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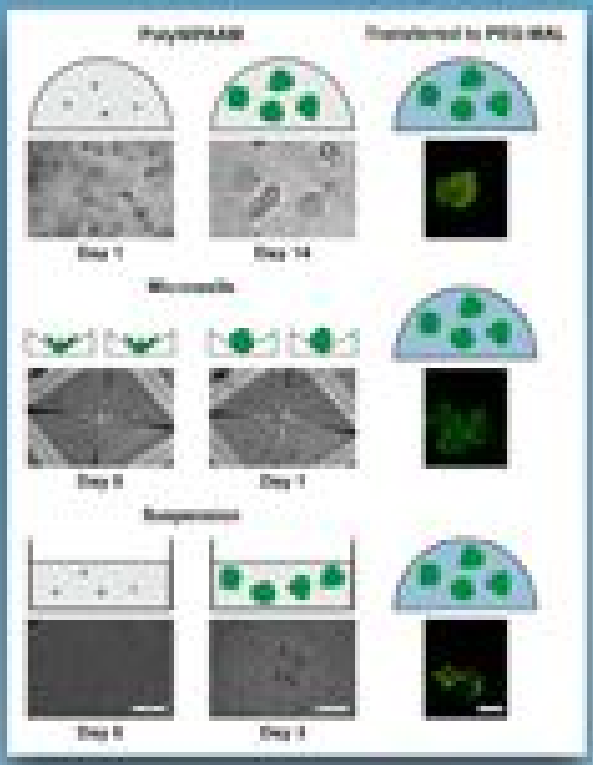

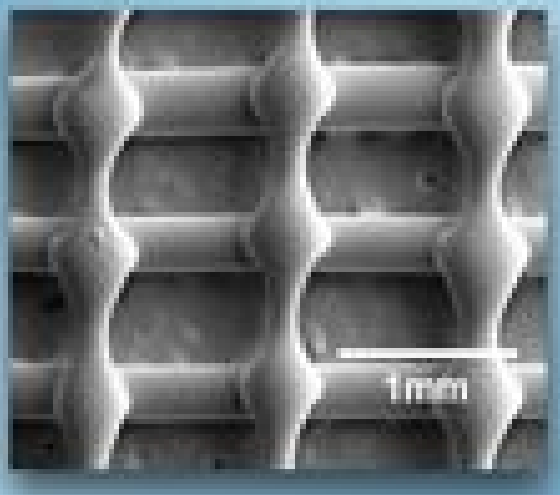

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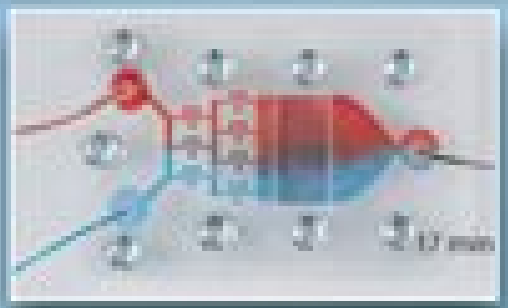

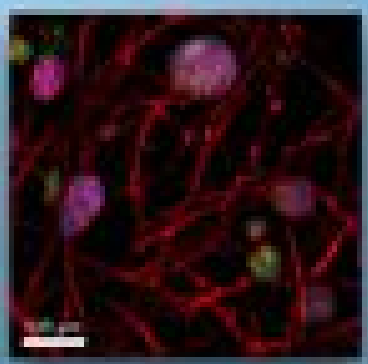

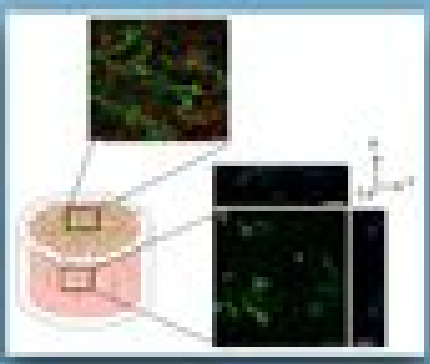

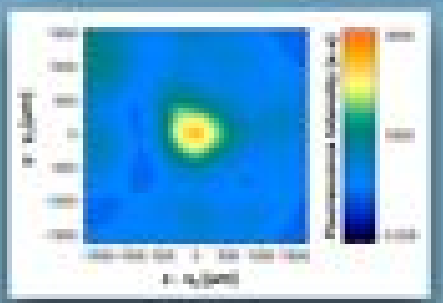

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Bio-interactions and Biocompatibility

Inactivation of HIV-1 Infection through Integrative Blocking with Amino Phenylboronic Acid Attributed Carbon Dots

Yu Yu Aung, Alfinda Novi Kristanti, Siti Qamariyah Khairunisa, Nasronudin Nasronudin, and Mochamad Zakki Fahmi

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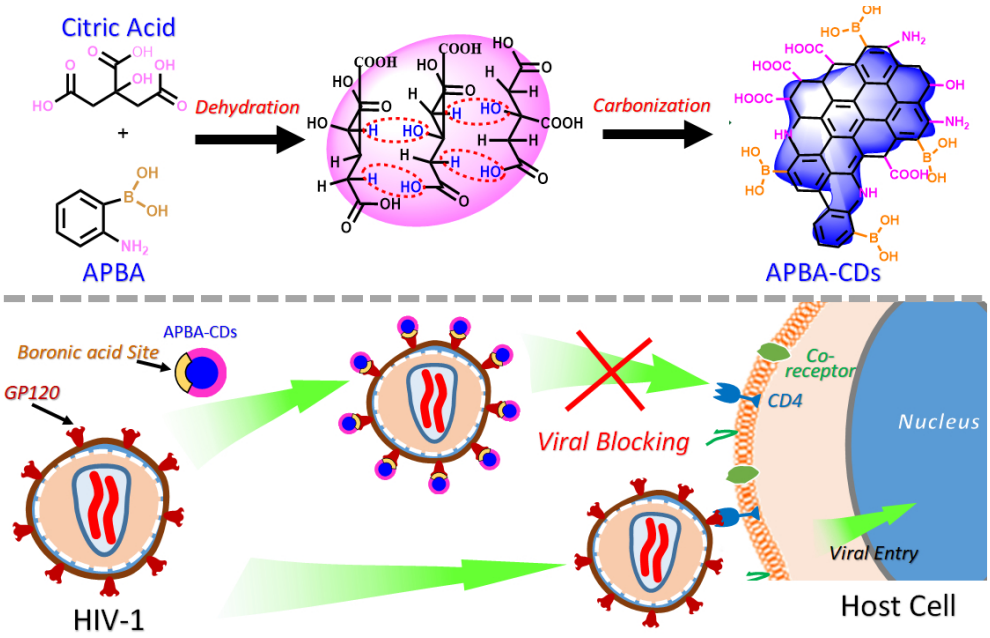

Scheme 1. Synthesis process of APBA-CDs via carbonization and role of this APBA-CDs block HIV-1 infection onto MOLT-4 cell.

200x126mm (144 x 144 DPI)

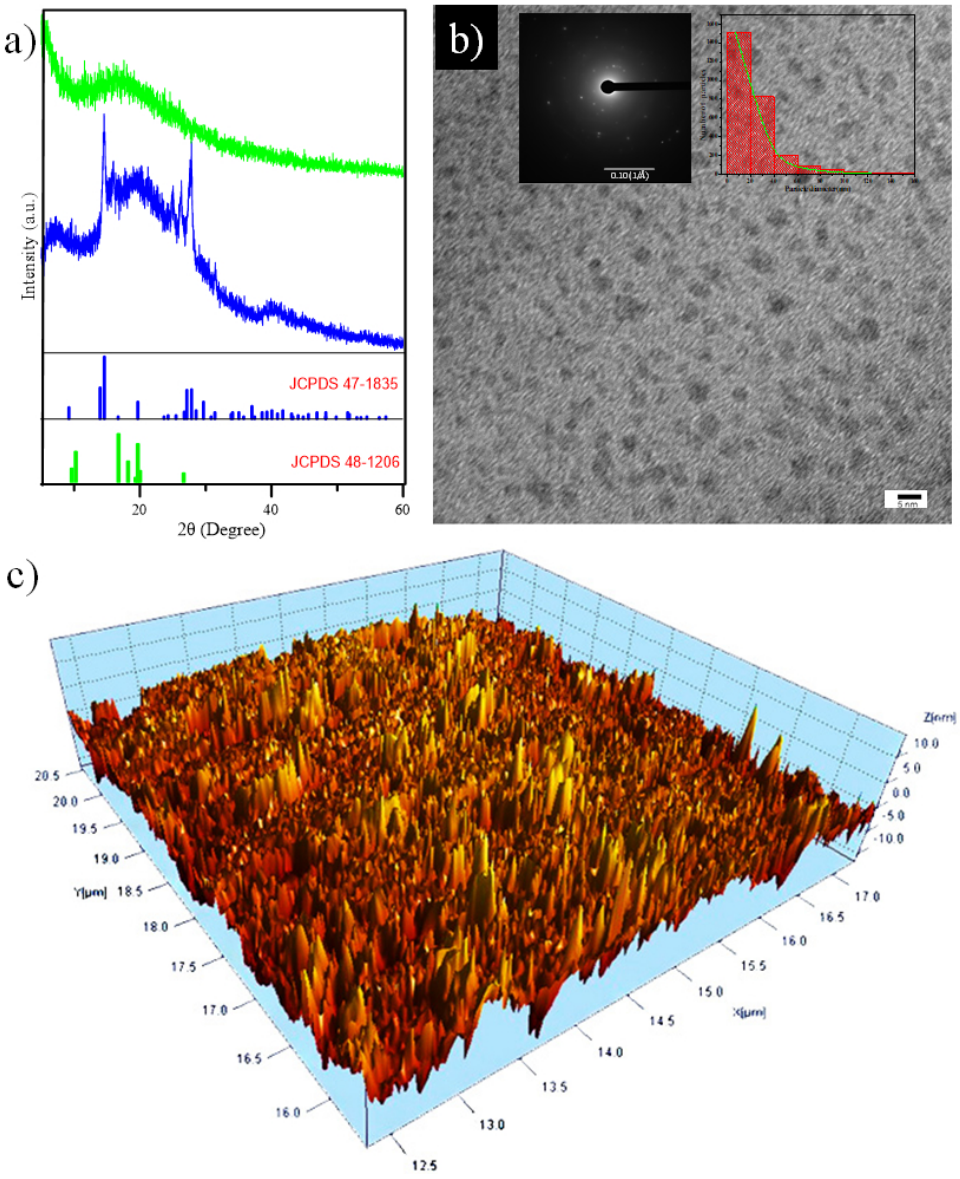

Figure 1. a) XRD pattern of nature CACDs (green) and APBA-CDs (blue), which is confirmed with JCPDs crystal database. b) High Resolution TEM image of APBA-CDs with scale bar showing 5 nm. insert: SAED data of APBA-CDs and its size distributions histogram. c) AFM topographic images of APBA-CDs.

