The supramolecular chemistry of novel synthetic biomacromolecular assemblies

by

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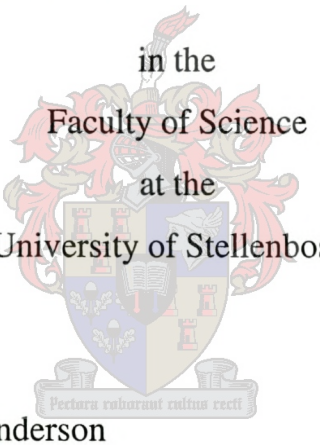

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

Over the past decade peptide bola-amphiphiles have been the subject of much attention because of their role as potential models of functionalised membranes and as new generation surfactants. In the quest for new surfactants a peptidomimetic-based approach was used to design a library of novel 'hybrid' bola-amphiphilic peptide surfactants derived from sapecin B and a model symmetrical oligo-glycine bola-amphiphile. The library was divided into different series, each one purpose-built; first, to investigate hierarchal supramolecular architecture and, second, to investigate potential antimicrobial activity. The bola-amphiphiles were synthesised using Fmoc-polyamide based solid phase peptide synthesis and purified *via* high performance liquid chromatography. The peptide hybrids were characterised using electrospray mass spectrometry, nuclear magnetic resonance, different modes of electron microscopy, Fourier-transform infrared spectroscopy and, in some cases, further studies were done using circular dichroism and bioactivity tests.

The model bola-amphiphile suberamide$(GG)_2$ was synthesised using peptide fragment condensation based on solid phase peptide synthesis. The synthesis is bi-directional (N→C and C→N) and versatile, making it possible to synthesis new dicarboxylic oligopeptide bola-amphiphiles and other analogous compounds. The product, suberamide$(GG)_2$, was purified using its inherent ability to self-assemble in an acidic solution.

Novel asymmetrical bola-amphiphiles composed of dipeptide head groups linked *via* an aliphatic ω–amino acid, serving as a hydrocarbon spacer, were also synthesized. Two small libraries of bola-amphiphiles were established — the first involved variation in ω–amino acid length and the other variation in the C-terminal amino acid. The bola-amphiphiles were self-assembled in either 0.1% trifluoroacetic acid or 0.1% triethylamine. Electron microscopy revealed the formation of a variety of higher order supramolecular architectures based on β-sheet self-assembly. FT-IR spectrometry indicated that interlayer and intralayer hydrogen bond networks, together with strong self-association, promoted by the hydrophobic effect and, in certain instances, electrostatic interactions, are responsible for the variety of supramolecular architectures. Variations in

the higher order structures can be attributed to amino acid composition, specifically length of ω-amino acid, nature of the C-terminal amino acid and the optimised solvent conditions used for the self-assembly process.

A third library of novel 'hybrid' bola-amphiphilic peptide surfactants, in which a cationic tripeptide motif from antimicrobial peptides was combined in a hybrid molecule containing a ω-amino acid residue, was established. These bola-amphiphiles displayed potent antimicrobial activity against both Gram-positive and Gram-negative bacteria; the analogues were as active or more active than the leader peptides yet, remarkably, displayed little or no appreciable haemolytic activity. These organopeptide bola-amphiphiles thus demonstrated selective toxicity towards bacteria. The hydrophobicity imparted by the ω-amino acid has contrasting effects on haemolysis and antimicrobial activity of the peptide analogues. The other unique feature of these peptides and their analogues is the fact they self-assembled into complex supramolecular architectures, composed primarily of β-sheets. Their self-assembly is primarily governed by hydrophobic interactions together with inter and intralayer hydrogen bonding. Electron microscopy clearly revealed higher order structures for both peptides and analogues. The generation of higher order supramolecular architecture is dependent on optimisation of β-sheet self-assembly whereas antimicrobial activity is dependent on the balance between net positive charge and optimum hydrophobicity of the peptide hybrids.

This study has demonstrated that it is possible to design hybrid peptide surfactants capable of producing higher order supramolecular architecture and improving the antimicrobial activity whilst reducing the haemolytic effect. The study and design of these versatile 'purpose-built' bio-inspired surfactants heralds a novel approach, one that shows tremendous potential.

Opsomming

Die afgelope dekade het bola-amfifiliese peptiede baie aandag geniet weens hulle rolle as potensiële modelle van gefunksionaliseerde membrane en as 'n nuwe generasie surfaktante. In die soeke na nuwe surfaktante is 'n peptiedomimetiese benadering gevolg om 'n biblioteek van nuwe "hibried" bola-amfifiliese peptiedsurfaktante van sapesien B en 'n simmetriese oligoglisien bola-amfifil af te lei. Die biblioteek is in verskillende reekse onderverdeel. Elke reeks is doelmatig vervaardig om ondersoek in te stel na twee aspekte, nl. die rangorde van die supramolekulêre strukture en die potensiële antimikrobiese aktiwiteit. Fmoc-poliamied gebaseerde soliedefase-peptied-sin-tese is aangewend vir die sintese van die bola-amfifile en hulle is met behulp van hoë doeltreffendheid vloeistofchromatografie gesuiwer. Die peptiedhibriede is gekarakteriseer met behulp van elekrosproei massaspektrometrie, kern-magnetiese resonansie, verskillende modusse elektronmikroskopie, Fourier-transform infrarooispektrometrie en, in sommige gevalle is verdere studies met sirkulêre dichroïsme en bioaktiwiteitstoetsing uitgevoer.

Die bola-amfifilsuberamied$(GG)_2$-model is met behulp van peptiedfragment-konden-sasie gesintetiseer gegrond op soliedefase-peptiedsintese. Dit sintese vind in twee rigtings plaas (N→C en C→N) en is veelsydig aangesien dit die sintese van sowel nuwe dikar-boksiel-bola-amfifile as ander analoë verbindings moontlik maak. Die produk, suber-amied$(GG)_2$, is gesuiwer met behulp van die verbinding se inherente vermoë tot self-montering in suur oplossings.

Nuwe assimetriese bola-amfifile, saamgestel uit dipeptiedkopgroepe, gekoppel *via* 'n alifatiese ω-aminosuur, wat as koolwaterstofspasieerder dien, is ook gesintetiseer. Twee klein bola-amfifilbiblioteke is saamgestel – die een het variasies in die ω-aminosuur se lengte omvat en die ander een variasies in die C-terminale aminosuur. Selfmontering van die bola-amfifile het plaasgevind in òf 0,1 % trifluorasynsuur òf 0,1 % trietielamien. Elektronmikroskopie het die bestaan van 'n verskeidenheid hoërorde supramolekulêre strukture, gegrond op β-plaatselfmontering, aangetoon. Uit FT-IR-spektrometrie blyk dit dat inter - en intralaag waterstofbinbdingsnetwerke en sterk selfassosiasie, lg. word bevorder deur die hidrofobiese effek en, in sekere gevalle, elektrostatiese interaksies, is verantwoordelik vir die verskeidenheid supramolekulêre strukture. Variasies in die

hoërorde strukture kan toegeskryf word aan aminosuursamestelling, in besonder die lengte van die ω-aminosuur, die aard van die C-terminale aminosuur en die geoptimiseerde oplosmiddelkondisies wat gebruik is vir die selfmonteringsproses.

'n Derde biblioteek nuwe "hibried" bola-amfifiliese peptiedsurfaktante, waarin 'n kationiese tripeptiedmotief uit antimikrobiale peptiede gekombineer is met 'n ω-aminosuurresidu, is geskep. Sommige van hierdie bola-amfifile het 'n kragtige antimikrobiese aktiwiteit teenoor sowel Gram-positiewe as Gram-negatiewe bakterieë gertoon. Die analoë strukture was aktief, of selfs meer aktief as die voorste peptiede maar het, verbasend genoeg, nie 'n beduidende hemolitiese aktiwiteit vertoon nie. Hierdie organopeptied bola-amfifil het dus 'n selektiewe toksisiteit teenoor bakterieë vertoon. Die hidrofo-bisiteit, as gevolg van die ω-aminosuur, het 'n resiproke effek op hemolise en die antimikrobiese aktiwiteit van die peptiedanaloë. Die ander uitstaande kenmerk van die peptiede en hulle analoë is die vermoë om te selfmonteer en komplekse supramolekulêre strukture, bestaande hoofsaaklik uit β-plate, te vorm. Hierdie selfmontering word in hoofsaak beheer deur hidrofobiese interaksies asook inter - en intralaagwaterstofbinding. Elektronmikroskopie het duidelik hoërorde strukture getoon by sowel dié peptiede as hulle analoë. Die ontwikkeling van hoërorde supramolekulêre struktuurvorms is afhanklik van die optimalisering van die β-plaatselfmontering. Daarteenoor is die antimikro-biese aktiwiteit afhanklik van die balans tussen die netto positiewe lading en die opti-male hidrofobisiteit van die peptiedhibriede.

Hierdie studie het getoon dat dit moontlik is om hibriedsurfaktante te ontwerp wat hoërorde supramolekulêre strukture te produseer en om die antimikrobiese aktiwiteit te verbeter terwyl die hemolitiese effek verminder word. Die studie en ontwerp van hier-die veeldoelige, "doelmatig-gesintetiseerde" biogeïnspireerde surfakante stel 'n unieke benadering daar, wat oor groot potensiaal beskik.

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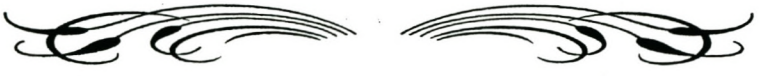
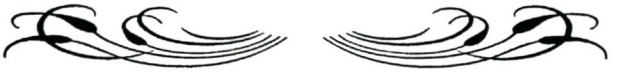


Dedicated with love and humility to Sat Purush



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List of Abbreviations and Acronyms

2D	two-dimensional
3D	three-dimensional
BLM	bilayer lipid membrane
BOP	benzotriazol-1-yl-oxy-tris-dimethylaminophosphonium hexafluorophosphate
C5	GG-NH$(CH_2)_5$CO-GG
C8	GG-NH$(CH_2)_8$CO-GG
C11	GG-NH$(CH_2)_{11}$CO-GG
^{13}C NMR	carbon-13 nuclear magnetic resonance
CD	circular dichroism
CD_3CN	deuterated acetonitrile
CID	collision induced dissociation/decomposition
CMC	critical micellular concentration
CV	cone voltage
Da	dalton
DCCI	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
Dhbt	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl
DIPCDI	diisopropylcarbodiimide
DIPEA	N, N-diisopropylethyl amine
DMF	N, N-dimethylformamide
DMSO-d_6	deuterated dimethyl sulphoxide
D_2O	deuterated water
E. coli	*Escherichia coli*

EDC	*N*-ethyl-*N*′-(3-dimethylaminopropylcarbodiimide)
EI	electron ionisation
EM	electron microscopy
E-SEM	environmental scanning electron microscopy
ESI	electrospray ionisation
ESMS	electrospray mass spectrometry
FAB	fast atom bombardment
FE-SEM	field emission scanning electron microscopy
Fmoc	N^9-fluorenylmethyloxycarbonyl
FT-IR	Fourier-transform infrared spectroscopy
G	glycine
GL	GG-NH$(CH_2)_8$CO-GL
Gly	glycine
GK	GG-NH$(CH_2)_8$CO-GK
GSED	gaseous secondary electron detector
^{1}H NMR	proton nuclear magnetic resonance
HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HC_{50}	peptide concentration leading to 50 % haemolysis
HOBt	1-hydroxybenzotriazol
HPLC	high performance liquid chromatography
IR	infrared
K	lysine
L	leucine
Leu	leucine
Lys	lysine
[M]	molecular ion

M	molar
MIC	minimum inhibitory concentration
mL	millilitre
MLM	monolayer lipid membrane
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
m/z	mass over charge ratio
NCCLS	National Committee for Clinical Laboratory Standards
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PP	GG-NH$(CH_2)_8$CO-KLK-NH_2
PyBOP®	benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate
PyBroP®	bromo-*tris*-pyrrolidino-phosphonium hexafluorophosphate
S. aureus	*Staphylococcus aureus*
SEM	scanning electron microscopy
SPSS	solid phase peptide synthesis
tBoc	N^{α}-*tert*-butyloxycarbonyl
tBu	t-butyl ester
TBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
TNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
TSTU	*O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	trifluoroethanol
µg	microgram
µM	micromolar

5L	KLKLLLLLKLK-NH_2
4L	KLKLLLLKLK-NH_2
3L	KLKLLLKLK-NH_2
5LA	KLK-$NH(CH_2)_{11}CO$-KLK-NH_2
4LA	KLK-$NH(CH_2)_8CO$-KLK-NH_2
3LA	KLK-$NH(CH_2)_5CO$-KLK-NH_2

"In Nature, hybrid species are usually sterile, but in science the reverse is often true. Hybrid subjects are often astonishingly fertile, whereas if a scientific discipline remains too pure it usually wilts." .....Francis Crick

Chapter 1

Introduction

Research was carried out into the design of novel bio-macromolecular assemblies for the creation of purpose-built assemblies and unique 2D and 3D hierarchal supramolecular architectures. This study contributes to the knowledge of design conceptualisation, synthesis, self-assembly and characterisation of supramolecular complexes involving bola-amphiphiles. In the past 10 years bola-amphiphiles have enjoyed much attention because their amphiphilicity allows for labile self-assembly and they also serve as model membranes.

1.1 Background

In an effort to take the concept of bola-amphiphiles further a purpose-designed peptide bola-amphiphile was selected. It was envisaged that functionalising the bola-amphiphile would lead to the creation of novel supramolecular architectures and yield surfactants that could be used for specific purposes. Literature reveals that bola-amphiphiles are capable of generating phenomenal supramolecular architectures; Shimizu et al.[1,2] demonstrated this admirably. There is, however, an additional feature of these bola-amphiphiles that has not yet been exploited to its full potential. The concept of bola-amphiphile-designed compounds has shown tremendous potential as antimicrobial agents[3] because of their natural tendency to disrupt membranes. Noting this, and the fact that there are naturally occurring antimicrobial peptides, it was envisaged that the incorporation of an integral structural motif of these antimicrobial peptides into a bola-amphiphile design should lead to the creation of hybrid bioactive organopeptide surfactants.

The search for a suitable antimicrobial motif led to the selection of a potent cationic bola-amphiphilic peptide based on a core peptide in sapecin B[4] that had potent antimicrobial activity[5] against *Staphylococcus aureus*, *Escherichia coli*, methicillin-resistant *S. aureus* and *Candida albicans* in liquid medium and demonstrated no appreciable haemolytic activity. Recent studies have shown that terminal basic motifs and the internal oligo-

leucine sequences of KLKLLLLLKLK-NH_2 play important roles in its antimicrobial activity[6]. In keeping with recent efforts to generate viable pharmaceutical therapies, focus was directed on "peptidomimetics", i.e. the creation of non-natural peptide hybrids[7]. This was accomplished by a simple modification of the peptide sequence; the hydrophobic oligo-leucine moiety was replaced with an ω-amino acid residue, mimicking the hydrophobic core of the original peptide. On this basis it was possible to establish a library of functionalised peptide bola-amphiphiles.

1.1.1 Peptide bola-amphiphiles

A bola-amphiphile is simply defined as a molecule in which two or more hydrophilic groups are connected by hydrophobic functions[8]. Various types of bola-amphiphiles have been synthesised and characterised, e.g. nucleotide bola-amphiphiles[9]. During the past decade there has been much interest in the field of peptide bola-amphiphiles[1,2,10,11]. Peptide amphiphiles lie at the interface of biology, chemistry, physics and engineering – hence research in this field is truly multidisciplinary research. Their potential extends into several different fields of science and technology, with applications that range from antibiotics to monomer design.

1.1.2 Self-assembly of bola-amphiphiles

Self-assembly in colloidal and interfacial systems is multifaceted. The types of compounds most frequently involved in self-assembly are surfactants (amphiphiles), polymers, nano- and micro-particles. Surfactant self-assembly is a major topic in colloid and interface science, and is of key importance in supramolecular chemistry[12]. Macromolecular systems of amphiphiles have received much attention because of their interesting physiochemical properties and their potential practical applications[9]. These systems are 'organised', because the amphiphilic molecules adopt a specific arrangement at surface interfaces, in colloidal aggregates and in membranes[8]. Designing molecular arrangements, involving a number of different interactions e.g. hydrogen bonding, hydrophobic interaction, leads to systems with amazingly unique properties, such as high tensile strength. Interaction between the different molecules gives rise to exciting new phenomena, such as pleochroism i.e. the absorption of different wavelengths of transmitted light.

1.2 Objectives

Essentially, the overall objective of this study was to investigate the use of peptide bola-amphiphiles in the design and synthesis of novel bio-macromolecular assemblies with two specific purposes in mind: first, to generate unique 2D or 3D hierarchal supramolecular architectures and, second, to explore the possibility of peptide bola-amphiphiles being considered for use as a new generation of antibiotics. Noting the aforementioned and the recent advances made in the design and construction of synthetic peptides[13] the specific objectives of this research were therefore:

(i) to design and synthesise a novel library of purpose-built peptide bola-amphiphiles;

(ii) to characterise these peptide surfactants;

(iii) to develop and optimise conditions for the self-assembly of these systems;

(iv) to characterise the bio-macromolecular assemblies obtained, i.e. the supramolecular chemistry of the assemblies;

(v) to explore the potential of these peptide bola-amphiphiles.

1.3 Scope

In order to meet the specific objectives of this research, a number of tasks were undertaken, as given below.

1.3.1 Design and synthesis of the peptide bola-amphiphiles

A library of purpose-built peptide bola-amphiphiles was designed. This library was focused on a synthetic cationic bola-amphiphilic peptide, based on a core peptide in sapecin B[4], and a model symmetrical bola-amphiphile synthesized by Grigoryan *et al*[14]. The library was divided into different series, each one purpose-built; first, to investigate hierarchal supramolecular architecture and, second, to investigate potential antimicrobial activity. The transition was from antimicrobial peptide → hybrid analogue → antimicrobial peptide / model bola-amphiphile precursor ← series II ← series I ← model bola-amphiphile (see Fig 1.1). The precursor was then subdivided into molecules that

resemble its building blocks (Series I and II). This subdivision was based on a model symmetrical bola-amphiphile synthesised by Grigoryan *et al.*[14], the potential for self-assembly of which was demonstrated by Shimizu *et al.*[1,2]. The library of peptide bola-amphiphiles was synthesised using solid phase peptide synthesis (SPPS) based on Fmoc chemistry[15]. The following is a schematic of the library used in this study.

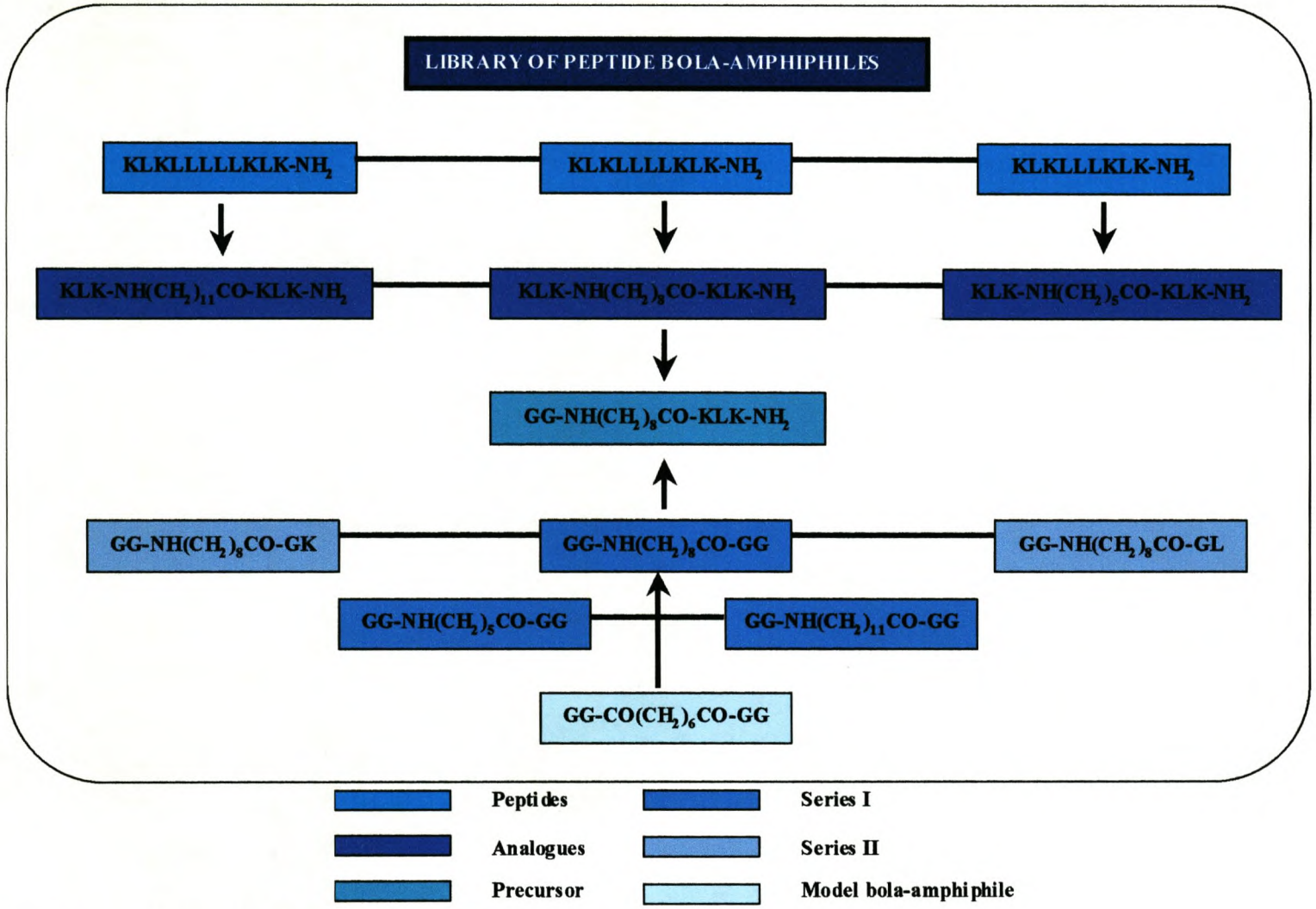


Figure 1.1 Schematic representation of the library of peptide bola-amphiphiles used in this study.

1.3.2 Characterisation of the peptide bola-amphiphiles

The structure of the peptide bola-amphiphiles was characterised using nuclear magnetic resonance (NMR) and electrospray mass spectrometry (ES-MS).

1.3.3 Optimisation of conditions for self-assembly

Aqueous solvent systems, modified with either trifluoroacetic acid (TFA) or triethylamine (TEA), were used in order to promote self-assembly of the different purified bola-amphiphiles.

1.3.4 Characterisation of the bio-macromolecular assemblies

Characterisation of the bio-macromolecular assemblies was conducted *via* a two-fold approach.

(i) **Microscopy**. Various microscopic techniques were employed to observe the supramolecular architectures obtained. Initial observations were made with phase-contrast light microscopy in order to determine whether self-assembly was occurring. This could easily be established by determining birefringence of the system. Environmental and field emission scanning electron microscopy were used for more detailed analysis of the supramolecular architectures obtained.

(ii) **Fourier-transform infrared spectroscopy**. Lyophilised samples of the self-assembled peptides were analysed by means of photo acoustic Fourier-transform infrared spectroscopy (FT-IR). This could be used to establish the internal molecular arrangements in the supramolecular complex. Savitsky-Golay[16] derivative function software was used to determine the second derivative spectra of the amide I bands in order to provide more detailed spectral information.

(iii) **Circular dichroism** (CD) was also used to characterise the biomacromolecular assemblies of the peptides and their analogues.

1.3.5 Potential uses of the peptide bola-amphiphiles

In order to assess the potential of these bola-amphiphiles as possible antibiotics, minimum inhibitory concentration (MIC) determinations were carried out using

Escherichia coli and methicillin-resistant *Staphylococcus aureus*. MICs were determined using the micro-dilution broth method, essentially following the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS)[17]. Haemolytic activity assays were used to demonstrate the selective toxicity of the peptides.

1.4 Layout of dissertation

Chapters 3 to 8 of this dissertation has been prepared in a format to facilitate submission to the selected journals:

(i) **Chapter 1.** Introduction

(ii) **Chapter 2.** Historical overview of the fields of supramolecular chemistry and peptide bola-amphiphiles.

(iii) **Chapter 3.** *Bi-directional solid phase synthesis of a model oligoglycine-based bola-amphiphile and purification by self-assembly.* This chapter describes in detail the synthesis and characterisation of the model bola-amphiphile used in this study. To be submitted to *Peptides*.

(iv) **Chapter 4.** *Monitoring oligoglycine bola-amphiphile nanotube self-assembly*. This chapter describes the synthesis, characterisation and self-assembly of the model bola-amphiphile. To be submitted to *Angewandte Chemie*.

(v) **Chapter 5.** *Self-assembly of an asymmetrical bola-amphiphile into defined 3D supramolecular architectures.* This chapter describes in detail, the synthesis, characterisation and self-assembly of [GG-NH$(CH_2)_5$CO-GG] one of the peptide bola-amphiphiles in series I. To be submitted to *Langmuir*.

(vi) **Chapter 6.** *The influence of structural and solvent conditions on the supramolecular architecture of novel peptide asymmetrical bola-amphiphiles.* This chapter describes synthesis, characterisation and self-assembly of the peptide bola-amphiphiles in series II and I. To be submitted to the *Journal of American Chemical Society*.

(vii) **Chapter 7.** *Bioinspired surfactants: Design, biological activity and self-assembly into supramolecular structures.* This chapter describes the synthesis, characterisation, self-assembly and potential uses of the designed peptide analogues. To be submitted to *BBA - Biomembranes.*

(viii) **Chapter 8.** Conclusions. *'Purpose-built' bio-inspired cationic surfactants.* This chapter is a concept article and summarises the highlights of this study.

1.5 References

1. Shimizu, T., Kogiso, M., Masuda, M., *Nature*., 1996, *383*, 487.

2. Kogiso, M., Okada, Y., Hanada, T., Yase, K., Shimizu, T., *Biochim et Biophys Acta*., 2000, *1475*, 346-352.

3. Jayasuriya, N., Bosak, S., Regen, S. L., *J. Amer. Chem. Soc*., 1990, *112*, 5844-5850.

4. Natori, S., *Antimicrobial Peptides, Ciba Foundation Symposium*, 1994, 123-134.

5. Alvarez-Bravo, J., Kurata, S., Natori, S., *Biochem. J*., 1994, *302*, 535-538.

6. Nakajima, Y., Alvarez-Bravo, J., Cho, J., Homma, K., Kanegasaki, S., Natori, S., *Febs. Lett*., 1997, *415*, 64-66.

7. Patch, J.A., Barron, A.E., *Curr. Opin. Chem. Biol.,* 2002, *6,* 872-877.

8. Fuhrhop, J.–H., Bach, R., *Advances in Supramolecular Chemistry*, 1992, *Vol. 2*, JAI Press Inc., p. 26.

9. Escamilla, G. H., Newkome, G. R., *Angew. Chem. Int. Ed. Engl*., 1994, *33*, 1937-1940.

10. Schneider, J., Messerschmidt, C., Schultz, A., Gnade, M., Schade, B., Luger, P., Bombicz, P., Hubert, V., Fuhrhop, J.–H., *Langmuir*, 2000, *16*, 8575-8584.

11. Matsui, H., Douberly, G. E., *Langmuir,* 2001, *17*, 7918-7922.

12. Kunitake, T., *Curr. Opin. Coll. Interf. Sci.,* 2001, *6*, 1-2.

13. Mayo, K. H., *Trends in Biotech.,* 2000, *18*, 212-217.

14. Grigoryan, N. A., Mndzhoyan Z. O., Kazaryan E. V., Ter-Zakharyan Z. Yu., Mndzhoyan, O. L., *Pharm. Chem. J*., 1992, *26*, 153-157.

15. Atherton, E., Fox, H., Harkiss, D., Logan, C. J., Sheppard, R. C., Williams, B. J., *J. Chem. Soc. Chem. Commun.,* 1978, 537-539.

16. Savitsky, A., Golay, J. E., *Anal. Chem.*, 1964, *36*, 1628-1639.

17. www.cmdr.ubc.ca/bobh/showmethod.php?methodid=79

Chapter 2

Historical overview

2.1 Introduction

For more than 150 years, ever since the synthesis of urea by Friedrich Wöhler in 1828[1], molecular chemistry has developed a vast array of highly sophisticated and powerful methods for the construction of complex molecular structures by the making or breaking of covalent bonds between atoms in a controlled and precise fashion. Chemists have used their knowledge to rival nature's ability to create everything from antibiotics to plastics. However, it is only in the last decade that they have begun to take advantage of another wonder of the natural world - the ability to form looser, but still highly specific links between small molecules, i.e. intermolecular forces, viz. hydrogen bonding. Intermolecular forces govern the assembly of molecules in living organisms, including holding DNA strands together in the familiar double helix and uniting the protective membrane that houses every cell. The exploitation of hydrogen bonding is one of the fastest growing areas of scientific research: supramolecular chemistry.

Lehn[2] first introduced the concept of, and the term, supramolecular chemistry in 1978. It was defined in the words, "Just as there is a field of *molecular chemistry* based on the covalent bond, there is a field of *supramolecular chemistry*, the chemistry of molecular assemblies and of the intermolecular bond." Since then it has been reformulated on various occasions, e.g., "Supramolecular chemistry may be defined as 'chemistry beyond the molecule', bearing on the organised entities of higher complexity that result from the association of two or more chemical species held together by intermolecular forces."[3] Supramolecular chemistry has grown into a major field and has fuelled numerous developments at the interface with biology and physics, thus giving rise to the emergence and establishment of supramolecular science and technology as a broad multidisciplinary and interdisciplinary domain, providing a highly fertile ground for the creativity of scientists from all fields. Beyond the molecule, supramolecular chemistry aims at developing highly complex chemical systems from components that interact by non-

covalent intermolecular forces[4,5]. Through the manipulation of these interactions it has become the chemistry of molecular information, involving the storage of information at a molecular level, in structural features, and its retrieval, transfer, and processing at the supramolecular level, by interactional algorithms operating through molecular recognition events based on well-defined interaction patterns[6] (hydrogen bonding arrays, sequences of donor and acceptor groups, ion co-ordination sites, etc.).

2.2 Supramolecular chemistry

Essentially, the goal of supramolecular chemistry is to gain progressive control over structural and dynamic features of matter through self-organization[4]. Supramolecular chemistry involves the design and investigation of pre-organised molecular receptors of various types, capable of binding specific substrates with high efficiency and selectivity.

