Trabajo de Fin de Grado

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Fluorescence of Molecules and Polymers

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Dedicado a mi abuelo Gregorio.
Que fomento mi interés por la ciencia,
tal vez sin darse cuenta al poner los
documentales de 'La 2' cuando
yo llegaba del colegio.

Acknowledgments

Quisiera expresar mi agradecimiento a mi tutor Fernando por ayudarme con el desarrollo del TFG y por presentarme a los investigadores del IPNA[1], que colaboraron con la síntesis de los derivados del NBD y parte del análisis de resultados. Y a Sergio uno de los alumnos de doctorado (tutorizado por Fernando Lahoz) por pasar parte de su tiempo en el laboratorio conmigo ayudándome y explicándome algunos detalles para tener en cuenta en los experimentos que se llevaron a cabo.

También a toda mi familia que siempre está apoyándome para que saque lo que me proponga. Y en especial a mis padres y mi hermano, por aguantarme cada vez que abro la boca para comentar algo científico y luego decirme la frase "Mientras lo entiendas tu genial", Jajaja.

Por último y no por ello menos importante, mis amigos, esos que se cuentan con los dedos de una mano (donde claramente importa más la calidad que la cantidad), por esos buenos ratos, apoyarme cuando no me encontraba bien y por esa amistad incondicional durante todo este tiempo, ya que todo lo que hace falta es eso tiempo y rodearte de aquellos que te hacen crecer a mejor.

> Imaginad que es una semilla ...
> todo lo que ha formado ese árbol gigantesco
> esta contenido dentro de esta diminuta semilla
> solo se necesita tiempo, un poco de sol, lluvia y VUALÁ.
>
> (Flik. Bichos: Una aventura en miniatura (1998))

[1] Alicia Boto, Dácil Hernández, Fernando Lobo (mirar [1]).

Abstract

Este trabajo se centra en el estudio de la fluorescencia de un conjunto de moléculas orgánicas, en concreto de un grupo de derivados del Nitrobenzoxadiazol (NBD), y en la utilización de uno de ellos para generar un fármaco con propiedades fluorescentes, para poder seguir la actuación del fármaco en el interior de las células.

Para dicha selección realizaremos una pequeña exposición sobre los conceptos necesarios para entender lo que intentamos determinar en este trabajo, como que es el fenómeno de fluorescencia, el Quantum Yield (QY), un fluoróforo, el espectro de absorción o emisión, entre otros.

Una vez expuestos esos conceptos necesarios se llevará acabo todo un estudio del espectro de absorción y de emisión de dichas muestras para compararlos con los de una muestra estándar y de esta manera obtener su QY, para ello se sigue una metodología experimental (bastante detallada en el trabajo).Pero para determinar cuál de esos compuestos es el adecuado, se analizan los datos obtenidos y una vez hecho, aquel que tenga el QY más alto y con un margen de error muy reducido será el candidato adecuado para unir con el fármaco.

A modo de conclusión, podría haber sido un éxito rotundo, si no hubiera aparecido el Covid-19, pero aun así se pudo hacer una gran parte del estudio que podría dar en el futuro a un posible nuevo trabajo mediante el desarrollo de los apartados que no se pudieron llevar acabo y las distintas ideas de propuesta como un estudio similar a los realizados para el FLTX1 (mirar apartado 3.4), pero usando el nuevo compuesto una vez sintetizado el fármaco fluorescente.

Abstract

En la introducción se trata el fenómeno de la fluorescencia, explicando cómo funcionan las transiciones de electrones desde niveles de energía más altos hasta su nivel fundamental. También se expone como la fluorescencia tiene un tiempo de vida y una eficiencia que dependen de varios mecanismos y como algunos disminuyen la intensidad de emisión. Por último, se introducen el concepto de QY (Quantum Yield) Standards y de fluoróforo bioquímicos, que son componentes moleculares con propiedades fluorescentes; además de mencionar algunos artículos donde se trabaja con derivados del NBD.

1 Introduction

1.1 Phenomenon of Fluorescence

Luminescence is the emission of light by a substance without being motivated by heat, so it is a form of cold radiation, it is the process of light emission whose origin does not lie exclusively in high temperatures It can be caused by chemical reactions , electrical energy, subatomic movements, or stress in a crystal [2].

Fluorescence is a particular type of luminescence, which characterizes substances that are capable of absorbing energy in the form of electromagnetic radiation and then emitting part of that energy in the form of electromagnetic radiation of different wavelength.

The luminescence is formally divided into two categories fluorescence and phosphorescence, depending on the nature of the excitation of the states. Typical fluorescence excitation occurs when an electron of the fundamental singlet pair S_0 goes to a higher energy level than S_1. Immediate desexitation during light emission of less than 10^{-8} s, called the fluorescence duration t, is also characteristic of the phenomenon [3].

The interesting thing is that the emitted light has a longer wavelength than the excitation radiation, this was first discovered by Sir George G. Stokes and then called Stokes Shift. The change in wavelength is related to a loss of energy during electronic excitation due to internal conversions, where the transfer of electrons to lower vibratory levels of S_0 and S_1 is caused by the release of thermal energy (non-radiant) just before or after the passage of the main beam from S_0 to S_1.

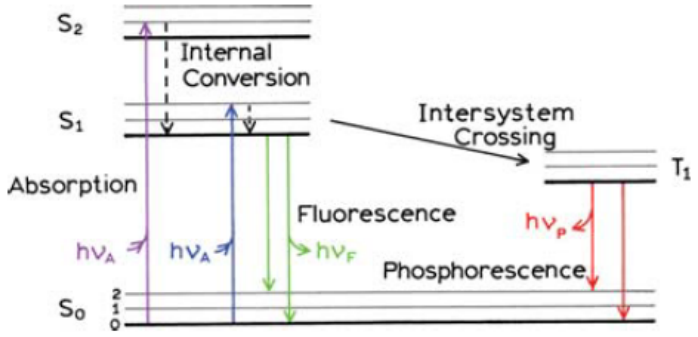


Figure 1: One form of Jablonski diagram [3].

The processes involved in the absorption and emission of light have usually been described in the Jablonski diagram. The Jablonski diagram is often used as a starting point to discuss the absorption and emission of light. The Jablonski diagram is used in different ways to compare the different types of molecular processes that can occur in excited states. These diagram are named after Professor Alexander Jablonski, nicknamed the father of fluorescence spectroscopy due to his many works.

It is seen in the Figure 1 the emission is normally independent of the excitation wavelength, after the absorption of a photon by a molecule in its fundamental state and the consequent population of excited electronic states, the radiative emission, both fluorescence and phosphorescence, happens from the excited state of lower energy of the molecule (Kasha's rule [4]).

Phosphorescence is a phenomenon similar to fluorescence, in which certain electrons are excited by light, passing a larger orbit of energy, and when they return to the resting state, they release some of this energy in the form of light. The difference between the two phenomena is that in phosphorescence the release of energy by means of photons occurs late, even when the stimulating source is no longer present, unlike fluorescence, in which the release of photons is almost immediate to their absorption.

In the case of phosphorescence, the substance can continue to emit light even hours after removing the stimulus, since the release of energy happens very slowly. We can say then, that phosphorescent substances have the ability to contain electromagnetic energy, even for a period not too long [5].

1.2 Fluorescence Lifetimes and Quantum yields

Fluorescence lifetimes and quantum yield are probably the most important properties of fluorophore. Quantum efficiency or quantum yields is the ratio between number of photons emitted front the number of photons absorbed. Substances with the highest quantum efficiency and the next unit show the brightest emissions. The lifetime is also important as it determines the time of available fluorophore for interaction or dispersion in the environment and, therefore, the available emission information [3].

The definition of quantum yield and lifetime are best illustrated with a simplified Jablonski diagram (Figure 2). In this diagram we do not explicitly illustrate the individual relaxation processes leading to the relaxed S1 state. Instead, we focus on the process responsible for returning to the fundamental state. In particular, we are interested in the emissive rate of the fluorophore (Γ) and its rate of nonradiative decay to S_0 (k_{nr}).

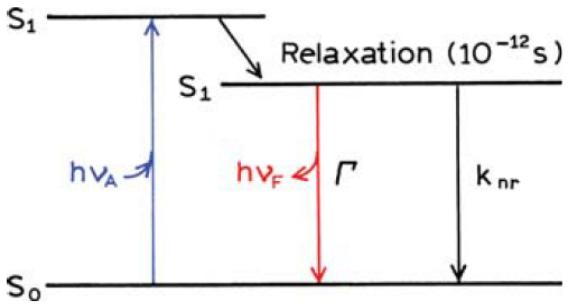


Figure 2: A simplified Jablonski diagram [3].

