1. Introduction and Outline

Due to the enormous plurality of biochemical reactions and the complex interplay of different compartments within a cell, proteins and enzymes belong incontrovertibly to the most important biomolecules of living organisms. Based on their structural variability they are able to accomplish a broad variety of functions like catalysis, regulation and transport of metabolites.[4] They play a major role in signal transduction networks and are involved in cell-reproduction as well as apoptosis. The broad functional spectrum fundamentally results from the ability to establish defined secondary structure elements. The importance of hydrogen-bonds between CO- and NH-groups in the formation of secondary structures was discovered by PAULING and COREY in 1951. They were able to show that α-helices and β-sheets are general and fundamental parts of most proteins.[5,6] The three dimensional spatial structure is additionally influenced by a variety of physico-chemical interactions, e.g. VAN-DER-WAALS interactions as well as π-stacking of aromatic residues within the sequence, respectively. Roughly one-third of all proteins could be considered to be integral membrane proteins.[7] They are mainly operating as active and passive carrier-proteins, ion-channels, and pores as well as in signal transduction pathways across membrane barriers. Likewise the membrane insertion of nascent proteins and posttranslational modifications like glycosylation were performed by membrane proteins. Hence, they are an interesting and more and more important target within pharmaceutical product development.

1.1 Membrane Properties

In the early seventies, SINGER and NICOLSON postulated the first model-structure of a biological membrane.[8] The so-called `fluid mosaic model´ describes the spatial orientation of phospholipids as a two-dimensional solution. Therefore, the hydrophobic acyl chains are assembled facing each other whereas the hydrophilic headgroups are orientated to the aqueous phase forming a natural boundary.

Elucidation of thermodynamic parameters of the assembly process are performed with simple model systems mimicking a natural membrane. However, a membrane is not a covalently linked bio-polymer, and thus, has to be assembled by a certain amount of lipid molecules. Therefore, a critical micelle concentration (*CMC*) has to be provided to induce self-assembly of membranes. The decrease in entropy caused by every aggregation process is compensated upon reaching the *CMC* by avoiding the hydrophilic/hydrophobic contact at the acyl-chain/water interface. A value of □10.5 kJ/(mol·nm^2) was calculated for this kind of interaction.[9] The first aggregates which are formed upon reaching the *CMC* are micelles as well as uni- and multi-lamellar vesicles. Decreasing the water concentration causes aggregation into a broad variety of structures which possess long range order.
The most common phases observed in various lipid systems are the *hexagonal phase* (*H1*), the *lamellar phase*, the *bicontinuous cubic phase*, the *inverted hexagonal phase* (H_{II}) and the *inverted cubic phase* (Q_{II}) (Fig. 1.1).[10]

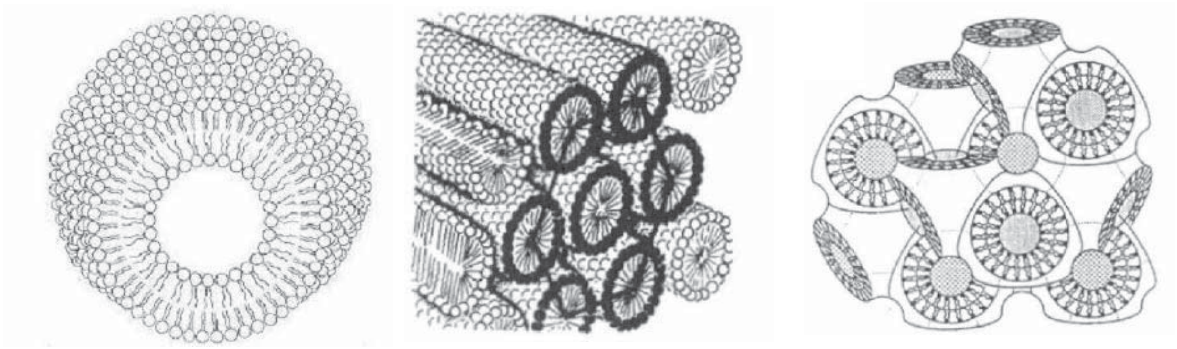

Fig. 1.1 Schematic representation of some lipid aggregates: Left) unilamellar vesicle; middle) hexagonal phase (H_{II}); right) inverted cubic phase (Q_{II}).[9] Figure is modified from reference source.

All experiments in the presented work were performed within the *lamellar phase* applying phosphotidylcholine lipids (e. g. dilauroyl-*sn*-glycero-3-phosphatidyl-choline (DLPC) and dimiristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC)), and therefore, the *lamellar phase* should be explained in more detail. Within the *lamellar phase* of a single lipid type, the arrangement of headgroups and lipid acyl chains can differ due to variation in temperature as well as variation in the relative humidity. The dependency on the temperature and relative humidity is expressed by the thermotrophic phase behavior.[11]
For example, a simple lipid with fully saturated acyl chains, such as DMPC, exhibits distinct thermodynamic transitions between the *gel* ($L_{\beta'}$), *rippled gel* ($P_{\beta'}$) and *liquid crystalline* (L_α) phases in water.[11] A common method used for investigation of such phase transitions is the *differential scanning calorimetry* (DSC).[11]

Figure 1.2 illustrates a typical DSC enthalpogram recorded for DMPC. Within the *gel phase* ($L_{\beta'}$) the acyl chains are tilted and packed into a quasi-hexagonal lattice.[12] The cross sectional plane per headgroup is increased with respect to fully extended acyl chains.

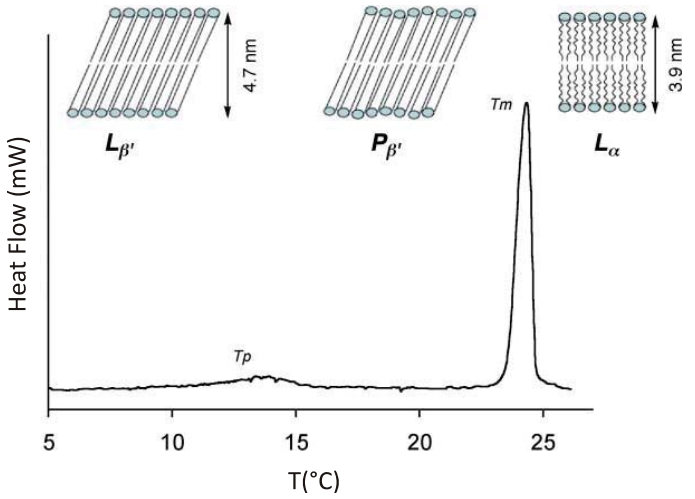

Fig. 1.2 Pre- and main-phase transitions observed for DMPC via *differential scanning calorimetry* DSC.[11] Figure is modified from reference source.

While increasing the temperature the pre-transition (T_p) occurs with enthalpies of the order of 3 kJ/mol.[11] Reaching the non-planar *rippled phase* ($P_{\beta'}$) the bilayer displays a periodic curved superstructure with a periodicity of 140-200 Å.[13] The interfacial area per headgroup is increased due to the displacement of every lipid molecule along the *z*-axis. Changing from *the rippled phase* to the *liquid-crystalline phase* (L_{α}) is denoted as main transition (T_m) with enthalpies in the range of 25 kJ/mol.[11] The formation of *gauche* rotamers becomes favorable due to the associated entropic decrease in free enthalpy. This so-called chain melting shortens the effective acyl chain length, and therefore, induces a lateral expansion and diminution of the bilayer.[14] In case of DMPC, both phase transitions are reversible. Since the *liquid-crystalline phase* is believed to be the most important in physiological relevant systems all described experiments were performed ensuring this fluid phase.

