Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

Green and shape-tunable synthesis of ellagic acid crystalline particles by tannic acid for neuroprotection against oxidative stress

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1. General Information

1.1 Chemicals. TA, N-hydroxysuccinimide (NHS) and Galantamine Hydrobromide were obtained from Aladdin Chemical Co. Ltd. Cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was purchased from Tianjin Guangfu Fine Chemical Research Institute. 3-(4-Morpholino) propanesulphonic acid (MOPS) was purchased from MREDA technology Inc. MOPS sodium salt, Epirubicin Hydrochloride, and Transferrin (TF) were purchased from Shanghai yuanye Bio-Technology Co., Ltd. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Accela ChemBio Co., Ltd. Phosphate-buffered saline (PBS, tablet), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,5,3',5'-tetramethylbenzidine (TMB), and 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Solarbio Science & Technology Co., Ltd., calcein-AM, PI, GSH, SOD and MDA kits was purchased from Beyotime Biotechnology (China). Ellagic acid was purchased from Macklin Biochemical Co., Ltd. Memantine Hydrochloride was purchased from Shanghai Haohong Biopharmaceutical Technology Co., Ltd. PC 12 cells were purchased from Shanghai Fuheng Biotechnology Co., Ltd. 9-AA were purchased from Sigma-Aldrich (St. Louis, MO, USA); Conductive indium tin oxide (ITO) slides (square resistance $\leq$ 5 Ω) were purchased from South China Technology Co., Ltd. (Shenzhen, China). Ultrapure water was obtained by a water purification system, which was purchased from Shanghai Laikie Instrument Co., Ltd.

1.2 Materials characterization. The morphologies of Cu-EA polymer and EA particles were investigated on an FEI-Tecnai G2 Transmission Electron Microscope (TEM) and JSM-6701F Scanning electron microscope. UV−vis spectra were studied by a Perkin-Elmer Lambda 35 spectrophotometer with a 1 cm pathlength quartz cuvette. The X-ray diffraction measurements of Cu-EA polymer were performed by a PHILP X'Pert PRO, using Cu Kα (λ = 1.542 Å) irradiation (40 kV, 40 mA) in the range of 2θ = 5-80°. NMR spectra were acquired with a Bruker Avance III 400

instrument. X-ray photoelectron spectroscopy (XPS) analysis was carried out using an ESCALAB 250Xi X-ray photoelectron spectrometer (Thermo Fisher scientific, USA). Thermogravimetric analyses (TGA) were performed on a STA 449C thermal analysis system with a nitrogen atmosphere at a heating rate of 10 °C/min from room temperature to 800 °C. Zeta potential and sizes of EA crystal particels were recorded by laser dynamic scattering instrument (Zetasizer Nano 3600, UK). Fluorescence images on cells were obtained on an Olympus Fluoview 1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Fluorescence spectra were acquired on a fluorescence spectrophotometer (Fluoromax-4, HORIBA, USA) with a slit width of 5.0 and 5.0 nm for excitation and emission. The content of Cu and Na in Cu-EA polymer samples was determined by Atomic Absorption Spectrometer (Jena ContrAA700). MALDI-TOF-MS and Imaging mass spectra were performed by an imaging mass spectrometer (iMScope TRIO Shimadzu, Kyoto, Japan). Flow cytometry was performed by CytoFLEX (Beckman Coulter).

1.3 Synthesis of Cu-EA coordination polymers. 340 mg of TA and 50 mg $CuSO_4 \cdot 5H_2O$ were dissolved in deionized water (100 mL) to make a homogeneous solution. Then, MOPS buffer (100 mM, pH 8) solution was used to accurately adjust the pH of above solution to 7.4. Then, the solution was bubbled with air and heated for 3 h at 100 °C under reflux. Then, it was natural cooling and kept still for three days at ambient temperature. The reaction process was monitored by UV-vis spectra and MS. The product was separated from the solution by centrifugation (12000 rpm, 10 min), and rinsed by deionized water for several times. Then, the precipitate was freeze-dried to afford dark-green product.

For further investing the formation mechanism of Cu-EA coordination polymer, gallic acid (340 mg) and epigallocatechin gallate (EGCG, 230 mg) were also selected as the precursor molecular to synthesis the Cu-EA polymer under same condition, and the reaction process was monitored by UV-vis spectra and MS.

1.4 Synthesis of EA particles. 2, 10, 20, 30, 40 and 50 μL of HCl solution (0.1M) and 50 μL of HCl solution (1 M) was added into 5 mL of Cu-EA aqueous solution (0.1 mg/mL) under stirring condition, respectively. The clear and transparent solution gradually becomes turbid, after stirred for 5 min, the products named as EA-1, EA-2, EA-3, EA-4 and EA-5 according to the added volume of HCl solution were separated from the solution by centrifugation (13000 rpm, 5 min), and rinsed by deionized water for several times. After freeze-dried, the particles were further characterized and analyzed.

1.5 ROS-scavenging ability of EA particles. The ability of EA particles to act like multiple enzymes was investigated, including catalase (CAT), and peroxidase (POD) activity.[1] The CAT-like activity of EA particles was assessed by observing the generated oxygen. Reactions containing H_2O_2 (30%) and EA-3 particles solutions (0, 1, 5, 10, 20, and 40 μg/mL) were incubated at 37 °C for 10 min to observe the production of oxygen bubbles. Then, the generated oxygen bubbles were eliminated and the reaction were continued to incubation for 10 min to observe the production of oxygen bubbles. The POD-like activity of EA particles was determined by a colorimetric method using TMB as the substrate in the presence of H_2O_2. The absorbance of the colored solution (at 650 nm for TMB oxidation products) was recorded after 10 min. Typically, reaction system containing EA-3 particles solution (0, 0.1, 0.2, 1, 5, 10, 20, 40 or 80 μg/mL), H_2O_2 (30%), TMB (1 mM) and acetate buffer solution were used to reveal the chromogenic reactions.

1.6 Chemical stability assessment. The chemical stability of EA particles was assessed by observing the morphology and fluorescence of EA particles in water and lysosomal mimic fluid.[1] The fresh prepared EA particle (50 μg/mL) was observed by CLSM under three different excitation wavelength (405, 488 and 559 nm). The EA particles were further stored for 6 months in water and observed by CLSM under same condition. Then, the EA particles were incubated in acetate buffer with pH 3.6

for 48 h, and the suspension was centrifuged and the precipitate was observed by CLSM to evaluate the changes of morphology and fluorescence.

1.7 Cell culture. Highly differentiated PC12 (rat adrenal medulla pheochromocytoma) cells were grown in DMEM medium containing 10% (v/v) fetal bovine serum and 1% penicillin /streptomycin (P/S). Cells were cultured in an incubator with a 5% CO_2 atmosphere at 37 °C and were subcultivated for further experiments when the cells were in the logarithmic growth phase.

1.8 Cellular uptake. PC12 cells were seeded in six-well culture plate and cultured in Dulbecco's modified eagle media (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2/air atmosphere. The cells were then treated with 50 μg/mL of EA-1, EA-3 and EA-5 at 37 °C for12 h, respectively. Then the medium was removed, and cells were washed with sterile PBS three times to remove any free material in the wells. 1 mL of 4% formaldehyde was added to each well to fix the cells at room temperature for 30 min. Then, the cells were stained with PI (5 μM) for 5 min. Finally, the cells were washed with PBS buffer before capturing images by using confocal laser scanning microscope (CLSM) and excited at 488 and 559 nm.

