Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

In vitro reconstitution of a cellular like environment using liposome for amyloid beta peptide aggregation and its propagation

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Materials:

Peptides and reagents: Beta - Amyloid (1 - 42), Beta - Amyloid (1 - 42) with HiLyte Fluor™ 488 labelled, Beta - Amyloid (1 - 42) with HiLyte Fluor™ 555 labelled were purchased from AnaSpec. Mineral oil, Dextran, Bovine serum albumin (BSA), 5(6)-Carboxyfluorescein (FITC), 1,1,1,3,3,3-Hexafluoro-2-propanol and DMEM medium for cell culture were purchased from Sigma-Aldrich. Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH and Wang resin (100-200 mesh) were purchased from Novabiochem. D-Glucose was purchased from Qualigens. Sucrose was purchased from SRL (Sisco Research Laboratory PVT. LTD). NaCl, KCl, KH_2PO_4, $Na_2HPO_4.2H_2O$, Diethyl ether (Et_2O), DMF (N, N′-Dimethylformamide), DCM (Dichloromethane), TFA (Trifluoroaceticacid) and chloroform (GR) were purchased from Merck. Piperidine was purchased from Spectrochem, India. Neutravidin and Fetal Bovine serum were purchased from Invitrogen. Avidin Rhodamine dye was purchased from Vectors laboratory.

Lipids: L-α-phosphatidylcholine (Egg, Chicken) (Egg PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG) were purchased from Avanti Polar Lipids. N-(6-tetramethyl rhodamine thiocarbamoyl)-1, 2-dihexadecanoyl-*sn*-glycero-3-phospho ethanolamine, triethylammonium salt (TRITC DHPE) and N-(Biotinoyl)-1, 2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (Biotin DHPE) were purchased from Invitrogen.

Control peptide: Water soluble FITC-GFE peptide was used as a control peptide in this manuscript. It was synthesized in our laboratory through solid phase peptide synthesis. Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH and 5(6)-Carboxyfluorescein were added sequentially on the Wang Resin(100-200 mesh). 20% piperidine in DMF was used for Fmoc deprotection. Finally, the peptide was cleaved of from the resin by using TFA in dichloromethane (DCM). The peptide was precipitated out in ice cold Et_2O, purified by HPLC and confirmed by MALDI mass.

All chemicals were used without further purification.

Cell: SH-SY5Y cell line was received as gift from Dr. S. C. Biswas, CSIR-IICB, Kolkata and cultured before use in our laboratory.

METHODS

Preparation of physiological relevant buffers:
The solution inside the liposome (Internal Buffer or IB) was prepared by mixing Phosphate buffer saline (PBS 1X: 140 mM of NaCl, 2.7 mM of KCl, 8.1 mM $Na_2HPO_4.2H_2O$ and 1.8 mM of KH_2PO_4 at pH 7.2), 2M sucrose solution, dextran solution (400 mg/ mL) in MilliQ water. The solution outside the liposome (External Buffer or EB) was prepared by mixing PBS (1X), 20% glucose in water and BSA solution (100 mg/mL) in PBS. Osmolarity of both the buffers were measured by Osmometer and osmolarity of EB was kept approximately 5-10 mOsm below than that of the IB for maintaining the shape of the liposomes. Iso-osmotic condition results change in the shape of the liposome[1]. We failed to prepare liposome when we maintained osmolarity of EB 5-10 mOsm higher than the IB.

Preparation of Lipid-oil mixture:
Chloroform solution of 2.5 mg of Egg PC/POPC, 0.5 mg of DOPS and 0.5 mg of POPG was taken in a 10 mL round bottomed flask. A thin film of lipid mixture was prepared by nitrogen flush. Round bottomed flask was kept for 40 min in high vacuum pump and then vacuum was released under nitrogen atmosphere. 5 mL of mineral oil was added into the round bottomed flask containing lipid film. It was sonicated for 30 min in cold water followed by incubation in hot air oven at 50 ^oC for 3 hours. Next, the flask was cooled down at room temperature and stored at 4 ^oC. 1% of biotin-DHPE and TRITC-DHPE were used in lipid mixture along with Egg PC/POPC, DOPS and POPG for preparation of liposome, containing biotin and TRITC. Biotin-DHPE lipid was used for immobilization of liposome on biotin surface and TRITC-DHPE lipid for visualization of the membrane of liposome.

