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TABLE OF CONTENTS

1 Executive Summary

1.1 Introduction

The BiOrigin project established critical design principles that can link a drug's molecular structure to its desired therapeutic effect. By way of example, the project has demonstrated the use of these principles for creating next-generation antibiotics, against which bacterial resistance is far less likely. The results of this research have enabled early impacts on commerce and healthcare, while improving precision and specificity with which therapeutically relevant biological functions can be measured and exploited.

1.2 The Problem

Diseases originate from molecular changes, as do their cures. Many disorders such as cystic fibrosis or hemophilia can be treated by editing single genes, while antimicrobials able to kill pathogenic bacteria and viruses can eradicate AIDS and tuberculosis. Cures are drugs which are specialist molecules that correct or destroy disease targets. Over the last decade treatments such as antibiotics have lost their effectiveness, in some cases leaving even commonplace infections untreatable, resulting in prolonged illness and greater risk of death. While this problem has been recognised to be among the most pressing challenges worldwide, the development of new treatments by pharmaceutical companies has failed to keep pace with needs.
New and innovative principles are required for the development of more effective methods and materials which are commercially attractive. At the same time, a measurement infrastructure is needed, that allows for monitoring, evaluation and screening of potential antibiotics so as to link biological activity and primary structure.

1.3 The Solution

In response to this problem, the BioOrigin project set out to provide a molecular rationale for the prediction of biological activity and demonstrate a systematic approach to acquire the experimental data needed for evaluation.

The project has determined a set of validated physical characteristics of molecular structures that guarantee the desired biological effects thereby enabling purely artificial designs and re-designs of novel and more efficient antimicrobials. In doing so it has successfully determined a model molecular template which predictably displays antimicrobial activity and has established underpinning methods and materials for the evaluation and screening of antimicrobial properties of biomolecules (small proteins) in natural and cellular environments (bacterial and human cells).

1.4 Impact

The project provides a demonstration by example of how to systematically build a biomolecular structure to elicit a desired mechanism of antimicrobial activity, which can serve, in turn, as the starting point for drug design and development. The established capabilities enable the prediction and monitoring of biological processes at the molecular and cellular level that are applicable for the development of novel diagnostics, antibiotics and biofilm-resistant materials.

The research has created a strong momentum for metrology to respond to the spread of antimicrobial resistance and is progressing towards a global impact in healthcare with a follow-up research program focusing on establishing high-throughput approaches to accelerate antimicrobial discovery and manufacture.

The project has initiated a new paradigm in antimicrobial discovery, enabling application of the developed approaches to other antimicrobial classes, to create a new pipeline of antimicrobial agents to be taken up by early adopters. The results have already been taken up by industry (SMEs and corporations), for example allowing one company to partially re-direct their R&D programme to enable entry into new markets, concentrating on predictive drug delivery and anti-infective technologies, and another organisation to commercialise novel molecular probes as highly efficient biosensors for magnetic resonance imaging (MRI).

2 Project context, rationale and objectives

2.1 Context and rationale

Relationships between the molecular origin of disease and therapeutic responses are sparse and fail to keep pace with the attempts to provide solutions to global health issues including antimicrobial resistance (AMR). Indeed, over the last 75 years we have relied heavily on antibiotics to cure everything from acne to pneumonia. These pillars of medical treatment are cheap and readily available, and have almost ruled out deaths from minor infections. However, over the last decade antibiotics have lost their effectiveness, in some cases leaving even commonplace infections untreatable.

The World Health Organisation (WHO) has identified AMR as one of the major global challenges to be fought with priority in the next future. In a recent fact sheet[1] it is stated, that AMR is 'an increasingly serious threat to global public health that requires action across all government sectors and society.' Multidrug-resistant tuberculosis (MDR-TB) and falciparum malaria are examples illustrating the dimension of the problem; resistance against some current anti-HIV drugs might be on the horizon. Likewise obvious, hospital-acquired infections, for example by methicillin-resistant Staphylococcus aureus (MRSA), constitute a significant risk to success of surgery and treatment resulting in prolonged illness, worse clinical outcome and even death, next to increased costs imposed on health systems.

Research efforts in industry are decreasing too. In the early 90's there were almost 20 active antibiotic R&D programmes, now there are only two by GlaxoSmithKline and AstraZeneca. Firstly, the everyday natural sources of antibiotic discovery, e.g. penicillin mould, have been exhausted. Secondly, many companies are seeing their licenses for antibiotics expire, meaning that the manufacture of these drugs, many of which are no longer effective, ceases to be profitable. Thirdly, there is a lack of commercial incentive for the development of antibiotics compared to other drugs. Antibiotics are not particularly profitable as they are used sparingly and only ever required short term in contrast to long-term courses of diabetes or heart failure medicine. Despite their significant importance for public health, antibiotics are not seen as a lucrative business. This situation demands fundamentally new solutions and such solutions have to be validated by robust and reproducible measurements.

It has been recognised, that next to a system of incentives devoted to the needs in the sector, development efforts should be backed by systematic fundamental research publicly funded so as to reduce risks that SME entering into the area are facing in the early stages of drug development. The project reported here contributes to this.

Traditional antibiotics interfere with the construction of bacterial cell walls or inhibit protein biosynthesis. To develop resistance to such treatments requires minor genetic mutations which bacteria can afford and readily develop. In contrast, making resistant cell membranes is a formidable task for bacteria as it requires the rebuilding of their entire genetic apparatus – an extremely high price to pay. Therefore, it is the membrane of a bacterial cell which remains its Achilles' heel. For this reason, this project has focused on membrane-active antibiotics (antimicrobial peptides, AMP) that principally target and destroy bacterial membranes thus making bacteria less likely to become resistant. However, exact mechanisms and rules of engagement with bacterial cells are still full of uncertainties.

Prominent examples of such peptides are *cecropins* (cecropia moth), *defensins* (mammalians) and *magainins* (amphibia). In contrast to 'classical' antimicrobials, these AMPs target microorganisms by the (net-) negative charge of their membrane, a trait which cannot easily be modified by simple mutation. This is held to be one of the main reasons that many such AMPs have been conserved structures in evolution, manifesting a host defence strategy of multicellular organisms for billions of years.[2] Based on this notion, it should be possible to 'tailor' peptides for clinical use that by far outlive the applicability period of conventional antibiotics, which is only a few decades.

First AMPs were discovered by chance only. So, for example, magainins were spotted as a source for antimicrobial resistance of African frogs (*Xenopus Laevis*) whose wounds healed even in microbially contaminated water-filled tanks to which the animals had been returned immediately after experiments including surgery.[3] Having recognised the potential of peptide-based antibiotics, a systematic screening of

[1] http://www.who.int/mediacentre/factsheets/fs194/en/
[2] Mangoni M, McDermott AM and Zasloff M, *Experimental Dermatology*, 2016, 25, 167–173
[3] Zasloff M, *Proc Natl Acad Sci USA*, 1987, 84, 5449-5453

peptide sequences (primary structures) for the few ones that display the desired biological activity is the way forward to the next generation of drugs.

Essential to this, a rational design is needed, starting from promising lead substances and exploiting design principles underlying the naturally occurring examples, either known or to be discovered. In order to learn design principles, availability of a metrological infrastructure comprising biophysical methods for characterisation of peptide-membrane interaction is an essential prerequisite.

Traditional measurement concepts and platforms fall short of providing answers, and so new and innovative principles are required for the development of more effective methods and materials. These are necessary for enabling any sustainable progress in drug design and development, and in particular in the area of antimicrobial resistance, given the global impact and urgency of the issue. With this in mind, the project dealt with the pre-validation of predictable and exploitable links between the structure and activity of antimicrobial agents. Consultations with stakeholders from industry, clinic and health research organisations identified two most pressing measurement challenges that this project addressed:

- Irreproducibility and inconsistency in the detail of what makes a better antibiotic. This was needed in order to provide reliable and predictable links between the structure and activity of last-resort membrane-active antibiotics,
- A lack of associated measurement tools and materials, particularly those that can allow for the monitoring, evaluation and screening of potential antibiotics in their natural cellular environments. This is needed to provide a validated rationale for drug design.

2.2 Objectives

Derived from this, the scientific and technical objectives for the BiOrigin project were to:

1. provide a set of *validated physical characteristics* that guarantee the desired biological effects thereby enabling purely artificial designs and re-designs of novel and more efficient antimicrobials,
2. establish *underpinning methods and materials* for the evaluation and screening of antimicrobial properties of biomolecules (small proteins) in natural and cellular environments (bacterial and human cells) at time and length scales of their therapeutic action,
3. develop *experimental and computational methods* to quantitatively determine the mechanistic performance and extent to which antimicrobials engage with their targets with the highest precision and accuracy (molecular to atomistic),
4. innovate *imaging methods* for the visual monitoring and imaging of antimicrobial action in real time thus allowing for the mechanistic elucidation of the antimicrobial action,
5. provide a *molecular rationale for the prediction of biological activity* by refining and optimising the experimental data with computational analyses.

3 Research results

3.1 Validated physical characteristics

The project applied systematic investigations of antimicrobial activity at the molecular and cellular levels. Model molecules were designed to experimentally obtain and validate physical characteristics that are responsible for antimicrobial activity. Such characteristics relate to the strength and speed of antimicrobial binding to bacteria, the selectivity of binding to bacterial cells as opposed to human cells, minimum concentrations necessary to kill bacteria and the like. The characteristics were then refined computationally and were subsequently used to build generic molecular templates (peptides) comprising different blocks of molecules (amino acids) that can be replaced with other blocks or different combinations of the same blocks to favour a particular biological function. These templates are now used to benchmark the activity of existing and emerging antibiotics and related antimicrobial agents.

Diseases are reducible to sources at the molecular level which need to be understood, consequently, in order to design the appropriate cure. The causality between chemical propensities of amino acids, their arrangement

within a peptide, and the secondary structure resulting then as most favourable, have been known long since, and prediction of structures from sequences is routine. This vastly holds true also for tertiary and quaternary structures (organisation of secondary structures into bigger domains or arrangement of different peptide chains within a complex protein).

BiOrigin, however, was focusing on the link between (primary) structure of short peptides and their interaction with both microbial and nonmicrobial cellular membranes.

Depending on the sequence, peptides can interact with membranes such as resulting in *(i)* membrane collapse comprising states of 'carpeting' the outer leaflet, subsequent membrane thinning and wormhole formation/poration (SMH-model), *(ii)* transduction through the membrane or *(iii)* fusion of separate membranes into one.

Namely, the first type of mechanism has been discussed with antimicrobial peptides while, to a lesser extent, transduction may be involved in antimicrobial activity too. Within BiOrigin, we focused to peptides which typically form α-helices on contact with bacterial membranes. Magainins, as first isolated and characterised by Zasloff, are typical representatives of the class.

