Subproject E1.2

Peptide-mediated transport of nanoparticles into cells

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E1.2 Ulrich

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Introduction and Summary

The overall goal of project area E1 is to develop molecular transporters and use them to carry nanoparticles into cells for observation, control or destruction of the biological target. It is not trivial to transport hydrophilic cargo across cellular membranes, because this barrier consists of a hydrophobic lipid bilayer. Certain peptides can translocate across membranes in a non-disruptive way, and some of them are able to carry along various types of cargo. In sub-project E1.2 we are studying the membrane-bound structures and molecular mechanisms of such cell penetrating peptides. Based on these findings, the most promising transporters are used to functionalize designated nanoparticles and deliver this cargo into cells. Several systems of interest have been selected here and will be presented here according to the following aims:

Aim 1: ***Characterize cell penetrating peptides in native biomembranes*** by ^{19}F-NMR and electron microscopy to understand their molecular mechanism of action

Aim 2: ***Deliver fluorophores/quantum dots into plant cells*** with cell penetrating peptides to visualize intracellular targets

Aim 3: ***Enhance the antimicrobial activity of silver nanoparticles*** against pathogenic bacteria by combining them with synergistic peptides

Aim 4: ***Improve the stability of cell penetrating peptides*** by covalent conjugation to nanoparticles and by chemical cyclization

Materials: Membrane-active peptides

For the applications listed above we have synthesized numerous representative types of peptides, belonging to different structural/chemical categories. They are traditionally classified as cell penetrating, antimicrobial or fusogenic, depending on their origin, but many of them exhibit an overlapping spectrum of biological functions. We consider all of them as membrane-perturbing and try to individually optimize and/or systematically screen them to achieve the desired activity against the particular target of choice. Besides several in-depth studies of selected peptide systems, we have also prepared a whole battery of sequences that are now available pre-functionalized with either a fluorophore (N-terminal carboxyfluorescein) and soon also with a suitable reagent for click chemistry (azide or acetylene moiety). The peptides of interest include:

- **MAG2** (Magainin-2): GIGKFLHSAKKFGKAFVGEIMNS
- **PGLa** (Magainin family): GMASKAGAIAGKIAKVALKAL-amide
- **MAP** (Model Amphiphilic Peptide): KLALKLALKALKAALKLA-amide
- **MSI-103** (α-helical model): KIAGKIAKIAGKIAKIAGKIA-amide
- **KIGAKI** (β-stranded model): KIGAKIKIGAKIKIGAKI-amide
- **BP100** (designer-made, optimized by screening): KKLFKKILKYL-amide
- **TEMP-A** (Temporin A): FLPLIGRVLSGIL-amide
- **MAX4** (Maximin 4): GIGGVLLSAGKAALKGLAKVLAEKYAN
- **ALM** (Alamethicin): acyl-UPUAUAQUVUGLUPVUUEQ-pheol
- **HIV-TAT**: GRKKRRQRRRPPQ
- **R9**: RRRRRRRRR
- **TP10** (Transportan): AGYLLGKINLKALAALAKKIL-amide
- **PEN** (Penetratin): RQIKIWFQNRRMKWKK

- **SAP** (Sweet Arrow Peptide): VRLPPPVRLPPPVRLPPP
- **PEP1**: KETWWETWWTEWSQPKKKRKV
- **NLS**: PKKKRKV
- **pVEC**: LLIILRRRIRKQAHAHSK-amide
- **SynB1**: RGGRLSYSRRRFSTSTGRA
- **FP23** (HIV fusion peptide): AVGIGALFLGFLGAAGSTMGARS
- **hLfP** (human Lactoferricin Peptide): KCFQWQRNMRKVRGPPVSCIKR
- **GS** (Gramicidin S): cyclo[PVOL^{D}FPVOL^{D}F]
- **SB056** (dendrimeric): WKKIRVRLSA
- **PxB** (polymyxin B): 6-methyloctanoyl-Dab-Thr-Dab-cyclo[Dab-Dab-^{D}Phe-Leu-Dab-Dab-Thr]
- **Cyclotide KB1** (Kalata B1): c^{4}[GLPVc^{1}[CGETc^{2}[CVGGTc^{3}[CNTPGC]^{1}TC]^{2}SWPVC]^{3}TRN]^{4}

