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Biocompatible and Blood-Brain Barrier Permeable Carbon Dots for Inhibition of A β Fibrillation and Toxicity, and BACE1 Activity

Xu Han^a, Zhifeng Jing^b, Wei Wu^c, Bing Zou^c, Zhili Peng^a, Pengyu Ren^b, Athula Wikramanayake^c, Zhongmin Lu^c, and Roger M. Leblanc^a

^aDepartment of chemistry, university of miami, coral gables, florida 33146, usa

^bDepartment of biology, university of miami, coral gables, florida 33146, usa

^cDepartment of biomedical engineering, the university of texas at austin, austin, texas 78712, usa

Abstract

Amyloid- β peptide (A β) fibrillation is pathologically associated with alzheimer's disease (AD), and this has resulted in the development of an A β inhibitor which is essential for the treatment of AD. However, the design of potent agents which can target upstream secretases, inhibit A β toxicity and aggregation, as well as cross the blood-brain barrier remains challenging. In, this research carbon dots for AD treatment were investigated *in vitro* using experimental and computational methods for the first time. the results presented here demonstrate a novel strategy for the discovery of novel antiamyloidogenic agents for AD treatments.

TOC image

The Blood-Brain Barrier Permeable C-Dots can deactivate the BACE1 and further inhibit A β fibrillation and toxic oligomer formation

Within biological systems, the information that directs hierarchical assembly is encoded in the structure of DNA and RNA, discovered by Watson and Crick, which induce the formation of elaborate natural materials.^{1,2} Peptides or proteins, expressed by these encoded genes, are the primary embodiment of biofunction, and the effective organization of their

natural assembly in response to environmental and structural changes demonstrates certain corresponding cellular functions. However, a misfolded peptide or protein can result in irreversible damage to the regular metabolism.³ It has been established for decades that highly ordered peptide or protein aggregates deposited in the brain can cause most neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's.⁴ Of all these amyloidoses, Alzheimer's disease (AD) is a leading cause of death worldwide, where no current cure, except for a therapy for delaying its onset, is available.⁵

The precise mechanism of AD pathogenicity remains to be established, but one considerable molecular etiology that underlies AD involves the aggregation of amyloid- β peptides (A β), A β 40 and 42.^{6,7} Both peptides are produced from sequential proteolytic cleavage of amyloid precursor protein by β - and γ -secretases.⁸ The resulting A β peptides can assemble into oligomers, also known as seeds, and induce other peptides to follow this misfolded process to further promote the formation of A β fibrils in an effective "catalytic" cycle.^{9,10} To redirect this "inaccurate" encoded pathway, the design of antiamyloidogenic agents for AD involves inhibiting the energetically driven assembly process of amyloid plaques by their binding towards A β monomer, oligomer, or filament. To achieve this aim, many peptides,¹¹ organic compounds,¹² and conjugated nanoparticles^{13, 14} have been examined with the aim of developing novel amyloid inhibitors. However, in addition to inhibiting A β fibril assembly, very few of these addressed other challenges which arise with AD treatments, such as the capability to: 1) target upstream secretases, β - or γ -secretases,⁸ to cut off the A β fibril generation resources, 2) reduce the toxicity of the A β 42 oligomer,¹⁵ and 3) penetrate the blood-brain barrier (BBB),¹⁶ where capillary endothelial cells are tightly interconnected to protect the central nervous system, for delivery to pathological tissues. Therefore, before the development of a potential drug for better AD treatment can be realized, there are strong motivations for a new antiamyloidogenic agent that can resolve these concerns.

Carbon dots (C-Dots), which were first discovered accidentally during the purification of carbon nanotubes, have recently emerged as a benign zero-dimensional nanomaterial¹⁷ with unique optical properties and biocompatibility.^{18,19} With either a top-down or bottom-up strategy, the prepared C-Dots exhibit promising applications in bioimaging,²⁰ optoelectronics,²¹ drug delivery,²² and biosensing.²³ Previously, human transferrin (HT) has been utilized to deliver the C-Dots across the BBB in a zebrafish model.²⁴ Inspired by this, C-Dots were used as a potential drug candidate for the design of new BBB-permeable nanomaterials for application in AD treatment for the first time. C-Dots possess large surface to volume ratios, and this low dimensionality nanomaterial is expected to redirect the partially unfolded A β by increasing the fibril steric hindrance as a result of their interaction with the A β monomer (Figure 1). In this research, this hypothesis was validated using both experimental and theoretical methods. The synthesized C-Dots were also found to inhibit the active site of the β -secretase 1 (BACE1) enzyme and reduce the toxicity of the A β fibril *in vitro*. In addition, combined with previous results, it was further proved that the C-Dots demonstrated a higher binding affinity towards the forebrain in a zebrafish model. These results demonstrated that there was an excellent potential application for further optimization of C-Dots as an antiamyloidogenic agent for AD treatment.

