Molecular rotors report on changes of live cell plasma membrane microviscosity upon interaction with beta-amyloid aggregates

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Amyloid deposits of aggregated beta-amyloid peptide A β (1-42) are a pathological hallmark of Alzheimer's disease. A β (1-42) aggregates are known to induce biophysical alterations in cells, including disruption of plasma membranes. We investigated the microviscosity of plasma membranes upon interaction with oligomeric and fibrillar forms of A β (1-42). Viscosity-sensing fluorophores termed molecular rotors were utilised to directly measure the microviscosities of giant plasma membrane vesicles (GPMVs) and plasma membranes of live SH-SY5Y and HeLa cells. The fluorescence lifetime of membrane-inserting BODIPY-based molecular rotor revealed a decrease of bilayer microviscosity upon incubation with A β (1-42) oligomers, while fibrillar A β (1-42) did not significantly affect the microviscosity of the bilayer. In addition, we demonstrate that the neuroprotective peptide H3 counteracts the microviscosity change induced by A β (1-42) oligomers, suggesting the utility of H3 as a neuroprotective therapeutic agent in neurodegenerative disorders, and indicating that ligand-induced membrane stabilisation may be a possible mechanism of neuroprotection during neurodegenerative disorders such as Alzheimer's disease.

Introduction

One of the markers of Alzheimer's disease (AD) is the aggregation and accumulation of amyloid- β peptide (A β) in brain tissue. A β is an extracellular protein that is produced by cleavage of the transmembrane amyloid precursor protein. According to the amyloid cascade hypothesis¹⁻³ it is mainly A β that is responsible for the pathological effects during AD. The plasma membrane is the cellular structure that A β mainly comes into contact with, providing a large surface for interactions. An increasing amount of evidence demonstrates that the properties and organisation of neuronal membranes play a key role during AD. In fact, the dominant hypothesis states that the primary cause of cytotoxicity may be the interactions of A β aggregates with plasma membranes, leading to disruptions of membrane properties and function, possibly affecting important processes such as transport across the bilayer.⁴⁻⁷ However, a unified explanation of the precise mechanism is still lacking. As a result, there is an increasing interest in the interactions of various amyloid structures with lipid membranes, both in model systems and *in cellulo*.

The mechanism and level of membrane disruption caused by

amyloid structures is thought to be determined by the specific characteristics of the aggregate species, as well as physicochemical properties of the membranes, such as charge or fluidity.⁸ Bilayer composition is another crucial factor affecting A β interactions.^{9,10} Since it is challenging to monitor these properties directly in the living brain, recent studies have focused on using model membrane systems such as large or giant unilamellar vesicles (LUVs or GUVs) to investigate the effect of A β on lipid bilayers.¹¹⁻¹⁸ However, the vesicles are made using an artificial mixture of phospholipids and other components and, as such, these model systems are usually oversimplified and may not reflect the true composition and behaviour of plasma membranes of living cells.¹⁹

Isolated membranes^{10,20-22} may present a better model, yet still suffer from disadvantages such as possible loss of membrane components during the isolation process.²³ Although these studies provided insight into how A β aggregates may influence phospholipid bilayers, studies in live cells and animal models are preferable in order to directly elucidate the mechanism of interactions of A β with plasma membranes.

Here we present a novel approach using small environmentally sensitive fluorophores termed "molecular rotors",²⁴⁻²⁶ to monitor viscosity changes in plasma membranes of live cells following the interaction with the A β protein. Membrane viscosity (from here on referred to as "microviscosity") can be defined as an extent of molecular order within the bilayer, that affects the motion of small molecules and macromolecules alike. Our previous work quantified the effect of microviscosity in model membranes (as measured by molecular rotors) on molecular diffusion rates and found strong correlations.²⁷ We presented evidence that microviscosity can be reliably estimated from the calibration of molecular rotor responses

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in bulk solvents, such as methanol/glycerol or castor oil mixtures.^{27,28}

The membrane microviscosity is a vital parameter for the function of the plasma membrane and a cell as a whole. Alterations of membrane microviscosity serve as markers of cell pathophysiology during aging²⁹ and diseases such as Alzheimer's,^{30,31} hyperlipoproteinemia³² and liver disease,³³ and red blood cell pathologies.³⁴ In this work we were able to directly monitor the effect of A β protein aggregates on the microviscosity of the plasma membranes in various cell types, and compare these observations to the effect of A β protein aggregates on model membranes.

Molecular rotors are fluorescent dyes in which both the fluorescence quantum yield and fluorescence lifetime strongly depend on the viscosity of the surrounding microenvironment.^{24–26} The behaviour of molecular rotors is described by the Förster-Hoffmann equation:³⁵

$$\phi_f = z\eta^\alpha \quad (1)$$

where ϕ is the quantum yield, z and α are constants, and η is viscosity.

Importantly, unknown fluorophore concentration and the optical properties of the microenvironment do not allow to use fluorescence intensity measurements for quantitative assessment of viscosity.²⁴ On the other hand, the use of fluorescence lifetime (τ_f) as a concentration-independent parameter allows quantitative determination of viscosity, even in cases where the concentration of the fluorophore is not known, such as in live cells.^{24,36}

We have recently reported a series of molecular rotors that were localised in plasma membranes of healthy and unperturbed live cells and could successfully probe its microviscosity.^{37–39} This work allowed for the first time to quantitatively and dynamically characterise the microviscosity of various types of cellular membranes and also to assess its heterogeneity. At the same time we have also used molecular rotors to monitor the changes in crowding during the aggregation of A β in the presence of live cells.⁴⁰ We found that beta-amyloid accumulates on the plasma membranes and adjacent to them, possibly contributing to the amyloid-induced cytotoxicity. We have also established, by monitoring the fluorescence lifetime of an A β -bound molecular rotor, that A β aggregation *in cellulo* proceeds via a different pathway, compared to the *in vitro* scenario in the absence of cells.⁴⁰

To the best of our knowledge, despite the perceived importance of A β -plasma membrane interactions, no direct data exist on membrane microviscosity of live cells in the presence of A β aggregates. Hence, in this work we use membrane-localised molecular rotor BODIPY 1³⁷ to investigate the effect of the interaction of aggregating beta-amyloid with plasma membranes on the plasma membrane microviscosity and organisation, in a dynamic and quantitative manner.

