



Study of the reactivity and properties of fluorescent carbon dots

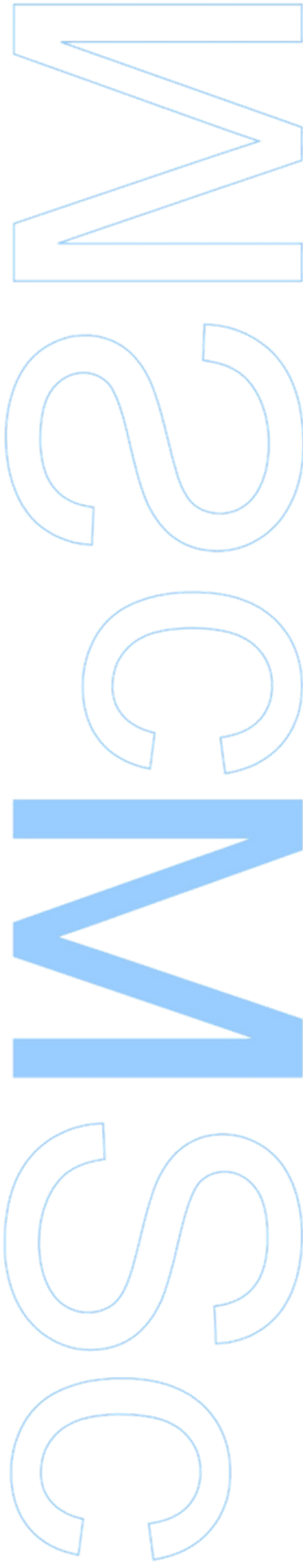
Ricardo Miguel Sá Sendão

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Master's degree in Biochemistry

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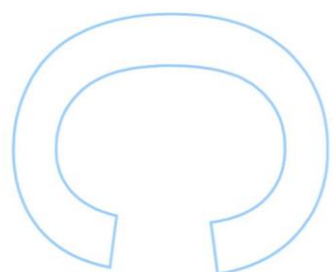
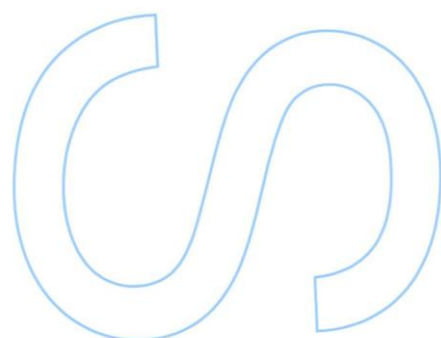
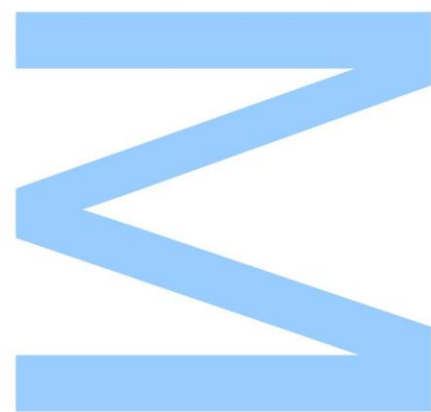
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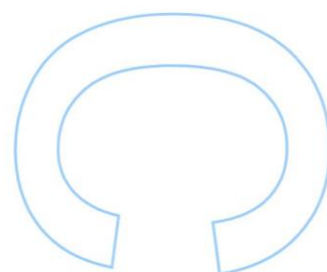
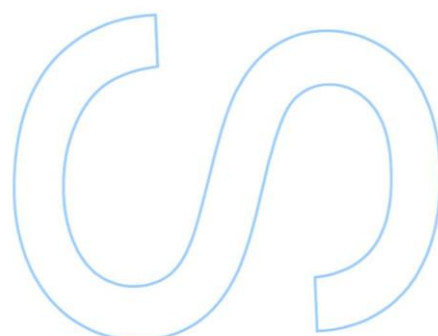
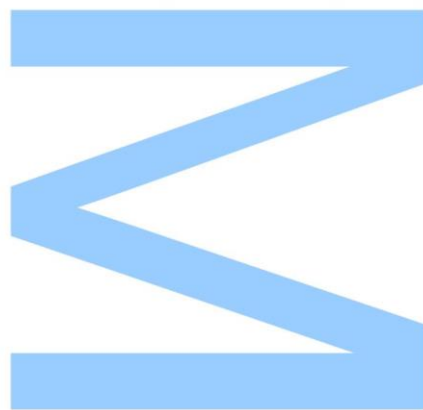
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO



Todas as _correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____



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Resumo

Carbon dots (CDs) são nanopartículas de carbono que possuem diversas propriedades óticas e electrónicas bastante vantajosas. Estas incluem uma alta fotoluminescência, absorção ótica de banda larga, baixa toxicidade, produção simples e de baixo custo, alta fotoestabilidade e estabilidade fotoquímica, são quimicamente inertes e apresentam boa solubilidade em água. Os CDs podem ter várias aplicações, entre as quais se encontram o uso em LEDs, *bioimaging*, sensores e biosensores, fotocatalise, terapia fotodinâmica, entre outros.

Uma das lacunas relativamente aos estudos de CDs é acerca do seu impacto no ambiente durante o ciclo de vida da nanopartícula. Dado que grande parte dos impactos originados durante o ciclo de vida de um nanomaterial advém da sua síntese, a avaliação do ciclo de vida (LCA) foi realizada relativamente à síntese de CDs usando as estratégias de síntese mais frequentemente utilizadas e usando como precursor ácido cítrico (um precursor extremamente comum) com a ocasional adição de ureia. Foi descoberto que de modo geral, quando a funcionalidade do CD (sob a forma do rendimento quântico, um parâmetro importante nos CDs) é usada como base da unidade funcional do estudo, sínteses por tratamento hidrotermal causam um maior impacto ambiental. Adicionalmente, a adição de ureia, devido ao grande aumento que causa no rendimento quântico da partícula originada, diminui largamente o impacto ambiental relativo associado à síntese.

Recentemente, alguns investigadores reportaram que parte da fotoluminescência anteriormente atribuída exclusivamente aos CDs resulta de produtos secundários moleculares fluorescentes (impurezas), que são produzidos durante a síntese dos CDs. Um CD, obtido a partir do tratamento por microondas de uma solução aquosa de ácido cítrico e ureia, e as impurezas formadas durante a sua síntese, foram caracterizados usando uma vasta gama de técnicas, incluindo HR-TEM, AFM, XPS, FT-IR, absorção UV-Vis, fluorescência e ESI-MS. Estudou-se o papel destes produtos fluorescentes na fluorescência de CDs na presença de nitrometano (uma molécula aceitadora de electrões) e difenilamina (dador de electrões). Observou-se que, quando presentes em conjunto numa mesma solução, o CD e as impurezas fluorescentes não se portam como duas espécies individuais. O resultado da co-existência destes componentes é mais do que um simples fenómeno aditivo das suas propriedades. Pelo contrário, apresentam um comportamento sinérgico em que a presença das impurezas afeta as propriedades óticas da nanopartícula em si mesma e vice-versa.

Por fim, o uso de CDs para aplicações catalíticas foi estudado. A reação de abertura do anel de um epóxido é um passo preliminar importante que teoricamente pode ser seguido por várias reações, como por exemplo conjugação com CO₂ ou reações de aminólise com compostos aminados. A capacidade de um CD baseado em 4-aminopiridina de causar a abertura do anel de um epóxido modelo (óxido de propileno) foi estudada e avaliada por RP-HPLC-DAD e estudos de fluorescência. Adicionalmente, a possibilidade de que à abertura do anel de um epóxido se possa seguir uma reação de aminólise quando na presença de anilina (composto aminado com um grupo NH₂) foi também estudada. O efeito da presença do CD no resultado da reação de aminólise foi avaliado através de GC-MS.

Palavras-chave: *carbon dots*, nanomateriais, síntese de nanopartículas, fluorescência, LCA, impacto ambiental, ciclo de vida, impurezas fluorescentes, sinergismo, reactividade, catálise, aminólise.

Deste trabalho resultaram dois artigos, sendo que um já se encontra publicado e outro encontra-se em revisão, em revistas científicas revistas por pares. Adicionalmente, deste trabalho também resultaram cinco comunicações dos resultados em conferências científicas a nível nacional e internacional.

Artigos:

- Ricardo M.S. Sendão, Maria del Valle Martínez de Yuso, Manuel Algarra, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Comparative Life Cycle Assessment of Bottom-Up Synthesis Routes for Carbon Dots Derived from Citric Acid and Urea*, Journal of Cleaner Production, *In revision*;
- Ricardo M.S. Sendão, Diana M.A. Crista, Ana Carolina P. Afonso, Maria del Valle Martínez de Yuso, Manuel Algarra, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Insight into the Synergistic Luminescence and Reactivity of Carbon Dots and Related Fluorescent Impurities*, Physical Chemistry Chemical Physics, 2019, 21, 20919-20926.

Comunicações:

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of CO₂ conversion into heterocyclic carbonates through organocatalysis*, XXIV Encontro Luso-Galego de Química, 2018, Porto (Portugal)
– Comunicação oral;

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of epoxide ring-opening reactions using carbon dots as organocatalysts*, 12º Encontro da Investigação Jovem da Universidade do Porto, 2018, Porto (Portugal) – Comunicação oral;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of the use of carbon dots as organocatalysts for epoxide ring-opening reactions*, 21st JCF Frühjahrssymposium and 2nd European Young Chemists Meeting, 2019, Brémen (Alemanha) – Comunicação em painel;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Insight into the interaction between fluorescent carbon dots and molecular by-products of their synthesis*, XXVI Encontro Nacional da Sociedade Portuguesa de Química, 2019, Porto (Portugal) – Comunicação oral;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Study of the interaction between fluorescent Carbon dots and the fluorescent by-products that result from their synthesis*, XXV Encontro Luso-Galego de Química, 2019, Santiago de Compostela (Espanha) – Comunicação oral.

Abstract

Carbon dots (CDs) are carbon-based nanoparticles that display several advantageous optical and electronic properties. Amongst these are included a high photoluminescence, broadband optical absorption, low toxicity, simple and low cost production, photostability, photo-chemical stability, chemical inertness and good water solubility. CDs can be used for several applications such as LEDs, bioimaging, sensing and biosensing, photocatalysis, photodynamic therapy, among others.

One of the gaps in the literature regarding CDs is information about the environmental impact caused during their life cycle. Given that the majority of the impacts originated during the life cycle of a nanomaterial result from their synthesis, a life cycle assessment (LCA) was made for the synthesis of CDs. This was done considering the most commonly used synthetic strategies while employing citric acid (an extremely common precursor) with the occasional addition of urea as precursors. It was observed that in general, when the functionality of the CD is considered (under the form of the quantum yield of fluorescence, an important parameter for CDs) and used as a base for the functional unit of the study, synthesis of CDs based in hydrothermal treatment cause the most pronounced environmental impacts. Furthermore, the addition of urea, which causes a great increase in the CD quantum yield of fluorescence, largely diminishes the relative environmental impact associated to the synthesis of the particle.

Recently, some authors reported that some of the photoluminescence previously attributed exclusively to CDs results from molecular fluorescent by-products (impurities) produced during the CD synthesis. A CD, obtained through the microwave-treatment of an aqueous solution of citric acid and urea, and the resulting impurities, were characterized using a range of techniques including HR-TEM, AFM, XPS, FT-IR, UV-Vis absorption, fluorescence and ESI-MS. The role of these fluorescent by-products in the fluorescence of CDs was studied in the presence of nitromethane (an electron-withdrawing molecule) and diphenylamine (an electron-donor). It was observed that, when present together in the same solution, the CD and the impurities do not behave as two separate species. The result from the co-existence of these components is more than just a simple additive phenomenon of their properties. Instead, they display a synergistic behaviour in which the presence of the impurities affect the optical properties of the nanoparticle and vice-versa.

Lastly, the use of CDs for catalytic applications was studied. The epoxide ring-opening reaction is an important preliminary step that theoretically can be followed by several reactions, such as conjugation with CO₂ or aminolysis reactions with aminated

compounds. The capacity of a 4-aminopyridine-based CD favoring the ring-opening in a model epoxide (propylene oxide) was assessed and evaluated by RP-HPLC-DAD and fluorescence studies. Additionally, the possibility of this ring-opening being followed by an aminolysis reaction when in the presence of aniline (aminated compound with an NH_2 group) was also studied. The effect of the CD presence in the reaction outcome was evaluated by GC-MS studies.

Keywords: carbon dots, nanomaterials, fluorescence, nanoparticles, environmental impact, LCA, life cycle, fluorescent impurities, synergism, reactivity, catalysis, aminolysis.

From this work resulted two scientific papers, one already published and another currently under revision, in peer-reviewed scientific journals. Additionally, from this work also resulted five communications in national and international scientific conferences.

Papers:

- Ricardo M.S. Sendão, Maria del Valle Martínez de Yuso, Manuel Algarra, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Comparative Life Cycle Assessment of Bottom-Up Synthesis Routes for Carbon Dots Derived from Citric Acid and Urea*, Journal of Cleaner Production, *In revision*;
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Communications:

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of CO_2 conversion into heterocyclic carbonates through organocatalysis*, XXIV Encontro Luso-Galego de Química, 2018, Porto (Portugal) – Oral communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of epoxide ring-opening reactions using carbon dots as organocatalysts*, 12º Encontro da Investigação Jovem da Universidade do Porto, 2018, Porto (Portugal) – Oral communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of the use of carbon dots as organocatalysts for epoxide ring-*

opening reactions, 21st JCF Frühjahrssymposium and 2nd European Young Chemists Meeting, 2019, Brémen (Germany) – Poster communication;

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Insight into the interaction between fluorescent carbon dots and molecular by-products of their synthesis*, XXVI Encontro Nacional da Sociedade Portuguesa de Química, 2019, Porto (Portugal) – Oral communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Study of the interaction between fluorescent Carbon dots and the fluorescent by-products that result from their synthesis*, XXV Encontro Luso-Galego de Química, 2019, Santiago de Compostela (Spain) – Oral communication.

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List of abbreviations

ACN – Acetonitrile;

CA – Citric acid;

CD – Carbon dot;

CD_{centrifuged} – Centrifuged CA,urea-based CD made by a microwave-assisted methodology;

CD_{dialyzed} – Centrifuged and dialyzed CA,urea-based CD made by a microwave-assisted methodology;

DPA – Diphenylamine;

DMF – Dimethylformamide;

DMSO – Dimethyl sulfoxide;

ENM – Engineered nanomaterial;

HOMO – Highest occupied molecular orbital;

K_{SV} – Stern-Volmer relationship constant;

LCA – Life cycle assessment;

LCI – Life cycle inventory;

LCIA – Life cycle impact assessment;

LUMO – Lowest unoccupied molecular orbital;

MeOH – Methanol;

NIR – Near infrared;

PAH – Polycyclic aromatic hydrocarbon;

PET – Photoinduced electron transfer;

QY_{FL} – Quantum yield of fluorescence;

QY_{FL}-FU – Functional unit of LCA study re-scaled with respect to the highest observed QY_{FL};

SWCNTs – Single-wall carbon nanotubes;

TDM – Transition dipole moment;

UV – Ultraviolet;

Water_{FI} – Wash waters from a CA,urea-based microwave-made CD dialysis.

1. Introduction

1.1. Carbon dots: origins and properties

Carbon is a black material that, until recently, was thought to be non-soluble in water or incapable of displaying fluorescent properties. However, while this is true for the bulk material, when nanomaterials based in carbon are produced, they display properties that greatly differ from those of the bulk material. [1-5] Several carbon-based nanomaterials are already known: single-wall and multi-wall carbon nanotubes, [2, 3, 6] nanodiamonds, [6] nanofibers, [5] graphene, [1, 2, 6] buckminsterfullerene [4] and more recently, carbon dots (CDs). Considering that each kind of carbon-based nanomaterial has specific properties that differ from those of the bulk material, and that each type of nanomaterial has properties that differ from the other kinds, there is a high potential for the development of carbon-based nanomaterials for new applications.

Quantum dots are nanoparticles that possess interesting optical and electronic properties. [7] CDs were first discovered in 2004 by Xu *et al.* [8] This novel group of nanomaterials was found during the production of single-wall carbon nanotubes (SWCNTs) when, after their fabrication through an arc discharge followed by purification through an electrophoretic method, two classes of nanomaterials were isolated from the crude soot originated during the synthesis. The components of those nanomaterials were a short tubular carbon structure and a mixture of fluorescent carbon-based nanoparticles derived from the SWCNTs synthesis, [8] being the latter later denominated as “carbon dots” by Sun *et al.* in 2006. [9] Since their discovery, CDs have become a regular field of interest for several research groups due to their properties and wide range of applications, being widely studied by the scientific community. For instance, due to their high photoluminescence, [10] an increased interest has arisen in several studies that aim to replace traditional fluorescent materials with fluorescent CDs, thus using their overall desirable properties. This resulted in an exponential growth in the number of scientific papers published regarding CDs since 2006. This was verified by Xiao and Sun, who observed the increase in the number of publications regarding CDs indexed in the Web of Science database using as keywords “carbon dots”, “C-dots”, “carbon nanodots” and “graphene quantum dots”, all of which are commonly used denominations for CDs. [11]

CDs are typically sized between 1 and 10 nm, [12, 13] although it's not uncommon to observe bigger sizes in CDs. [14, 15] They display a spherical or quasi-spherical shape with a core that might be amorphous or nanocrystalline, depending on the nanoparticle origin. [7, 12, 13] In fact, they are frequently described in terms of being a particle with a carbogenic core made by amorphous or crystalline parts with functional

groups on the surface. The particle core is mostly composed of graphitic carbon (sp^2 carbon) or graphene/graphene oxide sheets connected by sp^3 carbon atoms in between, organized in a diamond-like structure. [16, 17] On the surface, several functional groups can be found, such as amines, alcohols or carboxylic acids, [16, 18] which contribute for the CDs exceptional water solubility. Those functional groups also permit the CD to undergo further steps of passivation and functionalization as they provide a chemical base for the coupling of other molecules (e. g. ligands). These passivation and functionalization steps serve as a mean of enhancing the CD photoluminescent properties or modifying the CD physical properties and its interaction with other molecules, respectively. [16, 18] CDs possess tunable properties that depend on several factors, such as: variations in the precursors composition, the reaction conditions, the type of synthetic methodology employed and the post-synthetic treatment applied to the CDs. This means that the resulting nanoparticle will have a complex internal and external/surface structure and composition, which explains the extensive degree of variation obtainable regarding the optical properties displayed by CDs. To date, these properties are still elusive regarding their origin. [10]

CDs are known to possess many desirable properties such as high photoluminescence quantum yield (QY_{FL}), [12, 13, 19] a broadband optical absorption, [20] biocompatibility, [9, 16] low toxicity, [21] high photostability [18] and chemical stability, [22] and good water solubility. [12, 18] Additionally, CDs tend to have a lower toxicity than quantum dots (which are potentially toxic given that they use heavy-metal cores and can cause bioaccumulation), [23] while also displaying a larger Stokes shift, with the fluorescence wavelength maxima being able to greatly vary with the excitation wavelength. This means that changes in the excitation wavelength may greatly shift the emission spectrum. From this results that, if the CDs synthesis and post-synthetic treatments are done properly, absorption at wavelengths near the ultraviolet (UV) region can lead to emissions in the near-infrared (NIR) region. [24]

1.2. Carbon dots: applications

Because of their tunability and low toxicity, CDs are now commonly considered for use in fields for which quantum dots are usually less suited, such as the use for biological applications (e. g. imaging of tissues and organs). As seen in figure 1, they have already been used for applications such as bioimaging, sensing and biosensing, drug delivery systems and photodynamic therapy. Additionally, their properties and controlled synthesis also allow for CDs to be used in light emitting devices, nanothermometry, photocatalysis, and photovoltaic devices, among others.

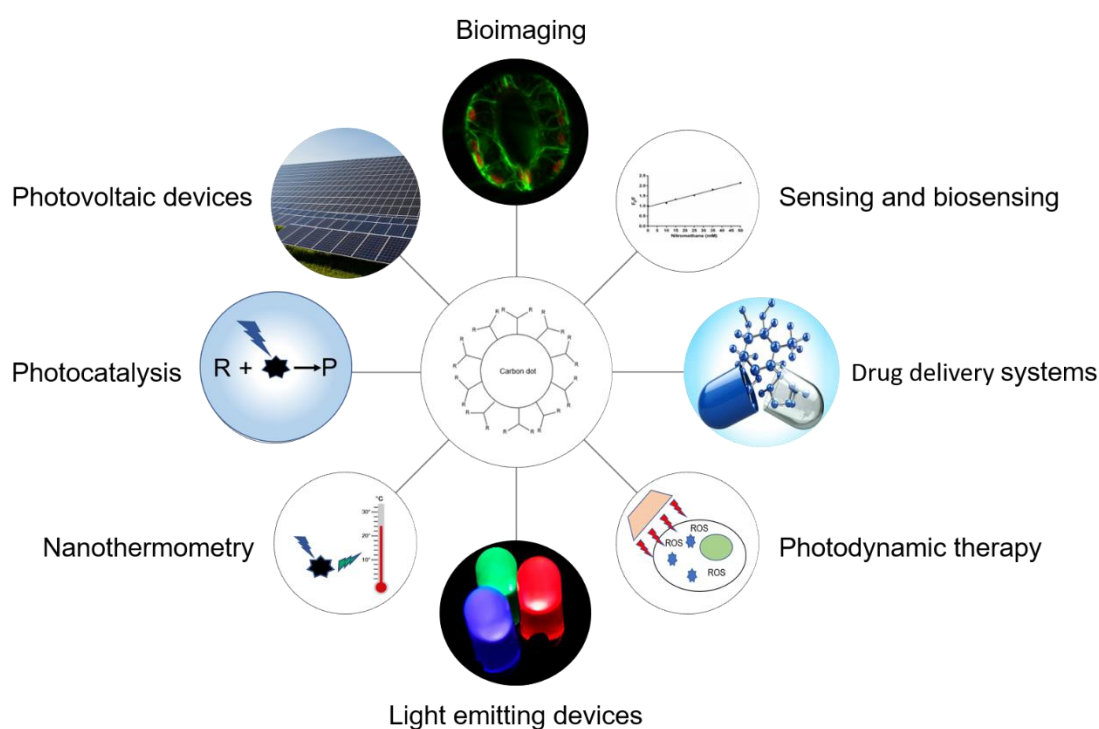


Figure 1 – General scheme of some of the most common applications for CDs.

➤ **Bioimaging** – consists in methods that non-invasively allow for the visualization of biologic processes in real time. It aims to interfere as little as possible with life processes while enabling the visualization of tissues, blood vessels, cells, and other biologic substrates in real time, usually through a signal output, such as the emission of radiation. In addition to their photoluminescence, stability, biocompatibility and resistance to metabolic degradation, CDs can be rapidly excreted from the body, [25] making them suitable candidates for applications in bioimaging. CDs have already been applied in the bioimaging of bacteria (e. g. *Escherichia coli*), [26] cell imaging, [27, 28] and even the *in vivo* bioimaging of cancer cells [29] or zebrafish embryos and larvae, [30] among others.

➤ Sensing and biosensing – one of the major applications for CDs is their use for sensing and biosensing. This means that the use of CDs allows for a given analyte in a complex mixture to be quantified through the analysis of changes in a given signal output that are induced by the presence of the analyte. In the case of CDs, the quantification is usually based on alteration in the nanoparticle's photoluminescence intensity. Reports have been made on the use CDs for the sensing of saccharides, [31, 32] metals, [28, 33] proteins, [34, 35] and other analytes of interest. [36-38]

➤ Drug delivery systems – mechanisms that aim to safely and effectively deliver an active pharmaceutical compound to its target site while preventing its degradation in order to reach therapeutical concentrations and obtain the desired effect. Nanoparticles, such as CDs, are being used in drug delivery systems to improve the solubility and half-life of drugs and to promote their accumulation at the target site. [39, 40] The presence of carboxylic acids or amine groups at the CD surface (a very common feature) promotes the interaction with drugs and other molecules through covalent interaction and amide linkage. This is very useful since it allows for the conjugation of CDs with the target drugs. [41, 42] CDs have been tested for the delivery of several drugs such as a conjugation of epirubicin and temozolomide, [39] doxorubicin, [43, 44] 5-fluorouracil [45] and benzofurans, [46] among others.

➤ Photodynamic therapy – is a type of treatment for cancer patients that uses photosensitizing agents and light to cause cellular death. [47, 48] This kind of therapy has gained more prominence in both research and clinical applications due to its numerous advantages. When compared to traditional therapies it has fewer side effects, an almost negligible skin phototoxicity, low damage to marginal tissues and is considerably less invasive (or non-invasive at all). [49-52] Photodynamic therapy is based on the interaction between a photosensitizer and the surrounding molecules. Localized irradiation excites the photosensitizer, which then interacts with the surrounding molecules, prompting the formation of reactive oxygen species that cause oxidative damage to cancerigenous cells. [53-55] He *et al.* reported the sucessful application of CDs as photosensitizers for photodynamic therapy. [56] Diketopyrrolopyrrole and chitosan-based CDs were prepared through an one-step hydrothermal synthesis. The resulting product was submitted to centrifugation and dialysis. The produced CDs were capable of generating reactive oxygen species in a satisfactory extent, thus inducing oxidative stress in the targeted cells that culminated with their death. [56] Furthermore, the CDs also displayed a very good biocompatibility and enhanced hydrophilic properties, making them suitable for applications *in vivo*. [56] In summary, He *et al.* demonstrated that their CDs could inhibit the growth of tumor cells

when submitted to laser irradiation (540 nm), proving their efficiency in photodynamic therapy. [56]

➤ Light emission devices – due to their low cost, unique optical features, chemical inertness and good aqueous solubility, CDs have attracted attention regarding their application in light emitting devices. One of the great difficulties regarding this area is the obtainment of high quality white-light. Materials that emit this kind of light are highly sought after due to their possible application in full-colour displays. The traditional route for the generation of white-light is based on the mixing of emitters that, independently and simultaneously, emit in the primary or complementary colours pairs, generating a low quality white-light. [57] Another alternative is the use of a phosphor, capable of converting monochromatic light from near-UV or UV radiation source into white-light, dispersed in a transparent medium. The majority of the high performance white-light emitting devices has the downside of using either expensive rare-earth-based phosphors or highly toxic Pd/Cd-based semiconductor quantum dots. [58-60] The use of CDs to overcome this difficulty regarding the emission of white-light is described in the work of Joseph and Anappara, who report the making of a graphite-based CD capable of converting the radiation of a UV light-emitting diode ($\lambda=365$ nm) into white-light (by the parameters of International Commission on Illumination). [61] The CDs were produced through the electrochemical exfoliation of graphite rods and purified by centrifugation and chromatographic separation. The CDs' UV-Vis absorption spectra presented a shoulder at 265 nm and an absorption tail which extends into the visible range. Furthermore, it presented a broadband emission covering a significant fraction of the visible range, with the CDs exhibiting white-light emission when excited at 365 nm. [61] To take advantage of this, their team inserted the CDs in a poly(methyl methacrylate) matrix, which was used to make caps for a light emitting diode emitting at 365 nm. The CD in the cap was capable of converting the UV radiation into white-light in a system that did not required highly toxic metals or expensive rare-earth phosphors. [61]

➤ Nanothermometry – temperature is a very sensitive parameter for several types of systems, be it physical, chemical or biological systems. This being said, a correct monitorization of the temperature of a system is quite important in several fields, such as photonic devices, microelectronics, biology and microbiology, medicine, among others. [62, 63] In particular, several processes at a cellular level are affected by temperature, which greatly varies depending on the cells, their biochemical processes and external stimulation. [64] Because of this, accurate ways of measuring the temperature in biological systems are highly sought after. Several nanoparticle-based thermal probes were reported. [65-67] Among the reported systems, the most suited operating principle for biological applications is, with all probability, noncontact

luminescence thermometry, which is based on the emission by a luminescent material in a temperature-dependent manner. Thermometers based on this mechanism were already made from several sources such as organic compounds, metal-based and rare-earth-doped nanoparticles, inorganic and hybrid phosphors, molecular fluorophores and semiconductor nanocrystals. [68-73] However, these luminescence-based systems present several disadvantages, which include cytotoxicity, low quantum yield of luminescence, unsatisfactory biocompatibility and poor photostability, making them unsuitable for application in biological systems. [65] Moreover, a dual emission temperature-dependent photoluminescence of CDs was already reported, establishing a basis for the use of CDs in this kind of temperature-measuring system. [74, 75] The application of CDs for nanothermometry was reported in 2017 by Kalytchuk *et al.*, who produced a N,S-doped CD based on citric acid (CA) and cysteine through hydrothermal treatment. [76] The CD did not affect the cellular viability and displayed a temperature-dependent photoluminescence lifetime, providing a sensitive and reliable nanothermometer for temperature measurements at cellular levels. [76] Moreover, due to their low toxicity, biocompatibility, solubility and stability, CDs can be accurately re-used as thermometers, without causing damage to the cells, as their photoluminescence decay (for temperatures between 15 and 45 °C) remained unchanged after seven cycles. [76] Overall, CDs displayed a temperature-dependent photoluminescence decay and were suitable for luminescence-based thermometry either *in vitro* or *in vivo*. [76]

➤ Photocatalysis – photocatalysts are materials that absorb light to bring them to higher energy levels, allowing them to provide that energy to a reacting substance in order to facilitate a chemical reaction. Metal-free photocatalysts have been under focus as potential alternatives to traditional metal-based catalysts. Due to the absence of heavy metals and the fact that light is an inexhaustible energy source, photocatalysts are cheaper, less toxic and less impactful towards the environment than their metal-based counterparts. [77, 78] Due to their properties, specially the enhanced photoluminescence, particular structure, aqueous solubility and the capacity to conduct photo-induced electron transfer (PET) reactions, CDs are potential candidates to be used as NIR light driven photocatalysts. [79, 80] The use of CDs for photocatalytic applications has been reported by several teams for different purposes, such as: selective oxidation of alcohols, [81] hydrogen production, [82, 83] reduction of nitroaromatics, [84] degradation of organic molecules, [85, 86] antibacterial activity, [87] among others.

