Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information for

Alzheimer's Amyloid-Beta Intermediates Generated by Polymer-Nanodiscs

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Supplementary contents:

Supplementary information

Discussion

Structural and mechanistic studies presented here revealed a symbiotic functional relationship for PMA and its associated membrane lipids. While PMA induces a rapid structural transition in $A\beta_{1-40}$, a controllable intermediate of $A\beta_{1-40}$ can be generated by varying the lipid composition and concentration in PMA-nanodiscs (Figs. 1 and 2 in main text). In addition, as evidenced from NMR results that showed a dispersed $^{1}H/^{15}N$ resonance distribution and an intermolecular $^{1}H/^{1}H$ correlation, polymer-nanodiscs can be used to track amyloid intermediates for real time measurements (Fig. 3 in main text). It is also remarkable to note that the ‘‘styrene-free’’ PMA-nanodiscs enable fluorescence and CD experiments for real-time characterization of amyloid aggregation and are useful for conformational analysis of amyloidogenic proteins embedded in a lipid bilayer.

While investigating the structure, dynamics and function of $A\beta_{1-40}$ in a membrane interface remains challenging due to the complexity, we have proposed the applicability of nanodiscs and macro-nanodiscs to decipher the molecular mechanism of the seeding reaction in real time. PMA encased lipid nanodiscs generate off-pathway lower-order aggregates of $A\beta_{1-40}$ directed by PMA and modulated by the lipid composition over time. Based on the NMR observation (Fig. 3 in main text), we propose a distinct structural ensemble of $A\beta_{1-40}$ intermediates. The toxicity of $A\beta_{1-40}$ oligomers was substantially lowered by nanodiscs and was dependent on the lipid properties and yielded distinct morphological phenotypes as revealed by TEM and cell assay (Fig. 4 in main text).[1]

The approach demonstrated in this study would enable the application of a variety of physical techniques for high throughput characterization of protein misfolding, nanodisc

encapsulated AD drug delivery and screening of small molecule compounds for the development of therapeutics. While the PMA-nanodiscs render a curvature-free lipid bilayer to probe the role of the lipid membrane in amyloid aggregation, further investigation to fully understand the mechanism of the polymer-belt induced formation of the β-sheet structure of Aβ and the use of PMA-nanodiscs on other amyloid proteins would be useful. Further development of different types of polymer belts like peptide and apolipoprotein-encased nanodiscs[2,3] for the stabilization of amyloid oligomers would be also beneficial for high-resolution structural studies.

Methods

Chemicals. All phospholipids used in this study, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG), sphingomyelin (SM) and gangliosides (GM1), were purchased from Avanti Polar Lipids, Inc® (Alabaster, AL). The polymethacrylate copolymer (PMA) was synthesized and purified at Nara Institute of Technology, Japan as reported elsewhere.[4] All other reagents were purchased from Sigma-Aldrich®.

Expression and purification of amyloid-$\beta_{1\text{-}40}$. Unlabeled and uniformly-^{15}N labeled $A\beta_{1\text{-}40}$ peptides were recombinantly expressed in *E. coli* BL21 (DE3). The $A\beta_{1\text{-}40}$ plasmid was a generous gift from Dr. Bernd Reif (Technical University of Munich, Germany). The expression and purification procedures of $A\beta_{1\text{-}40}$ were followed from elsewhere.[5,6] The purified $A\beta_{1\text{-}40}$ peptides were dissolved in 5% (v/v) NH_4OH and lyophilized at a concentration of 0.1mg/ml, then dissolved in buffer (10 mM sodium phosphate, pH 7.4) and sonicated for 30s followed by centrifugation at 14,000 $\times$ *g* for 15 min at 4 °C to remove

small aggregates. Unless otherwise stated, all experiments were performed using 10 mM sodium phosphate buffer, pH 7.4. The $A\beta_{1-40}$ oligomers were prepared using Ham's F-12 phenol free media as described elsewhere.[7]

Preparation of liposomes and nanodiscs. Large unilamellar vesicles (LUVs) were prepared as described elsewhere.[4,8] Briefly, the desired phospholipids were dissolved in 1:1 chloroform and methanol followed by evaporation under the continuous steam of nitrogen gas. The lipid film was kept under vacuum for 4 hours to remove residual trace of solvents. The dehydrated lipid film was hydrated in 10 mM sodium phosphate buffer (pH 7.4) followed by 5 minutes vortex mixing. The lipid mixture was subjected to several freeze-thaw cycles followed by extrusion using 100 nm polycarbonate membranes (Whatman, Clifton, NJ) to form LUVs. For the formation of nanodiscs, the lipid mixtures were suspended with a lipid to PMA ratio of 1:1.5 (w/w) followed by 5 minutes vortex mixing and were incubated for 15 minutes at 37 ºC while shaking. The solutions were vortexed and several freeze-thaw cycles were performed to homogenize the samples followed by incubation overnight at 37 ºC. The macro-nanodiscs (i.e., large nanodiscs with a diameter >15 nm) were prepared using a lipid to PMA ratio of 4:1 (w/w) using the above described methodology. Dynamic light scattering (DLS) measurements (Wyatt Technology Corporation) were performed to check the size of nanodiscs and macro-nanodiscs using a 1 µl quartz cuvette. The DLS measurements were performed at 25 ˚C and the scattering results (20 scans) were averaged.