According to SINGER and NICOLSON proteins as well as lipids are capable of free diffusion parallel to the membrane normal within the *liquid-crystalline phase*. To this day, many properties of biological membranes can be described with this simple model. A refinement of the model by introducing micro domains of specific compositions can explain phenomena like signal transduction and membrane trafficking.[15] The so-called *membrane rafts* can be promoted by complexation of Ca^{2+}-ions, the affinity of certain lipids to interact with proteins, variation of the temperature or by changing the pH value of the surrounding media.[16,17]

1.2 Peptides in Membranes

Despite strong efforts of many research groups, the complex interactions between membrane-spanning proteins and the lipid bilayer are still poorly understood. Usually simple model systems involving short peptides in artificial membrane systems are utilized to investigate basic principles of protein-lipid interactions. Pioneering work was performed by KILLIAN et al. introducing the so-called KALP and WALP model peptides.[18] The direct influence on the membrane morphology was shown applying these peptides to various model membranes each providing a different membrane thickness. The peptides consist of an alanine-leucine sequence that is flanked by lysine or tryptophan residues. In natural transmembrane-proteins, tryptophan residues were enriched at the membrane/water-interface, whereas charged lysine side chains are generally located more outwards.[19] Thus, the effective hydrophobic length of the model peptides can be elucidated from the minimal acyl chain length required to induce formation of the inverted hexagonal (H_{II}) phase. Furthermore, due to the influence of the bilayer thickness on the functional activity of a broad range of membrane proteins,[20] these model peptides represent a valuable tool for investigating a phenomena denoted as *hydrophobic mismatch*. Single lipid systems were frequently utilized for the investigation of the mismatch response.[21]

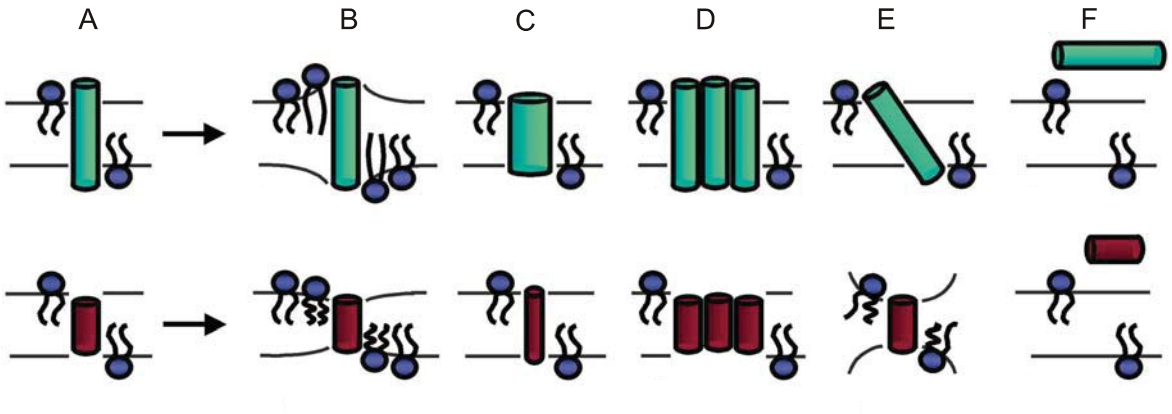

Fig. 1.3 A) Schematic representation of possible responses due to a positive or negative mismatch between a transmembrane α-helical peptide and a membrane. B) Lipid chain ordering, C) peptide backbone deformation, D) peptide oligomerization, E) peptide tilt in case of positive mismatch (upper row); adaptation of non-lamellar phases in case of negative mismatch (lower row), F) no lipid association.[22] Figure is modified from reference source.

If the effective hydrophobic length of a protein is too long to span the entire membrane, hydrophobic residues may stick out, and hence, become exposed to a polar environment. This positive mismatch can be prevented in several adaptations that avoid the energetic unfavorable situation (Fig. 1.3).
Modulation of the bilayer thickness or variation of the backbone conformation of the protein might be possible. Furthermore, the transmembrane protein may self-assemble to decrease the exposed hydrophobic surface. A tilting of the peptide with respect to the membrane normal can also reduce their effective hydrophobic length. The complete segregation of the protein from the bilayer could also be a plausible alternative.
On the contrary, when the lipophilic part of a membrane-spanning protein is too small to match the bilayer thickness, a negative mismatch occurs. Thus, similar adaptations are plausible except tilting of the protein. Also, the formation of a non-lamellar phase should only occur in a negative mismatch situation. Several experiments have been performed elucidating the described adaptations.[22] Effects referring to the acyl chain order were investigated applying ^{1}H-NMR experiments. It was shown that WALP peptides cause only slight decrease in chain order when applying negative mismatch situations. This might be due to the fact that ^{1}H-NMR experiments can show only the average chain order. However, estimated adaptations of lipids directly adjacent to the transmembrane peptide are not sufficient to completely relieve the mismatch.[23] Furthermore, changing of the peptide backbone also seems to be an unfavorable mismatch response. For example, the α-helical structure of WALP peptides was found to be highly conserved in different membrane systems.[24] Tilt angels can be determined by various methods. Most prominent techniques are ^{1}H- and ^{15}N-NMR experiments as well as *attenuated total reflection* FTIR (ATR-FTIR) measurements. Applying these methods, tilt angels in the range of 0-20° were measured.[22] Nevertheless, many mismatch situations can not be compensated by tilt angles below 20°. More likely seems to be the aggregation of incorporated peptide species as well as the formation of non-lamellar phases. The formation of linear peptide aggregates was proposed after fluorescence measurements and atomic force microscopy (AFM).[25,26] Also, transition to non-lamellar phases was observed depending on the precise extent of mismatch as well as the flanking residue. This remarkable mismatch response can be induced by relatively short Trp- and Lys-flanked peptides.[22] Common methods used for investigation are ^{31}P-NMR studies as well as sucrose density centrifugation analysis.[18]

Due to the reduced hydrophobic length of a more disordered bilayer in the inverted hexagonal phase (H_{II}), peptides are believed to span the distance between adjacent tubes as illustrated in Figure 1.4.

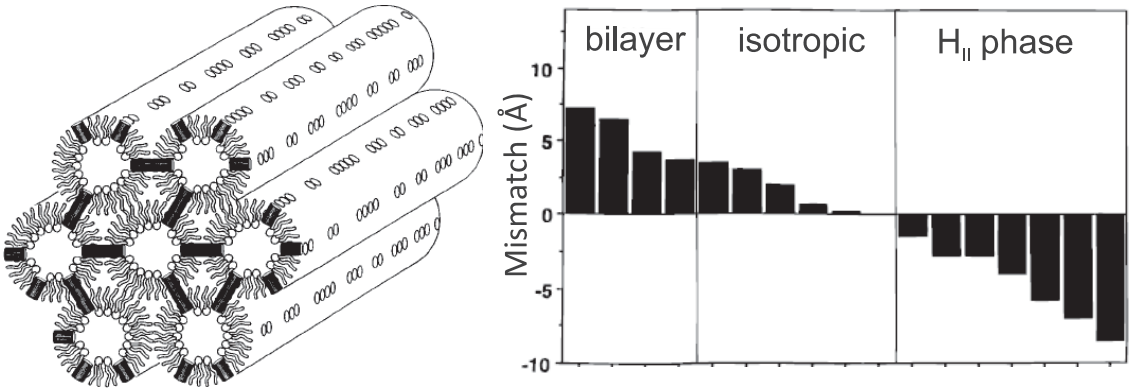

Fig. 1.4 Left) Schematic representation of induced *inverted-hexagonal phase* (H_{II}) by the WALP peptides. The tryptophan residues are located at the membrane-water interface. The monolayer thickness is much thinner than the thickness of the bilayer. Therefore the negative hydrophobic mismatch is compensated. Right) Positive and negative mismatch plotted against the phase preference of the lipid.[18,27] Modified picture is taken from reference sources.

Moreover, model peptides of different length with sequence AC-KKGL_nXL_mKKA-NH_2, where X denotes tryptophan or 3,5-dibromotyrosine, were utilized to investigate a similar assembly-behavior in response to changes in bilayer thickness.[28,29] Heterodimer formation was monitored via quenching of tryptophan fluorescence by 3,5-dibromotyrosine. Therefore, both peptides were mixed in artificial membrane systems followed by determination of association constants. The influence of varying numbers of leucine residues was relatively small compared to the significant increase in free energy of dimer formation while increasing the bilayer thickness.