Preparation of Liposome:
Mixture A: 100 µL lipid-oil mixture was placed on top of the 200 µL of EB and it was placed in an ice bath for 2 hours for the formation of a single lipid layer.
Mixture B: 5 µL of IB was added into the 600 µL of lipid-oil mixture and an emulsion was prepared using a Hamilton syringe and incubated at 4 ^oC for 5 min.
Now, 200 µL of **Mixture B** was loaded carefully into the **Mixture A** and incubated for 5 minutes at 4 ^oC. Then it was centrifuged at 4 ^oC for 12 minutes at the rate 110g, 5 minutes at the rate 350g and 3 minutes at the rate 560g sequentially. The upper oil phase was carefully aspirated with a pipette. The remaining solution in the vial was an EB containing liposomes. Glass capillary was filled with approximately 20 µL of EB and this solution was expelled into the vesicle solution. Now, liposome solution was aspirated by the glass capillary. Entrapment of Aβ peptides and other substances inside liposome was done by dissolving those substances in the IB.
Osmolarity of IB was kept higher than EB for all liposomes experiments in this manuscript.

Liposome was not formed following above method using single lipid such as Egg-PC/POPC/POPG/DOPS.

Construction of the flow chamber for microscopic observation of liposomes:
A flow chamber of 50 µL was constructed onto a microscopic glass slide using double sticky tape (Tesa, Hamburg, Germany) and cover slips[2]. Flow chamber was washed with EB before loading liposomes. Then liposomes were loaded into the flow chamber and it was sealed and observed under inverted fluorescence microscope and confocal microscope.

Immobilization of liposomes:
Biotin-DHPE lipid, with normal lipid mixture, was used during the preparation of lipid-oil mixture and biotin functionalized glass surface was used as counter glass during the preparation of flow chamber. Neutravidin solution (300 nM) in EB was loaded into the flow chamber and incubated for 10 min. After 10 min incubation flow chamber was washed with EB. Liposome solution was loaded into the flow chamber and observed under a confocal microscope (Scheme1I and Movie S5 & S8).

Attachment of two liposomes through biotin-neutravidin interaction:
Biotin-DHPE lipid, with normal lipid mixture was used during the preparation of lipid - oil mixtures. We prepared two types of liposomes containing red (Avidin Rhodamine) and green (5(6)-Carboxyfluorescein) dye separately and mixed them together with EB containing neutravidin (300 nM). This solution was loaded in the flow chamber and observed under a confocal microscope (Figure S2 and S3f).

Preparation of Beta-amyloid peptide stocks:
1.0 mg of Aβ42 peptide was dissolved in 100 µL of 1,1,1,3,3,3-Hexafluoro-2-propanol and 10 µL of aliquots were prepared and stored at -20 ^{o}C. Similarly 0.1 mg of Aβ42 labelled with HiLyte Fluor™ 488 peptide and Aβ42 labelled with HiLyte Fluor™ 555 were dissolved in 100 µL of 1,1,1,3,3,3-Hexafluoro-2-propanol and 10 µL of aliquots were prepared and stored at -20 ^{o}C.

Aβ peptide aggregation study inside of the liposome:
In an eppendrof tube 0.01 mg of Aβ42 peptide and 0.001 mg of Aβ42 with HiLyte Fluor™ 488 labelled peptides were taken as 1,1,1,3,3,3-Hexafluoro-2-propanol solution. The solution was dried by nitrogen flush. A mixture of 200 µL of IB and 0.7 µL of 1% NH_4OH (supplied along with the peptides from Anaspec as a peptide reconstitution solvent) solution was prepared and adjusting its osmolarity with sucrose (2M) solution. 28 µL of this solution was added to the tube containing the Aβ42 peptide mixture. Peptide mixture was dissolved by vortex and sonication. Now the solution contained the mixture of Aβ42 (~80 µM) and 488 HiLyte Fluor™ labelled Aβ42 (~8 µM) peptides in a 10:1 ratio and the solution has pH 7.4. After centrifugation at 12000 RPM for 5 minutes at 4 ^{0}C, the supernatant was used for the experiment. 5 µL of this solution was added into the 600 µL of lipid-oil mixture and an emulsion were prepared. Liposomes were prepared with this emulsion containing Aβ42 peptide inside the liposomes. It was incubated at 37 ^{0}C and images were captured by IX-81 confocal fluorescence microscope (Olympus) with X60 objective (Olympus, Hamburg, Germany) and an Andor iXon3 897 camera at 488 and 561 nm laser light (Figure 1, S4 and Movie S1). We have used non-aggregating FITC-GFE peptide in the control experiment following similar condition (Figure S5a-d).