Fluorescence assay of $A\beta_{1-40}$ aggregation. Thioflavin T (ThT) fluorescence assay was performed to monitor the aggregation kinetics of $A\beta_{1-40}$. Fisher 96-well polystyrene plates with a sample (5µM $A\beta_{1-40}$, 10µM ThT and variable concentration of nanodiscs) volume

of 100 µl/well were used for the fluorescence assay. The kinetics of amyloid formation was monitored at 3-min intervals using a microplate reader (Biotek Synergy 2) with an excitation and emission wavelengths of 440 and 485 nm, respectively at 25 °C (above the phase transition temperature of the DMPC containing nanodiscs). The kinetic parameters were obtained by fitting fibrillation curves to the following sigmoid equation.[9]

$$Y(t) = y_0 + \frac{A}{1 + \exp[-k(t - t_{0.5})]}$$

where y_0 is the pre-transition baseline, k is the apparent growth rate constant and $t_{0.5}$ is the half-time when ThT fluorescence reaches half of its maximum intensity. The lag time (t_{lag}) is defined as $t_{lag}=t_{0.5}-(1/2k)$.

Circular dichroism spectroscopy. Secondary structure evolution of $A\beta_{1-40}$ in presence of nanodiscs was recorded by far-UV circular dichroism (CD) using a JASCO (J820) spectropolarimeter. A light-path length (1 mm) cuvette with 25 µM $A\beta_{1-40}$ solution and a variable concentration of nanodiscs and macro-nanodiscs was used to monitor the secondary structure transition at 25 °C. CD spectra were averaged and expressed as the mean residue ellipticity [θ], after subtracting the signal from a solution without $A\beta_{1-40}$.

Transmission electron microscopy. Transmission electron microscopy (TEM) images of $A\beta_{1-40}$ and DMPC nanodisc or LUV mixed solutions were measured using a HITACHI H-7650 transmission microscope (Hitachi, Tokyo, Japan) at 25 °C following the protocols described elsewhere.[10] Sample solutions containing 5 µM $A\beta_{1-40}$ oligomers (prepared in F-12 media) or fibers (prepared in sodium phosphate buffer, pH 7.4) and oligomers incubated with nanodiscs or LUVs at 1:100 peptide:lipid molar ratio were spotted on

collodion-coated copper grid and incubated for 2 min followed by rinsing three times with Milli-Q water. The copper grid was next stained with 5 µl of 2% (w/w) uranyl acetate and the grid was rinsed three times with Milli-Q water after 1 min incubation.

Isothermal titration calorimetry analysis. Thermodynamic profiles of interactions between $A\beta_{1-40}$ and nanodiscs were measured using isothermal titration calorimetry (ITC) in a nano ITC (TA Instruments). We used anionic lipid containing nanodiscs for ITC where the $A\beta_{1-40}$ carries a net charge of -2.9 in the buffer solution, pH 7.4 at 37 °C. The peptide (in syringe) and lipids in nanodiscs (in cell) concentrations of 20 µM and 400 µM, respectively, were used for the titration experiment. A reference power of 10 µcal/s and an initial delay of 1800 s with a spacing time of 300 s were used for all titration experiments. ITC thermograms were collected over a total of 30 injections (7 µl per injections) with a stirring speed of 300 rpm, and were processed after baseline subtraction using NanoAnalyze (TA instruments).

NMR analysis. All NMR spectra were recorded on a 600 MHz Bruker Avance III spectrometer equipped with a z-axis gradient cryogenic probe. Unlabeled/^{15}N-labeled $A\beta_{1-40}$ peptides (70 µM) and d_{54}-DMPC/d_{54}-DMPG (4:1) nanodiscs (1.4 mM) were dissolved in 10 mM sodium phosphate (pH 7.4) buffer containing 90% H_2O/10% ^2H_2O. 2D ^{15}N-^{1}H SOFAST-HMQC (Bruker sfhmqcf3gpph pulse program)[11] spectra of $A\beta_{1-40}$ peptides were recorded at 10 and 25 ºC in buffer solution, and $A\beta_{1-40}$ mixed with nanodiscs (peptide/lipid=1:20) was recorded at 25 ºC. 1D and 2D NOESY spectra were measured for $A\beta_{1-40}$ in deuterated lipid nanodisc mixture (peptide/lipid=1:20) at 25 ºC with a mixing time of 60 ms and 300 ms, respectively. The NOESY experiments were recorded with 128 scans and 256 t1 points (States-TPPI). The SOFAST-HMQC spectra were recorded

with 64 scans with 200 t1 increments. ^{31}P NMR spectra of nanodiscs and multilamellar vesicles (MLVs) were acquired using an Agilent/Varian 400 MHz solid-state NMR spectrometer using 5 mm triple-resonance and double-resonance probes. The experimental conditions were set to 5 μs 90° pulse, 30 kHz ^{1}H continuous wave decoupling, 512 scans, and 6 s recycle delay. ^{31}P NMR spectra of nanodiscs/MLVs were referenced by setting the ^{31}P chemical shift of 100 % H_3PO_4 sample to 0 ppm. All NMR data were processed using TopSpin 3.5 (Bruker) and analyzed using Sparky.[12]

Cell viability assay. SH-SY5Y cells were plated in a 96-well plate and differentiated in Neurobasal A supplemented with GlutaMAX, B27, 1% penicillin/streptomycin, and 10 μM retinoic acid in a humidified incubator at 37 °C with an environment of 5% CO_2. Two-thirds of the media was replaced every 3 days prior to treatment. Cells were transduced with lentivirus encoding EGFP on day 5 of differentiation for neurite detection. Cells were next treated with 5 μM of $A\beta_{1\text{-}40}$ monomers, oligomers or fibers incubated with PMA-nanodiscs for 24 hours. Sodium phosphate buffer, pH 7.4 and F-12 media were used as negative control and staurosporine and nocodazole were used as positive control for cell-assay. On day 14, MTT cell proliferation assay (Promega, G4000) was performed to determine the toxicity of $A\beta_{1\text{-}40}$ oligomers in presence and absence of nanodiscs following the manufacturer protocol. 10 μL of the MTT dye solution was added to each well to label cells and incubated at 37 °C followed by addition of 50 μL DMSO. The absorbance of each well was then measured at both 570 nm and 620 nm. All cellular viability values were normalized to cells treated with F-12 media. Values reported are the average of five independent trials and the error is reported as the standard deviation of these averages.

