3. Concepts of Amyloid Forming Model Peptides

The use of model systems to study complex biological and biochemical mechanisms is as old as protein research itself. Especially chemists and chemical biologists, with their interest in molecular level understanding, have often used modeling approaches ranging from small biomimetic molecules to peptides, proteins, and DNA. In the context of amyloids, the term model is highly relative. Scale and complexity differ tremendously, depending on the particular issue that is being addressed. Investigations based on mutations in the primary structure of naturally occurring systems represent models as much as studies with completely *de novo* designed peptides and proteins do. Hence, *"the model lies in the scale of the modeler"* and their only commonality is the use of reductionist approaches.[80] Though, not to compare apples and oranges while addressing the development of amyloid forming models, this section highlights different strategies of *in vitro* amyloid models ranging from computational to empirical and *de novo* design approaches.

3.1 Why Models?

Figuring out the general properties of amyloids conspicuously highlights the advantages of *in vitro* model systems. Amyloids are non-crystalline deposits, derived from natural or nature related proteins which usually exhibit bad solubility and almost no sequence homology. Thus, there is a huge discrepancy between the resolution capability of available analytical techniques and the complexity of the amyloid forming system that is being addressed. In this regard it becomes obvious that models exhibit an extraordinary potential to circumvent these natural restrictions by creating amyloid forming systems for a specific purpose or technique. This facilitates studies of conformational transitions and amyloid assembly processes which provide valuable information that lead to a better understanding of the basic principles on a molecular level. Consequently, these studies can be used to generate prediction tools to identify aggregation prone sequence segments in natural proteins.[81] A detailed knowledge of the underlying principles of amyloid formation is also an essential requirement for the investigation and development of selective inhibitors.[82-84] Moreover, an understanding of amyloid formation processes on a molecular level can be applied for the development of novel peptide based nano-materials.[85,86]

In detail, there are particular design tools and features, just like in a construction kit that, when incorporated into amyloid models, can be used to generate certain properties and characteristics. For example, key sequences that are found in many naturally occurring proteins can be introduced into polypeptide and protein sequences to increase their amyloid association propensity.[87,88] This so-called "amyloid stretch hypothesis" is based on the finding that short segments with four to six amino acids are sufficient to force proteins with up to a twenty fold number of residues to form amyloid fibers. From the modeler's point of view, these findings enable the development of very short, clear and manageable amyloid forming sequences with a precisely predictable folding behavior. Furthermore, an incorporation of charged amino acids perceptibly enhances solubility in most cases and usually leads to a much better synthetic accessibility. On the other hand, charged amino acids can be used as trigger functions to respond to changes in the environmental conditions such as ionic strength and pH value. Additionally, the introduction of metal coordinating amino acids such as cysteine or histidine permits the generation of structural switches that can be triggered by the addition of metal ions. Thus, it is possible to generate folding models that change their conformation at will.

Recapitulating, these facts clearly emphasize the advantages of *in vitro* model peptides. They are easily accessible by SPPS, show good solubility, have a short, clear, and manageable sequence, and can be generated specifically for each application by using a construction-kit based design. Furthermore, since amyloid association appears to be a common feature for all proteins, independent whether naturally occurring or man-made, it becomes quite obvious why amyloid forming model peptides have drawn so much attention. Namely, they are so close to the natural system because they are a part of it.

3.2 Computational Approaches

Computational approaches for the investigation and prediction of protein structure and aggregation are widely used and range from very general and minimalist strategies to modeling systems with atomic level resolution. Recently, a very simplified Monte Carlo approach has been reported narrowing down the 20 proteinogenic amino acids to two types – hydrophobic and polar.[89] Hydrophobic amino acids have a tendency to be buried while polar amino acids desire to be exposed to water. The authors applied this simplification to study protein design, folding of unstructured molecules, and amyloid

formation. Interestingly, a comparison of the yielded conformations with experimentally obtained structures with similar hydrophobic polar patterns showed striking homologies. Although the degree of simplification is enormous, this example impressively shows how valuable such theoretical studies are. However, various modeling strategies are being reported in the field of amyloids which, in principle, can be divided into two classes. The first class addresses the prediction of aggregation propensities usually applying force field models and Monte Carlo simulations. On the other hand, many approaches focusing on amyloid formation from a molecular dynamic point of view have been reported and represent the second class. Applications of this strategy range from the modeling of structural changes and early aggregation events to the formation processes of complete fibrils. Due to the huge variety and the fact that these theoretical approaches are not primarily focused on the development of *in vitro* model systems they should not be discussed further in this context and have recently been reviewed elsewhere.[81,90]

Although it is not very common, computational methods also provide useful strategies for the design and/or identification of structural switches and amyloid forming models for *in vitro* studies. For example, the group of Serrano published a computational approach for the design of a series of self associating and β-sheet forming hexapeptides using an automatic protein design algorithm called PERLA.[91] Based on a lead structure derived from a naturally occurring protein, this algorithm enabled the identification of amino acid sequences with a high propensity to fold into homopolymeric six strand β-sheet assemblies. Subsequently, the obtained sequences were synthesized and characterized by various methods including CD spectroscopy, FTIR spectroscopy, electron microscopy, and X-ray diffraction. As expected, all of the designed peptides adopted a β-sheet conformation proven by CD spectroscopy but surprisingly failed to form amyloids in some cases. This effect and the further observation of differing fibril morphologies were found to perceptibly correlate with the net charge of the peptide. A net charge of zero yielded no fibrils although a β-sheet conformation was present, while charges of +1 as well as -1 resulted in the formation of amyloid assemblies. This was explained by the assumption that non-specific aggregation and fibril formation are competing phenomena. In the absence of charges β-sheets can pack against each other in many ways which result in faintly ordered amorphous aggregates. Contrarily, uncompensated charges within a β-strand preferably yield orientations with maximum charge distance and thus favor the highly ordered states found in fibrils. This study highlights that amyloid formation demands a delicate balance between hydrophobic

interactions, coulomb effects, and main chain hydrogen bonds. More importantly, the obtained data clearly indicate that β-sheet formation and amyloid association are not necessarily correlated.

Modeling approaches were also used to specify or identify sequences that exhibit structural conversions and conformational switches as observed during the amyloid formation process. The DeGrado group reported a computation based strategy that was used to design a secondary structure switch that shifts the conformation between random coil and a stable tetrameric coiled coil motif upon phosphorylation.[92] More recently, the group of Kuhlman described a strategy to create peptides that can be switched between two distinct folds by the addition of transition metal ions.[93] Therefore, the Monte Carlo protein design software "RosettaDesign" was used to optimize a single amino acid sequence for two protein structures, namely, a coiled coil and a zinc finger fold. The obtained sequence was later synthesized and characterized by various biophysical methods such as CD spectroscopy and analytical ultracentrifugation. It has been shown that the applied computation design strategy indeed yields a peptide that changes between a helical coiled coil conformation and a zinc finger structure upon addition of zinc or cobalt. Interestingly, rational, non computation based design approaches carried out in the Woolfson laboratory yielded a similar structural behaviour.[67] Thus, it appears likely that the zinc finger fold, which is merely defined by four metal binding cysteine or histidine residues in both studies, is only marginally influenced by the remaining residues of the sequence, so that the design of such switches is stunningly clear and simple.

3.3 Models Based on Natural Systems

Although some of the advantages of *in vitro* model systems highlighted above are not entirely valid in this context, naturally occurring amyloid forming proteins nevertheless provide a huge potential. They can be dissected, linked, modified, and subsequently used as model systems to study the process of amyloid formation and the resulting structure. Many approaches that profoundly differ regarding their methodology have been carried out in recent years. Applications range from detailed investigations of short segments with clear and manageable behavior to studies on rationally designed mutants of full length proteins. To classify this huge variety concisely, the following section is divided into two subsections distinguishing between empirical and rational approaches.

3.3.1 Empirical Approaches

Many of the empirical approaches depicted here represent very obvious strategies for using naturally occurring amyloid forming proteins as *in vitro* models. In another context, some of them would not even be considered to be a model at all, but due to their clear taxonomy they provide detailed information on the amyloid formation process and should, therefore, be mentioned here.

Investigating segments of amyloid forming proteins derived from natural sources is probably one of the most widespread strategies for the application of *in vitro* model systems. The group of Gazit used very short segments of the islet amyloid polypeptide (IAPP) and human calcitonin to investigate the influence of aromatic side chains on fibrillization.[37,87,94] These studies clearly showed that a minimal number of four residues is needed to form well ordered amyloid fibrils. More importantly, they hypothesized that the specific orientations of thermodynamically favored aromatic interactions provide specificity and directionality as well as an energetic contribution needed for the amyloid formation process. Besides this, Lynn and co-workers utilized the $A\beta_{(16-22)}$ segment to study protofilament lamination and the consecutive formation of higher order structures such as homonuclear bilayers and nanotubes.[95] These data highlight the potential of nature-derived segments for their application in the field of nanotechnology. In another approach, Radford and co-workers used several segments of β2-microglobulin as *in vitro* models to study their impact on the amyloid formation propensity of the full length protein.[96] Therefore, the β-sheet forming domains were identified on basis of the X-ray crystal structure synthesized independently and subsequently characterized by a multitude of biophysical methods. Interestingly, only one out of eight β-strands within the native β2-microglobulin structure yielded peptides that assembled into amyloid like fibrils. The obtained fibrils were furthermore shown to initiate the amyloid association of full length protein at various conditions. Due to the missing correlation between secondary structure propensity, length, pI, or hydrophobicity of the investigated peptides, the authors assumed an impact of the exceptionally high amount of aromatic residues within the amyloid forming peptide segments.[96] Analogous segment based strategies have been used to investigate the metal binding characteristics of amyloid forming proteins. Studies on $A\beta_{(10-20)}$ and $A\beta_{(13-21)}$ by Lynn and co-workers clearly showed that coordination of Zn^{2+} ions by histidine may act as a nucleus which initiates and accelerates the amyloid formation process.[66,97] Furthermore, structural conversions of $A\beta_{(1-16)}$ upon the addition of Zn^{2+} have been followed by solution state NMR spectroscopy at *in vitro*

ageing conditions.[98] These data showed that both Zn^{2+} coordination as well as isomerization caused by the ageing process have a strong impact on the structure of $A\beta_{(1-16)}$. Moreover, recent investigations of the recombinant human prion protein showed that the addition of Zn^{2+} and Al^{3+} accelerates the amyloid formation while Cu^{2+} and Mn^{2+} yield less aggregation, highlighting the tremendous impact of different transition metal ions.[99]

Besides these segment focused strategies, various studies applying scanning mutational analysis have been reported. The Wetzel group used proline, cysteine, and alanine scanning to characterize $A\beta_{(1-40)}$.[50,78,100] Especially in combination, these studies are very helpful for investigating structural stability and folding thermodynamics of amyloids. To gain information on the stability of the resulting fibrils, measurements on the critical concentration (c_r) have been applied. These measurements utilize the fact that $A\beta_{(1-40)}$ fibrils are in a dynamic equilibrium with residual monomeric $A\beta_{(1-40)}$ (see also section 2.2.3).[79] The amount of monomer in solution depends on the thermodynamics of the fibril elongation process and therefore represents the thermodynamic fibril stability. Since proline residues with their rigid, turn inducing and β-sheet breaking structure are highly disfavored in the extended β-strands of fibrils, destabilizations observed for proline mutagenesis, i.e., high values for c_r were interpreted to represent the impact of the proline on the ability of the peptide backbone to engage an extended β-chain.[50] From the obtained critical concentrations free energies ($\Delta\Delta G^{Ala\text{-}WT}$) have been calculated. Furthermore, the results obtained for the alanine scan were used to serve as a neutral control for the hydrophobicity of the proline side chain and yielded corrected $\Delta\Delta G^{Pro\text{-}Ala}$ values which, thus, exclusively show the backbone disrupting effect of proline. Due to the huge structural differences between alanine and proline, this assumption has to be questioned. However, the results obtained clearly identify positions that are sensitive and insensitive to proline substitution, which indicates the presence of β-strand forming segments and unordered domains in $A\beta_{(1-40)}$. These findings basically agree with solid state NMR observations and thus support the validity of this approach.

