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# From cow manure to bioactive carbon dots: a light-up probe for bioimaging investigations, glucose detection and potential immunotherapy agent for melanoma skin cancer†

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Bioactive carbon dots (C-dots) with ca. 4 nm were successfully produced with singular photophysical properties, low-toxicity and interesting immunological response. The optical properties of the C-dots were investigated and the "light-up" behaviour enabled them to be explored in glucose detection and bioimaging experiments (mitochondrial selective probe). C-dots were not selective to the tumour region and several fluorescent spots were visualized spread on animal bodies. The histology investigations showed that cancer-bearing mice treated with C-dots presented a large number of regions with necrosis and inflammatory infiltrates, which were not identified for cancer-bearing mice without the treatment. These results suggested that C-dots have the potential to be explored as an immune therapy agent for melanoma skin cancer.

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## Introduction

Cancer is a general term for a group of diseases characterized by the disorderly spread and growth of defective cells. It can be detected in any part of the body and its mortality rate is globally increasing. Those diseases are expected to be the major cause of death in the 21<sup>st</sup> century.

Nanotechnology has been an important allied on fighting cancer.<sup>1,2</sup> The versatility of nanomaterials in cancer therapy has contributed for recent innovations in drug delivery systems and enabled the creation of new therapeutic agents based on particle surface functionalization with molecules and biomolecules (nucleic acid strands, peptides, proteins or antibodies).<sup>3,4</sup> These sophisticated multifunctional nanosystems can contribute to more precise diagnostics and less invasive treatments.

It is well-known that compounds containing boronic acid-groups can be widely used in the diagnostic and treatment of different pathologies.<sup>5-8</sup> Several reports have described these compounds as selective taggants of glycoproteins, inhibitors of virus and bacterias, immunopotentiators and/or immunosensitization agents of tumour cells.<sup>9,10</sup> Boronic acid group can form highly-stable cyclic esters with *cis*-1,2 and 1,3-Lewis base molecules (alcohol and amine) in biological media. This important characteristic enabled the development of specific enzyme inhibitors that can be used for Alzheimer's disease and cancer treatments.<sup>11-13</sup> For instance, the peptidylboronic anti-neoplastic agent Bortezomib has been used in cancer treatment since 2003.<sup>14</sup>

Phenylboronic acid (PBA) belongs to a class of organic ligands that are widely employed for molecular recognition. Reports have indicated that PBA can target and reduce the proliferation process of prostate and breast cancer cells.<sup>15,16</sup> *In vivo* investigations demonstrated the effectiveness of nanoparticles coated with boronic groups in prolonging the survival time of cancer-bearing mice and reducing tumour growth.<sup>16,17</sup> PBA derivatives can also mimic the recognition properties of the lectins for tagging sugar fragments on cell surface.<sup>14</sup> Liu and co-workers produced a PBA-containing polymer which can reversibly capture and release cancer cells depending on the pH and glucose concentration.<sup>18</sup> In another example, Deshayes and co-workers have shown that a nanocarrier containing PBA

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fragments and a parent complex of the anticancer drug oxaliplatin was able to reduce the growth rate of both orthotopic and lung metastasis models of melanoma *in vivo*.<sup>13</sup>

Recent studies have demonstrated that malignant cell transformation, tumour growth, metastasis and the pessimistic prognosis in some cancer patients have been directly associated with the hypersialylation of the plasmatic cell membrane.<sup>19</sup> Aberrant Sialic Acid (SA) overexpression protects the cancer cell against immune recognition and eradication, and facilitates migration and tissue invasion.<sup>20</sup> Galvan and co-workers have confirmed the correlation between the SA levels and the progression of malignant melanoma in 25 patients.<sup>21</sup> In accordance with this study, patients in advanced melanoma stages presented high levels of SA in comparison with those in the early stage. Antibody and lectins have been widely explored for profiling the hyperexpression of SA onto the cell surface, while other strategies appeal for recombinant and SA-bind proteins for detecting anomalies in sialy glycans on cells and tissues.<sup>22</sup> However, these biomolecule-based strategies, that normally are expensive and very time-consuming, require complicated methods and costly equipments.