The fluorescence quantum yield is the ratio of the number of photons emitted to the number absorbed. The rate constants Γ and k_{nr} both depopulate the excited state, this affect directly to the quantum yield. The fraction of fluorophores that decay through emission, and hence the quantum yield, is given by:

$$Q = \frac{\Gamma}{\Gamma + k_{nr}} \tag{1.1}$$

The quantum yield can be close to unity if the radiationless decay rate is much smaller than the rate of radiative decay, that is $k_{nr} < \Gamma$. We note that the energy yield of fluorescence is always less than unity because of Stokes losses. For convenience we have grouped all possible non-radiative decay processes with the single rate constant k_{nr}.

The lifetime of the excited state is defined by the average time the molecule spends in the excited state prior to return to the ground state. Generally, fluorescence lifetimes are near 10 ns.

$$\tau = \frac{1}{\Gamma + k_{nr}} \tag{1.2}$$

Fluorescence emission is a random process, and few molecules emit their photons at precisely t = τ. The lifetime is an average value of the time spent in the excited state.

The lifetime of the fluorophore in the absence of nonradioactive processes, can simplify the equation because $k_{nr} = 0$ and in this case is called intrinsic or natural lifetime. The equation is:

$$\tau_n = \frac{1}{\Gamma} \tag{1.3}$$

In principle, the natural lifetime τ_n can be calculated from the absorption spectra, extinction coefficient, and emission spectra of the fluorophore. The radiative decay rate Γ can be calculated using

$$\begin{aligned} \Gamma &\simeq 2.88 \times 10^{-9} n^2 \frac{\int F(\overline{\nu})\, d\overline{\nu}}{\int F(\overline{\nu})\, d\overline{\nu}\overline{\nu}^3} \int \frac{\varepsilon(\overline{\nu})}{\overline{\nu}} d\overline{\nu} \\ &= 2.88 \times 10^{-9} n^2 \langle \overline{\nu}^{-3} \rangle^{-1} \int \frac{\varepsilon(\overline{\nu}) d\overline{\nu}}{\overline{\nu}} \end{aligned} \tag{1.4}$$

where F($\overline{\nu}$) is the emission spectrum plotted on the wavenumber (cm^{-1}) scale, $\epsilon(\overline{\nu})$ is the absorption spectrum, and n is the refractive index of the medium. The integrals are calculated over the $S_0 \leftrightarrow S_1$ absorption and emission spectra.In many cases this expression works rather well, particularly for solutions of polynuclear aromatic hydrocarbons.

However, there are numerous reasons why eq.1.4 can fail. This expression assumes no interaction with the solvent, does not consider changes in the refractive index (n) between the absorption and emission wavelength, and assumes no change in excited- state geometry.

The natural lifetime can be calculated from the measured lifetime (τ) and quantum yield which can be derived from eqs. 1.2 and 1.3.

$$\tau_n = \frac{\tau}{\mathrm{Q}} \tag{1.5}$$

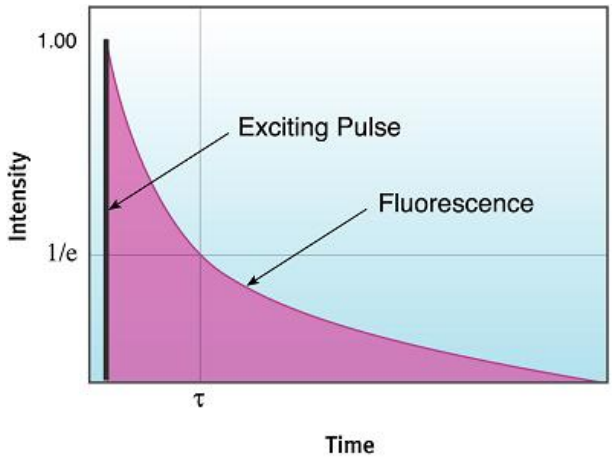


Figure 3: Example of lifetime curve [6].

The quantum yield and lifetime can be modified by factors that affect either of the rate constants (Γ or k_{nr}). During the excitation of the substance, some processes can affect the fluorescence [7]. Next, we briefly explain three of these processes:

- Quenching [7]: Destruction of the fluorescence produced by the collision of the fluorophore with other molecules (quencher) of the solution, but without distilling the fluorophore. This causes a decrease in fluorescence intensity.
- Photobleaching [8][9]: Is the photochemical destruction of a fluorophore. In microscopy, photobleaching can complicate the observation of fluorescent molecules, since at some point they can be destroyed by the exposure of light necessary to stimulate them to produce fluorescence.
- Rotational Diffusion [3]: The origin of anisotropy is the existence of transition moments for absorption and emission that lie along specific directions within the fluorophore structure. Upon excitation with polarized light the emission can become depolarized by a number of processes, the relative importance of which depends upon the sample under investigation. Rotational diffusion changes the direction of the transition moments and is one common cause of depolarization.

1.3 Quantum Yield Standards

In order to estimate the quantum yield of a fluorophore, it use quantum yield standard components, some of which are indicated in the Figure 4.

The quantum yields of these components are generally independent of the excitation wavelength. The quantum yield of unknown component is automatically calculated by comparing the integrated intensity of the wavelength of the unknown with that of the standard [3].

$$Q = Q_R \frac{I}{I_R} \frac{OD_R}{OD} \frac{n^2}{n_R^2} \tag{1.6}$$

The optical density is maintained below 0.05 to avoid the effects of the internal filter, or the optical densities of the sample and the reference (R) coincide with the excitation wavelength.

Compound	Solvent	λ_{ex} (nm)	°C	Q	Reference
Quinine sulfate	0.1 M H_2SO_4	350	22	0.577	45
		366	–	0.53 ± 0.023	46
β-Carboline[a]	1 N H_2SO_4	350	25	0.60	40
Fluorescein	0.1 M NaOH	496	22	0.95 ± 0.03	47
9,10-DPA[b]	cyclohexane	–	–	0.95	48
9,10-DPA	"	366	–	1.00 ± 0.05	49–50
POPOP[c]	cyclohexane	–	–	0.97	48
2-Aminopyridine	0.1 N H_2SO_4	285	–	0.60 ± 0.05	50–51
Tryptophan	water	280	–	0.13 ± 0.01	52
Tyrosine	water	275	23	0.14 ± 0.01	52
Phenylalanine	water	260	23	0.024	52
Phenol	water	275	23	0.14 ± 0.01	52
Rhodamine 6G	ethanol	488	–	0.94	53
Rhodamine 101	ethanol	450–465	25	1.0	54
Cresyl Violet	methanol	540–640	22	0.54	55

[a]β-carboline is 9H-pyrido[3,4-β]-indole.
[b]9,10-DPA, 9,10-diphenylanthracene.
[c]POPOP, 2,2'-(1,4-phenylene)bis[5-phenyloxazole].

Figure 4: Quantum Yield Standards [3].

where Q is the quantum yield, I is the integrated intensity, OD is the optical density, and n is the refractive index. The subscript R refers to the reference fluorophore of known quantum yield. In this expression it is assumed that the sample and reference are excited at the same wavelength, so that it is not necessary to correct for the different excitation intensities of different wavelengths.

This expression is mostly intuitive, except for the use of the ratio of refractive indices of the sample (n) and reference (n_R). This ratio has its origin in consideration of the intensity observed from a point source in a medium of refractive index n_i, by a detector in a medium of refractive index n_0 (Figure 5). The observed intensity is modified by the ratio $(n_i/n_0)^2$. While

the derivation was for a point source, the use of the ratio was found to be valid for many detector geometries.

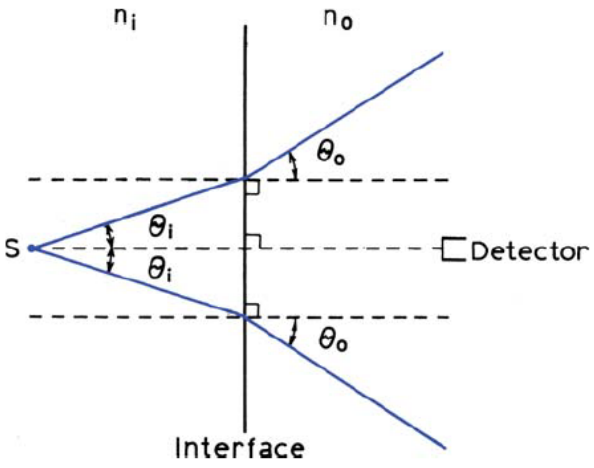
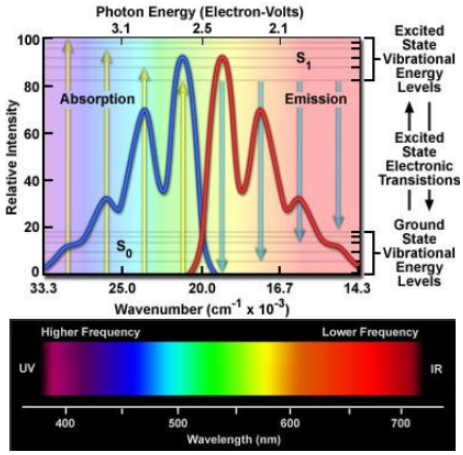


Figure 5: Refractive index effects in quantum yield measurements (n0 < ni) [3].