Results and Discussion

BODIPY 1 reports temperature modulations of the microviscosity of live cell plasma membranes

BODIPY 1 is a viscosity-sensitive molecular rotor that was previously shown to successfully partition in various lipid bilayers.²⁷ We have previously used a combination of Fluorescence Lifetime Imaging Microscopy (FLIM), Fluorescence Correlation Spectroscopy and molecular dynamics simulations to demonstrate that (i) all our BODIPY rotors localise in the lipid-tail region of the fluid-phase bilayers, irrespective of their charge and (ii) the lifetimes (microviscosity) of all BODIPY rotors show strong correlation to the diffusion coefficients recorded and simulated in model membranes at various temperatures.²⁷ As long as the calibration of rotors was performed in an appropriate bulk solvent system,²⁶ we are, therefore, confident that the fluorescence lifetimes of rotors are characteristic of the local packing arrangements of the lipid bilayer, that determine molecular diffusion in these locations.

Upon interaction with live cells BODIPY 1 was shown to selectively stain the plasma membranes³⁷ (Fig. 1a). This presents significant advantages compared to uncharged BODIPY rotors, which effectively internalise in live cells via endocytosis.^{37,39,41}

The time resolved fluorescence decays of BODIPY 1 conform to mono-exponential fitting and the fluorescence lifetime (τ_f) is sensitive to viscosity in the range between 3–1000 cP. The calibration plot of lifetime vs. viscosity recorded in methanol-glycerol mixtures (Fig. S1) follows Equation 2, a modified version of the Förster-Hoffmann plot:³⁷

Fig. 1 Characterising the microviscosity response of the fluorescence lifetime of BODIPY 1 in plasma membranes at different temperatures. **(a)** Schematic diagram showing the molecular structure of BODIPY 1 and its incorporation into the plasma membrane of a living cell. **(b)** Fluorescence lifetime maps of BODIPY 1 in the plasma membranes of SH-SY5Y cells at four different temperatures following mono-exponential fitting. **(c)** Histograms corresponding to FLIM images of SH-SY5Y plasma membranes at 4 different temperatures; each histogram was averaged from at least 8 different images and normalised to the peak value. **(d)** SH-SY5Y plasma membrane viscosity at different temperatures, calculated from the mean values of lifetime distributions in (c), according to Equation 2.

$$\log(\tau_f) = 0.49 \log(\eta) - 0.83 \quad (2)$$

where τ_f is fluorescence lifetime (ns) and η is viscosity (cP). Of note, the microviscosity values in cP reported further on in this work are relative to this methanol/glycerol calibration. Our previous work has established that the calibration is still valid in non-polar mixtures, such as castor oil, and can be used for quantitative prediction of diffusion coefficients in lipid bilayers.²⁷

Importantly, BODIPY **1** provides lifetime vs viscosity calibration that is temperature independent,^{26,42,43} i.e. is ideally suited for predictions of microviscosity in lipid membranes at variable temperature.

Previously, BODIPY **1** has reported average microviscosity of 270 cP at 20°C in the plasma membranes of epithelial SK-OV-3 cells.³⁷ Also, this probe was used to measure strong variations of membrane microviscosity with temperature, between 80-300 cP, in liquid-disordered-phase (L_d) 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) LUVs, in the temperature range between 37-10°C.²⁷ Significantly higher microviscosity was recorded in gel-phase 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) LUVs.

We first chose the neuroblastoma cell line SH-SY5Y, which is often used as a model system for studies of neurodegenerative diseases.⁴⁴ We tested the ability of BODIPY **1** to detect changes in plasma membrane microviscosity of SH-SY5Y cells upon variation in temperature. Chamber slides with SH-SY5Y cells incubated with BODIPY **1** were kept on a heated microscope stage and FLIM images were acquired at 37°C, 29°C, 22°C and 13°C.

As expected, the decays of BODIPY **1** in all images conformed to a monoexponential model (Fig. S2). The analysis of FLIM images (Fig. S1b+S2) revealed a significant change of microviscosity of plasma membranes of cells with temperature, with an overall distribution across an image described well by a Gaussian function (Fig. 1c).

The mean BODIPY **1** fluorescence lifetime calculated from values extracted from the Gaussian fit (Fig. S3) linearly decreased with increasing temperature (Fig. 1b, quantified in Fig. 1c,d), with microviscosity values between ca 280 cP (37°C) and 430 cP (13°C). These values were comparable to the 80-300 cP range observed for pure DOPC bilayers between 37-10°C,²⁷ while significantly higher values, ca 1000 cP, were measured for the inner membrane of *E. Coli* at 20 and 37°C,⁴⁵ and 1050-3500 cP between 37-10°C in porcine eye lens cell membranes.³⁹ The high microviscosity observed in the latter two systems is most likely the consequence of extremely high order of lipid organisation in these membranes.³⁹ At the same time, the mean microviscosity of SH-SY5Y plasma membranes is comparable to those previously reported for eukaryotic SK-OV-3 cells.³⁷

The effects of temperature on membranes seen in our experiments are in accordance with previous fluorescence spectroscopy studies, which demonstrated that membrane fluidity is reduced with decreasing temperature.⁴⁶⁻⁵⁰ Our results confirm the expectation that plasma membrane viscosity of live SH-SY5Y cells responds to temperature and that BODIPY **1** is capable of measuring these changes.