Furthermore, it seems to be the case that the association is phase-dependent, being much more pronounced in the *liquid-ordered phase* of a dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DOPC)/cholesterol mixture than in the *liquid-crystalline phase* of pure DOPC.[11]

All the presented model peptides and similar reported systems are designed as single membrane spanning α-helices. Nevertheless, a significant number of reported transmembrane proteins within the PDB provide a β-barrel structure, and therefore, model systems addressing the formation and folding of β-sheets within the membrane are required.[11]

WHITE et al. introduced a model peptide capable of forming β-sheets that consists of an alternating tryptophan-leucine sequence.[30] This peptide provides a monomeric-random coil structure in solution but forms a β-sheet aggregate when

incorporated into a membrane (Fig. 1.5). The partitioning property between water and the lipid phase was found to depend on the length and hydrophobicity of the model peptide. Peptides of sequence Ac-WL_4 showed only poor interactions with membranes, whereas the longer peptide Ac-WL_6 was found to have higher partition constants with lipid membranes. Further investigations utilizing Ac-WL_5 were performed applying IR and CD experiments. It seems to be the case that prior to the aggregation process the random coil monomer is inserted into the lipid environment. An equilibrium is formed by the unfolded monomer in the membrane and the β-sheet aggregate. Although folding mechanism inside the membrane correlates poorly with those of globular proteins, the observation of β-sheets in the lipid bilayer demonstrates the importance of polar interactions such as hydrogen bonds, within this environment.[11]

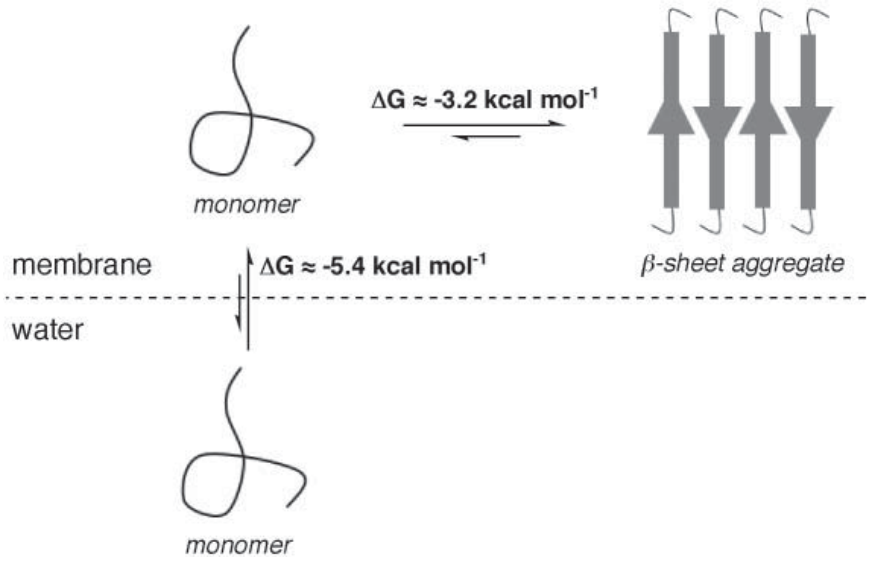

Fig. 1.5 Schematic representation of partitioning of AC-WL_5 at the bilayer/water interface followed by aggregation. The model peptide provides a monomeric random coil form in the aqueous phase. After incorporation, anti-parallel arrangement of β-strands maximizes the separation between the charged carboxyl termini.[11,30] Figure is taken from first reference source.

1.3 The Artificial D,L-Transmembrane Hairpin

β-Helices provide structural elements comparable to β-sheets. A D,L-alternating configuration of the peptide strand is mandatory to build up several β-helices. Here, all side chain residues point to the same direction which allows coiling up into a helix with all side chains pointing away from the interior.[31] The intensified investigation of the D,L-alternating structural motif started with the isolation of gramicidin A (gA) after enzymatic synthesis from *Bacillus Brevis*.[32] The natural antibiotic polypeptide provides a D,L-alternating sequence of 15 hydrophobic amino acids. In 1965, the primary structure HCO-VGALAVVVWLWLWLW-$NH_2(CH_2)$-OH[1] was determined by SARGES and WITKOP.[33] The antibiotic and anti-HIV activity of gA is based on the formation of ion channels across the membrane which are selective for monovalent cations such as K^+.[34] Gramicidin A is found in different conformations depending on its environment[35–40], and hence, a controversial discussion about the ion conducting form[39,41,42] with respect to the transport mechanisms[42–45] is still sustaining. Indeed, X-ray analysis provides a variety of conformations of gA depending on the crystallization conditions and additives.[42,43,46–48]

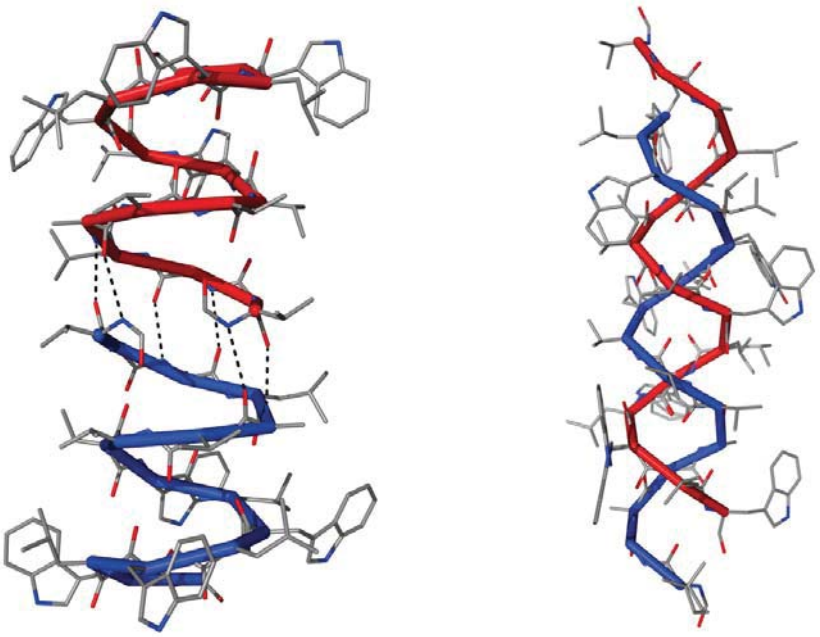

Fig. 1.6 Left) A solution NMR structure of gramicidin A. The head-to-head dimer consists of two right-handed $\beta^{6.3}$-helices recognized via six intermolecular hydrogen bonds (in SDS micellar environment, PDB id. 1GRM).[49] Right) An X-ray crystallographic structure of the left-handed dimer which is formed by antiparallel double helical $\beta^{5.6}$-helices (in benzene/methanol, PDB id. 1ALZ).[48]

[1] Underlined amino acids in this work denote D-amino acids

Most likely, it is considered that the active form requires the formation of a head-to-head dimer consisting of two $\beta^{6.3}$-helices recognized via intermolecular hydrogen bonds between the formylated *N*-termini.[35,50] NMR studies were applied determining a six-hydrogen-bond network within artificial lipid systems (Fig. 1.6).[34,51] Furthermore, the single-stranded $\beta^{6.3}$-helices seem to be the most favorable conformation within the membrane environment, whereas a broad diversity of double-helical conformations is found in organic solvents depending on additives such as different ionic species.[36,52] Usually, the narrow pores which result from the formation of double-stranded $\beta^{5.6}$-helices, do not provide any channel activity. Moreover, this conformation could be solely detected when gA is incorporated in poly-unsaturated lipid systems providing long acyl chains probably due to hydrophobic mismatch situations.[52,53] An overview of the physical data obtained by different methods is given in Table 1.1.