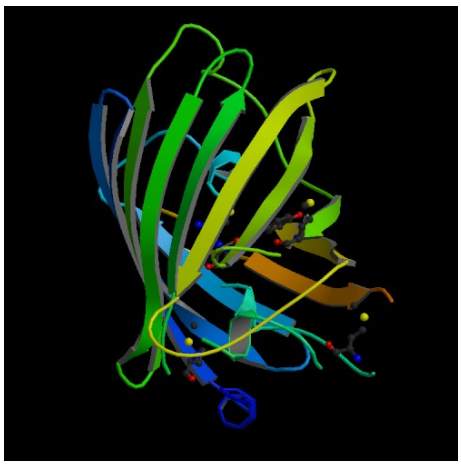
1.4 Biochemical Fluorophores

A fluorophore is a component of a molecule that makes it fluorescent. It is a functional group of the molecule that will absorb energy of a specific wavelength and will emit it again in a specific one of greater wavelength (that is, with less energy). The amount of energy emitted and its wavelength depend on both the fluorophore itself and its chemical environment [10].

This technology is particularly important in the field of biochemistry and protein studies. For example, the green fluorescent protein (or GFP) is a protein produced by the jellyfish Aequorea victoria, which emits fluorescence in the green zone of the visible spectrum. The gene encoding this protein is isolated and is commonly used in molecular biology as a marker [11].



(a) Electronic Absorption and Emission Bands [12].



(b) Green Fluorescent Protein [11].

Figure 6: (a) Electronic Absorption and Emission Bands.(b) Green Fluorescent Protein

We will study the NBD-X fluorescent molecule which can be seen below in the Figure 7. The nitrobenzoxadiazole (NBD) is a popular fluorescent membrane probe. However, understanding of important aspects of the photophysics of NBD remains incomplete, including the change observed in the NBD lipid emission spectrum at longer wavelengths after excitation at the red edge of the absorption spectrum (red-edge excitation shift or REES). REES of NBD lipids in membrane environments has been previously interpreted as reflecting restricted mobility of the solvent surrounding the fluorophore.

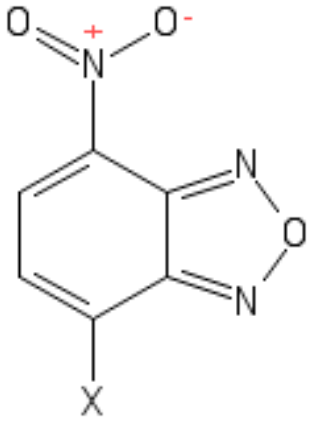


Figure 7: Molecule of NBD-X

We find in the journals more articles tall about the different uses of this

molecule, but mainly focused on its use for medicine, some of these papers are:

- Article 1: In this Letter, they demonstrate a laser emission produced by the coupling of the evanescent whispering gallery modes that resonate in a cylindrical microresonator to a newly developed gain medium. This medium is formed by attachment of a 7-nitrobenzo [c] [1,2,5]-oxadiazol-4-yl fluorescent tag to tamoxifen [13].
- Article 2: Dual-reactive and dual-quenching fluorescent probes based on thiolysis of NBD (7-nitro-1,2,3-benzoxadiazole) thioether/ether/amine for separate detection of H2S and Cys/Hcy were rationally designed [14].
- Article 3: They report the synthesis and self-assembly of fluorescent peptide amphiphiles (NBD-PA). It would facilitate the design of in situ pH detection systems as well as pH-responsive actuators for various applications in future [15].
- Article 4: The reactivity and selectivity of NBD-based dyes are discussed. The design principles of NBD-based probes are highlighted. The advantages and disadvantages of NBD-based probes are compared, and their biological applications are discussed [16].

Abstract

En este apartado se definen los distintos puntos en los que consistirá este trabajo para estudiar los derivados del NBD que nos proporcionarán en el IPNA y de qué manera se llevarán a cabo los distintos puntos desde la síntesis, la selección de un fluorescente estándar, las medidas y los cálculos para determinar el QY de los derivados hasta la síntesis de un fármaco fluorescente.

2 Objectives

The objective of this work is to study a series of NBD derivatives, in order to determine their QY and find which one has the best QY, select it and from there synthesize a drug (an anti-inflammatory) linked by covalent bonds to the derivative NBD and that it does not affect the therapeutic activity of the drug. And since it is a fluorescent anti-inflammatory, the activity of the drug in cells could be visually followed, using confocal microscopes or fluorescence microscopes.

2.1 Synthesis of NDB Derivatives

The synthesis of the NBD derivatives will be carried out in collaboration with our colleagues from the IPNA (CSIC La Laguna [1]) , where they will carry out all the chemical method necessary to synthesize said compounds (which are indicated in the Figure 13).

2.2 Selection of a Fluorescent Standard

For the selection of a standard, we search the available literature for articles or experimental methods of QY measurement, to see which different candidates for standard compounds will be better when determining QY.

2.3 Fluorescence Measurements to Determine QY

Next, the measurements will be made to determine the QY, specifically we will need a measure of the absorption and another measure of emission of all the compounds derived from NBD and the standard selected above.

2.4 Analysis of Results and Determination of QY

Once the pertinent measurements have been obtained, the results will be analyzed, where it will be studied whether there are any possible errors in the measurements to be able to carry it out again and check that the results make some sense. After confirming them, the QY determination will be carried out, using the data from the measurements and those that need to be searched in the bibliography.

2.5 Synthesis of a Fluorescent Drug

To finish, the NBD derivative with the best QY will be used, to bind it to an anti-inflammatory and see that it does not affect the therapeutic activity of the drug and that its activity can be followed.

Abstract

En este capítulo se describe la base teórica para entender cuáles son las medidas que necesitamos hacer si queremos medir el QY de un compuesto. Es decir lo que nos interesa medir en cualquier experimento de luminiscencia, como son el espectro de absorción, y la relación que existe entre el espectro de emisión (aparte del desplazamiento de la longitud de onda con respecto a la absorción en el espectro de emisión) y el de excitación. Además del conjunto de conocimientos previos que había a disposición gracias al conjunto de investigaciones y colaboraciones previas.

3 Theoretical Background

In any luminescence experiment, we will be interested in analyzing the emitted light, but we will also analyze what types of radiation causes the excitation of the material. We will begin by defining, for a system of three energy levels, the absorption, emission and excitation spectra of the luminescence.

3.1 Absorption Spectrum

All atoms and molecules absorb radiation of certain characteristic frequencies corresponding to the transitions of their electrons to excited states. The representation of these characteristic absorption frequencies is what is called the absorption spectrum.

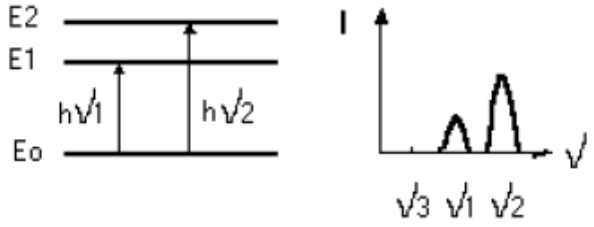


Figure 8: Diagram.

3.2 Emission Spectrum

The decay of electrons at lower energy levels as a result of de-excitation leads to the emission of radiation of certain frequencies corresponding to these transitions. These frequencies make up the emission spectrum.

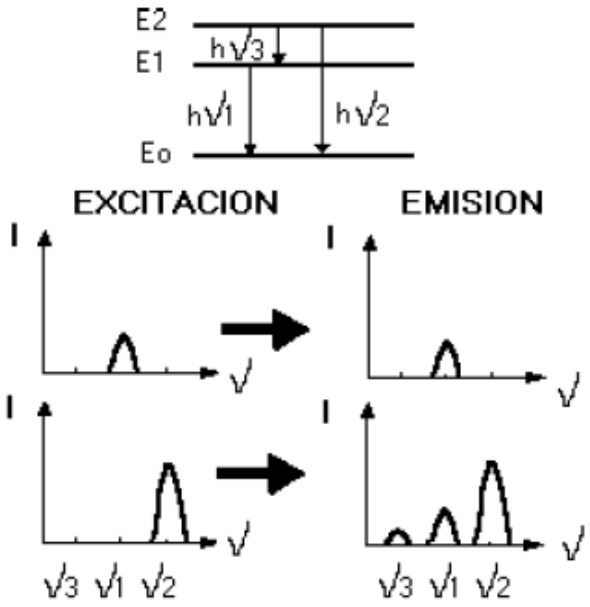


Figure 9: Diagram.

