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1 Introduction

1.1 Prion diseases

Prion diseases, also known as transmissible spongiform encephalopathies (TSE), are a class of infectious, progressive and fatal neurodegenerative disorders associated with the loss of cognitive skills and neuronal dysfunction in animals and humans ^[1-2]. Accumulation of misfolded proteinaceous particles (prions) is regarded as a prerequisite of TSEs ^[3].

TSEs can be etiologically classified as inherent, sporadic or iatrogenic ^[4]. Sporadic TSEs occur in animals without prion mutations, while inherent TSEs are accompanied by a rare mutation on the *Prnp* gene. Furthermore, prion diseases appear to be contagious parenterally and perorally ^[5-6]. Well-known examples of TSEs include bovine spongiform encephalopathy (BSE, cattle), scrapie (sheep, goat), kuru (human), Creutzfeld-Jacob-Disease (CJD, human), fatal familial insomnia (FFI, human), Gerstmann-Straussler-Scheinker syndrome (GSS, human) and chronic wasting disease (CWD, deer and elk) ^[7-13]. Currently, there are no known therapies for TSEs.

Already in the early 1950's, among the members of the Fore tribe in Papua New Guinea, a highly infectious TSE was described, named kuru. Following the eradication of cannibalism, the transmission of the disease stopped. Subsequent experiments were conducted using brain tissue samples of kuru victims by transferring these into primates in order to initiate spongiform encephalopathies. Tikvah Alper and John Stanley Griffith mainly carried out further investigations focusing on the infectious potential of the pathogens causing scrapie ^[14-15]. In doing so, they found out that the pathogens could not be eliminated using standard procedures such as UV or ionizing radiation that inactivated DNA- or RNA-based viral pathogens. With respect to these findings, Griffith postulated in 1967 "the protein-only" hypothesis, in which a protein was held as the main pathogen of TSEs ^[15].

However, it was not until 1982 that the term "prion" was coined by Stanley Prusiner ^[16]. In his seminal publication, he refers to a "small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids" as

prion. Prusiner and his coworkers could successfully isolate a partially Proteinase K (PK)-resistant isoform of the prion protein with a molecular weight of 27-30 kDa (PrP²⁷⁻³⁰) from infected brain tissues. These samples could only be isolated from infected animals and were not identified in healthy tissue samples.

In the following years, Edman sequencing of the N-terminal domain of PrP²⁷⁻³⁰ by Weissmann and coworkers led to identification of a peptide fragment that enabled cDNA library screening and molecular cloning of full length PrP [17-18].

Following the discovery of prions, scientists started to look at the pathophysiology of a variety of progressive, neurodegenerative diseases with similar indications. Although having distinct causes, all prion diseases involve a conformational change in the cellular (non-pathogenic) prion protein (PrP^C). Misfolding of PrP into pathogenic PrP^{Sc} (scrapie) is the initial step for the infection and disease progression (see section 1.3) [19]. Thus, the main attention to understand prion pathogenicity is drawn to PrP.

1.2 Prion Protein (PrP)

1.2.1 Biosynthesis of PrP^C

In 1985, a collaboration between the laboratories of Weissmann and Prusiner enabled the first identification of a gene encoding for PrP^C, termed *Prnp* gene, derived from syrian hamster [17]. Subsequently the same gene was identified in humans and other mammalian organisms, suggesting that this gene was highly conserved gene among different species [20].

The human *Prnp* gene is located on chromosome 20 comprising three exons. The open reading frame (ORF) lies completely within a single exon (exon 3) and encodes for a precursor protein of 254 amino acids [18, 21-22]. This precursor comprises a 22-amino acid (aa) length N-terminal signal peptide that allows the protein to enter the secretory pathway where the signal peptide is cleaved. Upon the cleavage of the signaling peptide, PrP can undergo *N*-glycosylation on asparagine residues N181 and/or N197 in the ER. In addition, a disulfide bridge between two cysteine residues C179 and C214 is formed. A second C-terminal signal sequence of 23 aa enables the attachment of a glycosylphosphatidylinositol

(GPI) anchor to the serine residue S231 in endoplasmic reticulum (ER) before transferring the mature, posttranslationally modified PrP to the Golgi apparatus. PrP^C is highly conserved and ubiquitously expressed in mammalian organisms. High expression levels can be found in neurons, lymphocytes and pancreatic islet cells [23-26]. High levels of the protein have also been found within the immune system, including natural killer (NK) cells, dendritic cells and monocytes [27-29]. The ubiquitous nature of PrP^C suggests an important function, whilst the exact physiological nature is still not fully understood (see below, Section 1.2.3.2).

1.2.2 Structure of PrP^C

Pan and coworkers were the first to explore the native structure of PrP^C using Fourier-transform infrared spectroscopy (FT-IR). PrP^C was purified from brain homogenates of syrian hamsters (Sha-PrP) and characterized via FT-IR, circular dichroism (CD) and electron microscopy (EM). The spectroscopic data showed that PrP^C has a high α -helical content (42 %) whereas the β -sheet content was almost negligible (3%) [30]. However, due to the limitations of access to sufficient yields of PrP^C upon purification, the three-dimensional structure of PrP^C could not be solved by either using NMR spectroscopy or X-ray crystallography.

To overcome this problem, other researchers used recombinant PrP (rPrP) that could be obtained in sufficient quantities for NMR measurements, and found that PrP^C and rPrP share a similar secondary structure [31-33]. Additionally, far-UV, near-UV and CD spectroscopy data suggested that rPrP is an excellent surrogate for PrP^C [34]. Over the course of the last two decades, the three dimensional structure of rPrP derived from different mammalian organisms has been characterized by means of NMR spectroscopy and/or crystallography [35-42].

Similarities in three-dimensional structures among different mammalian species could be predicted because of their highly conserved DNA sequences. The N-terminal part of PrP^C is mostly unstructured whereas the C-terminal domain is globular and comprises three α -helices alternating with two antiparallel β -strands [31]. As mentioned in section 1.2.1, PrP^C contains a disulfide bond that links helices 2 and 3, two *N*-glycosylation sites as well as a C-terminal GPI-anchor. The N-terminus is comprised of a polybasic region (residues 91-113) and an eight aa tandem repeat region called octarepeat region (PHGGGWGQ) with a high binding

affinity for copper ions (Cu^{2+}) and certain other divalent cations such as Zn^{2+} [43] and Mg^{2+} . The central fragment consists of a hydrophobic region (aas 113-135), which serves as a transmembrane domain [44-45]. Furthermore, a palindromic, alanine-rich sequence (residues 113-120) within this region tends to be highly conserved among different mammalian species (Figure 1).

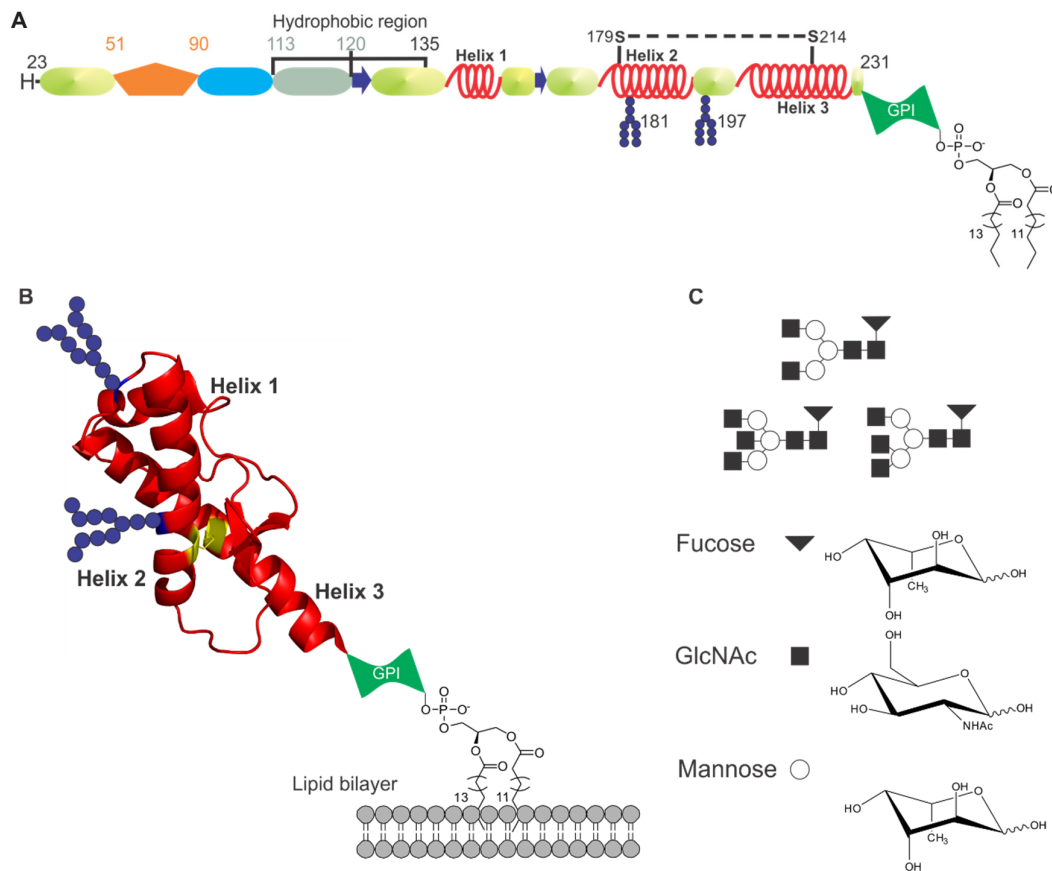


Figure 1: Secondary structure of posttranslationally modified PrP.

A: representative scheme of PrP containing the unstructured (light green), octarepeat (orange, aa 51-90), polybasic (blue, aa 91-113), alanine-rich (grey, aa 113-120) and hydrophobic regions (aa 113-135) as well as structured C-terminal domain with α -helices (red), β -sheets (purple arrows), glycans (dark blue circles) and the GPI-anchor (dark green). **B:** Tertiary structure of huPrP^C (aa 23-230) based on the NMR studies performed by Wütrich and coworkers [38]. Blue: glycans, yellow: disulfide bridge, green: GPI-anchor, Protein Data Bank (PDB) entry 1QLZ. The figures were modified using PyMOL, ChemBioDraw Ultra 12.0 and CorelDRAW X05. **C:** common glycosylation patterns of PrP^C and PrP^{Sc}.

To date, more than 30 mutations in the *Prnp* gene have been identified causing different genetic TSEs. Those vary from mutation or deletion of a single amino acid to introduction of residues into the polypeptide chain. Although this points to a potential link between the infectivity and the structural changes caused by certain

mutations in genetic TSEs [46-50], there has not been much biochemical data to prove this [51-52]. Moreover, the early hypothesis on destabilizing effects of point mutations on native PrP^C structure as well as their contribution to the conversion of PrP^C into PrP^{Sc} via stabilizing PrP^{Sc} structure was partially overturned in 1998 by the Surewicz group, who showed that only some of these mutations were able to destabilize the 3D structure of PrP^C [53].

1.2.3 PrP physiology

1.2.3.1 Trafficking of PrP^C

As described in section 1.2.1, PrP^C is as a GPI anchored glycoprotein. Upon translation, the protein enters the secretory pathway via the ER, where initial glycosylation and GPI anchor attachment occurs and correct folding is ensured [54-57]. While entering the secretory pathway, the N-terminal signaling peptide (~22 aa) is cleaved and the disulfide bridge is formed. After exiting the ER, PrP^C is transported to the Golgi apparatus where it undergoes multiple tripping and extensions of the glycan core and the GPI anchor to furnish the protein's native form [58]. There is evidence that while entering the Golgi apparatus, PrP^C is associated with detergent-resistant membranes (DRMs, also known as rafts) mainly via its GPI-anchor as well as the N-terminal domain [59-61]. Correctly folded and posttranslationally modified mature PrP^C is then transported to the cell surface, probably via exocytosis (Figure 2) [62-63].

Despite being the common form, extracellular PrP^C is not the only PrP variant found in cells. Mironov *et al.*, who described the first quantitative approach to the localization of PrP^C and highlighted the occurrence of PrP in cytosol of subpopulations of neurons in the hippocampus, neocortex and thalamus [64], identified two additional forms of transmembranal PrP, ^{Ntm}PrP and ^{Ctm}PrP. Both variants are not entirely translocated and cross the ER membrane insufficiently, which leads to their localization in the transmembrane regions. The ^{Ntm}PrP has its N-terminal region inside the ER with a C-terminal part spanning the transmembrane domain, whereas the ^{Ctm}PrP has the C-terminal part in the ER lumen and the N-terminal part on the transmembrane region [44, 65].

Moreover, there is strong evidence that the conformational change of PrP^C into PrP^{Sc} takes place on the outer leaflet of the plasma membrane, which is initiated

by the extracellular variant ^[66-68]. The Harris and Lehmann group have established that while PrP is constitutively internalized, the retrograde transfer of PrP^{Sc} could result in propagation of PrP^{Sc} inside the cell, which then will be transferred to the cell surface again ^[69-70]. This suggests a synergistic interplay of internalization process and retrograde transfer. From this point of view, the internalization process of PrP may play an important role in TSE occurrence and should be investigated further.

Although the PrP internalization is currently not completely understood, two main mechanisms are suggested to be involved in it, clathrin-mediated and clathrin-independent endocytosis (Figure 2). Clathrin is a self-polymerizing protein that consists of three heavy (~ 190 kDa each) and three light chains (~ 25 kDa each) that form a triskelion. The assembly of a triskelion via an adapter protein on cell membranes results in formation of lattices, leading to the formation of clathrin-coated vesicles (CCV) ^[71-72]. These vesicles carry the cargo protein either to recycling endosomes for repeated transport into the cell or to other cell compartments such as lysosomes for degradation ^[73]. An early insight into the internalization pathway of PrP^C was provided by Harris and coworkers, who used the chicken homologue of human PrP^C to show that the protein is trafficked continuously between the cell surface and the endocytic compartments during a 60 min measurement ^[74]. Later on, evidence that PrP^C is cycled between the plasma membrane and endosomes via clathrin coated pit mechanism could be produced based on this research ^[75-76]. Moreover, the 37-kDa human laminin receptor precursor (37-kDa LRP) was identified as the main cell surface receptor for PrP^C and its internalization through clathrin-coated pits by Weiss et al. ^[77-78]

As mentioned previously, PrP^C also gets internalized in a clathrin-independent manner. Clathrin-independent internalization occurs mainly via caveolae-mediated endocytosis. Caveolae are flask-shaped special lipid rafts, which are rich in cholesterol and sphingolipids. These form invaginations on the plasma membrane that can be endocytosed, and cycle cargo proteins in endosome-like vesicles (caveosomes) to late endosomes ^[79]. A similar mechanism has been proposed for caveolae-mediated endocytosis of PrP, although it is not completely clear whether the C-terminal GPI-anchoring part or the N-terminal raft-targeting region (aa 34-90) is located on the caveolae (Figure 2). Despite the fact that the mechanism of

caveolae-mediated endocytosis is not well understood, the synergistic interaction of both PrP internalization pathways has been highlighted recently [80].

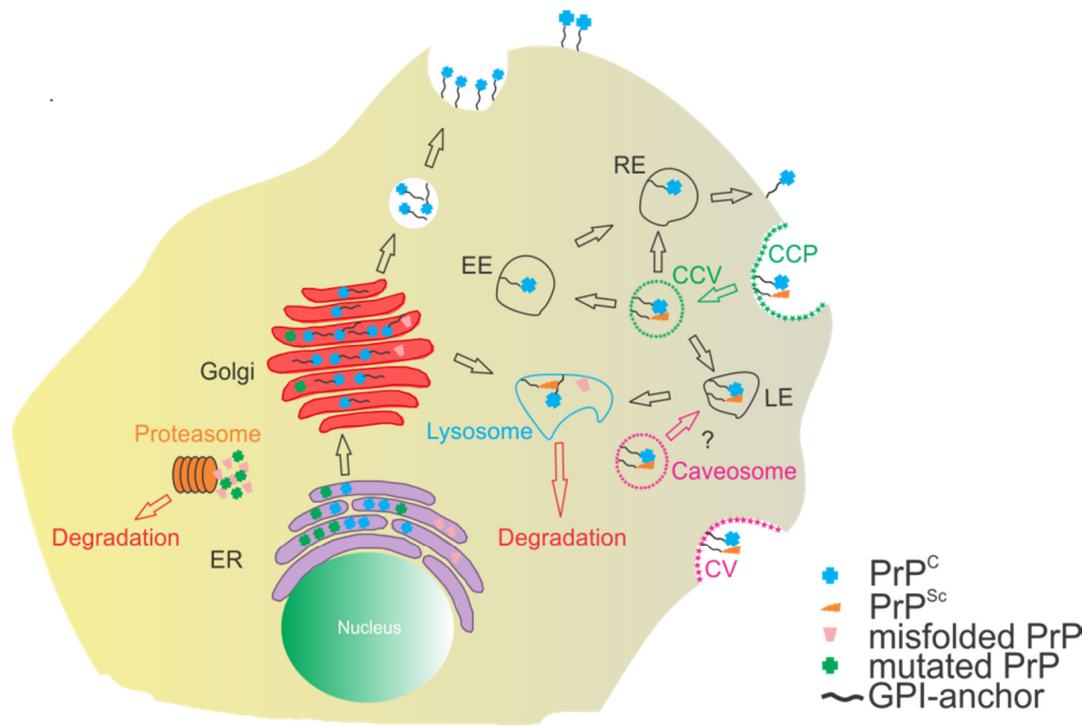


Figure 2: Trafficking of PrP

Following translation, PrP^C is transported to the ER where the N-terminal signaling peptide is cleaved. The attachment of GPI-anchor occurs herein. After several quality checks in the ER, posttranslationally modified PrP is first transported into the Golgi apparatus and then to the cell surface via vesicular transport. In the Golgi, it undergoes a series of refining modifications on the GPI-anchor core and possibly on sugars that are already attached to their respective glycosylation sites in the ER. PrP^C on the cell surface can be internalized in clathrin-coated vesicles or caveole-mediated endocytosis and then be transported into the cell membrane or degraded.

EE: early endosome, RE: recycling endosome, LE: late endosome, CCV: clathrin-coated vesicle, CCP: clathrin coated pit, CV: caveole.

1.2.3.2 Physiological functions of PrP^C

The pathological role of PrP^C via conversion into PrP^{Sc} is widely accepted and demonstrated in several experiments. Nevertheless, scientists still cannot fully explain the physiological functions of PrP^C despite the large number of attempts documented in many corresponding publications [81]. The ubiquitous nature of PrP^C makes it difficult to elucidate its role in mammalian physiology. In the last two decades, several functions of PrP have been postulated including immunomodulation [82-83], neuroprotection [84-85] and cell adhesion [86-87].

Perhaps, the most important clues to the function of the function of PrP^C can be found in its localization. Indeed, PrP^C is expressed by cells of both the innate and the adapted immune systems (IMS and AMS, respectively). The physiological role of PrP^C in cells of IMS such as monocytes and macrophages has been the subject of intense studies [88-90]. At first, PrP^C was first considered to decrease phagocytic activity and production of cytokines in macrophages [83]. However, recent *in vitro* studies with *Prnp*-deficient macrophage cell line from the bone marrow of Zrchl *Prnp*^{-/-} mice and human monocytes with membrane anchored PrP^C and a soluble version of PrP^C that is equipped with the Fc region of IgG, ruled out this theory [88, 91]. Furthermore, PrP^C was found to have an impact on the response of T lymphocytes to radical oxygen species (ROS)-mediated oxidative stress [92]. Aude-Garcia et al. used *Prnp*^{-/-} and *Prnp*^{+/+} thymocytes exposed to H₂O₂ to show that *Prnp*^{-/-} cells are more susceptible to oxidative stress. They also utilized *in vivo* assays to show the protecting role of PrP^C in conditions of oxidative stress using *Prnp*^{-/-} and *Prnp*^{+/+} mice [90].

As described in section 1.2.1, the majority of PrP^C is expressed in the central nervous system (CNS), which suggests a specific influence of PrP^C on neuronal activities. Based on this idea, early investigations were made to elucidate the role of PrP^C in CNS including determination of potential binding partners and the impact of PrP^C on preventing neuronal cell death [93-96]. In a more recent study, the interaction of PrP with stress-inducible-protein-1 (STI-1), a co-chaperone protein that is normally linking Hsp70 and Hsp90, resulted in generation of neuroprotective signals rescuing the cell from apoptosis [97]. Other publications supported the results on the neuroprotective nature of PrP by showing its interaction with different pro- and anti-apoptotic proteins [98-99]. Recent studies highlight the impact of PrP^C in both synaptic and neuronal signal transduction [100-103]. For example, Khosravani et al. demonstrated that PrP^C is inhibiting NMDA receptor via localizing on NR2D subunit and preventing the hyper-excitability [100].

The link between PrP^C and cell adhesion was first described by Schachner and coworkers [86] based on the work of Moulliet-Richard et al. using anti-PrP antibodies to determine physiological binding partners of PrP [104]. The outcome of this work was the characterization of the activation of Fyn, a membrane anchored tyrosine kinase, through PrP^C. Fyn is a regulator of neural cell adhesion molecule (NCAM)

mediated neurite outgrowth. These results initiated the hypothesis of the involvement of PrP^C in cell adhesion.

Schachner and coworkers found that PrP^C directly interacts with NCAM, stabilizes NCAM in lipid rafts and increases Fyn activation as well as NCAM-mediated neurite outgrowth [86].

Another potential clue to the function of PrP^C is its binding to bivalent copper (Cu²⁺), demonstrated *in vivo* by Kretzschmar and coworkers [105]. The N-terminal octarepeat region of PrP harbors up to five Cu²⁺ binding sites, and has been shown to protect against Cu²⁺ cytotoxicity in neurons and astrocytes [106]. Hereby it may increase the incorporation of Cu²⁺ into superoxide dismutase (SOD) or act as a SOD itself [107].

Overall, the physiological role of PrP^C is not fully understood. Identifying the influence of PrP^C on neuronal and immunological signaling pathways will give new insights into understanding of prion pathogenesis. Moreover, new findings that can distinguish the nature of PrP^C will probably result in understanding the toxicity and infectivity of PrP^{Sc}.

1.3 PrP^{Sc}

1.3.1 Conversion of PrP^C into PrP^{Sc}

TSEs involve the conformational change of predominantly α -helical PrP^C into β -sheet rich, misfolded PrP^{Sc} [108-109]. Convincing evidence has been accumulated to show that native PrP^C serves as a precursor for conversion into PrP^{Sc} via interacting with low amounts of template PrP^{Sc}, on the cell surface. This conversion has been demonstrated in several *in vitro* experiments and cell-free systems [66-67, 110-113].

In agreement with the protein-only hypothesis (see section 1.1), two models were proposed to describe the mechanism of conformational conversion of PrP^C into PrP^{Sc}, the template-directed refolding model and the seeding nucleation model (Figure 3). The first one postulates that the interplay between exogenous PrP^{Sc} and endogenous PrP^C is the driving force behind structural conversion. According to this hypothesis, a spontaneous conversion of PrP^C into PrP^{Sc} is prohibited by an energy barrier. The seeding nucleation model proposes an equilibrium state

between PrP^{C} and PrP^{Sc} and regards monomeric PrP^{Sc} to be non-contagious and non-pathogenic. The contagious agent would only be an assembly of highly ordered but aggregated PrP^{Sc} molecules. In the presence of these aggregates, further PrP^{Sc} monomers could be recruited, aggregated and form fibrils [114-116]. While both of these models explain some of the aspects of the interplay between PrP^{C} and PrP^{Sc} , the true mechanism is not yet fully uncovered. This will be discussed in more molecular detail in section 1.3.3.

While being identical in the amino acid sequence and carrying the same PTMs, both isoforms of PrP differ dramatically in their physiological properties, such as detergent solubility and proteinase K resistance. PrP^{C} is highly soluble and amenable to proteinase digestion, whereas PrP^{Sc} molecules tend to aggregate and form fibrils. Moreover, the latter species is resistant to proteinase K digestion [117-118]. Up to now, no high resolution structure of PrP^{Sc} is available due to its insolubility, inhomogeneity and tendency to aggregate [119].

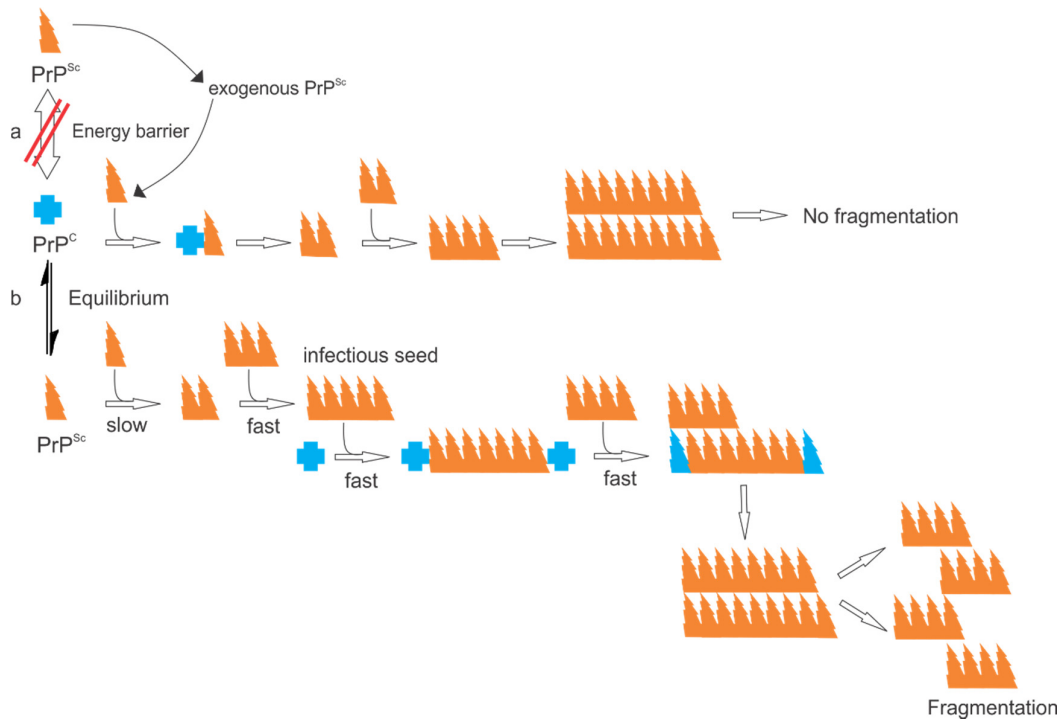


Figure 3: Possible mechanisms for prion propagation

a) Template directed refolding; b) seeding nucleation model. Blue cross: PrP^{C} monomer, orange lightning: PrP^{Sc} monomer.

1.3.2 Structure of PrP^{Sc}

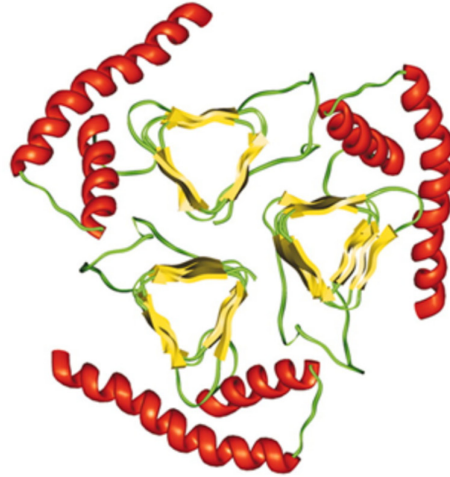
Despite the aforementioned absence of a high resolution structure of PrP^{Sc}, much structural information has been gathered by scientists over the recent years ^[120]. Indeed, it has been widely recognized that the conversion of PrP^C into PrP^{Sc} results in an increase of β -sheet content and PrP^{Sc} tends to form fibrillary assemblies with a cross β -strand secondary structure. Additionally, there are structural models for PrP^{Sc} based on low resolution electron microscopy, X-ray fiber diffraction, FT-IR, CD-spectroscopy and hydrogen/deuterium exchange mass spectrometry (HXMS) ^[120]. One of the most distinguished studies was conducted by Govaerts *et al.*, who surveyed all the known β -folds and used molecular modeling to come up with a low-resolution 2D PrP^{Sc} structure as a left-handed β -helix forming a trimer (figure 4A) ^[121]. Recently, Surewicz and coworkers demonstrated a rather different model using site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy ^[122]. The researchers used the “synthetic prion” that had been described by Legname *et al.*, a GPI anchor-less, non-glycosylated PrP²⁷⁻³⁰ variant isolated from prion-infected transgenic mice ^[123], and found that PrP^{Sc} does not contain any α -helices but is rather comprised of β -strands and short loops. This structural data points to an alternative conversion model, whereby the whole PrP^C molecule should be remodeled (figure 4B). This structural data is in conflict with previous data collected by FT-IR and CD measurements ^[108, 124].

1.3.3 *In vitro* conversion methods

As already described in section 1.3.1, a conformational change of PrP^C accompanies its conversion into PrP^{Sc} subsequently leading to pathogenicity. Hence, numerous investigations have been undertaken in an attempt to reveal the nature of this process. In this section, one of the most efficient and established methods will be discussed, the *in vitro* conversion of PrP^C.

The first successful *in vitro* conversion using radiolabeled PrP^C into PK-resistant PrP^{res} was described by Kacisko *et al.* ^[113] Nevertheless, this study and many of the following works had severe drawbacks, including the use of non-physiological conditions such as chaotropic buffers with high salt concentrations, and extremely low yields of PrP^{res}. Hence, the method is not ideal for further use.

A



B

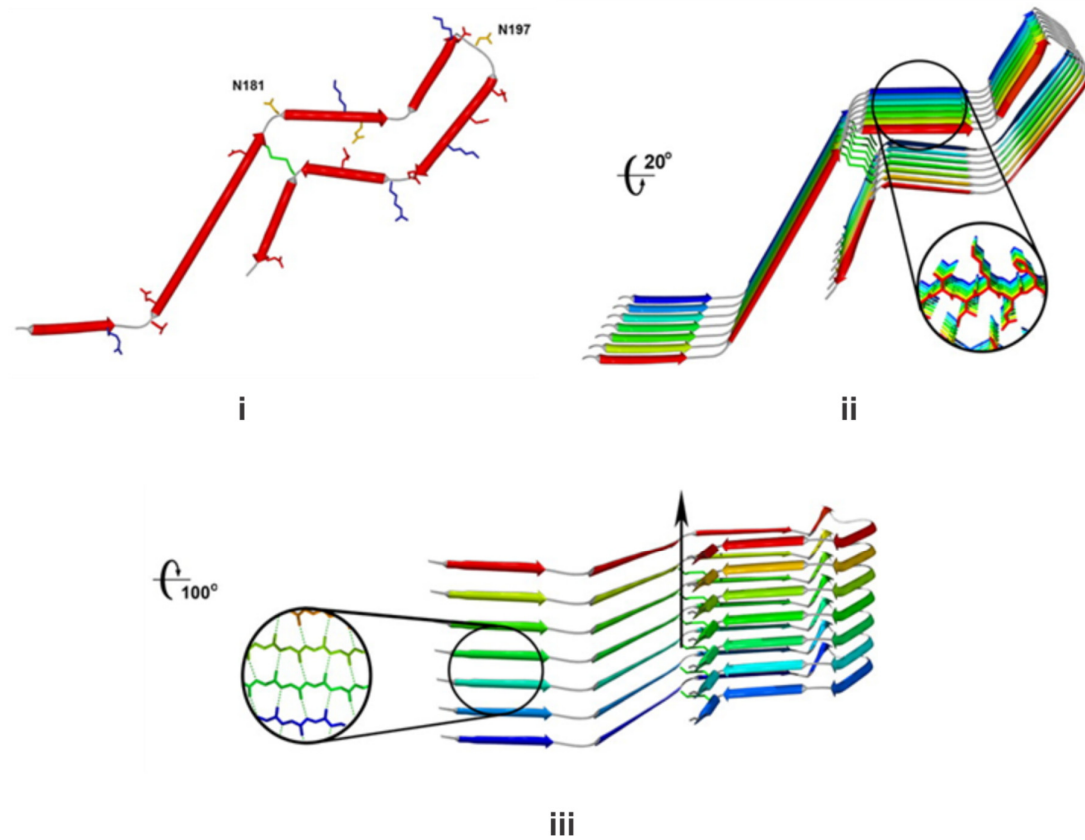


Figure 4: Possible 3D structures of PrP^{Sc} [121-122]

A: Trimeric PrP 27-30 model proposed by Govaerts *et al.*; B: Simple schematic representation of parallel, in-register alignments of PrP^{Sc} as demonstrated in Surewicz *et al.*, i) possible structure of PrP^{Sc} monomeric amyloid core (aa 159-219), ii) and iii) fibrillary assemblies from different angles demonstrating the typical β -cross structure.

Almost a decade later, Soto and coworkers developed the so-called “protein misfolding cyclic amplification assay” (PMCA). In this procedure, healthy brain homogenate containing endogenous PrP^C was mixed with several dilutions of PrP^{Sc} infected brain homogenates at 37°C. Sonication of the samples every hour for each cycle (5-40 cycles) initiated the amplification of PrP^{Sc}-like oligomers, which subsequently could convert freshly added non-infected brain homogenates into PrP^{Sc} as well. Therefore, this method is generally described as a seeded reaction. After the treatment, up to 97% PrP^{Sc} like protein oligomers could be detected in the original sample using the PK-assay [125]. In the last decade, PMCA has been extensively optimized. Thus, the assay is now automated, serial PMCA, and the introduced use of beads has enhanced the amplification rate and prevented the variability between distinct samples [126-128].

In the original work, Sabario *et al.* reported the higher efficiency of conversion while using brain homogenates rather than purified samples. This observation supports the hypothesis that not only the PrP^{Sc} but also other molecules might play a major role as cofactors in the PrP^C → PrP^{Sc} conversion [129]. The research using PMCA revealed that polyanions (specifically, RNA molecules), lipids and a proper PrP^C substrate promote the *in vitro* conversion, whereby the source of PrP^C substrate can be the cell lysate or purified PrP^C, even of recombinant origin [112, 130-134].

Further advances in seeded *in vitro* conversion has been made by Caughey and coworkers via developing the “quaking-induced conversion” (Quic) assay using accessible body fluids like cerebrospinal fluid as PrP^{Sc} seeds and recombinant PrP^C as substrate. The main difference to PMCA was the substitution of sonication with constant tube shaking [135]. In the last decade the Quic method has been modified to enable real-time monitoring of amyloid fibril formation (RT-Quic) via Thioflavin-T assay, a general method to visualize amyloid plaques using fluorescence [136-138].

In 2004, Legname and coworkers reported the first non-seeded prion generated from bacterially expressed N-terminally truncated recMoPrP89-230, referred to as “synthetic prion” [123]. Misfolding experiments carried out in chaotropic buffers generated amyloidogenic fibrils from Syrian hamster brain samples. Subsequently, these fibrils were applied intracerebral to transgenic mice Tg9949. This mouse model overexpresses MoPrP89-230 to produce 16 times more truncated PrP in comparison to the amount in the brain homogenate of a healthy Syrian hamster.

All mice treated with the recombinant amyloid fibrils showed signs of neurological disorders within 620 days. Interestingly, this result could not be fully reproduced by others. The prion generated *in vitro* from *E.coli* was indeed highly pure due to the chaotropic conditions for solubilization and purification processes. However, the infectivity of these synthetic prions in animal-based assays was low, which makes them unsuitable to reveal the underlying mechanism in PrP conversion^[139].

In 2010, Wang and colleagues generated a highly infectious prion derived from recombinant PrP using serial PMCA in the presence of liver RNA and anionic phospholipid POPG (1-palmitoyl-2-oleoylphosphatidylglycerol) after 17 cycles. The resulting PrP^{Sc}-like rPrP^{res} was able to convert glycosylated, endogenous PrP^C *in vitro* and *in vivo*. All mice inoculated with rPrP^{res} showed symptoms of TSEs within 130-180 days post infection (dpi) ^[134]. Additionally, it has been shown that genetic information of RNA is not necessary for prion infection and disease progression ^[140]. Despite various experiments done to support the theory that RNA contributes to *in vitro* conversion of prions, it remains unclear whether it acts as a cofactor or is directly involved in the infection by association with prions. Recent advances have been achieved using light-induced degradation of polyanionic factors with the use of serial PMCA ^[141]. Suppatapone *et al.* incorporated a synthetic polyanion that can be selectively degraded upon exposure to UV light, and found that polyanions indeed support the conversion but have no effect on the strain-specificity of different prion seeds. Moreover, a recent study supported these findings using RNase A to digest RNA during PMCA with different prion strains. The conversion efficiency after PMCA was significantly reduced regardless of the strain ^[142]. Based on this data, it can be proposed that RNA or polyanions act as cofactors and assist the misfolding of PrP^C. The role of PTMs in this process, on the other hand, is still unknown (Section 1.4.).

All the studies mentioned above have been performed with anchorless and heterogeneously glycosylated PrP^C variants. We recently showed the role of interactions between POPG and membrane-anchored rPrP on the misfolding process and PK-sensitivity ^[143]. In order to do that, we used three different PrP variants equipped with a synthetic dipalmitoylated membrane anchor (MA) and their non-lipidated counterparts, full length PrP (FL_PrP-MA: aa 23-231), N-terminally truncated PrP (T_PrP-MA: aa 90-231) and Δ CR_PrP where the central hydrophobic domain is missing (Δ CR_PrP-MA: aa 105-125 deleted). These PrP

variants was bound to biomembrane mimics formed out of neutral (DOPC), anionic (POPG) and more complex lipid compositions tightly. The secondary structure was influenced by the presence of the anchor mimic as well as the composition of the lipid membrane. A certain PK-resistance for FL-PrP mixed with POPG could also be detected, in accordance to previous work by Wang *et al* [144]. This study showed that recombinant PrP variants (rPrP) carrying a membrane-anchoring unit change the secondary structure upon membrane binding and may become PK-resistant under physiological conditions.

Overall, these findings suggest that it is essential to decipher the contribution of individual PTMs, especially N-glycosylation, and combinations thereof, to the folding and misfolding of PrP^C.

1.4 PrP and PTMs

PTMs of both the endogenous host-encoded PrP^C and the infectious PrP^{Sc} often play a crucial role in the onset and development of prion diseases. Here, key PTMs are discussed separately in detail.

1.4.1 N-Glycosylation of PrP^C

Asn-linked N-glycosylation of proteins is one of the most widespread and complex PTMs processed in the rough ER. A large variety of functions are linked to *N*-oligosaccharides, such as stabilization of protein secondary structure, protection against protease digestion, conferring backbone stability of proteins leading to assistance in correct folding, enhanced solubility, regulation of the immune response and cell-cell communication [55, 145-147]. Thus, it is of major interest to get detailed insights into the impact of *N*-glycans on PrP.

There are three main *N*-glycoforms of PrP, which are referred to as un-, mono- and di-glycosylated PrP with respect to which Asn residues are derivatized with glycans [148]. First steps in characterizing the glycosylation pattern of PrP^C and PrP^{Sc} were made by Haraguchi and coworkers, who treated the purified proteins with N-glycosidase F, then concluded glycosylation based on reduction of the total molecular weight of the treated proteins.

Following this crude analysis, glycoforms of PrP were shown to be highly heterogeneous by modern biochemical methods and mass spectroscopy [149-150]. More than 50 glycoforms of PrP^C and PrP^{Sc} could be characterized with an increased number of tri- and tetra-antennary glycans found on PrP^{Sc} using hydrazinolysis, fluorescence labeling or acetylation of glycans and glycosidase digestion followed by mass analysis. Further use of state of the art MS methods resulted in identification of the ratio of tri- and tetra-antennary glycans on Asn 180 and 196 of mouse PrP (moPrP), [150] revealing that Asn 196 has a higher content on tri- or tetra-antennary *N*-glycans compared to Asn 180.

One of the major questions in prion biology is the transmissibility between different prion strains. Important differentiation factors among various prion strains and TSEs are the incubation time, neuropathological patterns and differentiated glycoforms that were first characterized via electrophoretic methods [151]. In a seminal study, Collinge *et al.* used purified brain homogenate samples from sporadic CJD (sCJD) patients and detected three PK-resistant bands of PrP^{Sc} using SDS-gel electrophoresis. These strains were identified as un-, mono- and di-glycosylated PrP^{Sc}. Depending on the ratio between these three bands, there are major differences among pathological CJD phenotypes such as sCJD and variant CJD (vCJD). sCJD features predominantly the mono-glycosylated band whereas vCJD exhibits the di-glycosylated PrP^{Sc} as the most intense band. This observation led to considering glycoform ratios as suitable differentiation factors for different prion strains.[152]

Over the course of the last two decades, several research groups have been working on the identification of the impact of PrP glycosylation on prion pathogenesis. This work has led to the general assumption that *N*-glycosylation does not play a major role in conversion into misfolded form and prion infectivity [153-155]. However, due to the difficulties in characterizing heterogeneous glycoforms of PrP^C and PrP^{Sc} in different prion species via biochemical methods as well as very limited access to fully posttranslationally modified PrP^C and PrP^{Sc}, more evidence is urgently needed to confirm this assumption.

Indeed, Tuzi *et al.* recently reported that glycosylation of the host PrP^C has an indirect effect on prion infectivity [156]. The researchers used unique inbred lines of genetically modified mouse models harboring N→T mutations at either one or both

N-glycosylation sites of moPrP, N180 and N196. The major conclusion of this work was that glycosylation of host PrP, rather than PrP^{Sc}, is the crucial factor for the transmission of prion infectivity and influences the susceptibility to TSEs and the duration of showing pathological signs. However, in agreement with the results of previous studies [153-154], this work also highlighted that the glycosylation is not necessary for prion infectivity, i.e. the unglycosylated PrP has been proven to be infectious as well.

In 2008, Gambetti and coworkers described the discovery of a new sporadic TSE, protease-sensitive prionopathy (PSP^r) [157]. Like in familial CJDV180I, the absence of di-glycosylated PrP^{Sc} was reported, suggesting a degree of selectivity in glycosylation of PrP^C and conversion into PrP^{Sc}. Further *in vitro* and *in vivo* studies from the same group showed that not only di-glycosylated PrP^{Sc} was missing, but also mono-glycosylated PrP^C (at Asn181) was unable to be converted into PrP^{Sc} [158]. These results may be regarded as the first direct evidence of the impact of glycosylation on prion transmissibility.

1.4.2 GPI-anchoring of PrP

Under physiological conditions, PrP is tethered to the outer leaflet of the plasma membrane with a GPI anchor [159]. The GPI anchor consist of a common core structure but heterogeneous side chain and lipid moieties among different proteins. It has been widely accepted that the lipids interact with the hydrophobic part of the membrane and anchor the protein into the membrane via hydrophobic interactions. Similarly to other GPI-anchored proteins, PrP's GPI anchor can be cleaved by a member of the phospholipase C family, phosphatidylinositolphospholipase C (PI-PLC) [160]. As already mentioned in section 1.2.3., GPI-anchored proteins are usually concentrated in ordered membrane lipid microdomains, the so-called "rafts" [161]. Transport of PrP^C from the ER into the Golgi is accompanied by the localization of PrP^C in ordered membrane domains that contain sphingolipids and cholesterol. Interestingly, Bate *et al.* showed that depletion of cholesterol from biomembranes assists the correct folding of PrP and results in prevention of PrP^{Sc} formation, showing that the lipid composition has a profound effect on PrP conversion [162]. Moreover, the impact of the GPI anchor on the conversion of PrP^C into PrP^{Sc} has been widely investigated, and there is strong evidence that anchoring to the plasma

membrane is crucial for this process [67, 163-166]. Additionally, several publications highlight the link between the PrP's GPI anchor and ordered membrane domains [167-170]. Baron *et al.* reported a cell-free assay to convert radioactively labeled PrP^C into PrP^{Sc} at lipid rafts. The major finding of this study was that in the absence of PI-PLC, PrP^C in lipid rafts could not be infected with exogenously introduced PrP^{Sc}. These results highlight the fact that GPI-anchored PrP species stayed in ordered membrane domains based on their lipid compositions and are resistant to infection unless localized on the cell surface [169]. Further experiments by Baron and coworkers revealed that in the presence of the GPI anchor, PrP^C does not convert into PrP^{Sc} and remains localized in the ordered membrane domains unless i) PI-PLC liberated PrP^C from the GPI anchor or ii) a polymer support (PEG) was added in order to fuse two membrane fractions containing PrP^C and PrP^{Sc} [170]. In both cases, PrP^C could be converted into PrP^{Sc} suggesting that the conversion process is either occurring on the cell surface or during the internalization of PrP^C and PrP^{Sc}. Moreover, the localization in rafts tends to prevent the conversion into PrP^{Sc}. Despite being the focus of numerous publications, the exact impact of the GPI anchor and potential heterogeneities in its structure on prion pathogenicity is not completely understood.

Taken together, the research summarized in this section highlights the importance of the GPI anchor and other PTMs in TSEs, and the need to study their effect in more detail at the molecular level. Addressing these questions constitutes the main goal of the research described in this thesis.

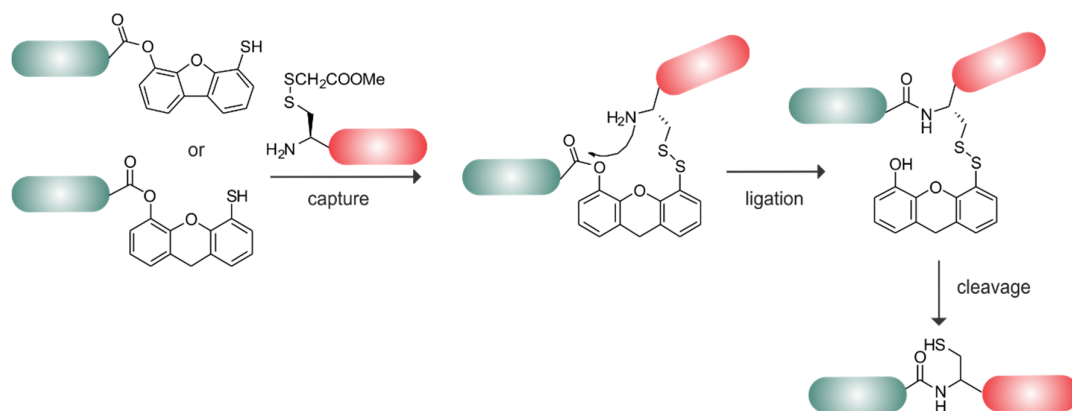
1.5 Semisynthesis of homogenously modified proteins

Low yields and harsh purification protocols limit the access to posttranslationally modified PrP. To decipher the impact of PTMs on prion infectivity and toxicity, new methods are needed to enable the access to posttranslationally modified PrP variants. Additionally, the heterogeneity of PTMs, as outlined in several parts of the introduction, is a major obstacle in determining the (patho-) physiological role of PrP. In order to solve this problem, a method combining the linkage of synthetic peptides and recombinant proteins can be used to obtain homogenously complex modified PrP. This method is generally referred to as semisynthesis of proteins ^[171-172]. In this section, recent advances in (semi-)synthetic methods to obtain modified peptides and proteins will be discussed.

1.5.1 Chemical synthesis of peptides and proteins

The total chemical synthesis of proteins has been one of the most challenging topics of organic chemistry in 20th century. In 1901, Emil Fischer reported the chemical synthesis of glycylglycine, which is regarded as the first synthetic peptide ^[173]. Over the course of the decades, temporary protecting groups such as carboxybenzyl (Cbz or Z) ^[174], *tert*-butoxycarbonyl (Boc) ^[175-176] and 9-Fluorenylmethoxycarbonyl group (Fmoc) ^[177] have been introduced to the α -amino group to hinder cross reactions and undesired side products. In 1963, Robert B. Merrifield described the solid phase peptide synthesis (SPPS) and enabled the generation of peptides (up to 50 aas) by synthesizing the polypeptide chain sequentially on a solid support ^[178]. Over the last 5 decades, SPPS has been refined by the introduction of orthogonal side chain protecting groups, activators, linkers and polymer-based solid supports ^[179-180]. Moreover, the automation of SPPS via synthesizers and the use of microwave and infrared heating improved the coupling time and decreased susceptibility to epimerization ^[181-182]. Nevertheless, major limitations of SPPS involve aggregation of peptides on solid support, insufficient coupling of amino acids, decrease in yield and solubility with growing chain length resulting a limitation in length of synthetic peptides (between 50-60 amino acids for non-optimized conditions) ^[181, 183]. As a result, the chemical

synthesis of proteins that have a median length between 300-500 aa ^[184] is not feasible with SPPS in one piece. Therefore, a convergent method had to be applied to connect unprotected peptide segments to enable the chemical synthesis of large proteins. Besides solid phase fragment condensation ^[185], one of the most promising early attempts has been introduced by Kemp and colleagues, the “prior thiol capture” method ^[186]. This method describes the attachment of a peptide to a temporary template, 6-hydroxy-4-mercaptodibenzopyran or –furan, on its C-terminus via an ester bond and caption of a second peptide via disulfide exchange of an N-terminal disulfide-activated cysteine by the free thiol moiety of the template (Scheme 1). Final cleavage of the template after ligation was achieved by the use of mild reducing agents ^[186-187]. After several modifications, the method has been optimized allowing ligation of two unprotected peptide segments forming a 39mer peptide ^[188]. Unfortunately, the limitation of peptide length and harsh cleavage conditions did not allow to expand the scope of this to chemical protein synthesis ^[189].