➤ Photovoltaic devices – a particular example regarding the application of CDs in photocatalysis is their use in photovoltaic devices. Titanium oxide (TiO₂) is a commonly used photocatalyst capable of splitting water into hydrogen fuel. [88] However, the main polymorphs of TiO₂, anatase and rutile, are only activated by UV light, limiting

their application range. Carbon doping, particularly the use of carbon-based nanostructures, was reported to be an efficient way of enabling and enhancing the visible light driven photocatalytic activity of the TiO_2 -based catalyst. [89-91] However, carbon nanotubes and graphene are quite difficult to disperse and are prone to aggregate. [92] The use of CDs for this purpose has already been reported for the making of CDs/anatase [82] and CDs/rutile [83] composite photocatalysts. Regarding the latter, Zhang *et al.* reported the making of N-doped CDs which were successfully combined with rutile TiO_2 , originating a CD/ TiO_2 composite displaying photocatalytic activity under visible light irradiation. [83] The application of N-doped CDs in conjugation with TiO_2 in metal-free dye-sensitized solar cells (a less expensive and more efficient alternative to the traditional silicon-based solar cells [93]) was reported. This kind of solar cells, despite having important benefits when compared to traditional silicon-based solar cells, requires either expensive, but highly efficient, rare metals, or simpler and cheaper, but less efficient, organic dyes that are prone to aggregate. [94-96] CDs, due to their properties, are potential substitutes for these components and can make for efficient, cheaper and less environmentally impactful photocatalytic systems, as was seen in the work developed by Zhang *et al.* [83]. The systems' performance when N-doped CDs were used was superior than when either component was used alone or when the CDs were nitrogen-free. This suggests a possible synergistic mechanism for the N-doped CD/ TiO_2 composites. [83] In summary, the introduction of N-doped CDs in the system enhanced the photocatalytic activity of TiO_2 and increased the performance of sensitized TiO_2 -based solar cells, proving it's potential for photovoltaic applications. [83]

1.3. Carbon dots: synthesis and fabrication

Regarding their fabrication, CDs can be synthesized through a varied array of methodologies that can be divided into two main groups: top-down and bottom-up methodologies (Figure 2). The techniques in the top-down group are based on breaking a bigger, macroscopic, carbon material (e. g. activated carbon or carbon nanotubes), into smaller particles with a nanometric size, followed by a surface treatment. This group includes techniques such as arc-discharge, [8] laser ablation or irradiation, [9] chemical exfoliation of graphite [97], ultrasonic treatment [98] and electrochemical shocking of carbon nanotubes. [99]

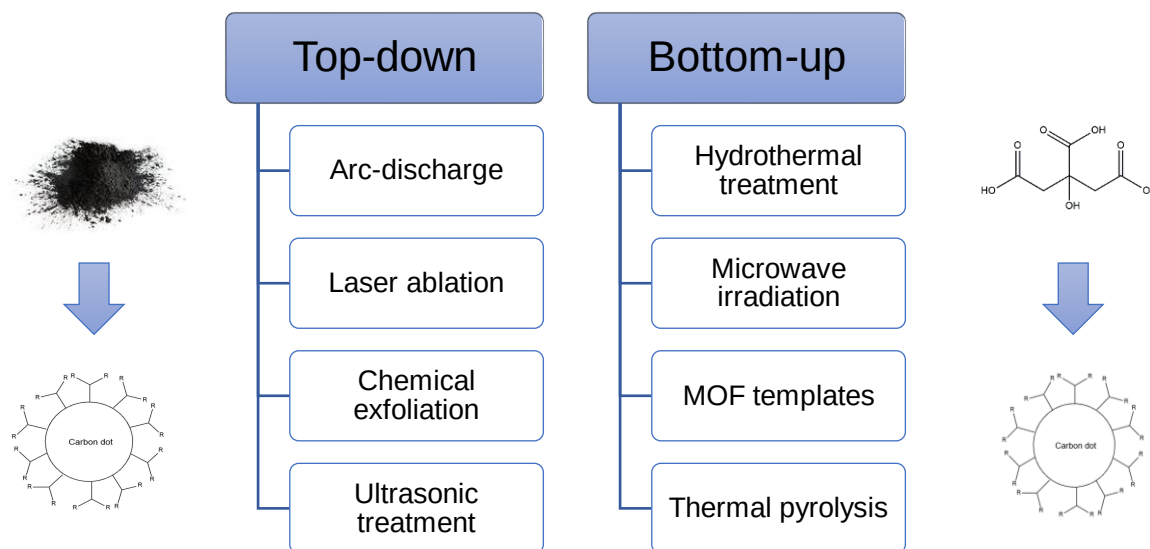


Figure 2 – Schematic list of some pathways of the two groups of synthetic methodologies for the fabrication of CDs.

Bottom-up methodologies work in the opposite way. They are based in the association of smaller carbon-based molecules, such as glucose or CA, into bigger nanoparticles, CDs. This group includes synthetic methodologies such as hydrothermal treatment, [100] microwave irradiation, [101] metal-organic framework templates [102] and thermal pyrolysis. [103] An important thing to retain is that no methodology is utterly superior to the others meaning that each synthetic route has advantages and demerits. When choosing a route, it needs to be taken into account what each technique can yield and how it will yield it. The synthetic procedure must be planned in accordance to what we expect the resulting CDs to do, as well as the available time, resources and equipment.

Because they are simpler, less expensive, relatively fast and usually do not require expensive equipments, bottom-up methodologies are usually preferred in detriment of top-down routes. Across all synthetic methodologies, the most commonly

used routes consist in the microwave irradiation or hydrothermal treatment of a carbon source with the occasional addition of compounds containing heteroatoms, usually nitrogen-sources, to the reactional mixture. [10, 32, 37, 100, 101, 104-109] CDs made by microwave irradiation were first reported by Zhu *et al.* in 2009. [101] The CDs were obtained using a solution of poly(ethylene glycol) (PEG-200) and saccharides (glucose and fructose). A microwave oven was used to irradiate the solution (2-10 minutes with a potency of 500W), resulting in CDs ranging from 2.57 ± 0.45 to 3.65 ± 0.6 nm in diameter, depending on the irradiation time. The resulting CDs displayed a high carbon and oxygen content, presenting hydrophilic groups that endowed the particle with a high water solubility. This methodology allows for relatively high QY_{FL} and an emission that can range from the deep UV to the NIR. [110-113]

On the other hand, the first hydrothermally synthesized CDs were reported by Yang *et al.* in 2011. [100] Briefly, a mixture of glucose and monopotassium phosphate was prepared in deionized water and transferred into a *teflon*-lined autoclave chamber. This was followed by a reaction period of 12h at 200 °C in an oven and further centrifugation and salt removal steps. By changing the molar ratios in the precursor mixture, the methodology permitted tunable emission wavelengths and particle sizes, which is important to obtain purpose-made CDs. In fact, by choosing the correct precursors and reactional conditions, it is possible to obtain hydrothermally made CDs capable of emitting in the whole visible range. [100, 114-117] In summary, because of the easyness and low cost of these two bottom-up strategies, associated to the tunable emission properties, microwave irradiation and hydrothermal treatment are frequently chosen methodologies for the fabrication of CDs with particular optical properties. Additionally, considering that when using natural bioresources these two routes usually do not require surface passivation after the synthesis (since the -OH groups are oxidized during the process), these CDs can be obtained and prepared in a one-step synthesis, thus reducing the time and resources required for its making. [118, 119]

Finally, in order to generate the carbogenic core, a carbon-source is always required for the production of CDs. One of the most commonly used carbon sources is CA, which is inexpensive and easy to obtain. Additionally, in order to enhance the CDs optical properties through heteroatoms doping, it is common to add a nitrogen-source (e. g. EDA) to the precursor mixture. [10, 112, 120] Several studies argue that, because of its chemical structure, CA is able to easily interact with amine groups. This promotes the formation of citrazinic acid and its derivatives, which are strong, blue emitting, photoluminescent fluorophores. [121-124] Nonetheless, the mechanism behind CDs' fluorescence is still poorly understood.

1.4. Carbon dots: fluorescence mechanisms

Several models and explanations have been proposed throughout the years to try and explain the enhanced optoelectronic properties displayed by CDs, amongst which photoluminescence is the most prominent. As for the proposed models, they include the quantum confinement effect and band gap emission, [114, 125] surface states emission, [108, 126, 127] carbogenic core and molecular fluorophores emission, [103, 121, 128] aggregate emission centers, [129, 130] emission by self trapped excitons, [131] and molecular emission by polycyclic aromatic hydrocarbons (PAH). [24] The referred mechanisms are explained underneath:

➤ Quantum confinement effect - this theory defends that CDs are confined in terms of size by the band gap existing between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). [132] As the particle size decreases, the energy gap between HOMO and LUMO grows larger, leading to higher energy requirements for the excitation of electrons from the HOMO into the LUMO (Figure 3). After excitation, the electron will relax and return to ground state with emission of light. Because of this, we can state that the energy gap determines the emission wavelength, and infer that the size of the nanoparticle determines the emission wavelength. [133] Smaller nanoparticles emit in shorter, more energetic wavelengths. A study case of this theory can be seen in the work of Yuan *et al.*, who, by controlling the synthetic methodology he chose, managed to obtain five differently sized CDs, ranging from 1.95 to 6.68 nm in diameter. [125] The CDs presented an excitation-independent photoluminescence with the emission peak varying in function of the CD size, indicating that the emission depends of the band gap emission. [125] The photoluminescence peak ranged from 430 to 604 nm (for sizes of 1.95 and 6.68 nm, respectively), which is consistent with the quantum confinement and band gap emission theory from which we infer that smaller particles emit at shorter, although more energetic, wavelengths. [125]

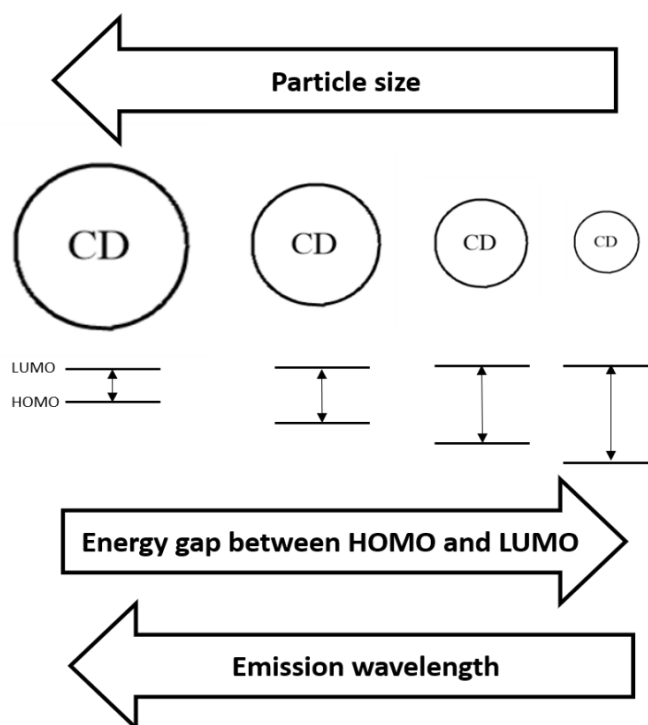


Figure 3 – Schematic representation of the quantum confinement and band gap emission mechanism of fluorescence.

➤ Surface states emission - commonly paired with the band gap emission mechanism, in a two factors theory, to explain the CDs mechanism of fluorescence. Chien *et al.* proposed a theory which stated that carboxylic groups (COOH) on sp^2 -hybridized carbons from graphene oxide could originate local distortions that resulted in a decrease of the energy gap. [134] Based on that theory, Ding *et al.* presented a model for CD emission defending that the nanoparticle emissive center is located on the surface of the nanoparticle. Additionally, it was mainly constituted by conjugated carbon atoms and bonded oxygen atoms, being the difference of energy between the HOMO and LUMO directly related with the degree of oxidation present at the particle surface. [127] As represented in Figure 4, an increased oxidation results in a decreased energy band gap between the HOMO and LUMO, meaning that the energy difference between HOMO and LUMO is diminished and that less energy is required to excite the electrons from the HOMO into the LUMO. This signifies that the CD emission will suffer a red-shift as the oxidation levels at the nanoparticle's surface increase. [127] This dependence on the state of the nanoparticle surface was also confirmed by tests made in acidic conditions. The authors observed that a surface charge modification (induced by the protonation and deprotonation caused by the different pH), caused a decrease in the fluorescence intensity and a shift in the emission spectra of a CD, proving that the CDs' surface composition has an impact in its emission. [127]

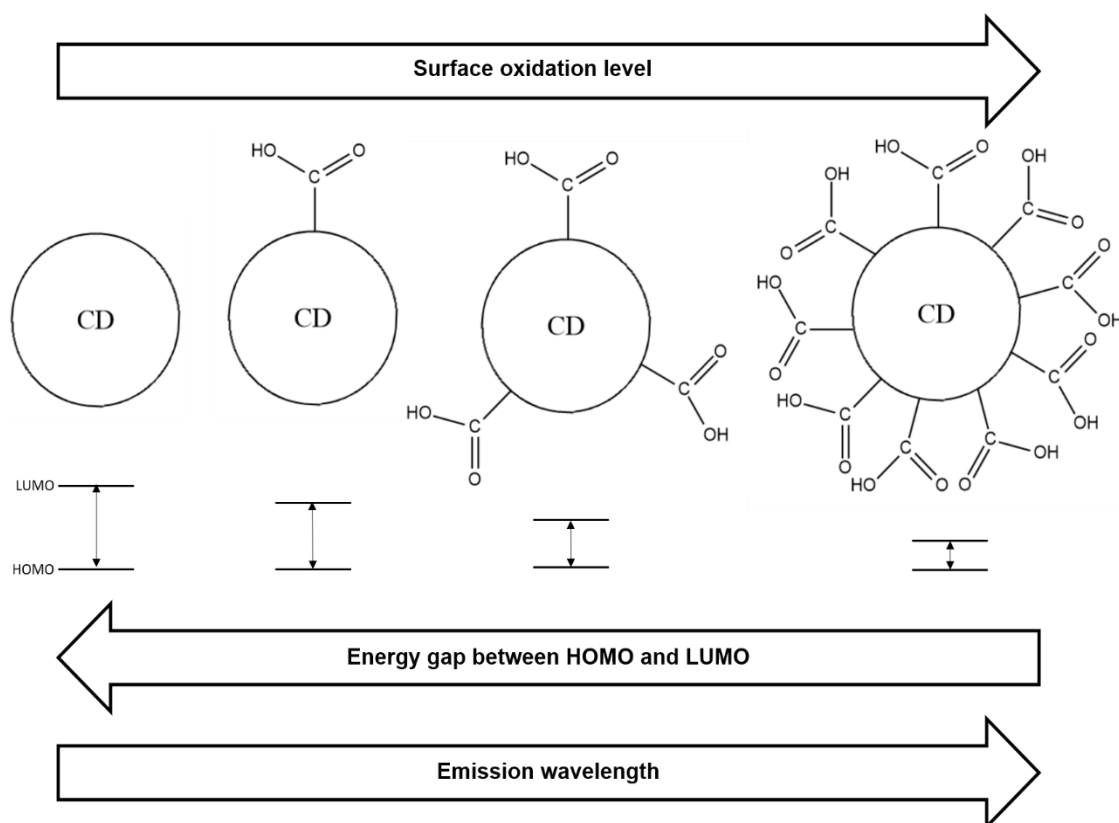


Figure 4 – Schematic representation of the surface states emission mechanism of fluorescence.

➤ Emission by molecular fluorophores - several authors have reported the presence of several fluorescent molecular by-products after the synthesis of CDs through bottom-up synthetic routes (arguably the most commonly used routes for the fabrication of CDs). [103, 121, 124, 128, 135, 136] Although this would partially explain the enhanced emission of an unpurified CD solution containing both the CD and the fluorescent impurities, it cannot be used to explain the intrinsic emission associated to CDs. While they can both be produced during the synthesis, the CD and the fluorescent impurities are entirely different entities (Figure 5), and even if the impurities are removed from the solution, the CDs still present an emission. Therefore, even though the presence of fluorescent impurities can greatly increase the fluorescence intensity of a CD solution, it will also mask the CD true emission. Kasprzyk *et al.* found the fluorescence of CA,urea-based CDs to be greatly influenced by the presence molecular fluorophores, which were nothing more than the fluorescent impurities formed during the CD synthesis. [128] Furthermore, since two different bottom-up strategies yielded different moieties in the resulting CD solution, these molecular fluorophores vary with the synthetic route. Hydrothermal treatment in a closed vessel originated blue emitting citrazinic acid and its derivatives, while microwave irradiation in solvent free conditions originated a green emitting compound named as HPPT. Either route produced CD solutions whose

fluorescence was influenced by these molecular fluorophores. [128] Similar results were found in 2011 by Krysmann *et al.*, who synthesized CA and ethanolamine-based CDs by a thermal pyrolysis approach and reported the overall emission to depend on the pyrolysis temperature. [103] The authors defend that, when the synthesis was made at 180 °C, it did not form CDs (seen by TEM and DLS), but instead formed some CD precursors that possessed a high QY_{FL} of ~50% (with excitation-independent emission). On the other hand, increasing the pyrolysis temperature to 230, 300 and 400 °C resulted in a diminished QY_{FL} (more commonly related to CDs), excitation-dependent emission and an increased carbon content, meaning that more carbonization occurred in the particles. In summary, when the pyrolysis temperature was low, the emission mostly resulted from molecular fluorophores. However, at higher temperatures, carbogenic cores became the main cause contributor for the emission. Moderate temperatures resulted in solutions with the emission being influenced both by the carbogenic core and the molecular fluorophores. The authors claimed that, even though both the carbogenic core and the amide-containing fluorophores could contribute for the emission at moderate temperatures, the CDs undergo further carbonization as the pyrolysis temperature increased, resulting in a higher proportion of less emissive carbogenic cores at the cost of the molecular fluorophores observed with lower temperatures. [103]

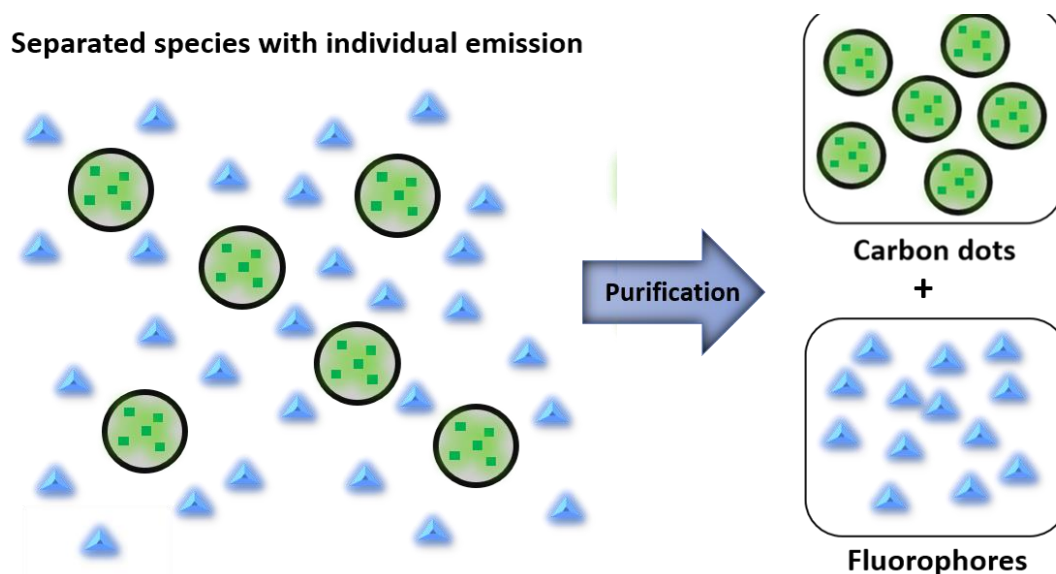


Figure 5 – Schematic representation of the emission by CDs allied to the emission of molecular fluorophores.

➤ Aggregate emission centers – researchers continued to explore aspects in the structure of CDs that could lead to photoluminescence. Two structure-based possibilities were proposed to explain photoluminescence: coupling between π electronic systems (efficient in near ranges) and dipole-dipole resonance between CDs, which can

occur at longer distances. Data from the work of Ghosh *et al.*, namely the parallel TDMS observed for single CDs, demonstrated that the excitonic interaction is, with all probability, responsible for the photoluminescence originated by the interaction of CDs. [137] According to Kasha *et al.*, only two types of aggregates can lead to π excitonic coupling: J- ($\theta = 0^\circ$, head-to-tail) and H-aggregates ($\theta = 90^\circ$, face-to-face) (Figure 6). [138] Parallel-aligned molecular dipoles ($\theta = 0^\circ$) can originate J-aggregates, which when compared to the respective monomer, present a smaller Stokes-shift and narrower absorption and photoluminescence spectra, albeit at longer wavelengths. [139] By its turn, $\pi - \pi$ stacked H-aggregates lead to the dimer excited state splitting into two energy levels (higher and lower energy excitonic states). [140] Given that the classic theory states that relaxation into lower excitonic states is forbidden, [138] $\pi - \pi$ stacked H-aggregates must be non-emissive (Figure 6), which contradicts the bright emission observed in some examples of H-aggregates. [141, 142] Considering this, Demchenko and Dekaliuk suggested that, unlike J-aggregates, excitonic coupling in H-aggregates could result from a cofacial stacking alignment originated by a weak van der Waals interaction, forming a structure which would resemble a sort of hybrid between J- and H-aggregates ($0^\circ < \theta < 90^\circ$). [140] Given this, a small rotation in one of the monomers in the H-aggregate could result in the probability of the transition into lower energy excitonic states being different from zero, thus allowing photoluminescence. Therefore, a small disorder in the structure could cause the transformation of a non-emissive H-aggregate into a highly emissive one. [140] For their theory, Demchenko and Dekaliuk proposed that CDs formed H-aggregates during their synthesis (by regular packing of graphene sheets) and that the cofacial positions of chromophores were in the CDs' surface. [140] Moreover, the authors claimed that surface functional groups, such as C=O and C=N, could modify the optoelectronic properties of CD-based H-aggregates. [140] A variation of this theory, presented by Sharma *et al.*, proposed that CDs emission resulted from several discrete electronic levels and that both J- and H-aggregates contributed to emission, displaying different excitation/emission bands and different responses to variations in the system (*e. g.* temperature). [143]

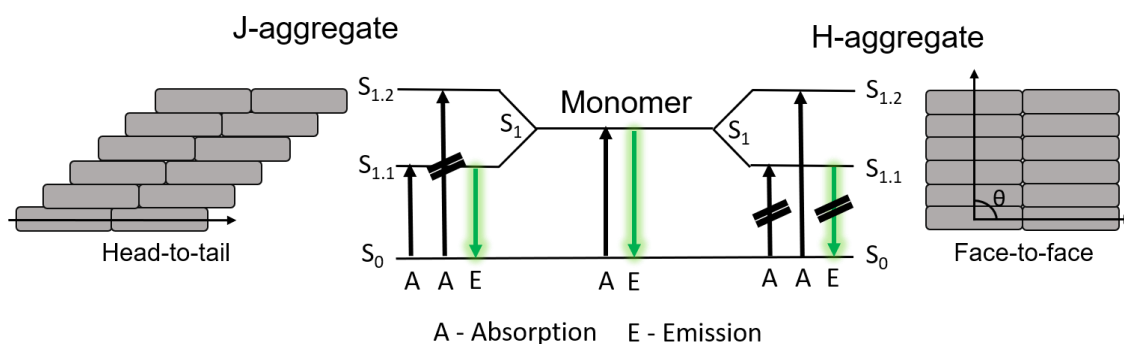


Figure 6 – Simplified schematic representation of J- and H-aggregates, including their organization and possible or forbidden electronic transitions.

➤ Emission by self-trapped excitons – in 2017, Xiao *et al.* claimed that CDs' emission resulted from a localized radiative recombination of self-trapped excitons, where momentum, energy and vibrational relaxation are suppressed by the presence of a strong local potential field. [131] An exciton (Figure 7) is the bound state of an electron and its respective electron-hole and is considered to be an elementary excitation of matter, capable of transporting energy without transporting charge. [144] Excitons can be formed when a material absorbs photons carrying an energy greater than its bandgap, occurring in both a localized (Frenkel exciton) or non-localized (Wannier-Mott exciton) manner. [145] In crystalline structures, self-trapped excitons can be a result from a strong interaction between excitons and phonons. Self-trapped excitons are surrounded by phonons (causing a local deformation of the lattice area around the exciton), which suppress the movement of excitons across the crystalline structure. The recombination of self-trapped excitons usually results in a broadband emission in the visible-light region, displaying a large Stokes shift when compared to the excitation wavelength. [146, 147] Considering the way excitons are formed and become self-trapped, self-trapped excitons are expected to present a linear response to increasing excitation power. [148, 149] Moreover, contrary to typical luminescence, after the emission, the system is not totally degraded and the information of the electronic system (spin, momentum, energy, *etc.*) is partially or entirely kept. [150] Knowing this, Xiao *et al.* performed a series of systematic experiments (time-resolved photoluminescence experiments, anisotropy spectroscopy and electric-field modulation spectroscopy), which yielded evidences that the emission of glucose and glucose,urea-based CDs, made through microwave irradiation, may result from the radiative recombination of self-trapped excitons. [131] The self-trapped exciton model was consistent with the steady-state and time-resolved optical spectroscopy analysis. [131] The authors hypothesize that the self-trapped exciton structure originates from a ruptured C-O bond and/or a peroxy radical bond, which would cause a localized distortion and a strong potential field, resulting in self-trapped excitons whose the radiative recombination would cause photoluminescence. [131]

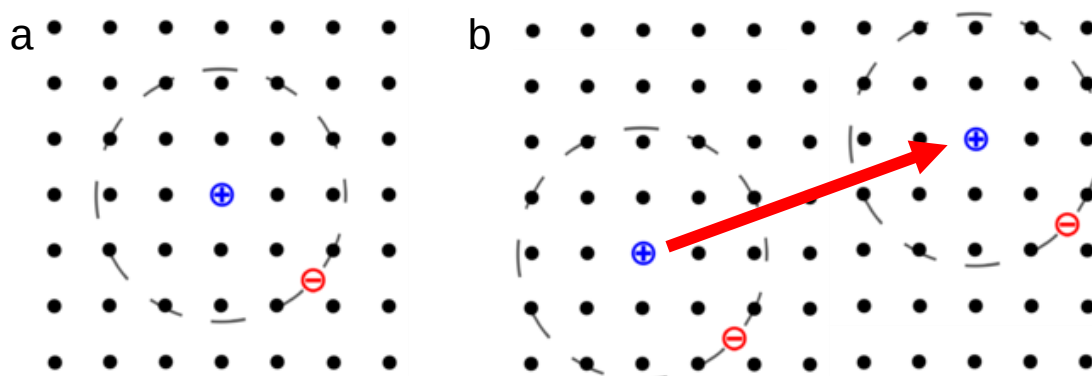


Figure 7 – Schematization of an exciton (electron (-) and electron-hole (+) pair), either when (a) localized or (b) non-localized and moving in the crystal lattice.

➤ PAH molecular emission – there is a general conviction that CDs' cores are comprised of sp^2 hybridized carbon nanodomains, that can be PAHs, embedded in a sp^3 hybridized carbon matrix. [82, 151, 152] A report by Fu *et al.* defends that the excitation-dependent behavior of CDs derives from the presence of several different PAHs in the CD structure. [24] Those PAHs are excited at different wavelengths and have slightly different energy gaps (resulting in different emission peaks), and thus are able to affect the CD overall emission (Figure 8). [24] The mentioned report was made using CA,EDA-based CDs, sized between 2 and 5 nm, made through hydrothermal treatment. The authors stated that the CDs could be considered as a type of organic molecular nanocrystal that contained several sp^2 carbon domains inserted in a sp^3 hybridized carbon matrix. [24] The presence of different PAH domains in the CDs was inferred from the fact that measurements made for a single CD presented an excitation-dependent photoluminescence, indicating that each CD possess multiple chromophores in its structure (PAHs in this case). [24] To test the emission by PAHs, Fu *et al.* tried to mimic the emission domain by employing three basic PAHs (anthracene, pyrene and perylene – chosen due to their relatively simple structures and for having absorption and emission spectra similar to those of the CDs) embedded in poly(methyl methacrylate) (used as a sp^3 hybridized carbon matrix). [24] The optical properties of the CD and its mimic were similar, and tests using that model were considered to be valid. Based on the comparison of the results obtained with the CD and the PAH-based model, the authors presented the following results: when excited at smaller wavelengths (under 400 nm), the PAHs with the largest bandgap (anthracene and pyrene) were excited while perylene (smaller bandgap) was incapable of strongly absorb radiation; the absorbing PAHs could contribute to the emission directly or by transferring energy into the smaller bandgaps (to perylene), which in turn would result in emission at longer wavelengths. [24] When excited at longer wavelengths (over 400 nm), PAHs with either small or large bandgaps could be excited directly, causing a red-shift in the emission. Increasingly higher excitation wavelengths led to a greater absorption from small bandgap PAHs and a decrease in the absorption by PAHs with larger bandgaps, resulting in a continual red-shift of the CDs' emission spectrum. [24] Even though the authors admit that their model is limited in terms of tested PAHs (many other PAHs might be responsible for affecting the emission of CDs), the study demonstrates that the contributions from different PAHs can effectively alter the CDs' photoluminescence. [24]

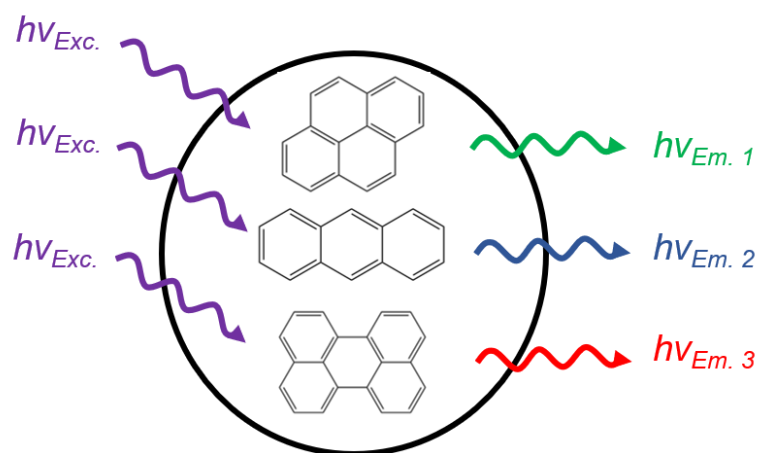


Figure 8 – Schematic representation of how the CDs emission can be affected by three PAHs (top to bottom: pyrene, anthracene and perylene) as a result of the different absorption wavelengths and energy gaps of each PAH (due to their structure).