In other words, if we radiate the sample with an energy of a certain frequency, which we can easily isolate by means of a monochromator, the material will emit at specific frequencies.

3.3 Luminescence Excitation Spectrum

We define the excitation spectrum as those frequencies of the incident radiation that allow us to obtain each of the frequencies corresponding to the emission spectrum.

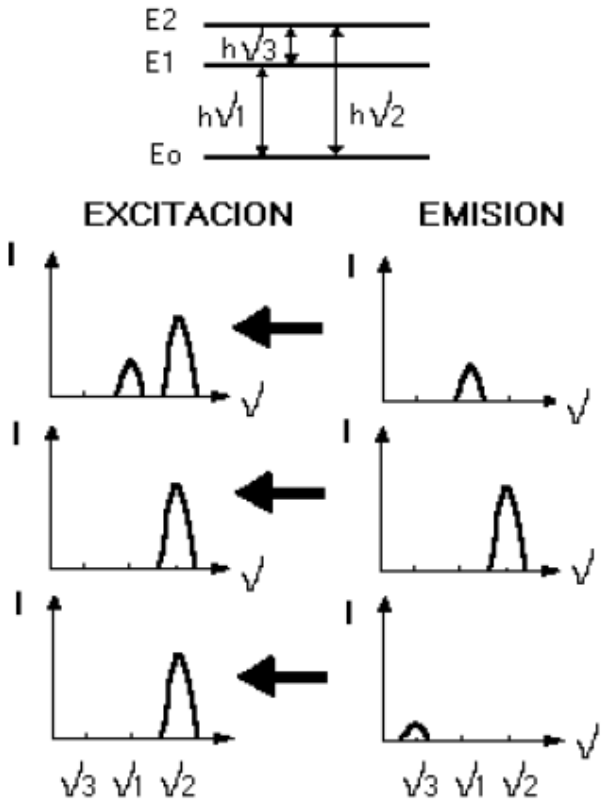


Figure 10: Diagram.

That is, if in the material emission spectrum we isolate a frequency line n, we want to know with what energies (and therefore, frequencies) we can radiate our material so that we can see that particular line (luminescence excitation spectrum).

3.4 Our Previous Results

As for other knowledge related to the study of NBD derivatives, to form a fluorescent molecule we already have a set of previous knowledge.

Due to the work carried out by a set of collaborations between different researchers on the study of a compound FLTX1, which is a fluorescent drug formed by the covalent binding of tamoxifen and nitro-2-1,3-benzoxadiazol-4-yl (NBD) dye, a common biomarker for lipids and hydrophobic environments.

We can research these new derivatives of NBD and count on the experience of all those previous investigations, to be able to work and analyze the results with confidence.

In these collaborations, we see that the study of this molecule has required the joint work of many study disciplines, the support of many departments, institutes and laboratories. Some articles on the different colorations to investigate FLTX1 are:

- Article 1: First article mentioned in the section 1.4.
- Article 2: In this article they conclude that the fluorescent derivative FLTX1 is not only a suitable probe for studies on the molecular pharmacology of tamoxifen, but also a potential therapeutic substitute to tamoxifen, endowed with potent antiestrogenic properties but devoid of uterine estrogenicity [17].

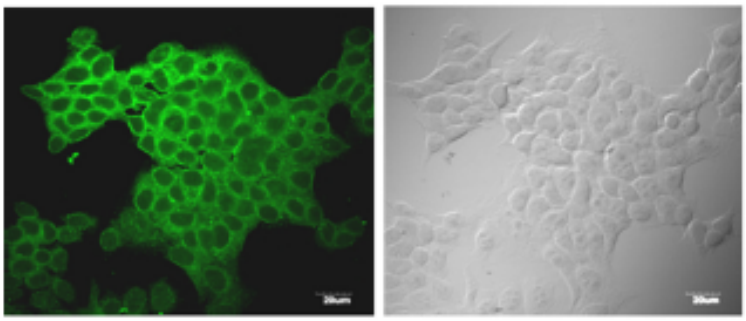


Figure 11: Specificity of FLTX1 labeling in MCF7 cells [17].

- Article 3: In this article, the agreement with an external amplified spontaneous emission (ASE) spatial propagation model, as well as the lack of optical feedback in the walls of the dilution cuvette confirms that ASE is the physicalmechanism that explains the high efficiency observed. The waveguide character and the polarization dependence of ASE are also studied [18].
- Article 4: In this article, they have demonstrated that chemically modified anticancer drugs can provide random laser (RL) when infiltrated in a biological tissue. These results show that RL could be obtained from other drugs, if properly marked with a fluorescent tag, which could be appealing for new forms of combined opto-chemical therapies [19].

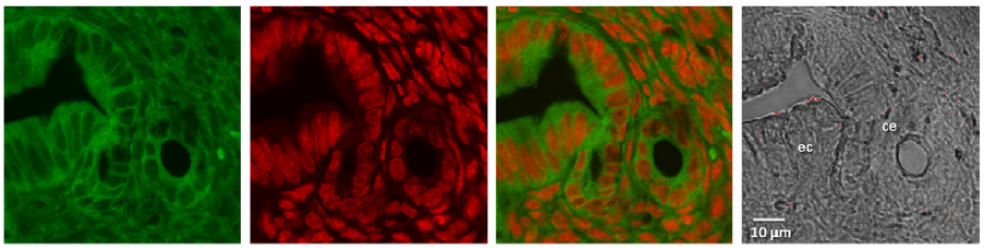


Figure 12: Confocal microphotographs of mouse uterine tissues labelled with FLTX1 solutions. Illustrated are, from left to right, FLTX1, propidium iodide, merged and phase contrast transmission images [19].

- Article 5: In this review, they will show its laser properties under three different configurations. First, as amplified spontaneous emission or mirrorless laser; second, through the evanescent field of WGMs of a ring resonator around an optical fiber; and finally as random laser in uterine tissues impregnated with the prodrug. Further, they observed another emergent property for FLTX1: this molecule, but not tamoxifen alone or NBD, was able to generate reactive oxygen species (ROS) upon irradiation. This property is extremely interesting as FLTX1 might be used for photodynamic therapy [20].

Abstract

En esta sección se describe la metodología llevada a cabo para realizar el experimento, aparte de indicar los equipos utilizados, también se indica como se prepararon las muestras y el proceso de medida llevado a cabo, indicando parámetros instrumentales y proporciones de los compuestos, una vez se tenían todas las medidas se procede al cálculo del QY y de su error usando las fórmulas indicadas, y utilizando un ordenador para agilizar el procedimiento de cálculo.

4 Methodology

So for the study of the fluorescence phenomenon of the samples we need to know the absorption and emission of the samples to compare them with a standard compound (see Figure 4).

Optical absorption measurements in the visible spectral range were performed in a spectrophotometer equipped with cuvette holders for measurement of solutions (Agilent Cary 5000 [21]). The photoluminescence spectra in the visible region and the decay of the fluorescence were obtained exciting with a 405/470 nm picosecond pulsed diode laser (Edinburgh Instruments EPL-405/470 [22]) with a typical pulse width of 80 ps.

The emission spectra and the fluorescence decays were recorded using a fluorescence spectrometer with a single photon counting multichannel plate photomultiplier and a dedicated acquisition software (Edinburgh Instruments LifeSpec II [23] and F900 software, respectively). All measurements were conducted at room temperature.

4.1 Samples Preparation

4.1.1 NBD Derivatives

The study samples (see Figure 13) were synthesized at the Instituto de Productos Naturales del CSIC.

