Colloids and Surfaces B: Biointerfaces 145 (2016) 251–256

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Crossing the blood–brain–barrier with transferrin conjugated carbon dots: A zebrafish model study

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ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 2 May 2016
Accepted 4 May 2016
Available online 5 May 2016

Keywords:
Blood–brain–barrier
Carbon dots
Central nervous system
Transferrin

ABSTRACT

Drug delivery to the central nervous system (CNS) in biological systems remains a major medical challenge due to the tight junctions between endothelial cells known as the blood–brain–barrier (BBB). Here we use a zebrafish model to explore the possibility of using transferrin-conjugated carbon dots (C-Dots) to ferry compounds across the BBB. C-Dots have previously been reported to inhibit protein fibrillation, and they are also used to deliver drugs for disease treatment. In terms of the potential medical application of C-Dots for the treatment of CNS diseases, one of the most formidable challenges is how to deliver them inside the CNS. To achieve this in this study, human transferrin was covalently conjugated to C-Dots. The conjugates were then injected into the vasculature of zebrafish to examine the possibility of crossing the BBB *in vivo* via transferrin receptor-mediated endocytosis. The experimental observations suggest that the transferrin-C-Dots can enter the CNS while C-Dots alone cannot.

The central nervous system (CNS), consisting of the brain and spinal cord, is responsible for integrating sensory information and responding accordingly. It has been well recognized that the CNS is protected by the complex and highly regulated blood–brain–barrier (BBB) which serves as a physiological checkpoint to allow the entry of selected molecules from the blood circulation into the CNS [1]. The BBB is primarily composed of capillary endothelial cells, which are closely interconnected by tight intercellular junctions [2]. While the capacity of the BBB to restrict permeability and transport into the CNS is protective, it also represents a formidable obstacle for the delivery of therapeutic molecules from the blood to the CNS. Studies show that less than 2% of small-molecule drugs can cross the BBB, and these drugs usually possess dual characteristics, *i.e.* molecular weight less than 500 g/mol and high lipophilicity [3,4]. Practically 100% of large-molecule drugs or nanoparticles targeted for the CNS diseases do not readily cross the BBB unless the surfaces of these drugs or nanoparticles are specifically modified for the uptake [5]. Because of the inability to adequately deliver therapeutic agents across the BBB, the current treatments for the CNS diseases remain extremely limited [6].

Compared with invasive drug delivery, non-invasive drug delivery to the CNS achieves better efficacy with lower dosage and side effects by taking advantage of the receptor-mediated transcytosis across the BBB [7]. Over the last few years, the development of a drug delivery system for crossing the BBB using nanoparticle carriers is among the most notable advances in the research of pharmaceutical research [8,9]. These carriers are usually conjugated to surface ligands which can be specifically bound by their receptors in the brain capillary endothelial cells [10]. Transferrin is one such ligand because the presence of highly expressed transferrin receptors on the endothelial cells have been shown to ferry transferrin-conjugates to the CNS [11,12]. The specific affinity between transferrin and the transferrin receptor as a mechanism to deliver therapeutics to the brain is an area of active exploration, although a precise understanding at the cellular and molecular level remains elusive [13,14]. It has been reported that transferrin-containing gold nanoparticles can reach the brain parenchyma

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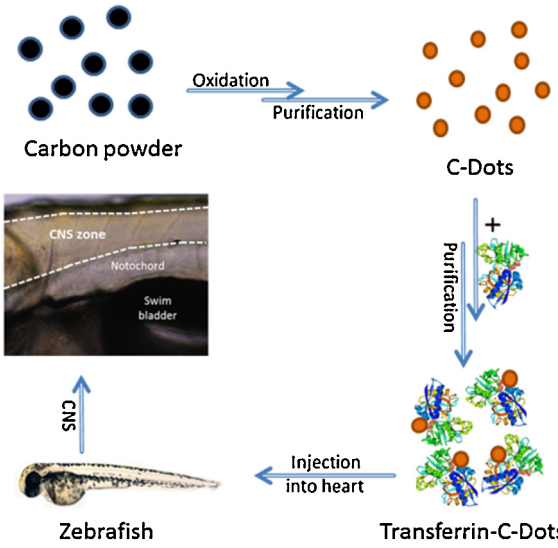
http://dx.doi.org/10.1016/j.colsurfb.2016.05.007
0927-7765/

when administrated systemically [11]. Most recently, an *in vitro* study using human brain cancer lines (LN229 and U87) shows that transferrin receptor–targeted gold nanoparticles could deliver the pro-drug into the mitochondria of the glioma cells [15]. In another study, Jiang et al. demonstrated that in addition to delivering compounds across the BBB, transferrin-conjugated superparamagnetic iron oxide nanoparticles could be internalized by C6 glioma cells and detected by magnetic resonance imaging (MRI) [16]. The delivery of nanoparticles with therapeutic effects in a cell or tissue specific manner may provide better efficacy and lower toxicity for disease treatment [17].

