Preparation, functionalization and characterization of engineered Carbon Nanodots

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Carbon-based Dots (CDs) and their functionalized (nano)composites have recently attracted a lot of attention, owing to their seemingly easy preparation and their numerous potential applications, ranging from biomedical (imaging and drug delivery) to (opto)electronics (*i.e.*, solar cells and LEDs). This protocol covers the synthesis, purification and functionalization of nitrogen-doped carbon nanodots (NCNDs). Firstly, we describe the bottom-up synthesis of NCNCDs by using molecular precursors (arginine and ethylene diamine), through a microwave hydrothermal procedure. We also provide guidelines for the purification of these materials, either through filtration, dialysis or low-pressure size-exclusion chromatography. Secondly, we outline post-functionalization procedures for the surface modification of NCNDs (such as alkylation and amidation reactions). Thirdly, we provide detailed instructions on the preparation of NCNDs with different properties (such as color emission, electrochemistry and chirality). Given the fast evolution in the preparation and application of CDs, issues that might arise from artifacts, errors and impurities should be avoided. In this context, the present protocol aims at helping synthesize high quality nanomaterials with high reproducibility, for various applications. Furthermore, even if CDs are prepared by different synthetic procedures, as well as from different molecular precursors, they can still be purified and characterized by following this protocol. The sample preparation takes various time frames, ranging from 2 to 12 days depending on the adopted synthesis and purification steps.

INTRODUCTION

Nanotechnology, the manipulation of matter with at least one dimension between 1 and 100 nanometers, has produced a multitude of nanomaterials and multiple applications[1–4]. The specific physical and chemical properties allow nanomaterials to be exploited in a variety of research areas and commercial products, ranging from electronics to medicine. Carbon, an element of many faces[5], is an example of how size, shape,

composition, surface topology, chemistry and morphology are pivotal for the development and application of nanomaterials and nano-based devices[6]. In recent years, carbon-based dots (CDs) have emerged because of their fine properties, different but equally interesting with respect to other carbon nanomaterials (such as carbon nanotubes and graphene)[7–12].

CDs are considered quasi-spherical nanoparticles, with size below 10 nm, comprising carbon, oxygen and hydrogen atoms. Next to their apparently harmless, abundant and inexpensive nature, they have shown intriguing photoluminescence (PL) properties, which has brought them the label of "carbon nanolights"[7] and has sparked numerous applications[12–20]. CDs can be prepared via two main routes: top-down and bottom-up approaches[21,22]. While the former consists mainly of cutting down larger carbon nanostructures (like carbon nanotubes and graphene), the latter route has shown the real potential of CDs by including numerous methods as well as a plentiful of small molecules as starting materials. For example, cheap precursors and accessible protocols can be used in a straightforward way, also as a way to teach nanotechnology and fluorescence at high-school and undergraduate level[23].

However, it is this widespread access to starting materials and protocols, as well as the apparent easy preparation of CDs, which may cause inconsistencies that need to be avoided for the CDs to truly shine as the next generation nanomaterials and to compete with inorganic-based quantum dots. Additionally, the literature present critical discrepancy in defining CDs, they should be classified depending on the presence of amorphous or crystalline structures, as well as their PL origin[24]. First of all, the family of carbon-based dots embraces mainly three types, namely carbon nanodots (CNDs), carbon quantum dots (CQDs) and graphene quantum dots (GQDs). Amorphous carbon-based nanoparticles without quantum confinement are called CNDs, while the presence of crystalline structures and quantum effects are present in spherical CQDs or in single-layer GQDs. Second of all, the widespread utilization and (mis)understanding of their properties can be due to vague (sometimes inadequate) synthetic protocols, as well as unsatisfactory (or lacking) purification and characterization procedures[25]. Finally, aggregation of the carbonaceous materials, as well as lack of size control and uniformity are common issues both for top-down and bottom-up procedures, which ultimately affect the quality of the prepared materials.

In recent years we have worked on the synthesis and purification of nitrogen-doped carbon nanodots (NCNDs)[26], the modification of their (chiro)optical[27,28] and electrochemical properties[29] and the preparation of (non)covalent hybrids[30–32] and nano-composite materials[33,34]. This work is based on our original procedure, which uses a two-component approach, together with a fast microwave-assisted hydrothermal synthesis[26]. More specifically, using arginine (Arg) and ethylenediamine (EDA) it is possible to prepare NCNDs that feature an amorphous core and an amino-rich surface. The two-component approach, given the different reactivity of the components, results in nanoparticles with the core originating from Arg, while the surface emerges from

EDA. The prepared nanodots are small, with a rather homogeneous size distribution (2.47 ± 0.84 nm), and show blue fluorescence (FLQY = 17%), after proper purification. Besides filtration and dialysis, NCNDs can be purified by using size-exclusion chromatography, which yields three distinct fractions of NCNDs that feature different size, surface functional groups and fluorescence properties (*e.g.,* the smallest fraction has also the highest PLQY = 46%). We have managed to expand this procedure in order to modulate and tailor the properties of CNDs to exhibit different fluorescence properties[28], to confer chirality[27], tuning their band gap[29], preparing donor-acceptor (supra)molecular assemblies[27,32], but also have exploited the amino-rich surface to prepare covalent hybrids[30,31] and composites with clay[33] or ionogels[34].

Overview of the procedure

Herein, Part 1 covers the synthesis, purification and characterization of NCNDs, tuning their properties and their surface functionalization. The procedure we describe could be particularly useful for researchers interested in or working in the field of carbon dots or nanotechnology in general. The major goal of this article is to guide researchers with a reliable and standard set of protocols for the hydrothermal synthesis of tailored CNDs, purification procedures and characterization steps needed for the preparation of high-quality nanomaterial. We hope that our protocols will aid in the utilization and understanding of CNDs properties, as well as eliminate error and artifacts that have occurred during the fast and intense research in the last years[25,35–40]. Furthermore, we expect our purification and quality control instructions to be employed also by researchers that are preparing other types of CDs, including carbon quantum dots and graphene quantum dots, as well as their hybrids or composite materials. For example, CNDs prepared through this route can be purified using size-exclusion chromatography that can be performed in a variety of solvents and therefore can be adapted to the solubility of CD-based nanoparticles.[41] Besides purification, the characterization procedures of CDs are in need of uniformity and therefore we propose proper absorption and emission protocols. Additionally, atomic force microscopy (AFM) characterization can provide useful information in the determination of CD size (height) and uniformity of the sample that cannot be easily identified with transmission electron microscopy (TEM) experiments. Indeed, CNDs are small nanoparticles with a low degree of crystallinity, have defects present and tend to aggregate, therefore making TEM not the best technique for morphological characterization.[9] We further describe, in Part 2, the post-synthetic modification/functionalization reactions that utilize the reactive amino-rich surface and then discuss on the quality control tools of the resulting modified CND materials. Finally, in Part 3, we illustrate how our synthetic reaction can be also used for the preparation of NCNDs with tunable properties (optical, chiroptical and electrochemical).

Experimental Design

This protocol presents a straightforward synthetic strategy to prepare NCNDs through a bottom-up approach, starting from commercial and low-cost precursors. Arginine (Arg) and ethylene diamine (EDA) are used as both carbon and nitrogen sources, allowing a simultaneous nitrogen doping and surface passivation during the synthetic process. NCNDs result from processes of condensation, polymerization and aromatization from the thermal carbonization of the precursors. We reported that, while Arg is mainly responsible for the NCND core, EDA leads to the formation of the outer shell of the nanodots. Our procedure involves the use of water as reaction medium and microwave-assisted (MW) conditions. This represents a useful synthetic approach that avoids long reaction times and has been successfully applied as an effective and user-friendly way for the preparation of various materials apart from CNDs. It has been already reported that the heating parameters, such as the heating time, can affect the size of the nanoparticles and their photophysical properties[42,43]. However, many microwave syntheses are carried out using domestic microwave ovens, where the reaction conditions are difficult to control, in terms of temperature or power, and therefore may suffer in terms of reproducibility. We use a MW reactor by applying a constant power and a narrow temperature interval during the course of the reaction (3 minutes). The MW parameters are optimized to obtain nanoparticles with an average size of *ca*. 2.5 nm and the uniform carbonization process limits the formation of aggregates, which can be removed through a simple filtration step, followed by appropriate purification procedures to remove small size by-products, including dialysis and size exclusion chromatography. The so produced NCNDs possess high solubility in water, as well as in common polar organic solvents and fluorescence emission in the blue region of the visible spectrum. Moreover, the surface, rich in primary (or secondary) amino groups, can be used for further functionalization reactions and make our nanodots water soluble carrier for hydrophobic molecules. Alternatively, the amino groups can be easily modified to tertiary amines or other functional groups for specific purposes.

