time using inexpensive equipment.^[29,30] Stimuli-responsive switch-on sensors can be used to enhance the signal-to-noise ratio but also to ensure specific detection of different ROS.^[31–33] Their general

drawback is an inherent concentration dependence, that can be overcome by monitoring the change of ratio of two fluorescence emissions.^[34–36] This type of sensors are referred to as ratiometric probes and a summary of such indicators for ROS is provided in Chapter 2, followed by a critical discussion of selected examples. As it was shown that extracellular ROS have a distinct function in inflammation,^[37] wound healing,^[38] and are important regulators in ROS-mediated apoptosis in tumor cells,^[39] discrimination between extra- and intracellular ROS is pivotal to better understand their complex and diverse functions. Most fluorescent ROS sensors are designed to detect only intracellular analytes. With the goal of expanding the detection possibilities to the extracellular space, we designed an extracellular ratiometric probe to allow real-time imaging of ROS. First, the intracellular hydrocyanines introduced by Kundu *et al.*^[40] were evaluated and compared to other commercially available ROS sensors, as reported in Chapter 3. Then, cell-free assays as well as cell-based models were established and used to show that ROS detection with hydrocyanines was possible. Additionally, a model fluorophore was selected for our study and was successfully modified to reduce internalization by cells. The sensor was made ratiometric by linking a stably emitting reference fluorophore to the ROS sensor (**Figure 1-3 a**). The spacer consisted of negatively charged glutamic acids and poly(ethylene glycol) (PEG) units to connect the two dyes yet keeping them spatially apart. The linker also increased the probe's solubility and reduced its cellular uptake. The synthesis and characterization of the sensor are described in Chapter 4, together with a discussion of its validation in sensing assays. To further improve our system, a peptide-based DNA mimic (peptide nucleic acid, PNA) was introduced in the linker, which allowed handling the two fluorophores separately and increased the modularity and versatility of the sensor design (**Figure 1-3 b**). The design, synthesis and validation of the flexible platform are outlined in Chapter 5. Overall, a prototype of a modular ratiometric probe was designed and synthesized. Our findings, summarized in Chapter 6, suggest that extracellular $O_2^{\cdot-}$ detection is possible with hydrocyanines using our ratiometric probes, but that their sensitivity is greatly decreased when coupled to a macromolecule. Generally, a strong influence of the medium was observed, which may complicate interpretation of cell and *in vivo* studies.

Figure 1-3: General design of the ratiometric sensors. a) Schematic representation of a ROS-sensor that is covalently linked to the reporter dye discussed in Chapter 4; b) Schematic representation of the modular approach using PNA to combine the building blocks outlined in Chapter 5.

Chapter 2

Ratiometric Fluorescent Probes to Detect Reactive Oxygen Species

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2.1 Introduction

Common means to study ROS include the analysis of specific biomolecule or lipid oxidations by mass spectrometry, the use of spin trapping techniques, as well as colorimetry-, chemiluminescence- and fluorescence-based imaging approaches.^[41–45] Fluorimetry is especially suitable for applications in cell culture and *in vivo*, as it allows real-time monitoring of the changes in analyte concentration in a non-invasive and relatively inexpensive way.^[30] The most widely used fluorophores (2',7'-dichlorofluorescein diacetate (DCFHDA), dihydrorhodamine 123 (DHR), hydroethidine (HE or DHE), hydroxyphenyl fluorescein (HPF)) react directly with ROS, switching from a non-fluorescent to a fluorescent state. Despite being routinely used to detect, and sometimes even quantify, specific ROS, these dyes react in fact with multiple ROS. Additionally, their turn-on mechanism is radical-mediated, which can lead to numerous side reactions and self-amplification of the fluorescence signal. Therefore, these probes allow the qualitative evaluation of the general redox-state of a sample rather than the quantitative monitoring of a specific analyte.^[46,47] For a more detailed discussion of the limitations of traditional probes and their applications in biological systems, the reader is referred to a review by C.C. Winterbourn.^[48]

With the aim of ameliorating ROS detection, new generations of fluorescent probes have been proposed in recent years.^[33,49,50] Various fluorophore scaffolds (*e.g.* rhodamine, coumarin, BODIPY, cyanine dyes) have been modified to obtain new sensors with different fluorescence excitation/emission wavelengths and physico-chemical properties.^[34,51] The turn-on response has also been progressively adjusted by fine-tuning the electronic properties of the analyte-reactive site, yielding more sensitive and specific sensors.^[36] Concurrently, nanoparticles and polymers have been introduced as ROS sensors, relying on their direct interaction with reactive species or their combination with small organic fluorophores.^[52–55] In general, the cytotoxicity of these emerging sensors - and especially of those based on polymers and nanoparticles - has been investigated and reported in the literature. In addition, probes equipped with organelle-targeting units, such as the mitochondrial targeting moiety triphenyl phosphine (TPP), have been implemented to provide spatially confined information on ROS production.^[56–58]

Newly developed sensors are primarily characterized by measuring their sensitivity and specificity in cell-free media. However, due to the intrinsic challenges in detecting ROS in biological systems, the promising properties observed in cell-free assays do not necessarily translate successfully in more complex *in vitro* or *in vivo* set-ups. Hence, sensor validation should be carried out in the

tissue culture setups and/or animal models for which they have been devised in order to more accurately estimate their potential in biological applications.^[48] In cell-based assays, cells are usually challenged with stimulants to induce a stress response with concomitant ROS production, *i.e.* the stimulation of murine macrophages RAW 264.7 by phorbol-12-myristate-13-acetate (PMA). In animal models, a stress response can also be induced by stimulants (*e.g.* lipopolysaccharides (LPS)) or by a genetically modified phenotype that results in altered ROS levels. Although *in vivo* monitoring would allow for the most complete evaluation of a sensor's potential, technical limitations are likely to arise from the short tissue penetration depth and the high tissue auto-fluorescence encountered at wavelengths below 650 nm. Thus, molecules emitting in the near infrared (NIR) region are generally preferred to enable more reliable analyte detection during *in vivo* sensing.^[59] Another relevant potential limitation when imaging biological systems is the fluctuation of fluorescence emission intensity stemming from concentration differences of the sensor, which can lead to flawed results.^[30] In ratiometric analyte detection this issue is overcome by establishing a ratio between two wavelengths (excitation or emission) thereby creating a signal read-out that is sensor concentration independent. A deceptive simple way to introduce a second fluorescence emission consists of adding a ROS-insensitive dye to a ROS-sensor, thereby obtaining a physical mixture of two fluorophores. Unequal tissue distributions of the two dyes can, however, lead to inaccurate data acquisition and, therefore, it should not be used for ratiometric sensing.^[60] Another possible strategy is the development of a ratiometric sensor from an intensity-based precursor. By coupling the sensor to a polymer or nanoparticle that either functions as anchor or also exhibits a reference emission, a ratiometric probe can be created. Alternatively, a shift in fluorescence emission wavelength can be detected. A frequently applied strategy is based on the Förster resonance energy transfer (FRET) effect, in which energy is transferred from a donor to an acceptor dye leading to a shift of fluorescence emission intensities. These probes consist of a dyad of two fluorophores or a fluorescent polymer/nanoparticle and a fluorophore, respectively. The reaction of an intensity-based sensor with the analyte of interest either enables or disables FRET leading to a shift of emission intensities. Alternatively, single small organic molecules may be used as ratiometric indicators in the cases where a shift in emission wavelength is detected upon reaction with the analyte through an internal charge transfer (ICT) mechanism.^[61,62]

Sensing of ROS has been achieved with ratiometric probes through very diverse approaches, each of them offering advantages as well as limitations depending on the intended application. In this chapter, we present a comprehensive summary of the advances made in the field, giving an overview of current ratiometric fluorescent probes for ROS available in literature and critically discussing selected approaches, with a special focus on their application in cell cultures and *in vivo* systems.

A class of fluorescent probes that will not be discussed in this review are the genetically encoded fluorescent sensors. These protein-based probes exhibit a change in ratiometric emission signal due to small conformational changes of the proteins as a response to their redox environment. As the conformational changes are non-permanent, reversible monitoring is possible. In addition, these probes are photostable, biocompatible and targeting of subcellular compartments was achieved. For a more detailed description on this class of probes, the reader is referred to other reviews on basic concepts and recent advances in the field.^[63–66]

2.2 Ratiometric fluorescent probes to detect ROS

2.2.1 Probes for H₂O₂

H₂O₂ is one of the most studied ROS and is known to be involved in mechanisms regulating health, ageing and disease. Compared to other reactive species, this two-electron oxidant reacts slowly with biological molecules (*e.g.*, with glutathione (GSH) $k = 0.9 \text{ M}^{-1}\text{s}^{-1}$) and has an approximate diffusion distance of 1.5 mm in the presence of cellular GSH concentrations (2 mM).^[15] Despite its intrinsic limitations (*vide supra*), DCFHDA has often been used to study H₂O₂. However, recurrent reports on its alleged unspecificity, over-oxidation and spontaneous auto-oxidation^[48] led to the introduction of novel sensors based on alternative detection strategies, such as the chemoselective oxidation of boronates. In this highly successful approach, a boronate ester is implemented in the structure of a dye to quench or alter its fluorescence emission after which H₂O₂ -but also other ROS- react with the boronate group and trigger a fluorescence turn-on response (**Figure 2-1**). Chang and coworkers synthesized and systematically studied boronate-based sensors, and applied many of them to biological systems.^[67] Their seminal work led to the widespread application of this new class of indicators, as confirmed by the use of boronate oxidations in all the ratiometric H₂O₂-sensing probes published to date with only one very recent exception (**Table 2-1**). An early development by Chang's research group was based on a 1,8-naphthalimide derivative (**PL1**) that shifted from 475 nm to 540 nm upon arylboronate oxidation, showing a 12-fold increase in ratiometric signal (I_{540}/I_{475}).^[68] Wen *et al.* used the same dye scaffold, but modified the H₂O₂ recognition site to a pinacol boronate ester (**NP1**), which resulted in a 20-times higher fluorescence ratio increase at the same H₂O₂ concentration after 1 h. Notably, even though a more pronounced change in signal ratio was observed for **NP1** in cell-free assays, its response in PMA-stimulated RAW 264.7 cells was similar to the one of **PL1** (1.3 vs. 1.5-fold increase), demonstrating that the direct translation of a sensor's potential cannot be assumed from *in vitro* to living systems.^[69] Furthermore, the amino substituent of **NP1** contained an additional azide to

Figure 2-1: Mechanism of H₂O₂ with boronate ester. Adapted from ^[67].

enable the coupling *via* click chemistry of peptides targeting specific subcellular compartments. Confocal laser scanning microscopy revealed that the fluorophore lacking targeting moieties (**NP1**) predominantly resided in the cytoplasm, whereas **Pep-NP1** and **Pep3-NP1** accumulated in the nucleus and the mitochondria respectively, due to their targeting peptides Pep and Pep3.^[69,70] **NP1** was further tested *ex vivo* on hippocampal cuts, on which it yielded a 1.5-fold increase after H₂O₂ addition. Even though emission intensity fluctuations can be minimized with ratiometric probes, high background noise due to tissue auto-fluorescence can complicate signal read-outs, especially for the blue and green lasers used with most of these probes. High-energy lasers can also damage the sample or induce a stress response, which is why NIR or two-photon dyes that can be excited with red lasers are more suitable for *in vivo* as well as *ex vivo* studies.^[51] Two ratiometric approaches have been designed using existing two-photon indicators equipped with an arylboronate ester as sensing unit. In comparison to the one-photon sensors **PL1** and **NP1**, the turn-on response of **PN1** and **SHP-Mito** in PMA-stimulated RAW 264.7 cells proved to be superior, and the pulsed 750 nm laser used allowed imaging in biological samples with a greatly reduced tissue auto-fluorescence. In principle, the lower background signal achieved with two-photon indicators should simplify the detection of H₂O₂ in hippocampal cuts. For **PN1**, however, this did not translate into a higher increase in ratiometric signal (1.2 to 1.6-fold) compared to the one-photon probes. A proof of concept was achieved with the mitochondrial targeting probe **SHP-Mito** that showed a 2.2-fold increase in ratiometric signal.^[71,72] In another targeted approach, Wu and coworkers proposed an elegant method to convey a fluorescein-based sensing unit to the mitochondria by means of a TPP moiety covalently linked to an amino-coated carbon dot. Although a modest 4.4-fold increase in the emission ratio (I_{550}/I_{457}) was achieved in cell-free assays, a striking 50-fold increase in fluorescence ratio was observed in PMA-stimulated RAW 264.7 cells.^[73] However, the reaction time of the nanoprobe with H₂O₂ was slow (>35 min), making it unsuitable for *in vivo* imaging. The same group proposed a similar system in which a faster responding sensing unit (3 min) was anchored to a carbon dot, resulting in a 34-fold change in the ratiometric signal in 20 mM HEPES buffer. Ratiometric imaging with the nanoprobe was demonstrated in biological systems through addition of H₂O₂ to L929 cells, as well as in five-day old zebrafish larvae, where it successfully detected doxorubicin-induced oxidative damage in the gut region (**Figure 2-2**).^[74]

Figure 2-2: Microscopy images of five-day old zebrafish larvae. (A) Brightfield and (B) fluorescence images of a zebrafish incubated with 0.1 mg mL^{-1} nanoprobe for 60 min. (C) Brightfield and (D) fluorescence images of a zebrafish incubated with DOX for 24 h and then with 0.1 mg mL^{-1} nanoprobe for 60 min. (E), (F) Brightfield and fluorescence images of a zebrafish incubated with DOX for 48 h and then incubated with the 0.1 mg mL^{-1} nanoprobe for 60 min. The white arrows indicate the location of the strongest ratiometric response due to endogenously generated H_2O_2 . (G), (H) Fluorescence microscopy images showing the ratio of fluorescence emission ($I_{\text{green}}/I_{\text{blue}}$) in pseudo-color. Scale bar: 200 μm . Adapted from Wu *et al.* with permission.^[74]

These promising examples illustrate the continuous progress made in the design of ratiometric probes to detect H_2O_2 , which has so far enabled remarkable achievements such as the targeting of specific intracellular compartments, and the *in vivo* application of a few sensors. While boronate-based probes may be widely applicable tools for H_2O_2 sensing, results should nevertheless be interpreted with caution. Kinetic studies have revealed that boronate oxidation is several orders of magnitude faster with HOCl ($k = 6.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) and ONOO^- ($k = 10^6 \text{ M}^{-1}\text{s}^{-1}$) than with H_2O_2 ($k = 2.2 \text{ M}^{-1}\text{s}^{-1}$).^[75] ONOO^- has not been tested as alternative oxidant in early approaches, and whereas the interference of HOCl has been investigated more frequently, this was generally done in HEPES buffer which is known to scavenge HOCl . Furthermore, sensitivity and selectivity evaluations in cell-free assays do not accurately reflect a probe's *in vitro* and/or *in vivo* detection power, especially when the studies have been carried out in solvent/buffer mixtures due to solubility issues of organic molecules. In general, the sensitivity and selectivity of a newly developed sensor should be tested in media that mimic biological systems more closely (*e.g.* addition of serum). Further biological

studies - carried out in various research laboratories and with different disease models - would also be needed to validate the general potential of these new probes.

Table 2-1: Ratiometric fluorescent probes for the specific detection of H₂O₂.

Structure	Name	Emission ratio	Maximum increase in emission ratio measured		Biological system / stimulant ^c	Ref.
			cell-free	biological		
H ₂ O ₂ – small molecules
RPF1	517/464	8-fold	n.a.	n.a.	[76]
PL1	540/475	12-fold	1.5-fold	RAW 264.7 / PMA and H ₂ O ₂	[68]
PN1	500/450 ^{TP}	17-fold	2.3-fold	RAW 264.7 / PMA, H ₂ O ₂	[71]
				1.2-1.6-fold	Hippocampal cuts / H ₂ O ₂
AC-bor	484/566	9.5-fold	qualitative	PC3 / H ₂ O ₂	[77]

SHP-Mito	545/470 ^{TP}	75-fold	3-fold	RAW 264.7 / PMA, H ₂ O ₂	[71]
				2.2-fold	Hippocampal cuts / H ₂ O ₂
DQHP	480/542	8.3-fold	n.a.	n.a.	[78]
Styryl-bor 1A	520/420	26.7-fold	n.a.	n.a.	[79]
D-BBO	510/405	73-fold ^a	n.a.	n.a.	[80]
HNA-bor	542/410	5-fold ^a	qualitative	RAW 264.7 / H ₂ O ₂	[81]
Quin-1c	527/415	80-fold ^a	5-fold ^a	HeLa, Jurkat / H ₂ O ₂	[82]
NP1	555/403	244-fold	6.5-fold	HeLa / H ₂ O ₂	[69]
				1.3-fold	RAW 264.7 / PMA	
				2-fold	A431 / EGF	
				1.5-fold	Hippocampal cuts / H ₂ O ₂
Pep-NP1	551/403	125-fold	semi-quantitative ^b	HeLa / H ₂ O ₂	

	DAT-B	574/500	8.3-fold ^a	n.a.	n.a.	[83]
	Pep3-Np1	555/646	12-fold	1.5-fold	HeLa / H ₂ O ₂	[70]
	BNBI	690/590	5.6-fold ^a	n.a.	n.a.	[84]
	ER-H₂O₂	558/458	22.6-fold	qualitative	HepG2 / PMA and nelfinavir	[85]
				qualitative	4T1 / Tm and nelfinavir	
	azaBDPBA	724/682	9.4-fold ^a	4.3-fold ^a	HeLa / H ₂ O ₂	[86]
				1.5-fold ^a	HeLa / PMA	
	RF₆₂₀	570/636	1720-fold	16-fold	HeLa / PMA	[87]
				14-fold	HeLa / EGF

SiOH2R

570/681

11-fold

4.26-fold

HeLa / PMA

H₂O₂ – nanoparticles and polymers **FI-BB**

522/420

26-fold^a

n.a.

n.a.

[88]

PF-FB

527/420

3.7-fold^a

n.a.

n.a.

[89]

G5-SNARF2-Ac[§]

520/650 & 590

n.a.

qualitative

RAW 264.7 / PMA

[90]

Mito-CD-PF1	525/457	4.4-fold ^a	50-fold ^a	L929 / H ₂ O ₂	[73]
				71-fold ^a	RAW 264.7 / PMA
CD-NP-BE	550/457	34-fold ^a	qualitative	L929 / H ₂ O ₂	[74]
				qualitative	Zebrafish / DOX
PMT-F17	525/440	10-fold ^a	semi-quantitative ^b	HeLa / H ₂ O ₂	[91]
				semi-quantitative ^b	RAW 264.7 / PMA	

^a: values calculated from graphs with plot digitizer, ^b: only color scale bar available ^c: compound used to induce or mimic stress response ^{TP}: two-photon probe, PMA: phorbol-12-myristate-13-acetate, DOX: doxorubicin, Tm: tunicamycin, EGF: epidermal growth factor, [§]dual responsive probe to H₂O₂ and pH, n.a.: not available.