149x176mm (144 x 144 DPI)

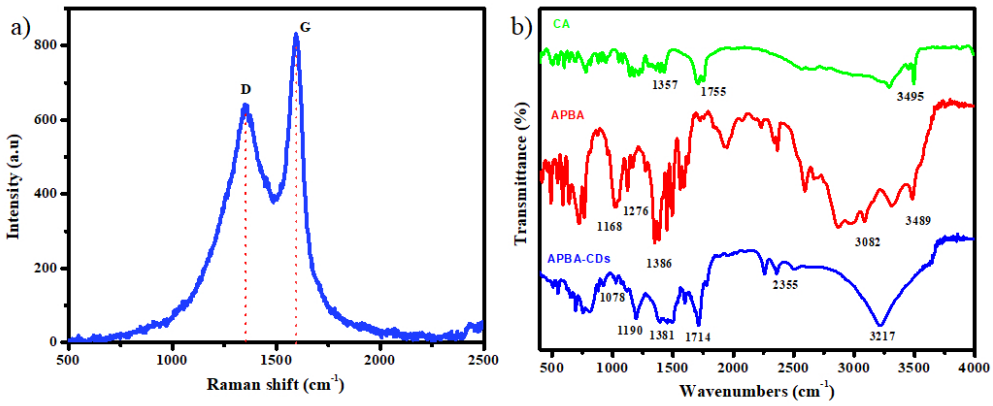

Figure 2. a) Raman spectrum of APBA-CDs and b) FTIR spectra of CACDs, bare APBA and APBA-CDs.

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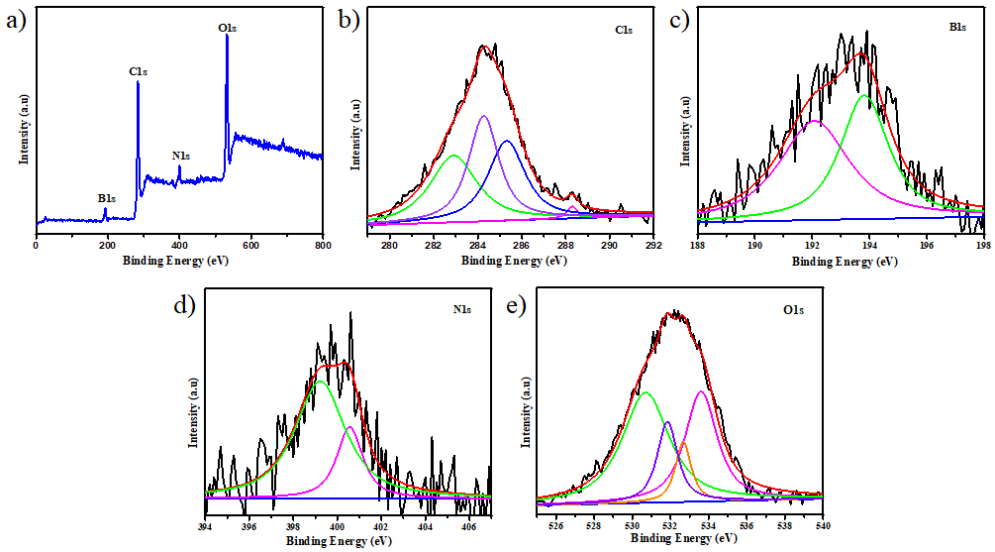

Figure 3. a) XPS survey spectrum of APBA-CDs and its high-resolution spectra on (b) C 1s, (c) B 1s, (d) N 1s and (e) O 1s, respectively.

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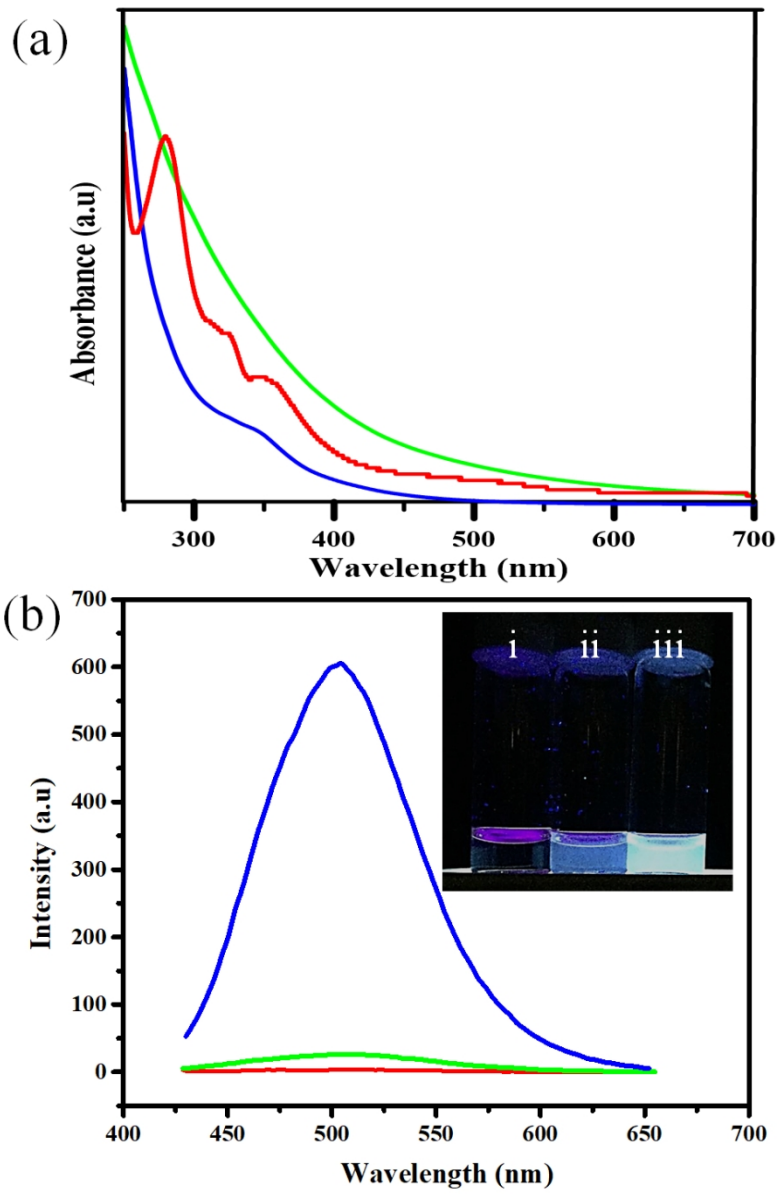

Figure 4. (a) UV-vis spectra and (b) PL emission spectra of the samples (50 μg/mL), including CACDs (green line), nature APBA (red line) and APBA-CDs (blue line). Insert: photograph images of water as a control (i), CACDs (ii), and APBA-CDs (iii) exposed with UV light (365 nm).

168x259mm (144 x 144 DPI)

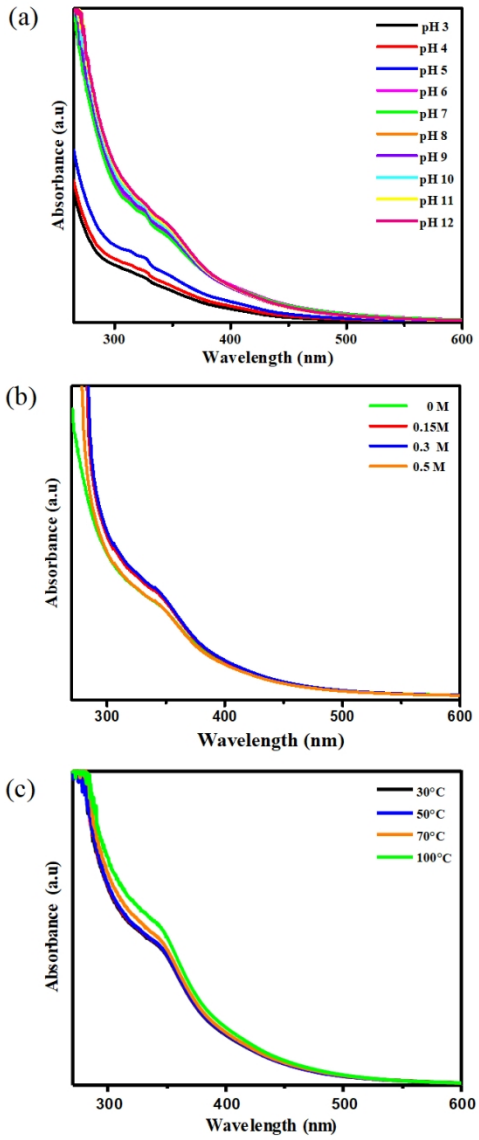

Figure 5. UV-Vis spectra of APBA-CDs solution after 24 h treated with varied pH value (a), NaCl concentration, and temperature (c).