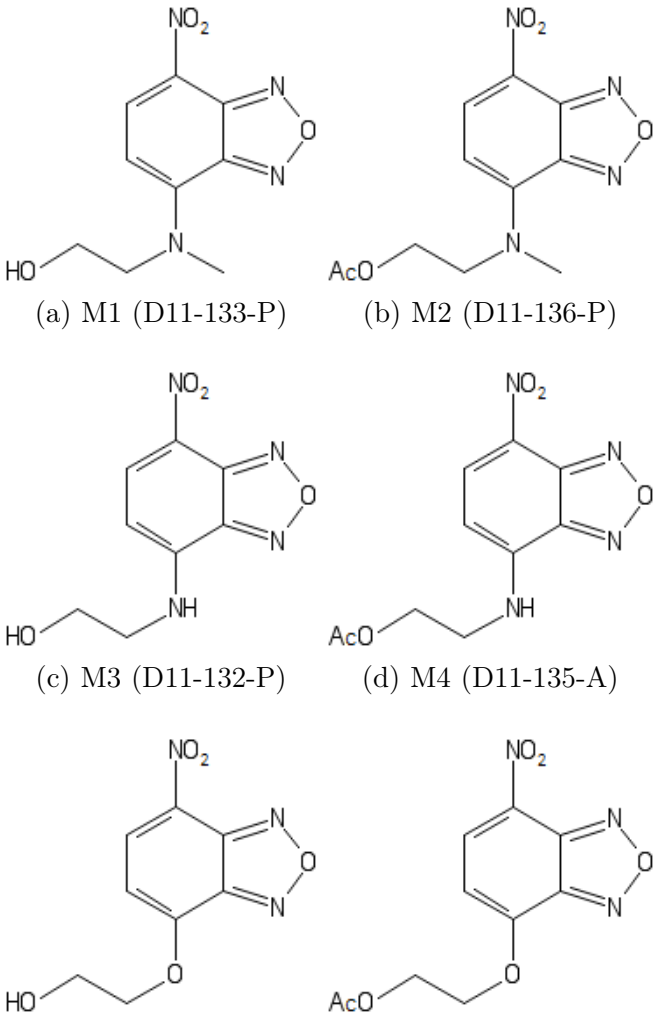


Figure 13: Samples.

4.1.2 Standard

This dissolution of standard was prepared from me, the reference sample was the fluorescein (see Figure 14). Specifically Fluorescein dissolved in NaOH with a concentration of 0.1M, in this way the comparison can be made with the study molecules and thus be able to measure the quantum yield of these [24].

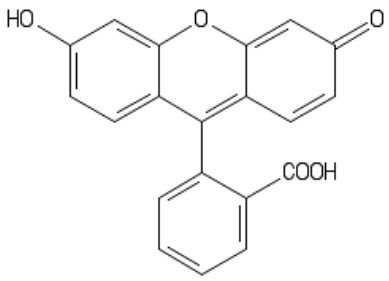


Figure 14: Fluorescein.

4.2 Process

Next, to start with the data collection, it is necessary to achieve an absorption measurement with the absorption peak around 0.04 for all the compounds (this is important to be able to neglect reabsorption effects in emission measurements, since the reabsorption of the emission could falsify the QY result), so the samples are diluted, because the absorption is related to the concentration (Beer's law [25]).

Once the solution is obtained with the appropriate concentration, the samples are introduced into the equipment (absorption) and we prepare the characteristics of the measurement that they want to do:

Matter	Start (nm)	Stop (nm)	Y Mode	Y min	Y max	Ave time (s)	Data Interval (nm)	Scan rate (nm/min)
Fluorescein	800.000	400.000	Abs	-0.50	2.00	1.000	1.000	60.000
M1	800.000	400.000	Abs	-0.50	2.00	1.000	1.000	60.000
M2	800.000	400.000	Abs	-0.50	2.00	1.000	1.000	60.000
M3	800.000	400.000	Abs	-0.50	2.00	1.000	1.000	60.000
M4	800.000	400.000	Abs	-0.50	2.00	1.000	1.000	60.000
M5	800.000	250.000	Abs	-0.50	2.00	1.000	1.000	60.000
M6	800.000	250.000	Abs	-0.50	2.00	1.000	1.000	60.000

Figure 15: Absorption measures characteristics.

When the parameters are defined, the measurement is launched, previously saving the file with the name of the sample M1 (it should be noted that to facilitate the treatment of the samples, the names of the samples were simplified to M1-M6), then the file was saved in ascii format for further processing.

As soon as we obtain the absorption curve, if we want to carry out the study of the emission, we must see where the absorption spectra are and for

this we resort to the graphs, to extract the frequency to which we have to excite the sample.

To start the emission measurements of the samples, the equipment must be prepared for the emission measurements, follow the steps (on the sheet) the equipment was turned on, the characteristics of the measurement were prepared and it was verified that the temperature of the equipment this at -20°C.

As some of the samples have a different absorption frequency than the rest, the laser source must be changed for a more suitable one, once assembled, the measurement parameters are prepared and launched, but before that, a filter must be placed to that the sensor signal is not contaminated, since the sample, when excited, emits in all directions.

Matter	Wavelength (nm) of the laser	Filter (nm)	Start (nm)	Stop (nm)	Step (nm/s)	Dwell Time (s)	Repeats
Fluorescein	470	495	480.00	750.00	1.00	1.00	1
M1	470	495	500.00	750.00	1.00	1.00	1
M2	470	495	500.00	750.00	1.00	1.00	1
M3	470	495	500.00	750.00	1.00	1.00	1
M4	470	495	500.00	750.00	1.00	1.00	1
Fluorescein	405	420	400.00	750.00	1.00	1.00	1
M5	405	420	425.00	750.00	1.00	1.00	1
M6	405	420	425.00	750.00	1.00	1.00	1

Figure 16: Emission measures characteristics.

It should be mentioned that it should not be measured in the measurement range, including the same wavelength as that in which the laser emits, since it could saturate the measurement and damage the equipment.

4.3 QY Calculation

Once all the measurements were made, the Python programming platform was used to make the necessary calculations for the QY, specifically the values of the I (which is the area under the emission curve in a range of wavelengths) were obtained for the samples as for reference, the value of A for the wavelength of the excitation source used for the emission experiment was also extracted too, to carry out the calculation of errors.

After extracting the necessary data and having searched the bibliography for other important data such as the QY of the reference (see Figure 4) and

the refractive indices (n) of the solvents used (DMSO [26] and water [27]), we perform the operation:

$$QY = QY_{STD}\frac{I}{I_{STD}}\frac{A_{STD}}{A}\frac{n^2}{n^2_{STD}} \tag{4.1}$$

$$\Delta QY = \left|\frac{QY}{QY_{STD}}\right|\Delta QY_{STD} + \left(\left|\frac{QY}{A_{STD}}\right| + \left|\frac{-QY}{A}\right|\right)\Delta A +$$

$$\left|\frac{QY}{I}\right|\Delta I + \left|\frac{QY}{I_{STD}}\right|\Delta I_{STD} + \left|2\frac{QY}{n}\right|\Delta n + \left|-2\frac{QY}{n_{STD}}\right|\Delta n_{STD} \tag{4.2}$$

Abstract

A continuación se presentan los resultados obtenidos para las distintas muestras utilizando las medidas obtenidas siguiendo el procedimiento indicado en el capítulo anterior. Además se presentan las distintas graficas de las medidas donde se pueden apreciar las diferencias entre un espectro de absorción y uno de emisión, y como los picos de absorción entorno a una frecuencia condicionaba el uso de una fuente laser para las medidas de emisión.

5 Results

The data obtained are those that appear in the following table 17.

Matter	Wavelength (nm) of the laser	Abs ± ΔAbs	I ± Error
Fluorescein	470	0.0129	1932310.7 ± 135.0
M1	470	0.0362	88793.0 ± 125.0
M2	470	0.0349	18359.3 ± 125.0
M3	470	0.0299	1091488.3 ± 125.0
M4	470	0.0360	102071.7 ± 125.0
Fluorescein	405	0.0009	677742.7 ± 175.0
M5	405	0.0269	440.3 ± 162.5
M6	405	0.0132	664.9 ± 162.5
Other date	$n_{Water} = 1.33 \pm 0$ n_{DMSO} (λ=470nm) = 1.4869 ± 0 n_{DMSO} (λ=405nm) = 1.4968 ± 0 $\Delta Abs = 5 \cdot 10^{-5}$		

Figure 17: Data used and QY results.

5.1 M1 (D11-133-P)

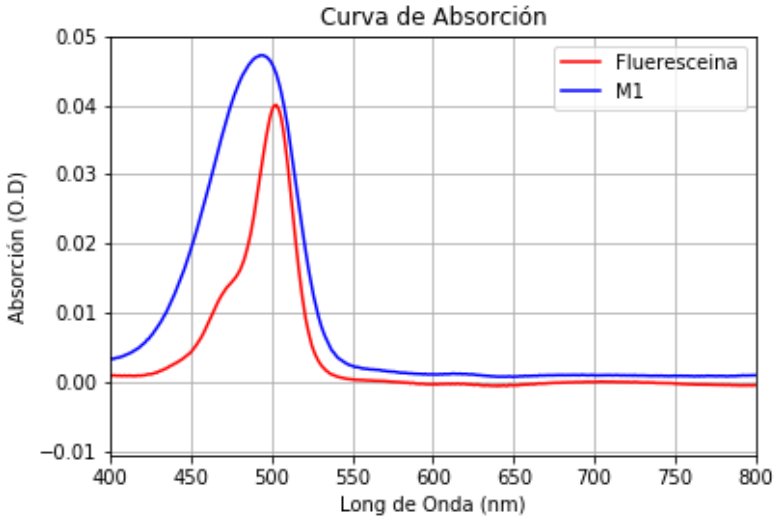


Figure 18: Absorption curve.