In order to characterise peptide-membrane interaction on the relevant scales of time (nanoseconds to minutes) and length (nanometers to micrometers) a network of complementary biophysical methods was established for the purpose and evaluated, comprising

- Optical Spectrometry (FTIR, CD and LD-spectrometry)
- Synchrotron-Radiation based FTIR-Spectrometry
- Chemical Cross-Linking Mass Spectrometry
- Atomic Force Microscopy (AFM) and Fluorescence microscopy
- (Nano-) Secondary Ion Mass Spectrometry (nanoSIMS)
- Nuclear Magnetic Resonance with hyperpolarised ^{129}Xe
- Solid State Nuclear Magnetic Resonance Spectroscopy (ssNMR)
- Molecular Dynamics Simulation (MD)

These, altogether, would provide the information needed to derive the conclusions about structure-activity relationship according to the design rationale pointed out in section 3.5.

Conclusion: Objective Achieved. The BiOrigin project has developed a generic approach for the discovery of 'design principles' for antimicrobial peptides. Based on a given peptide (template) it is now possible to predict the effect that will be induced by a targeted change of the peptide's formula. Such change, typically, would be the substitution of amino acids in certain positions of the original sequence. Then, biophysical methods are used to evaluate the difference in interaction of that peptide with microbial membranes, provoked in this way. Based on the experimental result, validity of the prediction (and particular design rule) is confirmed or rejected. Such systematic interrogation into design principles will help the development process for an optimised peptide-based drug from a lead molecule as a starting point. As a crucial prerequisite for this concept to perform, a set of biophysical methods has been optimised within BiOrigin enabling reliable and sensitive characterisation of differential peptide behaviour.

3.2 Underpinning methods and materials

The hallmark of the peptide-templates used (tilamin and amhelin) is that they are generic enough to apply to other applications including gene delivery, anticancer treatments and biosensors. The templates are structurally amenable and can accommodate other desired properties in order to support a new biological activity. For example, the antimicrobial activity of the template can be converted to a predominantly cell-penetrating activity that is necessary to deliver drugs into live cells. Because this capability allows converting one activity into another activity, be it similar or different, core methods that can validate the new acquired properties by comparing them with the original (benchmark) properties are necessary. To realise this, the project integrated physical, biological and imaging methods into one continuous methodology that can trace the desired biological activity down to the elementary structure of the template while providing step-by-step correlations between the mode of action, bacteria killing kinetics and molecular recognition.

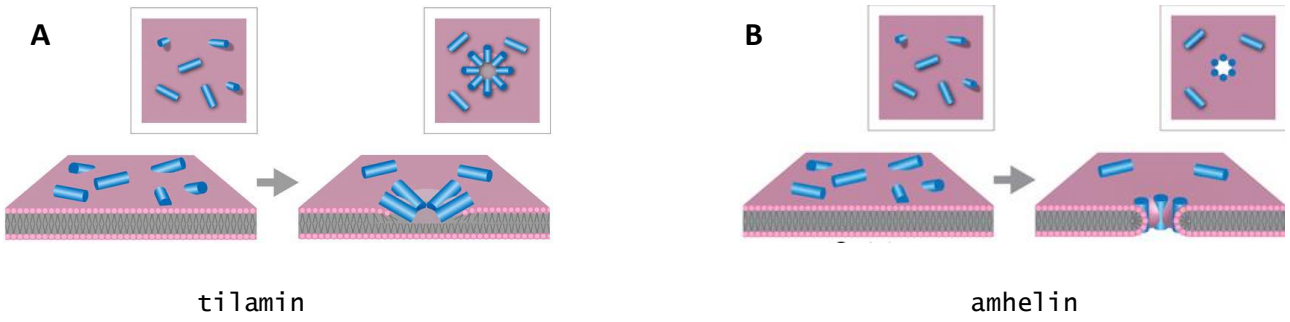

Figure 1. *Mechanisms postulated for destruction of microbial membrane mimics (anionic lipid bilayers): In the case of tilamin (A), collapse of the bilayer is initiated by exfoliation of the outer leaflet ('monolayer poration'), whereas pores are punched through the whole of the bilayer with amhelin, which ever grow by recruitment of further peptide molecules, until the membrane collapses.*

Materials to be used with the biophysical measurements/experiments

The peptides used for the investigations, amhelin and tilamin, next to a 'non-AMP' which was expected to be inactive are shown in Fig. 14 (section 3.5). These were produced using standard solid phase synthesis protocols, conjugated with fluorescent moieties and cryptophane-A xenon-cage, and shared between the project partners, as needed. Isotopomers of tilamin, needed for the GALA- (ssNMR) experiments were obtained by targeted substitution of alanines by α- CD_3 alanines. Standard protocols were applied and shared between the partners for preparation of unilamellar (phospholipid-) vesicles using 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) with 1,2-dilauroyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DLPG) and (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG). DLPC and POPC were used for construction of mammalian model membranes and DLPC/DLPG (3:1, molar ratios) and POPC/POPG (3:1, molar ratios) were used as bacterial model membranes.

FTIR and CD optical spectroscopy

Optical spectroscopy, though it does not provide spatial resolution down to the atomistic detail, it can be used to interrogate the secondary/tertiary structure of model compounds enabling distinction between random orientation ('random coil'), formation of β-sheets or α-helices and possibly tertiary contacts of these structural elements either within a peptide or between peptides molecules if aggregating.

Detection of any structural change on membrane contact (mammalian or microbial membrane) is vital in evaluation of potential antimicrobial activity of the chosen peptide templates as it is known that destruction of microbial membranes typically involves such changes as a prerequisite.

Within BiOrigin, optical/chiroptical methods were established as '*low resolution*' approaches (as opposed to imaging methods and NMR) and applied to provide insight into the topology or geometry of the peptide/membrane complexes formed. These were far-UV circular dichroism (far-UV CD), linear dichroism (LD) and Fourier transform infrared spectrometry, as complementary techniques with CD revealing helical structures, if present, by pronounced spectral details, LD probing the orientation of the peptide with respect to the membrane, while β-forms (sheets, turns) become apparent by a characteristic amide I band (C=O stretching, between 1600 and 1700 cm^{-1}).

In line with the expectation, both CD and LD spectra display features revealing that tilamin as well as amhelin to a significant extent take a helical structure in presence of microbial model membranes (DLPC/DLPG, 3:1), a propensity missing when a mammalian-like model membrane (DLPC only) is used instead. With the 'non AMP', at the same time, such secondary structure was apparent in neither case. CD Spectra recorded for tilamin in presence of DLPC/DLPG (anionic, microbial-membrane mimetic, AUV) are shown in Fig. 2 (left) as solid lines; for comparison, spectra are included too for phosphate buffer alone (dash-dotted line), DLPC (zwitterionic membrane mimetic, ZUV) without peptide, dotted, and DLPC/DLPG without peptide, dashed. Complementary, also the LD-spectrum for tilamin in microbial membrane-mimetic Fig. 2 (right, solid line) indicates helix formation to an appreciable extent as manifest by a flattened maximum at 220-230 nm, a negative dip at 205-215 nm. Particular features in the LD-spectrum allowed for conclusions about the approximate orientation of the (cationic) tilamin-peptide with respect to the membrane which was predominantly flat-on-membrane with a possible contribution from partial membrane insertion of the peptide.

α-Helix content, and extent of membrane-induced change of secondary structure, therefore, was found to depend on the amount ratio lipid-peptide for tilamin, increasing with the amount of lipid available per molecule: At a ratio of 100:1 average contents were found to be 68% (69%) for model membrane preparations using DLPC/DLPG (and POPC/POPG, resp.) compared to 41% (24.1%) if preparations at 40:1 ratio were measured instead. This basically confirms expectation. However, irrespective of the ratio used, all POPC/POPG samples appeared to be cloudy in the presence of tilamin. In contrast, for DLPC/DLPG the same was observed only for 40:1 lipid-peptide ratio. Notably, this was accompanied by the dampening of signal toward lower wavelengths, which suggests *(i)* light scattering typical of particulate systems, and *(ii)* increases of turbidity in solution upon peptide-lipid associations. An interlaboratory comparison between two of the partners revealed reproducibility of membrane preparation and incubation with the peptide and partly occurring cloudiness/turbidity of the samples to be sources of uncertainty that needed to be controlled to obtain agreement within 5%.

In addition, stopped flow CD and fluorescence measurements have been evaluated as method to detect early folding events for AMP on contact with membrane mimetic. With this technique, two solutions are rapidly mixed, one containing the peptide under investigation, the other one the model membrane, while monitoring changes of signals over time. Three different signals were recorded: CD at 222 nm (measuring regain of the secondary structure), CD at 280 nm (indicating changes in the environment of tyrosine and rigidity) and fluorescence ex. 280 nm (tyrosine rigidity/flexibility). As an example, Fig. 3A shows the decay of the 222 nm signal over a period of 10 s for tilamin (black), observable on interaction with microbial membrane mimetic (POPC/POPG), Fig 3B shows the (initial) appearance of tyrosine fluorescence within 0.2 s of the experiment. From curve-fitting and regression analysis it could be inferred that, tilamin adopts its final secondary structure to an extent of about 34% within 1 ms, which is the dead time of the instrument.

FTIR spectrometry, in contrast to CD and LD methods, did not provide features enabling an immediate distinction of peptide folding states and molecular orientation in presence of anionic and zwitterionic membrane mimics. This maybe owing to the fact that, signals in the region of the amide I and amide II bands indicative of peptide folding states are more difficult to uniquely identify from a spectrum dominated by the

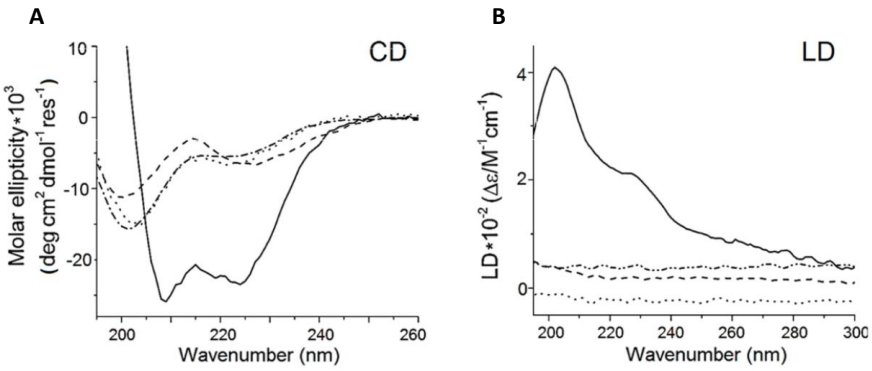

Figure 2. *(A) Circular dichroism (CD) spectra for tilamin in AUV (microbial membrane mimetic): solid line, in ZUV (zwitterionic membrane mimetic): dotted line, in buffer (without lipid): dash-dotted line, and non-AMP in AUV: dashed line. (B) Linear dichroism (LD) spectra for tilamin in AUV (solid line), ZUV (dotted line), non-AMP in AUV (dashed line) and ZUV (dash-dotted line). CD spectra indicate helix formation to an appreciable extent to occur only on interaction with microbial membrane model (AUV), with orientation of the helices more parallel to the membrane according to features in the LD spectrum.*

