Biomaterials 106 (2016) 98–110

Contents lists available at ScienceDirect

Biomaterials

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

Graphene quantum dots conjugated neuroprotective peptide improve learning and memory capability

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ARTICLE INFO

Article history:
Received 13 June 2016
Received in revised form
8 August 2016
Accepted 14 August 2016
Available online 17 August 2016

Keywords:
Graphene quantum dots
Neuroprotective peptide
Amyloid-β (Aβ)
Dendritic spine
Alzheimer disease (AD)

ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder and the most common form of dementia. Histopathologically is characterized by the presence extracellular neuritic plaques and with a large number of neurons lost. In this paper, we design a new nanomaterial, graphene quantum dots (GQDs) conjugated neuroprotective peptide glycine-proline-glutamate (GQDG) and administer it to APP/PS1 transgenic mice. The in vitro assays including ThT and CD proved that GQDs and GQDG could inhibit the aggregation of $A\beta_{1-42}$ fibrils. Morris water maze was performed to exanimate learning and memory capacity of APP/PS1 transgenic mice. The surface area of Aβ plaque deposits reduced in the GQDG group compared to the Tg Ctrl groups. Furthermore, newly generated neuronal precursor cell and neuron were test by immunohistochemical. Besides, neurons were impregnated by DiI using gene gun to show dendritic spine. Results indicated enhancement of learning and memory capacity and increased amounts of dendritic spine were observed. Inflammation factors and amyloid-β (Aβ) were tested with suspension array and ELISA, respectively. Several pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-33, IL-17α, MIP-1β and TNF-α) had decreased in GQDG group compared with Control group. Reversely, anti-inflammatory cytokines (IL-4, IL-10) had increased in GQDG group compared with Control group. Thus, we demonstrate that the GQDG is a promising drug in treatment of neurodegenerative diseases such as AD.

1. Introduction

Alzheimer's disease (AD) is characterized by the progressive loss of neurons [1]. One of the most widely accepted theory of AD is the amyloid hypothesis, featuring aggregation and fibril formation of amyloid-β (Aβ) peptides [2]. Studies have shown that Aβ is the possible biomarkers for the AD diagnose [3]. Aβ (38–43 residues) comes from proteolysis of amyloid precursor protein (APP), and $A\beta_{1-42}$ is the most toxic form presents in the brains of AD patients. The aggregation of Aβ in the brain is believed to be linked to the neuron apoptosis and loss of cognitive function observed in patients with AD [4]. Some studies indicate that Aβ can insert into the membrane and form ion channels and cause calcium overload; or it can alter the activity of NMDA receptors and modulating calcium influx [5].

Neuroinflammation is now well recognized as a prominent feature in Alzheimer's pathology and a potential target for therapy and prevention of this disease [6]. Inflammatory components

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http://dx.doi.org/10.1016/j.biomaterials.2016.08.021
0142-9612/

include microglia, cytokines and chemokines [7,8]. Evidence shows that accumulations of Aβ can cause inflammatory response, following the neurodegenerative condition [9]. Therefore, Aβ is a potential target for therapeutic intervention for AD.

Bioactive peptide which was defined as specific protein fragments have a positive impact on body functions [10]. A endogenous neuroprotective peptide glycine-proline-glutamate (Gly-Pro-Glu, GPE), which is N-terminal tripeptide of insulin-like growth factor I (IGF-1) [11], has been proved to be an effective peptide. The protective effects of GPE may be related with modulation of intracellular calcium signaling, inhibition of glutamate binding to NMDA receptors and protection against NMDA excitotoxicity [12]. GPE could also rescue cell death induced by Aβ and inhibit apoptotic [13]. These suggests that GPE could be an important factor implicated in the brain's protection [14].

Admittedly, the blood brain barrier (BBB) hampers the access of systemically administered drugs to the brain [15]. So the BBB permeability is one of the key factors for applications of biomaterials. In the area of biomedicine, graphene nano-particles and its derivatives have been highly anticipated to provide unique and new opportunities for the developments of novel nanocarriers for drug delivery [16]. Graphene quantum dots (GQDs), a new type of carbon-based nanomaterial, is a promising new material for drug carrier material owing to its small size, excellent solubility, large specific surface area and low cost [17,18]. GQDs exhibits no apparent toxicity in vitro and *in vivo* [19]. GQDs have been proved that could across the Madin-Darby canine kidney (MDCK) cell monolayer mainly through a lipid raft-mediated transcytosis [20]. Thus, GQDs may cross the BBB because of its small size. Moreover, GQDs are demonstrated to inhibit inhibitor for aggregation of $A\beta_{1\text{-}42}$ and rescue the cytotoxicity of Aβ oligomers [21].

In our study, we conjugated the GQDs with neuroprotective peptide GPE, we named the new nanomaterial as GQDG. We found GQDG can inhibit the $A\beta_{1\text{-}42}$ aggregation in vitro. Then transmission electron microscope, circular dichroism spectrum and Thioflavin-T assay was used to confirm the GQDG could prevent the aggregation of $A\beta_{1\text{-}42}$. Then GQDG was administrated to APP/PS1 transgenic mice by intravenous injection to observe its therapeutic effect through Morris water maze, immunohistochemical, ELISA, suspension array and diolistic labeling *in vivo*.