Methods: Structure-function analysis

In order to understand the mode of action and to be able to use a cell penetrating peptide in a rational way, it is essential to know its three-dimensional structure, its dynamic properties, its interaction with lipid molecules, and its tendency to self-assemble into specific complexes or to aggregate non-productively. However, very few peptides have been properly characterized so far in their biologically relevant membrane-bound state. Solid-state NMR spectroscopy is the method of choice for determining the detailed molecular conformation, alignment, and dynamic behaviour in oriented membrane samples [40,41,43,44]. Besides using conventional isotope labels (^{2}H, ^{15}N, ^{13}C), we have developed a highly sensitive ^{19}F-labeling scheme with several rationally designed amino acids that are now available as suitable substitutes for different side chains [7,12, 26,27,37]. These reporter groups have been optimized for solid-state NMR by fixing the ^{19}F-label in a rigid position relative to the peptide backbone, such that they yield unique orientational NMR-parameters for the 3D structure calculation [1,29,30,35,45].

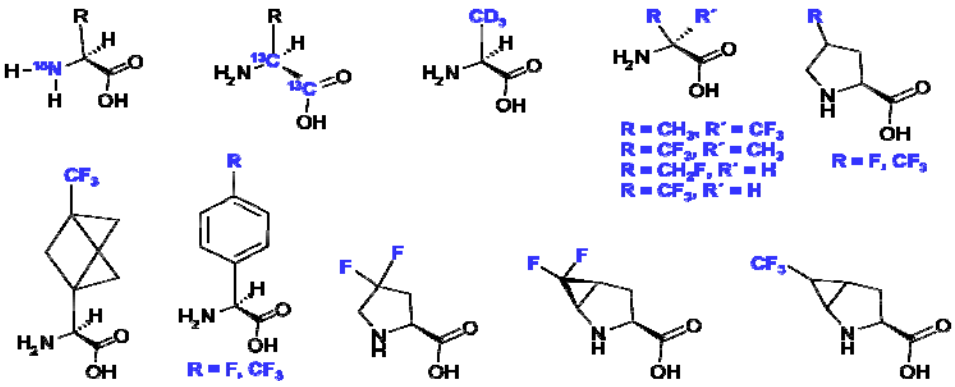

Figure 1: Conventional isotope labels for solid-state NMR (^{15}N, ^{13}C, ^{2}H), and specific ^{19}F-NMR amino acids that were designed in our group in collaboration with Igor Komarov, University of Kiev, Ukraine.

Additionally, qualitative information about the peptide conformation and orientation in membranes is obtained by circular dichroism [23], using the same kind of macroscopically oriented samples as for solid-state NMR. Various functional assays (cell uptake, minimal inhibitory concentration, fluorescence fusion, etc.) provide further information that helps to unravel the molecular mechanisms of how a peptide or peptide-cargo construct interacts with cell membranes [36].

Aim 1: Characterize cell penetrating peptides in native biomembranes

The interaction of a membrane-active peptide with a cell is critically determined by the type of membrane encountered, i.e. by the lipid composition, the phase state, the presence of other components (membrane proteins, carbohydrates), amongst other conditions such as pH, salt, serum, etc. [8,21,32]. Having successfully characterized these conditions for several peptides in a wide range of model membranes composed of synthetic phospholipids, the next step towards a biologically more relevant environment is to study native biomembranes. The low sensitivity of conventional isotope labels has been prohibitive for "in-cell" NMR so far, but our ^{19}F-labeling scheme promises several orders of magnitude gain in sensitivity. To observe the ^{19}F-labeled peptides in native biomembranes, we had to optimize the experimental procedures to harvest suitable membranes in 100 milligram amounts from different types of cells. Human erythrocytes were chosen to represent eukaryotic cells, protoplasts from *Bacillus migulanus* were selected as Gram-negative bacteria, and lipopolysaccharide (LPS) vesicles were prepared from *Escherichia coli* as Gram-negative outer membranes.