Cancer treatment based on boronic acid containing molecules have been widely reported.<sup>17</sup> For instance, Plopper and co-workers demonstrated that PBA is selective and effective to reduce viability and migration of prostate and breast cancer cells.<sup>23</sup> In another *in vitro* and *in vivo* investigation, PBA inhibited 57% of endocarcinoma 4T1 growth after intraperitoneal administration in mice.<sup>12</sup> Nagasaki and co-workers reported a PBA-decorated polymeric nanoparticle that is effective against a B16 melanoma-bearing mice model.<sup>24</sup>

Among the nanomaterials used against cancer, a new class of material has recently emerged and is showing promising results, that is, the carbon dots (C-dots).<sup>25–27</sup> C-dots are spherical nanoparticles with sizes typically smaller than 10 nm, composed by a graphitic core and covered by an amorphous carbonaceous edge.<sup>25,28</sup> C-dots modified with PBA moieties can present outstanding selectivity for glycoproteins and

antineoplastic properties that make them interesting nano-platforms for the development of theragnostic tools.<sup>29,30</sup> Based on our interest in the development of optical probes with more selective properties and cancer treatment, this work presents the synthesis, characterization and biological investigation of a simple luminescent platform based on surface-modified C-dots with PBA (PBAC-dots). We also report the exploration of PBAC-dots as a light-up probe for saccharide, bioimaging agent and a potential immunotherapy agent for melanoma skin cancer.

## Results and discussion

The production and passivation of C-dots were performed in accordance with our previous work using cow manure or glucose as the carbon sources.<sup>31</sup> 4-Formylphenylboronic acid (4-FPBA) was covalently bonded to primary amines of the passivated C-dots by Schiff base formation followed a reduction with sodium borohydride (see Scheme 1).

Fig. 1 shows high-resolution HRTEM images and particle size distributions acquired of the PBAC-dots. The spheroidal PBAC-dots are well-dispersed and presented narrow size distribution ranging from 2–6 nm with average diameter of  $4.2 \text{ nm} \pm 0.032 \text{ nm}$ . Fig. 1(a) exhibits a representative HRTEM image of an individual particle. PBAC-dots presents high crystalline structure with lattice parameter of  $2.2 \text{ \AA}$ , which agrees well with [100] lattice spacing of graphene along the [001] diffraction plane.<sup>32</sup>

The XPS spectrum of the PBAC-dots (Fig. 1(c)) clearly displays five elements, O, N, C, S and B, and their contents are 39.10%, 4.11%, 48.00%, 3.45% and 5.42%, respectively. The presence of boron onto PBAC-dots surface indicates that PBA moieties were successfully introduced in the synthesis process. The high-resolution XPS spectra are available in ESI.† C 1s spectrum presents three peaks at 289.4, 286.6 and 285.3 eV which can be assigned to the C=O, C=C and C–C bonds, respectively (Fig. S1(a)†). Fig. S1(b)† is the O 1s line that shows peaks at 532.4, 533.62 and 535.6 eV related to –C=O, C–O and