Scheme 1: Thiol capture mechanism

This early attempt was followed by the development of chemoselective ligation reactions that enable linkage of peptide segments selectively at their termini in aqueous buffer. Among others, the thioester ligation by Kent and coworkers ^[190], thiazolidine-mediated ligation by Tam and coworkers ^[191], oxime ligation first described by Rose ^[192], the disulfide-directed ligation reported in Baca et al. ^[193] and the thioether ligation by Alewood and coworkers ^[194] were accepted as powerful methods. Using the methods chemically modified and unmodified protein analogues could be obtained in good yield and purity (Scheme 2). Although the ligation products featured a non-native bond at the ligation site, they were fully

active and correctly folded compared to wild type recombinant counterparts. Nevertheless, the resulting chemically synthesized proteins comprised a non-native structure and are not considered as adequate models to study the molecular function of native proteins.

Thioester Ligation



Thioether Ligation



Thiazolidine Mediated Ligation



Oxime Ligation

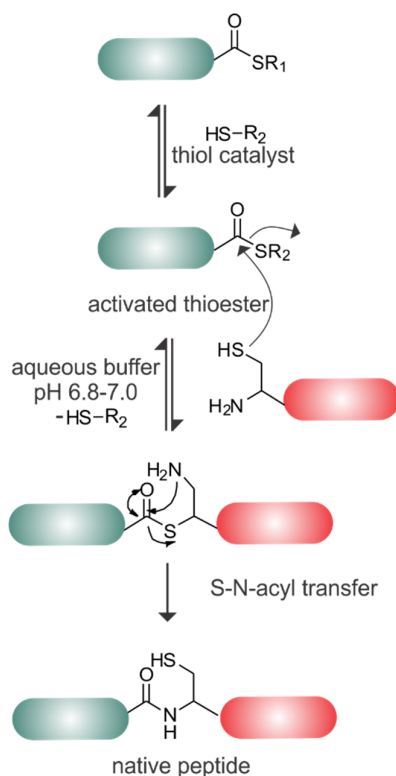


Scheme 2: Chemoselective ligation methods with non-native bonds on the ligation site

1.5.2 Native Chemical Ligation (NCL)

A major scientific breakthrough in chemoselective ligation chemistry was made in 1994, when Kent and coworkers described the total chemical synthesis of human interleukin 8 (IL-8) via a reaction that they termed native chemical ligation (NCL)^[171]. The reaction was based on Wieland's observation made in 1953. The researchers found that under neutral or basic pH a highly reactive aryl thioester could undergo an S→N acyl shift in the presence of cysteine^[195]. Kent and coworkers used an unprotected peptide carrying a C-terminal α-thioester prepared by Boc-SPPS, which was reacted with another unprotected peptide bearing an N-terminal cysteine in aqueous buffer under denaturing conditions. The first step of this ligation is reversible and yields a thioester-linked intermediate, which then undergoes a spontaneous S-to-N acyl shift in a second, irreversible step and results in the formation of a native peptide bond (Scheme 3). NCL is highly regio- and chemoselective, whereby it does not occur at other nucleophilic side-chain

groups of amino acids such as ϵ -amino moiety of lysine, hydroxyl side chains of aliphatic and aromatic amino acids or sulfhydryl groups of internal cysteines. The reaction conditions are mild and thioesters are stable at acidic or neutral pH. The reaction can be accelerated by transthioesterification with various thiol catalysts that generate highly reactive thioester intermediates *in situ*. Thus, recent advances in thiol catalyst development have shortened reaction times and increased the yields of ligation products dramatically [196-198].



Scheme 3: Native Chemical Ligation (NCL)

In addition to the nature of the thioester, an important aspect that has an impact on the progression of NCL reactions is the C-terminal amino acid carrying the thioester moiety. Hackeng and coworkers have investigated this aspect by testing all 20 proteinogenic amino acids at this position [199]. The outcome of this study can be summarized in two major points: firstly, a glycine thioester gave the fastest ligation reaction resulting in quantitative ligation. Secondly, amino acids with branched side chains such as leucine, valine, isoleucine, threonine and the secondary amine proline could not be converted into the desired ligation products even after long reaction times. NCL and its enhancements play a pivotal role in the research

described in this thesis, and will now be discussed in greater detail (Sections 1.5.2.1 and 1.5.2.2).

1.5.2.1 N-terminal cysteine surrogates

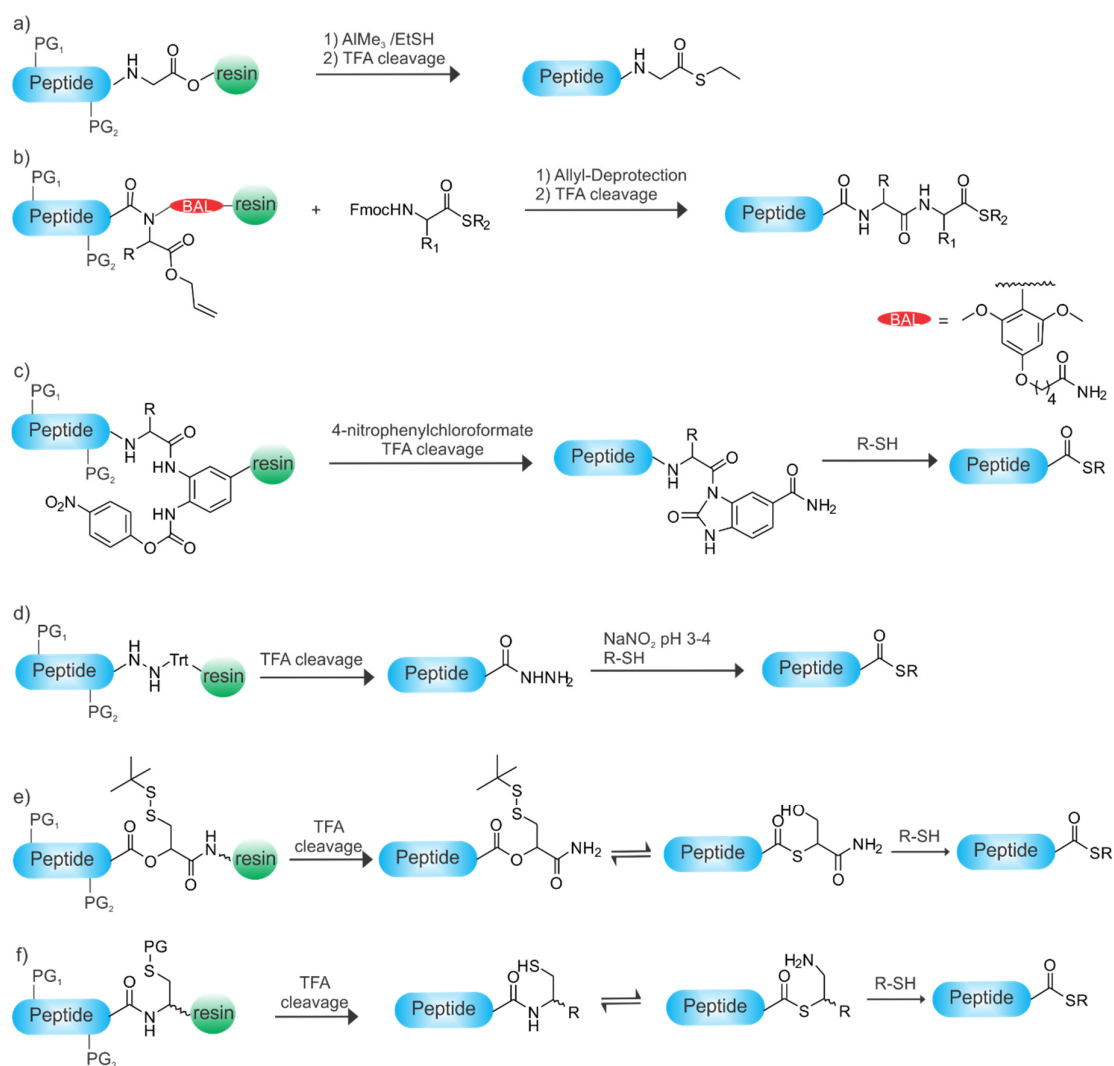
One of the major limitations for NCL reactions used to be need of a cysteine at the ligation site. As this amino acid is the second least abundant in native proteins ^[200], the requirement for it is a major limitation of NCL ^[201]. Fortunately, surrogates for cysteine have been synthesized and introduced into the N-termini of peptide segments to allow NCL-like chemoselective ligations. These surrogates have been able to give rise to proteinogenic amino acids at the ligation site upon ligation reaction. To date, the most successful method is the ligation-desulfurization chemistry first described by Yan and Dawson who used NCL reaction with a cysteine at the ligation site and converted it via reductive desulfurization into the native alanine, the second most abundant amino acid ^[202]. Danishefsky and coworkers improved the ligation-desulfurization reaction through the development of a metal-free, radical desulfurization method using water-soluble radical initiator VA-044 in combination with TCEP (tris(2-carboxyethyl)phosphine) and a sulfhydryl donor ^[203]. Inspired by these experiments, unnatural amino acids with β -, γ - and δ -sulfhydryl side chains have been developed and successfully ligated to peptide α -thioesters ^[204-209]. The non-native sulfhydryl has to be removed following the ligation to generate the corresponding native amino acid at the ligation site. Unfortunately, reductive desulfurization is non-selective and desulfurize all unprotected sulfhydryl groups in proteins and peptides. Recently, Payne and coworkers reported the chemical synthesis of various β - and γ -mercapto-amino acids, which they introduced into peptide segments and linked successfully to peptide- α -thioesters ^[210-212]. After the ligation, a selective desulfurization method using DTT and TCEP in excess gave rise to site-specific desulfurization of cysteine surrogates even in the presence of unprotected cysteine residues in the peptide sequence ^[211]. Furthermore, seleno-amino acids could be ligated and selectively deselenized into alanine or serine by the Payne and Metanis groups independently ^[213-215].

By virtue of the advances outlined in this section, the scope of NCL has been drastically expanded. The reaction enabled the total chemical synthesis of large proteins such as the 304 aa tetra ubiquitin^[216], both L- and D- isomers of 312 aa

GroEL/ES dependent protein, DapA ^[217], as well as posttranslationally-modified proteins, such as the chemokine Ser-CCL1 ^[218] or monoglycosylated GM2 ganglioside activator ^[219].

1.5.2.2 Peptide α -thioesters compatible with Fmoc-SPPS

Another important component of the NCL reaction is the C-terminal thioester. Peptide- α -thioesters can be readily synthesized via Boc chemistry. Unfortunately, the repeated exposure to acidic conditions associated with this method and the final cleavage of peptides from the resin by treatment with HF make it unsuitable for peptides with acid-labile modifications. Hence, for chemical synthesis of many modified proteins, there is a continuous need of different methods to generate

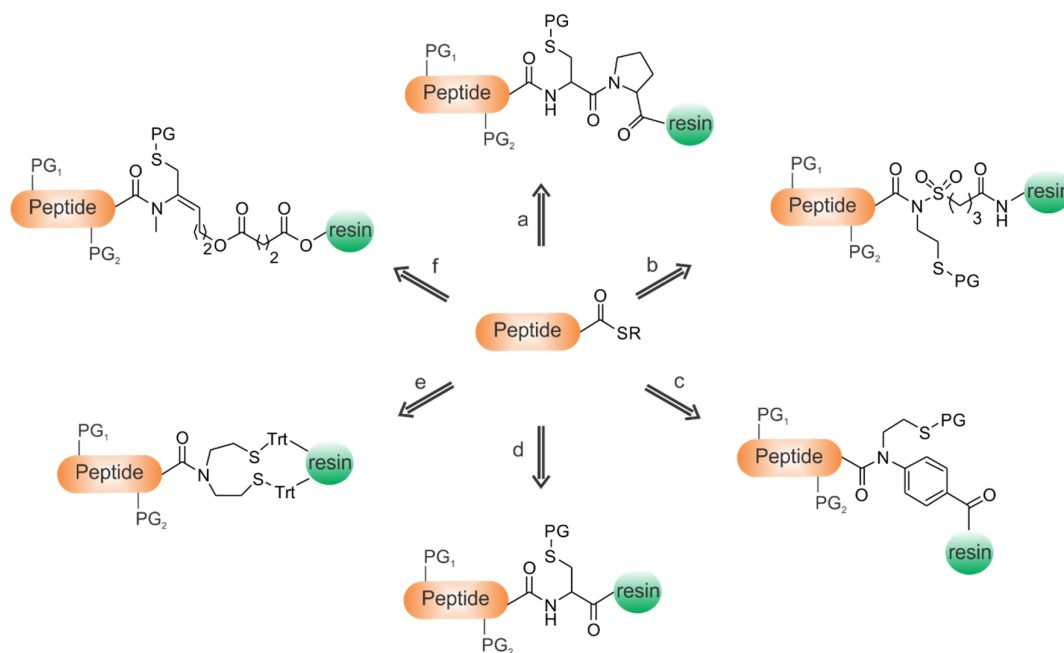


Scheme 4: Methods for generation of peptide- α -thioesters via Fmoc SPPS

PG: protecting group, Trt: Trityl.

peptide thioesters. Fmoc-SPPS chemistry provides milder conditions compared to that of Boc-SPPS, and is therefore an alternative method for the preparation of peptide- α -thioesters. However, under the Fmoc cleavage conditions with basic and nucleophilic piperidine, thioesters are unstable. This limits the use of resins with a C-terminal thioester bound to the peptide of interest. Several ways have been developed to overcome this problem. For example, resin-bound peptide esters and amides have been converted into thioesters ^[220-222] (Scheme 4a), peptide acyl donor activity has been increased via “safety-catch linkers” ^[223-228] (Scheme 4b-c), peptide hydrazides have been used as thioester precursors ^[229-230] (Scheme 4d) and peptide thioesters have been prepared via O- to S- ^[231-232] and N- to S-acyl shift ^[233-240] upon cleavage from the resin (Scheme 4e-f).

Among these techniques, the N- to S-acyl shift is considered by some to be the most promising method and therefore will be discussed in detail. This approach comprises the intramolecular nucleophilic attack of a sulfhydryl-group at the carbonyl of the C-terminal amide after the cleavage from the resin. The formation of a transient thioester is followed by the activation of the thioester moiety in presence of an external thiol catalyst, as discussed in section 1.5.2. A variety of linkers and “latent” thioesters has been used to give rise to C-terminal thioesters



Scheme 5: Generation of peptide- α -thioesters through N-to-S-acyl-shift

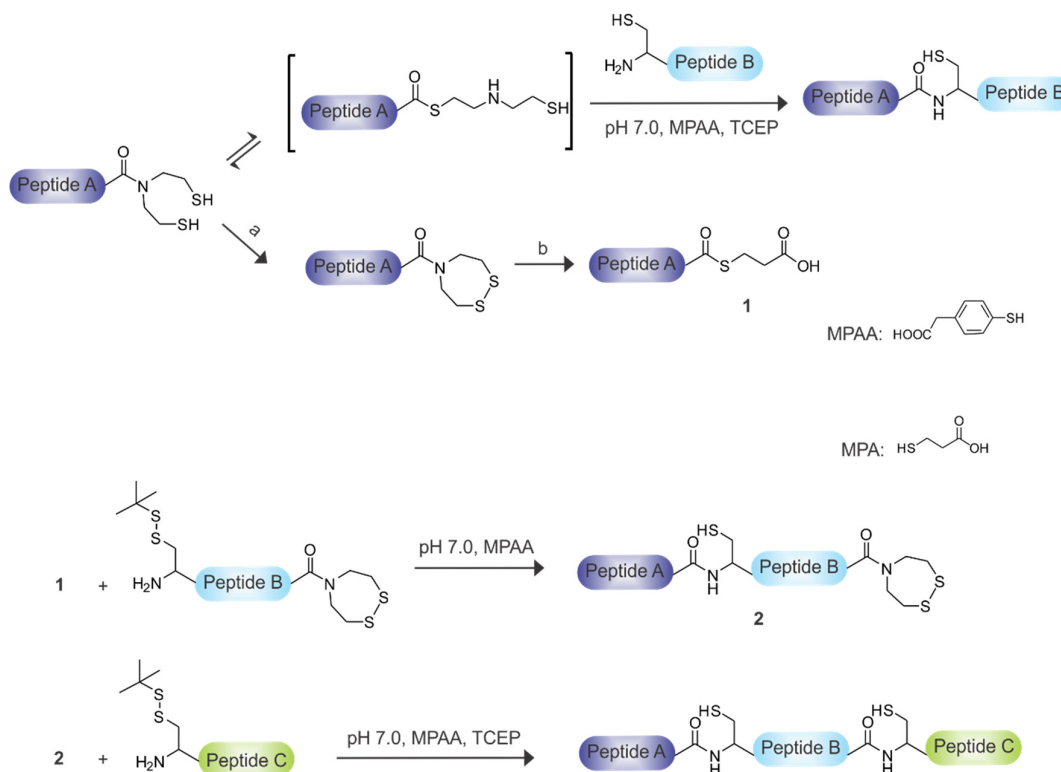
PG: protecting group

via N-to S acyl shift. For instance, Ollivier *et al.* reported a combination of a safety

catch linker and N- to S-acyl shift with an N-sulfonamide linker bound to the resin (Scheme 5b) ^[233].

In other examples based on such an approach, Kawakami et al. described the cysteine-proline-ester (CPE) method (Scheme 5a) ^[234], while Macmillan and coworkers used aliphatic thiol catalysts (3-mercaptopropionic acid, MPA) under acidic conditions to initiate a spontaneous N-to-S acyl migration and the formation of a C-terminal thioester ^[235] (Scheme 5d). Recently, the use of an unnatural amino acid with a C-terminal enamide harboring a protected thiol as a side chain and a free carboxylic acid for attachment to the peptide chain on resin has been reported (Scheme 5f). Upon cleavage of the thiol protecting group followed by the N-to-S acyl shift, the enamide is converted into an enamine, which then undergoes irreversible hydrolysis giving rise to a C-terminal thioester ^[239].

The most recent focus in this area is on one-pot sequential ligation strategies allowing NCL reactions via N-to-S acyl shift. The most noteworthy examples are the *N*-sulfanylethylanilide (SEAlide) linker described by Tsuda et al. (Scheme 5c), ^[236] as well as the bis(2-sulfanylethyl)amido (SEA) linker developed by Melnyk and coworkers ^[237] and also reported by Hou et al. (Scheme 5e) ^[238]. Both methods are Fmoc-SPPS compatible. Recently, SEAlide linker was used in the synthesis of glycosylated GM2 protein ^[219]. Nevertheless, the complex chemistry involved in the preparation of the linker is a major drawback of this method. In contrast, SEA-linker can be easily prepared under standard conditions in three synthetic steps, and switched between the so-called on and off state (SEA^{on} and SEA^{off}, respectively) by redox chemistry (for details see Scheme 6). In the SEA^{on} linker, the mercaptoethyl residues are reduced whereas in the SEA^{off} state a disulfide bond exists. With a suitable reduction agent (e.g. TCEP, DTT), SEA^{off} can be converted into SEA^{on} and used for sequential ligations. Interestingly, SEA^{off} peptide can also be converted into a peptide- α -thioester **1** when using 3-mercaptopropionic acid (MPA) and the ensuing thioester is suitable for standard NCL reaction (Scheme 6). In the presence of aryl thiols and in the absence of strong reduction agents during the ligation reaction, the SEA-linker remains in its off state and can be linked to **1** giving a SEA^{on} linked ligation product **2**. Once a reducing agent is added to the solution, conversion into SEA^{on} occurs within minutes, and the subsequent ligation step can be performed (Scheme 6).



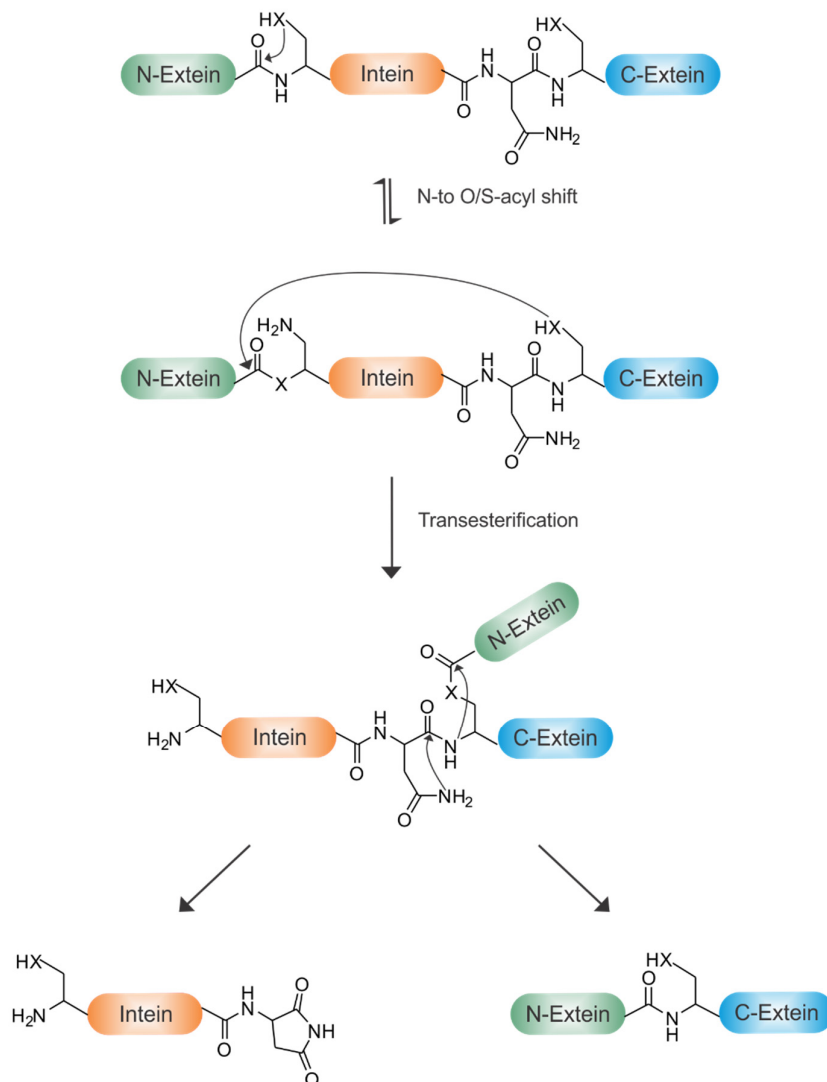
Scheme 6: One pot SEA ligation

Using this method, Melnyk and coworkers successfully completed the total chemical synthesis of the N-domain of biotinylated human HGF (hepatocyte growth factor) (128 aa) ^[241] and SUMO (small ubiquitin-like modifier) protein-peptide conjugates (96 aa) ^[242]. Not only the synthesis but also the conjugation strategy described in both publications is of interest. Overall, SEA-chemistry is an attractive approach for the preparation of homogenously modified peptides and proteins, and is utilized in this thesis.

1.5.3 Expressed Protein Ligation (EPL)

Despite being a very powerful technique, NCL has its limitations. The dependence on SPPS as a source of peptide segments automatically limits the size of ligation partners to 50-70 amino acids. Each additional ligation step dramatically reduces the overall yield of the target polypeptide. Additionally, the synthesis of peptide-thioester fragments can be time consuming.

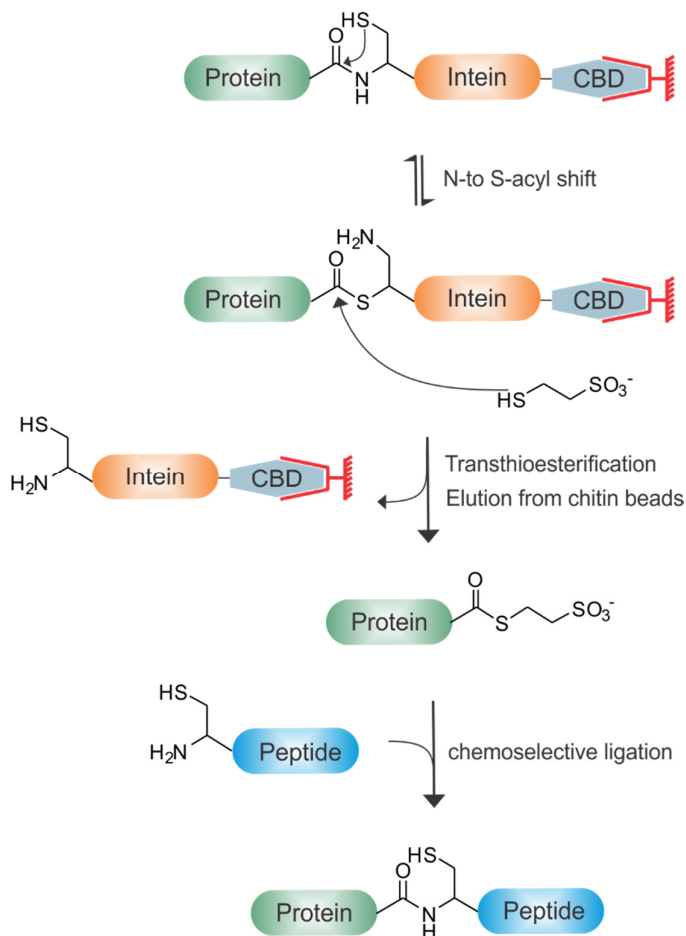
For the synthesis of proteins of large molecular weight, as well as multimeric proteins and hydrophobic sequences, Muir and Evans independently developed a powerful method termed expressed protein ligation (EPL) [172, 243]. This technique is



Scheme 7: Mechanism of protein trans-splicing

based on a natural process called protein splicing. Similarly, to RNA splicing, the protein fragment in the middle, intein, excises itself from the flanking N- and C-terminal domains, the exteins. During the cleavage, exteins are linked to each other by the formation of a new peptide bond. The mechanism is shown in scheme 7. In the first step, a nucleophilic side chain ($-OH$ of serine or $-SH$ of cysteine) of the N-extein undergoes an N-to-O or N-to-S shift driven by the autocatalytic activity of the intein. Hereby, a TXXH motif with threonine and histidine as basic residues

initiate the deprotonation of the nucleophilic side chain [244-245]. Furthermore, the conformational strain leads to the destabilization of the peptide bond favoring the nucleophilic attack [244]. The resulting thioester is in close proximity to the nucleophilic side chain of serine or cysteine and can be transferred to the side chain of the first amino acid on C-extein through trans(thio)esterification. The last step involves an S-to-N acyl transfer through the intramolecular rearrangement with the conserved Asn residue of intein giving rise to an intein with a C-terminal succinimide (Scheme 7) and ligated N-extein-C-extein with a native peptide bond (Scheme 7) [189, 246-247]. This step is assisted by a histidine residue of a highly conserved HNF motif Mutation of the C-terminal Asn of the intein as well as the N-terminal Cys/Ser of the C-extein to Ala prevents completion of the splicing process



Scheme 8: Expressed Protein Ligation (EPL)

at the ester/thioester, and enables the generation of a recombinant protein- α -thioester by the addition of an external thiol reagent in excess [248]. Furthermore, an affinity tag can extend the intein sequence and replace the C-extein, most

commonly the chitin binding domain (CBD), allowing the attachment of the recombinant protein-intein fusion construct to a solid support (chitin beads). There are two advantages of this method. First, the protein of interest (POI) can be isolated from a cell lysate without further purification steps, and second, the resulting protein- α -thioester can be freed from the solid support and used for NCL with any N-terminal cysteine-containing peptide segments (Scheme 8). In this way, EPL is an elegant combination of molecular biology and synthetic chemistry.

Over the course of the last seventeen years, EPL has been used for numerous applications such as immobilization of proteins on solid support ^[249-250], attachment of oligonucleotides ^[251-252] and polymers ^[253-254], incorporation of unnatural amino acids ^[255-256] as well as segmental isotope-labeling ^[257-258]. Among all of these applications, the most significant impact of EPL is by site-specific introduction of synthetic peptides harboring chemical and/or posttranslational modifications into recombinant proteins. Thus, EPL is the most commonly applied method for semisynthesis of posttranslationally modified proteins, and as such, it is highly relevant to the work described in this thesis.

1.5.3.1 Site-specific incorporation of homogenous posttranslational modifications to proteins through EPL

PTMs are often crucial for protein function ^[259]. To elucidate the role of proteins in physiological processes, the impact of their PTMs need to be deciphered. In general, PTMs exhibit a large degree of heterogeneity and are therefore very difficult to study. Much effort has been devoted to access homogeneously modified proteins. Unfortunately, both the recombinant expression and the total chemical synthesis of large, posttranslationally modified proteins are extremely challenging and usually result in low yields. Conventional recombinant expression of proteins in *E.coli* does not provide posttranslationally modified proteins. Other organisms, such as *S. cerevesia*, may provide posttraslationally modified POIs, however the yields are low and the exact chemical structure can severely differ. *In vivo* expression systems, such as human cell lines, are very suitable techniques for the expression of posttranslationally modified proteins but neither homogeneity of PTMs nor sufficient amount of POIs are guaranteed. EPL provides a powerful way to site specifically attach synthetic peptides carrying chemical or posttranslational modifications to recombinant protein-thioesters and thus obtain milligrams of

homogenously modified POIs. In 1998, Muir and coworkers generated in their initial experiment a phosphorylated semisynthetic kinase Csk through the site-specific incorporation of one phosphorylated tyrosine into the C-terminus of Csk via EPL [172].

Following this work, a number of PTMs have been introduced into different proteins site-specifically. One of the examples of the PTMs that have been studied is prenylation. For instance, Goody and coworkers investigated the impact of prenylation on Rab-GTPases and their yeast homologues, the Ypt-family [260-261]. It was previously known that C-terminal prenyl groups anchor these proteins to the membrane bilayer, where they contribute to intracellular trafficking [262]. To elucidate the 3D structure and specific effects of PTMs on protein-protein interactions of GTPases with other interacting proteins, sufficient amounts of such prenylated proteins were required. The task was technically challenging due to low solubility and stability of lipidated peptide and proteins in commonly used buffering systems. Nevertheless, Goody and coworkers were able to develop a semisynthetic route to generate mono- and diprenylated Ypt1 and Rab-GTPases. These semisynthetic homogenously lipidated Ypt1 variants were co-crystallized with Rab-GDI, which is a GDP dissociation inhibitor, to determine the mechanism of dissociation of Rab-GDIs from biomembranes [260].

Another important lipidation-related PTM is GPI-anchoring of proteins, which occurs at the C-terminus. First known chemoselective ligation reaction of a GPI anchor analogue to peptides was reported by Nakahara and colleagues [263]. The researchers synthesized a mannobiose-linked phosphoethanolamine and attached it to a model peptide- α -thioester involving the peptide sequence of a native GPI-anchored protein, CD52. Following this work, Grogan et al. reported the successful synthesis of a GPI anchor analogue bearing a flexible PEG linker instead of the oligosaccharide backbone [264]. This compound was ligated to a recombinantly expressed green fluorescent protein GFP thioester. More recently, Becker and coworkers performed the semisynthesis of N-terminally truncated PrP equipped with a GPI-anchor surrogate consisting of a peptide with two palmitoylated lysines for membrane anchoring and an N-terminal cysteine for NCL [265]. Additionally, in 2008 the same group reported the semisynthesis of a N-terminally truncated PrP variant with a synthetic GPI-anchor analog [266].

Another prolific area that enormously profit from the site-specific introduction of PTMs to proteins is histone biology. Very recently, Muir and coworkers published a review summarizing the advances in obtaining histone modifications using EPL. Various PTMs, such as ubiquitination, arginine mono- and dimethylation, phosphorylation and lysine acetylation have been successfully attached through EPL in order to study their physiological effects [267].

As already discussed in sections 1.5.2 and 1.5.3, a large number of modified peptides and proteins have been generated using these powerful techniques including glycoproteins. To the best of my knowledge, Bertozzi and coworkers performed the first total chemical synthesis of a homogenously glycosylated variant of the 82 aa O-glycoprotein dipterucin [268-269], while Yamamoto *et al.* reported the first chemical synthesis of a full length N-glycoprotein, monocyte chemotactic protein 3 (MCP 3), using both Boc- and Fmoc-SPPS as well as sequential ligation strategies [270]. As described in Section 1.5.2.1, the requirement of the N-terminal cysteine at the ligation site can be overcome using thiolated amino acids, which can later be desulfurized under mild conditions. A significant advantage of such ligation-desulfurization chemistry is the compatibility with saccharide moieties on peptides and proteins. Under the conditions of radical desulfurization, mono-, di- and more complex N- and O-saccharides remain intact. Hence, this method introduced more flexibility to chemical synthesis of glycosylated proteins and expanded the scope of glycoprotein chemistry extensively [201, 213].

Importantly, synthesis of glycoproteins requires sufficient amounts of complex oligosaccharides. To date, three major methods asserted themselves as useful: chemical synthesis [271], chemoenzymatic glycosylation [272] and isolation of oligosaccharides from natural sources [273-274]. While these days it is certainly possible to synthesize complex saccharide moieties, both the fully synthetic and chemoenzymatic procedure are very time consuming and the yields are only moderate. On the other hand, isolation of N-linked branched oligosaccharides from egg yolk is less time consuming and multimilligrams of material can be obtained. For instance, Kajihara and coworkers were able to isolate a sialoglycopeptide from egg yolk, purify it by HPLC and degrade the glycopeptide with proteinases to give rise to pure biantennary N-glycan-Asn building blocks [274].

In addition to N-glycosylated proteins, proteins and peptides can also be O-glycosylated (Section 1.4.1). The most common O-glycosylation pattern is the

attachment of *N*-acetylgalactosamine (commonly abbreviated to GalNAc) to the hydroxyl side chain of Ser/Thr/Tyr ^[275]. Unlike *N*-glycans, *O*-linked glycans do not have a conserved core structure. Mono- or disaccharides on single Ser/Thr/Tyr moieties are widespread and contribute to physiological effects of glycoproteins ^[276-277]. As a result, amino acids carrying simple (1-3 units) *O*-saccharides are widely used for the chemical synthesis of *O*-glycoproteins. Very recently, Bello *et al.* reported the synthesis of a photo-cleavable auxiliary and its application in chemoselective ligation reactions with *O*-glycosylated peptides ^[278]. As a model, a 20mer tandem repeat of mucin 1 (MUC1) was used. Homogenously glycosylated MUC1 peptide with the auxiliary on its N-terminus was successfully linked to non-glycosylated and glycosylated MUC1 variants. Subsequent enzymatic glycosylation with glycosyltransferases and cleavage of auxiliary via UV irradiation gave rise to good yields of homogenously glycosylated MUC1 variants. The auxiliary extended the repertoire of NCL for N-terminal glycine harboring peptides, since it enables the ligation at glycine.

One of the major challenges in glycoprotein synthesis is the total chemical synthesis of proteins that are both *O*- and *N*-glycosylated. Independently, the labs of Kent and Danishefsky worked on the solution to this synthetic challenge using erythropoietin (EPO) as the model protein ^[279-280]. In 2003, Kent and coworkers reported the total chemical synthesis of an EPO analog with branched and monodisperse polyethyleneglycol (PEG) as glycan surrogates and high in vivo activity ^[279]. In 2012, Kajihara reported the chemical synthesis of a homogeneously monoglycosylated EPO mutant ^[281]. In the same year, Kent and coworkers described the total chemical synthesis of a biologically active EPO variant with three N→K mutations on residues 24, 38 and 83 ^[282]. However, this variant lacks the *N*- and *O*-glycans. Very recently, Danishefsky and coworkers reported the highly anticipated first synthetic, homogenously glycosylated EPO, which showed in vitro activity ^[283-284]. Both methods involved the use of chemoselective, native ligation reactions. In addition to the achievements mentioned in this section, Unverzagt and coworkers were able to synthesize *N*-glycosylated proteins with complex glycan structures such as RNase C and a biologically active IL-6 derivative through semisynthesis ^[285-287]. These great advances show the enormous potential of chemoselective ligation chemistry and the broad spectrum

of applications to the synthesis of homogenously modified proteins in sufficient yields.

EPL continues to provide great insights into posttranslational modification of proteins. Therefore, using EPL in combination with other advances in the protein ligation will enable the access to homogenously *N*-glycosylated PrP variants in order to decipher their impact on prion pathogenicity

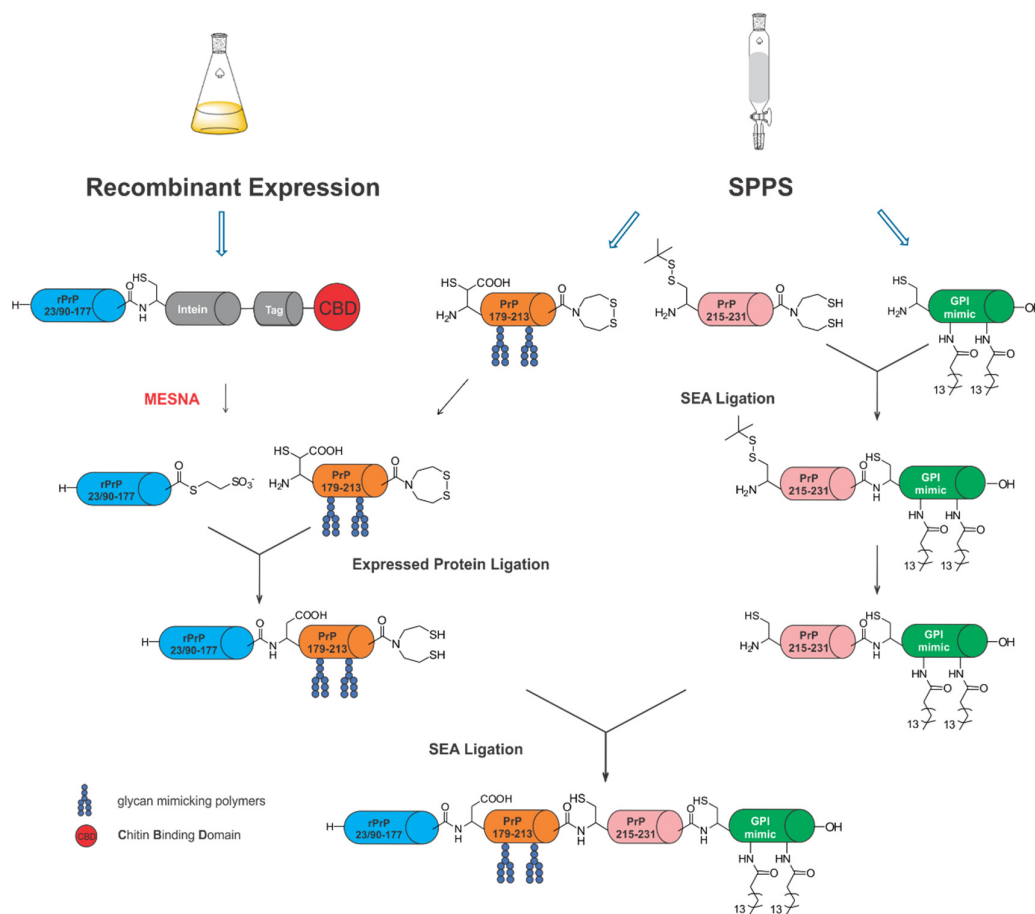
2 Objectives

Recent advances in prion research indicate that prions formed out of proteins different than PrP can cause certain neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson Disease (PD). Very recently, Prusiner and colleagues claimed the discovery of a new human prion, α -synuclein [288]. Moreover, Collinge and coworkers reported the first known case in transmission of amyloid β -pathology and cerebral amyloid angiopathy in humans [289]. Consequently, there is a strongly renewed interest in understanding prion transmission and toxicity. To understand prion pathogenicity, the contribution of PTMs on PrP infectivity and toxicity should be regarded as a key factor. As outlined the introduction, the GPI-anchoring is crucial for the conversion of PrP^C into PrP^{Sc}. Recent advances in prion glycobiology sustain the hypothesis that changes in glycan core and glycosylation level of PrP^{Sc} may play a role in certain prionopathies such as VPSPr [156-157, 290]. Nevertheless, the access to infectious and/or toxic prions as well as to PrP^C is very limited due to the difficulties in purification and isolation. Furthermore, isolated variants are not homogenous posttranslationally modified, which makes it very difficult to assess the impact of PrP in prion pathogenesis clearly.

To overcome this problem, I suggest to develop a semisynthetic route to access homogeneously modified PrP variants (Scheme 1). In order to achieve this goal, two PrP segments (aa 23-177 and aa 90-177) are chosen and cloned into plasmids carrying intein and affinity tag fusion protein coding sequence in order to give rise to protein α -thioesters. At the same time, I will synthesize peptides and peptide α -SEA-thioesters (aa 178-213 and aa 214-231) carrying chemical surrogates of native PTMs of PrP, which mimic the original modifications [265, 291-292]. Here, I will use monodisperse polyethyleneglycol (PEG) polymers as glycan mimics (at positions 181 and 197) and a palmitoylated peptide (called D5) for membrane anchoring at the C-terminus. For the introduction of PEG glycan mimics I will use 2,4-diaminobutyric acid (Dab) as well as 2,4-diaminopropionic acid (Dpr) instead of asparagine residues. The PrP variants will be modified with either one (position 181 or 197) or two glycan mimics. Linking of the GPI-anchor mimic and both

peptide segments will be achieved by NCL. To this end, I will synthesize peptides on SEA linkers as controllable thioester precursors, as described in Ollivier *et al.*^[237].

A native aspartate at position 178 will be replaced with β -mercapto-aspartate in order to enable EPL^[211]. This residue will be reduced selectively to aspartate without touching other cysteines after final chemoselective ligation reaction. Semisynthetic, homogenously modified PrP variants will be folded and characterized. Whilst biophysical characterization will be performed via far UV circular dichroism (CD) spectroscopy, aggregation assays will give insights into the biochemical properties of homogenously modified PrP variants.



Scheme 9: Semisynthetic strategy for the generation of homogenously modified PrP.

3 Materials and Methods

3.1 Chemicals

The following chemicals and solvents were purchased from commercial sources and used without further purification: Fmoc (Fluorenylmethoxycarbonyl)- and Boc (*tert*-Butyloxycarbonyl)-protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (Merck, Darmstadt); succinic anhydride (VWR, Vienna); TentaGel R PHB-Ser(tBu)-Fmoc, TentaGel R PHB-Met-Fmoc (Rapp polymers, Tuebingen); Fmoc-NH-PEG₂₇-COOH (Polypure, Oslo); acetonitrile (ACN), N,N-dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA) (Biosolve, Netherlands); 3-mercaptopropionic acid (MPA), 4-mercaptophenylacetic acid (MPAA), tris(2-carboxyethyl)phosphine (TCEP) (Sigma Aldrich, Vienna).

Bis(2-sulfanylethyl)amido (SEA)-linked resin was both generated via organic synthesis on a solid support and received as a kind gift from Prof. Oleg Melnyk, 4-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-4-oxo-3-((2,4,5-trimethoxybenzyl)thio)butanoic acid, Boc-Asp(tBu, STmob)-OH, was provided by Prof. Richard Payne ^[211].

3.2 Molecular Biology

All chemicals and reagents used for buffer preparation were purchased either from Sigma Aldrich (Vienna, Austria) or from VWR (Vienna, Austria). All buffers are prepared with Milli-Q water (ddH₂O).

Restriction enzymes, ligases and material used for cloning purposes were purchased from Promega (Mannheim, Germany) and New England Biolabs (Frankfurt, Germany). Purification kits from Qiagen (Hilden, Germany) and Thermo Fisher Scientific (Vienna, Austria) were used in this work.

All constructs were sequenced at Eurofin Genomics AT (Vienna, Austria). Antibodies for Western Blot detection were purchased from Sigma Aldrich (Vienna,

Austria), Merck Millipore (Darmstadt, Germany) and Santa Cruz Technologies (Heidelberg, Germany).

3.3 General protocols for molecular biology

3.3.1 Cloning of PrP cDNA sequences

Four different PrP cDNAs were each cloned into a modified pTXB3 low copy vector that contains an *Mxe*-Intein, a polyhistidine-tag (His₆) within the *Nco*I and *Sap*I restriction sites and a chitin-binding-domain (CBD). Hence, *Mxe*-Intein is linked to the C-terminus of the desired PrP construct. All true positive colonies were amplified in 5-10 mL LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl, autoclaved at 121°C for 30 min) at 37°C, glycerol stocks (1:1 in 40% glycerol in ddH₂O) were prepared and stored at -80°C.

3.3.1.1 PrP 23-178-*Mxe*Intein-His₆-CBD

The inserted DNA fragment was amplified from a plasmid previously constructed in our group: a pTWIN plasmid containing the coding sequence of human PrP residues 23-231 fused to *Mxe*-Intein-His₆-CBD [293-294].

Forward (5'-CACCATTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAAAGCGGCCA-3') and reverse primers (5'-GTGTGCACTAGTGCACTCTCCCGTGATGCAGTCGTGCACGAAGTTGTTCTGTTGC-3') were designed and the coding sequence for PrP residues 23-178 was amplified via PCR using the template 23-231-*Mxe*Intein-His₆-CBD mentioned above. The PCR product was purified (Qiagen PCR purification kit), analyzed via agarose gel electrophoresis, restricted with *Xba*I and *Spe*I and ligated to the adjacent vector pTWIN1 at those restriction sites after 18 h alkaline phosphatase treatment. The ligation mixture was then transformed into *E.coli* XL-1 strain (Amp^R) diluted in 1 mL LB-Medium, plated on an LB-agar-plate containing 100 µg/mL ampicillin and single colonies were picked for colony PCR as well as restriction control experiments to find the right colonies for gene sequencing and expression.

3.3.1.2 PrP 90-178-*Mxe*Intein-His₆-CBD

The inserted DNA fragment was amplified from an already existing: a pTXB3 plasmid containing the coding sequence of human PrP residues 90-231 fused to *Mxe*-Intein-His₆-CBD [265]. A similar strategy as described in section 3.3.1.1 was used for this cloning work.

Forward (5'-GATATAACCATGGGCCAAGGAGGGGGTACCCATAATCAGTGG-3') and reverse primers (5'-GTGTGCACTAGTGCATCTCCCGTGATGCAGTCGTGCACGAAGTTGTTCTGTTGC-3') were designed and the coding sequence for PrP residues 90-178 was amplified via PCR using the template mentioned above. The PCR product was purified (Qiagen PCR purification kit) and ligated into the vector pTXB3 at *Nco*I and *Spe*I restriction sites. The ligation mixture was then transformed into *E.coli* XL-1 strain (Amp^R) diluted in 1 mL LB-Medium, plated on an LB-agar-plate (100 µg/mL Ampicillin) and screened for positive colonies.

3.3.1.3 PrP 23-177-*Mxe*Intein-His₆-CBD

The plasmid containing this construct was generated using site-directed mutagenesis. The plasmid DNA pTXB3-PrP-23-178-*Mxe*Intein-His₆-CBD was isolated (Qiagen PCR purification kit), and a modified inverse PCR approach with the following primers was used: forward primer 90-177-F (5'-TGCATCACGGGAGATGCAC-3'), reverse primer 90-177-R (5'-GTGCACGAAGTTGTTCTGG-3'). In doing so, one base triplet coding for amino acid 178 could be deleted from the original sequence. The resulting PCR product was purified and digested with *Dpn*I (NEB) overnight. After repurification, the linearized plasmid DNA was phosphorylated at the 5' end with T4-Polynucleotide-Kinase PNK (NEB) and ligated with T4-Ligase (NEB) at 22°C for 18 h. The ligation mixture was transformed into *E.coli* XL-1 strain (Amp^R), grown on an LB-agar-plate (100 µg/mL Ampicillin), colonies were selected and analyzed via colony PCR.

3.3.1.4 PrP 90-177-*Mxe*Intein-His₆-CBD

The forward and reverse primers 90-177-F and 90-177-R were used to generate this construct via the same site directed mutagenesis strategy described in section 3.3.1.3. Colonies were analyzed using a colony PCR, restriction experiments with *Nco*I and *Spe*I and identified via sequencing.

3.3.1.5 His₆-ATPase(DnaK)-SortaseTag- PrP90-177-Mxe-Intein- His₆-CBD

In 2013, Chu *et al.* reported the recombinant expression of soluble PrP variants using the ATPase domain of DnaK for solubilization ^[294]. In order to raise the yield and enhance the solubility of recombinant PrP 90-177 fused to MxeIntein, a new strategy for generating soluble PrP-MxeIntein constructs in *E. coli* was developed. In doing so, the plasmid constructed by Chu and coworkers, pET30-His₆-ATPase(DnaK)-SortaseTag-PrP90-231-MxeIntein-His₆-CBD, was used for a modified inverse PCR with the forward and reverse primer 90-177-F and 90-177-R. The resulting PCR product was purified, digested with DpnI and religated upon to phosphorylation. For further details please see section 3.3.1.3

3.3.1.6 Sequencing

Selected clones were sent for DNA- sequencing with T7-promotor and T7-terminator primers to Eurofins Genomics AT (Vienna, Austria). The results were aligned with the theoretical sequence to confirm in-frame fusion of the inserted PrP genes and MxeIntein.

3.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The standard SDS-PAGE protocol used in this thesis is based on Lämmli *et al.* ^[295]. SDS-gels were cast either using a multi-casting chamber or TetraGel Mini gel casting systems (Biorad, Vienna). The components for separating and stacking gels are listed below. Stacking gel buffer and separating gel buffer contain Tris HCl in different concentrations (1.5 M and 0.5 M), 0.4 % (v/v) SDS and distinct pH values (8.0 and 6.8).

Table 1: 15% SDS-gel casting recipe

4 Gels	ddH ₂ O	30% Acrylamide	Buffer	SDS	10% APS	TEMED
Stacking Gel	6.3 mL	1.6 mL	1.2 mL	93 µL	93 µL	9.4 µL
Separating Gel	4.8 mL	10 mL	5.2 mL	209 µL	209 µL	9.4 µL

Upon the addition of 10% APS and TEMED, the separating gels were cast immediately due to the rapid polymerization and covered with few droplets of water.