Solvent:salt	Method	Pore/Channel	dh/hd	Hand	A/P	Periodicity	Length/Å
EtOH/Benzene	NMR	close pore	dh	left	A	5.6	36
Dioxane	NMR	-	dh	right	P	5.7	27
EtOH	X-ray	close pore	dh	left	A	5.6	35
MeOH:CsCl	X-ray	open pore	dh	left	A	6.4	26
MeOH/$CDCl_3$:CsCl	NMR	open pore	dh	right	A	7.2	27
MeOH:KSCN	X-ray	partially open p.	dh	left	A	6.4	26
MeOH:$CaCl_2$	NMR	blocked pore	dh	left	P	5.7	30
SDS micelles	NMR	open channel	hd	right	A	6.3	24
DMPC bilayer	ssNMR	open channel	hd	right	A	6.5	25

Tab. 1.1 Overview of different gramicidin A forms (double helix = dh, helical dimer = hd, antiparallel = A, parallel = P).[34]

Another D,L-alternating polypeptide providing anti-HIV and antibiotic properties just like gramicidin A was recently discovered by VÈRTESY et al. The 13-amino-acid feglymycin was isolated from *Streptomyces* cultures.[54] Due to the biological activity and unique amino acid sequence, which includes different non-natural residues, feglymycin represents a promising new class of anti-viral drugs. The primary sequence was primarily elucidated via mass spectrometry and NMR studies and afterwards confirmed by X-ray analysis (Fig. 1.7).[55]
Due to the high amount of the unnatural amino acids 4-hydroxyphenylglycine (Mpg) and 3,5-dihydroxyphenylglycine (Dpg) the antiparallel β-helix was found to be stabilized via intermolecular hydrogen bonds between phenolic hydroxy-groups. On the contrary to gramicidin A, the helical pitch varies around 9.0 residues per turn, and therefore, it is probably not long enough to span a biological membrane, and although the channel is wider, it is blocked by phenylalanine residues.[55] It seems to be more likely that feglymycin is feasible of penetrating the

membrane and to act as an ion carrier. Furthermore, the membrane insertion could be important in the HIV-inhibitory activity.[55]
The epimerization-probability of the unnatural amino acids included in feglymycin is significantly increased by the direct attachment of the aromatic moiety to the α-carbon, and thus, the first total synthesis was not published until 2009.[56] A stepwise coupling of every single amino acid does not seem to be possible, and therefore, a synthetic route involving four dipeptide building blocks and one tripeptide derivative was finally successful. Peptide fragments, which were obtained applying this synthesis strategy, were also subjected to antiretroviral tests. DETTNER et al. were able to show that some intermediates provide anti-HIV activity as well as a high cytotoxicity, whereas others show no activity at all. Considering all IC_{50}-values feglymycin provides a four-fold higher activity than the other tested fragments. Interestingly, the enantiomeric form of feglymycin showed also a high activity, and hence, the authors conclude, that the absolute configuration plays a minor role concerning activity.[56]

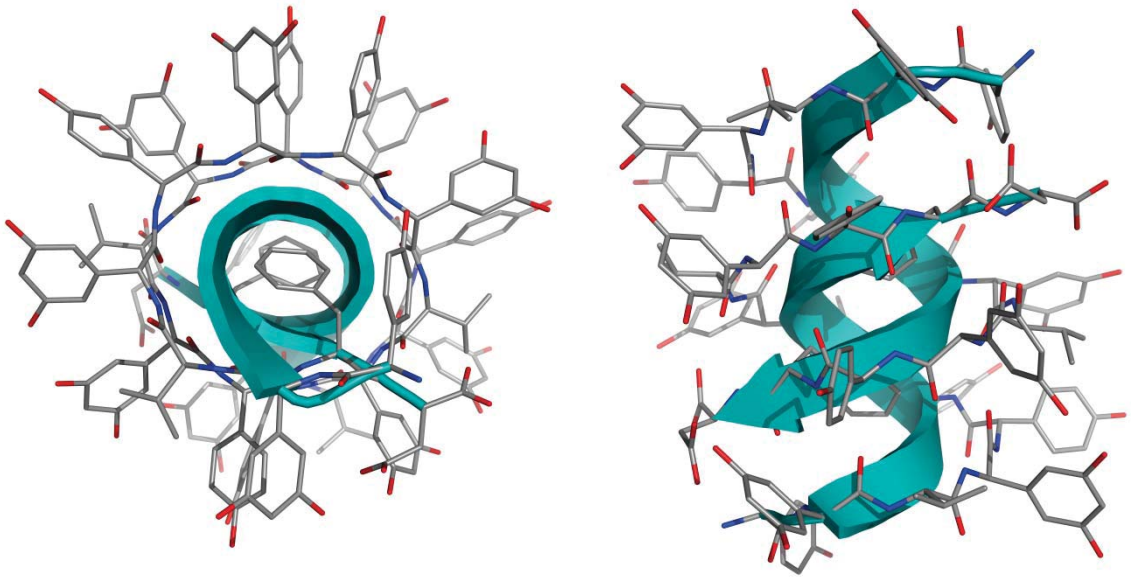

Fig. 1.7 X-ray structure of double-stranded antiparallel β-helix dimer of feglymycin (site and top view, PDB id. 1w7q).[55] The channel is blocked by phenylalanine.

Beside the appearance of D,L-alternating motifs found in natural products, different model peptides providing a broad variety of β-structures have been synthesized. The first X-ray structure to an atomistic resolution was obtained by LORENZI et al. after crystallizing the polypeptide Boc-(ValVal)$_4$-OMe.[57] A left-handed β$^{5.6}$-helix could be determined which was also persistent in chloroformic solution according to NMR spectroscopy.[58] Moreover, numerous experiments applying this and structural related D,L-alternating peptides were performed.[59–63] In conclusion, peptides with β- or γ-branched side chains highly favor β-helical conformations.[64] In contrast, peptides which are sterically less demanding show only a minor degree of β-helical pre-organization.[65]
Peptide nanotubes providing self-assembly probabilities were recently introduced by ALEXOPOULOUS et al.[2] The D,L-alternating tyrosine-oligomer H-(TyrTyr)$_4$Lys-OH was crystallized from aqueous solution and subjected to X-ray analysis.

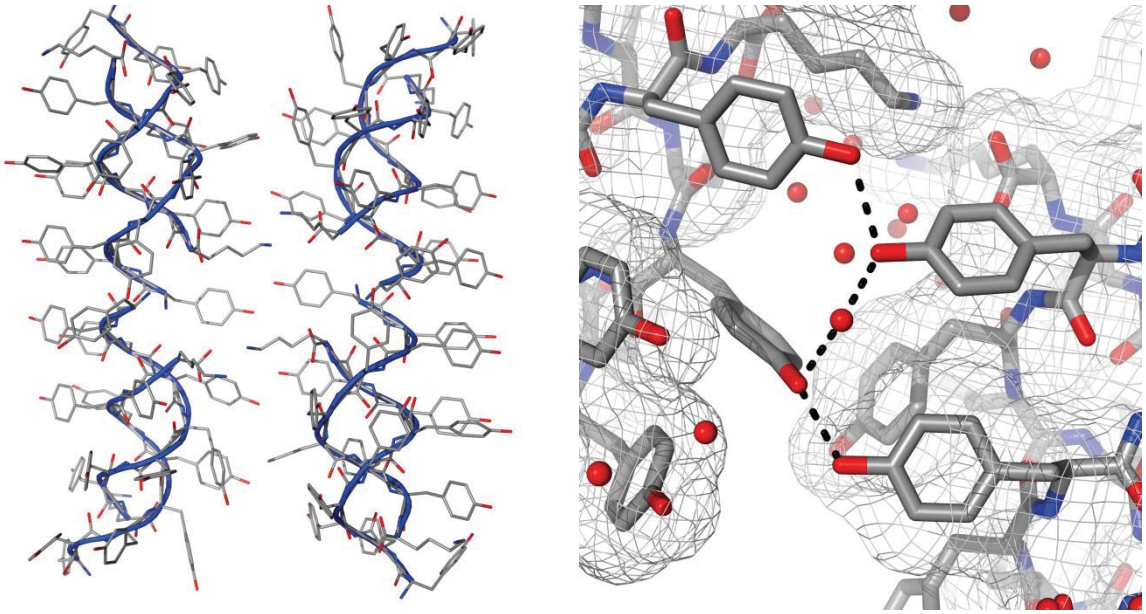

Fig. 1.8 Left) X-ray structure of H-(TyrTyr)$_4$Lys-OH forming a tubular nanotube network of antiparallel double stranded β$^{5.6}$-helices. Right) Tyrosine-water-tyrosine hydrogen bond network which links two symmetrical equivalents (PDB id: 1UNO).[2]