Carbon dots (C-Dots), a new type of nanoparticle with diameters below 10 nm, have recently emerged and attracted extensive attention for their unique optical properties and promising applications in drug delivery [18–20]. Li et al. observed *in vitro* the internalization of the transferrin conjugated C-Dots in the cancer cells HeLa [21]. More recently, we found that nontoxic C-Dots potently inhibit insulin fibrillation in aqueous solution [22]. In addition, our current research has also found that these C-Dots suppress fibrillation of amyloid beta peptides. It has been shown previously that proteins and peptides may share a common molecular mechanism to develop fibrils, a process associated with pathologies in CNS diseases including Alzheimer's and Parkinson's diseases [23,24]. However, to utilize the inhibiting effect of C-Dots on protein fibrillation, they need to be delivered first into the CNS by crossing the BBB. However, only one article up to now has been published on the topic that C-Dots can penetrate the BBB, targeting brain cancer glioma tissue [25]. The specialty of C-Dots in that publication may originate from the reagents of D-glucose and L-aspartic acid, both of which could be transported to cross the BBB by transporter- and/or receptor-mediated uptake [25]. It is unknown whether C-Dots with different surface properties can cross the BBB.

To address the challenge of crossing the BBB, we conjugated human transferrin to C-Dots to facilitate the delivery of C-Dots across the BBB from the circulation to the CNS. Zebrafish (Danio rerio) is a relatively complex vertebrate species with a high degree of physiological and genetic homology to humans. They also possess all major neurotransmitters, hormones, and receptors, including transferrin [26]. The remarkable anatomical and physiological conservation in the CNS development and function between zebrafish and amniotes (mouse and humans) has been demonstrated [27]. Therefore, a zebrafish model enables researchers to not only identify genes that might underlie human disease, but also to test and develop novel therapeutic agents *in vivo* [28]. Because of their small size and the relatively simple laboratory culture conditions required, it is much more straightforward to maintain zebrafish in what appear to be more natural conditions than possible to simulate in mammals such as mice [29]. Another advantage of zebrafish model is the transparence of the body, meaning that it is possible to follow the pharmacological treatment using non-invasive imaging techniques [29]. Particularly, previous studies also demonstrated that maturation of the zebrafish BBB occurred around 3 days post-fertilisation (d.p.f.), and that this BBB shared both structural and functional similarities with that of mammals [30,31]. Therefore, larval zebrafish at 6 d.p.f. with mature BBB were selected in our experiments as an *in vivo* model to examine the possibility of transporting C-Dots into the CNS. Transferrin, dye-transferrin or dye of fluorescein were covalently conjugated with C-Dots, and then the conjugates were injected to the zebrafish heart to examine the possibility of the receptor-mediated delivery in the CNS. Experimental observations confirmed that the transferrin-conjugated system facilitated entry of the C-Dots into the CNS.

A schematic summary of the experimental processes is illustrated in Scheme 1. The C-Dots used in this study were synthesized and characterized as in our previous studies [22]. The prepared C-Dots were characterized by UV–vis absorption, fluorescence,



Scheme 1. The experimental processes of carbon dots preparation from carbon powder, transferrin-C-Dots conjugation via EDC/NHS reaction, injection of conjugates to the zebrafish heart, and the confocal fluorescence microscopy imaging of the CNS zone.

infrared spectroscopy (IR), X–ray photoelectron spectroscopy (XPS), and transmission electron microscopy (TEM). The results from these spectroscopic and microscopic characterizations were almost identical to our previous study (Figure SI Figure SI-1, Supporting Information) [22]. Please refer to the Supporting Information (SI) for the experimental details and the characterizations. The average particle diameter of C-Dots is approximately 5 nm with abundant carboxylic groups distributed on the surface for possible chemical modifications with biomolecules [32].

In this study, transferrin, dye–labeled transferrin, or fluorescein were first covalently conjugated to C-Dots and tested for their ability to cross the BBB (Scheme 1). After the conjugating reaction, the conjugates were purified by a size exclusion chromatography column. The purified conjugates were systematically characterized by UV–vis absorption, fluorescence, circular dichroism (CD), matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy, and transmission electron microscopy (TEM). UV–vis absorption spectral differences shown in Fig. 1A indicate the successful conjugation of the transferrin-C-Dots and the dye-transferrin-C-Dots. The transferrin-C-Dots have absorption around 260 nm, while the dye-transferrin-C-Dots show a characteristic absorption peak at 594 nm of the fluorescent dye. Fig. 1B demonstrates that the fluorescence spectra of the transferrin-C-Dots display both the fluorescence of the protein transferrin (345 nm, black arrow) and C-Dots (excitation wavelength dependence, red arrows). The emission peak of transferrin at 345 nm is due to the presence of tryptophan residues when excited at 290 nm [33]. The excitation wavelength dependent photoluminescence is the key fluorescent characteristic of C-Dots [22,34–36]. The emission peak of C-Dots was shifted from around 515–595 nm when the excitation was shifted from 290 to 560 nm (Fig. 1B). Similarly, besides the strong fluorescence of the dye at 613 nm, the fluorescence spectra of the dye-transferrin-C-Dots also showed the characteristic shifting emission of C-Dots (red arrows) and transferrin (black arrow, Fig. 1C). It is worth noting that the emission of C-Dots at longer wavelength was overlapped by the much stronger fluorescence of the dye at 613 nm. That explains why the emission peak of C-Dots is not shown in Fig. 1C when excited at 520 and 560 nm. MALDI-TOF spectra also support the success of conjugation, as the mass determined were 79,224 Da of transferrin, 81,596 Da of dye-transferrin, 83,330 Da of transferrin-C-Dots, and 85,702 Da of dye-transferrin-