Fluorescent probes for selective detection of ozone in plasma applications

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Fluorescent Probes for Selective Detection of Ozone in Plasma Applications
Abstract

This thesis presents an overview of the research activities undertaken during my PhD under the supervision of Dr. F. Iza from the School of Electronic, Electrical and Systems Engineering and Dr. B. Buckley from the Chemistry Department at Loughborough University.

The thesis is divided as follows. The first chapter of the thesis presents an introduction to plasma and chemical probes as well as the motivation for developing fluorescent probes for plasma characterisation. Analytical techniques used during this work to analyse chemical substances are described in the second chapter. Results and discussions from the experiments are discussed in chapters 3 to 7. Conclusions and future work are presented in chapter 8. In chapter 9, experimental data is presented.

In the last century, plasma has attracted the attention of numerous researchers. Due to the wide-range of applications of this ionised gas, people from different fields have focused their effort on studying plasma. Low-temperature plasmas have received growing attention in the last 50 years when the development in cold plasma devices made them more controllable. Plasma played (and continues to play) a critical role in the fabrication process of integrated circuits and recent advances in the generation of low-temperature atmospheric-pressure plasmas have resulted in the emergence of new applications including treatment of temperature sensitive surfaces and biological targets.

During the first months at Loughborough I worked on the ozonolysis of various alkenes with air plasmas. This allowed me to familiarised myself with plasma as this was new to me and get a feeling of some of the challenges lying ahead. Nonetheless, the data I obtained was encouraging and I presented the results of batch and flow plasma-based ozonolysis of alkenes at the Technological Plasma Workshop held in Manchester in January 2012.

Once I had familiarised myself with the plasma system, I worked on synthesising fluorescent probes to detect ozone, one of the many reactive species that are typically
generated in oxygen containing plasmas. Details of the experiments conducted to date and most significant findings are discussed in this thesis.

Journal publications

2 C. Castello, F. Iza, B.R. Buckley; (in preparation).

Conference contributions

2 C. Castello, F. Iza, B.R. Buckley; Analysis of chemical species in plasma; *Electronic, Electrical and Systems Engineering (EESE) annual meeting*, 2012 (Loughborough, UK).
3 C. Castello, F. Iza, B.R. Buckley; Ozone Detection in Plasmas; *Electronic, Electrical and Systems Engineering (EESE) annual meeting*, 2013 (Loughborough, UK).
4 C. Castello, F. Iza, B.R. Buckley; Fluorescence Probe for Determining the Ozone Dose Delivered by Plasmas; *Technical Plasma Workshop*, 2013 (York, UK).
5 C. Castello, F. Iza, B.R. Buckley; Plasmas for Organic Synthesis and Chemical Probes for Plasma Diagnostics; *COST 1208 annual meeting*, 2014 (Lisbon, Portugal).
6 C. Castello, F. Iza, B.R. Buckley; Ozone Detection in Cold Plasmas by using Chemical Probes; *Electronic, Electrical and Systems Engineering (EESE) annual meeting*, 2014 (Loughborough, UK).
7 C. Castello, F. Iza, B.R. Buckley; Fluorescent Probes for Selective Detection of Ozone; *RSC Organic Division Midlands Meeting*, 2014 (Nottingham, UK).
9 C. Castello, F. Iza, B.R. Buckley; Probe for Determining Ozone in Low-temperature Atmospheric-pressure Plasmas; *Technical Plasma Workshop*, 2014 (Coventry, UK).
10 C. Castello, F. Iza, B.R. Buckley; Probe for Determining Ozone in Low-temperature Atmospheric-pressure Plasmas; *COST Action TD1208 “Electrical Discharges with liquids for Future Application”*, 2015 (Barcelona, Spain).
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1 Introduction

1.1 Plasma

1.1.1 Introduction

Plasma is an ionised gas, a gas in which a certain portion of the atoms or molecules are ionised. The word plasma comes from the Greek, πλασμα (plasma), which means ‘mouldable substance’. This term was coined by Irving Langmuir in 1927 when studying electronic devices based on ionised gases at General Electric Co.\textsuperscript{1} This gas with electrical properties, however, had been described earlier by Williams Crookes in 1879.\textsuperscript{2}

Plasma is the 4\textsuperscript{th} state of matter due to the differences between its properties and the properties of a gas. Plasma is electrically conductive since it has charged particles (free electrons and ions) and therefore, it responds strongly to electromagnetic fields. At sufficiently high frequencies, however, its response is more characteristic of a dielectric medium as charged particles cannot respond to fast changing electric fields due to their inertia. Plasma is the most abundant phase of matter in the universe: stars are plasma and much of the space between the stars is filled with plasma as well. On Earth, however, plasma is not as common as in the rest of the universe. An example of naturally occurring plasma on Earth is the lightning during a storm.

![Diagram of states of matter and phase changes](image_url)

\textbf{Figure 1:} Scheme of the states of matter and the processes to change between them.

If enough energy is placed in a system, a phase change may occur as matter moves to a more active state (\textbf{Figure 1}). Starting with matter in solid state, if this is heated to a certain temperature, a solid will melt and become a liquid. If additional energy is introduced in the system, continuing to heat it, the liquid will eventually evaporate and
become a gas. Finally, if more energy is introduced in the system, a point will be reached in which electrons break away from the neutral gas atoms (and/or molecules), ionising the gas. When the number of electrons/ions is high enough, the gas becomes plasma. Depending on the gas composition, the temperature needed to form plasma might vary significantly. For example, to ionise caesium the gas needs to be heated to ~4,000 K, whereas to ionise helium, ~20,000 K is required. As indicated above, plasma can be generated by heating a gas. However, this is not the only way of ionising a gas to form plasma. Plasma can also be produced by applying electric fields. These plasmas are often referred to as electrical discharges. In electrical discharges, energy is transferred primarily to electrons that are accelerated and heated to tens of thousands of Kelvin while neutral gas molecules remain close to room temperature because they are not affected by the applied electric field. A distribution of electrons that has a temperature of 10,000 K (~1eV) contains a significant fraction of electrons that have enough energy (1~15eV) to dissociate molecules of the feedstock gas into reactive species such as atoms, free radicals and excited species, and to ionise them to create plasma.

1.1.2 Types of Plasma

Plasmas can be classified by the degree of ionisation, plasma density, frequency, pressure or shape. Figure 2 shows a classification of space and laboratory plasmas as a function of their density and temperature. Based on the relative temperature of electrons, ions, and neutral species, plasmas are classified as ‘thermal’ and ‘non-thermal’, (also referred to as ‘hot’ and ‘cold’ plasmas, respectively). In all plasmas supported by an electric field, electrons gain energy from the electric field faster than the much heavier ions and as a result they heat up to several thousands of degrees before their environment heats up (energy transfer from electrons to neutrals via elastic collisions is very inefficient due to the large mass difference between electrons and gas molecules). These are non-thermal plasmas as they are not in thermodynamic equilibrium: electrons and neutrals have very different temperatures and therefore the system cannot be described by a single temperature value. On the other hand, in plasmas in which energy transfer from electrons to heavier ions and neutrals is more effective than cooling of ions and uncharged molecules (e.g. fusion plasmas, welding arcs), all particles reach the same temperature (typically several thousand Kelvin) and the plasma is said to be in thermal equilibrium.
Both nature-occurring and man-made plasmas spread across a wide range of plasma parameters (Figure 2). For example, stars are thermal plasmas very hot and very dense (with temperatures and densities beyond the limits of the graph in Figure 2) whereas plasmas in the interplanetary medium are cold and tenuous. The ionosphere, the layer in the earth’s atmosphere ionised by solar radiation that covers from around 50 to 1500 km in altitude, is another example of a weakly ionised low-temperature plasma with electron densities on the order of $10^4$ to $10^6$ cm$^{-3}$. Artificially produced plasmas can also be hot or cold. For example, high pressure welding arcs and laser plasmas are hot discharges close
to thermodynamic equilibrium whereas neon lights and plasmas employed for antimicrobial purposes are examples of cold non-thermal discharges.

The electron and gas temperatures are key factors that determine many of the plasma characteristics. Low gas temperature is required for the treatment of heat-sensitive materials and small changes in temperature (both electron and gas temperatures) can affect significantly the final chemical composition of the plasma and thereby its reactivity and efficacy.

Plasma sources come in many different sizes and shapes, such as small jets or large surface discharges. The dimensions of the devices are generally determined by the intended application, but it is also noted that chamber dimensions affect plasma properties.3

1.1.3 Applications

Low temperature plasmas are used in a variety of different fields. Applications of cold plasmas include lighting;6 surface modification;7 manufacture of integrated circuits;3 flat panel displays;8 water disinfection;9 and sterilisation and purification within the food and drug industries.10 In the last decade researchers have also investigated biomedical applications of low-temperature plasmas.11,12 In this kind of plasmas, due to their partially ionised nature, the specific energy content of the plasma is low as the energy content is dominated by the far more abundant neutral gas. This situation provides a unique set of conditions wherein plasma species in contact with surfaces can be non-destructive and beneficial. Some examples of the previously mentioned applications of non-thermal plasmas are briefly discussed below:

Lighting: Both, natural light and artificially produced light, come in most of the cases from plasma. We have talked about natural light sources such as stars and interstellar space in the introduction. Humankind has been producing light artificially since fire was controlled. Electric light started to be commercially available at the beginning of the 20th century when the incandescent lamps became economically viable for domestic, public and industrial use. Incandescent lamps, however, are not plasma but a filament heated by electric current until it becomes incandescent. This kind of lamps, are actually the only non-plasma light source widely used. Plasma based lamps can be classified in fluorescent lamps and high-intensity-discharge (HID) arc lamps. The first ones emit ultraviolet light
which is absorbed by a film of phosphor in the internal walls of the lamp and converted to visible light. On the other hand, HID lamps emit directly visible light. Compact fluorescent lamps and sodium-vapour lamps are examples of fluorescent lamps and HID lamps, respectively. These plasma lighting sources are much more effective than incandescent lamps. Plasma, however, may be superseded in the future by LED light sources as these have been improving steadily in terms of efficacy and performance in recent years and this trend is expected to continue as new materials, better manufacturing processes, and new configurations are developed.

Manufacture of integrated circuits: Manufacture of integrated circuits, a multi-billion dollar industry, is probably the plasma application with the highest societal impact. The information technology owes part of its huge development in the last decades to low-temperature plasmas since more than half the steps required to fabricate a modern chip involve plasma technology (e.g. substrate cleaning, etching, deposition, etc).

Coatings and Surface modification: Plasmas can be used to modify surface properties of materials without affecting their bulk properties. A large range of materials such as chips bags, car parts, wood, wool and synthetic fibres are routinely treated with plasmas to improve their hydrophilicity before being painted. Plasmas can also be used to deposit thin films to protect and/or confer new properties to a substrate. Plasma-deposited barrier coatings, for example, make containers impervious to liquids, gas tanks leak-proof and plastic bottles impervious to gas diffusion. Cleaning and sterilization of biomedical surfaces is yet another application of non-thermal plasmas in which surface properties are changed (toxins and microbes are eliminated) without affecting the bulk mechanical properties of the substrate.  

Flat panels display: During the last decade, plasma display panels (PDP) and plasma-illuminated liquid crystal displays (LCD) took over the display market dominated until then by the cathode ray tube (CRT). Plasma-driven displays grew to a multimillion dollar industry but as in the case of energy-efficient lighting, new LED-based products are emerging and outdating plasma-driven displays due to their higher efficacy and increasing performance.
Water disinfection: Conventional water treatments include the use of chlorine precursors, ozone and/or ultraviolet radiation to disinfect water. These methods have been proven to kill bacteria effectively but there are growing concerns about toxins such as volatile organic compounds that originate from industrial and agricultural wastewaters that are not decomposed by these conventional treatments. Plasma treatment of water has been proposed as a new advanced oxidation process for cleaning water that exploits the in-situ generation of reactive and oxidative species to destroy microorganisms and pollutants. This technology is particularly attractive because it does not require storage and handling of toxic chemicals, thereby mitigating adverse effects on the environment. Besides the growing number of lab-scale studies, pilot plans have also been developed to assess the efficacy and economic viability of this emerging technology.

Plasma medicine: Plasma medicine is an emerging scientific discipline that exploits the interaction between gas plasmas and biological or other targets for therapeutic purposes. One of the most promising applications of plasma in this field is sterilization/disinfection of surfaces such as living tissues and surgery tools since species generated in the plasma can have strong bactericidal properties. Laroussi et al., by exposing bacteria to atmospheric pressure plasma, demonstrated that the species generated in plasma create a lethal environment for microorganisms. Since then, plasma has been used to inactivate Gram-positive bacteria, Gram-negative bacteria, antibiotic resistant bacteria, viruses, fungi and spores. Plasma can therefore be used to sterilise implants and surgical instruments. Other applications of plasma in medicine are also being investigated. For example, plasmas are being investigated to treat pathologies including skin, gastrointestinal, cardiovascular and dental diseases as well as different forms of cancer. For most of the medical and healthcare applications mentioned above, the energy delivered to the biological target is critical since those targets are frequently living cells, and of course, living cells are temperature sensitive. For this reason, low-temperature plasmas are employed in this field. To prevent thermal damage to skin cells, the target temperature must remain below 60º C. In Figure 3 an example of a plasma device treating a human finger is shown.
Food industry: Treatment of food with cold plasma has also been studied during the last decade. With the development of cold plasmas that operate at atmospheric pressure without the need of vacuum technology, plasma has become economically viable. These plasma treatments can increase the safety for consumption and the shelf-life before spoilage.

Examples of thermal plasmas used in industry include the arcs of electrical torches for welding and cutting, which provide high performance cuts in materials such as steel. This devices use high-frequency, high-voltage sparks to ionise the gas and create very hot plasmas capable of melting the metal being cut. Arc torches are also used in waste treatment, pollution abatement and gas production. Plasma Spray Coating also relies on a high-temperature plasma to deposit ceramic coatings with thermal and anticorrosion properties to jet engines and turbine blades. This technique consists in melting or heating a powder in a plasma torch and spraying it onto a surface. Any material that can be melted without chemical decomposition can be used to form the coating.

1.1.4 Chemistry

Plasma contains a large number of different species that are generated as a result of collisional processes, such as excitation, ionization, attachment, recombination, detachment and charge exchange. The actual composition of a plasma depends on the nature of the original gas, its temperature, the discharge geometry and the amplitude and frequency of the applied voltage.

To better understand the processes involved in plasmas a small introduction about molecular energy levels will be useful. The ground state of a molecule is the state with
lowest energy, and all the others are called excited levels. The energy levels are quantified, which means the molecule can only adopt certain states with specific energy. The energy levels of the molecules are: translational, nuclear, rotational, vibrational and electronic from smaller to larger difference in energy between themselves. The separation in the translational, nuclear and rotational energy levels is smaller than KT, where K is the Boltzmann constant (K= 1.38x10^{-23} J K^{-1} or 8.62x10^{-5} eV K^{-1}) and T gas temperature. Therefore, at room temperature most of the molecules are in excited rotational states. On the other hand, the separation in the vibrational and electronic state is larger than KT and most molecules are in their ground electronic and vibrational energy levels at room temperature. In Figure 4, a typical energy level diagram of an atom or molecule showing two electronic states and associated vibrational and rotational energy levels is shown.

![Energy level diagram](image)

**Figure 4:** Typical energy diagram of an atom or molecule with two electronic states and associated vibrational and rotational energy levels.

During plasma formation in an electric discharge, free electrons are accelerated by the applied electric field and collisions of these energetic electrons with gas atoms/molecules free more electrons, which in turn are also accelerated in the electric field. This results in an avalanche process that continues until a steady state degree of ionization is reached. Charged particles accelerated in the electric field transfer their energy to the neutral species via collision with these particles. When a colliding particle absorbs sufficient energy, it is excited from the ground state to a higher energy level. The new species is then more reactive than the initial one and can react with other species in the plasma or with
surfaces in contact with the plasma. Due to the non-equilibrium conditions encountered in cold plasmas, very reactive environments can be generated while the gas temperature remains close to room temperature.

In the context of plasmas for biomedical applications, plasmas containing He and admixtures of O$_2$, N$_2$ and/or H$_2$O have received particular attention. Helium is typically used due its high thermal conductivity as this helps to maintain the plasma at close to room temperature. Oxygen, nitrogen and water admixtures are then introduced to create a reactive environment in the plasma. In plasma chemistry and in free radical biology, the terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) are used to refer reactive species containing oxygen and nitrogen, respectively. When the reactive species contains both nitrogen and oxygen, e.g. nitric oxide and peroxynitrite, the term RNS is used. Some of the most important ROS in terms of reactivity and presence in the atmosphere, biological samples and in plasma environments are atomic oxygen, singlet oxygen, superoxide radical, peroxide anion, ozone, hydrogen peroxide, hydroxyl radical and hydroxyl anion. The nature and the reactivity of these species will be explained in chapter 1.1.5.

Although the background gas may have a simple composition, a complex chemistry develops in plasmas. As an example, the results of a plasma chemistry model developed by D.X. Liu et al. shows that even if only He and O$_2$ are used as background gases, once the plasma strikes at least 21 species are generated and 267 reactions need to be taken into account in the ionised gas. These species include cations of helium and oxygen, anions as well as neutral species such as O$_3$ and exited species such as singlet oxygen. The number of generated species is increased notably if water is present in the background gas. Up to 55 species and 855 chemical reactions have been identified in He+O$_2$+H$_2$O plasmas. The chemistry of ionised air at atmospheric pressure is even more complex due its composition, that is, nitrogen 78%, oxygen 21%, argon 1%, water 0-4% and a small amount of CO$_2$. In air plasmas, other species containing nitrogen and carbon are generated in addition to the oxygen-derived species mentioned before.

1.1.5 ROS and RNS

When people talk about oxygen, it is normally referring to the diatomic molecule of oxygen in its ground state, O$_2$. As shown in Figure 5, molecular oxygen is a diradical with both unpaired electrons with the same spin. It is also called triplet oxygen. The compounds
which are in a triplet state do not react easily with other compounds in singlet state. Since most of the organic matter is in singlet state, the oxygen in the atmosphere is not dangerous to living organisms.

Figure 5: Molecular orbitals of triplet oxygen formed from atomic orbitals of two atoms of oxygen.

Four quantum numbers describe the electrons completely; the principal quantum number \( n \), azimuthal quantum number \( \ell \), magnetic quantum number \( m \), spin quantum number \( s \). Spin quantum number can be \( +\frac{1}{2} \) or \( -\frac{1}{2} \), spin numbers of the electrons are represented as arrows upwards or downwards as in Figure 5. In an atom or molecule, when all the electrons are paired or the sum of all their spin quantum numbers is zero, the total spin for the system is zero: \( S= 0 \). If it has an unpaired electron, it is said to be a radical, \( S= \frac{1}{2} \). If two electrons are unpaired with parallel spins (e.g. both with positive spin), \( S= 1 \). \( L \) is the spin-angular momentum vector given by \( L= 2S + 1 \). When the spin-angular momentum of a molecule is 1, 2 or 3, the molecules are singlet, doublet and triplet respectively. Molecules with a determined \( L \) react only with other molecules with the same momentum.

This small introduction about the 4th quantum number of the electrons allows us to understand the difference between triplet oxygen and singlet oxygen and the reactivity of these and other reactive species. Singlet oxygen is generated by exciting triplet oxygen. The only different between the two species is that the unpaired electrons are with parallel spin in triplet oxygen and antiparallel spin in singlet oxygen. This configuration, see
Figure 6, makes singlet oxygen to be an extremely hazardous compound to organic matter since organic molecules are in singlet states.

Atomic oxygen has two unpaired electrons in separate orbitals, making it susceptible to radical formation through successive reduction by addition of electrons (Figure 7). As Superoxide anion is formed by the addition of one electron to molecular oxygen. Since superoxide anion has a single unpaired electron and therefore it is a radical that can react very readily with organic matter. Upon two-electron reduction of molecular oxygen or one-electron reduction of superoxide anion peroxide is formed.

Figure 7: Lewis structure of some of the species derived from oxygen.
1.1.6 Diagnostics

Due to the different intended applications of plasma and its complexity, it is necessary to understand what is happening in the discharge in order to improve the plasma efficacy. In general, one wishes to know what species are generated in the plasma, in what concentration and through which mechanisms so that the plasma is operated under optimal conditions (temperature, humidity, gas composition, power, etc.).

It was Langmuir who developed the first diagnostic for plasmas. This diagnostic is called the Langmuir probe. It is a device which can determine physical properties of the plasma: electron temperature, electron density and electric potential. To determine these parameters, one or more electrodes are placed in the plasma and the current flowing through the probe at various potentials is measured. These probes have been used since Langmuir’s time and they are still routinely used nowadays. The measurement itself is fairly simple but the difficulty resides in the interpretation of the current-voltage graph obtained during the measurement. At the heart of this interpretation is the concept of plasma sheath. Langmuir developed the theory of plasma sheaths while trying to improve the lifetime of the filaments for tungsten-filament light-bulbs. Plasmas, which are quasi-neutral, i.e. the density of negatively charged particles and positively charged particles are almost identical, are joined to wall surfaces across thin positively charged layers. These layers called sheaths are formed due the absorption of electrons in the walls. Since the electrons have larger thermal velocity than ions, they are rapidly lost in the wall, leading to the formation of the plasma sheath.

One of the limitations of Langmuir probes is the fact that the probe itself must be in contact with the plasma and therefore it can perturb the discharge. This is particularly true for small plasmas such as those often encountered at atmospheric pressure. Furthermore, interpretation of Langmuir probe measurements in atmospheric pressure discharges is very challenging due to the lack of a well-established collisional sheath theory. However, there are others methods to characterise cold atmospheric-pressure plasmas that circumvent these limitations, e.g. spectroscopy.

Plasmas have also been studied for decades by optical absorption and emission spectroscopy in the visible, UV-visible and near infrared wavelength range. Fast electrons excite species in the gas and when these excited species fall back to lower energy levels, they emit photons. The measure of these emissions is a very powerful tool to characterise plasma as unlike Langmuir probes, optical emission spectroscopy is a non-invasive
method. For example, rotational temperature, which is a good indicator for gas temperature in atmospheric pressure discharges, can be determined by measuring some emissions related to molecular species such as N\textsubscript{2} in atmospheric air plasmas. Electric field intensity and electron densities in specific ranges can also be determined using optical emission spectroscopy, for example, by studying the Stark broadening of atomic lines.\textsuperscript{32} Density of excited species can be determined by measuring the optical emission of the plasma. Although some excited species emit photons readily, there are also excited species for which photon emission is a very unlikely process, i.e. involve forbidden transition. For example, excited OH molecules are readily detected in atmospheric pressure plasmas in which humidity is present whereas emission of singlet oxygen is extremely weak. Emission of singlet oxygen at 1270 nm, however, has been used to determine its concentration in some circumstances.\textsuperscript{33}

Although optical emission spectroscopy only inform about the population of excited species, absorption spectroscopy can be used to determine densities of ground state atoms/molecules (i.e. not emitting species). For example, ozone concentrations can be determined by UV-absorption in the Hartley band, from 200 to 300 nm. The maximum of this absorption is at around 254 nm and this band has been used for ozone quantification in numerous studies.\textsuperscript{34, 35} Absorption of infrared radiation can also be used to determine ozone concentration and Fourier Transformed Infrared (FTIR) spectroscopy has been used to verify that only ozone absorption is being detected in the UV analyses.\textsuperscript{35} Nitric oxide, hydroxyl radical and atomic oxygen have also been detected in plasma using optical and electrical diagnostics.\textsuperscript{36}

Laser-driven optical diagnostics provide an even more refined spectroscopic means of analysing plasmas as precise transitions can be targeted. For example, singlet oxygen can be directly measured using cavity ring-down spectroscopy (CRDS), which is a type of highly sensitive laser spectroscopy.\textsuperscript{37} CRDS enables measurement of absolute optical extinction by samples that scatter and absorb light. It has been widely used to study gaseous samples which absorb light at specific wavelengths, and in turn to determine mole fractions down to the parts per trillion levels. Ozone can also be measured by quantum cascade laser-absorption spectroscopy in the mid-infrared (IR) spectral region.\textsuperscript{34} Laser-induced fluorescence has also been used to analyse atomic oxygen in plasmas. In particular, Marinov \textit{et al.} measured atomic oxygen by two-photon absorption laser-induced fluorescence (TALIF). Atomic oxygen was excited using light with \(\lambda= 225.65 \text{ nm}\) and fluorescence was measured at 844.87 nm.\textsuperscript{38}
Beside optical means, plasma species can also be identified by mass-spectrometry. It is noted, however, that transport of short-lived species from an atmospheric-pressure discharge into the spectrometer is a significant challenge.