After completion of polymerization, stacking gels were cast and ten wells were generated using polycarbonate TetraGel MiniGel combs (Biorad, Vienna).

SDS-samples were prepared by diluting one volume of aqueous solution of protein with 1 volume of 2x SDS-loading buffer (0.5 M TrisHCl, SDS 6% (w/v), glycerol 35% (v/v), β -mercaptoethanol 3.5% (v/v) and bromophenol blue 0.05% (w/v), pH 6.8). Samples were boiled for 5 min at 95°C and loaded into the wells of stacking gel. Appropriate voltage (120 V or 250 V) was set and the electrophoresis was run for either 1 h (120 V) or 30 min (250 V). Protein bands were either detected with Coomassie Blue® or silver staining was performed [296]. For Coomassie Blue staining, a staining (0.1% (w/v) Coomassie R250, 10 % (v/v) Acetic acid, 45 % (v/v) methanol in ddH₂O) and destaining solution (10 % (v/v) Acetic acid, 40 % (v/v) methanol in ddH₂O) was used. After incubation of SDS gels in staining solution for 10-20 min on an orbital shaker, the gel was washed 3 times for 5-10 min with the destaining solution. The molecular weight was estimated via comparison with the protein standard Amersham Low Molecular Weight (LMW) Calibration Kit for SDS Electrophoresis (GE Healthcare, Vienna).

3.3.3 Semi-dry Western Blot Analysis

Western Blots were used to identify different PrP variants, His₆-tagged proteins as well as Flag-containing peptides and proteins. Anti His₆- and anti-FLAG antibodies were purchased from Sigma Aldrich (Vienna, Austria). Anti-PrP antibodies (M20 and 3F4) were either purchased from Santa Cruz Biotechnology (Heidelberg, Germany) or Sigma Aldrich (Vienna, Austria). The nitrocellulose membrane and the SDS gel were incubated in transfer buffer (3.0 g Tris HCl, 14.4 g glycine in 1 L of methanol: ddH₂O; 1:4) for 5 min, were placed between 6 pieces of filter paper on cathode and anode into Roth SEMI-DRY-BLOTTERMAXI (Roth, Germany). For each half square meter of the gel or membrane 1-1.5 mA was used (approx. 121 mA, 2 h) to transfer proteins from the gel onto the membrane.

After transferring the proteins, the membrane was soaked in blocking buffer (5% skim milk powder (w/v) with 0.05% Tween 20 (v/v) in TBS, pH 8.0) for 18 h at 4°C. The membrane was washed with TBS-T (TBS with 0.05% Tween 20) buffer and primary antibody in blocking buffer (diluted 1:10,000 for anti-PrP3F4, 1:3,000 Anti-FLAG and 1:8,000 Anti-His₆) was added. The membrane was incubated on an

orbital shaker for 1 h at room temperature (rt). Next, the membrane was incubated with TBS-T (6x5 min), secondary anti-mouse IgG, HRP linked antibody, was added and the membrane was incubated for another 1 h at rt. The secondary antibody can specifically bind to the primary antibody and is linked to HRP enzyme that can bind to its luminescent substrate contained in the ECL plus western blotting kit (GE healthcare, Germany). The membrane was rinsed with TBS-T (6x10 min), dried for 2 min and replaced in a transparent protector. Subsequently, the ECL plus western blotting kit was used to detect the protein bands on the membrane. Detection solutions A and B were mixed (40:1 v/v), pipetted onto the membrane containing protein bands and incubated for 3 min. The membrane was then imaged using Chemidoc Imaging Systems™ (Biorad, Germany) with a luminescent filter to visualize protein bands.

3.3.4 Expression and purification of recombinant PrP-MxeIntein fusion proteins

3.3.4.1 Recombinant protein expression

For the expression of recombinant PrP-MxeIntein fusion constructs three different *E. coli* expression strains were used, BL-21(DE3), Rosetta 2 and BL-21(DE3) RIL. All proteins were expressed in 2YT media (16 g/L trypton, 10 g/L yeast extract, 5 g/L NaCl) unless noted otherwise.

The plasmids pTXB3-PrP 23-177-MxeIntein-His₆-CBD and pTXB3-PrP 23-178-MxeIntein-His₆-CBD were transformed into chemically competent Rosetta 2 (DE3) cells (100 µg/mL Ampicillin and 30 µg/mL Chloramphenicol), pTXB3-PrP 90-177-MxeIntein-His₆-CBD and pTXB3-PrP 90-178-MxeIntein-His₆-CBD into BL-21(DE3) (100 µg/mL Ampicillin) and BL-21(DE3) RIL (100 µg/mL Ampicillin and 30 µg/mL Chloramphenicol). A single colony was picked for the subsequent expression strategy. An expression on small scale (50-200 mL 2YT-Medium) was done for each protein before upscaling (1-2 L 2YT). Glycerol stocks were prepared for further use. A 200 mL overnight culture (18 h, 37°C, and 170 rpm) was used for the inoculation of 2 L 2YT medium containing Ampicillin and Chloramphenicol (37°C, 170 rpm). The cells were grown until OD_{600nm} 0.6-1.0 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h. Cell growth was monitored by OD_{600nm} measurements during the expression at regular intervals.

Subsequently, cells were harvested by centrifugation (8,900 g, 15 min, and 4°C), washed with Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 8.0) and stored at -80°C for further usage.

Defrosted cell pellets were weighed, resuspended in lysis buffer (TBS) and lysed in a microfluidizer (Constant Systems TS). Lysed cells were centrifuged (50,000 g, 4°C, 30 min) to separate soluble and insoluble components. Both supernatant and pellet were analyzed via SDS-PAGE to determine whether the protein of interest (POI) is in the supernatant or pellet. All analyses performed for this thesis showed that all PrP-MxeIntein constructs were deposited in inclusion bodies (IB). Pellet containing POI was washed with TBS-T buffer (TBS with 0.1% Triton-X, VWR, Vienna) and centrifuged (50000g, 4°C, 30 min). After three washing and centrifugation cycles with TBS, the pellet was solubilized overnight (o.n.) in 15-30 mL of resuspension buffer (8 M Gnd-HCl, 50 mM Tris-HCl, pH 8.0) under continuous stirring.

3.3.4.2 Affinity purification and thiol induced intein cleavage

Resuspended POI was centrifuged (50000g, 4°C, 30 min), the supernatant was collected and loaded on Ni-NTA beads (5 mL, Qiagen) that are preequilibrated with washing buffer (6 M Gnd-HCl, 50 mM Tris-HCl, pH 8.0) at r.t. After incubation for 30 min, the flow through was collected and Ni-NTA beads were washed with 3-5 column volume of washing buffer. Subsequently, the beads were incubated with elution buffer (6 M Gnd-HCl, 50 mM Tris-HCl, 300 mM imidazole, pH 8.0). His-tagged protein was eluted with elution buffer (2 column volumes) and the column was washed with washing buffer (3 column volumes). All elution and wash fractions were combined separately for further analysis.

After concentration of PrP-MxeIntein fusion constructs using Amicon Ultra-15 Centrifugal Filter Units (VWR, Vienna), the buffer was exchanged to urea buffer (8 M urea, 50 mM TrisHCl pH 8.0) over PD-10 columns (GE Healthcare). The concentration of the resulting protein solution was determined using Nanodrop 2000 (VWR, Vienna). A solution of 2-mercaptoethanesulfonate (MESNA) in 50 mM Tris-HCl, pH 8.0 was added to the protein solution to reach a final buffer concentration of 4 M urea and protein concentration of ≤ 5 mg/mL. The mixture was gently stirred for 18 h at rt to initiate the cleavage of intein. The reaction was analyzed using SDS-PAGE. Upon completion, the reaction was quenched with

resuspension buffer, concentrated and protein α -thioester was purified over RP-HPLC.

3.3.4.3 Preparative RP-HPLC purification of thiol induced intein cleavage reactions

For the purification of the thiol assisted intein cleavage reaction, a Varian ProStar RP-HPLC System was used. The reaction mixture was loaded on a preparative C4 column (250 x 22 mm, 5 μ m particle size, Protein C4, Grace Vydac) that was pre-equilibrated with 5% buffer B (ACN + 0.08% TFA) and 95% buffer A (ddH₂O + 0.1% TFA). A linear gradient of buffer B from 30-90% (v/v) in 60 min was used to elute the protein α -thioesters from the column. Elution fractions containing the protein-thioester were combined; pooled and lyophilized. Subsequent analysis was done via analytical RP-HPLC, ESI-MS and SDS-PAGE to identify the protein α -thioesters.

3.3.5 Refolding of PrP variants

Based on the folding conditions described by Chu *et al.* [293-294], PEGylated and unPEGylated PrP variants were refolded as follows; lyophilized PrP variants were dissolved in denaturing conditions (6 M Gnd-HCl, 50 mM Tris-HCl, pH 8.0) and diluted stepwise to 2.5 M Gnd-HCl with folding buffer (20 mM Na-acetate, 0.3/3 mM GSSG/GSH, pH 5.0) at 4°C. The reaction mixture was incubated at 4°C at least for 2 d and dialyzed with Slide-A-Lyzer™ Dialysis Cassettes (MWCO 10 kDa, Thermo Fisher, Austria) against refolding buffer (500-fold sample volume), first for 3h then o.n. at 4°C. Resulting protein solution was transferred into an Eppendorf tube and centrifuged at 14,000 g, 4°C for 15 min to separate refolded and unfolded PrP variants. The supernatant was transferred into an Eppendorf tube and the concentration of POI was analyzed using a NanoDrop 2000 (VWR, Vienna, Austria). Typical concentrations were between 0.05-0.2 mg/mL.

3.3.6 Expression of a GPI-anchored protein in *S. cerevisiae*

For the generation of a homogenous GPI-anchor, a construct containing the N-terminal ER signal sequence Kre5, murine PrP (aa 23-230) with C-terminal His₆-Tag, TEV cleavage site as well as a FLAG-Tag and the GPI-anchoring segment of Gas1p has been transformed into YPH501 *S. cerevisiae* cells [292]. The overnight culture (200 mL, 18 h, 30°C, and 200 rpm) was used for the inoculation of 2 liter SC medium (drop out mix 2 g/L medium, yeast nitrogen base without amino acids 6.7 g/L medium and glucose 20 g/L) containing 0.1 mg/mL adenine for the prototrophic selection (37°C, 170 rpm). For this purpose, a leucine-drop out mix was used comprising 19 canonical amino acids excluding leucine (2 g each), meso-inositol (2 g) and p-aminobenzoic acid (0.2 g). The cells were grown until OD_{600nm} 0.8-1 and induced with 0.4 mM CuSO₄·5H₂O for 3 h. Subsequently, cells were harvested using centrifugation (6000 g, 15 min, and 4°C), washed with TBS+0.6 M sorbitol and centrifuged. Cell pellets were resuspended in lysis buffer (TBS+0.6 M sorbitol) and lysed with a microfluidizer. The lysate was centrifuged (50000 g, 4°C, 30 min) to separate supernatant and the pellet. The POI was expressed in IB. The pellet was washed with TBS-T buffer (TBS with 0.1% Triton-X, VWR, Vienna) and centrifuged at 50000 g, 30 min. After repeating wash and centrifugation cycle with TBS, the pellet was resuspended o.n. in 20 mL of resuspension buffer (8 M Gnd-HCl, 50 mM Tris-HCl, pH 7.8) under continuous stirring.

3.3.6.1 Purification of POI through affinity based purification

Resuspended POI was centrifuged at 50,000g, 4°C, 30 min and the supernatant was collected. Subsequently, the protein solution was loaded on Ni-NTA beads (5 mL, Qiagen) equilibrated with washing buffer (6 M Gnd-HCl, 50 mM Tris-HCl, 150 mM NaCl, pH 7.8). The solution was incubated for 30 min and the flow through was collected. Beads were washed with five column volumes of washing buffer and incubated with elution buffer (6 M Gnd-HCl, 50 mM Tris-HCl, 300 mM Imidazole, pH 7.8). His-tagged protein was eluted with elution buffer (2 column volumes) and the column was washed with washing buffer (3 column volumes). All elution and wash fractions were combined separately for further analysis.

3.3.6.2 Proteolytic cleavage of mPrP-HisTag fusion protein with TEV protease

In order to cleave the GPI-anchor containing C-terminal segment from the rest of the protein, a TEV protease was used. The PrP-Gas1p-GPI fusion protein was first dialyzed against 8 M urea containing TBS buffer. Then a stepwise dilution dialysis from 8 M to 6 M and finally 4 M urea containing TBS buffer was performed. TEV protease (dilution 1:10) and 5 mM DTT (dithiothreitol) was added to the protein solution and dialyzed against 2 M urea containing TBS with 5 mM DTT o.n.

3.4 General Protocols for Solid Phase Peptide Synthesis (SPPS)

SPPS was performed with Fmoc-protected amino acids (Fmoc-Aa-OH) on SEA-resin or Tentagel-MBHA-resin. The following side chain protecting groups were used; Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(StBut), Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc) and Tyr(tBu). The non-canonical amino acids racemic Boc-Asp(tBu, STmob)-OH, Fmoc--Dab(Mtt)-OH and Fmoc-L-Dpr(Mtt)-OH were used for chemoselective ligation or site selective modification of peptides. Pseudoproline dipeptides Fmoc-Glu-Ser($\psi^{\text{Me,Me}}\text{pro}$)-OH, Fmoc-Glu-Thr($\psi^{\text{Me,Me}}\text{pro}$)-OH, Fmoc-Ile-Thr($\psi^{\text{Me,Me}}\text{pro}$)-OH and Fmoc-Val-Thr($\psi^{\text{Me,Me}}\text{pro}$)-OH (Merck, Darmstadt) were used at suitable positions to prevent formation of secondary structures on the resin giving rise to higher yields [297-298].

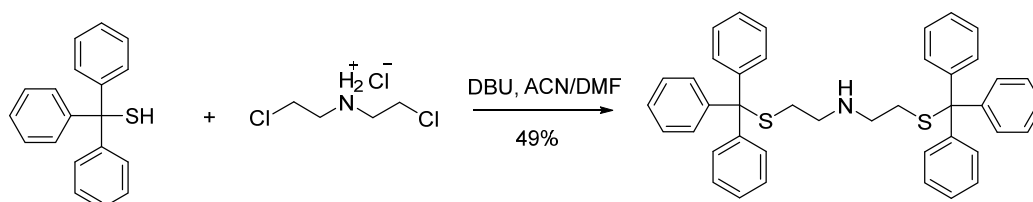
Peptides and peptide α -thioesters were synthesized either manually or on automated synthesizers, the PTI Tribute synthesizer (Protein Technologies, Inc., USA) and CEM Liberty Blue Automated Microwave Synthesizer (CEM, USA).

Fmoc-deprotection was achieved with 20% piperidine in DMF with cycles of three and seven minutes. All amino acids (2.5 eq.) were coupled using HBTU or HATU (2.38 eq.) and DIEA (5 eq.) for 30 min, unless stated otherwise.

Peptides were globally deprotected and cleaved from dried resin with a mixture of TFA, triisopropylsilane and water (92.5 : 5 : 2.5) for 3 h at rt. Precipitation of crude peptides was achieved by addition of three volumes of cold diethyl ether with subsequent centrifugation. After washing twice with ether, precipitated peptides were dissolved in either 5% or 50% ACN in water with 0.1% TFA and lyophilized

3.4.1 Synthesis of SEA-linked solid support (modified from Ollivier *et al.*) ^[237]

3.4.1.1 Bis(2-[triphenylmethyl)sulfanyl]ethyl)amine

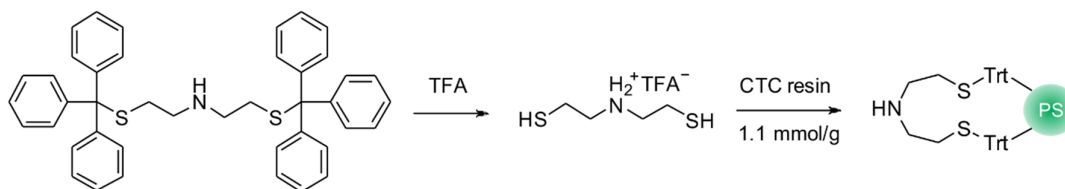


Scheme 10: Synthesis of Bis(2-[triphenylmethyl)sulfanyl]ethyl)amine

1,8-Diazabicyclo[5,4,0]undec-7-ene (DBU, 2.57 g, 16.8 mmol, 4 eq.) was added dropwise, under inert atmosphere to an ice cooled solution of triphenylmethanethiol (2.33 g, 8.4 mmol, 2 eq.) and bis(2-chloroethyl)amine hydrochloride (0.75 g, 4.2 mmol, 1 eq.) in a mixture of ACN/DMF (7/5; v/v; 40 mL). The mixture was stirred for 24 h and the reaction progress was monitored by TLC (petroleum ether: ethyl acetate: triethylamine; 8:2:0.1; R_f = 0.45). Upon completion of the reaction, the reaction mixture was evaporated under reduced pressure. The crude product was dissolved in approx. 100 mL of methylene chloride (CH_2Cl_2). The organic phase was washed with a 5 % aqueous solution of monobasic potassium phosphate (KH_2PO_4 ; 3 x 100 mL) and dried over magnesium sulfate (Mg_2SO_4). The solvent was removed under reduced pressure and the residue was purified by silica-gel chromatography (100-200 mesh, petroleum ether: ethyl acetate: triethylamine; 8:2:0.1) giving rise to bis(2-[triphenylmethyl)sulfanyl]ethyl)amine as a white powder (1.24 g, 49%).

All spectroscopic data is in agreement with previous results ^[299], ^1H NMR (CDCl_3) δ [ppm], 400MHz: 7.41-7.43 (m, 12H, Trt), 7.19-7.30 (m, 18H, Trt), 2.07-2.40 (m, 8H, CH_2), 1.81 (s, 1H, NH).

3.4.1.2 Generation of SEA-linked solid support



Scheme 11: Generation of SEA-linked solid support

Bis(2-[triphenylmethyl)sulfany]ethyl)amine was treated with a mixture of TFA/triisopropylsilane (97.5:2.5, v/v, 9.3 mL) for 30 min. Subsequently, the solution was evaporated under reduced pressure and the residual TFA was removed by co-evaporation with dry hexane (2x10 mL) under inert atmosphere. The yellowish solid was dissolved in dry DMF and added to chlorotriptyl chloride (CTC) resin using a syringe to prevent air oxidation. The mixture was gently stirred overnight at rt under inert atmosphere. Unreacted chloride functionalities were capped with methanol (0.1 mL) and 2.6-lutidine (0.28 mL) for 30 min. Finally, the resin was washed with DMF (6x2 mL), methanol (6x2 mL), DMF (6x2 mL), 5% (v/v) diisopropylethylamine (DIEA) in DMF (6x2 mL), DMF (6x2 mL) and methanol (6x2 mL). The resin was dried under vacuum overnight and stored at -20°C for further use.

3.4.2 Fmoc-SPPS on SEA-resin

3.4.2.1 Coupling of the first amino acid to SEA-resin

The attachment of the first Fmoc-Aa-OH to the SEA-resin was performed on 0.05 mmole scale. Fmoc-Aa-OH (0.5 mmol, 10 eq.) was dissolved in a solution of HATU (190 mg, 0.49 mmol, 10 eq.) in DMF (200 μ L). Subsequently, DIEA (260 μ L, 1.5 mmol) was added, the mixture was reacted for 1 min and transferred on the resin beads. After agitation for 3 h, the SEA-resin was washed with DMF (per g resin/ 10 mL), CH_2Cl_2 (per g resin/ 10 mL) and unreacted functionalities were capped by treating the resin with Ac_2O /DIEA/ CH_2Cl_2 (10: 5: 85 v/v, 2x10 min). The resin was then washed with CH_2Cl_2 and dried *in vacuo*.

The loading of the SEA-resin was determined through UV spectroscopy using Safas UVmc2 spectrometer (France) at 301 nm.

$$\frac{Abs * Volume\ of\ 20\%\ piperidine\ in\ DMF\ (mL)}{Extinction\ coefficient\ (units) * mass\ of\ resin\ (mg)} = Fmoc\ loading$$

3.4.2.2 Elongation of peptide sequences on SEA-resin

Standard Fmoc-Aa-OH coupling procedure described in section 3.4 was used. The deprotection and cleavage from dried resin was performed with TFA, triisopropylsilane, dimethylsulfide, thioanisole and water (90: 2.5: 2.5: 2.5: 2.5 v/v).

3.4.3 Purification and analysis of SEA-peptides

All peptides were purified by RP-HPLC using either a Waters Auto Purification HPLC/MS system (3100 Mass Detector, 2545 Binary Gradient Module, 2767 Sample Manager and 2489 UV/Visible Detector) or a Varian ProStar RP-HPLC system. For purification the following reversed phase columns (preparative and semi preparative) were used: Kromasil 300-10-C4 column (250 × 21.2 mm, 10 µm particle size), Kromasil 300-10-C4 column (250 × 10 mm, 10 µm particle size), Kromasil 300-5-C4 column (250 × 10 mm, 5 µm particle size), Grace Vydac C4 column (250 × 22 mm, 5 µm particle size and a Grace Vydac C4 column (250 × 10 mm, 5 µm particle size). If not indicated otherwise, peptides were dissolved in 6 M Gnd-HCl buffer (pH 4.7) and injected on the reversed phase columns. Elution was achieved by running linear gradients of buffer B (ACN + 0.05 % TFA) in buffer A (ddH₂O + 0.05 % TFA). Purified peptides were analyzed with mass spectrometry. Mass spectra were acquired by electrospray ionization MS (ESI-MS) operating in positive ion mode.

Analytical HPLC analysis of peptides was achieved using a Dionex Ultimate 3000 instrument on a RP- Kromasil-C4-column (300-5-C4, 150 × 4.6 mm, 5 µm particle size) or Thermo Fisher-C4-column (BioBasic-4, 150 × 4.6, 5 µm particle size) at a flow rate of 1 mL/min with a linear gradient from 5 to 65% buffer B (ACN + 0.08% TFA) in buffer A (ddH₂O + 0.1% TFA) over 30 min if not stated otherwise. Detection occurred at 214 and 280 nm wavelength.

3.4.4 Removal of Mtt(4-methyltrityl) from amino acid side chain

The peptidyl resin was washed with DCM and swollen in DCM for 3 h. A solution of 98% DCM, 1% TIS, 1% TFA (approx. 5 mL) was added to the peptidyl resin and agitated for 2 min giving rise to a yellow color. After washing of the peptidyl resin with DCM, this procedure was repeated until the yellowish color vanished upon addition of the DCM/TIS/TFA solution. For 0.05 mmol peptidyl resin, roughly 150 mL deprotection solution was applied. The resin was then washed with DMF and swollen for 2 h in DMF prior to further synthesis.

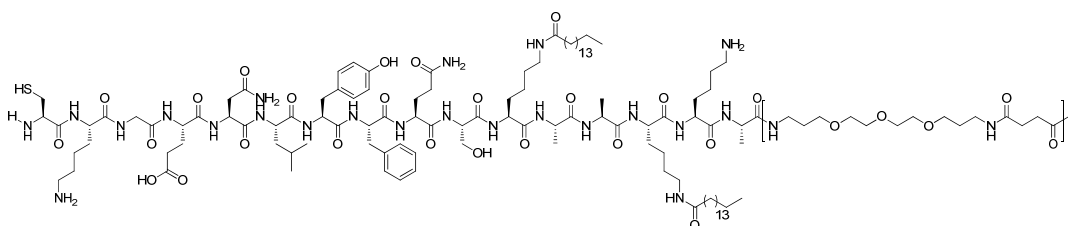
3.4.5 Coupling of Boc-Asp(*t*Bu, STmob)-OH to peptidyl SEA-resin

Boc-Asp(*t*Bu, STmob)-OH (2.0 eq.) was dissolved in a mixture of PyBOP (2.0 eq.), and NMM (4.0 eq.) in DMF (final concentration 0.1 M). The reaction mixture was then added to the resin (1.0 eq.) and rotated at rt for 20 h. Subsequently, the resin was washed with DMF (5 x 3 mL per g resin), DCM (5 x 3 mL per g resin), DMF (5 x 3 mL per g resin), and DCM (10 x 3 mL per g resin).

3.4.6 Radical desulfurization of Boc-Asp(*t*Bu, SH)-OH and cysteine

To a solution of peptide containing racemic β -mercapto-Asp in degassed buffer (6 M Gnd-HCl, 200 mM NaPi, 200 mM TCEP, adjusted to pH 7.3, 2.5 mM concentration of peptide), glutathione (40 mM) and 2,2'-azobis(2-methylpropionamidine)dihydrochloride (VA-050, 20 mM) were added sequentially as a solid. The solution was flushed with argon for an additional 10 min. The reaction vessel was incubated at 37°C on. The reaction was diluted with buffer A (1 mL) and immediately purified by RP-HPLC on Waters Auto Purification HPLC/MS system using Buffer A and Buffer B with a linear gradient of 30-90% B over 60 min. Fractions containing the product were collected, pooled, lyophilized and stored at -20°C for further use.

3.4.8 Fmoc SPPS of the lipidated D5 peptide



0.2 mmol of Fmoc-Ala-Wang-resin was swollen in DMF for 2 hours and Fmoc deprotection was achieved by treatment with 20 % (v/v) piperidine in DMF for 3 min and then 7 min. The resin was washed with DMF (1 min). Next, Fmoc-Lys(Mtt)-OH (2.5 eq.) was activated with HBTU (0.5 M in DMF, 2.38 eq.) and DIEA (5 eq.), transferred to the resin and stirred for 30 min. The Fmoc deprotection and coupling steps were repeated for other amino acids. After the coupling of Fmoc-Ser(tBu)-OH, palmitoylation of Mtt-protected Lys side chains was carried out.

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palmitoylation reaction was completed, the remaining amino acids of the peptide sequence were coupled as described above. Finally, the resin was washed with DCM, dried in a vacuum desiccator and stored at -20°C for further use. 100 mg of the peptide were cleaved from the resin using the method described in 3.4.

The lyophilized crude peptide was dissolved in 6 M Gnd-HCl (pH 4.7) and purified by RP-HPLC using a semi-preparative C4 column with elution gradient of 30 to 90% (v/v) of Buffer B over Buffer A for 60 min at r.t. Fractions containing the purified peptide were collected, pooled and lyophilized. Pure fractions were identified using analytical RP-HPLC and ESI-MS.

3.4.9 Coupling of Fmoc-NH-(PEG)₂₇-OH

The peptidyl resin was transferred into a syringe equipped with a frit. A solution of 0.5 M HATU in ACN/DMF (40:60 v/v) was prepared. Fmoc-NH-(PEG)₂₇-OH (2.75 eq.) was dissolved in this solution (2.5 eq. HATU) and 5 eq. DIEA was added. The solution was transferred into the syringe with the peptidyl resin and the syringe was rotated for 20 h. The resin was washed with DMF and subsequently with DCM.

3.5 General protocols for chemoselective ligation reactions

3.5.1 SEA Ligation (modified from Ollivier *et al.*) [300]

Gnd-HCl (573.24 mg, 6 mmol) and 4-mercaptophenylacetic acid (MPAA, 33.68 mg, 0.2 mmol) were dissolved in 0.1 M phosphate buffer (1 mL, pH 7.3). The pH was adjusted to 7.5 with 5 M NaOH.

SEA-peptide (2 mM) and N-terminal-cysteiny-peptide (4 mM) were dissolved together in ligation buffer. The reaction mixture was shaken at 37°C and 400 rpm under nitrogen atmosphere. The reaction progress was monitored by analytical RP-HPLC and LC-MS. For this, an aliquot (1 µL) of the reaction mixture was quenched with 5 % ACN in ddH₂O with 0.1%TFA (19 µL).

Upon the completion of ligation, the crude product was purified over RP-HPLC on Waters Auto Purification HPLC/MS system using Buffer A and Buffer B with a linear gradient of 5-45% B over 60 min.

3.5.2 Expressed Protein Ligation ^[172]

All EPL reactions were performed in ligation buffer (6 M Gnd-HCl, 100 mM NaP_i buffer and MPAA, 20-100 mM). Unless noted otherwise, 10-50 mM TCEP was used to eliminate disulfide formation during EPL. Prior to the ligation, the buffer was degassed with argon for 15 min. Recombinant protein α -thioesters (1-2 mM, 1 eq.) and N-terminal-cysteinyl-SEA-linked peptides (2-4 mM, 2 eq.) were dissolved in ligation buffer and incubated at 37°C with shaking (400 rpm). The progress of the reaction was monitored by analytical RP-HPLC and LC-MS.

The crude ligation product was purified by RP-HPLC on Waters Auto Purification HPLC/MS system using Buffer A and Buffer B with a linear gradient of 30-90% B over 60 min. Pure fractions were analyzed via ESI-MS, analytical RP-HPLC and SDS-PAGE.

3.6 Biophysical Methods

3.6.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

¹H NMR spectra were recorded with a 400 MHz spectrometer (Bruker, Austria) and chemical shifts are reported relative to the signals of residual CHCl₃ in deuterated solvent CDCl₃ or DMSO in d-DMSO for ¹H NMR spectroscopy. Multiplicities are defined as singlet (s), doublet (d), doublet of doublet (dd) and multiplet (m).

3.6.2 Circular Dichroism (CD)

Far UV-CD spectra were measured using a Chirascan Plus spectrometer (Applied Photophysics, UK) within a wavelength range of 190-260 nm. Unless stated otherwise, ten spectra with an acquisition time of 1 s for each protein in a 1 mm quartz cell at 1 nm resolution were acquired at rt and averaged. Typical protein concentrations were 0.1 mg/mL.

To determine the secondary structure, negative peak minima at 208 and 222 nm as well as a positive peak maximum at 193 nm for α -helices were considered, whereas a negative peak at 218 nm and a positive peak at 195 nm were used for the calculation of the percentage of well-defined anti-parallel β -sheets. The amount

of random coil structure was calculated using the maximum peak at 210 nm and minimum peak at 195 nm ^[301].

All calculations were done using CDNN software comparing at least 13 spectra from the database.

3.7 Biochemical assays

3.7.1 Aggregation assays with Thioflavin-T (ThT)

For the semi-automated aggregation assay modified from Baskakov *et al.* ^[302], 1mM ThT in ddH₂O and 2 M Gnd-HCl were added to a solution of refolded PEGylated and non-PEGylated PrP variants (minimum concentration of 0.1 mg/mL) in refolding buffer. The pH was adjusted to 6.0 and three samples with a volume of 160 μ L each of the reaction mixture was pipetted into three separate wells of a 96 well plate. To prevent long-term evaporation, a single mixing bead ($\varnothing$ = 2 mm, Merck) was placed into the well and the plate was covered with a sealer. Subsequently, the plate was incubated at 37°C under continuous agitation using a microplate-reader device (Biotek Synergy Mx). ThT fluorescence was recorded every 10 min with the excitation wavelength of 444 $\pm$ 9 nm and an emission wavelength of 485 $\pm$ 9 nm.

4 Results and Discussion

4.1 Semisynthesis of homogenously modified PrP variants

Semisynthesis of homogenously modified PrP variants was performed through the linkage of peptide segments carrying monodisperse polymeric glycan mimics to recombinantly expressed PrP α -thioesters via EPL. In forthcoming sections, the results of the semisynthetic route will be discussed in detail.

4.1.1 Generation of recombinant PrP α -thioesters

Despite being investigated extensively, it is still not entirely clear how α -helical PrP^C converts into β -sheet rich PrP^{Sc}. As outlined in section 1.3.3., certain aspects may play a role in this process including polyanions, lipid interactions and PTMs of PrP. However, it is very challenging to obtain sufficient amounts of PrP^C as well as PrP^{Sc} for investigating prion pathogenesis. Moreover, the heterogeneity of PTMs makes it difficult to assess the impact of them on prion pathogenicity. Based on this background, a semisynthetic method has been developed to access homogenously modified PrP variants.

4.1.1.1 Cloning and Expression of rPrP-Intein fusion constructs

Two PrP α -thioesters will be generated from PrP 23-178-*Mxe*Intein-His₆-CBD and PrP 90-178-*Mxe*Intein-His₆-CBD fusion constructs (Figure 6). These were cloned into pTXB3 plasmid containing *Mxe*Intein and HisTag coding sequences. *Mxe*Intein is an intein fragment expressed by the *Mycobacterium xenopi gyrA* gene. It can excise itself out of the flanking proteins through thiol-mediated cleavage giving rise to a protein α -thioester. The variant used in this thesis is a mutated version preventing transsplicing. All constructs were cloned according to the procedure described in Olschewski *et al.* [265], sequenced and aligned with the original sequences.

After transformation into the expression strain *E. coli* Rosetta 2 for PrP 23-178-*Mxe*Intein-His₆-CBD and *E. coli* RIL for PrP 90-178-*Mxe*Intein-His₆-CBD, the fusion

proteins were expressed in inclusion bodies, solubilized and purified under denaturing conditions (guanidine containing buffer) using Immobilized Metal Affinity Chromatography, IMAC.

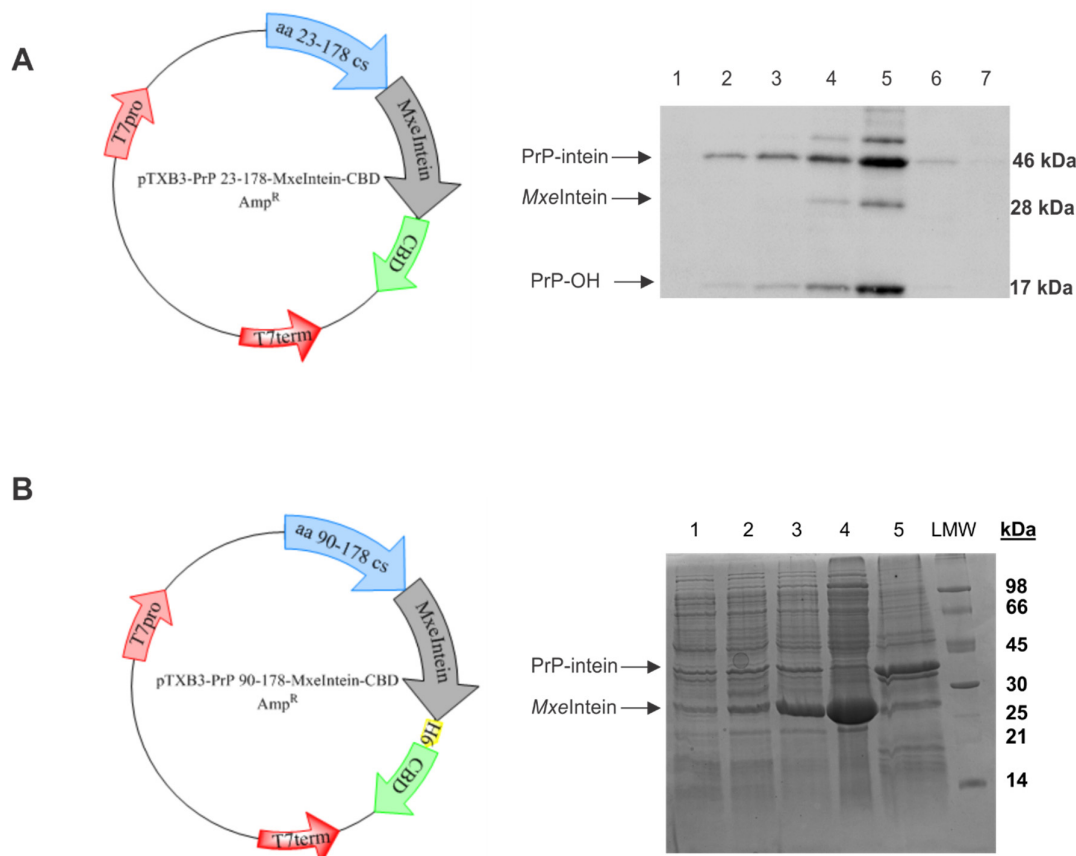


Figure 6: Expression of PrP 23/90-178-MxeIntein-His₆-CBD.

A: Left panel; Plasmid map of pTXB3-PrP 23-178-MxeIntein-His₆-CBD. Right panel; western blot analysis of expression in *E.coli* strain Rosetta 2 developed with 3F4 antiPrP antibody. Lane 1: negative control, lane 2: expression of PrP 23-178-intein fusion construct before IPTG induction, lane 3: 2 h after IPTG induction, lane 4: 4 h after IPTG induction, lane 5: collected cell pellet after cell lysis, lane 6: positive control, PrP 90-231-MxeIntein-His₆-CBD, lane 7: supernatant after cell lysis. **B:** Left panel; plasmid map of pTXB3-PrP 90-178-MxeIntein-His₆-CBD. Right panel; SDS-PAGE analysis of expression of PrP 90-178-intein fusion construct in *E.coli* strain RIL. Lane 1: before IPTG induction, lane 2: 2 h after IPTG induction, lane 3: 4 h after IPTG induction, lane 4: collected cell pellet after cell lysis, lane 5: supernatant after cell lysis, lane 6: low molecular weight marker (LMW).

4.1.1.2 Intein cleavage and generation of PrP α -thioesters

Purified PrP-intein fusion proteins were transferred into 8 M urea containing buffer and stepwise diluted in order to achieve partial folding of the intein. Stepwise dilution involves dilution of concentrated urea (8 M) down to 4 M urea containing buffer. At this concentration, intein is partially folded and the cleavage site is accessible for thiol catalysts. 300 eq. of MESNA was added to the protein solution

giving rise to the cleavage of intein-CBD fragment and PrP α -thioester. The cleavage reaction was monitored via SDS-PAGE (Figure 7). Three bands with different intensities could be detected. The band with the highest molecular weight (for PrP 23-178-Intein fusion 45.6 kDa, for 90-178-Intein fusion 39.1 kDa) represents the non-cleaved PrP-intein fusion protein and has the lowest intensity, the band at approximately 29 kDa corresponds to *Mxe*Intein-His6-CBD with the most intense band being the PrP α -thioester (17.0 kDa for PrP 23-178 α -thioester). The gel in Figure 7A also shows a premature intein cleavage for both PrP-intein fusion constructs after being transferred into urea containing buffer. Unfortunately, it was only possible to detect the thioester band for the PrP 23-178-intein fusion protein; PrP 90-178-intein fusion protein did not give rise to any cleavage reaction. This issue will be discussed later in more detail.

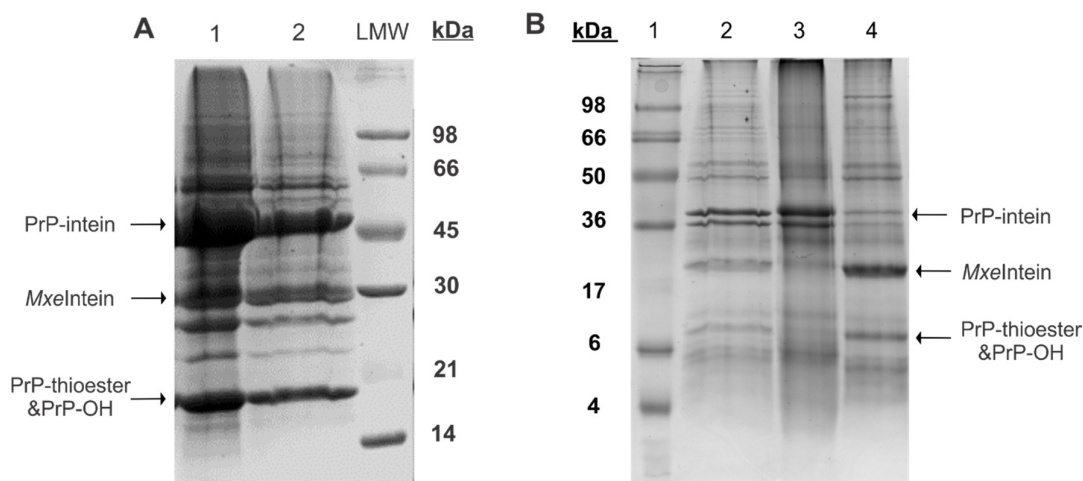


Figure 7: Thiol induced intein cleavage reaction

A: SDS PAGE analysis of intein cleavage reaction of PrP 23-178-MxeIntein-H6-CBD, lane 1: PrP-intein fusion construct before cleavage reaction, lane 2: cleavage reaction time 1d, lane 3: LMW. B: SDS PAGE analysis of intein cleavage reaction of PrP 90-178-MxeIntein-H6-CBD, lane 1: protein marker, lane 2: before cleavage reaction, lane 3: cleavage reaction time 1d, lane 4: cleavage reaction time 2d.

The cleavage reaction was quenched with Gnd-HCl buffer (pH 4.7), the mixture was then concentrated and purified by RP-HPLC to isolate the PrP α -thioester. PrP 23-178-MESNA α -thioester could be identified via analytical RP-HPLC and ESI-MS (Figure 8).

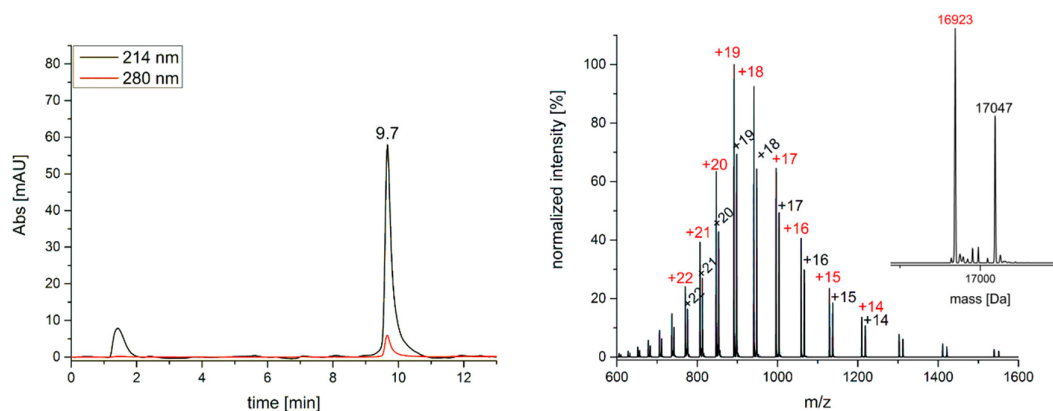


Figure 8: Characterization of purified PrP 23-178-MESNA thioester.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 23-178-MESNA thioester, linear gradient of 5-65% ACN in 10 min. Right panel: ESI-MS analysis and deconvoluted mass of purified PrP 23-178-MESNA thioester, expected mass: 17407 Da, observed mass: 16923 and 17047 Da by the presence of 22- to 14-fold charged ion peaks. 16923 Da corresponds to a C-terminally hydrolyzed variant of PrP 23-178 lacking the thioester.

The main product identified however was not the thioester but the hydrolyzed product carrying a carboxylic acid on the C-terminus. The HPLC trace also did not show a suitable separation for the hydrolyzed protein and protein α -thioester. This may be due to the fact that the C-terminal amino acid is an Asp residue. It has been previously reported that, Asp residues at the -1 position relative to an intein fragment tend to hydrolyze during thiol assisted cleavage. Moreover, the thioester moiety on the C-terminus has a susceptibility to migration to a β -carboxylic acid side chain [303-305].

As already outlined in this section, PrP 23-178 MESNA-thioester was obtained but not PrP 90-178 MESNA-thioester. Further cloning and expression experiments revealed that N- and C-terminally truncated PrP 90-178-intein fusion protein, once transformed into expression strains, is slightly cytotoxic and not suitable for long-term storage. Based on this, the hypothesis has been made that the construct damages the *E. coli* cells and lowers the yield of expression. Moreover, LC-MS and ESI-MS measurements showed that undesired truncation of PrP-intein fusion construct occurred during the expression and purification steps (data not shown). Taken together, PrP 90-178-intein fusion protein could not be transformed and expressed properly and was sensitive to premature degradation.

Due to the challenges described above, it was decided to change the recombinant expression strategy. In 2013, Payne and coworkers reported the synthesis of a fully protected β -mercapto-aspartate derivative, subsequent NCL and selective

desulfurization of this amino acid in the presence of unprotected cysteine residues [211]. Encouraged by these results, two new constructs, PrP 23-177-*Mxe*Intein-*His*₆-CBD and PrP 90-177-*Mxe*Intein-*His*₆-CBD were generated. These variants were shortened of one base triplet on the 3'-end that resulted in the deletion of the 178th aminoacid, aspartate, from the construct. Subsequently, these should enable the generation of C-terminal histidine thioesters upon intein cleavage. Indeed, the PrP-intein fusion proteins were successfully cloned and expressed (Figure 9). The band

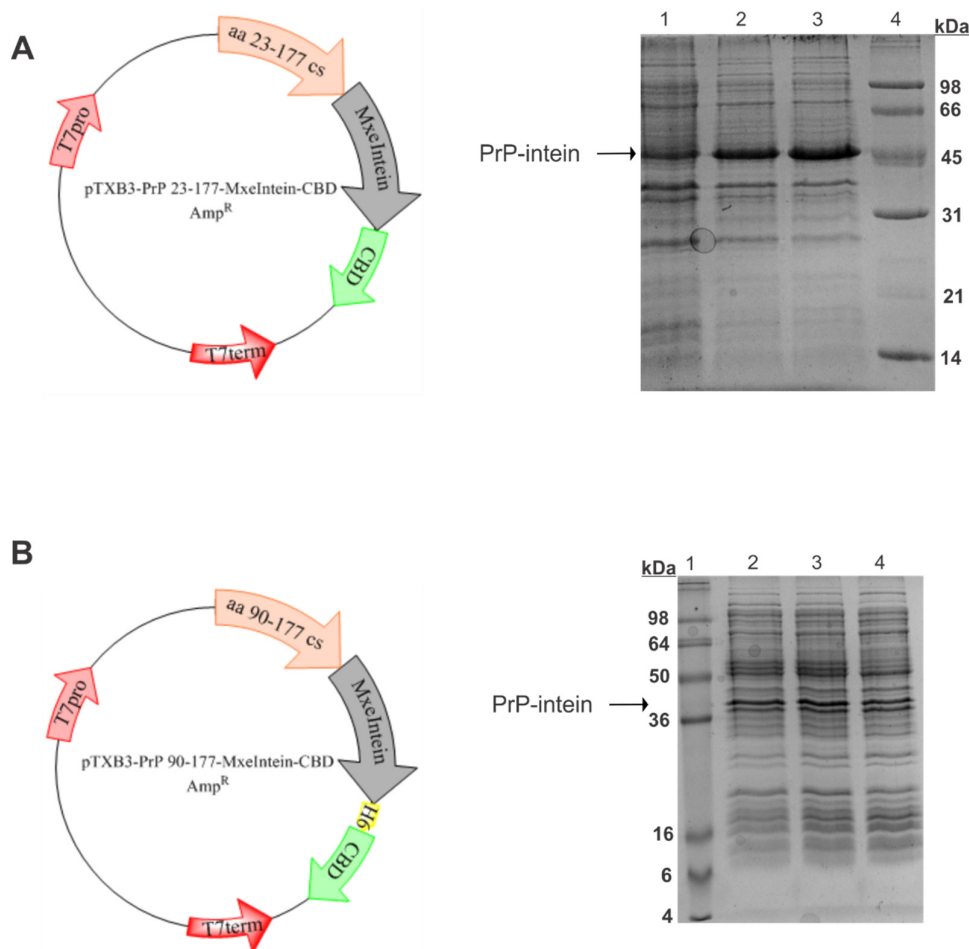


Figure 9: Expression of PrP 23/90-177-*Mxe*Intein-*His*₆-CBD.

A: Left panel; plasmid map of pTXB3-PrP 23-177-*Mxe*Intein-*His*₆-CBD. Right panel; SDS PAGE analysis of expression of PrP 23-177-intein fusion construct in *E.coli* strain Rosetta 2. Lane 1: expression before IPTG induction, lane 2: 3h after IPTG induction, lane 3: 5h after IPTG induction, lane 4: LMW. **B:** Left panel; plasmid map of pTXB3-PrP 90-177-*Mxe*Intein-*His*₆-CBD. Right panel; SDS-PAGE analysis of expression of PrP 90-178-intein fusion construct in *E.coli* strain BL-21 DE3. Lane 1: LMW, lane 2: before IPTG induction lane 3: 3h after IPTG induction, lane 4: 5h after IPTG induction.

at approximately 45 kDa corresponded to PrP 23-177-*Mxe*Intein-CBD (Figure 9 A, labelled as PrP-intein) and the intensity of the band increases over 5 h of

expression. In addition, PrP 90-177-*Mxe*Intein-H₆-CBD could be characterized by the presence of the band between 50-36 kDa (Figure 9 B, labelled as PrP-intein). Unfortunately, no overexpression of this construct could be achieved. Intein-cleavage was performed in the presence of MESNA either in one or in two days, and monitored by SDS-PAGE (Figure 10). Similar to the intein cleavage reaction described previously in this section, three bands could be detected. PrP 23-177 MESNA-thioester could be obtained quantitatively after one day (Figure 10 A), whereas generation of PrP 90-177 MESNA-thioester needed two days (Figure 10 B). Again, both intein-cleavage mixtures were purified via preparative RP-HPLC and thioester fractions were pooled.