Finally, our synthetic strategy can be extended in order to modify NCNDs properties. By using Arg, EDA and naphthalene derivatives as precursors, in appropriate ratios, it is possible to modulate the fluorescence emission affording nanodots that emit light across the entire visible spectrum. Alternatively, by using Arg and EDA, together with quinones as starting molecules, a redox library of CNDs can be prepared. Additionally, we describe the preparation of chiral CNDs by using as precursor a chiral diamine able to retain its chirality under the harsh conditions used.

Part 1: Preparation of NCNDs

Preparation of NCNDs. Carbon nanodots can be synthesized using a multi-component approach by hydrothermal microwave-assisted heating of arginine and ethylene

diamine. NCNDs can be synthesized in relatively large scale using fairly easy and inexpensive methods.

Purification of NCNDs. Removing large carbon aggregates or particles, as well as small byproducts from the synthesis is pivotal for obtaining high-quality nanomaterials. We describe how filtration/dialysis or size-exclusion chromatography can be employed for the purification of NCNDs. Finally, the nanomaterial is obtained as a powder, after lyophilization.

Quality control steps. It is important to check the quality of the prepared and purified NCNDs on the basis of various characterization approaches, such as UV-Vis (A) and fluorescence (B) spectra, Kaiser Test (C) and AFM microscopy (D). UV-Vis spectra of NCNDs in water should be recorded as quality check step, given that the UV peak absorbance (286 nm, with the absorption onset starting at 388 nm) and the mass absorption coefficient (~3.7 (g^{-1} L) cm^{-1} at 286 nm) indicates whether the synthesis and purification have been performed correctly. Fluorescence (FL) emission (with quantum yield) is probably the most important quality check. Broad FL emission and excitation dependence is a common phenomenon observed for CNDs. The fluorescence peaks of NCNDs in water shift from 356 nm to 474 nm when the excitation wavelength changes from 300 to 420 nm and the fluorescence intensity decreases as the peak redshifts. Kaiser test is used to estimate the amount of primary amino groups present on the surface of the NCNDs, a value of ~1350 μmol g^{-1} is expected if the nanoparticles have been synthesized, purified and lyophilized correctly. Finally, surface microscopy (AFM) is used to confirm the quasi-spherical shape, the small size (2.47 nm) as well as the uniform size distribution (± 0.84 nm).

Part 2: Post-functionalization of NCNDs

Chemical reactions and purification. The amino groups present on the surface of the NCNDs can be further modified to target specific applications. In this protocol we outline how to transform primary amino groups to tertiary ones, which we have exploited for electrochemiluminescence (ECL) purposes.[30] We also describe an amidation reaction, between NCND amino groups and molecules bearing carboxylic acid moieties. We use 5,10,15-tri(4-*tert*-butylphenyl)-20-(4-carboxyphenyl)porphyrin as an example of dye for the preparation of donor-acceptor hybrids.[31] However, this procedure can also applied for the preparation of various nanohybrids, including covalent attachment of luminophores[30] or biologically-active molecules[44].

Quality control steps. The successful modification or covalent attachment of molecules is confirmed by performing the same quality control steps as for the pristine materials, which include UV-Vis (A) and FL (B) spectra, Kaiser test (C) and AFM microscopy (D).

Part 3: Preparation of engineered NCNDs.

Preparation of NCNDs derivatives and purification. By employing the multi-component approach, it is possible to endow carbon nanodots with many properties, by using appropriate precursors. In this step, we outline how to tune the emission properties of NCNDs by employing chromophores (such as naphthalene dianhydride derivatives) that can react in the microwave-assisted hydrothermal conditions together with Arg and EDA as precursors. Then, we show how to confer chirality to the NCNDs by employing a chiral diamine (instead of EDA) as surface precursor. Additionally, the energy levels of NCNDs can also be tuned to produce nanomaterials with desired energy levels for applications, thus improving their photocatalytic performance, by incorporating quinones into their structures. Purification is performed through filtration and dialysis.

Quality control steps. The quality of the NCNDs derivatives is checked by employing the aforementioned characterization approaches, such as UV-Vis (A) and FL (B) spectra, Kaiser test (C) and AFM microscopy (D). Furthermore, depending on the targeted property, additional characterization techniques can be used. When energy levels of NCNDs are modified, it is appropriate to record cyclic voltammograms (E). On the other hand, when chiral NCNDs are prepared they can be characterized by recording their circular dichroism spectra (F).

MATERIALS

REAGENTS

Preparation of NCNDs

- Arginine (Arg, Fluorochem, cat. no. M03558, stored at 4 °C)
- Ethylenediamine (EDA, Sigma-Aldrich, cat. no. E26266, stored at RT) **! CAUTION** It is flammable, toxic and harmful upon ingestion, inhalation or skin contact.
- Milli-Q water (see Reagent Setup, ultrapure water)

Post-functionalization of CNDs

- *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl, Sigma-Aldrich, cat. no. 03449, stored at -20 °C) **! CAUTION** This compound is irritant.
- 4-(Dimethylamino)pyridine (DMAP, Sigma-Aldrich, cat. no. 107700, stored at RT) **! CAUTION** This compound is irritant and harmful.
- *N,N*-Dimethylformamide (DMF, anhydrous 99.8%, Sigma-Aldrich, cat. no. 227056, stored at RT) **! CAUTION** This compound is flammable, irritant and toxic.
- Paraformaldehyde (Sigma-Aldrich, cat. no. 158127, stored at 4 °C) **! CAUTION** This compound is carcinogenic, flammable, irritant and harmful.

- Formic acid (Sigma-Aldrich, cat. no. F0507, stored at RT) **! CAUTION** This compound is flammable, corrosive and toxic.

Preparation of NCNDs derivatives

- 2,6-Dibromonaphthalene-1,4,5,8-tetracarboxylic dianhydride (Br_2NDA, TCI Europe, cat. no. D4339, stored at RT) **! CAUTION** This compound is an irritant.
- 2,3-Dimethoxy-5-methyl-*p*-benzoquinone (Sigma-Aldrich, cat. no. 299561, stored at RT) **! CAUTION** This compound is irritant and harmful.
- (1*R*,2*R*)-(−)-1,2-Diaminocyclohexane (Sigma-Aldrich, cat. no. 346721, stored at RT) **! CAUTION** This compound is corrosive and harmful.
- (1*S*,2*S*)-(+)-1,2-Diaminocyclohexane (Sigma-Aldrich, cat. no. 346713, stored at RT) **! CAUTION** This compound is corrosive and harmful.

Quality checking

- Quinine hemisulphate salt monohydrate (suitable for fluorescence, Sigma-Aldrich, cat. no. 22640) **! CAUTION** This compound is irritant.
- Kaiser test kit (Ninhydrin test kit, Sigma-Aldrich, cat. no. 60017) **! CAUTION** This compound is flammable, harmful and toxic.
- Ferrocene (Sigma-Aldrich, cat. no. F408) **! CAUTION** This compound is flammable, toxic, corrosive and harmful.
- Tetrabutylammonium hexafluorophosphate (for electrochemical analysis, Sigma-Aldrich, cat. no. 86879)

EQUIPMENT

- Spatula (double headed micro-spoon/spatula; VWR International)
- Pressure vessels (10 mL microwave vessel, pyrex; 908535, CEM corporation)
- Vial cap (silicone/PTFE, 10 mL, Activent; 909210, CEM corporation)
- Stirring bar (Micro stir bar; 162810, CEM corporation)
- Pipette (100 µL, Pipetman P100, Gilson)
- Pipette tips (200 µL tip volume, Diamond Towerpack D200; Gilson)
- Microwave reactor (Discover-SP; CEM) running Synergy™ application software and connected to an air source
- Milli-Q ultra-pure water system (Milli-Q plus 185; Millipore) equipped with a purification cartridge (QPAK® 1 cat. no. CPMQ004R1; Millipore)
- Vacuum filtration system (25 mm glass vacuum filter holder and support; Millipore), filtration flask (Pyrex micro-filtering flask, 125 mL, Sigma-Aldrich) and pump (diaphragm pump; Vacuubrand)

