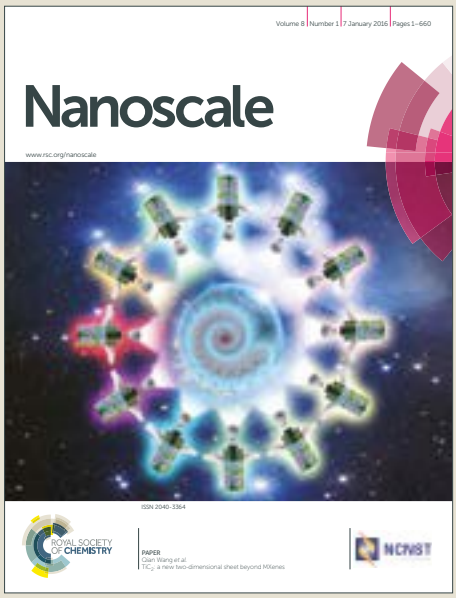
Nanoscale

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: M. Ortega-Muñoz, P. Vargas-Navarro, F. Hernandez-Mateo, A. Salinas-Castillo, L. F. Capitan-Vallvey, S. Pleselova, R. Salto, M. D. Giron, F. J. Lopez-Jaramillo and F. Santoyo-Gonzalez, *Nanoscale*, 2019, DOI: 10.1039/C8NR09459D.



ROYAL SOCIETY OF CHEMISTRY

rsc.li/nanoscale

View Article Online
DOI: 10.1039/C8NR09459D

Nanoscale

ARTICLE

Acid anhydride coated carbon nanodots: Activated platforms for engineering clicked (bio)nanoconstructs†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Mariano Ortega-Muñoz,[a] Paula Vargas-Navarro,[a] Fernando Hernández-Mateo,[a] Alfonso Salinas-Castillo,[b] Luis Fermin Capitan-Vallvey,[b] Simona Plesselova,[c] Rafael Salto-Gonzalez,[c] M. Dolores Giron-Gonzalez,[c] Francisco Javier Lopez-Jaramillo*[a] and Francisco Santoyo-Gonzalez*[a]

Activated carbon nanodots functionalized with acid anhydride groups (AA-CNDs) are prepared by the one-pot water-free green thermolysis of citric acid. As a proof of concept of their capabilities as appealing and versatile platforms for accessing engineering nanoconstructs, the as-prepared AA-CNDs have been reacted to yield clickable CNDs. Their click bioconjugation with relevant recognizable complementary clickable sugars has led to multivalent CNDs-based glyconanoparticles that are non-toxic and biorecognizable. The accessibility and intrinsic reactivity of AA-CNDs expand the current toolbox of covalent surface grafting methodologies and provide a wide range of potential applications for engineering (bio)nanoconstructs.

1. Introduction

Carbon nanodots (CNDs) are a leading-edge nanocarbon material that attract tremendous attention ever since their inception.[1,2] The facile preparation of these fluorescent quasi-spherical carbonaceous nanoparticles with size below 10 nm from a wide spectrum of cheap carbon sources and their unique optoelectronic/chemical properties, high-water solubility, excellent aqueous stability, outstanding biocompatibility and low toxicity are fine properties that make of CNDs promising candidates for numerous exciting (bio)applications.[1-4]

A crucial issue and valuable tool for the applicability and developing of high-performance CNDs-based materials is surface engineering aimed to surface passivation and surface (bio)functionalization of CNDs.[5-8] Surface passivation by a careful design of a coating layer of the carbogenic core of CNDs allows a controllable tuning of their fundamental physicochemical properties rendering them also stable and transportable in varying environments.[9] Furthermore, exploitation of the outward-facing endogenous functional groups of CNDs or those introduced by post-synthetic treatments or by one-step simultaneous passivation and functionalization procedures is pivotal for the construction of advanced fluorescent hybrid nanoarchitectures by conjugation with (bio)molecules.[9] Covalent-based methodologies dominate the decoration of CNDs where the incoming grafted (bio)molecules usually exploit carboxyl, carbonyl, hydroxyl and amine groups present on their surface as anchoring handles through robust and well-established post-synthetic methodologies, normally acylation reactions that requires activation strategies of such functional groups. However, despite the progress attained, new contributions aimed to expand the current panoply of surface reactive-group coatings of CNDs and facilitate their surface engineering are desirable.

Among the variety of techniques and carbon sources used for the fabrication of CNDs, bottom-up approaches based on citric acid (CA) are extremely popular because it is a cost-effective and time-saving strategy that uses an abundant and small molecular precursor as well as simple, low-cost, green, and efficient protocols.[10]One-step CA-based carbonization strategies use diverse combinations of passivation agents and processing schemes.[11] Partial or complete co-pyrolysis of CA, as the sacrificial carbon component, and amine-containing compounds, as surface modifiers, is a standard procedure where thermal condensation reactions and formation of amide linkages take place. According to literature, the use of water prior to the isolation of CNDs is common either during the thermal treatment (hydrothermal procedures) or in the processing which leads to water soluble carboxylate capped CNDs. However, direct utilization of as-prepared CNDs from CA in the absence of water and non-hydrolytic post-processing treatments is still unexplored. Previous investigations on the thermal decomposition of CA have evidenced the formation of diverse acid anhydride compounds.[12-14] Moreover, different studies related with the cross-linking of cotton with polycarboxylic acids have demonstrated the formation and reactivity of cyclic acid anhydride intermediates.[15-17] With this background, we hypothesized that water-free thermolysis of

[a] Department of Organic Chemistry, Biotechnology Institute, Faculty of Sciences, Campus Fuentenueva sn, University of Granada, 18071-Granada, Spain E-mail: fjljara@ugr.es and fsantoyo@ugr.es;
[b] Department of Analytical Chemistry, Faculty of Sciences, Campus Fuentenueva s/n, University of Granada, 18071-Granada, Spain
[c] Department of Biochemistry and Molecular Biology II, School of Pharmacy, Campus Cartuja 18071-Granada, University of Granada, Spain

†Electronic Supplementary Information (ESI) available: Experimental procedures, Figures S1-S9 and Tables S1-S5. See DOI: 10.1039/x0xx00000x

pure CA will yield the thermal dehydration of the acid carboxylic groups to render acid anhydride coated CNDs that will behave as activated platforms for further covalent functionalization and decoration.