The microviscosity of cell-derived giant plasma membrane vesicles

The mechanism of interaction of A β with membranes has been extensively studied using model membrane systems prepared through a bottom-up approach, such as large or giant unilamellar vesicles (LUVs or GUVs). However, the biological relevance of these studies is limited by the (over)simplified bilayer composition, which does not adequately reflect the composition of cell plasma membranes. We chose to use giant plasma membrane vesicles (GPMVs) as a model membrane system, since GPMVs are derived from cells by chemical induction of membrane blebbing⁵¹ and overcome the afore mentioned limitations, preserving a similar composition to plasma membranes.⁵²

First, we set off to compare the microviscosity of GPMVs with live cell plasma membranes, as this has not been done previously. GPMVs derived from HeLa cells were prepared according to the protocol of Sezgin et al. using NEM buffer as the vesiculating agent (Fig. S4),⁵³ we were not able to achieve membrane blebbing into GPMVs with the SH-SY5Y cell line.

To characterise the rheological properties of GPMVs, we incubated the vesicles with BODIPY **1** and obtained fluorescence lifetime images to determine bilayer microviscosity. In pure GPMVs at 37°C, the mean fluorescence lifetime of BODIPY **1** was 2.16 ± 0.22 ns (Fig. 2), corresponding to the microviscosity of 228 cP according to Equation 2. We next measured the microviscosities of plasma membranes of live HeLa and SH-SY5Y cells. At 37°C, the mean fluorescence lifetime of BODIPY **1** in HeLa cell membranes was 2.54 ± 0.26 ns (331 cP) and 2.36 ± 0.26 ns (285 cP) in SH-SY5Y cell membranes (Fig. 2).

In the comparison of live HeLa cells and HeLa-derived GPMVs, strikingly, live cell membranes were more viscous than the vesicles. The difference between the microviscosity of HeLa plasma membranes and HeLa-derived GPMVs was nearly 80 cP and statistically significant ($p < 0.001$, Fig. 2 and Fig. S5).

Fig. 2 The comparison of microviscosities at 37°C of GPMVs derived from HeLa cells (green) with the plasma membranes of live HeLa (red) and SH-SY5Y (blue) cells, overlaying the fluorescence lifetime-viscosity calibration data of BODIPY **1** in methanol-glycerol mixtures (grey). The mean microviscosity of HeLa GPMVs was significantly higher than that of live HeLa cell plasma membranes ($p < 0.001$). No significant difference was observed between membrane microviscosities of HeLa GPMVs vs SH-SY5Y plasma membranes ($p = 0.054$) or HeLa plasma membranes vs SH-SY5Y plasma membranes ($p = 0.159$); one-way ANOVA with Tukey's post-hoc test ($N = 64$, see Fig. S5 for details).³⁷

Fig. 3 Interactions of A β (1-42) oligomers with HeLa-derived giant plasma membrane vesicles (GPMVs) and plasma membranes of live HeLa cells. **(a,b)** FLIM of a GPMV at 37°C left untreated **(a)** or after 30 minutes of incubation with 10 μ M A β (1-42) oligomers **(b)**. **(c)** Fluorescence decays corresponding to the pixels marked by arrows in **(a)** and **(b)**. **(d)** Comparison of histograms of FLIM images **(a)** and **(b)**. **(e)** Mean fluorescence lifetime of BODIPY **1** in HeLa-derived GPMVs untreated (black) vs A β (1-42) oligomer-treated (red), unpaired t-test ($p < 0.001$, $N = 22$). **(f,g)** FLIM of HeLa cells left untreated **(f)** or incubated with 10 μ M A β (1-42) oligomers for 1 h **(g)**. **(h)** Fluorescence decays corresponding to the pixels marked by arrows in **(f)** and **(g)**. **(i)** The comparison of histograms of FLIM images **(f)** and **(g)**. **(j)** Mean fluorescence lifetime of BODIPY **1** in untreated (black) vs A β (1-42) oligomer-treated (red) HeLa cells (10 μ M, 0.5 - 1 hr incubation), unpaired t-test ($p = 0.011$, $N = 16$).

While the GPMVs are formed through membrane blebs and are supposed to have an identical lipid composition to their parent membrane, they may lack support of intact cortical cytoskeleton. Additional contributing factors may be the lack of lipid asymmetry in leaflets or phosphorylated lipids present in cells.^{19,54} Although GPMVs are the most adequate vesicle-based constructs for the experimental modelling of live cell membranes, maintaining the lipid and protein diversity of plasma membranes, our experiments demonstrate that these model membranes still do not fully reflect the true properties of the biological system.^{19,54}

Interestingly, the plasma membrane microviscosity of live SH-SY5Y cells at 37°C appeared to be 46 cP lower than that of HeLa plasma membranes ($p = 0.054$). This may contribute to the reason why SH-SY5Y cells do not bleb into GPMVs, since bleb nucleation is inversely dependent on membrane tension, which in turn is proportional to effective membrane microviscosity.⁵⁵ Importantly, in all three studied cell types (GPMVs, HeLa and SH-SY5Y) plasma membrane microviscosities fell into the linear range of the BODIPY **1** viscosity-lifetime calibration plot (**Fig. 2**), confirming that it was correct to use Equation 2 to estimate microviscosity values from fluorescence lifetimes.

Membrane microviscosity decreases upon the interaction with A β oligomers

It is believed that small soluble prefibrillar oligomeric intermediates are the most cytotoxic species during the aggregation of A β (1-42), inducing synapse loss and neuronal dysfunction.⁵⁶ Additionally, small oligomeric species are highly related to the propagation and infectivity of amyloids.⁵⁷⁻⁶⁰ The interaction of amyloid oligomers with membranes has been suggested as the primary mechanism of amyloid pathogenesis.^{5,6} However, the existing data on the effect of A β oligomers on plasma membranes

points to the existence of several scenarios of membrane disruption and cytotoxicity³⁰ and references therein). A β oligomers have been shown to attach to and assemble at the surface of live cell membranes^{61,62} and to increase membrane permeability for ion transport, the dominating hypothesis suggesting that this is due to pore formation across the bilayer.⁶³ Others have demonstrated membrane thinning and deformation caused by A β .^{18,64-67}

Although changes in fluidity of lipid bilayer upon interaction with A β have been suggested, the evidence is contradictory⁶⁸ (with both increase^{20,21,69} or decrease^{10,70,71} in fluidity reported). Here we set out to directly probe the microviscosity of the plasma membrane upon A β interaction with live cells and with GPMVs.

