Chiral modulation of amyloid beta fibrillation and cytotoxicity by enantiomeric carbon dots

Ravit Malishev,[a] Elad Arad,[a] Susanta Kumar Bhunia,[a] Shira haham-Niv,[b] Sofiya Kolusheva,[d] Ehud Gazit [b, c] and Raz Jelinek*[a,d]

[a.] *Department of Chemistry, and [d]Ilse Katz Institute for Nanotechnology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel.*

[b.] *Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel.*

[c.] *Department of Materials Science and Engineering, Iby and Aladar Fleischman Faculty of Engineering, Tel Aviv University, Tel Aviv 69978, Israel.*

**Author to whom correspondence should be addressed: razj@bgu.ac.il*

Experimental section:

Materials

Ethylene glycol, w O,O'-Bis(2-aminopropyl) polypropylene glycol-block-polyethylene glycol-block-polypropylene glycol1, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), sodium hydrosulfite, sodium phosphate, dialysis tubing (benzoylated, MWCO~ 2000 Da) were purchased from Sigma-Aldrich (Rehovot, Israel). D-Lysine and L-Lysine (99% purity) were purchased from Tzamal D-Chem Laboratories (Petach-Tikva, Israel). Aβ42 was purchased from AnaSpec (USA) in a lyophilized form at >95% purity. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (brain, porcine) and cholesterol (ovine wool, >98%) were purchased from Avanti Polar Lipids. A11 anti-oligomer antibody was obtained from Rhenium (Modi'in, Israel). Horseradish peroxidase conjugated anti-rabbit IgG (HRP) was purchased from ZOTAL (Tel-Aviv, Israel). 1,6-diphenylhexatriene (DPH) were obtained from Molecular Probes, Inc. (Eugene, Oregon).

Chiral Carbon Dot (C-dot) Preparation.

400 mg of each Lysine enantiomer were mixed with 10 mL ethylene glycol and 100 μL of O,O'-bis(2-amino propyl) polypropylene glycol-block-polyethylene glycol-block-polypropylene glycol1. The mixture was hydrothermally-treated at 170 ^{0}C for 2 hours for the formation of C-dots. The mixture was subsequently dialyzed in fractions of 5 mL in double

distilled water (18.2 MΩ cm) using dialysis tube (benzoylated, MWCO 2000 dalton) for 24 hours for purification. Synthesis yields were around 10%.

Peptide Preparation

Aβ42 was dissolved in HFIP at a concentration of 1 mg/mL and stored at −20 °C until use to prevent aggregation. For each experiment, the solution was thawed, and the required amount was dried by evaporation for 6−7 h to remove the HFIP. The dried peptide sample was dissolved in 10 mM sodium phosphate, pH 7.4, at room temperature.

Vesicle preparation.

Vesicles consisting of DOPC, sphingomyelin and cholesterol were prepared by dissolving the lipid components in chloroform/ethanol (1:1, v/v) and drying together in vacuo. Small unilamellar vesicles (SUVs; DOPC/sphingomyelin/cholesterol 0.67:0.08:0.25, mole ratio) were prepared in 10 mM sodium phosphate (pH 7.4) by probe-sonication of the aqueous lipid mixtures at room temperature for 10 min. Vesicle suspensions were allowed for 1 h at room temperature prior to usage.

High resolution transmission electron microscopy (HRTEM)

HRTEM samples were prepared by putting a drop of synthesized C-dots solution on a graphene-coated copper grid followed by drying. Samples were examined with a 200 kV JEOL JEM-2100F microscope.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded in the range of 190–260 nm at room temperature on a Jasco J-715 spectropolarimeter, using 1-mm quartz cuvettes. Solutions composed of 400 µL contained 25 µM Aβ42 in the absence or presence of -Lys-C-dots or L-Lys-C-dots. Spectra were recorded at t=0 and t=24 h. CD signals resulting from the buffer and D\L-Lys-C-dots were subtracted from each presented spectrum.

X-ray photoelectron spectroscopy (XPS)

XPS of the C-dots was performed on an X-ray photoelectron spectrometer ESCALAB 250 ultrahigh vacuum (1*10-9 bar) apparatus with an Al Kα X-ray source and a monochromator. The X-ray beam size was 500 µm and survey spectra was recorded with pass energy (PE) 150eV and high energy resolution spectra were recorded with pass energy (PE) 20eV. Processing of the XPS results was carried out using AVANTGE program.