Herein, we report a facile and high-output green procedure for the preparation of acid anhydride coated CNDs (AA-CNDs) through the bottom-up one-pot pyrolysis of CA under mild conditions. More important, taking advantage of the intrinsic activation of acid anhydrides we expand the current toolbox of covalent grafting methodologies for surface engineering of CNDs. We demonstrate that AA-CNDs are versatile platforms for accessing functional nanoconstructs. In particular, we exploit their reactivity to functionalize the surface of CNDs with clickable groups (alkyne, azide or vinyl sulfones) and perform ulterior click assembly of the prepared clickable CNDs with diverse complementary (bio)molecular components in a strategy that benefits of the prominent features of click chemistry.

2. Experimental

2.1. Chemical and Materials

Anhydrous citric acid (CA), 3-azido-1-propanamine, propargylamine, and dialysis membranes (14000 Da cutoff) were obtained from Sigma-Aldrich (St Louis, MO, USA). Commercial reagents were used as received without further purification. Clickable sugars were prepared following reported procedures in the literature: 2-propyn-1-yl-α-D-mannose,[18] 2-propyn-1-yl-α-D-galactose,[19] 2-azidoethyl α-D-mannopyranoside,[20] 2-propyn-1-yl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside,[21] and 2-azidoethyl-4-O-β-D-galactopyranosyl-β-D-glucopyranoside.[22]

2.2. Cell culture

CHO-k1 (ATCC CCL-61), Hela (ATCC CCL-2), RAW 264.7 (ATCC TIB-71) and CT26.WT (ATCC CRL-2638) cells were provided from the Cell Culture facility of the University of Granada. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine plus 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cell were seeded at 1.5 x 10^4 cells/well density in 48 well plates and incubated at 37 ºC for 24 h to reach a cell confluence of 80–90%.

2.3. Instrumentation

Optical rotations were recorded on a polarimeter (PerkinElmer Instruments Model 341) at room temperature. Fourier transform infrared (FTIR) spectra were obtained on a PerkinElmer FT-IR spectrometer Spectrum Two. The measurements were collected at room temperature. Samples were prepared by depositing powdered CNDs. Electron microcopy analysis was carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, with a high-resolution transmission electron microscopy (HRTEM) FEI TITAN G2 operating at 300 kV. The samples were prepared at room temperature in air by the deposition of a drop of aqueous solution of CNDs on a commercial 400 μm mesh carbon Cu-grid. X-ray photoelectron spectroscopy (XPS) analyses were carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, with a Kratos Axis Ultra-DLD Spectrometer using monochromatic Alkα radiation. X-ray powder diffraction (XRD) patterns were measured at Centro de Instrumentacion Cientifica, Universidad de Granada, using Cukα radiation with a BRUKER AXS D8 ADVANCE equipped with a LINXWYE detector. Elemental analysis (CNH) was carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, with a THERMO SCIENTIFIC FLASH 2000 series Analyzer. UV/Vis absorption spectra were obtained using a Specord 200 Plus (Analytik Jena). Fluorescence spectra were collected using a Varian Cary Eclipse luminescence spectrometer. Confocal microscopy was carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, using a Leica TCS-SP5 II multiphoton confocal microscope.

2.4. Thermal pyrolysis of CA and optimized synthesis of AA-CNDs.

To obtain information about thermal decomposition path of CA, the dehydration process and formation of the anhydride acid coated CNDs respecting to the heating duration, CA (10 g) was placed into an opened round flask (100 mL) and then introduced in an oil bath heated at 180 ºC. Samples were collected at different times directly from the flask over a range of 0-48 h without interruption of the heating and analyzed without further processing by Fourier transform infrared spectroscopy (FTIR). Spectra of the samples collected at different time of pyrolysis are depicted in Fig S1 and S2. CA melted within 5 m. Subsequently, the color of the liquid was changed from colorless to pale yellow, and then orange when t = 0.5 h, indicating the initial formation of CNDs. As the heating proceeded the orange liquid turned brown (within 1 h) to became a black solid after 24 h, suggesting carbonization. For the synthesis of AA-CNDs, CA (10 g) was heated at 180 ºC for 2 h to yield a brown residue of AA-CNDs (2.5 gr). The as-prepared CNDs were used without further purification.

2.5. Preparation of AA-CNDs(Na)

As-prepared AA-CNDs (0.5 g) were dissolved in aqueous NaOH (1 M, 20 mL) and kept at room temperature for 2 h. Then the solution dialyzed (MWCO = 14000 Da) against deionized water (12 h). Finally, the AA-CNDs(Na) were filtrated (pore size 0.22 μm) and lyophilized.