The methods briefly mentioned above provide a few examples of typical plasma diagnostics. Experimental measurements have also been complemented with computational studies to further advance our understanding of the physico-chemical processes in the discharges. In recent years, and in the context of plasmas for biomedical applications, a number of chemical probes initially derived for chemical and biological use are starting to be used by the plasma community as a means to unravel the chemistry at play in the discharges. Some of the recent work carried out in this field is reviewed in chapter 1.2.5.

### 1.2 Chemical Probes

#### 1.2.1 Introduction

Chemical probes are valuable tools for the investigation of biochemical processes, diagnosis of disease markers, detection of hazardous compounds, and for other purposes. When a direct sample analysis is not possible, for example, when the desired compound (analyte) does not have a signal or it is overlapped with other signals from other compounds in the same sample, then chemical probes can be used. These probes react with the analyte resulting in new compounds that are measurable, so that information regarding the desired analyte can be obtained.

Chemical probes consist in a binding subunit which reacts with the analyte and a signalling subunit. In optical probes, the reaction changes the electronic state of the molecule and consequently, the optical properties change providing a way to measure the target.

#### 1.2.2 Types of Chemical Probes

Chemical probes are normally classified by the method used to detect or quantify the analyte (see Figure 8). Electrical sensors are based on changes in electrical characteristics such as resistance and capacitance. Spin-traps are chemical probes that capture short lived radicals, which can then be detected by electron paramagnetic resonance spectroscopy.
Optical probes, however, are by far the most popular and are based in the measurement of absorbance or emission of light by a compound. Emission probes can be further classified based on the type of energy involved in the process. Colorimetric probes are based in changes in the colour of the probe and the product after the reaction.\textsuperscript{41} Chemiluminescence probes emit light as a result of a chemical reaction,\textsuperscript{42} and photoluminescence probes re-emit light received from an exciting source at a different wavelength.\textsuperscript{43} There are different kinds of photoluminescence probes depending on the time the molecules spend in the excited state. The most commonly used are fluorescence probes, which radiate within $\text{ns} - \mu\text{s}$ after being excited.\textsuperscript{43} Phosphorescence probes, on the other hand, take seconds, minutes or even hours to radiate.\textsuperscript{43}

Chemical probes can also be classified by the type of reaction involved in the probe: nucleophilic addition and substitutions as well as tandem reactions, which involve addition or substitution followed by other reactions such as elimination. These kinds of probes are used for analysing anionic and neutral species and could be used to quantify most plasma species of interest. Metal-promoted and metal-catalysed reactions, on the other hand, can be used to analyse metal analytes such as palladium or copper\textsuperscript{44} and could be used to determine traces of electrode material in plasmas.

![Chemical Probes Classification](image)

**Figure 8:** Classification of chemical probes by method used.

### 1.2.3 Potential and Challenges of Chemical Probes for Plasma Characterization

Although a variety of plasma diagnostic techniques have been developed to study plasmas (see chapter 1.1.6), most of these diagnostics cannot easily be applied to atmospheric pressure discharges.\textsuperscript{45} Furthermore, given the non-uniformity of atmospheric pressure discharges, it is not straightforward to link measurements in the gas phase at the
centre of the discharge with the fluxes of species hitting a target. Those techniques also require equipment which is expensive and the set-up can be difficult or may not be suitable for all applications. For these reasons, chemical probes can offer a new approach to characterise plasmas and in particular to determine the actual dose of reactive species delivered during plasma treatments.

When selecting a method for an analytical study, a few characteristics need to be taken into account: precision and accuracy, which measure the standard deviation and absolute error respectively; the sensitivity of the analysis which indicates the minimum amount of compound that can be detected; the selectivity of the method to avoid positive results from other compounds; the range of concentrations that can be detected; the stability of the probe which will determine its shelf-life and usage protocol; and finally the cost and time of the analysis. All of these are important characteristics when selecting an analytical method.

Sensitivity is likely to be an important aspect for plasma diagnostics as some plasma species are generated in very small concentrations. In addition, many plasma species have a relatively short life time and therefore these species need in-situ analysis. Selectivity, however, is arguably the most challenging requirement due to the large number of reactive species present in the ionised gas.

Fluorescent probes have been proven to be selective and very sensitive and in fact they have revolutionised biochemistry in recent years. Since many RNS generated in the plasma have also been detected by fluorescent probes in biological samples, fluorescent probes are likely to be the most promising probes for characterizing plasmas. Fluorescent probes could provide a convenient means to determine the dose of chemical species delivered by the plasma without making any assumption about the transport from the gas phase to the target. The measurements could be carried on while the plasma treatment is taking place or once the plasma has been switched off, eliminating electrical and optical noise from the plasma itself.

### 1.2.4 Probes for ROS and RNS in biological samples

Molecular oxygen (O\textsubscript{2}) is indispensable for the life of all aerobic organisms. It plays an essential role as an electron acceptor in mitochondrial electron transport\textsuperscript{47} and therefore in the generation of ATP. Reactive oxygen species may be generated during this or other electron transfer reactions. These species are harmful to the organism as they can damage
lipids, proteins and DNA. Therefore, defence mechanisms such as antioxidant enzymes are
developed by the organism to fight against these assaults. An imbalance between ROS
production and antioxidant capacity of the cell results in a situation known as oxidative
stress. Oxidative stress is believed to be involved in some diseases such as cancer,
inflammation, neurodegenerative injury and in aging. RNS such as NO and its higher
oxides are also involved in physiological and pathological processes. The growing
interest in free radicals in biology has caused the need to identify and quantify ROS and
RNS in biological samples.

In vivo and in situ analysis of reactive species using bioimaging techniques would be
useful to understand the production, transport and biochemical role of these species. As a
result of this need, a large number of chemical probes have been developed to monitor
ROS and RNS in biological samples. It turns out that many of these species are also
generated in atmospheric pressure plasmas and therefore existing chemical probes can be
used as a starting point for developing novel chemical methods for plasma characterization.
Existing probes, however, need to be tested on plasmas and their selectivity and sensitivity
need to be assessed in the presence of multiple plasma species. Several chemical probes
used to detect ROS and RNS that are also found in atmospheric pressure plasmas are
reviewed below.

In Table 1, at the end of this section, a summary of some of the most promising probes
for detecting ROS and RNS in plasmas is presented.

**Hydrogen peroxide:**

There are commercially available probes for H$_2$O$_2$: Amplex® Red, product of
Molecular Probes/Invitrogen, which is used to detect H$_2$O$_2$ and peroxidase activity in
biological samples and HyPer from Evrogen which is selective over ONOO$, NO$ and O$_2$$_2$. In addition researchers are trying to develop better probes in terms of sensitivity and
selectivity. Several probes containing boronate groups have been proposed since boronates
are selectively oxidised by H$_2$O$_2$. Imato and co-workers have also developed a probe
based on the reaction between hydrogen peroxide and triphenylphosphine. This probe,
however, is not selective against superoxide radical and NO. Maeda and co-workers have
developed pentafluorobenzene-sulfonyl fluorescein derivatives to analyse H$_2$O$_2$. Some of
the probes with boronate moiety, for example the compound shown in Scheme 1, and
probes containing sulfur were shown to be selective toward hydroxyl radical, superoxide
radical, peroxynitrite and singlet oxygen.
Scheme 1: Mechanism of the reaction between the non-fluorescent compound and hydrogen peroxide to give fluorescein.55b

Hydroxyl radical:
Aminophenyl fluorescein and hydroxyphenyl fluorescein (APF and HPF respectively) are commercial indicators for hydroxyl radical and peroxynitrite anion (APF also reacts with hypochlorite anion) developed by Nagano. These two probes are selective over singlet oxygen, hydrogen peroxide, nitric oxide and superoxide radical.58 Probes containing the nitroxide moiety have been developed to analyse \( \text{HO}^- \) in DMSO since DMSO reacts with hydroxyl radical to produce the radical CH₃. Methyl radical then reacts with the nitroxide functional group in the probe (see Scheme 2). The example showed on the right of this scheme was developed by Tang and co-workers, R is the fluorophore group, which gives the fluorescence to the compound. This probe showed high selectivity for hydroxyl radical over hydrogen peroxide, superoxide radical, nitric oxide, peroxynitrite, peroxy radical and singlet oxygen. The probe has low fluorescence while the product after the reaction is highly fluorescent. Maximum absorption is at 560 nm and the maximum emission wavelength is at 601 nm.59 These probes require DMSO as a solvent, which could be a limitation for some plasma applications. Other probes have been developed with good selectivity toward hydrogen peroxide over other ROS.60

\[
\text{Scheme 2: On the left, reaction between DMSO with hydroxyl radical. } \text{HO}^- \text{ probe reacts with the methyl radical on the right. } R \text{ is the fluorophore group used for the hydroxyl radical probes.}59
\]
Singlet oxygen:
Compounds with an anthracene moiety have been used as sensors for singlet oxygen based on the reaction between anthracene and this ROS to produce an endoperoxide.\(^{61}\) One of them is a commercially available compound called Singlet Oxygen Sensor Green (SOSG) which is selective against other ROS such as hydroxyl and superoxide radicals.\(^{62}\) The mechanism of this probe is shown in Scheme 3. This sensor, however, has a drawback: the product of the reaction is also a singlet oxygen photosensitizer and therefore the sensor produces singlet oxygen in small but not negligible amounts.\(^{63}\)

![Scheme 3: Mechanism of Singlet Oxygen Sensor Green.\(^{62}\)](image)

Superoxide radical:
Few compounds are commercially available to detect superoxide radical; MitoSOX Red does not react with other ROS or RNS present in mitochondria.\(^{64}\) Dihydroethidium, which is also called hydroethidine dihydroethidium is oxidized by superoxide to 2-hydroxyethidium. It is frequently used for mitochondrial superoxide detection (product from Invitrogen). Other fluorescent sensors have been developed to analyse superoxide radical in cells. For example, Tang and co-workers have developed a compound to analyse O$_2^\cdot\,$. This probe is selective over H$_2$O$_2$, $^1$O$_2$, HO$\cdot$, NO and ONOO$^-$ (see Scheme 4).\(^{65}\) The probe itself displays a low fluorescence and the product obtained after reaction with superoxide radical has a strong fluorescence. Excitation and emission wavelength for fluorescent measures with the probe are 485 and 559 nm respectively.

![Scheme 4: Superoxide radical probe developed by Tang.\(^{65}\)](image)
Ozone:

In 1989, Takeuchi, K. and Ibusuki, T. determined ozone in biological samples by chemiluminescence using Indigo-5,5’-disulfonate (IDS), Scheme 5. Wentworth, P. et al, however, have shown that IDS also reacts with $^1\text{O}_2$ giving the same product as the one given by ozone. The chemical pathway, however, is different.

\[ \text{Scheme 5: Reaction between IDS and O}_3. \]

Garner et al have recently developed a fluorogenic probe for ozone detection. They synthesised a derivative from 2’,7’-dichlorofluorescein (DCF), Scheme 6, which is not fluorescent. Once it reacts with ozone, however, a fluorescent compound is generated. This probe is sensitive and specific against other reactive oxygen species such as singlet oxygen, hydroxyl radical and hydrogen peroxide. They bubbled ozone through a solution of the probe in acetone and water (95/5) obtaining the fluorescent product in quantitative yield.

\[ \text{Scheme 6: Equilibrium structures of 2’,7’-dichlorofluorescein (DCF).} \]

The probe was synthesised from DCF in two steps with a yield of 64%. The mechanism of the ozone probe is shown in Scheme 7. The starting material is non-fluorescent since it has an ether moiety that inhibits the fluorescence. The terminal alkene reacts with ozone by ozonolysis, and the resulting aldehyde undergoes $\beta$-elimination to form the fluorescent product. The probe was exposed to singlet oxygen, hydrogen peroxide, hydroxyl radical and oxygen anion and it was concluded that the probe was selective. Fluorescence, however, was observed when an excess of these species was present in the environment. In particular, the compound reacted inside cells in the absence of exogenous ozone. This highlighted that the compound is not as selective as initially thought. A similar probe was synthesised to improve the selectivity by changing the hydroxymethyl group to
a methyl group to avoid possible oxidation of this moiety. Although this improved its selectivity, the new probe remained weakly fluorescent within the cells before exposure to exogenous ozone.  

![Scheme 7: Mechanism of the reaction of the fluorescent probe for ozone synthesised by Garner et al.](image)

A near-infrared fluorescent probe for monitoring ozone in cells has been recently proposed by Tang, B. and co-workers. They designed the probe using tricarbocyanine (Cy), a near-infrared fluorescent dye, and L-tryptophan (Trp), as an O₃ indicator. They called the new compound Trp-Cy. Trp-Cy and Cy have very different fluorescent excitation at about 630 and 770nm respectively and the λ_max of fluorescent emission for Trp-Cy is 770nm. The difference between the excitation and emission wavelengths avoids interferences improving the detection sensitivity. The synthesis of the ozone sensor consists in a one-step reaction which is shown in the Scheme 8.  

The near-infrared fluorescent probe was tested with different concentrations of ozone and good linearity between the ozone concentration and the fluorescence was observed in the range 0.05-7.0 µM of O₃. Trp-Cy was also exposed to chemical species typically encountered in intracellular environments: ROS such as single oxygen, hydrogen peroxide and hydroxyl radical; RNS such as nitric oxide; and biological antioxidants such as glutathione and ascorbic acid; and metals ions such as K⁺, Ca²⁺ and Mg²⁺. The results of these tests showed high selectivity of Trp-Cy towards ozone but the synthesis of the probe is not straight forward and of low yield.
Recently, in 2013, Zhang et al. reported a new resorufin-based ozone detection probe. This probe is claimed to be selective against hydrogen peroxide, singlet oxygen, superoxide radical, peroxynitrite and hydroxyl radical among others.  

Nitric oxide: 
There are a wide range of commercially available compounds to detect NO; a 2,3-Diamino-naphthalene derivative (DAN-1) has been used but has disadvantages such as short excitation/emission wavelengths, cytotoxicity, strong auto fluorescence, small extinction coefficient and insufficient solubility in neutral buffer. The o-phenylenediamine moiety has been used to detect NO since that moiety modulates fluorescent quantum yields. Nangano’s group has developed some probes for NO using this technique which is based in the formation of a triazole product in the presence of oxygen as is shown in Scheme 10. The diamino-fluorescein derivative developed by Kojima et al (DAF-2) is selective over NO$, NO_3^-$, O$_2^-$, H$_2$O$_2$ and ONOO$^-$. The same group developed an analogous
compound with rhodamine (DAR-2) instead of fluorescein which can detect NO in lower pH (pH=4). Anslyn and Shear have also developed a fluorescent probe for NO based on an N-nitrosation reaction, the reaction is shown in Scheme 10. This probe has high specificity for NO over other RNOS such as NO$_2^-$, NO$_3^-$, hydrogen peroxide, ozone, singlet oxygen and oxygen superoxide.

![Scheme 10: Formation of the triazole. Anslyn and Shear sensor for NO.](image)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Description of the probe</th>
<th>Ref.</th>
<th>Selective over:</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>Amplex Red, commercially available</td>
<td>53</td>
<td>Highly photosensitive</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>HyPer, commercially available</td>
<td>54</td>
<td>ONOO$^-$, NO, O$_2^-$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Boronated compound</td>
<td>55a</td>
<td>O$_2^-$, NO</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Boronated compound</td>
<td>55b</td>
<td>O$_2^-$, NO, ^1$O_2$, O$_3$, OH</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Triphenylphosphine</td>
<td>56</td>
<td>$^1$O$_2^-$, NO, poorly selective</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Fluorescein derivative with sulphur</td>
<td>57</td>
<td>O$_2^-$, ^1$O_2$, OH, ONOO$^-$</td>
</tr>
<tr>
<td>OH</td>
<td>Commercial probe, APF</td>
<td>58</td>
<td>H$_2$O$_2$, ^1$O_2$, ^1$O_2$, NO</td>
</tr>
<tr>
<td>OH</td>
<td>Contain nitroxide and needs DMSO</td>
<td>59</td>
<td>H$_2$O$_2$, ^1$O_2$, ONOO$^-$, NO</td>
</tr>
<tr>
<td>OH</td>
<td>Dimer of fluorescein derivative</td>
<td>60a</td>
<td>H$_2$O$_2$, ^1$O_2$, ONOO$^-$, NO</td>
</tr>
<tr>
<td>^1$O$_2^-$</td>
<td>Ma and co-workers probe</td>
<td>61</td>
<td>H$_2$O$_2$, OH, O$_2^-$</td>
</tr>
<tr>
<td>^1$O$_2^-$</td>
<td>Commercially available in Molecular Probes</td>
<td>62</td>
<td>O$_2^-$, OH</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Commercially available MitoSOX Red</td>
<td>64</td>
<td>Other ROS and RNS in mitochondria</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Rapidly-responsive probe</td>
<td>65</td>
<td>H$_2$O$_2$,  ^1$O_2$, ONOO$^-$, NO</td>
</tr>
<tr>
<td>O$_3$</td>
<td>Indigo, commercially available</td>
<td>42</td>
<td>O$_2^-$, H$_2$O$_2$, OH, ^1$O_2$</td>
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<td>O$_3$</td>
<td>Fluorescein derivative</td>
<td>67</td>
<td>O$_2^-$, H$_2$O$_2$, OH, ^1$O_2$</td>
</tr>
<tr>
<td>O$_3$</td>
<td>Near-infrared fluorescent probe</td>
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<td>H$_2$O$_2$, OH, ^1$O_2$, ONOO$^-$, NO, O$_3$, O$_2^-$</td>
</tr>
<tr>
<td>O$_3$</td>
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</tr>
<tr>
<td>NO</td>
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<td>71</td>
<td>NO$_2^-$, NO$_3^-$, O$_2^-$, H$_2$O$_2$, ONOO$^-$</td>
</tr>
<tr>
<td>NO</td>
<td>Rhodamine (DAR-2)</td>
<td>72</td>
<td>NO$_2^-$, NO$_3^-$, O$_2^-$, H$_2$O$_2$, ONOO$^-$</td>
</tr>
<tr>
<td>NO</td>
<td>Compound containing 2 amine groups</td>
<td>73</td>
<td>H$_2$O$_2$, OH, ^1$O_2$, ONOO$^-$, O$_3$, O$_2^-$</td>
</tr>
</tbody>
</table>

Table 1: Some of the developed chemical probes available for reactive species.

### 1.2.5 Probes for ROS and RNS in plasmas

As stated before, the compounds listed in chapter 1.2.4 have been used to quantify RONS in biological samples. However, some of them have already been tested also in plasma systems. This is the case of Amplex Red which has been used to quantify hydrogen
peroxide generated by a cold atmospheric-pressure plasma jet.\textsuperscript{53} This probe reacts with hydrogen peroxide giving the red-coloured and fluorescent product resorufin as shown in Scheme 11.

\begin{center}
\begin{tikzpicture}
  \node at (0,0) {\includegraphics[width=0.5\textwidth]{scheme11.png}};
  \node at (2.5,0) {Scheme 11: Reaction of Amplex Red with hydrogen peroxide resulting in resorufin.\textsuperscript{53}};
\end{tikzpicture}
\end{center}

This probe has possible interferences with thiols and it is air sensitive, the reactions is pH dependent. Other methods discussed in the plasma literature for the analysis of hydrogen peroxide include: iodometric titration, which consist in the reduction of peroxides by iodide ion\textsuperscript{74}, permanganate titration, other method to analyse the reactive specie in aqueous solutions\textsuperscript{75} and ammonium metavanadate (NH$_4$VO$_3$). Metavanadate reacts with hydrogen peroxide resulting in the formation of a red-orange compound with a maximum of absorbance at 450 nm.\textsuperscript{76}

Ozone generated in plasma has been quantified by using Indigo Carmine. For example, Pavlovich \textit{et al.} have measured aqueous ozone after plasma treatment of water.\textsuperscript{77} They used the indigo method as described by Bader and Hoigne.\textsuperscript{78} However, this method is not selective since it also reacts with singlet oxygen as mentioned in the previous chapter. Ozone has also been measured by HPLC (High Performance Liquid Chromatography) detecting muconic acid as product of phenol ozonolysis. The limitations are: Hydroxylated products of phenol are simultaneously formed upon ozonation, this method is pH dependent.\textsuperscript{79} Finally is also known that muconic acid is not only produced by ozone.

The search of a completely selective probe for these species is a difficult task since interferences from other reactive species normally appear. Probes to detect more than one reactive specie at the same time without distinguish between them have been used to quantify the oxidative level. For example, 2,7-dichlorofluorescein diacetate (DCFH-DA), is widely used for the detection of ROS in biological samples.\textsuperscript{41} DCFH-DA has also been used in plasma analysis.\textsuperscript{80} In this study, Ma \textit{et al.} measured intracellular ROS using the mentioned probe. In Scheme 12 is shown how the non-fluorescent DCFH-DA releases both diacetal moieties in the solution resulting in DCFH, which is also non-fluorescent. Finally, DCFH reacts with ROS to give the highly fluorescent DCF.
In the last years, the efforts of some researchers have been focussed in finding or developing probes to quantify RONS in plasma. In 2010, Oehmigen et al. published a study about the bactericidal effects of atmospheric pressure plasma in liquids. In 2013, Machala et al. published a work in RONS formation by plasma in water and their bactericidal effects using E. Coli. In both works, they monitored the concentration of hydrogen peroxide using the titanium sulphate colorimetric method. Sodium azide was added to the solution when nitrites were present to avoid hydrogen peroxide decomposition when adding the acidic solution of titanyl ions. In the second work, nitrites and nitrates were also measured by ion chromatography using HPLC system Shimadzu LC-10 Avp with UV (210 nm) and suppressed conductivity detection. DCFH-DA was used to measure peroxynitrite concentration. Although DCFH-DA is not specific to peroxynitrite, DCFH-DA is more sensitive to peroxynitrite than other ROS according to Possel et al.

Shortly after, Lukes et al., studying the chemistry and the bactericidal effects of a discharge plasma and the reaction of H_2O_2 with HNO_2 to give ONOO^−, used the same methods to control the concentration of H_2O_2, NO_2^- and NO_3^-.

