The impact of membrane composition on conformation and trafficking of lipidated prion protein (PrP) variants

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The impact of membrane composition on conformation and trafficking of lipidated prion protein (PrP) variants

Submitted by

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PhD thesis

A dissertation submitted in partial fulfilment of the requirements for the degree of
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Abstract

Prion protein (PrP) aggregation arises from the misfolding of the native cellular PrP (PrP^C) and is a key pathophysiologic event in fatal neurodegenerative prion diseases. To elucidate the mechanism of conversion of PrP^C into toxic PrP^{Sc}, homogeneous PrP constructs carrying a glycosylphosphatidylinositol (GPI) anchor mimic served as tools to directly observe PrP at the cellular membrane, the initial site of prion infection and PrP misfolding. The posttranslational modifications (PTMs) of PrP, especially the GPI anchor, have been shown to be crucially involved in PrP^{Sc} formation. To this end, fluorescently labeled, folded PrP variants equipped with GPI anchor-mimicking peptides were generated *via* a semisynthesis approach. Secondary structures of the resulting PrP constructs were assessed by circular dichroism (CD). All PrP variants exhibited typical far-UV CD spectra of predominantly α -helical proteins, in agreement with the structure of native PrP^C. *In vitro* aggregation, monitored by thioflavin T (ThT) fluorescence and proteinase K (PK) resistance, illustrated their use in understanding key steps of PrP pathology.

To study the PrP-membrane interaction, we monitored PrP association on artificial supported bilayers in quartz crystal microbalance-dissipation (QCM-D) experiments in real time, varying pH, lipid and salt compositions. Upon increasing the PrP concentration in close proximity of the bilayer by enhancing the initial electrostatic interaction, lipidated PrP variants, in particular the full-length PrP construct (aa 23-231), exhibited a higher binding affinity towards the bilayer compared to recombinant PrP. This finding corroborates the significance of the GPI anchor mimic in PrP-membrane interactions.

Further cell imaging experiments using super-resolution structured illumination microscopy (SR-SIM) combined with co-localization analysis confirmed the membrane localization of Cy5 labeled PrP constructs equipped with a GPI anchor mimic in neuron-like SH-SY5Y cells. The dynamic behavior of PrP within the ordered and disordered regions of the plasma membrane as well as the actin cytoskeleton was studied extensively with fluorescence correlation spectroscopy (FCS). Imaging total internal reflection-FCS (ITIR-FCS) measurements demonstrated that full-length PrP modified with a GPI anchor mimic affected the cholesterol-dependent membrane domains by making them more fluid. No such impact was observed for PrP without a GPI anchor

mimic. This emphasizes the significance of the interaction between the cellular membrane, PrP and its GPI anchor in cells.

By combining methyl- β -cyclodextrin (m β CD) treatments, a cholesterol-depleting agent, with addition of latrunculin A (LatA), an actin polymerization inhibitor, the interaction of lipidated PrP labeled with Cy5 in its membrane environment was studied. FCS revealed that PrP containing a GPI anchor mimic exhibits a lower (compared to free diffusing species), cholesterol-independent and cytoskeleton-dependent mobility. Manipulation of the link between PrP and the cytoskeleton by inhibiting actin polymerization induced an even slower diffusion of Cy5 labeled PrP. Typically, deceleration of diffusion is attributed to protein clustering, which could resemble the initial step of PrP aggregation. We propose that an intact actin cytoskeleton can act as a barrier for the conversion of PrP^C into PrP^{Sc} on cell membranes. Thus, the cytoskeleton-membrane association that immobilizes PrP *via* the actin cytoskeleton can be hypothesized to play a crucial role in the PrP^C-PrP^{Sc} conversion.

By taking advantage of site-selectively modified PrP variants and biophysical investigations of their interactions with membranes, including a complex live cell setting, we gained an improved understanding of the synergistic interplay between PrP, its GPI anchor, the cellular membrane and the cytoskeleton.

prion protein · GPI anchor · membrane interaction · semisynthesis · fluorescence correlation spectroscopy (FCS)

Zusammenfassung

Die Aggregation des Prion Proteins (PrP) hat ihren Ursprung in der Missfaltung des nativen zellulären PrPs (PrP^C) und ist ein Schlüsselereignis der fatalen neurodegenerativen Prion Krankheiten. Um den Mechanismus der Umwandlung von PrP^C in PrP^{Sc} aufzuklären dienten homogene PrP Konstrukte ausgestattet mit einem funktionellen Analogon des Glykosylphosphatidylinositol (GPI) Ankers als Werkzeuge um PrP direkt an der Zellmembran, dem ursprünglichen Ort der Prioninfektion und Umfaltung des PrPs, zu beobachten. Den posttranslationalen Modifikationen (PTM) von PrP, insbesondere dem GPI Anker, konnte eine bedeutende Rolle in der Bildung von PrP^{Sc} zugeschrieben werden. Aus diesem Grund wurden fluoreszenzmarkierte, gefaltete PrP Varianten ausgestattet mit einem funktionellen Analogon des GPI Ankers mittels eines semisynthetischen Ansatzes hergestellt. Die Sekundärstrukturen der resultierenden PrP Konstrukte wurden mittels Zirkulardichroismus (CD) analysiert. Alle PrP Varianten wiesen im fernen UV ein typisches CD Spektrum für überwiegend α -helikale Proteine in Übereinstimmung mit der nativen Struktur von PrP^C auf. *In vitro* Aggregation analysiert mittels Thioflavin T (ThT) Fluoreszenz und Proteinase K (PK) Resistenz veranschaulichte ihre Verwendung um Schlüsselschritte der PrP Pathologie zu verstehen.

Um die Membranwechselwirkung zu untersuchen verfolgten wir die PrP Assoziation an künstlichen Lipiddoppelschichten durch Experimente mit einer Quarzmikrowaage inklusive Dissipationsaufzeichnung (QCM-D) in Echtzeit bei variierendem pH, Lipid- und Salzzusammensetzungen. Durch die Erhöhung der PrP Konzentration in unmittelbarer Nähe zu der Lipiddoppelschicht über eine Verstärkung der initialen elektrostatischen Wechselwirkung wiesen die lipidierten PrP Varianten, insbesondere das volllängen PrP Konstrukt (aa 23-231), im Vergleich zu rekombinantem PrP eine höhere Bindungsaffinität gegenüber der Lipiddoppelschicht auf. Dieses Erkenntnis bekräftigt die Bedeutung des funktionellen Analogons des GPI Ankers in der PrP-Membran Wechselwirkung.

Weiterführendes Zell-Imaging unter Verwendung von supraauflösender struktureller Beleuchtungsmikroskopie (SR-SIM) kombiniert mit Co-lokalisationsanalyse bestätigte die Membranlokalisation der Cy5 markierten PrP Konstrukte ausgestattet mit einem funktionellen Analogon des GPI Ankers in neuronartigen SH-SY5Y Zellen. Das dynamische Verhalten von PrP wurde eingehend innerhalb der geordneten und

ungeordneten Regionen der Plasmamembran sowie des Aktinzytoskeletts mit Fluoreszenzkorrelationsspektroskopie (FCS) untersucht. Messungen mittels Imaging interner Totalreflexionsfluoreszenzkorrelationsspektroskopie (ITIR-FCS) zeigten, dass das volllängen PrP Konstrukt, modifiziert mit einem funktionellen Analogon des GPI Ankers, die Fluidität der cholesterolabhängigen Membrandomänen erhöhte. Für PrP ohne funktionelles Analogon des GPI Ankers konnte jedoch kein Effekt beobachtet werden. Dies unterstreicht die Bedeutung der Wechselwirkung zwischen der Zellmembran, PrP und dem GPI Anker in Zellen.

Durch Kombination einer Methyl- β -zyklodextrin (m β CD) Behandlung, einem cholesterolreduzierenden Wirkstoff, mit Zugabe von Latrunkulin A (LatA), einem Aktinpolymerisationsinhibitor, wurde die Wechselwirkung von lipidisiertem PrP markiert mit Cy5 in seiner Membranumgebung untersucht. FCS zeigte, dass PrP, das ein funktionelles Analogon des GPI Ankers enthält, eine geringere (im Vergleich zu frei diffundierender Spezies), cholesterolunabhängige und zytoskelettabhängige Mobilität aufweist. Manipulation der Verbindung zwischen PrP und dem Zytoskelett durch Inhibierung der Aktinpolymerisierung verursachte eine noch langsamere Diffusion von Cy5 markiertem PrP. Üblicherweise wird einer Diffusionsverlangsamung Proteinclustering zugeschrieben, das dem ersten Schritt der PrP Aggregation ähneln könnte. Wir behaupten, dass ein intaktes Aktinzytoskelett als Barriere für die Umwandlung von PrP^C in PrP^{Sc} an der Zellmembran wirken kann. Aus diesem Grund kann für die Zytoskelett-Membran Wechselwirkung, die PrP über das Aktinzytoskelett immobilisiert, eine entscheidende Rolle in der PrP^C-PrP^{Sc} Umwandlung angenommen werden.

Durch die Nutzung von positionsselektiv-modifizierten PrP Varianten und biophysikalischen Untersuchungen ihrer Wechselwirkungen mit Membranen, auch innerhalb einer komplexen *Live Cell* Umgebung, konnten wir ein verbessertes Verständnis des komplexen synergistischen Zusammenspiels zwischen PrP, seinem GPI Anker, der Zellmembran und des Zytoskeletts erlangen.

Prion Protein · GPI Anker · Membranwechselwirkung · Semisynthese · Fluoreszenzkorrelationsspektroskopie (FCS)

Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety, under the supervision of Univ. Prof. Dr. Christian F.W. Becker from the Institute of Biological Chemistry at the University of Vienna between March 2014 and May 2019.

I have duly acknowledged all the sources of information which have been used in the thesis.

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1. Introduction

1.1 Prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are incurable, neurodegenerative disorders affecting humans and animals.¹ They include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) of cattle, chronic wasting disease (CWD) of cervids, and several human diseases such as kuru, Creutzfeld-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). The disease progression is accompanied by the loss of cognitive skills and neuronal dysfunction, and can be of inherited, sporadic or iatrogenic origin.^{2,3} The central pathophysiologic event is ascribed to the conformational change of the cellular (PrP^C) into scrapie prion protein (PrP^{Sc}) that then not only propagates further PrP^C misfolding in neighbouring cells, but can also infect other organisms.⁴ Identification of the infective pathogen of prion diseases and its proof of transmissibility started in the 1950s. By ending cannibalism within the Fore tribe in Papua New Guinea, the transmission of kuru could be stopped. Experiments with transferring brain samples of these kuru victims into primates induced spongiform encephalopathies.⁵ Due to its infective property the pathogen was first assumed to be of nucleic acid-based, viral nature. However, the application of ultra-violet and ionizing irradiation failed to inactivate the agent, leading to the “protein-only hypothesis” by Griffith in 1967.^{6,7} Eventually in 1982 the term “prion” defining a small **proteinaceous infectious** particle was introduced by Prusiner during the course of discovering the prion protein.^{8,9} PrP 27-30 corresponding to the protease-resistant core of PrP^{Sc} with an apparent molecular mass of 27-30 kDa was isolated by enriching fractions from Syrian hamster (SHa) brain for scrapie infectivity.¹⁰⁻¹³ Successful Edman degradation paved the way for subsequent molecular cloning studies of the PrP gene.¹⁴⁻¹⁶ The linkage of PrP^{Sc} to prion diseases was recognized as an important feature of the protein, together with its role in transmission and pathogenesis of these illnesses.¹⁷ Thus, the main focus of elucidating prion pathogenicity is assigned to PrP (section 1.2). Understanding the key features in prion diseases can serve as paradigm for other neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Alzheimer’s (AD) and Parkinson’s disease (PD), that are characterized by misfolded proteins “prionoids” sharing the aggregation properties but being not strictly infectious.¹⁸⁻²⁰ As it happens, the latter statement might not be entirely correct. Recent prion research reported the

discovery of α -synuclein prions²¹ in multiple system atrophy (MSA) and iatrogenic AD with evidence of transmissibility of amyloid- β (A β),²² hence highlighting the need to understand prion transmission and toxicity even more.

1.2 Properties and structures of the prion protein (PrP)

1.2.1 Cellular prion protein (PrP^C)

High expression levels of PrP^C are found in the central nervous system (CNS), but it exists in other cell types and tissues, such as lymphoid organs, as well.²³⁻²⁶ Accessing the gene encoding SHaPrP^C, *Prnp*,^{14, 27} entailed its further identification in numerous other species and illustrated a highly conserved sequence.^{28, 29} The entire open reading frame (ORF) is contained within a single exon and primarily translates into a protein composed of 254 amino acids (aas).^{30, 31} The first 22 amino acids reflect an *N*-terminal signal sequence for PrP entering the secretory pathway. Upon its cleavage, glycosylation at asparagine residues and formation of a disulfide bond occur in the endoplasmic reticulum (ER). Lastly, cleavage of the *C*-terminal signal sequence facilitates the attachment of the glycosylphosphatidylinositol (GPI) anchor, providing mature, posttranslationally modified PrP at the outer leaflet of the cell membrane, typical for glycosylphosphatidylinositol anchored proteins (GPI APs).³² Interestingly, PrP can be found in three topologic forms at the ER. Apart from the fully translocated PrP, two transmembranal types occur with the *N*- or *C*-terminus facing the ER lumen, denoted as ^{Ntm}PrP or ^{Ctm}PrP, respectively.^{33, 34} Normally ^{Ntm}PrP and ^{Ctm}PrP only comprise a small portion of PrP^C, whereas an excess of ^{Ctm}PrP induces neurotoxicity. Neuronal cell death is caused in the absence of PrP^{Sc} formation, obviously by an aberrant metabolism of PrP^C. PrP^C mislocalization represents another mechanistic possibility for prion toxicity next to the alteration of PrP^C-mediated signaling and PrP-derived oligomeric species.²³ First structural studies on PrP^C isolated from brains of Syrian hamsters demonstrated a predominantly α -helical content.³⁵ As these measurements agreed well with subsequent spectroscopic data of recombinant PrP (rPrP), accessible in larger amounts, it was considered an appropriate surrogate in biochemical experiments,³⁶⁻³⁹ as well as in solving NMR and crystal structures of PrP.⁴⁰⁻⁴⁷ The PrP structure comprises an unstructured *N*-terminal (aa 23-120) and a globular *C*-terminal part (aa 121-231) (Scheme 1).

Scheme 1. Tertiary structure of PrP^C. The structure is based on NMR measurements of human PrP^C (aa 23-230) by Zahn *et al.* and was taken from the protein data bank (PDB) entry 1QLZ.⁴⁷

In more detail, the *N*-terminal segment consists of a nonapeptide (PQGGGGWGQ) followed by four octapeptide (PHGGGWGQ) repeats (OR) with a high affinity for copper,⁴⁸⁻⁵¹ and other divalent cations,⁵² adjacent to a charged cluster (CC) or polybasic region (Scheme 2). Depending on the concentration of the metal and pH, the OR region is capable to bind up to four copper ions in distinct coordination geometries.^{50, 53} Current estimates for K_d values vary between the micro- and femtomolar range.⁵⁰ The central hydrophobic domain (HD), comprising of aas 113-135, serves as a transmembrane domain³³ and includes a palindromic region (AGAAAAGA, aa 113-120) thought to be important in the PrP^C-PrP^{Sc} conversion.^{54, 55} Within the *C*-terminal region three α -helices (aa 144-154, 175-193 and 200-219) with two of them connected by a disulfide bond,⁵⁶ and a small anti-parallel β -sheet (aa 128-131 and 161-164) are present. As PTMs a *C*-terminal GPI anchor linked to serine 231 and two *N*-linked glycosylation sites at asparagines 181 and 197 exist. PrP^C can occur in non-, mono- and diglycosylated forms.^{57, 58} Variations in glycan structures attached to PrP may be differentially distributed depending on the areas of the CNS.⁵⁹ Molecular dynamics simulations indicate that the *N*-linked oligosaccharides located at two helices within the structured region of PrP contribute to its stabilization in generating a negative electrostatic field covering the helical surface,⁶⁰ thus impacting strain diversity and prion infection.⁶¹⁻⁶⁴ The *C*-terminal GPI anchor tethers PrP to the outer leaflet of the plasma membrane.⁶⁵

Scheme 2. Schematic outline of the primary PrP^{C} structure. Residue numbers correspond to hPrP^C.

It has been postulated that mutations in the *Prnp* gene facilitate the pathogenic process by destabilizing the tertiary structure of PrP^{C} . More than 30 mutations in *Prnp* could be linked to inherited prion diseases.⁶⁶ In affecting the primary sequence of PrP concomitant changes in its 3D structure may arise, and not cause, but influence a person's risk of developing a disease. Indeed, thermodynamic measurements of mutated PrP variants indicated destabilizing effects only for some of them.⁶⁷ For example by comparing the wild-type variant to the E200K mutant almost identical structures, but major perturbations of the surface electrostatic potential were found. This suggests that these defects cause abnormalities in PrP interactions and should be considered as key determinants in the misfolding process.⁶⁸

1.2.2 Scrapie prion protein (PrP^{Sc})

Scrapie prion protein (PrP^{Sc}) is the toxic, misfolded isoform of PrP. It is, as PrP^{C} , encoded by the *Prnp* gene and exhibits identical PTMs, but distinct structural, biochemical and physiological features.¹³ Despite a large interest in elucidating the structure of PrP^{Sc} , there is only limited data about its molecular details available.⁶⁹ To date, obtaining a high resolution structure of PrP^{Sc} has been impaired by its insolubility, propensity to aggregate and heterogeneity. Structural variations, such as differences in the glycosylation patterns, suggested to correlate with biochemical changes, including the extent of the proteinase K (PK) resistance, the electrophoretic mobility of the proteolytic fragments and the conformational stability, depend on the distinct strains and complicate the determination of PrP^{Sc} structure.⁷⁰ Besides, in agreement with discussions from the Prion 2018 round tables,⁷¹ the diversity of PrP assemblies

implicates that there may be no single PrP^{Sc} structure. Data generated by biochemical and -physical methods, such as spectroscopy analysis, electron microscopy and limited proteolysis, has led to several 3D structural models. Govaerts and colleagues suggested left-handed β -helices assembled into trimers, also known as the 4-rung β -solenoid model (Scheme 3).⁷² Based on ESR measurements Cobb *et al.* proposed a parallel in-register intermolecular β -sheet (PIRIBS) architecture where PrP^{Sc} consists of β -strands and short turns and/or loops with no residual α -helices.⁷³ Still, so far all models display discrepancies with experimental data.⁷⁴

*Scheme 3. 3D structural models for PrP^{Sc}. Trimeric PrP 27-30 depicted as 4-rung β -solenoid model (A) proposed by Govaerts *et al.*⁷² and parallel in-register intermolecular β -sheet (PIRIBS) model (B) suggested by Cobb *et al.*⁷³*

Notably, cryo electron microscopy (cryo-EM) is a technique providing high-resolution structures.^{75, 76} By rapidly cooling samples proteins can be observed in their native state. In 2016 Wille and colleagues⁷⁷ employed cryo-EM to analyze GPI anchorless PrP 27-30 amyloid fibrils. PrP 27-30 was purified from brains of transgenic mice infected with prions. Further inoculation of wt mice with the purified GPI anchorless PrP 27-30 confirmed the development of typical neurological signs of prion disease. The structure of GPI anchorless PrP 27-30 amyloid fibrils was found to agree with a 4-rung β -solenoid architecture. 3D reconstruction revealed two distinct protofilaments and an average molecular height of ~ 17.7 Å. However, Collinge, Wadsworth and co-workers⁷⁸ studied the structural features distinguishing infectious *ex vivo* mammalian prions from non-infectious fibrillar assemblies generated *in vitro*. Applying cryo-EM and atomic force microscopy (AFM) measurements non-infectious recombinant PrP fibrils were

identified as 10 nm wide single fibres with a double helical repeating substructure, agreeing with the structure described by Wille and colleagues.⁷⁷ Prion-infected transgenic mice replicate prions but they mainly develop PrP amyloid plaques which are not seen in prion-inoculated wt mice.^{79, 80} Caughey and co-workers⁸¹⁻⁸³ have described two morphologically distinct PrP fibril assemblies in prion-infected transgenic mice. Therefore, considering the lower infectivity titer of PrP 27-30 in the studies of Wille and colleagues⁷⁷ it appears that the more abundant, single non-glycosylated PrP fibrils, corresponding essentially to recombinant PrP, has been described rather than the infectious glycosylated PrP rods. Collinge, Wadsworth and co-workers⁷⁸ characterized infectious PrP rods, 20 nm in width, that contained two fibres, each with a double helical repeating substructure separated by a central gap of 8-10 nm. The gap between the paired fibres consists of irregularly structured material compositionally distinct to the protein surface. Thus, it was proposed as a location of the *N*-linked glycans of PrP. The structure of the infectious PrP rods differentiates them from all other protein assemblies so far studied in neurodegenerative diseases. This includes characterizations by cryo-EM of tau filaments from AD⁸⁴ and monoclonal immunoglobulin light chain (LC) fibrils from amyloid light-chain (AL) amyloidosis.⁸⁵ To date, cryo-EM studies of tau and AL represent the only structural data of fibrils directly extracted from human tissue under pathologic conditions. For tau paired helical and straight filaments could be identified with cores made of two identical protofilaments that adopt a combined cross- β / β -helix structure. AL fibrils were found to be helical with a single protofilament showing a cross- β architecture. It is widely accepted that during the PrP^C-PrP^{Sc} conversion the β -strand content increases vastly,^{86, 87} and the PK resistance of the “folded core” (aa ~90-231), as well.^{9, 14} Whereas PrP^C is dominated by α -helices, monomeric, soluble and highly susceptible to proteolytic digestion, PrP^{Sc} contains predominantly β -sheets (>43%),⁸⁷ aggregates into amyloid fibrils,⁸⁸ is insoluble in detergents and partially resistant to proteolysis.^{35, 89} These biochemical differences between the PrP isoforms appear to be associated with the changes of the secondary structure in PrP^{Sc}.

1.3 Physiology of the prion protein (PrP)

1.3.1 Function of PrP^C

Though the relevance of PrP^C in TSEs is widely accepted, its physiological function remains enigmatic. Studies with PrP knockout mice have failed on this regard. Transgenic mice lacking PrP were found to develop normally.^{90, 91} A multitude of functions has been ascribed to PrP in different tissues, cells and experimental settings, though not always without controversy or questionable reproducibility. Among others, PrP^C has been connected to developmental processes,⁹² cell adhesion,^{93, 94} neurite outgrowth, synapse formation,⁹⁵⁻⁹⁹ neuroprotection¹⁰⁰⁻¹⁰² and regulation of the circadian rhythm.¹⁰³ Moreover, there is evidence for PrP contributing to myelin maintenance,¹⁰⁴⁻¹⁰⁷ cellular homeostasis of divalent cations¹⁰⁸⁻¹¹⁰ and signaling events.¹¹¹⁻¹¹³ A more detailed discussion can be found in reviews by the group of Aguzzi.^{104, 114} Recently, other functions have been attributed to PrP^C. That is acting as a receptor for the aggregated proteins A β oligomers¹¹⁵⁻¹¹⁷ and α -synuclein.¹¹⁸ By mediating the uptake of A β and α -synuclein PrP^{Sc} is unable to replicate in their presence. Already 10 years ago it was suspected that reports on the function of PrP represent just specific aspects of a more complex physiological role of PrP^C.²³ Causes for the functional diversity of PrP^C might not only be its alternating transient binding partners in different cellular locations, but also its proteolytic processing.^{119, 120} For once, PrP fragmentation may inhibit association with some binding partners while possibly allowing new interactions with others. Then again, the cleaved products may act as soluble ligands facilitating protein interactions over large distances. These findings contribute to the biological complexity of the physiological function of PrP. In fact, four different but highly conserved cleavage events have been significantly characterized (Scheme 2).¹²¹⁻¹²³ During transport to the cell surface α -cleavage results in a soluble flexible *NI*- and a globular membrane-bound *CI* part. Myelin maintenance has been initially linked to this *CI* part derived from α -cleavage. Mice expressing PrP mutants not able to undergo α -cleavage suffered from chronic demyelinating polyneuropathy (CDP).^{105, 106, 124} Interestingly, recent work found a specific ligand role of the flexible *NI* part towards the G-protein coupled receptor *Adgrg6*, promoting myelin homeostasis.¹⁰⁷ During shedding PrP^C is released from the plasma membrane by a disintegrin and metalloproteinase (ADAM) enzyme, namely ADAM10, in a glycosylated form without the GPI anchor and designated as “shed PrP”.¹²⁵ Though

definite functions of “shed PrP” are not known to date, the shedding process regulates the membrane levels of PrP^C and thus its functions at the cell surface. Similar to α -synuclein,¹¹⁸ recent work by Jarosz-Griffiths *et al.*¹²⁶ found that the toxicity and cellular binding of A β oligomers can be reduced by shedding of PrP^C, thereby pointing towards a contribution as a receptor in AD. Moreover, PrP^C is expressed in immune cells as well, particularly on mast cells.¹²⁷ Upon activation of these cells the PrP^C shedding process is enhanced, proposing PrP involvement in the inflammatory mast cell response. Under pathological conditions and in response to oxidative stress, incidences of β -cleavage occurring around amino acid position 90 are increased.^{128, 129} Lastly, γ -cleavages restricted to unglycosylated PrP generate a large soluble N3 and a short C3 part by taking place in a region between amino acids 170 and 200. While prevalence and relevance of this cleavage requires further investigation, increased amounts of C3 in CJD brain samples suggest a pathophysiological role.¹³⁰

Despite multiple evidence of PrP in physiological processes, the functional diversity based on its manifold binding partners and proteolytic fragments complicate an exact definition of its physiological function. Yet successful elucidation of pathways and roles of PrP could help to understand its linkage to toxicity in prion diseases and to other neurodegenerative diseases.¹³¹

1.3.2 Trafficking of PrP^C

As the PrP function is closely intertwined with the cellular compartments where the protein is located, having a closer look at trafficking may assist in elucidating its involvement in patho- and physiological processes. PrP^C is tethered *via* its GPI anchor to the outer leaflet of the plasma membrane.⁶⁵ In 1993 data by Shyng *et al.*¹³² revealed constitutive cycling of PrP^C between the cell surface and endocytic compartments on varying times scales dependent on the cell line, as demonstrated in later work.¹³³ From the cellular membrane PrP^C can enter the cell *via* multiple pathways, mediated mainly by the unstructured N-terminal domain.^{134, 135} Evidence for a cooperation between clathrin^{133, 136, 137} and rafts¹³⁸⁻¹⁴⁰ in the internalization of PrP^C was found.¹⁴¹ Clathrin is a large, oligomeric protein assembling into lattice structures on the inner surface of the plasma membrane. Thereby, it causes the membrane to invaginate and pinch off to form clathrin-coated vesicles (CCVs), which can then fuse with other intracellular organelles.¹⁴² Though a clathrin-dependent internalization might appear unusual since

PrP lacks a cytoplasmic domain necessary for the direct interaction with clathrin and the adaptor protein, GPI APs can indeed enter the clathrin-dependent pathway upon interaction with transmembrane proteins possessing a clathrin-coated pit internalization signal.¹³⁹ Moreover, the endocytosis of PrP^C was found to be associated with the low-density lipoprotein receptor-related protein 1 (LRP1)^{137, 143} that belongs to a receptor family of cell-surface transmembrane proteins capable of binding a variety of ligands and internalizing *via* clathrin-coated pits.^{144, 145} As a non-classical clathrin-independent pathway the raft-dependent internalization route distinguishes caveolae-dependent and -independent endocytosis.¹⁴⁶ Caveolae are membrane invaginations, originating from the oligomerization of caveolins, their integral coat proteins, and are considered to be specialized raft domains.^{147, 148} Due to the presence of PrP^C in caveolae-like domains^{149, 150} and its co-localization with caveolin-1 (cav-1)^{138, 151} the involvement of caveolae in PrP^C endocytosis had been suggested earlier. To this end Sarnataro *et al.*¹⁵² could provide evidence that the raft-mediated pathway is not affected by caveolin expression. Still, PrP^C internalization was found to be impacted by cholesterol depletion and activation of the cell division control protein 42 homolog (Cdc-42), a member of the Rho family of GTPases being specifically involved in clathrin-independent endocytosis of GPI APs.¹⁵³ Additionally Sarnataro *et al.*¹⁵² reported that in co-immunoprecipitation studies of clathrin and PrP^C, the latter remained associated with detergent-insoluble microdomains. This fact supports a cooperation between rafts and clathrin in the internalization process. PrP^C susceptibility to various endocytic pathways could also be the basis for its neuroprotective and -degenerative functions.

1.4 Mechanism of PrP^C-PrP^{Sc} conversion

To date, despite considerable knowledge about the characteristics of the infective prion pathogen, its mechanism of replication and the molecular pathways leading to neurodegeneration are largely unknown. There is evidence from *in vitro* and transgenic mouse studies that the conversion to PrP^{Sc} implicates PrP^C-PrP^{Sc} interactions.^{79, 154-158} The rate of PrP^{Sc} formation and disease progression appears to be directly proportional to the level of PrP^C expression, indicated by PrP knockout mice not propagating scrapie infectivity and transgenic mice heterozygous for a disrupted PrP gene requiring prolonged incubation times upon prion inoculation.¹⁵⁹⁻¹⁶¹ In agreement with the

“protein-only hypothesis” these findings have raised two models explaining prion replication (Scheme 4). The template-directed refolding model by Prusiner¹⁶² proposes that a high-energy barrier prevents the spontaneous PrP^{C} - PrP^{Sc} conversion. Upon interaction, monomeric PrP^{Sc} induces PrP^{C} to convert into PrP^{Sc} . However, until now there is no experimental evidence for the existence of a stable PrP^{Sc} monomer.¹⁶³ PrP^{Sc} seeds in this prion propagation process are not considered essential. Alternatively, in the more accredited seeded nucleation model by Jarrett and Lansbury¹⁶⁴ a reversible thermodynamic equilibrium between PrP^{C} and PrP^{Sc} is postulated. In the presence of stable oligomeric PrP^{Sc} aggregates the conversion from PrP^{C} to PrP^{Sc} is favoured, thus making PrP^{Sc} aggregates (seeds) inevitable for prion spread. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei capable of recruiting further PrP^{Sc} . In fact, these soluble oligomers produced during the PrP amyloid aggregation have emerged as the primary neurotoxic species, supporting the seeded nucleation model.¹⁶⁵⁻¹⁶⁷

*Scheme 4. Models for the conversion of PrP^{C} into PrP^{Sc} . The model for template-directed refolding (**top**) and seeded nucleation (**bottom**) are depicted. Scheme was modified from Aguzzi *et al.*²³*

Ultimately, evidence for a direct PrP^{C} - PrP^{Sc} interaction in the conversion to PrP^{Sc} came from *in vitro* systems. Pioneering studies from Caughey and colleagues¹⁶⁸ succeeded within a cell free conversion (CFC) assay in the generation of protease-resistant (res), radioactive PrP^{res} from mixed PrP^{C} substrate and an excess of unlabeled PrP^{Sc} . This

in vitro PrP^{res} propagation recapitulates the species and strain specificity of prion transmission *in vivo*.^{168, 169} Mechanistically, it has identified structural factors underlying the species barrier and optimal conditions for the PrP^{res} formation.^{62, 170, 171} The ability to generate PrP^{res} not only from purified, but also recombinant protein,¹⁷² provides a unique opportunity to study prion propagation. CFC assays can be used as screening experiments as they have the potential to identify compounds directly inhibiting the PrP^C-PrP^{Sc} interaction or its subsequent conversion.^{173, 174} Still, the proportionally large amount of PrP^{Sc} seeds required to drive the CFC assay (PrP^{Sc}:PrP^C = 50:1) has prevented it from generating *de novo* infectivity.¹⁷⁵

A more efficient method for mimicking the autocatalytic replication of PrP^{Sc} was provided by Soto and colleagues³⁸ in affording a larger than 10-fold increase in PrP^{res} with the usage of a 1:100 ratio of PrP^{Sc} to PrP^C. By subjecting scrapie-infected and normal brain homogenate to the so-called protein misfolding cyclic amplification (PMCA) procedure PrP^{res} is amplified in cycles of sonication and incubation. Successive rounds of PMCAs and fragmentation of PrP^{Sc} rise the available amounts of replication-competent species.¹⁷⁶⁻¹⁷⁸ Thus, with automation this assay offers a promising diagnostic tool in pre-symptomatic blood screening,^{179, 180} and eventually it has facilitated the detection of *de novo* infectivity in hamsters.³⁸ However, the levels of infectivity still remain lower than with a similar quantity of brain-derived PrP^{Sc} and the usage of complex brain homogenate itself represents an obstacle in thoroughly elucidating the conversion and association of infectivity with PrP^{res}.¹⁸¹ Besides, the distinct efficiency differences between the CFC and PMCA assays applying purified and crude brain-derived PrP^{Sc} proposed that cellular accessory factors are involved in the generation of PrP^{res}. In fact, polyanionic molecules were identified as factors present in the brain homogenate that contribute to the conversion efficiency.¹⁸² Ongoing development of PMCA assays aiming to detect and early diagnose TSEs has led to the quaking-induced conversion (QuIC) method.¹⁸³ Sonication is replaced by reproducible and easier controllable shaking during the prion amplification process, which enables the application of standardized protocols. This accomplishment is reflected by the multiple variations currently available, such as standard (S-QuIC), real-time (RT-QuIC) and enhanced QuIC (eQuIC).¹⁸⁴⁻¹⁸⁷

Apart from the autocatalytic propagation of PrP^{Sc} another crucial hallmark of the PrP^C-PrP^{Sc} conversion is the *de novo* generation of infectivity. However, when inoculated into animals, rPrP fibrillar assemblies can range from being biologically inert

to fully infectious, pathogenic and transmissible in subsequent passages.^{37, 71, 188-190} Legname and coworkers¹⁹¹ inoculated transgenic mice expressing truncated PrP^C (aa 89-231) with amyloid fibrils formed from rPrP (aa 89-230). The outcome were low infectious titres and the affection of only that single line of transgenic mice Tg9949, probably due to the high expression and truncation of the transgene sequence enhancing the susceptibility to prion infection within the mice.⁷⁰ Hence, according to Supattapone,¹⁹² these highly concentrated samples of rPrP amyloid fibrils are not suitable in mimicking the infectious properties of PrP^{Sc}. In contrast, Wang *et al.*³⁹ succeeded in the formation of infectious *de novo* rPrP amyloid fibrils associated with neurological signs in wild-type mice after ~130 d. Here, PrP^{res} was formed in PMCA assays in the presence of negatively charged lipids, namely 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG). In their earlier work¹⁹³ they had shown that POPG promotes the conversion to PrP^{res} under physiological conditions. In further studies Wang *et al.*¹⁹⁴ could attribute a crucial role in the PrP-lipid interaction to the highly conserved middle region of PrP that induced conformational change.

