Hydrogen Bonding Interactions of Ferrocene-Peptides: From Molecule to Large Scale Assemblies

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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The main goal of this thesis was to explore the role of H-bonding interactions in ferrocene peptide conjugates at the molecular and supramolecular level. With the help of detailed spectroscopic and crystallographic studies, the intermolecular association of a range of conjugates was studied and described here. It was shown that C-terminal modifications directed the supramolecular assembly. In the case of Fc[CO-Gly-Val-OH]₂, the C-terminal carboxylate group directed intermolecular interactions, causing formation of a supramolecular architecture that was characterized by large solvent-filled hydrophobic channels. In the absence of this directional group, as was the case in Fc[CO-Leu-Val-OMe]₂ extended β-sheets were formed. Hierarchical self-assembly of disubstituted ferrocene peptide conjugates possessing Gly-Val-Phe and Gly-Val-Phe-Phe peptide substituents gave rise to nano- and micro-sized assemblies. Spontaneous self-assembly of Fc-peptides through intra- and intermolecular hydrogen bonding interactions induced supramolecular building blocks, which further associated to fibers, large fibrous crystals, and twisted ropes. Next, intermolecular H-bonding interactions were studied using a surface-based approach. A fragment of the amyloid-beta (Aβ) peptide was bound to a
gold surface through a C-terminal Cys. Various aspects of the peptide film were examined using different electrochemical and surface analytical techniques. The interaction of Congo red and of Lys-Leu-Val-Phe-Phe with the immobilized Aβ fragment was studied using electrochemical methods, showing responses that indicated intermolecular interactions. This surface approach was used to probe the interaction of a series of ferrocene peptides (Fc-CO-Leu-Val-Phe-Phe-OX and Fc-CO-Lys(Boc)-Leu-Val-Phe-Phe-OX with X=H and Me) with the surface-bound Aβ fragment. Biomolecular interactions between Fc-peptides and the Aβ-modified surface were studied by electrochemical methods. The current response of the Fc redox process was modulated by the interaction with the Aβ-modified surface.
Dedication

To my lovely parents, Hossein and Akram, who show their love and supports in every step of my life. And to my husband, Aryan, for his love, support and kindness.
Acknowledgments

I would like to acknowledge all the people who supported me during this Ph.D. work. First of all, I would like to thank my supervisor, Dr. Heinz-Bernhard Kraatz, for his support and guidance during the past five years. Without his support, this work would not have been a reality. I would like to thank all the past and present members of Kraatz group. Special thanks to Dr. Sanela Martić, Dr. Anas Lataifeh and Dr. Raheleh Partovi-Nia.

My gratitude goes to my supervisory committee both at Western University, Dr. Richard J. Puddephatt, Dr. Martin J. Stillman and at the University of Toronto, Dr. Robert Morris and Dr. Kagan Kerman.

I appreciate the assistance from the support staff (NMR lab, mass spectrometry lab, X-ray facility, chemistry stores and administration) of the department both at Western University and at the University of Toronto.

I would like to thank my parents and my husband for their patience, support and understanding on this wonderful journey.
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<tr>
<td>A</td>
<td>Absorption</td>
</tr>
<tr>
<td>Aa</td>
<td>Amino acid</td>
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<td>AC</td>
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<td>AD</td>
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<td>AFM</td>
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<td>BSB</td>
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Chapter 1
Introduction

The main focus of work described in this thesis was to explore the self-assembly properties of new ferrocene (Fc) peptide conjugates containing fragments of the hydrophobic region of the amyloid-beta (Aβ) peptide. This work highlights the ability of Fc to act as a scaffold allowing the formation of supramolecular assemblies. Some of these assemblies are hierarchical in nature, in which the blueprint of the supramolecular structure is already present in the molecule itself. Inter- and intramolecular hydrogen bonding interactions drive the organization into larger assemblies. In order to understand these processes driving peptide association, it is necessary to take a closer look at peptides and briefly introduce hydrogen bonding interactions and formation of secondary structural motifs. As part of this discussion, amyloidogenic peptides will be introduced, along with some methodologies used to investigate interactions and assembly formation.

1.1 Peptides

Most polypeptides and proteins are comprised of a sequence of twenty naturally occurring amino acids. Each amino acid contains a central α-carbon atom with an “R” group called a side chain, attached to the amino and carboxylic acid groups (Scheme 1.1a). According to the properties of their side chains, amino acids can be classified as hydrophobic or polar amino acids. However, there are some amino acids that do not fit into either of these categories (Gly, Pro and Cys).[1] The α-carbon can be in the S- or R-configuration resulting L- or D- amino acid, respectively (except for Cys).

Scheme 1.1 Chemical drawing of an α-amino acid with its “R” group in S-conﬁguration giving rise to L-amino acid (a), amino acids are connected by peptide or amide bonds to make a polypeptide chain (b). φ, ψ and ω are torsion angles which determine the secondary structure of peptides.

In a peptide chain, amino acids are linked by amide bonds in which each bond has three dihedral angles of interest φ \([\text{C}(=\text{O})-\text{N}^{\alpha}-\text{C}(=\text{O})]\), ψ \([\text{N}-\text{C}^{\alpha}-(=\text{O})-\text{N}]\) and ω \([\text{C}^{\alpha}-(=\text{O})-\text{N}-\text{C}^{\alpha}]\) (Scheme 1.1b). The number and sequence of amino acids within the peptide strand is called primary structure. Torsion angles, hydrogen bonding patterns and hydrophobic interactions determine the secondary structures formed by the peptide strand. Hydrogen bonds occur between the NH of an amide group and the oxygen of the carbonyl group with the distance N···O between 2.6 and 3.2 Å, to make three different secondary structures (α-helix, β-sheet and β-turn) induced by folding.

β-sheet and turn structures are the main focus of this chapter. β-sheets are the most common secondary structural motifs found in proteins. They are composed of at least two extended peptide strands which are aligned close to each other and are engaged in hydrogen bonding between the C=O groups of one strand and the NH groups of the adjacent strand. Two forms of β-sheets have been recognized in protein structures; parallel β-sheets are defined as peptide strands running in the same direction containing 12-membered hydrogen bonded rings (Scheme 1.2a) and antiparallel β-sheets which have strands running in opposite directions possessing 10- and 14-membered hydrogen bonded rings (Scheme 1.2b).
Scheme 1.2 Schematic representations of parallel (a) and anti-parallel β-sheets (b). In parallel β-sheets, peptide chains are aligned in the same direction forming a 12-membered H-bonded ring, whereas in antiparallel β-sheets peptide running in opposite direction forming 10 and 14-membered H-bonded rings. The common dihedral angles for parallel β-sheets are $\phi = -119^\circ$ and $\psi = 113^\circ$ and for antiparallel β-sheets are $\phi = -139^\circ$ and $\psi = 135^\circ$. The arrows show the direction of peptide strands from C- to N-terminus. Dashed lines indicate H-bonds.

Turn structures are important structural elements in peptides that result from folding of a peptide chain back on itself. Turns are classified according to their H-bonding ring size, number of amino acid and the associated peptide dihedral angles $\phi$ and $\psi$. $\beta$ turns (10-membered ring) and $\gamma$-turns (7-membered ring) are the most common forms of turn structures in peptides (Scheme 1.3a). $\beta$-turn occurs in peptide containing at least four amino acids with a distance $\leq$ 7 Å between $i$ and $i + 3$ amino acids. In contrast to turns, reverse turns with a $C$- to $N$-directionality (Scheme 1.3b), such as reverse $\gamma$-turn (5-membered ring) and reverse $\beta$-turn (8-membered ring) are not stable and infrequently reported in protein and peptide structures.
Scheme 1.3 Schematic representation of turns (a) and reverse turns (b). In β-turns, a 10-membered H-bonded ring is formed between the amide CO of the amino acid (i) and amide NH at amino acid (i + 3), whereas in reverse β-turn an 8-membered H-bonded ring is obtained from H-bonding between the amino acid (i + 3) and (i + 2).\textsuperscript{[7]} Dashed lines indicate H-bonds.

After this brief discussion of peptide secondary structures with a focus on β-sheet and turn structures, it will be useful to provide a short overview of synthetic methods used to prepare peptides and peptide conjugates.

1.2 Peptide Synthesis

A number of procedures exist that allow the condensation of two amino acids to form an amide or peptide bond. Both solution and solid phase peptide synthesis (SPPS) can be used.\textsuperscript{[8-11]} In a typical peptide coupling reaction, the carboxylic acid component of one amino acid is activated by peptide coupling reagents making it more reactive. The amino group of a second amino acid attacks the activated carboxylic group to form the desired dipeptide. Activation of the acid component of one amino acid 1.1 to the acyl chloride form 1.2 and the active ester forms (succinimidyl active ester 1.3 or benzotriazolyl active ester 1.4) can be achieved as shown in Scheme 1.4. Although, the activation step is necessary to allow the attack by the amino group, this step followed by the next coupling reaction can cause racemization at the carboxyl group. In this context, the choice of the appropriate coupling reagent is crucial to avoid racemization and other side reactions. For instance, active ester derivatives are known as reaction rate enhancer
and racemization suppressor and they can be achieved using carbodiimides or uronium reagents.\textsuperscript{[11]}

\textbf{Scheme 1.4} Common activation processes for amide bond formation. Activation of acid group of one amino acid 1.1 to acyl chloride form 1.2 or active ester forms (succinimidy active ester 1.3 or benzotriazoly active ester 1.4) using different coupling reagents followed by the reaction with amino group of second amino acid to form 1.5. Coupling reagents and conditions are (i) oxalyl chloride ((COCl)\textsubscript{2}) or thionyl chloride (SOCl\textsubscript{2}) in dry CH\textsubscript{2}Cl\textsubscript{2}, (ii) N-hydroxysuccinamide (HOSu), EDC in CH\textsubscript{2}Cl\textsubscript{2}, (iii) 1-hydroxybenzotriazole (HOBt), EDC in dry CH\textsubscript{2}Cl\textsubscript{2} or uronium reagent such as HBTU in dry CH\textsubscript{2}Cl\textsubscript{2}.

Among carbodiimides, \textit{N}-ethyl-\textit{N}''-(3-dimethylaminopropyl) carbodiimide (EDC) is more suitable for solution phase peptide synthesis due to its high solubility in organic solvent such as DCM (dichloromethane) and the solubility of its byproduct in aqueous solvents that can be easily removed during work up.\textsuperscript{[8]} Protection of the \textit{N}- or \textit{C}-terminal side of a peptide with proper protecting groups is critical in order to direct the synthesis toward a single product. For example, as shown in Scheme 1.4 the amino group of 1.1 is protected to prevent the formation of undesired cyclic and linear peptides as byproducts. The most common protecting groups for \textit{N}-terminal side of peptides are \textit{N}-\textit{tert}-butoxycarbonyl (Boc) or fluorenylmethyloxycarbonyl (Fmoc), while methyl, ethyl and benzyl esters are the \textit{C}-terminal protecting groups.\textsuperscript{[12]} Selective cleavage of protecting groups is critical in order to continue with peptide synthesis and at the same time minimize side reactions. For instance, the Boc group can be deprotected by
trifluoroacetic acid (TFA), whereas the ester groups can be removed under basic conditions using a mixture of water and organic solvents such as methanol.\textsuperscript{[2, 13]}

### 1.3 Ferrocene Peptide Conjugates

The design of peptide bioconjugates, as mimics of protein secondary structure, has attracted much attention, since protein folding and conformational changes play a significant role in molecular recognition and function of proteins.\textsuperscript{[14-16]} In this context, molecular scaffolds have been exploited to promote interactions between peptides resulting in specific structural motifs, such as α-helices, turns or β-sheet structures.\textsuperscript{[17]} A number of β-sheet scaffolds rely on strategies, including cyclizations of peptides,\textsuperscript{[18]} the coordination to metal ions,\textsuperscript{[15]} and the insertion of β-amino acids into the amino acid sequence.\textsuperscript{[19]} More recently, ferrocene (Fc) has been exploited as a scaffold. Using Fc scaffolds, it is possible to maintain the secondary structural motifs and to influence the optical and electrochemical properties of the conjugate. Fc has long been exploited as a redox active reporter due to its stability and reversible oxidation-reduction properties that are maintained in a biological environment. Not long after its discovery in 1950’s, Fc-labeled amino acids were the first bioconjugates to be studied,\textsuperscript{[20]} which paved the way towards other Fc-labeled peptides and proteins.\textsuperscript{[21]} Fc-carboxylic acid \textbf{1.6}, Fc-amine \textbf{1.7}, Fc-dicarboxylic acid \textbf{1.8}, Fc-amino acid \textbf{1.9} and Fc-diamine \textbf{1.10} are the most common forms of Fc-derivatives.

Synthetic approaches to synthesize Fc-peptide conjugates rely on peptide coupling methods. For example, Fc-carboxylic acid \textbf{1.6} and Fc-diamine \textbf{1.10} can be coupled to C- or N-protected amino acid or peptide respectively, in the presence of carbodiimide or uronium as activating reagents (Scheme 1.5).\textsuperscript{[22, 23]}
Scheme 1.5 Five ferrocene synthons, Fc-carboxylic acid 1.6, Fc-amine 1.7, Fc-dicarboxylic acid 1.8, Fc-amino acid 1.9 and Fc-diamine 1.10 (a), Synthesis of Fc-CO-Val-Phe-OMe 1.12 from Fc-carboxylic acid 1.6 and H-Val-Phe-OMe 1.11 using, (i) EDC, HOBt in CH$_2$Cl$_2$ \(^{[22]}\) (b), Synthesis of Fe-[NH-Ala-Boc]$_2$ 1.14 from Fc-diamine 1.10 and Boc-Ala-OH 1.13 using, (ii) EDC, HOBt in CH$_2$Cl$_2$ (c).\(^{[23]}\)

In the following sections we will explore the structural motifs and inherent properties of mono and disubstituted Fe-peptide conjugates.

1.3.1 Monosubstituted Ferrocene-Peptide Conjugates

Just as amino acids and peptides often assemble into extended supramolecular three-dimensional structures, monosubstituted Fe conjugates associate into larger aggregates. However, directing the self-assembly process is highly problematic and largely up to chance. Fc-carboxylic acid 1.6 and Fc-amine 1.7 serve as convenient starting materials for the synthesis of Fe-peptide
conjugates 1.15 and 1.16 (Scheme 1.6), both of which provide control over the directionality of the peptide. The supramolecular assembly of such Fc conjugates by intermolecular H-bonding, while interesting and aesthetically pleasing, is essentially not predictable. Self-assembly leads to chains or helical arrangements as is illustrated by the following examples. An intermolecular H-bonding network connects molecules of the Fc-amine conjugate Boc-Gly-NH-Fc (1.17) in the solid state resulting in the formation of linear chains. Formation of the 12-membered H-bonded rings in this compound resembles the H-bonding pattern observed in parallel β-sheets (Scheme 1.2a).[24]

**Scheme 1.6** Two ferrocene synthons, Fc-carboxylic acid 1.6 and Fc-amine 1.7 have been used for the synthesis of a large variety of Fc-peptide conjugates 1.15 and 1.16. The two synthons provide control over the directionality of the peptide attachment and thereby enable the introduction of the Fc group to either side of the peptide dipole. Fc-carboxylic acid conjugate 1.15 shows the N-terminus side of a peptide attached to the Fc group, whereas the Fc-amine conjugate 1.16 has the C-terminus of the peptide attached to the Fc moiety. In both cases conjugation is achieved through an amide linkage.

Similar H-bonding pattern is observed in Fc-CO-Gly_2OEt.[22] This is not surprising since the donor–acceptor sites in both complexes are identical, and only the directionality of the peptide attachment is affected. In contrast, the ferrocene-dipeptide conjugates Fc-L-Ala-L-Pro-NH-Py (1.18) align in an antiparallel manner having molecules connected through intermolecular N–H⋯N and N–H⋯O H-bonds, forming a nine-membered H-bonded ring (Figure 1.1b). [25]
**Figure 1.1** H-bonding patterns in two monosubstituted Fc-peptide conjugates. (a) Chemical drawing and molecular structure of Boc-Gly-NH-Fc (1.17) showing the H-bonding interactions leading to the formation of 12-membered H-bonded ring, the H-bond lengths are the $d(N\cdots O) = 2.844$ and $2.855$ Å. (b) Chemical drawing and molecular structure of Fc-L-Ala-L-Pro-NH-Py (1.18) showing the H-bonding interactions leading to the formation of 9-membered H-bonded ring, the H-bond lengths are the $d(N\cdots O) = 2.902$ (5) and $d(N\cdots N) = 3.153$ (7) Å. Dashed line indicate H-bonds.

Kenny has reported a series of structurally related peptide conjugates of ferrocenylbenzoic acid. Scheme 1.7 shows the interactions between two molecules of $N$-[meta-(ferrocenyl) benzoyl]alanineglycine ethyl esters (1.19). It is interesting to note that the H-bonding pattern established by this compound involved two $syn$ NH groups, which interact with two CO groups from an adjacent conjugate forming a 12-membered H-bonded ring. The molecules interact in a
head-to-tail helical fashion resulting in the formation of a helical structure. The Fc-glycyl-cystamine conjugate [Fc-Gly-CSA]$_2$ (1.20) is another interesting example of helical self-assembly in monosubstituted Fc conjugates.[27] H-bonding through the Fc-amide and cystamine functions involved interactions with the identical portion of two neighboring molecules. The two parts of the molecule displays a distinct H-bonding pattern involving 12-membered rings. As indicated in Scheme 1.7, one of the H-bonding interactions established involves a pair of H-bond acceptors/donors arranged in an unusual syn fashion resulting in a highly asymmetric H-bonding interaction.

![Scheme 1.7](image)

**Scheme 1.7** Two examples of H-bonding interaction in monosubstituted Fc-peptide conjugates. Two molecules of Fc conjugate N-[meta-(ferrocenyl)benzoyl]alanineglycine ethyl ester (1.19) interacting through H-bonding. The arrangement of the amide provides a syn alignment leading to the formation of a 12-membered H-bonded ring. Two molecules of [Fc-CO-Gly-CSA]$_2$ (1.20) showing two set of H-bonding patterns. One of the H-bonding interactions established in an unusual syn fashion. Dashed lines indicate H-bonds.

The Fc groups decorate a central H-bonded peptide core. Small changes, such as the substitution of Ala for Gly, have a profound effect on the arrangement of the donor and acceptor sites, which guides the H-bonding ability and the supramolecular assembly. [Fc-Ala-CSA]$_2$ is symmetrical. The unusual H-bonding found in the Gly analogue is lost.[28]
Figure 1.2 Molecular structure of Fc-CO-L-Ala-D-Pro-NH-4-Py (1.21) showing the intramolecular H-bonding between the NH adjacent to the pyridyl moiety and the CO proximal to Fc group of the same peptide chain and formation a 10-membered H-bonded ring. Dashed lines indicate H-bonds.

The first example of the monosubstituted Fc-peptide conjugates containing a β-turn structure was recently presented by Hirao and co-workers as depicted in Figure 1.2. Incorporation of a dipeptide strand with heterochiral sequence into Fc moiety led to the formation of a β-turn-like structure. In the solid state structure of Fc-CO-L-Ala-D-Pro-NH-4-Py (1.21, Py: pyridine), the formation of an intramolecular H-bonding (10-membered H-bonded ring) between the NH adjacent to the pyridyl moiety and the CO proximal to Fc group of the same peptide strand was observed resulting in a β-turn-like structure. [29]

1.3.2 Disubstituted Ferrocene-Peptide Conjugates

Unlike monosubstituted Fc-peptide conjugates, which lack the control over the self-assembly properties, their disubstituted analogues can be tailored with respect to the direction of the assembly and the H-bonding interactions between peptides. The two cyclopentadienyl (Cp) rings in Fc separated by 3.3 Å provide an ideal distance for promoting intramolecular hydrogen bonding between the peptide strands on the two Cp rings with the distance close to N⋯O
distance found in β-sheets. Employing different disubstituted Fc derivatives allows for the formation of different H-bonding patterns between peptide strands. For instance, Fc-peptides derived from the Fc-dicarboxylic acid (1.22) and the Fc-diamine (1.24) allow formation of 10- and 14-membered H-bonding rings, while those derived from Fc-amino acid (1.23) impose the 12-membered H-bonding constraints (Scheme 1.8). Moreover, the directionality of the peptide arms can be tailored through direct N- or C-terminal coupling to the peptides of interest. Due to the intramolecular H-bonding interactions in these conjugates, rotation about the Cp-Fe-Cp axis is restricted, leading to stable stereoisomers in which Fc moiety exhibits axial chirality. Recently, a systematic nomenclature was defined for Fc-disubstituted conjugates with chiral substituents which is based on the relative orientation of two Cp rings with respect to each other. Positional isomers can be defined by the torsion angle ω between peptide substituents on the two Cp rings.[7] For instance, a 1,1'-isomer is defined by an angle ω = -36° and ω = +36°, while a 1,2'-isomer is defined as -36° < ω > 108° (Scheme 1.9a and b).

Scheme 1.8 Illustration of the particular intramolecular H-bonding interactions in disubstituted Fc-peptide conjugates. Intramolecular H-bonding interactions in 1.22, 1.23 and 1.24 lead to the formation of 10-, 12-, and 14-membered H-bonding rings, respectively. Employing different disubstituted Fc derivatives, formation of peptide conjugates with parallel (1.22, 1.24) and antiparallel 1.23 alignment of peptide chains is possible.

In this context, the element of axial chirality of the Fc moiety can be described by using helical chirality descriptors P and M and by examining the relative orientation of the two peptide chains on the 1,n'-Fc motif. The P-isomer has the higher priority substituent in position 1 on the top Cp ring, while the lower priority substituent on the lower Cp ring in positions 2' or 3' giving a clockwise rotation applying Kahn-Ingold-Prelog rules. The M-isomer has lower priority substituents in positions 4' or 5' resulting in a counter-clockwise rotation. This gives rise to
isomers P-1,1', P-1,2', P-1,3', M-1,4' and M-1,5' (Scheme 1.9c). The effect of stereochemistry of peptide substituent on the axial chirality of Fe-peptide conjugates will be discussed later.

Scheme 1.9 Fc-axial chirality. (a) Schematic representation of torsion angle $\omega$ between peptide chains on the two Cp rings. (b) Possible orientations of the two substituents at two different Cp rings giving rise to different stereoisomers and (c) examples of stereoisomers considering the axial chirality and arrangement of the two substituents at the Cp rings.

The capability of Fe-dicarboxylic acid to act as a scaffold was first exploited by Herrick and co-workers who prepared a series of Fe-dicarboxylic acid peptide conjugates Fe[CO-Aa-OMe]$_2$ (Aa = Val, Phe, Pro). Using Fe[CO-Val-OMe]$_2$ (1.25) as a representative example of this group, the podant peptide substituents on the two Cp rings adopt a 1,2'-conformation allowing two equivalent intramolecular hydrogen bonding interactions between the amide NH of one strand and the carbonyl CO of the opposite strand to form a 10-membered H-bonded ring identical to that observed in $\beta$-turns (Scheme 1.10). Although theoretical calculations show that 1,2'-conformation or “Herrick” conformation is energetically favored, there are a number of Fe-conjugates that exhibit alternative conformations. For example, in the solid state structure of Fe[CO-Phe-OMe]$_2$ (1.26), the two peptide substituents also adopt a 1,2'-conformation, but the disposition of the two amide carbonyl groups allow only the formation of a single cross-strand H-bond. $\pi$-stacking of the aromatic phenyl groups presumably contributes to the stability of this
“van Staveren” conformation in the solid state. However, in solution conjugate 1.26 presumably adopts the more stable “Herrick” conformation. Fc[CO-Cys(Bn)-OMe]$_2$ (1.27) is an example of the “Xu” conformation in the solid state. The system adopts a 1,3'-conformation and lacks intramolecular H-bonding. Theoretical investigations revealed that the “Herrick” conformation is energetically favored over other conformations with an activation barrier for H-bond breaking and Cp ring rotation of 13-18 kJmol$^{-1}$.

Scheme 1.10 Different H-bonding patterns in Fc-dicarboxylic acid peptide conjugates. Fc[CO-Val-OMe]$_2$ (1.25) is an example of “Herrick” conformation with two equivalent intramolecular hydrogen bonding interactions between the amide NH of one strand and the carbonyl CO of the opposite strand. Fc[CO-Phe-OMe]$_2$ (1.26) and Fc[CO-Cys(Bn)-OMe]$_2$ (1.27) are examples of alternative conformations with one cross-strand H-bond (“van Staveren”) or no intramolecular H-bond (“Xu”), respectively.

Steric effects and the substitution at the peptide terminus may also induce conformational changes in Fc-peptide conjugates. For instance, in disubstituted Fc[CO-Asp$_n$]$_2$ dendrimers, the transition from the “Herrick” to alternative conformations is induced by increasing the size of the dendrimer unit. In addition, peptide C-terminal modification can affect the intra- and intermolecular H-bonding patterns, as is the case for the two Fc-Gly-OR conjugates. Upon ester deprotection of Fc[CO-Gly-OEt]$_2$, the conformation of Fc core changes from “Xu” to “Herrick” motif.
Fc-helical isomers possess a spectroscopic signature in the Fc region of the circular dichroism (CD) spectrum. Hirao and coworkers first recognized that induced axial chirality results in a distinct pattern for L- and D-amino acid conjugates of Fc.

Figure 1.3 (a) Molecular structures of Fc[CO-L-Ala-L-Pro-OEt]$_2$ (1.28) and Fc[CO-D-Ala-D-Pro-OEt]$_2$ (1.29). (b) Circular dichroism spectra of two Fc-peptide isomers and the representation of the $P$ (1.28) and $M$ (1.29) nomenclature used to describe the helicity of disubstituted Fc-peptides. Helicity is induced by intramolecular hydrogen bonding between two substituents at the ferrocene core. Generally L-amino acids impose $P$-helicity, while D-amino acids induce $M$-helicity. "Adopted and modified with permission from (T. Moriuchi, T. Hirao, Acc. Chem. Res. 2010, 43, 1040-1051). Copyright (2010), American Chemical Society".
To probe the effects of the amino acid and peptide chirality on the helicity of Fc-peptide conjugates, a variation of the D- and L-amino acid content was systematically explored.\textsuperscript{[38, 39]} Using Fc[CO-L-Ala-L-Pro-OEt]$_2$ (1.28) (Figure 1.3) as an example, a stable $P$-helical arrangement with two intramolecular H-bonds was observed in the crystal structure and in solution. The corresponding D-Ala conjugate 1.29, the enantiomer of 1.28, displays $M$-helicity. In this context, CD spectroscopy is essential for evaluating helical conformations in solution. In general, 1,$n'$-Fc conjugates with L-amino acids proximal to the Fc group exhibit a strong positive Cotton effect in the 400 – 500 nm range of the CD spectrum, indicative of $P$-helicity. The corresponding Fc-conjugates with D-amino acids proximal to Fc display a negative Cotton effect, indicative of $M$-helicity. Fc-conjugates with different peptide substituents on the two Cp rings generally adopt the helicity that is determined by the proximal amino acid and follow the rule “L-amino acids give rise to $P$-helicity; D-amino acids give rise to $M$-helicity”.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_4.png}
\caption{Chemical drawings and molecular structures of Fc[CO-L-Pro-L-Ala-NHPy]$_2$ (1.30) and Fc[CO-D-Pro-D-Ala-NHPy]$_2$ (1.31) showing the formation of $\gamma$-turn structure. Intramolecular H-bonding interactions between the aniline NH group and the CO group of Pro of the same peptide strand induce the formation of a 7-membered ring ($\gamma$-turn). The H-bonding distances in $\gamma$-turn motif are $d$(N···O) = 3.032 (6) Å and 2.996 (5) Å. Dashed line indicate H-bonds.}
\end{figure}
Hirao and co-workers also reported the first example of Fc-based γ-turn structures in Fc[CO-L-Pro-L-Ala-NHPy]$_2$ (1.30, Py: pyridine) and Fc[CO-D-Pro-D-Ala-NHPy]$_2$ (1.31, Py: pyridine) (Figure 1.4). A combination of Fc as a scaffold, Pro (Proline) as a known turn inducer, and Ala (Alanine) as an H-bond donor induces the formation of γ-turns. This results in backfolding of the peptide onto itself, in which intrastrand H-bonding interactions between the aniline NH and the CO group of Pro results in the formation of a γ-turn. Additional intramolecular H-bonding involving the Ala NH group is observed in the solid state, which helps to maintain the rigidity of the system.

Two basic methods are currently available enabling the formation of β-sheet-like structures in Fc-peptide conjugates. Cyclization of Fc-peptides is a useful method to form and stabilize a β-sheet conformation between peptides since it forces the two peptide strands into close proximity. This alignment of peptide strands and the resulting “Herrick” pattern of H-bonding jointly with the rigidity of the macrocycle set the conditions for intermolecular H-bonding. A series of cyclic Fc-peptide conjugates with the general formula Fc[CO-Aa-CSA]$_2$ (Aa = Gly, Ala, Val, Leu) and Fc[CO-Gly-Aa-CSA]$_2$ (Aa = Val, Ile) are now available. The presence of the cystamine linker, tying the two peptide strands, adds to the rigidity provided by the “Herrick” cross-strand interactions and establishes an interface for the intermolecular H-bonding.

**Figure 1.5** Chemical drawing and molecular structure of Fc[CO-Gly-CSA]$_2$ (1.32) showing the interaction of individual molecules. It is noteworthy to point out that the H-bonding interface on the edge of the macrocycles is not of sufficient size to allow more extensive H-bonding interactions. Dashed lines indicate H-bonds.
However, the β-sheet-like interactions are established on an intermolecular level. In the solid state, molecules of Fc[CO-Gly-CSA]₂ (1.32) associate by intermolecular H-bonding (Figure 1.5). It is this H-bonding interface on the edge of the molecule that was exploited for the design of a β-sheet interface. Extending the H-bonding interface within these molecules in a rational fashion is possible and favors the side to-side interaction between the peptide strands leading to the formation of an antiparallel strand alignment. It is important to point out that Gly as an Fc-proximal amino acid is critical in providing the necessary flexibility in this conjugate.

**Figure 1.6** Molecular structure of Fc[CO-Gly-Val-CSA]₂ (1.33) and a schematic representation of the pseudo-β-barrel. (a) The molecular structure of Fc[CO-Gly-Val-CSA]₂ (1.33) showing the intramolecular H-bonding between the peptide strands on the two Cp rings. (b) The formation of antiparallel β-sheet through intermolecular N(H)···O=C H-bonding. Molecules are arranged in a head-to-tail fashion. (c) Curvature of the molecular building blocks promotes H-bonding to form a β-barrel-like structure. (d) Tiling of cyclic ferrocene peptides is illustrated in this molecular surface representation of the barrel viewed along the c-axis. "Adapted with permission from (S. Chowdhury, D. A. R. Sanders, G. Schatte, H.-B. Kraatz, Angew. Chem. Int. Ed. 2006, 45, 751-754). Copyright (2006) Wiley-VCH Verlag GmbH & Co. KGaA."
The solid-state structure of Fc[CO-Gly-Val-CSA]$_2$ (1.33) clearly shows the intermolecular H-bond interactions and the peptide alignment (Figure 1.6). In this system, the three intramolecular H-bonds (Figure 1.6a) and the intermolecular contact in a head-to-tail fashion result in the formation of a 14-membered ring depicted in Figure 1.6b. Four molecules interact to give β-barrel (Figure 1.6c and d) and the eight β-strands running parallel to the axis of the barrel with an internal pore diameter of 8 Å combine into the pseudo-barrel and mimic other 8-stranded barrels found in biological systems.[42]

From the earlier discussion of peptide conjugates of Fc-dicarboxylic acids, it was learned that whereas these systems are rigid in proximity to the Fc core, they are highly flexible at the C-terminal ends of the conjugate. While cyclization adds rigidity to the molecule, it limits the validity of the conjugate as a model for an extended β-sheet-like structure. Recently, examples of extended β-sheet-like structure were reported for a series of 1,n′-Fc-tripeptide conjugates containing amino acids Gly, Val/Leu, and Cys(Bn).[43] The solid state structures of Fc[CO-Gly-Val-Cys(Bn)-OMe]$_2$ (1.34) is shown in Figure 1.7.

**Figure 1.7** Chemical drawing and molecular structure of the Fc[CO-Gly-Val-Cys(Bn)-OMe]$_2$ (1.34) showing intramolecular H-bonding motifs. The peptide chains are aligned in a parallel fashion allowing for the formation of H-bonding interactions between peptide strands. In addition to “Herrick” motif, a second set of H-bonding contacts exists between the two amino acids on the C-terminal of peptide chains. Dashed lines indicate H-bonds.
Contrary to Fc-dipeptide conjugates, the peptide chains in this class of examples are aligned in a parallel fashion. The choice of Gly as the first amino acid provides maximum flexibility to the system and aligns the two peptide strands to engage in additional interstrand H-bonding interactions akin to those found in sheet-like structures. The choice of Val or Ile, having high β-sheet propensities, is essential in promoting stable peptide alignments leading to intermolecular interactions towards a sheet-like supramolecular assembly.