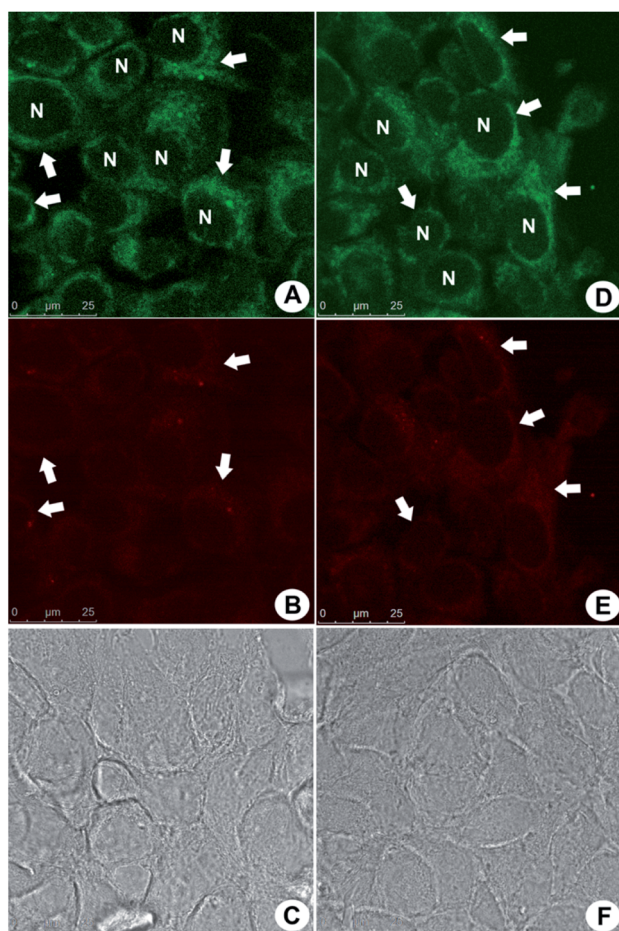
Scheme 1 Schematic approach adopted for production of PBAC-dots.





how it affects luminescence indicates an open field for further investigations.

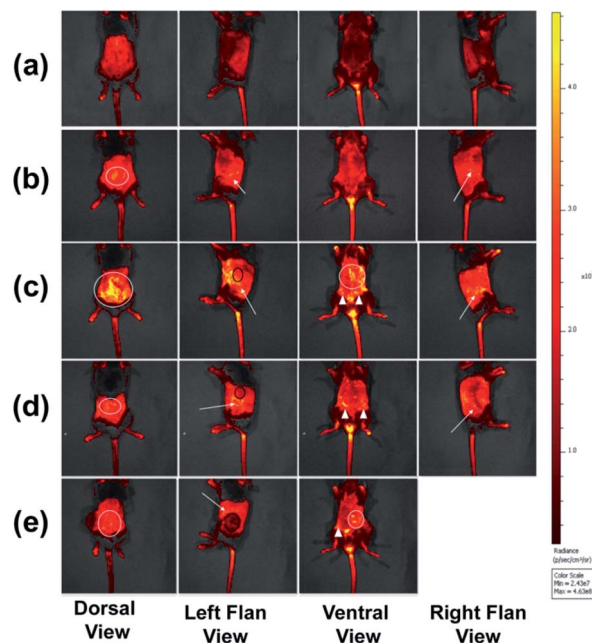
Usually, “light-down” probes might be inaccurate in sensing and might have limited work environment. On the other hand, “light-up” sensors are normally preferred due to their enhanced sensing capacity, especially in biological medium where the background fluorescence is highly problematic. Fig. 2(c) displays the emission intensity of **PBAC-dots** as a function of glucose concentration. Binding with glucose enhances emission intensities up to 6-fold relative to **PBAC-dots** and presents a good linear relationship ( $R^2 = 0.998$ ) as shown in Fig. 2(d). The presence of an electron-donor group in the chemical structure of boronic acid derivatives is well-established as a cornerstone for the design of “light-up” sensors.<sup>48,49</sup> Therefore, the antagonist behaviour presented by **PBAC-dots** in presence of glucose does not arise from a simple accident or haphazard. In fact, this emerges from a previous stratagem used in boronic acid derivatives.<sup>48–51</sup> In general, surface functionalization of nanoparticles is one of the most important limiting factors for biomedical applications, because it affects the cytotoxic properties, the dynamic of cell uptake and selectivity.<sup>52,53</sup>



**Fig. 3** MCF-7 breast cancer cells incubated with **PBAC-dots** and visualized on both the green and red channels. (A)–(C) Live cells. (D) and (E) Adhered cells. No staining could be detected inside the nuclei of the cells as shown by dark voids (N). (C) and (F) show the normal morphological aspects of the cells by phase contrast microscopy.