ζ-potential Measurement

ζ Potential was measured by Zetasizer (Zetasizer Nano ZS, Malvern, Worcestershire, UK). Every sample of 1 mL CQD solution, in concentration of 0.1 mg/mL, was measured for four replicates and averaged. Malvern DTS 1070 disposable capillary cuvette was used.

Cryogenic transmission electron microscopy (cryo-TEM)

Cryo-TEM imaging of aliquots taken from the same reaction mixtures used in the CD experiments after 24-h incubation was carried out as follows: A 3-μL droplet of the reaction mixture was deposited on a glow-discharged TEM grid (300 mesh Cu Lacey substrate grid; Ted Pella). The excess liquid was blotted with a filter paper, and the specimen was rapidly plunged into liquid ethane pre-cooled with liquid nitrogen in a controlled environment (Leica EM GP). The vitrified samples were transferred to a cryo-specimen holder (Gatan model 626) and examined at -181 ^{o}C using a FEI Tecnai 12 G2 TWIN TEM operated at 120 kV in low-dose mode. Grids were imaged a few micrometers under focus to increase phase contrast. The images were recorded with a Gatan charge-coupled device camera (model 794) and analyzed by Digital Micrograph software, Version 3.1.

Cell cytotoxicity experiments

SH-SY5Y cells (2×10^5 cells/mL) were cultured in 96-well micro plates (100 uL/well) and incubated overnight at 37°C. 100 μL of 10 μM Aβ42 in the absence or presence of D-Lys-C-dots or L-Lys-C-dots were added to each well. Following incubation for 24 hours at 37°C, cell viability was evaluated using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 20 μL of 5 mg/mL MTT dissolved in PBS was added to each well. After 4 hours' incubation at 37°C, 100 μL of the extraction buffer [20% SDS dissolved in a solution of 50% dimethylformamide and 50% DDW (pH 4.7)] were added to each well, and the plates were incubated again for 30 minuts. Finally, color intensity was measured using an ELISA reader at 570 nm. The data are presented as mean ± SEM.

Dot Blot Assay

Oligomers of Aβ42 were prepared in the absence or presence of bacoside A and probed by the oligomer-specific polyclonal antibody (pAb) A11 using a modification of previously described methods.[1] Briefly, HFIP-treated Aβ42 was dissolved in 60 mM NaOH at 2 mM. This solution was sonicated for 1 min followed by dilution with 10 mM phosphate buffer saline (PBS) to a final peptide concentration of 45 μM. The resulting solution was maintained at room temperature without agitation up to 1h. Periodically, 2-μL aliquots were applied to nitrocellulose membranes. The membranes were blocked for 1 h with 5%

nonfat milk in 10 mM Tris-buffered saline (TBS) followed by incubation with A11 at 1:1000 dilution in TBS containing 5% nonfat milk followed by appropriate horseradish peroxidase-linked secondary polyclonal antibodies and developed using an Enhanced Chemiluminescence (ECL) reagent kit (GE Healthcare).

Fluorescence anisotropy

The fluorescence probe DPH was incorporated into the SUVs (DOPC / sphingomyelin /cholesterol at 0.67:0.08:0.25 molar ratio) by adding the dye dissolved in THF (1 mM) to vesicles up to a final concentration of 1.25 μM. After 30 min of incubation at 28°C DPH fluorescence anisotropy was measured at 430 nm (excitation 360 nm) before and after addition of Aβ4, D-Lys-C-dots\L-Lys-C-dots, or their mixtures solution on a Fluorolog spectrofluorometer. Anisotropy values were automatically calculated by the spectrofluorimeter software using a conventional methodology. Results are presented as means ± SEM.

Fluorescence spectroscopy

Fluorescence emission of D-Lys-C-dots and L-Lys-C-dots were recorded using a FL920 spectrofluorimeter (Edinburgh Co., Edinburgh, UK).

Thioflavin T (ThT) kinetics assay

25 μM Aβ1–42 was incubated in presence or absence of 0.2mg/mL L/D-Lys-S-dots at 37°C. Aggregation of Aβ42 was measured by adding of 10 μL Thioflavin T solution (100 μM in phosphate buffer) to 90 μL of incubated Aβ42 solutions. The fluorescence intensity was measured at λex = 440 nm, λem = 490 nm, n = 3, and the fluorescence intensity of the C-dots/ThT solution was subscribed.