Three main themes outline the development of supramolecular chemistry[6]

(i) Molecular recognition – depends on design and pre-organisation, and implements information storage and processing.

(ii) Self-assembly/self-organisation – relies on design and implements programming and programmed systems.

(iii) Adaptation and evolution – is dependent on self-assembly through selection, in addition to design, and implements chemical diversity and "informed" dynamics.

2.2.1 Molecular recognition

Supramolecular chemistry has relied on pre-organisation for the design of molecular receptors, affecting molecular recognition, catalysis, and transport processes[4]. The concept of molecular recognition is defined by the energy and the information involved in the binding and selection of substrate(s) by a given receptor molecule. It may also involve a specific function[7]. It implies a pattern recognition process through a structurally well defined set of intermolecular interactions i.e. the (molecular) storage and (supramolecular) read out of molecular information. Supramolecular chemistry has provided new methods of chemical synthesis[8], establishing procedures for the

construction of supramolecular entities and providing supramolecular assistance to synthesis involving non-covalent positioning of the components followed by covalent bond formation[8,9]. Both methods will provide access to highly complicated non-covalent and covalent entities.

2.2.2 Self-assembly/self-organization

Beyond pre-organisation lies the design of programmed systems that self-assemble through explicit manipulation of molecular recognition features, thereby directing the build-up from their components of supramolecular species and devices[10-12]. Molecular self-assembly is defined as the spontaneous association of molecules, either separate or linked, under equilibrium conditions, to form structurally well-defined aggregates joined by non-covalent bonds[10,13]. The process of self-assembly is of great importance, both scientifically and technologically, for a number of reasons.

The most important one is that it is the essence of life. The cell contains an extensive range of complex structures, such as folded proteins, structured nucleic acids, molecular machines and many others that form by self-assembly[14]. The process of self-assembly also provides routes to a variety of materials with regular structures, e.g. molecular crystals[15], liquid crystals[16], and semicrystalline and phase-separated polymers[17]. Self-assembly also occurs widely in systems of components larger than molecules, and there is great potential for its use in materials and condensed matter science[11]. It has also emerged as the new synthetic strategy by which to generate nanostructures[18], i.e. the so-called bottom-up or engineering-up approach to nanoscale device fabrication[12].

The success of self-assembly in a molecular system is determined by five characteristics of the system[13]:

(a) Components: Components comprise the group of molecules or segments that make up the self-assembly system. Their interaction results in a transition from a less ordered state (solution, random coil, etc.) to a final state (crystal, folded macromolecule) that is more ordered.

(b) Interactions: Self-assembly occurs when molecules interact with one another through a balance of attractive and repulsive interactions. These interactions are

weak to medium strength and non-covalent (Van der Waals and Coulomb interactions, and hydrogen bonds). Relatively weak covalent bonds[19,20] i.e. co-ordination bonds, have also been reported to be significant in self-assembly.

(c) Reversibility (or Adjustability): In order to generate ordered structures the association between the components of the system must be reversible or be allowed to adjust their positions within an aggregate once it has formed. The strength of the bonds between the components must therefore be comparable to the forces tending to disrupt them.

(d) Environment: The self-assembly of molecules is usually carried out in solution or at an interface, to allow the required movement of the components. The interaction of the components with the environment is an integral part of this process.

(e) Mass transport and agitation: For self-assembly to occur, molecules must be mobile. In solution, thermal motion is largely responsible for bringing the molecules into contact. In nanoscale, mesoscopic, and macroscopic self-assembly systems, the components interact in ways similar to the way in which molecules interact. In designing such systems, the first challenge is assuring the mobility of the components. The choice of interactions between the components in the system, to allow them to reach equilibrium, is also important.

2.2.3 Adaptation and evolution

The combination of the features of supramolecular systems – information and programmability, dynamics and reversibility, constitution and diversity – has lead to the emergence of adaptive/evolutive chemistry[21]. Adaptive chemistry implies selection and growth under time reversibility. Implementing both design and selection, self-assembly offers adjustability (through self-correction, self-healing under internal dynamics); adjustability leads to adaptation (through reorganization under interaction with environmental effects); adaptation becomes evolution when acquired features are conserved and passed on. Adaptation is illustrated by functionally driven optimisation through selection from pools of dynamically interconverting supramolecular species[22]. Evolutive chemical systems suppose multiple dynamic processes with sequential

selection/acquisition/fixation steps and undergo progressive change of internal structure under the pressure of environmental factors.

Beyond programmed systems, and in line with evolutive chemistry, the next step in complexity comprises the design of chemical "learning" systems, systems that are not just instructed but systems that can be trained[23]. Supramolecular chemistry provides ways and means of progressively unravelling the complexity of matter through self-assembly. This leads to a science of complex matter, of informed, self-organized, evolutive matter, where the goal is to progressively discover, understand, and implement the rules that govern the evolution of matter from inanimate to animate and beyond - to ultimately acquire the ability to create new forms of complex matter[6].

2.3 Non-covalent molecular assemblies

2.3.1 Lipid membranes

Biological cell membranes are multi-component systems consisting of a fluid bilayer lipid membrane (BLM) and integrated membrane proteins. The main structural features of BLMs are determined by the wide variety of amphiphilic phospholipids whose hydrophilic head groups are exposed to water, while the hydrocarbon tails form the non-polar interior. The formation of BLMs in nature is the ultimate self-assembly, to form a permeability barrier acting mainly in functional compartmentalisation of cellular processes. BLMs also act as a medium for biochemical membrane processes such as photosynthesis, respiration and active ion transport. Synthetic non-covalent molecular assemblies mimic some aspects of biological structures or membrane reaction chains. In order to effectively mimic a membrane they must possess at least one of four important structural and functional properties of proteins[24]. These are (i) amphiphilic character, (ii) asymmetry, (iii) secondary amide group functionality and (iv) redox- and photochemically active cofactors. In a general sense, amphiphilic character gives access to organic media for the dissolution of hydrophobic molecules in bulk water. Asymmetry allows molecular ordering, in which sequences can identify with each other and self-assemble to form complex structures. Amide hydrogen bonds, or equivalent linear and two-sided interactions, cement these supramolecular assemblies. Dyes interact with sunlight, electrons and oxygen to bring power into the housings of molecular dimensions.

BLMs and analogous molecular assemblies are of significant importance in supramolecular chemistry.

Up until 1977, the non-covalent polymeric assemblies found in biological membranes rarely attracted any interest in supramolecular organic chemistry. Pure phospholipids and glycolipids were synthesised for biophysical chemists to investigate the physical properties of vesicles. Very few attempts were made to deviate from natural membrane lipids and to develop artificial membrane systems. However, in 1977, when Kunitake *et al.*[25] showed that didodecyl dimethylammonium bromide formed stable vesicles, it opened the way to simple and modifiable membrane structures. The following figure represents the very first synthetic amphiphile that formed membrane structures.

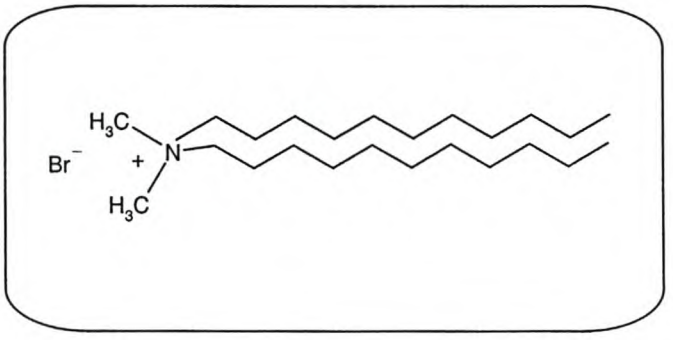


Figure 2.1 Chemical structure of the didodecyl dimethylammonium bromide, the first vesicle forming amphiphile synthesised by Kunitake *et al.*[25]

2.3.2 Bola-amphiphiles

Fuhrhop *et al.*[26] were largely responsible for stimulating research in the field of amphiphiles. They demonstrated the potential of amphiphile research in the quest for synthetic lipid membranes. In 1983, Fuhrhop *et al.*[27] took a significant step closer to preparing synthetic lipid membranes when they demonstrated that the arrangement of monolayer membranes (MLMs) made from bola-amphiphiles could be stereochemically controlled and that such membranes could be perforated with non-polymeric organic compounds[28,29]. Fuoss and Edelson[30] first introduced the term "bolaform electrolyte" in 1951, for a chain of hydrophobic groups connecting two ionic groups. A bola-amphiphile is simply defined as a molecule in which two or more hydrophilic groups are connected

by hydrophobic functions[31]. Important natural bola-amphiphiles[32-34] are the membrane lipids of thermophilic and acidophilic archaebacteria, e.g. *Sulfolobus solfataricus*. This bacterium grows best at 85°C and pH 2. The following is a structural representation of the first natural bola-amphiphile discovered.

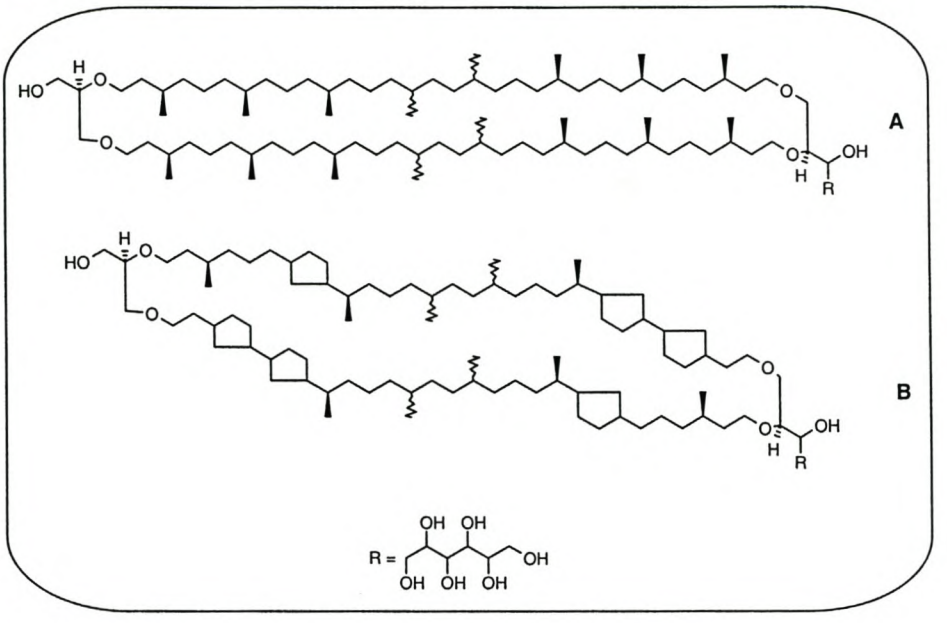


Figure 2.2 Chemical structure of the two macrocyclic tetraethers (**A** & **B**) found in the membrane lipids of thermophilic and acidophilic archaebacteria[34].

Much work has been done to explore the potential of the various forms of these bola-amphiphiles[30,34-38]. The following figure illustrates some of the different types of bola-amphiphiles synthesised to date.

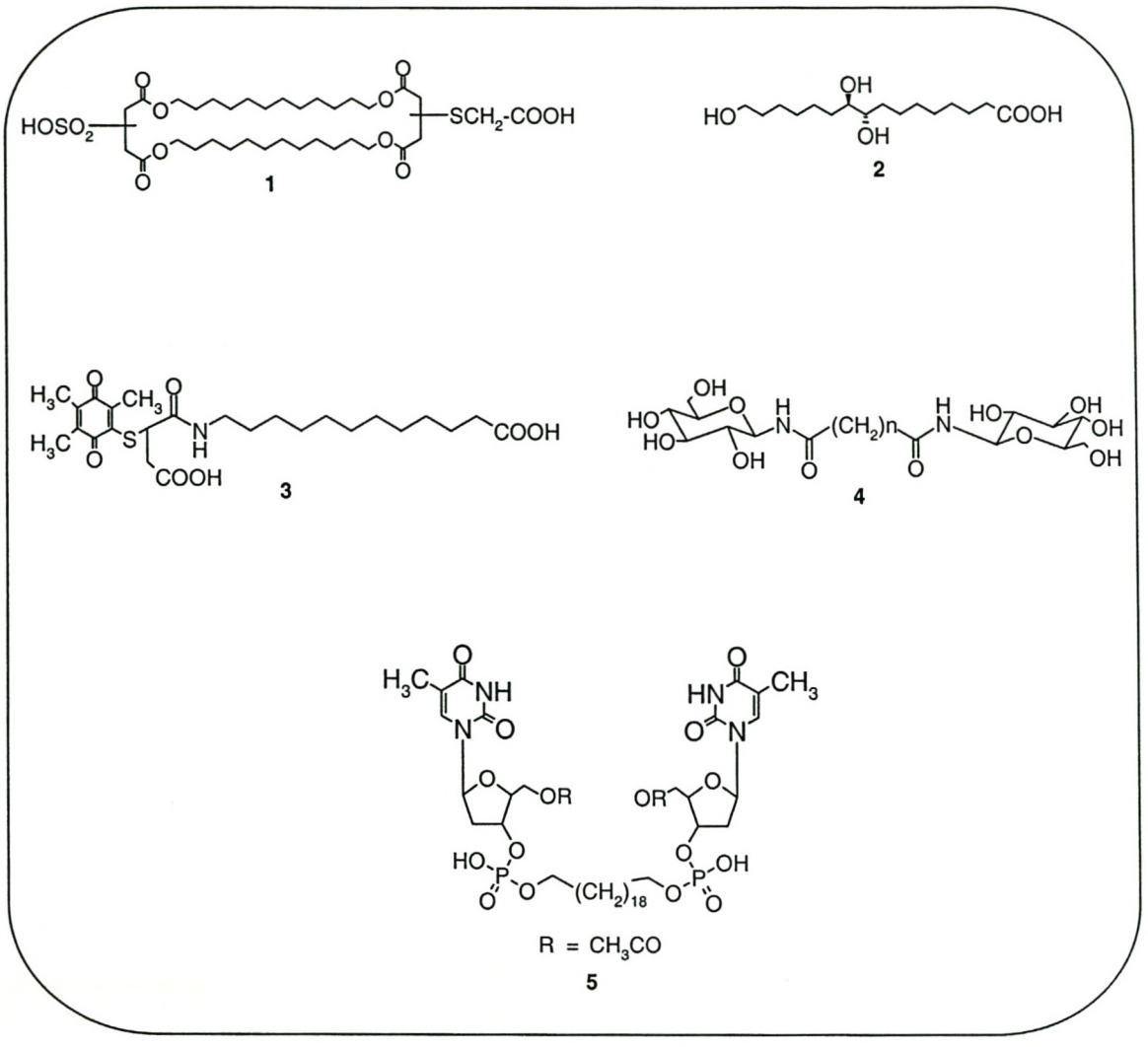


Figure 2.3 Examples of some of the bola-amphiphiles synthesized to date (**1**[31], **2**[35], **3**[36], **4**[37] and **5**[39]).

2.3.3 Peptide bola-amphiphiles

Peptide amphiphiles have been effectively used to create unique and fascinating supramolecular complexes[40-42]. The use of peptide bola-amphiphiles came to the fore when a group of Japanese chemists[43], using dicarboxylic oligoglycine bola-amphiphiles, reported vesicle assembly in microtubes. This marked the advent of a unique and fertile area of research. Significant progress has been achieved in exploring the potential of the various peptide bola-amphiphiles synthesised to date[43-50]. The following figure shows the various libraries of peptide bola-amphiphiles synthesised and characterised by Shimizu, to date.

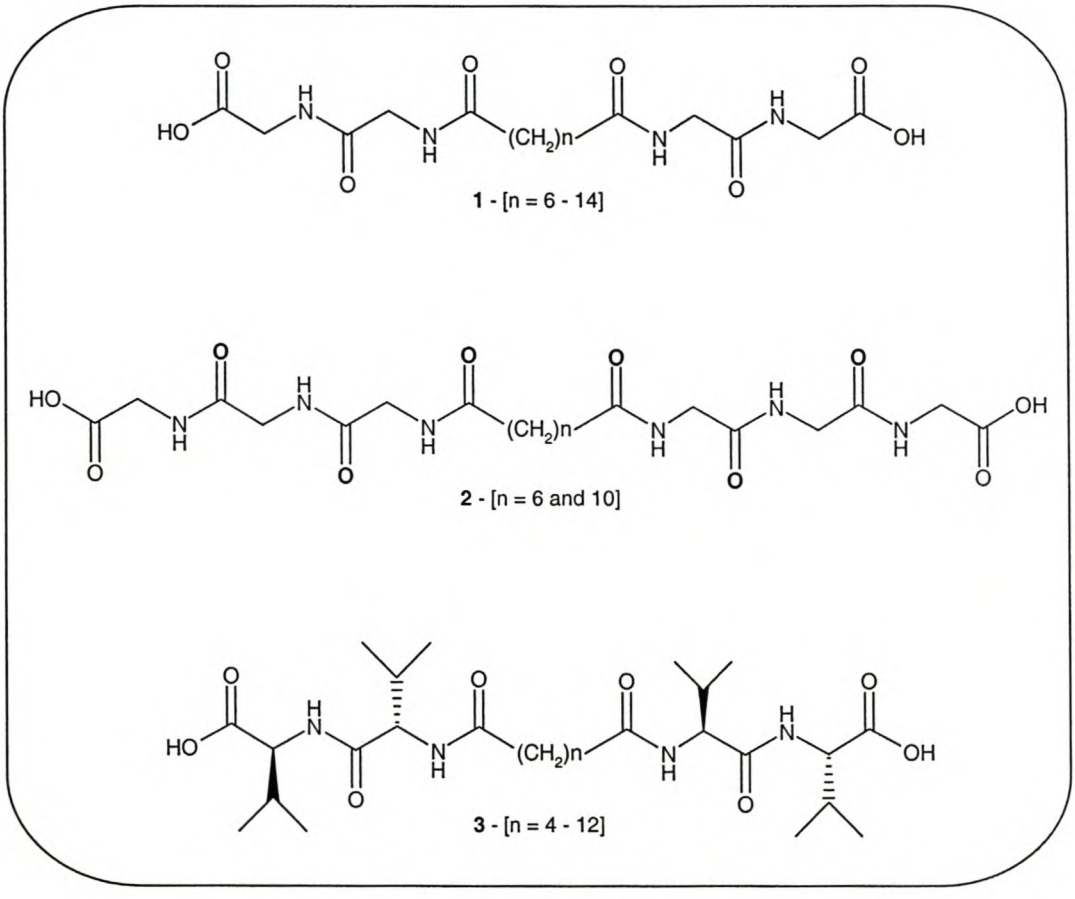


Figure 2.4 Libraries of peptide bola-amphiphiles synthesised and characterised by Shimizu *et al.* **1**[43,46,47] **2**[44] and **3**[45,48].

An interesting feature of these compounds is their ability to self-assemble into numerous types of morphologies, e.g. vesicles, fibres, spheroids and tubes. These bolaphiles also form nanotubes[49,50], comparable to those created by Ghadiri[51,52] using cyclic D,L-peptides.

The use of ω-amino acids as the hydrophobic functionality in the design of a library of peptide bola-amphiphiles[53,54] also produced excellent results. The following are examples of the latter synthesized using ω-amino acids in the design of bola-amphiphiles.

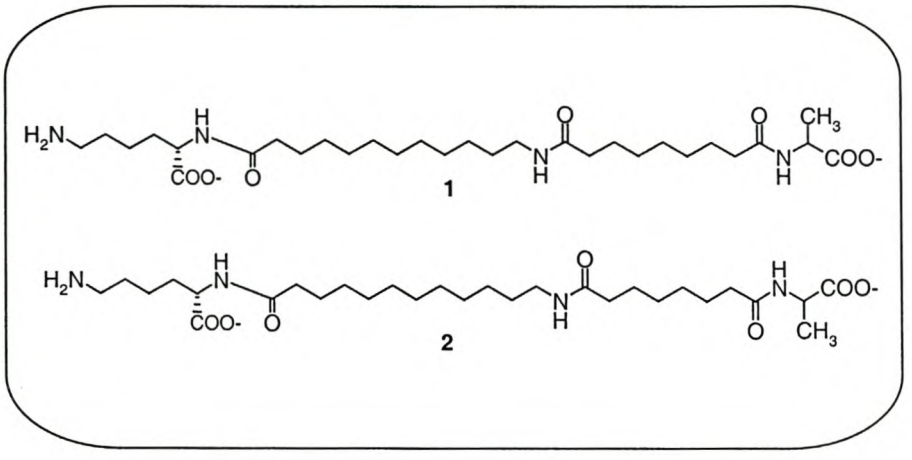


Figure 2.5 Peptide bola-amphiphiles synthesised by Fuhrhop *et al.*[54] using ω-amino acids.

The achievements of the various research groups involved in this relatively new field of peptide amphiphile/bola-amphiphile supramolecular chemistry to date have taken the concept of peptide bola-amphiphile design much closer to the creation of the ideal membrane mimic and enabled the formation of unique supramolecular architecture.

2.3.4 Natural cationic bola-amphiphilic peptides

Many insects produce antibacterial peptides in response to infection of bacteria or body injury[55]. Sapecins (sapecin, sapecin B and sapecin C) are a group of antimicrobial bacterial peptides isolated from the culture medium of an embryonic cell line, NIH-Sape-4, derived from *Sarcophaga peregina* (flesh fly)[56]. It consists of 40 amino acid residues and has bactericidal activity against various Gram-positive and Gram-negative bacteria. It also plays a role in insect development. One of the sapecin B homologues isolated from the culture medium of NIH-Sape-4 has a molecular mass and amino acid sequence very similar to that of charybdotoxin[57]. The following illustration shows the similarity between sapecin, sapecin B and charybdotoxin.

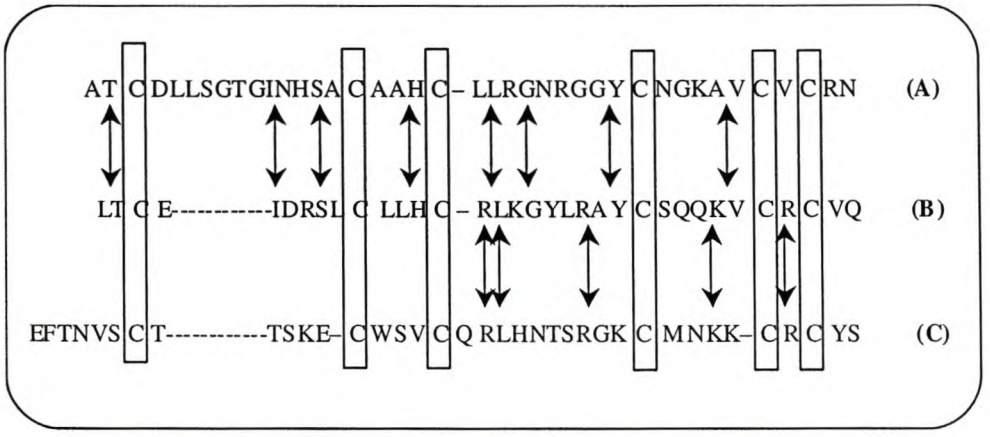


Figure 2.6 Amino acid sequences of sapecin **(A)**, sapecin B **(B)** and charybdotoxin **(C)**. Arrows indicate identical residues in the sequences.

To determine the active site of Sapecin B, the peptide was divided into four regions according to the structural model of charybdotoxin and amidated peptides corresponding to these regions were synthesized[58]. The following is an illustration of the fragmentation of sapecin B.

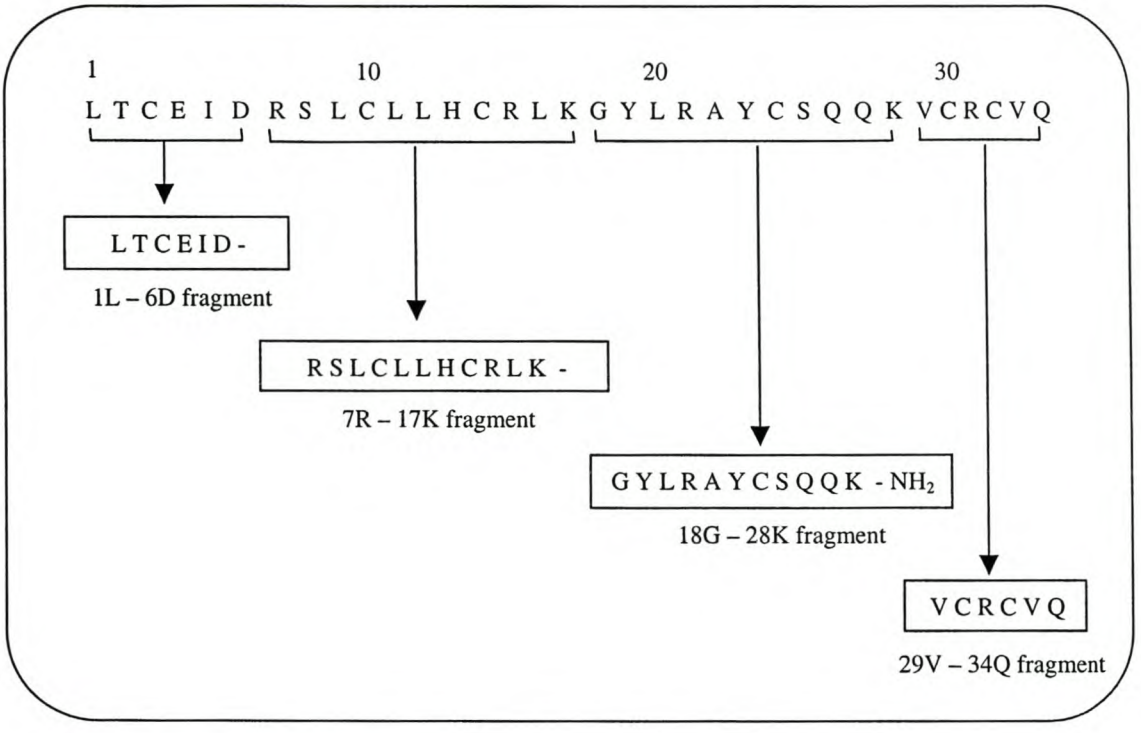


Figure 2.7 Fragmentation of sapecin B and synthetic peptides.

The 7R – 17K fragment, consisting of 11 amino acids residues, formed the α-helical region and the 29V – 34Q fragment the sheet region of sapecin B. The 7R – 17K fragment showed similar antibacterial activity to that of intact sapecin. The α-helical region of sapecin B is responsible for antibacterial activity whilst the other regions participated in the determination of antibacterial specificity of sapecin B, as this peptide demonstrated a significantly different spectrum of antibacterial activity[58] from that of sapecin B. Using the 7R – 17K fragment of sapecin B, peptides consisting of terminal polar basic motifs and internal hydrophobic oligo-leucine sequences were systematically synthesised to yield the potent antimicrobial cationic lead peptide used in this study[59], i.e. KLKLLLLLKLK-NH_2.

The field of peptide bola-amphiphiles is relatively new, one that shows tremendous potential, providing endless possibilities to create intricate, fascinating and unique forms of matter; the only limitation being is that there are none.

2.4 References

1. Wöhler, F., *Poggendorfs Ann. Physik.,* 1828, *12*, 253.

2. Lehn, J.-M., *Pure Appl. Chem.,* 1978, *50*, 871-892.

3. Lehn, J.-M., *Angew. Chem. Intl. Ed. Engl.,* 1988, *27*, 89-112.

4. Lehn, J.-M., *Supramolecular Chemistry: Concepts and Perspectives,* 1995, VCH, Weinheim, pp 11-16.

5. Lehn, J.-M., *Science,* 2002, *295*, 2400-2403.

6. Lehn, J.-M., *Proc. Natl. Acad. Sci.,* 2002, *99* (8), 4763-4768.

7. Lehn, J.-M., *Struct. Bonding.,* 1973, *16,* 1-69.

8. Whitesides, G. M., Simanek, E. E., Mathias, J. P., Seto, C. T., Chin, D. N., Mammen, M., Gordon, D. M., *Acc. Chem. Res.,* 1995, *28*, 37-44.

9. Fyfe, M. C. T., Stoddart, J. F., *Acc. Chem. Res.,* 1997, *30*, 393-401.

10. Whitesides, G. M., Mathias, J. P., Seto, C. T., *Science,* 1991, *254*, 1312-1319.

11. Whitesides, G. M., *Sci Amer.*, 1995, *273* (3), 146-149.

12. Philp, D., Stoddart, J. F., *Angew. Chem. Intl. Ed. Engl.,* 1996, *35*, 1154-1196.

13. Whitesides, G. M., *Proc. Natl. Acad. Sci.,* 2002, *99* (8), 4769-4774.

14. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J. D., *Molecular Biology of the Cell,* 1994, Garland, New York.

15. Schwiebert, K. E., Chin, D. N., MacDonald, J. C., Whitesides, G. M., *J. Amer. Chem. Soc.,* 1996, *118*, 4018-4029.

16. Schmidt-Mende, L, Fechtenkotter, A., Mullen, K., Moons, E., Friend, R. H., MacKenzie, J. D., *Science*, 2001, *293*, 1119-1122.

17. De Rosa, C., Park, C., Thomas, E. L., Lotz, B., *Nature*, 2000, *405*, 433-437.

18. Lowe, C. R., *Curr. Opin. Struc. Biol.,* 2000, *10*, 428-434.

19. Olenyuk, B., Whiteford, J. A., Fechtenkotter, A., Stang, P. J., *Nature*, 1999, *398*, 796-799.

20. Lehn, J.-M., *NATO ASI Ser. E.,* 1996, *320*, 511.

21. Lehn, J.-M., *Supramolecular Science: Where It Is and Where It Is Going,* 1999, Kluwer, Dordrecht, pp. 287-304.

22. Lehn, J.-M., *Chem. Eur. J.,* 2000, *6*, (12), 2097-2103.

23. Hohmann, W., Krauss, M., Schneider, F. W., *J. Phys. Chem.,* 1998, *102*, 3103-3111.

24. Fuhrhop, J.–H., Köning, J., *Membranes and Molecular Assemblies: The Synkinetic Approach,* 1994, The Royal Society of Chemistry, Cambridge, UK, pp. 1-7.

