chemistry proceedings

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Carbon Dots from *Porphyridium cruentum* Microalgae by High-Efficient Hydrothermal Approaches: Biocompatibility and Antioxidant Capabilities †

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† Presented at the 27th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-27), 15–30 November 2023; Available online: https://ecsoc-27.sciforum.net/.

Abstract: Fluorescent carbon dots (μAlgae-CDs) were successfully prepared from renewable *Porphyridium cruentum* biomass using a hydrothermal carbonization approach and ethylenediamine (ED) as a nitrogen additive. Structural and photophysical properties of the as-synthesized nanomaterials were evaluated using FTIR, UV-Vis, and fluorescence spectroscopies. The new μAlgae-CDs synthesized with a ratio of 0.16 of ED demonstrated good antioxidant properties by ABTS radical cation method and did not exhibit cytotoxicity against non-tumor Vero cells and tumor HeLa cells, showing potential application in bioimaging.

Keywords: carbon dots; microalgae; fluorescent; antioxidants; cytotoxicity

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Citation: Chouzende, I.; Costa, A.I.; Barata, P.D.; Martins, S.; Semedo, M.C.; Cardoso, F.M.H.; Lobo, M.L.; Prata, J.V. Carbon Dots from *Porphyridium cruentum* Microalgae by High-Efficient Hydrothermal Approaches: Biocompatibility and Antioxidant Capabilities. *Chem. Proc.* **2023**, *14*, 4. https://doi.org/10.3390/ecsoc-27-16074

Academic Editor: Julio A. Seijas

Published: 15 November 2023

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1. Introduction

Carbon dots (C-dots), a new "zero-dimensional" nanomaterial, have become one of the most prominent members of the carbon materials family due to their excellent luminescence, great photostability, small size, low toxicity, and biocompatibility [1]. In particular, green carbon dots derived from natural resources have received extensive attention due to their unique benefits, including abundant sources, cost-effectiveness, and environmental friendliness [2].

Bottom-up green approaches based on the use of renewable biomass for producing C-dots are particularly attractive since high-valued nanomaterials can be obtained from low-value precursors, contributing to a circular economy [3,4]. Regarding their unique properties, green C-dots have shown tremendous potential applications in several areas such as biomedicine, (bio)sensors, photocatalysis, optoelectronics, and bioimaging [1,5].

Among the various green sources explored, microalgae (μAlgae) have prominent advantages with respect to their ease in growth and ability to survive in extreme conditions of pH and temperature that make them a promising green low-cost feedstock for the large-scale synthesis of nanoparticles [6]. Additionally, their richness in compounds that

benefit human health, including lutein, astaxanthin, β-carotene, and unsaturated fatty acids, provide this biomass source with enormous potential to produce carbon materials with varied physicochemical and morphological properties [7,8].

In this work, we highlight the synthesis of green C-dots (μAlgae-CDs) via a one-pot hydrothermal treatment of *Porphyridium cruentum* biomass, a red marine microalgae. Results concerning the biocompatibility and antioxidant capabilities of the new nanomaterials will also be presented.

2. Materials and Methods

2.1. Materials

The biomass of *Porphyridium cruentum* microalgae used in the experiments was produced in flat-panel photobioreactors at Allmicroalgae's industrial facilities and was kept refrigerated at 5 °C until use. All reagents were used as received and the solvents were of analytical grade and/or were purified by standard methods. In all experiments, ultrapure water was used (Milli-Q, Millipore, Burlington, MA, USA).

2.2. Methods

A high-pressure reactor (Parr model 4560) equipped with pressure, temperature, and stirring sensors/controllers (Parr, model 4843), was used for hydrothermal carbonization of the biomass.

The structural characterisation of the as-prepared μAlgae-CDs was accomplished by FTIR on a Bruker Vertex 70 as KBr pellets, while for UV-Vis analysis the aqueous dispersions were directly employed after appropriate dilution on a Jasco UV V-750 spectrophotometer using 1 cm quartz cells.

Fluorescence spectra were obtained on a Perkin Elmer LS45 fluorimeter using a 1 cm quartz cuvette at 25 °C in right angle (RA) geometry. Fluorescence quantum yields (Φ_F) were measured in aqueous solutions using quinine sulphate in 0.01 M H_2SO_4 (Φ_F = 0.54) as a reference standard [9,10]. The optical density of the samples was kept below 0.05 at the excitation wavelength to prevent homo-inner filter effects. The microscopy fluorescence images were obtained on a fluorescence microscope Olympus BX51.