1.4.1 PrP^C-PrP^{Sc} conversion in cells

The findings regarding PrP^{res} formation in presence of POPG³⁹ support the possibility of the plasma membrane being the cellular localization of PrP^{Sc} formation as a posttranslational event. At this position contact between endogenous PrP^C and exogenous PrP^{Sc} can easily occur.¹⁹⁵⁻¹⁹⁹ This is supported by the finding that by releasing PrP^C from the cell surface or interrupting its transport to the plasma membrane prevents the formation of PrP^{Sc}.²⁰⁰⁻²⁰² More precisely both PrP isoforms were found to be associated with rafts.²⁰³⁻²⁰⁶ These are defined as highly dynamic microdomains wherein specific lipids stabilize larger lipid platforms and compartmentalize cellular processes at the membrane.²⁰⁷ Impairing the integrity of the cholesterol-enriched rafts associated with PrP by lowering the intracellular levels of cholesterol reduced the formation of PrP^{Sc} in infected cells.²⁰⁸ Moreover, PrP^C- and PrP^{Sc}-associated rafts were found to have distinct characteristics, as they can be separated from each other by solubilisation and flotation on density gradients.²⁰³ According to Campana *et al.*¹⁹⁵ this proposes that either the types of raft or the membrane association of each isoform have different characteristics. However, Baron *et al.*²⁰⁴ illustrated that the PrP^{Sc}-PrP^C conversion only takes place in the presence of fused PrP^{Sc}- and PrP^C-containing membranes, suggesting

that the two PrP isoforms need to be inserted into contiguous membranes. Alternatively, rafts were proposed to stabilize PrP in its conformation *via* a direct interaction with cholesterol. Thus, changes in the local lipid environment can mediate PrP conformation.²⁰⁹ Studies on model lipid bilayers regarding the impact of the PrP-lipid interaction on structure and affinity of PrP support the idea that predominantly α -helical PrP^C is stabilized upon binding to raft membranes, whereas binding to negatively charged lipid (non-raft) membranes leads to an increased β -sheet content.²¹⁰ Interestingly, the PrP-raft-association is mediated by the GPI anchor^{208, 211} and the *N*-terminal region of PrP.²¹² Unlike for a typical GPI-anchored protein, for PrP this raft-association occurs already earlier in the secretory pathway and appears to be involved in the maturation and folding process of PrP^C.^{213, 214} Alternatively to the plasma membrane the formation of PrP^{Sc} was suggested to involve additional cellular places. Immediately after PrP internalization the PrP^C-PrP^{Sc} conversion may occur in the endolysosomal compartment,²⁰⁰ in the Golgi apparatus and/or the ER following retrograde transport.^{215, 216} In infected cells, stimulation of retrograde transport towards the ER leads to an increase in PrP^{Sc} formation from PrP^C precursor,²¹⁷ suggesting that the ER may represent an amplification compartment for PrP^{Sc}.²¹⁸ Participation of the endocytic pathway is indicated by PrP^{Sc} accumulation in the late endosomes.^{200, 206, 219, 220} Still, as demonstrated by Goold *et al.*,¹⁹⁶ the plasma membrane is the initial site of prion conversion and consequently of most interest in studying the earliest events in prion infection and PrP misfolding.

1.4.2 Impact of GPI anchor on PrP^C-PrP^{Sc} conversion

Typically, PrP is attached to membranes by its GPI anchor.⁶⁵ A better understanding of the interplay between membranes, GPI-anchored PrP and PrP^C-PrP^{Sc} conversion is provided by work from Baron and Caughey.^{204, 205} First they studied the conditions necessary for PrP^{res} formation of PrP associated with detergent-resistant membranes (DRMs).²⁰⁴ Based on that, in cell-free conversion (CFC) assays Baron and Caughey²⁰⁵ investigated the impact of GPI-anchoring of PrP associated with model membranes on PrP^{res} formation. PrP was isolated by immunoprecipitation from mammalian cell lines expressing GPI-anchored and anchorless PrP, respectively.^{168, 221, 222} GPI-anchored PrP bound to liposomes could not be converted to PrP^{res} upon exposure to exogenous PrP^{res} in microsomes until phosphatidylinositol-specific phospholipase C (PI-PLC) was added

or the combined membrane fractions were treated with a membrane-fusing agent. These findings indicate for the initiation and propagation of PrP^{Sc} that at the membrane surface an insertion of PrP^{Sc} into the host cell membrane is necessary for the conversion. Whereas, if the conversion occurred extracellularly, PrP^C needed to be released from the cell membrane. In contrast, anchorless PrP bound to liposomes was converted to PrP^{res} without any treatments necessary. Hence, contradictory to PrP conversion occurring at the cellular membrane,²⁰⁰⁻²⁰² only the membrane-associated form containing PrP attached to a GPI anchor could resist the conversion induced by exogenous PrP^{res}. Moreover, Chesebro *et al.*⁷⁹ found that anchorless PrP results in infectious amyloid disease but without typical clinical TSE. Scrapie-infection of transgenic mice lacking GPI-anchored PrP^C lead to a formation of amyloid plaques in contrast to non-amyloid deposits, typically observed in wild-type mice. Although neuropathological lesions were induced, clinical manifestations were minimal. Surprisingly, the combined expression of anchorless and wild-type PrP accelerated the onset of clinical disease. This suggests that GPI-anchored PrP may be critically involved in the pathogenesis of prion diseases.²²³ Overall, the findings mentioned above indicate a major contribution of the GPI anchor in the toxicity of the PrP^C-PrP^{Sc} conversion.

1.5 Towards the elucidation of PrP conversion

As mentioned before in section 1.2.1 rPrP is an appropriate surrogate for PrP^C, as determined by spectroscopic measurements, including circular dichroism (CD), that eventually facilitated solving the NMR and crystal structures of PrP.⁴⁰⁻⁴⁷ However, rPrP can be a suitable representative only under certain conditions, including thioflavin T (ThT) fluorescence-based following of the aggregation process. Even though with this method, insights into the characteristics and kinetics of *in vitro* fibril formation have been gained,²²⁴ just recently the molecular basis of PrP replication was established in detail by applying a single-molecule fluorescence methodology to characterize individual aggregates. With total internal reflection fluorescence (TIRF) microscopy, Klenerman and colleagues^{225, 226} studied fibril fragmentation and elongation of individual murine PrP aggregates from seeded aggregation *in vitro*. PK-resistant PrP fibrils elongated until length-dependent fragmentation resulted in PK-sensitive fragments. This method allowed direct observation of heterogenous, transient, metastable oligomers during aggregation, found to be the most infectious PrP particles.²²⁷ Additionally, a

spreading model for aggregate propagation through the brain could be predicted and a framework established to start determining the main factors that control the rate of prion spreading in animals. In 2011, Goold *et al.*¹⁹⁶ analyzed a PrP knockdown (KD) neuroblastoma cell line expressing epitope-tagged PrP^C upon infection with exogenous PrP^{Sc}. After facing the limitation of immunological differentiation between PrP^{Sc} and PrP^C expressed on the recipient cell from cell lines susceptible to prion infection, epitope-tagged PrP^C appeared an elegant solution. However, several previous attempts had failed in generating PrP molecules capable of prion conversion, probably due to the sequence sensitivity in this process, particularly in certain key regions of the PrP molecule.²²⁸⁻²³¹ Eventually, out of eight different constructs Goold *et al.*¹⁹⁶ succeeded with a PrP-224AlaMYC construct, in which the tag is inserted within the C-terminal domain. A detailed analysis of the cells shortly after prion exposure demonstrated that PrP^{Sc} is formed on the plasma membrane. Furthermore, PI-PLC treatment effectively removed PrP^C from the plasma membrane of PrP-224AlaMYC cells and reduced the generation of PrP^{Sc}. However, immunostaining is only feasible on fixed cells and impedes dynamic studies revealing molecular details involved in the PrP conversion and propagation processes. ThT-based detection of pre-formed PrP amyloid fibrils applied in a cellular environment, cannot exclude ThT-binding to other structures unrelated to amyloid, and guarantee binding to all aggregates, as the binding mechanism is not fully understood yet.

To this end, labeling of PrP with an organic fluorophore is required for dynamic studies in cells. Recombinant PrP differs compared to PrP^C in a complete lack of PTMs causing distinct infectivity and membrane interaction characteristics. These properties can be mainly ascribed to the GPI anchor, tethering PrP to the cellular membrane, as demonstrated in various studies.^{79, 204, 205, 223, 232-234} Advances in semisynthetic strategies based on solid-phase peptide synthesis (SPPS),²³⁵ protein engineering, native chemical ligation (NCL)²³⁶ and expressed protein ligation (EPL),^{237, 238} have facilitated access to homogeneous membrane-anchored labeled PrP variants that allow to directly observe the biophysical properties of PrP upon interaction with the cellular membrane.

1.5.1 Semisynthesis strategies for PrP

To date, the majority of studies on the function and structure of PrP have been carried out with recombinant protein lacking all PTMs, including the GPI anchor, or with heterogeneous protein preparations isolated from mammalian cell lines.²³⁹⁻²⁴¹ Still, there have been attempts towards generating defined membrane-anchored PrP. Glockshuber²⁴² and Pinheiro²⁴³ with colleagues applied similar strategies, in which thiol-reactive lipids were attached to the C-terminus of recombinant PrP carrying a cysteine. However, further application of these PrP constructs in cell-based assays may be impeded by potential side reactions with thiol-containing compounds or internal cysteines of PrP, as the lipids were attached *via* disulfide bonds. Different strategies were utilized by Baskakov,²⁴⁴ Baldwin²⁴⁵ and Moroder²⁴⁶ with co-workers: Baskakov and colleagues²⁴⁴ applied maleimide chemistry to introduce a myristoyl chain at the C-terminus of genetically engineered PrP(S230C). This modification did not alter the structure of the protein. Interestingly, an increasing affinity of PrP for the cell membrane and a decreased extent of fibrillation was found. Baldwin²⁴⁵ and co-workers chemically synthesized a 106 residue “mini-prion” (PrP106) with SPPS and NCL connecting PrP (aa 90-141) to PrP (aa 178-231) by a peptide bond. NCL links in a selective reaction an unprotected peptide containing a C-terminal α -thioester to another peptide with an N-terminal cysteine.²³⁶ A membrane anchor made of a lipophilic group introduced at the C-terminus *via* an orthogonally side-chain-protected lysine was installed. Immunofluorescence analysis showed that only myristylated PrP peptides could be targeted to the cell-surface, unlike to acetylated PrP peptides. The group of Moroder²⁴⁶ applied click and ligation chemistry to obtain lipopeptides corresponding to the C-terminal PrP segment (aa 214-231). Confocal images of HeLa cells revealed a direct transfer of fluorescently labeled lipopeptides to the cellular membrane. Thus, lipopeptides can be considered eligible in mimicking the GPI anchor. Several semisynthetic strategies, based on EPL and protein *trans*-splicing (PTS) (Scheme 5), providing access to different posttranslationally modified PrP variants were developed by the Becker group (Scheme 6).^{233, 247-255}

Scheme 5. Mechanism of the EPL reaction (A) and PTS (B).

In the EPL^{237, 238} reaction a protein thioester obtained by recombinant technology is linked to a chemically synthesized peptide²³⁵ containing an *N*-terminal cysteine under formation of a native peptide bond. The mechanism proceeds equally to NCL.²⁵⁶ The reversible thioester rearranges by an irreversible *S*→*N* acyl shift to an amide bond at the ligation site in the presence of an excess of thiols mediating the reaction (Scheme 5A). The recombinant protein α -thioesters can be accessed using engineered inteins. Inteins are self-processing protein segments, which mediate protein splicing.^{237, 257} In the course of this intramolecular process the intein excises itself and joins the *C*- and *N*-terminal flanking protein segments (*C*- and *N*-extein). In more detail, a nucleophilic side chain, namely a hydroxy- or thiol-group for serine and threonine or cysteine residues, accomplishes an *N*→*O* or *N*→*S* acyl shift. Then, in a trans(thio)esterification the *N*-extein gets attached to a conserved *N*-terminal serine or cysteine of the *C*-extein. The instable branched intermediate is resolved *via* an intramolecular rearrangement involving a conserved asparagine residue of the intein producing an intein with a *C*-terminal succinimide, and an *O*→*N* or *S*→*N* acyl shift resulting in ligated exteins with a native bond at the ligation site.^{257, 258} Mutations of the *C*-terminal asparagine of the intein or the *N*-terminal cysteine, threonine or serine residue of the *C*-extein to alanine blocks the splicing process at the formed ester or thioester, which enables the generation

of a protein α -thioester by addition of an excess of a thiol, such as sodium 2-mercaptoethanesulfonate (MESNA), mercaptophenylacetic acid (MPAA) and thiophenol.²⁵⁹ Protein *trans*-splicing (PTS) is a process that relies on the assembly of two divided segments of inteins, so-called split-inteins, to form a functional intein. Upon re-assembly of the split-inteins PTS occurs and links the *N*- and *C*-exteins (Scheme 5B).²⁶⁰

Scheme 6. Semisynthetic strategies for PrP constructs, developed in the Becker laboratory.^{247, 253, 254}

The generation and biophysical characterization of PrP constructs containing a GPI anchor mimic started more than ten years ago in the Becker laboratory with work described in Olschewski *et al.*²⁴⁷ Two strategies based on the EPL approach provided rPrP^{Palm}, an *N*-terminally truncated T_PrP (aa 90-231) modified at the *C*-terminus with

chemically synthesized membrane anchor peptides (Scheme 6). At that time the protease-resistant PrP fragment comprising residues aa 90-231 had been considered as the structure crucially involved in TSEs. The GPI anchor mimics feature two palmitoyl modifications (Palm) that induce a high affinity towards DOPC liposomes and locate PrP in its native conformation to the detergent resistant domains (DRMs) of the cell membranes.²⁶¹ A tobacco etch virus (TEV) protease recognition site (ENLYFQ) facilitates to potentially release PrP from the membrane, a polyethyleneglycol polyamide oligomer (PPO) functions as solubilization tag,²⁶² and a fluorescent dye can be incorporated for tracking of the semisynthetic PrP *in vitro* and *in vivo*.

One strategy relies on the expression of rPrP in fusion with the GyrA intein and affinity tags, namely a His tag and a chitin-binding domain (CBD), and further cleavage with an excess of thiols to generate rPrP with a C-terminal thioester. This PrP-thioester is subjected to GPI anchor-mimicking peptides and reacts with the N-terminal cysteine residue in an EPL reaction to modified PrP (Scheme 6A). The second strategy is based on PTS by expressing rPrP fused to the N-terminal segment of the DnaE split intein (DnaE^N) and synthesis of its C-terminal segment (DnaE^C) linked to the GPI anchor-mimicking peptides.²⁶³ Both DnaE segments spontaneously associate when correctly folded and form a functional intein, which excises itself to give the desired modified PrP (Scheme 6B). Aggregation assays based on PK resistance and ThT binding²⁶⁴ revealed that vesicle-attached rPrP^{Palm} was less prone to convert into rPrP^{res} than rPrP in control experiments. Further, binding to zwitterionic DOPC liposomes indicated a very strong interaction for rPrP^{Palm} in contrast to rPrP. Indirect immunostaining of rPrP^{Palm} on cells was similar to patterns of PrP^C and unlike to rPrP. Together with extraction experiments of the cell membrane, this provided proof that soluble rPrP^{Palm} is attached to the membrane. This work demonstrated a similar membrane insertion for semisynthetic rPrP^{Palm} and PrP^C with a native GPI anchor. Next, Becker *et al.*²⁴⁸ developed a general synthetic strategy for the preparation of homogeneous GPI-anchored proteins, applied on rPrP. Different glycoforms of native PrP GPI anchors have been reported with the exact linkage positions and anomeric configuration of the oligosaccharide branch not defined, and no details about the lipid composition.²⁶⁵ In view of this structural uncertainty, a core GPI pseudopentasaccharide, containing three mannose (Man), a glucoseamine (GlcN) and

an inositol (Ino) glycan connected in an α -Man-(1 $\rightarrow$ 2)- α -Man-(1 $\rightarrow$ 6)- α -Man-(1 $\rightarrow$ 4)- α -GlcN-(1 $\rightarrow$ 6)-*myo*-Ino way, was selected. The incorporation of a cysteine residue on the 2-aminoethyl phosphate moiety of the GPI backbone prior to global deprotection provided a synthetic cysteine-tagged GPI anchor. In a following EPL reaction rPrP, T_PrP (aa 90-231), containing a C-terminal thioester, was linked to the synthetic GPI anchor. Analysis of the secondary structure of PrP attached to a synthetic GPI revealed that the CD curves are indistinguishable from the spectra of rPrP, and comparable to the spectra of PrP^C. Moreover, the CD spectra were found to agree with the spectra of rPrP^{Palm}. Thus, this confirms the successful application of the GPI anchor-mimicking peptides as an alternative to circumvent the elaborate synthesis of an artificial GPI anchor. Though the synthesis of the GPI anchor was succeeded, it remains a challenge to provide sufficient amounts. In contrast to rPrP, GPI-anchored PrP was found to quantitatively bind to DOPC vesicles. This emphasizes the contribution of GPI anchors in the membrane association of PrP.²⁴⁸ Noteworthy, the group of Silva^{266, 267} at MPI (Potsdam, Germany) is working on intein-based semisynthesis to obtain homogeneous GPI-anchored proteins, including PrP, using synthetic GPI anchors. Their strategy is based on rPrP fused to DnaE^N from *Nostoc punctiforme* (*Npu*)^{268, 269} reacting with DnaE^C linked to a synthetic GPI anchor.

The series of denaturation and renaturation steps required to obtain functional rPrP-intein fusion proteins as well as folded rPrP^{Palm} and GPI-anchored PrP severely limits the yield of the intein cleavage and the subsequent EPL reactions. These constructs are typically deposited in inclusion bodies in *E. coli*, probably due the hydrophobic nature of the membrane-anchored PrP and the overproduction that impedes proper folding, including the formation of the structurally important disulfide bridge.^{56, 270} Hence, to improve the semisynthetic access to posttranslationally modified PrP Chu *et al.*²⁵² developed a strategy for soluble expression of PrP-intein constructs in *E. coli*. Ultimately, the overexpression of T_PrP (aa 90-231) N-terminally fused to the ATPase domain of heat shock protein (Hsp) 70 DnaK chaperone succeeded in high quantities of soluble PrP. This approach offers an alternative way to produce posttranslationally modified PrP.

With robust semisynthetic strategies established the critical membrane attachment of PrP was studied by Chu *et al.*²³³ using three PrP variants, including full-length FL_PrP (aa 23-231), central hydrophobic region deleted Δ CR_PrP (aa 23-231 with Δ 105-125) and N-terminally truncated T_PrP (aa 90-231), all equipped with a C-terminal

membrane anchor. Interactions of the lipidated PrP constructs with phospholipid membranes demonstrated binding modes distinct from the non-modified rPrPs and impacts on the biochemical and conformational properties of PrP. Whereas non-modified rPrPs showed a conversion into β -sheet enriched structures upon interaction with anionic POPG vesicles, lipidated Δ CR_PrP and T_PrP retained their α -helical structure and lipidated FL_PrP partially converted into random coil. Evidence indicating pore formation of lipidated Δ CR_PrP was found in fluorescence-based assays and supported by patch clamp electrophysiological measurements of cells. Δ CR_PrP was previously found to be neurotoxic *in vivo*. Yet, expressed in cultured cells it is identically localized as wild-type PrP. Thus, altered binding interactions had been suggested to cause the deleterious signaling pathways.^{271, 272} Based on these results, critical roles for both C-terminal membrane attachment and the N-terminal domain of PrP have been suggested.

PTMs present in PrP comprise not only a C-terminal GPI anchor, but also N-glycosylations of asparagines at position 181 or 197, or at both.²⁷³ Different prion strains and prion-related diseases (TSEs) possess distinct glycosylation patterns of PrP. Studying the influence of these PTMs in prion pathogenesis has not been forthcoming mainly due to the heterogeneity of the sugars.^{58, 274} To that end, Shi *et al.*²⁷⁵ reported a strategy based on linking three segments of mPrP, namely recombinant aa 90-177S, aa 178-212 and aa 213-230, with the latter two obtained *via* SPPS, to enable the introduction of sugar moieties. Shortly after that Araman *et al.*²⁵³ demonstrated a semisynthetic approach to generate PrP variants modified with monodisperse polyethyleneglycol (PEG) units as mimics of N-glycans (Scheme 6C). Expressed rPrP (aa 23-177) with a C-terminal thioester affords in an EPL reaction with PEGylated peptides (aa 179-231), after selective desulfurization of the β -mercapto-aspartate at the ligation site, homogeneous PEGylated full-length PrP constructs. Interestingly, *in vitro* aggregation was abrogated for all PEGylated PrP constructs under conditions at which wild-type PrP aggregated. Furthermore, the addition of PEGylated PrP completely blocked aggregation of wild-type PrP. This has raised the question if large N-glycans interfere with aggregation *in vivo*. Recently, Mishra *et al.*²⁷⁶ introduced lactosyl and mannosyl glycans in huPrP (aa 90-231) at position 181 and 197 *via* mutated cysteine residues. In agreement with our results they found that glycosylated PrPs are less prone to spontaneous fibril nucleation.

In our previous approach (Scheme 6A) the introduced ligation site cysteine at the C-terminus of T_PrP (aa 90-231) was left un-desulfurized, which could potentially be problematic for the folding of PrP, as it involves the Cys178-Cys213 disulfide formation. Demonstrated by Matveenko *et al.*²⁵⁴ the utility of a phenacyl (PAC) protecting group for the protection of the cysteines within the larger recombinant PrP part allowed selective desulfurization of the introduced cysteine following to the EPL (Scheme 6D). Similar CD spectra of PrP containing a cysteine at the ligation site and an alanine proved that the introduced cysteine did not disturb the folding to native PrP.

In summary, with continuous improvement and systematical extension of our semisynthetic approach for homogeneous modified PrP constructs we provided a library of compounds characterized in regard of their biophysical and conformational properties, including their interaction with a lipidic environment. This strategy has the potential to generate homogeneously labeled posttranslationally modified PrP variants suitable for direct observation at the cellular membrane. Thus, it may reveal crucial steps and interactions involved in the PrP conversion.

2. Objectives

Prion diseases, including other neurodegenerative disorders, share as a common feature protein aggregation arising from misfolding of the native forms. Much knowledge of the cellular-to-scrapie prion protein (PrP^C-PrP^{Sc}) conversion process has come from recombinant PrP (rPrP) utilized as a surrogate, *in vitro* experiments, infectivity studies with transgenic mice and immunostaining of PrP^C in cells. Still, these approaches are limited in analyzing directly the earliest events of posttranslationally modified PrP at the cellular membrane, the initial site of prion infection and PrP misfolding. Especially the glycosylphosphatidylinositol (GPI) anchor has been shown to be crucially involved.^{204, 205}

To this end, we provide fluorescently labeled, folded PrP variants attached to peptides mimicking the physicochemical characteristics of the GPI anchor *via* a semisynthesis approach. We assess the conformational properties of the homogeneous PrP constructs using circular dichroism (CD), thioflavin T (ThT) and proteinase K (PK)-based assays. Towards studying the membrane interaction in a complex cellular environment, we proceed systematically with real-time monitoring of the PrP association on artificial lipid bilayers in quartz crystal microbalance-dissipation (QCM-D) experiments, varying pH, lipid and salt compositions.

Further imaging using super-resolution structured illumination microscopy (SR-SIM) combined with co-localization analysis is performed to confirm membrane localization of the Cy5 labeled PrP constructs in cells. In more detail, the dynamic behavior of PrP within the ordered and disordered regions of the plasma membrane as well as the actin cytoskeleton is studied applying fluorescence correlation spectroscopy (FCS). By combining methyl- β -cyclodextrin (m β CD) treatments, a cholesterol depleting agent, with addition of latrunculin A (LatA), an actin polymerization inhibitor, the interaction of PrP in its membrane environment can be understood more precisely.

Scheme 7. Strategy for directly studying PrP at the cellular membrane.

Hence, by taking advantage of site-selectively modified PrP variants and biophysical investigations of their interactions with membranes, including a complex live cell setting, we gain an improved understanding of the synergistic interplay between PrP, its GPI anchor, the cellular membrane and the cytoskeleton. Since these parameters influence the aggregation behavior of PrP, we can draw conclusions about its conversion into PrP^{Sc} on cell membranes.

3. Materials and Methods

3.1 Materials

3.1.1 Molecular biology

If not stated otherwise, chemicals for buffer preparation were purchased from Sigma-Aldrich (Vienna, Austria), Fisher-Scientific (Vienna, Austria), VWR (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Buffers were prepared using Milli-Q® water (dd H₂O). All plasmids were sequenced at Eurofin Genomics (Vienna, Austria) and Microsynth (Vienna, Austria). Chemical-competent cells were purchased from Merck Millipore (Darmstadt, Germany). Centrifugal filtration units used were Amicon Ultra-15 with a molecular weight cut-off (MWCO) of 30 and 10 kDa from Millipore (Darmstadt, Germany) and Vivaspin® 500 with MWCO of 3 kDa from Sartorius (Göttingen, Germany) or of 10 kDa from GE Healthcare (Freiburg, Germany). PD-10 Desalting Columns were purchased from GE Healthcare (Freiburg, Germany). Folding implements *N*-octyl- β -D-glucopyranoside (OG) and Slide-A-Lyzer™ cassettes with MWCO of 10 kDa were obtained from GERBU (Heidelberg, Germany) and Fisher-Scientific (Vienna, Austria). LMW marker was purchased from GE Healthcare (Freiburg, Germany). Mouse monoclonal antibodies for Western blotting were purchased either from Merck Millipore (Darmstadt, Germany), mAb 3F4 (aa 109-112), or from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany), mAb 6D11 (sc-58581; aa 93-109). Anti-mouse IgG-peroxidase ab was from Sigma-Aldrich (Vienna, Austria). As corresponding marker MagicMark™ XP Western Protein Standard from Novex, Life Technologies™, Fisher-Scientific (Vienna, Austria) was used. For transfer nitrocellulose blotting membrane (thickness 0.45 μ m) and for detection ECL plus western blotting kit were purchased from GE Healthcare (Freiburg, Germany). Proteinase K (PK) from *Tritirachium album* was acquired from Sigma-Aldrich (Vienna, Austria).

3.1.2 Chemicals

Cy5 dyes were either purchased from Lumiprobe (Hannover, Germany), Novartis (Nürnberg, Germany) or synthesized. 5(6)-Carboxyfluorescein as isomeric mixture of 61:39 was from Fisher-Scientific (Vienna, Austria). Monolipidated glycosylphosphatidylinositol (GPI) was provided by Roller from the group of Silva at MPI (Potsdam, Germany) based on a strategy they had developed earlier.²⁷⁷

Thioflavin T (ThT) and Phenylmethylsulfonyl fluoride (PMSF) were acquired from Sigma-Aldrich (Vienna, Austria). Benzamide derivatives as potential antiprion agents were provided by Fiorino from University of Naples Federico II (Naples, Italy).²⁷⁸ Lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased in lyophilized powder form from Avanti Polar Lipids (Alabaster, USA). All other chemical reagents and solvents were purchased in the highest available quality from Sigma-Aldrich (Vienna, Austria) or Fisher-Scientific (Vienna, Austria) if not noted otherwise.

3.1.3 Cell culture

SH-SY5Y cells were purchased from ATCC (Manassas, USA). Dulbecco's Modified eagle medium (DMEM) with high glucose content, its supplements fetal bovine serum (FBS), penicillin and streptomycin (PS) were obtained either from HyClone, GE Healthcare Life Sciences (South Logan, USA) and PAA Laboratories (Linz, Austria), or from GIBCO Invitrogen (Karlsruhe, Germany). 1× Phosphate buffered saline (PBS) without calcium and magnesium was purchased from BioWhittaker®, Lonza (Basel, Switzerland). 10× trypsin solution was from Sigma-Aldrich (St. Louis, USA). Hoechst 33258, CellMask™ green and deep red plasma membrane stains together with live cell imaging solution were purchased from Fisher-Scientific (Vienna, Austria). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C₁₈) and 1× Hanks' balanced salt solution (HBSS) were from Life Technologies (Grand Island, USA). Green fluorescent protein-glycosylphosphatidylinositol anchored protein (GFP-GPI AP) was received as a kind gift from Dangerfield, J. from Anovasia Pte Ltd (Singapore).^{279, 280} For electroporation, electrolytic (E-) and resuspension (R-) buffer were from Invitrogen (Carlsbad, USA). LifeAct-GFP transfected SH-SY5Y cells were kindly provided by the Wohland laboratory at NUS (Singapore).²⁸¹ Methyl-β-cyclodextrin (mβCD) and latrunculin A (LatA) were from Sigma-Aldrich (St. Louis, USA). Dyes for calibration Atto655, Atto565 and Atto488 were purchased from Sigma-Aldrich (St. Louis, USA). Microscopic measurements were performed using Nunc™ Lab-Tek™ 8-well chambers (Permanox, #1.0 borosilicate coverglass) from Thermo Scientific (St. Louis, USA), No. 1.0 35 mm glass-bottom dishes from MatTek (Ashland, US) and μ-slide 8-well ibiTreat chambers (#1.5 polymer coverslip,

tissue culture treated) from ibidi (Martinsried, Germany). 96-well TC plates (standard F, black, clear bottom, tissue culture treated, polystyrene) were obtained from Sarstedt (Nümbrecht, Germany). Pro-JectTM protein transfection reagent kit was purchased from Pierce Biotechnology (Rockford, USA). Mounting medium Roti[®]-Mount FluorCare was obtained from Roth (Karlsruhe, Germany).

3.2 General protocols for molecular biology

3.2.1 Protein expression & purification

α -Thioesters of FL_PrP (aa 23-231), Δ CR_PrP (aa 23-231 with Δ 105-125) and T_PrP (aa 90-231) were obtained according to previously established procedures in our group by Olschewski *et al.* and Chu *et al.*^{233, 247, 250} Briefly, pTWIN1 and pTXB3 plasmids containing constructs of PrP fused to *Mxe* GyrA intein, chitin binding domain (CBD), and an additional His-tag for T_PrP, were transformed into *E. coli* chemical-competent cells, Rosetta 2 and BL21(DE3)RIL, respectively. After expression in inclusion bodies fusion constructs were solubilized and purified under denaturing conditions using immobilized metal affinity chromatography (IMAC). MESNA-mediated intein cleavage and concomitant thioester formation proceeded in 4 M urea buffer (pH 8) supplemented with 500 mM sodium 2-mercaptoethanesulfonate (MESNA). Crude reaction mixtures were purified *via* preparative RP-HPLC. Desired fractions were identified *via* ESI-MS operating in positive ion mode, combined and following to lyophilization stored at -80°C. Purity of isolated α -thioesters was assessed *via* analytical RP-HPLC.

3.2.2 Folding of PrP variants

Folding of PrP variants into their native structure was accomplished according to a protocol from Chu *et al.*²³³ Unless otherwise stated, all steps were performed at 4°C. Solutions of denatured PrP variants in 6 M Gdn·HCl, 50 mM Tris-HCl buffer (pH 8) were stepwise diluted to 2.5 M Gdn·HCl, using 20 mM sodium acetate buffer (pH 5), containing a redox system of 0.3 and 3 mM oxidized and reduced glutathione (GSSG, GSH), and additionally 20 mM *N*-octyl- β -D-glucopyranoside (OG) for the lipidated constructs. After the folding reaction PrP variants were dialysed against 20 mM sodium acetate buffer (pH 5), with 20 mM OG, respectively, using Slide-A-LyzerTM cassettes with MWCO of 10 kDa. Precipitates of misfolded proteins were separated *via*

centrifugation at 14,000 g for 15 min. Supernatants containing folded PrP variants were stored at -80°C.

3.2.2.1 *Labeling of folded PrP variants*

In order to establish labeling conditions for folded lipidated T_PrP **6T** using maleimide functionalized sulfo-Cy5 **5c**, first reactions were performed with the corresponding lipidated peptide **1** adapting the manufacture's protocol. Peptide **1** (0.32 μ mol, 1eq), tris(2-carboxyethyl)phosphine (TCEP) (0.1 μ mol, 0.3eq) from a 1 mM stock solution in labeling buffer, sulfo-Cy5 **5c** (0.9 μ mol, 2.9eq) from a 50 mg/mL stock solution in dd H₂O were dissolved in a total volume of 100 μ L 20 mM sodium acetate buffer (pH 7.4) containing 20 mM OG and reacted at rt protected from light under shaking at 300 rpm for 17 h. For purification, the reaction mixture was diluted with acidic 6 M Gdn·HCl buffer (pH 4.7) and subjected to RP-HPLC. Desired fractions were identified *via* ESI-MS operating in positive ion mode, combined and following to lyophilization yielded in 0.3 mg (24%) of sulfo-Cy5 labeled peptide **2c**. Purity of was assessed *via* analytical RP-HPLC. For folded lipidated T_PrP **6T** (7 nmol, 1eq) in 20 mM sodium acetate buffer (pH 5), with 20 mM OG, prior labeling reaction dialysis was performed against 20 mM sodium acetate buffer (pH 7), with 20 mM OG, for 2 h at 4°C using Slide-A-Lyzer™ cassettes with MWCO of 10 kDa. After transfer into Eppendorf® tubes, sulfo-Cy5 **5c** (41 nmol, 6eq) from a 50 mg/mL stock solution in dd H₂O was added to a total volume of 130 μ L. The reaction proceeded at 4°C protected from light under shaking at 200 rpm for 22 h. Excess of sulfo-Cy5 **5c** was removed *via* dialysis against 20 mM sodium acetate buffer (pH 5), with 20 mM OG, at 4°C for 3 d using Slide-A-Lyzer™ cassettes with MWCO of 10 kDa. Purity of sulfo-Cy5 labeled T_PrP **7cr** was assessed *via* SDS-PAGE analysis using fluorescence-based detection.

3.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein analysis was carried out by SDS-PAGE based on a protocol from Laemmli *et al.*²⁸² using 15% acrylamide gels under reducing conditions. Gels were cast in a Mini-PROTEAN® Multi-Casting Chamber or a Tetra Handcast Module from Bio-Rad (Herkules, USA) with reagents as listed in Table 1.

gel	dd H ₂ O (mL)	30% acrylamide (mL)	10% SDS (μL)	10% APS (μL)	TEMED (μL)	buffer (mL)
stacking	3.6	0.9	53	53	5.3	0.7
separating	3.6	7.5	157	157	4.7	3.9

Table 1. Recipe for preparation of 2× SDS-gels. Buffer for stacking (pH 6.8) and separating gel (pH 8.8) consists of 0.4% (w/v) SDS, 0.5 and 1.5 M Tris-HCl, respectively.

Samples were treated in 1:1 (v/v) ratio with SDS-loading buffer (pH 6.8), containing 0.5 M Tris-HCl, 6% (w/v) SDS, 35% (w/v) glycerol, 3.5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue and for aggregated PrP samples additional 4.5 M urea, boiled at 95°C, and loaded onto the stacking gel. Prior to that any salts in higher concentrations had been removed *via* precipitation with 40% (v/v) trichloroacetic acid (TCA). Applied voltage on power source from VWR (Darmstadt, Germany) was set to 250 V for 35 min. Gels were stained with Coomassie Brilliant Blue R-250 or silver.²⁸³ Detection was performed on ChemiDoc™ MP Imaging System from Bio-Rad (Herkules, USA) with Image Lab 5.1 software.

3.2.4 Semi-dry Western blotting

Western blot samples containing PrP were obtained after cell lysis to demonstrate that the non-modified and the labeled PrP constructs stayed intact under the conditions used for the PrP-membrane measurements. The cells were washed twice with cold phosphate buffered saline (PBS), scraped off the plate, pelleted by centrifugation for 1 min at 14,000 rpm in a microcentrifuge, and lysed in a cold buffer of 0.5% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate (DOC) in PBS, as previously described.²⁴⁷ Samples were concentrated with TCA precipitation and further prepared according to the SDS-PAGE protocol in section 3.2.3. Based on a procedure from Towbin *et al.*,²⁸⁴ PrP variants were transferred in a Semi-Dry-Blotter MAXI from Roth (Karlsruhe, Germany) from SDS-gel onto a nitrocellulose membrane at 65 mA for 1 h. After blocking with a skim milk based TBS buffer (pH 7.5), containing 1.5 M NaCl, 500 mM Tris-HCl, 0.1% (v/v) Tween-20, 5% (w/v) skim milk powder, primary antibodies 3F4 (2 mg/mL) and 6D11 (0.1 mg/mL), respectively, in 10,000× and 500× dilutions were applied. For the detection of ΔCR_PrP variants missing aa 109-112 specific for 3F4 mAb only 6D11 as primary antibody can be used. Incubation of a secondary anti-mouse IgG-peroxidase antibody (10 mg/mL) in 50,000× dilution enabled detection with luminol-containing

solution A and peroxide-based solution B in a ratio of 1:1 on ChemiDocTM MP Imaging System from Bio-Rad (Herkules, USA) with Image Lab 5.1 software.

