Comparative study of the characteristics and fluorescent properties of three different biochar derived-carbonaceous nanomaterials for bioimaging and heavy metal ions sensing

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ABSTRACT

Three types of biochar (microalgae, rice straw and sorghum straw) from biomass thermal conversion production were tested for producing biochar-derived carbonaceous nanomaterials (BCN). BCN were obtained after using chemical depolymerisation and solvent extraction, NanoRefinery process. Microalgae biochar-derived carbonaceous nanomaterials (MAB-CN), rice straw biochar-derived carbonaceous nanomaterials (RSB-CN) and sorghum straw biochar-derived carbonaceous nanomaterials (SSB-CN) were characterised using spectroscopic and

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microscopic techniques. This characterisation evidenced significant differences among the three BCN with MAB-CN exhibiting greater structural differences compared to RSB-CN and SSB-CN. Biocompatibility, cellular uptake, and cellular localisation were evaluated using three yeast species, *Saccharomyces cerevisiae*, *Candida albicans*, and *Yarrowia lipolytica.* While all BCN were biocompatible, the degree of biocompatibility for each species was dependent on pH, BCN concentration and BCN type. Additionally, BCN were evaluated as transducers for the detection of 12 heavy metal ions. MAB-CN, RSB-CN, and SSB-CN had different responses to the 12 heavy metal ions. The SSB-CN/Cu (II) and the MAB-CN/Zn (II) combinations evidenced selectivity over the other metal ions with these combinations having limits of detection of 0.0125 μM and 9 μM, respectively. The results from this research pave the way for BCN novel applications for bioimaging and heavy metal ions sensing probes.

Keywords: Biochar; Carbonaceous nanomaterials; Heavy metal ions sensing; Bioimaging; Fluorescence probes;

1 INTRODUCTION

To achieve their objectives for growth, jobs and sustainability, the energy strategies of many governments around the world include, as a major component, the use of biomass as a sustainable source of electricity, heating, and biofuels. By 2030, the European Union aims to generate at least 27% of energy from renewable energy, and a minimum share of advanced biofuels of at least 6.8% [1]. Likewise, by 2030, the United States expects to sustainably produce 1 billion dry tons of non-food biomass and use them to expand the bioeconomy, contributing $260 billion and 1.1 million jobs to the US economy [2]. To achieve these goals,

a fundamental shift toward increased production of biofuels and renewable energy from biomass is required. Therefore, the current technologies for biomass transformation need to reach further levels of sophistication to maximise the value derived from biomass feedstocks and by-products obtained from their transformation [1,2].

Thermal conversion is one of the most important techniques for biofuels and bioenergy production. Gasification and pyrolysis processes are two core thermal conversion processes. Gasification is conducted at temperatures higher than 700 ºC, ambient or high pressure, and reduced oxygen concentration. Pyrolysis is conducted at lower temperatures (400–600 ºC), under higher pressure, and without oxygen. Both processes generate synthesis gas (syngas, 13–85%), biooil (5–75%), and biochar (10–30%). Syngas can be employed directly to generate electricity (combustion) or liquid fuels using the Fischer-Tropsch process [3]. Biooil can be upgraded to generate liquid biofuels or chemicals [4]. Biochar's principal applications are soil amendment [5-7] and activated carbons [8,9].

The upsurge in the worldwide goals for biofuels and bioenergy production will raise the number of industrial processes using thermal conversion for biomass transformation. Syngas and biooil are high value products employed for energy and biofuel generation, and their production rise can be easily managed. In contrast, the current lack of biochar applications makes it difficult to manage the massive amounts of biochar associated with the worldwide increase in thermal conversion processes. Therefore, it is critical to find new processes for the transformation of biochar into value-added products.

In recent years, new processes for the transformation of biochar into different added-value products have been reported. Humic and fulvic acids were generated as a product of the chemical and biological depolymerisation of cotton gin trash (CGT) biochar [10,11]. Similarly, humic substances were generated via alkaline depolymerisation of municipal solid waste (MSW) biochar. This work optimised and modelled humic acid production from MSW biochar using an artificial neural network [12]. CGT biochar chemical depolymerisation produced nano-silica as an additional material from biochar [11].

The production of carbon-based nanomaterials is one of the most recent developments in the production of add-value products from bioenergy production biochar. Placido et al. [13] recently reported the production and purification of carbonaceous nanomaterials from microalgae biochar by using chemical depolymerisation and solvent extraction (NanoRefinery). These nanomaterials were evaluated as a transducer for the detection of heavy metal ions in aqueous systems. The fluorescence emitted by the microalgae biochar-derived carbonaceous nanomaterials (MAB-CN) was quenched by four heavy metal ions, Ni (II), Pb (II), Cd (II), and Cu (II). The MAB-CN fluorescence reduction was dependent on the heavy metal ion concentration.

Biomass thermal conversion uses several feedstocks and diverse types of production processes. Therefore, the resulting biochar from these diverse processes have various chemical structures and properties. Carbon dots (Cdots) and other carbonaceous nanomaterials (CN) produced from other carbonaceous sources exhibit diverse physicochemical properties and variable biocompatibility [14-17]. Therefore, CN generated from different types of biochar are predicted to have diverse structures and properties. The effect of different feedstocks and

production processes on the structure and properties of biochar-derived carbonaceous nanomaterials (BCN) has not yet been studied. The objective of this research was to study three types of biochar ((microalgae, rice straw and sorghum straw)) for the production of BCN, and compare/contrast their physicochemical properties as well as their application as bioimaging fluorescent probes and as transducers for heavy metal ions detection in aqueous systems.

2 MATERIALS AND METHODS

2.1 Substrate

Microalgae, rice straw and sorghum straw biochars were the initial substrates for BCN production. Dr Sergio Capareda and his laboratory Bio-Energy Testing and Analysis Laboratory (BETA Lab) at Texas A&M University kindly donated all the biochars. Sorghum straw biochar (SSB) was obtained from sorghum straw in a fluidised bed/pyrolysis process at 500 °C for 30 min. Whereas, rice straw biochar (RSB) and microalgae biochar (MAB) were obtained in a pyrolysis process using a batch pressure reactor at 500 °C for 30 min (Series 4580 HP/HT, Parr Instrument Company, Moline, IL). After collecting the biochars from the reactor, they were crushed using a mortar and sieved using a 1 mm mesh.