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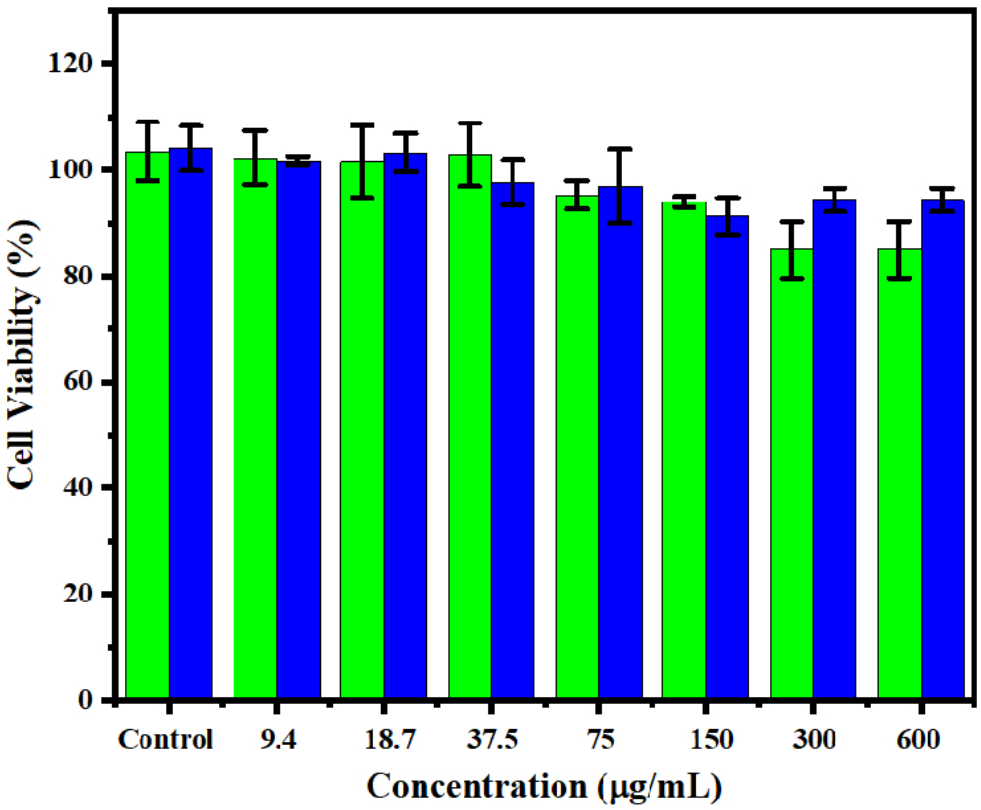

Figure 6. HeLa viability data post 24 h incubated with CACDs (green) and APBA-CDs (blue). The data is performed on the mean ± SD (n = 3).

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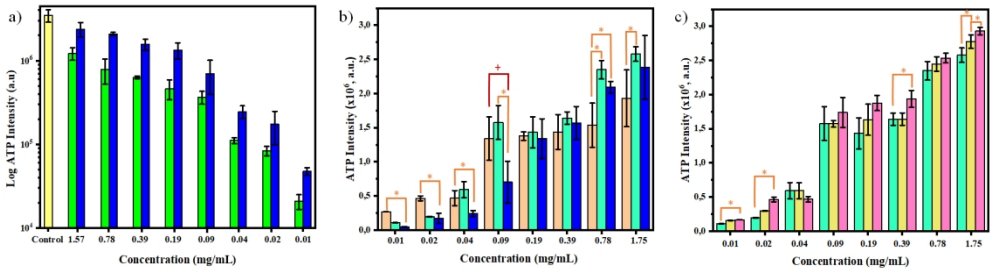

Figure 7. a) Logarithmic data of ATP intensity of MOLT-4 before (yellow bar) and after 24 h infected with MT-4/HIV with the addition of CACDs (green bars) and APBA-CDs (blue bars). b) ATP intensity MOLT-4 after 24 h infected with MT-4/HIV with the presence of Duviral (orange bars), APBA-CDs + Duviral (light green bars), and APBA-CDs (blue bars). c) ATP intensity MOLT-4 after 24 h infected with MT-4/HIV with the presence of APBA-CDs + varied Duviral concentration on 3 mg (light green bars), 6 mg (yellow bars), and 12 mg (pink bars). All of data presented as mean ± SD (n = 3) and t-test with +$p < 0.05$, *$p < 0.01$.

328x89mm (144 x 144 DPI)

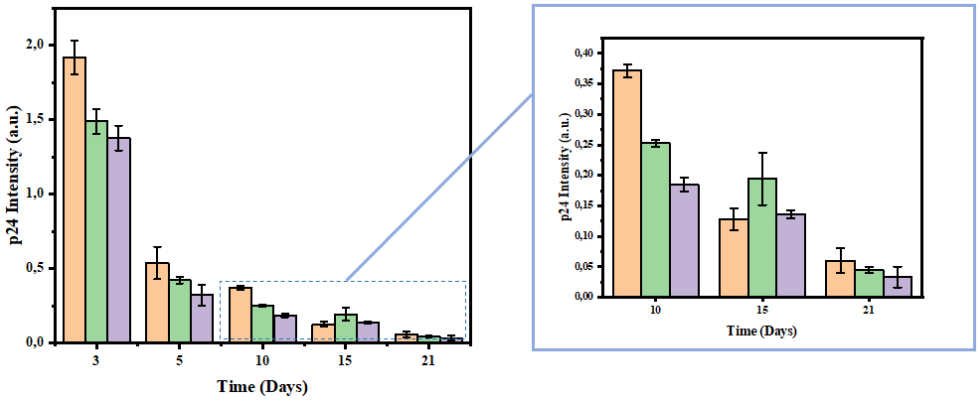

Figure 8. p24 Intensity of HIV-1 virus post-infect to MOLT-4 with the presence of CACDs (orange bars), APBA-CDs (green bars), and APBA-CDs + Duviral (21 mg, purple bars). High magnification for an adjusted area on blue dots box is show on the box. All of data presented as mean ± SD (n = 3)

177x72mm (144 x 144 DPI)

Inactivation of HIV-1 Infection through Integrative Blocking with Amino Phenylboronic Acid Attributed Carbon Dots

Yu Yu Aung[a], Alfinda Novi Kristanti[a], Siti Qamariyah Khairunisa[b], Nasronudin Nasronudin[b], Mochamad Zakki Fahmi,[a,c*]

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Abstract

Current antiretroviral HIV therapies continue to have problems related to procedural complications, toxicity, and uncontrolled side effects. In this study, amino phenylboronic acid-modified carbon dots (APBA-CDs) were introduced as a new nanoparticle-based on gp120 targeting that inhibits HIV-1 entry processes. Prolonged by simple pyrolysis for preparing carbon dots, this report further explores attributing amino phenylboronic acid on carbon dots, which prove the formation of graphene-like structures on carbon dots and boronic acid sites, thereby enabling the enhancement of positive optical properties through photoluminescent detection. Aside from performing well in terms of biocompatibility and low cytotoxicity (the CC_{50} reach up to 11.2 mg/mL), APBA-CDs exhibited superior capabilities in terms of prohibiting HIV-1 entry onto targeted MOLT-4 cells recognized by the delimitations of syncitia formation and higher ATP signal rather than bare carbon dots. The modified carbon dots also promote dual-action on HIV-1 treatment by both intracellularly and extracellularly viral blocking by combining with the Duviral drug, along with compressing p24 antigen signals that are better than APBA-CDs and Duviral itself.

Keywords : carbon dots, amino phenylboronic acid, HIV-1, viral entry, blocking infection

Introduction

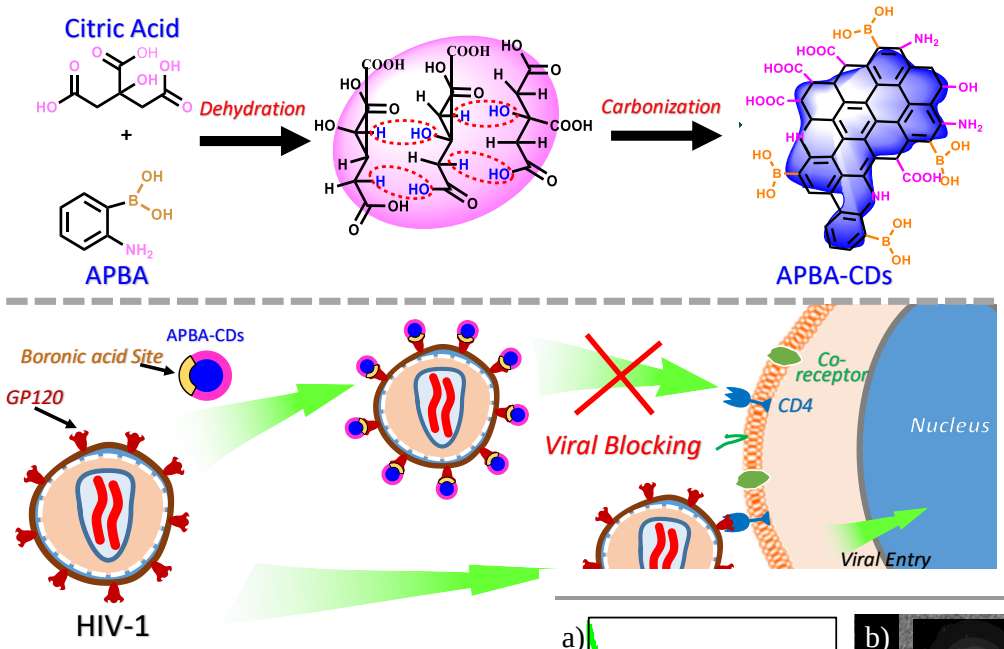
Acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) were discovered in 1983. Since then, HIV has presented a scientific challenge because a complete eradication strategy has not yet been successful.[1] Moreover, HIV/AIDS is currently a global and dangerous disease that has proliferated as an infectious and lethal disease among adults across the world.[2] As a result, it has been intensively investigated by scientists for over three decades.[1, 3-4] Currently, the most promising HIV drug applied has been a highly effective antiretroviral therapy (HAART). Antiretroviral drugs combine three or more types of drugs and have significantly extended the life expectancy of HIV-infected people over the years.[5-6] However, drug-resistant viral strains have made it so that infected people need take various pills daily.[6] Taking multiple drugs for HIV treatment sometimes limits the efficiency of this therapeutic process due to incompatibilities between drugs, toxicity, complex side effects, and obstacles to solvent solubility. Therefore, the exploration of new drugs that target HIV-1 through new methods of action are still required as next-generation efficient therapeutics.[3] HIV is an enveloped RNA virus, where the outside part of the virus contains two layers of lipids with various proteins outside; including gp120 and gp41 glycoproteins.[7] Many researchers have emphasized the advances of fundamental antiretroviral drugs in relation to the interactions of gp120 glycoprotein binding agents,[8-10] wherein the binding mechanism in the viral targeting process is highlighted by gp120 and CD4 receptor interactions. In the field of nanotechnology, many classes of nanomaterials have been widely used for biomedical active therapy for the living system.[2, 6, 11] Nanotechnology-based systems allow the development and modulation of the dispersion of hydrophobic and hydrophilic drugs simultaneously with precise targeting. The ability of nanoscale particles to enter different small tissue systems can have important uses in the clinical field, such as for therapy to treat cancer and virus-based disease.[12-13] In this respect, the surface design and modification of nanomaterial is a pivotal area. Highlighted with excellent biocompatibility, carbon dots (CDs) as part of nanotechnology are actively developed in the fields of bioimaging and biomedical. This carbon-based nanomaterial is also attributed with other attractive properties such as high fluorescence, low toxicity, and good solubility, which allows its application in major fields.[14]