2.2.2 Probes for HOCl

HOCl, commonly known as bleach, is frequently used as antimicrobial agent in households. In the body, HOCl is produced by myeloperoxidase from H_2O_2 and Cl^- , and its antimicrobial activity plays an important role during innate immune response. Its effects arise from diverse oxidation and chlorination reactions with biomolecules such as lipids, proteins or nucleic acids.^[6] With the aim of exploiting this oxidative power, various fluorescent sensors for HOCl have been proposed (**Table 2-2**). For instance, the spirolactam opening of rhodamine has been applied in HOCl and $\text{NO}^\bullet$ detection after having found wide-spread use in many metal sensing approaches. In Figure 2-3, the two main mechanisms for HOCl sensing are depicted. In one of them, the selective oxidation of a hydrazo group is exploited with subsequent release of the fluorophore by hydrolysis (**Figure 2-3 a**). In the other approach, a thiohydrazide is used instead, resulting in an irreversible oxadiazole formation (**Figure 2-3 b**).^[92] The spirolactam ring-opening was exploited in combination with a FRET mechanism in several ratiometric probes.^[93–104] **Rhod-TMC**, **Cou-Rho** and **RMClO-2**, for example, used coumarin as donor dye and employed a rigid six-membered ring to connect it to the acceptor fluorophore, *i.e.* the rhodamine-based sensor.^[93,95,96] Whereas a thiohydrazide was used in **Rhod-TMC**, the selective oxidation of the hydrazo group was exploited in **Cou-Rho** and **RMClO-2** using the same coumarin-rhodamine dyad with different leaving groups. In terms of ratiometric signal, **Cou-Rho** showed a lower increase in cell-free assays (25-fold) compared to **Rhod-TMC** (143-fold) and **RMClO-2** (150-fold), although these sensitivities did not quite translate in cellular assays. Indeed, when endogenously produced HOCl was monitored in RAW 264.7 macrophages,

Figure 2-3: Spirolactam ring-opening reaction by OCl^- adapted from ^[92].

all three probes showed comparable increases in ratiometric signal. It should, however, be mentioned that the macrophage stimulation was induced by different methods in each case. In the case of **Rhod-TMC**, the stimulation was achieved with LPS and PMA and the shift in emission signal was imaged qualitatively. For **Cou-Rho** only LPS was used, while for **RMClO-2** carbonyl cyanide m-chlorophenylhydrazone was chosen. Even though cell-free assays indicated a much higher sensitivity for **RMClO-2**, the increase in ratiometric signal for **Cou-Rho** and **RMClO-2** was in fact similar. Further studies in HeLa cells with **RMClO-2**, equipped with a TPP targeting moiety, revealed preferential accumulation of the sensor in mitochondria. Additionally, the basal levels of HOCl were studied in several cell lines and were found to be increased in cancer cells (HeLa, A549) compared to normal ones (HEK293, RAW 264.7). Such basic evaluation of cell lines can give valuable insights in how the metabolism of cancer cells may be altered. Yet, comparing different studies may sometimes be difficult, as experimental conditions can differ greatly. Taking into account that the rhodamine ring-opening is used to detect various endogenous analytes, a careful investigation of interferences of other compounds is necessary to estimate the biological significance of the detected signal. Similar cautions should be taken in the case of systems exploiting the oxidation of double bonds, as other strong oxidants (*e.g.* ONOO⁻ or HO[•]) show a comparable reaction behavior. An example for a sensing mechanism based on double-bond oxidation is the coumarin-piperazine (**CPBT**) platform, which showed a 123-fold increase in ⁻OCl sensing experiments and a 2.3-fold increase in stimulated RAW 264.7 cells. However, also a small interference with ONOO⁻ was detected in cell-free assays.^[105] A more successful example of a sensor with a mitochondria targeting unit that was based on double-bond oxidation (**PMN-TPP**) showed good selectivity over other ROS, an approximately 80-fold increase in fluorescence emission ratio (I_{522}/I_{640}) and a clear change in ratiometric signal during imaging of LPS-stimulated RAW 264.7 macrophages, but was not quantified. *In vivo* experiments with nude mice receiving a skin-pop injection of LPS followed by PMA were used to monitor the evolution of the two emission signals of **PMN-TPP** over 60 min. An increase in the ratio ($I_{\text{green}}/I_{\text{red}}$) of fluorescence emission signals was detected, which confirms that ratiometric imaging with **PMN-TPP** in animal skin is possible (**Figure 2-4**).^[106] Nevertheless, the used excitation wavelength ($\lambda_{\text{ex}} = 430 \text{ nm}$) is not optimal for animal imaging and is likely to create elevated background fluorescence, especially if other organs than the skin are studied. To improve *in vivo* imaging, the fluorescence excitation and emission wavelengths of the sensor should be shifted to the NIR region.^[51] Another hurdle that may descend from complex biological samples is the interference of hypobromous acid and

chloramines. These species are generally not tested but exhibit a similar reaction behavior and can therefore interfere with the data obtained.

Figure 2-4: Representative fluorescence, pseudo-color images of a nude mouse after skin-pop injection of LPS ($100 \mu\text{L} \times 1 \mu\text{g mL}^{-1}$) for 12 h and PMA ($50 \mu\text{L} \times 1 \mu\text{g mL}^{-1}$) injection for 30 min. Then a skin-pop injection of PMN-TPP ($50 \mu\text{L} \times 20 \mu\text{M}$) was applied and images were taken using an excitation laser of 430 nm, an emission filter of the DsRed channel (I_{red}) and an emission filter of the GFP channel (I_{green}). Adapted from Xiao *et al.* with permission.^[106]

Table 2-2: Ratiometric fluorescent probes for the specific detection of HOCl.

Structure	Name	Emission ratio	Maximal increase in emission ratio measured		Biological system / stimulant ^c	Ref.
			cell-free	biological		
HOCl – small molecule
Quinoxim	509/439	10-fold	n.a.	n.a.	[107]
AC-AMN	505/585	235-fold	semi-quantitative ^b	MCF-7 / NaOCl	[108]
Rhod-TMC	594/473	143-fold ^a	6-fold	Bel 7702 / NaOCl	[93]
				qualitative	RAW 264.7 / LPS+PMA
DPNO	430/354	6-fold	n.a.	n.a.	[109]
HRS1	578/501	3955-fold	n.a.	n.a.	[94]
Cy7-NR1	566/780	107-fold ^a	4 to 8-fold	HeLa / NaOCl	[110]
				semi-quantitative ^b	RAW 264.7 / NaOCl

BODIPY-DCDHF	520/629	760-fold	semi-quantitative ^b 2-fold	RAW 264.7 / NaOCl opsonized zymosan particles	[111]
Cou-Rho	470/580	25-fold	1.7-fold	RAW 264.7 / LPS	[95]
PIPT	430/505	12-fold	qualitative	A549	[112]
BRCIO	505/585	22-fold	qualitative	MCF-7 / NaOCl	[113]
TAM	485/630	17-fold ^a	qualitative	PBMC / NaOCl	[114]
RMCIO-2	570/476	150-fold	1.8-fold n.a.	RAW 264.7 / CCCP detection of basal ROS in RAW 264.7, HEK293, HeLa, A549	[96]
Rho-Dan	578/494	130-fold	n.a.	n.a.	[98]

PMN-TPP	522/640	80-fold ^a	qualitative qualitative	RAW 264.7 / LPS+PMA Nude mice / LPS+PMA	[106]
Naph-Rh	585/440	10-fold	4-fold	RAW 264.7 / LPS	[99]
Naph-1	435/360 ^a	19-fold ^a	n.a.	n.a.	[115]
AETU-HOCl	473/533	3-fold ^a	qualitative	RAW 264.7 / PMA	[116]
QC1	381/482	344-fold ^a	n.a.	n.a.	[117]
CRSH	580/470	6-fold ^a	1.8-fold	RAW 264.7 / LPS	[97]
STP-HOCl	520/470 ^{TP}	10-fold	40-fold 66-fold qualitative	HeLa / NaOCl RAW264.7 / LPS+PMA Liver cut / NaOCl	[118]

CARSH	580/490	5-fold ^a	1.7-fold	RAW264.7 / LPS	[100]
BRT	580/540	3-fold ^a	1.7-fold	RAW264.7 / LPS	[101]
HES-BODIPY	532/562	34-fold	8-fold	RAW264.7 / PMA	[119]
Lyso-HA	586/450	97-fold	10-fold qualitative	HeLa / NaOCl RAW264.7 / LPS+PMA	[102]
CPBT	480/580	123-fold	2.3-fold	RAW 264.7 / LPS+PMA	[105]
BDPOX	538/589	192-fold	semi-quantitative ^b	A549 / NaOCl	[120]
BMDP	464/546	205-fold ^a	qualitative	HeLa / NaOCl	[121]

	BFCIO	511/713	300-fold	qualitative	MCF-7, RAW264.7 / NaOCl	[103]
				qualitative	RAW264.7 / LPS+PMA	
	NR	582/532	19-fold	1.7-fold ^a	RAW264.7 / NaOCl	[104]
				1.5-fold ^a	RAW264.7 / LPS	
HOCl – nanoparticles						
	NP-2	575/750	1630-fold decrease ^a	qualitative	HeLa / NaOCl	[122]
	ThioRB- FITC- MSN	586/526	9-fold ^a	qualitative	L929 / NaOCl	[123]

^a Values calculated from graphs with plot digitizer, ^b Color indicating value, but no quantification ^c Compound used to induce or mimic stress response, ^{TP}two-photon dye, LPS: lipopolysaccharide, PMA: phorbol-12-myristate-13-acetate, CCCP: carbonyl cyanide *m*-chlorophenylhydrazone, n.a.: not available.

2.2.3 Probes for HO•

HO• is produced by metal-mediated cleavage of H₂O₂ and even though only small amounts are generated, this very reactive compound can have severe effects on biological samples, mainly by oxidizing biomolecules.^[124] Exploiting the susceptibility of DNA towards oxidative cleavage by HO•, a FRET-based ratiometric probe was designed linking two dyes (FAM and TAMRA) through two thymine nucleotides. Despite a promising ~40-fold increase in ratiometric signal of the sensor in cell-free assays, this setup has not found wide-spread use (**Table 2-3**).^[125] The lack of reliable organic turn-on probes to specifically detect HO• confirms that the high reactivity and short lifetime (*in vivo*: $t_{1/2} = \sim 10^{-9}$ s) of this radical is problematic.^[4] The strong oxidizing power of HO• also leads to the oxidative cleavage of cyanine dyes, which was investigated by Oushiki *et al.*^[126] This was the starting point for the design of a coumarin-hemicyanine platform **Cou-Cy-OH** in which a double bond oxidation induces a shift in the emission wavelength but does not lead to degradation. A 210-fold increase of the emission ratio (I_{651}/I_{495}) was observed and qualitative imaging in PMA-stimulated HeLa cells was achieved.^[127] Another approach coupled HPF, a traditional fluorophore for HO• and ONOO•, to gold nanocarriers so that the turn-on signal of HPF could be normalized to the constant emission of the nanoparticles. This **AuNC-HPF** sensor showed selectivity towards HO• with a small interference from ONOO•, representing a typical example of how turn-on fluorophores can be used in a nanoparticulate ratiometric sensor.^[128]

Table 2-3: Ratiometric fluorescent probes for the specific detection of HO[•].

Structure	Probe	Emission ratio	Maximum increase in emission ratio measured		Biological system / stimulant ^b	Ref.
			cell-free	biological		
HO• – small molecule
FAM-TAMRA	518/576	39-fold ^a	n.a.	n.a.	[125]
Cou-Cy-OH	651/495	210-fold	qualitative	HeLa / PMA	[127]
Naph-OH	418/552	100-fold	qualitative	RAW 264.7 / Fenton, PMA	[129]
HO• – nanoparticle and polymers
AFPAA PEBBLE	445/610	40% red. ^a	n.a.	n.a.	[130]
Coner-NR-Cou	450/528	2-fold ^a	qualitative	MCF-7 / H ₂ O ₂	[131]

	MEH-PPV-NP	497/437	5-fold	n.a.	n.a.	[132]
	AuNC-HPF	500-560/ 580-590	10-fold ^a	4-fold	HeLa / LPS	[128]
	CCA-APTES	455/620	2-fold	qualitative	HeLa / Fenton	[133]
	Si Qd Ce6	660/490	3-fold	qualitative	HepG2 - LPS	[134]

^a Values calculated from graphs with plot digitizer, ^b Compound used to induce or mimic stress response, LPS: lipopolysaccharide, PMA: phorbol-12-myristate-13-acetate, n.a.: not available.

2.2.4 Probes for $O_2^{\bullet-}$

$O_2^{\bullet-}$ imaging also suffers from similar problems; namely, specific fluorophores are difficult to design and the lifetime of the molecule is short ($t_{1/2} = 10^{-3}$ s).^[135] Interestingly, it is not the primary ROS itself that is the most dangerous reactive species but the fast dismutation of $O_2^{\bullet-}$ to H_2O_2 by SOD, and it is its diffusion-controlled recombination with $NO^{\bullet}$ to $ONOO^-$ that render it difficult to study. The widely used and commercially available fluorophore HE, for example, is known to not only react with $O_2^{\bullet-}$ leading to $2-OH-E^+$, but to also form a second oxidation product (E^+) with other ROS that has similar spectral properties. Due to the overlapping fluorescence emissions, these two products can only be distinguished by HPLC analysis.^[136,137] Nevertheless, HE has been used in various ratiometric sensors (**Table 2-4**). Tian and collaborators have designed a nanosensor in which HE was coupled to the surface of a carbon dot (**CD-HE**), allowing the turn-on response to be normalized to the constant emission of the nanoparticle ($I_{570-630}/I_{500-560}$). A 3-fold change in ratiometric signal was observed in cell-free assays, and LPS-treated HeLa cells led to a doubling of the ratio.^[138] The same group also introduced a nanoprobe to simultaneously detect pH and $O_2^{\bullet-}$ in the mitochondria. For this purpose, the pH and $O_2^{\bullet-}$ sensors (FITC, $\lambda_{em,max} = 520$ nm and HE, $\lambda_{em,max} = 630$ nm) and a mitochondria targeting moiety were grafted onto the surface of quantum dots ($\lambda_{em,max} = 800$ nm). An improved increase in ratiometric signal (5-fold) compared to **CD-HE** was observed. Addition of $O_2^{\bullet-}$ to RAW 264.7 macrophages triggered a 3.7-fold increase in fluorescence emission ratio and revealed a concomitant increase in pH.^[139] Our group has recently proposed a sensor to detect extracellular $O_2^{\bullet-}$ with improved flexibility. In this scaffold the reference dye Chromis500 was combined with the turn-on sensor^[40] hydrocyanine5. High flexibility was achieved by using a double stranded DNA analogue that allowed to combine the two fluorophores in a modular fashion. An up to 2-fold increase in the ratiometric signal was observed in cell-free assays, as well as with Caco-2 cells stimulated with H_2O_2 .^[140]

Table 2-4: Ratiometric fluorescent probes for the specific detection of $O_2^{\cdot-}$.

Structure	Probe	Emission ratio	Maximum increase in emission ratio measured		Biological system / stimulant ^b	Ref.
			cell-free	biological		
O ₂ ^{•-} – small molecules
HBT-1	470/375	58-fold	n.a.	n.a.	[141]
AIE-1	525/610	18-fold ^a	qualitative	HepG2 / PMA	[142]
O ₂ ^{•-} – nanoparticles and polymers
CD-HE	570-630/ 500-560	3-fold ^a	2-fold	HeLa / LPS	[138]
FMH NPs-HE	540-610/ 500-535	6-fold ^a	qualitative	HeLa / ROSup	[143]
FITC-HE-QD	600-670/ 730-800	5-fold ^a	4fold	RAW 264.7 / X/XO	[139]

C-PGA-HCy5	665/516	2-fold	n.a.	n.a.
C-PNA-HCy5	665/516	2-fold	1.8 to 2-fold	Caco-2 / AAPH, H ₂ O ₂

[140]

^a Values calculated from graphs with plot digitizer, ^b Compound used to induce or mimic stress response, LPS: lipopolysaccharide, AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride, n.a.: not available.

2.2.5 Probes for NO[•]

NO[•] has been recognized as an important messenger in the body for a long time. The physiological and pathological functions of this gaseous free radical have broadened extensively and now include cardiovascular signaling, neurological messenger functions and immune responsive activities. Two main classes of fluorescent sensors have been introduced, metal-based or FRET-based, both of them relying on the specific reaction of NO[•] to modulate fluorescence emission.^[42,144] In metal-based probes, the direct reaction with the metal center or close to the metal site is responsible for the change in fluorescence.^[145] To the best of our knowledge, however, this approach has not found wide-spread translation into ratiometric probe set-ups for NO[•]. The other class of sensors includes fluorophores equipped with a functional reactive group towards NO[•] and are mostly FRET-based (**Table 2-5**). The spirolactam ring opening, which is also used in ion and HOCl sensing, has been adapted to be specific towards NO[•].^[92] Instead of a hydrazine, an *o*-phenylamine was introduced, leading to the formation of benzotriazole upon reaction with NO[•] and followed by hydrolysis (**Figure 2-5**). Two FRET-based approaches have been introduced where ring-opening leads to a decrease of the donor and an increase of the rhodamine emission due to FRET. For instance, in **Cou-Rho-NO** coumarin was used as donor dye, whereas BODIPY was chosen in **BRP-NO**. The sensors showed a strong increase (>250-fold) in fluorescent emission ratio and ratiometric imaging in cells was achieved in both cases qualitatively. Further quantitative cell experiments would be necessary to more accurately estimate the potential of these probes for biological applications.^[146,147] Solubility issues of purely organic probes can often hamper the translation of novel imaging probes into *in vitro* or *in vivo* systems. With nanoparticles-based probes, the use of organic solvents can be avoided as particles suspension in aqueous media can be used. An example

Figure 2-5: Spirolactam ring opening reaction specific for NO adapted from ^[146].

of this is **CD-naph**, a carbon dot-based system to which an organic naphthalimide with an *o*-phenyldiamine group was anchored. In this setup, the reaction with NO lead to the formation of an aryltriazole that enabled FRET from the carbon dot to the naphthalimide moiety.^[148]

2.2.6 Probes for ONOO⁻

ONOO⁻ is the product of the diffusion-controlled reaction between NO[•] and O₂^{•-}, and plays an important role in signal transduction and antimicrobial activities.^[16] Nonetheless, it can also have deleterious effects at elevated concentrations, such as the oxidation of proteins, DNA or lipids.^[149] The fast reaction with arylboronates offers an excellent opportunity for preparing probes to study ONOO⁻ in biological systems.^[75,150] Surprisingly, only two ONOO⁻-detecting ratiometric probes have been published so far using arylboronates,^[151,152] and only a qualitative turn-on response has been reported in cell-based systems. The strong oxidizing power of ONOO⁻ has been harnessed to create many other set-ups that are based on a selective oxidation of the sensor, *i.e.* the oxidation of activated double bonds (**Table 2-6**). Exploiting the aforementioned pattern, **HMBT-Py** and **F₄₈₂** have shown >50-fold increases in cell-free experiments and good selectivity over other ROS and reactive sulfur species (RSS). However, the potential of these sensors differed greatly from each other when tested in more complex biological systems. While for **HMBT-Py** the potential could not be confirmed in HeLa nor in A549 cells,^[153] **F₄₈₂** was successfully translated to cellular systems, enabling the detection of endogenous ONOO⁻ in LPS+PMA-stimulated RAW 264.7 macrophages and THP-1 cells. Furthermore, imaging of inflammation in the mouse peritoneal cavity and in mouse paws was achieved with **F₄₈₂**, resulting in 10-fold and 5-fold increases, respectively.^[154] These two cases exemplify once again the discrepancies that can be found in performance measurements done in simplified sensing setups compared to more complex and biologically relevant environments, such as cell cultures or *in vivo* settings. Nevertheless, to improve *in vivo* imaging, sensors with emission wavelength in the NIR will be needed in order to minimize tissue damage and cell autofluorescence.^[51]

Table 2-5: Ratiometric fluorescent probes for the specific detection of NO[•].

Structure	Probe	Emission ratio	Maximum increase in emission ratio measured		Biological system / stimulant ^b	Ref.
			cell-free	biological		
NO [•] – small molecules
Fe complex	410/470	2-fold	n.a.	n.a.	[155]
Cou-Rho-NO	583/476	420-fold	qualitative	RAW 264.7 / LPS+IFN γ	[146]
BRP-NO	590/510	250-fold ^a	qualitative	MCF-7 / NO-donors	[147]
TPANO	502/416	18-fold	qualitative	Vero 76 / LPS	[156]
NO [•] – nanoparticles
carbon dot	CD-naph	535/455	6-10-fold ^a	qualitative qualitative	HeLa, L929 / NO-donor RAW 264.7 / NO-donor, LPS+ IFN γ	[148]

^a Values calculated from graphs with plot digitizer, ^b Compound used to induce or mimic stress response, LPS: lipopolysaccharide, IFN γ : interferon γ , n.a.: not available.