1.5. Objectives and scientific production

This project consisted in an extensive study about CDs. This included their properties and applications, their fabrication and purification, the impact they cause on the environment, the mechanisms responsible for their optical properties, their reactivity, among others. The project had three primary objectives:

- a. An evaluation of the impact caused in the environment by the production of six model CA,urea-based CDs made by different synthetic methodologies;
- b. A study regarding the effect caused by the presence of fluorescent molecular by-products in the fluorescence and reactivity of a model CA,urea-based CD;
- c. The assessment of a 4-aminopyridine-based CD catalytic potential for an epoxide ring-opening reaction followed by an aminolysis reaction.

From this work resulted two scientific papers, one already published and another currently under revision, in peer-reviewed scientific journals. Additionally, from this work also resulted five communications in national and international scientific conferences.

Papers:

- Ricardo M.S. Sendão, Maria del Valle Martínez de Yuso, Manuel Algarra, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Comparative Life Cycle Assessment of Bottom-Up Synthesis Routes for Carbon Dots Derived from Citric Acid and Urea*, Journal of Cleaner Production, *In revision*;
- Ricardo M.S. Sendão, Diana M.A. Crista, Ana Carolina P. Afonso, Maria del Valle Martínez de Yuso, Manuel Algarra, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Insight into the Synergistic Luminescence and Reactivity of Carbon Dots and Related Fluorescent Impurities*, Physical Chemistry Chemical Physics, 2019, 21, 20919-20926.

Communications:

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of CO₂ conversion into heterocyclic carbonates through organocatalysis*, XXIV Encontro Luso-Galego de Química, 2018, Porto (Portugal) – Oral communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of epoxide ring-opening reactions using carbon dots as*

organocatalysts, 12^o Encontro da Investigação Jovem da Universidade do Porto, 2018, Porto (Portugal) – Oral communication;

- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Mechanistic study of the use of carbon dots as organocatalysts for epoxide ring-opening reactions*, 21st JCF Frühjahrssymposium and 2nd European Young Chemists Meeting, 2019, Brémen (Germany) – Poster communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Insight into the interaction between fluorescent carbon dots and molecular by-products of their synthesis*, XXVI Encontro Nacional da Sociedade Portuguesa de Química, 2019, Porto (Portugal) – Oral communication;
- Ricardo M.S. Sendão, Joaquim C.G. Esteves da Silva, Luís Pinto da Silva, *Study of the interaction between fluorescent Carbon dots and the fluorescent by-products that result from their synthesis*, XXV Encontro Luso-Galego de Química, 2019, Santiago de Compostela (Spain) – Oral communication.

2. LCA study

2.1. Introduction

2.1.1. Environmental impacts of carbon dots as engineered nanomaterials

Engineered nanomaterials (ENMs) are materials with dimensions between 1 and 100 nm that are specifically built for a purpose. [153, 154] ENMs have different properties than those of the respective bulk materials, [153-155] being those properties a result of their increased surface area and quantum effects. Even though in recent years there was an increase in the development of such materials, the environmental impact caused by their synthesis and usage is still unclear. [156] A detailed study focusing on the impacts caused by ENMs is required in order to ensure their sustainable fabrication and environmentally conscious usage. [156, 157] A life cycle assessment (LCA) is a commonly used, and arguably the best suited, tool to assess, evaluate and quantify the environmental impacts caused by an ENM during its life cycle. [158-160] Although the use of LCAs to assess the impact of new kinds of nanotechnology is still on an early phase, several studies were already made for different carbon-based nanomaterials such as carbon nanotubes [161] and graphene oxide, [162] or metal-based nanoparticles, such as silver nanoparticles, [163] magnetic nanoparticles, [155] copper nanoparticles [164] and titanium oxide nanoparticles, [157] among others.

CDs, when developed for a specific purpose or utility, can be regarded as ENMs. Due to their properties, CDs have attracted a lot of attention for several applications, mainly because of them being a cheap and highly photoluminescent material. Despite that, so far, to the best of our knowledge, there hasn't been any study regarding the environmental impact caused by the production of this very important kind of ENM. This is worrying given that the synthesis of ENMs can be orders of magnitude higher than pharmaceuticals and fine chemicals in terms of resources and energy consumption, and lead to aggravated environmental impacts. [165] Another interesting factor is that the resources and energy consumed during the nanomaterial synthesis can be the major contributor to the environmental impacts caused during its entire life cycle. [135] Because of their rising importance and the lack of information about their toll on the environment, a LCA with respect to the fabrication of CDs (through different common synthetic routes and the most regularly used precursors) would fill a gap in the literature.

2.1.2. Life cycle assessment: scope, stages and limitations

LCA studies originated in the 1960s when concerns regarding the limitations of our planet raw materials and energy sources were raised. In order to better plan the future usage of our planet resources, a way to quantify and to account for the usage of resources, energy and the associated environmental impacts of the making and usage of a product (or process/activity) was searched for. [166] Curran states that “Life cycle assessment, or LCA, is an environmental accounting and management approach for assessing industrial systems. It considers all the aspects of resource use and environmental releases associated with the system, as defined by the function provided by a product, process or activity.” [166] LCA studies consider the entire life cycle of a product. During a LCA, all stages of a product life cycle are regarded as being interdependent, meaning that one stage of the cycle is required for the next stage to happen and that decisions made at any one point of the life cycle can change the outcome of other stages. [166-168] By other words, it is a technique used to assess the environmental impacts associated to all stages of a product life which include the extraction of the raw materials (removal of resources and energy from nature and associated impacts), fabrication of the product (materials manufacturing and product assembly), distribution (preparation, shipping and delivery), usage by the consumer, repair and maintenance (impacts associated to the useful life of the product from the point in which the consumer obtains the product, including the costs and impacts from product storage, use, reparation, refurbishing or similar procedures) and finally, at the end of its useful lifetime, the disposal or recycling of the product (environmental wastes ascribed to the disposing or recycling of a product). [169] A LCA study, when englobing all those stages, is considered to follow a cradle-to-grave approach. Despite evaluating the impacts of an entire life cycle, LCA studies should be regarded as a relative tool that should be used for comparisons, and not for the absolute evaluation of the environmental impact associated to a product. [166] LCA studies can contribute to compile a report regarding the costs and impacts of alternative courses of action, evaluate the impacts associated to different inputs and help designers to make more conscious decisions. [166, 168]

LCA studies are conducted in four main phases:

- 1) Statement of the goal and scope of the study - This serves to establish the context of the study and to define details such as the functional unit of the study, the system boundaries and limitations, and the environmental impact categories that are to be assessed and reviewed during the study. During this phase it is also decided how

should the results be presented in order to be meaningful and easily understood and which environmental impact categories are more or less relevant. Decisions regarding the goal and scope of the study will impact the way the study will be conducted and how impactful each step of the life cycle will be in the final results of the LCA; [166, 168]

2) Life cycle inventory (LCI) analysis – Consists in the creation of pathways flowing from and to nature during the life cycle of a product. [168] In the LCI phase of a LCA, all relevant data are collected and organized. It is a process of quantifying raw material and energy requirements, atmospheric and waterborne emissions, solid wastes production, and any other release into the environment during the life cycle of a product. [166, 168] It results in a list describing the type and quantity of the pollutants released during the life cycle as well as the amounts of raw materials and energy consumed. [166, 168] The input and output data is required for the construction of the model used in the study and represents all the components that are taken or released during the product life cycle, having an impact in the functional unit described in the goal of the study. Without the LCI phase there would be no base to evaluate comparative environmental impacts, or to assess potential improvements that could be made; [168]

3) Life cycle impact assessment (LCIA) – The LCIA tries to establish a linkage between the life cycle of a product or process and its potential environmental impacts. During this phase, the potential human and environmental impact of the environmental releases and the usage of water, electricity and raw materials is assessed. [166, 168] Differing from other types of environmental impact analysis, the LCIA does not try to quantify any specific impact related to a product. Instead, it tries to establish a linkage between the whole system and the impact analysis. It uses simplified models that are mostly derived from more sophisticated models used for individual impact categories. Those simplified models, while not being suitable for absolute quantifications of risks or impacts, allow for comparisons between different pathways with regard to their human and environmental impacts; [166, 168]

4) Life cycle interpretation – Last step in a LCA. It consists in a systematic assessment of the inventory analysis and impact assessment to correctly evaluate the information obtained during the LCI and LCIA phases. [166, 168] During this phase, assumptions and estimates that need to be made and included in the LCA can impact the conclusions drawn from the study. Because of the significant uncertainty introduced by the assumptions and estimates used, sometimes the researcher is not allowed to state that one alternative is better than another. Despite this, it still provides valuable information about the environmental impacts of each alternative, their magnitude and in

which stage of the life cycle they occur, revealing the vantages and disadvantages of each tested alternative for each stage. [166, 168] The main purpose of an LCA is to help in decision making by providing information about the environmental impacts associated with a process or a product, which is obtained by interpreting the results. This facilitates the selection of the optimal pathway for the desired product, taking into consideration the uncertainty and the assumptions used in the study. However, this interpretation of the results cannot account for the all three pillars associated with sustainability: while it can be seen as the environmental part, it cannot be used to assess the social and economic impacts, which are outside the scope of a LCA study. [166, 168]

Despite the versatility of a LCA study, there are some limitations that need to be considered: 1) gathering data can sometimes be problematic, in particular when the study is used with regard to applications in chemistry as the data required for the study may not yet be available for all the possible chemical compounds used in the study. Furthermore, considering that the data might slightly vary depending on the producer, a degree of uncertainty is introduced in the study; 2) LCA studies are unable to state which pathway for a product or a process is more cost-effective or productive. Giving useful information regarding the financial outcome associated to a process is outside the scope of an LCA, and thus the economic part is not assessed by the study; 3) while the study quantifies and categorizes the environmental impacts associated to the life cycle of a product, it comes to the decision maker to choose the pathway to follow; 4) assumptions and uncertainties regarding the study scope and parameters might greatly affect the final result. They must be considered during the interpretation phase in order to account for them.

2.1.3. Study objectives

To evaluate the environmental impact associated to the synthesis of CDs, several CD solutions were synthesized following six bottom-up synthetic routes. A LCA cradle-to-gate study was performed with regard to their synthesis. The cradle-to-gate approach (which include the stages of the life cycle from the extraction of the raw materials until the fabrication of the product) was chosen instead of the full cradle-to-grave approach given that, like mentioned above, most environmental impacts associated with the life cycle of an ENM result from its synthesis. [135, 165] Additionally, aside this being the first LCA study related to the life cycle CDs, the use of CDs at a commercial and industrial level is still on a early phase. Thus, there is a lack of information regarding the distribution, usage and disposal stages of the life cycle. To account for that, several

approximations and estimates would need to be made, introducing a high level of uncertainty in a full cradle-to-grave study, rendering it useless in terms of effective results. Considering that the data regarding the resource extraction (raw materials for precursors and energy) is readily available in databases, and that the data regarding the fabrication of the nanoparticle itself was obtained by us in a procedure designed by our team, the inputs for those stages have a low level of assumptions, reducing the uncertainty associated to the results.

The synthetic routes analyzed in the LCA are based in the two most widely used bottom-up methodologies: microwave irradiation and hydrothermal treatment. [104, 105] CA, a very commonly used precursor, was always used as a carbon-source. Additionally, in some cases, in order to enhance the particle optoelectronic properties, urea was also added to the mixture as a nitrogen source. Despite the selected synthetic routes being very common, there is a lack of data about the potential environmental impacts generated by those synthetic methodologies. Given this, the conclusions obtained in this study are not restricted to these specific syntheses in those specific conditions. They can be, and should be, taken into consideration for the fabrication of other CDs through similar strategies. The LCA can serve as a base to try and reduce the environmental impact of the synthesis while maintaining the effectiveness of the nanomaterial for the purpose it was designed for.

Our aim with this work was to evaluate, through a LCA approach, the potential environmental impacts caused by the synthesis of CDs. In the initial stage of the study, a simplified volume-based functional unit was considered. In later stages, a functional unit, more closely related with the functionality of the CD (based on its QY_{FL}), will also be considered. Such approach is justified by the fact that the functional benefits of ENMs must be considered, and that, sometimes, a more resource-consuming synthesis may be justified by a more functional ENM. When compared to recent ENM-related LCA studies, [170] which do not consider the functionality and benefits of the study's subject through an adapted functional unit, this proves to be an advantage.

2.2. Methods

The experimental section can be divided into several sub-sections which describe the routes for the synthesis of CDs (2.2.1.), the fluorescence characterization of the CDs (2.2.2.), the HPLC and XPS analysis of the CDs (2.2.3.), the scope and system boundaries for the LCA study (2.2.4.), the life cycle inventory data used (2.2.5.), details regarding the environmental impact evaluation (2.2.6.) and a sensitivity analysis (2.2.7.).

2.2.1. Carbon dots production

Bottom-up strategies were employed for the production of CDs, namely hydrothermal treatment and microwave irradiation. Three CDs were made by hydrothermal treatment while the remaining three were obtained through microwave irradiation. The CD fabrication schemes were designed by us and executed in a controlled manner. The general scheme is represented in Figure 9. The carbon-source was CA with the occasional addition of urea to CA-based precursor solutions.

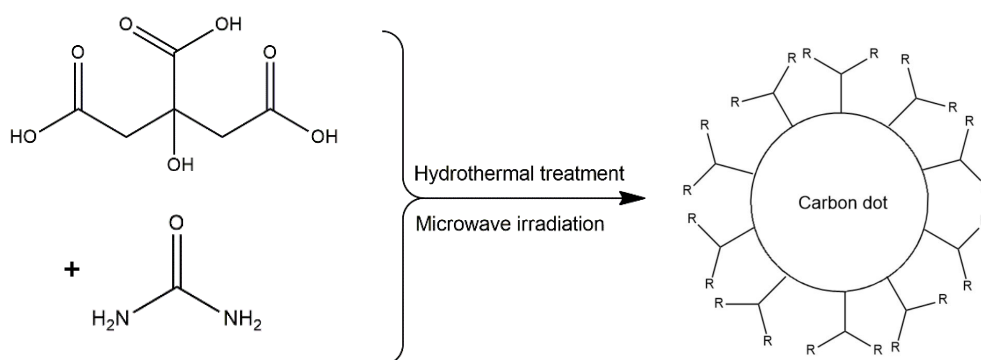


Figure 9 – General scheme for the production of CA- or CA,urea-based CDs for the LCA using two bottom-up methodologies: hydrothermal treatment and microwave irradiation.

For the hydrothermally made CA-based CDs, 100 g of CA were dissolved into 1 L of deionized water. The resulting solution was transferred into a closed *teflon* vessel with metal armoring and inserted in an oven at 200 °C for 2 or 4 hours (VWR DL 112 Prime), depending on the synthetic route. For the microwave-assisted synthesis, 100 g of CA were dissolved into 1 L of deionized water in a glass beaker. The beaker was taken into a domestic microwave (potency of 700 W) and the solution was submitted to microwave irradiation during a period of 5 or 10 minutes, depending on the synthesis. For either methodology, at the end of the reaction time, deionized water was added to complete the initial volume of 1 L. A centrifugation purification step, a simple and common purification step used for CDs, [31, 37] was performed for all CDs' solutions at 6000 rpm for 20 minutes in a MIKRO 220R centrifuge from Hettich. In two of the six

synthetic routes, urea was added to the mixture: 100 g of CA and 100 g of urea were dissolved in 1 L of deionized water. When the solution was clear it was taken either to an oven for 2 hours at 200 °C or submitted to microwave irradiation for 5 minutes (potency of 700 W). In both cases deionized water was added to restore the initial 1 L of solution, if required. A centrifugation purification step ensued (6000 rpm for 20 minutes).

Synthesis	Precursors	Treatment	Time	Vessel
A	CA	Hydrothermal, 200 °C	2h	Closed
B	CA	Hydrothermal, 200 °C	4h	Closed
C	CA, urea	Hydrothermal, 200 °C	2h	Closed
D	CA	Microwave irradiation	5 min	Open
E	CA	Microwave irradiation	10 min	Open
F	CA, urea	Microwave irradiation	5 min	Open

Table 1 – Summary of the synthetic routes used for the synthesis of the CDs used in the LCA. All CDs were prepared in an aqueous solution.

2.2.2. Fluorescence characterization of CDs

The fluorescence spectra of the CDs resulting from the six synthetic routes were obtained using a Horiba Jovin Yvon Fluoromax-4 spectrofluorimeter while employing the FluorEssence software to analyze the results. Standard 10 mm fluorescence quartz cells were used. The emission spectra were obtained with a 1 nm capture interval and 1 nm slit widths. Absorbance measurements were made using a UNICAM Helios Gamma in quartz cells. The QY_{FL} of the synthesized CDs was calculated by comparing the integrated fluorescence intensities and absorbance values displayed by the CDs with the values of quinine sulfate, a fluorophore with a high and known QY_{FL} . The QY_{FL} was calculated using the following equation:

$$QY_{FL} = QY_{FL}^{Ref} \times \frac{Grad}{Grad_{Ref}} \times \frac{\eta^2}{\eta_{Ref}^2}$$

Grad is the slope from the plot of the integrated fluorescence intensity *versus* absorbance and η is the refractive index of the medium, in this case, 1 ($\eta_{water} = 1$). The subscript *Ref* refers to quinine sulphate, which has a QY_{FL} of 54% ($QY_{FL}^{Ref} = 0.54$). [171]

2.2.3. HPLC and XPS characterization of CDs

The chromatographic analysis of the CA,urea-based CDs was performed using a reverse phase HPLC with a diode array detector (RP HPLC-DAD) chromatographic

system. The system employed a Thermo Scientific SpectraSystem P1000 pump, a Rheodyne manual injection valve, a SUPELCOSIL™ LC-18 column and a UV6000 LP diode array detector from Thermo Finnigan. A mobile phase composed of 30% acetonitrile (ACN) and 70% of a 1% ammonium acetate solution was used with a flow of 0.80 mL per minute.

X-Ray Photoelectron Spectroscopy (XPS) was carried out using a Physical Electronic PHI VersaProbe II spectrometer utilizing Al-K α monochromatic radiation with a hemispherical multichannel detector (53.6 W, 15 kV and 1486.6 eV). PHI SmartSoft software was used to analyze the results and the carbon C 1s signal, 284.8 eV, was used as a reference to obtain the binding energy values, using Gauss-Lorentz curves and Shirley-type background.

2.2.4. Study scope and system boundaries

This cradle-to-gate study aims to evaluate the potential environmental impacts of six different synthetic methodologies for the bottom-up production of CDs. The study considered the steps between the production of the raw materials (including electricity) and the production and purification of the nanoparticle. The study focused on the manufacturing of CDs at a laboratory scale and considers both the direct emissions caused by the CDs' synthesis and the upstream indirect impacts. The latter include the impacts resulting from the extraction of resources (production and purification of CA and urea) and production of the energy required for the synthesis. The flowchart depicted in Figure 10 describes the system boundaries, and both the background and foreground systems.

This work is related to three hydrothermal and three microwave-assisted syntheses of CDs. CA was always employed as a carbon source while urea was sometimes used as a nitrogen source. The production schemes mentioned in subsection 2.2.1. were developed by us and the synthesis and purification was performed as described. In a first stage, a volume-based functional unit of 1 L of aqueous CD solution was used to analyze and compare the environmental impacts associated to the synthesis. This was done considering that a volume or weight based functional unit would allow us to compare the impacts related to the production of an equivalent amount of nanoparticles. [155, 158] In a later stage, the environmental impacts were re-scaled by taking into account the QY_{FL} of the different CDs. This was required since a weight or volume based functional unit did not consider the functional utility of the CDs for the purpose they were produced for. In some cases, a more demanding synthesis in terms

of resources and energy may be justified by a greater functionality in the resulting CD. Given that the QY_{FL} of CD is a highly requested and desirable property for most, if not all, of the possible applications for CDs, it was chosen to be used as the normalization factor for the new functionality-based unit.

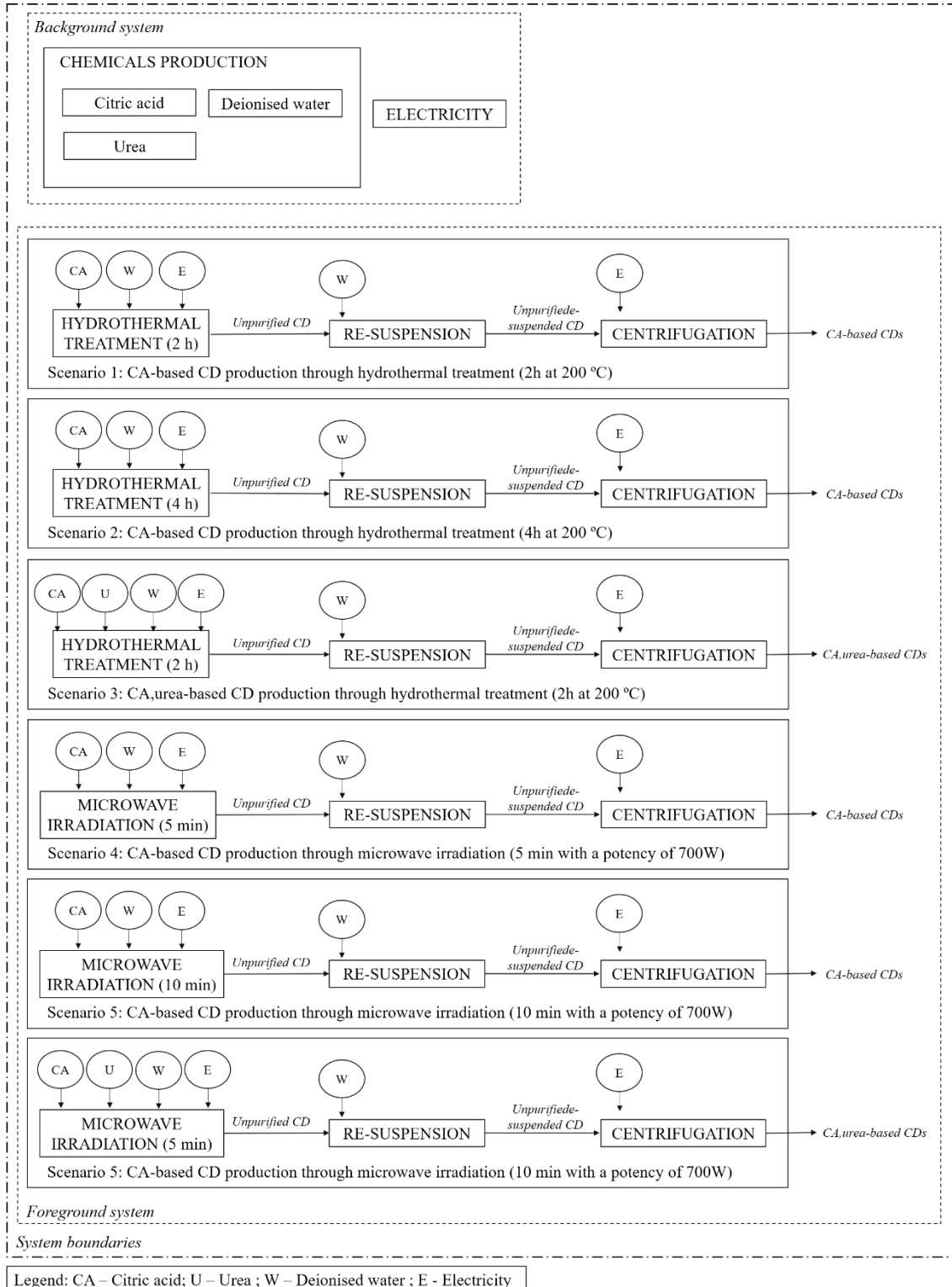


Figure 10 – Flowchart describind the background and foreground systems as well as the system boundaries of the LCA study.

2.2.5. Life cycle inventory data

Inventory (foreground) data from laboratory-scale synthetic methodologies designed by our group (as described in sub-section 2.2.1.) was used as the base for the evaluation of the environmental impacts. The life cycle inventory data for the foreground system consisted on the average data found in the Ecoinvent ® 3.4 database for the synthetic procedures (primary data).

The syntheses of the CDs consisted simply on the hydrothermal or microwave-assisted treatment of an aqueous solution of precursors during a determined amount of time using either an oven or a domestic microwave, respectively. The ensuing purification was a simple centrifugation. Therefore, the background system consisted only in the production of the chemical components used as raw materials (CA, urea and deionized water), and in the generation of the electricity required for the synthesis and purification processes.

The various processes and chemical resources included in this study were modelled with the following Ecoinvent ® 3.4 data: CA - citric acid {GLO} | market for; Urea – Urea, as N {GLO} | market for; Deionized water - Water, deionized, from tap water, at user {Europe without Switzerland} | market for; Electricity – Electricity, medium voltage {PT} | market for. The dataset used for electricity uses the available electricity data on the medium voltage level for Portugal for the year 2014, as described in the used database. The considered electricity combines the amount required for the use of the centrifuge during the purification with either the use of an oven (hydrothermal synthesis) or a domestic microwave (microwave-assisted synthesis). The centrifuge (MIKRO 220 R from Hettich) has a power supply of 230 V with a frequency of 50 Hz. The oven used for hydrothermal treatment is a VWR DL 112 Prime, which possessed a capacity of 112 L and a labeled power consumption of 2500 W. Microwave-assisted synthesis was performed using a model P70B17L-DE Electronia domestic microwave, which has a power consumption of 700 W.

The amounts used (inputs) of CA, urea and deionized water were 100 g, 100 g and 2 L, respectively. The amounts of electricity used are: 5.29 kWh for a hydrothermal treatment with a duration of 2 hours; 10.29 kWh for a hydrothermal treatment with a duration of 4 hours; 0.35 kWh for a microwave-assisted synthesis with an irradiation duration of 5 minutes; 0.42 kWh for a microwave-assisted synthesis with an irradiation duration of 10 minutes.

2.2.6. Environmental impact assessment

The performed LCA study is based on a cradle-to-gate approach, meaning it goes from the production of the precursor materials (CA, urea and deionized water) to the synthesis and purification of the CDs. Environmental impacts were modeled using the ReCiPe 2016 v1.1 LCIA, Hierarchist version. The impact potentials assessed in accord to this method were: global warming - human health (GW – HH), global warming - terrestrial ecosystems (GW – TE), global warming - freshwater ecosystems (GW - FE), stratospheric ozone depletion (SO), ionization radiation (IR), ozone formation - human health (OF – HH), fine particulate matter formation (FPM), ozone formation - terrestrial ecosystems (OF – TE), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TE), freshwater ecotoxicity (TET), marine ecotoxicity (MET), human carcinogenic toxicity (HC), human non-carcinogenic toxicity (HNC), land use (LU), mineral resource scarcity (MR), fossil resource scarcity (FR), water consumption - human health (WC – HH), water consumption - terrestrial ecosystem (WC – TE) and water consumption - aquatic ecosystems (WC – AE). The LCA study was performed by using the SimaPro 8.5.2.0 software.

2.2.7. Sensitivity analysis

Considering that the technological development of CDs at a laboratory scale is still on a fairly early stage, the assessment of the environmental impacts relative to their synthesis can have some associated uncertainty and be quite inaccurate. In order to try and determine such uncertainties for a scale-up of the process, a sensitivity analysis was performed. [172] This was done by considering several “What-if” scenarios which consisted on varying the inputs ($\pm 30\%$) for the amount materials and electricity in the synthesis of the CDs. Additional scenarios in which the precursors were changed altogether were also analyzed (CA substituted by glucose and urea substituted by EDA). This allows for an evaluation of the effect caused by some assumptions in the LCA results. [173]

2.3. Results

2.3.1. Carbon dots characterization

The fluorescence spectra of the six newly synthesized CDs solutions were analyzed and displayed in Figure 11. From the spectra analysis several things are observable. In either treatment, doubling the duration of the reaction time will have a slight impact in the photoluminescence of the resulting particles. With microwave-irradiation the fluorescence wavelength maxima suffers a ~ 27 nm blue-shift (~ 485 nm at 5 minutes *versus* ~ 458 nm at 10 minutes) when the irradiation time is doubled. For the hydrothermal synthesis, doubling the reaction time results in a ~ 21 nm blue-shift (~ 461 nm at 2 hours *versus* ~ 440 nm at 4 hours) on the emission wavelength of the particle.

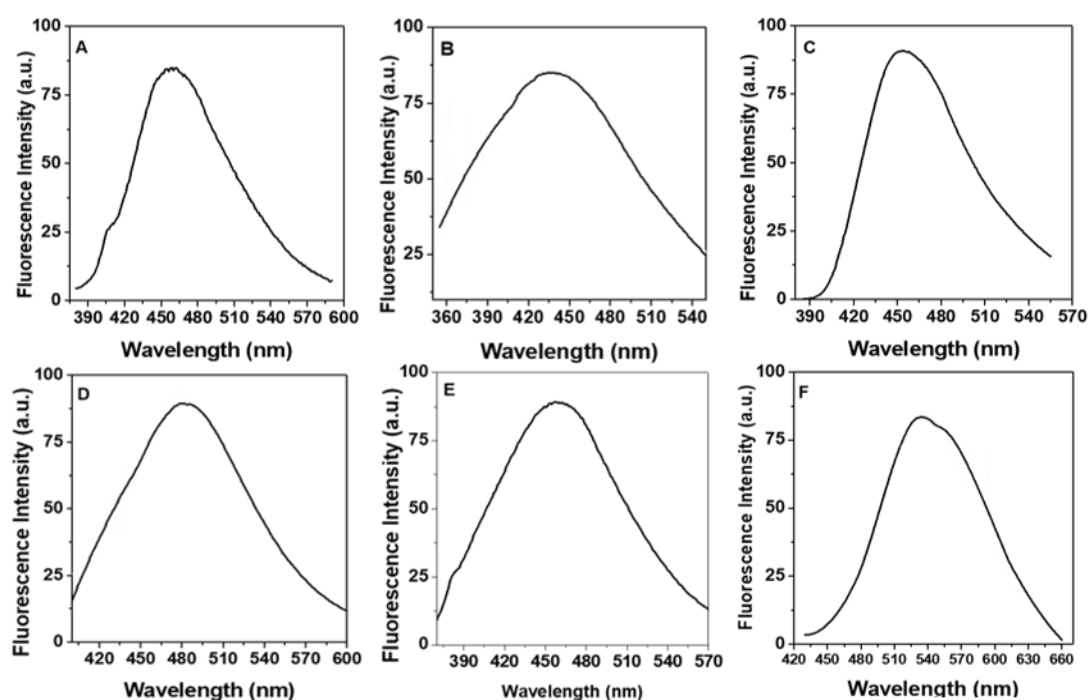


Figure 11 – Fluorescence spectra of the six synthesized CDs. A - Hydrothermal synthesis of CA-based CDs (2h at 200 °C); B - Hydrothermal synthesis of CA-based CDs (4h at 200 °C); C - Hydrothermal synthesis of CA,urea-based CDs (2h at 200 °C); D - Microwave-assisted synthesis of CA-based CDs (irradiated during 5 minutes); E - Microwave-assisted synthesis of CA-based CDs (irradiated during 10 minutes); F - Microwave-assisted synthesis of CA,urea-based CDs (irradiated during 5 minutes).