1.9 Antioxidant and Neuroprotective activity of EA particles in PC12 cells. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cell viability according to the manufacturer's instructions. Brief, PC12 cells were seeded in a 96 well culture plate at a density of 5000 cells mL^{-1}. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C in a humidified environment of 5% CO_2 for 1 day. To evaluate the biocompatibility of Cu-EA polymer and EA particles, cells were incubated with various concentrations of Cu-EA and EA particles (0, 5, 10, 20, 50, 100 and 200 μg/mL, dissolved in cell culture medium) for 24 h, respectively. An OS model for PC12 cell was established after incubated with H_2O_2 (1 mM) for 1 h. To assess the

preventive effects of Cu-EA polymer and EA particles, cells were first treated with Cu-EA and EA particles (50 μg/mL) with different morphology and size (EA-1, EA-3 and EA-5) for 24 h, and then treated with H_2O_2 (1 mM) for 30 min. For evaluate the concentration-dependent preventive effects of EA particles, cells were first treated with EA-3 particles (0, 1, 2, 4, 8 and 10 μg/mL, dissolved in cell culture medium) for 24 h, and then treated with H_2O_2 (1 mM) for 1 h. The cell viability was determined by a MTT assay according to the generally procedures and the relative cell viability (%) was expressed as a percentage of that of the control culture. The antioxidant and neuroprotective activity of EA particles in PC12 cells were also evaluated by detecting the activities of SOD, and the levels of GSH and MDA. In brief, PC12 cells were first treated with EA-3 particles (0, 5 and 10 μg/mL, dissolved in cell culture medium) for 24 h, and then treated with H_2O_2 (1 mM) for 1 h. The activities of SOD, and the levels of GSH and MDA were measured by using commercial kits and following the protocols from the manufacturers.

1.10 Cellular ROS detection and calcein-AM/PI dual staining. PC12 cells were seeded in 24-well plates at a density of 4×10^5 cells/well. The cells were divided into 4 groups: (1) the control group, (2) the H_2O_2 group, (3) the EA particle group, and (4) EA particles + H_2O_2 group. The control group without treatment, and the EA particle group treated with EA-3 (10 μg/mL) were all maintained for 24 h. The cells of H_2O_2 group treated with H_2O_2 (1 mM) for 1 h. The cells of EA particles + H_2O_2 group were first treated with EA-3 (10 μg/mL) for 24 h then treated with H_2O_2 (1 mM) for 1 h. After all treatments, the cells were washed with PBS three times and incubated with 10 μM DCFH-DA in fresh medium for 20 min at 37 °C. Then, the medium was removed, and the cells were washed three times with PBS. CLSM images were obtained and the fluorescence intensity of cells were collected and analyzed by flow cytometry. For calcein-AM/PI dual staining, after all treatments, the cells were stained with calcein-AM (2 μM) for 20 min and then treated with PI (5 μM) for 5 min, washed three times with PBS, and observed by using CLSM.

1.11 The drug loading capability of EA particles. Epirubicin Hydrochloride (EPI) was selected as model drug to evaluate the drug loading capability of EA particles due to its excellent water solubility and fluorescence properties.[2,3] In brief, EPI was dissolved in deionized water at a concentration of 1.0 mg/mL. EA particles were dispersed into deionized water at a concentration of 0.34 μg/mL. 50 μL of EPI solution and 0-400 μL of EA particle dispersions were mixed in a 1.5 mL of vials, respectively, and various volume of deionized water was added to maintain the volume of mixed solution to 1.0 mL. The vials were stirred for 1 h under dark conditions after being sealed tightly. The solutions were centrifuged and the supernatant of all samples were detected by fluorescence spectrophotometer. The drug loading efficiency were calculated as follows:

$$drug\ loading\ efficiency\ (\%) = \frac{m_1 - m_2}{m_1} \times 100$$

Where m_1 is the initial amount of EPI used for loading, m_2 is the amount of free EPI in supernatant.

For investigating galantamine hydrobromide and memantine hydrochloride loading capacity, galantamine hydrobromide and memantine hydrochloride were dissolved in deionized water at a concentration of 1.0 mg/mL, respectively. EA particles were dispersed into deionized water at a concentration of 1 mg/mL. 1 mL of drug solution and EA particle dispersions were mixed in a 5 mL of vials, respectively. The vials were stirred for 24 h at room temperature. The solutions were centrifuged and the precipitant of all samples were washed with deionized water three times and detected by MALDI-TOF-MSI.

1.12 Permeability assay of EA particles *in vitro*

Evaluation of blood-brain barrier (BBB) penetration using the parallel artificial membrane permeation assay (PAMPA) method refer to the previous method.[4] The pre-coated PAMPA plate system (Cat. No. 353015) (Coning, U.S.) used to perform

permeability assays. Carbamazepine and hydrocortisone purchased from Sigma were used as positive and negative control, respectively. EA-5 were dispersed in PBS at 4 mg/ml as stock solutions. The stock solution was diluted in PBS to a final concentration of 400 μg/ml. The solution was added to the donor wells (300 μl/well). PBS was added to the wells of the acceptor plate (200 μl/well). The filter plate was then coupled with the acceptor plate and the plate assembly was incubated at room temperature without agitation for 16 h. At the end of the incubation, 150 μl of supernatant was removed from the donor and acceptor well and quantified by HPLC with UV screening detector at 280 nm.

1.13 Synthesis of Transferrin (TF) modified EA particles (EA-TF).[1] 4 mg of TF was dissolved in 20 mL of DI water, and EDC (16 mg) was added under magnetic stirring for 15 min. Then, NHS (10 mg) and EA particle (0.5 mg/mL, 20 mL) were added to the mixture solution, and continue to stir for 72 h in the dark. The mixture was centrifuged at 10000 rpm for 10 min and washed three times with DI water to obtain EA-TF.

1.14 Evaluation of biodistribution of EA particles in brain. Animal care and handing procedures were according to the guidelines of the Regional Ethics Committee for Animal Experiments. Healthy C57BL/6 mice (25 weeks) were purchased from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Lanzhou, China). Mice were divided into three groups: (1) the control group (n=3); (2) the EA-5 particle group (n=3), in which mice were intraperitoneal administration of EA-5 particle (4 mg/mL, 200 μL); (3) The EA-TF group (n=3), in which mice were intraperitoneal administration of EA-TF particle (4 mg/mL, 200 μL). After 8 h administration, the mice were executed respectively and the brain of each mouse was collected and stored at -80 °C for Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI-TOF-MSI) analysis. The obtained brain of mouse was affixed to the frozen sample tray of slicer

using the OCT frozen section embedding agent. Subsequently, the brain tissues were sectioned to a specified thickness using a CM1950 cryostat (Leica, Wetzlar, Germany) at -20°C. These sections were then thawed and mounted on glass slides coated with conductive indium tin oxide (ITO). Sections on glass slides were used for MALDI-TOF-MSI analysis after returning to room temperature. The matrix was sublimated onto the tissue sections using the iMLayer matrix sublimation device (Shimadzu, Kyoto, Japan). The conditions for sublimation were as follows: a 9-AA matrix with a weight of 300 mg, a thickness of 0.9 μm, and a temperature of 220 °C. MALDI-TOF-MSI data were acquired using an iMScope TRIO imaging mass spectrometer (Shimadzu), which was equipped with an internal optical microscope and an Nd: YAG laser source (λ = 355 nm). Molecular images were captured and analyzed using Imaging MS Solution version 1.30 (Shimadzu). Ions between m/z range of 280-325 were measured in negative ionization mode. The laser spot diameter was set to 1 (10 μm), with a laser intensity of 45. The detector voltage was 1.89 kV, the sample voltage was 3.00 kV, the laser repetition rate was 1000 Hz, and the number of laser shots is 80. The pixel pitch was set at 45 × 45 μm.