- Filter membrane (0.1 µm, JV, Millipore)
- Dialysis tube (Float-A-Lyzer®, MWCO 0.5-1 kD, Spectrum Labs)
- Stirring bars (cylindrical, 60 mm length, diameter 9 mm; Sigma-Aldrich)
- Magnetic stirrer (RCT basic; IKA)
- Freeze-drier (CoolSafe55/10-Labogene)
- Size exclusion chromatography (Sephadex® LH-20; cat. no. LH20100, Sigma-Aldrich)
- UV-Vis spectrophotometer (Cary 5000 UV-Vis spectrophotometer; Agilent)
- Fluorimeter (Cary Eclipse Fluorescence Spectrophotometer; Agilent)
- UV/Fluorescence cuvettes (quartz, 4 polished windows, 10 × 10 mm light path, Hellma Analytics, 111-QS)
- AFM instrument (Multi-Mode V; Veeco)
- Mica substrate (Highest Grade V1 Mica 50 x 75 mm, 2 x 3", cat. no. 56-75, Ted Pella Inc.)
- AFM Probe (HQ:NSC19/AlBs probe, 65kHz; 0.5 N m^{-1}; MikroMasch)
- Standard cantilever + tip (HQ:NSC19/AlBs probe, 65 kHz; 0.5 N m^{-1}; MikroMasch)
- pH-meter (with electrode, PH 25+, Crison Instruments)
- Electrochemical workstation (either Autolab PGSTAT302N or CHI 750C)
- Electrochemical glass cell (CH Instruments, CH222) equipped with a Teflon cap (CH Instruments, CHI223)
- Glassy carbon (GC) working electrode (3 mm diameter, 66-EE047 Cypress Systems)
- Platinum wire Counter Electrode (CE, CH Instruments, CHI115)
- Quasi-reference (QRE) electrode consisting either of a silver wire (Sigma-Aldrich, cat. no. GF06769200) or non-Aqueous Ag/Ag+ reference electrode w/ porous Teflon tip (CH Instruments, CHI112)
- Electrode Polishing Kit (CH Instruments, CH120)
- CD Spectrometer (Jasco J-810, scanning rate 50 nm min^{-1}, data pitch 0.2 nm, D.I.T 2 s. Each CD spectrum is an average of at least five scans)

REAGENT SETUP

Ultrapure water. Bi-distilled water is fed to the Milli-Q ultra-pure water system, which produces Milli-Q water with 18.2 MΩ × cm resistivity.

Porphyrin synthesis. Prepare porphyrin according to previously reported protocol[31]. In brief, charge a dried two-necked round bottom flask (2.5 L) with 4-*tert*-butylbenzaldehyde (0.37 mL, 2.19 mmol), methyl 4-formylbenzoate (0.33 g, 2.19 mmol), freshly distilled pyrrole (0.30 mL, 4.38 mmol) and $CHCl_3$ (1.0 L, containing EtOH as stabilizer), under argon. Add $BF_3 \cdot OEt_2$ (0.36 mL, 2.91 mmol) and let stirring for 1 h under dark, then add *p*-chloranil (0.40 g, 0.33 mmol) and let stirring for another 1 h.

Quench the reaction with Et_3N (0.28 mL, 3.81 mmol). Concentrate, under reduced pressure, the reaction mixture, then pass it over a silica plug (SiO_2, Ø = 6 cm, *l* = 7 cm, CHX/CH_2Cl_2, 1:1 v/v). The pure porphyrin is separated from the mixture by column chromatography (SiO_2, 40-63 µm, Ø = 10 cm, *l* = 22 cm, dry load from CH_2Cl_2, CHX and then CHX/AcOEt 95:5), followed by crystallization with CH_2Cl_2/pentane (294 mg, 16% yield). The porphyrin ester is converted into its acid by refluxing overnight a mixture of the above-mentioned porphyrin (0.10 g, 0.12 mmol) in CH_3CH_2OH (0.05 L) and 2M aq. KOH (0.05 L). After cooling down, the mixture is acidified with 2M aq. HCl and then concentrated under reduced pressure. The porphyrin product is extracted with $CHCl_3$, the organic phase is washed with 1M aq. $NaHCO_3$ and brine, dried (Na_2SO_4) and removed under reduced pressure. Purification by chromatography (SiO_2, 40-63 µm, Ø = 4 cm, *l* = 15 cm, CH_2Cl_2 followed by CH_2Cl_2/CH_3OH 90:10) affords pure product (86 mg, 88% yield).

EQUIPMENT SETUP.

Microwave synthesis. Use the power cycling control option to irradiate at a defined power and bring the reaction to a maximum temperature (power interval) and then cool the sample until the minimum temperature (cooling interval) is reached. Program the power cycling method inserting the maximum amount of microwave power to be applied, the maximum and minimum temperatures, the power and cooling intervals (microwave maximum amount of time allowed to reach the maximum and minimum temperature) and the number of cycles (power intervals). The automated IntelliVent moves into right position to seal the vessel. The system begins heating and cooling through the number of cycles programmed and once completed begins to cool the vessel to permit its removal from the instruments. The microwave power, maximum and minimum temperatures, power and cooling intervals, power intervals and consequent total time of the reaction is specified for each synthesis.

Filtration system. Filtration of the reaction mixture from microwave is performed on a 0.1 µm membrane mounted between a glass funnel (25 mm) and base with an aluminum clamp. The filtration device, with the silicon stopper, is fitted on top of a vacuum flask and vacuum is applied with a pump.

Dialysis system. Float-A-Lyzer dialysis membranes need to be pre-wet before use and the reader should refer to the instructions (Spectra/Por® Float-A-Lyzer G2 Ready-to-Use Dialysis Devices, spectrumlab.com). Briefly, unscrew the cap, fill the device with 10% ethanol in water, cap and immerge the device in the same alcohol solution (2.0 L) for 10 minutes. Replace the alcohol solution with only water by removing the device, unscrewing the cap, removing the alcohol solution inside, flushing and then filling the device with water, screw the cap and then place the device in only water (2.0 L) for 20 more minutes. Repeat the last step. Then, load slowly the sample solution inside the device, place the floating ring and finally leave the device to dialyze, under slow stirring.

Size exclusion chromatography. Sephadex LH-20, supplied as dry powder, has to be swollen prior to use. The reader is encouraged to follow the instructions from GE Healthcare (56-1190-97 AD). Briefly, 32.0 g of Sephadex is weighted in a beaker and suspended in 128.0 mL of methanol (approximate bed volume 3.9-4.1 mL g^{-1}) and let swell for at least 3 hours, without stirring (as it may break the beads). The slurry is re-suspended and poured into the column (Ø = 2.4 cm, *l* = 56 cm) with one continuous motion, with the aid of a glass rod held against the column wall (in order to prevent introducing air bubbles). The column is left to pack/equilibrate overnight, making sure that it does not run dry, until the medium bed is stable. The column outlet is then opened until the solvent reaches the media bed, then the sample is loaded as a solution in methanol (~2 mL). The elution is carried out by gravity at room temperature (the lower the flow rate, the better the resolution; lower flow rate is especially recommended during the initial stages) and can be followed by using a handheld UV lamp (365 nm). The various fractions are checked by collecting UV-Vis e FL spectra and then collected.

Electrochemistry. Electrochemical characterization is carried out in a typical three-electrode glass cell (10 mL), composed of glassy carbon (GC) working electrode (Ø = 3 mm, 66-EE047 Cypress Systems), a platinum wire as counter electrode and a silver wire as quasi-reference (QRE) electrode. The potential of the reference electrode, in the case of silver QRE, is calibrated after each set of measurements using ferrocene/ferrocenium (Fc/Fc^+) redox couple as the internal standard (~1 mM). The GC electrode is stored in ethanol, and is polished before use with a 0.05 µm diamond suspension (Metadi Supreme Diamond Suspension, Buehler) and ultrasonically rinsed with deionized water for 10 minutes and ethanol for 10 minutes. The electrode is electrochemically activated in the background solution by means of several voltammetric cycles at 0.5 V s^{-1} between the anodic and cathodic solvent/electrolyte discharges. In the meantime, NCNDs are dissolved in solution of dry DMF/$TBAPF_6$ (0.1 M) by aid of ultrasonication (or periodical heating) (concentration of *ca*. 1 mg mL^{-1}). The cyclic voltammetries (CVs) are performed at different scan rates, ranging from 0.05 to 0.1 V s^{-1}.