To investigate the effect of A β (1-42) oligomers on vesicles and plasma membranes, A β (1-42) oligomers were prepared according to the published protocol⁴⁰; Thioflavin T intensity assay⁷² was used to confirm the correct course of A β (1-42) aggregation (**Fig. S6**). After the addition of A β (1-42) to chamber slides containing GPMVs in HBSS, fluorescence lifetime images were recorded to evaluate membrane microviscosity changes following interaction with the oligomers (see Materials and Methods for details of sample preparation and imaging). Each FLIM was taken in a new field of view of cells.

Sample FLIMs of GPMVs before and after incubation with A β (1-42) oligomers are shown in **Fig. 3a** and **Fig. 3b**, respectively. We found that incubation with A β (1-42) oligomers (approx. 30 min at 37°C) strongly decreased the mean fluorescence lifetime of BODIPY **1** in GPMVs to 1.76 ± 0.20 ns corresponding to the microviscosity of 151 cP (**Fig. 3e**, red), compared to the 228 cP mean microviscosity value of untreated GPMVs. We did not observe any changes in vesicle morphology after the incubation with oligomers (**Fig. 3b**), but the mean microviscosity of GPMVs upon incubation with oligomers was nearly 80 cP less than that of the control (untreated) GPMVs, indicating that A β (1-42) oligomer interaction with the

plasma-derived vesicles resulted in the decrease of the bilayer microviscosity.

Next, we incubated live HeLa cells with A β (1-42) oligomers and recorded FLIM images over 2 hours (Fig. 3 f,g); mean lifetimes from the histograms of several FLIM images were averaged to obtain the mean fluorescence lifetime before and after 0.5 - 1 hour of incubation with A β (1-42) (Fig. 3j). We observed a drop of microviscosity of the plasma membranes, with the mean lifetime of BODIPY 1 decreasing from 2.70 ± 0.15 ns to 2.49 ± 0.13 ns (Fig. 3j). The difference in fluorescence lifetime corresponds to a membrane microviscosity decrease by nearly 60 cP (from 375 cP to 317 cP), which is slightly less than the ca 80 cP membrane microviscosity difference observed for A β (1-42) oligomer interaction with HeLa-derived GPMVs (Fig. 3e,j). Of note, rather than a stepwise change, we observed a gradual decrease over the period of incubation with A β (1-42) oligomers (Fig. S7).

Cecchi *et al.* have demonstrated cell type-dependent variations in susceptibility to damage and apoptotic death caused by prefibrillar aggregates and shown that the degree of this susceptibility was inversely related to the membrane content of cholesterol.⁷³ In our study we were particularly interested in the effects of A β (1-42) oligomers on the membranes of SH-SY5Y cells, relevant as an *in vitro* model for studies of neurodegenerative diseases.⁴⁴ Again, we observed a reduction of BODIPY 1 fluorescence lifetime upon incubation of SH-SY5Y with A β (1-42) oligomers (Fig. 4a-c). The decrease of the mean lifetime of BODIPY 1 from the initial 2.52 ± 0.05 ns (control) to 2.32 ± 0.19 ns (A β treatment, Fig. 4c) corresponded to a drop from 326 cP to 275 cP (51 cP), comparable to that observed in the HeLa cell membranes (Fig. 3h). The membrane microviscosity again gradually decreased over the course of two hours of SH-SY5Y incubation of A β (1-42) oligomers (Fig. 4d).

Our results demonstrate that A β (1-42) oligomers interact with GPMVs, plasma membranes of HeLa and SH-SY5Y cells and reduce the bilayer microviscosity in all cases. This finding supports previous observations of oligomeric A β (1-42) increasing the molecular disorder of astrocyte membranes and of artificial membranes made of rat brain lipid extracts.⁶⁹ Accordingly, another study using a fluorescent pyrene probe shows that A β (1-40) oligomers increase the fluidity of isolated synaptic plasma membranes.^{20,21} A β oligomers are also known to increase the membrane permeability and transport of inorganic ions.^{17,74,75}

Interestingly, a study has shown that the increase of membrane conductance and enhanced ion passage occurs in the absence of pore or ion channel formation,⁶⁶ suggesting that oligomers enhance the ability of ions to cross membranes through a different mechanism, possibly by increasing membrane fluidity. In contrast, the increase of conductance was not observed in the presence of A β (1-42) fibrils.⁶⁶

Fig. 4 The interactions of A β (1-42) oligomers with plasma membranes of live SH-SY5Y cells. **(a)** FLIM image of BODIPY 1 in membranes of SH-SY5Y cells left untreated **(a)** or treated with 10 μ M A β (1-42) oligomers for 2 hr **(b)**; **(c)** Mean fluorescence lifetime of BODIPY 1 in untreated vs A β (1-42)-treated SH-SY5Y cells ($p = 0.018$, $N = 14$, unpaired t -test). **(d)** Gradual decrease of BODIPY 1 fluorescence lifetime over 2 hr of incubation of SH-SY5Y cells with 10 μ M A β (1-42) oligomers (red), compared to an untreated control (black). Time points were taken starting from $t = 0$ min after the addition of A β (1-42).

The change of membrane microviscosity caused by A β (1-42) oligomers may result in alterations of the biological functions of the bilayer; it is known that the physical state of the membrane influences the function of membrane proteins and affects important processes, such as the transduction of certain types of environmental stress, e.g. temperature and osmotic stress.⁷⁶ The precise mechanisms of interaction of A β (1-42) oligomers with the cell membranes were not the focus of this study; however, several potential scenarios can be suggested. Previous studies on supported membranes have shown deep hydrophobic penetration of A β (1-42) oligomers, resulting in the modification of the chemical composition of the membrane, possibly by disturbing the cholesterol distribution within the bilayer.⁷⁷ In astrocytes A β (1-42) has been shown to stimulate the trafficking of cholesterol from membranes into the Golgi apparatus.⁷⁸ Since it is known that cholesterol levels directly influence membrane lipid fluidity,⁷⁹ the decrease in membrane microviscosity observed in our experiments may have resulted from cholesterol redistribution in membranes upon the insertion of A β (1-42) oligomers.