Aβ peptide aggregation study in solution (without liposome):
Another control experiment using solution contained the mixture of Aβ42 (80 µM) and 488 HiLyte Fluor™ labelled Aβ42 (8 µM) peptides in a 10:1 ratio in absence of liposome was performed in a flow chamber following similar condition as mentioned above method and observed under IX-81 confocal fluorescence microscope (Olympus) with X60 objective (Olympus, Hamburg, Germany) and an Andor iXon3 897 camera at 488 nm laser light and DIC mode (Figure S9). We found peptide aggregates significantly at 120 minutes. But in case of non-aggregating FITC-GFE peptide in the control experiment following similar condition we did not observed similar aggregates even after four hours (Figure S8).

Transport of aggregates from liposome to liposome:
We have prepared two types of liposomes containing biotin lipid (Biotin-DHPE) following previously described method. One type of liposomes was prepared with IB containing 5 (6) -Carboxyfluorescein and another containing 10:1 mixture of Aβ42 (80 µM) and 555 HiLyte Fluor™ labelled Aβ42 peptide (8 µM) in IB. Now, two different liposomes solutions were mixed in EB containing 300 nM neutravidin. Liposomes were mixed and flowed into the flow chamber at 4 ^{0}C. Now, flow chamber was incubated at 37 ^{o}C and images were captured by IX-81 confocal fluorescence microscope (Olympus) with X60 objective (Olympus, Hamburg, Germany) and an Andor iXon3 897 camera at 488 and 561 nm laser light (Figure 3a-c, S2, S6, Movie S6, S7, S9 and S10).

Transport of aggregates from Liposome to SH-SY5Y cells:
Liposomes were prepared using previously described method containing 10:1 mixture of Aβ42 (80 µM) and 488 HiLyte Fluor™ labelled Aβ42 peptide (8 µM) in IB. 100 µL of liposome solution was diluted into 3000 µL of serum free DMEM medium and added into T-25 vial containing SH-SY5Y cells and incubated for 4h at 37 °C in the presence of 5% CO_2.[3] After that cells were washed with PBS buffer (pH

7.4) and viewed with a NIKON (Model Ti-U) inverted microscope (Figure 4a-c). We have used non-aggregating FITC-GFE peptide in the control experiment following similar condition (Figure S7 a-c).