Through a modification process, several carbon-based nanomaterials, such as graphene,[15-16] fullerene,[17-18] and carbon nanotube[19-20] were reported to be specifically targeting HIV. Molecular simulation reported by Zeng et al. informs that the six or five-ring structures on graphene oxide and graphene like structure can perform attraction to HIV-1 by physical interactions and disturb its activity.[21] This finding was supported Martinez et al. proving that activity of fullerene on protease inhibition of HIV-1 after modified with organic molecules.[22] This modification strengthen targeting process of fullerene onto HIV-1 by chemical interaction. Role of modification on carbon-based nanomaterials on enhance the force against HIV-1 was also reported by Iannazzo et al., where modification of Graphene quantum dots or CDs with non-nucleoside reverse transcriptase inhibitors (NNRTI) can increase its activity on HIV-1.[23] Thus, modification process that work for particular targeting ligand are a crucial factor in promoting effectiveness and the effectivity process, especially in medical fields. Recently, boronic acid-

modified materials have been used for numerous biomedical applications, such as for the treatment of diabetes, obesity, and cancer as well as HIV.[24] The unique boronic acids have an easy-to-bind hydroxyl site, such as on saccharide groups, and they potentially undergo changes in ionization transition materials used as potential HIV barriers, cell capture, and cultures in living systems.[6, 25-26]

Notably, the boronic acid-modified materials, which can combine reversibly with 1,2 and 1,3-cis-diols to form boronate ester, are considered a ligand that can rapidly interact with the receptor of gp120.[11, 27] Severin (2009) and Dhara et al. (2015) reported that nitrogen atoms effectively stabilize boronate ester forms and strengthen this cage bonding through a Lewis acid/base interaction.[28-29] Recently, the B-N dative bond on boronate ester was applied to a macromolecule and polymeric system,[30-31] as well as to a cross-coupling bond of complex organic compounds.[32] The use of boronic acid contained materials on has been reported to perform well in terms of affinity with particular DNA of the HIV virus; it is also predicted to accelerate the potential insertion process.[33] The existence of boronic acid on CD as a targeting ligand can be specifically activated in the viral cycle, benefiting from the better interaction of CDs by covalent, noncovalent and electrostatic interaction as well as its optical properties that allow to be used as HIV detection.[7] On our previous report, we modified CDs with p-Carboxylbenzene boronic acid and revealed the importance of boronate ester form on supporting CDs' attraction with HIV.[11] We further though to improve above study by modify CDs with molecule that owing boronic site and nitrogen element, because donation of lone pair from nitrogen to p-orbital of boron reinforce boronate ester formation.[34] Moreover, the enhancement of photoluminescent intensity of CDs by nitrogen doping was well-reported in several studies;[35-36] and supported by additional abilities such as tunable luminescence,[37] catalyst,[38] and molecular and ion detection.[39-40]

The above exposures indicated that the existence of boronic acid sites and nitrogen clearly had a positive impact on the CDs' performance. However, the modification of CDs by the



Scheme 1. Synthesis process of APBA-CDs via carbonization and role of this APBA-CD

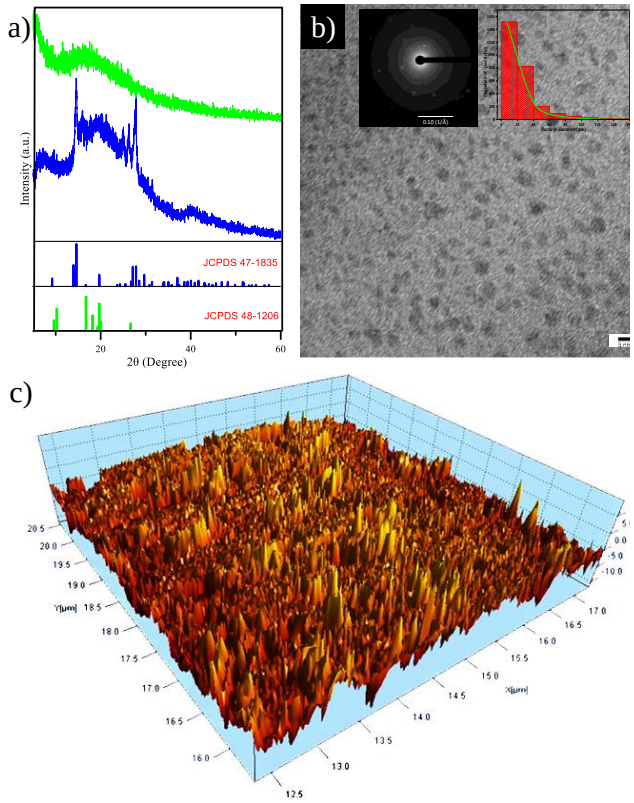


Figure 1. a) XRD pattern of nature CACDs (green) and APBA-CDs (blue), which is confirmed with JCPDs crystal database. b) High Resolution TEM image of APBA-CDs with scale bar showing 5 nm. insert: SAED data of APBA-CDs and its size distributions histogram. c) AFM topographic images of APBA-CDs.

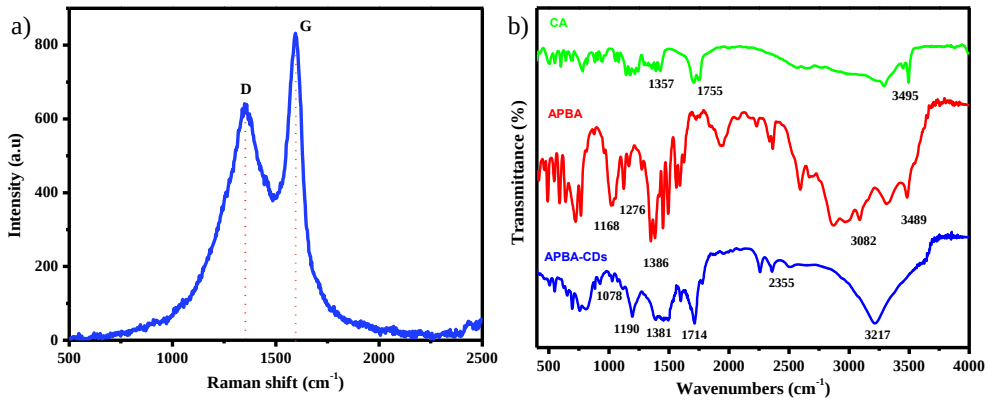


Figure 2. a) Raman spectrum of APBA-CDs and b) FTIR spectra of CACDs, bare APBA and APBA-CDs.

simultaneous addition of boronic acid and nitrogen features was not clearly investigated in the case of HIV treatment. In the present study, we reported the synthesis of CDs that featured simultaneously with boron and nitrogen elements. Citric acid (CA) and 2-aminophenylboronic acid (APBA) were used as carbon and nitrogen sources, respectively, to form amino phenylboronic acid-containing carbon dots (APBA-CDs). This study also applied direct carbonization via a simultaneously pyrolysis process to enable the simple and effective synthesis of APBA-CDs. Aside from basic characterizations of CDs, the potency of performed functional groups on both targeting and blocking agents of HIV replication have been highlighted in the present study, as well as viral entry aptitude.

Results and Discussion

Synthesis and characterization of functional APBA-CDs

This synthesis of amine- and boronic acid-functionalized CDs was executed using pyrolysis-based methods, which were further used as inhibitors of HIV and MOLT-4 cells interaction, as illustrated in Scheme 1. The pyrolysis process allows dehydration, which connects the compounds, and carbonization at a high temperature to guide the integration of citric acid and APBA to form a graphene oxide-like structure.

The crystalline phase of boronic acid-containing carbon dots was first proven by X-ray diffraction (XRD) analysis, as illustrated in Fig.1a. Pure carbon dots (CACDs) displaying broad bands located around 19.68° (d = 4.50 Å lattice spacing) revealed the nature of carbon dots and the higher interplanar spacing of C_{70} structure that reported close to graphitic carbon (JCPDS 48-1206).[41] The spectrum of the APBA-CDs still perform a broad signal at 19.68,° combined with several sharp peaks, these sharp peak similar to inderborite (JCPDS 47-1835) revealing boronic structure and the crystalline phase is more prevalent on the surface of APBA-CDs.[42] The morphology of APBA-CDs was then determined using high-resolution TEM (HRTEM); the results are presented in Fig. 1b. The obtained HRTEM image shows that the carbon dots have a natural graphene lattice structure with an average diameter of 5 nm. In addition, the crystal structural form of carbon dots was confirmed by analyzing SAED images as supporting HRTEM images. The atomic force microscopy (AFM) analysis (Fig. 1c) yielded a typical topographic image of the APBA-CDs that confirmed the HRTEM data stating that the diameter size of the obtained APBA-CDs was about 5 nm. This also confirms that these APBA-CDs contain many graphene layers.