Live, EGFP-expressing SH-SY5Y were imaged using a Leica SP8 confocal microscope using a 10X objective to detect neurites. Morphometric analysis of neurites was performed using the NeuronRead plugin for ImageJ.

Abbreviations:

Aβ	Amyloid-beta
AD	Alzheimer's disease
CD	Circular Dichroism
DLS	Dynamic Light Scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine
DMPG	1,2-dimyristoyl-sn-glycero-3-phosphorylglycerol
GM	Ganglioside
HMQC	Heteronuclear Multiple-Quantum Correlation
ITC	Isothermal Titration Calorimetry
LUV	Large Unilamellar Vesicles
MLV	Multilamellar Vesicles
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PMA	Polymethacrylate
SM	Sphingomyelin
SOFAST	Selective Optimized Flip Angle Short Transient
TEM	Transmission Electron Microscopy

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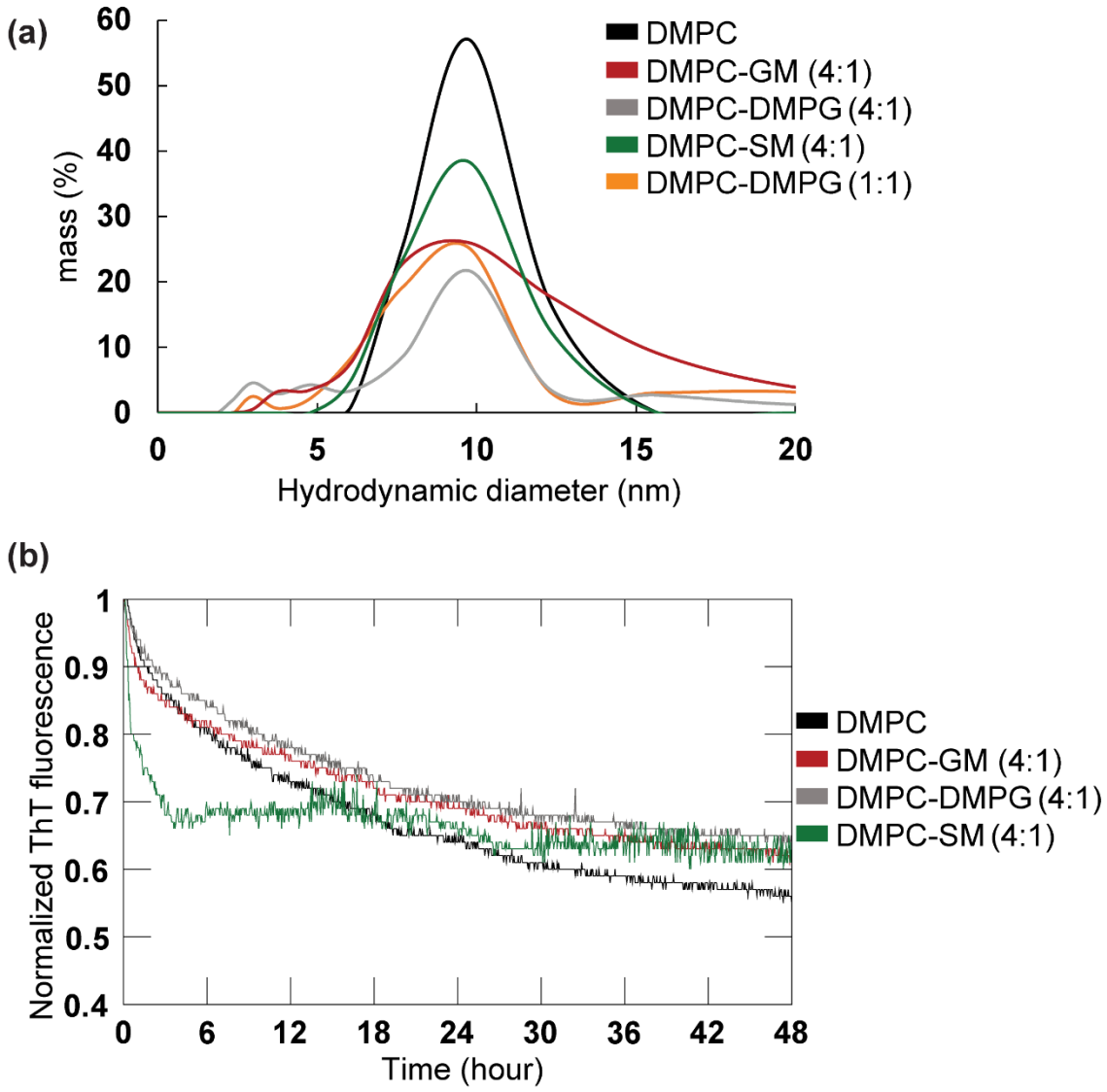

Figure S1. Size of PMA-nanodiscs and their activity on Aβ$_{1-40}$ aggregation. (a) Size distribution of different PMA encased nanodiscs measured by dynamic light scattering (DLS) at the indicated colors. The molar ratio of lipids in different nanodiscs are shown in parenthesis. (b) ThT fluorescence assay of PMA-nanodiscs (10 μM lipids) in presence of 20 μM ThT showing no ThT binding and no ThT fluorescence in the absence of amyloid-beta peptide.