Additionally, the inclusion of a nitrogen source (urea) in the precursor mixture originates a shift in the emission wavelength maximum. A ~ 50 nm red-shift occurs when compared to the CA-based CD with the same irradiation time while for the hydrothermal treatment a ~ 6 nm shift occurs when urea is added into the reaction. The emission maxima of CA-based CDs are tunable by controlling the reaction time (either the microwave irradiation time and the heating period in the hydrothermal treatment) and by adding a nitrogen source in the reactional mixture.

The QY_{FL} , an important property for CDs, was calculated and displayed in Table 2. Regarding the QY_{FL} , doubling the reaction time leads to very similar results either for hydrothermal treatment or microwave irradiation. The QY_{FL} for CA-based CDs are approximately 8% and 6%, respectively, with no significant observable difference with the extra reaction time. It is also observable that the addition of urea leads to a very significant increase in the QY_{FL} displayed by the CDs. This occurs irrespectively of the synthetic method: hydrothermal treatment ($\sim 49\%$ *versus* $\sim 8\%$) and microwave-treatment ($\sim 52\%$ *versus* $\sim 6\%$). These results, in conjugation with the ones describing the changes in the emission spectra (Figure 11), indicate that if a more intense and red-shifted emission is required, it could be obtained with the addition of urea in a synthesis made through microwave-irradiation.

Precursors	Methodology	QY_{FL}
CA	Hydrothermal, 2h at 200 °C	8.37%
CA	Hydrothermal, 4h at 200 °C	8.03%
CA + Urea	Hydrothermal, 2h at 200 °C	49.01%
CA	Microwave irradiated for 5 minutes	5.88%
CA	Microwave irradiated for 10 minutes	5.97%
CA + Urea	Microwave irradiated for 5 minutes	51.98%

Table 2 – QY_{FL} calculated for CA or CA,urea-based CDs synthesized either by microwave irradiation or hydrothermal treatment. The calculations were made using quinine sulphate as a reference fluorophore ($QY_{FL} = 54\%$).

After performing the photoluminescent characterization of the CDs and discovering the CDs with the highest QY_{FL} , we evaluated the purity of those CDs and made their characterization. The highest QY_{FL} was found in CA,urea-based CDs made either by hydrothermal treatment or microwave irradiation, as seen in Table 2. The purity of the CA,urea-based CDs was evaluated by reverse phase HPLC coupled to a diode array detector (RP-HPLC-DAD system). In the chromatograms presented in Figure 12, while the chromatogram of the hydrothermally produced CDs displays only a single peak (Figure 12a), the chromatogram obtained for the microwave-assisted CA,urea-based CD is comprised of multiple peaks (Figure 12b). This indicates that, while the purity of the hydrothermally produced CDs may be considered as satisfactory and they do not demand further purification steps, there is a presence of different composites in the microwave made CD solution. Those composites result from the microwave irradiation of the precursor solution, and further purification is required to remove them. Additional purification by more complex and resources/energy consuming steps (dialysis,

chromatographic separation, electrophoretic separations, among others) may be required in order to obtain an acceptable degree of purity.

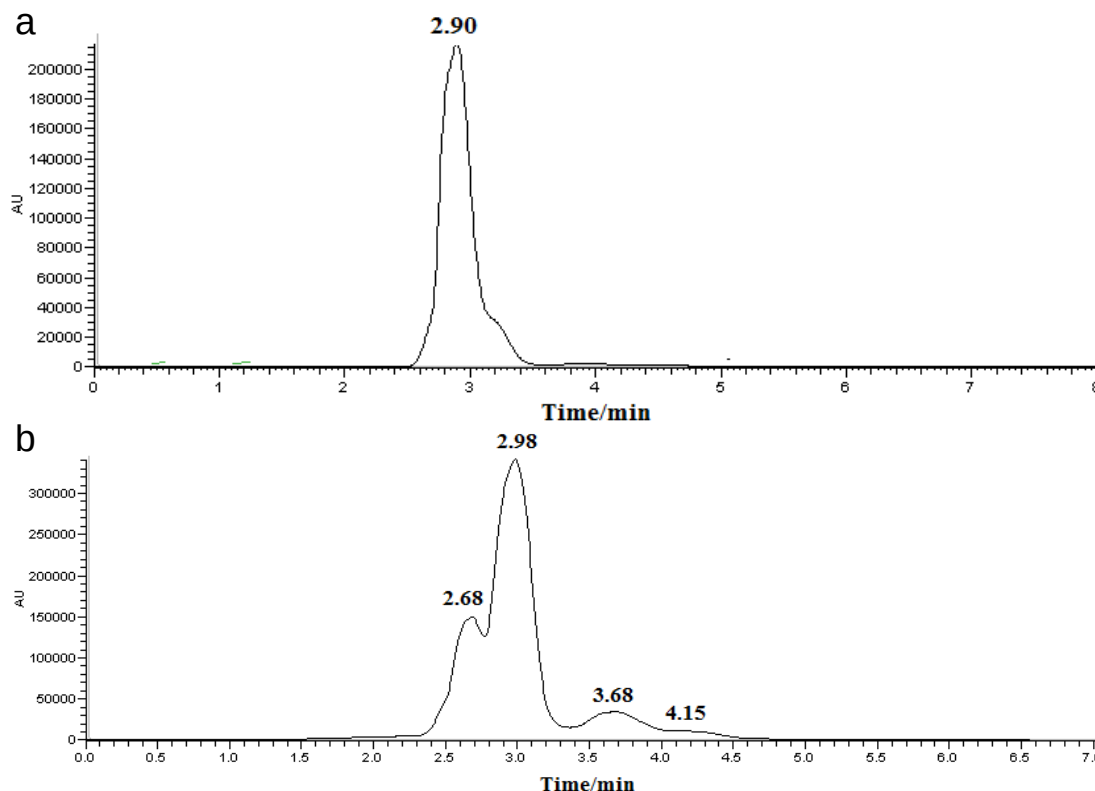


Figure 12 – RP-HPLC chromatograms of CA,urea-based CDs prepared by a) hydrothermal treatment (2 h at 200 °C) and b) microwave irradiation (5 minutes of irradiation with a potency of 700W).

In order to better understand the surface composition and electronic states of the elements present in the CA,urea-based CDs, a XPS analysis was performed and the results presented in Figure 13. After a microwave treatment of 5 minutes, the content on the particle surface in terms of C, N and O (in %) was 59.70, 16.75 and 23.55 %, respectively. By comparison, when the CD is produced through a hydrothermal procedure, the content of C, N and O (in %) was 55.74, 14.06 and 30.20 %, respectively. These results indicate that hydrothermal treatment, when compared with alternative, leads to an increased oxygen content on the CD surface, while causing a decrease in the presence of C and N atoms. This can be explained by oxidation processes that occur at the CD surface at 200 °C, when hydrothermal treatment is employed.

Considering that the CDs' survey scan presented peaks for three elements (C, N and O), a scan for the C 1s, O 1s and N 1s internal levels for deconvolution and chemical state was performed. Regarding the C 1s spectrum, for both CDs (hydrothermally-made and microwave irradiated), it splits into three peaks. Binding energies of 284.8 eV (54.53

%), 286.6 eV (11.95 %) and 288.3 eV (33.5 %) were observed for the CD made through microwave irradiation. The hydrothermally obtained CD displayed binding energies of 284.8 eV (54.79 %), 286.9 (17.1 %) eV and 288.6 eV (28.1 %). The observed C 1s peaks in either CD can be ascribed to C-C/C-H/adventitious carbon (284.8 eV), being adventitious carbon a layer of carbonaceous material normally found on the surface of air-exposed materials and usually comprised of hydrocarbon species with the occasional bond with an oxygen [174], C-O/C-N (286.6-286.9 eV) and C=O/O-C=O carbonyl/carboxylic groups (288.3-288.6 eV). The results show that hydrothermal treatment led to an increase in the C-O/C-N contribution while decreasing the C=O/O-C=O contribution comparatively to microwave treatment.

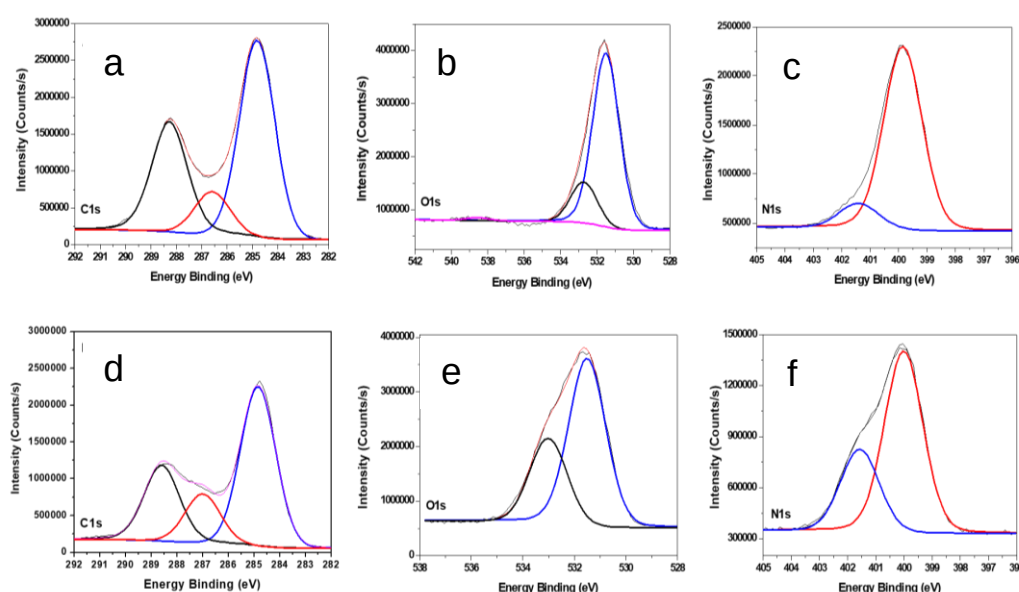


Figure 13 - XPS core level spectra of the CDs resulting from CA and urea after a 5 minutes microwave irradiation: a) C 1s b) O 1s and c) N 1s; and hydrothermal treatment for 2h at 200 °C: d) C 1s, e) O 1s and f) N 1s.

As for the O 1s spectrum, the CA,urea-based CDs made by microwave irradiation, when deconvoluted, presented a major peak at 531.5 eV (80.9 %) and a smaller one at 532.7 eV (18.1 %) that are due to carbonyl (C=O) linkage and C-O/O-C-O groups, respectively. The hydrothermal variant displays a similar spectrum profile with a higher C-O contribution, up to 32.42 %.

Finally, for the N 1s spectrum of the CD made through microwave irradiation, a major peak and a shoulder were found at 400.0 eV (88.9 %) and 401.5 eV (11.1 %), respectively. These can be ascribed to groups of amines/amides (400.0 eV) and protonated amines (401.5 eV). As for the hydrothermally treated CDs, they display an enhancement on the higher binding energy contribution (up to 30.5 %), which is explainable by the presence of protonated amine groups at the CDs' surface. The ratio

between the two binding energy peaks (400/401.5) goes from 8.03 in the microwave treatment to 2.3 with the hydrothermal treatment. This suggests the presence of a higher content of protonated amine groups on the CD surface after hydrothermal treatment, possibly due a greater degree of surface oxidation.

In summary, as seen by the CDs characterization, microwave-made CA,urea-based CDs might require further purification steps aside the initial centrifugation. This causes the process to be more energy and resource consuming while also being associated with the extra downside of decreasing the QY_{FL} of the CD solution (given that some by-products formed during the CD synthesis can positively contribute to the solution fluorescence). [135] When purity is desired, hydrothermally made CDs should be preferred as they present a satisfactory purity and a similar QY_{FL} .

2.3.2. LCA study

2.3.2.1. Synthesis comparison using a volume-based functional unit

In the first stage, a comparison between the six synthetic routes was made using a volume-based functional unit of 1 L of CD solution. This was made regarding the environmental impact displayed by the different synthetic procedures. The analysis for the routes based in hydrothermal treatment (Figure 14) demonstrated that electricity is the highest contributing resource for the majority of the environmental impact categories. The exceptions are the categories of marine eutrophication, stratospheric ozone depletion, land use and water consumption, for which the highest contributor is CA. Interestingly, doubling the reaction time, and therefore doubling the consume of electricity, only causes a small impact in the individual categories. Addition of urea to the precursor mixture results in small contributions, with the exception being the categories of terrestrial ecotoxicity, mineral and fossil resource scarcity and water consumption. The impact of deionized water seems to be almost negligible even for the categories closely related to water consumption.

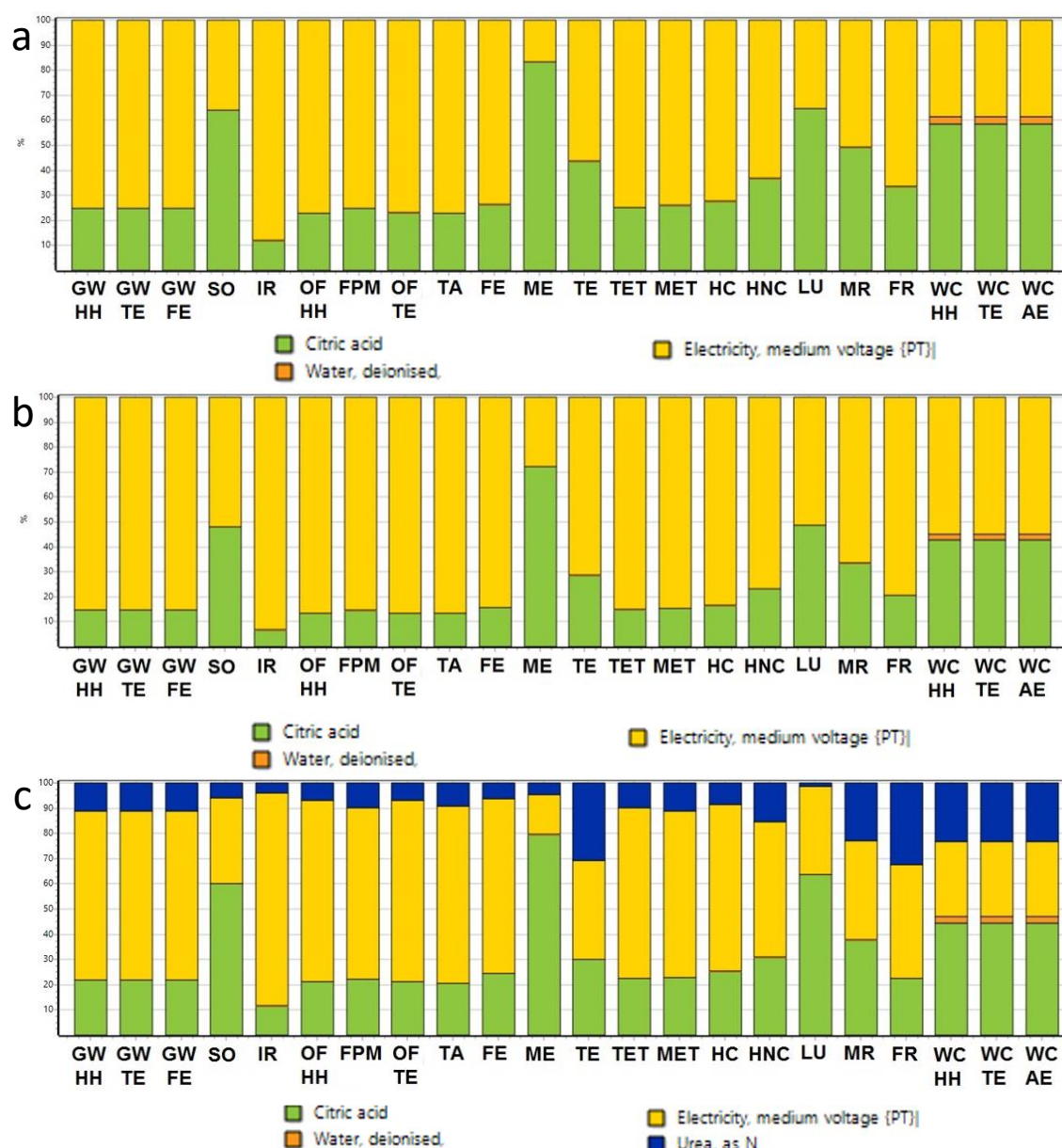


Figure 14 – Relative environmental impacts of CDs made through hydrothermal treatment using the ReCiPe2016 LCIA: a) CA-based CDs at 200 °C for 2 hours; b) CA-based CDs at 200 °C for 4 hours; c) CA,urea-based CDs at 200 °C for 2 hours. Abbreviations: global warming - human health (GW – HH), global warming - terrestrial ecosystems (GW – TE), global warming - freshwater ecosystems (GW – FE), stratospheric ozone depletion (SO), ionization radiation (IR), ozone formation - human health (OF – HH), fine particulate matter formation (FPM), ozone formation - terrestrial ecosystems (OF – TE), terrestrial acidification (TA), freshwater eutrophication (FE), marine eutrophication (ME), terrestrial ecotoxicity (TE), freshwater ecotoxicity (TET), marine ecotoxicity (MET), human carcinogenic toxicity (HC), human non-carcinogenic toxicity (HNC), land use (LU), mineral resource scarcity (MR), fossil resource scarcity (FR), water consumption - human health (WC – HH), water consumption - terrestrial ecosystem (WC – TE) and water consumption - aquatic ecosystems (WC – AE).

As for the results obtained with a microwave-assisted synthesis (Figure 15), they appear to be the opposite of those obtained with hydrothermally synthesized CDs. The use of electricity only causes small impacts, which is explainable when we consider the small time of irradiation (5 or 10 minutes). Additionally, doubling the reaction time results only in a slight impact in the individual categories. Because of this, CA now becomes the major contributor for all environmental impact categories, and the relative contribution of urea, when added, is now increased (mainly due to the significant reduction in the

contribution coming electricity consumption). In some categories, namely terrestrial ecotoxicity and fossil resource scarcity, the contribution of urea is equal or even superior to that of CA. The contribution from deionized water remains negligible.

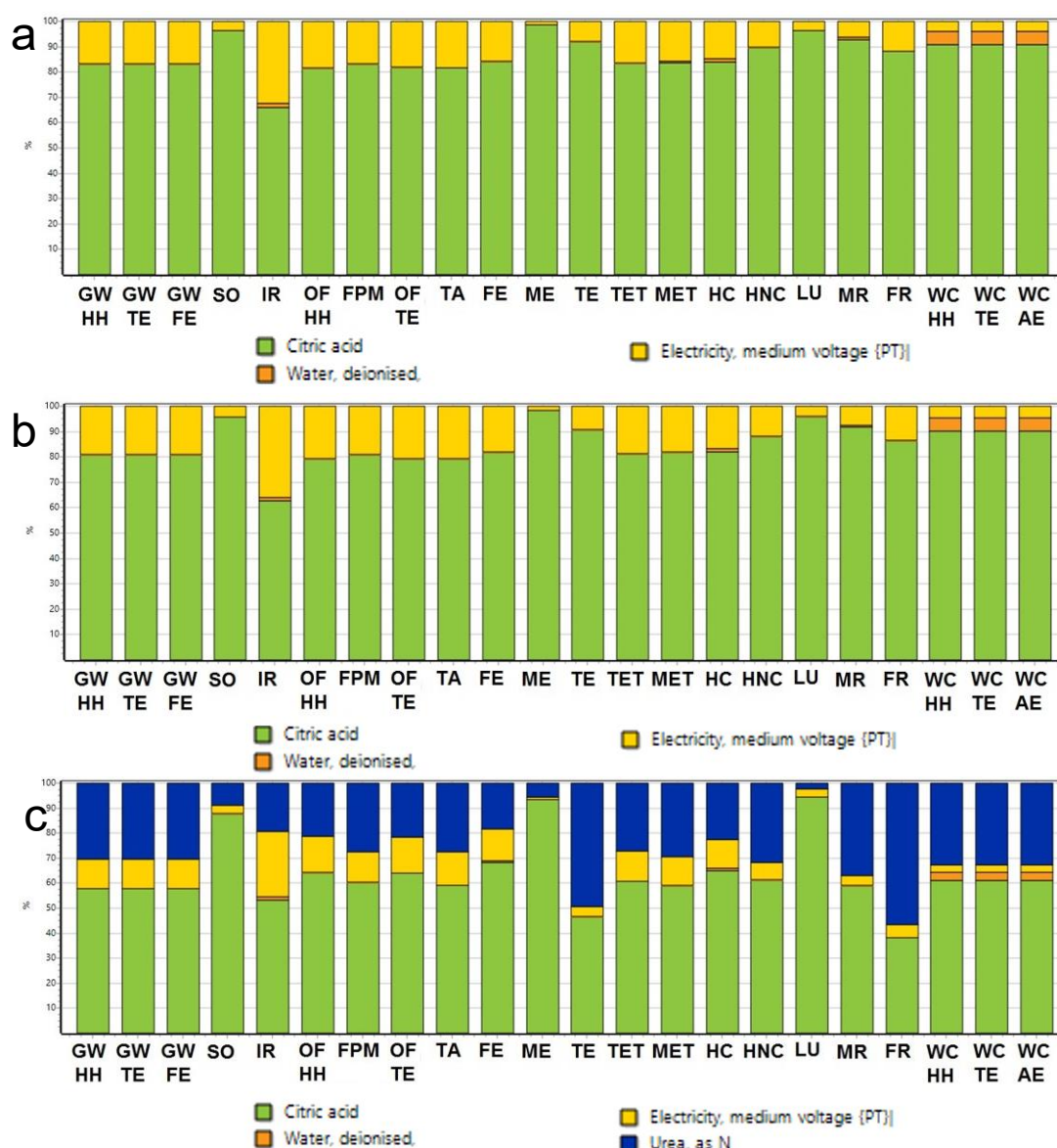


Figure 15 - Relative environmental impacts of microwave-assisted-synthesized CDs with the ReCiPe2016 LCIA: CA-based CDs under microwave irradiation for 5 minutes (a); CA-based CDs under microwave irradiation for 10 minutes (b); CA,urea-based CDs under microwave irradiation for 5 minutes (c). The abbreviations are the same as in Figure 14.

In order to facilitate the comparison between the results, and to present a summary of the obtained data, the general environmental impacts for the six synthetic routes are presented in Figure 16. The impacts appear summarized in three categories: human health, ecosystems and resources. In general, the use of microwave irradiation is less impactful than synthesis by hydrothermal treatment. Furthermore, doubling the hydrothermal treatment from 2 to 4 hours results in a significant increase of the impact for all three categories, while the impact from doubling the microwave-assisted synthesis

time is almost negligible. Finally, the addition of urea as a precursor results only in small effects in the human health and ecosystems categories. However, it can significantly increase the impacts associated to the usage of resources. The additional impact can be the equivalent of doubling the reaction time in the hydrothermal synthesis and, in a 5-minute microwave synthesis including urea, it results in impacts equivalent to those of a 2 hours hydrothermal synthesis using CA as precursor.

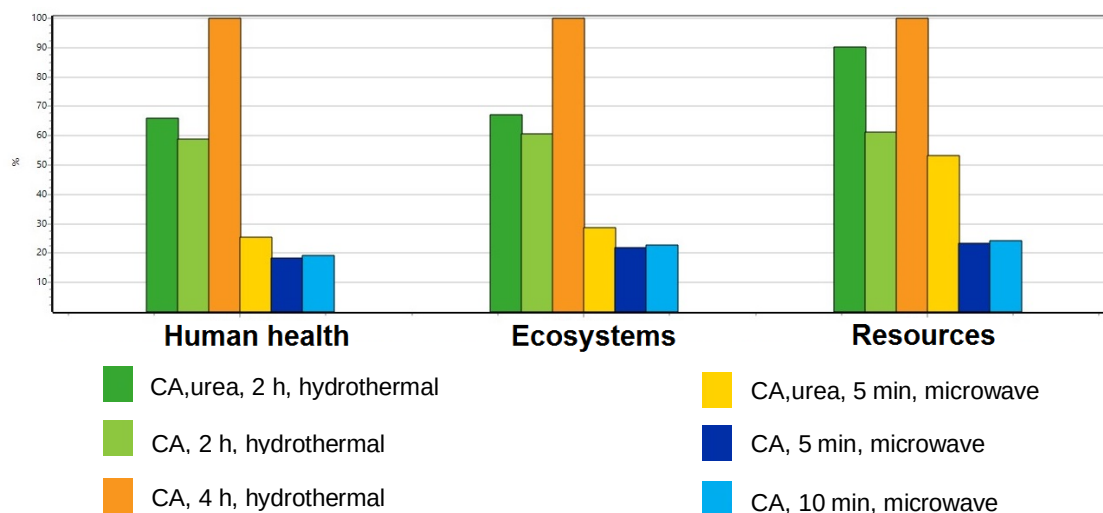


Figure 16 – Environmental profiles of the impacts caused by the synthesis of CDs using a volume-based functional unit of 1 L of CD solution. Environmental profiles were obtained using ReCiPe 2016 v1.1 as a LCIA, while toxicologic profiles were obtained using USEtox 2.02 as a LCIA.

2.3.2.2. Synthesis comparison using the QY_{FL} as a functional unit

The environmental (Figure 17) impacts profile for the six synthetic routes for CD synthesis were also compared based on a new functional unit obtained by using the CDs QY_{FL} as a re-scaling factor. The re-scaling was made by using the highest observed QY_{FL} (51.98% from the CA,urea-based microwave-made CDs) as a reference quantum yield, QY_{FL}^{REF} . The normalized functional unit ($QY_{FL}-FU$) was calculated by dividing QY_{FL}^{REF} by the QY_{FL} of each CD. The resulting $QY_{FL}-FU$ are displayed in Table 3.

Precursors	Methodology	QY_{FL}	$QY_{FL}-FU$
CA	Hydrothermal, 2h at 200 °C	8.37%	6.21
CA	Hydrothermal, 4h at 200 °C	8.03%	6.47
CA + Urea	Hydrothermal, 2h at 200 °C	49.01%	1.06
CA	Microwave irradiated for 5 minutes	5.88%	8.84
CA	Microwave irradiated for 10 minutes	5.97%	8.71
CA + Urea	Microwave irradiated for 5 minutes	51.98%	1.00

Table 3 – QY_{FL} and normalized quantum yield functional unit, $QY_{FL}-FU$, for the synthesized CDs.

As seen in Figure 17, after re-scaling, hydrothermal treatment still presents an environmental impact considerably higher than microwave-assisted synthesis. This means that hydrothermally-synthesized CA-based CDs are the worst option in terms of environmental impact, either when using a volume-based functional unit or a QY_{FL} -based unit. When urea was added as a nitrogen-source and a volume-based functional unit was considered, CA,urea-based CDs were the second (hydrothermal treatment) and fourth (microwave-assisted) worst options, irrespective of the environmental categories considered. When the QY_{FL} is considered, significant differences are observable and the CA,urea-based CDs become the first (microwave-assisted) and second (hydrothermal treatment) best options in terms of relative potential environmental impacts.

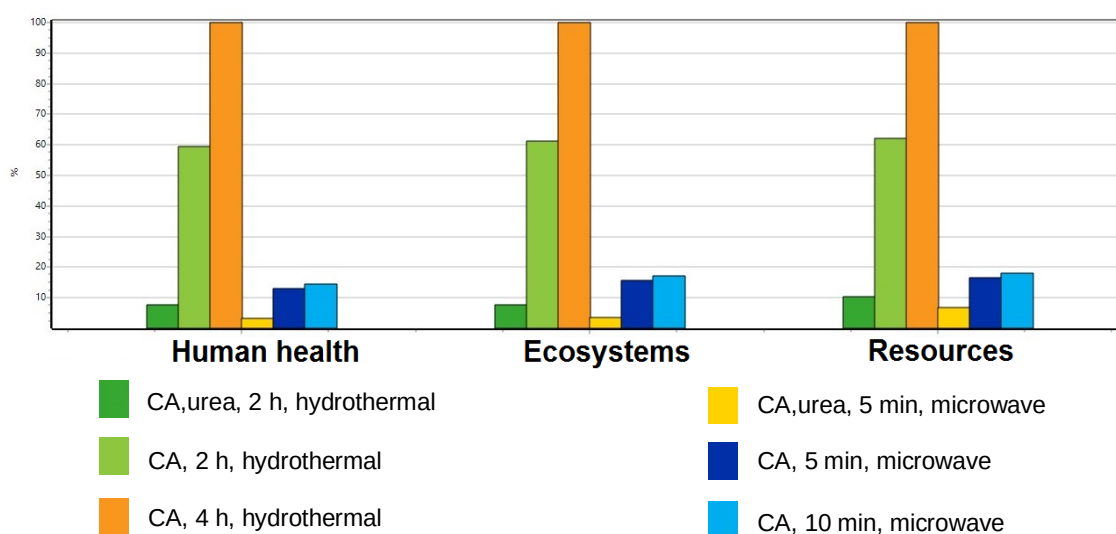


Figure 17 – Environmental profiles of the impacts caused by the six different synthetic routes for the synthesis of CDs, rescaled with consideration to the QY_{FL} of the resulting CDs. Environmental profiles were obtained using ReCiPe 2016 v1.1 as a LCIA, while toxicologic profiles were obtained using USEtox 2.02 as a LCIA.