3.3.2 Rational Approaches

One of the most commonly used strategies to rationally modify amyloid proteins derived from natural sources is site-directed mutagenesis. In contrast to the previously described scanning mutagenesis approaches, only selected positions within the se-

quence of the particular protein are substituted. Some examples will be mentioned here. To study the correlation between *in vitro* structure and *in vivo* infectivity of HET-s prion protein Ritter et al. used site-directed proline mutation with a selection strategy based on solid state NMR.[43] Substitutions at loop positions yielded an invariably higher infectivity than usually observed for aggregating prions while disrupting mutations within β-strands resulted in almost no infection. These data indicate the strong connection between structural elements and prion infectivity. Also several mutations at different positions within the sequence have been used to study full length Alzheimer's $A\beta_{(1-42)}$[36] Chiti et al. reported a very sophisticated strategy to investigate the effect of mutations that modify the charge state of a protein without influencing hydrophobicity and secondary structure propensity of the polypeptide chain.[101] Therefore, the very well investigated human muscle acylphosphatase (AcP), which is known to be non-amyloidogenic at ambient conditions but prone to amyloid formation at denaturating conditions was modified by introduction, replacement and inversion of charged residues. Interestingly, it was found that aggregation was favored by mutations yielding proteins with a net charge closer to neutrality. This indicates a strong correlation between overall charge and amyloid formation propensity. Furthermore, many disease-related mutations have been shown to exhibit similar charge reductions and, as a result, higher aggregation rates. Combining these results with the finding that completely uncharged proteins possess a lower tendency to form amyloids (see also section 3.2) emphasizes the fact that a delicate balance between hydrogen bonds, hydrophobic interactions as well as coulomb interactions is needed to adopt the well defined amyloid structure.[91] In a later study, Chiti et al. used these mutation based results to calculate free energy changes which were subsequently compared with that of other mutated amyloidogenic systems such as Amylin, Prion peptides α-Synuclein, and Aβ.[102] Independent on the origin of the mutation, a strong correlation between charge reduction and increased amyloid formation tendency indicates the strong impact of the physicochemical side chain characteristics.

Furthermore, site directed mutations allow introduction of potential binding sites as shown by Krishnan et al.[103] In a rather biological approach, cysteine variants of the 250 residue Sup35 yeast prion were expressed and either labeled with charged iodoacetate, fluorescence active pyrene and acrylodan, or used for cross linking experiments. The degree of labeling efficiency and the fluorescence properties of the dye provided information on solvent accessibility and environment of cysteine residues at certain positions. Furthermore, double cysteine mutants have been used to identify intermo-

lecular contacts in prion fibrils utilizing the effect of shifted fluorescence maxima of pyrene molecules which are located in close proximity to each other. Using this methodology, the authors were able to identify characteristic intermolecular contacts observed for early aggregation events, and thus lead to a better understanding of the complex processes that occur during prion amyloidogenesis.

Beside these mutation based strategies, Baldwin et al. used amyloids of an 86 residue SH3 domain as a scaffold to align metalloporphyrine binding cytochrome b_{562} segments.[104] Although the results have been discussed in the context of materials science, they provide insights into the structure of amyloids. An SH3 tandem repeat $(SH3)_2$ equipped with a poly-glycine linker was fused to the cytochrome segment and expressed in *E. coli*. In terms of dimension and morphology the resulting amyloids were similar to that observed for isolated SH3 segments. Binding studies showed that a huge amount of metalloporphyrine can be displayed on their surface. Thus it appears likely that the incorporated cytochrome segments are located in an easily accessible manner on the outside of the fibril. Conversely to this, Dolphin et al. used a non-natural decapeptide scaffold to align modified $A\beta_{(16-37)}Y_{20}K_{22}K_{24}$ segments (Figure 3.1).[105] Therefore, lysine side chains within the scaffold were covalently bound to the C-terminal end of the modified $A\beta_{(16-37)}Y_{20}K_{22}K_{24}$-strands yielding four well adjusted β-hairpins aligned parallel to each other (Figure 3.1a). In contrast to the free Aβ segments, which remained unordered for hours, ligated $A\beta_{(16-37)}Y_{20}K_{22}K_{24}$ formed fibrils in the same time range, as shown by CD spectroscopy and ThT staining. Interestingly, the kinetics of fibril formation did not possess the lag phase usually observed for Aβ, which points to the huge impact of the scaffold. Whether this template assisted four-strand alignment mimics the earlier nucleation event cannot be described satisfactorily yet and further studies are needed to clarify this interesting aggregation behavior.

Since aromatic residues are thought to be one of the dominating factors that force molecules to adopt amyloidogenic structures, Jack et al. used a short fragment of the islet amyloid precursor peptide ($IAPP_{(20-29)}$) as a model to investigate aromatic interactions on a molecular level by solid state NMR.[106] In order to achieve signals which are selective for aromatic π-π-stacking, phenylalanine rings were deuterated (D_5-$IAPP_{(20-29)}$) or modified with a ^{19}F tag at position 4 (^{19}F-$IAPP_{(20-29)}$). ^2H NMR measurements indicated that the majority of aromatic groups have limited motional freedom and are thus likely to be buried within layers of the adjacent β-sheets. To achieve information on short range distances between the Phe rings, distance dependent, intramolecular dipo-

lar couplings between ^{19}F-labelled rings and deuterated rings have been measured. These data showed that the ^{19}F rings centers are situated below 6.5Å from one or two ^{2}H-rings and provided evidence for the presence of a π-stacking arrangement. Furthermore, long range distances were estimated by a ^{19}F multiple quantum experiment on fibrils exclusively composed of ^{19}F-IAPP$_{(20-29)}$ and showed a spin correlation of at least eight residues. Although more distance constraints are needed to accurately determine aromatic spacing within fibrils, this study emphasizes the huge potential of ^{19}F as a sensitive nucleus for the measurement of long range contacts.

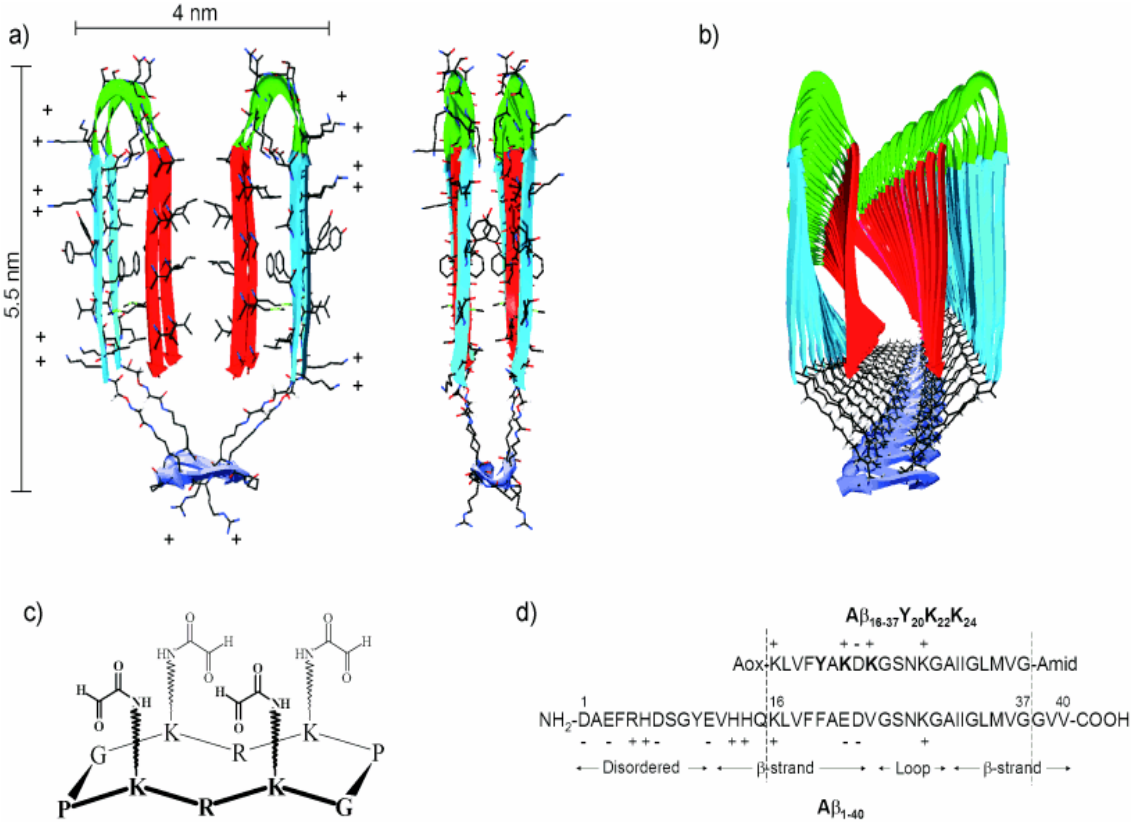


Figure 3.1. *(a) Modeled representation of (Aβ$_{(16-37)}$Y$_{20}$K$_{22}$K$_{24}$)$_4$, viewed along and perpendicular to the protofibril axis. (b) Representation of the quaternary structure for the (Aβ$_{(16-37)}$Y$_{20}$K$_{22}$K$_{24}$)$_4$ protofibril. (c) Chemical structure of the cyclic decapeptide scaffold with four glyoxyl-aldehyde-functionalized lysines. (d) Amino acid sequences of the Aβ$_{(16-37)}$Y$_{20}$K$_{22}$K$_{24}$ fragment and full-length Aβ$_{(1-40)}$. Mutated residues are shown in bold. Aox denotes the aminooxy function (according to G.T. Dolphin et al.)*[105]

Conversely to the previously described systems that were based on naturally occurring sequences with an intrinsic tendency to form amyloids, Van Hest and co-workers studied the self-assembly on basis of a peptide that does not form amyloids as an unmodified unit.[107] In order to introduce an aggregation propensity the GANPNAAG sequence, which was derived from a malaria parasite, was N-acetylated with alkyl chains differing in length. Modified with short acetyl residues (C_2-C_{12}) these peptides adopt an unfolded conformation at ambient conditions. Increasing the chain length of the alkyl chain to C_{14} yields a β-sheet conformation that reversibly unfolded upon temperature denaturation, while chain elongation to C_{16} results in a β-sheet peptide that was not able to be fully unfolded by heating up to 90°C. A similar behavior was observed for the corresponding C_{18} compound. During these experiments the authors noticed that an increased CD intensity of peptide C_{16} and C_{18} appears after cooling of the denatured samples which leads to the assumption that another, more rigid peptide structure is probably formed while cooling. Additionally, after heating above 90°C, these peptides did not unfold anymore at elevated temperatures. To shed light on this unexpected behavior, TEM was performed to investigate aggregates of heated and non-heated samples. Below a chain length of C_{14} exclusively amorphous aggregates have been observed, while samples of the corresponding C_{14}, C_{16}, and C_{18} analogs yielded untwisted fibrous aggregates with an hydrophobic exterior. Contrarily, after heating very rigid, twisted fibers have been observed which may be considered classical amyloids. Unfortunately, experiments to prove the presence of amyloids such as X-ray diffraction or staining with Congo red or ThT are missing. Nevertheless, it might be that the first fiber association step is nucleated by hydrophobic acetyl chain interactions, but once the amyloids are slowly formed after thermal unfolding and consecutive cooling, they dictate the stability and morphology of the resulting fibers. Thus, these data support the assumption that amyloids are thermodynamically stabilized aggregates.