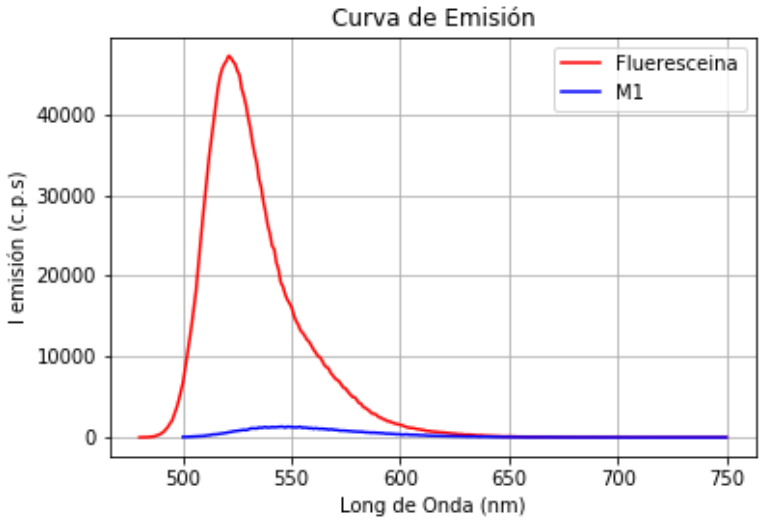


Figure 19: Emission curve.

The absorption spectrum was taken using the parameters in the table 15, for M1 with a concentration of 0.015mM, the absorption has a maximum centered at 485.9nm and 60nm half width at approximately mid-height; and

the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

As for the emission I, it was taken using the parameters in the table 16, we see that for M1 the emission spectrum is centered at 550nm and has a half width of approximately 60nm; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is a clear difference of a factor x difference.

5.2 M2 (D11-136-P)

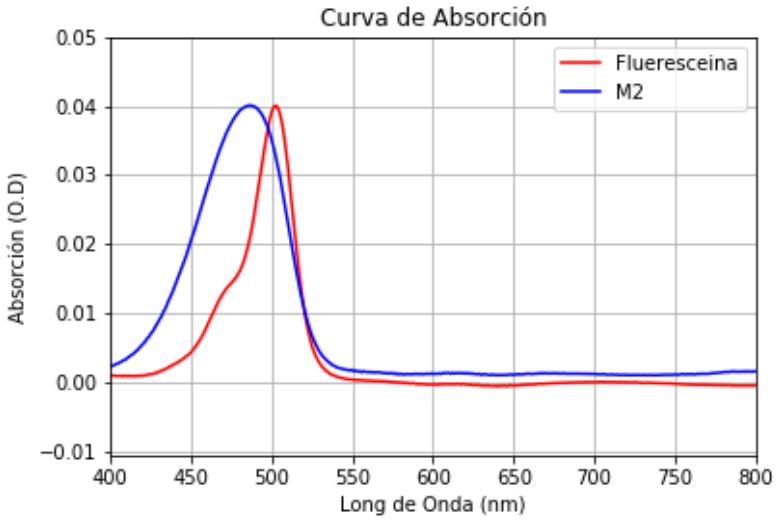


Figure 20: Absorption curve.

The absorption spectrum was taken using the parameters in the table 15, for M2 with a concentration of 0.005mM, the absorption has a maximum centered at 485.9nm and 65nm half width at approximately mid-height; and the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

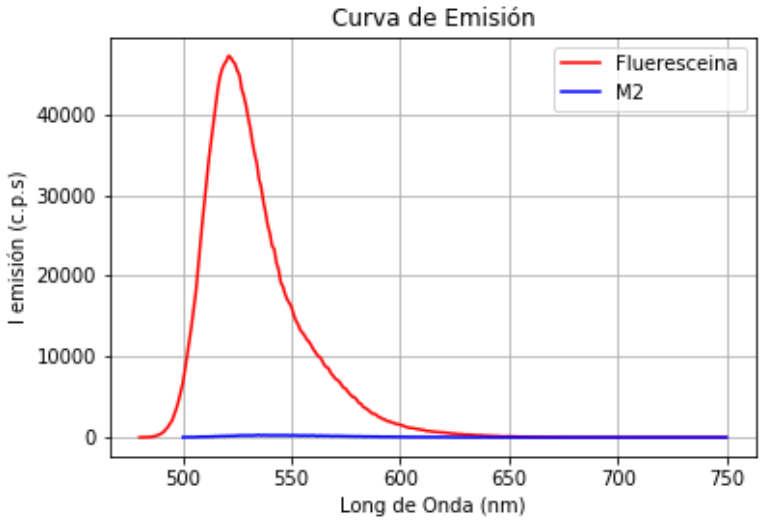


Figure 21: Emission curve.

As for the emission I, it was taken using the parameters of the table 16, we see that for the M2 the emission spectrum is not seen with the naked eye that there is emission; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is an immense difference.

5.3 M3 (D11-132-P)

The absorption spectrum was taken using the parameters in the table 15, for M3 with a concentration of 0.005mM, the absorption has a maximum centered at 480nm and 70nm half width at approximately mid-height; and the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

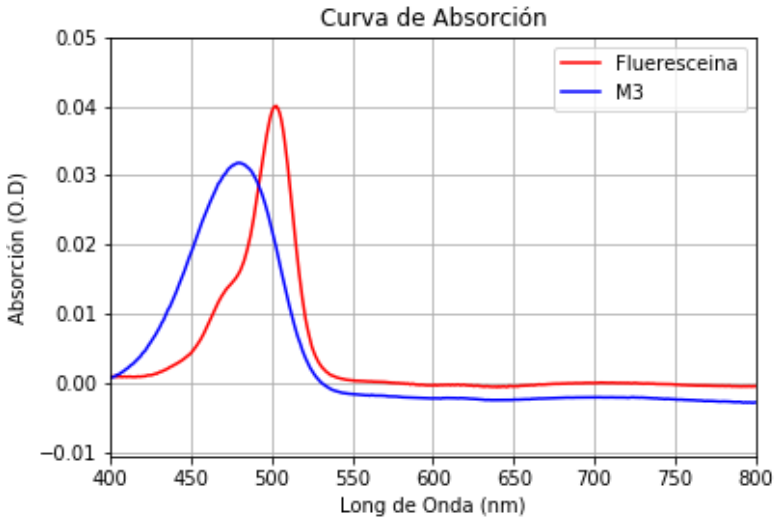


Figure 22: Absorption curve.

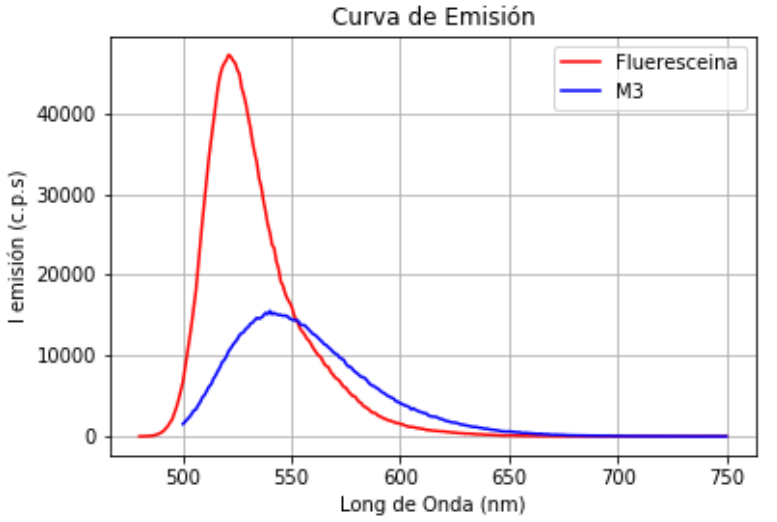


Figure 23: Emission curve.

As for the emission I, it was taken using the parameters in the table 16, we see that for M3 the emission spectrum is centered at 540nm and has a half width of approximately 75nm; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is a clear difference.