3.3 Chemical Methods

3.3.1 Solid phase peptide synthesis (SPPS)

SPPS was performed applying Fmoc-strategy on a pre-loaded Fmoc-A-Wang resin, in scales of 0.03-0.25 mmol to afford peptides based on the sequence H – CKGENLYFQSKAAKK-PPO₃-A – OH, according to a protocol from Olschewski *et al.*²⁴⁷ Coupled amino acids carried orthogonal side-chain protecting groups as follows: Lys(Boc), Lys(Mtt) and Lys(ivDde), respectively, Ala, Ser(tBu), Gln(Trt), Phe, Tyr(tBu), Leu, Asn(Trt), Glu(OtBu), Gly and Boc protected Cys(Trt). Synthesis was accomplished either manually in syringes and glass frits (pore size 2), or automatically using CEM Liberty BlueTM, Automated Microwave Peptide Synthesizer (CEM, Matthews, USA). For deprotection of Fmoc, a solution of 20% (v/v) piperidine in *N,N*-dimethylformamide (DMF) was used in cycles of 3 and 7 min. All amino acids were coupled for 30 min in amounts of 2.5eq and 4.8eq, respectively, either using 2.38eq 0.5 M (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in DMF and 5eq *N,N*-diisopropylethylamine (DIEA), or 4.5eq 0.5 M *N,N'*-diisopropylcarbodiimide (DIC) and 5eq 1 M Oxyma in DMF. To enhance solubility, polyethyleneglycol polyamide oligomer (PPO) as trimer was introduced, using first 10eq succinic anhydride in 5eq 0.5 M 1-hydroxybenzotriazole (HOBt) in DMF and 6eq DIEA, second 20eq 0.5 M 1,1'-carbonyldiimidazole (CDI) in DMF, and last 12eq of each, 0.5 M HOBt and 4,7,10-trioxatridecane-1,13-diamine. All steps were conducted for 30 min. In case of lipidation, ϵ -amino groups of lysine 11 and 14 were palmitoylated. After selective removal of orthogonal protective groups methyltrityl (Mtt) and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) with 1% (v/v) trifluoroacetic acid (TFA), 1% (v/v) triisopropylsilane (TIS) in dichloromethane (DCM) and 10% (v/v) hydrazine in DMF, respectively, palmitoylation was carried out with 20eq palmitoyl chloride, 20eq HOBt and 22eq triethylamine in DCM:DMF (3:1) solution for 12 h. Coupling of the dyes fluorescein and cyanine 5 (Cy5), respectively, to ϵ -amino group of lysine 2 was performed either with *in-situ* activation or by using them in their more reactive forms as succinimide esters.²⁸⁵ 5(6)-Carboxyfluorescein was attached using 10eq, and activated in 1:1:1 ratio with DIC

and 0.5 M HOBt in DMF. After incubation of 1 h, a solution of 20% (v/v) piperidine in DMF was applied to avoid side products resulting from multiple attachments of fluorescein.²⁸⁶ For Cy5 coupling over 1 h *in-situ* activation was applied using 1.5eq with 1.44eq 0.5 M HBTU in DMF and 3eq DIEA. However, for the acid-ester conversion Cy5 **4** (1eq) and **4a** (1eq), respectively, with *N*-hydroxysuccinimide (1.1eq) and *N,N'*-dicyclohexylcarbodiimide (1.1eq), were dissolved in anhydrous *N,N*-dimethylformamide under argon protected from light at 0°C. The reaction proceeded under continuous stirring over 12 h with steady increase of temperature to 25°C. *N,N*-dimethylformamide was removed *in vacuo*. The crude succinimide esters **5** and **5a** without further purification were coupled to the peptides for 12 h (Scheme 8).

Scheme 8. Synthesis of Cy5 succinimide esters 5, 5a and 5b. Reaction conditions: i = N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, N,N-dimethylformamide.

At the end of synthesis, peptides were cleaved off from resin through incubation with a solution of 92.5% (v/v) TFA, 5% (v/v) TIS, 2.5% (v/v) dd H₂O for 2.5 h. Crude peptides were obtained through precipitation with cooled diethyl ether, subsequent dissolving in acetonitrile:dd H₂O (1:1), 0.1% (v/v) TFA and lyophilization. For purification, semi- and preparative RP-HPLC was conducted. Crude peptides were dissolved in 6 M Gdn·HCl buffer (pH 4.7). Desired fractions were identified *via* ESI-MS operating in positive ion mode, combined and following to lyophilization stored at -20°C. Purity of isolated peptides was assessed *via* analytical RP-HPLC.

3.3.1.1 *Organic synthesis of fluorescent dye cyanine 5 (Cy5)*

Synthesis of the dye cyanine 5 **4** was performed in three steps, according to protocols from Yamane *et al.*, Zhang *et al.* and Korbel *et al.* Starting from 2,3,3-trimethyl-3*H*-indolenine **1** two indolium compounds, namely 1,2,3,3-tetramethyl-3*H*-indolium iodide **2**²⁸⁷ and 1-(4-carboxybutyl)-2,3,3-trimethyl-3*H*-indol-1-ium bromide **3**²⁸⁸, were generated and linked *via* the polymethine chain, providing 1-[4''-(1''-Carboxybutyl)]-1',3,3,3',3'-pentamethyl-indodicarbocyanine iodide, Cy5 **4** (Scheme 17 in section 4.1.2.2).²⁸⁹

Synthesis of 1,2,3,3-tetramethyl-3H-indolium iodide 2. 2,3,3-trimethyl-3*H*-indolenine **1** (3.2 mL, 20 mmol, 1eq) and iodomethane (1.5 mL, 20 mmol, 1eq) dissolved in 6 mL anhydrous dichloromethane (dried over molecular sieve: 4 Å) were heated to reflux for 4 h, under argon protected from light. The solvent was removed by evaporation, the residue filtered and washed with acetone. Drying *in vacuo* afforded 3.61 g (60%) of **2** as pale rose powder. ¹H-NMR (400 MHz, DMSO-*d*₆): 7.94–7.87 (m, 1H), 7.85–7.80 (m, 1H), 7.66–7.59 (m, 2H), 3.97 (d, *J* = 1.0, 3H), 2.76 (d, *J* = 1.0, 3H), 1.52 (s, 6H) ppm. HRMS (ESI): calc. for C₁₂H₁₆N⁺ 174.1277, found 174.1275.

Synthesis of 1-(4-carboxybutyl)-2,3,3-trimethyl-3H-indol-1-ium bromide 3. 2,3,3-trimethyl-3*H*-indolenine **1** (2 mL, 12 mmol, 1eq), 5-bromovaleric acid (2.5 g, 14 mmol, 1.2eq) and potassium iodide (0.21 g, 1.3 mmol, 0.1eq) in 120 mL acetonitrile were heated to reflux for 21 h. The solvent was removed by evaporation, the resulting suspension filtered, washed with diethyl ether and acetone. Drying under vacuum afforded 1.96 g (50%) of **3** as pale rose powder. ¹H-NMR (400 MHz, DMSO-*d*₆): 12.11 (br s, 1H), 8.00–7.94 (m, 1H), 7.87–7.82 (m, 1H), 7.66–7.60 (m, 2H), 4.47 (t, *J* = 5.2, 2H), 2.83 (s, 3H), 2.31 (t, *J* = 4.8, 2H), 1.90–1.81 (m, 2H), 1.70–1.61 (m, 2H), 1.53 (s, 6H) ppm. HRMS (ESI): calc. for C₁₆H₂₂NO₂⁺ 260.1645, found 260.1645.

Synthesis of 1-[4''-(1''-Carboxybutyl)]-1',3,3,3',3'-pentamethyl-indodicarbocyanine iodide, Cy5 4. 1-(4-carboxybutyl)-2,3,3-trimethyl-3*H*-indol-1-ium bromide **3** (0.60 g, 2 mmol, 1eq) and potassium acetate (0.41 g, 4.2 mmol, 2.1eq) were dissolved in 50 mL acetic anhydride under argon and heated to 110°C. Malonaldehyde bis(phenylimine) monohydrochloride (1.04 g, 4 mmol, 2eq) was added in portions over 30 min. The reaction was allowed to proceed for 1 h, then cooled to rt.

After addition of hexane, the mixture was concentrated under reduced pressure. To the crude intermediate **2** (1.56 g, 4.4 mmol, 2.2eq), potassium acetate (0.79 g, 8 mmol, 4eq), potassium iodide (6.64 g, 40 mmol, 20eq) and 50 mL anhydrous 1-butanol were added. The reaction mixture was heated to 95°C for 1 h under argon, then concentrated under reduced pressure. The residue was dissolved in dichloromethane. Subsequently, the organic phase was washed with H₂O, dried over anhydrous sodium sulfate, filtered and concentrated to a blue solid. The crude product was purified by flash chromatography, using dichloromethane:methanol (92:8) as eluant. Appropriate fractions were combined, the solvent removed and the product dried *in vacuo*, yielding in 71.0 mg (6%) of a blue solid with metallic red luster. ¹H-NMR (400 MHz, DMSO-*d*₆): 12.07 (br s, 1H), 8.33 (t, *J* = 8.6, 2H), 7.62 (d, *J* = 4.8, 2H), 7.45–7.36 (m, 4H), 7.29–7.22 (m, 2H), 6.55 (t, *J* = 8.2, 1H), 6.31 (d, *J* = 9.2, 1H), 6.27 (d, *J* = 9.2, 1H), 4.11 (t, *J* = 4.8, 2H), 3.6 (s, 3H), 2.28 (t, *J* = 4.8, 2H), 1.75–1.57 (m, 16H) ppm. MS (ESI): calc. for C₃₁H₃₇N₂O₂⁺ 469.29, found 469.20. Analytical HPLC *t*_R 31.0 min. TLC *R*_f 0.72 (CH₂Cl₂/CH₃OH 4:1 v/v).

3.3.2 Expressed protein ligation (EPL)

Ligations between PrP α-thioesters and either lipidated peptides or monolipidated GPI anchor proceeded at concentrations of 1-3 mM under argon at 37°C and shaking at 600 rpm. In the reactions yielding the lipidated PrP variants (**6-8_{FL}**, **6-8_{ΔCR}**, **6-8_T**) a 2.5-fold excess of the peptides was subjected to 1 mM of PrP α-thioester. For the generation of GPI anchor modified PrP **9_{FL}** both ligation partners were used in 3 mM concentrations. In general, the two components reacted in degassed buffers (pH 7.2) consisting of 6 M Gdn·HCl, 0.2 M Na₂HPO₄, with 20 mM TCEP and 30 mM 4-mercaptophenylacetic acid (MPAA) for 6 h, or as described previously²³³ with 2% (v/v) thiophenol for 24 h and quenching with 15% (v/v) β-mercaptoethanol. Progress was monitored by analytical RP-HPLC. The reaction was complete as judged by time-dependent HPLC traces revealing consistent integrated peak areas of the ligation products and LC/MS spectra exposing complete hydrolysis of the thioesters. Subsequently, the reaction mixture was diluted with acidic 6 M Gdn·HCl buffer (pH 4.7) and subjected to RP-HPLC. Desired fractions were identified *via* ESI-MS operating in positive ion mode, combined and following to lyophilization stored at -80°C. Purity of isolated lipidated PrP variants was assessed *via* analytical RP-HPLC.

3.3.3 HPLC and mass spectrometry

RP-HPLC was conducted on a Waters Auto Purification HPLC/MS system (3100 Mass Detector, 2545 Binary Gradient Module, 2767 Sample Manager, 2489 UV/Visible Detector) and a Varian ProStar HPLC system, respectively. On a semi- and preparative scale separation was achieved using Kromasil (300-10-C4, 250 × 21.2 mm and 250 × 10 mm, 10 µm particle size) and Grace Vydac C4 columns (250 × 22 mm and 250 × 10 mm, 5 µm particle size) running linear gradients of 30 to 60% for PrP α -thioesters, 30 to 90% for peptides and lipidated PrP variants of buffer B (acetonitrile + 0.05% TFA) in buffer A (dd H₂O + 0.05% TFA) over 60 min. Analytical LC/MS was performed using a Kromasil C4 column (300-5-C4, 50 × 4.6 mm, 5 µm particle size) at a flow rate of 1 mL/min running linear gradients of 5 to 65% or 5 to 95% over 10 min with buffer compositions and ascribed compounds as mentioned above. Analytical RP-HPLC was conducted on a Dionex Ultimate 3000 instrument using a Kromasil C4 column (300-5-C4, 150 × 4.6 mm, 5 µm particle size) at a flow rate of 1 mL/min running linear gradients of 5 to 65% for PrP α -thioesters and GPI-anchored PrP, 5 to 95% for peptides and lipidated PrP variants of buffer B (acetonitrile + 0.08% TFA) in buffer A (dd H₂O + 0.1% TFA) over 30 and 40 min, respectively. As required, HPLC traces were monitored at absorptions of 214 and 280 nm, at emissions of 650 nm and 517 nm, and with charged aerosol detector (CAD). The injection peak present in 0-5 min of the HPLC runs was cut-off in the depicted chromatograms. HPLC traces of the ligation time courses show only time periods comprising the peaks used for integration. Mass spectra from Waters Auto Purification HPLC/MS system were acquired by electrospray ionisation (ESI) operating in positive ion mode. Deconvolution was accomplished using the softwares MassLynx and MagTran. All raw data were exported and processed using OriginPro. ESI-MS with high resolution (HR) was conducted on a maXis classic ESI-Qq/oa-TOF MS instrument from Bruker Daltonik GmbH (Bremen, Germany) operating with -4500 V in positive and -3000 V in negative mode using acetonitrile:methanol:dd H₂O (v:v:v) 50:50:1 as solvent. HR for combined nanoHPLC/MS was performed on a LTQ Orbitrap Velos instrument from Fisher-Scientific (Vienna, Austria) and a Dionex Ultimate 3000 RSLCnano system using an AccucoreTM C4 column (150-C4, 500 × 0.075 mm, 2.6 µm particle size) at a flow rate of 0.3 µL/min running a linear gradient of 10 to 60% of buffer B (acetonitrile:dd H₂O:TFA (v:v:v) 80:20:0.1) in buffer A (acetonitrile:dd H₂O:TFA (v:v:v) 2:98:0.1) over 10 min. Matrix-assisted laser

desorption ionization (MALDI) spectra were acquired on a MALDI-TOF instrument from Bruker Daltonik GmbH (Bremen, Germany) in linear negative mode using a 3% 2,5-dihydroxybenzoic acid (DHB) matrix applying 39% laser power and 20×2155 V detector gain over a range of 700 to 2000 Da.

3.4 Biochemical & -physical Methods

3.4.1 Nuclear magnetic resonance (NMR) spectroscopy

Proton (^1H) NMR spectra were recorded at 25°C on a Bruker AVIII 400 spectrometer operating at 400 MHz. ^1H -NMR data are recorded as follows: chemical shift (δ) (multiplicity, coupling constant(s) J (Hz), relative integral) where multiplicity is defined as: (br) s = (broad) singlet; d = doublet; t = triplet; m = multiplet or combinations of the above. The residual protio-form of $(\text{CD}_3)_2\text{SO}$ (δ 2.50) was used as reference for ^1H -NMR spectra.

3.4.2 Circular dichroism (CD)

CD spectra were recorded on a Chirascan Plus CD-spectrophotometer from Applied Photophysics (Surrey, United Kingdom) using a micro cuvette with 1 mm path length from Hellma Analytics (Müllheim, Germany). Each spectrum was acquired at 25°C from 190 to 260 nm in 1 nm steps. In total, 10 measurements were averaged, and the background was subtracted. The raw data was exported from Pro-Data software and further processed using OriginPro. Spectra were analyzed regarding their secondary structure by the curve-fitting software CDNN from Böhm *et al.*²⁹⁰ For each measurement, a final protein concentration of 4-10 μM was used that previously had been determined by NanoDrop 2000c from Fisher-Scientific (Vienna, Austria). For folded recombinant, lipidated and labeled PrP variants, respectively, calculated molar extinction coefficients at 280 nm obtained from Expasy-ProtParam (<https://web.expasy.org/protparam>) were used in combination with MW values as follows: recombinant FL_PrP **5_{FL}** and $\Delta\text{CR_PrP}$ **5_{ACR}** 63,495 $\text{M}^{-1}\text{cm}^{-1}$, 23,200.6 and 21,306.4 Da; recombinant **5_T**, 27,515 $\text{M}^{-1}\text{cm}^{-1}$, 16,336.2 Da; FL_PrP **6_{FL}**, 64,985 $\text{M}^{-1}\text{cm}^{-1}$, 26,352.6 Da, Cy5 labeled **7_{FL}** 26,803.9 Da; $\Delta\text{CR_PrP}$ **6_{ACR}**, 64,985 $\text{M}^{-1}\text{cm}^{-1}$, 24,458.4 Da, Cy5 labeled **7_{ACR}** 24,909.7 Da; T_PrP **6_T** and **6_{Ala-T}**, 29,005 $\text{M}^{-1}\text{cm}^{-1}$, 19,488.2 and 19,456.2 Da, Cy5 labeled **7_T** 19,846.5 Da; fluorescein labeled FL_PrP **8_{FL}** and $\Delta\text{CR_PrP}$ **8_{ACR}** 76,441 $\text{M}^{-1}\text{cm}^{-1}$ (at pH 5), 26,710.9 and

24,816.7 Da; fluorescein labeled T_PrP **8_T**, 40,460.6 M⁻¹cm⁻¹ (at pH 5), 19,846.5 Da. For the estimation of the molar extinction coefficients of the fluorescein labeled variants **8_{FL}**, **8_{ΔCR}**, **8_T**, 11,456 M⁻¹cm⁻¹ determined at 280 nm for 5(6)-carboxyfluorescein in 20 mM sodium acetate, 20 mM OG buffer (pH 5) was taken into account as described by Gill *et al.*²⁹¹

3.4.3 Fluorescence spectroscopy

Fluorescence measurements were recorded on a FluoroMax-4 spectrofluorometer from Horiba Scientific (Tokyo, Japan) at 25 and 37°C, respectively, using a micro cuvette with 10 mm path length from Hellma Analytics (Müllheim, Germany). For following PrP aggregation, thioflavin T (ThT) was excited at 445 nm and emission was recorded at 480 nm. Data was collected with a slit width of 5 nm in three repetitions. The emission spectrum of fluorescein labeled ΔCR_PrP **8_{ΔCR}** was recorded from 500 to 550 nm with a slit width of 5 nm using an excitation wavelength of 490 nm. Data was collected in increments of 1 nm. The raw data was exported from FluorEssence software and processed using OriginPro.

3.4.4 *In vitro* aggregation assays & proteinase K (PK) digestion

Aggregation assays based on the fluorescence properties of thioflavin T (ThT) and proteinase K (PK) digestions were performed according to modified protocols from Breydo *et al.*²⁹² For the conversion folded PrP variants and buffer components were used in final concentrations of 0.5-20 μM PrP, 2 M Gdn·HCl, 50 mM Tris-HCl with 10 μM ThT at pH 6. Also, benzamide derivatives **6*h,i,m,o*** and **7*f,n,o*** (Scheme 9) dissolved in dimethyl sulfoxide (DMSO) were added in final concentrations of 10, 100, 500 μM in order to assess their potential antiprion activity.²⁷⁸ Overall, two different conversion setups were applied: *In-situ* addition of ThT to the aggregation mixture allowed a semiautomated following of the PrP conversion, whereas in the manual setup ThT was added *ex-situ* at each time point. The semiautomated conversion was conducted on a microplate reader from BioTek Synergy™ Mx (Vermont, USA) using 96-well plates from Greiner Bio-One (Frickenhausen, Germany) with continuous shaking at 37°C. Samples were measured in triplicates, per well supplemented with a glass bead (Ø of 2 mm) from Merck (Darmstadt, Germany) and covered with a plate sealer foil from VWR (Radnor, USA). Emission was recorded at 485 nm using 444 nm

as excitation wavelength, with band widths of 9 nm for both. Data was collected in intervals of 10 min with 10 measurements per data point at a read height of 8 mm. For the manual setup Eppendorf® tubes containing the PrP aggregation mixtures were incubated at 37°C with continuous shaking at 600 rpm. For monitoring the fluorescence intensity aliquots were withdrawn, diluted to 0.4 µM PrP using 20 mM sodium acetate buffer (pH 5) and ThT was added to a final concentration of 10 µM. Fluorescence was recorded as described in section 3.4.3. As negative control bovine serum albumin (BSA) was treated under the same conditions. Besides, resistance towards proteinase K (PK) digestion was evaluated for PrP in its native and aggregated form. Therefor PrP was incubated with PK in a ratio of 50:1 in 50 mM Tris-HCl buffer (pH 7.5) for 1 h at 37°C. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (PMSF) in acetonitrile to a final concentration of 5 mM. Samples were further analyzed with SDS-PAGE, subsequent Coomassie Brilliant Blue R-250 or silver staining and semi-dry Western blotting, respectively.

Scheme 9. Benzamide derivatives **6h,i,m,o** and **7f,n,o** evaluated for their antiprion activity.

3.4.5 Quartz crystal microbalance with dissipation (QCM-D) monitoring
Lipid deposition and PrP adsorption processes were monitored in real time utilizing a Q-Sense E4 instrument from Q-sense AB (Gothenburg, Sweden). Changes in resonance frequency and energy dissipation of a silicon oxide-coated quartz crystal sensor

substrate (model no. QSX303, 5 MHz) were recorded at the 3rd, 5th, 7th, 9th, and 11th overtones. Unless otherwise stated data from the 5th overtone is shown and was used for analysis. Prior experiments all sensor surfaces were treated with 2% SDS, dd H₂O, ethanol, dried with nitrogen and subjected to an oxygen plasma cleaner from Harrick Plasma (Ithaca, USA) at the maximum radiofrequency (RF) power. QCM-D measurements were conducted at 25°C under continuous flow at rates of 100 µL/min for all steps of the bilayer formation process and 100 or 50 µL/min for PrP adsorption as controlled by a Reglo Digital peristaltic pump from Ismatec (Glattbrugg, Switzerland). A stable lipid bilayer was formed according to an optimized protocol developed earlier by Cho *et al.*²⁹³⁻²⁹⁵ using solvent-assisted lipid bilayer (SALB) technique. In brief, the baseline of 10 mM Tris-HCl buffer containing 150 mM NaCl (pH 7.4) was acquired and an isopropanol exchange conducted. After that, lipids dissolved in organic solvents, DOPC in isopropanol and POPG in ethanol, respectively, mixed in molar ratios of 50/50 and 70/30 to a final concentration of 0.5 mg/mL were deposited on the substrate (Scheme 10).

Scheme 10. Structures of lipids used for bilayer formation, DOPC (upper) and POPG (lower), respectively. Net charges correspond to the state of neutral pH.

Re-exchange to 10 mM Tris-HCl buffer containing 150 mM NaCl (pH 7.5) induced bilayer formation. Typical values indicating the presence of successfully formed and stable bilayers showed frequency and dissipation shifts of -25.6 ± 0.55 Hz and $0.4 \pm 0.27 \times 10^{-6}$, respectively, corresponding to a hydrated mass of 450 ng/cm² and a thickness of 4.5 nm, as shown previously by Tabaei *et al.*²⁹³ To verify completeness and homogeneity 0.1 mg/mL BSA dissolved in 10 mM Tris-HCl buffer containing 150 mM NaCl (pH 7.5) was added. Frequency shifts of less than or only -1 ± 0.5 Hz imply that the formed bilayers are free from defects. Only SALB-formed layers fulfilling all

criteria mentioned above were further applied for PrP adsorption. After equilibration with 10 mM Tris-HCl buffer containing 150 mM NaCl, 2 mM sodium acetate (pH 7.4) and 20 mM sodium acetate buffer (pH 5), both supplemented with 2 mM OG for lipidated PrP variants, proteins diluted to final concentrations of 0.1, 0.5, 1 μ M into appropriate buffers mentioned before were adsorbed onto the lipid bilayer. Data was collected and analyzed using QtoolsTM software. Mass and thickness values were afforded applying Sauerbrey²⁹⁶ and Voigt-Voinova²⁹⁷ model, respectively. In compliance with the first one, characterizing the properties of the bilayer subjected to PrP was performed based on Equation 1, 2 and 3. Noteworthy the Sauerbrey relation describing a linear relationship between frequency shifts and mass changes is restricted in its application to thin and rigid films assuming the occurrence of a homogeneous layer with a spatially uniform density.

Equation 1. Sauerbrey equation with Δm as adsorbate mass, C_{QCM} as proportionality constant of 17.7 ng/cm² at 5 MHz, Δf as frequency shift and n as overtone number of the resonance frequency.

$$\Delta m = -C_{QCM} \frac{\Delta f_n}{n}$$

Equation 2. Definition of Sauerbrey thickness d_{eff} with 1,100 kgm⁻³ for the density ρ_{eff} .

$$d_{eff} = \frac{\Delta m}{\rho_{eff}}$$

Equation 3. Definition of Dissipation D. Dissipation occurs once the driving voltage to the crystal is shut off, with E_{lost} and E_{stored} being the lost energy during one oscillation cycle and total energy stored in the oscillator, respectively.

$$D = \frac{E_{lost}}{2\pi E_{stored}}$$

However, theoretically as soon as dissipation monitoring shows any viscoelastic behavior, meaning values larger than zero, the mass cannot be perfectly coupled to the oscillary motion, hence results in underestimated values. Therefore, further evaluation of the obtained results was accomplished using the viscoelastic Voigt-Voinova²⁹⁸ model. This method considers several harmonics. Based on that information it provides additional information about viscosity, elasticity and Newtonian fluid properties.

3.4.6 Microscopy in cells

3.4.6.1 *Cell culture, staining & treatment*

SH-SY5Y and stably transfected LifeAct-GFP SH-SY5Y cells were cultivated in DMEM medium supplemented with 10% FBS and 1% PS at 37°C in 5% (v/v) CO₂ humidified environment. For DiI-C₁₈ membrane staining of live cells, a stock solution in ethanol was prepared and its concentration determined using NanoDrop from Thermo-Scientific (Waltham, USA) assuming 144,000 M⁻¹cm⁻¹ as molar extinction coefficient.²⁹⁹ Dilutions with final concentrations of 20 nM and 75 nM in 1× HBSS for confocal and TIRF microscopy measurements, respectively, were added to the cells for 20 min at 37°C in 5% (v/v) CO₂ humidified environment. For the staining of cell nuclei and membrane using Hoechst 33258, CellMaskTM green and deep red, respectively, cells were incubated with dilutions in live cell imaging solution of 1:500 and 1:1000 for 30 min at 37°C in 5% (v/v) CO₂ humidified environment. Transfection of GFP-GPI AP by electroporation into live SH-SY5Y cells was accomplished on a NeonTM Transfection System from Life Technologies (Grand Island, USA). ~90% confluent cells grown in a 25 cm² culture flask were washed with 1× PBS and collected after trypsinization with 1× Trypsin solution *via* centrifugation for 3 min at 3,000 rpm using a 5810 centrifuge from Eppendorf®. Pelleted cells were re-suspended in 12.5 µL of R-buffer, mixed with ~1 µg of plasmid per 8× well chamber, drawn into a 10 µL Neon transfection tip and electroporated at 1,200 V for 20 ms with 3 pulses in a Neon transfection tube containing 3 mL of E-buffer. After transfection, cells were plated and grown in culture medium at 37°C in 5% (v/v) CO₂ humidified environment for 48 h. SH-SY5Y cells were washed twice with 1× HBSS before mounting them into the microscope chamber containing 1× HBSS as imaging medium. For drug treatments to inhibit actin polymerization and deplete cholesterol, respectively, the imaging medium was replaced with 3 µM LatA diluted from a 100 µM stock solution in DMSO and 3 mM mβCD, both dissolved in HBSS. If not otherwise stated folded PrP variants in sodium acetate buffer (pH 5), containing OG as required, were added to the cells 10× diluted in HBSS to a final concentration of 200 nM. For encapsulation of PrP by Pro-JectTM, PrP was treated according to the manufacture's protocol. For non-live measurements, cells were post fixed with formaldehyde (3.7% in PBS, 20 min) and mounted adding Roti®-Mount FluorCare.

3.4.6.2 Automated imaging

At first, the interaction between Cy5 labeled PrP variants and SH-SY5Y live cells was studied using Cytation™ 3 widefield cell imaging multi-mode reader from BioTek™ (Winooski, USA). Time-resolved fluorescence and brightfield images were captured with a charge-coupled device (CCD) digital camera using a filter-based system containing filter cubes for DAPI (360/40, 460/40, 400), GFP (485/20, 528/20, 510) and Cy5 (628/40, 685/40, 660) channel in combination with the corresponding light-emitting diode (LED) light sources at 365, 465 and 623 nm, respectively. Measurements were accomplished as triplicates in 96-well TC-plates with a Plan Fluorite objective (20×, NA 0.45) from Olympus (Tokyo, Japan) at 37°C in 5% (v/v) CO₂ humidified environment. Image tiles with 291 × 394 μm (height × width) of 1×5 in a montage were autofocused, exposed to the same settings of LED intensity, integration time and gain with 10, 100, 8.8 for brightfield, 10, 100, 5.9 for DAPI, 10, 337, 15.6 for GFP and 10, 100, 12.6 for Cy5 channel. Data processing was performed with Gen5 software from BioTek™ (Winooski, USA). For stitched images, reduced to 80%, threshold intensities and object areas were set to 2,000 and 5-50 μm for DAPI channel excluding edge objects and to 400 and 5-300 μm for GFP channel including edge objects. Within the mask built by CellMask™ green staining, cells were count based on Hoechst 33258 staining and PrP was characterized *via* its object sum integral that takes the total intensity change together with the area into account. Post-acquisition images were adjusted regarding their brightness and contrast, and background subtracted applying a rolling ball algorithm of 50 pixels under usage of the open source software ImageJ.^{300, 301}

3.4.6.3 Structured illumination microscopy (SIM) imaging

Widefield and super-resolution imaging using structured illumination microscopy (SIM) technique was conducted on a Zeiss 710 confocal laser scanning microscope (CLSM) with an Elyra PS.1 system (Oberkochen, Germany) at 37°C. More information on the SIM technique is provided in section 3.4.6.3.1. The 488 nm (for CellMask™ green excitation) or the 642 nm (for Cy5 excitation) HR-diode laser beam was focused on the sample by an oil immersion objective (Plan-Apochromat DIC M27, 63×, NA1.4). Fluorescence signal from the sample was spectrally filtered *via* an emission filter, namely BP495-575/LP750 for CellMask™ green or LP655 for Cy5 emission and

recorded by an electron multiplying charge-coupled device (EMCCD) camera Andor iXon 897. Images with a field of view of 1280×1280 pixels and pixel sizes of $64 \times 64 \times 110$ nm were acquired in 5 phases and rotations, using grating periods of 28 and 34 μm for CellMaskTM green and Cy5, respectively. Post-measurement processing was accomplished using Zen 2012 SP3 (black) software from Zeiss (Oberkochen, Germany) with the theoretical point spread function (PSF) set to a value of 2, a baseline cut, the SR frequency weighting set to 1, the noise filter (NF) to 4 (for Cy5) and 3 (for CellMaskTM green), and sectioning at 83/83/100. Eventually widefield and structured illumination images with a field of view of 2430×2430 pixels and pixel sizes of $32 \times 32 \times 110$ nm were obtained. For final image representation, widefield and SIM images were displayed either as a maximum intensity projection subjected to background subtraction applying a rolling ball algorithm of 50 pixels or using 3D shadow rendering, with both adjusted regarding their brightness and contrast. For quantitative co-localization analysis (section 3.4.6.3.2) regions of interest (ROIs) with 753×313 pixels were cut out.

3.4.6.3.1 Theory of SIM

Next to stimulated emission depletion (STED),³⁰² photoactivated localization microscopy (PALM)³⁰³ and stochastic optical reconstruction microscopy (STORM),³⁰⁴ SIM^{305, 306} is another super-resolution method that has been developed recently. All these techniques achieve resolutions beyond the diffraction limit of conventional microscopy. Resolution is defined as the minimum distance required to resolve two point objects upon convolution with the system's point spread function (PSF). PSF is a measure of the blurring of a static point object in the image plane that takes the shape of an Airy disk approximated by a 2D Gaussian function due to the diffraction of light caused by the objective lens. As defined by Abbe's laws and the Rayleigh-criterion the diffraction of the light at the objective focal plane dictates the maximum of resolution depending on the wavelength of illumination and the numerical aperture of the objective that itself depends on the refractive index of the medium (Equation 4).

Equation 4. Lateral and axial resolution as determined by Abbe and Rayleigh with λ_{em} as the wavelength of the emitted light, NA being the numerical aperture, n the refractive index of the medium and θ the collection angle of the objective.

Abbe's limit

$$d = \frac{0.5\lambda_{em}}{NA} = \frac{0.5\lambda_{em}}{n \sin \theta}$$

Rayleigh's criterion

$$d_{x,y} = \frac{0.61\lambda_{em}}{NA} = \frac{0.61\lambda_{em}}{n \sin \theta}$$

$$d_z = \frac{2\lambda_{em}}{NA^2}$$

For widefield microscopy the highest resolution achievable is of approximately 200 nm in the lateral (x,y) and 500 nm in the axial (z) dimension. For a widefield microscope, the illumination pathway consists of an excitation light source, which is filtered by appropriate excitation filters, reflected by a dichroic mirror and directed to the objective lens to generate a uniform widefield illumination across the entire field of view. The detection pathway comprises the emitted fluorescence from the entire depth of the sample collected by the same objective, transmitted across the dichroic mirror, narrowed by an emission filter and detected by an array detector (Scheme 11). Though offering short image acquisition time concomitant with low phototoxicity, the major limitation of widefield microscopy is the high fluorescence background caused by the out-of-focus light collected.

Scheme 11. Instrumental setup for widefield imaging.

Being a widefield technique, but with the lateral and axial resolution improved by a factor of ~ 2 to approximately 100 and 300 nm,³⁰⁷ SIM offers an excellent trade-off compared to the other SR methods regarding acquisition speed, need for specific staining agents and phototoxicity. Super-resolution is achieved by frequency shifting upon patterned widefield illumination and mathematical reconstruction. In the instrumental setup for SIM imaging (Scheme 12), laser light is directed through a polarizer and a diffraction grating. Its projection onto the specimen causes fluorescence emission from the sample that is captured by an EMCCD camera. In the sequential illumination steps the grating is shifted and rotated, so that the illumination patterns of the grating from the three (respectively five) diffraction orders interfere with the original pattern of the sample creating Moiré fringes. These contain high frequency information transformed down to low frequencies due to the superposition of the periodic (sinusoidal) structures, i.e. structures of high spatial frequency can generate a low spatial frequency in the overlap (Scheme 12).³⁰⁸ These frequency mixed patterns are further demodulated by digital reconstruction resulting in images with twice the resolution as compared to conventional widefield microscopy. More detailed information about the mathematical fundamentals building up the reconstruction process is described in publications by Heintzmann *et al.*^{309, 310} and Gustafsson *et al.*³¹¹

Scheme 12. Instrumental setup for SIM imaging & Moiré effect.

Though being a powerful technique, on the downside of SIM application its deconvolution algorithms can also cause artefacts as a result of ill-defined parameters of the system such as its PSF, strongly aberrated input images or over-deconvolution which poses the problem of data misinterpretation.³¹²

3.4.6.3.2 Co-localization analysis

To get precise local information of PrP within cells quantitative co-localization analysis using ImageJ^{300, 301} software with Just Another Co-localization Plugin (JACoP)^{313, 314} was performed on image stacks. Overlay methods provide a visual estimate of co-localizing events, but often lead to misinterpretation and are not appropriate for quantification purposes. Thus, several global statistic approaches performing intensity correlation coefficient-based (ICCB) analyses that offer complementary information were applied and compared. Pearson's correlation coefficient (PCC) describes the spread of the pixel distribution within a scatter plot in respect to the fitted line displaying the relationship between the intensities of the two images. Its value lies in the range of 1 to -1, with 1 for complete co-localization and -1 for exclusion (Equation 5).

Equation 5. Pearson's correlation coefficient (PCC) with A_i and B_i as the intensities of channel A and B, a and b as the corresponding mean intensities.

$$r_p = \frac{\sum_i (A_i - a) \cdot (B_i - b)}{\sqrt{\sum_i (A_i - a)^2 \cdot \sum_i (B_i - b)^2}}$$

To provide statistical significance to the calculated PCCs, Van Steensel's³¹⁵ and Costes'³¹⁶ approach were conducted. For the Van Steensel's cross-correlation function (CCF), the PCC is calculated while a shift of one of the images relative to the other is operated. If the resulting plot of the PCC as a function of the displacement shows a bell-shaped curve or a dip, co-localization or exclusion is identified. Depending on the position of the maximum being at 0 or shifted, partial co-localization can be determined. The height correlates with noise and/or differing fluorescence intensities. Costes' approach offers two possibilities, namely automatic thresholding together with a calculation of the respective PCC and by comparing a randomized to the original image an evaluation of the significance (*p*-value) of the PCC, meaning the probability of obtaining the specified PCC by chance. Another ICCB analyses is provided by the overlap coefficient, which is a variation of the PCC neglecting the average values and covers a range between 0 and 1. The overlap coefficient can also be described as a product of k_1 and k_2 , thereby enabling the identification of discrete co-localization, noise and background corruption affecting its value and of the PCC, as well (Equation 6).