A recent example of a two-photon ratiometric turn-on probe exemplifies the power of fluorescent imaging agents emitting in the near infrared. After screening 19 different fluorophores, the most sensitive and resistant candidates were combined as FRET pairs in **MITO-CC**. Evaluation of the probe in cell-free assays showed a 93-fold enhancement of ratiometric signal (I_{473}/I_{651}), a fast response (within 20 s) with a low detection limit (11.30 nM) and good selectivity over other ROS and RSS. **MITO-CC** successfully detected ONOO^- in HepG2 and RAW 264.7 cells as observed with a two-photon confocal microscope, and *ex vivo* stimulation of rat liver cuts with LPS resulted in a clear increase of ratiometric signal. Finally, leg skin sections of Kumming mice revealed a 3.1-fold increase in signal after LPS-induced inflammation (**Figure 2-6**).^[157] Overall, the recently developed probes for ONOO^- detection appear very promising, and further applications in biological systems are expected to arise and confirm their potential.

Figure 2-6: Imaging of ONOO^- with **MITO-CC** using two-photon confocal microscopic fluorescence images. (A) Subcutaneous injection of LPS ($200 \mu\text{L} \times 1 \mu\text{g/mL}$) into right leg of mice. After 12 h, **MITO-CC** ($20 \mu\text{L} \times 500 \mu\text{M}$) was subcutaneously injected. After 1 h, the leg skin of mice was sectioned after being anaesthetized. (B) Fluorescence images of **MITO-CC** in the normal and inflamed tissues. (C) Average $F_{\text{blue}}/F_{\text{red}}$ intensity ratios in panel B. Blue channel, $\lambda_{\text{em}} = 460\text{--}500 \text{ nm}$; red channel, $\lambda_{\text{em}} = 605\text{--}680 \text{ nm}$. $\lambda_{\text{ex}} = 800 \text{ nm}$. Scale bar: $200 \mu\text{m}$. Redrawn from Cheng *et al.* with permission.^[157]

Table 2-6: Ratiometric fluorescent probes for the specific detection of ONOO⁻.

Structure	Probe	Emission ratio	Maximum increase in emission ratio measured		Biological system / stimulant ^b	Ref.
			cell-free	biological		
ONOO ⁻ – small molecules
BOD-Se	680/572	225-fold	n.a.	n.a.	[158]
P2	480/580	25-fold ^a	n.a.	n.a.	[159]
CHCN	515/635	350-fold ^a	<div>qualitative</div> <div>qualitative</div>	WI38 VA13 / ONOO ⁻ RAW 264.7 / LPS+IFN γ +PMA	[160]
HMBT-Py	545/645	30-fold ^a	no increase	HeLa, A549 / SIN-1	[153]

PNCy3Cy5	560/660	324-fold	22 to 29-fold	RAW 264.7 / SIN-1, LPS+IFN γ	[161]
F482	510/606	50-fold	10-fold ^a	RAW 264.7 / LPS+PMA	[154]
				10-fold ^a	THP-1 / LPS+PMA	
				14-fold ^a	Mice / peritoneal cavity	
				5-fold ^a	Mice / inflamed paw
MITO-CC	473/651	93-fold	5.8-fold	HepG2 / LPS+IFN γ	[157]
				4.4-fold	RAW 264.7 / LPS+IFN γ	
				6.1-fold	rat liver cuts / LPS	
				3.1-fold	Kumming mice / LPS
4-MB	450/385	18-fold	n.a.	n.a.	[152]

ONOO⁻ – polymeric micelle polymeric
micelle

**CPP-
IRhB-
BzSe-Cy-
micelles**

810/575

15-fold
reduction

45% reduction

RAW 264.7 / SIN-1

[162]

^a Values calculated from graphs with plot digitizer, ^b Compound used to induce or mimic stress response, LPS: lipopolysaccharide, IFN γ : interferon γ , PMA: phorbol-12-myristate-13-acetate, SIN-1: 3-morpholino-sydnominine

2.2.7 Probes for HNO

HNO is the one-electron reduced and protonated form of NO[•]. It was not until evidence emerged that HNO may be produced endogenously, that its role in biological systems began to be investigated more thoroughly.^[163] As for the other ROS, HNO was discovered to participate in a wide range of processes, such as cardiovascular regulation, immune system modulation and neuronal activity.^[17] Similar to the probes for NO[•], HNO-sensing strategies have focused on either metal-based approaches or modified fluorophores, with only few ratiometric probes published up to date (**Table 2-7**).^[164–167] Among these, two-photon dye was combined with a FRET system to create the sensor **P-Np-Rhod**. Ratiometric imaging in HeLa cells and in frozen rat liver cuts showed a clear shift in emission wavelength, but was not quantified.^[167]

2.3 Ratiometric fluorescent probes to detect hROS and multiple ROS

Not all ratiometric sensors aim at detecting a single reactive species; some of them may also target a whole group of molecules, such as highly reactive oxygen species (ONOO⁻, HOCl and HO[•], **Table 2-8**). Shuhendler *et al.* introduced a semiconducting-based nanosensor that was equipped with a fluorescent NIR sensor for ONOO⁻ and HOCl, a chemiluminescent H₂O₂ substrate and the hepatocyte targeting moiety galactose. An 11-fold increase in ratiometric signal (I₆₈₀/I₈₂₀) was detected with this chemiluminescent sensor, and selective imaging of H₂O₂ was achieved. This nanosensor was successfully applied to a study of drug-induced hepatotoxicity in mice (using the analgesic and anti-pyretic acetaminophen, and the anti-tuberculosis agent isoniazid), where a 1.8- and 1.5-fold increase in ratiometric signal was detected, respectively.^[168] This study shows that NIR dyes are valuable tools to better understand the roles of ROS in animal models in a non-invasive manner.

Table 2-7: Ratiometric fluorescent probes for the specific detection of HNO.

Structure	Probe	Emission ratio	Increase in emission ratio		Biological system / stimulant ^c	Ref.
			cell-free	biological		
HNO – small molecules
Naph-HNO	546/418	4-fold ^a	semi-quantitative ^b	RAW 264.7 / AS	[164]
CF	517/470	5-fold ^a	qualitative	HeLa / AS	[165]
HBT	460/380	10-fold	n.a.	n.a.	[166]
P-Np-Rhod	541/448 ^T _P	50-fold ^a	qualitative	HeLa / AS	[167]
				qualitative	rat liver cuts / AS
Bod-Cou-HNO	463/592	63-fold	qualitative	SMMC-7721 / AS	[169]

^a Values calculated from graphs with plot digitizer, ^b Color indicating value, but no quantification, ^c compound used to induce or mimic stress response ^{TP} two-photon probe, AS Angeli's salt

Table 2-8: Ratiometric fluorescent probes for the specific detection of hROS and multi ROS.

Structure	Probe	Emission ratio	Increase in emission ratio		Biological system / stimulant ^b	Ref.
			cell-free	biological		
hROS – small molecule
PTZ-Cy2	470/598 ^a	[•] OH: turn-on ONOO ⁻ : turn-on ⁻ OCl: 30-fold ^a	qualitative	HeLa / PMA	[170]
hROS – nanoparticles and polymers
DEFN	435/565	[•] OH: 8-fold ^a ONOO ⁻ : 6-fold ^a ⁻ OCl: 4-fold ^a	qualitative	HeLa / H ₂ O ₂ , HOCl, SIN-1	[171]
				qualitative	HL60 / H ₂ O ₂	
				qualitative	RAW 264.7 / LPS, PMA
CdAuNC	455/565	[•] OH: 2.2-fold ^a ONOO ⁻ : 2-fold ^a ⁻ OCl: 1.5-fold ^a	qualitative	RAW 264.7 / LPS and PMA	[172]
CF-SPN	680/820	[•] OH: 1.4-fold ^a ONOO ⁻ : 11-fold ^a ⁻ OCl: 10-fold ^a	1.8-fold ^a 1.5-fold ^a	Mice, hepatotoxicity / APAP Mice, hepatotoxicity / INH	[168]

multi ROS

dLys-AgNC	450/640	H ₂ O ₂ : 35-fold •OH: 12-fold	qualitative	PC-3 / PMA	[173]
carbon nanotubes	SWCN T-NO	1004/1135	H ₂ O ₂ : 1.3-fold NO: 4-fold	qualitative	in plants	[174]
PN₆₀₀	620	ONOO ⁻ : qualitative •OCl: qualitative	n.a.	n.a.	[175]

^a Values calculated from graphs with plot digitizer, ^b Compound used to induce or mimic stress response, LPS: lipopolysaccharide, PMA: phorbol-12-myristate-13-acetate, SIN-1: 3-morpholino-sydnnonimine, APAP: analgesic and anti-pyretic acetaminophen, INH: isoniazid.

2.4 Ratiometric fluorescent probes to monitor the redox-state

Some probes do not aim at specific analyte detection but rather monitor the general redox state that can transiently change over time (**Table 2-9**). These ratiometric sensors show a shift in fluorescence emission upon reaction with oxidants such as ROS, which can be reversed when more reducing conditions are applied, leading to a reversible ratiometric probe that indicates a specific redox state when the ratio of the two wavelengths is used. The group of E. New has summarized and critically evaluated the progress in the field of small organic molecule redox sensors^[176,177] and proposed ratiometric probes to sense oxidative stress.^[178,179] Flavin, a redox-responsive fluorescent dye, has been used as acceptor in a FRET dyad in combination with different donors. When combined with a coumarin donor (**FCR1**), a 6-fold reduction of the fluorescence emission ratio could be detected upon addition of a reducing agent (e.g. NaBH₃CN). Even though ratiometric imaging in H₂O₂-stimulated HeLa cells was shown to be possible with this combination, the probe's reversibility was not studied.^[178] When combined instead with rhodamine as donor (**FRR2**), the probe yielded a 3-4-fold increase in fluorescence emission ratio after reducing agents were applied, and showed good reversibility with up to 7 reduction and oxidation cycles. LPS-stimulated macrophages showed a 1.7-fold increase, and the mitochondrial oxidative capacities of hematopoietic cells during different stages of embryonic development could be successfully monitored by fluorescence-activated cell sorting.^[179] These experiments show that small molecule probes can be successfully used to monitor transient changes in the redox state of cells.

Table 2-9: Ratiometric fluorescent probes for the specific detection of ROS.

Structure	Probe	Emission ratio	Change in emission ratio		Biological system / stimulant ^a	Ref.
			cell-free	biological		
Redox state
FCR1	520/470 ^{TP}	6-fold	qualitative	HeLa / <i>N</i> -acetyl cysteine, H ₂ O ₂	[178]
FRR2	530/460	7-fold	5-fold increase	RAW 264.7 / LPS	[179]
				qualitative	Hematopoietic cell differentiation	

^a Compound used to induce redox state, ^{TP} Two-photon probe

2.5 Conclusion

In the increasingly active field of ratiometric fluorescent sensors for ROS, new probes are constantly being introduced, usually with the aim of improving one or several features for a more reliable ROS detection (*e.g.* selectivity or sensitivity). Compared to the traditional and commercially available fluorophores, significant progress has been made with respect to selectivity and sensitivity. Nevertheless, ratiometric measurements are still inferior to intensity-based sensors, as the obtained detection limits are usually higher and specificity is often lower compared to turn-on probes. The fact that the introduction of new sensors has been favored over improving existing designs may explain their comparatively poor performance. The most promising systems are emission-ratiometric and are compatible with frequently used lasers lines (*e.g.* 488 nm, 640 nm or 750 nm). Whereas innovation in small organic fluorophores has centered around creating new chemical structures, nano- and polymer-based sensors preferably use existing fluorophores and focus on implementing them in a novel scaffold. Although small organic molecules exhibit a lower toxicity on cells and allow to obtain a well-defined structure, solubility issues in aqueous media, fast bleaching and low retention times in cell compartments can rise concerns. Fluorescent nanoparticles exhibit higher photostability but their possible toxicity is a major issue. Small organic sensors are often grafted to the nanoparticles, which allows a high loading of the sensors but also complicates the characterization of the probes. Additionally, the sensors' translation from cell-free assays to more physiologically relevant conditions has repeatedly proven to be challenging. Frequently, the sensing capacity of these new probes was remarkably reduced as soon as they were tested in more complex biological matrix. To enable the study of biological samples also *in vivo* the differences in the biodistribution profiles of small molecules and nanoparticles should be considered during the design process. Firstly, the route of administration can greatly affect the fate of the system and should be carefully chosen. Whereas both types of scaffolds can be equipped with targeting units, the general accessibility of certain tissues (*e.g.* brain tissue) can be greatly reduced with nanoparticles. Another major challenge for nanoprobe and small molecules is the tuning of their pharmacokinetics. Overall, the real potential of many newly developed sensors remains to be established through further biological studies. Additionally, even though ratiometric probes eliminate the issues associated with fluctuations in fluorescence intensity, calibration curves are required to correlate the ratiometric signal to an analyte concentration. Therefore, the quantification of a specific ROS is understandably more complicated in *in vivo* settings than in

cell culture applications, where feasibility has been demonstrated through several examples. In this regard, NIR sensors would greatly simplify *in vivo* applications, which are currently limited by the sub-optimal excitation and emission wavelengths used. To better understand the roles of ROS in biology, sensors with enhanced specificity, sensitivity and robustness are needed. The optimal design of a ratiometric probe may vary depending on the ROS of interest, that can be a single or multiple species. Even though promising sensing designs have been introduced for certain compounds, such as the use of boronate esters for the detection of H₂O₂, caution is still required as species are known that can interfere. In any case a systematic study of a specific scaffold should be greatly favored over the introduction of new designs to obtain a biologically well-tolerated sensor that allows for robust monitoring of ROS. While the newer generations of ratiometric fluorescent sensors have addressed some of these requirements, they have not yet found wide-spread use in general biological studies. This may be partly due to the limited availability of the newer probes, but also low solubility and aggregation of the sensor as well as difficulties in obtaining sufficient amounts limit their use in biological settings.

Chapter 3

Hydrocyanines

3.1 Introduction

Hydrocyanines are non-fluorescent sensors that can switch-on upon reaction with $O_2^{\bullet-}$ and $OH^{\bullet}$ but not with H_2O_2 .^[40,180] Sodium borohydride ($NaBH_4$) converts any cyanine dye into its corresponding hydrocyanine by reducing a double bond of the polymethine chain. The delocalized π -system can be restored upon reaction with ROS leading to a fluorescent turn-on response (**Figure 3-1**). Cyanine dyes are not only available with various reactive groups (*e.g.* NHS ester and maleimide) that allow to label biomolecules, polymers or nanocarriers,^[181] but also cover a wide range of emission wavelengths, namely from 500 nm to 810 nm. These favorable characteristics and the ease of synthesis of hydrocyanines from cyanine dyes make this class of sensors a promising tool to detect ROS. Hydrocyanines were used in various biological systems such as imaging of LPS- and implant-induced inflammation in mice or detection of retinal oxidative stress.^[40,182–189] Additionally, modification of hydrocyanines for *in vivo* imaging (derivatizing with PEG or coupling them to a nanocarrier) were reported.^[190,191] Our choice to use hydrocyanines for ROS sensing was based on these promising reports.

Figure 3-1: Reduction and re-oxidation cycle of cyanines and hydrocyanines, respectively, and nomenclature of (hydro)cyanine (H)Cy.

First, we tested the sensing behavior of seven different hydrocyanines towards $\text{HO}^\bullet$, and selected hydrocyanine5 (HCy5) as model fluorophore due to its promising turn-on response and its stability toward hROS. The sensing capacity of HCy5 towards $\text{O}_2^{\bullet-}$ and $\text{HO}^\bullet$ in aqueous media and in three cell-based assays was investigated. A stress response in cells and concomitant ROS production was stimulated with two different strategies: a) physical stress was applied to a cell monolayer and the stress response was monitored with HCy5 by fluorescent microscopy, b) the addition of chemical stimulants was used to induce elevated ROS levels in two cell models. An epithelial cell line (Caco-2) was challenged to mimic a stress response from intestinal epithelial tissue and the oxidative burst was triggered in human neutrophils to simulate an immune response in the intestine upon microbial invasion. Having evaluated the capacity of ROS detection with HCy5 in project-relevant *in vitro* models, the sensor was modified to minimize internalization by cells. The fluorophore was coupled to a long PEG molecule or to a highly charged poly(glutamic acid), and the uptake in differentiated Caco-2 monolayers was assessed by fluorescence activated cell sorting (FACS).

3.2 Experimental Section

3.2.1 Materials and Instruments

Acetic acid (AcOH), albumin (BSA, from bovine serum, $\geq 96\%$), acetonitrile (ACN), 2,2-azobis(2-methyl-propionamidine) dihydrochloride (AAPH), chloroform (CHCl_3), dichloromethane (DCM), *N,N*-diisopropylethylamine (DIPEA), dimethylformamide (DMF), diethylether (Et_2O), ethylacetate (EtOAc), iron(II) sulfate (FeSO_4 , heptahydrate), hydrochloric acid (HCl , 37 wt% aq.), potassium iodide (KI), methyl iodide (MeI), methanol (MeOH), sodium sulfate (Na_2SO_4 , anhydrous), sodium borohydride (NaBH_4), sodium chloride (NaCl), piperidine, pyridine (py, over molecular sieves), silica gel for flash column chromatography (SilicaFlash F60, 230-400 mesh), trifluoroacetic acid (TFA), 2',7'-dichlorofluorescein diacetate (DCFHDA), MeO-PEG(20k)- NH_2 , phorbol 12-myristate 13-acetate (PMA) and Sephadex G25[®] were purchased from Sigma Aldrich (Buchs, Switzerland). 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate (HATU) and dimethyl sulfoxide (DMSO, 99.8%, extra dry over molecular sieves) were obtained from Acros Organics (Geel, Belgium). 2,3,3-trimethylindolenine was purchased from TCI (Eschborn, Germany). 6-bromohexanoic acid and malondialdehyde bis(phenylimine) monohydrochloride were obtained from ABCR (Karlsruhe, Germany). Di-(4-carboxybenzyl) hyponitrite (SOTS-1) was bought from Cayman Chemical (Ann Arbor, MI). Acetic anhydride (Ac_2O) and hydrogen peroxide (H_2O_2) were obtained from Merck (Darmstadt, Germany). Fmoc-Glu(*t*Bu)-OH and Fmoc-Lys(Boc)-OH were purchased from Merck Millipore (Billerica, MA). Deuterated dimethyl sulfoxide ($\text{DMSO-}d_6$) was obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). Caco-2 cells were kindly provided by Dr. Vanessa Le-Moal (Université Paris-Sud). Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX[™] (high glucose), penicillin/streptomycin mixture, fetal bovine serum (FBS), phosphate buffered saline (PBS), 0.25% trypsin/EDTA, non-essential amino acids, Hank's balanced salt solution (HBSS), ACK lysing buffer were obtained from ThermoFisher Scientific (Waltham, MA). Cy3, Cy5, sulfo-Cy5, Cy5.5, Cy7 and Cy7.5 were purchased from Lumiprobe (Hannover, Germany). Dihydrorhodamine 123 (DHR123) was from Chemodex (St. Gallen, Switzerland). IRDye680 was obtained from Li-Cor Biosciences (Lincoln, NE). Fresh blood was received from Blutspende Zürich (Schlieren, Switzerland). Ficoll-Paque PLUS was obtained from GE Healthcare Life Sciences (Glattbrugg, Switzerland), MycoAlert[™] detection kit was purchased from Lonza (Visp, Switzerland).