3.4 De Novo Designed Models

Since many studies within the last 15 years have shown that *de novo* design approaches provide a huge potential for significant information on almost every biochemical issue, this method has gained increased acceptance. *De novo* design strategies are highly varied, ranging from small molecule mimicry of protein-ligand interactions to the design of complete enzymes with a well defined structure and enhanced activity. In its most challenging form, *de novo* design involves the “bottom-up” construction of a pro-

tein that should fold into a precisely defined 3-dimensional structure with a sequence which is unrelated to naturally occurring proteins. The complete range of applications has been described elsewhere and will not be highlighted here.[86,108,109] Due to the huge number of repeating patterns within fibrils an application of different *de novo* design strategies to generate amyloid forming model systems is self evident. In principle, two strategies have been developed during the last ten years which can be distinguished by their point of origin. The *de novo* design of amyloid forming models usually either starts with the implementation of repetitive patterns known from β-sheet folding or is based on the well investigated interaction principles of α-helical structures. Additionally, very recent studies on so-called switch peptides show that artificial bond rearrangement and migration could be used as amyloid model. The general principles and the outcome of these different approaches will be summarized and discussed in the following section.

3.4.1 β-Sheet Based Amyloid Models

Amyloids consist of extended β-strands which are aligned by hydrogen bonds along the fibril axis. Conversely, β-sheets do not necessarily yield amyloids.[91] Nevertheless, a β-sheet based design is one of the most obvious strategies for generating amyloid forming model peptides.[110] More importantly, β-sheet based approaches are widely used for the generation of self assembling peptides and proteins with nanotechnological applications. As these strategies are summarized elsewhere, only selected *de novo* systems will be highlighted here.[111]

The group of Hecht used a very minimalistic “binary code” approach to design different combinatorial libraries.[112-114] The design is based on the fact that all naturally occurring amino acids can be narrowed down to their intrinsic properties to divide them into two classes: polar (○) and nonpolar (●) (see also section 3.2). Applied for the classification of secondary structure elements, the “binary code” strategy can be used to point out intriguing general differences for α-helices and β-sheets. Helical structures usually possess a characteristic pattern of nonpolar residues (●) at every three and/or four positions which match the α-helical repeat of 3.6 residues per turn (●●○○●●○○●○○●●). Thus, peptides and proteins with this pattern fold into amphiphilic α-helices which assemble into intramolecular or intermolecular bundles resulting in a packing of the nonpolar faces.[115] On the other hand, amyloid forming peptides and

protein domains frequently show a rather simple, alternating pattern of polar and nonpolar amino acids (○●○●○●○). This results in a successive up-down-up-down alignment of polar and nonpolar residues which is characteristic for an extended β-sheet structure. Since the resulting strands possess both, a hydrophobic and a hydrophilic face, nonpolar residues are buried by aggregation into large assemblies which usually match the characteristics of amyloids. Early studies by Brack and Orgel on poly(Val-Lys) validate this concept.[116]

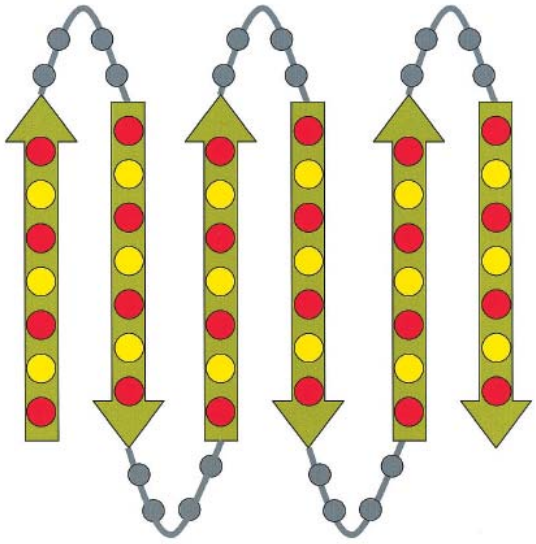


Figure 3.2. *The designed binary patterning for a combinatorial library of de novo proteins. Arrows designate β-strands, and gray loops designate reverse turns. Red circles represent positions that can be occupied by any of the polar amino acids His, Lys, Asn, Asp, Gln, or Glu. Yellow circles represent positions that can be occupied by any of the nonpolar amino acids Leu, Ile, Val, or Phe. Gray circles represent predefined turn residues (according to G. Xu et al.)*[113]

Using this structure based classification Hecht and co-workers developed a model system consisting of six antiparallel β-strands which were linked via five turns (Figure 3.2). After setting up this principle alignment, positions in the β-strands following the alternating pattern (○●○●○●○) were varied with His, Lys, Asn, Asp, Gln, Glu as polar and Leu, Ile, Val, Phe as nonpolar amino acids, respectively, yielding a combinatorial library.[114] From this library, several fibril forming, β-sheet rich proteins with an impressive amount of sequence heterogeneity have been obtained and demonstrate the protein like characteristics of these species and the validity of the design approach. Inter-

estingly, the fibril assembly process was shown to be reversible upon thermal denaturation, which lead the authors to assume that the obtained structure is dictated by thermodynamic stability and not kinetic trapping.[114] Additionally, more than 250,000 sequences from a database have been analyzed in order to identify binary patterns which are favored and disfavored in nature. The results clearly indicate that nature always disfavors alternating patterns, even in naturally occurring amyloids. This strongly supports the assumption that amyloid fibrils of proteins which are known from neurodegenerative diseases are doubtlessly misfolded species, since these proteins usually adobt globular structures capable of performing functions beneficial to the organism.

Furthermore, the obtained proteins have been used to study their folding behavior at the nonpolar/polar air/water interface in order to estimate their capability to serve as novel biomaterials.[113] Therefore, the protein was dissolved in helix inducing hexafluoroisopropanol, diluted with chloroform, and layered onto an air/water interface. Consequentially, the resulting structural conversion into amphiphilic β-sheet monolayers emphasizes the structure dictating role of the interface. Similar approaches for the generation of nanoscale-ordered monolayers at surfaces have been reported by other groups and are summarized elsewhere in an excellent review article.[111] More recently, the same binary pattern *de novo* approach was used to clarify the question whether amino acid substitutions that bury a charge at the hydrophobic intermolecular interface destabilize the fibril and subsequently prevent the oligomerization process.[112] Therefore, the alternating pattern (○●○●○●○) was interrupted by the introduction of one lysine residue at a central nonpolar position of the N-terminal or C-terminal strand (○●○K○●○). These second generation *de novo* proteins indeed formed monomeric β-sheet proteins and did not aggregate into fibrils at moderate concentrations. Since modifications were exclusively carried out at exterior strands of these β-sheet proteins, the authors assume that interruption of the hydrophobic face at these positions prevents amyloid association. It would be interesting to see if modifications at the internal strands of the sandwich (strand 3 and/or 4) yield similar results.

In an early study, Kelly and co-workers utilized an artificial bipyridine-scaffold that was modified with two antiparallel five residue peptide strands as mimicry for β-turns in β-hairpins.[117] Since bipyridine residues possess a high affinity for Cu^{2+} ion binding, this model was used to study the influence of Cu^{2+} coordination on the adapted conformation. Although these investigations were not focused on amyloids, the data clearly indicate that metal ion binding has the potential to nucleate the formation of β-hairpins and

their consecutive self-association. Later, the same group used a related strategy to study the occurrence of different aggregate morphologies that combine features of a binary peptide sequence pattern with a rigid, artificial dibenzofuran scaffold.[118,119] Two tetrapeptides with alternating Val-Thr sequence were coupled in a parallel manner via a linker to dibenzofuran molecules yielding peptidomimetic 1 (Figure 3.3 A). In the first and very detailed study this hairpin like molecule was shown to form fibrillar assemblies with different morphology depending on pH value and salt concentration. Isolated protofilaments, filaments, as well as twisted fibrils and ribbons were obtained. Based on these findings, the so-called interdigitated strand model was proposed to provide an explanation for the interaction between several protofilaments and the consecutive formation of structures with higher order morphology (Figure 3.3 B).

Applying this model, a protofilament of peptidomimetic 1 can be assumed to be a fiber that consists of interdigitated, antiparallel peptide strands with intermolecular hydrogen bonds perpendicular to strand direction and side chains, and the dibenzofurane scaffold parallel along the axis on the outside of the fibril. Strangely enough, this model does not explain why the observed fibrils and ribbons had widths that were multiples of 50-60Å rather than the expected 25-27 Å. Additionally inspired by investigations on Aβ, the authors realized that another quaternary structure was probably present. In contrast to classical β-hairpins, $A\beta_{(1\text{-}42)}$, peptide strands are turned inwards on themselves with intramolecular interactions between side chains, β-strands parallel to each other, and intermolecular hydrogen bonds along the fibril axis (see section 2.1.2.) In order to clarify whether such an alignment is present in fibrils obtained from peptidomimetic 1, different modifications have been carried out. These findings clearly indicate an internal protofilament structure that is distinctly different from the interdigitated strand model. Therefore, the authors have proposed the so-called side-chain hydrophobic collapse model as a possible explanation (Figure 3.3 C).[119] In this model, the peptide strands are aligned in a parallel manner with the scaffold perpendicular to the fibril axis. Since the dibenzofuran plane is exclusively presented on one side of the protofilament, filaments can be easily formed by interactions between these residues. As a result, a further formation of aggregates with higher order morphology can be directed by tuning interactions between the N-terminal functionalities of the peptide strands.