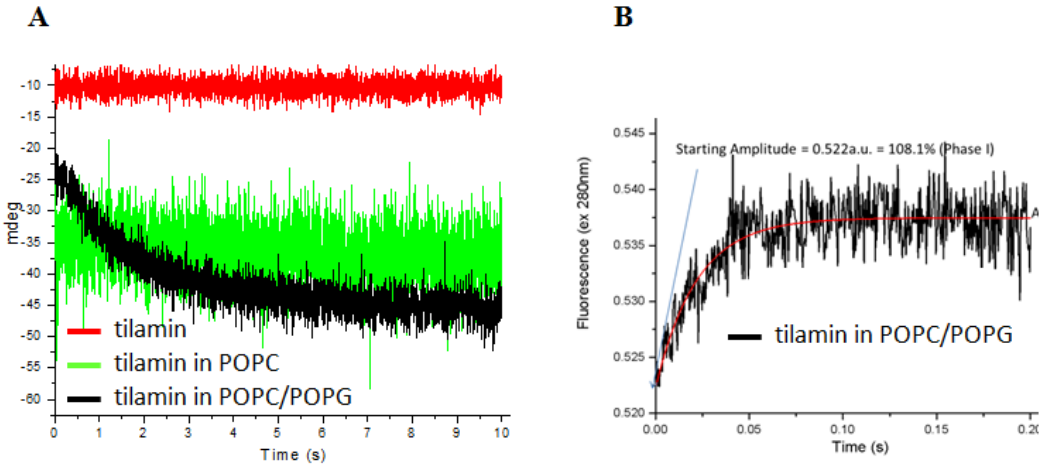

Figure 3. *Results of stopped-flow CD (A) and stopped flow fluorescence measurements (B). The wavelengths (222 nm for CD and 280 nm for fluorescence measurements) are such as to probe for time courses of regaining the secondary structure in (A) and changes in rigidity of the peptide on membrane contact with (B).*

excess liposome that is present at the same time. However, it could be demonstrated that exploiting the advantage in brightness/brilliance offered by synchrotron radiation compared to standard sources in combination with sensitivity achieved by applying a Focal Plane Array (FPA) detector plus multivariate data analysis basically points a way to identify spectral features that discriminate between antimicrobial peptides in buffer and on interaction with membranes. Experiments were done at the Metrology Light Source (PTB). In contrast to common detectors, the FPA (comprising 128 by 128 pixels) allowed for simultaneous collection of a large number of spectra. This multiplexing capability enables acquisition of high quality data of complex biological samples within collection of just minutes, and intensities are enhanced by two orders of magnitude compared to conventional radiation sources.[4] Better signal-to-noise ratios in combination with data analysis by Principal Components Analysis (PCA) would reveal spectral patterns characteristic for amhelin itself, human dermal fibroblasts (HDFa, used as substrate in this example) and the product of interaction of amhelin with HDFa. Among the spectral features presented as principal components, PC1-PC3, in Fig. 4, particularly PC3

[4] Müller R et al., *J Phys Conf Ser*, 2012, 359, 012004

and PC2 possess discriminating power. Transforming this back into the domain of wavenumbers, relevant spectral variations appeared in the spectral region between 900 cm^{-1} and 1500 cm^{-1}, where C=O, C-N and C-C stretching modes of esters, DNA molecules and amide I/II bands occur. Furthermore, the stretching vibrations observed in the peptide-treated HDFa cell spectra suggest the existence of DNA molecules that may have emerged from the cell nuclei.

Chemical cross-linking / mass spectrometry

Chemical cross-linking combined with mass spectrometric analysis of the products has emerged as a powerful approach to elucidation of the tertiary structure of proteins and for characterisation of binding interfaces of protein-protein complexes, constituting a tool thus, to get insight into topologies of biological objects at the molecular level. Bifunctional linkers (organic molecules with two activated sites) are used to probe for pairwise proximity of amino acids in the protein. Most of the time, amine-reactive linkers are used which interconnect two lysines, if close enough to one another, while the N-terminus can be engaged in such links as well. Typically, bottom-up proteomics workflows are used then to identify the amino acids that have been cross-linked. The length of the linker molecule imposes an upper limit on the distance between these amino acids, and with a sufficient number of links identified good approximation of the tertiary structure can be obtained.[5]

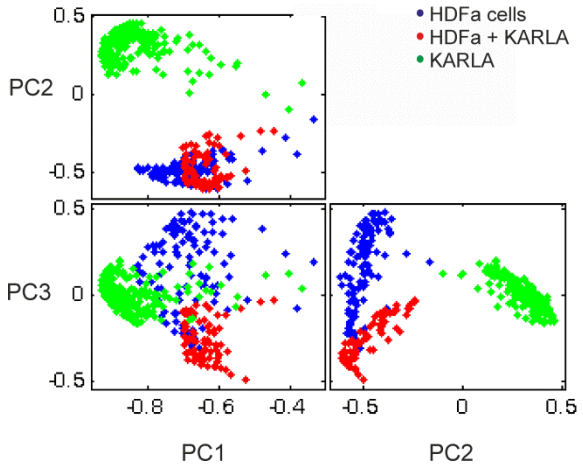

Figure 4. *Identification of characteristic spectral differences between tilamin ('KARLA', green dots), human dermal fibroblasts (HDFa, blue dots) and the product resulting from interaction of tilamin with HDFa (red dots) obtained by high sensitivity measurement using synchrotron radiation as source and PCA for multivariate data analysis.*

Within BiOrigin, cross-linking was applied to tilamin for the purpose to chemically stabilise peptide aggregates, such as being preserved in size after destruction of the original sample, allowing for their extraction/isolation and characterisation. Disuccinimidyl suberate (DSS), with a spacer length of about 11.4 Å, was employed as cross-linking reagent, in this study.

Distribution of molecular aggregates obtained for pure tilamin in buffer (-), as well as after 30 min incubation in DPLC (ZUV) and DPLC/DPLG (AUV), resp., are reproduced in Fig. 5A as results of (denaturing) SDS gel electrophoresis and in Fig. 5B as profiles from size exclusion chromatography (SEC). In Fig. 5A, the left lane shows the result for the standard substances used to calibration of the molecular mass (kDa, left-hand scale). Denominations (x*n*-) in Fig. 5A and 5B refer to the number of tilamin molecules corresponding to the pertaining molecular mass, and size thus, of the molecular aggregate.

Results of both, gel electrophoresis (A) and SEC (B) confirm that the bulk of tilamin molecules exists isolated in monomeric form in pure buffer (-), which does not change in presence of the zwitterionic (mammalian-like)

[5] Young M et al., *Proc Natl Acad Sci USA*, 2000, 97, 5802-5806

membrane mimetic (ZUV). A small fraction of peptide molecules forms dimers, indeed, but this is attributable to a small statistical chance for two molecules to get in sufficient proximity within the time scale of the cross-linking reaction.

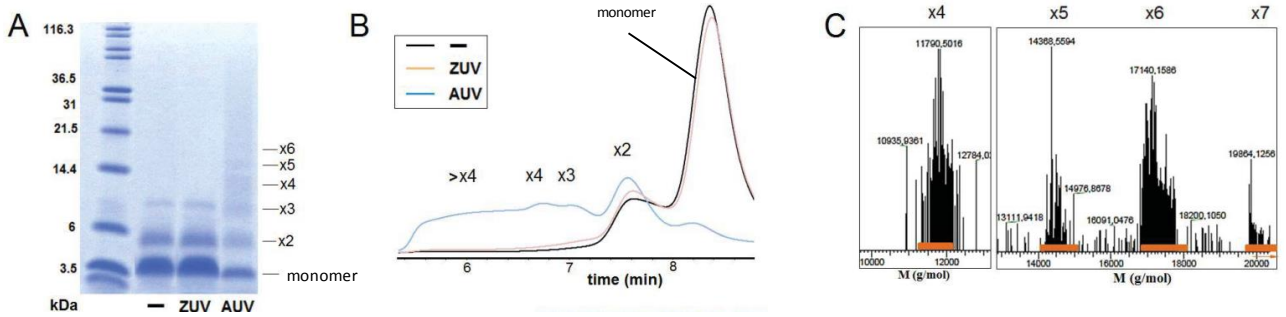

Figure 5. *Patterns of molecular aggregation of tilamin (-), in comparison to tilamin interacting with zwitterionic membrane mimetic (ZUV) and antimicrobial membrane mimetic (AUV) as revealed by gel electrophoresis (A) and further confirmed by size exclusion chromatography (B). Analysis of the corresponding fractions by mass spectrometry provides signals within the (molecular) mass ranges expected for cross-linked tetramers up to heptamers in the case of AUV.*

As opposed to this, if using the microbial membrane mimetic (AUV), little of monomer is remaining, and higher aggregates (> x4) are formed instead. Mass spectrometric analysis of the > x4- SEC fraction reveals signal clusters in the expected mass ranges (orange bars) for tetramers up to heptamers, as shown in Fig. 5C. Ranges are observed, rather than one distinct mass per aggregate, for the reason that each one of the aggregates (oligomer-types) exists as different variants, depending on the number of cross-links formed in the particular molecule.

Cross-linking mass spectrometry, as evaluated in BiOrigin for the particular purpose of detecting molecular aggregates, confirms a distinct behavior of tilamin depending on which type of (model) membrane. In addition to the structural changes observed with the chiroptical methods as a result of interaction with the microbial type of membrane, molecular aggregation can be stated based on the results of the cross-linking experiments. Using the linker (DSS) as a 'molecular ruler', and assuming, migration of the peptides during the reaction is negligible, distances may be inferred to be about 12 Å, maximally, between the amino groups of neighbouring peptides in such aggregates.

A limitation of the cross-linking / mass spectrometry approach it is, that the size of aggregates which may have formed tends to be underestimated, which is a consequence of the potentially incomplete cross-linking reaction with yields of less than 100% in each single step. Therefore, rather than indicating different pore sizes, the series of different oligomers might correspond to different endpoints of the reaction for a bigger aggregate quite as well. However, the method provides an independent proof for aggregation of tilamin as part or consequence of its interaction with microbial model membranes, complementing the information obtained with CD optical spectroscopy and the imaging techniques used within the project.

The following tasks have been achieved with respect to the objective:

- Peptide materials have been synthesised, chemically derivatised or isotopically modified according to the specific requirements of the set of biophysical methods to be applied within the project and shared between the project partners
- Unilamellar vesicles (ZUV, AUV) to be used as mammalian and bacterial membrane mimetics were prepared by the partners right before use according to a common protocol that had been optimised beforehand.
- CD-spectrometry indicates α-helices to be formed on interaction of both, tilamin and amhelin, with bacterial membrane mimetic as opposed to mammalian mimetic, or solutions of the peptides in buffer only. This is shown for the example of tilamin in Fig. 2A.