2.2 Chemicals

All chemicals were analytical grade: Potassium permanganate ($KMnO_4$) (Alfa Aesar), Acetone (Acros Organics), potato dextrose broth (PDB) medium (ForMedium). The heavy metal ions included: Nickel sulphate (Ni(II)) (Fisher Scientific), Copper sulphate (Cu (II)), Cadmium sulphate (Cd (II)), Lead Nitrate (Pb (II)), Cobalt nitrate (Co (II)) (Sigma–Aldrich), Barium chloride (Ba (II)) (Sigma–Aldrich), lithium acetate (Li (I)) (Sigma–Aldrich), iron

sulphate (Fe(II)) (Sigma–Aldrich), manganese chloride (Mn (II)) (Acros Organics), zinc sulphate (Zn (II)) (Sigma–Aldrich), silver nitrate (Ag (I)) (Sigma–Aldrich), sodium molybdate (Mo (VI)) (Sigma–Aldrich). Deionised and filtered (Milli–Q ultrapure water system with a 0.22 µm filter, Merck Millipore) water was utilised in all the procedures.

2.3 Biochar-derived carbonaceous nanomaterials preparation

The biochar depolymerisation reaction was as follows: 10% solutions of $KMnO_4$ were mixed with biochar (5%) in 125 mL Erlenmeyer flasks. The depolymerisation was performed at 120 °C for 1 h at 15 psi in an autoclave (Med 12, Selecta) [11]. After the chemical depolymerisation, the biochar solutions were centrifuged at 5000 rpm for 20 min at room temperature to separate the liquid and solid phases. The liquid phases were filtered using 0.22 µm filters (Millex) and refrigerated at 4 °C until use. The depolymerised biochar (solid phase) was dried in a convection oven at 105 °C for 24 h. The liquid phase was purified by repeated solvent extraction. Acetone was mixed with the liquid phase until the production of a second liquid phase [18,19]. The phases were separated by centrifugation at 5000 rpm for 20 min (Legend RT, Sorvall). The upper phase was withdrawn and roto-evaporated (miVAc Quattro concentrator, Genevac) until dry. After weighing, the solids were re-suspended in ultrapure water and ultrasonicated for 1 minute at 50% amplitude (200 W) (Branson, Emerson). The BCN were obtained after repeating the organic solvent precipitation process two additional times. The extracted BCN were suspended in water and kept at 4 °C until use.

2.4 Biochar-derived carbonaceous nanomaterials characterisation

The BCN were characterised with diverse spectroscopic and microscopic techniques. The BCN solutions were diluted to lower concentrations to facilitate characterisation. The fluorescence emission and excitation spectra of the BCN were determined on a Hitachi F2500 spectrophotometer. FT–IR spectra were collected using a Frontier FT-IR spectrophotometer with sampler (PerkinElmer) from 4000−600 cm^{-1}. The FT-IR spectra were analysed with Spectragryph software version 1.1 (Spectroscopy Ninja). UV-Vis absorption spectra were recorded using a U3310 spectrophotometer (Hitachi). Atomic force microscopy (AFM) images were captured on the BioScope AFM (BrukerCorporation) in ScanAssistant mode (tip radius nominal 2 nm and maximum 12 nm) and image analysis was performed using the Brucker NanoScope software package v8.15 (Bruker Corporation). For AFM imaging, the BCN were diluted to 100 ppm, filtered through a 0.2 μm filter and dried on mica substrate. The BCN size and zeta potential in solution were obtained using the Zetasizer Nano ZS (Malvern). The measurements were performed using 0.2 μm filtered solutions in a DTS1070 cell, with water as dispersant (Refractive Index: 1.330) and a BCN refractive index of 2.418 [20]. The size and zeta potential were obtained using the instrument's software.

2.5 Biocompatibility studies

The biocompatibility of the Biochar-derived carbonaceous nanomaterials (BCN) was studied in three yeast species: *Saccharomyces cerevisiae AH22*, *Candida albicans SC 5314*, and *Yarrowia lipolytica (*ATCC 46483*).* The yeast growth curve studies were performed in a Bioscreen C (Oy Growth Curves Ab Ltd). The instrument assessed five BCN concentrations (50, 100, 250, 500, 1000 ppm) in wells with 200 μL of PDB and 100 μL of 1×10^5 cells mL^{-1} inoculum with 3 replicates for each treatment. The cell concentration change in each well was

evaluated via optical density change at a wavelength of 600 nm for 72 h and 30 °C. The growth curves were also evaluated using BCN at pH 10, 7, and 3.

2.6 Cell Imaging

2.6.1 Biochar-derived carbonaceous nanomaterials bioimaging

The capabilities of BCN for cell bioimaging were tested in three yeast species (*S. cerevisiae, C. albicans,* and *Y. lipolytica*). Yeast species were initially cultured in PDB for 24 h at 30 °C and then inoculated with a BCN concentration of 250 ppm for 2 h at 30 °C. After incubation, the samples were centrifuged at 1000 rpm and washed with fresh PDB. This process was repeated twice. Finally, the samples were re–suspended in PDB at 1:10 of the original volume. After washing, the cells were imaged using confocal microscopy using a Zeiss LSM 710 confocal system with Zeiss AXIO Observer Z1 inverted microscope stand with transmitted light (HAL), Illuminator HXP 120C and laser illumination sources. The images were collected under bright field and 405 nm fluorescence excitation.

2.6.2 Bioimage Processing

To evaluate and identify differences in the fluorescence emitted by the BCN in the yeast cells, the images were analysed using the ImageJ software version 1.50i (Wayne Rasband, National Institutes Of Health, USA) and the SAS® Studio software 3.71 (University Edition, SAS Institute Inc., Cary, NC, USA). The analysis in image J software was performed on three separate images of each combination of yeast species and BCN type. Each image was processed to calculate the corrected total cell fluorescence (CTCF) through cell selection, fluorescence and area measurement, background correction, and CTCF calculation. The CTCF

was the response variable for the statistical analysis. As CTCF distribution did not follow a normal distribution, a $Y=X^{1/4}$ variable transformation was performed. The transformed variable was analysed in a two way non-balanced ANOVA because the yeast species and BCN type combinations had different sample sizes. the yeast species and BCN type were used as factors, and the three yeast species (*S. cerevisiae, C. albicans,* and *Y. lipolytica)* and the three BCN types (SSB-CN, RSB-CN and MAB-CN) as levels for each factor. The unbalanced ANOVA was calculated with the PROC GLM from the SAS® Studio software 3.71 (University edition, SAS Institute Inc., Cary, NC, USA).