Equation 6. Overlap coefficient and its squared product. The latter expression shows that the overlap coefficient is composed of two parts, k_1 and k_2 .

$$r = \frac{\sum_i A_i \cdot B_i}{\sqrt{\sum_i A_i^2 \cdot \sum_i B_i^2}}$$

$$r^2 = k_1 \cdot k_2$$

$$k_1 = \frac{\sum_i A_i \cdot B_i}{\sum_i A_i^2}$$

$$k_2 = \frac{\sum_i A_i \cdot B_i}{\sum_i B_i^2}$$

Manders' coefficients^{317, 318} are built by the ratio of the sum of intensities of co-localizing pixels from one channel and its integrated density. Pixels from image A are considered co-localized, if their intensity in channel B is above 0 (M_1), and vice versa (M_2). Values range from 0 to 1 for non- and complete overlapping events. Unlike the other coefficients' application to verify the presence of co-localization, additionally Manders' coefficients estimate the amount of co-localization and are suitable for comparative studies (Equation 7).

Equation 7. Manders' coefficients with $A_{i,coloc} = A_i$, if $B_i > 0$ and $B_{i,coloc} = B_i$, if $A_i > 0$.

$$M_1 = \frac{\sum_i A_{i,coloc}}{\sum_i A_i}$$

$$M_2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$$

An interpretable representation of co-localization was described by Li.³¹⁹ A set of two graphs presents the intensity correlation analysis (ICA) results. Normalized intensities (0-1) are plotted as a function of the product $(A_i - a)(B_i - b)$ for each channel, with the upper-case characters as the current pixel's intensity and the lower-case characters as the channel's mean intensity. For co-localizing pixels the covariance of both channels is positive, resulting in a dot cloud concentrated on the right side of $x = 0$. Non-co-localizing pixels are located on the left side. In case of ambiguous results the intensity correlation quotient (ICQ) can be determined. It is defined as the ratio of positive $(A_i - a)(B_i - b)$ products divided by the overall products subtracted by 0.5. Thus, resulting ICQs vary from co-localization with 0.5 to exclusion with -0.5, whereas random staining and images corrupted by noise give values close to 0.

3.4.6.4 Fluorescence correlation spectroscopy (FCS)

3.4.6.4.1 Theory of fluorescence correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) quantifies dynamical processes, such as diffusion, flow, chemical reactions and photophysical properties of fluorophores, which lead to fluctuations of the fluorescence signal due to the fluorescent particles moving in and out of the observation volume. The group of Rigler^{320, 321} integrated FCS in confocal microscopy generating femtoliter-sized observation volumes. Together with single-photon sensitive avalanche photodiode detectors and objectives with high numerical apertures, a considerable increase in the SNR was made possible. Thus, the applicability of FCS could be expanded to live cell measurements. Typical working concentrations are within 1 nM to 1 μ M and correspond to physiological protein expression levels in biological samples. In respect to PrP, this protein can be found in concentrations of ~ 1 μ M in cells.³²² The principle of FCS data analysis is illustrated in Figure 1. As fluorophores pass through the observation volume, the intensity time trace is recorded by a single-photon sensitive APD. Then, autocorrelation analysis is

conducted on the recorded intensity trace by measuring the self-similarity of the intensity trace at different defined lag times (τ). The area overlaps of the intensity trace with the same trace shifted at different lag times are calculated and converted into autocorrelation amplitudes at each corresponding lag time. Thereafter, the autocorrelation function (ACF) $G(\tau)$ is generated by plotting the autocorrelation amplitude against the lag time, and eventually fitted with an appropriate theoretical model (Figure 2 and section 3.4.6.4.2). The ACF has the maximum amplitude at $\tau = 0$ and decays with the lag time.

Figure 1. Principle of FCS data analysis. Fluorophores traversing the observation volume get excited and emit fluorescence (red dots) opposite to fluorophores outside the observation volume (black dots) (A). The fluorescence intensity time trace is recorded with intensity fluctuations $\delta I(t)$ and an average intensity of $\langle I(t) \rangle$ (B). Eventually, the autocorrelation process (C) using the intensity time traces results in the autocorrelation function (ACF) (D).

From the ACF the underlying dynamic processes can be obtained. First, its shape reflects the type of dynamics. For instance, diffusion has a hyperbolic decay profile. Second, the width of the ACF reveals the average residence time of the particles in the observation volume. Slower particles have a more significant overlap of the intensity time trace with itself at longer lag times, leading to a broadening and wider ACFs. In case of only diffusive processes involved the time particles need to pass through the observation volume, termed as the diffusion time (τ_D), can be determined. Assuming the particles to be spherical, τ_D is proportional to $\sqrt[3]{MW}$, the cubic root of the molecular weight (MW) of the particle. Third, the number of particles (N) is accessible. Increasing

concentration results in a decrease of the relative fluorescence fluctuations within the observation volume, i.e. N is inversely proportional to the amplitude of the ACF at $\tau = 0$. Typical values lie in the range of 0.1 – 1000.

Figure 2. Representative ACF fit applying a 2D diffusion with two components and a single triplet contribution model (2D, 2p1t).

3.4.6.4.2 Theoretical ACF determination and fit models for confocal FCS

For the computation of the normalized ACF $G(\tau)$ as a function of the lag time (τ), the fluorescence intensity $I(t)$ at time (t) emitted from fluorophores in the observation volume which have a fluorescence fluctuation $\delta I(t)$ around a time-averaged fluorescence of $\langle I(t) \rangle$ is statistically analyzed (Equation 8).

Equation 8. Normalized autocorrelation function (ACF).

$$G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle [\langle I(t) \rangle + \delta I(t)] [\langle I(t+\tau) \rangle + \delta I(t+\tau)] \rangle}{\langle I(t) \rangle^2}$$

$$= \frac{\langle \delta I(t)\delta I(t+\tau) \rangle}{\langle I(t) \rangle^2} + 1$$

Equation 9. Time-averaged fluorescence $\langle I(t) \rangle$ and fluorescence fluctuation $\delta I(t)$, with T_{total} as the total measurement time.

$$\langle I(t) \rangle = \frac{1}{T_{total}} \int_0^T I(t) dt$$

$$\delta I(t) = I(t) - \langle I(t) \rangle$$

Based on Fick's laws of diffusion, theoretical models of the ACF $G(\tau)$ can be described considering the average number of particles within the observation volume (N), the lag time (τ), the diffusion time (τ_D), the structure factor K and the convergence value of the ACF at long lag times (G_∞) with an expected value of 1,^{323, 324} as depicted in

Equation 10 and 11. How the theoretical fit models can be derived from Equation 8 and Equation 9 is outlined in numerous reviews.³²⁵⁻³²⁷ In the case of multiple diffusion components, $G(\tau)$ is expressed as the linear sums of the individual components weighted with their respective mole fractions (F_i) of the i^{th} species. For triplet state photodynamics F_{trip} refers to the fraction and τ_{trip} to the relaxation time of the triplet state.^{328, 329}

Equation 10. Mathematical models for 3D diffusion of a single component (1p) without (upper formula) and with a single triplet contribution (lower formula) for confocal FCS.

$$G_{3D,1p}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_D}\right)^{-1/2} + G_\infty$$

$$G_{3D,1p1t}(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{K^2 \tau_D}\right)^{-1/2} \left(1 + \left(\frac{F_{trip}}{1 - F_{trip}}\right) e^{-\frac{\tau}{\tau_{trip}}}\right) + G_\infty$$

Equation 11. Mathematical models for 2D diffusion with two components (2p) without (upper formula) and with a single triplet contribution (lower formula) for confocal FCS.

$$G_{2D,2p}(\tau) = \frac{1}{N} \left((1 - F_2) \left(1 + \frac{\tau}{\tau_{D1}}\right)^{-1} + F_2 \left(1 + \frac{\tau}{\tau_{D2}}\right)^{-1} \right) + G_\infty$$

$$G_{2D,2p1t}(\tau) = \frac{1}{N} \left((1 - F_2) \left(1 + \frac{\tau}{\tau_{D1}}\right)^{-1} + F_2 \left(1 + \frac{\tau}{\tau_{D2}}\right)^{-1} \right) \left(1 + \left(\frac{F_{trip}}{1 - F_{trip}}\right) e^{-\frac{\tau}{\tau_{trip}}} \right) + G_\infty$$

Triplet state relaxation of fluorophores (*trip*) is a dynamical process originating from changes in the excited singlet state population, i.e. intersystem crossing between singlet and triplet state. The formation and decay of this photoinduced transient non-fluorescent state is much faster than those caused by diffusion, thus, making it observable as an additional shoulder at the beginning of the ACF (Figure 2). Regarding Cy5 emission, another blinking process involved is the photo-induced isomerization (Scheme 13). Though this dye possesses several preferable features, such as excitation in the near-infrared (near-IR) range which is good for cell measurements due to the lower autofluorescence and higher transmittance through the cell media, next to relatively high fluorescence quantum yields, high photostability, low pH sensitivities and allows the

application of inexpensive, highly efficient laser diodes, its photophysical properties are rather complex.³²² In its monomeric form Cy5 exhibits low quantum yields of intersystem crossing and high quantum yields of the trans-cis isomerization upon excitation.

Scheme 13. Model of the photophysical behavior of Cy5. 1N_0 , 1N_1 , 3N_1 denote the ground singlet, the first excited singlet and triplet state of the thermodynamically stable all-trans conformation of the dye (left structure). 1P_0 , 1P_1 , 3P_1 are the corresponding states of the photo-isomerized cis conformation (right structure). Upon photoexcitation of 1N_0 the major process following to 1N_1 can be attributed to relaxation back to 1N_0 with fluorescence emission. Other processes occurring derive from intersystem crossing from 1N_1 to 3N_1 with a triplet state decay from 3N_1 to 1N_0 and photo-induced trans-cis isomerization from 1N_1 to 1P_0 . The same processes can be accounted for the photo-isomerized conformation.

The obtained diffusion coefficient can be assumed to follow the Stokes-Einstein equation given for spherical solute particles at a given temperature (Equation 12).

Equation 12. Stokes-Einstein equation where k_B is the Boltzmann constant, T the absolute temperature, η_v the viscosity of the solvent and r the hydrodynamic radius of the solute particle.

$$D = \frac{k_B T}{6\pi\eta_v r}$$

3.4.6.4.3 Confocal imaging & FCS

Both confocal imaging and FCS were conducted on an Olympus FV1200 laser scanning confocal microscope from Olympus IX83 (Tokyo, Japan) equipped with a Microtime 200 upgrade kit for single molecule detection from PicoQuant (Berlin, Germany) at 37°C. The 488 nm argon-ion (for Atto488 and EGFP excitation), the 543 nm helium-neon (for Atto565 and DiI-C₁₈ excitation) or the 635 nm (for Atto655 and Cy5

excitation) laser beam from Melles Griot (Singapore) was focused on the sample by a water immersion objective (UPLSAPO, 60×, NA1.2) from Olympus (Tokyo, Japan) after being reflected by a dichroic mirror (DM405/488/543/635 bandpass) from Olympus (Tokyo, Japan) and a scanning mirror unit (Scheme 14).

Scheme 14. Instrumental setup of confocal imaging and FCS.

The laser powers measured before the objective were in the range of 10 to 17 μW for all lasers (Table 2) and converted into excitation intensities according to Equation 13, as previously described by Sun *et al.*³³⁰

Equation 13. Calculation of the excitation intensity (E.I.) under certain laser power (L.P.) with ω_0 (μm) as the radial distance.

$$E.I. = \frac{L.P.}{\pi\omega_0^2}$$

	488 nm	543 nm	635 nm
Calibration dye	Atto488	Atto565	Atto655
D ($\mu\text{m}^2/\text{s}$)	400 ³³¹	400 ³³²	426 ^{333, 334}
L.P. (μW)	17	10	12
E.I. (kW/cm^2)	17	9	8

Table 2. Calibration dyes, measured laser powers – excitation intensities.

For confocal imaging, the system was operated in scan mode. Fluorescence signal from the sample was passed through the same objective, through a 120 μm pinhole in the image plane to block off out-of-focus light, spectrally filtered *via* a bandpass emission filter, namely BA505-525 for EGFP, BA560-620 for DiI-C₁₈ or BA655-755 for Cy5 excitation, from Olympus (Tokyo, Japan), and recorded by a photomultiplier (PMT) detector. Images with a field of view of 512×512 pixels and a pixel size of 138 nm were acquired at a scan rate of 12.5 $\mu\text{s}/\text{pixel}$.

For confocal FCS measurements, the system was de-scanned and operated in point mode. Fluorescence signal from the sample was passed through the 120 μm pinhole, spectrally filtered by an emission filter, namely 513/17 (for Atto488 or EGFP emission), 600/50 (for Atto565 or DiI-C₁₈ emission) or 680/42 (for Atto655 or Cy5 emission) from Semrock (Rochester, USA), and eventually recorded by a sensitive single-photon avalanche photodiode (SPAD) SPCEM-AQR-14 from PerkinElmer, Optoelectronics (Quebec, Canada). Signal detected by the avalanche photodiode (APD) was then processed online by the SymPhoTime 64 software correlator from PicoQuant (Berlin, Germany), giving the autocorrelation function (ACF). Post-measurement fitting was performed in the SymPhoTime 64 software or in Igor Pro 7 from WaveMetrics (Lake Oswego, USA) using the open-source FCS 2.1 script provided by the Wohland laboratory at NUS (Singapore). The total measurement time for the calibration dyes and on the cells was in the range of 30 to 40 s.

Prior to conducting FCS measurements on the samples, the system was aligned for performance of the highest SNR achievable by adjusting the pinhole position in x-, y- and z-directions. To obtain the maximum fluorescence intensity emitted by the respective calibration dyes, an FCS measurement was conducted to generate an ACF, which was then fitted to obtain the structure factor K (Equation 15) and the molecular brightness (η) in photon counts per particle per second (cps). The brightness itself is influenced by the absorption coefficient and quantum yield of the fluorophore and the detection efficiency of the instrument. The pinhole position was adjusted and FCS measurements repeated until a K value of 4-6 and the maximum η were achieved. As the water immersion objective used had a cover slip correction collar, also this was adjusted to optimize the brightness. Using the diffusion time of the calibration dye

($\tau_{D, calibration\ dye}$) with known diffusion coefficient and K values extracted from the FCS fits, the effective observation volume was then calculated applying Equation 14, 15 and 16.

Equation 14. Effective observation volume (V_{eff}) in confocal FCS for non-interacting particles, with $\vec{r}$ as the spatial coordinate and $MDE(\vec{r})$ the molecular detection efficiency.

$$V_{eff} = \frac{\left(\iiint_{-\infty}^{\infty} MDE(\vec{r}) dV \right)^2}{\iiint_{-\infty}^{\infty} (MDE(\vec{r}))^2 dV} = \pi^{\frac{3}{2}} \omega_0^2 z_0$$

Equation 15. Structure factor K is defined as the ratio of the axial (z_0) and radial (ω_0) distances of the laser focal spot at $1/e^2$ value of the maximum intensity at the focus of the observation volume.

$$K = \frac{z_0}{\omega_0}$$

Equation 16. Diffusion time and coefficient.

$$\tau_D = \frac{\omega_0^2}{4D}$$

The appropriate ACF models for fitting the FCS are chosen considering the weight of the noise from each data point, i.e. the standard deviation as described by Koppel³³⁵ and Wohland *et al.*³³⁶ For the calibration dye solutions, 3D diffusion fitting models with a single component (1p), 3D,1p, or with an additional single triplet contribution (1t), 3D,1p1t, were used (Equation 10). For the membrane measurements, the fitting models used were 2D diffusion with two diffusion components (2p), 2D,2p, or with an additional single triplet contribution, 2D,2p1t (Equation 11). As the photophysical processes of Cy5 are strongly environment sensitive, but not believed to change the diffusion properties drastically, they were neglected during the fitting of the ACF curves and a 2D, 2p model was applied on data from later lag times. The fit parameters are the average number of particles N , diffusion time τ_D , triplet relaxation time τ_{trip} and G_{∞} . The K values obtained from the calibration dye solutions were fixed for all sample measurements (Table 3). The apparent D of the sample (D_{sample}) was determined using the known D of the calibration dye ($D_{calibration\ dye}$, Table 2) together with the measured diffusion times of the sample and the calibration dye deriving from the fits of the ACF curve (Equation 17).

Equation 17. Relationship between diffusion times and coefficients of the sample and calibration dye.

$$D_{\text{sample}} = \frac{\tau_{D,\text{calibration dye}} \cdot D_{\text{calibration dye}}}{\tau_{D,\text{sample}}}$$

fit parameters	fitted values		
	Atto488	Atto565	Atto655
N	0.48 ± 0.000	0.79 ± 0.146	0.85 ± 0.021
τ_D (μs)	22.6 ± 0.51	23.9 ± 0.47	26.5 ± 0.13
τ_{trip} (μs)	3.5 ± 0.72	-	-
F_{trip}	0.2 ± 0.02	-	-
K	4.2 ± 0.12	4.7 ± 0.40	4.5 ± 0.54
G_{∞}	0.00036 ± 0.000243	-0.00013 ± 0.000421	-0.00009 ± 0.000748

Table 3. Typical values of parameters fitted with 3D, 1p and 3D, 1p1t models for FCS calibration using 2 nM Atto488, 2 nM Atto565 and 3.8 nM Atto655 dye in PBS (pH 7.4). The fitted values are given as value $\pm$ error of fit.

Cy5 FCS data in presence of LatA was provided by Ng Xue Wen from Wohland's group at NUS (Singapore).

3.4.6.4.4 Imaging total internal reflection-FCS (ITIR-FCS)

Quantitative images of FCS parameters were obtained applying ITIR-FCS technique on an inverted epi-fluorescence microscope from Olympus IX71 (Tokyo, Japan) equipped with a high NA oil-immersion objective (PlanApo, 100 $\times$, 1.45) from Olympus (Singapore) and a back illuminated EMCCD camera from Andor iXon 860 (128 $\times$ 128 pixels, South Windsor, US) at 37°C in 5% (v/v) CO₂ humidified environment. ITIR-FCS instrumentation and optimization of data acquisition was described previously.^{337, 338} The air-cooled 488 nm argon-ion (for EGFP and fluorescein excitation), the 532 nm (for DiI-C₁₈ excitation) or the 640 nm (for Cy5 excitation) laser beam from Spectra-Physics Lasers (Mountain View, US) and Cobolt Samba (Sweden) was focused on the sample after being reflected by a combination of tilting mirrors and a dichroic mirror (495LP, Z488/532RPC or 660DRLP). TIR was achieved by adjusting the same combination of tilting mirrors to control the incident angle of the laser beam. A stack of 30,000 or 50,000 images was collected by the CCD chip with 2 ms time resolution. Andor Solis was used as the acquisition software and operated in kinetic mode. The fluorescence intensity fluctuations at each pixel of an ROI with a typical size of 2.4 $\times$ 2.4 μm^2 containing 10 $\times$ 10 pixels on the sample were then bleach-corrected

applying the linear segment method, processed to ACFs and fitted according to Equation 18 for a one particle diffusion model by ImageJ^{300, 301} software using the ImFCS 1.48³³⁹ plugin to produce quantitative maps of the diffusion coefficients (D) and the number of particles (N).

Equation 18. ITIR-FCS fitting model for 2D diffusion with one component (1p).

$$G(\tau) = \frac{1}{N} \left[\text{erf} \left(p(\tau) + \frac{1}{p(\tau)\sqrt{\pi}} \left(e^{-(p(\tau))^2} - 1 \right) \right) \right]^2 + G_{\infty}; \quad p(\tau) = \frac{a}{2\sqrt{D\tau + \sigma^2}}$$

In the above equation, $G(\tau)$ is the ACF as a function of the correlation time (τ) and N , a , D and σ are the number of particles per pixel, pixel side length, diffusion coefficient and standard deviation of the Gaussian approximation of the microscope PSF respectively. a of the CCD chip in the camera is 24 μm which corresponds to 240 nm in the sample plane. G_{∞} is the convergence value of the ACF at very long correlation time. N , D and G_{∞} were used as fit parameters.

FCS diffusion law analysis^{340, 341} adapted for ITIR-FCS^{338, 342, 343} of the same image stack was performed to obtain the FCS diffusion law intercept (τ_0). The diffusion law describes the linear dependence of the diffusion time (τ_D) on the size of the observation area (A_{eff}) (Equation 20). By extrapolating the experimental FCS diffusion law plots to zero observation area the intercept values (τ_0) are obtained. A_{eff} at different bin sizes can be accurately determined by convoluting the detection area (A) of a given bin size (e.g. 1×1 , 2×2 , etc.), which is the area of the binned camera pixels, with the lateral PSF of the optical system (Equation 19). Mathematical theory is provided in publications from Wawrezinieck *et al.*³⁴⁰ and the group of Wohland.³³⁸

Equation 19. Effective Area (A_{eff}) in ITIR-FCS.

$$A_{\text{eff}}(\tau) = \frac{a^2}{\left(\frac{\sigma}{a\sqrt{\pi}} \left(e^{-\frac{a^2}{\sigma^2}} - 1 \right) + \text{erf} \left(\frac{a}{\sigma} \right) \right)^2}$$

Equation 20. Imaging FCS diffusion law.

$$\tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D}$$

The spatial dependence of the diffusion coefficients of membrane probes due to membrane structural features (section 4.4.3.1) elucidates the sub-resolution (<200 nm) plasma membrane organizational features. Extrapolation of the FCS diffusion law of τ_D against A_{eff} to zero observation area yields zero τ_0 for free diffusion, reflecting a direct proportionality of the diffusion time with observation area. However, in a heterogenous system where the diffusion is obstructed by membrane domains or meshwork, τ_0 is positive for hindered diffusion in domains or negative for hop diffusion due to cytoskeletal meshwork, respectively. Thus, the sign and magnitude of the FCS diffusion law intercept (τ_0) allows conclusions about the nature of the diffusive process. More information on the FCS diffusion law defining membrane dynamics dictated by existing membrane organization can be found elsewhere.^{340, 343}

ITIR-FCS allowed for the generation of various observation areas (A_{eff}) from a single measurement by pixel binning (1×1 to 3×3) after acquisition. TIRF images were taken using the same setup with 10 ms exposure time. All TIRF measurements were acquired at an EM gain of 300 (in a scale of 6-1000).

Data of **5_{ΔCR}** and **6_{ΔCR}** incubated on GFP-GPI AP transfected cells was provided by Ng Xue Wen from Wohland's group at NUS (Singapore).

4. Results & Discussion

4.1 Semisynthesis of PrP variants

To elucidate the properties of PrP containing a GPI anchor mimic and its membrane association, access to defined homogeneous, posttranslationally modified protein is needed. As this has not yet been possible, the majority of studies on the function and structure of PrP have been carried out with recombinant protein lacking the GPI anchor or heterogeneous protein preparations, with respect to *N*-glycosylation and the molecular structure of the GPI, isolated from mammalian cell lines.²³⁹⁻²⁴¹ Still, there have been attempts towards defined membrane-anchored PrP. Glockshuber and co-workers²⁴² attached a thiol-reactive phospholipid to the cysteine of a glycine linker extending the *C*-terminus of recombinant PrP. Thereby PrP is placed at a distance from the membrane surface in agreement with the flexible glycan moiety of the natural GPI anchor in PrP. Similarly, Pinheiro and colleagues²⁴³ coupled a GPI anchor mimetic to a genetically engineered cysteine residue of recombinant PrP. Independently, both laboratories reported that the PrP constructs retained their structural characteristics upon reconstitution in raft membranes, indicating that the raft environment itself protects the α -helical conformation of PrP. These examples demonstrate how the availability of membrane-attached rPrP facilitates systematic studying of PrP features in a native-like environment. However, further application of the PrP constructs mentioned above in cell-based assays may be impeded by potential side reactions with thiol-containing compounds or internal cysteines of PrP, as the lipids were attached *via* disulfide bonds. Baskakov and co-workers²⁴⁴ introduced a myristoyl chain at the *C*-terminus of PrP(S230C) applying maleimide chemistry and found that this hydrophobic substitution partially impaired the intrinsic ability of PrP to form amyloid fibrils. Still, also this lipid attachment is not stable *in vivo*.³⁴⁴ A different strategy based on click and ligation chemistry was applied by Moroder and co-workers²⁴⁶ to obtain lipopeptides corresponding to the *C*-terminal PrP fragment (aa 214-231). Confocal images of HeLa cells revealed a direct transfer of fluorescently labeled lipopeptides to the cellular membrane. This proved the eligibility of the lipopeptides in mimicking the GPI anchor. Baldwin and colleagues²⁴⁵ synthesized polypeptides comprising segments of PrP that were chemically modified with lipids at the side chain of a *C*-terminally introduced lysine. Immunofluorescence analysis showed that only myristylated PrP peptides could be targeted to the cell-surface, unlike to acetylated PrP peptides.

Semisynthesis represents a valuable tool that allows the attachment of a GPI moiety by a native peptide bond to recombinant PrP, thereby providing defined PrP modified with a GPI anchor. Demonstrated in our group by Olschewski *et al.*,²⁴⁸ PrP with a GPI anchor mimic performs similarly to recombinant PrP equipped with a synthetic GPI anchor. In subsequent studies Chu *et al.*²³³ worked on PrP variants containing a GPI anchor mimic with varying deletions in the sequence of the recombinant PrP part. These PrP variants interacted in different ways with phospholipid membranes depending on their sequence. The attachment of a GPI anchor mimic influences the biochemical and conformational properties of the constructs in a distinct way compared to recombinant PrP lacking this PTM, which emphasizes the need for homogeneously modified PrP in studying its structure and function.

The following three variants of full-length FL_PrP (aa 23-231), central hydrophobic region deleted Δ CR_PrP (aa 23-231 with Δ 105-125) and *N*-terminally truncated T_PrP (aa 90-231) were selected for elucidating the impact of electrostatic and hydrophobic interactions with membranes (section 1.5.1). We applied a robust semisynthetic strategy to selectively introduce fluorophores and obtained homogeneously labeled PrP variants containing GPI anchor mimics (Scheme 15). This allows to directly study trafficking at cellular membranes and to gain further insights into the protein's patho- and physiological function.

Scheme 15. Semisynthetic strategy for PrP variants. The key EPL reaction affords PrP variants by linking recombinantly expressed PrP thioesters to synthetic GPI anchor-mimicking peptides.

The strategy relies on the expression of rPrP in fusion with the GyrA intein and affinity tags, namely a His tag (only present in the T_PrP construct) and a chitin-binding domain (CBD), and further cleavage with an excess of thiols to generate rPrP with a C-terminal thioester. The PrP-thioesters (4_{FL, ΔCR, T}) are subjected to GPI anchor-mimicking peptides (1-3) and react with the N-terminal cysteine residue in an EPL reaction to modified PrP (6-8_{FL, ΔCR, T}) (Scheme 15). Mechanistic details of the EPL reaction are discussed in section 1.5.1.

4.1.1 PrP-thioesters

Protein α -thioesters of FL_PrP (aa 23-231), Δ CR_PrP (aa 23-231 with Δ 105-125) and T_PrP (aa 90-231) were generated by expression in *E. coli* (Figure 3). PrP variants fused to *Mxe* GyrA intein, a chitin binding domain (CBD), and an additional His-tag for T_PrP, were isolated from inclusion bodies.

Figure 3. Following expressions of PrP-Mxe GyrA intein(-His)-CBD fusion proteins with SDS-PAGE analysis. Lane 2, 4, 6 show FL_PrP (MW: 51,156 Da), Δ CR_PrP (MW: 49,262 Da) and T_PrP-Mxe GyrA intein(-His)-CBD (MW: 45,115 Da) before induction with IPTG and lane 3, 5, 7 after 22 h of IPTG induction. Lane 1 shows the bands of the LMW marker.

Solubilized proteins were subjected to nickel affinity chromatography giving the required purified fusion proteins. For FL and Δ CR_PrP variants, this purification method allowed taking advantage of the intrinsic affinity of the octarepeat region of PrP towards nickel (II) nitrilotriacetic acid (Ni(II)-NTA). For a detailed procedure, see section 3.2.1. The purified material was subjected to intein cleavage conditions using an excess of sodium 2-mercaptoethanesulfonate (MESNA). The reaction proceeded until 50, 35 and 29% of α -thioesters 4_{FL} , $4_{\Delta CR}$ and 4_T were formed, in relation to the corresponding fusion proteins. Percentages were determined based on the integrated intensity values of the bands from SDS-PAGE using Image Lab 5.1 software (Figure 4).

Figure 4. MESNA-mediated intein cleavage reactions followed by SDS-PAGE. PrP-Mxe GyrA intein(-His)-CBD fusion proteins, FL_PrP (left), Δ CR_PrP (middle) and T_PrP (right), in lane 2 subjected to MESNA-mediated intein cleavage conditions (lane 3). Lane 1 shows the LMW marker. Bands for FL_PrP-Mxe GyrA intein-CBD (MW: 51,156 Da), 4_{FL} (MW: 23,324 Da), Δ CR_PrP-Mxe GyrA intein-CBD (MW: 49,262 Da), $4_{\Delta CR}$ (MW: 21,430 Da), T_PrP-Mxe GyrA intein-His-CBD (MW: 45,115 Da), 4_T (MW: 16,459 Da), Mxe GyrA intein-CBD (MW: 27,938 Da) and Mxe GyrA intein-His-CBD (MW: 28,796 Da) could be detected.

HPLC purification gave PrP α -thioesters in high purity, as can be seen in Figure 5. For 4_{FL} , $4_{\Delta CR}$ and 4_T , respectively, deconvoluted mass spectra showed molecular weights of 23,324, 21,429, 16,459 Da in agreement with the calculated values. HPLC traces based on the detection of 214 and 280 nm absorption revealed single sharp peaks (Figure 5A). Isolation of PrP α -thioesters 4_{FL} , $4_{\Delta CR}$ and 4_T was achieved in high yields of 12, 13 and 8 mg/L *E. coli* medium.

Figure 5. Characterization of PrP α -thioesters. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, black: 214 nm, grey: 280 nm, baseline corrected) and ESI-MS spectra of isolated FL_PrP (B), ΔCR _PrP (C) and T_PrP (D) α -thioesters (4_{FL} , $4_{\Delta CR}$, 4_T).

4.1.2 SPPS of lipidated peptides

In its native structure, the GPI anchor consists of a phosphoethanolamine residue amide-linked to the C-terminus of a protein, three mannose residues, a glucoseamine and a phosphatidylinositol molecule. The latter comprises a glycerol backbone, two fatty acid tails and an inositol (Scheme 16A). Though the synthesis has already succeeded, it remains a challenge to provide sufficient amounts.³⁴⁵ To circumvent the elaborate synthesis of an artificial GPI anchor (Scheme 16B), peptides mimicking its physicochemical characteristics (Scheme 16C) were used.

Scheme 16. Structures of a native and artificial GPI anchor together with a GPI anchor mimic. **A** shows a common structure of a native GPI anchor from human erythrocyte acetylcholinesterase³⁴⁶ attached to the C-terminus of the protein. **B** is a chemically synthesized GPI from Silva *et al.*²⁷⁷ with a cysteine residue for NCL reactions. **C** shows the structure of the GPI anchor-mimicking peptide used for the semisynthesis of lipidated PrP constructs, previously developed by Olschewski *et al.*²⁴⁷

As already shown by Olschewski *et al.*^{247, 347} these doubly lipidated (green in Scheme 16C) mimics show similar structural, biochemical and cellular localisation properties as GPI-anchored PrP. Noteworthy in this context, the sequence features a tobacco etch virus (TEV) cleavage site ENLYFQ (red in Scheme 16C) for removal of the lipid anchor, used before to show association with liposomes for lipidated compared to recombinant PrP. Besides, this approach facilitates the generation of sufficient amounts and a straightforward incorporation of dyes for PrP trafficking (purple in Scheme 16C). Cy5 and fluorescein-based fluorophores were conjugated to the peptide *in-situ* activated or as reactive succinimide esters. In order to provide options to study the impact of the fatty acid chains, peptides missing palmitoyl groups (**3b**) were synthesized. To enable the determination of the exact aggregation state of PrP based on the quantification its fluorescence in cell assays a monomeric reference compound is needed. To this end, peptides being alanine substituted for cysteine (pink in Scheme 16C) at the N-terminus (**3a**) were generated, as these other than the respective peptides **3** exclude any possibility of dimer formation. As anticipated, this SPPS strategy

afforded milligrams of the required building blocks in high purity and yields of 20, 21, 16 and 40% (based on the amount of crude peptide) for the lipidated (**1**) and fluorescein (**3**), Cy5 labeled (**2**) and alanine substituted peptides (**3a** and **3b**), respectively (Table 4). For all peptides, HPLC traces showed single sharp peaks with >95% purity. Mass spectra could be deconvoluted to molecular weights in agreement with the respective calculated values (section 4.1.2.1).

peptide	synthesis scale (mmol)	Yield of				
		crude peptide		purified peptide		
		(mg)	(%)	(mg)	based on scale (%)	based on crude peptide (%)
1 , lipidated	0.25	470	59	91.8	12	20
2 , Cy5 labeled	0.03	30	28	6.3	6	21
2a , sulfo-Cy5 5a labeled	0.25	230	24	1.1	0.1	0.5
2b , sulfo-Cy5 5b labeled	0.25	150	16	2.8	0.3	2
3 , fluorescein labeled	0.03	20	19	4.1	4	20
3a , fluorescein labeled, A1C	0.03	50	48	8.1	8	16
3b , fluorescein labeled, A1C, non-lipidated	0.03	20	22	8.0	9	40

Table 4. Yields of GPI anchor-mimicking peptides **1**, **2**, **2a**, **2b**, **3**, **3a**, **3b** obtained by SPPS.

For sulfo-Cy5 modified peptides **2a** and **2b** the available amounts of the corresponding Cy5 compounds (**5a**, **5b**) were limited. Thus, in the coupling reactions the dyes could not be used in excess to the synthesis scale, as typical for SPPS. As a consequence of the incomplete peptide labeling HPLC purification was complicated. Resulting low yields of 0.5 and 2% (yield based on crude peptide) for the sulfo-Cy5 labeled peptides (**2a** and **2b**) in combination with noisier mass spectra (Figure 8, Figure 9B) motivated the synthesis of Cy5 **4**, as discussed in section 4.1.2.2.

4.1.2.1 Combined analysis data of peptides **1-3a-b**

HPLC chromatograms and mass spectra of lipidated, Cy5 and fluorescein labeled peptides are summarized in Figures 6-12.

Figure 6. Characterization of lipidated peptide **1**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, baseline corrected) and ESI-MS spectra (B) of lipidated peptide **1**.

Figure 7. Characterization of Cy5 labeled lipidated peptide **2**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra (B) of Cy5 labeled lipidated peptide **2**.

Figure 8. Characterization of sulfo-Cy5 labeled lipidated peptide **2a**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra (B) of sulfo-Cy5 labeled lipidated peptide **2a**.

Figure 9. Characterization of sulfo-Cy5 labeled lipidated peptide **2b**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra (B) of sulfo-Cy5 labeled lipidated peptide **2b**.

Figure 10. Characterization of fluorescein labeled lipidated peptide **3**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 492/517$ nm, baseline corrected) and ESI-MS spectra (B) of fluorescein labeled lipidated peptide **3**.

Figure 11. Characterization of fluorescein labeled lipidated peptide **3a**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 492/517$ nm, baseline corrected) and ESI-MS spectra (B) of fluorescein labeled lipidated A1C-substituted peptide **3a**.

Figure 12. Characterization of fluorescein labeled lipidated peptide **3b**. Analytical HPLC traces (A) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 492/517$ nm, baseline corrected) and ESI-MS spectra (B) of fluorescein labeled, A1C-substituted peptide **3b**.

4.1.2.2 Synthesis of Cy5

Synthesis of Cy5 was accomplished in three steps starting from 2,3,3-trimethyl-3*H*-indolenine **1**, an inexpensive precursor (Scheme 17). This approach afforded sufficient mg amounts of the dye to use it in excess for coupling reactions in SPPS.