Disubstituted Fc-dicarboxylic acid derivatives are characterized by a stable and reproducible redox signal with an $E_{1/2}$ potential in the 680-900 mV range vs Ag/AgCl (acetonitrile or dichloromethane). The inherent redox stability of Fc-peptides has been explored for the study of interactions with anions, cations and other guest molecules.$^{[29,44,45]}$

Unlike Fc-dicarboxylic acid, Fc-amino acid (Fca, 1.9) allows for the attachment of peptide chains in an antiparallel fashion, which more closely mimics the natural peptide turns. The molecular structure of Boc-L-Ala-Fca-L-Ala-L-Ala-OMe (1.35) reveals an antiparallel arrangement of the two peptide strands, allowing the formation of two intramolecular hydrogen bonds with 9- and 11-membered H-bonded rings (Figure 1.8).$^{[46]}

![Chemical drawing and molecular structure of the Boc-L-Ala-Fca-L-Ala-L-Ala-OMe (1.35)](image_url)

**Figure 1.8** Chemical drawing and molecular structure of the Boc-L-Ala-Fca-L-Ala-L-Ala-OMe (1.35) showing the expected interstrand H-bonding pattern. 9 and 11-membered H-bonded rings formed by intramolecular H-bonding between the proximal Fc-NH and the CO of Ala group. Dashed lines indicate H-bonds.
Introduction of an $\alpha$- (Gly, Ala, Val) and a $\beta$-amino acid into Fca also induces a well-defined secondary structure giving rise to 9- and 11-membered H-bonded rings.\cite{31, 47-49} As described previously in the discussion of Fc-dicarboxylic acid peptide conjugates, the amino acid proximal to the Fc group dictates the axial chirality of the Fc moiety. In Fca amino acid conjugates, the amino acids can be attached to either the C- or the N-terminal of Fca, potentially leading to different stereoisomers. One might assume significant differences in between having a given amino acid attached to the amino or carboxylic acid group of Fca. Studies on Fca-peptide conjugates clearly demonstrate that the helicity of the Fca core is dependent on the nature of the first amino acid attached to the Fca-amino group. For example, $M$- and $P$-helical conformations have been observed for Boc-Fca-L-Ala-OMe and Boc-Fca-D-Ala-OMe, respectively.\cite{50} In contrast, the conjugate 1.35, which possesses L-Ala at both terminal groups, adopts a $P$-helical conformation both in the solid state and in solution. Extended helical foldamers, containing repeating Fca-CO-Ala units, were reported to display a $\beta$-helical conformation.\cite{51} In these systems, $P$- and $M$-helicities have been observed for L- and D-Ala, respectively. The acetylated Ac-Fca-L-Val-L-Ile-OMe conjugate exhibits $P$-helical conformation in solution with a positive CD signal. \cite{31} The interesting differences between Boc- and Ac-terminated Fca conjugates were addressed by performing a systematic study of bisamide X-Fca-Aa-OMe and trisamide X-Aa-Fc-Aa-Aa-OMe (X = Ac, Boc; Aa = Gly, Ala, Val) systems. All trisamides exhibited two intramolecular H-bonds regardless of the protecting groups and the $\alpha$-amino acids. Moreover, the trisamides and bisamides are characterized by the positive $P$-helicity. While the effects of the protecting groups on the conformation were indiscernible, the increasing size of the amino acid side chain destabilized certain conformations.

Other Fca derivatives, such as Boc-Fca-Asp(OH)-OH have been explored with Fe[COOMe][CO-Arg-Arg-NH$_2$] conjugate towards understanding salt bridge-type interactions.\cite{52} In a 1:1 complex, the interaction between diarginine and aspartic acid leads to the formation of the ion-pair without the involvement of the amide backbone. These systems are useful models for naturally occurring salt-bridges which are found in enzymes such as lysozyme epitope and dihydroxylate reductase.\cite{53, 54} Alternatively, a $\beta$-lactam ring may be introduced in order to achieve additional H-bonding interactions and a potential synthetic handle for further modification, since $\beta$-lactams are known to serve as intermediates towards $\alpha$- and $\beta$-amino acids.\cite{55, 56} The lactam containing Fca conjugates Fe[CO-Ala-Alm]$_2$ and Boc-Fca-Ala-
Alm exhibit the intramolecular H-bonds between the two strands and an additional intrachain NHAlm⁻•COFca interactions resulting in the γ-turns and P-helicity arrangement. Expanding further on the asymmetrically substituted Fca, bioconjugates containing (S)-3-amino-2-methylpropanoic acid (Aib) were designed to probe the effects of the methylene group insertion on H-bonding pattern. The comparison between the Boc-Ala-Fca-Ala-OMe (1.36) and Boc-Aib-Fca-Ala-OMe (1.38) indicates the disordered conformation and preferred M-helicity in the latter case. For Boc-Aib-Fca-Aib-OMe (1.39), the two intramolecular H-bonds are greatly destabilized resulting in the inversion of helicity of the Fc group from P- to M-helicity (Scheme 1.11). Computational studies on these systems revealed that the configuration of the carbon atom of Aa1 in Aa1-Fca-Aa2, for α- and β-amino acids, directs the helicity of the Fc group.

Scheme 1.11 Chemical drawing and preferred conformations of Aib-Fc conjugates in solution. (a) Computational and experimental studies showed that P-helicity is stabilized if m = 0 and n = 0 or m = 0 and n = 1 as it was observed in Boc-Ala-Fc-Ala-OMe (1.36) and Boc-Ala-Fc-Aib-OMe (1.37), respectively, (b) while M-helicity is preferred conformation if m = 1 and n = 0 or m = 1 and n = 1 in the case of Boc-Aib-Fca-Ala-OMe (1.38) and Boc-Aib-Fca-Aib-OMe (1.39), respectively. Dashed lines indicate H-bonds.

The electrochemical properties of Fca derivatives differ from those of Fc-dicarboxylic acid analogues. Fca conjugates display a lower redox potential making it easier to oxidize, reflecting the increased electron density on the Fc core due to amino substitution proximal to Fc unit. E_{1/2} values fall in the 300-500 mV range vs. Ag/AgCl. The relatively low redox potential of Fca conjugates makes them useful for probing intermolecular interactions.
The Fc-diamine scaffold allows the attachment of two peptide strands in parallel fashion and is complementary to the Fc-dicarboxylic acid scaffold and provides access to 14-H-bonded ring. The H-bonded ring size increases from 10- to 12- to 14-membered when going from Fc-dicarboxylic to Fc-aminoacid to Fc-diamine conjugates, respectively. The first example of the symmetrically substituted Fc-diamine peptides was reported by Kraatz and co-workers,[23] and has since been explored for asymmetrically substituted analogues as well.[59, 60] The CD spectra of the two conjugates, containing D- or L- functionalities, reveal that they are enantiomers of each other. For example, the Fc[NH-D-Ala-Boc]_2 (1.40) in Scheme 1.12, containing D-Ala exhibits two intramolecular H-bonds between the amide groups connected to the Cp rings and the adjacent carbonyl of the Boc causing the M-helicity. The H-bonding pattern is dramatically influenced by the substitution of Cp rings and the helicity is achieved through the attachment of the first amino acid substituent regardless of the composition of the second modification. In a very recent study, Rapic and coworkers prepared a series of asymmetric 1,1′-diaminoferrocene conjugates of α-amino acids, such as Fe[NH-Aa-Boc][NH-Ac] (Aa = Gly, 1.41) (Aa = Gly, L-Ala, D-Ala, Val) (Scheme 1.12).[59] These systems assemble in weakly coordinating solvents such as CHCl_3, as well as in DMSO, with intramolecular H-bonds involving the NH groups proximal to the Fc core. The majority of the low-energy conformations possess a P-helical ferrocene chromophore for L-amino acid substituents so that chirality organization is already achieved by attaching one amino acid substituent at the 1,1′-diaminoferrocene central unit. The electrochemical behaviour of Fc-diamine is typically characterized by the quasi-reversible redox potential in the 300-400 mV range vs Ag/AgCl.

Scheme 1.12 Chemical drawings of two Fc-diamine peptide conjugates, Fe[NH-D-Ala-Boc]_2 (1.40) and Fe[NH-Gly-Boc][NH-Ac] (1.41). The presence of two intramolecular H-bonds between the NH amides of one strand and CO amides of opposite strand resulting in the helicity of Fc core. The helicity dependent on the nature of the first amino acid attached to the Fc-diamine core regardless of composition of second amino acid. Dashed lines indicate H-bonds.
H-bonding interactions in Fc-disubstituted peptide conjugate have been widely studied by $^1$H NMR and FT-IR spectroscopies. Generally, the chemical shift of amide protons engaged in strong H-bonding interactions are deshielded beyond 7 ppm. Temperature dependent $^1$H NMR can be applied to study the ability of amide protons to participate in intra- or intermolecular H-bonds,[34] whereas concentration dependent $^1$H NMR can distinguish between intra and intermolecular interactions.[43] The chemical shifts of amide protons (B and B') in conjugate 1.34 change by varying concentration indicating its engagement in intermolecular H-bonding interactions (Figure 1.9).

![Chemical structure and concentration dependent $^1$H NMR spectra of conjugate 1.34 in CDCl$_3$. Concentration from bottom to top 2, 1.6, 1.33, 1.14, 1.00, 0.98 and 0.8 mM. The Chemical shifts of amide protons (B and B') change dramatically by varying concentration indicating the involvement of NH protons in intermolecular H-bonds, whereas decreasing the concentration does not affect the chemical shifts of amide protons (A and A', C and C'). Adapted and modified with permission from (S. Chowdhury, G. Schatte, H.V.B. Kraatz, Angew. Chem. Int. Ed. 2008, 47, 751-754). Copyright (2008) Wiley-VCH Verlag GmbH & Co. KGaA.](image)

Another method was introduced by Rapic and co-workers to recognize which amide protons are involved in intra or intermolecular H-bonds. According to this method if the chemical shift variation of amide protons in DMSO-d$_6$ compared to CDCl$_3$ is small, the amide protons are considered to be engaged in intramolecular H-bonding interactions, which is unaffected by solvent effects.[59] If large chemical shift differences are observed, the amides are solvent
accessible and are able to engage in intermolecular interactions. In this case, concentration dependent NMR studies are generally necessary to confirm the presence of such intermolecular interactions. IR spectroscopy measurements are helpful to elucidate H-bonding in proteins, peptides and peptide conjugates. An absorption in the amide A region, below 3400 cm$^{-1}$, in a FT-IR spectra shows the involvement of amide protons in H-bonding interactions. Also appearance of a series of bands in the amide I and amide II regions of the FT-IR spectrum (1627-1639 cm$^{-1}$ and 1680-1690 cm$^{-1}$) can be related to a $\beta$-sheet structure.

1.4 Electrochemical Studies of Interactions of Fc-Peptide Derivatives with Biomolecules on Surfaces

The inherent redox property of the Fc moiety in Fc-peptides allows probing their interactions with peptides and proteins by monitoring changes in the electrochemical behavior of the Fc group. In these systems, the peptide aptamer is involved in specific interaction with an analyte, a molecule that is able to bind to the peptide motif, which induces or changes the electrochemical properties of the Fc/Fc$^+$ redox couple. In one general approach employed for Fc-biosensor design, the Fc-peptide is immobilized on an electrode surface and its response to the analyte is detected by electrochemical techniques. Lin and co-workers reported the enzymatic cleavage of a monosubstituted Fc-peptide conjugate on the surface based on the “signal off” sensing principle. The Fc-peptide conjugate containing the sequence Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-Cys was synthesized and immobilized on the gold surfaces through a C-terminal Cys thiol. The presence of a reversible redox signal with a formal potential of $E^\circ = 260$ mV vs Ag/AgCl, characteristic of the Fc group, confirmed the formation of the Fc-peptide film on the surface (Figure 1.10). Enzymatic hydrolysis of Fc-peptide at the Ala-Leu site in the presence of metalloproteinase-7, a hydrolytic enzyme, led to a significant signal decrease due to the removal of the Fc-tag from the electrode surface that can be detected electrochemically.

Examples of Fca-peptide conjugates immobilized on gold surfaces have also been explored for the purpose of studying peptide-protein interactions. The lower redox potential of Fca derivatives compared to Fc-dicarboxylate derivatives (~ 250 mV versus ~ 400 mV vs Ag/AgCl) makes them ideal candidates to study biomolecular interaction since the redox signal does not interfere with limitations due to solvent or ions present in complex mixtures.
**Figure 1.10** (a) Cyclic and (b) square-wave voltammograms of Fc-peptide film on gold surfaces before (red signal) and after (blue signal) interaction with metalloproteinase-7. Fc redox signal was decreased significantly after enzymatic hydrolysis of Fc-peptide. All potentials are referred to an Ag/AgCl reference electrode. "Adapted with permission from G. Liu, J. Wang, D. S. Wunschel, Y. Lin, J. Am. Chem. Soc., 2006, 128, 12382. Copyright (2006) American Chemical Society."

In this context, the unsymmetrically disubstituted Fca conjugates possessing the known papain peptide inhibitor, Gly-Gly-Tyr-Arg-OH, was immobilized on gold surfaces. Attachment of lipoic acid to the amino group of the Fca-peptide conjugate allowed for the film formation on the surfaces. Upon interaction of the Fca-peptide film with different concentrations of papain, the Fca signal shifted to higher potential and the signal intensity decreased, indicating a partial shielding of the Fca group by papain which limits the accessibility of the Fca group to the supporting electrolyte and renders its oxidation more difficult (Figure 1.11). Similar strategies were applied to the study of Fca-peptide interactions with HIV-1 related enzymes.

Electrochemical immunoassays involving Fc-labelled antibodies are another interesting approach to monitor the interaction between antigens and antibodies. In this approach, the antibody was immobilized on the surface and allowed to bind to the specific antigen, followed by the interaction of a Fc-labelled antibody which gave rise to the redox signal. The intensity of the redox signal is proportional to the concentration of the antigen.
Figure 1.11 Electrochemical detection of Fca-peptide and papain interactions on the gold surface. (a) Schematic representation of Fca-peptide on gold surface before and after interaction with papain enzyme. (b) Cyclic voltammograms of Fca-peptide film in the presence of different concentrations of papain enzyme. Interaction of counter ions (green) and Fc core is restricted in the presence of papain enzyme which led to an overall decrease in the current intensity. Potentials are referred to an Ag/AgCl reference electrode. Taken with permission from K. A. Mahmoud and H.-B. Kraatz, Chem. Eur. J., 2007, 13, 5885.

These examples clearly show the use of Fc-peptide conjugates for the study and detection of a range of interactions in peptides and proteins. Taking advantage of electrochemical properties of Fc core and biological relevant of peptide motifs, Fc-peptide conjugates are one of the useful systems to design biological sensors.

1.5 Hydrophobic Core of Amyloid β-Peptide

The next topic of focus will be on the peptide fragment that constitutes the hydrophobic region of the amyloid-β peptide (Aβ), which is hypothesized to play a critical role in aggregate formation and may present a possible target for aggregation inhibitors. Two pathological hallmarks of Alzheimer’s disease (AD), the most common form of dementia, are accumulation of plaques and neurofibrillary tangles outside and inside the neurons found in the brains of AD patients. The major component of plaques is amyloid-β peptide (Aβ) with 40 to 42 amino acids which is
formed from sequential proteolytic cleavage of a larger transmembrane precursor protein amyloid precursor protein (APP). High resolution X-ray diffraction and solid-state NMR measurements showed the presence of a cross-β structure that contains parallel, in register β-sheets. Studies confirmed that shorter fragments of Aβ, such as Aβ12-28, Aβ10-35, Aβ16-22, and Aβ34-42 also form amyloid fibrils. The crucial role of the peptide sequence Lys-Leu-Val-Phe-Phe (Aβ16-20) for self-assembly and self-recognition was first recognized by Tjernberg and co-workers. In studies investigating the role of this hydrophobic peptide sequence for binding to Aβ and its fragments, Tjernberg discovered that this hydrophobic peptide sequence is necessary for establishing interactions with Aβ. Only fragments containing this hydrophobic core including Aβ10-19, Aβ11-20 and Aβ12-21 were able to bind to full-length Aβ. Introduction of threonine (Thr) as a hydrophilic amino acid into the peptide sequence of the hydrophobic core resulted in a significant decrease in fibril formation, further illustrating the importance of the hydrophobic core for the initial peptide interactions, ultimately leading to fibril formation.

Transmission electron microscopy (TEM) studies showed that the Lys-Leu-Val-Phe-Phe (Aβ16-20) fragment itself formed fibrils in aqueous solution. Further studies based on electron microscopy, atomic force microscopy and small-angle scattering data reported the formation of fibers and nanotubes for acetylated Aβ16-22 (Ac-Lys-Leu-Val-Phe-Phe-Ala-Glu-NH₂) at different pH. Figure 1.12 (a and b) shows TEM images of acetylated Aβ16-22 nanofibers and nanotubes at pH 6 and pH 2, respectively, and the proposed model for fiber growth at the macromolecular level based on experimental evidence. As depicted in Figure 1.12c, association of peptide strands through H-bonding interactions leads to the formation of β-sheets. Lateral stacking of β-sheets through hydrophobic and electrostatic interactions gives rise to the formation of fibers. Hamley and co-workers studied the self-assembly properties of a range of peptide fragments derived from Aβ16-20 and reported distinct morphologies under different conditions. For example, Ala-Ala-Lys-Leu-Val-Phe-Phe self-associated to form twisted fibrils in water (Figure 1.13a), while in methanol formation of nanotubes was reported (Figure 1.13b). Replacement of the Ala residues with β-Ala gave rise to the formation of helical ribbons (Figure 1.13c).
Figure 1.12 TEM images of assemblies of acetylated Aβ_{16-22} at two different pH and proposed model for fiber formation. (a) TEM image of fibers obtained at pH 6 and (b) TEM image of nanotubes obtained at pH 2. (c) Proposed model for fiber formation. Peptide strands propagate through H-bonds to form β-sheets. Five β-sheets associate through hydrophobic and electrostatic interaction to give rise to the fiber formation. Each β-sheet is indicated with different colors. The distances between peptide strands and β-sheets are 5 and 10 Å, respectively. "Taken with permission from (A. K. Mehta, K. Lu, W. S. Childers, Y. Liang, S. N. Dublin, J. Dong, J. P. Snyder, S. V. Pingali, P. Thiyagarajan, D. G. Lynn, J. Am. Chem. Soc. 2008, 130, 9829-9835). Copyright (2008) American Chemical Society."

The ability of the Aβ_{16-20} fragment to self-assemble and bind to full-length Aβ suggests that the hydrophobic core and its derivatives may be attractive not only for interfering in the Aβ aggregation but also may allow disassembly of the formed Aβ aggregates. Initial work by Tjernberg and co-workers showed that this motif is capable of inhibiting Aβ fibril formation.\textsuperscript{[76]} Several groups have designed different peptides based on the hydrophobic core of Aβ. For example, modification of the peptide backbone in Lys-Leu-Val-Phe-Phe-Ala-Glu and Lys-Leu-Val-Phe-Phe motifs and replacement of amide protons with methyl groups (Scheme 1.13a) or replacement of amide bonds with ester bonds (Scheme 1.13b) blocked the H-bonding interactions between β-sheets and disrupted Aβ fibrillization.\textsuperscript{[83, 84]} These fragments are known to
form peptide strands with two different faces, one face with unmodified amide protons cable of engaging in H-bonding and the other possessing modified groups.

Figure 1.13 TEM images of assemblies obtained from Aβ_{16-20} derivatives in different solvents. (a) TEM image of assemblies of Ala-Ala-Lys-Leu-Val-Phe-Phe in water, formation of twisted fibrils with average width of 63 nm was observed. (b) TEM images of nanotubes with average width of 116 nm formed from the solution of Ala-Ala-Lys-Leu-Val-Phe-Phe in methanol. (c) cryo-TEM image of assemblies of βAla-βAla-Lys-Leu-Val-Phe-Phe in water, helical ribbons with the average diameter of 17.5 nm were formed. "Adapted and modified with permission from (V. Castelletto, I. W. Hamley, P. J. F. Harris, Biophys. Chem. 2008, 138, 29-35), Copyright (2008) Elsevier and (M. J. Krysmann, V. Castelletto, J. E. McKendrick, L. A. Clifton, I. W. H., P. J. F. Harris, S. M. King, Langmuir 2008, 24, 8158-8162), Copyright (2008) American Chemical Society) and V. Castelletto, I. W. Hamley, R. A. Hule, D. Pochan, Angew. Chem. Int. Ed. 2009, 48, 2317-2320), Copyright (2009) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim). "

Based on this arrangement, formation of H-bonds is blocked through the modified face and this can disrupt β-sheet formation. Other disruptors were incorporated to the hydrophobic core of Aβ to either solubilize aggregates or prevent H-bonding formation between the peptide strands. \[85-88\]

To study the interaction of Aβ with small molecules including peptides, CD spectroscopy and TEM are valuable experimental techniques that allow investigation of intermolecular interactions. While CD spectroscopy allows monitoring peptide and protein secondary/tertiary structures and effects of small molecule interactions in solution, TEM provides structural information about large aggregates that are formed as the result of intermolecular interactions. For instance, CD measurements of solutions of Aβ_{1-40} confirmed a conformational change from
random coil, indicated by a minimum around 200 nm, to a β-sheet structure with a minimum at 217 nm (Figure 1.14a), after 7 days of aging.\[85\]

![Chemical drawings of two peptide modulators based on Aβ fragments. (a) Chemical drawing of an Aβ\textsubscript{16-22} derivative having NMe instead of NH groups in alternating residues blocking the formation of H-bonding interactions between peptide strands. (b) Chemical drawing of an Aβ\textsubscript{16-20} derivative in which some of the amide bonds are replaced by ester bonds.](image)

**Scheme 1.13** Chemical drawings of two peptide modulators based on Aβ fragments. (a) Chemical drawing of an Aβ\textsubscript{16-22} derivative having NMe instead of NH groups in alternating residues blocking the formation of H-bonding interactions between peptide strands. (b) Chemical drawing of an Aβ\textsubscript{16-20} derivative in which some of the amide bonds are replaced by ester bonds.

In contrast, the CD spectra of Aβ\textsubscript{1-40} incubated in the presence of a peptide modulator, which was obtained by terminal modification of Aβ hydrophobic core, revealed the characteristics of both random coil and β-sheet structure after 7 days. This evidence clearly suggests that β-sheet formation is disrupted in the presence of the peptide modulator. TEM images clearly showed that the presence of the inhibitory peptide does not allow formation of mature Aβ fibers but instead only small amorphous aggregates were present as is shown in Figure 1.14b and c. This example illustrates the need for spectroscopic studies in combination with microscopy to elucidate interactions. In many cases, the formation of Aβ fibers is also monitored by measuring the spectroscopic properties of dyes that will only interact with Aβ fibers. Congo red (CR, \textbf{1.42}) and thioflavin T (ThT, \textbf{1.43}) are the two most common dyes which have been used to investigate Aβ aggregation (Scheme 1.14). \textbf{1.42} is a diazo dye generally used as a positive indicator of amyloid aggregation. CR-bound amyloids exhibit green birefringence under polarized light. A broad red-shift transition in CR absorption spectrum is another indicator of CR and Aβ interaction.\[89\]

Compound \textbf{1.43} is a benzothiazole-based dye that fluoresces at 485 nm upon binding to amyloid fibrils. ThT fluorescence measurement is an effective technique to probe the progression and kinetic of fibril formation.\[90\] Other techniques such as NMR and FT-IR spectroscopy, X-ray
diffraction and light scattering have been used extensively to study Aβ aggregation and inhibition in solution.\cite{91}

Surface-based methods can be applied to monitor Aβ inhibition and aggregation. Murphy and co-workers employed surface plasmon resonance (SPR) to evaluate the binding affinity of different peptide fragments derived from the hydrophobic core of Aβ on immobilized Aβ\textsubscript{10-35} peptide films.\cite{92} Their studies showed that the peptide fragment with the higher binding affinity had also higher inhibitor activity toward Aβ toxicity. Reflection–absorption infrared spectroscopy (RAIRS) and quartz crystal microbalance (QCM) were also used to study the growth of amyloid fibrils that were chemically bound to a gold surface.\cite{93, 94} Taking advantage of surface-bound peptide at low density, it is possible to decrease the difficulties that originate from the variability in the amyloid aggregation state involved in bulk solution measurements.

Figure 1.14  CD spectra and TEM images of Aβ\textsubscript{1-40} in the presence and absence of a peptide-based modulator. (a) CD spectra of peptide modulator (green line), Aβ\textsubscript{1-40} in the absence of modulator (red line) and in the presence of modulator (purple line) after 7 days of aging in the phosphate buffer (pH 7.4). The CD spectra of Aβ\textsubscript{1-40} in the absence of modulator exhibit a minimum at 217 nm consistent with β-sheet conformation. The CD spectra of Aβ\textsubscript{1-40} in the presence of modulator show different signature characteristic of both β-sheet and random coil. (b) TEM image confirmed the fibril formation for Aβ\textsubscript{1-40} after 7 days of aging in the absence of modulator, (c) while fibril formation is disrupted in the presence of modulator. "Adapted and modified with permission from (C. K. Bett, W. K. Serem, K. R. Fontenot, R. P. Hammer, J. C. Garno, ACS Chem. Neurosci. 2010, 1, 661-678). Copyright (2010) American Chemical Society."
Two literature reports outline the use of electrochemical techniques for probing Aβ aggregation in solution and on surfaces. In one case, oxidation of tyrosine residue is monitored and mediated by Aβ aggregation.[95] Work on Aβ_{1-40} and Aβ_{1-42} employed cyclic voltammetry (CV) and square wave voltammetry (SWV) to monitor the aggregation process. Ultimately, aggregation of Aβ buried the oxidizable Tyr residue, making it unavailable to supporting electrolyte, thereby preventing its oxidation. Time course studies allowed the evaluation of aggregation kinetics. The current signal of Tyr residue decreased dramatically after 300 and 750 min in the presence of Aβ_{1-42} and Aβ_{1-40} solutions, respectively. The second work exploits differential pulse voltammetry (DPV) and CV to monitor the change in the redox activity of compounds 1.43 and 1.44. The positively charged 1.43 and its uncharged derivative, BTA-1 ([2-(4’-(methylamino) phenyl) benzothiazole], 1.44) will bind to Aβ aggregates. The redox response after binding was evaluated.[96]

![Chemical drawings of CR (1.42), ThT (1.43) and BTA-1 (1.44).](image)

**Scheme 1.14** Chemical drawings of CR (1.42), ThT (1.43) and BTA-1 (1.44).

As shown in Figure 1.15, three electrochemical signals at 290, 600 and 810 mV were observed for 1.44 in phosphate buffer (pH 7.4) in the absence and presence of Aβ_{1-40} solution at 37 °C. Addition of Aβ solution decreased the peak currents at 600 and 810 mV immediately, whereas the signal at 290 mV decreased gradually over the 24 hours. The trend observed in the DP voltammogram of 1.44 in the presence of Aβ_{1-42} was slightly different.
Addition of Aβ₁₋₄₂ solution did not decrease the peak current immediately, whereas the rate of current decrease during the same period of time is much greater in the later case. This is expected since the rate of aggregation for Aβ₁₋₄₂ is faster than that of Aβ₁₋₄₀.

1.6 Research Hypothesis and Thesis Overview

The hypothesis of this thesis was that intermolecular interactions in peptide derived systems can be studies by electrochemical methods. Specifically, the aim was to explore the use of ferrocene-peptide conjugates and monitor the redox behaviour of the ferrocene group. It is hypothesized that the redox properties of the Fc group are affected by intermolecular interactions. For this purpose, my research explored the use of Fc-peptide conjugates as potential modulators for Aβ aggregation and probe interactions between the peptide motif of the conjugates and Aβ fragments by monitoring the changes in the redox properties of the Fc group.
As described in the previous sections, Fc has been recognized as a useful scaffold for the design of β-sheet and β-turn-like structures. Generally, Fc-peptides adopt the “Herrick” motif which has two interstrand H-bonding interactions linking the peptide chains in the most stable conformation. 31-33 This motif has been reported for a large number of Fc-dicarboxylic acid peptide conjugates and supports a rigid 1,2’-conformation. Although H-bonding interaction in Fc-dicarboxylic acid ester conjugates were investigated extensively, the corresponding carboxylates and the structural consequences of this chemical change have rarely been studied. Chapter 2 explores the structural consequences of C-terminal modifications. In a series of new Fc-peptide conjugates containing the amino acid valine, the effects of a carboxylate versus methylester substitution on the self-assembly properties of the Fc-peptide conjugates are explored. These differences are significant and have dramatic consequences at the supramolecular level. The presence of a carboxylate group drives association into a porous structure in which the constituting peptides form a hydrophobic channel, preventing formation of a more common β-sheet structure.

It was reported earlier that disubstituted Fc-peptides aggregate to form supramolecular architectures such as artificial β-helical peptides or β-barrels. 42, 51 This aggregation is driven by the self-assembly of molecular building blocks through intra- and intermolecular hydrogen bonding interactions. There is no reason why this self-assembly should not be extendable to the macroscopic level. Chapter 3 focuses on research that moves into this direction, exploiting hierarchical self-assembly of disubstituted ferrocene peptide conjugates, leading to the formation of nano- and micro-sized assemblies.

Next, we turned our attention to peptides in which H-bonding can be influenced and chose an Aβ fragment for this research. It was reported that peptides from the central hydrophobic core of Aβ are critical for its aggregation and self-recognition. 76 Several techniques such as TEM and CD, IR and fluorescence spectroscopies have been used to investigate Aβ aggregation and inhibition in solution. However, electrochemical studies to monitor Aβ aggregation and inhibition on surfaces are rarely reported. Taking advantage of a surface-bound Aβ fragment serving as the bio-recognition element, it was hoped to gain insight into the interactions by electrochemical means. Chapter 4 presents results of a detailed electrochemical study of the interactions of some known Aβ modulators with an Aβ fragment attached to gold surfaces through a C-terminal Cys.
This leads into a discussion of Fc-based modulators and their interactions with the Aβ film. Chapter 5 outlines the synthesis and characterization of four new Fc-peptide conjugates and their interactions with an Aβ fragment. Conventional solution studies of their interactions by CD spectroscopy and TEM were supplemented by electrochemical studies exploiting the current response of the Fc-redox process with Aβ film on gold surfaces.

Chapter 6 summarizes the results presented in this thesis and provides an outlook of future research.

1.7 References


Chapter 2
Hydrogen-Bonding Interactions in Ferrocene-Peptide Conjugates Containing Valine

As was described in Chapter 1, Fc-dicarboxylic acid has served as a structural scaffold that allows the design of Fc-peptide conjugates possessing β-sheet and turn-like structures. In most of these cases, it was shown that interactions occur via the peptide backbone, involving C=O···H-N hydrogen bonding interactions. The “Herrick” motif plays a critical role in providing structural rigidity to the Fc-peptide building blocks, while allowing intermolecular interactions, often leading to β-sheet formation. In this discussion, few reports described the influence of C-terminal modifications on the ability of Fc-peptides to self-associate. Here, the stage was set for a detailed discussion of supramolecular interactions of Fc-peptides in the subsequent chapters. This chapter explored the effect of C-terminal modification of Fc-peptides on their ability to engage in intra- and intermolecular interactions. Particular attention was paid to hydrophobic amino acids present in the hydrophobic core of Aβ. Hydrophobic amino acids including Leu, Val and Phe were selected due to their high propensity for the formation of β-sheet structure. Formation of channel structures for a Fc-peptide acid suggested that the self-association could be influenced by slight chemical modifications and/or environmental control, which would play a major role in subsequent chapters.

This chapter is reproduced with the permission from: S. Beheshti, A. Lataifeh, H.-B. Kraatz, “Hydrogen-Bonding Interactions in Ferrocene-Peptide Conjugates Containing Valine”, *J. Organomet. Chem. 2011*, 696, 1117-1125, Copyright © 2011, Elsevier. Major work described in this paper in terms of the experimental study and writing of the manuscript was carried out by me, with minor help from Dr. Lataifeh in the initial stages of the project. Prof. Kraatz is the lead author on this manuscript. The text below is a verbatim copy of the published paper.
2.1 Introduction

The desire to control the secondary structure of peptides has attracted much attention due to its potential importance in the design of functional peptidic materials\cite{1} and their role in guiding folding, misfolding\cite{2} and biochemical processes\cite{3} in proteins. For this purpose, molecular scaffolds have been successfully exploited to guide intramolecular peptide assemblies into specific secondary structures supported by hydrogen bonding (H-bonding).\cite{4} In this context, ferrocene (Fc) having two cyclopentadienyl (Cp) rings separated by 3.3 Å make it a suitable scaffold to support the formation of inter-strand hydrogen bonds between peptide substituents on the two Cp rings of the metallocene,\cite{5} allowing for the design of a range of β-sheet-like Fc-foldamers.\cite{6-8} In many of the resulting Fc-peptide conjugates,\cite{9-12} H-bonding adjacent to the Fc group involving the amide NH and CO of the first amino acid stabilizes the structure of the conjugate and was first proposed by Herrick.\cite{9} Hirao has reported a number of examples of Fc-dicarboxylic acid conjugates of dipeptides in which the amide of the Fc-proximal amino acid engages in intramolecular interaction of the “Herrick”-type, while the amide of the second amino acid is involved in intermolecular H-bonding interaction that ultimately leads to the formation of larger supramolecular arrangements.\cite{10} Recently extended β-sheet like structures were reported for Fc-dicarboxylic acid conjugates of tripeptides. These conjugates exhibit a second inter-strand H-bonding interaction in addition to “Herrick motif”.\cite{6} Most Fc-di- and tripeptide conjugates reported to date possess C-terminal esters, while the corresponding carboxylates have rarely been studied.

Fc-peptide conjugates adopting β-sheet-like conformations are potentially interesting molecular models for diseases in which β-sheet structure plays a structural role, such as in Alzheimer’s and Parkinson’s diseases. Alzheimer’s disease is associated with the aggregation of the amyloid β-peptide (Aβ) into fibrils in the brain.\cite{13} In order to elucidate the mechanism of aggregation and folding of Aβ, emphasis has been placed on identifying peptide sequences which are critical for the aggregation processes.\cite{14-16} Research has focused on peptides containing the hydrophobic sequence Aβ\textsubscript{16-22} (Lys\textsubscript{16}-Leu\textsubscript{17}-Val\textsubscript{18}-Phe\textsubscript{19}-Phe\textsubscript{20}-Ala\textsubscript{21}-Glu\textsubscript{22}), which is critical for the formation of the initial tangles, leading to the formation of mature peptide fibrils.\cite{17} Based on these considerations, a number of hydrophobic dipeptides Leu-Val (L-V), Val-Phe (V-F), Gly-Val (G-V) and tripeptide Val-Phe-Phe (V-F-F) were selected and conjugated to Fc-dicarboxylic acid in an attempt to study the interactions between the peptide substituents in soluble bioorganometallic
model systems. One of these systems possesses Gly as the Fc-proximal amino acid in order to provide maximum flexibility to the peptide strands, to facilitate inter-strand interactions and reduce potential steric strain in the resulting Fc-conjugate. We hope that these conjugates are more soluble than the hydrophobic peptides alone, which should ultimately enable us to study their interaction in solution and learn about the role of the hydrophobic peptide sequence in the aggregation of amyloid β. In this work, we focus on both Fc-peptide ester and Fc-peptide acid conjugates for most systems and report their properties including some interesting new supramolecular interactions which were previously not observed in Fc-peptide conjugates.