An antiparallel right-handed β$^{5.6}$-helix was determined providing a total length of 19.7 Å. Single strands are associated via seven intermolecular hydrogen bonds. Additionally, two remaining hydrogen bonds connect the dimer with the adjacent dimers along the helix axis (Fig 1.8). Also three tyrosines of one dimer are linked with symmetry equivalents or with each other via 17 water molecules. Due to an inner diameter of 2.23 Å (including the VAN-DER-WAALS radii) these nanotubes should be able to transport alkali-ions. Furthermore, terminal stacking in rods and the water-mediated interaction between these rods makes them strong

candidates for the membrane-channel formation.[2] A. KÜSEL developed and characterized on basis of the peptide shown in Figure 1.8 oligomers of sequence H-(PheTyr)$_n$TrpTrp-OH to investigate their conformation and interaction with artificial membranes.[3,13] The length of the peptides was varied between n = 3 and n = 5; a repetitive sequence of five PheTyr units was found to be best suitable for incorporation in model membrane systems such as DLPC and DMPC.

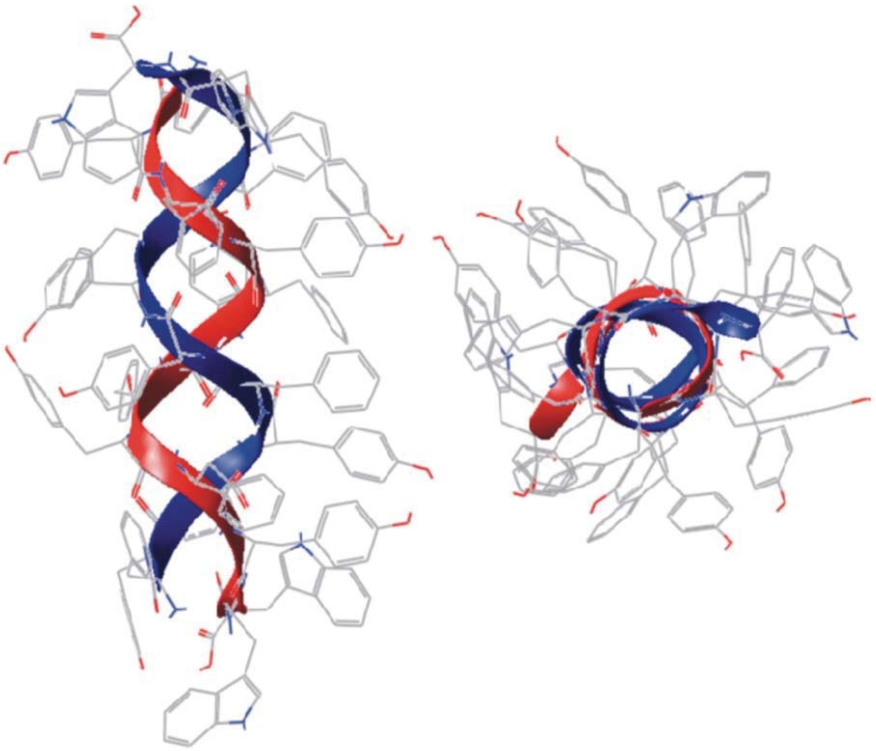

Fig. 1.9 Schematic representation of the proposed strutcture of H-(PheTyr)$_5$TrpTrp-OH.[66]

Figure 1.9 illustrates the ideal $\beta^{5.6}$-helix, based on PDB 1S10[67] after energy minimization (AMBER, MMCM)[3] expected to be formed by the homodimer H-(PheTyr)$_5$TrpTrp-OH after incorporation in a membrane environment.
Reconstitution in different bilayers was monitored via CD and FTIR spectroscopy. According to the recorded CD-spectra, which are characterized by a negative COTTON effect at 197 nm, a small positive signal at 207 nm, and an intense signal around 227 nm, the formation of $\beta^{5.6}$-helices was found to be deduced.[3] Additionally, FTIR measurements revealed, that the amide I band decreased from $\tilde{\nu}$ = 1646 cm^{-1} at 15 °C to 1636 cm^{-1} at 55 °C.[3] Absorption bands occurring at $\tilde{\nu}$ = 1656 cm^{-1} indicate $\beta^{5.6}$-helices, whereas $\beta^{4.4}$- and $\beta^{6.3}$- single helices are associated with bands at $\tilde{\nu}$ = 1631 cm^{-1} and 1641 cm^{-1}, respectively.[68,69] It seems to be the case that the conformation is changed from a single helix to double stranded β-structures. However, at low temperatures the FTIR data were considered to be in line with the respective CD spectra that did not show a

temperature dependency, whereas FTIR spectra recorded at an elevated temperature opened discussion for the existence of a structural equilibirium.[66] Additionally, X-ray reflectivity experiments were applied to elucidate the membrane spanning properties via the vertical density profile ρ(z).[3] Therefore, site-specific labeling with heavy-atoms like iodine was mandatory. This was realized in form of incorporating the 4-iodine-phenylalanine analogue. The vertical density profile ρ(z) can be determined via X-ray reflectivity utilizing aligned multilamellar lipid bilayers.[70,71] Elucidation of reflectivity measurements supports the conclusion that the terminally iodinated phenylyalanine is located at the membrane-water interface, and furthermore, the formation of transmembrane helices.

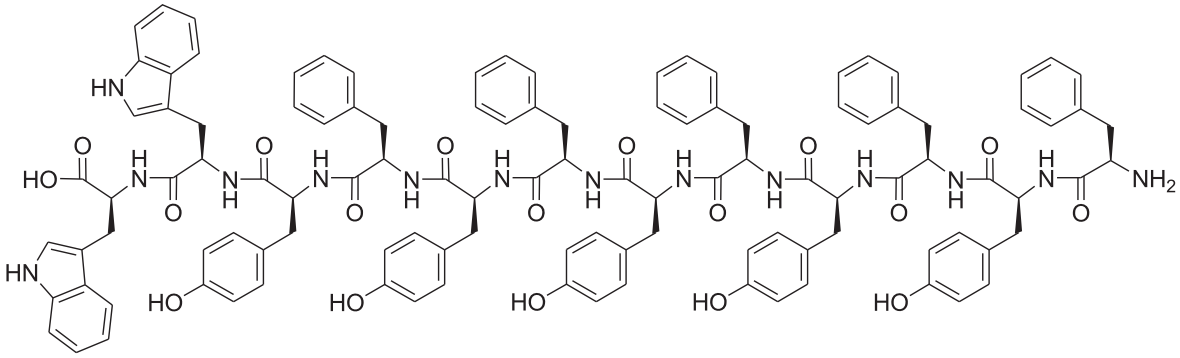

Fig 1.10 Molecular representation of H-(PheTyr)$_5$TrpTrp-OH.

The homodimer peptide of sequence H-(PheTyr)$_5$TrpTrp-OH (Fig. 1.10) was utilized by P. SCHNEGGENBURGER as a lead structure for the further design of a transmembrane domain regarding a peptidic model system, focusing on an outer membrane recognition with assembling effect on transmembrane helices.[66] As mentioned before, there is evidence for a structural equilibirium between single and double helical β-structures varying in helical pitch. Hence, in order to distinguish between these possible equilibria and the homodimer-homodimer interaction based on molecular recognition, two homodimer peptides were covalent connected.[66] In this regard, the formation of $\beta^{5.6}$-helices spanning the entire hydrophobic bilayer was preserved. Similar approaches were already performed for the gramicidin A channel. Here, two monomers were linked via special amino acid derivatives or tartaric acid residues resulting in a functional channel.[72–74] According to this concept, two homodimers were connected at the *C*- and *N*-terminus, respectively, applying different loop designs. Their structural properties were analysed via CD spectroscopy. As reported before, D-proline induces a stronger turn than the respective enantiomer L-proline.[75] Due to the 180° turn required for transmembrane helix formation, the D-Pro-Xxx-dipeptide β-turn motif was utilized.[66] A loop consisting of four amino acids with sequence -

-GKPG- was found to be the best promoter of a $\beta^{5.6}$-helix in artifical membrane systems (compare Fig 1.11).[66]

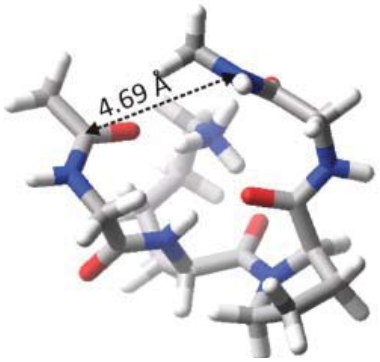

Fig. 1.11 Force field calculations of the comparable -GKPG- turn within hydrophobic environment providing a distance of approximately 4.7 Å.[66] Figure is taken from reference source.