Atomic Force Microscopy. An aqueous solution of NCNDs (concentration of *ca*. 0.1 mg mL^{-1}) is drop-cast on a mica substrate and left to dry overnight (and covered to avoid deposition of dust). Acquire images using tapping mode and a HQ:NSC19/AlBs probe (65kHz; 0.5 N m^{-1}). Save the acquired AFM images and then analyse them in Gwyddion 2.50 (freely available on http://gwyddion.net). The statistical analysis is performed on *ca*. 100 nanoparticles and the average size is calculated from the size histogram with curve fit to the data using a Gaussian model.

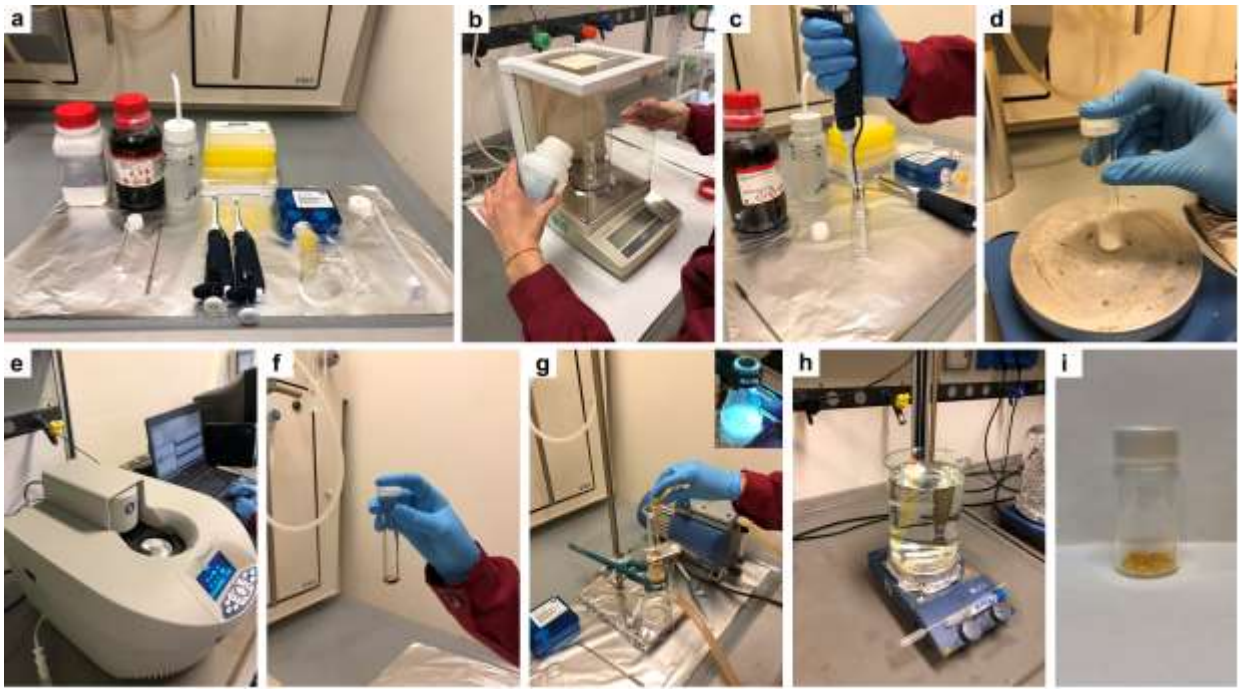

Figure 1. **Time-course illustration of key steps in the preparation of carbon nanodots**. A more detailed time-course is presented in Supplementary Figure 1. (**a**) Reagents and reaction setup for the synthesis of NCNDs. (**b**) Weigh the arginine powder in a microwave vessel. (**c**) Add liquids (milli-Q H_2O, followed by ethylenediamine). (**d**) Cap the reaction vessel and mix the components. (**e**) Microwave-assisted synthesis of NCNDs. (**f**) Reaction vessel after microwave irradiation. (**g**) Filter the crude reaction through a micro-filter (inset shows the filtered solution under 365 nm light). (**h**) Dialyze against pure water of the filtered solution. (**i**) Powder NCNDs after freeze-drying the dialyzed solution.

PROCEDURE

Part 1: Preparation of NCNDs ● TIMING 30 min

1. The first step is to prepare NCNDs by mixing Arg, EDA and H_2O in a microwave reaction vessel (**Figure 1a-g**).
    i. Weigh 87.0 mg of powdered Arg inside a microwave vessel (**Figure 1b**).
    ii. Add stir bar to the microwave vessel.
    iii. Add 100.0 µL of milli-Q H_2O with a micropipette (**Figure 1c**). ▲ **CRITICAL STEP** Water should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If water is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.
    iv. Add 33.0 µL of EDA with a micropipette. **! CAUTION** This step should be carried in a fumehood, while wearing gloves, googles and a lab coat. Dispose the tip in the appropriate waste container. ▲ **CRITICAL STEP** EDA should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If EDA is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.

v. Close the vessel using a microwave cap. **! CAUTION** Vessels and caps are designed for single use and should not be used more than once.
vi. Stir briefly (30 sec) on a magnetic stirrer (300 rpm) to mix the reagents (**Figure 1d**).
vii. Place the capped vial inside into the attenuator of the microwave reactor (**Figure 1e**) and use the following method – power cycling (12 cycles total-total time 180 seconds) of 200 W, 15 s power interval, 5 s cooling interval with 250 °C as max temperature and 240 °C as min temperature (Supplementary Figure 2).**? TROUBLESHOOTING**.
viii. Wet the filter membrane with milli-Q H_2O (using a washing bottle)
ix. Remove the cap from the vessel and add *ca*. 2 mL of milli-Q H_2O to the dark amber reaction mixture inside the microwave vessel (**Figure 1f**) and shake gently before filtering. Rinse the vial (*ca*. 2 mL) and filter again (**Figure 1g**). **! CAUTION** The cap should be removed from the vessel under a fumehood since gasses are formed during the reaction **■ PAUSE POINT** The obtained mixture can be stored at -20 °C before purification up to one week (the frozen solution can be most probably stored for longer times, but was not tested in our case).

Purification of NCNDs ● TIMING 1-4 d

2. Purification of NCNDs can be performed either by size exclusion chromatography or dialysis.

(A) Dialysis.

i. Transfer the filtrate (from the previous step) from the filtration flask to a graduated cylinder (or a 15 mL centrifuge or Falcon tube) and dilute with milli-Q H_2O up to the total volume of 10 mL. This solution is ready to be transferred to a dialysis tube. **▲ CRITICAL STEP** For effective dialysis purification, one dialysis tube can contain up to two filtered reaction mixtures from Part 1. The volume of the two filtrates combined should be 10 mL. Loading more material from more reaction mixtures in a single dialysis tube will result in ineffective purification.
ii. Before transferring, the dialysis device needs to be prepared (pre-wet). First prepare a 2.0 L of 10 % v/v EtOH in milli-Q H_2O solution inside a 2 L beaker. Transfer 10 mL of this solution inside a dialysis tube, close it and place it inside the 2 L beaker. Place a stir bar and stir (50-100 rpm) for 10 minutes. **? TROUBLESHOOTING**.
iii. Remove, uncap and flush the device with milli-Q H_2O and repeat step (*ii*) with only milli-Q H_2O.
iv. Remove, uncap and transfer the 10 mL of light-yellow filtrate solution to the emptied dialysis device and dialyze against pure milli-Q H_2O for 48 hours (**Figure 1h**).

v. Replace the milli-Q H_2O 5 times: next morning (after 12 hours), and then twice during the day (every 4-6 hours) and then twice the next day (every 4-6 hours).

vi. Transfer the solution from the dialysis tube and to a plastic Falcon tube (50 mL) and freeze it. ■ **PAUSE POINT** The tube can be capped and left at -20 °C for months without obvious changes in properties. Alternatively, freeze the solution by immerging the Falcon tube in liquid N_2. **! CAUTION** Liquid N_2 should be handled with insulating gloves, safety goggles and lab coat with long sleeves as it can cause severe frostbites (or eye damage) upon contact. Work in an open room since the release of N_2 can displace oxygen.

vii. Remove the plastic cap, cover the opening with parafilm tape and produce a series of small holes with a steel needle. Freeze-dry the solution for 24 hours. **? TROUBLESHOOTING**.

viii. Remove the sample from the freeze-drier, transfer (with the aid of a spatula) the yellow solid to a glass vial (previously weighted), then weigh the solid, pass a stream of Ar (or N_2), screw tightly the plastic cap on and cover it with parafilm (**Figure 1i**). **? TROUBLESHOOTING**. ■ **PAUSE POINT** The solid can be stored inside a desiccator (that block UV light) for at least 12 months without obvious changes in properties.