XBridge™ C18 column (5 μ m, 4.6 $\times$ 250 mm) was used from Waters Peptide Separation Technology (Baden-Dättwil, Switzerland). LaChrom Classic HPLC L-7200 and TLC plates Silica gel 60 F254 were provided by Merck (Zug, Switzerland) and UltiMate 3000 LC System (Dionex) by Thermo Fisher Scientific (Reinach, Switzerland). Samples were concentrated *in vacuo* on the Rotavapor R-210 and heating bath B-491 by Büchi (Uster, Switzerland). ^1H -NMR and ^{13}C -NMR spectra were recorded on Bruker AV 400. Chemical shifts are expressed in parts per million (ppm) and coupling constants are reported in hertz (Hz). Splitting patterns are indicated as follows: *br*, broad; *s*, singlet; *d*, doublet; *dd*, doublet of doublet; *t*, triplet; *q*, quartet; *m*, multiplet. High resolution mass spectrometry (HRMS)-ESI was recorded on Bruker solariX by the MS service of ETHZ. Solid phase peptide synthesis (SPPS) was implemented on the shaker KS125 basic by IKA Labortechnik (Staufen, Germany) in a peptide synthesis reactor 2.5 or 5 mL and frit SF-0500 by Torviq (Niles, MI). The plate reader infiniteM200 with Costar96 flat black well plates were provided by Tecan (Männedorf, Switzerland). CHRIST® Lyophilisator from Kuhner (Birsfelden, Switzerland), Varian Cary300 Bio UV-VIS Spectrophotometer (now Agilent, Basel, Switzerland) was used. FACS analysis was carried out on a FACSCanto™ Flow Cytometer (BD Biosciences, San Jose, CA).

3.2.2 Synthesis of Cy5

1,2,3,3-Tetramethyl-3H-indol-1-ium iodide 1

2,3,3-Trimethylindolenine (2.02 mL, 12.5 mmol, 1 eq) was diluted in 10 mL ACN. MeI (0.86 mL, 13.8 mmol, 1.1 eq) was slowly added and the reaction mixture was refluxed at 85 °C for 16 h. The purple solid was filtered and washed with Et₂O and DCM. A pink powder was obtained (3.38 g, 11.2 mmol, 90% yield).

^1H NMR (400 MHz, DMSO): δ = 7.97 – 7.88 (m, 1H), 7.88 – 7.79 (m, 1H), 7.69 – 7.57 (m, 2H), 3.98 (s, 3H), 2.77 (s, 3H), 1.53 (s, 6H); ^{13}C NMR (101 MHz, DMSO): δ = 195.82, 141.81, 141.37, 129.08, 128.58, 123.06, 114.89, 53.69, 34.47, 21.46, 13.90; HRMS-ESI calculated for [C₁₂H₁₆N] m/z = 174.1277, found: m/z = 174.1273. The characterization data are in agreement with those previously reported (Figure A1-2).^[192]

1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium iodide 2

2,3,3-Trimethylindolenine (1.6 mL, 10 mmol, 1 eq) and 6-bromohexanoic acid (1.95 g, 10 mmol, 1 eq) were dissolved in 6 mL ACN. KI (1.66 g, 10 mmol, 1 eq) was added and the reaction mixture was refluxed at 85 °C for 16 h. The liquid was decanted and solid KBr was

removed. The organic layer was concentrated under reduced pressure and precipitated in an EtOAc/DCM mixture. A pale pink powder was obtained (2.37 g, 5.9 mmol, 59% yield).

¹H NMR (400 MHz, DMSO): δ = 12.00 (s, 1H), 8.00 – 7.93 (m, 1H), 7.86 – 7.80 (m, 1H), 7.71 – 7.56 (m, 2H), 4.44 (t, J = 7.7 Hz, 2H), 2.83 (s, 3H), 2.22 (t, J = 7.2 Hz, 2H), 1.84 (m, 2H), 1.63 – 1.49 (m, 8H), 1.48 – 1.37 (m, 2H); **¹³C NMR** (101 MHz, DMSO): δ = 196.50, 174.31, 141.87, 141.05, 129.42, 128.95, 123.51, 115.48, 54.16, 47.41, 33.35, 26.94, 25.42, 24.01, 22.01, 13.96 **HRMS-ESI** calculated for [C₁₇H₂₄N₂O₂] m/z = 274.1802, found: m/z = 274.1800. The characterization data are in agreement with those previously reported (**Figure A3-4**).^[192]

Cyanine 5 (Cy5, 3)

2 (648 mg, 1.6 mmol, 1 eq) and malondialdehyde bis(phenylimine) monohydrochloride (556 mg, 2.5 mmol, 1.6 eq) were dissolved in 5 mL Ac₂O and the mixture was refluxed at 130 °C for 30 min. Then **1** (873 mg, 2.9 mmol, 1.8 eq) and 5 mL dry pyridine were added. The reaction was left stirring at room temperature for 16 h. The solvents were removed under reduced pressure at 60 °C. The blue solid was dissolved in DCM, washed with brine and 1 M HCl_{aq}. The combined organic layers were dried over Na₂SO₄ and removed under reduced pressure. The crude mixture was purified by silica column chromatography eluting with a gradient of 0 – 10% MeOH in DCM. 394 mg Cy5 **3** was obtained (0.76 mmol, 48% yield).

¹H NMR (400 MHz, DMSO): δ = 8.33 (t, J = 13.1 Hz, 2H), 7.62 (d, J = 7.4 Hz, 2H), 7.47 – 7.33 (m, 4H), 7.33 – 7.17 (m, 2H), 6.57 (t, J = 12.3 Hz, 1H), 6.29 (dd, J = 17.9, 13.9 Hz, 2H), 4.09 (t, J = 7.1 Hz, 2H), 3.60 (s, 3H), 2.24 – 2.11 (m, 2H), 1.74 – 1.61 (m, 14H), 1.61 – 1.49 (m, 2H), 1.46 – 1.31 (m, 2H). **¹³C NMR** (101 MHz, DMSO): δ = 173.25, 172.50, 154.00, 142.76, 142.02, 141.08, 141.01, 128.42, 128.35, 125.33, 124.73, 124.62, 122.43, 122.30, 111.04, 103.30, 103.02, 48.85, 43.23, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89, 33.88, 31.08, 27.17, 26.99, 26.70, 25.70, 24.33 **HRMS-ESI** calculated for [C₃₂H₃₉N₂O₂] m/z = 483.3006, found: m/z = 483.3003. The characterization data are in agreement with those previously reported (**Figure A5-6**).^[192]

3.2.3 Reduction of the cyanine dye

To cyanine (**Table 3-1**) in MeOH a bubbling NaBH_4 solution in MeOH (1 mg/mL) was added. The loss of color indicated the reduction of Cy to HCy5.

Table 3-1: Fluorescence excitation and emission wavelength used in the screening of (hydro)cyanines tested.

Dye	λ_{ex} (nm)	λ_{em} (nm)
(H)Cy3	525	570
(H)Cy5	620	665
(H)sulfo-Cy5	620	665
(H)IRDye 680RD	650	718
(H)Cy5.5	630	714
(H)Cy7.5	770	816
(H)Cy7	735	780

3.2.4 Re-oxidation with the Fenton's reagent - $\text{HO}^\bullet$ generation

Screening of commercial hydrocyanines: Ten μM hydrocyanine in MeOH were mixed with freshly prepared FeSO_4 stock solution (1 mM) in H_2O yielding a final concentration of 20 or 50 μM . Afterwards, H_2O_2 (stock solution: 10 mM) reaching a concentration of 200 or 500 μM , respectively, was added immediately. The fluorescence intensity was detected after 10 min.

Re-oxidation in H_2O : Ten μM imaging agent was mixed with freshly prepared FeSO_4 solution in H_2O yielding a final concentration of 20 or 50 μM . Afterwards, H_2O_2 reaching a concentration of 200 or 500 μM , respectively, was added immediately. The fluorescence intensity (Cy5 $\lambda_{\text{ex}} = 620$ nm, $\lambda_{\text{em}} = 665$, gain 200, DHR $\lambda_{\text{ex}} = 500$ nm, $\lambda_{\text{em}} = 535$, gain 100) was monitored every 5 min over 2 h at 37 °C on a plate reader in a black multi-well quartz plate (Hellma Analytics) equipped with a sealed quartz cover. All experiments were carried out in triplicate and independently repeated 3 times.

3.2.5 Re-oxidation with SOTS-1 – O₂^{•-} generation^[193,194]

Ten μM imaging agent were mixed with SOTS-1 (1 mg/mL in DMF) in H₂O (ratio DMF:H₂O 1:2, v/v) to generate up to 120 μM O₂^{•-}. O₂^{•-} generation follows a first order kinetic and is produced in 40 *mol%* according to literature.^[193,194] The indicated concentration corresponds to the maximal possible production of O₂^{•-} but might likely be lower in reality. The fluorescence intensity (Cy5 λ_{ex} = 620 nm, λ_{em} = 665, gain 150, DHR λ_{ex} = 500 nm, λ_{em} = 535, gain 100) was monitored as stated above.

3.2.6 Cell culture

Caco-2 cells were maintained in DMEM with GlutMAXTM (high glucose) supplemented with 15 *vol%* FBS, 1 *vol%* penicillin/streptomycin mixture (penicillin: 10,000 units/mL, streptomycin: 10,000 $\mu\text{g/mL}$) and 1 *vol%* non-essential amino acids at 37 °C in a 5 % CO₂ humidified atmosphere. The cells were used from passage 68 to 90. The cells were routinely tested negative for mycoplasma with the MycoAlertTM Detection Kit.

3.2.7 TEER measurement of differentiated Caco-2 cells

Caco-2 cells were seeded at 200,000 cell/mL in Corning transwellTM insert (0.4 μm , 12 mm diameter, 0.5 mL apical side, 1.5 mL basolateral side) and grown for 21 days. The transepithelial electrical resistance (TEER) was measured every 2-3 days using an EVOM epithelial voltohmmeter (World Precision Instruments) equipped with “chopstick” electrodes. The electrodes were incubated in Caco-2 growth medium for one hour before measurements. Resistance measurements were taken every 2-3 days for 3 weeks.

3.2.8 Histology of differentiated Caco-2 cells

Differentiated Caco-2 cells were grown in Corning transwellTM insert for 14 days as stated above. Then, growth medium was removed and the cells were fixed with Methacarn (60% MeOH, 30% chloroform, 10% acetic acid) for 60 min. After washing the insert with PBS, the membrane was cut out from the transwell with a scalpel and was transferred to 70% EtOH in water. The cell membranes were dehydrated through a series of alcohol washing steps and embedded in paraffin wax. Slices of 10 μm were cut and mounted on glass slides. The

hematoxylin and eosin staining was carried out using a routine staining procedure on a COT 20 (Medite AG) automated instrument.

3.2.9 Scratch-wounding assay with differentiated Caco-2 cells

Cells were grown in a 6-well plate for 15 days, so that the complete bottom of the well was covered with a differentiated Caco-2 monolayer. Wells were washed with PBS buffer and the wounding was carried out as one scratch using a glass pipette connected to the aspirator and the PBS was aspirated at the same time. A 20- μ M solution of HCy5 was added, and fluorescence images were recorded with a Leica DMI6000B microscope equipped with an EL6000 Hg light source every 5 min for 65 min.

3.2.10 Stimulation of non-differentiated Caco-2 cells

Caco-2 cells were seeded at a seeding density of 50,000 cells/well on 96-well microtiter plates. After 24 h, the growth medium was removed, and the cells were washed with 100 μ L PBS, followed by incubation with either a solution without stimulant, a 1-mM solution of AAPH or a 1-mM solution of H₂O₂ all of them containing 10 μ M of HCy5 or DHR. DCFHDA was pre-incubated with the cells in advance for 30 min. The fluorescence intensity was monitored with a plate reader at 37 °C for 2 h. HCy5: λ_{ex} = 620 nm, λ_{em} = 665 nm, gain: 200, DCFHDA: λ_{ex} = 485 nm, λ_{em} = 530 nm, gain: 80; DHR: λ_{ex} = 490 nm, λ_{em} = 534 nm, gain: 80.

3.2.11 Stimulation of human neutrophils

Human neutrophils were isolated from fresh blood samples obtained from Blutspende Zurich. The blood was diluted 1:2 (v/v) with HBSS. Granulocytes and erythrocytes were separated from plasma, lymphocytes, monocytes and platelets with a density gradient. The diluted blood sample was slowly added on top of a Ficoll-Paque PLUS layer and was centrifuged to separate the different components (300 $\times$ g, 4 °C, slow acceleration). Erythrocytes were lysed with ACK buffer (ratio 1:6 v/v) for 30 min on ice. After centrifugation (280 $\times$ g, 4 °C, 5 min), the pellet was re-suspended and washed with PBS. The procedure was repeated until the pellet was white/grey, indicating that the lysis of erythrocyte was complete. The white/grey pellet was re-suspended in FACS buffer (PBS with 2 mM EDTA and 0.5% BSA) and were used within 3 h. Neutrophils were incubated with DCFHDA (10 μ M), DHR (2 μ M) or HCy5 (20 μ M) at 37 °C

for 30 min. Then cells were washed with FACS buffer twice. PMA (200 nM) was added to stimulate neutrophil and fluorescence intensity was monitored with a plate reader at 37 °C for 2 h. HCy5: $\lambda_{\text{ex}} = 620$ nm, $\lambda_{\text{em}} = 665$ nm, gain: 200, DHR and DCFH: $\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 530$ nm, gain: 150.

3.2.12 PEGylation of cyanines

Cy5-NHS was mixed with an equimolar amount of MeO-PEG(20k)-NH₂ in dry DMSO and the reaction mixture was agitated at 800 rpm for 6 h at 30 °C. Ten-fold excess of H₂O was added, and the crude was lyophilized. Then the reaction was reconstituted in H₂O and purified with a Sephadex G25[®] size exclusion column. The absence of free Cy5 was verified by HPLC with an isocratic gradient of MeOH/PBS (80:20 v/v) and a C18 end capped column (**Figure A7**).

3.2.13 Quantification of modified cyanine dye

Cy5 conjugates were quantified with a UV spectrophotometer. Absorbance of Cy5 ($\lambda_{\text{abs,max}} = 649$ nm) was measured in MeOH and the concentration c was calculated using **Equation 3-1**, with $A_{\text{dye,max}}$ being the absorption at $\lambda_{\text{abs,max}}$ and ϵ_{dye} the extinction coefficient of the dye.

$$c_{\text{cyanine-conjugate}} = \frac{A_{\text{dye,max}}}{\epsilon_{\text{dye}} \times \text{path length}} \quad \text{Equation 3-1}$$

3.2.14 Synthesis of Cy5-Glu₉-Lys

SPPS was performed manually in a poly(propylene) syringe (2.5 and 5 mL) equipped with a frit at the bottom. All reactions were carried out on a vertical shaker at 600-800 rpm at room temperature. Rink amide resin was swollen in DCM for 30 min, and washed 5 times with DMF using 3 resin volumes. Fmoc was triple de-protected (1 min, 3 min, 15 min) with piperidine in DMF (20% v/v) using one resin volume and then washed 5 times with DMF using 3 resin volumes. Fmoc-Lys(Boc)-OH (5 eq) was pre-activated with HATU (4.9 eq) in DMF for 10 min. Then DIPEA (10 eq) was added and the mixture was transferred to the syringe and shaken for 60 min. The resin was washed 5 times with DMF using 3 resin volumes. This procedure was repeated 9 times for Fmoc-Glu(^tBu)-OH leading to resin-Lys(Boc)-Glu(^tBu)₉-Fmoc. After Fmoc deprotection, Cy5-OH (2.5 eq, **3**) was activated with HATU (2.4 eq) in DMF for 10 min.

DIPEA (5 eq) was added and the mixture was transferred to the syringe and shaken for 90 min. The resin was washed 5 times with DMF and 5 times with DCM using 3 resin volumes and was dried *in vacuo* for 16 h. The peptide was double-cleaved from the resin using a mixture of TFA:H₂O:TIS (*v/v*, 95:2.5:2.5) for 60 min. Lys-PGA-Cy5 was precipitated in ice-cold Et₂O and centrifuged at 4000 × *g* for 10 min at 4 °C. The blue pellet was dissolved in H₂O and lyophilized twice. The sample was analyzed by analytical HPLC and MS analysis (**Figure A8-9**).

3.2.15 Uptake in Caco-2 cells

Caco-2 cells were seeded in 12-well plate (66,000 cells/well) and grown for 14 days. Medium was exchanged every other day and the day before the experiment. Cells were washed with 1 mL pre-warmed PBS and trypsinized with 0.3 mL of 0.25% trypsin for 15 min at 37 °C. Cells were counted and 250,000 cells/mL and sample were used in the uptake experiment. After centrifugation at 300 × *g* for 5 min at 4 °C, cells were washed once with FACS buffer (PBS supplemented with 0.5% *w/v* BSA and 2 mM EDTA) and were then resuspended in FACS buffer containing 5 μM of Cy5 or Cy5-PEG or Cy5-PGA. The samples were incubated for 15 min at 37 °C in the dark. Cells were centrifuged (300 × *g*, 5 min, 4 °C) and washed once with FACS buffer, twice with an acid wash (0.2 M acetic acid, 0.5 M NaCl)^[195] and once with FACS buffer. Flow cytometry measurements (FACSCanto™ Flow Cytometer) were performed in FACS buffer. A minimum of 10,000 events per sample were recorded. The 633-nm laser was used for the Cy5 excitation and the APC channel to detect the fluorescence emission. All experiments were carried out in triplicates and three independent experiments.

3.3 Results and Discussion

3.3.1 Screening of different commercial cyanine dyes with Fenton's reagent

To test the turn-on response of different hydrocyanines, commercially available cyanine dyes were reduced with NaBH₄ in MeOH. The loss of color and fluorescence intensity indicated a successful reduction. The re-oxidation was carried out in MeOH by adding Fenton's reagent (FeSO₄ and H₂O₂) at two different concentrations, and the fluorescence intensity measured after 10 min was normalized to the sample containing the reduced dye but no Fenton's reagent **Equation 3-2**.

$$\text{Norm. fluorescence intensity} = \frac{\text{Fluorescence intensity of hydrocyanine+Fenton}}{\text{Fluorescence intensity of hydrocyanine}} \quad \text{Equation 3-2}$$

In this screening, the turn-on responses for HCy5, HCy5.5 and HCy7 were similar with a 5- to 7-fold increase for the lower OH[•] concentration and an 8- to 11- fold increase for the higher OH[•] concentration (**Figure 3-2**). HCy3 exhibited a 3- and 7-fold increase in signal intensity, respectively. These four sensors showed a concentration dependent behavior, which is in contrast to the turn-on response of HCy7.5 that was lower (2.5-fold) and similar for both OH[•] concentrations. The fluorescence intensity increase of dyes containing sulfates (sulfoCy5 and IRDye680) was small and no concentration dependent increase was detected. Generally, the observed effect was smaller than expected from literature.^[40,180] Cy5/HCy5 was used for all further studies due to its promising turn-on response and the presumably higher stability compared to Cy7/HCy7.^[126]

3.3.2 Synthesis of hydrocyanine5 (HCy5)

The selected fluorophore Cy5 was synthesized according to previous reports in literature (**Figure 3-3**).^[192] 2,3,3-Trimethylindolenine was alkylated with methyl iodide or 6-bromohexanoic acid in acetonitrile resulting in compounds **1** and **2**. The powders were obtained in good yields (90% and 59%, respectively) and analyzed by ¹H-, ¹³C-NMR. The data corresponded to accounts in literature (**Figure A1-4**).^[192]

2 was refluxed with malondialdehyde bis(phenylimine) in acetic anhydride for 30 min and, cooled down to room temperature. Pyridine and **1** were added to the reaction mixture and the suspension was stirred for 15 h. The dark blue crude mixture was dried under reduced pressure and purified by a silica gel column using a gradient of 0 - 10% MeOH in DCM. Cy5 **3** was

obtained in 30 - 48% yield as blue solid and was characterized by ^1H -, ^{13}C -NMR and spectrophotometric methods (**Figure A5-6**). The reduction of Cy5 was carried out in MeOH analogous to the commercial dyes in section 3.3.1.

Figure 3-2: Oxidation with $\text{OH}^\bullet$ generated by Fenton's reagent of hydrocyanines synthesized from commercially available cyanine dyes. Legend: $[\text{FeSO}_4]/[\text{H}_2\text{O}_2]$ in μM . (Mean + SD, $n = 3 - 10$).

Figure 3-3: Synthetic scheme of the synthesis of Cy5.