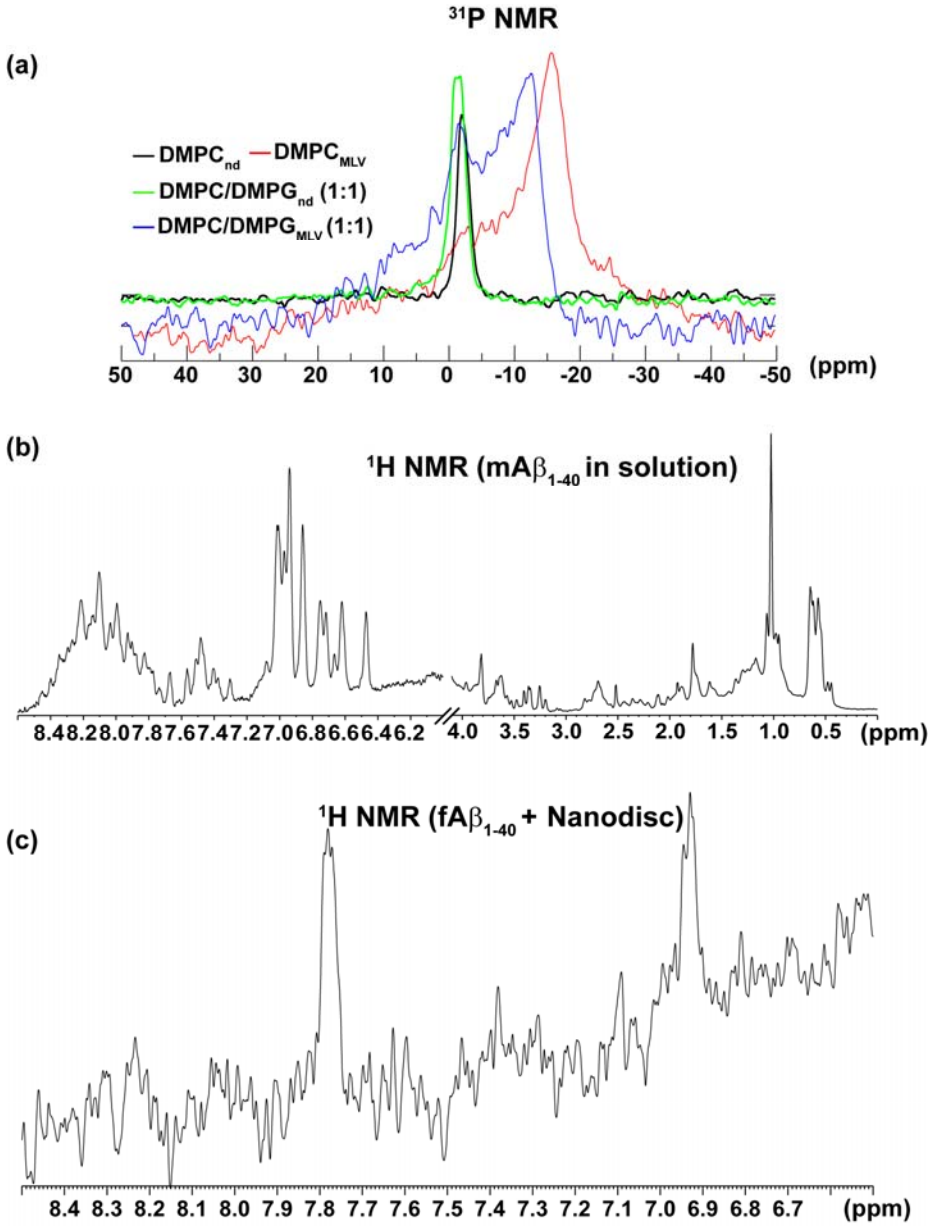

Figure S2. ^{1}H NMR spectra of Aβ_{1-40} and ^{31}P NMR spectra of PMA polymer based lipid-nanodiscs. (a) ^{31}P NMR spectra of polymethacrylate-copolymer encased DMPC or DMPC/DMPG (1:1) nanodiscs (nd) and multilamellar vesicles (MLVs) containing 5 mM phospholipids. ^{31}P NMR spectra under static conditions were acquired using a Bruker 400 MHz solid-state NMR spectrometer. (b) 1D ^{1}H NMR spectrum of 100 μM monomers of Aβ_{1-40} (mAβ_{1-40}) in solution. (c) 1D ^{1}H NMR spectrum of 100 μM Aβ_{1-40} fibers (fAβ_{1-40}) in presence of 2 mM DMPC:DMPG nanodiscs. The peptide was dissolved in 10 mM sodium phosphate buffer (pH=7.4) containing 10% D_2O, and the solution was used to acquire NMR spectrum shown in (b). The peptide solution was incubated to prepare fibers as explained in the sample preparation section, and then the fibers were incubated with 2 mM DMPC:DMPG nanodiscs to be used for acquiring the spectrum shown in (c). All solution NMR spectra were recorded at 25 °C on a Bruker 600 MHz NMR spectrometer using a cryoprobe.