### 2.2 Results and Discussion

The dipeptides Leu-Val (L-V), Val-Phe (V-F), Gly-Val (G-V) and the tripeptide Val-Phe-Phe (V-F-F), mimicking part of the hydrophobic sequence of the Aβ16-22 fragment, and their Fc-conjugates were prepared according to Scheme 2.1. Fc-dicarboxylic acid was synthesized as reported previously.\[^{18}\] All peptides were synthesized in reasonable yields from Boc-protected amino acids and amino acid esters by standard solution peptide coupling method using HBTU and HOBt as the coupling reagents.\[^{19-22}\] Next, the peptides were coupled to Fc-dicarboxylic acid using EDC/HOBt as coupling reagent, resulting in the formation of desired Fc-peptide conjugates 2.1-2.4. Ester deprotection of Fc-peptide ester 2.3 using molar excess of NaOH in a mixture of water/methanol results in the formation of the Fc-peptide acid 2.6, while conjugates 2.1 and 2.2 can be deprotected with LiOH in a mixture of water/THF to give the Fc-peptide acids 2.5 and 2.7. The Fc-conjugates were characterized by mass spectrometry and \(^1\)H, \(^{13}\)C NMR spectroscopies (see Appendix A). NMR assignments were made using 2D TOCSY and 2D HSQC and by comparison to literature data. Selected characterization data of Fc-peptide conjugates (2.1-2.7) are summarized in Table 2.1. It was not possible to find a common solvent for NMR characterization due to limited stability of compounds 2.1-2.4 in DMSO-d₆ and insolvability of compounds 2.5-2.7 in CDCl₃. The appearance of amide signals at the δ 6-9 region and the characteristic signals of Fc moiety at the δ 4-5 region shows the successful synthesis of Fc-peptide conjugates. For compounds 2.5-2.7, the appearance of a broad resonance around δ 12.60 reveals deprotection of ester groups and formation of the Fc-peptide acids.
Scheme 2.1 Synthesis of dipeptides, tripeptide and Fc-peptide conjugates. (i) HOBt (1.2 eq.), HBTU (1.2 eq.), Et$_3$N (1.5 eq.), CH$_2$Cl$_2$. (ii) (a) (v/v) TFA/CH$_2$Cl$_2$, (b) Boc-Aa-OH (1.2 eq.), HOBt (1.2 eq.), HBTU (1.2 eq.), Et$_3$N (1.5 eq.) in CH$_2$Cl$_2$. (iii) (a) (v/v) TFA/CH$_2$Cl$_2$, (b) Fe[COOH]$_2$ (1eq.), HOBt (2.2 eq.), EDC (2.2 eq.), Et$_3$N (2.5 eq.), CH$_2$Cl$_2$ (compounds 2.1-2.3). (iv) LiOH (3eq.) in H$_2$O:THF, HCl (compounds 2.5 and 2.7) or NaOH (3eq.) in H$_2$O:MeOH, HCl (compound 2.6). (v) (a) (v/v) TFA/CH$_2$Cl$_2$, (b) Fe[COOH]$_2$ (1eq), HOBt (2.2 eq.), EDC (2.2 eq.), Et$_3$N (2.5 eq.), CH$_2$Cl$_2$ (compound 2.4).

The presence of a set of Cp signals in a ratio of 1:1:1:1 follows the typical pattern of a disubstituted Fc corresponding to the four inequivalent Cp protons, except for compound 2.6 which exhibits a ratio of 2:2. This is presumably due to the presence of the Gly proximal to the Fc group reducing the effect of chiral induction of the distal amino acid on the Fc core resulting in magnetic equivalence. Similar signal ratio (2:2) was reported for Fe[CO-Gly-OH]$_2$.[12a] Representative $^1$H NMR spectra of compounds 2.1 and 2.4 are shown in Figure 2.1. The $\alpha$-protons are observed at $\delta$ 4.63 and 4.57 for conjugate 2.1 and $\delta$ 4.71 ($\alpha$-proton signals of two Phe residues are overlapping) and at $\delta$ 4.27 for conjugate 2.4. As is expected for these conjugates, two ($\delta$ 8.40 and 6.33), and three ($\delta$ 7.72, 7.03, 6.16) separate resonance for the amide NHs are observed for conjugates 2.1 and 2.4, respectively.
Table 2.1 Selected spectroscopic parameters of Fc-peptide conjugates 2.1-2.7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (m/z)</th>
<th>$^1$H NMR (ppm)</th>
<th>$^{13}$C NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (calc.)</td>
<td>δ of NH</td>
<td>δ of Cp protons</td>
</tr>
<tr>
<td>2.1</td>
<td>[M + Na]$^+$ 749.3184 (749.3189)</td>
<td>8.40, 6.33</td>
<td>4.88, 4.80, 4.42, 4.29</td>
</tr>
<tr>
<td>2.2</td>
<td>[M]$^+$ 794.95055 (794.29781)</td>
<td>8.12, 6.54</td>
<td>4.87, 4.79, 4.45, 4.33</td>
</tr>
<tr>
<td>2.3</td>
<td>[M]$^+$ 614.2 (614.2)</td>
<td>8.70, 6.65</td>
<td>4.86, 4.83, 4.43, 4.40</td>
</tr>
<tr>
<td>2.4</td>
<td>[M + Na]$^+$ 1111.4265 (1111.4244)</td>
<td>7.72, 7.03, 6.16</td>
<td>4.77, 4.66, 4.43, 4.37</td>
</tr>
<tr>
<td>2.5</td>
<td>[M + Na]$^+$ 721.2889 (721.2876)</td>
<td>8.52, 8.50</td>
<td>4.83, 4.72, 4.39, 4.31</td>
</tr>
<tr>
<td>2.6</td>
<td>[M + Na]$^+$ 609.1804 (609.1623)</td>
<td>8.41, 8.35</td>
<td>4.78, 4.20</td>
</tr>
<tr>
<td>2.7</td>
<td>[M + Na]$^+$ 789.2600 (789.2563)</td>
<td>8.47, 7.85</td>
<td>4.78, 4.77, 4.29, 4.26</td>
</tr>
</tbody>
</table>

Figure 2.1 $^1$H NMR spectrum of Fc[CO-Leu-Val-OMe]$_2$ (2.1) (a) and $^1$H NMR spectrum of Fc[CO-Val-Phe-Phe-OMe]$_2$ (2.4) (b) (c = 5 mM, CDCl$_3$). * Denotes the residual CHCl$_3$. For compound 2.4, the residual CHCl$_3$ signal is overlapped with the aromatic H of the Phe residue. Characteristic signals of Fc are observed at δ 4-5. Two amide resonances at δ 8.40 and 6.33 for conjugate 2.1 and three amide resonances at δ 7.72, 7.03 and 6.16 for conjugate 2.4 is observed.

In general, the involvement of amide protons in H-bonding will cause deshielding of these protons beyond δ 7.00.$^{[23, 24]}$ As shown in Figure 2.1, the amide protons NH$_a$ proximal to the Fc
group in conjugates 2.1 and 2.4 are shifted more downfield in comparison to the amide protons that are far from Fc. This downfield shift is presumably due to the involvement of these protons in intramolecular H-bonding interactions. In order to investigate the H-bonding ability of the systems, variable temperature (VT) $^1$H NMR measurements were carried out in CDCl$_3$ for compounds 2.1-2.4. A series of VT $^1$H NMR spectra of Fe[CO-Leu-Val-OMe]$_2$ 2.1 in the temperature range of 243-303 K is shown in Figure 2.2. As the temperature increases, the resonance of the amides NH shifts upfield. The assignment of amide NH protons is shown in Scheme 2.2 and the result of the VT $^1$H NMR is summarized in Table 2.2 (see Appendix A for plot of chemical shifts versus temperature).

Figure 2.2 VT $^1$H NMR spectra of Fe[CO-Leu-Val-OMe]$_2$ (2.1) (c = 10 mM, the temperature range 243-303 K). The temperature coefficients of compound 2.1 reveal the involvement of both amide protons in hydrogen bonding (-3.7 ppb K$^{-1}$ for NH$_a$ and -4.7 ppb K$^{-1}$ for NH$_b$, see Appendix A for plot of chemical shift versus temperature). * Denotes the residual CHCl$_3$. 
Scheme 2.2 Labelling scheme used for assignments of amide protons of compounds 2.1-2.4, there are only two type of amide protons in compounds 2.1-2.3 (NH\textsubscript{a} and NH\textsubscript{b}), while for compound 2.4, there are three different amide protons (NH\textsubscript{a}, NH\textsubscript{b} and NH\textsubscript{c}).

The amide protons that are involved in intramolecular or intermolecular hydrogen bonding experience temperature dependent chemical shifts.\textsuperscript{[25]} For example, the temperature coefficients of compound 2.1 (see Figure 2.2) reveal the involvement of both amide protons in H-bonding (-3.7 ppb K\textsuperscript{-1} for NH\textsubscript{a} and -4.7 ppb K\textsuperscript{-1} for NH\textsubscript{b}). The trend for compounds 2.2-2.4 is similar but the NH proximal to Fc displays a higher temperature dependence suggesting stronger H-bonding. Overall, our values compare favourably with other temperature behaviour observed for Fc-peptide conjugates involved in H-bonding interactions.\textsuperscript{[11; 26]} Next it was important to evaluate the nature of the H-bonding interactions. For this purpose, the concentration effect of Fc-peptide conjugate on the chemical shift of the amide NH was monitored. Generally, amide NH that is involved exclusively in intermolecular H-bonding should exhibit more significant concentration dependent chemical shifts compared to those involved in intramolecular H-bonding interactions. Our results show that the chemical shifts of the proximal amide NH\textsubscript{a} in Fc-conjugates 2.1 (see Figure 2.3) and 2.4 (see Appendix A) did not change appreciably with concentration in the range of 1-40 mM. In contrast, the resonances of NH\textsubscript{b} in conjugate 1 (see Figure 2.3) and NH\textsubscript{b} and NH\textsubscript{c} in conjugate 2.4 (see Appendix A) are greatly affected by concentration and shift to lower field at higher concentration. These results suggest the engagement of amide protons NH\textsubscript{b} in compound 2.1, NH\textsubscript{b} and NH\textsubscript{c} in compound 2.4 in intermolecular H-bonding interactions. Unfortunately, the insolubility of compounds 2.5-2.7 in CHCl\textsubscript{3} prevented us from studying their H-bonding behaviour. These compounds are soluble only in solvents that directly compete with the H-bonding of the peptide substituents (DMSO or CH\textsubscript{3}OH).
Table 2.2 Summary of VT $^1$H NMR results for compounds 2.1-2.4 (NMR spectra were recorded in CDCl$_3$ solution in the temperature range of 243-303 K at a Fe-peptide concentration of 10 mM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H NMR, $\delta$ H (ppm) /CDCl$_3$</th>
<th>$\Delta\delta/\Delta T$, NH (ppb/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>NH$_a$ (8.40), NH$_b$ (6.33)</td>
<td>NH$_a$ (-3.7), NH$_b$ (-4.7)</td>
</tr>
<tr>
<td>2.2</td>
<td>NH$_a$ (8.12), NH$_b$ (6.54)</td>
<td>NH$_a$ (-6.8), NH$_b$ (-1.8)</td>
</tr>
<tr>
<td>2.3</td>
<td>NH$_a$ (8.70), NH$_b$ (6.65)</td>
<td>NH$_a$ (-5.1), NH$_b$ (-4.3)</td>
</tr>
<tr>
<td>2.4</td>
<td>NH$_a$ (7.72), NH$_b$ (7.03), NH$_c$ (6.16)</td>
<td>NH$_a$ (-6.9), NH$_b$ Phe$_1$ (-2.6), NH$_c$ Phe$_2$ (-1.2)</td>
</tr>
</tbody>
</table>

Figure 2.3 Concentration dependent $^1$H NMR spectra of Fe[CO-Leu-Val-OMe]$_2$ (2.1) showing amide region (concentration range 1-40 mM). The chemical shift of the proximal amide NH$_a$ did not change appreciably with concentration while the resonance of NH$_b$ is affected by concentration and shift to lower field at higher concentration. * Denotes the residual CHCl$_3$.

Next, the helicity of the Fe-conjugates was examined by circular dichroism (CD) spectroscopy.$^{[27-31]}$ The helicity of the Fe group is induced by the restricted rotation about the Cp(centroid)-Fe-Cp(centroid) axis as a result of inter-strand H-bonding between the two podant peptide chains on the two Cp rings.$^{[29]}$ Generally, for Fe-dicarboxylic acid peptide conjugates, a
positive Cotton effect at the absorption of the Fc at about 480 nm is associated with a $P$-helical structure (generally induced by L-amino acids), while a negative Cotton effects are interpreted as being indicative of a $M$-helical structure (induced by D-amino acids). In general, three different conformations have been reported for Fc-peptide conjugates in solution based on the presence of two intramolecular H-bonds (“Herrick” conformation), one intramolecular H-bond (“semi-Herrick” or van Staveren conformation) and no intramolecular H-bond involving the Fc-proximal amides.[29-31] The CD spectra of compounds 2.1-2.4 (in CH$_2$Cl$_2$) and 2.5-2.7 (in CH$_3$OH) were measured and the results are summarized in Table 2.3. Figure 2.4 shows the CD spectra of compounds 2.1, 2.4 and 2.5. As expected for L-amino acid conjugates of Fc-dicarboxylic acid, a positive Cotton effect is observed at 484, 483 and 485 nm for 2.1, 2.4 and 2.5, respectively which indicates an ordered $P$-helical conformation of the Fc moiety, presumably as a result of sufficiently strong interstrand H-bonds between the peptide strands to maintain $P$-helicity. Other bands observed between 300 and 420 nm are characteristic of metal-centered transitions. The CD spectral patterns for all conjugates correlate nicely with results reported for other Fc-peptide conjugates possessing a “Herrick” conformation.[29, 30]

![Figure 2.4 CD spectra of Fc[CO-Leu-Val-OMe]$_2$ (2.1) (-) in CH$_2$Cl$_2$, Fc[CO-Val-Phe-Phe-OMe]$_2$ (2.4) in CH$_2$Cl$_2$ (- - -) and Fc[CO-Leu-Val-OH]$_2$ (2.5) in CH$_3$OH (···) (c = 1.1 mM). A positive Cotton effect at Fc region (484 nm for conjugate 2.1, 483 nm for conjugate 2.4 and 485 nm for conjugate 2.5) is indicative for a $P$-helical configuration of the peptide strands around Fc moiety as a result of two inter-strand H-bonds between peptide chains.](image-url)
Table 2.3 Summary of CD data of Fc-peptide conjugates in CH₂Cl₂ and CH₃OH*(c =1.1 mM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>λₘₐₓ/nm</th>
<th>M₀ × 10⁴/deg cm⁻² mol⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>484 (+6.5)</td>
<td>418 (-2.6)</td>
</tr>
<tr>
<td>2.2</td>
<td>481 (+6.2)</td>
<td>417 (-1.6)</td>
</tr>
<tr>
<td>2.3</td>
<td>481 (+6.0)</td>
<td>417 (-1.2)</td>
</tr>
<tr>
<td>2.4</td>
<td>483 (+3.9)</td>
<td>416 (-1.1)</td>
</tr>
<tr>
<td>2.5*</td>
<td>485 (+3.5)</td>
<td>419 (-1.9)</td>
</tr>
<tr>
<td>2.6*</td>
<td>488 (+1.9)</td>
<td>419 (-1.0)</td>
</tr>
<tr>
<td>2.7*</td>
<td>479 (+3.8)</td>
<td>411 (-0.92)</td>
</tr>
</tbody>
</table>

Figure 2.5 a, b) ORTEP diagram of Fc-peptide conjugates Fc[CO-Leu-Val-OMe]₂ (2.1) and Fc[CO-Gly-Val-OH]₂ (2.6). Ellipsoids are drawn at the 30% probability level. Hydrogen atoms are omitted for clarity. H-bonds are shown as dashed. Intramolecular H-bonding distances are N2···O6, 2.934(3) Å and N3···O3, 3.071(3) Å for Fc-peptide conjugate 2.1 (a) and N8···O11′and N8′···O11, 2.840(5) Å for compound 2.6 (b). The intramolecular H-bonding patterns are identical in both compounds. However the orientation of second amino acids is different in these two compounds probably due to the flexibility of Gly as the first amino acid and engagement of C-terminal carboxylates of conjugate 2.6 in extensive intermolecular H-bonding (see Figure 2.7).
In order to investigate the structures of the Fc-conjugates, single crystal X-ray diffraction studies were carried out on compounds **2.1** and **2.6**. Compound **2.1** was crystallized by slow diffusion of hexanes into a dichloromethane solution in the monoclinic space group P1 21 1 with two independent molecules Fe1 and Fe2 in the unit cell. One of these molecules (molecule with Fe1) is presented in Figure 2.5a (molecule with Fe2 is shown in Appendix A). The single-crystal X-ray structure of Fc-conjugate **2.1**, shown in Figure 2.5a, confirmed the presence of close contacts between the amide oxygen CO of Leu and the amide nitrogen NH on the Leu of the adjacent Cp ring (d(N2···O6) = 2.934(3) Å and d(N3···O3) = 3.071(3) Å, Figure 2.5a). This pattern is in accordance with “Herrick” H-bonding involving two intramolecular H-bonds. Compound **2.6** also was crystallized by slow diffusion of hexanes into a solution of methanol in the tetragonal space group P4322. An ORTEP view of conjugate **2.6** is shown in Figure 2.5b. Again, the structure is stabilized by two C2-symmetrical intramolecular H-bonds between CO (Gly) and NH (another Gly) of each dipeptide chain (d(N8···O11') = 2.840(5) Å). Similar structural features have been reported for other Fc-dipeptide conjugates in the solid state. In addition to intramolecular H-bonding interactions, the compounds also engage in intermolecular H-bonding interactions giving rise to supramolecular assemblies. It is important to emphasize that there are significant differences between the two compounds at the supramolecular level. In the case of compound **2.1**, molecules arrange in a head-to-tail fashion forming infinite chains linked by intermolecular H-bonding interactions between podant peptide chains of adjacent Fc-peptide molecules resulting in the formation of a 14-membered intermolecular H-bonded ring between the Fc-peptide conjugates (see Figure 2.6b). N···O distances between Fe1 and Fe2 are d(N5···O4) = 2.846 (3) Å and d(N1···O12) = 3.015 (3) Å, whereas they are d(N4···O13') = 2.967 (3) Å and d(N8'···O5) = 2.930 (3) Å for Fe1 and Fe2'. Similar intermolecular H-bonding distances were reported for other Fc-dipeptide conjugates. For compound **2.6**, the presence of Gly as the Fc-proximal amino acid provides the maximum flexibility to the peptide strands. The allowed torsion angles for Gly occupies the larger region in the Ramachandran plot compared to Leu, which presumably gives rise to the significant differences in the relative disposition of the second amino acid in conjugates **2.1** and **2.6** as shown in Figure 2.5. Presumably the flexibility of the Gly residue as well as the presence of the free acid group in compound **2.6** play a role, resulting in different intermolecular H-bonding interactions for conjugate **2.6**.
Figure 2.6 (a) ORTEP diagram of conjugate 2.1 showing intramolecular H-bonding interactions, (b) ORTEP diagram of conjugate 2.1 showing 1-D intermolecular H-bonding. Hydrogen atoms are omitted for clarity and dashed lines represent H-bonds. Ellipsoids are drawn at the 30% probability level. Each dipeptide strand forms a 14-membered intermolecular H-bonded ring with the podant dipeptide chain of another molecule of the different type, resulting infinite chains of Fe1 and Fe2 throughout the lattice. The intermolecular H-bond distances are $d(N5\cdots O4) = 2.846(3)$ Å, $d(N1\cdots O12) = 3.015(3)$ Å, $d(N4\cdots O13') = 2.967(3)$ Å and $d(N8'\cdots O5) = 2.930(3)$ Å.

The Fe-C=O establishes intermolecular H-bonding interaction with the acid O-H of an adjacent neighbouring molecule with a distance of $d(O7\cdots O16'') = 2.558(5)$ Å (Figure 2.7b). A similar H-bonding pattern was reported for Fe[CO-Gly-OH]$_2$. The Val NH groups are also involved in intermolecular H-bonding with Val CO groups of a neighbouring molecule with a $d(N12\cdots O15'''') = 3.043(6)$ Å (Figure 2.7b).
Figure 2.7 (a) ORTEP diagram of conjugate 2.6 showing intramolecular H-bonds, (b) ORTEP diagram of 2.6 showing 1-D intermolecular H-bonds. Hydrogen atoms are omitted for clarity and dashed lines represent H-bonds. Ellipsoids are drawn at the 30% probability level. Flexibility of Gly as first amino acid and involvement of C-terminal of peptide strands in free acid H-bonds would cause different intermolecular H-bond patterns for conjugate 2.6. Intermolecular H-bonds are between the Fe-C=O moieties and the acid O-H of adjacent neighbours (d(O7···O16'') = 2.558(5) Å) and valine NH groups with valine CO groups of neighbouring molecules (d(N12···O15'' = 3.043(6) Å). (c) Crystal packing of Fe-peptide conjugate 2.6, showing the formation of hydrophobic voids at the center of the stacks. The major void space in the structure of 2.6 is occupied by three hexane molecules along the columnar void (see Appendix A).
Table 2.4 Structural parameters of Fc-conjugates 2.1 and 2.6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Angle (°)</th>
<th>Fe–C (Å)</th>
<th>Cp–amide (Å)</th>
<th>Amide (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω</td>
<td>θ</td>
<td>β</td>
<td>(C–C)</td>
</tr>
<tr>
<td>2.1 (Fc 1)</td>
<td>73.5</td>
<td>2.5</td>
<td>5.3/2.2a</td>
<td>2.020(2)-2.053(3)</td>
</tr>
<tr>
<td>2.1 (Fc 2)</td>
<td>70.6</td>
<td>2.1</td>
<td>6.2/5.4a</td>
<td>2.023(2)-2.067(3)</td>
</tr>
<tr>
<td>2.6</td>
<td>72.9</td>
<td>6.3</td>
<td>5.7</td>
<td>1.994(7)-2.041(11)</td>
</tr>
</tbody>
</table>

a disubstituted derivative; the first values corresponds to the substituent with higher atomic label (see Appendix A for atomic labelling); b amide proximal to Fe.

Crystal packing of conjugate 2.6 shows the formation of hydrophobic channels formed by the interplay of intermolecular H-bonding interactions. However, these are not empty but occupied by three hexane molecules along the columnar void (Figure 2.7c, and see Appendix A).

Some selected structural parameters of conjugate 2.1 and 2.6 are summarized in Table 2.4. The torsion angle between the substituents (ω), 73.5° and 70.6° for 2.1 and 72.9° for (2.6) are close to the value observed for 1,2′-disubstituted conformations.[29] The tilt angle (θ) between Cp rings is (2.5°, 2.1°) for 2.1 and (6.3°) for 2.6 and confirms the co-planarity of two Cp rings. The twist angle between the planes of the amide bonds and the Cp rings (β) are small (5.3°/2.2° and 6.2°/5.4°) for 2.1 and (5.7°) for 2.6 resulting the electronic interactions between the π-system of amides and Cp rings. Our result compare well with results reported for Fc dipeptide conjugates.[11, 26]

2.3 Experimental

2.3.1 General Remarks

All syntheses were carried out in air unless otherwise indicated. Dichloromethane (CH₂Cl₂) used for synthesis was distilled and dried (CaH₂) prior to use. The coupling reagents used for amide bond formation, N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), 1-hydroxybenzotriazole hydrate (HOBt) and O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (Advanced ChemTech) were used as received. All amino acids
used were purchased from Advanced ChemTech. All other chemicals were used as received and purchased from Sigma-Aldrich. Fc-dicarboxylic acid Fe[COOH]$_2$ was synthesized as reported previously.$^{[18]}$ Products were purified by column chromatography, on a column packed 15-25 cm high with 230-400 mesh silica gel. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 spectrometer operating at 400.1 MHz for $^1$H NMR and 100.6 MHz for $^{13}$C NMR. Chemical shifts (δ) were reported in ppm for both $^1$H NMR and $^{13}$C NMR spectra in relation to tetramethylsilane (TMS, δ = 0). All $^1$H NMR and $^{13}$C NMR spectra are referenced to CHCl$_3$ (signals at δ = 7.27 and δ = 77.00, respectively) or non-deutero impurity in DMSO-d$_6$ (signals at δ = 2.50 and δ = 39.50, respectively) and coupling constants J were measured in Hz. 2D-COSY experiments were used to complete assignments in the $^1$H NMR spectra. Variable temperature and concentration dependent $^1$H NMR measurements have been carried out on a Varian Inova 400 (400 MHz). Mass spectrometry analysis was carried out using a Finnigan MAT 8400 time-of-flight mass spectrometer (TOF-MS) using high resolution electron impact (HR-EI) or high resolution electrospray ionization (HR-ESI). UV-vis data were recorded in CH$_2$Cl$_2$ and CH$_3$OH solution (0.5 mM) on a Cary 300 UV-vis spectrophotometer (Varian) using quartz cuvette with 1 cm path length (200-700 nm, 1 nm resolution). The CD spectra were recorded on a Jasco J-810 circular dichroism spectropolarimeter using a cell with a path length of 1 cm. The CD spectra are an average of three accumulations in CH$_2$Cl$_2$ and CH$_3$OH. The spectra were further smoothed using means-movement algorithm with a convolution width of 25-point supplied with the JASCO software. Ellipticity maxima ($\lambda_{\text{max}}$) are given in nm. Molar ellipticity coefficients ($M_\theta$) were calculated as $M_\theta = \theta/10(c\times l)$, where the ellipticity ($\theta$) is in millidegrees, sample concentration $c$ (1.1 mM) and pathlength $l$ in centimetres (1 cm), thus giving deg cm$^2$ mol$^{-1}$ for $M_\theta$. The dipeptides, Boc-Leu-Val-OMe,$^{[19]}$ Boc-Val-Phe-OMe,$^{[20]}$ Boc-Gly-Val-OMe,$^{[21]}$ and Boc-Phe-Phe-OMe$^{[22]}$ were prepared as described before using HBTU and HOBT. Their spectroscopic properties were identical to those reported in the literature.

2.3.2 Synthesis of Tripeptide

2.3.2.1 Boc-Val-Phe-Phe-OMe

Boc-Phe-Phe-OMe (1.0 g, 2.3 mmol) was dissolved in dry CH$_2$Cl$_2$ and treated with trifluoroacetic acid (TFA, 3.0 mL) in CH$_2$Cl$_2$ (3.0 mL) for 30 min. The TFA and CH$_2$Cl$_2$ were
removed in vacuo. The resulting residue was redissolved in dry CH$_2$Cl$_2$ and cooled in an ice bath prior to the addition of triethylamine (Et$_3$N). To this a solution of activated Boc-Val-OH, prepared from Boc-Val-OH (0.41 g, 1.9 mmol), Et$_3$N (0.40 mL), HBTU (0.87 g, 2.3 mmol) and HOBt (0.35 g, 2.3 mmol) in dry CH$_2$Cl$_2$ was added. The mixture was stirred for 12 hours. The solution was washed with aqueous solutions of saturated NaHCO$_3$, citric acid (10%), again saturated NaHCO$_3$ and finally distilled water. The organic phase was collected and traces of water were removed using anhydrous Na$_2$SO$_4$. The product was purified by column chromatography (silica gel, EtOAc: hexanes 1:1) to yield white solid compound (0.70 g, 70 %). MS (ESI): Calc for C$_{29}$H$_{39}$N$_3$O$_6$ 525.2; Found 525.6 [M]+.

$^1$H NMR (CDCl$_3$) : δ = 7.21 (m, 8H, CH$_{Ar}$ Phe), 6.96 (m, 2H, CH$_{Ar}$ Phe), 6.51 (d, 1H, NH Phe), 6.22 (d, 1H, NH Phe), 4.93 (d, 1H, NH Boc), 4.71 (m, 1H, CH$_{a}$ Phe), 4.70 (m, 1H, CH$_{a}$ Phe), 3.89 (m, 1H, CH$_{a}$ NH Boc), 3.65 (s, 3H, ester OCH$_3$), 3.03 (m, 4H, CH$_{2}$β Phe), 2.06 (m, 1H, CH$_{β}$ Val), 1.43 (s, 9H, CH$_3$ Boc), 0.87 (dd, 3H, (CH$_3$)$_2$ Val), 0.77 (dd, 3H, (CH$_3$)$_2$ Val).

2.3.3 General Synthesis of Ferrocene-Peptide Conjugates 2.1-2.7

To a stirring mixture of Fc-dicarboxylic acid (0.27 g, 1.0 mmol), triethylamine (0.34 mL, 2.5 mmol) in dry CH$_2$Cl$_2$ (50 mL), HOBt (0.33 g, 2.2 mmol) and EDC·HCl (0.42 g, 2.2 mmol) were added. In a separate flask Boc-(Aa)$_n$-OCH$_3$ in dry CH$_2$Cl$_2$ were deprotected by treatment with TFA. The TFA and CH$_2$Cl$_2$ were removed in vacuo. The resulting residue was redissolved in CH$_2$Cl$_2$, cooled in an ice bath prior to the addition of triethylamine and is then added to the reaction mixture. After 12 hours, the reaction mixture was washed with subsequent aqueous solutions of saturated NaHCO$_3$, citric acid (10%), again saturated NaHCO$_3$ and finally distilled water. The organic phase was collected and traces of water were removed using anhydrous Na$_2$SO$_4$. After concentration of the solution under reduced pressure, the crude product was purified by flash column chromatography in a chloroform-methanol solvent mixture. The solvent was then removed completely under reduced pressure and orange solids were obtained. Complete hydrolysis of the methyl ester moieties was performed by reacting LiOH or NaOH (3 eq.) in H$_2$O-THF or H$_2$O-MeOH. The solution was acidified with HCl to pH 2-3 and the precipitate was collected and washed with water and dried to give a brown solid.
2.3.3.1 Fc[CO-Leu-Val-OMe]$_2$ (2.1)

Boc-Leu-Val-OMe (0.75 g, 2.2 mmol), silica gel column, CHCl$_3$: CH$_3$OH (92:8 v/v), Yield: (0.43 g, 60%). MS (HR-ESI): calcd for C$_{36}$H$_{54}$FeN$_4$NaO$_8$ 749.3189; found 749.3184 [M + Na]$^+$. $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.40$ (d, $^3$J$_{HH} = 7.42$ Hz, 2H, NH Leu), 6.33 (d, $^3$J$_{HH} = 8.99$ Hz, 2H, NH Val), 4.88 (s, 2H, H$_{Cp}$), 4.80 (s, 2H, H$_{Cp}$), 4.63 (m, br, 2H, CH$_\alpha$ Val), 4.57 (m, br, 2H, CH$_\alpha$ Leu), 4.42 (s, 2H, H$_{Cp}$), 4.29 (s, 2H, H$_{Cp}$), 3.76 (s, 6H, ester OCH$_3$), 2.25 (m, 2H, CH$_\beta$ Val), 1.80 (m, 4H, CH$_2\beta$ Leu), 1.45 (m, 2H, CH$_\gamma$ Leu), 0.89 (m, 12H, (CH$_3$)$_2$ Val). $^{13}$C NMR (CDCl$_3$): $\delta = 174.8$, 172.3 (CO$_{amide}$), 170.4 (CO$_{ester}$), 75.9 (C$_{ipso}$), 71.5, 71.0 (C$_m$), 70.1, 70.0 (C$_p$), 57.3, 52.6 (CH$_\alpha$ Leu & CH$_\alpha$ Val), 52.1 (OCH$_3$), 39.7 (CH$_\gamma$ Leu), 31.4 (CH$_\beta$ Val), 24.8 (CH$_2\beta$ Leu), 22.9, 21.4 ((CH$_3$)$_2$ Leu), 19.1, 18.0 ((CH$_3$)$_2$ Val). UV–vis: $\lambda_{max}$ (nm) ($\varepsilon$ (cm$^{-1}$ M$^{-1}$)): 443 (396).

2.3.3.2 Fc[CO-Val-Phe-OMe]$_2$ (2.2)

Boc-Val-Phe-OMe (0.83 g, 2.2 mmol), silica gel column, CHCl$_3$: CH$_3$OH (92:8 v/v), Yield: (0.47 g, 60%). MS (HR-ESI): calcd for C$_{42}$H$_{50}$FeN$_4$O$_8$ 794.29781; found 794.95055 [M]. $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.12$ (d, $^3$J$_{HH} = 8.60$ Hz, 2H, NH Val), 7.30-7.24 (br, m, 6H, CH$_{Ar}$ Phe), 6.54 (d, $^3$J$_{HH} = 7.42$ Hz, 2H, NH Phe), 4.91 (m, 2H, CH$_\alpha$ Phe), 4.87 (s, 2H, H$_{Cp}$), 4.79 (s, 2H, H$_{Cp}$), 4.45 (s, 2H, H$_{Cp}$), 4.33 (s, 2H, H$_{Cp}$), 4.22 (m, 2H, CH$_\alpha$ Val), 3.69 (s, 6H, ester OCH$_3$), 3.26-3.13 (m, br, 4H, CH$_2\beta$ Phe), 2.12 (m, 2H, CH$_\beta$ Val), 0.98 (dd, $^3$J$_{HH} = 6.64$ Hz, 6H, (CH$_3$)$_2$ Val). $^{13}$C NMR (CDCl$_3$): $\delta = 173.1$, 171.5 (CO$_{amide}$), 170.5 (CO$_{ester}$), 135.4, 129.4, 128.7, 127.2 (all CH$_{Ar}$ Phe), 76.4 (C$_{ipso}$) 71.6, 71.2 (C$_m$), 70.1, 69.8 (C$_p$), 60.5 (CH$_\alpha$ Val), 53.6 (CH$_\beta$ Phe), 52.1 (OCH$_3$), 38.0 (CH$_2\beta$ Phe), 29.5 (CH$_\beta$ Val), 19.7, 19.5 ((CH$_3$)$_2$ Val). UV–vis: $\lambda_{max}$ (nm) ($\varepsilon$ (cm$^{-1}$ M$^{-1}$)): 448 (323).