- LD-spectrometry, additionally, indicates a component of parallel orientation for tilamin with respect to the membrane.
- FTIR with conventional radiation source less readily provided information for biophysical characterisation of the investigated model systems.
- FTIR if using synchrotron radiation, as available at the Metrology Light Source at PTB, in combination with multivariate data analysis, was identified as potential way forward, offering exceptional sensitivity by Focal Plane Array (FPA) detection.
- Chemical cross-linking mass spectrometry was explored and established as a method to stabilise higher molecular peptide-aggregates that were formed on contact with microbial membrane mimetic such that they could be isolated and subsequently characterised by size/mass fingerprinting.

Conclusion: Objective Achieved. Model antimicrobial peptides (referred to as *amhelin* and *tilamin*) have been designed (see Fig. 14, section 3.5). Replacement of amino acid lysine (K) by arginine (R) in defined positions on turning from amhelin to tilamin was hypothesized to induce the modified peptide (tilamin) to follow a different mechanism of attacking a microbial membrane. A number of various biophysical methods has been set up and validated on purpose to spot differences in behaviour of the peptides. Both antimicrobial-like (cellular-) membrane models and mammalian membrane mimetics were specified as substrates to probe for peptide-membrane interaction. Using optical and chiroptical spectrometry, significant changes could be detected as happening with both peptides once in contact with antimicrobial membrane surfaces, as opposed to mammalian kind of membrane models. This would confirm validity of the peptide-templates specified. In contrast, a non-antimicrobial peptide that had been specified as negative control (non AMP, see Fig. 14), did not display such interaction in either case. A novel technical option evaluated in this context consisted of the use of synchrotron radiation as a source for characterisation by FTIR-spectrometry in combination with Focal Plane Array (FPA)-detection enabling enhanced sensitivity. Another state-of-the-art technology was adapted to the purposes of BiOrigin and utilised to obtain complementary information and introduced for such purpose is the reference chemical cross-linkers as ‘molecular rulers’ to measure proximity of peptides occurring on contact with antimicrobial membrane models. The latter approach proved to be useful with testing the hypothesis of clustering of antimicrobial peptides in general.

3.3 Experimental and computational methods

As one of the main tasks of BiOrigin, structure-activity correlations for the model peptides (tilamin and amhelin, see Fig.14, section 3.5) were validated with nearly atomistic detail using high resolution methods including synchrotron radiation and magnetic resonance (MR). With this level of detail, the molecular behaviour of an antibiotic or another drug can be quantified and is the most relevant to drug design as it describes precise drug-target interactions. Each measurement methodology, which uses a unique physical principle, contributed a critical piece of information that was adapted to develop computational algorithms to predict and model therapeutic mechanisms with atomistic accuracy. This strategy provided the first metrological validation of antibiotic action and underpins a reference methodology for the screening and validation of antibiotics.

Biophysical methods to be used for characterisation of the model AMPs were selected such as providing overlapping information so as to validate and independently reproduce results of one method by the other(s), but also to provide complementary results so as to acquire as complete as possible a characterisation of (differential) behavior of the peptides. Some of the methods, as e.g. FTIR- and CD optical spectroscopy have become standard tools in the area, but many of them had to either be adapted to the present purpose or were developed to this. Noteworthy, molecular dynamics computations (MD) used to simulate peptide-lipid interaction and solid state nuclear magnetic resonance spectrometry (ssNMR) were available within BiOrigin. These are approaches providing ‘high-resolution’ kind of information and had to be optimised to application to the model peptide templates. Beyond this, cross-linking/mass spectrometry, though lower in resolution was developed and is capable of independently confirming presence/absence of membrane-induced peptide

aggregates, confirming thus the results by the imaging methods and ssNMR. A unique capability available within BiOrigin is the option of carrying out FTIR micro-spectroscopic investigations using synchrotron radiation (SR). Using a Focal Plane Array (FPA) detector in combination with multivariate analysis of the large data sets generated, spectral differences elicited by peptide-cell interaction could be identified which render the kind of technique a novel option to biophysical characterisation of such and similar biological materials.

A further innovative experimental approach evaluated within BiOrigin is nuclear magnetic resonance using hyperpolarised xenon. This technology is based on the orders of magnitude enhanced sensitivity of laser-polarised ^{129}Xe and bears high potential for the detection of rare events or species in biomaterials or even organisms. The application to low concentrations of tilamin and non-AMP peptides interacting with liposomes gave proof of the feasibility of qualitative and quantitative investigations of AMP activity by this novel methodology. Conditions for treatment of live cells in a xenon NMR investigation were defined and were complemented by an in-depth study of peptide-cell interaction by fluorescence microscopy.

BiOrigin performed measurements in live bacteria and their membranes as well as on live human cells and human cell membranes to demonstrate selectivity to antibiotics. For the first time, it was shown and validated that it is the position and structure of antibiotics in microbial membranes that are responsible for antimicrobial activity, which can thereby be made more selective and stronger. In accordance with our predictions, model antibiotics elicited faster and broad-spectrum antimicrobial responses when compared to commercial antibiotics daptomycin, vancomycin or polymyxins, which kill only particular types of bacteria. Yet, the visual evidence of how antimicrobials interact with bacteria has been elusive before this project, whereas seeing antimicrobial effects in real time has far-reaching benefits for antimicrobial therapy and diagnostics. Therefore, BiOrigin pushed the boundaries of existing capabilities by developing innovative imaging methods to give the first visual evidence of antibiotic action. The imaging capabilities included high-speed atomic force microscopy, magnetic resonance molecular imaging and mass-spectrometry chemical imaging, which provided important insights into antimicrobial mechanisms and the distribution of antibiotics in bacteria. These findings are already proving crucial for the development of effective antimicrobials and equally for the diagnosis of infectious diseases. The capabilities are beyond the state of the art and hold a considerable promise for use in hospitals while their use for commercial development is being probed in collaboration with industry.

Conclusion: Objective Achieved. A network of experimental methods has been established enabling a comprehensive characterisation of a candidate antimicrobial peptide (or comparison with another such peptide). This has been achieved by involvement of vastly complementary (while partly redundant) measurement principles. The biophysical methods engaged provide a characterisation of the biological system mapping the relevant scales of length and time (i.e., from micrometres down to Ångström and minutes to nanoseconds, resp.). The combination of biophysical methods used within BiOrigin demonstrate an approach for detection of design rules or optimisation of antimicrobial peptide drugs. A particularly noteworthy result is the enhancement that has been achieved with reference to cutting-edge techniques such as computer-based molecular modelling (MD) and solid-state NMR.

3.4 Innovative imaging methods

FTIR and CD- optical spectroscopy and cross-linking mass spectrometry are of 'lower-resolution' quality in that optical spectroscopy does not provide information about topologies of membrane-peptide complexes formed and damages induced to the membrane whereas aggregates detected by mass spectrometry cannot *per se* be taken as proof for pores to be formed in the membrane. For that reason, a number of different imaging and microscopy methods have been evaluated with respect to their performance in characterisation of the peptide-membrane model systems within BiOrigin. These methods would add both, an idea about geometries and shapes of structures formed on membranes and the time scale on which these occur. In addition, imaging methods were applied to live cells, to ascertain how far conclusions about peptide-membrane interaction, which are based on model- mammalian and antimicrobial membranes, would be applicable to the more complex conditions encountered with real biological systems. Next to imaging techniques which basically have been well established before, as high-speed atomic force microscopy (AFM) and fluorescence microscopy, highly innovative novel approaches have been exploited, namely, mass- spectrometry based chemical imaging and magnetic resonance on cryptophane-caged hyperpolarised xenon.

Atomic Force Microscopy (AFM) and nanoscale resolution secondary ion mass spectrometry (nanoSIMS)

The information obtained with lower resolution methods, particularly CD and LD spectroscopy, additionally supported by the observed cross-linked aggregates, suggests folding-mediated poration by tilamin in response to microbial membrane-binding.

Images acquired by AFM provide a visual proof for this assumption. The experiment differs from the previous lower-resolution investigations in that supported lipid bilayers (SLB) were used as substrates in place of unilamellar vesicles (ZUV and AUV, resp.) in buffer solution. The lipid is deposited on appropriate supports so as to provide an anionic bilayer mimicking a planar microbial-type membrane. Flatness to within ~0.1 nm in the unperturbed state of the bilayer is a noteworthy advantage offered by the preparation method used, as it enables high precision measurement of any profile minted into the membrane on interaction with the peptide.

Both, tilamin and amhelin were characterised by AFM on purpose to identify differential behavior of both model peptides as was hypothesised to result from the systematic and targeted modification of amino acids between both sequences (shown in Fig. 14, section 3.5). One such result, as it was obtained by in-air AFM, is shown in Fig. 6. In that case, DLPC/DLPG in 3:1 molar ratio was used for preparation of the bilayers. In the upper panel (Fig. 6A), an in-air image is reproduced as obtained after 15 min incubation of such SLB with tilamin (10 µM, pH 7.4), leftmost: control without peptide; the lower (Fig. 6B) panel shows a corresponding result for amhelin (10 µM, pH 7.4, 30 min). Profile plots shown in the picture have been recorded along the green and the red lines, respectively.

There are two striking differences between both of the investigated peptides: With amhelin (Fig. 6B), large pores of varying size are formed which might point to a continuous growth over time by recruitment of further

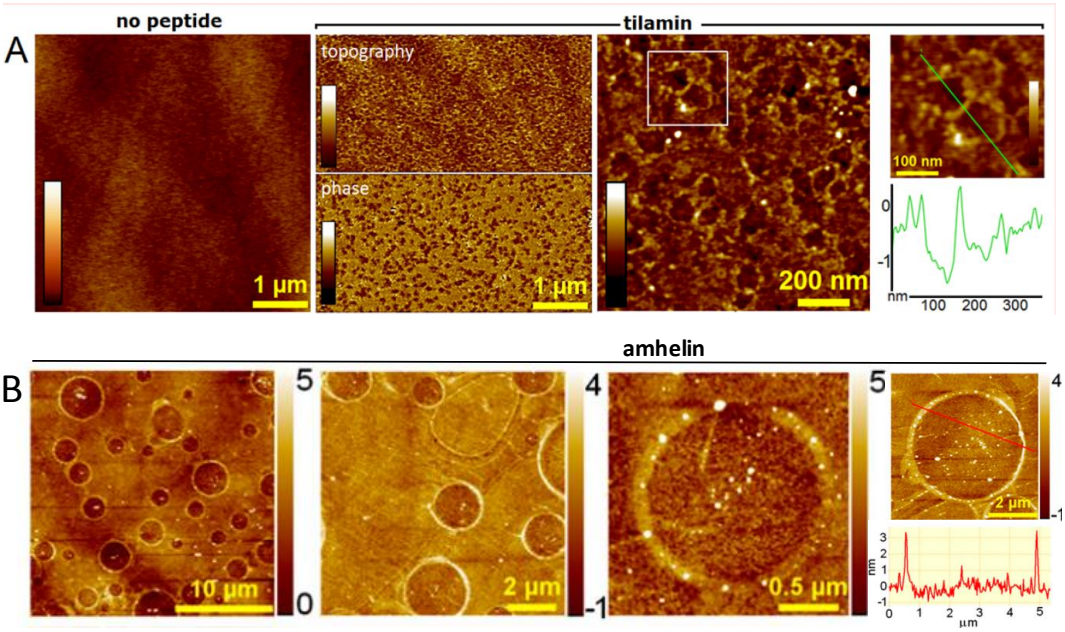

Figure 6. *Poration of supported lipid bilayers (SLB) prepared from DLPC/DLPG, 3:1 (anionic, microbial membrane mimetic) by tilamin (A) and amhelin (B) observed by atomic force microscopy (AFM).*

peptide molecules, whereas pores (or membrane damages) keep much smaller and rather tend to fuse into heterogeneous networks in the case of tilamin (Fig. 6A). Also, these networks are complete within 15 min of incubation with tilamin solution, as apparent from AFM in aqueous solution (not shown).