The performance of **PBAC-dots** probe in *in vitro* bioimaging experiments was also evaluated using MCF-7 cells (Fig. 3). **PBAC-dots** displayed an intense fluorescent signal in confocal microscope at both green and red channels. In addition, no cytotoxicity evidence was observed. MTT assays obtained also for other cell lines (Fig. S2†) showed no deleterious effect.

The fluorescent profile obtained in cells after **PBAC-dots** incubation indicates selective mitochondrial staining (Fig. 3). It is noteworthy to mention the fact that **PBAC-dots** were not found inside the nuclei of the cells, contrasting with previous results from **C-dots** passivated exclusively with ethylenediamine (**C-dot-NH<sub>2</sub>**),<sup>31</sup> which were selective to nucleoli acid components inside the nuclei. Many works have depicted that **C-dots** are in general noncytotoxic and can be easily internalized by the cell.<sup>54</sup> However, Havrdova and co-workers demonstrated the toxicity-dependence of **C-dots** as function of surface charge.<sup>55</sup> In this study, negatively charged **C-dots** have shown almost no cytotoxicity for high concentrations (0.1 mg mL<sup>-1</sup>). Cytotoxicity of **PBAC-dots** on melanoma (B16F10) and fibroblast (NIH3T3) murine cells was evaluated in three different concentrations (0.01, 0.05 and 0.1 mg mL<sup>-1</sup>) during 24 h, and afterwards MTT assays were performed to evaluate the cell viability. The only concentration that showed significant toxicity was 0.1 mg mL<sup>-1</sup> for B16F10 and NIH3T3 cells ( $p < 0.01$ ). The apparent low



**Fig. 4** Representative *in vivo* fluorescence bioimaging for anesthetized C57BL/6 mice (a) control; (b) non-tumour mice acquired 4 h after administration of **PBAC-dots** (0.16 mg mL<sup>-1</sup>); (c) melanoma-bearing mice 4 days after tumour induction and 4 h after administration of **PBAC-dots** (0.16 mg mL<sup>-1</sup>); (d) melanoma-bearing mice 8 days after tumour induction and 4 h after administration of **PBAC-dots** (0.16 mg mL<sup>-1</sup>); (e) melanoma-bearing mice 14 days after tumour induction and 4 h after administration of **PBAC-dots** (0.16 mg mL<sup>-1</sup>). White circles indicate fluorescence on dorsal regions. White arrows emphasize fluorescence on the kidney region. White triangles indicate the fluorescence in ureter regions and black circles indicate the tumour region.



cytotoxicity of **PBAC-dots** can be justified by the presence of negative surface charges centred at boronic groups at physiological pH.<sup>56</sup>

The “light-up” characteristic of **PBAC-dots** and the high complexing affinity at pH 7.4 between the boronic and sialic acid groups open up the opportunity to explore this nanoprobe as a tumour imaging agent using *in vivo* models.<sup>18,56</sup> It is well-known that melanoma murine B16F10 cells are tremendously aggressive, can metastasize from a primary subcutaneous site to the lungs and present high levels of sialic acid expressed on their membrane.<sup>57</sup> Hence, to validate the feasibility of the *in vivo* bioimaging probe, experiments on skin cancer-bearing mice were conducted. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of University of Brasilia (UnB) and experiments were approved by the Animal Ethics Committee of UnB. The subcutaneous injection of  $10^5$  B16F10 cells resulted in tumour growth at the injection site. Fluorescence bioimaging of C57BL/6 mice can be seen in Fig. 4.