2.2.1. Synthesis of Carbon Dots (CDs)

The biomass was fed into an inox vessel with water and ethylenediamine (ED) as an additive in several ratios. The hydrothermal carbonization of the biomass was achieved at the desired temperature (200–250 °C) for a certain period (4–6 h), under air-equilibrated conditions, using wet biomass (after removing the nutrient via centrifugation) or dry biomass achieved from the wet biomass.

The as-synthesized μAlgae-CDs were cooled to room temperature and the reaction mixture was filtered through a 0.2 µm cellulose membrane, yielding aqueous dispersions. Low-polarity molecular species were removed by liquid–liquid extraction with CH_2Cl_2/AcOEt.

2.2.2. In Vitro Cytotoxicity Assays and Cell Imaging

The viability of Vero (ATCC™ CCL-81) and HeLa (ATCC™ CCL-2) cell lines was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [11]. Cells were seeded in a 96-well plate in Dulbecco's Modified Eagle Medium (GIBCO®, Thermo Fisher Scientific). After spreading, cells were treated with the μAlgae-CDs concentrations of 0.02–4000 µg mL^{-1} and incubated at 37 °C under a 5% CO_2-enriched atmosphere. After incubation for 18 h, an MTT solution was added into each well and microtiter plates were incubated. The concentration of blue formazan crystals produced by live cells by reduction of MTT in mitochondria were determined by UV-Vis spectroscopy. The absorption spectrum was acquired, and the absorbance was read at 570 nm using 650 nm as reference.

Cellular uptake of μAlgae-CDs was observed by fluorescence microscopy.

2.2.3. Evaluation of Antioxidant Capacity

The antioxidant capacities of the μAlgae-CDs were evaluated using the ABTS radical cation method [12]. The reaction was conducted at room temperature in the dark for 6 min and the absorbance was measured at 655 nm. The radical scavenging effect was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_c - A_s) \times 100/A_c$$

where A_c is the absorbance of the negative control and A_s is the absorbance of the sample solution. Trolox was used as positive control.

3. Results and Discussion

3.1. Synthesis and Surface Characterization

The μAlgae-CDs were synthesized using a one-step hydrothermal method in a high-pressure reactor using an aqueous suspension from *Porphyridium cruentum* biomass (4.0 g) and ED as the nitrogen source (ED/biomass ratio = 0.16) under stirring. After a dwell time of 6 h at 250 °C, the resultant material was filtrated through a cellulose membrane, resulting in a yellowish-brown liquor.

The FTIR spectrum of μAlgae-CDs (Figure 1) showed an intense broad band at 3430 cm^{-1} from O-H, with a shoulder near 3270 cm^{-1} and N-H stretching, and weak bands at ~2930–2858 cm^{-1}, assigned to C-H bonds. The presence of an intense band at 1656 cm^{-1} is attributed to conjugated C=O and carboxylate functionalities, and C=C stretching. C-H bending bands at 1440 cm^{-1} (CH_2) and 1370 cm^{-1} (CH_3) are also present.

