Additive interaction of carbon dots extracted from soluble coffee and biogenic silver nanoparticles against bacteria

Patricia F. Andrade¹, Gerson Nakazato², Nelson Durán¹,³,⁴,*

¹Institute of Chemistry, University of Campinas (UNICAMP), Campinas, SP, Brazil.
²Biology Sciences Center, Londrina State University (UEL), Londrina, Brazil.
³NanoBioss, Institute of Chemistry, UNICAMP, Campinas, SP, Brazil.
⁴Brazil. Nat. Nanotechnol. Lab. (LNNano-CNPEM), Campinas, SP, Brazil.

*duran@iqm.unicamp.br

Abstract. It is known the presence of carbon dots (CDs) in carbohydrate based foods. CDs extracted from coffee grounds and instant coffee was also published. CDs from soluble coffee revealed an average size of 4.4 nm. CDs were well-dispersed in water, fluorescent and we have characterized by XPS, XRD analysis, fluorescence and by FTIR spectra. The MIC value by serial micro-dilution assays for CDs on S. aureus ATCC 25923 was 250 μg/mL and E. coli ATCC 25922 >1000 ug/mL. For silver nanoparticles biogenically synthesized was 6.7 μg/mL. Following the checkerboard assay with combining ½ MIC values of the MICs of 125 μg/mL of carbon dots and 3.4 μg/mL of silver nanoparticles, following the fractionated inhibitory concentration (FIC) index methodology, on S. aureus gave a fractionated inhibitory concentration (FIC) value of 1.0, meaning additive interaction. In general, the unfunctionalized CDs showed to be inefficient as antibacterial compounds, however the CDs extracted from Coffee powder and together silver nanoparticles appeared interesting as antibacterial association.

1. Introduction
Carbon dots (CDs) are carbon based photoluminescent structures, which may be applied in a wide range of areas. Photoelectronic, and electronic properties, photoacoustic imaging, bioimaging application as agents for targeted drug delivery, photodynamic therapy and in light emitting diodes, biocompatibility and biomedical applications, as gene and drug delivery nanocarriers, in particular as anticancer agents in nanomedicine. Also de CDs are non toxic compared with quantum dots (QDs) [1]. Various synthetic methods have been developed for the preparation or extraction of carbon dots, including natural products extraction [1]. Moreover, Durán et al. [1] showed that it is possible to extract CDs, under mild condition, from complex matrices. In this way, processed foods have a high potential as CDs sources.

Sk et al. [2] were the first to verify this hypothesis by extracting CDs from carbohydrate based foods, such as bread, jaggery, sugar caramel, corn flakes and biscuits. The CDs extracted were amorphous and fluorescent. Amongst the processed food, coffee grounds [3] and instant coffee [4], such as Nescafe®, are an interesting source. Coffee based carbon dots are usually attracted considerable attention because of their fascinating merits, including low cytotoxicity, good
biocompatibility, excellent solubility and nitrogen present in its surface which strongly enhances the quantum yield.

CDs, in spite of its photoluminescence, in general exhibit very low cytotoxicity in several cell cultures\(^5\), and even in \textit{in vivo} assays. For this reason, there has been an increasing interest in using it as levels for images in many diseases [6,7]. However, some reports deals with their antibacterial activities.

Water soluble CDs extracted from rice straw [8] did not exhibit any toxicity against sewage water bacteria up to 0.5 mg mL\(^{-1}\). In this work, a hydrothermal method was used for the preparation of water dispersible CDs by using \textit{S. officinarum} juice as carbon source. The growth curve of \textit{E. coli} showed that CDs were non-toxic up to 400 mg mL\(^{-1}\) for this bacterium [9].

Raw soot CDs passivated with a PEG2000N solution (CDs-NH\(_2\)) were applied to a \textit{Salmonella typhimurium} mutagenicity test and showed no detectable mutagenicity up to 0.1 mg/plate under the experimental conditions\(^6\). Amino-functionalized carbon dots were synthesized in the presence of glycerin and PEG diamine and this material over 3.000 μg mL\(^{-1}\) showed no inhibition of carbon dots on \textit{E. coli} culture [10]. Bare CDs (0.3 mg mL\(^{-1}\)) showed no zone of inhibition against Gram negative \textit{Pseudomonas aeruginosa} or Gram positive \textit{Bacillus subtilis} [11]. However, multifunctional CDs with glucose and poly(ethyleneimine) (PEI), which were further quaternized with benzyl bromide exhibited a significant antibacterial activity against Gram positive and Gram negative bacteria with a MIC around 16 μg mL\(^{-1}\) [12].