2. Supplementary Figures.

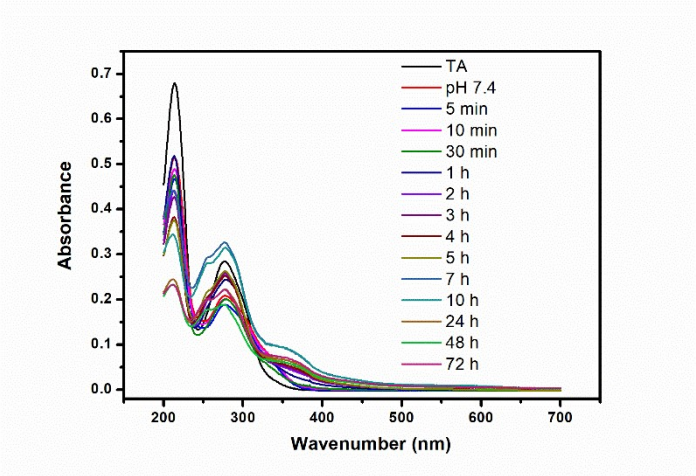

Figure S1. Changes of UV-Vis spectra during the synthesis process.

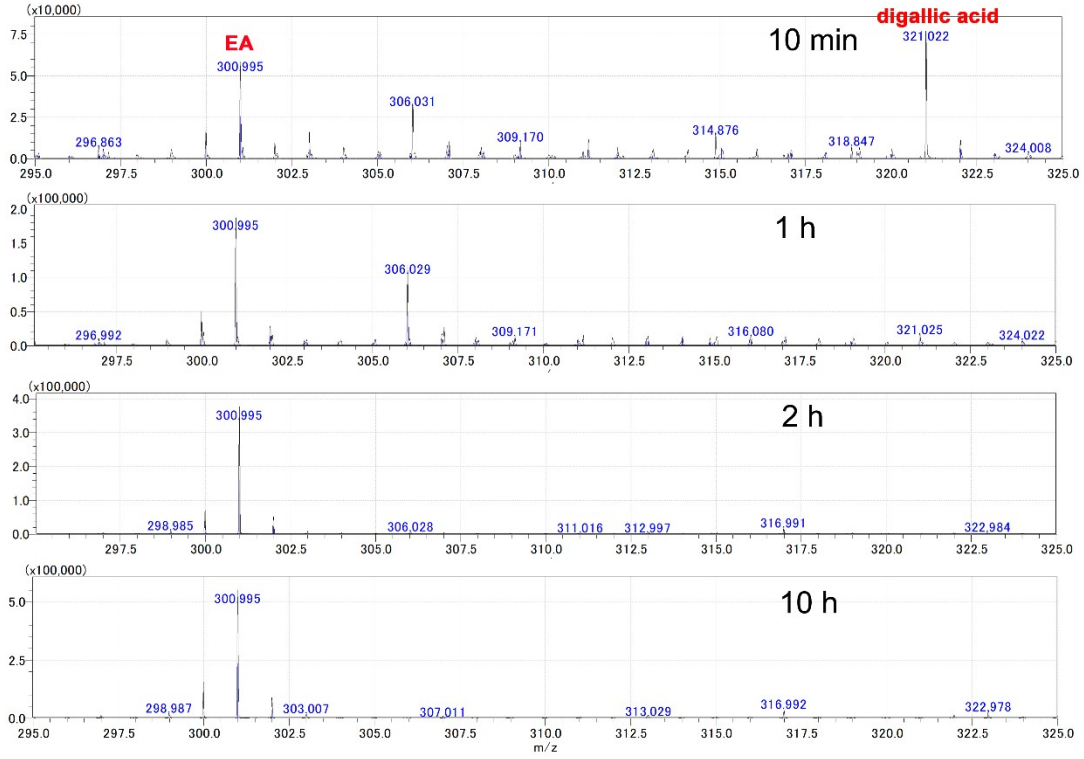

Figure S2. Changes of MALDI-TOF-MS during the synthesis process using 9AA matrix in negative ionization mode.

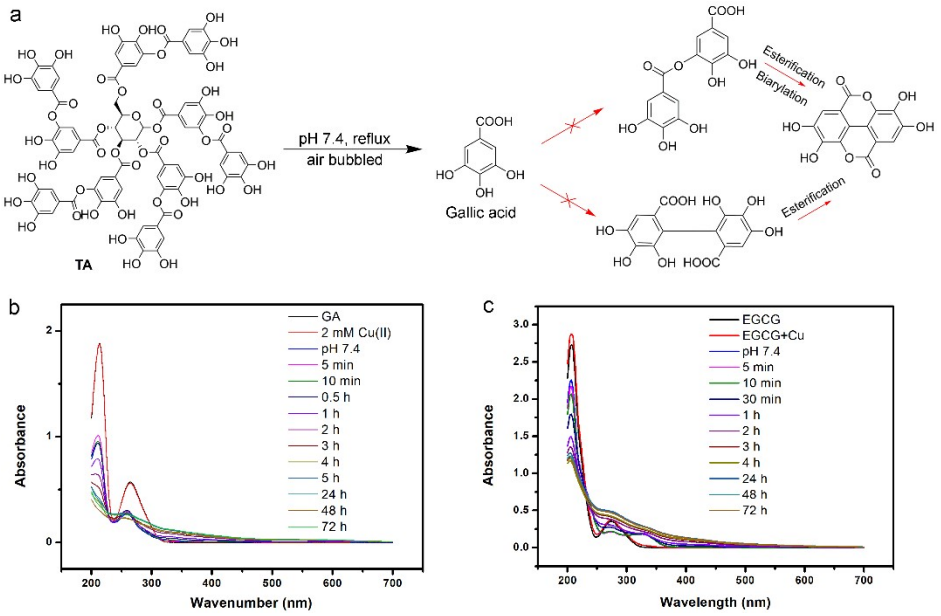

Figure S3. (a) The possible formation mechanism of Cu-EA polymer underwent gallic intermediate. (b) Changes of UV-Vis spectra by using gallic acid as precursor. (c) Changes of UV-Vis spectra by using EGCG as precursor.

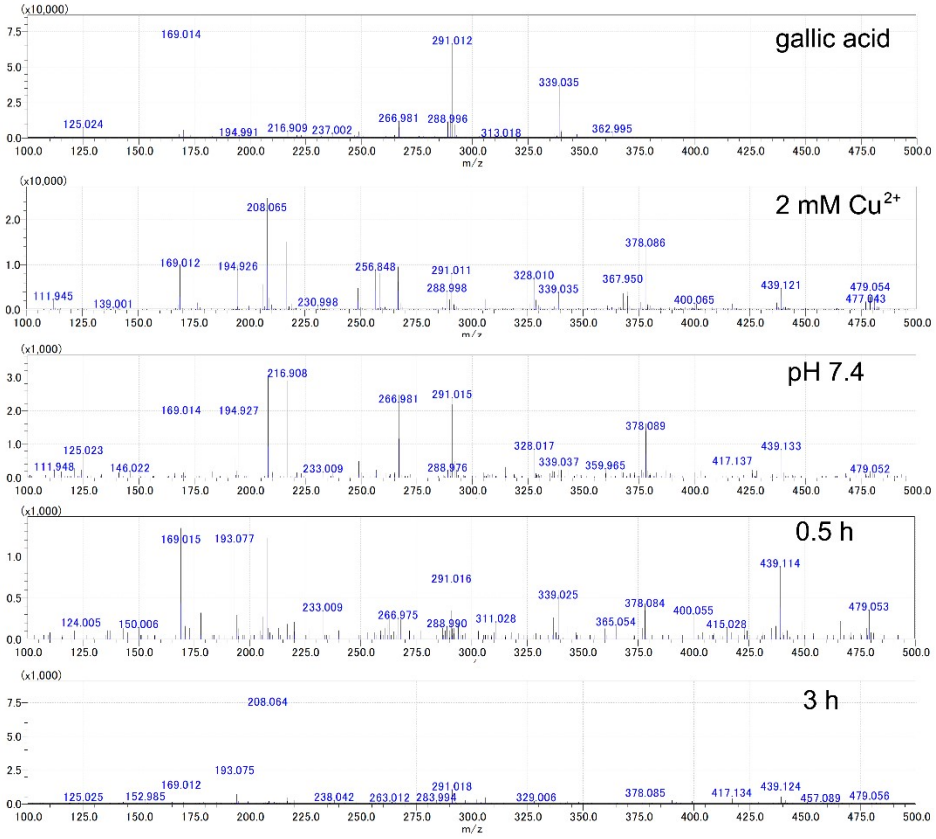

Figure S4. Changes of MALDI-TOF-MS by using gallic acid as precursor using 9AA matrix in negative ionization mode.