5.4 M4 (D11-135-A)

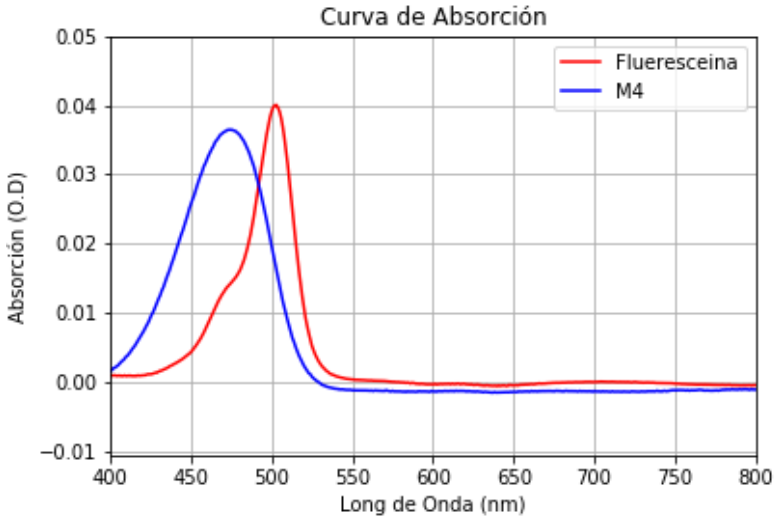


Figure 24: Absorption curve.

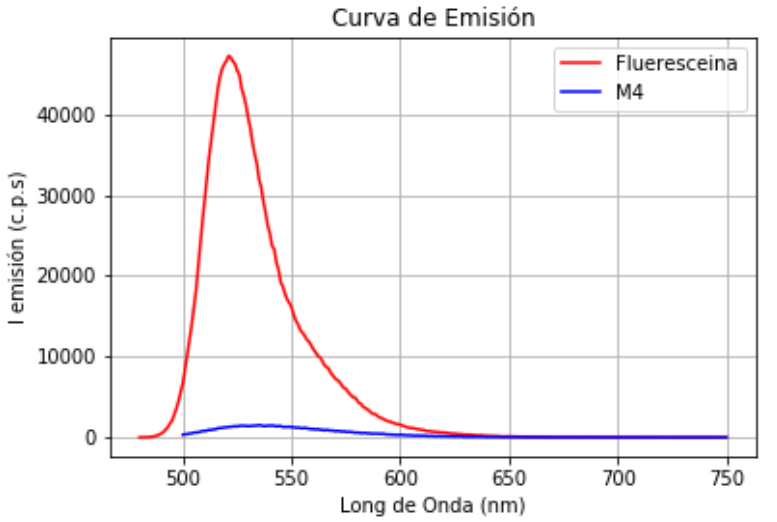


Figure 25: Emission curve.

The absorption spectrum was taken using the parameters in the table 15, for M4 with a concentration of 0.005mM, the absorption has a maximum centered at 474nm and 60nm half width at approximately mid-height; and

the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

As for the emission I, it was taken using the parameters in the table 16, we see that for M4 the emission spectrum is centered at 535nm and has a half width of approximately 70nm; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is a clear difference of a factor x difference.

5.5 M5 (D11-134-P)

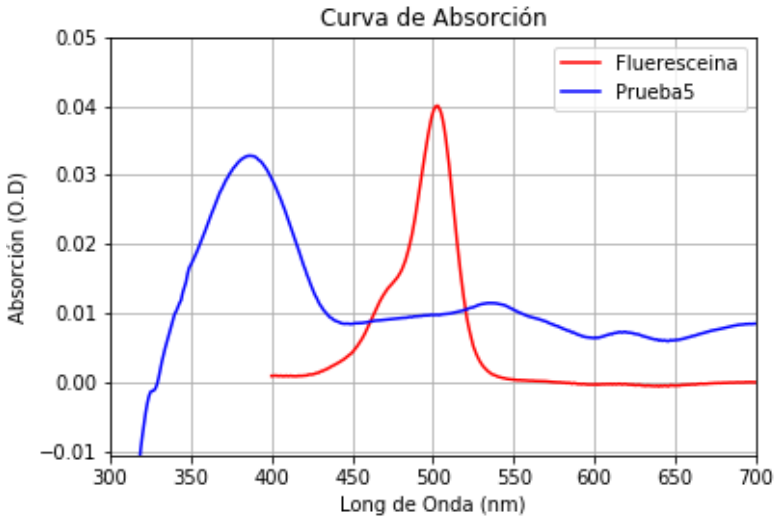


Figure 26: Absorption curve.

The absorption spectrum was taken using the parameters in the table 15, for M5 with a concentration of 5μM, the absorption has a maximum centered around 390nm (for this reason we must change the laser to one with a wavelength closer to the absorption wavelength of the sample, in this case we use the 405nm laser for emission measurement) and 70nm half width at approximately mid-height ; and the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

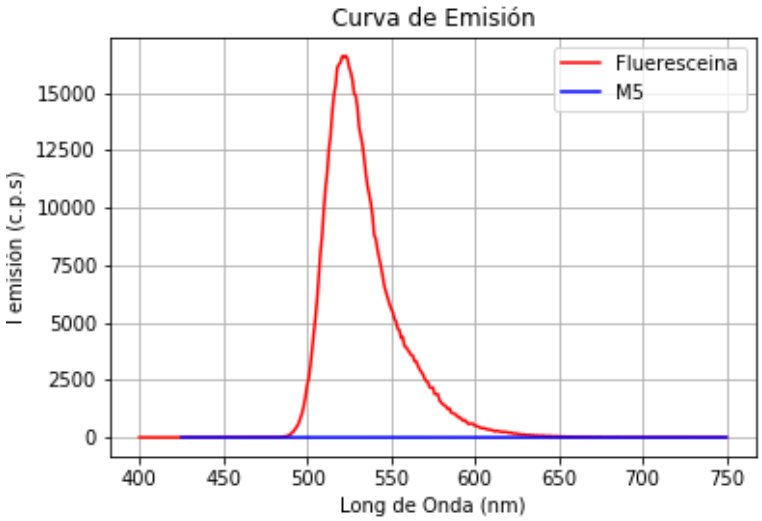


Figure 27: Emission curve.

As for the emission I, it was taken using the parameters of the table 16, we see that for the M5 the emission spectrum is not seen with the naked eye that there is emission; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is an immense difference.

5.6 M6 (D11-137-P)

The absorption spectrum was taken using the parameters in the table 15, for M6 with a concentration of 5μM, the absorption has a maximum centered at 385nm (we also use the 405nm laser for emission measurement) and 60nm half width at approximately mid-height; and the fluorescein has the maximum at 500nm and a half width of approximately 35nm, therefore they are comparable.

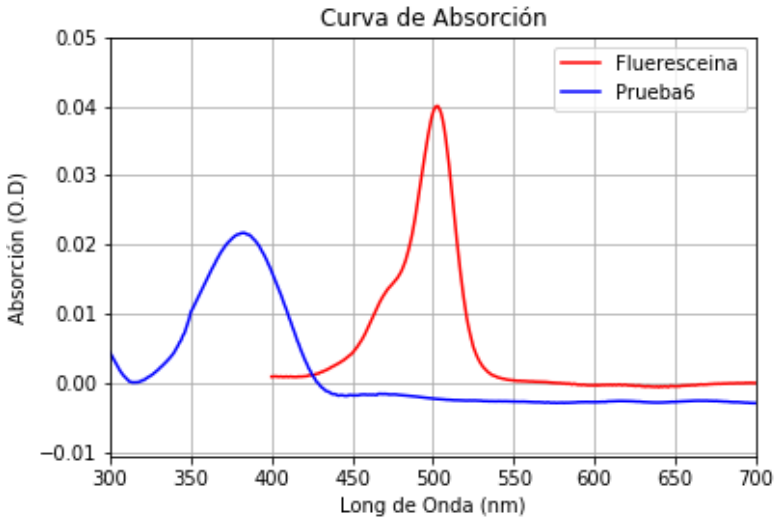


Figure 28: Absorption curve.

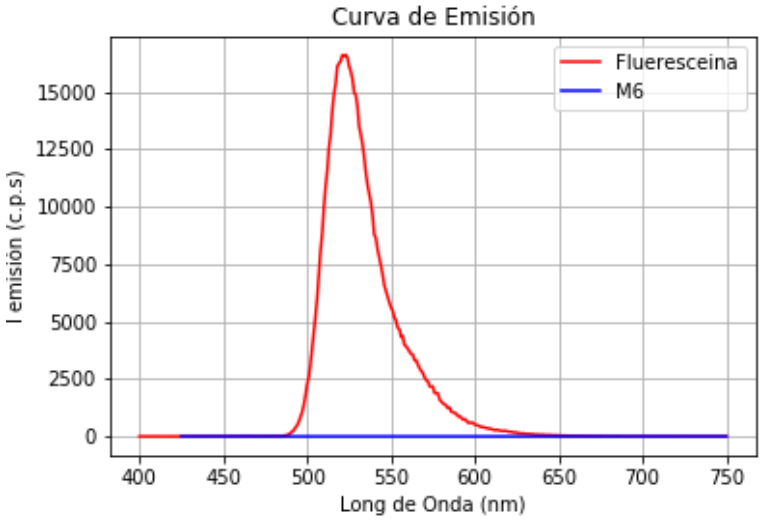


Figure 29: Emission curve.