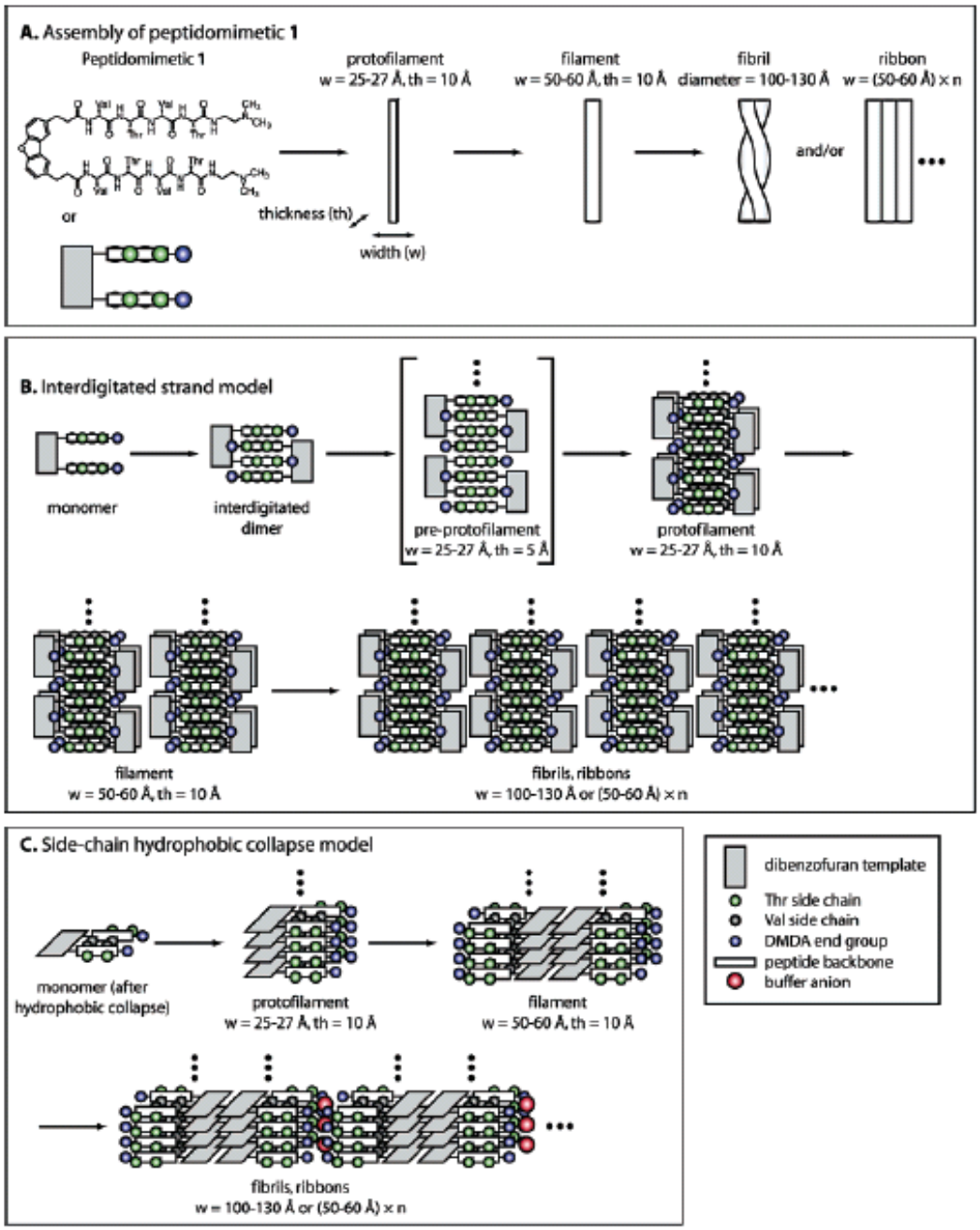


Figure 3.3. *(A) Structure of peptidomimetic 1 and its assemblies (protofilaments, filaments, fibrils, and ribbons). (B) Interdigitated strand model for the assembly of peptidomimetic 1. The backbone amides are in the plane of the white rectangles representing the peptide strands; neighboring white rectangles therefore imply intermolecular hydrogen bonding. (C) Side chain hydrophobic collapse model for the assembly of peptidomimetic 1. In contrast to the interdigitated strand model, hydrogen bonds are formed perpendicular to the dibenzofuran templates (according to S. Deechongkit et al.)*[119]

Size and nature of the scaffold can be changed to arrest the assembly process at the protofilament stage. Thus, this model enables a rich variety of ways to rationally alter the assembly morphology of such artificial systems. Design approaches for materials science and peptide engineering applications could especially benefit from this knowledge. From a more general point of view, moreover, these data suggest that hydrophobic as well as charged interactions between different protofilaments may determine aggregate morphology.

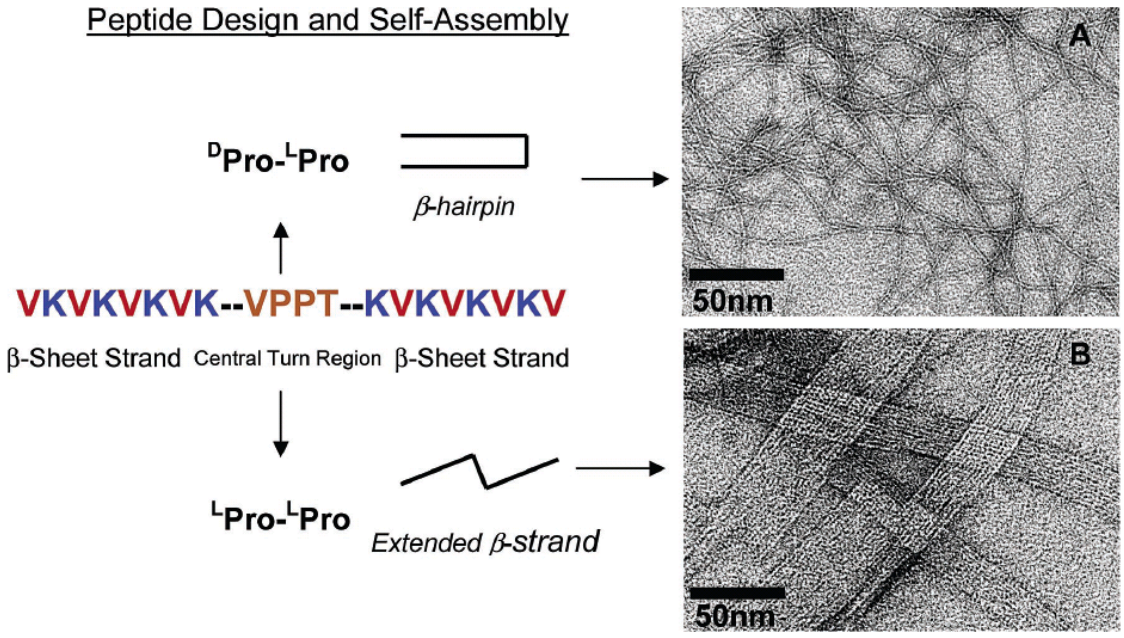


Figure 3.4. *Effect of turn sequence on peptide conformation and self-assembled material. Peptides with ^{D}Pro-^{L}Pro in the central turn region adopt a β-hairpin conformation and reversibly self-assemble into β-sheet rich hydrogels, whereas peptides with ^{L}Pro-^{L}Pro in the central region adopt an extended peptide conformation and irreversibly assemble into amyloid fibril precipitates. (A) TEM of negatively stained hydrogel of $(VK)_4$-V^{D}PPT-$(KV)_4$ revealing entangled fibrils each 3nm wide. (B) TEM of negatively stained precipitates showing the highly laminated, ribbonlike morphology of $(VK)_4$-VPPT-$(KV)_4$ fibrils (according to M.S. Lamm et al.)*[120]

Another approach addressing the aggregate morphology was reported by the Pochan group.[120-122] Based on an alternating Val-Lys pattern, several model peptides have been designed and studied intensively in terms of folding behavior and assembly into β-sheet rich hydrogels. The peptides consist of two β-sheet forming strands flanking a

Val-Pro-Pro-Thr sequence which was shown to form a type II' turn (Figure 3.4 A).[122] This sequence adopts an amphiphilic β-hairpin conformation that subsequently assembles into β-sheet rich hydrogels consisting of well defined, twisted nanofibrils. Various components within the peptide sequence (hydrophobic residues, polar residues, turn sequence) have been rationally modified in order to create functionalities which react upon changed environmental conditions such as pH and temperature.[121,122] Alteration of these parameters yielded unfolding and, as a result, re-dissolvation of the gel, which indicates the reversibility of this process. Contrarily, substitution of the ^{D}Pro residue at the central turn region with the L-enantiomer strongly inhibits the formation of the type II' turn and, thus, prevents formation and association of a β-hairpins.[120] The resulting peptide adopts an extended β-sheet conformation and assembles into fibrils with intermolecular hydrogen bonds and highly laminated, non-twisted, tape-like morphology. Unlike for the previously described peptides, these aggregates were stable at a wide range of temperatures and pH values and their formation has furthermore been shown to be entirely irreversible as observed for classical amyloids. Thus, the authors assume that this unique behavior is a direct result of the central Val-Pro-Pro-Thr unit. Beside potential applications in the field of nanotechnology these results impressively point the importance of turn regions for the amyloid formation characteristics of peptides and proteins with more than 15 residues.

In a very sophisticated theoretical model, Boden and co-workers classified the tendency of amyloid forming peptides and proteins to assemble into fibrillar aggregates with a higher order morphology.[65,123] Figure 3.5 shows proposed models for this hierarchical self-assembly pathway with the following principles: Amyloid forming peptides exist as unfolded species in solution below a critical concentration. Above this concentration, rod-like monomers, so-called chiral rod-like units, are formed as precursors which consecutively self-assemble via hydrogen bonds, and form long, twisted tapes (Figure 3.5 c and c'). Like the rod-like monomers, these tapes possess two complementary surfaces that are able to interact with different affinities and, thus, aggregate at elevated peptide concentrations. This association process strictly follows a hierarchical pathway with a sequence of untwisting, lamination, and twisting events that consecutively yield ribbons, fibrils, and fibers (Figure 3.5 d, e, f and d', e', f'). In order to compare the aggregation tendency of different peptides, several parameters have been defined to calculate phase diagrams. To prove this theory, several eleven residue peptides that are rich in glutamine were designed to obtain intermolecular hydrogen bonds that promote fibril formation (see section 2.1.2). The experiments clearly indicate that

the designed peptides behave differently but in accordance to the proposed theoretical model of an hierarchical step-by-step assembly, which suggests that the behavior proposed in the theoretical model might be a general characteristic for many amyloid forming species.[123] Further investigations on peptides that contained charged residues as trigger functions and, therefore, react upon altered ionic strength and pH values have been carried out.[65] Since these trigger functions instantaneously increase the absolute concentration of peptide molecules that can serve as rod-like monomeric precursors, the hierarchical assembly process is disrupted and several higher order species are simultaneously generated.