Scheme 17. Overview of Cy5 **4** synthesis. Reaction conditions: i = CH_3I , CH_2Cl_2 anh.; ii = 5-bromovaleric acid, KI, CH_3CN ; iii = KOAc, Ac_2O , malonaldehyde bis(phenylimine) monohydrochloride, 1-butanol anh.

Two indolium parts were generated in reactions of the indolenine compound **1** with the corresponding halogenoalkanes in yields of 60 and 50% for **2** and **3** using protocols from Yamane *et al.*²⁸⁷ and Zhang *et al.*²⁸⁸, respectively. Although the linkage *via* the polymethine chain, according to a procedure from Korbel *et al.*,³⁴⁸ gave 71 mg of pure Cy5 **4**, this corresponds to only 6% isolated yield. The low yield is caused by a lack of selectivity of the reaction resulting in mixtures of methyl- (**2**) and acid-group modified indolium (**3**) compounds to linked to themselves and to each other, respectively.

Isolation of the intermediate and subsequent coupling of the second indolium as described by Jung *et al.*³⁴⁹ could be a promising alternative.

Figure 13. Characterization of Cy5 **4**. Analytical HPLC traces (**A**) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra (**B**) of crude and isolated Cy5 **4**, respectively. The mass spectrum of the crude material shows a peak at m/z of 383.2 that can be assigned to two indolium **2** compounds bridged *via* the polymethine chain.

The purity of Cy5 **4** was assessed by HPLC and mass analysis (Figure 13), together with NMR spectroscopy (sections 3.3.1.1 and 8.2).

4.1.3 Expressed protein ligation with GPI anchor mimics

With the ligation partners in hand, their assembly was accomplished using expressed protein ligation (EPL).²³⁷ Following a nucleophilic attack of the *N*-terminal thiol group of the GPI anchor mimic towards the *C*-terminal thioester of the expressed PrP segments, the irreversible *S*→*N* acyl shift lead to the formation of a native amide bond linking the two parts (Scheme 5 in section 1.5.1). Depending on the nucleophilic properties, pKa values, performance as leaving group and water solubility of the added thiol the reaction rate could be tuned.³⁵⁰ For the synthesis of full-length lipidated PrP (**6FL**), using thiophenol, as described previously,²³³ and 4-mercaptophenylacetic acid (MPAA) resulted in very similar conversions of 68 and 62%, but the reactions were complete after 24 and 6 h, respectively (Figure 14). Completion of the EPL reactions was determined by time-dependent HPLC traces showing no further increase in the integrated peak areas of the ligation products. Besides, LC/MS spectra exposed complete hydrolysis of the thioesters, which impeded any further product formation. Thus, for obtaining lipidated and labeled PrP variants MPAA as ligation mediator was used.

Figure 14. Comparison of EPL time courses using different thiols as ligation mediators. In the ligation reaction of FL_PrP α -thioester **4_{FL}** and lipidated peptide **1** using the thiols MPAA and thiophenol, an increase of the ligation product **6_{FL}** is accompanied by a decrease of **1**, as determined by integrated peak areas of HPLC traces at 214 nm. For MPAA, a conversion up to ~60% of the ligation product **6_{FL}** is achieved ~4 times faster.

However, ligations proceeding at pH 7.2 inevitably led to partial hydrolysis of PrP thioesters. These hydrolysis products were isolated as reference compounds lacking any C-terminal modifications. Ensuing HPLC purification afforded non-modified and lipidated, labeled PrP variants in yields of 9 to 42% (Table 5). The purity of PrP constructs was assessed *via* SDS-PAGE and analytical RP-HPLC, showing sharp bands and single peaks with >95% purity. Mass spectra could be deconvoluted to molecular weights in agreement with the calculated values (section 4.1.3.1).

PrP variant	isolated yield (%)	conversion at 214 nm (%)
6_{FL} , lipidated FL*	42	68
6_{FL} , lipidated FL	35	62
7_{FL} , Cy5 labeled FL	14	60
8_{FL} , fluorescein labeled FL	23	31
5_{FL} , non-modified FL	39	n.d.
6_{ΔCR} , lipidated ΔCR	19	64
7_{ΔCR} , Cy5 labeled ΔCR	23	62
8_{ΔCR} , fluorescein labeled ΔCR	9	50
5_{ΔCR} , non-modified ΔCR	11	n.d.
6_T , lipidated T	21	45
7_T , Cy5 labeled T	37	30
8_T , fluorescein labeled T	19	28
5_T , non-modified T	14	n.d.

Table 5. Yields of PrP variants. Conversion of the ligations was determined by integrated peak areas of HPLC traces in relation to the GPI anchor-mimicking reactants **1**, **2** or **3**, respectively. Moreover, non-modified (**5_{FL}**, **5_{ΔCR}**, **5_T**) PrP constructs could be isolated due to hydrolysis of the corresponding thioesters. *Thiophenol used as thiol additive.

4.1.3.1 Combined analysis data of all generated PrP constructs

SDS-gels, HPLC chromatograms and mass spectra of PrP constructs are displayed in the subsequent Figures 15-22, respectively. The SDS-gels show dimer bands of the PrP constructs in varying intensity depending on the sample preparations, the storage and buffer conditions. This behavior has been previously described for PrP.^{233, 253} Western blots were performed after the cell assays to demonstrate that the labeled (**7_{FL}**, **7_{ΔCR}**), as well as the non-modified PrP constructs (**5_{FL}**, **5_{ΔCR}**, **5_T**), in amounts as little as 10-100 pg,²⁸⁴ depending on the applied antibody,^{351, 352} stayed intact under the conditions investigated. No cleavage products, e.g. from PrP cleavage at the cell membrane could be detected.

Figure 15. SDS-PAGE analysis of non-modified PrP variants. Non-modified PrP variants analyzed with Western blot (6D11 mAb) (left) and Coomassie staining (right): lane 1, MagicMark™ XP and LMW marker, respectively; lane 2, **5_{ΔCR}** (MW: 21,306 Da); lane 3, **5_{FL}** (MW: 23,201 Da); lane 4, **5_T** (MW: 16,336 Da) and its dimer (**5_T-5_T**).

Figure 16. SDS-PAGE analysis of lipidated PrP variants. Lipidated PrP variants subjected to Coomassie staining show next to lane 1 with the LMW marker bands for lipidated **6_{FL}** (MW: 26,353 Da) (left gel), **6_{ΔCR}** (MW: 24,458 Da) (middle gel) and **6_T** (MW: 19,488 Da) (right gel).

Figure 17. SDS-PAGE analysis of Cy5 labeled PrP variants. Cy5 labeled PrP variants analyzed with fluorescence-based detection (lane 3), but also with Western blot (3F4 and 6D11 mAb) and Coomassie-staining show bands for 7_{FL} (MW: 26,804 Da) (top left), $7_{\Delta CR}$ (MW: 24,910 Da) (bottom), 7_T (MW: 19,940 Da) (top right) and their corresponding dimers ($7_{FL}-7_{FL}$, $7_{\Delta CR}-7_{\Delta CR}$, 7_T-7_T) next to the MagicMark™ XP and the LMW marker (lane 1), respectively.

Figure 18. SDS-PAGE analysis of fluorescein labeled PrP variants. Fluorescein labeled PrP variants analyzed with fluorescence-based detection (lane 3) and Coomassie-staining (lane 2) show bands for 8_{FL} (MW: 26,711 Da) (top left), $8_{\Delta CR}$ (MW: 24,817 Da) (bottom), 8_T (MW: 19,847 Da) (top right) and their corresponding dimers ($8_{FL}-8_{FL}$, 8_T-8_T) next to the LMW marker (lane 1).

Figure 19. Characterization of non-modified PrP variants. HPLC traces (**A**, **C**, **E**) ($\lambda_{abs} = 214+280$ nm, baseline corrected) and ESI-MS spectra of isolated non-modified PrP variants FL_PrP (**B**), ΔCR_PrP (**D**) and T_PrP (**F**) (**5_{FL}**, **5_{ΔCR}**, **5_T**).

Figure 20. Characterization of lipidated PrP variants. HPLC traces (A, C, E) ($\lambda_{abs} = 214+280$ nm, baseline corrected) and ESI-MS spectra of isolated lipidated PrP variants FL_PrP (B), ΔCR_PrP (D) and T_PrP (F) (**6_{FL}**, **6_{ACR}**, **6_T**).

Figure 21. Characterization of Cy5 labeled lipidated PrP variants. HPLC traces (A, C, E) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra of Cy5 labeled lipidated PrP variants FL_PrP (B), Δ CR_PrP (D) and T_PrP (F) (7_{FL}, 7 _{Δ CR}, 7_T).

Figure 22. Characterization of fluorescein labeled lipidated PrP variants. HPLC traces (**A**, **C**, **E**) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 492/517$ nm, baseline corrected) and ESI-MS spectra of lipidated fluorescein labeled PrP variants FL_PrP (**B**), Δ CR_PrP (**D**) and T_PrP (**F**) (**8_{FL}**, **8_{ΔCR}**, **8_T**).

4.1.3.2 Expressed protein ligation with a monolipidated glycosylphosphatidylinositol (GPI)

In addition to generating PrP variants containing GPI anchor mimics, we wanted to use our optimized EPL approach with MPAA to access PrP with a synthetic GPI anchor, as previously described by Olschewski *et al.*²⁴⁸ So far, different glycoforms of PrP GPI have been reported with the exact linkage positions and anomeric configuration of the oligosaccharide branch not defined, and no details about the lipid composition.²⁶⁵ In view of this structural uncertainty, a core GPI pseudopentasaccharide, containing three mannose (Man), a glucoseamine (GlcN) and an inositol (Ino) glycan connected in an α -Man-(1 $\rightarrow$ 2)- α -Man-(1 $\rightarrow$ 6)- α -Man-(1 $\rightarrow$ 4)- α -GlcN-(1 $\rightarrow$ 6)-*myo*-Ino way, was selected (Scheme 18). We anticipated that monolipidated GPI (**4**) afforded by the group of Silva²⁷⁷ with a cysteine residue in a disulfide bond with β -mercaptoethanol, incorporated by extending the ethanolamine group, will react with FL_PrP α -thioester (**4_{FL}**) to give GPI modified PrP (**9_{FL}**). In one of the last steps of the synthesis of **4** the benzyl protecting groups were removed by hydrogenolysis. In traces this led to the reduction of the incorporated cysteine residue to an alanine. The cleaved protecting groups of the cysteine residue were precipitated as salts by adding β -mercaptoethanol. At the same time cysteine of the GPI anchor formed a heterodisulfide with β -mercaptoethanol. The amphiphilic nature of the GPI anchor hampered further purification processes. However, the quality of the crude was sufficient for the EPL reaction. As carbohydrates typically exhibit a weak UV absorption, another detection method for monitoring the progress of the EPL reaction was required. To this end, a charged aerosol detector (CAD) was helpful. The CAD-HPLC trace of monolipidated GPI showed a sharp peak at t_R of 29.3 min (Figure 23A). High resolution mass analysis (Scheme 18) and MALDI (Figure 23B) agreed with the calculated MW values of 1,461.5 and 1,385.4 Da for the monolipidated Cys-tagged GPI with and without disulfide and their corresponding sodium adducts 1,484.5 and 1,408.4 Da. However, traces of reduced Ala-tagged GPI with MW values of 1,353.3 and 1,376.3 Da (+sodium) could also be detected.

HRMS (ESI), positive mode: calc. for $C_{55}H_{106}N_3O_{33}P_2S_2^+$ 1,462.5620; $C_{55}H_{105}N_3NaO_{33}P_2S_2^+$ 1,484.5439; $C_{53}H_{102}N_3O_{32}P_2^+$ 1,354.5916; $C_{53}H_{101}N_3NaO_{32}P_2^+$ 1,376.5736; found 1,462.5610; 1,484.5446; 1,354.5989 and 1,376.5722 Da.

HRMS (ESI), negative mode: calc. for $C_{55}H_{104}N_3O_{33}P_2S_2^-$ 1,460.5474; $C_{55}H_{103}N_3NaO_{33}P_2S_2^-$ 1,482.5294; $C_{55}H_{103}N_3O_{33}P_2S_2^{2-}$ 729.7701; $C_{55}H_{102}N_3NaO_{33}P_2S_2^{2-}$ 740.7610; $C_{53}H_{100}N_3O_{32}P_2^-$ 1,352.5771; $C_{53}H_{99}N_3NaO_{32}P_2^-$ 1,374.5590; $C_{53}H_{99}N_3O_{32}P_2^{2-}$ 675.7849; found 1,460.5476; 1,482.5308; 729.7715; 740.7639; 1,352.5754; 1,374.5554 and 675.7835 Da.

Scheme 18. HRMS data and structure of monolipidated GPI with cysteine in a heterodisulfide bond with β -mercaptoethanol.

Figure 23. Characterization of monolipidated GPI. CAD-HPLC trace (A) and MALDI spectrum (B) of monolipidated GPI (**4**) used in ligation reaction with FL_PrP α -thioester (**4_{FL}**).

The EPL reaction between the PrP thioester **4_{FL}** and monolipidated GPI (**4**) proceeded to 69% conversion after 5 h (Figure 24). However, isolation of the formed FL_PrP-GPI (**9_{FL}**) turned out to be challenging due to similar retention times of the ligation product and starting material **4_{FL}**.

Figure 24. Following the EPL reaction between 4_{FL} and 4 by HPLC. The ligation was monitored over 5 h via HPLC traces (CAD signal, baseline corrected) and values of the corresponding integrated peak areas. Peaks at t_R of 25.1 min can be attributed to the formation of product 9_{FL}. Peaks at 29.1, 29.3, 29.5 min can be assigned to a mixture of monolipidated Cys-tagged GPI in (4) and lacking a disulfide bond, and reduced Ala-tagged GPI, respectively. The ligation is finished after 5 h with 69% of 9_{FL}. Integration of peak areas was accomplished with the software OriginPro using CAD HPLC traces.

Still, we managed to successfully demonstrate that 9_{FL} was formed and isolated in high purity (Figure 25). The HR-ESI MS spectrum of 9_{FL} could be deconvoluted to 24,568 Da agreeing with the calculated mass.

Figure 25. HR-ESI MS spectrum of FL_PrP-GPI 9_{FL}. Labeled charge states in mass spectrum deconvolute to a molecular weight of 24,568 Da in agreement with the calculated value of 24,568 Da.

However, no further experiments could be performed due to the limited amounts of 9_{FL} available, that were only sufficient for HR-ESI MS analysis.

4.1.3.3 Labeling of folded lipidated PrP variants

To provide labeled PrP variants, we also developed another strategy requiring only small amounts of dye. We envisioned that the non-native cysteine residue of the peptide mimicking the GPI anchor reacts selectively in a maleimide reaction. Though, two more cysteines are present within the sequence of PrP, in its native folded state these thiol groups are blocked within a disulfide bond. For establishing a labeling protocol, first lipidated peptide **1** was used. The reaction proceeded with 1 mM of peptide concentration in a sodium acetate buffer containing 20 mM OG at pH 7.4, using 2.9eq of dye, but also 3.3eq of TCEP. It afforded, following HPLC purification, sulfo-Cy5 **5c** labeled **2c** in amounts of 0.3 mg, corresponding to 24% of theoretical yield, and in high purity. HPLC traces showed a single sharp peak and the deconvoluted mass spectrum a mass of 3,934.0 Da in agreement with the calculated value (Figure 26).

Figure 26. Characterization of sulfo-Cy5 labeled lipidated peptide **2c**. Analytical HPLC traces (**A**) ($\lambda_{\text{abs}} = 214+280$ nm, $\lambda_{\text{exc/em}} = 630/650$ nm, baseline corrected) and ESI-MS spectra (**B**) of isolated sulfo-Cy5 **5c** labeled lipidated peptide **2c**. Labeled charge states in mass spectrum deconvolute to a molecular weight of 3,934.0 Da in agreement with the calculated value of 3,934.0 Da.

Motivated by this result, a similar procedure was applied for selectively labeling the whole protein. First, to achieve suitable buffer conditions for the reaction, folded lipidated T_PrP **6r** was dialyzed against 20 mM sodium acetate buffer, with 20 mM OG, pH 7.4 from pH 5. For the labeling reaction, the protein in concentrations of 0.05 mM was subjected to 6eq of sulfo-Cy5 **5c** dye. Successful labeling could be confirmed using SDS-PAGE with fluorescence-based detection and Coomassie staining, showing for both detection methods a band at the size of sulfo-Cy5 labeled T_PrP **7cr** (MW 20,250 Da) (Figure 27). However, removal of excess dye turned out to be rather challenging. Using dialysis, even after 3d still significant amounts of free dye were

found. As keeping the protein in its folded state is a prerequisite for the selectivity of the reaction, possible methods for separating non-bound Cy5 are limited. Thus, this approach was neglected. Noteworthy, a possibility to prove the labeling selectivity could have been using T_PrP **6_{Ala-T}** as a negative control (for more information see section 1.5.1).²⁵⁴ With the cysteine substituted for alanine in the GPI anchor-mimicking moiety no band at the size of sulfo-Cy5 labeled T_PrP should be detectable in the fluorescence channel of the SDS-gel. However, we continued using labeled peptides in the EPL reaction for generating all PrP variants containing fluorophores, as described in the sections above 4.1.3.1 and 4.1.3.2.

*Figure 27. SDS-PAGE analysis of sulfo-Cy5 labeled T_PrP **7c_T**. Coomassie stained lane 1 and 2 show LMW marker and sulfo-Cy5 labeled T_PrP **7c_T** (MW 20,250 Da) at ~20 kDa. Lane 2 subjected to fluorescence-based detection results in lane 3, showing the Cy5 fluorescence of **7c_T**, but also bands in the low molecular range corresponding to the labeling reagent, sulfo-Cy5 maleimide **5c**. PrP structure was taken from PDB 1QM1 of NMR data from hPrP (aa 90-230), modified using PyMOL, ChemDraw 15.0 and CorelDRAW X7 softwares.*

4.2 Secondary structure & *in vitro* aggregation

PrP^C can be converted into PrP^{Sc}. In the latter form, it exhibits a distinct secondary structure and novel properties. Hence, a more detailed characterization of the conformation of the generated PrP constructs is important. The following sections address the secondary structure analysis of the full-length FL_PrP, central hydrophobic region deleted Δ CR_PrP and N-terminally truncated T_PrP variants modified with a GPI anchor mimic compared to recombinant PrP, and, in addition, characteristics of PrP subjected to aggregation prone conditions.

4.2.1 Native secondary structure of PrP

Most recombinantly expressed PrP variants are deposited in inclusion bodies in *E. coli*, probably due the hydrophobic nature of the membrane-anchored protein and the overproduction that impedes proper folding, including the formation of the structurally important disulfide bridge of PrP.^{56, 270} To obtain the native structure comprising a Cys179-Cys214 disulfide bond, PrP variants were subjected to folding conditions previously optimized by Chu *et al.*²³³ This procedure provided folded non-modified (**5_{FL}**, **5_{ACR}**, **5_T**), lipidated (**6_{FL}**, **6_{ACR}**, **6_T**, **6_{Ala-T}**), Cy5 (**7_{FL}**, **7_{ACR}**, **7_T**) and fluorescein labeled PrP variants (**8_{FL}**, **8_{ACR}**, **8_T**) in yields ranging from 37 to 78% (Table 6).

PrP variant	folding yield (%)	PrP variant	folding yield (%)
5_{FL} , non-modified FL	50	8_{ACR} , fluorescein labeled Δ CR	57
6_{FL} , lipidated FL	78	5_T , non-modified T	37
7_{FL} , Cy5 labeled FL	69	6_T , lipidated T	57
8_{FL} , fluorescein labeled FL	45	6_{Ala-T} , lipidated T*	23
5_{ACR} , non-modified Δ CR	73	7_T , Cy5 labeled T	64
6_{ACR} , lipidated Δ CR	42	8_T , fluorescein labeled T	62
7_{ACR} , Cy5 labeled Δ CR	51		

Table 6. Folding yields of PrP variants. Non-modified (**5_{FL}**, **5_{ACR}**, **5_T**), lipidated (**6_{FL}**, **6_{ACR}**, **6_T**, **6_{Ala-T}**), Cy5 (**7_{FL}**, **7_{ACR}**, **7_T**) and fluorescein (**8_{FL}**, **8_{ACR}**, **8_T**) labeled PrP variants could be obtained in their native structure after folding. *Folding yield was determined after the sample had been concentrated. Low amounts of **6_{Ala-T}** were available. Thus, to reach the minimal concentration necessary for CD measurements, first the sample had to be concentrated.

In accordance with previous measurements,^{35, 40, 233, 353, 354} all PrP variants exhibit typical far-UV CD spectra of predominantly α -helical proteins with a characteristic double minimum at 208 and 222 nm (Figure 28). The CD spectra of constructs with

modifications, namely a membrane anchor (**6**) and fluorescent labels (**7**, **8**), are very similar to the ones of non-modified PrP (**5**). This indicates that the incorporated C-terminal GPI anchor mimic in combination with fluorophores does not effect the overall secondary structure of PrP.

Figure 28. Far-UV CD spectra of PrP variants. The secondary structures of lipidated, Cy5 and fluorescein labeled FL_PrP **6_{FL}**, **7_{FL}**, **8_{FL}** (**A**), Δ CR_PrP **6_{ΔCR}**, **7_{ΔCR}**, **8_{ΔCR}** (**B**) and T_PrP **6_T**, **6_{Ala-T}**, **7_T**, **8_T** (**C**) variants are compared to their corresponding non-modified PrP variants **5_{FL}**, **5_{ΔCR}**, **5_T**.

Moreover, according to CDNN software used for analysis of the distribution of secondary structure elements, significant α -helical levels of up to 37%, next to random coil levels of around 30% contribute to the structure but only minor amounts of β -sheet contents down to 16%. This is consistent with 36% content of α -helices for PrP^C, as shown by Safar *et al.*³⁵³ (Table 7). However, variations in the α -helical and β -sheet levels with the PrP batches have been observed, depending on the exact concentrations of PrP and the glutathione-based redox system.²³³ Minimal differences from 0.3/3 mM of oxidized and reduced glutathione led to less α -helical contents.^{355, 356} Still, any misfolded forms of PrP were eliminated in a previous centrifugation step. We argue that

the correct disulfide linkage is achieved for all PrP constructs based on similar CD results for the non- and modified PrP variants that agree with the findings by Maiti *et al.*⁵⁶ These findings connect the formation of the disulfide bridge and PrP conformation by using PrP with cysteines replaced by alanine residues (C179A/C214A) in structural studies. Additional evidence for this conclusion comes from the identical CD spectrum of **6_{Ala-T}**,²⁵⁴ a variant with the introduced cysteine at the ligation site reduced to alanine (for more information section 1.5.1). Thus, **6_{Ala-T}** contains only two native cysteines as wt PrP^C and rules out any possibility for incorrect intramolecular disulfide formation.

PrP variant	α -helix	β -sheet	β -turn	random coil
5_{FL} , non-modified FL	32.6	19.2	17.4	30.8
6_{FL} , lipidated FL	27.3	23.8	17.6	31.3
7_{FL} , Cy5 labeled FL	34.6	17.0	17.2	31.3
8_{FL} , fluorescein labeled FL	25.5	25.5	17.4	31.6
5_{ACR} , non-modified Δ CR	29.5	20.9	17.5	32.1
6_{ACR} , lipidated Δ CR	22.1	29.1	17.2	31.5
7_{ACR} , Cy5 labeled Δ CR	21.3	30.7	17.3	30.7
8_{ACR} , fluorescein labeled Δ CR	26.1	24.1	17.3	32.5
5_T , non-modified T	37.4	15.8	16.5	30.3
6_T , lipidated T	29.9	20.9	17.5	31.7
6_{Ala-T} , lipidated T	28.4	20.6	16.9	34.0
7_T , Cy5 labeled T	26.5	22.0	16.9	34.5
8_T , fluorescein labeled T	34.8	15.6	16.6	33.0

Table 7. Calculated percent levels of secondary structure elements from far-UV CD data of PrP variants, using CDNN software.

4.2.2 *In vitro* PrP aggregation

As for other neurodegenerative disorders, the progression of prion diseases is characterized by advancing neuronal loss and the presence of PrP aggregates.²²⁴ A common technique to study the kinetics of fibril formation *in vitro* is based on using the benzothiazole salt thioflavin T (ThT) (Scheme 19). Upon binding to cross- β -sheet-rich structural elements in fibrils the fluorescence quantum yield of ThT increases by several orders of magnitude, mainly ascribed to a rotation barrier between the benzothiazole and aminobenzoyl rings relative to each other in the excited state. Thus, quantification of fibril formation is facilitated.³⁵⁷

Scheme 19. Structure of Thioflavin T (ThT).

Our group has previously evaluated the impact of the folding state^{252, 253} and the attachment of a GPI anchor mimic²⁴⁷ by monitoring aggregation. Chu *et al.*²⁵² found a different aggregation behavior for recombinant solubilized and refolded PrP variants compared to denatured PrP. Findings by Olschewski *et al.*²⁴⁷ showed a delay in the aggregation process for lipidated over non-modified PrP. Araman *et al.*²⁵³ studied PrP variants modified with polyethyleneglycol (PEG) units as mimics for *N*-glycans. Only small changes to the secondary structure were induced when compared to wt PrP. Intriguingly, *in vitro* aggregation of PEGylated PrP was abrogated under conditions at which wt PrP aggregated. Here, in using a protocol based on Breydo *et al.*,²⁹² the aggregation course of recombinant, folded FL_PrP **5_{FL}** in different concentrations was investigated. The kinetics of fibril formation were monitored by *in situ* incubation with ThT in a microplate reader. As anticipated, we could show that increasing PrP concentrations correlate with rising fluorescence intensities and decreasing lag times (Figure 29). Exact values of ThT fluorescence depend on several factors, including yields of fibril formation,³⁵⁸ differences in ThT binding affinity and purity of PrP. Thus, ThT fluorescence intensity values allow assessing the yield and quality of formed fibrils. In the aggregation course after reaching a plateau, fluorescence intensities often showed a decline that seems to occur due to sorption of the fibrils to the plate walls and/or partial aggregation into clumps.

concentration (μM)	n	average intensity $\pm$ SD	average lag time (h) $\pm$ SD
0.5	2	262 ± 44.5	$102 (\sim 4 \text{ d}) \pm 9.9$
4.3	3	935 ± 279.8	11 ± 2.1

Figure 29. Influence of PrP concentration on aggregation. Aggregation courses (A) resulted in higher maximum intensities and shorter lag times (B) for 4.3 μM PrP compared to 0.5 μM .

To rule out the possibility of ThT directly affecting the aggregation and to further assess the characteristics of the formed PrP fibrils, additional measurements were performed. At specific time points during the aggregation process aliquots of the aggregation mixture were withdrawn, ThT added and the fluorescence determined with a spectrofluorometer (Figure 30A, B). In this setup PK digestion experiments were feasible too. Here PrP conversion proceeded similarly to the process with ThT being present throughout the aggregation. Besides, FL_PrP $\mathbf{5}_{\text{FL}}$ in its native folded state was completely degraded by the endopeptidase K, contrary to the aggregated species showing a resistant fragment ($\sim$ aa 90-230) with the size of ~ 16 Da, typical for PrP^{Sc}.^{292, 359} Surprisingly, only a short time after incubation with the aggregation buffer (30 min), PK-resistant bands appeared. However, at the same time no increase in ThT fluorescence was observed. This could be due to the formation of β -oligomers,³⁶⁰

showing lower ThT-binding capacities, but nevertheless limited PK digestion (Figure 30C).

Figure 30. Following the aggregation process of PrP via ThT-based fluorescence and PK digestion. During the aggregation process of 11 μ M FL_PrP 5_{FL}, at different time points samples were withdrawn, and either after (*ex situ*) addition of ThT subjected to fluorescence measurements (A, B), or PK digested (C) (ratio PrP:PK = 50:1). Fluorescence values at 480 nm were extracted from the recorded fluorescence spectra (A) and depicted as a function of time (B). In C, Coomassie stained SDS-gels show correctly folded (left) and aggregated 5_{FL} (right) prior and after PK treatment. For aggregating 5_{FL}, lane 3-9 are PK digested samples from the aggregation process, withdrawn at time points: 0.5 (lane 3), 2.5 (lane 4), 6 (lane 5), 23 (lane 6), 25 (lane 7), 28 (lane 8) and 47 h (lane 9).

4.2.2.1 Benzamide derivatives as potential inhibitors of PrP aggregation

According to previous findings by Fiorino *et al.*,²⁷⁸ the benzamide derivatives **6h,i,m,o** and **7f,n,o** act as potential antiprion agents (Scheme 9 in section 3.4.4). In “scanning for intensely fluorescent targets” (SIFT) assays these compounds showed strong binding affinities to PrP^C, thus preventing its association with PrP^{Sc}, and supposedly its subsequent conversion. Also, determined by cell lysis and PK digestion the amount of PrP^{Sc} accumulating in scrapie-infected mouse neuroblastoma (ScN2a) cells was reduced upon incubation with the inhibiting compounds. Thus, we anticipated to find direct evidence for the inhibition of PrP^C conversion following this process *in vitro* based on

ThT fluorescence. To this end, different concentrations of non-modified FL_PrP **5_{FL}** and antiprion agents were subjected to aggregation applying a protocol based on Breydo *et al.*²⁹² To elucidate whether DMSO, a solvent required for dissolving the benzamide compounds, affects the aggregation course, its addition together with increasing PrP concentrations was studied (Figure 31).

concentration (μ M)	n	average intensity $\pm$ SD	average lag time (h) $\pm$ SD
1.3	3	122 $\pm$ 53.9	137 (~6 d) $\pm$ 10.2
2.6	1	366	54
4.3	3	606 $\pm$ 279	25 $\pm$ 8.7
11	3	1822 $\pm$ 240.4	26 $\pm$ 7.0

Figure 31. Influence of concentration of PrP in buffers supplemented with DMSO on aggregation.

As observed before (Figure 29), increasing PrP concentration correlated with rising fluorescence intensities and decreasing lag times. Moreover, in encompassing a broader range of concentrations a linear correlation between PrP concentration and the fluorescence intensity, meaning the amounts of formed fibrils, was detected. Also, PrP concentration showed an inversely proportional exponential dependence with the lag

time, i.e. the onset of aggregation. As anticipated, we found that the presence of DMSO only showed a minimal impact on the fibril formation (Figure 31).

Therefore, we continued testing the influence of inhibitors at 10 μM concentrations, as previously used in SIFT assays,²⁷⁸ on the aggregation of 4.3 μM PrP. All compounds, except **6o** and **7n**, induced longer lag times of 30 to 48 h, compared to 25 h for just PrP (Table 8). Compound **7f** showed using even higher PrP concentrations of 11 μM a delay of the onset of aggregation with 37 h, compared to 26 h for only PrP. However, the fluorescence intensities observed displayed higher values, which could, among several other factors, indicate a stronger binding affinity towards ThT once the aggregation process had been initiated.²⁹²

compound	compound c (μM)	PrP c (μM)	PrP ratio to compound (1:x)	n	average intensity $\pm$ SD	average lag time (h) $\pm$ SD
7o	10	1.3	7.7	2	310 $\pm$ 19.8	135 $\pm$ 8.5 (~6 d)
7o	100	1.3	76.9	2	269 $\pm$ 55.2	143 $\pm$ 0.0 (~6 d)
7o	500	1.3	384.6	2	405 $\pm$ 123.7	116 $\pm$ 9.2 (~5 d)
7o	10	2.6	3.8	2	792 $\pm$ 870.4	66 $\pm$ 32.5
7o	100	2.6	38.5	2	648 $\pm$ 491.4	47 $\pm$ 0.0
6h	10	4.3	2.3	3	1065 $\pm$ 514.5	34 $\pm$ 9.3
6i	10	4.3	2.3	2	1562 $\pm$ 369.1	35 $\pm$ 4.2
6m	10	4.3	2.3	2	1534 $\pm$ 145.7	38 $\pm$ 9.2
6o	10	4.3	2.3	3	951 $\pm$ 481.9	21 $\pm$ 2.0
7f	10	4.3	2.3	2	854 $\pm$ 787.0	30 $\pm$ 14.8
7n	10	4.3	2.3	2	1080 $\pm$ 196.6	23 $\pm$ 9.9
7o	10	4.3	2.3	2	946 $\pm$ 579.8	48 $\pm$ 5.7
7o	10	11	0.9	2	2274 $\pm$ 126.6	20 $\pm$ 4.2
7n	10	11	0.9	2	2149 $\pm$ 869.0	37 $\pm$ 4.2
7f	10	11	0.9	3	2621 $\pm$ 626.6	11 $\pm$ 2.1

Table 8. Testing benzamide derivatives **6h,i,m,o** and **7f,n,o** for their potential antiprion activity during the PrP aggregation process. Fluorescence intensities and lag times under varying conditions were evaluated and compared to values resulting from PrP aggregation without compounds.

As the antiprion agent **7o** showed the most promising results by delaying the lag time of PrP aggregation (PrP at 4.3 μM concentration), it was chosen for additional experiments using lower concentrations of PrP together with higher amounts of the inhibitor (up to its solubility limit). 10 μM of **7o** showed with decreasing concentrations in steps of 10, 4.3, 2.6 and 1.3 μM PrP extending lag times and decreasing fluorescence intensities. In order to find conditions for the maximum inhibition of PrP aggregation the amounts of **7o** were enhanced at lower PrP concentrations. Indeed, at 1.3 μM PrP rising the

concentration of **7o** from 10 to 100 μM caused a further reduction of the fluorescence intensity and extension of the lag time. Yet, using higher concentrations of **7o** (500 μM) or PrP (2.6 μM) seemed to reverse this effect. This may be due to a specific concentration limit above which increasing concentrations of compounds, PrP or inhibitor, present within the aggregation solutions favor the aggregation process.

We anticipated to find direct evidence for the inhibition of PrP^C conversion by the benzamide derivatives **6h,i,m,o** and **7f,n,o** based on findings by Fiorino *et al.*²⁷⁸ Our attempt to work within concentration ranges and ratios used in their previous PrP^C-PrP^{Sc} association and PrP^{Sc} accumulation assays²⁷⁸ (low nM PrP concentration with 10 μM inhibitor in ratios of 1:10 000 for PrP:inhibitor) was impeded by at least micromolar concentration of PrP required for *in vitro* aggregation (Table 8). Taken together, a conclusive statement and comparison to previous assays²⁷⁸ giving straightforward results is rather difficult due to the complexity and sensitivity of the aggregation process, and in this regard the impossibility of using same concentration conditions. Considering these aspects, delays in lag times, even if not showing a clear correlation with concentration, can be seen as convincing in regard of the antiprion activity of the benzamide compounds.

4.3 Lipid bilayer interactions

As a GPI-anchored protein PrP^C is predominantly found at the outer leaflet of the neuronal cell membrane.^{25, 346} As discussed in section 1.4 the GPI anchor can impact PrP^{Sc} formation. Moreover, several studies indicate that the membrane environment is a key element in PrP^C conversion into toxic PrP^{Sc}.^{201, 209} Methods to release PrP^C from the cell surface or to disrupt the transport of PrP^C to the plasma membrane show complete prevention or reduction of the conversion.^{200, 202} Due to the complexity of biological membranes, and since protein-membrane interactions are often governed by the physicochemical properties of the lipid bilayer, using simplified model membranes over neuronal cells or cell extracts is often preferred to enable initial studies. Previous work performed in our group by Chu *et al.*²³³ demonstrates that PrP variants containing a GPI anchor mimic show different interaction modes with phospholipid liposomes, depending on sequence variations and lipid composition. This association influences the biochemical and conformational properties of the constructs in a distinct way compared to recombinant PrP lacking this posttranslational modification.