2.7 Heavy metal ions quenching assays

Stock solutions of the metal ions were prepared at concentrations of at least 25 mM and for BCN at concentrations of 1000 ppm. All the solutions were prepared using deionised and 0.22 μM filtered water. The metal ions titration quenching studies utilised BCN solutions of 50 ppm diluted from the 1000 ppm solutions. The fluorescence of the BCN solution was measured and then the metal ions solutions were added to the cuvette containing BCN (50 ppm) to reach a concentration of 50 μM. Then, the fluorescence of metal/ BCN solution was measured. The reduction in fluorescence was calculated as fluorescence reduction percentage (%) (see Equation 1). Metal ions titration quenching studies were determined using the metal ions with highest effect in the BCN fluorescence. Cu (II) and Hg(II) were used at concentrations from 0.0125 μM to 50 μM. Whereas, Zn (II) was prepared at concentrations between 0.0125 μM to 1000 μM. The concentration range was selected to include the minimum regulatory limit for these metal ions and concentrations reported on wastewaters effluents. The heavy metal ion solution was added to the cuvette containing BCN starting from 0.0125 μM up to 50 μM or 1000 μM. Fluorescence spectra were collected after each

heavy metal ion aliquot was added. The reduction in fluorescence was calculated as fluorescence reduction percentage (%) (see **Equation 1**).

$$Fluorescence\ reduction\ \% = \left(\frac{FL_0 - FL_{HMt}}{FL_0}\right) \times 100 \qquad \textbf{Equation 1}$$

Where FL_0 is the BCN fluorescence without the addition of heavy metal ions and FL_{HMt} corresponds to the BCN fluorescence after a specific concentration of heavy metal ions was added.

3 RESULTS

3.1 Biochar chemical depolymerisation

The chemical depolymerisation of MAB, RSB and SSB produced modification of their chemical structure. These modifications were followed by FT-IR spectroscopy (**Figure 1**). The three non-depolymerised biochar spectra display similar bands between 400 and 500 cm^{-1}, 900 and 1200 cm^{-1}, 1700 and 1250 cm^{-1} and 2800 and 3000 cm^{-1}. A signal at 455 cm^{-1} was shared by all the non-depolymerised biochars and it was associated with the presence of silica in the biochar. The silica found in MSB and RSB was explained by the composition of the raw material, which has significant amounts of silica in their composition [21,22]. In contrast, the presence of silica in SSB was explained by the presence of remaining bed material from the fluidised bed pyrolysis process [23,24]. The MAB peaks at 873 and 1415 cm^{-1} were more intense than RSB and SSB. These signals demonstrated a structure with greater amounts of aromatic compounds in MAB than that of RSB and SSB. Likewise, the RSB and SSB peaks at 775, 1027 and 1415 cm^{-1} demonstrated a structure rich in carbon molecules linked to oxygen and hydrogen atoms. The loss of intensity and sharpness in the peaks related to carbon linkages,

such as 775, 873, 1078, 1415, 2920 and 2851 cm^{-1}, evidenced modification of the biochars' structure and release of carbonaceous compounds into the liquid phase.

The non-depolymerised MAB FT-IR spectrum included ten signals at 455, 705, 873, 1027, 1415, 1574, 2851, and 2920 cm^{-1}. The strongest signals corresponded to 450, 873, 1027, and 1415 cm^{-1}. The strong and sharp signals observed at 873 cm^{-1} (aromatic C–H), 1415 cm^{-1} (C=O, C–C ring stretch), 2851 cm^{-1} (C–H aliphatic) and 2920 cm^{-1} (C–H aromatic and unsaturated) were the key signatures of the MAB spectra. In contrast, the depolymerised MAB spectrum exhibited three bands (400 to 700, 800 to 1200, and 1200 to 1700 cm^{-1}) dominating the peak profile. The 400 to 700 cm^{-1} band contained a new strong peak at 415 cm^{-1} connected with potassium presence (K–OH). The bands of 800 to 1200, and 1200 to 1700 cm^{-1} shared signals with the non-depolymerised MAB. The chemically depolymerised MAB carbon associated peaks (873, 1027, 1415 and 1574 cm^{-1}) showed considerable reduction in the intensity and sharpness of the peaks. In a like manner, the carbon related peaks at 2851 and 2020 cm^{-1} were also reduced considerably. The reduction in the carbon related peaks demonstrates the reduction in carbonaceous linkages resulting from the depolymerisation process, and the possible release of carbonaceous compounds to the liquid phase.

The non-depolymerised RSB FT-IR spectrum had eight peaks at 455, 775, 873, 1078, 1415, 1574, 2851 and 2920 cm^{-1}. The most intense band associated with carbon linkages was the band between 900 and 1200 cm^{-1} with a maximum at 1078 cm^{-1} (C–OH hydroxyl). The band between 1700 and 1250 cm^{-1} contained two strong signals, 1415 cm^{-1} (C=O, C–C ring stretch) and 1574 cm^{-1} (C=O, COO^{-}). The aromatic signals at 873, 2851 and 2920 cm^{-1} were present, but were less intense than MAB. However, the signal at 775 cm^{-1} was sharper and more intense.

On the other hand, the chemically depolymerised RSB spectrum had three principal changes in their spectra compared with the non-depolymerised biochar. First, a significant increase between 400 and 600 cm^{-1} with a max at 415 cm^{-1} (K–OH). Second, an intensity reduction in the band between 800 and 1200 cm^{-1}. Third, the disappearance of the signal at 775 cm^{-1}. The aromatic signals at 873, 1415, 1574, 2851 and 2920 cm^{-1} decreased significantly between spectra, although they were still observed in the depolymerised RSB spectrum. The RSB depolymerisation reaction produced a reduction of intensity in the signals associated with carboxyl, hydroxyl and methyl linkages, indicating possible release of this type compounds into the liquid phase.