Furthermore, the influence of modifications at the *C*- and *N*-terminus was part of the investigation. It turned out that both ends are not suitable for further functionalization. Free, and thus, charged termini (under physiolocial conditions) seem to be mandatory for transmembrane helix formation.[76] A correlation of a decreased helical content with missing terminal COULOMB interactions was observed via CD spectroscopy (Fig. 1.12). This explains the decreased CD signal for the hairpin system compared to the homodimer system providing two additional charges.

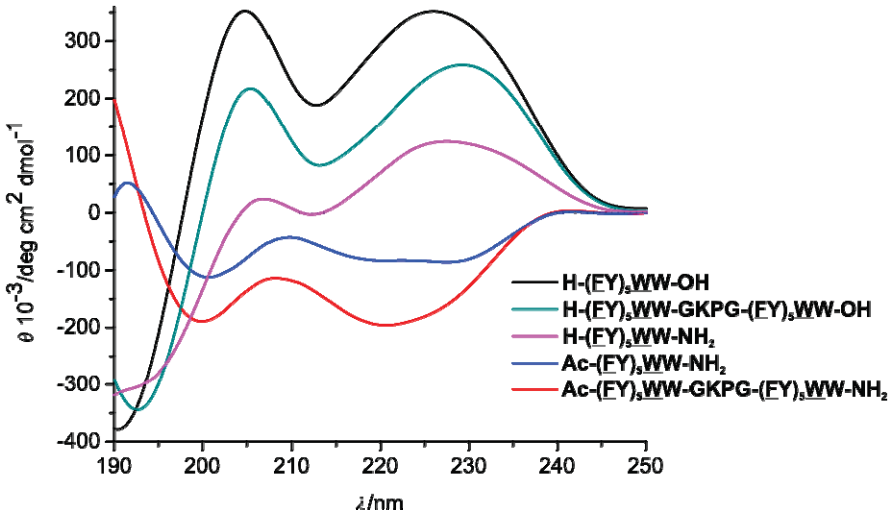

Fig. 1.12 CD spectroscopic analysis of homodimer and hairpin transmembrane peptide. Additionally, the effect of substituting the terminal charge at *C*- and *N*-terminus is shown.[66] Figure is taken from reference source.

Figure 1.13 illustrates the optimized transmembrane hairpin **1** which is considered as lead structure within this work. The hairpin should be utilized as a well-defined, membrane spanning model system. Functionalization with recognition units located in the membrane adjacend water-layer or within the bilayer should result in assembling of the transmembrane helices. Furthermore, the assembly process should be monitored via FRET measurements.

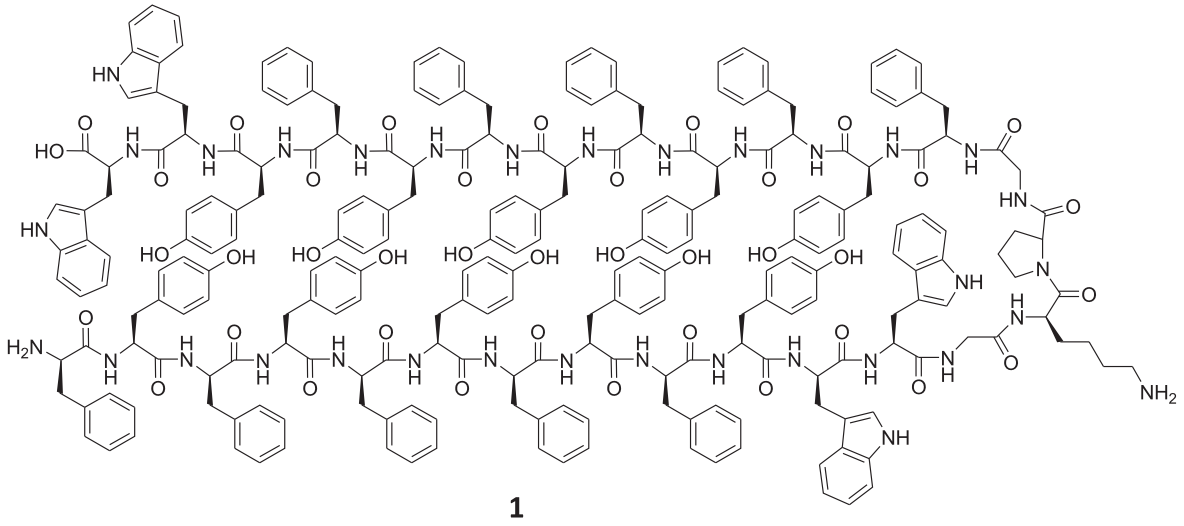

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Fig. 1.13 Molecular representation of hairpin construct **1** considered as lead structure within the presented work.

1.4 Theoretical Background and Evaluation of FRET Data

The dimerization state of the fluorescence probe-labeled hairpin molecules (section 2 and 3) with different recognition units was addressed via FRET (FÖRSTER Resonance Energy Transfer) experiments. FRET-measurements are an established and versatile tool used in a wide range of biological applications. FRET has been applied to an increasingly vast number of systems, discussed in many reviews.[77–80] With this method it is possible to determine distances between a donor and acceptor molecule (FRET pair) in a range of up to 8 nm. A donor fluorophore denotes a molecule which is excited by UV/VIS-radiation and performs a non-radiative energy transfer to an acceptor molecule.[81] The acceptor species itself is excited to the S_1 state enabling fluorescence emission of the acceptor.[82] This mechanism does not require a close contact state of donor and acceptor fluorophores usually mandatory for quenching of molecular fluorescence by dynamic or statistical processes.[83] The non-radiative energy transfer is described as resonance phenomenon according to the FRET theory. The excitation energy of the donor molecule, treated as a classical harmonic oscillator, can only be transferred to the acceptor oscillator if the acceptor`s $S_0 \rightarrow S_1$ excitation frequency is similar to the frequency of the donor (the process should not be confused with the occurrence of re-absorbation).[66] This implies an overlap of the acceptors absorption spectrum with the emission spectrum of the donor as indicated by the emission maxima wavelengths of the FRET-pair (NBD/TAMRA) with 537 nm and 550 nm, respectively, applied within this work.[66]
As realized by FÖRSTER, the rate of energy transfer k drops as the sixth power of the separation between a donor and an acceptor r:

$$k = \left(\frac{1}{\tau}\right)(R_0/r)^6 \qquad \text{eq. 1.1}$$

where τ is the fluorescence lifetime of the donor and the constant R_0 is known as the FÖRSTER radius (substance specific critical distance) at which the energy transfer process provides an effectivity of 50%.[77,82] By reaching the critical FÖRSTER radius, the spontaneous deactivation of the excited donor has the same probability as the non-radiative energy transfer.

$$E = 1 - (I/I_0) = \frac{1}{1+(r/R_0)^6} \qquad \text{eq. 1.2}$$

The FRET efficiency E, the ratio between the non-radiative transferred energy to the totally absorbed energy by the donor, can be estimated via measuring the fluorescence intensities of the donor in presence (I) and absence (I_0) of an

acceptor.[84] Solving equation 1.2 for r points out, that FRET-experiments allow the estimation of discrete distances r between two fluorophores.

$$r = R_0 \cdot \left(\frac{I}{I_0 - I}\right)^{\frac{1}{6}} \qquad \text{eq. 1.3}$$

The critical FÖRSTER radius depends on spectroscopic properties as well as on the spatial orientation of the FRET pair. For high efficiency, there has to be a large overlap between the emission spectrum of the donor and the absorbtion spectrum of the acceptor, a high fluorescence quantum yield of the donor and a favorable orientation between the transition dipole moments of the donor and acceptor fluorophores.[77] Orientation between the donor and acceptor fluorescence transition dipole moment is given by the factor κ^2.[77]

$$\kappa^2 = (cos\theta_T - 3cos\theta_D cos\theta_A)^2 = (sin\theta_D sin\theta_A cos\emptyset - 2cos\theta_D cos\theta_A)^2 \qquad \text{eq. 1.4}$$

The respective angels θ are illustrated in Figure 1.14.