(B) Size exclusion chromatography

i. Transfer the filtrate from Part 1-Step 1(ix) from the filtration flask to a single-neck 50 mL round-bottom flask and remove water under reduced pressure. Recover the yellow oily residue with MeOH (*ca*. 2 mL). The solution is ready to be transferred to a SEC column.

ii. The column outlet is opened until the solvent reaches the media bed and the sample is loaded. The elution can be carried by gravity and can be followed by using a handheld UV lamp. The slower is the flow rate, the better is the resolution and slower flow rates is especially recommended during the initial stages. **? TROUBLESHOOTING**.

iii. The various fractions are checked by collecting UV-Vis and FL spectra and then grouped.

iv. Transfer the solutions to single-neck 50 mL round bottom flasks and remove MeOH under reduced pressure with the temperature of the water bath at 40 °C.

v. Take up the residues with H_2O and freeze-dry, as outlined in Step 2A, vi-viii.

Quality control steps ● TIMING 1-2 d

3. Before using the CNDs or before proceeding with post-functionalization reactions, it is important to ensure the quality of the prepared materials. Possible control tests include recording UV-Vis (A) and FL (B) spectra, Kaiser test (C) and AFM microscopy (D).

(A) UV-visible absorbance spectra ● TIMING 1-2 h

i. Record the UV-Visible absorption spectra (240-700 nm) for CNDs aqueous solution samples. The dialyzed CNDs should present a characteristic band at ~285 nm. Make sure to plot the mass absorption coefficient ((g^{-1} L) cm^{-1}): prepare at least 3 different samples at different concentrations of CNDs (recommended concentrations are between 1-10 g L^{-1}) and record the UV-Vis spectra. Then plot the absorbance (at 285 nm *vs*. concentration). Make sure that the mass absorption coefficient is ~3.7 (g^{-1} L) cm^{-1} (at 285 nm). **? TROUBLESHOOTING**.

(B) Fluorescence emission spectra ● TIMING 1-2 h

i. Record the emission spectra (310-650) for the CNDs aqueous solution samples, with increments of 10 nm in the excitation wavelength. For dialyzed CNDs, a broad emission centred at 356 nm is observed when the sample is excited at the optimal excitation wavelength (*i.e*. 300 nm). When the excitation wavelength changes from 300 to 420 nm, the emission intensity decreases and red-shifts from 356 to 474 nm.

ii. Determine the quantum yield (ϕ_f) of the CNDs according to previously published protocol[45]. Briefly, the relative fluorescence quantum yield relies on the comparison of the integral emission spectra between the CNDs sample and the standard (quinine sulphate), measured under identical conditions with the standard's absorbance matching that of the sample at the chosen excitation wavelength.

(C) Kaiser test ● TIMING 1-2 h

i. Run a Kaiser test according to a published procedure[46]. In a typical test, weigh 1.00 mg of CNDs. Add 75 µL of the kit solution of phenol (80% in EtOH) and 100 µL of the kit solution of KCN (in H_2O/pyridine) and sonicate the solution for 2 minutes. Subsequently, add 75 µL of the kit solution of ninhydrin (6% in EtOH), and heat the mixture at 120 °C for 10 minutes. Cool the solution to room temperature, dilute it at a known concentration with EtOH (60% in H_2O) to record the absorbance spectrum. A control solution is prepared in the same way, but without the CNDs, for background correction of the absorbance. Finally, calculate the amine loading (in µmol g^{-1}) by using the absorbance at 570 nm (ε = 15000 M^{-1} cm^{-1}) as an average of at least two different tests. **? TROUBLESHOOTING**.

(D) AFM microscopy ● TIMING 1 d

i. Prepare the substrate by cutting a small piece (~5 × 5 mm) of mica.

ii. Cleave mica by inserting a sharp edge into a corner of the mica sheet and gently separate the layers.

iii. Drop-cast an aqueous solution of CNDs (~0.1 mg mL^{-1}) and let the substrate to dry overnight.

iv. Use AFM to image the quasi-spherical shape of CNDs deposited on the mica substrate. MultiMode AFM standard operating procedures can be followed[47].

Part 2: Post-functionalization and purification of CNDs ● TIMING 2-6 d

4. According to specific needs and applications, the NCNDs can be modified by post-functionalization reactions. Herein we report two different reactions that exemplify the surface amine modifications of the NCNDs (A) carbodiimide coupling with molecules bearing carboxylic groups and (B) reductive methylation (Eschweiler-Clarke reaction)[48,49].

(A) Amidation reaction ● TIMING 1-2 d

i. Charge a 25 mL dry two-necked flask with 5,10,15-tri(4-*tert*-butylphenyl)-20-(4-carboxyphenyl)porphyrin (44.8 mg, 0.05 mmol), DMAP (12.2 mg, 0.10 mmol) and dry DMF (5.0 mL), under Ar. **! CAUTION** This reaction should be carried in a fume hood, while wearing gloves, googles and a lab coat.
ii. Cool the solution down to 0 °C using a water-ice bath and then add EDC·HCl (19.2 mg, 0.10 mmol)
iii. Let the mixture stir for 30 minutes and, during this time, prepare a NCNDs dispersion (25.0 mg) in dry DMF (5.0 mL) by sonication (and periodical heating), under Ar. **? TROUBLESHOOTING**.
iv. Add the NCNDs solution to the activated acid-EDC porphyrin solution by syringe, under Ar.
v. Leave the solution to warm-up gradually to r.t. and let it stir overnight, under Ar.
vi. Transfer the solution to a single-neck 50 mL round bottom flask and remove the DMF under reduced pressure (toluene can be added to accelerate the evaporation).
vii. Recover the residue with MeOH and purify the materials by using size-exclusion chromatography (as detailed in Step 2B). NCND-porphyrin covalent conjugate is obtained as purple solid after lyophilization (18.1 mg).

(B) Reductive alkylation reaction ● TIMING 2-4 d

viii. Prepare a formalin solution[50] (37% w/V, 3.0 mL, 0.01 mol) and transfer it to a 25 mL single-neck flask equipped with a reflux condenser. **! CAUTION** This step should be carried in a fume hood, while wearing gloves, googles and a lab coat.
ix. Add formic acid (4.0 mL, 106.0 mmol), followed by NCNDs as powder (80.0 mg) to the formalin solution, warm up and keep the reaction mixture at 101 °C for 48 hours.
x. Cool down the solution to room temperature and concentrate the mixture under reduced pressure. **! CAUTION** The excess formaldehyde should be removed with a rotary evaporator in a fume hood.

xi. The residues are taken up with H_2O and dialyzed against pure water as outlined in Step 2A. Methylated NCNDs (mNCNDs) are obtained as brownish solid after lyophilization (47.5 mg)

Quality control steps ● TIMING 1-2 d

5. CNDs that were post-functionalized are now characterized to confirm the successful outcome of the reactions. Control steps include recording UV-Vis (A) and fluorescence (B) spectra, Kaiser Test (C) and AFM microscopy (D), as outlined in Steps 3A-D.

Part 3: Preparation and purification of NCNDs derivatives ● TIMING 30 min-4 d

6. Various NCNDs derivatives can be prepared by following the procedure outlined in **Step 1** (Preparation of NCNDs), by varying the precursors. As an example, we outline how to tune (A) the electrochemical, (B) emission or (C) chiroptical properties of NCNDs. These properties can be modified by employing either quinone, a chromophore or chiral precursors in the reaction mixture. As a general procedure, first add the solids, followed by liquids to the reaction vial.