2. Materials and methods

2.1. Preparation of materials

2.1.1. Preparation of graphene oxide

Natural graphite powder (<150 μm, Aldrich) was used for graphene oxide (GO) synthesis by the modified Hummers method [22]. An additional preoxidation procedure was performed before the GO preparation. Solution contains H_2SO_4 (30 mL), $K_2S_2O_8$ (10 g), and P_2O_5 (10 g) was used to dissolve the graphite powder (8 g) and the temperature was control at 80 °C. The dark mixture was isolated, cooled at room temperature overnight and then diluted with ultrapure water to be neutral. After that the graphite was preoxidized and then put into cold H_2SO_4 (180 mL) added with $NaNO_3$ (4 g). We kept the temperature below 10 °C and added $KMnO_4$ (24 g) in to the mixture slowly. The mixture was then stirred at around 40 °C for 8 h. Then we added 400 mL water and heated the mixture over 90 °C for 15 min, large amount of water and 30% H_2O_2 solution was added to stop the reaction. The mixture was filtered and washed with 1:8 HCl solution (800 mL). Lastly, after ultrapure water washed for three times, GO product was collected.

2.1.2. Preparation of GQDs

GQDs were synthesized using the method by published before [23]. Briefly, GO was dissolved in *N*, *N*-Dimethylformamide (DMF) with the concentrations of 30 mg/mL. Ultrasonication was used to treat the complex for 30 min (120 W, 100 kHz). The complex was added to a poly(tetrafluoroethylene) (Teflon)-lined autoclave (40 mL) and heated at 200 °C for 4 h. Then the container was cooled to 25 °C temperature by water. The product contained black sediments and brown transparent supernatant, and the black sediments (GQDs) were collected and washed by water. PBS was used to conserve the GQDs.

2.1.3. GQDs and GPE coupling

The conjunction between GQDs and GPE was realized by the 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide and *N*-hydroxysuccinimide (EDC/NHS) coupling reaction [24]. Briefly, 8 mg of EDC and 12 mg of NHS were added into GQDs solution (10 mL). We added 10 mg of GPE (ChinaPeptide Co., Ltd, Shanghai, China) into the solution and the reaction was conducted under stirring for 3 h at room temperature. To remove residual EDC and NHS, the solution was dialyzed (MWCO, 500–1000) for 72 h (dialysate was replaced every 12 h).

2.2. Animals

APPswe/PS1dE9 double transgenic mice (APP/PS1 mice) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) (strain type B6C3-Tg [APPswe, PSEN1dE9] 85Dbo/J, stock number 004462). In this study, 36 SPF 6-month-old male APP/PS1 double transgenic (Tg) mice were used (weight = 27.70 g ± 3.47 g), 12 age-matched wild-type (Wt) littermates were used as controls. All experimental procedures involving animals were performed according to the regulations of the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University, Guangzhou, China. We kept all animals under specific pathogen–free (SPF) conditions on a 12-h light, 12-h dark cycle, and food and water were provided to the mice *ad libitum*.

The GQDG was resolved in the 0.01 M PBS with the final concentration of 200 μg/mL. We randomLy divided the mice into 4 groups: transgenic control (Tg Ctrl, n = 12), transgenic GQDG (GQDG, n = 12), transgenic PBS (PBS, n = 12), wild type control (Wt Ctrl, n = 12). Mice were administered GQDG or 0.01 M PBS every day for 4 weeks. All of the mice were administered 50 mg/kg body weight 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, USA) each day for the first 5 days of administration.

2.3. In vitro assays

2.3.1. Thioflavin-T assay

The Thioflavin-T (ThT) fluorescence method was used [25], and $A\beta_{1\text{-}42}$ (Sigma-Aldrich, USA) was dissolved in phosphate buffer (PB, pH 7.4, 0.01 M) to give a 50 μM solution. GQDG was firstly dissolved in PB at a concentration of 200 μg/mL. After incubating at 37 °C for 48 h, ThT (5 μM in 50 mM glycine-NaOH buffer, pH 8.50) was added. Fluorescence was measured at 450 nm and 485 nm. Each sample was examined in triplicate. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated.

2.3.2. Transmission electronic microscopy (TEM)

$A\beta_{1\text{-}42}$ samples were prepared in PB (pH 7.4) at a concentration of 50 μM. Then the $A\beta_{1\text{-}42}$ samples were incubated with or without 200 μg/mL GQDG for 48 h. To detect the structure of these $A\beta_{1\text{-}42}$ samples, 5 μl of samples to be imaged were spotted on 300-mesh Formvar-carbon coated copper grid and stained with 1% uranyl formate for 1 min. Afterwards, samples were air dried and observed under the transmission electron microscope (FEI Inc., USA) with a voltage of 80 kV.