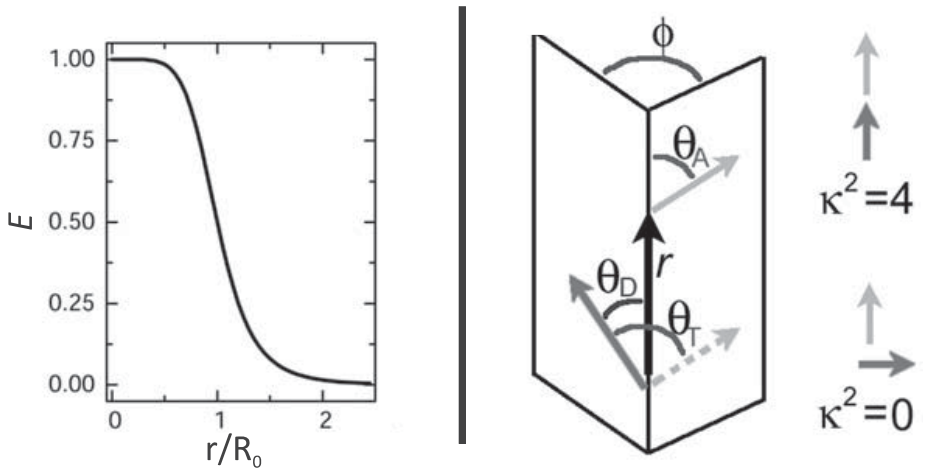

Fig. 1.14 Left) The efficiency is plotted against the fluorophore separation r (normalized to the critical FÖRSTER radius R_0). The major sensitivity is reached for r within a factor of 2 of R_0. Geometry of transition dipole moments of the FRET-pair is illustrated right. The donor fluorophore dipole moment (dark gray) is at angle θ_D to the vector r connecting both dipole moments; the acceptor`s dipole (light gray) is at angle θ_A to r. θ_T denotes the angle between the dipoles of donor and acceptor. Orientation of dipole moments giving maximal and minimal energy transfer: Maximally, parallel and co-linear dipoles have κ^2= 4; minimally, dipoles that are perpendicular to each other and oriented at 0 and 90° to r have κ^2= 0.[77] Figure is modified from reference source.

A lack of information about κ^2 is often a limiting factor in interpreting FRET-experiments. However, the situation is often simplified if both fluorophores are randomly oriented.[77] This limits κ^2 to the range of 1/3 to 4/3 after averaging over random acceptor orientations.[85] Thus, R_0 can be calculated applying equation 1.5 (see refs. for details of the derivation and underlying physics):[77,85,86]

$$\left(\frac{R_0}{[cm]}\right) = (8.79 \cdot 10^{-25}) k^2 n^{-4} Q_D J(\lambda) \qquad \text{eq. 1.5}$$

With the index of fraction n of the medium (often assumed to be 1.4 in biological applications) and the overlap integral $J(\lambda)$ given by the following equation:

$$J(\lambda) = \int_0^\infty I^0(\lambda)\epsilon_a(\lambda)\lambda^4 d\lambda \qquad \text{eq. 1.6}$$

where λ denotes the wavelength and ε_A the extinction coefficient of the acceptor. Typical values of R_0 are in the range of 20-60 Å. The frequently used FRET-pair NBD and 5(6)-TAMRA is well suitable for experiments dealing with the association of membrane spanning peptides (Fig. 1.15).[84,87–90] FRET experiments were not only carried out in detergent micelles but also in vesicels.[91,92]

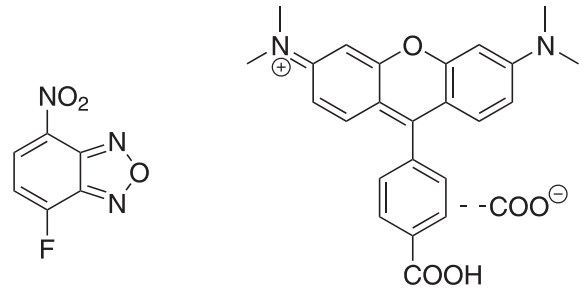

Fig. 1.15 The FRET pair NBD-F and 5(6)-TAMRA.

With the previous estimated FÖRSTER radius of 51 Å this FRET pair was chosen within the presented work to determine the dimerization of the sterically demanding hairpin systems.[93] Therefore, modified hairpin TMDs with the respective recognition unit were incorporated into large unilamellar vesicles (LUVs) and FRET-experiments were carried out to assess the oligomeric state of the constructs.

The dependence of the fluorescence emission intensity of donor (NBD)-labeled peptides on the mole fraction of acceptor (TAMRA)-labeled peptides in a lipid bilayer enables the determination of subunits in the peptide assembly.[89]

Whether the observed FRET-effect belongs to a peptide assembly or is caused by statistical effects, respectively, can be estimated upon mathematical evaluation of the data. Thus, the applied model is based on four assumptions:[93]

- labeling does not influence the aggregation of the peptides
- all peptides are located in the lipid bilayer
- the interaction of the peptides is random
- one acceptor is capable of quenching all donors within one aggregate

A binomial distribution of quenched (N_Q) and non-quenched (N_D) donors is given by a random number of acceptors and donors within the vesicle. The recorded relative fluorescence intensity can be expressed via equation 1.7.

$$\frac{I}{I_0} = 1 - \left(1 - \frac{f_Q}{f_D}\right)\frac{N_Q}{N_D} \qquad \text{eq. 1.7}$$

Where I is the fluorescence intensity in presence and in absence of an acceptor I_0. The molar fluorescence of non-quenched and quenched donors is denoted by f_D as well as f_Q, respectively. To describe a monomer-dimer equilibrium the following equation can be formulated.

$$\frac{I}{I_0} = \left(\frac{I}{I_0}\right)_m \cdot \frac{n_m}{n_0} + \left(\frac{I}{I_0}\right)_d \cdot \left(1 - \frac{n_m}{n_0}\right) \qquad \text{eq. 1.8}$$

$$n_m = \frac{N_m}{n_{Lipid}} \qquad n_0 = \frac{N_0}{n_{Lipid}}$$

The recorded fluorescence intensity can be defragmented into terms of fluorescence intensities resulting from the monomeric species $(I/I_0)_m$ and from the dimeric species $(I/I_0)_d$. N_m denotes the number of monomeric peptides, n_0 the total amount of used peptides and n_{Lipid} the amount of lipid molecules. Due to the occurrence of FRET without the formation of aggregates, $(I/I_0)_m$ can be estimated applying the approximation of WOLBER and HUDSON:[94]

$$\left(\frac{I}{I_0}\right)_m = A_1 \exp\left(-k_1 \chi_A \frac{N_0}{A} R_0^2\right) + A_2 \exp\left(-k_2 \chi_A \frac{N_0}{A} R_0^2\right) \qquad \text{eq. 1.9}$$

where A_1, A_2, k_1, k_2 are constants and A is the area of one vesicle. The constants are chosen for R_e/R_0 = 0, with R_e being the distance of the closest approach.[94] The molar fraction of the acceptor is given by χ_A. The area of a DLPC lipid was