25. Okahata, J., Kunitake, T., *J. Amer. Chem. Soc.*, 1977, *99*, 3860-3861.

26. Fuhrhop, J.–H., Heifrich, W., *Chem. Rev.,* 1993, *93* (4), 1565-1582.

27. Fuhrhop, J.–H., Mathieu, J., *J. Chem. Soc., Chem. Commun.*, 1983, 144-145.

28. Fuhrhop, J.–H., Liman, U., *J. Amer. Chem. Soc.,* 1984, *106*, 4643-4644.

29. Fuhrhop, J.–H., Liman, U., David, H., *Angew. Chem. Intl. Ed. Engl.,* 1985, *24*, 339-340.

30. Fuoss, R. M., Edelson, D. J., *J. Amer. Chem. Soc.,* 1951, *73*, 269-273.

31. Fuhrhop, J.–H., Bach, R., *Advances in Supramolecular Chemistry*, 1992, *Vol. 2*, JAI Press Inc., pp. 25-63.

32. Gliozzi, A. Rolandi, R. De Rosa, M. Gambacorta, A. *J. Membr. Biol.,* 1983, *75*, 45-56.

33. Gliozzi, A., Paoli, G., Rolandi, R. De Rosa, M. Gambacorta, A. *J. Electroanal. Chem.,* 1982, *141*, 591-601.

34. Gulik, A., Luzzati, V., De Rosa, M., Gambacorta, A., *J. Mol. Bio.*, 1985, *182*, 131-149.

35. Fuhrhop, J.–H., Fritsch, D., *Acc. Chem. Res.,* 1986, *19*, 130-137.

36. Escamilla, G. H., Newkome, G. R., *Angew. Chem. Int. Ed. Engl.*, 1994, *33*, 1937-1940.

37. Shimizu, T., Masuda, M., *J. Amer. Chem. Soc.,* 1997, *119*, 2812-2818.

38. Nakazawa, I., Masuda, M., Okada, Y., Hanada, T., Yase, K., Asai, M., Shimizu, T., *Langmuir,* 1999, *15*, 4757-4764.

39. Iwaura, R., Yoshida, K., Masuda, M., Yase, K., Shimizu, T., *Chem. Mater.,* 2002, *14*, 3047-3053.

40. Hartgerink, J. D., Beniash, E., Stupp, S. I., *Science,* 2001, *294*, 1684-1688.

41. Niece, K. L., Hartgerink, J. D., Donners, J. J. J. M., Stupp, S. I., *J. Amer. Chem. Soc.,* 2003, *125*, 7146-7147.

42. Claussen, R. C., Rabatic, B. M, Stupp, S. I., *J. Amer. Chem. Soc.,* 2003, *125*, 12680-12681.

43. Shimizu, T., Kogiso M., Masuda, M., *Nature*, 1996, *383*, 487.

44. Kogiso, M., Ohnishi, S., Yase, K., Masuda, M., Shimizu T. *Langmuir*, 1998, *14*, 4978-4986.

45. Kogiso, M., Hanada, T., Yase, K., Shimizu, T., *Chem. Commun.*, 1998, 1791-1792.

46. Kogiso M., Masuda, M., Shimizu, T., *Supramol. Chem.,* 1999, *9*, 183-189.

47. Shimizu, T., Ohnishi, S., Kogiso. M., *Angew. Chem. Int. Ed. Engl.*, 1998, *37*, 3260-3262.

48. Kogiso, M., Okada, Y., Hanada, T., Yase, K., Shimizu, T., *Biochim et Biophys Acta.*, 2000, *1475*, 346-352.

49. Matsui, H., Gologan, B., *J. Phys. Chem. B.,* 2000, *15*, 3383-3386.

50. Matsui, H., Douberly, G. E., *Langmuir,* 2001, *17*, 7918-7922.

51. Ghadiri, M. R., Granja, J. R., Milligan, R. A., McRee, D. E., Khazanovich, N., *Nature*, 1993, *366*, 324-327.

52. Ghadiri, M. R., Kobayashi, K., Granja, J. R., Chadha, R. K., McRee, D. E., Khazanovich, N., *Angew. Chem. Int. Ed. Engl.*, 1995, *34*, 95-98.

53. Fuhrhop, J.–H., Spiroski, D., Boettcher, C., *J. Amer. Chem. Soc.,* 1993, *115*, 1600-1601.

54. Schneider, J., Messerschmidt, C., Schultz, A., Gnade, M., Schade, B., Luger, P., Bombicz, P., Hubert, V., Fuhrhop, J.–H., *Langmuir*, 2000, *16*, 8575-8584.

55. Jayasuriya, N., Bosak S., Regen, S. L., *J. Amer. Chem. Soc.*, 1990, *112*, 5844-5850.

56. Natori, S., *Antimicrobial Peptides, Ciba Foundation Symposium*, 1994, 123-134.

57. Kim, J., Iwai, H., Kurata, S., Takahashi, M., Masuda, K., Shimada, I., Natori, S., Arata, Y., Sato, K., *Febs. Lett.,* 1994, *342*, 189-192.

58. Yamada, K., Natori, S., *Biochem. J.*, 1993, *291*, 275-279.

59. Yamada, K., Natori, S., *Biochem. J.*, 1994, *298*, 623-628.

Chapter 3

Bi-directional solid phase synthesis of a model oligoglycine-based bola-amphiphile and purification by self-assembly

Abstract

Peptide fragment condensation based on solid phase peptide synthesis was used to synthesise bola-amphiphilic compounds containing a *bis*-acyl moiety. The method was developed using a model bola-amphiphile, suberamide$(GG)_2$. The peptide synthesis is bi-directional (N→C and C→N) and versatile, enabling the synthesis of new dicarboxylic oligopeptide bola-amphiphiles and other analogous compounds. The product, suberamide$(GG)_2$, was purified using its inherent ability to self-assemble in an acidic solution.

3.1 Introduction

Fuoss and Edelson[1] first introduced the term "bolaform electrolyte" in 1951 for a hydrophobic chain connecting two ionic moieties. Essentially, a bola-amphiphile is a molecule consisting of two hydrophilic species connected by a hydrophobic tether. Since the discovery of bola-amphiphiles, many compounds of a similar chemical nature have been synthesised and characterised[2-7]. These amphiphilic compounds display two very unique properties, viz. if designed in a particular way they tend to have biological activity, as well as the propensity to self-assemble[3]. In 1996 Shimizu *et al.*[4] indicated the possibility of vesicle assembly in microtubular structures made up of synthetic oligoglycine-based bola-amphiphiles. These dicarboxylic glycylglycine bola-amphiphiles self-assemble in a variety of ways, such as by the formation of vesicles, fibres and spheroids[5]. The formation of these assembly patterns is pH dependent in aqueous dispersions[6].

Taking into account that many peptides have a natural tendency to self-assemble, we investigated the synthesis of symmetrical and non-symmetrical peptide bola-amphiphiles of varying length. To achieve this, we adapted a solid phase synthetic route to synthesise a model bola-amphiphile, suberamide$(GG)_2$ (Fig. 3.1B), where G represents the amino acid glycine, one of the bola-amphiphiles created by Grigoryan *et al.*[7] using solution phase synthesis. Solution phase methods depend heavily on the unpredictable solubility properties of the peptide intermediates, while solid phase synthesis offers the ease of intermediate purification from starting materials and by-products by simple filtration and washing steps of resin bound intermediates. Furthermore, the ease of synthesising very complex peptides, selectively protected on their functional side-chains, and even large peptide libraries by solid phase makes solid phase the methodology of choice in conventional peptide synthesis.

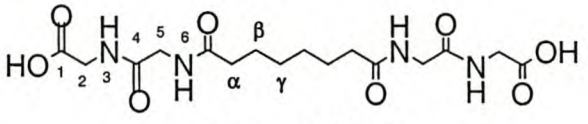
The synthetic strategy used here is based on the solid phase peptide synthesis (SPPS) protocol developed by Atherton, Sheppard and Dryland[8], where the peptide is anchored with an acid labile ester bond and chain elongation proceeds from the C-terminus towards the N-terminus. The protocol employs a protection scheme in which a base-labile N^9-fluorenylmethyloxycarbonyl (Fmoc) group, requiring only mild removal conditions[9], is used to protect the α-amino group of the incoming amino acid. The structure of bola-amphiphiles, including dicarboxylic acids, requires an adaptation of conventional SPPS synthetic protocol to a bi-directional synthetic route (Fig. 3.1). The first part of the synthesis is in the C→N direction, leaving the second carboxyl group of dicarboxylic acid linker open for the following part of the synthesis in the N→C direction. The reversal of the synthesis direction to the N→C direction poses some problems and early studies in peptide synthesis chemistry showed that most of the N→C synthesis strategies resulted in optically impure peptides[10]. Numerous synthetic strategies have since been introduced to address this anomaly[11-17]. We report here on the use of one of the strategies[17] to synthesise novel peptide bola-amphiphiles. The choice of method depended on the solubility of the peptide fragment being attached in the N→C direction.

In order to synthesise in the N→C direction, the resin-bound carboxyl-group must be activated and the carboxyl-group of the incoming amino acid must be reversibly protected to prevent polymerisation. *In situ* activation of the carboxyl group of the resin-bound

linker by, for example, methods using phosphonium based (BOP, PyBOP®, PyBroP®)[18,19,20], uronium based (HBTU, TBTU, TNTU, TSTU)[21] or carbodiimide (DCCI, DIPCDI, EDC)[22] activation reagents is quite feasible if the α-carboxyl group of the incoming amino acid is reversibly protected (see footnote* for abbreviations). Phosphonium based reagents react with the α-carboxyl groups in the presence of a base to form the highly active benzotriazolyl ester[23]. Combining 1-hydroxybenzotriazol (HOBt) with uronium-based reagents or carbodiimides also leads to an extremely active benzotriazolyl ester of the carboxyl group[23]. HOBt is also a so-called configuration-trapping agent that limits racemisation to less than 0.4% for most amino acids[24].

The reversible protection of the α-carboxyl group of the incoming amino acid could be quite complex if introduced into the base-labile Fmoc-synthesis strategy. Pre-activation of the carboxyl group by forming a more stable active ester, such as a pentafluorophenyl ester[25] should eliminate the necessity of protecting the α-carboxyl group of the incoming amino acid. Alternatively, the use of the carbodiimide EDC offers the possibility of pH-control of the activation and coupling reactions, thus also eliminating the necessity of protecting the incoming carboxyl group.

Here the first bi-directional solid phase synthesis, using a water-soluble carbodiimide, namely *N*-ethyl-*N'*-(3-dimethylaminopropylcarbodiimide) (EDC) for the N→C synthesis step, of a model oligoglycine bola-amphiphile (**A**) is reported.



(A)

Suberamide(GG)$_2$

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* Abbreviations: BOP: benzotriazol-1-yl-oxy-*tris*-dimethylaminophosphonium hexafluorophosphate; PyBOP®: benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate; PyBroP®: bromo-*tris*-pyrrolidino-phosphonium hexafluorophosphate; HBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TNTU: 2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate; TSTU: *O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate; DCCI: dicyclohexylcarbodiimide; DIPCDI: diisopropylcarbodiimide; EDC: *N*-ethyl-*N'*-(3-dimethylaminopropylcarbodiimide)

3.2 Experimental

3.2.1 Materials

NovaSyn® KA (0.15 milli-equivalent/g), NovaSyn® TGT (0.2-0.26 milli-equivalent/g), 1-hydroxybenzotriazol (HOBt) and benzotriazol-1-yloxy-*tris*-pyrrolidinophosphonium hexafluorophosphate (PyBOP®) were obtained from Novabiochem Co. (Luzern, Switzerland). Fmoc-Gly-OH was from Advanced ChemTech (Louisville, USA). *N,N*-dimethylformamide (DMF, 99.5%), glacial acetic acid, diethyl ether (99.5%), glycylglycine (>98%), sodium dihydrogen phosphate dihydrate (99%) and disodium hydrogen phosphate dihydrate (99%) were from Merck (Darmstadt, Germany). 2-Methylbutan-2-ol (*t*-amyl alcohol; 98%) was from BDH Chemicals (Poole, UK). *N,N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA, >98%), piperidine (98%), suberic acid (>98%), pyridine (98%), piperidine (98%), *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC) and aluminum oxide (>98%) were from Sigma Chemical Co. (St. Louis, USA). Acetonitrile (HPLC-grade, UV cut-off 190 nm) was from Romil LTD (Cambridge, UK). Analytical grade water was prepared by filtering glass distilled water through a Millipore Milli Q® water purification system.

3.2.2 Bola-amphiphile synthesis

The *in situ* activation of the carboxyl groups of Fmoc-Gly-OH and suberic acid linker was accomplished using PyBOP®, a relatively low cost reagent, HOBt as catalyst and DIPEA as base[11].

Part A: Synthesis of R-O-GG-CO(CH_2)$_6$COOH (A): The synthesis was accomplished on a polydimethylacrylamide resin encapsulated in Kieselguhr (R). Conventional SPPS, based on the Fmoc chemistry, was used with freshly distilled amine-free DMF as solvent[26] and 20% high quality distilled piperidine in DMF as base for removal of the Fmoc group after each coupling step. The first Gly residue was coupled to the resin with an acid labile ester bond. Activation of the incoming carboxyl-group was accomplished by using PyBOP® as activation agent, with freshly distilled DIPEA as base and HOBt as catalyst. The PyBOP® reagent was used as follows: a fivefold molar equivalent (in terms of resin capacity) of both Fmoc-Gly-OH (or suberic acid) and HOBt were dissolved in a minimum amount of DMF, a fivefold molar equivalent of PyBOP® was dissolved

separately in a minimum of DMF and mixed with a tenfold molar equivalent DIPEA, and both were mixed thoroughly with the resin. In our shake flask method the total volume of DMF was limited to <1.5 mL/gram of resin. The reaction time of the coupling steps was approximately 60 minutes for coupling of the Gly-residues and overnight for the dicarboxylic acid linker. The coupling time depended on the completeness of acylation as determined by the Kaiser test[27] and Fmoc test[9] after the coupling of the Gly-residues, and a picric acid test[28] after the coupling of the dicarboxylic acid linker. After the coupling of the linker the resin was washed with DMF and diethyl ether then thoroughly dried under vacuum at room temperature.

The scheme below illustrates the general solid phase synthesis strategy employed.

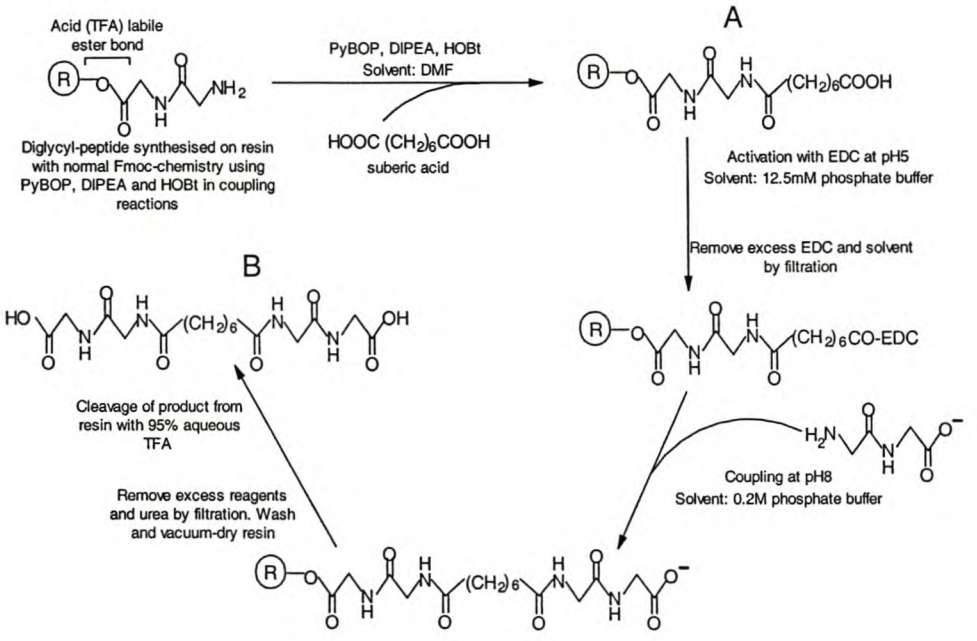


Figure 3.1 A scheme depicting the general solid phase synthesis strategy employed, based on the synthesis of the model bola-amphiphile suberamide$(GG)_2$.

Part B: Synthesis of Suberamide$(GG)_2$ (B in Fig. 3.1): The unprotected glycylglycine fragment was attached to R-O-GG-CO$(CH_2)_6$COOH (**A** in Fig. 3.1) using a two-step carbodiimide method[16]. A ten times molar equivalent of EDC was dissolved in 250 µL analytical grade water and added to the resin swollen in 250 µL of 25mM phosphate buffer (pH 5.0). After fifteen minutes activation, the excess EDC was removed from the resin and the suspension of activated resin was added drop-wise to 1.5 mL glycylglycine in 0.2M phosphate buffer (pH 8.0). The resin mixture was gently shaken for 36 hours where after it was washed on a sintered glass filter with water, *t*-amyl alcohol, acetic acid, again *t*-amyl alcohol and finally with peroxide-free diethyl ether, and then thoroughly dried under vacuum. To monitor the coupling reaction, a sample of the resin (~5-10 mg) was taken after 30 hours, washed and treated with TFA (as described in 3.2.3). The TFA-product mixture was then removed and evaporated under nitrogen. The residual product was made up in 50% acetonitrile and analysed using electrospray ionisation mass spectrometry (ESMS).

3.2.3 Removal of peptides from the solid phase resin

The peptides were cleaved from the resin with 95% TFA and 5% H_2O as scavenger. The peptide resin was treated for three to six hours with four-bed volumes 95% TFA. After cleavage, the resin was removed by filtration and washed with 95% TFA, acetic acid and analytical grade water. The combined filtrate containing the bola-amphiphile was dried under high vacuum on a Buchi Rotavapor at 40 - 45°C, resuspended in 50% acetonitrile and lyophilised.

3.2.4 Purification of Suberamide$(GG)_2$

The synthesis product was purified using the unique self-assembly properties of the bola-amphiphile product. A 4 mg/mL solution was prepared in 0.1% TFA and the solution was left undisturbed for a period of five days. The self-assembled solution was then centrifuged, using a bench top PicoFuge™, for five minutes and the supernatant carefully removed. The resulting pellet was dissolved in 50% acetonitrile and lyophilised.

3.2.5 Analysis of synthesis products

Electrospray ionisation mass spectrometry (ESMS) was performed using a Micromass triple quadrupole mass spectrometer fitted with an electrospray ionisation source. The carrier solvent was 50% (v/v) acetonitrile in analytical grade water delivered at a flow rate of 20 µL/minute during each analysis, using a Pharmacia LKB 2249 pump. Ten µL of the sample solution (0.2 mg in 1.0 mL of a 50% CH_3CN:0.05% aqueous TEA solution or 50% CH_3CN) was introduced into the ionisation source using a Rheodyne injector valve. A capillary voltage of 3.5 kV was applied throughout and the source temperature was set at 80°C. The skimmer lens offset was 5 V and the cone voltage was varied between 50 and 60 V. Data acquisition was in the negative or positive mode, scanning the first analyser (MS_1), through m/z = 100 to 1500 m/z at a scan rate of 675 atomic mass units/second. Averaging spectra across the elution peak and subtracting the background produced representative spectra. The instrument was calibrated using the ion spectrum of polyethylene glycol acquired under similar conditions.

To obtain the fragmentation patterns the $[M+H]^-$ molecular specie was selected in MS_1 and subjected to collisionally induced decomposition (CID). These experiments were

conducted using the same ionisation parameters as described before. Argon was introduced into the collision cell at (1.8 ± 0.2) x 10^{-3} millibar and the collision energy setting was 30 eV. Data acquisition was in the negative mode, scanning the second analyser (MS_2), through *m/z* = 10 to 450 *m/z*, at a scan rate of 220 atomic mass units/second.

^1H NMR and ^{13}C NMR spectra of suberamide$(GG)_2$ in DMSO-d_6 were recorded on a Varian VXR 300 MHz instrument. Assignments were made empirically.

3.3 Results and discussion

The synthesis of suberamide$(GG)_2$ was conducted according to the general protocol outlined in Fig. 3.1. In the synthesis of the bola-amphiphile the coupling of the first amino acid [i.e. Gly (G)] with an acid-labile ester bond to the resin was >99%, according to an analytical Fmoc test[9]. The elongation of each of the peptide chains in the N→C direction was successful, as monitored by the Kaiser test[27]. Coupling of the dicarboxylic acid linkers overnight led to >99% coupling efficiency in each case, as determined by an analytical picric acid test[28].

By activating the free carboxyl group of the linker on the resin it is possible to synthesise the second peptide unit (even complex sequences) beforehand, in the normal C→N direction and couple it as a protected or unprotected peptide fragment (depending on the sequence) in the N→C direction. Although it was possible to pre-activate the free carboxyl group of the resin-bound linker by forming its pentafluorophenyl ester, the unpredictable solubility of some of the incoming peptides such as glycylglycine in suitable organic solvents made this a less attractive option. Using PyBOP® as activation agent in this step would necessitate the incoming peptide fragment to be protected in order to limit polymerisation. Unprotected glycylglycine (GG) dipeptide was readily available, but GG tended to be highly soluble in water and poorly soluble in most organic solvents. Having investigated the phosphonium based, carbodiimide based and active ester activation procedures for the N→C direction synthesis step, a modified two-step carbodiimide method, using the water-soluble carbodiimide, EDC (Fig. 3.1) was decided upon. The choice of method was based on the solubility of the peptide fragment being

attached in the N→C direction. The EDC method, commonly used in peptide fragment condensation reactions[17], utilises the water-soluble properties of the carbodiimide with activation and coupling proceeding at different pH-values (pH 5 and 8) in order to limit unwanted side reactions and polymerisation. The first step at pH 5, together with low phosphate buffer concentration (12.5mM final concentration), favours the activation of the free carboxyl group of the linker and minimise phosphate competition for EDC. Adjustment to pH 8 by addition of excess phosphate (0.2M final concentration) at the time of the addition of the glycylglycine peptide fragment prevents the activation of the incoming carboxyl groups on the incoming peptide by the excess carbodiimide. This allows the preferential formation of the peptide bond between the activated carboxyl groups of the acid and the amino group of the incoming peptide fragment. To limit the activation of the carboxyl group of the incoming peptide, the excess EDC was removed by repeated washing of the resin after the initial activation step. The high concentration of phosphate (0.2M) in the second step favours the reaction of EDC with phosphate. The resulting reaction yields a urea derivative. Phosphate thus quenches any residual EDC, further minimising activation of the carboxyl group of the incoming peptide.

The peptide bola-amphiphile was characterised by electrospray ionisation mass spectrometry (ESMS; Fig. 3.2) and NMR spectroscopy. Signal assignments refer to the system illustrated in **A** and were made empirically.

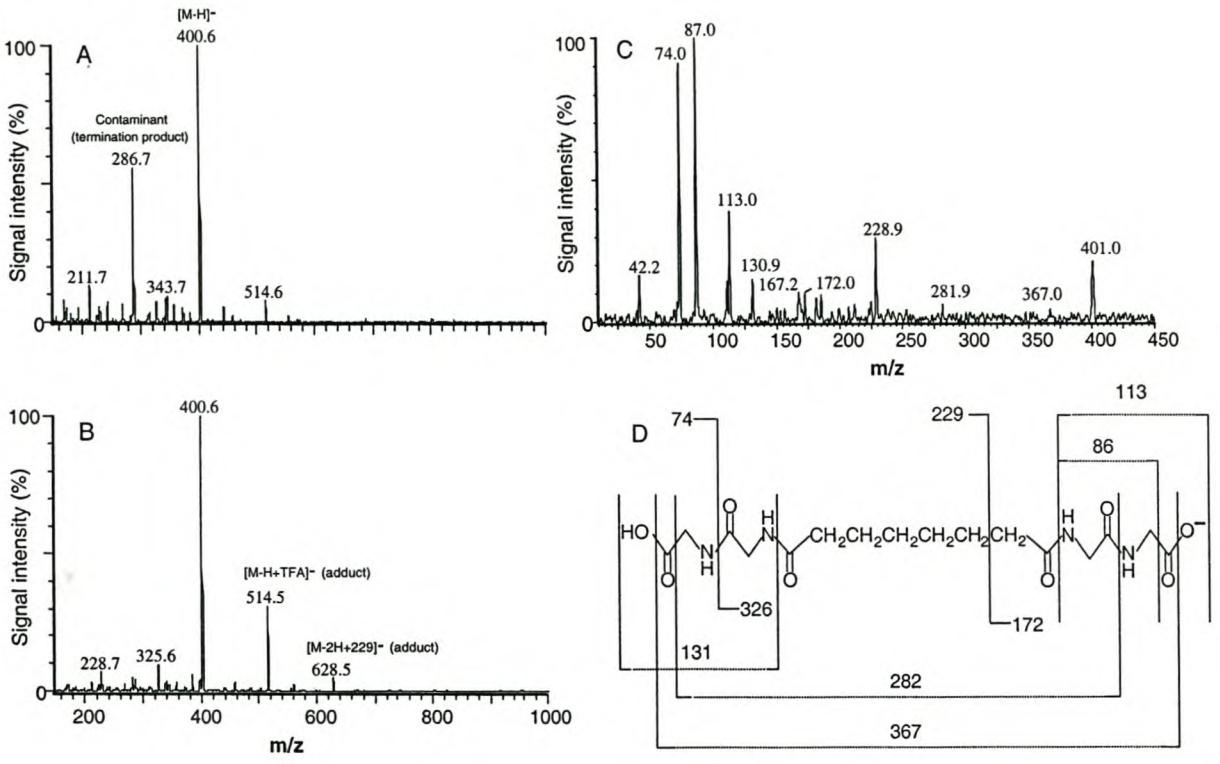


Figure 3.2 ESMS spectra of the products from the self-assembly purification step: supernatant (A) and self-assembled product in pellet (B). Spectrum (C) depicts the CID fragmentation pattern of $[M-H]^-$ after selection in MS_1. Insert (D) shows the allocation of the fragments.

3.3.1 Purification

ESMS analysis of the self-assembled product and supernatant from the purification verified the efficiency of this extremely simple purification protocol (Fig. 3.2). The major contaminant is HO-GG-CO$(CH_2)_6$COOH at m/z = 287, indicating incomplete coupling of the second Gly-Gly unit, remained in solution and could thus easily be removed (Fig. 3.2A). In the purified sample the major molecular ions were $[M-H]^-$ with m/z = 400.6 (required 401.3967) and a TFA adduct (m/z = 514.5) (Fig. 3.2B). The spectrum in Fig. 3.2C shows the CID fragmentation pattern of the m/z = 401 molecular ion. The expected fission at the amide bonds was observed and other product ions were from the loss of a carboxyl group or an OH group (Fig. 3.2 D). The product ion with m/z = 228.7 was attributed to the fission of the C-C bond neighbouring the amide carbonyl of the linker.

3.3.2 NMR data

The synthesised bola-amphiphile was fully characterised by NMR spectroscopy. NMR data collected for the compound also indicated that there was incomplete coupling in the last step of the original synthetic sequences. Signals observed at δ 2.0 - 2.2 in the ^{1}H-NMR for the α-CH_2 of the diacid linker are diagnostic, for indicating the degree of coupling of the peptide fragment. The signals observed for the half-reacted diacid, i.e. α-CH_2 of the free acid, and the superimposed α-CH_2s of the amide groups of both product and half-reacted diacid were compared and all the signals were adjusted accordingly with respect to integration.

Suberamide(GG)$_2$: Mass yield 88%; *m/z*. 400.67 [(M-H)$^-$; $C_{16}H_{26}N_4O_8$ required 401.40]; ^{1}H-NMR δ_H (300 MHz; DMSO-d_6; TMS) 1.21 - 1.29 (4H, m, 2 x γ-CH_2), 1.43 - 1.55 (4H, m, 2 x β-CH_2), 2.13 (4H, t, J = 7.4 Hz, 2 x α-CH_2), 3.45 (4H, d, J = 5.5 Hz, 5_G x 2), 3.51 (4H, d, J = 5.5 Hz, 2_G x 2); 8.13 (4H, t x 2, J = 5.4 Hz, 2 x 3_G and 2 x 6_G); δ_C (75 MHz; DMSO-d_6; TMS). 24.97 (suberic β x 2); 28.40 (suberic γ x 2); 35.17 (suberic α x 2); 41.84 (*C*H_2COO x 2); 40.93 (CONH*C*H_2CO x 2); 169.81 (CH_2CH_2*C*ONH x 2); 171.68 (CH_2*C*OO x 2); 173.09 (*C*ONHCH_2CO x 2).

3.4 Conclusions

Although SPPS is an established method for the synthesis of peptides, the bola-amphiphilic peptide under consideration in this study requires a different synthetic approach. For the first part of the synthesis conventional SPPS suffices (Fig. 3.1). However, after coupling of the dicarboxylic acid the synthesis must proceed in the reverse direction. This was done by using fragment condensation with an EDC method that limits polymerisation, even if unprotected peptide fragments are used. The EDC method not only represents a very simple, cheap and efficient way to attach peptide fragments in the N→C direction but also addresses the issue of solubility of the peptide fragments used in the synthesis of the bola-amphiphilic peptides.

The advantages of this bi-directional solid phase synthesis method are its flexibility, versatility in terms of bola-amphiphile design (*i.e.* to synthesise both symmetrical and asymmetrical peptide bola-amphiphiles) and the possibility to apply it to a convergent

synthesis protocol. Such an approach provides countless possibilities for the synthesis of "peptido"-organic compounds and even compounds with multiple peptide arms in which the direction of the peptide bond is reversed in a part of the compound. The self-assembly properties of the synthesised compound were investigated. Results are presented in Chapter 4.

Finally, a particularly important conclusion from our results is that the inherent ability of some molecules to self-assemble can be used as a purification method for compounds with self-assembly properties. Crystallisation has long been an established purification method in organic chemistry. Some organic components, however, are difficult to crystallise, especially larger molecules such as peptides and polypeptides. Many other compounds[29] and peptides[30,31] have been observed to self-assemble, but the possibility of self-assembly as a purification method for peptides and analogous compounds has gone, until now, unrecognised.

3.5 References

1. R. M. Fuoss and D. J. Edelson, 1951, *J. Amer. Chem. Soc.*, **269**, 73.

2. J. H. Fuhrhop and D. Fritsch, 1986, *Acc. Chem. Res.*, **19**, 130.

3. J. H. Fuhrhop and W. Heifrich, 1993, *Chem. Rev.*, **93**, 1565.

4. T. Shimizu, M. Kogiso and M. Masuda, 1996, *Nature*, **383**, 487.

5. M. Kogiso, S. Ohnishi, K. Yase, M. Masuda and T. Shimizu, 1998, *Langmuir*, **14**, 4978.

6. H. Matsui and B. Gologan, 2000, *J. Phys. Chem. B.*, (104), **15**, 3383.

7. N. A. Grigoryan, Z. O. Mndzhoyan, E. V. Kazaryan, Z. Yu. Ter-Zakharyan and O. L. Mndzhoyan, 1992, *Pharm. Chem. J.*, (2), **26**, 153.