O_3 was also monitored by using the iodometric method. This method is based on the reaction of ozone with potassium iodide to give iodine. Iodine reacts with sodium thiosulfate. Ozone can then be measured by determining the amount of sodium thiosulfate consumed, see Figure 9.

\[ \text{KI} + \text{O}_3 + \text{H}_2\text{O} \rightarrow \text{I}_2 + \text{O}_2 + \text{KOH} \]

\[ 3\text{I}_2 + 6\text{S}_2\text{O}_3^{2-} \rightarrow 6\text{I}^- + 3\text{S}_4\text{O}_6^{2-} \]

**Figure 9:** Iodometric method.
1.3 This thesis

1.3.1 Relevant Species

The work undertaken as part of this PhD thesis has been motivated by the need to better understand the chemistry at play in low temperature plasmas used for biomedical applications. It is currently accepted that ROS and RNS are the main plasma species responsible for the response elicited by biological targets after atmospheric-pressure plasma treatments. These species can be generated in air plasmas as well as in plasmas of noble gases with admixtures of O₂ and/or N₂. The main RONS generated in these plasmas are nitric oxide, peroxynitrite, atomic oxygen, singlet oxygen, superoxide radical, ozone, hydrogen peroxide, hydroxyl radical and hydroxyl anion; ozone being the species focus of this PhD. These species have been identified in various plasma systems and their relevance for biological targets is well-documented in the free radical biology literature. Chemical probes for most of these species have been developed in the past and remarkable progress has been achieved in molecular biology over the last decade. Most of these probes, however, have been developed for use in biological targets and not in plasma environments.

Plasmas present a new challenge for the probes in terms of selectivity due the large number of reactive species typically present in the ionised gas. In addition, species such as atomic oxygen have not been considered in selectivity studies of chemical probes, neither probes have been developed for their detection.

1.3.2 Ozone

Ozone (O₃) is one of the reactive species generated in oxygen containing plasmas. It is an allotrope of oxygen and in fact it was the first allotrope of a chemical element to be recognised. Ozone is neutral but it is a polar molecule that is represented as a hybrid of its two most stable Lewis structures (see Scheme 13).

\[ \text{Scheme 13: Lewis structures of ozone.} \]
In the 18th century, the strong ozone odour was associated with electricity because it was noticed in lightning and in electrical machinery. It was not until 1839 that Christian Schönbein proposed that the odour came from a new chemical compound. Following its discovery, ozone became subject of great interest for the scientific community. The oxidizing properties of this compound and reactions between this and some organic compounds were quickly recognised. Also the harmful effects in human health were soon discovered.

In the mid-19th century Schönbein developed a way to measure ozone using test papers impregnated with starch-iodine. Aqueous potassium iodide (KI) methods were developed by Andrews in 1856 and Fremy and Beckerel in 1852 and iodide-based probes remain in use today. These probes, however, are not very selective as other oxidising species such as halogens, H₂O₂ and some oxides of nitrogen also liberate I₂ from KI. Therefore, although iodide-based probes can be used to measure ozone when it is known a priori that this is the only oxidant present, they have limited use for plasma characterization.

The development of the emission spectrometer by Bunsen and Kirchhoff in the 1859 contributed to the study of ozone in the atmosphere. In 1879, Cornu observed that the intensity of the sun’s radiation dropped off rapidly at wavelengths below ~300 nm. Moreover, he realised that this effect was larger during the sunrise and during the sunset. He argued that this was due to the presence of a substance in the atmosphere that absorbed light at that range of wavelengths and attributed the contrast between different times of the day to an increase in the path of the light through the atmosphere at dawn and dusk. Shortly after, in 1881, Hartley linked this absorption in the atmosphere with ozone by measuring the ultraviolet absorption of the gas produced by electric discharges.

Over the years these spectrometric methods have been improved and adapted for different environments such as lower atmosphere, stratosphere, living cells and plasmas. The maximum of absorbance in the ozone spectrum is at 260 nm and the extinction coefficient is 2900 M⁻¹ cm⁻¹ according to the experiments carried out by Kilpatrick and Herrick in 1955. Due to the instability and volatility of ozone, it is difficult to determine its molar absorptivity in water. Different values, from 2600 to 3600 M⁻¹ cm⁻¹, have been published. The values used during this work are the ones obtained by Kilpatrick and Herrick and corroborated by Hoigne and Bader in 1976 (λ_max = 260 nm and ε = 2900 M⁻¹ cm⁻¹).

Ozone is a powerful oxidant, much more powerful than the diatomic molecule of oxygen. It has a short half-life of 30-40 min in atmospheric conditions, but this time
depends on the temperature, humidity and air speed. The half-life time of ozone decreases substantially in aqueous conditions. The stability of ozone in solution is also dependent on pH. The more acidic the solution, the longer the half-life time of ozone is. In the experiments carried out by Hoigne and Bader, the half-life time of ozone in a pH 4 aqueous solution, at 25 °C, reached 350 min.

This allotrope of oxygen has different roles depending in the environment in which is present: in the stratosphere, ozone prevents damaging ultraviolet light for reaching the Earth surface. In the lower atmosphere, however, ozone is an air pollutant linked to photochemical smog and climate change and for this reason it has been measured since its discovery. Ozone, along with other reactive oxygen species such as singlet oxygen and hydrogen peroxide, is naturally produced by biological systems as a means of destroying bacteria. Moreover, it is believed that the powerful oxidizing properties of ozone may also be a contributing factor of inflammation.

Due to its oxidant properties, ozone is used as a bactericidal agent to kill bacteria in swimming pools, and to disinfect water, hospitals and food packaging. It is also widely used in the pharmaceutical industry and in the synthesis of lubricants and other organic compounds. In organic chemistry, this compound acts as a powerful electrophile. Ozone attracts electrons and it can cleave carbon-carbon double bonds in a process known as ozonolysis. This process involves several steps and the most widely accepted mechanism of the reaction was first proposed by Criegee in 1953 (see Scheme 14).

In the first step, a 1,3 dipolar cycloaddition creates a primary ozonide that is very unstable due the low energy of the two oxygen-oxygen bonds (140 kJ mol⁻¹ ~ 1.5eV). This compound immediately decomposes by a reverse 1,3-dipolar cycloaddition to its corresponding carbonyl oxide and carbonyl (aldehyde or ketone). Then, a second cycloaddition gives the final ozonide, which is more stable than the first since it has only one oxygen-oxygen bond. Nonetheless, this second ozonide is also unstable and in fact quite explosive, so for the reaction to be of practical use the secondary ozonide needs to
undergo a final decomposition step. Various compound families can be obtained depending on the initial alkene used in the ozonolysis and the reducing power of the reagent used in the final decomposition step.

\[ \text{Scheme 15: Products obtained using different reagent in the last step of ozonolysis.}^{101} \]

**Scheme 15** shows a few common ozonolysis reactions. Using dimethylsulfide or triphenylphosphine as a reducing agent, ozonolysis results in aldehyde and ketone formation; using more powerful reducing agents, such as NaBH\(_4\), ozonolysis produces alcohols; and adding oxidizing compounds, such as H\(_2\)O\(_2\), ozonolysis results in the formation of carboxylic acids and ketones. As an example, **Scheme 16** shows the mechanism of the last step of the reaction using dimethyl sulfide as reducing agent.

\[ \text{Scheme 16: Mechanism of the last step in ozonolysis using dimethyl sulfide.}^{101} \]

Since the ozonides generated as intermediates in the ozonolysis of alkenes are unstable and explosive, ozonolysis batch processes are generally avoided due to safety reasons. This has triggered a growing interest in flow ozonolysis as a way to avoid the accumulation of large amounts of hazardous intermediates.\(^{102, 103}\)

**1.3.3 Plasma Source**

For the studies presented in this thesis, dielectric barrier discharges (DBDs) have been used to generate the atmospheric-pressure plasmas. DBDs are generated between two electrodes with at least one of them being covered by a dielectric material. Two different
DBD devices have been used in this work: one of them is a plasma surface device and the other is a plasma jet. Whereas the surface plasma described earlier can be used to treat large surfaces, the plasma jet is better suited for the selected treatment of small areas.

In Figure 10 a scheme of the plasma surface device is shown. A picture of the actual device is shown in Figure 11. In the plasma surface device, the dielectric layer (1.5 mm thick, 7 cm diameter) is sandwiched between two electrodes. PTFE and alumina have been used as dielectric material, with the latter providing a more reliable operation. PTFE dielectrics failed after a few hours of operation, making necessary to stop the experiment to replace the dielectric material. On the contrary, alumina dielectrics have been in operation for over 3 years without a single failure. The ground electrode consists of a stainless steel mesh placed under the dielectric material and it is around this electrode that plasma forms. The high voltage electrode is placed on the other side of the dielectric material. It is made out of aluminium tape and its diameter (3 cm) is smaller than that of the dielectric to prevent flashing between the high voltage electrode and the ground mesh around the dielectric edges. The plasma applicator has been designed so that it can sit on a Petri dish, creating an enclosed volume (“chamber”) for the plasma treatment to proceed. The applicator has a diameter of 7.5 cm. Samples to be exposed to the plasma were placed either directly on a Petri dish or inside the chamber in containers of different sizes (2-6 cm diameter). A magnetic stirrer was used in all cases to guarantee consistent mixing. In this set up, the plasma forms around the ground mesh 2-4 cm away from the sample itself and therefore the treatment is a remote afterglow treatment in which long-lived chemical species such as ozone are expected to drive the chemical reactions.

![Figure 10: Plasma surface experimental setup.](image)

A home-built 1-10 kV<sub>p-p</sub> 10 kHz sinusoidal power supply has been used to generate the plasmas. The source was operated in continuous wave mode as well as in modulated mode,
the latter to minimize the temperature rise of the device and to control the average production rate of oxidative species. Discharges have been sustained in air for most experiments but other atmospheres such as mixtures of helium and oxygen have also been used.

Figure 11: Actual picture of the plasma device. Insert in the image shows the plasma source in operation.

The other plasma source employed in this study is an atmospheric-pressure DBD plasma jet. This device was used only for the characterisation of Probe 1 (see section 4.2). The device consists of a ceramic body with a ceramic tube inside (internal diameter 2mm). The high voltage electrode is wrapped around the ceramic tube in the middle of the body (see Figure 12). The ceramic tube acts as dielectric barrier as it separates the electrode from the plasma itself. The ground electrode is located at the bottom of the system, under the glass vial where the solution being treated is held. In this device background gas is flown through the ceramic tube and the plasma forms inside the tube. The jet can be operated above a liquid sample but also be inserted in the liquid itself, forcing the afterglow plasma to bubble through the liquid (see Figure 12). The plasma jet has been driven by the same power supply as the surface plasma device albeit operating at higher frequency (20kHz).
1.3.4 Aim and Objectives

The aim of this research was to synthesise, calibrate and test novel specific, selective and sensitive fluorescent chemical probes to quantify the flux of ozone delivered from an oxygen-containing plasma to a target in a fast, practical and inexpensive way. Traditional plasma diagnostics characterise the plasma gas phase, but for atmospheric pressure discharges it is difficult to link experimental information on the gas phase to the actual flux of species experienced by a target. This is due to the non-homogeneity of atmospheric pressure discharges and their complex dynamics.

To achieve this goal the following objectives were set:

1. Familiarization with plasma systems: Various plasma systems and arrangements were used to get a grasp about plasma generation and application of plasma to liquid samples. In particular, ozonolysis of various alkenes with air plasmas were performed to familiarise myself with the plasma device and the plasma itself. As discussed in chapter 1.3.2, ozonolysis is the reaction between ozone and a double bond. This reaction can in principle be used for the detection of ozone but the analysis of the resulting products does require complex analytical techniques such
as NMR that would not be readily available in plasma laboratories. The experiments carried out confirmed that ozone is generated in atmospheric air plasmas, that plasma can be used to trigger ozonolysis reactions and that both batch and flow plasma systems could be developed.

2. Identification and assessment of known chemical probes for ozone detection: A thorough search in the literature of compounds and methods employed to analyse ozone was carried out to identify the most promising ones for plasma applications. We proceeded to buy the chemicals when commercially available and synthesised the probes when these had only been reported in the scientific literature and were not commercially available yet. These compounds were then exposed to ozone to assess their performance.

3. Synthesis of new ozone probes: Due to the limitations of existing chemical probes, new compounds were proposed, synthesised and tested. Five fluorescent probes have been developed in the course of this PhD. Once synthesised, the probes and the products generated after ozone exposure were isolated and thoroughly analysed (absorbance, fluorescence, high-definition mass spectrometry, chromatography, nuclear magnetic resonance, crystallography, etc) to gain an understanding on the mechanisms and performance of the probes. Probes were then exposed to other reactive oxygen species likely to be present in plasma applications to assess their selectivity and fluorescence measurements were compared with UV absorption measurements of ozone in the gas phase.
2 Chemical Analysis

In this chapter, the chemical analysis methods used in this work to identify compounds and monitor progress of chemical reactions are briefly introduced. Since no single method can be used to uniquely identify compounds and reactions, various chemical analysis methods had to be used together to advance our understanding.

2.1 Chromatography

Chromatography encompasses a collection of laboratory techniques for the separation of chemical compounds. The term chromatography comes from the Greek words ‘chroma’ and ‘graphien’, which mean ‘colour’ and ‘to write’, due to the fact that the first mixtures to be separated were plant pigments.

Chromatographic separation relies on the different affinity (e.g. polarity, hydrogen bonds, Van der Waals interactions) between a stationary phase and the different compounds being separated as they are carried in a mobile phase. Chromatographic techniques can be classified in liquid (LC) and gas chromatography (GC) based on whether the mobile phase containing the mixed sample is a liquid or a gas. Liquid chromatographic techniques such as thin layer chromatography (TLC) and column chromatography have been routinely used during the course of this thesis to monitor progress of chemical reaction and to separate products. Gas chromatography has also been used, albeit less extensively, because this technique is only applicable to low molecular weight compounds.

2.1.1 Thin Layer Chromatography (TLC)

TLC is a liquid chromatographic technique in which the stationary phase consists of a layer of solid particles, typically silica or alumina, spread on a glass plate. A sample of the mixture to be separated is placed on the plate, which is subsequently dipped into a solvent (the mobile phase). As the mobile phase is drawn up the TLC plate, different compounds in the sample mixture travel different distances according to their solubility and how strongly they interact with the stationary phase as compared to the mobile phase. The technique is fast and can be used to identify compounds and to qualitatively monitor the progression of a reaction. Figure 13 shows a graphical representation of a TLC plate with...
some starting material (SM) and product (P) from the reaction of DCF with 4-bromobutene. This reaction is the 1st step for the synthesis of Probe 1, which shall be introduced in chapter 4. A few minutes after dipping the TLC plate in the mobile phase (a 1:1 mixture of ethyl acetate and petroleum ether), different compounds have travelled up the plate different distances and they can be identified as different spots on the plate. These spots can be visualised under a UV lamp as plates are normally doped with a fluorescent material. The separation depends on the polarity of the compound, its interaction with the silica (stationary phase), and its solubility in the liquid phase. In the example considered here, the reaction yields two compounds, which are labelled as ‘Main product’ and ‘By-product’ in Figure 13 and the reaction has come to an end as no starting material is detected in P.

The ratio between the distance travelled by a compound and the distance travelled by the solvent is known as the retention factor (R_f) of the compound and this quantity does not change from experiment to experiment when the same solvent and the same TLC plate type are used.

TLC can be preparative or analytical, i.e. it can be used to separate compounds for further processing (preparative) or to measure the relative proportions of analytes in a mixture (analytical). An example of a large preparative-TLC plate is shown in Figure 14. In this case, four products can be identified and separated after the chromatography.
2.1.2 Column chromatography

Column chromatography has been used in this work to separate and purify individual chemical compounds from mixtures. Its principle of operation is similar to that of the TLC plate and it relies on different compounds moving at different rates through a stationary phase (e.g. silica). Instead of having a thin layer of stationary phase deposited on a plate, in column chromatography a bed of stationary phase is placed within a tube to create a column (see Figure 15). A layer of the sample to be analysed is then placed on the top of the column. This layer should be as thin as possible to improve the quality of the separation. Before the mobile phase is introduced in the column, a layer of sand on top of the sample can be introduced to avoid disturbing the materials when introducing the mobile phase. A solvent or a mixture of solvents with appropriate polarity to separate the compounds is used as a mobile phase. The mobile phase is forced through the column, and since different compounds in the sample travel at different speed depending on their solubility, polarity and interaction with the stationary phase, they can be separated at the end of the column.

Fractions of the eluent coming out from the column are collected in different containers and these are then analysed via TLC to determine their composition (see Figure 15). The volume of these fractions is important. Too large fractions would lead to different products being collected together and therefore requiring subsequent separation. On the other hand, too small fractions would result in loss of product as fractions will need to be combined after the column.

Figure 14: Preparative TLC of the mixture obtained in the synthesis of Probe 5 (see chapter 4) before and after running the chromatography respectively.
2.1.3 Gas Chromatography (GC)

GC is a chromatographic technique in which the mobile phase is a gas instead of a liquid. GC can be used to separate compounds that can be vaporized without decomposition and in practice it is limited to low molecular weight compounds. This technique was used during the plasma-driven ozonolysis reactions (chapter 3). Vaporized samples are passed through a stationary phase and different compounds travel at different speed depending on their polarity and interaction with the stationary phase. Gases leaving the GC column are then analysed continuously typically by infrared spectroscopy (IR) or mass spectrometry (MS). Chromatographic data is then presented as a graph of detector response against retention time, which is called a chromatogram.

2.2 Elemental analysis

One of the techniques used to determine the elemental composition of unknown compounds has been combustion analysis. This analytical technique was used to provide quantitative information about the carbon, hydrogen and nitrogen content in unknown compounds. A sample of purified unknown compound is burnt in an excess of oxygen and the combustion products (carbon dioxide, water and nitric oxide) are trapped and measured. From the amount of combustion products detected, the elemental composition of the unknown compound can be calculated.
2.3 Spectroscopy

Spectroscopy is the study of the interaction between matter and electromagnetic (EM) radiation and this has been extensively used in this thesis to gain an understanding of the molecular structure of compounds and their reaction mechanisms. EM energy is absorbed or emitted by matter in a discrete amount, or quanta, as molecules transition from one energy level to another. When a molecule absorbs electromagnetic radiation, the molecule is excited to a higher energy level. On the other hand, when a molecule relaxes to a lower energy level, it radiates. The frequency of the radiation absorbed or emitted corresponds to the energy difference between the two energy levels involved in the transition and these can represent changes in translational, nuclear, rotational, vibrational and/or electronic state.

The energy (\( \varepsilon \)) and the frequency (\( \nu \)) of the absorbed/emitted radiation are related by the Planck relation \( \varepsilon = h \nu \), where \( h \) is the Planck constant. As a result, it is possible to relate the frequency of the EM spectrum interacting with a sample (Figure 16) to the energy difference of the two molecular states involved in the interaction. From a complete investigation, it is possible to identify compounds.\(^{104}\)

![Electromagnetic Spectrum](image)

**Figure 16** Electromagnetic Spectrum.

In chemistry, most of the spectroscopic methods involve irradiating a sample and determining the absorbed radiation. These absorption methods, however, are not the only techniques; fluorescence and chemiluminescence in which emitted radiation is measured are also widely used. It is also noted that plasmas emit light as a result of the relaxation of excited molecules in the ionised gas and this contain valuable information to characterise the plasma (chapter 1.1.6).
2.3.1 Nuclear magnetic resonance spectroscopy (NMR)

NMR is one of the most revolutionary spectroscopic methods used routinely by chemists in all branches of science. NMR can be used to verify the presence of known compounds as well as to determine the structure of unknown chemicals.

As the name of the technique suggests, NMR spectroscopy studies the interaction of EM radiation with atomic nuclei in the presence of an external magnetic field. NMR spectroscopy can detect only nuclei which have a non-zero nuclear spin momentum (I≠0). Examples of nuclei with non-zero nuclear spin momentum include $^1$H (I=1/2), $^{13}$C (I=1/2), $^{19}$F (I=1/2), $^{11}$B (I=3/2), $^{17}$O (I=5/2), $^2$H (I=1) and $^{14}$N (I=1). The most commonly studied nucleus is by far $^1$H. In this thesis, proton ($^1$H) and carbon ($^{13}$C) NMR spectroscopy have been extensively used.

In the presence of an external magnetic field, degenerate energy levels split, a phenomenon known as Zeeman Splitting, creating a low-energy and a high-energy state with energies slightly below and slightly above the energy of the original degenerate state. The energy difference between the split levels increases with the applied magnetic field and it turns out that a very strong magnetic field is required to split up the energy levels sufficiently as for the two levels to have significantly different populations. For this reason, the field strength of the applied magnetic field in an NMR machine is around 2 to 10 Tesla. For comparison, the earth’s magnetic field has a field strength of only 2 x $10^{-5}$ Tesla.

Once the nuclei are in the low energy state, they are irradiated with radio frequency radiation that, if of the correct frequency, will be absorbed leading to the excitation of nuclei from the low energy level to the higher energy level. The electromagnetic frequency required for this transition depends on the nucleus in question and the applied magnetic field strength as this determines the energy difference between the split levels. In modern NMR machines, the frequency ranges from 60 to 1000 MHz.

When the excited nuclei fall back to the lower energy level, the radiated energy is detected by a radio receiver. By sweeping the frequency of the EM radiation while keeping the magnetic field strength constant a spectrum is obtained. This spectrum indicates the conditions in which the radio frequency is resonantly absorbed and re-emitted and this information can be used to assess the structure of compounds. In order to be able to compare data from different NMR machines, a standard reference substance, tetramethylsilane (TMS) is commonly used. It is customary to plot the frequency axis as ‘chemical shift’, $\delta$, in parts per million (ppm) instead of in frequency:
\[ \delta \text{(ppm)} = \frac{f(Hz) - f_{TMS}(Hz)}{f_{TMS}(MHz)} \]

where \( f \) is the frequency of the EM radiation and \( f_{TMS} \) is the resonant frequency of the protons in TMS. With the above definition, the origin \( \delta = 0 \) ppm corresponds to the resonant frequency of the protons in TMS (Figure 17). Most of the protons in organic samples resonate at higher frequencies than the protons in TMS (chemical shifts \( \delta > 0 \)) because in TMS protons experience a reduced magnetic field due to the shielding provided by the high electron density that surrounds them. For example, protons from a carboxylic acid or an aldehyde group appear at high chemical shifts (typically \( \delta = 9-13 \) ppm) because there is a smaller electron density surrounding them due to the presence of oxygen, an electronegative atom that attracts electrons.
Figure 17: $^1$H NMR and $^{13}$C NMR of Probe 2 (This compound has been designed and synthesised during this project and will be introduced in chapter 4).
2.3.2 Infra-red spectroscopy (IR)

In this thesis, IR absorption has been used as a technique to identify functional groups in unknown compounds and to validate the synthesis of compounds for which their IR spectrum has previously been reported.

The IR region of the spectrum is associated with changes in molecular vibrational energy. Each molecule has a number of vibrational modes (\(3N-6\) for a non-linear molecule containing \(N\) atoms and \(3N-5\) for a linear molecule) and for each vibrational mode a number of quantised vibrational energy levels exist. These vibrational modes and quantified energy levels give rise to absorption bands in the infrared spectrum (radiation with wavelength of 2.5-100 µm, wavenumber of 4000 cm\(^{-1}\) to 100 cm\(^{-1}\) and energy in the 0.01-0.5 eV range). Each molecule has its own characteristic infrared spectrum and this can be used to identify compounds. The spectrum of a large molecule, however, can be quite complex and involve a large number of bands (see Figure 18). In this spectrum from Probe 1 we can see the O-H bond from the carboxylic acid group at around 3200 nm, few peaks around 3000 from C-H bonds and the C=O bonds at 1628 and 1608 nm. Probe 1 is introduced in chapter 4.

Furthermore, when various compounds are present in the sample, the overlapping spectra of each of the individual compounds can be quite difficult to separate from each other.