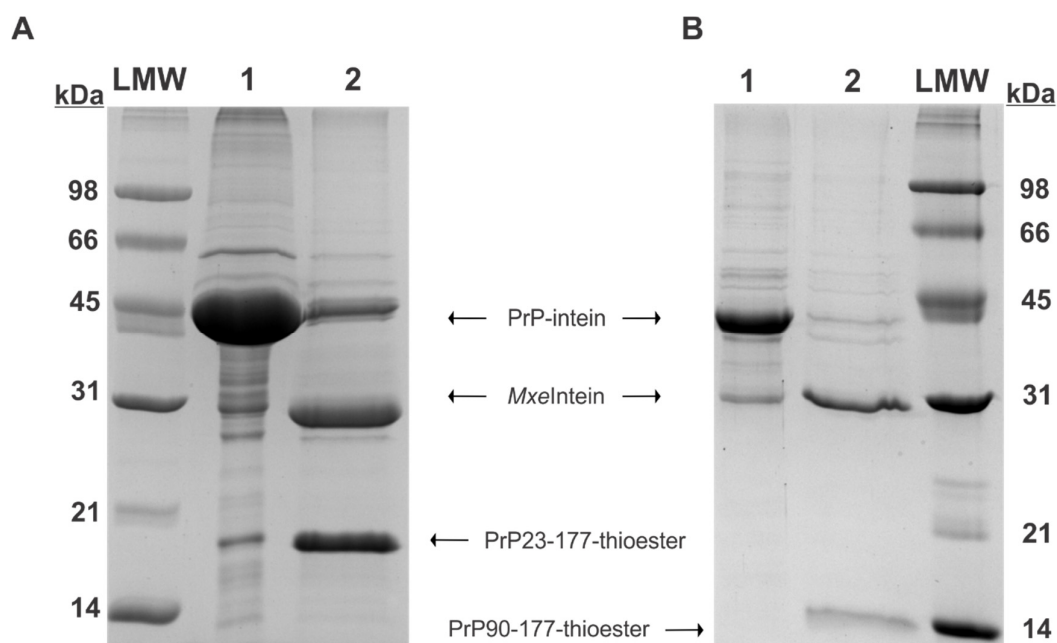


Figure 10: Thiol assisted cleavage reaction of 23/90-177-*Mxe*Intein-H₆-CBD

A: SDS PAGE analysis of intein cleavage reaction of PrP 23-177-*Mxe*Intein-H₆-CBD, lane 1: PrP-intein fusion construct before cleavage reaction time, lane 2: 1d of cleavage reaction. B: SDS PAGE analysis of intein cleavage reaction of PrP 90-177-*Mxe*Intein-H₆-CBD, lane 1: PrP-intein fusion construct before cleavage reaction, lane 2: 2d of cleavage reaction time

Interestingly, only trace amounts of hydrolyzed protein could be detected, which makes PrP 23-177 MESNA- and 90-177 MESNA-thioesters suitable for further ligation reactions. The purity of PrP 23-177 MESNA-thioester could be determined via analytical RP-HPLC chromatogram (Figure 11 A). One sharp peak at 22.1 min was observed. The ESI-MS spectrum as well as the deconvoluted spectrum (Figure 11 B) showed the mass of the desired product in high purity (expected mass: 16932.6 Da, observed mass: 16933 Da). As an additional proof, SDS-PAGE

was utilized. One intense band between 21 and 14 kDa could be detected, whereas a faint band between 31 and 45 kDa was also shown. The faint band corresponded to a dimerized version of the PrP 23-177 MESNA thioester (Figure 11 C).

The analytical RP-HPLC chromatogram of PrP 90-177 MESNA thioester showed a very sharp peak at 22.4 min (Figure 12 A). The ESI-MS spectrum and the deconvoluted mass spectrum provided the proof for the presence of the desired product (expected mass: 10068.4 Da, observed mass: 10069 Da) by the detection

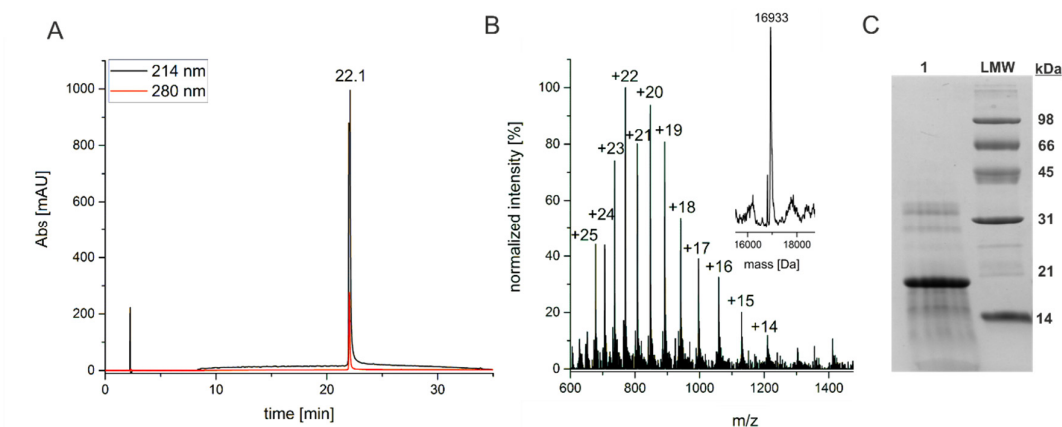


Figure 11: Characterization of PrP 23-177-MESNA thioester.

A: analytical C4 RP-HPLC chromatogram of PrP 23-177-MESNA thioester, linear gradient 5-65% ACN in 30 min. B: ESI-MS (left) and deconvoluted (right) spectrum of PrP 23-177-MESNA thioester, expected mass: 16,931.6 Da, observed mass: 16,933 Da by the presence of 25- to 14-fold charged ion peaks. C: SDS-PAGE analysis of PrP 23-177 MESNA thioester, lane 1: PrP 23-177-SR MESNA thioester, lane 2: LMW.

of 14- to 6-fold charged ion peaks (Figure 12 B). Interestingly, the SDS-PAGE analysis showed a very intense band at approx. 14 kDa, which did not correspond to the expected molecular weight of 10068 Da. However, this was also seen for the intein cleavage reaction suggesting that the PrP 90-177 MESNA thioester band retards on the SDS gel (Figure 12 C, lane 1). A band at approx. 20 kDa was also detected. This band was most likely dimerized PrP 90-177 MESNA-thioester variant. Hence, it was concluded that the desired product was observed.

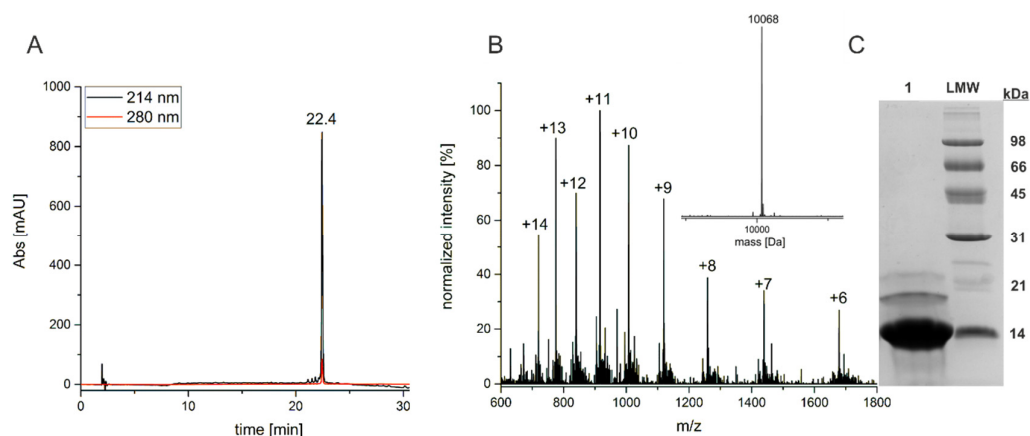
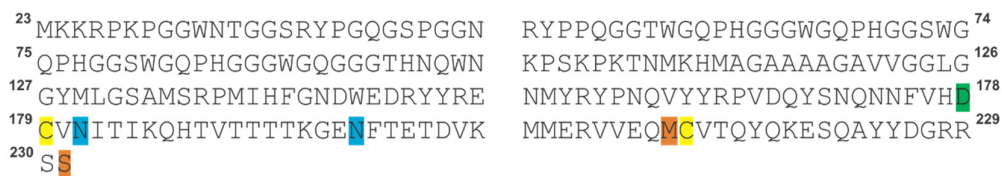


Figure 12: Characterization of PrP 90-177-MESNA thioester.

A: analytical C4 RP-HPLC chromatogram of PrP 90-177-MESNA thioester, linear gradient 5-65% ACN in 30 min. B: ESI-MS (left) and deconvoluted (right) spectrum of PrP 90-177-MESNA thioester, expected mass: 10,068.4 Da, observed mass: 10,069 Da by the presence of 14- to 6-fold charged ion peaks. C: SDS-PAGE analysis of PrP 90-177 MESNA thioester, lane 1: PrP 90-177-SR, MESNA thioester, lane 2: LMW.

4.1.2 Generation of PrP peptide α -thioesters

Simultaneously to the preparation of recombinant PrP thioesters, a library of peptides and peptide-thioesters carrying monodisperse polymeric glycan mimics has been synthesized. These peptides are middle fragments comprising amino acids 178-213 and 214-231 of PrP 23-231 (Scheme 12).



Scheme 12: Primary sequence of PrP 23-231 with possible ligation sites

yellow: cysteine residues, orange: amino acids for introduction of SEA moieties, green: β -mercapto aspartate, blue: Asn181 and Asn197 for introduction of glycan mimics

Upon preparation of recombinant PrP α -thioesters, a library of peptides and peptide-thioesters carrying monodisperse polymeric glycan mimics were synthesized (Scheme 9). All synthetic peptides carry an N-terminal cysteine or cysteine surrogates for further chemoselective ligation reactions. The choice of the primary peptide sequence depends on the presence of cysteine and asparagine residues. Cys178 and Cys214 can be used for NCL reactions. Furthermore, Asn181 and Asn197 can be modified with glycans or glycan mimics.

Two different core peptides and peptide α -thioesters were designed. One of the peptides must carry an N-terminal cysteine or cysteine surrogate, both Asn residues and a C-terminal thioester, whereas the other peptide also has an N-terminal cysteine and a C-terminal thioester. The presence of controllable C-terminal thioesters is important for chemoselective ligation reactions to link both peptides and subsequently to the GPI-anchoring segment. All the peptides described in the forthcoming section were derived from these designed core peptides.

For the syntheses of peptide α -thioesters, three different thioester precursors were used: SEA, α -hydrazide and mercaptopropionate. The forthcoming sections will summarize the results of the SPPS of these different PrP peptides and peptide α -thioesters.

4.1.2.1 PrP 179-213-SEA



Figure 13: PrP 179-213-SEA

In order to generate C-terminal SEA containing peptides, the SEA linker was synthesized as described in section 3.4.1. Optimization of the synthesis led to higher loading values for SEA-linked tritychloride resin (0.1-0.2 mmol/g resin). First, a peptide, which comprises amino acids 179 to 213 of human PrP and carries the C-terminal SEA-linker (Figure 13), has been synthesized.

Following synthesis, the peptide-thioester should be ligated to a peptide comprising residues 214 to 231 of human PrP with and without the GPI anchor mimic. The peptide, referred to as PrP 179-213-SEA, was synthesized both manually and on an automated synthesizer via SPPS. Pseudoprolines (residues 182-183, 189-190 and 200-201 in Scheme 12) were used to prevent secondary structure formation on the resin and improve yields as well as synthetic quality. After completion of the synthesis, the desired product could be detected by analytical RP-HPLC and ESI-MS after a test cleavage (Figure 14).

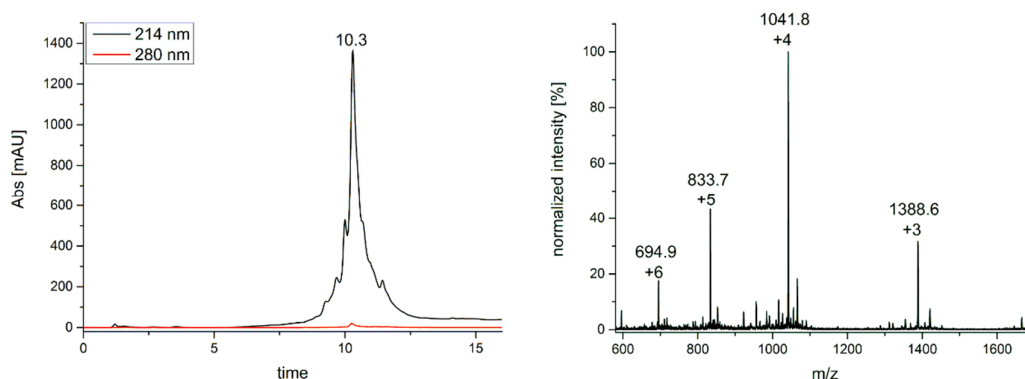


Figure 14: Characterization of PrP 179-213-SEA.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 179-213-SEA. Right panel: ESI-MS spectrum of crude PrP 179-213-SEA; expected mass: 4159.9 $[M+H]^+$, observed mass: 4162.8 $[M+H]^+$; 694.9 $[M+6H]^{6+}$, 827.2 $[M+5H]^{5+}$, 1033.6 $[M+4H]^{4+}$ and 1377.7 $[M+3H]^{3+}$.

As described by Ollivier *et al.*, SEA-linked peptides can be transformed into peptide α -thioesters with an aliphatic thioester moiety. Under acidic conditions, the SEA linker first converts into the peptide SEA-thioester because the equilibrium between amide and thioester is shifted towards thioester. Then, 3-mercaptopropionic acid (MPA) is able to attack the thioester moiety (for detailed information see section 1.5.2.2). In order to obtain PrP 179-213-MPA-thioester, we used MPA in excess as described in section 3. The conversion was successful with an overall synthesis yield of 5% after purification. The product was characterized via analytical RP-HPLC and ESI-MS (Figure 15).

The RP-HPLC chromatogram shows one sharp peak at 10.4 min. The right product could be characterized by the presence of 3- to 5-fold charged ion peaks (1377.7, 1033.6 and 827.2 Da) in the ESI-MS spectrum (Figure 15, right panel).

Even though we were able to obtain the product with the correct mass (expected mass 4128.9 Da, observed mass 4131.0 Da), poor yields and poor purity of the product was limiting for further use in NCL reactions. Therefore, forthcoming studies has been done with PrP 179-213-SEA peptides.

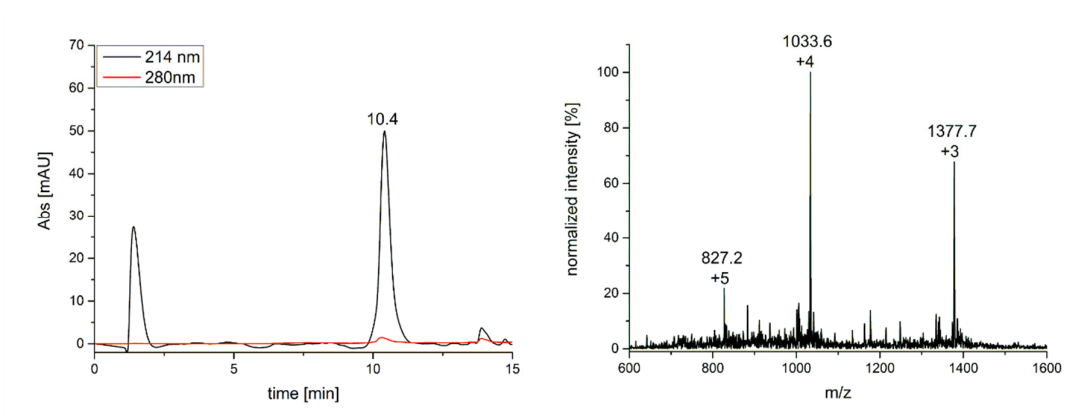


Figure 15: Characterization of purified PrP 179-213-MPA-thioester.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 179-213-MPA-thioester. Right panel: ESI-MS spectrum of crude PrP 179-213-MPA-thioester, linear gradient 5-65% ACN in 10 min; expected mass: 4128.9 $[M+H]^+$, observed mass: 4130.4 $[M+H]^+$; 827.2 $[M+5H]^{5+}$, 1033.6 $[M+4H]^{4+}$ and 1377.7 $[M+3H]^{3+}$.

4.1.2.2 PrP 214Cys(*StBu*)-231-SEA

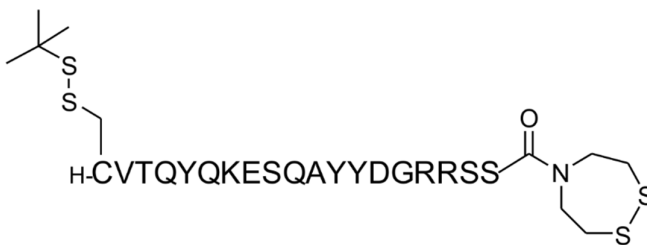


Figure 16: PrP 214-Cys(*StBu*)-231-SEA^{off}

Once PrP 179-213-MPA and PrP 179-213-SEA thioesters were obtained, further PrP peptide α -thioesters have been synthesized. PrP 214-231-SEA peptide was synthesized in order to attach it either to the lipidated D5 peptide (see also section 3.4.8) or to the GPI anchor analogue expressed in yeast (for further information, see section 3.3.5) for membrane anchoring. PrP 214-231-SEA peptide comprises an N-terminal cysteine side chain-protecting group, disulfide *tert*-butyl (Figure 16), and was synthesized both manually and on an automated synthesizer. One of the major problems with this peptide was aspartimide formation during the synthesis or TFA cleavage. The aspartimide was formed between Asp226 and Gly227. The formation could be characterized via piperidine formation on the ester side chain of aspartate or epimerization of the peptide. In order to overcome this problem 5% v/v formic acid in Fmoc removal solution (20% piperidine in DMF) was used as described in Michels *et. al* [306]. In doing so, the aspartimide formation was decreased down to 10%. Nevertheless, even this low amount could be a potential

contamination during NCL reaction. To solve this problem, a new strategy was utilized, which will be discussed in section 4.1.2.3.

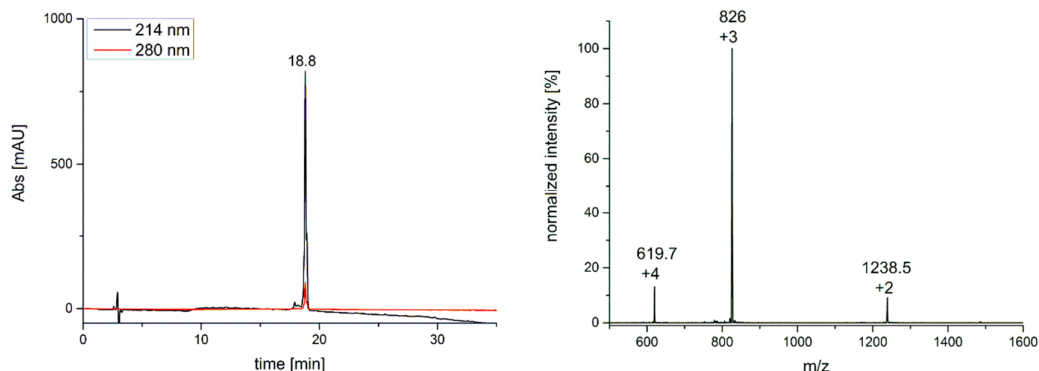


Figure 17: Characterization of PrP 214Cys(StBu)-231-SEA^{off}

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 214Cys(StBu)-231-SEA^{off}, linear gradient 5-65% ACN in 30 min. Right panel: ESI-MS spectrum of purified PrP 214(StBu)Cys-231-SEA^{off}; expected mass: 2475.1 [M+H]⁺, observed mass: 2475.1 [M+H]⁺; 619.7 [M+4H]⁴⁺, 826.0 [M+3H]³⁺ and 1238.5 [M+2H]²⁺.

Upon synthesis, the peptide was cleaved from the resin and purified via preparative RP-HPLC. The final characterization was done by analytical RP-HPLC and ESI-MS (Figure 17). The peptide thioester has been obtained in a very good purity and yield (> 95% purity, ~ 52.3% yield). The HPLC chromatogram (Figure 17, left panel) showed one sharp peak at 18.8 min and the ESI-MS spectrum corresponded well with the expected mass of 2475.1 by the presence of 4-to 2-fold charged ion peaks (619.7, 826.0 and 1238.5 Da).

4.1.2.3 PrP 178(β-mercapto-Asp)-213-SEA



Figure 18: PrP 178(β-mercapto-Asp)-213-SEA^{off}

To adapt the new synthesis strategy described in 4.1.1.2 the required synthetic peptides were changed as well. Payne and coworkers reported the synthesis of an unnatural aminoacid, Boc-Asp(tBu, STmob)-OH at the same time as the problems mentioned above were encountered. Cleavage of side chain protecting groups under acidic conditions gives rise to (D,L)-β-mercapto-aspartic acid (β-mercapto-

Asp). Interestingly, the researchers demonstrated that unprotected β -mercapto-Asp can be reduced to aspartic acid selectively even in the presence of cysteine residues in the same peptide. They postulated that the bond dissociation energy (BDE) of the C-S bond for β -mercapto-Asp should be lower than the BDE of C-S in cysteine. Indeed, computational calculations led to the observation that the BDE of C-S in β -mercapto-Asp was 10 kJ/mol lower than the BDE of C-S in cysteine or deprotonated β -mercapto-Asp [211].

Based on these findings, a new peptide was designed with β -mercapto-Asp at the N-terminus and a SEA latent thioester at the C-terminus (178 β -mercapto-Asp-213-SEA^{off}, Figure 18). After automated synthesis of PrP 179-213-SEA, Boc-Asp(tBu,STmob)-OH was manually coupled to this peptide under conditions described in section 3.4.5. Subsequently, the peptide was cleaved from the resin as described in section 3.4 for SEA containing peptides and analyzed via LC-MS (Figure 19).

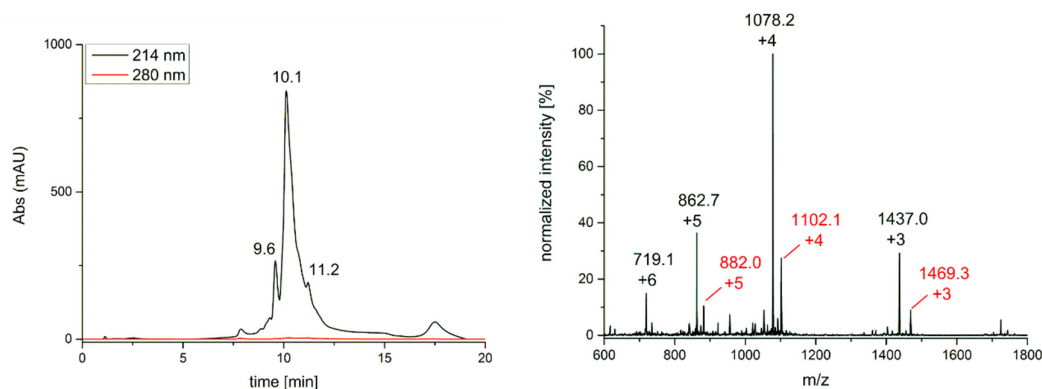


Figure 19: LC-MS analysis of crude 178 β -mercapto-Asp-213-SEA.

Right panel: analytical C4 RP-HPLC chromatogram of crude 178 β -mercapto-Asp-213-SEA, linear gradient 5-65% ACN. Left panel: corresponding mass spectrum to the RP-HPLC peak at 10.1 min, expected mass: 4306.9 Da, observed mass: 4308.8 Da $[M+H]^+$, 719.1 $[M+6H]^{6+}$, 862.7 $[M+5H]^{5+}$, 1078.2 $[M+4H]^{4+}$ and 1437.0 $[M+3H]^{3+}$. The red series correspond to the mass of an TFA ester adduct with an increase in mass of 96 Da.

As can be seen in Figure 19, the major peak 10.1 min and the corresponding mass spectrum confirm 178 β -mercapto-Asp-213-SEA as the main product. In addition, another peptide with a molecular weight of 4404 Da, which appears to be the corresponding peptide TFA-ester adduct, co-eluted with the desired product. Further analysis of a minor peak at 9.6 min lead to the observation that a byproduct carrying one *tert*-butyl group was also present. After RP-HPLC purification, the byproduct was eliminated. Purified sample was analyzed via analytical RP-HPLC

and ESI-MS (Figure 20). The product was obtained in a good yield (22%) and purity (>95%).

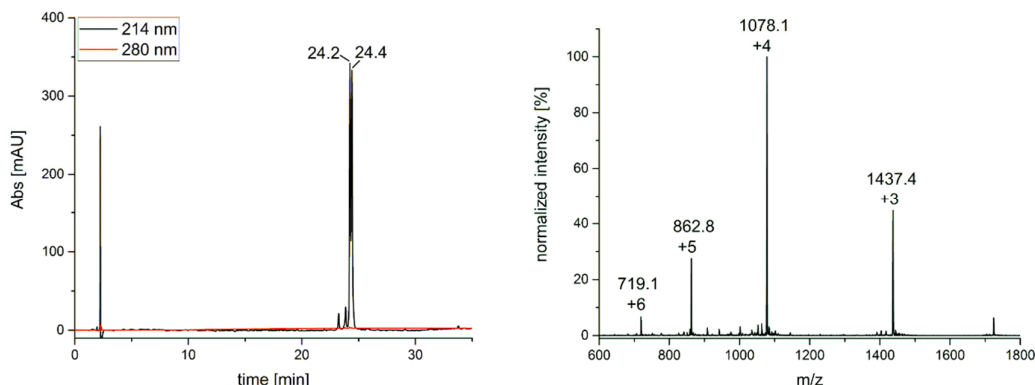


Figure 20: Characterization of purified 178β-mercapto-Asp-213-SEA.

Left panel: analytical C4 RP-HPLC chromatogram of purified 178β-mercapto-Asp-213-SEAthioester. Right panel: ESI-MS spectrum of 178β-mercapto-Asp-213-SEA, expected mass: 4306.9 Da, observed mass: 4308.6 Da $[M+H]^+$, 719.1 $[M+6H]^{6+}$, 862.8 $[M+5H]^{5+}$, 1078.1 $[M+4H]^{4+}$ and 1437.4 $[M+3H]^{3+}$.

The HPLC chromatogram of purified product shows two main peaks at 24.2 and 24.4 min. The corresponding mass spectrum showed clearly that both species have the same molar mass by the presence of 3- to 6-fold charged ion peaks (1437.4, 1078.1, 862.8 and 719.1 Da). Introducing racemic β-mercapto-Asp leads to epimers that can be separated by RP-HPLC. Since the mercapto moiety would be reduced upon ligation, the peptide thioester could be used for further NCL reactions.

4.1.2.4 PrP 178(β-mercapto-Asp)-181(Dab)-213-SEA

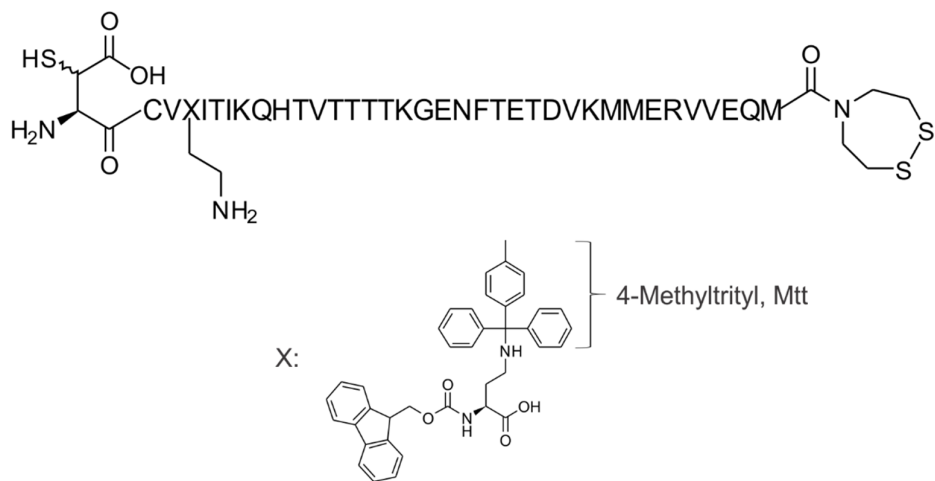


Figure 21: PrP 178(β-mercapto-Asp)-181(Dab)-213-SEA

PrP comprises two Asn residues, Asn181 and Asn 197, both of which can be modified with N-glycans [148-149]. It is a major synthetic challenge to attach complex glycans to Asn residues of synthetic peptides as described in section 1.5.3.1. Here, in order to attach glycan and glycan mimics to synthetic peptides, an unnatural amino acid with suitable orthogonal sidechain protecting groups (PG) on N- α -Fmoc-L-diaminobutanoic acid (Fmoc-Dab-OH) was introduced as an Asn surrogate. This amino acid has the same number of carbon atoms on the sidechain units as on Asn and can therefore be considered as a suitable surrogate. For our purposes, the 4-methyltrityl (Mtt) PG on the amine side chain was the most favorable. This PG can be cleaved under mild acidic conditions (1% TFA) and the resulting free amine moiety can be modified with glycan and glycan mimics.

The previously synthesized PrP peptide fragment, PrP Fmoc-184-213-SEA, was used for the attachment of Fmoc-Dab(Mtt)-OH on solid support. Introduction of one pseudoproline, Fmoc-Ile-Thr($\psi^{\text{Me,Me}}$ pro)-OH, into the peptide sequence and subsequent coupling of Fmoc-Dab(Mtt)-OH to its N-terminus via Fmoc/OtBu chemistry was followed by a test cleavage from the SEA resin under acidic conditions. The resulting product was analyzed via LC-MS. As shown in Figure 22, the crude product not only contains the desired peptide (expected mass: 3943.9 Da, observed mass: 3944.0 Da) by the presence of multiple charged ion peaks but also a minor side product with a mass loss of 100 Da.

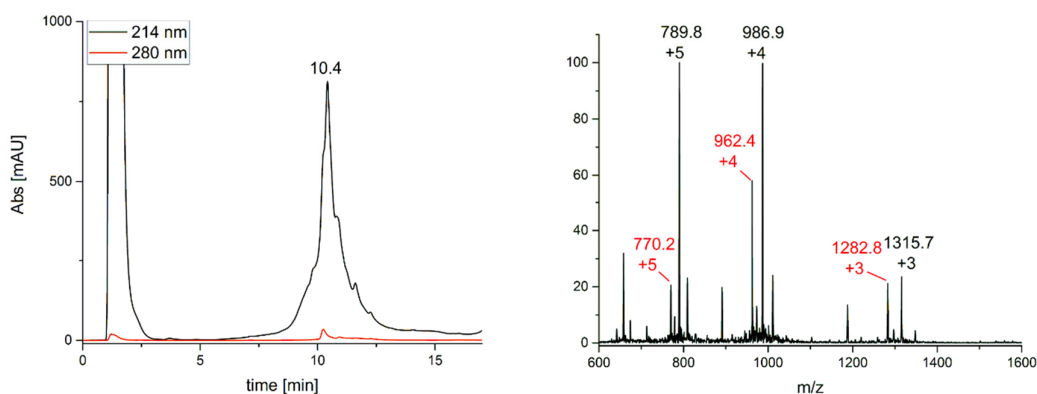


Figure 22: Characterization of crude Fmoc-181Dab-213-SEA via LC-MS.

Left panel: analytical C4 RP-HPLC chromatogram of crude Fmoc-181Dab-213-SEA, linear gradient 5-65% ACN in 10 min. Right panel: mass spectrum corresponding to the peak at 10.4 min, expected mass: 3943.9 Da, observed mass: 3944.0 Da, 789.9 [M+5H]⁵⁺, 986.9 [M+4H]⁴⁺, 1315.7 [M+3H]³⁺. The red series corresponds to PrP 182-213-SEA lacking the N-terminal Dab (770.2 [M+5H]⁵⁺, 962.4 [M+4H]⁴⁺, 1282.8 [M+3H]³⁺)

Once attached, Dab increases the molecular weight of the peptide by ~100 Da. Based on this, it was postulated that Dab was only partially coupled to the peptide chain on solid support. Another observation was the lack of the N-terminal Fmoc group even though it was not removed by 20% piperidine in DMF after attachment of Dab and prior to test cleavage. After Dab was attached to the polypeptide chain, two more amino acids were coupled in order to obtain the PrP peptide containing residues 179-181Dab-213-SEA. After the final attachment and test cleavage, the product was analyzed via LC-MS. Unfortunately it was found that the attachment of two N-terminal residues was not successful; only the mass of PrP 181(Dab)-213-SEA and the side product with a molecular weight of 100 Da less could be found (Figure 23).

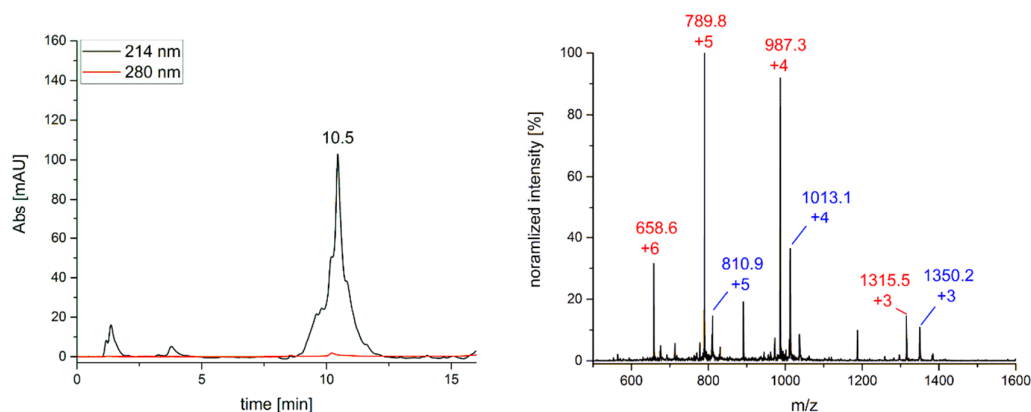


Figure 23: Characterization of 179-181Dab-213-SEA via LC-MS.

Left panel: analytical C4 RP-HPLC chromatogram of crude 179-181Dab-213-SEA. Right panel: mass spectrum corresponding to the peak at 10.4 min, expected mass: 4293.0 Da, observed mass: red series, 790 $[M+5H]^{5+}$, 987 $[M+4H]^{4+}$, 1315 $[M+3H]^{3+}$. The red series corresponds to PrP 181Dab-213-SEA. The mass of the blue series corresponds to the full length peptide sequence without Dab (811 $[M+5H]^{5+}$, 1013 $[M+4H]^{4+}$, 1350 $[M+3H]^{3+}$.)

No attempts to attach amino acids 180 and 179 worked. Consequently, two major problems had to be addressed; how to obtain the desired peptide and how to prevent undesired Fmoc cleavage. Using different batches of Fmoc-Dab(Mtt)-OH did not work either, suggesting that coupling of Dab was the major problem. To address this problem, a proof of concept study was performed. In order to find, if Dab coupling was complete, a 8-mer peptide sequence was designed, H-GEXFTETD-OH, where X was replaced by Dab(Mtt), side chain Mtt protected 2,4-diaminopropionic acid (Dpr(Mtt)) or Lys(Mtt). After synthesis, peptides were

cleaved from the resin under acidic conditions and analyzed via LC-MS (Figure 24).

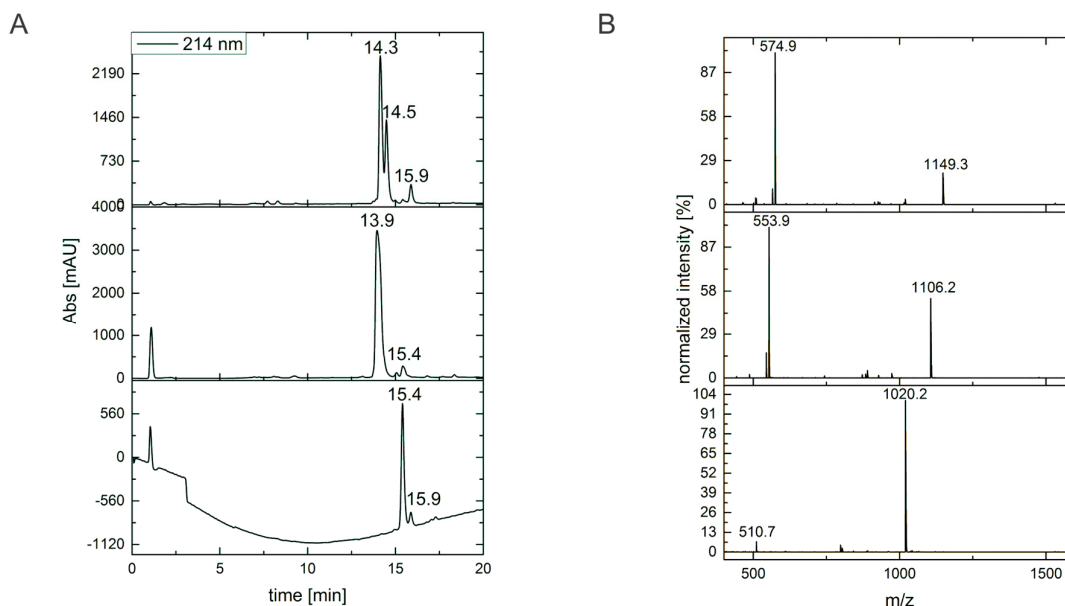


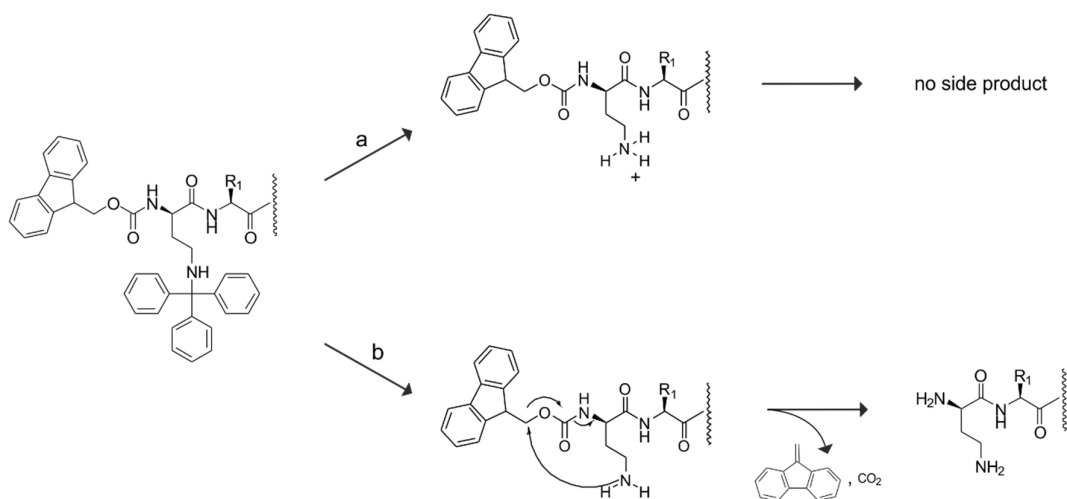
Figure 24: Characterization of 8-mer peptides, H-GEXFTETD-OH, via LC-MS.

From bottom to top; X: Dab, Dpr and Lys. A: analytical C4 RP-HPLC chromatogram of 8-mer Peptides, linear gradient 5-65% ACN in 20 min, B: Corresponding mass spectra to main peaks at 15.4, 13.9 and 14.3 min.

As shown in Figure 24, Dab coupling was not complete. The expected mass could not be observed, the only mass that could be found was the mass of the 7mer peptide without Dab (1020.0 Da). Lys(Mtt) incorporation worked better (Figure 24, upper panel) but gave rise to the 7mer truncated peptide as well, which could be identified under the side peak at 14.5 min. The mass spectrum of the main peak corresponded to the mass of desired product (expected mass: 1149.7, observed mass: 1149.3 Da). The most promising amino acid in this study was Dpr with almost no side products (Figure 24, middle panel). The right product could be identified as the main product with a good purity (expected mass: 1105.5, observed mass: 1106.2 Da). According of this data, Dpr was chosen to use for further syntheses.

In 2001, Albericio and coworkers reported a study, where they tested the ability of N-terminal amino acids containing a primary amine on the side chain to cleave the N- α -Fmoc PG [307]. The side chain *pKa* values dominated to the ability to cleave the Fmoc group. However, premature cleavage only occurred, when the researchers used unprotected amine side chain under standard conditions. In

contrast, the approach described in this thesis involves the protection of the amine side chain of Dab with an acid labile PG. Furthermore, under acidic cleavage conditions, if there is a free amine, it will be definitely protonated. This should prevent a nucleophilic attack of the amine on the electrophilic carbon atom (scheme 13, a). The only possibility was that the premature Fmoc cleavage occurred during the coupling process (scheme 13, b). One possible explanation for this could be the use of Fmoc-Dab(Mtt)-OH in excess. During the subsequent coupling cycle, remaining amount of Dab would be deprotected. However, the pK_a value of the free amine group on C- α atom is less than the one of the δ -amino moiety making N-terminal deprotected Dab less basic. Having that in mind, another possibility would be insufficient purity of Fmoc-Dab(Mtt)-OH. This has been analyzed via ESI-MS and $^1\text{H-NMR}$ (data not shown). ESI-MS analysis showed the right mass with small amount of impurities and $^1\text{H-NMR}$ spectrum of Fmoc-Dab(Mtt)-OH was satisfactory. In addition, the use of new batches did not result in the formation of the right product. Thus, for the introduction of modifications at position 181, Dab was not the suitable amino acid.



Scheme 13: Hypothetical mechanism for premature Fmoc cleavage with deprotected Dab amine side chain.

a: under acidic conditions, b: under neutral conditions.

at either one or both residues 181 and 197 instead of Dab. In the forthcoming sections of this thesis this amino acid will be referred to as Fmoc-Dpr(Mtt)-OH or the unprotected version as Dpr.

4.1.2.6 PrP 178(β -mercapto-Asp)-181(PEG₂₇)-213-SEA

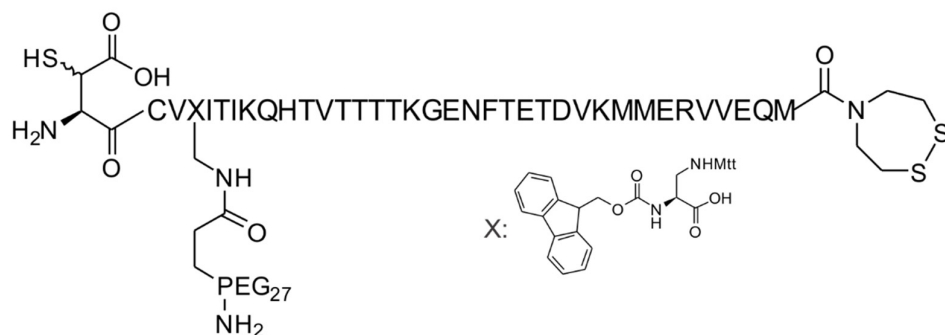


Figure 27: PrP 178(β -mercapto-Asp)-181(PEG₂₇)-213-SEA

The precursor peptide-thioester, PrP 182-213-SEA, was synthesized previously. Fmoc-Dpr(Mtt) was introduced manually at the N-terminus followed by the attachment of Boc-Asp(tBu, STmob)-OH, referred here to as β -mercapto-Asp. Due to simplicity reasons, the term β -mercapto-Asp will be used for both protected and unprotected amino acid (for primary sequence see Figure 27).

For a test cleavage, a small amount of the peptidyl resin was treated under acidic conditions. The resulting crude peptide was analyzed via LC-MS (Figure 28). The HPLC chromatogram shows one major peak with a retention time of 14 min. Two different species could be assigned from the mass spectrum of this peak. One of these is the desired product (expected mass: 4278.9 Da, observed mass: 4280.4 Da) and could be characterized via 3- to 6-fold charged ion peaks (1427.8, 1071.2, 857.2 and 714.0 Da). The other one (red series), with a mass difference of + 96 Da, is most likely a covalently bound TFA ester.

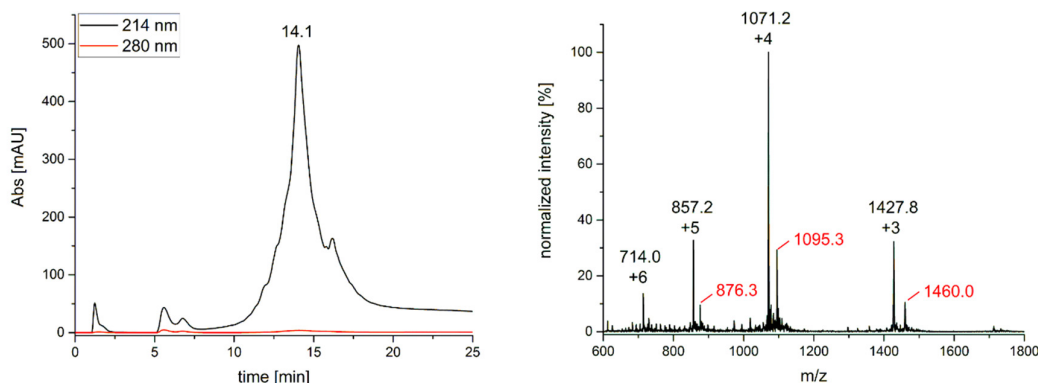


Figure 28: Characterization of crude PrP 178(β -mercapto-Asp)-181Dpr-213-SEA via LC-MS.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 178(β -mercapto-Asp)-181Dpr-213-SEA, linear gradient 5-65% ACN in 10 min. Right panel: mass spectrum corresponding to the peak at 14.0 min, expected mass: 4278.9 Da, observed mass: 714.0 $[M+6H]^{6+}$, 857.2 $[M+5H]^{5+}$, 1071.2 $[M+4H]^{4+}$, 1427.8 $[M+3H]^{3+}$. The red series corresponds to a TFA ester adduct of the product with an increased mass of 96 Da.

With the designed product in hand, the Mtt protecting group on the amino side chain of Dpr was cleaved under mild acidic conditions as described in section 3.4.4. O-(2-carboxyethyl)-O'-[2-(Fmoc-amino)ethyl]heptacosaeethylene glycol, Fmoc-NH-PEG₂₇-OH, was then coupled to the free amino side chain of Dpr. PEG₂₇ was chosen as a glycan mimic because of the similarity in physicochemical properties of this polymer to glycans. Furthermore, PEG₂₇ enhances the solubility of a protein or peptide, is monodisperse and can be easily coupled to peptides using standard aa coupling conditions. After the coupling and final Fmoc deprotection, the crude peptide thioester was cleaved from the resin and analyzed via LC-MS (Figure 29). The HPLC chromatogram of the crude peptide shows one main peak at 10.3 min. The mass spectrum corresponding to main peak clearly showed three mass series. The desired product was obtained as the main fraction (expected mass: 5584.8 Da, observed mass: 5586.0 Da) and two side products with additional 56 Da and 96 Da were also present. The side product with additional 56 Da may correspond to a *t*-butyl ester protecting group on an hydroxyl side chain, whereas the series with 96 Da could be the covalently bound TFA ester on a carboxylic side chain.

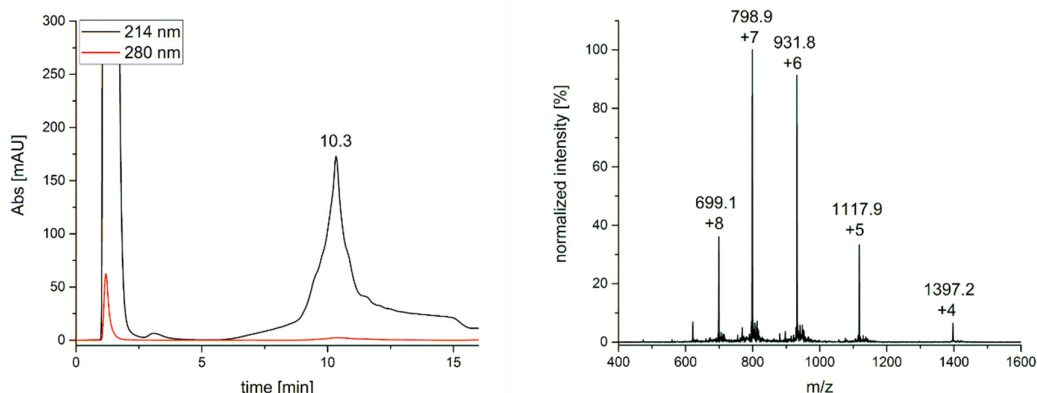


Figure 29: Characterization of crude PrP 178(β-mercapto-Asp)-181PEG₂₇-213-SEA

Left panel: Analytical C4 RP-HPLC chromatogram of crude PrP 178(β-mercapto-Asp)-181PEG₂₇-213-SEA, linear gradient 5-65% ACN in 10 min. Right panel: Mass spectrum corresponding to the peak at 10.3 min, expected mass: 5584.8 Da, observed mass: 699.1 [M+8H]⁸⁺, 798.9 [M+7H]⁷⁺, 931.8 [M+6H]⁶⁺, 1117.9 [M+5H]⁵⁺, 1397.2 [M+4H]⁴⁺.