Results

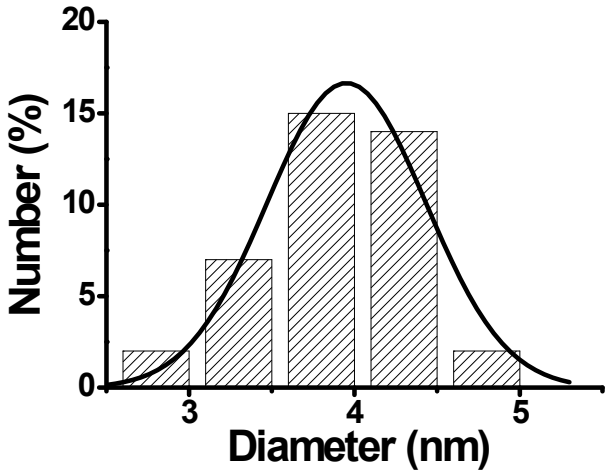

Figure 1, SI: Lysine C-dots size distribution. Carbon dots present diameter of 3.9 ± 1.1 nm (n=50).

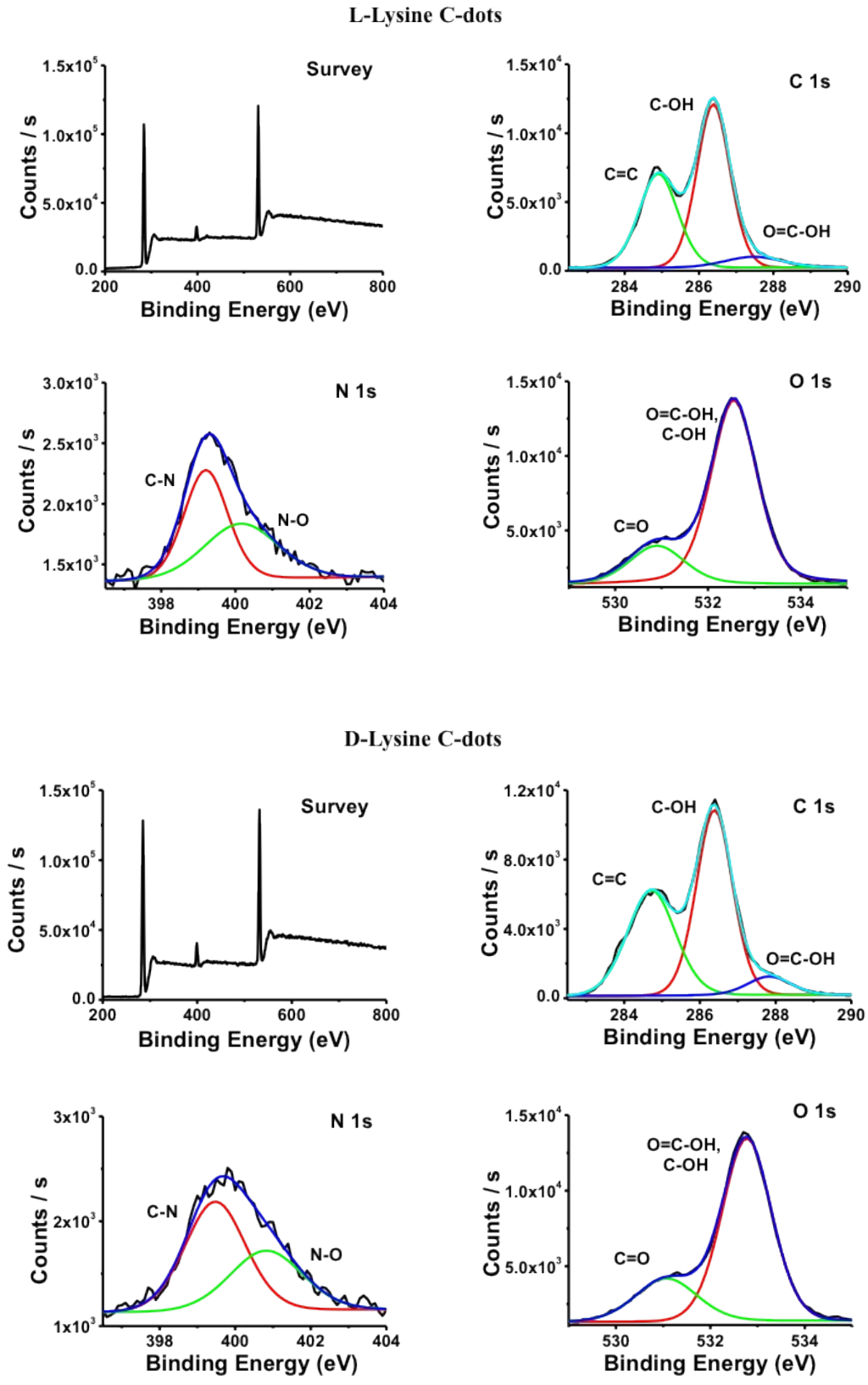

Figure 2, SI: **X-ray photoelectron spectroscopy (XPS) analysis of D-Lys-C-dots and L-Lys-C-dots.**