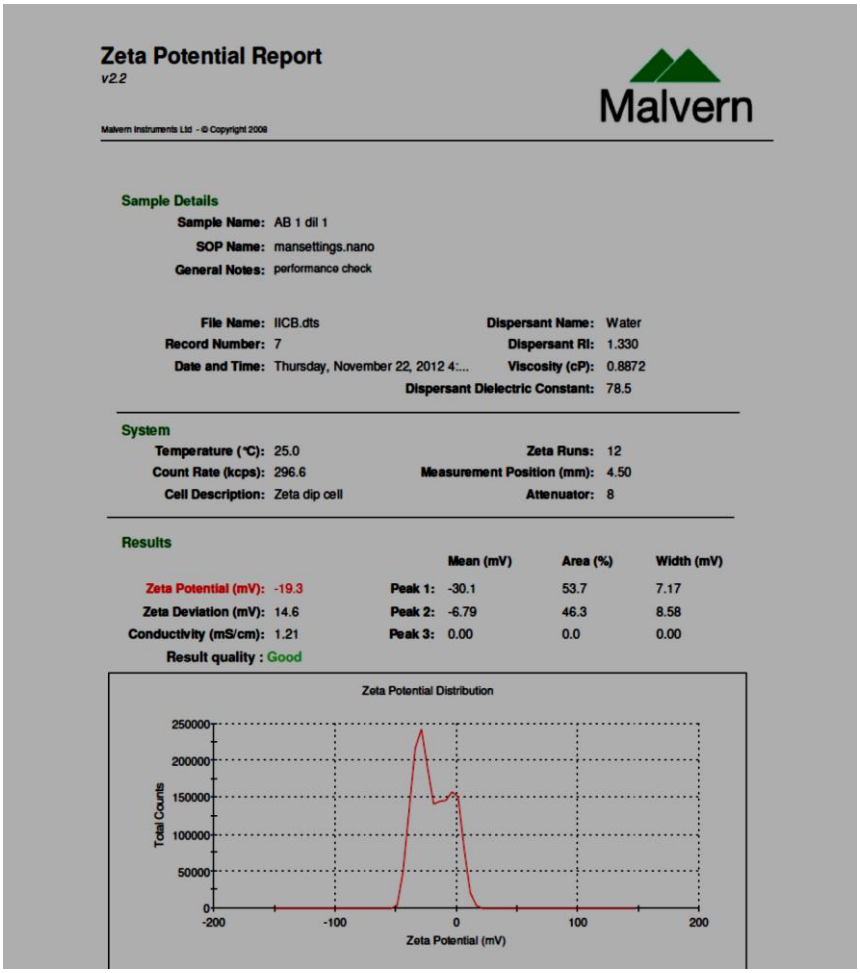

Figure S1. Zeta potential of liposomes reveals negative charge of the membrane.

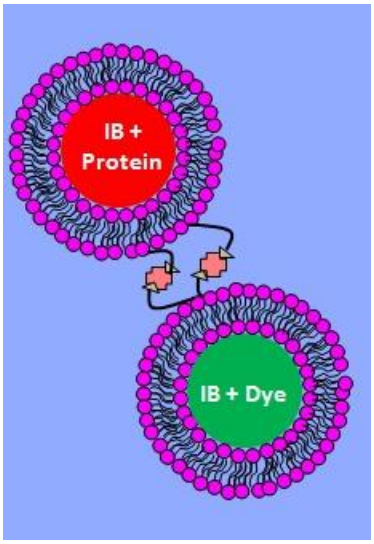

Figure S2. Schematic representation of two attached liposomes through biotin-neutravidin interaction.