Fortunately, both species could be eliminated during RP-HPLC purification of the crude peptide. The resulting purified peptide α-thioester was lyophilized and analyzed via analytical RP-HPLC and ESI-MS (Figure 30). The HPLC chromatogram shows two peaks with similar retention times, 24.3 min and 25.2 min respectively. The ESI spectrum of the same sample however corresponds to the pure peptide with the desired mass, 5584.6 Da. The occurrence of these two peaks

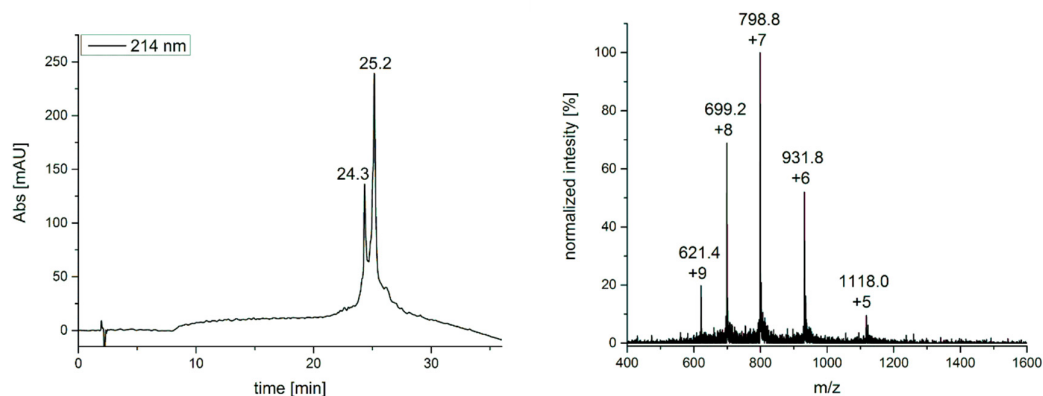


Figure 30: Characterization of purified 178β-mercapto-Asp-181PEG₂₇-213-SEA

Left panel: analytical C4 RP-HPLC chromatogram of purified 178β-mercapto-Asp-181PEG₂₇-213-SEA, linear gradient 5-65% ACN in 30 min. Right panel: ESI-MS spectrum of purified 178β-mercapto-Asp-181PEG₂₇-213-SEA, expected mass: 5584.8 Da, observed mass: 5584.6 [M+1H]⁺, 621.4 [M+9H]⁹⁺, 699.0 [M+8H]⁸⁺, 798.8 [M+7H]⁷⁺, 931.8 [M+6H]⁶⁺, 1118.0 [M+5H]⁵⁺.

could be explained that the SEA linker easily converts into the thioester form under acidic pH. At acidic pH, the equilibrium between thioester and amide favors the thioester. However, some amide-linked peptide still exists. This has been observed

by Melnyk and coworkers in early experiments ^[237]. The overall yield was 51.2% and the purity based on the integrated RP-HPLC area for both peaks was > 95%.

4.1.2.7 PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA

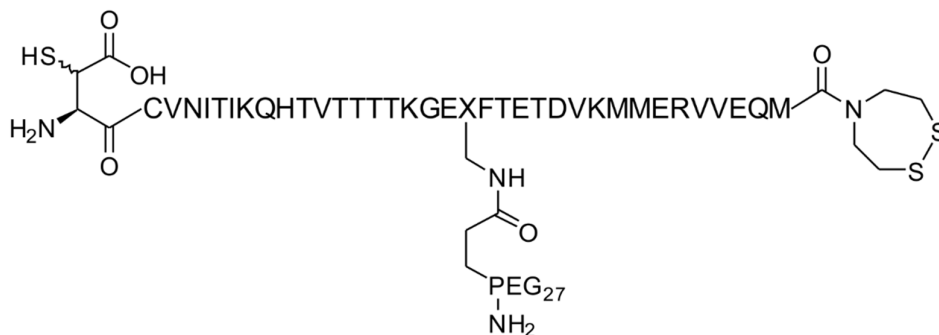


Figure 31: PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA

The synthetic route was analogous to that described in section 4.1.2.7. After the attachment of Dpr(Mtt) a small amount of the peptide was cleaved from the resin and analyzed via LC-MS (Figure 32 A). The HPLC chromatogram (Figure 32 A) shows one major peak and three minor peaks. The product with the correct mass (expected mass: 2399.1 Da, observed mass: 2399.9 Da) was found in the main peak (Figure 32 B). In addition, a second peak giving rise to the same mass spectrum was detected (Figure 32 C). This peak (t =16.23 min) corresponds most likely to the amide from of the SEA-linker as described in section 4.1.2.6. Furthermore, the other two minor peaks at 15.4 min (Figure 32 D) and 15.0 min (Figure 32 E) had corresponding mass spectra with a mass of 2415.2 Da, which corresponds to a mass increase of 16 Da compared to main product. This can be explained by the oxidation of methionine. In the sequence, there are three methionines. The susceptibility of methionine towards oxidation upon acidic treatment has been described elsewhere ^[308-309].

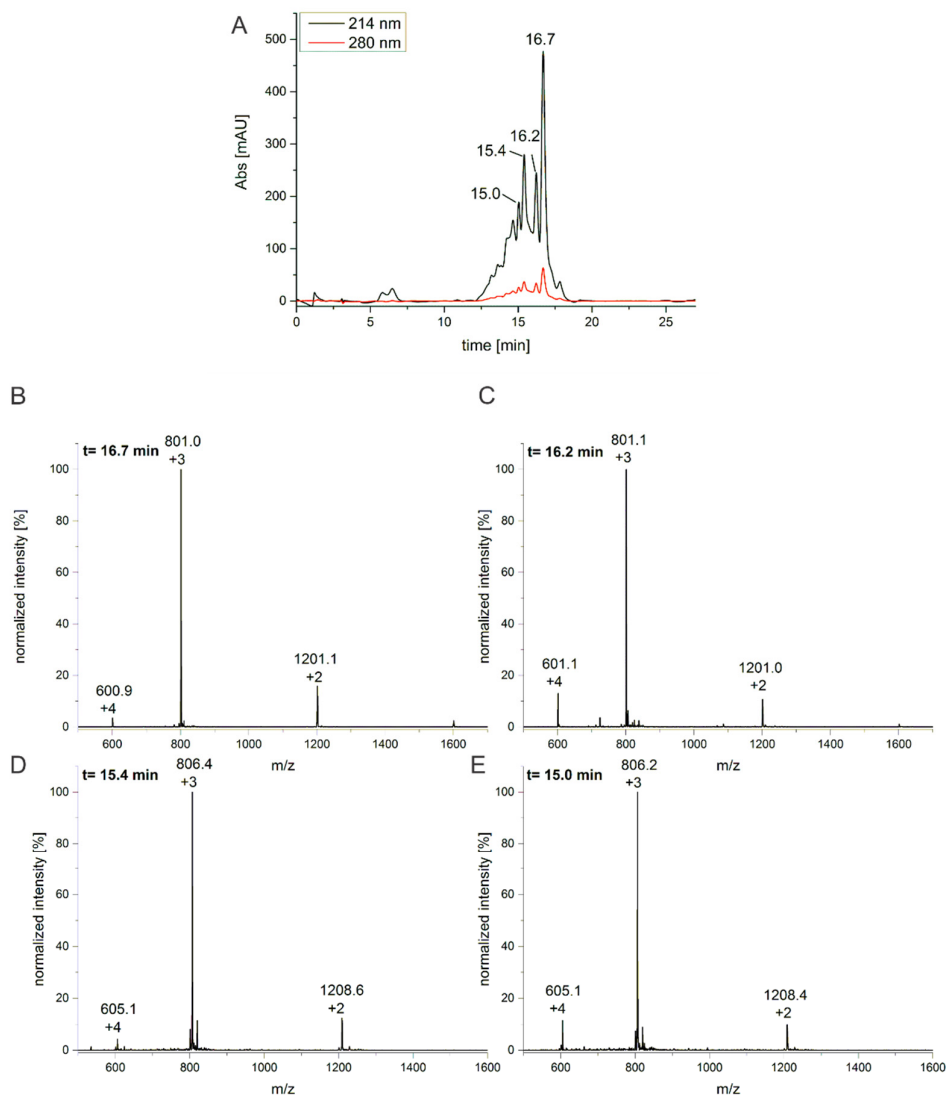


Figure 32: Characterization of crude PrP 197Dpr-213-SEA via LC-MS.

A: analytical C4 RP-HPLC chromatogram of crude PrP 197Dpr-213-SEA, 5-65% ACN in 20 min.

B-E: mass spectrum corresponding to the peak at 16.7, 16.2, 15.4 and 15.0 min

Encouraged by these results, PrP 179-197-Dpr(Mtt)-213-SEA was synthesized and characterized via LC-MS (Figure 33). Two peaks with different mass spectra were observed. The main peak at 9.6 min showed the desired product (expected mass: 4132.0 Da, observed mass: 4133.6 Da) by the presence of multiple charged ion peaks. The side peak at 8.9 min showed a slight increased mass of 4149.4 Da, which corresponds to the mass of the desired product with one oxidized methionine.

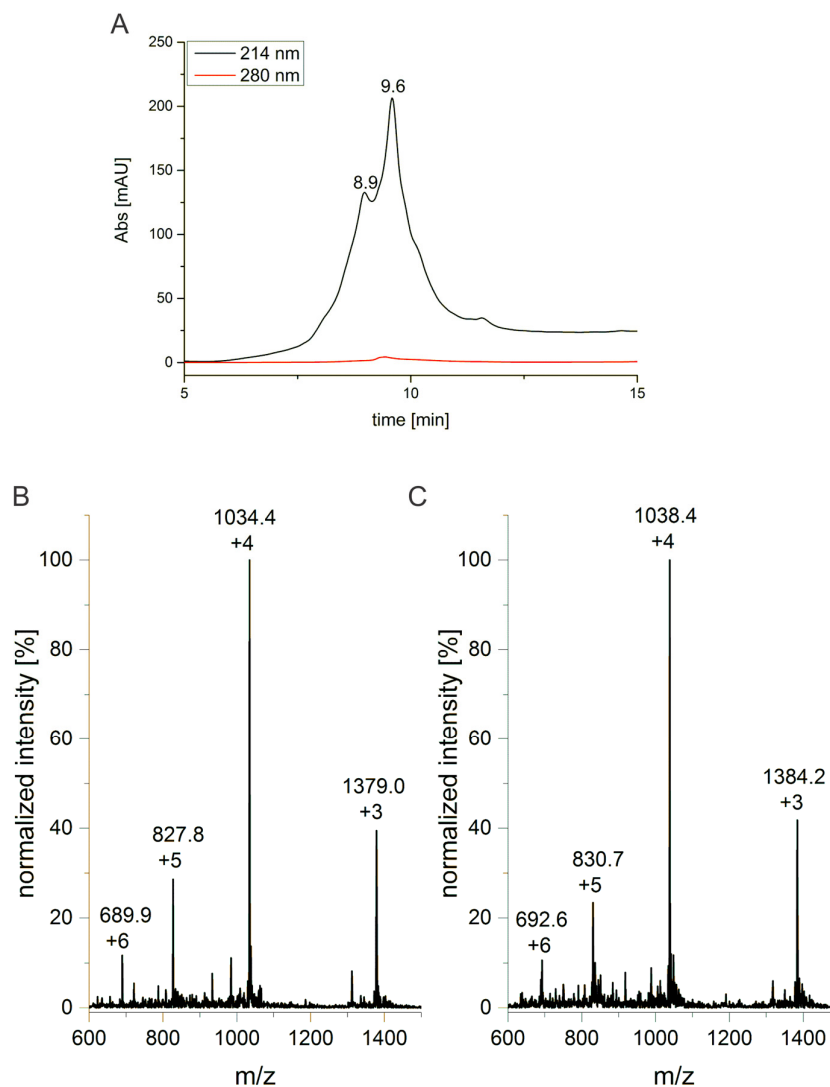


Figure 33: Characterization of crude PrP 179-197(Dpr)-213-SEA via LC-MS.

A: analytical C4 RP-HPLC-chromatogram of crude PrP 179-197(Dpr)-213-SEA, 5-65% ACN in 10 min. B: mass spectrum corresponding to the peak at 9.6 min, expected mass: 4132.0 Da, observed mass: 689.9 [M+6H]⁶⁺, 827.8 [M+5H]⁵⁺, 1034.4 [M+4H]⁴⁺, 1379.0 [M+3H]³⁺. C: expected mass: 4132.0 Da, observed mass: 692.6 [M+6H]⁶⁺, 830.7 [M+5H]⁵⁺, 1038.4 [M+4H]⁴⁺, 1384.2 [M+3H]³⁺.

Because the amount of oxidized species was negligible, the synthesis was continued with the attachment of β -mercapto aspartate and subsequently with Fmoc-NH-PEG₂₇-OH on the side chain of Dpr. The crude peptide was analyzed by LC-MS (Figure 34). Although the desired peptide (expected mass: 5584.8 Da, observed mass: 5585.4 Da) was obtained as the main product, another side product with an excess mass of 16 Da (red series) was detected. This species is most likely the peptide thioester harboring one oxidized methionine. For further

experiments, we used a modified cleavage cocktail K as described by Hackenberger [309]. In doing so, not only the rate of methionine oxidation could be decreased but also the yield of the SEA peptide cleaved from resin compared to the results of the cleavage with the SEA cleavage cocktail as described in section 3.4.2.

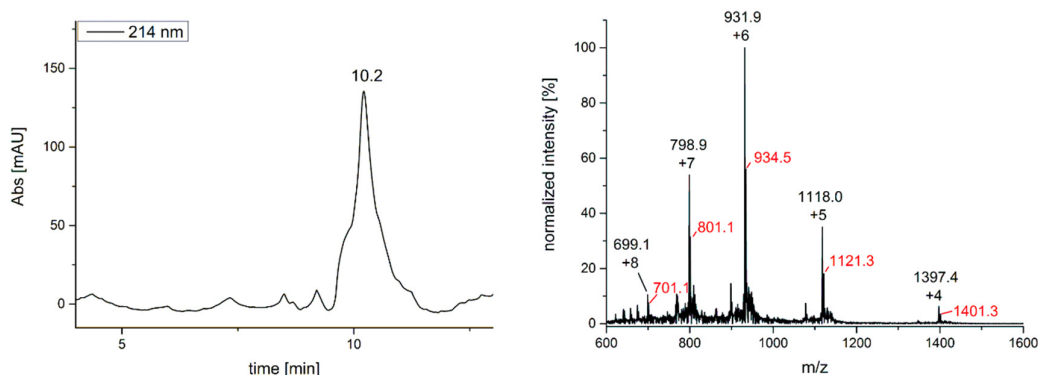


Figure 34: Characterization of crude PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA, 5-65% ACN in 10 min. Right panel: mass spectrum corresponding to the peak at 10.2 min, expected mass: 5584.8, observed mass: 5585.4 [M+H]⁺, 699.1 [M+8H]⁸⁺, 798.9 [M+7H]⁷⁺, 931.9 [M+6H]⁶⁺, 1118.0 [M+5H]⁵⁺, 1397.4 [M+4H]⁴⁺. The red series of multiple charged ion peaks shows the mass of an methionine oxidized species with an additional mass of 16 Da.

The crude mixture was purified via preparative RP-HPLC, pooled and lyophilized. The purified product was analyzed by analytical RP-HPLC and ESI-MS (Figure 35). The analytical RP-HPLC chromatogram showed clearly that the desired product could be obtained. One sharp peak at 24.4 min was detected and the purified product was further analyzed via ESI-MS. The mass spectrum corresponds well with the expected results (expected mass: 5584.8 Da, observed mass: 5585.4 Da) by the detection of 4- to 7-fold charged ion peaks (1397.1, 1181.1, 931.9 and 798.6 Da). The deconvoluted spectrum showed a mass of 5585 Da, which corresponds with the mass of the desired product (sequence can be seen on Figure 31). The overall yield was 20.3 % and the purity of the peptide based on the integrated peak area was > 95%.

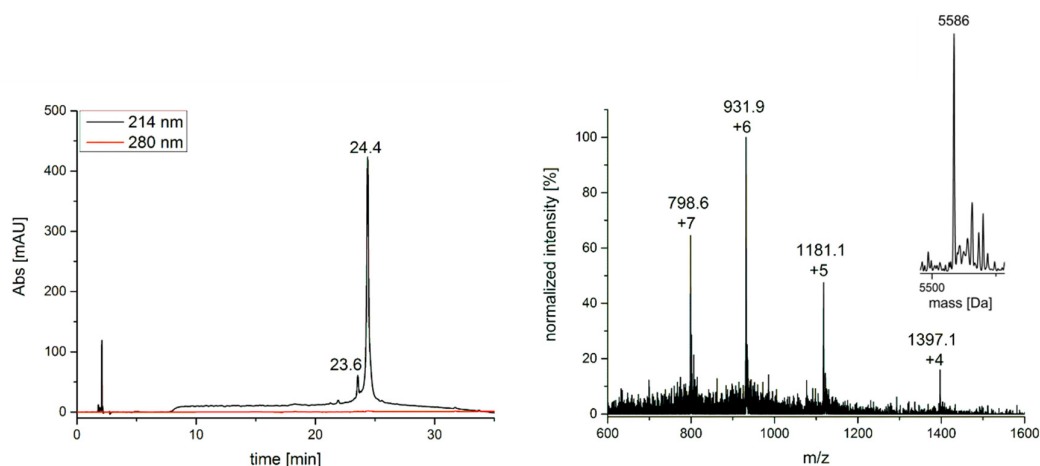


Figure 35: Characterization of purified PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA
 Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β -mercapto-Asp)-197(PEG₂₇)-213-SEA, 5-65% ACN in 10 min. Right panel: ESI-MS spectrum, expected mass: 5584.8, observed mass: 798.6 [M+7H]⁷⁺, 931.9 [M+6H]⁶⁺, 1181.1 [M+5H]⁵⁺, 1397.1 [M+4H]⁴⁺.

4.1.2.8 PrP 178(β -mercapto-Asp)-181&197(PEG₂₇)-213-SEA

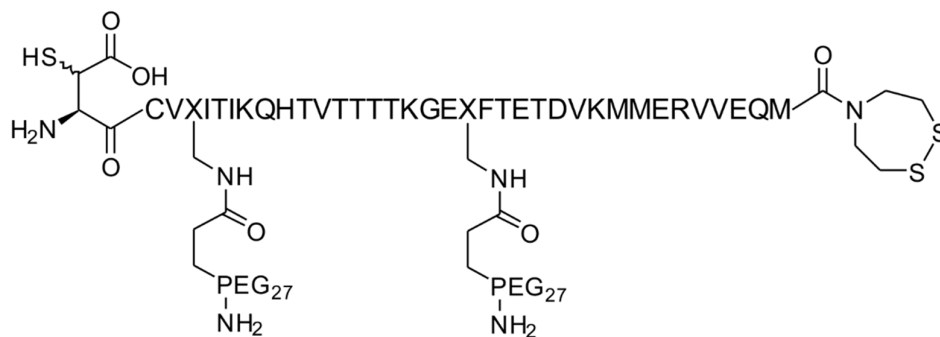


Figure 36: PrP 178(β -mercapto-Asp)-181&197(PEG₂₇)-213-SEA^{off}

PrP-SEA peptides modified at both positions 181 and 197 (Figure 36) were synthesized by replacing both Asn181 and Asn197 with Dpr(Mtt), removal of the Mtt protecting groups, and coupling of PEG₂₇ to the Dpr side chains, as described for the singly-modified PrP thioesters (section 4.1.2.7). To prove that the desired peptide thioester was synthesized, a test cleavage was performed and the product was analyzed via LC-MS (Figure 37). There are two peaks visible in the RP-HPLC chromatogram with retention times 6.0 min and 10.7 min. For the minor peak no ESI spectrum was detectable whereas the mass spectrum of the major peak at 10.7 min showed that the right product (expected mass: 6858.6 Da, observed mass: 6860.6 Da) could be obtained with the detection of 6- to 10-fold charged ion

peaks (1144.6, 981.0, 858.7, 763.3 and 687.2 Da). In addition, a side product with an excess mass of 96 Da was observed suggesting that a TFA ester was formed.

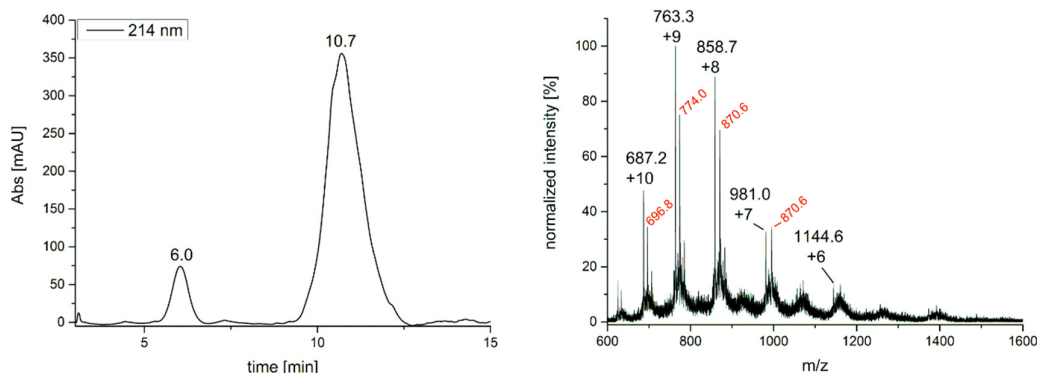


Figure 37: Characterization of crude PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-213-SEA via LC-MS.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-213-SEA, 5-65% ACN in 10 min. Right panel: mass spectrum corresponding to the peak at 10.7 min, expected mass: 6858.6, observed mass: 6860.7 [M+H]⁺, 687.2 [M+10H]¹⁰⁺, 763.3 [M+9H]⁹⁺, 858.7 [M+8H]⁸⁺, 981.0 [M+7H]⁷⁺, 1144.6 [M+6H]⁶⁺. The red series of multiple charged ion peaks shows the mass of a peptide TFA adduct with an increase of 96 Da in molecular weight.

Fortunately, the TFA ester adduct could be separated from the desired product during preparative RP-HPLC. The final product was characterized upon purification via analytical RP-HPLC and ESI-MS (Figure 38). Two sharp peaks at 24.9 and 25.1 min were observed. The ESI-MS spectrum showed 5- to 10-fold charged ion peaks (1373.2 Da, 1144.4 Da, 981.3 Da, 858.7 Da, 763.2 Da and 687.1 Da) for the right

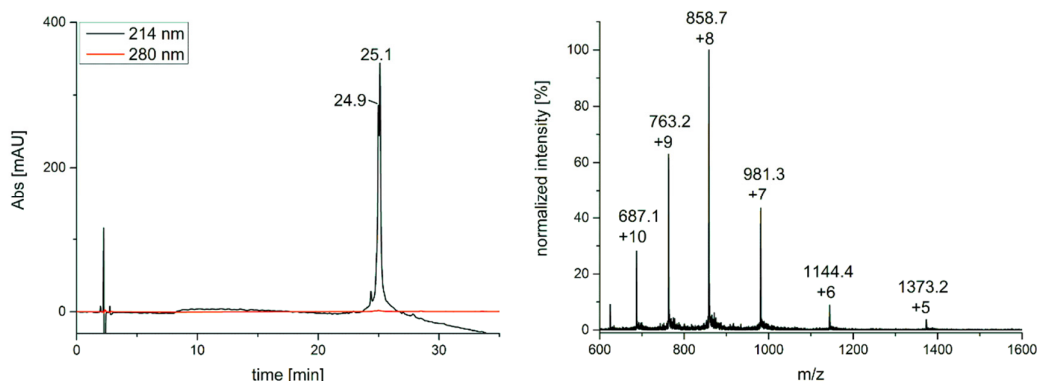


Figure 38: Characterization of purified PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-213-SEA.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-213-SEA, 5-65% ACN in 10 min. Right panel: ESI-MS spectrum of purified PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-213-SEA., expected mass: 6858.6, observed mass: 6860.1 Da, 687.1 [M+10H]¹⁰⁺, 763.2 [M+9H]⁹⁺, 858.7 [M+8H]⁸⁺, 981.3 [M+7H]⁷⁺, 1144.4 [M+6H]⁶⁺, 1373.2 [M+5H]⁵⁺.

product (purity > 95%, yield 21.7%). As described in section 4.1.2.6, the peaks correspond to peptide thioester epimers with D- or L- β -mercapto aspartate on their N-termini.

4.1.2.9 PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231-SEA

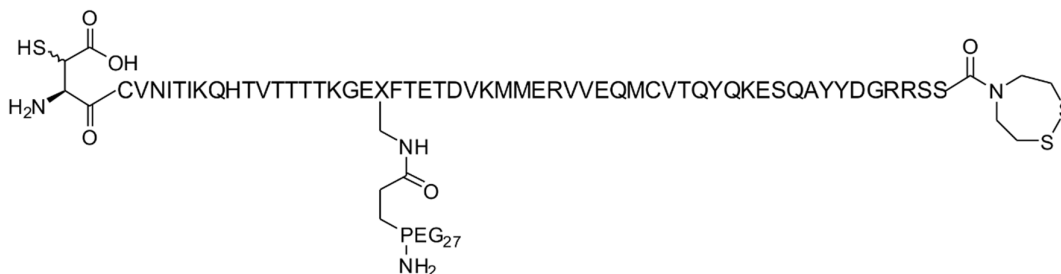


Figure 39: PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231-SEA

In order to generate an alternative to the ligation of two peptide α -thioesters and push the limits of SPPS of modified SEA peptides, a general approach similar to the one described in section 4.1.2.7 has been utilized to synthesize a 53mer peptide thioester with one monodisperse polymeric glycan mimic (PEG₂₇) at position 197 (Figure 39). Although the synthesis was successful, the yields were low (< 1%, < 1 mg of purified peptide thioester) and the purity was moderate (Figure 40, right panel). Therefore, the method to obtain larger peptide thioesters had to be modified. In doing so, a new approach was adopted, which has been described in Bello *et al.* in 2015 and is based on the conversion of peptide- α -hydrazides in peptide thioesters [310].

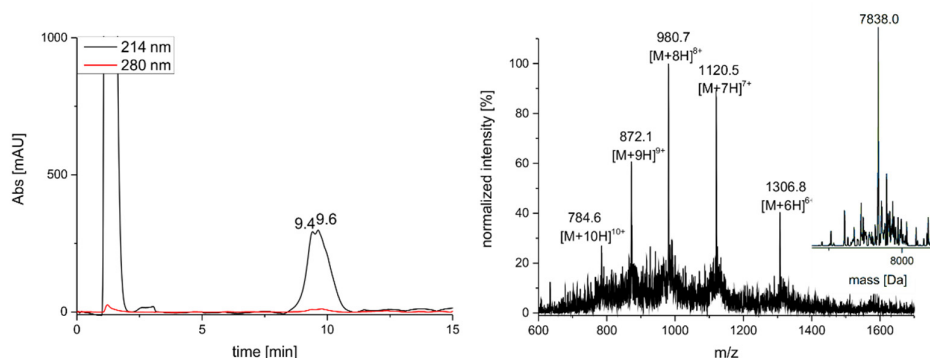
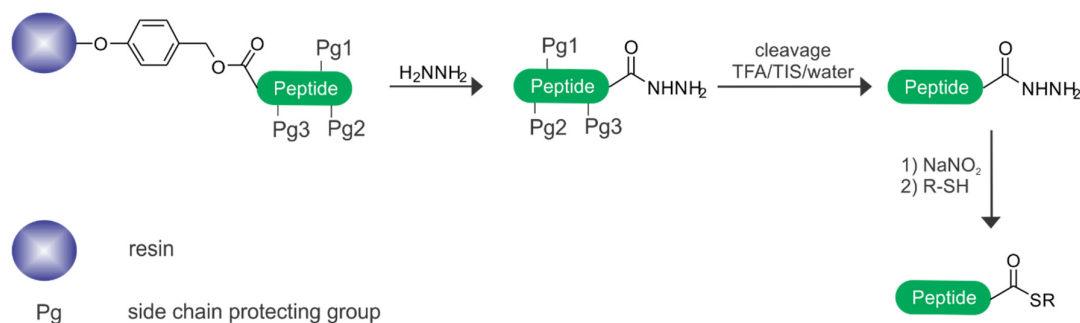


Figure 40: Characterization of purified PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231-SEA.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231-SEA, linear gradient 5-65% ACN in 10 min. Right panel: ESI-MS spectrum of purified PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231-SEA, expected mass: 7838.2, observed mass: 784.6 [M+10H]¹⁰⁺, 872.1 [M+9H]⁹⁺, 980.7 [M+8H]⁸⁺, 1120.5 [M+7H]⁷⁺, 1306.8 [M+6H]⁶⁺. deconvoluted mass spectrum; 7838.0 Da.

4.1.3 Synthesis of PrP peptide- α -hydrazides

In 2011, Lei Liu and coworkers reported the total chemical synthesis of Cssl through one-pot sequential ligation with peptide α -hydrazides as C-terminal thioester precursors and N-terminal cysteinyl peptides [229]. The researchers were able to synthesize peptide α -hydrazides via Fmoc-SPPS. The solid support was treated with 4-nitrophenylcarbonochloridate and reacted with hydrazine to give rise to hydrazinecarboxylate linked Wang resin. The peptide α -hydrazide was then cleaved from the resin under strong acidic conditions. Following this work, Bello *et al.* reported a simple and efficient method for the generation of peptide α -hydrazides using a low loading Wang-TenTaGel resin with good swelling properties [310]. The researchers used 5% $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$ either in methanol or in $\text{H}_2\text{O}/\text{ACN}$ 1:1 mixture to cleave fully sidechain protected peptide from the resin as a peptide α -hydrazide (Scheme 13). In addition, 1M hydrazine in THF was used for peptides with Val, Ile and Thr on their C-terminus, since these did not give good yields for the cleaved peptide α -hydrazides using 5% hydrazine in aqueous solution.



Scheme 14: Generation of peptide α -hydrazides and their conversion into peptide α -thioesters.

Two model peptides have been chosen to synthesize peptide α -hydrazides with the method described above, PrP peptide α -hydrazide comprising aa 214-231 and aa 179-213. These peptides are referred to as PrP 214-231- NHNH_2 and PrP 179-213- NHNH_2 in the forthcoming sections.

4.1.3.1 PrP 214-231-NHNH₂



Figure 41: PrP 214-231-NHNH₂

Peptide 214-231 (Figure 41) was prepared via standard Fmoc SPPS on a TentaGel resin and analyzed via LC-MS after test cleavage (Figure 42). The HPLC chromatogram shows two broad and two sharp peaks at 3.7 and 5.9 min as well as at 7.2 and 8.5 min. For each peak, the corresponding MS spectrum was recorded. The corresponding mass spectrum to 3.7 can be seen in Figure 42. As one can see, the correct mass was observed (expected mass: 2268.0 Da, observed mass: 2267.6 Da). At 5.9 min, the mass spectrum shows the correct mass plus 252 Da, which corresponds to a non-cleaved 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting group on the guanidine side chain of an arginine. No mass has been observed for the peak at 7.2 min whilst the corresponding spectrum to the peak at 8.5 min showed a series of multiple charged ion peaks. These belong to PEG, which comes off with the peptide from the resin upon cleavage and does not have a major effect on the purity of the peptide.

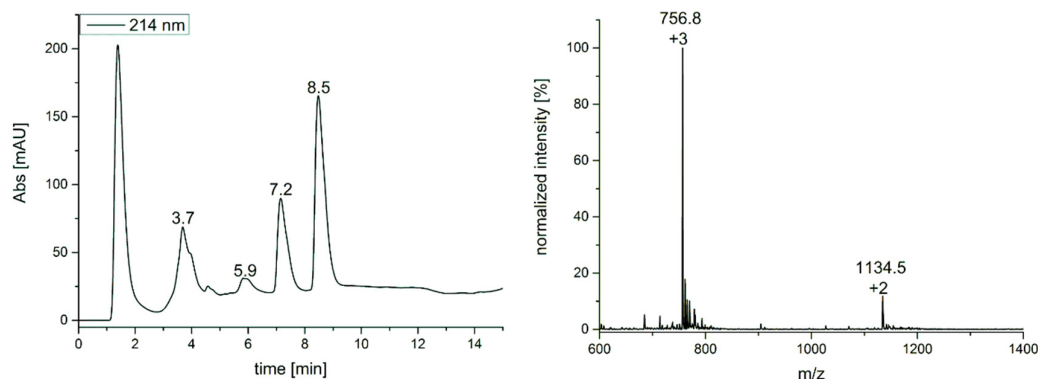


Figure 42: Characterization of crude PrP 214-231.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 214-231, 5-65%B ACN in 10 min. Right panel: mass spectrum corresponding to the peak at 3.7 min, expected mass: 2268.0, observed mass: 756.8 $[M+3H]^{3+}$, 1134.5 $[M+2H]^{2+}$.

Having the desired peptide, the fully side chain protected peptide α -hydrazide should be cleaved. In order to do that, two small portions of the peptidyl resin (each 20 mg) has been treated with two different reagents, 5% H₂NNH₂·H₂O in water and

1M hydrazine in THF. The cleavage reaction took place overnight under continuous agitation. Both reaction mixtures were diluted 1:1 with aqueous buffer and analyzed via LC-MS (Figure 43). The HPLC chromatogram of the reaction mixture in THF (Figure 43 A) shows a broad peak at 13.6 min. The desired product with a molecular weight of 4549.3 Da could not be obtained according to the corresponding mass spectrum (Figure 43 B). The main product observed had a molecular weight of 4471.5 Da, which presumably corresponds to a peptide α -hydrazide plus sodium, with one additional hydrazide, probably on an aspartic or glutamic acid side chain. In addition, one *tert*-butyl side chain PG most likely came off. All the other peaks (7.8 and 8.6 min) were analyzed, but failed to give a mass. The UV absorption of the reaction mixture in water/ACN (1:1) on RP-HPLC (Figure 43 C) has a lower intensity, but the retention times of the peaks are comparable with those of the reaction mixture in THF. The main peak at 14.3 min had a corresponding mass spectrum with only one molecular weight. The mass of the crude product, 4471.5 Da, was recorded by the presence of 4- to 3- fold charged

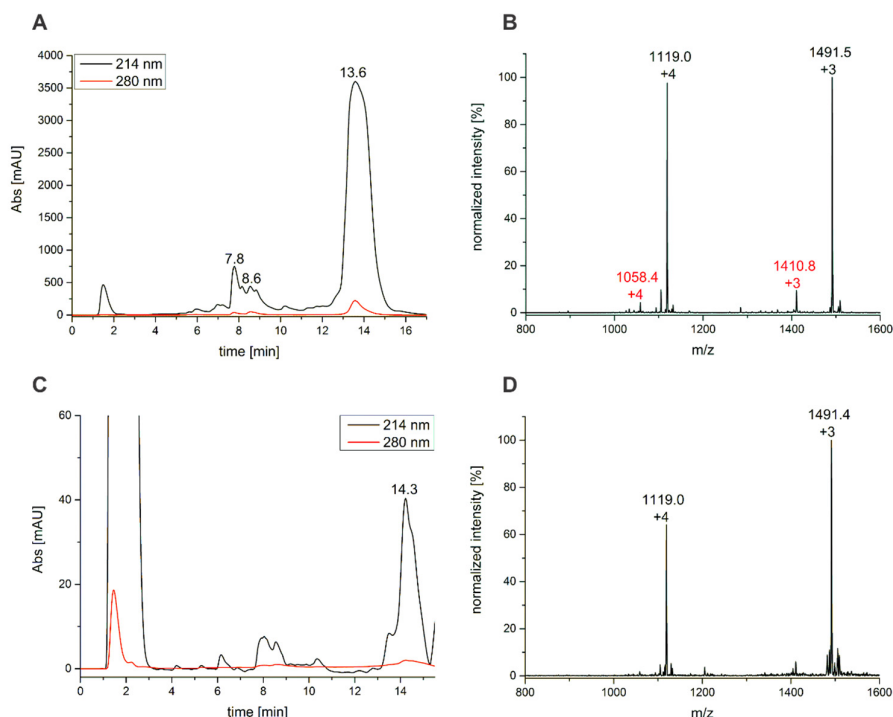


Figure 43: Characterization of the product of hydrazine cleavage reaction after 20h.

A: analytical C4 RP-HPLC chromatogram of crude cleavage product in 1M hydrazine/THF, linear gradient 5-65% ACN in 10 min. B: corresponding mass spectrum to the peak at 13.6 min, expected mass: 4549.3 Da, observed mass: 4471.5 Da $[M+Na]^+^{23+}$. C: analytical C4 RP-HPLC chromatogram of crude cleavage product in 50:50 mixture of water and ACN, linear gradient 5-65% ACN in 10 min. D: corresponding mass spectrum to the peak at 14.3 min expected mass: 4549.3 Da, observed mass: 4471.5 Da.

ion peaks (1119.0 and 1491.4 Da). Both mass spectra (Figure 43 B&D) are almost identical and probably correspond to side chain hydrazinolyated species. Since no clear result was obtained, the hydrazine cleavage solutions had to be evaporated and the resulting crude peptide had to be deprotected with a mixture of TFA/TIS/H₂O (95:2.5:2.5) in order to find whether mono- or dihydrazinolyated peptides was obtained. Furthermore, to confirm that hydrazine cleavage was quantitative, the resin also was treated with the same solution. After ether precipitation, no product could be detected.

In contrary, the crude peptides evaporated from the hydrazine cleavage solutions could be analyzed via LC-MS (Figure 44). The HPLC chromatogram of the crude product which has been evaporated from 1M hydrazine in THF, shows a broad peak as the major peak at 3.8 min with a slight shoulder at 3.5 min (Figure 44 A). The corresponding mass spectrum (Figure 44 B) shows the presence of two products. The dominant product tends to be the peptide dihydrazide recognized by the presence of four- to two-fold ion charged peaks (574.9, 766.1 and 1148.6 Da). The side product has a mass of 2321.7 Da, which corresponds well to the mass of desired peptide α -hydrazide (2282.4 Da) with a potassium adduct ion (+ 38 Da). Nevertheless, it was still unclear, if the monohydrazide peptide carries the hydrazide on its C-terminus or on a side chain. The HPLC chromatogram of fully deprotected peptide hydrazide generated with 5% hydrazine in water (Figure 44, C) shows two major peaks, at 3.8 min and 7.3 min. The mass spectrum that belongs to the peak at 7.3 min did not show any mass. However, the corresponding mass spectrum to the peak at 3.8 min shows two series of products (Figure 44 D). The main product is the peptide dihydrazide proven by the presence of 4- to 2-fold charged ion peaks (574.9, 766.2 and 1148.6 Da). The side product has a mass of 2392.0 Da, which corresponds to a TFA ester adduct on an acidic side chain. No peptide monohydrazide could be obtained. All attempts to optimize the reaction conditions failed.

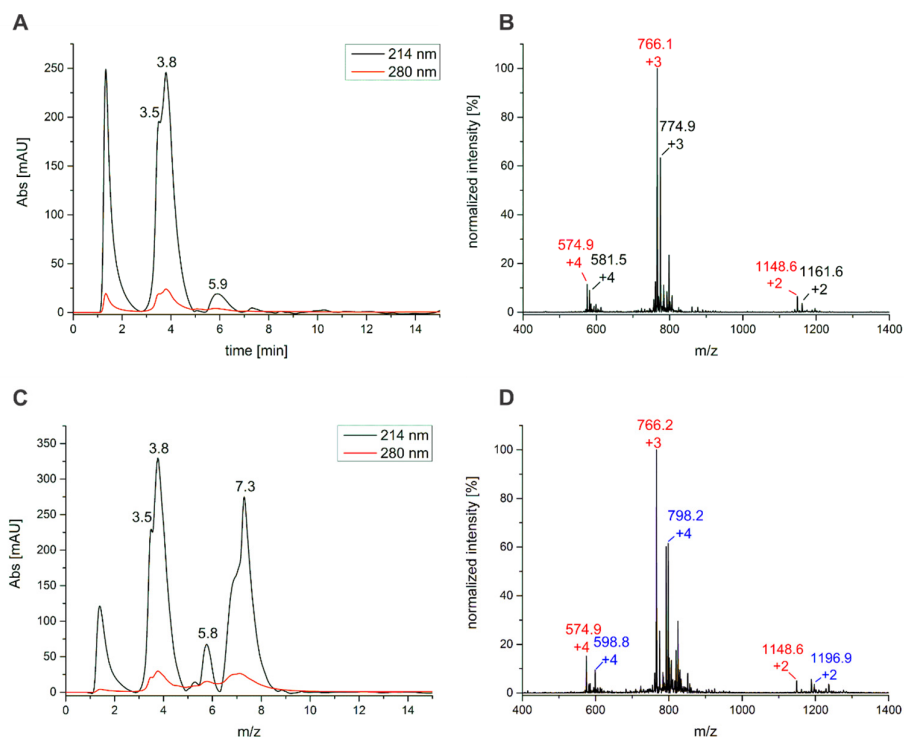


Figure 44: Characterization of deprotected PrP 214-231-NH₂ via LC-MS.

A: analytical C4 RP-HPLC chromatogram of crude product evaporated from 1M hydrazine/THF solution and treated with TFA/TIS/H₂O, linear gradient 5-65% ACN in 10 min. B: corresponding mass spectrum to the peak at 3.8 min, expected mass: 2282.4 Da, observed mass: 2295.3 Da. The black series may show the desired product with a potassium ion adduct (2321.8 Da). C: analytical C4 RP-HPLC chromatogram of crude product evaporated from 5% hydrazine in water and ACN and treated with TFA/TIS/H₂O, linear gradient 5-65% ACN in 10 min. D: corresponding mass spectrum to the peak at 3.8 min, expected mass: 2282.1 Da, observed mass: 2295.3 Da. The blue series shows a mass with additional 96 Da, which corresponds to a TFA ester adduct.

4.1.3.2 PrP 179-213-NH₂



Figure 45: PrP 179-213-NH₂

In order to generate PrP 179-213-NH₂ (Figure 45), the corresponding peptide has been synthesized using Fmoc/tBu chemistry and characterized via LC-MS (Figure 46). Two series of products has been identified, the desired product (expected mass: 4040.9 Da, observed mass: 4042.0 Da) and a side product with an additional mass of 57 Da, which corresponds well with a *tert*-butyl or *tert*-butylester adduct. This modification has been observed with other peptides several times however, it could always be eliminated after preparative

RP-HPLC. Because the strategy followed for the generation of PrP 214-231-NHNH₂ did not give rise to the desired product but to multiply hydrazinolyated products, milder conditions have been tried to generate PrP 179-213-NHNH₂. For this purpose, two cleavage solutions were prepared, 3% hydrazine in THF and 3% hydrazine in THF. Unfortunately, both cleavage reactions did not give rise to any product, which could be detected via LC-MS.

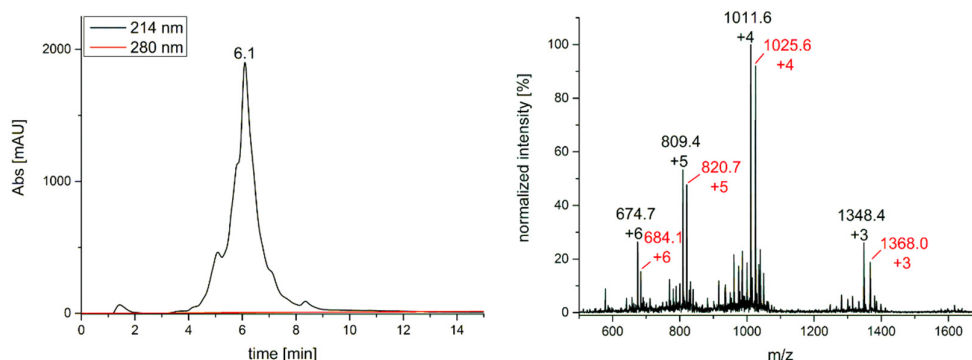


Figure 46: Characterization of crude PrP 179-213.

Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 179-213, linear gradient 5-65%B in 10 min. Right panel: corresponding mass spectrum to the peak at 6.1 min, expected mass: 4040.9 Da, observed mass: 674.7 [M+6H]⁶⁺, 809.4 [M+5H]⁵⁺, 1011.6 [M+4H]⁴⁺, 1348.4 [M+3H]³⁺. The red series shows the mass of a peptide TFA ester adduct with additional 96 Da.

In order to achieve better cleavage conditions, the strategy was changed. Former cleavage solutions with 5% v/v hydrazine in THF or water were used. The samples were treated as described in section 4.1.3.1 and analyzed via LC-MS. Unfortunately; the peptide observed could only be dissolved partially in different solvent mixtures and no elution peak for a peptide hydrazide could be detected. The cleaved crude product is a 35mer peptide with all the side chain protecting groups, which makes it very hydrophobic and hard to dissolve in common solvents used for RP-HPLC. Therefore, the cleavage mixtures were evaporated and the peptide hydrazide was cleaved under acidic conditions as described in section 4.1.3.1. At the same time, the peptide on resin was also cleaved under acidic conditions to compare yields. The unprotected peptide samples both from the supernatant of the hydrazine cleavage and from subsequent resin cleavage were analyzed via LC-MS (Figure 47). The HPLC chromatogram of both peptide samples showed one major peak at approx. 9.7 min. For both chromatograms,

mass spectra corresponded to the desired mass for a fully unprotected peptide monohydrazide (expected mass: 4054.9, observed mass: 4056.3 Da).

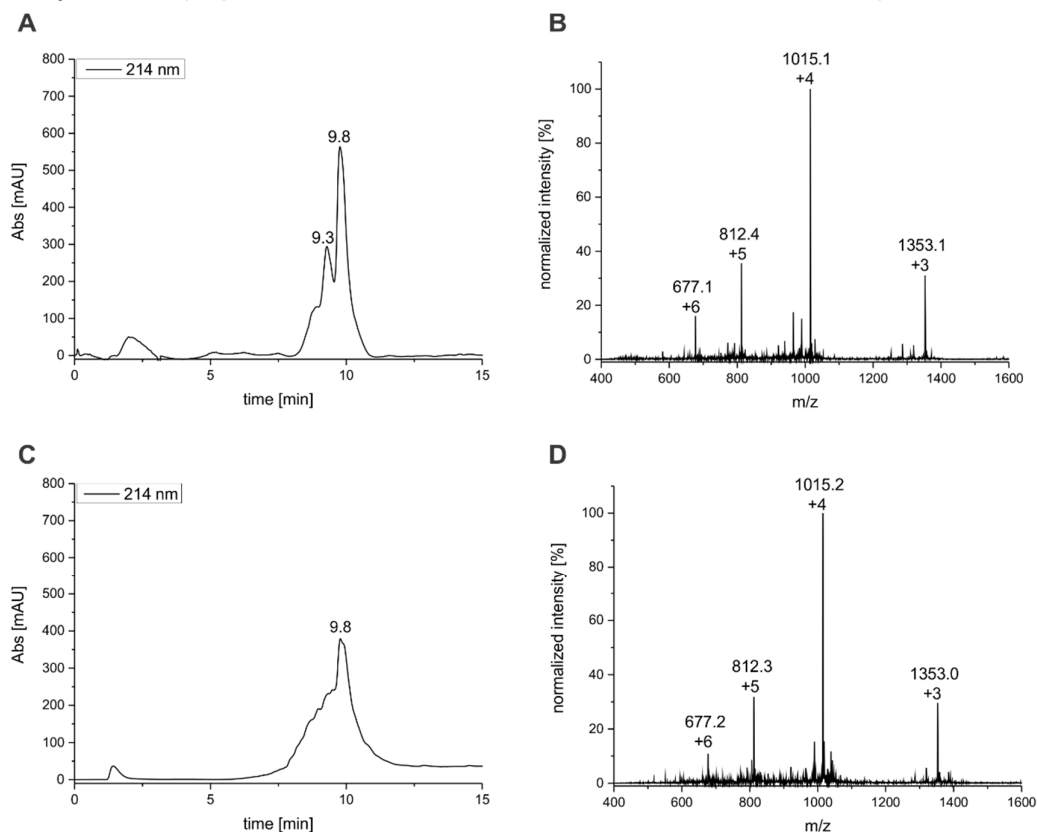


Figure 47: Characterization of fully deprotected crude PrP 179-213-NHH2 via LC-MS.

A: analytical C4 RP-HPLC of supernatant after TFA treatment, 5-65% ACN in 10 min. B: analytical C4 RP-HPLC of precipitated peptide after TFA treatment, 5-65% ACN in 10 min. C: corresponding mass spectrum to the peak at 9.7 min from A, expected mass: 4054.9, observed mass: 4056.8 Da, 677.2 [M+6H]⁶⁺, 812.3 [M+5H]⁵⁺, 1015.2 [M+4H]⁴⁺, 1353.0 [M+3H]³⁺. D: corresponding mass spectrum to the peak at 9.7 min from A, expected mass: 4054.9, observed mass: 4056.8 Da, 677.1 [M+6H]⁶⁺, 812.4 [M+5H]⁵⁺, 1015.1 [M+4H]⁴⁺, 1353.1 [M+3H]³⁺.

Nevertheless, this peptide also contains glutamic acid residues, which could undergo a hydrazinolysis. Moreover, the presence of peptide monohydrazide on the resin after hydrazine cleavage was unexpected. It can be speculated that either the peptide monohydrazide was cleaved from the resin but could not be completely dissolved in ACN/H₂O buffer due to its high hydrophobicity or a side chain ester group was modified with the hydrazine.

Taken together, hydrazine cleavage method works very well with smaller peptide fragments but is not completely suitable for generation of larger PrP peptide α -thioesters as well as with peptides carrying *tert*-butylesters on β -carboxy units.

Current efforts to develop suitable hydrazine cleavage conditions for larger and/or hydrophobic peptide segments are being made by our working group.

4.1.4 Synthesis of PrP peptides

To eliminate the effect of the GPI anchor on PrP conversion, the C-terminus of homogenously glycosylated PrP should not contain any GPI anchor analogues. Based on this, peptides comprising aa 178-231 should be synthesized (see also scheme 12). The aa at position 178 was substituted with β -mercapto aspartate, the positions 181 and/or 197 were equipped with monodisperse polymeric glycan mimics attached to Dpr. As already mentioned in section 1.5., branched and non-branched PEG chains asserted themselves as very suitable surrogates of glycans. In this section, the results of the chemical synthesis of PrP peptides harboring glycan mimics, more specific PEG₂₇, will be discussed.