assumed to be 0.63 nm^2.[95] The relative fluorescence intensity of dimeric peptide species is given by

$$\left(\frac{I}{I_0}\right)_d = 1 - \chi_A \qquad \text{eq. 1.10}$$

The dissociation constant K_D in a monomer-dimer equilibrium is definded as

$$K_D = \frac{n_m^2}{0.5(n_0 - n_m)} \qquad \text{eq. 1.11}$$

K_D is calculated in units of peptide/lipid mole fraction (MF). Nevertheless, beside a monomer-dimer equilibrium, a monomer-trimer equilibrium has to be considered as well. The equation for this case reads:

$$\frac{I}{I_0} = m\left(\frac{I}{I_0}\right)_m + (1-m)\left[1 - \chi_D^2\left(\frac{2}{\chi_D}\right)\left(\frac{\chi_A}{\chi_D}\right) - \left(\frac{\chi_A}{\chi_D}\right)^2\right] \qquad \text{eq. 1.12}$$

where m is the peptide fraction of the monomeric state and 1- m that of the oligomeric state. With χ_A+χ_D+χ_u= 1, where χ_D denotes the mole fraction of the donor and χ_u that of the non-labeled peptide species. Also the dissociation constant K_D can be expressed in a more general form:

$$K_D = \frac{(m\chi_p)^n}{(1-m)\chi_p/n} \qquad \text{eq. 1.13}$$

where χ_p is the lipid-to-peptide ratio and n the number of species in the oligomer. Applying a global fit analysis, the dissociation constant can be estimated assuming a FÖRSTER radius of R_0 = 5.1.[93]

1.5 Outline

Molecular recognition of biomolecules are fundamental and essential processes in every living cell. The mechanism behind these processes is often highly complex, and hence, in most cases still poorly understood. Simplified model systems can help to clarify the molecular details and basic understanding.
The main focus of this study is to create different model systems to address and elucidate the recognition process, and thus, the induced assembly of artificial transmembrane peptides. Therefore, functionalization of the recently developed transmembrane hairpin **1** (section 1.3) with different kinds of molecular recognition systems should be performed. As mentioned before, hairpin **1** is a structural well characterized, membrane spanning polypeptide, which provides a high affinity to form $\beta^{5.6}$-helices. Thus, it seems to be a promising candidate to test different molecular recognition units.
According to HALL, two approaches have to be considered concerning the assembly of transmembrane proteins (Fig. 1.16).[1] Interactions can either take place within the membrane environment or be performed by luminal domains.

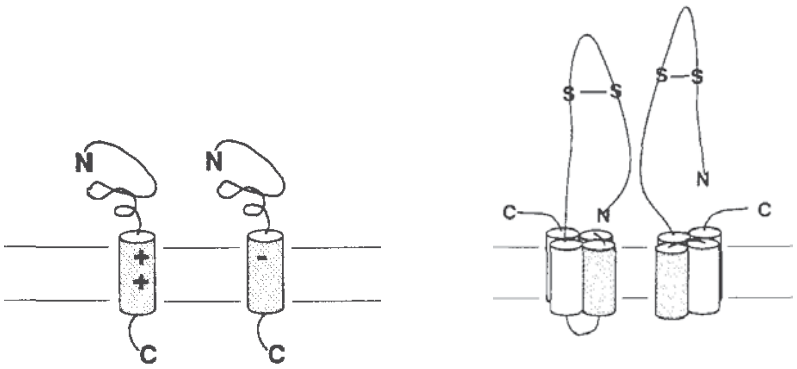

Fig. 1.16 Schematic representation of how transmembrane proteins achieve assembly via molecular recognition. Left) Association of the T-cell receptor subunits by charge-charge interactions. Right) Luminal domains perform recognition and drive assembly of acetylcholine esterase.[1] Modified picture is taken from reference source.

Model systems addressing selective recognition outside of the membrane bilayer are rarely reported. Within the first part of this work, an oligomerization of artificial transmembrane constructs should be achieved via metal-complex formation in the membrane adjacent water layer. Therefore, a metal-ion chelating ligand had to be synthesized and covalently attached to construct **1**. Also fluorescence probes had to be attached for investigation of a possible aggregation via FRET. Furthermore, control experiments require fluorescently labeled derivatives lacking the recognition unit to elucidate the effect of helix-helix interactions. Additionally, these constructs should be utilized in a collaborative

project with P. SCHNEGGENBURGER. Within this project a short peptide nucleic acid (PNA) oligomer was considered as recognition unit.[66]

The goal of the first part contains:

I. Synthesis of ligands providing metal-chelating properties in combination with different linkers
II. Orthogonal peptide synthesis including functionalization with recognition units and fluorescence probes at selected positions
III. Incorporation of modified or control hairpin derivatives in artificial lipid systems
IV. Analysis of the aggregation process via FRET assays

Aim of the second project is the creation of a model system, which addresses recognition within the membrane. Again, hairpin **1** is considered as lead structure in the further design. According to the association of T-cell receptor subunits by electrostatic interactions, charged amino acids should be incorporated in the center of the transmembrane hairpin. Moreover, the uncharged polar amino acid aspargine should be incorporated to study a possible homodimerization via hydrogen bonds. As a third possibility, the utilization of alanyl-PNA building blocks was considered.[96] Until now, the base pairing properties of these nucleobase-functionalized amino acid derivatives have not been investigated within a lipid bilayer. Thus, incorporation into the well-defined transmembrane hairpin **1** should enable the elucidation in a hydrophobic environment.
The in-membrane pore formation by adopting $\beta^{5.6}$-double-helices should be investigated applying CD spectroscopy. Also experiments using fluorescence probes to determine the dynamic aggregation process via FRET should be performed.

Purpose of the second part:

I. Synthesis of hairpin derivatives with the possibility to incorporate inner-membrane recognition units
II. Incorporation of different recognition units as well as attachment of fluorescence probes
III. Characterizing the membrane incorporation properties of the modified constructs with respect to formation of $\beta^{5.6}$-helices
IV. Analysis of the aggregation process via FRET assays

In addition to the aggregational studies in the first two parts, orientational studies as well as distance measurements are considered as a further milestone within this work. Therefore, the homodimer structure H-(PheTyr)$_5$TrpTrp-OH should be equipped with the spin label 1-oxyl-2,2,5,5,-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL) at the position three, four and five. These homodimers should be incorporated in LUVs followed by analysis with CD-spectroscopy. EPR-measurements will be performed by GIUSEPPE SICOLI in the group of MARINA BENNATI at the MPI for Biophysical Chemistry, Göttingen.

The goal of the third part involves:

I. Peptide synthesis of homodimer constructs providing cysteine residues for post synthetic modifications
II. Site directed spin labeling of homodimer structure H-(PheTyr)$_5$TrpTrp-OH at the position three, four and five
III. Characterizing the membrane incorporation properties of the modified constructs with respect to formation of $\beta^{5.6}$-helices

As described above, PNA duplex formation will be utilized to direct the assembly of two hairpin constructs. Variation of the PNA sequence might allow the formation of higher aggregates such as PNA triple helices or guanine tetrad assemblies. Therefore, incorporation of fluorescent PNA analogues could be applied, to follow the assembly process and for monitoring the folding into various topologies. As recently reported, attachement of a vinyl group at guanine position 8 provides fluorescent properties of the nucleobase.[97] To take advantage of the micro environment sensitive fluorescence of 8-vinylguanine also in PNA oligomers, a novel 8-vinylguanine PNA building block should be incorporated into PNA oligomers, and investigated in PNA/PNA, PNA/DNA duplex formation and within a PNA/RNA quadruplex fold. Furthermore, this derivative could be incorporated into the recognition sequence used before. A deeper understanding of the assembly process could be achieved by comparing the fluorescence read out with FRET experiments.

Agenda of the fourth part:

I. Synthesis of an 8-vinylguanine PNA building block and determination of the extinction coefficient as well as quantum yield
II. Testing the incorporation properties into short PNA sequences
III. Hybridization experiments with complementary PNA, DNA or RNA strands.
IV. Incorporation into recognition sequences to monitor the assembly of TMDs