Considering the potential application of CDs as antibacterial agents and the simple processing to achieve CDs from processed foods, the aim of our work was to extract the different fractions (separated by size) of non-functionalized CDs from the soluble coffee Nescafe\(^\circledR\), characterize and determine its antibacterial activity.

Herein, CDs from soluble coffee revealed an average size of 5.7 nm. They were well-dispersed in water, photoluminescent and characterized by X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD) and Fourier-transform infrared (FTIR) spectra. The cytotoxicity study showed that the CDs from coffee did not cause any toxicity to cells at concentrations up to 20 mg mL\(^{-1}\) and may applied to cells and fish imaging\(^5\).

2. Methods

\subsection*{2.1 Materials}

Nescafe\(^\circledR\) Original instant coffee powder was purchased from at the supermarket of Brazil. Quinine sulfate and sulfuric acid were purchased from Sigma Aldrich and was synth, respectively. The aqueous solutions were prepared in deionized water.

\subsection*{2.2 Carbon dots extraction from instant coffee}

The preparation process for carbon dots was carried out following a modified method of Jiang et al. [4]. Briefly, 4 g of Nescafe\(^\circledR\) Original instant coffee powder were added to 20 mL of distilled water at 90°C and the mixture vigorously stirred for 10 minutes. Afterwards, the mixture was centrifuged at 14,000 rpm for 20 min 3 time to remove particles in suspension. The resulting supernatant was further filtered through a 0.22 μm membrane to remove agglomerated particles. Finally the filtrate was purified by Sephadex G-25-40 gel filtration chromatography with deionized water as eluent. All fractions, containing carbon dots with different mean diameter, were accompanied by fluorescence measurements. The fractions obtained (~120 mL) were then lyophilized and stored at 4°C for further characterization and use.

\subsection*{2.3 Biosynthesis of silver nanoparticles}
The *Fusarium oxysporum* (*F. oxysporum*) strain used was the following: 07 SD, from ESALQ-USP Genetic and Molecular Biology Laboratory-Piracicaba, S.P., Brazil. The fungal inoculates were prepared in a malt extract 2% and yeast extract 0.5% at 28 °C in Petri dishes. The liquid fungal growth was carried out in the presence of yeast extract 0.5% at 28 °C for 6 days. The biomass was filtrated and resuspended in sterile water. Approximately 10 g of *F. oxysporum* biomass was taken in a conical flask containing 100 mL of distilled water, kept for 72 h at 28 °C and then the aqueous solution components were separated by filtration and/or centrifugation. To this solution, AgNO$_3$ (10$^{-2}$ M) was added and kept for 72 hours at 28 °C [13].

2.4. Association of silver nanoparticles and carbon dots

To evaluate the antibacterial effects and interactions of CD nanoparticles combined with AgNPs produced by *F. oxyporum* against *Methicillin-resistant Staphylococcus aureus* (MRSA), assays of microdilution (described below) in double-antimicrobial gradient were performed.

To perform these experiments, solutions containing the minimum inhibitory concentration (MIC) of CDs and various concentrations of AgNPs (including MIC) were prepared.

2.5. Characterization

The fluorescence measurements were obtained by a fluorescence spectrophotometer (Varian, Palo Alto, USA) equipped with a 1 cm quartz cell at room temperature, used a wavelength 370 nm of the sample with excitation and emission slits of 5 mm, acquisition of emission spectra in the interval 300-700 nm.

The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectra was obtained from solid using an Agilent Spectrometer, operating at a 4 cm$^{-1}$ resolution, with 16 scans between 500 and 4000 cm$^{-1}$.

CDs morphology, size and size distribution was investigated in a JEOL JEM-2100F URP transmission electron microscope operating at 200 kV, equipped with a 2k x 2k Gatan CCD camera. For that, fractions 12 and 14 were dripped on top of carbon coated copper grids and left to dry.

Carbon dots X-ray diffraction (XRD) measurements were carried out on an X-ray diffractometer (Shimadzu XRD 7000). The patterns with Cu K$_\alpha$ radiation ($\lambda = 0.15406$ nm) at a 40 kV voltage, 30 mA current with a 2° min$^{-1}$ scanning rate were recorded in the 5°-90° 2$\theta$ region.