Even when full identification of a compound is not possible, IR spectroscopy can be used to identify functional groups since particular bonds have very characteristic bands which always appear in a very narrow range of frequencies. For example, carbonyl groups have a peak around 1700 cm\(^{-1}\).
2.3.3 Ultraviolet-visible absorbance spectroscopy UV-VIS

Radiation and absorption in the UV-VIS region of the spectrum (radiation with wavelength of 100-800 nm and energy in the 1.5-12 eV range) are related to electronic transitions and provide a characteristic fingerprint of a compound. In an atom, a change in electronic state gives rise to a narrow spectral line but in a molecule such a change is, in general, accompanied by changes in vibrational and rotational levels giving rise to a band system instead of a spectral line.

The part of the molecule that is responsible of its colour is known as the chromophore and changes in the visible properties of the molecule are related to excitation/deexcitation of electrons in this part of the molecule. Chromophores almost always arise in one of two forms: conjugated π systems (also known as resonant systems) and metal complexes. Functional groups or atoms attached to the chromophore change the wavelength or the colour intensity of a compound even though they do not absorb in the visible themselves.43
In this thesis UV-VIS absorption spectroscopy has been used as a means to detect and quantify concentration of compounds. The setup for these experiments is quite simple. A sample is irradiated with light and the light passing through the sample is collected for further analysis. Transmittance \( T \) at a particular wavelength is then calculated by dividing the intensity of light that is detected when the sample is present by the intensity that is detected without sample. Based on this transmittance measurement, the absorbance \( A \) can be calculated as \( A = -\log_{10} T \). Transmittance and absorbance are dimensionless; however, they are often reported in percentage or absorbance units (UA). Absorbance can be used as a qualitative tool for detecting the presence of a particular compound and as a quantitative tool for determining the concentration of the chromophore.

The Lambert-Beer law relates the absorbance \( A \) with the concentration of the chromophore \( c \), the path length (distance covered by the light in which the chromophore is present, \( l \)) and the extinction coefficient \( \varepsilon \). The extinction coefficient is a unique characteristic of a compound and it has a different value at different wavelengths. Mathematically, the Lambert-Beer law is given by \( A = c l \varepsilon \). In this equation, absorbance can be measured experimentally, \( l \) is given by the dimensions of the experimental setup, and if the extinction coefficient \( \varepsilon \) of a compound is known then its concentration can be inferred. This technique has been used in this thesis for measuring the ozone concentration in gas phase produced by air plasma and the Indigo Carmine concentration in solution.

From a practical point of view, it is noted that the path length of the experimental setup should be chosen so that the absorbance measurement lay in the range of 0.1-2 to avoid loss of accuracy. Absorbance measurements are taken normally at the wavelength of max absorbance as at that point not only values are larger but variations are smaller due to the shape of the curve. An example of the absorbance of a 0.04 M Indigo Carmine solution is shown in Figure 19. Indigo carmine is a widely known compound used to determine ozone among other oxidative species and it will be further discussed in chapter 4.66
2.3.4 Fluorescence spectroscopy

During this project, fluorescence has been used as a qualitative tool, to detect a compound and to complement other methods to determine chemicals. It has been used also as quantitative tool to quantify ozone by measuring the fluorescence of the product of ozone with various chemical probes (chapter 4).

Luminescence is the emission of light as a response to an input of energy such as thermal, electrical or chemical energy and fluorescence is a type of photoluminescence, i.e. emission of light after the absorption of shorter wavelength (more energetic) photons. Fluorescence is distinguished from other types of photoluminescence by the fact that the excited molecule returns to the ground state immediately after excitation; the time spent in the excited state being typically of the order of $10^{-8}$ s.\textsuperscript{43} Fluorescence measurements can therefore provide information about processes with high time resolution.

To perform quantitative measurements, however, it is necessary to obtain a calibration curve prior to the experiment. This is the case because the fluorescence signal detected in the experiments will depend not only on the dimensions of the experimental setup but also on the wavelength and intensity of the incident radiation. This can be done by measuring the fluorescence of solutions with known concentrations of the fluorescent compound in the same setup to be used during the experiments. Once the calibration curve fluorescence vs concentration has been determined, it is possible to relate the fluorescence of solutions with unknown concentration of fluorescent material to their actual concentration.

In Figure 20, the fluorescence of one of the probes used during this thesis, Probe 1 (chapter 4), is shown before and after ozone exposure. The original probe is virtually non-
fluorescent and after it reacts with ozone, it becomes highly fluorescent. Not only the intensity of the fluorescence changes but also the wavelength at which the maximum fluorescence occurs shifts, an indication that the two samples contain different compounds.

Figure 20: Fluorescence of Probe 1 before and after ozone exposure. λ<sub>ex</sub> = 497 nm.

2.3.5 X-ray crystallography

With this analysis method, the actual three-dimensional (3D) structure of a crystalline solid can be determined. A crystal of the substance under investigation is exposed to radiation in the X-ray region of the electromagnetic spectrum (Figure 16). This is energetic radiation (0.1-100keV) with wavelength in the range of 0.01-10nm. Since the wavelength of the incident radiation is comparable to the dimensions of the crystal atomic structure, the incident beam is diffracted. The interferences created by the diffracted beams can then be analysed to determine the elements present in the molecule, their position and the length and angle of their bonds.

Despite this valuable information, the analytical method is expensive and requires the growth of a sizeable pure crystal of the substance under analysis, which in itself can be problematic. For this reason, X-ray crystallography has only been used to characterize the 3D structure of novel compounds.
2.4 Mass spectrometry (MS)

MS studies the molecular weight of the molecule and provides information about the fragmentation processes which occur when a molecule is disrupted by ionization. Charged molecules and fragment ions produced from a sample exposed to ionizing conditions are accelerated by an electric field and separated according to their mass-to-charge ratio in the presence of a magnetic field. By analysing the mass-to-charge ratio of the molecule and its fragments, it is possible to infer the exact composition of the compound, i.e. number of atoms of each element present in the molecule.\textsuperscript{104} MS has been used in this thesis to help determining the composition of unknown compounds and as a detection method in gas chromatography experiments (GC-MS). An example of MS spectrum is shown in Figure 21. In the figure is shown the spectrum of the actual compound synthesised in the lab, above, and below is shown the theoretical spectrum from the correspondent composition. The error between our point and the theoretical one is -2.27 ppm. Error between -4 and 4 ppm is required to confirm the composition.

Figure 21: MS spectrum of Probe 3 (Introduced in chapter 4).
3 Plasma-driven Ozonolysis

As discussed in chapter 1.3.2, ozonolysis is the reaction between ozone and a double bond. Using the surface plasma system described in chapter 1.3.3, air plasmas have been used to trigger ozonolysis of several alkenes. The seven reactions considered in this study are shown in Scheme 17.

Although the ozonolysis reactions in Scheme 17 can in principle be used to detect ozone, the yields obtained were not very high for the alkenes to be sensitive ozone probes. More importantly the analysis of the resulting products required complex analytical techniques that would not be readily available in plasma laboratories. For this reasons, these compounds were not pursued as chemical ozone probes.

 Nonetheless, the results presented in this chapter indicate that a plasma-driven ozonolysis reactor is feasible and that both batch and continuous systems could be developed.

Scheme 17 Alkenes used as starting material in air plasma-driven ozonolysis reactions and the intended products. Acetone was used as solvent and Ph3P as reducing agent after plasma exposure. The alkenes are: trans-stilbene (1), 1,2-dihydro-4-phenylnaphthalene (3), 4-methoxystyrene (5), 4-tert-butylecyclohe-1-enylbenzene (7), styrene (9) and 1-methoxy-4-(prop-1-en-1-yl)benzene (10) and phenanthrene (11).
3.1 Experiments in Batch-mode

In all reactions shown in Scheme 17, the desired product was obtained after exposure of alkene samples to an atmospheric-pressure air plasma and the conversion increased with increasing plasma treatment time. For example, for the case of 1,2-dihydro-4-phenynaphthalene (3), 100% conversion of 11.5 mg, 0.006 mmol, in a 37 cm$^2$ glass dish was observed after ~50 minutes (see Figure 22).

![Figure 22: Temporal evolution of the ozonolysis reaction of 1,2-dihydro-4-phenynaphthalene and of the temperature in the plasma chamber.](image)

Ozone thermal decomposition increases rapidly with temperature$^{106}$ and therefore, it is important to operate the plasma at low temperature for effective production of ozone. It is noted, however, that the plasma source used in this study was not optimized for ozone generation and no attempt was made to improve it as that was not within the scope of the thesis. Not only is important to keep the temperature low for effective ozone generation, but also to prevent accidents with potentially explosive intermediate compounds generated during the ozonolysis reaction. For this reason, the temperature in the plasma chamber was monitored with a thermocouple during the experiments. The temperature increased rapidly during the first few minutes and a steady state temperature of ~44°C was reached after ~15 minutes (see Figure 22). It is likely, however, that higher temperatures were reached near the mesh in the plasma itself. Humidity also decreases the stability of ozone but it was not measured in this study.
3.1.1 Rate-determining Step

To determine whether the ozonolysis reaction was limited by the amount of ozone generated in the plasma or the diffusion of the ozone to the liquid, experiments were performed in which samples with different open areas were exposed to the same plasma. It was observed that larger exposure area resulted in faster conversion (see Figure 23), indicating that the reaction was limited by the diffusion of ozone to the liquid rather than by the amount of ozone that was being generated. This suggests that active transport of the plasma afterglow to the liquid will result in faster conversion. This could be achieved, for example, by generating the plasma in a tube and having the plasma afterglow bubbled through the liquid. Besides an improved transport, flowing gas through the plasma would also help in keeping the temperature low and prevent “poisoning” of the plasma atmosphere.\textsuperscript{107} This poisoning occurs in closed reactors such as the one employed in this work as NO and NO\textsubscript{2} build up and catalytically quench oxygen atoms and destroy ozone molecules.

![Figure 23: Conversion versus exposure area for a 16 min plasma treatment.](image)

Each experiment was repeated at least 3 times and the standard deviation in the conversion percentage is indicated with error bars in Figure 23. A significant variability can be observed, and this is attributed to variations in the plasma even though the plasma source was left to cool and cleaned in between experiments. DBDs are known for their
variability and in our case this was further accentuated by the fact that the PTFE dielectric layer used in our devices failed after a few hours of operation. This required replacing the dielectric layer and live electrode, and since parts were cut and assembled by hand, variability in the operation of the plasma system was inevitable. Nevertheless a clear trend can be observed and it can be concluded that the ozonolysis was transport limited.

### 3.1.2 Yields

Although the initial alkenes could be completely consumed after the plasma treatment, this does not imply that they had undergone ozonolysis. To make sure that the desired products were obtained, TLC and $^1$H NMR spectroscopy were performed. Furthermore, to obtain quantitative yield information, $^1$H NMR spectra were normalised against the signal of a known amount of m-dimethoxybenzene (13, Figure 24) that was added to the NMR samples. The process followed here is similar to that followed by Wang, M. et al, who used p-dimethoxybenzene to quantify products by NMR spectroscopy.

![Figure 24: m-Dimethoxybenzene: used as reference material in NMR.](image)

In brief, NMR samples were prepared in deuterated chloroform that contained a known amount of m-dimethoxybenzene. m-dimethoxybenzene was directly added to the deuterated chloroform to minimise errors and the products obtained from the plasma treatments dissolved completely in this solution. This is important for the validity of the process.

To determine the actual amount of product obtained after the plasma treatment, the areas under different peaks in the NMR spectrum are compared. m-Dimethoxybenzene has a peak with a chemical shift of 3.73 ppm which integrates the 6 protons of the methoxy groups (see Figure 24). By comparing this signal (IntR) with the integration of a peak corresponding to one proton of the product (IntP), the actual amount of product in the sample (P) can be calculated as follows:
where \( R \) is the amount of reference material introduced in the sample. The equation above assumes that 1 mmol of hydrogen corresponds to 1 mmol of product. If this is not the case, the calculation can easily be adapted.

![Scheme 18: General conditions for ozonolysis for the reactions in Table 2.](image)

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Starting material structure</th>
<th>Plasma exposure</th>
<th>Yield</th>
<th>Literature Yield</th>
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<td>8.6%</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="structure_3" /></td>
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<td>21.1%</td>
<td>28% (^{109})</td>
</tr>
<tr>
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<td><img src="image" alt="structure_5" /></td>
<td>16min</td>
<td>33.0%</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><img src="image" alt="structure_7" /></td>
<td>16min</td>
<td>34.7%</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
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<td>2.6%</td>
<td>6.3% (^{103})</td>
</tr>
<tr>
<td>10</td>
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<td>84% (^{110})</td>
</tr>
<tr>
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<td>2.2%</td>
<td>70% (^{109})</td>
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</table>

30min*: Plasma operated at 50% duty cycle: 20ms ON and 20 ms OFF

Table 2: Yield of air plasma-driven ozonolysis reactions of different alkenes.
The yield is calculated by comparing the actual amount of product obtained in the process (P) with the maximum theoretical amount that could be obtained if all the starting material was converted to the desired product (T). Since the stoichiometry of the reaction is 1 mmol of starting material to 1 mmol of product, the theoretical amount of product is the same as the moles of starting material. The yield of the reaction in percentage is then given by 100xP/T. The yields of the 7 reactions shown in Scheme 17 are shown in Table 2. The mechanism and conditions are shown in Scheme 18.

The yields are consistently lower than those reported in the literature for conventional ozonolysis. This is in part attributed to the evaporation of starting material/product during the experiments. It was observed that during the experiments material deposited on the chamber walls and this was hard to collect. Although all the alkenes and aldehydes used in the experiments have a boiling point of at least 140 ºC, energetic particles from the plasma can favour the evaporation of the samples even though the sample remains at a much lower temperature.

In addition, it is also possible that aldehydes generated in the ozonolysis process undergo further reaction with plasma species, which would result in a reduction of the apparent yield of the reaction. This possibility is supported by the following result: when the ozonolysis product of the alkenes 5 and 10 (Scheme 17), 4-methoxybenzaldehyde, was exposed to plasma for 16 min, only 61% of the aldehyde was recovered despite the boiling temperature of the aldehyde being 248 ºC.

3.2 Experiments in Continuous-mode

Intermediate ozonides can be explosive and therefore it is of interest to examine the feasibility of an ozonolysis plasma system operated in flow. Such a system would prevent the accumulation of large amounts of dangerous material, reducing the risks and cost of the ozonolysis process. To that end, flow ozonolysis of 1,2-dihydro-4-phenynaphthalene (1) was carried out using a semipermeable Teflon AF-2400 tube that allows the passage of ozone but prevents the passage of the solution. A picture of the setup of the experiment is shown in Figure 25. The tube was placed inside the plasma chamber and the alkene was slowly injected at one end of the tube and collected at the other after having been exposed to the plasma species. The internal diameter of the tube used in this study was 0.6 mm, its length 80 cm, and the flow used in the experiment was set at 0.508 cm³/h. As a result, the
time the compound spent in contact with the plasma was 27 min and the contact surface 
\(\sim 15 \text{ cm}^2\).

The conversion in the flow experiment was \(\sim 8\%\) versus the 8.6% obtained in the batch 
experiment (Table 2). The exposure time required to obtain this conversion, however, was 
longer in the flow experiment (27 minutes vs 16 minutes) and it was performed with a 
larger contact area (15 cm\(^2\) vs 12.5 cm\(^2\)). The slower conversion in the flow experiment is 
attributed to the slow diffusion of ozone through the tube wall. Larger conversion is 
expected if the length of the tube is increased or the flow is decreased as these would result 
in longer exposure time to plasma to compensate for the slower diffusion through the tube 
wall. It is noted that starting material remained in the solution after the flow experiment, 
suggesting that larger yield could be obtained in the flow system.

Figure 25: Flow experimental setup.
4 Ozone Probes

Although ozonolysis reactions (chapter 3) can be used to determine the presence of ozone, the quantification of the resulting aldehyde is time consuming and requires specialised instruments (e.g. NMR) normally not available in a plasma laboratory. On the other hand, optical spectroscopic methods are routinely used to diagnose plasmas and therefore an absorbent or fluorescent probe could be easily adopted by the plasma community. Furthermore, absorbance and fluorescence methods are much more sensitive than NMR spectroscopy and of the two; fluorescence spectroscopy is more sensitive than UV-visible absorption spectroscopy.

A widely-used absorbance probe has been used in this work as a reference. Indigo Carmine, which is commercially available, absorbs light at 610 nm and reacts with ozone resulting in a colourless product that is transparent at that wavelength. The decline in absorbance at 610 nm can therefore be used to quantify ozone concentration. The rest of the probes synthesized in this work, however, were fluorescent probes. The first fluorescent probe we worked with was developed by Garner et al.\textsuperscript{67}, a dichlorofluorescein (DCF) derivative and it was claimed to be specific towards ozone. Then, three more fluorescent probes were developed in our laboratory. Those probes are also DCF derivatives as the first one. Finally, a similar probe of that developed by Tang, B. and co-workers\textsuperscript{68} and based on tricarbocyanine instead of DCF was synthesised and tested. In Figure 26, all these compounds used to quantified ozone are presented.
Figure 26: Ozone probes used during this project.
4.1 Indigo Carmine

5,5'-Indigodisulfonic acid sodium salt, also known as Indigo Carmine, is a derivative from Indigo dye, a dye originally extracted from plants such as *Indigofera tinctoria*. Nowadays, however, these compounds are mainly obtained by synthesis and they are commercially available and inexpensive.

Indigo has been used since ancient times. For example, there are evidences that Egyptians used this compound to tint fibres around 4000 years ago and the first description found of a method to dye with Indigo dates back to 700 BC. These dyes continue to be used nowadays and for example, both Indigo and Indigo Carmine are used as dyes to colour blue jeans. Other uses of Indigo Carmine include pH and redox indicators in chemistry, colorant in the food industry (E132 in Europe used for example in icing sugar), and ozone indicators.

The reaction of this blue compound with ozone gives an uncoloured product and therefore changes in colour or absorbance at a particular wavelength can be used to quantify ozone exposure. The reaction is shown in Scheme 19.

![Scheme 19: Scheme of the reaction between Indigo Carmine and ozone.](image)

**Figure 27** shows the results of a series of experiments in which the absorbance at 610nm of solutions with different concentrations (0.1-0.6 mM) of Indigo Carmine was measured. The relation between the absorbance at 610 nm and the concentration of Indigo Carmine is linear in the range investigated and the extinction coefficient ($\epsilon$) was determined to be 20103 M$^{-1}$ cm$^{-1}$, which agrees with that reported in the literature.
In Figure 28, the absorbance of Indigo Carmine is measured after the addition of different amounts of ozone. Ozone was generated in an ozone generator fed with oxygen and bubbled through pure water. The dissolved ozone concentration was determined by UV absorbance at 254 nm. As expected, the absorbance at 610 nm due to Indigo Carmine decreases with ozone concentration and a good linearity can be observed in this experiment. Therefore, it can be concluded that in the absence of any other reactive species, Indigo Carmine is a good probe for quantitative determination of ozone.

Despite these good properties, it will be shown later in chapter 5 that Indigo Carmine is not selective to quantify ozone and similar reactions take place with other ROS such as singlet oxygen.
4.2 Probe 1

The fluorescent probe we chose as a starting point was first developed by Garner et al. and it was chosen for its alleged ozone selectivity and sensitivity and the fact that it had also been used in biological studies. The probe was synthesised from 2',7'-dichlorofluorescein (DCF) by the two-step process used by the Koide group (Scheme 20 and Scheme 21). The first step was the addition of two molecules of 4-bromobutene to DCF (16) to synthesise but-3-en-1-yl 2-(6-(but-3-en-1-yloxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)-benzoate (17). This reaction had a 91% yield after 17 hours. In the second step (Scheme 21), the ester, 17, was reduced to the primary alcohol using DIBAL. After column chromatography and recrystallization with hexanes, the desired final product, i.e. the probe, 18, was synthesised. Some problems were encountered in the second step when separating the product from impurities. On reading other papers from Koide and co-workers with a similar reaction, we realised that the amount of DDQ after the reduction could be the problem since they have reported a 25 fold less amount of this compound for a similar reaction. After repeating the reaction with less DDQ we obtained improved results: better yield and more purity. For this reaction, the best yield after column chromatography and recrystallization has been 58%.

![Diagram](attachment:image.png)

Scheme 20: First step of the synthesis of the Koide’s fluorescent probe.
Absorbance and fluorescence spectra of 18 were measured and compared with the ones reported in the literature to validate the synthesis process. The probe does not have absorbance in the visible spectrum and it is nearly non fluorescent, as reported by Garner et al.\(^6\)\(^7\) The \(^1\)H NMR spectrum and the melting point (169-170\(^\circ\)C) also indicated that 18 had been synthesised. Optical and NMR spectra of this compound can be found in the experimental section (chapter 9).

Once the probe was synthesised, it was exposed to a He+O\(_2\) plasma (99.5:0.5). Probe 1 had been reported to produce 19, see Scheme 22, in the presence of ozone in quantitative yield.\(^6\)\(^7\) After a 30-min plasma treatment, however, \(^1\)H NMR analysis showed that reaction had taken place but multiple compounds were being generated. This early result suggested that the probe is not selective enough to be used to characterise plasmas. Although undesirable, the result was not surprising because it is known that oxygen containing plasmas produce a large number of oxidizing species. Nevertheless, to narrow down the possible reactions, an ozone generator was used to limit the number of reagents the probe could react with. Surprisingly, the \(^1\)H NMR of samples exposed to ozone from an ozone generator showed similar spectra as the ones that had been exposed to plasma. Two ozone generators (Ozone Generator 500MM, Fischer and Ambiox, Ozone Generators & Systems) were used and similar results were obtained with both devices. Ozone generators were fed with pure oxygen. The results with the ozone generators questioned our understanding of the chemical probe and triggered further analysis. It is noted that although similar results were obtained with both ozone generators, the Ambiox device is more powerful than the Fischer device and required ~10x shorter treatment times to get similar results.

To isolate and analyse the products, the probe was exposed to ozone. TLC readings suggested that two main products were being generated when the probe was exposed to ozone. NMR spectra also indicated the presence of multiple compounds despite the quantitative yield reported in the literature.\(^6\)\(^7\)
Although the TLC plate suggested two main byproducts, three products are obtained after separation of the mixture by column chromatography:

1. Product (19): This is the expected product. Evidence of the structure of the product was given by comparing $^1$H NMR, MS, absorbance and fluorescence with the data showed by Garner. This data can be found in chapter 9.

2. Product (20): This product always comes out the column chromatography with the expected product (19).

3. Product (16): Evidence of the generation of DCF was given by TLC, comparing commercial DCF with the obtained byproduct. Both of them have a retention factor $R_f= 0.32$ (50% EtOAc in petroleum). $^1$H NMR spectra were also identical and high-resolution mass spectrometry showed a peak at 422.9792 when m/z calculated for $C_{20}H_{10}O_5Cl_2$ [Na+H]$^+$ is 422.9798. Fluorescence of both products was also measured giving $\lambda_{em}= 522$ and 523 nm for commercial DCF and our product respectively ($\lambda_{ex} = 502$ nm). The reaction was repeated several times obtaining similar results.

Scheme 22: Reaction between Probe 1 and ozone and product from ozone overexposure.

Monitoring the reaction using TLC made it possible to stop the reaction just when all the probe is consumed. In this way, a possible over oxidation of the probe could be avoided. In experiments with shorter ozone exposure, DCF was not observed and only 19 and small traces of product 20 were found in the NMR spectra. On the other hand, overexposure of the probe to ozone results in abundant DCF generation, which suggests that in the absence of probe material, ozone oxidises 19 to yield DCF. This is indicated in Scheme 22.
It is noted, that DCF 16 was also observed after the He+O₂ DBD plasma treatment. With this plasma, however, DCF was being generated even when there was probe remaining suggesting that oxidation of 19 was triggered by a ROS other than ozone or the solution was not well stirred. To further study this fact, the plasma jet introduced in section 1.3.3 was used. After the treatment with the plasma jet, DCF was not detected if there was probe still in the mixture. This fact indicated that the problem with the DBD plasma was the way of stirring or the amount of solvent.