(A) Tuning the electrochemical properties of NCNDs. ● TIMING 30 min-4 d

i. Weigh 87.0 mg of powdered Arg inside a microwave vessel.
ii. Add 45.5 mg of powdered 2,3-dimethoxy-5-methyl-*p*-benzoquinone to the microwave vessel.
iii. Add stir bar to the reaction vessel.
iv. Add 100.0 µL of milli-Q H_2O with a micropipette (**Figure 1c**). **▲ CRITICAL STEP** Water should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If water is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.
v. Add 33.0 µL of EDA with a micropipette. **! CAUTION** This step should be carried out in a fumehood, while wearing gloves, googles and a lab coat. Dispose the micropipette tip in the appropriate waste container. **▲ CRITICAL STEP** EDA should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If EDA is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.
vi. Close the vial using a microwave cap. **! CAUTION** Vessels and caps are designed for single use and should not be used more than once.
vii. Stir briefly (30 sec) on a magnetic stirrer (300 rpm) to mix the reagents.
viii. Place the capped vial inside the attenuator of the microwave reactor and use the following method – power cycling (12 cycles total – total time 180

seconds) of 200 W, 15 s power interval, 5 s cooling interval with 250 °C as max temperature and 240 °C as min temperature (Supplementary Figure 3). **? TROUBLESHOOTING**.

ix. Wet the filter membrane with milli-Q H_2O (using a washing bottle)

x. Remove the cap from the vessel and add *ca*. 2 mL of milli-Q H_2O to the dark amber reaction mixture inside the microwave vessel and shake gently before filtering. Rinse the vial (*ca*. 2 mL) and filter again (twice or until all the material is collected). **! CAUTION** The cap should be removed from the vessel in a fumehood since gasses are formed during the reaction **■ PAUSE POINT** The obtained mixture can be stored at -20 °C for one week without obvious changes in properties. **TROUBLESHOOTING**.

xi. Dialyze against ultra-pure water as outlined in Step 2 (Purification of NCNDs) and finally lyophilize to obtain NCNDs derivative as powder material (63.4 mg).

(B) Tuning the emission properties of NCNDs. **● TIMING 30 min-4 d**

i. Weigh 20.0 mg of powdered Arg inside a microwave vessel.

ii. Add 43.0 mg of powdered Br_2NDA to the microwave vessel.

iii. Add stir bar to the reaction vessel.

iv. Add 260.0 µL of milli-Q H_2O with a micropipette. **▲ CRITICAL STEP** Water should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If water is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.

v. Add 4.0 µL of EDA with a micropipette. **! CAUTION** This step should be carried out in a fumehood, while wearing gloves, googles and a lab coat. Dispose the tip in the appropriate waste container. **▲ CRITICAL STEP** EDA should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If EDA is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.

vi. Close the vial using a microwave cap. **! CAUTION** Vessels and caps are designed for single use and should not be used more than once.

vii. Stir briefly (30 sec) on a magnetic stirrer (300 rpm) to mix the reagents.

viii. Place the capped vial inside into the attenuator of the microwave reactor and use the following method – power cycling (18 cycles – total time 200 seconds) of 300 W, 15 s power interval, 5 s cooling interval with 230 °C as max temperature and 220 °C as min temperature (Supplementary Figure 4).

ix. Wet the filter membrane with milli-Q H_2O (using a washing bottle)

x. Remove the cap from the vessel and add *ca*. 2 mL of milli-Q H_2O to the dark red reaction mixture inside the microwave vessel and shake gently before filtering. Rinse the vial (*ca*. 2 mL) and filter again. **! CAUTION** The cap should

be removed from the vessel under a fume hood since gasses are formed during the reaction.

xi. The pH of the filtrate solution is then adjusted to pH = 7.2 (by using a 0.2 M aqueous HCl solution) and filtered again. ■ **PAUSE POINT** The obtained mixture can be stored at -20 °C for one week without obvious changes in properties.

xii. Dialyze against ultra-pure water as outlined in Step 2A (Purification of NCNDs) and finally lyophilize to obtain NCNDs derivative as powder material (16.5 mg).

(C) Tuning the chiroptical properties of NCNDs. ● **TIMING 30 min-4 d**

i. Weigh the powdered 87.0 mg of powdered Arg inside a microwave vessel.

ii. Add 57.0 mg of powdered (*R,R*)- or (*S,S*)-CHDA the microwave vessel. ▲ **CRITICAL STEP** CHDA is a light-yellow solid that is hygroscopic. The weighting should be done quickly or under moisture-free conditions.

iii. Add stir bar to the reaction vessel.

iv. Add 100.0 µL of milli-Q H_2O with a micropipette (**Figure 1c**). ▲ **CRITICAL STEP** Water should be added to the bottom of the vial. Please avoid touching the walls of the vial with the pipette tip. If water is misplaced, the microwave heating will not result in the preparation of NCNDs with anticipated results.

v. Close the vial using a microwave cap. **! CAUTION** Vessels and caps are designed for single use and should not be used more than once.

vi. Stir briefly (30 sec) on a magnetic stirrer (300 rpm) to mix the reagents.

vii. Place the capped vial inside into the attenuator of the microwave reactor and use the following method – power cycling (12 cycles – total time 180 seconds) of 200 W, 15 s power interval, 5 s cooling interval with 250 °C as max temperature and 240 °C as min temperature (Supplementary Figure 5).

viii. Wet the filter membrane with milli-Q H_2O (using a washing bottle)

ix. Remove the cap from the vessel and add *ca*. 2 mL of milli-Q H_2O to the dark amber reaction mixture inside the microwave vessel and shake gently before filtering. Rinse the vial (*ca*. 2 mL) and filter again. **! CAUTION** The cap should be removed from the vessel under a fume hood since gasses are formed during the reaction ■ **PAUSE POINT** The obtained mixture can be stored at -20 °C for one week without obvious changes in properties.

x. Dialyze against ultra-pure water as outlined in Step 2A (Purification of NCNDs) and finally lyophilize to obtain NCNDs derivative as powder material (CNDs-*R*: 20.5 mg; CNDs-*S*: 22.0 mg).

Quality control steps. ● **TIMING 1-2 d**

7. As in the case of NCNDs, before proceeding with further experiments, it is important to ensure the quality of the materials. The control tests include, once again, recording UV-Vis (A) and fluorescence (B) spectra, Kaiser Test (C) and AFM microscopy (D). Additionally, the electrochemical properties of the CNDs can be probed with cyclic voltammetry (E) and the chiroptical properties are investigated by circular dichroism (F):

(E) Cyclic Voltammetry ● TIMING 1-4 h

i. Cyclic voltammetry measurements can be set-up and performed according to published procedures[51,52].
ii. Measure a cyclic voltammogram of a NCNDs solution (*ca*. 1 mg mL^{-1} NCNDs concentration, dissolved by heating and sonication cycles) in dry and degassed 0.1 M $TBAPF_6$ DMF solution, at 100 mV s^{-1}, with GC electrode (Ø = 3 mm), silver wire QRE electrode and platinum wire counter electrode.
iii. When using a QRE electrode, ferrocene/ferrocenium (Fc/Fc^+) redox couple is added as the internal standard (to obtain a concentration of *ca*. 1.0 mM) as the last measurement, at 100 mV s^{-1}.
iv. Finally, the energy levels (the HOMO and LUMO) of the CNDs can be calculated from the onset oxidation potential ($E_{onset,ox}$) and the onset reduction potential ($E_{onset,red}$), using published procedures[53,54].

(F) Circular Dichroism ● TIMING 30 min-2 h

i. Equipment preparation and data collection can be performed by following the relevant parts from previously published procedures[55,56].
ii. Prepare in a quartz cuvette (10 mm light path) a *ca*. 0.65 mg mL^{-1} solution of chiral CNDs in ultrapure H_2O.
iii. Record the CD spectra as an average of at least five scans, with a scanning rate of 50 nm min^{-1}, data pitch 0.2 nm, digital integration data 2 s.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Table 1. Troubleshooting table.