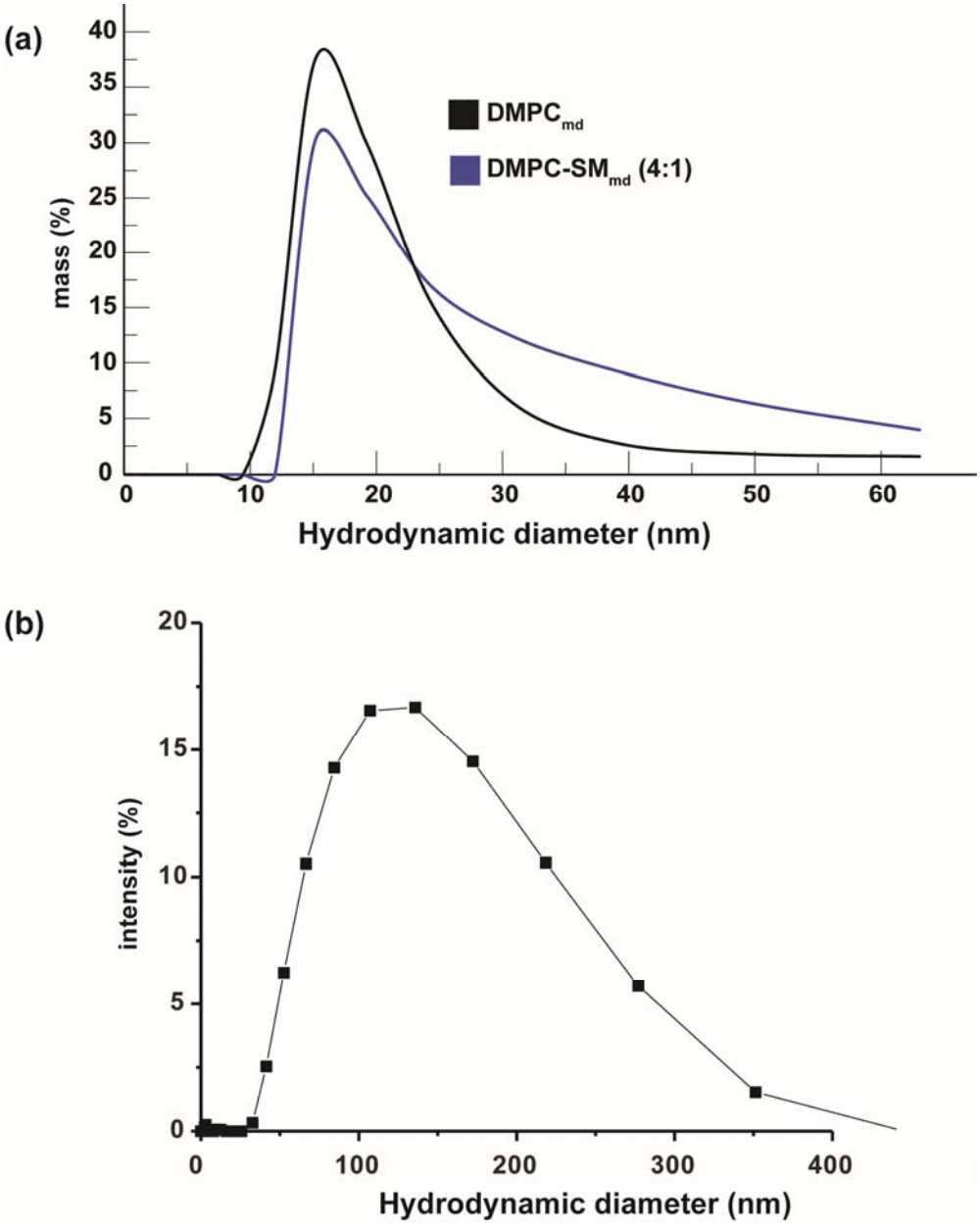

Figure S3. Size distribution of PMA-macro-nanodiscs and DMPC LUVs by DLS. Size distribution of (a) DMPC and DMPC/sphingomyelin (4:1) macro-nanodiscs (md) and (b) DMPC LUVs measured by dynamic light scattering (DLS).