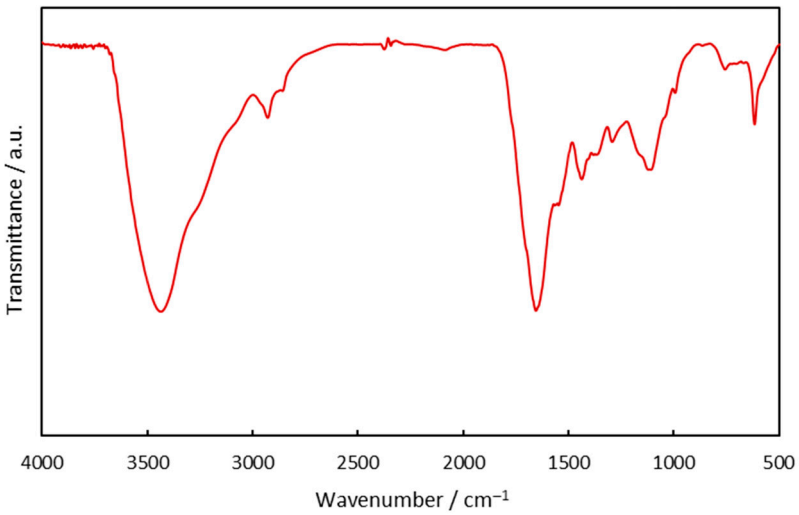


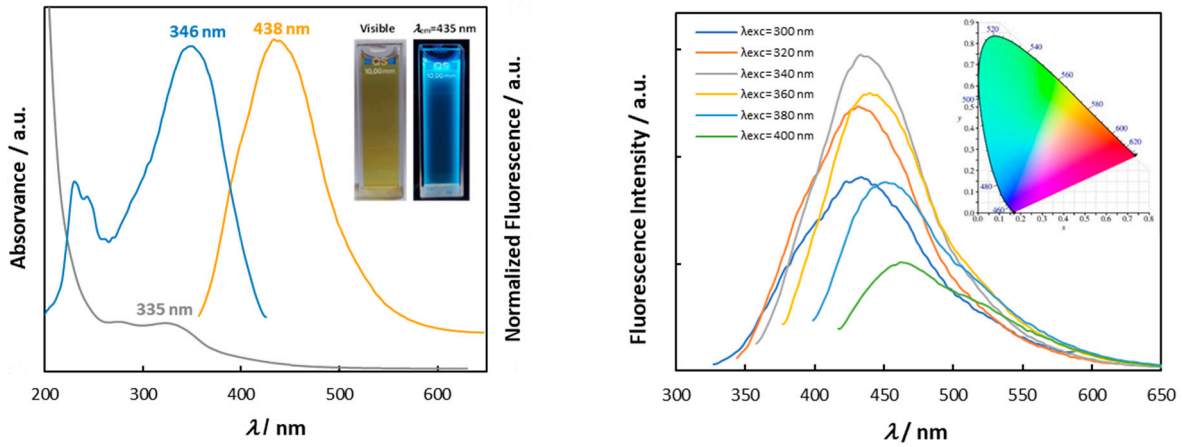
Figure 1. FTIR spectrum of the synthesized μAlgae-CNDs (KBr).

3.2. Optical Characterization

Aqueous dispersions of μAlgae-CDs were prepared in appropriate concentrations for the optical assays. The UV-Vis absorption spectrum of μAlgae-CDs showed a long absorption edge in both the UV and visible regions (300–600 nm) (Figure 2a).

The aqueous dispersions of μAlgae-CDs displayed a maximum excitation and emission at 346 and 438 nm, respectively (Figure 2a), and exhibited a blue color when irradiated with 366 nm ultraviolet light (Figure 2a, inset).

The μAlgae-CDs synthesized with ED as a nitrogen source using wet and dry biomass of *P. cruentum* showed relevant fluorescence quantum yields ($0.07 < \Phi_F < 0.16$), revealing a strong dependence between λ_{exc} and λ_{em} (Figure 2b) and excellent photostability (not shown).



(**a**) (**b**)

Figure 2. (**a**) UV-Vis (grey), excitation (blue, monitored at 435 nm), and emission (orange; λ_{exc} = 340 nm) spectra of μAlgae-CDs in aqueous dispersion (0.1 mg mL^{-1}) (inset: photograph of μAlgae-CDs showing emission colors); (**b**) emission spectra of μAlgae-CDs with different excitation wavelengths (inset: CIE chromaticity diagram).

3.3. Antioxidant Performance of µAlgae-CDs

An important property of C-dots is their antioxidant activity, i.e., the ability to act as a free radical scavenger. The antioxidant capacity of μAlgae-CDs at 5 mg mL^{-1} was evaluated with the ABTS radical inhibition assay (Table 1).

Table 1. Antioxidant activity and quantum yield of μAlgae-CDs.

Entry	Antioxidant Activity/%	Φ_F (λ = 340 nm)
1	94.2 ± 0.47 [1]	0.065
2	38.3 ± 0.08 [1]	0.159

[1] Values are mean ± SD for three determinations for antioxidant activity.

The results showed that the μAlgae-CDs obtained from wet biomass (Entry 1, Table 1) had better biological properties, even though they revealed a lower quantum yield as compared to the carbon dots achieved from dry biomass (Entry 2, Table 1).

3.4. Toxicity Evaluation and Cell Imaging

Using MTT assays, the biocompatibility of μAlgae-CDs against two epithelial cell lines, Vero (kidney epithelial cells extracted from an African green monkey) and HeLa (human cervical tumor cells), was evaluated over a concentration range of 0.02 to 4000 µg mL^{-1} (Figures 3 and 4, respectively).

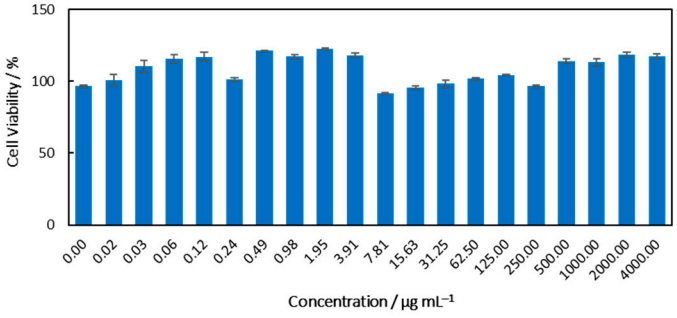


Figure 3. Cell viability of μAlgae-CDs at different concentrations for Vero cell line.