As a second difference, profile depths measured display a clear and regular pattern in the case of amhelin with depth of about 2.7 nm, while dips in the membrane are roughly 1.4 nm with tilamin. This difference lead

to the postulation of two different mechanisms occurring with amhelin and tilamin as illustrated in Fig. 7. According to this, tilamin destructs microbial membrane mimics (AUV) by penetrating the upper layer only as opposed to transmembrane poration as observed with amhelin. Therefore, the profile depth observed is rather compatible with half the thickness of the bilayer (~3.2 nm with SLB using DLPC/DLPG, 3:1, and 4 nm with POPC/POPG, 3:1). In order to account for the observed behavior, the assumption is made that tilamin takes a tilted orientation so as to accommodate the peptide with its expected axial length of about 3.15 nm in helical conformation.

Next to AFM, nanoSIMS was applied to the SLBs in order to confirm results with an independent and complementary imaging method. Basically, the samples are bombarded using a 16 kV primary $^{133}Cs^+$ beam and generated secondary ions are registered over a defined area on the sample. If incorporating isotope-labeled nitrogen (^{15}N in place of ^{14}N) into the peptides, their distribution will become visible as sites of elevated ^{15}N. NanoSIMS does not provide topographical information as AFM does. However, in the present context it provides a proof that the structures to be seen on the surface really are elicited by the model peptides, as only these produce elevated local abundances in ^{15}N. The images obtained (exemplified in Fig.8) confirm the conclusions drawn before, based on AFM. Note that, isotope abundance is visualised by hue- scales in these images ranging from blue (normal abundance) up to red (highest in ^{15}N). For amhelin (Fig. 8A), it can be seen that the peptide is concentrated at the edges of the pores while the pores

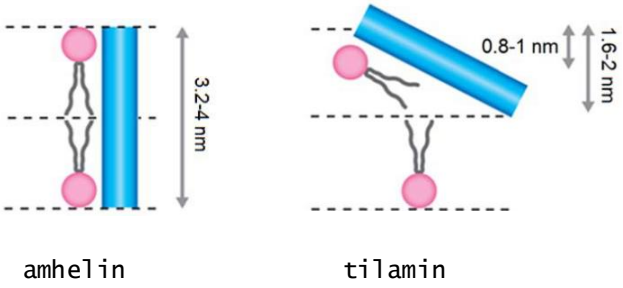

Figure 7. *Proposed mechanisms for poration of AUV (microbial membrane mimetic) proposed on the basis of the results by atomic force microscopy (AFM): Amhelin 'punches' through the bilayer as a whole (A), whereas tilamin interacts with the upper leaflet only.*

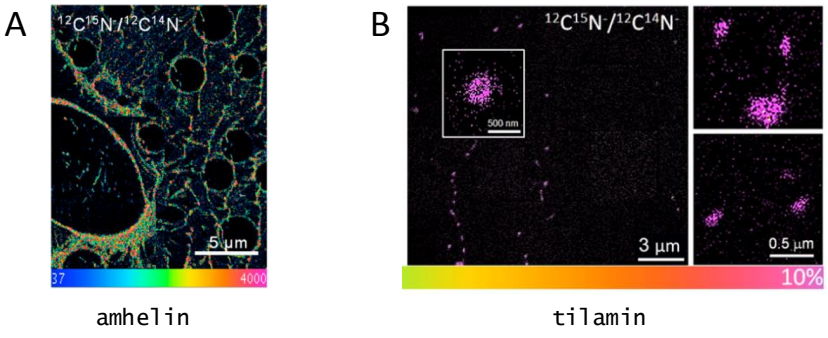

Figure 8. *NanoSIMS-images confirm the different patterns of poration for amhelin (A) and tilamin (B) as observed before with AFM, but additionally provides a proof, that peptides (colored areas) are either concentrated at the pore-walls (A) or form networks of clustered tiny porations. Distribution of peptides is unambiguously revealed by presence of $^{12}C^{15}N^-$ -ions.*

themselves are virtually empty. Apparently also, the peptide content increases with increasing pore sizes. With tilamin (Fig. 8B), on the other hand, clusters composed of comparatively very small pores (presumably restricted to the upper leaflet of the bilayer) are formed instead, which is in line with the findings by AFM. Next to the large clusters there many isolated small pores, and not all of them may even be visible with nanoSIMS owing to the limited lateral resolution (< 100 nm) that can be achieved with the experimental setup. However,

the 'shotgun'- kind of distribution further supports the assumption of isolated tiny damages to be formed with tilamin which do not laterally grow into (comparatively) huge pores as with amhelin, but rather continually spread covering the whole of the surface.

Biological activities of the designed peptides (tilamin and amhelin)

At this point, in order to test biological relevance of the membrane-destructive activity found for both of the model AMPs, their effects on live bacteria were evaluated. Minimum Inhibitory Concentrations (MIC) are shown in Tab. 1 for tilamin and amhelin in comparison with cecropin B (a naturally occurring AMP, originally identified in moths) next to the commercial membrane-active antibiotic drugs daptomycin and polymyxin B. The peptide used for negative control (non-AMP, see Fig. 14, section 3.5) was inactive (MIC >> 250 µM). None of the peptides displayed haemolytic activity, as was tested using human erythrocytes, which is a prerequisite for potential applicability as a drug.

As apparent from the low MIC, both of the peptides show appreciable activity against bacteria in general and appear to be competitive, at least, in this respect to cecropin B and the two (example-) commercial drugs. According to these data, tilamin equally appears to inhibit bacterial growth in a broad spectrum of bacteria not regardless of basic differences in construction of cell walls associated if proceeding from Gram-positive bacteria as substrates to Gram-negative cells. This is the case with amhelin as well, except, perhaps, for S. *aureus.*

Growth inhibition, however, provides just one parameter for characterising biological activities of AMPs. But it is falling short, e.g., of mapping the time scale relevant for peptide action which is determined by the (proteolytic) lifetime of such peptides in biological systems, which is minutes, as opposed to duration of the inhibition experiments (24 h). Complementary, therefore, cell-death was monitored for *E. coli* over 45 min incubation time by LIVE/DEAD® *Bac*Light™ viability assays using fluorescence microscopy for detection. With this type of assay, wavelength of fluorescence switches on damage of the cell membrane such that the fraction of 'dead' (damaged) cells can be determined by counting the spots of the respective color from the image. It could be demonstrated that, tilamin at MIC induces collapse of cell membranes to an extent of 80% within 45 min which would be higher a rate than with amhelin or cecropin B, yielding 27% and 25% dead cells, resp., within this time.

	tilamin	***amhelin***	***cecropin B***	***daptomycin***	***polymyxin B***
	Minimum inhibitory concentration (MIC), μM				
P. aeruginosa	4.0	12.3	1.6	>100	<1
S. aureus	8.8	>50	>100	7.7	$\leq$ 50
E. coli	10.2	3.0	<1	>100	<1
B. subtilis	<1	6.2	>50	7.7	1.5
S. enterica	3.1	6.2	25	>100	>100
E. faecalis	3.1	6.2	25	>100	>100
M. luteus	<1	7.1	<1	15.4	<1

Table 1. *Minimum inhibitory concentrations of the studied model peptides in comparison to cecropin B (naturally occurring host defence peptide) as well as daptomycin and polymyxin B (two commercial peptide-antibiotics). Red: Gram negative, blue: Gram positive and green: Gram-variable bacteria. Low MIC-values indicate strong inhibitory effect on growth of the particular type of bacteria.*

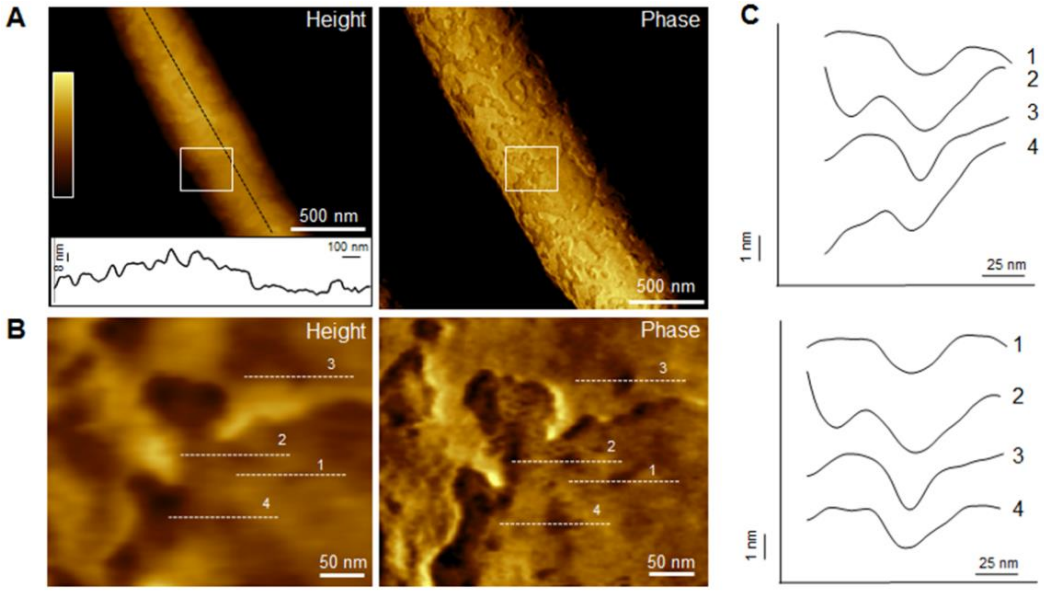

Figure 9. *AFM of E. coli cells in water after15 min incubation with tilamin. (A) Images of a cell shown in height- and phase modes; profile shown in the left picture was measured along the black dotted line. (B) Zoom into the white squares shown in (A). (C) Height profiles taken along the white-dotted lines shown in (B); upper: original profiles, lower: after local background subtraction.*

Evidence for the bacteria-killing effect on the cellular level, eventually, was obtained by time-lapse AFM of *E. coli* cells in water. Images acquired after 15 min of incubation with tilamin indicate lesions with ~8 nm steps which roughly corresponds to the thickness of the outer membrane (3.7-4.1 nm) plus the peptidoglycan layer (4 nm) in Gram-negative bacteria as *E. coli*. The results, as shown in Fig. 9, fit well to the assumption of exfoliation of the outer membrane and poration in both outer and inner membrane.