2.3.3.3 Fc[CO-Gly-Val-OMe]$_2$ (2.3)

Boc-Gly-Val-OMe (0.63 g, 2.2 mmol), silica gel column CHCl$_3$: CH$_3$OH (92:8 v/v), Yield: (0.42 g, 70%). MS (EI): calcd for C$_{28}$H$_{38}$FeN$_4$O$_8$ 614.2; found 614.2 [M]. $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.70$ (t, $^3$J$_{HH} = 5.86$ Hz, 2H, NH Gly), 6.65 (d, $^3$J$_{HH} = 8.21$ Hz, 2H, NH Val), 4.86 (s, 2H, H$_{Cp}$), 4.83 (s, 2H, H$_{Cp}$), 4.59 (m, 2H, CH$_\alpha$ Val), 4.43 (s, 2H, H$_{Cp}$), 4.40 (s, 2H, H$_{Cp}$), 4.01 (s,
2H, CH₂ Gly), 3.88 (s, 2H, CH₂ Gly), 3.78 (s, 6H, ester OCH₃), 2.23 (m, 2H, CH^β Val), 1.00 (dd, 3J_HH = 7.03 Hz, 12H, (CH₃)₂ Val). ^13C NMR (CDCl₃): δ = 172.2, 171.4 (COamide), 170.6 (COester), 75.7 (ipso Cp), 71.5, 71.3 (CH⁻m Cp), 70.6, 70.3 (CH₀ Cp), 57.6 (CH² Val), 52.2 (OCH₃), 42.9 (CH₂ Gly), 31.35 (CH² Val). 

UV–vis: λₘₐₓ (nm) (ε (cm⁻¹ M⁻¹)): 443 (334).

2.3.3.4 Fc[CO-Val-Phe₁-Phe₂-OMe]₂ (2.4)

Boc-Val-Phe-Phe-OMe (1.1 g, 2.2 mmol), Silica gel column, increasing proportions of CH₃OH (2-5)% in CHCl₃, Yield: (0.54 g, 50%). MS (HR-ESI): calcd for C₆₀H₆₈FeN₆O₁₀ 1111.4244; found 1111.4265 [M + Na]^+.

^1H NMR (400 MHz, CDCl₃): δ = 7.72 (d, 3J_HH = 8.21 Hz, 2H, NH Val), 7.30-7.19 (m, br, 8H, CH⁻Ar Phe), 7.03 (d, 3J_HH = 7.82 Hz, 2H, NH Phe¹), 6.89 (m, 4H, CH⁻Ar Phe), 6.16 (d, 3J_HH = 7.42 Hz, 2H, NH Phe²), 4.77 (s, 2H, H⁻Cp), 4.71 (m, br, 4H, CH^α Phe), 4.66 (s, 2H, H⁻Cp), 4.43 (s, 2H, H⁻Cp), 4.37 (s, 2H, H⁻Cp), 4.27 (m, 2H, CH^α Val), 3.66 (s, 6H, ester OCH₃), 3.24 (m, 2H, CH² Phe), 3.03 (m, 6H, CH² Phe), 2.16 (m, 2H, CH^β Val), 1.00 (d, 3J_HH = 6.64 Hz, 6H, (CH₃)₂ Val), 0.86 (d, 3J_HH = 6.64 Hz, 6H, (CH₃)₂ Val). ^13C NMR (CDCl₃): δ = 172.8, 171.1, 170.5 (COamide), 170.0 (COester), 136.3, 135.5, 129.5, 129.1, 128.7, 128.5, 127.1 (all CH⁻Ar Phe), 77.2 (Cp¹pso), 71.6, 71.4 (Cp⁻m), 70.6, 69.9 (Cp₀), 60.5 (CH² Val), 54.7, 53.4 (CH² Phe), 52.2 (OCH₃), 38.3, 37.9 (CH² Phe), 30.0 (CH² Val), 19.4, 19.3 ( (CH₃)₂ Val). UV–vis: λₘₐₓ (nm) (ε (cm⁻¹ M⁻¹)): 444 (128).

2.3.3.5 Fc[CO-Leu-Val-OH]₂ (2.5)

Fc[CO-Leu-Val-OMe]₂ (0.72 g, 1.0 mmol), Yield: (0.41 g, 60%). MS (HR-ESI): calcd for C₃₄H₅₀FeN₄NaO₈ 721.2876; found 721.2889 [M+Na]^+.

^1H NMR (400 MHz, DMSO-d₆): δ = 12.60 (s, br, 2H, COOH), 8.52, 8.50 (m, 4H, NH Leu & NH Val), 4.83 (s, 2H, H⁻Cp), 4.72 (s, 2H, H⁻Cp), 4.63 (m, 2H, CH^α Leu), 4.39 (s, 2H, H⁻Cp), 4.35 (m, 2H, CH^α Val), 4.31 (s, 2H, H⁻Cp), 2.13 (m, 2H, CH² Val), 1.75 (m, 2H, CH² Leu), 1.61 (m, 2H, CH² Leu), 1.39 (m, 2H, CH⁻β Leu), 0.94 (d, 3J_HH = 6.64 Hz, 12H, (CH₃)₂ Val), 0.85 (m, 12H, (CH₃)₂ Leu). ^13C NMR (DMSO-d₆): δ = 174.5 (COacid), 172.6, 168.7 (COamide), 76.6 (Cp¹pso), 71.1, 70.8 (Cp⁻m), 69.5, 69.4 (Cp₀), 56.9,
51.0 (CH\(^a\) Leu & CH\(^a\) Val), 39.7 (CH\(^a\) Leu), 30.1 (CH\(^b\) Val), 24.4 (CH\(^2\)\(^b\) Leu), 23.1, 20.7 ((CH\(^3\))\(^2\)\(^b\) Leu), 19.1, 17.9 ((CH\(^3\))\(^2\) Val). UV–vis: \(\lambda_{\text{max}}\) (nm) (\(\varepsilon\) (cm\(^{-1}\) M\(^{-1}\))): 433 (390).

### 2.3.3.6 Fc[CO-Gly-Val-OH]\(_2\) (2.6)

Fc[CO-Gly-Val-OMe]\(_2\) (0.65 g, 1.0 mmol), Yield: (0.36 g, 60%). MS (HR-ESI): calcd for C\(_{26}\)H\(_{34}\)FeN\(_4\)NaO\(_8\) 609.1623; found 609.1804 [M+Na]\(^+\). 1H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 12.69\) (br, 2H, COOH), 8.41 (m, 2H, NH Gly), 8.35 (m, 2H, NH Val), 4.78 (s, 4H, H\(_{Cp}\)), 4.20 (s, 4H, H\(_{Cp}\)), 4.22 (s, 2H, CH\(^a\) Val), 3.80 (s, 4H, CH\(_2\) Gly), 2.10 (s, 2H, CH\(^b\) Val), 0.98 (s, 12H, (CH\(^3\))\(^2\) Val). 13C NMR (DMSO-\(d_6\)): \(\delta = 173.2, 173.0\) (CO acid), 171.9, 171.4, 171.0 (CO amide), 75.2 (C\(_{ipso}\)), 71.4, 70.8 (C\(_{m}\)), 70.1, 69.7 (C\(_{o}\)), 52.5 (CH\(_2\) Gly), 52.2, 52.1 (CH\(^a\) Val), 50.8, 49.7 (CH\(^b\) Val), 28.9 28.7, 27.5, 27.3 ((CH\(_3\))\(^2\) Val). UV–vis: \(\lambda_{\text{max}}\) (nm) (\(\varepsilon\) (cm\(^{-1}\) M\(^{-1}\))): 439 (411).

### 2.3.3.7 Fc[CO-Val-Phe-OH]\(_2\) (2.7)

Fc[CO-Val-Phe-OMe]\(_2\) (0.79 g, 1.0 mmol), Yield: (0.45, 60%). MS (ESI): calcd for C\(_{40}\)H\(_{46}\)FeN\(_4\)NaO\(_8\) 789.2563; found 789.2600 [M+Na]\(^+\). 1H NMR (400 MHz, DMSO-\(d_6\)): \(\delta = 12.67\) (s, br, 2H, COOH), 8.47 (d, \(^3J_{HH} = 7.82\) Hz, 2H, NH Phe), 7.85 (d, \(^3J_{HH} = 8.99\) Hz, 2H, NH Val), 7.26-7.19 (m, br, 10H, CH\(_Ar\) Phe), 4.78 (s, 2H, H\(_{cp}\)), 4.77 (s, 2H, H\(_{cp}\)), 4.48 (m, 2H, CH\(^a\) Phe), 4.29-4.26 (m, br, 6H, H\(_{cp}\) CH\(^b\) Val), 3.06 (2H, CH\(_2\) of Phe), 2.95 (m, 2H, CH\(^b\) of Phe), 2.07 (m, 2H, CH\(^b\) Val), 0.89 (\(t, ^3J_{HH} = 6.25\) Hz, 12H, (CH\(_3\))\(^2\) Val). 13C NMR (CDCl\(_3\)): \(\delta = 172.6\) (CO acid), 172.0, 168.6 (CO amide), 137.4, 129.0, 128.1, 126.4 (all CH\(_Ar\) of Phe), 77.0 (C\(_{ipso}\)), 71.6, 71.3 (C\(_m\)), 70.0, 69.0 (C\(_o\)), 58.2, 53.5 (CH\(^a\) Val & CH\(^a\) Leu), 36.6 (CH\(_2\) Phe), 29.6 (CH\(^b\) Val), 19.2, 18.9 ((CH\(_3\))\(^2\) Val). UV–vis: \(\lambda_{\text{max}}\) (nm) (\(\varepsilon\) (cm\(^{-1}\) M\(^{-1}\))): 434 (395).

### 2.3.4 X-ray crystallographic Data Collection and Refinement of the Structures

Orange crystals were obtained by slow diffusion of hexanes into a solution of compound 2.1 in CH\(_2\)Cl\(_2\). Yellow brownish crystals were grown by a slow diffusion of hexanes into a methanolic solution of compound 2.6 at room temperature.
Table 2.5 Summary of crystallographic data for Fc[CO-Leu-Val-OMe]$_2$ (2.1) and Fc[CO-Gly-Val-OH]$_2$ (2.6)

<table>
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<th>2.1</th>
<th>2.6</th>
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<tbody>
<tr>
<td>Identification code</td>
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<td>08123</td>
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<tr>
<td>Empirical formula</td>
<td>C$<em>{36}$H$</em>{54}$FeN$_4$O$_8$</td>
<td>C$<em>{26}$H$</em>{34}$FeN$_4$O$_8$</td>
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<td>150 (2)</td>
<td>150 (2)</td>
</tr>
<tr>
<td>λ / Å</td>
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<td>0.71073</td>
</tr>
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<td>tetragonal</td>
</tr>
<tr>
<td>Space group</td>
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<td>P4$_3$2$_2$</td>
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<tr>
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<td>12.5711 (5)</td>
<td>16.749 (2)</td>
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<td>b / Å</td>
<td>17.6392 (7)</td>
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<td>17.1800 (7)</td>
<td>12.862 (3)</td>
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<td>α / °</td>
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<td>90</td>
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<tr>
<td>β / °</td>
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<td>90</td>
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<tr>
<td>γ / °</td>
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<td>3608.1 (10)</td>
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<td>4</td>
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<tr>
<td>D$_{calc}$ / Mg m$^{-3}$</td>
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<td>M / mm$^{-1}$</td>
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<td>2.04–25.03</td>
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<td>–13 &lt; h &lt; 14</td>
</tr>
<tr>
<td></td>
<td>–23 &lt; k &lt; 23</td>
<td>0 &lt; k &lt; 19</td>
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<tr>
<td></td>
<td>–23 &lt; l &lt; 20</td>
<td>0 &lt; l &lt; 15</td>
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<td>Refractions collected</td>
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<td>28080</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>19517 [R(int) = 0.0465]</td>
<td>3182 [R(int) = 0.0580]</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>multi-scan</td>
<td>multi-scan</td>
</tr>
<tr>
<td>Max. / Min. transmission</td>
<td>0.9616 / 0.9608</td>
<td>0.894 / 0.966</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least squares on F</td>
<td>Full least squares matrix on F</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
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<td>3182 / 0 / 178</td>
</tr>
<tr>
<td>Goodness-of-fit on F$^2$</td>
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<td>0.985</td>
</tr>
<tr>
<td>Final R indices [I &gt; 2σ(I)]</td>
<td>R1 = 0.0453, wR2 = 0.1155</td>
<td>R1 = 0.0875, wR2 = 0.2237</td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>R1 = 0.0552, wR2 = 0.1225</td>
<td>R1 = 0.1227, wR2 = 0.2463</td>
</tr>
<tr>
<td>Largest diffraction peak and hole</td>
<td>0.849 and −0.626 e Å$^{-3}$</td>
<td>0.512 and −0.483 e Å$^{-3}$</td>
</tr>
</tbody>
</table>

Both compounds were mounted on glass fibers. Data were collected at 150 K on a Bruker Kappa Apex II diffractometer using Mo-K$_\alpha$ radiation for 2.1 and on a Nonius Kappa-CCD area detector diffractometer for 2.6. The crystal data and refinement parameters are summarized in Table 2.5. SHELXS-97 (Sheldrick, 2008)$^{33}$ (for 2.1) and SHELXTL/PC V6.14 for Windows NT (Sheldrick, G.M., 2001)$^{34}$ (for 2.6, see Appendix A) were used to solve the structures by direct methods. For compound 2.1, the final R value was 0.0453 for 17158 reflection with I > 2σ(I). For compound 2.6, the final R value was 0.0875 for 1968 reflection with I > 2σ(I).
2.4 Conclusions

The synthesis and spectroscopic properties of a series of Fc-peptide ester and Fc peptide acid conjugates containing hydrophobic amino acids from part of hydrophobic region of amyloid beta peptide are reported. There are evidences that H-bonds play an important role in the stability of beta amyloid aggregates. The presence of H-bonds in our compounds is confirmed in solution and some cases solid states using spectroscopic techniques. The solid-state structures of Fc[CO-Leu-Val-OMe]$_2$ (2.1) and Fc[CO-Gly-Val-OH]$_2$ (2.6) confirmed formation of “Herrick” conformation (anti-parallel β-sheet like). However, there are significant differences between two compounds at the supramolecular level in the solid state. The flexibility of Gly as a Fc-proximal amino acid and the presence of the free acid group give rise to the significant differences in intermolecular H-bonding interactions of compound 2.6. In compound 2.1, the intermolecular H-bonding interactions arrange molecule in a head-to-tail fashion forming infinite chains, while crystal packing of 2.6 shows the formation of hydrophobic channels resulting from involvement of free acid and NH Val in intermolecular H-bonds. This particular association of Fc-peptide conjugates is as of yet unprecedented for this class of compounds and has to be compared with Ghadiri’s cyclic octapeptides composed of alternating D and L amino acids forming flat rings that associate into continuous peptide tubes with an inner diameter of 7-8 Å. More work in this area is necessary to probe the generality of this assembly pattern for Fc-peptide acids. The Fc-peptide systems reported here may have a potential to interact with the hydrophobic part of Aβ. We are now exploring the interaction of some of the Fc-peptide conjugates with different fragments of Aβ in solution and on surfaces. Work is also under way to study longer peptide fragments of Aβ, their assembly properties and their potential to interact with Aβ.

2.5 Supporting Information

Supporting information for this chapter is provided in Appendix A.
2.6 References


As was discussed in Chapter 2, the supramolecular structure of Fc-peptides could be manipulated by C-terminal modification of peptide strands. In addition, our results suggested that a particular amino acid sequence had a role to play in determining supramolecular interactions since it determined dihedral angles in the peptide and spatial relationships of α-substituents, thus influencing the orientation of H-bonding groups. Clearly, intermolecular H-bonding interactions directed the self-assembly process, resulting in the formation of β-sheets and supramolecular framework structures possessing hydrophobic channels. In this chapter, we exploited concepts developed in Chapters 1 and 2 and extended them to the macroscopic scale. Spontaneous self-assembly of Fc-peptides through intra- and intermolecular hydrogen bonding interactions gave rise to fibers, large fibrous crystals, and twisted ribbons, some of which were micrometers in length. Hierarchical association of Fc-peptide conjugates was critical. Several factors including solvent composition and the nature of amino acids affected Fc-peptide assembly at the macroscopic level.

This chapter is to be published material from: S. Beheshti, S. Martić, H.-B. Kraatz, “Hierarchical Organization of Ferrocene-peptides”, *Chem. Eur. J.* 2012, 18, 9099-9105. All work described in this paper was carried out by me. I wrote the first draft of the manuscript. Dr. Martic assisted with data interpretation and editing of the manuscript. The text below is a *verbatim* copy of the paper.
3.1 Introduction

The molecular self-assembly of peptides and proteins are driven by intra- and intermolecular interactions. These interactions have been explored extensively aiming at unravelling the formation of large assemblies from molecular precursors.\cite{[1]-[4]} The scale of the self-association may span several orders of magnitude from nano- to micrometers, in which molecular precursors, guided by predetermined ordering, assemble in a hierarchical form into discrete objects.\cite{[5, 6]} Numerous examples of such assemblies exist in nature including the tobacco mosaic virus, microtubules and amyloidogenic fibrils.\cite{[7]-[12]} Self-assembly is typically directed by inter- and intramolecular contacts through hydrogen bonding (H-bonding), hydrophobic interactions, and \(\pi-\pi\) stacking amongst others. In this context, bio-inspired synthetic peptides offer a structural handle towards the design of hierarchical structures such as nanofibers, nanotubes and vesicles.\cite{[13, 14]} For example, Ghadiri demonstrated the assembly of the cyclopeptide, \(cyclo[-(D-Ala-Glu-D-Ala-Gln)_2-]\) possessing an alternating D, L-amino acid sequence, into channels, ultimately giving rise to a highly porous peptide 3D-architecture.\cite{[15]} H-bonding interactions are critical for the formation of these tubular structures. The smallest dipeptide Phe-Phe forms gel-like nanostructures in which \(\pi-\pi\) stacking in addition to H-bonding is critical. In organic solvents, this dipeptide forms nanofibers while nanotubes are produced in aqueous solutions, which reinforces the role of the solvent in the overall assembly.\cite{[16, 17]} Upon sonication, the protected tripeptide Boc-Val-Phe-Phe-OMe (Boc = \(N\)-tert-butoxycarbonyl) forms nanofibers composed of \(\beta\)-sheets which resemble amyloidic peptide aggregates.\cite{[18]} In addition to the environmental factors, the peptide self-assembly can be influenced by using molecular scaffolds, which often contribute to \(\beta\)-sheet stability and conformational flexibility or rigidity. The assembly properties of a range of bipyridine (bipy) peptide conjugates serve as examples.\cite{[19]-[21]} For instance, the bipy conjugate (Boc-\{\((N\text{-Cbz})\text{-Lys}\}\text{-Phe-Ala})_2-2,2\text{'}-bipy (Cbz = carboxybenzyl), prepared from Boc-\{\((N\text{-Cbz})\text{-Lys}\}\text{-Phe-Ala-COOH with 5,5\text{'}-dibromomethyl-2,2\text{'}-bipyridine, forms a network of nanofibers, which displays an architecture that is reversibly switchable from fibers to vesicles by heat and back to fibers by ultrasound.\cite{[22]} The introduction of the ferrocene (Fc) group as a part of the peptide scaffold provides control over the secondary structure of the molecular precursor by promoting cross-strand H-bonding between podant peptide chains into a locked conformation,\cite{[23]-[26]} which is thermodynamically stable.\cite{[27, 28]} Work by Hirao showed chirality organization in which the helicity of Fc-peptides
dictates the directional properties of the peptide conjugate.\cite{29} Other Fc-peptides self-assemble into β-barrel-like shapes, driven by the inherent H-bonding blueprint.\cite{30}

**Scheme 3.1** Proposed schematic representation of the hierarchical assembly of Fc-peptides into higher ordered supramolecular structures. A) In the crystallographically determined structure of Fc[CO-Gly-Val-Phe-OMe]$_2$ (3.2), the peptide substituents are involved in intramolecular H-bonding, making it a rigid building block. B) The Fc-peptide is also characterized by the intermolecular H-bonding between Fc-peptide precursors giving rise to 2D peptide sheets with peptide backbone separation of 0.47 nm. C-D) In the crystal packing, the peptide sheets associate laterally through hydrophobic interactions giving rise to extended pleated sheets. E) SEM images of compound 3.2 prepared from dichloromethane/hexane (1:2) show the formation of twisted ropes composed of crystalline platelets. We propose that, the molecular offsets and defects during crystal growth may induce the formation of the helical turns during the stacking of pleated sheets (D). F) SEM images of compound 3.2 obtained from chloroform/hexane (1:2) reveal the formation of crystalline cuboids which are made up of fibrillar bundles composed of pleated sheets (D).
The recognition interface in Fc-peptides which possess the H-bonding information directs the assembly into larger structures. This is one of the underlying principles of supramolecular organization of these Fc-peptides, in some cases leading to chirality organized systems. However, here we go beyond earlier reports and explore the hierarchical assembly of Fc-peptide conjugates and their growth into macroscopic objects (Scheme 3.1).

In our system, it is possible to tune the morphological outcome of Fc-peptides based on the amino acid variation from Gly-Val-Phe in compound 3.2 to the Gly-Val-Phe-Phe in compound 3.3. The association of Fc-peptides in solution and solid state was studied by a range of characterization techniques from nano- to micromolecular scale. The H-bonding and hydrophobic interactions between the peptide strands drive their self-assembly and influence the morphological outcome as seen in Scheme 3.1. The hierarchical association of Fc-peptides gives rise to nanofibers networks, twisted ropes or rectangular cuboids depending on the peptide sequence and solvent composition.

3.2 Results and Discussion

3.2.1 Design and Synthesis

The compounds Fc[CO-Gly-Val-Phe-OMe]$_2$ (3.2) and Fc[CO-Gly-Val-Phe-Phe-OMe]$_2$ (3.3) were synthesized as depicted in Scheme 3.2. The amino acid H$_2$N-Phe-OMe was coupled to Fc[CO-Gly-Val-OH]$_2$ (3.1) using O-benzotriazole-$N,N,N',N'$-tetramethyl-uronium-hexafluorophosphate (HBTU) as a coupling reagent to give compound 3.2. Compound 3.3 was synthesized from NH$_2$-Phe-Phe-OMe, after Boc-Phe-Phe-OMe deprotection with trifluoroacetic acid (TFA), and coupling to 3.1. Compounds were purified by column chromatography and obtained as orange solids in 50-60% yields and were characterized by mass spectrometry, $^1$H, $^{13}$C NMR, infrared (IR) and circular dichroism (CD) spectrosopies (see Appendix B). NMR assignments were made using gradient correlation spectroscopy (gCOSY) and heteronuclear single quantum correlation (HSQC) spectrosopies and by comparison to the literature. In the $^1$H NMR spectra of compounds 3.2 and 3.3, the presence of the characteristic signals of the disubstituted Fc at the $\delta$ 4-5 region and amide proton signals at the $\delta$ 6-9 region shows the successful synthesis of the compounds. The $^{13}$C NMR further confirmed the formation of the desired Fc-peptides (see Appendix B).
3.2.2 Self-assembly of Fc-peptides in Solution

In order to evaluate the ability of the amide protons to involve in H-bonding in solution a series of variable temperature dependent $^1$H NMR measurements in the range of 253-303 K were carried out in CDCl$_3$ for compounds 3.2 and 3.3. With an increase in the temperature, chemical shifts of all amide protons shifted upfield indicating loss of interaction at elevated temperatures. This confirms the involvement of all amide protons in intra or intermolecular H-bonding for conjugates 3.2 and 3.3. The temperature coefficients of amide protons of compound 3.2 were measured from the slope of the plot of chemical shift versus temperature (-2.0 ppb K$^{-1}$ for NH$_{Gly}$, -3.0 ppb K$^{-1}$ for NH$_{Phe}$ and -4.0 ppb K$^{-1}$ for NH$_{Val}$, see Appendix B). A similar temperature response was observed for compound 3.3 and the temperature coefficients of amide protons were -4.1 ppb K$^{-1}$ for NH$_{Gly}$, -3.1 ppb K$^{-1}$ for NH$_{Phe}$, -4.3 ppb K$^{-1}$ for NH$_{Val}$ and -1.6 ppb K$^{-1}$ for NH$_{Phe}$.

Scheme 3.2 Synthesis of Fc-peptide conjugates (3.2 and 3.3). (i) HOBT (2.2 eq.), HBTU (2.2 eq.), Et$_3$N (2.5 eq.), CH$_2$Cl$_2$. (ii) NH$_2$-Phe-OMe (2.2 eq.) (compound 3.2). (iii) Boc-Phe-Phe-OMe (2.2 eq.), (v/v) TFA/CH$_2$Cl$_2$ (compound 3.3).
This compares well with other H-bonded Fc-peptide conjugates that are associated through H-bonding. Next, the effects of concentration of Fc-peptide on the chemical shift of amide protons were monitored in order to distinguish inter- from intramolecular H-bonding interactions. Typically, the chemical shift of amide protons engaged in intermolecular H-bonding are affected by changing the concentration, while amide protons involved in intramolecular interactions are not affected. For compound 3.2, no change was observed for the chemical shifts of amide protons of Gly and Phe in the concentration range of 6-20 mM. In contrast, the chemical shift of amide protons of Val is affected significantly by varying the concentration of Fc-peptide (see Appendix B). The same study of compound 3.3 shows the involvement of amide protons of Val and Phe in intermolecular H-bonding interactions (see Appendix B). Overall, our results suggest that the conjugates 3.2 and 3.3 may potentially form larger soluble aggregates in solution. A diffusion-ordered spectroscopy (DOSY) NMR was carried out to probe this hypothesis. We first performed DOSY-NMR experiments on compounds 3.2 and 3.3 in DMSO, a strong H-bonding acceptor solvent which has been shown to interfere with intermolecular H-bonding and thus compounds should not be associated in solution. The diffusion coefficients $D_{avg}$ for compounds 3.2 and 3.3 obtained in a given solvent by averaging the $D$ values of uncompromised peaks (without solvent overlap, see Appendix B). We observe $D_{avg}$ values of $1.31 \times 10^{-10} \pm 0.08$ m$^2$ s$^{-1}$ and $1.14 \times 10^{-10} \pm 0.08$ m$^2$ s$^{-1}$ for compounds 3.2 and 3.3 in DMSO-d$_6$, respectively. A relatively small difference in $D_{avg}$ of 3.2 and 3.3 is due to the larger size of the latter. Next, we calculated the expected diffusion coefficients of two compounds in CDCl$_3$ using the $D_{avg}$ obtained for compounds in DMSO-d$_6$ and the inverse dependence between $D$ and solvent viscosity ($\eta$). The comparison of calculated $D$ values ($4.85 \times 10^{-10}$ m$^2$ s$^{-1}$ for 3.2 and $4.09 \times 10^{-10}$ m$^2$ s$^{-1}$ for 3.3) and experimental $D_{avg}$ values in CDCl$_3$ ($5.62 \times 10^{-10} \pm 0.21$ m$^2$ s$^{-1}$ for 3.2 and $4.87 \times 10^{-10} \pm 0.16$ m$^2$ s$^{-1}$ for 3.3) suggests that 3.2 and 3.3 exist predominantly as monomers in this solvent. While the presence of H-bonding is evident from the concentration- and temperature-dependent NMR studies, under the experimental conditions there is no clear evidence of discrete aggregate formation from DOSY-NMR experiments (see Appendix B). Presumably, weak intermolecular interactions disfavour the formation of stable discrete soluble aggregates that can be probed by NMR spectroscopy. Next, the helicity of Fc-conjugates 3.2 and 3.3 was evaluated in solution (chloroform or dichloromethane) by circular dichroism (CD) spectroscopy (see Appendix B). Helicity of the Fc core in these types of conjugates is induced by the presence of intramolecular H-bondings between the peptide substituents on the two Cp
rings. As a result, the helicity leads to the strong CD signals in the absorption region of Fc moiety. A negative CD signal at ~500 nm is due to the presence of intramolecular H-bonding in conjugates 3.2 and 3.3, which locks the Fc core into a particular helical conformation and thus induces helical chirality. This has been previously observed for other Fc-peptides. The CD spectra of 3.2 and 3.3 in the peptide region (230-300 nm) display two minima at 231 and 254 nm for 3.2 and 237 and 258 nm for 3.3. The minimum at ~230 nm can be assigned to the stacking of aromatic Phe amino acids due to the peptide self-assembly. In solution, Fc-peptides exhibit intramolecular H-bonding and potentially self-assemble through intermolecular interactions.

3.2.3 Hierarchical organization of Fc-peptides in the Solid State
In order to elucidate the assembly of Fc-peptide conjugates in the solid state, a series of experiments were carried out that allowed us to probe deeper into their associative properties and monitor the organization at the nano- and microscopic level. Compound 3.2 crystallizes in the orthorhombic space group P2_12_12_1 and was obtained by slow diffusion of hexane to a chloroform solution. Figure 3.1A shows the crystal structure of Fc-conjugate 3.2, which indicates close contacts between amide groups on the two peptide strands in line with intramolecular H-bonding interactions between the amide oxygen CO of Gly of one strand and the amide nitrogen NH on the Gly of the other strand (d(N4···O2) = 2.758 Å and d(N1···O7) = 2.958 Å. This pattern is known as “Herrick” motif previously observed for 1,n’-Fc-peptide conjugates. In addition to “Herrick motif”, a second set of intramolecular H-bonding interaction between the amide nitrogen NH of Phe of one strand and the ester CO of Phe of opposite strand (d(N3···O9) = 3.141 Å and d(N6···O5) = 3.115 Å, Figure 3.1A) adds to the rigidity of the Fc-peptide building block. The presence of Gly as a flexible amino acid proximal to Fc moiety provides enough flexibility to our system to align peptide strands in a parallel fashion allowing for extended intramolecular H-bonding between the peptide strands. In Figure 3.1B, intermolecular H-bonding interactions (d(N2···O6*) = 2.821 Å and d(N5···O1*) = 2.988 Å) connect each molecule to two other molecules to form a network at the supramolecular level through H-bonding. Formation of a 14-membered H-bonded ring at intermolecular level resembles H-bonding in anti-parallel β-sheet-like structures. H-bonding interactions of compound 3.2 in the solid state match our findings about the H-bonding patterns of this conjugate in solution.
Figure 3.1  A) ORTEP diagram of compound 3.2 showing four intramolecular H-bonding interactions $d(\text{N}4\cdots\text{O}2) = 2.758 \text{ Å}$, $d(\text{N}1\cdots\text{O}7) = 2.958 \text{ Å}$, $d(\text{N}3\cdots\text{O}9) = 3.141 \text{ Å}$ and $d(\text{N}6\cdots\text{O}5) = 3.115 \text{ Å}$. B) ORTEP diagram of conjugate 3.2 showing 1D intermolecular H-bonding interactions $d(\text{N}2\cdots\text{O}6^*) = 2.821 \text{ Å}$ and $d(\text{N}5\cdots\text{O}1^*) = 2.988 \text{ Å}$ leading to the formation of a 1D tape. C) View along a-axis showing solvent molecules ($\text{2\times CHCl}_3 + \text{2\times hexane}$) in a hydrophobic pocket created by the association of molecular building blocks and lined by the phenyl group of the Phe residues. D) View along b-axis showing the association of molecules of 3.2 into 2D pleated sheet. The gray overlay indicates the direction of intermolecular H-bonding in an antiparallel fashion. E) View down the c-axis showing association to form zigzag chains of adjacent pleated sheets interacting laterally. In this figure, all hydrogen atoms and solvents are omitted for clarity (with exception of view c) and dashed lines represent H-bonds.

The amide protons of Gly and Phe are involved in intramolecular H-bonding, while Val amide protons are exclusively engaged in intermolecular interactions. Notably, in the extended supramolecular packing diagram, compound 3.2 forms hydrophobic pockets composed of Phe side chains pointing towards solvent molecules (Figure 3.1C). Looking down the b axis in Figure 3.1D, hydrophobic interactions between peptide sheets give rise to non-planar pleated sheet.
view along the c axis (Figure 3.1E) shows that individual peptide pleated sheets are stacked with respect to each other in a zigzag fashion.

Spectroscopic and crystallographic evidence show a strong preference for individual Fc-peptide molecules to form ordered structures through a combination of intra- and intermolecular H-bonding and hydrophobic interactions. We expect that this molecular organization at the molecular and supramolecular level may translate into the microscopic realm. For this purpose, we carried out extensive scanning electron microscopy (SEM) studies, which provide clear evidence for the formation of ordered structures. For these studies, we explored solvent conditions used for crystal growth and spectroscopic analysis to retain the interactions identified earlier. In chlorinated solvents, no evidence of hierarchical growth was observed by SEM. To induce assembly of the Fc-peptides, a chlorinated solvent/hexane mixture (1:2) was employed since the compounds are soluble in chlorinated solvents and insoluble in hexane. Under the given experimental conditions, the addition of excess hexane promotes fast self-assembly, which differs from the conditions used for the single crystal growth (slow diffusion of hexane into chlorinated solvent). SEM images of assemblies of compound 3.2 in the mixture of dichloromethane/hexane (1:2) show clear evidence of crystalline platelets associating into longer structures (Figure 3.2A). These long crystalline fibers possess an inherent helical twist presumably brought about by screw dislocations, which has been previously identified during the crystal growth due to offsets and packing errors. The rates of crystal growth may affect the crystal packing and result in the crystal lattice defects. Stress during the crystal growth may also induce dislocations and crystal imperfections. Upon further growth, the helical pitch along the strand is evident in Figure 3.2B. The formation of longer twisted strands, tens of micrometers in length, presumably originates from twisting around an axis, giving rise to mature molecular ropes with a helical pitch (~2 µm) (Figure 3.2C and D). SEM images of assemblies of compound 3.2 in the mixture of chloroform/hexane (1:2) show distinctly different behaviour (Figure 3.2E-H). We observed the formation of rectangular crystalline cuboids (Figure 3.2H). Distinct cuboids (Figure 3.2G-H) with several micrometers in length are made up of fibrillar bundles. Figure 3.2F shows the view along the edge of cuboid and fiber stacking along its longitudinal axis. Higher magnification in Figure 3.2E shows the formation of fibrous network on the surface of cuboids. Our studies indicate that solvent composition may have a profound effect on the morphological outcome. Significant solvent effects on the morphology of assemblies have been observed for
dipeptide Phe-Phe.\textsuperscript{[16, 17]} We next studied the self-assembly of compound 3.3, in order to investigate the effect of the second Phe residue in the Fc-tetrapeptide conjugate. Figure 3.3A and B show SEM images (at different magnification level) of compound 3.3 prepared from chloroform/hexane (1:2). The conjugate displays a nanofibers network. The nanofibers are 20-50 nm in width and can exceed several micrometers in length. The morphology of these nanofibers is similar to the morphology of fibers obtained from short Aβ\textsubscript{16-22} peptide.\textsuperscript{[12]}

**Figure 3.2** SEM images of assemblies of compound 3.2 in a mixture of dichloromethane/hexane (1:2) (A-D). A) The crystalline platelets associate to give a cylinder-like strand structure. B) The structure is characterized by inherent helicity. C-D) During further growth the twisted ropes are formed as mature structures. The helicity is ascribed to screw dislocation\textsuperscript{[42, 43]} or solvent properties.\textsuperscript{[16, 17]} SEM images of assemblies of compound 3.2 in the mixture of chloroform/hexane (1:2) at different magnifications (E-H). G-H) Mature rectangular cuboids are formed with several micrometers in length. F) View along the edge showing peptide fibers oriented along the longitudinal axis of cuboids. E) Magnified SEM of the cuboid surface showing the fibrous network. Scale bar is 2 µm.
Changing the solvent mixture from chloroform/hexane to dichloromethane/hexane has little effect on the overall morphology of the assemblies of conjugate 3.3 (Figure 3.3C and D). The differences in the morphology of assemblies formed from compounds 3.2 and 3.3 highlight the importance of additional H-bonding and hydrophobic interactions due to the presence of the additional Phe group present in conjugate 3.3. Increase in the number of hydrophobic residues may favour fiber formation.\cite{47} For example, Val-Phe-Phe sequence preferentially forms nanofiber networks\cite{18} while the Val-Phe dipeptide does not assemble into any ordered structures.\cite{48} However, the presence of Fc group and Gly residue dramatically influences the overall self-assembling properties of our systems. Hence, the morphological differences observed for compounds 3.2 and 3.3 may be ascribed to the extra Phe amino acid residue.