In performing sensitive quartz crystal microbalance-dissipation (QCM-D) experiments with real-time monitoring, we anticipated to gain further quantitative insights into PrP-membrane interactions. In contrast to surface plasmon resonance (SPR) spectroscopy, another common surface-based technique, QCM-D allows additional characterization of the viscoelastic properties of the adsorbed film. By measuring the changes in frequency of a resonating quartz crystal, mass variations are reported combined with energy dissipation revealing the softness, hence viscoelasticity, of the adsorbed film. Supported lipid bilayers consisting of POPG and DOPC lipids were generated by the solvent-assisted lipid bilayer (SALB) method according to an optimized protocol developed earlier by Cho *et al.*²⁹³⁻²⁹⁵ The principle of SALB formation is based on findings from Hohner *et al.*,³⁶¹ demonstrating that a gradual increase in the solvent water fraction of inverted micelles formed by lipids dissolved in organic solvents causes the formation of micelles and monomers that are capable to adhere onto the substrate. In agreement with Figure 32, the process of forming planar bilayer platforms started with aqueous buffer equilibration of the QCM-D setup, followed by an exchange with isopropanol (at 5 min). Then (at 20 min), lipids dissolved in organic solvents (DOPC in isopropanol and POPG in ethanol), mixed in molar ratios

of 50/50 and 70/30 to a final concentration of 0.5 mg/mL, were deposited onto the substrate. After incubation for 10 min (at 30 min), a rapid re-exchange to the aqueous buffer solution induced bilayer formation. Stable bilayers showed frequency and dissipation shifts of -25.6 ± 0.55 Hz and $0.4 \pm 0.27 \times 10^{-6}$, respectively, corresponding to a hydrated mass of 450 ng/cm^2 and a thickness of 4.5 nm, as shown previously by Tabaei *et al.*²⁹³

Figure 32. *SALB* formation. QCM-D frequency (A) and dissipation shifts (B) were recorded at the 3rd, 5th, 7th, 9th and 11th overtone as a function of time during bilayer formation.

With supported lipid bilayers (SLBs) established, QCM-D measurements were utilized to track PrP adsorption under variation of pH values, ionic strength and the amount of negatively charged lipids within the bilayer. Buffers of 20 mM sodium acetate and of 10 mM Tris-HCl with 2 mM sodium acetate, 150 mM NaCl, both supplemented with 2 mM OG for the lipidated PrP variants, were used for measurements at pH 5, mimicking the endosomal compartments³⁶² with low ionic strength, and at pH 7.5 under physiological, cytoplasmic conditions. The PrP variants possess isoelectric points (pIs) between 8.8 to 9.6, therefore have a positive net charge under acidic and neutral conditions. As previous experiments^{233, 363, 364} on the interaction of PrP variants with liposomes consisting of non-charged compared to anionic lipids showed stronger binding affinities for the latter one, we were interested in studying a possible effect of the fraction of POPG, by raising it from 30 to 50%. POPG contents higher than that impede stable, reproducible bilayer formation due to charge-based repulsion. The corresponding QCM-D traces are depicted in section 4.3.1.1 in Figures 35-37 and Tables 9-11, with an overview of the results in Figure 33.

However, no notable impact of the increase in negative membrane surface charge could be observed. This is probably due to a too small variation of the POPG content, barely affecting the overall surface charge of the bilayer in an experimental setup studying protein interaction under continuous flow. Previously Cho and co-workers³⁶⁵ estimated surface charges of SLBs based on zeta potential measurements of lipid vesicles of equivalent lipid membrane composition. The zeta potential for 70/30 DOPC/POPG vesicles was -50.4 ± 2.9 mV. Evans *et al.*³⁶⁶ found potentials around -40 mV for negatively charged DOPG vesicles, a negatively charged lipid structurally related to POPG. These similar values point towards minor changes in zeta potentials and surface charges for lipid compositions of 70/30 and 50/50 DOPC/POPG.

At physiological pH and ionic strength, on a membrane with a non-raft, negatively charged composition we observed a tendency for a better adsorption of non-modified (**5_{FL}**, **5_{ACR}**) compared to lipidated PrP variants (**6_{FL}**, **6_{ACR}**) (Figure 33B, D). In previous studies this lipid composition has induced changes of the secondary structure of PrP.^{210, 233, 364} Unlike to PrP variants lacking a GPI anchor mimic lipidated PrP variants did not undergo conformational changes into β -sheet-rich structures.²³³ In addition to slowing down PrP aggregation in a way that inhibits conformational changes, the lipid anchor may also lead to less binding of PrP to negatively charged lipids. Here, frequency and dissipation shifts for non-modified (**5_{FL}**, **5_{ACR}**, **5_T**) and lipidated PrP constructs (**6_{FL}**, **6_{ACR}**, **6_T**) in the range of -4.5 to -13.3 Hz, 0.4 to 1.8×10^{-6} and -2.4 to -4.6 Hz, 0.7 to 1.2×10^{-6} occurred (Tables 9-11 in section 4.3.1.1). It has been suggested by Sanghera *et al.*²¹⁰ that following to an initial electrostatic interaction directed by the *N*-terminal tail of PrP hydrophobic lipid-PrP binding takes place.^{212, 233} Thus, at continuous flow conditions with no enhancement of the electrostatic interactions we propose that the initial binding step is kinetically disfavoured for lipidated PrP variants compared to non-modified PrP variants. The presence of the GPI anchor mimic seems to slow down the interactions of the *N*-terminal tail with negatively charged membranes. Intriguingly, the difference between the lipid-adsorbed amounts of the T_PrP variants (**5_T**, **6_T**), missing the *N*-terminal domain, was significantly smaller. This confirms the importance of the *N*-terminal domain in leading PrP to membranes.

Figure 33. Frequency and energy dissipation shifts of membrane association of non-modified and lipidated PrP variants at pH 5 with low ionic strength (left, **A** and **C**) and at physiological pH 7 (right, **B** and **D**). Changes in frequency and energy dissipation after protein adsorption of non-modified 5_{FL} , $5_{\Delta CR}$, 5_T and the corresponding lipidated constructs 6_{FL} , $6_{\Delta CR}$, 6_T on bilayers with POPG/DOPC in ratios of 30/70 and 50/50, respectively, are depicted. Error bars indicate the average values and standard deviations of two to five independent measurements.

At pH 5, mimicking the endosomal compartments, together with low ionic strength, hence dominating electrostatic interactions, significantly greater frequency shifts covering a broad range of -16.6 to -55.3 Hz for PrP adsorption occurred (Figure 33A, C). At low ionic strength electrostatic interactions between protein and the polar phospholipid headgroups become more dominant. The increased PrP adsorption at acidic pH agrees with previous findings by Morillas *et al.*³⁶³ and Henriques *et al.*³⁶⁷ Human PrP constructs of rPrP (aa 23-231), (aa 23-145) and (aa 90-231) showed increased binding at pH values from 7 to 4. At pH 5 compared to pH 7 the positive net charge of PrP increases.³⁶⁸ Moreover, the impact of ionic strength on PrP-lipid binding

appeared to be more significant than pH changes.^{363, 367} Enhanced electrostatic interaction led to an increased PrP concentration in close proximity to the bilayer, that enabled studying the role of the hydrophobic interactions in greater detail.

Under these conditions lipidated (**6_{FL}**, **6_T**) PrP variants tended to be adsorbed more efficiently than non-modified variants (**5_{FL}**, **5_T**). Twice the molar amount of lipidated **6_{FL}** compared to non-modified **5_{FL}** bound to the membrane, indicating a stronger affinity once the initial electrostatic interaction had been established. Yet, the binding of non-modified and lipidated Δ CR_PrP (**5_{ΔCR}**, **6_{ΔCR}**) appeared to be identical. Previously when using Δ CR_PrP associated with liposomes, the lipidated form was capable of pore formation, whereas the non-modified protein was not.²³³ It is common that pore-forming proteins beyond a specific concentration limit remove lipids from the bilayer and adsorb to a proteolipid or a protein film.^{369, 370} Such an behavior may provide an explanation for the similar values of non-modified and lipidated Δ CR_PrP (**5_{ΔCR}**, **6_{ΔCR}**). We hypothesize that concomitant with pore formation the loss of lipids results in similar shifts for lipidated **6_{ΔCR}** to non-modified **5_{ΔCR}**, the latter showing less membrane binding and no lipid removal. However, no dramatic changes in the viscoelastic properties indicating pore formation could be observed. This could be due to the “lipid-PrP exchange process” happening very fast, as indicated previously by measurements based on cation influx.²³³

The observed changes in the dissipation of 0.3 to 1.2×10^{-6} suggest a rigid conformation for the membrane-associated lipidated and non-modified PrP constructs. The values are similar to the ones obtained at physiological buffer conditions. An exception is **6_{FL}** with slightly higher final values of 3.0 ± 1.16 and $1.5 \pm 0.67 \times 10^{-6}$ (Table 9 in section 4.3.1.1). These increased dissipation values indicate a distinct binding mode to the bilayer for full-length PrP equipped with a GPI anchor mimic compared to the other PrP variants. Within this specific environment, comprising negatively charged lipids, pH 5 and low ionic strength, a viscoelastic character may be attributed to membrane-associated lipidated FL_PrP. Previous studies postulated that the endosomal pathway is involved in PrP^{Sc} formation and accumulation in neuronal cells.^{195, 362} Acidic pH agrees with endosomal conditions. A viscoelastic character of membrane-associated **6_{FL}** can be attributed to protein binding in an oligomeric state. Thus, conditions inducing aggregation on bilayers for PrP containing a GPI anchor mimic may have been identified.

To gain additional insights on the bilayer integrity and properties of membrane-associated PrP variants, mass and thickness values were compared and calculated applying the Sauerbrey²⁹⁶ and viscoelastic Voigt-Voinova²⁹⁷ model, respectively (Tables 14, 15 in section 4.3.1.1). More detailed information about the Sauerbrey and Voigt-Voinova model is provided in section 3.4.5. At physiological pH and ionic strength, only slightly increased values for fits based on Voigt-Voinova were obtained, indicating that the Sauerbrey model was valid as well. A rigid conformation of membrane-associated PrP could be re-confirmed, as already minor shifts in dissipation had suggested. At pH 5, almost indistinguishable mass and thickness values for both models were determined (Figure 34). This originates from the low ionic strength and indicates a very tight and rigid bilayer-PrP complex.

Noteworthy, the frequency and dissipation shifts obtained at pH 5 conditions before and after re-equilibration to pH 7 buffer conditions used for the bilayer fabrication were the same, confirming that the bilayer was intact, though subjected to low ionic strength solutions.

Figure 34. Mass and thickness shifts of membrane association of non-modified and lipidated PrP variants at pH 5 with low ionic strength (left, **A** and **C**) and at physiological pH 7 (right, **B** and **D**). Changes in mass and thickness of the formed bilayers consisting of 50/50 and 30/70 POPG/DOPC, respectively, after protein adsorption are calculated applying the Sauerbrey and viscoelastic Voigt-Voinova model. Error bars indicate the average values and standard deviations of two to five independent measurements.

All PrP variants once bound to the membrane demonstrated a strong irreversible association, as shown previously.³⁶⁴ After PrP adsorption at pH 5 and pH 7 conditions, variations of the ionic strength (between 3 M and no NaCl) did not show any impact on frequency or dissipation shifts (data not shown). This finding is best explained by a strong hydrophobic interaction between PrP and lipids that remains unaffected under varying buffer conditions. The usage of NaOH, capable to disrupt weak electrostatic interactions, removed ΔCR , but also lipids, as indicated by an additional frequency gain of $41 \pm 10\%$ ($n = 3$) relative to the normalized frequency decrease caused by PrP

binding. Basic pH of 9.5 without any other change of the buffer composition did not disrupt the PrP-lipid binding either, but led to conformational changes of $5_{\Delta CR}$, as can be seen in Figure 40 in section 4.3.1.1.

Taken together, we could show that our PrP constructs interact with negatively charged lipid bilayers under physiological pH and ionic conditions forming stable PrP-lipid complexes. This strong binding could be assigned to hydrophobic interactions. The PrP-lipid association was further increased in an acidic, low ion strength environment that enhances the electrostatic interactions. Upon increasing the PrP concentration in close proximity of the bilayer by enhancing the initial electrostatic interaction, lipidated PrP variants (6_{FL} , 6_T), in particular the full-length PrP construct (6_{FL}), exhibited a higher binding affinity towards the bilayer compared to recombinant PrP. In agreement with other studies^{203, 204,204, 205, 233} these findings corroborate the significance of the GPI anchor in PrP-membrane interactions.

4.3.1.1 *Combined data from QCM-D monitoring of PrP adsorption on SLB platforms*

This section comprises the QCM-D traces of PrP variants subjected to lipid compositions of POPG/DOPC in ratios of 30/70 and 50/50 at physiological and acidic pH with distinct ionic strength. Data for non-modified and lipidated FL, ΔCR and T_PrP constructs are found in Figures 35-37 and Tables 9-11. Different PrP bulk concentrations at physiological pH and ionic strength revealed a diffusion-limited protein uptake (Figures 38, 39 and Tables 12, 13). Calculated mass and thickness values after PrP adsorption according to the Sauerbrey and Voigt-Voinova model are summarized in Tables 14 and 15. Changes in pH from 7.5 to 9.5 of the buffer solution conducted with the intention to disrupt the PrP-lipid binding, eventually resulted in a conformational change of membrane-associated $5_{\Delta CR}$ in Figure 40.

Figure 35. Following the SLB adsorption of FL_PrP constructs. Lipidated (left, A and C) and non-modified (right, B and D) FL_PrP constructs were subjected to membrane environments at and acidic physiological pH.

FL_PrP	pH	POPG/DOPC (%)	n	relative f (Hz) $\pm$ SD	relative D (10^{-6}) $\pm$ SD
non-modified, 5_{FL}	5	30/70	4	-23.6 $\pm$ 9.48	0.5 $\pm$ 0.35
	5	50/50	3	-16.6 $\pm$ 6.85	0.3 $\pm$ 0.37
	7	30/70	4	-6.0 $\pm$ 4.65	0.7 $\pm$ 0.91
	7	50/50	3	-7.8 $\pm$ 7.10	1.3 $\pm$ 1.81
lipidated, 6_{FL}	5	30/70	3	-55.3 $\pm$ 3.73	3.0 $\pm$ 1.16
	5	50/50	2	-42.5 $\pm$ 8.19	1.5 $\pm$ 0.67
	7	30/70	4	-2.9 $\pm$ 0.83	1.0 $\pm$ 0.34
	7	50/50	5	-3.0 $\pm$ 2.83	0.8 $\pm$ 1.33

Table 9. QCM-D measurement response after accomplished 5_{FL} and 6_{FL} adsorption applying different SLB platforms, pH and ionic strength conditions.

Figure 36. Following the SLB adsorption of Δ CR_PrP constructs. Lipidated (left, A and C) and non-modified (right, B and D) Δ CR_PrP constructs were subjected to membrane environments at acidic and physiological pH.

Δ CR_PrP	pH	POPG/DOPC (%)	n	relative f (Hz) $\pm$ SD	relative D (10^{-6}) $\pm$ SD
non-modified, 5_{ACR}	5	30/70	3	-30.0 ± 9.95	0.7 ± 0.61
	5	50/50	2	-38.3 ± 5.87	0.8 ± 0.37
	7	30/70	5	-13.3 ± 4.45	1.8 ± 1.48
	7	50/50	3	-11.4 ± 1.12	0.8 ± 0.44
lipidated, 6_{ACR}	5	30/70	2	-27.9 ± 10.81	0.7 ± 0.80
	5	50/50	2	-39.4 ± 14.46	1.0 ± 0.84
	7	30/70	5	-3.5 ± 2.68	0.7 ± 0.30
	7	50/50	2	-2.4 ± 0.62	1.1 ± 0.18

Table 10. QCM-D measurement response after accomplished 5_{ACR} and 6_{ACR} adsorption applying different SLB platforms, pH and ionic strength conditions.

Figure 37. Following the SLB adsorption of T_{PrP} constructs. Lipidated (left, **A** and **C**) and non-modified (right, **B** and **D**) T_{PrP} constructs were subjected to membrane environments at acidic and physiological pH.

T_{PrP}	pH	POPG/DOPC (%)	n	relative f (Hz) $\pm$ SD	relative D (10^{-6}) $\pm$ SD
non-modified, 5r	5	30/70	3	-25.6 ± 2.79	0.3 ± 0.19
	5	50/50	2	-26.4 ± 5.81	0.3 ± 0.18
	7	30/70	5	-4.5 ± 0.53	0.4 ± 0.22
	7	50/50	2	-5.9 ± 0.02	0.5 ± 0.11
lipidated, 6r	5	30/70	2	-30.0 ± 4.55	0.8 ± 0.21
	5	50/50	2	-39.4 ± 8.79	1.2 ± 0.97
	7	30/70	2	-3.6 ± 0.02	1.2 ± 0.21
	7	50/50	2	-4.6 ± 4.18	1.1 ± 1.85

Table 11. QCM-D measurement response after accomplished $5r$ and $6r$ adsorption applying different SLB platforms, pH and ionic strength conditions.

We investigated the effect of the bulk protein concentration of non-modified $5_{\Delta CR}$ and lipidated 6_T on membrane association (Figures 38, 39). For both cases, with and without the lipid anchor, concentration-dependent frequency shifts were monitored, indicating that the rate of protein uptake was limited by the diffusion in the bulk solution. The rigidity of membrane-associated PrP variants increased with rising bulk protein concentration.

Figure 38. Concentration dependence of lipidated 6_T on membrane association. Changes in frequency and energy dissipation upon interaction with POPG/DOPC (30/70) at pH 7 using bulk concentrations of 0.5 and 1 μM 6_T are depicted. Error bars indicate the average values and standard deviations of two independent measurements.

c (μM)	n	relative f (Hz) $\pm$ SD	relative D (10^{-6}) $\pm$ SD
0.5	2	-3.6 $\pm$ 0.02	1.2 $\pm$ 0.21
1	2	-6.0 $\pm$ 0.11	0.3 $\pm$ 0.07

Table 12. Comparison of QCM-D measurement response using different bulk concentrations of 6_T , together with POPG/DOPC in a ratio of 30/70 at pH 7.

Figure 39. Concentration dependence of non-modified 5_{ACR} on membrane association. Changes in frequency and energy dissipation upon interaction with POPG/DOPC (30/70) at pH 7 using bulk concentrations of 0.1 and 1 μM 5_{ACR} are depicted. Error bars indicate the average values and standard deviations of three to five independent measurements.

c (μM)	n	relative f (Hz) $\pm$ SD	relative D (10^{-6}) $\pm$ SD
0.1	3	-4.1 ± 1.29	0.8 ± 0.31
1	5	-7.9 ± 3.22	0.22 ± 0.35

Table 13. Comparison of QCM-D measurement response using different bulk concentrations of 5_{ACR} , together with POPG/DOPC in a ratio of 30/70 at pH 7.

PrP	POPG/ DOPC (%)	n	Sauerbrey mass (ng/cm ²) ± SD	viscoelastic mass (ng/cm ²) ± SD	Sauerbrey thickness (nm) ± SD	viscoelastic thickness (nm) ± SD
5 _{FL}	50/50	3	291 ± 120.1	283 ± 136.1	2.6 ± 1.09	2.6 ± 1.24
5 _{FL}	30/70	4	413 ± 170.9	523 ± 340.0	3.8 ± 1.55	5.0 ± 3.54
6 _{FL}	50/50	2	745 ± 143.6	741 ± 162.4	6.8 ± 1.31	6.7 ± 1.48
6 _{FL}	30/70	3	969 ± 65.4	967 ± 61.3	8.8 ± 0.59	8.8 ± 0.56
5 _{ACR}	50/50	2	672 ± 102.9	686 ± 113.3	6.1 ± 0.94	6.2 ± 1.03
5 _{ACR}	30/70	3	526 ± 174.4	528 ± 172.1	4.8 ± 1.59	4.8 ± 1.57
6 _{ACR}	50/50	2	691 ± 253.6	699 ± 257.3	6.3 ± 2.31	6.4 ± 2.34
6 _{ACR}	30/70	2	489 ± 189.7	499 ± 176.7	4.4 ± 1.72	4.5 ± 1.61
5 _T	50/50	2	462 ± 101.9	471 ± 101.1	4.2 ± 0.93	4.3 ± 0.92
5 _T	30/70	3	450 ± 49.0	452 ± 62.6	4.1 ± 0.45	4.3 ± 0.66
6 _T	50/50	2	691 ± 154.2	702 ± 137.5	6.3 ± 1.40	6.4 ± 1.25
6 _T	30/70	2	527 ± 79.8	539 ± 73.5	4.8 ± 0.73	4.9 ± 0.67

Table 14. Calculated shifts in mass and thickness at pH 5 after accomplished adsorption of non-modified and lipidated PrP constructs, using the Sauerbrey and viscoelastic Voigt-Voinova model.

PrP	POPG/ DOPC (%)	n	Sauerbrey mass (ng/cm ²) ± SD	viscoelastic mass (ng/cm ²) ± SD	Sauerbrey thickness (nm) ± SD	viscoelastic thickness (nm) ± SD
5 _{FL}	50/50	3	137 ± 124.5	279 ± 350.5	1.2 ± 1.13	2.8 ± 3.53
5 _{FL}	30/70	4	106 ± 81.6	288 ± 248.9	1.0 ± 0.74	2.9 ± 2.51
6 _{FL}	50/50	5	54 ± 49.7	141 ± 100.1	0.5 ± 0.45	1.4 ± 1.00
6 _{FL}	30/70	4	51 ± 14.6	90 ± 71.0	0.5 ± 0.13	0.9 ± 0.73
5 _{ACR}	50/50	3	200 ± 19.6	218 ± 41.7	1.8 ± 0.18	2.1 ± 0.51
5 _{ACR}	30/70	5	234 ± 78.1	796 ± 392.3	2.1 ± 0.71	7.8 ± 3.99
6 _{ACR}	50/50	2	42 ± 10.9	168 ± 16.5	0.4 ± 0.10	1.7 ± 0.16
6 _{ACR}	30/70	5	61 ± 47.1	105 ± 43.6	0.6 ± 0.43	1.0 ± 0.44
5 _T	50/50	2	104 ± 0.3	223 ± 170.1	0.9 ± 0.00	2.2 ± 1.77
5 _T	30/70	4	82 ± 7.3	83 ± 6.2	0.8 ± 0.07	0.8 ± 0.06
6 _T	50/50	2	81 ± 73.3	169 ± 204.6	0.7 ± 0.67	1.7 ± 2.05
6 _T	30/70	2	64 ± 0.3	91 ± 31.1	0.6 ± 0.00	0.9 ± 0.35

Table 15. Calculated shifts in mass and thickness at pH 7 after accomplished adsorption of non-modified and lipidated PrP constructs, using the Sauerbrey and viscoelastic Voigt-Voinova model.

The change in pH from 7.5 to 9.5 of the buffer solution induced a conformational change of membrane-associated 5_{ACR} , accompanied by a decrease in viscosity and shear viscosity (data not shown). This state was characterized by spreading frequency shifts within several harmonics, increased dissipation as well as large differences in calculated mass and thickness values applying the Sauerbrey and viscoelastic Voigt-Voinova model (Figure 40B, D). With respect to initial values, mass and thickness increased according to Sauerbrey and Voigt-Voinova from 60 to 70% and 180 to 170%, respectively.

Figure 40. Impact of low ionic strength and pH on membrane-associated 5_{ACR} Frequency and dissipation shifts (left, **A** and **C**) at several harmonics showed after PrP adsorption (step 1) onto the POPG/DOPC (30/70) bilayer, followed by a re-exchange to the initial buffer conditions at pH 7 (step 2) and incubation with buffers absent of salt (step 3), a deficiency of impact changing the ionic strength with re-exchange to initial pH 7 buffer conditions (step 4). Buffers of pH 9.5 (step 5) displayed after re-exchange back to the initial buffer conditions at pH 7 (step 6) slightly decreasing and spreading frequency shifts (**A**) together with increasing dissipation values (**C**). Application of Sauerbrey and Voigt-Voinova models resulted in higher mass and thickness values (**B** and **D**).

4.4 Studying PrP in its cellular environment

PrP^C is a GPI-anchored protein processed in the rough endoplasmic reticulum (ER) and after travelling through the Golgi apparatus it is found on the outer leaflet of the plasma membrane. However, the precise localization in neurons remains enigmatic due to conflicting data obtained by different techniques.¹⁹⁵ Characterizing the exact localization of PrP^C on the cell membrane and its conversion into the toxic form PrP^{Sc} is important for identifying the mechanisms underlying this process and parameters impacting it. To address this, we started with a simple automatic system providing an overview of PrP added to a large number of cells. Widefield imaging, supported by super-resolution structured illumination microscopy (SR-SIM), with co-localization analysis confirmed the membrane localization of PrP. In addition, complementary confocal imaging and fluorescence correlation spectroscopy (FCS) measurements on live cells provided an insight into the diffusion behavior of PrP. These experiments provided new insights into the relationship between PrP mobility on the membrane and the cellular membrane organization.

4.4.1 Automated imaging

The first step to systematically assess the localization and behavior of PrP in neuron-like SH-SY5Y cells was imaging on microplates. Thereby, we gained an overview of how Cy5 labeled PrP variants containing a GPI anchor mimic interacted with the cellular membrane within a live cell environment. This approach allowed rapid and multiple measurements with quantifiable estimations of PrP-cell membrane binding. Additionally, it offered a convenient route to establish conditions for further microscopy studies with SH-SY5Y cells.

We found that our lipidated, Cy5 labeled constructs **7_{FL}** and **7_{ΔCR}** were located at the cell membrane. Figure 41A and C clearly show the high specificity of these PrP variants for the cell membrane. Images look similar to data generated by Breydo *et al.*,²⁴⁴ in which SH-SY5Y cells were incubated with myristoylated PrP for 1 h and immunostained.

Quantification of the amounts of Cy5 labeled PrP at the cellular membrane was accomplished with average object sum integrals. These values describe the sum of Cy5 intensities on the cell membrane, which is defined by the area of the CellMaskTM green intensities, divided by the number of cells determined by Hoechst 33258 staining.

Within 1 h of incubation amounts of **7_{FL}** and **7_{ΔCR}** with average object sum integrals of 0.6×10^7 and 1.2×10^7 RIU per cell were determined (Figure 42). However, these amounts were too low for membrane localization analysis in imaging studies using higher magnifications.

Longer incubation times resulted in more PrP on the membrane but also in increased aggregation of PrP in solution. For **7_{FL}** the average object sum integral of 0.6×10^7 increased to 0.7×10^7 RIU per cell after 1 or 1.5 h of incubation, respectively. Out-of-focus PrP in solution forming clusters can be seen as brighter larger dots in Figure 41A for **7_{FL}** and in Figure 41C for **7_{ΔCR}** addition.

In order to accelerate the process of PrP transfer from solution to the cell membrane and to achieve a more homogenous accumulation, we employed the cationic liposome-based transfection agent Pro-JectTM. According to the manufacture's protocol this reagent encapsulates proteins based on non-covalent interactions, and thereby does not interfere with the biological activity.^{371, 372} Previously Pro-JectTM had been used successfully for transferring lipidated **6_T** onto the plasma membrane of mouse neuroblastoma N2a cells by Olschewski *et al.*²⁴⁷ Similar to transiently transfected cells with PrP^C carrying a 3F4 epitope, lipidated **6_T** could be detected on the membrane of fixed cells by immunostaining with the 3F4 mAb. For non-modified **5_T** less efficient binding could be observed.

Microplate assays with Pro-JectTM showed an increased and homogenous accumulation on the cell membrane for **7_{ΔCR}** and **7_{FL}**, as can be seen in Figure 41B and D. Quantification revealed 5- to 7-fold higher amounts of **7_{ΔCR}** and **7_{FL}** at the cell membrane after 1 h with values similar to the GPI anchor-mimicking peptide **3** without Pro-JectTM.

Figure 41. Images of SH-SY5Y cells incubated with 7_{FL}, 7_{ΔCR} and peptide 3 in the presence and absence of Pro-Ject™ acquired after 1 h. Hoechst 33528 staining of the nuclei (left), CellMask™ green staining of the cell membrane (middle) and Cy5 fluorescence of PrP variants (right). In F, control images without any staining show brightfield (left) and Cy5 fluorescence (right).

Noteworthy, ProJect™ also increased the membrane transfer of peptide 3 based on confocal imaging (data not shown). Compared to 7_{FL} more 7_{ΔCR} was localized at the cellular membrane by adding PrP with and without Pro-Ject™ (Figure 42).

Additional control measurements without staining the nuclei and the cell membrane were performed using brightfield and Cy5 imaging to exclude any stainings artefacts affecting PrP localization within cells (Figure 41F).

PrP	Pro-Ject™	average object sum integral (RIU) per cell	SD (RIU)
7 _{FL}	-	0.6×10^7	0.34×10^7
7 _{ΔCR}	-	1.2×10^7	0.17×10^7
7 _{FL}	+	4.4×10^7	1.19×10^7
7 _{ΔCR}	+	5.6×10^7	1.33×10^7
3	-	5.7×10^7	0.70×10^7

Figure 42. Cy5 object sum integrals of PrP variants on the membrane of SH-SY5Y cells after 1 h incubation. Error bars indicate standard deviations of three (n = 3) independent measurements.

With these experiments of PrP-membrane binding and optimization of the delivery of PrP to cell membranes, we have successfully set the framework for our further studies.

4.4.2 PrP-membrane co-localization studied by structured illumination microscopy (SIM) imaging

To precisely localize PrP^C on the cell membrane is important for identifying pivotal parameters and mechanisms underlying the conversion into its toxic isoform PrP^{Sc}. To this end, microscopy together with specific compartment labeling dyes and co-localization analysis enabled quantification and statistical interpretation of localization data of our labeled PrP variants (7_{FL}, 7_{ΔCR}) within a complex biological environment.

As the veracity of co-localization is limited by the dimensions defined by the optical system, we compared images acquired with widefield and SIM technique. SIM provides super-resolution (SR) information, i.e. it narrows down the uncertainty of physical dimensions implied by the limits of resolution from the optical system. The gain of resolution can be directly measured by the full width at half maximum (FWHM) values of the peak intensity profiles extracted from the images acquired (Panels **C** and **D** in Figures 45, 47, 49 of section 4.4.2.1). More detailed information about the SIM technique is provided in section 3.4.6.3.1.

Images of neuron-like SH-SY5Y cells were acquired with the widefield and SIM technique. Prior to fixation these cells were incubated for 1 h with Cy5 labeled **7_{FL}**, **7_{ΔCR}** or the GPI anchor-mimicking peptide **3** in presence of the transfection agent Pro-Ject™ and Cellmask™ green stained membranes (Figures 44-49 in section 4.4.2.1). As described in more detail in section 3.4.6.1 SH-SY5Y cells were first subjected to a membrane staining procedure, incubated with PrP, and after washing with HBSS buffer fixed according to a fixation protocol.

For quantification and statistical interpretation of co-localization between PrP constructs and the cellular membrane in the three-dimensional space Pearson's (r_p or PCC) and Manders' (M_1 , M_2) coefficients^{317, 318} were calculated and evaluated with thresholds and significances (p -values) determined by the Costes' approach.³¹⁶ Detailed information regarding co-localization analysis can be found in section 3.4.6.3.2. In Costes' automatic thresholding, initial limits are set to the maximum intensity of each channel and progressively decremented. PCCs are calculated for each increment of thresholded image pairs. The final Costes' thresholds are then set to values below which PCC is zero or negative. Additional statistical significance by Costes *et al.*³¹⁶ was introduced in comparing a randomized to the original image and evaluating the significance (p -value) of the PCC, meaning the probability of obtaining the specified PCC by chance.

The application of widefield microscopy resulted in PCCs and overlap coefficients (r) close to 1 for **7_{FL}**, **7_{ΔCR}** and **3**, indicating complete co-localization. Interestingly, SIM reconstruction yielded lower PCC and r values in the mid-range, illustrating how the uncertainty due to diffraction of light affected the quantitative measurements of molecular interactions (Figure 43D).

Additional evidence for co-localization could be determined with Manders' (M_1 , M_2) coefficients close to 1. These coefficients were statistically evaluated as highly probable according to p -values by Costes' of 100% (Table 16).

PrP	technique	r_p	M_1	M_2	p -value	ICQ	r	k_1	k_2
7_{FL}	widefield microscopy	0.68	1.00	0.95	100	0.38	0.89	0.56	1.42
7_{ΔCR}		0.79	1.00	1.00	100	0.29	0.96	0.96	0.97
3		0.91	1.00	0.98	100	0.45	0.96	1.32	0.70
7_{FL}	SIM	0.27	0.94	0.91	100	0.27	0.56	0.70	0.44
7_{ΔCR}		0.44	0.87	0.89	100	0.28	0.66	0.40	1.10
3		0.54	0.95	0.95	100	0.39	0.73	0.97	0.55

Table 16. Co-localization analysis of **7_{FL}**, **7_{ΔCR}**, **3** on the cell membrane of SH-SY5Y cells. Pearson's (r_p), Manders' (M_1 , M_2) and overlap (r with k_1 , k_2) coefficients were calculated and evaluated with thresholds and significances (p -values) determined by the Costes' approach using ImageJ software^{300, 301} with the JACoP^{314, 373} plugin. Li's intensity correlation quotients (ICQ) from the respective intensity correlation analyses (ICA) of the two channels are listed as well.

Based on the intensity correlation analysis (ICA) representations by Li³¹⁹ (Figures 44-49 in section 4.4.2.1) intensity correlation quotients (ICQ) values close to 0.5 were identified (Table 16). ICA shows a set of two graphs capable of intensity representation and co-localization assessment. Normalized intensities (0-1) are plotted as a function of the covariance for each channel. For all cases, including widefield and SR images of **7_{FL}**, **7_{ΔCR}** and **3**, distributions were situated on the right side of the y-axis in agreement with co-localizing pixels (Panels **F**, **G** in Figures 44, 46, 48 and Panels **E**, **F** in Figures 45, 47, 49 of section 4.4.2.1).

Plots of the Van Steensel's³¹⁵ cross-correlation functions (CCFs) were obtained by shifting one of the images relative to the other and afforded bell-shaped curves with their maxima located at $\delta x = 0$ for widefield and SIM images. Hence, complete co-localization was confirmed. The different heights can be assigned to noise and/or distinct fluorescence intensities during image acquisition and processing (Figure 43).

Figure 43. Van Steensel's cross-correlation functions (CCFs) and Costes' evaluation data for 7_{FL} , $7_{\Delta CR}$, 3 on the cell membrane of SH-SY5Y cells acquired using widefield and SIM technique. **A** displays CCFs for 7_{FL} , **B** CCFs for $7_{\Delta CR}$ and **C** CCFs for 3 . **D** shows the Pearson's correlation coefficients (PCCs) from the original and randomized images used in the determination of the significance or p -value. All p -values were 100%.

Taken together, microscopy applying different imaging techniques combined with quantitative and statistical co-localization analysis verified membrane localization of the Cy5 labeled PrP constructs containing a GPI anchor mimic in SH-SY5Y cells.

4.4.2.1 Combined widefield & SIM images with data from the co-localization analysis

This section contains the full widefield and SR images of membrane binding of Cy5 labeled 7_{FL} , $7_{\Delta CR}$, and 3 , and the regions of interest (ROIs) chosen for co-localization analysis (Panels **A**, **B**, **C**, **D** in Figures 44, 46, 48 and Panels **A**, **B** in Figures 45, 47, 49). Intensity profiles illustrate the resolution gain obtained by SIM over widefield technique (Panels **C**, **D** in Figures 45, 47, 49). Projections of the Costes' masks reflect the co-localization determined by the automatic thresholds (Figures 44, 46, 48E). ICA plots

by Li^{319} are depicted in Panels **F**, **G** in Figures 44, 46, 48 and Panels **E**, **F** in Figures 45, 47, 49.

Figure 44. SR images of SH-SY5Y cells incubated with 7_{FL} . Images show the CellMask™ green (**A**) and Cy5 fluorescence (**B**) of the membrane and the peptide. **C** and **D** are the ROIs used in the co-localization analysis. In **E** a maximum intensity Z-projection of the Costes' mask (white: co-localization, black: background, red: Cy5 fluorescence, green: CellMask™ green fluorescence) illustrates the co-localization based on calculated thresholds according to Costes' statistical significance algorithm.³¹⁶ Intensity correlation analysis (ICA) plots by Li^{319} represent the co-localizing pixels of the Cellmask™ green fluorescence with Cy5 in **F** and of the Cy5 fluorescence with Cellmask™ in **G**.

Figure 45. Widefield images of SH-SY5Y cells incubated with 7_{FL}. The ROIs show the CellMask™ green (A) and Cy5 fluorescence (B) of the membrane and the peptide used in the co-localization analysis. C and D illustrate the resolution gain for the CellMask™ green and Cy5 channel with the FWHM values of the intensity profiles that are extracted from the images acquired with widefield and SIM technique. Intensity correlation analysis (ICA) plots by Li³¹⁹ represent the co-localizing pixels of the CellMask™ green fluorescence with Cy5 in E and of the Cy5 fluorescence with CellMask™ in F.