4.1.4.1 PrP 178(β -mercapto-Asp)-181(PEG₂₇)-231

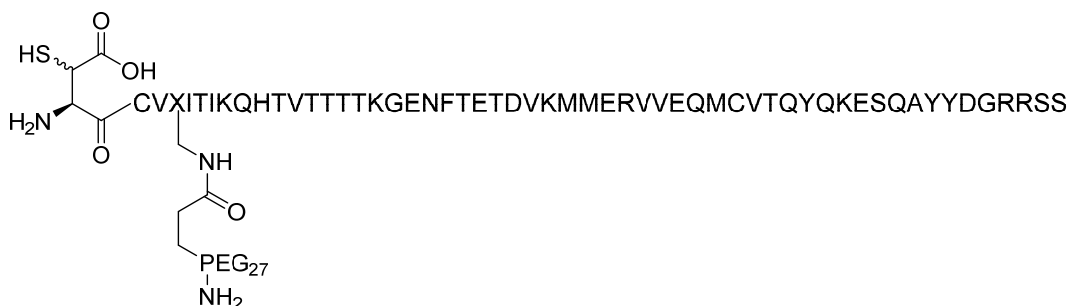


Figure 48: PrP 178(β -mercapto-Asp)-181(PEG₂₇)-231

Similar to SPPS of peptide α -thioesters described in section 4.1.2, PrP 178(β -mercapto-Asp)-181(PEG₂₇)-231 (Figure 48) has been prepared via automated Fmoc SPPS. A small amount of final product was cleaved from the solid support under acidic and reducing conditions utilizing a modified reagent K as described by Hackenberger and coworkers^[309]. The crude peptide was characterized via LC-MS (Figure 49). The HPLC chromatogram shows a broad peak at 8.6 min. In the corresponding mass spectrum two different mass series could be detected. The black series is the major product with the expected mass of 7717.4 Da with 6- to 10-fold charged ion peaks (1287.4, 1103.7, 965.9, 858.6, 772.8 and 702.6 Da) whilst the red series corresponds to a side product with a mass difference of +96

Da. As already discussed in section 4.1.2.3, the increase in mass (+96 Da) corresponds to a TFA ester adduct.

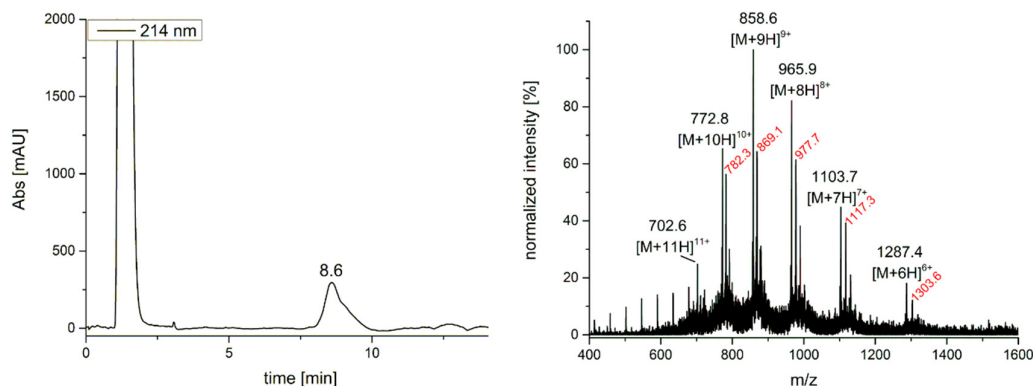


Figure 49: Characterization of crude PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231 via LC-MS. Left panel: analytical C4 RP-HPLC chromatogram of crude PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231, linear gradient 5-65% ACN in 10 min. Right panel: corresponding mass spectrum to the peak at 8.6 min, expected mass: 7718.8 Da, observed mass: 7718.2 Da, 702.6 [M+11H]¹¹⁺, 772.8 [M+10H]¹⁰⁺, 858.6 [M+9H]⁹⁺, 965.9 [M+8H]⁸⁺, 1103.7 [M+7H]⁷⁺, 1287.4 [M+6H]⁶⁺. The red mass series corresponds to a TFA ester adduct of the final product with a increase mass of 96 Da.

Upon purification via RP-HPLC, the final product was identified using analytical RP-HPLC and ESI-MS (Figure 50). One sharp peak at 23.9 min could be observed in the HPLC chromatogram. The corresponding ESI-MS spectrum shows the correct mass by from 11- to 5-fold charged ions (1544.9, 1287.2, 1103.3, 965.7,

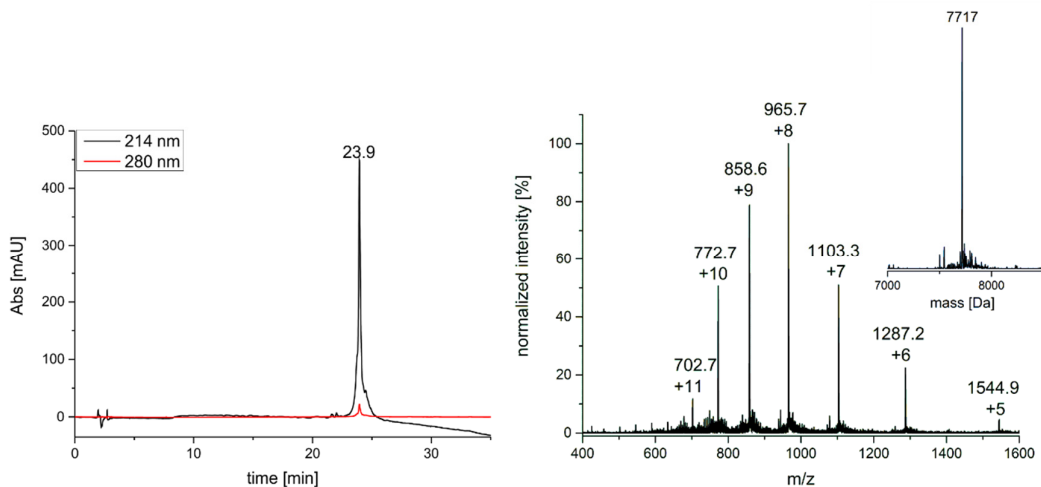


Figure 50: Characterization of purified PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231. Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231, linear gradient 5-65% ACN in 30 min. Right panel: ESI-MS spectrum of purified PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231, expected mass: 7718.8 Da, observed mass: 702.7 [M+11H]¹¹⁺, 772.7 [M+10H]¹⁰⁺, 858.6 [M+9H]⁹⁺, 965.7 [M+8H]⁸⁺, 1103.3 [M+7H]⁷⁺, 1287.2 [M+6H]⁶⁺, 1544.9 [M+5H]⁵⁺.

858.6, 772.7 and 702.7 Da). The final product could be obtained in a good yield and purity (22%, > 95%).

4.1.4.2 PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231

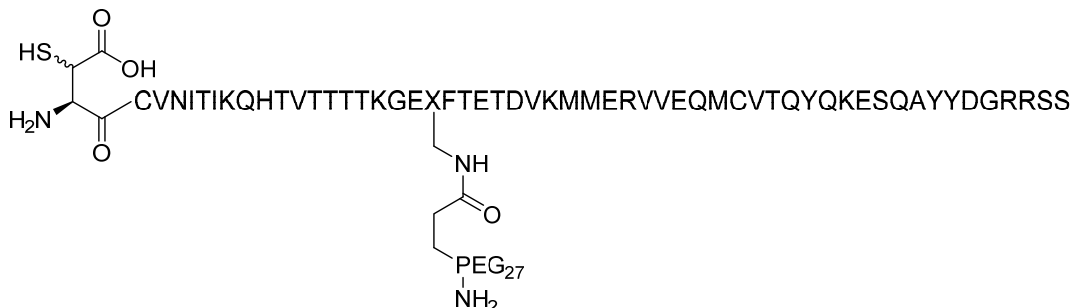


Figure 51: PrP 178(β -mercapto-Asp)-197(PEG₂₇)-231

The synthetic route was analogous to that of PrP 178(β -mercapto-Asp)-181(PEG₂₇)-231 (Figure 51). After the attachment of Fmoc-NH-PEG₂₇-OH on position 197, the peptide has been deprotected with piperidine in DMF to remove the Fmoc group on the PEG moiety and cleaved from the resin. Upon preparative RP-HPLC purification, fractions were pooled, lyophilized and characterized via analytical RP-HPLC and ESI-MS (Figure 52). The analytical RP-HPLC chromatogram has a very sharp peak at 24.1 min. The corresponding ESI-MS spectrum clearly showed the presence of the right product by the presence of from 11- to 5-fold multiple charged ions (772.7, 858.5, 965.6, 1103.5, 1287.1 and 1544.5). The product has been obtained in good yield and purity (22%, > 90%). In Figure 52, two small peaks at 22.3 and 22.5 min can be seen. These peaks were isolated and analyzed via ESI-MS. No mass spectrum could be detected. Nevertheless, for the calculation of the purity of the final product, these minor peaks were considered as impurities and were brought in the calculation.

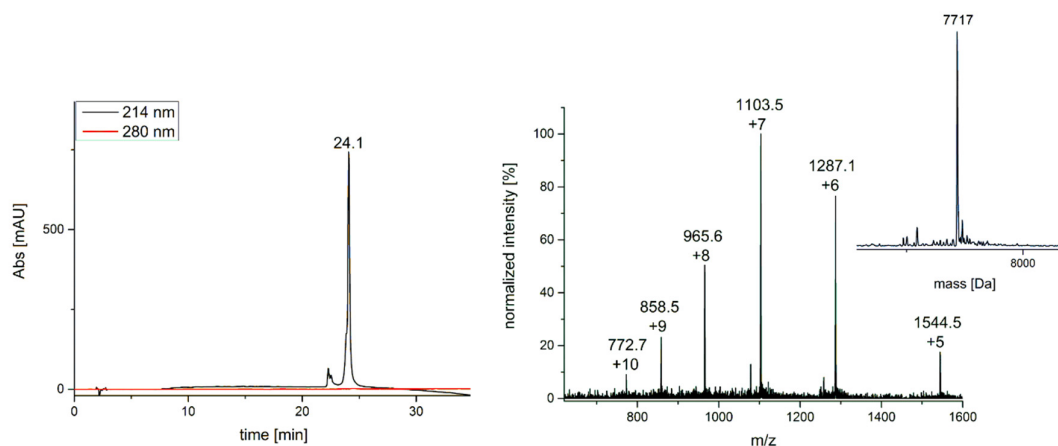


Figure 52: Characterization of purified PrP 178(β-mercapto-Asp)-197(PEG₂₇)-231.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β-mercapto-Asp)-197(PEG₂₇)-231, linear gradient 5-65% ACN in 30 min. Right panel: ESI-MS spectrum of purified PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231, expected mass: 7718.8 Da, observed mass: 772.7 [M+10H]¹⁰⁺, 858.5 [M+9H]⁹⁺, 965.6 [M+8H]⁸⁺, 1103.5 [M+7H]⁷⁺, 1287.1 [M+6H]⁶⁺, 1544.5 [M+5H]⁵⁺. The deconvoluted spectrum showed a mass of 7717 Da.

4.1.4.3 PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-231

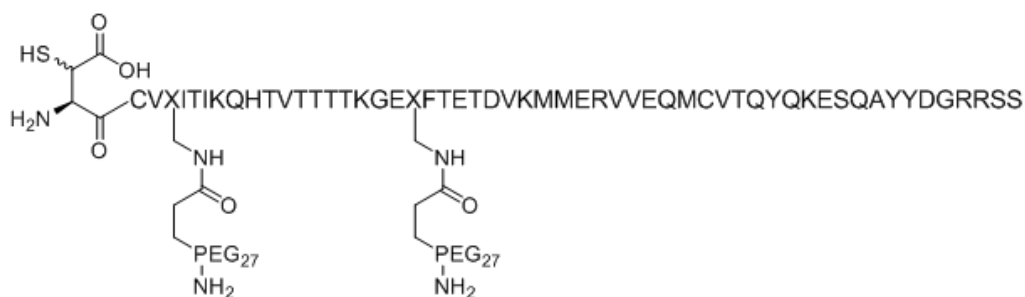


Figure 53: PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-231

The peptide (Figure 53) has been prepared similarly to both mono-PEGylated peptides. After preparative RP-HPLC purification, the peptide was characterized via analytical RP-HPLC and ESI-MS (Figure 54). The analytical RP-HPLC chromatogram shows one peak at 24.1 min with a shoulder at 23.9 min. The ESI-MS spectrum showed very clearly that the expected product was found (expected mass: 8995.3, observed mass: 8995) by the presence of 12- to 6-fold charged ion peaks (705.4, 818.7, 900.5, 1000.4, 1125.3, 1285.9 and 1500.1 Da). The product has been obtained in good yields and purity (15%, > 90%).

The presence of the shoulder can be explained by the racemic nature of β-mercapto aspartate. The peak was isolated and detected via ESI-MS giving rise to the desired mass of 7718 Da. In addition, two minor peaks at 22.1 and 22.4 min

were detected. These minor peaks did not give any mass spectrum. For the calculation of purity of the final product, the shoulder peak and the minor peaks were considered as impurities.

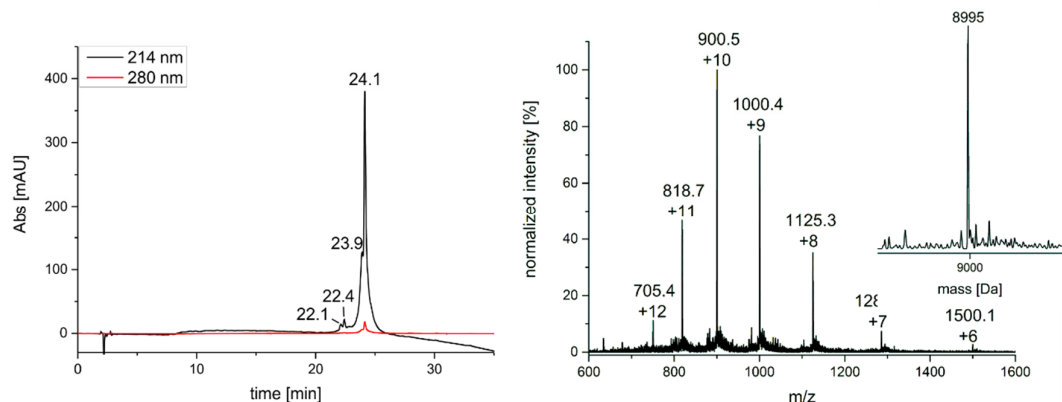


Figure 54: Characterization of purified PrP 178(β-mercapto-Asp)-181&197(PEG₂₇)-231.

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 178(β-mercapto-Asp) 181&197(PEG₂₇)-231, linear gradient 5-65% ACN in 30 min. Right panel: ESI-MS spectrum of purified PrP 178(β-mercapto-Asp)-181(PEG₂₇)-231, expected mass: 8995.3 Da, observed mass: 705.4 [M+12H]¹²⁺, 818.7 [M+11H]¹¹⁺, 900.5 [M+10H]¹⁰⁺, 1000.4 [M+9H]⁹⁺, 1125.3 [M+8H]⁸⁺, 1285.9 [M+7H]⁷⁺, 1500.1 [M+6H]⁶⁺. The deconvoluted spectrum showed a mass of 8995 Da.

4.1.5 Generation of GPI anchor analogues

In 2008, a synthetic GPI anchor with a cysteine modification was linked to a recombinant PrP α-thioester via EPL enabling the semisynthesis of PrP variants with homogenous GPI modifications [266]. Despite being an elegant method, the chemical synthesis of GPI anchor derivatives is limited and time consuming due to the complex nature of the compound. Hence, alternative and less complex methods had to be utilized for membrane anchoring of proteins.

In order to achieve membrane anchoring for homogeneously PEGylated PrP variants, two routes to obtain GPI anchor were envisioned. One route implies the recombinant expression of a GPI anchored protein in *S. cerevisiae* and has been described by Engelhard and coworkers [292]. In order to obtain the product, an ER signal sequence, Kre5, the murine full length PrP or eGFP, a hexa-His tag, a TEV protease site, FLAG tag and the GPI anchoring segment (17 aas) of Gas1p, a GPI-anchored yeast protein, as well as the GPI signal peptide of Gas1p has been cloned into a p425Cup1 plasmid (Figure 55). For our purposes, we used murine

full length PrP. This construct will be referred to as Kre5-mPrP-His₆-FLAG-Gas1p-GPI.

The second route is based on the synthesis of a peptidyl-GPI anchor surrogate. This lipidated peptide has been used previously established as a membrane anchor for PrP replacing the GPI anchor [265]. Both GPI anchor analogues carry a cysteine on their N-terminus for chemoselective ligation reactions. In this section, the results of the syntheses of both membrane anchors will be discussed in detail.

4.1.5.1 Recombinant expression of Kre5-mPrP-His₆-FLAG-Gas1p-GPI

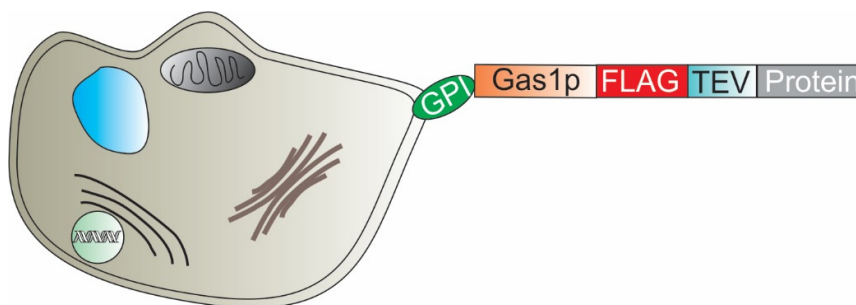


Figure 55: GPI anchored Gas1p fusion protein under physiological conditions.

Kre5 and a GPI anchoring signal peptide are cleaved after translation and the protein is expressed in fusion with His-Tag, TEV cleavage site, FLAG-Tag, Gas1p signal peptide and a GPI anchor. The fusion protein is attached to the yeast cell membrane via the GPI anchor.

The prototrophic selection medium SC was pre-cultured with a sample from a glycerol stock of YPH501 *S. cerevisia* cells transformed with Kre5-mPrP-His₆-FLAG-Gas1p-GPI and induced with copper(II)sulfate. As described in section 3.3.6, the construct was expressed in inclusion bodies. To solubilize the protein pellet after cell lysis, denaturing conditions were used. Upon solubilization, IMAC was utilized and fractions were analyzed via SDS-PAGE and western blot (WB) (Figure 56 A). As it can be seen in figure 56, the fusion protein could be detected as the only His-tagged protein in the elution fraction and only negligible amounts of POI was found in the flow through. The same trend has been observed by detection with an antiFLAG antibody (Figure 56 B). The elution fraction was then dialyzed from 8 M to 2 M urea containing buffer in three steps to enable the subsequent TEV cleavage reaction. In order to monitor the TEV cleavage reaction, samples after 24h of reaction time were blotted (Figure 56 C&E). The reaction mixture was centrifuged in order to separate precipitated protein from the reaction mixture. The protein band detected with antiHis should show a decrease in MW,

whilst the protein band detected with antiFLAG should show a decrease in band intensity. As expected, antiHis WB analysis showed a quantitative cleavage reaction (Figure 56 C). Additionally, antiFLAG detection showed ~ 80% proteolytic cleavage efficiency (Figure 56 E).

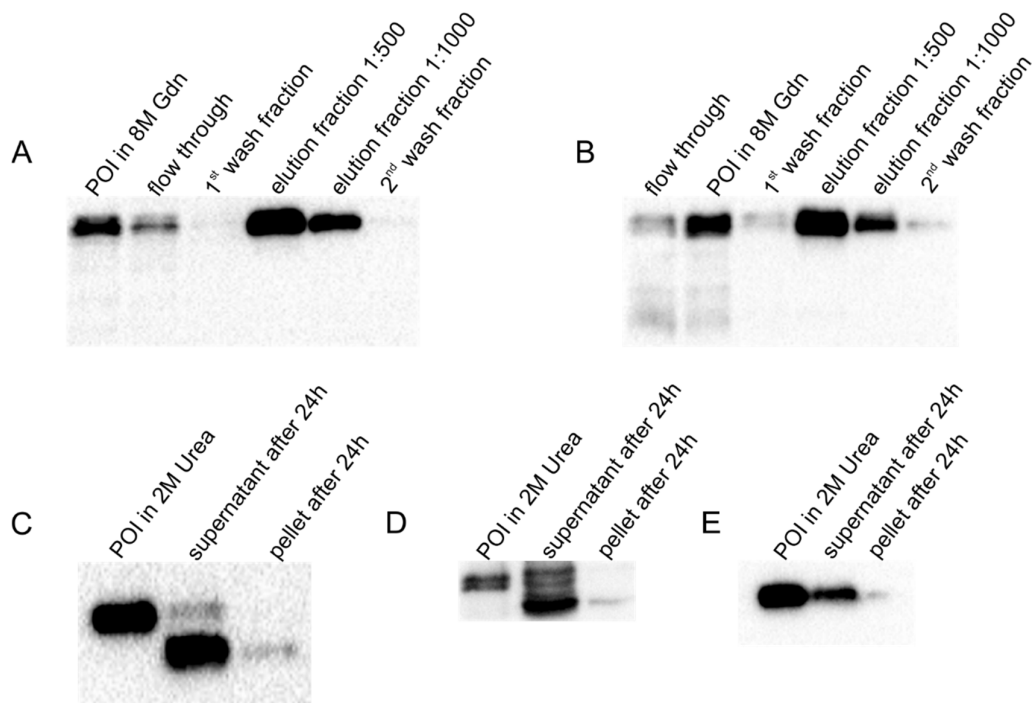
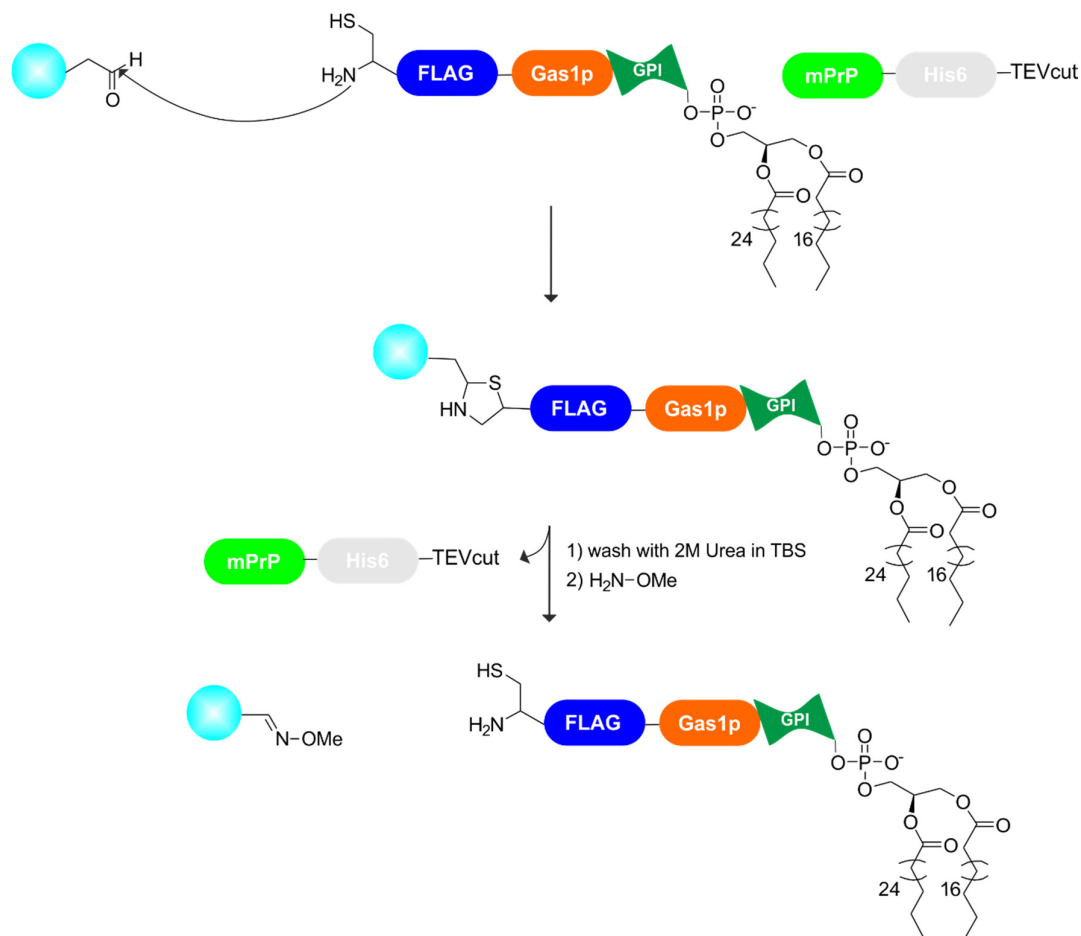


Figure 56: Western blot analysis of purification and TEV proteolysis reaction.

A: WB detection of IMAC purified POI with antiHis antibody, elution fraction was either diluted 1:500 or 1:1000. B: WB detection of IMAC purified POI with antiFLAG antibody, elution fraction was either diluted 1:500 or 1:1000. C: WB detection of TEV proteolysis reaction with antiHis antibody. D: WB detection of TEV proteolysis reaction with antiPrP 3F4 antibody. E: WB detection of TEV proteolysis reaction with antiFLAG antibody. Supernatant refers to the cleavage solution of TEV proteolysis reaction after 24 h. Pellet refers to centrifuged pellet from the cleavage solution after 24 h.

Finally, antiPrP WB analysis (Figure 56 D) showed similar results compared to that of antiHis detection suggesting that a cleavage reaction with good yields was achieved. Unfortunately, the SDS gel was not suitable to show the low molecular weight band at 4.8 kDa, which corresponds to the MW of the GPI-anchoring segment.

Schumacher *et al.* reported the successful generation of this GPI anchor analogue [292]. Nevertheless, there was no information on the amount of the isolated compound and no subsequent ligation reactions were done. Nevertheless, the product was successfully isolated and characterized. One isolation method described in this publication was the use of so-called cysteine covalent capture beads [311]. The method is based on the nucleophilic attack of the sulfhydryl group of cysteine on the highly electrophilic aldehyde carbon atom on the resin. In doing so, a thiazolidine is formed, which is stable in the pH range of 4.5-8. The



Scheme 15: Mechanism of cysteine covalent capture.

thiazolidine can be cleaved via competitive elution with *O*-methylhydroxylamine (Scheme 15). Unfortunately, the isolation procedure did not give the desired product. Here, no product could be obtained or identified via analytical C4 and C18 RP-HPLC or ESI-MS. This is most likely due to the hydrophobic nature of the GPI anchor. It tends to interact very strongly with hydrophobic sidechains of the stationary phase.

4.1.5.2 SPPS of lipidated GPI anchor mimic

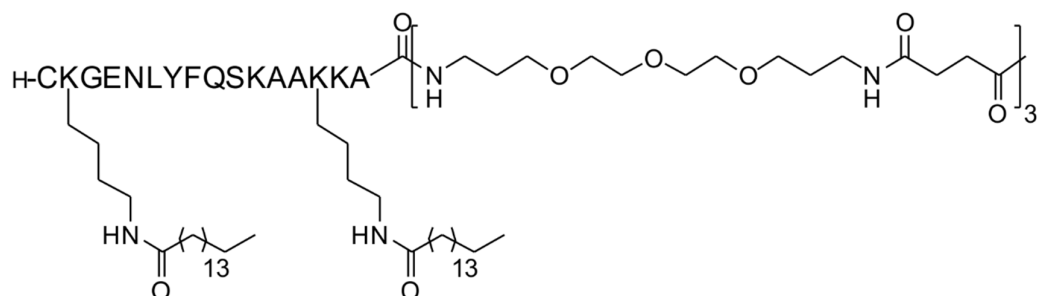


Figure 57: lipidated GPI anchor mimic

In 2007, Becker and coworkers showed that the dipalmitoylated peptide shown in Figure 57 enables the attachment of murine PrP variants to artificial and cellular membranes as well as lipid bilayers ^[265]. The researchers used EPL to attach the GPI anchor surrogate into PrP. The C-terminal PEG unit enhanced the solubility of the peptide in aqueous buffers. Recently, we described the incorporation of human PrP variants equipped with this membrane anchor (MA) consisting of more complex lipids that mimic neuronal membranes ^[143]. In virtue of these findings, the MA has been chosen for further *in vitro* membrane attachment studies.

As outlined in section 3.4.8, the method described in Olschewski *et al.* has been modified and the peptide has been synthesized in 0.2 mmol scale ^[266]. Upon completion of the synthesis, MA has been analyzed via analytical RP-HPLC and ESI-MS (Figure 58).

The RP-HPLC chromatogram showed a sharp peak at 34.1 min. The corresponding ESI-MS spectrum proved that the right peptide (expected mass: 3168.1 Da, observed mass: 3169.5 Da) could be observed in a good purity (> 95%) and yield (47.6%) by the presence of 3-to 6-fold charged ion peaks (1057.5, 793.4, 635.0 and 529.4).

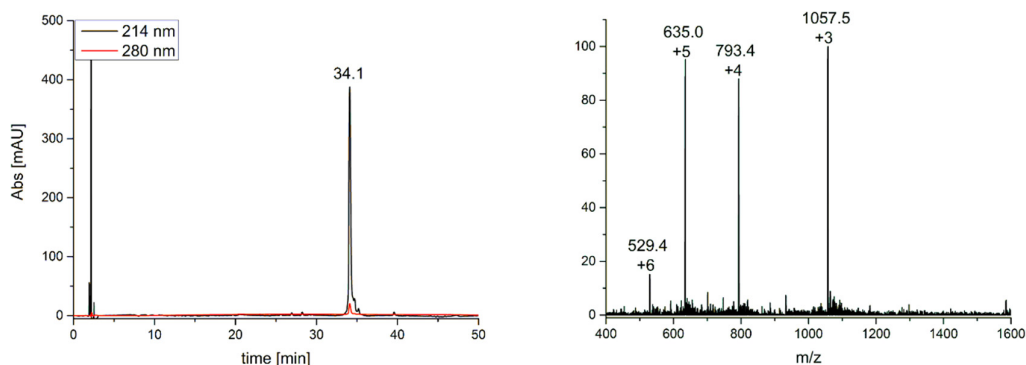


Figure 58: Characterization of MA peptide via analytical RP-HPLC and ESI-MS.

Left panel: analytical C4 RP-HPLC chromatogram of purified MA peptide, linear gradient 5-95%B in 45 min. Right panel: ESI-MS spectrum of purified D5, expected mass: 3168.1, observed mass: 3169.5 Da, 529.4 [M+11H]⁶⁺, 635.0 [M+5H]⁵⁺, 793.4 [M+4H]⁴⁺, 1057.5 [M+3H]³⁺.

4.1.6 Expressed Protein Ligation

4.1.6.1 PrP 23-178(β -mercapto-Asp)-213-SEA^{off}

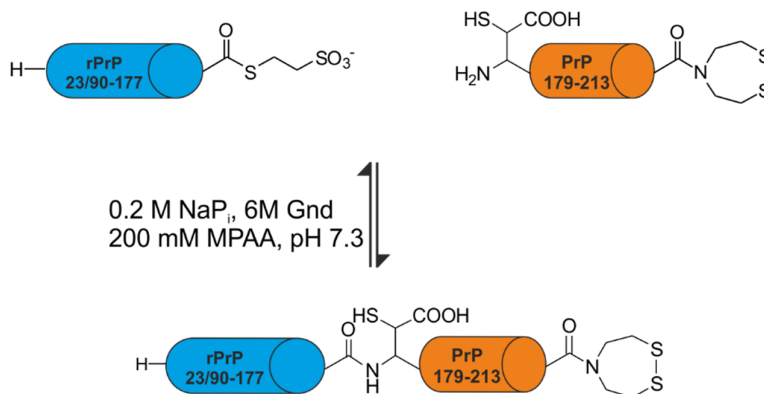


Figure 59: EPL of recombinant PrP thioesters to SEA peptides

For the introduction of glycan mimics and GPI anchor into full length PrP (aa 23-231), recombinantly generated PrP 23-177-MESNA thioester and latent peptide α -thioesters carrying the monodisperse, linear polymer modifications should be linked to each other. In order to do this, an EPL strategy was utilized (Figure 59). It was important that the SEA handle on the C-terminus of ligation product should remain intact after the first ligation reaction because the second ligation reaction with the peptide segment harboring the GPI anchor analogue will occur subsequently. Additionally, the SEA handle should not be converted into SEA^{on}; otherwise, a self-ligation and cyclization reaction might occur. Therefore, no additional reducing agent was used during the ligation reactions. As described in

section 3.5 in detail, a guanidine based aqueous buffer was used for the ligation reaction in the presence of excess thiol catalyst, MPAA. Since MPAA does not convert SEA^{off} into SEA^{on} as described by Melnyk and coworkers, it was a suitable catalyst [299].

One eq. (2 mM) of recombinant thioester were reacted with one eq. (2 mM) of 178(β -mercapto-Asp)-213- SEA^{off} in an aqueous buffer under denaturing conditions. The reaction was complete ($> 90\%$ conversion) after 3 h (Figure 60). Interestingly, all starting material was consumed (Figure 60, A, A* and B) but the UV absorption of ligation product (Figure 60, C) was lower than expected. This might be due to the fact, that the sample was highly diluted before applying it to the column. In addition, a part of the ligation product might be eluted with the MPAA

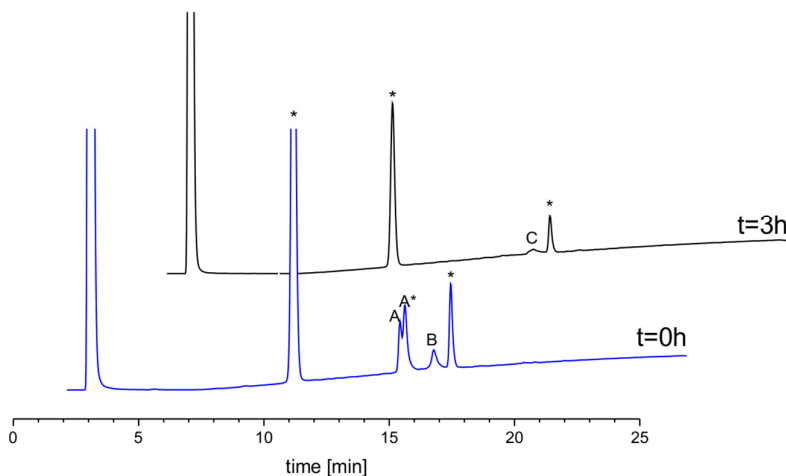


Figure 60: Ligation of PrP 23-177-MESNA thioester with 178(β -mercapto-Asp)-213-SEA

A: PrP 23-177 α -thioester, A*: PrP 23-177 α -MPAA thioester B: PrP 178(β -mercapto-Asp)- 213-SEA, C: ligation product. The reaction was monitored during 3 h of reaction time. *: MPAA. Chromatograms were recorded on a RP-C4 column with a linear gradient 5-65% ACN in 20 min.

peak (Figure 60, *).

To prove, that the desired product was obtained, the ligation mixture was purified using semi preparative RP-HPLC. Purified product was analyzed via analytical RP-HPLC and ESI-MS (Figure 61). The analytical RP-HPLC chromatogram showed two sharp peaks at retention times 23.3 and 23.7 min. The racemic nature of β -mercapto-Asp might be an explanation for the occurrence of two peaks, since SEA handle was not reduced. The ESI-MS spectrum showed one species of product by the presence of 32- to 19-fold charged ion peaks (expected mass: 21,097.2 Da,

observed mass: 21,097 Da). The product was obtained in a very good purity (> 95%) and yield (78 %).

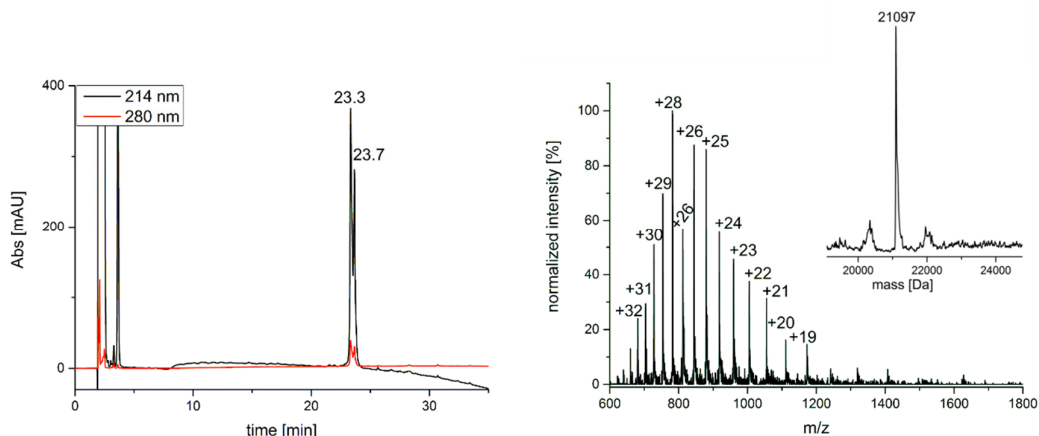


Figure 61: Characterization of purified PrP 23-178(β -mercapto-Asp)-213-SEA

Left panel: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-213-SEA, linear gradient 5-65%B ACN in 30 min, Right panel: ESI-MS spectrum and deconvoluted mass of purified PrP 23-178(β -mercapto-Asp)- 213-SEA, expected mass: 21,097.2 Da, observed mass: 21,097 Da thirty-two to nineteen fold multiple charged ion peaks were detected.

4.1.6.2 PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}

The ligation reaction was carried out under similar conditions as described in section 4.1.6.1. Two eq. of peptidyl-SEA (4 mM) and one eq. PrP 23-177-MESNA thioester (2mM) was reacted over a course of 2 h. The reaction progress was monitored via analytical RP-HPLC (Figure 62).

The UV trace at 214 nm showed that the desired product (Figure 62 peak C) is formed over the course of two hours, whereas the starting materials, recombinant protein thioester (Figure 62, A) and PrP 178(β -mercapto-Asp)-181PEG₂₇-213-SEA (Figure 62, B) have almost been fully consumed. Nevertheless, after 3 h still 25% of A could be detected. This peak has been analyzed and identified as the C-terminally hydrolyzed recombinant PrP thioester and could therefore not react. The occurrence of two separate peaks for the ligation product is most likely due to the reduction of SEA^{off} $\rightarrow$ SEA^{on}.

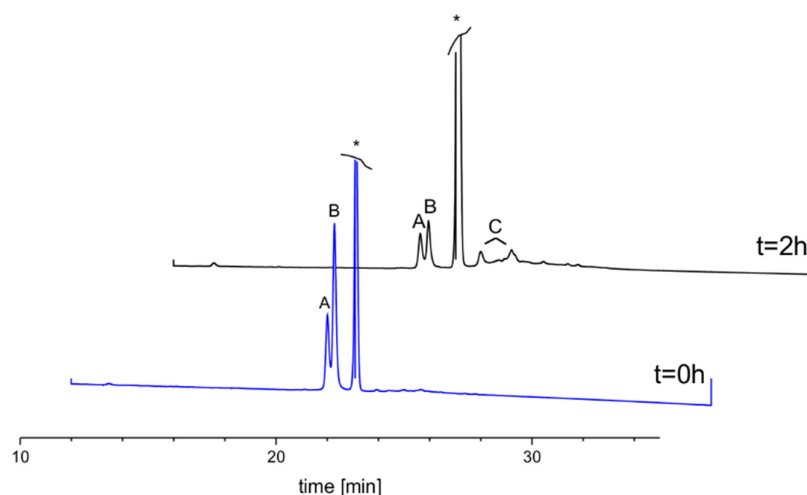


Figure 62: Ligation of PrP 23-177-MESNA thioester with 178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}.

A: PrP 23-177 α -thioester, B: PrP 178(β -mercapto-Asp)-181PEG₂₇-213-SEA, C: ligation product. The reaction was monitored during 2h of reaction time. *: MPAA. Chromatograms were recorded on a C4 column with a linear gradient 5-65% ACN in 30 min.

The crude product C was purified via semi preparative RP-HPLC, pooled and lyophilized. The final product has been analyzed via analytical RP-HPLC and ESI-MS (Figure 63). The RP-HPLC chromatogram (Figure 63 A) shows clearly one sharp peak at 21.9 min with a good purity (> 95%) and three small peaks. These peaks were isolated and analyzed via ESI-MS. No mass was observed. Therefore, these peaks are considered not as impurities of the sample.

The ESI-MS spectrum of 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA has a high background noise (Figure 63 B). Nevertheless, the desired product could be obtained and characterized once it is deconvoluted (Figure 63 C). The expected mass is 22377.2 Da, whereas the observed mass was 22374 Da. The overall yield of PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA was 20.1% and the peptide has been obtained in good purity (~ 95%).

Interestingly, only one sharp peak at 21.9 min could be characterized as the desired product. We hypothesized, that not only the balance between SEA^{off} $\rightarrow$ SEA^{on} was important but also the conversion of amide into the thioester under acidic pH as described by Melnyk and coworkers. The thioester isoform is more favorable at higher pH values. Moreover, we obtained different elution behavior for

different SEA peptides by using different buffer solutions (data not shown). Still, no clear trend could be determined.

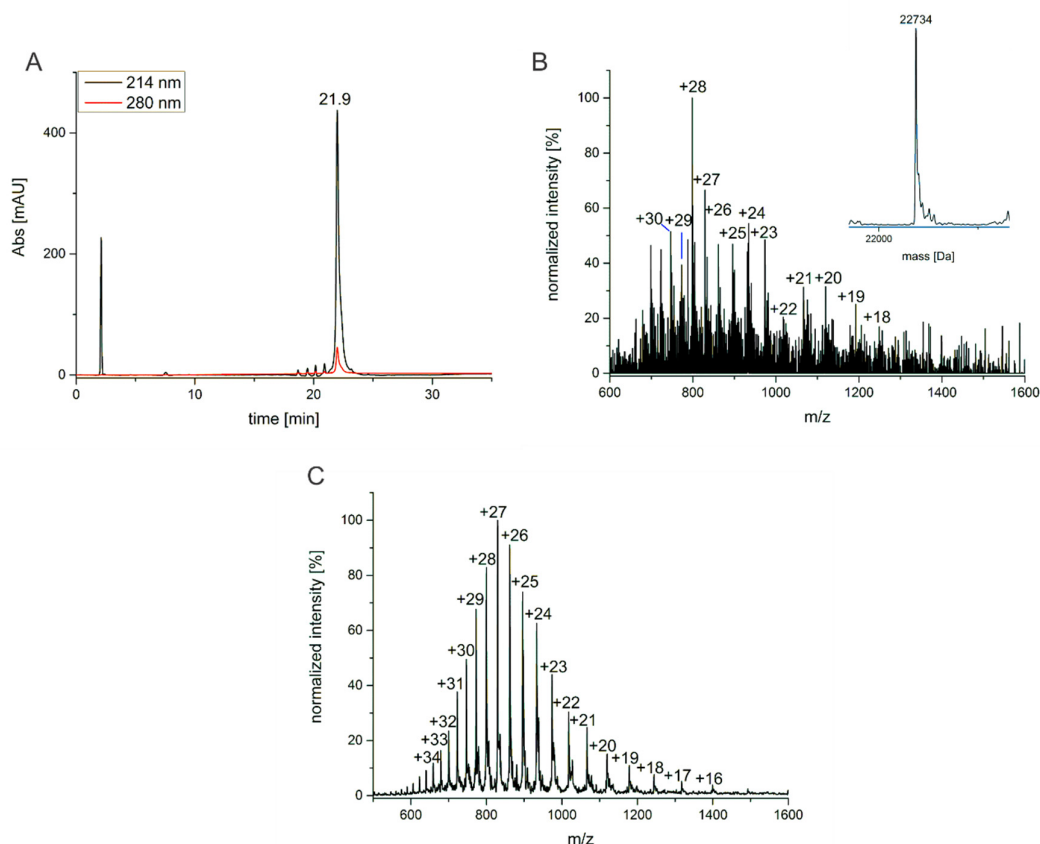


Figure 63: Characterization of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}
 A: analytical C18 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}, linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}, expected mass: 22377.2 Da, observed mass: 22374 Da, 30- to 18-fold multiple charged ion peaks were detected. C: back calculated MS spectrum and deconvoluted mass of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-213-SEA^{off}.

4.1.6.3 PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA^{off}

PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA has been obtained analogously to the procedure described in section 4.1.5.1. The reaction progress was monitored via analytical RP-HPLC (Figure 64). Recombinant protein α -thioester (Figure 64, A) and SEA^{off} peptide (Figure 64, B) were fully consumed after 2 h, and the crude product (Figure 64, C) was purified via preparative RP-HPLC after the ligation reaction was finished.

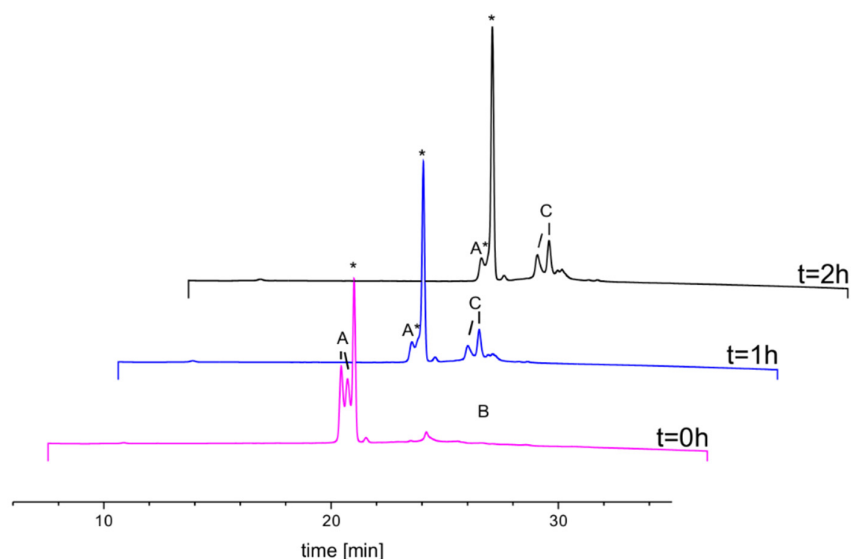


Figure 64: Ligation of PrP 23-177-MESNA thioester with 178(β-mercapto-Asp)-197PEG₂₇-213-SEA^{off}.

A: PrP 23-177 α-thioester, B: PrP 178(β-mercapto-Asp)-197PEG₂₇-213-SEA, C: ligation product. The reaction was monitored during 2h of reaction time. *: MPAA. Chromatograms were recorded on a C4 column with a linear gradient 5-65% ACN in 30 min.

The purified product has been characterized via analytical RP-HPLC and ESI-MS (Figure 65). The RP-HPLC chromatogram showed one peak at 24.1 min (Figure 65 A) and the ESI-MS spectrum corresponded to the right mass by the presence of 18- to 32-fold charged ion peaks (Figure 65 B). The deconvoluted mass and the back calculation of the spectrum showed that the right product was observed (expected mass: 22377.2 Da, observed mass: 22386.0 Da). The peptide was obtained with a good overall yield (~22%) and purity (> 90%).

Even though, two peaks for the right product were detected during reaction monitoring, the purified product was eluted as one peak. MPAA, even after partial precipitation for reaction monitoring, tends to coelute with PrP 23-178(β-mercapto-Asp)-197PEG₂₇-213-SEA. Moreover, since no reducing agent was present, products containing MPAA disulfides result in an increase in elution time. During RP-HPLC purification, these adducts could be eliminated by the use of catalytical amounts of reducing agents (e.g. TCEP) to reduce the disulfide bond between MPAA and cysteine residues. The conversion of SEA^{off} → SEA^{on} was not disturbing since no cyclization of the product can occur due to the lack of N-terminal Cys or Ser residues.

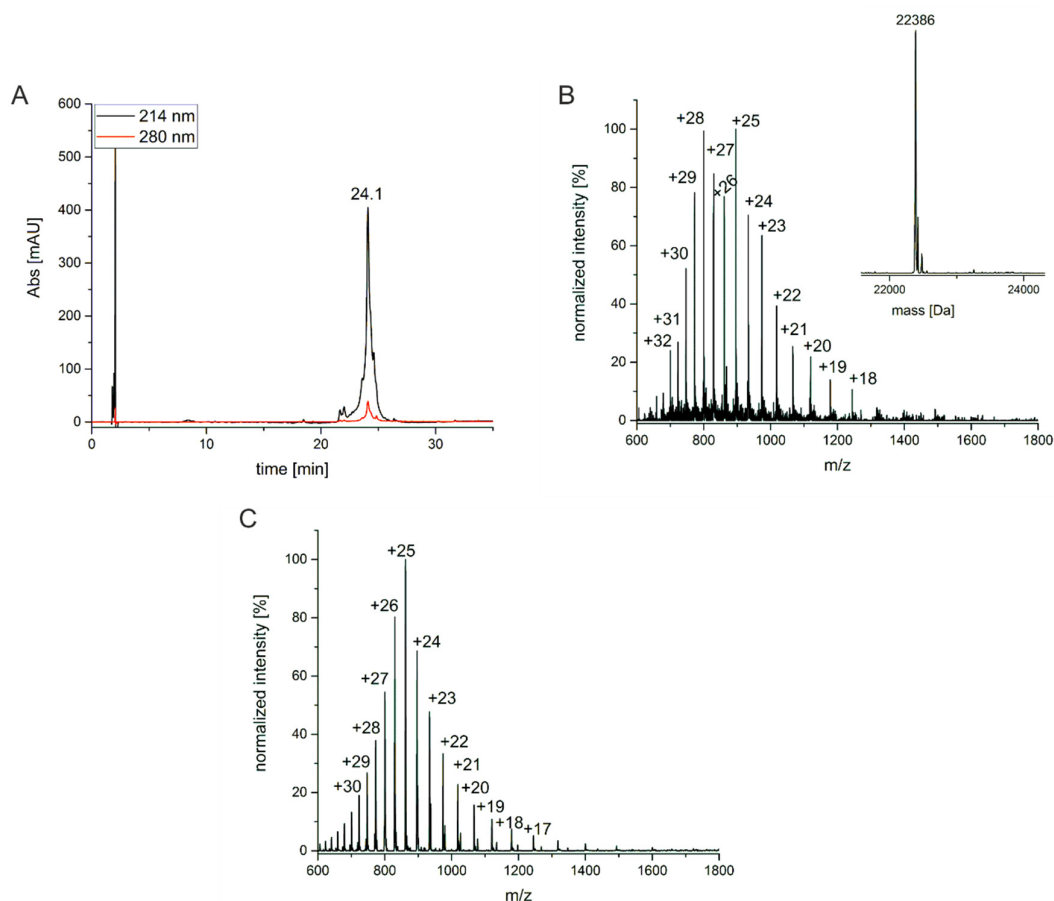


Figure 65: Characterization of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA, linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS and deconvoluted mass spectrum of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA, expected mass: 22377.2 Da, observed mass: 22386 Da, C: back calculated MS spectrum of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-213-SEA.