It has been observed that product 19 leads to 20 when 19 is left for 20 days dissolved in methanol in a glass flask. The resulting compound has a similar structure to 19 according to the NMR spectrum since both of them have the same number of hydrogen atoms. On the other hand, if this product is stored in the dark, no degradation is observed after 20 days. The decomposition of 19 also seems to be catalysed by silica, as performing a chromatography column or stirring the product with silica bids for 24h results in the formation of 20. This explains the presence of 3 main compounds after the column even though the TLC plate suggests the presence of only 2 compounds. It has also been observed that the product 19 decomposes in the silica of the TLC plates and the spots disappear within 24h (evaporation of the product cannot explain this since the melting point of the product is 270 ºC).

After numerous experiments with the product of Probe 1 after ozonolysis, it was observed that the two products obtained were in equilibrium. The fact that the ratio of both products changed depending on the treatment indicates that both of them exist as a mixture of ring-opened (19) and ring-closed (20) compounds, see Scheme 23. Fortunately, on two occasions we were able to gain almost pure samples of both compounds. Due to this, it has been possible to analyse and characterise 19 and 20. In general, however, we often observed a mixture of the two.

Scheme 23: Equilibrium between products 19 and 20.
4.3 Probe 2

The reaction between Probe 1 and ozone works well, but it has some drawbacks: a mixture of two compounds in equilibrium obtained after ozone exposure and reaction of the ozonolysis product with ozone resulting in formation of DCF (16). Under plasma conditions, DCF is obtained even while there is Probe 1 still in the mixture, which represents an important drawback for ozone quantification in plasmas.

Due to those drawbacks, we started to work on synthesising a new probe that would yield an oxidation resistant fluorescent product. Since one of the main issues from Probe 1 was the DCF obtained after ozone overexposure and even during the exposure with plasma, we conceptualized a probe that is a DCF derivative that yields directly DCF after ozone exposure. This can be achieved if the oxidation state of the alcohol group in Probe 1 is changed to a carboxylic acid.

In order to synthesise the desired compound, Probe 2, we started from DCF. The first step of the synthesis is the addition of two molecules of 4-bromobutene to dichlorofluorescein as for Probe 1. In the second step, sodium hydroxide or lithium hydroxide was used to carry out the basic hydrolysis of the ester resulting from the first step. This second step leads to Probe 2, compound 21. Different conditions were tested until satisfactory results were obtained, see Table 3. The best yield obtained was 79% using lithium hydroxide as base. The synthesis of Probe 2 is shown in Scheme 24.

Scheme 24: Synthesis of Probe 2, 21.
<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Eq.</th>
<th>Solvent</th>
<th>Time</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaOH</td>
<td>100</td>
<td>H₂O</td>
<td>30 min</td>
<td>17</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>2</td>
<td>NaOH</td>
<td>200</td>
<td>H₂O:MeOH (1:1)</td>
<td>90 min</td>
<td>21</td>
<td>&lt;15%</td>
</tr>
<tr>
<td>3</td>
<td>NaOH</td>
<td>15</td>
<td>H₂O:1,4-dioxane (3:7)</td>
<td>90 min</td>
<td>21</td>
<td>&lt;15%</td>
</tr>
<tr>
<td>4</td>
<td>LiOH</td>
<td>3</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>40 min</td>
<td>21</td>
<td>26%</td>
</tr>
<tr>
<td>5</td>
<td>LiOH</td>
<td>2</td>
<td>H₂O:1,4-dioxane (1:1)</td>
<td>60 min</td>
<td>21</td>
<td>52%</td>
</tr>
<tr>
<td>6</td>
<td>LiOH</td>
<td>1</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>400 min</td>
<td>unfinished</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>LiOH</td>
<td>1.5 + 0.3</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>240 min</td>
<td>21</td>
<td>72%</td>
</tr>
<tr>
<td>8</td>
<td>LiOH</td>
<td>1.6</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>40 min</td>
<td>21</td>
<td>72%</td>
</tr>
<tr>
<td>9</td>
<td>LiOH</td>
<td>1.6</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>40 min</td>
<td>21</td>
<td>79%</td>
</tr>
<tr>
<td>10</td>
<td>LiOH</td>
<td>4.2</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>45 min</td>
<td>21 +22</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>LiOH</td>
<td>4 + 2</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>90 min</td>
<td>21 + 22</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>LiOH</td>
<td>6</td>
<td>H₂O:1,4-dioxane (1:2)</td>
<td>90 min</td>
<td>22</td>
<td>37%</td>
</tr>
</tbody>
</table>

Table 3: Second step for the synthesis of Probe 2.

An important amount of an unexpected product (22) was generated during the synthesis of Probe 2 (21), Scheme 24. This new compound was obtained especially when a large excess of the base was added to the mixture. Six equivalents of lithium hydroxide lead to the highest yield achieved of this compound (37%) (see Table 3).

Probe 2 is expected to react with ozone giving an ozonide which would yield directly DCF. DCF is well-known to be fluorescent and DCF derivatives are non-fluorescent when the phenolic hydroxyl group is alkylated\(^{113}\). We also know that DCF does not show the keto-enol tautomerism in these conditions since we have been using it for earlier experiments.

After testing the probe 2 (21) with ozone generated by an ozone generator and triethylamine (Scheme 25), it was verified that indeed DCF is obtained. Although the triethylamine step is not required for DCF to form, it was found that for both probe 1 and probe 2, triethylamine promotes cleavage of the side chain driving the reaction faster to completion. After ozone overexposure, DCF is still the main compound found by \(^1\)H NMR analysis. DCF decomposes when exposed to ozone slower than probes 1 and 2. It is noted that the addition of triethylamine needs to be subsequent to the ozone exposure as the presence of the base quenches ozone and prevents the ozonolysis of the probe.

Probe 2 is more fluorescent than Probe 1 but once exposed to ozone, the fluorescence of the product, DCF, is similar to the product obtained from probe 1. The maximum absorbance of DCF occurs at a wavelength of 502 nm, and the maximum fluorescence when illuminated at 502nm is at 522 nm.
As shown in Scheme 24, during the synthesis of Probe 2, a byproduct was generated which also has the butene moiety attached to the aromatic phenol. Since we had the isolated product and it would react with ozone we decided to investigate its potential as an ozone probe. Ozone reacts with the double bond as it does with the other two probes, resulting in the loss of the moiety attached to the oxygen. But in this case, neither the byproduct nor the ozonolysis product were fluorescent and therefore were no longer considered them for ozone quantification.

### 4.4 Probe 3

With the idea of having more probes to compare and to choose from, other DCF derived probes were conceptualized and synthesized. We decided to synthesise a new probe in which, instead of having an alcohol or carboxylic acid attached to the aromatic ring on the top, we will have a methyl ester.
In order to synthesise the methyl ester probe, four possible ways were considered, see **Scheme 26**: With methanol in acidic conditions or sodium methoxide starting from the butene ester (17), methyl iodide in basic conditions from Probe 2, or using diazomethane. The two options starting from Probe 2 are based in the possible equilibrium of this compound with the ring-opened structure, see **Scheme 27**.

The first attempt for the synthesis was adding iodomethane and potassium hydroxide to Probe 2, **Scheme 28**. Probe 2 dissolved in DMF was mixed with iodomethane in the presence of potassium hydroxide under nitrogen.

The reaction did not work as expected due to the fact that the lactone, Probe 2, was not in equilibrium with the carboxylic acid; i.e. compound (21’, Scheme 27) was not present in the mixture. For this reason the methyl iodide reacted directly with the phenoxy group.

Since Probe 2 as starting material did not yield the desired compound, we decided to try with another reaction. The next starting material we used was an intermediate synthesized during the synthesis of Probe 1 (17). For doing the transesterification, the initial ester was dissolved in MeOH and the mixture was heated under reflux. Unfortunately this was not successful either as the starting material remained intact.
Sulphuric acid was then added to the mixture to catalyse the reaction. With the acidic conditions a range of products were obtained, as shown in Scheme 29. These included the desired product (methyl ester), Probe 4 (compound 26) and a number of products in which the methyl group from methanol was incorporated in different parts of the molecule.

The large number of products obtained in the reaction made the yield low and complicated the purification steps. In addition, the reaction does not go to completion due to the equilibrium between the two esters even though an important excess of methanol was used. Therefore an alternative synthesis process was investigated.

Scheme 29: Reaction of the butene ester (17) in methanol catalysed with acid and main products obtained.

Sodium metoxide was used to do the transesterification starting from the ester (17), Scheme 30. The sodium methoxide was generated in the laboratory just before the reaction to make sure it was fresh and ready for the reaction. A solution of NaOMe 1M was prepared by dissolving 230 mg of Na (10 mmol) in 10 mL of dry MeOH. Sodium was weighed in hexane and subsequently poured directly with the methanol to avoid the reaction of sodium with the water in the environment. This solution was added to the ester. Under these conditions, the reaction leads to the desired product with an excellent yield, 94% after column chromatography.

Unfortunately the product contained impurities even after purification. Recrystallization of the solid was carried out using different solvents: DCM, EtOAc and
MeOH but the impurities remained with the product in all cases. Using $^1$H NMR line ratios, the purity of the product was estimated to be 92%.

Scheme 30: Synthesis of Probe 3.

In order to test the new probe, Probe 3 was exposed to ozone. Triethylamine was added after ozone exposure as before and fluorescence of the product was measured. The product turned from nearly non-fluorescent to highly fluorescent after ozonolysis proving that the expected reaction took place, see Scheme 31. The fluorescent product has its peak of absorption at 507 nm and the maximum of emission at 525 nm when excited at 507 nm.

Scheme 31: Ozonolysis of Probe 3.

4.5 Probe 4

The last DCF derivative exposed to ozone in this project was the one obtained accidentally in the first attempt to synthesise Probe 3. The reaction between Probe 2 and iodomethane in the presence of potassium hydroxide leads to the addition of the methyl group to the oxygen in the phenol group, Scheme 32.
This new compound has two alkyl groups attached to the phenolic hydroxyl groups. For this reason it is expected to be less fluorescent than the other probes synthesised before which have just one of the hydroxyl groups alkylated.

In Table 4, the most representative attempts to synthesise Probe 3 and Probe 4 are listed.

<table>
<thead>
<tr>
<th>Entry</th>
<th>SM</th>
<th>Solvent</th>
<th>Reagent</th>
<th>Eq.</th>
<th>Temp.</th>
<th>Time</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>DMF</td>
<td>MeI/KOH</td>
<td>1.5/1.5</td>
<td>20º C</td>
<td>2 h</td>
<td>21</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>DMF</td>
<td>MeI/KOH</td>
<td>3.0/3.0</td>
<td>20º C</td>
<td>24 h</td>
<td>26</td>
<td>81%</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>DMF</td>
<td>MeI/KOH</td>
<td>3.0/3.0</td>
<td>20º C</td>
<td>3 h</td>
<td>26</td>
<td>90%</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>MeOH</td>
<td>-</td>
<td>-</td>
<td>reflux</td>
<td>5 h</td>
<td>17</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>MeOH</td>
<td>H₂SO₄</td>
<td>50</td>
<td>reflux</td>
<td>20 h</td>
<td>25</td>
<td>40%</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>MeOH</td>
<td>H₂SO₄</td>
<td>200</td>
<td>reflux</td>
<td>45 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>MeOH</td>
<td>H₂SO₄</td>
<td>20</td>
<td>reflux</td>
<td>22 h</td>
<td>25</td>
<td>40%</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>MeOH</td>
<td>H₂SO₄</td>
<td>10</td>
<td>reflux</td>
<td>17 h</td>
<td>25</td>
<td>43%</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>MeOH</td>
<td>NaOMe</td>
<td>1</td>
<td>50º C</td>
<td>90 min</td>
<td>25</td>
<td>94%</td>
</tr>
</tbody>
</table>

Table 4: Synthesis of Probes 3 and 4, compounds 25 and 26, respectively.

Probe 4 was exposed to ozone and treated with triethylamine, as shown in Scheme 33, to afford the ozonolysis product (compound 31). Fluorescence was measured after the reaction to confirm that was being generated.

Scheme 32: Synthesis of Probe 4.

Scheme 33: Ozonolysis of Probe 4.
This compound, Probe 4, is non-fluorescent. Unlike the other dichlorofluorescien derivatives used as ozone probes during this work, Probe 4 has a methoxy group attached to an aromatic ring instead of a hydroxyl group. This fact affects to the fluorescence of the probe, especially to the fluorescence of the product. The product after ozonolysis is slightly fluorescent while the other 3 probes described above lead to highly fluorescent products. The product has its maximum absorption at 485 nm and emits at 518 nm.

4.6 Probe 5

With the purpose of having a non-DCF derivative probe to compare with the previously synthesised ones, Trp-Cy, mentioned in the introduction was chosen. At the time of preparing the reaction, we realised that it would be easier to start from a commercially available compound (Cy). This chemical, instead of an ethyl group, has a propyl moiety attached to both nitrogen atoms, see Figure 29. We decided to use this similar chemical since this part of the molecule does not affect to its optical properties and it should not affect to the reactivity with ozone. Furthermore, it would make the synthesis much more effective.

![Chemical structure](image)

Figure 29: Chemical used to synthesise the ozone probe by Tang and co-workers, on the left and the commercially available compound used to synthesise Probe 5, Cy.

Compound 33 was mixed with 34 in DMF and H₂O in order to synthesise the desired product (Probe 5). See Scheme 34.
We did not expect the reaction to work very well since the yield reported by Tang and co-workers is 35%. Different conditions were applied to the reaction between Cy and L-tryptophan until satisfactory results were obtained, see Table 5.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>Eq. of Trp</th>
<th>Temp.</th>
<th>Atm.</th>
<th>Time</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMF/H₂O</td>
<td>1.6</td>
<td>70°C</td>
<td>N₂</td>
<td>20 h</td>
<td>33</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>1.6</td>
<td>150°C</td>
<td>Air</td>
<td>6 h</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>DMF/H₂O</td>
<td>1.6</td>
<td>80°C</td>
<td>N₂</td>
<td>24 h</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>DMF/H₂O</td>
<td>1.6</td>
<td>100°C</td>
<td>Air</td>
<td>20 h</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>1.6</td>
<td>100°C</td>
<td>N₂</td>
<td>35 h</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>DMF/H₂O</td>
<td>8</td>
<td>80°C</td>
<td>N₂</td>
<td>18 h</td>
<td>33 + 36</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>DMF/H₂O</td>
<td>1.6</td>
<td>80°C</td>
<td>Ar</td>
<td>18 h</td>
<td>33 + 36</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>DMF/H₂O</td>
<td>1.6</td>
<td>80°C</td>
<td>N₂</td>
<td>18 h</td>
<td>33 + 36</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>DMF/H₂O</td>
<td>7</td>
<td>80°C</td>
<td>N₂</td>
<td>24 h</td>
<td>33 + 36</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>DMF/H₂O</td>
<td>10</td>
<td>80°C</td>
<td>N₂</td>
<td>65 h</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>DMF/H₂O</td>
<td>10</td>
<td>80°C</td>
<td>Ar</td>
<td>65 h</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>DMF/H₂O</td>
<td>10</td>
<td>80°C</td>
<td>Ar</td>
<td>24 h</td>
<td>36</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>13</td>
<td>DMF/H₂O</td>
<td>10</td>
<td>80°C</td>
<td>N₂</td>
<td>16 h</td>
<td>33 + 36</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>DMF</td>
<td>10</td>
<td>80°C</td>
<td>N₂</td>
<td>4 h</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>DMF/H₂O</td>
<td>10</td>
<td>75°C</td>
<td>N₂</td>
<td>48 h</td>
<td>36</td>
<td>10%</td>
</tr>
<tr>
<td>16</td>
<td>DMF/H₂O</td>
<td>5</td>
<td>80°C</td>
<td>Ar</td>
<td>26 h</td>
<td>35 + 36</td>
<td>14%/9%</td>
</tr>
<tr>
<td>17</td>
<td>DMF/H₂O</td>
<td>20</td>
<td>80°C</td>
<td>Ar</td>
<td>16 h</td>
<td>35 + 36</td>
<td>33%/13%</td>
</tr>
</tbody>
</table>

Table 5: Synthesis of Trp-Cy probe.
The reaction was carried out under nitrogen conditions at the beginning and only starting material was recovered from the chromatography. The temperature was increased and the inert atmosphere was removed obtaining not product at all even though all the starting material disappeared. The reaction was then carried out in N₂ again and at lower temperatures obtaining some product in one of the attempts, but the crudes of the reaction were very dirty. After four unsuccessful attempts with N₂, the reaction was done under argon. ¹H NMR of the crude looked cleaner when an Ar atmosphere was used. However, a byproduct was being synthesised in the same reaction, see Scheme 35. This compound was isolated and characterised. It is the result of the addition of formaldehyde with Cy.

![Scheme 35: Scheme of the generation of the main byproduct in the synthesis of Probe 5.](image)

The equivalents of tryptophan were increased in order to favour the desired reaction. Finally, 33% yield was obtained using 20 equivalents of Trp, Ar atmosphere at 80 ºC. This yield, while low, is comparable with the yield obtained in the literature.

Probe 5 was exposed to ozone and the product after ozonolysis was isolated and characterised. Unexpectedly, the reaction did not happen as anticipated (Scheme 36) and no fluorescent product was observed when ozone was bubbled through a solution containing the probe. To make sure that the product was not being destroyed by ozone overexposure, ozone was also first dissolved in water and measured by UV absorption at 260 nm and then added to Probe 5 in a proportion 1:1. Even in this case, fluorescent product was not observed and the ¹H NMR did not show the expected product.
Scheme 36: Ozonolysis of Probe 5.
5 Selectivity Studies

The probes introduced in chapter 4 were exposed to ozone and other RONS in order to assess their selectivity. The results of these experiments are reported in this chapter. DCF, being the product after ozonolysis of Probe 2, was also exposed to study its stability in the presence of ozone and other reactive species. Probes were also overexposed to ozone to study the degradation after the ozonolysis and the kinetics of the ozonolysis reactions of Probe 1 and 2 were studied by exposing the probes to ozone for different periods of time.

5.1 Source of reactive species

Some of the most relevant reactive species generated in oxygen containing plasmas and also in biological environments were considered in the selectivity studies. All the probes were exposed to ozone (O₃), singlet oxygen (¹O₂), superoxide radical (O₂⁻), hydroxyl radical (OH), hydrogen peroxide (H₂O₂), hypochlorite (ClO⁻) and peroxynitrite (ONOO⁻). Optical measurements were taken after the treatments to study possible interferences in ozone analysis.

These reactive species are in general unstable and short lived. Some of them such as hydrogen peroxide and sodium hypochlorite are commercially available in aqueous solution using stabilizers. Solutions of 35% and 14%, respectively, were used in this study. The rest of the reactive species, however, have to be generated \textit{in situ}.

Singlet oxygen can be generated by the reaction between hydrogen peroxide and molybdate ion. In basic conditions, hydrogen peroxide reacts with molybdate ion resulting in water and diperoxomolybdate. This ion provides singlet oxygen in quantitative yield. The process is shown in Scheme 37.

\[
2 \text{H}_2\text{O}_2 \quad \text{MoO}_4^{2-} \quad \rightarrow \quad 2 \text{H}_2\text{O} \quad \text{MoO}_6^{2-} \\
\text{MoO}_6^{2-} \quad \rightarrow \quad \text{MoO}_4^{2-} \quad ¹\text{O}_2
\]

\textbf{Scheme 37:} Generation of singlet oxygen from hydrogen peroxide and sodium molybdate.\textsuperscript{114}
Superoxide anions were obtained from potassium superoxide. This salt contains K⁺ and O₂⁻ ions linked by ionic bonds and in solution the superoxide radical is released. Potassium superoxide is soluble in DMSO but it reacts in water resulting in potassium hydroxide and oxygen. Potassium superoxide should be handled with care as it can undergo explosive reactions with a variety of substances including water, acids and organics.

The well-known Fenton reaction was used to generate hydroxyl radical. Iron sulphate heptahydrated and hydrogen peroxide are mixed in equal concentrations resulting in Iron (III), the desired hydroxyl radical and water, see Scheme 38.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

Scheme 38: Fenton reaction.

Peroxynitrite anion can be generated by the reaction between acidified hydrogen peroxide with sodium nitrite. Sodium hydroxide is added immediately after the nitrite in order to stabilise the solution. The concentration of the ONOO⁻ in the resulting solution is calculated by measuring absorbance at 302 nm (ε = 1670 M⁻¹ cm⁻¹). Peroxynitrite anion can be generated by the reaction between acidified hydrogen peroxide with sodium nitrite. Sodium hydroxide is added immediately after the nitrite in order to stabilise the solution. The concentration of the ONOO⁻ in the resulting solution is calculated by measuring absorbance at 302 nm (ε = 1670 M⁻¹ cm⁻¹).116

Ozone was generated by feeding high purity oxygen into an AMBIO3X, Ozone Generator. The ozone coming from the ozone generator was bubbled through pure water at 0 °C for 10 min and the concentration of dissolved O₃ was determined by UV absorbance at 260 nm (ε = 2900 M⁻¹ cm⁻¹).94 The concentration of ozone reached in these solutions is around 25 to 30 µM. It is noted that the ozonated water needs to be used immediately due to the rapid loss of ozone (around 5 µM during the time of the additions, which is less than 3 min.)