The SSB spectrum had nine peaks at 455, 775, 873, 1078, 1320, 1415, 1574, 2851 and 2920 cm^{-1}. The most intense peaks were 455, 1078, 1415 and 1574 cm^{-1}. The 455 cm^{-1} signal is associated with potassium linkages and the final three are correlated with carbon linkages between aromatic carbons and with substituents such as hydroxyl, carboxyl, or ester. In contrast to non-depolymerised SSB, the chemically depolymerised SSB spectrum had four significant changes. First, a significant rise between 400 and 600 cm^{-1} with a max at 415 cm^{-1} (K–OH).and two shoulders. Second, an intensity reduction in the band between 850 and 1200 cm^{-1} including a shift in the maximum signal wavenumber from 1078 cm^{-1} to 1027 cm^{-1}. Third, the complete reduction of the signals at 873, 1078, 2851 and 2920 cm^{-1}. Fourth, a significant reduction of the signals at 775, 1415 and 1574 cm^{-1}. Aromatic, carboxylic and hydroxyl linkages participated the most in the depolymerisation reaction, which indicates possible release of compounds with these linkages into the liquid phase.

3.2 Biochar-derived carbonaceous nanomaterials characterisation.

The liquid phases obtained from the biochar depolymerisation were mixed with an organic solvent sequentially until obtaining BCN. The liquid phases obtained differed among the three biochars. The RSB and SSB generated a liquid with a dark brown colour while MAB produced a dark orange liquid. After the purification process, all the BCN solutions had yellowish and light brown colours. The BCN yield varied for each material, evidencing the effect in the initial feedstock and the production process. The highest yield (BCN g/ Biochar g) was obtained by MAB-CN (13%), followed by SSB-CN (7%) and RSB-CN (4%). Lower yields can be increased by including more biochar depolymerisation cycles.

Figure 2 illustrates the characterisation of the MAB-CN. AFM microscopy (**Figure 2a**) was employed to study MAB-CN topography. The particles height had a normal distribution confirmed by the Kolmogorov-Smirnov test (**Annex 1, Supplementary material**). The MAB-CN had an average height of 4.7 ± 0.96 nm with a minimum height of 2.9 nm and a maximum height of 7.3 nm (**Figure 2b**). The MAB-CN had a lateral dimension of 68 ± 25 nm, with the smallest lateral dimension of 38 nm and the maximum lateral dimension of 153 nm. The AFM section (diagonal line white line) described the height and distance among particles. The section included particles of different heights, but in quantities similar to the height distribution (**Figure 2c**). The spectroscopic characterisation was performed using fluorescence, UV-Vis and FTIR spectroscopy. MAB-CN emission and excitation spectra at various pH. MAB-CN exhibited their maximum excitation and emission wavelengths at 328 and 400 nm, respectively. The particles emitted fluorescence when excited up to 450 nm, where an increase in the excitation wavelength produced a reduction in the fluorescence emitted and a corresponding increase in the emission wavelength (**see Annex 2, supplementary material**). The MAB-CN pH studies exhibited a small variation (±2%) in the magnitude of the emitted fluorescence. In contrast, the peak of excitation scan fluorescence (328 nm) decreased around 2% after each pH unit reduction from pH 8 to 5. The maximum emission and excitation wavelengths were not

affected by the pH changes (**see Annex 2, supplementary material).** The MAB-CN' FTIR spectrum (**Figure 2e**) indicated a mixture of chemical bonds (**see Annex 2, supplementary material**). However, the majority of the wavenumbers and the strongest signals were associated with the presence of carbon linkages (648, 719, 1413, 1561, 1667, 2957, 2933 and 2871 cm^{-1}). Bonds associated with aromatic carbons were the strongest signals (1561, 1413 cm^{-1}) with C–H bonds, C–O or C=O bonds and aromatic bonds comprised 62% of the wavenumbers identified. Additionally, the MAB-CN FTIR spectra demonstrated the probable presence of sulphur (1013 and 648 cm^{-1}), nitrogen (1377 cm^{-1}) and silica (753, 404 and 511 cm^{-1}) linkages. The hydrodynamic diameter and zeta potential in solution of MAB-CN (**see Annex 2, supplementary material**) described molecules with a hydrodynamic diameter of approximately 200 nm. The zeta potential described negatively charged molecules with moderate stability (-39.9 mV).

Figure 3 exhibits the spectroscopic and morphologic characterisation of RSB-CN. The AFM images (**Figure 3a**) described a wide range of heights and lateral dimensions. The RSB-CN average height was 6.7 ± 2.8 nm with a minimum height of 3.3 nm and a maximum height of 16 nm (**Figure 3b**). The particle height distribution did not fit a normal distribution (Annex 1, Supplementary material). However, the majority of the RSB-CN heights (89%) were below 10 nm. The RSB-CN average lateral dimension was 95.8 ± 47.4 nm with a maximum of 319.7 nm and a minimum of 45.1 nm. The AFM section (**Figure 3c**) described a horizontal section (white line) in which it was possible to identify the different particles heights in the sample. RSB-CN fluorescence spectra (**Figure 3d**) showed the maximum emission and excitation signals at 420 and 330 nm, respectively. The excitation spectra contained a series of small peaks that became sharper with the pH reduction. At alkaline pH, the peaks formed a band from 300 to 350 nm, with three peaks at 340, 330 and 313 nm where the 340 nm peak was largest. From pH 6 to pH

3, the strongest excitation peak was observed at 330 nm. The emission peak sharpness changed with the pH reduction, but the maximum emission wavelength was located at 420 nm for all pHs. The pH strongly influenced the emission and excitation fluorescence generated by RSB-CN. The pH reduction created a 7.5% linear increase in both the emission and excitation fluorescence for each pH unit reduced. The difference between the fluorescence emitted by RSB-CN at pH 8 and pH 3 was almost 40% (**Annex 2, supplementary material)**. The RSB-CN FTIR spectrum (**Figure 3e**) had signals grouped in three large bands from 400 to 1100 cm^{-1}, from 1100 to 1800 cm^{-1} and from 2000 to 4000 cm^{-1}. The most intense signals were located in the 1100 to 1800 cm^{-1} with three peaks at 1563 (C–C stretching, C=C aromatic stretching), 1393 ($–COO^{-}$ symmetrical vibrations), and 1367 cm^{-1} (−COOH). The 400 to 1100 cm^{-1} band included half of the spectrum' peaks and diverse functional groups such as C–O and C=O bonds, S–C bonds, aromatic signals, and Si–O bonds (see **Annex 2, supplementary material**). The 2000 to 4000 cm^{-1} band comprised three wide signals with a flat peak revealing the presence of OH and C–H linkages in the RSB-CN structure. The FTIR spectrum indicated nanoparticles rich in aromatic structures with a significant amount of substituents especially, carbonyl hydroxyl and methyl groups. The hydrodynamic diameter and zeta potential in solution of RSB-CN (see **Annex 2, supplementary material**) described molecules with a hydrodynamic diameter of approximately 200 nm and a large negative zeta potential (-65.8 mV) indicating particles with high stability in solution.