2.6. Synthesis of clickable CNDs (Az-CNDs and Ak-CNDs)

A solution of the as-prepared AA-CNDs (0.5 g) and the clickable amines (8.5 mmol) 3-azido-1-propanamine (0.98 g), or propargylamine (0.55 mL) in anhydrous MeOH (5 mL) was magnetically stirred overnight (16 h). Unreacted reactants were removed by dialysis (MWCO = 14000 Da) against i) deionized water (1 h), ii) 5% HCl (3 h), deionized water (1 h), iii) 0.5 M NaOH (3 h), and iv) deionized water (12 h). Then the dialyzed clickable CNDs (Az-CNDs and Ak-CNDs) were filtered (pore size 0.22 μm) and lyophilized to yield a yellow powder.

2.7. Synthesis of clickable vinyl sulfone CNDs (VS-CNDs)

A solution of the as-prepared AA-CNDs (0.5 g) and N,N'-dimethylethylenediamine (750 mg, 8.5 mmol) in anhydrous

View Article Online
DOI: 10.1039/C8NR09459D

MeOH (5 mL) was magnetically stirred overnight (16 h). Unreacted reactants and solvent were removed under vacuum. The crude was dissolved in anhydrous MeOH (5 mL) and reacted with a solution of divinylsulfone (3 g, 25.5 mmol) and triethylamine (0.859 g, 8.5 mmol) in anhydrous MeOH (5 mL). The mixture was magnetically stirred overnight (16 h). Unreacted reactants were removed by dialysis (MWCO = 14000 Da) against i) deionized water (1 h), ii) 5% HCl (3 h), deionized water (1 h), iii) 0.5 M NaOH (3 h), and iv) deionized water (12 h). The dialyzed VS-CNDs were filtered (pore size 0.22 μm) and then lyophilized to yield a yellow powder.

2.8. Synthesis of clicked glycosylated CNDs (Glyco-CNDs)

Method A: A solution of the clickable CNDs (Ak-CNDs orAz-CNDs) (0.2 g) in DMF (5 mL) and the corresponding complementary clickable alkyne (1-3) or azide sugar (4-6) (0.4 mmol) was irradiated in presence of $(EtO)_3P{\cdot}CuI$[23] (12 mg) for 15 min at 300 W and 80 °C in a Milestone Star Microwave Labstation. Unreacted reactants were removed by dialysis (MWCO = 14000 Da) against deionized water (12 h). The dialyzed clicked glycosylated CNDs (Glyco-CNDs) were filtered (pore size 0.22 μm) and lyophilized.

Method B: A solution of the clickable VS-CNDs (0.2 g.) in DMF (5 mL) was reacted with the thiolated GlcNAc 7 (0.4 mM) at room temperature for 16 h. Unreacted reactants were removed by by dialysis (MWCO = 14000 Da) against deionized water (12 h). The dialyzed clicked glycosylated CNDs (Glyco-CNDs) were filtered (pore size 0.22μm) and lyophilized.

2.9. Study of the interaction between glyco-CNDs and lectins

ELLA analysis was carried out on ELISA plates coated with Man, Lac or GlcNAc. Wells were coated with Man or Lac by incubation either commercial mannan or lactose functionalized polymer[24] in 100 mM carbonate buffer pH 9.6 (5 μg/mL, 200 μL, 5 μg/mL) at 4 ºC overnight. Coating with Glc-NAc was carried out by reaction of vinyl sulfone functionalized Glc-NAc with commercial amino functionalized plates.[25] Once coated, wells were washed with 300 μL/well of PBST buffer (3 x 3 min) to remove the excess of saccharide and further incubated with PBSTA (PBST supplemented with 0.1% (w/v) BSA) at room temperature for 30 min to minimize the lectin-well inespecific interaction. Then, wells were incubated with 200 μL of a solution of HRP coupled lectin (0.25 μg/mL ConA supplemented with 1 mM $CaCl_2/MnCl_2$, 0.2 μg/mL PNA, 40 ng/mL WGA) in PBST buffer at 37 ºC for 1 h. The unbound lectin was washed with PBST (3 x 300 μL/well for 3 min) and then wells were incubated with serial dilutions of methyl-α-D-mannopyranoside (MADM) (ConA), Lac (PNA), GlcNAc (WGA) or the corresponding glyco-CNDs at 37 ºC for 1 h. Wells were washed PBST (3 x 300 μL/well for 3 min) and revealed by incubation with 200 μL/well of 100 mM Na_2HPO_4, 50 mM citrate pH 5, 0.04% (w/v) o-phenylenediaminedihydrochloride, 0.05% (v/v) H_2O_2 at 37 ºC for 30-45 min. The reaction was stopped by addition of 1.5 M H_2SO_4 (100 ul/well) and the resulting optical density was measured with a Sunrise absorbance reader (Tecan). The IC_{50} values were estimated from the plot of percentage of displacement of the HRP-lectin bound to the coated well versus the concentration of glyco-CNDs.

2.10. Cytotoxicity assay

Cytotoxicity of the CNDs derivatives was evaluated in CHO-K1, CT26.WT and Hela cells after 24 h of incubation with 0, 50, 100 and 250 μg/mL of different CNDs. Percentage of cell viability (with respect to unexposed cells) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method which correlates the cellular metabolic activity with the number of viable cells in culture. Results are reported as % viability based on the untreated control cells normalized to 100% viable.