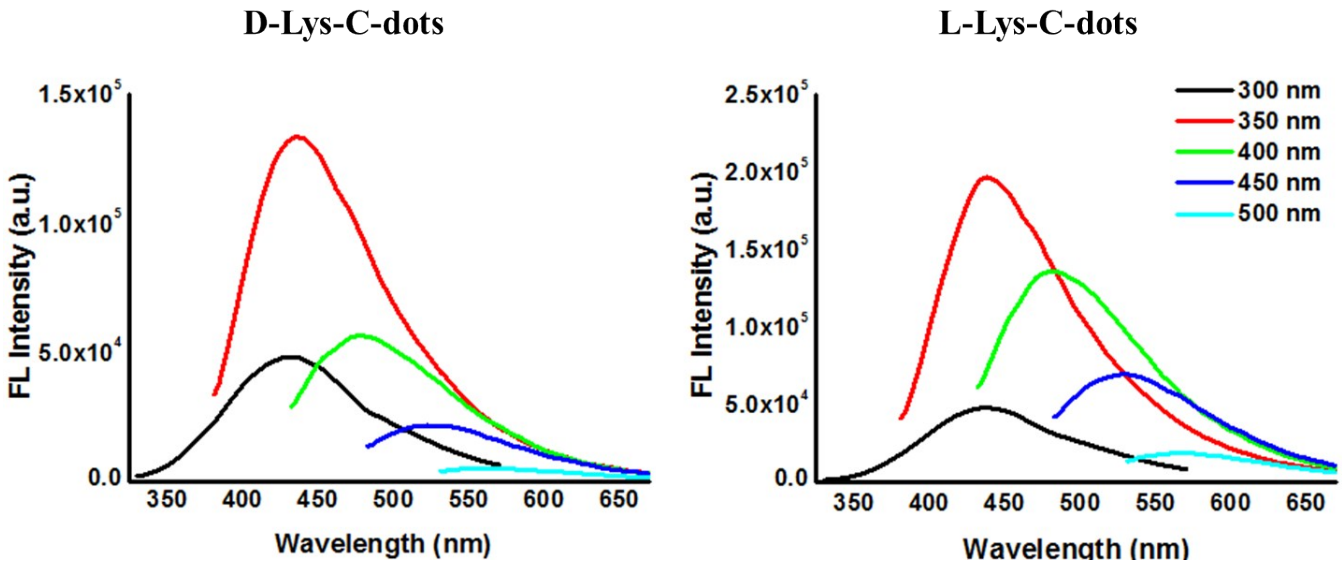

Figure 3, **SI: Fluorescence emission of D-Lys-C-dots and L-Lys-C-dots.** Photoluminescence spectra of D-Lys-C-dots and L-Lys-C-dots excited at different wavelengths, recorded in phosphate buffer.