5.2 Preparation of stock solutions

Stock solutions were prepared to facilitate the selectivity studies. Table 6 lists all the stock solutions prepared for these studies. The solutions were prepared and used within 2 months except for the solutions containing hydrogen peroxide and sodium hypochlorite, which were prepared and used in the same day. Ozone and peroxynitrite solutions were prepared just before they were needed in the experiments.
### Table 6: Preparation of the stock solutions for selectivity studies. Conc. is the final concentration of the stock solution.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reagent</th>
<th>MW (mol/L)</th>
<th>Quantity</th>
<th>Solvent</th>
<th>V (mL)</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Indigo Carmine</td>
<td>466.35</td>
<td>4.6393 mg</td>
<td>DMSO</td>
<td>1.990</td>
<td>5 mM</td>
</tr>
<tr>
<td>B</td>
<td>Probe 1</td>
<td>441.30</td>
<td>1.1438 mg</td>
<td>DMSO</td>
<td>2.590</td>
<td>1 mM</td>
</tr>
<tr>
<td>C</td>
<td>Probe 2</td>
<td>455.29</td>
<td>0.9001 mg</td>
<td>DMSO</td>
<td>1.980</td>
<td>1 mM</td>
</tr>
<tr>
<td>D</td>
<td>Probe 3</td>
<td>468.05</td>
<td>1.1618 mg</td>
<td>DMSO</td>
<td>2.480</td>
<td>1 mM</td>
</tr>
<tr>
<td>E</td>
<td>Probe 4</td>
<td>468.05</td>
<td>0.8902 mg</td>
<td>DMSO</td>
<td>1.900</td>
<td>1 mM</td>
</tr>
<tr>
<td>G</td>
<td>Compound 11</td>
<td>509.38</td>
<td>0.9815 mg</td>
<td>DMSO</td>
<td>1.925</td>
<td>1 mM</td>
</tr>
<tr>
<td>H</td>
<td>DCF</td>
<td>401.20</td>
<td>0.8329 mg</td>
<td>DMSO</td>
<td>2.075</td>
<td>1 mM</td>
</tr>
<tr>
<td>I</td>
<td>NaMoO₄·2H₂O</td>
<td>241.95</td>
<td>6.27 mg</td>
<td>pH 10 BS</td>
<td>2.590</td>
<td>10 mM</td>
</tr>
<tr>
<td>J</td>
<td>NaMoO₄·2H₂O</td>
<td>241.95</td>
<td>45.63 mg</td>
<td>pH 10 BS</td>
<td>1.885</td>
<td>100 mM</td>
</tr>
<tr>
<td>K</td>
<td>KO₂</td>
<td>71.10</td>
<td>1.24 mg</td>
<td>DMSO</td>
<td>1.745</td>
<td>10 mM</td>
</tr>
<tr>
<td>L</td>
<td>KO₂</td>
<td>71.10</td>
<td>16.09 mg</td>
<td>DMSO</td>
<td>2.265</td>
<td>100 mM</td>
</tr>
<tr>
<td>M</td>
<td>FeSO₄·7H₂O</td>
<td>278.02</td>
<td>6.99 mg</td>
<td>H₂O</td>
<td>2.515</td>
<td>10 mM</td>
</tr>
<tr>
<td>N</td>
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<td>48.36 mg</td>
<td>H₂O</td>
<td>1.740</td>
<td>100 mM</td>
</tr>
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<td>O</td>
<td>H₂O₂ 35 %</td>
<td>34.02</td>
<td>10.0 mL</td>
<td>H₂O</td>
<td>-</td>
<td>11.6 M</td>
</tr>
<tr>
<td>P</td>
<td>H₂O₂ (N)</td>
<td>34.02</td>
<td>1.0 mL</td>
<td>H₂O</td>
<td>9.0</td>
<td>1.16 M</td>
</tr>
<tr>
<td>Q</td>
<td>H₂O₂ (O)</td>
<td>34.02</td>
<td>1.0 mL</td>
<td>H₂O</td>
<td>9.0</td>
<td>0.116 M</td>
</tr>
<tr>
<td>R</td>
<td>H₂O₂ (P)</td>
<td>34.02</td>
<td>1.0 mL</td>
<td>H₂O</td>
<td>9.0</td>
<td>0.012 M</td>
</tr>
<tr>
<td>S</td>
<td>NaOCl 14%</td>
<td>74.44</td>
<td>25 μL</td>
<td>H₂O</td>
<td>4.975</td>
<td>10 mM</td>
</tr>
<tr>
<td>T</td>
<td>NaOCl (R)</td>
<td>74.44</td>
<td>250 μL</td>
<td>H₂O</td>
<td>4.750</td>
<td>100 mM</td>
</tr>
<tr>
<td>U</td>
<td>H₂O₂ 35 %</td>
<td>34.02</td>
<td>0.516 mL</td>
<td>H₂O</td>
<td>9.484</td>
<td>0.6 M</td>
</tr>
<tr>
<td>V</td>
<td>HCl 35 %</td>
<td>36.46</td>
<td>0.618 mL</td>
<td>H₂O</td>
<td>9.382</td>
<td>0.7 M</td>
</tr>
<tr>
<td>W</td>
<td>NaNO₂</td>
<td>69.00</td>
<td>0.414 g</td>
<td>H₂O</td>
<td>10</td>
<td>0.6 M</td>
</tr>
<tr>
<td>X</td>
<td>NaOH</td>
<td>40.00</td>
<td>12 g</td>
<td>H₂O</td>
<td>100</td>
<td>3 M</td>
</tr>
</tbody>
</table>

5.3 **Preparation of final solutions**

Since fluorescence analysis are more sensitive than absorbance analysis, two different processes are needed for the selectivity experiments: one of them with higher amounts of the probe and reactive species will be applied to Indigo Carmine, whose concentration is measured by absorbance spectroscopy, and the other process, with smaller amounts, will be applied to the fluorescent probes.
Process 1 below explains the preparation of the solutions for the Indigo Carmine’s selectivity study (Entry A in Table 6) whereas process 2 describes the process for the rest of compounds under investigation (entries B-H in Table 6).

### 5.3.1 Process 1: Selectivity study of Indigo Carmine

Blank: **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Ozone: O3 aq. (30 µM (2.0 mL), [O3]final= 12 µM) was added to **A** (50 µL, [IC]final= 50 μM). pH 7 PBS (3.0 ml) were added to the solution.

Singlet Oxygen: **J** (15 µL, [NaMoO4·2H2O]final= 300 µM) and **O** (5 µL, [H2O2]final= 11.6 mM) were added to **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (3.0 ml) were added to the solution.

Superoxide: **L** (15 µL, [KO2]final= 300 μM) was added to **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (3.0 ml) were added to the solution.

Hydroxyl radical: **N** (15 µL, [FeSO4·7H2O]final= 300 µM) and **Q** (15 µL, [H2O2]final= 350 µM) were added to **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (3.0 ml) were added to the solution.

Hydrogen peroxide: **O** (10 µL, [H2O2]final= 23.2 mM) was added to **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (3.0 ml) were added to the solution.

Hypochlorite: **T** (15 µL, [NaOCl]final= 300 µM) was added to **A** (50 µL, [IC]final= 50 μM). H2O (0.4 mL) and pH 7 PBS (3.0 ml) were added to the solution.

Peroxynitrite: ONOO⁻ (700 µM (2.0 mL), [ONOO]final= 280 µM) was added to **A** (50 µL, [IC]final= 50 μM). H2O (0.15 mL) and pH 7 PBS (3.0 ml) were added to the solution.

### 5.3.2 Process 2: Selectivity study of fluorescent probes and products

Blank: **B-H** (5 µL, [probe]final= 1 μM). H2O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Ozone: O3 aq. (20 µM (0.2 mL), [O3]final= 0.8 µM) was added to **B-H** (5 µL, [probe]final= 1 μM). H2O (0.2 mL) and pH 7 PBS (4.6 ml) were added to the solution.
Singlet Oxygen: I (15 µL, [NaMoO₄·2H₂O]_{final}= 30 µM) and P (5 µL, [H₂O₂]_{final}= 1.16 mM) were added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Superoxide: K (15 µL, [KO₂]_{final}= 30 µM) was added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Hydroxyl radical: M (15 µL, [FeSO₄·7H₂O]_{final}= 30 µM) and R (15 µL, [H₂O₂]_{final}= 35 µM) were added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Hydrogen peroxide: P (10 µL, [H₂O₂]_{final}= 2.32 mM) was added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Hypochlorite: S (15 µL, [NaOCl]_{final}= 30 µM) was added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.4 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Peroxynitrite: ONOO⁻ (700 µM (0.25 mL), [ONOO\^-]_{final}= 35 µM) was added to B-H (5 µL, [probe]_{final}= 1 µM). H₂O (0.15 mL) and pH 7 PBS (4.6 ml) were added to the solution.

Selectivity graphs from the different probes and also DCF treated with RONS are shown in Figure 30 to Figure 32. The concentrations of the reactive species added for Process 1, Indigo Carmine (IC), are: [O₃]= 12 µM, [¹⁰₂]= [O₂]= [OH]= [OCl⁻]= 300 µM, [H₂O₂]= 23.2 mM and [ONOO⁻]= 280 µM. For Process 2, i.e. for all the compounds but IC, the concentrations are: [O₃]= 0.8 µM, [¹⁰₂]= [O₂]= [OH]= [OCl⁻]= 30 µM, [H₂O₂]= 2.32 mM and [ONOO⁻]= 35 µM. Experiments were performed in triplicate and the error bars shown in the figures represent the standard deviation of the values based on the three repetitions.

As shown in Figure 30, the absorbance of the solution with Indigo Carmine drops when this is exposed to ozone as desired. This is in agreement with the results shown in chapter 4.1. Unfortunately, the absorbance also drops when the solution is exposed to singlet oxygen, hydroxyl radical and peroxynitrite indicating that this commercially available probe is unfortunately not selective for ozone. Therefore Indigo Carmine cannot be used reliably as an ozone probe in the presence of these other reactive species.\(^{66,67}\)

5.4 Results

Selectivity graphs from the different probes and also DCF treated with RONS are shown in Figure 30 to Figure 32. The concentrations of the reactive species added for Process 1, Indigo Carmine (IC), are: [O₃]= 12 µM, [¹⁰₂]= [O₂]= [OH]= [OCl⁻]= 300 µM, [H₂O₂]= 23.2 mM and [ONOO⁻]= 280 µM. For Process 2, i.e. for all the compounds but IC, the concentrations are: [O₃]= 0.8 µM, [¹⁰₂]= [O₂]= [OH]= [OCl⁻]= 30 µM, [H₂O₂]= 2.32 mM and [ONOO⁻]= 35 µM. Experiments were performed in triplicate and the error bars shown in the figures represent the standard deviation of the values based on the three repetitions.

As shown in Figure 30, the absorbance of the solution with Indigo Carmine drops when this is exposed to ozone as desired. This is in agreement with the results shown in chapter 4.1. Unfortunately, the absorbance also drops when the solution is exposed to singlet oxygen, hydroxyl radical and peroxynitrite indicating that this commercially available probe is unfortunately not selective for ozone. Therefore Indigo Carmine cannot be used reliably as an ozone probe in the presence of these other reactive species.\(^{66,67}\)
Figure 30: Selectivity study of Indigo Carmine. $\lambda_{\text{abs}}= 610$ nm. $[O_3]= 12$ µM, $[{^{1}O_2}]= [O_2^-]= [OH^-]= [OCl^-]= 300$ µM, $[H_2O_2]= 23.2$ mM and $[ONOO^-]= 280$ µM

The behaviour of the fluorescent probes is shown in **Figure 31**. Probe 1 shows very good selectivity towards ozone and these results agree with data published by Garner et al.\textsuperscript{67} The fluorescence of the solution drops slightly when it is exposed to singlet oxygen and peroxynitrite, suggesting that the probe may be slowly destroyed by these reactive species. There is no significant fluorescence increase for any of the reactive species other than ozone as desired although a very minor increase is noted for hydroxyl radicals. Based on the difference in the increase of fluorescence (~80 times larger for $O_3$ than for OH) and the difference in concentration (~37 times larger for OH than for $O_3$), the probe would be selective for hydroxyl radical concentrations of up to ~1000x larger than that of ozone.

Probe 2 behaves similarly to probe 1 but it has a higher fluorescence (compare the Blank columns for the two probes in **Figure 31**). As for probe 1, fluorescence increases significantly only when the probe is exposed to ozone and it decreases when the probe is in the presence of singlet oxygen and peroxynitrite. The decrease in fluorescence in the presence of $^{1}O_2$ and ONOO is further discussed in chapter 5.5.

Probe 3 has a very similar behaviour to Probe 1 and comparable quantitative values in terms of fluorescence. It has, however, slightly worse selectivity towards OH.

Probe 4 has very similar qualitative respond to the other 3 fluorescent probes but its ozonolysis product is ~30 times less fluorescent. Nonetheless, the probe itself is also less fluorescent, making the probe a valid ozone sensor for higher ozone concentrations. In fact, Probe 4 seems to be the one with the highest selectivity towards ozone as no increase in fluorescence is observed when the probe is exposed to any of the other reactive species.
Figure 31: Selectivity of fluorescent probes. $[O_3]=0.8 \, \mu M$, $[^1O_2]=\left[O_2\right]=\left[OH\right]=\left[OCl^{-}\right]=30 \, \mu M$, $[H_2O_2]=2.32 \, \text{mM}$ and $[ONOO^-]=35 \, \mu M$. (a) Probe 1. $\lambda_{ex}=502 \, \text{nm}$, $\lambda_{em}=522 \, \text{nm}$. (b) Probe 2. $\lambda_{ex}=502 \, \text{nm}$, $\lambda_{em}=522 \, \text{nm}$. (c) Probe 3. $\lambda_{ex}=507 \, \text{nm}$, $\lambda_{em}=525 \, \text{nm}$ and (d) Probe 4. $\lambda_{ex}=485 \, \text{nm}$, $\lambda_{em}=518 \, \text{nm}$.
The intermediate product (**Compound 17**) in the synthesis of Probe 1 (also Probe 2, 3 and 4) has been also exposed to ozone and other reactive species. Its response (see **Figure 32**) is similar to that of the other fluorescent probes. Fluorescence increases significantly only when the compound is exposed to ozone and it reaches similar fluorescence intensities. However, since ozone could attack either (or both) of the two double bonds in the side chains, would difficult the study of this compound as a quantitative ozone probe.

**Figure 32**: Selectivity study of compound 17. $\lambda_{ex} = 503$ nm, $\lambda_{em} = 523$ nm. $[O_3] = 0.8 \mu M$, $[^1O_2] = [O_2] = [OH.] = [OCl^-] = 30 \mu M$, $[H_2O_2] = 2.32$ mM and $[ONOO^-] = 35 \mu M$.

Finally, **Figure 33** shows the stability of DCF when exposed to different reactive species. DCF is the product of ozonolysis of Probe 2 and although it is more stable than the product of Probe 1, it is found that it degrades slowly in the presence of $O_3$, OH and ONOO$^-$.  

**Figure 33**: Study of stability of DCF. $\lambda_{ex} = 502$ nm, $\lambda_{em} = 522$ nm. $[O_3] = 0.8 \mu M$, $[^1O_2] = [O_2] = [OH.] = [OCl^-] = 30 \mu M$, $[H_2O_2] = 2.32$ mM and $[ONOO^-] = 35 \mu M$. DCF was exposed to these conditions for 24 h. at rt.
In summary, the four fluorescent probes synthesized as part of this work are more selective than Indigo Carmine. The four probes have similar qualitative behaviour and are selective to ozone against a significant number of other reactive species. However, some quantitative differences among the four probes exist. Probe 4 is the least fluorescent of them all although its increase in fluorescence when it is exposed to ozone is similar to that of Probe 1 and compound 17: ~20 fold. For Probe 2, however, the increase is only ~4 times and for Probe 3 it is 43 times. Based on the increase in fluorescence, Probe 3 would be the best probe. However, Probe 3 is the least selective against OH and therefore if large concentrations of OH are to be expected, the other probes would be a better choice. Probe 4, despite its low fluorescence, seems to be the most selective probe of all as increase in fluorescence has only been observed after ozone exposure.

Notwithstanding that other reactive species may be encountered in plasma applications, the selectivity study reported here provides some encouraging results.

5.5 Singlet Oxygen and peroxynitrite: Hydrogen peroxide in basic conditions

As shown in the selectivity studies, all the DCF-derived probes (Probe 1-4) and also DCF lose fluorescence when exposed to singlet oxygen and peroxynitrite (see Figure 31). This loss of fluorescence suggests that the probes react with these species, probably destroying its DCF core.

In order to further study these reactions, Probe 1 and 2 were exposed to $^1\text{O}_2$ generated this time in a singlet oxygen generator instead of using sodium molibdate in basic conditions. A schematic of the singlet oxygen generator is showed in Figure 34. $^1\text{O}_2$ is generated by photosensitization, a reaction to light that is mediated by a light-absorbing molecule, i.e. a photosensitizer, than in this case was meso-tetraphenylporphyrin (Figure 34). When the photosensitizer is illuminated with violet light, it is excited and these excited photosensitizer molecules effectively transfer excitation energy to oxygen molecules to generate singlet oxygen.

In the experiments, the photosensitizer was added to the probe and O$_2$ bubbled through the solution. Two cooling units were used: one of them with water at 0 ºC for the reactor and the other one with methanol at -5 ºC to avoid any loss of solvent or product through the gas output.
Neither of the probes showed changes in fluorescence after the exposure to singlet oxygen, and that no reaction took place was further confirmed by \(^1\text{H} \text{NMR}\). Samples analysed after 2 and 24 hours of exposure showed the starting material. This result contradicts the one obtained previously when singlet oxygen was generated chemically (Figure 31) and suggests that the degradation of the probes may be due to the conditions in which the experiment is performed rather than to the presence of singlet oxygen. Chemically, \(^1\text{O}_2\) was produced from sodium molybdate in a pH= 10 solution mixed with hydrogen peroxide.\(^{67,114}\)

To investigate this, Probe 1 and Probe 2 were exposed to the following conditions (Figure 35): 1= blank, 2= MoO\(_4\) in pH 10 and H\(_2\)O\(_2\), 3= ONOO\(^-\) in NaOH aq. (pH 12), 4= MoO\(_4\) in pH 10, 5= pH 10, 6= NaOH aq. (pH 12), 7= pH 10 and H\(_2\)O\(_2\), 8= NaOH aq. (pH 12) and H\(_2\)O\(_2\).

Probe 1 did not show significant changes in fluorescence, in agreement with the results of Garner \textit{et al.}\(^{67}\) On the other hand, Probe 2 loses its fluorescence under certain conditions but not others. Careful analysis of Figure 35 reveals that Probe 2 loses its fluorescence whenever it is exposed to hydrogen peroxide in basic conditions.

These conditions were used when singlet oxygen and peroxynitrite were chemically produced and these are two cases in which fluorescence of Probe 2 decreased (Figure 31).
Although we have been able to check the selectivity against singlet oxygen obtained from the singlet oxygen generator, it remains unclear whether peroxynitrite reacts with the probes or not. It is possible that the loss of fluorescence shown in Figure 31 is solely due to the presence of hydrogen peroxide in basic conditions without contribution from peroxynitrite. Further studies, however, are required to elucidate this.

**Figure 35:** Fluorescence intensity of Probe 1 and 2 exposed to different conditions: 1= blank, 2= MoO$_4$ in pH 10 and H$_2$O$_2$, 3= ONOO’ in NaOH aq. (pH 12) and H$_2$O$_2$, 4= MoO$_4$ in pH 10, 5= pH 10, 6= NaOH aq. (pH 12), 7= pH 10 and H$_2$O$_2$, 8= NaOH aq. (pH 12) and H$_2$O$_2$. 
6 Application Method

The intended application of the probes synthesized as part of this thesis (chapter 4) is quantification of ozone in plasmas and other environments such as biological samples and atmospheric air. These would require detection of ozone both in gas phase as well as in liquid phase. Therefore, two application methods have been envisaged for the probes: in solution and in dry conditions. For solutions, the probe will be dissolved in an appropriate solvent and ozone will be bubbled through the liquid or diffused through the gas/liquid interphase. For applications where the presence of liquids is not appropriate, the compound will be deposited on a supporting substrate and used in dry conditions.

6.1 In solution

Figure 36 shows the fluorescence of the products obtained after ozonolysis of probe 1 and probe 2 when used in solution. In both cases, 10 mg of the probe were dissolved in 50 ml acetone/water (95/5) and ozone generated by an ozone generator was bubbled through the solutions. The fluorescence of the solutions was measured at different time intervals to capture the kinetics of the reaction. Both probes have similar kinetics, with the product of Probe 1 being slightly more fluorescent than that of Probe 2. Both of them degrade slowly once the probe has been consumed (see also discussion in chapter 4.2). This application method was used to perform all the selectivity studies carried out in chapter 5.

![Figure 36: Fluorescence of the solution versus time of ozone exposure.](image-url)
6.2 Dry conditions

Two methods were developed to deposit probe material on supporting substrates for their use in dry conditions. These methods are described below.

6.2.1 Immersion

In order to place probe material on the surface of a supporting substrate we first used a method similar to the one used by Maruo et al. for detecting ozone in air. Maruo et al. used Indigo Carmine, Orange I (4-[(4-hydroxy-1-naphtyl)azo]benzenesulfonic acid sodium salt) and Orange II (4-[(2-hydroxy-1-naphtyl)azo]benzenesulfonic acid sodium salt) to detect ozone and they placed the dyes onto 20mmx20mm cellulose sheets that were 0.26mm thick, had a retention particle diameter of 5 μm and a density of 125 g/m². The cellulose sheets were immersed for 30 seconds in 1.9 mM solutions of the respective dye and then allowed to dry in nitrogen atmosphere for 1 day for the solvent to evaporate. After this preparation procedure, the impregnated paper was exposed to gas mixtures containing between 30 and 150 ppb levels of ozone¹¹⁷,¹¹⁸ and ozone concentrations were determined by assessing changes in the colour of the paper.

In our case, instead of using the reflection of the compound as Mauro et al., fluorescent measurements were carried out to determine the ozone concentration. Before using the probes, commercially available DCF was deposited onto cellulose substrates to optimize the deposition conditions: solution concentration, time of immersion, and drying conditions. Figure 37 shows the fluorescence of 20mm×50mm cellulose sheets (qualitative filter paper with particle retention of 5-10 μm of diameter) that have been immersed in a solution of DCF, 0.45 mM, in water-acetone (15-10) for different times from 5 to 30 seconds. After the immersion the papers were allowed to dry for 1 day in dark before fluorescence was measured. Fluorescence was found to decrease with increasing immersion time. These unexpected results might be due to changes in the surface of the filter paper and require further investigation.
It was noted that the fluorescence of the impregnated paper decreases quickly if exposed to ambient air and light in the lab but could be stored for several days in the dark. Fluorescence would decrease ~2% in 8 minutes when left exposed to air and light in the lab but only 1.6% after 4 days when stored in the dark. For longer storage periods, inert atmosphere and low light and temperature conditions would be advisable.

Based on the results obtained with DCF impregnated papers, Probe 2 was deposited onto cellulose sheets by immersing them into a solution of 10 mg of Probe 2 in 100 mL of acetone/water for 30 seconds and allowing the papers to dry in the dark for 1 day. For each set of experiments, all papers were immersed simultaneously in the same bath to reduce variability due to changes in probe concentration and immersion time. Impregnated papers were then exposed to ozone and the fluorescence measured. The probe was exposed to ozone by blowing the gas through a vessel containing the paper. The maximum fluorescence reached was too low when compared with earlier experiments and this was due to the ozonolysis reaction taking too long to finish. As explained in chapter 4, the second step of the ozonolysis, i.e. the cleavage of the side chain, is slow and hinders real time measurements. To address this issue, the papers were immerse in a pH 7 buffer solution containing triethylamine (Et$_3$N) to dissolve the probe and ensure a fast conversion to the final fluorescence product.

The fluorescence of the obtained solution was then measured and the results are shown in Figure 38. The time evolution of the fluorescence is similar to that obtained previously when the probe was used in solution (Figure 36). The reaction time, however, is much faster when papers are used due to the reduced amount of probe available on the paper and
the enhanced transfer of ozone from the gas phase to the probe. For this reason, in these experiments the flow of ozone coming out of the ozone generator was diluted in nitrogen.

![Figure 38: Fluorescence of Probe 2 versus time of ozone exposure.](image)

Despite repeated attempts to improve repeatability and reduce the error bars in Figure 38, significant variability remained when the immersion procedure was used and an alternative preparation method was explored: Drops.

### 6.2.2 Drops

Due to the large variability obtained with the immersion method above a new impregnation method was developed. The error in the previous experiments was in part due to the immersion process since even samples without ozone exposure had large variability in fluorescence. This suggested that different amount of material was being deposited in each paper and a means of depositing a consistent amount of probe in each sample was required to improve reproducibility.

Instead of immersing the paper in a solution, a drop of the probe solution was dropped on each paper. In this way, the amount of product deposited on each paper can be easily calculated and consistently applied, due to the repeatability in drop volume\(^{119}\) and solution concentration.

One drop, 5.625 μL, of a solution 1.7 mM of Probe 2 was deposited onto six filter papers (4.36 μg per sample) and the average fluorescence intensity was measured to be
109.1 with a standard deviation of 6. This represents a significant improvement with respect to the immersion method as for similar experiments, the standard deviation with the immersion method was 20. The variability in fluorescence remained low even for samples left to dry for different periods of time. The standard deviation of 11 samples left to dry between 5 seconds and up to 2 hours remained at 6.0, demonstrating that this new preparation method is more robust than the immersion.