2.11. Hemolytic assay

Human red blood cells (RBCs) concentrated (O+) were provided by the Clinical Analysis School of Granada University (Spain). RBCs were washed with PBS and pelleted by centrifugation (10000 xg, 10 min, 4°C) 3 times. The final pellet was diluted 1:10 (v/v) in PBS and used in the hemolytic assays. For the assay, 100 μL of RBCs were incubated at room temperature for 1, 3, 6 and 24 hours with different solutions CDNs to a final concentration of 250 and 500 μg/mL. After the incubation, cells were centrifuged (10000 xg, 10 min, 4°C) and the supernatants were collected. The hemolysis was evaluated by the absorbance of the supernatants at 540 nm, using the values of the cells maintained in deionized water and in PBS to set the 100 and 0 value.

2.12. Cellular uptake of CNDs derivatives.

Cells were seeded in 24-well plates at a density of $4.5x10^5$ cells/well and 24 h later cells were incubated with the different CNDs at a final concentration of 250μg/mL for 6 h. Cells were washed three times with PBS and lysed with 200 μL of 0.5% Triton X-100 in PBS. Fluorescence was measured in a JASCO FP−8300 spectrofluorometer with 2.5 nm band windows at 326 nm excitation and 454 nm emission wavelengths. Fluorescence was normalized to the protein concentration and the background fluorescence of non-incubated cells was subtracted. Bio-Rad Protein Assay was used to estimate the protein concentration.

2.13. Confocal Microscopy.

Hela, RAW 264.7 and CT26.WT cells were seeded onto coverslips in 12-well plates at a density of $9x10^5$ cells/well and incubated at 37 ºC to reach a 80% confluence. Then, cells were incubated with different CNDs at a final concentration of 250 μg/mL for 6 h. Afterwards, cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and confocal microscopy was performed on a Leica TCS-SP5 II multiphoton confocal microscope. Excitation laser lanes and non-overlapping detection channels were selected for the detection of no fluorescent signal from control cells (i.e. incubated with buffer). A pinhole of 1 Airy unit was used. Images were acquired at a 1024 x 1024 pixel resolution. Series were acquired in the xyz mode. Data were processed using Leica AF software package.

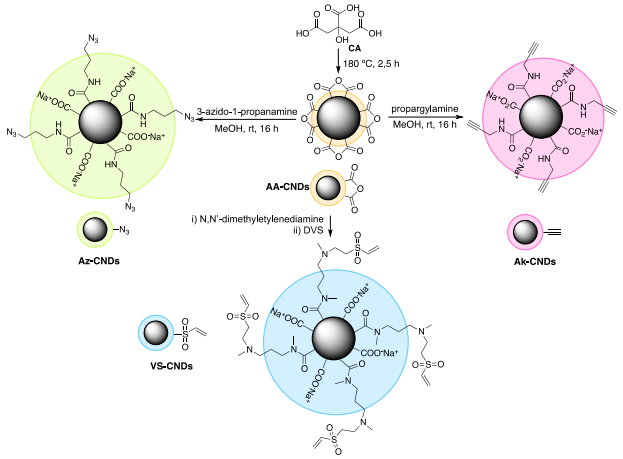
2.14. Statistical Analysis.

Results are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's test as appropriate. $P < 0.05$ was considered statistically significant.

Results and discussion

3.1. Synthesis and characterization of AA-CNDs

The surface modification confers to CNDs better properties and expands the fields of application. Covalent modifications, dominated by amide coupling reactions due to the predominant presence of carboxyl and amino surface groups, require multi-step synthetic schemes and activation protocols that may yield side products.[26] We hypothesized that water-free thermolysis of pure CA will yield the thermal dehydration of the acid carboxylic groups to render **AA-CNDs** that will behave as activated platforms for further covalent functionalization and decoration. To validate our hypothesis, we designed an exploratory assay based on reported reactions conditions for the pyrolysis of CA using 180 ºC under air-open conditions.[27] Samples at different reaction time points were collected and analyzed by FTIR without any processing to evaluate the reaction progress respecting to the time of thermolysis (Fig. 1 and Fig. S1, ESI†). It was observed that the typical O-H stretching signals of CA (3500 and 3292 cm^{-1}) broadened and diminished until vanishing after 48 h. In parallel, the C=O stretching peaks of carboxylic groups (1744 and 1699 cm^{-1}) transformed initially into a single signal at 1708 cm^{-1}that evolves to an increasing overtime double signal at 1846 and 1764 cm^{-1}. These vibration signals are the characteristic spectral signature of the symmetric and asymmetric C=O stretching of acid anhydrides and their presence supports our hypothesis that pyrolysis of CA yields carbonization with concomitant transformation of the carboxylic groups into acid anhydrides. Considering that our results revealed that the anhydride signature weakened at long times of pyrolysis (Fig. S2,ESI†) and that it has been reported that fluorophore molecules formed at the first stages of the carbonization of CA are also consumed with the time,[28] we focused on the material obtained after 2 h of heating that were designated as **AA-CNDs** (Scheme 1).



Scheme 1. Preparation of AA-CNDs and their transformation in clickable CNDs

The survey of the X-ray photoelectron spectrum (XPS) confirmed that the surface of **AA-CNDs** was composed of C and O elements (Fig. S3 and Table S1, ESI†). The fine structure spectrum of C_{1s}showed peaks corresponding to graphitic (284.5 eV) and oxygenated carbon atoms (288.2 eV)[29] while the O_{1s}spectrum exhibited a broad peak (530-534 eV) that envelops the C=O and C-O signals.[29]

The morphology and size of the **AA-CNDs** particles were investigated (Fig. 1). Despite the fact that the standard aqueous processing of CNDs (hydrolysis with aqueous NaOH, dialysis and lyophilization) is non-compatible with the anhydride functionalization of **AA-CNDs**, we applied this processing for comparative purposes and obtained named **AA-CND(Na)**. X-ray Powder Diffraction (XRD) analysis showed a broad band between 2θ= 20º – 50º (data not shown) which suggests a small particle size and an amorphous nature for the **AA-CND(Na)**.[30] However, high-resolution transmission electron microscopy (HR-TEM) revealed spherical particles with an average diameter of 3.2±0.7 nm and a diffraction pattern that supports the crystalline nature of **AA-CNDs(Na)**with an interplanar spacing of 0.314 nm in the range of the interplanar spacing of graphite (Fig. 1).