8. E. Atherton and R. C. Sheppard, 1989, *Solid phase synthesis: A practical approach*, In: The practical approach series, IRL Press, Oxford University Press, Oxford.

9. L. A. Carpino and G. Y. Han, 1972, *J. Org. Chem.*, **37**, 3404.

10. M. Bodansky, 1984, *Principles of peptide synthesis*, (Eds. K. Hafner, C. W. Rees, B. M. Trost, J. Lehn, P. Von Ragné Schleyer and R. Zahradnik), Springer Verlag, Berlin, pp. 202-227.

11. A. Johansson, E. Akerblom, K. Ersmark, G. Lindeberg and A. Hallberg, 2000, *J. Comb. Chem.*, **2**, 496.

12. N. Mihila, J. Bódi, A. Gömöry and H. S. Vargha, 2001, *J. Pep Sci.*, (7), **10**, 565.

13. N. Thieriet, J. Alsina, F. Guibe and F. Albericio, 2000, *Peptides for the New Millennium,* (Eds G. B. Fields, J. P. Tam and G. Barany), Kluwer Academic Publisher, Dordrecht, pp. 78-79.

14. N. Thieriet, F. Guibe and F. Albericio, 2000, *Org. Lett.*, **2**, 1818.

15. P. Wang, M. Landon, R. Layfield, R. J. Mayer and R. Ramage, 1999, *Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries,* (Ed. R. Epton), Mayflower Scientific Limited, Birmingham, pp. 415-416.

16. Y. D. Huang, S. Z. Yang, Y. M. Feng and C. I. Niu, 1999, *J. Pept. Res.*, **54**, 18.

17. M. B. Davis and J. F. Preston, 1981, *Anal. Biochem.*, **116**, 402.

18. B. Castro, J. R. Dormey, G. Elvin, C. Selve, 1975, *Tetrahedron Lett.*, 1219.

19. J. Coste, D. Le-Nguyen, B. Castro, 1990, *Tetrahedron Lett.*, **31**, 205.

20. J. Coste, M. N. Dufour, A. Pantaloni, B. Castro, 1990, *Tetrahedron Lett.,* **31**, 669.

21. R. Knorr, A. Trzeciak, W. Bannwarth, D. Gillessen, 1989, *Tetrahedron Lett.*, **30**, 1927.

22. M. Fujino , T. Fukuda, S. Kobayashi, M. Obayashi, 1974, *Chem. Pharm. Bull.*, **22**, 1857.

23. T. Hϕeg-Jensen, M. H. Jakobsen, C. D. Olsen, A. Holm, 1991, *Tetrahedron Lett.*, **32**, 7617.

24. D. S. Kemp, 1979, *Racemisation in peptide synthesis, In: The peptides: Analysis, synthesis, biology* (Eds. E. Gross, J. Meienhofer) Academic Press, New York, pp. 1-10.

25. E. Atherton, L. Cameron, R. C. Sheppard, 1988, *Tetrahedron*, **44**, 843.

26. J. M. Stewart, J. D. Young, 1984, *Solid phase synthesis*, 2nd edition, Pierce Chem. Co. Rockford, Illinois, pp. 69-70.

27. E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, 1970, *Anal. Biochem.*, **34**, 595.

28. B. F. Gisin, 1972, *Anal. Chim. Acta.*, **58**, 248.

29. D. Philp, J. F. Stoddart, 1996, *Angew. Chem. Intl. Ed. Engl.,* **35**, 1154.

30. M. R. Ghadiri, K. Kobayashi, J. R. Granja, R. K. Chadha, D. E. McRee, N. Khazanovich, 1995, *Angew. Chem. Int. Ed. Engl.*, **34**, 93.

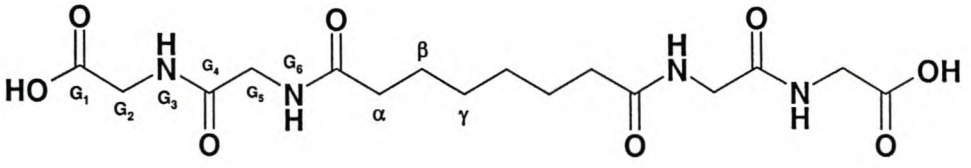
31. J. Schneider, C. Messerschmidt, A. Schultz, M. Gnade, B. Schade, P. Luger, P. Bombicz, V. Hubert, J.–H. Fuhrhop, 2000, *Langmuir*, **16**, 8575.

Chapter 4

Monitoring oligoglycine bola-amphiphile nanotube self-assembly

4.1 Introduction

Bola-amphiphiles have been the subject of much attention because of their role as ideal models of functionalised membranes[1]. Oligoglycine bola-amphiphiles, introduced by Grigoryan *et al.* [2] as possible anti-staphylococcic agents, have gained much prominence in the field of self-assembly [3-5]. In the quest for new surfactants our focus has centred on the design and synthesis of peptide bola-amphiphiles using solid phase peptide synthesis (SPPS), with the oligoglycine bola-amphiphile **(A)** serving as model in our design strategy.



(A)

We report here a new synthetic strategy for the model bola-amphiphile (**A**), solvent conditions for the optimised time required for self-assembly and, most importantly, monitoring self-assembly *via* environmental scanning electron microscopy (E-SEM) and electrospray mass spectrometry (ESMS).

4.2 Results and Discussion

4.2.1 Synthesis

Several researchers have synthesised the model bola-amphiphile **(A)** using solution phase synthesis[2-4]. This synthetic strategy has many limitations, especially in terms of versatility of peptide design. The synthesis of the model bola-amphiphile (**A**) required an adaptation

of conventional SPPS synthetic protocol to a bi-directional synthetic route. The synthesis of [HO-GG-CO(CH_2)$_6$CO-GG-OH] **(A)** was accomplished on Novasyn® KA resin *via* Fmoc chemistry[5] using freshly distilled amine-free DMF as solvent and distilled piperidine (20% in DMF) as base for the removal of the Fmoc group after each coupling step. For the first part of the synthesis, in the C→N direction, the two-glycine residues together with the dicarboxylic acid were attached to the resin *via* conventional SPPS, leaving the second carboxylic acid group of the dicarboxylic acid linker open for the following part of the synthesis in the N→C direction. To link the glycylglycine to the second carboxylic acid group the EDC (*N*-ethyl-*N'*-(3-dimethylaminopropylcarbodiimide) method, commonly used in peptide fragment condensation reactions[6], was used. The advantage of this method is that it utilises the water-soluble properties of the carbodiimide, with activation and coupling proceeding at different pH-values (pH 5 and 8 respectively). This allows the preferential formation of the peptide bond between the activated carboxylic acid group of the acid and the amino group of the incoming glycylglycine fragment, thereby limiting unwanted side reactions and polymerisation. The solubility of glycylglycine in water made this synthetic route, the method of choice here. The peptide was cleaved from the resin with 95% TFA as scavenger and then lyophilised.

4.2.2 Self-assembly (ESMS and E-SEM)

A number of solvents were used for self-assembly but 0.1% TFA in water proved to be most suitable for the promotion of self-assembly. A solution of the bola-amphiphile (10 mM, pH 2.4) in 0.1% TFA was prepared. The solution was left undisturbed at room temperature for up to seven days and samples were analysed at predetermined intervals using E-SEM and ESMS. The first sample was analysed after 7 hours. From observation 7 hours was found to be the optimal time period required for the solution to progress from clear to opaque. The results revealed the formation of monomers, dimers and trimers in solution, indicating the onset of self-assembly (Fig. 4.1). This was validated by the E-SEM image (Fig. 4.2A) obtained after 7 hours.

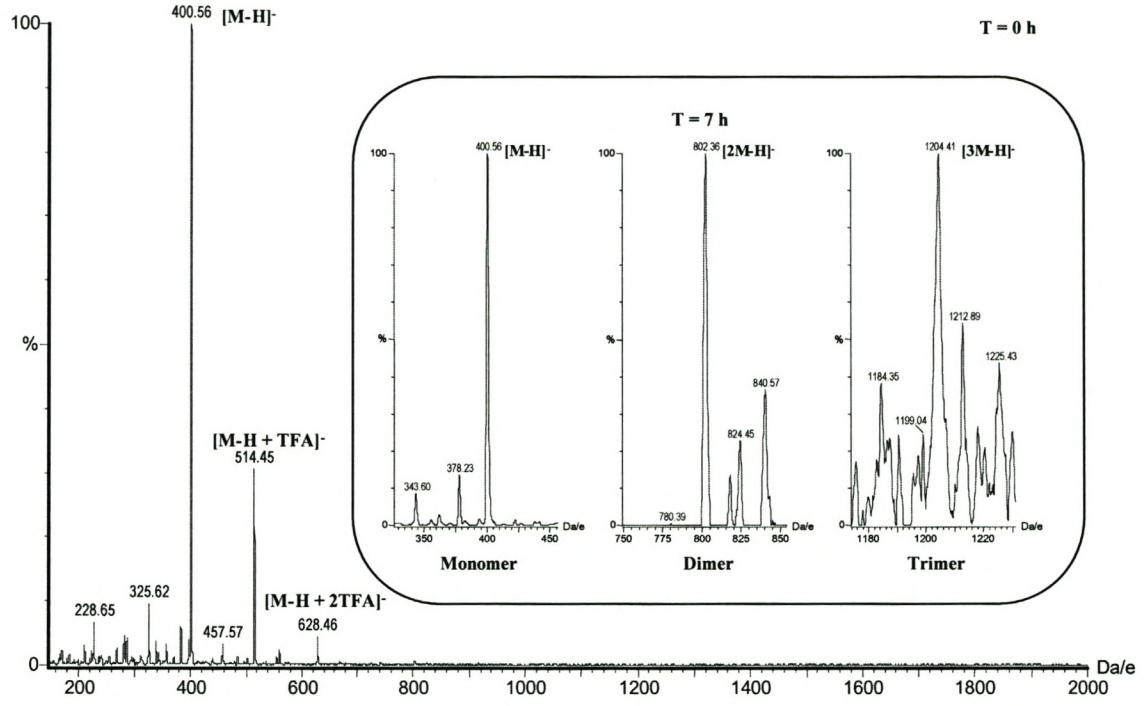


Figure 4.1 ESMS spectra of suberamide$(GG)_2$ at T = 0 and T = 7 h illustrating the formation of monomers, dimers and trimers.

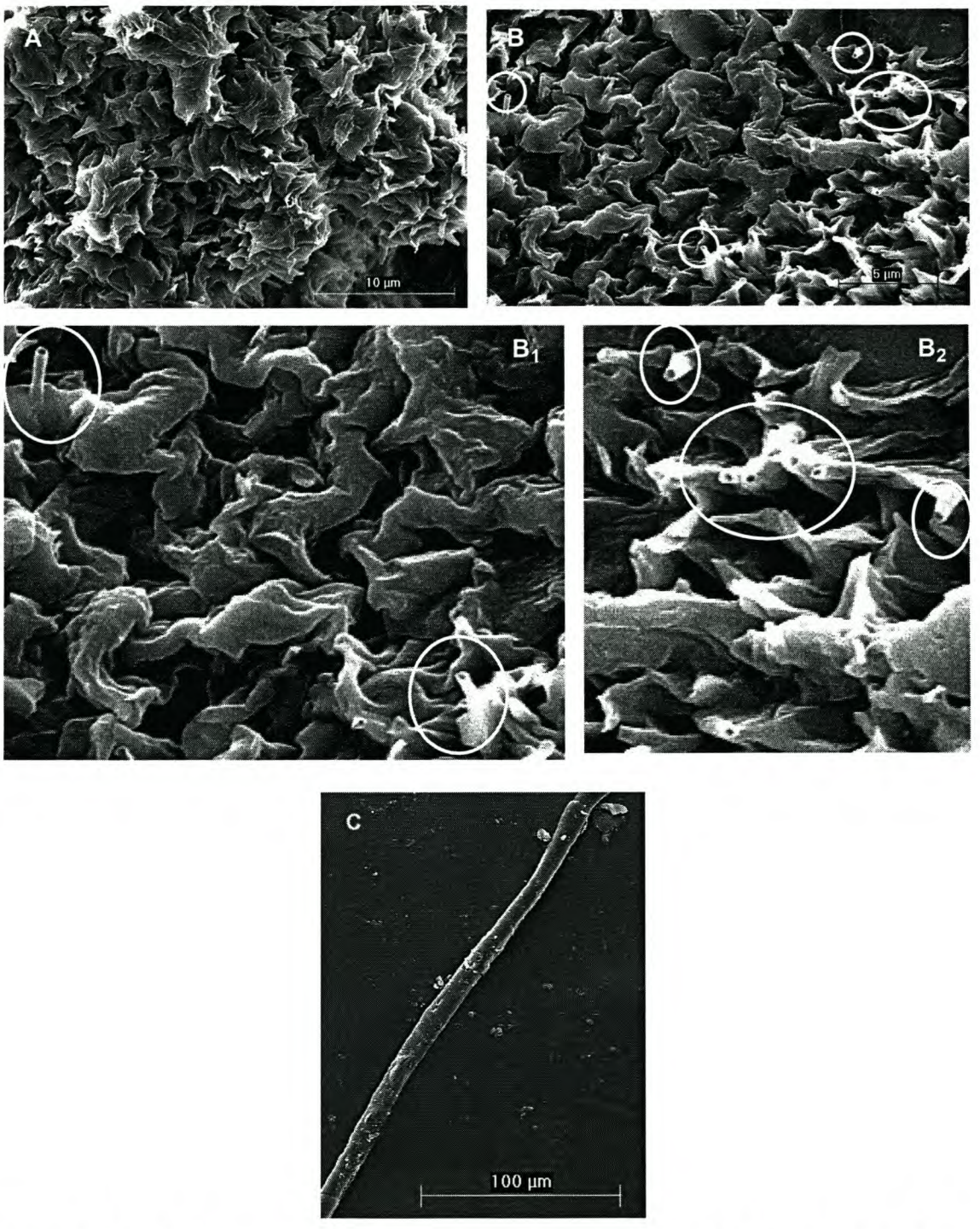


Figure 4.2 Environmental scanning electron microscopy (E-SEM) images of the self-assembly process of (**A**). A – self-assembly into monolayers after 7 hours; B – self-assembly into nanotubes after 24 hours; $B_{1,2}$ – magnified images of B illustrating nanotube formation and C – microtube after 7 days.

According to earlier reports[3,4], the time required for self-assembly was 7 – 14 days – and we obtained results within a day. This could be attributed to the promotion of strong

intralayer hydrogen bonding through the use of 0.1% TFA. TFA promotes strong intralayer hydrogen bonding by acting as dehydration and/or bridging agent by direct interaction with the peptide. The solvent also promotes more effective hydrophobic interaction of the oligomethylene chain by "drying" out the flanking peptide backbone i.e. it "out-competes" the water molecules attached to the peptide backbone[7].

4.2.3 Fourier-transform infrared spectroscopy (FT-IR)

A distinct feature of bola-amphiphiles is that the initiation of self-assembly is usually marked by the formation of monolayers[8]. These layers were clearly evident from the electron micrograph obtained after a period of 7 hours (Fig. 4.2A).

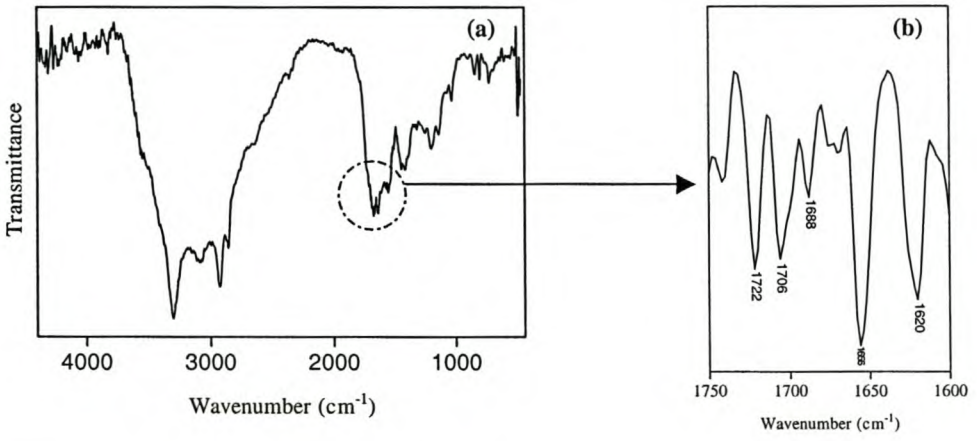


Figure 4.3 FT-IR spectrum of the lyophilised peptide self-assembly fibres of (**A**). (**a**) FT-IR spectrum in the region 500-4500 cm^{-1} and (**b**) second derivative of spectrum of (**A**) in the amide I region.

Monolayer formation is a result of intralayer hydrogen bonding via an inherent self-recognition process[9] as indicated by N-H stretching bands at 3301 and 3083 cm^{-1}, an amide II band at 1554 cm^{-1} [Fig. 4.3 (a)] and amide I bands [Fig. 4.3(b)][10].

In addition, the low pH of the solution possibly results in the shortening of the intralayer hydrogen-bonds, and this, in turn, induces a tilt in the molecular arrangement and makes the β-sheet surface more convex[4] The short oligomethylene spacer also enhances this

phenomenon. This tilt causes the β-sheet to fold and form nanotubes (Figs. 4.2B, $4.2B_1$ and $4.2B_2$).

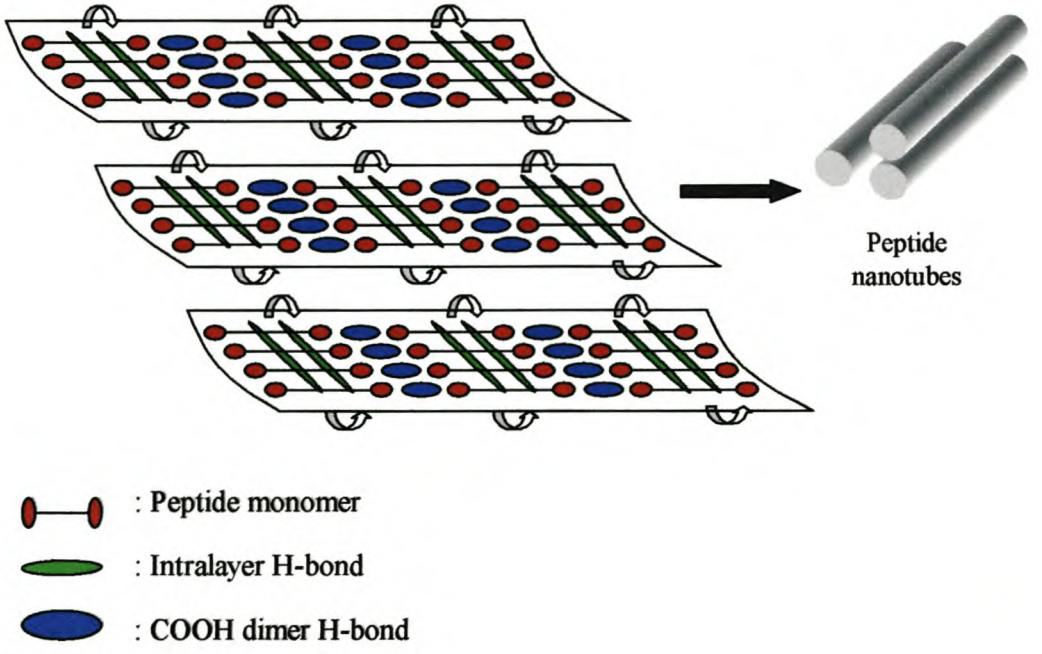


Figure 4.4 Proposed structure of self-assembly of the peptide monomers of **(A)**.

With time, inter- and intralayer hydrogen-bonding[12] (1710 and 1728 cm^{-1}, respectively) between the carboxyl groups results in the extension of the β-sheet, and this translates into longer and wider tubes (Fig. 2C). CH_2 antisymmetric and symmetric bands at 2925 and 2852 cm^{-1} respectively, suggest the prevalence of *trans* configuration[11] of the oligomethylene spacer in the microtube. To verify the authenticity of the supramolecular complex, lyophilised samples of the self-assembled peptide were subjected to ESMS analysis.

4.2.4 Characterisation of suberamide$(GG)_2$ (A)

Yield 88%. $[M-H]^-$ required *m/z* 401.39, found 401.13. ^{1}H-NMR δ_H(300 MHz; DMSO-d_6; TMS) 1.23 (2H, m, γ-CH_2); 1.47 (2H, m, β-CH_2); 2.13 (2H, t, *J* 7.4, α-CH_2); 3.45 (2H, d, *J* 5.5, G5); 3.51 (2H, d, *J* 5.5, G2); 8.13 (2H, t, *J* 5.4, G3, and G6).

4.3 Conclusions

To our knowledge this is the first study of real-time self-assembly with visual evidence to support the many theories postulated in terms of bola-amphiphile self-assembly. The choice of 0.1% TFA as the solvent medium for the self-assembly of the bola-amphiphile significantly reduced self-assembly time. Nanotubes were obtained within a day and a microtube in 24 hours, as opposed to 14 days. Real-time self-assembly is of paramount importance as it clearly illustrates the process of self-assembly i.e. the transition from monolayers → nanotubes → microtube. This result is of high significance for it facilitates a better understanding of β-sheet self-assembly which has far reaching implications in fields of both medicine and material science. An improved understanding of β-sheet self-assembly will not only enable supramolecular chemists to design more complex and purpose driven systems but take science closer to the ever elusive goal of 'coded' self-assembly.

4.4 References

1. J.-H. Fuhrhop, H. Bartsch, D. Fritsch, *Angew. Chem. Int. Ed. Engl.*, **1981**, *20*, 804-805.

2. N. A. Grigoryan, Z. O. Mndzhoyan, E. V. Kazaryan, Z. Yu Ter-Zakharyan, O. L. Mndzhoyan, *Pharm. Chem. J.*, **1992**, 2, *26*, 153-157.

3. M. Kogiso, S. Ohnishi, K. Yase, M. Masuda, T. Shimizu, *Langmuir,* **1998**, *14.* 4978-4986.

4. H. Matsui, G. E. Douberly, *Langmuir,* **2001**, *17*, 7918-7922.

5. E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, B. J. Williams, *J. Chem. Soc. Chem. Commun.*, **1978**, 537-539.

6. M. B. Davis, J. F. Preston, *Anal. Biochem.,* 1981, **116**, 402-407.

7. R. Rajan, P. Balaram, *Int. J. Pept. Prot. Res.*, **1996**, *48*, 328-336.

8. G. H. Escamilla, G. R. Newkome, *Angew. Chem. Int. Ed. Engl.*, **1994**, *33*, 1937-1940.

9. J.-M. Lehn, *Supramolecular Chemistry*, VCH, Verlagsgesellschaft, Weinheim, **1995**, p. 139.

10. S. Krimm, J. Bandekar, *Adv. Protein. Chem.*, **1986**, *38*, 181-365.

11. R M. Kogiso, Y. Okada, T. Hanada, K.Yase, T. Shimizu, *Biochim et Biophys Acta.*, **2000**, *1475*, 346-352.

12. G. Snyder, H. L. Strauss, *J. Phys. Chem.,* **1982**, *86*, 5145-5150.

Chapter 5

Self-assembly of an asymmetrical bola-amphiphile into defined 3D supramolecular architectures

Abstract

A novel synthetic, peptide-based, asymmetrical bola-amphiphile was synthesized using solid phase peptide synthesis. The bola-amphiphile was composed of oligoglycine head groups linked *via* an aliphatic ω–amino acid, which serves as a hydrocarbon spacer. The self-assembly properties of the bola-amphiphile in 0.1% trifluoroacetic acid were studied by light microscopy, scanning electron microscopy, and infrared spectroscopy. Light microscopy results indicated the formation of ribbons, while further analysis with electron microscopy revealed that the ribbons were composed of well-defined fibrils with an average diameter of 2.3 μm, which further associated into bundles of ~ 10.5 μm in diameter. Using low and high vacuum modes of environmental scanning electron microscopy it was possible to observe the supramolecular morphogenesis of the bola-amphiphile: from multilayer lamellar sheet-like structures to a well-defined bundle of fibrils. Fourier-transform infra-red spectrometry revealed that vectorial formation of interlayer and intralayer hydrogen bond networks, together with strong self-association, promoted by the hydrophobic effect and electrostatic interactions, are responsible for the well-defined 3D supramolecular architectures, i.e. the self-assembly of β-sheets into higher order quaternary structures.

5.1 Introduction

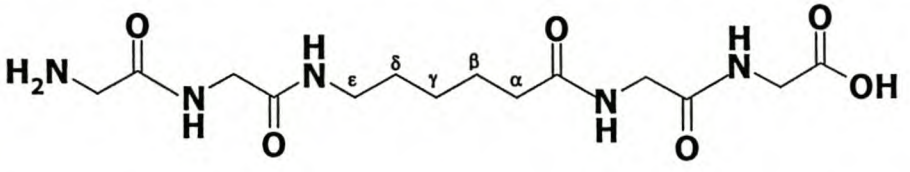
Bilayer lipid membranes (BLMs) of biological cells comprise predominantly phospholipids[1] and functional membrane proteins. These proteins form the functional components of, for example, life-supporting systems such as oxidative phosphorylation

and photosynthesis. These protein systems have an asymmetric distribution and this in BLMs is an absolute requirement for the fulfilment of these tasks[2]. In the quest for the creation of functionalized synthetic lipid membranes, without using phospholipids, two approaches have been formulated: first, the formation of BLMs using hydrophobic compounds with reactive head groups such as photo-reactive diazo dyes[3] or, second, replacing the BLMs by monolayered analogues (MLMs). Asymmetrical bola-amphiphiles with head groups of different sizes, charges, or water solubilities are ideal and have been successfully used for the formation of MLMs[3]. The lipid membranes of thermophilic and acidophilic bacteria, e.g. *Sulfolobus solfataricus,* represent the most important natural bola-amphiphiles and the first MLMs[4]. Many types of asymmetric bola-amphiphilic compounds have been synthesized and studied in the quest for functionalized synthetic lipid membranes and to explore their tremendous potential[5].

In 1996 Shimizu *et al.*[6] indicated the possibility of vesicle assembly in microtubular structures made up of synthetic oligoglycine-based bola-amphiphiles. The dicarboxylic-acid linked- glycylglycine bola-amphiphiles self-assembled into various supramolecular complexes: vesicles, fibers and spheroids[7]. The formation of the assembly patterns in aqueous dispersions was pH dependent[8]. Fuhrhop *et al.*[9] then introduced the use of diamide bola-amphiphiles composed of α,ω-amino acid linkers and amino acid head groups to generate higher order structures. Matsui *et al.*[10], using glycylglycine bola-amphiphiles, indicated the possibility of organizing peptide nanotubes into bundles through the use of metal co-ordination bridges.

Here we report on the use of a novel synthetic peptide-based asymmetrical bola-amphiphile [GG-NH$(CH_2)_5$CO-GG] **(A)**, composed of an oligoglycine group at both the C- and N-terminals of an ω–amino acid, to generate bundles of fibrils, without the use of metal co-ordination bridges. This is the first report of a dipeptide-based bola-amphiphile using a ω–amino acid moiety as a lipophilic linker.

The bola-amphiphile was synthesized using bench-top solid-phase peptide synthesis



(A)

(SPPS). This afforded more flexibility and versatility in terms of bola-amphiphile design. Solvent conditions were adjusted to produce optimal conditions for self-assembly. The self-assembly structures and supramolecular complexes were evaluated by Fourier transform infrared (FT-IR) spectroscopy and by environmental scanning electron microscopy (E-SEM) under different conditions.

5.2 Experimental

5.2.1 Materials

NovaSyn® KA (0.15 milli-equivalent/g), HOBt (1-hydroxybenzotriazole) and benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosphonium hexafluorophosphate (PyBOP®) were obtained from Novabiochem Co. (Luzern, Switzerland). Fmoc-Gly-OH and Fmoc-6-Ahx-OH were from Advanced ChemTech (Louisville, USA). *N,N*-dimethylformamide (DMF, 99.5%) and glacial acetic acid were from Merck (Darmstadt, Germany). *N,N*-diisopropylethyl amine (DIPEA) and trifluoroacetic acid (TFA, >98%) were from Sigma Chemical Co. (St. Louis, USA). Acetonitrile (HPLC-grade, UV cut-off 190 nm) was from Romil LTD (Cambridge, UK). Analytical grade water was prepared by filtering glass-distilled water through a Millipore Milli Q® water purification system.

5.2.2 Peptide synthesis

The synthesis of [GG-NH$(CH_2)_5$CO-GG] **(A)** was accomplished using conventional solid-phase peptide synthesis (SPPS), based on Fmoc chemistry[11]. The synthesis was carried out on a polydimethylacrylamide resin encapsulated in Kieselguhr. Freshly distilled amine-free DMF was used as solvent and distilled piperidine (20% in DMF) was used as base for the removal of the Fmoc group after each coupling step. The first Gly residue was coupled to the resin *via* an acid-labile ester bond. Thereafter, activation of each incoming carboxyl group was achieved by using PyBOP® as an activation agent, with freshly distilled DIPEA as base and HOBt as catalyst. The peptides were cleaved from the resin with 95% TFA and 5% H_2O as scavenger. The peptide resin was treated for between 3 and 6 hours with the TFA cleavage mixture. After cleavage, the resin was removed by filtration and washed consecutively with 95% TFA, acetic acid and analytical

grade water. The combined filtrate, containing the bola-amphiphile, was dried under high vacuum on a rotary evaporator at 40-45°C, resuspended in 50% acetonitrile and lyophilized.

5.2.3 Self-assembly

A stock solution of the bola-amphiphile (10 mM, pH 2.4) in 0.1% TFA was prepared. The solution was then left undisturbed at room temperature for up to seven days. Samples of it were then analyzed using phase contrast light microscopy, environmental scanning electron microscopy (E-SEM) and FT-IR spectroscopy.

5.2.4 Polarized-light optical microscopy

For light microscopy measurements, a drop of the dispersion containing the fibrous assemblies was placed on a glass slide and covered with a cover slip. The samples were examined using a phase-contrast light microscope (Nikon OPTIPHOT-POL, 40X, 100X, 200X, 400X objective). The micrograph images were recorded with a CCD Colour Video Camera (National WV-CD110 NC) and monitored on an 11-inch colour monitor (National WV-CD110). The picture images were stored using a video capturing card.