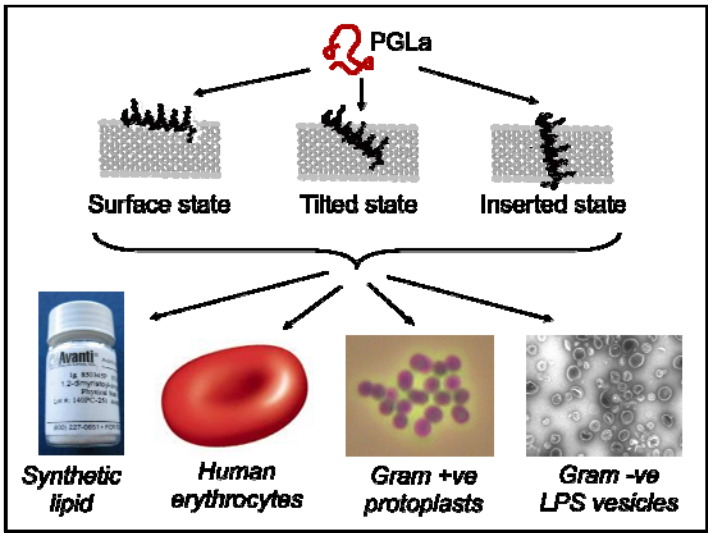

Figure 2: Interaction of the antimicrobial peptide PGLa with membranes: Upon binding to the lipid bilayer it folds as an amphiphilic α-helix, which remains surface-bound at low peptide concentration, but is able to tilt into and insert across the membrane at elevated concentration to form an oligomeric pore. Having characterized this stepwise re-alignment in various synthetic model membranes first, the peptide could be studied in native biomembranes from various representative types of cells.

The purity and lamellar state of these membranes were monirored by ^{31}P-NMR, and numerous washing steps were required before they could be deposited as macroscopically oriented samples for the ^{19}F-NMR analysis. The resulting spectra showed the typical fingerprint triplet lineshapes as in model membranes, whose analysis yielded a surface-alignment of PGLa [42]. An additional powder contribution in erythrocyte membranes implied some non-specific agglomeration, and a pronounced isotropic component suggested a higher binding affinity of the cationic peptide for bacterial membranes than for erythrocytes, in good agreement with their charge characteristics. Since no inserted state was found for PGLa, pore-formation *in vivo* appears to be a transient event involving a toroidal wormhole mechanism.

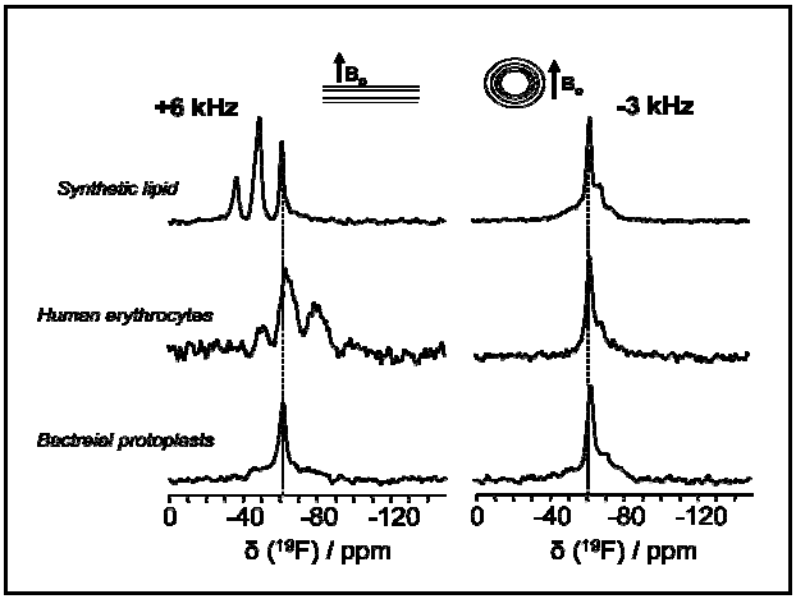

Figure 3: For the first time the structure and molecular alignment of a peptide could be determined in native biomembranes. The solid-state NMR analysis was possible due to the unique sensitivity of the ^{19}F-labels and the lack of a natural abundance background. The helical peptide PGLa was found to be aligned on the membrane surface.