The photoluminescence spectrum of processed **AA-CND(Na)** showed an excitation and emission peak at 326 and 454 nm, respectively (Fig. S4, ESI†) the fluorescence quantum yield (FLQY) being estimated as 4%. This value is higher than that reported for CNDs obtained from pyrolysis of CA at 180º C for 40 h[27] and within the range of those for CNDs obtained at shorter pyrolysis times.[31]

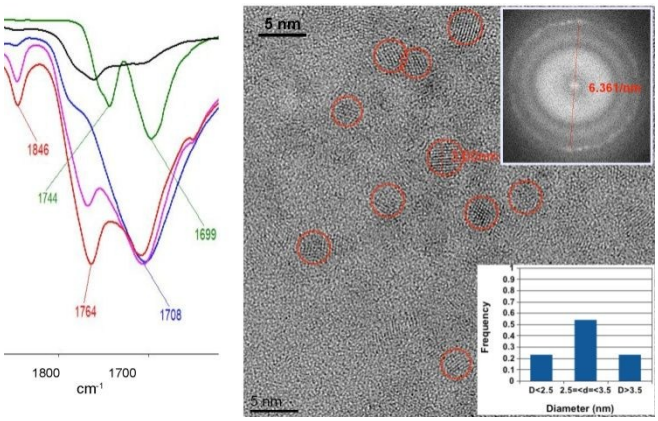


Fig 1. Left: detail of the 1650-1850 cm^{-1} region of the FTIR spectra of the CA (green) and the resulting products from the thermolysis at 180 ºC for 20 min (blue),1 h (magenta), 2 h (red) and 48 h (black). Right: HRTEM of the AA-CNDs(Na) showing the estimation of the interplanar spacing and the particle size distribution (inserts)

3.2. Synthesis and characterization of clickable CNDs

After proving successful preparation and characterization of **AA-CNDs**, their reactivity was exploited to transform them into clickable CNDs as a highly valuable proof of concept that demonstrates the synthetic potential of the acid anhydride surface functionalization. Carbon nanomaterials have been

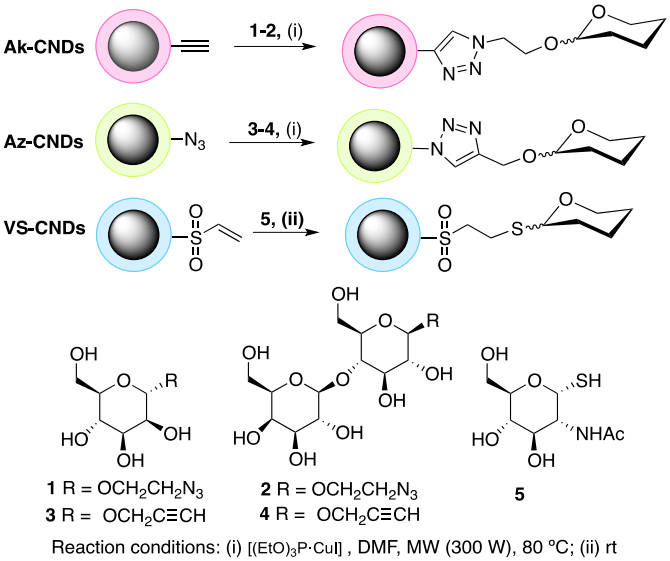
benefited, as a wide variety of diverse materials, by the outstanding characteristic of click-chemistry for surface functionalization without adversely altering their chemical structure and electronic properties. Click chemistry-mediated functionalization has been applied to satisfy special needs on graphene, graphene oxide and carbon nanotubes and limited to the widespread prevalent use of the CuAAC.[32] However, preparation and use of clickable CNDs are scarce.[33,34] The as-prepared **AA-CNDs** were treated at room temperature with clickable amines, namely, 3-azido-1-propanamine and propargylamine, without any coupling agent (Scheme 1). This simple procedure allows the covalent grafting of clickable handles thought the formation of amide bonds. After standard aqueous processing to remove unreacted reactants, the corresponding azide and alkynylated CNDs, designated as **Az-CNDs** and **Ak-CNDs**, were obtained in excellent yields.