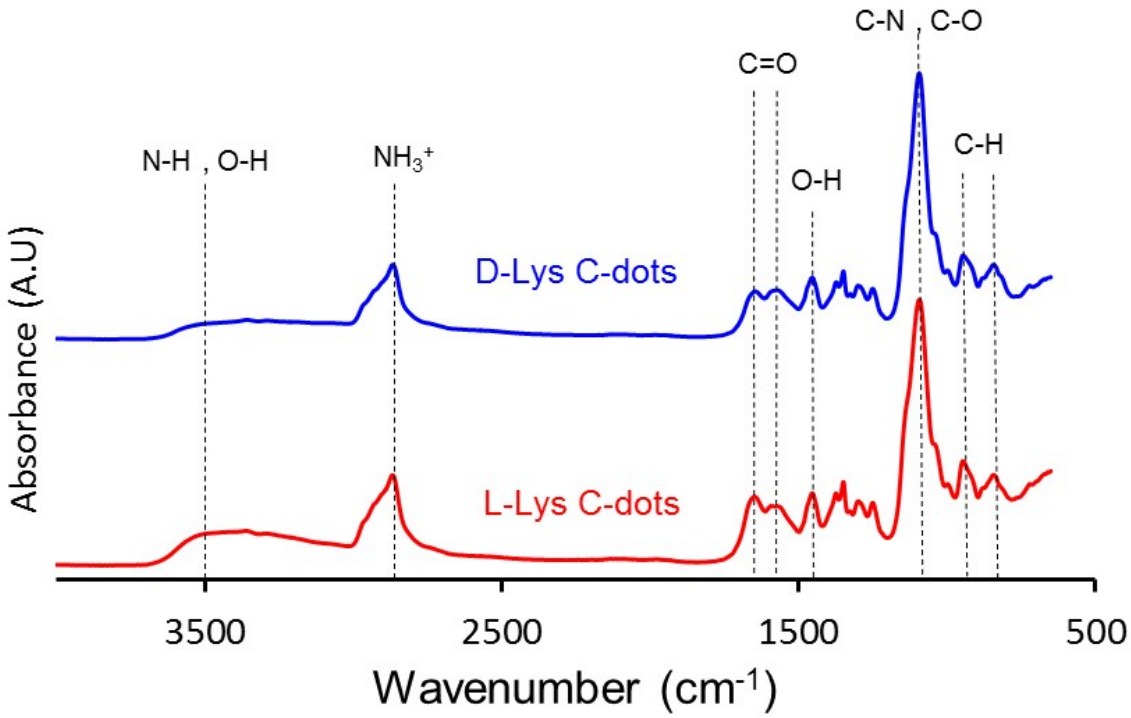

Figure 4, SI: Fourier Transform Infra-red Spectroscopy of D-Lys-C-dots and L-Lys-C-dots. Both spectra show similar typical amine and carboxyl functional groups, indicating lysine presence on the C-dots surface. Both spectra showing same functional groups, including primary amine and carboxylic acid. The peak at ~3500 cm-1 corresponds to N-H/O-H stretching, peak at ~1650 cm-1 and ~1550 cm-1 for C=O bond, peak at ~ 1095 cm-1 corresponds to the C-O, C-N bonds. ATR-FTIR spectroscopy was performed on a NICOLET 6700 FTIR spectrometer. Peak intensity was normalized according to maximal peak value.

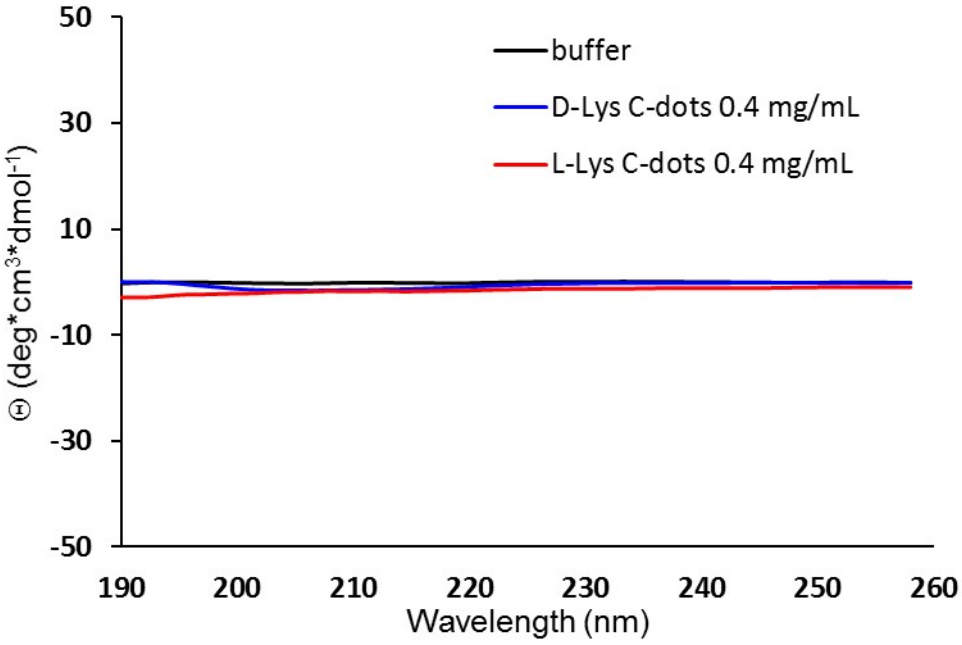

Figure 5, SI: Lysine C-dots Circular Dichroism spectra at low concentration of 0.4 mg/mL.