Figure 46. SR images of SH-SY5Y cells incubated with $7_{\Delta CR}$. Images show the CellMask™ green (A) and Cy5 fluorescence (B) of the membrane and the peptide. C and D are the ROIs used in the co-localization analysis. In E a maximum intensity Z-projection of the Costes' mask (white: co-localization, black: background, red: Cy5 fluorescence, green: CellMask™ green fluorescence) illustrates the co-localization based on calculated thresholds according to Costes' statistical significance algorithm.³¹⁶ Intensity correlation analysis (ICA) plots by Li³¹⁹ represent the co-localizing pixels of the CellMask™ green fluorescence with Cy5 in F and of the Cy5 fluorescence with CellMask™ in G.

Figure 48. SR images of SH-SY5Y cells incubated with **3**. Images show the CellMask™ green (**A**) and Cy5 fluorescence (**B**) of the membrane and the peptide. **C** and **D** are the ROIs used in the co-localization analysis. In **E** a maximum intensity Z-projection of the Costes' mask (white: co-localization, black: background, red: Cy5 fluorescence, green: CellMask™ green fluorescence) illustrates the co-localization based on calculated thresholds according to Costes' statistical significance algorithm.³¹⁶ Intensity correlation analysis (ICA) plots by Li³¹⁹ represent the co-localizing pixels of the CellMask™ green fluorescence with Cy5 in **F** and of the Cy5 fluorescence with CellMask™ in **G**.

Figure 49. Widefield images of SH-SY5Y cells incubated with **3**. The ROIs show the CellMask™ green (A) and Cy5 fluorescence (B) of the membrane and the peptide used in the co-localization analysis. C and D illustrate the resolution gain for the CellMask™ green and Cy5 channel with the FWHM values of the intensity profiles that are extracted from the images acquired with widefield and SIM technique. Intensity correlation analysis (ICA) plots by Li³¹⁹ represent the co-localizing pixels of the Cellmask™ green fluorescence with Cy5 in E and of the Cy5 fluorescence with Cellmask™ in F.

4.4.3 Membrane organization in the presence of PrP studied by imaging and FCS

Super-resolution microscopy confirmed the membrane localization of our PrP constructs containing a GPI anchor mimic. This paved the way to study the mutual relation between PrP and the cellular membrane organization on a molecular level within living cells. Details of the membrane structure and mechanisms responsible for its dynamic organization are provided in section 4.4.3.1.

4.4.3.1 Cellular plasma membrane organization

The cellular plasma membrane is a highly dynamic complex fluid structure with several levels of organization at different spatiotemporal scales. It is composed of a lipid bilayer with numerous proteins either bound to the surface or spanning the membrane and coupled, *via* some of these proteins, to the cortical actin cytoskeleton. Artificial lipid bilayers³⁷⁴ and cells³⁷⁵ were demonstrated to have lateral fluidity. The fluidity of the lipid matrix is influenced by lipid-lipid, lipid-protein and protein-protein interactions as well as cytoskeleton confinement.³⁷⁶ Due to this lateral heterogeneity the membrane organization cannot be described by individual models but requires a combination of multiple models,³⁷⁷ as outlined in the three-tiered mesoscale (2-300 nm) domain organization model proposed by Kusumi *et al.*³⁷⁸ (Figure 50).

Following a hierarchical architecture, the first tier consists of membrane compartments with cell-dependent³⁷⁹ sizes of 40-300 nm in diameter formed due to partitioning of the entire plasma membrane by the actin cytoskeleton (fence) and transmembrane proteins anchored to the cytoskeleton (pickets). As described in the picket-fence model by Fujiwara *et al.*^{380, 381} the hydrodynamic friction of the immobilized transmembrane proteins causes the diffusion of membrane components around them to slow down. Thus the physical and diffusion barrier provided by the cytoskeleton and the immobilized transmembrane proteins give an explanation for lipids in the plasma membrane possessing diffusion rates 5-100 times smaller than that compared to model membranes.^{382, 383} Plasma membrane molecules undergo short-range free diffusion within a fence with a diffusivity similar to free diffusion in model membranes, as described by the fluid mosaic model,³⁸⁴ but also slower long-range diffusion across multiple fences. The second tier consists of membrane raft domains of 2-20 nm in diameter. The membrane raft model was eventually proposed by Simons and Ikonen,³⁸⁵ after studies had found non-Brownian diffusion³⁸⁶ and non-ionic detergent (Triton-X-100) resistant membrane (DRM) constituents.³⁸⁷ The *Keystone Symposium on Lipid Rafts and Cell Function* provided a general definition of membrane rafts as small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts sometimes are stabilized to form larger platforms through protein-protein and protein-lipid interactions.³⁸⁸ The topic of rafts is still controversial due to the lack of direct evidence for their existence. The continuous revision of the definition of rafts is a result of their varying physical properties.^{389, 390} Nevertheless, the presence of membrane domains with diverse physical properties has

been established indirectly by different biophysical techniques.³⁹¹ Lastly, the third tier comprises dynamic protein complexes, being smallest in size with 3-10 nm. The dynamic interplay between these three mesoscale domains regulates membrane function and processes cooperatively.

Figure 50. Model for plasma membrane organization. Scheme was adopted from Ng *et al.*³⁴³

Thus, in the highly dynamic and spatially heterogeneous membranes, their lipid composition and membrane properties influence the nanoscale organization and function of membrane proteins. Hence, it is important to investigate the mobility of both biomolecules, proteins and lipids, within the membrane to find a potential link to their functionality.³⁷⁷

4.4.3.2 Membrane organization studied by confocal imaging & FCS

Assessing diverse molecular dynamics in live specimen is a challenge well met by fluorescence correlation spectroscopy (FCS). By measuring dynamic processes such as diffusion and interactions within typical time scales of micro- to milliseconds, FCS provides a quantitative approach to identify potential mechanisms at the molecular level. This technique utilizes the detection of spontaneous temporal fluorescence fluctuations to generate an autocorrelation function (ACF) *via* statistical analysis and mathematical transformation. From the ACF a multitude of measurement parameters, including diffusion coefficients and the state of aggregation, can be extracted. For further details regarding FCS measurements and analysis see section 3.4.6.4.1. In our studies, FCS measurements were conducted at *z*-positions below the upper membrane at the cell boundary. For the external addition of PrP to SH-SY5Y cells, insufficient PrP localization at the bottom of the cell membrane was observed under the conditions applied (see section 3.4.6.4.1). This could be due to the adherence of the cells to the cover slip which forms a physical barrier, together with the slow diffusion and strong

anchoring of PrP to the membrane once attached. Pre-incubation of cells with PrP during the cell splitting process did not lead to satisfactory PrP-membrane binding either. This is probably caused by the distinct characteristics, such as decreased levels of plasma membrane activity, of undifferentiated SH-SY5Y cells compared to major neurons and differentiated SH-SY5Y cells.³⁹²

Confocal FCS measurements were performed on labeled PrP and membrane probes to assess membrane diffusion and impact on organization. In all confocal measurements, PrP refers to the Cy5 labeled construct **7_{FL}**. Unless otherwise stated cells were incubated with PrP and ProjectTM for 30 min, washed with HBSS buffer and then subjected to staining protocols or drug treatments. It should be noted that measurements at the upper membrane were occasionally hampered by PrP in solution that freely diffused and showed a stronger tendency to aggregate, resulting in a large number of spikes present in the fluorescence intensity traces complicating ACF analysis. This was rectified by segmenting the intensity trace in the absence of large fluorescence spikes and computing the ACFs thereafter.

For assessing PrP interactions with the cellular membrane, we have analyzed PrP mobility together with the diffusion of different membrane probes, that are associated with several levels of membrane organization. These include the fluid matrix enriched with unsaturated phospholipids, the cholesterol-dependent membrane domains and the actin cytoskeleton. The organic dye DiI-C₁₈ served as a membrane marker for the outer leaflet exhibiting free diffusion with negligible domain confinement.³⁹³⁻³⁹⁵ Domain confinement describes the energy a probe needs to escape from the middle of the membrane domain and results in different trapping and diffusion times of the probe. In contrast, the green fluorescent protein-glycosylphosphatidylinositol anchored protein (GFP-GPI AP) resides on the outer leaflet primarily in cholesterol-dependent domains. Their composition includes membrane rafts that are mainly enriched with sphingolipids and glycosphingolipids, next to cholesterol. The cholesterol-dependent domains are suggested to impact and regulate cellular processes such as membrane trafficking, endocytosis, signal transduction and host-pathogen interactions.^{146, 396} Importantly, palmitoylation, including GPI-anchoring, of proteins was suggested to play a major role in the targeting of proteins to membrane rafts and subsequently leads to enhanced signalling.^{391, 397} Compared to the fluid matrix enriched with unsaturated phospholipids, the cholesterol-dependent domains are more ordered and tightly packed, thus impeding the mobility of GFP-GPI AP. However, the integrity of these more ordered domains can

be disrupted by incubation with methyl- β -cyclodextrin (m β CD) leading to a faster diffusion of GFP-GPI AP.^{299, 395} Cyclodextrins can modify the cholesterol content of cell membranes. Though the mechanism is not completely established, cyclodextrins are capable to extract cholesterol from cell membranes probably due to the presence of a relatively hydrophobic cavity.³⁹⁸ In this study a concentration of 3 mM m β CD was applied that does not affect the viability of cells.³⁹⁹ Moreover, the plasma membrane is compartmentalized by the actin cytoskeleton into membrane compartments that act as diffusion barriers to membrane biomolecules, as described by the picket-fence model (section 4.4.3.1).^{380, 381} Partial depolymerization induced by addition of latrunculin A (LatA) was observed to reduce the trapping time of these compartments.³⁸¹ Noteworthy with respect to PrP, recent studies suggest the involvement of the dynamic cortical actin in the nanoclustering of GPI-anchored proteins on the plasma membrane.⁴⁰⁰⁻⁴⁰³

Taken together, the dynamics of the PrP interaction with the cellular membrane of SH-SY5Y cells were assessed using DiI-C₁₈ as a freely-diffusing marker, GFP-GPI AP with m β CD for the cholesterol-dependent domains and LifeAct-GFP transfected SH-SY5Y cells treated with LatA for the impact of the actin cytoskeleton, all in combination with the diffusion of Cy5 labeled PrP itself. Previously it has been shown that in SH-SY5Y cells the diffusion of DiI-C₁₈ is unaffected by cholesterol removal and depolymerization of the actin cytoskeleton using the drugs m β CD and LatA, respectively.^{299, 395, 404} This demonstrates that the compounds affect specific membrane domains.

The confocal FCS data obtained from Cy5 labeled PrP 7_{FL} on SH-SY5Y cell membrane was fitted with a two-component model (2D, 2p) to yield the diffusion coefficients and fractions of two diffusive species, namely a fast component representing 7_{FL} in the extracellular space ($D \sim 30 \mu\text{m}^2/\text{s}$, fraction $\sim 60\%$), and a slow component corresponding to membrane-bound 7_{FL} ($D \sim 0.6 \mu\text{m}^2/\text{s}$, fraction $\sim 40\%$). Similar values were obtained in SH-SY5Y cells, subjected to no treatment, staining (DiI-C₁₈), transient (GFP-GPI AP) and stable transfection (LifeAct-GFP). Noteworthy, the fraction of the slow component (F_{slow}) increased upon addition of Pro-JectTM reflecting its importance in delivering PrP to the cell membrane (Table 17).

cells	D_{slow} ($\mu\text{m}^2/\text{s}$)	D_{fast} ($\mu\text{m}^2/\text{s}$)	F_{slow}	N_m	N_{cells}
SH-SY5Y	0.67 ± 0.386	28.93 ± 10.729	0.40 ± 0.259	3	2
SH-SY5Y*	0.49 ± 0.280	36.55 ± 12.785	0.29 ± 0.199	2	1
DiI-C ₁₈ stained SH-SY5Y	0.59 ± 0.035	15.25 ± 5.266	0.53 ± 0.070	2	1
GFP-GPI AP SH-SY5Y	0.60 ± 0.130	30.69 ± 12.877	0.36 ± 0.248	5	4
LifeAct-GFP SH-SY5Y	0.64 ± 0.107	25.06 ± 24.157	0.36 ± 0.164	11	7

Table 17. Average D_{slow} and D_{fast} values for **7_{FL}** at the cell membrane. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS. ***7_{FL}** delivered to the cells without the usage of Pro-JectTM.

Based on FCS measurements, the following findings in context with specific membrane domains could be gained and will be discussed in more detail:

- i) PrP containing a membrane anchor affects the membrane organization of SH-SY5Y cells

Subsequent to PrP incubation (30 min) staining of the cell membrane with DiI-C₁₈ and FCS measurements of the dye were conducted. This sequence of treatment was preferred to restrict self-aggregation of the organic dye, which increases with time of incubation on cells. FCS data was fitted applying a one- and two-component model with a single triplet contribution. In good agreement with previous measurements for untreated SH-SY5Y cells, diffusion coefficients of $2.84 \pm 0.408 \mu\text{m}^2/\text{s}$ for DiI-C₁₈ could be determined.^{299, 395, 404} However with increasing time, i.e. after ~ 2 h 30 min, a second slower component ($D \sim 0.7 \mu\text{m}^2/\text{s}$, fraction $\sim 80\%$) was observed in the absence of **7_{FL}**, which most likely occurs due to the self-aggregation propensity of the membrane dye.⁴⁰⁵ With increasing time and aggregation events data sets fitted better with the two-component model (2D, 2p1t) opposed to the one-component model (2D, 1p1t). Upon addition of **7_{FL}**, for both two-dimensional fitting models with a single triplet contribution and one or two particles (2D, 1p1t and 2D, 2p1t), the diffusion coefficient of DiI-C₁₈ was reduced. Besides, already at shorter times after DiI-C₁₈ staining, i.e. after ~ 20 min, more ACF curves fitted better to the 2D, 2p1t model (Figure 51 and Table 18). Thus, the FCS data indicates that PrP enhances the domain confinement within the fluid matrix enriched with unsaturated phospholipids.

Figure 51. Average diffusion coefficients and representative ACFs of DiI- C_{18} in SH-SY5Y cells incubated with 7_{FL} , compared to untreated cells. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements for confocal FCS.

DiI- C_{18}	fit	D ($\mu\text{m}^2/\text{s}$)	D_{slow} ($\mu\text{m}^2/\text{s}$)	F_{slow}	τ_{trip} (μs)	F_{trip}	N_m	N_{cells}
Ctrl	2D, 1p1t	2.84 ± 0.408			4.2 ± 2.94	0.29 ± 0.158	12	6
+PrP	2D, 1p1t	1.97 ± 0.182			2.8 ± 1.30	0.30 ± 0.164	3	1
Ctrl	2D, 2p1t	5.32	0.68	0.77	15.2	0.17	1	1
+PrP	2D, 2p1t	3.97 ± 0.694	0.48 ± 0.190	0.82 ± 0.087	2.3 ± 1.81	0.16 ± 0.150	2	2

Table 18. Confocal FCS results of the DiI- C_{18} fluorescence in SH-SY5Y cells incubated with 7_{FL} compared to untreated cells. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS.

In agreement with the FCS results, confocal images show aggregation of the membrane dye DiI- C_{18} upon addition of 7_{FL} (Figure 52).

Figure 52. DiI- C_{18} stained SH-SY5Y cells incubated with 7_{FL} . Confocal images show DiI- C_{18} and Cy5 fluorescence with their respective brightfield channels for non-treated cells (top) and cells subjected to PrP (bottom).

However, we cannot report on whether the induced aggregation of the free diffusing DiI-C₁₈ is caused *via* a direct or indirect interaction with PrP. We speculate that PrP indirectly affects the free diffusing compartments based on our measurements with m β CD, a cholesterol-depleting agent, and LatA, an actin polymerization inhibitor, described below. These provide direct evidence for PrP accumulating in ordered membrane domains, which is in good agreement with other studies.^{196, 406-408}

Next, SH-SY5Y cells transiently transfected with GFP-GPI AP were incubated with 7_{FL} and subjected to the cholesterol-depleting agent methyl- β -cyclodextrin (m β CD). FCS data of GFP-GPI AP was fitted applying a two-component model with a single triplet contribution. In good agreement with previous measurements^{299, 395, 404} the fitting model yielded D values of ~ 0.5 and $20 \mu\text{m}^2/\text{s}$, with $\sim 50\%$ contribution of each. The slow component corresponds to a membrane-bound fraction and the fast component probably to a cytosolic fraction, as shown for plasma membrane targeting, labeled enhanced GFP (PMT-EGFP).⁴⁰⁹ Data by Bag *et al.*⁴¹⁰ showed that the domain confinement of GFP-GPI AP was removed reversibly by addition of m β CD with the highest effect after ~ 30 min. This time-dependent abolishment of the domain confinement is possibly caused by only partial cholesterol removal during m β CD saturation⁴¹⁰ or by subsequent lipid recruitment as a cellular response to recover the plasma membrane.⁴¹¹

To study the interaction of PrP in cholesterol-rich domains two experimental setups were used:

- 1) Cells were incubated with PrP, washed with HBSS and m β CD was added (Figure 53A). Upon membrane binding PrP increased the fluidity of the cholesterol-dependent domains. However, subsequent addition of m β CD did not lead to increasing D values of GFP-GPI AP after 30 min as previously described (left in Figure 53A, upper part in Table 19). This missing increase of D values of GFP-GPI AP may indicate that PrP sequesters cholesterol upon membrane binding. The diffusion of Cy5 labeled 7_{FL} remained unaffected by m β CD treatment and retained a D value of $\sim 0.6 \mu\text{m}^2/\text{s}$ for the membrane-bound component (right in Figure 53A, B and Table 20).
- 2) To investigate whether cholesterol extraction prior to PrP addition impacts its diffusion, cells were incubated first with m β CD for 15 min (time of half maximum effect) with subsequent exchange for a solution containing PrP and m β CD (Figure 53B).

This resulted in reversible changes in the diffusion of GFP-GPI AP after 30 min as expected for m β CD addition (left in Figure 53B, bottom part in Table 19). Still, the diffusion of Cy5 labeled 7_{FL} remained unaffected by this (right in Figure 53A, B and Table 20).

Figure 53. Average diffusion coefficients and representative ACFs of GFP-GPI AP and 7_{FL} on cells subjected to cholesterol-depleting m β CD. **A** shows diffusion coefficients of cells treated with m β CD subsequent to PrP incubation. **B** shows diffusion coefficients of cells treated with m β CD prior simultaneous PrP and m β CD addition. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements for confocal FCS. **C** shows the structure of m β CD. In **D**, representative ACF curves reflect the increasing diffusion coefficients of GFP-GPI AP upon PrP and m β CD addition that result in narrower ACF curves.

GFP-GPI AP	D_{slow} ($\mu\text{m}^2/\text{s}$)	D_{fast} ($\mu\text{m}^2/\text{s}$)	F_{slow}	τ_{trip} (μs)	F_{trip}	N_m	N_{cells}
untreated	0.53 ± 0.085	19.66 ± 4.527	0.51 ± 0.062	24.3 ± 10.45	0.17 ± 0.040	8	7
+PrP	0.63 ± 0.027	31.49 ± 4.416	0.43 ± 0.145	16.8 ± 11.80	0.17 ± 0.013	4	4
+m β CD (~30 min)	0.43 ± 0.042	37.97 ± 8.839	0.36 ± 0.245	10.7 ± 5.80	0.23 ± 0.065	5	4
untreated	0.52 ± 0.019	18.25 ± 4.616	0.48 ± 0.047	23.4 ± 8.28	0.17 ± 0.029	2	2
+m β CD+PrP (~30 min)	0.79 ± 0.028	29.35 ± 4.868	0.50 ± 0.009	18.7 ± 3.60	0.19 ± 0.016	3	3
+m β CD+PrP (>30 min)	0.57 ± 0.110	29.21 ± 0.995	0.44 ± 0.022	18.8 ± 2.07	0.19 ± 0.022	3	3

Table 19. Average D_{slow} and D_{fast} values for GFP-GPI AP. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS.

7_{FL}	$D_{slow} (\mu m^2/s)$	$D_{fast} (\mu m^2/s)$	F_{slow}	N_m	N_{cells}
7_{FL}	0.60 ± 0.130	30.69 ± 12.877	0.36 ± 0.248	5	4
+ m β CD	0.57 ± 0.106	63.82 ± 24.480	0.48 ± 0.230	5	4
7_{FL} + m β CD	0.66 ± 0.006	90.04 ± 4.307	0.48 ± 0.309	2	1

Table 20. Average D_{slow} and D_{fast} values for 7_{FL} subjected to m β CD. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS.

Based on these measurements we conclude that PrP may sequester cholesterol upon membrane binding and that PrP dynamics are not affected by cholesterol depletion of the plasma membrane.

ii) Cholesterol-rich domains affect the membrane accumulation of PrP

Moreover, m β CD induced cholesterol depletion impacted PrP accumulation on the cellular membrane (Figure 54). After 40 min of 7_{FL} incubation, significantly higher Pearson's correlation coefficients (PCC) were observed for co-localization between GFP-GPI AP and Cy5 labeled 7_{FL} of untreated compared to m β CD pre-treated SH-SY5Y cells (Figure 55 and Table 21). Thus, disturbances in the integrity of the cholesterol-rich domains influence the PrP-membrane binding. This finding agrees with other studies^{196, 210, 406, 408} highlighting the necessity of cholesterol for PrP localization at the cell surface and the role of lipid rafts.

Figure 54. 7_{FL} incubated on GFP-GPI AP transfected SH-SY5Y cells non- and pre-treated with $m\beta CD$. Confocal images show GFP-GPI AP and Cy5 fluorescence with their respective brightfield channels for untreated cells (**top**) and cells subjected to $m\beta CD$ (**bottom**) after 5 min of PrP addition.

Figure 55. Pearson's correlation coefficients (PCCs) for 7_{FL} and GFP-GPI AP subjected to $m\beta CD$. PrP accumulation on the cell membrane was compared after 40 min of PrP incubation for untreated cells and cells subjected to $m\beta CD$ prior PrP addition.

pre-incubation with $m\beta CD$	r_p	p -value	N
-	0.34 ± 0.052	100	8
20 min	0.14 ± 0.014	100	3

Table 21. Co-localization analysis between 7_{FL} and GFP-GPI AP subjected to $m\beta CD$ treatment. The PrP-membrane accumulation was quantified for untreated and $m\beta CD$ pre-treated SH-SY5Y cells using Pearson's coefficients (r_p) and significances (p -values) determined by the Costes' approach. The values were calculated applying ImageJ software^{300, 301} with JACoP^{313, 314} plugin.

iii) Actin cytoskeleton dependence of PrP membrane diffusion

Recent other studies of GPI APs, such as the folate receptor and CD52, have shown a strong anchoring of the proteins to the plasma membrane and also the involvement of the actin cytoskeleton.⁴⁰⁰⁻⁴⁰³ To address such an involvement of the cytoskeleton in PrP interactions with the cellular membrane, partial actin depolymerization was induced by latrunculin A (LatA) treatment.³⁹⁴ However, the cytoskeleton disruption did not render PrP containing a GPI anchor mimic more mobile. It caused a decrease in the diffusion coefficients from 0.6 to 0.4 $\mu\text{m}^2/\text{s}$ for the membrane-bound components (Figure 57 and Table 22). Slower dynamics associated with clustering have been described previously for other peptides and proteins, including PrP itself.^{412, 413} We propose that these slower dynamics of the Cy5 labeled PrP containing a GPI anchor mimic (**7_{FL}**) at the membrane are attributed to PrP clustering, which could resemble the initial step of PrP aggregation. By inhibiting the actin polymerization a physical and diffusion barrier provided by the cytoskeleton was degraded and enabled PrP to aggregate on the cellular membrane. Thus, these findings suggest that an intact actin cytoskeleton can act as a barrier for the conversion of PrP^C into PrP^{Sc} on cell membranes.

Figure 56. **7_{FL}** incubated on LifeAct-GFP transfected SH-SY5Y cells. Confocal images with GFP (A) and Cy5 fluorescence (B).

Figure 57. Average D_{slow} and D_{fast} values for 7_{FL} in LifeAct-GFP transfected SH-SY5Y cells for un- and LatA-treated cells. LatA (structure in B) treated cells were measured for 20-75 min after LatA addition. Control measurements were conducted for the same period of time of PrP incubation. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements for confocal FCS.

7_{FL}	D_{slow} ($\mu m^2/s$)	D_{fast} ($\mu m^2/s$)	F_{slow}	N_m	N_{cells}
Ctrl	0.55 ± 0.151	35.83 ± 1.638	0.29 ± 0.120	3	2
-LatA	0.64 ± 0.107	25.06 ± 24.157	0.36 ± 0.164	11	7
+LatA	0.36 ± 0.120	10.06 ± 3.009	0.42 ± 0.287	3	2

Table 22. Confocal FCS results of the Cy5 fluorescence from 7_{FL} in LifeAct-GFP transfected SH-SY5Y cells for un- and LatA-treated cells. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS.

Overall, studying the dynamic processes of the PrP-membrane interaction on a molecular level with confocal FCS revealed aggregation of the fluid matrix enriched with unsaturated phospholipids induced by PrP containing a GPI anchor mimic (7_{FL}). Increasing diffusion of GFP-GPI AP located in the ordered membrane domains together with less PrP-membrane accumulation caused by cholesterol depletion attributes an important role to cholesterol-dependent domains in PrP-membrane binding. Moreover, FCS revealed that PrP containing a GPI anchor mimic (7_{FL}) exhibits a lower (compared to free diffusing species), cholesterol-independent and cytoskeleton-dependent mobility. Manipulation of the link between PrP and the cytoskeleton by inhibiting of actin polymerization induced an even slower diffusion of Cy5 labeled PrP. Typically, deceleration of diffusion is attributed to protein clustering.^{412, 413} We propose that an intact actin cytoskeleton can act as a barrier for the conversion of PrP^C into PrP^{Sc} on cell membranes. Thus, a crucial role of the cytoskeleton-membrane association that immobilizes PrP *via* the actin cytoskeleton can be hypothesized in the PrP^C-PrP^{Sc} conversion.

Taken together, we gained an improved understanding of the synergistic interplay between PrP, its GPI anchor, the cellular membrane and the cytoskeleton (Figure 58).

Figure 58. Schematic overview of mβCD and LatA effects on the PrP-membrane interaction for PrP containing a GPI anchor mimic.

4.4.3.3 Bottom membrane organization studied with ITIR-FCS

As imaging total internal reflection (ITIR)-FCS measurements of the bottom membrane provide large continuous areas, FCS diffusion law analysis can be used. More information about ITIR-FCS and FCS diffusion law analysis is provided in section 3.4.6.4.4. A continuous membrane image area is required for the binning of pixels to generate the points on the FCS diffusion law plot at various binned areas. Thus, FCS diffusion law analysis presents information of the sub-resolution membrane organization, as described in section 4.4.3.1. Unlike to a confocal FCS setup,⁴¹⁴⁻⁴¹⁷ no special optical design to control the laser beam size or multiple sequential measurements at different spot sizes are required.³³⁸ With huge statistics of data due to several ACFs obtained from one measurement (Figure 60A, B) and spatial information crucial for elucidating the heterogeneity in molecular dynamics ITIR-FCS is a good and fast technique to study the bottom membrane organization.

Therefore, for studying the sub-resolution membrane organization at the lower membrane upon PrP accumulation, ITIR-FCS was applied to compare non-modified

(**5_{FL}**, **5_{ACR}**, **5_T**) and lipidated PrP constructs (**6_{FL}**, **6_{ACR}**, **6_T**). The organic dye DiI-C₁₈ served as marker for the free diffusion within the fluid matrix enriched with unsaturated phospholipids and GFP-GPI AP was used for the cholesterol-dependent membrane domains.

However, ITIR-FCS is limited to a boundary of ~100 nm in axial dimension between sample and cover slide. As discussed above in section 4.4.3.2 external addition of fluorescently labeled PrP to SH-SY5Y cells resulted in PrP localized to a large extent at the cell boundary and top membrane, but only limited at the bottom membrane. This could be re-confirmed with TIR imaging depicting fluorescein labeled **8_{FL}** at the cell border (Figure 59). Thus, for ITIR-FCS PrP constructs without a fluorescent dye and in 5× times higher concentrations of 1 μM were used, as compared to confocal measurements.

Figure 59. Images of SH-SY5Y cells incubated with **8_{FL}** acquired in epi and TIR mode.

As anticipated and agreeing with the confocal FCS measurements DiI-C₁₈ showed larger diffusion coefficients compared to GFP-GPI AP. The additional application of FCS diffusion law analysis provided information on the type of membrane organization with FCS diffusion law intercepts (τ_0) around zero for the free diffusion of DiI-C₁₈ and positive values of ~0.4 indicative for the domain confinement of GFP-GPI AP.

Figure 60. ITIR-FCS measurements of DiI-C₁₈ stained and GFP-GPI AP transfected SH-SY5Y cells incubated with non- and lipidated PrP, 5_{FL} and 6_{FL}. Data are mean values $\pm$ SD of the number of ACFs.

Upon PrP incubation with non- and lipidated 5_{FL} and 6_{FL} DiI-C₁₈ stained SH-SY5Y cells exhibited a minor reduction in the D values concomitant with a slight increase of τ_0 (Figure 60A, C and Table 23). However, as cells were stained prior and not after protein addition we cannot clearly distinguish between this increase in confinement within the disordered membrane region originating from PrP or the self-aggregating properties of DiI-C₁₈, as opposed to the confocal FCS measurements. For the GFP-GPI AP diffusion different behaviors for PrP lacking (5_{FL}) and containing a GPI anchor mimic (6_{FL}) were observed. Whereas lipidated PrP caused an increase of the D values of GFP-GPI AP accompanied by decreasing τ_0 values, no changes occurred for non-modified PrP (Figure 60B,D and Table 23). This disruption of the cholesterol-dependent membrane domains upon 6_{FL} agrees with increasing diffusion coefficients found for the labeled 7_{FL} in confocal FCS measurements. Thus, both full-length PrP constructs containing a GPI anchor mimic (6_{FL}, 7_{FL}) affected the cholesterol-dependent domains by making them

more fluid. This indicates that GPI anchor (mimic) presence induces a distinct PrP-membrane interaction and corroborates its important role.

	PrP	$D (\mu\text{m}^2/\text{s})$	$\tau_0 (\text{s})$	N_m	N_{cells}
DiI-C ₁₈	-	1.82 ± 0.224	0.06 ± 0.020	7	4
	6_{FL}	1.67 ± 0.270	0.08 ± 0.027	7	1
	-	2.08 ± 0.194	0.02 ± 0.017	6	4
	5_{FL}	1.91 ± 0.177	0.04 ± 0.022	13	2
GFP-GPI AP	-	0.39 ± 0.069	0.49 ± 0.072	7	4
	6_{FL}	0.68 ± 0.200	0.30 ± 0.087	14	2
	-	0.51 ± 0.018	0.36 ± 0.078	2	1
	5_{FL}	0.50 ± 0.087	0.35 ± 0.086	5	1

*Table 23. ITIR-FCS data of DiI-C₁₈ stained and GFP-GPI AP transfected SH-SY5Y cells incubated with non- and lipidated PrP, **5_{FL}** and **6_{FL}**. Data are mean values $\pm$ SD of the number of ACFs.*

Additional measurements using central hydrophobic region deleted $\Delta\text{CR_PrP}$ and *N*-terminally truncated T_PrP variants lacking (**5_{ACR}**, **5_T**) and containing a GPI anchor mimic (**6_{ACR}**, **6_T**) did not indicate any conclusive changes on DiI-C₁₈ stained and GFP-GPI AP transfected SH-SY5Y cells (data in section 8.5).

Taken together, ITIR-FCS measurements agree well with confocal FCS data and provided complementary information on the sub-resolution bottom membrane organization and the significance of the GPI anchor. Whereas, the full-length PrP variant not modified with a GPI anchor mimic (**5_{FL}**) did not impact the diffusion of the ordered domains of the plasma membrane, full-length variants containing a GPI anchor mimic (**6_{FL}**, **7_{FL}**) affected the cholesterol-dependent domains by making them more fluid. In showing different impacts on the cholesterol-dependent membrane domains for non- and modified FL_PrP the significance of the introduced PTM in the physiological membrane interaction of PrP is highlighted.

5. Conclusion

The elucidation of the pathophysiologic events in prion diseases, involving the conformational change of cellular (PrP^C) into scrapie prion protein (PrP^{Sc}) that further propagates PrP^C misfolding and aggregation, is challenging. So far, experimental studies have been limited in directly analyzing the earliest events of posttranslationally modified PrP at the cellular membrane, the initial site of prion infection and PrP misfolding, by a lack of suitably modified PrP variants.

To this end, fluorescently labeled, folded PrP variants equipped with glycosylphosphatidylinositol (GPI) anchor-mimicking peptides were generated *via* a semisynthesis strategy. By optimizing the conditions of the key expressed protein ligation (EPL) reaction, the reaction rate was increased. Besides, its application could be extended towards the formation of full-length PrP (aa 23-231) containing a synthetic GPI anchor. Secondary structures of the homogeneous PrP constructs were assessed by circular dichroism (CD). All PrP variants exhibited typical far-UV CD spectra of predominantly α -helical proteins. Thus, the incorporated C-terminal GPI anchor mimic in combination with the fluorophore did not effect the overall secondary structure of PrP. *In vitro* aggregation, monitored by thioflavin T (ThT) fluorescence and proteinase K (PK) resistance, illustrated normal aggregation behavior.

To study the PrP-membrane interaction in detail, we monitored PrP association on artificial supported bilayers in quartz crystal microbalance-dissipation (QCM-D) experiments in real time, varying pH, lipid and salt compositions. We found that the PrP constructs interacted with negatively charged lipid bilayers at physiological pH and ionic strength forming PrP-lipid complexes unable to dissociate due to strong hydrophobic interactions. The PrP-lipid association was further strengthened by enhancing the initial electrostatic interaction in an acidic, low ionic environment that led to increased PrP amounts in close proximity to the bilayer. Thereby, lipidated PrP variants, in particular the full-length PrP construct, exhibited a higher binding affinity towards the bilayer compared to recombinant PrP. This finding corroborates the significance of the GPI anchor mimic in PrP-membrane interactions.

Further cell imaging using super-resolution structured illumination microscopy (SR-SIM) combined with co-localization analysis confirmed the membrane localization of Cy5 labeled PrP constructs equipped with a GPI anchor mimic in neuron-like SH-SY5Y cells. The dynamic behavior of PrP within the ordered and disordered regions of the plasma membrane as well as the cytoskeleton was studied extensively with

fluorescence correlation spectroscopy (FCS). Imaging total internal reflection-FCS (ITIR-FCS) measurements demonstrated that full-length PrP modified with a GPI anchor mimic affected the cholesterol-dependent membrane domains by making them more fluid. No such impact was observed for PrP without a GPI anchor mimic. This emphasizes the significance of the interaction between the cellular membrane, PrP and its GPI anchor in cells.

By combining methyl- β -cyclodextrin (m β CD) treatments, a cholesterol-depleting agent, with addition of latrunculin A (latA), an actin polymerization inhibitor, the interaction of lipidated PrP labeled with Cy5 in its membrane environment was studied. FCS revealed that PrP containing a GPI anchor mimic exhibits a lower (compared to free diffusing species), cholesterol-independent and cytoskeleton-dependent mobility. Manipulation of the link between PrP and the cytoskeleton by inhibiting actin polymerization induced an even slower diffusion of Cy5 labeled PrP. Typically, deceleration of diffusion is attributed to protein clustering, which could resemble the initial step of PrP aggregation. We propose that an intact actin cytoskeleton can act as a barrier for the conversion of PrP^C into PrP^{Sc} on cell membranes. Thus, the cytoskeleton-membrane association that immobilizes PrP *via* the actin cytoskeleton can be hypothesized to play a crucial role in the PrP^C-PrP^{Sc} conversion.

By taking advantage of site-selectively modified PrP variants and biophysical investigations of their interactions with membranes, including a complex live cell setting, we gained an improved understanding of the synergistic interplay between PrP, its GPI anchor, the cellular membrane and the cytoskeleton.