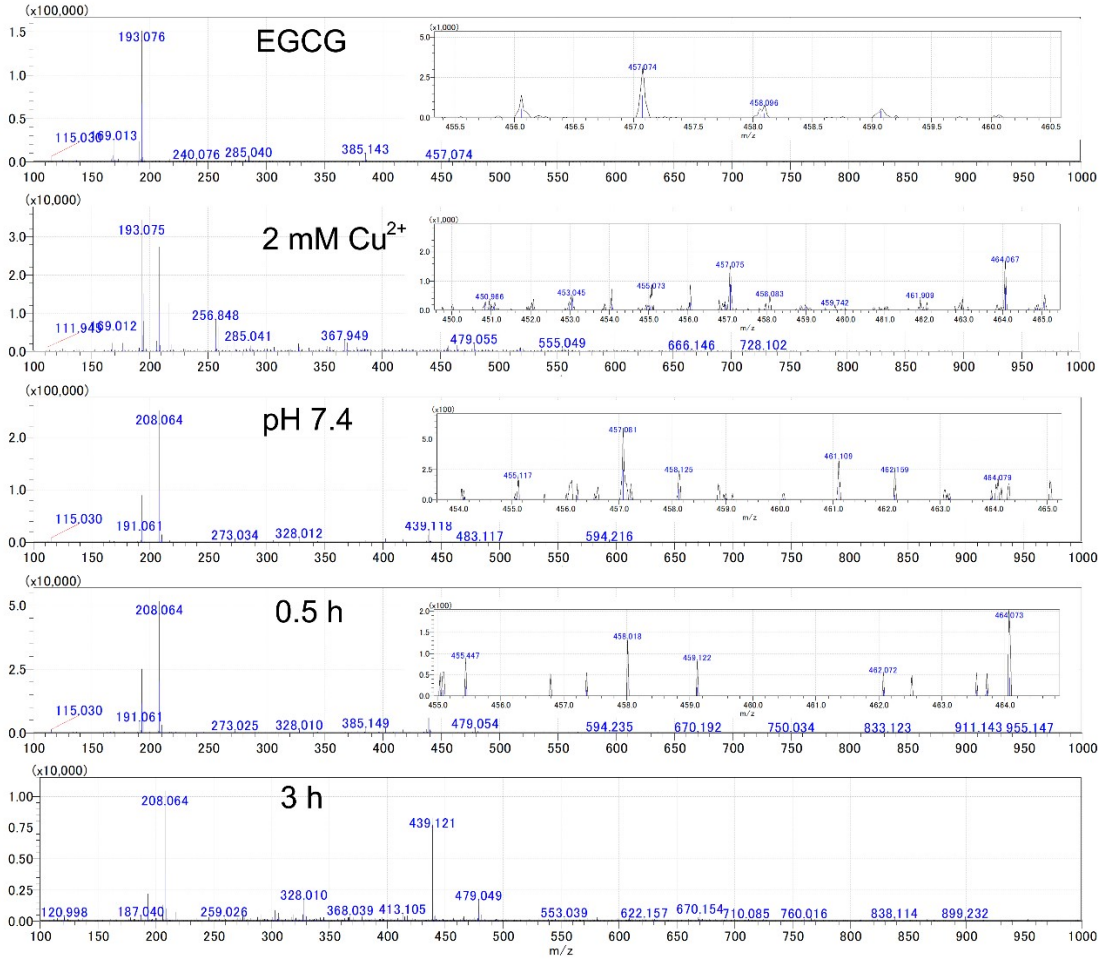

Figure S5. Changes of MALDI-TOF-MS by using EGCG as precursor using 9AA matrix in negative ionization mode.

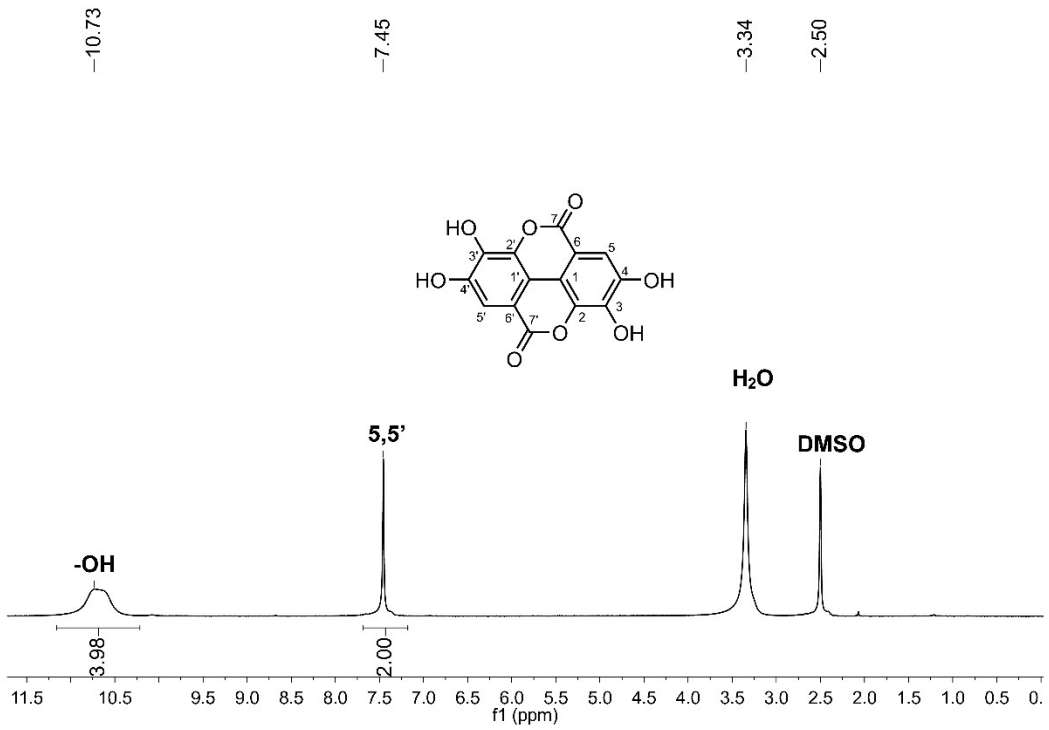

Figure S6. [1]H NMR of light-yellow precipitate obtained from Cu-EA polymer treated with HCl (DMSO-d_6, 400 MHz, 298K).