A β fibrils do not significantly affect membrane microviscosity of live SH-SY5Y cells

One of the potential causes of discrepancies in the reported effects of A β aggregates on membranes may arise from the differences in the aggregation states of A β and/or the differences in the physico-chemical designs of the probes used for the measurements. Thus, we used the same molecular rotor probe (BODIPY 1) to directly compare the effects of A β (1-42) oligomers and fibrils on the microviscosity of SH-SY5Y cell plasma membranes.

Upon a week-long maturation of A β (1-42) fibrils from a 50 μ M monomeric A β (1-42) solution, fibrils were added to live HeLa or SH-SY5Y cells and incubated for 2 hours. In HeLa cells, we observed an increase of microviscosity (Fig. 5a), in contrast to the detected decrease of microviscosity in the A β (1-42) oligomer experiments (Fig. 3). In SH-SY5Y cells, incubation with A β (1-42) fibrils had no significant effect on membrane microviscosity (Fig. 5b), although the trend follows the same direction as in Fig. 5a. Thus, A β (1-42) fibrils and oligomers had significantly different effects on membrane microviscosity both in HeLa and SH-SY5Y cells, suggesting that these two aggregated forms of A β (1-42) may interact with cell membranes in different manners. The exact mechanism of these interactions is beyond the scope of this study, however, we can hypothesise that small oligomeric aggregates cause higher permeation of the bilayer, in accordance with previous findings.⁸⁰ While large A β (1-42) fibrils can certainly successfully attach to the surfaces of bilayers, it appears from our data that they are not able to significantly alter the diffusion rates of small molecules within the lipid tail region of the bilayer (which is a function of microviscosity).

Neuroprotective peptide H3 reverts the A β oligomer-induced decrease of membrane microviscosity in SH-SY5Y cells

A number of neuroprotectants have been identified which are able to counteract deleterious effects of extracellular neurotoxins, including A β aggregates. However, most research of neuroprotective agents in AD has focused on intracellular signalling launched by the neuroprotectants; only a few explored their effect on cell membranes. In one study, neuroprotective cyclodextrins were shown to reduce membrane cholesterol level and up-regulate A β clearance⁸¹; another study found that neuroprotectant curcumin reduces A β membrane insertion by attenuating A β -membrane interactions and membrane damage.⁸² Docosahexaenoic acid (DHA, an omega-3 polyunsaturated fatty acid) was shown to have neuroprotective effects in AD models and to modulate the organisation and various biophysical properties of plasma membranes.⁸³ However little is known about the effects of neuroprotective agents on plasma membrane microviscosity. Therefore, we set off to investigate whether a previously characterised neuroprotectant (i) can affect basal microviscosity of cell membranes and (ii) can modulate/counteract microviscosity alterations induced by A β .

As a neuroprotective agent, we used a small peptide termed H3, representing an active motif from the sequence of the neurotrophic S100A4 protein.⁸⁴ As we have previously shown, both S100A4 and H3 protect neurons from apoptosis and oxidative stress *in vitro* and in animal models of brain trauma, epilepsy and nerve degeneration.^{84,85} These effects are mediated by several plasma membrane receptors triggering intracellular signalling cascades^{84,86}; however, the effect of S100A4 or its derivatives on biophysical properties of cell membranes has not yet been investigated.

Fig. 5 Fluorescence lifetime of BODIPY 1 in plasma membranes before (black) and after (red) 2 hr incubation of 10 μ M A β (1-42) fibrils with (a) live HeLa cells and (b) live SH-SY5Y cells. Mean fluorescence lifetimes in HeLa cells were analysed by an unpaired t-test and found to be significantly different ($p = 0.03$, $N = 13$). A β (1-42) fibrils had no significant effect on the mean fluorescence lifetime of BODIPY 1 in SH-SY5Y cells ($p = 0.417$, $N = 24$).

We therefore tested whether treatment with the neuroprotective H3 peptide affects membrane microviscosity of non-treated and/or A β -treated SH-SY5Y cells. As an *in vitro* model, we have chosen the SH-SY5Y cell line which, in contrast to HeLa, has neural origin and is commonly used in studies of neurotoxicity, oxidative stress,⁸⁷ and neurodegenerative disorders, in particular Parkinson's and Alzheimer Diseases^{44,88,89}, thus representing a relevant system to investigate the membrane effects of a neuroprotective agent.

As can be seen from the Fig. 6a, incubation of SH-SY5Y with the H3 peptide had no significant effect on membrane microviscosity. We next pre-treated SH-SY5Y cells with H3 for 6 hours and challenged them with A β (1-42) oligomers. Membrane microviscosity was analysed by FLIM of our molecular rotor BODIPY 1 (Fig. 6b). In accordance with our previous results (Fig. 4), treatment with A β (1-42) oligomers alone decreased the microviscosity of the membrane (reduction in fluorescence life time by ca 10%, Fig 6b, red). However, in cells pre-treated with the H3 peptide, the mean microviscosity (Fig 6b, blue) did not significantly differ from that of the untreated controls.

Fig. 6 (a) Fluorescence lifetime of BODIPY 1 in plasma membranes of SH-SY5Y cells at room temperature (black) compared to the lifetime after overnight incubation of 20 μ g/ml H3 peptide with SH-SY5Y cells at room temperature (blue), demonstrating that the protein does not significantly alter plasma membrane microviscosity as sensed by BODIPY 1 (unpaired t-test, $p = 0.57$, $N = 17$). (b) The effect of A β (1-42) oligomers on plasma membranes of live SH-SY5Y cells are reverted by incubation with the H3 peptide. H3-treated SH-SY5Y cells subjected to A β (1-42) oligomers for two hours (blue) maintain similar membrane microviscosity values as untreated cells (black, $p = 0.342$, $N = 11$), whereas treatment with A β (1-42) in the absence of H3 decreases membrane microviscosity (red, $p < 0.001$, $N = 10$).