The existence of a graphene-like structure on APBA-CDs was examined through Raman spectroscopy, as indicated in Fig. 2a. The Raman spectrum of the APBA-CDs exhibits D band at 1370 cm^{-1}, which is related to the formation of sp^3 hybridized carbon atoms and the higher G band at 1578 cm^{-1}, referring to the presence of sp^2 hybridized carbon on the APBA-CDs. Generally, the Raman spectrum ID/IG ratio of APBA-CDs is 0.86, which confirms that the disordered graphene contains both sp^2 to sp^3 hybridized carbon. Furthermore, the ratio on the Raman spectrum indicates a high degree of graphitization for a higher impact quality of carbon-based nanomaterials (CDs).[43] The next FTIR analysis compared CA, APBA, and APBA-CDs spectra, which were recorded as 400 to 4000cm^{-1} in Fig. 2b. The FTIR spectrum of CA exhibits peaks at 3495, 1755, and 1357 cm^{-1}, which can be attributed to O-H, C=O, and C-H stretching, respectively. The FTIR spectra of APBA shows peaks at 3489, 3082, and 1562 cm^{-1}, which have been confirmed to correspond to -NH_2, C-H, and N-H bending vibrations. The FTIR peaks at 1386, 1276, and 1168 cm^{-1} can be assigned to bands resulting from B-O, C-N, and B-OH vibrations. There is no C=O peak in the APBA spectrum. Therefore, in the spectrum of APBA-CDs, the hydroxyl group peak becomes sharp and is located at around 3217 cm^{-1} indicating mostly on nitrogen vibration. The major peaks of APBA-CDs are found at 2355, 1782, 1381, 1190, and 1078 cm^{-1}, corresponding to N-H, C=O, B-O, and B-OH bending, and C-B stretching vibrations, respectively. The data above clearly indicate that the boron is doped on the CDs' part. Furthermore, the XPS data supporting the FTIR results confirm the existence of carbonyl, hydroxyl, and amine groups on the

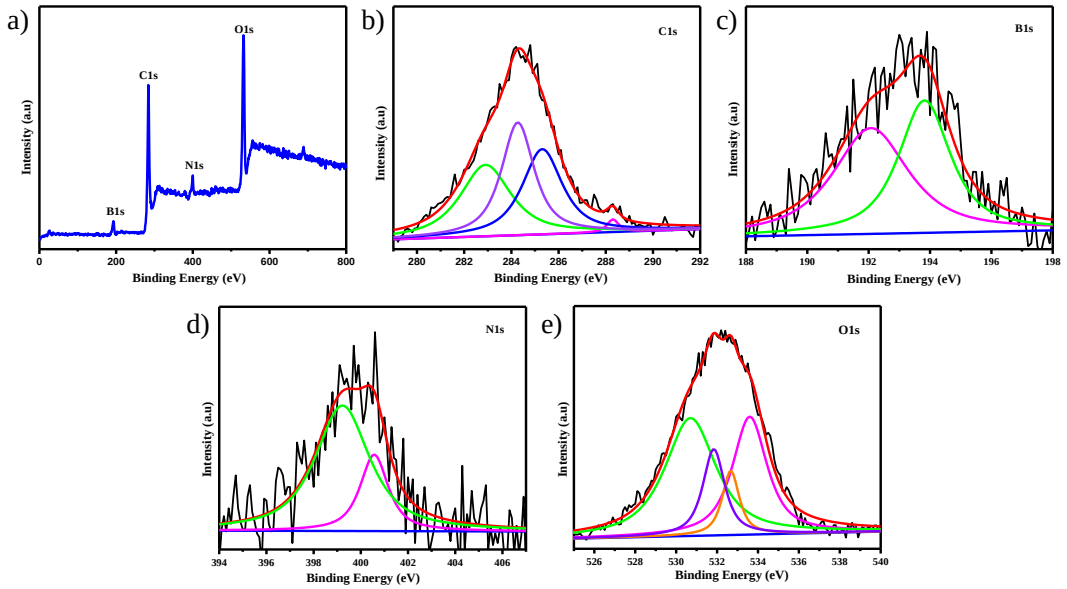


Figure 3. a) XPS survey spectrum of APBA-CDs and its high-resolution spectra on (b) C 1s, (c) B 1s, (d) N 1s and (e) O 1s, respectively.

synthesized APBA-CDs, which can be used as key functional groups for further modifications.

To confirm the chemical structure and performed functional groups on CDs, X-ray photoelectron spectroscopy (XPS) was performed. The high-resolution spectrum of APBA-CDs contains four peaks, namely C1s, B1s, N1s, and O1s (as furnished in Fig. 3). The C1s peak can be deconvoluted into four components with different binding energy (Fig. 3). The peaks located at 283.0 eV, 289.3eV, 285.3 eV, and 288.3 eV, can be attributed to the C-B bond for sp^2 on (C-C)/(C=C), sp^3 on(C-N) and COOH bonds, respectively; these indicate that most of the carbon atoms are located in a conjugated lattice.[44-45] The B1s spectrum of APBA-CDs, which is assigned to the binding energies at 191.6 eV (B-OH) and 193.82eV (B-O), confirmed the presence of the boronic acid site of CDs.[46-48] The high-resolution XPS spectrum of N1s includes two peaks. The binding energy at 399.2 eV can be attributed to N-H bonds, and at 400.6 eV, it can be associated with pyrrolic N bonds on the APBA-CDs.[26, 49] The binding energies of O1s spectrum at 531.9, 532.7, and 534 eV are confirmed to correspond to the O-H, C-O, and B-O bonds (on the boronic acid form) on the CDs, respectively.[46-47, 50] All XPS results strongly suggest that APBA-CDs perform N-H functional groups and maintain the its boronic acid site.

For further characterization, the UV-Vis analysis of APBA-CDs performance shoulder peak as excitation of CDs on 312-358 nm (supporting information, Fig. S1a); this peak indicates n-π* transitions of APBA CDs. In comparison with APBA and nature CDs, the UV-Vis spectra showed bands at 264-281 nm and 312-358 nm on both APBA-CDs and CACDs, corresponding to π-π* and n-π* transitions (Fig. 4a). In particular, APBA-CDs also showed an absorption band below 262 nm, which is related to the π-π* transition of C=C, while the broad absorption band at ~350 nm can be assigned to a n-π* transition of C=O and C=N vibrations on the surface of APBA-CDs. The PL emission band of APBA-CDs is more intense than bare APBA and CACDs (Fig. 4b). Increasing emission intensity due to the addition of nitrogen and boron elements was supported separately in previous reports.[51-53] It can be the first advantage for conjugating APBA onto CDs, which initiate increasing QY to 75% (APBA-CDs)

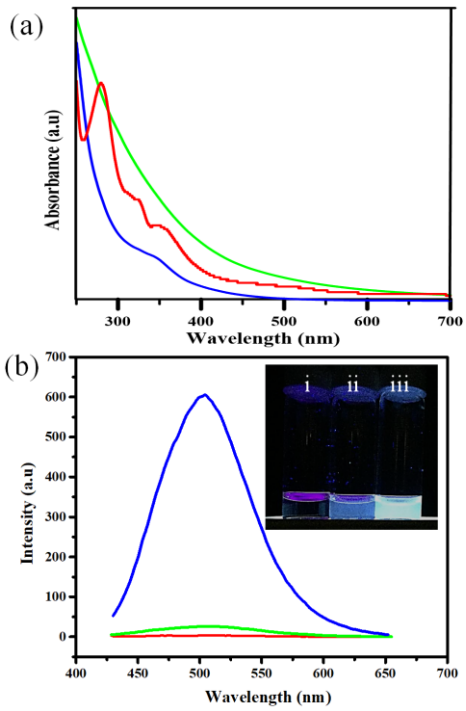


Figure 4. (a) UV-vis spectra and (b) PL emission spectra of the samples (50 µg/mL), including CACDs (green line), nature APBA (red line) and APBA-CDs (blue line). Insert: photograph images of water as a control (i), CACDs (ii), and APBA-CDs (iii) exposed with UV light (365 nm).

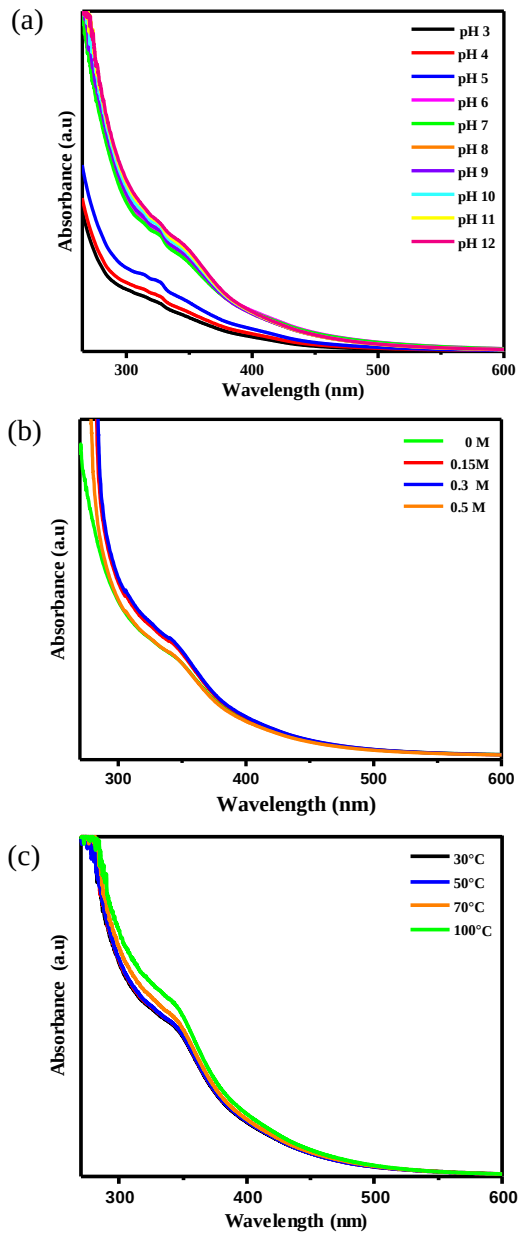


Figure 5. UV-Vis spectra of APBA-CDs solution after 24 h treated with varied pH value (a), NaCl concentration, and temperature (c).

from 26% (CACDs). Moreover, this broad emission band can be attributed to the complicated band structure and random energy levels of the CDs.[54] While excitation wavelength is varied, the maximum emission of APBA-CDs become to be shifted (supporting information, Fig. S1b), where increasing the excitation wavelength drove the emission to red-shift. APBA-CDs also showed the strongest fluorescence at a 400-nm excitation wavelength. Furthermore, the prepared APBA-CDs exhibited a moderately excitation-dependent emission spectrum ranging from 320 to 400 nm. This phenomenon was quite common on CDs due to the combination of surface traps, edge state, and size effects.[55-56] The unique PL properties of CDs are the most interesting and important effects in terms of analyzing PL derivatives and adjusting the properties of CDs for next novel applications.[57]

Colloidal stability (pH, ionic strength, and temperature effect)

The next investigation focused on the colloidal stability of as-prepared APBA-CDs such the pH activity, ionic strength, and temperature effects of APBA-CDs. Fig. S2 (Supporting Information) shows the stability of APBA-CDs at pH 3-12 for 24 h indicated no precipitates at pH 6-12 for 24 h. The molecular interaction, which was highlighted with hydrogen bonding and the electrostatic interaction, was quite intense in the APBA-CDs induced destruction of the colloidal structure and following precipitate on the nanoparticle. Destruction of colloidal system of APBA-CDs solution was also investigated by UV-Vis absorption analysis (Fig. 5a), in which all spectra performed regular spectra of APBA-CDs; however, the spectra on pH 3-5 exhibited a slight deformation pattern and it was confirming by a coagulation on the samples. This statement on occurring coagulation of samples was also confirmed by assess turbidity value on each sample. Fig. S3a show turbidity value for APBA-CDs on varied pH, where commonly on range 2.13 to 2.88 NTU for pH 6-12 and increase up to from 4.41 to 7.89 NTU from pH 5-3. This increasing tends to instability of APBA-CDs on low pH and supporting above data from UV-vis spectra. Moreover, the structure of APBA-CDs was stable at varied salt concentrations up to 0.5 M and varied temperature conditions up to 100°C, as illustrated in Fig. S2b-c (supporting information) and 5b-c. Even on high-salted concentration and temperature, the solutions were still clear and the spectra that almost similar one to another indicating no sufficient change on APBA-CDs solution after 24 h treatment. Again, these also confirmed with turbidity assessment (Fig S3b&c), where no significant enhancement of its turbidity value. This colloidal stability data obviously suggested that obtained APBA-CDs was stable on circumstance condition of human body. Thus, suitable to be used on the chemo/ biological application.