Successful click functionalization of CNDs was confirmed by XPS (Fig. S3, ESI†). The high resolution N_{1s} spectra show new signals absent in **AA-CNDs** that clearly reveal the presence of different nitrogen atoms at the surface: a single signal at 398 eV in the case of **Ak-CNDs** and two more signals at 399 and 403 eV for **Az-CNDs**, the latter being assigned to the electron-poor azide nitrogen.[35] Moreover, the O_{1s} spectra also revealed that the broad signal of **AA-CNDs** splits into two signals at 530 and 534eV for the clickable CNDs. Additionally, the C_{1s} spectrum of **Az-CNDs** exhibited a shoulder at 286.5 eV attributable to C-N_3 carbon atoms.[35] XPS spectra also allowed an evaluation of the degree of click functionalization of the CNDs through a quantitative analysis of the percentage of atoms at the surface (Table S1, ESI†). From the obtained data, the N/C ratio is estimated as 0.32 and 0.04 for **Az-CNDs** and **Ak-CNDs**, respectively. Providing that **Az-CNDs** should contain 4-fold more N than **Ak-CNDs,** this result points to a higher click functionalization for **Az-CNDs** respecting to **Ak-CNDs** and suggests a lower reactivity of propargylamine towards acid anhydrides. Elemental analysis confirmed the higher degree of azide functionalization that was estimated as 1.3 µmol/mg versus 0.6 µmol/mg for the alkyne functionalization (Table S2, ESI†). An additional evidence of this different degree of functionalization is the strong peak of Na_{1s} at 1069 eV in the **Ak-CNDs** spectrum (Fig. S3, ESI†) and the Na/O ratio (0.30 and 0.49 for **Az-CNDs** and **Ak-CNDs**, respectively). Na atom is the counterion of the carboxylate group resulting from the aperture of acid anhydrides when amides are formed and also the counterion of the two carboxylate groups formed in the hydrolysis of unreacted acid anhydrides during the dialysis. Hence, the lower the reactivity of the amine, the higher the number of unreacted anhydride groups and the higher the observed content of Na counterion (Table S1, ESI†).

Despite the goodness of CuAAC, a major drawback of the methodology is the toxicity of Cu(I), particularly in a biological milieu, that has led to the development of alternative metal-free click-chemistry strategies.[36-38] In this context and to further demonstrate the capabilities of **AA-CNDs**, we expanded the range of clickable CNDs by preparing vinyl sulfone (VS) functionalized CNDs (**VS-CNDs**).The efficiency and versatility displayed by the Michael-type additions of VS with aminated and thiolated nucleophiles have been successfully exploited in diverse click metal-free (bio)conjugation scenarios.[39] **AA-CNDs** were thus transformed in **VS-CNDs** by a straightforward two-step one-pot procedure based on reaction with N,N'-dimethyletylenediamine followed by treatment with divinyl sulfone (DVS) and standard aqueous processing (Scheme 1). The functionalization was assessed by the HR-XPS spectra that showed the S_{2p} peak at 167 eV, typical of oxidized organic sulfur, and a double peak at 401 and 398 eV corresponding to the N_{1s} signature of the amine- and amide-type nitrogen atoms (Fig. S5, ESI†). The S/N ratio from two independent XPS analysis was 0.79±0.05, which is higher than the expected 0.5 ratio, but within the experimental error, suggesting a high functionalization with the VS group (Table S2, ESI†). The content of N was estimated by elemental analysis as 3.2 µmol/mg. Providing that one half of the N atoms react with **AA-CND** to form amides, the VS functionalization may be in the range of the 1.3 µmol/mg of azide functionalization estimated for **Az-CNDs**.

3.3. AA-CNDs: platforms for engineering clicked (bio)nanoconstructs

To exemplify the potential of clickable CNDs as versatile platforms for accessing high performance engineered clicked (bio)nanoconstructs, we next prepared CNDs-based multivalent glyconanoparticles by reaction with complementary clickable functionalized sugars (Scheme 2).Multivalent glycoconjugated nanomaterials are well-stablished biomimetic tools for studying carbohydrate-lectin interactions to ascertain the roles that they play.[40] In this endeavor, the value of glycated based-quantum dots in glyconanotechnology has been demonstrated their as outstanding hybrid fluorescent probes that synergistically combines the unique optical properties of the core nanocrystals with the biological activity and specificity of the coated biomolecules.[41] However, glyco-CNDs have deserved limited attention until nowadays.[42-44] With this perspective, the bioconjugation of clickable CNDs was performed with selected biorecognizable sugars (Man,Lac and GlcNAc) adequately



Scheme 2. Preparation of engineering clicked glyco-CNDs

functionalized with azide, alkyne or thiol groups at the anomeric position and prepared following reported procedures.[40-44] CuAAC reactions of **Ak-CNDs** and **Az-CNDs** carried out using well-established microwave assisted protocols and [$(EtO)_3P{\cdot}CuI$] as catalyst[23] (Scheme 2). Furthermore, the click Michael-type conjugation of **VS-CNDs** with thiolated sugars **7** was easily achieved by direct mixture of the reagents[39] (Scheme 2). The successful glyco decoration was assessed in the case of **Az-CNDs** by FTIR. Spectra showed the disappearance of the characteristic stretching signal of azides at 2095 cm^{-1} while preserving other significant signals: 1653 cm^{-1} (amide C=O stretching), 1553 cm^{-1} (carboxylate C=O asymmetric stretching) and 1393 cm^{-1} (carboxylate C=O symmetric stretching) (Fig. S6, ESI†).[45] The sugar coating of the glyco-CNDs was quantified by standard phenol-sulfuric acid method that showed values in the range of 0.7-1.3 µmol/mg, except for those functionalized with GlcNAc whose estimation was not reliable because of the poor absorbance[46] (Table S3, ESI†).

Concerning the optical properties of glyco-CNDs, it is observed that although they retain the fluorescence (Table S4, ESI†,) the grafting of non-fluorescent carbohydrate to the fluorescent core renders lower FLQY as a consequence of the decrease of the fluorescence per unit of mass, in particular for the case of Lac-CNDs. Interestingly the enhancement of the FLQY observed for **Ak-CNDs** seems to be related to the alkyne function since it vanished when clicked and transformed into a triazole. Considering that the grafted sugars are chiral, the rotatory power of glyco-CNDs was measured (Table S3, ESI†). These data indicate that they are optically active materials.