The morphology and particle size of the AgNPs were investigated in a Carl Zeiss Libra 120 Plus (with a $\Omega$-filter in column) transmission electron microscope, operated at an 80 kV acceleration voltage and using a tungsten thermionic source. An Olympus camera with iTEM software was used for image.

2.6. Determination of the minimum inhibitory concentration (MIC)

MIC was determined by serial micro-dilution assays in 96-well microplates, as suggested by the CLSI 15. In brief, single colonies of bacterial cultures (on *Staphylococcus aureus* ATCC 25923 – *S. aureus* and *Escherichia coli* ATCC 25922 – *E. coli*) grown in Mueller-Hinton agar (Sigma-Aldrich, USA) media were diluted in saline solution and adjusted to 0.5 on the MacFarland scale, which corresponds to 1.5 x 10$^8$ CFU (colony forming units)/ml. Then, the bacterial suspensions were diluted in Mueller-Hinton broth (Difco, USA) and plated in 96-well microplates at a density of 5.0 x 10$^5$ CFU/well. Finally, different concentrations of the analyzed compounds were added to each well to determine the MIC values. The plates were incubated at 37°C for 18 h, and then the optical density values at 600 nm were determined using a Bio-Rad Microplate Reader model 3550. MIC was determined at total inhibition of growth after 24 h incubation. All assays were carried out in triplicate [14].
To evaluate the MIC values of the association between both antimicrobials, the fractionated inhibitory concentration (FIC) index was used as described by Chin et al. [15].

\[ \text{FIC} = \frac{\text{MIC(Cc)}}{\text{MIC(Ca)}} + \frac{\text{MIC(Sc)}}{\text{MIC(Sa)}} \]

where MIC(Cc) is the MIC of CDs nanoparticles used combined with the AgNPs, MIC(Ca) is the MIC of carbon dot nanoparticles used alone, MIC(Sc) is the MIC of the AgNPs used combined with carbon dots and MIC(Sa) is the MIC of the AgNPs used alone. FIC indexes were interpreted as follows: FIC ≤ 0.5 = synergic interaction; 0.5 < FIC ≤ 1.0 = additive interaction; 1.0 < FIC ≤ 4.0 = no interaction; FIC > 4.0 = antagonist interaction.

3. Results and discussions

Carbon dots (CDs) extracted from the soluble coffee Nescafé® were further purified by Gel Permeation chromatography (GPC) using deionized water as eluent. Every 120 mL fraction of the eluted material was accompanied by fluorescence spectroscopy. Figure 1a shows excitation and emission CDs spectra in water. The strongest emission peak was located at 460 nm with an excitation wavelength in 370 nm. Therefore, the GPC fractions analyses were taken at the optimal excitation wavelength of 370 nm. Figure 1b shows the fluorescence spectra of fraction 12 to 21, in which CDs are present. The highest concentration of CDs in fraction 12 is verified by the fluorescence intensity at 460 nm. The above results indicate that not only carbon dots were successfully obtained but also that it possess stable and strong fluorescence in water, which may serve as a biosensor. Prior to investigating its fluorescence and phosphorescent properties, it was necessary to determine the carbon dots size and composition.

Figure 1. (a) Fluorescence emission spectra of carbon dots with excitation wavelengths in aqueous solution. Inset: C-dot containing solution in room (left) and ultraviolet (right) light, (b) Fluorescence emission spectra at room temperature of carbon dots at excitation wavelength 370 nm, obtained of different fractions.

Figure 2 shows the FTIR spectra of the Fraction 12 CDs, which showed bands centered at 3457 cm\(^{-1}\), 3260 cm\(^{-1}\), 1637 cm\(^{-1}\), 1371 cm\(^{-1}\) and 1023 cm\(^{-1}\). These bands reveal the surface functional groups present at the CDs. The band centered at 3457 cm\(^{-1}\) are assigned to stretching vibrations of O-H.
and N-H, while the appearance of a band at 3260 cm\(^{-1}\) can be assigned to the bending vibrations of C-N groups. The bands at 1637 cm\(^{-1}\), 1371 cm\(^{-1}\) and 1023 cm\(^{-1}\) correspond to the vibrations of C-O-C bonds, CH\(_2\) and C-N, respectively. Therefore, the FTIR results indicated that the surfaces of carbon dots were full hydrophilic, and presented hydroxyl, carbonyl and amine groups. The hydrophilic surface is probably the reason for the good dispersibility in water.