To visualize directly the damaging effect of PGLa and other peptides on the membrane structure and morphology of living cells, the NMR studies were complemented by electron microscopy in collaboration with Dagmar Gerthsen (Z-project). Membrane blisters and craters are induced by the membrane-active peptides, and the ensuing osmotic influx causes the cells to burst [48].

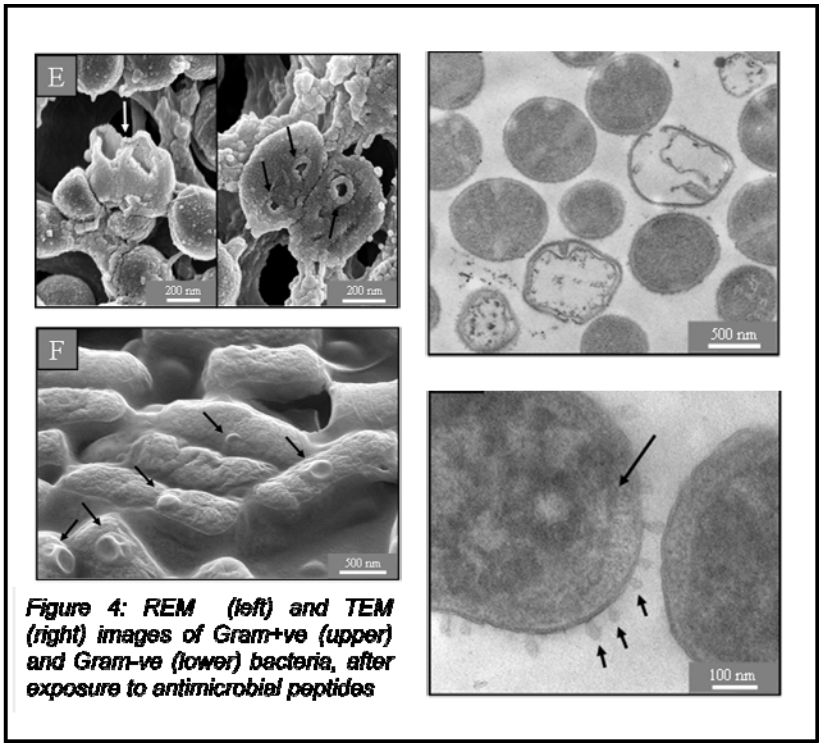

Figure 4: REM (left) and TEM (right) images of Gram+ve (upper) and Gram-ve (lower) bacteria, after exposure to antimicrobial peptides

Aim 2: Deliver fluorophores/quantum dots into plant cells

BP100 is a new designer-made peptide from our collaboration partner Miguel Castanho (University of Lisbon, Portugal), which has been optimized as an antimicrobial agent against plant pathogens by medium-throughput screening. It has an exceptionally short sequence, which makes it attractive for functionalization with other peptidic units, such as the actin-targeting sequence Lifeact. We thus examined its cell penetrating properties and toxicity against plant cells in collaboration with Peter Nick (project E1.5) to find that BP100 has indeed a potent dual antimicrobial and cell penetrating action [52]. When tagged with the actin-binding sequence Lifeact, the construct is selectively targeted to actin filaments and allows the cytoskeleton to be visualized.