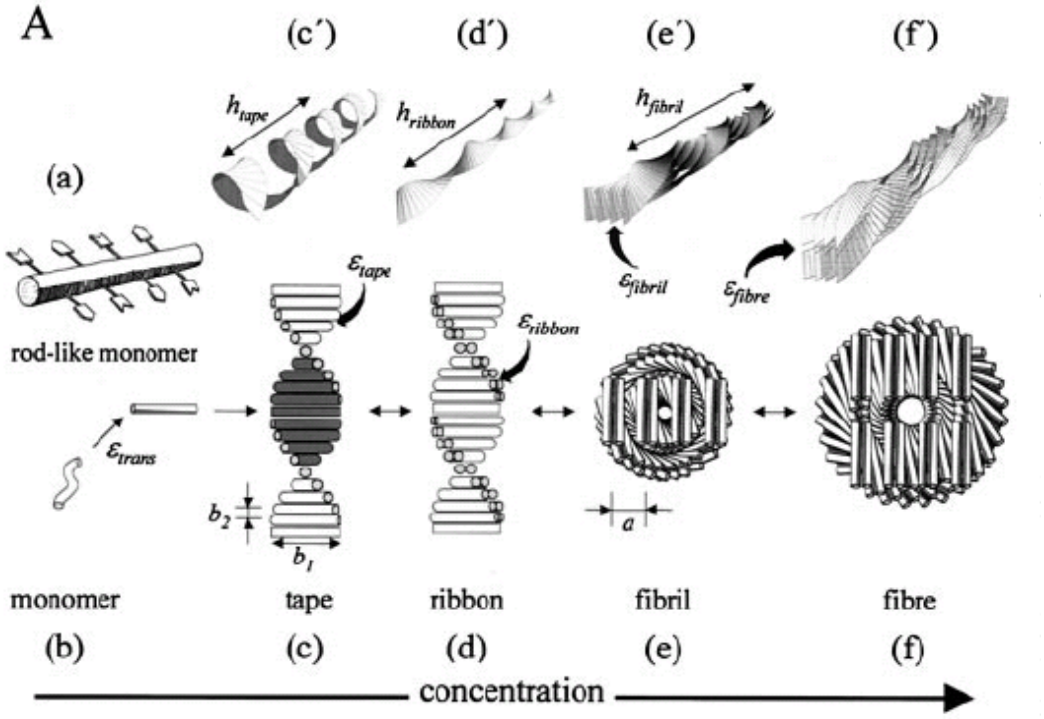


Figure 3.5. *Model of the hierarchical self-assembly of chiral rod-like units with drafts representing local arrangements (c-f) and the corresponding global equilibrium structure (c'-f'). In a solution of chiral molecules rod like monomers with complementary surfaces (a) are formed, that align to form tapes (c). The black and white surfaces of the rod (a) are reflected in the sides of the helical tapes (c), which is chosen to curl toward the black side (c'). The outer sides of the twisted ribbon (d), the fibril (e), and the fibre (f) are all white. One of the fibrils in the fibre (f') is drawn with a darker shade for clarity. (e and f) The front views of the edges of fibrils and fibres, respectively (according to A. Aggeli et al.)*[123]

3.4.2 Amyloid Forming Coiled Coils

In the native state, most of the naturally occurring peptides and proteins that convert into amyloids possess a defined structure that provides beneficial functions for the organism. During the amyloid formation process, these molecules at least partially unfold, subsequently refold, and associate into fibrillar species (see section 2.2.1). This conversion appears to be the most critical and sensible event during the course of amyloid association. A model peptide based investigation of the unfolding, refolding, and association process consequently requires systems that provide a well defined, non-amyloidogenic starting conformation. In contrast to β-sheet based systems, which are often focused on structural aspects of the resulting filaments and fibrils (see section 3.4.1), such models exhibit the potential to provide information on conformational transitions that occur at different stages of the amyloid formation process.

The most frequently used structural motif here is the α-helical coiled coil folding motif. Coiled coils are very common in nature and have therefore been extensively studied in the last 15 years.[124-127] Approximately 3-5% of amino acids in naturally occurring peptides and proteins are involved in the formation of coiled coil structures. α-Helical coiled coils typically consist of two to seven right-handed amphiphilic α-helices which are wound around each other forming a left-handed superhelical twist.[128] Figure 3.6 shows the helical wheel diagram and a schematic model of a dimeric parallel coiled coil. The primary structure of each helix is characterized by a periodicity of seven residues, the so-called 4-3 *heptad* repeat which is commonly denoted $(abcdefg)_n$. Positions a and d are typically occupied by apolar residues (Leu, Ile, Val, Met) that form a special interaction surface at the interface of the helices by hydrophobic core packing (“knobs-into-holes”). These hydrophobic interactions provide the main thermodynamic driving force for the coiled coil formation and furthermore dictate the oligomerization state (Figure 3.6 yellow).[129] In contrast, the positions e and g are frequently occupied by charged amino acids (most commonly Glu, Lys, and Arg) that form interhelical ionic interactions and, therefore, determine the parallel or antiparallel helix orientation (Figure 3.6 red and blue).[130] Polar residues are often found in the remaining *heptad* repeat positions b, c, and f, which are located solvent exposed at the opposite side of the motif. Since this structural motif is the essential basis for the peptides presented in this work, further information regarding the design principles and their implementation into amyloid forming model peptides are summarized in section 6.

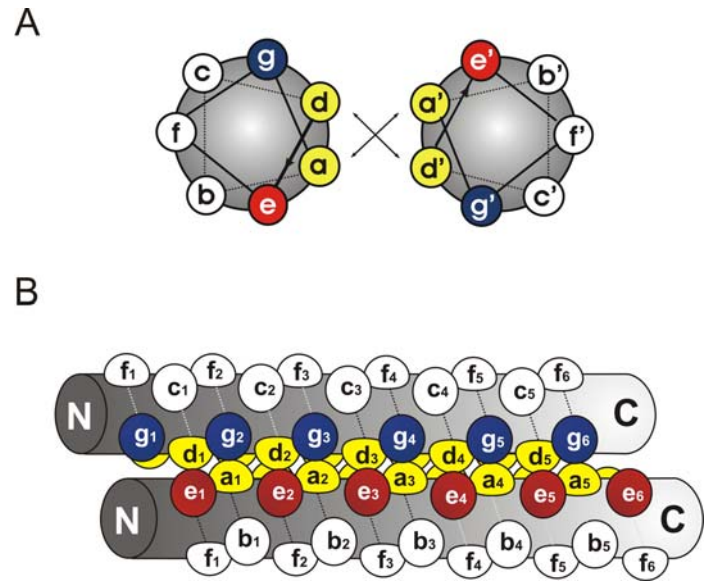


Figure 3.6. *Helical wheel diagram (A) and schematic model (B) of a parallel dimeric coiled coil. Yellow: hydrophobic residues at positions a and d; red/blue: complementarily charged residues at positions e and g; white: solvent exposed residues at the b, c, and f position.*

The idea to combine design features of α-helices and β-sheets is not new and was first described by Mutter and co-workers in 1991.[131,132] Although these groundbreaking studies do not utilize the principles of the classical coiled coil design, as has been mostly seen in recent studies, and were not focused on the generation of amyloid forming models, they nevertheless provide a basis for the generation of α→β conformational switches. Figure 3.7 summarizes how to fuse the design principles of α-helices and β-sheets. The design of these 16 amino acid peptides starts with an alternating pattern of hydrophobic (Leu) and hydrophilic (Lys or/and Glu) residues known from β-sheets (Figure 3.7 I), which is an unfavorable arrangement for helical folding (Figure 3.7 II). In order to improve the propensity to adopt a helical structure, certain positions on one side of the helical cylinder have been modified with alanine residues which are neither polar nor hydrophobic. This results in a primary sequence which is amphiphilic in nature with respect to an α-helical as well as a β-sheet conformation (Figure 3.7 III and IV). Based on these principles, four peptides with either lysine, glutamic acid, or a mixture of both as polar residues have been synthesized and studied at different pH values. All these peptides showed a pH-induced, reversible conformational transition from unfolded or helical structures to β-sheet conformations and vice versa. Since the secondary structure of these peptides changed upon alteration of the pH value, they have been termed “switch peptides.”

Later in 1996, the group of Gellman showed in a quite similar approach that the redox state also has a huge impact on the secondary structure of peptides.[133] Therefore, the fact that the intrinsic physicochemical properties of methionine side chains are perceptibly shifted from hydrophobic to polar upon oxidation to the corresponding sulfone or/and sulfoxide state has been utilized. Incorporation of methionine residues at the hydrophobic face within the helical wheel yields a peptide with a mainly helical conformation at reductive conditions. Contrarily, side chains of these residues are converted into polar species at oxidative conditions and receive a peptide that follows an alternating hydrophobic-hydrophilic-pattern instead and therefore folds into amphiphilic β-sheets. Although the term amyloid was not mentioned in both studies, the results of both switch peptide classes indicate that subtle changes in the environment have a huge impact on the amphiphilic nature of peptides and are therefore able to change the entire peptide conformation.

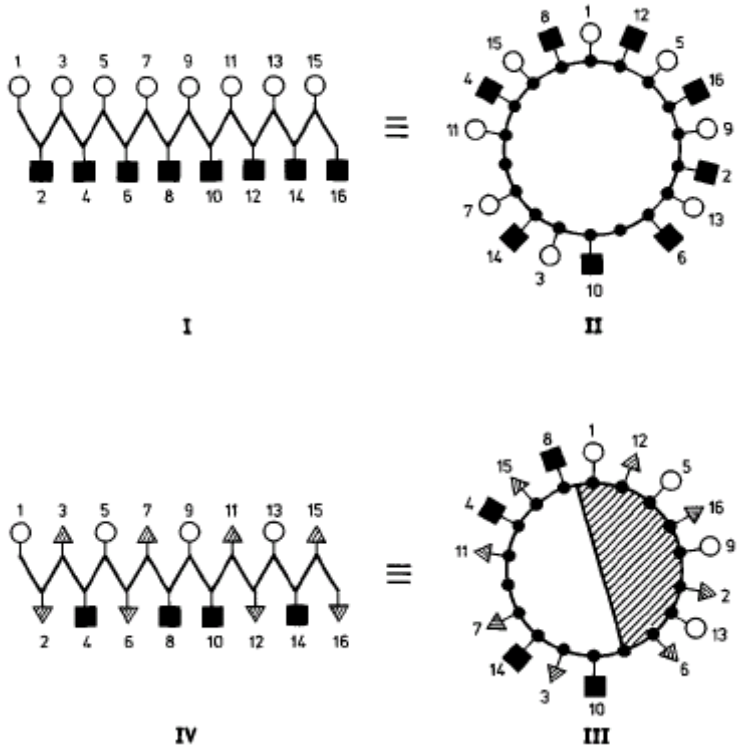


Figure 3.7. *Design of switch peptides. Left: schematic representation of a β-sheet conformation for the starting sequence (I) and the final switch peptide sequence (IV). Right: helical wheel diagram of the starting sequence and a final switch peptide sequence. Cycles, squares, and triangles denote hydrophilic, hydrophobic, and neutral residues respectively (according to M. Mutter et al.)*[131,132]

The first coiled coil based model system that focused on conformational transitions with respect to amyloid formation was developed by the group of Mihara.[134,135] This system has been investigated comprehensively and was used for various experiments and applications regarding the amyloid formation process.[134-140] The design is based on two 14 residue peptide strands that follow the characteristic pattern of an α-helical coiled coil (Figure 3.8).[135] These strands were C-terminally linked via a β-alanine spacer and an additional cysteine disulfide bridge. Furthermore, adamantane groups were coupled to the N-terminal end of each peptide strand to serve as a hydrophobic defect that initiates the conformational change and resulting amyloid formation. Capturing these hydrophobic residues by β-cyclodextrin complexation and substitutions with aliphatic chains differing in length demonstrated the validity of this concept.[139]