3.3.3 Sensing of $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$

Sensing experiments were carried out with the freshly synthesized HCy5. $\text{OH}^\bullet$ detection was repeated in an optimized set-up, where the turn-on response of the dye was monitored over 2 h in water in a quartz plate with a cover. The re-oxidation with the Fenton's reaction reached its maximum after approximately 20 - 30 min and revealed a 30- and 45-fold increase in fluorescence intensity (**Figure 3-4 a**). For the higher concentration (50/500 μM) an over-oxidation was observed, which led to a loss of fluorescence intensity after 30 min (**Figure 3-4 b**).^[126] The turn-on response was stronger compared to the commercial Cy5, and might be due to a higher purity of the freshly synthesized fluorophore. The re-oxidation capacity in this experiment corresponded to the one reported by Kundu *et al.* but could only be reproduced to such an extent after extensive optimization of the published protocol and very stringent experimental settings.

$\text{O}_2^{\bullet-}$ detection was investigated by using a $\text{O}_2^{\bullet-}$ generator (SOTS-1).^[193,194] The fluorescence intensity of Cy5 was measured for 2 h by sampling the reaction mixture and measuring the aliquots with the plate reader. Three different $\text{O}_2^{\bullet-}$ concentrations were monitored (4, 20 and 40 μM) and a 3-, 22- and 84-fold increase in fluorescence intensity was observed after 1 h (**Figure 3-5 a**). At the highest concentration, the fluorescence intensity increased so dramatically, that after 40 min the detector was saturated and the sample could not be measured with the same instrument settings (**Figure 3-5 b**). For the other two concentrations, a non-linear response was observed.

The same cell-free sensing experiments were carried out with a rhodamine-based ROS sensor DHR, a commercially available fluorophore that has been widely employed for measuring the respiratory burst of neutrophils.^[196] Even though it was mainly used to detect $\text{O}_2^{\bullet-}$, it is not specific for one single ROS but reacts *via* a radical based mechanism with many reactive species.^[46] In the $\text{OH}^\bullet$ sensing experiments using the Fenton's reagent, a maximal turn-on response of 1.4-fold at the highest concentration was observed (**Figure 3-6 a**). Similar results in a cell-free set-up were obtained by Henderson *et al.* who showed that the fluorescence intensity only slowly increased upon H_2O_2 addition, but was extremely accelerated when catalase or horseradish peroxidase was added.^[197] $\text{O}_2^{\bullet-}$ sensing with SOTS-1 proved to be more sensitive for DHR reaching a 8-fold increase. However, a three times higher concentration than tested for HCy5 was required (**Figure 3-6 b**).

Overall, oxidation of HCy5 with $O_2^{\bullet-}$ and $OH^{\bullet}$ was possible, and the detected increase in fluorescence intensity was greater than for the well-established DHR. The turn-on response of the synthesized HCy5 was similar to the initial publication of *Kundu et al.* Considering these promising result of the cell-free assays, the potential of HCy5 to detect a stress response in cell-based experiments was investigated.

Figure 3-4: Sensitivity of synthesized HCy5 (10 μ M) towards $OH^{\bullet}$ generated by the Fenton's reaction ($[FeSO_4]/[H_2O_2]$) and H_2O_2 . a) Normalized fluorescence intensity after 20 min. b) Time course of the re-oxidation for 2 h. Legend: $[FeSO_4]/[H_2O_2]$ in μ M. (Mean $\pm$ SD, n = 9).

Figure 3-5: Sensitivity of synthesized HCy5 towards $\text{O}_2^{\bullet-}$ generated with SOTS-1, a) Normalized fluorescence intensity after 20 min, b) Time course of the re-oxidation for 2 h. Legend: [$\text{O}_2^{\bullet-}$] in μM (Mean $\pm$ SD, $n = 6$).

Figure 3-6: Sensitivity of DHR in cell-free assays, a) $\text{OH}^{\bullet}$ sensing using four different concentrations of Fenton's reagent, b) $\text{O}_2^{\bullet-}$ radical sensing with SOTS-1 at four different concentrations (Mean $\pm$ SD, $n = 3$).

3.3.4 Scratch-wounding experiment with differentiated Caco-2 cells

During intestinal inflammation, recruitment of neutrophils is one of the first line defense mechanisms of the body as explained in Chapter 1. ROS generation by phagocytic NADPH oxidases is one of the major sources of these transient molecules, but it was shown that this enzyme is also expressed in epithelial cells.^[20] A well-established model to mimic epithelial cells of the intestine are differentiated Caco-2 cells, which form a polarized monolayer when cultured for two weeks. For this project, Caco-2 cells were the model cell line chosen to not only study stress responses towards various stimuli, but also to assess sensor uptake.

A typical characteristic of Caco-2 differentiation is the formation of tight junctions between single cells, which decreases the permeability of the monolayer. The differentiation process was followed by measuring the increase in resistance of the cell monolayer on a tissue culture insert. To validate our cell model, the unit area resistance was measured during 21 days. After a constant increase of resistance for the first 14 days, a plateau was reached and the cells were considered as ready for the experiments (**Figure 3-7 a**). The same tissue culture inserts were fixed and embedded in paraffin to examine the morphology of the monolayer by histological cuts (10 μm). Pictures were taken after hematoxylin and eosin staining and polarized cells are clearly visible on the support (**Figure 3-7 b**).

Figure 3-7: Characterization of differentiated Caco-2 monolayer, a) TEER measurement over 3 weeks (Mean $\pm$ SD, $n=3$), b) Hematoxylin and eosin stained histological cuts (10 μm) of differentiated Caco-2 cells grown on a transwell insert.

A stress response was first induced by wounding the differentiated Caco-2 monolayer. This physical stress was applied by scratching the cell monolayer with a glass pipette. Subsequently a 20- μ M HCy5 solution was added, and the fluorescence intensity around the wound was monitored for 65 min with a microscope (**Figure 3-8 a-c**), and three randomly chosen areas on the scratch border were quantified (**Figure 3-8 d**). A constant increase of fluorescence intensity was observed during the first 40 min, which reached a plateau after 1 h at a 2- to 3-fold fluorescence intensity increase. This experiment suggested that the cells at the border of the injury were affected by the physical wounding and showed a stress response that could be detected with HCy5. Encouraged by these results, an experimental set-up in which all cells were affected and that allowed to use a plate reader was established. Unfortunately, differentiated Caco-2 cells are very insensitive to many stimuli and it was not possible to stimulate a differentiated monolayer. Thus, non-differentiated Caco-2 cells were used for the second stimulation assay.

Figure 3-8: Stress response of a differentiated Caco-2 monolayer to a physical injury. The cell monolayer was wounded with a glass pipette and HCy5 (20 μ M) was monitored for 65 min to follow the stress response, a) t = 0 min, b) t = 30 min, c) t = 65 min, d) quantification of colored areas depicted in a).

3.3.5 ROS detection in non-differentiated Caco-2 cells

Addition of stimulants that trigger a stress response is another way to generate ROS in a tissue culture model. After a screening of possible stimulants, H₂O₂ and AAPH were used to induce elevated levels of ROS. The performance of HCy5 was compared to the two oxidation-sensitive dyes DCFHDA and DHR, that are regularly used in biological systems to detect ROS. Twenty-four hours after seeding, H₂O₂ or AAPH were added, and the dye response was monitored for 2 h with the plate reader at 37 °C. Both stimulants induced a turn-on response of all three fluorophores. The data was normalized to the fluorescence intensity of non-stimulated cells incubated with the same fluorophore (**Equation 3-3**):

$$\text{Norm. fluorescence intensity} = \frac{\text{Fluorescence intensity with stimulated cells}}{\text{Fluorescence intensity with non stimulated cells}} \quad \text{Equation 3-3}$$

Addition of AAPH led to a steady rise in fluorescence for all three dyes reaching a 8-, 14- and 12-fold intensity increase for HCy5, DCFHDA and DHR, respectively (**Figure 3-9 a**). With H₂O₂ the maximal increase was observed after 20-30 min and a 6-, 3- and 16-fold enhancement of fluorescence intensity was detected for HCy5, DCFHDA and DHR, respectively (**Figure 3-9 b**). Overall, HCy5 performed similar to the commercially available ROS sensors, but bore the advantage of emitting light at a wavelength with less background fluorescence ($\lambda_{\text{em,Cy5}} = 665$ nm, $\lambda_{\text{em,DCFHDA,DHR}} = 520\text{-}530$ nm). In contrast to cell-free assays, DHR exhibited a stronger increase than HCy5 for cells stimulated with any of the two stimulants.

Figure 3-9: Time courses of the normalized fluorescence intensities with non-differentiated Caco-2 cells with HCy5, DCFHDA and DHR, a) with 1 mM AAPH addition, b) with 1 mM H₂O₂ stimulation (Mean $\pm$ SD, n = 9-21).

3.3.6 Human neutrophils

To simulate an immune response during intestinal inflammation and to show that also ROS generated from neutrophils can be detected, human neutrophils were isolated from fresh blood samples and stimulated with PMA. Granulocytes and erythrocytes can be separated from other blood components by a density gradient and subsequently erythrocytes are lysed to obtain the granulocyte that are to a large extent neutrophils. After pre-incubation of the fluorophores with neutrophils at 37 °C, 200 nM PMA was added to induce the respiratory burst of these immune cells. The fluorescence intensity increase of HCy5, DCFHDA and DHR was monitored for 2 h with a plate reader at 37 °C. The fluorescence data was normalized as explained for the stimulation assay of non-differentiated Caco-2 cells. The fluorescence intensity increased steadily for the first 40-60 min and remained constant afterwards (**Figure 3-10**). DHR exhibited the highest increase (8-fold) and DCFHDA and HCy5 behaved similar for more than an hour reaching a 4-fold increase. In contrast to DCFHDA and DHR, a small decrease of normalized fluorescence intensity was observed for HCy5 at late time points. Nevertheless, all three dyes can be used to monitor the oxidative burst of human neutrophil in a plate reader set-up and similar sensitivity can be expected.

Figure 3-10: Stimulation of human neutrophils with 200 nM PMA. Fluorescence intensity of HCy5, DHR and DCFH was monitored for 2 h with a plate reader (Mean $\pm$ SD, n = 6).

3.3.7 Modification of cyanine dyes and uptake in differentiated Caco-2 cells

Having shown with three cell-based and two cell-free assays that ROS detection with HCy5 was readily achieved, the next step was to modify the sensor to minimize uptake of the probe by cells. PEGylation of small hydrophobic molecules has been a strategy to increase solubility and prolong their biological half-life.^[198] Applying the same strategy to HCy5, an amine terminated PEG 20k was reacted with a Cy5-NHS ester. After purification with a size exclusion column, Cy5-PEG was obtained (**Figure A7**). The effect of the reducing agent (NaBH₄) on the re-oxidation capacity was investigated, and no influence of the amount of NaBH₄ used to reduce Cy5-PEG to HCy5-PEG was observed (**Figure 3-12 a**). In all cases, the turn-on response was dramatically reduced compared to the HCy5 and a 2.5-fold increase in fluorescence intensity was achieved maximally (**Figure 3-12 a**). A similar reduction of sensitivity was found in O₂^{•-} sensing using SOTS-1 and only a 2.6-fold enhancement of fluorescent intensity was detected (**Figure 3-12 b**). However, ROS detection was still possible and, therefore, we tested the internalization by differentiated Caco-2 cells. After 1 h incubation at 37 °C of a trypsinized monolayer, significantly less PEG-Cy5 was taken up compared to the uncoupled Cy5 (**Figure 3-11**), confirming that attaching a hydrophilic polymer to a small organic fluorophore can increase water solubility and decrease internalization by cells. With the aim of designing a ratiometric probe, the broad weight distribution of PEG may impose problems during purification and characterization. Therefore, the PEG 20k was replaced by a well-defined, negatively charged peptide, namely a poly(L-glutamic acid) sequence (PGA) consisting of 9 negatively charged residues. The peptide was synthesized by manual SPPS using a Rink-amide resin. Cy5 was coupled to the N-terminus of the peptide directly on the resin. After obtaining the soluble peptide (**Figure A8-9**), the uptake of Cy5-PGA was tested to investigate whether a 10 times smaller negatively charged peptide had the same effect on internalization as PEG 20k. The behavior of Cy5-PGA was similar to Cy5-PEG, and the uptake in cells was lower compared to the uncoupled Cy5 (**Figure A10**).

Figure 3-12: Sensitivity of HCy5-PEG in cell-free assays, a) $\text{OH}^\bullet$ radical sensing using four different concentrations of Fenton's reagent and increasing excess of reducing agent. Legend: amount of NaBH_4 used, b) $\text{O}_2^{\bullet-}$ sensing with SOTS-1 generating 20 μM of ROS (Mean $\pm$ SD, $n = 3$).

Figure 3-11: Uptake in differentiated Caco-2 cells (Mean $\pm$ SD, $n = 9$).

3.4 Conclusion

After an initial screening, (H)Cy5 was selected as model sensor for this study. The *in vitro* behavior of this indicator was first investigated in two cell-free assays by generating $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$. A strong increase in fluorescence was observed, and was much more prominent than with the control sensor DHR. Three cell-based assays were established to validate the ROS detection capacity of (H)Cy5. The border of a scratch wound in a differentiated Caco-2 monolayer lighted up when incubated with the ROS sensor. A stress response was observed with non-differentiated stimulated Caco-2 cells and stimulated human neutrophils. In both cases the control dyes DCFHDA and DHR showed a comparable behavior as HCy5. To obtain an extracellular probe, Cy5 was first modified with a large PEG to reduce uptake by cells. This was readily achieved, but the ROS sensitivity was greatly reduced compared to the uncoupled (H)Cy5. A better defined, smaller and negatively charged PGA was then synthesized to obtain a platform to build up a ratiometric probe. Cy5-PGA also stayed mainly extracellular and, therefore, represented a valuable starting point for the design of a ratiometric fluorescent probe.

Chapter 4

Chromis-PGA-(PEG₁₀)_x-(H)Cy5

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4.1 Introduction

As the fluorescence intensity of turn-on probes, such as (H)Cy5, is inherently dependent on their concentration, undesired signal fluctuation due to concentration differences or sample heterogeneity can occur. An elegant design strategy to circumvent this issue are ratiometric probes, which use a ratio of two fluorescence intensities to obtain a concentration-independent measurement and a change in ratio is usually detected during sensing experiments. Combining a turn-on sensor with a stably emitting fluorophore, that does not react with the analyte of interest (*e.g.* ROS), is one possibility to create these two fluorescence intensities.

By appending a second dye to the extracellular PGA-(H)Cy5 probe presented in Chapter 3, two fluorescence emission from the same probe – one being ROS sensitive and the other one being ROS-insensitive – could be obtained. To enable incorporation of this reference fluorophore, an additional glutamic acid and a lysine residue were added to the C-terminus of the peptide moiety. The ROS-insensitive indicator was then coupled to the primary amine of the lysine side chain yielding a ratiometric fluorescent probe (**Figure 4-1 a**).

Considering that the generation of HCy5 required reducing conditions, which may affect the ROS-insensitive dye, the choice of the reporter fluorophore had to be carefully evaluated. Thus, the stability of several fluorophores towards NaBH₄ was tested. Chromis500 proved to be the most resistant candidate and was used as reporter dye in the ratiometric fluorescent probes designed in this study.

The well-defined bio-polymeric probe was synthesized by manual SPPS and the number of PEG₁₀ spacers was successively increased to minimize the interactions between the fluorophores (**Figure 4-1 b**). The uptake of the optimized ratiometric probe in differentiated Caco-2 monolayers was assessed and the behavior of the same indicator was studied in aqueous media. Additionally, the reduction with NaBH₄ was investigated by monitoring the fluorescence emission of both dyes to ensure that only the Cy5 emission is quenched and the reporter dye remains fluorescent. Finally, validation of the sensing capacity towards HO[•], O₂^{•-} and H₂O₂ was assessed in cell-free assays. The main findings are summarized and discussed at the end of the chapter and possible improvements are proposed.

Figure 4-1: Design of the ratiometric probe Chromis-PGA-(PEG₁₀)_x-(H)Cy5, a) Schematic illustration, b) Chemical structure.

4.2 Experimental Section

4.2.1 Materials

Additional to the materials listed in Chapter 3 following compounds were used: 2-chlorotrityl-Gly-OH resin and Atto 465 were purchased from Sigma Aldrich (Buchs, Switzerland). Fmoc-Lys(Boc)-OH were purchased from Merck Millipore (Billerica, MA). Fmoc-NH-PEG₁₀-CH₂CH₂-OH was obtained from ChemPep Inc. (Wellington, FL). Chromeo™ 488 carboxylic acid was purchased from Active-Motif (La Hulpe, Belgium). 3-bodipy-propanoylaminocaproic acid *N*-hydroxysuccinimide ester (BODIPY) was from Molecular Probes (now Thermo Fisher, Reinach, Switzerland). BDP FL ester was purchased from Lumiprobe (Hannover, Germany). Tris(hydroxymethyl)aminomethane (TRIS) was obtained from BioSolve (Dieuze, France).

4.2.2 Screening of reporter dyes

Different amounts (0, 5, 10, 20 and 50 eq) of NaBH₄ in MeOH were added to a dye solution (100 μM) in MeOH. The fluorescence intensity of each dye was measured on a plate reader. BDP: λ_{ex} = 488, λ_{em} = 525 nm, Chromis 500N: λ_{ex} = 470, λ_{em} = 516 nm, Atto 465: λ_{ex} = 430, λ_{em} = 498 nm, BODIPY: λ_{ex} = 490, λ_{em} = 523 nm. The experiment was repeated 3 times in triplicates. The data were normalized to the sample with no NaBH₄.

4.2.3 Synthesis of Chromis-PGA-(PEG₁₀)_x-Cy5

Peptide synthesis

Solid phase peptide synthesis (SPPS) was performed as explained in 3.2.14. Instead of a Rink amide resin, a 2-chlorotrityl-Gly-OH resin was used and one Fmoc-Lys(Boc)-OH and ten Fmoc-Glu(^tBu)-OH were coupled to the resin, leading to resin-Gly-Lys(Boc)-Glu(^tBu)₁₀-Fmoc.

After Fmoc de-protection, Fmoc-NH-PEG₁₀-CH₂CH₂-OH (2.5 eq) was activated with HATU (2.4 eq) in DMF for 10 min. Then DIPEA (5 eq) was added and the mixture was transferred to the syringe and shaken for 90 min. The resin was washed 5 times with DMF using 3 resin volumes. This procedure was repeated x times (x = 0-6).

Cy5-OH coupling and cleavage of the peptide from the resin was carried out as for PGA-Cy5. All compounds were analyzed with HRMS-MALDI and used without further purification.

Chromis-NHS coupling

Lys-PGA-(PEG₁₀)_x-Cy5 ($x = 0-6$) was dissolved in dry DMF (200 μ L), then DIPEA (10 eq) and Chromis500-NHS (3 eq) were added. The reaction was stirred for 5 h at room temperature. 5 mL H₂O were added and the reaction mixture was lyophilized. The product was dissolved in H₂O and extracted with Et₂O until the organic phase was colorless. The crude product was purified by semi-preparative HPLC (XBridge™ C₁₈ 5 μ m 4.6 mm $\times$ 250 mm, 1 mL/min flow rate, isocratic with water/ACN (+ 0.1 % TFA, 80:20 v/v) 5 min followed by a linear gradient to 20:80 water/ACN (v/v) in 20 min). The purity was verified by analytical HPLC and HRMS-MALDI was recorded for all compounds.

4.2.4 Quantification

Chromis-PGA-PEG₀₋₆-Cy5 were quantified as stated in 3.2.13.

4.2.5 Energy transfer measurement

A fluorescence emission scan of Chromis-PGA-(PEG₁₀)₀₋₆-Cy5 at a 20 μ M was measured ($\lambda_{ex} = 470$).

The critical distance R_0 in Ångström was calculated by **Equation 4-1**.^[199]

$$R_0 = 0.211 \left[\frac{\kappa^2 \Phi_D J(\lambda)}{n^4} \right]^{\frac{1}{6}} \quad \text{Equation 4-1}$$

κ^2 is the orientation factor and was assumed to be 2/3. Φ is the quantum yield of the donor ($\Phi_{\text{Chromis500}} = 1$). n is the refractive index of the medium (H₂O = 1.33) and $J(\lambda)$ is the spectral overlap integral of the fluorescence donor spectrum and the acceptor absorption spectrum (**Equation 4-2**)

$$J(\lambda) = \int_0^\infty \varepsilon_A(\lambda) \lambda^4 F_D(\lambda) d\lambda \quad \text{Equation 4-2}$$

The overlap was calculated using a|e-UV-Vis-IR Spectral Software 2.2 (FluorTools, www.fluortools.com).