Figure 4 depicts the spectroscopic and morphologic characterisation of SSB-CN. The AFM morphologic characterisation (**Figure 4a**) evidenced an average height of 2.5 ± 1.7 nm with a minimum of 0.4 nm and a maximum of 9.2 nm. The particle height's distribution did not fit a normal distribution (**Annex 1, Supplementary material**) as it was a positive skewed distribution (skewness: 1.85) (**Figure 4b**). In this distribution, 90% of the particles had a height

below 5 nm and 50% below 2 nm. The lateral dimension average of the particles was 54.6 ± 43.5 nm with a minimum lateral dimension of 17.6 nm and a maximum lateral dimension of 223.3 nm. The AFM section analysis (**Figure 4c**), exhibits a horizontal section (white line) with a majority of particles below 5 nm, corresponding with the height distribution. The fluorescence spectra (**Figure 4d**) revealed the maximum excitation peak around 310 nm and the maximum emission peak at 420 nm. **A** pH decrease caused an increase in SSB-CN fluorescence of almost 10% between pH 8 and pH 4, with the increase linear between pH 8 and 5 (see **Annex 2, supplementary material**). pH 3 generated a 6% reduction in the emission fluorescence versus pH 4. The excitation fluorescence increased with a reduction from pH 8 to pH 5, and reduced from pH 4 and pH 3. The maximum emission wavelength was constant at all pHs. Whereas, the maximum excitation wavelength shifted 8 nm at pH 3. The SSB-CN FTIR spectrum (**Figure 4f**) had three bands at 400 to 1100, 1100 to 1800 and 2800 to 4000 cm^{-1}. The most intense signals were 1562 and 1395 cm^{-1} and the maximum peaks in the 1100 to 1800 band cm^{-1}. These peaks were associated with the presence of aromatic compounds and carbonyl groups. The 400 to 1100 cm^{-1} band comprised wavenumbers correlated with functional groups such as aromatic, carbonyl, C–H, C–S and O–Si (see **Annex 2, supplementary material**). The band between 2800 and 4000 cm^{-1} contained two peaks, indicating hydrogenation in the SSB-CN structure. The SSB-CN hydrodynamic diameter was on average below 150 nm and the majority of the particles were in only one distribution peak (see **Annex 2, supplementary material**). SSB-CN had a large negative zeta potential (-63 mV) indicating particles with high stability in solution .

SSB-CN and RSB-CN AFM images exhibited a more intersected configuration, which resembled a honeycomb organisation. These levels of organisation can be related to chemical interactions, such as between BCN itself or the mica and the BCN, or to BCN structural changes

associated with water removal. The fluorescence spectra provided one of the most significant differences among the three BCN. The SSB-CN, RSB-CN and MAB-CN had Stokes shifts of 109 nm, 90 nm and 72 nm, respectively. The pH effect on the emission and excitation spectra differed as well. In SSB-CN and RSB-CN, decreasing the pH increased the emission and excitation fluorescence while MAB-CN were not affected by pH changes. The increase in the fluorescence is likely associated with the structure of these nanomaterials since SSB-CN and RSB-CN are richer in carboxylic and hydroxyl groups than MAB-CN. As these groups are commonly identified as fluorophores for carbonaceous nanomaterials [25,26], changes in the pH will modify the carboxylic and hydroxyl groups by producing dissociation and association of the hydrogen atoms. As illustrated by the FTIR spectra, the BCN had an aromatic structure with several types of substituents in their structure. The principal differences among the three FTIR spectra were observed in the number and intensity of the peaks and shoulders between 400 and 1100 cm^{-1} and between 1200 and 1800 cm^{-1}. The three BCN shared the signals at 1561, 1008, 701, 646 and 620 cm^{-1}. All these signals are carbon bonds involved in aromatic rings, carbonyl linkages and S–C linkages. These signals indicated the prominence of aromatic groups in BCN structures, which is a constant component on Cdots from lignocellulosic material [27]. A significant difference was observed between 1200 and 1500 cm^{-1}. MAB-CN had a max peak at 1413 cm^{-1} with four shoulders. RSB-CN had two maximum peaks at 1393 and 1367 cm^{-1} without shoulders. SSB-CN had only a maximum signal at 1395 cm^{-1} with three shoulders. Additionally, the relationship between the two peaks between 1200 and 1800 cm^{-1} is another indicator of structural differences. In MAB-CN, the 1800 cm^{-1} peak was significantly greater than the 1200 cm^{-1} peak, while in RSB-CN and SSB-CN both peaks have similar sizes. RSB-CN and SSB-CN had a considerable number of common peaks, but with different intensity and sharpness. The majority of uncommon signals in the RSB-CN spectrum were from shoulders or bands associated with hydroxyl and C–H bonds (2800-2200, 1800-1900,

1688, 1617, 1438, and 880 cm^{-1}). The uncommon bands in the SSB-CN correlated with aromatic C–H and S–O bonds. The presence of sulphur, nitrogen and silica bonds in all the samples indicate that the BCN had a different composition than other carbonaceous nanomaterials such as Cdots or graphene carbon dots but with similar optical properties as other nanomaterials from lignocellulosic material [16]. All the BCN had moderate to high negative zeta potential indicating their facility to interact with positive particles such as heavy metal ions.

3.3 Biocompatibility studies

The effect of the BCN in the yeast growth is summarised in **Table 1**. Additionally, the growth curves from the biocompatibility studies for each yeast species are in the **Annex 3** of the supplementary material. At all pH, yeast species, and BCN types, concentrations of 100 ppm or below did not generate significant changes in the yeasts' growth curves. MAB-CN produced various effects in the three yeast species. MAB-CN did not modify the *Y. lipolytica* growth curves at any pH or MAB-CN concentrations. In contrast, *S. cerevisiae and C. albicans* evidenced modifications in their growth curves. *S. cerevisiae* growth was inhibited at pH 10 and concentrations above 100 ppm. The growth inhibition was correlated with the increase of the MAB-CN concentration. At pH 7, a slight inhibition occurred at 500 and 1000 ppm. However, the inhibition did not correlate with the MAB-CN concentration. At pH 4, the only inhibition was observed at 1000 ppm and was similar to that observed at pH 7. *C. albicans* was inhibited at 250, 500 and 1000 ppm at basic and neutral pH, 1000 ppm and 500 ppm generated considerable inhibition. At pH 4, MAB-CN at 1000 ppm inhibited *C. albicans* growth. However, the inhibition was less significant than the other pHs. In general, at acid pH the yeast species experienced less inhibition.