Fluorescence microscopy

Given toxicity to bacteria, as exemplified for *E. coli* (see Fig. 9), further confirmation was sought that the primary target of the attack of the AMP is the lipid bilayer. AFM- investigations based on SLBs provide a good deal of evidence for this, indeed, but SLBs are planar and not ideally modelling the shape of real cells. Giant unilamellar vesicles (GUV) are a better approximation in this respect and used as minimum membrane models of biological cells, as they are the only system, presently, in which membrane mediated interactions can separately be studied.[6] GUV are composed of a phospholipid bilayer membrane and an aqueous interior, and do not provide interior targets for the AMP and lack any other membrane components focusing the experiment to the phospholipid bilayer, therefore. In order to visualise integrity/disintegration of GUV (POPC/POPG, 3:1) a technique of selective staining bilayers and interior, with different dyes each, was applied and stability monitored using fluorescence microscopy. Fluorescence micrographs shown in Fig. 10 reveal membrane-destructive activity of tilamin against GUV, as expected, within a time scale compatible with the expected 'proteolytic lifetime' of the AMP and similar to the time scale of destruction of *E. coli* cells observed with time-lapse AFM (see Fig. 9).

Further to this, distribution of tilamin was measured for different cell lines (mouse fibroblasts, human fibrosarcoma cells and breast cancer cells). The peptide was conjugated with a cyanine dye, so as to enable visualisation of its dislocation by fluorescence imaging. As opposed to the expectation based on the results from the experiments with ZUV as membrane mimetic, enrichment of the peptide and clustering at the cell membranes was observed, as shown in Fig. 11A for human fibrosarcoma cells. This would happen within about 15 minutes, while local tilamin concentrations did continually increase (Fig. 11B). As concluded from

[6] Semrau S and Schmidt T, *Soft Matter*, 2009, 5, 3174-3186

experiments at significantly higher tilamin concentrations than those shown in Fig. 11, there appears to be a pathway for the peptide to transgress into the cytoplasm, which, however could not be identified as to what mechanism it would occur. These experiments indicate that, investigations focused to interaction of AMP with liposomes as membrane mimetics, as within BiOrigin, reflect just one aspect of the complex mechanisms pursued in defence against microbes. The latter, apart from membrane poration, may involve interference with cytoplasmic membrane septum formation, cell-wall and nucleic acid synthesis as well as inhibition of enzymatic activity and protein synthesis.[7]

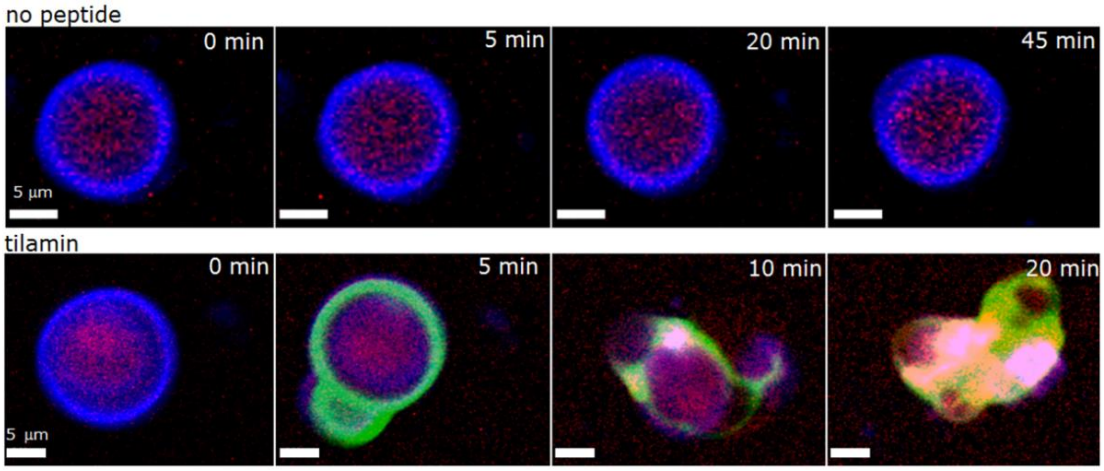

Figure 10. *Tilamin-induced lysis of giant unilamellar vesicles (GUVs). GUVs are used as 'lipid-bilayer-only' cell models. Membrane and interior become visible in blue and red, resp., by a selective staining technique applied. The status is tracked by taking fluorescence micrographs at different times elapsed. While the GUVs are stable in absence of the peptide, rapid disintegration of the (anionic) GUV is provoked by tilamin.*

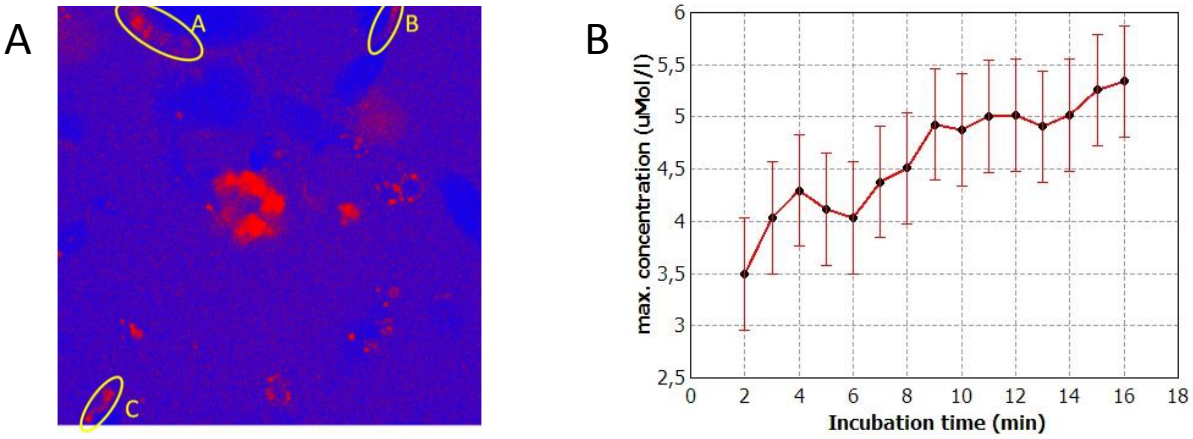

Figure 11. *(A) Distribution of tilamin interacting with human fibrosarcoma cells visualised by fluorescence microscopy. Clusters predominantly form at cell membranes; at higher concentrations (not shown) the peptide may be translocated into the cytosol too. (B) Increase of local tilamin concentration over time for cluster C shown in (A).*

[7] Brogden KA, *Nat Rev Microbiol*, 2005, 3, 238-250

Nuclear Magnetic Resonance with hyperpolarised ^{129}Xe

The Enhancement of the polarisation of the spin/1/2 isotope ^{129}Xe up to five orders has become well established a technique during the last years, thus making the noble gas a formidable probe of unprecedented sensitivity for nuclear magnetic resonance (NMR). Currently, fields of application particularly in the area of biomedicine are explored. Within BiOrigin, the feasibility of NMR spectroscopy and imaging using hyperpolarised ^{129}Xe (hpXe) for the investigation of AMP was tested.

One obstacle, hampering applicability of xenon itself to the purpose, is owing to its chemical inertness, rendering the binding to specified targets a difficult task. To circumvent the rather unspecific binding of bare xenon, the concept of xenon-based biosensors was developed.[8] The idea is to trap xenon in a molecular cage which itself may get functionalised by a reactive agent, as is illustrated in Fig. 12 (upper left). In BiOrigin the molecule cryptophane-A (CrA) was chosen as xenon host and conjugated with the model peptide tilamin through a maleimide linker (CrA-tilamin, Fig. 12 lower left).

Indeed, the CrA-hpXe host-guest system was found basically to be suitable for application as biosensor in NMR as exemplified in Fig. 12 (right) for tilamin-CrA. Spectral 'fingerprints' of tilamin-CrA, which had been synthesised for this purpose, characteristically change depending on the molecular environment and are thus a probe thus for peptide-membrane interaction. For reference, non-AMP-CrA (see Fig. 14, section 3.5, for non-AMP) was included into the studies as well. The following conclusions could be drawn:

(i) Tilamin strongly interacts with AUV (anionic, microbial-membrane mimetic) but no interaction was apparent with ZUV (zwitterionic, mammalian-membrane mimetic). This is in line with what was seen by different methods before (CD-spectrometry, cross-linking mass spectrometry, AFM, nanoSIMS).

(ii) At the same time, no interaction of non-AMP was detected with neither of microbial or mammalian membrane mimetic (AUV and ZUV, resp.).

(iii) As by the spectra acquired, the biosensor may be speculated to be anchored solely by the CrA-moiety in the bilayer while the peptide residual, strongly positively charged is still surrounded by solvent (water), thus directed away from the liposome surface, or only very mildly interacts through polarisation effects with the neutral liposome surface.

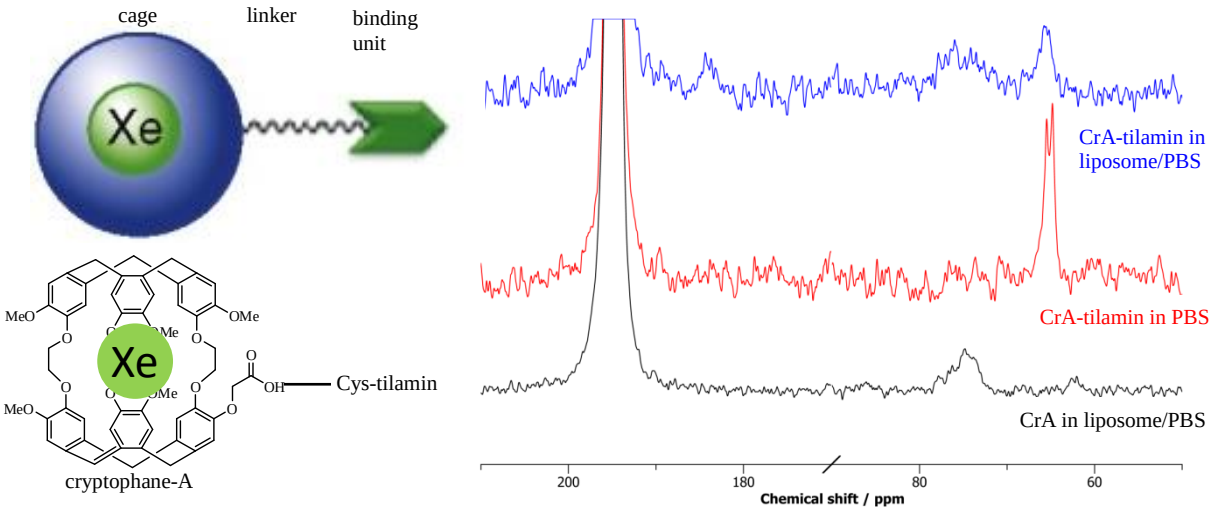

Figure 12. *The conjugate of the molecular cage cryptophane-A and the peptide tilman (left) is a biosensor for the investigation of peptide membrane interactions by NMR with hyperpolarised xenon. This biosensor largely partitions into the membrane of liposome in physiological solution which may be quantified by way of comparison of the respective NMR signals (right).*

[8] Spence MM et al., *Proc Natl Acad Sci USA* 98, 10654 (2001)

The findings may be taken as a first proof-of-concept for future applicability in magnet-resonance molecular imaging (MRMI) of such kind of interactions. The hyperpolarised xenon, as necessary if pursuing this approach, introduces an extra contribution to the uncertainty in quantifications of spectral signals due to fluctuations in the production and supply of the hpXe during an NMR experiment. The relative uncertainty in the signal intensity from this source was estimated as 1% (standard deviation from mean for a large number of individually acquired signals of hpXe dissolved in water under nominal identical conditions) and is deemed to be fit for the intended purpose, therefore.