The relative FRET efficiency (E_{rel}) was calculated from the emission scans recorded (**Equation 4-3**).

$$E_{rel} = \frac{I_A}{I_D + I_A} \quad \text{Equation 4-3}$$

The approximate length of the spacer (R) was calculated with **Equation 4-4**.

$$R = R_0 \sqrt[6]{\frac{1-E_{rel}}{E_{rel}}} \quad \text{Equation 4-4}$$

4.2.6 Dilution of probe

Cy5-(PEG₁₀)₆-PGA-Chromis was diluted to seven concentrations (1, 3, 5, 10, 20, 30, 50 μ M) in 100 mM TRIS buffer (pH = 8.5). Fluorescence intensity was measured at $\lambda_{ex/em}$ = 620/665 nm (Cy5, gain 150) and $\lambda_{ex/em}$ = 470/516 nm (Chromis500, gain 80). The ratio of the measured fluorescence intensities was calculated by dividing fluorescence intensity at 665 nm through intensity at 516 nm ($I_{Cy5}/I_{Chromis}$).

4.2.7 Reduction of Chromis-PGA-(PEG₁₀)₆-Cy5

To Chromis-PGA-(PEG₁₀)₆-Cy5 **4** in Tris buffer (pH 8.5) a bubbling NaBH₄ solution (1 mg/mL) in MeOH was slowly added to the blue dye solution. The loss of blue color indicated that the reduction occurs. The fluorescence intensity was measured and the reduction was considered complete if the value obtained was < 1000 (λ_{ex} = 620, λ_{em} = 665, gain 150).

4.2.8 Re-oxidation with the Fenton's reagent

HO[•] sensing experiments were carried out as described in 3.2.4.

4.2.9 Re-oxidation with SOTS-1^[193,194]

O₂^{•-} sensing experiments were carried out as described in 3.2.5.

4.2.10 Uptake in differentiated Caco-2 monolayers

Cells were seeded into 96-well plates (Nunc) at a density of 5.8 x 10⁵ cells/mL and grown for 14-16 days to form a confluent, differentiated monolayer. Medium was exchanged every other day. The growth medium was removed and the monolayer was washed with pre-warmed PBS (100 μ L/well). 100 μ L dye solution (10 μ M in PBS) was added for 2 h at 37 °C. The dye solution was removed and the cells were washed with PBS, twice treated with an acid wash (0.2 M acetic acid and 0.5 M NaCl, pH 2.5),^[195] and washed with PBS. To prepare the cells for flow cytometry, they were detached using 100 μ L trypsin (0.25 % Trypsin-EDTA) for 15 min.

The cells were collected in medium and centrifuged ($300 \times g$, 4°C , 5 min). The supernatant was discarded and each cell pellet was washed once. Flow cytometry measurements (FACSCanto™ Flow Cytometer) were performed in PBS supplemented with 0.5 % w/v BSA and 2 mM EDTA. A minimum of 10,000 events per sample were recorded. The 488-nm laser was used for the Chromis excitation and the FITC channel to detect the fluorescence emission. The 633-nm laser was used for the Cy5 excitation and the APC channel to detect the fluorescence emission. All experiments were carried out in triplicates and two independent experiments.

4.2.11 Statistical Analysis and Coefficient of variation

All data are presented as Mean $\pm$ SD and the coefficient of variation (CV) is SD divided by the Mean. The one-way ANOVA (parametric or non-parametric depending on the distribution of the sample groups) test combined with a Dunnett's test was applied for comparison of different groups to a control group. The significant difference was assigned at a p-value of <0.05 . (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$).

4.3 Results and Discussion

4.3.1 Screening of ROS-insensitive dye

The use of HCy5 as ROS-sensor offered the major advantage of introducing the ROS-sensing ability by a reduction step shortly before the sensing experiments, which simplified the synthesis of the probe. However, this also implied that the reference dye should not be affected by the reducing conditions. In a preliminary screening, several possible reporter fluorophores were exposed to NaBH₄ and their stability was assessed with a plate reader by measuring the residual fluorescence intensity. The data were normalized to the initial fluorescence intensity value obtained of the same dye when no NaBH₄ was added. In contrast to all other tested dyes (BODIPY Fl, BDP Fl and Atto465, **Figure 4-2 a**) that completely lost their fluorescence properties, Chromis500 retained more than 90% of its initial fluorescence intensity upon addition of 50 equivalents NaBH₄ (**Figure 4-2 b**). Chromis500 (Chromis) is neutral derivative of the BODIPY family, which are known to be highly fluorescent and exhibit sharp absorption and emission peaks. They were shown to be insensitive to solvent polarity and stable towards variations in pH.^[200] These favorable characteristics and the fact that its derivatives, that span the visible spectrum, are readily available, led to the routine use of these fluorophores in bio-imaging. Based on our own findings and its frequent use in literature, Chromis was selected as reporter dye for this study.

4.3.2 Synthesis, optimization of spacer length and characterization

The introduction of Chromis into PGA-Cy5, presented in section 3.3.7, required a few modifications of the chemical structure and synthesis conditions. The spacer was assembled with manual standard SPPS (**Figure 4-2 a**) containing one lysine and ten glutamic acid residues (poly(glutamic acid), PGA). Cy5-OH was coupled to the N-terminus of the last glutamic acid directly on the resin as stated in Chapter 3. However, a 2-chlorotrityl-Gly-OH resin instead of a Rink amide resin was used to obtain higher yields when the peptide was cleaved from the resin. The primary amine of the introduced lysine amino acid was later used to condensate activated Chromis to the spacer-Cy5 derivative (**Figure 4-2 b**) obtaining Chromis-PGA-(PEG₁₀)₀-Cy5.

a)

b)

Figure 4-2: a) chemical structure of BDP, Bodipy FL, Atto 465 and Chromis500-NHS, b) effect of NaBH₄ on different reporter dye candidates. (n = 3, Mean ± SD).

Figure 4-3: Synthesis of Cy5-(PEG₁₀)_x-PGA-Chromis a) Standard Fmoc solid phase peptide synthesis, b) Coupling of Chromis500 to the peptide.

During spectrophotometric characterization, two fluorescence emission peaks were observed upon excitation of Chromis at 470 nm. The lower wavelength emission at 510 nm was consistent with the usually obtained spectrum of Chromis and the second emission peak at 670 nm corresponded to Cy5 fluorescence (**Figure 4-4**, green line). The appearance of a Cy5 emission peak at 670 nm, indeed without exciting the cyanine dye, was attributed to a fluorescence resonance energy transfer (FRET) from Chromis to Cy5.^[201] A FRET effect was undesired for this system as it only occurs when the ROS sensor is switched-on, considerably complicating the final signal read-out. Taking into account that FRET efficiency is inversely proportional to the sixth power of the distance from donor to acceptor,^[201] the inter-fluorophore distance was increased subsequently. A library of probes with one to six discrete PEG₁₀ units

Figure 4-4: Effect of spacer length on FRET efficiency in **4**, $\lambda_{\text{ex}} = 470$ nm, inset: calculation of the approximate length of the spacer unit. Increasing the number of PEG₁₀ spacers resulted in a reduction of E_{rel} and an increased distance between the two dyes.

was prepared to compare the effect of spacer length. The PEG₁₀ units were inserted after the PGA and synthesis was carried out as stated for Chromis-PGA-(PEG₁₀)₀-Cy5 (**Figure 4-3**). All ratiometric probes were purified by semi-preparative HPLC and the obtained compounds were analyzed by HPLC and MALDI mass analysis (**Figure A11-31**). As anticipated, a pronounced reduction of FRET was observed with increasing number of PEG₁₀ units (**Figure 4-4**). By calculating the relative energy that was transferred and considering the spectral overlap of the fluorophores, an approximate distance between the dyes was calculated (**Figure 4-4 box**). Incorporating six PEG₁₀ units increased the distance from 56 Å to over 70 Å and resulted in a negligible FRET effect. Thus, Chromis-PGA-(PEG₁₀)₆-Cy5 **4** was used in all further experiments. Absorption and fluorescence emission spectra did not change compared to the uncoupled fluorophores, exhibiting a sharp absorption and fluorescence emission peaks around 500 nm and 650 nm (**Figure 4-5**).

Figure 4-5: Absorbance scan of Chromis-PGA-(PEG₁₀)₆-Cy5 **4** (black line) and Chromis-PGA-(PEG₁₀)₆-HCy5 **5** (red line) and fluorescence emission scans of **4** (dashed black lines). Chromis λ_{ex} =470 nm, Cy5 λ_{ex} =620 nm.

4.3.3 Chromis-PGA-(PEG₁₀)₆-Cy5 in Tris-buffered medium

To study the behavior of Chromis-PGA-(PEG₁₀)₆-Cy5 in Tris-buffer, the fluorescence emissions of Cy5 and Chromis at different concentrations of the probe were measured and the fluorescence emission ratio $I_{\text{Cy5}}/I_{\text{Chromis}}$ was calculated. The ratio stayed constant at concentrations $<10\ \mu\text{M}$, but decreased for concentration $>10\ \mu\text{M}$ (**Figure 4-6**). A decrease in fluorescence emission ratio implied that the emission intensity of Cy5 did not increase to the same extent as the one of Chromis, when the concentration of the probe was increased, indicating possible aggregation and concomitant quenching of Cy5. Therefore, the highest concentration of Chromis-PGA-(PEG₁₀)₆-(H)Cy5 used in all further experiments was $10\ \mu\text{M}$.

Figure 4-6: Fluorescence intensity ratio $I_{\text{Cy5}}/I_{\text{Chromis}}$ measured in Tris-buffer (pH = 8.4) at seven different concentrations (1, 3, 5, 10, 20, 30 and $50\ \mu\text{M}$, $n = 9$, Mean $\pm$ SD).

4.3.4 Uptake of Chromis-PGA-(PEG₁₀)₆-Cy5 in differentiated Caco-2 monolayers

Having synthesized a well-defined dual-emissive probe with a minimal FRET effect, uptake studies of Chromis-PGA-(PEG₁₀)₆-Cy5 in differentiated Caco-2 cells were effectuated. After 1h incubation of the free dyes and **4** at $3\ \mu\text{M}$, the cell monolayer was trypsinized and analyzed by flow cytometry (**Figure 4-7**). A considerably reduced uptake of **4** in comparison with the uncoupled dyes Chromis and Cy5 was observed, confirming that the probe remains largely extracellular. In a last synthetic step, the ROS-sensing ability was introduced by reducing Cy5 to HCy5.

Figure 4-7: Uptake of Chromis-PGA-(PEG₁₀)₆-Cy5 **4** Cy5 and Chromis in differentiated Caco-2 cells at 3 μ M after 1 h at 37 °C, a) bar plot of the fluorescence intensity normalized to the intensity of the blank (Mean + SD, n = 9), b) representative flow cytometry diagrams, left: filter for Cy5, right: filter for Chromis500.

4.3.5 Reduction of Chromis-PGA-(PEG₁₀)₆-Cy5 **4**

The effect of the reducing agent on the ratiometric molecule was investigated by adding different equivalents of NaBH₄ to a solution of **4**. The fluorescence intensity of Chromis almost doubled, then plateaued and stabilized at higher intensity also during all further experiments (**Figure 4-8**). On the contrary, the Cy5 emission was rapidly suppressed, which confirmed the successful conversion into the ROS-sensor **5** without losing the spectral properties of the reporter dye. The remaining FRET effect present in Chromis-PGA-(PEG₁₀)₆-Cy5 **4** is disabled upon reduction of Cy5 to HCy5 and may partly account for the increase in Chromis intensity observed during NaBH₄ addition.

Figure 4-8: a) effect of NaBH₄ on Chromis and Cy5 (10 μ M, Mean $\pm$ SD, n = 9), b) emission of Chromis over 2 h in Chromis-PGA-(PEG₁₀)₆-HCy5 **5**.

4.3.6 Sensing of HO $\cdot$ and O₂ $\cdot^-$ radicals and specificity of **5**

The ROS-sensing properties of **5** were validated using the same cell-free assays as established for (H)Cy5 in Chapter 3. First HO $\cdot$ was generated using the Fenton's reagent and the increase in fluorescence intensity ratio $I_{\text{Cy5}}/I_{\text{Chromis}}$ was monitored at 10 μ M. In contrast to the free (H)Cy5, a minor increase in (H)Cy5 signal and a negligible increase in ratiometric signal was observed during the experiment (1.1- to 1.3-fold, **Figure 4-9**). Two different concentrations (50 and 100 μ M) of O₂ $\cdot^-$ were generated to investigate the sensing capacity of **5**. After 1 h a 1.9- and 2.3-fold increase of the fluorescence intensity ratio ($I_{\text{Cy5}}/I_{\text{Chromis}}$) was observed (**Figure 4-10**) and the ratio at a physiologically relevant concentration^[202] (50 μ M) showed a low coefficient of variation (CV, 5%). However, the conjugation of HCy5 with the polymer reduced its sensitivity towards the detection of both ROS significantly compared to free (H)Cy5. Upon addition of H₂O₂ at 200 μ M, no increase in fluorescence intensity ratio was observed, resulting in a specific detection of O₂ $\cdot^-$ over HO $\cdot$ and H₂O₂ (**Figure 4-11**). These findings show that the optimized setup of the covalently linked ratiometric ROS-sensing probe Chromis-PGA-(PEG₁₀)₆-HCy5 allows specific superoxide detection.

Figure 4-9: Sensing hydroxyl radicals of Chromis-PGA-(PEG₁₀)₆-HCy5 **5** (10 μM) with the Fenton's reaction ($[\text{FeSO}_4]/[\text{H}_2\text{O}_2]$). Legend: $[\text{FeSO}_4]/[\text{H}_2\text{O}_2]$ in μM . control: 2 at 10 μM . Mean $\pm$ SD (n = 9).

Figure 4-10: $\text{O}_2^{\bullet-}$ sensing with **5** a cell-free assay at 10 μM a) bar plot of the 60 min time point (Mean + SD), b) time course over 120 min (Mean $\pm$ SD, n = 9).

Figure 4-11: Specificity of Chromis-PGA-(PEG₁₀)₆-HCy5 **5** (10 μM). Hydroxyl radicals ($\cdot\text{OH}$, 200 μM , generated with the Fenton's reaction (20 μM FeSO_4 and 200 μM H_2O_2)) and hydrogen peroxide (H_2O_2 , 200 μM) showed no increase in fluorescence intensity ratio even though the concentration used was the double of superoxide concentration ($\text{O}_2^{\cdot-}$, 100 μM generated with SOTS-1). For $\text{O}_2^{\cdot-}$ more than a doubling of the ratio was observed. (Mean + SD, $n = 9$).

4.4 Conclusion

Altogether, an extracellular ratiometric probe using (H)Cy5 as ROS sensor was successfully designed. The BODIPY-analogue Chromis500 was identified as optimal reporter dye due to its resistance towards NaBH_4 . To minimize the FRET effect between Chromis and Cy5, discrete PEG₁₀ were introduced to increase the distance between the dyes. Six PEG₁₀ units reduced the effect sufficiently and Chromis-PGA-(PEG₁₀)₆-(H)Cy5 showed a negligible FRET effect. The fluorescence intensity ratio of this probe was constant up to 10 μM . Uptake studies in differentiated Caco-2 cells revealed that, the introduction of a second hydrophobic fluorophore did not change the overall internalization behavior of the probe compared to PGA-Cy5 and the extracellular probe was reduced without affecting the reporter dye fluorescence. Finally, the selectivity of the coupled (H)Cy5 shifted towards superoxide detection and it was shown, that the newly developed probe is specific for this primary ROS. The sensitivity of the Chromis-PGA-(PEG₁₀)₆-(H)Cy5 was, however, greatly reduced compared to the free (H)Cy5. With the aim of gaining more control of the dyes (*e.g.* reduction of Cy5), a modular unit that allowed to combine fluorophores as last step of synthesis was introduced in a next step.

Chapter 5

Chromis-PNA:PNA-(PEG₁₀)_x-HCy5

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5.1 Introduction

With the aim of improving our first prototype Chromis-PGA-(PEG₁₀)₆-Cy5 **4**, the design of the probe was upgraded. The reduction of Cy5 to HCy5 without affecting the reporter dye presented a major challenge and restricted the choice of ROS-insensitive fluorophores considerably. To avoid complications during this step, a modular unit that allowed to combine the two dyes at the end of the synthetic procedure was introduced. By using complementary strands of a DNA/RNA analogue, namely peptide nucleic acid (PNA), the assembly of the final probe was achieved by a simple hybridization step (**Figure 5-1**). In PNA the nucleobases are connected to an uncharged polyamide backbone with the same dimensions as DNA or RNA and, therefore, allows helix formation (**Figure 5-2**).^[203] Due to the lack of electrostatic repulsion, PNAs form stronger Watson-Crick base pairing than DNA or RNA. The polyamide backbone stabilizes the oligomers in biological media and to enzyme degradation.^[203–206] They were first introduced in 1991^[207] and have been used in many different areas of research such as FISH probes,^[208] aptamer sensors^[209] and bio-barcode systems.^[210,211] Owing to their pseudo-peptidic backbone, PNA synthesis is compatible with SPPS and amino acids can be appended to a PNA strand. By derivatizing both strands at the 5' end either with a lysine residue or with the PGA-(PEG₁₀)_x-(H)Cy5 sensing unit (**Figure 5-1, inset**) a modular probe was created.

Figure 5-1: Schematic illustration of the modular ratiometric sensors, inset: Chemical structures of the building blocks.

The optimization of the distance between the fluorophores was carried out by implementing discrete PEG₁₀ units as explained in Chapter 4. Hybridization of the probe was followed by re-assessment of the probe's extracellular nature by uptake studies in differentiated Caco-2 monolayers. The ROS sensing capacity towards $\cdot\text{OH}$ and $\text{O}_2^{\cdot-}$ was investigated in the established cell-free assays and detection of ROS-generated in cell culture was monitored. The main findings are discussed and compared to Chromis-PGA-(PEG₁₀)₆-(H)Cy5.

Figure 5-2: Chemical structure of the backbone of DNA and PNA.

5.2 Experimental Section

5.2.1 Materials

Additional to the materials listed in Chapter 3 and 4 following compounds were used: Trizma[®] acetate, *m*-cresol and HO-Ala-2-chlorotrityl resin were purchased from Sigma Aldrich (Buchs, Switzerland). Hexafluoro-2-propanol (HFIP) was bought from Fluorochem Ltd (Derbyshire, UK). Ac-Lys-gcattggcat and resin-cgtaaccgta-O-NH₂ were purchased from Panagene (Daejong, Korea).^[212]

5.2.2 Synthesis of Chromis-PNA

400 nmol Ac-Lys-gcattggcat (1.2 mg, 1 eq) were dissolved in 50 μ L dry DMSO. 350 μ L Chromis500-NHS stock solution in dry DMSO (2 mg/mL, 1.2 μ mol, 3 eq) and 0.3 μ L DIPEA (2 μ mol, 5 eq) were added. The mixture was shaken for 4 h at room temperature. After diluting with 7 mL of ultra-pure H₂O the reaction mixture was lyophilized. The resulting yellowish solid was dissolved in water/ACN (60:40, v/v) and purified by semi-preparative HPLC (XBridgeTM Prep BEH130 C₁₈ 5 μ m (10 $\times$ 150 mm), 4.4 mL/min flow rate, isocratic with water/ACN (+ 0.1 % TFA, 60:40, v/v) 5 min followed by a linear gradient to 40:60 water/ACN (v/v) in 20 min. The purity was verified by analytical HPLC (XBridgeTM C₁₈ 5 μ m 4.6 mm $\times$ 250 mm) and a HRMS-MALDI was recorded.