Step	Problem	Possible Reason	Possible Solution
1(vii)	Different reaction profile from Supplementary Figure 2	(i) Incorrect amount of reagents inside the vial or (ii) incorrect microwave heating	(i) Add reagents to the bottom of the microwave vial and be sure they are not misplaced on the walls (ii) Turn on the microwave at least 30 minutes before

			running the reaction and be sure that the compressed air source is open and well connected
2A(ii)	Dialysis is not performed properly	(i) The dialysis membrane was punctured with a glass pipette (ii) The dialysis membrane was not conditioned property	(i) Use plastic pipettes when transferring the solution from/to dialysis tubes (ii) Condition the dialysis membrane according to specification: soaking the membrane less or more than the specified time can result in an unsatisfactory dialysis
2A(vii)	The NCNDs are not a powder	(i) Presence of starting materials (ii) The lyophilization was not efficient	(i) Check and be sure that synthesis/purification have been done correctly (ii) Freeze-dry again for longer time and being sure that it was well freeze.
2A(viii)	The NCNDs become oil on weighting	The NCNDs are highly hygroscopic	(i) Weigh the sample quickly (ii) Weigh the sample under N_2 stream (iii) Add a small amount of water and freeze-dry again
2B(ii)	NCNDs fractions are not separated	(i) Too much sample was loaded (ii) Not enough bed volume or fast flow rates (iii) The column, left equilibrating overnight, run dry	(i) The sample volume should be around 1-2% of the total bed volume (ii) Prepare a higher column and use recommended flow rates of 1-10 cm h^{-1}, in accordance with specification of the medium (iii) Make sure to tightly seal the column with enough solvent and that the column is not leaking
3A(i)	The mass absorption coefficient is not ~3.7 (g^{-1} L) cm^{-1} (at 286 nm) or the absorption profile is different	(i) NCNDs are hygroscopic and the weigh is incorrect (ii) Incorrect synthesis, dialysis and/or lyophilization	(i) Quickly weigh the solid or under N_2 stream (water adsorption results in higher weights) (ii) Follow the step-by-step procedures taking into account the troubleshooting

			steps
4A(iii)	The solutions are suspensions	(i) NCNDs are not solubilized in DMF	(i) Heat the NCNDs suspension, it is stable at 120 °C, up to 30 min (ii) Few drops (*ca*. 4 drops) of MeOH can be added to improve the solubility of NCNDs
6A(viii)	The total reaction time is not 180 seconds	The total reaction time is affected by the reactivity of the employed quinone: if the power/cooling interval is insufficient to reach the minimum/maximum temperature the instrument will skip the cooling/power cycle and continue the next heating/cooling cycle until the minimum/maximum temperature is reached	Use the same quinone or complete the 12 cycles in less or more than 180 seconds, depending on the quinone employed
6A(x)	The reaction mixture is stuck on the walls of the microwave vessel	Stronger carbonization could be observed using quinones as starting materials	Adding water and sonicating (for at least 15 minutes) helps recovering most of the material form the vessel

● TIMING

Part 1: Preparation, purification and quality control of NCNDs 2-6 d

Preparation of NCNDs, 30 min

Purification of NCNDs, 1-4 d

Quality control steps, 1-2 d

Part 2: Post-functionalization and purification of NCNDs, 2-6 d

Amidation reaction, 1-2 d

Reductive alkylation reaction, 2-4 d

Quality control steps, 1-2 d

Part 3: Preparation and purification of NCNDs derivatives,

Tuning the electrochemical properties of NCNDs, 30 min-4 d

Characterization of NCNDs derivatives, 1-2 d

Tuning the emission properties of NCNDs, 30 min-4 d

Tuning the chiroptical properties of NCNDs, 30 min-4 d

Quality control steps, 1-2 d

ANTICIPATED RESULTS

Part 1: Preparation, purification and quality control of NCNDs.

The NCNDs prepared by step-by-step procedure in Steps 1-3 are obtained as a yellow solid. These carbon nanoparticles are highly soluble in water (up to 80 mg mL^{-1}). The aqueous solution appears yellow in daylight at a concentration of 0.5 mg mL^{-1} while emits blue luminescence upon excitation under a 365 nm UV lamp and have an absorption band at 286 nm, with the absorption onset starting from 388 nm (**Figure 2a**). They are fluorescent in the blue region of the UV-Vis spectrum (the fluorescence peaks shift from 356 nm to 474 nm when the excitation wavelength changes from 300 to 420 nm and the fluorescence intensity decreases as the peak red shifts) (**Figure 2b**) and their size is determined by AFM to be 2.47 nm with homogenous size distribution (± 0.84 nm) that obey to a gaussian distribution with full width at-half-maximum (fwhm) of 1.98) (**Figure 2c-e**) Furthermore, the surface of the NCNDs is rich of primary (and secondary) amino groups, which is determined by the Kaiser test (1350 μmol g^{-1}).

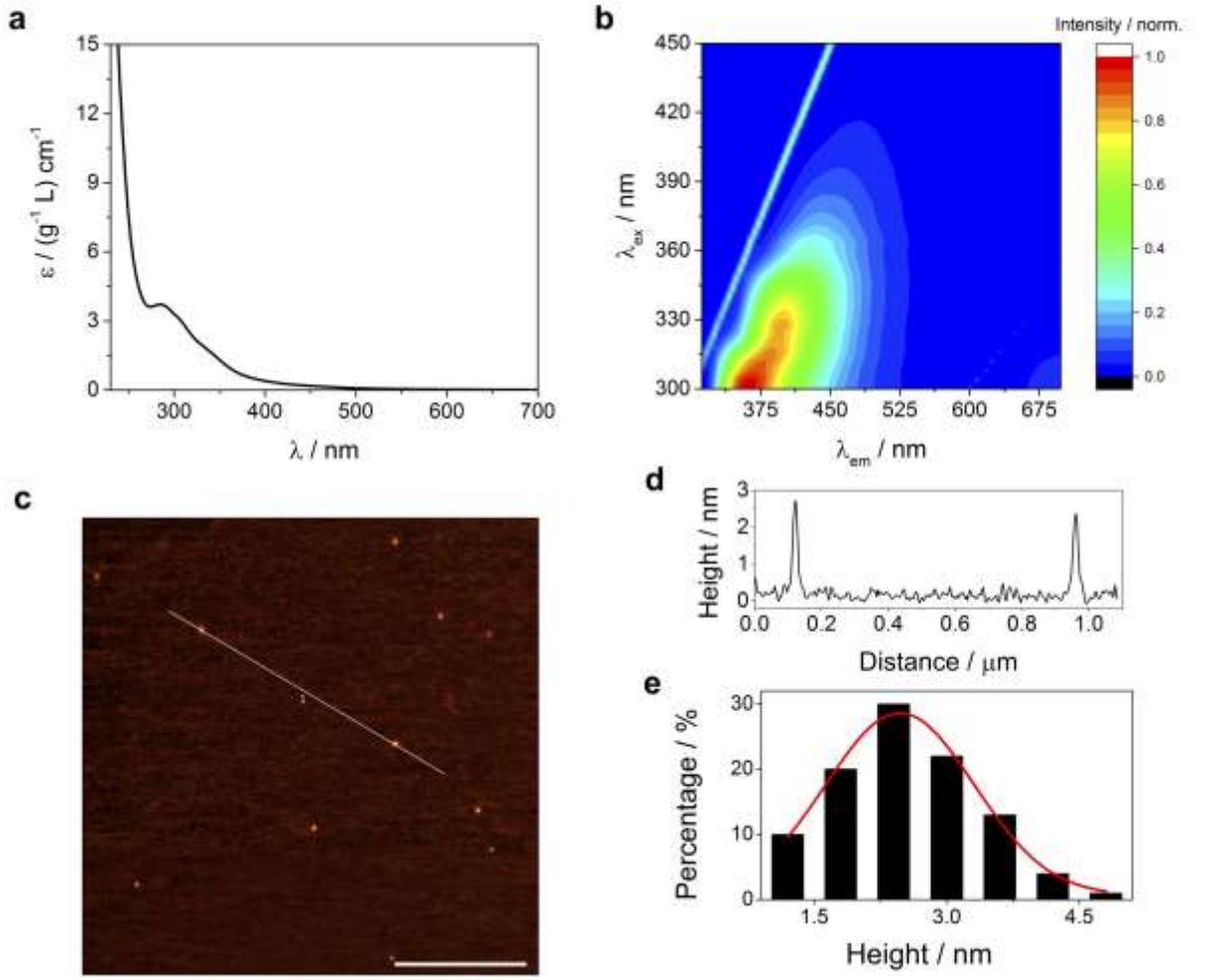

Figure 2. Characterization of NCNDs. (**a**) UV-Vis spectrum in water (298 K). (**b**) Fluorescence matrix scan in water (298 K). (**c**) Tapping mode AFM image (1.7 × 1.7 μm) from a drop-cast aqueous solution on a mica substrate (scale bar, 500 nm) . (**d**) Height profile. (**e**) Size histogram with curve fit to the data using a Gaussian model.