All C57BL/6 mice treated with **PBAC-dots** presented several fluorescent spots spread all along animal bodies. The bioimaging of healthy C57BL/6 mice treated with **PBAC-dots** was used as control as shown in Fig. 4(a). It was possible to observe the fluorescence on the kidneys and ureter regions, which is a clear indication of the **PBAC-dots** excretion *via* the urinary system. Licciardello and co-workers found that carbon quantum dots had been mainly eliminated by kidneys after 60 minutes of their application.<sup>58</sup> The histological studies demonstrated no abnormalities on brain, kidney, spleen, lung and liver tissues, such as necrosis, apoptosis or haemorrhage

(see Fig. 5(b)). Haematological and serum biochemistry assays are in good agreement with the histological result since they did not show evidence of toxicological disturbances in healthy mice treated with **PBAC-dots** (Fig. S28–S30†).

The bioimages of the cancer-bearing mice acquired 4 h after **PBAC-dots** administration ( $0.16 \text{ mg mL}^{-1}$ ), Fig. 4(c)–(e), show a dispersive fluorescent pattern suggesting that the probe is not selective to the tumour region. Other *in vivo* fluorescent images acquired after 2, 6, 24 and 48 h of 0.16 and  $0.32 \text{ mg mL}^{-1}$  administrations of **PBAC-dots** presented the same behaviour and are available in Fig. S3–S26.† It was possible to observe luminescent spots on kidney and ureter regions.

The histopathological images of the organs are shown in Fig. 5. The studies demonstrated no evidence of tissue damage on the brain and kidneys for all cancer-bearing mice treated with **PBAC-dots**, whereas inflammatory focus and white pulp hyperplasia were observed in liver and spleen tissues, respectively. On lung tissue, there are areas of fibrosis and inflammatory infiltrate.

Fig. 6 shows the histological analysis of the tumour tissues. Surprisingly, after the treatment with **PBAC-dots** a large number of areas of necrosis and inflammatory infiltrate were revealed, which were not identified for cancer-bearing mice without the treatment (Fig. 6(b)–(d)).

Similar results were reported when **C-dots** and graphene-oxide were used in photodynamic therapy,<sup>59,60</sup> however, **PBAC-dots** were able to introduce tumour lesion without external stimulus. Haematological assays were also used to shed light on this astonishing therapeutic effect of the **PBAC-dots**. The major haematological parameters are similar to those presented by the control group, except for the number of white blood cells in cancer-bearing mice whose blood samples were collected 30 days after **PBAC-dots** administration. This alteration can be interpreted as a response of immunological system for the

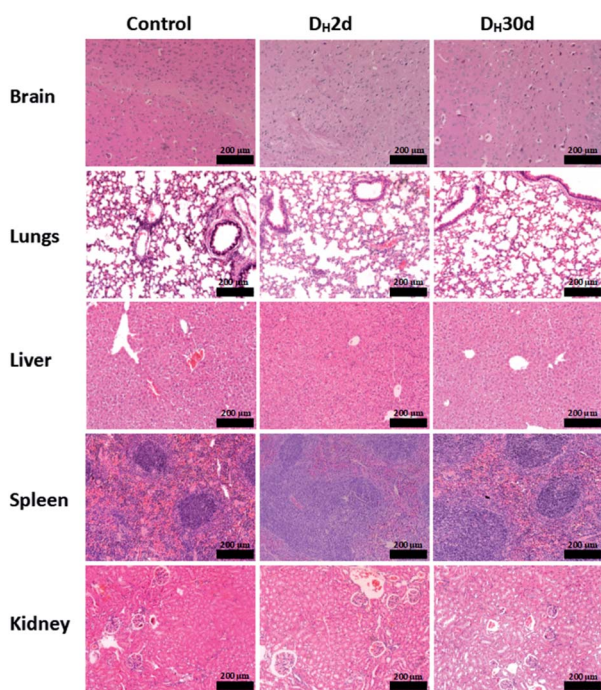


Fig. 5 Histological images of the brains, lungs, livers, spleens and kidneys of the control and cancer-bearing mice treated with **PBAC-dots**.