C-Dots and HT – C-Dots conjugates were prepared according to procedures previously published in the literature.^{24,25} All characterizations using ultra violet-visible (UV-vis), mass spectrometry, and transmission electron microscopy (TEM) were consistent with previous reported results. Initially, a fluorescence resonance energy transfer (FRET) assay was employed to examine the effect of C-Dots on the inhibition of BACE1 activity. In this assay, the substrate was attached to a fluorescence dye and a quenching group on each side, which significantly reduces the fluorescence of the substrate because of the FRET. Upon the cleavage of the substrate by the BACE1, the fluorescence of the substrate was enhanced as a result of the disturbance of the FRET. As shown in Figure 2a, when incubating C-Dots in the FRET assay, the substrate fluorescence intensity decreased, and the higher the concentration of C-Dots, the more effective the inhibition of C-Dots on BACE1 activity. However, one concern arose with the FRET between the C-Dots and the dye. To address it, 10 $\mu\text{g/mL}$ of C-Dots were added to the BACE1 substrate after 3 h incubation. The fluorescent intensity was determined in a similar way to the test without C-Dots (Figure S10), which clearly revealed no effect of C-Dots on dye quenching in the FRET assay. As a control, graphene oxide (GO) was selected to test whether the two-dimensional carbon materials exhibited the same effect. Figure S10 shows that GO contributes nothing towards the inhibition of BACE1 activity. Therefore, the synthesized C-Dots can specifically bind to the activation site of BACE1 to block the pocket for further fluorescence enhancement.

To evaluate the inhibition activity of C-Dots against A β fibrillation, different concentrations of C-Dots were prepared for the thioflavin T (ThT) binding assay, where ThT can specifically recognize the β structure inside the peptide to obtain a locked conformation, and exhibit enhanced fluorescence upon binding to amyloid fibrils so as to alter the fluorescent spectrum with the growth of fibrils. In the absence of C-Dots, the ThT profile of A β 42 showed a faster aggregation process, increasing immediately until it reached a plateau at around 4.5 h. When C-Dots were introduced at 0 h incubation, it illustrated that 1) the suppression of A β 42 can be observed with only 2 $\mu\text{g/mL}$ C-Dots, and 2) the fluorescence intensity decreased when the concentration of C-Dots was increased to saturation. However, one critical question that needs to be answered is how C-Dots inhibit A β fibrillation. It was speculated that C-Dots could interact with the A β monomer at a very early stage before the critical nucleation concentration was reached. To address this, it was observed that adding 10 $\mu\text{g/mL}$ C-Dots after 1 h incubation (Figure S3a), promoted a faster A β 42 aggregation than that in the presence of 10 $\mu\text{g/mL}$ C-Dots before incubation, which strongly indicates that the C-Dots interact with A β 42 monomers to redirect fibril assembly at an early stage. The ThT profile of A β 40 demonstrated similar results (Figure S2 and S3b). In addition, the theoretical data below also conclude the same results. TEM images were obtained after mixing A β 42 and A β 40 with 10 $\mu\text{g/mL}$ of C-Dots. Figure S9 shows that the size of the A β -C-Dots complex (~14 nm) is larger than that of the C-Dots (~6 nm) and A β alone which could indicate the binding of C-Dots to A β at an early stage. Both A β 40 and A β 42, therefore, can be inhibited by C-Dots in a dose dependent manner. The ThT assay suggests the potential use of C-Dots for AD treatment, but the effect of nontoxic C-Dots (Figure S11) on cytotoxicity of A β 42 fibrils also needs to be addressed. Sea urchin embryos were selected as a model system, because 1) it has extreme sensitivity towards hazardous materials, and 2) it has been widely used for static acute toxicity testing. Aliquots of

different ThT assay samples at 5 h were collected for the test. As shown in Figure S7, A β 42 fibrils reduced the rate of an embryo with normal development to 51%. If more C-Dots were added, the rate of an embryo with normal development of sea urchin embryos would be restored, which strongly indicates that the formation of A β 42 toxic species can be delayed.