Part 2: Post-functionalization, purification and characterization of NCNDs.

The surface amino groups of the NCNDs are modified through chemical reactions and we use amidation and alkylation reactions as examples. In the first case, the amino groups from the NCNDs and carboxylic acids from the porphyrin dye are combined through a carbodiimide condensation reaction. In the second case, the primary amino groups are converted in tertiary ones through reductive alkylation. In both cases, the post-functionalized NCNDs are purified and, after lyophilization, obtained as solids.

The covalent attachment of the porphyrin is confirmed by recording UV-Vis and fluorescence spectra of the hybrid material – the typical Soret and Q bands of the porphyrin are visible in absorption[26], while electronic interaction is confirmed in emission[31]. The number of amino groups on the surface is decreased when compared

to the pre-functionalized NCNDs, while AFM shows the size of the hybrid to be around 5 nm[31].

The reaction to afford NCNDs with tertiary amino groups on the surface can be monitored with the Kaiser test (26 μmol g^{-1} after completing the reaction)[30]. The changes observed in their photophysical properties are consistent with the NCNDs surface modification. While the UV-Vis absorption and FL emission spectra did not show significant differences compared to the one of NCNDs, the CV of mNCNDs exhibits a new peak at +0.80 V vs SCE that can be attributed to the presence of tertiary amines.

Part 3: Preparation, purification and characterization of NCNDs derivatives.

The electrochemical, emission and chiral properties of NCNDs are tailored by following the procedures described in Steps 6-7. Tuning these properties includes using the multi-component approach and hydrothermal microwave-assisted synthesis already described in Step 1. Modifying the electrochemistry is accomplished by adding quinones to the Arg and EDA starting materials in the synthesis[29], while emission is tuned by adding core-substituted naphthalene dianhydrides as chromophore along Arg and EDA[28], and finally chirality is conferred by employing chiral cyclohexanediamine instead of EDA[27].

The NCNDs derivatives are purified and finally obtained as solids. In addition to standard characterization techniques, supplemental quality control steps are performed based on the tailored property. For example, addition of quinones to the synthesis results in NCNDs that are more easily to reduce, as evidenced by the cyclic voltammograms (**Figure 3a**), and a higher absorptivity and a red-shifted absorption onset (**Figure 3b**). Tuning the emission properties of NCNDs by employing core-substituted naphthalene dianhydrides results in a red-shifted emission spectrum (**Figure 3c**). Finally, preparing chiral NCNDs is evidenced by recording circular dichroism spectra (**Figure 3d**).

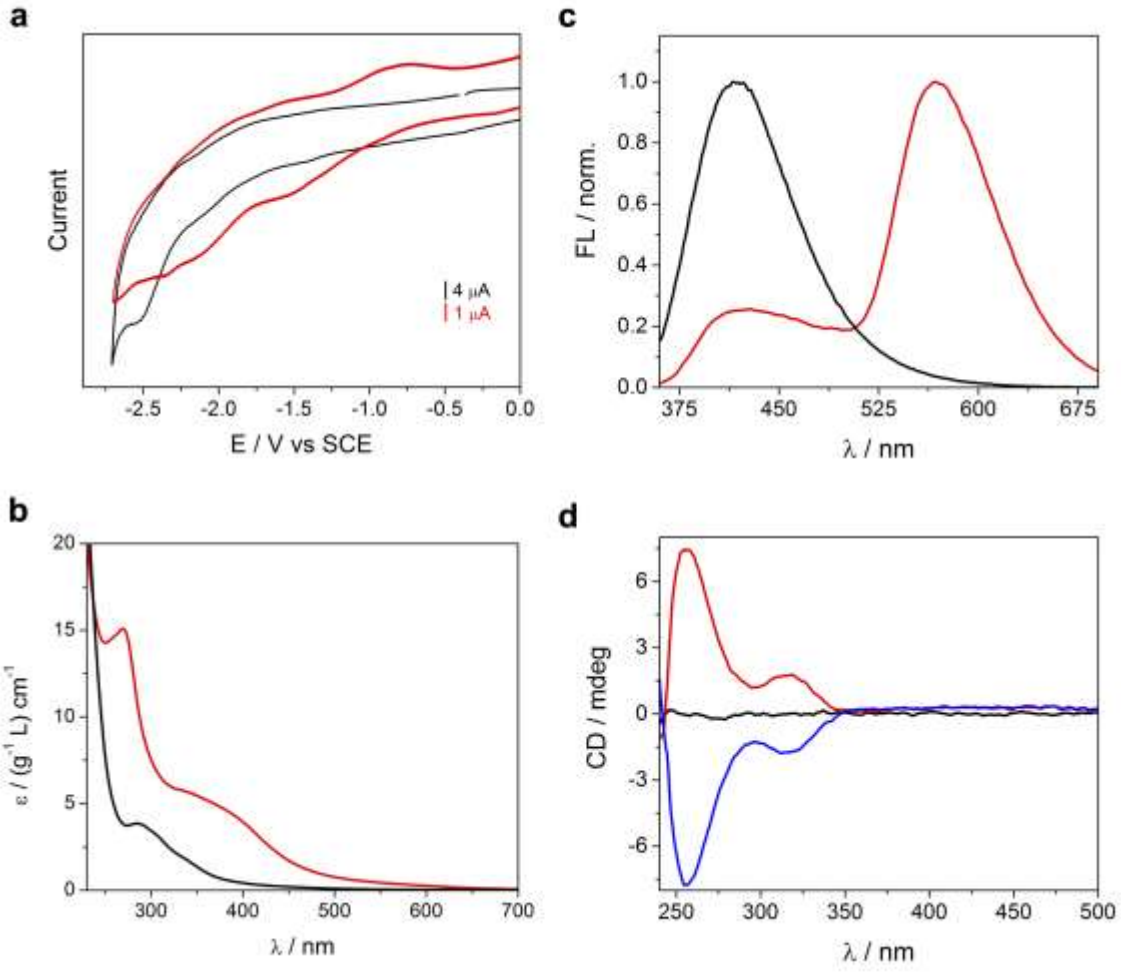

Figure 3. Characterization of NCNDs derivatives. (**a**) Cathodic CVs (in 0.1M $TBAPF_6$ DMF solution, GC electrode, scan rate = 100 mV s^{-1}, Pt wire used as counter electrode and the potential is referenced to SCE, at room temperature) and (**b**) UV-Vis spectra in water (298K) of NCNDs from Part 1 (black line) and CNDs prepared from 2,3-dimethoxy-5-methyl-*p*-benzoquinone (red line) from Part 3A. (**c**) Normalized emission spectra (350 nm as excitation wavelength) of NCNDs from Part 1 (black line) and CNDs and CNDs prepared from Br_2NDA (red line) from Part 3B. (**d**) ECD spectra of NCNDs from Part 1 (black line) and CNDs-*S* and CNDs-*R* (red and blue line, respectively).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary.

Data availability

The main data supporting the findings of this study are available within the article and its Supplementary Information file. Additional data are available from the corresponding authors upon request.

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Acknowledgments

This work was supported by the University of Trieste, INSTM, AXA Research Fund, the Spanish Ministry of Economy and Competitiveness MINECO (project CTQ2016-76721-R), Diputación Foral de Gipuzkoa program Red (101/16) and ELKARTEK bmG2017 (Ref: Elkartek KK-2017/00008, BOPV resolution: 8 Feb 2018).

Author Contributions

L.Đ. and F.A. designed, performed the experiments and wrote the manuscript. M.P. planned the research, co-wrote the manuscript and secured the funding.

Competing Interests

The authors declare no competing interests.

Additional Information

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