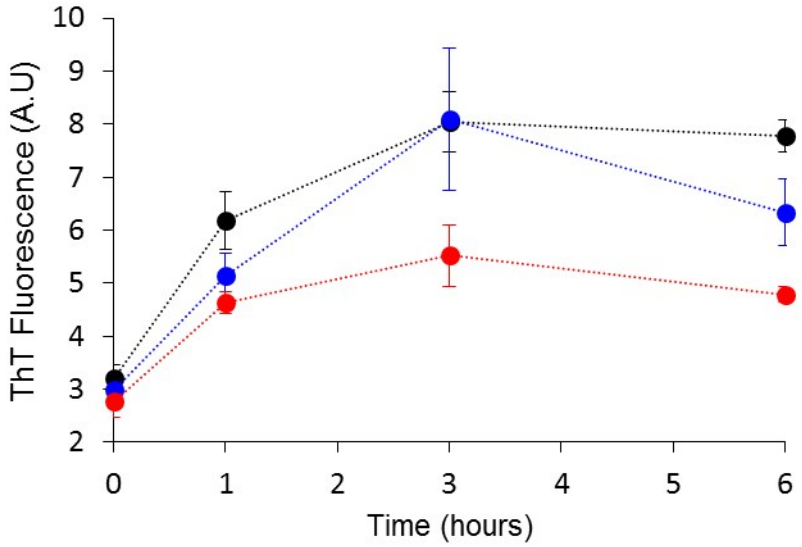

Figure 6, SI: Aggregation of Aβ42 in presence or absence of Lys-C-dots. Sample containing L-Lys C-dots (*red* spectrum) shows decreased fluorescence in comparison to sample with D-Lys C-dots (*blue* spectrum) and without C-dots (*black* spectrum). Sample containing L-Lys C-dots shows decreased fluorescence and indicates lower agregate content.

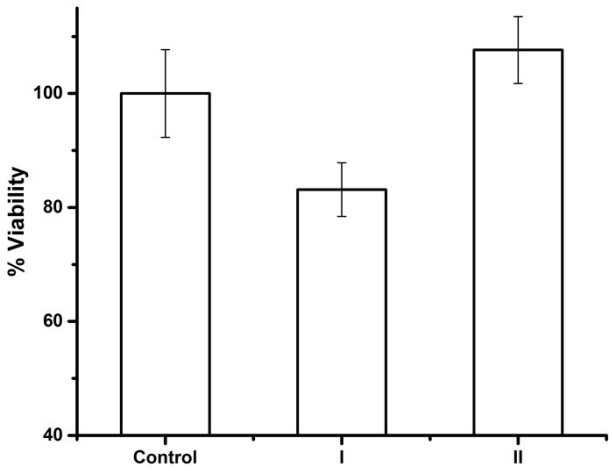

Figure 7, SI: Treated SH-SY5Y cells were incubated with medium containing D-Lys-C-dots (0.4 mg/ml, **I**) and L-Lys-C-dots (0.4 mg/ml, **II**).

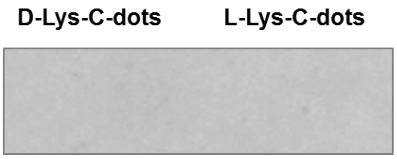

Figure 8, SI: Immunoreactivity assay of D-Lys-C-dots and L-Lys-C-dots using pAb A11.

Lys-C-dots	ζ-potential (Mv)
D-Lys	-2±1
L-Lys	-5.9±0.4

Table 1, SI: **Electrostatic properties of D-Lys-C-dots and L-Lys-C-dots.** ζ-potential of D-Lys-C-dots and L-Lys-C-dots was measured in aqueous solution.

References

1. R. Kayed, E. Head, J. L. Thompson, T. M. McIntire, S. C. Milton, C. W. Cotman and C. G. Glabe, *Science*, 2003, **300**, 486-489.