![Figure 3.3](image)

**Figure 3.3** SEM images of assemblies of compound 3.3 obtained from a mixture of chloroform/hexane (1:2) (A-B) and dichloromethane/hexane (1:2) (C-D). B and D) Formation of nanofibers network. A and C) Magnified SEM images showing the nanofibers that are 20 - 50 nm in width and can exceed several micrometers in length. The scale bar corresponds to 1 µm.
Next, the focus turned to the nature of interactions of Fc-peptides in an effort to evaluate the structural motifs present in the assemblies of compounds 3.2 and 3.3. For this purpose, assemblies of compound 3.2 and 3.3 prepared identically to the SEM preparations were investigated by attenuated total reflectance (ATR) FT-IR spectroscopy and powder X-ray diffraction (PXRD). For all solvent compositions, the Fc-peptides exhibited two signals in the amide I region from 1600-1700 cm\(^{-1}\). For example, the two signals at 1632 and 1675 cm\(^{-1}\) for compound 3.2 are characteristic of the formation of a \(\beta\)-sheet arrangement of the peptide moieties.\(^{49}\) In the case of compound 3.3, a strong peak at 1636 cm\(^{-1}\) and a weak peak at 1683 cm\(^{-1}\) are characteristic of anti-parallel \(\beta\)-sheet structure (see Appendix B).\(^{50}\)

PXRD experiments of conjugate 3.2 and 3.3 prepared identically to the SEM preparation show a series of intense diffraction signals suggesting the crystallinity of the assemblies. Importantly, an intense peak at \(2\theta = 18.7^\circ\) with a d spacing of 0.47 nm was observed, which is associated with \(\beta\)-sheet-like structures and corresponds to the spacing between peptide strands as shown in Scheme 3.1B.\(^{22, 51}\) Additional intense signal observed at d spacing of 0.94 nm may correspond to the repeat of peptide strands.\(^{52}\) The peaks in the 0.37-0.38 nm range are assigned to the van der Waals distance of side chains of packed peptides. Signals in the 0.50-1.0 nm range are assigned to the inter-sheet spacing between the Phe side chains.\(^{6, 22}\) These structural distances are in agreement with our single X-ray analysis of conjugate 3.2. The PXRD pattern of assemblies of compound 3.3 obtained from chloroform/hexane show less crystallinity and display the characteristic \(\beta\)-sheet signature peak at \(2\theta = 18.9^\circ\) (see Appendix B). Unlike in compound 3.2, the presence of a peak at small range angle PXRD at \(2\theta = 1.85^\circ\) at d = 4.8 nm for compound 3.3 reveals the presence of a bilayered packing of the \(\beta\)-sheet-like conformation. This structural feature was previously observed for nanofiber peptide networks.\(^{22}\)

### 3.3 Experimental

#### 3.3.1 General Remarks

All syntheses were carried out in air. Dichloromethane (CH\(_2\)Cl\(_2\)) used for synthesis was distilled and dried (CaH\(_2\)) prior to use. The coupling reagents used for amide bond formation, 1-hydroxybenzotriazole hydrate (HOBt) and \(O\)-benzotriazole-\(N,N,N',N'\)-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and all amino acids were purchased from Advanced ChemTech.
All other chemicals were used as received and purchased from Sigma-Aldrich. The dipeptide N-tert-butoxycarbonyl Boc-Phe-Phe-OMe and Fe[CO-Gly-Val-OH]$_2$ were synthesized as reported previously.$^{[34, 53]}$ All products were purified by column chromatography, on a column packed 15-25 cm high with 230-400 mesh silica gel (Silicycle, Quebec City, PQ).

### 3.3.1.1 NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz spectrometer. Chemical shifts (δ) were reported in ppm for both $^1$H NMR and $^{13}$C NMR spectra in relation to tetramethylsilane (TMS, δ = 0). All $^1$H NMR and $^{13}$C NMR spectra are referenced to CHCl$_3$ (signals at δ = 7.27 and δ = 77.00, respectively) and coupling constants $J$ were measured in Hz. 2D-gCOSY experiments were used to complete assignments in the $^1$H NMR spectra. Variable temperature and concentration dependent $^1$H NMR measurements have been carried out on an Agilent DD2 500 MHz spectrometer. DOSY-NMR spectroscopy experiments were performed with a Varian S-400 NMR spectrometer (concentration = 10 mM).

### 3.3.1.2 Mass Spectrometry

Mass spectrometry analysis was carried out using an AB/Sciex QStar mass spectrometer with an ESI source.

### 3.3.1.3 Circular Dichroism (CD) Spectroscopy

The CD spectra were recorded on a Jasco J-810 circular dichroism spectropolarimeter. The spectra are an average of three accumulations in chloroform and dichloromethane. The spectra were further smoothed using means-movement algorithm with a convolution width of 11-point supplied with the JASCO software. Ellipticity maxima ($\lambda_{\text{max}}$) are measured in nm. Molar ellipticity coefficients ($M_\theta$) were calculated as $M_\theta = \theta/10(c\times l)$, where the ellipticity ($\theta$) is in millidegrees, sample concentration $c$ (1.0 mM) and pathlength $l$ in centimetres (0.1cm), giving deg cm$^2$ mol$^{-1}$ for $M_\theta$. 
3.3.1.4 Electrochemical Measurement
All measurements were performed with a Ag/AgCl/3M KCl as reference electrode, Pt wire as an auxiliary electrode and glassy carbon (with 0.02 cm$^2$ surface area) as a working electrode. For solution measurements, compounds were dissolved in CHCl$_3$ at 0.1 mM in the presence of 0.1 M tetrabutylammonium perchlorate. For the characterization of bulk material, a droplet of 10 µL of sample was dropped onto an inverted glassy carbon electrode and allowed to dry. The samples were prepared in CHCl$_3$, CH$_2$Cl$_2$ and in the chlorinated solvent/hexane (1:2) mixtures. Analysis was carried out using 2 M NaClO$_4$ as supporting electrolyte. Cyclic voltammetry was performed at 20 s quiet time (equilibration time), potential range 0.3-1.0 V and 100 mV s$^{-1}$ scan rates. Square-wave voltammetry was carried out at amplitude of 0.025 V, frequency of 15 Hz, quiet time at 20 s and in the 0-1.2 V potential range.

3.3.1.5 Sample Preparation
Solutions of compound 3.2 and 3.3 in chloroform, dichloromethane, chloroform/hexane (1:2) and dichloromethane/hexane (1:2) at concentration of 5mg/1mL were prepared. Assemblies were formed in chloroform/hexane and dichloromethane/hexane solutions within several seconds.

3.3.1.6 Infrared Spectroscopy
Attenuated total reflectance (ATR) FT-IR spectra were recorded on Bruker Alpha-P instrument with a diamond ATR unit at 4.0 cm$^{-1}$ resolution. 32 scans were averaged for data recorded from 400 to 4000 cm$^{-1}$.

3.3.1.7 Scanning Electron Microscopy (SEM)
The morphologies of assemblies were investigated by SEM. 5 µl of Solutions of compound 3.2 and 3.3 in chloroform, chloroform/hexane, dichloromethane and dichloromethane/hexane were deposited on a smooth silicon surface adhered to SEM grid and dried for 20 min. SEM images were collected at 0.5 and 2 kV with a Hitachi S-5200 SEM and Leo 1530 SEM.
3.3.1.8 X-ray crystallographic Data Collection and Refinement of the Structures

Orange crystals were obtained by slow diffusion of hexane into a solution of compound 3.2 in chloroform. Data were collected at 150 K on a Bruker Kappa Apex II diffractometer using Mo-K\textsubscript{α} radiation. The crystal data and refinement parameters are summarized in Table S4. The SHELXTL/PC V6.14 for Windows NT (Sheldrick, G.M., 2001) suite of programs was used to solve the structure by direct/Patterson methods. Subsequent difference Fourier syntheses allowed the remaining atoms to be located. The final $R$ value was 0.0783 for 10113 reflection with $I > 2\sigma(I)$.

3.3.1.9 Powder X-Ray Diffraction

The sample was prepared from suspension of compound 3.2 and 3.3 in chloroform/hexane (1:2). Few drops were deposited onto a zero-background Si plate. After evaporation of the solvent, the residue forms flat film, which is suitable for X-ray measurements. X-ray diffraction measurements were performed on an automated Siemens/Bruker D5000 diffractometer. The system is equipped with a high power line focus Cu-k\textsubscript{α} source operating at 50 kV/35 mA. A solid-state Si/Li Kevex detector was used for removal of k-beta lines. The diffraction patterns were collected on a $\theta$/2$\theta$ Bragg-Brentano reflection geometry with fixed slits. A step scan mode was used for data acquisition with step size of 0.02$^\circ$ $2\theta$ and counting time of 1.5 s. per step. Data processing (peaks data assessment, phase composition) was carried out with Bruker AXS software Eva\textsuperscript{TM} v.8.0.

3.3.2 General Synthesis of Ferrocene-Peptide Conjugates 3.2-3.3

To a stirring mixture of Fc[CO-Gly-Val-OH]\textsubscript{2} (3.1) (0.58 g, 1.0 mmol), triethylamine (0.34 mL, 2.5 mmol) in dry CH\textsubscript{2}Cl\textsubscript{2} (50 mL), HOBt (0.33 g, 2.2 mmol) and HBTU (0.83 g, 2.2 mmol) were added. NH\textsubscript{2}-Phe-OCH\textsubscript{3} (0.53g, 2.5 mmol) in dry CH\textsubscript{2}Cl\textsubscript{2} is added to initial solution to give compound 3.2. For preparing compound 3.3, Boc-Phe-Phe-OMe in dry CH\textsubscript{2}Cl\textsubscript{2} was deprotected by treatment with TFA. The TFA and CH\textsubscript{2}Cl\textsubscript{2} were removed in vacuo. The resulting residue was redissolved in CH\textsubscript{2}Cl\textsubscript{2} cooled in an ice bath prior to the addition of triethylamine and is then
added to the initial mixture. After 12 hours, the reaction mixture was washed with subsequent aqueous solutions of saturated NaHCO₃, citric acid (10%), again saturated NaHCO₃ and finally distilled water. The organic phase was collected and traces of water were removed using anhydrous Na₂SO₄. After concentration of the solution under reduced pressure, the crude product was purified by flash column chromatography in a CHCl₃-CH₂OH solvent mixture. The solvent were then removed completely under reduced pressure and orange solids were obtained.

3.3.2.1 Fc[CO-Gly-Val-Phe-OMe]₂ (3.2)

Silica gel column, increasing proportions of CH₃OH (0-2)% in CHCl₃. Yield: (0.54 g, 60%).

ESI-MS: calcld for C₄₆H₅₆FeN₆O₁₀ 909.3; found 909.4 [M+H]. ¹H NMR (300 MHz, CDCl₃): δ = 8.59 (t, 2H, NH Gly), 7.32-7.28 (br, m, 5 H, CH₄ Ar Phe), 7.24 (br, m, 5 H, CH₄ Ar Phe) 6.94 (d, 3JHH = 7.82 Hz, 2H, NH Phe), 6.55 (d, 3JHH = 8.60 Hz, 2H, NH Val), 4.91 (m, 4H, HCP), 4.88-4.86 (m, br, 2H, CHα Phe ), 4.47 (m, 2H, HCP), 4.43 (m, 2H, HCP), 4.35 (m, br, 2H, CHα Val), 4.00 (m, br, 2H, CH₂ Gly), 3.79 (m, br, 2H, CH₂ Gly), 3.74 (s, 6H, ester OCH₃), 3.21 (m, 4H, CHβ Phe), 2.24 (m, 2H, CHβ Val), 0.96 (d, 3JHH = 6.64 Hz, 6H, (CH₃)₂ Val), 0.78 (d, 3JHH = 7.03 Hz, 6H, (CH₃)₂ Val). ¹³C NMR (CDCl₃): δ = 172.1, 171.8, 170.9, 170.8, 136.1, 129.1, 128.6, 127.1, 75.6, 71.7, 71.4, 70.7, 70.4, 58.8, 53.5, 52.4, 43.3, 37.4, 30.3, 19.3, 17.2. CV (0.1 mM in 0.1 M TBAP in CH₂Cl₂), E½ = 818 ±1 mV.

3.3.2.2 Fc[CO-Gly-Val-Phe-Phe-OMe]₂ (3.3)

Silica gel column, increasing proportions of CH₃OH (0-2)% in CHCl₃. Yield: (0.60 g, 50%).

ESI-MS: calcld for C₆₄H₇₄FeN₈O₁₂ 1203.4; found 1203.5 [M+H]. ¹H NMR (300 MHz, CDCl₃): δ = 8.63 (t, 2H, NH Gly), 7.52 (d, 2H, NH Phe), 7.32-7.29 (br, m, 6 H, CH₄ Ar Phe), 7.25-7.17 (br, m, 10 H, CH₄ Ar Phe), 6.98 (m, 4 H, CH₄ Ar Phe), 6.79 (d, 3JHH = 7.82 Hz, 2H, NH Val), 6.44 (d, 3JHH = 8.60 Hz, 2H, NH Phe), 4.97 (m, 2H, HCP), 4.75-4.67 (m, br, 2H, CHα Phe ), 4.63 (m, 4H, HCP ), 4.41 (m, 2H, HCP), 4.29 (m, br, 2H, CHα Val), 3.90 (m, br, 2H, CH₂ Gly), 3.79 (m, br, 2H, CH₂ Gly), 3.67 (s, 6H, ester OCH₃), 3.21 (m, 4H, CH₂ Phe), 2.97 (m, 4H, CH₂ Phe), 2.34 (m, 2H, CHβ Val), 0.93 (d, 3JHH = 6.64 Hz, 6H, (CH₃)₂ Val), 0.63 (d, 3JHH = 7.03 Hz, 6H, (CH₃)₂ Val). ¹³C NMR (CDCl₃): δ = 172.2, 171.6, 171.4, 171.1, 170.7, 137.1, 135.4, 129.3, 129.1, 128.5,
CV (0.1 mM in 0.1 M TBAP in CH$_2$Cl$_2$), $E_{1/2} = 818 \pm 1$ mV.

### 3.4 Conclusions

Combined spectroscopic and microscopic analysis indicate that Fc-peptides self-assemble into hierarchical structures. Individual molecules engage in intra- and intermolecular H-bonding interactions to produce peptide sheets (Scheme 3.1). The common feature of the extended self-association is the lateral packing of peptide $\beta$-sheets. Depending on the structure of the Fc-peptide and the solvent composition, the direction of supramolecular growth may be influenced. Variety of morphologies ranging from cuboids, twisted ropes to nanofibers network were observed. The propensity for supramolecular and hierarchical organization of Fc-peptides makes them ideal building blocks towards designing functional biomaterials.

### 3.5 Supporting Information

Supporting information for this chapter is provided in Appendix B.

### 3.6 References


Chapter 4

Study of Amyloid β-Peptide (Aβ_{12-28}-Cys) Interactions with Congo Red and β-Sheet Breaker Peptides Using Electrochemical Impedance Spectroscopy

Ferrocene peptides are able to engage in intermolecular interactions and are at the same time redox active. In essence, intermolecular interactions between biological (or biologically inspired) targets can be tailored to suit a particular need or application. In the following two chapters, peptide interactions were explored in greater detail with a focus on the interactions between a target that was bound to a surface and a peptide in solution. Intermolecular interactions were front and center of this work and electrochemical methods were exploited to monitor these interactions. As was described in Chapter 1, peptides derived from the hydrophobic core of Aβ had the ability to interact with full length Aβ and interfere with its aggregation. In the present chapter, Aβ_{12-28}, a fragment of the amyloid β peptide, was immobilized on gold surfaces via a C-terminal Cys, resulting in the formation of a thin film, which was studied using electrochemical techniques. Work detailed in this chapter focused on the interactions of surface-supported Aβ_{12-28} with the beta-breaker peptide Ac-Lys-(N-Me-Leu)-Val-(N-Me-Phe)-Phe-NH₂ and Congo red. Work described in this chapter sets the stage for subsequent work on the interaction of Fc-peptides with Aβ which is the focus of Chapter 5.

This chapter is reproduced with the permission from: R. Partovi-Nia, S. Beheshti, Z. Qin, H. S. Mandal, Y. -T. Long, H. H. Girault, H.-B. Kraatz, “Study of Amyloid β-Peptide (Aβ_{12-28}-Cys) Interactions with Congo Red and β-Sheet Breaker Peptides Using Electrochemical Impedance Spectroscopy”, Langmuir 2012, 28, 6377-6385. I co-authored this paper and I was the main contributor to the experimental work. In addition, I was involved in writing the first draft and was involved in final editing of the manuscript.
4.1 Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by the presence of extracellular deposits of amyloid proteins and plaques, composed primarily of toxic aggregates of amyloid β-peptide (Aβ) protein.\textsuperscript{[1-3]} Aβ exists in many aggregation states, ranging from dimers and trimers to fibrils and plaques, and there is increasing evidence that oligomers may be the primary pathogenic species.\textsuperscript{[4]} Fibrils were initially targeted as the species responsible for neuronal toxicity and cell death. More recently, growing evidence suggests that much smaller, soluble oligomeric Aβ species correlate better with severity of AD than plaques (fibrils). Several small molecules have been reported to modulate the formation of Aβ fibrils.\textsuperscript{[4-12]} For many of these compounds, the mechanisms of action are only vaguely understood. The most frequently studied of such molecules is Congo red [CR (Scheme 4.1a)].\textsuperscript{[13, 14]} However, the details of its binding mechanism and influence on protein aggregation are still not well understood.

β-sheet breaker peptides (BSB) constitute a class of inhibitors that are designed to specifically bind the Aβ peptide while preventing and reversing its conversion to a β-sheet-rich aggregated structure.\textsuperscript{[10,15]} Tjerberg and colleagues showed that Aβ\textsubscript{16-20} is the most important region for Aβ protein–protein interaction\textsuperscript{[16]}, in agreement with previous reports from several groups using Aβ mutations, which demonstrated that the central hydrophobic domain of Aβ was responsible for protein aggregation.\textsuperscript{[8,17,18]} Tjerberg’s studies involved the Aβ\textsubscript{16-20} pentapeptide and demonstrated that it is able to bind to Aβ\textsubscript{1-40} and inhibit the formation of amyloid fibrils.\textsuperscript{[16]} However, Aβ\textsubscript{16-20} spontaneously aggregates into amyloid-like fibrils, and thus its use as an inhibitor may be problematic. Therefore, several groups have modified this sequence to produce peptide derivatives containing the self-recognition motif, along with disrupting elements that might enhance their inhibitory activity. For example, N-methylated peptide derivatives of Aβ\textsubscript{16-20} have been reported that are able to bind to Aβ and disrupt its fibril formation.\textsuperscript{[19]}

In the present work, we make use of C-terminal cysteine-linked Aβ\textsubscript{12-28} (H-Val\textsubscript{12}-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys\textsubscript{28}-Cys-OH) peptide and assemble thin films on gold surfaces in order to study the interactions with the BSB peptide, Ac-Lys\textsubscript{16}-(N-Me-Leu)-Val-(N-Me-Phe)-Phe\textsubscript{20}-NH\textsubscript{2} (Scheme 4.1b), and CR using electrochemical techniques.

The 17 amino acid fragment, Aβ\textsubscript{12-28}, peptide is a particularly attractive system, as it is toxic to the cell and forms amyloid fibrils similar to those found for the full-length peptide.\textsuperscript{[20]} Graslund
and co-workers studied the conformational properties of the Aβ_{12-28} peptide using a combination of spectroscopic techniques. They found a certain fraction of Aβ_{12-28} is in a monomeric state with a dominating random coil conformation. The monomers are in equilibrium with heterogeneous fractions of aggregates of various sizes, and these are partly in β-sheet conformation.\textsuperscript{[20, 21]} Previous studies have shown that the kinetic of aggregation of Aβ is dependent on peptide length.\textsuperscript{[22]} According to previous report,\textsuperscript{[23]} aggregation of full length of Aβ (Aβ_{1-42} and Aβ_{1-43}) was very fast (complete within a few minutes after dilution in phosphate buffer), whereas aggregation of Aβ_{12-28} was quite slow (10–30 days).\textsuperscript{[24]}

\textbf{Scheme 4.1} Molecular structure of (a) Congo red (CR) and of (b) a beta-sheet breaker peptide (BSB)

Previous studies of adsorbed amyloid peptides evaluated peptide aggregation by surface plasmon resonance (SPR),\textsuperscript{[25]} reflection–absorption infrared spectroscopy (RAIRS)\textsuperscript{[26]} and atomic force microscopy (AFM).\textsuperscript{[27]} Such an approach, while effective, does not provide control over the aggregation of the system and in the event of strong peptide-surface attraction, a condensation of one or several condensed peptide layers at the surface becomes possible. At higher peptide
concentrations, the formation of macroscopic amorphous or ordered peptide aggregates becomes possible in the bulk solution. In addition, conformations of peptides might be affected by the underlying surface substrate, which in turn will affect the formation of aggregates.\textsuperscript{[28, 29]} We want to stress that none of these studies make use of chemically modified surfaces in which an amyloid peptide is chemically attached to the surface.

In the present work, the focus is on the interaction of BSB and CR with films of the amyloid peptide fragment A\textsubscript{β}12-28-Cys, in which the C-terminal cysteine group allows film formation on gold surfaces. Electrochemical impedance spectroscopy (EIS) studies\textsuperscript{[30]} allow to monitor changes in impedance of the system as a function of BSB and CR addition, which can then be interpreted in terms of changes in the interface and charges in the electron transfer across the modified electrode/solution interface.

4.2 Results and Discussion

4.2.1 Film Characterization

CV and SWV were employed to characterize the film by monitoring the electron transfer process on the A\textsubscript{β}12-28-Cys peptide film on gold electrodes in the presence of [Fe(CN)\textsubscript{6}]\textsuperscript{3+/4-} as a redox probe. In order to facilitate the A\textsubscript{β}12-28-Cys peptide immobilization on the gold electrode surface, this process was carried out in a buffer solution. The isoelectric point (pI) of A\textsubscript{β}12-28 peptide is 7.9,\textsuperscript{[31]} so at pH 7.4 it is globally uncharged by having 3 positive and 3 negative charges. The CVs obtained for bare and modified gold electrodes are presented in Figure 4.1. Electrochemical analysis showed that well-packed peptide films were formed by means of assembling cysteine-terminated peptides onto the gold-electrode.
Figure 4.1 (a) Cyclic voltammograms and (b) square-wave voltammograms of bare gold (solid line) and gold modified with Aβ_{12-28}-Cys (dotted line) after 72 h incubation of bare gold in 50 μM Aβ_{12-28}-Cys. The solution composition was 5 mM [Fe(CN)_{6}]^{3/-4-} (1:1) in phosphate buffer (50 mM Na₂HPO₄, 50 mM KH₂PO₄, pH 7.4) at scan rate of 0.1 V/s. The voltage range was -0.1 to 0.6 V.

The voltammograms obtained for the peptide-coated electrode lacked the characteristic redox waves observed in the voltammogram of the stripped gold-electrode, suggesting that densely packed peptidic films are formed.

### 4.2.2 EIS Analysis

EIS has been exploited in sensor applications, biological cell analysis, and clinical analysis. However, it has been rarely applied to the investigation of the interaction of molecules with Aβ peptide. In EIS, a small sinusoidal voltage is applied and the respondent current is collected within a frequency range, allowing the evaluation of the impedance, Z, which may give information about the underlying system.

Figure 4.2 shows the typical Nyquist plot of a bare and peptide modified gold electrode in the phosphate buffer (50 mM Na₂HPO₄, 50 mM KH₂PO₄, pH 7.4), containing 5 mM [Fe(CN)₆]^{3/-4-} as a redox probe at an applied potential of 250 mV vs Ag/AgCl in a frequency range of 0.1 Hz to 100 kHz. In this figure, the highest and lowest frequencies are measured and the characteristic
frequency (the frequency at which the imaginary component of the impedance is at its maximum) have been labelled, since the frequency dependence is obscured in a Nyquist plot.\[33\] As shown in Figure 4.2, the bare gold electrode exhibits impedance behaviour that is characteristic of a mass diffusion controlled electron transfer process.\[34\]

Figure 4.2 Representative Nyquist plots (-$Z_{\text{im}}$ vs $Z_{\text{re}}$) for (▲) bare gold electrode, (●) after 72 h incubation of bare gold electrode in 50 µM $\text{Aβ}_{12-28}$-Cys peptide in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4). Measured data are shown as symbols with calculated fit to the equivalent circuit (Figure 4.3b) as solid lines. Impedance spectra obtained in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4), containing 5 mM $[\text{Fe(CN)}_6]^{3^-/4^-}$ (1:1) as a redox probe, at a formal potential of 250 mV vs Ag/AgCl, frequency range from 100 kHz to 0.1 Hz, and AC amplitude of 10 mV.

We will focus on the impedance behavior of $\text{Aβ}_{12-28}$-Cys peptide-modified gold electrode. The semicircle at higher frequencies, corresponding to limited electron-transfer process of $[\text{Fe(CN)}_6]^{3^-/4^-}$, occurred after the incubation of the gold electrode in $\text{Aβ}_{12-28}$-Cys peptide. This insulating layer on the electrode introduces a barrier to interfacial electron transfer.\[35,36\] The $\text{Aβ}_{12-28}$-Cys peptide covers the gold surface, effectively blocking the Faradaic current of the redox process of $[\text{Fe(CN)}_6]^{3^-/4^-}$. The EIS data were consistent with the results obtained from CV
and SWV experiments (Figure 4.1) and provide further evidence of peptide film formation on the gold surface and show increases in the resistance to charge transfer due to a densely packed film. In Figure 4.3a, the charge transfer resistance, $R_{ct}$, and the double layer capacitance, $C_{dl}$, are associated with the reduction of $[\text{Fe(CN)}_6]^{3-/4-}$ on the active surface; $R_{A\beta}$ and $C_{A\beta}$ are the charge transfer resistance for electron transfer through the film and capacitance of the peptide film, and $R_s$ is the uncompensated resistance of solution. When the surface is blocked such that all electron transfer reactions must occur through the film, $R_{A\beta} \gg R_{ct}$ the model of Figure 4.3a can reduce to Figure 4.3b where the total resistance, $R_t$, and total capacitance, $C_t$, are given by the equations:

$$R_t = \frac{R_{ct}}{\theta}$$

(1)

$$C_t = (1 - \theta)C_{A\beta} + \theta C_{dl}$$

(2)

Where $\theta$ is the fraction of the active sites of the surface, which simply relates to the fraction of the peptide coverage by $(1 - \theta)$.

In fitting the EIS data by the equivalent circuit Figure 4.3b, the total capacitance ($C_t$) was replaced by a constant phase element (CPE) to account for time constant dispersion as a result of surface inhomogeneity. The CPE is a phenomenological term defined as:

$$CPE = \left[ Y_0 (j\omega)^{\alpha} \right]^{-1}$$

(3)

where the parameters $Y_0$ and $\alpha$ are independent of frequency $\omega$, and $j^2 = -1$. When $\alpha = 1$, the CPE is identical to a capacitor and $Y_0 = C$. When $\alpha$ is close to unity, the CPE may be converted to capacitance by the equation derived by Brug$^{[37]}$

$$C_t = \left[ Y_0 \left( \frac{1}{R_s} + \frac{1}{R_t} \right)^{-1} \right]^{\alpha}$$

(4)
4.2.3 The Interaction of CR and BSB with Aβ₁₂₋₂₈-Cys Peptide Film

The principal components of amyloid plaques are the fibrillar aggregates of β-amyloid (Aβ) peptides (39–43 amino acids), which are the main constituents of amyloid plaques in the brains of people suffering from neurodegenerative disease. Aβ readily aggregates into fibrils and plaques. Earlier reports indicate that the Aβ₁₂₋₂₈ fragment forms fibril aggregates that are toxic. EIS allows us to monitor the changes occurring at the peptide interface presumably due to molecular interactions with CR and BSB. One has to bear in mind that EIS measurements have to be used in conjunction with other physical measurements described below.
Figure 4.4 Faradic impedance spectra of for Aβ12-28-Cys peptide film after (▲) 30 min, (●) 60 min, (■) 90 min and (+) 120 min interaction with 5 mM of (a) Congo red (CR) and (b) beta-sheet breaker (BSB) peptide in 50 mM phosphate buffer (pH 7.4). The impedance spectra were recorded from 100 kHz to 0.1 Hz. Measured data are shown as symbols with calculated fit to the equivalent circuit (Figure 4.3b) as solid lines. Impedance spectra obtained in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4), containing 5 mM [Fe(CN)$_6$]$_{3/4}^-$(1:1) as a redox probe, at a formal potential of 250 mV vs Ag/AgCl, frequency range from 100 kHz to 0.1 Hz, and AC amplitude of 10 mV.

The impedance spectra of Aβ12-28-Cys peptide-modified gold electrodes after interaction with 5 mM of CR and BSB are shown in Figure 4.4a and b, respectively. The electrodes were incubated with the compounds and then studied at different interaction times: 30, 60, 90 and 120 min. The measurements were carried out in an electrolyte solution containing 50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4 and 5 mM [Fe(CN)$_6$]$_{3/4}^-$. It is important to point out that the impedance behaviour of the peptide film after incubation with CR or BSB is significantly different. In the case of CR the impedance decreased with the interaction time, while it increased upon incubation with the BSB peptide. The equivalent circuit (Figure 4.3b) was used to fit the experimental data and shown in the Figure 4.4a and b as solid lines. It is obvious that the circuit fit the data quite well up to a frequency limit after which mass transport or other processes dominate. The results were summarized in Table 4.1 for the fittings of Figure 4.4a and 4.4 b. In both cases, $C_t$ appears not to change significantly with interaction time. As illustrated in eq 2, the total capacitance ($C_t$) is composed of two components, the capacitance of the peptide film ($C_{Aβ}$) and the double layer capacitance ($C_{dl}$).
Table 4.1 Values of the equivalent circuit elements shown in Figure 4.3b for Aβ12-28-Cys peptide film after different interaction time with 5 mM CR and BSB. The solution composition was 5 mM \([\text{Fe(CN)}_6]^{3-/4-}\) (1:1) in phosphate buffer (50 mM \(\text{Na}_2\text{HPO}_4\), 50 mM \(\text{KH}_2\text{PO}_4\), pH 7.4) at 250 mV (vs. Ag/AgCl). Errors given are standard deviations for three different day measurements and fitting error was less than 5%.

<table>
<thead>
<tr>
<th>Time/min</th>
<th>(R_s / \Omega \text{ cm}^2)</th>
<th>(C_t / \mu \text{F cm}^{-2})</th>
<th>(R_t / \Omega \text{ cm}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
<td>BSB</td>
<td>CR</td>
</tr>
<tr>
<td>0</td>
<td>3.6 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>19.6 ± 2.6</td>
</tr>
<tr>
<td>30</td>
<td>3.2 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td>24.2 ± 3.9</td>
</tr>
<tr>
<td>60</td>
<td>3.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>25.7 ± 4.3</td>
</tr>
<tr>
<td>90</td>
<td>3.5 ± 0.2</td>
<td>3.2 ± 0.5</td>
<td>25.9 ± 2.0</td>
</tr>
<tr>
<td>120</td>
<td>5.5 ± 0.3</td>
<td>4.1 ± 0.9</td>
<td>19.2 ± 0.6</td>
</tr>
</tbody>
</table>

When the ratio of the active sites \(\theta\) changes during the interaction, the first and second term of eq. 2 change in the opposite direction leaving the total capacitance virtually unchanged. However, the total resistance \(R_t\) changed during the interaction, but differently in the case of CR or BSB. Since the rate of \([\text{Fe(CN)}_6]^{3-/4-}\) reduction on gold would not be expected to be influenced by CR/BSB interaction, these changes would basically reflect the change in the fraction of active sites during the interaction. The fractional active sites of surface will change as the consequence of reactions of CR or BSB with peptide. Though the EIS cannot provide the absolute values of the fraction of the active sites, the ratio of \(\theta/\theta_0\) will represent the change in peptide packing with interaction time, which can be obtained via eq. 5:

\[
\frac{\theta(t)}{\theta_0} = \frac{R(0)}{R(t)}
\]

where \(\theta_0\) is the fraction of the active area prior to reaction. When \(\theta/\theta_0 > 1\), the reaction increases the active area, on the other hand the active area decreases when \(\theta/\theta_0 < 1\). Figure 4.5 shows \(\theta/\theta_0\) as a function of the interaction time of the peptide film with CR and BSB. A significant increase is observed for CR as the interaction time is increased. This indicates that the electron transfer ability of redox probe \([\text{Fe(CN)}_6]^{3-/4-}\) was largely improved by the insertion of CR to the peptide film since the \(\theta/\theta_0\) ratio as a function of time is attributed to the electrostatic repulsion between the solution-based redox probe and the electrode surface.
However, the behaviour in the presence of BSB is rather different. The ratio of $\theta/\theta_0$ decreases with reaction time. This is an indication of a diminished electron transfer from the solution to the electrode surface. Therefore, it is more difficult for the redox probe $[\text{Fe(CN)}_6]^{3-/4-}$ to approach the electrode as compared with the absence of BSB interaction. A decrease in the $\theta/\theta_0$ ratio over time is presumably caused by a tighter aggregation caused by the interaction with the BSB peptide. Upon longer BSB interactions, the Aβ$_{12-28}$-Cys peptide film becomes more dense and crowded. The difference in the electrochemical behavior of the interaction of CR and BSB with Aβ film on the surface might be related to the difference in the mechanism of their interaction with Aβ. According to the literature, two different binding sites (the possible sites on Aβ peptide for CR interaction) have been suggested for CR. In the site with higher affinity, CR orients itself in an antiparallel fashion with respect to the β-sheets and the sulfonate residues of CR align with N-terminus of peptides to provide ionic interactions between negatively charged sulfonate acid groups of CR and positively charged N-terminus of peptide strands. The second binding site with lower affinity is at the end of fibrils or oligomers and CR orients itself parallel to the β-sheets. Earlier molecular dynamic simulations demonstrated that CR prefers binding antiparallel to the β-sheets which shows that ionic interaction plays an important role in the interaction of CR with
amyloid aggregation. Thus, when CR is inserted into the antiparallel β-sheets, it presumably enlarges the distance between the carbonyl oxygen and the amide nitrogen and disrupts the hydrogen bonding between β-sheets. Unlike CR, peptides derived from hydrophobic core of Aβ (Lys-Leu-Val-Phe-Phe) favor binding parallel to the β-sheets at the end of fibrils or oligomers. The interaction of Lys-Leu-Val-Phe-Phe derivatives is presumably through hydrophobic interaction, hydrogen-binding and π-π stacking with their corresponding residues in the amyloid fibrils or oligomers. Hence, it is reasonable to believe that after a long time, BSB will accumulate more and bind on the peptide film.