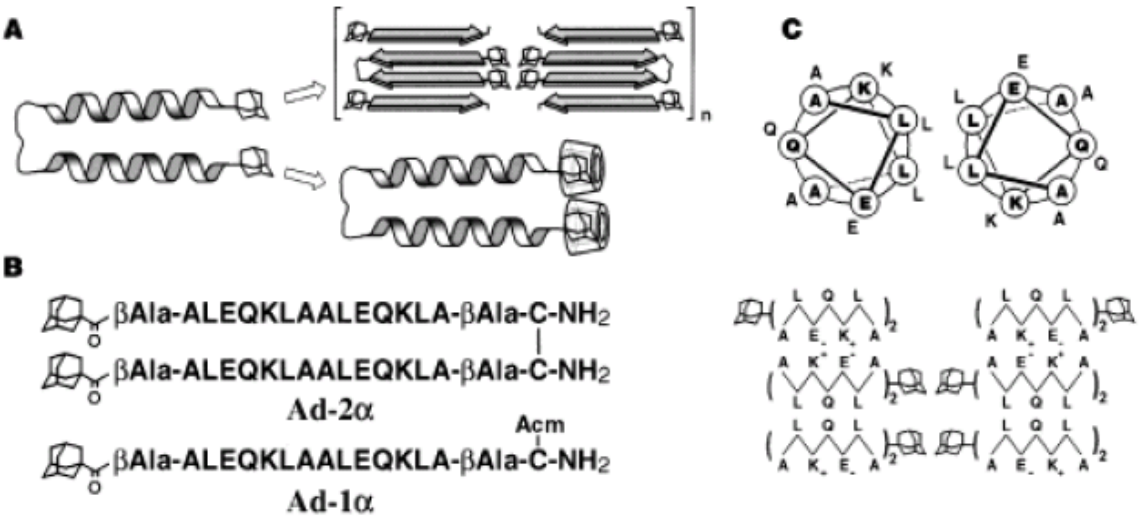
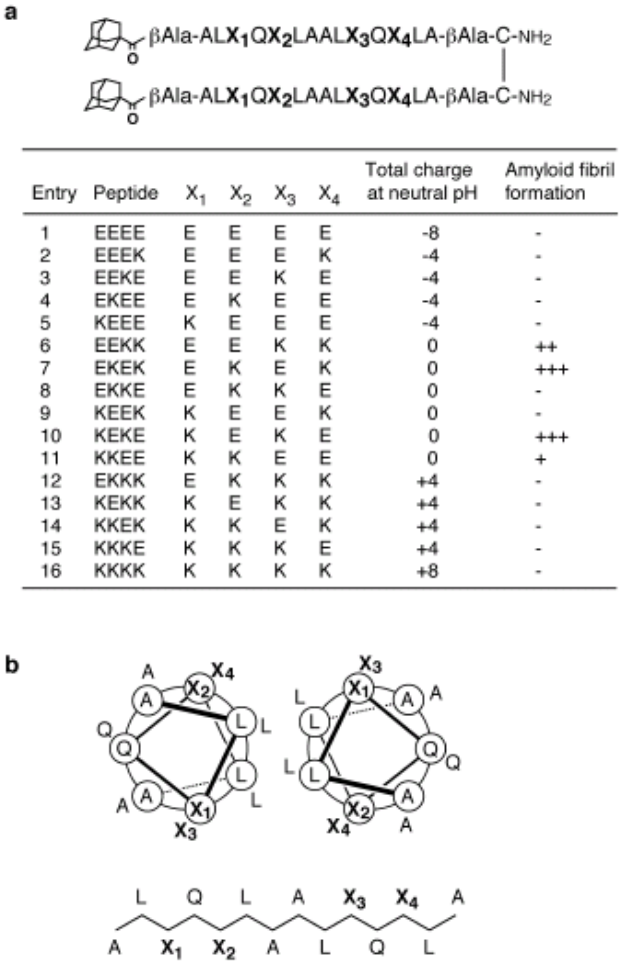


Figure 3.8. *Design of the α-β transitional peptide with a hydrophobic defect. (A) Schematic representation of the α-β transition of peptide Ad-2α and prevention by β-cyclodextrin. (B) Amino acid sequences of the peptides 1α and 2α with a 1-adamantanecarbonyl group as hydrophobic defect. (C) Helical wheel diagram (top) of the coiled coil form and an illustration of the β-sheet structure of the 14 residue core in the 2α-peptide (according to Y. Takahashi et al.)*[135]

The first and very extensive results showed that peptide Ad-2α indeed undergoes an α→β structural transition with consecutive amyloid formation within hours at neutral aqueous conditions. CD spectroscopy, dye binding, and EM measurements proved the appearance of amyloid-like aggregates which are formed by a nucleation dependent process as expected for a classical amyloid elongation reaction. Furthermore, the impact of differing pH values, salts and TFE concentrations was investigated. Summarizing these results, the authors came to the assumption that clustering caused by the hydrophobic defect may form unstable α-helical aggregates, which would then initiate the cooperative conversion into huge assemblies that are stabilized by intermolecular interactions.[135] Thus, the initial α-helical structure and its stability appear to be important determinants for the α→β transition and the consecutive amyloid formation in general.



Entry	Peptide	X_1	X_2	X_3	X_4	Total charge at neutral pH	Amyloid fibril formation
1	EEEE	E	E	E	E	-8	-
2	EEEK	E	E	E	K	-4	-
3	EEKE	E	E	K	E	-4	-
4	EKEE	E	K	E	E	-4	-
5	KEEE	K	E	E	E	-4	-
6	EEKK	E	E	K	K	0	++
7	EKEK	E	K	E	K	0	+++
8	EKKE	E	K	K	E	0	-
9	KEEK	K	E	E	K	0	-
10	KEKE	K	E	K	E	0	+++
11	KKEE	K	K	E	E	0	+
12	EKKK	E	K	K	K	+4	-
13	KEKK	K	E	K	K	+4	-
14	KKEK	K	K	E	K	+4	-
15	KKKE	K	K	K	E	+4	-
16	KKKK	K	K	K	K	+8	-

Figure 3.9. *(a) Designed peptides in the peptide library and their ability to form amyloid fibrils individually. (b) A helical wheel drawing of the peptide as coiled coil form and a β-strand drawing of the core 14 residue peptide (according to Y. Takahashi et al.)*[137]

Based on these findings each of the four charged residues within one strand was permuted by either lysine or glutamic acid to yield a combinatorial library of peptides with differing amyloid formation tendency.[137,138] Dissolved individually, only the minority of the obtained peptides formed amyloids at ambient conditions, which emphasizes a certain impact of Coulomb interactions on the fibril formation process.[138] Figure 3.9 shows the amyloid formation ability of the different peptides from the library.[137] Interestingly, although most of the peptides do not form amyloids if dissolved individually, mixtures of complementarily charged peptides do. For example, the EKKE / KEEK pairing, in which each species is unable to form fibrils independently, associates into amyloids in an equimolar mixture. Similar effects have been observed for pairings of EEEK/EKKK, EEKE/KEKK, EKEE/KKEK, and KEEE/KKKE, which indicates an antiparallel intermolecular arrangement within the amyloids or their intermediates. Similar investigations have been carried out for heteromeric mixtures of three or four peptide species. Recapitulating these results, the so-called “complementary assembly model” was proposed (Figure 3.10).

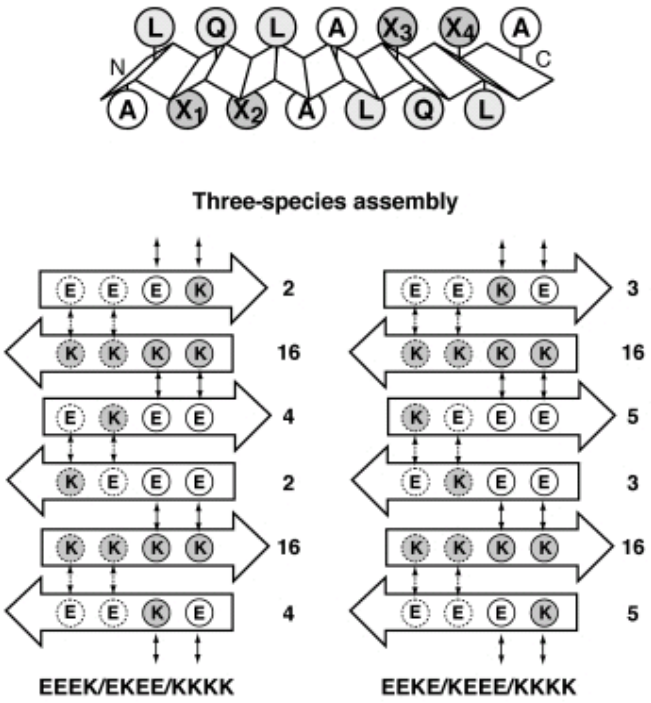


Figure 3.10. *Complementary assembly model of peptide triplets EEEK/EKEE/KKKK and EEKE/KEEE/KKKK. The β-strands are arranged in an antiparallel manner to maximize charge interactions. Solid circles denote residues on the front side, while dashed circles denote residues on the back side of the β-sheet. Hydrophobic residues are located on the opposite side. (according to Y. Takahashi et al.)*[137]

In this model, the charged faces of one β-strand and the hydrophobic faces on the opposite side are aligned in an antiparallel manner to the next strand, resulting in maximum overlapping between hydrophobic residues and complementary pairing between charged residues. In all these studies, only peptides or peptide mixtures following this rule of a complementary assembly formed amyloid fibrils. This indicates the significant impact of hydrophobic as well as electrostatic interactions on the amyloid association process. Nevertheless, although these results appear obvious and logical, a detailed characterization of the internal structure of these fibrils with methods such as solid-state NMR is still missing. In principle, an interdigitated alignment as proposed by Kelly et al. for another amyloid model (see section 3.4.1) could also serve as explanation. Furthermore, it is still unclear whether hydrogen bonds are intramolecularly formed as is known for classical β-hairpins or in an intermolecular manner as shown for many naturally occurring amyloids (see section 2.1.2). Additionally, the general applicability of the results obtained from these *de novo* designed peptides could be questioned, since the impact of the non-natural modifications must not be underestimated.

The studies conducted by the Mihara group, however, demonstrated that amyloid formation is promoted by electrostatic as well as hydrophobic interactions and, more importantly, that a heteromolecular fibril association is possible. In the future, this fact might afford the development of strategies for a site specific incorporation of functional groups or structural probes (such as isotopes, fluorescence tags, or spin labels) into amyloids. More recent studies utilizing the same model system have shown that binding cationic analogs of Ad-2α (e.g. Ac-KKKK-2α) can inhibit the amyloid formation process of Ad-EKEK-2α.[140] Furthermore, amyloids obtained from the monomeric, non-modified EKEK sequence can serve as a template for the self-replicating chemical ligation reaction of its fragments (EK-SBzl and CEK). Especially in the context of bioengineering, these findings of a self-catalytic function are of outstanding interest and outline the enormous potential of amyloids as novel biomaterials.[136]

In contrast to the previously described amyloid model that contained non-natural building blocks and linkers, Teplow and co-workers exclusively used proteinogenic amino acids for the *de novo* design of a 38-residue peptide with a similar α-helix-turn-α-helix structure (αtα).[141] At acidic conditions this peptide adopts a stable, protein like helix-turn-helix conformation and a completely unfolded structure was observed at highly basic conditions. Adjusting the pH to neutral conditions yields a partially unfolded conformation with a high α-helical content. Interestingly, elevated temperatures induced

the association into amyloids, but only at neutral conditions where the peptide is partially unfolded. The well folded helical conformation at acidic conditions as well as the entirely unfolded structure at basic conditions did not show any fibril formation. Thus, the authors came to the assumption that partially folded intermediates that do not necessarily contain an observable β-sheet structure can mediate the initial steps in amyloid formation. More importantly, investigations regarding the cytotoxicity and proteolytic stability of αtα fibrils clearly showed that amyloid formation does not implicitly indicate toxicity. Since the important role of early aggregates on the toxicity of amyloids[9] was not known at that time, these findings were interpreted as the result of different types of fibrils with unique biological properties. From the current point of view they rather appear to be a result of structural differences at the early stages of amyloid formation.