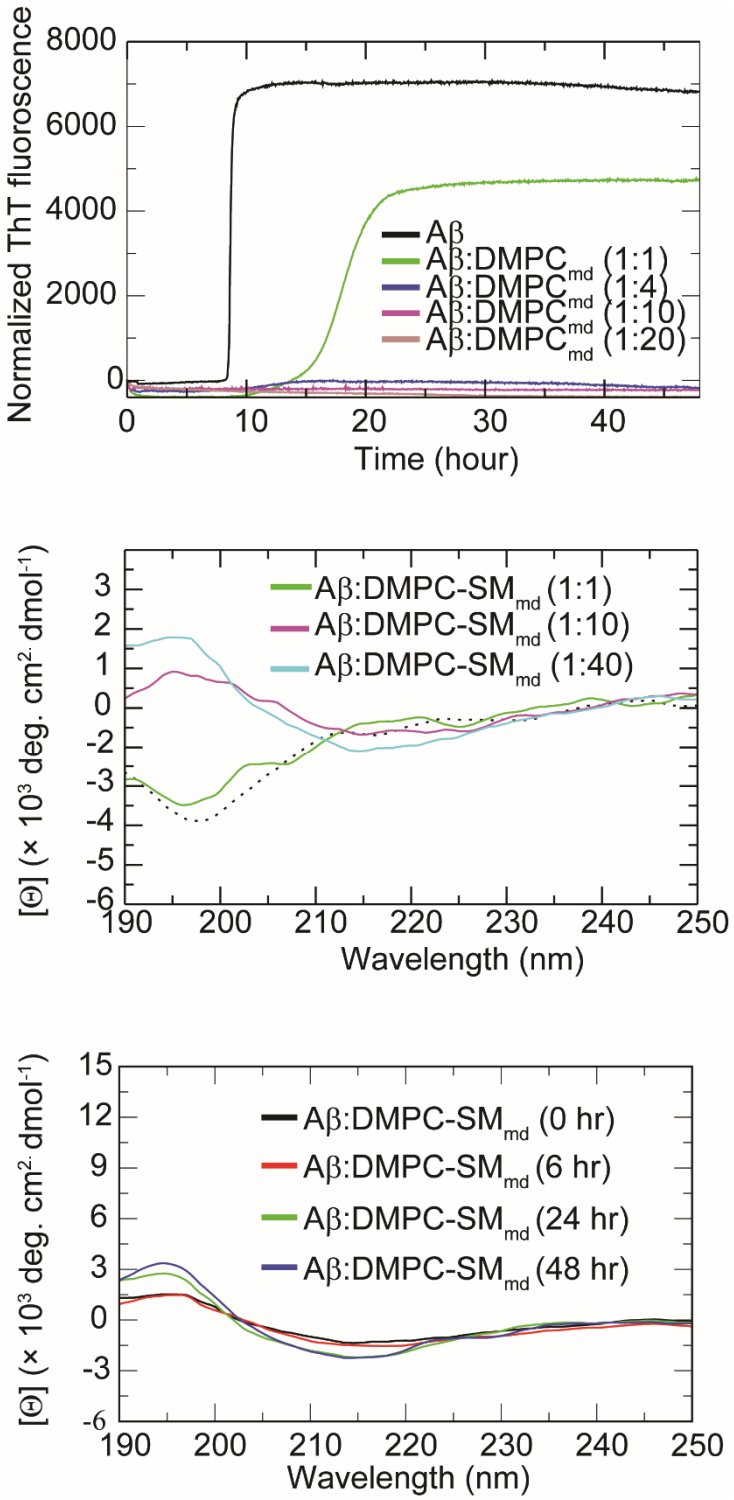

Figure S4. Effect of macro-nanodiscs on Aβ$_{1-40}$ aggregation and conformation. ThT dye based fluorescence assays of Aβ$_{1-40}$ (5 μM) aggregation in DMPC macro-nanodiscs at the indicated peptide:lipid molar ratios (top). CD spectra showing titration of Aβ$_{1-40}$ (25 μM) with DMPC/sphingomyelin (4:1) macro-nanodiscs (md) at the indicated peptide/lipid molar ratios (center), and conformational changes as a function of time (bottom).

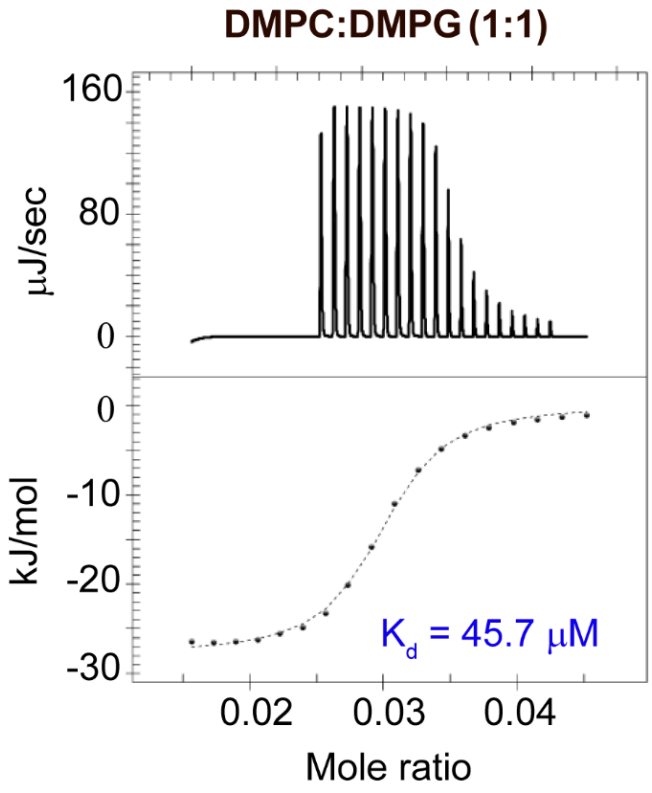

Figure S5. Kinetics analysis of binding between Aβ$_{1-40}$ and DMPC/DMPG nanodiscs using ITC. Thermograms showing the binding kinetics between Aβ$_{1-40}$ and DMPC/DMPG (1:1) nanodiscs at a peptide (20 μM) to lipid (400 μM) molar ratio of 1:20. The peptide was dissolved in 10 mM sodium phosphate buffer (pH=7.4).

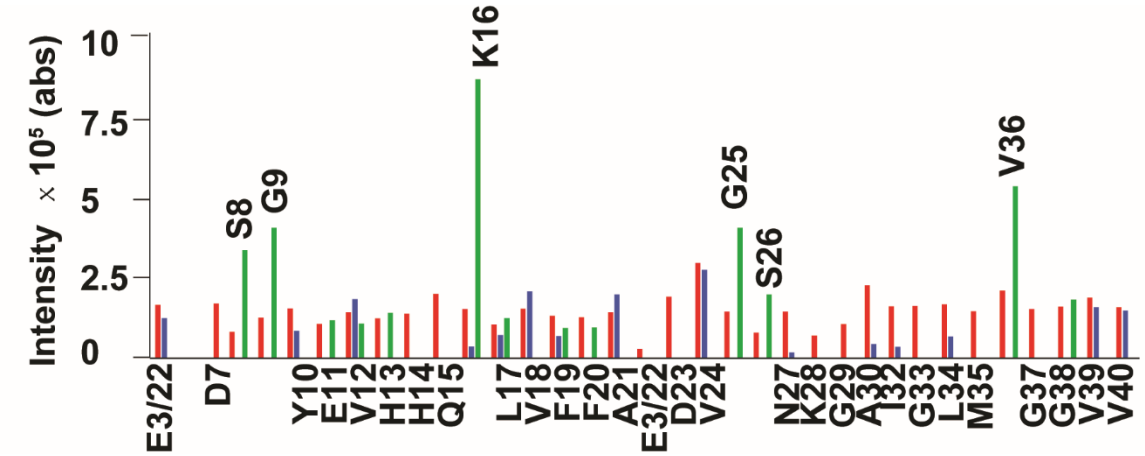

Figure S6. Intensity change of $A\beta_{1\text{-}40}$ resonances upon binding to nanodiscs measured from 2D SOFAST-HMQC. NMR signal intensities of the 2D $^{1}H/^{15}N$ SOFAST-HMQC spectra of $A\beta_{1\text{-}40}$ in solution obtained at 10 ºC (red) and 25 ºC (blue). Monomers of the $A\beta_{1\text{-}40}$ peptide was dissolved in 10 mM sodium phosphate buffer solution (pH=7.4) containing 10% D_2O. Peak intensities measured from $A\beta_{1\text{-}40}$ in presence of PMA-based (4:1 DMPC:DMPG) nanodiscs (1.4 mM lipids) at 25 ºC (green). The observed differences between solution and nanodisc samples is discussed in the main text. NMR spectra were recorded on a 600 MHz Bruker NMR spectrometer using a cryoprobe.