Cytotoxicity evaluation

Cytotoxicity assessment is crucial aspect on showing the feasibility of APBA-CDs in biomedical applications. We subsequently studied the effect of various concentrations of CACDs and APBA-CDs (1 to 400 µg/ ml) on the viability of HeLa cell (human cervical cancer cell) by using Cell-Counting Kit 8 (CCK-8) assay. The assessment in Fig. 6 shows the HeLa viability post 24 h treatment with varied concentrations of CACDs and APBA-CDs. The cytotoxicity data reveals, even the concentration of CACDs and APBA-CDs is enhanced up to 600 µg/ml, the cell viability percentage still over 80% means the carbon dots not harms the cell. Further investigation of cytotoxic effect is also revealed the CC_{50} of both CACDs and APBA-CDs are 19.9 and 354.8 mg/mL, respectively (Fig. S4, Supporting Information). These data strongly indicate that the CDs sample

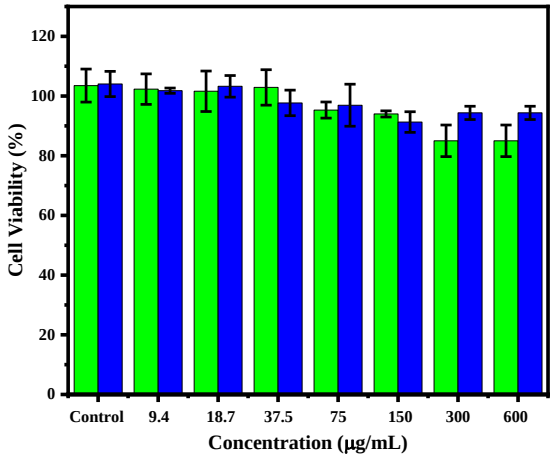


Figure 6. HeLa viability data post 24 h incubated with CACDs (green) and APBA-CDs (blue). The data is performed on the mean ± SD (n = 3).

exhibited a low cytotoxic effect and feasible for biomedical applications.

Cytopathic inhibition efficiency of APBA-CDs

The key factor of HIV-1 infection is the attraction of gp120 site on the virus with the CD4 receptor /chemokine co-receptor on the surface of the host MOLT-4 cells. To prove it, we explore potential attraction on HIV-1 compared with MOLT-4 by tracking fluorescence properties owing by APBA-CDs with flowcytometry method (Fig. S5, supporting Information). We used MOLT-4 cell and MT-4 infected HIV-1 as represent of the virus due to the instrument cannot detect flow of object as small as virus. The data on Fig. S5 showed that fluorescent intensity of APBA-CDs on MT-4/HIV-1 was significantly higher than MOLT-4 cell. Even the CDs exist on cell by physical attraction on membrane surface and forward to insert on MOLT-4 cell, but the number was quite low compared with it is on the HIV-1. This finding proves the role of APBA-CDs on its antiviral mechanism that more likely to interact with HIV-1 instead of the cell, along with approve the assumption the attraction of gp120 and boronic acid site on strengthen the virus and CDs attraction.

Next on cytopathic assessment, we used syncitia assay for the first assessment in this study. This assessment is a quantitative assay based on syncitia formation. We first cultured HIV-1 on an MT-4 cell as an infected cell and further introduced it to MOLT-4 as another kind of T Cell, the infection route of MT-4/HIV-1 to MOLT-4 will produce syncitia as a cytopathic effect; where it cannot happen if we just add bare MT-4 or another kind of T cells. Therefore, the Syncitia formation was a significant indicator of infection with HIV. The investigation of the inhibition ability of unmodified carbon dots (CACDs) and APBA-CDs are depicted in Fig. S6 (supporting information). By comparison with bare MOLT-4 cells as a negative control and bare MT-4/HIV cell as a positive control, the production of Syncitia was compressed after the 24 h incubation of MT-4/HIV-1 with CACDs and APBA-CDs at low concentration (0.02 mg/mL). The figures confirm that, even though it still appears on treated CACDs and APBA-CDs, the syncitia number does not grow substantially like the positive control. To ensure the inhibition activity of CACDs and APBA-CDs, we further investigated the process by tracing the amount of intracellular ATP on the normal cell using a ToxGlow ATP marker. It is well-understood that the infection of MOLT-4 cells by MT-4/HIV-1 will destroy the organelle structure of the cell and decrease the number of ATP as the energy source of the live cell. Therefore, the amount of syncitia will opposite intracellular ATP. The result in Fig. 7a demonstrates that decreasing of cellular ATP follow the increasing concentration of CACDs and APBA-CDs. In fact, the ATP intensity can reach similar to negative control (bare MOLT-4) when the concentration of the carbon dots is set on 1.57 mg/mL. Even CACDs provide active sites (belonging to hydroxyl and carboxyl functional groups) that allow physical interaction with gp 120; the existence of boronic acid on APBA-CDs can improve the attraction with the HIV, affecting its higher ATP intensity CACDs. The boronic acid site on APBA-CDs raised the possibility of forming chemical bonding with gp120 that further obstructs viral infection better than merely physical interaction. Szunerits *et al*. have explained that the carboxyl- and boron-containing hydroxyl groups are good ligands; it can be attracted to amino moieties of reverse transcriptase containing HIV-through H-bonding and covalent bonding and block the reverse transcriptase phase of the viral life cycle.[58-59]

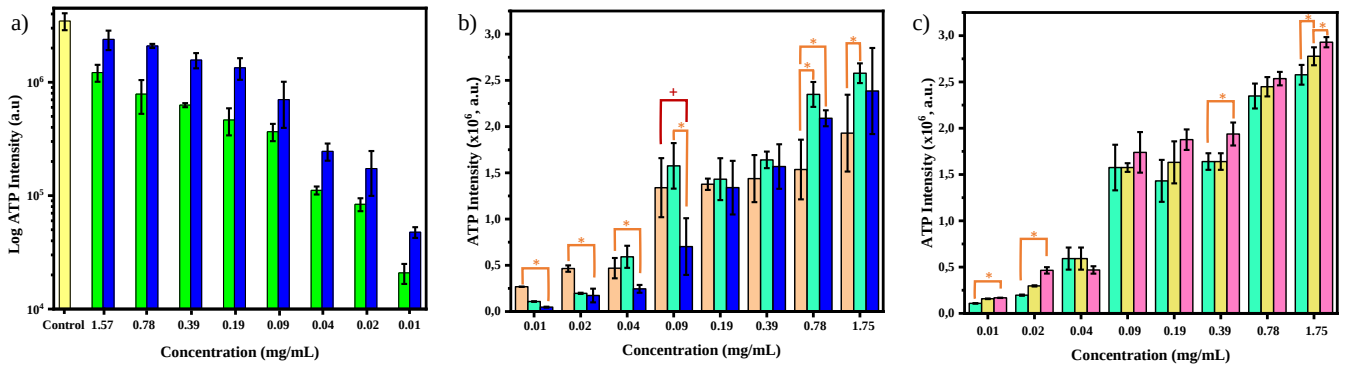


Figure 7. a) Logarithmic data of ATP intensity of MOLT-4 before (yellow bar) and after 24 h infected with MT-4/HIV with the addition of CACDs (green bars) and APBA-CDs (blue bars). b) ATP intensity MOLT-4 after 24 h infected with MT-4/HIV with the presence of Duviral (orange bars), APBA-CDs + Duviral (light green bars), and APBA-CDs (blue bars). c) ATP intensity MOLT-4 after 24 h infected with MT-4/HIV with the presence of APBA-CDs + varied Duviral concentration on 3 mg (light green bars), 6 mg (yellow bars), and 12 mg (pink bars). All of data presented as mean ± SD (n = 3) and t-test with $^{+}p < 0.05$, $^{*}p < 0.01$.

The determination of EC_{50} values (on Fig. S7, supporting information) are reflected in the data in Fig. 7a, where CaCDs and APBA-CDs result in EC_{50} of about 3.72 and 0.48 mg/mL, respectively. Compared with EC_{50} reports from previous studies (Table S1, Supporting Information), APBA CDs perform relatively well in terms of EC_{50}. Furthermore, APBA-CDs has a superior Selectivity Index value (up to 3720.93). This highest value reveals that APBA-CDs can be effectively used in both in vivo and medical clinics by saving drugs, offering a wide variability of concentrations for multi-complex treatment and minimizing side effects during the treatment.[60]

The performance of APBA-CDs as a viral infection agent in this study is then engaged with Duviral as a commercial HIV-1 drug to explore its potential combination. Duviral was a multicomponent drug that mainly consisted of Lamivudine and Zidovudine, which both synergy on work as nucleoside reverse transcriptase inhibitors (NRTIs) by intracellularly block enzyme initiating the transcriptase process. Fig. 7b illustrates that APBA-CDs works more effectively at high concentration than the others, while Duviral demonstrates working wells at low concentrations. This phenomenon could occur because APBA-CDs work extracellularly by touching the virus surface, which could be related to the amount of gp120 sites. Therefore, at low concentrations, the HIV binding on APBA-CDs was limited. However, the combination of APBA-CDs with Duviral performs better in terms of diminishing the infection. This finding enables a new innovation for HIV clinical treatment, since it can optimize the inhibition process. Indeed, the combination of lamivudine and zidovudine does not cure HIV well, but the addition of APBA-CDs will create a multi-action drug that can intracellularly and extracellularly attack HIV. This is then improved by evaluating the cytopathic effect of the virus against APBA-CDs with varied Duviral concentrations (Fig. 7c). Although it shows irregular data at low concentrations, increasing the Duviral concentration on APBA-CDs enhances the ATP intensity at high concentrations (over 0.09 mg/mL), meaning the cytopathic effect is prohibited, and the infection can be avoided.