RSB-CN exhibited an inhibitory effect at alkaline pH and concentrations of 500 ppm and 1000 ppm. *S. cerevisiae* and *Y. lipolytica* were partially inhibited at 500 ppm and completely inhibited at 1000 ppm. In contrast, *C. albicans* was completely inhibited at both concentrations. At neutral and acidic pH, the RSB-CN concentrations tested did not inhibit *C. albicans*, but the log phase of the curves were less sharp with the pH rise. In *S. cerevisiae* and acidic pH, RSB-CN did not produce inhibition at any concentration. At concentration above 250 ppm and neutral pH, RSB-CN generated a low inhibition in *S. cerevisiae*. *Y. lipolytica* at neutral and acid pH was not inhibited by any concentration of RSB-CN

SSB-CN was the most bio-compatible material, as *S. cerevisiae, C. albicans, and Y. lipolytica* were not inhibited at any of the pHs and SSB-CN concentrations. The changes in the growth curves patterns were associated with the pH changes instead of the concentration or presence of SSB-CN. This evidenced SSB-CN's favourable characteristic as it can be used at any concentrations at neutral and alkaline pHs without generating inhibition.

3.4 BCN bioimaging

Figure 5 displays confocal fluorescence microscopy images recorded after 2 h of growth with BCN. The image illustrated BCN uptake by the three yeast that depended on a combination of BCN type and yeast. **Figure 5a** describes the effect of MAB-CN in the three yeast species. *S. cerevisiae* exhibited a less intense signal with the fluorescence observed throughout the entire cell. *C. albicans* fluorescence was localised in a cellular organelle for some cells and distributed the entire cell possibly indicating multiple uptake/distribution processes. *Y. lipolytica*

fluorescence was localised in one of the cytoplasmic organelles. RSB-CN fluoresced in all the yeast (**Figure 5b**) with a varied localisation and fluorescence intensity dependent on the yeast. *S. cerevisiae* fluorescence localisation was low with small points inside the cells. This can be associated with some interaction between RSB-CN and molecules in the cytosol. *C. albicans* evidenced a diverse distribution of RSB-CN inside the cells. However, it was possible to identify particles concentrated in specific zones in the cells. In *Y. lipolytica,* RSB-CN exhibited a well-localised fluorescence inside the cells demonstrating the introduction of these materials in a specific organelle. SSB-CN exhibited fluorescence in all the yeast species (**Figure 5c**). In *S. cerevisiae,* SSB-CN had fluorescence throughout the entire cell. In *C. albican and Y. lipolytica,* SSB-CN the fluorescence was localised in cellular compartments. The control using only PDB did not generate any fluorescence either associated with the BCN or any autofluorescence from the cells.

The CTCF differences among the combinations of yeast species and BCN types were analysed with a two-way non-balanced ANOVA (**Annex 3, supplementary material**). As the ANOVA *p-value* (<0.0001) was lower than the alpha (0.05), at least one of the 9 combinations of BCN and yeast species were different. Additionally, the two main factors (yeast: (<0.0001 and BCN: (<0.0001) and the interaction between factors (YEAS*BCN: 0.0018) were significant for the model. As the interaction between the factors was significant, the interaction plots (**Figure 6**) were necessary to analyse the fluorescence emitted by the yeast cells with each BCN. **Figure 6a** describes how the yeast species were influenced by each BCN. *S. cerevisiae* had the lowest CTCF for all the BCN. *C. albicans* and *Y. lipolytica* had similar CTCF when grown with RSB-CN and SSB-CN. In contrast, the cultures with MAB-CN had a CTCF significantly higher in *Y. lipolytica* than *C. albicans*. Moreover, MAB-CN was the only BCN presenting significant CTCF differences among the three yeast species CTCF. **Figure 6b** depicts the effect of each

BCN in the yeast species. RSB-CN exhibited the lowest CTCF in all the yeast species. *C. albicans* had the highest CTCF with SSB-CN and was significantly different from RSB-CN and MAB-CN, which had a similar CTCF. In contrast, *Y. lipolytica* and *S. cerevisiae* generated the largest CTCF when mixed with MAB-CN and SSB-CN. In those BCN, the CTCF was not significantly different. MAB-CN present advantages as a future discrimination probe since the yeast species' CTCF varied. However, the SSB-CN exhibited the highest CTCF in all the yeast, making this BCN the most appropriate for fluorescence imaging

3.5 Heavy metal ions detection in aqueous systems

The interactions between 12 heavy metal ions and the three BCN is depicted in **Figure 7.** Hg (II) and Cu (II) ions quenched MAB-CN significantly, having fluorescence reduction percentages of 41.5% and 27%, respectively (**Figure 7a**). Pb (II), Ni (II), Co (II) and Ag (I) ions quenched MAB-CN in percentages between 10% and 15%. Mn (II), Mo (IV), Li (I), and Ba (II) ions did not quench the MAB-CN fluorescence. In contrast with the other heavy metal ions, Zn (II) ions increased MAB-CN fluorescence significantly (15%). RSB-CN was significantly quenched by Cu (II) and Pb (II) ions with fluorescence reduction percentages of 39% and 29%, respectively (**Figure 7b**). Similar to MAB-CN, the second tier of quenching included metal ions with fluorescence reduction percentages between 10% and 15% including Ni (II), Co (II), Fe (II) Hg (II), Mn (II) and Ag (I). Cu (II) (43%) was the only heavy metal ion that significantly quenched SSB-CN fluorescence. Pb (II) ions obtained the second highest quenching with 15%, while the rest of the heavy metal ions achieved fluorescence reductions below 10%. The significant difference between the quenching obtained by Cu (II) ions and the other metal ions indicates a selectivity between SSB-CN and Cu (II) ions that was not observed in the other BCN (**Figure 7c**). Similar to MAB-CN, Mo (IV), Li (I), and Ba (II) ions did not

quench RSB-CN and SSB-CN. Whereas, Zn (II) ions increased the fluorescence emitted by RSB-CN and SSB-CN, but the fluorescence rise in those BCN was 50% and 75% lower than the rise in MAB-CN, respectively. The lowest heavy metal ions quenching in all the BCN was obtained by Mo (IV), Li (I) and Ba (II) ions.