5.2.5 Environmental scanning electron microscopy (E-SEM)

A 5 μL sample of the self-assembled stock solution of the bola-amphiphile was placed on a support film coated with silver, and mounted on a specimen stub. The peptide fibres were washed thoroughly with de-ionized water and excess water was blotted off with filter paper. The samples were then air-dried and viewed in E-SEM mode using a peltier cooling stage. The stage was cooled to 5°C at a pressure of 5-6.5 torr, at a relative humidity of between 75 and 100%. By adjusting the water vapour pressure in the chamber and the temperature of the cooling stage it was possible to increase or decrease the water level in and on the surface of the sample. Images were obtained using the gaseous secondary electron detector (GSED). All samples were viewed using a Philips XL 30 E-SEM at 15 kV accelerating voltage. For coated samples, a 4 nm layer of Au/Pd alloy was deposited onto the samples using an E5100 Polaron sputter coater. The samples were viewed in the high vacuum mode at 10-15 kV accelerating voltage.

5.2.6 FT-IR spectroscopy

A freeze-dried self-assembly sample was analyzed by means of photo acoustic Fourier transform infrared spectroscopy (FT-IR). The photo acoustic detector used was a MTEC model 300 unit that was coupled to a Perkin Elmer Paragon 1000. The following parameters were used for the determination of each spectrum: mirror velocity (OPD), 0.1 cm/s; resolution, 8 cm^{-1}; source aperture, maximum; number of scans, 128; sample reference, carbon black and detector gas atmosphere, helium. A typical scan required fifteen minutes. The infrared spectrum was scanned from wave number 4000 to 450 cm^{-1} and was mathematically adjusted to compensate for the photo acoustic effect.

5.2.7 Electrospray mass spectrometry (ESMS)

ESMS was performed using a micromass triple quadrupole mass spectrometer fitted with an electrospray ionization source. The carrier solvent was 50% (v/v) acetonitrile in analytical grade water. A capillary voltage of 3.5 kV was applied throughout and the source temperature was set at 80^0C. The skimmer lens offset was 5 V and the cone voltage was varied between 50 and 60V. Data acquisition was in the negative mode, scanning the first analyzer (MS_1) through m/z = 100 to 1500 m/z at a scan rate of 675 atomic mass units/second, averaging spectra across the elution peak and subtracting the background produced representative spectra. The instrument was first calibrated using the ion spectrum of polyethylene glycol acquired under similar conditions.

5.2.8 NMR

The ^{1}H NMR spectrum [δ, J values in Hz] of freshly dissolved bola-amphiphile in 50% CD_3CN was recorded on a Varian Inova 600 MHz instrument.

5.3 Results

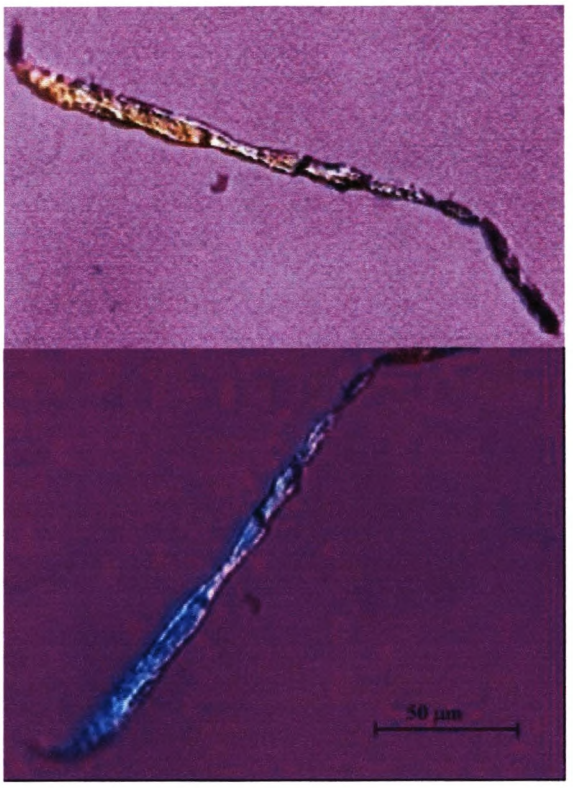


Figure 5.1 Birefringence of the self–assembly of **(A).** Anisotropic behaviour observed using polarized-light microscopy (25°C in 0.1% TFA, 7 days).

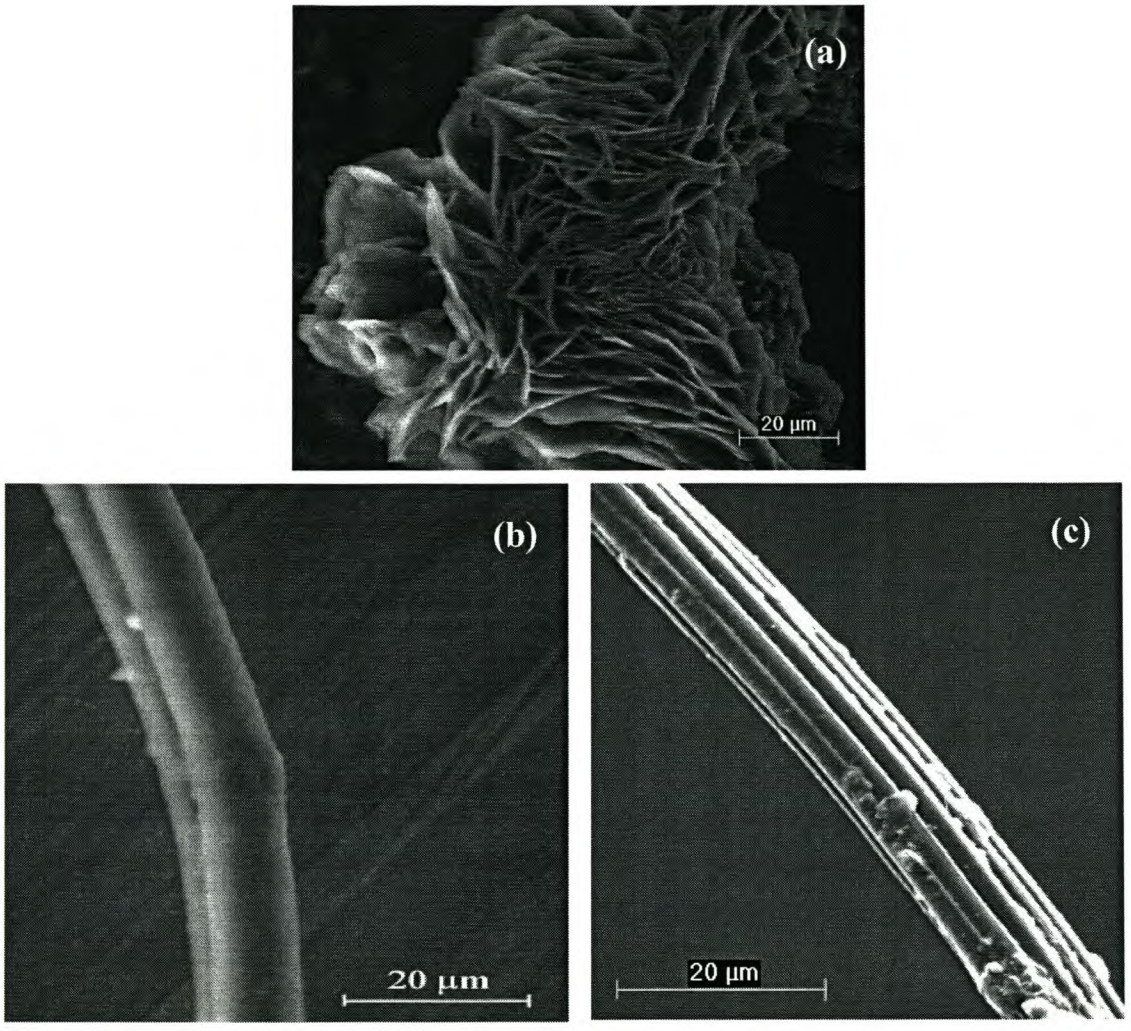


Figure 5.2. Self-assembly of bola-amphiphile **(A)** using E-SEM under different conditions over time: (a) thin platelet-like sheets arranged perpendicular to the surface (5°C, 45% relative humidity, 7 hour self-assembly); (b) fibrils associating with each other to form a bundle of fibrils (5°C, 60% relative humidity, 7 day self-assembly), and (c) bundle of fibrils (high vacuum, 7 day self-assembly).

5.3.1 Polarized-light optical microscopy

Samples of the stock solution of the bola-amphiphile were first analyzed after a period of 7 days. Polarized-light optical micrographs of self-assembled material (Fig. 5.1a/b) indicated the formation of a birefringent ribbon-like structure.

5.3.2 Environmental scanning electron microscopy (E-SEM)

A fresh stock solution of **(A)** was prepared and samples were analyzed in E-SEM mode. The E-SEM micrograph was most intriguing: there was a random arrangement of thin platelet-like sheets, arranged perpendicularly. This supported earlier reports on glycyl-glycine bola-amphiphiles[12,13]. E-SEM micrographs (Fig. 5.2b) indicated the formation of fibrils (~10.7 μm in diameter), associated with each other to form a bundle of fibrils. An attempt to improve the resolution of the micrograph by changing the water vapour pressure within the chamber proved to be ineffective. The samples were therefore coated and viewed under high vacuum. The resulting micrograph (Fig. 5.2c) indicated fibrils of 2.3 μm in diameter that formed a bundle 10.5 μm in diameter. Variations in tube diameter detected in the different E-SEM modes were attributed to swelling in water.

5.3.3 Characterization of GG-NH-$(CH_2)_5$-CO-GG

Yield 85%, $[M-H]^-$ $C_{14}H_{24}N_5O_6$ required *m/z* 358.37, found 358.16; ^{1}H-NMR δ_H(600 MHz; 50% CD_3CN) 1.23 (2H, q_n, *J* 7.7, γ_{CH_2}); 1.40 (2H, q_n, *J* 7.2, δ_{CH_2}); 1.50 (2H, q_n, *J* 7.5, β_{CH_2}); 2.20 (2H, t, *J* 7.2, α_{CH_2}); 3.09 (2H, q, *J* 6.4, ε_{CH_2}); 4.20→4.40 (α-protons of 4x glycine-residues underneath the water signal); 7.53, 7.69, 7.93 (amide NH-signals). (q_n – quintuplet)

5.3.4 FT-IR spectroscopy

Dried self-assemblies were analyzed by FT-IR to establish the internal molecular arrangements in the supramolecular complex (Fig. 5.3).

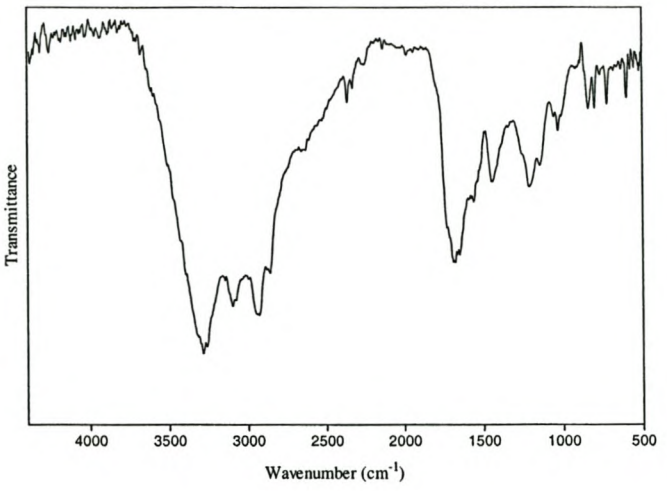


Figure 5.3 FT-IR spectrum of the dried self-assembly from **(A)** in the region 500-4500 cm^{-1}.

The Savitsky-Golay[14] derivative function software was used to determine the second derivative spectra of the amide I bands (Fig. 5.4) in order to obtain more detailed spectral information.

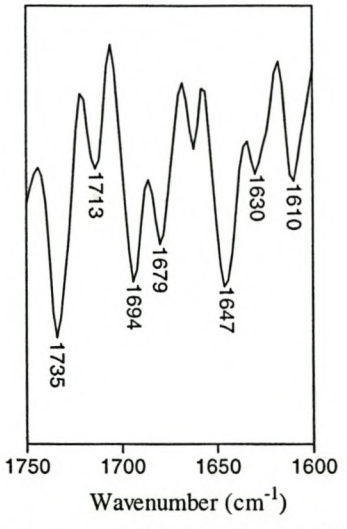


Figure 5.4 Second derivative spectrum of **(A)** in the amide I region.

The N-H stretching vibration band at 3290 cm^{-1}, together with the amide I bands at 1694, 1647 and 1630, and amide II bands at 1555 and 1534 cm^{-1} indicated the formation of hydrogen bonds resulting in the formation of β-sheets[15,16]. The IR band observed at 1679

cm^{-1} could represent residual TFA. However, this may also indicate that TFA is part of the self-assembly complex as a bridging agent, due to its unique amphiphatic properties conferred by the three F-atoms and the carboxyl group. The CH_2 scissoring band produced a sharp peak at 1439 cm^{-1} indicating that's its packing mode was triclinic or hexagonal[17,18], furthermore the CH_2 antisymmetric and symmetric bands at 2940 and 2854 cm^{-1}, respectively suggested the presence of a high trans conformational population[19,20]. FT-IR bands at 1735 and 1713 cm^{-1} indicated lateral and bifurcated hydrogen bonds[21] of the terminal carboxyl group, respectively. FT-IR analysis proved that intralayer and lateral hydrogen bonds contributed significantly to the stabilization of the β-sheets.

5.4 Discussion

It is proposed that the self-assembly process can be subdivided into five transition phases:

Monolayer → β-sheet → β-sheet sandwich → fibril → bundle of fibrils

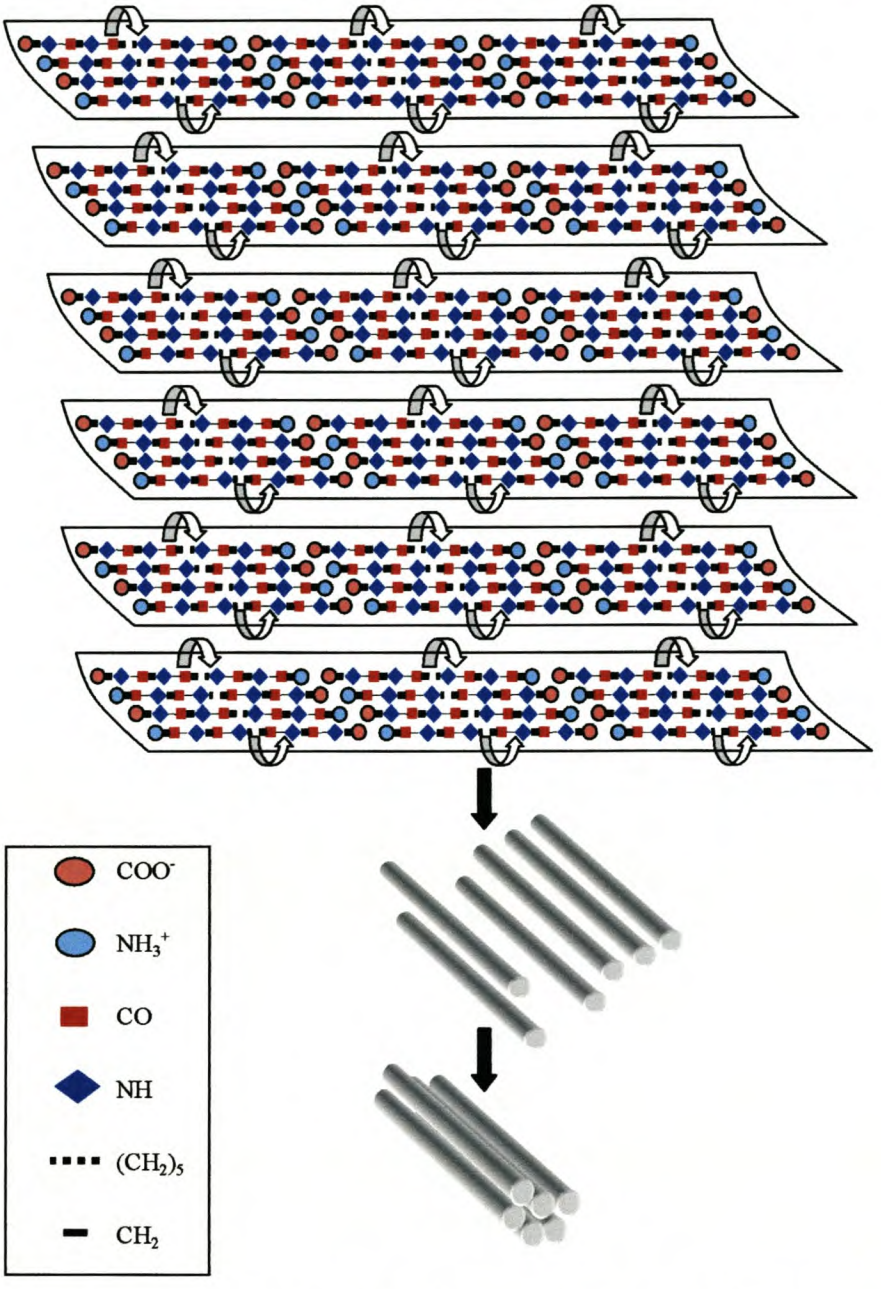


Figure 5.5 Schematic illustration of the self-assembly transitional phases.

All of the above phases will be strongly influenced by the solvent medium. After testing a number of solvent conditions it was found that 0.1% TFA, due to its action with the peptide, was an ideal solvent for the supramolecular morphogenesis of the bola-amphiphile. The use of TFA resulted in a shift in the conformational equilibrium state of the bola-amphiphile towards compact states that contained internally hydrogen-bonded amides[22] i.e. TFA promoted strong intralayer hydrogen bonding. TFA also enhances the hydrophobic interaction of the oligomethylene chain for it "out-competes" the water molecules attached to the flanking peptide backbone[22].

The morphogenesis dictates that the bola-amphiphile initially self-assembles into a monolayer *via* an inherent molecular recognition process[23]. The monolayer represents the basic building block of the supramolecular complex. These layers then self-associated to form β-sheets *via* the hydrophobic effect and electrostatic interactions. Although there were both parallel and anti-parallel β-sheets present in our self-assembly structures, as determined by FT-IR, the interface strand will possibly adopt an intermediate conformation and the sheets will then assume the general appearance of the predominant hydrogen bonding type[24]. Intralayer and interlayer H-bond formation are responsible for the extension of the β-sheet and the formation of lamellar monolayer sheets[25], respectively. The low pH of the solution possibly resulted in the shortening of the intralayer and interlayer H-bonds and, in turn, induced a tilt in the molecular arrangement making the β-sheet surface more convex[8]. The short oligomethylene spacer also may have enhanced this phenomenon, resulting in the formation of fibrils. On the basis of the amphiphilicity of the lamellar sheets, it is expected that the individual sheets will subsequently dimerize, through association of their hydrophobic faces, to generate a dimeric β-sandwich structure[24], which may laterally associate *via* intermolecular β-sheet formation to form a bundle of fibrils. Hydrophobic and electrostatic interactions contribute to the stability of the fibrils and their bundles.

5.5 Conclusions

Using SPPS a novel asymmetrical peptide bola-amphiphile was synthesized. Employing a simple self-assembly strategy a well-defined 3D supramolecular complex was generated. The supramolecular morphogenesis of the complex was a function of β-sheet self-assembly governed by intra- and interlayer H-bonds and a strong self-associative effect promoted by electrostatic interactions and a hydrophobic effect. Results of this work facilitate a better understanding of β-sheet self-assembly and its use to generate higher order supramolecular complexes. Furthermore, this bola-amphiphile can serve as leader compound or scaffold for more complex structures with specific active functions. Having prepared a library of related bola-amphiphiles, future work will focus on studying the self-assembly properties of these compounds, some of which have specific membrane related activities.

5.6 References

1. Fuhrhop, J.-H.; Fritsch, D. *Acc. Chem. Res.*, **1986**, *19*, 130-137.

2. Rothman, I.; Lenard, I. *Membr. Asym. Sci.*, **1977**, *195*, 743-753.

3. Fuhrhop, J.-H.; Bartsch, H.; Fritsch, D. *Angew. Chem. Int. Ed. Engl.*, **1981**, *20*, 804-805.

4. Gliozzi, A.; Rolandi, R.; De Rosa, M.; Gambacorta, A. *J. Membr. Biol.*, **1983**, *75*, 45-56.

5. Escamilla, G. H.; Newkome, G. R. *Angew. Chem. Int. Ed. Engl.*, **1994**, *33*, 1937-1940.

6. Shimizu, T.; Kogiso, M.; Masuda, M. *Nature*, **1996**, *383*, 487.

7. Kogiso, M.; Ohnishi, S.; Yase, K.; Masuda, M.; Shimizu T. *Langmuir*, **1998**, *14*, 4978-4986.

8. Matsui, H.; Gologan, B. *J. Phys. Chem., B.*, **2000**, *15*, 3383-3386.

9. Schneider, J.; Messerschmidt, C.; Schultz, A.; Gnade, M.; Schade, B.; Luger, P.; Bombicz, P.; Hubert, V.; Fuhrhop, J.–H. *Langmuir*, **2000**, *16*, 8575-8584.

10. Matsui, H.; Douberly, G. E. *Langmuir,* **2001**, *17*, 7918-7922.

11. Atherton, E.; Fox, H.; Harkiss, D.; Logan, C. J.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc. Chem. Commun.*, **1978**, 537-539.

12. Shimizu, T; Kogiso, M.; Masuda, M. *J. Amer. Chem. Soc.*, **1997**, *119*, 6509-6510.

13. Kogiso, M.; Masuda, M.; Shimizu, T. *Supramol. Chem.*, **1998**, *9*, 183-189.

14. Savitsky, A.; Golay, J. E. *Anal. Chem.*, **1964**, *36*, 1628-1639.

15. Toniolo, T.; Palumbo, M. *Biopolymers*. **1977**, *16*, 219-224.

16. Krimm, S.; Bandekar, J. *Adv. Protein. Chem.*, **1986**, *38*, 181-365.

17. Parikh, A. N.; Schively, M. A.; Koo, E.; Seshadri, K.; Aurentz, D.; Mueller, K.; Allara, D. L. *J. Amer. Chem. Soc.*, **1997**, *119*, 3135-3143.

18. Yamada, N.; Okuyama, K.; Serizawa, T.; Kawasaki, M.; Oshima, S. *J. Chem. Soc., Perkin Trans. 2,* **1996**, 2707-2714.

19. Snyder, R. G.; Strauss, H. L. *J. Phys. Chem.,* **1982**, *86*, 5145-5150.

20. Masuda, M.; Shimizu, T. *Carbohydr. Res.* **1997**, *302*, 139-14

21. Kogiso, M.; Hanada, T.; Yase, K.; Shimizu, T. *Chem. Commun.*, **1998**, 1791-1792.

22. Rajan, R.; Balaram, P. *Int. J. Pept. Prot. Res.*, **1996**, *48*, 328-336.

23. Lehn, J.-M. *Supramolecular Chemistry*, Anton, U., Ed.; VCH Verlagsgesellschaft, Weinheim, **1995**, p 139.

24. Nesloney, C. L.; Kelly, J. W. *Biorg & Med. Chem.*, **1996**, *4*, 739-766.

25. Kogiso, M.; Okada, Y.; Hanada, T.; Yase, K.; Shimizu, T. *Biochim et Biophys Acta.*, **2000**, *1475*, 346-352.

Chapter 6

The influence of structural and solvent conditions on the supramolecular architecture of novel peptide asymmetrical bola-amphiphiles

Abstract

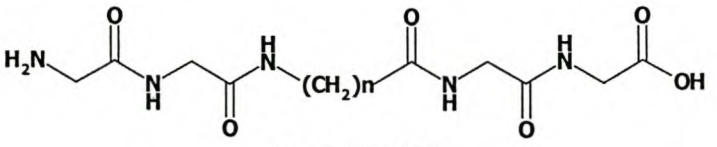
Solid phase peptide synthesis was used to synthesise novel peptide-based asymmetrical bola-amphiphiles. These bola-amphiphiles were composed of dipeptide head groups linked *via* an aliphatic ω–amino acid, serving as a hydrocarbon spacer. Two small libraries of bola-amphiphiles were designed and synthesized — the first involved variation in ω–amino acid length and the second involved variation in the C-terminal amino acids. The self-assembly properties of the bola-amphiphiles in 0.1% trifluoroacetic acid or 0.1% triethylamine were studied by scanning electron microscopy (SEM), field emission SEM (FE-SEM) and Fourier transform infrared spectroscopy (FT-IR). The EM results indicated the formation of a variety of higher order supramolecular architectures based on β-sheet self-assembly. FT-IR spectrometry revealed that interlayer and intralayer hydrogen bond networks, together with strong self-association, promoted by the hydrophobic effect and, in certain instances, electrostatic interactions, are responsible for the supramolecular architectures. Variations in the higher order structures can be attributed to amino acid composition, specifically length of ω-amino acid, nature of the C-terminal amino acid and the optimised solvent conditions used for the self-assembly process.

6.1 Introduction

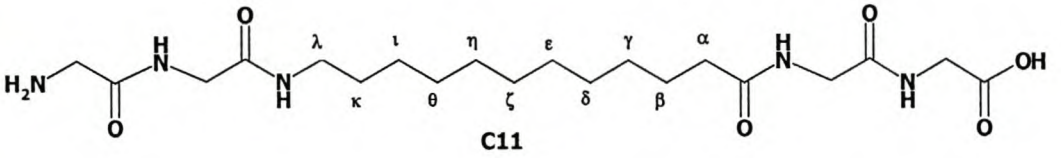
Peptide bola-amphiphiles reached prominence in 1996 when Shimizu *et al.*[1] reported vesicle assembly in microtubes using dicarboxylic oligoglycine bola-amphiphiles. This

marked the advent of a unique niche area in surfactant research. A number of libraries of peptide bola-amphiphiles, including dicarboxylic acids of different lengths and varying amino acid compositions, have since been synthesized and characterized[2,3]. The supramolecular architectures formed by these peptide bola-amphiphile macromolecular assemblies included vesicles, fibres, microtubes and spheriods[2,3]. Dicarboxylic oligoglycine bola-amphiphiles also have the ability to chelate metals and form nanotubes[4]. Fuhrhop *et al.*[5] introduced the use of ω-amino acids, instead of dicarboxylic acids, into the design of peptide bola-amphiphiles and obtained supramolecular architectures very similar to those achieved by Shimizu *et al.*[2,3]. However, up until this point in time, the design of peptide surfactants was limited because solution phase synthetic techniques were employed. Solid phase peptide synthesis (SPPS), on the other hand, affords one the opportunity to exploit the full potential of the various amino acids available.

Here we report on the use of SPPS together with ω-amino acids as the hydrophobic linker to generate two libraries (series I and II) of synthetic, peptide-based asymmetrical bola-amphiphiles. The use of SPPS allows particular flexibility in terms of syntheses of peptide bola-amphiphiles designed for self-assembling in distinctive higher order supramolecular architectures. Series I was established to evaluate the effect of the length of the ω-amino acid on supramolecular architecture i.e. the hydrophobic effect of the ω-amino acid.



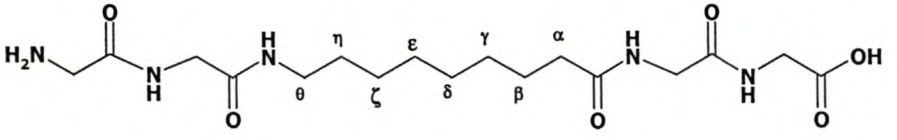
n = 5, 8 and 11
(C5, C8, C11)



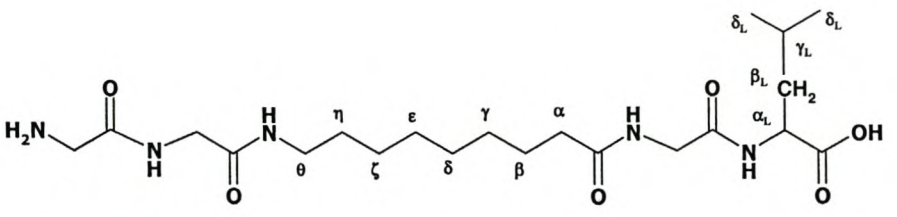
C11

Series I

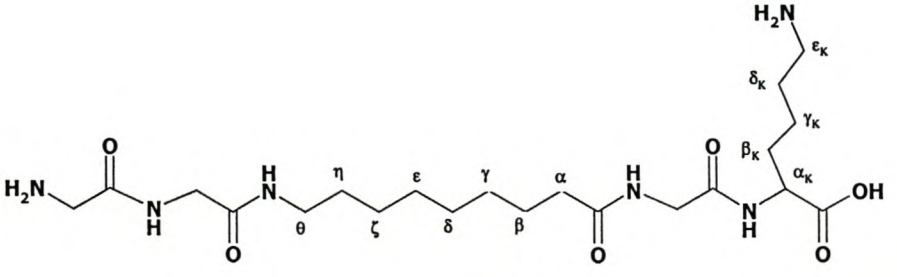
The second library, series II, was established to investigate the influence of the variation of the C-terminal amino acid composition on the morphology of the bio-macromolecular assemblies formed.



(GG)



(GL)



(GK)

Series II

The incorporation of the ω-amino acid functionality in the design of the peptide bola-amphiphile added a new dimension to the self-assembly process — the possibility of extra intramolecular hydrogen bonding between the atoms in the amide backbone could promote a more effective hydrophobic interaction between the hydrocarbon chains and therefore lead to more stable higher order structures. To further aid the self-assembly process, we selected to use 0.1% TEA or 0.1% TFA in water as the bola-amphiphile solvent. The supramolecular complexes of the purified peptide bola-amphiphiles were

evaluated by Fourier-transform infrared (FT-IR) spectroscopy, by scanning electron microscopy (SEM) and by field emission SEM (FE-SEM).

6.2 Experimental

6.2.1 Peptide synthesis and purification

The syntheses of the peptide bola-amphiphiles (see above for structures and abbreviations) were accomplished using conventional SPPS, based on Fmoc chemistry[6]. The synthesis was carried out on NovaSyn® KA resin. The peptides were purified using a semi-preparative C_{18} Polygosil HPLC column (irregular particle size, 60 Å pore size, 250 mm x 10 mm). A linear gradient over 13 minutes (flow rate 3 mL/min) was used, starting with 20% eluant A (0.1% TFA in water) and ending with 100% eluant B (90% acetonitrile and 10% A). Product elution was monitored with a UV detector at 254 nm. ESMS and NMR were used to characterise the purified peptide.

6.2.2 Analysis of peptides

^{1}H NMR spectra [δ, *J* values in Hz] in 50% CD_3CN in H_2O or DMSO-d_6 were recorded with a Varian Inova 600 MHz instrument (q_n refers to quintuplet and s_b to a broad singlet.). Electrospray ionisation mass spectrometry (ESMS) was performed using a Micromass triple quadrupole mass spectrometer fitted with an electrospray ionisation source. FT-IR spectroscopic measurements were made using a photo acoustic detector (MTEC model 300 unit) coupled to a Perkin Elmer Paragon 1000.