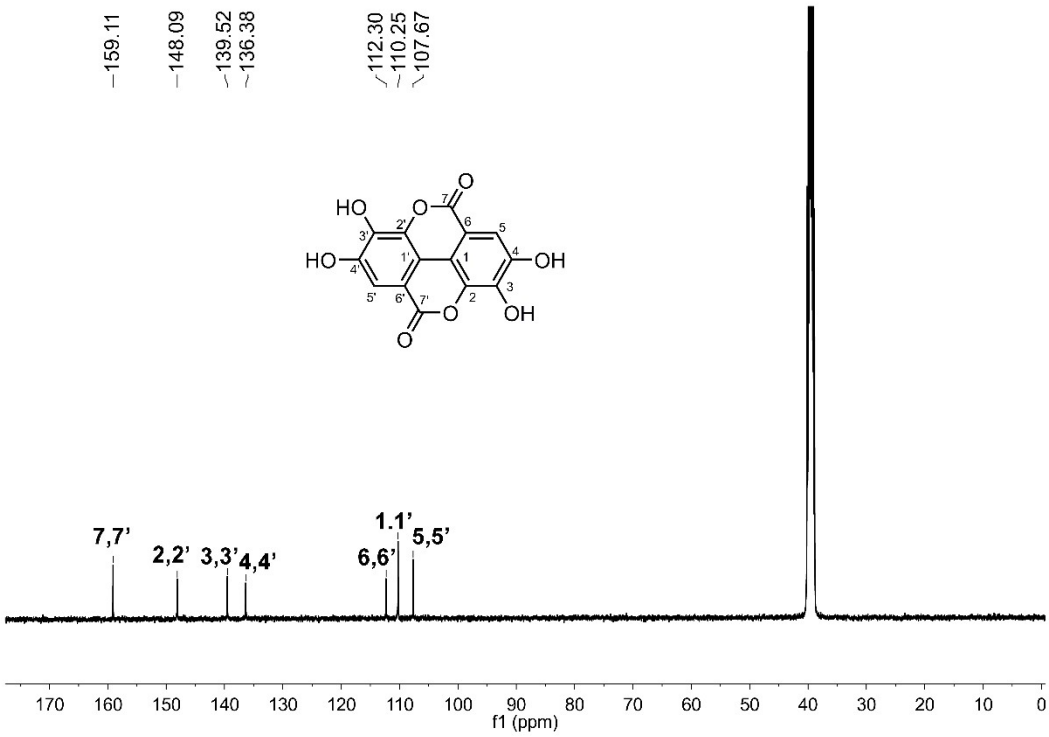

Figure S7. [13]C NMR of light-yellow precipitate obtained from Cu-EA polymer treated with HCl (DMSO-d_6, 100 MHz, 298K).

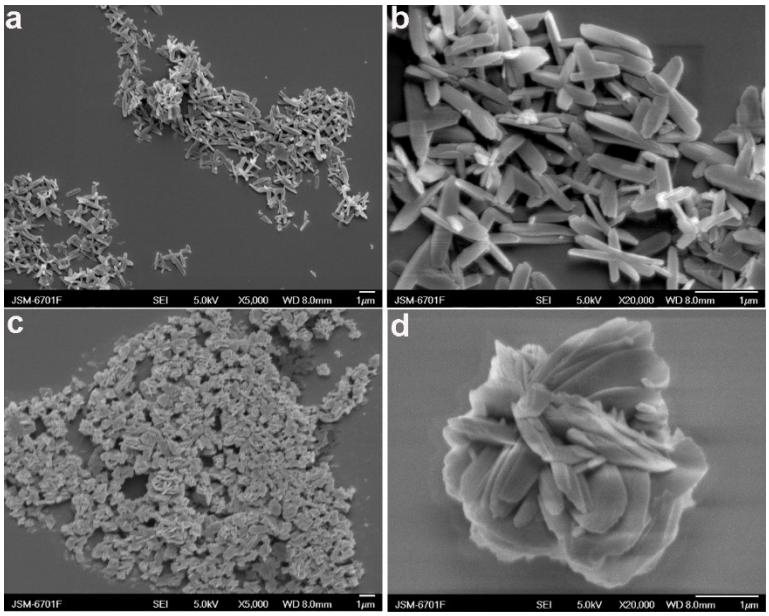

Figure S8. SEM images of EA particles after adding HCl solution with different concentration, 0.1 M (a and b) and 1 M (c and d).

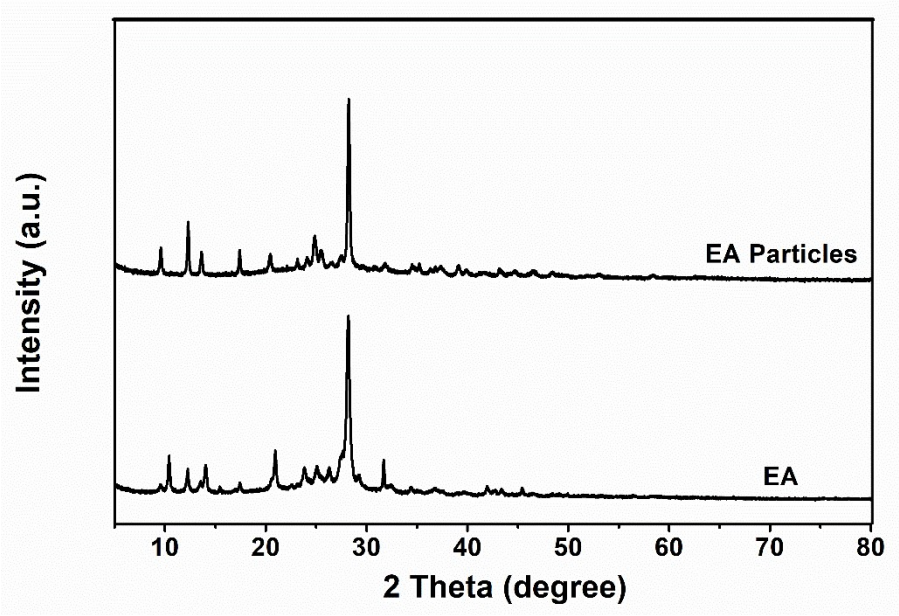

Figure S9. XRD pattern of EA crystalline particles and commercial EA.

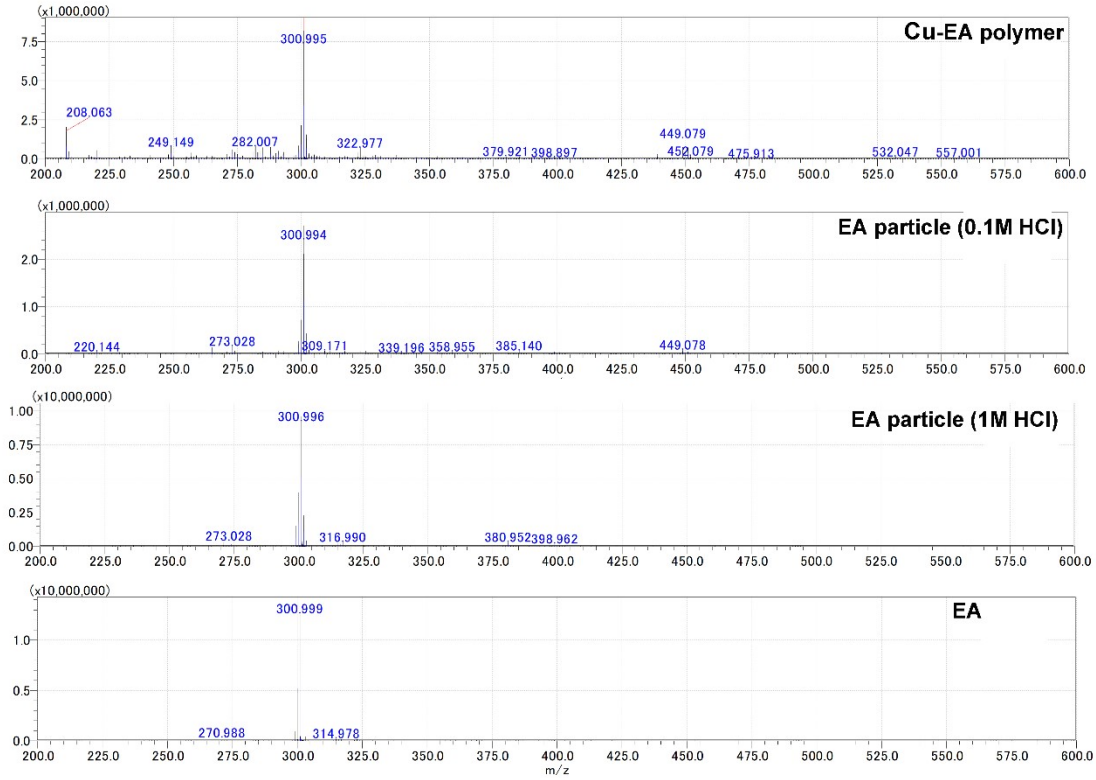

Figure S10. MALDI-TOF-MS of Cu-EA polymer, EA particles and EA using 9AA matrix in negative ionization mode.