4.1.6.4 PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-213-SEA^{off}

PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-213-SEA has been generated analogously to the ligation method described in sections 4.1.6.1 and 4.1.6.2. The progress of the reaction has been monitored via analytical RP-HPLC (Figure 66). Surprisingly, a high content of product was formed after 2 min of reaction time (Figure 66, C). Further investigations clarified that the desired product was eluted at the same retention time as MPAA. To eliminate coelution of MPAA, a prolonged gradient was used during RP-HPLC purification (5-65% B in 60 min), which resulted in separation of the desired product and MPAA. The purified ligation product was analyzed via analytical RP-HPLC and ESI-MS (Figure 67).

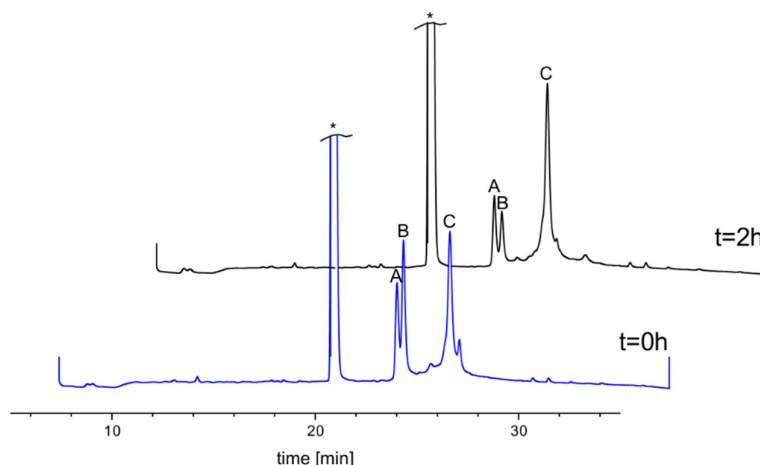


Figure 66: Ligation of PrP 23-177-MESNA thioester with 178(β-mercapto-Asp)-181&197 PEG₂₇-213-SEA^{off}.

A: PrP 23-177 α-thioester, B: PrP 178(β-mercapto-Asp)-181&197PEG₂₇-213-SEA, C: ligation product+MPAA. The reaction was monitored during 2h of reaction time. *: MPAA. Chromatograms were recorded on a C4 column with a linear gradient 5-65% ACN in 30 min.

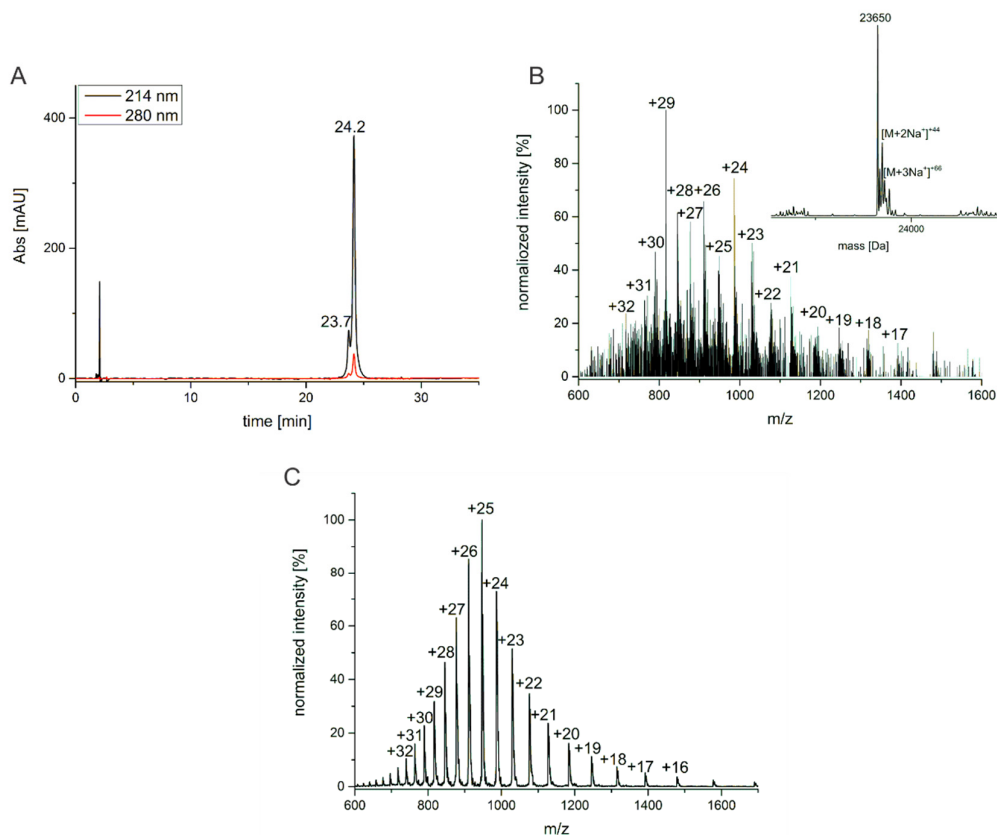


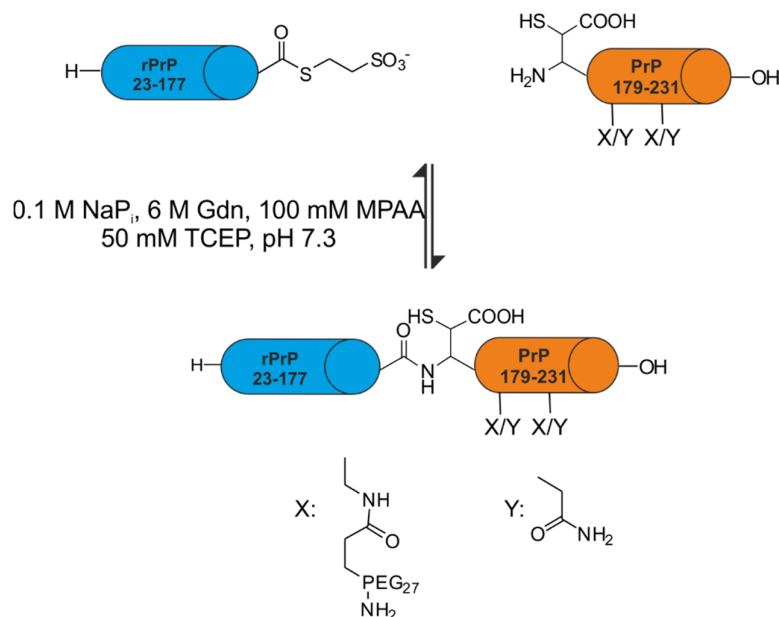
Figure 67: Characterization of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇-213 SEA^{off}.

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇-213-SEA, linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum and deconvoluted mass of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇-213 SEA, expected mass: 23654.2 Da, observed mass: 23650 Da, C: back calculated MS spectrum of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇-213-SEA.

The analytical RP-HPLC chromatogram showed one major peak at 24.2 min (Figure 67 A). The ESI-MS spectrum showed one major mass species even though the spectrum has a relative noisy background (Figure 67 B). The deconvoluted spectrum and the back calculation clarified that the desired product was observed by the detection of 32- to 17-fold charged ion peaks (Figure 67, C, expected mass: 23652.2 Da, observed mass: 23650, 23672 $[M+Na]^{23+}$, 23694 $[M+2Na]^{46+}$). The ligation product was obtained in a moderate yield ($\sim 10.7\%$) but very good purity ($>95\%$).

The presence of a minor peak at 23.7 min (Figure 67 A) could be explained by the presence of a peptide epimer due to the racemic nature of β -mercapto-aspartate, since the SEA handle on the peptide was not reduced and the ESI-MS spectrum showed one major mass species.

4.1.6.5 PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231



Scheme 16: EPL of recombinant PrP thioesters to peptide SEA-thioesters. Positions 181 and 197 are either both or only one position is PEGylated.

For the chemoselective ligation of recombinant PrP α -thioesters to PEGylated PrP peptides with N-terminal β -mercapto-Asp, a similar procedure to that described in 4.1.6.2 has been utilized (Scheme 16). 1.2 mM of protein thioester and 1 mM of PEGylated peptide were reacted within two hours to give quantitative yields. The reaction was monitored via analytical RP-HPLC (Figure 68).

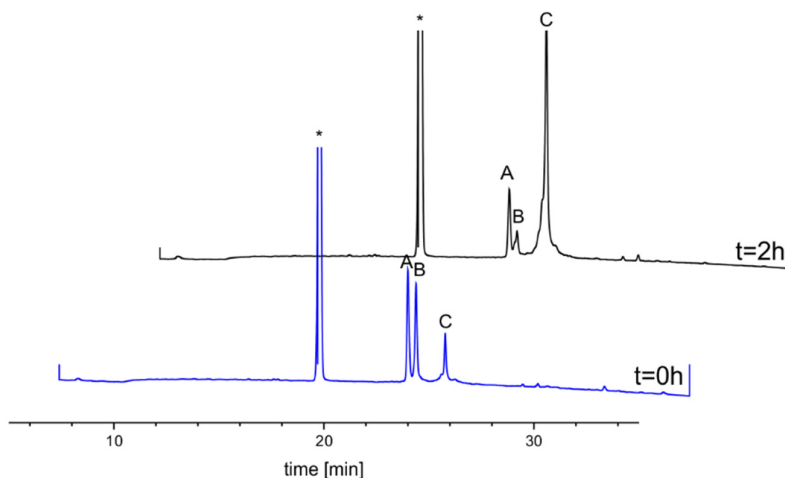


Figure 68: Ligation of PrP 23-177-MESNA thioester with 178(β -mercapto-Asp)-181-PEG₂₇ 231.

A: PrP 23-177 α -thioester, B: PrP 178(β -mercapto-Asp)-181PEG₂₇-231, C: ligation product+MPAA. The reaction was monitored during 2 h of reaction time. *: MPAA. Chromatograms were recorded on a C4 column with a linear gradient 5-65% ACN in 30 min.

The RP-HPLC chromatograms showed that the amount of product (Figure 68, C) increases dramatically within two hours while the amount of starting materials, protein thioester and PrP peptide (Figure 68, A and B respectively), decreases. The conversion yield based on the integrated peak area was calculated as $\sim 92\%$. The crude peptide was identified via LC-MS, purified using semi preparative RP-HPLC and pooled. The purified product was analyzed via analytical RP-HPLC and LC-MS (Figure 69). The analytical RP-HPLC chromatogram (Figure 69 A) shows clearly one sharp peak at 23.5 min highlighting that only one product was obtained. The corresponding mass spectrum to the peak at 23.5 min shows the presence of the right product by the detection of 32- to 14-fold charged ion peaks (Figure 69 B, calculated mass: 24509.4, observed mass: 24508 Da). In addition, the back calculated mass spectrum (Figure 69 C) and the deconvoluted mass spectrum highlighted that the right product was obtained. SDS-PAGE was applied as an alternative analysis method (Figure 69 D). The ligation product (lane 2) was compared to the PrP thioester (lane 1) and a single band between 30 kDa and 21 kDa was obtained. The overall yield of PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231 was 45.8%.

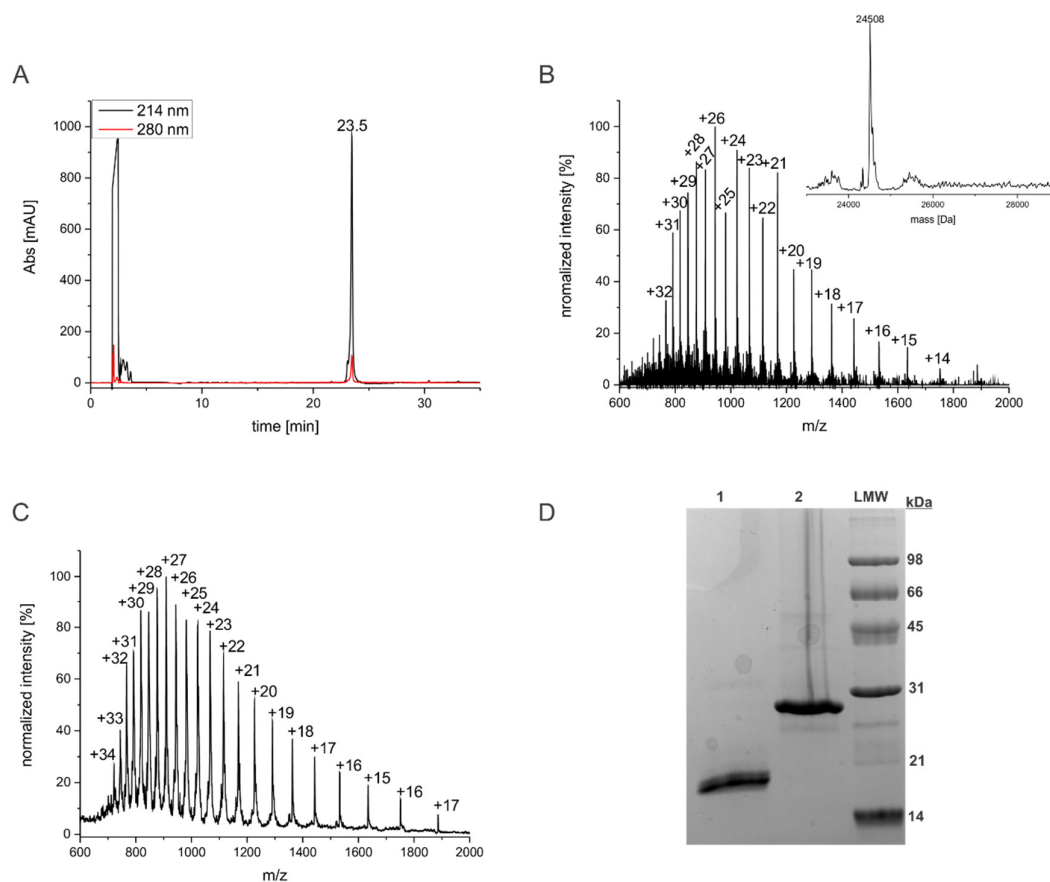


Figure 69: Characterization of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231, linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231, linear gradient C: corresponding mass spectrum to the peak at 9.9 min of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231, expected mass: 24509.4 Da, observed mass: 24508 Da, D: back calculated MS spectrum and deconvoluted mass of purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231. E: SDS-PAGE analysis of purified 23-178(β -mercapto-Asp)-181PEG₂₇-231, lane 1: PrP 23-177-MESNA thioester, lane 2: purified PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231, lane 3: LMW.

4.1.6.6 PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231

Similar to the synthesis of PrP 23-178(β -mercapto-Asp)-181PEG₂₇-231, 1 mM of recombinant protein thioester and 2 mM of 178(β -mercapto-Asp)-197PEG₂₇-231 peptide were mixed in a guanidine containing aqueous buffer (pH 7.2) and reacted for 3 h. The progress of the ligation was monitored via analytical RP-HPLC (Figure 70). Already at beginning of the reaction a small portion of the ligation product (Figure 70, C) was formed. After three hours, the reaction was finished (> 90% ligation product relative to the integrated peak area) and the crude product was purified via semi preparative RP-HPLC, pooled and lyophilized.

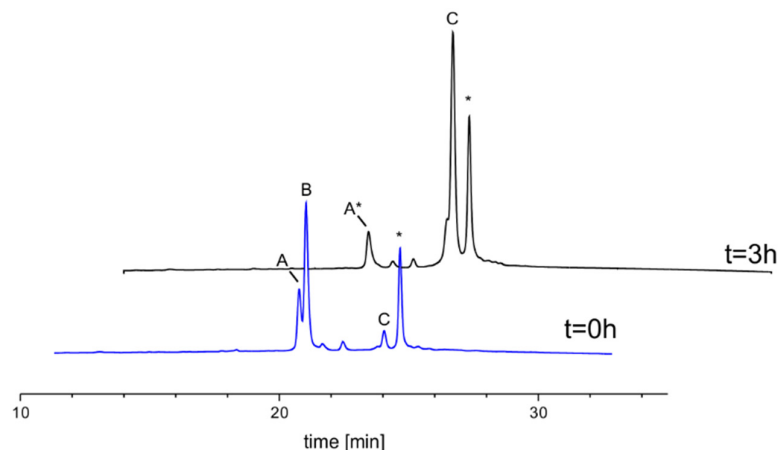


Figure 70: Ligation of PrP 23-177-MESNA thioester with 178(β -mercapto-Asp)-197-PEG₂₇-231.

A: PrP 23-177 α -thioester, B: PrP 178(β -mercapto-Asp)-197PEG₂₇-231, C: ligation product. The reaction was monitored during 3h of reaction time. *: MPAA, A*: hydrolyzed PrP 23-177-OH. Chromatograms were recorded on a C4 column with a linear gradient 5-65% ACN in 30 min.

The purified ligation product was identified using analytical RP-HPLC, ESI-MS and SDS-PAGE (Figure 71). The analytical RP-HPLC chromatogram (Figure 71 A) featured three peaks with following retention times, 22.3, 22.5 and as the major peak 24.1. All peaks were analyzed and only the peak at 24.1 min gave a mass spectrum. Additionally, an ESI-MS spectrum of purified product was recorded (Figure 71 B). The mass spectrum and the deconvolution (Figure 71 C) shows clearly that the right product was obtained (calculated mass: 24509.4 Da, observed mass: 24512 Da) by the detection of 34- to 19-fold charged ion peaks. SDS-PAGE analysis of the purified ligation product (Figure 71, D, lane 2) compared to PrP thioester (Figure 71 D, lane 1) was an additional proof for the presence of the right product with a band between 31 and 21 kDa and a very faint band between 45 and 66 kDa (probably dimerized PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231, MW ~ 49 kDa). The product has been observed in good yields (44.3%) and purity (> 95%).

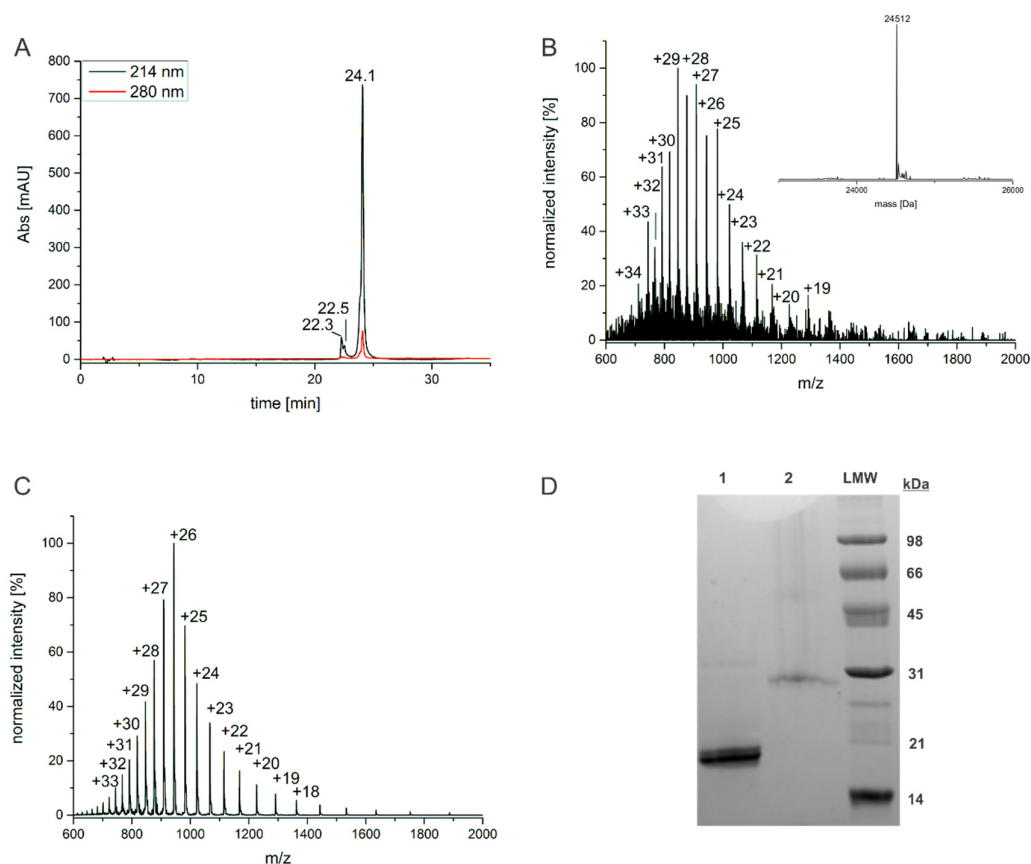


Figure 71: Characterization of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231, linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231, expected mass: 24509.4 Da, observed mass: 24512 Da, multiple charged ion peaks were detected. C: back calculated MS spectrum and deconvoluted mass of purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231. D: SDS-PAGE analysis of purified 23-178(β -mercapto-Asp)-197PEG₂₇-231, lane 1: PrP 23-177 MESNA thioester, lane 2: purified PrP 23-178(β -mercapto-Asp)-197PEG₂₇-231, lane 3: LMW.

4.1.6.7 PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-231

The chemoselective ligation reaction was carried out as described in section 4.1.6.6 with a slight excess of PrP 178(β -mercapto-Asp)-181&197PEG₂₇-231 (2 mM, 2eq.). The reaction progress was monitored via analytical RP-HPLC (Figure 72). Already in the first minutes of ligation, the product C was formed (ligation yield at $t=0$ h, 23.4%). After 3 h the protein thioester was fully consumed (only PrP 23-177-OH, hydrolyzed protein thioester was left) and the amount of B was decreased into 50%, which gave rise to a quantitative conversion of PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-231 (> 95% conversion yield).

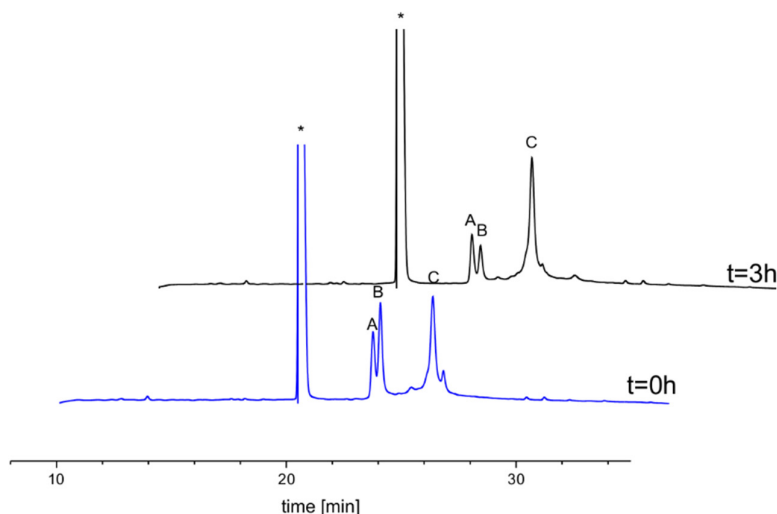


Figure 72: Ligation of PrP 23-177-MESNA thioester with 178(β -mercapto-Asp)-181&197PEG₂₇-231.

A: PrP 23-177 α -thioester and PrP 23-177-OH, B: PrP 178(β -mercapto-Asp)-181&197PEG₂₇-231, C: ligation product. The reaction was monitored during 3h of reaction time. *: MPAA. Chromatograms were recorded on a C4 column with a linear gradient of 5-65% ACN in 30 min.

The crude product was purified via semi preparative RP-HPLC, pooled and lyophilized. Purified PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-231 was analyzed via analytical RP-HPLC, ESI-MS and SDS-PAGE (Figure 73).

The analytical RP-HPLC chromatogram showed a peak at 22.8 min with a slight shoulder (Figure 73 A), and the ESI-MS spectrum highlighted one mass series by the presence of 33- to 18-fold charged ion peaks (Figure 73 B, expected mass: 25786.6 Da, observed mass: 25787 Da). Deconvoluted mass and back calculated spectrum (Figure 73 C) were in good agreement with the ESI-MS data. SDS-PAGE analysis of purified 23-178(β -mercapto-Asp)-181&197PEG₂₇-231 showed two bands, one band appeared to be the desired product (Figure 73 D, lane 2, intense band) with an apparent molecular weight below 31 kDa. The other one (Figure 73 D, lane 2, faint band) had approximately the two-fold more molecular weight (between 66 and 45 kDa) than the right product suggesting that it is the dimerized PrP 23-178(β -mercapto-Asp)-181&197PEG₂₇-231. The final product was obtained in a good yield after RP-HPLC purification (40.8%).

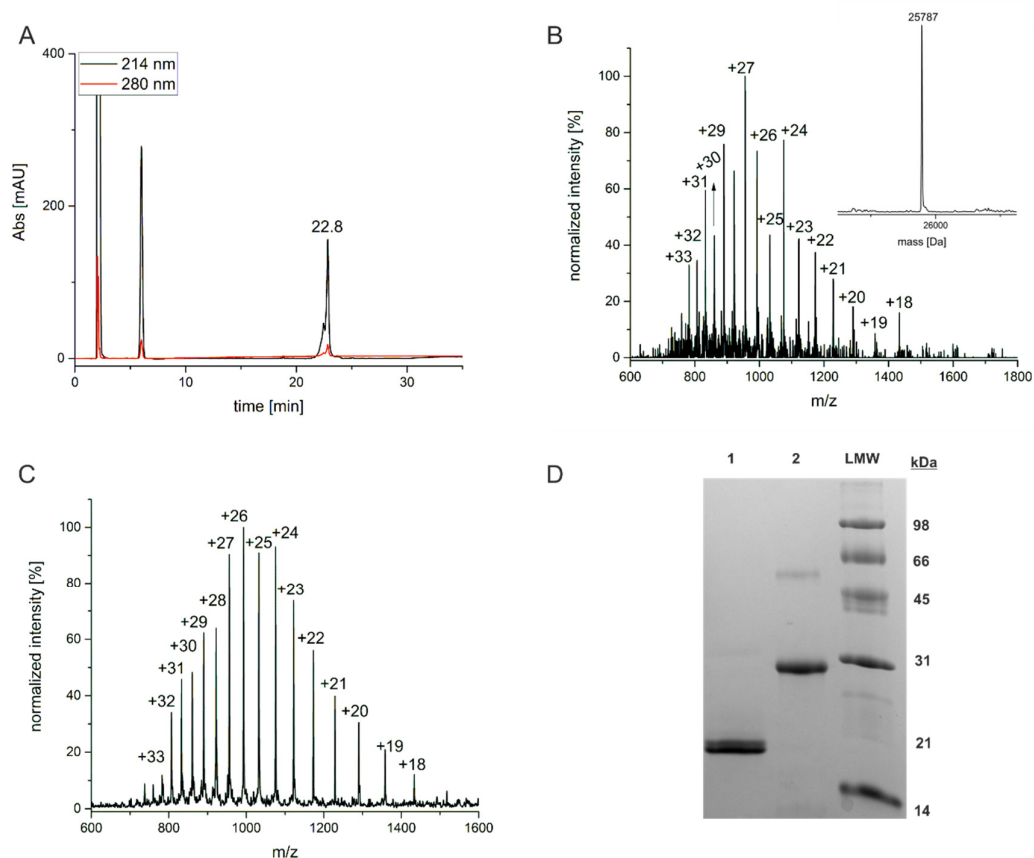
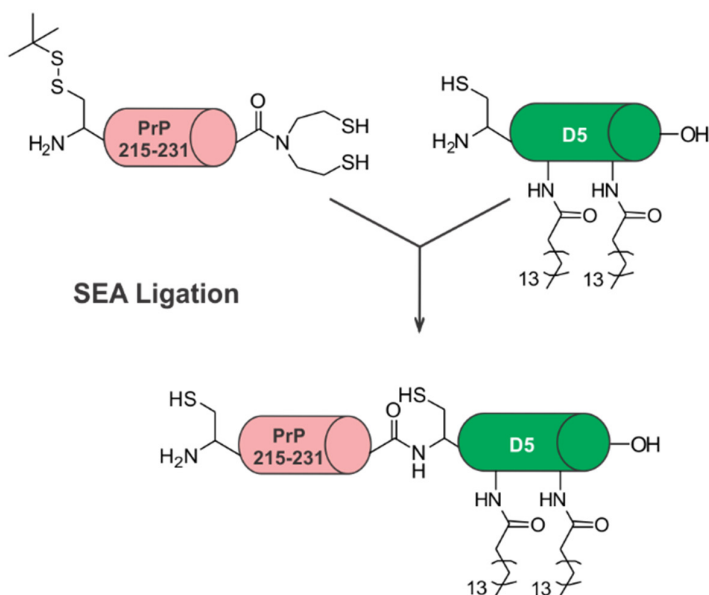


Figure 73: Characterization of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇₋₂₃₁

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇₋₂₃₁ in denaturing buffer (6M Gnd, pH 4.7), linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇₋₂₃₁, expected mass: 25786.6 Da, observed mass: 25787 Da, multiple charged ion peaks were detected. C: back calculated spectrum and deconvoluted mass of purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇₋₂₃₁. D: SDS-PAGE analysis of purified 23-178(β-mercapto-Asp)-197PEG₂₇₋₂₃₁, lane 1: PrP 23-177-MESNA thioester, lane 2: purified PrP 23-178(β-mercapto-Asp)-181&197PEG₂₇₋₂₃₁, lane 3: LMW.

4.1.6.8 PrP 214-231-MA

Membrane attachment of PrP is considered as a critical event on prion formation as outlined in several parts of this thesis. As described in section 4.1.5.2, Becker and coworkers reported the synthesis of a peptidic GPI anchor mimetic. This peptide has been synthesized and used for chemoselective ligation reactions. Furthermore, *in vitro* membrane attachment of homogenously PEGylated PrP variants with a GPI-anchoring unit will be carried out. For our purposes, an NCL strategy has been developed (Scheme 17).



Scheme 17: Ligation of PrP 214-231-SEA^{on} to membrane anchor peptide

Due to the low solubility of the MA peptide, the ligation reaction was carried out in the presence of 6 M Gnd-HCl and PrP 214Cys(*StBu*)-231-SEA^{on} was used in excess (2 eq.). The reaction progress was monitored via LC-MS (Figure 74, upper panel). The ligation product (Figure 74, upper panel, C) is formed already after 5 h, however the reaction progress was too slow (no completion after 36 h) and the yield was very low (< 3%, ~ 0.3 mg). For this ligation reaction, 20 mM SEA^{on} peptide and 10 mM MA peptide was used. Subsequent 214-231-MA ligations were done in smaller scales not giving rise to desired product. The only product was hydrolyzed peptide thioester.

Based on this, we postulated that higher concentrations of peptide (> 10 mM) and peptide thioester (> 20 mM) are necessary for this SEA ligation. The small amount of peptide, which has been isolated, was characterized via ESI-MS (Figure 74, lower panel). The correct mass could be found (expected mass: 5419.6, observed mass: 5420.9) by the presence of 8- to 3-fold charged ion peaks (678.8, 775.5, 904.3, 1085.2, 1356.6 and 1808.0 Da).

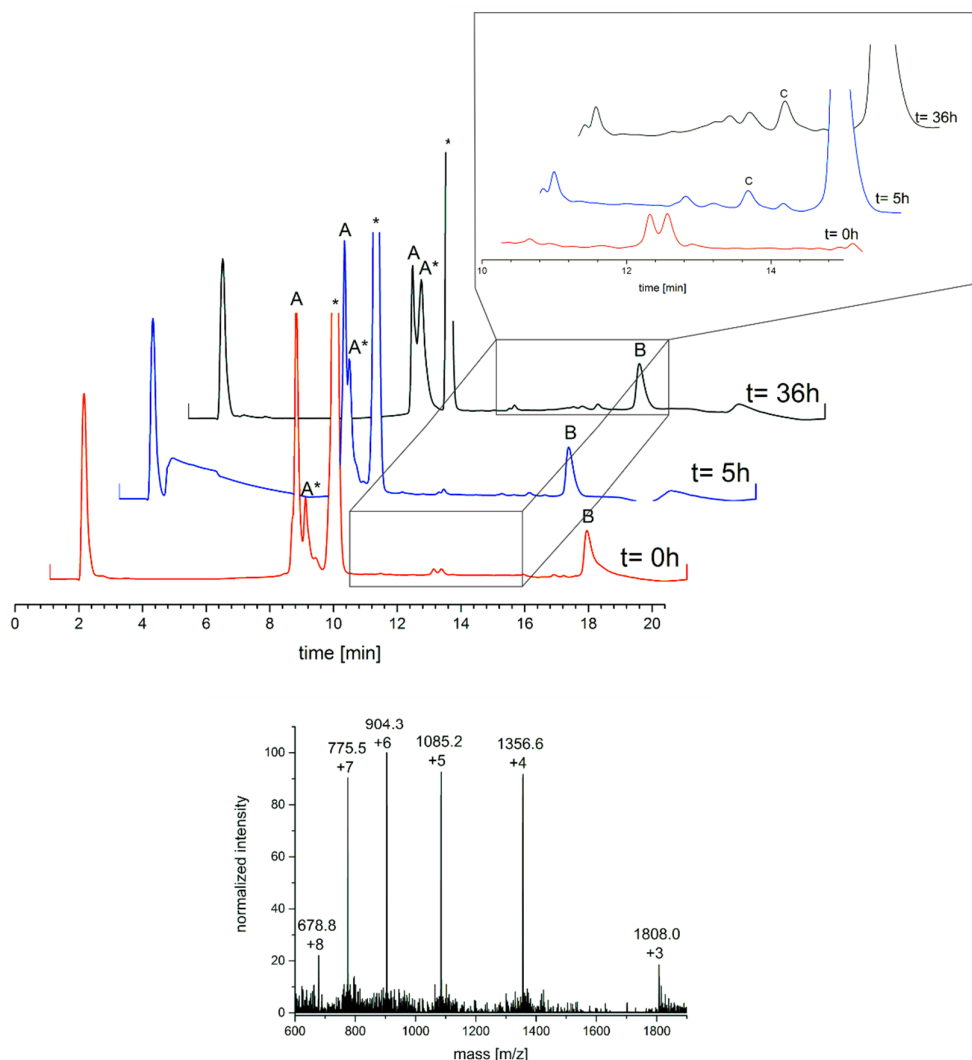


Figure 74: Ligation of PrP 214(*StBut*)Cys-231-SEA^{on} with lipidated D5 peptide

A: PrP 214(*StBut*)Cys-231-SEA^{on}, B: D5 peptide, C: ligation product. The reaction was monitored during 36 h of reaction time. *: MPAA. Chromatograms were recorded on a C4 (t=0 h) and C18 (t= 5 h and 36 h) column with a linear gradient 5-65% ACN in 10 min. Upper right: t= 10-15 min zoomed in.

4.1.7 Desulfurization of modified FL-PrP derivatives

The need of cysteine at the ligation site has been considered as a major limitation of NCL reactions. As outlined in section 1.5.2.1, researchers have synthesized and successfully introduced cysteine surrogates at the ligation site via SPPS. One of the synthetic cysteine surrogates is β -mercapto-Asp, which can be selectively desulfurized in the presence of other cysteine residues. In this section, results on selective desulfurization of PEGylated and non-PEGylated β -mercapto-Asp containing PrP derivatives will be discussed.

4.1.7.1 PrP 23-181PEG₂₇-231

In order to desulfurize the β -mercapto moiety of the β -mercapto-Asp in 23-178(β -mercapto-Asp)-181PEG₂₇-231 selectively, the protocol described in section 3.4.7 was utilized. The PrP variant was dissolved in the desulfurization buffer with excess DTT. Under rigorous shaking at 66°C, the reaction progress was monitored via LC-MS. The crude product was purified via semi preparative RP-HPLC and characterized through LC-MS and analytical RP-HPLC (Figure 75). Selectively desulfurized PrP 23-181PEG₂₇-231 was obtained in a good yield (51.2% after purification) and a good purity (~ 90% relative to the integrated peak area). Nevertheless, some issues had to be addressed. Firstly, the analytical RP-HPLC run of the purified product did not give a clean UV trace (Figure 75 A). In other studies in our group, using a long PEG chain, even covalently attached to a peptide, resulted in elution of impurities from C4 and C18 analytical columns. Secondly, the ESI-MS spectrum had a very high background-to-noise ratio (Figure 75 B). Fortunately, it was possible to deconvolute the mass spectrum (Figure 75 C) and characterize the protein as the main product by the presence of 33- to 18-fold charged ion peaks (expected mass: 24477.6, observed mass: 24476 Da). In addition, SDS-PAGE analysis showed a single band between 31 and 21 kDa (Figure 75 D, lane 2), which corresponded well with the findings mentioned above. No dimerized PrP 23-181PEG₂₇-231 was detected in the mass spectrum or on native PAGE. This may be an indirect proof of disulfide formation between Cys 179 and Cys214.

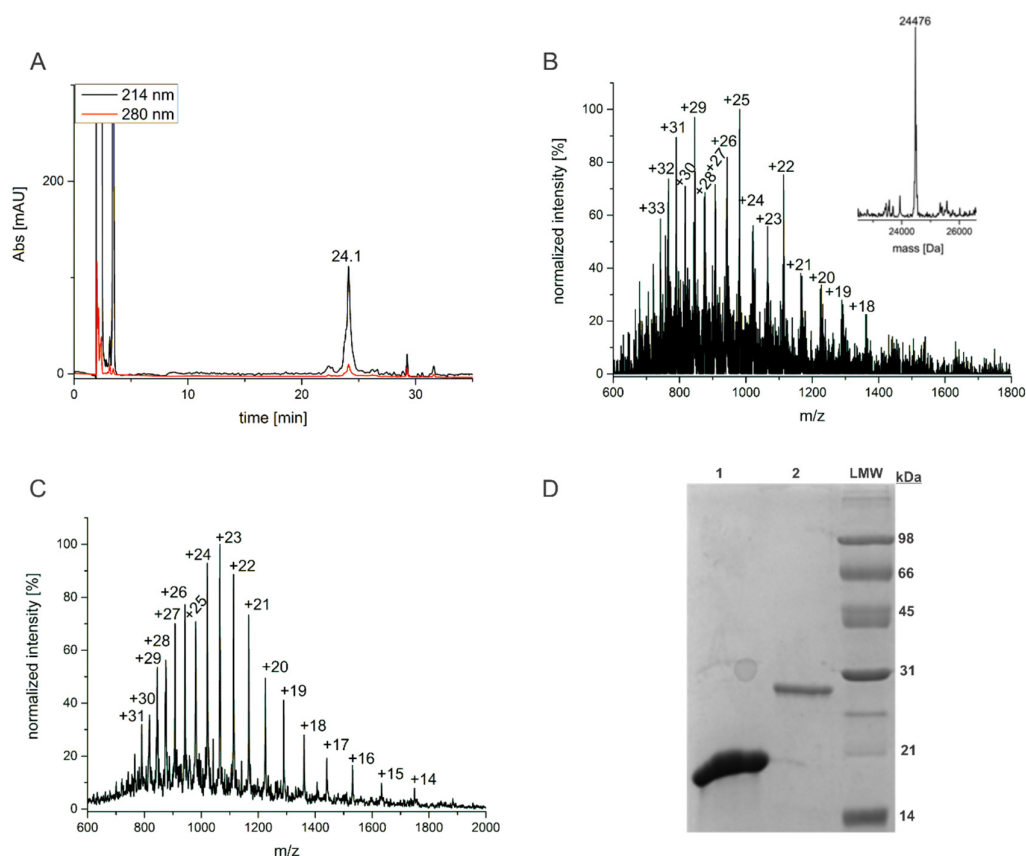


Figure 75: Characterization of purified PrP 23-181PEG₂₇₋₂₃₁

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-181PEG₂₇₋₂₃₁ in denaturing buffer (6M Gnd, pH 4.7), linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-181PEG₂₇₋₂₃₁, expected mass: 24476.6 Da, observed mass: 24476 Da, multiple charged ion peaks were detected. C: back calculated spectrum and deconvoluted mass of purified PrP 23-181PEG₂₇₋₂₃₁. D: SDS-PAGE analysis of purified 23-181PEG₂₇₋₂₃₁, lane 1: PrP 23-177-MESNA thioester, lane 2: purified PrP 23-181PEG₂₇₋₂₃₁, lane 3: LMW.

4.1.7.2 PrP 23-197PEG₂₇₋₂₃₁

PrP 23-178 β -mercapto-Asp-197PEG₂₇₋₂₃₁ was selectively desulfurized using the same strategy as described in section 4.1.7.1. Hereby, 0.03 μ mol of PrP 23-178 β -mercapto-Asp-197PEG₂₇₋₂₃₁ was used and the reaction progress was monitored through LC-MS. The reaction was finished after 8 h; the crude product was purified over analytical RP-HPLC and lyophilized. The purified PrP 23-197PEG₂₇₋₂₃₁ was characterized via analytical RP-HPLC, ESI-MS and SDS-PAGE (Figure 75). The sample was dissolved in a denaturing buffer (6 M Gnd-HCl, pH 4.7) due to solubility problems, nevertheless the analytical RP-HPLC chromatogram showed one sharp peak at 23.5 min (Figure 76 A), the corresponding mass spectrum however showed a noisy background with a clear trend of one dominant mass pattern (Figure 76 B). In order to get more information on the final product, the spectrum was

deconvoluted. After deconvolution, a clear isotopic pattern (32- to 18-fold charged ion peaks), which corresponded nicely to the desired product, could be observed (expected mass: 24476.6 Da, observed mass: 24476, Figure 76 C). SDS-PAGE analysis also showed a clear defined band between 31 and 21 kDa without dimerization products (Figure 76 D, lane 2). The protein sample was compared to the corresponding MESNA thioester. The band on the SDS gel showed for the protein thioester, two well defined bands, one at approx. 17 kDa the other one between 31 and 45 kDa. Most likely, the higher band corresponded to a PrP dimer.

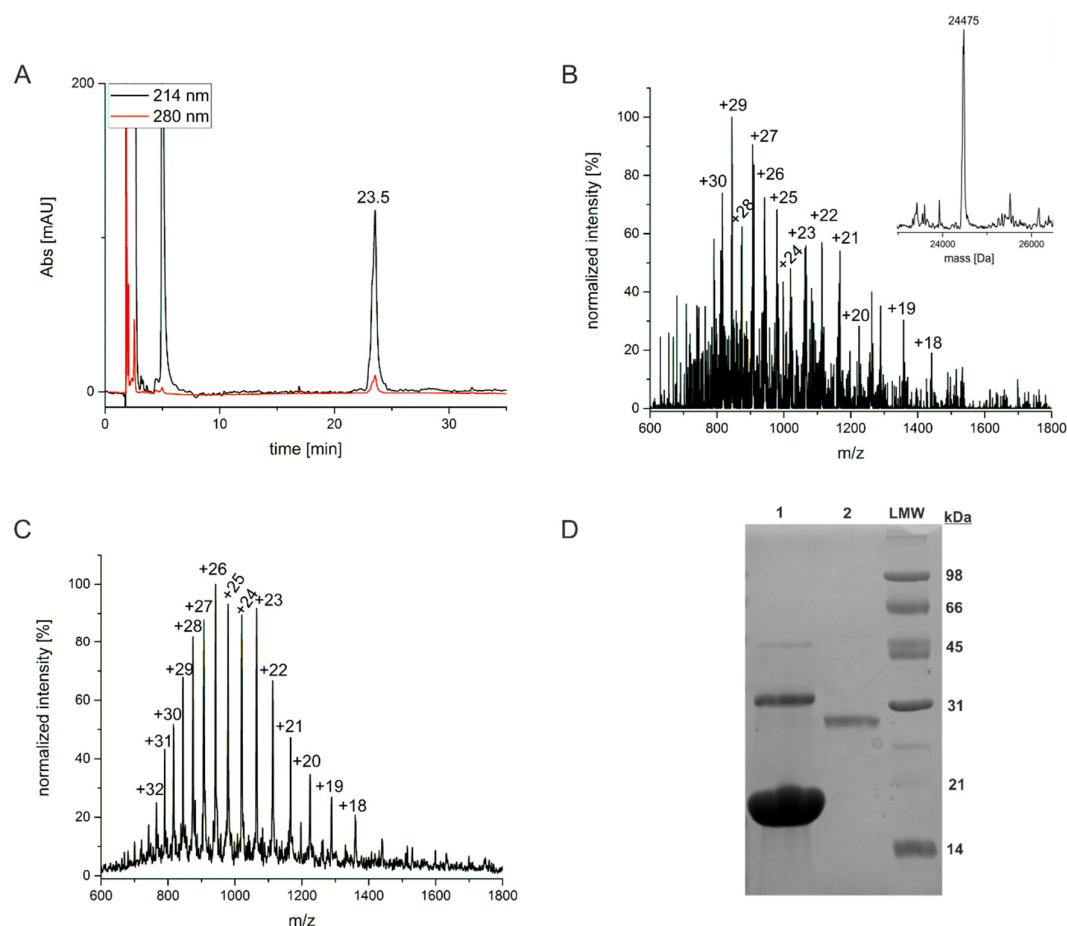


Figure 76: Characterization of purified PrP 23-197PEG₂₇-231

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-197PEG₂₇-231 in denaturing buffer (6M Gnd, pH 4.7), linear gradient 5-65%B ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-197PEG₂₇-231, expected mass: 24476.6 Da, observed mass: 24476 Da, multiple charged ion peaks were detected. C: back calculated spectrum and deconvoluted mass of purified PrP 23-197PEG₂₇-231. D: SDS-PAGE analysis of purified 23-197PEG₂₇-231, lane 1: PrP 23-177-MESNA thioester, lane 2: purified PrP 23-197PEG₂₇-231, lane 3: LMW.

4.1.7.3 PrP 23-181&197PEG₂₇-231

The procedure to desulfurize the β -mercapto moiety on aspartate was analogous to the method described in sections 4.1.7.1 and 4.1.7.2. The desulfurization reaction was monitored via LC-MS and the crude product was purified via C18 analytical RP-HPLC. Purified PrP 23-181&197PEG₂₇-231 was lyophilized and subsequently analyzed via analytical RP-HPLC, ESI-MS and SDS-PAGE (Figure 77).

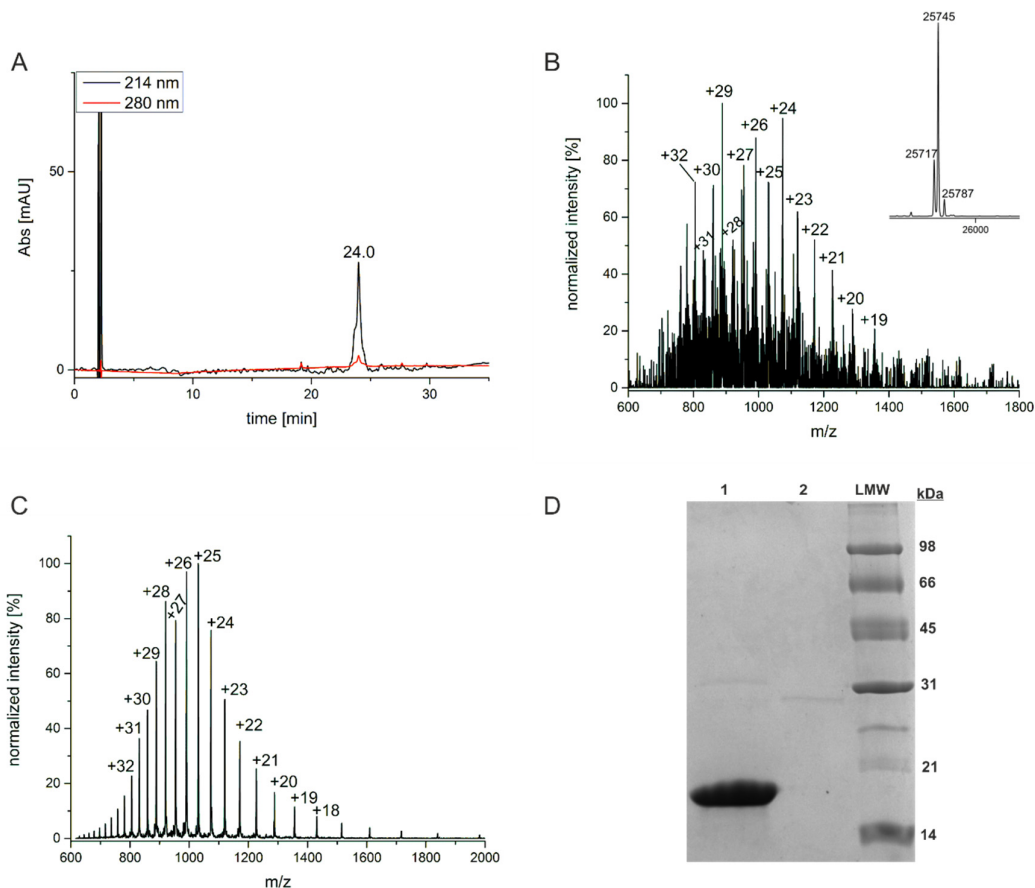


Figure 77: Characterization of purified PrP 23-181&197PEG₂₇-231

A: analytical C4 RP-HPLC chromatogram of purified PrP 23-181&197PEG₂₇-231 in 5% ACN with 0.1% TFA, linear gradient 5-65% ACN in 30 min, *baseline corrected*. B: ESI-MS spectrum of purified PrP 23-181&197PEG₂₇-231, expected mass: 25754.6 Da, observed mass: 25754 Da, multiple charged ion peaks were detected. C: back calculated spectrum and deconvoluted mass of purified PrP 23-181&197PEG₂₇-231. D: SDS-PAGE analysis of purified 23-181&197PEG₂₇-231, lane 1: PrP 23-177-MESNA thioester, lane 2: purified PrP 23-181&197PEG₂₇-231, lane 3: LMW.