6.2.3 Self-assembly

4 mg/mL (in 0.1% TFA) solutions were prepared of the bola-amphiphiles in series I and II, with the exception of GK where 0.1% TEA was used instead of 0.1% TFA. The solutions were then left undisturbed at room temperature for a period of up to seven days. Samples were analysed using SEM, FE-SEM and characterised by FT-IR spectroscopy.

6.2.4 SEM and FE-SEM

A 5 μL sample of the stock solution was placed on a support film coated with carbon and mounted on a specimen stub. The peptide fibres were washed thoroughly with de-ionised

water and excess water was blotted off with filter paper. The samples were then air-dried at room temperature. Field emission scanning electron micrographs were obtained using a LEO 1525/1530 at 5-10 kV accelerating voltage. For SEM, the samples were viewed using a Philips XL 30 E-SEM in the high vacuum mode at 10-15 kV accelerating voltage. Prior to EM imaging a 4 nm layer of Au/Pd alloy was deposited onto the samples using a sputter coater.

6.3 Results and discussion

6.3.1 NMR characterization of the bola-amphiphiles

GG-NH-$(CH_2)_5$-CO-GG: Yield 85%, $[M-H]^-$ $C_{14}H_{24}N_5O_6$ required *m/z* 358.37, found 358.16; ^{1}H-NMR δ_H(600 MHz 50% CD_3CN) 1.23 (2H, q_n, *J* 7.7, γ_{CH_2}); 1.40 (2H, q_n, *J* 7.2, δ_{CH_2}); 1.50 (2H, q_n, *J* 7.5, β_{CH_2}); 2.20 (2H, t, *J* 7.2, α_{CH_2}); 3.09 (2H, q, *J* 6.4, ε_{CH_2}); 4.20→4.40 (4 x α_{Gly}, underneath H_2O signal); 7.53, 7.69, 7.93 (amide NH-signals).

GG-NH-$(CH_2)_8$-CO-GG: Yield 66%, $[M-H]^-$ $C_{17}H_{30}N_5O_6$ required *m*/z 400.45, found 400.19; ^{1}H-NMR δ_H(600 MHz DMSO-*d*$_6$) 1.21 (8H, s_b, γ - ζ_{CH_2}); 1.36 (2H, m, η_{CH_2}); 1.48 (2H, m, β_{CH_2}); 2.11 (2H, t, *J* 7.4, α_{CH_2}); 3.02 (2H, m, θ_{CH_2}); 3.59 (2H, -C*H*$_2$-COOH, underneath H_2O signal); 3.60 (2H, m, α_{Gly}); 3.65 (2H, d, *J* 5.7, α_{Gly}); 3.70 (2H, m, α_{Gly}); 7.51, 7.87, 8.29 (amide NH-signals).

GG-NH-$(CH_2)_{11}$-CO-GG: Yield 64%, $[M-H]^-$ $C_{20}H_{36}N_5O_6$ required *m/z* 442.53, found 442.23; ^{1}H-NMR δ_H(600 MHz DMSO-*d*$_6$) 1.23 (14H, s_b, $\gamma - \iota_{CH_2}$); 1.37 (2H, m, κ_{CH_2}); 1.48 (2H, m, β_{CH_2}); 2.10 (2H, t, *J* 7.5, α_{CH_2}); 3.20 (2H, m, λ_{CH_2}); 3.47 (2H, d, *J* 4.5, -C*H*$_2$-COOH); 3.56 (2H, s_b, α_{Gly}); 3.66, 3.70 (2 x 2H, 2 x s_b, 2 x α_{Gly}); 7.48, 7.88, 8.36 (amide NH-signals).

GG-NH-$(CH_2)_8$CO-GK: Yield 91%, $[M-H]^-$ $C_{27}H_{34}N_6O_6$ required *m/z* 471.58, found 471.17; ^{1}H-NMR δ_H(600 MHz 50% CD_3CN) 1.21 (8H, s_b, γ - ζ_{CH_2}); 1.28 (2H, q_n, *J* 7.8, γ_K); 1.38 (2H, q_n, *J* 7.1, η_{CH_2}); 1.49 (2H, q_n, *J* 7.1, β_{CH_2}); 1.54 (2H, m, δ_K); 1.59 (1H, m, β_{Kb}); 1.75 (1H, m, β_{Ka}); 2.18 (2H, t, *J* 7.6, α_{CH_2}); 2.85 (1H, s_b, ε_K); 3.08 (2H, q, *J* 5.9,

θ_{CH_2}); 3.71 (1H, s, α_K) 3.76 (2H, d, *J* 5.8, α_{Gly}); 3.79 (2H, d, *J* 5.9, α_{Gly}) 4.14 (H_2N-C*H*$_2$-CO underneath H_2O signal); 7.60 (2H, m), 7.80 (1H, t, *J* 5.5), 8.18 (1H, s_b), amide NH-signals respectively.

GG-NH-(CH$_2$)$_8$CO-GL: Yield 90%, $[M-H]^-$ $C_{21}H_{38}N_5O_6$ required *m/z* 456.56, found 456.20; ^{1}H-NMR δ_H(600 MHz 50% CD_3CN) 0.79 (2 x 3H, 2 x s, 2 x δ_L); 1.20 (8H, s_b, γ - ζ_{CH_2}); 1.40 (2H, s_b, η_{CH_2}); 1.49 (5H, s_b, β_{CH_2},β_L,γ_L); 2.20 (2H, t, *J* 6.9, α_{CH_2}); 3.08 (2H, m, θ_{CH_2}); 3.72 (2H, s, H_2N-C*H*$_2$-CO) 3.77, 3.80 (2 x 2H, 2 x d, *J* 5.3, 2 x α_{Gly}); 4.34 (1H, s_b, α_L); 7.43, 7.65, 7.80, 7.81 (amide NH-signals).

6.3.2 SEM (S) and FE-SEM (FS)

Self-assembly solutions of the different bola-amphiphiles were analysed after an incubation period of up to 7 days. The EM images of supramolecular architectures of series I, [GG-NH$(CH_2)_n$CO-GG], where n = 5 (C5), 8 (C8) and 11 (C11), are given in Fig 6.1.

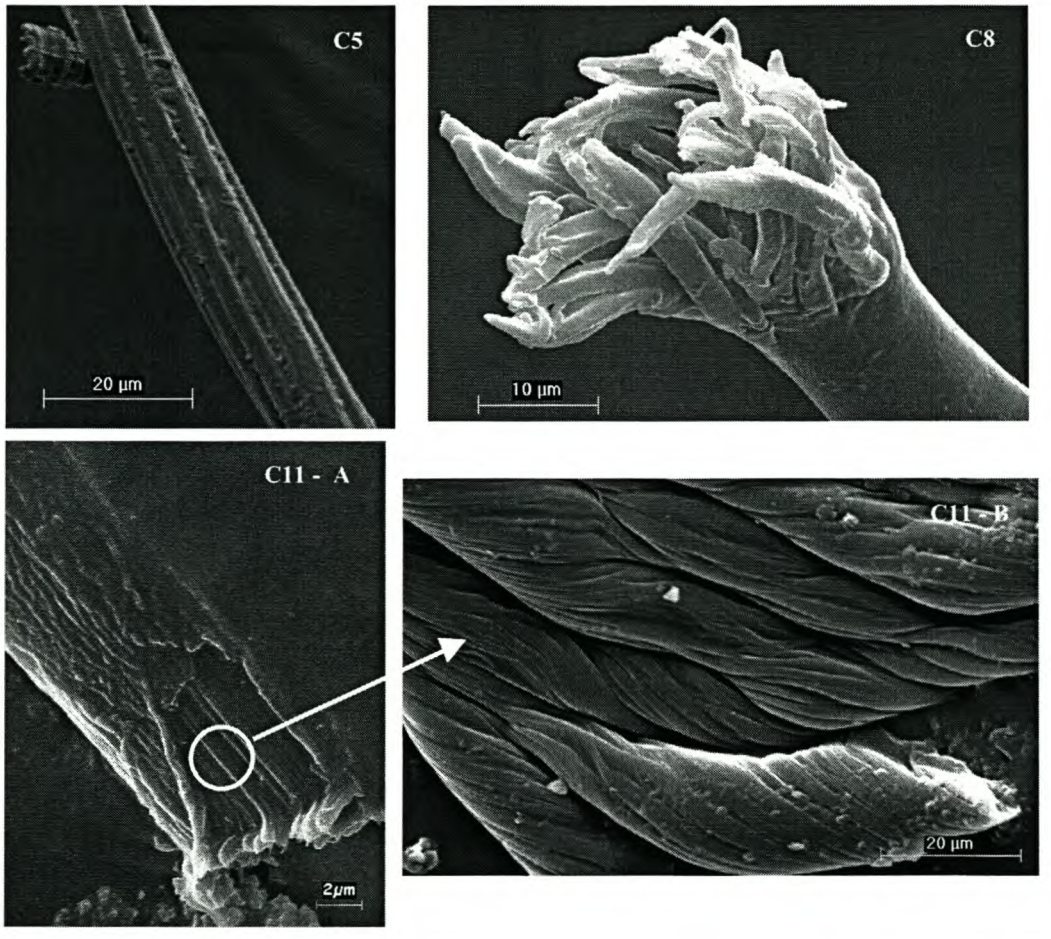


Figure 6.1 EM images of the bolaphiles **(GG)**, (C5), (C8) and (C11).

An interesting feature of the images is the apparent change in morphology of the supramolecular architecture as the length of the ω-amino acid increases from 5 *via* 8 to 11. For C5 (S) we have a distinct bundle of fibrils, were the fibril was approximately 2.3 µm in diameter and the bundle 10.5 µm in diameter. However, for C8 (S) we have a

bundle of sheet-like fibrils with varying widths. For C11 the supramolecular complex is composed of sheet-like structures stacked on top of each other (C11 – A) (FS). The SEM image (C11 – B) represents a detailed illustration of the structural characteristics of the sheet-like structures of C11. The sheets vary in width and length, with the thickness of the sheets being in the order of magnitude of a few hundred nanometers. An increase in the length of ω-amino acid length resulted in the supramolecular complex going from a bundle of fibrils (C5) (S) to a bundle of sheet-like fibrils (C8) (S) and finally to a stack of sheet-like structures (C11) (FS). A variation in the C-terminal amino-acid composition i.e. series II, produced the supramolecular structures shown in Fig 6.2.

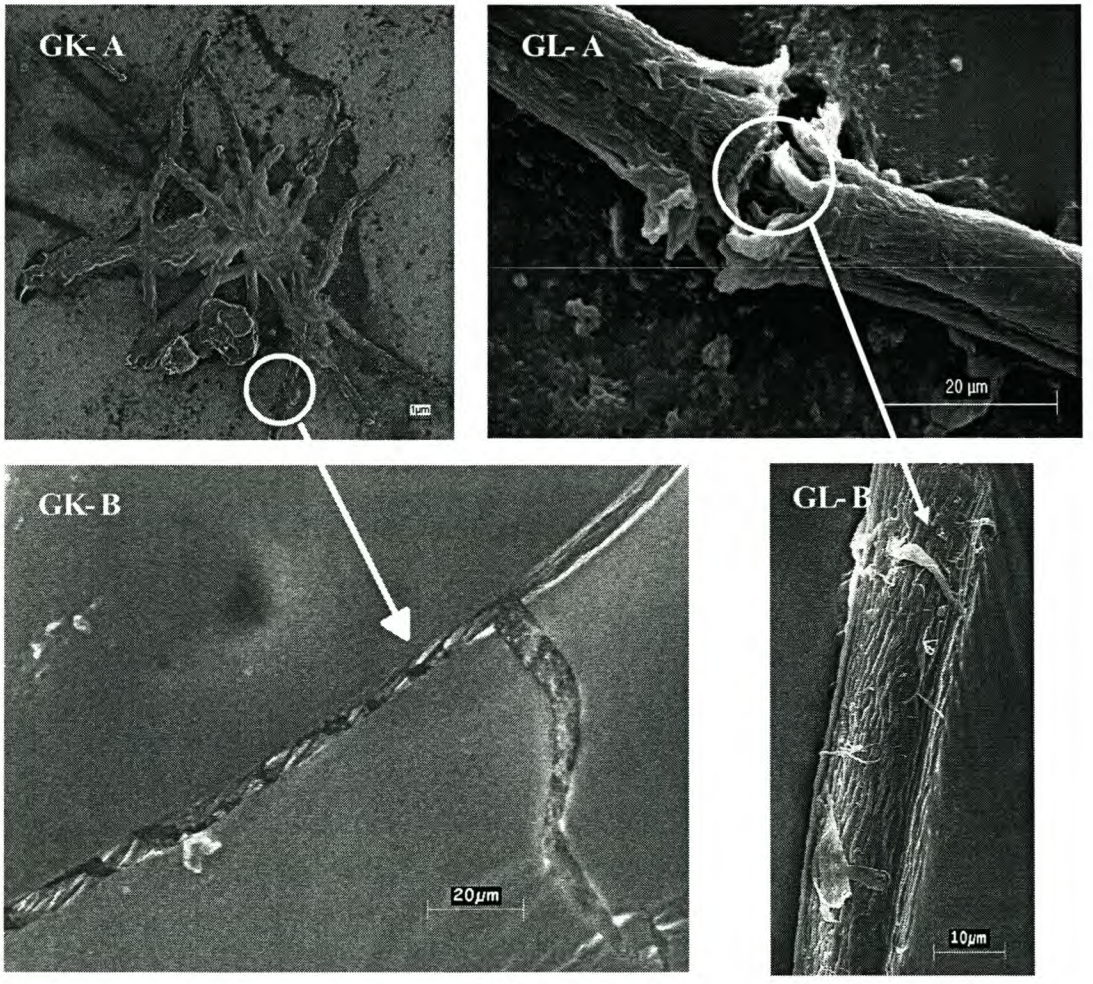


Figure 6.2 EM images of the bola-amphiphiles [GG-NH$(CH_2)_8$CO-GX], where X = K and L **(Series II)**, and X = G in **Series I**

Series II. The images obtained for the series II bola-amphiphiles ([GG-NH$(CH_2)_8$CO-GX], where X = K and L (Series II), and X =G in Series I, are presented in Fig 6.2. From the electron micrographs it is clear that variation of the C-terminal amino acid has a pronounced effect on the morphology of the supramolecular complex obtained. For GL, the introduction of the leucine moiety results in the formation of a bundle of intertwined microfibrils, as can be seen in GL – B (FS), approximately 1 μm in width, encased in a tubular structure composed of a number of sheet-like structures of varying width (GL – A) (S). We investigated the inclusion of 0.1% TEA in the bola-amphiphile solvent in an attempt to optimise self-assembly conditions for the lysine moiety in GK. This strategy proved to be successful, as can be seen in Fig 6.2. This allowed us to assess the influence of lysine on the morphology of the complex formed, although the influence of incorporated TEA in the structure must not be underestimated. Sheet-like structures twisted into rope-like structures were produced. GK – A represents an uncoated FE-SEM image whereas GK – B a coated FE-SEM image. The difference in size of the complexes obtained in GK – A and B can be attributed to the flattening of the complex as a result of the sputter coating. A unique feature of GK is that it is composed of a core structure comprising twisted sheets wrapped within a twisted sheet (GK - B).

6.3.3 FT-IR Spectroscopy

Lyophilised self-assemblies were analysed by FT-IR to establish the internal molecular arrangements in the supramolecular complex. FT-IR absorption bands and their assignments for the two series are given in Table 6.1

Table 6.1 FT-IR bands (cm^{-1}) observed for the fibres of the bola-amphiphilic peptides

Assignment	C5[a]	C8[a]	C11[a]	GL[a]	GK[a]
NH str.	3287	3280	3281	3282	3278
	3098	3089	3099	3081	3086
CH_2 antisymmetric str.	2940	2925	2922	2932	2924
CH_2 symmetric str.	2858	2856	2855	2858	2852
CH_2 scissoring	1439	1452	1411	1411	1418
Amide II	1555	1557	1554	1554	1554

[a]Amide I bands determined from second derivative spectra (See Fig 6.3)

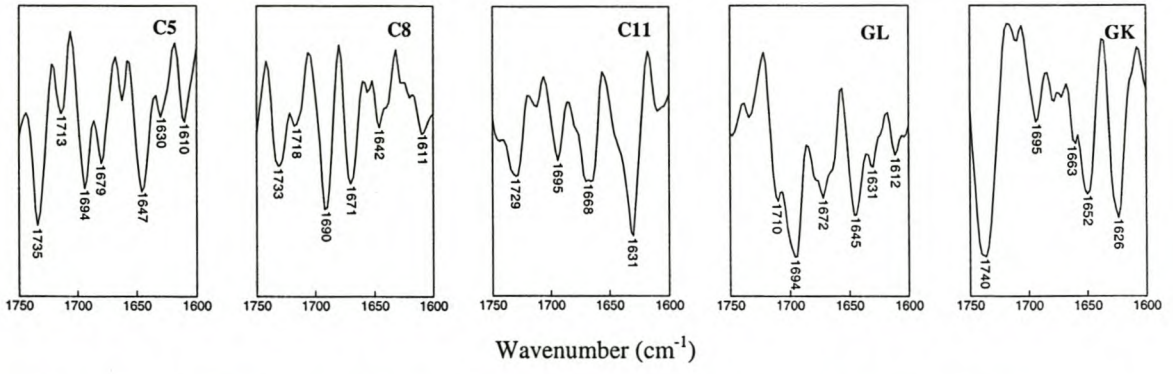


Figure 6.3 Second derivative spectra of the bola-amphiphiles in the amide I region.

The Savitsky-Golay[7] derivative function software was used to determine the second derivative spectra of the amide I bands (Fig 6.3 and Table 6.1) in order to provide more detailed spectral information.

The N-H stretching vibration band, together with the amide I and II bands, indicated the existence of hydrogen bonds[8,9]. Deformation vibration bands of the methylene groups i.e. CH_2 symmetric and antisymmetric bands, revealed the packing mode of the oligomethylene chain[10]. The presence of amide I bands at around 1710 and 1730 cm^{-1} indicated the possibility of inter- and intralayer hydrogen bonds of the terminal carboxylic acid group, respectively. A band in the 1670-1680 cm^{-1} region usually indicates residual TFA[11]. The residual TFA is probably from the HPLC purification and the self-assembly solvents or possibly part of the supramolecular complex. Amide I bands in the region of 1630 and 1695 cm^{-1} indicate anti-parallel β-sheets, whereas IR bands in the region of 1640 cm^{-1} indicate parallel β-sheet self-assembly.

6.3.3.1 Series I

The FT-IR bands observed for C5 and C8 are very similar in the amide I region. The N-H stretching bands clearly indicate the possibility of two or more types of hydrogen bonding. This is further substantiated by the presence of both parallel and anti-parallel β-sheets as indicated by the amide I bands (Fig 6.3, C5 and C8). Based on the intensities of the IR bands in the amide I region (Fig 6.3), C5 has both parallel and antiparallel β-sheets whereas for C8 it is predominantly antiparallel β-sheet formation. The similarity of the CH_2 antisymmetric and symmetric bands, together with the CH_2 scissoring band, indicate that the oligomethylene chain has a high *trans* conformational population[10] and that its packing mode is triclinic or hexagonal[12]. Intense FT-IR bands in the 1740 - 1710 cm^{-1} region indicate that inter- and intralayer hydrogen bonds[13], more so intralayer, of the terminal carboxyl group are prominent in series I. In essence, the basis of the supramolecular architecture for C5 and C8 is heterogeneous β-sheet i.e. anti- and parallel β-sheet self-assembly and intralayer hydrogen bonding. However, it must be noted that although we have heterogeneous β-sheet self-assembly for C8 we tend to have predominantly anti-parallel β-sheet self-assembly. In the case of C11 there is homogeneous β-sheet self-assembly, i.e. antiparallel β-sheet formation and intralayer hydrogen bonding of the carboxyl group. It appears that C8 represents the transition from C5 to C11.

6.3.3.2 Series II

The N-H stretching bands, CH_2 symmetric and antisymmetric bands are very similar to those of series I. The other interesting similarity is that of the CH_2 scissoring bands to that of C11, where there is homogeneous antiparallel β-sheet association. The distinct feature of GK is the appearance of a band at 1740 cm^{-1}, which usually marks the presence of a free acid, and the absence of bands in the 1700 – 1730 cm^{-1} region. This implies that we have no intra- and interlayer hydrogen bonding of the carboxylic acid group. This holds true, as TEA was used for the self-assembly process i.e. a highly basic medium. In addition, we have predominantly homogeneous antiparallel β-sheet association as determined from the intensity of the characteristic antiparallel bands observed in the second derivative spectra (Fig 6.3, GK). The presence of a 1652 cm^{-1} band can be ascribed to the presence of the twisted sheets observed (Fig 6.2, GK). In the case of GL we have heterogeneous β-sheet association. A shoulder at 1710 cm^{-1} indicates interlayer hydrogen bonding between the carboxylic acid groups, which allows for the extension of the β-sheet, i.e. sheet-like structures encasing the bundle of fibrils (Fig 6.2, GL - A).

6.4 Conclusions

It is proposed that the self-assembly process for the bola-amphiphiles can follow five different transition phases:

Monolayer → β-sheet → β-sheet sandwich/stacking →
fibril/ sheet-like fibril → bundle of fibrils

These phases are dependent on the ω-amino acid and C-terminal amino acid of the peptide and are also strongly influenced by the solvent medium. After testing a number of solvent conditions, it was found that 0.1% aq. TFA is an ideal solvent for the supramolecular morphogenesis of these bola-amphiphiles. The use of TFA probably resulted in a shift in the conformational equilibrium state of the bola-amphiphile towards compact states that contain internally hydrogen-bonded amides[14], i.e. TFA promotes strong intralayer hydrogen bonding by acting as dehydration and/or bridging agent by direct interaction with the peptide.

TFA also promotes more effective hydrophobic interaction of the oligomethylene chain by "drying" out the flanking peptide backbone and also creates a more favourable environment for intrapeptide hydrogen bonding by out-competing the water molecules attached to the peptide backbone[14]. As bridging agent, the F-groups of TFA could interact with the hydrophobic core of the bola-amphiphile and expose its carboxylate/carboxylic acid for either electrostatic interaction or hydrogen bonding with the next molecule or layer.

We propose that for C5, the morphogenesis dictates that the bola-amphiphile initially self-assembles into a curved monolayer *via* an inherent molecular recognition process[15]. The monolayer represents the basic building block of the supramolecular complex. These layers then self-associate to form β-sheets *via* the hydrophobic effect and electrostatic interactions. Although there are both parallel and anti-parallel β-sheets present in our self-assembled structures, as observed by FT-IR, the interface strand will possibly adopt an intermediate conformation and the sheets then assume the general appearance of the predominant hydrogen bonding type[16]. Intra- and interlayer H-bond formation is responsible for the extension of the β-sheet and the formation of lamellar monolayer sheets[4]. The low pH of the solution or the TFA could result in the shortening of the intralayer and interlayer H-bonds. This, in turn, could induce a tilt in the molecular arrangement and makes the β-sheet surface more convex[3]. The short oligomethylene spacer enhances this phenomenon, resulting in the formation of well-defined fibrils (Fig 6.1). On the basis of the amphiphilicity, it is expected that the individual fibrils will subsequently associate *via* their hydrophobic faces, to generate a bundle of fibres[16].

A very similar self-assembly mechanism for C8, C11 and GL is envisaged, although the increase in the length of the ω-amino acid lessened the curving effect on the β-sheet surface. As a result we have the formation of multi-layered sheet-like structures in the supramolecular architecture of all these compounds. The self-assembly mechanism is therefore directed by the hydrophobic interaction of the oligomethylene chain. This is evident from the FT-IR data, where we have the following β-sheet transition phases for series I:

Heterogeneous (C5) → Heterogeneous ~ Homogeneous (C8, GL) → Homogeneous β-sheet (C11)

This can also be observed from the EM images (Figs. 6.1 and 6.2) where we have the following transition with the increase in oligomethylene chain length:

Fibrils → Sheet-like fibrils → Sheets

A common feature of the supramolecular architectures obtained for series I is that hydrophobic and electrostatic interactions contribute to their stability. This is true for GL and GK, however, the variation in morphology can also be attributed to the length of the aliphatic side chains of Leu and Lys. Packing of the hydrophobic side chains of amino acids, such as Leu, on the surface of the β-sheet, results in the sheet twisting[17]. In the case of GL this twisting phenomenon most probably results in the formation of microfibrils (Fig 6.2, GL – B), whereas in the case of GK we have the formation of twisted β-sheets (Fig 6.2, GK). The absence of TFA (and presence of TEA) in the self-assembly solvent of GK may also have an influence on the supramolecular morphology of this cationic bola-amphiphile. However, the presence of a uncharged ε-amino group in the side chain of Lys may be of a major importance in the unique rope-like twisting observed in the supramolecular fibril of GK. Computational studies have indicated that the twisting is favoured by intra- and inter-chain non-bonded interactions between the amino acid side chains, which are optimised in the twisted structures[18]. Twisting of β-sheets is prohibited by intra-chain electrostatic interactions, as the most favourable orientation of peptide dipoles occurs in the non-twisted structure. The stability of the twisted sheets is enhanced by the fact that they are composed of unbranched residues (Gly and Lys). This is amiably demonstrated by both the FT-IR results (1652 cm^{-1}) (Fig 6.3) and EM images (Fig 6.2) of GK. Thus, the stability of twisted β-sheets is dependent on the packing efficiency of the strands of the β-sheet (intra- and inter-strand interactions) and is determined by the amino acid composition of the strands and the frequency of the various intra- and inter-strand neighbouring pairs i.e. sheet wrapped around a core structure composed of twisted sheets associated with one another.

Highly defined supramolecular architecture is dictated by optimum structural composition and solvent conditions e.g. the C5 bundle of fibrils is determined by ω-amino acid length and TFA whereas the GK twisted rope-like structure is governed by C-terminal amino acid, TEA and ω-amino acid length. In summary, incorporation of ω-amino acid functionality, variation of the C-terminal amino acid and optimisation of solvent

conditions can be used as variables for the generation of novel supramolecular architectures of asymmetrical peptide bola-amphiphiles.

6.5 References

1. Shimizu, T.; Kogiso M.; Masuda, M. *Nature*, **1996**, *383*, 487.

2. Kogiso, M.; Ohnishi, S.; Yase, K.; Masuda, M.; Shimizu T. *Langmuir*, **1998**, *14*, 4978-4986.

3. Kogiso, M.; Okada, Y.; Hanada, T.; Yase, K.; Shimizu, T. *Biochim et Biophys Acta.*, **2000**, *1475*, 346-352.

4. Matsui, H.; Douberly, G. E. *Langmuir,* **2001**, *17*, 7918-7922.

5. Schneider, J.; Messerschmidt, C.; Schultz, A.; Gnade, M.; Schade, B.; Luger, P.; Bombicz, P.; Hubert, V.; Fuhrhop, J.–H. *Langmuir*, **2000**, *16*, 8575-8584.

6. Atherton, E.; Fox, H.; Harkiss, D.; Logan, C. J.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc. Chem. Commun.,* **1978**, 537-539.

7. Savitsky, A.; Golay, J. E. *Anal. Chem.*, **1964**, *36*, 1628-1639.

8. Toniolo, T.; Palumbo, M. *Biopolymers*. **1977**, *16*, 219-224.

9. Krimm, S.; Bandekar, J. *Adv. Protein. Chem.*, **1986**, *38*, 181-365.

10. Snyder, R. G.; Strauss, H. L. *J. Phys. Chem.,* **1982**, *86*, 5145-5150.

11. Aggeli, A.; Bell, M.; Boden, N.; Keen, J. N.; McLeish, C. B.; Nyrkova, I.; Radford, S. E.; Semenov, A. *J. Mater. Chem.,* **1997**, *7*, 1135-1145.

12. Parikh, A. N.; Schively, M. A.; Koo, E.; Seshadri, K.; Aurentz, D.; Mueller, K.; Allara, D. L. *J. Amer. Chem. Soc.*, **1997**, *119*, 3135-3143.

13. Kogiso, M.; Hanada, T.; Yase, K.; Shimizu, T. *Chem. Commun.*, **1998**, 1791-1792.

14. Rajan, R.; Balaram, P. *Int. J. Pept. Prot. Res.*, **1996**, *48*, 328-336.

15. Lehn, J.-M. *Supramolecular Chemistry*, Anton, U., Ed.; VCH Verlagsgesellschaft, Weinheim, 1995, p 139.

16. Nesloney, C. L.; Kelly, J. W. *Biorg. & Med. Chem.*, **1996**, *4*, 739-766.

17. Vtyurin, N.; *Proteins: Struct., Funct., Genet.,* **1993**, *15*, 62-70.

18. Chou, K. C.; Némethy, G.; Scheraga, H. A. *Biochem.,* **1983**, *22*, 6213-6221.

Chapter 7

Bioinspired surfactants: Design, biological activity and self-assembly into supramolecular structures

Abstract

A peptidomimetics based approach was used to design a library of novel 'hybrid' bola-amphiphilic peptide surfactants in which a cationic tripeptide motif from antimicrobial peptides was combined in a hybrid molecule containing an ω-amino acid residue. These bola-amphiphiles displayed potent antimicrobial activity against both Gram-positive and Gram-negative bacteria; the analogues were as active or more active than the leader peptides yet, remarkably, displayed little or no appreciable haemolytic activity. These organopeptide bola-amphiphiles thus demonstrated selective toxicity towards bacteria. The hydrophobicity imparted by the ω-amino acid has contrasting effects on haemolysis and antimicrobial activity of the peptide analogues. The other unique feature of these peptides and their analogues is the fact they self-assembled into complex supramolecular architectures, composed primarily of β-sheets. Their self-assembly is primarily governed by hydrophobic interactions together with inter and intralayer hydrogen bonding. Electron microscopy clearly revealed higher order structures for both peptides and analogues. The results obtained herald a new approach for the design of 'purpose-built' hybrid peptide surfactants.