A schematic view of the interactions of Aβ12-28-Cys peptide film on gold with CR and BSB is presented in Scheme 4.2b-c. Qualitatively, it can be concluded that CR breaks the film, as shown by the decrease of the film resistance, likely by increasing its porosity, whereas BSB makes the film more compact and thicker, increasing the resistance.

**Scheme 4.2** (a) A schematic view showing the Aβ12-28-Cys peptide adsorption onto gold surfaces followed by the interactions with (b) Congo red (CR) and (c) beta-sheet breaker peptide (BSB).

The EIS results are in agreement with the CV observations, which showed that the current decreases/increase with interaction of BSB and CR, corresponding to the observed increase/decrease in ability of redox probe [Fe(CN)6]3-/4- (see Appendix C). The response of Aβ12-28-Cys peptide film for different concentrations of CR and BSB were also investigated (see Appendix C). The EIS results showed that at lower concentration of BSB, a smaller diameter of the semicircle was observed, and as the concentration increased the resulting diameter of the semicircle increased. The opposite is observed in the case of the interaction of CR with the peptide film.
Fourier transform reflection absorption infrared spectroscopy (FT-RAIRS) provides structural information and a spectroscopic signature for β-sheet structures. Infrared spectra of BSB and CR are shown in Appendix C. Our Aβ_{12-28}-Cys peptide modified gold substrates showed the presence of an intense band at 1624 cm\(^{-1}\) and a weak band at 1692 cm\(^{-1}\) in the amide I region (Figure 4.6a) indicating that the peptides are arranged predominantly as an antiparallel β-sheet. Antiparallel β-sheets are commonly characterized by a pair of amide I bands at 1615-1620 cm\(^{-1}\) and at 1680-1690 cm\(^{-1}\).\(^{45}\) An additional band at 3260 cm\(^{-1}\) is related to NH groups involved in β-sheet like H-bonds.\(^{46}\) Figure 4.6b also shows that there are differences between the spectra of BSB treated Aβ_{12-28}-Cys modified gold substrate and Aβ_{12-28}-Cys modified gold substrate. The bands at 1624 cm\(^{-1}\) and 1692 cm\(^{-1}\) showed little change in positions, rather a slightly enhanced absorbance for BSB treated Aβ_{12-28}-Cys modified gold substrate indicating the presence of β-sheet conformation. These bands were completely absent for CR treated Aβ_{12-28}-Cys modified gold substrate (Figure 4.6c).

**Figure 4.6** FT-RAIR spectra of Aβ_{12-28}-Cys modified gold substrates before (a) and after interacting with 50 µM BSB peptide (b) and CR (c), separately for 120 min in phosphate buffer (50 mM Na\(_2\)HPO\(_4\), 50 mM KH\(_2\)PO\(_4\), pH 7.4).
Instead, the appearance of the new band at 1677 cm\(^{-1}\) and the absence of H-bonded NH groups clearly demonstrated a conformational change to the random coil state. Frequencies above 1660 cm\(^{-1}\) have been assigned to random coil structures of peptide and proteins.\(^{47, 48}\) The amyloidogenic properties of A\(\beta_{12-28}\)-Cys and its interaction with CR and BSB were also confirmed in solution by transmission electron microscopy (TEM) and Thioflavin T (ThT) fluorescence. TEM of aggregated/precipitated peptide samples obtained from aqueous solutions showed the typical fibrillar structure of \(\beta\)-amyloid peptides (Figure 4.7a). Equimolar amounts of the BSB peptide and CR were added separately to solutions of preformed fibrils of A\(\beta_{12-28}\)-Cys, incubated for 4 days and imaged by TEM. Our results show that preformed fibrils maintain their integrity with the BSB peptide (Figure 4.7c) but addition of CR causes a complete dissolution (Figure 4.7b). These observations are consistent with previous studies.\(^{13,16}\) BSBs prevent the fibril formation in a freshly prepared solution of amyloid peptides when used in equimolar quantities.\(^{16}\) But the dissolution of a significant fraction of preformed fibrils occurs in the presence of a high excess (up to 20 times) of BSBs\(^{10}\) possibly because of the stabilization of the monomeric species and shift of the dynamic equilibrium that exists between the fibrils and different active species.\(^{9}\)

![Figure 4.7](image-url)

**Figure 4.7** (a) Transmission electron microscopy (TEM) image observation of A\(\beta_{12-28}\)-Cys peptides in the absence of CR and BSB, (b) after addition of CR and (c) after addition of BSB incubated for 4 days in phosphate buffer (50 mM Na\(_2\)HPO\(_4\), 50 mM KH\(_2\)PO\(_4\), pH 7.4).

Thioflavin T (ThT) fluorescence has been widely used for probing A\(\beta\) aggregation and inhibition.\(^{49, 50}\) Monitoring the ThT fluorescence at 485 nm, which occurs after its binding to
amyloid fibril, is an effective method to probe Aβ fibril formation. Solutions of CR and BSB were added to the preformed Aβ₁₂₋₂₈-Cys fibrils in a 1:1 (CR or BSB: Aβ₁₂₋₂₈-Cys) molar ratio. Upon addition of CR to Aβ₁₂₋₂₈-Cys, the ThT fluorescence of Aβ₁₂₋₂₈-Cys samples decreased immediately. This dramatic decrease might not directly be explained as the reduction of Aβ fibrils due to the competition of CR and ThT to bind to the same binding sites at Aβ₁₂₋₂₈-Cys. Therefore we assumed that ThT fluorescence reading in the presence of CR might be bias. Similar behaviour was previously reported for ThT fluorescence of Aβ samples in the presence of other dyes such as resveratrol.⁵¹ In contrast to CR, the ThT fluorescence of Aβ solutions slightly increased following the addition of BSB to amyloid fibrils (BSB: Aβ 1:1, see Appendix C). This increase is in good agreement with our electrochemical and TEM results.

4.3 Experimental

4.3.1 General Remarks

K₃[Fe(CN)]₆ and K₄[Fe(CN)]₆ were purchased from Aldrich. NaOH, H₂SO₄, KCl, KH₂PO₄, ethanol, Congo red (CR) and thioflavin T (ThT) were obtained from Sigma. Aβ₁₂₋₂₈-Cys and BSB were purchased from AnaSpec (USA). All solutions were prepared with deionized water (Millipore Milli-Q, 18 MΩcm resistivity).

In the present studies we have chosen to work at pH 7.4, where aggregation and fibrillogenesis occurs for Aβ₁₂₋₂₈-Cys. The estimated isoelectric point of the Aβ₁₂₋₂₈-Cys is about pI 7.9.⁵¹ Dissolving the peptide up to mM concentrations directly into an ice-cold aqueous solvent at pH 7.4, which is close to the isoelectric point of peptide, gives a sample with reproducible and stable spectral properties. A pH 7.4 phosphate buffer in aqueous solution was prepared with 50 mM Na₂HPO₄, 50 mM KH₂PO₄. Solution of the redox probe (5 mM), [Fe(CN)]₆³⁻/⁴⁻, was prepared with 1:1 molar ratio of K₃[Fe(CN)]₆ and K₄[Fe(CN)]₆ in phosphate buffer at pH 7.4. Aβ₁₂₋₂₈-Cys peptide stock solution was prepared in 50 mM phosphate buffer solution pH 7.4 and stored at -20 °C or diluted with buffer at pH 7.4 to prepare solutions of different concentrations, which were stored at 4 °C. The electrodes were protected from water evaporation and kept at 4 °C for 72 h.
4.3.2 Peptide Immobilization on the Surface

Gold electrodes (99.99% (w/w) polycrystalline) were purchased from CH Instruments Inc. (TX, USA). Prior to experiments, the gold electrode was immersed for 5 min into a piranha solution (1:3, v/v, 30% H₂O₂, 18 M H₂SO₄). The electrode was then polished with wet 0.05 µm alumina slurry on a flat pad for at least 2 min.

Upon rinsing with Millipore water, the electrodes were then dipped in 0.5 M KOH solution, cycled between -0.1 and -1.5 V (vs. Ag/AgCl) at a scan rate of 0.1 V s⁻¹. At the completion of the scan the electrode was once again rinsed with Millipore water. The electrode was cleaned by electrochemical sweeping in 0.5 M H₂SO₄ by cycling at a scan rate of 0.1 V s⁻¹ from a potential of -0.1 to +1.6 V (vs. Ag/AgCl) until a stable gold oxidation peak at 1.1 V was obtained.

Subsequently, the gold electrode was placed in ethanol for 5 min with ultrasonication, and then dried with N₂ flow. The clean gold electrode was incubated with 50 µM Aβ₁₂₋₂₈-Cys peptide in phosphate buffer solution (50 mM Na₂HPO₄, 50 mM KH₂PO₄, pH 7.4) for 72 h, at 4 ºC. Afterwards, the electrode was rinsed with phosphate buffer solution and dried with N₂ flow. Subsequently, the peptide-modified electrode was incubated with 5 mM solution of CR and BSB in phosphate buffer solution (50 mM, pH 7.4) at room temperature.

4.3.3 Electrochemical Instrumentation and Measurements

All electrochemical studies, including cyclic voltammetry (CV), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS) were performed with an electrochemical analyzer (CH Instruments 660B, TX, USA) connected to a personal computer. All measurements were carried out at room temperature in an enclosed and grounded Faraday cage. A conventional three-electrode system was used, comprising a peptide-modified gold electrode as a working electrode, a platinum wire as a counter electrode, and Ag/AgCl/3M KCl as a reference electrode. The reference electrode was always isolated from the cell by a miniature salt bridge (agar plus KNO₃) to avoid the leakage of the Cl⁻ ions from the reference electrode to the measurement system. The open-circuit potential (OCP) of the system was measured prior to all electrochemical experiments to prevent sudden potential changes in the film. All electrochemical measurements were started from OCP and were carried out in 50 mM phosphate
buffer solution pH 7.4 and 5 mM [Fe(CN)₆]³⁻/⁴⁻. All CV experiments were performed at a scan rate of 0.1 V s⁻¹ in the range from -0.1 V to +0.6 V. SWV experiments were carried out in the same range as CV with a step potential of 5 mV, pulse amplitude of 25 mV, and a frequency of 15 Hz. The EIS measurements were recorded within the frequency range of 0.1 Hz to 100 kHz at the formal potential of the redox couple [Fe(CN)₆]³⁻/⁴⁻ (250 mV) with AC amplitude of 10 mV. The experimental EIS data were fitted to an appropriate equivalent circuit using the software ZView 3.2c by Scribner Associates Inc.

4.3.4 Surface Characterization

FT-RAIRS spectroscopy was carried out using a Thermo Nicolet NEXUS 670 FT-IR. A peptide film was prepared by adsorption of the peptide at the cysteine thiol group onto the gold substrate. A 100 nm thick gold substrate prepared by electron-beam deposition with a prior 5 nm thick titanium adhesion layer on a cleaned Si wafer having a 1 µm thick SiO₂ layer. Prior to incubation of Aβ₁₂⁻₂₈-Cys peptide on the gold-coated silicon, few drop of piranha solution (H₂SO₄ 70%:H₂O₂ 30% = 3:1, v/v) was deposited for 2 min on the surface then the gold-coated silicon surface was washed and sonicated sequentially in methanol and deionized water for 10 min each. Finally, the electrode was dried with N₂ flow. Aβ₁₂⁻₂₈-Cys film was prepared by soaking a clean gold substrate for 72 h, at the 4 ºC temperature in a phosphate-buffered solution (50 mM Na₂HPO₄, 50 mM KH₂PO₄, pH 7.4) of the peptide (0.1 mg/mL). The same molar as peptide were used for 60 min interaction of CR and BSB with peptide film.

4.3.5 ThT Fluorescence Assay

Solutions of Aβ₁₂⁻₂₈-Cys (50 µM, dissolved in 50 mM phosphate buffer) was incubated for fibril growth for 4 days. Solutions of CR and BSB were added to the Aβ₁₂⁻₂₈-Cys fibril sample in 1:1 (CR or BSB: Aβ₁₂⁻₂₈-Cys) molar ratio. Thioflavin T (ThT) was added to the samples to the final concentration of 50 µM in 96-well plates. The resulting ThT fluorescence of samples was measured at an emission of 485 nm using the excitation wavelength 440 nm using a Bio-Tek Synergy HT Multimode Microplate Reader.
4.3.6 Transmission Electron Microscopy

Samples were prepared in 50 mM phosphate buffer solution (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4), then dried onto carbon-coated nickel grids for characterization by transmission electron microscopy (TEM) (JEOL 1200 EX) operated at 80 kV. TEM was used for the characterization of Aβ$_{12-28}$-Cys peptide solution samples in the course of aggregation.

4.3.7 Optimization of Experimental Conditions

In order to establish optimal conditions for the peptide film formation, cyclic voltammetry (CV) was carried out for an electro-active species such as [Fe(CN)$_6$]$^{3+/4-}$ at a film of Aβ$_{12-28}$-Cys on gold electrodes. The peptide film on the electrode surface is globally uncharged and does not affect the electron transfer from the negatively charged redox ions, such as [Fe(CN)$_6$]$^{3+/4-}$, to the electrode.

The extent of kinetic hindrance to the electron transfer process increases with the increasing coverage and thickness and the decreasing defect density of the barrier.$^{[52]}$ The influence of incubation time on CV signal was investigated for modified gold electrode with Aβ$_{12-28}$-Cys in 5 mM [Fe(CN)$_6$]$^{3+/4-}$ solution. The results show (see Appendix C, Figure C.1) that the current of the modified electrodes decreased with the increment of incubation time, and then levelled off after 60 h, implying that the Aβ$_{12-28}$-Cys modified electrodes were saturated with Aβ$_{12-28}$-Cys. Therefore, 72 h was selected as the optimum incubation time. Figure C.2 (see Appendix C) shows the effect of Aβ$_{12-28}$-Cys peptide concentration on cyclic voltammogram response after 72 h incubation on Au electrodes. By increasing the concentration of Aβ$_{12-28}$-Cys peptide from 5 nM to 50 µM, the measured current of Au electrode was decreased corresponding to the concentration of Aβ$_{12-28}$-Cys peptide. When the concentration of Aβ$_{12-28}$-Cys peptide is higher than 20 µM, changes in current response become sluggish, which might be attributed to the limitation of active sites (Appendix C). Thus, Aβ$_{12-28}$-Cys peptide concentration of 50 µM (103 µg/mL) was chosen in the experiment for film preparation.
4.4 Conclusions

In this work, we demonstrated that, in addition to more classical spectroscopic techniques, electrochemical methods including electrochemical impedance spectroscopy provide some useful information about differences in the interactions of CR and BSB with Aβ\textsubscript{12-28}-Cys peptide immobilized on gold surfaces and in fact can be used to monitor this interaction. However, the net result of the interaction is fundamentally different indicating differences in the interaction and in film structure upon exposure. CR appears to cause loss of film integrity making the film more permeable to solution based redox probes. In contrast, BSB appears to integrate into the film and cause an increase in the resistance to charge transfer. We interpret these differences in terms of structural differences in the film structure. CR appears to lead to porous peptide films whereas the opposite is the case for BSB. FT-RAIRS studies further support the interaction of peptide films with CR or BSB. Clearly our studies indicate the value of EIS measurements for monitoring interactions of Aβ disrupting molecules with peptide films. Non-electrochemical techniques, such as TEM and ThT fluorescence measurements provide complementary information that support our chemical understanding gained from our electrochemical investigations. Additional studies are ongoing screening of larger peptide libraries and their abilities to interact with peptide films.

4.5 Supporting Information

Supporting information for this chapter is provided in Appendix C.

4.6 References


Chapter 5
Electrochemical “Signal-on” Reporter for Amyloid Aggregates

In Chapter 4, a surface-based approach to study of biomolecular interactions of an amyloid-beta (Aβ) peptide chemically linked to a surface with a peptide in solution was explored. It was clearly demonstrated that the intermolecular interactions gave rise to an electrochemical signature that was indicative of the interaction. In the present chapter, we built on this work and attached a redox probe covalently to the solution-based peptide to facilitate detection of the interaction. We took advantage of Fc as a redox probe, which when attached to the β-sheet breaker peptide should enable us to monitor the interaction of the Fc-peptide conjugate with the Aβ fragment chemically linked to a gold surface. For this purpose, a series of monosubstituted Fc-peptide conjugates containing variations of the hydrophobic core Lys-Leu-Val-Phe-Phe (Aβ

This chapter is reproduced with the permission from the peer reviewed version of the paper: S. Beheshti, S. Martić, H.-B. Kraatz, “Electrochemical “Signal on” Reporter for Amyloid aggregates”, ChemPhysChem 2012, 13, 542-548, Copyright © 2012, Wiley-VCH. I performed the majority of the experimental work and carried out writing of the initial draft manuscript, which was improved in an iterative approach. The text below is a verbatim copy of the published paper.
5.1 Introduction

Understanding the aggregation of amyloid β-peptides (Aβs) is a key for probing their neurotoxic effects and designing drug molecules that interfere with the formation of amyloid oligomers, protofibrils, fibrils, and plaques. Studies have shown that early stage aggregates contribute to Aβ neurotoxicity and are potentially viable drug targets.[1-3] Several fragments of Aβ, such as Aβ18-28,[4] Aβ10-35,[5] Aβ16-22,[6, 7] and Aβ16-20[8] have been shown to form amyloid fibrils. Recent studies on the hydrophobic core Aβ16-20 suggested that the Lys-Leu-Val-Phe-Phe peptide sequence is critical for fibril formation.[9-11] Hilbich et al. confirmed that in Aβ16-20, the Leu-Val-Phe-Phe motif is crucial for β-sheet formation and reported that substitution of this sequence with hydrophilic amino acids reduces the amyloid formation.[12] Tjernberg et al. studied the binding ability of different fragments of Aβ1-40 to the full peptide ranging from 3 to 10 amino acids and concluded that sequence Aβ16-20 is present in all fragments that strongly bind to Aβ1-40.[13] Several modifications of Lys-Leu-Val-Phe-Phe and Lys-Leu-Val-Phe-Phe-Ala-Glu have been reported.[14-16] For example, N-methylation of the amide protons in Lys-Leu-Val-Phe-Phe and Lys-Leu-Val-Phe-Phe-Ala-Glu resulted in the formation of peptides which are able to bind to Aβ1-40 and disrupt its fibril formation. Aggregation and inhibition have been studied in vitro by a plethora of techniques including transmission electron microscopy (TEM), and circular dichroism (CD), infrared (IR), and fluorescence spectroscopies.[17-20] While these techniques address the underlying molecular aspects, immunoassays involving antibodies specific to Aβ bridge the gap to the clinic.[21, 22] Alternatively, robust, facile and low-cost electrochemical methods are useful for probing Aβ fibrilization and its inhibition. For example, the oxidation of a Tyr residue present in Aβ has been exploited to monitor its aggregation in solution and on surfaces.[23, 24] Similarly, electrochemical methods are useful for probing metal ion effects on Aβ aggregation.[25] More recently, the redox activities of the benzothiazoles thioflavin T, a fluorescence dye used to monitor Aβ fibrils ex vivo and in vitro, and its uncharged derivative BTA-1, were exploited to probe Aβ association in solution.[26]

Towards developing new electrochemical biosensors for probing Aβ-type interactions and inhibitor screening, we propose to use a series of bioorganometallic conjugates[27] of the consensus sequence (Lys)-Leu-Val-Phe-Phe which was shown to be critical for fibril formation,[10, 11, 13] and has the potential to disrupt aggregation and even causes dissolution of mature Aβ fibrils.[13] To probe disruption of Aβ aggregation, we prepared ferrocene (Fc)-peptide
conjugates 5.3-5.6 of (Lys)-Leu-Val-Phe-Phe as shown in Scheme 5.1. The attachment of the Fc group to the N-terminal side of the peptide provides a convenient handle to detect the interaction between the Fc-peptide conjugates and Aβ electrochemically. The charge of the Fc-peptide conjugates can be influenced by C-terminal modification and may affect the interaction. Here, we present an electrochemical approach, demonstrating for the first time that Fc-conjugates 5.3-5.6 bind to Aβ12-28 immobilized on gold surfaces generating a “signal-on” electrochemical response. Our complementary solution results show that the inhibitory potency of the Fc-peptide conjugates influences Aβ12-28 aggregation and coincides with their ability to interact with Aβ12-28 surfaces.

5.2 Results and Discussion

5.2.1 Synthesis and Characterization of Fc-peptides

Four monosubstituted Fc-conjugates were synthesized as depicted in Scheme 5.1. Briefly, the tetrapeptide Boc-Leu-Val-Phe-Phe-OMe,\textsuperscript{[28]} Aβ17-20, was synthesized using HBTU and HOBt as coupling reagents. The peptide was then coupled to Fc-carboxylic acid or Fc-CO-Lys(Boc)-OH in similar manner, resulting in the formation of compounds 5.3 and 5.5, respectively (Scheme 5.1). Methyl ester deprotection of compounds 5.3 and 5.5 using 10 molar excess of NaOH in a mixture of H₂O/CH₃OH results in the formation of compounds 5.4 and 5.6 (Scheme 5.1). Compounds 5.3-5.6 were characterized by mass spectrometry, \(^1\)H, \(^{13}\)C NMR and IR (see Appendix D, Figure D.1-D.9). A distinct set of cyclopentadienyl (Cp) signals in the \(^1\)H NMR spectrum, in a ratio of 5:2:1:1, is typical for the mono substituted Fc group.\textsuperscript{[29]} The chemical shift of an unsubstituted second Cp ring appears as a singlet around 4.2 ppm, while the meta and ortho proton signals of substituted Cp ring are located further downfield. The appearance of a broad resonance around \(\delta\) 12.76 reveals the successful deprotection of the ester groups and formation of the Fc-peptide acids compounds 5.4 and 5.6.

The electrochemical properties of Fc-peptides 5.3-5.6 in CH₃CN include half-potentials, \(E_{1/2}\), in the 580 – 620 (10) mV range, a \(i_{pc}/i_{pa}\) ratio close to unity and a potential difference, \(\Delta E\), in the 50-90 mV range which indicate a one-electron reversible redox process. Notably, Fc-tetrapeptide methylester conjugate 5.3 exhibits a higher current density than the deprotected free acid
analogue 5.4 in Figure 5.1a. Importantly, the inclusion of a Boc-protected Lys residue, next to the Fc group in conjugates 5.5 and 5.6, has little effect on the redox potential (see Appendix D, Figure D.19). Deprotected Fc-pentapeptide conjugate 5.6 exhibits a slight cathodic redox shift compared to the methyl ester protected congener. The diffusion coefficients were also reduced in going from Fc-tetrapeptides (13-22 × 10⁻⁵ cm² s⁻¹) to Fc-pentapeptides (7-13 × 10⁻⁵ cm² s⁻¹).

**Scheme 5.1** Synthesis of Fc-peptide conjugates 5.3-5.6. (i) HOBt (1.2 eq.), HBTU (1.2 eq.), Et₃N (1.5 eq.), CH₂Cl₂. (ii) Boc-Leu-Val-Phe-Phe-OMe (1.2 eq.), (v/v) TFA/CH₂Cl₂ (compounds 5.3 and 5.5). (iii) NaOH (10eq.) in H₂O:CH₃OH (compounds 5.4 and 5.6).

Initially, the hydrogen bonding (H-bonding) ability of Fc-conjugates 5.3-5.6 was studied in solution and the solid state. The secondary structure of Fc-peptide conjugates in CH₃CN solution was assessed by CD spectroscopy. In general, a strong β-sheet signature with a minimum at 214 nm was observed.[⁷] Variable temperature CD experiments for compounds 5.3-5.6 show that the CD intensity at 214 nm is decreasing dramatically with increasing temperature from 25 °C to 70 °C, suggesting weakening of hydrogen bonds at higher temperatures (see Appendix D, Figure D.11). Next, the CD spectra of compounds 5.3-5.6 were measured in a mixture of 50% CH₃CN/tris buffer at pH 8.5 in order to enhance the solubility of Fc-peptides while maintaining an aqueous environment. In 50% CH₃CN /tris buffer, the CD spectra of compounds 5.3 and 5.4 are dominated by two minima at 214 nm and 232 nm (Figure 5.1b). The minimum at 214 nm
indicates a β-sheet structure. The second minimum at 232 nm results from the stacking of aromatic amino acids and was previously reported for modified Aβ peptide fragments in aqueous solution.

The Amide I region in the FT-IR spectra for compounds 5.3 and 5.4 (peak at 1633 cm\(^{-1}\) with a shoulder at 1683 cm\(^{-1}\) for compound 5.3 and peaks at 1637 and 1685 cm\(^{-1}\) for compound 5.4) support the assignment of a β-sheet structure (Figure 5.1c). Similar trends were observed in the CD and FT-IR spectra of Fc-conjugates 5.5 and 5.6 (see Appendix D, Figure D.12).

![Figure 5.1](image)

**Figure 5.1** (a) Cyclic voltammograms of 5.3 (−) and 5.4 (− − −) (0.1 mM in 0.1 M TBAP in CH\(_3\)CN). Scan rate 100 mV s\(^{-1}\), Pt wire auxiliary electrode, Ag/AgCl reference electrode and glassy carbon as working electrode. (b) Circular dichroism spectra of 5.3 (−) and 5.4 (− − −) in 50% CH\(_3\)CN/ tris buffer, pH 8.5: the first minimum at 214 nm is consistent with β-sheet structure and the second minimum at 232 nm is due to the stacking of aromatic amino acids. (c) FT-IR spectra of 5.3 (−) and 5.4 (− − −) in the Amide I region showing two peaks at ~1630 and ~1680 cm\(^{-1}\) which are associated with β-sheet structure.
5.2.2 Study of the Interactions of Fc-peptides with Aβ_{12-28}

Due to the ability of Fc-conjugates to undergo H-bonding in solution and solid state as evidenced by CD and IR studies, the inhibitory effects of 5.3-5.6 on the self-assembly of Aβ_{12-28} were first investigated in solution. Instead of probing the full Aβ, we chose Aβ_{12-28} Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Val-Gly-Ser-Asn-Lys, one of the short Aβ peptide fragments, which possesses the central hydrophobic core of Aβ, Lys-Leu-Val-Phe-Phe and displays a slower self-association. Furthermore, this sequence is easier to handle since its aggregation rate is slower than that of the full length Aβ peptide. Earlier studies reported that this sequence provides a reliable model for early state aggregation of Aβ.[32] Solutions of Aβ_{12-28} (50 µM) and Aβ_{12-28}/Fc-conjugate (1:2 molar ratio) mixtures were aged for 7 days at room temperature and then the CD spectra and TEM images were recorded. The CD spectrum of monomeric Aβ_{12-28} displays a strong minimum near 200 nm consistent with a random coil structure. After 7 days of aging, the CD spectrum of Aβ_{12-28} displays a negative signal at 228 nm indicating structural change from random coil to β-sheet (Figure 5.2a). The red-shifted minimum for β-sheet (minimum at 228 nm instead of minimum at 214 nm) have been reported for other β-sheet peptides and might be related to the unusual pitch of the β-sheet.[33, 34] In contrast, the aged mixtures of Aβ_{12-28}/Fc-conjugates exhibit different CD signatures characteristic of random coil and β-sheet (Figure 5.2b). We propose that the interactions of compounds 5.3-5.6 with Aβ_{12-28} disrupt or delay Aβ_{12-28} aggregation and result in the formation of alternative aggregates.

TEM was used to probe the aggregates in the presence and absence of Fc-conjugates 5.3-5.6. Aβ_{12-28} forms long, straight, unbranched filaments that are characteristic of amyloid fibrils[35] (Figure 5.2c) after 7 days of aging. The fibrils are 7 to 17 nm wide and several micrometers in length. In the presence of conjugates 5.3-5.6 dramatically different morphologies were observed. In the case of Aβ_{12-28}/5.3 and Aβ_{12-28}/5.4, a network of short fibrils and spherical particles were evident (Figure 5.2d and e). The population of spherical particles in the case of Aβ_{12-28}/5.4 was lower than for Aβ_{12-28}/5.3. This difference in the fibrillar morphologies of Aβ_{12-28}/5.3 and Aβ_{12-28}/5.4 might be due to the presence of carboxylate group at the C-terminal of compound 5.4.
Figure 5.2 (a) Circular dichroism spectra of Aβ_{12-28} monomer (50 µM) (˗) and Aβ_{12-28} after 7 days of aging (···) in 50% CH$_3$CN/ tris buffer at pH 8.5. The Aβ_{12-28} after 7 days of incubation exhibited a CD spectrum with a minimum around 228 nm that was consistent with β-sheet structure. (b) Circular dichroism spectra of Aβ_{12-28}/5.3 (˗), Aβ_{12-28}/5.4 (˗ ˗ ˗), Aβ_{12-28}/5.5 (···) and Aβ_{12-28}/5.6 (˗ · ˗) mixtures (1:2 molar ratio) in 50% CH$_3$CN / tris buffer at pH 8.5 after 7 days. When Aβ_{12-28} was aged in the presence of compounds 5.3-5.6, different CD signature was observed. (c) TEM images of assemblies present in 7-day-old CD samples of Aβ_{12-28} (50 µM) showing the formation of long, unbranched fibrils. (d and e) TEM images of assemblies present in 7-day-old CD samples Aβ_{12-28}/5.3 and Aβ_{12-28}/5.4 mixtures, respectively. (f and g) TEM images of 7-day-old CD samples of Aβ_{12-28}/5.5 and Aβ_{12-28}/5.6, respectively. No fibrils were observed for the mixture of Aβ_{12-28}/5.6. TEM images may not be representative of the whole sample. Scale bars are 100 nm.
Spherical particles were previously reported for a mixture of Aβ₁–₄₀ with a Lys-Leu-Val-Phe-Phe peptide based inhibitor which has a neutral mini PEG (polyethylene glycol) groups at its C and N- termini. Incubations of Aβ₁₂–₂₈ with conjugates 5.5 and 5.6 result in the formation of mostly spherical particles (Figure 5.2f and g). In the case of Aβ₁₂–₂₈/5.6, no fibrils were observed. Presumably, the presence of Boc-protected Lys in the case of compounds 5.5 and 5.6 increases the overall hydrophobicity of the Fc-conjugates and leads to stronger hydrophobic interaction with Aβ₁₂–₂₈. These observations are in line with results indicating that hydrophobic and ionic interactions play an important role in aggregation of Aβ. These effects may be responsible for the dramatic differences in propensities of 5.3-5.6 to interfere with Aβ aggregation.

Fluorescence studies with thioflavin T (ThT) are used to assess the formation/inhibition of Aβ fibrils. However, it was previously shown that certain peptides that form amyloid fibrils do not induce thioflavin T fluorescence. In our system, the results from thioflavin T assay were inconclusive despite the fact that Aβ₁₂–₂₈ forms amyloid fibrils as shown by electron microscopy. We attempted to use the solution electrochemistry to study interactions between excess Fc-peptides and Aβ₁₂–₂₈ at low concentrations. However, no significant changes in the redox signals of Fc-peptides were observed upon aging of the Aβ₁₂–₂₈/Fc-peptide mixtures. Alternatively, surface electrochemistry may allow for probing the biomolecular peptide interactions, which is presented next.

### 5.2.3 Surface-Based Electrochemical Sensing of the Fc-peptide Interactions with Aβ₁₂–₂₈-Cys Films

We probed the ability of Fc-conjugates 5.3-5.6 to interact with Aβ₁₂–₂₈ films electrochemically. Surface based electrochemical methods, which take advantage of surface-bound bio-recognition elements, can provide insight into biomolecular interactions. In the present system, Aβ₁₂–₂₈ is bound to a gold surface through a C-terminal Cys (Aβ₁₂–₂₈-Cys) and the Fc-peptide conjugates 5.3-5.6 serve as biomolecular probes. An electrochemical response is expected only when Fc-peptides 5.3-5.6 strongly interact with the Aβ bio-recognition surface. To our knowledge this is the first example of a surface electrochemical study involving Aβ and Fc-peptides. Initial studies were performed in order to evaluate Aβ films on gold surfaces in the absence of Fc-peptides. The amyloid surface used throughout the study was formed by incubating clean gold electrodes (0.02
cm² surface area) in 50 µM Aβ₁₂₋₂₈-Cys solution in tris buffer (pH 8.5) for 3 days. The long incubation time allowed for surface binding, film reorientation and aggregate formation. The Aβ surface coverage, $\Gamma_{A\beta}$, was estimated by electrochemical desorption in a basic solution to be $~4 \times 10^{-10}$ (1) mol cm⁻². The amount of charge arising from the reduction current was estimated by integrating the reduction peak.¹³⁹ Electrochemical surface characterization indicated the formation of a well-packed Aβ film on gold surfaces, and was performed by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in the presence of the [Fe(CN)₆]³⁻⁻⁻⁻ redox couple. Figure 5.3a shows the current density observed for bare electrode and a significant reduction in the current following the incubation with Aβ₁₂₋₂₈-Cys, which indicates Aβ₁₂₋₂₈-Cys film formation. In addition, electrochemical impedance measurements, shown in Figure 5.3b, depict a significant increase in the resistance after film formation. Electrochemical data were fitted to the electrochemical circuit (inset top left) consisting of an ohmic resistance, $R_s$, of the electrolyte solution, the electronic charge transfer resistance, $R_{CT}$, in parallel with the constant phase element, CPE, and in parallel with the finite length Warburg constant, $W$. The $R_s$ reflects the resistance between amyloid-modified gold electrode and the reference Ag/AgCl electrode. CPE value is associated with the interface between the assembled peptide film and the electrolyte solution. In line with expectations for film formation on gold surfaces, $R_{CT}$ dramatically increases following the immobilization of the Aβ film. Our results showed that Aβ₁₂₋₂₈-Cys forms tightly packed films on gold surfaces after 3 days of incubation.