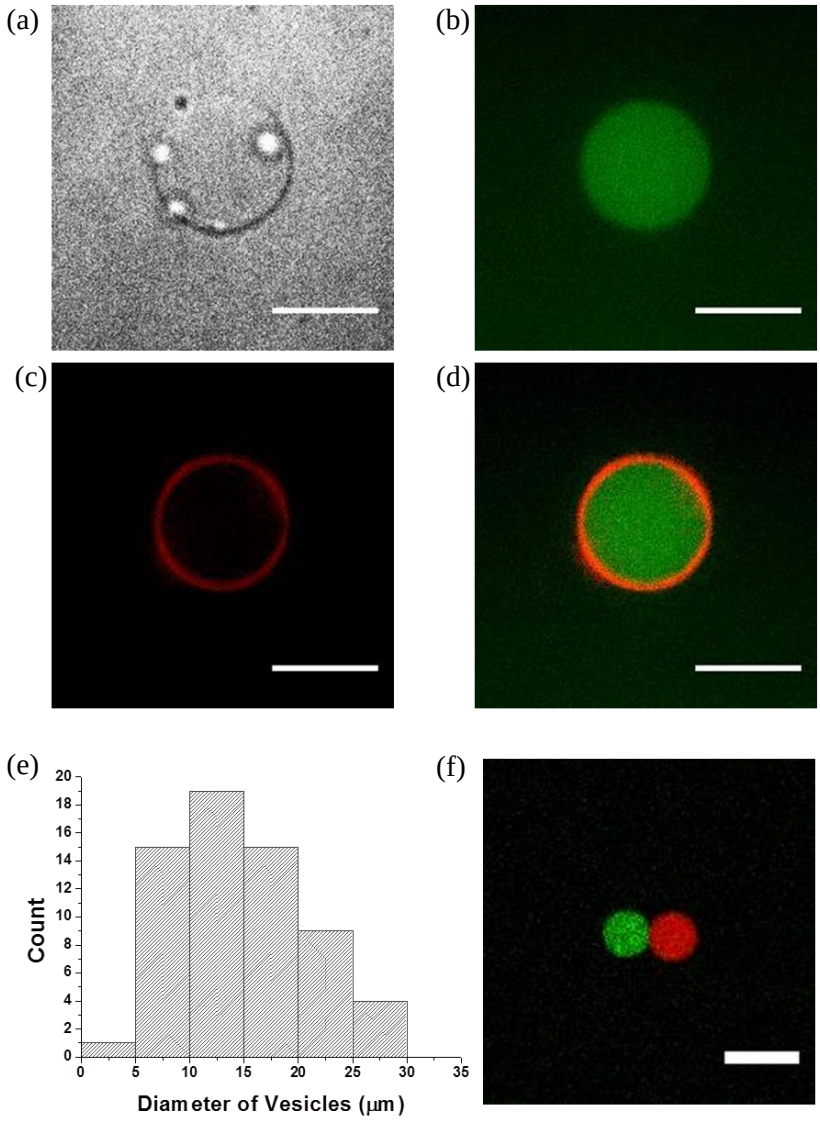

Figure S3. Characterization of Liposome. Confocal images of liposome containing Aβ42 and Hilyte TM 488 labeled Aβ42 peptides (in 10:1 ratio) in (a) DIC mode; (b) 488 nm (green); (c) 561 nm (red) laser lights; (d) merged image of red and green channels. Scale bar corresponds to 20 μm. (e) Distribution of size of liposomes, which shows diameter of liposomes vary from 4 to 30 μm; (f) confocal image shows attachment of two liposomes by biotin-neutravidin noncovalent interaction containing green solution of 5-(6)-carboxyfluorescein in one liposome and red solution of rhodamine dye in another liposome. Scale bar corresponds to 10 μm.

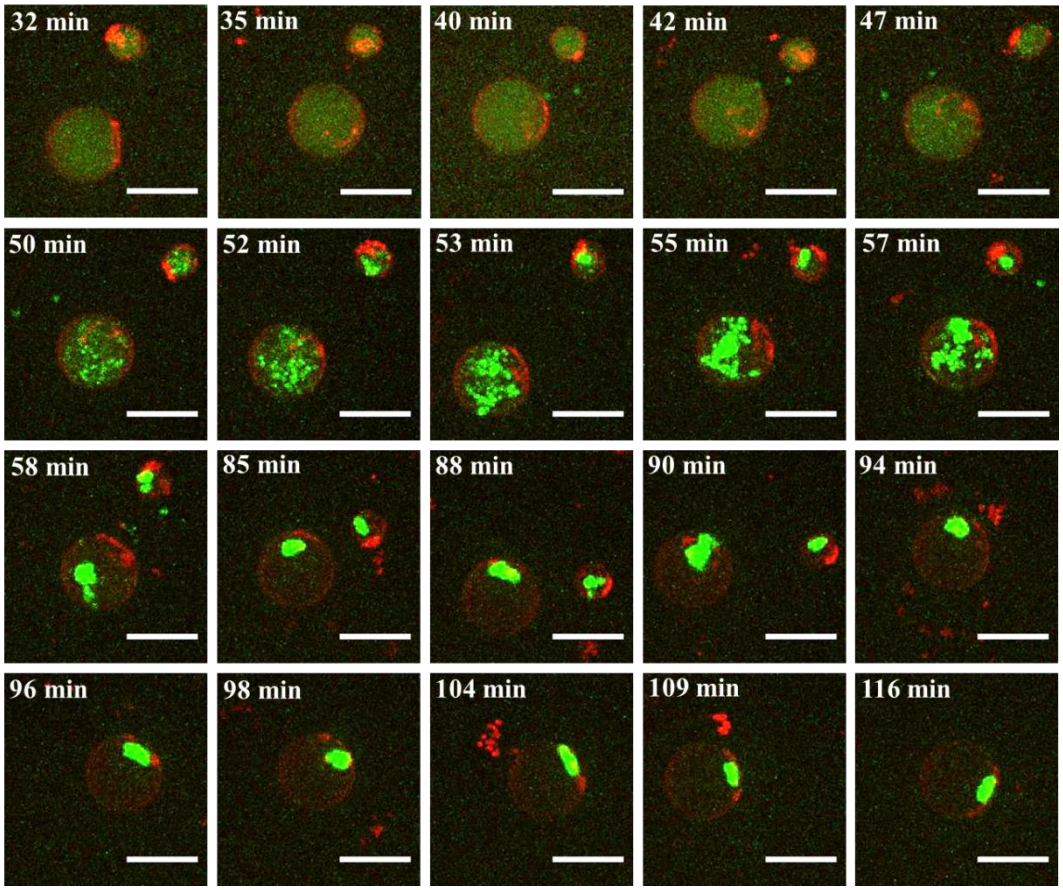

Figure S4. Time lapse images from a merged movie at 37 ^{o}C from different time period reveal time dependent aggregation of Aβ42 peptide inside liposome and the departure of aggregates from liposome by pore formation on the membrane. Scale bar corresponds to 20 µm.