**Figure 2.** Carbon dots FTIR spectrum.

To investigate the CDs size and morphology, TEM micrographs were taken from fractions 12 and 14. Figure 3a shows that the CDs, indicated by white arrows, are spherical and well dispersed. Fractions 12 and 14 presented the same morphology, distribution and average size. The histogram in Figure 3b was calculated from 360 measurements of fractions 12 and 14 CDs. These measurements yielded an average diameter of 5.7 ± 2.5 nm.

**Figure 3.** Carbon dots TEM micrograph (a), diameter histogram (b) The total number of particles counted for the histogram was 358.

High resolution TEM (HR-TEM) from a single fraction 14 carbon dot is shown in Figure 4a. The nanoparticle boundaries are highlighted by the white lines. It can be observed at the micrograph the atomic planes characteristic of crystalline subjects. The fast Fourier transforms (FFT), shown in Figure 4b, presents four white spots, which confirms the nanoparticle crystallinity. On the other hand,
the XRD measurements of fraction 12 CDs (Figure 4c) showed only an amorphous halo with maxima at $2\theta \approx 22^\circ$. The lack of crystalline peaks at the XRD, in spite of the HR-TEM crystallinity confirmation, is due to the small particle size. Since XRD only detects long-range order, the few nanometers diameter of the CDs is insufficient to produce detectable X-ray diffraction.

![Figure 4. HR-TEM micrograph (a) and its respective FFT (b) of a single fraction 14 carbon dot. XRD diffractogram of fraction 14 CDs (c).](image)

Fluorescence quantum yield (QY) of as prepared CDs is estimated by comparing the wavelength integrated intensity of testing sample to that of a standard (std). A solution of quinine sulfate in 0.01M H$_2$SO$_4$ (QY of 54% at 360 nm, $n = 1.33$) is used as a standard. The QY is determined by the following equation:

$$Q = Q_{std} \frac{I}{I_{std}} \frac{A_{std}}{A} \frac{n^2}{n_{std}^2}$$

Where Q is the quantum yield, I is the integrated emission intensity. A refer to absorbance, which is measured by a UV-Vis spectrophotometer, and n is refractive index of the solvent. The subscript std denotes the standard value. Specifically, the CDs is dissolved in deionized water ($n=1.33$) and excited under 360 nm. To avoid inner filter effect, the optical density must be below 0.1 at the excitation wavelength. 5 nm slit width is used for both excitation and emission. According to above equation, QY of the CDs is determined to be 11% (Table 1), which is better than previous reported ones (e.g., QY of 3.8% [3], 5.5% [4]) (Table 1).

| Sample         | Integrated emission Intensity (I) (365-700 nm) | Absorbance at 360 nm (A) | Quantum yield (Q) | Z$_{av}$ (nm) |
|----------------|-----------------------------------------------|--------------------------|-------------------|---------------|
| Quinine sulfate | 39033.20                                      | 0.04                     | 0.540             |               |
| CDs [this paper]| 34843.20                                      | 0.18                     | 0.110             | 5.7 ± 2.5     |
| CDs Coffee beans[3] | 0.038                                  | 5 ± 2                    |                   |               |
| CDs Coffee powder[4] | 0.055                                | 3-6                      |                   |               |

Table 1: Quantum yield of CDs
To investigate the antimicrobial activity of these CDs, the MIC against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 was calculated by the serial micro-dilution method and was found to be 250 μg mL\(^{-1}\) and >1000 μg mL\(^{-1}\), respectively.

On the other hand, the MIC of biogenic silver nanoparticles (AgNPs) on *S. aureus* ATCC 25923 was found to be 6.7 μg mL\(^{-1}\). The morphology, size, and size distribution of these AgNPs was also investigated by TEM. Figure 5a shows AgNPs with different morphologies (pseudo-spherical, triangular truncated, nanorods and platelets), while the histogram, Figure 5b, showed a mean AgNPs diameter value and standard deviation of 55 ± 18 nm. It is important to point out that AgNPs with an average size of 4 nm can be also observed. Further characterization of these nanoparticles, such as zeta potential and DLS measurements, can be found at the supplementary material.