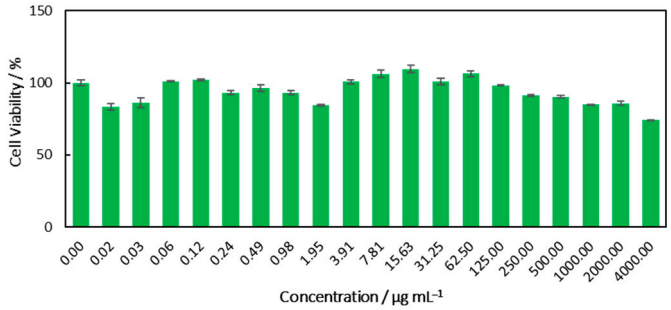


Figure 4. Cell viability of μAlgae-CDs at different concentrations for HeLa cell line.

Even for high levels of μAlgae-CDs, no significant cytotoxicity was observed for both cell lines, showing cell viability higher than 80%.

After incubation with μAlgae-CDs for 18 h at 37 °C, both cell lines were observed using fluorescence microscopy and the images in Figure 5 were collected.

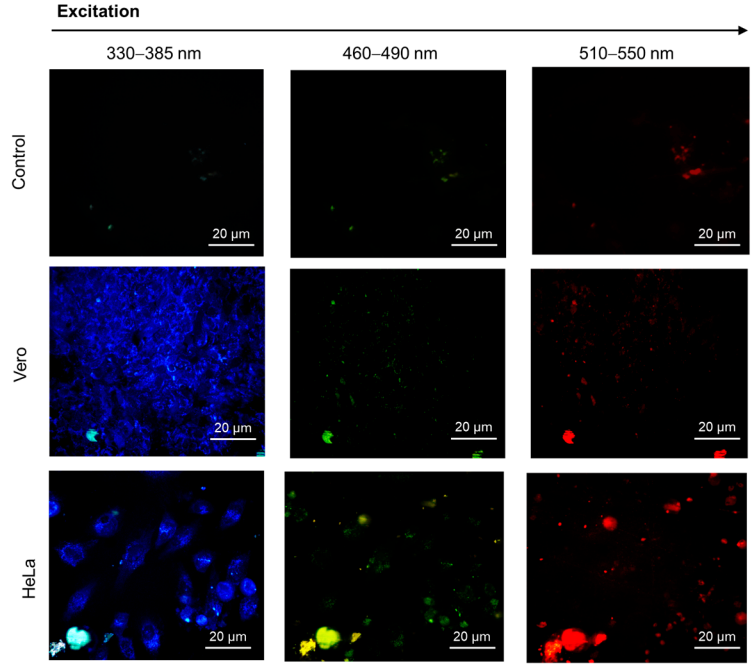


Figure 5. Optical microscopy fluorescence images of Vero and HeLa cell lines with μAlgae-CDs (400×).

4. Conclusions

The μAlgae-CDs were prepared from biomass of the red microalgae *Porphyridium cruentum* using an ecofriendly method and showed good photostability and relevant quantum yield.

The ABTS assay showed high efficacy in radical scavenging, suggesting good antioxidant properties of the μAlgae-CDs.

The μAlgae-CNDs did not exhibit cytotoxicity against Vero and HeLa cells, and by using fluorescence microscopy we observed that μAlgae-CNDs were internalized by both cell lines, opening up new applications for these nanomaterials.

Author Contributions: Conceptualization, A.I.C., J.V.P. and P.D.B.; methodology, J.V.P., F.M.H.C. and M.L.L.; investigation, I.C., F.M.H.C. and M.L.L.; resources, A.I.C.; data curation, A.I.C., I.C., M.C.S., P.D.B. and S.M.; writing—original draft preparation, A.I.C., M.C.S., P.D.B. and S.M.; writing—review and editing, A.I.C., J.V.P., M.C.S., P.D.B. and S.M. All authors have read and agreed to the published version of the manuscript.

Funding: We are grateful to Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES) for financial support (UIDB/00616/2023, UIDP/00616/2023 and UIDP/04035/2020), and Instituto Politécnico de Lisboa (IPL/IDI&CA_2022/µAlgae2Dot_ISEL).

Data Availability Statement: Data sharing is not applicable to this article.

Acknowledgments: We thank Allmicroalgae-Natural Products, S.A. for μAlgae biomass supply.

Conflicts of Interest: The authors declare no conflict of interest.

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