6. Outlook

The data generated here emphasizes defined lipidated PrP variants as important tools to directly study PrP at the cellular membrane, the initial site of prion infection and PrP misfolding. Beyond the data presented in this thesis, it will be important in future work to gain an improved understanding of the function and binding of PrP mutants comprising the *N*-terminal and the central hydrophobic domain. This could reveal sequence-dependent contributions to the initial step of PrP^C-PrP^{Sc} conversion on cell membranes. In more detail, dynamics of Cy5 labeled lipidated PrP constructs T_PrP (aa 90-231) and ΔCR_PrP (aa 23-231 with Δ105-125) should be studied in combination with membrane manipulations, such as cholesterol-depleting agents and actin polymerization inhibitors. Initial confocal FCS data can be found in section 8.6.

Working with labeled PrP variants may be extended towards a synthetic GPI anchor by optimizing the labeling protocol based on maleimide chemistry for folded PrP (section 4.1.3.3). Moreover, studying homogeneous glycosylated PrP variants equipped with a GPI anchor mimic based on a strategy developed by Araman *et al.*²⁵³ (Scheme 6C in section 1.5.1) could give insights on the function of all PTMs involved with PrP.

Another interesting application for the labeled PrP constructs could be the formation of labeled PrP^{res} in PMCA assays. Soto and colleagues at University of Texas Health Science Center in Houston (UTHealth, USA) are currently working towards this aim using our fluorescein labeled PrP construct (8_{FL}).

Addition of infectious PrP seeds to cells incubated with labeled PrP may allow direct oligomer and fibril observation and characterization in cells. Thus, further structural and mechanistic insights into PrP aggregation may be gained and potential species mediating cytotoxicity identified.

In summary, our labeled, lipidated PrP constructs permit several alternate ways to directly investigate PrP conversion *in vitro* and in cells. Thereby, this approach has the potential to elucidate the mechanism of prion conversion, transmission and pathogenicity. Understanding these key features in prion diseases can further serve as paradigm for other neurodegenerative diseases.

7. References

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8. Appendix

8.1 Nucleotide and amino acid sequences of genes

8.1.1 pTWIN1-*Mxe* GyrA intein-CBD murine FL_PrP (aa 23-231) via *XbaI/SpeI*

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1  TATGAAAAAGCGGCCAAAGCCTGGAGGGTG
31  GAACACCGGTGGAAGCCGGTATCCCGGGCA
61  GGGAAGCCCTGGAGGCAACCGTTACCCACC
91  TCAGGGTGGCACCTGGGGGCAGCCCCACGG
121 TGGTGGCTGGGGACAACCCCATGGGGGCAG
151 CTGGGGACAACCTCATGGTGGTAGTTGGGG
181 TCAGCCCCATGGCGGTGGATGGGGCCAAGG
211 AGGGGGTACCCATAATCAGTGAACAAGCC
241 CAGCAAACCAAAAACCAACATGAAGCATAT
271 GGCAGGGGGCTGCGGCAGCTGGGGCAGTAGT
301 GGGGGGCCTTGGTGGCTACATGCTGGGGAG
331 CGCCATGAGCAGGCCCATGATCCATTTTGG
361 CAACGACTGGGAGGACCGCTACTACCGTGA
391 AAACATGTACCGCTACCCTAACCAAGTGTA
421 CTACAGGCCAGTGGATCAGTACAGCAACCA
451 GAACAACCTTCGTGCACGACTGCGTCAATAT
481 CACCATCAAGCAGCACACGGTCACCACCAC
511 CACCAAGGGGGAGAACTTCACCGAGACCGA
541 TGTGAAGATGATGGAGCGCGTGGTGGAGCA
571 GATGTGCGTCACCCAGTACCAGAAGGAGTC
601 CCAGGCCTATTACGACGGGAGAAGATCCAG
631 CTG

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1  MKKRPKPGGWNTGGSRYPGQGSPGGNRYPP
31  QGGTWGQPHGGGWGQPHGGSWGQPHGGSWG
61  QPHGGGWGQGGGTHNQWNKPSKPKTNMKHM
91  AGAAAAGAVVGGLGGYMLGSAMSRPMIHFG
121 NDWEDRYRENMYRYPNQVYYRPVDQYSNQ
151 NNFVHDCVNITIKQHTVTTTTKGENFTETD
181 VKMMERVVEQMCVTQYQKESQAYYDGRSS

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8.1.2 pTWIN1-*Mxe* GyrA intein-CBD murine Δ CR_PrP (aa 23-231 with Δ 105-125) via *XbaI/SpeI*

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1  TATGAAAAAGCGGCCAAAGCCTGGAGGGTG
31  GAACACCGGTGGAAGCCGGTATCCCGGGCA
61  GGGAAGCCCTGGAGGCAACCGTTACCCACC
91  TCAGGGTGGCACCTGGGGGCAGCCCCACGG
121 TGGTGGCTGGGGACAACCCCATGGGGGCAG
151 CTGGGGACAACCTCATGGTGGTAGTTGGGG
181 TCAGCCCCATGGCGGTGGATGGGGCCAAGG
211 AGGGGGTACCCATAATCAGTGAACAAGCC
241 CAGCAAACCAGGCTACATGCTGGGGAGCGC

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271 CATGAGCAGGCCCATGATCCATTTTGGCAA
 301 GACTGGGAGGACCGCTACTACCGTGAAAA
 331 CATGTACCGCTACCCTAACCAAGTGTACTA
 361 CAGGCCAGTGGATCAGTACAGCAACCAGAA
 391 CAACTTCGTGCACGACTGCGTCAATATCAC
 421 CATCAAGCAGCACACGGTCACCACCACCAC
 451 CAAGGGGGGAGAACTTCACCGAGACCGATGT
 481 GAAGATGATGGAGCGCGTGGTGGAGCAGAT
 511 GTGCGTCACCCAGTACCAGAAGGAGTCCCA
 541 GGCCTATTACGACGGGAGAAGATCCAGCTG

1 MKKRPKPGGWNTGGSRYPGQSPGGNRYPP
 31 QGGTWGQPHGGGWGQPHGGSWGQPHGGSWG
 61 QPHGGGWGQGGGTHNQWNKPSKPGYMLGSA
 91 MSRPMIHFGNDWEDRYRENMYRYPNQVYY
 121 RPVDQYSNQNNFVHDCVNITIKQHTVTTTT
 151 KGENFTETDVKMMERVVEQMCVTQYQKESQ
 181 AYYDGRRSS

8.1.3 pTXB3-*Mxe* GyrA intein-His-CBD murine T_{PrP} (aa 90-231) *via*

NcoI/SapI

1 TGGGCCAAGGAGGGGGGTACCCATAATCAGT
 31 GGAACAAGCCCAGCAAACCAAAAACCAACA
 61 TGAAGCATATGGCAGGGGCTGCGGCAGCTG
 91 GGGCAGTAGTGGGGGGCCTTGGTGGCTACA
 121 TGCTGGGGAGCGCCATGAGCAGGCCCATGA
 151 TCCATTTTGGCAACGACTGGGAGGACCGCT
 181 ACTACCGTGAAAACATGTACCGCTACCCTA
 211 ACCAAGTGTACTACAGGCCAGTGGATCAGT
 241 ACAGCAACCAGAACAACTTCGTGCACGACT
 271 GCGTCAATATCACCATCAAGCAGCACACGG
 301 TCACCACCACCACCAAGGGGGAGAACTTCA
 331 CCGAGACCGATGTGAAGATGATGGAGCGCG
 361 TGGTGGAGCAGATGTGCGTCACCCAGTACC
 391 AGAAGGAGTCCCAGGCCTATTACGACGGGA
 421 GAAGATCCAGCT

1 GQGGGTHNQWNKPSKPKTNMKHMAGAAAAG
 31 AVVGGLGGYMLGSAMSRPMIHFGNDWEDRY
 61 YRENMYRYPNQVYYRPVDQYSNQNNFVHDC
 91 VNITIKQHTVTTTTKGENFTETDVKMMERV
 121 VEQMCVTQYQKESQAYYDGRRSS

8.1.4 pcDNA3-EGFP-His human GPI (from DAF, aa 345-381) *via**XhoI/XbaI*

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1  ATGAGCAAGGGCGAGGAGCTGTTCACCGGG
31  GTGGTGCCCATCCTGGTTCGAGCTGGACGGC
61  GACGTAAACGGCCACAAGTTCAGCGTGTCC
91  GGCGAGGGCGAGGGCGATGCCACCTACGGC
121 V AAGCTGACCCTGAAGTTCATCTGCACCACC
151 GGCAAGCTGCCCCGTGCCCTGGCCCCACCCTC
181 GTGACCACCCTGACCTACGGCGTGCAGTGC
211 TTCAGCCGCTACCCCGACCACATGAAGCAG
241 CACGACTTCTTCAAGTCCGCCATGCCCCGAA
271 GGCTACGTCCAGGAGCGCACCATCTTCTTC
301 AAGGACGACGGCAACTACAAGACCCGCGCC
331 GAGGTGAAGTTCGAGGGCGACACCCTGGTG
361 AACCGCATCGAGCTGAAGGGCATCGACTTC
391 AAGGAGGACGGCAACATCCTGGGGCACAAG
421 CTGGAGTACAACTACAACAGCCACAACGTC
451 TATATCATGGCCGACAAGCAGAAGAACGGC
481 ATCAAGGTGAACTTCAAGATCCGCCACAAC
511 ATCGAGGACGGCAGCGTGCAGCTCGCCGAC
541 CACTACCAGCAGAACACCCCCATCGGCGAC
571 GGCCCCGTGCTGCTGCCCCGACAACCACTAC
601 CTGAGTACCCAGTCCAACTGAGCAAAGAC
631 CCCAACGAGAAGCGCGATCACATGGTCCTG
661 CTGGAGTTCGTGACCGCCGCCGGGATCACT
691 CTCGGCATGGACGAGCTGTACAAGCACCAC
721 CATCACCACCACCCAAATAAAGGAAGTGGA
751 ACCACTTCAGGTACTACCCGTCTTCTATCT
781 GGGCACACGTGTTTCACGTTGACAGGTTTG
811 CTTGGGACGCTAGTAACCATGGGCTTGCTG
841 ACT
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1  MSKGEELFTGVVPILVELDGDVNGHKFSVS
31  GEGEGDATYGKLTLKFICTTGKLPVPWPTL
61  VTTLTYGVQCFSRYPDHMKQHDFFKSAMPE
91  GYVQERTIFFKDDGNYKTRAEVKFEGDTLV
121 NRIELKGIDFKEDGNILGHKLEYNYNSHNV
151 YIMADKQKNGIKVNFKIRHNIEDGSVQLAD
181 HYQQNTPIGDGPVLLPDNHYLSTQSKLSKD
211 PNEKRDHMLLEFVTAAGITLGMDELYKHH
241 HHHHPNKGSGTTS GTTRLLSGHTCFTLTGL
271 LGTLVTMGLLT
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8.2 NMR spectra

Figure 61. NMR spectrum of compound 2.

Figure 62. NMR spectrum of compound 3.

Figure 63. NMR spectrum of compound 4.

8.3 SDS-PAGE analysis (whole SDS-gels)

8.3.1 MESNA-mediated intein cleavage reactions

Figure 64. MESNA-mediated intein cleavage reaction of the FL_PrP variant followed by SDS-PAGE analysis. FL_PrP-Mxe GyrA intein-CBD fusion protein subjected to MESNA-mediated intein cleavage conditions, proceeds until 50% of α -thioester relative to the corresponding fusion protein is formed according to the integrated intensity values of the bands determined by Image Lab 5.1 software. Lane 1 shows the LMW marker, lane 2 FL_PrP-Mxe GyrA intein-CBD (MW: 51,156 Da), lane 3 unreacted fusion protein, cleaved Mxe GyrA intein-CBD (MW: 27,938 Da) and formed α -thioester of FL_PrP (MW: 23,324 Da). The LMW marker shows bands at 97, 66, 45, 30, 21, 14 kDa.

Figure 65. MESNA-mediated intein cleavage reaction of the Δ CR_PrP variant followed by SDS-PAGE analysis. Δ CR_PrP-Mxe GyrA intein-CBD fusion protein subjected to MESNA-mediated intein cleavage conditions, proceeds until 35% of α -thioester relative to the corresponding fusion protein is formed according to the integrated intensity values of the bands determined by Image Lab 5.1 software. Lane 1 shows the LMW marker, lane 2 Δ CR_PrP-Mxe GyrA intein-CBD (MW: 49,262 Da), lane 3 unreacted fusion protein, cleaved Mxe GyrA intein-CBD (MW: 27,938 Da) and formed α -thioester of Δ CR_PrP (MW: 21,430 Da). The LMW marker shows bands at 97, 66, 45, 30, 21, 14 kDa.

Figure 66. MESNA-mediated intein cleavage reaction of the T_PrP variant followed by SDS-PAGE analysis. T_PrP-Mxe GyrA intein-His-CBD fusion protein subjected to MESNA-mediated intein cleavage conditions, proceeds until 29% of α -thioester relative to the corresponding fusion protein is formed according to the integrated intensity values of the bands determined by Image Lab 5.1 software. Lane 1 shows the LMW marker, lane 2 T_PrP-Mxe GyrA intein-His-CBD (MW: 45,115 Da), lane 3 unreacted fusion protein, cleaved Mxe GyrA intein-His-CBD (MW: 28,796 Da) and formed α -thioester of T_PrP (MW: 16,459 Da). The LMW marker shows bands at 97, 66, 45, 30, 21, 14 kDa.

8.3.2 Isolated PrP variants

Figure 67. SDS-PAGE analysis of non-modified PrP variants and Cy5 labeled 7 Δ CR. Analysis is accomplished with Western blot based on 6D11 mAb interaction. Lane 1 shows the MagicMark™ XP marker with bands at 80, 60, 50, 30, 20 kDa; lane 2 7 Δ CR (MW: 24,910 Da), its dimer; lane 3-5 non-modified 5 Δ CR (MW: 21,306 Da), 5 $_{FL}$ (MW: 23,201 Da), 5 $_T$ (MW: 16,336 Da) and its dimer.

Figure 68. SDS-PAGE analysis of non-modified PrP variants. After Coomassie staining, lane 1 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa and lane 2-4 non-modified 5 $_T$ (MW: 16,336 Da), 5 Δ CR (MW: 21,306 Da) and 5 $_{FL}$ (MW: 23,201 Da).

Figure 69. SDS-PAGE analysis of lipidated FL_PrP 6 $_{FL}$ and fluorescein labeled 8 Δ CR. After Coomassie staining, lane 1 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa; lane 2 6 $_{FL}$ (MW: 26,353 Da) and lane 3 fluorescein labeled Δ CR_PrP 8 Δ CR (MW: 24,817 Da) and its dimer, with fluorescence-based detection, as well.

Figure 70. SDS-PAGE analysis of Cy5-labeled FL_PrP 7_{FL}. The upper gel shows detection based on 6D11 mAb interaction within a Western blot, the lower gel was recorded *via* fluorescence channel. Lane 1 shows the MagicMark™ XP marker with bands at 120, 100, 80, 60, 50, 30, 20 kDa, lane 2 7_{FL} (MW: 26,804 Da) and its dimer.

Figure 71. SDS-PAGE analysis of fluorescein labeled FL_PrP. Fluorescence-based detection shows in lane 1 fluorescein labeled 8_{FL} (MW: 26,711 Da) and its dimer.

Figure 72. SDS-PAGE analysis of fluorescein labeled FL_PrP. After Coomassie staining, lane 2 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa; lane 2 Cy5 labeled 7_{FL} (MW: 26,804 Da) and lane 3 fluorescein labeled 8_{FL} (MW: 26,711 Da), each construct with its dimer.

Figure 73. SDS-PAGE analysis of lipidated 6_{ACR} and 6_{T} , labeled 7_{T} and 8_{T} . The upper gel is Coomassie stained, whereas the middle and lower gels were recorded *via* fluorescein and Cy5 channel. Lane 1, 3 and 7 show the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa. In lane 2 is lipidated 6_{ACR} (MW: 24,458 Da), in lane 4 lipidated 6_{T} (MW: 19,488 Da), in lane 5 fluorescein labeled 8_{T} (MW: 19,847 Da) and its dimer, and lane 6 shows Cy5 labeled 7_{T} (MW: 19,940 Da) and its corresponding dimer.

Figure 74. SDS-PAGE analysis of Cy5 labeled 7_{ACR} . In lane 2, 7_{ACR} (MW: 24,910 Da) and its dimer are shown, detected with Coomassie (upper gel) and Cy5 channel (lower gel). Lane 1 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa.

8.3.3 PK digestion

Figure 75. PK digestion of folded non-modified $FL_PrP\ 5_{\text{FL}}$. Folded 5_{FL} prior (lane 2) and subjected to (lane 3) PK treatment in a ratio $PrP:PK = 50:1$. Lane 1 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa

8.3.4 Cy5 labeling of folded PrP

Figure 76. SDS-PAGE analysis of sulfo-Cy5 labeled T_PrP 7cr. In lane 2, sulfo-Cy5 labeled T_PrP 7cr (MW 20,250 Da) is shown, detected with Coomassie (upper gel) and Cy5 channel (lower gel). Besides, fluorescence-based detection results in additional bands in the low molecular range corresponding to the labeling reagent, sulfo-Cy5 maleimide 5c. Lane 1 shows the LMW marker with bands at 97, 66, 45, 30, 21, 14 kDa.

8.4 Time courses of EPL reactions

Figure 77. Following the EPL reaction between 4_{FL} and **1** by HPLC using two different thiols as ligation mediator. The ligation was monitored via absorbance at 214 and 280 nm (baseline corrected and normalized) over 3.2 and 23 h for MPAA (A, B) and thiophenol (C, D), respectively. Peak areas of the formed lipidated FL_PrP 6_{FL} at t_R of 25.5 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **1** at 30.0 min.

thiol	t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)	
		6_{FL}	1	6_{FL}	1
thiophenol	0	0	100	0	100
	2	33	67	48	52
	5.5	50	50	64	36
	23	68	32	79	21
MPAA	0	0	100	0	100
	1.2	46	54	63	37
	3.2	59	41	72	28
	4.5*	62	38	78	22

Table 24. Following the EPL reaction between 4_{FL} and **1** by HPLC using two different thiols as ligation mediator. For MPAA, according to the absorbance at 214 and 280 nm the ligation is finished after 4.5 h with 62 and 78% of formed lipidated FL_PrP 6_{FL} , whereas in case of thiophenol the reaction requires 23 h to be completed. Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214 and 280 nm. *HPLC traces are not shown due to shifts in retention times caused by usage of different columns.

Figure 78. Following the EPL reaction between $4_{\Delta CR}$ and **1** by HPLC. The ligation was monitored over 5 h via absorbance at 214 (A) and 280 (B) nm (baseline corrected and normalized). Peak areas of the formed lipidated ΔCR _PrP $6_{\Delta CR}$ at t_R of 26.5 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **1** at 31.8 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)	
	$6_{\Delta CR}$	1	$6_{\Delta CR}$	1
0	0	100	0	100
1	41	59	62	38
2	53	47	71	29
3	59	41	76	24
4	62	38	78	22
5	64	36	80	20

Table 25. Following the EPL reaction between $4_{\Delta CR}$ and **1** by HPLC. According to the absorbance at 214 and 280 nm the ligation is finished after 5 h with 64 and 80% of formed lipidated ΔCR _PrP $6_{\Delta CR}$. Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214 and 280 nm.

Figure 79. Following the EPL reaction between **4_T** and **1** by HPLC. The ligation was monitored over 4.5 h via absorbance at 214 (A) and 280 (B) nm (baseline corrected and normalized). Peak areas of the formed lipidated T_PrP **6_T** at t_R of 25.2 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **1** at 31.8 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)	
	6_T	1	6_T	1
0	0	100	0	100
2	32	68	43	57
3.5	40	60	53	47
4.5	45	55	57	43

Table 26. Following the EPL reaction between **4_T** and **1** by HPLC. According to the absorbance at 214 and 280 nm the ligation is finished after 4.5 h with 45 and 57% of formed lipidated T_PrP **6_T**. Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214 and 280 nm.

Figure 80. Following the EPL reaction between 4_{FL} and 2 by HPLC. The ligation was monitored over 6 h via absorbance at 214 (A) and 280 (B) nm and emission at 650 (C) nm (baseline corrected and normalized). Peak areas of the formed Cy5 labeled FL_PrP 7_{FL} at t_R of 30.7 min increase with time proceeding and are accompanied by a decrease ascribed to peptide 2 at 36.5 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	7_{FL}	2	7_{FL}	2	7_{FL}	2
0	0	100	0	100	0	100
1	37	63	35	65	4	96
2	45	55	46	54	7	93
3	51	49	49	51	8	92
4	54	46	54	46	9	91
5	56	44	56	44	10	90

Table 27. Following the EPL reaction between 4_{FL} and 2 by HPLC. According to the absorbance at 214 and 280 nm and the emission at 650 nm the ligation is finished after 5 h with 56 and 10% of formed Cy5 labeled FL_PrP 7_{FL} . Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 650 nm.

Figure 81. Following the EPL reaction between 4_{ACR} and **2** by HPLC. The ligation was monitored over 5.5 h via absorbance at 214 (A) and 280 (B) nm and emission at 650 (C) nm (baseline corrected and normalized). Peak areas of the formed Cy5 labeled $\Delta\text{CR_PrP } 7_{\text{ACR}}$ at t_R of 30.9 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **2** at 36.5 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	7_{ACR}	2	7_{ACR}	2	7_{ACR}	2
0	0	100	0	100	0	100
1	34	66	22	78	4	96
2	49	51	37	63	6	94
3	55	45	44	56	7	93
4	59	41	49	51	8	92
5	60	40	49	51	10	90
5.5	62	38	50	50	11	89

Table 28. Following the EPL reaction between 4_{ACR} and **2** by HPLC. According to the absorbance at 214 and 280 nm and the emission at 650 nm the ligation is finished after 5.5 h with 62, 50 and 10% of formed Cy5 labeled FL_PrP 7_{FL} . Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 650 nm.

Figure 82. Following the EPL reaction between 4_T and **2** by HPLC. The ligation was monitored over 5 h via absorbance at 214 (A) and 280 (B) nm and emission at 650 (C) nm (baseline corrected and normalized). Peak areas of the formed Cy5 labeled T_PrP 7_T at t_R of 29.0 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **2** at 34.3 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	7_T	2	7_T	2	7_T	2
0	0	100	0	100	0	100
1	14	86	6	94	4	96
2	22	78	10	90	6	94
3	26	74	12	88	8	92
4	28	72	13	87	9	91
5	30	70	14	86	9	91

Table 29. Following the EPL reaction between 4_T and **2** by HPLC. According to the absorbance at 214 and 280 nm and the emission at 650 nm the ligation is finished after 5 h with 30, 14 and 9% of formed Cy5 labeled T_PrP 7_T . Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 650 nm.

Figure 83. Following the EPL reaction between 4_{FL} and 3 by HPLC. The ligation was monitored over 5 h via absorbance at 214 (A) and 280 (B) nm and emission at 517 (C) nm (baseline corrected and normalized). Peak areas of the formed fluorescein labeled FL_PrP 8_{FL} at t_R of 26.7 min increase with time proceeding and are accompanied by a decrease ascribed to peptide 3 at 32.8 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	8_{FL}	3	8_{FL}	3	8_{FL}	3
0	4	96	4	96	1	99
1	16	84	14	86	3	97
2	22	78	20	80	4	96
3	26	74	22	78	5	95
4	28	72	26	74	5	95
5	31	69	29	71	6	94

Table 30. Following the EPL reaction between 4_{FL} and 3 by HPLC. According to the absorbance at 214 and 280 nm and the emission at 517 nm the ligation is finished after 5 h with 31, 29 and 6% of formed fluorescein labeled FL_PrP 8_{FL} . Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 517 nm.

Figure 84. Following the EPL reaction between 4_{ACR} and 3 by HPLC. The ligation was monitored over 5 h via absorbance at 214 (A) and 280 (B) nm and emission at 517 (C) nm (baseline corrected and normalized). Peak areas of the formed fluorescein labeled $\Delta\text{CR_PrP } 8_{\text{ACR}}$ at t_R of 24.6 min increase with time proceeding and are accompanied by a decrease ascribed to peptide 3 at 29.1 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	8_{ACR}	3	8_{ACR}	3	8_{ACR}	3
0	0	100	0	100	0	100
2	44	44	44	56	9	91
4	50	50	49	51	12	88
5	50	50	50	50	14	86

Table 31. Following the EPL reaction between 4_{ACR} and 3 by HPLC. According to the absorbance at 214 and 280 nm and the emission at 517 nm the ligation is finished after 5 h with 50 and 14% of formed fluorescein labeled $\Delta\text{CR_PrP } 8_{\text{ACR}}$. Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 517 nm.

Figure 85. Following the EPL reaction between **4_T** and **3** by HPLC. The ligation was monitored over 5 h via absorbance at 214 (A) and 280 (B) nm and emission at 517 (C) nm (baseline corrected and normalized). Peak areas of the formed fluorescein labeled T_{PrP} **8_T** at *t_R* of 25.0 min increase with time proceeding and are accompanied by a decrease ascribed to peptide **3** at 29.1 min.

t (h)	integrated peak area at 214 nm (%)		integrated peak area at 280 nm (%)		integrated peak area from emission (%)	
	8_T	3	8_T	3	8_T	3
0	0	100	0	100	0	100
2	17	83	13	87	4	96
4	25	75	20	80	6	94
5	28	72	23	77	7	93

Figure 86. Following the EPL reaction between **4_T** and **3** by HPLC. According to the absorbance at 214 and 280 nm and the emission at 517 nm the ligation is finished after 5 h with 28, 23 and 7% of formed fluorescein labeled T_{PrP} **8_T**. Integration of peak areas was accomplished with the software OriginPro using HPLC traces at 214, 280 and 517 nm.

Figure 87. ESI-MS spectrum of lipidated sulfo-Cy5 labeled ΔCR_PrP ($7b_{\Delta CR}$). Labeled charge states in mass spectrum deconvolute to molecular weight of 25,081 Da in agreement with the predicted value of 25,084 Da.

Figure 88. Emission spectrum of fluorescein labeled ΔCR_PrP $8_{\Delta CR}$ (5.6 μM), recorded from 500 to 550 nm with a slit width of 5 nm using an excitation wavelength of 490 nm, shows a maximum at 518 nm.

8.5 ITIR-FCS data

A

B

Figure 89. ITIR-FCS measurements of DiI- C_{18} stained SH-SY5Y cells incubated with non- and lipidated PrP. The left (A) and right figures (B) show Δ CR_PrP and T_PrP constructs, respectively. Data are mean values $\pm$ SD of the number of ACFs.

Figure 90. ITIR-FCS measurements of GFP-GPI AP transfected SH-SY5Y cells incubated with non- and lipidated PrP, 5_{ΔCR} and 6_{ΔCR}. Data are mean values $\pm$ SD of the number of ACFs.

8.6 Confocal FCS data

Figure 91. SH-SY5Y cells incubated with $7_{\Delta CR}$ after 35 min. Confocal images show GFP-GPI AP and Cy5 fluorescence with the respective brightfield channel.

GFP-GPI AP	D_{slow} ($\mu m^2/s$)	D_{fast} ($\mu m^2/s$)	F_{slow}	τ_{trip} (μs)	F_{trip}	N_m	N_{cells}
untreated	0.74 ± 0.058	25.14 ± 0.020	0.56 ± 0.055	17.7 ± 0.612	0.15 ± 0.023	2	2
+ $7_{\Delta CR}$	0.72 ± 0.024	35.78 ± 4.341	0.55 ± 0.012	14.9 ± 0.085	0.17 ± 0.041	2	2
Cy5 - $7_{\Delta CR}$	0.62	13.97	0.38			1	1

Table 32. Confocal FCS results of the GFP-GPI AP and Cy5 fluorescence from $7_{\Delta CR}$ in SH-SY5Y cells. Data are mean values $\pm$ SD of the number of ACFs that is identical to the number of measurements (N_m) for confocal FCS.

9. Abbreviations

aa	Amino acid
ab	Antibody
A β	Amyloid- β
ACF	Autocorrelation function
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
Adgrg6	Adhesion G-protein coupled receptor G6
AFM	Atomic force microscopy
AL	Amyloid light-chain
ALS	Amyotrophic lateral sclerosis
APD	Avalanche photodiode
APS	Ammonium persulfate
ATPase	Adenosine 5'-triphosphatase
BP	Bandpass
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CAD	Charged aerosol detector
CBD	Chitin binding domain
CCD	Charge-coupled device
CCF	Cross-correlation function
CCV	Clathrin-coated vesicle
CD	Circular dichroism
Cdc-42	Cell division control protein 42 homolog
CDP	Chronic demyelinating polyneuropathy
CFC	Cell free conversion
CJD	Creutzfeld-Jakob disease
CLSM	Confocal laser scanning microscope
CNS	Central nervous system
cps	Counts per second
cryo-EM	Cryo electron microscopy
CWD	Chronic wasting disease
Cy5	Cyanine 5

DAF	Decay-accelerating factor
DAPI	4',6-Diamidino-2-phenylindole
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCM	Dichloromethane
DHB	2,5-Dihydroxybenzoic acid
DIC	<i>N,N'</i> -Diisopropylcarbodiimide
DIC	Differential interference contrast
DIEA	<i>N,N</i> -Diisopropylethylamine
DiI-C ₁₈	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DLS	Dynamic light scattering
DM	Dichroic mirror
DMEM	Dulbecco's Modified Eagle Medium
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
DOC	(Sodium) deoxycholate
DOPC	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPG	1,2-Dioleoyl- <i>sn</i> -glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
DRM	Detergent-resistant membrane
E-buffer	Electrolytic buffer
<i>E. coli</i>	<i>Escherichia coli</i>
EGFP	Enhanced GFP
E.I.	Excitation intensity
EMCCD	Electron multiplying charge-coupled device
eQuIC	Standard quaking-induced conversion
EPL	Expressed protein ligation
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
ESI-Qq/oa-TOF	Hybrid quadrupole orthogonal accelerated time-of-flight mass spectrometer
MS	spectrometer
ESR	Electron spin resonance
FBS	Fetal bovine serum

FCS	Fluorescence correlation spectroscopy
FFI	Fatal familial insomnia
Fmoc	Fluorenylmethyloxycarbonyl
Fps	Frames per second
FTD	Frontotemporal dementia
FWHM	Full width at half maximum
GFP	Green fluorescent protein
GFP-GPI AP	Green fluorescent protein-glycosylphosphatidylinositol anchored protein
GPI	Glycosylphosphatidylinositol
GSH	Reduced glutathione
GSS	Gerstmann-Sträussler-Scheinker syndrome
GSSG	Oxidised glutathione
HBSS	Hanks' balanced salt solution
HBTU	(2-(1 <i>H</i> -Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	High performance liquid chromatography
HOBt	1-Hydroxybenzotriazole
Hsp	Heat shock protein
ICA	Intensity correlation analysis
ICQ	Intensity correlation quotient
ICCB	Intensity correlation coefficient-based
i.e.	Id est
IR	Infrared
ITIR-FCS	Imaging total internal reflection-FCS
JACoP	Just Another Co-localization Plugin
KD	Knockdown
K _d	Dissociation constant
LatA	Latrunculin A
LC	Light chain
LED	Light-emitting diode
L.P.	Laser power
LP	Lowpass

LRP1	Low-density lipoprotein receptor-related protein 1
LTQ	Linear trap-quadrupole
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption ionization
m β CD	Methyl- β -cyclodextrin
MESNA	(Sodium) 2-mercaptoethanesulfonate
MPAA	4-Mercaptophenylacetic acid
MS	Mass spectrometry
MSA	Multiple system atrophy
Mtt	Methyltrityl
MWCO	Molecular weight cut-off
<i>Mxe</i>	<i>Mycobacterium xenopi gyrA</i>
NA	Numerical aperture
NaCl	Sodium chloride
Na ₂ HPO ₄	Disodium hydrogen phosphate
NCL	Native chemical ligation
NF	Noise filter
NMR	Nuclear magnetic resonance
<i>Npu</i>	<i>Nostoc punctiforme</i>
NTA	Nitrilotriacetic acid
OG	<i>N</i> -octyl- β -D-glucopyranoside
OR	Octapeptide repeats
ORF	Open reading frame
PAC	Phenacyl
PALM	Photoactivated localization microscopy
PBS	Phosphate buffered saline
PCC	Pearson's correlation coefficient
PD	Parkinson's disease
PDB	Protein data bank
PEG	Polyethyleneglycol
pI	Isoelectric point
PI-PLC	Phosphatidylinositol-specific phospholipase C
PIRIBS	Parallel in-register intermolecular β -sheet

PK	Proteinase K
PMCA	Protein misfolding cyclic amplification
PMSF	Phenylmethanesulfonyl fluoride
PMT	Photomultiplier
PMT-EGFP	Plasma membrane targeting, labeled EGFP
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
PPO	Polyethyleneglycol polyamide oligomer
PrP	Prion protein
PrP ^C	Cellular prion protein
PrP ^{res}	(PK-)-resistant prion protein
PrP ^{Sc}	Scrapie prion protein
PS	Penicillin and streptomycin
PSF	Point spread function
PTM	Posttranslational modification
PTS	Protein <i>trans</i> -splicing
QCM-D	Quartz crystal microbalance with dissipation monitoring
QuIC	Quaking-induced conversion
R-buffer	Resuspension buffer
RF	Radiofrequency
ROI	Region of interest
RP	Reversed phase
rPrP	Recombinant prion protein
RT-QuIC	real-time quaking-induced conversion
rt	Room temperature
SALB	Solvent-assisted lipid bilayer
ScN2a	Scrapie-infected mouse neuroblastoma cells
SD	Standard deviation
SHa	Syrian hamster
SIFT	Scanning for intensely fluorescent targets
SIM	Structured illumination microscopy
SLB	Supported lipid bilayer
SNR	Signal-to-noise ratio

SPAD	Single-photon avalanche photodiode
SPR	Surface plasmon resonance
SPPS	Solid phase peptide synthesis
S-QuIC	standard quaking-induced conversion
SR	Super-resolution
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	<i>N,N,N',N'</i> -Tetramethyl ethylenediamine
TEV	Tobacco etch virus
TFA	Trifluoroacetic acid
ThT	Thioflavin T
TIS	Triisopropylsilane
TLC	Thin layer chromatography
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TSEs	Transmissible spongiform encephalopathies
Vis	Visible
XAS	X-ray absorption spectroscopy

Curriculum Vitae (CV)

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EDUCATION

2014-2019	Doctoral studies within International Graduate School (IGS) in BioNanoTechnology (BioNanoTech) joint PhD program of BOKU-AIT-NTU <i>Thesis: The impact of membrane composition on conformation and trafficking of lipidated prion protein (PrP) variants.</i> Advisor: Univ. Prof. Dr. Christian F.W. Becker (University of Vienna) Co-Advisor: Prof. Dr. Wolfgang Knoll (AIT, Vienna) & Assoc. Prof. Nam-joon Cho (NTU, Singapore)
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PARTICIPATION AT CONFERENCES

2017	<i>7th Austrian Peptide Symposium (AtPS) in Vienna, Austria</i> Poster: Semisynthetic lipidated prion protein variants in membrane environments.
2017	<i>Tethered Membrane (TethMem) conference in Vienna, Austria</i> Oral and poster presentation: Impact of membrane-association on semisynthetic lipidated prion protein variants.
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2016	<i>6th Austrian Peptide Symposium (AtPS) in Vienna, Austria</i> Poster: Impact of membranes on semisynthetic lipidated prion protein variants.
2016	<i>Prion conference in Tokyo, Japan</i> Poster: Semisynthesis of lipidated prion protein variants.
2015	<i>5th Austrian Peptide Symposium (AtPS) in Vienna, Austria</i> Poster: Semisynthesis of lipidated prion protein variants.
2015	<i>Tethered Membrane (TethMem) conference in Singapore</i> Poster: Semisynthesis of lipidated prion protein variants. The poster was awarded as one of the top 3 posters.
2015	<i>12th German Peptide Symposium (GPS) in Darmstadt, Germany</i> Poster: Semisynthesis of lipidated prion protein variants. The poster was selected for an award.
2014	<i>4th Austrian Peptide Symposium (AtPS) in Salzburg, Austria</i> Poster: Semisynthesis of lipidated prion protein variants.

PUBLICATIONS

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