**Figure 39** shows the results of an experiment in which probe 2 deposited on filter paper was exposed to ozone. 12 points were taken between 0 and 500 seconds, all of them in triplicate. As expected, the graph shows a clear tendency of increasing fluorescence with exposure time. The error between the repetitions is attributed to the variability of the porosity and surface topology of the paper. Variability in the ozone content produced by the ozone generator cannot be ruled out at this stage either. Nevertheless, the method of depositing a drop of solution onto the filter paper is found to be more consistent and reproducible than the immersion method described in section 6.2.1.

![Figure 39](image)

**Figure 39:** Fluorescence intensity at 521 nm (au) of the paper with the probe after exposure to ozone versus time of exposure to ozone (seconds).
7 Correlation between Fluorescence Probe Measurements and Ultraviolet Absorption Spectroscopy

In this chapter, the fluorescence of Probes 1 to 4 is compared with the ozone dose determined by ultraviolet absorption spectroscopy. Good correlation is found for Probes 1, 2 and 3 but Probe 4 seems to fail when used in a plasma environment.

7.1 Experiment Set-up

The set-up used for the correlation experiments is shown in Figure 40. The experiment set-up consists of a power supply which is connected to the plasma device by a high voltage cable (red) and a ground cable (white). The plasma source used is the surface DBD source described in chapter 1.3.3, which is housed in a cylindrical gas chamber 43 mm high and 75 mm in diameter. An optical fibre coming from a UV LED source (260nm) is connected to the chamber through a collimating lens and diametrically opposite to it another optical fibre is used to collect the UV light and send it to the spectrometer. A solution with the probe under test is placed in the gas chamber and it is stirred with a magnetic bar while being exposed to plasma.

Figure 40: Experimental set-up for the correlation experiments.
Before exposing the probes to plasma, the amount of ozone generated by the plasma source was determined by UV absorption spectroscopy (see chapter 2.3.3 and discussion below in chapter 7.2.1) under different operation conditions. Frequency and modulation on/off time (duty cycle, DC) were varied during these experiments in order to identify appropriate conditions for testing the fluorescent ozone probes.

Ozone density was monitored during 15 minutes with a duty cycle varying from 5 to 35 %. The modulation period in all these tests was fixed at 400 ms. The frequency used in these experiments was 9.7±0.1kHz and ozone concentration was measured every 2 seconds. The graph showing the ozone density versus time for the different duty cycles is shown in **Figure 41**.

![Figure 41: Ozone density versus time. Each line represents the ozone density for a different duty cycle.](image)

At low duty cycles (<15%), the ozone concentration in the chamber increases rapidly and reaches a steady state value that is maintained for the at least 15 minutes. In this regime, as the duty cycle increases the steady state value also increases (compare traces for the 5% and 10% duty cycle in **Figure 41**). As the duty cycle is increased further, however, a maximum ozone concentration is reached and then the ozone concentration in the chamber decreases. This decrease is attributed to ozone thermal decomposition as the temperature in the plasma source increases in time at high duty cycles and a discharge poisoning mechanism where the accumulation of nitrogen oxides leads to the quenching of ozone.
The optimum duty cycle for maximum ozone production in this system during a 15 minute treatment can be calculated by integrating the ozone concentration over time. The result of this calculation is shown in Figure 42. For a 15 min treatment, a 25% duty cycle leads to the largest production of ozone.

**Figure 42**: Production of ozone during 15 min using different duty cycles.

**Figure 43** shows the fluorescence of Probe 1 (40 µL 0.5 mM in dimethyl sulfoxide, DMSO) as a function of the time it was exposed to air plasma created at ~10.0kHz and a 25% duty cycle. Although these conditions result in the largest ozone production (**Figure 42**), this is not ideal for the probe characterization because as it is shown in **Figure 43** the probe gets consumed very rapidly. As seen in previous chapters, once all Probe 1 has reacted, ozone attacks the fluorescent product and the fluorescence of the solution decreases (**Figure 36**). When exposed to plasma, the attack to the fluorescent product actually occurs even when probe remains in the solution, suggesting that plasma species other than ozone degrade the fluorescent product. This attack explains the low fluorescence observed in **Figure 43** (maximum of ~320 when values larger than 1000 are obtained when the probe is exposed to only ozone) and the simultaneous presence of unreacted probe, fluorescent product and DCF in NMRs of Probe 1 solutions partially exposed to plasma.
To limit the amount of probe required for the experiments, milder conditions in which less ozone is produced were selected. Data shown in the rest of the chapter was obtained when the plasma was operated at 11.5 kHz (slightly out of resonance to decrease the plasma intensity and minimize variability in between runs), with a modulation period of 500 ms and a duty cycle of 0.1%. These conditions resulted in slower ozone production and therefore extended the time required for the probe to be consumed.

7.2 Ultraviolet Absorption Measurements

7.2.1 Ozone concentration in the gas phase

Using the setup presented in section 7.1, the ozone concentration in the gas chamber can be measured by ultraviolet absorption spectroscopy. Light emitted by a UV LED source (260nm) travels through one of the optical fibres, reaching the gas chamber. The light then crosses the chamber where it may be partially absorbed by the gases present in the chamber. The transmitted light is then collected at the other side of the chamber and guided through another optical fibre to the spectrometer (Figure 40). From the intensity of the light measured by the spectrometer, the ozone concentration in the chamber can be determined. According to Lambert-Beer law (see discussion in chapter 2.3.3),

\[ A = c l \varepsilon \]

where \( A \) is the absorbance, \( c \) the concentration of the chromophore (ozone in this case), \( l \) the path length given by the chamber dimensions (7.5 cm), and \( \varepsilon \) the extinction coefficient.

Figure 43: Fluorescence intensity versus ozone dose for Probe 1, with a plasma on time of 25%.
at the relevant wavelength. Therefore, the ozone concentration can be calculated as:

\[ c = \frac{A}{l \varepsilon} \]. This calculation assumes that only ozone absorbs at 260nm, and assumption that is valid for the gases encountered in air plasma. The extinction coefficient of ozone at room temperature for \( \lambda = 260 \text{ nm} \) is \( \varepsilon = 1.075 \times 10^{-17} \text{ cm}^2 \text{ molecules}^{-1} \) \(^{1,120} \) and the absorbance is directly obtained during the experiment as \( A = -\log_{10} T \), where \( T \) is the ratio of the light intensity received by the spectrometer at any given time divided by the intensity received when no ozone is present in the chamber, i.e. initial conditions.

### 7.2.2 Estimated ozone dose

It is possible to estimate the dose of ozone to a liquid sample placed in the gas chamber by assuming that transport of ozone to the liquid interphase is governed by diffusion. This is a sensible approximation for the current setup as the system is closed, i.e. no gas flow is imposed, and the plasma is operated at room temperature. In this case, the flux of ozone to the sample (\( \Gamma \)) depends on the density of ozone in the gas phase (\( n \)) and the ozone thermal velocity (\( v_{th} \)): \(^3\)

\[
\Gamma = \frac{1}{4} n v_{th} ; \quad v_{th} = \sqrt{\frac{8 K_b T}{m \pi}}
\]

where the thermal velocity \( v_{th} \) is related to the Boltzmann constant (\( K_b \)), the temperature (\( T \)) and the mass (\( m \)). For ozone (\( M_{O_3} = 48 \text{ amu} = 7.97 \times 10^{-26} \text{ kg} \)) at 20º C (293 K), the thermal velocity is found to be 363.77 m/s.

Therefore, the dose of ozone delivered to the liquid sample can be estimated by integrating the flux through the interphase area (\( A \)) over time.

\[
\text{dose} = \int_0^t \Gamma A \, dt = \int_0^t \frac{1}{4} n v_{th} A \, dt = \frac{1}{4} v_{th} A \int_0^t n \, dt = \frac{1}{4} v_{th} A \sum_0^t n \Delta t
\]

For the samples used in these experiments, the area exposed to the gas phase was \( A = r^2 \pi = 1.25^2 3.1416 = 4.90874 \text{ cm}^2 \) and ozone concentration measurements were collected every second.
Figure 44: Dose of ozone estimated from UV measurements versus plasma exposure time.

**Figure 44** shows the estimated dose of ozone based on the ozone concentration in the gas phase as a function of the plasma exposure time. As expected, the dose increases with time, more slowly at the beginning as ozone builds up initially in the chamber (see **Figure 41**). It is noted that there is a significant variation from run to run in the ozone dose for a given exposure time. This variability is attributed to the DBD plasma system and the short duty cycles used in the experiments. Nonetheless, by correlating simultaneously obtained fluorescence measurements with dose estimates based on gas phase measurements, it is possible to overcome this variability.

### 7.3 Fluorescent Probe Measurements

Fluorescent Probes 1 to 4 (chapter 4) were exposed to a DBD plasma for different times in order to study the linearity between the change in fluorescence intensity and the dose of ozone. The compounds, 40 µL 0.5 mM, were diluted in pure water, 1 mL, before plasma exposure and the plasma was operated at 11.5kHz with a modulation period of 500 ms at a duty cycle of 0.1 %. Probe solution samples were prepared in glass vials 1.00 cm high and 2.50 cm in diameter and placed at the bottom of the gas chamber. After the exposure, the samples were transferred to a 10-mL volumetric flask and diluted, washing the remains in the glass vial, with pure water, 4 mL. One hour later, one drop of Et₃N:H₂O was added to the sample and the solution was diluted to 10 mL. Finally, fluorescence of the resulting solutions was measured.
7.3.1 Probe 1

The first compound studied was Probe 1. In Figure 45, fluorescence of the sample versus the estimated ozone dose is shown. The fluorescence increases linearly at small doses and then it reaches a maximum fluorescence once no more probe is available.

![Figure 45: Fluorescence intensity versus ozone dose for Probe 1.](image)

In Figure 46 the linear range is shown. The lineal fit has a coefficient of determination ($R^2$) of 0.87 when all the points in the triplicate experiment are taken into account.

![Figure 46: Fluorescence intensity versus ozone dose for Probe 1, linear range.](image)
7.3.2 Probe 2

Figure 47 shows the fluorescence of Probe 2 as a function of the estimated ozone dose. This compound, synthesised for first time in our lab, have different behaviour than Probe 1. It reaches higher fluorescence and has as a result a larger linear range. This suggests that Probe 2 and its product are more stable in the presence of plasma than Probe 1, as for the latter the fluorescence dropped by more than a factor of 4 when exposed to plasma (compare Figure 36 and Figure 43).

As shown in Figure 48, the linearity is good with a coefficient of determination ($R^2$) of 0.97.
7.3.3 Probe 3

Probe 3 was also exposed to plasma under the same conditions as Probe 1 and 2. In Figure 49 the correlation between the fluorescence measurements and the ozone dose estimated by UV spectroscopy is shown. The behaviour is similar to the previous probes.

![Figure 49: Fluorescence intensity versus ozone dose for Probe 3.](image)

As shown in Figure 50, the linearity for the reaction between Probe 3 and ozone in plasma conditions is similar to the one with Probe 1 ($R^2=0.87$).

![Figure 50: Fluorescence intensity versus ozone dose for Probe 3, linear range.](image)
7.3.4 Probe 4

The last compound tested to see the correlation between ozone dose and fluorescence of the sample was Probe 4. The results are shown in Figure 51. The weak fluorescence of this probe is found to be unrelated to the ozone dose from the plasma, likely due to the degradation of the fluorescent product by other plasma species.

![Figure 51: Fluorescence intensity versus ozone dose for Probe 4.](image-url)
8 Conclusions and Future Work

8.1 Ozonolysis with plasma:

- Alkenes were oxidised to aldehydes when exposed to air plasma. That means that ozone is being generated in this plasma and ozonolysis can be carry out in ionised air.
- The yields of the ozonolysis were considerably lower than figures reported in the literature. The plasma system, however, had not been optimised for ozone production.
- Evaporation of solvent and products was identified as one of the potential problems in the plasma systems used in this work but this problem could be addressed by careful engineering of the system, e.g. use of condensers and gas recycling.
- Despite the large number of reactive oxygen species (ROS) generated in air plasmas, the devices used deliver a remote plasma treatment in which only long lived species reach the sample. As a result the NMR analyses are quite clean. This could also be favoured by some of the byproducts being volatile.
- The reaction rate increased with the treatment surface area, suggesting that the reaction was limited by diffusion of ozone to the sample and therefore enhanced transport would increase the efficiency of these systems.
- Batch and flow plasma-based ozonolysis have been demonstrated.

8.2 Fluorescent probes:

- Koide’s fluorescent probe for ozone (Probe 1) was synthesised successfully.67
- It has been found that when Probe 1 is exposed to ozone, the probe yields three main compounds: The expected fluorescent product, the lactone and DCF.
- The expected fluorescent product decomposes with light and in the presence of silica, which hinders its isolation with conventional columns.
- The fluorescent product of Probe 1 is oxidized by ozone to DCF once the probe has been consumed. More importantly, in He+O2 and in air plasmas, DCF is
formed before the probe is fully consumed, suggesting that the product reacts with plasma species other than ozone.

- Promising new fluorescein derivative probes (Probes 2-4) have been developed, characterized and compared with colorimetric probes (Indigo Carmine).
- All new probes reacted quantitatively with ozone but there were slightly differences in terms of sensitivity and selectivity to ozone as well as in stability in a plasma environment.
- The linearity of the fluorescent probes has been studied by correlating the fluorescence to ozone concentration determined by ultraviolet absorption spectroscopy.

**8.3 Future work:**

- Further selectivity studies are required to confirm selectivity over singlet oxygen, peroxynitrite and other RONS present in plasmas.
- Detection of other RONS in the DBD plasma used in this study.
- Synthesis of probes to measure other species generated in oxygen plasma such as singlet oxygen, superoxide radical and atomic oxygen which have been studied in biological samples.
- Combination of multiple probes to detect various compounds simultaneously.\(^{121}\)
- Skin models will be used to gain not only an understanding of the dose received from the plasma but also its penetration in to biological targets. Probes embedded in agar could provide a first skin model since its mechanical properties and pH can be matched to those of human skin.\(^{122,123}\)
9 Experimental

General Information:

Commercial dry solvents were used in all reactions except dichloromethane which was distilled from CaCl₂. Purification of compounds was carried out using flash column chromatography over silica gel or by recrystallization. Thin layer chromatography using silica gel absorbent was carried out with Merck TLC Silica gel 60 F₂₅₄ aluminium sheets. Apollo Scientific ZEOprep 60 Silica gel was used for column chromatography.

Anhydrous reactions were carried out in oven-dried glassware and under an atmosphere of nitrogen or argon. Ozone was generated by feeding high purity oxygen (99.5%) into a Fischer Ozone Generator 500MM or into an AMBIO₂X, Ozone Generators & Systems. The Plasma sources employed in this research are described in chapter 1.3.3.

Melting points were determined on a Stuart Scientific SMP3 melting point apparatus and are uncorrected. Elemental analyses were determined on a Perkin Elmer 2400 CHN Elemental Analyser in conjunction with a Sartorius Cubis MSA2.7S0TRDM Ultra-Micro Balance 2.1g x 0.1µg. Infrared spectra were recorded on a Perkin-Elmer 100 FT-IR spectrometer as KBr pellets. ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were recorded on a Bruker DPX-400 or on a Jeol ECS-400 as solutions in CDCl₃ with tetramethylsilane as the internal standard unless otherwise specified. Chemical shifts are given in parts per million (ppm). Multiplicity is denoted as singlet (s), doublet (d), triplet (t), quartet (q) or multiplet (m). Coupling constant (J) values are given in hertz (Hz). High-resolution mass spectrometry was carried out on a Thermo Exactive Benchtop Orbitrap MS coupled to Advion TriVersa NanoMate injection system using electrospray ionisation (ES) from a methanol solution of the analyte.

Sartorius Cubis MSA2.7S0TRDM Ultra-Micro Balance 2.1g x 0.1µg with motorized glass draft shield was used to prepare samples for fluorescence analysis.

Absorption spectra were acquired in Quartz Cells 10mm pathlength UV/VIS on a Shimadzu UV-Visible Spectrophotometer UV-1601PC. Fluorescence spectra were recorded in a Quartz Fluorimeter Cell 10mm pathlength on a Perkin-Elmer Luminiscence Spectrometer LS SOB. The samples were excited at 497 nm and the emission intensities were collected from 500 to 650 nm.

All chemicals were purchased from Sigma or Alfa-Aesar and used as supplied.
General procedure for ozonolysis in atmospheric air plasma (A)

3-(2-Benzoylphenyl)propanal:\[^{109}\]

A solution of 1,2-dihydro-4-phenynaphthalene (11.6 mg, 0.056 mmol) in acetone (6 mL) in a glass disk was exposed to plasma over 16 min reaching a temperature of 31 °C in the ionised gas near the solution. The mixture was transferred to a 50 mL round bottom flask and Ph3P (15.4 mg, 0.059 mmol) was added to the mixture. After stirring at rt for 10 min the solvent was removed under reduced pressure. The product was analysed and quantified by NMR using m-dimethoxybenzene as an internal standard (8.4 mg, 0.061 mmol). The yield of the reaction was calculated to be 21%. \(^{1}\)H NMR (400 MHz, CDCl\(_3\), 293 K): \(\delta\) 9.66 (t, \(J = 1.2\) Hz, 1H), 7.00-8.00 (m, 9H), 2.92 (t, \(J = 8\) Hz, 2H), 2.72 (td, \(J = 8, 1.2\) Hz, 3H).

3-(Tert-butyl)-6-oxo-6-phenylhexanal:

According to procedure A, 3-(tert-butyl)-6-oxo-6-phenylhexanal was obtained in 34.7% yield by reaction between 4-tert-butylcyclohe-1-enylbenzene (4) (12.1 mg, 0.057 mmol) in plasma and adding Ph3P (15.4 mg, 0.059 mmol) after plasma exposure. \(^{1}\)H NMR (400 MHz, CDCl\(_3\), 293 K): the peak in \(\delta = 9.75\) ppm indicates that the aldehyde was generated.
**Benzaldehyde:**

a) Benzaldehyde was obtained in 8.6% yield as oil by reaction of trans-stilbene (2) (9.9 mg, 0.055 mmol) in plasma and adding Ph$_3$P (14.5 mg, 0.055 mmol) after plasma exposure, according to general procedure A. $^1$H NMR (400 MHz, CDCl$_3$, 293 K): δ 10.0 (s, 1H), 7.85-7.88 (m, 2H), 7.60-7.64 (m, 1H), 7.46-7.54 (m, 2H).

b) This compound was also obtained in 2.6% yield by reaction of styrene (5) (6.3 mg, 0.060 mmol) in plasma and adding Ph$_3$P (15.9 mg, 0.061 mmol) after plasma exposure, according to general procedure A.

**p-Methoxybenzaldehyde:**

a) p-Methoxybenzaldehyde was obtained in 33.0% yield by reaction of p-methoxystyrene (3) (9.8 mg, 0.073 mmol) in plasma and adding Ph$_3$P (20.2 mg, 0.077 mmol) after plasma exposure, according general to procedure A. $^1$H NMR (400 MHz, CDCl$_3$, 293 K): δ 9.85 (s, 1H), 7.80 (d, J= 8.8 Hz, 2H), 6.97 (d, J= 8.8Hz, 2H), 3.81 (s, 3H).

b) This compound was also obtained in 36.0% yield by reaction of 1-methoxy-4-(prop-1-en-1-yl)benzene (6) (15.8 mg, 0.106 mmol) in plasma over 30 min by turning the plasma 20/40 ms (on/off) and adding Ph$_3$P (28.0 mg, 0.106 mmol) after plasma exposure, according to general procedure A.
Potassium carbonate (2.88 g, 20.8 mmol) and 4-bromobutene (2.1 mL, 20.7 mmol) were added to a stirred solution of 2’,7’-dichlorofluorescein (2.76 g, 6.88 mmol) in DMF (25 ml) in a 100-mL round bottom flask at rt. The reaction mixture was stirred at 70 ºC over night. The mixture was introduced in a beaker with 500 mL of distilled water. The resulting precipitate was collected on a Büchner funnel by suction filtration and washed with water (250 mL). The solid was dissolved in DCM. Dried over anhydrous magnesium sulphate and filtered. The solvent was removed under reduced pressure and the solid was dried on a vacuum line to obtain a red solid (3.19 g, 91% yield); Mp= 182-183 ºC [184-185 ºC]. IR (KBr pellet): 3077, 3033, 2927, 1718, 1591, 1520, 1278 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\), 293 K): δ 8.31 (dd, J= 7.6, 1.2 Hz, 1H), 7.73 (td, J=7.6, 1.2 Hz, 1H), 7.28 (dd, J=7.6, 1.2 Hz, 1H), 7.03 (s, 1H), 7.02 (s, 1H), 6.94 (s, 1H), 6.59 (s 1H), 5.94 (ddt, J=17.2, 10.4, 6.8 Hz, 1H), 5.59 (ddt, 17.2, 9.6, 6.8 Hz 1H), 5.23 (dd, J= 17.2, 1.2 Hz, 1H), 5.17 (dd, J= 10.4, 1.2 Hz, 1H), 5.00-5.01 (m, 1H), 4.97 (m, 1H), 4.21 (t, J= 6.8 Hz, 2H), 4.07-4.17 (m, 2H), 2.66 (q, J=6.8 Hz, 2H), 2.22 (q, J=6.8 Hz, 2H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\), 293 K): δ 177.7, 164.9, 158.6, 157.8, 152.5, 149.7, 135.2, 133.5, 133.3, 133.2, 133.1, 131.6, 130.4, 130.3, 130.2, 128.0, 127.4, 120.5, 118.1, 117.6, 117.4, 114.9, 105.7, 100.7, 69.3, 64.5, 33.1, 32.6. HRMS (ES) m/z calculated for C\(_{28}\)H\(_{23}\)\(^{35}\)Cl\(_2\)O\(_5\) [M+H]\(^+\) 509.0917, found 509.0906 (-2.24 ppm).
6-(But-3-en-1-yloxy)-2,7-dichloro-9-(2-(hydroxymethyl) phenyl) -3H-xanthen-3-one, (Probe 1): 67