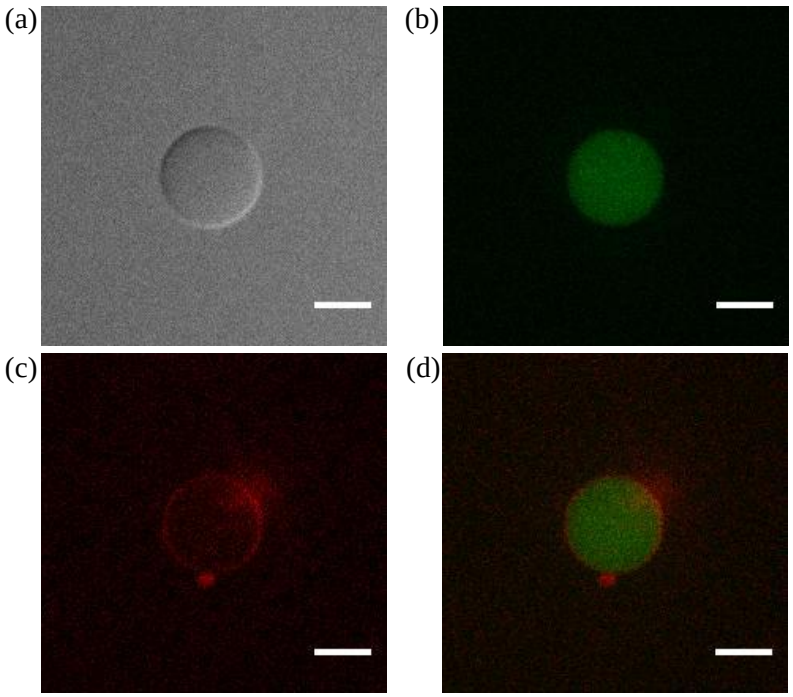

Figure S5. Confocal images of control experiment with FITC-GFE non-aggregating peptide. (a) DIC mode; (b) 488 nm channel; (c) 561 nm channel and (d) merged image of green and red channels. Images indicate that absence of aggregate formation in case of FITC-GFE peptide after 2 hours of incubation at 37 ^{o}C. Scale bar corresponds to 10 µm.

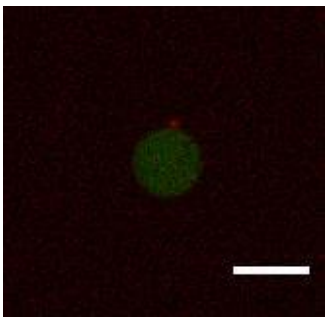

Figure S6. Image reveals that red coloured aggregate was landed on green liposome. Scale bar corresponds to 10 µm.

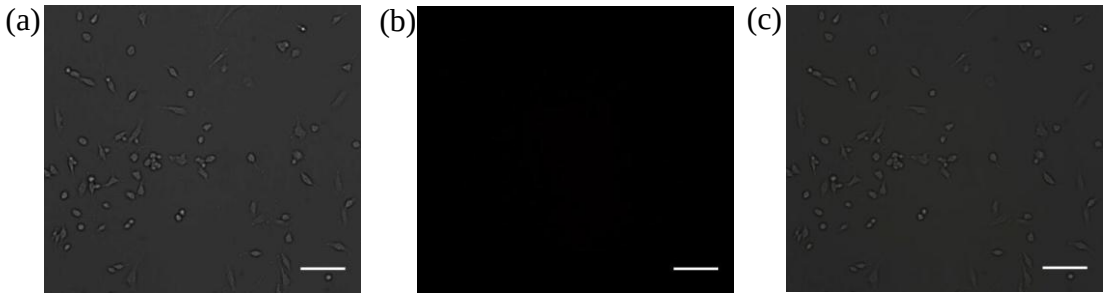

Figure S7. Fluorescence microscopic images of control experiment using non-aggregating FITC-GFE peptide and SH-SY5Y neuron cell line. (a) DIC mode image; (b) 488 nm channel; (c) overlay images of DIC and 488 nm channels. Scale bar corresponds to 100 µm.