7.1 Introduction

Surfactant design and development is a highly fertile domain of research in polymer science. The quest for novel surfactants is inexhaustible, for there is always scope for new molecules designed for specific purposes and innovative work. Current developments in surfactant research are inspired by their applications in nanotechnology, utilization in complex formulations and bioactive materials, and the increasing demand for biodegradable surfactants[1]. To address this ever-increasing demand for novel surfactants the focus of this work is the design, development and synthesis of bola-amphiphilic

peptide surfactants. Essentially, a bola-amphiphile (or bolaphile) is a molecule consisting of two hydrophilic moieties connected by a hydrophobic tether. Since the discovery of bolaphiles, many compounds of a similar chemical nature have been synthesized and characterized[2-4]. Most recently peptide bolaphiles have been prepared[5-7]. These amphiphilic compounds display two very unique properties if designed in a particular way; they tend to have biological activity[8], as well as the propensity to self-assemble[4]. Bolaphile-designed compounds have shown tremendous potential; such compounds may be possible antimicrobial agents[9] because of their natural tendency to disrupt membranes. Current research efforts in the field of drug design include the design of bioactive substances that will meet the requirements of being both "soft" compounds (low toxicity), as defined by Bodor[10], and immunomodulating[11]. Noting the aforementioned and the fact that there are many naturally occurring antimicrobial peptides, it became apparent that the incorporation of a crucial structural motif of the latter into a bolaphile design should lead to the creation of a hybrid peptide with antimicrobial activity. The search for an antimicrobial motif led us to a potent cationic amphiphilic peptide, KLKLLLLLKLK-NH_2, based on a core peptide in sapecin B isolated from *Sarcophaga peregina* (flesh fly)[12]. This peptide was found to be effective, not only against Gram-positive bacteria, but also Gram-negative bacteria and fungi[12]. It has also been shown to be active against *Trypanosoma cruzi*, the pathogen of Chagas' disease, which is one of the most serious parasitic diseases in Central and South America[13]. Recent studies have shown that terminal basic motifs and the internal oligo-leucine sequences play important roles in its antimicrobial activity[12]. In keeping with recent efforts to generate viable pharmaceutical therapies, we focused on "peptidomimetics" i.e. the creation of non-natural organopeptide hybrids[14]. This was accomplished by a simple modification of the peptide sequence; the hydrophobic oligo-leucine moiety was replaced with its ω-amino acid counterpart. On this basis the following library of compounds was established:

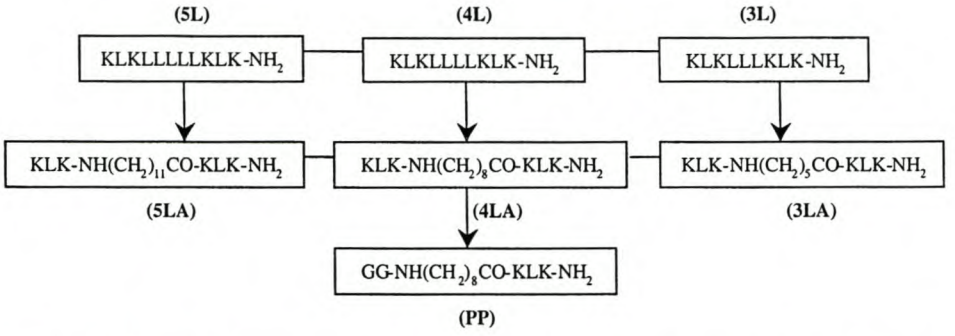


Figure 7.1 Schematic representation of the peptidomimetics approach used in this study.

Generally, peptides have proved to be inferior drug candidates because of their low oral bioavailability, potential immunogenicity and poor *in vivo* stability[14], as in the case of the leader peptide, KLKLLLLLKLK-NH_2[12]. However, we envisaged that by retaining the critical biophysical characteristics of the peptide in these analogues, i.e. the KLK and KLK-NH_2 motifs, and incorporating authentic bolaphile design by employing the use of a ω-amino acid to replace the hydrophobic oligo-leucine moiety, we would not only maintain, but possibly improve, the antimicrobial efficacy, whilst overcoming the many downstream limitations associated with peptide pharmaceuticals[15,16]. In addition, a library of this nature affords one the opportunity to identify the contribution of some components and identify important structural parameters crucial to activity.

7.2 Experimental

7.2.1 Peptide synthesis

The synthesis of peptide amides, analogues and precursor was accomplished using conventional solid phase peptide synthesis (SPPS), based on Fmoc chemistry[17]. The synthesis was carried out on Pepsyn® KB resin. The synthesis products were purified using a semi-preparative C_{18} Polygosil HPLC column. The purified products were characterised by electrospray mass spectrometry (ESMS) and nuclear magnetic resonance (NMR) spectroscopy.

7.2.2 Self-assembly

A solution of the bola-amphiphile (4 mg/mL, pH 9.2) in 0.1% TEA was prepared. The solution was then left undisturbed at room temperature for a period of up to seven days. Samples were then visualised using field emission scanning electron microscopy (FE-SEM) and analysed by photo acoustic Fourier transform infrared spectroscopy (FT-IR) and circular dichroism (CD).

7.2.3 FE-SEM

A 5 μL sample of the stock solution was placed on a support film coated with carbon and mounted on a specimen stub. The peptide fibres were washed thoroughly with de-ionised water and excess water was blotted off with filter paper. The samples were then air-dried at room temperature. Scanning electron micrographs were obtained using a LEO 1530 at 5-10 kV accelerating voltage. Prior to FE-SEM imaging a 4 nm layer of Au/Pd alloy was deposited onto the samples using a sputter coater.

7.2.4 Fourier-transform infrared spectroscopy

Lyophilised self-assembly samples were analysed by means of photo acoustic Fourier-transform infrared spectroscopy (FT-IR). Measurements were made using a photo acoustic detector MTEC model 300 unit, coupled to a Perkin Elmer Paragon 1000.

7.2.5 Circular dichroism

CD measurements were made using a Jasco J-810 spectrophotometer. All CD spectra were recorded from 270 to 180 nm in quartz cells of 2 mm path length at room temperature. The scan speed was 100 nm/min and the concentration of peptides was 0.2 mg/mL. Peptides were dissolved in 10 mM phosphate buffer (pH 7.4) (PBS), PBS + 50% TFE and 0.1% TEA.

7.2.6 Antimicrobial testing protocol

The antimicrobial testing procedure used was based on the microtitre broth dilution method recommended by the National Committee of Laboratory Safety and Standards (NCLSS), modified for cationic antimicrobial peptides by R.E.W Hancock Laboratory[18]. Target cells subjected to the peptides and analogues were *Escherichia coli* SPE 001 and methicillin resistant *Staphylococcus aureus* (MRSA) SPM 101.

7.2.7 Assay of haemolytic activity of antimicrobial peptides

Bovine erythrocytes were prepared by allowing erythrocytes to stand overnight on Alsevers solution (pH 7.2) at 4°C and then washed with 150 mM phosphate buffered saline (PBS) (pH 7.2). The cells were diluted to a final concentration of 1.8 x 10^8/mL using 150 mM PBS. The erythrocytes were then incubated with increasing quantities of each peptide, dissolved in 50% acetonitrile, for 30 minutes at 37°C in 150 mM PBS. After centrifugation, the absorption at 405 nm of the supernatants was measured to detect released haemoglobin. Melittin and Gramicidin S were used as positive controls.

7.3 Results and discussion

7.3.1 NMR characterisation and ESMS analysis of the analogues and precursor

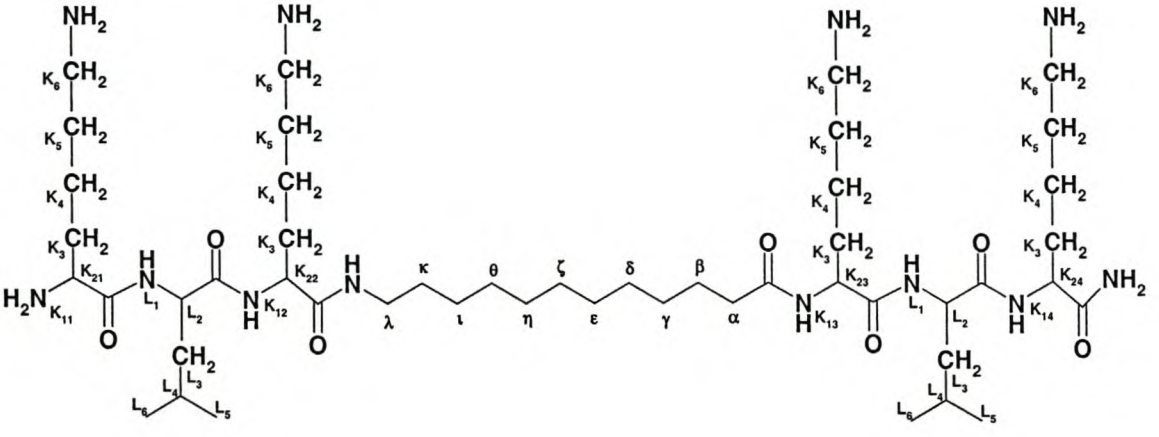


Figure 7.2 Scheme indicating atom identification for NMR discussion (5LA).

GG-NH(CH$_2$)$_8$CO-KLK-NH$_2$: Yield 85%. $[M+H]^+$ $C_{31}H_{61}N_9O_6$ required *m/z* 656.89, found 656.44; ^{1}H-NMR δ_H(600 MHz; D_2O) 0.83, 0.87 (2 x 3H, 2 x d, *J* 6.1, $L_{5\ and\ 6}$); 1.23 (8H, s_b, γ - ζ_{CH_2}); 1.30 – 1.46 (6H, m, 2 x K_4 and η_{CH_2}); 1.48 – 1.56 (4H, m, L_3 and β_{CH_2}); 1.56 – 1.64 (5H, m, L_4 and 2 x K_3); 1.64 – 1.82 (4H, m, 2 x K_5); 2.22 (2H, t, *J* 6.9, α_{CH_2}); 2.94 (4H, q_n, *J* 5.9, 2 x K_6); 3.13 (2H, q, *J* 6.7, θ_{CH_2}); 3.83 (2H, s, H_2N-C*H*$_2$-CO), 3.89 (2H, s, α_{Gly}); 4.21 (2H, q, *J* 6.3, K_{21}/K_{22}); 4.30 (1H, m, L_2).

KLK-NH(CH_2)$_5$CO-KLK-NH_2: Yield 96%. $[M+H]^+$ $C_{42}H_{84}N_{12}O_7$ required *m/z* 870.21, found 869.64; ^{1}H-NMR δ_H(600 MHz; D_2O) 0.77, 0.80, 0.83 (2 x 3H and 6H, 3 x d, *J* 6.0, 2 x $L_{5\ and\ 6}$); 1.19 (2H, m, γ_{CH_2}); 1.22 – 1.44 (10H, m, 4 x K_4 and δ_{CH_2}); 1.44 – 1.54 (6H, m, 2 x L_3 and β_{CH_2}); 1.54 – 1.64 (10H, m, 2 x L_4 and 4 x K_3); 1.64 – 1.81 (8H, m, 4 x K_5); 2.17 (2H, t, *J* 7.3, α_{CH_2}); 2.89 (8H, q_n, *J* 5.5, 4 x K_6); 3.05 (2H, m, ε_{CH_2}); 3.87 (1H, m, K_{24}), 4.09 (1H, m, K_{21}); 4.16 (2H, m, K_{22} and K_{23}); 4.26 (2H, m, 2 x L_2).

KLK-NH(CH_2)$_8$CO-KLK-NH_2: Yield 95%. $[M+H]^+$ $C_{45}H_{90}N_{12}O_7$ required *m/z* 912.29, found 911.74; ^{1}H-NMR δ_H(600 MHz ; D_2O) 0.83, 0.85, 0.89, 0.89 (4 x 3H, 4 x d, *J* 6.1, 2 x $L_{5\ and\ 6}$); 1.23 (8H, s_b, $\gamma - \zeta_{CH_2}$); 1.28 – 1.48 (10H, m, 4 x K_4 and η_{CH_2}); 1.48 – 1.60 (6H, m, 2 x L_3 and β_{CH_2}); 1.60 – 1.70 (10H, m, 2 x L_4 and 4 x K_3); 1.70 – 1.88 (8H, m, 4 x K_5); 2.22 (2H, t, *J* 7.3, α_{CH_2}); 2.94 (8H, m, 4 x K_6); 3.10, 3.15 (2 x 1H, 2 x m, θ_{CH_2}); 3.93 (1H, m, K_{24}), 4.14 (1H, m, K_{21}); 4.22 (2H, m, K_{22} and K_{23}); 4.32 (2H, m, 2 x L_2).

KLK-NH(CH_2)$_{11}$CO-KLK-NH_2: Yield 91%. $[M+H]^+$ $C_{48}H_{96}N_{12}O_7$ required *m/z* 954.37, found 953.71; ^{1}H-NMR δ_H(600 MHz ; D_2O) 0.85, 0.87, 0.90 (2 x 3H, 6H, 2 x d, m, *J* 6.1, 2 x $L_{5\ and\ 6}$); 1.23 (14H, s_b, $\gamma - \iota_{CH_2}$); 1.28 – 1.50 (10H, m, 4 x K_4 and κ_{CH_2}); 1.50 – 1.62 (6H, m, 2 x L_3 and β_{CH_2}); 1.62 – 1.72 (10H, m, 2 x L_4 and 4 x K_3); 1.72 – 1.90 (8H, m, 4 x K_5); 2.24 (2H, t, *J* 7.2, α_{CH_2}); 2.96 (8H, q_n, *J* 7.6, 4 x K_6); 3.12, 3.16 (2 x 1H, 2 x m, λ_{CH_2}); 3.95 (1H, m, K_{24}), 4.16 (1H, m, K_{21}); 4.23 (2H, m, K_{22} and K_{23}); 4.33 (2H, m, 2 x L_2).

Peptides were characterized using ESMS:

KLKLLLKLK-NH_2, Yield 85%, $[M+H]^+$ $C_{54}H_{106}N_{14}O_9$ required *m/z* 1096.53, found 1095.84.

KLKLLLLKLK-NH_2, Yield 60%, $[M+H]^+$ $C_{60}H_{117}N_{15}O_{10}$ required *m/z* 1209.69, found 1208.68.

KLKLLLLLKLK-NH_2, Yield 58%, $[M+H]^+$ $C_{66}H_{128}N_{16}O_{11}$ required *m/z* 1322.85, found 1322.17.

7.3.2 FT-IR spectroscopy

FT-IR analysis of the lyophilised self-assembled peptide fibres was used to determine the molecular arrangement in the different supramolecular complexes.

Table 7.1 FT-IR bands observed for the fibres of the precursor and the peptide analogues.

Analogues	PP	3LA	4LA	5LA
NH str.	3266	3294	3284	3283
	3087	3066	3074	3068
CH_2 antisymmetric str.	2954	2952	2934	2928
CH_2 symmetric str.	2870	2871	2868	2862
Amide II	1543	1543	1543	1543
CH_2 scissoring	1468	1464	1460	1470

[a]Amide I bands determined from second derivative spectra (See Fig 7.1)

Table 7.2 FT-IR bands observed for the peptide fibres

Peptide	3L	4L	5L
NH str.	3264	3266	3267
	3088	3084	3087
Amide II	1548	1545	1546

[a]Amide I bands determined from second derivative spectra (See Fig 7.1)

The N-H stretching vibration band, together with the amide I and II bands, indicated the formation of hydrogen bonds[19,20]. The Savitsky-Golay[21] derivative function was used to determine the second-derivative spectra of the amide I bands (Fig 7.1) in order to provide more detailed spectral information.

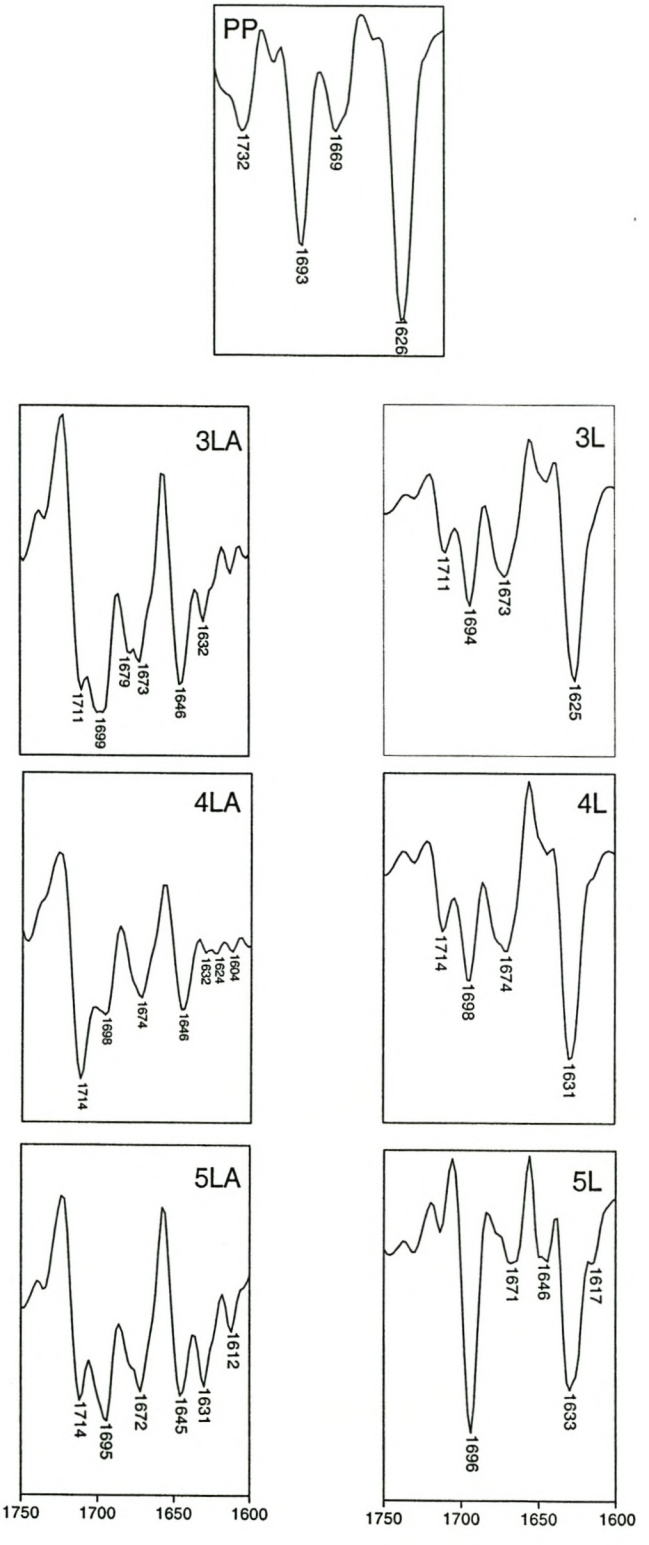


Figure 7.1 Second derivative spectra of the dried self-assemblies of the precursor, analogues and peptide in the amide I region.

Deformation vibration bands of the methylene groups displayed the packing mode of the oligomethylene chain[22]. The CH_2 antisymmetric and symmetric bands (Table 7.1) of the precursor and the peptide analogues are relatively larger than those of the oligomethylene chains having a high *trans* conformational proportion. Therefore, the oligomethylene spacers are in a highly disordered state. This is duly reflected by the lack of a 'defined' supramolecular complex formed by either the analogues or the precursor (Fig 7.2a and b).

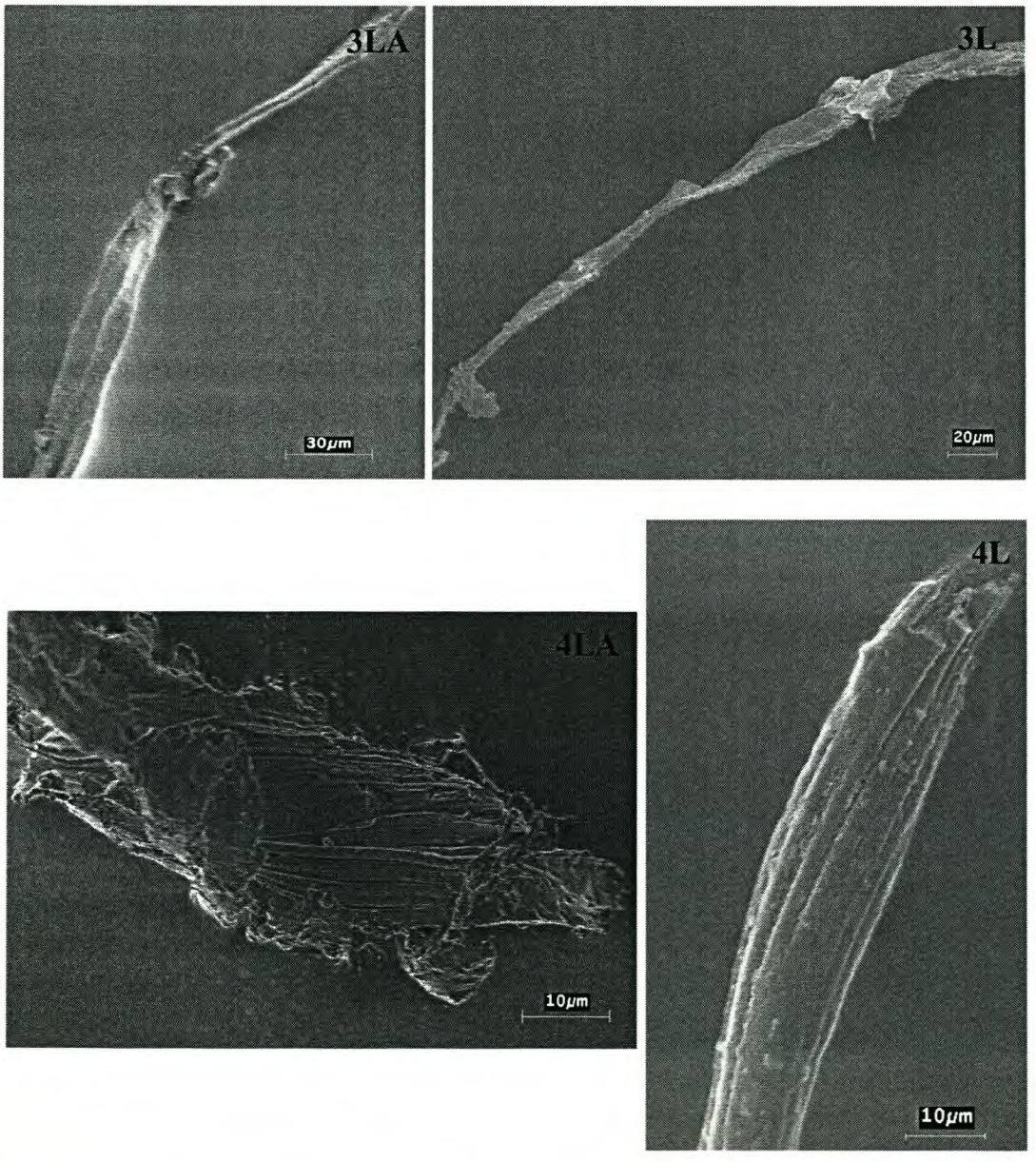


Figure 7.2a. Self-assembly of the peptides (3L and 4L) and their corresponding analogues (3LA and 4LA) in 0.1% TEA, observed using FE-SEM.

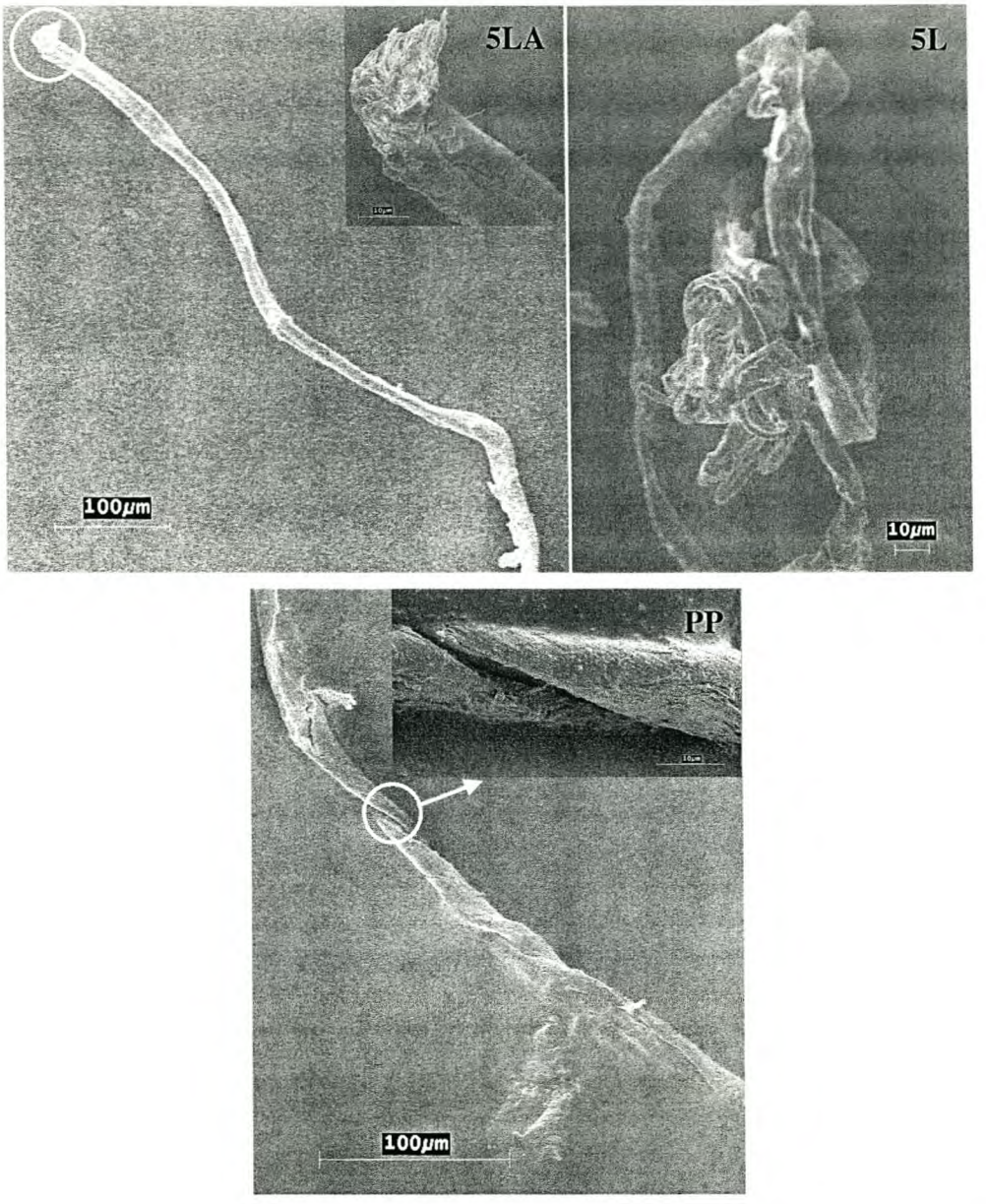


Figure 7.2b. Self-assembly of the peptide (5L), its corresponding analogue (5LA) and precursor in 0.1% TEA, observed using FE-SEM.

The presence of amide I bands at around 1710 cm^{-1} and 1730 cm^{-1} indicated the possibility of inter- and intralayer hydrogen bonds of the terminal amide group, respectively. A band around the 1670-1680 cm^{-1} region usually indicates residual TFA[23], possibly from HPLC purification solvents. It is well established that amide I bands in the region of 1630 and 1695 cm^{-1} indicate antiparallel β-sheet formation whereas IR bands in the region of 1640 cm^{-1} indicate parallel β-sheet self-assembly. The N-H stretching and

amide II (Table 7.1) and I bands (Fig 7.1) of the precursor are typical of an antiparallel β-sheet network. The FT-IR band at 1732 cm^{-1} indicated lateral hydrogen bonds[24] of the C-terminal amide group. This is responsible for the multilayered twisted sheet formation revealed in the FE-SEM micrograph of the precursor of the peptide analogue (PP), (Fig 7.2b). The 'twist' in the sheets can be ascribed to polyglycine II conformation imparted by the glycine residues[25]. In the case of the analogues the N-H stretching band (Table 7.1) and amide I band (Fig 7.1) are comprised of multiple bands, suggesting the formation of two or three types of hydrogen-bond networks. The FT-IR bands in the amide I region are of similar intensity and are characteristic of both antiparallel and parallel β-sheets, indicating heterogeneous β-sheet self-assembly for the analogues. FT-IR bands observed at 1710-1715 cm^{-1} represent bifurcated hydrogen bonds[24], i.e. amide-amide dimer formation, which results in an extension of the β-sheets. Electron micrographs of 4LA and 5LA (Fig 7.2a and b) reveal that the complexes comprise self-associated β-sheets in the order of nanometers, whereas 3LA self-assembles into a ribbon-like structure (Fig 7.2a). Since their supramolecular complexes comprise both parallel and antiparallel β-sheets (Table 7.1), the interface strand most probably adopts an intermediate conformation and the sheets will then assume the general appearance of the predominant hydrogen bonding type[26]. In the peptides, the N-H stretching and amide II (Table 7.2) and the intensity of the amide I bands (Fig 7.1) are characteristic of an antiparallel β-sheet network. FT-IR bands in the 1710-1715 cm^{-1} region indicate the possibility of bifurcated hydrogen bond formation between the terminal amide head groups, very similar to that observed for the analogues. FE-SEM images of 4L and 5L (Fig 7.2a and b) indicate the formation of higher order supramolecular structures whereas for 3L we have the formation of a ribbon-like structure (Fig 7.2a). More defined supramolecular structures are possibly due to the intramolecular H-bonding between the somewhat more rigid peptide backbone. A notable similarity between the peptides and the analogues in the case of 4L/4LA and 5L/5LA is that higher order structures were formed, whereas in the case of 3L/3LA simple ribbon-like structures were formed.

7.3.3 Circular dichroism

In order to obtain more data on the self-assembly of the library of peptides the current techniques were complemented by the use of CD. CD experiments were carried out in various solvents to study the behaviour of the peptides in different environments.

Experiments conducted in 0.1% TEA gave an excellent indication of the type of secondary structure involved in the self-assembly process (Fig 7.3).