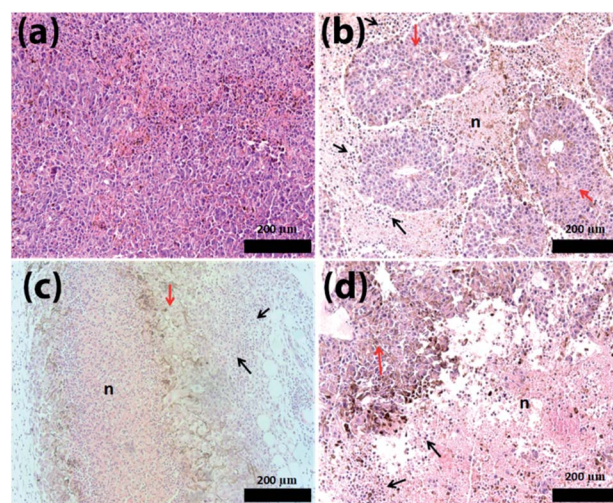


Fig. 6 Histological images of the tumour. (a) Cancer-bearing mice without **PBAC-dots** treatment. Histological images of the tumour-bearing mice treated  $0.16 \text{ mg mL}^{-1}$  of **PBAC-dots** after 4 (b), 8 (c) and 14 (d) days of cancer induction. The “n” indicates necrosis regions, red and black arrows indicate intact tumoral tissue and Immunological cells, respectively.



higher cancer stage, since previous reports have demonstrated that **C-dots** presented non toxicological effects on haematological parameters after 28 days of treatment.<sup>60</sup> Lymphogram results showed a growth of lymphocyte percentage for cancer-bearing mice euthanized 2 and 30 days after treatment, (Fig. S28†). Therefore, **PBAC-dots** can either interact with the tumour and stimulate the immune system to respond more strongly against the disease, or can interfere indirectly on cancer cells abilities to keep themselves undetectable by the immune system.<sup>61</sup> Both hypotheses are plausible for explaining the lesion on the tumour.

Bortezomib (Velcade) is a kind of dipeptide boronic acid that acts as a proteasome inhibitor and was approved by the U.S. Food and Drug Administration (FDA) for use in therapies for a myriad of cancers. The boronic group of the Bortezomib is associated with the protease inhibition that contributes to sensitizing solid tumours to T-cell-mediated killing, stabilizing T-cell activation, and thus improving the outcome of adoptive T-cell therapy of cancer.<sup>62–65</sup> Tumour cells are apparently more susceptible to proteasome inhibition than normal cells caused by the loss of checkpoint mechanisms that occurs during tumorigenesis. Bortezomib and **PBAC-dots** have the boronic acid group incorporated in their structures, thus allowing us to highlight the importance of this acid to the observed outcomes. The results presented in this work also indicate the potential of **PBAC-dots** as checkpoint inhibitor.

Mechanistic knowledge of the cancer sensitization induced by **PBAC-dots** is vital for expanding its application in different cancers and pathologies, however, for that, it would be required more specific studies on a molecular level base.

## Conclusions

In summary, it was demonstrated that the strategy adopted to modify the **C-dots** surface with PBA moieties resulted in a nontoxic light-up probe for glucose detection in aqueous solution, that was well tolerated by mice. It was also determined that **PBAC-dots** can induce necrosis in tumour tissues in cancer-bearing mice. Cell imaging experiments indicated the preference for mitochondria and it could be visualized in both green and red channels. The haematological studies showed a significant increase of lymphocyte that can be associated with immunological stimulation. Future works will be focused on elucidating the antitumoral mechanism of **PBAC-dots**.

## Author contributions

These authors contributed equally.

## Conflicts of interest

There are no conflicts to declare.

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