The following tasks have been achieved in combined application of different imaging methods to characterisation of peptide interaction with membrane mimetics or real cellular membranes:

- Atomic Force Microscopy (AFM) has proven effective as to visualising different patterns of peptide-membrane interaction and mechanisms of membrane disruption as observed between both model peptides (tilamin and amhelin). Additionally, it provides information on poration depths profiles which can be used to distinguish between bilayer poration and monolayer exfoliation.
- Imaging nano- Secondary Ion Mass Spectrometry (nanoSIMS) further contributes a two-dimensional map of distribution of the investigated peptide. In these images, the peptide selectively is recognised by 'heavy' variants of the ions recorded, if isotope-labeled peptides are used. Structures seen, therefore, unambiguously confirm pores identified in AFM to be induced by that peptide (or not).
- Giant unilamellar vesicles (GUV) with selectively stained bilayer and interior, resp., are appropriate models for separate characterisation of membrane destructive activity of model peptides, as potential other pathways of cell destruction are excluded with GUV; timed experiments using fluorescence microscopy can be used to characterise the time scale of disintegration of the bilayer.
- Conjugation of peptides with cryptophane-A (CrA) moieties could be an option to use hyperpolarised xenon as sensitive probe for the peptide in combination with detection by Nuclear Magnetic Resonance (NMR) and may be a starting point to further development into methods for in vivo-imaging of distribution and of such peptides.

Conclusion: Objective Achieved. The work on innovative imaging methods within BiOrigin meets the general project objectives. New scientific and technological knowledge was generated and consolidated using complementary approaches. Firstly, by optical and atomic force microscopy the antimicrobial activity of peptides could be visually followed in the course of time by a series of snapshot two-dimensional images. These, in combination with nano-SIMS imaging, provide an idea about geometries and shapes of pores formed on microbial membranes and the time scale in which they emerge. In this way, qualitative and quantitative information about a new interaction mechanism with model membranes and live cells could be obtained. Secondly, the magnetic resonance of hyperpolarised xenon was explored for the imaging of antimicrobial activity to set the stage for the technological development of a novel imaging modality. Basic instrumentation and methods were developed and enabled the acquisition of relevant data which could be validated independently within BiOrigin. Xenon-based magnetic resonance imaging on peptide activity now appears feasible while completion of the technology is still awaited.

3.5 Molecular rationale for prediction of biological activity

The results acquired by the biophysical methods, 'low resolution', as FTIR and CD optical spectroscopy, chemical cross-linking mass spectrometry (section 3.2) in combination with molecular-scale and real time-imaging techniques (AFM, nanoSIMS, fluorescence microscopy, section 3.4) suggest a changeover in biological activity to occur as a consequence of the differences in amino acid sequence (see Fig. 14) between amhelin and tilamin. It is hypothesised, that tilamin provides a structural motif with the propensity to insert into the upper (outer) leaflet only of the lipid bilayer of reconstituted or live bacterial membranes forming monolayer pits which coalesce resulting in membrane exfoliation and collapse of the bilayer eventually, and cell death,

therefore, within minutes. In contrast to this, amhelin forms through-membrane pores which continually grow by successive recruitment of further peptide molecules into the existing pores. As both, tilamin and amhelin are likely to be of similar length (~3.15 nm in helical structure) this implies accommodation of tilamin in a tilted orientation with respect to the surface of the bilayer, as opposed to vertical 'punching' with amhelin. These putative differences had been illustrated in Figs. 1 and 7.

The postulate of a tilted insertion of tilamin into the upper leaflet of the lipid bilayer could be further substantiated by oriented solid state Nuclear Magnetic Resonance Spectrometry (ssNMR), a state-of-the-art technique available to the consortium within BiOrigin by the University of Oxford (REG2) and molecular-dynamics (MD) simulations contributed by the University of Edinburgh (REG1). Particularly, geometric analysis of labeled alanines (GALA) would yield an overall tilt of 70°, with respect to the membrane normal, for the peptide in AUV prepared from DLPC/DLPG, 3:1. An equilibrium ensemble of angles observable within the time scale of NMR and is shown in Fig. 13A. A similar distribution of tilt angles resulted from MD-simulations for a single tilamin helix within 250-500 ns after being inserted into a DLPC/DLPG (3:1) bilayer. At the same time, transmembrane configurations of oligomer pores turned out not to be stable in such simulations, but would move toward one leaflet, thus forming rather symmetrical monolayer pores (as shown in Fig. 13B).

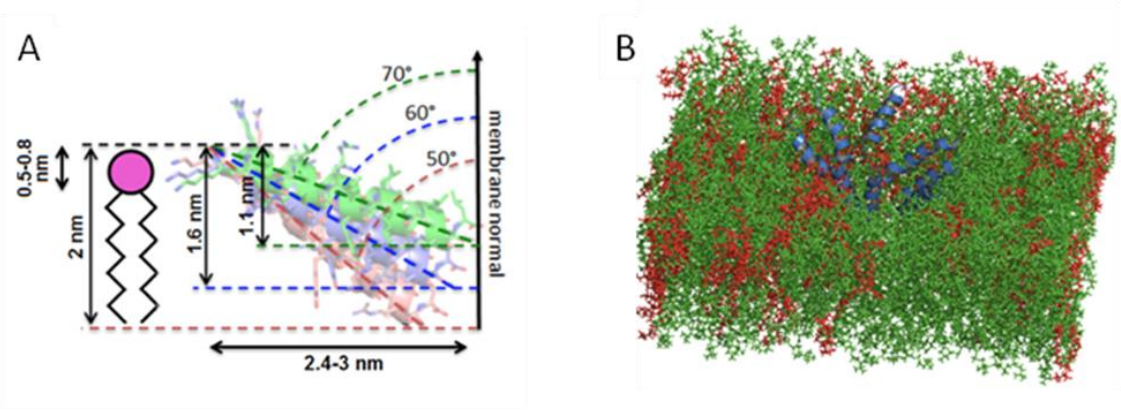

Figure 13. *(A) Ensemble of tilt angles for tilamin interacting with AUV (microbial membrane mimetic) as derived from NMR-measurement of ^{2}H-quadrupolar splittings in a GALA-experiment and MD simulations, and insertion depths and horizontal projections (peptide spans) concluded from this. Left: Phospholipid with about 2 nm length and a headgroup (pink) of 0.5-0.8 nm. (B) Example low-oligomer pore constructed from the results of coarse-grained molecular dynamics simulations in a modeled bilayer (POPC/POPG, 3:1). Tilamin: blue, POPC: green, POPG: red.*

The derived insertion angles, given the length is 3.15 nm of the helix, translate to insertion depths of 1.2-2.0 nm as shown in Fig. 13A. This is in satisfactory agreement with pore depths of 1.2-1.8 nm as observed by Atomic-Force Microscopy (AFM, see Fig. 6). The projected span of a tilted helix in the membrane (2.4-3 nm, see Fig. 13A) suggests a minimum diameter of ~5-6 nm for a pore, which is consistent with the diameters of the smallest pores observed in AFM. The GALA-experiment, unfortunately could not resolve the orientation of the helix as to which end, N-terminus or C-terminus, is inserted into the bilayer. At all events, ssNMR spectra, acquired in another measurement using POPC/POPG, 3:1, deuterated at the headgroup either or at the fatty acid chain of POPG, revealed tilamin to exert greater disordering impact on lipid headgroup regions compared to the chains. This is indicative of close charge interplays of tilamin which are maintained more efficiently if the peptide takes a tilted position rather than totally perpendicular.

In conjunction, these results corroborate fundamentally different modes of peptide-membrane interaction and mechanisms of disruption to prevail with amhelin and tilamin, which had been investigated as model peptides to investigate structure-activity relations as task of BiOrigin. This validates the design rationale underlying the construction of the two model peptides with primary structures (amino acid sequences) as shown in Figure 14.

Figure 14. *Peptide template and model antimicrobial peptides (tilamin and amhelin) studied within BiOrigin. The non-AMP was expected to be inactive and used as negative control.*

In the first template (grey letters), *C* indicates a cationic residue, *N* a (neutral) polar or small and *H* a hydrophobic residue to be used in construction of the peptide. The arrangement of hydrophobic residues (leucines, L, were used exclusively) into *i+3* and *i+7* repeats imparts a desired propensity to helix formation, which is further amplified by insertion of *C*- and *N*-residues in between these sites. Cationic residues (lysine, K, blue, and arginine, R, dark blue) were placed in the specified positions so as to obtain two slightly different peptides, *tilamin* and *amhelin*. As a negative control, the non AMP shown was used, which is lacking cationic residues, and particularly contains negatively charged glutamate residues (E, shown in red).

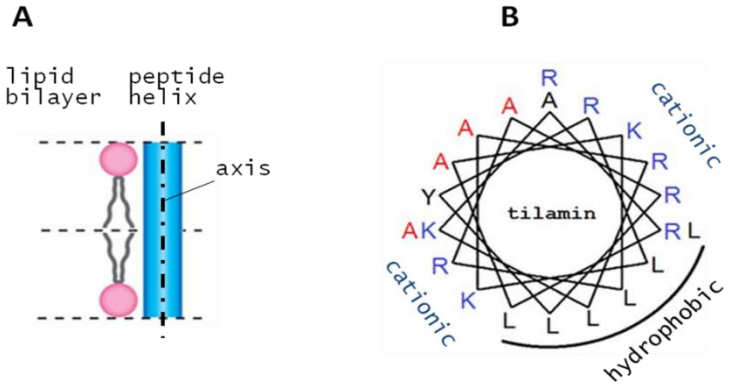

Figure 15. *Design of the model peptides: (A) Sequence length (21 amino acids) was chosen such as spanning through a typical lipid bilayer(3.2-4 nm) if forming a helix of 6 turns. (B) Arrangement of cationic and hydrophobic residues into separate faces on adopting helical structure shown for the example of tilamin. The view is top-down along the axis of the helix (helical-wheel representation).*

The length of 21 amino acids was chosen such as to make a helix of ~6 turns, corresponding to an axial length of 3.2-4 nm, which dimensionally is compatible with a typical lipid bilayer, as shown in Fig. 15A.