To assess the potential bioapplications of the synthesized CNDs, cytotoxicity, interaction with lectins and cell recognition capabilities were evaluated. Results showed that CNDs have low cytotoxicity (concentrations up to 250 µg/mL), and that they do not produce significant hemolysis (Fig. S7 and S8, ESI†). In addition, glyco-CNDs were biorecognizable by lectins, the affinity being between 2 and 3 orders of magnitude higher than the corresponding sugar (i.e MADM for ConA, Lac for PNA and GlcNAc for WGA) (Table S5, ESI†,). Furthermore, as expected from the ELLA assay, glyco-CNDs showed an enhanced uptake when incubated with cell lines that express surface receptors for different sugars (Man receptor in RAW264.7 cells and Lac receptors in the CT26.WT cells) (Fig. S9, ESI†).

4. Conclusions

In conclusion, we have developed a facile and high-output strategy for the synthesis of fluorescent acid anhydride coated CNDs and exploited their intrinsic reactivity for the direct functionalization of their surface. As a paradigm of their potential they were turned into clickable CNDs and further transformed into glyco-CNDs that are nontoxic and biorecognizable by cells.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

View Article Online
DOI: 10.1039/C8NR09459D

This work was supported by grantCTQ2017-86125-P (Ministerio de Economía. Industria y Competitividad, Spain). Authorsalsoacknowledge "Unidad de Excelencia de Química Aplicada a Biomedicina y Medio Ambiente", University of Granada (Spain).

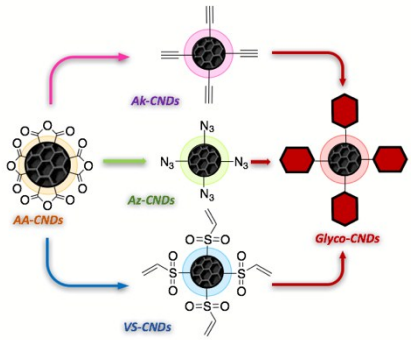
Notes and references

1. G. Hong, S. Diao, A. L. Antaris and H. Dai, *Chem. Rev.*, 2015, **115**, 10816-10906.
2. S. Y. Lim, W. Shen and Z. Gao, *Chem. Soc. Rev.*, 2015, **44**, 362-381.
3. Y. Wang and A. Hu, *J. Mater. Chem. C*, 2014, **2**, 6921-6939.
4. P. G. Luo, S. Sahu, S.-T. Yang, S. K. Sonkar, J. Wang, H. Wang, G. E. LeCroy, L. Cao and Y.-P. Sun, *J. Mater. Chem. B*, 2013, **1**, 2116-2127.
5. F. Yan, Y. Jiang, X. Sun, Z. Bai, Y. Zhang and X. Zhou, *Microchim Acta*, 2018, **185**, 424.
6. W. Liu, C. Li, Y. Ren, X. Sun, W. Pan, Y. Li, J. Wang and W. Wang, *J. Mater. Chem. B*, 2016, **4**, 5772-5788.
7. K. Dimos, *Curr Org Chem*, 2016, **20**, 682-695.
8. C. Ding, A. Zhu and Y. Tian, *Acc. Chem. Res.*, 2014, **47**, 20-30.
9. Y. Park, J. Yoo, B. Lim, W. Kwon and S. W. Rhee, *J. Mater. Chem. A*, 2016, **4**, 11582-11603.
10. D. Shan, J.-T. Hsieh, X. Bai and J. Yang, *Adv. Healthcare Mater.*, 2018, **0**, 1800532.
11. J. Schneider, C. J. Reckmeier, Y. Xiong, M. von Seckendorff, A. S. Susha, P. Kasak and A. L. Rogach, *J. Phys. Chem. C*, 2017, **121**, 2014-2022.
12. D. Wyrzykowski, E. Hebanowska, G. Nowak-Wiczk, M. Makowski and L. Chmurzyński, *J. Therm. Anal. Calorim.*, 2011, **104**, 731-735.
13. B. A. J. Noordover, R. Duchateau, R. A. T. M. van Benthem, W. Ming and C. E. Koning, *Biomacromolecules*, 2007, **8**, 3860-3870.
14. M. M. Barbooti and D. A. Al-Sammerrai, *Thermochim. Acta*, 1986, **98**, 119-126.
15. C. Q. Yang, *Test. Res. J.*, 2001, **71**, 201-206.
16. G. Xiaohong and C. Q. Yang, *Test. Res. J.*, 2000, **70**, 64-70.
17. Q. Yang Charles and X. Wang, *J. Polum. Sci. Pol. Chem.*, 2000, **35**, 557-564.
18. H. Pohlit, M. Worm, J. Langhanki, E. Berger-Nicoletti, T. Opatz, and H. Frey, *Macromolecules* 2017, **50**, 9196-9206.
19. V. Percec, P. Leowanawat, H.-J. Sun, O. Kulikov, C. D. Nusbaum, T. M. Tran, A. Bertin, D. A. Wilson, M. Peterca, S. Zhang, N. P. Kamat, K. Vargo, D. Moock, E. D. Johnston, D. A. Hammer, D. J. Pochan, Y. Chen, Y. M. Chabre, T. C. Shiao, M. Bergeron-Brlek, S. André, R. Roy, H.-J. Gabius and P. A. Heiney, *J. Am. Chem. Soc.* 2013, **135**, 9055-9077.
20. S. Park and I. Shin, *Org. Lett.* 2007, **9**, 1675-1678.
21. T. Hasegawa, M. Umeda, M. Numata, C. Li, A.-H. Bae, T. Fujisawa, S. Haraguchi, K. Sakurai and S. Shinkai, *Carbohydr. Res.* 2006, **341**, 35-40.
22. O. Blixt, D. Vasiliu, K. Allin, N. Jacobsen, D. Warnock, N. Razi, J. C. Paulson, S. Bernatchez, M. Gilbert and W. Wakarchuk, *Carbohydr. Res.* 2005, **340**, 1963-1972.
23. F. Perez-Balderas, M. Ortega-Munoz, J. Morales-Sanfrutos, F. Hernandez-Mateo, F. G. Calvo-Flores, J. A. Calvo-Asin, J. Isac-Garcia, F. Santoyo-Gonzalez, *Org. Lett.* 2003, **5**, 1951-1954.
24. M. Ortega-Muñoz, J. Morales-Sanfrutos, F. Perez-Balderas, F. Hernandez-Mateo, M. D. Giron-Gonzalez, N. Sevillano-Tripero,