In order to probe the interactions between the Aβ film and Fe-conjugates as depicted in Figure 5.3c, the Aβ₁₂₋₂₈-Cys films were incubated in 5 mM solutions of Fe-conjugates 5.3-5.6 (CH₃CN/tris buffer, pH 8.5) for 1 day, followed by stringent washing to remove any physisorbed Fe-peptides. Background-subtracted cyclic voltammograms of Aβ₁₂₋₂₈-Cys films on gold surfaces after incubation with conjugates 5.3-5.6 are presented in Figure 5.4a and b and clearly display the strong redox signals for Fe-peptide 5.4 and 5.6 (for original CV data see Figure D.15, Appendix D). The film of Aβ₁₂₋₂₈-Cys/5.4 (Figure 5.4a) exhibits a formal potential, $E^0 = 439$ (5) mV vs Ag/AgCl, a potential difference $\Delta E = 130$ (5) mV and a peak current ratio $i_{pa}/i_{pc}$ close to unity. In addition, the Aβ₁₂₋₂₈-Cys/5.6 film (Figure 5.4b) exhibited a significant electrochemical response characterized by $E^0 = 448$ (5) mV, $\Delta E = 40$ (5) mV and a peak current ratio $i_{pa}/i_{pc} \sim 0.9$. Upon electrochemical cycling in the 0.2-0.6 V potential range vs Ag/AgCl, the electrochemical signal observed for the film in the presence of conjugates 5.4 and 5.6 improves with cycling.
suggesting reorganization of the films due to the specific interactions (Figure D.16 and D.17, Appendix D).

**Figure 5.3** (a) Cyclic voltammograms of bare gold electrode (-) and gold electrode modified with Aβ_{12-28}-Cys amyloid film (–––). (b) Electrochemical impedance spectra of gold electrode modified with Aβ_{12-28}-Cys amyloid film. Inset (top right) represents the Nyquist plot (−Z_{im} vs Z_{re}) of impedance spectra of bare electrode. Inset (top left) depicts the equivalent circuit used for fitting the measured data shown as symbols to the proposed circuit solid lines. Pt wire auxiliary, Ag/AgCl reference and Aβ_{12-28}-Cys modified gold electrode as working electrode in [Fe(CN)$_6$]$^{3-}/^{4-}$. (c) Illustration of the surface electrochemical approach used for probing interactions of Aβ$_{12-28}$-Cys amyloid film (left) with compounds 5.3-5.6 (right).
From the variation of the peak potentials with the scan rates, according to the Laviron equation, we estimated the heterogeneous electron transfer rate constants, $k_s$, to be in the range of 0.06 – 0.59 s$^{-1}$ (see Appendix D, Figure D.20). The background-subtracted square-wave voltammograms (SWVs) of the Aβ$_{12-28}$-Cys/5.4 and Aβ$_{12-28}$-Cys/5.6 films are presented in the Figure 5.4c and d, respectively. The current density reproducibility determined from the SWVs was in the 60 - 90 % range for Aβ$_{12-28}$-Cys/5.4 and Aβ$_{12-28}$-Cys/5.6 films (see Appendix D, Figure D.21).

![Figure 5.4](image)

**Figure 5.4** (a) Background-subtracted cyclic voltammograms of Aβ$_{12-28}$-Cys films on gold surfaces after the incubation with Fc-conjugates 5.3 (· · ·) and 5.4 (−). (b) Background-subtracted cyclic voltammograms of Aβ$_{12-28}$-Cys films on gold surfaces after the incubation with Fc-conjugates 5.5 (· · ·) and 5.6 (−). Significant current density was observed for free acid ferrocene conjugates 5.4 and 5.6. (c) Background-subtracted square-wave voltammograms of Aβ$_{12-28}$-Cys films on gold surfaces after the incubation with Fc-conjugates 5.3 (· · ·) and 5.4 (−). (d) Background-subtracted square-wave voltammograms of Aβ$_{12-28}$-Cys films on gold surfaces after the incubation with Fc-conjugates 5.5 (· · ·) and 5.6 (−). Significant redox signals were observed for 5.4 and 5.6 at 440 and 464 mV, respectively. Pt wire auxiliary, Ag/AgCl reference and Aβ$_{12-28}$-Cys modified gold electrode as working electrode in 2 mM NaClO$_4$. 
The slight distortions in the SWVs are due to the background-subtraction model used to generate the curves. Negligible current responses were observed for Aβ_{12-28}-Cys films in the presence of 5.3 and 5.5. In the case of compounds 5.4 and 5.6, the interaction between these compounds and Aβ_{12-28}-Cys is presumably supported by strong electrostatic interactions between the carboxylate groups at the C-terminal of conjugates and positively charged residues of Aβ_{12-28}-Cys. This electrostatic force in combination with hydrophobic interaction results in strong interactions between Fc-peptides 5.4 and 5.6 with films. The lack of charged groups in 5.3 and 5.5 leads to a relatively weak binding to Aβ_{12-28}-Cys films and in turn produces negligible signal. No redox signal was observed for Aβ films in the presence of ferrocene and unrelated ferrocene amide conjugates.

The surface electrochemistry results agree with the data in solution discussed earlier (vide supra), which indicated a stronger interaction of the free acid derivatives 5.4 and 5.6 with Aβ_{12-28}. In solution, this interaction affects fibrillization of Aβ_{12-28}, while on surfaces, it results in a measurable electrochemical response. Solution and surface results for the methyl esters 5.3 and 5.5 indicate some interactions with Aβ_{12-28}-Cys, but at a weaker level compared to that of the free acids 5.4 and 5.6.

We can speculate that ionic interactions involving the basic amino acids of Aβ_{12-28} and the carboxylate of the Fc-peptide conjugates 5.4 and 5.6, dominates the surface interactions. Presumably, in Aβ_{12-28}-Cys films, the basic residues are readily accessible from the solution.

**5.3 Experimental**

**5.3.1 General Remarks**

All syntheses were carried out in air unless otherwise indicated. Dichloromethane (CH₂Cl₂) used for synthesis was distilled and dried (CaH₂) prior to use. The coupling agents used for amide bond formation, 1-hydroxybenzotriazole hydrate (HOBt) and O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (Advanced ChemTech) were used as received. All amino acids used were purchased from Advanced ChemTech. Aβ_{12-28} for solution studies and Aβ_{12-28}-Cys for surface studies were purchased from American peptide and Anaspec,
respectively. All other chemicals were used as received and purchased from Sigma-Aldrich. Fe-carboxylic acid\[^{41}\] and Fe-CO-Lys(Boc)-OMe\[^{42}\] was synthesized as reported previously.

5.3.1.1 **NMR Spectroscopy**

The NMR spectra were recorded on a Varian Mercury 400 spectrometer operating at 400.1 MHz for \(^1\)H NMR and 100.6 MHz for \(^13\)C NMR. Chemical shifts (δ) were reported in ppm for both \(^1\)H NMR and \(^13\)C NMR spectra in relation to tetramethylsilane (TMS, δ = 0). All \(^1\)H NMR and \(^13\)C NMR spectra are referenced to non-deutero impurity in DMSO-d\(_6\) (signals at δ = 2.50 and δ = 39.50, respectively).

5.3.1.2 **Mass Spectrometry**

MS measurements were carried out on Finnigan MAT 8400 time-of-flight mass spectrometry (TOF–MS) using electrospray soft chemical ionization (HR-ESI).

5.3.1.3 **IR Spectroscopy**

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on Nicolet Impact 400 FT-IR spectrophotometer (Nicolet Analytical Instrument) at a 2.0 cm\(^{-1}\) resolution, 21 ± 1 °C. One thousand scans were averaged for data recorded from 400 to 4,000 cm\(^{-1}\).

5.3.1.4 **CD Spectroscopy**

The CD spectra are an average of eight accumulations at 23 ± 1 °C in CH\(_3\)CN or mixture of 50% CH\(_3\)CN/tris buffer (pH 8.5). The spectra were further smoothed using means-movement algorithm with convolution width of 25-point supplied with the JASCO software. Molar ellipticity (M\(_{\theta}\), deg cm\(^2\) mol\(^{-1}\)) calculated using M\(_{\theta}\) = θ\(_{obs}\)/10lc, where θ\(_{obs}\) is ellipticity measured in millidegrees, c is the sample concentration, l is the optical path length of the quartz cuvette in centimeters (0.1 cm). The solvent background is subtracted.
5.3.1.5 Transmission Electron Microscopy

Samples (20 µL) were spotted onto carbon-coated nickel grids and left for 2 min. The grids were then blotted with filter paper to remove excess buffer, and the sample was stained with 2% Uranyl acetate. Grids were blotted again and air-dried before analysis on a transmission electron microscope (Phillips CM10 microscope) operating at a voltage of 80 kV.

5.3.2 Sample Preparation for Fibril Formation

The powdered Aβ_{12-28} peptide was first dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg mL^{-1}. The solution was shaken at 4 °C for 2 h in a sealed vial for further dissolution and was then stored at -20.0 °C as a stock solution. Before use, the solvent HFIP was removed by evaporation under a gentle stream of nitrogen, and the peptide was dissolved in mixture of CH₃CN/tris buffer (pH 8.5). 50 µM Aβ_{12-28} solutions in the presence of 100 µM of Fc-conjugates were aged at room temperature for 7 days. As a control 50 µM Aβ_{12-28} solution was also aged in the absence of Fc-conjugates.

5.3.3 Electrochemical Studies

Electrochemical experiments were performed on CH Instrument potentiostat 660B (Austin, TX). Solution studies of 0.1 mM compounds were performed in the presence of 0.1 M tetrabutylammonium perchlorate in CH₃CN or CH₃OH. Glassy carbon electrode (CH Instruments Inc.) with the 0.02 cm² surface area was used as the working electrode, Pt wire as the auxiliary electrode and Ag/AgCl/3M KCl as the reference electrode.

5.3.3.1 Preparation of Aβ-Modified Gold Electrode

Gold disk electrodes (CH Instruments Inc.) of 0.02 cm² surface area were used as working electrodes. The gold electrodes were cleaned firstly in piranha (3:1 %v H₂SO₄: H₂O₂) then rinsed with milliQ water. Next, electrodes were hand polished over 0.05 µm alumina slurry and sonicated in milliQ water. Electrochemical cleaning was performed by linear sweep in 0 to -1.5
V potential range in 0.5 M KOH solution. Final electrochemical cleaning was done in 0.5 M H₂SO₄ by cycling in 0 to 1.5 V range until the stable redox potential was observed. The gold electrodes were then sonicated for 20 minutes in freshly distilled ethanol. Clean gold electrodes were then incubated in 50 µM Aβ₁₂₋₂₈ peptide (tris buffer pH 8.5) for 3 days at 5 °C. Electrodes were rinsed with buffer and then incubated in 5 mM solutions of compounds 5.3 and 5.5 (CH₃CN: tris buffer 75:25 %v/v) and compounds 5.4 and 5.6 (CH₃CN: tris buffer 50:50 %v/v) for 1 day at 5 °C. Subsequently, the electrodes were rinsed with CH₃CN and H₂O prior to electrochemical measurements.

5.3.3.2 Electrochemical Surface Studies

Surface electrochemistry was performed using a three electrode cell: amyloid-modified electrode as working electrode, Pt wire as auxiliary electrode and Ag/AgCl as reference electrode. All measurements were performed in 2 mM NaClO₄. Cyclic voltammetry was performed at 100 mV s⁻¹ and in 0.2-0.7 V range. Square-wave voltammetry was performed in the 0.2-0.7 V potential range, 15 Hz frequency and 0.025 V amplitude. All electrochemical impedance spectroscopy experiments were conducted in the frequency range of 100 kHz to 0.1 Hz and with AC amplitude of 5 mV. The measured EI spectra were fitted with the help of equivalent circuit using ZSimpWin 3.22 (Princeton Applied Research) and the data were presented in Nyquist plots.

5.3.4 Synthesis of Ferrocene-Peptide Conjugates 5.3-5.6

The Boc-Leu-Val-Phe-Phe-OMe was synthesized as reported before using HBTU and HOBt as coupling reagents.[28] The tetrapeptide was then coupled to Fc-carboxylic acid or Fc-CO-Lys(Boc)-OH using HBTU and HOBt. The products were purified by flash column chromatography in a CHCl₃-CH₃OH solvent mixture to give compounds 5.3 and 5.5. Complete hydrolysis of the methyl ester moieties in compounds 5.3 and 5.5 was performed by reacting NaOH (10 equiv.) in H2O/CH₃OH and results in the formation of compounds 5.4 and 5.6, respectively. All the compounds were characterized by mass spectrometry, ¹H, ¹³C NMR and IR (see Appendix D).
5.4 Conclusions

In conclusion, we demonstrated that Fc-CO-(Lys)-Leu-Val-Phe-Phe peptides disrupt Aβ aggregation in solution. Clear differences in the ability to interact with Aβ were observed between Fc-peptide methyl esters and the corresponding free acids, underlining the importance of the balance between ionic and hydrophobic forces. Our electrochemical studies of Aβ surfaces represent a complementary platform to study Aβ interactions with these conjugates, and demonstrate the utility of the electrochemical methods for probing biomolecular interactions involved in amyloid research. Importantly, our system provides a “signal-on” response in case of a favourable interaction between the Fc-peptide probe and the Aβ surface. Currently, the electrochemical surface approach is being optimized for screening of Aβ inhibitor candidates and may help in identifying potential therapeutic agents for Alzheimer’s disease.

5.5 Supporting Information

Supporting information for this chapter is provided in Appendix D.

5.6 References


Chapter 6
Conclusions and Future Works

In the work described in this thesis, new Fc-peptide conjugates possessing hydrophobic peptides were designed and their self-assembly properties were explored. The following chapter provides a short summary of some of the major results and insights and gives an outlook on future research.

6.1 Summary and Conclusions

Chapter 2 focused on the effect of C-terminal modifications on the molecular and supramolecular structures of Fc-peptides. The profound influence of C-terminal modification on the intermolecular H-bonding interactions of Fc-peptides was demonstrated through comparing a series of Fc-peptide ester conjugates and their corresponding carboxylates. The presence of a carboxylate group directed association into a porous structure containing hydrophobic channels filled with solvents. In contrast, self-assembly of Fc-peptide ester conjugates led to the formation of a common β-sheet structure. In addition, it was noted that the intramolecular H-bonding pattern did not change upon C-terminal modification. The “Herrick” motif, creating a rigid 1,2’-Fc-peptide core through cross-strand H-bonding, is the most stable conformation in both cases. This study highlights the important role of terminal modifications in the self-assembly process of Fc-peptide conjugates and suggests a potential route to control the self-association of these conjugates at the supramolecular level.

Next, hierarchical self-assembly of Fc-dicarboxylic peptide conjugates containing the peptide Gly-Val-Phe and Gly-Val-Phe-Phe was studied. Results in Chapter 3 showed that the molecules associate into larger structures through H-bonding, which continue to associate into features that can be monitored by microscopy and thus provide a transition from the molecular to the macroscopic level. The presence of helical chirality in the assemblies was observed by CD spectroscopy. SEM measurements indicated the formation of a remarkable range of morphologies including helical ropes, crystalline cuboids and network of nano fibers depending
on the nature of peptide motif and solvent compositions. Further studies demonstrated that these assemblies were formed from β-sheet structures. The present findings may contribute to the understanding of the hierarchical structural arrangement in Fc-peptide conjugates having potential applications in the designing functional biomaterials.

The ability of Fc-peptides to engage in intermolecular H-bonding interactions and their redox activity guided us to evaluate peptide interactions by electrochemical methods in greater detail; these results were described in Chapter 4. In this study, a peptide film composed of Aβ12-28 was immobilized on gold surfaces via a C-terminal Cys and its interaction with a beta-sheet breaker peptide (BSB) and Congo red (CR), a non-peptide based breaker, were studied in solution. Results showed interactions between the surface bound Aβ12-28 and small molecules in solution led to a change in the electrochemical signal that was indicative of specific interactions. It was demonstrated that BSB incorporated into the peptide film on the surface through H-bonding and hydrophobic interactions and made the peptide film more compact. In contrast, the interaction of CR with peptide film disrupted the H-bonding contacts between the peptide strands and made the peptide film more porous. This work highlights the utility of surface-based electrochemical methods to evaluate Aβ interactions.

In Chapter 5, we expanded the surface-based approach to include a study of the interactions of Fc-peptide conjugates with Aβ12-28 films on gold surfaces. We took advantage of the self-recognition ability of the (Lys)Leu-Val-Phe-Phe peptide sequence and redox activity of the Fc group to monitor peptide interactions using electrochemical techniques. A redox signal was observed in the case of Fc-peptide acid after interaction with Aβ fragment on the surface. It was noted that C-terminal deprotection of Fc-peptide ester conjugates improved the interaction with Aβ on the surface. Solution studies provided complementary information about the importance of hydrophobic and ionic forces for the interactions between Aβ12-28 and Fc-peptides.

### 6.2 Proposed Future Work

The work described in this thesis gives rise to a number of questions that should be addressed in the future. These include issues related to investigating ferrocene amino acids and of ferrocene diamine peptide derivatives and their self-assembly properties in greater detail. To date there has
been no detailed study on the factors driving self-assembly or even hierarchical assembly of such systems. The synthesis of such conjugates is relatively simple and there are some examples of such conjugates described in the literature.\textsuperscript{1, 2} This work may include N- and/or C-terminal modification and/or a detailed investigation into the influence of a particular amino acid on the assembly. A schematic view of Fe amino acid and Fe diamine peptide conjugates is provided in Scheme 6.1.

\textbf{Scheme 6.1} Proposed Fe-diamine (a) and Fe amino acid peptide conjugates (b, c).

Such a study should include a detailed evaluation of the environmental factors that contribute to the assembly process. Work on the hierarchical assembly described in Chapter 3 has demonstrated the morphology of Fe-peptide conjugates can be influenced. While initial DOSY-NMR studies suggested that no large discrete assemblies are formed in solution at a concentration of 10 mM, higher concentrations might allow the observation of such discrete species. Dynamic light scattering should provide insight into the presence of assemblies and also about the size of such supramolecular structures.\textsuperscript{3-5}

Lastly, Chapters 4 and 5 demonstrated the use of electrochemical techniques to monitor the interaction of small molecules with surface-bound peptides. In the context of monitoring Aβ interactions, future efforts may explore Fe-peptide conjugates containing polar amino acids as well as hydrophobic amino acids. The presence of polar amino acids may increase their overall solubility in aqueous solution, while maintaining their inhibitory potential. Previous studies showed that peptides with polar amino acids such as Glu and Arg, in addition to the hydrophobic core of Aβ, could disrupt Aβ aggregation.\textsuperscript{6}
6.3 References


Figure A.1 ORTEP diagram of conjugate 2.6 showing labeling of atoms. Hydrogen atoms are omitted for clarity and dashed lines represent H-bonds. Ellipsoids are drawn at the 30% probability level.
Figure A.1 ORTEP diagram of conjugate 2.1 (Fe 1) showing numbering of atoms (a), ORTEP diagram of conjugate 2.1 (Fe 2) showing numbering of atoms (b). Hydrogen atoms are omitted for clarity and dashed lines represent H-bonds. Ellipsoids are drawn at the 30% probability level.

Figure A.2 ORTEP diagram of conjugate 2.1 (Fe 1) showing numbering of atoms (a), ORTEP diagram of conjugate 2.1 (Fe 2) showing numbering of atoms (b). Hydrogen atoms are omitted for clarity and dashed lines represent H-bonds. Ellipsoids are drawn at the 30% probability level.
Figure A.3 Chemical shift (δ) versus temperature (T) plot of NH resonances for Fc[CO-Leu-Val-Phe-OMe]₂ (2.1).
Figure A.4 VT $^1$H NMR spectra showing amide region of Fc[CO-Val-Phe-OMe]$_2$ (2.2) (c = 10 mM, the temperature range 243-303 K) (a), chemical shift (δ) versus temperature (T) plot of NH resonances (b). * Denotes the residual CHCl$_3$. 
Figure A.5 VT $^1$H NMR spectra showing amide region of Fc[CO-Gly-Val-OMe]$_2$ (2.3) ($c = 10$ mM, the temperature range 243-303 K) (a), chemical shift ($\delta$) versus temperature (T) plot of NH resonances (b). * Denotes the residual CHCl$_3$. 
Figure A.6 VT $^1$H NMR spectra showing amide region of Fc[CO-Val-Phe-Phe-OMe]$_2$ (2.4) (c = 10 mM, the temperature range 243-303 K) (a), chemical shift (δ) versus temperature (T) plot of NH resonances (b).
Figure A.7 Concentration dependent $^1$H NMR spectra of Fc[CO-Val-Phe-Phe-OMe]$_2$ (2.4) showing amide region (concentration range 1-40 mM).

Figure A.8 $^1$H NMR spectrum of Fc[CO-Val-Phe-OMe]$_2$ (2.2) (5 mM) in CDCl$_3$. * Denotes the residual CHCl$_3$. 
Figure A.9 $^1$H NMR spectrum of Fc[CO-Gly-Val-OH]$_2$ (2.6) (5 mM) in DMSO-$d_6$. * Denotes the non-deutero impurity in DMSO-$d_6$. 
Figure A.10  $^{13}$C NMR spectrum of Fc[CO-Leu-Val-OMe]$_2$ (2.1) (45 mM) in CDCl$_3$. The spectrum shows signals for amide and ester carbonyl at $\delta$ 180-160. The characteristic set of signals belongs to Fc group has appeared at the chemical shift range $\delta$ 76-70. The alpha carbons appear at $\delta$ 58-52 and beta carbons at $\delta$ 32-24.
Figure A.11 $^{13}$C NMR spectrum of Fc[CO-Val-Phe-Phe-OMe]$_2$ (2.4) (45 mM) in CDCl$_3$. The spectrum shows signals for amide and ester carbonyl at $\delta$ 180-160. The characteristic set of signals belongs to Fc group has appeared at the chemical shift range $\delta$ 78-71. The alpha carbons appear at $\delta$ 60-53 and beta carbons at $\delta$ 39-30.
Figure A.12 $^{13}$C NMR spectrum of Fe[CO-Leu-Val-OH]$_2$ \textit{(2.5)} (45 mM) in DMSO-d$_6$. The spectrum shows signals for amide and acid carbonyl at $\delta$ 180-160. The characteristic set of signals belongs to Fc group has appeared at the chemical shift range $\delta$ 77-69.

A.1 X-ray Crystallographic Data of Compound \textit{2.6}

For compound \textit{2.6}, the SHELXTL/PC V6.14 for Windows NT (Sheldrick, G.M., 2001) suite of programs was used to solve the structure by direct methods. Subsequent difference Fourier syntheses allowed the remaining atoms to be located. Only half of the molecule was located and refined, the remainder was generated by symmetry. The molecules were linked by hydrogen bonding to form doughnuts consisting of 4 molecules. Further bonding linked these doughnuts together via H-bonding. The molecule was very well ordered but fitting the solvent was problematic. Ultimately it was decided to use “Squeeze” to address the problem. All of the non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atom positions were calculated geometrically and were included as riding on their respective carbon atoms.
The largest residue electron density peak (0.512 e/Å³) was associated with the Cp ring. Full-matrix least squares refinement on F² gave $R_1 = 8.75$ for 2$\sigma$ data and $wR_2 = 24.63$ for all data. The final solution was submitted to the IUCR checkCIF program and had three Alert level A’s associated with the solvent void and the fact that the Flack parameter was negative. Since SHELX demands a positive value the parameter shift to standard uncertainty ratio was high.

SQUEEZE reduced the model with one solvent from 15.0 % to 8.75 %. The area SQUEEZED was relatively large, 28.4% (1023.4 Å³ of a total volume of 3608.2 Å³). The total electron count of the voids in the cell is 148 e-. The unknown solvent is most likely hexane (50 e-), allowing approximately 3 molecules in the columnar void.

**Figure A.13** Electron density map of crystal packing of compound 2.6 before squeezing the structure. Green shows the electron density of solvents inside the hydrophobic voids. Win Coot (Crystallographic Object-Oriented Toolkit) 0.6.1 was used to draw above picture using unsqueezed files.
Appendix B: Supporting Information for Chapter 3

Figure B.1 $^1$H NMR spectrum of Fe[CO-Gly-Val-Phe-OMe]$_2$ (3.2) in CDCl$_3$ (*Denotes for CHCl$_3$ residue).
**Figure B.2** $^{13}$C NMR spectrum of Fe[CO-Gly-Val-Phe-OMe]$_2$ (3.2) in CDCl$_3$. 
Figure B.3 g COSY NMR spectrum of Fe[CO-Gly-Val-Phe-OMe]$_2$ (3,2) in CDCl$_3$. 
Figure B.4 $^1$H NMR spectrum of Fc[CO-Gly-Val-Phe-Phe-OMe]$_2$ (3.3) in CDCl$_3$ (*Denotes for CHCl$_3$ residue).

Figure B.5 $^{13}$C NMR spectrum of Fc[CO-Gly-Val-Phe-Phe-OMe]$_2$ (3.3) in CDCl$_3$. 
Figure B.6 g COSY NMR spectrum of Fe[CO-Gly-Val-Phe-Phe-OMe]₂ (3.3) in CDCl₃.
Figure B.7 Variable temperature $^1$H NMR spectra of compound 3.2 showing amide region (c = 10 mM, the temperature range 253-303 K) (A). The chemical shift of all amide protons NH$_{\text{Gly}}$ (a), NH$_{\text{Phe}}$ (b) and NH$_{\text{Val}}$ (c) shifted to upfield upon increasing the temperature. Plot of chemical shift versus temperature for amide protons of compound 3.2 (B). The temperature coefficients were calculated from the slope of the plot (-2.0 ppb K$^{-1}$ for NH$_{\text{Gly}}$ (a), -3.0 ppb K$^{-1}$ for NH$_{\text{Phe}}$ (b) and -4.0 ppb K$^{-1}$ for NH$_{\text{Val}}$ (c)).
Figure B.8 Concentration dependent $^1$H NMR spectra of compound 3.2 showing amide region ($c = 6\text{–}20\text{ mM}$) (A). The chemical shifts of NH$_{Gly}$ (a) and NH$_{Phe}$ (b) did not change by varying the concentration. In contrast, NH$_{Val}$ (c) shifted to upfield upon decreasing the concentration. Plot of chemical shift versus concentration for NH$_{Val}$ (c) (B).
Figure B.9 Variable temperature $^1$H NMR spectra of compound 3.3 showing amide region (c = 10 mM, the temperature range 253-303 K) (A). The chemical shift of all amide protons NH$_{Gly}$ (a), NH$_{Phe}$ (b), NH$_{Val}$ (c) and NH$_{Phe}$ (d) shifted to upfield upon increasing the temperature. Plot of chemical shift versus temperature for amide protons (B). The temperature coefficients were calculated from the slope of the plot (-4.1 ppb K$^{-1}$ for NH$_{Gly}$ (a), -3.1 ppb K$^{-1}$ for NH$_{Phe}$ (b), -4.3 ppb K$^{-1}$ for NH$_{Val}$ (c) and -1.6 ppb K$^{-1}$ for NH$_{Phe}$ (d)). Significant Broadening of the amide peaks was observed below 273 K.
Figure B.10 Concentration dependent $^1$H NMR spectra of compound 3.3 showing amide region (c = 6-20 mM) (A). The chemical shifts of NH$_\text{Gly}$ (a) and NH$_\text{Phe}$ (b) did not change by varying the concentration. In contrast, NH$_\text{Val}$ (c) and NH$_\text{Phe}$ (d) shifted to upfield upon decreasing the concentration. Plot of chemical shift versus concentration for NH$_\text{Val}$ (c) and NH$_\text{Phe}$ (d) (B).
The diffusion coefficients determined for each peak were used to calculate the average diffusion coefficient ($D_{AVG}$).

**Figure B.11** Partial DOSY-NMR spectrum of compound 3.2 in DMSO-$d_6$. (A) Experimental diffusion coefficients determined for each peak were used to calculate the average diffusion coefficient ($D_{AVG}$). (B) 2D representation of the partial DOSY-NMR for 3.2 in DMSO-$d_6$. 

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$D_{AVG} = 1.31 \pm 0.079$
Figure B.12 Partial DOSY-NMR spectrum of 3.2 in CDCl$_3$. (A) Experimental diffusion coefficients determined for each peak were used to calculate the average diffusion coefficient ($D_{\text{avg}}$). (B) 2D representation of the partial DOSY-NMR for 3.2 in CDCl$_3$. 

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$D_{\text{avg}} = 1.31 \pm 0.079$
Figure B.13 Partial DOSY-NMR spectrum of 3.3 in DMSO-d$_6$. (A) Experimental diffusion coefficients determined for each peak was used to calculate the average diffusion coefficient ($D_{avg}$). (B) 2D representation of the partial DOSY-NMR for 3.3 in DMSO-d$_6$. 
Figure B.14 Partial DOSY-NMR spectrum of 3.3 in CDCl$_3$. (A) Experimental diffusion coefficients determined for each peak was used to calculate the average diffusion coefficient ($D_{\text{avg}}$). (B) 2D representation of the partial DOSY-NMR for 3.3 in CDCl$_3$.

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Figure B.15 CD spectra of 3.2 (-) and 3.3 (---) in CHCl₃ (c = 1mM). (A) CD spectra of 3.2 (-) and 3.3 (---) in the range of 300-600 nm, (B) CD spectra of 3.2 (-) and 3.3 (---) in the peptide region (230-300nm).

Figure B.16 Attenuated total reflectance (ATR) FT-IR spectra of assemblies of compound 3.2 obtained from chloroform/hexane in the amide I region (A). The presence of two signals at 1632 and 1675 cm⁻¹ are characteristic of a β-sheet like structure. The XRD pattern of assemblies of compound 3.2 obtained from chloroform/hexane (B). The presence of two intense peaks at 0.47 nm and 0.94 nm reveal the formation of β-sheet like structure.
Table B.1 Experimental XRD data of assemblies of compound 3.2 obtained from chloroform/hexane

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<td>14.45</td>
<td>6.12</td>
<td>15.6</td>
<td>31</td>
<td>24.57</td>
<td>3.62</td>
<td>22.5</td>
</tr>
<tr>
<td>15</td>
<td>15.17</td>
<td>5.84</td>
<td>23.2</td>
<td>32</td>
<td>25.65</td>
<td>3.47</td>
<td>15.9</td>
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<tr>
<td>16</td>
<td>15.49</td>
<td>5.72</td>
<td>49</td>
<td>33</td>
<td>26.69</td>
<td>3.34</td>
<td>15</td>
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<tr>
<td>17</td>
<td>15.67</td>
<td>5.65</td>
<td>51.7</td>
<td>34</td>
<td>28.41</td>
<td>3.14</td>
<td>59.3</td>
</tr>
</tbody>
</table>

Figure B.17 Simulated XRD of compound 3.2 calculated from single X-ray crystallography data
Figure B.18 Attenuated total reflectance (ATR) FT-IR spectra of assemblies of compound 3.3 obtained from chloroform/hexane (A). A strong peak at 1636 cm$^{-1}$ and a weak peak at 1683 cm$^{-1}$ are characteristic of anti-parallel β-sheet structure. The XRD pattern of assemblies of compound 3.3 obtained from chloroform/hexane in wide and small angle range (B).

Table B.2 Experimental XRD data of assemblies of compound 3.3 obtained from chloroform/hexane

<table>
<thead>
<tr>
<th>No</th>
<th>2θ°</th>
<th>d, Å</th>
<th>I/I₀, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.81</td>
<td>23.16</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>5.4</td>
<td>16.34</td>
<td>34.8</td>
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<tr>
<td>3</td>
<td>6.97</td>
<td>12.67</td>
<td>90.2</td>
</tr>
<tr>
<td>4</td>
<td>7.91</td>
<td>11.17</td>
<td>56.1</td>
</tr>
<tr>
<td>5</td>
<td>10.6</td>
<td>8.34</td>
<td>41.2</td>
</tr>
<tr>
<td>6</td>
<td>14.7</td>
<td>6.02</td>
<td>35.9</td>
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<tr>
<td>7</td>
<td>18.98</td>
<td>4.67</td>
<td>33</td>
</tr>
</tbody>
</table>
**Figure B.19** SEM images of assemblies of compound 3.2 in the mixture of chloroform/hexane. Scale bar is 2µm.

**Figure B.20** SEM images of assemblies of compound 3.2 in the mixture of dichloromethane/hexane. Scale bar is 2µm.
Figure B.21 SEM images of assemblies of compound 3.3 in the mixture of chloroform/hexane. Scale bar is 1µm.