The metal ions with the highest quenching in each BCN were used to evaluate the correlation between heavy metal ion concentration and BCN fluorescence reduction or increase (**Figure 8**). The RSB-CN and SSB-CN emission fluorescence spectra at different concentrations of Cu (II) ions are shown in **Figures 8a** and **8b**. The limit of detection (LOD) for the RSB-CN and Cu (II) ions and SSB-CN and Cu (II) combinations was 0.5 μM. The Stern-Volmer plot for these combinations evidenced a liner correlation (**Figure 8a and 8b embedded figures**). A linear Stern-Volmer plot indicates collisional quenching and can be modelled using the Stern-Volmer equation: $F0/F = 1 + K_{SV}[Q]$, where Ksv is the Stern-Volmer quenching constant and [Q] is the concentration of the quencher molecule, in this case the Cu (II) ions. The SBB-CN/Cu(II) combination had a K_{SV} of 0.017 L.μMol-1 while the RSB-CN/Cu(II) combination had a K_{SV} of 0.0162 $L.\mu Mol^{-1}$. A larger Ksv indicates a larger interaction between heavy metal ions and the fluorophores. Therefore, the greater quenching observed in SSB-CN/Cu (II) is explained by the higher SSB-CN's Ksv. As Hg (II) was the metal ion with the largest fluorescence reduction in MAB-CN, the effect of Hg (II) ions concentration on MAB-CN concentration was evaluated (**Figure 8c**). The LOD for the MAB-CN/ Hg (II) combination was 0.4 μM. The Stern-Volmer plot for the MAB-CN/ Hg (II) combination evidenced a nonlinear behaviour with a downward curvature (**Figure 8c embedded figure**). Such curves are obtained by pure collisional quenching when some of the fluorophores are less accessible than others [28,29]. The non-linear downward behaviour depends of diverse variables and could not be empirically modelled. Although, the fluorescence reduction percentage obtained by MAB-

CN/Hg (II) had a similar percentage as the RSB-CN/Cu(II) and SBB-CN/Cu(II), the difference between their Sten-Volmer plots evidenced a lower interaction between the MAB-CN/Hg (II) than the RSB-CN/Cu(II) and SBB-CN/Cu(II).

As the Zn (II) ions produced a fluorescence increase, the influence of the Zn (II) ions concentration was evaluated using the MAB-CN/Zn (II) combination. This combination was selected because it achieved the highest fluorescence increase. The Zn (II) ions did not increase the MAB-CN fluorescence at concentrations below 5 μM (**Figure 8d**). At 5 μM, the fluorescence increased until 1000 μM. However, the fluorescence increase from 500 μM to 1000 μM was less than 15% of the total fluorescence rise. The limit of detection for this ion was 9 μM with a range of detection between 10 and 1000 μM. As the MAB-CN fluorescence increased, the Stern-Volmer plots could not been used. Therefore, the fluorescence increase percentage (%) (**Equation 2**) was calculated to describe the interaction between MAB-CN and Zn (II) ions (**Figure 8d embedded image**). In the concentration range between 5 and 1000 μM, the MAB-CN fluorescence and Zn (II) ions were correlated with a logarithmical equation ($Y = 7.0187\ln(x) - 12.773$, $R^2 = 0.9814$). As the model is an empirical approach, it was not possible to correlate the constants with measurable properties from the Zn (II) ions or MAB-CN.

4 DISCUSSION

This is the first article showing the versatility of chemical depolymerisation and solvent extraction (NanoRefinery) for producing biochar-derived carbonaceous nanomaterials from different feedstocks (rice straw, sorghum straw and microalgae) and different thermal conversion processes. These carbonaceous nanomaterials had different optical and chemical

properties, evidencing the importance of the original biochar feedstock and the production process in the resulting materials. The effect of the thermal conversion process conditions, such as reactor type, heating rate, final temperature, residence time, catalyst presence, oxygen concentration etc. are significant variables that can affect the type of carbonaceous nanomaterials produced. In this case, MAB and RSB were obtained with batch pyrolysis whereas SSB was obtained with fluidized bed pyrolysis. However, it was not possible to identify specific properties associated with the initial processing conditions. Further studies are necessary to understand the details of the interaction between process conditions and feedstock for the combined production of bioenergy and carbonaceous nanomaterials.

Biochar from bioenergy production used as a raw material for the production of nanomaterials has the advantages of utilising a high variety of wastes, being coupled with bioenergy production, and generating a diversity of carbonaceous nanomaterials with different properties. These differences can be tuned to develop new types of renewable nanomaterials and novel application such as the treatment of polluted water or bioimaging. BCN exhibited different heights and lateral dimensions, and different chemical groups in their structure. In all cases, the materials had a high negative zeta potential that can be associated with the ability to interact with heavy metal ions, which generally have positive charge. Further research needs to be focused on the modification of BCN, BCN applications and the development of other types of nanomaterials.

Microalgae, rice straw and sorghum straw have been utilised for the production of other carbonaceous nanomaterials. Microalgae carbon dots were obtained from eutrophic algal bloom (EAB-Cdots) and microwave thermolysis [30]. Rice straw has been employed for the production of carbon dots [14] and a combination of silica and carbon dots materials [27].

Whereas, sorghum straw has been used for producing Cdots as a tool for detecting chromium (Cr^{3+}) ions in aqueous media [31]. In contrast with SSB-CN, sorghum straw carbon dots detected Cr^{3+} ions via fluorescence enhancement instead of quenching.