Moreover, the Woolfson group developed a huge number of different coiled coil based models.[67,142-144] Although only one of these studies is focused on the generation of *in vitro* amyloid models, they nevertheless provide valuable information for the design of coiled coil based conformational switches. In order to study the α→β structural transition, a series of peptides was created by a positive design approach containing features of a dimeric α-helical coiled coil and a β-hairpin.[143] Figure 3.11 shows the design principles and the obtained sequences.[143] Using the established rules of coiled coil design with hydrophobic residues at the a and d position and charged residues at e and g position, Template-α was designed to form a parallel dimeric coiled coil. Furthermore, the *heptad* repeat was flanked by cysteine residues, which, at oxidizing conditions, form intramolecular disulfide bonds that move the strands in close proximity to each other and, therefore, favor the alternative β-hairpin conformation. As expected, Template-α forms a dimeric coiled coil at moderate concentrations (100μM) and neutral buffer conditions. Furthermore, thermal unfolding experiments showed an almost completely reversible α-helix → random coil → α-helix transition. Since threonine has a higher β-sheet propensity than glutamine, the six f positions were replaced in order to reduce the stability of the α-helical coiled coil. Temperature induced denaturation experiments confirmed this assumption. Interestingly, cooling down the sample after thermal unfolding at higher peptide concentration (300μM) yielded a structural transition from an unfolded conformation to a β-sheet. Additionally, electron microscopy revealed the presence of aggregates with characteristic amyloid morphology. To clarify whether this amyloid formation is a direct result of the coiled coil unfolding process, Template-αTu was synthesized. This peptide contains an uncapped C-terminus and a free carboxyl group at the C-terminus to destabilize the helical structure. At ambient

conditions and high concentrations (300µM), an unfolded conformation was indeed obtained, but unexpectedly no fibrils were formed. Again amyloid formation has only been observed at elevated temperatures and the transition temperature strangely increased from 70°C (Template-αT) to 80°C. Thus, the authors came to the assumption that there is no correlation for this system between destabilization of the native conformation and fibrillogenesis. Furthermore, the question arose whether the transition is affected by an increase of the β-hairpin propensity at oxidizing conditions. This was the case, since the oxidized forms of Template-α, Template-αT and Template-αTu exhibited a structural conversion, even at lower concentrations, but elevated temperatures were still necessary. This requirement of thermal unfolding highlights the involvement of the hydrophobic effect which has previously been discussed as major contributor to amyloid formation.[134,135]

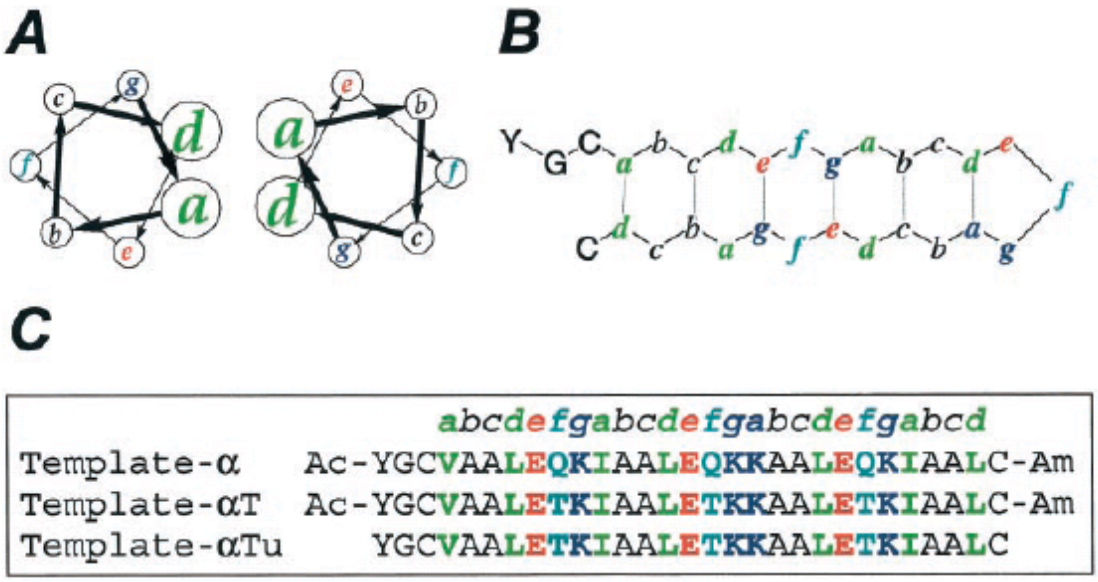


Figure 3.11. *Design principles and designed peptide sequences. (A) and (B): Schematic representations of a heptad repeat sequence configured onto an helical wheel and a β-hairpin. (C): Designed peptide sequences. Green: hydrophobic residues, dark blue: positively charged lysine, red: negatively charged glutamate, light blue: positions where Gln to Thr substitutions were made (according to B. Ciani et al.)*[143]

With quite similar rational design approaches, the same group generated several model peptides that, depending upon different environmental conditions, were able to adopt two stable conformations. In one study, disulfide bridges were again utilized to

switch between a dimeric coiled coil and an α-helical hairpin conformation.[142] Furthermore, design attempts combining the general features of the coiled coil folding motif and the zinc-finger structure highlighted the impact of transition metal binding on the resulting secondary structure (see also section 3.2). More importantly, several experiments from this lab proved the existence of fibrous coiled coil assemblies with a keratin-like super structure.[144,145] Although these α-helical fibrils are not directly related to amyloids, they provide valuable information for understanding coiled coil interactions and assembly pathways and should therefore be mentioned here.[86,144-146] Additionally, working with coiled coil based amyloid models always requires consideration of such fibers as an alternatively formed structure. The design is based on the principles of coiled coil folding. Figure 3.12 shows the design idea, the resulting sequence, and a computer modeling structure of the self-assembling fiber.[144]

Two 28 residue peptides, SAF-p1 and SAF-p2 have been designed for a heteromeric assembly with “sticky ends.” In both peptides, isoleucine was placed at position a, and leucine at position d to encourage a dimeric oligomerization. Oppositely charged residues at e and g position were used to promote a staggered heterodimeric alignment with complementary overhangs (Figure 3.12 B grey). Additionally, asparagine residues introduced at different a positions preferentially pair with each other in the core and therefore ensure the off-set register of the two peptides. Both peptides when dissolved individually possessed a random coil conformation, while their mixture, even at low concentrations of 10μM, exhibited a characteristic α-helical CD signature. Electron microscopy measurements revealed that long fibers are indeed formed in this mixture. Interestingly, in contrast to classical amyloid fibrils, these fibers showed a branched morphology. Furthermore, X-ray diffraction data clearly indicated that the obtained assemblies are not amyloids, since neither the characteristic 4.7Å nor the 10Å reflection was present. Instead, a meridional reflection at 5.13Å, that represents the distance between two coiled coil turns and an equatorial reflection at 9.2Å characterizing the spacing between alpha-helices in coiled coils has been observed. Heteromeric assemblies of peptides equipped with different fluorescence dyes, on the other hand, showed polarity for these fibers.[146] This is consistent with the sticky-end assembly model, since α-helices justified in one direction along a fiber are supposed to possess a dipole moment different from zero. Recent investigations highlighted that the thickness and morphology of these fibers can be controlled by altering the conditions under which they are grown.[145] Thus, the enormous potential of these SAF-peptides to serve as self assembling nano-materials is fairly obvious.

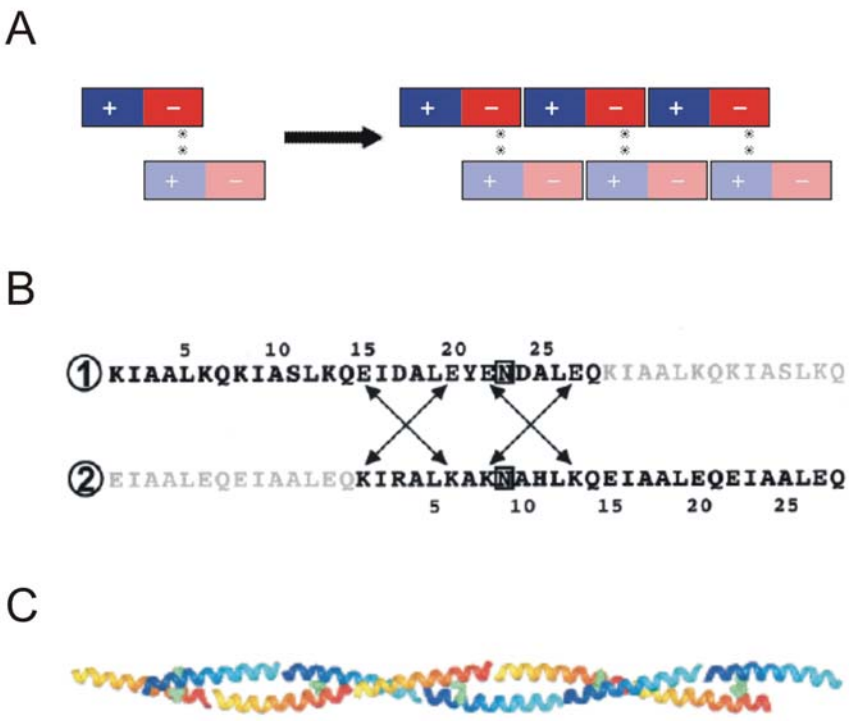


Figure 3.12. *Sticky end assembly of coiled coil peptides. (A) Design principles with electrostatic interactions depicted in red and blue and asparagines pairings denoted by an asterisk. (B) Sequence of the peptides SAF-p1 (1) and SAF-p2 (2) as a coiled coiled heteromer with sticky ends. Arrows indicate electrostatic attractions. (C) computer modelling structure of the designed self-assembling fiber. SAF-p1 is colored yellow-to-red and SAF-p2 is colored blue-to-cyan (according to M.J. Pandya et al.)*[144]

Analogous α-helical fibers have also been designed and investigated by the group of Kajava.[147,148] Several methods ranging from circular dichroism to electron microscopy and X-ray diffraction have been applied for their characterization. In contrast to the previously described heteromers, these peptides were based on a homo-oligomeric coiled coil pentamer with a hydrophobic core that exclusively contained leucine residues. As a result, the obtained fibers exhibited a width that correlated with the characteristic cross section of a five-stranded helical arrangement.

Getting back to the field of classical amyloids, Kammerer and collaborators more recently described the design and characterization of several amyloid forming model peptides which were based on native-like dimeric or trimeric coiled coils.[149-151] In principle, this approach is comparable to those reported by the groups of Mutter and Gellman for non-amyloidogenic switch peptides, because it fuses the characteristics of two structural motifs – amphiphilic α-helices and β-sheets. Figure 3.13 shows the general design strategy.