H3 counteracted the microviscosity-decreasing effect of A β (1-42) oligomers on plasma membranes of the SH-SY5Y cells. Resolving the precise mechanisms behind this effect was beyond the scope of this study; however, several potential scenarios may exist. Like its parent protein S100A4, the H3 peptide is a multimeric molecule and was synthesized as a tetramer consisting of four identical peptide chains attached to a lysine backbone. H3 binds to its plasma membrane targets with nanomolar to low micromolar affinity and low dissociation rate^{84,86} and could thus cluster cell surface receptors initiating 'background' pro-survival signalling and/or increasing membrane stability. Additionally, H3 could be internalised together with its binding partners and exert its biological effects acting from the cytoplasm, as S100A4 is known to bind intracellular targets that regulate cytoskeleton dynamics, thereby affecting cell morphology and motility.⁹⁰ Together, these findings suggest that neurotrophic agents may also exert their protective effects by counteracting membrane changes induced by neurotoxins. The characterisation of the underlying molecular mechanisms presents a fascinating field for future research.

Conclusions

The presence of aggregated A β (1-42) is connected with synapse failure and neuronal dysfunction during Alzheimer's disease and is known to induce biophysical alterations in cells. We set out to investigate the microviscosity of plasma membranes upon interaction with different forms of A β (1-42). We considered it important to perform live cell studies, rather than use model systems such as unilamellar vesicles or cell membrane homogenates. Hence, we have utilised a membrane-localised molecular rotor BODIPY 1 to directly measure the microviscosity of live cell membranes. We investigated the plasma membranes of different cell lines, compared our observations with those in model membranes, and separated the effects of A β (1-42) oligomers and fibrils on plasma membranes of neuroblastoma cells. In all studied samples we found that A β (1-42) oligomers decrease the bilayer microviscosity, while the fibrillar A β (1-42) did not significantly affect this parameter. In addition, we investigated the plasma membrane effects of the neuroprotective peptide H3 and demonstrated that it counteracts the microviscosity change induced by A β (1-42) oligomers. These results suggest an additional mechanism behind the pro-survival effect of the H3 peptide and indicate that neuroprotectant-induced membrane stabilisation may be an important factor contributing to effective neuroprotection in pathologies such as Alzheimer's disease.

Experimental section

Preparation of A β (1-42) peptides

A β (1-42) was purchased from rPeptide in vials containing 1mg of protein film, which was resuspended in 1,1,1,3,3,3-Hexafluoro-2-propanol (Sigma-Aldrich), aliquoted and dried under nitrogen flow into films containing 0.2 mg A β (1-42),

which were stored at -20°C. For each aggregation experiment, one 0.2 mg A β (1-42) film was resuspended in HFIP, sonicated for 5 minutes at room temperature, vortexed for 1 minute and solvent evaporated under nitrogen flow. The resulting film was resuspended in 200 μ L of anhydrous DMSO, vortexed and stirred for 1 minute. Gel filtration using Zeba 5K MWCO spin-exchange columns was done in order to replace DMSO with 10 mM HEPES. Absorption spectra were recorded immediately to determine the concentration of A β (1-42), which was then diluted to 50 μ M. To obtain A β (1-42) oligomers the solution was incubated for 2 hours at room temperature; to obtain fibrils, the solution was incubated for 7 days.

Cell culture, incubation of cells with A β , dye and H3 peptide

SH-SY5Y and HeLa cells (European Collection of Cell Cultures) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% Foetal Calf Serum. Cells were grown in T-25 flasks for 4-5 days to reach 80% confluency. For each experiment, cells were seeded into ibiTreat 8 well μ -slides (Ibidi) in 300 μ L of culture media at 100,000 cells per well, and allowed to grow to confluence overnight prior to adding A β (1-42). For each experiment, media in a well was replaced with 200 μ L of Mg²⁺ and Ca²⁺ free HBSS (Sigma-Aldrich). 50 μ L of the A β (1-42) HEPES solution was added to each well containing 200 μ L of buffer, such that the final concentration of A β (1-42) in each well was 10 μ M. Prior to imaging, BODIPY 1 stock solution in DMSO was added to the well to a final concentration of 0.8 μ M and mixed gently by pipetting in and out several times. Temperature in the wells was equilibrated and checked with a thermocouple. The H3 peptide (sequence KELLTRELPFLGKRT) was synthesized as a tetramer composed of four monomers coupled to a lysine backbone (Schafer-N, Denmark). Tetramerization was previously found to be necessary for the neuritogenic activity of S100A4.⁹¹ For the experiments with H3 peptide, SH-SY5Y cells were incubated for 6 hours in culture media containing 20 μ g/ml H3. Afterwards, A β (1-42) oligomers were added to the cells (apart from control wells), incubated for 2 hours, and membrane viscosity was analysed by BODIPY 1.

Preparation of giant plasma membrane vesicles

Giant plasma membrane vesicles were prepared according to published protocol⁵³ using 2 mM NEM as vesiculating agent. HeLa cells cultured in DMEM w/ FCS in T-25 flasks were brought to 80% confluence and incubated with 3-4 ml of vesiculating buffer for 90 minutes before gently collecting the buffer with floating GPMVs.

Absorption and fluorescence spectra acquisition

Agilent 8453 UV-Vis spectrophotometer was used to acquire absorption spectra and FluoroMax4 spectrofluorimeter (Horiba) with a Xenon lamp as an excitation source was used

to acquire fluorescence spectra. Spectra were corrected for wavelength-dependent efficiency of the light source and sensitivity of the detector. Quartz cuvettes with 1 cm path length were used.