In summary, when the QY_{FL} is considered in the analysis, CA,urea-based CDs are the best option in terms of environmental and toxicologic impacts, irrespective of the synthetic approach. This was not the case when the QY_{FL} was not used to rescale the results, demonstrating that in the case of purpose-made ENMs, a parameter that allows the LCA to consider the functionality of the nanomaterial is required. Additionally, the results demonstrate that in general, hydrothermal treatment causes greater environmental and toxicologic impacts than microwave-assisted synthesis. Hence, microwave-assisted synthesis should be preferred in detriment of hydrothermal treatment, which is the worst option in terms of impact for either functional unit.

2.3.3. Sensitivity evaluation

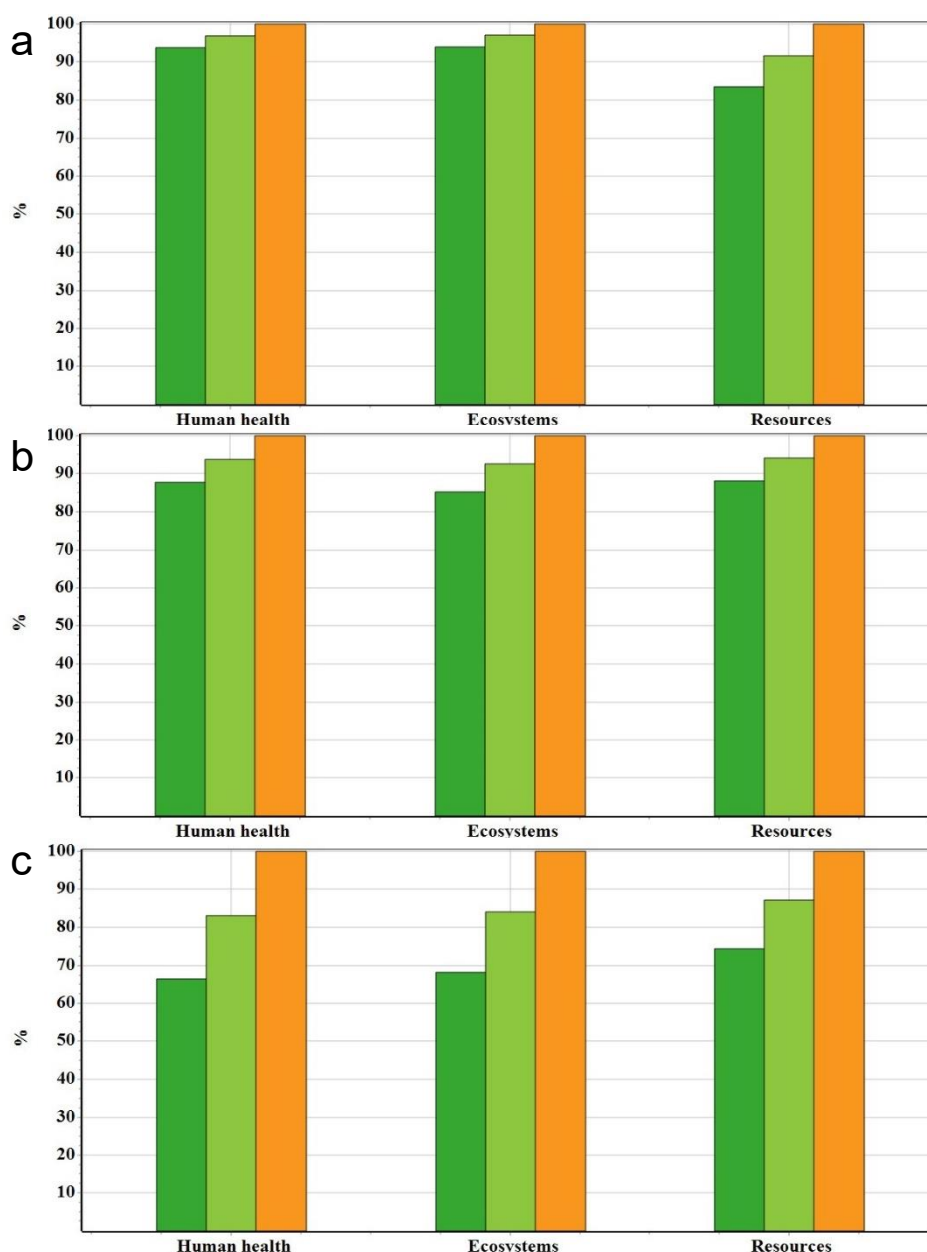


Figure 18 – Comparative environmental profiles regarding the variation of the urea (a), CA (b) and electricity (c) inputs by $\pm 30\%$ for the hydrothermal synthesis of CA,urea-based CDs. Dark green bars refer to variations of -30%, light green bars refer to base levels, and orange bars refer to variations of 30%.

A sensitivity analysis was performed with regard to the two best performing synthetic methodologies in the LCA when a QY_{FL} -based functional unit was used: CA,urea-based CDs made by either hydrothermal treatment or microwave-assisted synthesis. The tested scenarios consisted on altering the input values for the electricity and raw materials (CA and urea) by $\pm 30\%$ and altering the precursors used for the synthesis (glucose instead of CA and EDA instead of urea). Both glucose [175] and EDA [36] are commonly used precursors for the synthesis of CDs.

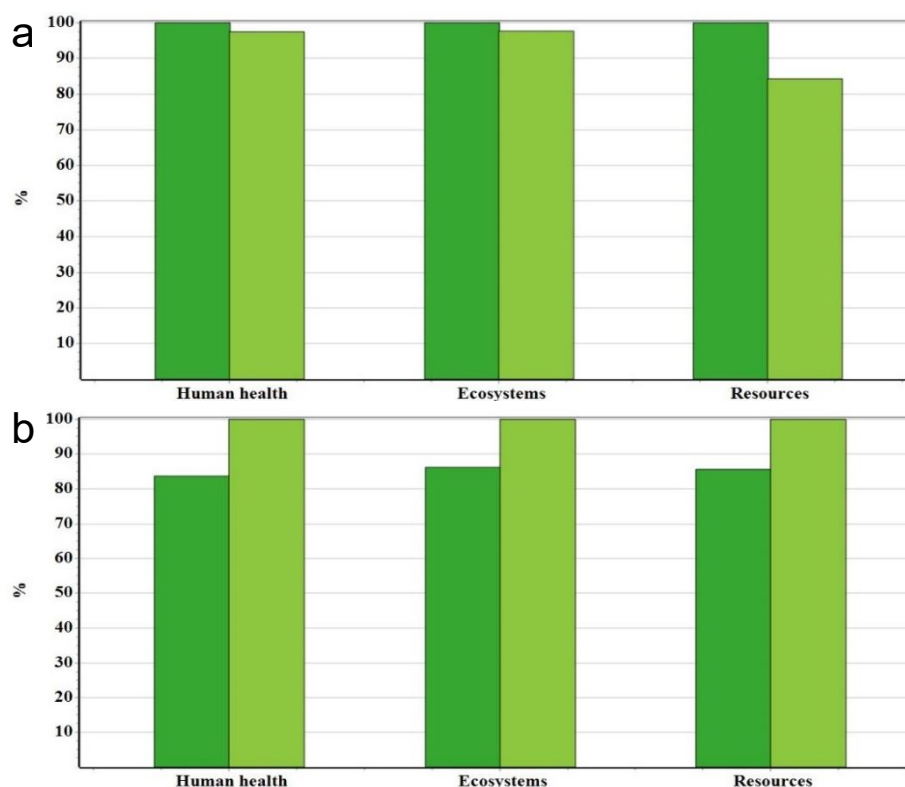


Figure 19 – Environmental profiles for the hydrothermal synthesis of CA,urea-based CDs when (a) urea is replaced by an equal amount of EDA and (b) CA is replaced by an equal amount of glucose.

Figure 18 presents the results obtained for the hydrothermally made CA,urea-based CDs when the inputs of CA, urea and electricity are changed by $\pm 30\%$. Figure 19a and 20b display the LCA results when EDA is used as a nitrogen source instead of urea, and when glucose is used as a carbon source instead of CA, respectively. A 30% change in the amount of urea (Figure 18a) has a negligible effect in the human health and ecosystem impact categories ($\pm 3\%$) while resulting in a more noticeable effect for the resources impact category ($\pm 8\%$). Knowing this, it is not very surprising that using EDA instead of urea resulted in significant changes only in the resources category (Figure 19a), in which the impact increased by 16%, while the other categories only saw a variation of 3%. Alterations of $\pm 30\%$ on the input of CA (Figure 18b) results in moderate effects ($\pm 6\%$ for all three categories), while replacing it by glucose altogether led to a 15% decrease in all impact categories (Figure 19b). Finally, altering the energy input by $\pm 30\%$ results in significative alterations for all the environmental impact categories (Figure 18c). The impact in the human health and ecosystems categories varies by $\pm 16\%$ while the impact in the resources category varies by $\pm 13\%$. For hydrothermal synthesis both electricity and CA appear to be quite relevant sensitive factors capable of influencing the environmental impacts.

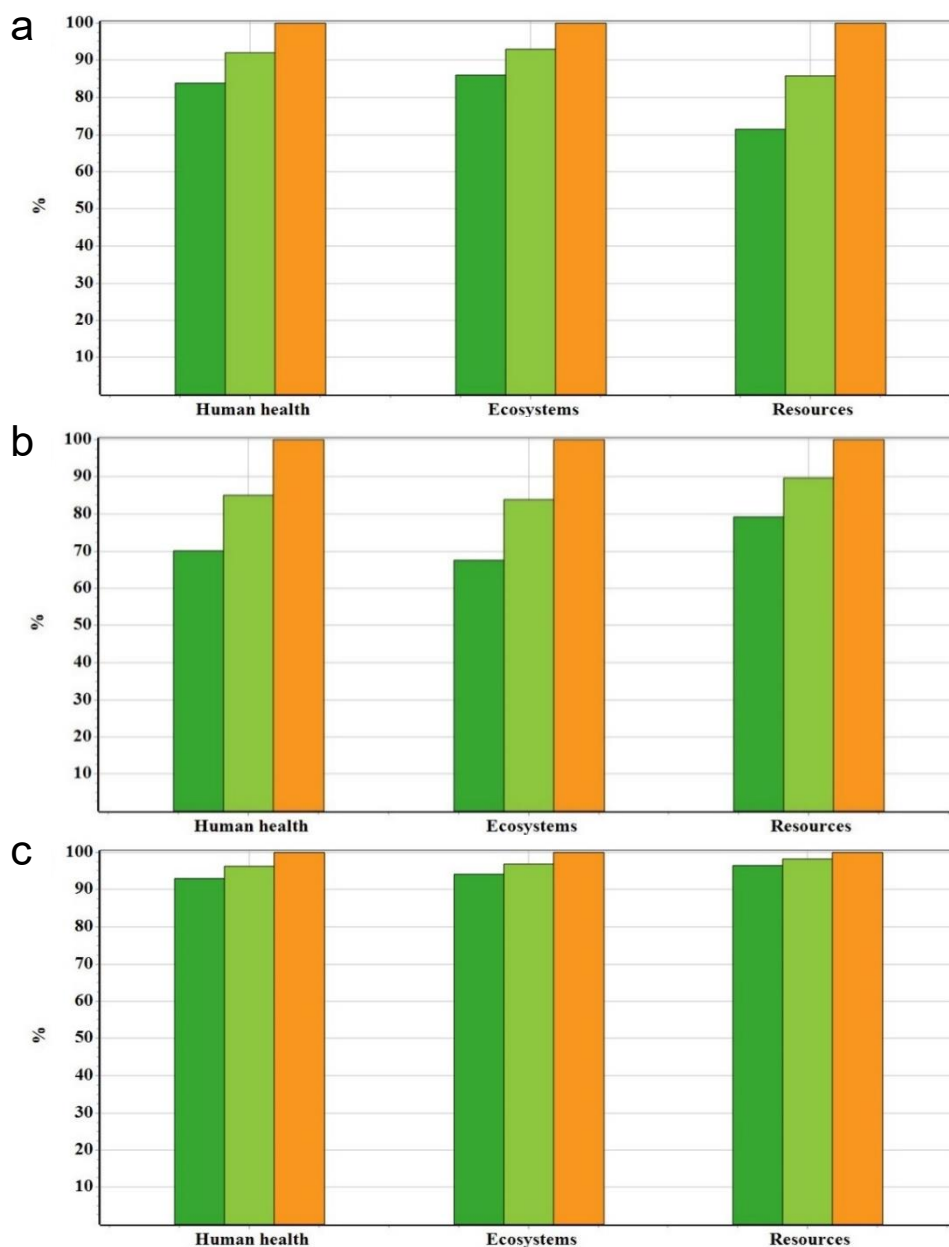


Figure 20 – Comparative environmental profiles regarding the variation of the urea (A), CA (B) and electricity (C) inputs by $\pm 30\%$ for the microwave-assisted synthesis of CA,urea-based CDs. Dark green bars refer to variations of -30% , light green bars refer to base levels, and orange bars refer to variations of 30% .

On the other hand, in the microwave-assisted synthesis of CA,urea-based CDs, altering the electricity input has an almost negligible effect ($\pm 3\%$) in the three tested environmental impact categories (Figure 20c). Instead, changing the amount of CA by $\pm 30\%$ (Figure 20b) led to pronounced changes in the impacts (between $\pm 10\%$ and $\pm 16\%$), while replacing CA by glucose altogether (Figure 21b) resulted in significant decreases for all three impact categories: 44% for human health, 33% for ecosystems and 27% for resources. Finally, changing the input of urea (Figure 20a) results in moderate changes in the human health and ecosystems categories ($\pm 8\%$ and $\pm 7\%$, respectively) and causes a more relevant change in the resources impact category

($\pm 14\%$). The replacement of urea by EDA in the study (Figure 21a) originated a quite considerable increase in the resources category (26%) while causing a more moderate increase in the human health and ecosystems categories (6%). Summarizing, for the microwave-assisted synthesis of CA,urea-based CDs, the use of CA appears to be the most sensitive factor in terms of the overall environmental impact. Additionally, urea, due to the low contribution of electricity, appears to have a significant impact for the resources impact category.

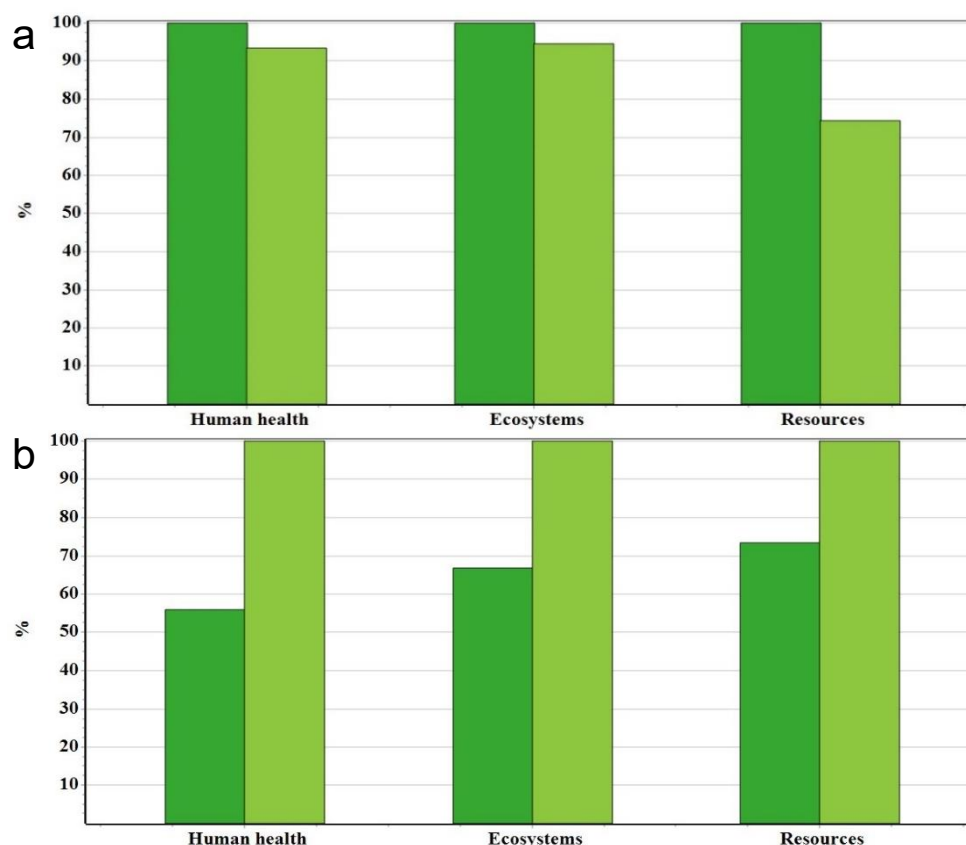


Figure 21 – Environmental profiles for the microwave-assisted synthesis of CA,urea-based CDs when (a) urea is replaced by an equal amount of EDA and (b) CA is replaced by an equal amount of glucose.

Despite the results presented in this section showcasing the environmental impacts induced by changes in the system, it is noteworthy that the QY_{FL} of a nanomaterial is highly dependent on its structure, which in turn depends on the identity and molar ratio of the chemicals used as precursors. Therefore, although the sensitivity analysis showed that CA and urea are sensitive factors for the resulting environmental impacts, more detailed studies are required before a variation of their ratio, or their replacement, can be recommended. Given that changes in these specific parameters will most likely alter the QY_{FL} of the resulting nanoparticle and offset the expected environmental benefits originated by the high QY_{FL} , the resulting relative impacts will possibly differ and the specific outcome can not be predicted without further studies.

2.4. Conclusions

This study was the first LCA-based assessment regarding representative synthesis strategies for CDs and the environmental impact they cause. The studied synthetic methodologies included hydrothermal treatment and microwave irradiation, two widely used bottom-up strategies. Aqueous solutions of CA and urea were employed as the precursors. Those represent, arguably, the most common and relevant synthetic routes for the production of CDs.

The environmental impact results were obtained by taking into consideration two different units: a simple volume-based functional unit (which does not account for the CD functionality) and a QY_{FL} -based functional unit that allows for the CDs' performance to be considered. During the first stage of the study, analysis using the volume-based unit demonstrated that hydrothermal treatment and microwave-assisted synthesis have different environmental impact profiles. Using this unit, the most suitable option in terms of overall environmental impacts would be CA-based CDs synthesized through microwave irradiation. This kind of synthesis has lower impacts because of the smaller reaction times (decreasing the impacts derived from obtaining electricity) and the absence of urea in the precursor solution. While for hydrothermal treatment the use of electricity has the highest contribution for the majority of the tested categories, this was not the case with microwave-assisted synthesis. For this kind of route, the resources used, in particular CA, are responsible for the majority of the impacts caused. The predominance of the impact caused by electricity in hydrothermal synthesis is explainable by the large duration of the syntheses (hours instead of minutes). In hydrothermal treatment, adding urea has little impact in the individual environmental impact categories, while for microwave-assisted synthesis it results in major contributions. However, urea has a significant impact for resources irrespectively of the synthetic methodology: the addition of urea in hydrothermal based synthesis results in an impact equivalent to doubling the duration of the synthesis period in a microwave-assisted synthesis; in microwave-assisted synthesis, addition of urea to a 5-minute synthesis will result in an impact similar to a 2 hours hydrothermal synthesis. In summary, when considering the environmental impact alone, the rank order for the tested synthetic routes is: CA-based microwave-made CDs (either with 5 or 10 minutes irradiation duration) > CA,urea-based microwave-made CDs (5 minutes irradiation duration) > CA-based CDs made by hydrothermal treatment (2 hours at 200°C) > CA,urea-based CDs made by hydrothermal treatment (2 hours at 200 °C) > CA-based CDs made by hydrothermal treatment (4 hours at 200 °C).

However, when a QY_{FL} -based functional unit is chosen, the rank order among the different synthetic routes changes. CA,urea-based CDs, made by either approach, become the best option in terms of environmental impacts. This is majorly due to their very high QY_{FL} , which offsets the relative impacts associated to the synthesis. But, as seen by chromatographic analysis, while hydrothermal treatment results in a reasonably pure CD solution, microwave-irradiation causes the formation of several moieties in the CD solution. To remove them, additional and more complex steps of purification are required, resulting in a higher cost in terms of energy and resources. Furthermore, additional purification steps might cause a decrease in the QY_{FL} , decreasing the functional potential of these CDs. In general, the data presented demonstrates that, while the use of electricity during the synthesis is the main contributor for hydrothermal synthesis, CA contributes the most for the impacts resulting from a microwave-assisted synthesis.

A sensitivity analysis considering several scenarios was also performed towards the goal of decreasing the environmental impacts resulting from the synthesis of CDs. The tested scenarios consisted on varying the inputs of each component (CA, urea and electricity), or by replacing the raw materials with similar compounds (glucose for CA and EDA for urea). For hydrothermal-assisted synthesis, electricity is the most sensitive factor when changes are introduced in its input. On the other hand, changes on the amounts of CA and urea do not result in any significant alterations in the associated environmental impacts. However, the replacement of CA by glucose altogether leads to a very significative reduction in the associated impacts. This reduction is even more prominent when the CDs are synthesized by a microwave-assisted method, which is expected, given that CA is a more sensitive parameter for this kind of synthesis than for hydrothermal treatment. Urea is also a sensitive parameter for microwave-assisted synthesis, and its replacement by EDA increased the associated environmental impacts. Finally, due to the low amounts of energy required, electricity is not a sensitive factor for the microwave-assisted synthesis of CDs. The effect caused by the use of deionized water is negligible for both methodologies.

In summary, nitrogen-doping strategies originate great benefits in terms of the QY_{FL} of the resulting nanoparticle. From this results an offsetting of the environmental impacts associated CDs' synthesis, either by hydrothermal treatment or microwave irradiation. Furthermore, it was observed that the carbon source used as a precursor is a critical factor in both kinds of methodologies. Considering that, it should become a focus of interest in future studies related to a cleaner production of CDs.

3. Effect of molecular fluorophores in the fluorescence and reactivity of CDs: insight into a hybrid synergistic effect

3.1. Introduction

CDs possess several desirable properties, amongst which their high photoluminescence is one of the most desirable. Even though CDs have been extensively studied during recent years, the origin of their photoluminescence is still a matter of active debate. [19, 176] Several models have been proposed: quantum confinement effect and band gap emission, [114, 125] surface states emission, [108, 126, 127] self-trapped excitons, [131] among others. Recently there has been an interest about the role of molecular fluorophores in the CD optimal photoluminescent properties. As was reported by several researchers, the synthesis of CDs through bottom-up methodologies, which include the most commonly used routes for the fabrication of CDs, can lead to the formation of several fluorescent molecular by-products. [103, 121, 124, 128, 135, 136] Successful separations of the fractions containing the CD and the molecular by-products demonstrated the CD fraction to be weakly fluorescent while the fluorescent by-products are strongly fluorescent. [121, 124, 128, 135, 136] This suggests that the fluorescence commonly associated to CDs can be highly influenced (even masked) by the presence of fluorescent molecular by-products in the solution that are formed during the CD synthesis. This would consist in a setback to our knowledge regarding these materials.

Knowing this, a better understanding regarding the effect of those fluorescent impurities in the fluorescence displayed by a CD solution is required. With this work we aim to assess if, when present and co-existing in the same solution, the CD and the fluorescent impurities behave as two separated species with a well-defined individual fluorescent behavior, or if, on the other hand, upon synthesis, the CD and the impurities interact and originate some kind of synergy. If the interaction between CD and fluorescent by-products does in fact originate a synergistic effect, this could lead to the fabrication of novel hybrid materials that would have different properties than those of the individual components alone.

Towards this objective, CA,urea-based CDs were made through microwave irradiation and were characterized through different techniques (AFM, HR-TEM, FT-IR,

XPS, UV-Vis, ESI-MS, UV-Vis and fluorescence spectroscopy). The CD solution was further fractioned into three samples: the CD, the fluorescent impurities and a mixture which contained both the CD and the fluorescent impurities. Studies were also performed with electron-withdrawing and electron-donor probes to assess if the CD and the fluorescent impurities would present an individual photoluminescence. It was observed that, when co-existing in the same solution, the CD and the fluorescent impurities do not display individual photoluminescent properties. Instead, they interact and originate a synergistic effect that differs from the sum of the properties of the individual species. Efficient purification steps are required if we intend to observe CDs' emission without it being masked by the emission of the strongly photoluminescent impurities. Despite that, when the impurities co-exist with the CD, a potential arises for the fabrication of novel hybrid materials (composed by CD – molecular fluorophores) with properties different than those of the individual species alone.

3.2. Methods

3.2.1. CD samples production

CDs were fabricated through the microwave irradiation of a CA and urea aqueous solution. Briefly, 0.5 g of CA and 0.5 g of urea were dissolved in 5 mL of deionized water in a glass beaker. The solution was submitted to microwave irradiation for a duration of 5 minutes (potency of 700 W in a domestic microwave). In the end, 5 mL of deionized water were used to re-suspend the product from the synthesis, yielding a CD solution. Purification steps followed the synthesis: centrifugation (13 000 rpm for 10 minutes) and dialysis (3 days, membrane cut-off of 1000 Da). While centrifugation is a simple and commonly used process to remove suspended particles in a solution through gravitational force, it is not capable of separating the CD from the soluble impurities produced during the CD synthesis (due to them being low-weight compounds). [31, 37, 135] A more complex purification step had to ensue. Dialysis is a process based on the separation of the components of a mixture in function of their size: particles bigger than the molecular weight cut-off (MWCO) of the dialysis bag membrane cannot exit the bag (as they cannot cross the bag membrane), while particles smaller than the MWCO can exit the bag, separating themselves from the bigger particles (Figure 22).

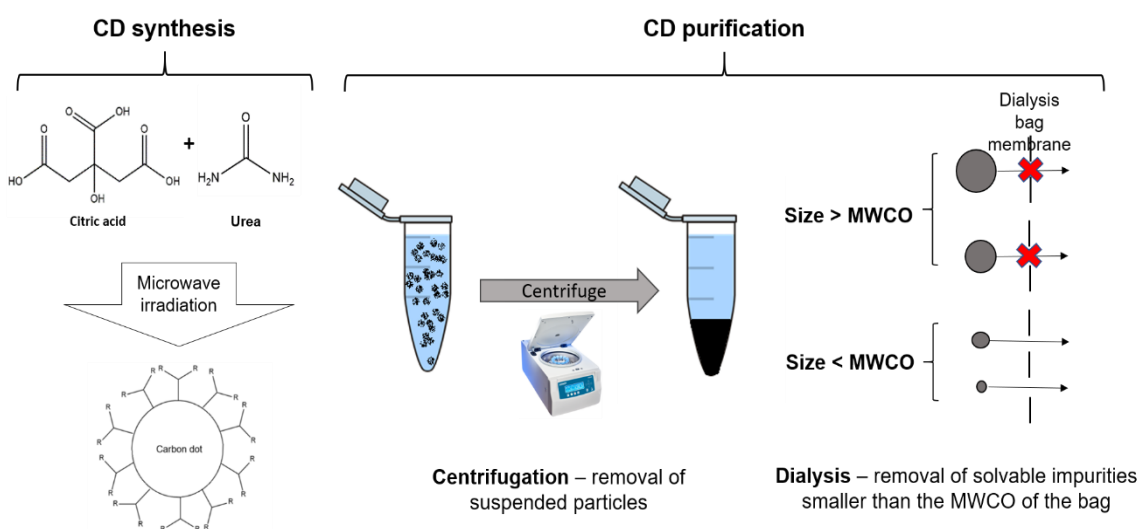


Figure 22 – Schematic representation of the synthesis and purification steps for the preparation of a CD solution.

Three samples were obtained from the initial CD solution (Figure 23): a centrifuged CD sample ($CD_{\text{centrifuged}}$), a centrifuged and dialyzed CD sample (CD_{dialyzed}) and a sample of dialysis wash waters from the CD dialysis ($Water_F$). The centrifugation had a duration of 10 minutes and was made at 13000 rpm. The dialysis was carried using a Float-A-Lyzer®G2 Dialysis Device with a MWCO of 1000 Da from SPECTRUM®. The dialysis process ran continuously for 3 days with regular changes in the dialysis wash

waters. The Water_{FI} sample corresponds to the first wash waters collected from the dialysis, before they were changed with fresh deionized water.

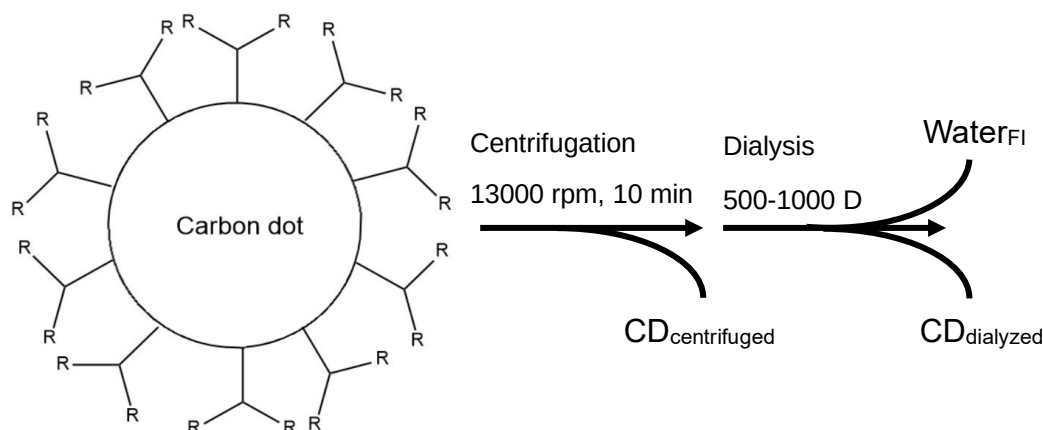


Figure 23 – Pathway for the obtention of the different fractions of CA,urea-based CD made by microwave irradiation. Centrifugation was made at 13000 rpm for 10 minutes and dialysis was made in a 500-1000 D dialysis bad during 3 days with regular changes in the wash waters. The Water_{FI} sample corresponds to the first dialysis wash waters, collected before any subsequent change.

3.2.2. CD-based samples analysis and characterization

AFM analysis was carried out using a Veeco Metrology Multimode / Nanoscope IVA by tapping. A silica plate was used to deposit the sample for analysis and an AFM R-TESP cantilever was used. The software used for the AFM data analysis was NanoScope. Suspensions of CDs were analyzed by high-resolution TEM (HR-TEM) under a FEI Talos F200X.