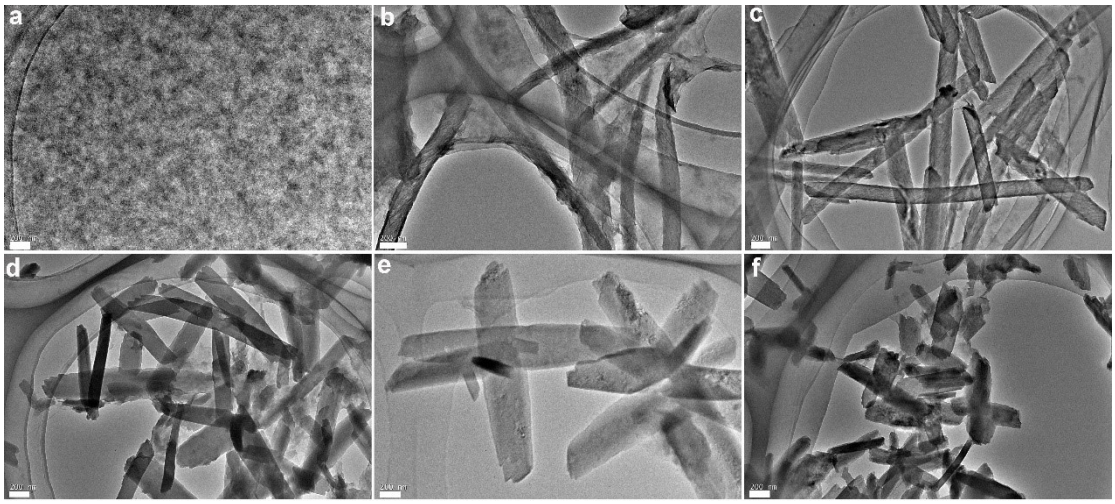

Figure S11. TEM images of Cu-EA polymer (a) and EA particles with adding different amount of HCl (0.1 M). 10 μL (EA-1, b), 20 μL (EA-2, c), 30 μL (EA-3, d), 40 μL (EA-4, e) and 50 μL (EA-5, f).

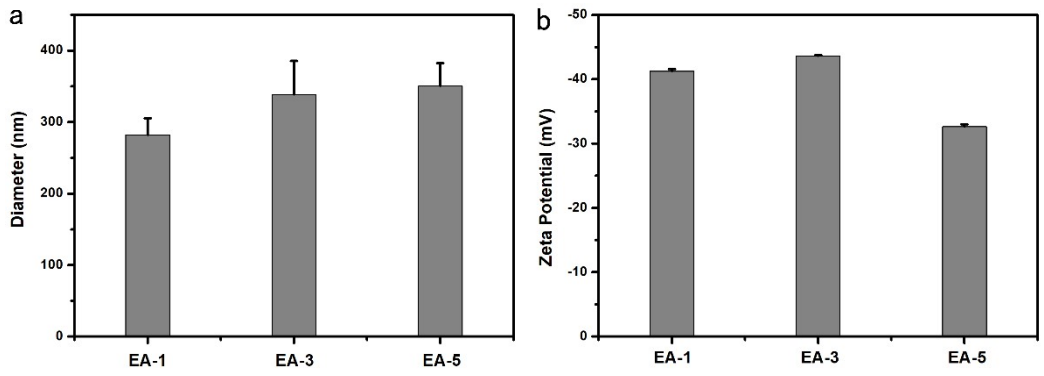

Figure S12. The hydrodynamic diameter (a) and zeta potential (b) of EA particles. the data are presented as the mean ± SD (n = 3)

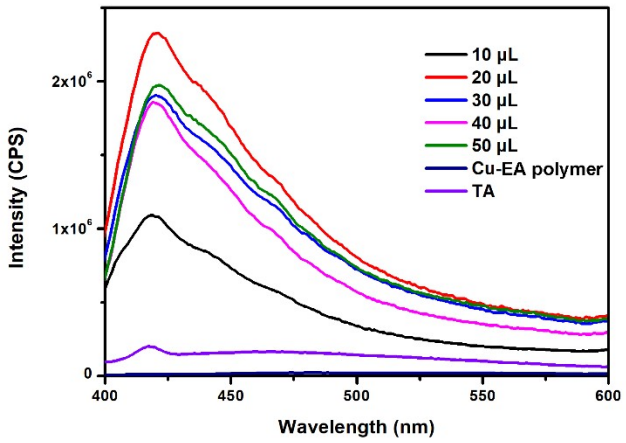

Figure S13. Fluorescence emission spectra of TA, Cu-EA polymer and EA particles with adding different volume of HCl solution obtained at excitation wavelength of 360 nm.

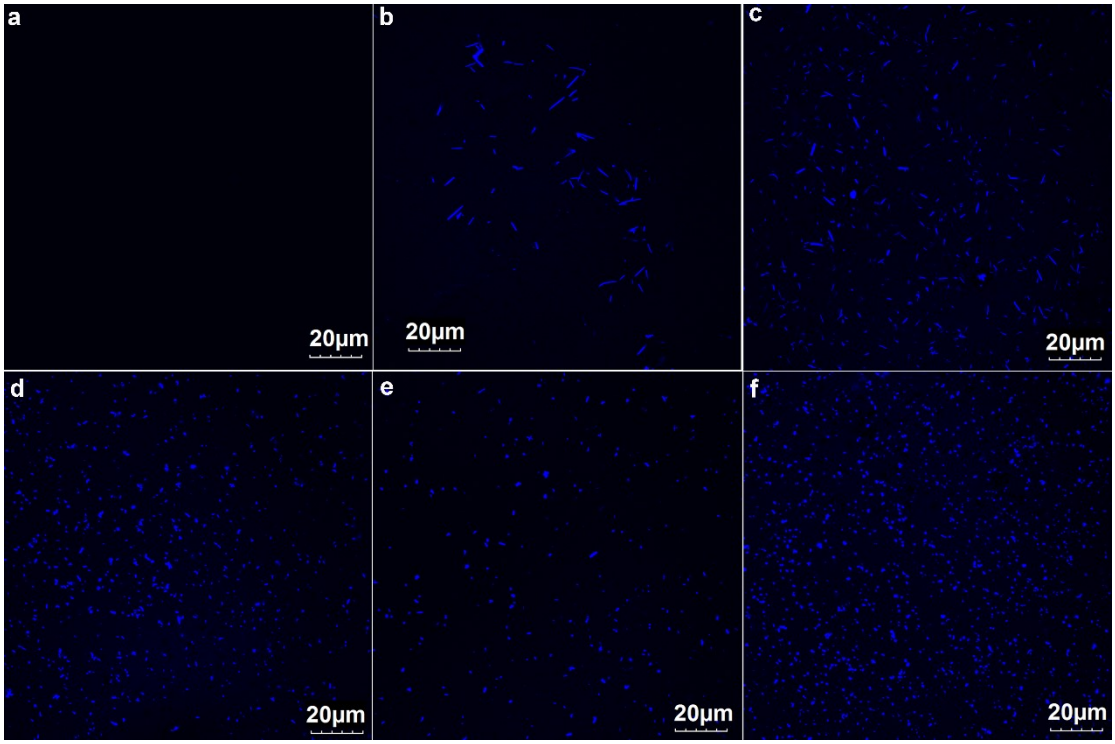

Figure S14. CLSM images of EA particles excited at 408 nm. (a) Cu-EA polymer, (b) EA-1, (c) EA-2, (d) EA-3, (e) EA-4, and (f) EA-5.