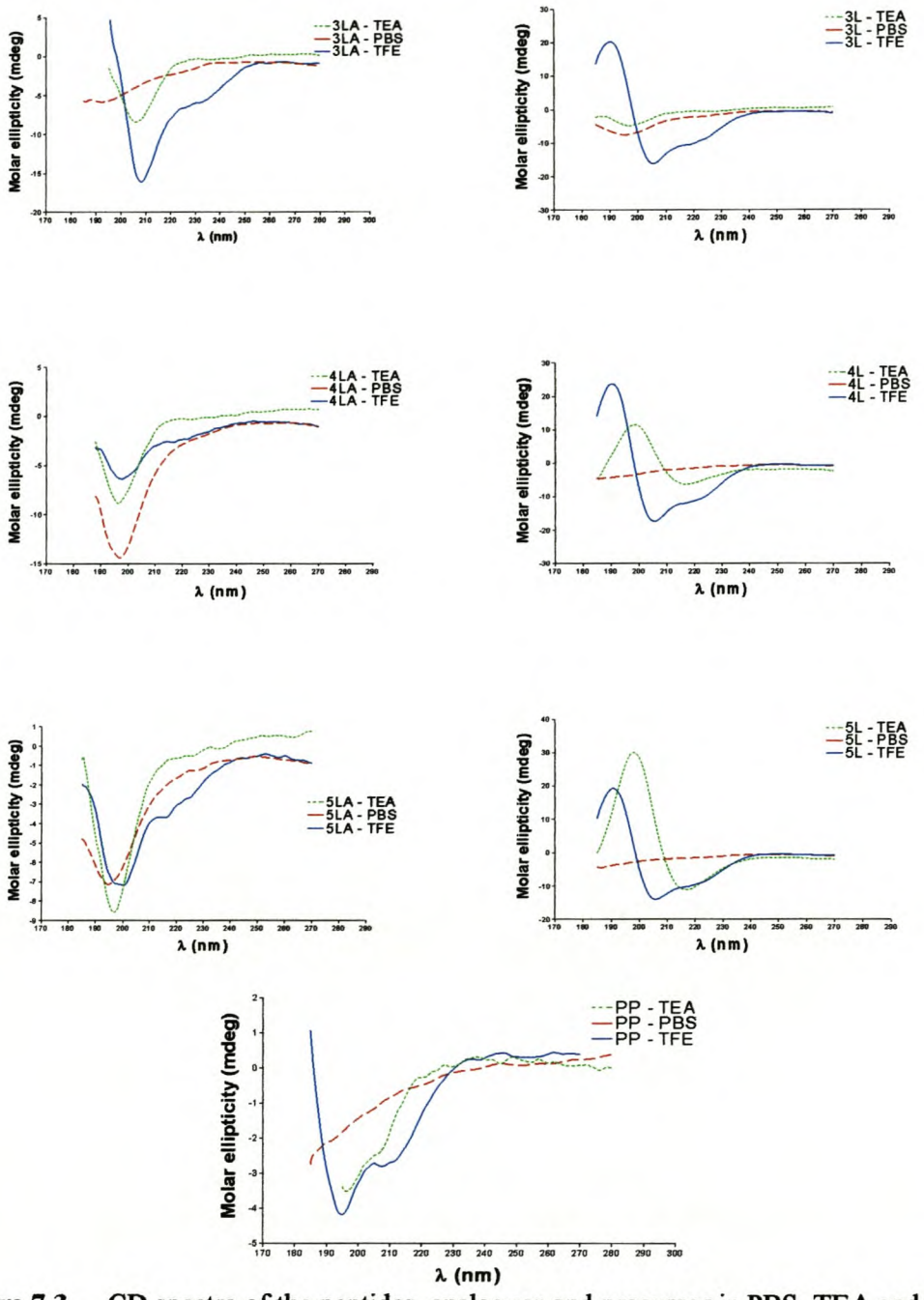


Figure 7.3 CD spectra of the peptides, analogues and precursor in PBS, TEA and TFE.

A distinct feature of the results is that the analogues (4LA and 5LA) assumed a random structure in 0.1% TEA (minima between 194-199.6 nm), whereas the peptides (4L and 5L) formed β-sheets (Fig 7.3) (maxima 196.6-198.2 nm and minima 212.6-216 nm)

with some random structure (minima 191.2-196 nm). 3L and 3LA were very similar in that both had predominantly random structures (Fig 7.3). The trend observed here is very similar to that observed with FE-SEM.

CD of samples was then conducted in TFE and PBS to assess the peptides' behaviour when passing from a watery medium to a hydrophobic environment (Fig 7.3). Results from this study helped us to predict the possible conformational change of the peptides upon interaction with a target cell membrane. The induction of a secondary structure (α-helix or β-sheet) upon membrane interaction has been shown to be crucial in the activity of most antimicrobial peptides[27,28]. All the peptides and analogues had random structures in PBS. The analogues also had random structures in TFE, as indicated by the positive Cotton effect at 193-201 nm. The CD spectra of leader peptides in TFE, however, exhibited negative Cotton effects at 209.8-213 nm and 221-224 nm, and a positive Cotton effect at 180-181 nm, which pointed to structures with a major α-helical content. Some random structure was also present, as indicated by a minimum in the region of 195-196 nm. An unexpected CD spectrum was observed for the precursor peptide (PP), indicating that the PP structure in TFE contained either some α-helical structures or mimicked a α-helix. However, the PP-spectrum's maximum (178.8 nm) and minima (195.0, 209.2 and 220 nm) were slightly blue-shifted from those of the leader peptides and it also had a much lower intensity (<50%).

7.3.4 Biological activity

The antimicrobial results obtained for the peptides, analogues and precursor (Table 7.3) correlated well with the CD results, in the sense that the three leader peptides, which exhibited α-helical behaviour in TFE, were highly active against *E. coli* and MRSA.

Peptide or analogue	*E. coli* MIC μM (μg/mL)	*MRSA* MIC μM (μg/mL)	Erythrocytes HC_{50} μM[§]
5L	12 (16)	0.4 (0.5)	>>200
4L	26 (32)	2 (2)	140
3L	7 (8)	2 (2)	143
5LA	67 (64)	17 (16)	194
4LA	4 (4)	0.5 (0.5)	>>200
3LA	18 (16)	1 (1)	>>200
PP	24 (16)	1 (0.5)	>>200
Gram S	20*	ND	22
Melittin	ND	ND	2

* Concentration of 50% inhibition (personal communication M Vlok)

§ Calculated from dose-response curves (Fig 7.4)

Table 7.3 Biological activity of the precursor, peptides and their analogues

The precursor of the peptide analogue (PP), which also demonstrated α-helical structure, showed similar activity levels to that of the leader peptides. The analogues were also highly active. They were comparable to the leader peptides and in some cases even more active, even though they assumed a random structure in TFE. This implies that their mode of action probably differs from that of the leader peptides. The general antibacterial activity sequence of the peptides can be summarised as follows: **3L ~ 4LA > 5L ~ 3LA > 4L ~ PP > 5LA**. Further, all of the peptides were more active against the Gram-positive MRSA than the Gram-negative *E. coli*, an indication of selectivity towards the former.

Another significant result of this investigation was that 5L and the hybrid analogues, except 5LA, demonstrated no appreciable haemolysis of bovine erythrocytes (Table 7.3 and Fig.7.4).

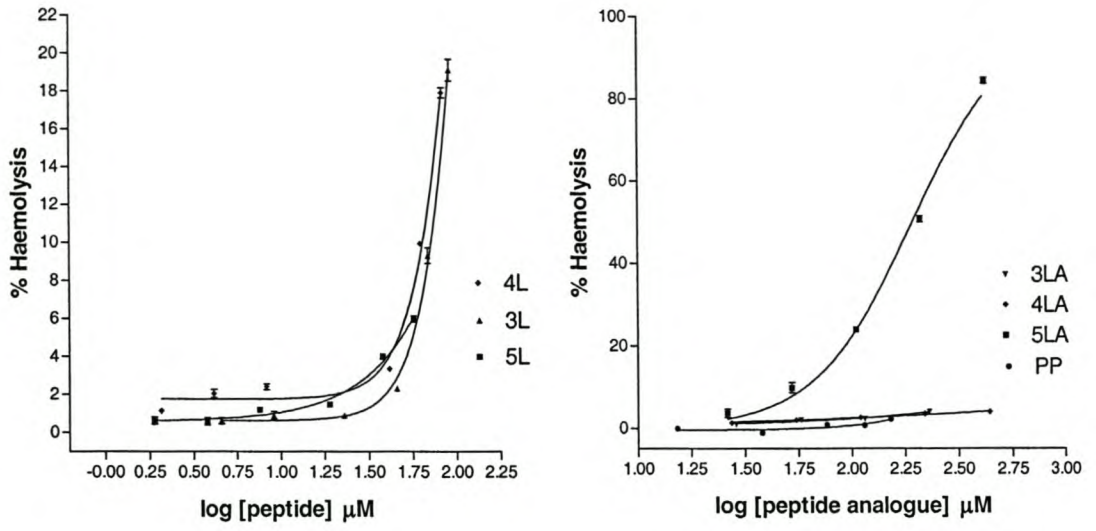


Figure 7.4 Haemolytic activity of the leader peptides, their analogues and the precursor.

3L and 4L were the most haemolytic and for 5LA a HC_{50} (concentration causing 50% haemolysis) just less than 200 µM was found. The haemolytic activity of the latter three, however, was between 7 and >70 times less active than gramicidin S (Gram S) and melittin. For 5LA there is a direct contrast between its antimicrobial activity (least active) and haemolytic activity (most active of hybrid peptides). This is probably because of the effect of the longer hydrophobic ω-amino acid incorporated in this peptide hybrid. Blondelle *et al.*[29] observed similar trends when they increased the hydrophobicity of model peptides by substituting lysine with leucine.

7.4 Conclusions

A general mechanism for the self-assembly of the precursor, analogues and peptides into supramolecular complexes can be described as follows:

Monolayers → β-sheet → multilayered β-sheets

The interaction of TEA with the peptide made 0.1% TEA an ideal solvent for the self-assembly of the peptides, i.e. it promoted strong intralayer hydrogen bonding. A characteristic feature of bola-amphiphiles is their ability to form monolayers[2]. The formation of monolayers marks the onset of the self-assembly process. These monolayers

are formed *via* a self-recognition process[30], which can occur in one of two ways: first by virtue of peptide backbone interaction through intramolecular hydrogen bonding (H-bonding) or, second, via hydrophobic interaction of the ω-amino acid linker. These monolayers then self-associate *via* inter and intramolecular H-bonding to from β-sheets. The extension and the formation of multilayered β-sheets can be attributed to inter and intralayer H-bonding between the terminal amide head groups.

The self-assembly of the peptides and analogues may be crucial for bioactivity, because the biological target of our peptides and analogues is most probably the bacterial cell membrane. It has been shown that at least 18-20 amino acid residues are required for a membrane active peptide to span the average lipid bilayer[31,32]. The peptides and analogues comprise only 7-11 residues, so they probably form some type of a self-assembly complex to disrupt and/or form pores[33] in the bacterial membrane. In the case of the leader peptides, it has been established that their antimicrobial activity is due to membrane perturbation and possibly the formation of ion channels in the bacterial membrane[34]. In the case of the analogues, we propose that they also initially associate with negatively charged cell wall components *via* electrostatic interactions[35], possibly also out-competing Ca^{2+} and Mg^{2+} from their cross-bridges[15]. It has been hypothesised for other cationic peptides that the affected cell wall may then be prone to form 'cracks' which then allow the peptides to pass through self-promoted uptake[36]. The uptake of the analogues into the cell membrane most probably does not depend on the formation of an amphipathic α-helix[27], as for the leader peptides, but may be driven by hydrophobic interaction involving the ω-amino acid functionality. We envisage that the mode of action on the cell membrane of these analogues may follow the "carpet" model[37] or, more simply, it may work "detergent-like". It is, however, important to note that if the analogue (or peptide) is too hydrophobic it tends to prematurely self-associate in an aqueous medium and this will prevent its transport to the microbial membrane target. This was clearly reflected in the higher MIC values of 5LA (Table 7.3). The hydrophobicity phenomenon is also of paramount importance in the self-assembly process i.e. the longer the length of the ω-amino acid the more complex the supramolecular architecture. One can therefore conclude that optimal bola-amphiphilicity is required for both self-assembly and activity; where bola-amphiphilicity is a function of hydrophilicity and length of the hydrophobic domain of the molecule.

In general the peptide analogues probably do not interact appreciably with mammalian cells, yet they do have potent antimicrobial activity. Our results clearly indicate that the hybrid organopeptide surfactants, especially 3LA and 4LA, display selective toxicity toward bacteria. This, together with their low molecular weights, make them ideal candidates in the development of therapeutic drugs. The design and study of these peptide analogues marks a novel approach, one that shows tremendous potential, in the quest for developing new generation pharmaceutical drugs and also creating novel biomaterials. Continued active exploration of diverse backbone and side chain chemistries, and connectivities in peptide surfactants offer the potential for obtaining self-organized materials with greater chemical diversity, bioactivity and biostability than natural peptides. Subtle modifications in design will also be beneficial in understanding their properties on a molecular level. These systems span the disciplines of molecular biology, bioengineering, chemistry and polymer science.

7.5 References

1. Chevalier, Y. (2002), *Curr. Opin. Coll. Interf. Sci., 7*, 3-11

2. Fuhrhop, J.-H., and Fritsch, D. (1986), *Acc. Chem. Res., 19*, 130-137.

3. Fuhrhop, J.-H., Bartsch, H., and Fritsch, D. (1981), *Angew. Chem. Int. Ed. Engl., 20*, 804-805.

4. Escamilla, G. H., and Newkome, G. R. (1994), *Angew. Chem. Int. Ed. Engl., 33*, 1937-1940.

5. Kogiso, M., Ohnishi, S.; Yase, K., Masuda, M., and Shimizu, T. (1998), *Langmuir, 14*, 4978-4986.

6. Matsui, H., and Gologan, B. (2000), *J. Phys. Chem. B., 15*, 3383-3386.

7. Schneider, J., Messerschmidt, C., Schultz, A., Gnade, M., Schade, B., Luger, P., Bombicz, P., Hubert, V., and Fuhrhop, J. –H. (2000), *Langmuir, 16*, 8575-8584.

8. Grigoryan, N. A., Mndzhoyan, Z. O., Kazaryan, E. V., Ter-Zakharyan, Z. Yu., and Mndzhoyan, O. L. (1992), *Pharm. Chem. J.*, (2), *26*, 153-157.

9. Jayasuriya, N., Bosak, S., and Regen, S. L. (1990), *J. Amer. Chem. Soc., 112*, 5844-5850.

10. Bodor, N., Kaminsky, J. J., and Selk, S. (1980), *J. Med. Chem., 23*, 469-480.

11. Jahnová, E., Ferenčík, M., Nyulassy, S., Devínsky F., and Lacko, I. (1994), *Immunol. Lett., 39,* 71-75.

12. Alvarez-Bravo, J., Kurata, S., and Natori, S. (1994), *Biochem. J., 302*, 535-538.

13. Shimada, J. N., Natori, S., and Aoki, T. (1998), *Parasit. Intl., 47,* 203-209.

14. Patch, J. A., and Barron, A. E. (2002), *Curr. Opin. Chem. Biol., 6,* 872-877.

15. Hancock, R. E.W., (1997), *Lancet, 349*, 418-422.

16. Rautenbach, M., and Hastings J. W. (1999), *Chim Oggi/Chem., Today, Nov/Dec,*

81-89.

17. Atherton, E., Fox, H., Harkiss, D., Logan, C. J., Sheppard, R. C., and Williams, B. J. (1978), *J. Chem. Soc. Chem. Commun.*, 537-539.

18. www.cmdr.ubc.ca/bobh/methods.php

19. Doan, V., Köppe, R., and Kasai, P. H. (1997), *J. Amer. Chem. Soc.*, *119*, 9810-9815.

20. Krimm, S., and Bandekar, J. (1986), *Adv. Protein. Chem.*, *38*, 181-365.

21. Savitsky, A., and Golay, J. E. (1964), *Anal. Chem.*, *36*, 1628-1639.

22. Snyder, R. G., and Strauss, H. L. (1982), *J Phys. Chem., 86*, 5145-5150.

23. Aggeli, A., Bell, M., Boden, N., Keen, J. N., McLeish, C. B., Nyrkova, I., Radford, S. E., and Semenov, A. (1997), *J. Mater. Chem., 7*, 1135-1145.

24. Kogiso, M., Hanada, T., Yase, K., and Shimizu, T. (1998), *Chem. Commun.*, 1791-1792.

25. Navarro, E., Tereshko, V., Subirana, J. A., and Puiggalí, J. (1995), *Biopolymers, 36,* 711-722.

26. Nesloney, C. L., and Kelly, J. W. (1996), *Biorg & Med. Chem.*, *4*, 739-766.

27. Epand, R. M., and Vogel, H.J. (1999), *Biochim. Biophys. Acta., 1462*, 11-28.

28. Blondelle, S. E., Lohner, K., and Aguilar, M-I. (1999), *Biochim. Biophys. Acta., 1462*, 89-108.

29. Blondelle, S. E., and Houghten R. A. (1992), *Biochem.*, *31*, 12688-12694.

30. Lehn, J.-M. (1995), *Supramolecular Chemistry.*, (Anton, U., Ed.), p 139, VCH Verlagsgesellschaft, Weinheim.

31. Kiyota, T., Lee, S., and Sugihara, G. (1996), *Biochem., 35*, 13196-13204.

32. Lear, J. D., Wasserman, Z, R., and DeGrado, W. F. (1988), *Science, 240*, 1177-

1181.

33. Anzai, K., Hamasuna, M., Kadona, H., Lee, S., Aoyagi, H., and Kirino, Y. (1991), *Biochim. Biophys. Acta.*, *1064*, 256-266.

34. Hirakura, Y., Alvarez-Bravo, J., Kurata, S., Natori, S., and Kirino, Y. (1996), *J. Biochem., 120*, 1130-1140.

35. Liu, L.-P., and Deber, C. M., (1997), *Biochem., 36*, 5476-5482.

36. Hancock, R. E. W., Falla, T., and Brown, M. M. (1995), *Adv. Microbial. Physiol., 37*, 135-175.

37. Shai, Y., (1999), *Biochim. Biophys. Acta.*, *1462*, 55-70.

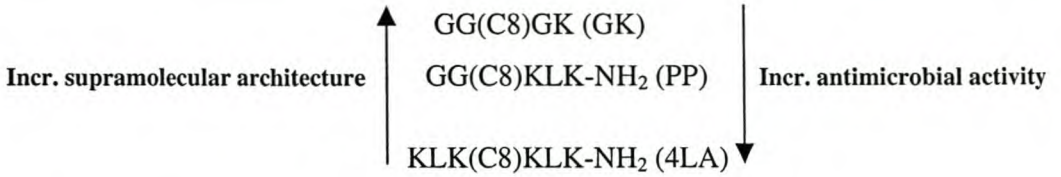
Chapter 8

'Purpose-built' bio-inspired cationic surfactants

8.1 Conclusions

The molecular machinery of nature is unrivalled by conventional manufacturing technology and has much to offer in demonstrating how to recognise, organise, functionalise and assemble new molecular materials and tools for applications in everything from electronics to medicine. This potential will only be realised fully when the ability to design specific protein, polynucleotide or peptide motifs with preordained functions becomes commonplace. This dissertation is the first report of a study in which versatile, purpose-built bio-inspired cationic surfactants with both remarkable self-assembly properties and antimicrobial activity were designed *via* a "peptidomimetics" approach, i.e. the creation of non-natural peptide mimics[1].

Our cationic peptide surfactant/bola-amphiphiles were designed using motifs from KLKLLLLLKLK-NH_2 (5L), derived from sapecin B[2] (refer to Chapter 7), and Shimizu's [3] oligo-glycine bola-amphiphiles (refer to Chapters 3 and 4). In essence, these amphiphilic molecules are functionalised membrane mimics[4]. The peptide hybrids in our study were designed with two specific purposes in mind: one, to generate hierarchal supramolecular architecture and secondly, to create the novel antibiotics where the combination of both properties would epitomize unparalleled versatility. A library of ten hybrid peptide bola-amphiphiles, including 5L and two shorter peptides and Shimizu's oligo-glycine bola-amphiphile were synthesised using Fmoc-polyamide based peptide synthesis[5] and purified via high performance liquid chromatography. The library was characterised using electrospray mass spectrometry (ESMS), nuclear magnetic resonance (NMR), different modes of electron microscopy and Fourier-transform infrared spectroscopy (FT-IR). In some cases further studies were done using circular dichroism for structural analysis and bioactivity tests. From these results the following three hybrid peptides from the library resemble the compounds with the most unique characteristics:



Where C8 = $-NH(CH_2)_8CO-$

For bola-amphiphiles without lysine, the optimum solvent for self-assembly was found to be 0.1% TFA, and for those with lysine in its structure, 0.1% TEA was used. Large self-assembly complexes were achieved in less than a day for most of the compounds, and in some cases within seven hours (refer to Chapter 4). The bola-amphiphiles self-assembled to form β–sheets that continued to self-assemble into higher order supramolecular complexes (refer to Chapters 5-7). The resulting supramolecular complexes that were observed represent highly ordered structures. These structures are primarily composed of monolayers[6], which self-assemble *via* an inherent molecular-recognition process[7] to form β sheets, that in turn self-associate to form the complexes. FT-IR spectroscopy of the supramolecular complexes indicated that the structures comprise heterogeneous/homogeneous β–sheets and in some cases intra- and interlayer hydrogen bonding between the C-terminal head groups. The stability of the complexes can also be ascribed to hydrophobic and electrostatic interactions. It is proposed that the self-assembly process for the bola-amphiphiles can follow five different transition phases:

Monolayer → β-sheet → β-sheet sandwich/stacking →
fibril/ sheet-like fibril → bundle of fibrils

The general trend for the supramolecular complexes of the three representative peptides is:

GK (twisted β-sheets) → **PP** (β-sheets with some twist) → **4LA** (flattened β-sheets)

The presence of a uncharged ε-amino group in the side chain of Lys is of major importance in the unique rope-like twisting observed in the supramolecular fibril of GK. Computational studies have indicated that the twisting is favoured by intra- and inter-chain non-bonded interactions between the amino acid side chains, which are optimised in the twisted structures[8]. The stability of the twisted sheets is enhanced by the fact that they are composed of amino acids with unbranched side chains (Gly and Lys). Thus, the

stability of twisted β-sheets is dependent on the packing efficiency of the strands of the β-sheet (intra- and inter-strand interactions) and is determined by the amino acid composition of the strands and the frequency of the various intra- and inter-strand neighbouring pairs, i.e. sheet wrapped around a core structure composed of twisted sheets associated with one another. The supramolecular complex of PP on the other hand comprises an antiparallel β-sheet network where lateral hydrogen bonds[9] of the C-terminal amide group are responsible for the multilayered twisted sheet formation. The 'twist' in the sheets can be ascribed to polyglycine II conformation imparted by the glycine residues[10]. However, in the case of 4LA FT-IR, analysis indicated that the supramolecular complex is comprised of heterogeneous β-sheet self-assembly where bifurcated hydrogen bonds[9], i.e. amide-amide dimer formation, is responsible for an extension of the β-sheets. In essence, we achieved "self-assembly of self-assemblies", which may represent a most basic example of supramolecular morphogenesis at its highest expression, namely the generation of biological structure in the course of the development of living organisms.

In the exploration of further potential of the peptides, we analysed the activity of some of the 5L derived bola-amphiphiles on selected bacteria and bovine erythrocytes. The antibacterial activity of some of the 5L derived hybrid peptides and even PP indicated that the peptide bola-amphiphiles were more effective against methicillin resistant *Staphylococcus aureus* than *Escherichia coli* (refer to Chapter 7). The antimicrobial activity of most of the amphiphiles was similar to that of the leader peptide (5L) and in some cases such as 4LA appreciably higher. A distinct feature of these peptide hybrids is that they are relatively non-haemolytic. No appreciable haemolysis of bovine erythrocytes under conditions in which both mellitin and gramicidin S caused 100% haemolysis (refer to Chapter 7) was observed. 4LA demonstrated little or no haemolytic activity up to levels of 400 μg/mL. The hybrid peptide surfactants probably do not interact appreciably with mammalian cells in general, yet they do have potent antimicrobial activity, i.e. these bola-amphiphilic peptide surfactants display selective toxicity to bacteria. The general trend for the antimicrobial activity: **GK <<< PP ~ 5L < 4LA**, where the net charges of the peptides vary from +1 to +5 with an increase in the number of lysine residues. As found by other investigators[11], our studies also indicated that a sensitive balance of electrostatic and hydrophobic interactions determines the antimicrobial activity of the peptides. We also found that the reinforcement of electrostatic interactions by

modification of the positive peptide charge, together with a conservation of a high permeabilizing efficiency based on a well developed hydrophobic domain, enhances antimicrobial activity and improves prokaryotic selectivity[12]. Retaining the critical biophysical characteristics of the peptide in these analogues we not only maintained, but improved, the antimicrobial efficacy, whilst possibly overcoming the many downstream limitations associated with peptide pharmaceuticals[13,14]. This, together with their low molecular weights, makes the analogues and precursor ideal candidates in the development of therapeutic drugs.

To summarise, the self-assembly of GK epitomises the design of peptide hybrids for the generation of higher order supramolecular architecture, whereas 4LA represents a remarkably versatile surfactant capable of generating higher order supramolecular architecture and serving as a potent biocompatible antimicrobial agent. The generation of higher order supramolecular architecture is dependent on optimisation of β-sheet self-assembly, whereas antimicrobial activity is dependent on the balance between net positive charge and optimum hydrophobicity of the peptide hybrids.

This study has demonstrated that it is possible to design hybrid peptide surfactants capable of producing higher order supramolecular architecture and improving the antimicrobial activity whilst reducing the haemolytic effect by using a simpler, economically more viable system than that suggested by Ghadiri *et al*[15]. The study and design of these versatile 'purpose-built' bio-inspired surfactants marks a novel approach, one that shows tremendous potential. The antimicrobial activity of the peptide hybrids and the generation of supramolecular architectures from these synthetic peptide assemblies, together with an understanding of the molecular mechanisms that govern their formation, provide a conceptual breakthrough towards the design of new innovative drugs and therapies to address the global crisis of antibiotic resistance, and offers the potential for self-organised materials with greater chemical diversity, bioactivity and biostability than natural peptides.

8.2 Future prospects and recommendations

The two patents[16,17] that evolved from this study can best articulate future prospects and recommendations stemming from this study.

8.2.1 Peptide hybrid nanomaterials

Compounds with the following structural motif are potentially useful for generating higher order supramolecular architecture and nanomaterials,

$$(XG)_m(R^{\Phi})R_1$$

Where

(i) X = H

- Lys*, Orn*
- Gly, β-Ala
- Leu*, Val*
- Phe*, Tyr*

* L or D amino acid

(m = 0-10)

(ii) R^{Φ} = -NH-$(CH_2)_n$-CO- , n = 2 – 12; in addition: unsaturated, functionalised and branched analogues of R^{Φ}

(iii) $R_1 = (GX)_m$ or $[GX(R^{\Phi})GX]_n$, $n = 1 \rightarrow \infty$

Other possible structures include cyclic analogues and polymers of the structures.

The applications of these hybrid peptides extend into many fields:

- Surfactants – bioinspired materials, paint, cosmetics, polymers, etc.
- Pleochroic material – applications in solar cells and photo switches, etc.
- High tensile strength material

- Nanotechnology
- Antimicrobial agents (see 8.2.2)

8.2.2 Antimicrobial peptides and peptide hybrids

Compounds with the following structural motif can possibly serve as novel antimicrobial agents,

[BΦ or ΦB or BΦB][R^{Φ} and/or $(\Phi)_n$][BΦ or ΦB or BΦB]

Where

(i) B = Lys*, Orn*

(ii) Φ = Gly, β-Ala
- Leu*, Val*
- Phe*, Tyr*

*L or D amino acid

(iii) R^{Φ} = -NH-$(CH_2)_n$-CO- , n = 2 – 12; in addition: unsaturated, functionalised and branched analogues of R^{Φ}

N and C – terminal ends of the hybrids can also be modified by using N – terminal blocking (amide) or C – terminal blocking/modification (ester or amide).

Other possible structures include polymers and cyclic analogues of the structures. In addition there is also the possibility of combinations/repeats of [BΦ / ΦB / BΦB] on N- and/or C-terminal of **[R^{Φ} or $(\Phi)_n$]**.

Potential applications of these novel hybrid antimicrobial peptides include:

- Immobilised peptide/polymer with antimicrobial activity
- Sterile catheters, wound dressings, filters
- Antimicrobial agents

- Human bacterial infections (topical and systemic)
- Fungal infections (animal and plant)
- Viral infections (topical and systemic)
- African sleeping sickness (systemic)
- Malaria (systemic)

8.3 References

1. Patch, J. A., Barron, A. E. *Curr. Opin. Chem. Biol.*, **6**, 872-877, 2002.
2. Alvarez-Bravo, J., Kurata, S., Natori, S. *Biochem. J.*, **302**, 535-538, 1994.
3. Fuhrhop, J.–H., Bach, R., *Advances in Supramolecular Chemistry*, 1992, *Vol. 2*, JAI Press Inc. pp. 25-63.
4. Shimizu, T., Kogiso, M., Masuda M. *Nature,* **383**, 487-488, 1998.
5. Atherton E., Sheppard, R. C. *Solid phase synthesis: A practical approach*, In: The practical approach series, IRL Press, Oxford University Press, Oxford, 1989.
6. Fuhrhop, J.-H., Fritsch, D. *Acc. Chem. Res.*, **19**, 130-137, 1986.
7. Lehn, J.-M. *Supramolecular Chemistry*, VCH Verlagsgesellschaft. Weinheim. p. 139 1995.
8. Chou, K. C., Némethy, G., Scheraga, H. A. *Biochem.,* **22**, 6213-6221, 1983.
9. Kogiso, M., Hanada, T., Yase, K., Shimizu, T. *Chem. Commun.,* 1791-1792, 1998.
10. Navarro, E., Tereshko, V., Subirana, J. A., Puiggalí, J. *Biopolymers,* **36**, 711-722, 1995.
11. Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W. L., MacDonald, D. L., Beyermann, M., Bienert, M. *FEBS Lett.,* **403**, 208-212, 1997.
12. Dathe, M, Wieprecht, T. *Biochim. et. Biophys. Acta.,* **1462**, 71-87, 1999.
13. Hancock, R. E.W. *Lancet,* **349**, 418-422, 1997.
14. Rautenbach, M., Hastings J. W. *Chim Oggi/Chem., Today. Nov/Dec.* 81-89, 1999.
15. Lopez, S.-F., Kim, H.-S., Chol, E. C., Degado, M., Granja, J. R., Khasanov, K., Kraehenbuehl, K., Long, G., Weinberger, D. A., Wilcoxen, K. M., Ghadiri, R. M. *Nature,* **412**, 452-455, 2001.

16. Naidoo, V. B., Sanderson, R. D., Rautenbach, M. *Peptide hybrid nanomaterials*, patent PA 136179-S, 2003.

17. Naidoo, V. B., Sanderson, R. D., Rautenbach, M. *Antimicrobial peptides and peptide hybrids*, patent PA 136058-S, 2003.

***A final thought*.....**

In the words of the artist-scientist:

Leonardo da Vinci: " .....dove la natura finisce di produrre le sue spezie, l'uomo quivi comincia con le cose naturali, con l'aiutorio di essa natura, a creare inifinite spezie.....". ("Where nature finishes producing its own species, man begins, using natural things and with the help of this nature, to create an infinity of species.....".)