FT-IR analysis was performed using a PerkinElmer® Spectrum Two FT-IR spectrometer and the PerkinElmer Spectrum 10.5.3 software. Direct injection ESI-MS was made using a Thermo Finnigan™ LCQ™ Deca XP Max (Thermo Electron Corporation, Waltham, USA) mass spectrometer. This device is based on an electrospray interface used as ionization source and a quadruple ion trap for MSⁿ experiments. It was used as follows: spray voltage of 5 kV; capillary voltage of ±15 V; capillary temperature of 300 °C. ESI-MS results were analyzed using the Thermo Xcalibur software. X-Ray Photoelectron Spectroscopy (XPS) was carried out using a Physical Electronic PHI VersaProbe II spectrometer utilizing Al-Kα monochromatic radiation with a hemispherical multichannel detector (53.6 W, 15 kV and 1486.6 eV). PHI SmartSoft software was used to analyze the results and the carbon C 1s signal, 284.8 eV, was used as a reference to obtain the binding energy values.

For the absorption studies, the samples' absorption spectra were recorded and normalized with regard to the highest absorbance value observed for each sample. The equipment used was a VWR® UV3100PC spectrophotometer and standard 10 mm

quartz cells. The CDs emission and their fluorescent properties were analyzed using a Horiba Jovin Yvon Fluoromax-4 spectrofluorimeter and fluorescence quartz cells as described in the LCA chapter. The emission spectra with varying excitation wavelengths were obtained to evaluate the dependence on the excitation wavelength of the CD emission. The same sample concentration was maintained throughout the analysis. Additionally, the samples' emission spectra were recorded with the CD dissolved in several organic solvents (ACN, DMF, dimethyl sulfoxide (DMSO) and methanol (MeOH)). The pH effect in the emission was also evaluated by analyzing the emission spectrum when in the presence of 0.01 M of NaOH or 0.01 M of HCl, to assess what basic or acidic conditions would do, respectively. In all the above-mentioned tests, the concentration of CD-based sample was maintained at 0.04 mg mL⁻¹.

Further on, to evaluate the effect of electron-donor and electron-withdrawing molecules on the samples' emission, studies were made by analyzing the effect in the samples fluorescence induced by the presence of diphenylamine (DPA) and nitromethane, respectively. The effect caused by DPA in the samples fluorescence was evaluated by preparing several mixtures with the same amount of CD sample and varying concentrations of DPA, and analyzing the effect caused by each concentration of DPA. The DPA concentrations varied from 0 to 100 μM while the dilution of the CD-based samples was kept constant (concentration of 0.04 mg mL⁻¹). The variation of the fluorescence is represented by F_0/F , which is calculated by dividing the emission intensity of a blank sample by the emission intensity of each sample (for each different concentration of DPA). Fluorescence tests with a neutral electron-withdrawing molecule, nitromethane, were also performed in a similar way. Concentrations of nitromethane ranging from 0 to 50 mM were mixed with a constant amount of CD_{centrifuged}, CD_{dialyzed} and Water_{FI} (0.04 mg mL⁻¹). An additional test was performed and consisted in the combination of a constant amount of CD_{dialyzed} (0.04 mg mL⁻¹) with different concentrations of Water_{FI} (ranging from 0.002 to 0.04 mg mL⁻¹). The nitromethane-induced quenching was analyzed and displayed as F_0/F .

3.3. Results and dicussion

Considering the objective of understanding how the CD and the fluorescent impurities that resulted from its synthesis would interact, three samples were prepared: a sample containing both the CD and the impurities, a sample with the CD alone and a third sample containing only the fluorescent impurities. The first sample results from a simple purification of the CD solution after its synthesis (by centrifugation), and therefore was named as CD_{centrifuged}. Given that centrifugation is capable of removing the supended impurities but not low-weight compounds, [31, 37, 135] it is not enough to separate the fluorescent impurities from the CD. Therefore, CD_{centrifuged} contains both the CD and the fluorescent impurities. The other two samples, containing the individual species, were separated through a dialysis, in which one component was kept inside the dialysis bag while the other exited the bag during the process. The fraction kept inside the bag was expected to be composed of high molecular weight species that could not exit the bag due to their size (greater than the MWCO of the dialysis bag, 1000 Da), which are expected to be the CDs themselves. [128, 135] The fraction that exited the bag is comprised of low-weight molecular species, which we expect to be the fluorescent impurities. [128, 135] Those samples, containing the CD or the fluorescent impurities, were named CD_{dialyzed} and Water_{FI}, respectively.

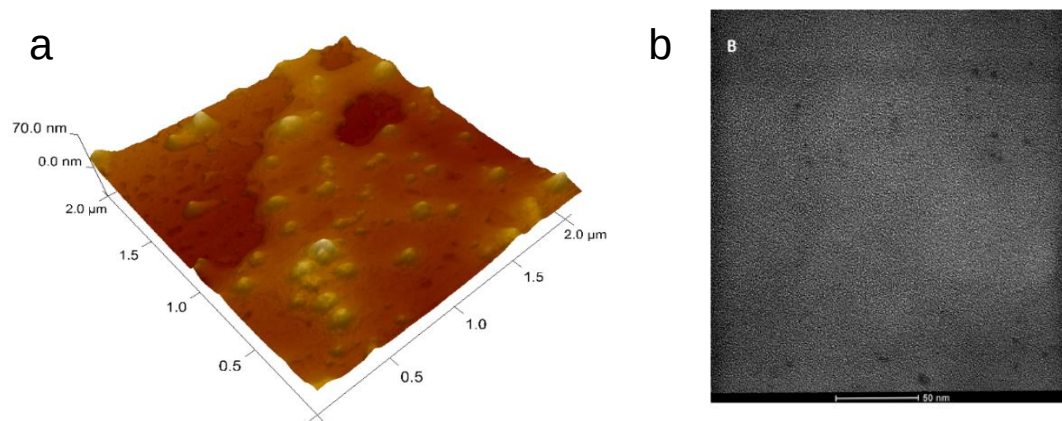


Figure 24 – a) AFM 3D image of CD_{dialyzed} in a silica plate; b) HR-TEM image of the CD_{dialyzed}.

One of the initial steps in this study was the structural analysis of the nanoparticle when in the presence (CD_{centrifuged}) or in the absence (CD_{dialyzed}) of fluorescent impurities. Through AFM analysis of the CD_{dialyzed} sample (Figure 24a), the size of the CDs was estimated to be 23.1 ± 0.5 nm. While CDs are commonly sized below 10 nm, nanoparticles with sizes up to 30 nm are not uncommon to be found. [14, 15] Despite that, when the morphology of the particles was analyzed by HR-TEM (Figure 24b), well dispersed nanoparticles with uniform spherical shape and a medium diameter size of 6.5

nm were observed. Thus, we can assume that some aggregation occurred during the AFM experiments causing the observation of a size bigger than what was observed by HR-TEM.

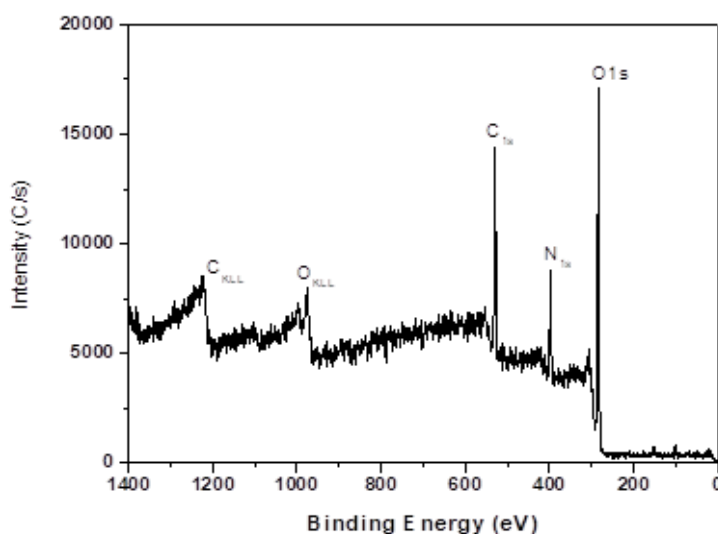


Figure 25 – Survey XPS spectra of the obtained CDs.

XPS analysis was carried out to characterize the surface composition of both the CD_{centrifuged} and CD_{dialyzed} (Figures 25 and 26). The full survey XPS spectra (Figure 25), which presents the same profile for both samples, displays predominant peaks for C 1s, N 1s and O 1s. The obtained XPS mole fraction for the elements in the CD_{centrifuged} was 59.70% for carbon, 16.75% for nitrogen and 23.55% for oxygen. A more detailed study about the internal levels of C 1s, O 1s and N 1s was performed (Figure 26), consisting in the deconvolution of the spectrum and a quantitative analysis of the possible groups composition. The deconvolution of the C 1s spectrum results in three peaks with binding energies of 284.8 eV (54.53%), 288.3 eV (33.50%) and 286.6 eV (11.95%). These binding energies can be attributed to C-C/C-H adventitious carbon (284.8 eV), C-O/C-N (286.6-286.9 eV) and C=O/O-C=O carbonyl/carboxylic groups (288.3-288.6 eV), respectively. The core level of N 1s displayed a predominant peak at 400 eV (80.9%) with a shoulder at 401.5 eV (11.1%). These can be respectively ascribed to amine/amide groups and to protonated amines. Last, the deconvolution of the O 1s spectrum yielded two peaks: a dominant peak at 531.5 eV (80.9%) due to C=O linkage, and a smaller peak at 532.7 eV (18.1%) that results from C-O/C-O-C groups. The CD_{dialyzed} yielded similar results with a slight increase in the XPS mole fraction of C (59.70% to 62.57%) and decreased N and O fractions (16.75% to 15.47% and 23.55% to 21.97%, respectively). In summary, the XPS analysis indicates the surface composition of both CDs to be quite identical. Purification seems to have a very limited effect on the CD surface composition, as there are no noticeable differences in the surface composition after the dialysis.

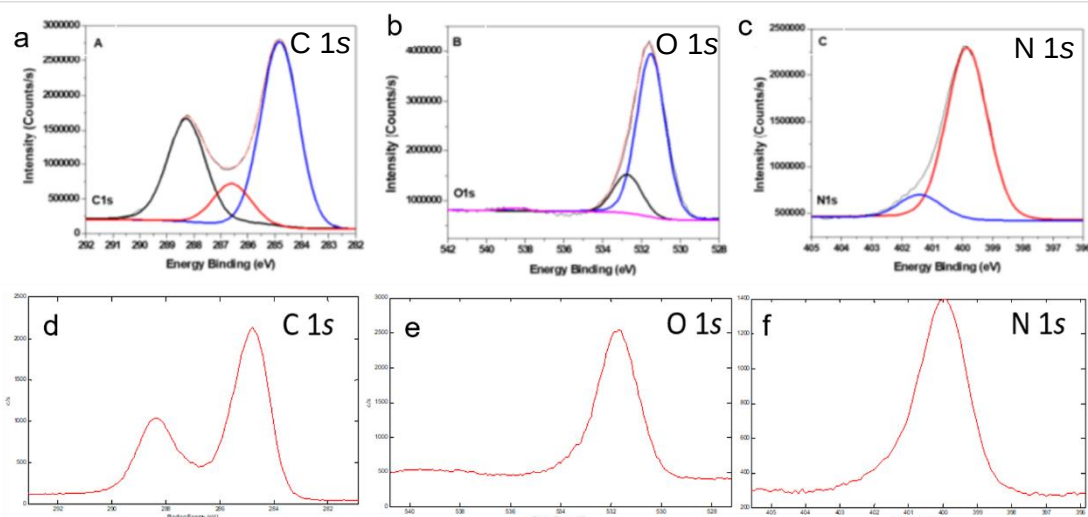


Figure 26 - CD_{centrifuged} XPS core level spectra for a) C 1s; b) O 1s and c) N 1s. CD_{dialyzed} XPS core level spectra for d) C 1s; e) O 1s and f) N 1s.

XPS results are supported by the analysis made by FT-IR of the surface groups of both the CD_{centrifuged} and CD_{dialyzed} samples (Figure 27). FT-IR analysis resulted in quite similar spectra for both samples. In the group frequencies, a band at 3300 cm^{-1} , indicates the presence of O-H and N-H groups. Additionally, both CD_{centrifuged} and CD_{dialyzed} display peaks at 1655 cm^{-1} (ascribed to C=C stretching vibrations or primary amides bending vibrations), 1575 cm^{-1} (N-H bending vibrations for secondary amides), 1350 cm^{-1} (O-H bending vibrations) and at 1185 cm^{-1} (commonly attributed to C-N stretching vibrations from amines). In summary, the FT-IR spectra profile for CD_{centrifuged} and CD_{dialyzed} can almost be overlapped, indicating an identical composition in terms of surface functional groups for both samples.

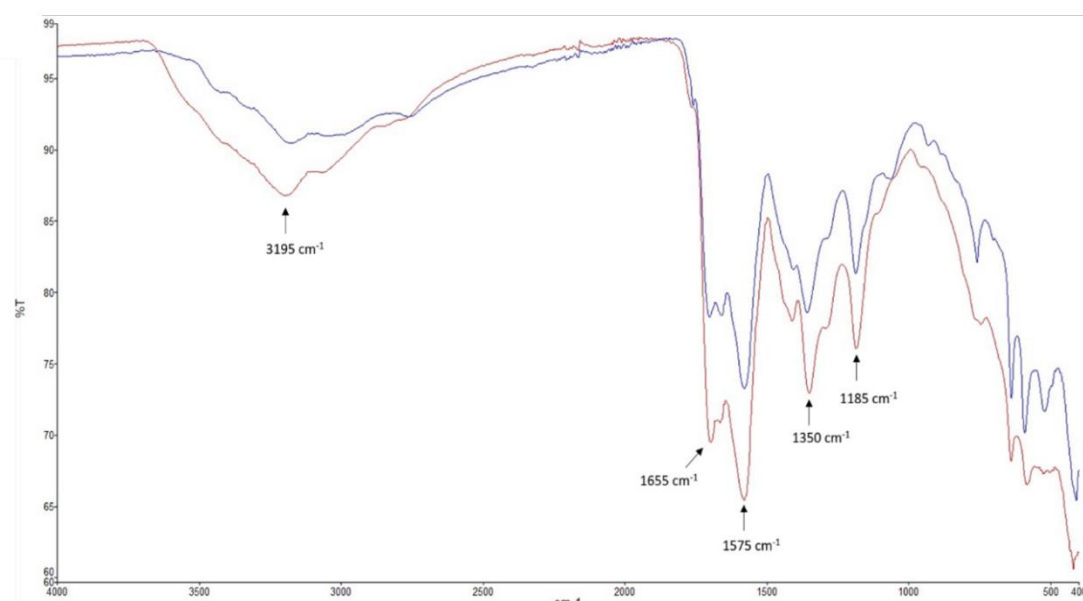


Figure 27 – FT-IR spectra obtained for CD_{centrifuged} (blue plot) and CD_{dialyzed} (red plot).

Despite the fact that data from XPS and FT-IR indicates a high degree of similarity between $CD_{centrifuged}$ and $CD_{dialyzed}$ (atleast at a surface level), the optical analysis does not support this similarity. The UV-Vis spectrum obtained for $CD_{dialyzed}$ (Figure 28a) displayed a main absorption band at 340 nm and a small shoulder at 245 nm. These values can be ascribed to $n-\pi^*$ and $\pi-\pi^*$ transitions that correspond to C=C and C=N bonds, respectively. [143, 177] A small and less well-defined band is observable at 410 nm. As for the UV-Vis spectrum from the $CD_{centrifuged}$, it presents two shoulders (at 245 nm and 275 nm), unlike the $CD_{dialyzed}$ which only presents one (245 nm). Additionally, unlike in the $CD_{dialyzed}$ spectrum, the band at 410 nm is well defined and presents a higher relative absorption than the band at 340 nm.

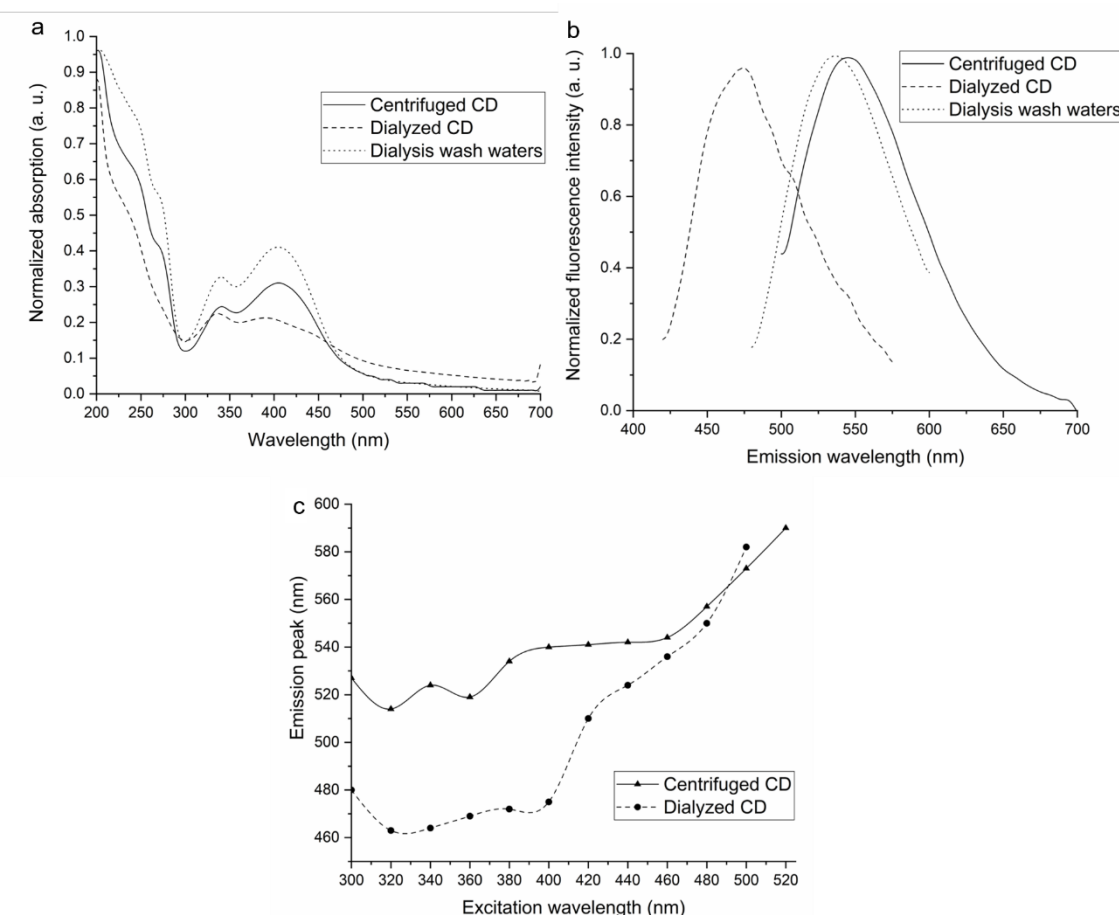


Figure 28 - a) Normalized absorption spectra for the $CD_{centrifuged}$, $CD_{dialyzed}$ and $Water_{FI}$ samples; b) Emission spectra for the $CD_{centrifuged}$, $CD_{dialyzed}$ and $Water_{FI}$ samples; c) Variation observed in the emission peak (highest emission intensity of the respective spectrum) with different excitation wavelengths for $CD_{centrifuged}$ and $CD_{dialyzed}$ samples.

Aside the differences regarding their absorption, the most significant optical difference between the samples occur in their emission profiles (Figure 28b). While the $CD_{centrifuged}$ displays an emission maximum located at 540 nm, the emission of $CD_{dialyzed}$ is blue-shifted, presenting its peak at 475 nm. The samples maximum excitation wavelength also differs, with the $CD_{centrifuged}$ being optimally excited at 410 nm and the $CD_{dialyzed}$ at 380 nm. These results suggest that the dialysis purification process, to which

the CA,urea-based CD was submitted, can significantly alter the samples' photoluminescent properties. However, it is worthy of note that either sample ($CD_{\text{centrifuged}}$ and CD_{dialyzed}) presents an emission that depends on the excitation wavelength (Figure 28c). Furthermore, both samples appear to have similar emission wavelengths for higher excitation wavelengths.

The differences observed in the optical properties of the $CD_{\text{centrifuged}}$ and CD_{dialyzed} can be attributed to the presence of fluorescent impurities in the $CD_{\text{centrifuged}}$ sample. Analysis by both UV-Vis and fluorescence spectroscopy of the $Water_{F1}$ sample results in a spectrum similar to the $CD_{\text{centrifuged}}$ sample, while differing from the CD_{dialyzed} sample. $Water_{F1}$ is optimally photo-excited at 410 nm and emits at 540 nm, having an absorption spectrum with the same bands observed for the $CD_{\text{centrifuged}}$. These measurements suggest that the optical properties observed in the $CD_{\text{centrifuged}}$ might not result from the CD itself, but instead result from the presence of fluorescent impurities produced during the CD synthesis, that are present in both the $CD_{\text{centrifuged}}$ and $Water_{F1}$ samples. Essner *et al.* reported that the bottom-up synthesis of CDs can cause the formation of highly fluorescent molecular by-products (mono-, oligo- or polymeric in nature) that can be responsible for the majority of the fluorescence observed in unpurified CD samples. [135] Furthermore, Essner *et al.* also reported that a simple centrifugation might not be enough to remove these fluorescent impurities from the sample, and that the more intense fluorescence originated by them might mask the true emission of the nanoparticle. [135] Thus, from the data we obtained so far, it appears that the $CD_{\text{centrifuged}}$ optical properties are affected by the fluorescent impurities formed during the synthesis. On the other hand, the CD_{dialyzed} sample, which aside the initial centrifugation had an additional and more complex dialysis purification, is free of impurities and therefore displays its own optical properties (absorption and emission).

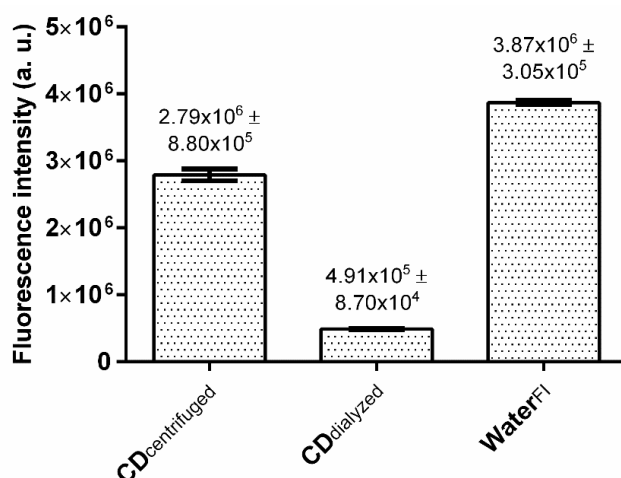


Figure 29 - Fluorescence intensity of $CD_{\text{centrifuged}}$, CD_{dialyzed} and $Water_{F1}$ in deionized water. $CD_{\text{centrifuged}}$ and $Water_{F1}$ were excited at 410 nm while the CD_{dialyzed} was excited at 380 nm.

The fluorescence intensity of the CD_{centrifuged}, CD_{dialyzed} and Water_{F1} in aqueous solution was compared (Figure 29), with all three samples being in the same concentration (1 mg mL⁻¹). It was observed that, while the CD_{centrifuged} and Water_{F1} present emission intensities of the same order of magnitude, their emission is 6 to 8 times higher than the emission of the CD_{dialyzed}. This is in accord with recent literature which states that, while the fluorescent impurities are strongly fluorescent, the CDs themselves are only weakly fluorescent. [121, 124, 128, 135, 136] This data further supports that the fluorescence observed for the CD_{centrifuged} is strongly influenced by the fluorescent impurities, which mask the CD true emission.

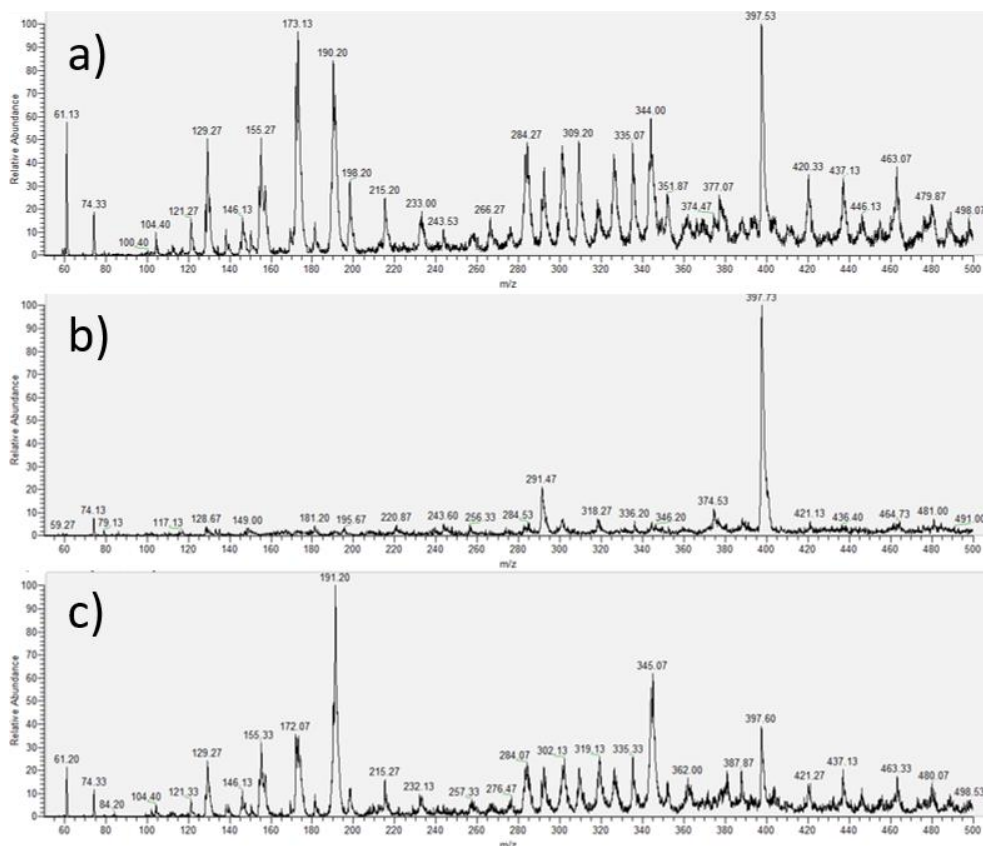


Figure 30 – Direct-injection ESI-MS with positive ionization mode spectra for the different CA,urea-based microwave-made samples: a) CD_{centrifuged}; b) CD_{dialyzed} and c) Water_{F1}.

The CD-based samples were submitted to direct-injection ESI-MS analysis. The resulting mass spectra (either in the positive or negative ionization modes) are presented in Figures 30 and 31. When analyzing in the positive ionization mode, the mass spectrum of the CD_{centrifuged} (range between 50.0 *m/z* and 500.0 *m/z*) displays three predominant peaks with *m/z* values of 171.13, 190.20 and 397.53 and several medium sized peaks (Figure 30a). The mass spectrum of the CD_{dialyzed} sample in positive ionization mode displays just one dominant peak with a *m/z* value of 397.73, with small peaks at 291.47

and 374.53 m/z (Figure 30b). Lastly, Water_{FI} positive ionization mode mass spectrum (Figure 30c) is nearly identical to the spectrum obtained for the CD_{centrifuged}, with the only difference being a reduced predominance of the peak at 397.73 m/z .

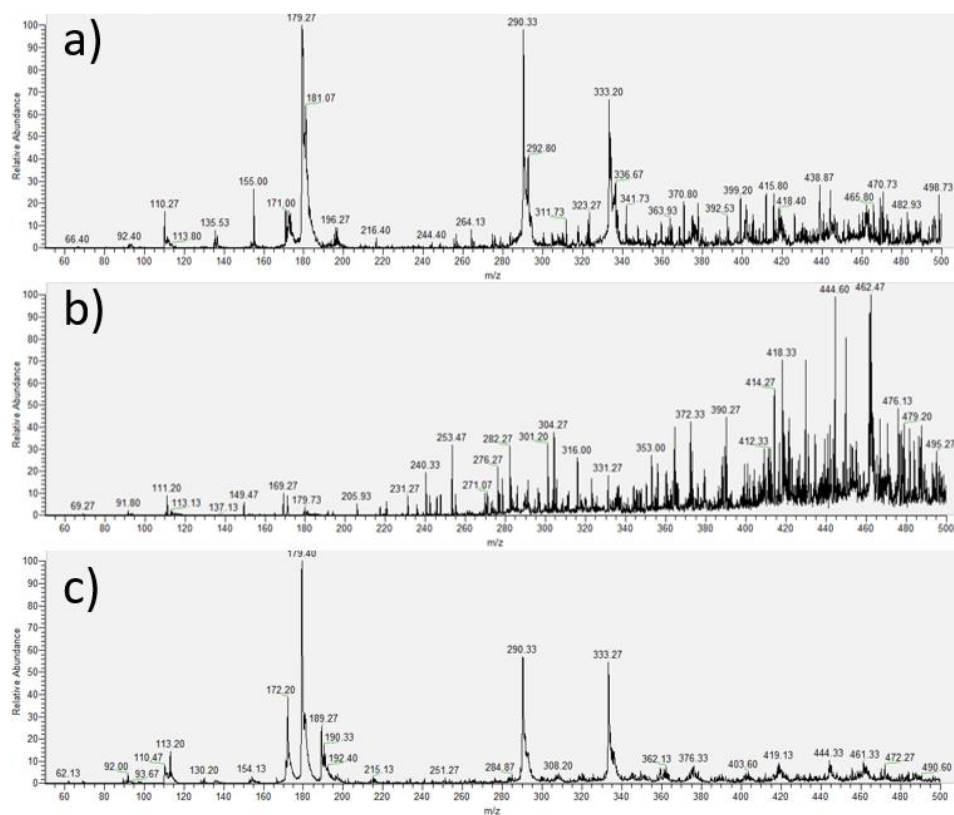


Figure 31 – Direct-injection ESI-MS with negative ionization mode spectra for the different CA,urea-based microwave-made samples: a) CD_{centrifuged}; b) CD_{dialyzed} and c) Water_{FI}.