A solution of but-3-en-1-yl 2-(6-(but-3-en-1-yloxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoate, (11), (2.05 g, 4.0 mmol) in dried CH₂Cl₂ (15 mL) in a 100-mL round bottom flask was purged with N₂ over 10 min while stirring. DIBAL (14 mL, 1M in hexanes, 14 mmol) was added dropwise over 15 min at -78 ºC under N₂. After 2 h stirring the mixture at rt, Et₂O (20 mL) and sat. NH₄Cl aq. (6 mL) were added to the mixture at 0 ºC. After 30 min stirring at rt DDQ (1.0 g, 4.4 mmol) was added to the mixture at 0 ºC, the colour changed from orange to dark green. The mixture was stirred for 30 min at rt before filter through Celite using EtOAc to extract the product until no product appeared on TLC. Silica gel chromatography of the crude (5-10% EtOAc in hexanes) afforded the product as light orange solid (1.41 g, 79% yield). The product was recrystallised with hexane obtaining a light orange solid (1.03 g, 58% yield); Mp= 169-170 ºC [167-168 ºC]. IR (KBr pellet): 3231, 2921, 2869, 1628, 1608 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 7.38-7.43 (m, 2H), 7.26-7.31 (m, 1H), 6.90 (s, 1H), 6.88 (s, 1H), 6.86 (s 1H), 6.81-6.83 (m, 1H), 6.74 (s, 1H), 5.92 (ddt, J = 17.2, 10.4, 6.4 Hz, 1H), 5.32 (s, 2H), 5.19 (dd, J = 17.2, 1.6 Hz 1H), 5.13 (dd, J = 10.4, 1.6 Hz, 1H), 4.08 (t, J = 6.8 Hz, 2H), 2.61 (td, J = 6.8, 6.4 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 154.9, 151.9, 150.1, 149.6, 143.9, 138.6, 133.8, 129.5, 128.8, 128.6, 123.7, 121.0, 118.2, 118.1, 117.5, 117.1, 115.5, 103.6, 101.1, 83.1, 77.3, 72.3, 68.6, 33.3. HRMS (ES) m/z calculated for C₂₄H₁₉⁵Cl₂O₄ [M+H]+ 441.0655, found 441.0653 (-0.32 ppm).
A solution of the ether (12) (100 mg, 0.227 mmol) in 30 mL of acetone: water (95: 5) was cooled to 0 °C. Ozone generated in an AMBIOX ozone generator was bubbled through the solution for 1 min. KI (15 mg, 0.090 mmol) was added. The mixture was stirred for one hour and solvent was removed under reduced pressure. Silica gel chromatography of the crude (10-75% EtOAc in hexanes) afforded the product as an orange solid (83 mg, 95% yield). $^1$H NMR (400 MHz, CDCl$_3$, 293 K): δ 7.43-7.49 (m, 2H), 7.33 (t, J= 7.6 Hz, 1H), 6.91 (s, 1H), 6.84-6.86 (m, 2H), 6.81 (s, 1H), 6.78 (s, 1H), 5.31 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, 293 K): δ 155.3, 151.1, 145.4, 140.1, 130.6, 129.8, 124.6, 122.2, 118.1, 117.5, 104.4, 84.7, 73.1 (although 20 peaks expected, only 13 peaks detected); HRMS (ES) m/z calculated for C$_{20}$H$_{13}$$^{35}$Cl$_2$O$_4$ [M+H]$^+$ 387.0185, found 387.0177 (-2.22 ppm).
2',7'-Dichloro-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diol:

A solution of the ether (12) (100 mg, 0.227 mmol) in 30 mL of acetone: water (95: 5) was cooled to 0 °C. Ozone generated in an AMBIOX ozone generator was bubbled through the solution for 1 min. KI (15 mg, 0.090 mmol) was added. The mixture was stirred for one hour and solvent was removed under reduced pressure. Silica gel chromatography of the crude (10-75% EtOAc in hexanes) afforded the product as an orange solid (83 mg, 95% yield). The product was dissolved in MeOD d₄ and kept 20 days in the NMR tube before being analysed. $^1$H NMR (400 MHz, CDCl₃, 293 K): δ 7.67 (d, J= 7.6 Hz, 1H), 7.56 (t, J= 7.6 Hz, 1H), 7.44 (t, J= 7.6 Hz, 1H), 7.15 (d, J= 7.6 Hz, 1H), 6.93 (s, 2H), 6.50 (s, 2H), 4.24 (s, 2H); $^{13}$C NMR (100 MHz, CDCl₃, 293 K): δ176.6, 158.8, 154.3, 140.9, 132.6, 131.1, 130.3, 129.8, 129.2, 129.0, 128.7, 112.5, 105.1, 62.6.
A solution of the ether (12) (100 mg, 0.227 mmol) in 30 mL of acetone: water (95: 5) was cooled to 0 °C. Ozone generated in an AMBIOX ozone generator was bubbled through the solution for 10 min. KI (15 mg, 0.090 mmol) was added. The mixture was stirred for one hour and solvent was removed under reduced pressure. Silica gel chromatography of the crude (10-75% EtOAc in hexanes) afforded the product as an orange solid (37 mg, 40% yield). IR (KBr pellet): 3478, 1731, 1627, 1606 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.07 (d, J= 7.6 Hz, 1H), 7.33 (t, J= 7.6 Hz, 1H), 7.79 (t, J= 7.6 Hz, 1H), 7.27 (d, J= 7.6 Hz, 1H), 6.86 (s, 2H), 6.64 (s, 2H); ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 169.4, 155.3, 152.2, 150.8, 135.6, 130.2, 128.1, 126.4, 124.7, 123.7, 116.8, 110.8, 103.4, 82.6; HRMS (ES) m/z calculated for C₂₀H₁₀Cl₅O₅Na [M+Na]^+ 422.9798, found 422.9792 (-1.29 ppm).
3’-(But-3-en-1-yloxy)-2’,7’-dichloro-6’-hydroxy-3H-spiro[isobenzofuran-1,9’-xanthen]-3-one, (Probe 2):

A 100-mL round bottom flask was charged with the ester (11) (1.02 g, 2.0 mmol) and LiOH (75 mg, 3.1 mmol). H$_2$O (10 mL) and 1,4-dioxane (20 mL) were added. The mixture was stirred with strong reflux for 40 min. Solvents were removed in rotary evaporator. The product was dissolved in H$_2$O (100 mL) and HCl aq. 35% was added (8 drops) to acidify the solution until pH= 3. An orange precipitate was formed in the flask. The product was extracted with EtOAc (6 x 100 mL). The combined organic layers were dried over MgSO$_4$ and filtered. EtOAc was removed under reduced pressure and the solid was dried on a vacuum line to afford 1.02 g of the crude. Silica gel chromatography of the crude (10% - 50% EtOAc in petrol) afforded the product as orange solid (720 mg, 79% yield). The product was recrystallized by diffusion with EtOAc and hexane obtaining a white solid (537 mg, 59% yield). Mp= 213-214 ºC. IR (KBr pellet): 3259, 3079, 2938, 1734, 1628, 1608 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$, 293 K): δ 8.06 (dd, J= 7.4, 1.2 Hz, 1H), 7.72 (td, J= 7.4, 1.2 Hz, 1H), 7.68 (td, J= 7.4, 1.2 Hz, 1H), 7.16 (dd, J= 7.4, 1.2 Hz, 1H), 6.92 (s, 1H), 6.80 (s, 1H), 6.72 (s, 1H), 6.72 (s, 1H), 6.63 (d, J= 10.4 Hz, 1H), 5.14 (d, J= 6.8, 1.6 Hz, 2H), 2.63 (qt, J = 6.8, 1.2 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, 293 K): δ 169.0, 156.0, 153.1, 152.2, 151.1, 150.6, 135.5, 133.6, 130.3, 128.7, 128.0, 126.4, 125.5, 123.9, 118.7, 117.7, 116.0, 112.4, 111.2, 104.1, 101.3, 81.9, 68.7, 33.2; elemental analysis calculated for C$_{24}$H$_{16}$Cl$_2$O$_5$ (%): C 63.31, H 3.54, N 0.00; found: C 63.62, H 3.42, N 0.10; HRMS (ES) m/z calculated for C$_{24}$H$_{17}$Cl$_2$O$_5$ [M+H]$^+$ 455.0448, found 455.0442 (-1.11 ppm).
2',7'-Dichloro-3',6'-dihydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (2',7'-dichlorofluorescein):

A solution of Probe 2 (110 mg, 0.241 mmol) in 60 mL of acetone: water (95: 5) was cooled to 0 ºC. Ozone generated in an AMBIOX ozone generator was bubbled through the solution for 3 min (100% power). Et₃N (15 mg, 0.090 mmol) was added and the colour turned from bright yellow to orange. The mixture was stirred for one hour and solvent was removed under reduced pressure to afford the product as an orange solid. The crude was purified by column chromatography (20% - 100% EtOAc in petrol) to afford the desired product (92 mg, 95% yield). IR (KBr pellet): 3480, 1734, 1596, 1491, 1433, 1266 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.08 (d, J= 7.6 Hz, 1H), 7.85 (t, J= 7.6 Hz, 1H), 7.78 (t, J= 7.6 Hz, 1H), 7.28 (d, J= 7.6 Hz, 1H), 6.87 (s, 2H), 6.65 (s, 2H); ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 169.4, 155.3, 152.2, 150.8, 135.6, 130.2, 128.1, 126.4, 124.7, 123.7, 116.8, 110.8, 103.4, 82.6.
2-(4-(But-3-en-1-yloxy)-5-chloro-2-hydroxybenzoyl) benzoic acid:

The ester (11) (500 mg, 0.98 mmol) was dissolved in 1,4-dioxane (8 mL) in a 50-mL round bottom flask and the solution was heated to reflux. A solution of LiOH (140 mg, 5.85 mmol) in H₂O was added. The mixture was stirred with reflux 1.5 hours. Solvents were removed in rotary evaporator. The product was dissolved in H₂O (100 mL) and HCl aq. 35% was added (14 drops) to acidify the solution until pH= 2. The orange precipitate was extracted with EtOAc (4 x 75 mL). The combined organic layers were dried over MgSO₄ and filtered. EtOAc was removed under reduced pressure and the solid was dried on a vacuum line to afford 508 mg of the crude. Silica gel chromatography of the crude (20% - 80% EtOAc in petrol) afforded the product as orange solid (177 mg, 52% yield). The product was recrystallized by diffusion with EtOAc and hexane obtaining a white solid (126 mg, 37%). Mp= 169-171 °C; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 12.30 (s, 1H), 8.15 (dd, J= 7.6, 1.2 Hz, 1H), 7.71 (td, J= 7.6, 1.2 Hz, 1H), 7.61 (td, J= 7.6, 1.2 Hz, 1H), 7.35 (dd, J= 7.6, 1.2 Hz, 1H), 7.02 (s, 1H), 6.55 (s, 1H), 5.91 (ddt, J = 17.2, 10.4, 6.8 Hz, 1H), 5.20 (dm, J = 17.2 Hz, 1H), 5.14 (dd, J = 10.4, 1.6 Hz, 1H), 4.13 (t, J = 6.8 Hz, 2H), 2.62 (q, J = 6.8, 2H); ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 200.2, 170.2, 163.9, 160.6, 140.2, 133.4, 133.4, 132.9, 131.4, 130.0, 127.4, 127.3, 117.8, 114.0, 113.5, 101.5, 68.6, 33.1; elemental analysis calculated for C₁₈H₁₅ClO₅ (%): C 62.35, H 4.36, N 0.00; found: C 62.00, H 4.00, N 0.17; HRMS (ES) m/z calculated for C₁₈H₁₅³⁵ClO₅Na [M+Na]+ 369.0500, found 369.0496 (-1.27 ppm).
2-(5-Chloro-2,4-dihydroxybenzoyl)benzoic acid:

The alkene (100 mg, 0.29 mmol) was dissolved in acetone: H₂O (60 mL, 95:5). Ozone was bubbled through the solution for 3 min. After ozone exposure, Et₃N (3 drops) was added. The solution was stirring overnight. Solvents were removed under reduced pressure. Silica gel chromatography of the crude (80% EtOAc in hexanes – 50% MeOH in EtOAc) afforded the product as pale brown solid (60 mg, 71% yield). ¹H NMR (500 MHz, MeOD d₄, 293 K): δ 8.07 (dd, J= 7.5, 1.5 Hz, 1H), 7.63 (m, 2H), 7.35 (dd, J= 7.5, 1.5 Hz, 1H), 7.02 (s, 1H), 6.48 (s, 1H) ; ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 204.0, 172.0, 165.4, 162.4, 141.9, 136.0, 135.7, 132.9, 131.9, 131.6, 128.9, 116.7, 113.9, 105.6.
Methyl 2-(6-(but-3-en-1-yloxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoate, (Probe 3):

![Methyl 2-(6-(but-3-en-1-yloxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoate](image)

NaOMe 1M in MeOH (1 mL) was added to the initial ester (510 mg, 1.00 mmol) and MeOH (3 mL) was added to the mixture. The solution was stirred at 50 ºC. After 1.5 h, HCl 1M (0.5 mL) was added to neutralize the solution. H2O (50 mL) was added to the mixture and the product was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine and dried over MgSO4, the slurry was filtered and the MgSO4 was washed with DCM to recover the entire product. Solvents were removed on a rotary evaporator and the product was dried on a vacuum line. The crude (481 mg) was purified by column chromatography (25% – 66% EtOAc in petrol) to afford an orange solid (440 mg, 94% yield). Mp= 241-245 ºC IR (KBr pellet): 3071, 3036, 2926, 1724, 1592, 1520, 1278 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.32 (dd, J= 7.6, 1.2 Hz, 1H), 7.80 (td, J= 7.6, 1.2 Hz, 1H), 7.74 (td, J= 7.6, 1.2 Hz, 1H), 7.30 (dd, J= 7.2, 1.2 Hz, 1H), 7.03 (s, 1H), 7.02 (s, 1H), 6.93 (s, 1H), 6.60 (s, 1H), 5.93 (ddt, J= 16.8, 10.0, 6.4 Hz, 1H), 5.24 (d, J= 16.8 Hz, 1H), 5.17 (d, J= 10.0 Hz, 1H), 4.21 (t, J= 6.4 Hz, 2H), 3.71 (s, 3H), 2.67 (q, J= 6.4 Hz, 2H); ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 177.9, 165.3, 158.7, 157.9, 152.6, 149.9 135.3, 133.9, 133.3, 131.6, 130.5, 130.3, 129.9, 128.0, 127.4, 120.5, 118.2, 117.6, 115.0, 105.8, 100.8, 69.3, 52.6, 33.1; HRMS (ES) m/z calculated for C₂₅H₁₉³⁵Cl₂O₅ [M+H]⁺ 469.0604, found 469.0593 (-2.27 ppm).
Probe 3 (25 mg, 0.05 mmol) was dissolved in acetone: H₂O (60 mL, 95:5). Ozone, 20% power, was bubbled through the solution for 2 min at 0 °C. Solvents were removed under reduced pressure. Silica gel chromatography of the crude (66% EtOAc in hexanes – 50% MeOH in EtOAc) afforded the product as orange-red solid (20 mg, 90% yield). IR (KBr pellet): 3449, 1721, 1636 cm⁻¹. ¹H NMR (400 MHz, MeOD d₄, 293 K): δ 8.32 (d, J = 7.6 Hz, 1H), 7.88 (t, J = 7.6 Hz, 1H), 7.82 (t, J = 7.6 Hz, 1H), 7.44 (d, J = 7.6 Hz, 1H), 6.96 (s, 2H), 6.65 (s, 2H), 3.66 (s, 3H) ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 165.9, 157.4, 154.9, 134.2, 132.6, 130.8, 130.4, 130.2, 129.9, 128.3, 127.4, 127.4, 111.2, 103.7, 51.6. HRMS (ES) m/z calculated for C₂₁H₁₃Cl₂O₅ [M+H]+ 415.0135, found 415.0132 (-0.50 ppm).
The ester (11) (500 mg, 0.98 mmol) was dissolved in MeOH (50 mL). H₂SO₄ 95% (0.5 mL, 10 mmol) was added and the solution was stirred with reflux for 17 h. Solvents were removed in rotary evaporator. 50 mL of H₂O were added. NaOH 1M was added dropwise to neutralise the slurry. The product was extracted with EtOAc (4 x 50 mL). The combined organic layers were washed with brine and dried over MgSO₄. Solvents were removed under reduced pressure and the solid was dried on a vacuum line. The crude, 505 mg, was purified by column chromatography (20% EtOAc in petrol) and the lactone was obtained as a byproduct. It was recrystallized with EtOAc and hexane to afford the colourless compound (18 mg, 4% yield). Mp> 300 ºC. IR (KBr pellet): cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.06 (d, J= 6.8 Hz, 1H), 7.70 (m, 2H), 7.15 (d, J= 7.6 Hz, 1H), 6.81 (s, 1H), 6.74 (s, 1H), 3.95 (s, 3H); ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 168.9, 156.7, 152.2, 150.8, 135.6, 130.4, 128.9, 126.5, 125.5, 123.9, 118.4, 111.5, 100.4, 81.9, 56.6; elemental analysis calculated for C₁₈H₁₅ClO₅ (%): C 61.56, H 3.29, N 0.00; found: C 61.43, H 3.17, N 0.13; HRMS (ES) m/z calculated for C₂₂H₁₅⁵Cl₂O₅ [M+H]⁺ 429.0291, found 429.0290 (-0.24 ppm).
2',7'-Dichloro-3'-hydroxy-6'-methoxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one, (Probe 4):

A solution of the phenol (100 mg, 0.22 mmol) in DMF (2.5 mL) was treated with ground KOH (34 mg, 0.61 mmol). MeI (40 µL, 0.63 mmol) in DMF (1 mL) was added dropwise over 5 min at 0 ºC. The reaction was stirred at rt for 3 h. The solution was diluted with 25 mL of H₂O and extracted with EtOAc (3 x 25 mL). The combined organic layers were washed with brine and dried over MgSO₄. Solvents were removed under reduced pressure and the solid was dried on a vacuum line to yield the yellow solid crude. Silica gel chromatography of the crude (10 % – 50 % EtOAc in petrol) afforded the product as pale yellow solid (93 mg, 90 %). Mp= 185-187 ºC IR (KBr pellet): 3082, 2929, 1763, 1628, 1609 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.06 (d, J= 7.2 Hz, 1H),  7.70 (m, 2H),
7.15 (d, J= 7.2 Hz, 1H), 6.79 (s, 1H), 6.78 (s, 1H), 6.73 (s, 2H) 5.93 (ddt, J= 16.8, 10.0, 6.8 Hz, 1H), 5.21 (dm, J= 16.8 Hz, 1H), 5.15 (dm, J= 10.0 Hz, 1H), 4.11 (t, J= 6.8 Hz, 2H), 3.94 (s, 3H), 2.63 (q, J= 6.8 Hz, 2H); ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 169.0, 156.7, 156.0, 152.2, 150.9, 150.7, 135.6, 133.7, 130.4, 128.8, 128.8, 126.5, 125.5, 123.9, 118.8, 118.4, 117.8, 111.5, 111.4, 101.2, 100.4, 82.0, 68.7, 56.5, 33.3; elemental analysis calculated for C₂₅H₁₈Cl₂O₅ (%): C 63.98, H 3.87, N 0.00; found: C 64.36, H 4.05, N 0.18; HRMS (ES) m/z calculated for C₂₅H₁₉³⁵Cl₂O₅  [M+H]⁺ 469.0604, found 469.0593 (-2.32 ppm).
2',7'-Dichloro-3'-hydroxy-6'-methoxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one:

Probe 4 (25 mg, 0.05 mmol) was dissolved in acetone: H₂O (60 mL, 95:5). Ozone, 20% power, was bubbled through the solution for 2 min at 0 °C. Solvents were removed under reduced pressure. Silica gel chromatography of the crude (50% EtOAc in hexanes –EtOAc) afforded the product as orange-red solid (14 mg, 64% yield). IR (KBr pellet): 3260, 2926, 2854, 1760, 1736, 1628, 1609 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 8.07 (d, J= 6.8 Hz, 1H), 7.71 (m, 2H), 7.17 (d, J= 7.2 Hz, 1H), 6.93 (s, 1H), 6.82 (s, 1H), 6.73 (s, 1H), 6.72 (s, 1H), 3.95 (s, 3H) ; ¹³C NMR (100 MHz, CDCl₃, 293 K): δ 169.0, 156.8, 153.2, 152.2, 151.1, 150.8, 135.6, 130.4, 128.8, 128.1, 126.4, 125.6, 123.9, 118.4, 116.2, 112.5, 111.3, 104.2, 100.6, 81.9, 56.6. HRMS (ES) m/z calculated for C₂₁H₁₃.Cl₂O₅ [M+H]+ 415.0135, found 415.0133 (-0.45 ppm).
2-((E)-(2-((1-Carboxy-2-(1H-indol-3-yl)ethyl)amino)-3-(((Z)-3,3-dimethyl-1-propylindolin-2-ylidene)methyl)-cyclohex-2-en-1-ylidene)methyl)-3,3-dimethyl-1-propyl-3H-indol-1-iium iodide, Trp-Cy, (Probe 5):

L-Trp (4.084 g, 20.00 mmol) and Cy (668 mg, 1.00 mmol) were placed in a 100-mL round bottom flask. The flask was purged with argon for 15 min. DMF (32.5 mL) and H₂O (25 mL) were added to the mixture. The solution was stirred at 80 °C for 16 h. H₂O (80 mL) was added and the product was extracted with EtOAc (4 x 100 mL). The combined organic layers were washed with brine (100 mL) and dried over MgSO₄. Solvents were removed under reduced pressure and the crude was dried on a vacuum line to afford 505 mg of an orange solid. The crude was purified by column chromatography (0% - 33% MeOH in EtOAc) obtaining the blue solid (280 mg, 33% yield). Mp= 143-145 °C IR (KBr pellet): 2934, 2971, 1529, 1456, 1295, 1170, 1128 cm⁻¹; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 7.67 (d, J= 12.8 Hz, 2H), 7.47 (d, J= 8 Hz, 1H), 7.38 (d, J= 7.6 Hz, 1H), 7.23-7.19 (m, 3H), 7.13 (d, J= 7.6 Hz, 2H), 7.02-6.96 (m, 3H), 6.91 (t, J= 7.6 Hz, 1H), 6.77 (d, J= 8.0 Hz, 2H), 5.44 (d, J= 12.8 Hz, 2H), 4.91 (m, 1H), 3.67 (t, J= 7.2 Hz, 4 H), 3.60 (m, 2H), 2.40-2.33 (m, 2H), 2.13-2.09 (m, 2H), 1.75 (q, J= 7.2 Hz, 4H), 1.58 (s, 6H), 1.34 (s, 6H), 1.00 (t, J= 7.2 Hz, 6H); ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 167.5, 166.9, 143.2, 140.2, 137.1, 136.4, 128.1, 127.8, 124.8, 122.3, 122.1, 121.0, 120.6, 118.5, 118.5, 111.7, 108.0, 94.1, 62.8, 47.4, 44.5, 29.0, 28.6, 28.2, 25.1, 21.2, 20.0, 11.7 (missing 2 quaternary carbons); HRMS (ES) m/z calculated for C₄₇H₅₅N₄O₂ [M-I]⁺ 707.4320, found 707.4301 (-1.90 ppm).
L-Trp (4.084 g, 20.00 mmol) and Cy (668 mg, 1.00 mmol) were placed in a 100-mL round bottom flask. The flask was purged with argon for 15 min. DMF (32.5 mL) and H₂O (25 mL) were added to the mixture. The solution was stirred at 80 °C for 16 hours. H₂O (80 mL) was added and the product was extracted with EtOAc (4 x 100 mL). The combined organic layers were washed with brine (100 mL) and dried over MgSO₄. Solvents were removed under reduced pressure and the crude was dried on a vacuum line to afford 505 mg of an orange solid. The crude was purified by column chromatography (0% - 33% MeOH in EtOAc) obtaining the blue solid (91 mg, 13% yield). Mp= 105-107 °C; ¹H NMR (400 MHz, CDCl₃, 293 K): δ 7.46 (d, J= 13.2 Hz, 2H),  7.28 (m, 4H), 7.07 (t, J= 7.6 Hz, 2H), 6.89 (d, J= 8.0 Hz, 2H), 5.64 (d, J= 13.2 Hz, 2H) 3.82 (t, J= 7.4 Hz 4H), 3.67 (s, 6H), 2.50 (t, J= 6.4 Hz, 4H), 1.86 (m, 6H), 1.67 (s, 12H),  1.04 (t, J= 7.4 Hz, 6H); ¹³C NMR (100 MHz, MeOD d₄, 293 K): δ 178.4, 167.4, 143.1, 140.4, 140.1, 128.2, 122.8, 122.1, 121.7, 108.6, 94.0, 47.9, 47.7, 44.9, 29.6, 25.4, 21.6, 20.1, 11.7. HRMS (ES) m/z calculated for C₃₈H₅₀N₃ [M-I]⁺ 548.3999, found 548.3984 (-2.81 ppm).
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