By primary structure (amino acid sequence) both, tilamin and amhelin will form separate segments (faces) of type ‘polar’ and ‘non polar’ if folding into helices, as apparent from projection shown in Fig. 15B for the example of tilamin. This helical wheel is a map of positions/orientations of the individual residues if viewed along the helical axis shown in Fig. 15A. Forming these faces is thought to maximise the number of contacts between negative phospholipid headgroups and the peptide and to therefore favor the hypothesised ‘S-state’ with the peptide first attached parallel to the membrane, which is precursor to subsequent formation of pores.

As a core result of BiOrigin, it has been demonstrated how targeted change of primary (amino acid-) sequence, as exemplified by tilamin and amhelin, can be used to change the biological activity of AMPs and the mechanism pursued in destruction of microbial membranes.

It could be shown in BiOrigin that, both, tilamin and amhelin, as expected, display antimicrobial activity and attack microbial membranes with formation of helical structures which would not be the case with zwitterionic

membrane mimetic, so potentially toxicity is reduced towards mammalians, or multicellular animals in general. However, a change in the mechanism of membrane destruction had been postulated and was found found to be elicited by replacement of arginine (R) by lysine (K) in tuning the sequence on converting tilamin to amhelin as schematically illustrated in Fig. 14.

In the first case (tilamin), the peptide turns from initially being randomly distributed over the membrane into small clusters where the peptide molecules take a tilted position with respect to the membrane. This, in turn, results in (local) exfoliation of the outer leaflet of the membrane-forming bilayer. The pits formed, destabilise the membrane as a whole, as exposing the hydrophobic core of the membrane to the surrounding water molecules, which is hypothesised to be the driving force for the eventual collapse of the whole membrane, which is observed. With amhelin, in contrast, a pore formation is apparent involving holes punched through the membrane as a whole as an initial state, and membrane collapse proceeds then by continued recruitment of further amhelin molecules, enlarging thus the pores formed until the point of membrane collapse.

The finding that, transmembrane poration, as with amhelin, is not a necessary prerequisite for antimicrobial activity is new and points to a distinct mechanism not previously discussed. Tilamin or similar sequence motifs, therefore may become starting templates for development of novel antibiotics.

In summary, the design rationale followed to draft peptide templates according to the desired effect on mammalian or microbial membranes (bilayer poration or monolayer exfoliation) was:

- A strategy of 'inverse protein folding'[9] was applied to designing the model peptides used within BiOrigin, i.e., given the biological mechanism of peptide-membrane interaction, the underlying essential traits, or sequence motifs were sought for.
- A combination of neutral polar or small (N), polar cationic (C) and hydrophobic residues (H), with repeats of the sequence pattern *CNCHNCH* as shown in Fig. 14 induce the arrangement of the peptide into amphipathic helices on contact with negatively charged (microbial type) of phospholipid bilayers. This condition is fulfilled with both of the peptides, tilamin and amhelin.
- In order to further support this, all amino acids used have high helical propensity.
- Using three such heptads (*CNCHNCH*) results in an axial length of ~3.15 nm of such a helix, which is well compatible with the thickness of bilayers of typically 3-4 nm (see Fig. 15A). A sequence length of about 21 amino acids is sufficient to make such a motif.
- Replacement of Arginine (R) with tilamin in place of Lysine (L), however, induces the peptide not to form pores, through the whole of the bilayer, but to switch into a mode of tilted insertion into the upper leaflet only (see Fig. 7, section 3.4).
- By the triplicate of *CNCHNCH*- heptads, same-type residues (spaced as $i, i+3,$ *and* $i, i+4$ see Fig.14) are arranged in the same segment if viewed along the helical axis (Fig. 15B). This is meant to facilitate co-operative assembly in lipid bilayers while maintaining peptide-peptide interfacial contacts.
- As a consequence of such substitution, a mechanism of destruction of the upper leaflet is prevailing then that appears to result in stronger antimicrobial activity if compared to amhelin.
- Even distribution of arginines along the tilamin sequence supports preferential interaction with the upper lipid-layer by enabling networks of electrostatic interactions to be formed along the helix while inserting into membranes.
- Increased frequency of arginines in the middle part of the sequence is to arrest its interfacial binding.
- Hemolytic effects are minimised by splitting the polar face into two unequal sub-faces via a neutral alanyl (A) cluster placed opposite to the hydrophobic face (L), see Fig. 15B. By this way, the hydrophobic face is kept shorter than typically with venom peptides as melittin.
- Tyrosine (Y) as incorporated into the alanyl- cluster is to help the peptide adapting to the curvature of membrane after disruption.

[9] Yue K and Dill KA, *Proc Natl Acd Sci USA*, 1992, 89, 4163-4167.

The rationale was demonstrated in the design of experimental antibiotics, antimicrobial materials and was also probed to reveal unknown antimicrobial resistance mechanisms. Model antimicrobials derived from this rationale performed as predicted. Examples include antibiotics predicted to form pores in bacteria (Figure) and antimicrobial materials designed to resist biofilm formation while supporting the growth of human cells. Matched by experimental validation, computational predictions provide an ever-evolving basis for designing next-generation antimicrobial agents. Thus, the rationale enables limitless prediction-validation cycles leading to constant improvements in drug design. After consultations with key industry stakeholders (IBM, GSK, Malvern Cosmeceutics, Novabiotics, CEM Corp and others) the rationale was tested to design bespoke antibiotics with broad spectrum antimicrobial activities while having no effects on human cells, and was also used for the formulation of bacterial membrane materials that are now developed as high-throughput platforms for screening antibiotics.

Conclusion: Objective Achieved. The strategy for systematic investigation into design principles using a spectrum of complementary biophysical methods to experimentally test assumptions about structure-activity relationships for antimicrobial peptides, has been successfully demonstrated with the example of the model substances (amhelin and tilamin). It could be shown that, by solely replacing a small number of amino acids a qualitative turn could be induced in the way a peptide attacks and destroys lipid vesicles (as simplified models for microbial membranes). By this way a structural motif was deduced, which elicits strong antimicrobial activity by insertion into the outer leaflet of microbial membranes as opposed to poration through the whole bilayer. Re-iterating this approach, tilamin may provide a starting point for further optimisation toward a particular application in drug design, or for the exploration of further rationales for targeted drug design.

4 Actual and potential impact

The project is a scene setter for the direct and real-time measurements of biomolecular processes of therapeutic relevance. BiOrigin is indeed an exemplary success in delivering high value outcomes within this short period of time. The strong group of industrial stakeholders provided the project with a necessary focus from the start, which proved to be essential for the successful trial of real-world applications.

4.1 Dissemination activities

The established capabilities enable the prediction and monitoring of biological processes at the molecular and cellular level that are applicable for the development of novel diagnostics, antibiotics and biofilm-resistant materials. Research was published in co-authorship with industry and clinicians, which demonstrates an early, pre-impact interest from stakeholders. The project has had an exemplary number and quality of outputs including papers in high-impact scientific journals such as *PNAS, JACS and JBC*, while being featured in industry-oriented and public publications including *Microscopy & Analysis, Drug Discovery World* and *The Times*. A full list of papers published as a result of this project are listed in Section 5 below.

In addition to written papers, project partners presented the results orally at 15 industrial, clinical and scientific conferences worldwide, including the AVS 59th International Congress in Florida USA, the 6th International conference on drug discovery and therapy in Dubai, CLINAM 2014 in Switzerland, and SNI 2014, Germany. Apart from original scientific papers presenting the direct output of the project, a RSC-Series entitled *Amino Acids, Peptides and Proteins,* co-edited by a scientist from one of the partners (NPL), was utilised to highlight important aspects and backgrounds of the project in the format of focused book chapters. Reviews given therein were about prescriptive and biofunctional peptide design (2012 & 2013), computer-modelling of folding and tertiary contacts of peptides (2012), tools and technologies in peptidome analysis (2013) and cross-linking/ Mass-Spectrometry techniques for analysis of peptides and proteins (2013), cmp. publication list.

The consortium has also had a good interaction with Standards bodies such as BIPM, BSI, NIST and NIH as well as the German Ministry of Economics and Energy, with partners regularly presenting results and providing information to the relevant Technical Committees. For example, rationale and strategy pursued in BiOrigin were presented to and discussed at a joint Workshop of the Organic-Analysis and Bioanalysis Working Groups

of the CCQM (Consultative Committee for Amount of Substance). CCQM is a panel of expert representatives of National Metrology Institutes taking care of Metrology in Chemistry and Biology, and one of the advisory boards to BIPM. Preliminary results and experiences have also been contributed to discussions conducted in preparation of the re-structuring of CCQM, which was undertaken in order to better address contemporary needs in life sciences.

The project results have also been fed into a published documentary standard at BSI relating to advanced medicinal products

4.2 Overall and long-term impact

The project has generated a number of know-hows, which have started being taken up by industry (SMEs, corporations) as entry capabilities to new markets and are being developed as screening platforms for antibiotics in collaboration with EU clinicians. For example, the discovery of a new mechanism of membrane damaging peptide activity will certainly impact future research on antimicrobial agents. Also, the concerted application of a number of physico-chemical measurement techniques to pin down the relevant measurands may be accepted as one of the most substantive strategies in the field. Overall, BiOrigin has created a strong momentum for metrology to respond to the spread of antimicrobial resistance and is progressing towards a global impact in healthcare with a follow-up research program focusing on establishing high-throughput approaches to accelerate antimicrobial discovery and manufacture. The program has attracted important partnerships with academia (Cambridge, Edinburgh, Oxford, UCL), industry (IBM, GSK, CEM, Ingenza), NMIs (NIST, PTB, LNE, LGC, NIBSC) and world-leading research organisations (Turing and Crick Institutes, NIH, Antibiotic Action, EC JRC, EMA).

4.3 Immediate Impact

Some examples of short-term impacts include the design and production of novel molecular probes for magnetic resonance imaging which are being proposed as highly efficient biosensors. A European company was involved in these activities over the whole project and was thus enabled to generate such biosensors. This new capability is anticipated to increase the company's market share and is already providing it with a competitive edge with know-hows generated by the project.

Another example is a company who collaborated at later stages of the project and who are active in the R&D and sales of membrane-based cosmetics with products sold in Europe, the US and Japan. The project results allowed the company to partially re-direct their R&D programmes to enable entry into other markets thus expanding their product portfolio. Predictive drug delivery and anti-infective technologies are of particular interest to the company.

In addition, a European leader in industrial biotechnology with clients in pharmaceutical and chemical industry is collaborating in the commercialisation of high-throughput methods developed in the project. They place a particular emphasis on *in-situ* metrology of molecular processes inside live cells for the development of high-throughput drug discovery technologies.

These and other examples at different stages of development, from consultations to implementation, manifest the impact the project has had through the introduction of a predictive rationale for drug design and development. The project has initiated a new paradigm in antimicrobial discovery, which is undergoing a next R&D stage of applying the developed approaches to other antimicrobial classes to create a new pipeline of antimicrobial agents to be taken up by early adopters.

5 List of Publications

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Crain J, Martyna GJ et al., Molecular-Scale Remnants of the Liquid-Gas Transition in Supercritical Polar Fluids, *Phys. Rev. Lett*., 115(2015)117801

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