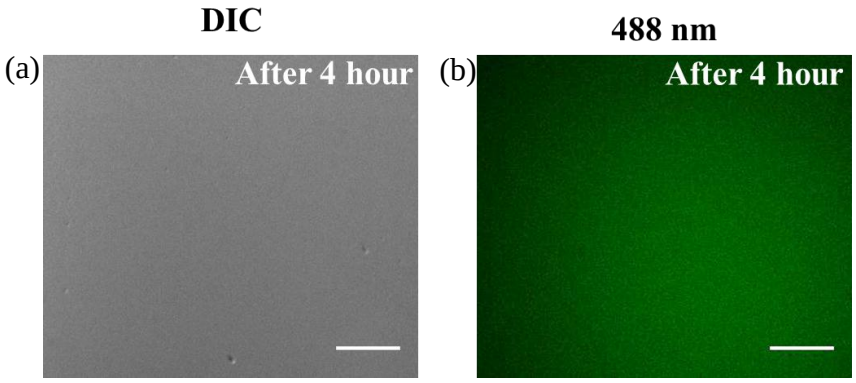

Figure S8. Fluorescence microscopic images of control experiment using non-aggregating FITC-GFE peptide in a flow chamber in absence of liposome. (a) DIC mode image and (b) 488 nm channel. Scale bar corresponds to 20 µm.