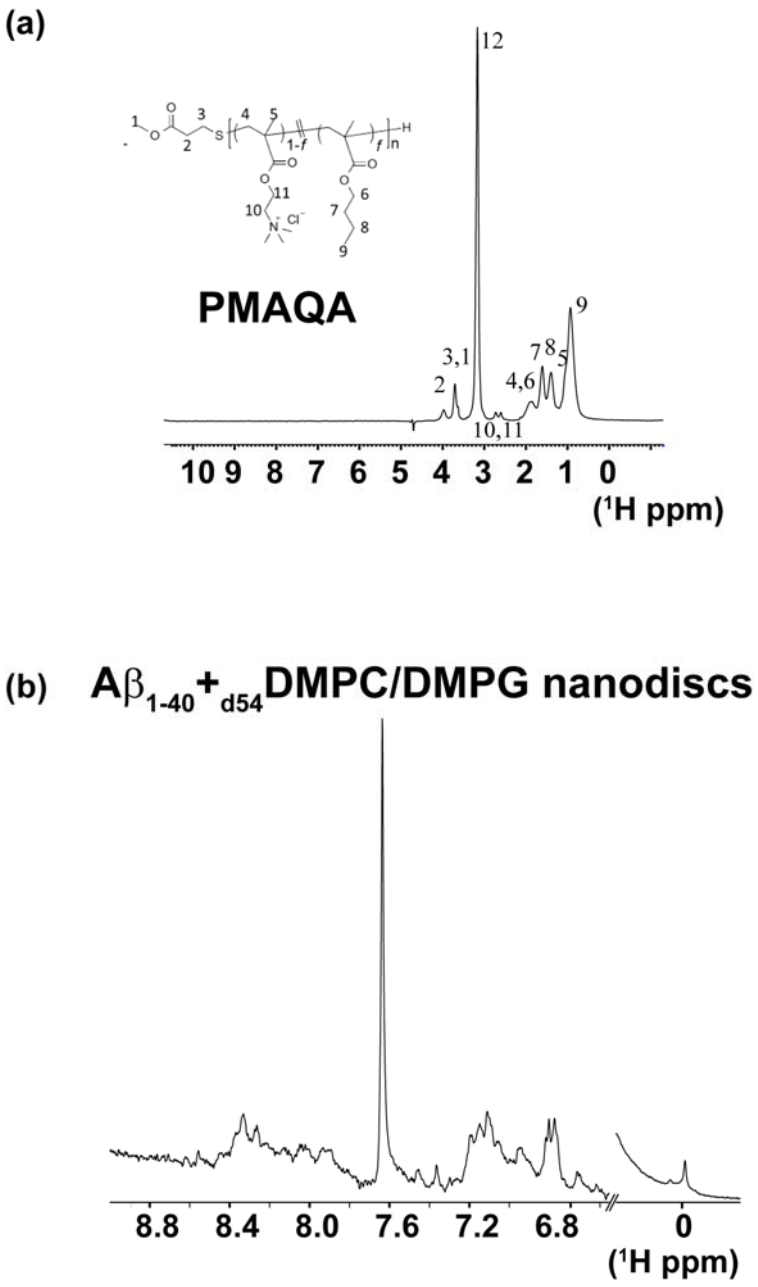

Figure S7. ^{1}H NMR spectra of PMAQA (a) and PMAQA-DMPC/DMPG nanodiscs (containing deuterated lipids) interacting with $A\beta_{1\text{-}40}$ (b). NMR spectra were recorded on a 600 MHz Bruker NMR spectrometer at 25 ºC.