To attain a clearer perception of the effect of C-Dots on the conformational transition of A β , far-UV circular dichroism spectroscopy (CD) was utilized to monitor the secondary structure of A β 42 over time (Figure 2c and 2d). The A β peptides mainly adopt α -helical or random coil in the native conformation, whereas they undergo a conversion to β -strand during fibrillation process. Because of the effect of PBS buffer on the CD, both A β peptides spectra are in the form of disturbance but are still distinguishable. As illustrated in Figure 2d, in the absence of C-Dots, the initial secondary structure of A β 42 is a random coil with a representative negative band at around 198 nm. After two hours, this peak diminished and shifted slightly to approximately 207 nm, indicating the conformational switch of A β 42. Upon further aggregation, a negative valley, which was assigned to the β sheet of the mature peptide fibril, appeared at approximately 215 nm. In the presence of 10 μ g/mL C-Dots, it exhibited a similar conformational transition as shown in Figure 2c. However, the structure of A β 42 did not completely convert to β sheet conformation after incubation for two hours, showing that C-Dots can potentially delay the conformational transition of A β 42. For A β 40 alone, Figure S5a shows a similar secondary structure to that of A β 42. With further peptide aggregation, bands shrink and there is a significant decrease in the negative absorption at around 198 nm, suggesting severe conformational changes. The final content of the β strand at 5 h was much more than that of the initial A β 40. In the presence of 10 μ g/mL C-Dots, A β 40 showed a similar conformational transition with a delayed fibrillation process as shown in Figure S5b, where a structural shift was observed when compared to the 2 h curve shown in Figure S5a and S5b. Thus, it is seen that C-Dots can partially suppress the fibrillation of A β 40 and 42.

The ability of C-Dots to inhibit A β aggregation was also investigated using atomic force microscopy (AFM), where the formation of the A β 42 and 40 fibrils can be directly observed and monitored (Figure 2e, 2f and S5). AFM images were obtained in the absence and presence of C-Dots, and the inhibition effect of C-Dots on A β peptide fibrillation was consistent with the ThT profiles and CD spectra. As shown in Figure 2e and S5a, long A β 42 and 40 fibrils were apparent after 4.5 h incubation in the absence of C-Dots, whereas less fibrils with a small amount of short protofibrils appeared during the incubation with C-Dots. In this research, all results indicate the inhibition effect of C-Dots on A β fibrillation and BACE1 activity. To test which of these C-Dots target first, a FRET assay with a mixture of C-Dots and A β was utilized. In Figure S10, it is shown that 10 μ g/mL of C-Dots does not block the activity site of BACE1 in the presence of 10 μ M A β , because of the enhancement of fluorescence in the presence of C-Dots and A β conjugates. However, when the concentration increased, C-Dots began to inhibit BACE1 activity in the presence of 10 μ M A β . This suggests that inhibition by C-Dots of the amyloid aggregation is the pre-dominant activity. As a control (Figure S10), A β 42 or A β 40 alone showed no effect on inhibition of BACE1 activity. Therefore, C-Dots show favorable targeting towards A β rather than BACE1, even though they can block A β fibrillation and BACE1 activity.

The experimental results revealed that C-Dots can inhibit A β fibrillation. To obtain more details about the A β fibrillation by C-Dots, molecular dynamics simulations with the Martini coarse-grained force field²⁶ were performed to study the interaction between C-Dots and A β monomers, which has been a valuable tool for the study of A β peptides.^{27–29} In this study, the surface of C-Dots has a high oxygen content,²⁴ which indicates high hydrophilicity. In order to see the role of the hydrophilic surface, C-Dot models with both hydrophilic and hydrophobic C-Dots were constructed.

The A β monomer can bind to the C-Dot with either a hydrophilic or hydrophobic surface, but the binding modes and the structures of the A β monomer are distinct. The A β monomer has more contact with the hydrophilic surface than with the hydrophobic surface. Most of the residues can interact with the hydrophilic surface through the backbone, whereas fewer residues have contact with the hydrophobic surface (Figure S12 and S13), and isoleucine (ILE), leucine (LEU) and phenylalanine (PHE) bind to the hydrophobic surfaces mainly through the side chains. As a result, the A β monomer forms more extended structures on the hydrophilic surface (Figure 3), and the structures on the hydrophobic surface are more compact. Analysis of the radius of gyration shows that the structural flexibility of the A β monomer is also higher on the hydrophilic surface. Previous experimental and computational studies suggest that increased flexibility inhibits fibril formation.^{28,30} Therefore, the extended structure and flexibility of the A β monomer induced by the hydrophilic C-Dots are possible mechanisms for the inhibition of fibrillation.