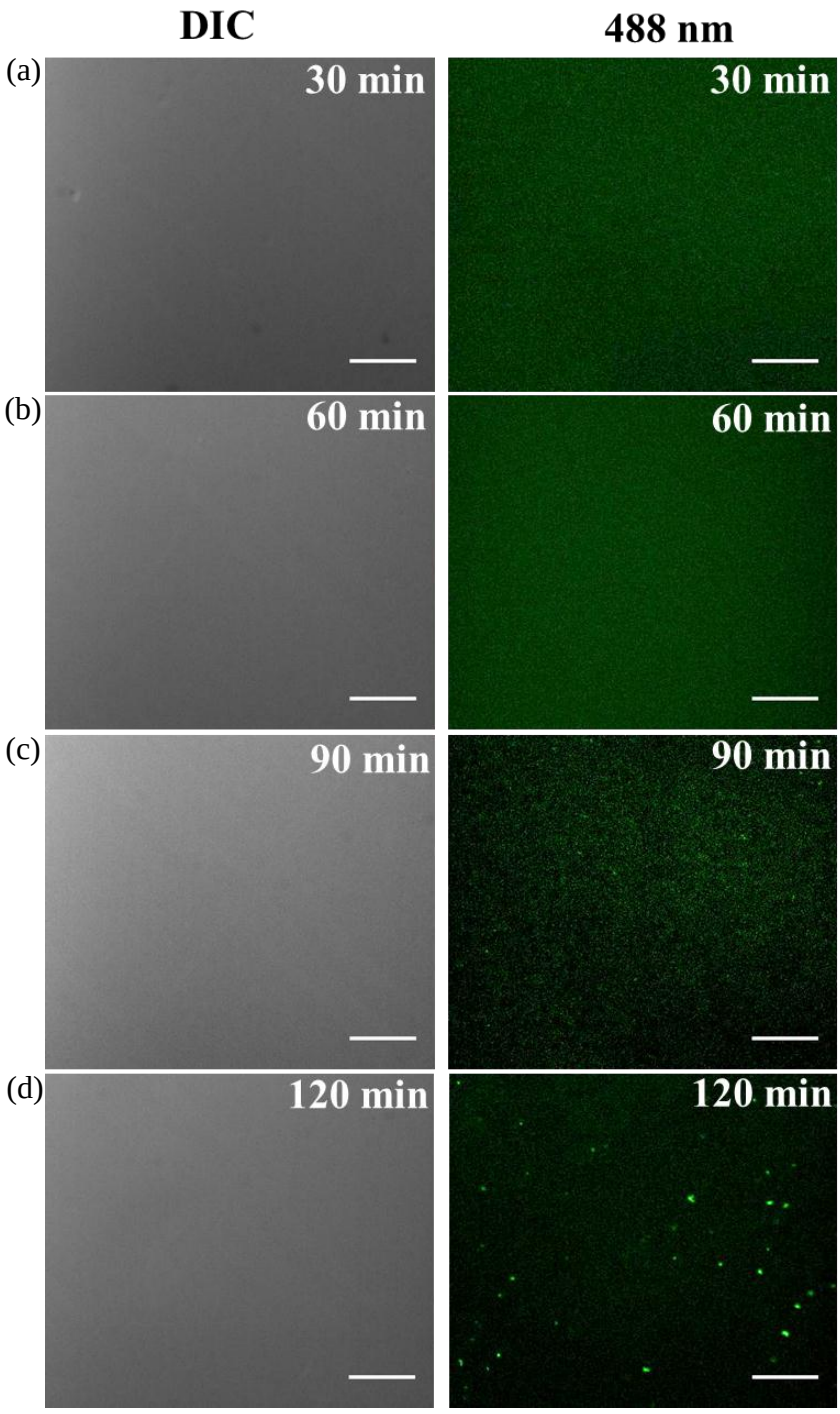

Figure S9. Images captured in DIC and 488 nm laser light from different time period of a control experiment using 10:1 mixture of Aβ42 and HiLyte Fluor™ 488 labelled Aβ42 in a flow chamber in absence of liposome at 37 ^{o}C reveal time dependent aggregation of Aβ42 peptide in absence of liposome is slow. (a) 30 min; (b) 60 min; (c) 90 min and (d) 120 min. Scale bar corresponds to 20 µm.

Supplementary Movies:

Movie S1. Aβ peptide aggregation was occurred inside the liposome. Z-Stack time lapse movie was captured for two hours at 37 ^{o}C incubation. Red colour appears from fluorescence of TRITC-Lipid and green colour from 488 labelled Aβ peptide.

Movie S2. 3D animation movie of a giant liposome after 2 h incubation at 37 ^{o}C, indicates that green colour aggregated Aβ peptide binds with TRITC labelled red coloured lipid membrane.

Movie S3. Time lapse movie in green (from Aβ peptide) and red (TRITC-Lipid) channel indicates rupture of liposome after Aβ peptide aggregation at 37 ^{o}C

Movie S4. Time lapse movie of **Movie S3** in DIC mode clearly shows rupture of liposomes.

Movie S5. Merged (DIC, green and red) time lapse movie of immobilized biotinylated liposomes onto neutravidin immobilized surface. Aβ peptide aggregates and moves inside liposome at 37 ^{o}C but liposome was fixed on surface.

Movie S6. Initial time lapse movie of two liposomes containing different coloured fluorophore (green was carboxyfluorescein and red was Aβ peptide labelled with 555 fluorophore) at 37 ^{o}C incubation condition.

Movie S7. 3D animation of z-stack of a liposome where red aggregates entered into carboxyfluorescein contained liposome after 2 h incubation at 37 ^{o}C.

Movie S8. Aβ peptide aggregation was occurred inside the liposome contain POPC instead of EggPC, Biotin-DHPE, DOPS and POPG. Green colour from HiLyte Fluor™ 488 labelled Aβ42 peptide.

Movie S9. Time lapse movie of two attached liposomes, one containing carboxyfluorescein (green) and other containing 10:1 mixture of Aβ peptide and HiLyte Fluor™ 555 labelled Aβ42 at 37 ^{o}C incubation condition.

Movie S10. Time lapse movie of aggregated Aβ peptide (Red) attached outside the green coloured liposome membrane containing carboxyfluorescein at 37 ^{o}C incubation condition.

Reference.

1. B. L. S. Mui, P. R. Cullis, E. A. Evans and T. D. Madden, *Biophys. J.* 1993, **64**, 443.
2. P. Bieling, I. A. Telley, J. Piehler, T. Surrey, *EMBO Rep.* 2008, ***9***, 1121.
3. Y. V. Mahidhar, M. Rajesh, A. Chaudhuri, *J. Med. Chem.* 2004, ***47***, 3938.