In this work, BCN biocompatibility experiments demonstrated that SSB-CN were the most biocompatible material as none of the yeast species, in any of the conditions evaluated, exhibited a modification in their growing curves. This result is comparable with other carbonaceous nanomaterials that did not demonstrate a toxic effect on yeast [32]. *Y. lipolytica* was the most compatible yeast species as only RSB-CN concentrations of 500 ppm and 1000 ppm at alkaline pH were able to inhibit these yeast. *S. cerevisiae* and *C. albicans* were affected by RSB-CN and MAB-CN at alkaline pH and neutral pH. In all BCN, acidic pH was associated with yeast resistance to higher concentrations of carbonaceous nanomaterials. This is principally associated with yeast's physiological conditions where acidic pH is the most favourable condition for growing this type of microorganisms. At all pH, MAB-CN was the only nanomaterial able to inhibit the growth of *S. cerevisiae* and *C. albicans* using concentrations of 1000 ppm. This result opens the door to a possible application of MAB-CN as an antifungal. The concentrations that achieved inhibitory effect by MAB-CN are below the concentrations that achieved antifungal effect in *Pichia pastoris* using citric acid-derived carbon dots (25 mg mL^{-1}= 25000 ppm) [33] and close to the concentrations of Vitamin C derived-Carbon dots (300μg mL^{-1}= 300 ppm) with antifungal effect in *Rhizoctonia solani* and *Pyricularia grisea* [34]. At neutral pH, the MAB-CN inhibitory effect can be achieved with a lower concentration (500 ppm) evidencing the potential of this carbonaceous nanomaterial as an antimicrobial. Future work will focus on the evaluation of BCN as antimicrobial agents and the mechanisms associated with the antimicrobial effect.

This article proved that yeast species had a differential uptake and localisation of BCN. The differential uptake was identified by the differences in the fluorescence emitted by the BCN inside the yeast species. Differential uptake of carbonaceous nanomaterials (Cdots and CN) has been previously demonstrated in human and bacterial cells. In human cells, these differences were employed to differentiate between healthy and cancerous cells. Whereas in bacterial cells, it was utilised to differentiate between live and dead cells [35] as well as gram positive and gram negative bacteria [36]. In yeast species, to our knowledge, this is the first research reporting the differential uptake of carbonaceous nanomaterials. As evidenced by the confocal images (**Figure 5**), the BCN localisation inside the yeast cells also varied with some yeast localising these compounds in cellular organelles (*C. albicans* and *Y. lipolytica*) while others distributed them in the whole cell (*S. cerevisiae*). Additionally, these results showed the effective internalisation of BCN into the yeast's cytosols and organelles, indicating the possible use of BCN as nano-carriers for drug delivery or for imaging specific organelles. The differences, in localisation and uptake, reported in this article are the initial steps for developing fast microbial identification methods based on the combination of BCN and the different interactions between microbial species and the BCN.

BCN interact with various heavy metal ions. The different quenching levels and dynamics registered by each heavy metal ion/BCN combination can be correlated with the chemical, electronic and vibrational characteristics of each material [37]. SSB-CN had the most selective quenching as it only had high quenching with Cu (II) ions. Whereas, MAB-CN was selective for Zn (II) detection as it was the only heavy metal ion producing a fluorescence enhancement. Selectivity is a common property in other types of carbonaceous nanomaterials such as Lotus root-derived carbon dots, chocolate derived Cdots and pigeon feathers Cdots, which were selective to Hg (II), Pb (II) and Fe(III), respectively [38-40]. Compared with

these materials SSB-CN had similar limits of detection and a slightly wider range of detection. The high selectivity evidenced by these materials make them the most promising BCN for developing a sensing method to detect Cu (II) and Zn (II) in aqueous systems. BCN-CN can be used as a heavy metal ion detection probe. However, other strategies are necessary to improve the selectively in detection of heavy metal ions using these materials. Some of these strategies include the addition of phosphorous or nitrogen groups, introduction of a secondary set of materials, and the use of multivariate statistics and additional sets of measurements [13]. BCN structure is rich in C–O, C=O and C-OH linkages, these functional groups with unshared electron pairs are responsible for forming coordination bonds with heavy metal ions and producing the fluorescence reduction. The fluorescence increase observed in all the BCN with some heavy metal ions is a significant result as the increased fluorescence by the interaction with CNs has only been reported in Cdots synthesised from rice using a microwave assisted method [41]. The chemical interaction between Zn (II) and other carbonaceous compounds for enhancing the fluorescence is associated with linkages to nitrogen groups (amide and amine) and carbon oxygen linkages with free electron pairs (C=O) [42]. The presence of some nitrogen groups was evidenced in the FT-IR spectra. However, the most significant signals come from carbon linkages with free electron pairs. As the nitrogen groups were lower than the C=O groups, it is possible that the fluorescence enhancement followed similar interactions as other fluorescent compounds such as fluorescein, coumarin and rhodamine [43-45]. In these compounds, the fluorescence enhancing interactions have a reduced participation of nitrogen compared with the C=O linkages. Future work will focus on evaluating the combination of BCN and heavy metal ions with multivariate analysis for improving their selectivity, the evaluation of matrices for easy and portable detection of heavy metal ions, and the evaluation of BCN as probes for the detection of biomarkers.

5 CONCLUSIONS

This work demonstrated the significant effects of initial biochar feedstock and production process on the final physicochemical properties as well as biocompatibility, bio-imaging, and heavy metal sensing applications of BCN. The three types of BCN exhibited different optical and chemical characteristics. However, the SSB-CN and RSB-CN were more similar than MAB-CN. The biocompatibility between yeast species and BCN depended of the BCN type, pH and BCN concentration. SSB-CN did not produce a negative effect to the yeast species at any of the conditions evaluated. RSB-CN had a negative effect at alkaline pHs, In contrast, MAB-CN inhibited the growth of *S. cerevisiae* and *C. albicans* at all the tested pHs and concentrations above 500 ppm and evidenced its possible use as an antifungal agent. All the BCN were suitable as a bioimaging probe for yeast bioimaging and had different fluorescence intensity and the localisation depending of the yeast cells. The intensity of the signals and lack of toxicity of SSB-CN suggest this nanomaterial as the most suitable for bioimaging applications. On the other hand, an initial investigation of BCN as heavy metal ions sensors demonstrated the possible use of SSB-CN and MAB-CN as transducers for the detection of Cu (II) and Zn (II) ions, respectively. Cu (II) selectively quenched SSB-CN (LOD 0.4 μM) and Zn (II) enhanced MAB-CN fluorescence (LOD 9 μM). This research is the first steps to understand the differences between BCN and further utilise them to develop novel and sustainable methods for cell bioimaging and chemical compounds detection.

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FIGURES AND TABLES LIST