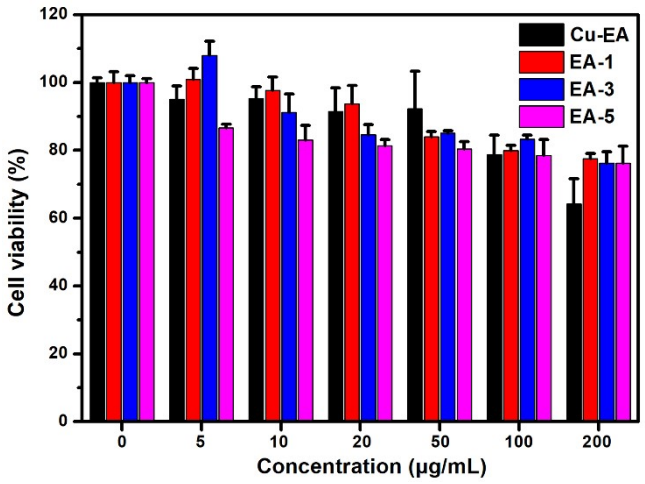

Figure S15. Cytotoxicity assay of PC12 cell after 24 h of incubation with Cu-EA polymer and different EA particles.

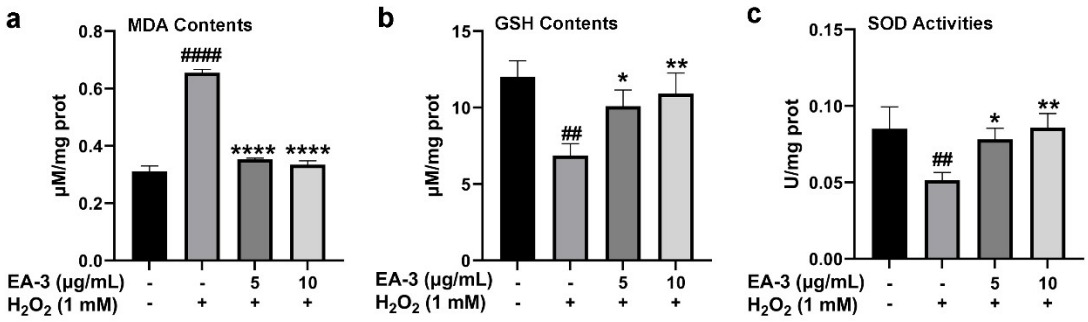

Figure S16. The levels of MDA (a), GSH (b) and activities of SOD (c) of PC12 cells pretreated with EA-3 particles (5 and 10 μg/mL) and then incubated with H_2O_2 (1 mM). For all graphs, the data are presented as the mean ± SD and statistical significance was calculated by Ordinary one-way ANOVA; (##) $p < 0.01$ and (####) $p < 0.0001$ vs control group; *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ vs H_2O_2-treated group.

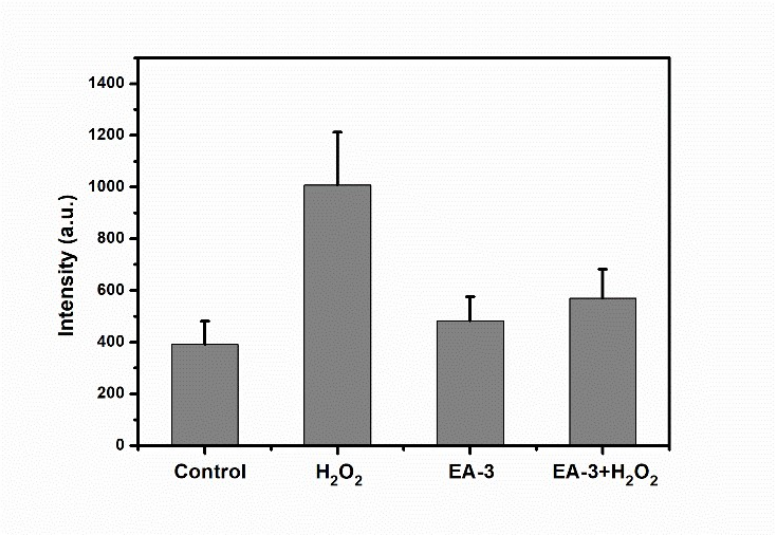

Figure S17. Semi-quantitative analysis of fluorescence intensity in cells by measuring 60 randomly selected cells corresponding to CLSM images in Figure 7, the data are presented as the mean ± SD.

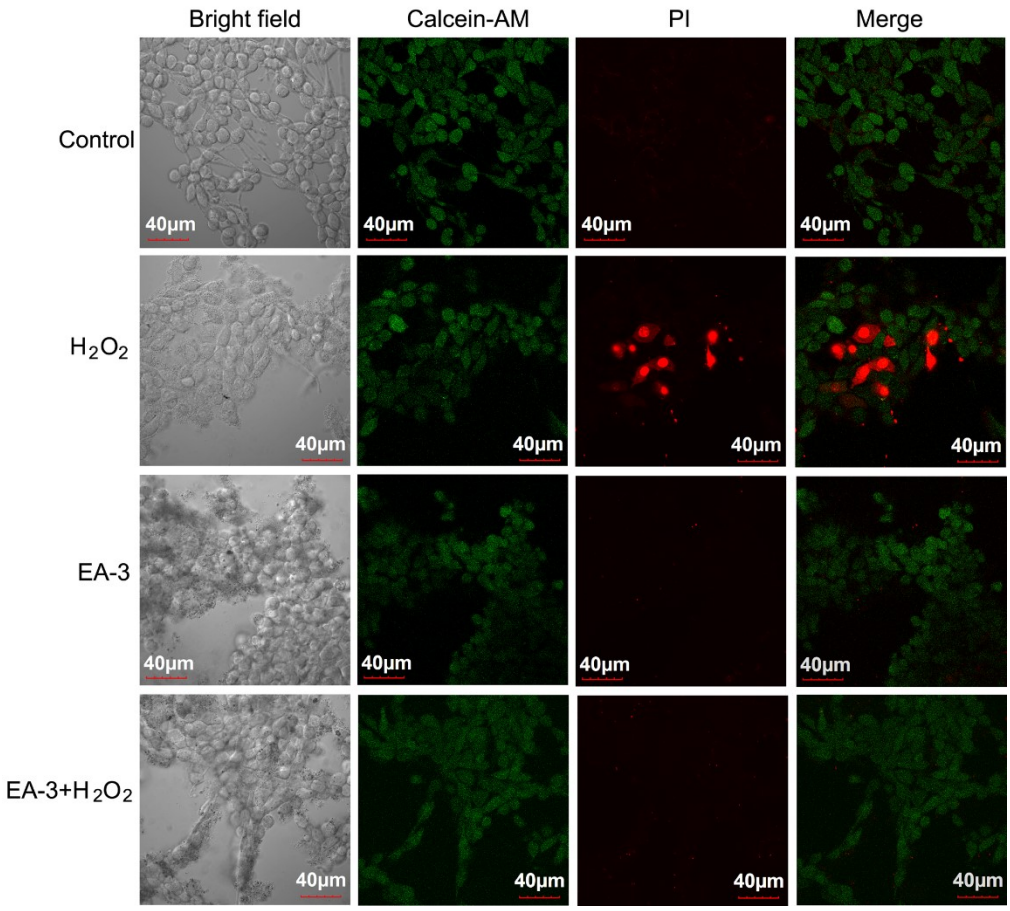

Figure S18. Live/dead assay of PC12 cells after different treatments. Green colors represent the live cell, and red color represent dead cells.