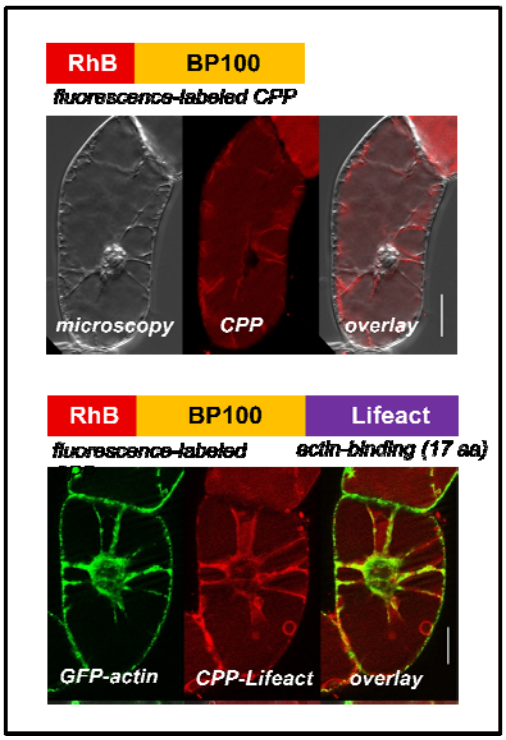

Figure 5: RhodamineB-labeled BP100 is able to enter tobacco cells and distributes homogeneously in the cytosol (upper). When coupled to Lifeact, it binds to actin filaments (that have been recombinantly labeled with green-fluorescent-protein; lower).

To employ BP100 for further applications in plants (quantum dots, magnetic nanoparticles, etc.), it is important to know its structure and understand its membrane-perturbing mode of action. Inspection of its primary sequence (see first page) does not allow a straightforward guess. On the one hand, the high charge density resembles the cell penetrating peptide HIV-TAT, which is unstructured but can form inverted micelles in membranes [9], whereas the charge distribution suggests that BP100 can fold as an amphiphilic α-helix to penetrate membranes via a toroidal wormhole (like Magainin-2, or PGLa in Figure 2) [35]. Yet again, according to our biological assays (summarized in Figure 5), its functional profile resembles most closely the model peptide MAP, which has a pronounced tendency to aggregate as β-sheets [22]. We thus determined the three-dimensional structure of BP100 in lipid bilayers by ^{19}F-NMR. With the aid of molecular dynamics (MD) simulations [14], an α-helix was found to be present in a surface-bound state. It can be concluded that BP100 does not aggregate and enters cells via a transient wormhole mechanism.

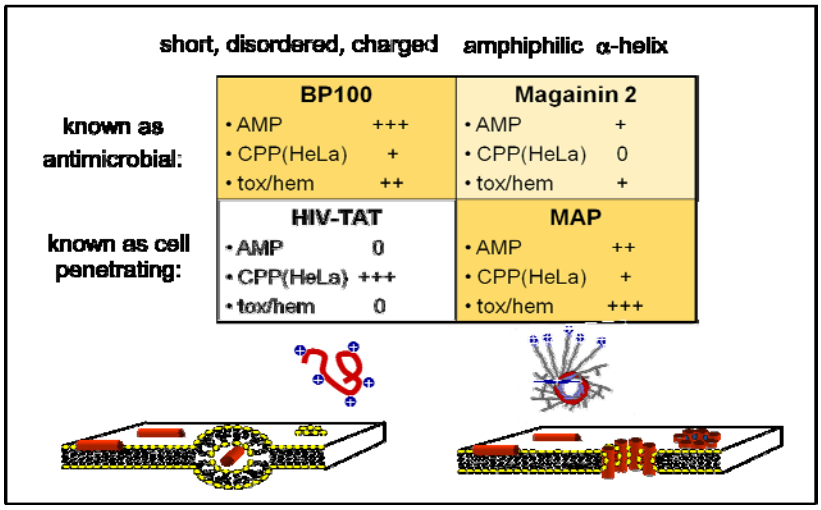

Figure 5: BP100 shares characteristic features with different classes peptides, which permeate membranes by different mechanisms. (AMP: antimicrobial action, CPP: uptake into HeLa cells, toxicity, and hemolysis).

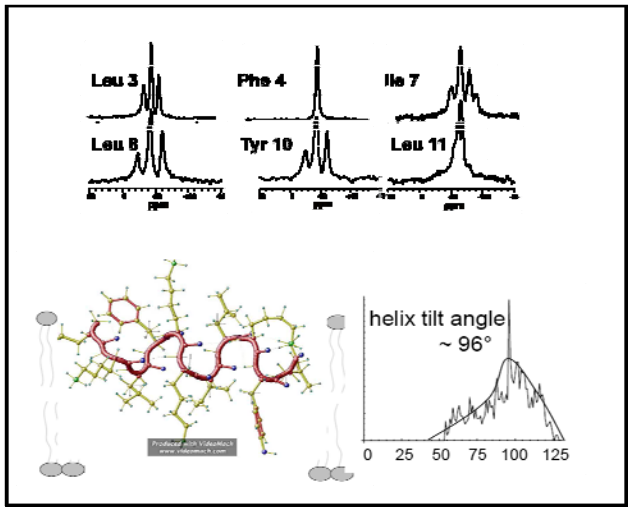

Figure 6: ^{19}F-NMR spectra of selectively labeled BP100. The NMR-constrained MD structure analysis yielded a surface-bound α-helix.