R. Salto-Gonzalez and F. Santoyo-Gonzalez, *Org. Biomol. Chem*. 2007, **5**, 2291–2301.
25. F. J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Hernandez-Mateo and F. Santoyo Gonzalez, *Bioconjugate* Chem. 2012, **23**, 846-855.
26. E. Valeur and M. Bradley, *Chem. Soc. Rev.* 2009, **38**, 606-631
27. B. C. M. Martindale, G. A. M. Hutton, C. A. Caputo and E. Reisner, *J. Am. Chem. Soc.*, 2015, **137**, 6018-6025.
28. S. Zhu, X. Zhao, Y. Song, S. Lu and B. Yang, *Nano Today*, 2016, **11**, 128-132.
29. N. Thongsai, Y. Nagae, T. Hirai, A. Takahara, T. Uchiyama, K. Kamitani and P. Paoprasert, *Sensor Actuat. B-Chem.*, 2017, **253**, 1026-1033.
30. K. Spyrou and P. Rudolf, in *Functionalization of Graphene*, ed. V. Georgakilas, Wiley-VCH Verlag GmbH & Co., Weinheim, Germany, 2014, ch. 1, pp. 1-20.
31. S. Wang, Z.-G. Chen, I. Cole and Q. Li, *Carbon*, 2015, **82**, 304-313.
32. H. J. Salavagione, M. A. Gomez-Fatou, G. Martinez, C. Marco and G. J. Ellis, in *Chemical Functionalization of Carbon Nanomaterials*, eds. V. K. Thakur and M. K. Thakur, CRC Press, Boca Raton, 2016, ch. 16, pp. 399-429.
33. M. X. Gao, L. Yang, Y. Zheng, X. X. Yang, H. Y. Zou, J. Han, Z. X. Liu, Y. F. Li and C. Z. Huang, *Chem. Eur. J.*, 2017, **23**, 2171-2178.
34. Y. Zhou and Z. Ma, *Sensor Actuat. B-Chem.*, 2016, **233**, 426-430.
35. T. Heinrich, C. H. H. Traulsen, E. Darlatt, S. Richter, J. Poppenberg, N. L. Traulsen, I. Linder, A. Lippitz, P. M. Dietrich, B. Dib, W. E. S. Unger and C. A. Schalley, *RSC Adv.*, 2014, **4**, 17694-17702.
36. P. Ostrovskis, C. M. R. Volla, M. Turks and D. Markovic, *Curr. Org. Chem.*, 2013, **17**, 610-640.
37. J. M. Baskin and C. R. Bertozzi, *Aldrichim. Acta*, 2010, **43**, 15-23.
38. J. M. Baskin and C. R. Bertozzi, in *Click Chemistry for Biotechnology and Materials Science*, ed. J. Lahann, John Wiley & Sons Ltd., New Delhi, India, 2009, ch. 3, pp. 29-51.
39. J. Morales-Sanfrutos, J. Lopez-Jaramillo, M. Ortega-Munoz, A. Megia-Fernandez, F. Perez-Balderas, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *Org. Biomol. Chem.*, 2010, **8**, 667-675.
40. P. Bojarova and V. Kren, *Biomaterials Sci.*, 2016, **4**, 1142-1160.
41. C. R. A. Cunha, A. D. P. R. Oliveira, T. V. C. Firmino, D. P. L. A. Tenório, G. Pereira, L. B. Carvalho, B. S. Santos, M. T. S. Correia and A. Fontes, *Biochim. Biophys. Acta*, 2018, **1862**, 427-439.
42. I. P.-J. Lai, S. G. Harroun, S.-Y. Chen, B. Unnikrishnan, Y.-J. Li and C.-C. Huang, *Sensor Actuat. B-Chem.*, 2016, **228**, 465-470.
43. S. A. Hill, D. Benito-Alifonso, D. J. Morgan, S. A. Davis, M. Berry and M. C. Galan, *Nanoscale*, 2016, **8**, 18630-18634.
44. C. I. Weng, H. T. Chang, C. H. Lin, Y. W. Shen, B. Unnikrishnan, Y. J. Li and C. C. Huang, *Biosens. Bioelectron.*, 2015, **68**, 1-6.
45. J. Oomens and J. D. Steill, *J. Phys. Chem. A*, 2008, **112**, 3281-3283.
46. M. Dubois, K. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Nature*, 1951, **168**, 167.

View Article Online
DOI: 10.1039/C8NR09459D

TABLE OF CONTENTS



Reactive acid anhydride coated carbon nanodots (AA-CNDs), prepared by the one-pot water-free green thermolysis of citric acid, are a versatile gateway for engineering clicked (bio)nanoconstructs