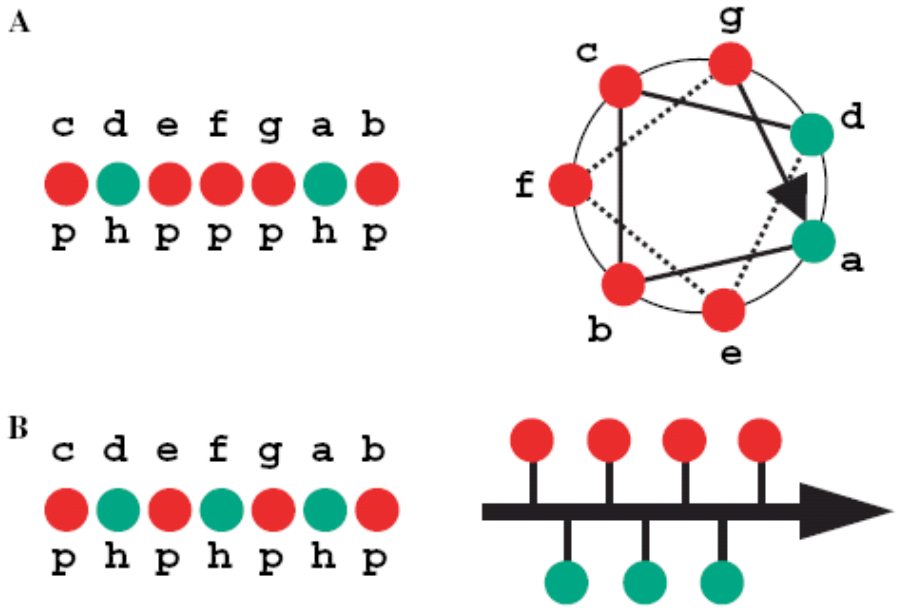


Figure 3.13. *Design of an amyloid forming, dimeric coiled coil. (A) To match the amphipathic α-helical coiled-coil pattern of 3.5 residues per turn, polar (p, red spheres) and nonpolar (h, green spheres) amino acids are placed at the a and d positions of the heptad repeat. (B) The binary pattern of amphipathic β-strands is obtained by placing polar and nonpolar residues in an alternating manner. Short stretches of such binary sequence patterning can be superimposed onto the coiled coils heptad repeat incorporating hydrophobic residues at solvent exposed f positions (according to R.A. Kammerer et al.)*[149]

As in the previously described studies, the coiled coil *heptad* repeat pattern was combined with the strategy of a β-sheet preferring alignment of alternating hydrophobic and hydrophilic residues. Starting with an idealized design of a 17 residue coiled coil peptide (Figure 3.13 A), hydrophobic alanine, leucine, or methionine residues have been incorporated at f position. Since these positions are exposed to the solvent on the out-

side of the helix and usually occupied by polar amino acids, an incorporation of non-polar residues would represent an unfavorable state for α-helical folding. Additionally, replacing these residues yields *heptad* repeat compartments (cdefgab*)* following the binary pattern of hydrophobic and hydrophilic residues, which are known to induce amyloids (Figure 3.13B). A systematic approach classifying the impact of different amino acids at f positions of coiled coil peptides has been described very recently.[152] However, applying this strategy, peptides with either a dimeric or a trimeric coiled coil oligomerization state have been designed, synthesized, and characterized by various methods. All peptides formed stable coiled coil structures at ambient conditions and associated into amyloids exclusively at elevated temperature. Especially the model peptide based on a trimeric coiled coil was studied extensively.[150] The helical structure was solved by X-ray crystallography and the corresponding amyloid form was studied by several techniques such as X-ray diffraction, electron microscopy, and solid state NMR spectroscopy. These experiments support the presence of a classical laminated cross-β structure (see section 2.1.2) and yielded high resolution data which were subsequently used as a basis for a structural model describing the internal geometry within a protofilament. In this model, the peptide strands are fully extended and aligned in an antiparallel manner and with a slight register offset. Additionally, hydrophobic interactions, as well as salt bridges between residues of adjacent strands, appear to govern the well defined relative geometry in protofibrils and their laminated higher order derivatives.

To recapitulate, coiled coils and helical peptides in general have recently attracted interest for the development and investigation of structural switches and *in vitro* amyloid models with a protein like conformation in the native state and a well defined amyloid structure in the aggregated form. Different environmental factors such as altered pH, oxidative conditions, and the presence of metal ions have been shown to trigger conformational transitions in coiled coils. Nevertheless, unnatural modifications and conditions that are far away from nature often limit the general validity. In all the described systems the transition into amyloids was triggered by elevated temperatures precluding the direct observation of amyloid formation processes at native-like conditions. This emphasizes the necessity for a further development of coiled coil based amyloid models that are able to mimic structural conversions and fibril formation processes under natural conditions.

3.5 Switch Peptides

In 2004, Mutter and co-workers reported a groundbreaking strategy for the generation and investigation of structural conversions in peptides and proteins.[153,154] Although, this *"*switch-peptide in *statu nascendi"* approach is not primarily focused on the development of *in vitro* amyloid models, it is valuable for understanding of conformational transitions and protein aggregation pathways and should therefore be mentioned here. Figure 3.14 shows the general conception of the switch peptide strategy.

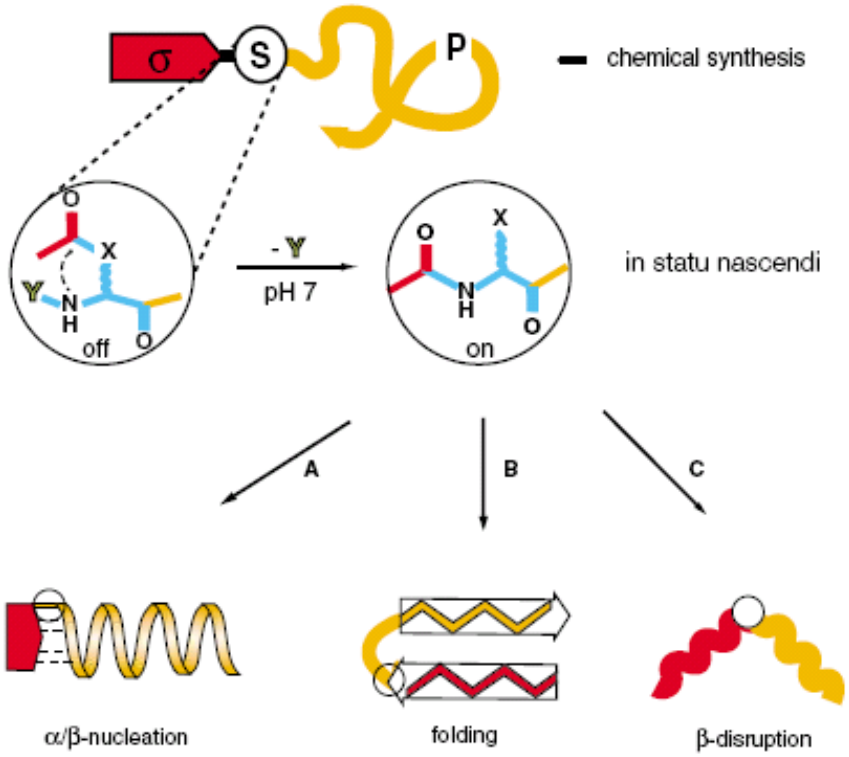


Figure 3.14. *In statu nascendi induced conformational transitions. By removal of Y (enzymatic or by change of pH), X-N acyl migration is triggered. This leads to the formation of a regular peptide bond between σ and P and results in a change in conformation, typically from a flexible random-coil (S_{off}) to a folded (S_{on}) state. By application of peptidomimetics and templating effects, these conformation changes can be used to study the s-induced nucleation (A), onset (B), and disruption (C) of secondary and tertiary structures (according to M. Mutter et al.)*[153,154]

In the context of peptide conformations, the term "*in statu nascendi*" (the Latin phrase means literally "in the status birth," i.e., being just born) describes the very early structural state of a peptide during the process of folding. In other words, the *statu nascendi* represents the conformational state of a peptide or protein which is present at time point zero during the dynamic process of structure formation. In order to conserve this particular molecular arrangement, the artifice of a newly formed peptide bond provided by site-directed X-N acyl migration has been applied. Therefore, two peptide segments (σ and P) were coupled via a switchable building block (Figure 3.14 S) with an ester (X=O) or thioester (X=S) bond and a protected α-amino group. At these S_{off} conditions both segments adopt their conformation independently from each other, since they are connected artificially and non-peptidic. Photolytic, enzymatic, or pH-induced cleavage of the protecting group (S_{on}) Y initiates an X-N acyl migration process that yields a new peptide bond. As a result, the two peptide fragments are no longer disrupted by the non-natural building block and, thus, rapidly respond with the formation of the full length peptide structure.

The switch peptide strategy is an excellent tool for various applications, ranging from nucleated conformations to folding and structure disruption studies (Figure 3.14 A, B, and C, respectively).[153-157] Since the onset of the folding process is clearly defined by the *"statu nascendi"* conditions, this approach is especially beneficial for investigations addressing the dynamics and kinetics of the amyloid formation process (Figure 3.14 B). Hence, the random coil to β-sheet conformational transitions of several peptide segments from naturally occurring amyloid forming proteins, such as the Amyloid β (14-24) peptide and the islet-protein-derived NFGAIL sequence, have been studied utilizing the switch peptide methodology.[153-155] Moreover, recent investigations from the Mutter group showed that fibrils of the Amyloid β (14-25) fragment can be redissolved using α-helix inducing elements.[156,158] Therefore, the helix nucleating Ncap residue (acetylated, cyclic pentapeptide KARAD) was coupled to the Aβ fragment via a serine derived, N-protected switch element. Subsequently, the peptide was dissolved and the resulting amyloids were clearly characterized by several methods. Interestingly, the formation of a new peptide bond by removal of the protecting group from the switch element redissolved the preformed fibrils as shown by electron microscopy and ThT staining experiments. Additionally, the structure of the resulting peptides was shown to be rich in α-helix. These experiments strongly support the findings from the amyloid recycling hypothesis (see section 2.2.3) and, therefore, emphasize the dynamic nature of amyloid fibrils. Similar structure disruption could be used as tools for the investigation of the

molecular mechanisms of amyloid formation *in vivo* and be utilized for developing novel therapeutic strategies in the future.

Another advantage of the switch peptide methodology has been applied in the field of SPPS, often referred to as so-called “click peptides.”[159-162] Difficult sequences which aggregate on the solid support during the synthetic build up can be equipped with switchable elements at critical positions. After cleavage from the resin and a usually much easier purification, these peptides can be transferred into their native conformation by activation of the switch element’s X-N acyl migration trigger. These approaches have been very well established in recent years and are widely used for the synthesis of peptides, such as Amyloid β (1-42), which are badly or even not accessible by SPPS.[159-161] Additionally, recent investigations showed that the esterification procedure (X=O) which is used for the introduction of the switch element can be performed by peptide synthesizers affording a fully automated synthesis of switch containing sequences.[163]

In a rather materials science related approach, the group of Boerner used the switch peptide methodology for the peptide guided self-assembly of an organo soluble peptide-polymer conjugate.[164,165] Therefore, amyloid inducing Thr-Val-Thr-Val segments were coupled via depsi-peptide bonds and subsequently equipped with a poly(n-butyl acrylate) polymer chain. Dissolved in methanol and without activation of the switch, the peptide adopted an unordered conformation that did not exhibit any ordering effects on the polymer chains and, thus, yielded a solution with the biophysical properties of a liquid. In contrast, increasing the pH value above 6 triggered the O-N acyl migration and, as expected, resulted in the formation of fibrils that aligned the fastened polymer chains. This ordering effect changed the biophysical properties of the methanolic solution tremendously and yielded gel-like characteristics. This study exemplifies the enormous potential of the switch peptide method for the development of novel peptide based biomaterials.