Viral assessment

To provide comprehensive data and ensure the viral inhibition potency of obtained CDs, the investigation subsequently focused *in-vitro* on determining the existing virus by tracing the p24 antigen. This antigen is a protein that constructs most of the HIV capsid; thus, high concentrations of p24 antigen on blood indicates a high number of active HIV undertaking the infection. Fig. 8 presents the p24 antigen level of the treated MOLT-4 that was post-infected with MT-4/HIV-1. In general, the figure confirms that the p24 concentration had obviously decreased by the 15th day after being treated with 1.75 mg/mL of CACDs, APBA-CDs, and APBA-CDs+Duviral (21 mg).

The alighting of p24 intensity by time indicates the desisting of p24 release that regularly accompany infection process on the cell substrate and it concerning to deactivation of HIV-1 infection. The reduction pattern of p24 by all of the samples (Fig.8) suggests similarities with previous findings, where APBA-CDs+Duviral (21 mg) is dominantly lower than the others, and it still works after 21 days. These results confirm that the viral activity of APBA-CDs is stronger than the viral activity of CACDs and that it be better when combined with the Duviral commercial drug to perform synergetic treatment.

Conclusion

In the present work, we reported potential applications of carbon dot nanoparticle and its boronic acid modification for inactivating the HIV-1 infection. Even prepared with a simple pyrolysis process through decarboxylation and self-assembly reactions, this report further confirms the formation of a graphene-like structure on carbon dots that improve its low toxicity, biocompatibility, and high stability properties under

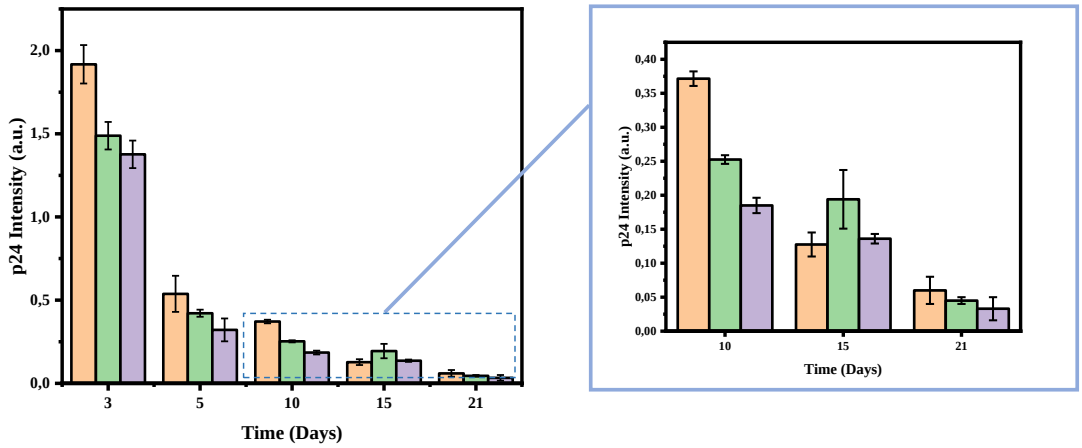


Figure 8. p24 Intensity of HIV-1 virus post-infect to MOLT-4 with the presence of CACDs (orange bars), APBA-CDs (green bars), and APBA-CDs + Duviral (21 mg, purple bars). High magnification for an adjusted area on blue dots box is show on the box. All of data presented as mean ± SD (n = 3).

varied conditions. Attributing boron and nitrogen elements on APBA-CDs increases its fluorescence emission, even significantly enhancing its ability to prevent the insertion of HIV-1 into targeted cells by engaging gp120 and prohibiting syncitia formation. Combination with Duviral made APBA-CDs perform a multi-task action in terms of inactivating the HIV-1 infection through intracellular and extracellular blocking, as proven by the ATP intensity pattern and decrease in p24 antigen. All results data confirm the therapeutic efficiency of using APBA-CDs; this can be a fundamental reference for further improvements in complex HIV therapies and one solution for the current outbreak of disease caused by the other analog virus.

Experimental section

Materials

All the chemicals used in the experiments are commercially available analytical grade products and were used without any specific purification. Citric acid (CA, 99%), 2-Amino phenylboronic acid (APBA, 97%), sodium hydroxide (NaOH, 97%), hydrochloric acid (HCl, 37%), sodium chloride (NaCl, 99.5%), sulphuric acid (H_2SO_4, 99.9%) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Cell Counting Kit-8 (CCK-8) kit was purchased from Dojindo Molecular Technologies (Rockville, USA). RPMI-1640 medium and Dulbecco's Modified Eagle medium (DMEM) was purchased from Wako Pure Chemical Corporation (Osaka, Japan). Viral Tox-Glo assay (G8941) was purchased from Promega (Wasconsin, USA).

Synthesis of APBA-CDs

Amino phenylboronic acid conjugated carbon dots were synthesized from anhydrous citric acid (CA) and 2-amino phenylboronic acid (APBA) via pyrolysis as previously reported.[26] Experimentally, 216 mg of CA and 184 mg of APBA were calcinated on isolated reactor at 270°C for 4h. The obtained sample was further naturally cooled at stable room temperature and dissolved with 1M NaOH solution. After neutralized the solution using HCl, the obtained samples are sign as APBA-CDs.

Cells and Viral Incubation

The human acute T lymphoblastic leukemia cell (MOLT-4/ MT-4) and human cervical cancer cell (HeLa cell) lines were cultured as monolayers in RPMI-1640 medium and Dulbecco's Modified Eagle medium (DMEM) by using supplemented nutrient such as 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 µg ml^{-1}streptomycin. These cells were incubated under stable temperature at 37°C and humidified condition of 5% CO_2.

HIV-1 virus was obtained from the infected peripheral blood mononuclear cells (PBMCs) by applying phytohemagglutinin (PHA, 10µg ml-1). This virus introduced to MT-4 cells resulting infected MT-4 (MT-4/HIV-1), while MOLT-4 further used as targeted cell for MT-4/HIV-1 infection as well as control cell. The cells were co-cultured in a developed culture medium for three days in a 5 % CO_2 incubator. The MOLT-4 cell, MT-4 cell, and HeLa cell, and HIV-1 virus were obtained from Surabaya, Indonesia.

Cytotoxicity assessment

Cell cytotoxicity was quantified by referring its viability using the colorimetric CCK-8 Cell Proliferation Regent. The CCK-8 assay is based on the WST-8 (highly water-soluble tetrazolium salt), which produces the coloring formazan from the living tissue. In the step one, the APBA-CDs samples was firstly added with the DMEM medium. Afterwards, the cultured HeLa cells were added in the 96 well plate with density 2×10^5 cells per µL in 100 µL sample combined with medium condition. After 24 h incubation, CCK-8 reagents (10 µL) was added to each well plate and incubated for 4 hours under stable temperature 37 °C in a 5% CO_2. After incubation, the cells were examined the absorbance of each sample using an ELISA microplate reader at the 450 nm. The cytotoxicity activity of APBA-CDs was also expressed at half of the cytotoxicity concentration (CC_{50}) and was examined with origin software.

Cytopathic effect and Antiviral assay

The infection process will produce Syncitia as result of cytopathic effect and decrease of ATP number of host cell. Therefore, the number of Syncitia can be used to assess and quantify antiviral efficiency of the synthesized APBA-CDs samples. In this assay, we design the infection by mix MT-4/HIV-1 cells and MOLT-4 cells (4×105 cell per µl), only MOLT-4 cell as common T cell that produce syncitia while it infected by MT-4/HIV-1 cells. For, cytopathic effect inhibition assessment on sample, the adjusted concentrations of carbon dots samples were combined firstly introduced to MT-4/HIV-1 cells (2×104 cell per µL)) and incubated for 24 h at temperature 37 °C and 5% CO_2. Subsequently, MOLT-4 cells (4×105 cell per µl) was added to the mixture and incubated for 24 h for allowing infection process. The infection will promote syncitia number as cytopathic effect and decrease adenosine triphosphate (ATP) of MOLT-4 cell. Thus, the number of syncitia were observed by optical microscope and counted by comparing absorbance intensity of adenosine triphosphate (ATP) on adjusted sample with negative control using Viral ToxGlo G8941 kit (Promega Corp., Madison USA). Further improvement on this Syncitia assay, the performance of APBA-CDs also be combined and compared with Duviral (Kimia Farma Co Ltd., Jakarta) as conventional HIV drug. The Origin software was used to express the inhibiting activity and determining EC_{50} counted value via single dose response.

The investigation on CDs attraction on HIV-1 and cell were carried out based on flowcytometry assessment. In detail, the virus represented on MT-4/HIV-1 and MOLT-4 cell were prepared on 6-well culture plate and cultured with RPMI medium. the APBA-CDs solution was then added to the well and adjusted the concentration up to 50 µg/mL by adding RPMI medium and incubated for 24 h. The MT-4/HIV-1 and MOLT-4 cell was further collected by centrifugation (1000 rpm, 10 min), washed twice with Phosphate Buffered Saline buffer, and dispersed with DI water. The emission of APBA-CDs on both MT-4/HIV-1 and MOLT-4 cell were tracked by Guava® easyCyte 5 Benchtop Flow Cytometer (Merck Millipore, Darmstadt Germany) using blue excitation laser (488 nm) and default setting on emission green fluorescence (525 nm/PM1).