Aim 3:Enhance the antimicrobial activity of silver nanoparticles

We intend to use antimicrobial peptides to enhance the intrinsic antimicrobial activity of silver, in order to fight pathogenic bacteria that are multiresistant against conventional antibiotics. As outlined in the last CFN-report (2008), we had started by simply mixing the two components and determining the minimal inhibitory concentrations (MIC) for different ratios of peptide to silver. Evaluation of the fractional inhibitory concentration (FIC) index indicates whether there is genuine synergy or only additive effects. That way we had found that PolymyxinB acts synergistically with silver against all Gram-negative bacteria tested [34]. Interestingly, the same activity was observed for silver ions (Ag^+) as for commercial silver nanoparticles (AgNP) with a diameter of 25 nm, based on the same metal weight.

To examine any toxic side effects in therapeutic applications, we also carried out hemolysis assays in an analogous way. Here, the use of Ag^+ in combination with antimicrobial peptides increased the intrinsic hemolytic activities of both partners. On the other hand, hemolysis was not enhanced in combination with AgNP, and even at extremely high concentration the nanoparticles themselves remained non-hemolytic. We may conclude that one major advantage of AgNP over a solution of Ag^+ ions is the absence of hemolytic side effects of the nanoparticles.

The next step after mixing and co-administering peptides together with nanoparticles is their covalent coupling. We thus prepared silver and gold nanoparticles *in situ* and conjugated them to various peptides via a cystein-pentaglycine linker at their N- or C-terminus. The <10 nm conjugates were characterized (UV-VIS, dynamic light scattering, metal core size from TEM). Accurate amino acid analysis allowed us to estimate the number of peptides-per-particle as typically between 20 and 50, which represents the first analysis of this kind to our knowledge. Examination of the peptide conformation by circular dichroism showed that the preferentially α-helical peptides maintained this structure when conjugated to the nanoparticles. Likewise, in collaboration with M. Franzreb (KIT) we could show that the enzyme penicillin acylase also maintains its intact structure and full catalytic function when coupled to silica nanoparticles [18].

Antimicrobial tests of the peptide-AgNP conjugates revealed that the peptides maintain their activity, which is remarkable in view of the fact that one terminus is surface-tethered [33].