As it can be clearly seen on the analytical RP-HPLC chromatogram (Figure 77 A) only one major peak could be detected at 24.0 min. Despite being slightly noisy, the ESI-MS mass spectrum of purified PrP 23-181&197PEG₂₇-231 showed the

correct mass by the presence of 32- to 19-fold charged ion peaks (expected mass: 25754.6 Da, observed mass: 25754 Da, Figure 76 B). Both, the back calculated and the deconvoluted spectrum provided more evidence for the presence of the desired product (Figure 77 C). However, if zoomed in, the deconvoluted spectrum revealed that not only the desired product was observed but also a double desulfurized PrP variant. This was expected, since harsh reaction conditions and excess of DTT may cause a globular reduction of sulfhydryl groups as described by Payne and coworkers [211]. Fortunately, the amount of twice-desulfurized product was < 20% compared to mono-desulfurized product. Additionally, the protein was going to be refolded. After refolding, all misfolded protein variants. Which are not able to form the required disulfide bridge, should aggregate. In doing so, double desulfurized PrP variant and the right product could be separated easily via centrifugation.

4.2 Biophysical characterization of homogenously PEGylated PrP variants

4.2.1 Folding of homogenously PEGylated PrP variants

Recombinant PrP variants are generally expressed in inclusion bodies as insoluble proteins [312]. Once expressed in inclusion bodies, PrP molecules are denatured. To overcome this problem, homogenously PEGylated PrP variants should be folded. Hence, a stepwise dilution strategy of purified PEGylated PrP variants from 6 to 2 M Gnd-HCl in 50 mM Tris (pH 8.0) with refolding buffer and subsequent dialysis against the refolding buffer (20 mM NaOAc, pH 5.0) was utilized. Reduced and oxidized glutathione (3 mM: 0.3 mM) was added to the refolding solution in order to achieve the correct disulfide formation between Cys179 and Cys214. The ratio of reduced to oxidized glutathione was determined by Chu *et al.* and has been applied for full length and N-terminally truncated PrP variants. Since the PrP variants used in this thesis were derived from the PrP variants mentioned above, the ratio described in Chu *et al.* has been used for the refolding experiments [294]. Upon refolding, the concentration and yield of refolding for different samples has been determined. Below, typical concentrations and yields are presented (Table 2). All PEGylated PrP variants were recovered in good (folding-) yields. The folding

yields have been calculated through the determination of volume after folding and measuring the protein concentration at 280 nm.

Table 2: Determination of concentration and yield after folding of PEGylated PrP variants.

<i>variant</i>	<i>[c] in 6 M Gnd-HCl</i>	<i>[c] in 20 mM NaOAc</i>	<i>Folding yield</i>
23-181PEG ₂₇ -231	0.2 mg/mL	0.17 mg/mL	85% (0.17 mg)
23-197PEG ₂₇ -231	0.3 mg/mL	0.2 mg/mL	63% (0.2 mg)
23-181&197PEG ₂₇ -231	0.27 mg/mL	0.2 mg/mL	74% (0.2 mg)

4.2.2 Secondary structure analysis via CD spectroscopy

To determine whether the PEGylated PrP variants have a defined secondary structure after refolding, CD measurements have been carried out. To the best of our knowledge, these are the first PrP variants with site specifically attached, homogenous PEG₂₇. PEG derivatives may enhance the solubility and assist to increased conformational stability [313]. Moreover, many proteins does not show alterations in the secondary structure after covalent attachment of PEG chains [313-314]. Therefore, we expected to see only minor changes upon PEG₂₇ addition to PrP and the PEGylated variants will adopt an α -helix rich confirmation similar to the wild type (wt) FL-PrP (comprising aas 23-231). However, CD measurements revealed that the α -helical content was dramatically decreased in comparison to wt, recombinant FL-PrP. Table 3 presents the percentage of α -helices, β -sheets and random coils determined via CD measurements. All calculations were done using CDNN software.

Table 3: Distrubition of secondary structure elements among different homogenous PEGylated PrP species [%]

<i>PrP variant</i>	<i>α-helices</i>	<i>β-sheet antiparallel</i>	<i>β-sheet parallel</i>	<i>β-turn</i>	<i>random coils</i>	<i>Σ</i>
23-181PEG ₂₇ -231	27.3	9.9	7.3	15.3	40.3	100.1
23-197PEG ₂₇ -231	28.6	6.8	4.7	21.6	38.6	100.3
23-181&197PEG ₂₇ -231	12.3	26.1	5.2	20.8	37.6	102.0
23-231	38.0	8.0	5.9	16.3	31.8	100.0
SHaPrP ^C	28.0	*	*	7.0	57.0	101.0

* The results were taken from Elfrink *et al.* [315] and based on NMR calibrated FTIR spectra evaluation, antiparallel β -sheet contents were combined, 9.0%.

Figure 78 shows CD spectra of site-specific and homogenously PEGylated PrP variants. In combination with the results from Table 3, an unexpected secondary structure for homogenously PEGylated PrP derivatives was observed. As described in section 1.2.2, PrP^C and recombinant PrP share a common α -helix rich secondary structure. Previous work from our group also indicated, that semisynthetic lipidated and non-lipidated PrP^C variants adopt a predominantly α -helical structure [143, 265-266, 294]. However, the CD measurements showed that site-selective and homogenously PEGylated PrP variants are less α -helical than wt PrP. Compared to wt PrP, mono-PEGylated PrP variants PrP 23-181PEG₂₇₋₂₃₁ (Figure 78 A) and PrP 23-197PEG₂₇₋₂₃₁ (Figure 78 B) had 11.3 and 9.4% less α -helicity, whereas di-PEGylated PrP variant PrP 23-181&197PEG₂₇₋₂₃₁ (Figure 78 C) had a dramatic decrease of 25.7%. Interestingly, for mono-PEGylated PrP variants compared to PrP 23-231, only a slight increase in β -sheets and β -turns could be assessed suggesting that more random coiled structure was adapted. However, di-PEGylated PrP showed a very high β -sheet content (31.3%). This was an increase of 17.4% in β -sheet structure. This finding is of interest, because PrP^{Sc} is β -sheet rich and related to that, tends to aggregate into amyloid fibrils [108-109].

New advances in PEGylation of peptides and proteins showed that the length and position of PEGylation plays a major role in stabilization of the native structure [314]. Furthermore, there is proof that PEGylated proteins generally retain their native-like folding and PEGylation provides an increase in conformational stability [313-314, 316-317]. However, one prominent example, G-CSF, has been reported to show a decrease in conformational stability and slight increase in aggregation sensitivity upon PEGylation [318]. Moreover, hen egg white lysozyme (Lyz), once PEGylated, featured lower values at 205 nm compared to non-PEGylated, wt Lyz at the same molar concentration [319]. This pointed to a minor loss of α -helical content.

Since no other, homogenously PEGylated PrP variants exist, only a comparison to glycosylated PrP is possible (Table 3). In 2008, Gerwert and coworkers described the structural changes of membrane bound PrP^C [315]. The protein was fully equipped with native PTMs, diglycosylation and GPI-anchor respectively. In this study, membrane bound, posttranslationally modified SHaPrP^C (0.4 μ M) showed a similar secondary structure to the recombinant anchorless PrP in solution. The α -

helical content was 28%, β -sheet 9%, β -turn 7% and random coil 57%. Compared to our results (table 3), SHaPrP^C showed a similar α -helicity. The content of β -sheet in SHaPrP^C was slightly lower than of PrP 23-181PEG₂₇-231 (2.5%) and PrP 23-197PEG₂₇-231 (8.2%). Di-PEGylated PrP consists of 3.5-fold more β -sheets than SHaPrP^C. The percentage for β -turns in mono- and di-PEGylated PrP variants were generally 2- to 3-fold higher than in SHaPrP^C. Taken together, the α -helicity of PEGylated PrP variants is comparable with that of fully posttranslationally modified SHaPrP^C, whereas the β -turn content was dramatically increased in PEGylated PrP variants.

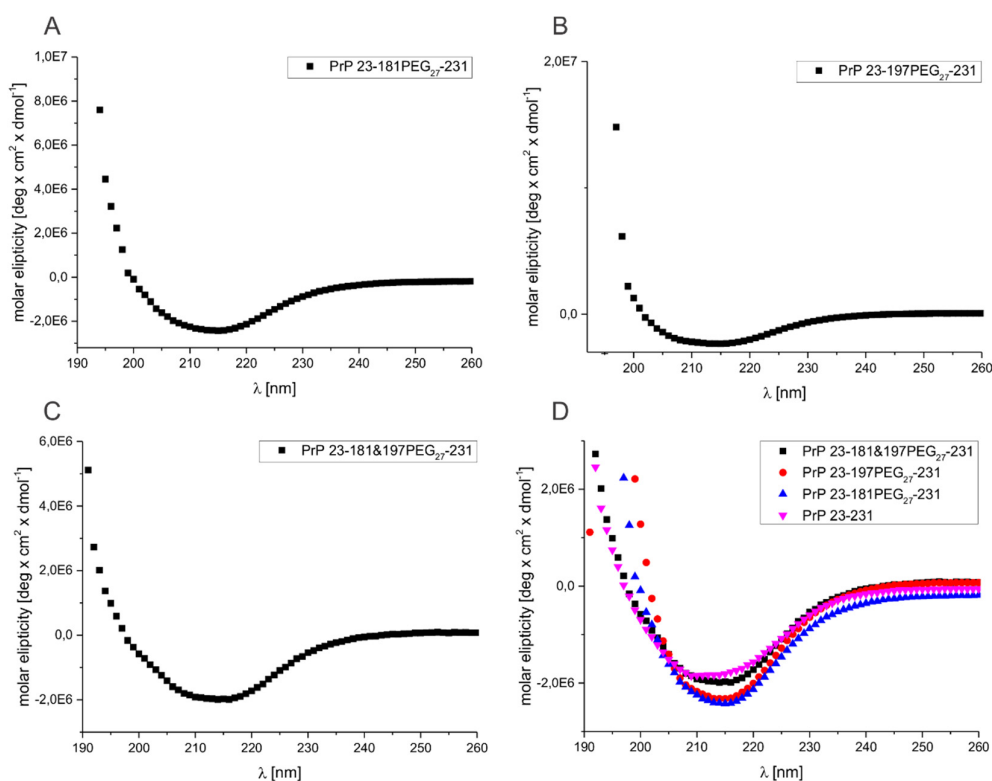


Figure 78: CD spectra of site-selective, homogenously PEGylated PrP variants.

A: CD spectrum of PrP 23-181PEG₂₇-231, [c]=8 μ M (0.2 mg/mL), B: CD spectrum of PrP 23-197PEG₂₇-231, [c]=8 μ M (0.2 mg/mL), C: CD spectrum of PrP 23-181&197PEG₂₇-231, [c]=7.8 μ M (0.2 mg/mL), D: Comparison of all recorded CD spectra of site-specific and homogenously PEGylated PrP variants and non-modified PrP 23-231 [c]=4.3 μ M (0.1 mg/mL), All spectra were recorded from 190-260 nm.

PEGylation of critical positions, which should carry complex *N*-glycans, on PrP, has never been performed before. From this point of view, large PEG polymers might enhance the solubility and assist to increase conformational stability but there is no proof that the PEG₂₇ units, which have been used in this thesis, can help to stabilize a more α -helical, native-like structure. To elucidate the reasons for these

change in folding, more biophysical characterization experiments need to be performed including FT-IR and NMR spectroscopy.

4.3 Biochemical characterization of homogenously PEGylated PrP variants

4.3.1 ThT fluorescence aggregation assay

Prion diseases accompany with the formation of amyloid fibrils, also called plaques. In section 1.3.1, two models for amyloid formation have been discussed with noting the high susceptibility of PrP^{Sc} to aggregation. The ThT fluorescence aggregation assay is a reliable method to measure the degree of protein aggregation *in vitro*. First discovered in 1959, ThT is a benzothiazole derivative of aniline [320]. Despite being not understood completely, ThT binding to fibrils shifts its excitation (385 nm → 450 nm) and emission maximum (445 nm → 482 nm) dramatically. This makes ThT a valuable agent for visualizing aggregation and fibril formation [321].

For the aggregation assay, we modified the protocol described in Breydo *et al.* [322]. Homogenously PEGylated PrP variants were diluted to a final minimum concentration 0.1 mg/mL (~ 4 µM) with a Gnd-HCl/Tris buffer. The final buffer composition was 2 M Gnd-HCl in 50 mM Tris, pH 6. Bovine Serum Albumin (BSA) and refolded PrP 23-231 were used as negative and positive controls, respectively. As it can be seen on Figure 79, mono and di-PEGylated PrP variants are more resistant to aggregation than PrP 23-231. Non-modified, wt PrP 23-231 starts to aggregate after ~ 83 h, whereas the PEGylated counterparts were not showing any ThT fluorescence signal changes (Figure 79 A-C). Furthermore, a negative control (Figure 79 D), BSA, was used to estimate the fluorescence intensity for aggregation prone proteins that do not form fibrils. Compared to mono- (Figure 79 A&B) and di-PEGylated PrP variants (Figure 79 C), no significant change in fluorescence intensity was observed. Thus, mono- and di-PEGylated PrP variants described in this thesis are resistant to *in vitro* aggregation under these conditions compared to their non-modified counterpart PrP 23-231. This can be related to the presence of large and covalently bound PEG₂₇ chains and is in good agreement with the results shown in Bosques *et al.* [323] as well as Katorcha *et al.* [324]. In the

first work, the researchers showed that the core *N*-glycan structure stabilizes the disulfide formation of PrP and cause a retardation of the aggregation rate. Katorcha et al. reported that native, sialylated PrP^C is not a preferable substrate for PMCA when compared to its desialylated counterpart. Both publications showed the positive impact of *N*-glycans in structural and physicochemical stability of PrP.

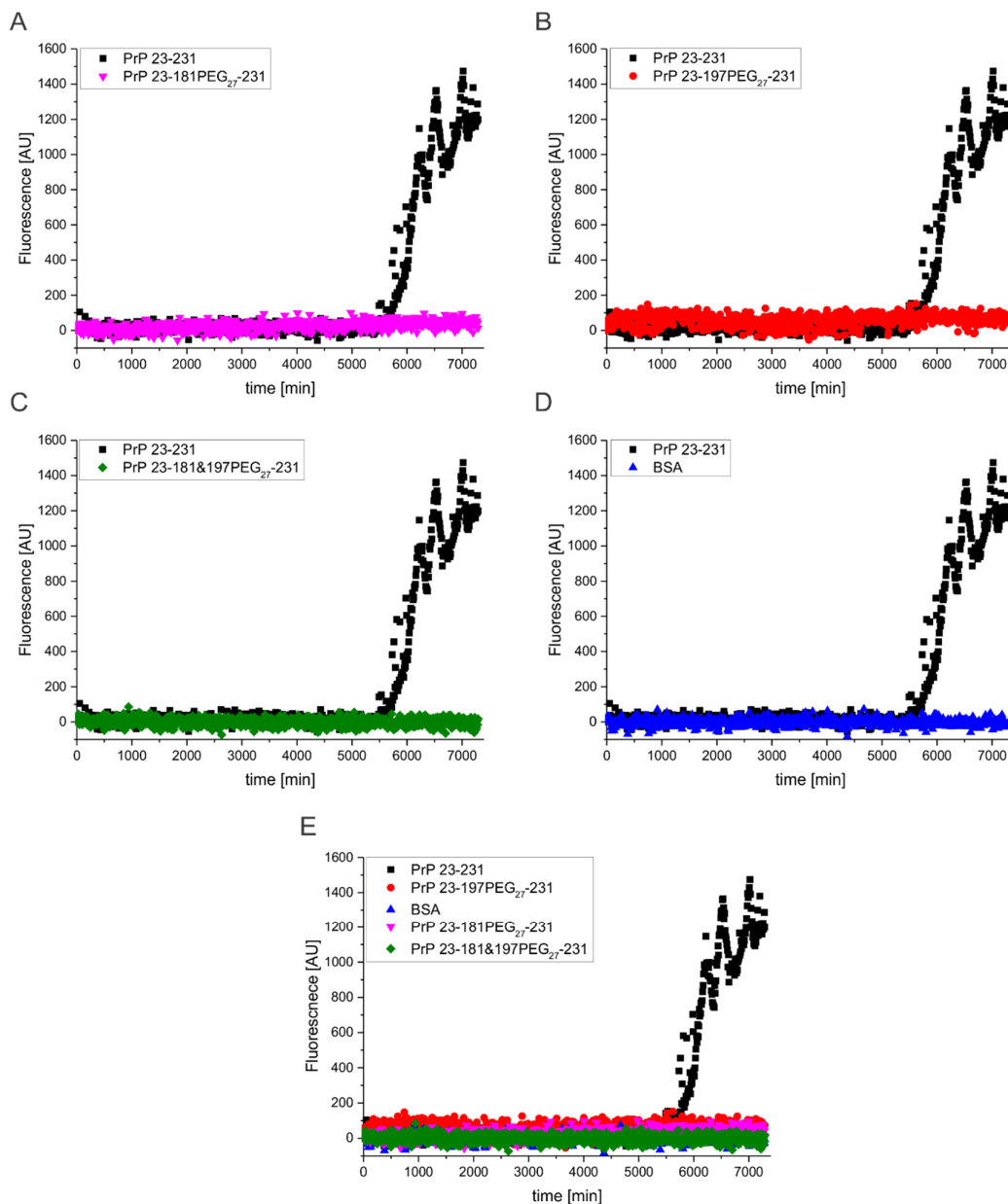


Figure 79: ThT fluorescence measured at 37°C, pH 6 in NaOAc buffer.

All data were recorded at an excitation wavelength of 444 ± 9 nm and an emission wavelength of 485 ± 9 nm. All samples were used at a concentration of 0.1 mg/mL (~4 mM). A: fluorescence spectrum of PrP 23-231 and PrP 23-181PEG₂₇-231, B: fluorescence spectrum of PrP 23-231 and PrP 23-197PEG₂₇-231, C: fluorescence spectrum of PrP 23-231 and PrP 23-181&197PEG₂₇-231, D: fluorescence spectrum of PrP 23-231 and negative control BSA, E: Comparison of all recorded fluorescence spectra.

A large number of publications highlighted the stabilizing effects of polydisperse, covalently bound PEG chains on secondary structure and thermal aggregation properties of proteins ^[313-314, 317]. Recently, Price and coworkers described that site-specifically attached, homogenous PEG increases the conformational stability and folding rate of the WW domain of pin 1 protein depending on the PEG chain length ^[316]. In addition, site-specific PEGylation of G-CSF led to increased thermal stability and decreased aggregation sensitivity ^[314].

Taken together, site-specific and covalently bound homogenous PEG₂₇ modifications on PrP resulted in no susceptibility for aggregation. To the best of our knowledge, the main reason for prion formation is the conformational change from α -helical PrP^C into β -sheet rich PrP^{Sc}. Our results suggest, that a decrease in α -helical content and an increase in β -sheet and/or random coil does not necessarily lead to the aggregation of PrP once the conformation is stabilized by PEG.

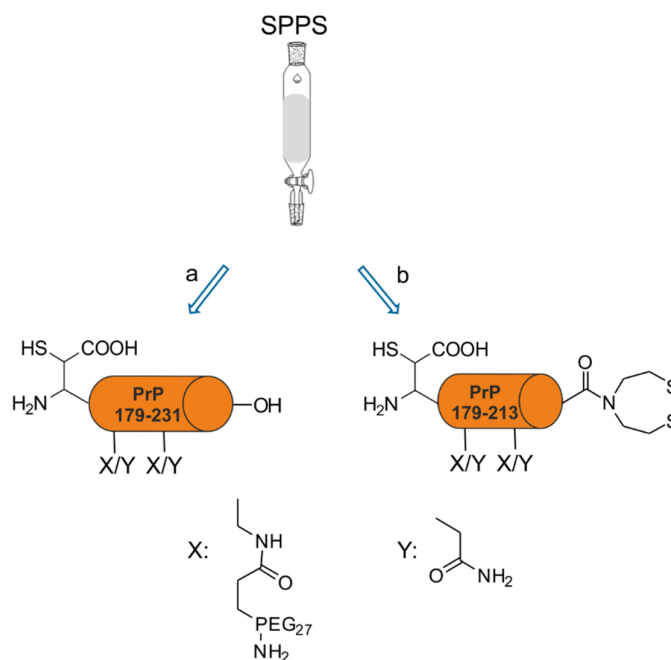
5 Summary

Transmissible spongiform encephalopathies (TSEs) are incurable, fatal and neurodegenerative diseases caused by misfolded prion protein (PrP) aggregates. It is still not entirely understood, how these aggregates are formed and when or why the conversion of cellular PrP (PrP^C) into pathogenic scrapie PrP (PrP^{Sc}) occurs. To date, PrP is accepted as the only pathogen causing TSEs. Recent results sustained this theory but extended it by adding the influence of cofactors such as polyanions and lipids. Moreover, there has been convincing proof that PrP is anchored on the outer leaflet of the plasma membrane via a GPI anchor and that the conversion into PrP^{Sc} occurs there. In addition, PrP^C is to a large extent *N*-glycosylated, different prion strains have distinct glycosylation patterns and the rate of glycosylation of PrP^{Sc} has a major impact in certain prion disease as described in section 1.4.1. These advances made it clear that posttranslational modifications (PTMs) on PrP may favor the conversion of PrP^C → PrP^{Sc}. Therefore, homogenous posttranslationally modified PrP variants are needed as unique tools to investigate the impact of PTMs on the pathogenesis of prion diseases. However, the broad heterogeneity of PTMs between different prion strains and within PrP variants from one source made it very challenging to assess the impact of PTMs on prion pathogenicity. So far, all investigations of the (patho-)physiological effect of heterogenous *N*-glycans on PrP did not give rise to conclusive results.

To this end, we aimed at developing a new and straightforward extension of our previous semisynthetic approaches to access to homogeneously posttranslational modified PrP variants. In this thesis, we described the semisynthesis of site specifically and homogeneously PEGylated PrP variants at positions 181 and 197. We successfully generated two recombinant PrP α -thioesters comprising amino acids 23/90-177 in very good yields (11 mg/L and 1.5 mg/L culture). Furthermore, we utilized Fmoc SPPS to obtain PrP peptides and latent peptide α -thioesters (SEA peptides) for subsequent use in chemoselective ligation reactions. Due to the major challenges in synthesis of homogenous *N*-glycans and their introduction into PrP peptide sequences, we decide to use monodisperse and linear PEG₂₇ as an *N*-glycan surrogate, mostly due to the physicochemical properties of this polymer. In

addition, using one kind of a glycan mimic would give rise to homogenously modified PrP variants. PEGylation is a standard procedure to enhance the solubility and half-life of proteins and peptides and site-specific, homogenous PEGylation has been used as a glycan surrogate in synthetic erythropoiesis protein (SEP) giving rise to increased serum protein levels and hematopoietic *in vivo* activity.

Non-, mono- or di-PEGylated PrP peptides and SEA peptides comprising the unnatural amino acid β -mercapto aspartate on their N-terminus were successfully synthesized (Scheme 18).



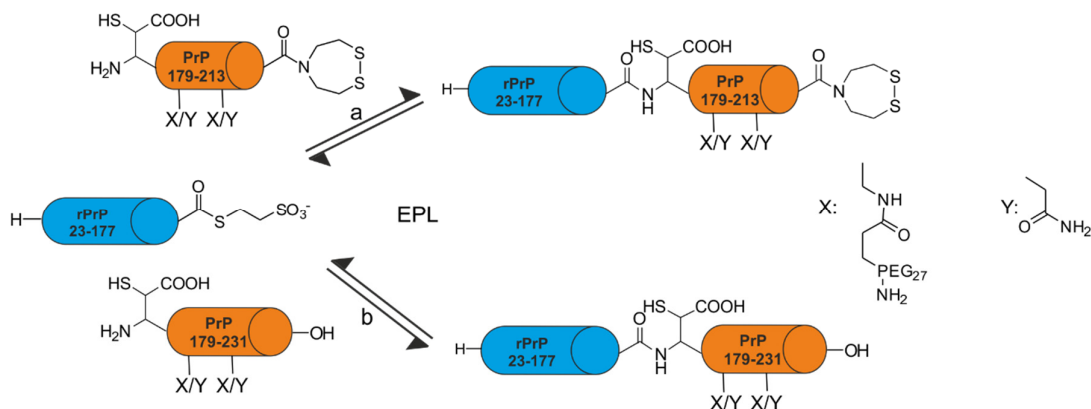
Scheme 18: Scheme of SPPS strategy.

a: Fmoc-SPPS of PrP peptides carrying either one or both PEG₂₇ units at positions 181 and 197,
b: Fmoc-SPPS of PrP SEA-peptides carrying either one or both PEG₂₇ units at positions 181 and 197. X: covalently bound PEG₂₇ unit, Y: side chain of Asn.

N-terminal β -mercapto aspartate was necessary for subsequent chemoselective ligation reactions and should be desulfurized upon ligation. All peptides and SEA peptides were generated in good yields (15-51%) and high purity (>90-95%).

With these components, expressed protein ligation (EPL) reactions were performed to link recombinant PrP 23-177 thioesters to non-, mono- or di-PEGylated PrP peptides (Scheme 19). Fast reaction times and good yields for ligating recombinant protein thioesters to modified PrP peptides were recorded. Furthermore, synthetic SEA^{on/off} peptides were ligated to recombinant PrP 23-177 thioester to enable further NCL reactions with a GPI mimicking membrane anchor.

All ligations gave rise to good to satisfactory yields (11-78%) and high purity (>90-95%).



Scheme 19: Scheme of EPL reactions.

a: EPL of recombinant PrP 23-177-MESNA thioester with PrP 178(β-mercapto-Asp)-X/Y-213-SEA b: EPL of recombinant PrP 23-177-MESNA thioester with PrP 178(β-mercapto-Asp)-X/Y-231. X: covalently bound PEG₂₇ unit, Y: side chain of Asn. The peptides carry on either one or both positions (181 and 197) the PEG₂₇ unit.

Even though the focus of this thesis was on *N*-glycosylation of PrP, we also worked on the generation of GPI anchors and mimics thereof. One GPI anchor was of recombinant origin and has been generated in *S. cerevisiae*. Recombinant expression and purification of a fusion protein also carrying this construct was successful. However, we failed to isolate the desired construct in sufficient amounts. A second strategy involving a peptidyl-GPI anchor derivative has been adopted. The product was successfully synthesized, characterized and linked to a PrP SEA^{off} peptide comprising aas 214-231. Unfortunately, isolated yield for this reaction were not sufficient for further ligation reactions (<3%, 0.3 mg).

Based on these findings, we decided to work on homogenously PEGylated PrP variants without a membrane anchor to investigate the effects of PEGylation on PrP structure and aggregation properties. The non-canonical aa β-mercapto-aspartate had to be desulfurized after ligation. Since PrP has two critical cysteine residues in its primary sequence, we used the method described in section 3.4.7 for selective desulfurization of β-mercapto aspartate. After several optimization attempts, we could isolate three homogenous and site-specific PEGylated PrP variants (Table 4).

Table 4: Overall and folding yields of PEGylated PrP variants

variant	amount	overall yield	folding yield
PrP 23-181PEG ₂₇ -231	0.6 mg	60%	85%
PrP 23-197PEG ₂₇ -231	0.75 mg	75%	63%
PrP 23-181&197PEG ₂₇ -231	0.57 mg	57%	74%

In order to fold these proteins, we performed a stepwise-dilution refolding strategy. Folded PrP variants were characterized via CD spectroscopy and analytical RP-HPLC. Previous NMR, CD and FT-IR experiments showed that recombinant PrP and PrP^C share a common, predominant α -helical structure. In addition, experiments from our group with semisynthetic PrP variants with and without GPI anchoring units showed similar results. Homogenous and site-specific PEGylated PrP variants described in this thesis showed less α -helical content compared to semisynthetic non-PEGylated PrP. In order to determine whether the conformational changes had an effect on aggregation behavior of PEGylated PrP variants, a ThT fluorescence-based aggregation assay was carried out. Mono- and di-PEGylated PrP variants showed no tendency for aggregation under conditions at which folded PrP 23-231 aggregated. Therefore, we concluded that PEGylated PrP variants are less prone to aggregation linking PEGylation to a higher conformational stability. Additionally, steric hindrance of covalently bound PEG₂₇ may prevent β -sheet formation and the high solubility of PEGylated PrP variants may decrease their susceptibility to aggregation.

6 Outlook

Future efforts should address to three major points; i) development of one pot ligation-desulfurization strategies, ii) linkage of membrane anchors on C-terminus and iii) attachment of native-like *N*-glycans to PrP.

The chemoselective ligation conditions are optimized but a large-scale ligation reaction will be performed and a one-pot ligation desulfurization strategy established. Furthermore, more complex but homogenous PEG units should be attached to Dpr residues in order to mimic branched *N*-glycans. In virtue of these attempts, chemical or enzymatic *N*-glycosylation of PrP variants should be considered as the next step. First attempts to achieve this can be done via introduction of a mono- or diglycosylated Asn into PrP 178 β -mercapto-Asp-213-SEA^{off} peptide at positions 181 and/or 197. Alternatively, isolation of a sialoglycopeptide (SGP) from egg yolk as described in Kajihara *et al.* [275] and its introduction into PrP peptides and peptide α -thioesters can be utilized. An enzymatic approach involving the linkage of an *N*-glycan-oxazoline derivative to a mono-glycosylated Asn residue can also be performed.

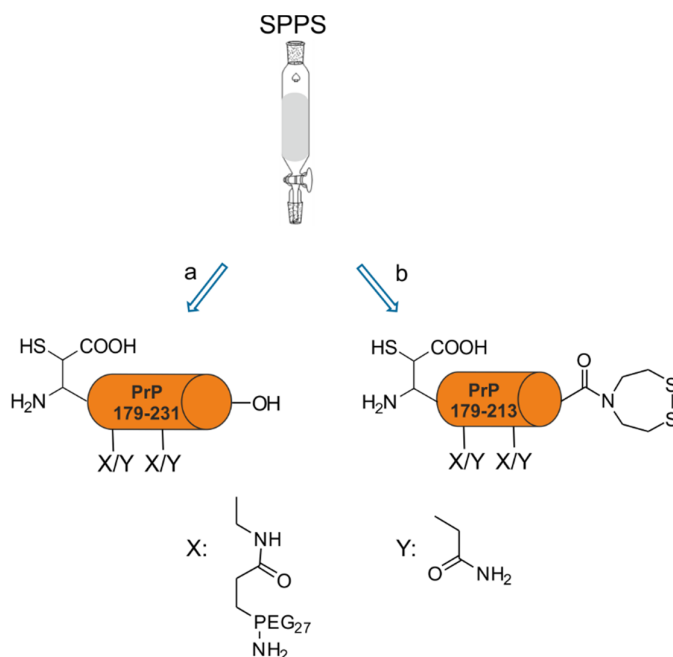
Not only glycosylation but also membrane attachment of PrP should be considered as an important aspect. For this purpose, ligation of PrP 214-231 SEA^{off} peptide with the peptidyl GPI anchor surrogate D5 must be scaled up and optimized. Upon optimization, homogeneously glycosylated PrP variants equipped with a GPI anchor analogue can be characterized via CD and FT-IR spectroscopy. Biochemical assays such as ThT fluorescence aggregation assay and PK digestion assays will give more insights into prion formation and pathogenesis

7 Zusammenfassung

Transmissible spongiforme Enzephalopathien (TSE) sind neurodegenerative Krankheiten, die nicht heilbar sind und tödlich verlaufen. Diesen Erkrankungen liegen die fehlgefalteten Aggregate des Prion Proteins (PrP) zu Grunde. Es ist noch nicht komplett verstanden, wie diese Aggregate gebildet werden und wann bzw. warum die konformationelle Umwandlung von zellulärem PrP (PrP^{C}) ins pathogene scrapie PrP (PrP^{Sc}) geschieht. Derzeit ist PrP das einzige anerkannte Pathogen, welches TSEs hervorrufen kann. Neue Studien erweitern diese Theorie, wobei der Einfluss von Kofaktoren wie Polyanionen und Lipiden bei der Entstehung von PrP^{Sc} berücksichtigt werden. Außerdem gibt es überzeugende Beweise, dass PrP an der Außenseite der Zellmembran durch einen GPI verankert wird und die konformationale Konversion von PrP^{C} in PrP^{Sc} dort passiert. PrP^{C} ein *N*-glykosyliertes Protein, wobei unterschiedliche Prionen-Stämme verschiedene *N*-Glykan-Muster aufweisen. Die Glykosylierungsrate von PrP^{Sc} , wie in 1.4.1 beschrieben, spielt eine entscheidende Rolle bei manchen TSEs. Diese Befunde verdeutlichen, dass posttranslationale Modifikationen (PTMs) von PrP bei der Einleitung der Konformationsänderung von PrP^{C} in PrP^{Sc} eine beträchtliche Rolle spielen können. Folglich werden möglichst homogene, posttranslational modifizierte PrP-Varianten in genügender Quantität gebraucht, um diese als Werkzeuge zu benutzen, damit man den Einfluss von PTMs in der Pathogenese der Prionen-Erkrankungen untersuchen kann. Auf Grund der großen Heterogenität in der PTMs bei den natürlich vorkommenden, unterschiedlichen Prionen-Stämmen und den damit verbundenen PrP Varianten ist es sehr schwierig, den Einfluss der PTMs auf die Pathogenität zu untersuchen. Bisher wurden die (patho-)physiologischen Effekte in sehr heterogenen Gemischen von posttranslational modifizierten PrP-Varianten untersucht, jedoch lieferten diese keine überzeugenden Ergebnisse.

Im Rahmen dieser Dissertation haben wir eine neue Strategie entwickelt, welche den Zugang zu homogenen, posttranslational modifizierten PrP-Varianten ermöglicht und eine vorherige Semisynthese-Strategie ergänzt. Dabei stand besonders die Semisynthese von ortsspezifisch (an Positionen 181 und 197)

PEGylierten PrP-Varianten im Mittelpunkt. Zwei rekombinante PrP α -thioester, welche die Aminosäuren 23 bzw. 90-177 umfassen, wurden in guten Ausbeuten erhalten (11 mg/L Kultur für PrP 23-177 und 1.5 mg/L Kultur für PrP 90-177). Ferner wurden PrP Peptide und PrP-Thioester Vorläufer (SEA Peptide) mittels Fmoc Festphasenpeptidsynthese hergestellt, um diese in chemoselektiven Ligationsreaktionen zu benutzen. Auf Grund der aufwendigen organisch-chemischen Synthese für homogene *N*-Glykane und deren Einbau in PrP Sequenzen, wurde monodisperses, lineares PEG₂₇ als Glykanmimetikum verwendet. Dieses Polymer weist ähnliche physikochemische Eigenschaften auf wie Oligosaccharide. Um die Homogenität der Modifikation zu gewährleisten, wurde nur eine Sorte von Glykanersatz genutzt. PEGylierung ist ein Standardverfahren, um die Löslichkeit und Serumhalbwertszeit von Proteinen und Peptiden zu erhöhen. Homogene PEGylierung wurden bei der Synthese von „Synthetic Erythropoiesis Protein (SEP)“ erfolgreich verwendet und hat eine Zunahme der Serumwerte für SEP sowie der hämatopoetischen Aktivität *in vivo* zu Folge. Unmodifizierte sowie mono- und di-PEGylierte PrP Peptide und SEA Peptide mit der unnatürlichen Aminosäure β -mercapto-Aspartat (Schema 18) wurden in guten Ausbeuten (15-51%) und Reinheit (>90-95%) erhalten.

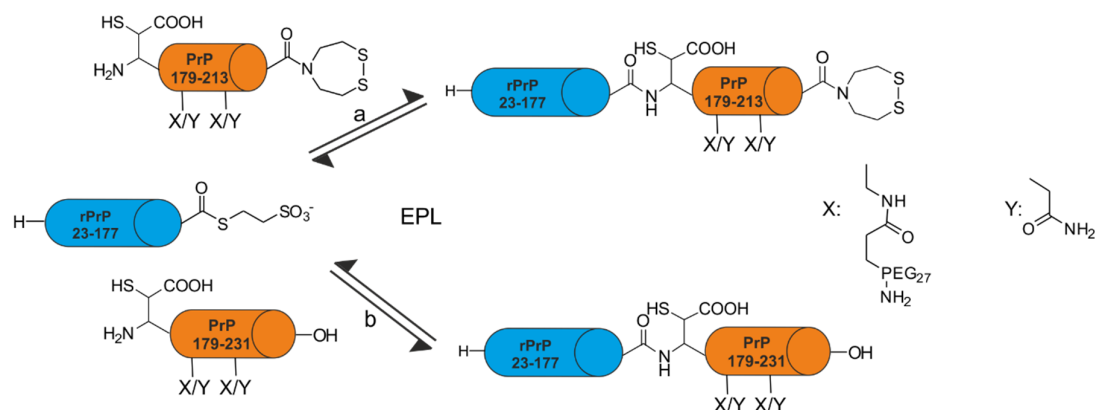


Schema 18: Strategie für Fmoc-Festphasensynthese.

a: Fmoc-Festphasensynthese von PrP Peptiden, welche entweder eine oder zwei PEG₂₇ Einheiten besitzen (Aminosäuren 181 und 197), b: Fmoc-Festphasensynthese von PrP SEA-Peptiden, welche entweder eine oder zwei PEG₂₇ Einheiten besitzen (Aminosäuren 181 und 197)
X: kovalent gebundene PEG₂₇ Einheit Y: Seitenkette von Asn.

Ein N-terminales β -mercapto-Aspartat ermöglicht die folgende chemoselektive Ligationsreaktion und kann nach der Ligation spezifisch desulfuriert werden, ohne Cystein-Reste zu verändern.

Mit diesen Komponenten wurden Expressed Protein Ligation (EPL) Reaktionen durchgeführt, um PrP 23-177-Thioester mit den nicht-, mono- und di-PEGylierten PrP Peptiden zu verknüpfen (Schema 19). Des Weiteren wurden synthetische SEA Peptide mit rekombinantem PrP 23-177-Thioester ligiert, um anschließend native chemische Ligation (NCL) Reaktionen mit einem GPI-Anker Mimetikum zu ermöglichen. Alle Ligationsprodukte wurden in guter bis zufriedenstellender Ausbeute (11-78%) und Reinheit (>90-95%) erhalten.



Schema 19: Schematische Darstellung von EPL Reaktionen.

a: EPL des rekombinanten PrP 23-177-MESNA Thioesters mit PrP 178(β -mercapto-Asp)-X/Y-213-SEA Peptid; b: EPL des rekombinanten PrP 23-177-MESNA Thioesters mit dem PrP 178(β -mercapto-Asp)-X/Y-231. X: kovalent gebundene PEG₂₇ Einheit, Y: Seitenkette von Asn. Die Peptide tragen entweder eine oder beide PEG₂₇ Einheiten in Positionen 181 und 197.

Obwohl die Modifikation von PrP mit *N*-Glykanen bzw. mit deren Mimetika das Hauptaugenmerk dieser Dissertation war, habe ich mich auch mit der Entwicklung von GPI-Ankern und deren Mimetika beschäftigt. In *S. cerevisiae* wurde ein GPI Anker mit einem Fusionsprotein und verschiedene Affinitätstags exprimiert. Die Isolierung des GPI Ankers in ausreichenden Mengen konnte im Rahmen dieser Arbeit jedoch nicht bewerkstelligt werden. Eine zweite Strategie sah die Synthese eines Peptidyl-GPI Anker Mimetikums vor, welches erfolgreich synthetisiert und gereinigt werden konnte. Ferner wurde es mit dem SEA Peptid PrP 214-231 ligiert, wobei die Ausbeuten dieser Ligation (< 3%, 0.3 mg) nicht genügend waren und somit keine weiteren Ligationsreaktionen möglich waren.

Auf Grund der oben beschriebenen Ergebnisse, wurden im Rahmen dieser Arbeit homogene, PEGylierte PrP Varianten ohne Membranverankerung hergestellt und untersucht. Dabei wurden die Einflüsse der PEGylierung auf die Sekundärstruktur und das Aggregationsverhalten von PrP untersucht. Die unnatürliche Aminosäure β -mercapto-Aspartat wurde nach den oben aufgeführten Ligationsreaktionen zum nativen Aspartat desulfurisiert werden, ohne die zwei natürlichen Cystein-Reste, die zur Ausbildung einer Disulfidbrücken notwendig sind, zu verändern. Nach zahlreichen Optimierungen wurden drei homogene und ortsspezifisch PEGylierte PrP Varianten isoliert (Tabelle 6).

Tabelle 6: Isolierte Ausbeuten von ungefalteten und gefalteten PEGylierten PrP Varianten

PrP Variant	Quantität	Ausbeute	Faltungsausbeute
PrP 23-181PEG ₂₇ -231	0.6 mg	60%	85%
PrP 23-197PEG ₂₇ -231	0.75 mg	75%	63%
PrP 23-181&197PEG ₂₇ -231	0.57 mg	57%	74%

Um diese Proteine zu falten, wurde eine Verdünnungsstrategie (6 M $\rightarrow$ 2 M Gdn-HCl) verwendet. Korrekt gefaltete PEG₂₇-PrP Varianten wurde mit Hilfe von CD Spektroskopie und analytische RP-HPLC charakterisiert. Vorangegangene NMR, CD und FT-IR Experimente haben gezeigt, dass rekombinant, volllängen PrP und PrP^C eine hauptsächlich α -helikale Sekundärstruktur aufweisen. Wir haben in ähnlichen Experimenten mit Membran-Anker-tragenden semisynthetischen PrP Varianten ähnliche Ergebnisse erzielt. Die im Rahmen dieser Dissertation hergestellten, homogenen und ortsspezifisch PEGylierten PrP Varianten zeigen allerdings weniger α -helikalen Anteil als die unmodifizierten, semisynthetischen und nicht-PEGylierten PrPs.

Um den Einfluss dieser Konformationsveränderungen sowie die grundsätzlichen Effekte der PEGylierung auf PrP zu studieren, habe ich einen Aggregationsassay verwendet, in dem ThT (Thioflavin T) als Fluoreszenzmarker benutzt wurde. Mono- und di-PEGylierte PrP Varianten zeigten dabei keine Aggregation, wobei unmodifiziertes, semisynthetisches PrP 23-231 unter denselben Bedingungen aggregiert ist. Daraus kann schlussfolgert werden, dass die PEGylierten PrP Varianten weniger anfällig für Aggregation sind, da vermutlich PEGylierung diesen Proteinen eine höhere konformationale Stabilität verleiht. Ferner könnte der

sterische Anspruch von kovalent gebundenen PEG₂₇-Einheiten die β -Faltblatt Bildung verhindern oder die hohe Löslichkeit von PEGylierten PrP-Varianten die Aggregationsanfälligkeit vermindern.

8 Abbreviation

37-kDa LRP: 37-kDa human laminin receptor precursor

°C:	degree Celsius
aa:	amino acid
ACN:	acetonitrile
AD:	Alzheimer's Disease
AMS:	adapted immune system
AU:	arbitrary unit
BDE:	bond dissociation energy
Boc:	<i>tert</i> -butyloxycarbonyl
BSA:	Bovine Serum Albumin
CBD:	chitin binding domain
Cbz:	carboxybenzyl
CCV:	clathrin-coated vesicles
CD:	circular dichroism
CJD:	Creutzfeld-Jacob-Disease
CNS:	central nervous system
CPE:	cysteine-proline-ester
CWD:	chronic wasting disease
d:	day(s)
Dab:	2,4-diaminobutyric acid
DCM:	dichloromethane
DBU:	1,8-Diazabicyclo[5,4,0]undec-7-ene
ddH ₂ O:	MilliQ-water
DIEA:	<i>N,N</i> -diisopropylethylamine
DMAP:	4-dimethylaminopyridine
DMF:	<i>N,N</i> -dimethylformamide
DTT:	D,L- dithiothreitol
dpi:	days post infection
Dpr:	2,4-diaminopropionic acid
DRM:	detergent-resistant membrane

EM:	electron microscopy
EPL:	expressed protein ligation
EPO:	erythropoietin
EPR:	electron paramagnetic resonance
ER:	endoplasmic reticulum
ESI-MS:	Electrospray ionization mass spectrometry
eq.:	molar equivalent
FFI:	fatal familial insomnia
Fmoc:	9-Fluorenylmethoxycarbonyl
FT-IR:	Fourier-transform infrared spectroscopy
GalNAc:	<i>N</i> -acetylgalactoseamine
Gnd-HCl:	Guanidine Hydrochloride
GPI:	glycosylphosphatidylinositol
GSH:	reduced glutathione
GSS:	Gerstmann-Straussler-Scheinker syndrome
GSSG:	oxidized glutathione
h:	hour
HATU:	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxidhexafluorophosphate
HBTU:	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate
HGF:	hepatocyte growth factor
His ₆ :	hexahistidine-tag
HXMS:	hydrogen/deuterium exchange mass spectrometry
IBs:	inclusion bodies
IL-8:	interleukin 8
IMS:	innate immune system
IPTG:	isopropyl-β-D-thiogalactopyranoside
kDa:	kilo-Dalton
LC-MS:	liquid chromatography-mass spectrometry
M:	molar
MCP 3:	monocyte chemotactic protein 3
MESNA:	2-mercaptoethanesulfonate
Mg ₂ SO ₄ :	magnesium sulfate

Abbreviation

min:	minutes
mM:	millimolar
MPA:	3-mercaptopropinociacid
Mtt:	4-methyltrityl
MUC1:	mucin 1
<i>Mxe</i> :	<i>Mycobacterium xenopii</i>
NaPi:	sodium phosphate
NCAM:	neural cell adhesion molecule
NCL:	native chemical ligation
NK:	natural killer
NMM:	4-Methylmorpholine
NMR:	Nuclear Magnetic Resonance
OD:	optical density
o.n:	over night
ORF:	open reading frame
PD:	Parkinson Disease
PDB:	Protein Data Bank
PEG:	polyethylene glycol
PI-PLC:	phosphatidylinositolphospholipase C
PK:	proteinase K
PMCA:	protein misfolding cyclic amplification assay
POI:	protein of interest
POPG:	1-palmitoyl-2-oleoylphosphatidylglycerol
PrP ^C :	cellular prion protein
PrP ^{res} :	PK-resistant PrP
PrP ^{Sc} :	prion protein scrapie
PSPr:	protease-sensitive prionopathy
PTM:	posttranslational modification
PyBOP:	benzotriazol-1-yl-oxytrypyrrolidinophosphonium-hexafluorophosphate
Quic:	quaking-induced conversion
RML:	Rocky Mountain Laboratories
ROS:	radical oxygen species
RP-HPLC:	reverse phase high performance liquid chromatography

rPrP:	recombinant PrP
rt:	room temperature
RT-Quic:	real time quaking-induced conversion
SDSL:	site-directed spin labeling
SDS-PAGE:	sodiumdodecylsulfate polyacrylamide gel electrophoresis
SEA:	bis(2-sulfanylethyl)amido
SEAlide:	<i>N</i> -sulfanylethylanilide
SGP:	sialoglycopeptide
Sha:	Syrian hamster
SOD:	superoxide dismutase
SPPS:	solid phase peptide synthesis
STI-1:	stress-inducible-protein-1
SUMO:	small ubiquitin-like modifier
TBS:	Tris buffered saline
TCEP:	tris(2-carboxyethyl)phosphine
TEV:	tobacco etch virus
TFA:	trifluoroaceticacid
ThT:	thioflavin T
TIS:	triisopropylsilane
TSE:	transmissible spongiform encephalopathy
UV:	ultra violet
v/v:	volume per volume
w/v:	weight per volume

One- and three letter code of the 20 canonical amino acids

Alanine:	A	(Ala)
Arginine:	R	(Arg)
Asparagine:	N	(Asn)
Aspartic acid:	D	(Asp)
Cysteine:	C	(Cys)
Glutamine:	Q	(Gln)
Glutamic acid:	E	(Glu)
Glycine:	G	(Gly)
Histidine:	H	(His)

Abbreviation

Isoleucine	I	(Ile)
Leucine	L	(Leu)
Lysine	K	(Lys)
Methionine	M	(Met)
Phenylalanine	F	(Phe)
Proline	P	(Pro)
Serine	S	(Ser)
Threonine	T	(Thr)
Tryptophan	W	(Trp)
Tyrosine	Y	(Tyr)
Valine	V	(Val)

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