To deep improving the antiviral activity of APBA-CDs, the amount of HIV on treated samples were accessed by detecting the number of P24 protein as HIV specific antibody using RETRO-TEK HIV-1 p24 Antigen ELISA kit (Zeptometrix Corp., New York USA) up to 22 days. Experimentally, all the polystyrene microtiter plates were coated with 200µl HIV-ICX/CRX, the CDs samples were subsequently added to each

well containing viral antigen with final concentration of sample was 1.75 mg/mL and incubated overnight at 37 ℃. The unreacted antigen and impurities were washed off with 300 µl PBS solution. Next, 100 µl HIV-1 p24 detector antibody was introduced to each well and incubated again for 1h at 37 ℃, after which they were washed with PBS to remove the unbound impurities from the plate. Then, 100 µl Streptavidin-Peroxidase was added into each well and incubated for 30min at 37 ℃, followed by washing with washing solution (PBS). 100 µl of substrate buffer solution was added into microwells and incubated for 30 min at room temperature. Finally, the Sandwich immunocomplexes were used to determine the optical density of HIV-1 p24 antigen for 3, 5, 15 and 21 days later with microplate reader (450 nm). The concentration of P24 antigen further determined following calibration curve of standards prepared on the kit. All experimental data were collected Triply.

Statistical Analysis

In the analysis, containing the concentration of cytotoxicity reducing 50% of cell viability (CC_{50}) and Efficiency concentration inducing 50% of cell viability (EC_{50}), brought out by using dose response mode on non-linear fitting curve of OriginPro 2016 64Bit software (version 8.0724, Origin lab Inc., Northampton, MA). All data had the advantage to be performed and compared the means by using a sample *t*-test.

Other characterizations

The samples were also characterized by transmission electron microscopy (TEM). A dilute APBA-CDs solution was dripped onto copper grids (200 mesh) covered with a thin Formvar-carbon film and completely dried in air at 25℃. High-resolution TEM (HR-TEM) images were recorded using a Tecnai G2 F20 (Philips, Holland) instrument. Physicochemical analysis was performed by XRD (X-ray diffraction) and the spectrum was investigated using a Rigaku Smart Lab X-ray diffractometer with the Cu Kα 1 line (l = 1.54 Å). Furthermore, AFM was used to characterize the samples. AFM images were recorded using a scanning probe SPM-9600 (Shimadzu Co., Japan). XPS (X-ray photoelectron) patterns were recorded using a VG ESCA scientific theta probe spectrometer with an Al-Kα (1486.6 eV) X-ray source and 28eV pass energy. Raman spectrum was recorded using a T-64000 Horiba Jobin-Yvon LABRAMHR with a focal length of 800 mm. UV-vis absorption spectra were recorded using Perkin Elmer Lamda-25. Colloidal stability of CDs on varied pH, NaCl concentration, and temperature were investigated by TB1 turbidimeter instrument (Velp Scientifica, Italy). Photoluminescence (PL) spectra were observed using a Shimadzu RF-6310PC spectrofluorometric. Quantum yield percentage (%QY) combining UV-vis and PL spectra was determined following previous report.[61] Fourier transform infrared (FTIR) spectrum was recorded using FTIR spectrophotometer (Shimadzu, Japan).

Supporting Information

Figure S1. a) UV-Vis Spectra of APBA CDs. Insert: photographs of solution APBA-CDs under daylight (i) and UV lamp(ii). (b) Photoluminescence spectra of APBA-CDs at varied excitation wavelength (320-400nm).

Figure S2. The Photograph images of APBA-CDs at pH variation (from 3 to 12) and time (a); varied NaCl concentration (from 0 to 0.5 M) and time (b); and temperature (from 30 to 100°C) (c).

Figure S3. Graph of turbidity values of APBA-CDs after 24 h on varied (a) pH, (b) NaCl concentration, and (c) temperature.

Figure S4. a) Comparison cell viability of MOLT-4 cancer cells after 24 h incubation with CACDs (green line) and APBA-CDs (blue line). CC_{50} values from fitted curve are resulted from doses response mode on Origin software, which show at 4.26 and 5.55 mg/mL for each log [Sample] or at 19.9 and 354.8 mg/mL for each [Sample], respectively.

Figure S5. Comparison fluorescent intensity of APBA-CDs on (a) MOLT-4 cell and (b) HIV-1 infected MT-4 cell after 24 h incubation performed by flowcytometry method using blue laser with PM1 set to 525 nm.

Figure S6. Microscopical images of a) MOLT-4 cell and b) MT-4/HIV-1 cell as control. c) microscopical images of MOLT-4 cell after post 24 h infected with MT-4/HIV-1 with addition of CACDs (c) and APBA-CDs (d). The syncitia is showed as non-spherical bubbles on figure b to d.

Figure S7. Inhibition curve MT-4/HIV-1 on MOLT-4 after 24 h with presence of CACDs (green line) and APBA-CDs (blue line). EC_{50} values is determined the curve fitting using doses response mode on Origin software, which show at 0,57 mg/mL and -0.32 mg/mL for each log [Sample] or at 3.72 mg/mL and 0.48 mg/mL for each [Sample], respectively.

Table S1. Comparison EC_{50} and CC_{50} values from previous studies and current report.

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Competing interests

The authors declare no competing interest on this report.

Abbreviation used

Amino phenylboronic acid-modified carbon dots (APBA-CDs); glycol protein 120 (gp120); Acquired immunodeficiency syndrome (AIDS); human immunodeficiency virus (HIV); highly effective antiretroviral therapy (HAART); concentration of cytotoxicity reducing 50% of cell viability (CC_{50}); Efficiency concentration inducing 50% of cell viability (EC_{50}); carbon dots (CDs); cluster of differentiation 4 (CD4); carbon dots (CDs); Citric acid (CA); 2-aminophenylboronic acid (APBA); X-ray diffraction (XRD); citric acid carbon dots (CACDs); high-resolution Transmission Electron Microscope (HRTEM); selected area diffraction (SAED); atomic force microscopy (AFM); X-ray photoelectron spectroscopy (XPS); Photoluminescence (PL); Cell-Counting Kit 8 (CCK-8); nucleoside reverse transcriptase inhibitors (NRTIs);

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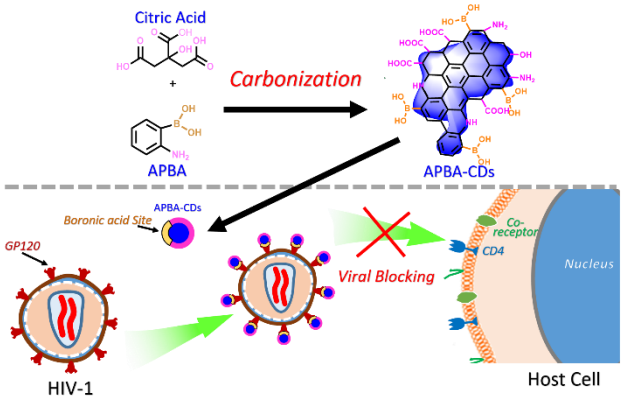

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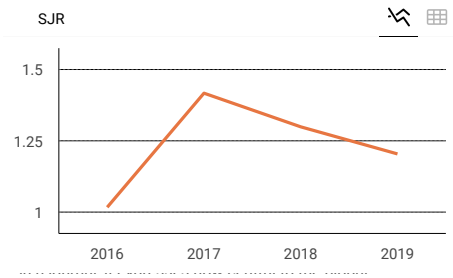

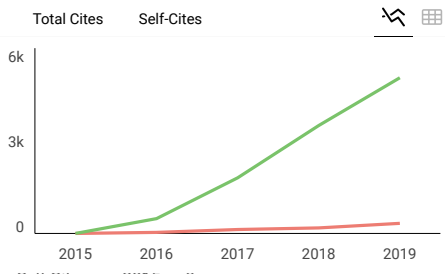

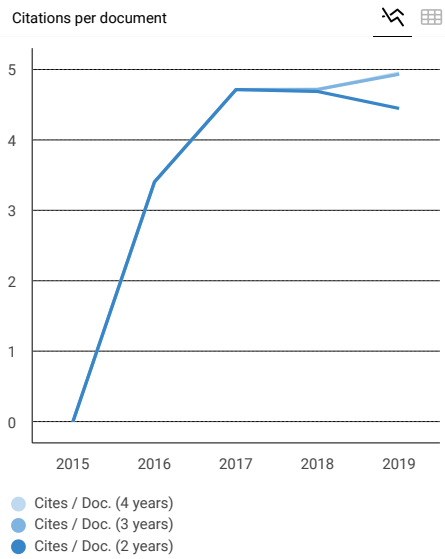

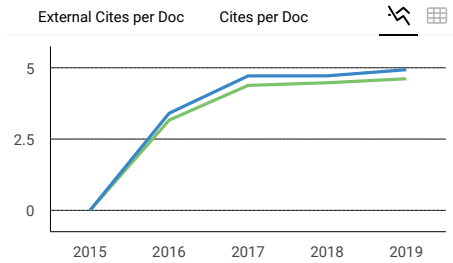

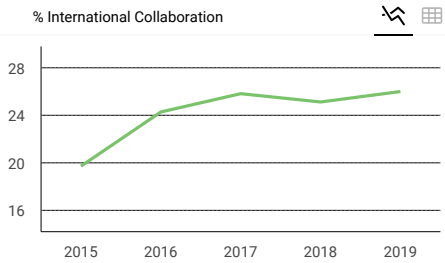

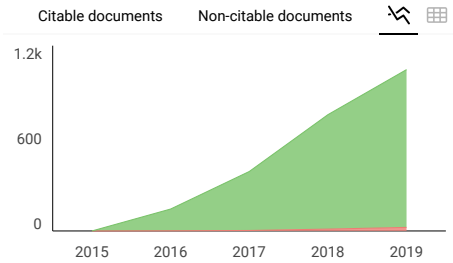

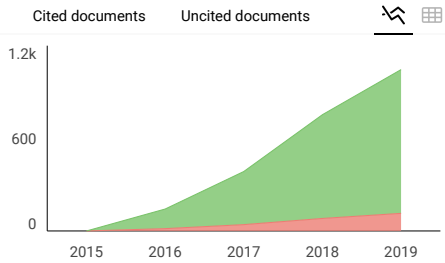

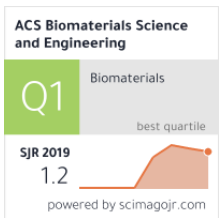

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