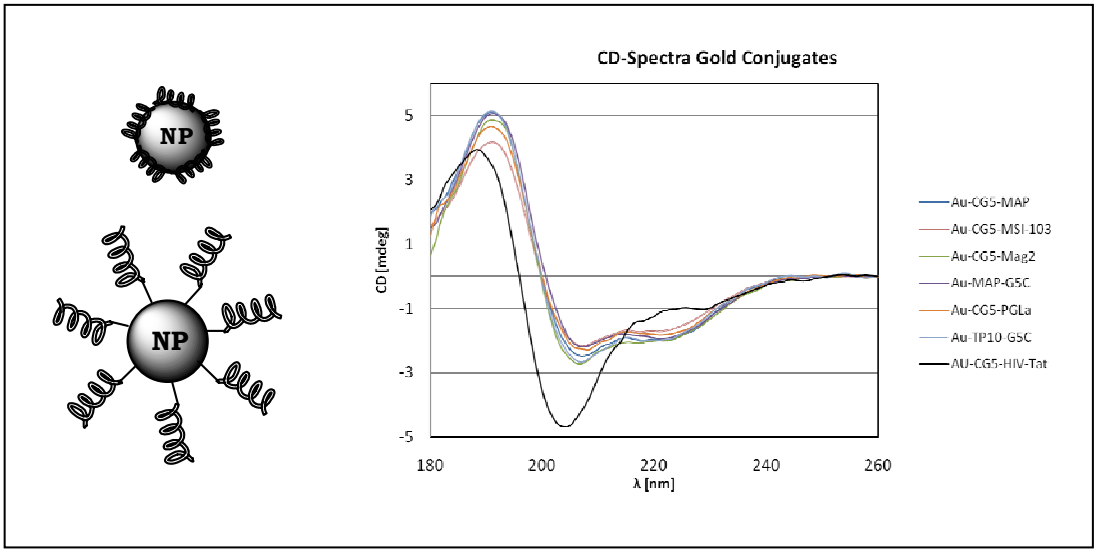

Figure 7: CD analysis showed that numerous membrane-active peptides assume their preferred secondary structure when covalently conjugated to Ag and Au nanoparticles. (Only HIV-TAT is intrinsically unstructured).

Aim 4: Improve the stability of cell penetrating peptides

One of the main challenges in therapeutic applications of peptides is to prevent degradation by proteases and combat their low stability in blood serum against temperature, pH, and during storage [28]. Given that the peptide-nanoparticle conjugates had a promising biological activity, we tested their stability by enzymatic digestion with trypsin (which is present in blood serum and cleaves after cationic residues). In contrast to the free peptides, which were degraded within an hour, the

nanoparticle conjugates remained stable. These results suggest that peptide-AgNP conjugates are are promising antimicrobial drugs against multiresistant bacteria, and that peptide-Au conjugates may serve as stable cell penetration agents.

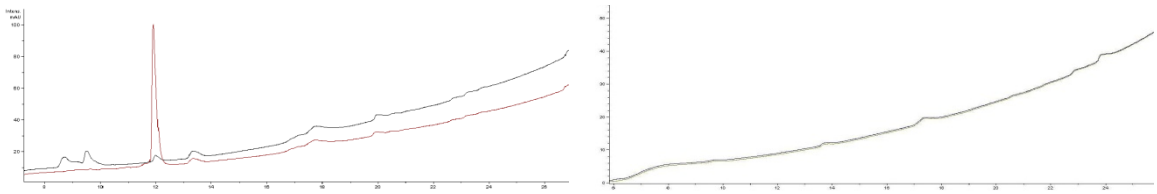

Figure 8: The HPLC traces show that free BP100 is rapidly degraded by protease (left), while the BP100-AuNP conjugate remains stable (right).

Peptides are highly to degradation when unfolded, which tends to be the natural state in solution for most peptides with free termini. However, a naturally occurring class of cyclic peptides, the so-called cyclotides, are remarkably stable and can be administered orally without degradation. The cyclotide Kalata B1 (see first page) has a tightly knotted structure, constrained by three disulfide bridges, and its amphiphilic character suggests a membrane-perturbing activity. One of our more recent aims is to employ Kalata B1 as an inert cell penetrating agent, and to incorporate some specific targeting sequences into its scaffold in collaboration with David Craik, University of Queensland, Australia. As a first step, we thus characterized the uniformly ^{15}N-labeled peptide in the membrane-bound state using PISEMA NMR. To cope with the considerable spectral overlap, we had to develop a data analysis programme that fits a set of calculated signals to the experimental intensity distributions in the broadened spectra. A rough model for the alignment of Kalata B1 in the membrane was obtained, and a more detailed analysis using selectively ^{19}F-labeled peptides is in progress.

5. Service for other CFN Projects

- NMR of peptoids and Carbon-Organic-Frameworks (COFs) with **S. Bräse (C5.2, E1.1)**
- Peptides are used to transport nanoparticles into plant cells with **P. Nick (E1.5)**
- MAS NMR characterization of nanoparticles with **C. Feldmann (C3.12)**
- MAS NMR characterization of battery materials with **S. Indris and H. Hahn (F3.3)**
- NMR characterization of channel proteins MscL and TatA with **S. Grage (E3.4)**
- MD simulations of membrane-bound peptides with **W. Wenzel** (**C5.1**)
- Peptides are coupled to nanoparticles of **L. Fruk (JRG)** by click-chemistry
- Coiled-coil peptides for **D. Wedlich (E2.2)** to functionalize surfaces reversibly
- EM on bacteria and functionalized nanoparticles with **D. Gehrtsen (Z-project)**

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