Similar patterns can be observed on the mass spectra for the negative ionization mode (Figure 31). The CD_{centrifuged} mass spectrum (Figure 31a) is composed by several dominant peaks with m/z values below 400.0 and medium peaks in the 350.0-500.0 range. The CD_{dialyzed} spectrum (Figure 31b) presents predominant peaks in the 350.0-500.0 m/z region, while Water_{FI} (Figure 31c) again displays no peaks in that region, having peaks similar than those observed with the CD_{centrifuged} sample in the 50.0-350.0 m/z region. This mass spectroscopy analysis is not enough to identify the fluorescent impurities present in the CD_{centrifuged} and Water_{FI} samples. Nonetheless, it confirms our assumption that the bottom-up synthesis of CDs produces low-weight molecular fluorescent species alongside the nanoparticle. Centrifugation alone is not enough to separate those fluorescent species from the CD, and more complex steps (dialysis for instance) must be considered to separate the low-weight fluorescent impurities from the CDs.

Despite not being enough to unambiguously identify the fluorescent impurities, we wish to refer that in the negative ionization mode ESI-MS analysis for the CD_{centrifuged} and Water_{FI} samples (Figures 31a and 31c, respectively), the predominant peak in the spectrum occurs at a m/z value of 179.27. This peak can be ascribed to a compound called 4-hydroxy-1H-pyrrolo[3,4-c]pyridine-1,3,6(2H,5H)-trione (named as HPPT, molecular weight of 180 g mol⁻¹), which was identified by Kasprzyk *et al.* [128] as being a fluorescent by-product of the synthesis of CDs though a microwave-assisted methodology. HPPT displays an absorption peak at 410 nm and has a bright green emission, which is similar to the properties displayed by both the CD_{centrifuged} and the Water_{FI} samples. [128] Worthy of note is that the peak at ~179 m/z is not significant in the mass spectrum of the CD_{dialyzed} sample, and HPPT was possibly removed from the initial CD solution during the dialysis. Thus, it is likely that, before being removed, HPPT was one of the main responsables for masking the CD emission.

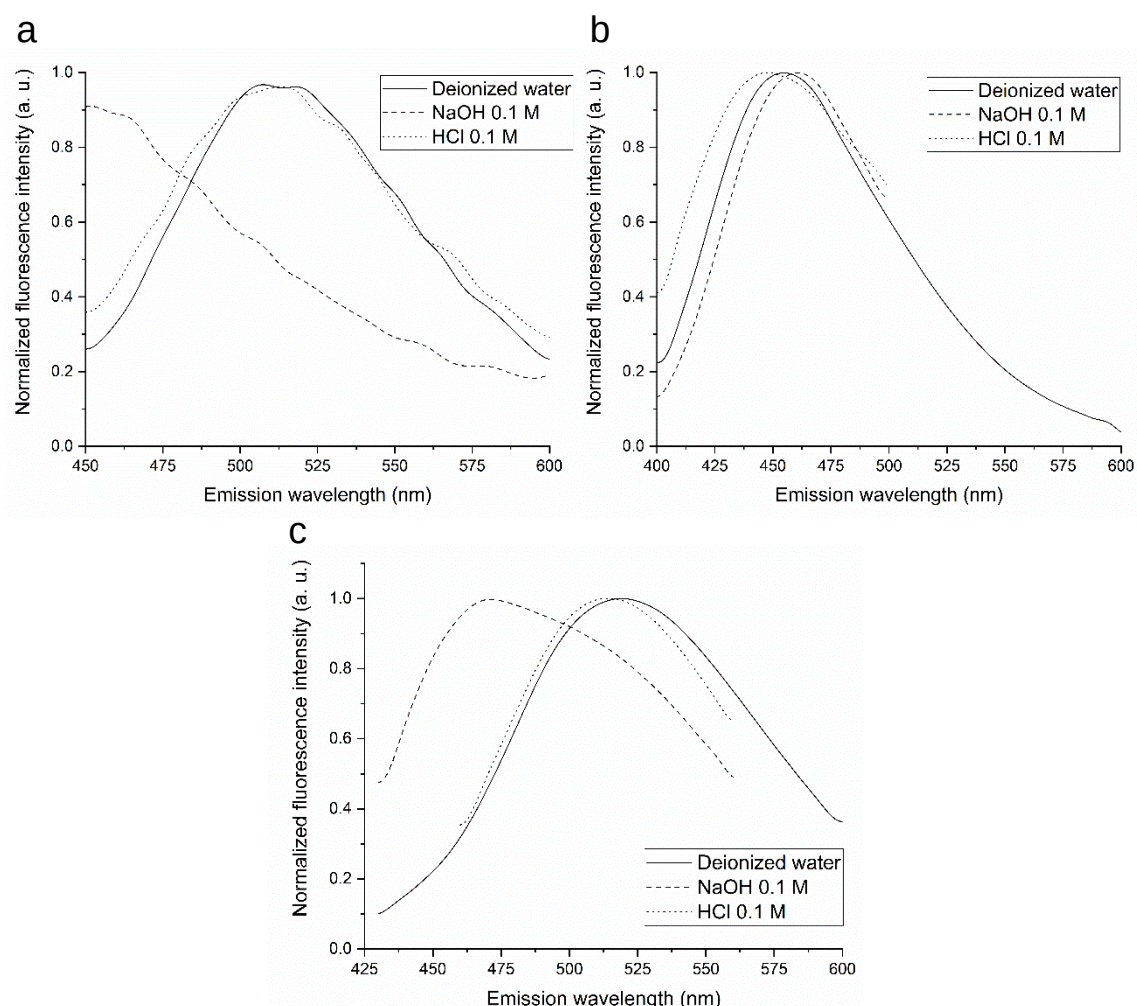


Figure 32 – Normalized emission spectra in deionized water, a 0.01 M NaOH solution or a 0.01 M HCl solutions for a) CD_{centrifuged}; b) CD_{dialyzed} and c) Water_{FI}.

Measuring the fluorescence of the three samples at different pH conditions (Figure 32) further supported the theory that, if not removed, the fluorescent impurities produced during the bottom-up synthesis of CDs can mask the signal of the CD itself. This experiment was done by measuring the fluorescence of the samples in simple deionized water, in an aqueous solution acidified with HCl (0.1 M) and in an aqueous solution basified with NaOH (0.1 M). Similar to previous experiences, the emission profile observed for the $CD_{\text{centrifuged}}$ and $Water_{FI}$ samples are quite similar in these conditions (Figure 32a and 32c). Both of them display a green emission in water and acidic conditions with a blue-shift occurring when basified conditions are introduced. By its turn, the CD_{dialyzed} sample does not show any significant shift in the emission peak between the different pH conditions tested (Figure 32b). The only observable difference was a slight broadening in the emission band induced by acidic pH conditions.

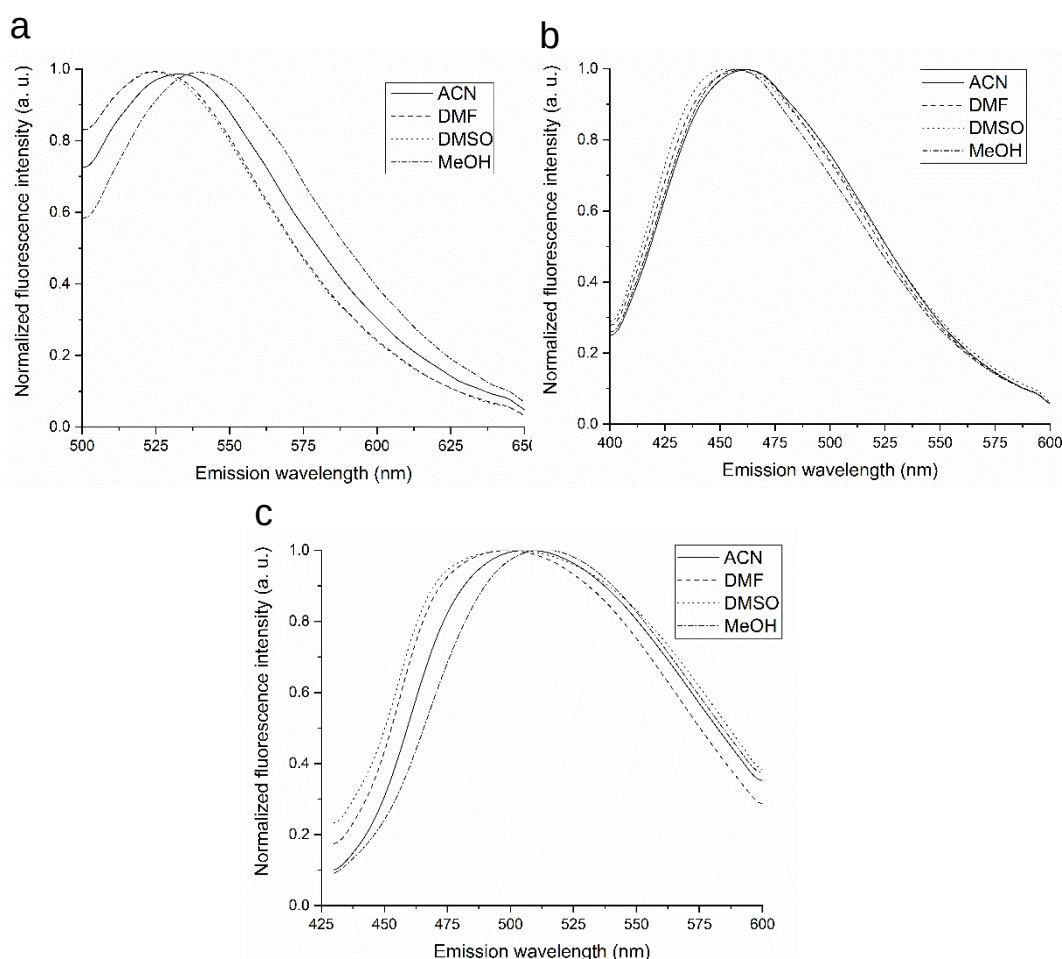


Figure 33 - Normalized emission spectra for a) $CD_{\text{centrifuged}}$, b) CD_{dialyzed} and c) $Water_{FI}$ in the presence of several organic solvents, namely ACN, DMF, DMSO and MeOH.

The fluorescence spectra of $CD_{\text{centrifuged}}$, CD_{dialyzed} and $Water_{FI}$ in different organic solvents, namely ACN, DMF, DMSO and MeOH, was also recorded and the resulting spectra are presented in Figure 33. Despite their different properties and characteristics,

the solvents had an almost negligible effect on the emission spectrum of the CD_{dialyzed} (Figure 33b). This could be an indicator that the fluorescent moieties of the CD are inside the nanoparticle, and therefore are not exposed to the external environment and are not affected by the different solvents. [178, 179] On the opposite way, the emission spectra of the Water_{FI} sample (Figure 33c), when recorded in the tested organic solvents, undergoes significant blue-shifts when compared to the 540 nm emission peak observed in an aqueous solution. The emission peak is located at 510 nm in ACN, 500 nm in DMF and DMSO, and 515 nm in MeOH. The blue-shift suggests that the fluorescent moieties are exposed to the external microenvironment, and therefore are subjected to the effect induced by the tested solvents. This data indicates a difference between the CD_{dialyzed} and the Water_{FI} samples regarding their interaction with the external molecular microenvironment. By other words, the CD itself and the fluorescent impurities respond differently towards the surrounding microenvironment. Worthy of note is the fact that the solvents cause only an intermediate effect in the CD_{centrifuged} sample (Figure 33a), when compared to the CD_{dialyzed} and Water_{FI} samples. A blue-shift occurs in the emission maxima wavelengths, but is not as significant as the one observed with Water_{FI} (540 to 525-530 nm *versus* 540 to 510-520 nm, respectively). This leads us to think that while some of the fluorescent moieties present in the CD_{centrifuged} are exposed to the external medium, they are more shielded from their action than those in the Water_{FI} sample, as observable by the smaller and less significative blue-shift in their emission. Therefore, the presence of the CD in the CD_{centrifuged} sample is capable of affecting the fluorescent properties of the fluorescent impurities, namely the way they interact with the external environment.

So far, the results obtained show that, during the microwave-assisted synthesis of CDs fluorescent molecular impurities are produced alongside the CD. Due to them being strongly photoluminescent while the CDs are only weakly fluorescent, the impurities are capable of masking the CD signal itself. However, these results are not able to show that if, when co-existing in a solution, the resulting properties are the simple combination of the effects and properties of the individual species (CD plus fluorescent impurities) or if, on the other hand, the CD and the fluorescent impurities interact to generate a synergistic effect. To assess this, we evaluated the excited state reactivity of our CD-based samples towards electron-donors and electron-withdrawing molecules. This was performed by analyzing the fluorescence intensity variation in the presence of different concentrations of DPA (electron-donor) and nitromethane (electron-withdrawing). By comparing the responses of the individual species (CD and fluorescent impurities in the CD_{dialyzed} and Water_{FI} samples, respectively) with those of the CD_{centrifuged}

containing both species in co-existence, we were able to assess the effect induced by the co-existence of both species. Namely, we aimed to observe if, when present in the same solution, the CD and the fluorescent impurities interacted to generate a signal different from the one resulting from the sum of the individual species' properties.

Our next step consisted in an assessment of the samples' photochemical responses when in the presence of nitromethane (Figure 34). Nitromethane is a known electron-withdrawing molecule, and for that reason it can be used as a non-ionic electron-acceptor probe to study PET reactions in CDs. The analysis of the results presented in Figure 34 demonstrates that nitromethane induces quenching for all samples ($\text{CD}_{\text{centrifuged}}$, $\text{CD}_{\text{dialyzed}}$ and Water_{FI}), irrespective of the excitation wavelength, suggesting that all samples may be capable of realizing PET reactions if acting as an electron-donor. The evolution of the samples' emission intensity as the nitromethane concentration increases follows a Stern-Volmer relationship. Moreover, the nitromethane-induced quenching in the fluorescence of the $\text{CD}_{\text{dialyzed}}$ sample (Stern-Volmer constant, K_{SV} of $23.7 \pm 0.1 \mu\text{M}^{-1}$) is significantly more efficient than the quenching induced in the fluorescence of either the $\text{CD}_{\text{centrifuged}}$ (K_{SV} of $5.4 \pm 0.2 \mu\text{M}^{-1}$) or the Water_{FI} (K_{SV} of $6.1 \pm 0.4 \mu\text{M}^{-1}$) samples. The fact that the K_{SV} values for the $\text{CD}_{\text{centrifuged}}$ and Water_{FI} samples are so similar suggests that the responses observed in the $\text{CD}_{\text{centrifuged}}$ might result from, or be masked by, the fluorescent impurities.

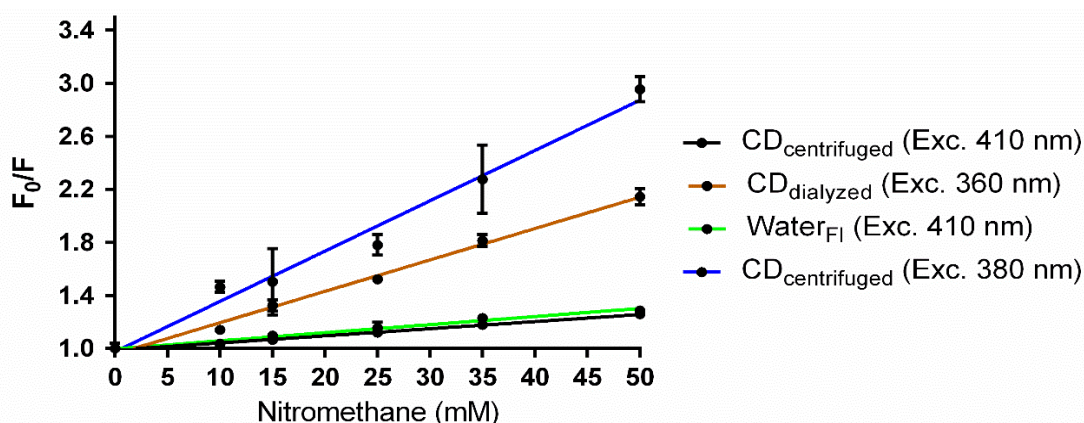


Figure 34 - F_0/F values in the presence of the CD-based samples with different concentrations of nitromethane (0-50 mM) and excitation wavelengths: black - $\text{CD}_{\text{centrifuged}}$ excited at 410 nm; orange - $\text{CD}_{\text{dialyzed}}$ excited at 360 nm; green - Water_{FI} excited at 410 nm; blue - $\text{CD}_{\text{centrifuged}}$ excited at 380 nm.

The effect of DPA, a strong electron-donor and known redox indicator, on the samples' fluorescence was also analyzed (Figure 35). DPA can be used to study the samples' potential regarding PET reactions when acting as electron-acceptors. Considering the results obtained so far, we were expecting that the emission profiles of $\text{CD}_{\text{centrifuged}}$ and Water_{FI} (Figures 35a and 35c) would differ from that of the $\text{CD}_{\text{dialyzed}}$

sample (Figure 35b). There were some differences between $CD_{centrifuged}$ and $CD_{dialyzed}$: the $CD_{centrifuged}$ fluorescence suffered quenching in the type of a Stern-Volmer relationship (K_{SV} of $0.0054 \mu M^{-1}$), while the $CD_{dialyzed}$ sample displayed an enhancement (increase of the emission intensity, the opposite of quenching) of fluorescence intensity when in the presence of DPA. This was expected given the results described above. But, unlike we expected, DPA had no effect whatsoever in the emission of the $Water_{FI}$ sample. These results suggest that the DPA-induced quenching observed in the fluorescence of the $CD_{centrifuged}$ does not originate in the fluorescent impurities present both in the $CD_{centrifuged}$ and $Water_{FI}$ samples. Additionally, given that the $CD_{dialyzed}$ sample, which contains the CD alone, suffered enhancement when DPA was added, the DPA-induced quenching observed in the $CD_{centrifuged}$ sample also cannot derive from the CD itself. The CD and the fluorescent impurities, when combined in a solution, yield a different result in the presence of DPA than the ones observed for both individual components by themselves. Therefore, the CD and the fluorescent impurities can interact and generate a synergistic effect, leading to a result different of the one originated by their individual forms.

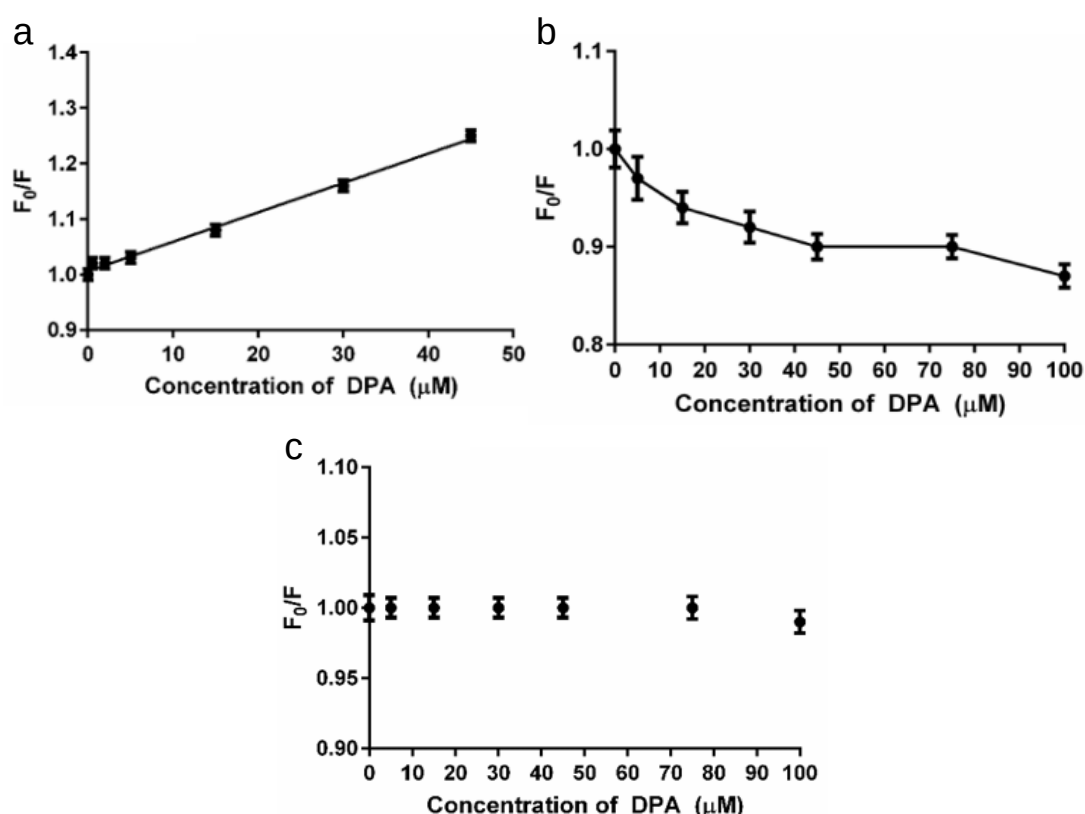


Figure 35 - F_0/F values of $CD_{centrifuged}$ (a), $CD_{dialyzed}$ (b) and $Water_{FI}$ (c) in the presence of increasing concentrations of DPA.

Considering this, if we re-evaluate the results obtained in the nitromethane assays (Figure 34), a synergistic effect can also be found. More specifically, if we use an excitation wavelength of ~ 380 nm instead of the original excitation wavelength of 410

nm, the fluorescence spectra of the CD_{centrifuged} sample displays a shoulder at ~460 nm that can be ascribed to the emission of the CD itself (Figure 36). Thus, we measured the response of the CD_{centrifuged} towards nitromethane by using an excitation wavelength of 380 nm and measuring the emission intensity at 460 nm (Figure 34). This should permit us to evaluate the response of the CD itself (similar to the CD_{dialyzed}), even when in the presence of the fluorescent impurities also contained in the CD_{centrifuged} sample. In this case, with the new wavelengths, the emission of the CD_{centrifuged} sample displays a significant quenching effect that follows a Stern-Volmer relationship when in the presence of nitromethane. This is similar to the response obtained with the CD_{dialyzed} sample, as observable in Figure 34. However, the calculated K_{SV} ($38.0 \pm 2.5 \mu\text{M}^{-1}$) was almost the double of the one obtained for the CD_{dialyzed} (K_{SV} of $23.7 \pm 0.1 \mu\text{M}^{-1}$), and around 7-fold higher than those obtained previously for the CD_{centrifuged} and Water_{FI} when excited at 410 nm, K_{SV} of $5.4 \pm 0.2 \mu\text{M}^{-1}$ and $6.1 \pm 0.4 \mu\text{M}^{-1}$, respectively. Therefore, the presence of fluorescent impurities is capable of increasing the nitromethane-induced quenching of the CD itself, even though they themselves do not suffer a significant quenching in its presence. As neither component alone suffers a quenching this significant, the more acute quenching does not result only from an additive phenomenon of each species' properties. Instead, it must originate from a synergistic effect resulting from the interaction of the nanoparticle with the fluorescent impurities.

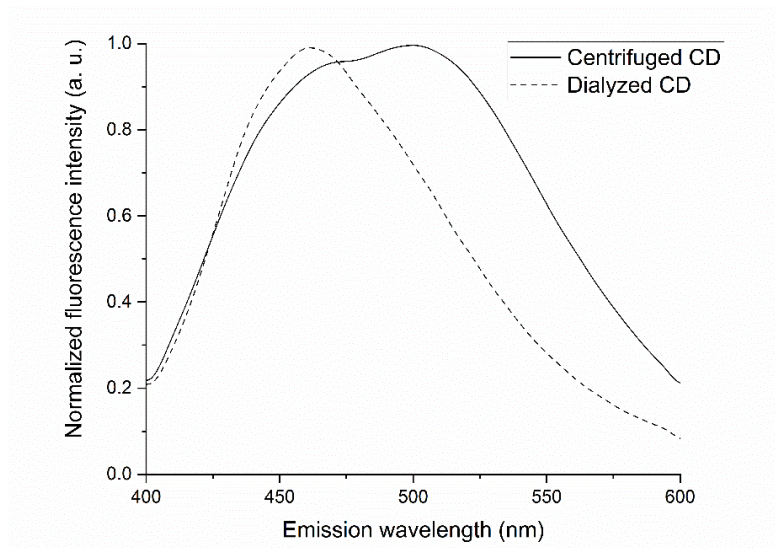


Figure 36 – Emission spectra in deionized water of CD_{centrifuged} and CD_{dialyzed} when excited at 380 nm.

Finally, in order to assess if the fluorescent impurities were capable of modulating the CDs' photochemical reactivity, the response of the CD_{dialyzed} towards nitromethane was analyzed in the presence of increasing concentrations of Water_{FI}. The results are presented in Figure 37. The addition of fluorescent impurities (Water_{FI} sample) to

CD_{dialyzed} does in fact increase the nitromethane-induced quenching extent in the CD emission. However, this increase of the quenching diminishes when increasingly higher concentrations of fluorescent impurities are used. Our theory is that the fluorescent impurities interact with the CD, becoming loosely bound to the nanoparticle surface by non-covalent interactions (such as π - π stacking and electrostatic interactions). This prompts the formation of a kind of hybrid material that possesses synergistic properties, such as was seen by the increase of the nitromethane-induced quenching extent. However, increasing the concentration of fluorescent impurities beyond a certain threshold could mask the surface of the CD, preventing the interaction with the quencher, which would ultimately lead to a decrease in the nitromethane-induced quenching extent. This would be an offsetting of the effect generated by the synergy between the CD and the fluorescent impurities.

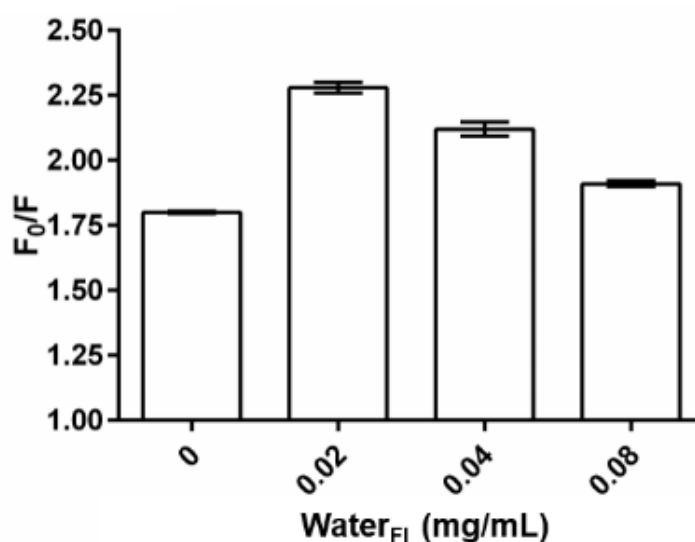


Figure 37 - Variation of F_0/F values of CD_{dialyzed} samples (0.04 mg mL^{-1}) in the presence of nitromethane (45 mM), with the addition of successively higher concentrations of Water_{FI} ($0.02 - 0.08 \text{ mg mL}^{-1}$).

3.4. Conclusion

The structural, morphologic and optical properties of CA,urea-based CDs made through microwave irradiation were characterized. From an initial CD solution, three fractions were obtained: a centrifuged CD sample (CD_{centrifuged}) containing both the CD and fluorescent impurities, a dialyzed CD sample (CD_{dialyzed}) containing only the CD and the wash waters from the CD dialysis (Water_{FI}), which contained only the fluorescent impurities. Several experimental techniques were employed during the samples' characterization, namely AFM, XPS, FT-IR, ESI-MS, UV-Vis and steady-state fluorescence spectroscopy.

During the study we demonstrated that the microwave-assisted synthesis of CA,urea-based CDs results in the production of green-emitting molecular fluorophores. Those fluorophores are formed alongside the CD and, because of their strong photoluminescence, can mask the emission of the blue-emitting CDs. ESI-MS results suggest that the fluorescent impurities are comprised mainly by a compound named HPPT. This compound is removed during the dialysis process of the initial CD, as confirmed by the almost total absence of its corresponding peak in the ESI-MS spectrum of the CD_{dialyzed} sample. Moreover, our results show that both the CD and the fluorescent impurities have different properties and react differently to parameters such as the medium pH or the surrounding molecular microenvironment.

Finally, when both the fluorescent impurities and the CDs are present in the same solution, they do not behave as two separate and individual species. Instead, they interact to generate a hybrid photoluminescence, presenting different properties and excited state reactivity than those resulting from the additive effect of both species by themselves. We hypothesize that the CDs and the fluorescent impurities can co-exist in solution and form loosely bound supramolecular complexes due to non-covalent interactions (such as electrostatic interactions and π - π stacking). These interactions are easily disrupted during purification processes, such as dialysis, prompting them to only be observable in the CD_{centrifuged} sample, which is the only sample that is not submitted to dialysis. While not denying the need for a thorough purification and characterization of these kind of materials, these results suggest a possibility for the development of novel hybrid materials composed of CDs and their related fluorescent impurities. The new hybrid materials would likely possess improved properties when compared to the components alone, and could possibly be used for new applications.

4. Carbon dots for catalytic applications in epoxide ring-opening and aminolysis reactions

4.1. Introduction

Over the past few years, catalytic processes that do not require metallic catalysts have gained momentum as being a greener alternative to traditional organic synthesis. [77, 78] Due to several of their properties, such as a good water solubility, chemical inertness, photostability, physico-chemical stability and a low toxicity, CDs have some advantages when compared to other catalysts. Because of this, CDs can be used as a versatile component in a catalytic system.

A CD capable of catalyzing the ring-opening reaction on epoxides, without the need of light as an energy source, was developed. Epoxides are highly reactive cyclic ethers with a three-atom ring that are used for several purposes, being the preparation for aminolysis one of them. Common applications of this kind of reactions include the degradation of poly(ethylene terephthalate) plastics [180] and the synthesis of peptides: the reaction of a primary or secondary amine with a carboxylic acid results in the formation of an amide; given that this specific reaction allows the formation of bonds between different amino acids, it is widely used for the commercial synthesis of peptides. [181, 182] Aside aminolysis reactions, the ring-opening reaction of epoxides is also important for other applications, one example being the conversion of carbon dioxide into heterocyclic carbonates. [183, 184]

Our objective with this work was to test the effect caused by the presence of 4-aminopyridine-based CDs in the outcome of an aminolysis reaction. Tests with a model epoxide and several chemicals with different functional groups demonstrated that the CD could influence reactions when in the presence of aminated molecules (e. g. aniline). Considering this and the interest of aminolysis reactions, more detailed studies about our CD's capacity to enhance an aminolysis reaction extent were performed. Regarding this, we predict that the aminolysis reaction, improved by the presence of CDs, would allow for the production of coupled compounds between an open epoxide molecule (oxyanion) and aminated compounds, such as aniline, through the mechanism depicted in Figure 38. Considering that after the ring-opening reaction, the nucleophile is detached from the open epoxide molecule, the reaction would not inutilize the CD, making it possible for it to be re-used, interacting with more epoxide molecules and giving continuation to the reaction.