The key process to the success of the design of inhibitors is targeting the delivery of BBB-impermeable nanomaterial or molecules into the brain. To achieve this aim, HT was previously used to deliver the C-Dots across the BBB in a zebrafish model.²⁴ In this research, the same procedure was followed. Briefly, C-Dots loaded with HT were injected into the heart of anesthetized zebrafish at five days post-fertilization (dpf), where the maturation of its BBB developed as early as 3 dpf.²⁴ Confocal fluorescence microscopy was used to examine the brain sections of zebrafish in three dimensions by measuring the normalized fluorescence intensity after 5 h injections. Figure 4a demonstrates that the C-Dots can target the forebrain of zebrafish, and this is compared to the dorsal (Figure 4b) and the ventral (Figure 4c) section images. This finding will provide more clues about using C-Dots as a BBB-impermeable nanomaterial for targeting tissues. In addition, the C-Dot conjugates were still active in deactivating BACE1 and retarding A β fibrillation (Figure S4). Combining these results with those of previous studies, these results strongly suggest that C-Dots can be delivered across the BBB via a transferrin receptor-mediated mechanism and that they would further help AD treatments.

Conclusions

In conclusion, the excellent inhibitory effect of C-Dots on A β 42 and 40 fibrillations was verified using a ThT assay, CD, and AFM (Table S1). Molecular dynamics simulations showed that the hydrophilic surface of C-Dots can help A β fibrillation inhibition. C-Dots were also found to be effective in inhibiting BACE1 activity and delaying the formation of A β 42 toxic species *in vitro*. Given the tunable properties of C-Dots, transferrin was engineered onto their surface for delivery and crossing BBB in a zebrafish model. Combined

with previous results, HT managed to deliver the C-Dots across the BBB to the forebrain section of the zebrafish. As far as is known, this is the first time that C-Dots have been applied for AD treatment in terms of: 1) A β fibrillation inhibition, 2) target up-stream secretases, 3) reducing the toxicity of the A β 42 fibril, and 4) BBB penetration. Thus, the C-Dots presented in this work display an excellent potential for application in the pharmaceutical industry or other areas for the treatment of Alzheimer's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
A schematic overview of a) Aβ fibrillation with conformational transition from monomer to fibril, and b) the effect of C-Dots on the inhibition of Aβ fibrillation.

Figure 2.

a) Inhibitory effect of different concentrations of C-Dots on BACE1 activity determined using the FRET assay. b) Kinetics of 10 μM of A β 42 fibrillation: fluorescence intensity of ThT at 485 nm as a function of incubation time at 37 $^{\circ}\text{C}$ in 25 mM phosphate buffered saline (PBS), pH 7.4 with 0, 2, 5, 10 $\mu\text{g/mL}$ of C-Dots. The ThT fluorescence measurement was repeated three times for each sample. The error bars indicate the standard error of the mean. Far-UV circular dichroism spectra of 10 μM A β 42 incubated with c) 10 $\mu\text{g/mL}$ C-Dots, and d) without C-Dots in 25 mM PBS pH 7.4 at 0, 2, 5 h. AFM images (size: $2.5 \times 2.5 \mu\text{m}$) of 10 μM A β 42 incubated at 37 $^{\circ}\text{C}$ in 25 mM PBS, pH 7.4, e) in the absence of C-Dots, and f) in the presence of 10 $\mu\text{g/mL}$ C-Dots.

Figure 3. A β monomer binds to the C-Dot with a) hydrophilic surface and b) hydrophobic surface. Cyan spheres are the SC4 (aromatic carbon) and red spheres are Qa/P1 (COO⁻, C=O, OH) beads in the C-Dot. Pink sticks represent the backbone of A β 42 (PDB code: 1Z0Q).

Figure 4. Confocal fluorescent images of a) brain, b) dorsal, c) ventral section and (d) lateral view of zebrafish after 5 h injection with C-Dots-HT conjugates.