5.2.3 Synthesis of PNA-(PEG₁₀)_x-Cy5

Protected peptide-cyanine conjugate HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)_x-Cy5

Solid phase peptide synthesis (SPPS) was performed as explained in 3.2.14 and 4.2.3. Instead of a 2-chlorotrityl-Gly-OH resin a 2-chlorotrityl-Ala-OH resin was used and to obtain the fully protected peptide, the peptide was double-cleaved from the resin using 40% HFIP in chloroform (v/v, 3 mL/100 mg resin) for 60 min. The collected liquid was evaporated under a stream of N₂ at 30 °C. After dissolving in ACN:H₂O (4:1, v/v) the peptide was lyophilized. This procedure was repeated once for x = 0-3 and twice for x = 4. The product was used without further purification. HRMS-MALDI was recorded for all protected peptide-Cy5 conjugates.

Coupling of HO-Ala-Glu₁₀-PEG_x-Cy5 to resin-cgtaaccgta-O

cgtaaccgta-O-NH₂ was purchased on a Rink amide resin from Panagene. The resin was swollen in DCM for 30 min and washed 5 times with DMF using 3 resin volumes afterwards.

For x = 0-3: 3 eq HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)_x-Cy5 was pre-activated with 2.9 eq HATU in DMF for 10 min. 6 eq DIPEA were added and the mixture was transferred to the resin and shaken at 700 rpm for 2 h. The resin was washed 5 times with DMF and 5 times with DCM using 3 resin volumes and was dried *in vacuo* for 16 h.

For x = 4: 3 eq HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)_x-Cy5 was pre-activated with 2.9 eq HATU in DMF for 10 min. 6 eq DIPEA was added and the mixture was transferred to the resin and shaken at 1200 rpm for 1 h at 75 °C using microwave radiation. The resin was washed 5 times with DMF and 5 times with DCM using 3 resin volumes. Then it was dried *in vacuo* for 16 h.

The PNA-Ala-Glu(^tBu)₁₀-(PEG₁₀)_x-Cy5 was cleaved from the resin using 3 mL TFA : *m*-cresol (1:4, v/v) for 3 h. The liquid volume was reduced under a stream of N₂ and precipitated in ice-cold Et₂O. The immediately formed blue solid was separated by centrifugation at 3000 × *g* for 10 min at 4 °C. The crude product was purified by semi-preparative HPLC (XBridge™ C₁₈ 5 μm, 4.6 mm × 250 mm, 1 mL/min flow rate, isocratic with water/ACN (+ 0.1% TFA, 80:20 v/v) 5 min followed by a linear gradient to 20:80 water/ACN (v/v) in 20 min. The purity was verified by analytical HPLC.

5.2.4 Quantification of PNA-dye conjugates

PNA-dye conjugate concentration (*c*_{PNA}) were calculated according to **Equation 5-1** using the absorbance at 260 nm (*A*₂₆₀), the sequence specific extinction coefficient (*ε*₂₆₀) and a correction factor for the dyes.

$$c_{PNA} = \frac{A_{260} - A_{dye,max} \times \text{correction factor}}{\epsilon_{260}} \quad \text{Equation 5-1}$$

$$\epsilon_{260}(\text{Ac-Lys-gcattggcat}) = 102.1 \text{ mL}/(\mu\text{mol} \times \text{cm})$$

$$\epsilon_{260}(\text{O-atgccaatgc}) = 101.9 \text{ mL}/(\mu\text{mol} \times \text{cm})$$

$$\text{Cy5: correction factor} = 0.05, A_{dye,max} = 649 \text{ nm}$$

$$\text{Chromis500: correction factor} = 0.01, A_{dye,max} = 496 \text{ nm}$$

5.2.5 Hybridization

Equimolar amounts of the PNA-dye conjugates were mixed in PBS buffer (pH = 7.4) and heated to 90 °C for 1 min. The sample was cooled down slowly to 4 °C. The hybridization was visualized by a native PAGE (18% acryl amide gel, running buffer: Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate, 1 mM EDTA, pH = 8.0). The gel was imaged with an IVIS instrument at the wavelength of the dyes used (Chromis500: 470 nm/530 nm; Cy5: 650 nm/700 nm).

5.2.6 Energy transfer measurement

The different Chromis-PNA:PNA-(PEG₁₀)₀₋₄-Cy5 were hybridized and the native PAGE was run as described above. The band of the hybridized probe was excised from the gel and transferred to a 0.5 mL Eppendorf tube with a hole. This was placed in a 1.5-mL Eppendorf tube and centrifuged for 10 min at $18,210 \times g$. The crushed gel was transferred to a glass vial and the product was extracted in 100 μ L running buffer for 16 h. 80 μ L sample were transferred to a multi-well quartz plate (Hellma Analytics). Energy transfer between the dyes was measured by excitation at the lower wavelength (for Chromis500/Cy5 $\lambda_{ex} = 470$ nm) and recording of a fluorescence scan (for Chromis500/Cy5 from 490 nm – 800 nm) on a plate in a black multi-well quartz plate (Hellma Analytics). The distance between the dyes was calculated as described 4.2.5.

5.2.7 Reduction of the cyanine dye

PNA-(PEG₁₀)₃-Cy5 was dissolved in H₂O and a bubbling 1 mg/mL NaBH₄ solution in MeOH was slowly added to the blue dye solution. The loss of blue color indicated that the reduction occurs. The fluorescence intensity was measured and the reduction was considered complete if the value obtained was < 1000 ($\lambda_{ex} = 620$, $\lambda_{em} = 665$, gain 150).

5.2.8 Re-oxidation with the Fenton's reagent

HO[•] sensing experiments were carried out as described in 3.2.4.

5.2.9 Re-oxidation with SOTS-1^[193,194]

O₂^{•-} sensing experiments were carried out as described in 3.2.5.

5.2.10 Uptake in differentiated Caco-2 monolayers

Uptake studies were carried out as explained in 4.2.10.

5.2.11 Stimulation of Caco-2 cells^[213]

ROS production was induced and monitored as stated in 3.2.10

5.2.12 Statistical Analysis, Coefficient of variation

Results were analysed and the coefficient of variation was calculated as described in 4.2.11.

5.3 Results and Discussion

5.3.1 Synthesis and optimization of PNA-dye conjugates

To gain a better control over the fluorophores, a double stranded PNA was implemented in the linker, thereby creating two building blocks each of them bearing one dye. To simplify the synthesis of the PNA-dye conjugates, the PNA strands were inserted between the lysine residue and the PGA-(PEG₁₀)_x-(H)Cy5, leading to a Lys-PNA and a PNA-peptide-Cy5 conjugate (**Figure 5-1 inset**). The PNA sequence used in this work consisted of ten nucleotides and was shown to have a high melting temperature with its complementary DNA strand ($T_m=333.2-334.6$ K).^[212] The primary amine of the lysine-modified strand (Ac-Lys-gcattggcat, Lys-PNA) underwent reaction with NHS-activated Chromis under basic conditions in solution resulting in PNA-Chromis (**Figure 5-3 a**). After semi-preparative HPLC purification, PNA-Chromis was characterized with analytical HPLC, MALDI-MS (**Figure A32-33**) and spectrophotometric methods (**Figure 5-4**).

Figure 5-3: a) Synthesis of PNA-Chromis b) Synthesis of the protected Ala-Glu(^tBu)₁₀-(PEG₁₀)_x-Cy5 (x = 0-4) and coupling of the protected peptide-Cy5 to PNA-resin.

Figure 5-4: Absorbance (solid line, left axis) and fluorescence emission ($\lambda_{\text{ex}} = 470$ nm, dashed line, right axis) scan of PNA-Chromis.

The complementary PNA strand was directly modified at the N-terminus on the Rink amide resin with PGA-(PEG₁₀)_x-Cy5 moiety. To couple the peptide to the N-terminus of PNA-resin, the protected peptide-Cy5 was synthesized by manual SPPS (**Figure 5-3 b**) using the same synthesis conditions as described in Chapter 3 and 4. To obtain the fully protected peptide cleavage conditions from the 2-chlorotrityl resin were changed to 40% HFIP. Interestingly, the loss of exactly one *tert*-butyl protecting group was observed, when glutamic acid was directly attached to the 2-chlorotrityl resin, but by introducing the helix inducing alanine residue this issue could be solved.^[214] The fully protected peptide-Cy5 was analyzed with MALDI-MS (**Figure A34-38**) and to study the impact of spacer length on FRET efficiency, the number of PEG₁₀ units was varied from zero to four (**Figure 5-3 b**, $x = 0-4$). The five protected peptidic units were reacted with the PNA linked to a resin using common coupling conditions at room temperature for (PEG₁₀)₀₋₃ and microwave assisted conditions at 70 °C for (PEG₁₀)₄. The PNA-peptide-Cy5 conjugates (PNA-(PEG₁₀)_x-Cy5) were purified and analyzed as PNA-Chromis (**Figure A39-48**). Annealing of the strands resulted in the ratiometric sensor in the unreduced form and was verified by a native PAGE (**Figure 5-5**) using an excess PNA-Chromis. Due to a lack of charge, PNA-Chromis did not show any motility on the gel and is visible as blue band. The single strands of PNA-(PEG₁₀)₀₋₄-Cy5 move more than the hybridized probes

Chromis-PNA:PNA-(PEG₁₀)₀₋₄-Cy5. A molecular weight dependent motility of the single strand but also of the hybridized compounds analyzed.

Figure 5-5: Motility of Chromis-PNA:PNA-(PEG₁₀)_x-Cy5 on a native PAGE (loading 10 μ M). Excess PNA-Chromis is visible as a first band in all hybridized probes and in the lane of Chromis-PNA alone. The single PNA-(PEG₁₀)_x-Cy5 strands migrate more than the hybridized probes Chromis-PNA:PNA-(PEG₁₀)_x-Cy5 due to their lower molecular weight. The motility of the single strands PNA-(PEG₁₀)_x-Cy5 and the hybridized probes Chromis-PNA:PNA-(PEG₁₀)_x-Cy5 are molecular weight dependent. Blue: Chromis, grey: Cy5, white: overlay.

To study the effect of spacer length on FRET efficiency in these probes, they were purified with a native PAGE. The band corresponding to the hybridized probe was excised from the gel and extracted overnight. The energy transfer was measured by excitation of Chromis at 470 nm and recording a fluorescence emission scan. The observed differences between the various spacer length were small (**Figure 5-6**). Calculation of the approximate distance revealed that the distance between the dyes of all PNA probes was bigger than the one observed in Chromis-PGA-(PEG₁₀)₆-Cy5 **4** (72-80 Å vs. 71 Å, **Figure 5-6**). Chromis-PNA:PNA-(PEG₁₀)₃-Cy5 **6** exhibited the smallest energy transfer and was, therefore, used in all further experiments.

Characterization of Cy5-(PEG₁₀)₃-PNA was carried out as for all the other strands with HPLC and MALDI-MS (**Figure A45-46**). Spectrofluorometric analysis of the assembled probe Cy5-(PEG₁₀)₃-PNA:PNA-Chromis is presented in **Figure 5-7**.

Chromis-PNA:PNA-(PEG₁₀)_x-Cy5	E_{rel}	R [Å]
Chromis-PNA:PNA-(PEG ₁₀) ₀ -Cy5	0.171	74
Chromis-PNA:PNA-(PEG ₁₀) ₁ -Cy5	0.119	79
Chromis-PNA:PNA-(PEG ₁₀) ₂ -Cy5	0.190	72
Chromis-PNA:PNA-(PEG ₁₀) ₃ -Cy5	0.115	80
Chromis-PNA:PNA-(PEG ₁₀) ₄ -Cy5	0.128	78

Figure 5-6: Effect of spacer length on FRET efficiency, $\lambda_{\text{ex}} = 470$ nm. The effect of the spacer length on the FRET efficiency was small. Only a minor improvement from PNA-(PEG₁₀)₀ to PNA-(PEG₁₀)₃ was observed. Black box: Calculation of the length of the spacer unit using the FRET scans.

Figure 5-7: Absorbance scans of Cy5-(PEG₁₀)₃-PNA:PNA-Chromis **6** (black solid line, left axis) and **7** (red solid line, left axis) and fluorescence emission scans of Cy5-(PEG₁₀)₃-PNA:PNA-Chromis **3** (dashed black lines, right axis) and HCy5-(PEG₁₀)₃-PNA:PNA-Chromis **6** (dashed red line, right axis). Chromis λ_{ex} =470 nm, Cy5 λ_{ex} =620 nm.

5.3.2 Uptake studies of Chromis-PNA:PNA-(PEG₁₀)₃-Cy5

The uptake of Chromis-PNA:PNA-(PEG₁₀)₃-Cy5 in differentiated Caco-2 cells was assessed by incubating the free dyes and **6** for 1 h, followed by flow cytometer analysis. The internalization of **6** was significantly reduced compared with the free dyes Cy5 and Chromis (**Figure 5-8**), confirming that the probe design still prevents uptake by cells, although the overall hydrophilicity of the sensor was altered by introducing the relatively hydrophobic PNA moiety.

Figure 5-8: Representative flow cytometry diagrams of the uptake of Chromis-PNA:PNA-(PEG₁₀)₃-Cy5 **6** in differentiated Caco-2 monolayers after 1 h of incubation at 37 °C. Left: filter for Cy5, right: filter for Chromis. Chromis-PNA:PNA-(PEG₁₀)₃-Cy5 **6** shows a lower intensity for both filters than the free dyes.

5.3.3 Reduction and annealing of Chromis-PNA:PNA-(PEG₁₀)₃-HCy5

To verify the successful annealing of the two strands and to show that it is not affected by the reduction of the cyanine dye, a native PAGE was used to analyze the reduced **7** and the non-reduced sensor **6** (**Figure 5-9**). The yellow band, being an overlay of green (Chromis) and red (Cy5), confirmed annealing of the modified strands. The lack of other bands or a band corresponding to Cy5-(PEG₁₀)₃-PNA (red band) showed that most of the probe was successfully hybridized in a 1:1 ratio and no undesired structures are built (*e.g.* triplex). Chromis-PNA does not have negative charges and, thus, did not show any electrophoretic mobility. The same position of the green (**4**) and yellow band (**3**) indicated that both imaging probes have similar molecular weights and negative charges, which proved that the reduction of Cy5 to HCy5 did not affect the hybridization of the ratiometric PNA probe.

Figure 5-9: Native PAGE of Chromis-PNA:PNA-(PEG₁₀)₃-HCy5 **7**, Chromis-PNA:PNA-(PEG₁₀)₃-Cy5 **6** and Cy5-(PEG₁₀)₃-PNA (single strand), Chromis: green, Cy5: green, overlay: yellow.

5.3.4 Sensing of HO[•] and O₂^{•-} radicals in cell free assays

In analogy to Chromis-PGA-(PEG₁₀)₆-(H)Cy5, detection of HO[•] was attempted. A maximal increase in fluorescence intensity ratio of 1.2-fold was observed, that could not even be detected after 2 h (**Figure 5-10**). The O₂^{•-} generation by SOTS-1 was monitored at 50 and 100 μM and revealed a 1.3-fold and 2.2-fold increase, respectively, after 1 h (*c*_{probe} = 10 μM, **Figure 5-11**). The CV was low for both concentrations (4% and 3%). These results suggest, that the sensing behavior compared with Chromis-PGA-(PEG₁₀)₃-(H)Cy5 were not affected by the introduction of the modular unit and that also for the modular ratiometric prototype, the sensing behavior was shifted towards specific O₂^{•-} detection.

Figure 5-10: Sensing of $HO^\bullet$ with Chromis-PNA:PNA-(PEG₁₀)₃-HCy5 **7** with the Fenton's reaction ($[FeSO_4]/[H_2O_2]$). The bar plot presents the increase at $t = 60$ min. The sensing was monitored for 2 h. Legend: $[FeSO_4]/[H_2O_2]$ in μM . (Mean $\pm$ SD, $n = 6$).

Figure 5-11: Sensing of $O_2^{\bullet-}$ with Chromis-PNA:PNA-(PEG₁₀)₃-HCy5 **7** monitored over 2 h. A steady increase of $O_2^{\bullet-}$ was detected, showing that variation of concentration can be monitored for at least 2 h. The bar plot shows the increase at $t = 60$ min. Control: 4. (Mean $\pm$ SD, $n = 6$).

5.3.5 Stimulation of non-differentiated Caco-2 cells

As O₂^{•-} detection was possible, oxidative stress sensing *in vitro* was investigated using 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and H₂O₂-stimulated Caco-2 cells. AAPH showed a 1.4-fold increase after 1 h with a CV of 6%. A steady increase was observed reaching the same level as H₂O₂-stimulated cells after 2 h. For H₂O₂-stimulated cells a doubling of the ratio was observed immediately with a CV of 10% (**Figure 5-12**). Overall, the sensing capacity of **7** was as good as the covalently linked ratiometric probe **5** in the cell-free assays, which corroborates that the design of modular platform increases the flexibility of the sensor with equal ROS detection ability. Additionally, **7** was successfully used to detect extracellular oxidative stress in cell culture.

Figure 5-12: Sensing with Chromis-PNA:PNA-(PEG₁₀)₃-HCy5 **7** of oxidative stress generated by stimulated Caco-2 cells monitored over 2 h. The bar plot represents the increase at t = 60 min. (Mean ± SD, n = 9).

5.4 Conclusion

In summary, we have modified the ratiometric probe described in Chapter 4 by implementing a PNA double strand to obtain a modular ROS sensor that can be assembled under mild conditions just before the sensing experiments. The number of PEG₁₀ units did not have a significant effect on FRET efficiency, that was very low in general and smaller than observed for Chromis-PGA-(PEG₁₀)₆-(H)Cy5. Chromis-PNA:PNA-(PEG₁₀)₃-(H)Cy5 was selected for further experiments, due to its favorable characteristics and its uptake in differentiated Caco-2 cell monolayers was studied. The internalization of the modular probe was greatly reduced compared to the uncoupled dyes (>20-fold decrease). As for Chromis-PGA-(PEG₁₀)₆-(H)Cy5 sensing selectivity was shifted towards O₂^{•-} and a was not significantly altered by the modular PNA unit introduced. Additionally, ROS detection in cell culture was achieved with non-differentiated Caco-2 cells. Even though the sensitivity of our modular ratiometric ROS-probe is lower when compared to HCy5 in cell-free as well as in cell-based assays, O₂^{•-} detection was exemplified and was, indeed, similar to the commercially available hydrocyanines.^[215] This modular platform can be used to build-up a library with other reference dyes or sensor fluorophores and can be combined flexibly by a simple hybridization step.

Chapter 6

Conclusion and Outlook

ROS are short-lived molecules that are endogenously produced, for example by neutrophils during the respiratory burst in host defense. In this first-line defense mechanism of the innate immune response, ROS exert important antimicrobial activities against intruders and the dysregulation of these events can have deleterious effects on health. Over the last twenty years, the spectrum of action of these transient molecules has been broadened with an ever-increasing number of signaling functions in stem cell differentiation, cancer and ageing, among others. The growing interest in ROS calls for reliable and specific detection methods to study them, which, due to the molecules' transient nature and similar chemical reactivity, imposes a substantial challenge.

The development of fluorescent probes for the detection of ROS has received considerable attention, with new sensors being constantly introduced. Although the design of most probes have aimed at ROS imaging in the intracellular space, selective detection in the extracellular milieu can also give important insights, as it has been shown that the function of ROS can vary depending on their location (*e.g.* in tumorigenesis). Thus, the study presented in this thesis aimed at designing an extracellular fluorescent ROS-sensor that addressed some of the usual obstacles encountered by fluorescent probes. To minimize signal fluctuations and to eliminate artefacts, a ratiometric probe was created by linking a ROS-indicator and a reference dye with a charged bio-polymeric moiety. The linker enabled the modification of the probe's properties (*e.g.* uptake by cells) and the use of an existing indicator for intracellular ROS detection.