FLIM acquisition and analysis

Fluorescence lifetime imaging microscopy (FLIM) was performed via time-correlated single-photon counting (TCSPC) technique, using a confocal laser scanning microscope (Leica, SP5 II). A mode-locked femtosecond Ti:Sapphire laser (Coherent, Chameleon Vision II) tunable over the 680–1080 nm range (140 fs pulse duration, 80 MHz) was used as the excitation source, operated at 880 nm. A PMC-100-1 photomultiplier tube (Hamamatsu) and a SPC-830 single-photon counting card (Becker-Hickl) were used for data acquisition. Fluorescence was collected between 500 and 580 nm; short-pass filters of 700 and 715 nm were used. FLIM acquisition time was usually 180 seconds. Samples on chamber slides were mounted in the microscope chamber heated by a thermostat (Lauda GmbH, E200). SHG signal from urea crystals on a glass cover slide was used to obtain the instrument response function (IRF).

Exponential fitting was done in SPCI software (Becker-Hickl) using the nonlinear least squares method (NLLSM) and reconvolution algorithm. The χ^2 value and randomness of residuals were used to indicate the goodness of fit. Fluorescence decays were judged to be mono-exponential and followed the equation

$$I(t) = \sum_{i=1}^n \alpha_i \exp\left(-t/\tau_i\right) \quad (3)$$

where I is fluorescence intensity, t is time, and α_i are the amplitudes and τ_i the fluorescence lifetimes of the n exponentially decaying components (1 in this case). The threshold for each pixel was set to at least 100 fluorescence counts in the decay peak, pixels with fewer counts were not included in the analysis. The binning parameter was set to 1 or 2 in the case of images with lower intensity. Data was further processed with OriginPro 8.6.

Statistical analysis

Statistics were performed using IBM SPSS Statistics software by two-tailed non-paired t -test or one-way ANOVA with Tukey's HSD post-test to identify statistically significant groups. Unless indicated otherwise, results are expressed as mean $\pm$ SD, * p < 0.05; ** p < 0.01; *** p < 0.001.

Conflicts of interest

There are no conflicts to declare.

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References

- J. A. Hardy and G. A. Higgins, *Science*, 1992, **256**, 184–185.
- J. A. Hardy and D. J. Selkoe, *Science*, 2002, **297**, 353–356.
- D. J. Selkoe, *Physiol. Rev.*, 2001, **81**, 741–766.
- H. A. Lashuel et al., *Nature*, 2002, **418**, 291.
- C. G. Glabe, *Neurobiol. Aging*, 2006, **27**, 570–575.
- R. Kaye et al., *Science*, 2003, **300**, 486–489.
- J. N. Kanfer, G. Sorrentino and D. S. Sitar, *Neurochem. Res.*, 1999, **24**, 1621–1630.
- E. Evangelisti et al., *J. Cell Sci.*, 2012, **125**, 2416–2427.
- C. M. Yip, A. A. Darabie and J. McLaurin, *J. Mol. Biol.*, 2002, **318**, 97–107.
- S. A. Waschuk et al., *J. Biol. Chem.*, 2001, **276**, 33561–33568.
- I. Mikhalyov et al., *Biophys. J.*, 2010, **99**, 1510–1519.
- J. J. Kremer, D. J. Sklansky and R. M. Murphy, *Biochemistry*, 2001, **40**, 8563–8571.
- P. T. Wong et al., *J. Mol. Biol.*, 2009, **386**, 81–96.
- M. Morita et al., *Phys. Chem. Chem. Phys.*, 2014, **16**, 8773–8777.
- K. Sasahara, K. Morigaki and K. Shinya, *Phys. Chem. Chem. Phys.*, 2013, **15**, 8929.
- M.-S. Lin et al., *Colloids Surfaces B Biointerfaces*, 2007, **58**, 231–236.
- G. Valincius et al., *Biophys. J.*, 2008, **95**, 4845–4861.
- J. J. Kremer et al., *Biochemistry*, 2000, **39**, 10309–10318.
- E. Sezgin et al., *Nat. Rev. Mol. Cell Biol.*, 2017, **18**, 361–374.
- R. P. Mason et al., *J. Biol. Chem.*, 1999, **274**, 18801–18807.
- N. A. Avdulov et al., *J. Neurochem.*, 2002, **68**, 2086–2091.
- W. E. Müller et al., *Amyloid*, 1998, **5**, 10–15.
- W. Stoeckenius and D. M. Engelman, *J. Cell Biol.*, 1969, **42**, 613–646.
- M. K. Kuimova, *Phys Chem Chem Phys*, 2012, **14**, 12671–12686.
- M. A. Haidekker and E. A. Theodorakis, *Org. Biomol. Chem.*, 2007, **5**, 1669–1678.
- A. Vyšniauskas and M. K. Kuimova, *Int. Rev. Phys. Chem.*, 2018, **37**, 259–285.
- M. R. Dent et al., *Phys. Chem. Chem. Phys.*, 2015, **17**, 18393–18402.
- Y. Wu et al., *Phys. Chem. Chem. Phys.*, 2013, **15**, 14986.
- R. J. Reiter et al., *Mech. Ageing Dev.*, 1999, **110**, 157–173.
- X. Yang, S. Askarova and J. C.-M. Lee, *Mol. Neurobiol.*, 2010, **41**, 138–148.
- A. M. Aleardi et al., *J. Bioenerg. Biomembr.*, 2005, **37**, 207–225.
- S. Müller et al., *Atherosclerosis*, 1990, **83**, 231–237.
- J. S. Owen et al., *J. Lipid Res.*, 1982, **23**, 124–132.
- S. Hollán, *Haematologia (Budap.)*, 1996, **27**, 109–27.
- T. Förster and G. Hoffmann, *Zeitschrift für Phys. Chemie*, 1971, **75**, 63–76.
- M. K. Kuimova et al., *J Am Chem Soc*, 2008, **130**, 6672–6673.
- I. López-Duarte et al., *Chem. Commun.*, 2014, **50**, 5282–5284.
- M. R. Dent et al., *Chem. Commun.*, 2016, **52**, 13269–13272.
- P. S. Sherin et al., *Chem. Sci.*, 2017, **8**, 3523–3528.
- M. Kubánková et al., *Biomaterials*, 2017, **139**, 195–201.
- L. E. Shimolina et al., *Sci. Rep.*, 2017, **7**, 41097.
- A. Vyšniauskas et al., *Chem. Sci.*, 2015, **6**, 5773–5778.
- A. Vyšniauskas et al., *Phys. Chem. Chem. Phys.*, 2017, **7**, 25252–25259.
- A. Krishna et al., *BMC Genomics*, 2014, **15**, 1154.
- J. T. Mika et al., *Biophys. J.*, 2016, **111**, 1528–1540.
- M. Sinensky, *Proc Natl Acad Sci*, 1974, **71**, 522–525.
- A. R. Cossins, J. Christiansen and C. L. Prosser, *Biochim. Biophys.*