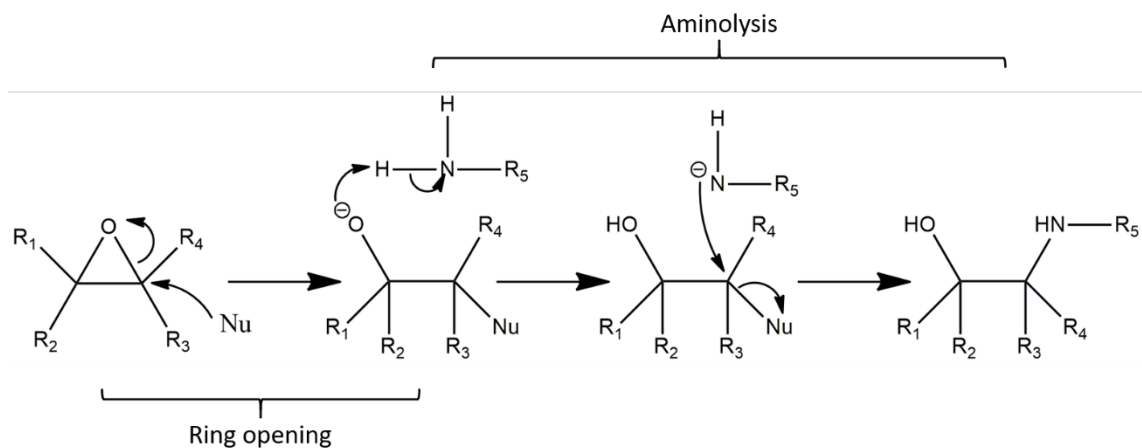


Figure 38 – Mechanistic view of the possible epoxide ring-opening reaction in the presence of a nucleophile (CD) followed by an aminolysis reaction with aminated compounds.

4.2. Methods

4.2.1. Carbon dot production and size characterization

For the application in catalytic purposes, a 4-aminopyridine-based CD was manufactured by hydrothermal treatment. 0.3 g of 4-aminopyridine were dissolved into 5 mL of deionized water, obtaining a clear solution. The solution was transferred into a closed reaction vessel made out of *teflon* with metal armoring to prevent vessel deformation due to high temperature. The vessel was then inserted into an oven at 200 °C for 2 hours. At the end of that period, the resulting products were collected and, if needed, re-suspended in 5 mL of deionized water. Centrifugation was performed at 13000 rpm for 10 minutes. AFM analysis were carried out as mentioned before.

4.2.2. Evaluation of the catalytic potential

Three techniques were employed to assess the 4-aminopyridine-based CD catalytic potential: RP HPLC-DAD, fluorescence studies and GC-MS. Following the CD fabrication, these techniques were applied to evaluate the CD capacity to open an epoxide molecule ring through a nucleophilic attack (RP HPLC-DAD and fluorescence studies), and the capacity in enhancing a follow-up aminolysis reaction resulting in the coupling between the open epoxide molecule and an aminated compound (evaluated by GC-MS).

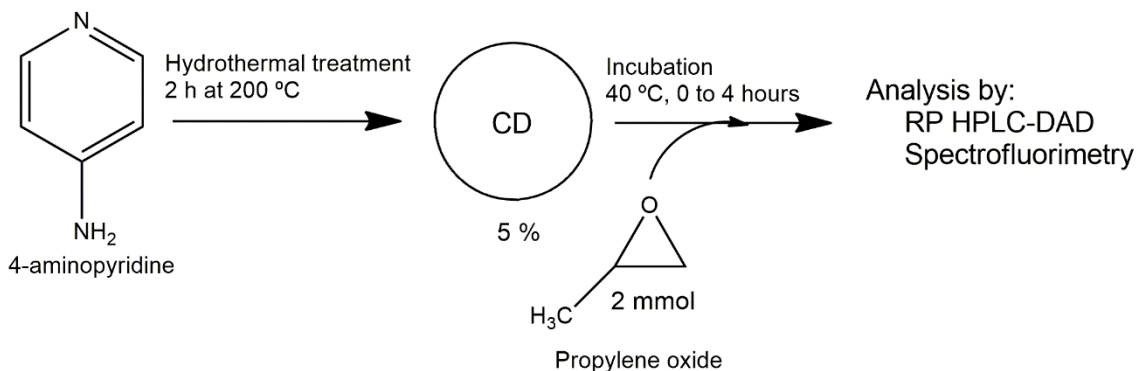


Figure 39 – Scheme of the methodologies used to assess a 4-aminopyridine-based CD capacity to catalyze the ring-opening reaction of a model epoxide (propylene oxide).

To evaluate the epoxy ring-opening reaction, samples that were a mixture of CD solution with a model epoxide, propylene oxide, were prepared as schematized in Figure 39. 2 mmol of propylene oxide were mixed with CD solution (5% in the mixture) and deionized water was added to complete the volume of 1 mL. The samples were incubated at 40 °C with agitation during several different times (ranging from 0 to 4 hours). When ready, samples were analyzed by RP HPLC-DAD and the chromatogram

profile evolution was evaluated. The change in the evolving peaks (with the increasingly higher incubation periods) was observed, quantified and plotted. Given that propylene oxide has no absorption in the UV-Vis region, it did not appear in the chromatogram. Therefore, all the information obtained from the HPLC system refers only to the CD. The HPLC system used was the same as the one described in the LCA chapter.

Fluorescence studies were also made for the same samples: the initial excitation and emission peaks were recorded and compared with the peaks resulting from a 4 hours incubation period. The excitation and emission maximum wavelengths were compared and have been displayed in a fluorescence matrix. The fluorescence properties were analyzed using a Horiba Jovin Yvon Fluoromax-4 spectrofluorimeter in the same way as described in the LCA chapter.

As results from previous studies displayed some potential for the aminolysis reaction, GC-MS was employed to study the effect caused by the CDs' presence in an aminolysis reaction. This technique would serve to search for the m/z values expected for either the reagents and our predicted coupled products. Towards that intent, 1 mL samples comprised of 1 mmol of propylene oxide or allyl glycidyl ether, 5% CD and 1 mmol of aniline were prepared. ACN was used to complete the total volume given that aniline has poor solubility in water. The samples were incubated at temperatures ranging from 30 to 60 °C during 24 hours periods. The resulting products were analyzed by GC-MS and the expected masses were searched for. The percentage of aniline alone and the coupled product were quantified by calculating the area of the corresponding peaks and comparing it to the total area formed by the peaks corresponding to aniline and the coupled product. GC-MS analysis were carried with a Thermo Scientific TRACE™ 1300 with a TG-5MS column (60 meters with an internal diameter of 0.25 millimeters) and an ISQ mass detector.

4.3. Results

4.3.1. Epoxide ring-opening reaction

A structural analysis of the CD was performed in an initial stage. From the synthesis, hydrothermally produced CDs with an estimated mean size of 18 ± 2 nm were obtained, as quantified from AFM data (Figure 40). The CD alone when analyzed in a RP-HPLC-DAD system, before any products can be formed, displays just one major peak with a shoulder at around 7.3 minutes, as observed in Figure 41a for a 0 hour reaction time. Despite being a different CD, this is concordant with the conclusions obtained in previous chapters, which demonstrated that a hydrothermally-based synthesis resulted in a CD solution with a satisfactory degree of purity.

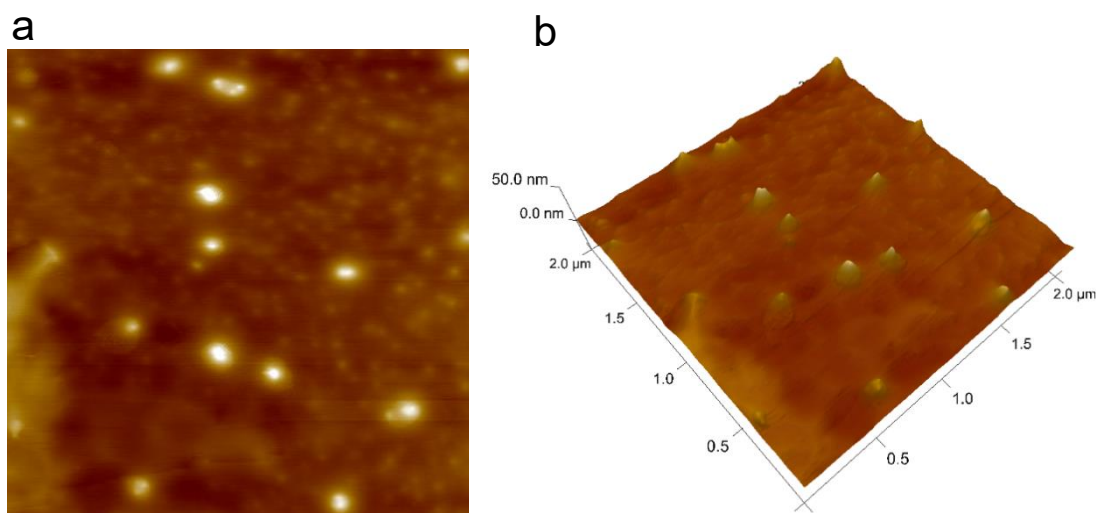


Figure 40 – AFM images of a 4-aminopyridine-based CD made by hydrothermal treatment: a) 2D image and b) 3D topologic image.

Regarding the reaction between CD and epoxide itself, as the reaction time increases, a change in the chromatogram profile is observable. The emergence of new peaks is observable while others gradually disappear. In fact, the major initial peak without incubation time, peak 2, gradually disappears as the reaction progresses (Figure 41a and 41b). In its place, two new peaks, peaks 3 and 4, emerge noticeably. This suggests that a gradual change might have occurred in the CD as the reaction progressed, resulting in a change in its structure, represented by the different profiles in the chromatograms. Some sort of interaction must occur between the 4-aminopyridine-based CD and propylene oxide in order to change the particle structure. Additionally, even though peak 2 was still about one fifth of the total area of the chromatogram by the time the reaction was stopped at 4 hours, the absorption wavelengths associated to that peak are slightly different (Figure 41c). Thus, even though the retention times for peak 2 are similar between the samples, the species eluting marked by those peak are at least

slightly different. Because of that, for an incubation period of 4 hours, we can assume that almost all the CD in the initial mixture had reacted with propylene oxide. From the reaction resulted a change in its structure that was translated into a different chromatogram profile.

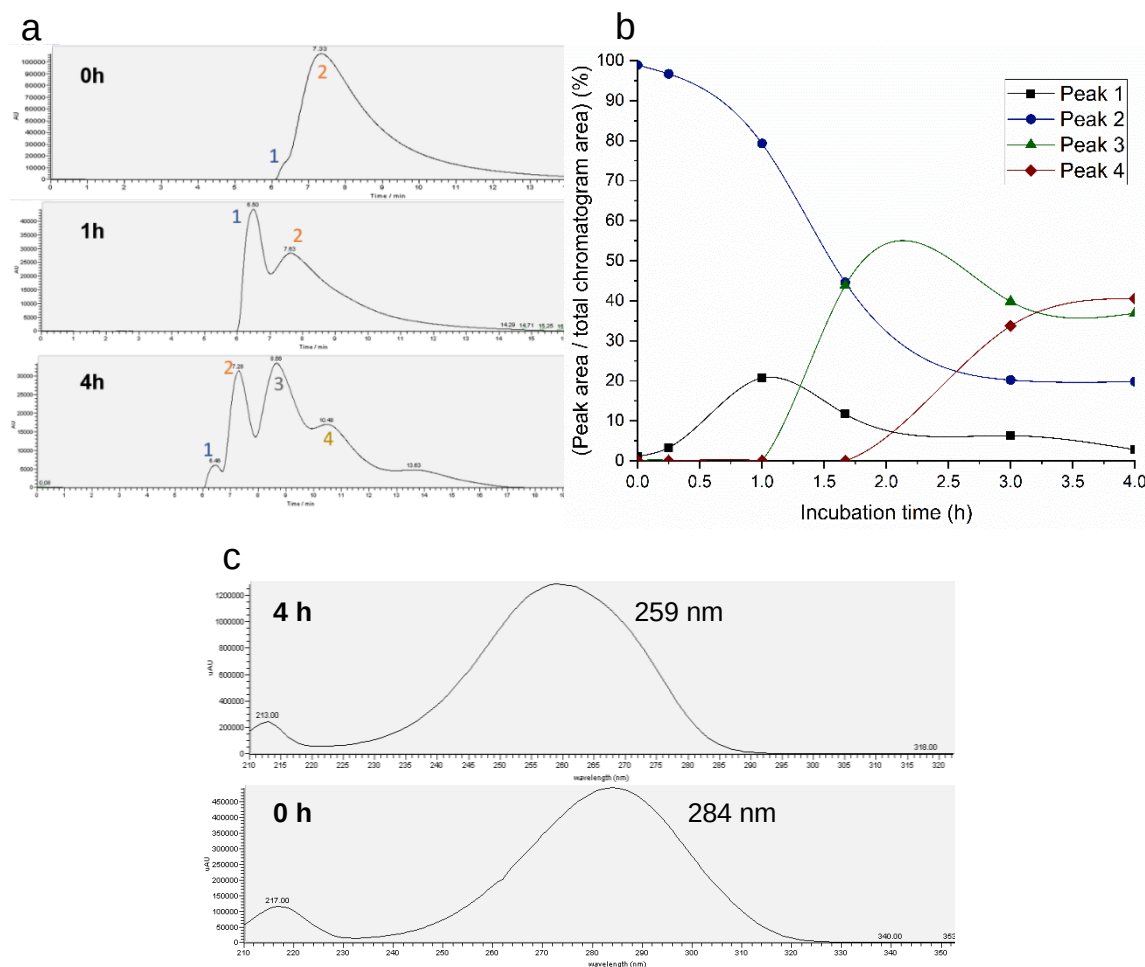


Figure 41 – a) RP-HPLC-DAD chromatogram for a mixture of fixed amounts of propylene oxide and 4-aminopyridine-based carbon, with incubation periods of 0, 1 and 4 hours at 40 °C; b) graphical representation of the variation of the ratio between the area of a specific peak and the total chromatogram area in function of the incubation time; c) UV-Vis absorption spectra for peak 2 at 0 and 4 hours.

Studies regarding the CD fluorescence further confirmed the changes induced by the interaction with propylene oxide. The CDs' excitation and emission patterns, either before and after reacting with propylene oxide for 4 hours, were analyzed. As observed in Figure 42, the patterns changed by the end of a 4 hours incubation period in the presence of propylene oxide. Before reacting, the CD displays two emissive centers ($\lambda_{exc} = 290$ nm with $\lambda_{em} = 380$ nm and $\lambda_{exc} = 300$ nm with $\lambda_{em} = 440$ nm), meaning that our CD was capable of emitting in two different, well separated, wavelengths. After the reaction, both those emissive centers disappeared and a third one emerged with $\lambda_{exc} = 380$ nm and $\lambda_{em} = 480$ nm, which are wholly different wavelengths from those presented by the same CD before the incubation time. Therefore, our assumption that a significant change

occurs in the CD is further confirmed. The interaction with propylene oxide caused a change in the CD structure, culminating in different photo-physical properties before and after reacting with the epoxide, as observed by the change in the excitation and emission wavelengths.

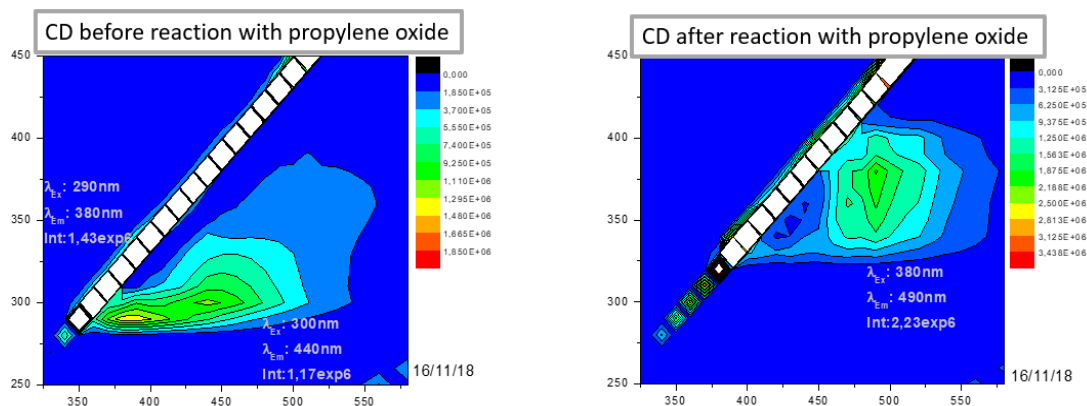


Figure 42 – 4aminopyridine-based hydrothermally-made CD excitation and emission patterns obtained through a 3D fluorescence analysis (emission spectra made at successively higher excitation wavelengths).

4.3.2. Aminolysis follow-up reaction

In order to assess the CDs' capacity to enhance the aminolysis reaction, GC-MS studies were made and the m/z values of aniline and our expected coupling product were searched for. Aniline was chosen given that it yielded positive results in the preliminar tests and presented an exposed amine group. As for epoxides, in order to obtain additional data besides the already tested simple model epoxide (propylene oxide), a more complex epoxide molecule, allyl glycidyl ether, was also used to see if the reaction would depend on the epoxide molecule structure itself. Figure 43 is a schematic representation of the expected result of the reaction between aniline and the two tested epoxides. It also displays the masses of either component alone and the masses of the products resulting from the coupling between aniline and the epoxides.

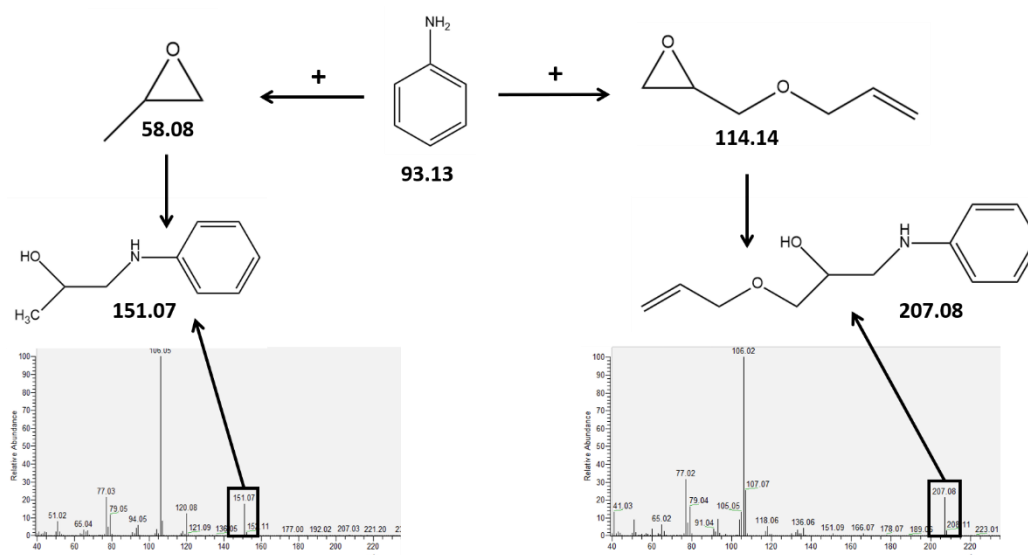


Figure 43 – Representation of the coupling between aniline and two different kinds of epoxides: propylene oxide and allyl glycidyl ether. The displayed m/z values correspond to either the precursors or the coupled products and were searched for in the MS spectra in order to determine which GC peak corresponded to which compound.

By analyzing the results obtained through GC-MS we were able to determine the proportion of the formation of coupled products between aniline and epoxides. By comparing the peaks ascribed to aniline to the peaks corresponding to the product, we were able to calculate the percentage of aniline that was coupled with the epoxide. By varying the incubation temperature and the quantity of CD present in the mixture, we evaluated the changes induced by alterations in each of those parameters. The results are presented in Tables 4 and 5:

Epoxide	% CD	Aniline (%)	Product (%)
Propylene oxide	5	53.9	46.1
	10	49.8	50.2
	15	44.5	55.5
Allyl glycidyl ether	5	43.6	56.4
	10	43.6	56.4
	15	36.6	63.4

Table 4 – Aniline coupling percentage with two different epoxides in the presence of different quantities of 4-aminopyridine-based CD (5 to 20% of the estimated number of epoxide molecules present in the mixture).

Table 4 represents the rate of coupling between aniline and two epoxides in the presence of different concentrations of CD. Considering that the tests were made using varying quantities of CD, they can be used to observe the effect induced in the aminolysis reaction by increasingly higher concentrations of CD. Observing the data from the Table 4, it is easily noticed that, as expected, increasing the concentration of the CD present in the sample will result in an increase of the coupling between aniline and epoxides.

This is easily explainable given that the CD is being used as a catalyst and increasing the concentration of a catalyst in a reaction, to a certain limit, will increase the reaction rate. In our case, increasing the quantity of CD will result in an increase of the rate at which aniline is coupled with the epoxide, thus increasing the aminolysis reaction rate.

Epoxide	Temperature (°C)	Aniline (%)	Product (%)
<i>Propylene oxide</i>	30	46.1	53.9
	40	49.8	50.2
	60	54.7	45.3
<i>Allyl glycidyl ether</i>	30	32.6	67.4
	40	43.6	56.4
	60	46.7	53.3

Table 5 – Aniline coupling percentage with two different epoxides when incubated at different temperatures for a period of 24 h. The quantity of CD was kept constant at 10% the estimated number of epoxide molecules present in the mixture.

Table 5 displays the coupling extent when the mixture is incubated at different temperatures, ranging from 30 to 60 °C, while the CD quantity is maintained constant. It allows us to observe the effect caused in the reaction by temperature. Unlike expected, as the temperature rises, the coupling extent diminishes. This is unexpected considering that increasing the incubation temperature (therefore increasing the energy provided to the system), should lead to a faster coupling between the components present in the reactional mixture. We hypothesize that, when the CD is loosely bonded to the epoxides as a nucleophile, they form a complex that can be disrupted when excessive amounts of energy are provided. The excess of energy that results from higher temperatures can disrupt this complex and inhibit the epoxide ring-opening step catalyzed by the CD to a certain extent, and therefore, the first reactional step, which is required for the following aminolysis, would happen at a lower rate, resulting in a decreased coupling extent between aniline and the oxyanion obtained during the ring-opening step.

4.4. Conclusions

A 4-aminopyridine-based CD with a mean size of 18 nm was obtained through hydrothermal treatment. By acting as nucleophile, the CD was capable of catalyzing the ring-opening reaction in a model epoxide, resulting in the formation of reactive oxyanions. Additionally, it was found that this could be followed by an aminolysis reaction that led to the coupling between the oxyanion resulting from the epoxide ring-opening and aminated compounds, such as aniline.

The chromatographic analysis of the CD before the reaction yielded a chromatogram presenting a solo peak with a small shoulder. After successively higher incubation periods with propylene oxide, that sole peak gradually disappeared while two new peaks emerged. This indicates that, during the incubation period, the CD interaction with propylene oxide resulted in alterations in the nanoparticle's structure. This was evidenced by the different chromatogram profiles and the different absorption spectra profiles for the major initial peak (for incubation periods of 0 and 4 hours). These results were further confirmed by fluorescence studies in which the CD displayed different photo-physical properties, before and after reacting with propylene oxide for 4 hours. Considering the combined results of the chromatographic analysis and the fluorescence studies, we can assume that during the incubation period an interaction occurs between the CD and the epoxide molecule, possibly resulting in the formation of an oxyanion.

GC-MS studies demonstrated that our CD was capable of enhancing the coupling extent between aniline and epoxide in a manner affected by the CD quantity and the incubation temperature. As expected, higher CD quantities result in higher coupling extents given that there are more particles to promote the epoxide ring-opening reaction. On the other hand, an increase in the incubation temperature results in lower coupling rates. We hypothesize that our CD forms a complex with the epoxide when acting as nucleophile. Considering that higher temperatures result in greater amounts of energy being provided to the mixture, the excess of energy might disrupt the complex, resulting in a partial inhibition in the CDs' capacity to catalyze the ring-opening step, thus limiting the reaction.

Finally, since aniline was here used as the aminated compounds due to being relatively simple and having an exposed amine groups, studies with more complex aminated molecules are required. If they display potential, it could be a source of new synthetic pathways for the production of several molecules and peptides, widening the possibilities for the development of new compounds and synthetic methodologies based on the use of metal-free catalysts.

5. Conclusions

This work consisted on a study regarding CDs and their properties, characteristics, impact and applications. The project had three main objectives: an assessment of the impacts caused in the environment by the bottom-up synthesis of CDs; a study about the influence of molecular fluorescent impurities in the fluorescence and excited state reactivity of a model CA,urea-based CD; and a study about the application of 4-aminopyridine-based CDs for catalytic applications, namely the ring-opening reaction of epoxides followed by aminolysis reactions. Some final remarks regarding each objective are mentioned below.

5.1. CDs' synthesis life cycle assessment

A LCA study regarding six bottom-up synthetic strategies for the synthesis of CA and CA,urea-based CDs was performed. For a volume-based functional unit, electricity is the major contributor for most environmental impact categories for a hydrothermal synthesis, while for microwave-assisted synthesis, the main contributor is the impact associated with the use of resources. Overall, the addition of urea had a significative effect. When only the environmental impact was considered, microwave-made CA-based CDs result in the lowest impacts while the highest derive from hydrothermal syntheses.

When the CDs' functionality is considered (by introducing a QY_{FL} -based functional unit), the addition of urea in either methodology significantly lowers the relative environmental impact, mainly because it greatly increases the QY_{FL} of the resulting CDs. However, despite the similar QY_{FL} between the routes, the purity degrees of the obtained CDs vary: hydrothermal treatment yields a satisfactory degree of purity while microwave-treatment causes the formation of several moieties in the resulting CD solution. In that case, additional and more complex purification steps would be required, resulting in higher costs in terms of energy and resources (increasing the associated impacts). Furthermore, as they cause the removal of fluorescent impurities that could positively contribute for the emission, those steps of purification would likely cause a decrease in the QY_{FL} of the CD solution, increasing the relative environmental impact.

Finally, as observed by sensitivity studies, electricity was found to be the most sensitive factor for hydrothermal syntheses, while the use of resources was the most relevant for microwave-assisted syntheses. Additionally, changes in the inputs of the precursors or the replacement of the raw materials altogether could greatly influence the

associated environmental impacts. In summary, CA,urea-based CDs are the best option to follow, irrespective of the synthetic methodology. Nitrogen-doping strategies result in great benefits in terms of QY_{FL} , thus offsetting the environmental impacts associated with the synthesis of the nanoparticles. Additionally, the carbon source used in the synthesis is a critical point for either methodology. Because of that, future studies regarding the cleaner production of CDs should focus on that point.

5.2. Fluorescent impurities influence in the properties and excited state reactivity of CDs

The second part of our work consists in a study regarding the effect of fluorescent impurities in the properties and excited state reactivity of CDs. To this end, three CD-based samples (with different components – fluorescent impurities and/or CD) were submitted to characterization and fluorescence studies.

The microwave-assisted synthesis of CA,urea-based CDs was demonstrated to result in the production of green-emitting fluorescent molecular by-products, capable of masking the fluorescence of the blue-emitting CDs themselves. ESI-MS studies suggest that the impurities are mainly a compound named HPPT, which is removed during the dialysis, as the corresponding peak disappears after the dialysis of the initial CD solution. Our results demonstrate that the CD and the impurities have different properties and react differently to alterations in parameters such as the medium pH or the surrounding molecular microenvironment.

Finally, it was found that, when co-existing in the same solution, the CD and the fluorescent impurities do not behave as two individual species. Instead, their interaction originates a synergistic effect with different excited state reactivity and properties than those resulting from an additive effect of the individual components alone. We believe that this opens the possibility for the making of novel hybrid materials, composed of CDs and fluorescence impurities. They would display new and improved properties, thus allowing them to be used for new applications.

5.3. CDs' catalytic potential for epoxide ring-opening and aminolysis reactions

The last segment describes the potential application of CDs for catalytic applications. A 4-aminopyridine-based CD was prepared and characterized. It could improve the rate of the ring-opening reaction on a model epoxide by acting as a nucleophile. RP HPLC-DAD studies demonstrated that as it reacted with propylene oxide during successively higher incubation periods, the CD underwent a gradual change. This is evidenced by a gradual change in the chromatogram profile and in the peaks area ratio (when compared to the total chromatogram area), which indicate that our CD suffers a gradual change that results in the alteration of its properties. By the end of a 4 hours incubation period, the CD had changed to such an extent that the major initial peak absorption wavelength was shifted by ~25 nm. Moreover, the CD excitation and emission patterns, before and after an incubation period of 4 hours, changed: the initial two emissive centers disappeared while a third one emerged with higher excitation and emission wavelengths.

After studying the interaction between CDs and propylene oxide, GC-MS studies were performed to evaluate the CDs' capacity to enhance the coupling between aniline and an oxyanion derived from the epoxide ring-opening reaction. The CD could improve the aminolysis reaction outcome in a manner depending on the CD quantity and the incubation temperature. While the influence of the CD quantity is easily explainable by the higher number of particles that are present to interact with the epoxide, the decreasing effect observed with higher temperatures was unexpected. We hypothesize that, while acting as a nucleophile, the CD forms a complex with some of the intervenients in the reaction, which might be easily disrupted. The excess of energy associated to higher reaction temperatures could dissociate the complex, resulting in a partial inhibition of the CDs capacity to catalyze the ring-opening step, thus limiting the coupling reaction extent.

While this study is still on an early stage and more detailed experiments are still required, the CDs' catalytic potential for aminolysis reactions with epoxides could be employed in the synthesis of peptides and new coupled molecules. This would widen the possibilities for the development of new synthetic methodologies based in metal-free catalysts.

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