As presented in Chapter 3, our choice of hydrocyanines as model ROS-sensor was based on promising reports in literature showing that not only a high fluorescence increase could be observed with such probes, but also that imaging was possible in various *in vivo* systems. Our evaluations with HCy5 revealed that a high increase in fluorescence could indeed be obtained in cell-free assays generating HO[•] and O₂^{•-}. However, very stringent experimental conditions were required and small variations in the protocol resulted in a 10-fold decrease of the turn-on response. A possible explanation for these observations could be that as with cyanines, hydrocyanines could form H-aggregates in water and buffer, especially at higher concentrations, altering their sensing behavior.^[216] Oxidation of HCy5 was also followed in cell-based assays, where a clear fluorescence increase was observed. Physical stress induced on differentiated epithelial cell monolayers by scratch-wounding showed that the cells at the border of the injury produced ROS. Stress response mimicking was also achieved by addition of stimulants to non-differentiated Caco-2 cells with subsequent monitoring of the turn-on response of the sensor. HCy5 successfully sensed the oxidative stress induction and its detection

capacity was found to be similar to that of other fluorophores frequently used in ROS sensing (DHR and DCFHDA). To estimate the potential of the sensor, the respiratory burst of neutrophils was chosen to imitate a relevant situation occurring in host defense, as explained in Chapter 1. After isolating human neutrophils from fresh blood, the respiratory burst was chemically induced with PMA and subsequently detected with HCy5 as well as the control dyes DHR and DCFHDA. ROS production was observed with all three dyes and their turn-on responses were found to be comparable, further supporting our findings in Caco-2 cells.

Encouraged by these results, the cyanine dye was modified with a hydrophilic macromolecule to transform it into an extracellular sensor. PEGylation or modification with PGA reduced the internalization by Caco-2 cells compared to the free dye. Unfortunately, a concomitant decrease in sensing capacity was observed.

A major obstacle in fluorescence spectroscopy *in vitro* and *in vivo* arises from signal fluctuation due to sample heterogeneity and concentration differences. This issue has been frequently addressed by implementing a second fluorescence emission in the probe, and also our extracellular probe design was adapted to take advantage of an additional fluorescence signal.

In Chapter 4, we linked the intracellular ROS-sensor HCy5 to a reporter fluorophore that provided a second fluorescence emission. An advantage of using hydrocyanines as ROS indicator was the possibility to modify the cyanine dye and introduce the ROS-sensing capacity as a last step in the synthesis by reducing cyanine to hydrocyanine. However, this synthetic strategy introduced challenges such as guaranteeing the stability of all building blocks towards the reducing conditions. Indeed, selecting a reporter dye with high stability towards NaBH_4 proved to be crucial. The BODIPY-analogue Chromis500 was the most resistant fluorophore among the tested dyes, and was used as model reporter compound. By linking the two hydrophobic dyes Chromis500 and Cy5 with a well-defined, hydrophilic peptide-PEG spacer, the ratiometric probe was rendered predominantly extracellular. The spacer was improved in Chapter 5 by introducing a modular unit consisting of complementary PNA strands, which enabled the separate handling of the dyes (*e.g.* cyanine reduction) and a simple combination of the fluorophores as a last step. Also in this case, synthesis led to a well-defined bio-polymeric ratiometric ROS sensor. This modular approach offers an optimal starting point to build up a library with different sensors and reporter dyes that could be combined on-demand for specific experiments.

Given their macromolecular size and hydrophilic nature, the ratiometric probes were not internalized by differentiated Caco-2 monolayers and in both cases, functional extracellular ROS-sensors were obtained. The detection specificity of the ratiometric probes shifted towards $O_2^{\cdot-}$ in cell-free assays, thereby creating a specific probe for $O_2^{\cdot-}$. The modular sensor was also tested in tissue culture and a doubling in ratiometric signal was observed. A summary of our main results is provided in **Table 6-1**.

Table 6-1: Summary of the main results of this Ph.D. thesis.

Sensor	<i>In vitro</i> localization	Maximal fluorescence intensity increase
HCy5	Intracellular	Cell-free, $O_2^{\cdot-}$: 84-fold ^a Caco-2: scratch: 2-3-fold AAPH: 8-fold H_2O_2 : 6-fold Neutrophils: 4-fold
HCy5-(PEG₁₀)₆-PGA-Chromis	Extracellular	$O_2^{\cdot-}$, cell-free: 2.3-fold ^b
HCy5-(PEG₁₀)₃-PNA:PNA-Chromis	Extracellular	$O_2^{\cdot-}$, cell-free: 2.2-fold ^b Caco-2: AAPH: 2-fold H_2O_2 : 2-fold

^a: $[O_2^{\cdot-}] = 20 \mu M$, ^b: $[O_2^{\cdot-}] = 100 \mu M$

Despite the interesting results obtained with our modular prototype for sensing extracellular $O_2^{\cdot-}$, derivatization of HCy5 seemed to decrease the switch-on ability of the HCy5 in cell-free assays.^[40,180] Indeed, as also reported by two other groups who modified hydrocyanines for imaging purposes (derivatizing HCy5 with PEG^[190] or coupling them to a nano-carrier^[191]), the detected response towards $O_2^{\cdot-}$ and $HO^{\cdot}$ was lower compared to the original report.^[190,191] Taken together, these findings suggest that coupling the (hydro)cyanines to a macromolecule may influence the fluorescence properties, either due to potential effects on the *cis/trans* isomerization of the polymethine chain and/or the sensing ability, accounting for the reduced

effect.^[181] Nevertheless, it is worth noting that in the cell assays the increase in the fluorescence signal obtained with the ratiometric probe was in the same order of magnitude than that reported with the commercially available HCy5 (ROStar™ 650).^[215] This would indicate that the switch-on response of unconjugated HCy5 is highly influenced by experimental settings and needs carefully controlled conditions to obtain reliable read-outs, an observation that we also made during our validation studies in Chapter 3.

Figure 6-1: Sensing hydroxyl radicals of Chromis-PGA-(PEG₁₀)₆-HCy5 (10 μM) with the Fenton's reaction ($[FeSO_4]/[H_2O_2]$) in presence of 50 mg/mL BSA. Legend: $[FeSO_4]/[H_2O_2]$ in μM . control: 2 at 10 μM . (Mean $\pm$ SD, n = 9).

Even though the detection capacity of our ratiometric probes was reduced compared to HCy5, the detection of ROS in cell-based assays was possible. To more accurately evaluate the full potential of our sensors, *in vivo* studies would be a valuable addition. With the presented prototypes, however, the selected fluorophores pose a major limitation for *in vivo* applications as they do not emit in the NIR and especially Chromis should be replaced by a higher wavelength dye. Additionally, the limited sensitivity of our probes may also be an issue for their *in vivo* use. Nevertheless, a preliminary sensing experiment was carried out in presence of BSA, a solubilizing agent abundantly present in blood. The HO $\cdot$ detection capacity of Chromis-PGA-(PEG₁₀)₆-HCy5 was tested in presence of BSA and to our surprise, an up to 3-fold increase in ratiometric signal was observed (**Figure 6-1**). This indicates that the turn-on behavior of hydrocyanines – coupled or not – is greatly dependent on the solubilizing power of the used medium, and also that the aggregation of the hydrophobic dye may reduce the sensing behavior. Recent findings from Chattaraj *et al.* corroborate these data, as they reported that even quinones

can oxidize a hydrophobically modified hydrocyanine derivative if they are completely solubilized in the oil that is used as imaging medium.^[217]

To summarize, after careful validation of HCy5 as ROS-sensor, an extracellular ratiometric ROS-sensing probe was developed. In addition, a novel platform for analyte sensing was introduced for the first time, using PNA-PGA-(PEG₁₀)_x as combinatorial unit. Due to the PNA spacer, the building blocks may be chosen with high versatility, as the fluorophores are combined with a simple hybridization step. Specific O₂^{•-} chemodosing revealed that detection of oxidative stress in cell-free assays as well as cell culture is possible and is comparable for the modular ratiometric imaging probe **7** and the covalently linked sensor **5**. Although the sensitivity towards O₂^{•-} of the proposed fluorophores could be further improved, the concept of a general modular platform for extracellular ratiometric sensing shows potential and could be expanded to other analytes.^[34]

Cyanine5 (Cy5)

Figure A1: ¹H-NMR spectrum of 1,2,3,3-tetramethyl-3H-indol-1-ium iodide **1**.

Figure A2: ¹³C-NMR spectrum of 1,2,3,3-tetramethyl-3H-indol-1-ium iodide **1**.

Figure A3: ¹H-NMR spectrum of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium iodide **2**.

Figure A4: ^{13}C -NMR spectrum of 1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium iodide 2.

Figure A5: ^1H -NMR spectrum of Cy5 3.

Figure A6: ^{13}C -NMR spectrum of Cy5 3.

Cy5-PEG

Figure A7: HPLC analysis of Cy5 (left) and Cy5-PEG (right). The absence of a peak at 4.8 min corresponding absorbing at 650 nm was detected to verify successfully size-exclusion chromatography with Sephadex™ G25.

Cy5-PGA

Figure A8: HPLC analysis of Cy5-PGA: Absorbance chromatogram (left) and fluorescence chromatogram (right, $\lambda_{\text{ex}}=620$ nm, $\lambda_{\text{em}}=665$ nm).

Figure A9: MS analysis of Cy5-PGA.

Figure A10: Representative histogram for the uptake of Cy5-PGA, Cy5-PEG and Cy5 in differentiated Caco-2 monolayers.

Characterization of molecules in Chapter 4

Lys-PGA-(PEG₁₀)_x-Cy5

Table A1: Expected m/z of the Lys-PGA-(PEG₁₀)₀₋₆-Cy5.

Lys-PGA-(PEG ₁₀) _x -Cy5	MW [g/mol]
Lys-PGA-(PEG ₁₀) ₀ -Cy5	1960.55
Lys-PGA-(PEG ₁₀) ₁ -Cy5	2471.66
Lys-PGA-(PEG ₁₀) ₂ -Cy5	2983.27
Lys-PGA-(PEG ₁₀) ₃ -Cy5	3494.88
Lys-PGA-(PEG ₁₀) ₄ -Cy5	4006.49
Lys-PGA-(PEG ₁₀) ₅ -Cy5	4518.10
Lys-PGA-(PEG ₁₀) ₆ -Cy5	5029.71

Figure A11: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₀-Cy5.

Figure A12: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₁-Cy5.

Figure A13: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₂-Cy5.

Figure A14: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₃-Cy5.

Figure A15: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₄-Cy5.

Figure A16: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₅-Cy5.

Figure A17: HRMS-MALDI of Lys-Glu₁₀-(PEG₁₀)₆-Cy5.

Chromis-PGA-(PEG₁₀)_x-Cy5**Table A2:** Expected m/z of the Chromis-PGA-(PEG₁₀)₀₋₆-Cy5.

Chromis-PGA-(PEG ₁₀) _x -Cy5	MW [g/mol]
Chromis-PGA-(PEG ₁₀) ₀ -Cy5	2413.40
Chromis -PGA-(PEG ₁₀) ₁ -Cy5	2925.01
Chromis -PGA-(PEG ₁₀) ₂ -Cy5	3436.62
Chromis -PGA-(PEG ₁₀) ₃ -Cy5	3948.22
Chromis -PGA-(PEG ₁₀) ₄ -Cy5	4459.83
Chromis -PGA-(PEG ₁₀) ₅ -Cy5	4971.44
Chromis -PGA-(PEG ₁₀) ₆ -Cy5	5483.05

Figure A18: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₀-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A19: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₀-Cy5.

Figure A20: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₁-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A21: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₁-Cy5.

Figure A22: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₂-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A23: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₂-Cy5.

Figure A24: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₃-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A25: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₃-Cy5.

Figure A26: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₄-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A27: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₄-Cy5.

Figure A28: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₅-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A29: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₅-Cy5.

Figure A30: Verification of purity by HPLC analysis of Chromis-PGA-(PEG₁₀)₆-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) c) absorbance at 500 nm d) absorbance at 650 nm.

Figure A31: HRMS-MALDI of Chromis-PGA-(PEG₁₀)₆-Cy5.

Characterization of molecules in Chapter 5

Lys-PNA

Figure A32: Verification of purity by HPLC analysis of PNA-Chromis500. a) Fluorescence detection ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$) b) absorbance at 500 nm c) absorbance at 260 nm.

Figure A33: HRMS MALDI of PNA-Chromis500.

Protected peptide-cyanine conjugate**Table A3:** Expected m/z of Ala-Glu(^tBu)₁₀-(PEG₁₀)₀₋₆-Cy5.

(PEG ₁₀) _x -Cy5	MW [g/mol]
(PEG ₁₀) ₀ -Cy5	2406.98
(PEG ₁₀) ₁ -Cy5	2918.59
(PEG ₁₀) ₂ -Cy5	3430.20
(PEG ₁₀) ₃ -Cy5	3941.81
(PEG ₁₀) ₄ -Cy5	4453.42

Figure A34: HRMS-MALDI of HO-Ala-Glu₁₀(^tBu)-(PEG₁₀)₀-Cy5.

Figure A35: HRMS-MALDI of HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)₁-Cy5.

Figure A36: HRMS-MALDI of HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)₂-Cy5.

Figure A37: HRMS-MALDI of HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)₃-Cy5.

Figure A38: HRMS-MALDI of HO-Ala-Glu(^tBu)₁₀-(PEG₁₀)₄-Cy5.

PNA-Ala-Glu₁₀-(PEG₁₀)_x-Cy5 conjugates

Table A4: Expected m/z of PNA-PEG₀₋₄-Cy5

PNA-(PEG ₁₀) _x -Cy5	MW [g/mol]
PNA-(PEG ₁₀) ₀ -Cy5	4684.69
PNA-(PEG ₁₀) ₁ -Cy5	5196.30
PNA-(PEG ₁₀) ₂ -Cy5	5707.91
PNA-(PEG ₁₀) ₃ -Cy5	6219.52
PNA-(PEG ₁₀) ₄ -Cy5	6731.12

Figure A39: Verification of purity by HPLC analysis of PNA-(PEG₁₀)₀-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) b) absorbance at 640 nm c) absorbance at 260 nm.

Figure A40: HRMS MALDI of PNA-(PEG₁₀)₀-Cy5.

Figure A41: Verification of purity by HPLC analysis of PNA-(PEG₁₀)₁-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) b) absorbance at 640 nm c) absorbance at 260 nm.

Figure A42: HRMS-MALDI of PNA-(PEG₁₀)₁-Cy5.

Figure A43: Verification of purity by HPLC analysis of PNA-(PEG₁₀)₂-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) b) absorbance at 640 nm c) absorbance at 260 nm.

Figure A44: HRMS-MALDI of PNA-(PEG₁₀)₂-Cy5.

Figure A45: Verification of purity HPLC analysis of PNA-(PEG₁₀)₃-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 620 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) b) absorbance at 640 nm c) absorbance at 260 nm.

Figure A46: HRMS-MALDI of PNA-(PEG₁₀)₃-Cy5.

Figure A47: Verification of purity by HPLC analysis of PNA-(PEG₁₀)₄-Cy5. a) Fluorescence detection ($\lambda_{\text{ex}} = 620$ nm, $\lambda_{\text{em}} = 665$ nm) b) absorbance at 640 nm c) absorbance at 260 nm.

Figure A48: HRMS-MALDI of PNA-(PEG₁₀)₄-Cy5.

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List of Abbreviations

AAPH	2,2-azobis(2-methyl-propionamidine) dihydrochloride
Ac ₂ O	acetic anhydride
ACN	acetonitrile
AcOH	acetic acid
APAP	analgesic and anti-pyretic acetaminophen
AS	Angeli's salt, sodium α -oxyhyponitrite
Boc	butyloxycarbonyl
BODIPY	boron-dipyrromethene
BSA	bovine serum albumin
CCCP	carbonyl cyanide <i>m</i> -chlorophenylhydrazone
CGD	chronic granulomatous disease
CHCl ₃	chloroform
CONER	coumarin-neutral red nanoparticle
cpYFP	circular permuted yellow fluorescent protein
DCFHDA	2',7'-dichlorofluorescein diacetate
DCM	dichloromethane
DHE	dihydroethidine
DHR	dihydrorhodamine 123
DIPEA	<i>N,N</i> -diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOX	doxorubicin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Et ₂ O	diethylether

EtOAc	ethylacetate
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
FeSO ₄	iron(II) sulfate
FITC	fluorescein
Fmoc	fluorenylmethoxycarbonyl
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
GI	gastro intestinal
GSH	glutathione
H ₂ O ₂	hydrogen peroxide
H ₂ O	water
HATU	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HE	hydroethidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HFIP	hexafluoro-2-propanol
HNO	nitroxyl
HPF	hydroxyphenyl fluorescein
IBD	inflammatory bowel disease
IFN γ	interferon γ
¹ O ₂	singlet oxygen
HO $\cdot$	hydroxyl radical
HOCl	hypochlorous acid
KI	potassium iodide

LPS	lipopolysaccharides
MeI	methyliodide
MeOH	methanol
Na ₂ SO ₄	sodium sulfate
NaBH ₄	sodium borohydride
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NIR	near infrared
NO [•]	nitric oxide
NO ₂	nitrogen dioxide
NOX	subunit of NADPH oxidase
NP	nanoparticle
O ₂ ^{•-}	superoxide
O ₃	ozone
ONOO ⁻	peroxynitrite
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEBBLE	photonic explorer for bioanalysis with biologically localized embedding
PEG	poly(ethylene glycol)
PGA	poly(glutamic acid)
PMA	phorbol-12-myristate-13-acetate
PNA	peptide nucleic acid
py	pyridine
QD	quantum dot
roGFP	redox-sensitive green fluorescent protein
ROS	reactive oxygen species
SIN-1	3-morpholino-sydnnonimine
SOD	superoxide dismutase

SOTS-1	di-(4-carboxybenzyl) hyponitrite
^t Bu	<i>tert</i> -butyl
TAMRA	5-carboxytetramethylrhodamine
TEER	transepithelial resistance
TFA	trifluoroacetic acid
Tm	tunicamycin
TP	two-photon
TPP	triphenyl phosphine
TRIS	tris(hydroxymethyl)aminomethane

Curriculum Vitae

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Education

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09/2011 – 12/2012	Master of Science, Chemistry , ETH Zurich, Switzerland Master’s thesis title: <i>“Synthesis of IP5-polymer analogues targeting toxins A and B of C. Difficile”</i> Advisors: Prof. Dr. Bastien Castagner and Prof. Dr. Jean-Christophe Leroux
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Professional Experiences

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Scientific Contributions

Publications

Andina D., Leroux J.-C. & Luciani P. Ratiometric Fluorescent Probe to Detect Reactive Oxygen Species. *Chem. - A Eur. J.* (2017) doi:10.1002/chem.201702458.

Gong Y., **Andina D.**, Nahar S., Leroux J.-C., Gauthier M. A. Releasable and Traceless PEGylation of Arginine-rich Antimicrobial Peptides. *Chem. Sci.* **8**, 4082-4086 (2017).

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Oral Presentations

Andina D., Leroux J.-C., Luciani P., A Ratiometric Fluorescent Probe for the Specific Detection of Neutrophil-Induced Reactive Oxygen Species. *Doktorandentag Institute of Pharmaceutical Sciences*, ETH Zurich, Switzerland, February 17, **2016**.

Andina D., Brambilla, D., Zivko C., Leroux J.-C., Luciani P. Design of a Ratiometric Fluorescent Probe Library for Specific Detection of Reactive Oxygen Species in Inflamed Intestine, *WMIC 2015*, Honolulu, Hawaii, September 2-5, 2015.

Andina D., Leroux J.-C., Luciani P., Fluorescent Probes for Specific Detection of Reactive Oxygen Species in Inflamed Intestine, *Zurich-Geneva-Basel Joint Seminar Series on Drug Formulation & Delivery*, Basel, Switzerland, January 23-24, 2015.

Poster presentations

Andina D., Brambilla D., Leroux J.-C., Luciani P.; Fluorescent probes for detection of reactive oxygen species in inflamed intestine; *Materials and Processes Graduate Symposium*, ETH Zurich, Switzerland, May 12, 2014.

Andina D.; Brambilla D., Leroux J.-C., Luciani P.; Contrast agents for imaging intra- and extracellular reactive oxygen species in inflamed intestine; *International Conference and Workshop on Biological Barriers 2014*, Saarbrücken, Germany, February 16-21, 2014.

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