Figure B.22 SEM images of assemblies of compound 3.3 in the mixture of dichloromethane/hexane. Scale bar is 1µm.
Table B.3 Electrochemical data of compounds 3.2 and 3.3 (0.1 mM in 0.1 M TBAP in CHCl₃). $E_{1/2}$, $\Delta E$ in mV and diffusion coefficient, $D$, in $\times 10^{-4}$ cm$^2$ sec$^{-1}$. Glassy carbon working electrode, Ag/AgCl reference, Pt wire is auxiliary.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_{1/2}$</th>
<th>$\Delta E$</th>
<th>$i_c/i_a$</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>818 ± 1</td>
<td>68 ± 6</td>
<td>0.97 ± 0.05</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>3.3</td>
<td>818 ± 1</td>
<td>72 ± 5</td>
<td>0.92 ± 0.06</td>
<td>1.0 ± 0.6</td>
</tr>
</tbody>
</table>

Figure B.23 A) Cyclic voltammogram of the thin film of compound 3.2 on carbon electrode (solution of compound 3.2 in CHCl₃ was deposited on glassy carbon electrodes). B) Change in the potential estimated from the square wave voltammograms for different deposits on glassy carbon electrodes as a function of solvent compositions ($\Delta$potential was obtained by subtracting the potential values observed for chlorinated solvent/ hexane mixtures from those in chlorinated solvent only): (a) compound 3.2 (CHCl₃) (b) compound 3.2 in CH₂Cl₂ (CH₂Cl₂) (c) compound 3.3 (CHCl₃) (d) compound 3.3 (CH₂Cl₂) (Ag/AgCl reference, Pt wire is auxiliary, 3.3 M NaClO₄ as electrolyte).
Table B.4 Electrochemical parameters estimated from cyclic voltammograms for bulk material obtained for compounds 3.2 and 3.3 as deposited on glassy carbon electrode. Solvent composition represents a specific chlorinated solvent or chlorinated solvent/ hexane mixture. (Ag/AgCl reference, Pt wire is auxiliary, 2 M NaClO₄).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Compound 3.2 ((\text{CHCl}_3/\text{CH}_2\text{Cl}_2))</th>
<th>Compound 3.2 ((\text{CHCl}_3/\text{CH}_2\text{Cl}_2)^a)</th>
<th>Compound 3.3 ((\text{CHCl}_3/\text{CH}_2\text{Cl}_2))</th>
<th>Compound 3.3 ((\text{CHCl}_3/\text{CH}_2\text{Cl}_2)^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E°</td>
<td>660 ± 3 / 648 ± 2</td>
<td>655 ± 3 / 657 ± 4</td>
<td>668 ± 5 / 663 ± 8</td>
<td>655 ± 8 / 670 ± 8</td>
</tr>
<tr>
<td>ΔE</td>
<td>102 ± 8 / 86 ± 5</td>
<td>73 ± 8 / 67 ± 5</td>
<td>61 ± 3 / 60 ± 5</td>
<td>89 ± 12 / 74 ± 1</td>
</tr>
<tr>
<td>(i_a/i_c)</td>
<td>0.91 ± 0.03 /</td>
<td>0.99 ± 0.17 / 1.06</td>
<td>0.98 ± 0.11 /</td>
<td>0.98 ± 0.03 /</td>
</tr>
<tr>
<td></td>
<td>0.97 ± 0.04</td>
<td>± 0.04</td>
<td>0.64 ± 0.09</td>
<td>0.68 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\)Hexane was added to each solvent
<table>
<thead>
<tr>
<th></th>
<th>3.2</th>
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<tr>
<td>Identification code</td>
<td>B11024</td>
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<tr>
<td>Empirical formula</td>
<td>C\textsubscript{53}H\textsubscript{57}Cl\textsubscript{3}FeN\textsubscript{6}O\textsubscript{10}</td>
</tr>
<tr>
<td>Formula weight</td>
<td>1100.25</td>
</tr>
<tr>
<td>( T / K )</td>
<td>150(2) K</td>
</tr>
<tr>
<td>( \lambda / \AA )</td>
<td>0.71073 \AA</td>
</tr>
<tr>
<td>Crystal system</td>
<td>orthorhombic</td>
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<tr>
<td>Space group</td>
<td>P 2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}</td>
</tr>
<tr>
<td>( a / \AA )</td>
<td>16.6723(18)</td>
</tr>
<tr>
<td>( b / \AA )</td>
<td>16.6720</td>
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<tr>
<td>( c / \AA )</td>
<td>19.214(2)</td>
</tr>
<tr>
<td>( \alpha / \degree )</td>
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</tr>
<tr>
<td>( \beta / \degree )</td>
<td>90</td>
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<tr>
<td>( \gamma / \degree )</td>
<td>90</td>
</tr>
<tr>
<td>( V / \AA^3 )</td>
<td>5340.7(8)</td>
</tr>
<tr>
<td>( Z )</td>
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</tr>
<tr>
<td>( D_{\text{calc}} / \text{g cm}^{-3} )</td>
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</tr>
<tr>
<td>Absorption coefficient / mm(^{-1} )</td>
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</tr>
<tr>
<td>( F(000) )</td>
<td>2296</td>
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<tr>
<td>Crystal size / mm</td>
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<td>( \theta ) range for data collection / \degree</td>
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<tr>
<td></td>
<td>-24 &lt; \textit{k} &lt; 24</td>
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<tr>
<td></td>
<td>-28 &lt; \textit{l} &lt; 28</td>
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<td>Independent reflections</td>
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<tr>
<td>Max. / Min. transmission</td>
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<td>Refinement method</td>
<td>Full-matrix least squares on ( F^2 )</td>
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<tr>
<td>Data / restraints / parameters</td>
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<tr>
<td>Goodness-of-fit on ( F^2 )</td>
<td>1.105</td>
</tr>
<tr>
<td>Final ( R ) indices [I &gt; 2\sigma (I)]</td>
<td>( R_1 = 0.0783, \text{ wR}2 = 0.1973 )</td>
</tr>
<tr>
<td>( R ) indices (all data)</td>
<td>( R_1 = 0.1478, \text{ wR}2 = 0.2333 )</td>
</tr>
<tr>
<td>Largest diffraction peak and hole</td>
<td>0.865 and -1.112 e\textsubscript{A}^{-3}</td>
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</table>
Appendix C: Supporting Information for Chapter 4

Figure C.1 The influence of incubation time on cyclic voltammograms of a gold electrode incubated with 50 µM $\alpha$β$_{12-28}$-Cys. Cyclic voltammograms were run in a 5 mM $[\text{Fe(CN)}_6]^{3-/4-}$ (1:1) solution in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4) at scan rate of 0.1 V s$^{-1}$. The voltage range was -0.1 to 0.6V.

Figure C.2 The influence of $\alpha$β$_{12-28}$-Cys concentration on cyclic voltammogram responses after 72 h incubation. CVs were run in 5 mM $[\text{Fe(CN)}_6]^{3-/4-}$ (1:1) solution in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4) at scan rate of 0.1 V s$^{-1}$. The voltage range was -0.1 to 0.6 V.
Figure C.3 Plot of current intensity vs time for a gold electrode incubated with 50 µM Aβ_{12-28}-Cys in a 5 mM [Fe(CN)_6]^{3-/4-} (1:1) solution prepared in phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4) at scan rate of 0.1V s^{-1}. All potentials are given vs. Ag/AgCl reference electrode.

Figure C.4 Plot of current intensity vs concentration of Aβ_{12-28}-Cys for a gold electrode incubated with Aβ_{12-28}-Cys after 72 h incubation. CV runs in a 5 mM [Fe(CN)_6]^{3-/4-} (1:1) solution prepared in phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4) at scan rate of 0.1V s^{-1}. All potentials are given vs. Ag/AgCl reference electrode.
Figure C.5 Cyclic voltammograms of Aβ_{12-28}-Cys peptide film alone (a), after 30 min interaction with (b) 5 mM β-sheet breaker (BSB) and (c) 5 mM Congo red (CR). The solution composition was 5 mM [Fe(CN)_6]^{3+/4-} (1:1) in phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4) at scan rate of 0.1Vs^{-1}. All potentials are given vs. Ag/AgCl reference electrode.

Figure C.6 (a) Electrochemical impedance spectra of Aβ_{12-28}-Cys peptide film in 5 mM [Fe(CN)_6]^{3+/4-} (1:1) in phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4) after 1 h treatment with (●) 1, (▲)100, (■) 5000 and (+) 10000 μM solutions of Congo Red (CR). (b) (●) 100, (▲)1000, (■) 5000 and (+) 10000 μM solutions of β-sheet breaker (BSB). Measured data are shown as symbols with calculated fit to the equivalent circuit (Figure 4.3b) as solid lines. Impedance spectra obtained in phosphate buffer (50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4), containing 5 mM [Fe(CN)_6]^{3+/4-} (1:1) as a redox probe, at a formal potential of 250 mV vs Ag/AgCl, frequency range from 100 kHz to 0.1 Hz, and AC amplitude of 10 mV.
Figure C.7 FT-IR spectra of (a) 50 µM BSB and (b) CR, separately for 120 min in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4).

C.1 X-ray Photoelectron Spectroscopy Analyses

The XPS analyses were carried out with a Kratos AXIS Nova spectrometer using a monochromatic Al-K$_\alpha$ source (15mA, 14kV). XPS can detect all elements except hydrogen and helium, probes the surface of the sample to a depth of 5-7 nanometres, and has detection limits ranging from 0.1 to 0.5 atomic percent depending on the element. The instrument work function was calibrated to give a binding energy (BE) of 83.96 eV for the Au 4f$_{7/2}$ line for metallic gold. The Kratos charge neutralizer system was used on all specimens. Survey scan analyses were carried out with an analysis area of 300 x 700 microns and pass energy of 160 eV. High resolution analyses were carried out with an analysis area of 300×700 microns and pass energy of 20 eV. Spectra were analysed using CasaXPS software (version 2.3.14). The peptide film preparation was similar to those for FT-RAIRS.

X-ray photoelectron spectroscopy (XPS) was used to obtain detailed information about the chemical composition of Aβ$_{12-28}$-Cys peptide film and its interaction with BSB and CR.
The XPS peaks of S$_{2p}$ for A): Aβ$_{12-28}$-Cys peptide; (B): Aβ$_{12-28}$-Cys peptide with Congo red (CR); (C): Aβ$_{12-28}$-Cys peptide with β-sheet breaker (BSB) were fitted and deconvoluted to give the chemical shift data of the components within the coated molecules, respectively. As shown in Figure C.8 (A), two dominant peaks located at ~161.5 and ~162.9 eV with an area ratio of 2:1 and a peak separation of ~1.4 eV were observed in the S$_{2p}$ spectra, which could be assigned to the S atom bound on the gold surface$^{[1]}$. Moreover, two additional peaks are observed at lower binding energies in Figure C.8 (B), that is, at 167.4 and 168.7 eV for the S$_{2p3/2}$ and S$_{2p1/2}$ components, respectively. These peaks can be ascribed to the sulfonic acid groups (-SO$_3$-) compensated by the Congo red. This confirmed the Aβ$_{12-28}$-Cys peptide anchors at the gold surface. Figure C.8 (C) is similar to Figure C.8 (A), because there is no sulfur atom from β-sheet breaker.

**Figure C.8** High-resolution XPS spectra of S$_{2p}$ for (A) Aβ$_{12-28}$-Cys peptide; (B) Aβ$_{12-28}$-Cys peptide with Congo red (CR); (C) Aβ$_{12-28}$-Cys peptide with β-sheet breaker (BSB). Open circles stand for experimental raw data, red solid lines are for the total fits, black lines are for the component-fitted peaks, and green lines are for the baselines.
Figure C.9 shows the XPS spectra of C$_{1s}$ for (A): Aβ$_{12-28}$-Cys peptide; (B): Aβ$_{12-28}$-Cys peptide with Congo red (CR); (C): Aβ$_{12-28}$-Cys peptide with β-sheet breaker (BSB). We observed two peaks in the C$_{1s}$ spectra at binding energies of ~285.0 and ~288.5 eV.\cite{2} The C$_{1s}$ peak was divided into three peaks: highly antiscreened peptide N-C=O carbons and carboxyl groups of amino acid (a); C-N carbons of the peptide backbone (b); aliphatic C-C carbons (c). Following the condensation reaction between β-Amyloid and CR, the XPS spectra (Figure C.9 (B)) of the lower binding energy at 284.5 eV for aliphatic C-C carbons increased markedly after CR immobilization. Meanwhile, for Aβ$_{12-28}$-Cys peptide with β-sheet breaker system, we found the area of the higher binding energy at 288.0 eV for carboxyl groups become larger than Aβ$_{12-28}$-Cys peptide after BSB immobilization.

Figure C.9 High-resolution XPS spectra of C$_{1s}$ for (A) Aβ$_{12-28}$-Cys peptide; (B) Aβ$_{12-28}$-Cys peptide with Congo red (CR); (C) Aβ$_{12-28}$-Cys peptide with β-sheet breaker (BSB). Open circles stand for experimental raw data, red solid lines are for the total fits, black lines are for the component-fitted peaks, and green lines are for the baselines. The data of C$_{1s}$ are fit with three components: highly antiscreened peptide N-C=O carbons and carboxyl groups of amino acid (a), C-N carbons of the peptide backbone (b) and aliphatic C-C carbons (c).
The results from XPS spectra for N$_{1s}$ (Figure C.10) and O$_{1s}$ were not good to explain the change after CR/BSB modification because there are N and O elements in three compounds.

**Figure C.10** High-resolution XPS spectra of N$_{1s}$ for (A) Aβ$_{12-28}$-Cys; (B) Aβ$_{12-28}$-Cys peptide with Congo red (CR); (C) Aβ$_{12-28}$-Cys peptide with β-sheet breaker (BSB). Open circles stand for experimental raw data, red solid lines are for the total fits, and green lines are for the baselines.
Figure C.11 (a) Nyquist and (b-c) Both Bode plots of Aβ_{12-28}-Cys peptide film in 5 mM [Fe(CN)$_6$]$^{3-/4-}$ (1:1) in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4). Impedance spectra obtained in phosphate buffer (50 mM Na$_2$HPO$_4$, 50 mM KH$_2$PO$_4$, pH 7.4), containing 5 mM [Fe(CN)$_6$]$^{3-/4-}$ (1:1) as a redox probe, at a formal potential of 250 mV vs Ag/AgCl, frequency range from 100 kHz to 0.1 Hz, and AC amplitude of 10 mV.

Figure C.12 ThT fluorescence of preformed fibrils (4 days) (Aβ$_{12-28}$-Cys, 50 µM) and Aβ$_{12-28}$-Cys mixed with CR (1:1 molar ratio) immediately after addition of CR. Values represent means ± standard deviation (n = 3).
Figure C.13 ThT fluorescence of preformed fibrils (4 days) (Aβ\textsubscript{12-28}-Cys, 50 µM) (square line) and Aβ\textsubscript{12-28}-Cys mixed with BSB (1:1 molar ratio) (circle line). Values represent means ± standard deviation (n = 3).

C.2 References


Appendix D: Supporting Information for Chapter 5

D.1 Synthesis and Characterization

D.1.1 Synthesis of Fc-CO-Lys(Boc)-OH (5.2)

To a solution of FcVCOVLys(Boc)VOMe (0.47 g, 1mmol) in methanol (100 mL) was added dropwise NaOH (0.40 g, 10 mmol) at room temperature. The reaction mixture was stirred for 14 hours. Solvent was removed in vacuo and the brown solid residue was dissolved in water and washed with dichloromethane. Concentrated hydrochloric acid was added to aqueous solution at 0 °C and the precipitate was collected, washed with water and dried to give a brown solid (0.27 g, 60%). MS (HR-ESI): calcd for C_{22}H_{30}FeN_{2}O_{5} 481.1402; found 481.1385[M+Na]^+. \(^1\)H NMR (400 MHz, DMSO-d\(_6\)):\(\delta = 12.52\) (s, br, 1H, COOH), 7.83 (d, \(^3\)J\(_{HH}\) = 7.82 Hz, 1H, NH Lys), 6.80 (m, 1H, NH Boc), 4.90 (s, 1H, H\(_{cp}\)), 4.84 (s, 1H, H\(_{cp}\)), 4.35 (s, 2H, H\(_{cp}\)), 4.29 (m, 1H,\(\alpha\)-H), 4.20 (s, 5H, H\(_{cp}\)), 2.90 (s, 2H, CH\(_2\)), 1.75 (s, 2H, CH\(_2\)), 1.39 (s, 2H, CH\(_2\)), 1.35 (s, 11H, CH\(_3\) Boc+ CH\(_2\) Lys).

Figure D.1 \(^1\)H NMR spectrum of Fc-CO-Lys(Boc)-OH (5.2) in DMSO-d\(_6\).
D.1.2 General Procedure for the Synthesis of Fc-Peptides 5.3-5.6

To a stirring mixture of Fc-carboxylic acid (0.23 g, 1.0 mmol) or Fc-CO-Lys(Boc)-OH (0.45, 1 mmol), triethylamine (0.2 mL, 1.5 mmol) in dry CH₂Cl₂ (50 mL), HBTU (0.45 g, 1.2 mmol) and HOBt·H₂O (0.18 g, 1.2 mmol) were added. In a separate flask Boc-Leu-Val-Phe-Phe-OMe in dry CH₂Cl₂ were deprotected by treatment with TFA. The TFA and CH₂Cl₂ were removed in vacuo. The resulting residue was redissolved in CH₂Cl₂, cooled in an ice bath prior to the addition of triethylamine and is then added to the reaction mixture. After 14 hours, the reaction mixture was washed with subsequent aqueous solutions of saturated NaHCO₃, citric acid (10%), again saturated NaHCO₃ and finally distilled water. The organic phase was collected and traces of water were removed using anhydrous Na₂SO₄. After concentration of the solution under reduced pressure, the crude product was purified by flash column chromatography in a chloroform-methanol solvent mixture. The solvent were then removed completely under reduced pressure and orange solids were obtained. Complete hydrolysis of the methyl ester moieties was performed by reacting NaOH (10 equiv.) in H₂O-MeOH as described for Fc-CO-Lys(Boc)-OH.

D.1.2.1 Fc-CO-Leu-Val-Phe-Phe-OMe (5.3)

Boc-Leu-Val-Phe-Phe-OMe (0.76 g, 1.2 mmol), Silica gel column, increasing proportions of CH₃OH (0-2)% in CHCl₃, yield (0.45 g, 60%). MS (HR-ESI): 773.2977 calcd for C₄₁H₅₀FeN₄NaO₆; found 773.2984 [M+Na]⁺. ¹H NMR (400 MHz, DMSO-d₆): δ = 8.41 (d, 3J_HH = 7.42 Hz, 1H, NH), 8.05 (d, 3J_HH = 8.21 Hz, 1H, NH), 7.78 (d, 3J_HH = 8.21 Hz, 1H, NH), 7.62 (d, 3J_HH = 8.99 Hz, 1H, NH), 7.28-7.15 (m, br, 10 H, CH_ar Phe), 4.89 (s, 1H, H_cp), 4.86 (s, 1H, H_cp), 4.55 (m, 1H,α-H), 4.47 (m, 1H,α-H), 4.35 (s, 2H, H_cp), 4.16 (s, 6H, H_cp+ α-H), 3.55 (s, 3H, ester OCH₃), 2.95 (m, 3H, CH₂ β Phe), 2.69 (m, 1H, CH₂ β Phe), 1.88 (m, 1H, CH β Val), 1.66 (m, 2H, CH₂ β Leu), 1.42 (m, 1H, CH³ Leu), 0.89 (d, 3J_HH = 6.25 Hz, 6H, (CH₃)₂ Val), 0.74 (m, 6H, (CH₃)₂ Leu). ¹³C NMR (DMSO-d₆): δ = 172.1, 171.5, 171.0, 170.4, 169.0, 137.4, 137.0, 129.0, 128.9, 128.2, 127.9, 126.5, 126.1, 76.0, 70.0, 69.2, 68.3, 57.2, 53.4, 53.2, 51.7, 51.1, 37.5, 36.6, 30.9, 24.3, 23.2, 21.2, 19.1, 18.0.
Figure D.2 $^1$H NMR spectrum of compound 5.3 in DMSO-d$_6$.

Figure D.3 $^{13}$C NMR spectrum of compound 5.3 in DMSO-d$_6$. 
D.1.2.2 Fc-CO-Leu-Val-Phe-Phe-OH (5.4)

Fc-CO-Leu-Val-Phe-Phe-OMe (0.18 g, 0.25 mmol), yield (0.092 g, 50%). MS (HR-ESI): 759.28216 calcd for C_{40}H_{48}FeN_{4}NaO_{6}; found 759.28257 [M+Na]^+. \(^1\)H NMR (400 MHz, DMSO-d_{6}): \(\delta = 12.76\) (s, br, 1H, COOH), 8.23 (d, \(^3\)J_{HH} = 7.03 Hz, 1H, NH), 8.04 (d, \(^3\)J_{HH} = 8.21 Hz, 1H, NH), 7.77 (d, \(^3\)J_{HH} = 7.82 Hz, 1H, NH), 7.63 (d, \(^3\)J_{HH} = 8.60 Hz, 1H, NH), 7.25-7.15 (m, br, 10 H, CH\_Ar Phe), 4.89 (s, 1H, H\_cp), 4.86 (s, 1H, H\_cp), 4.55 (s, 1H,\_\(\alpha\)-H), 4.44 (m, 1H,\(\alpha\)-H), 4.35 (s, 2H, H\_cp), 4.16 (s, 6H, H\_cp\(\alpha\)-H), 2.97 (m, 3H, CH\_\(\beta\) Phe), 2.69 (m, 1H, CH\_\(\beta\) Phe), 1.87 (m, 1H, CH\_\(\beta\) Val), 1.66 (m, 2H, CH\_\(\beta\) Leu), 1.40 (m, 1H, CH\^\gamma\ Leu), 0.87 (m, 3H, (CH\_3)\_2 Val), 0.83 (m, 3H, (CH\_3)\_2 Val), 0.73 (s, 6H, (CH\_3)\_2 Leu). \(^13\)C NMR (DMSO-d\_6): \(\delta = 172.4, 172.0, 170.7, 170.4, 168.3, 137.5, 137.2, 129.0, 128.1, 127.9, 126.3, 126.1, 76.0, 70.0, 69.2, 68.3, 57.2, , 53.2, 53.0, 51.0, 37.4, 36.6, 30.9, 24.3, 23.2, 21.2, 19.1, 18.0.

**Figure D.4** \(^1\)H NMR spectrum of compound 5.4 in DMSO-d\_6.
D.1.2.3 Fc-CO-Lys(Boc)-Leu-Val-Phe-Phe-OMe (5.5)

Boc-Leu-Val-Phe-Phe-OMe (0.76 g, 1.2 mmol), Silica gel column, increasing proportions of CH$_3$OH (0-2)% in CHCl$_3$, yield (0.29 g, 30%). MS (HR-ESI): 1001.44525 calcd for C$_{52}$H$_{70}$FeN$_6$NaO$_9$; found 1001.44634 [M+Na]$^+$. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ = 8.41 (d, $^3$J$_{HH}$ = 7.42 Hz, 1H, NH), 8.00 (m, 2H, NH), 7.68 (d, $^3$J$_{HH}$ = 8.21 Hz, 1H, NH), 7.61 (d, $^3$J$_{HH}$ = 8.99 Hz, 1H, NH), 7.28-7.16 (m, br, 10 H, CH$_{Ar}$ Phe), 6.78 (m, 1H,NH Boc), 4.90 (s, 1H, H$_{cp}$), 4.85 (s, 1H, H$_{cp}$), 4.56 (s, 1H,\(\alpha\)-H), 4.46 (m, 1H,\(\alpha\)-H), 4.39 (m, 1H, H$_{cp}$\(\alpha\)-H), 4.35 (s, 3H, H$_{cp}$\(\alpha\)-H), 4.17 (s, 5H, H$_{cp}$), 4.09 (s, 1H, \(\alpha\)-H), 3.04-2.87 (m, 6H, CH$_2$\(\beta\) Phe+ CH$_2$ Lys), 2.73 (m, 1H, CH$_2$\(\beta\) Phe), 1.86 (m, 1H, CH$_{\beta}$ Val), 1.65 (m, 4H, CH$_2$\(\beta\) Leu+ CH$_2$ Lys), 1.46-1.37 (m, 5H, CH$_{\beta}$ Leu+CH$_2$ Lys), 1.35 (s, 9H, CH$_3$Boc), 0.83 (dd, $^3$J$_{HH}$ = 6.64 Hz, 6H, (CH$_3$)$_2$ Val), 0.70 (m, 6H, (CH$_3$)$_2$ Leu). $^{13}$C NMR (DMSO-$d_6$): $\delta$ = 172.1, 171.5, 170.9, 170.3, 169.0, 155.5, 137.4, 136.9, 129.0, 128.2, 127.9, 126.5, 126.1, 77.2, 76.0, 69.9, 69.3, 68.3, 57.3, 53.5, 53.2, 52.6, 51.7, 50.9, 40.5, 37.5, 36.6, 31.3, 30.6, 29.2, 28.2, 24.0, 23.1, 21.6, 19.0, 18.0.

Figure D.5 $^{13}$C NMR spectrum of compound 5.4 in DMSO-$d_6$. 
**Figure D.6** $^1$H NMR spectrum of compound 5.5 in DMSO-d$_6$.

**Figure D.7** $^{13}$C NMR spectrum of compound 5.5 in DMSO-d$_6$. 
D.1.2.4 Fc-CO-Lys(Boc)-Leu-Val-Phe-Phe-OH (5.6)

Fc-CO-Lys(Boc)-Leu-Val-Phe-Phe-OMe (0.10 g, 0.10 mmol), yield (0.048 g, 50%), $\delta = 12.76$ (s, br, 1H, COOH), 8.20 (s, br, 1H, NH), 7.98 (m, 2H, NH), 7.66 (d, $^3J_{HH} = 8.21$ Hz, 1H, NH), 7.60 (d, $^3J_{HH} = 8.60$ Hz, 1H, NH), 7.25-7.12 (m, br, 10 H, CH$_{Ar}$ Phe), 6.76 (s, br, 1H,NH Boc), 4.88 (s, 1H, H$_{cp}$), 4.83 (s, 1H, H$_{cp}$), 4.54 (s, 1H,$\alpha$-H), 4.39 (m, 2H,$\alpha$-H), 4.35 (s, 3H, H$_{cp+}$ $\alpha$-H), 4.15 (s, 5H, H$_{cp}$), 4.07 (s, 1H, $\alpha$-H), 3.05-2.86 (m, 6H, CH$_2^{\beta}$ Phe+ CH$_2$ Lys), 2.68 (m, 1H, CH$_2^{\beta}$ Phe), 1.82 (m, 1H, CH$_{\beta}$ Val), 1.61 (m, 4H, CH$_2^{\beta}$ Leu+ CH$_2$ Lys), 1.44-1.37 (m, 5H, CH$_{\gamma}$ Leu+CH$_2$ Lys), 1.33 (s, 9H, CH$_3$Boc), 0.81 (dd, $^3J_{HH} = 6.25$ Hz, 6H, (CH$_3$)$_2$ Val), 0.67 (m, 6H, (CH$_3$)$_2$ Leu). $^{13}$C NMR (DMSO-d$_6$): $\delta = 171.5, 170.9, 170.3, 169.0, 137.3, 129.0, 128.1, 127.9, 77.2, 76.0, 69.9, 69.3, 68.3, 57.3, 53.3, 50.8, 30.6, 29.2, 28.2, 24.0, 23.1, 21.6, 19.0, 18.0.

Figure D.8 $^1$H NMR spectrum of compound 5.6 in DMSO-d$_6$. 
**Figure D.9** $^{13}$C NMR spectrum of compound **5.6** in DMSO-d$_6$.

**Figure D.10** Cyclic voltammograms of Fc-conjugates **5.3-5.6** (0.1 mM in 0.1 M TBAP in CH$_3$OH). Scan rate 100 mV s$^{-1}$, Pt wire auxiliary electrode, Ag/AgCl reference and glassy carbon electrode as working electrode.
Figure D.11 Variable Temperature Circular dichroism spectra of compounds 5.3 (a), 5.4 (b), 5.5 (c) and 5.6 (d) in CH$_3$CN. Signal intensity at 214 nm is decreasing dramatically with increasing temperature from 25 °C to 70 °C, suggesting the weaker hydrogen bonds at higher temperature. The compound reverted to its original structure when cooled to 25 °C (as shown for compounds 5.3 and 5.4).
Figure D.12 Circular dichroism spectra of compounds 5.5 (-) and 5.6 (-- --) in 50% CH$_3$CN/tris buffer pH 8.5, the first minimum is consistent with β-sheet structure and the second minimum at ~ 232 nm is due to the stacking of aromatic amino acids (a). FT-IR spectra of 5.5 and 5.6 in the Amide I region showed two peaks at ~1635 and 1689 cm$^{-1}$ which are associated with β-sheet structure (b).
Figure D.13 (a, b) TEM images of assemblies present in 7-day-old CD samples of $\text{A}^\beta_{12-28}/5.3$ mixture (1:2) and (c, d) $\text{A}^\beta_{12-28}/5.4$ mixtures (1:2). Scale bars are 100nm.
Figure D.14 (a, b) TEM images of 7-day-old CD samples of $\text{A}\beta_{12-28}/5.5$ mixture (1:2) and (c, d) $\text{A}\beta_{12-28}/5.6$ mixture (1:2). Scale bars are 100 nm for (a and b) and 500 nm for (c and d).
Figure D.15 (a-b) Cyclic voltammograms and (c-d) square-wave voltammograms of gold electrode modified with Aβ<sub>12-28</sub>-Cys film and subsequent incubation with bioconjugates 5.3-5.6. Scan rate 100 mV s<sup>-1</sup>, Pt wire auxiliary, Ag/AgCl reference and Aβ<sub>12-28</sub>-Cys-modified gold electrode as working electrode.
Figure D.16 (a) Cyclic voltammograms as a function of scan rate and (b) plot of cathodic and anodic current densities versus scan rate for \(A\beta_{12-28}\)-Cys-modified gold electrode incubated with Fe-conjugate 5.4.
Figure D.17 (a) Cyclic voltammograms as a function of scan rate and (b) plot of cathodic and anodic current densities versus scan rate for $\text{A}^{\beta}_{12-28}$-Cys-modified gold electrode incubated with Fe-conjugate 5.6.
Figure D.18 Cyclic voltammograms of Aβ_{12-28}-Cys/5.4 film as a function of number of cycles: (a) 2 CV scans and (b) 8 CV scans in 2 mM NaClO_4. Scan rate 100 mV s^{-1}, Pt wire auxiliary electrode, Ag/AgCl reference electrode and Aβ_{12-28}-Cys modified gold electrode as working electrode.

Figure D.19 Cyclic voltammograms of Fc-conjugates 5.3-5.6 (0.1 mM in 0.1 M TBAP in CH_3CN). Scan rate 100 mV s^{-1}, Pt wire auxiliary electrode, Ag/AgCl reference electrode and glassy carbon electrode as working electrode.
Figure D.20 Plot of $E_p$ as a function of the logarithm of the scan rates for $\text{A}\beta_{12-28}$-Cys-modified gold electrode incubated with (a) Fc-conjugate 5.4 and (b) Fc-conjugate 5.6. The values of the electron transfer coefficient, $\alpha$, and the heterogeneous rate constant, $k_s$, were estimated from the plots.
Figure D.21 Plot of current densities as a function of the film type for the Aβ_{12-28}-Cys-modified gold electrode incubated with (a) Fc-conjugate 5.4 and (b) Fc-conjugate 5.6. The current densities were determined from the background-subtracted square-wave voltammograms and the error bars represent the duplicate measurements performed on two electrodes for each film.
Table D.1 Solution electrochemical data of Fc-conjugates 5.3-5.6 (0.1 mM in 0.1 M TBAP in CH$_3$OH). $E_{1/2}$, Δ$E$ in mV and diffusion coefficient, $D$, in × 10$^{-5}$ cm$^2$s$^{-1}$.

<table>
<thead>
<tr>
<th>Fc-conjugate</th>
<th>$E_{1/2}$</th>
<th>Δ$E$</th>
<th>$i_{pa}/i_{pa}$</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>593 ± 0.8</td>
<td>81 ± 3</td>
<td>1.07 ± 0.01</td>
<td>18.3 ± 1.1</td>
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<tr>
<td>5.4</td>
<td>594 ± 0.5</td>
<td>59 ± 2</td>
<td>1.10 ± 0.07</td>
<td>12.5 ± 3.0</td>
</tr>
<tr>
<td>5.5</td>
<td>593 ± 1.5</td>
<td>67 ± 1</td>
<td>1.10 ± 0.03</td>
<td>10.1 ± 4.3</td>
</tr>
<tr>
<td>5.6</td>
<td>585 ± 1.6</td>
<td>67 ± 3</td>
<td>1.09 ± 0.03</td>
<td>14.8 ± 5.8</td>
</tr>
</tbody>
</table>

Table D.2 Solution electrochemical data of Fc-conjugates 5.3-5.6 (0.1 mM in 0.1 M TBAP in CH$_3$CN). $E_{1/2}$, Δ$E$ in mV and diffusion coefficient, $D$, in × 10$^{-5}$ cm$^2$s$^{-1}$.

<table>
<thead>
<tr>
<th>Fc-conjugate</th>
<th>$E_{1/2}$</th>
<th>Δ$E$</th>
<th>$i_{pa}/i_{pa}$</th>
<th>$D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>600 ± 0.6</td>
<td>62 ± 4</td>
<td>1.06 ± 0.02</td>
<td>22.3 ± 0.7</td>
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<tr>
<td>5.4</td>
<td>580 ± 0.8</td>
<td>68 ± 2</td>
<td>1.05 ± 0.08</td>
<td>13.2 ± 2.2</td>
</tr>
<tr>
<td>5.5</td>
<td>620 ± 2.0</td>
<td>64 ± 3</td>
<td>0.90 ± 0.20</td>
<td>12.7 ± 3.5</td>
</tr>
<tr>
<td>5.6</td>
<td>603 ± 1.8</td>
<td>95 ± 3</td>
<td>0.96 ± 0.03</td>
<td>7.64 ± 3.6</td>
</tr>
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</table>

Table D.3 Equivalent circuit element values for the impedance spectroscopy employed for surface characterization of bare gold electrode and Aβ$_{12-28}$-Cys film immobilized on gold electrode. Relative standard errors in percents are shown in the bracket.

<table>
<thead>
<tr>
<th>Surface Coverage</th>
<th>$R_s$ (Ω cm$^2$)</th>
<th>$CPE$ (µF cm$^2$)</th>
<th>$n$</th>
<th>$R_C$ (Ω cm$^2$)</th>
<th>$W$ (µF$^{0.5}$ cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare gold</td>
<td>0.024 (13.5)</td>
<td>9.3 (3.2)</td>
<td>0.8</td>
<td>24 (1.0)</td>
<td>0.002 (2.3)</td>
</tr>
<tr>
<td>Aβ$_{12-28}$ amyloid</td>
<td>7.699 (4.0)</td>
<td>2.83 (9.1)</td>
<td>0.9</td>
<td>3760000 (11)</td>
<td>0.001 (76)</td>
</tr>
</tbody>
</table>
List of Publications