As for the emission I, it was taken using the parameters of the table 16, we see that for the M6 the emission spectrum is not seen with the naked eye that there is emission; and for fluorescein we see that the emission spectrum is centered at 525nm and has a half width of 30nm, here there is an immense difference.

Abstract

En este capítulo se procede a analizar los datos y cálculos llevados a cabo para determinar cuál de entre todos los derivados del NBD es el que tiene un mejor QY. Para ello se realizan los cálculos del QY pero también se analizan las gráficas de emisión las cuales incluso sin realizar cuentas ya nos aportan información. También para dicho análisis hay que tener en cuentas la estructura química ya que juega un papel importante dentro del estudio, pues aporta matices a la hora de estudiar que molécula es mejor.

6 Discussion of Results

The data obtained are those that appear in the following table 30. The equation 4.1 was used to calculate QY. And the equation 4.2 was used to calculate the error of the QY.

Matter	Wavelength (nm) of the laser	Abs ± ΔAbs	I ± Error	QY ± Error
Fluorescein	470	0.0129	1932310.7 ± 135.0	0.95 ± 0.03
M1	470	0.0362	88793.0 ± 125.0	0.0194 ± 0.0007
M2	470	0.0349	18359.3 ± 125.0	0.0042 ± 0.0002
M3	470	0.0299	1091488.3 ± 125.0	0.2893 ± 0.0108
M4	470	0.0360	102071.7 ± 125.0	0.0225 ± 0.0009
Fluorescein	405	0.0009	677742.7 ± 175.0	0.95 ± 0.03
M5	405	0.0269	440.3 ± 162.5	$(2.6155 \pm 1.2729)\ 10^{-5}$
M6	405	0.0132	664.9 ± 162.5	$(8.0486 \pm 2.8520)\ 10^{-5}$
Other date	$n_{Water} = 1.33 \pm 0$ n_{DMSO} (λ=470nm) = 1.4869 ± 0 n_{DMSO} (λ=405nm) = 1.4968 ± 0 $\Delta Abs = 5 \cdot 10^{-5}$			

Figure 30: Data used and QY results.

Here we indicate the calculations made for each of the samples, replacing the data in the table.

$$QY_{M1} = 0.95 \frac{88793.0}{1932310.7} \frac{0.0129}{0.0362} \frac{1.4869^2}{1.33^2} = 0.0194 \tag{6.1}$$

$$QY_{M2} = 0.95 \frac{18359.3}{1932310.7} \frac{0.0129}{0.0349} \frac{1.4869^2}{1.33^2} = 0.0042 \tag{6.2}$$

$$QY_{M3} = 0.95\frac{1091488.3}{1932310.7}\frac{0.0129}{0.0299}\frac{1.4869^2}{1.33^2} = 0.2893 \tag{6.3}$$

$$QY_{M4} = 0.95\frac{102071.7}{1932310.7}\frac{0.0129}{0.0360}\frac{1.4869^2}{1.33^2} = 0.0225 \tag{6.4}$$

$$QY_{M5} = 0.95\frac{440.3}{1932310.7}\frac{0.0009}{0.0269}\frac{1.4968^2}{1.33^2} = 2.6155 * 10^{-5} \tag{6.5}$$

$$QY_{M6} = 0.95\frac{148.417}{1932310.7}\frac{0.0009}{0.0132}\frac{1.4968^2}{1.33^2} = 8.0486 * 10^{-5} \tag{6.6}$$

It is observed with the naked eye with the emission graphs (see Figures 27 y 29), without the need to perform any operation, the QY result for samples M5 and M6, we can consider it to be a null QY, since the curve is flat; if we calculate we see that it is a factor 10^{-5}. This means that the chemical structures (see Figure 13) in which the NBD binds to an oxygen with ramifications are not good for emission to take place.

Among the remaining samples (M1-M4), if there is a clearly appreciable emission when observing the emission graphs, because we can see Gaussian curves (see Figures 19, 21, 23 y 25).

When calculating, we see that the QY are quite good for these other samples and we see that the NBD bound to an N or an NH with ramification they give rise to a good emission. But among them, the one that gives better results is the one that it has the NH.

The molecules with the highest QY are M3 and M4, the only difference between these two molecules is the radical at the end of the linker. Which from the point of view of molecular electronics should not influence as much because they are quite far from the main molecule that is NBD, and the electron cloud should not be so influenced.

This difference between the QY for these molecules could indicate that there was perhaps degradation of the M4 sample and it would be convenient to repeat the measurement to check if there is variation or not. In case that when repeating it, there was not a significant variation of the QY, we would confirm that there was no degradation and that the previous measurement was correct.

Therefore, what we would have to do before continuing to move forward, is to perform a new synthesis of compound M4 (which would be carried out

by our colleagues at IPNA), once done we must repeat all the previous procedures described in the Methodology section 4 to get the QY and compare.

As a result of this discussion of the results, sample M3 is chosen (see Figure 13c), to join it to the drug. The synthesis will be carried out by colleagues from the IPNA. And once it is done, the study of the complex will also be carried out, to see if it continues to maintain its fluorescent properties and thus continue its activity.

Unfortunately, during the development of this work, we have been involved in the health emergency caused by the COVID-19 crisis, a type of coronavirus that has led the population to confinement in their homes to curb the contagion rate. This state of alarm was declared on March 14, 2020, in the following link to the "Agencia Estatal Boletín Oficial del Estado" (see here [28]), we see the limitations on the freedom of movement of people by the which could not continue developing the project.

Specifically, the measures related to M4 (after its synthesis to check whether or not a degradation occurred, which could not be done either), in addition to the synthesis of the compound formed by the NBD derivative (M3) and the drug, they could not develop, as planned from the beginning, until before the quarantine took place.

Abstract

A modo de conclusión, se puede decir que el proyecto ha sido casi un éxito, pues se ha podido llevar a cabo la gran mayoría del trabajo, a excepción de una comprobación y el último apartado de los objetivos 2.5, pues apareció el Covid-19, pero aun así se pudo hacer un estudio y análisis de los datos para dar un candidato de entre todos eso derivados del NBD para la síntesis de un fármaco fluorescente, actividad que se podría hacer como parte de otro proyecto, al igual que un posible estudio matemático de la nube de electrones del NBD y sus posibles linkers.

7 Conclusions and Outlook

In this work, the phenomenon of fluorescence has been discussed, giving information on how it works and what are the mechanisms that cause it to stop having fluorescence, and all this, to study whether a set of NBD derivatives were good candidates to form a compound with a drug, to continue its journey through the body.

And like all work in which you want to measure fluorescence, it is necessary to know several of the concepts related to them, such as the absorption and emission spectra. That together with another set of data on the refractive indices of solvents, allowed us to carry out the QY accounts, that would indicate which of all those NBD derivatives would be the best candidate to use in the synthesis of a fluorescent drug.

But like all good experimental physicists, one cannot neglect to carry out a study of the possible errors that could have been committed, and then act accordingly, either discarding and repeating the measures that could have been wrong or that gave strange results, as in the case of M4 samples.

All these procedures have allowed us to know which of all those derivatives was the best candidate, specifically the M3 sample, although if the Covid-19 health crisis had not taken place, could have carried out the checks for M4 and the synthesis of the fluorescent drug.

This work has also allowed me to work with a fluorescent compound and its derivatives (which are new compounds without studying), which is kind of exciting for someone with my curiosity. This experience has also allowed me to reinforce and expand my knowledge in some areas of knowledge such as the characterization of materials, spectroscopy, laboratory work, chemistry, since I had to understand the theoretical foundations, but also know how to

analyze the implications of the results obtained and how it influences when deciding the candidates for an experiment..

As a possible future extension of this work, I can mention the realization of the sections mentioned above that could not be carried out, that is, the synthesis of the M4 to check the measurements and the synthesis of the fluorescent drug with the sample with the best QY .

It would also include a mathematical study of how the electron cloud is modified by making small variations in the chemical structure of the molecule. In order to make a better selection of candidate linkers to generate new derivatives of NBD and that do not greatly modify its fluorescent properties. Starting from the electronic cloud of the NBD, and studying the electronic cloud of the linkers to then "join" them as if it were a kind of "sp" link.

In addition, a study similar to those carried out for FLTX1, mentioned in section 3.4, could be carried out, but using the new compound once the fluorescent drug has been synthesized.

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