- Acta*, 1978, **511**, 442–452.
- 48 D. J. Pehowich et al., *Biochemistry*, 1988, **27**, 4632–4638.
- 49 I. Horváth et al., *Biochemistry*, 1998, **95**, 3513–3518.
- 50 V. Chmyrov et al., *Anal. Chem.*, 2015, **87**, 5690–5697.
- 51 T. Baumgart et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 3165–3170.
- 52 B. Bauer, M. Davidson and O. Orwar, *Angew. Chemie Int. Ed.*, 2009, **48**, 1656–1659.
- 53 E. Sezgin et al., *Nat. Protoc.*, 2012, **7**, 1042–1051.
- 54 K. R. Levental and I. Levental, *Curr. Top. Membr.*, 2015, **75**, 25–57.
- 55 G. T. Charras et al., *Biophys. J.*, 2008, **94**, 1836–1853.
- 56 S. T. Ferreira et al., *Front. Cell. Neurosci.*, 2015, **9**, 191.
- 57 C. Haass and D. J. Selkoe, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 101–112.
- 58 M. Ahmed et al., *Nat. Struct. Mol. Biol.*, 2010, **17**, 561–567.
- 59 J. D. Harper and P. T. Lansbury, *Annu. Rev. Biochem.*, 1997, **66**, 385–407.
- 60 M. Sakono and T. Zako, *FEBS J.*, 2010, **277**, 1348–1358.
- 61 S. Nag et al., *Biophys. J.*, 2010, **99**, 1969–1975.
- 62 P. Narayan et al., *J. Am. Chem. Soc.*, 2013, **135**, 1491–1498.
- 63 M. Kawahara and Y. Kuroda, *Brain Res. Bull.*, 2000, **53**, 389–397.
- 64 Y. Shai, *Biochim. Biophys. Acta*, 1999, **1462**, 55–70.
- 65 J. A. Hebda and A. D. Miranker, *Annu. Rev. Biophys.*, 2009, **38**, 125–152.
- 66 R. Kaye et al., *J. Biol. Chem.*, 2004, **279**, 46363–46366.
- 67 Y. Sokolov et al., *J. Gen. Physiol.*, 2006, **128**, 637–647.
- 68 W. Gibson Wood et al., *Biochim. Biophys. Acta - Biomembr.*, 2003, **1610**, 281–290.
- 69 J. B. Hicks et al., *Biochim. Biophys. Acta - Biomembr.*, 2008, **1778**, 2512–2519.
- 70 W. E. Müller et al., *Brain Res.*, 1995, **674**, 133–136.
- 71 I. Peters et al., *Biochim. Biophys. Acta*, 2009, **1788**, 964–972.
- 72 M. R. H. Krebs, E. H. C. Bromley and a M. Donald, *J. Struct. Biol.*, 2005, **149**, 30–37.
- 73 C. Cecchi, *J. Cell Sci.*, 2005, **118**, 3459–3470.
- 74 H. Hartmann, A. Eckert and W. E. Müller, *Biochem. Biophys. Res. Commun.*, 1994, **200**, 1185–1192.
- 75 Y. Oyama et al., *Jpn. J. Pharmacol.*, 1995, **68**, 77–83.
- 76 D. A. Los and N. Murata, *Biochim. Biophys. Acta - Biomembr.*, 2004, **1666**, 142–157.
- 77 V. Rondelli et al., *Sci. Rep.*, 2016, **6**, 20997.
- 78 U. Igbavboa et al., *Neuroscience*, 2009, **162**, 328–338.
- 79 R. A. Cooper, *J. Supramol. Struct.*, 1978, **8**, 413–430.
- 80 T. L. Williams and L. C. Serpell, *FEBS J.*, 2011, **278**, 3905–17.
- 81 J. Yao et al., *J. Exp. Med.*, 2012, **209**, 2501–2513.
- 82 A. Thapa et al., *Langmuir*, 2013, **29**, 11713–11723.
- 83 S. C. Dyll, *Front. Aging Neurosci.*, 2015, **7**, 52.
- 84 O. Dmytriyeve et al., *Nat. Commun.*, 2012, **3**, 1197.
- 85 M. Moldovan et al., *Mol. Med.*, 2013, **19**, 43–53.
- 86 S. Pankratova et al., *Theranostics*, 2018, **8**, 3977–3990.
- 87 N. H. Gay et al., *Neurochem. Res.*, 2018, **43**, 619–636.
- 88 H. Xicoy, B. Wieringa and G. J. M. Martens, *Mol. Neurodegener.*, 2017, **12**, 10.
- 89 L. Agholme et al., *J. Alzheimer's Dis.*, 2010, **20**, 1069–1082.
- 90 M. Kriajevska et al., *J. Biol. Chem.*, 1998, **273**, 9852–9856.
- 91 V. Novitskaya et al., *J. Biol. Chem.*, 2000, **275**, 41278–41286