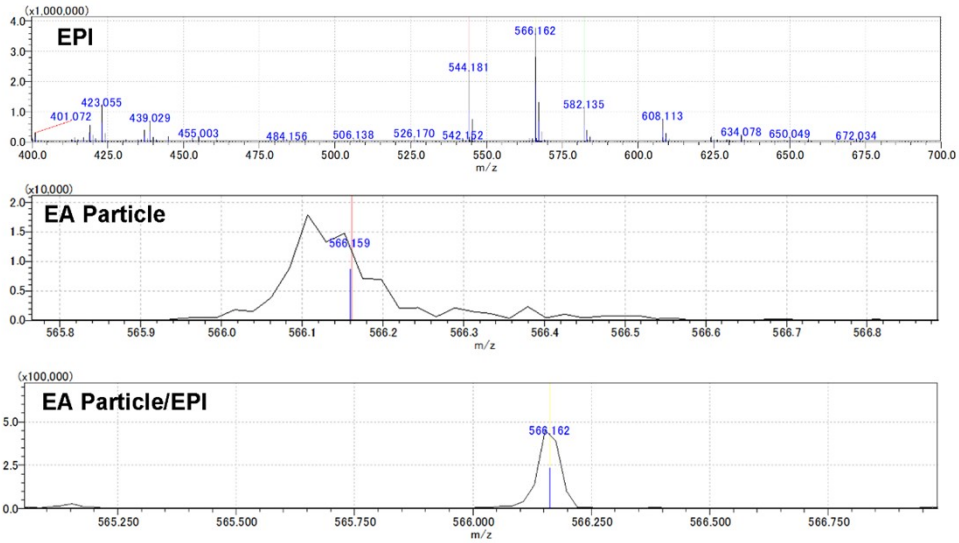

Figure S19. The MALDI-TOF-MS spectrum acquired from EPI, EA particles and EA particle/EPI using CHCA matrix in positive ionization mode.

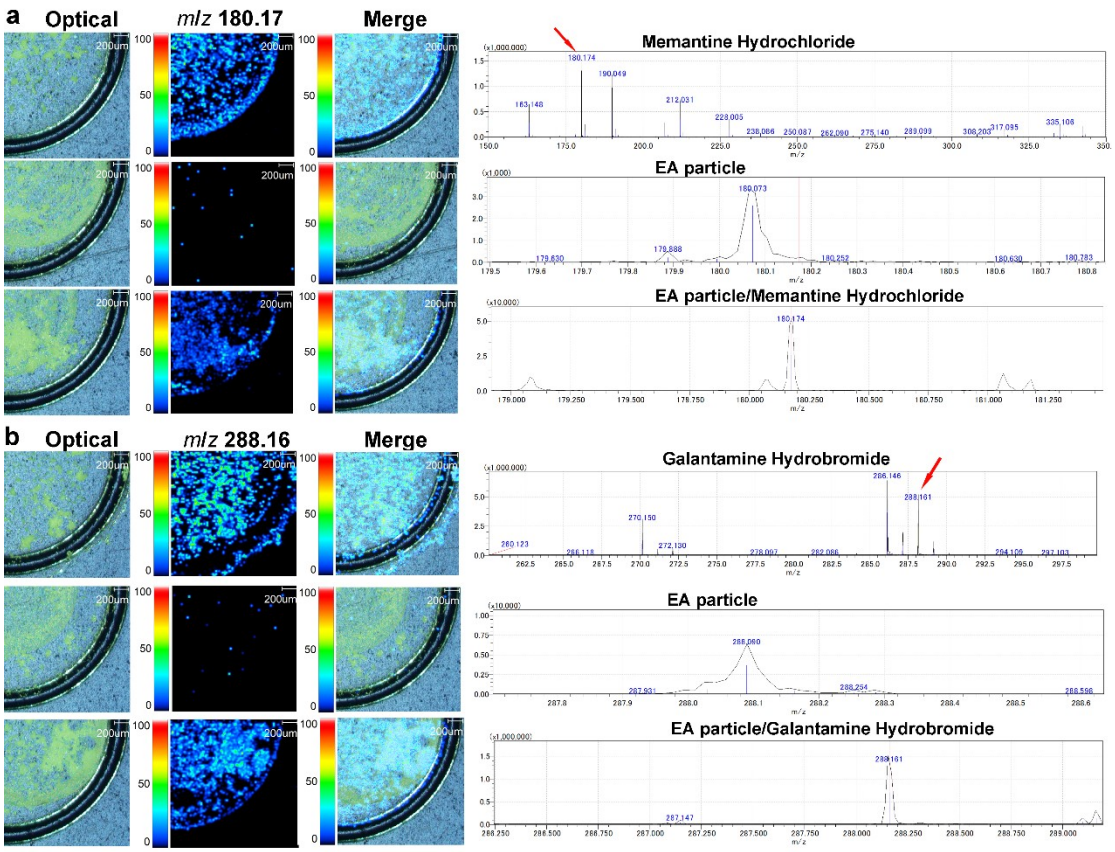

Figure S20. (a) MALDI-TOF-MSI (left) and MALDI-TOF-MS (right) analysis of memantine hydrochloride, EA particle, and EA particle/ memantine hydrochloride, The MS signal at *m*/*z* 180.17 was selected as diagnostic ion. (b) MALDI-TOF-MSI (left) and MALDI-TOF-MS (right) analysis of galantamine hydrobromide, EA particle, and EA particle/galantamine hydrobromide, The MS signal at *m*/*z* 288.16 was selected as diagnostic ion.

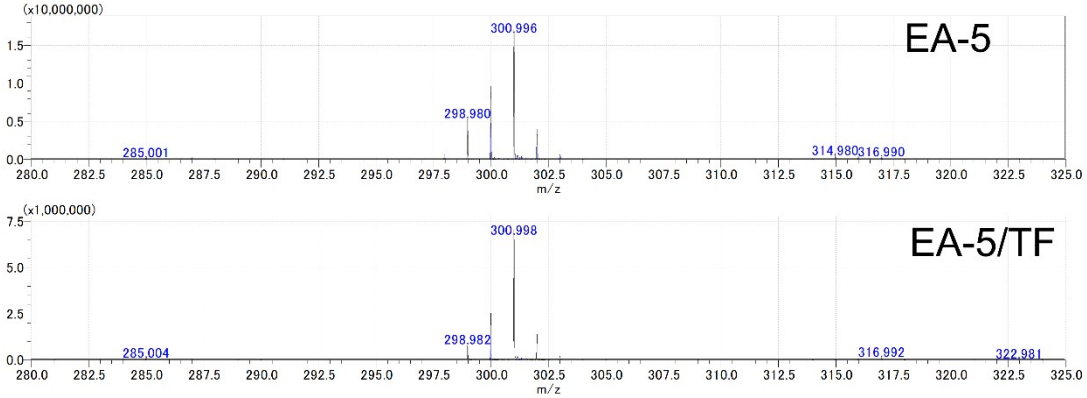

Figure S21. The MALDI-TOF-MS spectrum acquired from EA-5 particle and EA-5/TF particle using 9AA matrix in negative ionization mode.

Table S1

Permeability results of EA particles from the PAMPA-BBB assay.

Sample	*Pe* (10^{-6}cm·s^{-1})	**prediction**
EA	7.71	CNS+
Carbamazepine	10.8	CNS+
Hydrocortisone	1.9	CNS-

Samples with permeabilities Pe > 4.7×10^{-6} cm·s^{-1} could cross the BBB by passive diffusion.

Reference

1. D. Zhao, Y. Tang, X. Suo, C. Zhang, Y. Dou, J. Chang, A dual-targeted multifunctional nanoformulation for potential prevention and therapy of Alzheimer's disease, The Innovation, 2021, 2(4), 100160.

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