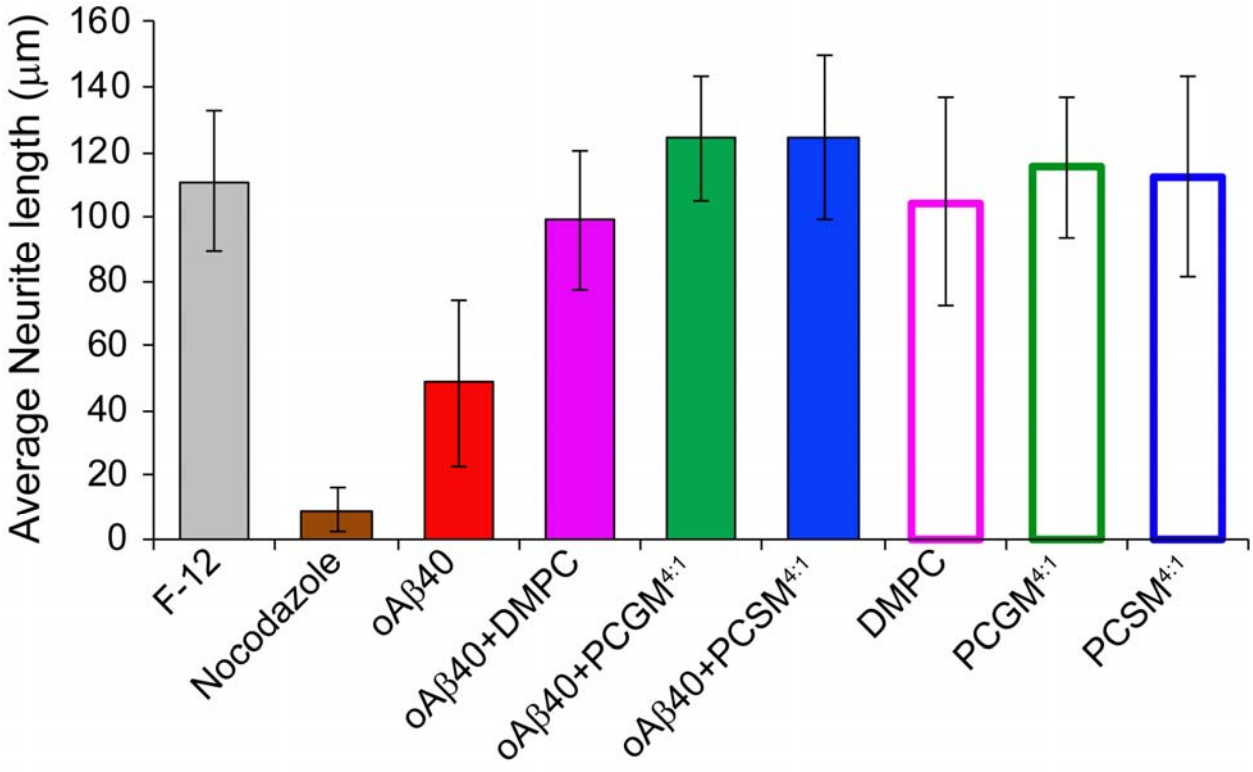

Figure S8. Neuritic length analysis of SH-SY5Y cells treated with 5 μM $A\beta_{1\text{-}40}$ oligomers in presence or absence of nanodiscs (DMPC; PCGM: DMPC/GM; PCSM: DMPC/SM). oAβ denotes Aβ oligomers.

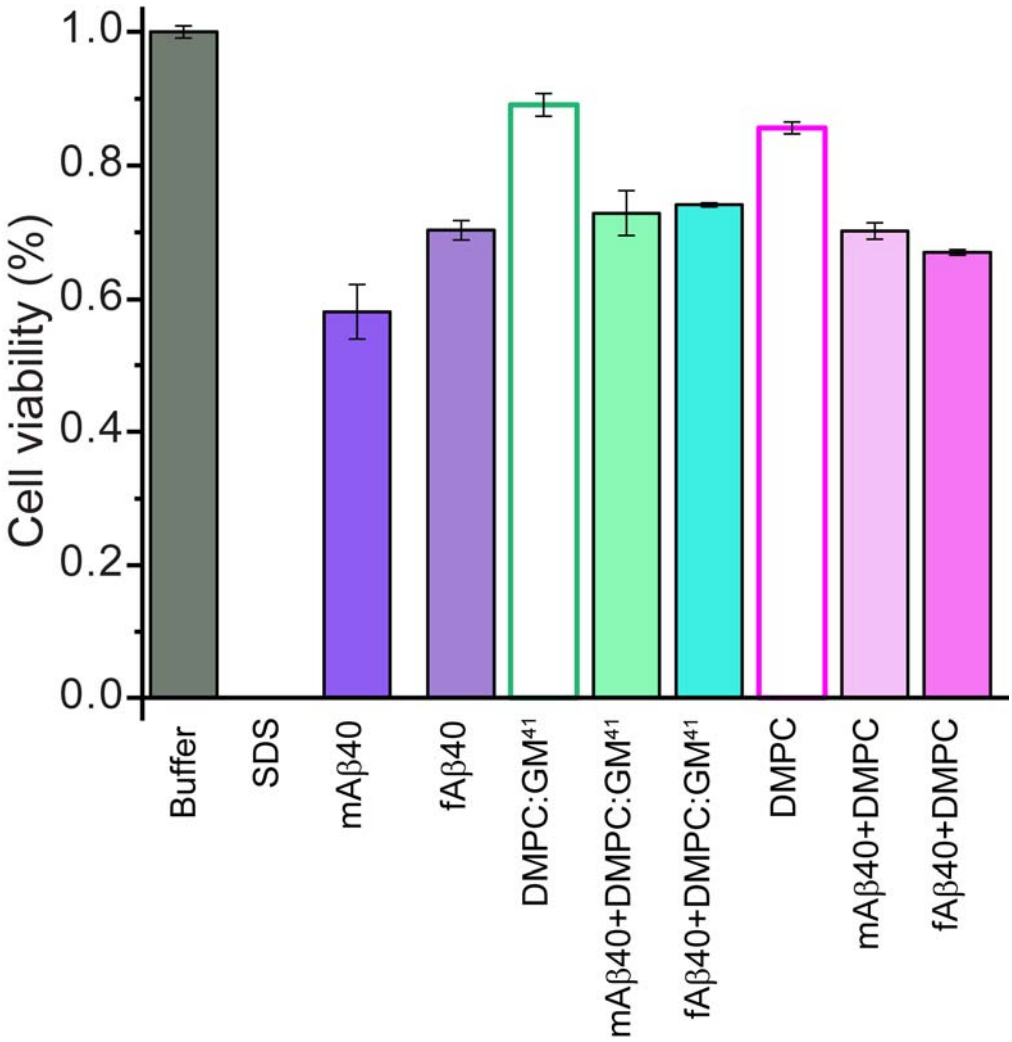

Figure S9. MTT assay showing the formazan absorbance expressed as a measure of cell viability from SH-SY5Y cultured cells treated with freshly dissolved $A\beta_{1\text{-}40}$ monomers or fibers mixed with nanodiscs (DMPC; PCGM: DMPC/GM; PCSM: DMPC/SM). mAβ and fAβ denote Aβ monomer and fiber, respectively.