![Figure 5. AgNPs TEM micrograph (a) and diameter histogram (b). The total number of particles counted for the histogram was 198.](image-url)

Chin et al. [15] used the checkerboard assay methodology to combine two different antimicrobial agents. In this work, the combination of ½ MIC value of the CDs (125 μg mL\(^{-1}\)) and ½ MIC value of the AgNPs (3.4 μg mL\(^{-1}\)) through the checkerboard methodology acting on *S. aureus* gave a fractionated inhibitory concentration value of 1.0 which showed a remarkable synergy between these antimicrobial agents.

The only CDs that showed a better antibacterial activity was from a complex CDs produced by glucose and poly(ethyleneimine) (PEI), and posterior quaternized with benzyl bromide. This CDs exhibited a significant antibacterial activities against Gram (+) and Gram (-) bacteria with a MIC around 16 μg mL\(^{-1}\) [12].

### 4. Conclusions

CDs nanoparticles were isolated and characterized from soluble coffee (~6 nm). The CDs exerted antibacterial activity alone (MIC of 250 μg mL\(^{-1}\) on *S. aureus* ATCC 25923) and exhibited an additive effect using a checkerboard assay (combination of ½ MIC) with AgNPs biogenically synthesized. These data of the presence of CDs in human drinks may help their potential applications in cell tracking and more practical biological applications. This is a relevant result, since the CDs from different sources, such as rice straw sources [8], from hydrothermal method of CDs synthesis from sugar cane source [9], from CDs from raw soot [6], from CDs synthesized from glycerin and PEG-diamine [10] or from Gum Arabic by microwaves method [11] did not exhibited any toxicity against different kinds of bacteria as shown in this manuscript.
These data of the presence of CDs from soluble coffee, apparently without any toxic effect on humans, could be an important nanomaterial for applications in cell tracking and more practical biological applications.

Acknowledgements

Support from FAPESP, CNPq, INOMAT (MCTI/CNPq), NanoBioss (MCTI) and Brazilian Network on Nanotoxicology (MCTI/CNPq) are acknowledged. The authors also acknowledged the Brazilian National Nanotechnology Laboratory (LNNano-CNPq) and the Electron Microscopy Laboratory (LME-LNNano) staff for TEM technical support and to Prof. T.D.Z. Atvars from Institute of Chemistry, University of Campinas, Brazil for the preliminary results on fluorescence measurements.

References

[1] Durán N, Simões MB, Moraes ACM, Fávaro WJ and Seabra AB 2016 J. Biomed. Nanotechnol. 12 1323
[2] Sk MP, Jaiswal A, Paul A, Ghosh SS and Chattopadhyay A 2012 Sci Rep 2 383
[3] Hsu PC, Shih ZY, Lee CH and Chang HT 2012 Green Chem. 14 917
[4] Jiang C, Wu H, Song X, Ma X, Wang J and Tan M 2014 Talanta 127 68
[5] Wang Y, Anilkumar P, Cao L, Liu JH, Luo PG, Tackett II, KN., Sahu S, Wang P, Wang X and Sun YP 2011 Exper. Biol. Med. 236 1231
[6] Wang K, Gao Z, Gao G, Wang Y, Shen G and Cui D 2013 Nanoscale Res. Lett. 8 122
[7] Zheng ZT, Ananthanarayanan A, Luo KQ and Chen P 2015 Small 11 1620
[8] Mandal TK and Parvi N. 2011 J. Biomed. Nanotechnol. 7 846
[9] Mehta VN, Jha S and Kailasa SK 2014 Mat. Sci, Eng. C 38 20
[10] Krishna AS, Radhakumary C, Antony M and Sreenivasan K 2014 J. Mater. Chem. B 2 8626
[11] Thakur M, Pandey S, Mewada A, Patil V., Khade M, Goshi E and Sharon M. 2014 J. Drug Deliv. 2014 Article ID 82193.
[12] Dou Q, Fang X, Jiang S, Chee PL., Lee, TC and Loh XJ 2015 RSC Adv. 5 46817
[13] Durán N, Marcato PD, Alves OL, De Souza GIH and Esposito E 2005 J. Nanobiotechnol. 3 1
[14] CLSI (Clinical and Laboratory Standards Institute) 2013 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard, M07-A8, eighth edition. CLSI, Wayne, PA, USA
[15] Chin NX, Weitzman I and Della-Lata P 1997 Antimicrob. Agents Chemother. 41 850